Short Communication

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The NS3 protein of rice hoja blanca virus suppresses RNA silencing in mammalian cells

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The NS3 protein of the tenuivirus rice hoja blanca virus (RHBV) has previously been shown to represent the viral RNA interference (RNAi) suppressor and is active in both plant and insect cells by binding short interfering RNAs (siRNAs) *in vitro*. Using a firefly luciferase-based silencing assay it is described here that NS3 is also active in mammalian cells. This activity is independent of the inducer molecule used. Using either synthetic siRNAs or a short hairpin RNA construct, NS3 was able to significantly suppress the RNAi-mediated silencing of luciferase expression in both monkey (Vero) and human (HEK293) cells. These results support the proposed mode of action of NS3 to act by sequestering siRNAs, the key molecules of the RNAi pathway conserved in all eukaryotes. The possible applications of this protein in modulating RNAi and investigating the proposed antiviral RNAi response in mammalian cell systems are discussed.

RNA interference (RNAi) is a conserved eukaryotic gene regulation mechanism comprising endonucleoytic cleavage of long double-stranded RNA (dsRNA) into short interfering RNA (siRNA) molecules of 21-26 nt by an enzyme designated Dicer (Bernstein et al., 2001). After incorporation of the siRNAs in RNA-induced silencing complexes (RISC), present in the cytoplasm, they are unwound and the retained (guide) strand is used for sequence-specific recognition and degradation of RNA targets (reviewed by: Sontheimer, 2005). Although most eukaryotes encode a functional RNAi pathway with conserved parts, there are differences between the kingdoms (Dykxhoorn et al., 2003). Over time, different biological processes involving this mechanism have been identified (reviewed by: Herr, 2005; Sontheimer, 2005; Tomari, 2005; Voinnet, 2005).

In plants, nematodes and insects, RNAi has been demonstrated to serve as an innate antiviral defence response, but RNAi has not been confirmed to operate as such in mammalian cells (Li et al., 2002; Voinnet, 2001; Zambon et al., 2006). To counteract this antiviral response, many plant and insect viruses encode proteins that interfere with the RNAi pathway. These proteins, denoted RNAi suppressors, have been identified and characterized in negative- and positive-stranded RNA viruses as well as DNA viruses (reviewed by: Lecellier & Voinnet, 2004). Similar to the RNAi suppressors of plant and insect viruses, some proteins of human infecting viruses have also been demonstrated to act like this. The NS1 protein of human influenza A virus was shown to act as an RNAi suppressor

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protein in plants and insect cells (Bucher et al., 2004; Li et al., 2004). Next to the cross-kingdom activity by human infecting viruses, some intraspecies RNAi suppression was demonstrated, too. The human immunodeficiency virus type 1 (HIV-1) Tat protein, for example, shows RNAi suppression activity in cultured human cells (Bennasser et al., 2005). A considerable number of viruses are able to infect hosts belonging to two different kingdoms such as plants and insects or insects and mammals. Rice hoja blanca virus (RHBV; genus Tenuivirus) for example infects rice and is propagatively transmitted by an insect vector, the plant hopper Tagosodes orizicolus (Ramirez et al., 1993; Ramirez et al., 1992). The virus has an ambisense RNA genome which is divided among four segments (Fig. 1a). As RHBV replication takes place in both plants and insects (Falk & Tsai, 1998) it is likely to induce antiviral RNAi in

We have previously identified the RNA 3-encoded NS3 protein (Fig. 1a) as an RNAi suppressor protein active in plants (Bucher *et al.*, 2003) and recently its RNAi suppressor activity in cultured insect cells was established (Hemmes *et al.*, 2007). As NS3 has a high affinity for 21 nt siRNAs (Hemmes *et al.*, 2007), it is tempting to propose that the protein acts by sequestering siRNAs. If this would be the exclusive biochemical activity of NS3 to perform its RNAi suppressor function it should also be operational in mammalian cells.

To investigate this, a pEF5/V5-based expression plasmid was constructed by fusing the NS3 gene to the maltose-binding protein gene (yielding MBP-NS3) using Invitrogen's gateway technology. As negative controls, pEF5/V5-based expression plasmids encoding the MBP alone or fused to a mutant of the NS3 open reading frame

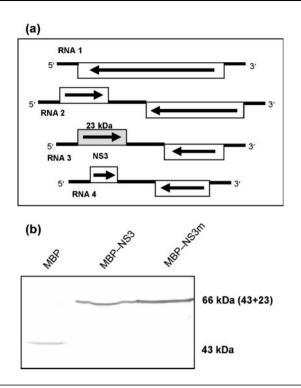


Fig. 1. Schematic presentation of the RHBV genome and transient expression of its NS3 protein. (a) The RHBV RNA genome consists of a fully negative-stranded RNA 1 and 3 ambisense genome segments (RNA 2-4). (b) Expression in Vero cells of MBP, MBP-NS3 or MBP-NS3 mutant (MBP-NS3m); confirmed by Western blotting.

(NS3m), in which three lysine residues, K173/K174/K175 were substituted with alanines, were constructed. NS3m has a significantly decreased affinity for siRNA and shows no RNAi suppressor activity in plants (H. Hemmes and others, unpublished data). Vero cells were transfected with the expression plasmids and 24 h post-transfection (p.t.) the expression of MBP and MBP-tagged NS3 proteins was verified by Western blotting (Fig. 1b).

Next a mammalian cell reporter gene RNAi assay was developed based on firefly luciferase (Fluc). To this end, Vero and HEK293 cells were co-transfected with plasmids encoding Fluc and a short hairpin RNA (shRNA) construct (Paddison et al., 2002) specifically targeting Fluc (shFluc) or a scrambled shRNA. To ensure comparability, a Renilla luciferase (Rluc) expression vector (pRL-CMV; Promega) was used as the internal control. Luciferase expression levels were determined 48 h p.t., using the Dual luciferase assay (Promega). Cells co-transfected with Fluc- and shFluc-encoding plasmids showed a drastic decrease in Fluc expression levels, which was not observed in cells expressing scrambled shRNA (Fig. 2a). This decrease was dependent on the amount of the RNAi inducer (data not shown) reaching a maximum silencing of approximately 80% of the original Fluc expression level. At this level, it was not possible to further increase silencing of the Fluc expression by adding more RNAi inducer plasmid.

To validate the assay, Vero cells were co-transfected with Fluc, shFluc or a scrambled shRNA and the tombusvirus P19 siRNA-binding RNAi suppressor-encoding plasmid, known to be active in plant and mammalian cells (Dunoyer et al., 2004; Lakatos et al., 2004). To observe the most optimal RNAi suppression of P19, a sh-construct concentration was chosen not over saturating the RNAi pathway and giving a silencing of approximately 60% at 48 h p.t. Using this set-up the observed luminescence in silenced cells expressing P19 was significantly higher than in cells expressing MBP, whereas non-silenced cells did not show differences irrespective of the presence or absence of P19 (Fig. 2a).

These results demonstrate that the designed experimental set-up can be used to determine RNAi suppressor activity in mammalian cells. However, it should be noted that the RNAi suppression conferred by the P19 protein was not as high as previously reported in a different experimental system (Dunover et al., 2004). We next tested the RNAi suppressor activity of NS3 in Vero cells (Fig. 2b). Again a significant and reproducible (partial) recovery of the luminescence was observed in the presence of wild-type NS3, either tagged or untagged, indicating that this protein is able to suppress RNAi in mammalian cells, as was observed previously in plant and insect cells (Hemmes et al., 2007). The silenced cells transfected with NS3m (expressed at a similar level as wild-type NS3; Fig. 2c) showed no significant increase in luminescence. Using HEK293 cells instead of Vero cells, similar results were obtained for NS3 (Fig. 2b) and P19.

Instead of shRNAs, synthetic siRNAs are also used for specific gene silencing in cultured mammalian cells (Elbashir *et al.*, 2001). Since Dicer action is most probably not required for the activity of siRNAs, their use can give more detailed information about the mode of action of NS3 in the RNAi pathway. In the literature, siRNAs are more potent RNAi inducers than shRNA constructs (Paddison *et al.*, 2002). Whereas shRNAs homologous to Fluc yielded a maximal silencing effect of 80 %, siRNAs achieved over 95 % of silencing (Fig. 3a). As expected, a concentration-dependent decrease in the Fluc expression was detected in cells transfected with Fluc plasmid DNA and Fluc-specific siRNAs (siLuc) compared to cells transfected with Fluc and the scrambled siRNAs (data not shown).

Having demonstrated sequence-specific silencing using synthetic siRNAs (Fig. 3a), timing of NS3 action was investigated. As it proved impossible to suppress silencing at the highest siRNAs concentrations, optimal conditions for measuring RNAi suppression were determined. RNA silencing was not suppressed in cells in which the NS3 plasmid was co-transfected with the Fluc plasmid and siLuc (Fig. 3b). However, a significant increase in Fluc expression was observed in cells initially transfected with the NS3 plasmid, and sequentially transfected after 24 h with Fluc and siLuc (Fig. 3c).

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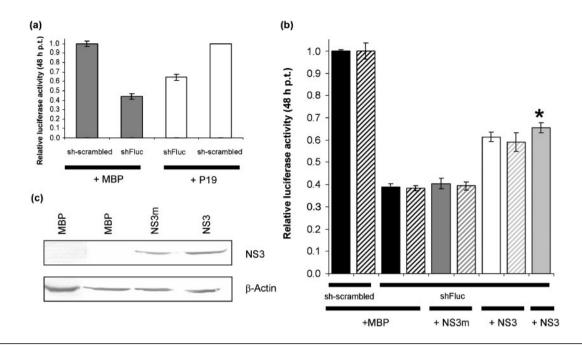


Fig. 2. Effect of NS3 expression on shRNA-induced silencing. HEK293 (dashed, panel b) or Vero (solid) cells were cotransfected with Fluc, a non-specific (sh-scrambled) or specific (shFluc) Fluc shRNA, and either MBP or P19 (a), MBP-NS3 mutant (NS3m) or NS3 (b), either MBP-tagged or untagged (*). Each treatment was performed in duplicate in at least two independent assays and the Fluc activity was normalized to Rluc for each of these treatments. (c) Western blotting confirmed expression of NS3 constructs. Immunological detection of β-actin served as loading control.

This indicates that the NS3 protein needs to be present prior to the accumulation of siRNAs as already reported for the B2 RNAi suppressor of flock house virus in insect cells (Li et al., 2004). It is apparent that NS3, due to its higher affinity for double-stranded siRNA, may extract siRNAs from the intermediate RISC complexes (R1 and RLC), where the siRNAs are still double stranded. However, single-stranded siRNAs present in mature, preassembled RISC complexes cannot be dissociated by NS3; consequently NS3 cannot compete for single-stranded siRNA in these complexes (Hemmes et al., 2007). Although, Dicer action is, most probably, not needed if siRNAs are used as inducer molecules it cannot be ruled out that NS3 acts by inhibition of Dicer needed in hairpin transfections. Data obtained from the well established in vitro Drosophila Dicer cleavage assay (Bernstein et al., 2001) showed no inhibition of Dicer by the addition of the bacterial produced MBP-NS3 protein, even at high concentrations (Fig. 3d).

Altogether, these findings suggest that the siRNA-binding capacity of NS3 is responsible for the observed RNAi suppressor activity in mammalian cells. This is confirmed by the observed decrease of the NS3 RNAi suppressor activity by an increasing siRNA concentration, resulting in a higher silencing signal. The combined presence of the pre-transfected protein as well as the siRNA in one single cell was investigated by cyto-immunological detection of the protein and transfection of rhodamine-labelled siRNA.

This revealed exclusively doubly transfected cells at a transfection efficiency of approximately 60 %.

Taken together, our data suggest that the RNAi suppressor activity of NS3 in mammalian cells is strongly dependent on the high affinity for 21 nt dsRNA, but independent of the used inducer molecules. Furthermore, the NS3 RNAi suppressor activity is not restricted to plant and insect cells, but is extended to mammalian cells. The majority of RNAi suppressors studied so far bind either long or short dsRNA (Lakatos *et al.*, 2006; Merai *et al.*, 2006), representing conserved molecules of the RNAi pathway in all eukaryotic organisms.

Incapacitating such essential molecules within the RNAi pathway offers a suppressor protein the possibility to be active in different host organisms and reduces the chance of these different hosts to evade the RNAi suppression. On the other hand, it implies that relatively high amounts of RNAi suppressors are needed in the cytoplasm of cells to reach a good level of suppression when compared with a presumed suppressor protein that would inhibit specific proteins of the RNAi machinery. Alterations in the host protein that interacts with the latter type of RNAi suppressor, though, may easily result in loss of RNAi suppression and hence avirulence. Regarding the fact that RHBV is facing antiviral RNAi in both plant and insect hosts, it is not surprising that NS3 interferes with the shared part of the RNAi pathways and not with a specific protein of one host. This hypothesis is in line with the

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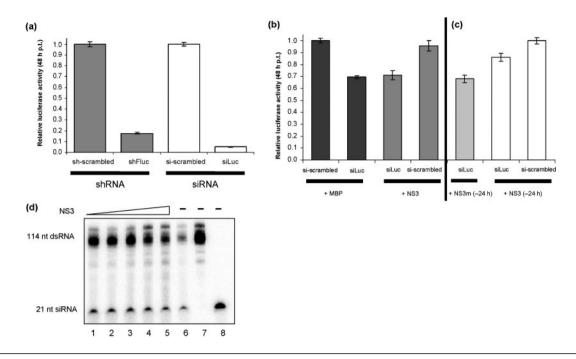


Fig. 3. (a) siRNA- or shRNA-based silencing in Vero cells co-transfected with either specific (luc) or unspecific (scrambled) RNAi inducer, together with Fluc and Rluc plasmids. (b) Effect of co-transfected NS3. Vero cells were co-transfected with vectors encoding Fluc, Rluc and either MBP or MBP-NS3 as well as siRNA specific (siLuc) or unspecific (si-scrambled) for Fluc. (c) Effect of 24 h pre-transfected NS3. Vero cells were transfected with Fluc and Rluc expression vectors, together with siRNA either specific against Fluc (siLuc) or unspecific (si-scrambled). Fluc activity was normalized to Rluc activity independently for each treatment. The resulted relative luciferase activity is a representative of at least two independent assays, whereas each assay was performed in duplicate. (d) Effect of NS3 on Dicer activity *in vitro*. Radioactively labelled dsRNA (lane 7) was cleaved into siRNA (lane 8) in *Drosophila* embryo extract in the absence (lane 6) or increasing presence of bacterial purified MBP-NS3 protein (lanes 1–5).

observation that the NS3 RNAi suppressor activity is also exhibited in mammalian cells. As the effects of synthetic siRNAs and a shRNA construct were similar, it is tempting to assume that NS3 acts downstream of both RNAi inducer molecules or on the inducer molecule itself. This is in line with our model where NS3 captures and sequesters siRNA (Hemmes *et al.*, 2007).

Using plant, insect or mammalian cell-based assays, a number of innate immunity suppressors, like interferon antagonists, encoded by mammalian viruses have been demonstrated to have RNAi suppressor activity. Some of the best studied examples so far are NS1 of influenza A virus (Bucher *et al.*, 2004; Li *et al.*, 2004), VP35 of Ebola virus, E3L of vaccinia virus, Tat of HIV-1, NSs of La Crosse virus, Tas of primate foamy virus-1 and C of hepatitis virus C (Haasnoot *et al.*, 2007; Schutz & Sarnow, 2006; Soldan *et al.*, 2005). Furthermore, it has been shown that the interferon antagonists VP35, NS1 and E3L are RNAi suppressors in human cells that are capable of restoring the production of a HIV-1 strain defective in the Tat gene (Haasnoot *et al.*, 2007).

These results indicate that RNAi, like the interferon pathway, may be an important innate antiviral defence

response in mammals, and that mammalian viruses, similar to plant and insect viruses, need to counteract this response in order to replicate. Although not studied in great detail, the RNAi suppressors from mammalian viruses seem to bind longer dsRNAs with a higher affinity than siRNAs. Besides serving as RNAi inducers, long cytoplasmic dsRNAs induce the replication-dependent antiviral interferon pathway in mammalian cells (Kato et al., 2005; Marques et al., 2006). It is therefore difficult to separate these two pathways as well as to unravel the effect of long dsRNA-binding proteins with respect to both pathways. However, using a protein, like NS3, exclusively binding siRNA, provides a promising strategy to study the possible presence of an antiviral RNAi pathway in mammalian cells. The use of NS3 could also give information about the relative importance of each antiviral pathway, by determining its complementing effect on replication level of viruses defective in their innate antiviral suppressor protein.

Next to the possibility to reveal novel aspects of the virushost interaction, the *in trans* complementation of viruses defective in their own innate antiviral suppressor genes opens the chance of virus particle production in plants, mammalian and insect cells, for example, for attenuated

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vaccine strains. Until now, a problem faced during the production cycle of attenuated viruses, most probably having defects in their innate antiviral suppressor genes, are the low virus titres reached in culture. In insect cells it was shown that virus titres can be increased by complementation, using either the virus' own suppressor protein or even cross-kingdom suppressor proteins (Li et al., 2004). Recent results have shown that this is also possible by using cell lines stably expressing an RNAi suppressor protein, such as Tat of HIV-1. The main drawback of using mammalianencoded RNAi suppressors, like VP35 and NS1, may in this case be the risk of wild-type rescuing due to recombination. The use of the NS3-based producer cell lines would avoid such drawbacks and improve the biosafety of such an approach. Further research is needed to confirm the potential application of NS3 in mammalian virus research and production strategies.

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