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NOTE

Characterization of *Sulfolobus islandicus* rod-shaped virus 2 gp19, a single-strand specific endonuclease

Andrew F. Gardner · David Prangishvili · William E. Jack

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Abstract The hyperthermophilic *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2) encodes a 25-kDa protein (SIRV2gp19) annotated as a hypothetical protein with sequence homology to the RecB nuclease superfamily. Even though SIRV2gp19 homologs are conserved throughout the rudivirus family and presumably play a role in the viral life cycle, SIRV2gp19 has not been functionally characterized. To define the minimal requirements for activity, SIRV2gp19 was purified and tested under varying conditions. SIRV2gp19 is a single-strand specific endonuclease that requires Mg^{2+} for activity and is inactive on double-stranded DNA. A conserved aspartic acid in RecB nuclease superfamily Motif II (D89) is also essential for SIRV2gp19 activity and mutation to alanine (D89A) abolishes activity. Therefore, the SIRV2gp19 cleavage mechanism is similar to previously described RecB nucleases. Finally, SIRV2gp19 single-stranded DNA endonuclease activity could play a role in host chromosome degradation during SIRV2 lytic infection.

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A. F. Gardner (✉) · W. E. Jack
New England Biolabs, Inc., 240 County Road,
Ipswich, MA 01938, USA
e-mail: gardner@neb.com

D. Prangishvili
Biologie Moléculaire du Gène chez les Extrêmophiles,
Institut Pasteur, 25 rue du Docteur Roux,
75724 Paris Cedex 15, France

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Introduction

Sulfolobus islandicus rod-shaped virus 2 (SIRV2) infects the archeaon *Sulfolobus islandicus* at both extreme temperature (70–80°C) and acidity (pH 3) and has been the focus of structural, genomic, and transcriptional studies (Blum et al. 2001; Kessler et al. 2004; Peng et al. 2001; Prangishvili et al. 2006a). The SIRV2 genome has been completely sequenced; however, the majority of open reading frames (ORFs) are annotated as hypothetical proteins that share little sequence homology to other proteins in GenBank (Blum et al. 2001; Prangishvili et al. 2006b). Thus, a key step in understanding SIRV2 biology will be assigning biochemical and biological functions to hypothetical SIRV2 proteins.

SIRV2gp19 was previously annotated as a hypothetical nuclease based on amino acid similarity to the RecB nuclease superfamily (Aravind et al. 2000; Dillingham and Kowalczykowski 2008). SIRV2gp19 homologs are conserved in related thermophilic rudiviruses including *Acidianus* rod-shaped virus 1 (ARV1gp17), *Sulfolobus islandicus* rod-shaped virus 1 (SIRV2gp12) and *Stygiolobus* rod-shaped virus (SRV ORF199) suggesting a common function (Supplemental Figure S1). Interestingly, SIRV2gp19 shares sequence similarity with CRISPR-associated (cas4) nucleases from *Sulfolobus* and suggests a common ancestor (Jansen et al. 2002) (Supplemental Figure S1). SIRV2gp19 shares conserved RecB nuclease superfamily motifs including a Motif II (GxhD) aspartic acid that coordinates a divalent metal ion essential for nuclease activity, Motif III

(hhE/DhK) lysine that interacts with a phosphate oxygen on the DNA backbone, and a cysteine-rich motif (Jansen et al. 2002; Singleton et al. 2004; Wang et al. 2000; Yu et al. 1998). To define the function of SIRV2gp19, this study examines the minimal requirements for SIRV2gp19 biochemical activity and substrate specificity and proposes a role in the SIRV2 life cycle.

Materials and methods

Enzymes

All restriction endonucleases, modifying enzymes, DNA polymerases, nucleotides, DNA ladders, and expression vectors were from New England Biolabs.

Strains

E. coli strains for cloning (NEB 5-alpha) and expression (SHuffle® T7 Express) were from New England Biolabs.

MBP-SIRV2gp19 gene synthesis, cloning and purification

To improve protein expression, a synthetic SIRV2gp19 gene was codon optimized to reflect the codon usage of *E. coli* rather than the native *S. islandicus*. SIRV2gp19 gene was synthesized by PCR amplification of overlapping oligonucleotides (Czar et al. 2009).

To assemble a template for SIRV2gp19 gene synthesis, an equimolar amount (1 μM) of each overlapping oligonucleotide (Supplementary Table S1) was combined in 1× Standard Taq Buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and then serially diluted by twofold. PCR reactions (50 μL) were assembled as follows: 1× Phusion Master Mix (containing dNTPs, HF reaction buffer, and Phusion DNA polymerase), 0.5 μM Forward Primer (Supplementary Table S1, primer 1), 0.5 μM Reverse Primer (Supplementary Table S1, primer 10), and SIRV2gp19 gene synthesis oligonucleotide template mixtures. Reactions were cycled in a PCR instrument (98°C 2 min followed by 25 cycles of 98°C 10 s, 65°C 15 s, 72°C 30 s, followed by a final extension step at 72°C for 30 s). A band corresponding to the SIRV2gp19 gene (405 bp) was gel purified. The SIRV2gp19 codon-optimized PCR product was cloned into expression vector pMAL-c4X (New England Biolabs) digested with *Xmn*I to create a construct (pEPI) encoding an N-terminal Maltose Binding Protein (MBP)-SIRV2gp19 fusion protein. The sequence of plasmid pEPI was verified by DNA sequencing.

For MBP-SIRV2gp19 expression and purification, SHuffle® T7 Express *E. coli* was transformed with plasmid pEPI. A 1 L SHuffle® T7 Express *E. coli*/pEPI culture was grown at 37°C to mid-log phase (OD_{600} = 0.5), whereupon protein expression was induced by addition of 0.4 mM IPTG. Cells were then incubated at 37°C for 5 h, and cells were collected by centrifugation. The cell pellet was suspended in 0.2 L Buffer A (20 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 1 mM EDTA) and lysed by sonication. Cell debris was removed by centrifugation and the supernatant was applied to a 15 mL amylose column. The column was washed with 0.15 L Buffer A. MBP-SIRV2 Hjr was eluted with 30 mL Buffer A containing 10 mM maltose. MBP-SIRV2gp19 purification was monitored by 4–20% SDS-PAGE analysis. Fractions containing MBP-SIRV2gp19 were pooled, dialysed against storage buffer (0.1 M KCl, 10 mM Tris–HCl, pH 7.4 @ 25°C, 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol) and stored at −20°C.

SIRV2gp19 site-directed mutagenesis, expression, and purification

SIRV2gp19/D89A expression plasmid (plasmid: pEPJ) was constructed by PCR mutagenesis using Phusion Site-Directed Mutagenesis kit (New England Biolabs) using the following primers:

forward (D89A): pTCG CAT TGC TAT CGT TTG TGG CAA CG;
reverse: pCCA GAG ATC TTC ATG CCT TCG ATT TCG.

Plasmids were screened for the correct D89A mutation by DNA sequencing. MBP-SIRV2gp19/D89A was expressed and purified as described above.

SIRV2gp19 and SIRV2gp19/D89A nuclease activity

Single- and double-stranded DNA endonuclease and exonuclease activities were monitored as described previously (Hirano et al. 2006). Quantities of MBP-SIRV2gp19 and MBP-SIRV2gp19/D89A (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 pmol) were incubated with 0.5 pmol of circular single-stranded M13mp18 DNA (ssM13), 0.25 pmol linear double-stranded phiX/HaeIII DNA, or 0.25 pmol circular double-stranded DNA (pBR322 or double-stranded M13mp18) in 1× ThermoPol Buffer (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 @ 25°C) in a 20 μL reaction for 1 h at 55°C. DNA products were separated by 0.7% agarose gel electrophoresis.

To confirm that SIRV2gp19 is an endonuclease, its activity was further characterized using a synthetic single-stranded oligonucleotide labeled on the 5′ or 3′ end with a 6-carboxyfluorescein (FAM)-label for detection (Supplemental Table S1). MBP-SIRV2gp19 (0.2 μM) was incubated

with 3′ or 5′ FAM-labeled oligonucleotide (1 μM) in NE-Buffer 4 (50 mM potassium acetate, 20 mM Tris–acetate, pH 7.9 @ 25°C, 10 mM magnesium acetate, and 1 mM dithiothreitol) at 55°C. *E. coli* exonuclease I (0.05 μM), a single-stranded exonuclease, was incubated with 3′ or 5′ FAM-labeled oligonucleotide (1 μM) in exonuclease I reaction buffer (67 mM glycine–KOH, pH 9.5 @ 25°C, 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol) at 37°C. Single-stranded endonuclease, Mung Bean Nuclease (0.3 μM), was incubated with 3′ or 5′ FAM-labeled oligonucleotide (1 μM) in Mung Bean Nuclease buffer (50 mM sodium acetate, pH 5.0 @ 25°C, 30 mM NaCl, and 1 mM $ZnSO_4$) at 30°C. Reaction aliquots were sampled at the indicated times and reaction was halted by the addition of an equal volume of 10 mM EDTA in formamide. Reaction products were separated by 15% denaturing PAGE and fluorescence was detected by a GE Typhoon scanner.

Requirements for SIRV2gp19 nuclease activity

To determine the effect of salt concentration, pH dependence and cation requirements on SIRV2gp19 activity, the nuclease assay as described above was repeated with single-stranded M13mp18 in varying reaction buffers. NaCl concentration was varied from 0 to 0.5 M (in a buffer with 10 mM Tris–HCl, pH 7.0 and 2 mM $MgCl_2$). Tris–HCl or Bis–Tris–propane (10 mM) was used in the pH range of 5.0–10 (with 50 mM NaCl and 2 mM $MgCl_2$). $MgCl_2$ was substituted by 2 mM $MnCl_2$, $CoCl_2$, $ZnSO_4$, or $CaCl_2$, in buffer containing 10 mM Tris–HCl, pH 7.0 and 50 mM NaCl.

Results

SIRV2gp19 substrate specificity and requirements for cleavage activity

The similarity to the RecB nuclease superfamily led us to test SIRV2gp19 nuclease activity on a panel of DNA substrates to determine substrate specificity. Circular double-stranded M13mp18 DNA and linear double-stranded phiX174/HaeIII DNA were not substrates for SIRV2gp19 cleavage (Fig. 1a, b). SIRV2gp19 cleaved circular single-stranded M13mp18 DNA into smaller disperse fragments, appearing as a smear rather than discrete fragments, suggesting that SIRV2gp19 has minimal sequence or structure specificity (Fig. 1c).

To confirm that SIRV2gp19 is a single-stranded endonuclease, activity on a single-stranded oligonucleotide was tested. Oligonucleotide degradation by SIRV2gp19 was compared to degradation by a single-strand 3′–5′

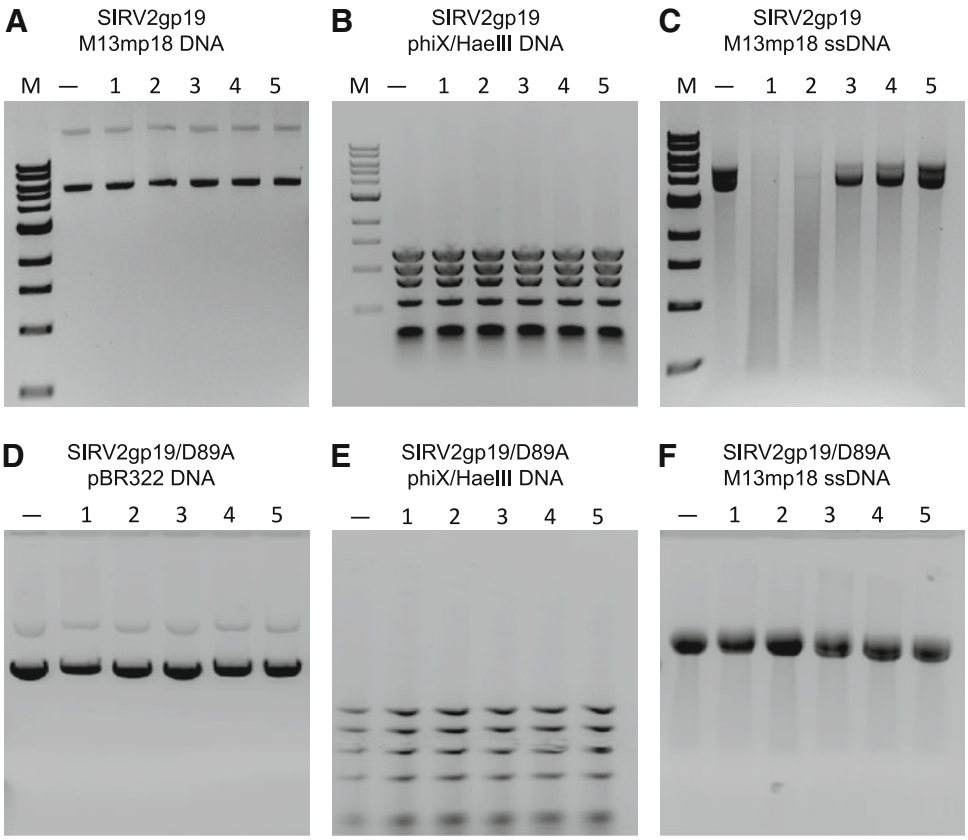

Fig. 1 SIRV2gp19 cleaves single-stranded DNA. MBP-SIRV2gp19 nuclease activity was measured by incubating MBP-SIRV2gp19 dilutions (*lanes 1–5* 1, 0.5, 0.25, 0.125, or 0.0625 pmol) with 1 μg of **a** circular double-stranded M13mp18 RF I DNA, **b** linear double-stranded phiX/HaeIII DNA, or **c** circular single-stranded M13mp18 DNA in 1× ThermoPol Buffer for 1 h at 55°C. SIRV2gp19/D89A was assayed for nuclease activity as described above with substrates **d** circular double-stranded pBR322 DNA, **e** linear double-stranded phiX/HaeIII DNA, or **f** circular single-stranded M13mp18 DNA. As a control, DNA was incubated in the absence of MBP-SIRV2gp19 or MBP-SIRV2/D89A (−). Reaction products were separated by 0.7% agarose gel electrophoresis. The NEB 1 kb DNA ladder (*M*) as a reference

exonuclease (*E. coli* exonuclease I) and a single-strand endonuclease (Mung Bean Nuclease) (Fig. 2). As previously shown (Brody et al. 1986), *E. coli* exonuclease I processively degrades single-stranded oligonucleotides in the 3′–5′ direction releasing dNMPs (Fig. 2). Mung Bean Nuclease degrades single-strand oligonucleotides by cleavage at internal sites (Fig. 2) (Johnson and Laskowski 1968). Similar to Mung Bean Nuclease, SIRV2gp19 degrades single-strand oligonucleotides by cleavage at internal sites (Fig. 2), confirming that SIRV2gp19 is a single-strand specific DNA endonuclease.

Requirements for SIRV2gp19 single-stranded DNA cleavage

The reaction conditions for SIRV2gp19 were investigated using single-stranded M13mp18 DNA as a substrate. Despite the acidic growth environment, the internal pH of host *Sulfolobus islandicus* is neutral. Therefore, it was of interest to determine whether SIRV2gp19 had a pH optimum mimicking the neutral intracellular pH or the acidic extracellular environment. SIRV2gp19 activity optimum reflects the host intracellular pH and cleaves cruciform DNA with a broad pH range between pH 7.0 and 10.0, with inhibition occurring below pH 7.0 (Table 1). Similar to previous studies (Kushner et al. 1971), $MgCl_2$ is a required cofactor for SIRV2gp19 cleavage and cannot be substituted by $MnCl_2$, $CaCl_2$, $ZnSO_4$ or $CoCl_2$ (Table 1). NaCl is not required for SIRV2gp19 activity and inhibits activity at higher concentrations of NaCl (>100 mM) (Table 1).

SIRV2gp19/D89A and its biochemical characterization

Previous studies identified a conserved aspartic acid in Motif II of the RecB nuclease superfamily that coordinates a Mg^{2+} required for nuclease activity (Singleton et al. 2004; Wang et al. 2000; Yu et al. 1998). To test if this conserved Motif II aspartic acid is also essential for SIRV2gp19 activity, Asp89 was mutated to alanine (SIRV2gp19/D89A). SIRV2gp19/D89A nuclease activity was completely abolished on M13mp18 circular single-stranded DNA as well as double-stranded DNA substrates (Fig. 1).

Discussion

SIRV2gp19, previously annotated as a hypothetical RecB nuclease, is a single-stranded specific DNA endonuclease. Mutagenesis of conserved SIRV2gp19 Motif II aspartate to alanine (D89A) abolishes nuclease activity, presumably by disrupting Mg^{2+} coordination in the active site. As little sequence or structure specificity was observed with the SIRV2gp19 nuclease, it could act with other components to direct nuclease activity like the related archaeal cas4

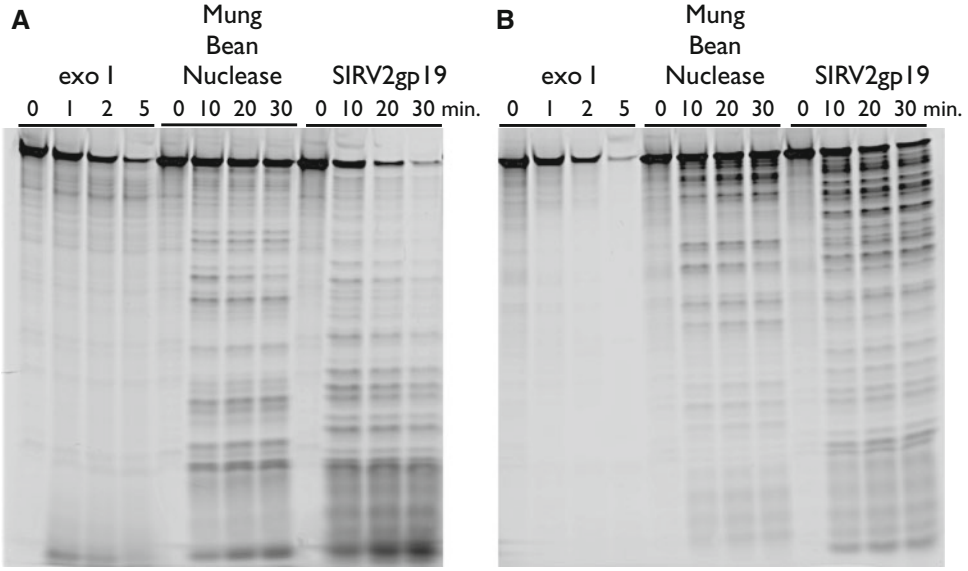

Fig. 2 SIRV2gp19 is a single-strand specific endonuclease. Nuclease activity was characterized using a synthetic single-stranded oligonucleotide labeled on the **a** 5′ or **b** 3′ end with a fluorescent FAM-label for detection. MBP-SIRV2gp19 (0.2 μM) was incubated with **a** 5′ or **b** 3′ FAM-labeled oligonucleotide (1 μM) in NEBuffer 4 at 55°C. *E. coli* exonuclease I (exo I) (0.05 μM) was incubated with **a** 5′ or **b** 3′ FAM-labeled oligonucleotide (1 μM) in exonuclease I reaction buffer at 37°C. Mung Bean Nuclease (0.3 μM) was incubated with **a** 5′ or **b** 3′ FAM-labeled oligonucleotide (1 μM) in Mung Bean Nuclease buffer at 30°C. Reaction aliquots were sampled at the indicated times and reaction was halted by the addition of EDTA (10 mM) in formamide. Reaction products were separated by 15% denaturing PAGE and fluorescence detected by a GE Typhoon scanner

Table 1 Minimal requirements for SIRV2gp19 activity

[NaCl] (mM)	SIRV2gp19 activity[a]	pH	SIRV2gp19 activity[a]	Cation (2 mM)	SIRV2gp activity[a]
0	+	5	−	$MgCl_2$	+
50	+	6	−	$MnCl_2$	−
100	+	7	+	$ZnSO_4$	−
200	−	8	+	$CoCl_2$	−
300	−	9	+	$CaCl_2$	−
400	−	10	+		
500	−				

[a] Single-stranded endonuclease activity (+) or no detectible activity (−)

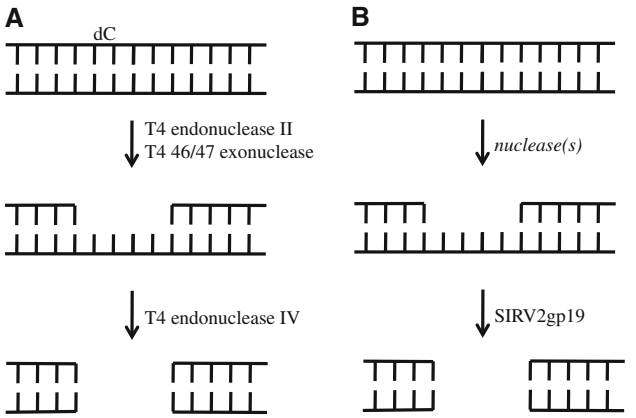

Fig. 3 A model of SIRV2gp19 host chromosome during SIRV2 infection. Bacteriophage T4 or SIRV2 infection triggers an enzymatic cascade that degrades of the host chromosome (Bize et al. 2009; Parson and Snustad 1975). **a** T4 endonuclease II creates nicks in dCMP regions of the host chromosome. T4 46/47 exonuclease removes mononucleotides at gaps to create single-stranded DNA regions. Then T4 endonuclease IV cleaves these single-stranded gaps to fragment DNA. **b** If SIRV2 degrades its host chromosome by a similar mechanism as bacteriophage T4, then a predicted nuclease could first create single-stranded gaps in the host chromosome. This nuclease has not been identified. Then SIRV2gp19, a single-stranded endonuclease, could cleave single-stranded gaps and fragment the host chromosome

nuclease involved in CRISPR processing (Haft et al. 2005; Jansen et al. 2002). Alternatively, SIRV2gp19 could act as a broad spectrum nuclease, presumably to degrade DNA. Broad degradative function has been observed in the metabolism of other bacteriophages, e.g., bacteriophage T4 (Parson and Snustad 1975). Bacteriophage T4 degrades host chromosomal DNA by a combination of several nucleases (Fig. 3). First, a double-strand specific endonuclease (T4 endonuclease II) makes nicks in double-stranded host DNA. Then 46/47 exonuclease catalyzes the removal of mononucleotides from the nicks to expose gapped, single-stranded DNA regions. Finally, a single-stranded endonuclease (T4 endonuclease IV) recognizes and cleaves single-stranded gapped regions to create double-stranded breaks, leading to a collapse of the host genome (Parson and Snustad 1975).

Consistent with a model of host chromosome degradation (Bize et al. 2009), one might expect SIRV2 to encode double-strand specific nucleases akin to T4 endonuclease II and 46/47 exonucleases to create single-stranded gaps in the host chromosome. Such an endonuclease has not yet been identified in SIRV2. However, SIRV2gp19, a single-strand endonuclease, could catalyze the second step of the degradation process since it could cleave single-stranded gapped regions similar to T4 endonuclease IV (Fig. 3). Therefore, in spite of differences in hosts and environments, mechanisms for core viral processes such as host chromosome degradation could converge. Assigning functions to additional proteins annotated as hypothetical will be essential to more completely understand SIRV2 core functions.

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