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Inhibition of RNAse A family enzymes prevents degradation and loss of silencing activity of siRNAs in serum

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ABSTRACT

Small interfering RNAs (siRNA), RNA duplexes of ~21 nucleotides, offer a promising approach to specifically degrade RNAs in target cells by a process termed RNA interference. Insufficient in vivo-stability is a major problem of a systemic application of siRNAs in humans. The present study demonstrated that RNAse A-like RNAses degraded siRNAs in serum. The susceptibility of siRNAs towards degradation in serum was strongly enhanced by local clustering of A/Us within the siRNA sequence, i.e. regions showing low thermal stability, most notably at the ends of the molecule, and by 3′-overhanging bases. Importantly, inhibition of RNAse A family enzymes prevented the degradation and loss of silencing activity of siRNAs in serum. Furthermore, the degradation of siRNAs was considerably faster in human than in mouse serum, suggesting that the degradation of siRNAs by RNAse A family enzymes might be a more challenging problem in a future therapeutic application of siRNAs in humans than in mouse models. Together, the present study indicates that siRNAs are degraded by RNAse A family enzymes in serum and that the kinetics of their degradation in serum depends on their sequence. These findings might be of great importance for a possible future human therapeutic application of siRNAs.

1. Introduction

Small interfering RNAs (siRNA), short double-stranded RNAs (dsRNAs) with two-base 3′-overhangs, serve to specifically degrade RNAs in target cells by a process termed RNA interference [1–3]. siRNAs cause post-transcriptional gene silencing by virtue of their sequence complementary to target RNAs. One strand of the siRNAs is incorporated into a multicomponent nuclease, termed RNA-induced silencing complex (RISC) [4,5], which then targets and cleaves specifically RNA that is complementary to the siRNA. This raises the prospect of harnessing this potent and specific gene-silencing mechanism for biomedical research and therapy. Indeed, siRNAs have been successfully used to inhibit viral-induced liver-cell inflammation [6], human immunodeficiency virus replication [7,8] and oncogenic K-*ras* allele-induced tumorigenesis [9]. Despite these proofs of principle, there are serious obstacles to negotiate on the way to a therapeutic application of siRNAs in humans, which include difficulties with delivery, bio-stability, pharmacokinetics, and off-target effects [10].

A possible therapeutic application of the siRNAs in humans requires that siRNAs remain active under physiological conditions during their systemic application. In the blood siRNAs are exposed to serum RNAses known to degrade single-stranded RNAs within seconds, whereas dsRNAs are poor substrates of most RNAses. A few studies have

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investigated the stability of siRNAs in serum, but produced remarkably heterogeneous results [11–15]. Possible reasons for these discrepant results include a lack of knowledge concerning the RNAses that degrade the siRNAs in serum, variable serum-stability of the siRNAs depending on their sequence, and the presence or absence of overhangs. The elucidation of the processes that limit the silencing activity of the siRNAs in serum might be of great importance for a possible future therapeutic application of siRNAs and may result in feasible strategies to prevent loss of biological activity upon their systemic application in mammals. The present study demonstrates that RNAse A-like RNAses degrade siRNAs in serum, that the susceptibility of siRNAs towards degradation in serum was strongly enhanced by local clustering of A/Us in the siRNAs and by 3′-overhanging bases, and most importantly, that inhibition of RNAse A family members prevented the degradation and loss of silencing activity of siRNAs in serum. These findings might be of great importance for a possible future human therapeutic application of siRNAs.

2. Materials and methods

2.1. RNAs and annealing

The RNAs used in the present study were obtained as single strands from Dharmacon (Lafayette, CO). For annealing, complementary and deprotected single-stranded RNAs were incubated at 60 °C for 45 min. Then, the RNAs were allowed to cool down to room temperature for 30 min. The duplex RNAs were desalted according to the protocol of the manufacturer (Dharmacon, Lafayette, CO) and dissolved in siRNA buffer.

Sequences of the siRNAs used in the present study:
STAT3 siRNA:

5′-UGAGUUGAAUUAUCAGCUUdTdT-3′
3′-dTdTACUCAACUUAAUAGUCGAA-5′

STAT3 19 bp dsRNA:

5′-UGAGUUGAAUUAUCAGCUU-3′
3′-ACUCAACUUAAUAGUCGAA-5′

Plk1 siRNA:

5′-AGACCUACCUCCGGAUCAAdTdT-3′
3′-dTdTUCUGGAUGGAGGCCUAGUU-5′

β-Catenin siRNA:

5′-UGCCGUUCGCCUUCAUUAUdTdT-3′
3′-dTdTACGGCAAGCGGAAGUAAUA-5′

The sequences of further siRNAs used in the present study are illustrated in Fig. 2.

2.2. siRNA degradation assay and RNA gel electrophoresis

A 1.5 μg duplex RNA was incubated in 15 μl of human serum (prepared from healthy donors by centrifugation without addition of coagulating/anti-coagulating reagents) or mouse serum (freshly prepared) for various duration at 37 °C. Where indicated, 40–70 units of RNAseOUT™ (Invitrogen) were added to the serum prior to the addition of the duplex RNAs. Alternatively, the siRNAs were incubated in the presence of the indicated amount of recombinant RNAse A (Sigma, St. Louis, MO).

For extraction of the duplex RNAs from the sera, 20 μl of RNAse-free water, 3.5 μl 2 M NaAc (pH 5.2) and 100 μl phenol were sequentially added to the sera. Following incubation on ice for 10 min and centrifugation at 20,000 × *g* for 8 min, the supernatants were collected and mixed with an equal volume of chloroform. After an additional centrifugation (20,000 × *g* for 3 min), the supernatants were electrophoretically separated in 20% polyacrylamide gels. RNAs were visualized by silver staining.

2.3. Cell culture and transfection of Huh7 cells

Huh7 cells were grown on 24-well tissue culture plates in RPMI 1640-medium supplemented with 10% FCS and antibiotics (Invitrogen) to 60–70% confluence. One hour prior to the transfection, the cells were switched to serum-reduced medium (Opti-MEM® I, Invitrogen). The duplex RNAs were transfected into the cells using 1 μl of Lipofectamine™ 2000 (Invitrogen) according to the recommendation of the manufacturer. After 6 h of incubation, the medium was replaced by complete medium.

2.4. Immunodepletion of RNAse A-like RNAses from serum

Sera were preincubated with protein G-Sepharose (Amersham-Pharmacia, Piscataway, NJ) for 1 h. The supernatants were divided in two aliquots. One aliquot was incubated with an anti-RNAse A antibody (Acris Antibodies, Hiddenhausen, Germany), the other aliquot with an equal amount of an irrelevant antibody (anti-STAT3, Santa Cruz, Santa Cruz, CA) for 2 h at 4 °C with gentle rocking, followed by the addition of protein G-Sepharose (Amersham-Pharmacia) and continuation of the incubation for 1 h. After brief centrifugation, the supernatants were used for the siRNA degradation assay or transfection.

2.5. Protein electrophoresis and immunoblotting

The samples were matched for protein concentration and separated on SDS polyacrylamide gels as described previously [16]. Gel-resolved proteins were electrophoretically transferred to nitrocellulose membranes, and immunoblotting with anti-STAT3 or anti-Plk1 (BD Biosciences, San Diego, CA) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA) as described recently [17]. Equal loading of the lanes on the nitrocellulose membranes was controlled by Ponceau S staining.

2.6. Reproducibility of the results

Results are representative of at least three experiments performed on different occasions.