Detection of Leishmania RNA Virus 1 Proteins

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Polyclonal antiserum was raised against the peak viral fraction of a sucrose gradient from LRV1-4-infected cells and used in Western immunoblot analysis to identify viral proteins from various isolates. Consistent with this result, in vitro-translated protein from cloned RNA was immunoprecipitated with the same antiserum. The putative capsid at times appeared as a doublet; relative amounts of the two species varied, depending on the method of purification.

Some strains of Leishmania have been shown to harbor an infection caused by a double-stranded-RNA (dsRNA) virus (7, 9). This infection is believed to be persistent, since no extracellular virus has been observed and attempts to stably infect uninfected strains of Leishmania have failed. Electroporation of virus particles into uninfected and heterologously infected strains of Leishmania produces only a transient infection (1). Recently, Stuart and colleagues have reported the entire molecular organization of LRV1-1 (6). The 5,284-nucleotide dsRNA genome of this virus contains three open reading frames (ORFs) on the mRNA plus strand. The complete amino acid sequence of ORF1, which has no significant amino acid homology to any known protein, is 72 amino acids long. The predicted amino acid sequence of ORF3 has motifs characteristic of viral RNA-dependent RNA polymerases (3) and could encode a 98-kDa protein. ORF2 presumably encodes the major viral coat protein (approximately 82 kDa) and overlaps ORF3 by 71 nucleotides, suggesting a +1 translational frameshift to produce a gag-pol type of fusion protein (6). Sequencing of LRV1-4 (5) has confirmed a molecular organization similar except for the presence of two ORFs in the place of ORF1 at the 5′ end. None of the ORFs have been directly shown to translate proteins, although striking similarities to the yeast dsRNA killer virus system suggest strongly that at least ORF2 and ORF3 are translated. However, prior to this report, no proteins associated with viral infection or viral particles had been identified.

Since Western immunoblot analysis can detect lower levels of proteins than conventional staining methods, antiserum was generated against a crude sucrose gradient fraction of extract from infected cells (MHOM/BR/75/M4147), corresponding to the peak of viral dsRNA. Sucrose gradients were prepared as described previously (11). This 1-ml fraction was emulsified with an equal volume of Freund’s complete adjuvant and injected subcutaneously along the back of a female New Zealand White rabbit. The rabbit was bled by the ear vein every 4 weeks immediately prior to a booster injection. Each booster injection consisted of 0.5 ml of an identical viral sucrose fraction in 1.5 ml of Freund’s incomplete adjuvant. One month after the third booster injection, the final antiserum was collected.

Since this antiserum was generated against a crude sucrose gradient fraction from infected-cell extracts, we expected a significant host protein background. To minimize the effect of this background, the antiserum was preclarified with uninfected-cell extracts. Approximately 3 × 10⁶ uninfected cells (MTAM/BR/80/M6244) were washed with phosphate-buffered saline (PBS), resuspended in 1 ml of TMN (10 mM Tris [pH 7.5], 5 mM MgCl₂, 150 mM NaCl), and sonicated thoroughly. A 1-ml portion of antiserum was mixed with this sonicate and rocked overnight at 4°C. The cell debris and immune complexes were removed by centrifugation at 12,000 rpm in a tabletop Eppendorf centrifuge at 4°C.

This antibody was used in a Western blot assay to detect viral proteins. A portion (10 μl) of the 1-ml sucrose gradient fraction which corresponded to the peak of viral dsRNA was electrophoresed on a sodium dodecyl sulfate–7% polyacrylamide gel. This was then transferred to a polyvinylidene difluoride membrane (Millipore Immobilon-P) with a Hoefer Semi-Phor electrottransfer unit. The blot was blocked overnight at 4°C in PBS with 0.05% Tween 20 (TPBS) plus 1% Carnation nonfat dry milk. The blot was incubated in a 1/500 dilution of preclarified antiserum in TPBS plus 5% milk for 3 h at room temperature and washed for 45 min in TPBS at room temperature with at least five changes of wash. The blot was then incubated in a 1/5,000 dilution of affinity-purified goat anti-rabbit immunoglobulin G (Promega) for 90 min in TPBS plus 5% milk and washed as before. The alkaline phosphatase reaction was performed according to the manufacturer’s instructions (Promega). Three bands were observed (Fig. 1). The major band migrates at approximately 82 kDa, the size predicted for the putative capsid protein from sequence information for ORF2 (6). Frequently seen in various amounts below the major band is slightly faster migrating species. A minor band migrates at approximately 180 kDa, the size predicted for a capsid polymerase fusion protein. The detection of this band was the first evidence for the generation of a fusion protein as predicted from sequence information (6).

Further proof of the viral origin of these proteins was obtained by performing Western blot analysis on fractions from a sucrose gradient, showing that these proteins cosedimented with the viral dsRNA. A 400-ml culture of late-log-phase virally infected (MHOM/BR/75/M4147) or uninfected (MTAM/BR/80/M6244) cells was washed, resuspended in lysis buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid] [pH 7.4], 300 mM KCl, 10 mM MgCl₂), and lysed with 1% Triton X-100 by a method modified from that of Kapfer and Beverley (4). The nucleo
and cell debris were removed by brief centrifugation, and the cell extract was loaded on a 10 to 40% sucrose gradient in HCN buffer (20 mM HEPES [pH 7.4], 5 mM CaCl₂, 150 mM NaCl). The gradients were centrifuged at 36,000 rpm in an SW41 Beckman rotor for 135 min at 4°C and collected in 1-ml fractions. The pellet from each gradient was resuspended in 1 ml of HCN buffer. Ten percent (100 μl) of each 1-ml fraction was phenol-chloroform extracted, ethanol precipitated, electrophoresed on 0.7% agarose, and stained with ethidium bromide (Fig. 2A and C). One percent (10 μl) of each fraction was mixed with 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and loaded on SDS–7% PAGE gels. These gels were transferred to polyvinylidene difluoride membranes, and Western blot analysis was performed as described above (Fig. 2B and D).

The nucleic acid distribution was similar in infected (Fig. 2A) and uninfected (Fig. 2B) cells, except for the distinct viral 6-kb dsRNA band which peaked at fractions 3 and 4 in the infected cells. As expected, the Western blots show similar distribution of the host background at the top of the gradient but the uninfected cells lack the putative capsid band which, like the viral RNA, peaks at fractions 3 and 4. The putative capsid band cosegregated with the dsRNA of the virus as predicted. Western blot analysis was also performed on other *Leishmania braziliensis* strains and *L. guyanensis* strains which carry the viruses MHOM/SR/80/CUMC1-1A, MCHO/BR/80/M6200, WR677 (originally derived from MHOM/BR/75/M4147), and MHOM/BR/75/M2904 (Fig. 3). We see similar distributions of the putative capsid protein in all of the strains tested. None of the recognized proteins could be detected by classical staining methods, because of either instability or the low abundance of these proteins.

Since the polyclonal sera cross-reacted with the capsid of every virus from *L. braziliensis* and *L. braziliensis guyanensis* tested, it was possible that the sera would also recognize the viral capsid from a newly identified virus, LRV2-1, from an Old World strain of *Leishmania major* (MHOM/SU/73/5ASKH). Cells were collected and analyzed as described above. Ethidium bromide staining of nucleic acids shows that the viral band has a size and a sedimentation pattern in a sucrose gradient similar to those of the other viral isolates (Fig. 4A). Unlike those of the other isolates, however, the capsid is not recognized by the polyclonal sera (Fig. 4B). While the positive control is clearly visible on the Western blot (Fig. 4B, lane +), the host background seen in other gradients is not seen in MHOM/SU/73/5ASKH (the host strain for LRV2-1), presumably because of the great differences between New World and Old World strains of *Leishmania*. Also, unlike the capsids of the other viral isolates, the viral capsid appears in great enough quantity to be detectable by Coomassie blue stain (Fig. 4C). A portion (10 μl) of each sucrose gradient fraction was electrophoresed on an SDS–8% PAGE gel, stained overnight with 0.25% Coomassie brilliant blue R250, and destained for several hours with 50% methanol–10% glacial acetic acid. Interestingly, the size of the putative capsid protein of MHOM/SU/73/5ASKH appears to be similar to those of the other viral isolates, and the protein also appears as a doublet. The capsid of LRV2-1 is, therefore, very divergent from those of the New World viral isolates but may have a similar coding strategy.

The putative capsid ORF (ORF2) of LRV1-4 was cloned in
its entirety into a single vector (5). T3 (sense) and T7 (antisense) transcripts synthesized from the clone were translated in vitro, and the antibody was used to precipitate the translated protein. T7 transcripts were generated by using T7 polymerase (New England Biolabs) in a mixture containing 40 mM Tris [pH 7.5], 2 mM spermidine, 30 mM dithiothreitol, 4 mM each nucleoside triphosphate, 0.5 mM m7GpppG (Boehringer Mannheim), 1 μg of linear template, and 100 U of polymerase in a 50-μl total volume. T3 transcripts were synthesized under identical conditions but with 50 mM NaCl and T3 polymerase (Stratagene) added. Incubations were done for 90 min at 37°C. The DNA template was then digested for 10 min with 2 U of RQ1-DNase (Promega). Transcripts were translated in vitro by the Promega wheat germ extract system. Samples of 5 μg (Fig. 5A, lanes 4, 5, 7, and 8) or 0.5 μg (lanes 3 and 6) of transcripts were added to each translation reaction mixture with either 75 mM (lanes 3, 4, 6, and 7) or 95 mM (lanes 5 and 8) potassium acetate. One microgram of brome mosaic virus RNA (Fig. 5A, lane 2) was translated in the presence of 100 mM potassium acetate. A translation reaction with 100 mM potassium acetate without added RNA (Fig. 5A, lane 1) was also run. The products from in vitro translation show that sense but not antisense transcripts generated a product of the expected size.

The in vitro-translated product was immunoprecipitated by the virus polyclonal antiserum used in the Western blots (Fig. 5B). Protein A-Sepharose beads CL4B (Sigma) were prepared according to the manufacturer’s instructions and allowed to bind 100 μl of either the polyclonal virus antiserum or rabbit preimmune serum for 45 min at room temperature. These beads were washed and incubated with the in vitro-translated product at 4°C for 3 h. Unbound antigen was removed by extensive washing, and bound antigen was eluted by boiling in SDS-PAGE sample buffer. These products were electrophoresed on an SDS-8% PAGE gel and analyzed by autoradiography. In vitro-translated products were immunoprecipitated by immune (Fig. 5B, lane 3) but not preimmune (lane 2) serum. Both preimmune and immune sera precipitated background levels identical to those of brome mosaic virus translated products (data not shown).

A very faint, more slowly migrating species also appears above the major band. This minor band migrates more slowly than the capsid band seen in Western blot analysis. This was shown by superimposition of the autoradiogram from in vitro-translated products on a Western blot (Fig. 5C). The in vitro-translated product (Fig. 5C, lane 2) and fraction 4 from a CsCl density gradient (lane 1) were electrophoresed on an SDS-8% PAGE gel and transferred to a polyvinylidene difluoride membrane, and Western blot analysis was performed as before. The dried Western blot was analyzed by autoradiography, and the exposed X-ray film was superimposed on the Western blot. The major band of the radiolabelled translated product corresponds to the more slowly migrating capsid band from Western blot analysis. The faint, more slowly migrating, in vitro-translated band could be the product of an unpredicted upstream initiation or a read-through product due to the inclusion of the predicted frameshift region and a portion of ORF3 in the clone from which the transcripts were templated. It is also notable that the in vitro-translated ORF2 does not produce a faster-migrating species of the size seen on Western blots, indicating that any potential cleavage of the capsid protein does not occur under the conditions used in the translation.

RNA polymerase activity is associated with viral particles (10). However, as reported previously, distinct differences in viral polymerase activity have been observed, depending on the method of purification (11). Particles purified on CsCl
density gradients show altered viral polymerase activity compared with that of particles purified on sucrose gradients. Specifically, CsCl-purified particles show three differences: loss of replicase activity, while retaining transcriptase activity (11), loss of the ability to synthesize a 314-nucleotide subgenomic transcript (2), and sensitivity of the polymerase activity to proteinase K treatment (11) (whereas sucrose-purified particles have proteinase-K-resistant polymerase activity). To determine whether there is a corresponding difference in protein distribution in CsCl versus sucrose-purified particles, Western blot analysis was performed. CsCl-purified particles (Fig. 6, lanes 4 and 5) lose most of the 180-kDa protein band that sucrose-purified particles retain (lanes 2 and 3). There is also a corresponding increase in the faster-migrating putative capsid species not seen in large amounts in cell extracts (Fig. 6, lane 6) or sucrose-purified particles (lanes 2 and 3). One possible explanation for the altered protein distribution is cleavage of the capsid protein and concomitant leakage of the fusion protein from the particle. Cleavage of the capsid protein at the N terminus would result in the loss of a lysine-arginine-rich region which could potentially interact with the viral RNA, possibly generating the observed changes in polymerase activity. However, because of the low abundance of capsid protein, no further information about the generation of the faster-migrating capsid species has been obtained.

We report here the first direct evidence of the expression of viral gene products from a Leishmania RNA virus. Previous sedimentation studies show that the virus must be encapsidated (8). While the prediction of an RNA-dependent RNA polymerase from ORF3 is made on the basis of significant homology to the RNA-dependent RNA polymerase domains of yeast dsRNA killer virus (3, 6), the prediction of a capsid protein from ORF2 is made primarily by a process of elimination, as there is no homology with other known nucleocapsid proteins. Western blot analysis supports the idea that the capsid protein is an 82-kDa protein, as predicted from sequence information. This report also demonstrates the presence of a protein of approximately the correct size to include both the putative capsid protein and the polymerase protein; however, as yet there is no direct evidence that the 180-kDa protein is a capsid-polymerase fusion protein. It remains to be determined how the capsid doublet seen in some Western blots is generated and whether it is a true reflection of gene expression in infected cells or a function of purification.

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REFERENCES


