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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.

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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.

3. Results

3.1. siRNAs are degraded via stable intermediates in serum

The elucidation of the influence of serum on the integrity of siRNAs and the processes that limit their silencing activity is of great importance for a possible future therapeutic application of siRNAs and is likely to result in feasible strategies to prevent loss of biological activity upon their systemic application in mammals. To explore the mode of degradation of siRNAs in serum, synthetic 21 bp siRNAs containing 3′-dTdT-overhangs (against STAT3) were incubated in mouse or human serum for various duration at 37 °C followed by their extraction and separation in polyacrylamide gels. The RNAs were visualized in the gels by silver-staining. In human serum, the siRNA became progressively shortened up to degradation intermediates considerably shorter than the 19 bp blunt end dsRNA (Fig. 1A and C). Because mouse models are widely used to investigate the possible therapeutic potential of siRNAs [6,18,19], we also examined the stability of siRNAs in mouse serum. The degradation of the siRNA in mouse serum differed from that in human serum in that the kinetics of degradation was slower. After 2–3 h of incubation, the RNA extracted from mouse serum migrated only slightly faster than the intact siRNA and instead comigrated with a synthetic 19-mer blunt end dsRNA (Fig. 1B and C). These data indicate that systemic application of siRNAs in mice may not reflect their degradation in human serum, in that a possible future therapeutic application of siRNAs in humans may require a higher biostability of the siRNAs than essential for down-modulation of target RNAs in mice.

3.2. 3′-Overhanging bases enhance the susceptibility of siRNAs to degradation by serum RNAses

To determine the influence of the 3′-overhangs on the stability of siRNAs in serum, we investigated the effect of the two different sera on the integrity of the 19 bp blunt end dsRNA whose sequence corresponded to the STAT3-siRNA. This blunt ended dsRNA was more resistant towards degradation in the two different sera in comparison to the siRNA (Fig. 1A and B). The increased stability of the 19 bp blunt end dsRNA compared to the corresponding siRNA was particularly striking in human serum (Fig. 1A). These data indicate that the 3′-overhangs enhanced the susceptibility of siRNAs to degradation by serum RNAses.

3.3. The degradation of siRNAs strongly depends on their sequence

To investigate the influence of the sequence of the siRNA on its degradation in human serum, different siRNAs were exposed to human serum for 2 h followed by their extraction and separation in polyacrylamide gels. As illustrated in Fig. 2, the kinetics of degradation of the siRNAs strongly depended on their sequence. siRNAs containing only few A/U at the ends of the molecule (siRNAs 1 and 2) were very stable, showing only minimal degradation after an incubation of 2 h in human serum. siRNA molecules containing four consecutive A/Us at the 5′-end of the antisense strand were relatively degradation-resistant in human serum (Fig. 2, siRNAs 3 and 4). However, further destabilization of the 5′-end of the antisense strand of the siRNA molecule resulted in considerably shorter degradation products (siRNAs 5 and 6) or even in a degradation into products smaller than 13 bp (siRNA 7) within 2 h of incubation. Degradation of the siRNA into products of less than 13 bp was also observed for a siRNA enriched in A/U at both ends of the molecule (siRNA 8). These data indicated that the susceptibility of siRNAs towards degradation in serum depended on their sequence and was strongly increased by thermodynamic instability preferably at the ends of the molecule. A low thermodynamic stability in the antisense strand of the siRNA is nevertheless required for preferable incorporation of the antisense strand into the RISC complex and thus siRNA activity [5,20–22].

3.4. Inhibition of RNAse A-like enzymes ablates degradation of siRNAs in serum

Our data indicating that active siRNAs characterized by 5′-flexibility in the antisense strand tended to be unstable in serum prompted us to study the enzymatic activities that degrade siRNAs in serum. Pancreatic type RNAses, for which bovine RNAse A is the prototype, possess helix-destabilizing activity towards dsRNA and digest dsRNA, although to a much lower rate than single-stranded RNA [23]. Human pancreatic RNAse (HP-RNAse), which is structurally and functionally very similar to bovine RNAse A, accounts for approximately 70% of RNAse activity contained in human serum [24–26]. Thus, members of the RNAse A family were good candidates to mediate degradation of siRNAs in serum. Therefore, we studied the effect of RNAse A family inhibition by RNAseOUT™ on serum-induced siRNA degradation. As illustrated in Fig. 3A and B, RNAseOUT™ completely prevented the degradation of all different siRNAs tested (as shown for STAT3, Plk1 and β-catenin) upon their exposure to human or mouse serum for 2 h. Similar results were obtained with RiboLock and RNAsin, two other commercially available inhibitors of pancreatic type RNAses (data not shown). These data suggest that RNAse A family enzymes constitute the major enzymatic activity that degraded siRNAs in serum and that specific inhibition of these RNAses strongly increased the half life of siRNAs in serum.

3.5. Inhibition of RNAse A-like RNAses prevents serum-induced loss of silencing activity of siRNAs

The RNAi activity declines dramatically when the RNA duplex length drops below 19 bp [13,27]. To investigate if RNAse A family enzyme inhibition protects siRNAs from inactivation in serum, the siRNA raised against STAT3 was exposed to human serum for 15 min or 2 h, followed by their extraction. Equal amounts of the extracted RNAs as well as of non-exposed siRNAs were transfected into Huh7 cells. Seventy-two hours after transfection, down-modulation of STAT3 was determined by immunoblotting. As shown in Fig. 3C, RNAseOUT™ completely prevented the loss of silencing activity of the siRNA upon their preincubation in human serum. RNAseOUT™ also prevented the serum-induced loss of RNAi

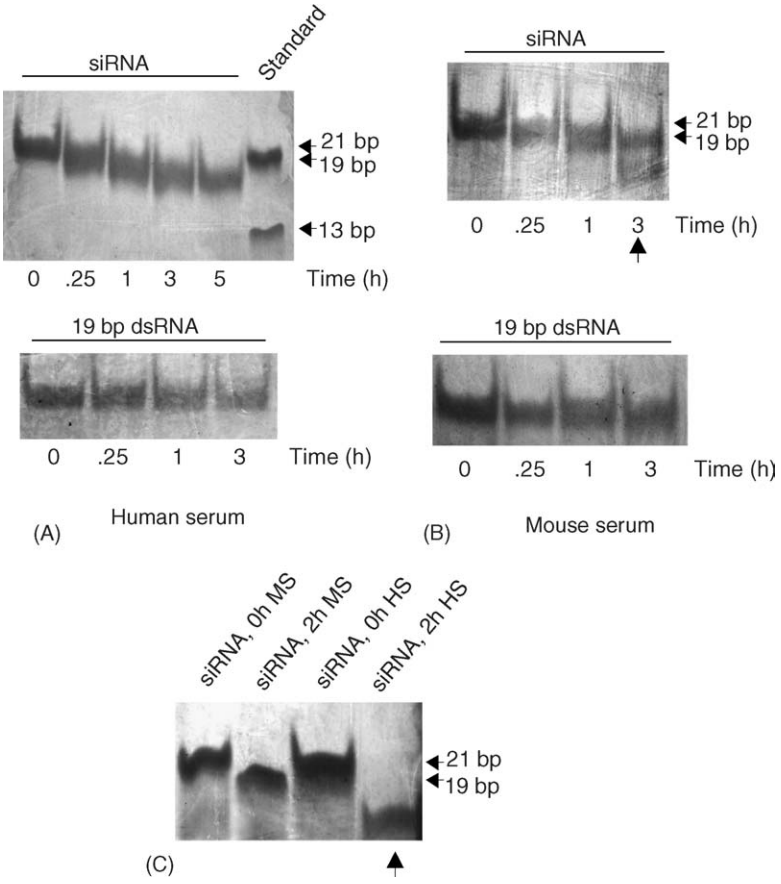


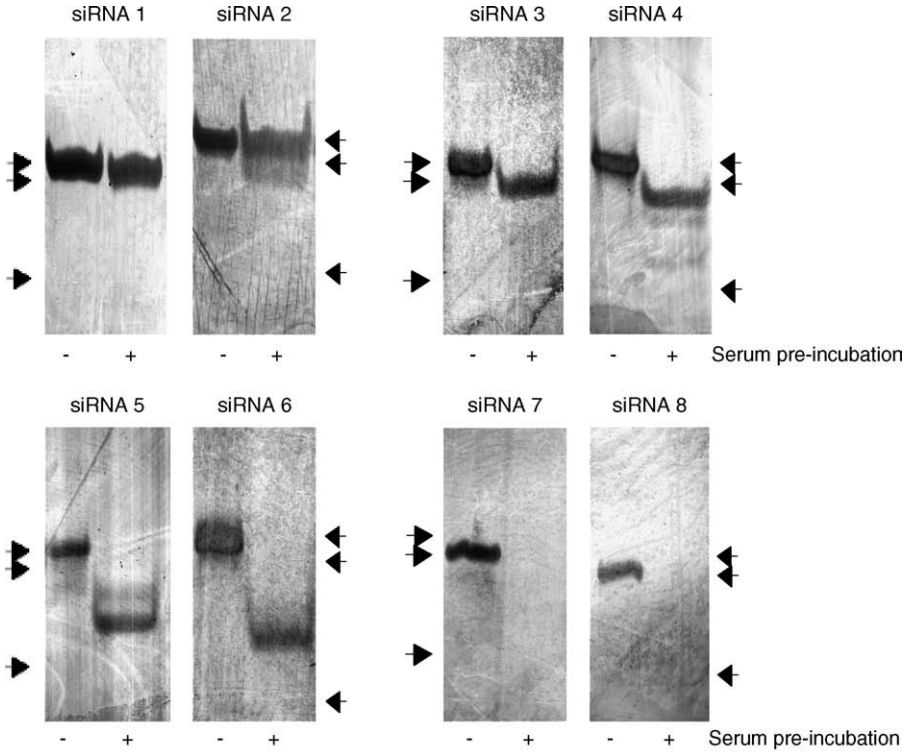
Fig. 1 – Degradation of siRNA against STAT3 in sera of different origin. A chemically synthesized siRNA against STAT3 and a 19 bp dsRNA were incubated in human (A and C) or mouse serum (B and C) for the indicated duration at 37 °C. Thereafter, the RNAs were extracted and separated in non-denaturing polyacrylamide gels. As molecular weight standards, non-treated 21, 19 and 13 bp dsRNAs were co-electrophorezed (A, last lane). Horizontal arrows indicate the migration of 21 bp siRNA, 19 bp and 13 bp dsRNA, respectively, which had not been exposed to serum. Vertical arrows refer to the respective lanes also marked by arrows in Fig. 4. HS, human serum; MS, mouse serum.

activity of a siRNA against polo-like kinase 1 (Plk1) (Fig. 3D), a promising target for an anti-tumor therapy [28,29]. These data indicate that inhibition of RNAse A family enzymes prevented the loss of silencing activity of siRNAs in serum, which might be of great importance for a possible future therapy with siRNAs in humans.

Because the two bases 3′-overhangs appeared to sensitize the siRNAs for serum-induced degradation, one strategy to reduce the rate of degradation in serum would be to use blunt end dsRNAs instead of siRNAs containing 3′-overhanging bases. However, this may reduce their silencing activity [27]. To investigate if the prevention of the loss of the 3′-overhangs of the siRNAs is important for the biological activity of the duplex RNAs, we compared the silencing activity of the STAT3-siRNA containing two bases 3′-overhangs with that of the corresponding 19 bp blunt end dsRNA. We found that in agreement with the results of Tuschl and colleagues [27], the 21 bp siRNA containing the 3′-overhangs was approximately two times more potent in eliciting RNAi than the 19 bp blunt end dsRNA (Fig. 3E), indicating that the prevention of the loss of the 3′-overhangs of siRNAs in serum may enhance their silencing efficacy in humans.

3.6. *Depletion of RNAse A immunoreactivity prevents the degradation of siRNA in serum*

Antibodies raised against bovine RNAse A recognize RNAses in human serum, indicating the presence of RNAses closely related to bovine RNAse A in human serum. Most notably, HP-RNAse has a similar molecular mass as RNAse A, is recognized by anti-RNAse A antibodies and constitutes a substantial proportion of all RNAses contained in human serum [24–26]. To investigate if RNAse A family enzymes are detectable in human serum that could mediate the degradation of siRNAs, we examined human serum for the presence of RNAse A immunoreactivity using an antibody raised against bovine RNAse A. Using this antibody, a band at 14 kDa comigrating with bovine recombinant RNAse A was identified (Fig. 4A). Thus, the data of the present study are compatible with the notion that HP-RNAse is the major enzymatic activity that degrades siRNAs in serum. Depletion of RNAse A immunoreactivity from human serum ablated the degradation of the siRNA in this serum (Fig. 4B), supporting the notion that RNAse A-like enzymes mediate the degradation of siRNAs.



siRNA	Sequence
1	5′- AGCUGAUAUUGAUGGACAG(dTdT) -3′ 3′- (dTdT)UCGACUAUAACUACCUGUC -5′
2	5′- GUGAAGUCAACAUGCCUGC(dTdT) -3′ 3′- (dTdT)CACUUCAGUUGUACGGACG -5′
3	5′- CCACGUCUGUAAUCCUUUA(dTdT) -3′ 3′- (dTdT)GGUGCAGACAUUAGGAAAU -5′
4	5′- GGACAGGAGUGACACUAUU(dTdT) -3′ 3′- (dTdT)CCUGUCCUCACUGUGAUAA -5′
5	5′- CCGAAGAGCCCAUUUACAU(dTdT) -3′ 3′- (dTdT)GGCUUCUCGGGUAAAUGUA -5′
6	5′- UGCCGUUCGCCUUCAUUAU(dTdT) -3′ 3′- (dTdT)ACGGCAAGCGGAAGUAAUA -5′
7	5′- AGGUGGUAGUUAAUAAA(dTdT) -3′ 3′- (dGdG)UCCACCAUCAAUUAUUU -5′
8	5′- AACUAUUUCCGCAAUUA(dTdT) -3′ 3′- (dTdT)UUGAUAAAGGCGUUAAU -5′

Fig. 2 – The degradation of siRNAs strongly depends on its sequence. Different siRNAs were incubated in human serum for 2 h at 37 °C. Thereafter, the RNAs were extracted and separated in non-denaturing polyacrylamide gels. The arrows denote the position of non-treated 21, 19 and 13 bp dsRNAs co-electrophorezed as molecular weight standards.

3.7. *Exposure of siRNAs to recombinant RNAse A mimics serum-induced degradation of siRNA*

To obtain further information on the functional properties of the RNAses that degrade siRNAs in serum, we compared the degradation of siRNAs in serum with that obtained upon their exposure to recombinant RNAse A. Notably, the degradation pattern of the siRNA in human and mouse sera could be mimicked by 0.5 ng/μl (compare Fig. 1B, last lane marked by an arrow with Fig. 4C, lane marked by left arrow) and 0.1 ng/μl (compare Fig. 1B, lane marked by an arrow with Fig. 4C, last lane) of recombinant RNAse A, respectively. Furthermore, similar to the stability of the duplex RNAs in serum, degradation of the blunt end dsRNA by recombinant RNAse A required approximately 50-fold higher amounts of RNAse A than essential for the degradation of the 21-mer siRNA (Fig. 4C and D). Together, these data support the notion that RNAse A-like RNAses constitute the major enzymatic activity that

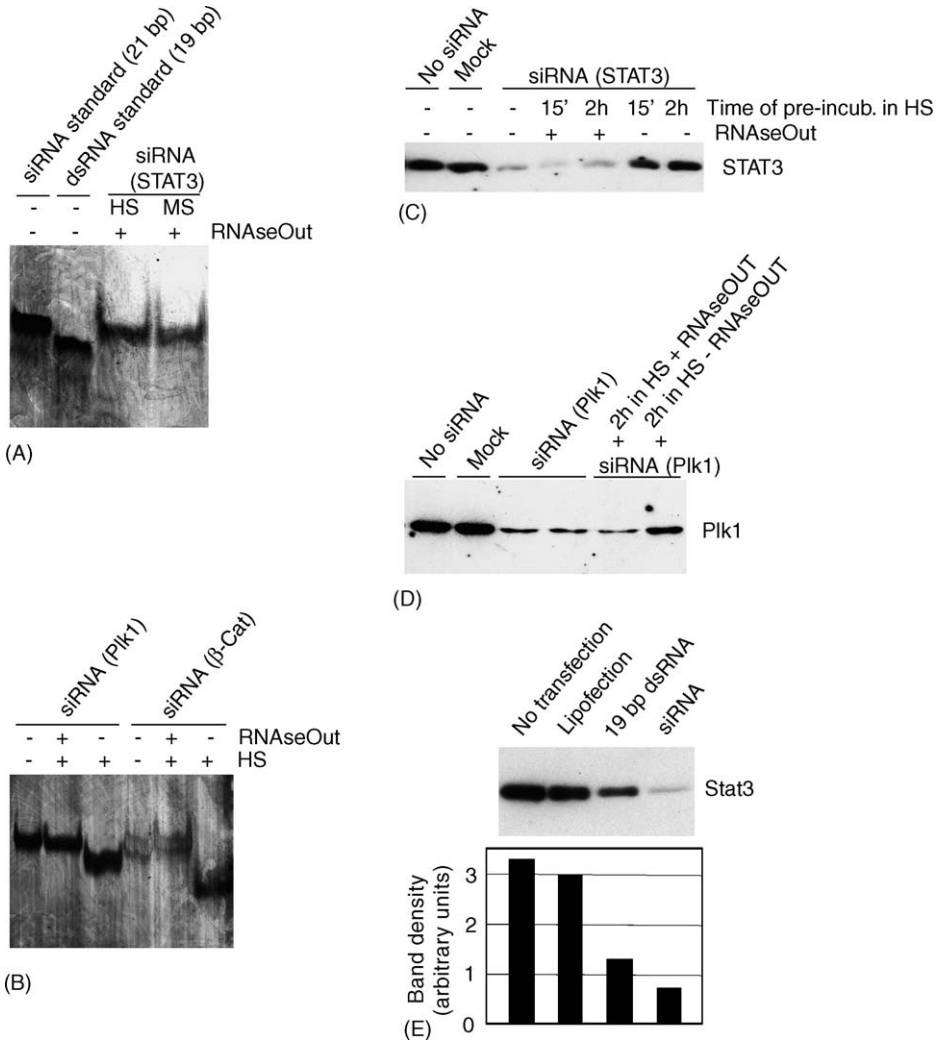


Fig. 3 – RNAseOUT™ prevents degradation and the loss of silencing activity of siRNAs in serum. siRNAs of STAT3 (A), Plk1 or β-catenin (β-Cat) (B) were incubated in human (HS) or mouse (MS) for 2 h at 37 °C in the presence or absence of RNAseOUT™ (4 U/μl serum). Non-treated siRNA and the 19 bp dsRNA were co-electrophorezed as molecular weight standards. The RNAs were visualized by silver-staining. (C) and (D) siRNA raised against STAT3 (C) or Plk1 (D) were preincubated in human serum (HS) for the indicated duration, followed by their extraction. The extracted as well as siRNAs, which had not been preincubated in serum, were transfected into Huh7 cells. Seventy-two hours (C) or 48 h (D) after transfection, the expression of STAT3 and Plk1 was determined by immunoblotting. (E) 3′-Overhangs increase the gene-silencing effect of siRNA compared to the corresponding 19 bp blunt end dsRNA. A 20 nM of siRNA raised against STAT3 or the 19 bp dsRNA were transfected into Huh7 cells by lipofection. Forty-eight hours after transfection, STAT3 expression in the cells was determined by immunoblotting.

degrades siRNAs in serum and their inhibition may thus provide a feasible strategy to prevent loss of the silencing activity of siRNAs in the blood circulation.

4. Discussion

The elucidation of the processes that limit the silencing activity of the siRNAs in human 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 major findings of the present study are that (1) RNAse A-like RNAses degrade siRNAs in serum, (2) 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 (3), 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 the development of a possible future therapeutic application of siRNAs in humans.

The present study shows that siRNAs are less stable in human compared to mouse serum. An important implication of these findings is that the systemic application of siRNAs in mice may not reflect their degradation in human serum, in which siRNAs were considerably more rapidly degraded.

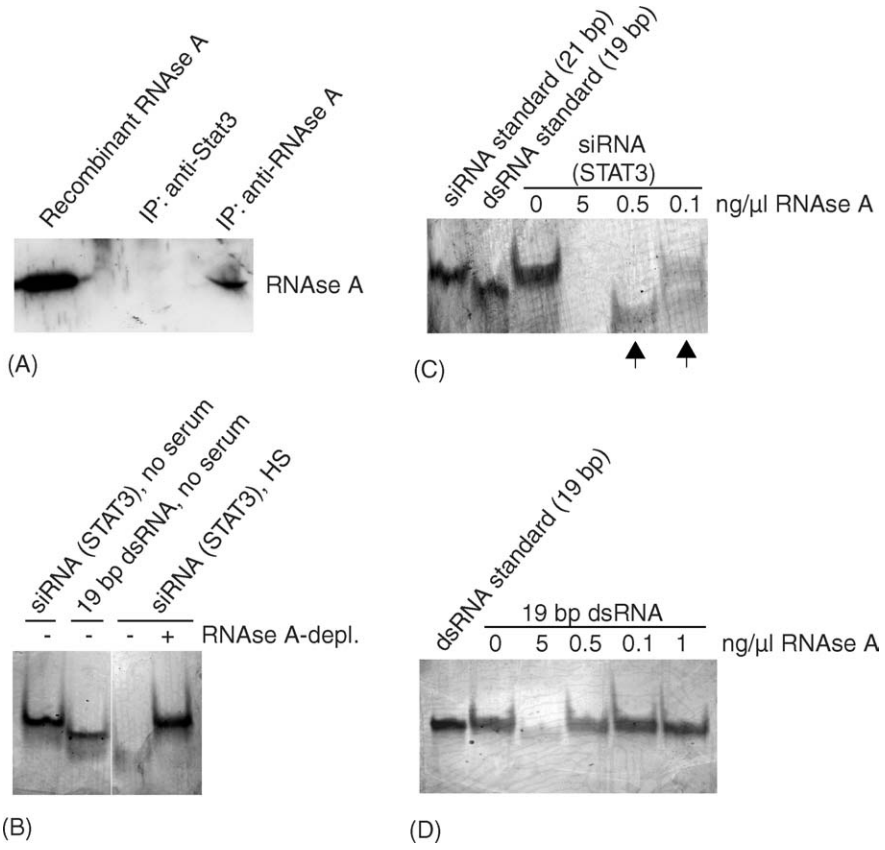


Fig. 4 – Depletion of RNAse A immunoreactivity prevents serum-induced degradation of siRNAs, whereas recombinant RNAse A mimics it. (A) Presence of RNAse A immunoreactivity in human serum. A 100 μl of serum was incubated with protein G-sepharose for 1 h. The supernatant was immunoprecipitated with 10 μg anti-RNAse A or an irrelevant antibody (anti-STAT3 IgG) for 2 h at 4 °C followed by the addition of protein G-sepharose. Immune complexes as well as recombinant RNAse A were analyzed by anti-RNAse A immunoblotting. (B) Human serum (HS) was depleted with anti-RNAse A (+) or an irrelevant antibody (−) as described in Section 2. Then, the STAT3 siRNA was incubated in these sera for 2 h, followed by RNA extraction, gel separation and silver staining. (C) and (D) The STAT3 siRNA (C) or the 19 bp dsRNA (D) were incubated with the indicated amount of recombinant RNAse A for 2 h followed by the extraction of the RNA, electrophoretic separation and silver staining.

When siRNAs are injected i.v. by the high-pressure method as frequently performed to test the effects of siRNA upon their systemic application [6,18,19], the siRNAs are likely to escape degradation in the circulation of the mouse because of the short time of exposure of the siRNAs to serum RNAses due to their rapid uptake into the target cells. Thus, the present study suggests that a possible future therapeutic application of siRNAs in humans may require a higher biostability of the siRNAs than essential for down-modulation of target RNAs in mice, which are frequently used as experimental model to study systemic siRNA therapy.

The data of the present study further indicate that the stability of siRNAs in serum depended on the presence of 3′-overhangs as well as on the sequence. In agreement with the results of Tuschl and colleagues [27], but different from the study of Czauderna et al. [13], the 21 bp siRNA containing the 3′-overhangs was approximately two times more potent in eliciting RNAi than the 19 bp blunt end dsRNA, indicating that the prevention of the loss of the 3′-overhangs of siRNAs in serum may enhance their silencing efficacy in humans. Furthermore, the susceptibility of siRNAs towards degradation in serum depended on their sequence and was strongly enhanced by thermodynamic instability preferably at the ends of the molecule. Nevertheless, a low thermodynamic stability in the antisense strand of the siRNA favors selective incorporation of the antisense strand into the RISC complex, which is a pre-requisite for high silencing activity and avoidance of off-target effects due to the incorporation of the sense-strand into the RISC complex [5,20–22,30]. Thus, designing siRNAs for high stability in serum will considerably restrict the choice of functional siRNAs within the target RNA sequence. Our findings that the degradation of the siRNAs in serum strongly depended on the sequence of the siRNA and on the presence of 3′-overhanging bases may reconcile the heterogeneity of the results on the stability of siRNAs in serum described in different studies [11–15]. Furthermore, different modes of preparation of the sera may explain variations in the kinetics of degradation of similar siRNAs in different studies. Thus, we found that the addition of EDTA, citrate or heparin to the blood to prevent blood coagulation inhibited the degradation of the siRNAs in the plasma prepared from this blood (unpublished observation).

Another important finding of the present study is that RNAse A family enzymes constitute the major enzymatic activity that degraded siRNAs in serum and that specific inhibition of these RNAses can increase the half life of siRNAs in serum. This is concluded from our findings that immunodepletion of RNAse A from serum or specific inhibition of RNAse A family enzymes prevented the degradation and loss of silencing activity of the siRNAs. In agreement with our data, HP-RNAse is recognized by anti-RNAse A antibodies and constitutes a substantial proportion of all RNAses contained in human serum [24–26]. Thus, HP-RNAse might be the major enzymatic activity that degrades siRNAs in human serum.

RNAse A family enzymes have a much higher affinity for single-stranded RNA than for dsRNA [23]. Our result that 3′-overhangs and A/U rich domains preferably at the ends of the siRNAs conferred the siRNAs susceptible to degradation by RNAse A-like RNAses is in agreement with the properties of RNAse A and its proposed mechanism of action on dsRNA: the 3′-overhangs may allow RNAse A to bind to and then to digest single-stranded domains generated by thermal fluctuation within the dsRNA secondary structure, thus allowing its degradation [23]. The extent of single strand formation by thermal fluctuation highly depends on the A/U content of that particular sequence and thus may explain why A/U rich stretches render siRNAs susceptible for degradation by serum-derived RNAses.

The present study is the first to show that siRNAs are degraded by RNAse A-like enzymes in serum and that the kinetics of degradation depended on the sequence of the siRNA. Furthermore, our finding that inhibition of RNAse A family enzymes prevented the degradation of siRNAs as well as loss of their silencing activity in serum could be of great importance for the development of feasible strategies to prevent inactivation of siRNAs upon their systemic application in mammals, including humans. This might be accomplished by several strategies, including inhibition of the RNAse A family enzymes that degrade siRNAs in the blood circulation, chemical modification of the siRNA [11–15,31,32] or their complexation with carriers such as cationic liposomes, PEI or Atelocollagen [33–37]. In combination with strategies which improve the ability of siRNAs to penetrate into cells, the inhibition of the degradation of siRNAs may allow the use of much lower siRNAs concentrations in a possible future therapeutic application in humans. This is important to avoid off-target effects of siRNAs, most notably the interferon response [38,39], or activation of Toll-like receptors [40].

In summary, the present study shows that siRNAs were degraded by RNAse A-like enzymes in serum, that the kinetics of degradation depended on the sequence of the siRNA, and that inhibition of RNAse A family enzymes prevented the loss of silencing activity of siRNAs in serum. These findings could be of great importance for the development of a feasible strategy to prevent loss of activity of siRNAs upon their systemic application in mammals, most notably in humans.

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