Selective Cytoplasmic Translocation of HuR and Site-specific Binding to the Interleukin-2 mRNA Are Not Sufficient for CD28-mediated Stabilization of the mRNA*

Received for publication, November 10, 2003, and in revised form, January 28, 2004
Published, JBC Papers in Press, March 12, 2004, DOI 10.1074/jbc.M312306200

Yuko Seko‡, Hooman Azmi, Robert Fariss§, and Jack A. Raghets
From the Laboratory of Immunology and the Biological Imaging Core, NEI, National Institutes of Health, Bethesda, Maryland 20892

The interleukin-2 mRNA is a labile transcript containing AU-rich elements that is transiently stabilized by CD28 receptor signaling. For a number of proto-oncogenes and cytokines, the HuR protein has been shown to avidly bind the AU-rich elements that confer instability upon their mRNAs. HuR was originally thought to participate in mRNA degradation but subsequent studies indicated that it actually functions to stabilize mRNA. Binding of HuR to the mouse interleukin-2 mRNA has not been studied. We tested if HuR binds the interleukin-2 mRNA and whether such binding is related to CD28-mediated stabilization of the mRNA. First, we confirm that T cell receptor signaling, which is sufficient to induce interleukin-2 transcription, also triggers translocation of HuR from the nucleus to the cytoplasm. Interestingly, T cell receptor-triggered translocation is selective as heterogeneous nuclear ribonucleoprotein A1 does not shuttle under the same conditions. Engagement of both the T cell and CD28 receptors, which enhance interleukin-2 transcription and induce stabilization of the mRNA, did not further increase the level of cytoplasmic HuR. Using an in vitro binding assay, we demonstrate that HuR binds the interleukin-2 mRNA and localize binding to a sequence downstream of the single nonameric AU-rich element that is present in its 3′-untranslated region. However, we conclude that HuR binding to the interleukin-2 mRNA, both in vitro and in vivo, is not associated with alterations in mRNA stability.

It has long been recognized that post-transcriptional control of cytokine and proto-oncogene expression is an important facet of their regulation. The rapid decay of many cytokine and proto-oncogene mRNAs is dependent upon AU-rich elements (AREs) present within their 3′-untranslated region (UTR) (1, 2). Several cytokine mRNAs also appear to contain ARE independent destabilizing sequence elements (3). The interleukin (IL-2) 2 mRNA, which encodes a key regulatory cytokine and the principal mitogen of the immune system, contains an ARE in its 3′-UTR (4–6). The stability of the IL-2 mRNA, like that of several lymphokine mRNAs, is regulated in vivo through CD28 receptor stimulation (4, 6–9). CD28-mediated stabilization of the IL-2 mRNA is transient, with the mRNA subsequently decaying at a rate similar to that observed in the absence of CD28 signaling. CD28 receptor signaling also up-regulates transcription of the IL-2 gene. Because transcription of IL-2 is NF-AT dependent, the calcineurin inhibitor cyclosporin A (CSA) completely blocks both TCR-induced and CD28 up-regulated transcription (4, 9, 10). By contrast, CD28-mediated stabilization of the IL-2 mRNA appears to have both CSA-sensitive and -resistant components, which may represent an interspecies difference between mouse and human (4, 6, 9). In human T cells, the IL-2 mRNA-binding proteins nucleolin, YB-1, and NF90 appear to be components of a CSA-sensitive CD28 pathway that is in part mediated through JNK activation (11–13). By contrast, in mouse T cells, p38 rather than JNK appears to be activated in response to CD28 signaling (14). This finding is supported by the observation that the JNK1/2 knockout mouse does not have a defect in IL-2 production (15). To date, no RNA-binding proteins have been identified that participate in the CSA-resistant CD28 pathway.

HuR was one of the first RNA-binding proteins shown to increase mRNA stability (16–19). It is expressed in a wide variety of cell types, including T lymphocytes where HuR nucleocytoplasmic shuttling is triggered by T cell activation (20, 21). Originally shown to bind the ARE present in the 3′-UTR of the c-fos and IL-3 mRNAs, HuR was subsequently found to bind the ARE of a number of critically regulated mRNAs (22). Initially, HuR was thought to participate in mRNA degradation (23, 24). However, prompted by the report that HuB stabilizes the GLUT1 mRNA, subsequent studies utilizing overexpression of sense or antisense HuR constructs in transfected cell lines indicated that HuR is actually involved in mRNA stabilization (16–19, 25–29).

HuR is not known to bind the IL-2 mRNA, but its affinity for AREs, ability to stabilize several other cytokine mRNAs, and its cytoplasmic translocation upon T cell activation suggested that it potentially could participate in the regulation of IL-2 mRNA half-life. We have now examined the role of HuR in the CSA-resistant, CD28-mediated stabilization of IL-2 mRNA in mouse T cells. To do so using an in vivo system unperturbed by manipulation of HuR expression or the use of transcriptional inhibitors, we examined HuR binding to the IL-2 mRNA in AE7 cells, a normal CD4+ T cell clone. These cells do not proliferate spontaneously; they must be induced to divide by stimulation through the TCR and CD28 receptors. Proliferation is transient and the cells subsequently enter a resting state until they are again stimulated. The IL-2 gene is not
HuR Binds the IL-2 mRNA

expressed in resting AE.7 cells but is rapidly induced by stimulation through the TCPR. CD28 signaling stabilizes the IL-2 mRNA, levels of which rise for several hours following stimulation and then plateau before declining and eventually disappearing (4, 9, 10). These characteristics make AE.7 cells an ideal model system for the study of both T cell activation and IL-2 mRNA stability.

Whereas HuR-H11032 was expressed in both resting and activated T cells, we find that it is selectively translocated from nucleus to cytoplasm in response to TCPR but not CD28 signaling. HuR binds the IL-2 mRNA both in vitro and in vivo but not to the nonameric ARE within the 3'-UTR. We observe that HuR binding to the IL-2 mRNA in vitro reflects differences in the steady state level of the mRNA and find no evidence to support a role for HuR binding in the CD28-mediated stabilization of the mRNA.

EXPERIMENTAL PROCEDURES

Cell Culture and Stimulation—AE.7, a normal CD4+ Th1 mouse T cell clone (30) was used for all experiments. Maintenance, stimulation, and CSA treatment of AE.7 cultures were performed as described previously (4). In brief, antibody stimulations were carried out following Ficoll gradient purification of resting cells and resuspension in fresh media at 1 × 10^6/ml. Antibodies were titrated and used at a concentration that elicited maximal IL-2 secretion from the AE.7 cells. Costar plates (Costar, Cambridge, MA) were coated overnight at 37 °C with 0.5 μg/ml of the anti-TCPRβ antibody. The anti-CD28 antibody was added (i.e. –1:1000) directly to the costimulated cell samples. Establishment of the stably transfected IL2XAC AE.7 line was described previously (4).

Antibodies—The monoclonal antibodies H57-S97 (anti-TCPRb) and 37.51 (anti-CD28), a gift from J. Allison, were partially purified from ascites preparations by ammonium sulfate precipitation (31, 32). Rabbit antibodies to hnRNP C1/C2 (N-16) and hnRNP A1 (Y-15) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to HuR were a gift from H. Furneaux (19F12) or from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Pierce or Vector Laboratories Inc. (Burlingame, CA).

Conical Microscopy—AE.7 cells were stimulated for 4 h on Lab-Tek chamber slides (Nalge Nunc, Naperville, IL). Culture medium was removed and the cells were washed with phosphate-buffered saline (Digene, Beltsville, MD) for 5 min. Cells were fixed in freshly prepared 4% formaldehyde in phosphate-buffered saline, pH 7.3, for 10 min at room temperature and washed three times with phosphate-buffered saline for 10 min. To permeabilize cells and reduce nonspecific labeling, cells were incubated for 20 min in immunolabeling buffer/phosphate-buffered saline, 0.5% Fraction V bovine serum albumin (Sigma), 0.2% Tween 20 (Bio-Rad), and 0.05% sodium azide (Sigma), pH 7.3, supplemented with 5% normal goat serum (KPL, Gaithersburg, MD). The cells were incubated for 60 min at room temperature with anti-HuR antibody (3A2: Santa Cruz Biotechnology) diluted to 10 μg/ml in immunolabeling buffer. The cells were washed in immunolabeling buffer three times for 15 min and incubated for 30 min with an Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). Nuclei were labeled with 1 μg/ml 4,6-diamidino-2-phenylindole (Molecular Probes). Filamentous actin was labeled with 2.5 units/ml of Alexa 568-conjugated phallolidin (Molecular Probes).

Immunofluorescent actin was labeled with 2.5 units/ml of Alexa 568-conjugated phallolidin (Molecular Probes). The samples were washed with immunolabeling buffer three times for 15 min and protected from photobleaching with Gel/Mount (Biomed) diffused on slides prior to placement of coverslips. Images were collected on a Leica SP2 laser scanning confocal microscope (Exton, PA) using a Leica 63X–1.32NA UV-corrected planapo objective. All fluorescent dyes were imaged with 106 cpm) incubated in a final volume of 10 μl of lysis buffer for 30 min at room temperature. In all experiments, 2.5 μg of heparin sulfate (Sigma) and 2 μg of total Escherichia coli RNA (Ambion) were added to the binding reaction as nonspecific competitors. Ten μl of RNA-bound protein in an open microcentrifuge tube was captured by UV cross-linking (250 mJ for 10 min at 0 °C) using a Stratagene UV Cross-linker (Stratagene, La Jolla, CA) and then digested with RNase A/T1 using 0.25/10 units, respectively (Ambion), at 37 °C for 15 min. After addition of 20× SDS sample buffer (Invitrogen/Novex, Carlsbad, CA) the reaction was heated at 95 °C for 5 min and fractionated by SDS-PAGE on 12% or 4–20% acrylamide gels.

Western Blot—Protein lysates (0.8 million cell equivalents per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, P, Millipore, Bedford, MA) using an XCell II Blot Module (Invitrogen). The membrane was then blocked with 5% powdered milk in TBST (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.05% Tween 20) and probed with anti-HuR antibody (19F12, 1:2000) at room temperature for 2 h. The membrane was washed 5 times in TBST and then incubated with 100% peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 2 h (Vector Laboratories) and detection was performed using WestPico enhanced chemiluminescence reagents (Pierce). Western blots for hnRNP proteins were performed as for HuR except that 2.5 million cell equivalents were used per lane and primary antibodies from Santa Cruz were used at a 1:2000 dilution in TBST with 1% powdered milk.

UV Cross-linking and HuR Immunoprecipitations—RNA-protein binding and UV cross-linking were performed as described above except that the binding reaction (400 μg of protein in 200 μl) was divided into 10 tubes prior to UV cross-linking. Afterward, the tubes were pooled.
HuR Binds the IL-2 mRNA

**RESULTS**

**TCR but Not CD28 Signaling Selectively Increases the Level of Cytoplasmic HuR**—To first determine whether HuR is expressed and translocated in the A.E7 clone as has been reported in mouse splenocytes, equal cell equivalents of nuclear and cytoplasmic extracts were transferred to a polyvinylidene difluoride membrane following SDS-PAGE and were sequentially probed for hnRNP C1/C2, hnRNP A1, and HuR (Fig. 1B). No hnRNP C1/C2 was detected in the cytoplasm demonstrating that the presence of HuR in the cytoplasmic fraction was not because of nuclear contamination. Interestingly, hnRNP A1, a known nucleocytoplasmic shuttling protein, was also undetectable in the cytoplasm, indicating that TCR signaling selectively triggers translocation of HuR. Because of the greater amount of lysate that was used in this experiment, HuR is overloaded in this blot compared with Fig. 1A.

To demonstrate that HuR translocation in vivo occurs in response to T cell activation, A.E7 cells were adhered to chamber slides and left unstimulated or activated for 4 h. Following staining for HuR, actin, and DNA, it is apparent that in resting cells HuR is largely restricted to the nucleus, but very faint staining can be seen in the cytoplasm (Fig. 2A). Following T cell activation, HuR is clearly visible in the cytoplasm, where much of it appears to be distributed in a somewhat reticular pattern (Fig. 2E).

**HuR Binds the IL-2 mRNA in Vitro**—To test whether HuR binds the IL-2 mRNA, a UV cross-linking assay was performed using a uniformly radiolabeled probe and cytoplasmic lysate from resting T cells. Following UV cross-linking and RNase digestion, multiple proteins are detected that appear to bind the IL-2 mRNA 3'-UTR in vitro (Fig. 3A). Control reactions performed without cytoplasmic lysate or UV cross-linking did not produce any bands upon autoradiography. Comparison to the bands detected with an antisense probe indicated that three proteins (52, 49, and 32 kDa) bind the IL-2 mRNA 3'-UTR specifically. The two largest proteins were consistently detected despite their faint signal. The binding specificity of two additional proteins (44 and 40 kDa) was inconclusive as they also gave weak and variable signals with the antisense probe. The smallest and most prominent IL-2 3' UTR-binding protein has an apparent molecular mass (32 kDa) close to that of HuR. This pattern was qualitatively the same in activated T cells.

To determine whether the 32-kDa IL-2 mRNA-binding protein was HuR, UV cross-linking was used in conjunction with HuR immunoprecipitation (IP). In Fig. 3B, cytoplasmic lysates from TCR- and CD28-stimulated cells before (lane 1) and after (lane 2) depletion of HuR by IP were Western blotted and probed for HuR. Under the conditions employed, IP depleted nearly all of the HuR from the lysate loaded in lane 2. When the same lysates were used in a UV cross-linking assay (Fig. 3C), the intensity of the 32-kDa band was markedly reduced in the HuR-depleted lysate (lane 2) compared with the HuR replete lysate (lane 1). Immunoprecipitation of the UV cross-linked lysate shown in lane 1 with anti-HuR antibody prior to SDS-PAGE and autoradiography yields a prominent band of 32 kDa (Fig. 3C, lane 3). Control IP with normal mouse serum did not produce a band. These results demonstrate that the 32-kDa protein is HuR and that HuR binds to the IL-2 3'-UTR.

To quantitate HuR binding activity, the UV cross-linking assay was performed under conditions of linear binding. Binding of HuR to the IL-2 3'-UTR probe was found to be linear between 2.5 and 7.5 µg of added cytoplasmic protein from activated T cells. In Fig. 4 the assay was performed in triplicate with 5 µg of cytoplasmic protein from resting and stimulated cells. The amount of protected probe was quantitated using a PhosphorImager. Whereas the HuR binding activity was mark-
HuR Binds Downstream of the Nonamer ARE in the IL-2 mRNA—Having demonstrated that HuR binds the IL-2 mRNA 3′-UTR, it was of interest to determine the nucleotide sequence to which HuR binds. The sequence of the IL-2 mRNA 3′-UTR is shown in Fig. 5A. It has been previously demonstrated that 3′-UTR sequences confer instability upon the mRNA. The IL-2 mRNA is somewhat unusual among cytokine mRNAs in that it contains only a single (AUUU), 3′ dimer (n = 2) or nonamer ARE, although it has multiple isolated AUUUA elements dispersed throughout its 3′-UTR. This prompted us to first perform site-directed mutagenesis of the dimer and compare the binding activity of HuR to this mutant and the wild type IL-2 mRNA. As shown in Fig. 5B, this UU → GG mutation did not affect HuR binding to the IL-2 mRNA. To confirm this observation, we tested binding to three additional probes. As shown in Fig. 5C, HuR binding to a 3′-UTR probe truncated at nt 601 (lane 2) or a probe containing the intact dimer (lane 3) was no greater than the background (lane 5). These data indicate that HuR binds downstream of nt 605 and the (AUUU), 3′ dimer sequence. This was confirmed by testing binding to a probe that spans nt 606–656 and thus lacks the dimer (Fig. 5A). Binding of this probe (Fig. 5C, lane 4) was comparable with that of the wild type probe (Fig. 5C, lane 1), demonstrating that HuR binds between nt 605 and 656, downstream of the nonamer ARE.

HuR Binds the IL-2 mRNA in Vivo—To demonstrate that HuR binds the IL-2 mRNA in vivo, IP of HuR from cytoplasmic lysates of resting, TCR- and CD28-stimulated cells was performed in duplicate and followed by quantitative real time RT-PCR. The IL-2 mRNA is not present in resting T cells and as expected, no amplicon was detected following IP RT-PCR of resting T cell lysates. Likewise, no amplicon was detected when activated T cell lysates were immunoprecipitated with normal mouse serum. As shown in Fig. 6, 5-fold more IL-2 mRNA is immunoprecipitated by anti-HuR antibody from TCR- and CD28-stimulated T cells than from cells stimulated through the TCR alone. An aliquot of the same IP subjected to immunoblotting showed that equal amounts of HuR had been immunoprecipitated from both activated lysates.

HuR Binding in Vivo Reflects Steady State Levels of the IL-2 mRNA—Whereas the amount of HuR protein and HuR IL-2 mRNA binding activity in vitro are very similar in cytoplasmic lysates from T cells stimulated for 4 h via the TCR or TCR and CD28 receptors, the amount of HuR bound IL-2 mRNA in vivo is 5-fold greater in TCR- and CD28-stimulated cells (Fig. 6). The difference observed in vivo could reflect the steady state level or the half-life of the IL-2 mRNA, both of which are greater in TCR- and CD28-stimulated T cells at 4 h. To distinguish between these two possibilities without using transcriptional inhibitors or overexpressing HuR, we took advantage of the fact that CD28 signaling stabilizes the IL-2 mRNA transiently. Whereas there is little if any IL-2 mRNA decay during the first 5 h following CD28 costimulation, between 5 and 7 h the mRNA decays at a rate similar to that seen in cells stimulated via the TCR alone. This can be revealed by blocking IL-2 transcription at different times following T cell activation. In Fig. 7, CSA was added to parallel cultures after 3, 4, or 5 h of TCR and CD28 stimulation and IL-2 mRNA was quantitated at the time of CSA addition (0 min) and again 120 min later. As can be seen by comparing the relative decrease in the amount of IL-2 mRNA in samples treated with CSA at 3 h (○) versus those treated at 5 h (△), CD28-mediated stabilization of the mRNA is transient. Meanwhile, IL-2 promoter activity in TCR- and CD28-stimulated T cells appears to remain constant throughout this time interval (9). Importantly for our experiments, the change in half-life around 5 h does not have a strong impact on the steady state level of the IL-2 mRNA in TCR- and CD28-stimulated cells until after 7 h (4, 9, 10). We reasoned that if there is a causal relationship between HuR binding and the stability of the IL-2 mRNA, then diminished mRNA stability should be associated with a reduction in HuR binding. Three approaches were pursued to determine whether the greater amount of HuR bound IL-2 mRNA detected in TCR- and CD28-stimulated cells in vivo reflects the steady state level or the half-life of the IL-2 mRNA. First, we examined lysates prepared at various times following TCR and CD28 stimulation to determine whether there is a decrease in HuR binding activity in vitro that corresponds to the in vivo decline in IL-2 mRNA stability that occurs after 5 h. As shown in Fig. 8, HuR binding activity in vitro is stable from 3 to 7 h following TCR and CD28 stimulation. Immunoblotting revealed that there were equivalent amounts of HuR present in these lysates. While not directly pertinent to this analysis, we also observed that CSA had no effect on the level of HuR or its in vitro binding activity. Second, we performed IP RT-PCR at various times following TCR and CD28 stimulation. In the time course shown...
in Fig. 9A, steady state levels of cytoplasmic IL-2 mRNA (○) rise and then level off in TCR- and CD28-stimulated cells. Again, as can be revealed by blocking transcription after 3 h of stimulation, IL-2 mRNA (●) stability changes markedly after 5 h, exhibiting in this experiment a half-life of 200 min between 3 and 5 h that is reduced to 58 min between 5 and 7 h. By contrast, we observed (Fig. 9B) that the amount of IL-2 mRNA immunoprecipitated by anti-HuR mAb (Δ) does not decline between 5 and 7 h, as would have been expected if there is a causal relationship between HuR binding and the stability of the IL-2 mRNA. Immunoblotting revealed that equivalent amounts of HuR had been immunoprecipitated at the various time points. This finding indicates that HuR binding to the IL-2 mRNA reflects the steady state level of the mRNA and does not support a role for HuR binding in IL-2 mRNA stabilization.

Third, we studied the in vivo binding of HuR to a deleted form of the IL-2 mRNA (IL2XAC) that cannot be stabilized by CD28 signaling. In contrast to the IL-2 mRNA, the IL2XAC mRNA is rapidly degraded between 3 and 5 h in TCR- and CD28-stimulated cells; which is reflected in the reduced steady state levels of the mRNA (Fig. 9C). Despite the inability of this mRNA to be stabilized by CD28 signaling, HuR binds the IL2XAC mRNA in vivo and the amount of IL2XAC mRNA immunoprecipitated by anti-HuR mAb parallels the steady state level of the mRNA (Fig. 9D). Immunoblotting revealed that equivalent amounts of HuR had been immunoprecipitated at the various time points. This result corroborates that HuR binding reflects the steady state level of the IL-2 mRNA and demonstrates that such binding is not sufficient to stabilize the mRNA.

**DISCUSSION**

In the present study we set out to ask whether HuR binds the IL-2 mRNA and if so whether such binding is involved in CD28-mediated stabilization of the mRNA. We first demonstrated that HuR is expressed in a mouse CD4^+^ T cell clone that is known to faithfully mimic normal T cell activation. Our results are consistent with earlier reports that HuR is present in mouse spleen and thymus and undergoes nucleocytoplasmic shuttling upon T cell activation (21, 35). We show that, in contrast to the IL-2 mRNA, the level of cytoplasmic HuR is dependent upon TCR but not CD28 signaling. The present data would suggest that HuR translocation from the nucleus to the cytoplasm in T cells is triggered by TCR but not CD28 signaling (16, 21, 36). HuR binding to the granulocyte-macrophage colony-stimulating factor mRNA *in vitro* has been reported to be increased in cytoplasmic lysates from activated human T cells but the levels of HuR protein and its subcellular translocation were not investigated (37). We also observed that hnRNP A1, a known nucleocytoplasmic shuttling protein, is present in the...
nucleus of T cells but is not translocated upon T cell activation. This result was surprising as hnRNP A1 has been reported to be present in the cytoplasm of human lymphocytes stimulated for 20 h with phytohemagglutinin (38). The absence of cytoplasmic hnRNP A1 in T cells stimulated for 4 h may be attributable to a number of differences between the experiments, such as the nature of the stimulatory signal. It has been reported that in NIH3T3 cells, hnRNP A1 nucleocytoplasmic shuttling can be triggered by stimuli that activate the extracellular signal-regulated kinase, but not the mitogen-activated protein, kinase pathway (39). The earlier time point used in our experiments, while more consistent with the temporal accumulation of IL-2 mRNA than the 20-h time point used in earlier phytohemagglutinin experiments, may also account for the difference. Nonetheless, our result demonstrates that TCR signaling selectively triggers the translocation of nucleocytoplasmic shuttling proteins.

Having found that HuR binds the IL-2 mRNA both in vitro and in vivo, we mapped the binding site to the 3'-UTR of the IL-2 mRNA. Among cytokine mRNAs, IL-2 is somewhat unusual in that its AU-rich 3'-UTR contains multiple isolated AUUUA elements but only a single AUUUA dimer or nonamer.
the mouse IL-2 mRNA. Sequences between nt 605 and 656 in HuR binding and decay of HuR to the IL-2 mRNA (data not shown). We are presently in vitro binding assay almost completely inhibits the binding of which we have mapped HuR binding. Second, we observed that ability of HuR to bind the mouse but not the human IL-2 mRNA contains the AUUUA dimer and two isolated AUUUA pentanucleotides, one of which is embedded within a U-rich region. Many studies have demonstrated the importance of overlapping AUUUA multimers in the decay of ARE containing mRNAs (41–43). Vakalopoulou et al. (24) demonstrated that insertion of three copies of the AUUUA sequence reduced the accumulation of β-globin mRNA, whereas the same insert carrying a series of U to G substitutions had little effect on mRNA levels. They also showed that U to G mutations within either the AUUUA motif or the U-rich flanking sequences abrogated binding to a 32-kDa protein, which is presumed to be HuR. Mah et al. (20) demonstrated that HuR binds an AUUUA trimer that is essential for destabilization of the IL-3 mRNA and Bohjanen et al. (44) found that an AUUUA trimer, but not a dimer, is sufficient for HuR binding. Through both deletional analysis and site-directed mutagenesis we have demonstrated that HuR does not bind the nonameric ARE within the IL-2 mRNA 3′-UTR and have localized binding between nt 605 and 656. Our result is surprising in light of the report by Shim et al. (11) that HuR does not bind the human IL-2 mRNA 3′-UTR. Whereas Raghavan et al. (37) did report indirect evidence that HuR binds weakly to a human IL-2 probe, we found the data to be equivocal at best. At least two differences between our work and the earlier publications might account for the apparent ability of HuR to bind the mouse but not the human IL-2 mRNA. First, between the human and mouse IL-2 3′-UTR there are multiple nucleotide substitutions in the region to which we have mapped HuR binding. Second, we observed that the use of total yeast RNA rather than E. coli RNA in the in vitro binding assay almost completely inhibits the binding of HuR to the IL-2 mRNA (data not shown). We are presently working to further define the role of the AUUUA dimer and sequences between nt 605 and 656 in HuR binding and decay of the mouse IL-2 mRNA.

Signaling through the CD28 receptor critically regulates IL-2 mRNA stability. We sought to determine whether there was an association between HuR binding to the IL-2 mRNA and stabilization of the message via CD28 receptor signaling. Having found equal amounts of cytoplasmic HuR protein in TCR and TCR- and CD28-stimulated cells, we tested whether there might be changes in HuR binding activity under different states of T cell activation. Using the IL-2 mRNA 3′-UTR as a probe, we found that in vitro HuR binding activity was the same in cytoplasmic lysates from TCR and TCR- and CD28-stimulated T cells. Furthermore, HuR binding activity in vitro was the same in lysates prepared between 3 and 7 h following TCR and CD28 stimulation; a period during which CD28-mediated IL-2 mRNA stability changes dramatically in vivo. By contrast, the amount of HuR bound IL-2 mRNA in vivo was greater in TCR- and CD28-stimulated T cells than in cells stimulated through the TCR alone.

The greater amount of HuR bound IL-2 mRNA found in vivo could reflect differences in the half-life of the IL-2 mRNA or its steady state level, both of which are greater in TCR- and CD28-stimulated T cells. To distinguish between these possibilities we took advantage of the transient nature of CD28-mediated IL-2 mRNA stabilization. Our results clearly show that the amount of HuR bound IL-2 mRNA in vivo does not decline in association with the abatement of CD28-mediated stabilization; consistent with the proposal that HuR binding to the IL-2 mRNA is constitutive and reflects the steady state level of the mRNA. In addition, the in vivo binding of HuR to a mutated IL-2 mRNA, which cannot be stabilized by CD28 signaling, reflects the steady state level of that mRNA as well. The binding of HuR to this mutated mRNA also demonstrates that HuR binding cannot be sufficient for stabilization of the wild type IL-2 mRNA. Collectively, our results in vitro and in vivo suggest that HuR does not play a role in stabilization of the IL-2 mRNA, although it specifically binds the 3′-UTR.

Originally, HuR was postulated to have a role in mRNA degradation (20). This hypothesis was predicated on the finding that HuR specifically binds with high affinity the ARE of the c-fos, IL-3, and other mRNAs but fails to bind mutated forms of their AREs that are incapable of inducing mRNA decay (20, 23, 45). However, because the demonstration that HuR overexpression does not enhance degradation but rather stabilizes ARE-containing mRNAs, evidence has accumulated that HuR plays a role in mRNA stabilization (25–29), For example, HuR has been reported to stabilize tumor necrosis factor-α (25) and nitric-oxide synthase II mRNAs (27). Furthermore, Ming et al. (26) demonstrated that HuR could overcome the destabilizing effect of tristetraprolin on the IL-3 mRNA. Conversely, lowering endogenous HuR levels through expression of antisense HuR has been reported to reduce the half-lives of the cyclin-dependent kinase inhibitor p21 mRNA (29) and cyclins A and B1 mRNAs (28). All these studies utilized systems in which normal HuR levels had been perturbed by transfection of sense or antisense HuR expression vectors. As has been pointed out by several investigators, such experiments are open to alternative interpretations. It is possible that HuR actually plays a role in degradation, but that overexpression results in the sequestration or displacement of other factors that are needed for mRNA decay (22, 46). Whereas we found no evidence that HuR plays a role in the CD28-mediated stabilization of the IL-2 mRNA in our systems, our results may have no bearing on earlier reports that overexpression of HuR is associated with stabilization of the c-fos and other mRNAs whose half-life is known not to be regulated by CD28 signaling (8, 18, 36). With regard to those CD28-regulated mRNAs that have been reported to be stabilized by overexpression of HuR, we are unaware of any report demonstrating that the CD28-
mediated stabilization of those mRNAs is a direct consequence of CD28 signaling and not secondary to increased signaling through the IL-2 or other cytokine receptors. It was recently reported that when such an mRNA (granulocyte macrophage-colony stimulating factor) is ectopically expressed in NIH3T3 cells it is not stabilized by overexpression of HuR and others have shown that the stability of the granulocyte-macrophage colony-stimulating factor mRNA can be regulated through IL-7 receptor signaling (46, 47). We have also found that CD28 up-regulation of interferon-γ is dependent on IL-2 receptor signaling (48). As it has been demonstrated that sequences outside of the 3′-UTR are required for stabilization of the IL-2 mRNA, our results are also consistent with the possibility that constitutive binding of HuR is necessary but not sufficient for CD28-mediated stabilization of the mRNA (4, 6).

It should be noted that, following earlier reports that HuB is involved in translational regulation, HuR has recently been demonstrated to regulate translation of p53 (19, 49–51). In so far as there is no discordance between the amount of IL-2 mRNA and the amount of IL-2 protein in the presence and absence of CD28 signaling, there is nothing to suggest that there is translational regulation of IL-2 expression. It has also been reported that the stability of some mRNAs is coupled to translation but this is not the case for IL-2 (10).

IL-2 mRNA stabilization through CD28 receptor signaling is a critical post-transcriptional control that regulates T cell activation and the very nature of the immune response (52–54). This is the first report to ascertain the role of HuR in mRNA stabilization without perturbing the level of HuR in the system under study. Our findings indicate that binding of HuR to an mRNA need not be associated with its stabilization and suggest that CSA-resistant CD28-mediated stabilization proceeds through a pathway that may be independent of HuR binding. Because it is known that CD28 signaling stabilizes only a...
subset of ARE containing mRNAs, it remains to be seen whether our findings with regard to the role of HuR in CD28-mediated mRNA stabilization will be generalizable or are particular to the IL-2 mRNA (8). The regulated and selective translational activation of the IL-2 mRNA that we observe strongly suggests that it has a specific function in T cell activation; which we conjecture is related to nuclear export of the IL-2 mRNA. Whereas studies are underway to elucidate the role of HuR binding to the IL-2 mRNA, this report further defines the mechanisms involved in CD28-mediated mRNA stabilization and highlights an important exception to the widely held view that HuR binding acts to stabilize mRNAs.

Acknowledgments—We thank H. Furneaux and J. Allison for the kind gift of anti-HuR and anti-CD28 antibodies, respectively, J. Lee for performing site-directed mutagenesis, C. Roy for generating the puMuTX plasmid, V. Sivaraman for assistance with the H-2KXAC experiments, and S. Haynes for critical reading of the manuscript.

REFERENCES
1. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1670–1674
2. Shaw, G., and Kamen, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 14721–14725
3. Ragheb, J. A., Deen, M., and Schwartz, R. H. (1999) J. Immunol. 163, 120–129
4. Chen, C. Y., Xu, N., and Shyu, A. B. (2002) J. Biol. Chem. 277, 6417–6423
5. Dong, C., Yang, D. D., Tournier, C., Whitmarsh, A. J., Xu, J., Davis, R. J., and Flavell, R. A. (2000) Nature 405, 91–94
6. Sivaraman, V., Sivaraman for assistance with the H-2KXAC experiments, and S. Haynes for critical reading of the manuscript.


downloaded from http://www.jbc.org/ by guest on July 27, 2018
Selective Cytoplasmic Translocation of HuR and Site-specific Binding to the Interleukin-2 mRNA Are Not Sufficient for CD28-mediated Stabilization of the mRNA

Yuko Seko, Hooman Azmi, Robert Fariss and Jack A. Ragheb

J. Biol. Chem. 2004, 279:33359-33367.
doi: 10.1074/jbc.M312306200 originally published online March 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312306200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 43 of which can be accessed free at http://www.jbc.org/content/279/32/33359.full.html#ref-list-1