FasL Expression in Activated T Lymphocytes Involves HuR-mediated Stabilization

Gillian L. Drury‡, Sergio Di Marco§, Virginie Dormoy-Raclet§1, Julie Desbarats‡, and Imed-Eddine Gallouzi§2

From the §Department of Biochemistry and Rosalind and Morris Goodman Cancer Center and the ‡Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6, Canada

Received for publication, April 25, 2010, and in revised form, July 14, 2010. Published, JBC Papers in Press, July 30, 2010, DOI 10.1074/jbc.M110.137919

A prolonged activation of the immune system is one of the main causes of hyperproliferation of lymphocytes leading to defects in immune tolerance and autoimmune diseases. Fas ligand (FasL), a member of the TNF superfamily, plays a crucial role in controlling this excessive lymphoproliferation by inducing apoptosis in T cells leading to their rapid elimination. Here, we establish that posttranscriptional regulation is part of the molecular mechanisms that modulate FasL expression, and we show that in activated T cells FasL mRNA is stable. Our sequence analysis indicates that the FasL 3’-untranslated region (UTR) contains two AU-rich elements (AREs) that are similar in sequence and structure to those present in the 3’-UTR of TNFα mRNA. Through these AREs, the FasL mRNA forms a complex with the RNA-binding protein HuR both in vitro and ex vivo. Knocking down HuR in HEK 293 cells prevented the phorbol 12-myristate 13-acetate-induced expression of a GFP reporter construct fused to the FasL 3’-UTR. Collectively, our data demonstrate that the posttranscriptional regulation of FasL mRNA by HuR represents a novel mechanism that could play a key role in the maintenance and proper functioning of the immune system.

Fas and Fas ligand (FasL)3 are a transmembrane receptor ligand pair of the TNF receptor and TNF family, primarily involved in maintaining the homeostasis of the immune system by eliminating antigen-activated lymphocytes which consequently limits the magnitude and duration of the immune response (1). Fas/FasL mediates this effect by triggering an apoptotic response in these cells. This response involves recruiting the adaptor protein FADD to the intracellular tail of Fas via an interaction with a death domain. In turn, the FasL-Fas-FADD complex recruits procaspases 8 and 10 via homotypic death effector domain interactions leading to caspase cleavage and apoptosis (1). The importance of Fas and FasL is evidenced by the fact that defects in their expression trigger excessive lymphoproliferation resulting in loss of immune tolerance and autoimmune diseases (1–4). Although the Fas receptor is constitutively expressed in most tissues, FasL is restricted to activated lymphocytes and sites of immune privilege (1). This supports the idea that FasL but not Fas is the limiting factor in the Fas/Fasl-induced signaling pathways.

The disruption of Fas/Fasl signaling pathways by spontaneous mutations in mice or in human patients has been associated with diseases such as systemic lupus erythematosus or autoimmune lymphoproliferative syndrome (1, 3). Likewise, an increase in FasL-mediated apoptosis of normal, Fas-bearing, bystander cells causes certain immunopathologies such as hepatitis, which is linked to excessive T cell activation (1). Hence, a tight regulation of FasL expression during T cell activation is critical to maintain the homeostasis and the proper functioning of the immune system.

During the past decade, the majority of studies have focused on delineating the molecular mechanisms that modulate FasL expression at the transcriptional level. Several factors, such as NF-AT, NF-κB, and IRF-1, have been shown to activate the transcription of the fasl gene directly (5–7). It is well established for other TNF family members such as TNFα, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and several interleukins, that although transcription is tightly regulated, the amount of mRNA produced does not correlate with the protein expression levels (8). In many cases, although the steady-state levels of these messages remain unchanged, the protein levels increase significantly in response to extracellular stimuli (9, 10). This increase is because the expression of these mRNAs is also regulated posttranscriptionally at the level of subcellular localization, mRNA turnover, and translational efficiency. These posttranscriptional effects are mediated mainly by AU-rich elements (AREs) in the 3’-untranslated regions (3’-UTRs) of these and other messages (11–13). AREs are known to regulate a variety of transiently expressed cytokines during T cell activation. These include IFNγ, GM-CSF, CD83, TNFα, and CD40L (14–16). This regulation is due to the stabilization of these messages by a mechanism that involves their association with ARE-binding proteins such as HuR (12, 17, 18).

HuR belongs to the ELAV (embryonic lethal abnormal vision) family of RNA-binding proteins that contains three other members, HuB, HuC, and HuD (19). Of the four ELAV family members, only HuR is ubiquitously expressed, and it is particularly well expressed in primary and secondary lymphoid tissues such as the thymus, spleen, and the gut (20, 21).
Recently, it has been shown that in a tissue-specific knock-out mice, disrupting the hur gene in T lymphocytes causes a severe defect in their maturation (22). Indeed, although these mice have a wild type thymic microenvironment, the HuR+/− thymocytes of these mice are unable to undergo positive selection, negative selection, and thymic egress (22). The defect in positive selection is attributed to an alteration in the T cell receptor signaling pathway. Likewise, the defect in thymic egress can be explained by defects in chemokine signaling required in this process such as the TNF receptor family members including Fas (22).

The observations described above and the fact that FasL belongs to the TNFα family of cytokines raised the possibility that the expression of FasL could depend on posttranscriptional events involving ARE-binding proteins such as HuR. In this study we addressed this question and showed that the expression of FasL mRNA contains AREs strikingly similar in structure to those of TNFα. Our data demonstrate that via these AREs, FasL mRNA associates with HuR and that this association is absolutely required for its expression. We also discuss the functional relevance of posttranscriptional regulation of FasL and its impact on T lymphocyte maturation.

**EXPERIMENTAL PROCEDURES**

** Constructs**

The human Fasl 3’-UTR was PCR-amplified, and a 5’-BamHI site followed by stop codon and a 3’-HindIII site were introduced (for primer sequences see supplemental Materials and Methods). This PCR fragment was inserted into a pEGFP-C2 vector (Clontech) between the BglII and HindIII sites downstream of GFP. The GST and GST-HuR constructs were previously described (23). All plasmids were prepared using the plasmid maxiprep kit (Qiagen) according to the manufacturer’s instructions.

The fusion proteins were purified as described in (23, 24) with the following modifications. The proteins were eluted from the glutathione-agarose beads with three applications of 20 mM glutathione elution buffer (10 mM for the first elution, and 20 mM for the second and third elutions). Proteins were then dialyzed overnight against phosphate-buffered saline at 4 °C. The 20 mM glutathione eluates were the most pure (as determined by SDS-PAGE) and were used in all experiments.

**Cell Culture, Treatments, and Transfections**

Jurkat cells (E6 clone) (American Type Culture Collection (ATCC)) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) augmented with 10% FBS (Invitrogen). Jurkat cells were stimulated with PHA (Sigma) at 1 µg/ml or at 50 ng/ml for the times indicated. RNA stability curves were generated by treatment of cells with actinomycin D (Sigma) at 5 µg/ml. Transfections were performed in 12-well plates using 1 µg of plasmid DNA and TransPass RV (New England Biolabs) according to the manufacturer’s instructions.

HEK 293 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Multicell) supplemented with 10% FBS (Sigma). HEK 293 cells were stimulated with 50 ng/ml PMA for the times indicated. Transfections were performed in 10-cm² dishes with 8–16 µg of plasmid DNA and Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Transfections of siRNA were performed with 60 nM duplexes (siHuR or siCtrl)/10-cm² cell culture dish, using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions (25). HEK 293 cells were transfected at 20% confluence then retransfected with siRNA 24 h later. Knockdown was assessed 52 h after the first transfection.

**RNA Immunoprecipitation and RT-PCR**

**Preparation of mRNA (mRNP) Complexes—Immunoprecipitation and RNA preparation were performed as previously described (26) with several modifications. Briefly, cell extracts were prepared from PHA-stimulated Jurkat cells or PMA-stimulated HEK 293 cells. mRNP lysate (400 µl) was precleared with 8 µl of protein G-Sepharose, and the precleared lysate was subsequently divided for each immunoprecipitation (IP). IPs were performed using antibodies to HuR (3A2) and IgG1 isotype control antibody (Sigma). Specific messages associated with HuR were defined using RT-PCR.

RT-PCR—RNA was isolated from the immunoprecipitated mRNP complexes using the ChargeSwitch RNA isolation kit (Invitrogen) scaled to 20% of the manufacturer’s instructions. Purified RNA was eluted in 30 µl of water, and 4 µl was reverse-transcribed using the Senscript reverse transcription kit (Qiagen) according to the manufacturer’s protocol in 20 µl of final volume. Subsequently, 2 µl of cDNA was PCR-amplified with HotStarTaq (Qiagen) using actin, FasL, or GFP cDNA-specific primers (see supplemental Materials and Methods). The sequences of the primers, as well as the PCR conditions, are described in the supplemental Materials and Methods.

**Gel Shift**

The FasL RNA probes were produced by in vitro transcription as described previously (27). Regions of Fasl 3’-UTR were PCR-amplified with sense primers containing the T7 promoter in 5’ (see supplemental Materials and Methods for primer sequences). The purified PCR fragment was used as a template for transcription using [32P]UTP with T7 RNA polymerase (Promega) according to the manufacturer’s instructions. The RNA binding assay was performed with purified 300 ng of GST and GST-HuR as described previously (18) except that upon incubation the RNA-protein complex was not treated with RNase T1.

**Northern Blotting**

Northern blot analysis was performed as described (28). 15 µg of RNA was used and was isolated with the RNase Plus extraction kit (Qiagen) according to the manufacturer’s instructions. After transferring to a Hybond-N membrane (Amersham Biosciences) and UV cross-linking, the blot was hybridized with GFP or 18 S rRNA probes prepared with [32P]dCTP by random priming with Ready-to-go DNA labeling beads (GE Healthcare) according to the manufacturer’s instructions. PCR-amplified fragments of GFP and 18 S rRNA were used to generate labeled probes. After hybridization, the membranes were washed and subsequently exposed on BioMax films (Kodak).
**HuR Posttranscriptionally Regulates FasL mRNA**

**Fluorescence-activated Cell Sorting**

Cells were counted by using a hemocytometer and adjusted to a concentration of $1 \times 10^6$ cells/ml. 0.5 ml of cells was washed three times in 1.5 ml of PBS and then resuspended in 0.5 ml of PBS with 2% FBS. Acquisition of data was done on a FACScan (Becton Dickinson), and the analysis of these data was performed with Flowjo software.

**Western Blotting**

Total cell extracts were prepared as described (27). Western blotting was performed as described previously (23). The blots were probed with antibodies to HuR (3A2), tubulin (Developmental Studies Hybridoma Bank), and GFP (JL8; Clontech).

**Immunofluorescence**

Immunofluorescence was performed as described previously (29). Anti-HuR (3A2) was used at a 1:1500 dilution in 1% goat serum/PBS. HuR was detected using a 1:500 diluted Alexa Fluor 488-conjugated goat anti-mouse IgG polyclonal antibody. DAPI staining 1:20,000 was performed after secondary antibody. A Zeiss Axiovision 3.1 microscope was used to observe the cells with a 63 $\times$ oil objective, and an Axiocam HR (Zeiss) digital camera was used for immunofluorescence photography.

**Quantitative PCR**

RNA was isolated for quantitative RT-PCR by using the ChargeSwitch RNA isolation kit (Invitrogen) scaled to 20% of the manufacturer’s instructions. RNA was quantitated using the Ribogreen kit (Molecular Probes), and 150 ng was reverse transcribed using the Sensiscript reverse transcription kit (Qiagen). Subsequently, the cDNA was PCR-amplified with primers described in supplemental Materials and Methods, using the Quantitect SYBR Green kit (Qiagen) in a Corbett Rotor Gene 31132

**RESULTS**

**FasL mRNA Has a Short Half-life in Activated T Cells**—Previous reports have shown that FasL mRNA is rapidly induced in T lymphocytes upon T cell receptor engagement and mitogen stimulation (1). To define whether this up-regulation was associated with a stabilization of the FasL mRNA, we first assessed its steady-state levels in T cells exposed to various activators. Jurkat T cells were treated with either PHA, a lectin that non-specifically aggregates cell surface receptors or PMA, a PKC agonist (1, 30). We observed a rapid increase in the level of FasL mRNA at 3 h of PHA treatment. In contrast, PMA treatment had a smaller effect on the steady-state levels of FasL mRNA (Fig. 1A). Interestingly, levels of FasL mRNA return to baseline within 6–12 h of PHA stimulus, indicating that FasL is transiently expressed in response to lectins similarly to other cytokines. In the absence of PHA or PMA, however, the FasL mRNA was hard to detect (Fig. 1A). Next, we determined the half-life of FasL mRNA under these conditions. We performed actinomycin D pulse-chase experiments (31) and determined the half-life of the FasL mRNA using quantitative RT-PCR analysis. Jurkat cells were treated with PHA or PMA for 3 h to induce maximal FasL mRNA expression and then treated with 5 $\mu$g/ml actinomycin D (ActD) treatment for 0, 15, 45, 60, 120, and 240 min. The half-life of FasL mRNA is indicated by the 50% line and corresponds to $-60$ min upon PHA and $>240$ min upon PMA. Fas ligand mRNA was detected by quantitative RT-PCR and was normalized to GAPDH mRNA expression as described above.

**3'-UTR of FasL mRNA Binds to HuR in an ARE-dependent Manner**—The stabilization of cytokine mRNAs is usually mediated by U-rich elements such as AREs located in their 3'-UTR...
HuR Posttranscriptionally Regulates FasL mRNA

Due to the effects of PMA or PHA treatment on FasL expression (Fig. 3C), however, regions 5 and 6, which are particularly U-rich (supplemental Fig. 2A), show a better binding to HuR (Fig. 3C, compare lanes 15 and 18 with lanes 3, 6, 9, and 12). We also showed that this interaction is competed away by an excess of the same unlabeled probes (Fig. 3D) but not with an excess of unlabeled R2 probe (supplemental Fig. 3).

Next, we mapped regions 5 and 6 more precisely, to determine the number of HuR binding sites in these regions. Regions 5 and 6 of the FasL 3′-UTR were further subdivided into six 50-nucleotide subregions (Fig. 4A). RNA electromobility shift assay experiments as described above showed strong interactions between HuR and some of the subregions, notably subregions 5.2, 5.3, 6.1, 6.2, and 6.3 (Fig. 4B). Therefore, our results indicate that there are at least four distinct U-rich HuR binding sites in regions 5 and 6 in addition to the weaker binding sites in regions 1, 3, and 4 (Fig. 3C). Furthermore, because the binding of HuR to these fragments can be competed by the same unlabeled probes (Fig. 4C), these interactions seem to be specific. Together, these observations argue that HuR binds directly to the 3′-UTR of FasL in an ARE-dependent manner.

HuR Protein Is Required for Expression of an mRNA Containing FasL 3′-UTR—The data described above suggest that ARE sequences could collaborate with HuR to ensure the rapid expression of FasL mRNA during T cell activation. Hence, we assessed whether the expression of FasL mRNA depends on HuR in cells treated with activators such as PMA or PHA. Ideally, we would have liked to test this possibility in the context of the full-length FasL message by following its expression in the presence or absence of HuR. However, expressing the full-length FasL mRNA caused massive cell death in different cell lines, including Jurkat cells regardless of stimulus (data not shown). Therefore, we used GFP reporter constructs in which we fused the FasL 3′-UTR to the GFP coding sequence. The entire FasL 3′-UTR was included in the reporter construct because any or all of the HuR binding sites described in Figs. 3 and 4 could mediate regulatory effects. Surprisingly, flow cytometry experiments showed that the level of expressed GFP was reduced by >55% in untreated Jurkat cells transfected with GFP-FasL 3′-UTR compared with GFP alone (Fig. 5A). This is probably due to other RNA-binding proteins which, in the absence of any stimulus, bind the FasL 3′-UTR in trans and promote its rapid decay. To eliminate the possibility that poor transfection efficiency in Jurkat cells led to the evaluation of a selected population, we transfected HEK 293 cells with the same constructs and obtained similar results by flow cytometry (Fig. 5B). In agreement with GFP protein levels, Northern blot analysis showed that in the absence of any treatment, the amount of GFP-FasL 3′-UTR mRNA in HEK 293 cells was significantly decreased compared with the GFP control (Fig. 5, C and D). These experiments suggest that in the absence of extra-cellular stimulus, the FasL 3′-UTR mediates the rapid decay of the GFP reporter mRNA.

Due to the effects of PMA or PHA treatment on FasL expression, we investigated whether these actions were recapitulated on the expression of the GFP-conjugated FasL 3′-UTR. The GFP-FasL 3′-UTR or GFP plasmids were transfected into HEK 293 cells which were then treated or not with PMA. It is well established that HEK 293 cells activate the PKC pathway in...
HuR Posttranscriptionally Regulates FasL mRNA

A Immunoprecipitation

|          | UV: |
|----------|-----|
| IgG      | +   |
| αHuR     | -   |
| β-actin  |     |
| FasL     |     |

B RT-PCR

|          | 1 | 2 | 3 | 4 |
|----------|---|---|---|---|
| FasL 3'UTR | 850 bp |

C Probe:

| GST: | R1 | R2 | R3 | R4 | R5 | R6 |
|------|----|----|----|----|----|----|
| -    | +  | -  | -  | -  | -  | -  |
| -    | +  | -  | -  | -  | -  | -  |
| GST-HuR: | - | - | - | - | - | - |

D Radio-labeled Probes:

| GST: | R2 | R5 | R6 |
|------|----|----|----|
| +    | -  | -  | -  |
| GST-HuR: | - | - | - |
| Cold Probes: | - | - | - |

HuR-C

Free Probe

FIGURE 3. Fas ligand mRNA associates with HuR via U-rich sequences in the 3'-UTR. A, Jurkat cells stimulated with 1 μg/ml PHA for 2 h were either exposed to UV for 4 min (34) or not. These cells were then used to prepare total cell extracts. HuR was immunoprecipitated from Jurkats with the anti-HuR (3A2) antibody (lanes 2 and 4). A mouse IgG1 antibody was used as an isotype-matched specificity control (lanes 1 and 3). IP was followed by RT-PCR for FasL (upper) and the β-actin mRNA (lower) that was used as a positive control. Representative blots of two independent experiments are shown. B, diagram indicates the location and length of probes for the gel shift assay. C, HuR associates with regions R1 (lane 3), R3 (lane 9), R4 (lane 12), R5 (lane 15), and R6 (lane 18) of the Fasl 3'-UTR. Gel shift binding assays were performed by incubating 300 ng of purified GST or GST-HuR protein with radiolabeled probes (see B). Radiolabeled probe-GST-HuR complexes (HuR-C) are indicated with an asterisk. D, increasing concentrations of unlabeled probes for the regions R5 and R6 were incubated with GST-HuR in the presence of radiolabeled probes from the same regions. HuR-Cs are indicated by asterisks. GST was incubated with radiolabeled R2 (lane 1), R5 (lane 4), and R6 (lane 12) probes as negative controls. These gel shifts were performed with 300 ng of purified GST or GST-HuR and with 0.01 × (lanes 6 and 14), 0.1 × (lanes 7 and 15), 1 × (lanes 8 and 16), 10 × (lanes 9 and 17), and 100 × (lanes 10 and 18) amounts of unlabeled probe. C and D, representative gel shift blots of two independent experiments are shown.

response to PMA but not to PHA (35). Additionally, our stability experiments presented in Fig. 1B clearly showed that PMA has a much stronger stabilizing effect on the half-life of the FasL mRNA than PHA. Therefore, to mimic the effect seen in activated Jurkat cells, we decided to treat the HEK 293 cells with absence of PMA. We were able to deplete HuR expression by >55% (Fig. 6E). Our experiments showed that in these HuR-depleted cells, PMA treatment did not affect GFP expression; however, it failed to induce the expression of GFP-Fasl 3'-UTR mRNA (Fig. 6, F and G). It is interesting to note that the steady-
HuR Posttranscriptionally Regulates FasL mRNA

A

FasL 3′-UTR 850 bp

R5 (160bp) | R6 (150bp)

R5.1 | R5.2 | R5.3 | R6.1 | R6.2 | R6.3

B

| Probe: |
|-------|
|      |
| R5.1 |
| R5.2 |
| R5.3 |
|      |

| GST: |
|      |
|      |

| GST-HuR: |
|         |
|         |

| HuR-C |
|       |

| Free probe |
|           |

| Gel Shift |
|          |
|          |

C

| Radio-labeled Probes: |
|----------------------|
|                      |
| R5.2 |
| R5.3 |
| R6.2 |
| R6.3 |

| GST: |
|      |
|      |

| GST-HuR: |
|         |
|         |

| Cold Probes: |
|             |
|             |

| HuR-C |
|       |

| Free Probe |
|           |

| Gel Shift |
|          |
|          |

FIGURE 4. HuR binds to specific U-rich regions of the FasL mRNA. A, diagram indicates the location and length of probes for gel shift assay. B, HuR forms complexes (HuR-C) with regions 5.2 (lane 6), 5.3, (lane 9), 6.1 (lane 12), 6.2 (lane 15), and 6.3 (lane 18), indicated by asterisks. Gel shift binding assay was performed by incubating 300 ng of purified GST or GST-HuR protein with radiolabeled probes as indicated. C, HuR-Cs (*) formed with radiolabeled R.5.2, R.5.3, R.6.2, and R.6.3 were competed away with the same unlabeled probes. These gel shifts were performed with 300 ng of purified GST or GST-HuR, [32P]UTP-labeled probes as described above and with 0.01 × (lanes 4, 12, 20, and 28), 0.1 × (lanes 5, 13, 21, and 29), 1 × (lanes 6, 14, 22, and 30), 10 × (lanes 7, 15, 23, and 31), and 100 × (lanes 8, 16, 24, and 32) amounts of cold probe. B and C, representative gel shift blots of two independent experiments are shown.

state levels of GFP mRNA alone seem also to depend on HuR expression in the presence or absence of PMA treatment (Fig. 6F, compare lanes 5 and 6 with lanes 9 and 10). These observations indicate that HuR is indeed required for the increased expression of GFP-FasL 3′-UTR mRNA in PMA activated HEK 293 cells.

DISCUSSION

Regulation of cytokine mRNAs by posttranscriptional mechanisms at the level of splicing, subcellular localization, stability, and translational efficiency is requisite for their appropriate expression (38). However, this level of regulation has never been investigated for FasL. In this study, we demonstrate that the expression of the FasL mRNA is regulated posttranscriptionally by a mechanism that involves the HuR protein. The molecular mechanisms behind these effects are still unknown. Our observations suggest that although FasL and TNFα mRNAs have little sequence similarity in the first ARE, the secondary structure of the hairpin required for the recruitment of ARE-binding proteins (33) is conserved in addition to considerable sequence and structural homology in the second ARE (Fig. 2). This indicates that these two TNF superfamily members could be posttranscriptionally regulated by similar mechanisms. Indeed, consistent with TNFα, our study shows that HuR associates strongly and specifically with two regions of the FasL 3′-UTR which span both putative AU-rich regulatory regions, including the fragment (6.2) which contains the AUUUA pentamer. Although this suggests that HuR regulates the half-life of FasL mRNA via association with these two AREs, the fact that HuR also interacts with other U-rich elements in the FasL 3′-UTR harbors two ARE sequences that are similar in structure and U content to the TNFα AREs. Similarly to the TNFα message, HuR directly binds to FasL mRNA in an ARE-dependent manner. This binding depends on the activation of PKC-induced pathways that in turn trigger the rapid cytoplasmic accumulation of HuR leading to the expression of FasL mRNA. Therefore, collectively, our data support a model whereby HuR protein plays a key role in the transient expression of FasL mRNA in response to PKC activators. This suggests that HuR could be involved in T lymphocyte activation and selection which directly affects the homeostasis of the lymphoid system and the duration of the immune response.

HuR is typically involved in relocating, stabilizing, and modulating the translational efficiency of ARE-containing mRNAs. In the case of TNFα, there are two AREs in the 3′-UTR. Interestingly, these AREs mediate distinct posttranscriptional effects on TNFα message (18, 33). The first ARE binds HuR and is responsible for LPS-mediated TNFα expression. Although normally this region mediates the destabilization and the translational repression of TNFα mRNA in macrophages, upon LPS stimulus, this ARE also allows for the stabilization of the TNFα message via HuR (18, 39). Although it has been suggested that ARE2 modulates TNFα mRNA expression and protein abundance (18, 39), the molecular mechanisms behind these effects are still unknown. Our observations suggest that although FasL and TNFα mRNAs have little sequence similarity in the first ARE, the secondary structure of the hairpin required for the recruitment of ARE-binding proteins (33) is conserved in addition to considerable sequence and structural homology in the second ARE (Fig. 2). This indicates that these two TNF superfamily members could be posttranscriptionally regulated by similar mechanisms. Indeed, consistent with TNFα, our study shows that HuR associates strongly and specifically with two regions of the FasL 3′-UTR which span both putative AU-rich regulatory regions, including the fragment (6.2) which contains the AUUUA pentamer. Although this suggests that HuR regulates the half-life of FasL mRNA via association with these two AREs, the fact that HuR also interacts with other U-rich elements in the FasL
**HuR Posttranscriptionally Regulates FasL mRNA**

**FIGURE 5. FasL 3’-UTR decreases the mRNA and protein levels of a GFP reporter in both Jurkat and HEK 293 cells.** A and B, Jurkat (A) and HEK 293 (B) cells were transfected with pEGFP-C2 control and pEGFP-C2-FasL 3’-UTR. Flow cytometric analysis of GFP expression was performed 24 h after transfection. C, RNA was isolated from the pEGFP-C2- lane 1 and pEGFP-C2-FasL 3’-UTR- lane 2 transfected HEK 293 cells for Northern blotting for GFP and 18 S rRNA control. D, levels of GFP mRNA were quantified using the ImageQuant software program (Molecular Dynamics). Levels were then standardized against 18 S levels and plotted as the percentage ± the S.D. (error bar) of three independent experiments.

3’-UTR (Fig. 3) argues that association with multiple regions could also be required for the HuR-mediated activation of FasL mRNA expression. The expression of the TNFα mRNA in macrophages (38) may likewise parallel the up-regulation of FasL mRNA expression mediated by HuR driven by mitogenic stimulus that activates posttranscriptional mechanisms such as mRNA relocation and/or stabilization.

Here, we show that PKC activation correlates with the transient expression of FasL mRNA (Fig. 6). Cell treatments with the PKC agonist PMA lead to the expression of FasL or the GFP-FasL 3’-UTR mRNAs in a mechanism that involves their association with HuR in an ARE-dependent manner. This is consistent with previous reports showing that HuR is phosphorylated by PKCα at serines 158 and 221 (40). Interestingly, Ser221 is located within the hinge region of HuR that is known to regulate its nucleocytoplasmic shuttling (29). The activation of PKC has been recently tied to the relocation of HuR to the cytoplasm and the stabilization of target mRNAs in this subcellular compartment (41). This is concordant with previous findings, showing that T cell activation via PKC pathways triggers the HuR-mediated cytoplasmic translocation of the CD83 mRNA, and this involves the phosphorylation of the HuR protein ligand APRIL (15). Our results raise the possibility that FasL mRNA could be regulated the same way because PKC activation causes HuR to relocalize to the cytoplasm, and this correlates with an increase in its association with a reporter mRNA fused to the FasL 3’-UTR (Fig. 6, C and D). Indeed, protein ligands such as pp32 and APRIL have been shown to regulate the export of HuR and some of its mRNA targets in different cell systems (41). Hence, the PKC-mediated phosphorylation of HuR may potentially provide a mechanism for FasL mRNA not only to be stabilized but also to be rapidly translocated to the translation machinery in the cytoplasm for protein synthesis. Exploring the implication of HuR protein ligands such as pp32 and APRIL in regulating the cellular movement of FasL mRNA in activated T cells could help us better understand how the expression of this message is modulated during normal conditions and during the activation of an immune response.

Although there is currently some controversy toward the functions of FasL in the immune system (1), there is a consensus that the Fas/FasL signaling pathway is required for immune tolerance and homeostasis as evidenced by natural mouse mutants and autoimmune lymphoproliferative syndrome patients (1).

In addition, there have been several studies linking defects in Fas induced apoptosis to autoimmune diseases such as systemic lupus erythematosus. One study in particular links the number of microsatellite repeats in the FasL 3’-UTR to systematic lupus erythematosus (42). Interestingly, this microsatellite repeat is located in region 5.2, which we show binds HuR very strongly (Fig. 4). Thus, it is possible that HuR-mediated posttranscriptional regulation of FasL is important for the maintenance of immune tolerance.

Our observations indicate that despite the high level of expression of HuR protein in unstimulated cells, the FasL is rapidly degraded (Fig. 1). It is possible that under these conditions, the FasL mRNA remains in the nucleus where it associates with factors known to promote the AU-rich-mediated mRNA decay pathway (43). There are several well-characterized activators of AU-rich-mediated mRNA decay that are members of the CCCH zinc finger protein family, which includes proteins such as tristetraprolin (TTP), butyrate response factor 1 (BRF1), and KH-type splicing regulatory protein (KSRP) (44–47). It has been shown that under different growth conditions HuR competes for binding to AREs with KSRP and TTP proteins (41). In addition, HuR has been described as a key player in the transfer of some of its target transcripts from processing bodies (a site of mRNA decay) to polysomes (a site of translation) (48). Thus, it is possible that in
FIGURE 6. Expression of the GFP-FasL 3′-UTR mRNA and protein in conditions of PKC activation is mediated by HuR. A, HEK 293 cells were transfected with either GFP (filled bars) or GFP-FasL 3′-UTR (open bars). These cells were treated for 4 h with 50 ng/ml PMA 16 h after transfection. GFP protein expression was assessed by flow cytometry, and the values that are relative to GFP expression in untreated cells were plotted as the percentage ± the S.E. (error bars) of three independent experiments. B, HEK 293 cells were transfected with plasmids expressing GFP or GFP-FasL 3′-UTR as described in A. Total mRNA from untreated (0) (lanes 1 and 6) or cells treated with 50 ng/ml PMA for 2, 4, 6, and 8 h (lanes 2-5 and 7-10) were harvested and used for Northern blot analysis with specific radiolabeled DNA probes against GFP mRNA and 18 S as loading control. Representative blots of three independent experiments are shown. C, HEK 293 cells were left untreated (panels 1 and 2) or treated for 4 h with 50 ng/ml PMA (panels 3 and 4), fixed, permeabilized, and stained with 3A2 (anti-HuR) followed by anti-mouse conjugated to Alexa Fluor 488 (panels 1 and 3) and DAPI (panels 2 and 4). A single representative field for each cell treatment of three independent experiments is shown. Scale bars, 20 μm. D, RNA IP of extracts from HEK 293 cells stimulated with PMA 50 ng/ml for 1 (lanes 1 and 5), 2 (lanes 2 and 6), 4 (lanes 3 and 7), and 8 (lanes 4 and 8) h with 3A2 (anti-HuR) (lanes 5–8) antibody or mouse IgG1 isotype control (lanes 1–4). Immunoprecipitation was followed by RT-PCR for GFP (upper) and β-actin (lower) as a positive control. E and F, HEK 293 cells were mock-, siHuR-, or control siRNA (siCtrl)-transfected at 0 and 24 h. This was followed by GFP or GFP-FasL 3′-UTR transfection and PMA treatment for 4 h. E, total cell extracts from HEK 293 cells treated with siRNA and transfected with GFP-FasL 3′-UTR and treated or not with PMA as described above were collected and used for Western blotting with the anti-HuR and tubulin antibodies. F, total mRNA was prepared from the same samples described in E or from cells transfected with GFP plasmid and was used for Northern blot analysis with GFP and 18 S rRNA radiolabeled probes. G, levels of GFP mRNA were quantified and standardized as described in Fig. 5D. Levels for each treatment were plotted as the percentage ± S.D. (error bars) of two independent experiments.
HuR Posttranscriptionally Regulates FasL mRNA

stimulated T cells, where PKC pathways are activated, binding of HuR to the FasL mRNA is favored, leading to its stabilization and rapid translation. Therefore, assessing the implication of proteins such as TTP, BRF1, and KSRP in regulating FasL mRNA expression before and after stimulation and defining how their function could be counterbalanced by HuR could provide new strategies to control T lymphocyte responses under normal and or pathological conditions. This may lead to the identification of novel therapeutic targets/tools for the treatment of autoimmune diseases.

Acknowledgment—We are grateful to Xian Jing Lian for technical assistance.

REFERENCES

1. Strasser, A., Jost, P. J., and Nagata, S. (2009) *Immunity* 30, 180–192
2. Karray, S., Kress, C., Cuvellier, S., Hue-Beauvais, C., Damotte, D., Babinet, C., and Lévi-Strauss, M. (2004) *J. Immunol.* 172, 2118–2125
3. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992) *Nature* 356, 314–317
4. Senju, S., Negishi, I., Motoyama, N., Wang, F., Nakayama, K., Nakayama, K., Lucas, P. J., Hatakeyama, S., Zhang, Q., Yonehara, S., and Loh, D. Y. (1996) *Int. Immunol.* 8, 423–431
5. Chow, W. A., Fang, J. L., and Krammer, P. H. (1999) *Eur. J. Immunol.* 29, 3017–3027
6. Beutler, B., Korchin, N., Milsark, I. W., Luedke, C., and Cerami, A. (1986) *Science* 232, 977–980
7. Jacob, C. O., Lee, S. K., and Strassmann, G. (1996) *J. Immunol.* 156, 3043–3050
8. Jacobs, D. B., Mandelin, A. M., 2nd, Giordano, T., Xue, I., Malter, J. S., Jacobs, D. B., Mandelin, A. M., 2nd, Giordano, T., Xue, I., Malter, J. S., and Eberhardt, W. (2007) *Cell Biol. 20* 2165–2173
9. von Roretz, C., and Gallouzi, I. E. (2008) *J. Cell Biol.* 181, 189–194
10. von Roretz, C., and Gallouzi, I. E. (2008) *J. Cell Biol.* 181, 189–194
11. von Roretz, C., and Gallouzi, I. E. (2008) *J. Cell Biol.* 181, 189–194
12. Katsanou, V., Dimitriou, M., and Kontoyiannis, D. L. (2006) *Ernst Schering Found. Symp. Proc.* 37–57
13. Ford, L. P., Watson, J., Keene, J. D., and Wülsz, J. (1999) *Genes Dev.* 13, 188–201
14. Ford, L. P., Watson, J., Keene, J. D., and Wülsz, J. (1999) *Genes Dev.* 13, 188–201
15. Ford, L. P., Watson, J., Keene, J. D., and Wülsz, J. (1999) *Genes Dev.* 13, 188–201