Viroplasm Protein P9-1 of Rice Black-Streaked Dwarf Virus Preferentially Binds to Single-Stranded RNA in Its Octamer Form, and the Central Interior Structure Formed by This Octamer Constitutes the Major RNA Binding Site

Jianyan Wu,^a Jia Li,^a Xiang Mao,^b Weiwu Wang,^c Zhaobang Cheng,^d Yijun Zhou,^d Xueping Zhou,^{e,f} Xiaorong Tao^a

Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), Department of Plant Pathology, Nanjing Agricultural University, Nanjing, People's Republic of China^a; College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, People's Republic of China^b; College of Life Sciences, Nanjing Agricultural University, Nanjing, People's Republic of China^c; Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, People's Republic of China^d; State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou, People's Republic of China^e; State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China^f

The P9-1 protein of Rice black-streaked dwarf virus (RBSDV) is an essential part of the viroplasm. However, little is known about its nature or biological function in the viroplasm. In this study, the structure and function of P9-1 were analyzed for *in vitro* binding to nucleic acids. We found that the P9-1 protein preferentially bound to single-stranded versus double-stranded nucleic acids; however, the protein displayed no preference for RBSDV versus non-RBSDV single-stranded ssRNA (ssRNA). A gel mobility shift assay revealed that the RNA gradually shifted as increasing amounts of P9-1 were added, suggesting that multiple subunits of P9-1 bind to ssRNA. By using discontinuous blue native gel and chromatography analysis, we found that the P9-1 protein was capable of forming dimers, tetramers, and octamers. Strikingly, we demonstrated that P9-1 preferentially bound to ssRNA in the octamer, rather than the dimer, form. Deletion of the C-terminal arm resulted in P9-1 no longer forming octamers; consequently, the deletion mutant protein bound to ssRNA with significantly lower affinity and with fewer copies bound per ssRNA. Alanine substitution analysis revealed that electropositive amino acids among residues 25 to 44 are important for RNA binding and map to the central interior structure that was formed only by P9-1 octamers. Collectively, our findings provide novel insights into the structure and function of RBSDV viroplasm protein P9-1 binding to RNA.

The replication and assembly of viruses are often concentrated in specific locations within infected cells. For the viruses in the *Reoviridae* family, distinctive electron-dense structures appear throughout the cytoplasm of infected cells at early time points after viral entry. These cytoplasmic structures are called viroplasms (*Rotavirus*, *Phytoreovirus*, and *Fijivirus*) (1–3), viral factories (VFs) (mammalian *Reovirus*) (4), or viral inclusion bodies (VIBs) (*Orbivirus*) (5). These inclusion bodies are believed to be the sites at which viral replication and assembly occur. In previous studies, nonstructural proteins from each virus were shown to be required for the formation of cytoplasmic structures, and they are regarded as key players in viral morphogenesis. In orbiviruses such as bluetongue virus (BTV), the VIBs consist predominantly of a nonstructural protein, NS2, that is responsible for recruiting both the core proteins and newly synthesized transcripts (6–8). In phytoreoviruses such as rice dwarf virus, the nonstructural protein Pns12 forms viroplasm-like structures (3). In contrast, rotaviruses encode two nonstructural proteins, NSP2 and NSP5, that must be coexpressed to form viroplasms (9, 10). However, only NSP2 contributes to the binding of the innermost capsid protein (11), the RNA-dependent RNA polymerase (12), and to the binding of single-stranded RNA (ssRNA) (13). In the mammalian *Reoviridae* family genera, μ NS alone is sufficient to form VF structures (14), and it recruits σ NS into the VFs (15). The μ NS protein acts as a scaffold to recruit viral core surface proteins (15), while σ NS binds preferentially to ssRNA (16, 17).

Rice black-streaked dwarf virus (RBSDV), a member of the genus *Fijivirus* in the family *Reoviridae*, causes serious diseases in rice and maize fields in China (18). The virus, transmitted by *Laodel-*

phax striatellus in a persistent, circulative-propagative manner (19), has a double-shelled, icosahedral capsid approximately 75 to 80 nm in diameter and contains 10 segments of double-stranded RNA (dsRNA). The nucleotide sequences of the RBSDV genome range in size from 1.8 to 4.5 kb (20, 21). The core particle of RBSDV comprises at least four proteins, P1 (RNA-dependent RNA polymerase), P2 (major core protein) (20, 21), P3 (putative capping enzyme) (22), and P8 (minor core protein) (1). The outer layer of the RBSDV particle consists of two proteins, P4 (B-spike) and P10 (major capsid protein) (1, 23). RBSDV encodes at least three nonstructural proteins, P6, P7-1, and P9-1. P6 possesses a silencing suppressor activity (24), P7-1 has been characterized as a putative tubular protein, and P9-1 accumulates in viroplasms (22). P6 and P9-1 have been shown to interact and colocalize with each other (25). Viroplasm protein P9-1 has also been shown to interact with itself *in vitro* and *in vivo* (26), and its crystal structure was recently determined, showing that neighboring dimers interact with each other to form the octamer structure (27). Expression of P9-1 in the absence of other viral proteins leads to the formation of viroplasm-like inclusions in the *Arabidopsis* protoplast (26) and

Received 12 August 2013 Accepted 17 September 2013

Published ahead of print 25 September 2013

Address correspondence to Xiaorong Tao, taoxiaorong@njau.edu.cn, or Xueping Zhou, zzhou@zju.edu.cn.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.02264-13

in Sf9 insect cells (27). RBSDV P9-1 viroplasm formation has been shown to be necessary for viral replication. Transgenic rice plants designed to target the expression of viroplasm protein P9-1 are immune to infection by RBSDV (28).

Although the P9-1 protein is known to be an essential part of the viroplasm, little is known about the nature or biological function of RBSDV P9-1 in the viroplasm. One of the major biological functions of viroplasm proteins is that they can recruit viral RNA transcripts for the synthesis of genomic dsRNA. In this study, the structure and function of P9-1 were analyzed for *in vitro* binding to nucleic acids by using gel mobility shift assays. We found that the P9-1 protein bound preferentially to single-stranded versus double-stranded nucleic acids; it did not show any preference for binding to RBSDV ssRNA versus non-RBSDV ssRNA. Using gel mobility shift assays and discontinuous blue native gel analysis, we demonstrated that P9-1 preferentially binds to ssRNA as an octamer rather than as a dimer. Alanine substitution analysis further revealed that electropositive amino acids among residues 25 to 44 are important for RNA binding and that these RNA binding sites map to the central interior structure that was formed only by P9-1 octamers. Our findings provide novel insights into the structure and function of RBSDV P9-1.

MATERIALS AND METHODS

Construction of plasmids. The full-length open reading frame of the P9-1 gene was amplified from the cDNA of an RBSDV Lianyungang isolate (accession number [KC875238](#)) and cloned into bacterial expression vector pET28c (Novagen, Darmstadt, Germany) and pXT99, which was engineered to contain a *Tobacco etch virus* (TEV) cleavage site based on pET28. Site-directed mutagenesis was used to replace amino acids (aa) 25 to 44 or 319 to 327 or single sites at R26, R39, K43, and K44 individually or jointly with alanine by using the two-step PCR procedure described by Higuchi et al. (29). Deletion mutant P9-1 proteins were generated by direct PCR amplification with combinations of specific primer pairs ([Table 1](#)); the products were then inserted into pET28c. The templates for *in vitro* transcription, i.e., the plus or minus strand of full-length (1,900 nucleotides [nt]) RBSDV S9 (S9-1900nt), the 5'-terminal fragment (150 nt) of S9 (S9-150nt), and full-length S4 of *Rice stripe virus* (RSV) (2,157 nt), were amplified from the cDNA of RBSDV or RSV. For each amplification, the upstream primer possessed a T7 promoter sequence and the downstream primer contained a SpeI restriction site ([Table 1](#)). The PCR products were cloned into the pMD19-T vector (TaKaRa, Dalian, China), and positive clones were selected by forward insertion. All of the primers used in this study are described in [Table 1](#).

Protein expression and purification. To express the wild-type and mutant forms of P9-1, each plasmid was transformed into Rosetta (DE3). The cells were cultured to reach an optical density at 600 nm (OD_{600}) of 0.6 and then induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 20°C for 12 h. The cells were harvested and lysed by sonication for 20 min on ice in suspension buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0). The lysed cells were centrifuged at 10,000 rpm for 1 h. The supernatants were incubated with 1 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose at 4°C for 2 h and then loaded onto a chromatographic column (Bio-Rad, Hercules, CA). After the Ni-NTA column was washed three times with 20 mM imidazole wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 8.0), the proteins were eluted with 250 mM imidazole elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.0). After dialysis of the protein into storage buffer (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0), the sample was dispensed into aliquots and stored at -70°C until use. To purify the tag-free P9-1 protein, we removed the N-terminal His tag from the 6 $\times$ His-tagged P9-1 protein that was expressed from pXT99-P9-1 by using a recombinant 6 $\times$ His-TEV protease as described by Tropea et al. (30).

Western blot analysis. The proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The antigen on the membrane was probed with a rabbit anti-6 $\times$ His tag antibody (1:1,000 dilution; made in this lab) and recognized with alkaline phosphate (AP)-coupled goat anti-rabbit IgG (Sigma; 1:10,000 dilution). The membrane blot was developed in 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) substrate solution (Shenggong, Shanghai, China).

Discontinuous blue native gel electrophoresis and size exclusion chromatography (SEC) analysis. Discontinuous blue native gel electrophoresis was carried out as described by Niepmann and Zheng (31). The protein samples were mixed with loading buffer (100 mM Tris-Cl [pH 8.0], 40% glycerol, 0.5% Coomassie brilliant blue G-250) and incubated for 10 min at room temperature. The mixtures were analyzed on a 4 to 16% polyacrylamide gradient gel with 100 mM histidine (adjusted to pH 8.0 with Tris base) as the cathode buffer. The gels were destained with several changes of 7.5% acetic acid and 5% ethanol.

SEC analysis was carried out with a Superdex 200 prep grade (pg) column (GE Healthcare, Shanghai, China) as described by Akita et al. (27). The column was equilibrated with buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. SEC was performed at a flow rate of 0.5 ml/min. Elution was monitored at a UV absorbance of 280 nm. The standards used to calibrate the column were blue dextran, ferritin, catalase, conalbumin, ovalbumin, and carbonic anhydrase (GE Healthcare, Shanghai, China).

***In vitro* transcription and RNA labeling.** The plasmid DNA used as the template was linearized with SpeI. RNA transcripts were labeled with digoxigenin (DIG)-UTP (Roche, Shanghai, China) with a T7 riboprobe *in vitro* transcription system (Promega, Shanghai, China). Each transcription reaction mixture was set up to include the supplied buffer; 1 mM (each) ATP, CTP, and GTP; 0.65 mM UTP; 0.35 mM DIG-11-UTP; 500 ng of linearized template; 20 U of RNase inhibitor; and 40 U of T7 polymerase. For the preparation of unlabeled transcripts, 2.5 mM (each) ATP, CTP, GTP, and UTP were used for each reaction. The reactions were carried out for 2 h at 37°C. Two units of RNase-free DNase I was then added, and the mixture was incubated at 37°C for 30 min. Following extraction with phenol-chloroform, the transcripts were precipitated with dehydrated alcohol in the presence of 300 mM sodium acetate (pH 5.2). The transcript pellets were then washed with 70% alcohol, air dried at room temperature, and resuspended in RNase-free, double-distilled H_2O (ddH_2O). The concentrations of RNA transcripts were determined by measurement of the OD_{260} with a NanoDrop 1000 spectrophotometer (Thermo Electron, Shanghai, China).

Gel mobility shift assay. In a typical binding assay, increasing amounts of P9-1 or mutant proteins were incubated in 10 μl of binding buffer (45 mM Tris, 45 mM boric acid, pH 8.4) with 3.1 nmol of DIG-11-UTP-labeled ssRNA S9-1900nt or 39 nmol ssRNA S9150nt. The reaction mixtures were incubated for 10 min at room temperature, and then 5 μl of loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 15% Ficoll 400 in 0.5 $\times$ Tris-borate-EDTA [TBE] buffer) was added. The reaction mixtures were separated by electrophoresis in 1% agarose with 0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). All buffers were prepared with diethyl pyrocarbonate-treated ddH_2O to eliminate potential RNase activity.

The gel was electroblotted onto a nylon membrane (Hybond N+; GE Healthcare, Shanghai, China). The ssRNA was fixed to the membranes with 0.25 J/cm² of UV light (UVP, Upland, CA). The electrophoretic retardation of the DIG-labeled RNAs was detected with DIG-AP Fab fragments (Roche, Shanghai, China), and the blots were developed with BCIP-NBT substrate solution (Shenggong, Shanghai, China).

Competition assays. A DIG-labeled S9 full-length RNA probe (3.1 nmol) was mixed with various amounts of unlabeled competitors; 4.6 μmol of P9-1 was then added, and the mixtures were incubated for 10 min at room temperature. For one set of reactions, P9-1 was combined with DIG-labeled RNA probe and incubated for 10 min as described earlier,

TABLE 1 Primers used to make RBSDV P9-1 constructs in this study

Clone and primer	Primer direction ^a	Sequence ^b	Purpose
pET28c-P9-1			
XT420	F	CGGGATCCCGATGGCAGACCAAGAGCGGAG	Amplification of RBSDV P9-1 and cloning into pET28c
XT421	R	CCGCTCGAGTCAAACGTCCAATTTCAAG	
pET28c-P9-1 ^{ΔC9}			
XT420	F	CGGGATCCCGATGGCAGACCAAGAGCGGAG	Amplification of P9-1 ^{ΔC9} and cloning into pET28c
XT736	R	CCGCTCGAGTCAAATAACGCAATCAACATTGCC	
pET28c-P9-1 ^{ΔC23}			
XT420	F	CGGGATCCCGATGGCAGACCAAGAGCGGAG	Amplification of P9-1 ^{ΔC23} and cloning into pET28c
XT939	R	CCGCTCGAGTCAGAATTTACGATATCTCTTG	
pMD19-S9-1900-Sense			
XT545	F	CGTCAGAAAT ~~taatacgactcactatagg~~ AAGTTTTTTAGCCTGGAAGT	Amplification of plus strand of full-length RBSDV S9 (1,900 bp) and cloning into pMD19-T vector
XT297	R	GGACTAGTGACGATAGCTGTAGCCGGCTTG	
pMD19-S9-1900-Antisense			
XT558	F	CGTCAGAAAT ~~taatacgactcactatagg~~ ACGATAGCTGTAGCCGGCTTG	Amplification of minus strand of full-length RBSDV S9 (1,900 bp) and cloning into pMD19-T vector
XT296	R	GGACTAGTAAGTTTTTTAGCCTGGAAGT	
pMD19-S9-150			
XT545	F	CGTCAGAAAT ~~taatacgactcactatagg~~ AAGTTTTTTAGCCTGGAAGT	Amplification of the 5' terminal fragment of S9 (150 bp) and cloning into pMD19-T vector
XT696	R	GGACTAGTAGAAGTCGATGAATTTGTG	
pMD19-S4			
XT52	F	CGTCAGAAAT ~~taatacgactcactatagg~~ ACACAAAGTCCAGGGCATTG	Amplification of full-length RSV S4 (2,157bp) and cloning into pMD19-T vector
XT126	R	GGACTAGTACACAAAGTCAGGGCATATC	
pXT99-P9-1			
PV251	F	GGAATTCCATATGGCAGACCAAGAGCGGAG	Amplification of RBSDV P9-1 and cloning into pXT99
XT421	R	CCGCTCGAGTCAAACGTCCAATTTCAAG	
pET28c-P9-1-Bind1m			
XT879	F	GGCTGCAGCTGCTGCGGCAGCGGCTGCGGCTGCAGCAGCGATCCCCCTACTCGATGACG	Alanine substitution of amino acid residues 25–44 by site direct mutagenesis based on pET28c-P9-1
XT878	R	CTGCCGCAGCAGCTGCAGCCGCTGCAGCTGCCGCAGCTGCTGTTGGTCGTTTTTAATT	
pET28c-P9-1-Bind2m			
XT879	F	GGCTGCAGCTGCTGCGGCAGCGGCTGCGGCTGCAGCAGCGATCCCCCTACTCGATGACG	Alanine substitution of amino acid residues 319–327 by site direct mutagenesis based on pET28c-P9-1
XT878	R	CTGCCGCAGCAGCTGCAGCCGCTGCAGCTGCCGCAGCTGCTGTTGGTCGTTTTTAATT	
pET28c-P9-1-Bind3m			
PV252	F	AAACGACCAACCAAAATGCAAACACAAATTCATCG	Alanine substitution of amino acid R26 by site direct mutagenesis based on pET28c-P9-1
PV253	R	CGATGAATTTGTGTTTGCATTTGGTTGGTCGTTT	
pET28c-P9-1-Bind4m			
PV306	F	CTCAATCTACTGAAAATGCTCTTTCAACCAAGAA	Alanine substitution of amino acid R39 by site direct mutagenesis based on pET28c-P9-1
PV307	R	TTCTTGTTGAAAGAGCATTTTCAGTAGATTGAG	
pET28c-P9-1-Bind5m			
PV308	F	AATCGTCTTTCAACCGCAAAAATCCCCCTACTCG	Alanine substitution of amino acid K43 by site direct mutagenesis based on pET28c-P9-1
PV309	R	CGAGTAGGGGGATTTTTCGGTTGAAAGACGATT	
pET28c-P9-1-Bind6m			
PV310	F	CGTCTTTCAACCAAGGCGATCCCCCTACTCGATG	Alanine substitution of amino acid K44 by site direct mutagenesis based on pET28c-P9-1
PV311	R	CATCGAGTAGGGGGATCGCCTTGGTTGAAAGACG	

(Continued on following page)

TABLE 1 (Continued)

Clone and primer	Primer direction ^a	Sequence ^b	Purpose
pET28c-P9-1-Bind7m			
PV407	F	CGTCTTTCAACCGCAGCGATCCCCCTACTCGAT	Alanine substitution of amino acid residues K43 and K44 by site direct mutagenesis based on pET28c-P9-1
PV408	R	ATCGAGTAGGGGGATCGCTGCGGTTGAAAGACG	
pET28c-P9-1-Bind8m			
PV254	F	CTCAATCTACTGAAAATGCGCTTTC AACCGCGGCAATCCC	Alanine substitution of amino acid residues R39, K43 and K44 by site direct mutagenesis based on pET28c-P9-1
PV255	R	CCTACTCGATG CATCGAGTAGGGGGATTGCCGCGGTTGAAAGCGCATTTC AGTAGATTGAG	
pET28c-P9-1-Bind9m			
PV252	F	AAACGACCAACCAATGCAAACACAAATTCATCG	Alanine substitution of amino acid residues R26, R39, K43 and K44 by site direct mutagenesis based on pET28c-P9-1-Bind8m
PV253	R	CGATGAATTTGTGTTTGCAATTTGGTTGGTCGTTT	
pET28c-P9-1 ^{ΔC23} -Bind9m			
XT420	F	CGGGATCCCGATGGCAGACCAAGAGCGGAG	Amplification of P9-1 ^{ΔC23} -Bind9m and cloning into pET28c
XT939	R	CCGCTCGAGTCAGAAATTTACGATATCTCTTG	

^a F, forward; R, reverse.
^b BamHI site is underlined, XhoI site is in bold, NdeI site is both underlined and in bold, SpeI site is in italics, and T7 promoter is in lowercase and underlined.

after which the competitor was added and the samples were incubated for an additional 10 min. The competitors used for competition between ssRNA and dsRNA and between ssDNA and dsDNA were as follows: ssRNA, full-length *in vitro*-transcribed S9 RNA transcript; dsRNA, annealing product of the plus and minus strands of the full-length S9 RNA; ssDNA, M13mp18 phage (NEB, Beijing, China); dsDNA, PCR amplification product of full-length RBSDV S9. The samples were separated electrophoretically and visualized as described for the gel mobility shift assay. The bound RNA was quantified as described by Gillian et al. (17). The ratios of unlabeled competitor to labeled RNA were calculated from masses of nucleic acid.

Crystal structure visualization and analysis. The RBSDV P9-1 crystal structure (3VJJ) (27) was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (www.rcsb.org). Visualization and analysis of the amino acid residues on the crystal structure were carried out with the PyMOL program.

RESULTS

P9-1 protein binds preferentially to single-stranded rather than double-stranded nucleic acids. To obtain large amounts of P9-1 protein for biochemical characterization, we cloned full-length RBSDV P9-1 into pET28c. After induction, the N-terminally 6×His-tagged recombinant P9-1 protein was purified with Ni-NTA resin and separated by 10% SDS-PAGE. The expression of high levels of a 40-kDa protein was observed in the fraction eluted by 250 mM imidazole buffer (Fig. 1A). The nature of the purified protein was further verified with 6×His antiserum. A Western blot assay showed that the purified P9-1 protein reacted specifically with the antiserum (Fig. 1A).

The RNA binding activity of purified P9-1 was examined with an agarose gel electrophoretic mobility shift assay (EMSA). DIG-labeled RNA transcripts representing full-length plus strand ssRNA of the RBSDV S9 genome were incubated with the P9-1 protein. As shown in Fig. 1B, the DIG-labeled S9 ssRNA was retarded by the addition of P9-1 in the EMSA. Addition of protease K caused the DIG-labeled ssRNA to shift back to the fast-mobility band (Fig. 1B). The DIG-labeled S9 ssRNA was not retarded by the

addition of bovine serum albumin (BSA) (Fig. 1B), suggesting that P9-1 was able to bind to ssRNA.

To compare the affinities of P9-1 for ssRNA and other nucleic acids, we performed competition assays. DIG-labeled S9 RNA was combined with unlabeled competitor nucleic acids (ssRNA, dsRNA, ssDNA, or dsDNA) before the addition of P9-1. The RNA-protein complexes were then analyzed by agarose gel mobility shift assay (Fig. 2A). Both ssRNA and ssDNA competed efficiently with the labeled ssRNA probe for P9-1 binding, whereas dsRNA and dsDNA competed inefficiently (Fig. 2A). For example, when 30-fold more ssRNA or ssDNA competitor was added, only 22% ± 3% or 21% ± 5% of the labeled S9 RNA was bound,

FIG 1 Purification and RNA binding activity of RBSDV P9-1. (A) Expression and purification of the P9-1 protein. Purified protein was separated by 10% SDS-PAGE and visualized by either Coomassie blue staining (left, 10 μg P9-1 protein) or by Western blotting (right, 100 ng P9-1). The blot was probed with 6×His rabbit antiserum (1:1,000 dilution). (B) RNA binding activity of RBSDV P9-1. The DIG-labeled full-length RNA transcript of RBSDV S9 (20 ng, 3.1 nmol) was incubated in the absence (leftmost lane) or presence (second lane from the left) of purified P9-1 protein (2 μg, 4.6 μmol) or BSA (2 μg, 2.88 μmol) (rightmost lane). For the third lane from the left, 50 ng of proteinase K was added after the incubation of P9-1 with the probe and the sample was incubated for 10 min at room temperature. Probe-protein complexes were separated by electrophoresis on a 1% agarose gel, and the blot was detected by an AP-linked anti-DIG monoclonal antibody (1:10,000 dilution).

FIG 2 Competition and specificity assays of P9-1. (A) Increasing amounts of unlabeled competitor ssRNA, dsRNA, ssDNA, or dsDNA were mixed with 3.1 nmol DIG-labeled S9-1900nt ssRNA; 4.6 μ mol of purified P9-1 was added to each sample, and the sample was incubated for 10 min at room temperature. Samples were subjected to electrophoresis and visualized as described in the legend to Fig. 1B. (B) The assay was performed as described for panel A, except that the unlabeled competitor ssRNAs were from RBSDV S9 and RSV S4. For one set of samples, P9-1 protein was added to the DIG-labeled RNA before the addition of competitor S9 ssRNA. Each data point represents the mean of three experiments, and error bars represent the standard deviation of the mean.

respectively. In contrast, when 30-fold more dsRNA or dsDNA competitor was added, $94\% \pm 4\%$ or $91\% \pm 5\%$ of the labeled RNA was bound, respectively. Hence, P9-1 bound efficiently to single-stranded, but not double-stranded, nucleic acids.

P9-1 protein does not bind preferentially to RBSDV rather than non-RBSDV ssRNA. The sequences at the 5' and 3' termini of all 10 RBSDV RNAs are quite conserved (20, 21) and therefore may be involved in distinguishing RBSDV from non-RBSDV RNA for binding by P9-1. To test whether P9-1 exhibits binding activity specific for RBSDV RNAs representing the full length of the RBSDV S9 plus strand (1,900 nt) and non-RBSDV sequences derived from RSV S4 (2,157 nt). DIG-labeled S9 RNA was incubated with unlabeled competitor RNA (RBSDV S9 or RSV S4) before the addition of P9-1. The RNA-protein complexes were then analyzed in a 1% agarose gel mobility shift assay.

In this assay, both RBSDV S9 and RSV S4 competitor RNAs competed to similar levels with DIG-labeled S9 RNA for binding to P9-1 (Fig. 2B). For example, when 20-fold more RBSDV S9 or RSV S4 competitor RNA was added, only $30\% \pm 5\%$ or $34\% \pm 4\%$ of the DIG-labeled S9 RNA was bound by P9-1. When 30-fold more RBSDV S9 or RSV S4 competitor RNA was added, only $15\% \pm 5\%$ or $19\% \pm 4\%$ of the DIG-labeled S9 RNA was bound. These results suggest that the P9-1 protein does not have a preference for binding to RBSDV sequences.

To investigate the stability of preformed RNA-protein complexes, DIG-labeled S9 RNA was incubated with P9-1 before the addition of competitor RNA. As shown in Fig. 2B, competitor RBSDV ssRNAs did not displace DIG-labeled S9 ssRNA that was already bound to P9-1, even when a 30-fold excess of competitor RNAs was added. These data indicate that once P9-1 bound to RNA, it formed a stable complex that could not be easily disrupted by the subsequent addition of other RNA.

Multiple units of P9-1 protein bind to ssRNA. To investigate the nature of the P9-1 protein that binds to ssRNA, the DIG-labeled full-length RNA transcript of RBSDV S9-1900nt (3.1 nmol) was incubated with increasing amounts of P9-1 protein (Fig. 3A). As the amount of P9-1 was increased from 0.037 to 0.37 μ mol, the S9 ssRNA band shifted drastically. When the amount of P9-1 was further increased from 0.37 to 2.94 μ mol, the mobility of the S9 ssRNA was progressively retarded and clearly distinguishable, different-size complexes were observed. With larger amounts of P9-1 (5.74 to 22.97 μ mol), a lowest-mobility complex was formed, accumulated, and not further retarded by the addition of more protein. The gradual shifting of the RNA band suggested that multiple units of P9-1 could bind to the 1,900-nt S9 ssRNA. To further examine this property of P9-1 and because smaller ssRNAs might better display intermediate-size complexes if P9-1 binds to the ssRNA as multiple units, a 150-nt RNA transcript (39 nmol) was incubated with increasing amounts of P9-1 (Fig. 3B). Indeed, intermediate-size complexes began to form upon the addition of 0.037 to 0.18 μ mol of P9-1 protein (Fig. 3B). When 0.18 to 0.37 μ mol of P9-1 protein was added, another intermediate, retarded band was formed; consequently, the smaller, intermediate-size complexes started to disappear. When the amount of P9-1 was further increased from 0.37 to 2.94 μ mol, different-size complexes were again distinguishable. Upon the addition of more P9-1 protein (5.74 to 22.97 μ mol), the 150-nt ssRNA was no longer retarded. These results suggested that multiple units of P9-1 protein could bind to RNA. A similar RNA retardation pattern has also been reported for viroplasm protein NSP2 of *Rotavirus* and σ NS of *Reovirus* (13, 17).

P9-1 forms a range of high-order oligomers. To investigate what type of high-order oligomers P9-1 can form, a discontinuous blue native polyacrylamide gradient gel was used. Discontinuous blue native protein gel electrophoresis, which maintains the native protein conformation and protein complex quaternary structure, allows the separation of proteins according to their size and shape (31). Discontinuous blue native PAGE is a powerful tool for the analysis of protein-protein interactions and protein oligomer formation. It has been widely used in protein complex analysis in numerous studies (32–36). To examine the oligomer formation of P9-1, we analyzed purified, 6 $\times$ His-tagged P9-1 on a 4 to 16% blue native polyacrylamide gradient gel. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was loaded as a size marker. According to the sizes of the GAPDH oligomers, P9-1 was capable of forming

FIG 3 Formation of gradually shifted higher-order complexes by P9-1 and RNA. (A) Increasing amounts of P9-1 were incubated with 3.1 nmol of DIG-labeled RBSDV S9-1900nt ssRNA. The complexes were resolved by electrophoresis on a 1% agarose gel, and blots were visualized with anti-DIG antibody. (B) Gel mobility shift assays were performed as described for panel A, except that the DIG-labeled ssRNA was a 150-nt ssRNA from the 5' terminus of RBSDV S9 (39 nmol). The positions of unbound (free) probe and gradually shifted higher-order complexes are indicated.

a range of oligomers, from low order to high order (Fig. 4A). The major bands corresponded to 80, 160, and 320 kDa, suggesting that P9-1 forms dimers, tetramers, and octamers. Size exclusion chromatography analysis further confirmed that P9-1 can form a similar pattern of high-order oligomers (Fig. 4B).

Both discontinuous blue native PAGE and chromatography analysis demonstrated that viroplasm protein P9-1 forms dimers, tetramers, and octamers. However, chromatography experiments performed by Akita et al. showed that the vast majority of the protein assembled into an octamer (27). Our pET28c-P9-1 construct expressed His-tagged P9-1 with 34 extra amino acids at its N terminus. His-tagged P9-1 expressed by Akita et al. has an extra 21 amino acids at its N terminus. To determine whether the difference in the dominant form of P9-1 protein is due to the number of extra amino acids at its N terminus, we also constructed pXT99-P9-1, which expressed His-tagged P9-1 with an extra 21 aa at the N

terminus as described by Akita et al. (27). However, in both blue native PAGE and chromatography experiments, pXT99-P9-1 formed a pattern of dimers, tetramers, and octamers similar to that of pET28c-P9-1 (Fig. 4C and D). An alternative possibility is that the extra amino acids at the N terminus of P9-1 affect the dominant form of P9-1 oligomers; thus, we purified tag-free P9-1 and found that tag-free P9-1 formed a pattern of oligomers very similar to that of pXT99-P9-1 and pET28c-P9-1 (Fig. 4D). Although a slightly stronger octamer band was observed for tag-free P9-1, the dominant forms of oligomers were still dimers and tetramers (Fig. 4D). Because P9-1 with or without the tag formed similar oligomers, the His-tagged protein expressed by pET28c-P9-1 was used in all of the subsequent experiments.

P9-1 prefers to bind to ssRNA as an octamer. Given observations that P9-1 forms dimers, tetramers, and octamers, and together with the detection of the gradually shifted RNA band over

FIG 4 P9-1 protein preferentially binds to ssRNA as an octamer. (A) Oligomer formation analysis of P9-1 by discontinuous blue native PAGE. Purified P9-1 protein (80 μ g) was applied to a 4 to 16% polyacrylamide gradient gel; 10 μ g of GAPDH was loaded as a molecular size marker (lane M). Dimer, tetramer, and octamer positions are indicated. (B and C) Size exclusion chromatography analysis of oligomer formation by pET28c-P9-1 (B) and pXT99-P9-1 (C). Each purified protein (1 ml of 10 mg/ml) was loaded onto the Superdex 200 pg column. (D) Discontinuous blue native PAGE analysis of oligomer formation by pET28c-P9-1, pXT99-P9-1, and tag-free P9-1. pET28c-P9-1 and pXT99-P9-1 expressed His-tagged P9-1 containing 34 and 21 aa, respectively, at its N terminus. The dimer, tetramer, and octamer positions are indicated. (E) Dimer step binding model to describe the formation of the P9-1-RNA complex. More than one unit of viroplasm protein can bind each molecule of RNA. Each binding unit of P9-1 is likely to be a dimer of this protein. The step binding of the dimer results in the maximum number of protein units (octamer) binding to RNA molecules to form the saturated complex. (F) Preference analysis of different oligomers of P9-1 that bind to ssRNA by discontinuous blue native PAGE. Increasing amounts of 150-nt ssRNA were incubated with fixed amount of P9-1 (11.48 μ mol), and the complexes were resolved with a 4 to 16% polyacrylamide gradient gel. (G) Octamer binding model for the formation of the P9-1-RNA complex. In this model, the octamer preferentially binds to ssRNA. With increasing amounts of P9-1, more and more P9-1 octamers bind to the unoccupied spaces of the ssRNA until they become saturated.

the range of P9-1 amounts added, there are two models that can be proposed to interpret these results. The first is the dimer step binding model proposed earlier by Gillian et al. and Taraporewala et al. for reovirus σ NS and rotavirus NSP2 (17, 28). In this model (Fig. 4E), more than one unit of viroplasm protein can bind to each molecule of the RNA. Each binding unit of viroplasm protein is hypothesized to be a small oligomer of that protein (a dimer for P9-1). In addition, the saturated complex represents RNA molecules that could each have been bound by the maximum number of protein units (an octamer for P9-1).

To determine whether the dimer step binding model (Fig. 4E) represents P9-1 binding to ssRNA, we incubated a fixed amount of P9-1 protein (11.48 μ mol) with increasing amounts of 150-nt ssRNA and analyzed the reaction products in a 4 to 16% blue native polyacrylamide gradient gel. With increasing amounts of ssRNA, surprisingly, the dimer and tetramer bands did not shift in an obvious manner, while the octamer band shifted dramatically (Fig. 4F). For example, the addition of 3 μ mol ssRNA reduced the intensity of the octamer band somewhat. When the ssRNA amount was increased to 9 μ mol, the octamer was completely gone. At the same concentration of ssRNA, the intensity of the tetramer and dimer bands did not change. We observed a slight reduction in the dimer and tetramer bands only when the ssRNA amount was increased to 18 μ mol (Fig. 4F). These data suggest that the octamer is the form that either is required for RNA binding or binds to RNA with very high affinity.

These findings strongly support an octamer binding model (Fig. 4G), in that the octamer of P9-1, rather than the dimer, is the form that prefers to bind to ssRNA. With increasing amounts of P9-1, some of the P9-1 octamer binds to ssRNA; then, more octamer binds to the unoccupied space of the ssRNA until the binding space of the ssRNA is completely saturated. Eventually, this forms the saturated, lowest-mobility P9-1–ssRNA complex.

The P9-1 C-terminal arm deletion mutant protein no longer forms an octamer and binds to ssRNA with significantly lower affinity. To further examine the octamer binding model, we took advantage of the P9-1 crystal structure (27), in which the C-terminal arm formed by each dimer interacts with the adjoining dimer, with each holding the other at the “shoulder” to form the octamer (Fig. 5A and B). On the basis of our observations that P9-1 preferentially binds to ssRNA as an octamer, we hypothesized that deletion of the C-terminal arm could cause the oligomeric state of P9-1 to shift from a higher to a lower order; subsequently, the mutant protein would bind ssRNA with lower affinity and fewer copies of the P9-1 mutant protein would bind per ssRNA. To test this hypothesis, we generated two C-terminal arm deletion mutant proteins, P9-1 Δ C9 and P9-1 Δ C23 (Fig. 5C); their oligomer formations were determined by discontinuous blue native PAGE. As shown in Fig. 5D, both C-terminal arm deletion mutant proteins produced a high-intensity band corresponding to the dimer and a lower intensity band corresponding to the tetramer; additionally, neither formed an octamer.

To determine the RNA binding capability of P9-1 C-terminal arm deletion mutant proteins, a fixed amount of S9 RNA transcripts (3.1 nmol) was incubated with increasing amounts of P9-1 deletion mutant proteins and analyzed in an agarose gel mobility shift assay. Wild-type P9-1 protein was loaded onto the same gel for comparison. As shown in Fig. 5E and F, when the amount of protein was increased, the mobility of the ssRNA–wild-type P9-1 complex was progressively retarded, whereas that of the ssRNA–

P9-1 Δ C9 or ssRNA–P9-1 Δ C23 complex was retarded less (Fig. 5E and F).

In the preliminary salt gradient experiments, we found that the salt started to outcompete the DIG-labeled probe for the binding of wild-type P9-1 when the NaCl concentration was increased to 300 mM (data not shown). To further examine whether the RNA binding affinity of the P9-1 Δ C9 and P9-1 Δ C23 mutant proteins is much different from that of the wild-type protein, a gel mobility shift assay was performed in the presence of 200 mM NaCl. As shown in Fig. 6A, the ssRNA–P9-1 Δ C9 or ssRNA–P9-1 Δ C23 complex band was retarded significantly less than the wild-type complex band in the presence of 200 mM NaCl. These data suggest that the dimer or tetramer form of P9-1 does bind to ssRNA, whereas the octamer has much stronger binding affinity for ssRNA than the dimer or the tetramer does.

To further investigate the RNA binding activity of octamer formation-defective mutant proteins, a fixed amount of P9-1 Δ C9 or P9-1 Δ C23 protein (11.48 μ mol) was incubated with increasing amounts of 150-nt ssRNA and analyzed by 4 to 16% blue native PAGE. With increasing amounts of ssRNA, the dimer and tetramer bands of P9-1 Δ C9 or P9-1 Δ C23 protein did not shift (Fig. 6B and C). Band intensity was not reduced even in the presence of up to 18 μ mol ssRNA. These results suggested that the binding affinity of the dimer or tetramer form of P9-1 to ssRNA might be low.

In summary, deletion of the C-terminal arm of P9-1 yielded mutant forms that no longer formed an octamer; consequently, the mutant proteins bound to ssRNA with significantly lower affinity and fewer copies were bound per ssRNA.

Amino acids 25 to 44 are the critical RNA binding sites and map to the central interior structure formed by the octamer of P9-1. By using the online RNA binding residue prediction software BindN (<http://bioinfo.ggc.org/>), we found that amino acid residues 25 to 44 and 319 to 327 were predicted to be major RNA binding sites on P9-1. The precise role of these predicted residues was examined by replacing amino acid residues 25 to 44 (Bind1m) and 319 to 327 (Bind2m) with alanines (Fig. 7A), and oligomer formation by the mutant proteins was determined by discontinuous blue native PAGE. As shown in Fig. 7B, the aa 25 to 44 substitution mutant protein (Bind1m) formed an oligomeric structure comparable to that formed by wild-type P9-1, except that the Bind1m mutant protein lacked octamer formation. The oligomeric state of the Bind2m mutant protein, however, was drastically affected. Although the Bind2m mutant protein could form dimers, tetramers, and octamers, the amount of each was significantly reduced; in addition to these changes, Bind2m formed more monomers than wild-type P9-1 did.

To check the RNA binding activity of the Bind1m and Bind2m mutant P9-1 proteins, a fixed amount of S9-1900nt ssRNA (3.1 nmol) was incubated with increasing amounts of P9-1 alanine mutant proteins and analyzed by agarose gel mobility shift assay. Wild-type P9-1 protein was loaded onto the same gel as a positive control. As the amount of P9-1 Bind1m was increased from 0 to 1.47 μ mol, the S9 ssRNA band did not shift (Fig. 7C). Only when the amount of P9-1 was increased to 2.94 μ mol did the S9-1900nt ssRNA band display some retardation, but the retarded band was significantly lower than the wild-type complex band (Fig. 7C). The S9-1900nt ssRNA band was not further retarded by the addition of larger amounts of P9-1 protein (data not shown). These data suggest that P9-1 Bind1m almost lost the ability to bind ssRNA. Compared with the binding behavior of P9-1 Bind1m,

FIG 5 P9-1 C-terminal arm deletion mutant proteins no longer form octamers and bind to ssRNA with lower affinity. (A) Visualization of the C-terminal arm in the crystal structure of the P9-1 dimer. The C-terminal 23 aa are in blue on the P9-1 dimer. (B) Visualization of the C-terminal arm in the crystal structure of the P9-1 octamer. Each dimer is depicted in one of four colors. The C-terminal arm is boxed. The C-terminal arm formed by each dimer interacts with the adjoining dimer, with each holding the other at the “shoulder” to form an octamer. (C) Construct map of wild-type RBSDV P9-1 and the two C-terminal deletion mutant proteins. P9-1, wild-type P9-1; P9-1^{ΔC9}, deletion of the last 9 aa of the C terminus (aa 339 to 347); P9-1^{ΔC23}, deletion of the last 23 aa of the C terminus (aa 325 to 347). The blue box depicts the C-terminal arm of P9-1 in the construct map. (D) Oligomer formation analysis of the C-terminal deletion mutant proteins by discontinuous blue native PAGE. Eighty micrograms of protein was loaded into each lane, and wild-type (Wt) P9-1 was loaded as a positive control. The monomer, dimer, tetramer, and octamer positions are indicated. (E and F) Gel retardation analysis of the RNA binding capacities of the P9-1^{ΔC9} and P9-1^{ΔC23} deletion mutant proteins. Increasing amounts of P9-1^{ΔC9} or P9-1^{ΔC23} were incubated with 3.1 nmol of S9-1900nt ssRNA, and the mixtures were applied to a 1% agarose gel. Wild-type P9-1 was loaded onto the same gel as a positive control. The blots were probed as described in the legend to Fig. 1B.

that of P9-1 Bind2m was very different. As the amount of P9-1 Bind2m was increased from 0 to 0.74 μmol, the S9-1900nt ssRNA band did not shift (Fig. 7D). When the amount of P9-1 Bind2m was increased from 1.47 to 2.94 μmol, the S9-1900nt ssRNA band

shifted drastically (Fig. 7D), suggesting that P9-1 Bind2m has lower binding affinity.

When we mapped aa 25 to 44, which are critical for RNA binding, onto the crystal structure of P9-1, we found that they corre-

FIG 6 RNA binding analyses of C-terminal arm deletion mutant P9-1 proteins by salt competition and blue native PAGE. (A) EMSA analysis of the RNA binding capacities of the P9-1^{ΔC9} and P9-1^{ΔC23} deletion mutant proteins by salt competition. Increasing amounts of P9-1^{ΔC9} or P9-1^{ΔC23} were incubated with 3.1 nmol of S9-1900nt ssRNA in the presence of 200 mM NaCl, and the mixtures were applied to a 1% agarose gel. (B and C) RNA binding analysis of dimer and tetramer species of the P9-1^{ΔC9} (B) and P9-1^{ΔC23} (C) deletion mutant proteins by discontinuous blue native PAGE. A fixed amount of P9-1^{ΔC9} or P9-1^{ΔC23} (11.48 μmol) was incubated with increasing amounts of S9-150nt ssRNA, and the complexes were resolved by 4 to 16% gradient PAGE.

spond to the disordered region of the crystal structure. So the flanking amino acids in this region (aa 20 and 42 to 44, KTKK) were mapped onto the P9-1 crystal structure instead. Notably, these amino acid residues were mapped to a central interior structure that was formed only by the octamer of P9-1 (Fig. 7E and F).

Electropositive amino acids among residues 25 to 44 are important for RNA binding. Among residues 25 to 44 are four positively charged amino acids, R27, R39, K43, and K44. To investigate whether these amino acids are responsible for RNA binding, we used an alanine-scanning strategy to mutate each positively charged amino acid individually or jointly (Fig. 8A). A fixed amount of S9 RNA transcript (3.1 nmol) was incubated with either 0.74 or 2.94 μmol of each alanine-scanning mutant protein. Compared with the wild-type protein, all of the single alanine mutant proteins, R26A (Bind3m), R39A (Bind4m), K43A (Bind5m), and K44A (Bind6m), had the same RNA binding affinity as wild-type P9-1 (Fig. 8A and B). While the double alanine mutant protein, K43A-K44A (Bind7m), had a less-retarded RNA band, the triple alanine mutant protein, R39A-K43A-K44A (Bind8m), had a continuously less-retarded band. The quadruple alanine mutant protein, R26A-R39A-K43-K44A (Bind9m), which had a band that was even less retarded, lost most of its ability to

bind to RNA (Fig. 8A and B). These data demonstrated that the positively charged amino acids among residues 25 to 44 are important for P9-1 binding to ssRNA.

The oligomer formation of alanine-scanning mutant proteins was also determined by discontinuous blue native PAGE. As shown in Fig. 8C, all single alanine mutant proteins, R26A (Bind3m), R39A (Bind4m), K43A (Bind5m), and K44A (Bind6m), formed an octamer that was comparable to that of wild-type P9-1. However, the double alanine mutant protein (Bind7m) formed a weak octamer band. Most strikingly, the triple alanine (Bind8m) and quadruple alanine (Bind9m) mutant proteins were unable to form octamers. These data, together with the above-described findings that the Bind1m mutant protein lacks octamer formation, suggested that those positively charged amino acids may also be required for the formation of the octamer, a structure that has a higher affinity for ssRNA.

To further investigate whether those positively charged amino acids represent RNA binding sites, we introduced the quadruple alanine mutations into P9-1^{ΔC23}, which was unable to form an octamer but still bound to RNA to a certain degree. A fixed amount of S9 ssRNA (3.1 nmol) was incubated with increasing amounts of P9-1^{ΔC23} quadruple alanine mutant protein (P9-

FIG 7 The major RNA binding sites map to the central interior structure formed by the octamer of P9-1. (A) Construct map of P9-1 RNA binding mutant proteins. Amino acids 25 to 44 and 319 to 327 were predicted to be possible RNA binding sites by the BindN software. In the Bind1m and Bind2m mutant proteins, aa 25 to 44 and 319 to 327, respectively, were all mutated to alanines. The blue box depicts aa 25 to 44 and the green box depicts aa 319 to 327 in the construct map. (B) Oligomer formation analysis of RNA binding mutant proteins by discontinuous blue native PAGE. Eighty micrograms of protein was loaded into each lane, and wild-type (Wt) P9-1 was loaded as a control. The dimer, tetramer, and octamer positions are indicated. (C and D) RNA binding activities of P9-1 Bind1m and Bind2m mutant proteins determined by gel mobility shift assay. Increasing amounts of Bind1m or Bind2m mutant protein were incubated with 3.1 nmol S9-1900nt ssRNA, and mixtures were separated on a 1% agarose gel with wild-type P9-1 loaded as a positive control. The blots were probed as described in the legend to Fig. 1B. (E) Mapping of aa 25 to 44 and 319 to 327 in the P9-1 dimer crystal structure. (F) Visualization of aa 25 to 44 in the P9-1 octamer crystal structure. Amino acid residues 21 to 41 were disordered in the crystal structure of P9-1, so the flanking amino acids of this region (aa 20 and 42 to 44; KTKK) were mapped in the crystal structure and are shown in cyan. Amino acid residues 319 to 327 are shown in green.

FIG 8 Electropositive amino acids among aa 25 to 44 are important for RNA binding. (A) Alanine-scanning mutagenesis of the positively charged amino acids among aa 25 to 44. Bind3m, Bind4m, Bind5m, and Bind6m represent, respectively, the R26A, R39A, K43A, and K44A single alanine substitution mutant proteins. Bind7m, Bind8m, and Bind9m, respectively, are the double (K43A-K44A), triple (R39A-K43A-K44A), and quadruple (R26A-R39A-K43-K44A) alanine substitution mutant proteins. (B) RNA binding activity analysis of P9-1 alanine-scanning mutant proteins by gel mobility shift assay. S9-1900nt ssRNA (3.1 nmol) was incubated with 0.74 or 2.94 μmol of each alanine-scanning mutant protein, and mixtures were applied to a 1% agarose gel. The results of the gel retardation analysis are to the right of each mutagenesis in panel A. The symbols + + + + to + indicate the RNA binding levels, from strong to weak, of the mutant proteins. Wt, wild type. (C and E) Oligomer formation analysis of alanine-scanning mutant proteins (C) and P9-1^{ΔC23} quadruple alanine substitution mutant protein

1^{ΔC23}-Bind9m) and analyzed in an agarose gel mobility shift assay. As shown in Fig. 8D and E, the P9-1^{ΔC23}-Bind9m mutant protein was still able to form dimers and tetramers; however, it completely lost its RNA binding ability, suggesting that the electropositive amino acids among residues 25 to 44 are important for RNA binding.

DISCUSSION

In this study, we demonstrated that the P9-1 protein preferentially bound to single-stranded versus double-stranded nucleic acids; however, the protein displayed no preference for RBSDV versus non-RBSDV ssRNA. We further revealed that the P9-1 protein forms dimers, tetramers, and octamers. Strikingly, RBSDV P9-1 preferentially bound to ssRNA in the octamer form rather than in the dimer form. The electropositive amino acids among residues 25 to 44 were demonstrated to be the major RNA binding sites and mapped to a central interior structure formed only by the P9-1 octamer.

P9-1 binds to the full-length RBSDV S9 ssRNA in a non-sequence-specific manner, although S9 RNA contains both the 5'- and 3'-terminal conserved end sequences. It is plausible that other cofactors increase the RNA binding specificity of RBSDV P9-1 *in vivo*; the best candidate is the P6 protein, which has been shown to interact and colocalize with P9-1 (25). Another possibility is that viral mRNAs are retained and concentrated in the viroplasm factories, whereas cellular mRNAs are excluded, obviating the need for viroplasm-associated P9-1 to preferentially bind viral sequences.

By using both discontinuous blue native PAGE and chromatography analysis, we found that viroplasm protein P9-1 is capable of forming dimers, tetramers, and octamers. Whenever P9-1 was expressed with the N-terminal 6×His tag or without the tag, the dominant forms of the protein were dimers and tetramers. However, in chromatography experiments by Akita et al. (27) with expressed P9-1 His tagged at its N terminus, the vast majority of the protein assembled as octamers. The reason for the differences in the dominant form of P9-1 protein between our results and those of Akita et al. (27) remains unknown and needs to be determined. The existence of the dimers, tetramers, and octamers may indicate an equilibrium balance of these high-order oligomers, suggesting that in the P9-1 oligomerization process, two dimers interact with each other to form a tetramer that further polymerizes to form an octamer. Perhaps the difference between our results and those of Akita et al. (27) are due to a difference in the equilibrium of oligomers caused by an unknown factor(s) such as the use of different RBSDV isolates, different concentrations of P9-1 preparation, etc.

The P9-1 protein of *Mal de Río Cuarto virus* (MRCV), a counterpart of the P9-1 protein of RBSDV, has also been shown to self-associate, giving rise to different high-molecular-weight oligomers when expressed in bacteria (37). The RBSDV P9-1 protein shares 63% amino acid identity with the P9-1 protein of MRCV, which is in the same genus (*Fijivirus*) and family (*Reoviridae*) as RBSDV. The amino acid similarity between the two viruses sug-

gests that their P9-1 proteins may have similar biochemical properties, such as nonspecific RNA binding activity (37).

In the discontinuous blue native gel assay, the band corresponding to the octamer was dramatically reduced, while a slight reduction in the intensity of the dimer and tetramer bands was observed only with the addition of large amounts of RNA. The reduced intensity of the dimer and tetramer bands could be due to the increased binding of the octamer to the RNA, as this could result in a shift in equilibrium from low-order oligomers to the octamer form, while, on the basis of the results of the EMSA of the C-terminal deletion mutant proteins, dimer and tetramer species are likely to be responsible for RNA binding and the associated mobility shift. The C-terminal deletion mutant proteins did not display a reduction in dimer or tetramer bands with a larger amount of RNA in the discontinuous blue native gel, probably because the RNA-to-protein mole ratio is still low in the blue native gel assay. With the high RNA-to-protein mole ratio used for the C-terminal deletion mutant protein EMSA, binding did occur. These findings are consistent with the notion that dimer and tetramer species may bind to RNA but that the affinity of the octamer is much stronger than that of the dimer or tetramer.

According to the crystal structure of P9-1, proteins with the C-terminal arm deleted should not form higher-order oligomers; however, these deletion mutant proteins do form tetramers. Although we do not know how the deletion mutant proteins form tetramers, perhaps they polymerize by using other interacting surfaces. The residual RNA binding capacity of the deletion mutant proteins may have arisen from assembled octamers that were derived from the tetramers and were not detectable on the gel. However, the residual RNA binding capacity of the C-terminal deletion mutant proteins may also be derived from the binding activity of dimers or tetramers.

Amino acid residues 25 to 44, which were critical for RNA binding, were mapped to a central interior structure of the octamer of P9-1 (Fig. 7E and F), consistent with the finding that the C-terminal arm deletion mutant protein was unable to form an octamer that bound to the RNA with lower affinity. Although dimers and tetramers have the same amino acid sequence, they do not bind well to RNA. This amino acid region may undergo a conformational change when P9-1 forms an octamer because only the octamer, not the dimer or the tetramer, forms the central interior structure that is capable of binding to RNA. It is interesting that residues 25 to 44 were disordered in the crystal structure of P9-1; perhaps this disordered region becomes ordered when it binds to RNA.

The RNA binding capability of the mutant protein with alanine substitutions at residues 25 to 44 (Bind1m) suggests that the amino terminus of P9-1 is important for RNA binding. The amino-terminal portions of the *Orthoreovirus* protein σNS (16) and BTV protein NS2 (38) have also been shown to be essential for ssRNA binding. However, for rotavirus NSP2, in addition to the highly conserved residues in the NSP2 enzymatic cleft, the C-terminal helix and other basic residues outside the enzymatic cleft account for sequence-independent RNA binding of NSP2 (39).

(P9-1^{ΔC23}-Bind9m) (E) by discontinuous blue native PAGE. Fifty micrograms of protein was loaded into each lane. The dimer, tetramer, and octamer positions are indicated. (D) Gel retardation analysis of the RNA binding capacities of the P9-1^{ΔC23} quadruple alanine substitution mutant protein (P9-1^{ΔC23}-Bind9m). Increasing amounts of P9-1^{ΔC23}-Bind9m mutant protein were incubated with 3.1 nmol S9-1900nt ssRNA, and mixtures were applied to a 1% agarose gel, with P9-1^{ΔC23} as a positive control. The blots were probed as described in the legend to Fig. 1B.

The aa 25 to 44 alanine mutant P9-1 protein has some residual RNA binding activity. An alternative RNA binding region of P9-1 may also be involved in ssRNA binding. Such a binding site could be the region containing aa 319 to 327, as the replacement of these amino acids with alanines significantly reduced its RNA binding affinity. However, oligomer formation by this mutant protein was substantially changed; thus, we cannot rule out the possibility that its lower binding affinity was also caused by the conformational change due to the mutation.

Akita et al. suggested earlier that the internal pore of the P9-1 octamer might be important for RNA binding (27). Our alanine-scanning mutant proteins further demonstrate that the positively charged amino acids among residues 26 to 44 are critical RNA binding sites, and they map to the central interior structure of octameric P9-1. Previous cryoelectron microscopy studies with the rotavirus NSP2 octamer have shown that ssRNA binds to the highly electropositive grooves that run diagonally across the structure, while ssRNA does not bind to the central pore of the rotavirus NSP2 octamer (40). Our finding strongly implies that the electropositive amino acids along the central interior structure of the octamer, instead of the internal pore itself, may be the real RNA binding sites of RBSDV P9-1. Surprisingly, both the mutant protein with alanine substitutions at residues 25 to 44 (Bind1m) and the quadruple-mutant protein with alanine substitutions at four positively charged amino acids among residues 26 to 44 (Bind9m) of RBSDV P9-1 no longer form octamers. The importance of the corresponding positively charged amino acids in octamer/oligomer formation by rotavirus NSP2 or other viroplasm proteins has not been reported previously. It is not known how these amino acids contribute to the formation of octamers. The electropositive amino acids among residues 26 to 44 of RBSDV P9-1 may map to sites needed to form an octamer, a structure that may be essential for RNA binding. Our finding leads to an important new concept that the positively charged amino acids among residues 26 to 44 are not only required for RNA binding but also essential for octamer formation. The broad implications of this finding await investigations of the structure and function of the viroplasm protein from other viruses in the family of *Reoviridae*.

In summary, the results presented here reveal that the octamer of P9-1, rather than the dimer, preferentially binds to ssRNA. Deletion of the C-terminal arm of P9-1 produces a mutant protein that is unable to form an octamer; consequently, the deletion mutant protein bound to ssRNA with significantly lower affinity and in fewer copies. The positively charged amino acids among residues 25 to 44 are the critical RNA binding sites, which mapped to a central interior structure in the P9-1 octamer. In light of these results, we propose a unifying model of the structure and function of RBSDV viroplasm protein in which the octamer of P9-1 is an important form that gradually binds to ssRNA to form a saturated complex (Fig. 4G).

ACKNOWLEDGMENTS

This work was supported by the Program for New Century Excellent Talents in University (NCET-12-0888), the National Program on Key Basic Research Project of China (973 Program, 2014CB138400), the National Natural Science Foundation of China (31222045 and 31171813), the Special Fund for Agro-Scientific Research in the Public Interest (201303021 and 201003031), and the Natural Science Foundation of Jiangsu Province (SBK201222198).

REFERENCES

1. Isogai M, Uyeda I, Lee BC. 1998. Detection and assignment of proteins encoded by rice black streaked dwarf fijivirus S7, S8, S9 and S10. *J. Gen. Virol.* 79(Pt 6):1487–1494.
2. McNulty MS, Curran WL, McFerran JB. 1976. The morphogenesis of a cytopathic bovine rotavirus in Madin-Darby bovine kidney cells. *J. Gen. Virol.* 33:503–508.
3. Wei T, Shimizu T, Hagiwara K, Kikuchi A, Moriyasu Y, Suzuki N, Chen H, Omura T. 2006. Pns12 protein of rice dwarf virus is essential for formation of viroplasms and nucleation of viral-assembly complexes. *J. Gen. Virol.* 87:429–438.
4. Sharpe AH, Chen LB, Fields BN. 1982. The interaction of mammalian reoviruses with the cytoskeleton of monkey kidney CV-1 cells. *Virology* 120:399–411.
5. Eaton BT, Hyatt AD, White JR. 1987. Association of bluetongue virus with the cytoskeleton. *Virology* 157:107–116.
6. Kar AK, Bhattacharya B, Roy P. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Mol. Biol.* 8:4. doi:10.1186/1471-2199-8-4.
7. Theron J, Huismans H, Nel LH. 1996. Site-specific mutations in the NS2 protein of epizootic haemorrhagic disease virus markedly affect the formation of cytoplasmic inclusion bodies. *Arch. Virol.* 141:1143–1151.
8. Thomas CP, Booth TF, Roy P. 1990. Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. *J. Gen. Virol.* 71(Pt 9):2073–2083.
9. Fabbretti E, Afrikanova I, Vascotto F, Burrone OR. 1999. Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo. *J. Gen. Virol.* 80(Pt 2):333–339.
10. Mohan KV, Muller J, Som I, Atreya CD. 2003. The N- and C-terminal regions of rotavirus NSP5 are the critical determinants for the formation of viroplasm-like structures independent of NSP2. *J. Virol.* 77:12184–12192.
11. Prasad BV, Rothnagel R, Zeng CQ, Jakana J, Lawton JA, Chiu W, Estes MK. 1996. Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. *Nature* 382:471–473.
12. Kattoura MD, Chen X, Patton JT. 1994. The rotavirus RNA-binding protein NS35 (NSP2) forms 10S multimers and interacts with the viral RNA polymerase. *Virology* 202:803–813.
13. Taraporewala Z, Chen D, Patton JT. 1999. Multimers formed by the rotavirus nonstructural protein NSP2 bind to RNA and have nucleoside triphosphatase activity. *J. Virol.* 73:9934–9943.
14. Broering TJ, Parker JS, Joyce PL, Kim J, Nibert ML. 2002. Mammalian reovirus nonstructural protein microNS forms large inclusions and colocalizes with reovirus microtubule-associated protein micro2 in transfected cells. *J. Virol.* 76:8285–8297.
15. Miller CL, Arnold MM, Broering TJ, Hastings CE, Nibert ML. 2010. Localization of mammalian orthoreovirus proteins to cytoplasmic factory-like structures via nonoverlapping regions of μ NS. *J. Virol.* 84:867–882.
16. Gillian AL, Nibert ML. 1998. Amino terminus of reovirus nonstructural protein sigma NS is important for ssRNA binding and nucleoprotein complex formation. *Virology* 240:1–11.
17. Gillian AL, Schmechel SC, Livny J, Schiff LA, Nibert ML. 2000. Reovirus protein σ NS binds in multiple copies to single-stranded RNA and shares properties with single-stranded DNA binding proteins. *J. Virol.* 74:5939–5948.
18. Chen SX, Zhang QY. 2005. Advance in researches on rice black-streaked dwarf disease and maize rough dwarf disease in China. *Acta Phytotaphytica Sin.* 32:97–103.
19. Shikata E, Kitagawa Y. 1977. Rice black-streaked dwarf virus: its properties, morphology and intracellular localization. *Virology* 77:826–842.
20. Zhang H, Chen J, Lei J, Adams M. 2001. Sequence analysis shows that a dwarfing disease on rice, wheat and maize in China is caused by rice black-streaked dwarf virus. *Eur. J. Plant Pathol.* 107:563–567.
21. Zhang HM, Chen JP, Adams MJ. 2001. Molecular characterisation of segments 1 to 6 of Rice black-streaked dwarf virus from China provides the complete genome. *Arch. Virol.* 146:2331–2339.
22. Supyani S, Hillman BI, Suzuki N. 2007. Baculovirus expression of the 11 mycoreovirus-1 genome segments and identification of the guanylyltransferase-encoding segment. *J. Gen. Virol.* 88:342–350.
23. Liu H, Wei C, Zhong Y, Li Y. 2007. Rice black-streaked dwarf virus outer

- capsid protein P10 has self-interactions and forms oligomeric complexes in solution. *Virus Res.* 127:34–42.
24. Zhang L, Wang Z, Wang X, Li D, Han C, Zhai Y, Yu J. 2005. Two virus-encoded RNA silencing suppressors, P14 of beet necrotic yellow vein virus and S6 of rice black streak dwarf virus. *Chin. Sci. Bull.* 50:305–310.
  25. Wang Q, Tao T, Zhang Y, Wu W, Li D, Yu J, Han C. 2011. Rice black-streaked dwarf virus P6 self-interacts to form punctate, viroplasm-like structures in the cytoplasm and recruits viroplasm-associated protein P9-1. *Viol. J.* 8:24.
  26. Zhang C, Liu Y, Liu L, Lou Z, Zhang H, Miao H, Hu X, Pang Y, Qiu B. 2008. Rice black streaked dwarf virus P9-1, an alpha-helical protein, self-interacts and forms viroplasms in vivo. *J. Gen. Virol.* 89:1770–1776.
  27. Akita F, Higashiura A, Shimizu T, Pu Y, Suzuki M, Uehara-Ichiki T, Sasaya T, Kanamaru S, Arisaka F, Tsukihara T, Nakagawa A, Omura T. 2012. Crystallographic analysis reveals octamerization of viroplasm matrix protein P9-1 of rice black streaked dwarf virus. *J. Virol.* 86:746–756.
  28. Shimizu T, Nakazono-Nagaoka E, Akita F, Uehara-Ichiki T, Omura T, Sasaya T. 2011. Immunity to rice black streaked dwarf virus, a plant reovirus, can be achieved in rice plants by RNA silencing against the gene for the viroplasm component protein. *Virus Res.* 160:400–403.
  29. Higuchi R, Krummel B, Saiki RK. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* 16:7351–7367.
  30. Tropea JE, Cherry S, Waugh DS. 2009. Expression and purification of soluble His(6)-tagged TEV protease. *Methods Mol. Biol.* 498:297–307.
  31. Niepmann M, Zheng J. 2006. Discontinuous native protein gel electrophoresis. *Electrophoresis* 27:3949–3951.
  32. Brindley MA, Plemper RK. 2010. Blue native PAGE and biomolecular complementation reveal a tetrameric or higher-order oligomer organization of the physiological measles virus attachment protein H. *J. Virol.* 84:12174–12184.
  33. Eubel H, Braun HP, Millar AH. 2005. Blue native PAGE in plants: a tool in analysis of protein-protein interactions. *Plant Methods* 1:11. doi:10.1186/1746-4811-1-11.
  34. Fristedt R, Vener AV. 2011. High light induced disassembly of photosystem II supercomplexes in Arabidopsis requires STN7-dependent phosphorylation of CP29. *PLoS One* 6:e24565. doi:10.1371/journal.pone.0024565.
  35. Kikuchi S, Hirohashi T, Nakai M. 2006. Characterization of the preprotein translocon at the outer envelope membrane of chloroplasts by blue native PAGE. *Plant Cell Physiol.* 47:363–371.
  36. Mine A, Takeda A, Taniguchi T, Taniguchi H, Kaido M, Mise K, Okuno T. 2010. Identification and characterization of the 480-kilodalton template-specific RNA-dependent RNA polymerase complex of red clover necrotic mosaic virus. *J. Virol.* 84:6070–6081.
  37. Maroniche GA, Mongelli VC, Peralta AV, Distefano AJ, Llauger G, Taboga OA, Hopp EH, del Vas M. 2010. Functional and biochemical properties of Mal de Rio Cuarto virus (Fijivirus, Reoviridae) P9-1 viroplasm protein show further similarities to animal reovirus counterparts. *Virus Res.* 152:96–103.
  38. Zhao Y, Thomas C, Bremer C, Roy P. 1994. Deletion and mutational analyses of bluetongue virus NS2 protein indicate that the amino but not the carboxy terminus of the protein is critical for RNA-protein interactions. *J. Virol.* 68:2179–2185.
  39. Hu L, Chow DC, Patton JT, Palzkill T, Estes MK, Prasad BV. 2012. Crystallographic analysis of rotavirus NSP2-RNA complex reveals specific recognition of 5' GG sequence for RTPase activity. *J. Virol.* 86:10547–10557.
  40. Jiang X, Jayaram H, Kumar M, Ludtke SJ, Estes MK, Prasad BV. 2006. Cryoelectron microscopy structures of rotavirus NSP2-NSP5 and NSP2-RNA complexes: implications for genome replication. *J. Virol.* 80:10829–10835.