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IL-17 contributes to inflammatory response in part by promoting enhanced expression of chemokines, such as CXCL1, by prolonging the $t_{1/2}$ of this constitutively unstable mRNA. Although IL-17 is a weak stimulus for transcription of the *CXCL1* gene, it strongly potentiates message accumulation via stabilization when the mRNA is transcribed in cells stimulated with TNF. In myeloid cells, LPS-induced CXCL1 mRNA stabilization is dependent on AUUUA-containing sequence motifs that are recognized by the RNA binding protein tristetraprolin (TTP). Using deletion and site-specific mutagenesis, we report that IL-17–mediated stabilization of CXCL1 mRNA in nonmyeloid cells depends on a sequence that does not contain the AUUUA motif. Furthermore, a specific two-nucleotide mutation within this region markedly abrogates sensitivity for IL-17–mediated stabilization. Consistent with this finding, the IL-17–sensitive sequence does not exhibit increased instability in the presence of TTP, and CXCL1 mRNA remains unstable and can be stabilized in response to treatment with IL-17 in embryo fibroblasts from mice in which the *TTP* gene has been deleted. Whereas the RNA binding protein KSRP has been shown to participate in regulating the instability of human CXCL8 mRNA, inhibitory RNA-based reduction in KSRP does not effect the instability mediated by the IL-17–sensitive sequence motif. These findings suggest that IL-17–mediated chemokine mRNA stabilization in nonmyeloid cells uses a mechanism that is distinct from that operating to control AU-rich mRNA stability in myeloid cells. *The Journal of Immunology*, **2010, 184: 1484–1491.**

Interleukin-17 is now widely recognized as an important regulatory cytokine participating in chronic and acute inflammatory responses that are often associated with autoimmune disease (1–3). IL-17 is known to promote enhanced expression of multiple proinflammatory cytokines, particularly members of the CXC chemokine family that function to recruit neutrophils at sites of acute injury and infection. IL-17 isoforms A and F operate through interaction with a dimeric cell surface receptor that has been linked with multiple downstream signaling events, including the activation of a number of transcription factors, chief among which is NFκB (2, 4, 5). The magnitude of NFκB and the associated transcriptional response to IL-17 is, however, modest by comparison with other well-studied proinflammatory cytokine signals (6, 7). Indeed, IL-17 is known to function cooperatively with other stimuli (i.e., TNF) to promote strong chemoattractant and cytokine gene expression (7, 8). Multiple laboratories have reported recently that IL-17 can promote enhanced gene expression by prolonging the $t_{1/2}$ of normally unstable cytokine and chemokine mRNAs transcriptionally induced by TNF (7, 9–11).

Many inflammatory cytokine and chemokine mRNAs are known to be unstable with half-lives in the range of minutes to several hours (12–14). This characteristic prevents the accumulation of potentially dangerous gene products under inappropriate conditions. In some cases, however, the instability mechanisms can be transiently overcome in response to extracellular stimulation, allowing rapid accumulation of mRNA and associated protein production. Both instability and stimulus-induced stabilization are properties dependent on the nucleotide sequence usually located within the 3′ untranslated region of the mRNA (UTR). Perhaps best studied are adenine–uridine-rich sequence elements (AREs) found in many cytokine and chemokine mRNA 3′UTRs (14, 15). The most commonly recognized sequence includes a central AUUUA pentameric motif often flanked by additional U or A residues (16). These sequence motifs are recognized by RNA binding proteins that function to promote either enhanced decay or stability. Multiple genes encoding proteins with binding specificity for AREs have been identified, cloned, and studied (17–22). Those known to promote enhanced decay include tristetraprolin (TTP), AUF-1 (also known as hnRNP D), and KSRP (a splicing enhancer also associated with mRNA instability), whereas HuR (a ubiquitously expressed member of the ELAV family) is associated with increased stability. TTP exhibits the most stringent specificity for the AUUUA motif and appears to function by promoting association of decapping and exonuclease activities with ARE-containing mRNAs in subcellular loci known as P bodies (23–25). Studies of a number of specific mRNAs including those encoding TNF, COX2, and CXCL8 have demonstrated that the pentamer sequences are able to confer both instability and sensitivity to stabilization in response to extracellular stimuli (21, 26–28). There is also evidence that AU-rich regions that have no AUUUA pentamers are capable of promoting rapid decay, although the contribution of such sequences to stimulus-induced stabilization has not been extensively investigated (29–31).

The mouse chemokine CXCL1 mRNA has served as a model for the study of mechanisms leading to message instability and IL-17–dependent stabilization (7, 32). The 3′UTR from CXCL1 mRNA

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Abbreviations used in this paper: Act D, actinomycin D; ARE, adenine–uridine-rich sequence element; Dox, doxycycline; MEF, mouse embryonic fibroblast; siCTRL, control small interfering RNA; siKSRP, siRNA targeting KSRP; siRNA, small interfering RNA; TIR, Toll IL-1 receptor; TTP, tristetraprolin; UTR, untranslated region of mRNA.

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confers both instability and potent sensitivity for stimulus-dependent prolongation of $t_{1/2}$ (33–35). This mRNA contains a clustered series of four overlapping AUUUA motifs along with three isolated pentamer elements within ~600 nucleotides composing the 3′UTR. The mRNA exhibits a $t_{1/2}$ of ~30–45 min that can be prolonged 2- to 4-fold following stimulation through multiple Toll IL-1 receptor (TIR) family members as well as by IL-17 (6, 7, 34–36). The AUUUA motifs within the CXCL1 3′UTR confer sensitivity to the destabilizing protein TTP and indeed, in macrophages from mice deficient for the TTP gene, CXCL1 mRNA remains highly stable (37). The ability of IL-17 to prolong TNF-induced CXCL1 expression is seen largely in nonmyeloid cell populations where TTP expression could be relatively low (21, 38, 39). Moreover, the signaling pathways used by IL-17 appear to be at least partially distinct from those used by TIR family receptors (6). In this study, we have evaluated the sequence within the CXCL1 3′UTR that is necessary to confer sensitivity to IL-17 in several nonmyeloid cell populations. The results demonstrate that the 3′UTR of CXCL1 contains an AUUUA-free segment that provides TTP-independent instability, which can be markedly stabilized in response to stimulation with IL-17.

Materials and Methods

Reagents

DMEM, RPMI 1640, Dulbecco's PBS and antibiotics were obtained from Central Cell Services of the Lerner Research Institute (Cleveland, OH). Neomycin sulfate (G418), formamide, dextran sulfate, MOPS, diethylpyrocarbonate, actinomycin D (Act D), salmon sperm DNA, and protease inhibitor mixture were purchased from Sigma-Aldrich (St. Louis, MO). FBS was purchased from Atlas Biologicals (Fort Collins, CO). Doxycycline (Dox) and the vector pTRE2 were obtained from Clontech Laboratories (Palo Alto, CA). Random priming kits were purchased from Stratagene (Cedar Creek, TX). RNase-free DNAse was obtained from Promega (Madison, WI). Nylon transfer membrane was purchased from Micron Separation (Westboro, MA). Superfect and Polyfect Transfection Reagents were obtained from Qiagen (Valencia, CA) and Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH). Recombinant human IL-17, mouse TNF, and mouse IL-17 were purchased from R&D Systems (Minneapolis, MN). Dupont-New England Nuclear (Boston, MA) was the source for (α-[^{32}P])-dCTP. Protogel, Sequagel (acrylamide, N, N-methylene bis-acrylamide, urea), and related buffers were obtained from National Diagnostics (Atlanta, GA). Protein assay reagents were purchased from Bio-Rad Laboratories (Richmond, CA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Ab against KSRP was kindly provided by Robert Gherzi (University of Genoa, Italy). Control or KSRP-specific double stranded small interfering RNA (siRNA) oligonucleotides were obtained from Dharmacon (Lafayette, CO). The KSRP target sequence was 5′-GAGAUCAACCGGAGAGCAAGA-3′, as described previously (20).

Plasmids

Radiolabeled cDNA probes for use in northern hybridization analysis were prepared from plasmids containing fragments of GAPDH, CXCL1, or IκBζ in the Bluescript vector as described previously (40). Plasmids used to drive expression of different versions of CXCL1 mRNA were prepared in pTRE2 (Clontech). The parent clone was created by insertion of the full CXCL1 5′UTR and coding region (357 nucleotides) into the *Bam*[^{1}H]/Not1 sites of pTRE2, and the 3′UTR was provided from the rabbit β-globin gene. Additional constructs were created by excising the rabbit β-globin region with Xba1 and Sap1, and different versions of the CXCL1 3′UTR sequence were inserted in the remaining *Eco*RV site. The full length CXCL1 3′UTR contains 591 residues (Fig. 1). The Δ1 clone contains residues 467–949 (numbered according to National Center for Biotechnology Information reference sequence NM_008176.3). The sequential deletions from the 5′ end of the Δ1 fragment were made at residues 718 (Δ3), 805 (Δ4), and 866 (Δ5) and extended 3′ to residue 949. An internal deletion construct was made between residues 805 and 866 (Clu-P3). The mt1, mt2, and mt3 mutations were UU to CG transitions in residues 819–820, 851–852, and 860–861, respectively, and were mutated in the CXCL1(Δ4) version using oligonucleotide site-directed mutagenesis as described previously (34, 38). The mt2 motif was also mutated in the CXCL1(Δ1) version. The full length human CXCL8 3′UTR was cloned from IL-1 stimulated HeLa cell total RNA by RT-PCR with primers flanking the beginning and end of the sequence, but without the polyA tail. A plasmid encoding the full-length human TTP cDNA containing a hemagglutinin epitope tag under control of the CMV promoter (pCMV.hTTP.tagHA) was provided by Dr. Perry Blackshear (21).

Cell culture and transfection

The HeLa Tet-Off cell line was obtained from Clontech and was maintained as described previously (7). Transient transfections were done using Polyfect Transfection Reagent according to the manufacturer's protocol; 293 Tet-Off cells were used as described previously (36). Double-stranded siRNA oligonucleotides were transfected using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Mouse embryo fibroblasts were prepared from littermates of TTP$^{+/-}$ heterozygote crossings as described previously (37), and the genotype for each embryo was determined as described above. Mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin as described previously (41).

Measurements of RNA stability

HeLa Tet-Off or 293 Tet-Off cells were transfected and after 3 h, subdivided into 60-mm dishes and rested for 18 h before individual treatments. CXCL1 mRNA transcription was terminated by the addition of Dox (1 μg/ml) and total RNA was prepared at the indicated times using Tri-Reagent following the manufacturer's instructions. Total RNA preparations were digested with RNase-free DNase to eliminate residual plasmid DNA before analysis of specific mRNA content by northern blot hybridization as described previously (35, 42). In experiments using MEFs, cell cultures were grown to confluence treated with various stimuli for the indicated times as described in the text. Decay was assessed following the addition of Act D (5 μg/ml) for various times before preparation of total RNA and analysis of CXCL1 and GAPDH mRNA levels as described above. Autoradiographs were quantified using the National Institutes of Health Image software and the levels of CXCL1 mRNA were normalized to those for GAPDH mRNA in each sample.

Results

Previous work has shown that TTP and AUUUA-containing sequence regions are necessary for instability of CXCL1 mRNA in primary mouse macrophages and in TTP-deficient cultured nonmyeloid (HEK293) cells transfected with TTP (37). Nevertheless, CXCL1 mRNA in which all AUUUA motifs were mutated retained instability even in the absence of TTP. Therefore, we wished to identify what regions of CXCL1 mRNA 3′UTR were required for TTP-independent instability and for sensitivity to IL-17–mediated stabilization. For this purpose we have evaluated CXCL1 3′UTR sequence motifs using HeLa Tet-Off cells, in which IL-17 promotes a strong stabilization signal (7). Consistent with previous studies, a CXCL1 transgene containing a region of the 3′UTR spanning residues 467–949 (fragment Δ1; Fig. 1) exhibits strong transgene expression in HeLa Tet-Off cells and when the cultures are treated with Dox, transgene transcription is terminated and the mRNA decays rapidly (Fig. 2) (33). The addition of IL-17 can prolong the $t_{1/2}$ of this message from 25 min to >50 min. The blots from three separate experiments were quantified and the data used to calculate the $t_{1/2}$ for the mRNA with and without stimulation. The mean $t_{1/2}$ data are also presented (Fig. 2*C*). The three remaining AUUUA pentamers in the Δ1 construct have been shown to contribute to instability and LPS-induced stabilization through the action of TTP in mouse macrophages (37). To determine whether these motifs are required for the instability that is sensitive to the action of IL-17, a construct was prepared in which all three remaining pentamers within the Δ1 construct were mutated by substituting CG for two of the three internal U residues (AUUUA to AUCGA) and transfected in HeLa Tet-Off cells (Fig. 2). The mRNA derived from this construct exhibited only modest loss of instability and retained strong sensitivity to IL-17, compared with the wild type CXCL1 mRNA. This finding demonstrates that AUUUA pentamer motifs are not required for the stabilization of CXCL1 mRNA in response to IL-17.

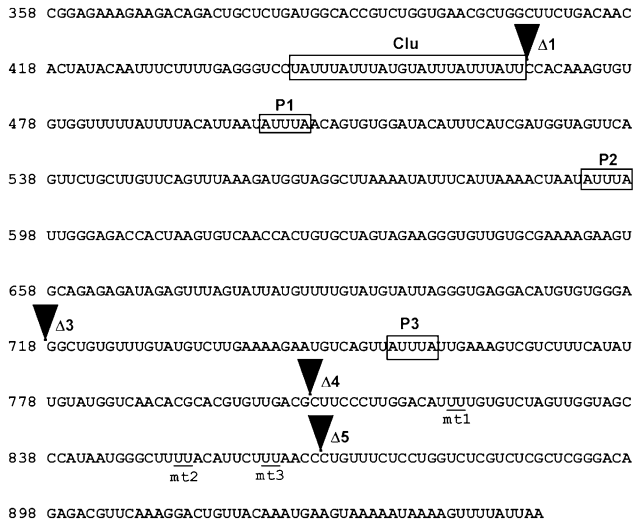

FIGURE 1. Sequence of the CXCL1 mRNA 3′UTR. The 591 nucleotide CXCL1 3′UTR is illustrated. The sequence contains seven separate AUUUA motifs (boxed). Four of these are contained within a cluster that includes two sets of two overlapping pentamers (Clu). The remaining three independent pentamers are designated as *P1*, *P2*, and *P3*. Three separate UU residues selected for mutagenesis are underlined labeled *mt1*, *mt2*, and *mt3*. The positions at which deletions Δ1, Δ3, Δ4, and Δ5 were made are indicated by arrows.

To more precisely identify the regions of the CXCL1 3′UTR that are responsible for sensitivity to IL-17–mediated stabilization, HeLa Tet-Off cells were transfected with a series of plasmids encoding tet-regulated 3′UTR deletion mutants of CXCL1 mRNA. The $t_{1/2}$ of each was monitored in the absence or presence of IL-17 (Fig. 3). Consistent with the prior experiment, incremental removal of segments of 3′UTR sequence containing AUUUA motifs produced modest but incomplete reduction of instability (i.e., prolonged the $t_{1/2}$) yet retained the sensitivity for IL-17–mediated stabilization. Whereas the mean $t_{1/2}$ of mRNA derived from the Δ1 construct was 25 min and could be increased to 51 min in cells treated with IL-17, the $t_{1/2}$ of a message derived from the Δ4 construct, which contains no AUUUA motif, was 60 min and could be enhanced to 135 min in the presence of IL-17 (Fig. 3*B*). The loss of instability but sparing of stimulus-sensitive stabilization suggests that removal of sequences containing the AUUUA motif eliminates IL-17–insensitive instability motifs while the remaining sequence retains a distinct region that confers IL-17–sensitive instability. Deletion of a fragment from the Δ4 construct between residues 718 and 805 (generating the Δ5 construct) resulted in a dramatic loss of instability. Finally, a construct (Clu-P3) with an internal deletion between residues 718 and 805 generates a message exhibiting intermediate instability that has lost sensitivity for IL-17–driven stabilization. Hence, the region between residues 718 and 805 is required for both instability and IL-17 sensitivity.

To identify critical residues necessary for IL-17–sensitive instability within the CXCL1 mRNA 3′UTR, we introduced mutations in several uridine residues within the 70 nucleotide portion of the Δ4 fragment that retains this activity (Fig. 1). Three separate mutants were created, in each of which two uridine residues were changed to CG. Although mRNA containing mt3 produced no significant change in either instability or stabilization in response to IL-17, the mt1 (at residues 819–820) and the mt2 (at residues 851–852) both produced substantial loss of instability (Fig. 4*A*). When the mt2 mutation was examined in the context of the larger Δ1 fragment, instability was modestly reduced but IL-17 sensitivity was fully abrogated (Fig. 4*B*). These results demonstrate convincingly that these sites within the instability region are critical for AUUUA-independent instability and IL-17 sensitivity.

We have previously shown that CXCL1 mRNA instability and LPS-mediated stabilization in mouse macrophages are dependent on the 7 AUUUA sequences and expression of TTP (37). To determine whether the IL-17–sensitive instability of CXCL1 mRNA requires TTP, we evaluated the ability of IL-17 to stabilize TNF-induced CXCL1 mRNA in MEFs obtained from matched wild type and TTP-deficient mice. Cultures were stimulated with either TNF or IL-17 alone or TNF in combination with IL-17 for 1 h followed by the addition of Act D, and total RNA was prepared at the indicated times to determine the residual levels of CXCL1 and GAPDH mRNA (Fig. 5*A*). In wild type MEFs, TNF-stimulated CXCL1 mRNA was highly unstable ($t_{1/2}$ = 18 min), whereas the addition of IL-17 alone produced only a modest signal but with a prolonged $t_{1/2}$ (90min). The combination of TNF and IL-17 results in a marked increase in starting mRNA levels and

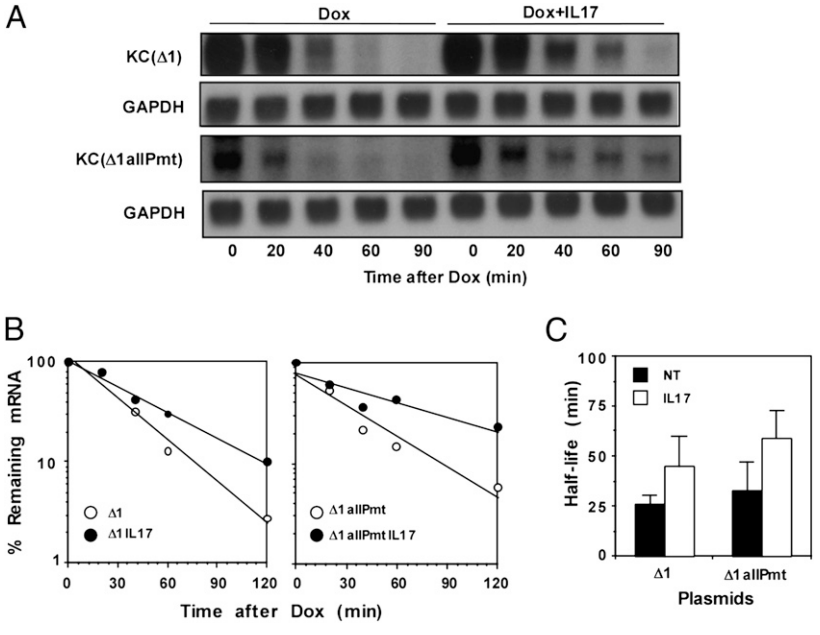

FIGURE 2. CXCL1 mRNA instability and IL-17–induced stabilization do not require AUUUA motifs. *A*, HeLa Tet-Off cells were transiently transfected with pTRE2 containing the 5′UTR and coding region of CXCL1 with a fragment spanning residues 467–949 of the 3′UTR. The three AUUUA pentamers were either wild type [CXCL1(Δ1)] or mutated to AUCGA [CXCL1(Δ1allPmt)]. After overnight culture, plates were treated with Dox alone or in combination with IL-17 (25 ng/ml). Incubation continued for the indicated times prior to preparation of total RNA and analysis of CXCL1 and GAPDH mRNA levels by northern hybridization. *B*, The autoradiographs were quantified (levels of CXCL1 mRNA normalized to those for GAPDH) and presented graphically. *C*, The mean $t_{1/2}$ of CXCL1 mRNA in either untreated or IL-17–stimulated cells was calculated from the slope of the decay plot for each of three experiments ±1 SD.

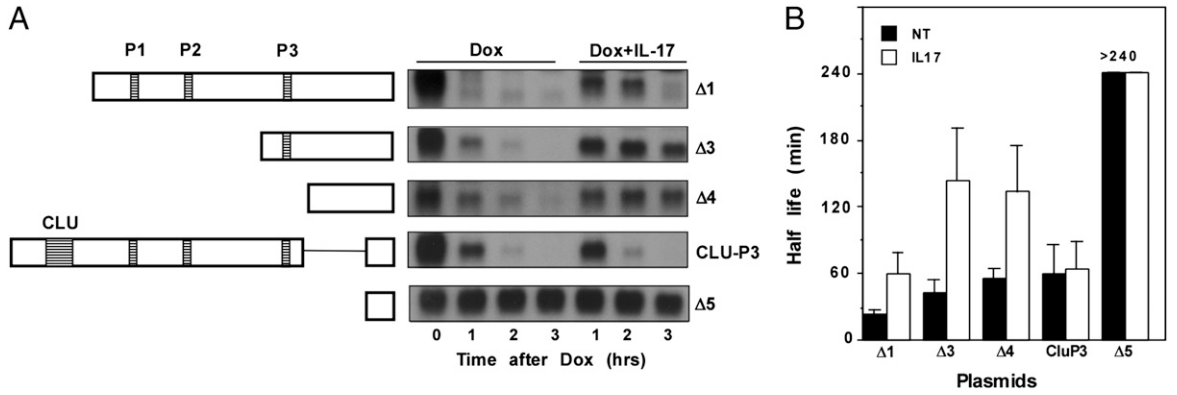

FIGURE 3. Identification of the IL-17–sensitive instability region in CXCL1 mRNA. *A*, HeLa Tet-Off cells were transiently transfected with pTRE2 containing the CXCL1 5′UTR and coding region with the indicated segments of the 3′UTR (as described in *Materials and Methods*). After overnight culture, plates were treated with Dox alone or in combination with IL-17. Incubation continued for the indicated times prior to preparation of total RNA and analysis of CXCL1 and GAPDH (not shown) mRNA levels by northern hybridization. *B*, The mean $t_{1/2}$ of each mRNA species in either untreated or IL-17–stimulated cells was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD.

substantial stabilization ($t_{1/2}$ = 90 min). In TTP-deficient MEFs, TNF-induced CXCL1 mRNA remains unstable ($t_{1/2}$ = 40 min), athough relative to wild type cells, the $t_{1/2}$ is prolonged, suggesting that a portion of the instability of the endogenous message is determined by TTP. Importantly, even in the absence of TTP IL-17 is able to promote substantially prolonged $t_{1/2}$ either alone or in combination with TNF. Hence IL-17–mediated stabilization of CXCL1 does not depend on TTP-mediated instability, rather it appears to act through a TTP-insensitive motif. To determine whether the stabilization of other IL-17–inducible mRNAs is also independent of TTP, we examined the $t_{1/2}$ for IκBζ mRNA in MEFs from $TTP^{+/+}$ and $TTP^{-/-}$ MEFs (Fig. 5*B*). In both wild type and TTP-deficient cells, TNF-induced IκBζ mRNA is highly unstable and its $t_{1/2}$ can be prolonged by including IL-17 with TNF.

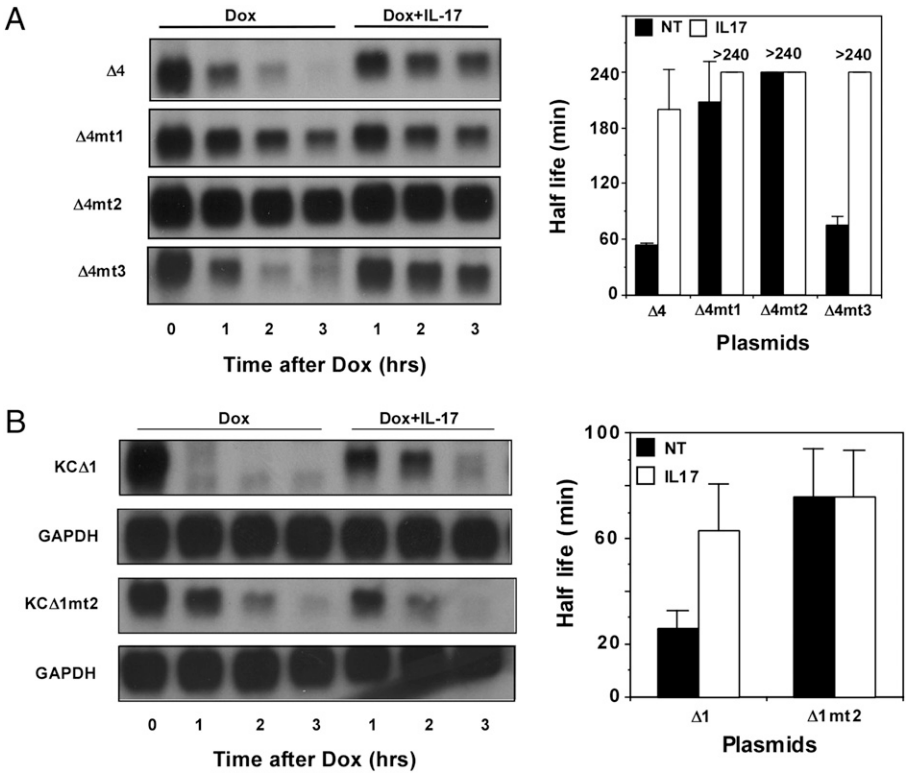

FIGURE 4. Specific nucleotide sequence requirements within the IL-17–sensitive CXCL1 mRNA instability region. *A*, HeLa Tet-Off cells were transiently transfected with pTRE2 containing the CXCL1 5′UTR and coding region linked with one of four versions of the Δ4 fragment of CXCL1 3′UTR, including a wild type version and three separate mutants described in *Materials and Methods* and Fig. 1. After overnight rest, the cultures were treated with Dox alone or Dox with IL-17 for the indicated times prior to preparation of total RNA and analysis of CXCL1 and GAPDH (not shown) mRNA by northern hybridization. Autoradiographs were quantified and the mean $t_{1/2}$ of each mRNA species in either untreated or IL-17–stimulated cells was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD. *B*, HeLa Tet-Off cells were transiently transfected with pTRE2 containing the CXCL1 5′UTR and coding region linked with either wild type Δ1 fragment or Δ1 containing the mt2 mutation (as in *A*). After overnight rest, the cultures were treated with Dox alone or Dox with IL-17 for the indicated times prior to preparation of total RNA and analysis of CXCL1 and GAPDH mRNA by northern hybridization. Autoradiographs were quantified and the mean $t_{1/2}$ of each mRNA species in either untreated or IL-17–stimulated cells was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD.

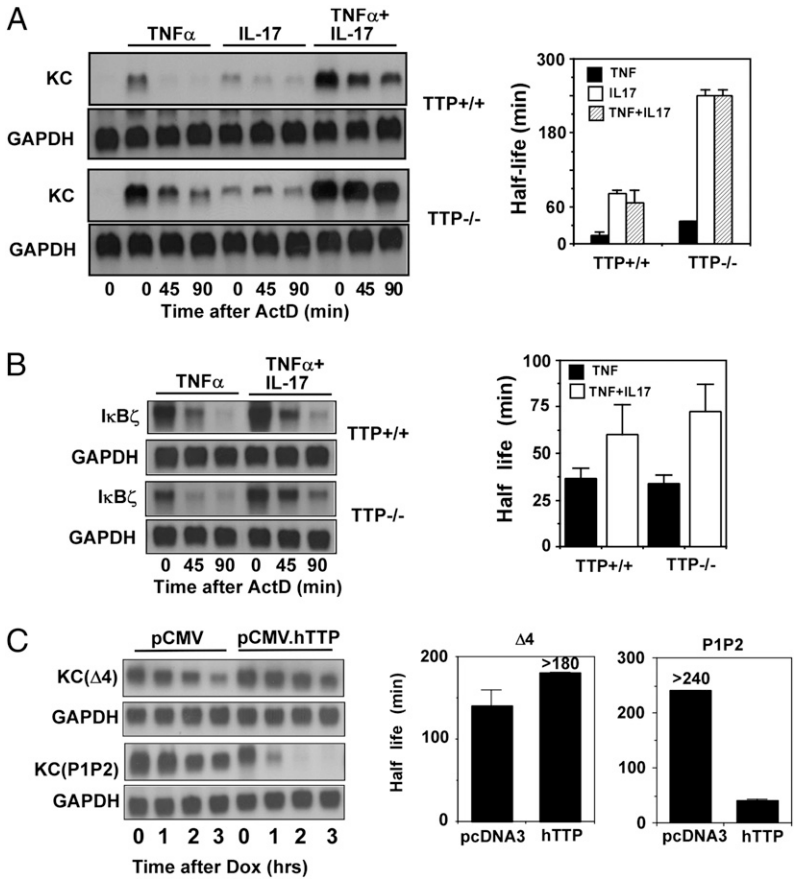

FIGURE 5. The IL-17–sensitive instability region in CXCL1 mRNA is not sensitive to TTP. *A*, Wild type and TTP$^{-/-}$ MEFs were treated with TNF (10 ng/ml), IL-17 (25 ng/ml), or TNF + IL-17 for 1 h prior to the addition of Act D (5 μg/ml). Total RNA was prepared immediately after the indicated incubation times, and CXCL1 and GAPDH mRNA levels were determined by northern blot hybridization. Autoradiographs were quantified, and the mean $t_{1/2}$ of each mRNA species in each treatment condition was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD. *B*, Wild type and TTP$^{-/-}$ MEFs were treated with TNF (10 ng/ml) alone or in combination with IL-17 (25 ng/ml) for 1 h prior to the addition of Act D (5 μg/ml). Total RNA was prepared immediately after the indicated incubation times, and IκBζ and GAPDH mRNA levels were determined by northern blot hybridization. Autoradiographs were quantified and the mean $t_{1/2}$ of each mRNA species in each treatment condition was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD. *C*, 293 Tet-Off cells were transiently cotransfected with pTRE2 containing the CXCL1 5′UTR and coding region linked with the P1P2 or Δ4 fragments of CXCL1 3′UTR and either pCMV or pCMV.hTTP.tagHA. After overnight rest, the cultures were treated with Dox for the indicated times prior to preparation of total RNA and analysis of CXCL1 and GAPDH mRNA by northern hybridization. Autoradiographs were quantified, and the mean $t_{1/2}$ of each mRNA species in either empty vector or TTP-transfected cells was calculated from the slope of decay plots (not shown) for each of three experiments ±1 SD.

To directly assess whether the IL-17–sensitive sequence fragment is a target for TTP-mediated destabilization, the CXCL1(Δ4) construct was cotransfected into TTP-deficient HEK293 Tet-Off cells (33, 43) with an expression plasmid encoding human TTP (pCMV.hTTP.tagHA) or empty pCMV. After overnight culture, Dox was added and mRNA levels were determined at the indicated times (Fig. 5*B*). The coexpression of TTP did not result in more rapid decay of mRNA derived from the CXCL1(Δ4) construct; indeed, the decay appears to be somewhat prolonged by the presence of TTP. Another construct containing a segment of the CXCL1 3′UTR with two isolated pentamers (nucleotides 468–633, CXCL1[P1P2]) is relatively stable in HEK293 Tet-Off cells, but is degraded rapidly when coexpressed with TTP, demonstrating the destabilizing activity of the transgenic protein.

In addition to TTP, other RNA binding proteins exhibiting specificity for ARE motifs have been identified, and in some cases have been shown to possess the ability to modulate the rate of mRNA decay in a sequence-specific fashion (18–20, 22). In particular, we wanted to explore the possible contribution of the protein KSRP because it has been implicated recently in the regulation of mRNA encoding another neutrophil targeting human chemokine (IL-8) (44). To explore the possible role of KSRP in mediating decay of mRNA containing the CXCL1(Δ4) sequence, we used siRNA to deplete the expression of KSRP and determined the effect on the $t_{1/2}$ of CXCL1 mRNA. HeLa Tet-Off cells were transfected with siRNA targeting KSRP for 24 h before a second transfection with either the CXCL1(Δ4) plasmid or a plasmid encoding the CXCL1 coding region linked with the full 3′UTR from human IL-8 as a positive control. The KSRP-specific siRNA produced substantial reduction in the targeted protein, compared with a control siRNA (Fig. 6*A*). Although this treatment modulated the instability of the mRNA containing the CXCL8 3′UTR, there was no impact on the $t_{1/2}$ of mRNA containing the IL-17–sensitive region of CXCL1 mRNA (Fig. 6*B*, 6*C*).

Discussion

IL-17 is known to enhance the expression of several proinflammatory cytokines in part through mechanisms that prolong the $t_{1/2}$ of the short-lived mRNAs (7, 9–11). The instability of such mRNAs and the modulation of decay by multiple extracellular stimuli has been linked with the presence of AU-rich motifs within the 3′UTRs, particularly those containing the pentameric sequence AUUUA (14–16). Such sequences appear to function via interaction with RNA binding proteins that can modulate the process of mRNA degradation (17–22). In prior studies, we demonstrated that the expression of CXCL1 mRNA, which contains multiple AUUUA motifs, is highly sensitive to the action of the destabilizing protein TTP in primary macrophages (37). In these cells, TTP is the major mediator of instability and is likely to be the target for the stabilizing action of stimuli acting through Toll IL-1 family receptors such as LPS. In the current study, we wished to determine whether these same sequences and RNA binding proteins were responsible for CXCL1 mRNA instability that is subject to stabilization in response to IL-17 in nonmyeloid cells. The results demonstrate: 1) the AUUUA motifs within the CXCL1 mRNA 3′UTR do not confer IL-17–sensitive mRNA instability; 2) the IL-17–sensitive instability motif resides within a ~60-nucleotide segment located near the 3′ end of the 3′UTR and contains four critical uridine residues at two separate locations; and 3) the IL-17–sensitive region of CXCL1 3′UTR is not a target for the action of TTP, and TTP is not required for IL-17 to prolong CXCL1 mRNA $t_{1/2}$; and 4) reduction in level of the ARE-binding protein KSRP does not alter instability driven by the IL-17–sensitive sequence motif. Together, these findings support the existence of an instability mechanism controlling CXCL1

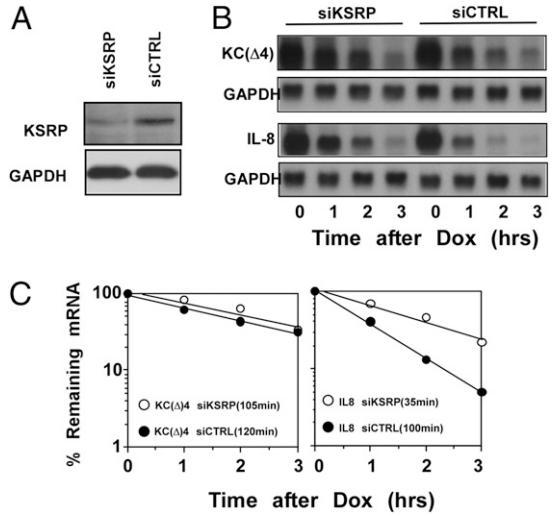

FIGURE 6. Depletion of KSRP by siRNA does not alter the function of the IL-17–sensitive instability region of CXCL1 mRNA. *A*, HeLa Tet-Off cells were transfected with a control siRNA (siCTRL) or siRNA targeting KSRP (siKSRP); 24 h later, the cultures were transfected with plasmids encoding tet-regulated CXCL1(Δ4) or human CXCL8. After overnight culture, levels of KSRP or GAPDH protein were determined in protein extracts from these cells by western blot analysis. *B*, Separate cultures were treated with Dox for the indicated times prior to determination of CXCL1 and GAPDH mRNA levels by northern hybridization. *C*, Autoradiographs were quantified and mRNA half lives, determined as described in *Materials and Methods*, are shown in each graph. Results are representative of two separate experiments.

mRNA decay that is sensitive to modulation by IL-17 and is distinct from the AUUUA/TTP-dependent stabilization mechanisms that control CXCL1 mRNA in LPS-stimulated macrophages.

There are now many reports describing the sequence determinants of instability, particularly within mRNAs encoding important components of the inflammatory response (12–15). Although these studies have frequently identified the canonical AUUUA sites as critical to promoting rapid mRNA degradation, there are also multiple examples of both noncanonical AREs and non-ARE motifs that can confer instability (15, 26, 28, 29, 45). In similar fashion, there are multiple studies demonstrating enhanced stability of unstable mRNAs following stimulation, and several of these have provided detailed characterization of the responsible sequences (26, 28, 46). These include mRNAs encoding COX2, CXCL8, and GM-CSF. In these cases, one or more core AUUUA pentamers have been reported to be a requisite feature of the regulatory element. The IL-17–sensitive regulatory site in CXCL1 mRNA identifies a cytokine mRNA sequence responsible for instability and stimulus sensitivity that does not depend either fully or partially on the canonical core pentamer sequence. It is noteworthy that this site contributes both instability and IL-17 sensitivity. In the Δ4 construct, mutations eliminate instability, hence we cannot assess IL-17 sensitivity. Nevertheless, in constructs containing other instability determinants (Clu-P3, Δ1; Figs. 3, 4), the deletion or mutation of this new element only modestly reduces instability, but does fully abrogate the ability of IL-17 to prolong $t_{1/2}$. The ability of IL-17 to stabilize IκBζ mRNA is also retained in TTP-deficient MEFs, indicating that this property is not unique to CXCL1. Finally, preliminary observations in our laboratory indicate that the IL-17–sensitive instability regions of human CXCL2 and CXCL3 do not require AUUUA pentamers (T. Hergen, M. Novotny, and T. Hamilton, unpublished observations). Indeed, sequence analysis of the full 3′UTR of IκBζ and the IL-17 sensitive regions of human CXCL2 and CXCL3 and mouse CXCL1 3′UTRs does not reveal any conserved motifs. Moreover, inspection of predicted folding conformations of these sequences does not identify any common features. Whereas these findings are consistent with the possibility that there are multiple IL-17–sensitive instability motifs and corresponding mechanisms, we cannot rule out an unidentified common feature linking all.

TTP is among the best-characterized ARE-binding proteins and is known to be responsible for promoting decay of multiple inflammation-linked mRNAs including TNF and GM-CSF (17, 47, 48). Indeed, TTP exhibits the capacity to promote instability of CXCL1 mRNA via the seven AUUUA pentamer sequence motifs and CXCL1 mRNA expression is markedly enhanced via prolongation of $t_{1/2}$ in macrophages from mice in which the TTP gene has been deleted (37). The requirement for AUUUA motifs in TTP-dependent decay of CXCL1 mRNA is supported by studies defining the sequence recognition specificity of TTP, in which the highest-affinity recognition sites were demonstrated to contain three or four uridine residues flanked by at least one adenine (49, 50). Interestingly, the two mutations that compromise instability and IL-17–induced stabilization are within sequence regions exhibiting this characteristic, but do not appear to be targets for TTP. More important perhaps is the finding that TTP-deficient MEFs retain strong sensitivity for IL-17–induced CXCL1 mRNA stabilization, indicating that TTP is not necessary for this response to IL-17. This latter idea is supported by recent studies showing that the signaling pathways downstream of the IL-17R that couple with mRNA stabilization do not require TRAF6 or activation of p38 MAP kinase pathway and are distinct from those used by several different TIRs (40). Furthermore, the IL-17–induced expression of IκBζ, an important transcription factor in the IL-17 response, involves mRNA stabilization that is independent of TRAF6/p38 and AUUUA/TTP (11, 40).

KSRP is an ARE-binding protein that has also been reported to participate in regulating mRNA instability (20, 44, 51, 52). This splicing regulatory factor has been implicated in regulating the instability of myogenic transcripts during the differentiation of myocytes and in control of β-catenin mRNA decay (51, 52). Of particular relevance, KSRP has recently been linked with control of CXCL8 mRNA stability (44). Our findings confirm this latter observation; however, they also indicate that KSRP does not contribute to the instability mediated by the IL-17–sensitive motif in CXCL1 mRNA.

Because the findings indicate that neither TTP nor KSRP are required for IL-17–sensitive instability, we have attempted to identify a protein that could distinguish between wild type and inactive mutant sequences from the region of CXCL1 mRNA responsible for IL-17–senstive instability, using either UV-crosslinking or RNA affinity chromatography. These strategies have identified proteins that associate with the RNA sequence, but there were no detectable differences observed when the inactive mutant forms were used. This negative outcome could reflect that the level of the specific protein is below a detectable level under the conditions of the experiment or that the protein might interact at sites that are not sensitive to UV-mediated crosslinking. Moreover, it is possible that binding specificity is not the sole determinant of functional outcome. In this regard, it is noteworthy that the folding conformations of the wild type and inactive mutant versions of the CXCL1 3′UTR are indistinguishable.

Our results support the concept that the control of ARE-containing mRNA turnover is likely to involve multiple independent mechanisms. In the case of CXCL1 mRNA, it is now clear that the AUUUA motifs confer instability that is mediated by TTP and regulated in response to TIR stimulation, whereas the 3′ localized non-AUUUA–containing motif confers instability that is subject to

stabilization in response to IL-17. Moreover, the TTP-dependent mechanism appears to be dominant in myeloid cell populations such as macrophages, and the IL-17–sensitive mechanism appears to more restricted to nonmyeloid cell types (e.g., HeLa and MEF cells). Interestingly, it also appears that both mechanisms (TTP-dependent and IL-17–sensitive) can operate within the same cell population (e.g., MEFs). Whereas the physiologic significance of the IL-17–sensitive mechanism remains to be explored experimentally, it is likely to be important in IL-17–dependent pathogenesis. In addition, it may be an important regulatory mechanism operating during the early stages of inflammatory stimulation in tissue sites with a paucity of resident macrophages when CXC chemokines are expressed predominantly by nonmyeloid cells resident within such sites. Finally, CXC chemokines are frequently expressed in epithelial and stromal cell-derived tumor cells, where they have been implicated as contributing to the survival and spread of tumors (53). Because such cells may be relatively deficient in expression of TTP, TTP-independent mechanisms for regulation of chemokine mRNA stability could predominate in such settings.

Disclosures

The authors have no financial conflicts of interest.

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