Biochimica et Biophysica Acta 1779 (2008) 712–719

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

TNF-α stimulation inhibits siRNA-mediated RNA interference through a mechanism involving poly-(A) tail stabilization

Johann Mols [a,b], Arjen van den Berg [a], Motoyuki Otsuka [a], Min Zheng [c], Jianming Chen [a], Jiahuai Han [a,*]

[a] Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, 92037, CA, USA
[b] Viral Development Unit, R&D Department, GlaxoSmithKline Biologicals, 15, rue de l'institut, B-1340 Rixensart, Belgium
[c] The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, China

ARTICLE INFO

Article history:
Received 14 December 2007
Received in revised form 21 March 2008
Accepted 22 March 2008
Available online 1 April 2008

Keywords:
MicroRNA
siRNA
RNA interference
mRNA stability
TNF
Ago2
Poly(A) tail

ABSTRACT

The control of mRNA stability is a complex biological process that involves numerous factors, including microRNA (miRNA) and short interfering RNA (siRNA). Here, we show that short interfering RNA (siRNA) and microRNA share some similarities in their response to cellular stress. miR16 expedites the degradation of mRNAs containing AU-rich elements (ARE) in their 3′ untranslated region (UTR). si20 is an siRNA designed to target a non-ARE sequence in the TNF 3′UTR. We found that both si20 and miR16/ARE-mediated degradation of mRNAs can be inhibited by stimulating cells with different stresses. By analyzing TNF-α stimulation-mediated stabilization of si20- and miR16-targeted mRNA, we show that this stabilization is not caused by modifying si20 and miR16 loading into Ago2 complexes, or mRNA targeting to Ago2, but by inhibiting mRNA deadenylation. This is the first report showing that a specific siRNA-mediated mRNA degradation can be regulated by inflammatory stimuli, and that deadenylation is involved in this siRNA-mediated mRNA decay.

1. Introduction

Gene expression is tightly controlled at various levels, from gene transcription to protein posttranslational modifications [1–3]. Deregulation of gene expression can result in dramatic phenotypes leading to pathological states [4–8]. The control of mRNA expression levels and translation have been highlighted as major control checkpoints for transiently expressed molecules like cytokines [9–11]. In cells of the innate immune system, inflammatory stimuli can be associated with an increase in mRNA translation and stability [12–15]. AU-rich elements (ARE), present in the 3′ untranslated region (3′UTR) of many unstable mRNAs, control mRNA degradation through the recruitment of ARE-binding proteins having various biological activities [14,15]. Amongst ARE-binding proteins that were studied and characterized at the functional level, some are destabilizing factors like tristetraprolin (TTP), some are stabilizing factors like the ELAV-related protein HuR [16,17]. Even more strikingly, the ARE and poly(A) binding protein AUF-1 has several isoforms: p37, p40, p42, and p45 that act in an antagonistic way in the control of ARE-mediated mRNA decay [17].

Research undertaken in our laboratory demonstrated that ARE-mediated mRNA degradation involves short non-coding endogenous RNA molecules called microRNAs (or miRNAs) [18]. In human cells, ARE-mediated mRNA degradation is triggered by the interaction of miR16 with a hexameric ribonucleotide sequence that exists in several ARE sequences. From a thermodynamic point of view, the interaction between miR16 and the hexamer is weak and unlikely to occur in vivo without the help of other factors. [18]. The biogenesis of miRNAs and short interfering RNAs (siRNAs) share some common features in their maturation steps, processing by Dicer to mature 20–23 nucleotide miRNA/siRNAs and subsequent loading into Ago-containing complexes. Genes of miRNA are transcribed into large miRNA precursors, matured in the nucleus by Drosha to imperfect hairpin-like pre-miRNAs. These pre-miRNA precursors are then translocated to the cytosol by Exportin-5. Dicer further processes the hairpin structures to imperfect double-stranded RNA duplexes that are transferred to RISC [20–23]. After loading into RISC, the passenger strand of the duplex RNA is destroyed by Ago2 as a consequence of the duplex RNA thermodynamic polarity, while the guide strand is retained into RISC to direct RNAi. The synthesis of siRNA from pSuper vectors is quite similar to the endogenous synthesis of miRNAs. Transcription of siRNA is performed by RNA polymerase III, and the perfectly matching hairpin is transferred into the cytosol where Dicer, present in the RISC-loading complex, generates a duplex RNA. This duplex is then transferred to RISC and the passenger strand is destroyed by Ago2 as a result of the same thermodynamic considerations as for the maturation of miRNAs [20–23].

* Corresponding author.
E-mail address: jhan@scripps.edu (J. Han).

1874-9399/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagrm.2008.03.007

siRNA and miRNA are strikingly similar in structures and synthesis pathways; however, studies have also pointed out their different functions. It is well known that the ARE-mediated mRNA degradation, in which miR16 is involved, is subjected to regulation by inflammatory signals. Here we present evidence to demonstrate that siRNA-mediated mRNA decay is also subject to regulation by inflammatory signals. We show that TNF stimulation can inhibit siRNA-mediated RNA degradation by suppression of deadenylation.

2. Material and methods

2.1. Constructs

CMV-ARE and CMV-ΔARE were constructed by PCR amplification from the pBBB-TNF-3′UTR and pBBB-TNF-ΔARE vectors, as described earlier [19]. The forward primer contained a NotI restriction site whereas the reverse primer contained an XhoI restriction site. Both PCR amplification products were introduced into CMV-driven 3xFlag vectors by ligation into NotI and XhoI sites, generating the CMV-ARE and CMV-ΔARE plasmids. The Tet-ARE and Tet-ΔARE plasmids were generated by exchanging the CMV promoter for the Tetracycline responsive promoter from the CMV-ARE and CMV-ΔARE plasmids, respectively. Cloning of si20 and miR16 were performed by ligating duplex oligos into the BamHI and XhoI of pSuper vector in a similar way as was done by the Flemington lab, which is further described on their website (http://www.flemingtonlab.com). The sequence of si20 is ggttgcctctgtctcagaat. The sequence of miR16 is described in our previous work [18]. Flag-Ago2 was from Thomas Tuschl (Rockfeller Institute, New York, NY).

2.2. Cells and cell culture

HEK293T and HeLa Tet-off cells were grown at 37 °C in a water-saturated atmosphere containing 5% CO_2. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and further supplemented with 1% of a mixture of 100×-concentrated penicillin–streptomycin–glutamine solution (Invitrogen, Carlsbad, CA). Both cell lines were maintained in 10 cm dishes and passed at ~80% confluence. All transfections were performed using Lipofectamine 2000 (Invitrogen) following the procedure described by the manufacturer.

2.3. Quantitative RT-PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR reactions were performed with the one-step RT-PCR master mix reagents from Applied Biosystems (Foster City, CA) with the following primers: gtgacaagctgcacgtggat,; acagcacaataaccagcacgtt and Taqman® probe; FAM-ctgagaacttcagctcctgggc-. Quantitative RT-PCR was normalized against GAPDH, as described in our previous work [18]. Detection was performed using an ABI 7900HT fast instrument (Applied Biosystems).

2.4. Northern-blots

The procedure to perform Northern-blots is described elsewhere [24]. Briefly, Northern-blots were run on agarose gels containing formaldehyde. RNA was transferred to nitrocellulose membranes by capillary transfer. Hybridizations were performed with full-length RNA probes. Hybridizations were conducted at 70 °C in a rotisserie oven for up to 18 h. Membranes were washed until a specific signal appeared. Anti-sense full-length RNA probes were internally labeled with [α-^{32}P] UTP and generated with T7 polymerase (Roche Applied Science, Indianapolis, IN), following the manufacturer's recommendations and as described in previous publications [24].

2.5. Primer extension

Primer extensions were performed as in our previous work [18].

2.6. mRNA degradation experiments and TNF-α treatment

HeLa Tet-off cells were grown overnight in a 100 mm plate until reaching 80–85% confluence, and then they were transfected with 20 μg of Tet-ARE or Tet-ΔARE with pSuper-si20. Cells were then cultured for 15 additional hours, collected and redistributed in 12 different wells among two 6-well plates. Cells were grown for an additional 24 h. In the first set of six wells, PBS was added 15 min prior to doxycycline treatment (10 μg/ml), which was added for 0, 0.25, 0.5, 1, and 2 h to block transcription. In a second set of six wells,

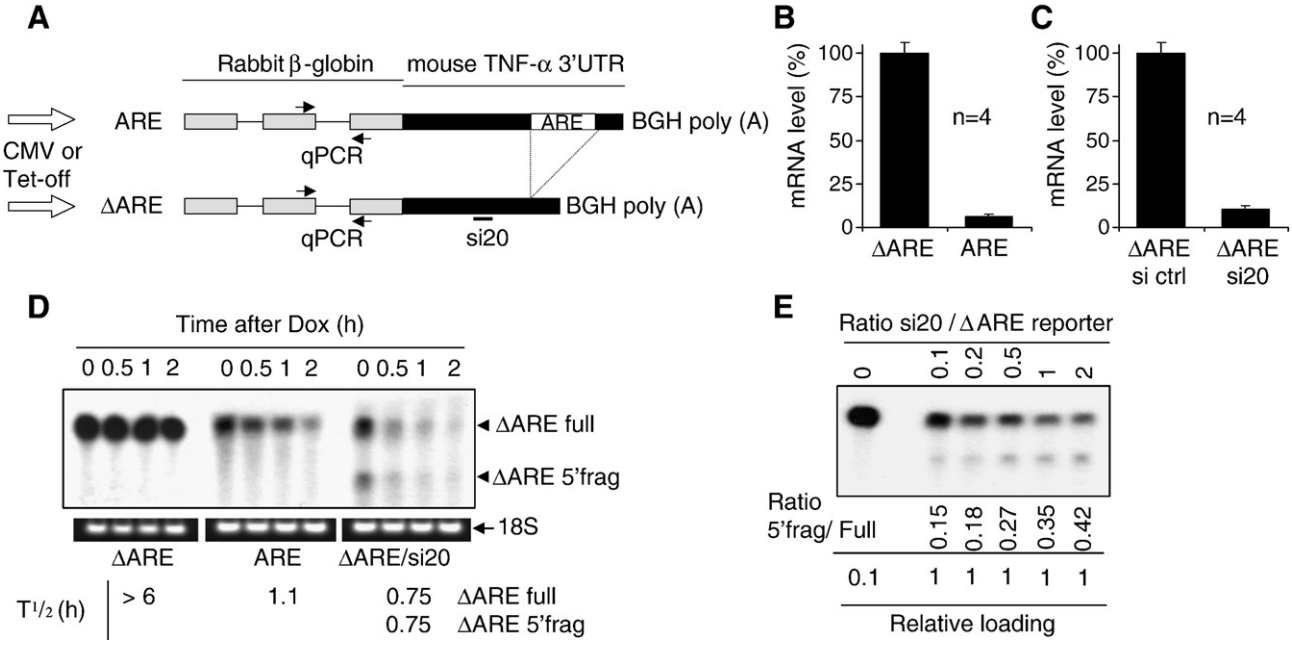

Fig. 1. ARE and ΔARE reporter constructs and their expression in mammalian cells. A) Schematic representation of the ARE and ΔARE constructs. The full-length mouse TNF-α 3′UTR was added downstream of the rabbit β-globin gene (ARE reporter). The AU-rich elements in the TNF-α 3′UTR were deleted to generate the ΔARE reporter. Both the ARE and ΔARE reporters were constructed under the control of the cytomegalovirus (CMV) or Tet promoters. The quantitative RT-PCR detection system spans intron 2. si20-targeting site and AU-rich elements (ARE) are localized in the 3′UTR of the constructs. Bovine growth hormone poly (A) signal (BGH poly (A)) was used. B) HEK-293T cells were transfected with CMV-ΔARE (ΔARE) or CMV-ARE (ARE) plasmids and cultured for 40 h. Total RNA was extracted with Trizol® reagent. Quantitative one-step RT-PCR assays were then run to measure ARE and ΔARE mRNA. ARE and ΔARE mRNA levels were normalized against GAPDH mRNA levels. Results are expressed as the mean of 4 independent experiments, the error bar represents the standard deviation. C) HEK-293T cells were cotransfected with CMV-ΔARE and pSuper-si20 (ΔARE si20), or with CMV-ΔARE and pSuper-si ctrl (ΔARE si ctrl). Samples were analyzed as in B). D) HeLa Tet-off cells were transfected with pcDNA3 and Tet-ΔARE (ΔARE); pcDNA3; Tet-ARE (ARE); or pSuper si20 and Tet-ΔARE (ΔARE/si20) and cultured for 40 additional hours. Doxycycline was then added to block transcription, and total RNA was isolated with Trizol. Reporter mRNA degradation was analyzed by Northern-blotting and 18S ribosomal RNA was utilized to control RNA loading. Half-lives were analyzed using the phosphoimager. E) HeLa Tet-off cells were transfected with a total of 5 μg of plasmid DNA. The mass ratio of pSuper-si20 and Tet-ΔARE plasmids were 0, 0.1, 0.2, 0.5, 1, and 2. pcDNA3 was utilized to normalize a total of 5 μg plasmid per transfection. Cells were then cultured for 40 additional hours before total RNA was isolated using Trizol. Reporter mRNA was analyzed by Northern-blotting and the ratios of 5′frag/Full were analyzed using the phosphoimager.