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HuR Is Required for IL-17–Induced Act1-Mediated CXCL1 and CXCL5 mRNA Stabilization

Tomasz Herjan,* Peng Yao,† Wen Qian,* Xiao Li,‡ CaiNi Liu,* Katarzyna Bulek,* Dongxu Sun,* Wen-Pin Yang,§ Jun Zhu,§ Aiqing He,§ Julie A. Carman,§ Serpil C. Erzurum,‖ Howard D. Lipshitz,§ Paul L. Fox,† Thomas A. Hamilton,* and Xiaoxia Li*

IL-17, a major inflammatory cytokine plays a critical role in the pathogenesis of many autoimmune inflammatory diseases. In this study, we report a new function of RNA-binding protein HuR in IL-17–induced Act1-mediated chemokine mRNA stabilization. HuR deficiency markedly reduced IL-17–induced chemokine expression due to increased mRNA decay. Act1-mediated HuR polyubiquitination was required for the binding of HuR to CXCL1 mRNA, leading to mRNA stabilization. Although IL-17 induced the coshift of Act1 and HuR to the polysomal fractions in a sucrose gradient, HuR deficiency reduced the ratio of translation-active/translation-inactive IL-17–induced chemokine mRNAs. Furthermore, HuR deletion in distal lung epithelium attenuated IL-17–induced neutrophilia. In summary, HuR functions to couple receptor-proximal signaling to posttranscriptional machinery, contributing to IL-17–induced inflammation. The Journal of Immunology, 2013, 191: 640–649.

Interleukin-17 (also known as IL-17A) is a key proinflammatory cytokine produced mainly by a distinct subset of CD4+ Th cells called Th17 (1–3). IL-17 is required for host defense against extracellular microorganisms (3–6) and has been linked to the development and pathogenesis of many autoimmune disorders, including rheumatoid arthritis, multiple sclerosis, psoriasis, and asthma (7–12). Deficiency in IL-17 leads to diminished Ag-specific T cell–mediated immune responses, including allergen-induced pulmonary inflammation and airway hyperresponsiveness (13, 14). The major function of IL-17 is to coordinate local tissue inflammation by promoting production of proinflammatory and neutrophil-mobilizing cytokines and chemokines, including IL-6, CSF2, TNF-α, IL-1, CXCL1, CCL2, CXCL2, CCL7, and CCL20, resulting in the infiltration of inflammatory cells, such as neutrophils, monocytes, and lymphocytes.

IL-17 signals through a heterodimeric receptor complex composed of IL-17RA and IL-17RC, members of the IL-17R family (15, 16). Both IL-17RA and IL-17RC belong to a SEFIR protein family, which is defined by the presence of a conserved cytoplasmic SEFIR domain (17). Act1 (also known as CIKS) is an essential component in IL-17 signaling and required for IL-17–dependent immune responses (15, 18, 19). Act1 is also a member of the SEFIR protein family, containing a SEFIR domain at its C terminus (20). Upon IL-17 stimulation, Act1 is recruited to IL-17R through a SEFIR-dependent interaction. Furthermore, Act1 possesses a U-Box domain that is functionally required for its E3 ligase activity. Upon IL-17 stimulation, Act1, together with the Ubc13–Uev1A E2 complex, exerts K63-linked polyubiquitination of TRAF6 (21, 22). This ubiquitination event is required for TRAF6-mediated activation of TAK1 and the IKK complex, resulting in activation of transcription factor NF-κB and subsequent NF-κB–dependent transcription of proinflammatory and neutrophil-mobilizing cytokines and chemokines. Although IL-17 regulates gene transcription, it also induces gene expression by stabilizing otherwise unstable mRNAs of proinflammatory genes (23). Recently, we reported that, following IL-17 stimulation, a phosphorylated form of Act1 forms a complex with TRAF2 and TRAF5 (24). This phosphorylation event and complex formation are functionally required for the stabilization of CXCL1 and CSF3 mRNA.

Many cytokine and chemokine mRNAs exhibit very short half-lives due to the presence of adenine and uridine–rich sequence elements (AREs) located within their 3′ untranslated regions (UTRs) (25–27). Therefore, the regulation of mRNA stability is an important control of inflammatory gene expression (28). The AREs within the 3′ UTR can be recognized by RNA-binding proteins that function to mediate the sequential deadenylation, decapping, and, ultimately, exonucleolytic degradation of the RNA (29–31). These ARE-binding proteins include AUF1 (hnRNP D), tristetraprolin, butyrate response factors, and KH domain–containing splicing regulatory factor KSRP (32, 33). We recently identified a novel mRNA destabilizing factor, called SF2/ASF, which is linked to IL-17 signaling through its interaction with Act1 and TRAF5 (24). SF2/ASF binds chemokine mRNAs in unstimulated cells, whereas the SF2/ASF–mRNA interaction is markedly diminished following IL-17 stimulation. SF2/ASF promotes chemokine mRNA decay, and its depletion results in prolonged mRNA t1/2.

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Abbreviations used in this article: ARE, adenosine and uridine–rich sequence element; ARE-mRNA, adenosine and uridine–rich sequence element containing mRNA; BAL, bronchoalveolar lavage; ES, embryonic stem; HA, hemagglutinin; IKK, inducible inhibitor of NF-κB (IκB) kinase; KO, knockout; MEF, mouse embryonic fibroblast; RNP, ribonucleoprotein; UTR, untranslated region; WT, wild-type.

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The important question is what happens to the IL-17-induced chemokine mRNAs after their dissociation from SF2/ASF; that is, how the chemokine mRNAs are actually stabilized and translated. In this study, we report that ARE-binding protein HuR plays a critical role in IL-17-induced chemokine mRNA stabilization. IL-17 stimulation induced the interaction of HuR with Act1 and TRAF5 but not SF2/ASF. Although Act1 is required for IL-17-induced dissociation of SF2/ASF from mRNA, IL-17 induced the HuR–mRNA interaction in an Act1-dependent manner. HuR depletion substantially impaired IL-17–induced CXCL1 and CXCL5 expression due to increased mRNA decay. Furthermore, upon IL-17 stimulation, Act1 mediated K63-linked polyubiquitination of HuR, which is required for HuR–mRNA interactions and HuR-mediated chemokine mRNA stabilization. Moreover, IL-17 stimulation induced the coshift of Act1 and HuR toward the fractions of polysomes in a sucrose gradient, implicating the importance of the Act1–HuR complex in moving the mRNAs to the translation-active state. The impact of HuR on IL-17–induced chemokine mRNA stabilization had important biological consequences, because HuR deficiency attenuated IL-17–induced neutrophilia and pulmonary inflammation in vivo.

Materials and Methods

Reagents

Abs against Act1, GAPDH, HuR, SF2, TRAF2, TRAF5, o-tubulin, and β-actin were from Santa Cruz Biotechnology. Anti-hemagglutinin (HA) and anti-ubiquitin Ab were from Sigma; anti–pro-SP-C Ab was from Upstate. Adenoviruses encoding GFP and Cre-GFP were obtained from Vector Biolabs. Purified GST-HuR was purchased from Novus Biologicals. Cell culture, mouse embryonic fibroblasts (MEFs) and HeLa and HeLa Tet-Off cells were as previously described (21, 34). Reporter vectors and anti–pro-SP-C Ab were from Molecular Probes. For ELISA assay, CXCL1 production was measured using a 96-well plate reader (Molecular Devices). For real-time PCR, a SYBR Green PCR Master Mix kit (Applied Biosystems) was used.

Affymetrix GeneChip Microarray analysis

A total of 200 ng RNA was used for target labeling, and the target preparation was done on a Biomek FXP (Beckman Coulter, Brea, CA) using a GeneChip HT 3’ IVT Express Kit (Affymetrix, Santa Clara, CA) and following the manufacturer’s recommendation. Labeled cRNA were hybridized on an Affymetrix GeneChip HT-MG-430PM-96 (Affymetrix). All array hybridization, washing, and scanning were performed on GeneTitan (Affymetrix), according to the manufacturer’s recommendations. Three independent biological replicates were analyzed in each experiment, which yielded very similar results. The t test was used to assess significance, with p < 0.05 deemed significant.

Immunoprecipitation

Cells were lysed in buffer A (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 10 mM NaF; 2 mM DTT, 1 mM sodium orthovanadate, 2 mM EGTA, 20 μM aprotinin, 1 mM PMSF) or, to prevent possible protein–protein interaction, in buffer B (buffer A plus 0.1% SDS and 0.5% deoxycholate). Cell extracts were incubated with 1 μg different Abs overnight at 4˚C with 20 μl protein A Sepharose beads. After incubation, beads were washed four times with lysis buffer, separated by SDS-PAGE, and analyzed by Western blot.

In vitro ubiquitination assay

The polyubiquitination assays were performed in 10 μl reaction volume in buffer (20 mM Tris HCl [pH 7.5], 2 mM ATP, 5 mM MgCl2) at 37˚C for 1 h, together with the following components provided accordingly: 100 ng E1, 100 ng E2, 100 ng individual E3 (GST-TRAF6 or GST-Act1), and 5 μg ubiquitin. For Act1-mediated polyubiquitination of HuR, in addition to E1, E2, and ubiquitin, 100 ng GST-HuR was added as substrate, together with Act1 in the same reaction buffer.

Quantitative real-time PCR

Total RNA was isolated with TRizol reagent (Invitrogen). Real-time PCR was performed using a SYBR Green PCR Master Mix kit (Applied Biosystems). The following primers were used: β-actin, 5′-GTGTCACATATGGG-GAACG-3′ (forward) and 5′-ACGGATGTCAACGTCACACT-3′ (reverse); CXCL1, 5′-TTAGGTTGAGCATTGTTG-3′ (forward) and 5′-AAGATTGTCACAGAGGAAAGCCT-3′ (reverse); GFPT2, 5′-GCCCTATTTGGAAGATG-3′ (forward); and HuR, 5′-GCCAACTCGTCTAGATCGACTT-3′ (forward) and 5′-GGGTCTACAGGGTATC-3′ (reverse). The relative expression levels were calculated using the comparative ΔCt method.

Intranasal instillation of IL-17

Mice were anesthetized with isoflurane. Carrier-free, murine rIL-17 (R&D Systems) resuspended in sterile saline (0.9%) was instilled into the nasal cavity using a blunt needle and 1-ml syringe. Lungs were collected and snap-frozen immediately in a liquid nitrogen container. Total RNA was obtained using TRIzol (Invitrogen) and an OMNI TH tissue homogenizer (Omni International). H&E staining was performed on lung sections after fixation in 10% neutral-buffered formalin and paraffin embedding. Paraffin-embedded lung sections were stained with H&E to evaluate inflammation. For frozen sections, lungs were embedded in OCT (Tissue-Tek) and snap-frozen in liquid nitrogen. Sections (10 μm) were incubated with anti-HuR (1:100) and anti–pro-SP-C (1:100). Ags were visualized following incubation with fluorescein-conjugated secondary Abs (Molecular Probes). For ELISA assay, CXCL1 production was measured using DuetSet ELISA Development Systems (R&D Systems), following the manufacturer’s instructions.

Subcellular fractionation

Confluent cells in 15-cm plates, untreated or treated with IL-1 (1 ng/ml) for various times, were resuspended in 1 ml ice-cold hypotonic buffer (10 mM
HEPES [pH 7.4], 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT) and homogenized on ice with 45 strokes of a Dounce homogenizer. Unlysed cells, nuclei, and cell debris were pelleted by centrifugation at 1000 × g for 5 min five times. Soluble (supernatant, S100) and particulate (pellet, P100) fractions were generated by centrifugation at 100,000 × g for 1 h.

RNA-binding assays

The ability of HuR, SF2, and Act1 to bind to RNA in vivo was assessed as described previously (33). Briefly, cells were transiently transfected with tetracycline-response element-regulated CXCL1-3’UTR A4 and CSF2-3’UTR reporter constructs. Twenty hours after transfection, 2 × 10⁶ cells were trypsinized, washed twice, and resuspended in 10 ml ice-cold PBS. Cells were fixed in 0.1% formaldehyde for 15 min at room temperature, whereupon the cross-linking reaction was stopped with glycine (pH 7; 0.25 M). The cells were then washed twice with ice-cold PBS, resuspended in 2 ml RIPA buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, and proteinase inhibitors), and sonicated. The lysate was centrifuged (15 min, 4°C, 16,000 × g), and 1 ml each supernatant was immunoprecipitated overnight at 4°C, using protein G–agarose beads preincubated with 20 μg anti-HuR or anti-Act1 Ab. The beads were washed five times with 1 ml RIPA buffer and resuspended in 150 μl elution buffer (50 mM Tris-Cl [pH 7.5], 1% SDS, 0.5 M salt, 0.5 M DTT). Cross-linking was reversed by incubation at 70°C for 45 min. RNA was purified from immunoprecipitates with TRI Reagent and treated with RNase-free DNase, and 10% of the total RNA sample was reverse transcribed with Moloney murine leukemia virus reverse transcriptase. Two microilters (10%) of the reverse transcriptase product was subjected to quantitative real-time PCR. The primers for CXCL1 coding region were 5’-CTGGCCACAGGGGCGCCTATC-3’ (forward) and 5’-GGACACCTTTAGCATCTTT-3’ (reverse). Primers for GAPDH were 5’-GTGTTGTTGTGACAGGAGCATTGCT-3’ (forward) and 5’-GACAGAGGAGGACATTGCT-3’ (reverse).

Adenoviral infection

Primary kidney epithelial cells were divided into 60-mm dishes and infected by exposing them to media containing 2 × 10⁶ infectious units/ml overnight.

**Statistical analysis**

The data are presented as the mean + SD. The significance of the difference between two groups was determined by the Student t test.

**Results**

HuR depletion diminishes IL-17–induced expression of CXCL1 and CXCL5 but not CSF2

Although IL-17–induced Act1-mediated signaling induces transcriptional activation of genes encoding inflammatory molecules, it also affects their production at the posttranscriptional level. Many cytokine and chemokine mRNAs are known to have half-lives shortened by mechanisms that depend on AREs located in the 3’ UTR. To investigate the mechanism for Act1-dependent mRNA stabilization during IL-17 signaling, we used coimmunoprecipitation assays to search for possible interactions of Act1 with ARE-binding proteins that have been implicated in the regulation of mRNA stability. Through the search, we found that IL-17 stimulation induced the interaction of endogenous Act1 with mRNA stabilizing protein, HuR (Fig. 1A). HuR was previously shown to inhibit mRNA decay through its cooperation with other ARE-stabilizing protein, HuR (Fig. 1A). HuR was previously shown to inhibit mRNA decay through its cooperation with other ARE-binding proteins. To identify the potential HuR target mRNAs induced by IL-17, we performed array analysis of kidney epithelial cells from HuR flox/flox mice infected with adenovirus encoding GFP or GFP-Cre. Cre-mediated deletion reduced the expression of HuR protein by ~95% (Fig. 1B). We found that expression of a subset of IL-17–induced genes was substantially reduced in HuR-deficient cells compared with that in the control cells (Fig. 1C). By real-time PCR, we confirmed that CXCL1 and CXCL5 mRNA accumulation was markedly diminished in HuR-deficient cells compared with that in control cells, whereas the accumulation of CSF2 mRNA was unaffected (Fig. 1D).

**FIGURE 1.** HuR depletion diminishes IL-17–induced expression of CXCL1 and CXCL5 but not CSF2. (A) Cell lysates from HeLa cells that were treated or not with IL-17 (50 ng/ml) for the indicated time were immunoprecipitated (IP) with anti-Act1, anti-HuR, and IgG control, followed by Western blot analysis (IB) with anti-HuR and anti-Act1. (B) Western blot analysis of HuR and β-actin in lysates of primary mouse kidney epithelial cells isolated from HuRfloxflox mice infected with a GFP-encoding adenovirus (Control) or Cre-GFP-encoding adenovirus (Cre). (C) Affymetrix microarray analyses of primary kidney epithelial cells infected as in (B), left untreated or stimulated with IL-17 (50 ng/ml) for the indicated time. A heat map is shown for the IL-17 induction of the expression levels in HuR-deficient cells compared with that in control cells. (D) Real-time PCR analysis of Cxcl1, Cxcl5, and Csf2 in mouse primary kidney epithelial cells infected as in (B), which were left untreated or stimulated with IL-17A (50 ng/ml) for the indicated time. The results are presented as fold induction of the expression levels in treated samples over the untreated samples. Data are representative of three independent experiments (error bars show SD). WCL, Whole-cell lysates.
HuR binds and stabilizes CXCL1 and CXCL5 mRNAs in response to IL-17 stimulation

We next measured the impact of HuR deficiency on IL-17–induced mRNA stabilization. WT and HuR-deficient kidney cells were pretreated for 0.5 h with TNF-α to promote inflammatory gene transcription and subsequently were treated for 0.5–3 h with actinomycin D (to block transcription) along with IL-17 (for mRNA stabilization). Although CXCL1 and CXCL5 mRNA was induced to a similar extent in WT and HuR-deficient kidney cells after the initial treatment with TNF-α, the mRNAs decayed more rapidly in HuR-deficient kidney epithelial cells than in WT cells (Fig. 2A). However, the stability of CSF2 mRNA was unaffected by HuR deficiency. These results indicated that HuR is necessary for IL-17–mediated stabilization of CXCL1 and CXCL2 mRNAs.

We recently reported that SF2/ASF bound CXCL1 chemokine mRNA in unstimulated cells to mediate mRNA decay, but the SF2 (ASF)–mRNA interaction was much lower after stimulation with IL-17. To determine whether HuR directly mediates IL-17–induced mRNA stabilization, we examined the binding of HuR to CXCL1 mRNA with and without IL-17 stimulation. We used reporter constructs containing the coding region of CXCL1 with a truncated 3’ UTR of either CXCL1 (CXCL1-3’UTR Δ4) or CSF2 (CSF2-3’UTR) under the control of a tetracycline-response element. These constructs were transfected into HeLa Tet-Off cells stably expressing the tetracycline-controlled trans activator. Cells extracts of the transfected cells, which were treated or not with IL-17 for 90 min, were immunoprecipitated with anti-HuR, anti-SF2, or IgG. The amounts of HuR- and SF2-bound KCΔ4 and KC-GM-CSF mRNAs, relative to the abundance of GAPDH mRNA, were determined by real-time PCR. IL-17 treatment resulted in a 4-fold increase in the abundance of HuR-bound CXCL1-3’UTR Δ4 mRNA, whereas SF2 bound CXCL1-3’UTR Δ4 mRNA in untreated cells and was dissociated from the transcript in IL-17–treated cells (Fig. 2B). Neither HuRnor SF2-bound CSF2-3’UTR was altered in response to IL-17 stimulation, which is consistent with the fact that HuR and SF2 deficiency did not have much impact on CSF2 mRNA stability.

HuR is ubiquitinated upon IL-17 stimulation

It was noted that IL-17 stimulation induced slower migrating forms of HuR, implicating IL-17–dependent HuR posttranslational modification (Fig. 3A). The laddering feature of the modified HuR suggests possible ubiquitination of HuR. To test this, HeLa cells were transfected with HA-tagged ubiquitin, followed by treatment with IL-17. The cell lysates were immunoprecipitated with anti-HuR Ab and immunoblotted with anti-HA, anti-HuR, and anti-ubiquitin Abs. These results showed that IL-17 induced the ubiquitination of HuR (Fig. 3B). Previous studies suggested that, although HuR is localized primarily in the nucleus, extracellular stimuli can promote its translocation from the nucleus to the cytosol. We wondered where HuR is modified and whether IL-17 stimulation has any impact on HuR’s subcellular localization. HeLa cells, which were treated or not with IL-17, were fractionated into membranes and cytosol and subsequently analyzed with Abs against HuR and Act1. The Na+/K+ ATPase and α-tubulin were used as markers for the membrane and cytosol fractions, respectively. Although unmodified HuR was found in both membrane and nuclear fractions before IL-17 treatment, the modified HuR induced by stimulation with IL-17 was found only in the mem-

**FIGURE 2.** HuR binds and stabilizes CXCL1 and CXCL5 mRNAs in response to IL-17 stimulation. (A) Real-time PCR analysis of Cxcl1, Cxcl5, and Csf2 in primary kidney epithelial cells, infected as in Fig. 1B, which were pretreated for 2 h with TNF (10 ng/ml), followed by actinomycin D (5 μg/ml) alone (NT) or in combination with IL-17 (50 ng/ml) for the indicated time. The results are presented as decay over time (left panels) and as half-life (right panels). (B) HeLa Tet-Off cells, transfected with the CXCL1 (KCΔ4) and CSF2-3’UTR reporter constructs, were left untreated (0) or were treated for 90 min with IL-17, followed by RNA immunoprecipitation with anti-HuR or anti-SF2 (ASF) and real-time RT-PCR analyses of KCΔ4 and KC-GM-CSF mRNAs. Results are presented as relative to results obtained by immunoprecipitation with nonspecific IgG. Data are representative of three independent experiments. Error bars represent SD.
brane fraction and not in the cytosol or nucleus (Fig. 3C). In contrast, we reported previously that IL-17 induces Act1 phosphorylation through the activation of inducible inhibitor of NF-κB (IkB) kinase (IKK); also known as IKKe. Interestingly, the IL-17–induced phosphorylated Act1 (slower migration) was colocalized with the modified HuR in the membrane fraction, whereas unmodified Act1 was found in both membrane and cytosol fractions before IL-17 treatment (Fig. 3C). The colocalization of Act1 with HuR is consistent with the IL-17–induced complex formation between Act1 and HuR (Fig. 1A), implicating a possible role for Act1 in HuR modification. To determine the importance of Act1 in IL-17–induced polyubiquitination of HuR, we examined several primary cell types from WT and Act1-deficient mice, including MEFs, kidney epithelial cells, and bone marrow macrophages. We found that, although IL-17 induced HuR polyubiquitination in WT cells, Act1 deficiency abolished IL-17–dependent HuR modification (Fig. 3D, data not shown).

Act1-mediated HuR polyubiquitination is required for HuR–mRNA interaction

We reported previously the E3 ligase activity of Act1 that exerts K63-linked polyubiquitination of TRAF6 to mediate NF-κB activation (20). Because Act1 was required for IL-17–induced HuR polyubiquitination, we hypothesize that Act1 might also function as an E3 ligase for HuR. We found that overexpression of Act1 in HeLa cells induced polyubiquitination of HuR in unstimulated cells and further enhanced IL-17–induced HuR modification, suggesting the ability of Act1 to mediate HuR ubiquitination (Fig. 4A). To test whether Act1 can directly ubiquitinate HuR, we performed an in vitro polyubiquitination assay. Recombinant Act1 was able to use the Ubc13/Uev1A E2 complex to specifically catalyze polyubiquitination on HuR in this in vitro assay (Fig. 4B), suggesting that Act1 is likely an E3 ubiquitin ligase for HuR. Although TRAF6 was used as a positive control, the specificity of the assay was also confirmed by different control reactions (Fig. 4B). Moreover, we found that WT ubiquitin and ubiquitin mutant K48R (Lys48 mutated to Arg), but not K63R (Lys63 mutated to Arg), were able to mediate HuR polyubiquitination in vivo upon Act1 overexpression (Supplemental Fig. 2). These results indicate that Act1 is a bona fide E3 ligase for HuR.

We showed previously that Act1 contains a U-box–like region and is a member of the U-box type E3 ubiquitin ligase family. Therefore, it is important to determine whether the U-box of Act1 is required for IL-17–induced HuR polyubiquitination. Although restoration of Act1-deficient MEFs with retroviral Act1 induced HuR polyubiquitination and rendered IL-17–induced HuR modification, U-box deletion mutant of Act1 failed to mediate HuR ubiquitination (Fig. 4C). Overexpression of WT Act1, but not U-box mutant of Act1, induced HuR–mRNA interaction (Fig. 4D). Furthermore, CXCL1 mRNA stability was reduced in Act1-deficient MEFs reconstituted with the D-Ubox mutant of Act1 compared with that of WT Act1 (Fig. 4E). Taken together,
these results indicate the importance of Act1-mediated HuR polyubiquitination for HuR’s RNA binding and stabilizing activity.

*Act1–TRAF2/5–SF2 and Act1–TRAF2/5–HuR are two independent complexes*

We recently reported that IKKi-mediated Act1 phosphorylation plays a critical role in directing the interaction of Act1 with TRAF2/5, which is required for the formation of the Act1–TRAF2/5–SF2 complex, leading to dissociation of SF2 from mRNA. In this study, we show that the interaction of Act1 with HuR is essential for the binding of HuR to mRNA. We wondered whether and what TRAF molecules are involved in this process. Through coimmunoprecipitation experiments, we found that IL-17 induced the interaction of HuR with TRAF2 and TRAF5 but not TRAF6 (Fig. 5A, data not shown). Because both SF2 and HuR interact with Act1–TRAF2/5, the important question is whether SF2 and HuR are in the same complex. It is interesting to note that we failed to coimmunoprecipitate HuR with SF2 (Fig. 5B), suggesting that these two RNA-binding proteins are not in the same complex. These results suggest that Act1–TRAF2/5–SF2 and Act1–TRAF2/5–HuR are probably two independent complexes. Although Act1–TRAF2/5 facilitates the dissociation of SF2 from mRNA, Act1–TRAF2/5 enhances the interaction of HuR with mRNA, thereby stabilizing mRNA.

*IL-17–induced shift of Act1–HuR to polysomes facilitates the translation of ARE-containing mRNAs*

It is well known that ARE-containing mRNAs (ARE-mRNAs) follow HuR trafficking to polysomes for the transcripts to be translated. Because IL-17 induced the interaction of Act1 with HuR to promote HuR–ARE-mRNAs interaction, we hypothesize that IL-17 stimulation might induce the shift of HuR–ARE-mRNAs to the polysomes to facilitate the translation. Therefore, we examined the distribution of HuR in a sucrose gradient, which was used to separate translation-inactive free ribosomes from translation-active polysomes (Fig. 6B). Interestingly, increased amounts of HuR were detected in the fractions of translation-active polysomes from lysates of IL-17-treated cells compared with untreated cells.
Surprisingly, Act1 was also detected in the same polysomal fractions as HuR, suggesting that the Act1–HuR complex is associated with translation-active polysomes (Fig. 6C). In support of this, IL-17 treatment resulted in a 2-fold increase in the abundance of Act1-bound CXCL1 mRNA (Fig. 6D). We then wondered whether the IL-17–induced association of Act1–HuR with the polysomal fractions has any impact on the translation of IL-17–induced chemokines. Compared with WT kidney epithelial cells, the HuR-deficient cells had more CXCL1 mRNAs in the translation-inactive pool than in the translation-active pool. As a result, the ratio of IL-17–induced CXCL1 mRNAs in translation-active versus translation-inactive pools was reduced in HuR-deficient cells.

**FIGURE 5.** IL-17 induces the formation of a complex containing Act1, TRAF2, TRAF5, and HuR. (A) Cell lysates from HeLa cells that were treated or not with IL-17 (50 ng/ml) for the indicated time were immunoprecipitated (IP) with anti-TRAF2, anti-TRAF5, anti-TRAF6, and IgG, followed by Western blot analysis (IB) with anti-HuR, anti-TRAF2 anti-TRAF5, and anti-TRAF6. (B) Cell lysates from HeLa cells that were treated or not with IL-17 (50 ng/ml) for the indicated time were immunoprecipitated (IP) with anti-Act1 and anti-HuR and IgG, followed by Western blot analysis (IB) with anti-SF2, anti-Act1, and anti-HuR. Data are representative of three independent experiments. WCL, Whole-cell lysates.

**FIGURE 6.** IL-17–induced shift of Act1-HuR to polysomes facilitates the translation of ARE-mRNAs. (A) UV-absorbance profile of RNP and polysome complexes separated on a sucrose density gradient. (B and C) Cytoplasmic extracts of HeLa cells that were untreated (NT) or treated with IL-17 (50 ng/ml) for 90 min were fractionated through a 10–50% sucrose gradient, as described in Materials and Methods. (B) Western blot analyses of HuR, Act1, SF2, TRAF2, TRAF5, and GAPDH in sucrose gradient fractions (for corresponding UV-absorbance profiles see Supplemental Fig. 3; the fraction numbers correspond to lanes in the Western blots). (C) The ratios of HuR, Act1, and GAPDH from translation-active (fractions 4–9) and translation-inactive (1–4) pools. (D) HeLa Tet-Off cells transfected with the CXCL1-3'UTR Δ4 reporter plasmid were treated or not with IL-17 for the indicated time, followed by RNA immunoprecipitation with anti-Act1 and real-time RT-PCR analyses of KCΔ4 mRNA. Results are presented relative to results obtained by immunoprecipitation with nonspecific IgG. (E) Primary kidney epithelial cells isolated from HuR^{lox/lox} mice, infected as in Fig. 1B, were treated with IL-17 (50 ng/ml) for 90 min and fractionated as in (A). CXCL1, CSF2, and GAPDH mRNAs from translation-active pools and translation-inactive pools were analyzed by quantitative RT-PCR and normalized to β-actin. The ratios of mRNAs from translation-active/inactive pools are shown. Data are representative of three independent experiments. Error bars represent SD.
cells compared with WT cells (Fig. 6E). These results suggest that HuR is not only important for mRNA stabilization of a subset of IL-17–induced ARE-mRNAs but is also essential for their translation.

HuR depletion reduces IL-17–induced pulmonary inflammation

The main function of IL-17 is to coordinate local tissue inflammation via the upregulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines. We previously reported that epithelial-derived Act1 is required for IL-17–induced neutrophilia in the airway and for allergic pulmonary inflammation (36). Furthermore, IKKi, the kinase that mediates IL-17–induced mRNA stabilization, is also required for IL-17–induced neutrophilia and pulmonary inflammation. Because our study showed that HuR functions downstream of IKKi, mediating chemokine mRNA stabilization ex vivo, we examined the importance of HuR in this in vivo inflammation model. The SFTPC (Surfactant Protein C or SP-C) gene promoter was used to generate the SP-C-rtTA/tetO-CMV-Cre–transgenic mice that allow doxycycline-regulated expression of Cre recombinase in the distal-lung respiratory epithelium. HuR<sup>Box/wt</sup> mice were bred onto the SP-C-rtTA/(tetO)7CMV-Cre to generate conditional distal lung–specific HuR-deficient mice [SP-C-rtTA/(tetO)7CMV-Cre-HuR<sup>flox/wt</sup> ] and control mice ([SP-C-rtTA/(tetO)7CMV-Cre-HuR<sup>flox/flox</sup> ] (Supplemental Fig. 4). These HuR conditional and control mice were administered doxycycline for 1 wk, followed by treatment with rIL-17 via intranasal injection. Twenty-four hours after challenge, the mice were analyzed for BAL fluid cells and lung inflammation. Infiltration of cells, especially neutrophils, was significantly reduced in conditional distal lung–specific HuR-deficient mice [SP-C-rtTA/(tetO)7CMV-Cre-HuR<sup>flox/flox</sup> ] compared with that in control mice [SP-C-rtTA/(tetO)7CMV-Cre-HuR<sup>flox/wt</sup> ] (Fig. 7A). Histological analysis of lung tissue showed that HuR deficiency in distal lung epithelium attenuated IL-17–induced neutrophilia and pulmonary inflammation in vivo, demonstrating the biological importance of HuR.

Discussion

IL-17, a signature cytokine produced by pathogenic Th17 cells, plays a critical role in the pathogenesis of many autoimmune inflammatory diseases. Although IL-17 induces transcriptional activation of inflammatory genes, it also impacts their production at the posttranscriptional level (23). Elucidation of signaling mechanisms that govern IL-17–induced gene expression and protein production is crucial for the development of new therapeutic strategies to attenuate this major proinflammatory pathway. In this study, we report a new function of RNA-binding protein HuR in IL-17–induced Act1-mediated chemokine mRNA stabilization. HuR depletion substantially impaired IL-17–induced CXCL1 and CXCL5 expression due to increased mRNA decay, in contrast to the prolonged mRNA half-life in cells deficient in SF/ASF, a destabilizing factor in the IL-17 pathway. Although the interaction of Act1 with SF2 results in dissociation of SF2 from CXCL1 mRNA, IL-17–induced Act1–HuR interaction and subsequent Act1-mediated HuR ubiquitination promote the binding of HuR to CXCL1 mRNA, leading to mRNA stabilization. Although Act1 used the same TRAFs (TRAF2/5) to form the Act1–TRAF2/5–SF2 and Act1–TRAF2/5–HuR complexes, HuR and SF2/ASF interaction was not detected, indicating the action of two distinct complexes in the IL-17–induced mRNA stabilization process. Furthermore, IL-17 stimulation induced the coshift of Act1 and HuR toward the polysomal fractions in a sucrose gradient, implicating the importance of the Act1–HuR complex in moving the mRNAs to the translation-active state. Moreover, HuR deficiency in distal lung epithelium attenuated IL-17–induced neutrophilia and pulmonary inflammation in vivo, demonstrating the biological importance of HuR.

We showed previously that IKKi is activated upon IL-17 stimulation and acts as a critical upstream kinase of Act1 (34). The serine in the Act1 molecule targeted by IKKi is located adjacent to a putative TRAF-interacting consensus motif (P/S/T/A-X-Q/E-acidic/polar). IKKi deficiency and mutation of Act1–Ser<sup>311</sup> abolished IL-17–induced Act1-mediated chemokine mRNA stabilization, whereas the Act1–TRAF6–NF-kB axis was retained. Consistent with this, although IL-17 induced the interaction of HuR with Act1, TRAF2, and TRAF5, HuR–TRAF6 interaction was not detected, indicating the action of two distinct complexes in the IL-17–induced mRNA stabilization process. Furthermore, IL-17 stimulation induced the coshift of Act1 and HuR toward the polysomal fractions in a sucrose gradient, implicating the importance of the Act1–HuR complex in moving the mRNAs to the translation-active state. Moreover, HuR deficiency in distal lung epithelium attenuated IL-17–induced neutrophilia and pulmonary inflammation in vivo, demonstrating the biological importance of HuR.

FIGURE 7. HuR regulates IL-17–induced gene expression in vivo. (A) Total BAL fluid and differential cell counts in samples from HuR<sup>Box/wt</sup> (HuR WT) and HuR<sup>Box/flox</sup> (HuR KO) mice expressing SP-CrtTA/tetO-CRE (<i>n</i> = 6/group) administered doxycycline for 1 wk and then left unchallenged (Control) or challenged for 24 h by intranasal injection of IL-17 (0.5 μg). (B) Lung sections of saline-challenged (NT) and IL-17–challenged mice, as described in (A) (H&E; original magnification ×100). (C) ELISA of CXCL1 in BAL fluid from control or IL-17–challenged mice, as described in (A). (D) Real-time PCR analysis of Cxcl1 in lung tissue from control or IL-17–treated mice, as described in (A). Results are presented in arbitrary units relative to the expression of β-actin mRNA. Data are representative of two independent experiments. Data are mean and SD in (A), (C), and (D). *<i>p</i> < 0.05, two-tailed <i>t</i> test.
interaction was not detected. It is possible that when HuR is recruited to the Act1–TRAF2/5 complex, a conformational change takes place, allowing direct contact between Act1 and HuR for Act1 to mediate HuR polyubiquitination.

Although polyubiquitination with the ubiquitin linked through Lys48 targets a protein for proteasomal degradation, polyubiquitination chains linked through Lys63 of ubiquitin mediate protein–protein interactions and cell signaling (37). We showed previously that Act1 uses the Ubc13–Uev1A E2 complex to exert K63-linked polyubiquitination of TRAF6, which is required for TRAF6-mediated activation of TAK1 and the IKK complex, resulting in activation of transcription factor NFκB21. In this study, we found that Act1, together with the Ubc13–Uev1A E2 complex, ubiquitinates HuR, which is critical for HuR to bind and stabilize chemokine mRNAs. Interestingly, a previous study showed that heat shock potently induces HuR ubiquitination, followed by proteasome-mediated HuR protein degradation, implicating Lys48-linked HuR polyubiquitination (38). It is important to note that IL-17 stimulation does not induce HuR degradation, consistent with the fact that Act1 mediates K63-linked polyubiquitination through the Ubc13–Uev1A E2 complex. Future studies are required to investigate additional IL-17–induced HuR modification, especially phosphorylation, because phosphorylation often proceeds ubiquitination.

The important question is how HuR stabilizes mRNAs. HuR was shown to bind and increase the stability of a large number of functionally different ARE-containing transcripts, including c-Fos, c-Myc, cyclooxygenase-2, TNF-α, and IL-3 (39–42). HuR, a member of the embryonic lethal abnormal vision family of RNA-binding proteins, contains three classical RNA-binding domains (RNA-recognition motifs) implicated in ARE recognition (43). It was proposed that HuR stabilizes ARE-mRNAs by competing with destabilizing proteins for common binding sites, thus preventing the recruitment of deadenylases and exonucleases and the degradation of the transcripts (44, 45). In this study, we show that IL-17 stimulation induced the dissociation of destabilizing factor SF2 from CXCL1, whereas association of HuR with the transcript increased. Importantly, HuR and SF2 were not present in the same complex, suggesting that HuR may compete with SF2 for binding to the ARE-mRNAs in response to IL-17 stimulation. One novel finding is that Act1-mediated HuR ubiquitination facilitated the RNA binding of HuR. Future studies are required to elucidate the molecular mechanism for how the ubiquitination of HuR might affect its RNA binding.

It is known that HuR binding to its target mRNAs contributes to their protection from degradation, as well as favors their translation (46). We found that IL-17 stimulation induced the increased association of HuR with polysomal fractions, promoting protein translation. It is intriguing that IL-17 treatment also induced the shift of Act1 to the polysomal fractions and formation of the Act1–mRNA complex. Because there is no detectable RNA-binding domain in Act1, it is likely that the association of Act1 with RNA is through HuR or other RNA-binding proteins. Additionally, it is intriguing that the IL-17–dependent modified forms of Act1 and HuR were colocalized in the membrane (P100) fraction, which could represent polysomal-containing rough endoplasmic reticulum. Future studies are required to investigate the detailed mechanism by which Act1–HuR associates with polyosomes and promotes protein translation of ARE-mRNAs.

It is important to note that HuR deficiency in distal lung epithelium led to decreased IL-17–induced neutrophilia and lung inflammation. The decreased inflammation phenotype correlated with decreased levels of CXCL1 (a potent neutrophil chemokine) in the BAL fluid and lung. These results are consistent with our previous findings about the critical role of epithelial-specific Act1 signaling in IL-17–mediated lung inflammation (36). Taken together, these results suggest that HuR-mediated chemokine mRNA stability has an important impact on IL-17–induced neutrophilia and pulmonary inflammation. As a future study, it will be critical to evaluate the importance of HuR in a more physiologically relevant airway-inflammation model. Because HuR is also a downstream molecule for other inflammatory pathways, including TLR signaling, it is important to identify pathway-specific HuR targets to evaluate the impact of HuR, in a pathway-specific manner, on the pathogenesis of airway inflammation.

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Disclosures

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References


