Published in final edited form as:

Cytokine. 2010 ; 52(1-2): 116–122. doi:10.1016/j.cyto.2010.04.003.

Diversity in Post-Transcriptional Control of Neutrophil Chemoattractant Cytokine Gene Expression

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Abstract

Regulation of neutrophil chemokine gene expression represents an important feature in tissue inflammation. While chemokine gene transcription through the action of NF κ B is recognized as an essential component of this process, it is now clear that post-transcriptional mechanisms, particularly the rates of decay of mature cytoplasmic mRNA, provides an essential component of this control. Chemokine and other cytokine mRNA half-life is known to be controlled via adenine-uridine rich sequence motifs localized within 3' untranslated regions (UTRs), the most common of which contains one or more copies of the pentameric AUUUA sequence. In myeloid cells AUUUA sequences confer instability through the action of RNA binding proteins such as tristetraprolin (TTP). The resulting instability can be regulated in response to extracellular stimuli including Toll like receptor ligands that signal to control the function of TTP through pathways involving the activation of p38 MAP kinases. Recent findings indicate that substantial mechanistic diversity is operative in non-myeloid cells in response to alternate pro-inflammatory stimuli such as IL-17. These pathways target distinct instability sequences that do not contain the AUUUA pentamer motif, do not signal through p38 MAPK, and function independently of TTP.

Keywords

chemokine; gene regulation; mRNA stability; signal transduction

INTRODUCTION

Inflammation is a complex multi-step process that operates to protect the host organism from the consequence of injury and infection and to orchestrate the restoration of normal tissue architecture and function [1]. A major feature of the inflammatory response involves the trafficking of professional inflammatory cell populations into affected tissue sites and this is regulated, in part, via the action of chemoattractant cytokines or chemokines [2,3]. Amongst the earliest responses to injury is the elevated expression of chemokines that specifically recruit granulocytic leukocytes, particularly neutrophils [2–4]. The major neutrophil-directed chemokines are members of the CXC chemokine family and include CXC ligands 1–3 and 5–8. These proteins all share the feature of a specific three amino acid motif containing glutamine,

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leucine and arginine immediately preceding the defining CXC motif. With the exception of CXCL8 or Interleukin-8, these proteins are all recognized by a single receptor (CXCR2). IL-8 is also recognized by another receptor protein (CXCR1) and both receptors are members of the G protein coupled receptor family. It is interesting that so many separate genes have evolved encoding what appear to be functionally redundant proteins and this suggests the importance of the process of regulating inflammatory cell trafficking [5]. Another rationale for such apparent redundancy is the need to encode variability into the regulation of expression such that the same function could be induced or suppressed in a relatively broad selection of physiologic or pathophysiologic circumstances. Indeed, this may be an important concept since the regulation of expression for the different neutrophil chemokines exhibits some significant differences with respect to cell type and stimulus sensitivity [6–9].

The inflammatory recruitment of neutrophils to a site of injury or infection occurs rapidly and is usually transient [1,2,4,10]. Moreover, the infiltration of tissues by neutrophils poses the potential for unnecessary tissue damage [1,4]. Hence there is substantial need to stringently regulate the events that govern neutrophil recruitment in both positive and negative fashion. Regulation of chemokine gene expression is achieved through modulation at multiple stages in the process including transcription, mRNA translation, and mRNA degradation [11–13]. The transcriptional regulation of the different ELR-CXC chemokine genes exhibits many common features but also some significant differences that remain poorly understood in mechanistic terms. The promoters for IL-8, and the GRO family (CXCL1-3) members have been explored in some detail in cells stimulated with pro-inflammatory agents acting through Toll like receptor family members or pro-inflammatory cytokines such as IL-1 and TNF α [13–15]. They are all controlled, at least in part, via the presence of one or two potent NF κ B sites located within a short distance of the transcription start site. In addition, the NF κ B dependent induction of these genes can be strongly suppressed by both type I and type II IFNs and depends upon activation of STAT1 [11,16,17]. The mechanistic basis for the inhibitory action of IFNs and STAT1 on chemokine transcription remains incompletely understood. Of interest, these genes are not equivalently induced in all cell types indicating that there must also be cell lineage dependent controls that provide additional specificity in regulation of transcription [8,9].

While rapid up-regulation of transcriptional activity on neutrophil-specific chemokine genes is recognized as an essential aspect of the initiation of the early inflammatory response, the mRNAs encoding these products are also known to exhibit short half-lives that serve to rapidly eliminate the message and produce the transient nature of the expression burst [12,18–21]. Indeed, the rate of mRNA degradation is so fast that the accumulation of mRNA can be severely limited in the absence of stimulus-driven mechanisms to stabilize the mRNA resulting in increased abundance and protein production. Indeed, the instability of IL-8 and the GRO family chemokines was recognized early after their identification and they have served as models for understanding the mechanism associated with the control of mRNAs encoding short-lived cytokines and growth factors [18,21,22].

The following sections will first present a brief overview of the basic machinery involved in mRNA degradation, the contribution of nucleotide sequences that determine selective message behavior, and the RNA binding proteins that recognize such sequences. The remainder of the discussion will provide a more detailed consideration of the control of neutrophil-specific chemokine mRNA instability with emphasis on mechanisms distinguished by sequence requirements, binding proteins and signalling pathways through which extracellular stimuli promote the prolongation of half-life.

mRNA DEGRADATION: BASIC MACHINERY, INSTABILITY SEQUENCES, AND RNA BINDING PROTEINS

Basic Machinery

Much of our knowledge of the molecular basis for general control of mRNA decay has emerged from studies in yeast though many of the principles appear to be operative in mammalian cells as well [23–25] (see figure 1). mRNAs are protected from exonucleolytic degradation on the 5' and 3' ends by the 5' 7-methyl-guanosine cap and the poly A tail, respectively, each of which is bound by proteins with specificity for these structures. Mechanisms targeting the degradation of mRNAs focus upon the removal of either or both of these structures through the action of deadenylases or decapping enzymes leaving the mRNA body susceptible to 5'to3' or 3'to5' directed exonucleases. While both decapping/5'-3' and deadenylation/3'-5' mechanisms appear to be operative (and are not mutually exclusive), substantial evidence indicates that poly A tail shortening is a critical regulated step in vertebrate cell populations. Cytoplasmic structures termed P bodies have been reported to represent sites for mRNA degradation and these have been shown to contain decapping enzymes and 5'to3' exonuclease activity [26]. However recent work suggests that many mRNAs are also degraded while ribosome bound [27]. A schematic illustration of general mRNA catabolism is presented in Figure 1.

Instability Sequences

It has been known for more than 2 decades that the short half life exhibited by many cytokine mRNAs is dependent upon nucleotide sequence within the mRNA and generally within the 3' UTR. Early studies on mRNAs encoding GM-CSF and TNF α demonstrated that adenine and uridine rich sequences (termed AU rich elements or AREs), particularly containing the pentameric sequence AUUUA, can confer instability to otherwise stable messages [28,29]. Indeed, bioinformatics analysis has identified as many as 4000 ARE containing sequences in the human genome [30]. ARE sequences are structurally heterogeneous and have been classified into at least three categories based upon the presence and distribution of the nucleotide pentamer AUUUA [31]. Class I and II AREs contain multiple copies of the AUUUA motif either in isolation (class I) or in clusters (class II) while class III AREs contain AU rich regions but no AUUUA structure.

While most inflammatory cytokine genes exhibit a potent transcriptional burst following appropriate stimulation, the short half-lives conferred by ARE sequence motifs within the mRNAs can severely limit message accumulation and hence extracellular stimuli often also promote mRNA stabilization [12,25,32,33]. In particular, ligands for the Toll Interleukin 1 Receptor (TIR) family such as IL-1 and LPS have been shown by a number of laboratories to increase the stability of ARE-containing cytokine mRNAs [21,34–37] though the rate of decay and the relative sensitivity to stimulus vary substantially suggesting mechanistic heterogeneity [38,39].

RNA binding proteins

The sequence specific control of mRNA decay involves, in many cases, the participation of proteins that recognize and bind to ARE sequence motifs [25,33,40,41]. Over the last 15 years a number of laboratories have identified broadly expressed proteins that can specifically bind to ARE motifs [42–47]. One of the first ARE-binding proteins identified was AUF1 (also known as hnRNP D) and multiple lines of evidence indicate that one or more alternatively spliced isoforms of this gene product participate in either stabilization or destabilization of selective mRNAs [46,48,49]. One of the mammalian isoforms of the embryonic lethal abnormal visual (elav) gene family (HuR) exhibits both high specificity for ARE sequences and has been shown to stabilize multiple ARE-containing mRNAs [50,51]. A third well

documented ARE binding protein, termed KH domain splicing regulatory factor (KHSRP or KSRP), has been shown to regulate the decay of several mRNAs [52–54]. KSRP can bind multiple ARE-containing mRNAs and has the capacity to interact with and recruit components of the RNA decay machinery (deadenylases, decapping enzymes, exonucleases) to the messenger ribonucleoprotein complex [55,56]. Perhaps the most thoroughly studied ARE-binding protein is TTP, a zinc finger protein that exhibits high binding specificity for RNAs containing 3–4 uridine residues flanked by at least 1 adenine (or the nonamer structure A/UA/UUUUAA/UA/U) [57,58], is induced by LPS in mouse macrophages, and has been implicated in control of ARE-mediated mRNA decay in several experimental settings [43, 44,59,60]. Most convincingly, mice deficient in the TTP gene exhibit a systemic inflammatory syndrome that results from dysregulation of TNF α gene expression due to enhanced TNF α mRNA stability [61].

REGULATION OF NEUTROPHIL-SPECIFIC CHEMOKINE mRNA HALF LIFE

Role AUUUA and TTP

The role of instability in determining the expression patterns of CXC chemokine mRNAs and the ability to modulate their half-life by extracellular stimulation was recognized in early studies of regulation and these messages have served as models for analysis of the control of mRNA stability during inflammation [18–22]. Initial studies focused substantial attention on the contribution of regions containing the AUUUA pentamer within the 3'UTR and demonstrated by mutation and deletion analysis that such sequences were responsible for instability and further, that such instability could be abrogated in cells exposed to TIR ligands [20,21,34]. The ARE binding protein TTP has been shown to recognize such motifs with high specificity [57,58] and appears to be a major mediator of CXCL1 (KC) mRNA instability in mouse macrophages since the message remains almost completely stable in LPS-stimulated cells obtained from TTP-deficient mice [62]. The AUUUA sequence motifs are responsible for such function since TTP-dependent degradation of mouse CXCL1 mRNA is lost if the 7 AUUUA motifs are mutated. Hence the AUUUA structures found in many neutrophil-directed CXC chemokines appear to be important determinants of instability in myeloid cells through the action of TTP.

Signaling pathways

The ability of extra-cellular stimuli to regulate inflammatory chemokine mRNA decay rates was first observed in the early 90s [18,22] and responses initiated by ligands for Toll Interleukin 1 Receptor (TIR) family members have been observed in multiple settings [21,34,35]. These findings have been extended to include a wide selection of short-lived mRNAs and to more precisely appreciate the molecular mechanisms through which such signaling events ultimately couple to mRNA stabilization [63–65]. TIRs initiate signaling via the recruitment of one or more adaptor proteins that interact with the cytoplasmic TIR domain including Myd88, TIRAP, TRIF, and TRAM [66,67]. Interestingly, while TLRs 2,4,5,7/8, and 9 all can promote stability of CXC chemokine mRNAs, TLR3, the TIR that recognizes extracellular or endosomal doublestranded RNA does not [37]. This selective ability of different TIRs to couple with the mRNA stabilization mechanisms correlates with differential use of the adaptor proteins; Myd88 and TIRAP but not TRIF or TRAM are able to signal mRNA stabilization.

Signaling events downstream of Myd88 in the TIR pathways include the recruitment of members of the IL-1 Receptor Associated Kinase (IRAK) family, particularly IRAKs 1,2, and 4 [68–70]. Interestingly, TIRs can couple with a diverse subset of outcome measures at least some of which are independent of the kinase activity of IRAKs [68,71,72]. In macrophages from mice in which the IRAK4 gene has been substituted with a kinase-inactive version, TIR-induced activation of κ B-dependent gene transcription is intact but signaling for mRNA

stabilization is compromised [72]. IRAK1/2 are reported to link to downstream signaling endpoints through interaction with TRAF6, a member of the TNF receptor associated factor family known to be necessary for signaling from TIRs to the activation of the transcription factor NF κ B [70,73–77]. This involves the interaction of TRAF6 with TAK1 which also connects with p38 MAP kinase and its downstream kinase mitogen activated protein kinase activated protein kinase (MK)2 and 3. These latter kinases have been shown to play a critical role in TIR-induced stabilization of chemokine and other cytokine mRNAs in a variety of settings [21,36,78–80]. Multiple studies have demonstrated that modulation of the p38 MAP kinase cascade either by genetic manipulation or pharmacologic inhibition can significantly alter the half life of both IL-8 and KC, particularly in myeloid cells [21,62,63,81]. Moreover, the MK2/3 enzymes have been shown to be critical determinants of the stability of chemokine mRNAs including KC using mice deficient in both genes [79,82]. TTP has been shown to be a substrate for MK2 with two critical serine phosphate acceptors identified (S52, S178) [60, 82–84]. Phosphorylation of TTP on these sites has been reported to alter nuclear to cytoplasmic distribution, protein stability, P body association, and ultimately the destabilizing function of TTP. Understanding of the role of TTP as a target of this pathway provides an example where the signal transduction from stimulus to endpoint for mRNA stability control is now understood in concept [60]. Signaling pathways that couple with chemokine mRNA stabilization are illustrated in Figure 3.

Distinct control of CXC chemokine mRNA stability in non-myeloid cell populations

Though the evidence supports the role of AUUUA pentamers, TTP, and the TRAF6/p38/MK2 signaling pathways in regulating CXC chemokine mRNA half life and expression levels in myeloid-derived cell populations including macrophages and neutrophils, several observations suggested that the control of these particular chemokine mRNAs might exhibit additional complexity. Though macrophages (and other myeloid cell populations including neutrophils) are able to express KC upon stimulation, the early expression of this gene within injured tissues is derived from non-myeloid cell populations including epithelial and stromal cells [9] and hence it is necessary to examine the behavior of CXCL1 mRNA in these cell types. In this context, it has been shown that IL-17 is also a potent stimulus for stabilization of chemokine mRNAs, particularly in non-myeloid cell types [85]. IL-17 is produced predominantly by a subpopulation of T cells known as Th17 and contributes to potent pro-inflammatory activity associated with a number of acute and chronic inflammatory diseases in part by potent induction of neutrophil-specific chemokine gene expression in non-myeloid cell populations including epithelial and stromal cell populations [86–88]. Furthermore, though IL-17 alone is a relatively weak stimulus for KC expression, it is able to cooperate effectively with TNF α to promote CXC chemokine gene expression [85,89]. The mechanisms involved in cooperativity of TNF α and IL-17 appears to reflect the role of TNF α in chemokine transcription and IL-17 in stabilization of the chemokine mRNA [90].

KC mRNA is highly unstable and stimulus-sensitive in non-myeloid cells that may express little or no TTP [91] suggesting that TTP/AUUUA-independent determinants of mRNA instability and stimulus sensitivity may operate in such settings. Not surprisingly, the half life of KC mRNA mutated in all 7 AUUUA pentamers is not sensitive to TTP but the mRNA retains significant instability that can be abrogated in cells treated with IL-1 α and IL-17 [62,92]. Moreover, removal of all 7 pentamers results in only partial reduction in instability but markedly increased the sensitivity to stabilization in response to IL-1 or IL-17 treatment demonstrating that stimulus-sensitive instability is determined by a non-AUUUA containing region of the KC 3'UTR [91,92]. The deletion of the sequence containing the 7 AUUUA motifs leaves a region of approximately 155 nucleotides that retains both instability and sensitivity for stabilization in cells stimulated with IL-1 or IL-17 [91,92]. Furthermore, KC mRNA from which the non-AUUUA motif has been deleted exhibits AUUUA-dependent instability that

cannot be prolonged in response to stimulation. Hence this region contains AUUUA-independent determinants of instability and stimulus-sensitivity that operate in the non-myeloid cell environment.

Because the AUUUA pentamer sequences in KC mRNA that are necessary for sensitivity to TTP were not required for instability in non-myeloid cells (HeLa, HEK293), we reasoned that TTP-independent mechanisms may contribute to the control of KC mRNA stability in such cell types. Indeed, preliminary observations using TTP-deficient mouse embryo fibroblasts show that KC mRNA remains unstable and can be stabilized following treatment with either IL-17 or IL-1 α [92] [and unpublished findings]. Moreover, the sequence motif in the KC mRNA 3'UTR that confers IL-1 α -sensitive instability is not sensitive to TTP [62]. Collectively these studies of KC (CXCL1) in mouse cells support the hypothesis that at least two mechanistically distinct instability mechanisms regulate neutrophil chemokine mRNA decay, that these depend upon separate 3'UTR sequences and distinct RNA binding proteins, and that they may operate in cell lineage restricted fashion (see model in Fig 2). While TTP appears to be responsible for promoting the instability of KC mRNA in myeloid cell populations via recognition of one or more of the 7 AUUUA motifs, an AU rich region that is insensitive to TTP regulates the instability of this mRNA in non-myeloid cell types including stromal fibroblasts and epithelial cells.

IL-17-induced responses depend upon two receptor chains (IL-17RA and IL-17RC) [93]. IL-17RA contains a SEFIR domain in the intracellular region and is able to interact with another SEFIR domain-containing adaptor protein termed Act1, which is necessary for most responses to IL-17 [75,76]. While Act1 can interact with TRAF6 and this is necessary for its ability to promote activation of NF κ B, TRAF6 is not necessary for mRNA stabilization pathway in response to IL-17 [94]. This is also observed with IL-1 α at least in non-myeloid cells, as both IL-1 and IL-17 can promote enhanced stability of KC mRNA even in embryo fibroblasts from mice deficient in TRAF6 [89]. Hence the signaling pathways from both IL-17R and the TIRs bifurcate upstream of TRAF6 with one pathway linking with the activation of NF κ B through TRAF6 and a TRAF6-independent pathway connecting with mRNA stabilization.

The finding that TRAF6 was not required for IL-17- or IL-1-induced mRNA stabilization raises questions regarding the role of the p38 MAP kinase cascade in these responses. Furthermore, while KC mRNA half-life is prolonged from approximately 20 to 40 minutes in TNF α -stimulated wild type mouse embryo fibroblasts as compared to cells deficient in MK2/MK3, both IL-1 and IL-17 can still promote stabilization even in the absence of both MK2 and MK3 [89]. Collectively, these findings support the existence of multiple distinct signaling pathways for stabilization of neutrophil chemokines that vary with the cell type and stimulus (Figure 3).

Future perspectives on post-transcriptional control of chemokine mRNA stability

Transcription is recognized as a key control point in the regulation of gene expression in many physiologic and pathophysiologic settings including inflammation. Indeed, though obvious, it is worth stating that post-transcriptional control mechanisms are of little value in the absence of mechanisms that ensure transcriptional activity. Nevertheless, mechanisms that govern the multiple steps downstream of the generation of primary gene transcripts are receiving increased attention as potential targets of regulatory mechanism and therapeutic intervention. These include not only the degradation of mRNAs but also their splicing, polyadenylation, nuclear-cytoplasmic transport, cytoplasmic localization, and translation. Moreover, the identification of specific mechanisms for post-transcriptional control and the definition of the participating proteins have enabled genetic manipulation strategies that reveal the significant impact that these processes have in determining patterns of specific gene expression.

These approaches have provided increasingly detailed understanding of the general steps involved in degradation of mRNA. In turn there is increasing realization that each of these steps may be subject to independent controls and that there is likely to be substantial diversity or heterogeneity in control mechanisms that target specific mRNAs. While there is a tendency to consider the control of mRNA half life by AU rich sequence as a single process, the more detailed studies underway in multiple laboratories are beginning to demonstrate that regulation of ARE-based mRNA half life may actually involve multiple mechanistically distinct processes. These can be distinguished principally by dependence on different nucleotide sequence motifs and/or RNA binding proteins. In this regard, it seems likely that the mechanisms involved in controlling the decay of a specific mRNA sequence may differ depending upon the cell population and may be differentially sensitive to distinct extracellular stimuli. For example, different members of the ELR-CXC chemokine family appear to show considerable differences in sensitivity to different pathways of mRNA half-life control. Considerations that are likely to be additional sources of complexity include the increased array of RNA binding proteins that can recognize specific mRNA sequence motifs, the almost certain complexity of the messenger ribonucleoprotein complexes, the subcellular localization or concentration of process-specific components (e.g., P bodies and stress granules), and the likely participation of micro RNA machinery in the process. Finally, it is evident that the behavior of a specific mRNA may reflect the action of many mechanisms based upon the presence of multiple regulatory motifs, each a target for a distinct pathway, with the overall outcome reflecting the sum of the individual parts.

Abbreviations

UTR	untranslated region
TTP	tristetraprolin
ARE	AU rich element
T IR	Toll Interleukin 1 Receptor
KSRP	KH domain splicing regulatory factor
IRAK	IL-1 associated receptor kinase
TRAF	TNF receptor associated factor
MK	MAP kinase activated protein kinase

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Figure 1. Mechanisms of mRNA degradation

Mature mRNAs exist within the cytoplasm as ribonucleoprotein particles and exhibit a number of common features. These include interaction with proteins that recognize the poly A tail or the 5' 7meG CAP structure, each of which may help protect the RNA from exonuclease. mRNA degradation is believed to initiate primarily upon removal of the poly A tail via specific deadenylase enzymes. Independently or sequentially, enzymes specific for decapping the 5' end of the mRNA may be engaged. These two activities then enable the exonucleolytic degradation of the mRNA in either 3' to 5' or 5' to 3' directions. The rate at which these enzymatic actions occur on selected mRNAs may be determined by the presence of specific sequence motifs that serve as recognition and binding sites for proteins that can recruit one or more components requisite to the degradation process.

Figure 2. Distinct sequences motifs in the KC mRNA 3'UTR determine instability and stimulus sensitivity in myeloid and non-myeloid cell types

KC mRNA 3'UTR contains 7 separate AUUUA containing regions (4 in a cluster near the 5' end and 3 isolated pentamers). These sites confer instability, at least in part through recognition by the destabilizing protein tristetraprolin (TTP). In non-myeloid cells, TTP or related family members may also operate but a second instability mechanism can also be demonstrated. The mechanism(s) responsible for recognition of this activity remain to be defined in molecular terms.

Myeloid Cells

Non-Myeloid Cells

Figure 3. Signaling pathways leading to mRNA stabilization

In myeloid cells, chemokine mRNA instability is dependent upon AUUUA-containing ARE motifs that are recognized by TTP while in non-myeloid cells, AUUUA-free sequences produce comparable instability through undefined RNA binding protein(s). TIR signaling couples to mRNA stabilization via the adaptor protein MyD88 with IRAK4 and IRAK1 kinases, TRAF6, TAK1, and the p38 MAP kinase cascade. IL-17 signals in non-myeloid cells through the adapter protein Act1 and couples with a second mRNA stabilization pathway that operates independently of TRAF6 and p38/MK2. This second pathway also appears to be utilized by IL-1 in non-myeloid cells and segregates from the NFκB activation pathway at IRAK1. These pathways link to an instability/stabilization mechanism that depends upon non-AUUUA containing sequence and does not require TTP. The details of this second pathway, including the specific target nucleotide sequence motifs, the RNA binding proteins, and the signaling events remain to be identified.