Structure of the RNA-Binding Domain of Nodamura Virus Protein B2, a Suppressor of RNA Interference[†,‡]

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ABSTRACT: Protein B2 from Nodamura virus (NMV B2), a member of the Nodavirus family, acts as a suppressor of RNA interference (RNAi). The N-terminal domain of NMV B2, consisting of residues 1−79, recognizes double-stranded RNA (dsRNA). The 2.5 Å crystal structure of the RNA-binding domain of NMV B2 shows a dimeric, helical bundle structure. The structure shows a conserved set of RNA-binding residues compared with flock house virus B2, despite limited sequence identity. The crystal packing places the RNA-binding residues along one face of symmetry-related molecules, suggesting a potential platform for recognition of dsRNA.

RNA interference (RNAi), an endogenous mechanism of gene silencing based on sequence-specific recognition of target mRNA, is thought to have evolved as a way of maintaining genomic integrity against transposable elements and viruses. Suppression of RNAi by viruses is a key clue to the evolutionary origins of RNAi as an antiviral defense, and viral suppressors of RNAi are widespread (*1*). Not surprisingly, viruses have arrived at different solutions for suppressing RNAi; thus, studying the structure and function of these proteins is valuable in improving our understanding of the host RNAi machinery and the complex array of viral−host interactions. Structures of suppressors have revealed different modes of RNAi suppression by these proteins. Tombusvirus P19 specifically recognizes post-Dicer processed siRNAs, by means of aromatic residues that "measure" the size of the siRNA (*2, 3*). Protein B2 from flock house virus (FHV B2), 2b protein from tomato aspermy virus (TAV 2b), and P21 of beet yellows virus appear to bind to double-stranded RNA (dsRNA) as well as siRNAs and may act as more generalized suppressors, able to interact with the RNAi pathway at different points (*4*−*9*).

Nodamura virus (NMV), a member of the Alphanodavirus subgroup of the Nodavirus family of animal viruses, is unusual in that it is capable of infecting insects and vertebrates. During replication, a subgenomic RNA3 is generated, containing two overlapping open reading frames (ORFs), one of which encodes the B2 protein. Expression of NMV B2 (137 amino acids, 15.0 kDa) leads to accumulation of viral RNA in mammalian and insect cells (*10, 11*). Studies have shown that NMV protein B2 (NMV B2) acts as a suppressor of RNAi (*12*). Among members of the Alphanodaviruses, the B2 proteins have limited sequence identity; for example, FHV B2 and NMV B2 are less than 30% identical. Surprisingly, the Cucumovirus family of plant viruses also possesses a similar genomic arrangement, with a subgenomic ORF encoding RNAi suppressor protein 2b. Despite the related genomic arrangement of the B2 and 2b proteins in these two families and their related functions, their protein sequences share little sequence similarity. To improve our understanding of how NMV B2 suppresses RNAi, we identify the RNA-binding domain of NMV B2 and report its crystal structure at 2.5 Å resolution.

Full-length NMV B2, expressed as a maltose binding protein (MBP) fusion, aggregated in solution. On the basis of sequence alignments of B2 proteins in the Alphanodaviruses, a construct consisting of amino acids 1−79 of NMV B2 was expressed and purified. The purified protein runs as a dimer on a gel filtration column, as estimated on the basis of protein standards, and the expressed construct is thermally stable, with a melting temperature of 60 °C as monitored by circular dichroism at 222 nm (Figures 1 and 2 of the Supporting Information). A gel shift assay was performed to determine the RNA binding properties of truncated NMV B2. Fluorescein-labeled 25-nucleotide double-stranded siRNA was titrated with increasing concentrations of protein, showing a clear gel shift indicative of RNA binding (Figure 3 of the Supporting Information). The results are consistent with experiments showing that GST-tagged, full-length NMV B2 recognizes dsRNA (*12*). Binding experiments with FHV B2 also localize RNA binding to the N-terminal domain. Thus, this truncated NMV B2 construct forms a RNA-binding domain, although additional binding determinants may lie in the 58 C-terminal amino acids of the protein.

Crystals of truncated NMV B2 grew in space group $P2_12_12_1$ and diffracted to 2.5 Å resolution. The crystals contain one dimer in the asymmetric unit, in agreement with the gel filtration data. The structure was determined by molecular replacement and refined to an R_{free} of 29.3% (Table 1 of the Supporting Information). Nearly all residues of truncated NMV B2 are defined and in good electron density in the structure, except for the first five to six residues in both chains and the residues constituting the affinity tag (Figure 4 of the Supporting Information). The structure of NMV B2 is an elongated dimer measuring approximately 55 Å × 10 Å × 15 Å in size, with the monomers interacting with each other in a head-to-tail fashion, forming a four-

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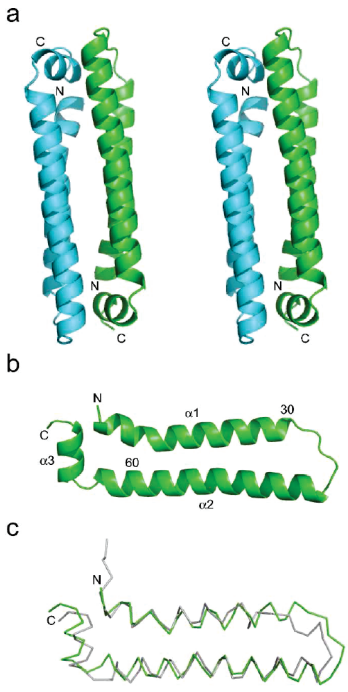

FIGURE 1: Structure of the RNA-binding domain of NMV B2. (a) Stereoview of the NMV B2 dimer. The two monomers are colored green and blue. (b) Structure of the NMV B2 monomer. (c) Structural alignment of FHV B2 (gray) and NMV B2 (green). The monomers align with an α-carbon rmsd of 1.5 Å.

helix bundle at its core (Figure 1a). The NMV B2 monomer is composed of three α-helices. Helix α1 encompasses residues Ala 7−Gln 30, with a kink at conserved residues Leu 14 and Pro 15. Helix α2, from Pro 37 to Leu 65, packs in an antiparallel fashion against helix 1. Helix α3, composed of residues Pro 68−Leu 74, is oriented orthogonal to helices ±1 and ±2 (Figure 1b). The dimeric arrangement of NMV B2 is nearly 2-fold symmetric. The two monomers of NMV B2 differ from each other with an α-carbon root-mean-square deviation (rmsd) of 0.48 Å. Differences between the two monomers are primarily found in the region between residues 32 and 37, which connect α1 and α2.

The dimer is stabilized primarily by hydrophobic interactions, with a total interface area of 1350 Å^2 per monomer, involving approximately half of the amino acids in the protein. The hydrophobic core of NMV B2 is well-defined, with a number of conserved aliphatic residues packing against one another. The helical packing seen in NMV B2 is unusual and nearly collinear, with helices intersecting at an angle smaller than the normal 18−21° crossing angle necessary for the canonical knobs-into-holes packing (*13*). Other members of the Alphanodavirus B2 proteins may therefore assume an oligomeric arrangement similar to that of NMV B2 and FHV B2.

Although NMV B2 is less than 30% identical to FHV B2, both proteins adopt a similar four-helix bundle structure capped by two short helices at the C-termini. The structures of unliganded FHV B2 and NMV B2 differ by an overall rmsd of 1.5 Å over 64 α-carbon positions (Figure 1c). A comparison of residues in FHV B2 found to be involved in RNA recognition with their structural paralogs in NMV B2 shows a significant amount of conservation (Table 2 and Figure 5 of the Supporting Information and Figure 2). Of the 10 RNA-interacting residues, 50% are identical, far higher

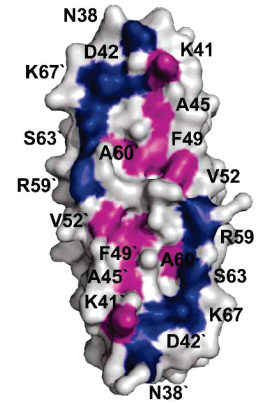

FIGURE 2: RNA-binding surface of NMV B2. Residues in NMV B2 identical to the RNA-binding residues in FHV B2 are colored blue, and NMV B2 residues corresponding to other RNA-binding residues in FHV B2 are colored purple. The orientation of the molecule is the same as in Figure 1a.

than the overall level of identity of 28% between the two proteins. Two additional residues in FHV B2 found to contact RNA via van der Waals interactions, Cys 44 and Met 55, are Phe 49 and Ala 60 in NMV B2. In both FHV B2 and NMV B2, this combination of a small and large residue forms a continuous hydrophobic patch, with similar accessible surface areas (163 Å^2 for NMV B2 and 169 Å^2 for FHV B2). Furthermore, the RNA-binding residues are clustered on α2 and are contributed from both monomers of the dimer, suggesting that a minimal dimer is required for RNA binding by B2 proteins. Therefore, it is likely that NMV B2, FHV B2, and other Alphanodavirus B2 proteins all recognize dsRNA in a similar fashion. Members of the Betanodavirus subgroup of B2 proteins, despite being RNA-binding proteins and suppressors of RNAi, are shorter in length and unrelated in sequence and appear to be monomeric in solution (Figure 5 of the Supporting Information) (*14*). No structures for these B2 proteins have been determined to date.

One model proposed for suppression of RNAi by the Alphanodavirus B2 proteins involves the sequestration of the RNA by surrounding B2 molecules (*12*). Although the crystal structure of the FHV B2−RNA complex, crystallized as a 1:1 stoichiometric mixture of B2 dimer and RNA, showed a 1:1 complex, the authors proposed that FHV B2 could further interact with the target RNA, creating larger macromolecular complexes with oligomers of B2 bound to RNA (*5*). The crystal packing observed in our structure suggests that this may be possible, as the principal crystallographic interface of the unit cell involves NMV B2 dimers packed in a staggered fashion, with an approximately 28 Å translational shift between adjacent molecules (Figure 3a). The total interface area is 460 Å^2 per molecule and involves 18 residues. Three of these residues, Arg 59, Leu 65, and Pro 68, are identical in FHV B2. The interactions governing the crystal contacts are primarily hydrophobic and nonspecific and may therefore be flexible and plastic. To spatially relate the observed crystal packing to possible modes of RNA binding, the putative RNA-binding residues of NMV B2 were mapped onto this crystallographic "oligomer". Surprisingly, the residues all line one face within the crystal (Figure 3b). So it is notable that despite the inherent symmetry of the helical bundle, NMV B2 molecules in the crystal appear to associate in a fashion in which the RNA-binding residues

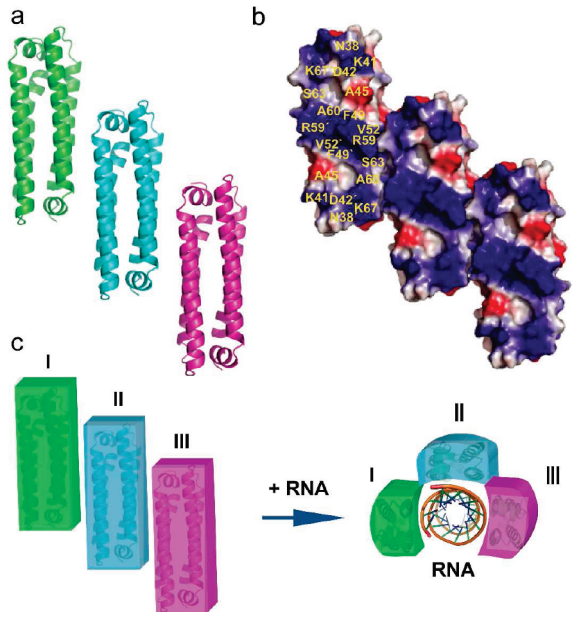

FIGURE 3: Crystal packing and potential mode of interaction between NMV B2 and RNA. (a) Packing of adjacent NMV B2 dimers within the crystal, with symmetry-related molecules colored green, blue, and purple. (b) Electrostatic representation of NMV B2 dimers, from red (negative) to blue (positive). The orientation of the molecule is the same as in Figures 1a and 2 and panel a. Putative RNA-binding residues in NMV B2 are labeled in yellow, corresponding to the two monomers. (c) Model for higher-order interaction of NMV B2 with dsRNA as suggested by the crystal packing.

are in a productive binding orientation. The arrangement of B2 molecules seen in the crystal is a linear one, so a permuted set of interactions may exist when forming around a curved surface such as that of dsRNA (Figure 3c). To accommodate the pitch of A-form RNA (30 Å per turn) and curvature of the RNA, the NMV B2 molecules may pack against each other in a less staggered fashion than in the crystal. Despite these differences, the hydrophobic, nonspecific nature of the crystal contact suggests that this may indeed be possible and that packing interactions involving α2 helices on adjacent dimers may also occur in a higher-order NMV B2−protein complex. This arrangement may be further influenced by the presence of the C-terminal tail of NMV B2. Further experiments will be necessary to test these hypotheses.

A comparison of the structure of NMV B2 with that of Cucumovirus suppressor TAV 2b highlights a number of significant similarities and differences. It is remarkable that the two viral families, with very different host ranges, encode the B2 and 2b proteins in a similar genomic arrangement. Both the B2 and 2b genes lie in a frameshifted ORF at the 3′-end of the RNA polymerase gene, and in spite of a low degree of sequence similarity between B2 and 2b, both proteins suppress RNAi. Alphanodavirus B2 proteins and TAV 2b from the Cucumoviruses are similar in length and recognize dsRNA, albeit with different binding arrangements (*5, 7*). TAV 2b is disordered in solution, adopting a helical structure in the presence of dsRNA, while FHV and NMV B2 are structured both in the absence and in the presence of dsRNA (*6, 8*). Despite these structural differences, the mode of RNAi suppression by B2 and 2b appears to be similar. Both proteins are able to recognize a variety of dsRNA species in a sequence-independent manner and are able to suppress RNAi at the pre-Dicer and post-Dicer stages of the pathway.

The results shown in this study have demonstrated that the NMV B2 suppressor adopts a helical bundle structure, and that Alphanodavirus B2 proteins likely recognize dsRNA in a similar manner, despite limited sequence identity. The packing of NMV B2 in the crystal supports the possibility of higher-order complexes with dsRNA. Nevertheless, additional experiments and structures will be necessary to present a full picture of RNA binding and suppression of RNAi by NMV B2. Furthermore, this study has identified a highly stable, RNA-binding domain that can potentially be developed as a tool for the study of small RNAs within cells.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures, Figures 1−5, and Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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