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Short communication

Characterization of structural proteins of *Solenopsis invicta* virus 1

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ABSTRACT

Purification of *Solenopsis invicta* virus 1 (SINV-1) from its host, *S. invicta*, and subsequent examination by electron microscopy revealed a homogeneous fraction of spherical particles with a diameter of 30–35 nm. Quantitative PCR with SINV-1-specific oligonucleotide primers verified that this fraction contained high copy numbers of the SINV-1 genome. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the SINV-1 purified fraction revealed three major and one minor protein bands. The protein bands were labeled VP1 (40.8 ± 1.4 kDa), VP2 (35.7 ± 2.8 kDa), VP3 (25.2 ± 1.8 kDa), and VP4 (22.2 ± 2.5 kDa) based on mass. N-terminal sequence was acquired successfully for VP1, VP2, and VP3, but not VP4, and delineated each capsid protein within the 3′-proximal open reading frame of SINV-1. Positional organization of the viral proteins within the SINV-1 structural polyprotein was consistent with dicistroviruses (when based on sequence similarity). Blastp analysis of SINV-1 VP1, VP2, and VP3 revealed significant identity with corresponding structural capsid proteins of positive-strand RNA viruses, particularly acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV). Amino acid residues about the scissile bonds for VP1 and VP3 were consistent with dicistroviruses and insect-infecting picorna-like viruses. N-terminal sequencing of VP2 also established that translation initiation of the SINV-1 structural polyprotein was mediated by an internal ribosomal entry site and is AUG-independent.

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Solenopsis invicta virus 1 (SINV-1) is the first virus reported to infect the red imported fire ant, *S. invicta* (Valles et al., 2004; Valles and Strong, 2005). This virus appears to be confined to the *Solenopsis* genus as an asymptomatic, chronic infection that may, under certain conditions, kill the host (Valles et al., 2007a). SINV-1 was found to infect the midgut epithelial cells of larval and adult stages of the red imported fire ant (Hashimoto and Valles, 2007). SINV-1 is a positive-strand RNA virus exhibiting characteristics consistent with viruses in the *Dicistroviridae* (Mayo, 2002). The monopartite, bicistronic genome is comprised of 8026 nucleotides containing two open reading frames (ORFs), each encoding a polyprotein (Valles et al., 2004). The 5′- and 3′-proximal ORFs exhibit similarity with non-structural (i.e., RNA-dependent RNA polymerase, helicase, and protease) and structural proteins, respectively, of positive-strand RNA viruses (Koonin and Dolja, 1993). To provide further evidence, beyond sequence similarity, that the 3′-proximal ORF encodes the structural polyprotein and to establish the proteins comprising the viral capsid, SINV-1 was purified and the coat proteins separated by SDS-PAGE and subjected to N-terminal sequencing. The amino acid sequences were then used to identify the position of each capsid protein in the translated SINV-1 genome.

SINV-1-infected fire ant colonies were identified by RT-PCR with SINV-1-specific oligonucleotide primers (Valles and Strong, 2005). RT-PCR was also conducted with oligonucleotide primers specific to *S. invicta* virus 2 (SINV-2, Valles et al., 2007b). Ants determined to be infected exclusively with SINV-1 were used in this study.

SINV-1 was purified from infected ants by centrifugation as described by Ghosh et al. (1999) and modified by Valles et al. (2004). Purified SINV-1 particles were negatively stained with 2% phosphotungstic acid, pH 7, and examined with a Hitachi H-600 transmission electron microscope (Hitachi, Pleasanton, CA) at an accelerating voltage of 75 kV.

Purified SINV-1 was denatured in an equal volume of 60 mM Tris–HCl containing 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue by heating to 100 °C for 3 min in dry block heater. Proteins from the denatured sample were separated on a 12% resolving SDS polyacrylamide gel at 200 V for 1 h. After electrophoresis, the gel was equilibrated in transfer buffer (10 mM $NaHCO_3$/3 mM Na_2CO_3, pH 9.9 with 20% methanol and 0.1% SDS) for 20 min. The gel was electroblotted at 350 mA for 2 h onto a polyvinylidene fluoride membrane (Immobilon-PSQ; Millipore, Bedford, MA). The membrane was stained with 0.2% Coomassie blue and destained with 40% methanol/5% acetic acid, then submitted to the Interdisciplinary Center for Biotechnology Research (University of Florida) for N-terminal sequencing analysis (Edman degradation). Preparations from four different SINV-1-

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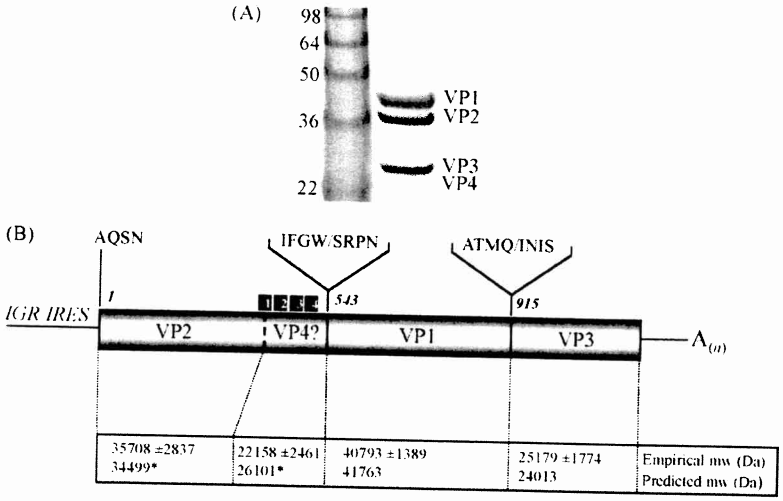

Fig. 1. (A) SDS-PAGE of purified SINV-1. Molecular weights from standards are indicated to the left. Three major bands corresponding to VP1, VP2, and VP3, and one minor band (VP4) are indicated on the right. N-terminal sequence was acquired for VP1, VP2, and VP3, but not VP4. (B) Diagrammatic representation of the 3′-proximal ORF of SINV-1. Amino acid residues about the scissile bonds are depicted by a forward slash and the first residue position of each fragment is indicated. *The putative predicted molecular weight for VP2 and VP4 were determined by using four regions (indicated by white numerals within a black background) with similarity to the VP1/VP3 cleavage site. Amino acid residues about these potential scissile bonds (/) are as follows: 1: 305-ATMQ/TAWG, 2: 359-HAMQ/DLIN, 3: 415-VEMQ/LDAY, and 4: 485-ATMQ/VQAE.

infected *S. invicta* colonies were completed—two of which were submitted for sequence analysis. All four preparations were evaluated by SDS-PAGE to estimate the molecular weight of the capsid proteins.

Isopycnic centrifugation in equilibrated CsCl (1.35 g/ml) yielded a blue–white, iridescent band with a density of 1.337 g/ml. Scanning electron microscopy of this fraction revealed spherical particles with diameters of 30–35 nm consistent with a previous report (Valles et al., 2004). QPCR with SINV-1-specific oligonucleotide primers verified this fraction contained high copy numbers of the SINV-1 genome (Hashimoto et al., 2007). SDS-PAGE analysis of this fraction produced three major and one minor protein bands consistently (Fig. 1A). The protein bands were labeled VP1, VP2, VP3, and VP4 based on mass (Fig. 1A). N-terminal sequence was acquired successfully for VP1, VP2, and VP3, but not VP4 (Table 1). The empirically derived sequences for VP1, VP2, and VP3 were recognized in the SINV-1 3′-proximal ORF (Fig. 1B) and were largely in agreement with the predicted amino acid sequences (GenBank Accession YP164441).

The predicted molecular weight of VP3 (24.0 kDa) was determined by using the empirically derived N-terminal sequence as the start point (amino acid residue 915) and the end of the ORF as the stop point (residue 1126); this predicted molecular weight was within the standard deviation of the SDS-PAGE empirically derived molecular weight (25.2 ± 1.8 kDa). Similarly, the predicted molecular weight of VP1 was determined by using the empirically derived N-terminal sequence as the start point (residue 543) and the amino acid residue just upstream of the empirically derived N-terminal sequence of VP3 as the end point (residue 914). With these start and stop points, the VP1 predicted molecular weight (41.8 kDa) was in agreement with the empirically derived molecular weight (40.8 ± 1.4 kDa).

Reconciling the empirically derived and predicted VP2 was problematic. Although N-terminal sequencing clearly established the start point for VP2 at amino acid residue 1, the stop point was unknown because no sequence was obtained for the protein we labeled VP4 (ostensibly downstream of VP2). The start point for VP2 provides evidence that translation initiation of the SINV-1 structural polyprotein is methionine-independent and internal ribosomal entry site (IRES)-mediated (Sasaki and Nakashima, 2000). SINV-1 structural polyprotein initiation occurs at the GCU (position 4423–4425) codon encoding an alanine, which is consistent with dicistroviruses (Pisarev et al., 2005). Indeed, Jan (2006) correctly predicted the alanine start site for SINV-1 VP2 based on characterization of the intergenic IRES. The predicted molecular weight of the capsid protein starting at amino acid residue 1 and ending at the amino acid residue just upstream of VP1 (residue 542) was 60.6 kDa. This value is 41% larger than the empirically derived molecular weight determined for VP2 (35.7 ± 2.8 kDa). Obviously, an additional scissile bond(s) is present between amino acid residue 1 (VP2 start) and 543 (VP1 start) most likely representing the VP2/VP4 cleavage point. Insect-infecting picorna-like viruses exhibit a general polyprotein organization in which VP0 (comprised of VP2 + VP4) is upstream of VP1 and VP3, and VP4 is

Table 1
Comparison of empirically derived and predicted N-terminal VP1, VP2, and VP3 sequences from SINV-1

SINV-1 protein	Acquisition	N-terminal sequence[a]	Identity with SINV-1 predicted sequence (%)
VP1	Empirical	SYPNDMELVTSL	83
VP1	Empirical	GYPNDVEL	50
VP1	*Predicted*	***SR*PNDME*K*VTSL**	
VP2	Empirical	AQSNNAN	100
VP2	*Predicted*	***AQSNNAN***	
VP3	Empirical	INKSNEHSGN	60
VP3	Empirical	INKSNEAGN	67
VP3	*Predicted*	***IN*ISNEAS*ID***	

[a] Bold case indicates empirically derived residues observed in the corresponding predicted sequence of the SINV-1 3′-proximal ORF.

an N-terminal extension of VP3 (Sasaki et al., 1998; Nakashima et al., 1999; Liljas et al., 2002). It appears that SINV-1 conforms to this structural polyprotein organization in that VP4 is downstream of VP2. Indeed, the amino acid sequence immediately upstream of VP1 (amino acid residue 478–542) exhibited similarity with VP4 from dicistroviruses (e.g. Kashmir bee virus (KBV): 31% identity). In picornaviruses, cleavage of VP0 to VP2 and VP4 is thought to occur after capsid assembly and auto-catalytically because no protease has been detected within the assembled capsid (Hogle et al., 1985). However, examination of the SINV-1 structural polyprotein for regions similar to the known scissile bonds observed by N-terminal sequence analysis revealed four regions (noted in Fig. 1B) with similarity to the sequence at the VP1/VP3 junction (i.e., ATMQ/INIS). These putative cleavage sites and the corresponding predicted molecular weight of VP2 (start point of amino acid 1) produced were (1) 308/309 (34.5 kDa), (2) 362/363 (40.5 kDa), (3) 418/419 (47.1 kDa), and (4) 488/489 (55.3 kDa). The first of these potential cleavage sites (at amino acid residues 308/309) would yield a protein with a molecular weight (34.5 kDa) close to the empirically derived value observed for VP2 (35.7 ± 2.8 kDa). Cleavage at this site results in a second, downstream protein with a molecular weight of 26.1 kDa, a value close to the empirically derived 22.2 ± 2.5 kDa for the observed VP4 (Fig. 1). However, it is obvious from SDS-PAGE results that VP4 was not present in an equimolar concentration compared with VP1-VP3. This result was somewhat anticipated based on characteristics associated with the pseudo-T = 3 virion architecture of picorna-like viruses (Le Gall et al., 2008). The capsid of picorna-like viruses is characteristically comprised of three main proteins; observation of a fourth protein is reported inconsistently. SINV-1 VP4 may be released during capsid assembly or required for RNA encapsidation or stabilization as reported for some mammalian picornaviruses (Rossmann and Tao, 1999). The amino acid sequence (residues 309–477) between VP2 and VP4 (as defined by similarity with KBV and acute bee paralysis virus (ABPV)) appears to be unique to SINV-1 because no sequences with significant identity were observed by blast analysis (Altschul et al., 1997). This result also supports VP4 assignment because these proteins are unrelated to the other capsid proteins (Le Gall et al., 2008).

When examined by size, the positional organization of the capsid proteins in the SINV-1 3′ ORF did not follow the pattern exhibited by most known dicistroviruses. Although VP0 (VP2 + VP4) was at the N-terminus of the ORF, and VP1 and VP3 were downstream of VP0, VP1 was found between VP0 and VP3. This organization was also reported for the deformed wing virus (DWV), an iflavirus (Lanzi et al., 2006). However, when based on sequence similarity, the positional organization of the SINV-1 capsid proteins was consistent with the majority of the dicistroviruses (i.e., NH_2-VP2-VP4-VP3-VP1). Another similarity between SINV-1 and DWV is an unusually large VP1. Picorna-like and related virus VP1 homologues are typically no larger than 35 kDa (Liljas et al., 2002). However, DWV exhibited the largest reported mass for VP1 at 46.7 kDa while SINV-1 VP1 is 41.7 kDa.

Comparative analyses of SINV-1 structural polyprotein sequences at the VP4/VP1 and VP1/VP3 cleavage sites were consistent with other dicistroviruses and unclassified picorna-like insect-infecting viruses (Liljas et al., 2002). Amino acid residues G_{541}, S_{543}, and P_{545} at the VP4/VP1 cleavage site and Q_{914} at the VP1/VP3 cleavage site were conserved. Not surprisingly, these sites exhibited highest identities with KBV, ABPV, and Israeli acute paralysis virus (IAPV) (de Miranda et al., 2004; Maori et al., 2007). Examination of the 5′-proximal ORF (non-structural proteins) of SINV-1 revealed 4 sites ($ATMQ_{267}$VGDE, $ANMQ_{620}$MWKD, $ARMQ_{852}$ICLD, and $DVMQ_{1029}$KVHE) with sequence similarity to the SINV-1 VP1/VP3 ($ATMQ_{914}$/INKS) scissile bond that could serve as potential cleavage sites. These sites were found outside the conserved regions of the protease, helicase, and RNA-dependent RNA polymerase.

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