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Regulation of mRNA stability by CCCH-type zinc-finger proteins in immune cells

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Abstract

Current studies using knockout mice have revealed that some Cys–Cys–Cys–His (CCCH)-type zinc-finger proteins, namely tristetraprolin (TTP), Roquin and Regnase-1, play important roles in the immune system. These proteins are closely associated with the fate of their target RNAs in normal immune responses. However, the functions of many RNA-binding proteins have not been characterized precisely. To understand the molecular mechanisms of RNA metabolism in the immune system, investigation of TTP/Roquin/Regnase-1 might provide new knowledge. In this review, we will discuss the current understanding of these proteins in immune regulation and homeostasis and discuss RNA metabolism in the immune system.

Keywords: inflammatory cytokine, mRNA stability, RNA-binding protein, zinc-finger protein

Introduction

Inflammation is predominantly mediated by pro-inflammatory cytokines including TNF-α, IL-1β and IL-6. Upon induction of inflammation, various immune cells rapidly respond to the pathogens and cellular stresses (1). Primary signals triggering inflammation are recognized by pattern-recognition receptors (PRRs) such as TLRs, RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs) (2–4). PRRs recognize, for example, the bacterial product LPS (5, 6) and activate downstream signaling pathways through activation of transcription factors such as activator protein 1 (AP1), CCAAT/enhancer-binding proteins (C/EBPs), interferon regulatory transcription factors (IRFs) and nuclear factor κ light-chain-enhancer of activated B cells (NF-κB).

These transcription factors precisely control the mRNA expression level of pro-inflammatory cytokines at several steps (7). In this regulation, RNA-binding proteins (RBPs) are involved in the mechanism of mRNA decay (8). Sequence motifs containing adenine- and uridine-rich (AU-rich) elements (AREs) are found within the 3′-untranslated region (UTR) of various cytokine mRNAs including TNF-α (9). ARE-binding proteins play roles in degradation or stabilization of mRNAs (10–13). Currently, it is known that three RBPs—tristetraprolin (TTP), Roquin and Regnase—perform tightly controlled post-transcriptional regulation of cytokine mRNAs. These proteins are known as zinc-finger (ZnF) proteins (ZFPs). Although ZFPs were originally characterized as DNA-binding proteins, it was subsequently found that some member proteins, such as Cys–Cys–Cys–His (CCCH)-type ZFPs, are capable of binding to target RNAs (14).

So far, there are 60 known CCCH-type ZFPs in mice and humans (15). ZFPs are involved in RNA metabolism in innate immunity (16). However, most of them have not yet been characterized and their biological functions are unknown. To understand inflammation and autoimmunity, we require elucidation about how RBPs play their important roles in the multiple steps of mRNA biogenesis and degradation associated with immune responses (16, 17). In this review, we discuss the current important findings of CCCH-type ZFPs, TTP, Roquin and Regnase in immune regulation. We will also discuss these proteins from the perspective of RNA metabolism.

Tristetraprolin

TTP (Zfp36) is a member of the Zfp36 gene family that consists of three genes (*Zfp36*, *Zfp36l1* and *Zfp36l2*) and a rodent-specific *Zfp36l3* gene (18) (Fig. 1). It was discovered as a gene that is rapidly induced by insulin stimulation (19) and is well characterized in immune functions (20). The expression of TTP is induced by inflammatory modulators such as TNF-α, LPS, glucocorticoids, insulin and IFN-γ (21–24).

TTP directly binds to AREs in its target mRNAs to promote destabilization and decay by recruiting deadenylation and decapping complexes (10, 11, 25) (Fig. 2). The tandem-repeat CCCH-type ZnF domain of the TTP protein recognizes a specific sequence in AREs (AUUUA) in the 3′-UTR of mRNAs. TTP is a component of a negative-feedback loop that controls TNF-α production by destabilization of its

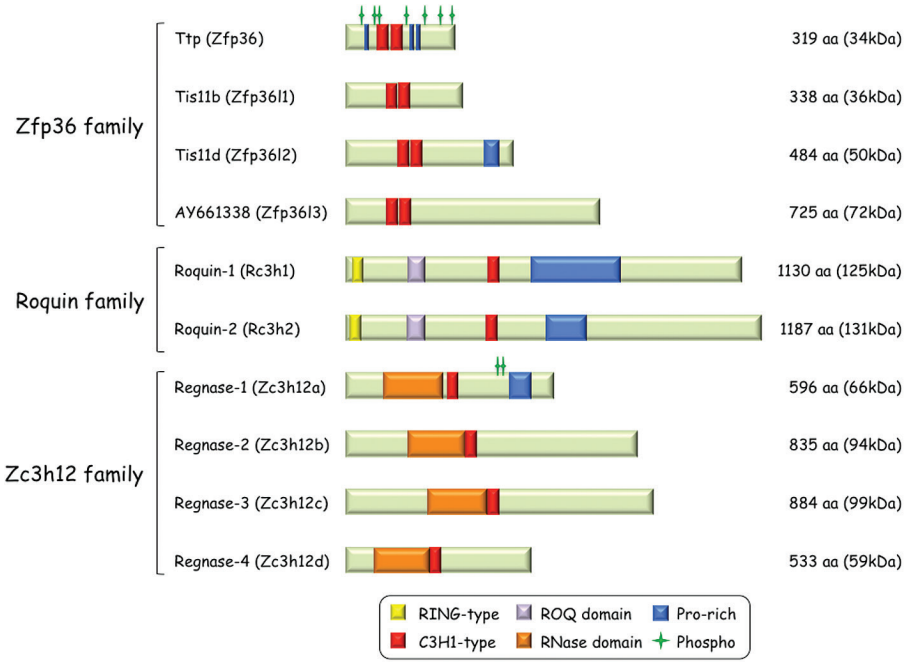

Fig. 1. Structural properties of murine Zfp36, Roquin and Regnase family member proteins. The conserved CCCH(C3H1)-type ZnF domains are shown in red boxes. For the Roquin family members, the RING-type ZnF domain and the ROQ domain are shown in yellow and purple boxes, respectively. RNase domains of Regnase family proteins are indicated in orange boxes. The proline (Pro)-rich regions are represented in blue boxes. The phosphorylation sites involved in known signaling pathway are indicated in green four point stars.

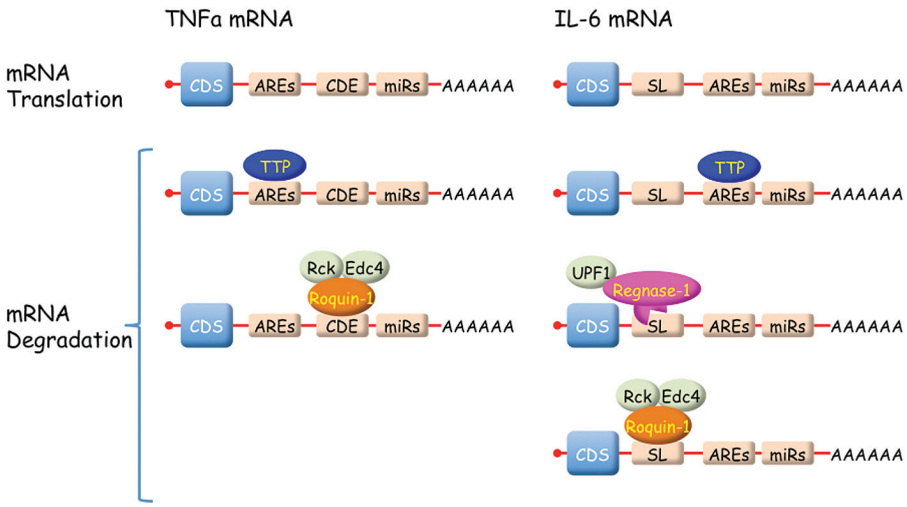

Fig. 2. Regulation of the 3′-UTRs of TNF-α and IL-6 mRNAs by CCCH-type ZnF proteins. TTP can bind to AREs and promote mRNA decay. Roquin-1 can bind to the CDE and promote mRNA degradation through recruitment of Rck and Edc4. Regnase-1 binds to the SL (stem–loop) region in the 3′-UTR of IL-6 mRNA and promotes its degradation in the early phase of the inflammatory response through the recruitment of RNA helicase and ATPase, UPF1. In the late phase, Roquin-1 also targets to the same region and promotes IL-6 mRNA decay. There are some miRNA-binding sites in the 3′-UTR of both mRNAs. These miRs are not shown in the correct positions. CDS, coding sequence.

mRNA (11, 12). TTP knockout mice show severe, complex syndromes such as cachexia, arthritis and autoimmunity (26, 27), indicating that TTP is involved in dampening excessive inflammation through TNF-α production. Indeed, both TNF-α transgenic mice (28) and TNF-α-ΔARE mice (in which ARE sequences are deleted) (29) have phenotypes similar to TTP knockout mice. The phenotype of young TTP-deficient mice is attenuated by either treatment with an anti-TNF-α antibody or backcrossing with TNFR1 (*Tnfrsf1a*) knockout mice (26, 27). Therefore, the TTP–TNF-α axis is a crucial mechanism for immune homeostasis.

TTP is a phosphoprotein, and phosphorylation of TTP by p38/MAPKAP kinase 2 (Mapkapk2) affects the mRNA decay pathway along with relocation from stress granules (SGs) to P-bodies (PBs) (30–33). TTP is relocated to SGs in the stress response following delivery of its target mRNAs (30, 34, 35). SGs and PBs are cytoplasmic foci that are independently regulated in the signal. Therefore, this mechanism is important

to understand how TTP-mediated mRNA degradation rapidly occurs. Moreover, TTP negatively regulates the NF-κB pathway by impairment of the signaling cascade (36–39). Other *Zfp36* gene family members also have various predicted phosphorylation sites, but their functions remain unknown.

Destabilization of mRNA by TTP has been reported for not only TNF-α mRNA but also various mRNA transcripts such as inflammatory cytokines, including IL-2 (40), IL-6 (24, 41, 42) and GM-CSF (43), IFN-γ (44), the anti-inflammatory cytokine IL-10 (45, 46) and some chemokines (24, 47). Taken together, these studies imply that TTP plays important roles in TNF-α production and directly binds to AREs in the 3′-UTR of mRNAs for ARE-related decay.

Roquin

Roquin (*Rc3h1*) was discovered as a genetic mutant in the Roquin gene locus (48). Mutant mice (sanroque/Roquin$^{san/san}$: M199R) develop autoimmune phenotypes accompanied by activation of follicular T helper (T_{fh}) cells and spontaneous germinal center formation (48). Roquin-1 possesses a single CCCH-type ZnF domain and is a RING-type ZnF E3 ubiquitin ligase (Fig. 1). Roquin-2 (*Rc3h2*) was identified as an E3 ubiquitin ligase required for reactive oxygen species-induced ubiquitination and degradation of apoptosis signal-regulating kinase 1 (ASK1/Map3k5) (49). Both Roquin-1 and Roquin-2 are functional E3 ligases with similar structures and overlapping functions, but their activities are not identical or redundant (50). The importance of the RING-type ZnF domain is supported by knockout mouse studies (51, 52). Recently, it was shown that the RING-type ZnF domain directly binds to the catalytic α1 subunit of AMP-activated kinase (AMPK/Prkaa1), a central regulator of cellular metabolism and an inhibitor of the mammalian target of rapamycin (mTOR/Mtor) signaling pathway.

This new metabolic pathway through the RING-type ZnF domain is important for selectively promoting T_{fh} cell responses (53). Roquin has a unique domain, ROQ (~300 amino acid residues), near the CCCH-type ZnF domain (Fig. 1). The M119R mutation in Roquin$^{san/san}$ is located in this domain. The ROQ domain binds to the stem–loop-forming constitutive decay element (CDE), which is located downstream of the ARE in the 3′-UTR of TNF-α (13, 54) (Fig. 2), facilitating degradation of the transcript through interactions with the enhancer of mRNA-decapping protein 4 (Edc4), which is a decapping enzyme, and Rck, which is a helicase (55). Therefore, Roquin participates in the regulation of TNF-α mRNA decay in a manner similar to TTP through different elements in the same mRNA.

Roquin-1 modulates the IκB kinase (IKK)–NF-κB pathway by repressing ubiquitin enzyme A20, which is an important negative regulator of inflammation (56). In that study, it was shown, by PAR-CLIP (photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation) experiments, that Roquin-1 binds to thousands of mRNAs, mostly located in 3′-UTR of target mRNAs. This study predicted how Roquin-1 coordinates its target RNAs *in vivo*.

Roquin$^{san/san}$ mice express a high level of inducible T-cell co-stimulator (ICOS) on T cells, which is required for T_{fh} cell development and expansion (57). Therefore, the elevated amount of ICOS is responsible for the lupus phenotype in Roquin$^{san/san}$ mice (58). Roquin-1 knockout mice exhibit perinatal lethality within 6 h after birth (51). CD4-specific Roquin-1 knockout mice have elevated ICOS expression and impaired T_{fh} cell expansion and do not show autoimmunity (51). These results support the conclusion that Roquin post-transcriptionally down-regulates ICOS mRNA expression (55, 58, 59).

Similar to Roquin-1 knockout mice, conventional Roquin-2 knockout mice also show postnatal lethality. However, CD4-specific Roquin-2 knockout mice show no effects on immune cell homeostasis (60). Roquin-1 and Roquin-2 double-knockout mice develop an autoimmune disease phenotype with induced differentiation of T_{fh} cells and expansion of germinal centers similar to Roquin$^{san/san}$ mice, suggesting that both proteins have redundant functions in ICOS expression of T_{fh} cells (60). Furthermore, CD4-specific double-knockout mice show severely enhanced T_h17 cell differentiation, developing severe lung inflammation and gastritis (61).

Taken together, these studies demonstrate that Roquin controls the expression level of ICOS mRNA in activated $CD4^+$ T cells (48).

Regnase-1

Regnase-1 (*Zc3h12a*/*Mcpip1*) was identified as a rapidly induced gene in response to stimulation via TLR ligands (62). It possesses a PilT-N terminal (PIN)-like RNase domain harboring a CCCH-type ZnF domain (Fig. 1). Conventional Regnase-1 knockout mice show a spontaneous severe phenotype of autoimmune inflammatory disease along with elevated serum immunoglobulin levels and auto-antibodies. Regnase-1-deficient mice exhibit significantly high levels of IL-6 and IL-12p40 production in response to TLR ligands compared with control mice. Regnase-1 binds to a conserved stem–loop element in the 3′-UTR of IL-6 mRNA, but not AREs, and directly degrades target mRNAs via its intrinsic RNase activity. Interestingly, this activity selectivity acts on limited target mRNAs, such as IL-1β, IL-6 and IL-12p40 mRNA, but not TNF-α. Thus, the mechanism through which Regnase-1 degrades target mRNAs is essential to maintain immune homeostasis.

Regnase-1 is predominantly expressed in immune tissues such as the spleen, thymus, lymph nodes, lungs and intestines (63, 64). After LPS or IL-1β stimulation, Regnase-1 is phosphorylated through activation of the IKKα–IKKβ complex and an unknown kinase (63) (Fig. 3). Phosphorylated Regnase-1 rapidly undergoes ubiquitin- and proteasome-mediated degradation through E3 ligase degradation in response to stimulation by IL-1β or TLR ligands, but not TNF-α (63). The expression level of Regnase-1 mRNA is also negatively controlled by Regnase-1 itself, which targets its own stem–loop element in the 3′-UTR and promotes its degradation. Upon degradation of Regnase-1 protein, IL-6 mRNA can accumulate and be expressed stably. These data indicate that the Regnase-1-mediated regulatory mechanism is important to control inflammation.

CD4-specific conditional Regnase-1 knockout mice also show autoimmunity arising from aberrant proliferation of

effector T cells (65). Regnase-1 is critical to prevent autoimmunity because of the intrinsic properties of T cells. It appears that Regnase-1 degrades various target mRNAs in other cell types. In T cells, Regnase-1 directly regulates decay of immunoregulatory mRNAs, such as IL-2, OX40 and c-Rel, which are important to maintain normal effector functions. Furthermore, in TCR signaling, formation of the CARMA1–BCL-10–MALT1 (CBM) complex is critical for NF-κB activation via the IKK complex. The arginine-specific protease MALT1 directly cleaves Regnase-1 at R111 in response to TCR stimulation. This activation cascade causes protein degradation to control the amount of Regnase-1 at the protein level (Fig. 4).

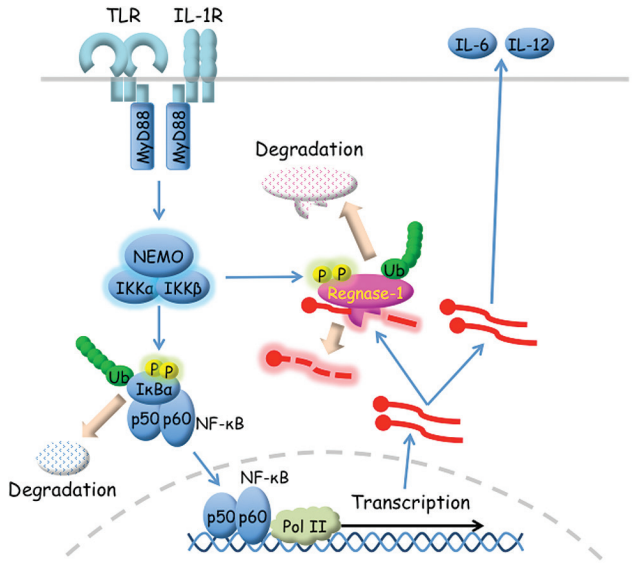

Fig. 3. Post-transcriptional regulation of Regnase-1. Regnase-1 post-transcriptionally destabilizes IL-6/IL-12p40 mRNAs. Stimulation of TLR or IL-1R triggers association with myeloid differentiation primary-response protein 88 (MyD88) and then the IKK complex (NEMO–IKKα–IKKβ) phosphorylates both IκBα and Regnase-1, causing ubiquitin proteasome-mediated degradation, leading to nuclear translocation of NF-κB (p50–p65 heterodimer) and induction of transcription of its target genes. This activation facilitates the rapid and robust production of inflammatory cytokines such as IL-6 and IL-12.

Regnase-1 and Roquin-1 regulate overlapping sets of inflammation-related mRNAs such as IL-6 through a common stem–loop structure and pyrimidine–purine–pyrimidine sequence in the loop (66). However, their mechanism is spatiotemporally distinct in cells. Regnase-1 specifically cleaves and degrades translationally active mRNAs and recruits the RNA helicase and ATPase UPF1 (Fig. 2). Conversely, Roquin-1 controls translationally inactive mRNAs in a UPF1-independent manner. It is interesting that Regnase-1 regulates a set of mRNAs. This aspect may provide novel insights into autoimmune diseases and inflammatory conditions. Regnase-1 also collaborates with Roquin to control T_h17 differentiation by repression of T_h17 cell-promoting genes (61). Recently, it was shown that Regnase-1 is involved in IL-17-mediated signaling and inflammation (67, 68). It appears that this negative mechanism is not mediated by the 3′-UTR of target genes. Therefore, how Regnase-1 precisely regulates other candidate genes requires further investigation.

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) that repress protein expression through binding to the 3′-UTR of target mRNAs. This post-transcriptional control mechanism is highly conserved in numerous eukaryotes (69, 70). Thus, the regulation of miRNAs is involved in the control of cellular mechanisms including immune-mediated inflammation. The small stretch (seed) sequence of six to eight nucleotides at the 5′-end of miRNAs is important for recognition of their target mRNAs. This seed sequence is capable of pairing to many target mRNAs. Therefore, one miRNA can simultaneously control various mRNAs.

Similar to RBPs, the regulation of miRNAs also contributes to mRNA stability. ARE-mediated decay of TNF-α mRNA is dependent on both TTP and miR-16 (71). Roquin directly binds to miR-146a that is known to repress ICOS mRNA in T cells. Roquin also binds to Argonaute 2 (Ago2), which

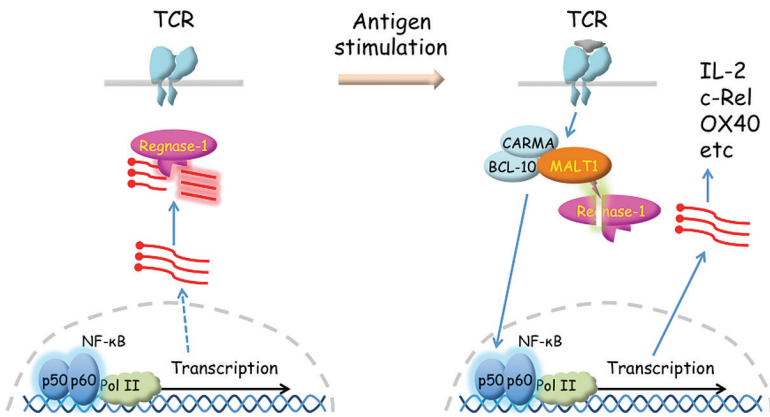

Fig. 4. Control of T-cell activation by Regnase-1. In resting T cells, Regnase-1 is constitutively expressed and suppresses abnormal activation via mRNA decay (left). After TCR stimulation, Regnase-1 is cleaved by the protease MALT1, a component of the CBM complex. As a result, Regnase-1-target mRNAs encoding immunoregulatory genes such as c-Rel, OX40 and IL-2 are stably expressed to allow an appropriate immune response (right).

is a component of the RNA-induced silencing complex (RISC) (72), implying that miRNA homeostasis also shares the Roquin-mediated mechanism. It would be interesting to determine whether TTP, Roquin and/or Regnase-1 participate in miRNA homeostasis via the close element in the 3′-UTR of mRNAs (Fig. 2). From the viewpoint of post-transcriptional control, CCCH-type ZFPs may regulate either the steady-state or the first round of translation of some target RNAs in cooperation with miRNAs. We need to understand precisely the relationship between CCCH-type ZFPs and each miRNA in miRNA biogenesis.

Conclusions

The regulation of inflammatory cytokines is strictly controlled to maintain immune homeostasis. Dysregulation of the post-transcriptional machinery leads to increases in inflammatory cytokine production by modulating the mechanism of mRNA stability and translation. After the discovery that *cis*-acting element AREs in the 3′-UTR of TNF-α mRNA are regulated by binding RBPs such as TTP, AUF1 (ARE/poly-U binding degradation factor 1 and heterogeneous nuclear ribonucleoprotein U), and HuR (ELAV1), it is expected that other cytokine mRNAs are also regulated by a similar mechanism. At present, the CCCH-type ZFP-mediated molecular mechanism provides a new insight into how inflammatory cytokines are intrinsically controlled to maintain immune homeostasis through multiple regulatory steps. Indeed, knockout mice show development of systemic inflammatory responses and autoimmune disease. Therefore, further study focusing on RNA metabolism of target RNAs in various cell types may reveal the details of its molecular mechanisms as well as therapeutic targets in inflammation.

During the preparation of this manuscript, Fu and Blackshear published a review focusing on current known members of CCCH-type ZFPs (73). This paper also helps us to understand the knowledge of RBPs in immune responses and future perspectives.

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