The RNA-stabilizing Protein HuR Regulates the Expression of \( \zeta \) Chain of the Human T Cell Receptor-associated CD3 Complex*

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T cell dysfunction is crucial to the pathogenesis of systemic lupus erythematosus (SLE); however, the molecular mechanisms involved in the deficient expression of the T cell receptor-associated CD3 \( \zeta \) chain in SLE are not clear. SLE T cells express abnormally increased levels of an alternatively spliced isoform of CD3 \( \zeta \) that lacks a 562-bp region in its 3′-untranslated region (UTR). Knockdown of HuR resulted in decreased expression of the CD3 \( \zeta \) chain, whereas overexpression led to the increase of CD3 \( \zeta \) chain levels. Additionally, overexpression of HuR in human T cells resulted in increased mRNA stability of CD3 \( \zeta \). Our results identify the 3′-UTR of CD3 \( \zeta \) as a novel target for the mRNA-stabilizing protein HuR. Thus, the absence of two critical AREs in the alternatively spliced CD3 \( \zeta \) 3′-UTR found in SLE T cells may result in decreased HuR binding, representing a possible molecular mechanism contributing to the reduced stability and expression of CD3 \( \zeta \) in SLE.

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organs, including the joints, skin, kidneys, and brain. Several signaling abnormalities of the immune system have been described in SLE and are thought to be central in the pathophysiology of this disease (1). Our laboratory has extensively studied the intracellular signaling of SLE T cells and identified abnormal proximal as well as distal components of the signal transduction machinery. A common molecular feature of T cells from SLE patients is a unique rewiring of the T cell receptor wherein expression of the \( \zeta \) chain, a critical intra-cellular signaling component of the CD3 complex, is decreased and is substituted by the Fc receptor \( \gamma \) (FcR\( \gamma \)) chain. This leads to a robust calcium influx once the cell is activated via the T cell receptor, whereas interleukin (IL)-2 production remains abnormally low (2–4). Replenishment of the CD3 \( \zeta \) in SLE T cells in vitro restores IL-2 production and reduces intracellular calcium flux (5), thus suggesting a central role for CD3 \( \zeta \) in the T cell defect.

Multiple mechanisms have been found to contribute to the decreased expression of CD3 \( \zeta \) in SLE T cells. First, the transcriptional activity of the CD3 \( \zeta \) promoter is limited because of limited binding of the enhancer E-74-like factor (Eli)-1 (6) and increased binding of the repressor cAMP-response element modulator \( \alpha \) (7), which is increased in SLE T cells. Second, CD3 \( \zeta \) is degraded by caspase 3, which is expressed at increased levels in SLE T cells (8). Third, CD3 \( \zeta \) mRNA in SLE T cells is produced in many alternatively spliced forms lacking coding regions that may result in non-functional CD3 \( \zeta \) chain (9, 10). Finally, in SLE T cells an alternatively spliced form of CD3 \( \zeta \) mRNA that lacks a 562-base-long (672–1233 nucleotide) region in its 3′-untranslated region (UTR) is produced in increased levels (9). This AS CD3 \( \zeta \) mRNA is significantly more unstable and has a lower protein expression than the wild-type (WT) CD3 \( \zeta \) mRNA (9, 11). The WT CD3 \( \zeta \) mRNA bears three AUUUU motifs or AREs at positions 636, 705, and 985 in its 3′-UTR, henceforth referred to as ARE1, ARE2, and ARE3, respectively. The AS 3′-UTR is missing the ARE2 and ARE3 regions that are contained in the 672–1233 stretch that is splice-deleted in SLE T cells (Fig. 1). Both these elements are independently important for the mRNA stability and protein expression of the CD3 \( \zeta \) chain. A mutation of either ARE2 or ARE3 led to a reduced expression of the CD3 \( \zeta \) chain whereas a mutation in ARE1 did not (12). The exact mechanisms by which the ARE2 and ARE3 promote CD3 \( \zeta \) mRNA stability are unknown.

AREs are the best known stability elements regulating mammalian messages and are known to control mRNA stability of various cytokines, transcription factors, and growth factors. ARE-binding proteins alter the fate of mRNA by regulating the deadenylation and decapping processes of mRNA decay (13, 14, 15).
HuR Regulates CD3γ Expression in Human T Cells

**A**

**CD3**

mRNA with WT 3'-UTR

1

566 672

ARE1 ARE2 ARE3

Splice-deleted

1233 1472

**CD3**

mRNA with AS 3'-UTR

566

672

1233 1472

**B**

**WT 3’-UTR**

AUUUA AUUUA AUUUA

(ARE2 oligonucleotide, 5'-CUCCUGCUGUAAA-UUUAAGCUUUGACGCU-3'; ARE3 oligonucleotide, 5'-CUCCUGCUUUGACGCUUUGACGU-3'; control oligonucleotide, 5'-AAGAU-GUCCAUGUCAGUCUGAC-3'), respectively. AREs 1–3 at positions 636, 705, and 985 are indicated as ARE1, ARE2, and ARE3, respectively.

**FIGURE 1.** A, schematic showing the CD3γ mRNA with full-length 3'-UTR (top) and alternatively spliced 3'-UTR (bottom). The black box represents exons I–VII. The gray shaded box represents exon VIII, a major portion of which (bases 566–1472) encodes the 3'-UTR. The AUUUA pentamers at positions 636, 705, and 985 are indicated as ARE1, ARE2, and ARE3, respectively. B, schematic showing the nucleotide sequence of wild-type 3'-UTR of CD3γ. AREs 1–3 at positions 636, 705, and 985 are in boldface and the predicted HuR-binding sites are italicized and underlined.

14). ARE-binding proteins such as Hu-antigen R (HuR) can stabilize the message by competing with destabilizing proteins (15–17) or redirecting transcripts from P bodies to polysomes (18), whereas proteins such as ARE/poly(U)-binding/degradation factor 1 (AUF1), K homology splicing regulatory protein, and tristetraprolin (TTP) can destabilize mRNA via the mRNA decay machinery.

The Hu family of RNA-binding proteins has four members. HuR (also known as HuA) is ubiquitously expressed, whereas HuB, HuC, and HuD are neuron-specific in their expression. HuR is one of the best studied ARE-binding proteins and has been shown to stabilize/ regulate numerous genes, including tumor necrosis factor-α (13, 19), granulocyte monocyte-colony-stimulating factor (20), cyclooxygenase 2 (COX-2) (21), c-myc (22), c-fos (22), cyclins A, B1, and D1 (23), and vascular endothelial growth factor (24). A predominantly nuclear protein, HuR is known to shuttle between the nucleus and cytoplasm (20). HuR has been proposed to bind ARE-containing mRNA in the nucleus, and to remain bound during mRNA transport to the cytoplasm thereby protecting the mRNA from degradation (25).

Based on our previous findings that ARE2 and ARE3 are critical for mRNA stability and protein expression of CD3γ, we hypothesized that proteins binding to ARE2 and ARE3 regulate the expression of CD3γ by promoting the stability of CD3γ mRNA. We show here for the first time that the embryonic lethal abnormal vision (ELAV)-like protein HuR binds to the ARE2 and ARE3 bearing regions of the CD3γ 3'-UTR and regulates the CD3γ protein expression and mRNA stability.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—Jurkat T cells (ATCC, Manassas, VA) were maintained in culture under sterile conditions in a 37 °C incubator with 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics. Peripheral blood T cells were purified using the Rosette Sep kit (Stem Cell Technologies Inc.) according to the manufacturer’s instructions. Antibodies to HuR, CD3γ, NFATc, and goat anti-mouse horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin and FLAG antibodies were purchased from Sigma; β-tubulin antibody was from Upstate (Millipore, Billerica, MA); TTP was from Abcam Inc. (Cambridge, MA), and the antibody to AUF1 has been described before (26).

**Plasmid Transfections**—The pcDNA3.1-HuR-C-FLAG expression vector was a kind gift from Dr. Joan Steitz (Yale University). Transient transfections of peripheral blood primary T cells were carried out using the Nucleofector system (Amaxa Inc., Gaithersburg, MD). Briefly, 5 × 10^6 cells were resuspended in 100 μl of nucleofector solution. Plasmid DNA (1.5–2 μg per 10^6 cells) was added, and cells were transfected using the nucleofector U-014 program. Cells were rescued immediately after transfection in pre-warmed RPMI medium + 10% fetal bovine serum in a 6-well culture plate.

**Oligonucleotide Pulldown Assay**—Custom-synthesized 25-base biotin-labeled ARE2 and ARE3 bearing RNA oligonucleotides and random scrambled control RNA oligonucleotides were purchased from Integrated DNA Technologies (Corvallis, IA). Sequences of the oligonucleotides are as follows: ARE2 oligonucleotide, 5'-UGCUUUGGUUAUUUAGCUCCAAA-3'; ARE3 oligonucleotide, 5'-CUCUCCCCUAAAGUAUUUAAGCUUUGACGUUUGACGCU-3'; control oligonucleotide, 5'-AAGAUGUCCAUGUCAGUCUGAC-3'.

Streptavidin magnetic beads (PureBiotech LLC, Middlesex, NJ) were washed three times in Hepes-buffered saline (HBS-P) wash buffer (0.01 M Hepes buffer, pH 7.4, 0.15 M NaCl, 0.005% (v/v) Surfactant P-20, and 5 mM MgCl2; Biacore, Inc., Piscataway, NJ). 40 pmol of biotinylated RNA oligonucleotides were incubated with 30 μl of washed streptavidin magnetic beads in binding buffer (wash buffer with 0.2 mg/ml bovine serum albumin, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM diithiothreitol) for 30
min at room temperature. Magnetic bead-bound oligonucleotides were selected using a magnetic particle separator (Pure-Biotech, LLC). Jurkat cell nuclear lysates (~700 μg) were pre-treated with polynucleosine-cytosine (dl-DC) (50 μg/400 μg of lysate) and 16 units of RNasin/100 μg lysate in binding buffer at room temperature for 15 min. 100 μl of bead oligonucleotide was added to the nuclear lysate and incubated at room temperature for 1 h. The magnetic particle separator was used to selectively bind oligo-protein complexes, whereas unbound proteins were washed three times with binding buffer. Each wash fraction was collected and labeled Wash 1, 2 (Fig. 2A, lanes 4, 5, 9, and 10), and 3. Binding buffer with 0.3 μM NaCl was used for a pre-elute wash. Bound proteins were eluted from the oligonucleotides using 10 mM glycine/HCl, pH 2.0 (Biocare, Inc., Piscataway, NJ). Three eluate fractions were collected and labeled Elute 1, 2, and 3 (Fig. 2A, lanes 1–3 and 6–8). Washes and eluates (13 μl) were loaded onto a 12-well 1.0-mm 4–6% Bistris NuPAGE pre-cast gel (Invitrogen) and run at 200 V for 35 min. Silver staining of the gel was carried out according to instructions in the Silver Express staining kit (Invitrogen). Specific bands were excised and washed twice in 50% acetonitrile.

Mass Spectrometry Protein Identifications—Peptide sequencing and protein identification were performed by the Mass Spectrometry core facility at Beth Israel Deaconess Medical Center Boston as follows.

Gel bands from SDS-PAGE were excised washed and cut into 1-mm³ pieces. Cysteine residues were then reduced with 10 mM dithiothreitol for 30 min at 56 °C and alkylated with 10 mM iodoacetamide in the dark for 45 min. Gel bands were then washed and dried in a SpeedVac concentrator. 250 ng of modified trypsin Promega (Madison, WI) in 50 mM ammonium bicarbonate, pH 8.3, was added, and the protein bands were digested overnight at 37 °C. Peptides were extracted from the gel pieces with 20 mM ammonium bicarbonate following 40% acetonitrile, 2% formic acid to a final elution volume of 65 μl.

A 4-μl aliquot of peptide mixture was injected onto a micro-capillary reversed-phase liquid chromatography tandem mass spectrometry system using a self-packed 75-μm inner diameter × 10-cm length C₁₈ column at a flow rate of ~300 nl/min. Data-dependent MS/MS spectra were collected using a Thermo Scientific LTQ two-dimensional linear ion trap mass spectrometer operated in the positive ion mode. MS/MS spectra were searched versus a reversed nonredundant protein database from NCBI using the Sequest® algorithm. Identified data base peptide sequences were validated using score cutoffs as well as manual inspection to ensure that the sequences were consistent with the typical b- and y-series fragment ions.

RNA Electrophoretic Mobility Shift Assays—20 pmol of RNA oligonucleotides were end-labeled with [³²P]ATP (PerkinElmer Life Sciences) in a 10× T4 Kinase buffer (Fisher), with T4 polynucleotide kinase, and RNasin for 1 h at 37 °C. The reaction was terminated with Tris-HCl, pH 8.0, buffer and radiolabeled probes were separated using microspin columns (GE Healthcare). Jurkat cell nuclear lysates were incubated with antibody or excess cold (unlabeled) probe in a 5× high density TBE buffer with 1× KCl, 500 μg of poly(dI-dC), and RNasin on ice for 15 min. 20 pmol of [³²P]ATP-labeled RNA probes were added, and reaction mixtures were incubated at room temperature for another 15 min and then electrophoresed on a 6% DNA retardation gel at 110 V for 45 min. Gels were fixed in a solution containing 10% acetic acid and 10% methanol for 10 min, dried in a gel dryer for 1 h at 80 °C under vacuum, exposed overnight to a phosphor screen, and scanned using a PhosphorImager scanner (GE Healthcare).

Short Interfering (si) RNA Knockdown Studies—A pair of siRNAs against HuR Hs_ELAVL1_10 (target sequence CAG AAA CAT TTG AGC ATT GTA) and Hs_ELAVL1_9 (target sequence CAC AGT GAA GTT TGC AGC CAA) and control nonsilencing rhodamine siRNA (target sequence AAT TCT CCG AAC GTG TCA CGT) were purchased from Qiagen (Valencia, CA). siRNA knockdown experiments were carried out using the Amaza Nucleofector system. Briefly, 5 × 10⁶ Jurkat cells were resuspended in 100 μl of cell line Nucleofector solution (Amaza Inc. Gaithersburg, MD), and transient transfection of 1.5–2 μg of control or HuR-specific siRNA was carried out using the Nucleofector program C-016. Cells were immediately rescued in 2 ml of prewarmed RPMI + 10% fetal bovine serum medium in 6-well plates and rested in a 37 °C incubator. Cells were harvested, and cytoplasmic and nuclear lysates were made 24–48 h post-transfection.

Western Blotting—Cytoplasmic and nuclear extracts were prepared using Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA) and Buffer C (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors 5 mM sodium fluoride (NaF), 4 mM sodium vanadate (Na₃VO₄), aprotinin, leupeptin, 1 mM dithiothreitol, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. 5 × 10⁶ cells were pelleted and resuspended in 400 μl of Buffer A and kept on ice for 15 min. 25 μl of Nonidet P-40 was added; the suspension was vortexed for 10 s and immediately centrifuged at 13,200 rpm for 1 min. The supernatant was collected and labeled as the cytoplasmic fraction, and the pellet was washed with Buffer A. After discarding the supernatant, 50 μl of Buffer C was added to the pellet, shaken on a rotator for 15 min at 4 °C, and then centrifuged at 13,200 for 10 min. The supernatant was collected and labeled as the nuclear fraction. Extracts were resolved on a 4–12% Bistris NuPAGE precast gel at 200 V for 35 min and transferred to polyvinylidene difluoride membrane at 30 V for 75 min. Membranes were blocked in 5% nonfat milk in Tris-buffered saline with Tween 20 (TBS-T) buffer for 1 h followed by incubation with primary antibody (1:1000) for 1 h, washed three times with TBS-T, incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h, washed three times with TBS-T buffer, incubated for 1 min with ECL reagents (Amersham Biosciences), and visualized by autoradiography.

mRNA Expression Studies—Total mRNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) from primary T cells according to the manufacturer’s instructions. 0.1–1 μg of total RNA was reverse-transcribed into single-stranded cDNA using the avian myeloblastosis virus reverse transcriptase-based system from Promega (Madison, WI) according to the manufacturer’s instructions. The primers (Beth Israel Deaconess Medical Center Genomics core) for quantitative real time PCR of wild-type CD3ζ 3′-UTR were as follows: forward, 5′-AGT GGC TTC ACT CCT GCT GT 3′ (sense bp 963–982),
and reverse 5′-GCC TAG GCT CCT TTC CAT CT 3′ (antisense bp 1096–1077), according to the numbering of Weissman et al. (27). This primer pair amplifies the splice deleted region of the 3′UTR specific to the wild-type isoform. GAPDH was used as a control, and the primers were as follows: forward, 5′-CAA CTA CAT GGT TTA CAT GTT CC-3′, and reverse, 5′-GGA CTG TGG TCA TGA GTC CT-3′. The reverse transcription product cDNA was diluted 2-fold, and SYBR green master mix (Roche Applied Science) was used for amplification of CD3ζ or GAPDH control. Real time PCR amplification was carried out in a LightCycler 480 (Roche Applied Science) as follows: initial denaturation cycle at 95 °C for 5 min, 40 cycles of amplification with denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s, 1 cycle of melting curves at 95 °C for 15 s, 65 °C for 2 min, and 97 °C continuous and a final cooling step at 37 °C for 30 s. All samples were run in triplicate and average threshold cycle (Ct) values were used to calculate relative mRNA expression by the ΔΔCt relative quantification analysis method. For semi-quantitative PCR of wild-type, primers used were forward, 5′-AGC CTC TGC CTC CCA GCC TCT TCC GAG-3′ (sense bp 34–62), and reverse, 5′-CCT GCA CAT GGT ACA GTT CAA TGG TG-3′ (antisense bp 1233–1205), and primers for AS-3′ were forward, 5′-AGC CTC TGC CTC CCA GCC TCT TCC GAG-3′ (sense bp 34–62), and reverse, 5′-CAT CTT CTG GCC CTT CAG TGG CTG AGA AGA GTG AA-3′ (antisense bp 1235–1234 and 671–694; this primer spans both sides of the alternative splice site and is specific for the AS-3′ isoform) according to the numbering of Weissman et al. (27). PCR was carried out in a Bio-Rad thermal cycler as follows: initial denaturation at 94 °C for 4 min, 40 cycles at 94 °C for 45 s; 67 °C for 1 min; 72 °C for 2 min and a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.2% agarose gels and visualized with ethidium bromide staining. Quantitation of the PCR product was done using the Quantity1 software (Bio-Rad).

**RESULTS**

**Identification of Putative Proteins Binding to ARE2 in the 3′-UTR of CD3ζ Chain—**The 3′-UTR of CD3ζ contains three ARE, two of which (ARE2 and ARE3) are missing in SLE T cells. Chowdhury et al. (12) showed that a mutation of the AUUUA in either ARE2 or ARE3 of CD3ζ 3′-UTR led to a significant reduction in the mRNA stability and protein expression of CD3ζ, indicating that the ARE2 and ARE3 regions positively regulate the integrity of CD3ζ mRNA. Because SLE T cells express very low levels of the wild-type CD3ζ mRNA, we used Jurkat T cells that express this transcript abundantly. To identify proteins that bind to the two AREs and function to stabilize CD3ζ mRNA, we used custom-synthesized biotin-conjugated RNA oligonucleotide encoding the ARE2. This oligonucleotide contained the sequence 693–717 of the CD3ζ 3′-UTR, including the AUUUUA pentamer at position 705. A random scrambled 25-base-long sequence with similar GC content was used as the control. The ARE2-bearing oligonucleotide or the control oligonucleotide were incubated with Jurkat cell nuclear extracts, and as shown in Fig. 2A, after washing off the unbound proteins (Wash 1 and Wash 2), several binding proteins were eluted from the ARE2 oligonucleotide (elute 1 and elute 2) with a 10 mM glycine/HCl, pH 2.0, solution, and eluates were labeled elute 1 and elutes were used to elute 1 and elutes were resolved on a 4–12% BisTris denaturing gel followed by silver staining. B, eluates and wash 1 from the ARE2 oligonucleotide pulldown assay were electrophoresed on a 4–12% BisTris denaturing gel, transferred to a polyvinylidene difluoride membrane, and subsequently blotted to anti-HuR antibody. C, amino acid sequence of HuR showing peptide matches (in boxes) in the 36-kDa protein complex from the ARE2 oligonucleotide pulldown assay.

**Densitometry and Statistical Analysis—**Densitometric analysis of the Western blots was performed with the Quantity 1 software (Bio-Rad). Statistical analyses were performed using the Student’s t test.
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A–C, Jurkat cell nuclear extracts were incubated with 32P-labeled RNA oligonucleotide spanning ARE2 in the absence (lane 1) or presence (lane 2) of HuR antibody or an unrelated NFATc antibody (lane 3). D, Jurkat cell nuclear extracts were incubated with a 32P-labeled RNA oligonucleotide spanning ARE2 in the absence (lane 1) or presence of 20-fold excess of unlabeled (cold) ARE2-bearing (lane 2) or ARE3-bearing (lane 3) or nonspecific control (lane 4) or ARE1-bearing (lane 5) oligonucleotides. E, Jurkat cell nuclear extracts were incubated with a 32P-labeled RNA oligonucleotide spanning ARE3 in the absence (lane 1) or presence of 20-fold excess of unlabeled (cold) ARE3-bearing (lane 2) or ARE3-bearing (lane 3) or nonspecific control (lane 4) or ARE1-bearing (lane 5) oligonucleotides. Jurkat cell nuclear extracts were incubated with 32P-labeled oligonucleotides spanning ARE3 in the absence (lane 6) or presence of anti-TTP antibody (lane 7), or anti-AUF1 antibody (lane 8) or an unrelated anti-NFATc antibody (lane 9). The asterisk in A, lane 2, denotes the supershifted RNA-protein complexes. Results are representative of three independent experiments.

HuR Regulates the Protein Expression of CD3ζ Chain—Because HuR bound to the oligonucleotides containing ARE2 and ARE3, and HuR is known to stabilize mRNAs containing ARE, we hypothesized that HuR regulates the expression of CD3ζ chain. To address this hypothesis, we used siRNA to knock down HuR in Jurkat cells and analyzed the effect on the expression of CD3ζ chain. Nonsilencing rhodamine siRNA was used as control. HuR was completely knocked down in the cytoplasm and was partially decreased in the nucleus (Fig. 4A). Moreover, there was a 44 ± 10% (p = 0.01) decrease in CD3ζ chain expression (Fig. 4B and C) upon knockdown of HuR. To confirm the biological relevance of HuR in the regulation of CD3ζ expression, we used human peripheral blood T cells for the overexpression of HuR with a HuR expression vector. Cells were transfected for 3, 6, and 24 h to determine the kinetics of

36-kDa band showed the presence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (5 peptide match), hnRNP A0 (three peptide match), and hnRNP A2/B1 (three peptide match). The 50-kDa band revealed a five peptide match with hnRNP G, whereas the 45-kDa band contained hnRNP A3 (seven peptide match), hnRNP AB (four peptide match), and hnRNP A2/B1 (three peptide match). Among these proteins, HuR is one of the best described proteins for its role in mRNA stability. Additionally, analysis of the CD3ζ 3′-UTR sequence predicted HuR-binding sites (28) in the vicinity of both the ARE2 and ARE3 regions (Fig. 1B, underlined and italicized). Hence we chose to analyze the role of this protein in regulating CD3ζ. Western blot with an HuR-specific antibody showed the presence of this protein in the eluate of the ARE2 oligonucleotide pulldown but not in the eluate of the control oligonucleotide (Fig. 2B). These data show that HuR may bind to the ARE2-containing region of CD3ζ 3′-UTR.

HuR Binds to the ARE2 and ARE3 Bearing Regions of the 3′-UTR of CD3ζ—Because our mass spectrometry results indicated a potential interaction between HuR and the ARE2-bearing region of the 3′-UTR of CD3ζ, we performed RNA electrophoretic mobility shift assays using ARE2, ARE3, and ARE1-containing oligonucleotides and Jurkat cell nuclear extracts. As seen in Fig. 3A, there was a specific band that could be supershifted upon addition of anti-HuR antibody (Fig. 3A, lane 2) but not with the addition of an unrelated NFATc antibody (lane 3). This indicated that HuR bound specifically to this ARE2-containing oligonucleotide. In a similar assay using ARE3-bearing oligonucleotide (Fig. 3B), the addition of HuR-specific antibody (lane 2) but not an unrelated NFATc antibody (lane 3) abolished the formation of the RNA-protein complexes as compared with lane 1, indicating the presence of HuR in these complexes as well. We performed the same assay using a radiolabeled ARE1-bearing RNA oligonucleotide and did not observe the formation of RNA-protein complexes (Fig. 3C).

We also found that the ARE2-binding protein complexes (Fig. 3D, lane 1) were outcompeted by the addition of excess cold ARE2 oligonucleotide (Fig. 3D, lane 2) and abolished by the addition of excess cold ARE3 oligonucleotide (Fig. 3D, lane 3). The addition of excess cold control (Fig. 3D, lane 4) or ARE1 (Fig. 3D, lane 5) oligonucleotides did not affect the ARE2-protein complexes. In a similar assay using radiolabeled ARE3 oligonucleotide (Fig. 3E, lane 1), we were able to abrogate the binding using excess cold ARE3 (Fig. 3E, lane 2) but not excess cold ARE2 oligonucleotides (Fig. 3E, lane 3). Again, the addition of excess cold control (Fig. 3E, lane 4) or ARE1 (Fig. 3E, lane 5) oligonucleotides did not alter the ARE3-protein complexes. These results indicate that HuR specifically binds to the ARE2 and ARE3 but not to the ARE1-bearing regions of CD3ζ 3′-UTR. The observed differences in the binding of HuR to the ARE2- and ARE3-bearing oligonucleotides in vitro may be due to differences in the tertiary conformations of these complexes.

Because HuR is known to compete with RNA-binding proteins that destabilize mRNA (such as AUFI), we examined whether ARE2 and ARE3 are binding sites for such proteins. We found that neither AUFI nor TTP (another RNA-destabilizing protein) bind to ARE3- (Fig. 3E, lanes 7 and 8) or ARE2 (data not shown)-bearing oligonucleotides.
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HuR plasmid expression. As shown in Fig. 5A, the HuR expression was at its highest level at 6 h after transfection. Importantly, CD3ζ chain levels as measured by Western blot were increased by 75 ± 25% (p = 0.01) in the HuR-transfected cells as compared with control plasmid-transfected cells (Fig. 5B). These results indicate that HuR positively regulates CD3ζ chain expression in T cells.

HuR Regulates the mRNA Stability of Wild-type but Not AS Form of CD3ζ—Because HuR was identified as a CD3ζ mRNA-binding protein and a regulator of CD3ζ protein expression, we asked whether CD3ζ mRNA stability is regulated by HuR. Because we showed that HuR binds to the ARE2 and ARE3 regions but not to the ARE1 region of CD3ζ mRNA, we also tested whether HuR affected the mRNA stability of the AS form of CD3ζ that lacks the ARE2 and ARE3. After transient transfection of primary T cells with an HuR expression vector or control plasmid for 6 h, cells were treated with the transcription inhibitor actinomycin D to prevent new RNA synthesis, and total mRNA was isolated 0, 1, 3, and 4 h later. Reverse transcription-PCR analysis showed very stable levels of CD3ζ mRNA even at 3 and 4 h post-actinomycin D in HuR-overexpressing cells (Fig. 5C, filled squares) as compared with the control plasmid-treated cells (Fig. 5C, open circles). On the other hand, HuR overexpression had no significant effect as expected on the mRNA expression levels of the AS form of CD3ζ (Fig. 5D). These data indicate that HuR positively regulates the mRNA stability of wild-type CD3ζ by binding to ARE2 and ARE3 regions in the 3′-UTR.

DISCUSSION

In this study, we have several novel findings. First we show that several putative proteins bind to the ARE2 bearing region of the 3′-UTR of CD3ζ. Second we show that the well known mRNA-stabilizing protein HuR binds to the ARE2 and ARE3 of CD3ζ 3′-UTR. Finally we demonstrate that HuR regulates protein as well as mRNA levels of CD3ζ in human T cells.

As we have recently shown, mutations in the ARE2 or ARE3 regions of the 3′-UTR reduce the mRNA stability and protein expression of CD3ζ (12); therefore, we postulated that proteins binding to this region may promote the integrity of CD3ζ mRNA. In our ARE2 oligonucleotide pulldown assay (Fig. 2), several proteins were isolated, including the ELAV like-1 protein, HuR, and various members of the hnRNP class of proteins. Because HuR is best known for its mRNA-stabilizing function, we chose to study the effect of this protein on CD3ζ stability. We found that siRNA knockdown of HuR decreased the CD3ζ protein expression, whereas HuR overexpression led to an increase in CD3ζ chain levels (Figs. 4 and 5). The effect of HuR on CD3ζ chain levels seems to be mediated via stabilization of the transcript (Fig. 5C).

Although our data show that HuR positively regulates CD3ζ expression, its effect on the levels of CD3ζ is partial but significant. One possibility is that the effect of actinomycin D inhibition on the transcription of the HuR-expressing plasmid results in the limited availability of HuR, and hence, at the later time points, the difference in CD3ζ mRNA levels between the two sets of cells is modest. It is also likely that HuR is not the only protein influencing the mRNA stability of CD3ζ and that other ζ mRNA-binding proteins cooperate with HuR to mediate this regulation. Our preliminary results from the oligonucleotide pulldown assay revealed that hnRNPs also may bind to the ARE2 bearing region of CD3ζ 3′-UTR. Because hnRNPs play various roles in mRNA metabolism, including splicing (29), stability (30), transport (31), and translation (32), we speculate that they may also be involved in the regulation of CD3ζ mRNA processing.

It has also been shown that the RNA-stabilizing protein HuR and -destabilizing protein AUF1 (or hnRNP D) can compete for binding the same transcript on common as well as distinct non-
overlapping sites and thus determine the fate of the target mRNA (15, 33, 34). Our results show that CD3ζ 3'UTR ARE2 and ARE3 are not binding sites for this protein. Nevertheless, further studies are needed to address whether CD3ζ mRNA is another such target for these two proteins with opposing function, and if a balance between the two determines the fate of the transcript. HuR is also involved in the regulation of translation (35, 36), and whether CD3ζ translation is also regulated by HuR is not known.

Although HuR expression levels have been correlated with advanced stages of malignancy, including cancers of the breast, colon, lung, and ovary (37–40), its role in autoimmune disease pathophysiology is unknown. Post-transcriptional mechanisms regulate a variety of T cell cytokines and signaling molecules, suggesting a role for mRNA stabilizing/destabilizing proteins in inflammatory conditions (41). In the case of T cells from patients with SLE, we and others (10, 42, 43) have shown that the mRNA levels of important signaling molecules such as CD3ζ and transcription factors such as c-Fos and cAMP-response element modulator are altered. It is therefore possible that post-transcriptional regulatory mechanisms play a role in the aberrant behavior of SLE T cells.

In the case of CD3ζ mRNA, SLE T cells express an abnormally spliced isoform that lacks critical cis regulatory regions resulting in increased decay of the message. Here, our data show that this may be due to the inability of this particular alternatively spliced isoform to bind HuR. In addition, HuR is known to regulate the mRNA stability of the transcription factor c-Fos, which is significantly reduced in SLE T cells, leading to decreased activator protein (AP)-1 transcriptional activity and IL-2 expression (42). At this point, we do not know whether the decreased levels of c-Fos are purely because of transcriptional mechanisms or whether the altered mRNA stability is also contributing. Therefore, further studies are needed to determine the potential role of HuR in SLE T cells.

Besides regulation of mRNA, HuR was recently identified in the supra-spliceosomes in endogenous pre-mRNA processing complexes purified from live HeLa cells (44), suggesting that HuR may also be involved in alternative splicing. Because the

**FIGURE 5.** HuR overexpression leads to increased CD3ζ protein expression by regulating mRNA stability. Peripheral blood T cells were transfected with 2 μg/10^6 cells of control plasmid (pcDNA) or a FLAG-tagged HuR-expressing plasmid (pHuR). A, 3, 6, and 24 h post-transfection, cells were harvested, and whole cell lysates were used in Western blots for HuR, FLAG epitope, and CD3ζ. Membranes were re-blotted for β-tubulin as loading control. B, quantitative analysis of 6 h of protein expression of HuR (left graph) and CD3ζ (right graph) expressed as a ratio to β-tubulin expression. Error bars represent mean ± S.E. C and D, 6 h post-transfection, cells were treated with actinomycin D (5 μg/ml), and cells were harvested 0, 1, 3, and 4 h later. Total RNA was reverse-transcribed, and the wild-type (C) and AS forms (D) of CD3ζ were amplified by quantitative real time PCR and semi-quantitative PCR and normalized to GAPDH. Results are shown from one representative experiment of three independent experiments. In three independent experiments, the expression of normalized WT CD3ζ was 1.7 ± 0.38-fold higher at 3 h and 2 ± 0.5-fold higher at 4 h in HuR-overexpressing cells as compared with control cells (C). The fold change in expression of normalized AS CD3ζ in three independent experiments was 1.1 ± 0.1, 1 ± 0.1, and 0.8 ± 0.35 at 1, 3, and 4 h, respectively, in HuR-overexpressing cells as compared with the control cells (D).
HuR Regulates CD3ζ Expression in Human T Cells

3′-UTR of CD3ζ undergoes alternative splicing in SLE T cells, leading to an increased expression of the short AS isoform, it is possible that HuR may be involved in this processing of CD3ζ mRNA. Interestingly, analysis of the sequence of CD3ζ 3′-UTR shows that the ARE2 region at position 705 that binds HuR is in close proximity to the upstream splice site at position 671–672. In conclusion, the mRNA-stabilizing protein HuR binds to the ARE2 and ARE3 bearing regions of CD3ζ 3′-UTR and partially regulates CD3ζ protein and mRNA expression. The absence of this HuR binding region in the AS CD3ζ mRNA predominantly expressed in SLE T cells may help explain its increased decay in these cells.

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