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Multiple Targets for Suppression of RNA Interference by Tomato Aspermy Virus Protein 2B[†]

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Received July 9, 2008; Revised Manuscript Received September 30, 2008

ABSTRACT: Viral suppressors of RNA interference (RNAi) appear to have evolved as a response to this innate genomic defense. We report the nucleic acid binding properties of the *Cucumovirus* RNAi suppressor tomato aspermy virus protein 2B (TAV 2B). Using total internal reflection fluorescence spectroscopy (TIRFS), we show that TAV 2B binds double-stranded RNA corresponding to siRNAs and miRNAs, as well as single-stranded RNA oligonucleotides. A number of positively charged residues between amino acids 20 and 30 are critical for RNA binding. Binding to RNA oligomerizes and induces a conformational change in TAV 2B, causing it to form a primarily helical structure and a 4:2 protein−RNA complex.

RNA interference (RNAi), an ancient mechanism for gene silencing triggered by recognition of dsRNA, is thought to have emerged as a way of safeguarding the genome against mobile genetic elements and the infection of viruses, and thus is a way of maintaining genomic integrity (*1−5*). Therefore, it is not surprising that viruses have evolved different strategies for suppressing the host RNAi response in the form of viral suppressor protein. These viral suppressors are widespread, having been identified in a number of different viral families. Not surprisingly, they generally share little sequence homology with one another, although they appear to exist as oligomers built upon an ∼100−200-amino acid protomer.

Tomato aspermy virus, a member of the Cucumoviruses, encodes protein 2B (TAV 2B, 95 amino acids, ∼11.3 kDa) that acts as an RNAi suppressor. Intriguingly, a similar genomic arrangement is seen in RNAi suppressors in the Nodaviruses, a family of viruses that can infect both plants and animals, such as Flock house virus b2 (FHV b2). The 2B and b2 proteins are both derived from a frame-shifted open reading frame (ORF) within the RNA polymerase gene (*6*). In spite of this genomic similarity, the 2B and b2 proteins

share little sequence identity, and it is not well understood how the *Cucumoviru*s 2B proteins suppress RNAi. To address this question, we report the characterization and oligonucleotide binding properties of TAV 2B and discuss possible modes of suppression of RNAi by this protein.

Full-length TAV 2B expressed poorly and was marginally soluble. On the basis of a sequence alignment with other 2B suppressors of the Cucumoviruses, and previous domain swapping experiments, a truncated construct consisting of amino acids 1−71 (TAV71) was expressed and purified (*7, 8*). Affinity-purified TAV71 migrates as a monomeric species on a Superdex 200 gel filtration column, at a physiological salt (150 mM) concentration (data not shown).

The high percentage of basic residues in the primary sequence suggested that it may bind nucleic acids. To investigate the potential oligonucleotide binding properties of TAV71, total internal reflection fluorescence spectroscopy (TIRFS) was used to probe the binding of fluorescently labeled oligonucleotide substrates to TAV71 in real time. For this purpose, TAV71 is immobilized onto a sensor chip through its His tag (*9, 10*). The fluorescently labeled oligonucleotide is excited by the evanescent wave emanating from the surface. Thus, a signal is recorded only when the labeled molecule is bound to protein immobilized on the surface (Figure 1 of the Supporting Information).

Suppression of RNAi may occur through a direct interaction with siRNAs. Plants have a distribution of different siRNA lengths; shorter species (21−23 nucleotides) are involved in the RNAi response, while longer siRNAs (25−27 nucleotides) are associated with transcriptional silencing and the spread of silencing. To determine if TAV71 binds siRNAs, and if so, whether there was a length preference for this recognition, fluorescently labeled siRNAs of 21, 25, and 27 nucleotides were probed for binding to TAV71 immobilized on a Ni-NTA chip. At a given protein concentration on the surface, the observed fluorescence amplitude is ∼3-fold higher for the 21-nucleotide siRNA (400 mV) than for 25- and 27-nucleotide siRNAs (110 and 130 mV, respectively). As a control, no appreciable fluorescence signal is observed in the absence of immobilized protein (Figure 2 of the Supporting Information). Thus, TAV71 recognizes siRNAs and preferentially binds 21-nucleotide siRNA compared to 25- and 27-nucleotide siRNAs (Figure 1a). The

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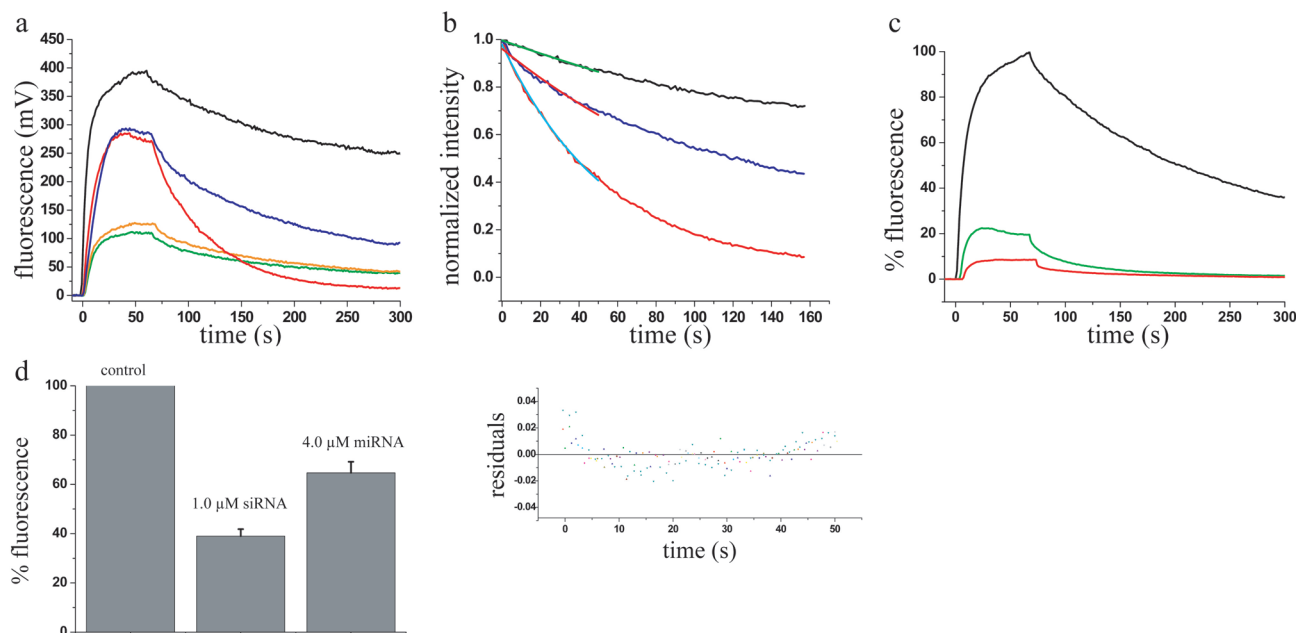|| Institute of Biochemistry.

FIGURE 1: Interaction of different RNAs with TAV71 as probed by TIRFS. (a) Sensorgram of kinetics of binding of 21-nucleotide (black), 25-nucleotide (orange), and 27-nucleotide (green) siRNAs and 21-nucleotide (red) and 30-nucleotide (blue) ssRNAs to TAV71, plotted as a function of fluorescence (millivolts) vs time (seconds). (b) Single-exponential fit of the early phase of dissociation, for 21-nucleotide siRNA (black), 30-nucleotide ssRNA (blue), and 21-nucleotide ssRNA (red). (c) Binding of RAKA (green) and KAKA (red) mutants to 21-nucleotide siRNA, normalized to the wild type (black). (d) Competition with unlabeled siRNA, showing the specificity of binding. The maximum fluorescence in the wild type is normalized to 100% in the histogram (left), with 1.0 μM unlabeled siRNA (center) and 4.0 μM unlabeled miRNA (right).

length preference is similar to that of P19, which recognizes 21-nucleotide siRNAs (*11*).

We next tested the ability of TAV71 to bind single-stranded nucleic acids, as these may represent mimics of mRNA. Both 30-nucleotide ssRNA and 21-nucleotide ss-RNA are bound by immobilized TAV71, albeit with different kinetic profiles (Figure 1a). A fit of the curves shows markedly faster dissociation in the case of the shorter ssRNA (0.018 s^{-1} for the 21-mer vs 0.0068 s^{-1} for the 30-mer), compared with 0.0029 s^{-1} in the case of the double-stranded 21-mer siRNA (Figure 1b). Thus, TAV71 is able to bind ssRNA, although with a preference for longer species. Notably, TAV71 binds to its ligands only when a certain critical concentration of the protein (~2−3 ng/mm^2) is present on the chip. Any concentration of the protein below that critical level shows no binding to the oligonucleotides (Figure 3 of the Supporting Information). This implies that oligomerization of TAV71 is required for binding to these oligonucleotides. Due to the relatively high concentration of protein on the chip, interpretation of the kinetics is complicated by the high probability for rebinding of the ligands to the protein at such high surface concentrations. To minimize this possibility, fitting of the dissociation curves was done in the early phase of dissociation, where rebinding of the ligands to the protein is minimized.

The *Cucumovirus* 2B proteins contain a particularly rich stretch of highly conserved, basic residues between amino acids 20 and 30. To further examine the roles of specific residues that may be involved in siRNA binding, two sets of double mutants of TAV71, K21A/K22A (KAKA) and R26A/K27A (RAKA), were assayed for their ability to bind 21-mer siRNA. The level of binding to siRNA was significantly reduced in both mutants, compared to that of wild-type TAV71 under similar conditions on the chip (Figure

1c). In the case of the two double mutants, equilibrium is reached quickly, and the amplitude of fluorescence is reduced, indicative of a weaker binding affinity. These residues are therefore important for the recognition of RNA.

To check the specificity of binding, immobilized TAV71 was injected with unlabeled siRNA mixed with 200 nM labeled siRNA with an identical sequence. At 1.0 μM unlabeled siRNA, the level of binding to labeled siRNA is significantly reduced (Figure 1d). Thus, the interaction of siRNA with TAV71 is specific and unaffected by the presence of the label.

Having established the specificity of the TAV71−siRNA interaction, we next examined whether miRNA can also bind to TAV71. Immobilized TAV71 was injected with 4.0 μM unlabeled miRNA (*Arabidopsis* miR-171b), mixed with 200 nM labeled siRNA. There was a significant decrease in the level of binding of labeled siRNA (Figure 1d). This shows that in addition to siRNAs, miRNAs are also able to bind to TAV71 (Figure 1d).

To investigate the oligomeric properties of TAV71 in the presence of siRNA, 4.0 μM unlabeled 21-mer siRNA was incubated with 40 μM TAV71, and the mixture was applied to an analytical Superdex-200 gel filtration column. Compared to protein alone and siRNA alone, a new peak appears at ~67 kDa, and the magnitude of the siRNA peak is diminished, indicative of siRNA binding and an oligomeric TAV71−siRNA complex (Figure 4 of the Supporting Information).

LILBID mass spectrometry (*12*) was used to determine the molecular mass of the complex. This method is useful for resolving the masses of noncovalent protein and nucleic acid complexes. For the TAV71−siRNA complex, the major peak runs as a molecular mass of 68900 ± 700 Da, which corresponds to a 4:2 protein−RNA complex [calculated mass
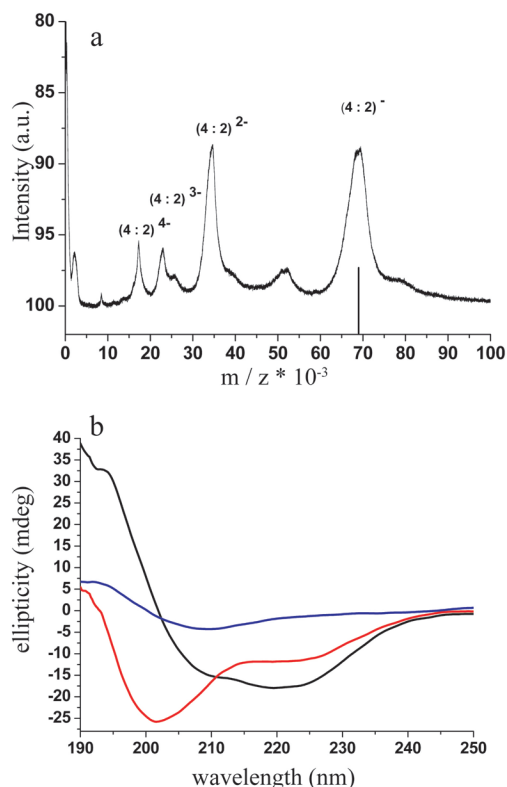
FIGURE 2: Oligomerization and secondary structure of the TAV71−siRNA complex. (a) LILBID mass spectrometry of the TAV71−siRNA complex, showing a mass of 68.9 kDa with up to four net charges corresponding to a 4:2 stoichiometry. (b) CD spectra of TAV71 (6 μM) in the absence (black) and presence of 3 μM siRNA (maroon). The spectrum for siRNA alone (3 μM) is blue.

of 69.6 kDa (Figure 2a)]. This mass is in good agreement with the gel filtration data and the recently published structure (Figures 4 and 5 of the Supporting Information) (*13*). The stoichiometry of the complex was independently determined by analytical gel filtration with UV−vis spectroscopic detection using fluorescently labeled siRNA, showing a 2:1 molar stoichiometry (Figure 6 of the Supporting Information).

Conformational changes in TAV71 that occur upon binding of RNA were probed using circular dichroism (CD). A CD spectrum of TAV71 alone (6 μM) shows a high percentage of random coil structure. Upon incubation at a 2:1 ratio with siRNA, the protein assumes a primarily helical secondary structure, with an approximate secondary structure content of 60% α-helix, 7% β-strand, and 33% random coil (Figure 2b). Upon incubation with 30-mer ssRNA, TAV71 forms a similarly ordered secondary structure (Figure 7 of the Supporting Information). Thus, on the basis of mass spectrometry and CD data, we conclude that binding to RNA orders and oligomerizes TAV71. It is worth noting, however, that the presence of the C-terminal residues may influence this oligomerization behavior.

The data presented here demonstrate that TAV 2B is able to bind a variety of different RNA species, such as siRNAs, miRNAs, and ssRNA. These results suggest that suppression of RNAi by TAV 2B may occur via targeting of different stages of the RNAi pathway. TAV 2B falls under the category of more general RNAi suppressors, with potentially multiple targets for suppression, similar to P21 (*14*). Al-

though only a limited number of suppressors have been biochemically characterized, they can be divided into ones that are specific, such as P19, and more generalized suppressors, as described in this study (*11*).

A comparison of TAV 2B and FHV b2 shows some remarkable similarities. Both proteins have a two-domain structure, with an N-terminal region involved in RNA binding, and are primarily helical in secondary structure content (*6*). Thus, it is notable that these two proteins, in spite of having little sequence similarity, have arrived at similar solutions for suppressing RNAi.

Of particular interest are viral suppressors that also bind miRNAs. We also showed that miRNAs are bound by TAV 2B, a property that has been characterized in the NS3 suppressor from rice hoja blanca virus, as well as CMV 2B. Binding of miRNAs by viral suppressors is potentially detrimental to the host (*15*). The suppression effect of TAV 2B, and perhaps in the case of other suppressors, is likely to be fine-tuned for the balance between viral reproduction and host survival. Further studies of this and other suppressors, in their native context, will be necessary for improving our understanding of these proteins and the complex interplay between virus and host.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures and Figures 1−7. This material is available free of charge via the Internet at http://pubs.acs.org.

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