The perplexities of the ZC3H12A self-mRNA regulation*

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The mechanisms regulating transcript turnover are key processes in the regulation of gene expression. The list of proteins involved in mRNAs’ degradation is still growing, however, the details of RNase-mRNAs interactions are not fully understood. ZC3H12A is a recently discovered inflammation-related RNase engaged in the control of proinflammatory cytokine transcript turnover. ZC3H12A also regulates its own transcript half-life. Here, we studied the details of this regulation. Our results confirm the importance of the 3’UTR in ZC3H12A-dependent ZC3H12A mRNA degradation. We compared the mouse and human stemloop structures present in this region and discovered that the human conserved stemloop structure is not sufficient for ZC3H12A-dependent degradation. However, this structure is important for the ZC3H12A mRNA post-transcriptional regulation. Our studies emphasize the importance of the neighboring features of the identified stem-loop structure for its biological activity. Removal of this region together with the stem-loop structure greatly inhibits the ZC3H12A regulation of the investigated 3’-untranslated region (3’UTR).

Key words: ZC3H12A/MCPIP1; RNase; transcript turnover; inflammation.

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INTRODUCTION

ZC3H12A (zinc finger CCCH-type containing 12A) is a recently discovered protein involved in the attenuation of inflammation (Matsushita et al., 2009). Its importance in this process was shown by the use of a mouse model. Zc3h12a−/− mice spontaneously develop severe autoimmune inflammatory disease and most of them die within 12 weeks of birth (Matsushita et al., 2009). The negative regulation of the inflammatory response by ZC3H12A depends on its dual RNase and deubiquitinase activities which allow this single protein to act at two levels of the cellular response – signal transduction (ZC3H12A negatively regulates JNK and NF-κB signaling by deubiquitinating the TRAF proteins) and mRNA decay (Liang et al., 2010; Matsushita et al., 2009). Its RNase activity specifically shortens the half-life of mRNAs of a subset of proinflammatory cytokines such as interleukins: IL1β, IL-6, IL-12p40 and IL-2, or transcription factor c-Rel (Li et al., 2012; Matsushita et al., 2009; Mizgalska et al., 2009; Uehata and Akira, 2013). Transcripts are not the only targets of this RNase. ZC3H12A also cleaves terminal loops in precursor miRNAs (pre-miRNAs) (Suzuki et al., 2011) and viral RNA (Lin et al., 2013; Lin et al., 2014; Liu et al., 2013).

The level of ZC3H12A mRNA is rapidly upregulated in macrophages in response to Toll-like receptors (TLRs) and IL-1β receptor activation (Matsushita et al., 2009). Additionally, signaling from TLRs and IL-1R influences the ZC3H12A protein level. Stimulation of these receptors leads to the activation of the IKK complex resulting in phosphorylation and subsequent ubiquitination, followed by proteasomal degradation of ZC3H12A. The protein reappears within two hours after stimulation (Iwasaki et al., 2011). Such mechanism allows for temporal synthesis of proinflammatory cytokines within the period of time when ZC3H12A is degraded and then ZC3H12A-dependent degradation of proinflammatory transcripts can be switched on.

The mechanisms responsible for mRNAs’ degradation by ZC3H12A have been studied as well. It is documented that the CCCH-type zinc finger domain is engaged in the interaction of ZC3H12A with RNA. Following binding, another domain, the NYN/PIN-like domain, is involved in endonucleolitic activity of the protein (Iwasaki et al., 2011; Matsushita et al., 2009; Mino et al., 2015; Yokogawa et al., 2016). ZC3H12A was reported to associate with a set of mRNAs containing a stemloop structure within their 3’UTRs, with IL-6 to be the most prominent target (Matsushita et al., 2009). Results obtained by our group confirmed a direct interaction of ZC3H12A with IL-6 mRNA in a single cell (Kochan et al., 2015). However, our knowledge about the features of the sequences present in 3’UTR which influence the binding is very poor.

Recently, ZC3H12A involvement in the regulation of its own mRNA turnover has been identified, presenting an intrinsic auto-regulatory mechanism (Iwasaki et al., 2011; Mino et al., 2015; Mizgalska et al., 2009). To understand exactly which elements are crucial for ZC3H12A-dependent degradation, in the paper presented here we performed detailed analyses of this transcript focused not only on the stem-loop structures but also on their neighboring features. Unexpectedly, our results suggest, in contrast to published data, that the conserved stem-loop structure present in human ZC3H12A 3’UTR is not sufficient for ZC3H12A-dependent degradation.

MATERIALS AND METHODS

Cell culture. HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 1 g/L D-glucose

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Abbreviations: IL, interleukin; UTR, untranslated region; TLR, Toll-like receptor; ZC3H12A, Zinc finger CCCH-type containing 12A.
The plasmid for expression of human ZC3H12A (Invitrogen, USA), supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), and 10% (v/v) FBS (Biowest, France), at 37°C in a humidified atmosphere containing 5% CO2.

**Construct preparation.** The pLuc-ZCH12A-3’UTR construct encoding the firefly luciferase transcript fused with the ZCH12A 3’UTR fragment, was prepared by insertion of PCR amplified ZCH12A 3’UTR (NM_025079.2) into the pmirGLO vector (Promega, USA). Briefly, the 3’UTR fragment of ZCH12A was amplified from cDNA prepared from HeLa cells using PfuUltra II Fusion HS DNA Polymerase (Agilent, USA), and the following primers: forward 5’ATCGCTAGCGCTGCCCTGGGCTGG-3’, and reverse 5’ATCTCTAGAGATGTTTACAGAGATTTAGGACT-3’. After agarose gel electrophoresis, the PCR product corresponding to ZCH12A 3’UTR fragment was excised, purified, cleaved with NheI and XbaI (NEB, USA), and inserted using T4 DNA ligase (NEB, USA) into the pmirGLO vector linearized with NheI and XbaI and dephosphorylated with CIP (NEB, USA). The obtained construct was verified by restriction digestion and Sanger sequencing (Genomed, Polska).

The plasmids encoding the firefly luciferase transcript fused with the ZCH12A 3’UTR fragment devoid of stem-loop structures 1(ΔSL1), 2(ΔSL2) or both(ΔSL1+ΔSL2), were prepared by site-directed mutagenesis using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, USA), according to the manufacturer’s instructions, with pLuc-ZCH12A-3’UTR as template for ΔSL1 and ΔSL2 constructs, or ΔSL1 for the ΔSL1+ΔSL2 construct. The following primers were used: dSL1 sense 5’GAAGGACGATCCACCTGGTTGTCGCTGTTAGTCTTAAGAAGAAGGG-3’, dSL1 anti-sense 5’TCTCCCTTTAACACTAGACCAAAGGTGATGCCTTC-3’, dSL2 sense 5’CTATTTCTTCTTACCTGCTGTACGGAGGCCCTCCTGCTCTA-3’, and dSL2 anti-sense 5’TAGACGGAGGCGCCTTTTGACAGAGAAGATTGAC-3’. The plasmids encoding the firefly luciferase transcript fused with 3’ truncations of the ZCH12A(D141N), does not lead to a decrease in luciferase activity (firefly/Renilla) and in some cases for clearer presentation normalized to 1.0 for the control sample. Experiments were conducted in technical duplicates and represent at least two biological replicates.

**RESULTS AND DISCUSSION**

To assess the influence of ZC3H12A on its own mRNA in the human system, we cotransfected HeLa cells with the pLuc-ZCH12A-3’UTR construct encoding the firefly luciferase transcript fused with the human ZCH12A 3’UTR. The expression vector coding for RNase-inactive mutant of ZC3H12A, referred to as ZC3H12A(D141N) was obtained with QuikChange II XL site directed mutagenesis kit (Agilent, USA) using plasmid coding for WT form as a template and the following primers: M1-D141Nsense: 5’AGACCGTGGTCTCATACTGGGAGCAAGCTGGTGG-3’ and M1-D141Nantisense: 5’CCACGGTGTGCTGGGACTGATTGACACCTGGTCTT-3’. The final vectors were verified by Sanger sequencing.

**RNA isolation and cDNA preparation.** Total RNA was isolated from HeLa cells using the Chomczynski method (Chomczynski and Sacchi, 2006). Briefly, cells were lysed with the GTC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol) and total RNA was isolated using subsequent acid phenol-chloroform extraction and isopropanol precipitation. RNA quality was assessed using standard agarose gel electrophoresis under denaturing conditions. RNA quantity was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For cDNA preparation, 1 μg of the obtained total RNA was incubated with M-MLV reverse-transcriptase (Promega, USA), oligo(dT)15 primer (Genomed, Poland), and processed according to the manufacturer’s instructions.

**Transfection and reporter gene assays.** HeLa cells were seeded in 24-well plates at density of 1 × 104 cells/well. 24-h later, the cells were transfected with plasmid DNA using the Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer’s instructions. The total amount of 0.8 μg of plasmid DNA per well was used, including 0.7 μg of the luciferase-coding reporter vector and 25 ng of the ZC3H12A expression vector or the empty control plasmid. The quantity of DNA/well was normalized using an empty pcDNA3.1/mycHisA vector (mock DNA; Invitrogen, USA). 24-hs post-transfection, the cells were lysed and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA), according to the manufacturer’s instructions. For transfection efficiency normalization, the Renilla luciferase encoded on the same plasmid as the firefly luciferase (pmirGLO) was used. Luciferase data are presented as relative luciferase activity (firefly/Renilla) and in some cases for clearer presentation normalized to 1.0 for the control sample. Experiments were conducted in technical duplicates and represent at least two biological replicates.
ciferase activity, although it was expressed at the same level as the wild type ZC3H12A (not shown), clearly indicating that the NYN domain is crucial for this activity (Fig. 1). Similarly to wild-type variant, no effect of ZC3H12A(D141N) over-expression on the empty pmirGLO was observed.

To specify exactly which fragment of the human ZC3H12A 3'UTR is responsible for the observed ZC3H12A-dependent regulation, we aligned ZC3H12A 3'UTR fragments from various mammalian species using ClustalW2 (Larkin et al., 2007) and visualized their secondary structures using the mfold web server (Zuker, 2003) (Fig. 2). The analysis of the sequences and secondary structures reveals that the stem-loop identified by Iwasaki and others (2011) as the one responsible for ZC3H12A-dependent regulation in the murine model is also present in human ZC3H12A 3'UTR (SL1). However, the 3'UTRs differ slightly as an additional stem-loop structure (SL2), similar to SL1, is present only in the human 3'UTR and not in the murine one (Fig. 2B). The similarity between both stem-loops (SL1 and SL2) in human ZC3H12A 3'UTR and their close proximity, prompted us to verify whether the ZC3H12A-dependent regulation of ZC3H12A mRNA relies solely on SL1, as was shown in the murine model, or maybe also on the additional SL2, which is present only in the 3'UTR of human origin.

In order to identify the role of each stem-loop within in the ZC3H12A 3'UTR in our system, we prepared reporter constructs lacking each stem-loop (ΔSL1 or ΔSL2) or lacking both of them (ΔSL1+ΔSL2), and assessed whether they are also regulated in a ZC3H12A-dependent manner (Fig. 3). Surprisingly, removal of neither SL1 nor SL2 abrogated the decrease in luciferase activity observed upon ZC3H12A over-expression. The effect was also unchanged when both of the stem-loops were deleted (ΔSL1+ΔSL2; Fig. 3C). Although all of the constructs we used did not stop to respond to ZC3H12A over-expression, the SL1 is of great importance, as removal of this stem-loop in both single (ΔSL1) and double (ΔSL1+ΔSL2) mutant constructs increased the luciferase activity approximately 5-times. The SL2 did not show such an effect, as its removal did not change the luciferase activity (Fig. 3D). This indicates that SL1, conserved between the analyzed species, is important in ZC3H12A mRNA post-transcriptional regulation, but seems to be dispensable for ZC3H12A-protein dependent regulation in the human system.

As removal of SL1 or SL2 did not result in irresponsiveness of ZC3H12A 3'UTR to ZC3H12A over-expression, we decided to prepare a series of truncations of the 3'UTR from the 3' side and test which fragment will no longer be regulated in a ZC3H12A-dependent manner (Fig. 4A). The obtained results clearly indicate that removal of the fragment between 220-257 nts within the 3'UTR, greatly diminishes the responsiveness to ZC3H12A over-expression. Surprisingly, this fragment contains the SL1 stem-loop, which when solely removed (ΔSL1) did not abrogate the 3'UTR regulation. However, both constructs, D3 and ΔSL1, indicate that the region containing SL1 is crucial for the regulation of ZC3H12A 3'UTR (Fig. 3D and Fig. 4B).

Our studies indicate that the mechanisms underlying the regulation of transcript stability by ZC3H12A still needs to be elucidated. Although the removal of the SL1 structure does not disturb the observed regulation, the truncation studies reveal that the 220–257 nts fragment containing this structure is crucial for ZC3H12A-dependent regulation in the human system. The stem-loop structure corresponding to the human SL1 was already...
Figure 3. ZC3H12A regulates its own transcript regardless of the absence of identified stem-loop structures. (A, B) To uncover the exact element within the ZC3H12A 3'UTR required for ZC3H12A-dependent regulation, we used site-directed mutagenesis to remove the stem-loop structures indicated in Figure 2. The obtained constructs are schematically depicted in (A) along with modified sequences presented in (B). (C) HeLa cells were co-transfected with wildtype pLucZC3H12A-3'UTR, pLuc-ZC3H12A-3'UTRΔSL1, pLuc-ZC3H12A-3'UTRΔSL2 or pLuc-ZC3H12A-3'UTRΔSL1+ΔSL2 (constructs schematically depicted in (A)) and ZC3H12A expression vector or empty control vector. 24 hrs after transfection, the firefly and Renilla luciferase activity in cell lysates was measured. Luciferase data are presented as relative luciferase activity (firefly/Renilla) and normalized to 1.0 for the control samples. Removal of the SL1 structure does not suppress the effect of ZC3H12A over-expression on its own transcript. Removal of the SL2, absent in the murine transcript, also does not impede the observed effect. Nor does the removal of both structures. (D) However, the SL1 is clearly important in the post-transcriptional transcript regulation as is visible when non-normalized relative luciferase values for control samples from (C) are plotted. Plots show mean results from three independent experiments ± S.D., **p<0.01, ***p<0.001. Two-way ANOVA with Bonferroni post-hoc test (C) or two-tailed t-test (D) was used.

Figure 4. Fragment between nucleotides 220–257 within the 3'UTR of ZC3H12A mRNA is crucial for its regulation by the ZC3H12A protein. (A) Truncation studies using naturally occurring restriction sites within the human ZC3H12A 3'UTR reveal that fragment spanning nucleotides 220 and 257 is indispensable for ZC3H12A-dependent regulation. HeLa cells were co-transfected with wild-type pLucZC3H12A-3'UTR, pLuc-ZC3H12A-3'UTRΔD1, pLuc-ZC3H12A-3'UTRΔD2 or pLuc-ZC3H12A-3'UTRΔD3 (constructs schematically depicted next to the graph) and ZC3H12A expression vector or empty control vector, and 24 hrs after transfection the firefly and Renilla luciferase activity in cell lysates was measured. Luciferase data are presented as relative luciferase activity (firefly/Renilla) and normalized to 1.0 for the control samples. Comparison of the non-normalized relative luciferase values for control samples of the truncation variants presented in (A) reveals that the fragment between nucleotides 220–257 within the 3'UTR of ZC3H12A transcript is crucial for the post-transcriptional mRNA regulation. Plots show mean results from three independent experiments ± S.D., ***p<0.001. Two-way ANOVA with Bonferroni post-hoc test (A) or two-tailed t-test (B) was used.
shown to be important for ZC3H12A-dependent regulation in the murine system. However, Iwasaki et al. (2011) used a different approach for studying the influence of murine Zc3h12a on self-mRNA. Instead of deletion of the structure, they created substitution mutations within the stem of the structure which, supposedly by inhibition of the stem-loop formation, impeded the response to ZC3H12A over-expression. Whether this approach would result in a loss of response of ZC3H12A 3'UTR to ZC3H12A needs more detailed studies. However, our data (data not shown) indicate that also in the murine Z3h12a 3'UTR the deletion of the stem-loop does not abrogate the response to human ZC3H12A over-expression. The answer why there are discrepancies between results presented in this study and the ones presented by Iwasaki et al. (2011) is as yet unknown. One of the possibilities is that ZC3H12A binding to substrates does not solely rely on the stem-loop but also on some other sequences/structures in its vicinity. These may not change in deletion constructs but may be disrupted by introducing substitutions which can potentially cause the RNA to fold differently. The other possibility is that the substrate specificity of human and murine ZC3H12A proteins is not exactly the same in terms of the stem-loop structure. What is more, the RNA in silico folding does not represent the “in-cell” conditions where the presence of RNA binding proteins, divalent ions etc. may influence the folding and the final RNA structure. Nonetheless, this study shows that defining a transcript instability elements is cumbersome and extreme care must be taken during experimental design and setup.

The authors declare no competing interests.

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