Binding and processing of small dsRNA molecules by the class 1 RNase III protein encoded by sweet potato chlorotic stunt virus

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Sweet potato chlorotic stunt virus (SPCSV; genus Crinivirus, family Closteroviridae) causes heavy yield losses in sweet potato plants co-infected with other viruses. The dsRNA-specific class 1 RNase III–like endoribonuclease (RNase3) encoded by SPCSV suppresses post-transcriptional gene silencing and eliminates antiviral defence in sweet potato plants in an endoribonuclease activity-dependent manner. RNase3 can cleave long dsRNA molecules, synthetic small interfering RNAs (siRNAs), and plant- and virus-derived siRNAs extracted from sweet potato plants. In this study, conditions for efficient expression and purification of enzymically active recombinant RNase3 were established. Similar to bacterial class 1 RNase III enzymes, RNase3-Ala (a dsRNA cleavage-deficient mutant) bound to and processed double-stranded siRNA (ds-siRNA) as a dimer. The results support the classification of SPCSV RNase3 as a class 1 RNase III enzyme. There is little information about the specificity of RNase III enzymes on small dsRNAs. In vitro assays indicated that ds-siRNAs and microRNAs (miRNAs) with a regular A-form conformation were cleaved by RNase3, but asymmetrical bulges, extensive mismatches and 2'-O-methylation of ds-siRNA and miRNA interfered with processing. Whereas Mg2+ was the cation that best supported the catalytic activity of RNase3, binding of 21 nt small dsRNA molecules was most efficient in the presence of Mn2+. Processing of long dsRNA by RNase3 was efficient at pH 7.5 and 8.5, whereas ds-siRNA was processed more efficiently at pH 8.5. The results revealed factors that influence binding and processing of small dsRNA substrates by class 1 RNase III in vitro or make them unsuitable for processing by the enzyme.

INTRODUCTION

Co-infection with sweet potato chlorotic stunt virus (SPCSV; genus Crinivirus, family Closteroviridae) and unrelated viruses causes severe diseases in sweet potatoes (Ipomoea batatas Lam.), because SPCSV eliminates the RNA silencing-based antiviral defence and precludes recovery of plants from virus infection (Cuellar et al., 2009; Gibson et al., 1998; Karyeija et al., 2000). RNA silencing operates via a conserved pathway triggered by dsRNA in plants and animals (Carthew & Sontheimer, 2009; Ding, 2010). The initiation of antiviral RNA silencing occurs when dsRNA, formed as an intermediate during viral RNA replication or present as secondary structures in viral ssRNA or viral gene transcripts, is recognized by specific Dicer-like endoribonucleases (DCLs). They process long dsRNA and produce small interfering RNAs (siRNAs), which are 21–24 nt in plants, contain 2 nt overhangs and 2'-O-methylation of ds-siRNA and miRNA interfered with processing. Whereas Mg2+ was the cation that best supported the catalytic activity of RNase3, binding of 21 nt small dsRNA molecules was most efficient in the presence of Mn2+. Processing of long dsRNA by RNase3 was efficient at pH 7.5 and 8.5, whereas ds-siRNA was processed more efficiently at pH 8.5. The results revealed factors that influence binding and processing of small dsRNA substrates by class 1 RNase III in vitro or make them unsuitable for processing by the enzyme.

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Three supplementary tables and four supplementary figures are available with the online version of this paper.
Viruses, in turn, encode silencing suppressor proteins, which are structurally divergent but can have the same targets in the RNA silencing pathway (Burgýán & Havelda, 2011; Deleris et al., 2006; Li & Ding, 2006).

Endogenous microRNAs (miRNAs) specifically involved in regulating gene expression arise from double-stranded precursor molecules that are transcribed from non-coding regions of the genome and processed by DCL1 in A. thaliana (Axtell et al., 2011). The passenger strand of miRNA is removed by the RNase H-like Argonaute, and the guide strand mediates mRNA cleavage or translational interference by pairing with the target transcript (Brodersen et al., 2008; He & Hannon, 2004). In plants, siRNAs and miRNAs are stabilized by a characteristic phosphate group at the 5' end and 2'-O-methylation at the 3' end (Dalakouras & Wassenegger, 2013; Yu et al., 2010). Viral silencing suppressor proteins can interfere with miRNA biogenesis, perturb regulation of gene expression, and cause developmental and physiological disturbances recognized as viral symptoms in plants (Burgýán & Havelda, 2011; Dunoyer et al., 2004).

SPCSV is an exceptional RNA virus because it encodes a class I RNase III-like endoribonuclease (RNase3) containing a single endoribonuclease domain (the RNase III signature motif) and a dsRNA-binding domain (Kreuze et al., 2002). RNase3 inhibits sense transcript-induced post-transcriptional gene silencing (Kreuze et al., 2005) and plays the key role in development of severe diseases in sweet potato plants co-infected with other viruses (Cuellar et al., 2009). A dsRNA cleavage-deficient mutant of RNase3 (RNase3-Ala) containing alanine residues in the place of glutamic and aspartic acid residues at positions 37 and 44, respectively, cannot suppress RNA silencing, suggesting a pivotal role for endoribonuclease activity in interference with silencing (Cuellar et al., 2009; Kreuze et al., 2005). No cellular class I RNase III proteins are known to play a role in RNA silencing pathways, but they are involved in the metabolism of cellular RNA (Comella et al., 2008; Giorgi et al., 2001; Spasov et al., 2002). The mechanism by which SPCSV RNase3 suppresses RNA interference (RNAi) is not fully elucidated, but RNase3 can cleave synthetic double-stranded siRNA (ds-siRNA) of 21, 22 and 24 bp in vitro to products of ~14 bp that are inactive in RNA silencing. Furthermore, RNase3 is able to process plant- and virus-derived siRNAs extracted from sweet potato plants (Cuellar et al., 2009). The aim of this study was to develop an efficient procedure for expression and purification of enzymically active RNase3 and to test its capacity to bind and process various types of small dsRNA substrates in vitro.

RESULTS

Adjustment of conditions for expression, purification and catalytic activity of RNase3

Expression of RNase3 in vitro using the procedure of Kreuze et al. (2005) resulted in relatively low yields of active RNase3. Therefore, protein expression and purification protocols were adjusted to enhance protein yield, stability and RNase3 purification. Exploiting 6×His-tag affinity to Ni-NTA, protein expression and purification resulted in homogeneous preparations of RNase3 and its mutant (RNase3-Ala) (Fig. 1a). The final yield was typically 0.5–3.0 mg purified RNase3 (ml eluate)\(^{-1}\) (Fig. 1a) and 80 mg purified RNase3 (1 starting culture)\(^{-1}\), indicating an ~15-fold higher RNase3 yield compared with the original protocol. Purified RNase3, in contrast to RNase3-Ala, processed 60, 200 and 700 bp dsRNA (Fig. 1b). However, RNase3 precipitated readily at the concentrations used in in vitro assays, which could not be prevented by adding glycerol. Furthermore, most of the common techniques used to exchange the buffer and concentrate the proteins (e.g. dialysis cassettes and cups, centrifugal units) from the highest-purity RNase3 fractions (3–5) obtained by elution of the 6×His-tagged RNase3 and RNase3-Ala from an affinity Ni-NTA resin resulted in uncontrolled protein precipitation. However, pooled fractions subjected to ammonium sulfate precipitation yielded a pellet that was insoluble in double-distilled H\(_2\)O (ddH\(_2\)O) and could hence be subjected to several washing steps. Buffers at pH 4.5 or 10.5 (acidic or alkaline shock) (Table S1, available in JGV Online) solubilized the pellet efficiently and, subsequently, pH could be increased (from pH 4) or lowered (from pH 10.5) without apparent precipitation of the protein. Thus, using the new protocol, pure and soluble RNase3 could be obtained in high amounts (Fig. 1c), typically at 1–1.3 mg ml\(^{-1}\). Most importantly, RNase3 was not denatured during precipitation. Protein pellets were stored at ~20 or ~80 °C until use.

In vitro assays to test RNase3 activity on various dsRNA substrates showed that long dsRNA (60, 200 and 700 bp) was readily processed at pH 7.5 (Fig. 1b) and pH 8.5 (60 bp dsRNA in Fig. 2c, lane 12), whereas small 22 nt ds-siRNA was processed efficiently only at pH 8.5 (Fig. S1). Subsequently, divalent cations were compared for their ability to support RNase3 activity in an end point assay at pH 8.5. Efficient cleavage of dsRNA substrates by RNase3 occurred using Mg\(^{2+}\), but processing was less efficient using Mn\(^{2+}\) or Zn\(^{2+}\); Ca\(^{2+}\) did not support RNA processing (Fig. 2a). RNase3-Ala did not process dsRNA, but migration of the 22 nt ds-siRNA substrate was retarded, which indicated that RNase3-Ala could bind the substrate (Fig. 2a). RNase3-Ala also bound the 60 bp dsRNA (Fig. 2b), in agreement with Kreuze et al. (2005). These results revealed that high-molecular-mass RNA–protein complexes were formed. They could be removed by adding proteinase K to the reaction mix (Fig. S2), after which ds-siRNA (Fig. S2, lane 7) and 60 bp dsRNA (Fig. S2, lane 10) each migrated normally by agarose gel electrophoresis. The well-characterized carnation Italian ringspot virus (CIRV) p19 protein, which suppresses RNAi by binding siRNA (Vargason et al., 2003), was included in the RNA-binding assay for comparison. p19 also formed high-molecular-mass RNA–protein complexes (Fig. S2,
determinants (Axtell et al., 2011). These obstacles can range from single-nucleotide mismatches to stretches of unpaired nucleotides that produce bulges. Symmetrical bulges (an even number of nucleotides unpaired on each strand) do not perturb the normal A-form rotation of RNA duplexes characterized by alternating minor and major grooves (Nicholson, 1999). However, A-form rotation is disturbed by asymmetrical bulges (an uneven number of nucleotides in the two strands). Therefore, the selection of small dsRNA substrates chosen for the assays included sequences with varying nucleotide content and length, degree of mismatches, type of bulge, 2 nt 5' or 3' overhang or methylation (Table S2). The degree to which the substrates were processed by RNase3 was assessed by comparing the intensity of RNA bands after agarose gel electrophoresis.

In three independent experiments, RNase3 processed all ds-siRNA substrates that consisted of random sequences, possessed a regular A-form rotation, contained 3' or 5' overhangs and were 19–24 nt in size (Figs 2c, 3a and S3). However, 2'-O-methylated ds-siRNA (Fig. 2c, lane 9) was processed less efficiently by RNase3 than the corresponding unmethylated ds-siRNA (Fig. 2c, lane 1). Furthermore, the 21 nt ds-siRNA containing only U and A residues (GS U/A, Table S2) was poorly processed (Fig. 2c, lane 11).

A selection of plant (A. thaliana) and human miRNAs derived from the Exiqon miRNA library were tested as substrates for RNase3 (Figs 2c and S3). Asymmetrical bulges altering regular A-form rotation inhibited processing (Fig. 3b) and high number of mismatches reduced digestion by RNase3, as demonstrated with hmiR9 (Fig. 3c). In contrast, single-nucleotide mismatches or stretches of mismatches that produced symmetrical bulges did not interfere with processing by RNase3 (Fig. 3a); however, GS5/6 21 nt miRNA (Fig. 2c, lane 3; Table S2) was not processed, even though only two symmetrical bulges were present. These results were obtained consistently in three (ds-siRNA and Arabidopsis miRNA) and two (human miRNA) independent experiments.

**RNase3 binds siRNA as a dimer**

Class 1 RNase III enzymes are expected to act as dimers because they contain only a single dsRNA-binding domain and one catalytic domain (Robertson et al., 1968). Dimerization of RNase3 has not been studied and was tested using recombinant 6×His-tagged RNase3, which was analysed by native PAGE or by SDS-PAGE followed by Western blotting in three experiments. The results of all experiments were consistent, indicating that, in both assays, RNase3 forms dimers and also tetramers in the absence of dsRNA substrate (Fig. S4).

In the RNA-binding experiments, processing of dsRNA would preclude detection of dsRNA binding and, therefore, catalytically inactive RNase3-Ala was included in the electrophoretic mobility shift assay (EMSA). The p19 protein of CIRV was included for comparison (Jin et al., 2010; Silhavy et al., 2002; Vargason et al., 2003). Retarded migration of the radiolabelled siRNA indicated that RNase3-Ala...
Small RNAs processed by viral class 1 RNase III

**DISCUSSION**

Only a few viruses are known to encode class 1 RNase III-like proteins exhibiting endoribonuclease activity (King...
et al., 2012). Besides SPCSV, they include *Paramecium bursaria* chlorella virus, which infects algae (Zhang et al., 2003), *Diadromus pulchellus* ascovirus (Stasiak et al., 2000), *Heliothis virescens* ascovirus (Hussain et al., 2010), which infect insects, and rock bream iridovirus, which infects fish (Zenke & Kim, 2008). The RNase III of HvAV-3e suppress RNA silencing (Hussain et al., 2010). However, RNA binding, catalytic activity and the biochemical conditions have not been characterized for most of the known viral RNase III-like proteins.

The results indicated that the virus-encoded RNase3 binds dsRNA as a dimer, which is the active form able to accommodate dsRNA binding and cleavage, and support the classification of RNase3 as a class 1 RNase III endoribonuclease (Blaszczyk et al., 2001). Binding of dsRNA was not dependent on RNase3 activity, as reported

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### Fig. 3. Grouping of the tested small RNA duplexes based on extent of processing by RNase3: efficiently processed (a), not processed (b) or moderately processed (c). Guide and passenger strands of miRNA are displayed in green and red, respectively. CH₃ indicates methylation of the 3'-OH end.
with the class 1 RNase III of *Escherichia coli* (Eco-R3) (Calin-Jageman & Nicholson, 2003). However, there is little information about binding of small 21 and 22 nt ds-siRNA by the bacterial class 1 RNase III enzymes and the RNase III-like proteins of viruses. The results of this study showed that RNase3-Ala binds ds-siRNA and miRNA, and Mn$^{2+}$ supported binding of 21 nt siRNA most efficiently. However, RNase3-Ala seemed to bind preferentially to 22 nt ds-siRNA, which is produced by DCL2, which provides surrogacy to the DCL4 pathway generating 21 nt siRNAs (Deleris et al., 2006). While 21 nt siRNAs play the main role in virus resistance, 22 nt siRNAs are important to antiviral silencing in systemically infected tissues. Both siRNAs move systemically through phloem in plants and act as systemic signals for silencing (Dunoyer et al., 2010; Garcia-Ruiz et al., 2010; Molnar et al., 2010; Seo et al., 2013). SPCSV infects only vascular tissues in sweet potato and may interfere with systemic signalling for silencing, which is suggested by inhibited recovery of sweet potato plants from virus infection when co-infected with SPCSV (Karyeija et al., 2000). In this scenario, RNase3 would suppress the DCL2 pathway (22 nt siRNA) and complement p22, a strong silencing suppressor that inhibits cell-to-cell movement of silencing signals (Kreuze et al., 2005), probably by suppressing the DCL4 pathway (21 nt siRNA) (Brosnan & Voinnet, 2011). This hypothesis gains support from the observed complementary action of p22 and RNase3 in suppression of RNA silencing (Kreuze et al., 2005).

RNase3 (Kreuze et al., 2005) and Eco-R3 (Lichner et al., 2003) suppress sense RNA-induced silencing in the leaves of *Nicotiana benthamiana*. RNA binding is not sufficient for suppression of silencing, but the catalytic activity on dsRNA is necessary (Cuellar et al., 2009; Lichner et al., 2003). RNase3 and Eco-R3 can cleave small ds-siRNA to products shorter than 19 nt (Cuellar et al., 2009), which are not bound by Argonaute proteins and cannot trigger assembly of the so-called ‘RNA-induced silencing complexes’ (Hammond et al., 2000; Montgomery et al., 2008; Tuschl et al., 1999). Cleavage of ds-siRNA or long dsRNA synthesized by host RNA-dependent RNA polymerase for amplification of silencing (Baulcombe, 2007; Garcia-Ruiz et al., 2010) suggests putative mechanisms for suppression of silencing by RNase3, but whether cleavage of small and long dsRNA is supported under similar biochemical conditions was not known before this study. Mg$^{2+}$ supports efficient cleavage of long dsRNA by RNase3 (this study; Kreuze et al., 2005) and Eco-R3 (Blaszczyk et al., 2001), rock bream iridovirus (Zenke & Kim, 2008) and HvAV-3e (Hussain et al., 2010) and supported RNase3-mediated cleavage of ds-siRNA and miRNA more efficiently than Mn$^{2+}$, which, in turn, provided RNase3 with the highest capacity to bind 21 nt ds-siRNA. RNase3 processed small dsRNA within a narrower pH range than long dsRNA. These results collectively suggest that specific biochemical conditions can differentially favour binding or processing of small or long dsRNA by RNase3 and suggest regulatory mechanisms of RNase3 activity.

Structural features such as bulges, mismatches and certain primary-sequence determinants in the long miRNA precursor dsRNA (pri-miRNA) interfere with proper processing by Dicers (class 3 RNase III) (Auyeung et al., 2013; Werner et al., 2010). Dicers do not act on small dsRNA, and little is known about whether any structural features could render small dsRNA molecules unsuitable as substrates for class 1 RNase III enzymes. Our results showed that RNase3 processed all tested ds-siRNA and miRNA substrates containing the regular A-form conformation, regardless of sequence or length, as expected for a class 1 RNase III enzyme (Nicholson, 1999). Asymmetrical bulges and a high degree of sequence mismatch in the dsRNA duplex conferred protection against cleavage by RNase3. In Eco-R3, bulge–helix–bulge structures in long dsRNA molecules inhibit processing but not binding (Calin-Jageman & Nicholson, 2003). Furthermore, dsRNA consisting only of U and A and the 2'-O-methylated ds-siRNA were processed poorly by RNase3, possibly due to disruption of the A-form rotation or steric interference. Indeed, siRNA and miRNA carrying a 2'-O-methyl group are protected against 3'-5' exonucleolytic activity and against uridylation activity which adds an oligo-U tail to the 3' ends of miRNAs (Li et al., 2005; Yu et al., 2010) and are hence stabilized *in vivo* (Cerutti & Ibrahim, 2010; Rüegger & Großhans, 2012; Yang et al., 2006). The results of these studies suggest that such modifications of siRNA and miRNA might partially protect them against the endoribonuclease RNase3, although the exact mechanism remains to be elucidated.

Taken together, our results suggest that optimal binding/processing of small dsRNA by RNase3 requires specific cations and pH range, which may be found in the various types of vascular cells (Karyeija et al., 2000) and subcellular environments. The study revealed structural factors that influence binding and processing of small dsRNA substrates by RNase3 *in vitro*, and also suggested additional, unknown factors that inhibit processing as implicated by GSS/6 21 nt miRNA. Selective action of RNase3 is indicated by the lack of any discernible morphological changes in the transgenic sweet potato plants that overexpress RNase3 (Cuellar et al., 2009). Substrate specificity could be advanced further by the action of RNase3 in interaction with host proteins, e.g. those involved in the RNA silencing pathway, as proposed for the selectivity of AGO1 (Mallory et al., 2009). Although the biological aspects of RNase3 activity *in vitro* require further study, the *in vitro* experiments carried out in this study provide general information concerning the interactions and activity of class 1 RNase III enzymes on small dsRNAs. Because RNase III homologues are encoded by viruses that infect algae, plants and animals, studies of viral RNase IIIIs may eventually help to design a means for controlling viruses in a wide range of host organisms.

**METHODS**

**Expression and purification of recombinant 6×His-tagged RNase3 and RNase3-Ala proteins from *E. coli***. RNase3 and
Fig. 4. EMSA to observe binding of ds-siRNA by RNase3 and RNase3-Ala (800 ng per reaction). The commercially available siRNA-binding CIRV p19 fusion protein (67 kDa) was included for comparison and as a size marker. All reactions in (a) to (f) were carried out at 37 °C, pH 8.5. (a) Incubation for 30 min with Mn²⁺ (10 mM MnCl₂) and 21 nt ds-siRNA or 16 bp dsDNA. Lane 1, RNase3 with 21 nt ds-siRNA, no cation added; lane 2, RNase3-Ala with 21 nt ds-siRNA, no cation added; lane 3, RNase3 with 21 nt ds-siRNA; lane 4, RNase3-Ala with 21 nt ds-siRNA; lane 5, RNase3 (1.6 μg) with 21 nt ds-siRNA; lane 6, RNase3-Ala (1.6 μg) with 21 nt ds-siRNA; lane 7, RNase3 with 16 bp dsDNA; lane 8, RNase3-Ala with 16 bp dsDNA. (b) Incubation for 30 min at 37 °C including 21 nt ds-siRNA and different cations at 10 mM. Lane 1, no cation added; lane 2, Mg²⁺; lane 3, Mn²⁺; lane 4, Zn²⁺; lane 5, Ca²⁺. (c) 22 nt ds-siRNA incubated for 180 min with different cations; cations in...
RNase3-Ala were translationally fused with a C-terminal 6×His tag by expression from the plasmids pET11d+ SPCSV RNase3-His and pET11d+ SPCSV RNase3-Ala-His (provided by M. Rajamäki, Department of Agricultural Sciences, University of Helsinki) in E. coli [strain BL21-CodonPlusBL21(DE3)-RIL] (Strategene) essentially as described by Kreuze et al. (2005); however, conditions for protein expression, purification and storage were adjusted. The transformed bacterial cells were induced by 0.1 mM IPTG and grown overnight at 20°C. Cells were lysed on ice for 2 h in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole), which contained lysozyme (1 mg ml−1; Sigma-Aldrich) and was supplemented with EDTA-free Proteinase Inhibitor Cocktail (Roche). Sonication was used (Misonix XL) and recombinant proteins were purified by chromatography with Ni-NTA agarose resin (Qiagen), loaded onto column in 300 mM NaCl, 50 mM NaH2PO4 and 500 mM imidazole. Protein fractions were eluted from the Ni-NTA chromatography with Ni-NTA agarose resin (Qiagen), loaded onto used (Misonix XL) and recombinant proteins were purified by chromatography with Ni-NTA agarose resin (Qiagen), loaded onto column in 300 mM NaCl, 50 mM NaH2PO4 and 500 mM imidazole.

Protein samples were concentrated by precipitation with saturated ammonium sulfate solution [61 g (NH4)2SO4 dissolved in 100 ml boiling ddH2O followed by cooling to room temperature]; 1 vol. protein sample was mixed with 4 vols 0.5 M EDTA (pH 8.0) and subjected to sterile filtration. All solutions were cooled to 4°C, and the protein concentration procedure was carried out in a cold room (4°C). Lysis buffer was added to pooled protein fractions to reach a volume of 9 ml. Saturated (NH4)2SO4/EDTA solution (6 ml) was added dropwise to the protein solution under continuous stirring at 500 r.p.m., and stirring was continued for 15–30 min. When the solution appeared cloudy (typically after a few hours), it was centrifuged at 20 000 g for 10 min, the supernatant was discarded and the white protein pellet was washed three times with 3 ml ddH2O. Protein suspension was distributed to several tubes, and the protein concentration procedure was carried out in a cold room (4°C). Saturated (NH4)2SO4/EDTA solution (6 ml) was added to 10 ml sample buffer (2× Tris/HCl-SDS, pH 7.0, SDS 5 % w/v; Anamed) and subjected to analysis by 9–12 % SDS-PAGE using Laemmli buffer (Laemmli, 1970) without initial thermal denaturation. A PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used for protein size estimation. Staining (10 % glycerol, 0.02 % bromophenol blue, 2 ml EDTA). A 4–20 % gradient TGX pre-cast gel (Bio-Rad) was used. Electrophoresis and destaining of the gel were carried out as described by Schägger & von Jagow (1991). NativeMark Unstained Native Protein Marker (Invitrogen) was used as a size standard.

Western blotting. Western blotting (Sambrook & Russell, 2001) of purified protein (500 ng) was carried out as described (Weinheimer et al., 2010). Immunodetection was done with polyclonal rabbit anti-RNase3 (dilution 1:500) (Kreuze et al., 2005) and HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich).

Preparation of small and long dsRNAs. Aliquots (10 µl) of complementary sense and antisense small RNA strands (Table S2) (stock 0.1 µg µl−1 in ddH2O; Sigma-Aldrich) were complementary with 1 µl RiboLock RNase Inhibitor (Fermentas), combined and incubated at 85°C for 15 min and cooled to room temperature. To assess annealing and concentration, 3 µl of the reaction was analysed by electrophoresis on a 3 % agarose gel. GS U/A was obtained as a duplex from Sigma-Aldrich. Human miRNAs were purchased from Exiqon A/S. Sequences and annotations were obtained from the miRbase database (University of Manchester, UK; http://www.mirbase.org). Secondary structures of siRNA and miRNA were predicted using the RNAhybrid Web tool (Krüger & Rehmsmeier, 2006; http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/).

Long dsDNA templates (Replicator RNAi kit manual; Finnzymes Thermo) for transcription were prepared with PCR using the plasmid pET 11d (+) SPCSV RNase3-His (Kreuze et al., 2005) as a template and the RNase3-specific forward primer SR3 T7 fwd in combination with the RNase3-specific reverse primers Phi6 SR3 rev 250 for the 60 bp dsRNA, Phi6 SR3 rev 250 for the 250 bp dsRNA, and Phi6 SR3 rev 700 for the 700 bp dsRNA (Table S3). dsDNA was purified using 2 M LiCl for precipitation, but, because it precipitates dsRNA of <100 bp inefficiently, two vols 100 % ethanol and 0.1 vol 3 M sodium acetate were added to the supernatant, followed by precipitation at −20°C for 30 min and centrifugation at 13 000 g (4°C) for 20 min to pellet dsRNA. The pellet was washed with 70 % ethanol and resuspended in 60 µl ddH2O.

End-point RNase activity assay. Cleavage of dsRNA molecules by RNase3 was tested in ‘cleavage buffer’ (2 µl 200 mM Tris/HCl, pH 7.5 or 8.5, 2 µl 500 mM NaCl, 4 µl 50 mM MgCl2, and ddH2O to reach the final volume of 20 µl), in which MgCl2 was replaced with CaCl2, ZnSO4 or MnCl2 (all at 10 mM final concentration) in some experiments. The buffer used for EMSA on CIRV p19 (see below) was included for comparison. dsRNA substrate (long dsRNA, ds-siRNA or miRNA) was applied at 200 ng per reaction containing 200 or 500 ng RNase3 or RNase3-Ala and incubated at 37°C for 30 min (long dsRNA) or up to 3 h (ds-siRNA and miRNA). The reaction was stopped by adding 1 µl 1 M EDTA. The samples were analysed by electrophoresis through a 3 % agarose gel. RNA was stained with ethidium bromide and visualized using UV Transphosphager (Biorad).

Fig. 4. (cont.) samples 1–5 are as in (b). (d) Samples and cations are as in (b) but incubation time was 180 min. (e) Binding of 21 nt ds-siRNA (GS9/10) by p19 (lane 1), in contrast to 22 nt-gfp ds-siRNA (lane 2) in the presence of Mg2+ at 10 mM. No binding of 22 nt-gfp ds-siRNA by the recombinantly active RNase3 was detected in the absence of added cations (lane 3) or in the presence of 10 mM Mg2+, Mn2+, Zn2+ and Ca2+ (lanes 4–7, respectively). (f) No binding of long dsRNA (60 bp) was detected by p19 (lane 2). RNase3 processed the 60 bp dsRNA in the presence of 10 mM Mg2+ (lane 4) but not Mn2+ (lane 5), and failed to bind and process the dsRNA without cations (lane 3). In lane 1, no protein was incubated with 60 bp dsRNA. Binding of 60 bp dsRNA was observed with and without cations added. (g) Far-Western blot analysis to detect protein–RNA complexes. RNase3 and RNase3-Ala were tested with 22 nt-gfp ds-siRNA, whereas p19 was tested using 21 nt ds-siRNA (GS9/10). Lane 1, no cross-linking; lane 2, cross-linking of protein and RNA; lane 3, cross-linking of protein and RNA including DTT in the reaction. The smear detected with RNase3 indicates cleavage of a portion of ds-siRNA. RNase3-Ala bound ds-siRNA as a monomer, dimer and tetramer (arrowheads).
BIS303PC). The existence of RNA–protein complexes was tested by adding 20 μg proteinase K (New England Biolabs) to the end-point activity assay reaction, followed by incubation at room temperature for 10 min and analysis by agarose gel electrophoresis.

**EMSA.** Sense and antisense RNA oligos (Table S3) were annealed as described above and treated with RNase H (NEB) to remove ssRNA (incubation at 37 °C for 45 min). dsRNA was purified using a QIAQuick Nucleotide Removal kit and eluted with 30 μl elution buffer (Qiagen). dsRNA oligonucleotides (2 μl) were phosphorylated with 20 U T4 polynucleotide kinase (NEB) and diluted with ddH2O to 200 counts per minute. The labelled dsRNA was purified using Quick Spin Sephadex G25 columns (Roche) and diluted with ddH2O to 200 counts per minute (measured with a Geiger counter; Berthold LB124). For EMSA, the dsRNA probe (2–5 μl) and 800 ng RNase3 or RNase3-Ala were incubated in the ‘cleavage buffer’ (see above) including different cations at 10 mM concentration at 37 °C for 3 h. Ten units of p19 (i.e. the amount of p19 that binds 100 ng siRNA at 25 °C in 1 h) (NEB) was incubated in buffer (83 mM Tris/HCl, pH 7.5, 2.5 mM MgCl2, 66 mM KCl, 100 mM NaCl, 0.1 M DTT and 0.2 % (w/v) Tween 20) at 37 °C for 3 h. UV irradiation (450 ml) was used to create covalent cross-links between RNA and proteins. The sample was mixed 1:1 (v/v) with 2 × TBE/urea loading buffer (89 mM Tris/ HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA), and the RNA–protein complexes were analysed as described (Lakatos et al., 2004).

The 60 bp dsRNA was prepared as described above and subjected to in vitro transcription in the presence of 5 × NTP mix comprising 10 mM each of ATP, CTP and GTP, 1 mM UTP and 1 μl [α-32P]UTP [800 μCi (29.6 MBq) mmol−1] (Perkin-Elmer) using an RNAi Replicator kit (Thermo Scientific) and purified. dsRNA was dissolved in 50 μl ddH2O and 3 μl was analysed by electrophoresis on a 1% agarose gel. Probes were diluted with ddH2O and tested as described for small dsRNA probes but with an incubation time of less than 2 h and using a running time of 2.5 h at 80 V.

**Far-Western blot assay.** Probe preparation (Table S3) and sample incubation were done as described for the small dsRNA probes in EMSA. RNA–protein complexes were resolved in a 9 % VarioGel agarose gel. Probes were diluted with ddH2O and tested as described above and treated with RNase If (NEB) to remove ssRNA (incubation at 37 °C for 10 min) and using a running time of 2.5 h at 80 V.

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