Specific In Vitro Cleavage of a *Leishmania* Virus Capsid–RNA-Dependent RNA Polymerase Polyprotein by a Host Cysteine-Like Protease

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Antibodies raised against baculovirus-expressed RNA-dependent RNA polymerase (RDRP) recognized a 95-kDa antigen and two smaller proteins in sucrose-purified *Leishmania* virus particles isolated from infected parasites. The 95-kDa antigen is similar in size to one predicted by translation of the RDRP open reading frame (ORF) alone. In an effort to reconcile in vitro observations of translational frameshifting on *Leishmania* RNA virus 1-4 transcripts, we have developed an in vitro cleavage assay system to explore the possibility that the fusion polyprotein is proteolytically processed. We show that coincubation a synthetic Cap-Pol fusion protein with lysates from *Leishmania* parasites yields major cleavage products similar in size to those encoded by the individual capsid and RDRP genes as well as the antigens detected in vivo. The major 82- and 95-kDa major cleavage products are specifically immunoprecipitated by capsid- or polymerase-specific antibodies, respectively, showing that cleavage occurs at or near the junction of the two functional domains. Protease inhibitor studies suggest that a cysteine-like protease is responsible for cleavage in the in vitro assay system developed here. From these results, we suggest that failure to detect a capsid-polymerase fusion protein produced by translational frameshifting in vivo may be due to specific proteolytic processing.

Members of the genus *Leishmania* are protozoan parasites and the causative agent of leishmaniasis in humans. Since the first report showing that some *Leishmania* strains harbor a double-stranded RNA (dsRNA) virus (36), similar viruses have been identified in at least 13 cultured strains of *Leishmania* (25, 34). All but one of the known *Leishmania* RNA viruses (LRVs) are restricted to members of the New World parasites *Leishmania braziliensis* and *L. guyanensis*. One virus, LRV2-1, has been found in an Old World parasite, *L. major* (5, 30). The viruses have been assigned to genus *Leishmanivirus* of the *Totiviridae* family (26).

Complete nucleotide sequences have been reported for LRV1-1 (34), LRV1-4 (29), and LRV2-1 (30). Sequence analysis of the viral genomes identifies two large conserved open reading frames (ORFs), designated ORF2 and ORF3, on the virus plus-strand RNA. The 5' end of the genome, highly conserved at the nucleotide level between LRV1-1 and LRV1-4, shows some divergence in that LRV1-1 has one small ORF (ORFx) while LRV1-4 has two small ORFs (ORF1 and ORFx). LRV2-1 has a single small ORF (ORF1) at the 5' end. The small ORFs exhibit no significant sequence homology to any known protein, and there are currently no data to suggest that the ORFs generate protein products. ORF2 was shown to encode the major 82-kDa viral coat protein when the baculovirus-expressed gene product self-assembled into virus-like particles in *Spodoptera frugiperda* Sf9 cells (4). The presence of viral RNA-dependent RNA polymerase (RDRP) consensus motifs (3) in ORF3, which is predicted to encode a 98-kDa protein product, suggests that ORF3 encodes the polymerase activity associated with gradient-purified virus particles. The existence of a Gag-Pol-type fusion protein in *Totiviridae* has been reported for the L-A virus of yeast (7, 10) and *Giardia*

virus (37), where the fusion protein is presumably generated via a –1 translational frameshift.

Previous attempts to demonstrate an LRV capsid-polymerase fusion protein have been unsuccessful. In an earlier study, a polyclonal antibody raised against purified viral particles recognized two proteins (82 and 180 kDa) (5) that were well matched with the predicted sizes of a capsid and a capsid-polymerase fusion protein, respectively. However, identical products were detected in Sf9 cells expressing only the viral capsid gene, demonstrating that the fusion-size protein is likely a capsid dimer rather than a capsid-polymerase fusion protein (4).

Sequence analysis has shown that viral ORF3 does not encode a consensus translation initiation sequence near its 5' end (17, 18). Also, the 71-nucleotide overlapping sequence between ORF2 and ORF3 in LRV1-4 encodes both a putative slippery translation site and predicted RNA pseudoknot structures shown to promote ribosomal frameshifting in other systems. The findings raise the possibility that a +1 translational frameshift produces a Gag-Pol type fusion protein in LRV1-4, as seen in retroviruses (14, 15), yeast L-A virus (7), and *Giardia* virus (37). Consistent with this possibility, Lee et al. (21) have provided in vitro evidence for a +1 ribosomal frameshift during translation of the overlapping gene sequences present on LRV1-4 transcripts. In the present study, we identify for the first time RDRP antigens associated with LRV1-4 particles and present other results demonstrating that a synthetic LRV Cap-Pol fusion protein encodes a protease-sensitive site that is cleaved by a host-encoded cysteine-like protease in vitro.

MATERIALS AND METHODS

Parasite strain and cell culture. *L. guyanensis* M4147 (MHOM/BR/75/M4147) and *L. braziliensis* M6244 (MTAM/BR/80/M6244) served as virus (LRV1-4)-infected and uninfected strains, respectively. Cells were grown at room temperature in M199 semidefined medium (GIBCO-BRL) supplemented with 5% fetal bovine serum (HyClone, Inc.) and 1% fresh, filter-sterilized human urine (12).

Construction of a recombinant RDRP baculovirus expression vector. First, two overlapping subclones, pCRE1-T12 and pCRE3-III', representing a com-

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plete LRV1-4 RDRP gene were constructed by reverse transcription-PCR with virus-specific primers (29). A 1.1-kb *EcoRI/HindIII* fragment from pCRE1-T12 was ligated along with an 1.6-kb *HindIII/BamHI* fragment from pCRE3-III' into *EcoRI/BamHI*-cut plasmid pVLGS to generate pVLGS-RDRP14. The pVLGS vector encodes an upstream glutathione S-transferase (GST) fusion partner in the polylinker of the baculovirus expression vector, pVL1393 (Invitrogen), and was kindly provided by Bruce Meyer (Harvard Medical School).

Generation of a full-length LRV1-4 cDNA clone and an in-frame capsid-polymerase fusion construct. A full-length cDNA copy of LRV1-4 was assembled from a panel of overlapping cDNA fragments as described below. Briefly, a 2.0-kb *NotI/NsiI* fragment derived from subclone pCRII5'-F2 (29) was ligated, along with a 0.7-kb *NsiI/AflIII*-cut PCR fragment produced with primers LRV4-R2 (5' GCACATTATTGCATGAC 3') and Gap2-rev (5' GATCCTCG AGGCTGAGGTAGTAGTCCTGAC 3'), into *NotI/AflIII*-cut pVL-RDRP14 (see above). The resulting plasmid (pVL-LRV14), which contains a full-length cDNA copy of LRV1-4 RNA, was digested with *XbaI/XhoI*, and the entire viral insert was transferred into *XbaI/XhoI*-cut pBluescriptII SK (pBSK-FULL14) under the control of the T7 transcriptional promoter. An in-frame capsid-polymerase gene fusion construct (pBSK-INFRAFRAME) was then derived by PCR with mutagenic primers (inframe 1 [5' CTCTGGTTCGGTACTGTATC 3'] and inframe 2 [GATACAGTACCGAACCAGAG 3']) by deleting one nucleotide from the overlapping region of ORF2 and ORF3 in pBSK-FULL14. The mutated nucleotide was verified by DNA sequencing.

Expression of the LRV1-4 RDRP ORF in Sf9 cells and production of polyclonal antisera. pVLGS-RDRP14 was transfected into Sf9 cells in the presence of wild-type linear AcMNPV (*Autographa californica* nuclear polyhedrosis virus) baculovirus DNA according to the manufacturer's recommendations (Linear AcMNPV DNA transfection module; Invitrogen). Recombinant virus was isolated from plaques lacking occlusion bodies and further purified as described previously (35). Presence of the LRV1-4 RDRP gene was confirmed by PCR with specific primers. The GST-RDRP fusion protein expressed in Sf9 cells was characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis with GST-specific antibody (Pharmacia Biotech). The recombinant GST-RDRP protein was isolated from a preparative SDS-polyacrylamide gel by electroelution (model 491 Prep Cell; Bio-Rad) and inoculated into New Zealand White rabbits. The final production bleed was precleared by incubation with lysates prepared from uninfected Sf9 cells.

Preparation of cell extracts and virus particles. *Leishmania* cells were harvested from late-log phase cultures, washed once with TMN (10 mM Tris [pH 7.5], 5 mM MgCl₂, 150 mM NaCl), and lysed in TMN supplemented with 1% (vol/vol) Triton X-100 (Sigma) at 4°C for 3 h. The lysed cells were clarified by centrifugation (15,000 × g for 10 min), and the supernatant was saved for processing as a cellular extract. For sucrose gradient fractionation, a 1-ml portion of the cellular extract was loaded onto an 11-ml sucrose gradient (10 to 40%, wt/vol) prepared in TMN buffer and centrifuged at 36,000 rpm in a Beckman SW41 rotor for 210 min at 4°C. Sucrose gradients were collected in 1-ml fractions and numbered from the bottom of the gradient. Virus particles were concentrated from gradient fractions by adding an equal volume of polyethylene glycol (PEG) 8000 solution (20%, wt/vol) prepared in 1 M NaCl. After 30 min of incubation at room temperature, the mixture was centrifuged at 12,000 × g for 10 min and washed once with 1 ml of TMN buffer. The precipitates were resuspended in 20 µl of TMN buffer and used in Western blot analysis.

Sf9 cells (2 × 10⁶/ml) were infected with recombinant baculovirus, prepared as described above, at a multiplicity of infection of 10. Infected cells were harvested at day 5 by centrifugation at 2,500 rpm (10 min) in a tabletop Beckman GP centrifuge, washed once in TMN buffer, and lysed in TMN supplemented with Triton X-100 as described above.

SDS-PAGE and Western blot analysis. Western blot analysis was performed as described previously (5), with minor modifications. Briefly, cellular extract was resolved by SDS-PAGE (10% polyacrylamide) and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon-P) with a Hoefer Semi-Phor electrotransfer unit. The blot was incubated overnight at room temperature in blocking solution (phosphate-buffered saline with 0.05% [vol/vol] Tween 20 [TPBS] plus 5% [wt/vol] Carnation nonfat dry milk). The blot was then incubated in a 1/5,000 dilution of precleared LRV1-4 capsid (4) or GST-RDRP (see above) antiserum in blocking solution for 60 min at room temperature. After extensive washing in TPBS, the blot was incubated in a 1/10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim) in blocking solution for 1 h at room temperature. The blot was washed extensively prior to the alkaline phosphatase color development reaction as recommended by the manufacturer (GIBCO-BRL).

RDRP assays. Viral polymerase assays were performed on sucrose gradient fractions as described by Widmer and Patterson (38) except that [α -³²P]UTP (Dupont-NEN) was used instead of [α -³²P]GTP. Sucrose gradient fractions were prepared as described above, and a 10-µl aliquot of each fraction was used in a 20-µl (final) reaction volume. The polymerase products were resolved on denaturing 6% polyacrylamide-7 M urea gel and visualized by autoradiography.

In vitro transcription and translation. In vitro transcription and translation reactions were performed by using a TnT coupled rabbit reticulocyte lysate system (Promega) according to the manufacturer's protocol. Briefly, 1 µg of plasmid DNA was incubated with the TnT reaction mixture for 1 h at 30°C in a 50-µl reaction mixture containing T7 RNA polymerase and [³⁵S]methionine

(Dupont-NEN). Reaction products were resolved on an SDS-polyacrylamide gel with 10% polyacrylamide (20). The dried gel was exposed overnight at room temperature to Biomax Xomat-AR film (Kodak).

Proteolytic cleavage assay. In vitro-produced transcripts were incubated in a 15-µl cleavage assay mixture containing 4 µl of in vitro-translated polyprotein, 4 µl of *Leishmania* cell extract, 1.5 µl of 0.2 M HEPES (pH 7.4) buffer, and 5.5 µl of distilled deionized H₂O. Reaction mixtures were incubated at 30°C for 40 min, and reactions were terminated by heating for 4 min at 100°C in SDS sample buffer (20). The products were resolved by SDS-PAGE (10% polyacrylamide) and visualized by autoradiography. For the time course experiment, equal volume aliquots were removed at different time points and processed as described above.

Immunoprecipitation of cleavage reaction products. Cleavage reaction products were characterized by immunoprecipitation with capsid- or RDRP-specific antiserum. Cleavage reaction products (20 µl) were incubated with 1 µl of polyclonal antibody for 1 h at 4°C in 100 µl (final volume) of TPBS and then mixed with an equal volume of protein A-Sepharose beads (0.2 g/ml; Pharmacia Biotech) pre-equilibrated in TPBS. Incubation was continued for 1 h to allow immune complex binding. Unbound proteins were removed by repeated (five times) centrifugation (1,500 × g for 5 min) in 200 µl of TPBS at room temperature. Bound proteins were eluted by heating in SDS sample buffer as described above. Eluted proteins were resolved by SDS-PAGE (10% polyacrylamide) and visualized by autoradiography.

Protease inhibitor studies. Cell lysates (5 µl) were preincubated for 20 min with 1 µl of the indicated protease inhibitor prior to addition to the cleavage assay. The following inhibitors were tested for effects on cleavage in the in vitro assay: 1,10-phenanthroline (Sigma), phenylmethylsulfonyl fluoride (Sigma), soybean trypsin inhibitor (Boehringer Mannheim), iodoacetamide (Sigma), and a protease inhibitor set from Boehringer Mannheim including antipain dihydrochloride, aprotinin, EDTA, leupeptin, Pefabloc SC, and phosphoramidon. Inhibitors were prepared according to manufacturer's recommendations and used at the indicated final concentrations for the cleavage assays.

RESULTS

Identification of RDRP antigens in sucrose-purified LRV1-4 particles. Previous studies using capsid-specific antisera (5) identified a protein of approximately 180 kDa in LRV1-4 particles isolated from virus-infected M4147 cells. The protein was presumed to be a capsid-polymerase fusion product until analysis of baculovirus-expressed capsid ORF alone revealed a protein of similar characteristics (4). To further investigate the possibility of a capsid-polymerase fusion protein in virus-infected parasites, we raised antisera against baculovirus (AcMNPV)-expressed LRV1-4 polymerase gene and used the immunological probe to characterize RDRP antigens in sucrose-purified virus particles.

A recombinant baculovirus containing a GST-fused RDRP gene was prepared as described in Materials and Methods and used to produce GST-RDRP protein in Sf9 cells. The GST-fusion protein expressed in Sf9 cells was characterized by SDS-PAGE and Western blot analysis of Sf9 cell lysates with GST-specific antiserum prior to inoculation of rabbits and the production of a polyclonal antiserum (data not shown). The antiserum was characterized for specificity by Western blotting against lysates from Sf9 cells expressing the recombinant protein prior to probing LRV particles. LRV1-4 particles were purified from virus-infected M4147 cells by sucrose density gradient centrifugation as described previously (38), resolved on SDS-polyacrylamide gels, transferred to a PVDF membrane, and incubated with antiserum raised against baculovirus-expressed capsid (4) or GST-RDRP protein (see above). Presence of virus in the samples was confirmed by ethidium bromide staining of viral dsRNA and by in vitro polymerase assays as previously described (38). Individual lanes (Fig. 1) contain unconcentrated or PEG-concentrated virus particles, as indicated. As previously reported (4), antiserum raised against baculovirus-expressed capsid protein recognized the 82-kDa capsid monomer as well as an 180-kDa antigen, presumed to be a capsid dimer, in either unconcentrated or PEG-concentrated sucrose gradient fractions (Fig. 1B, lanes 1 to 3). Antiserum raised against purified GST-RDRP fusion protein

FIG. 1. Western blot analysis with antisera raised against a recombinant RDRP protein identifies LRV1-4 Pol antigens in sucrose-purified virus particles. Lysates from virus-infected M4147 cells were fractionated over a 10 to 40% sucrose density gradient. Fractions 1 and 2 (numbered from the bottom of the gradient) were identified as virus-enriched by in vitro polymerase assays (Fig. 5B) and used for Western analysis. Duplicate samples were resolved by SDS-PAGE, electroblotted to a PVDF membrane, and incubated overnight with an antiserum raised against either baculovirus-expressed LRV1-4 capsid (A) or a GST-RDRP fusion protein (B) either before or after PEG concentration. Lanes contain 10 μ l of the following samples: unconcentrated gradient fraction 1 (lane 1), PEG-concentrated fraction 1 (lane 2), PEG-concentrated fraction 2 (lane 3), or gel-purified GST-RDRP fusion protein (lane 4). Arrowheads indicate the three antigens specifically recognized by an antiserum raised against a GST-RDRP fusion protein. Positions of size markers are indicated between the gels.

easily recognized the 120-kDa GST-RDRP control protein in lysates from Sf9 cells expressing the RDRP ORF (Fig. 1A, lane 4) but failed to detect any antigens in purified but unconcentrated virus (Fig. 1A, lane 1). The antiserum detects three antigens (95, 56, and 41 kDa) in PEG-concentrated virus samples; however, a predicted 180-kDa fusion capsid-polymerase fusion-size protein is not among them. The 95-kDa antigen

FIG. 2. An in vitro assay produces specific cleavage of a synthetic capsid-RDRP fusion protein. In vitro-produced (radiolabeled) LRV1-4 capsid-polymerase fusion protein (see Materials and Methods) was incubated for 40 min at 30°C with lysates from virus-infected M4147 (A) or uninfected M6244 (B, right) cells prior to examination by SDS-PAGE and autoradiography. Control incubations were in TMN buffer. In vitro-expressed luciferase served as a nonspecific cleavage substrate (B, left). Positions of size markers (lane M) (hash marks), uncleaved fusion protein (asterisk), luciferase (arrowhead), and the major 82- and 95-kDa cleavage products (arrows) are indicated.

FIG. 3. Loss of the 180-kDa fusion substrate correlates with the appearance of capsid- and RDRP-size peptides in the in vitro cleavage assay. In vitro-synthesized fusion protein was prepared and incubated with M4147 lysates as described for Fig. 1. Equal-volume aliquots were removed at the indicated time points and examined by SDS-PAGE and autoradiography as for Fig. 2. The fusion substrate was incubated for 60 min without M4147 cell extract (-) as a control. Positions of the 180-kDa cleavage substrate (asterisk) and the two major cleavage products (arrows) are indicated. Positions of size markers are shown on the left.

could be produced by translation of the RDRP ORF alone. The immunoreactive 56- and 41-kDa antigens could be produced either by internal translation initiation or by proteolytic processing of a larger RDRP antigen.

FIG. 4. Immunoprecipitation with specific antisera of in vitro cleavage reaction products identifies the 82- and 95-kDa major cleavages as the capsid and RDRP domains, respectively. The cleavage assay was performed as previously described in the absence (-) or presence (+) of M4147 lysates (left). Aliquots (20 μ l) of the cleavage reactions products were immunoprecipitated with pre-immune or capsid- or polymerase-specific antisera, and immune complexes were collected on protein A-coated Sepharose beads (see Materials and Methods). Bound antigens were eluted by boiling in sample buffer, resolved by SDS-PAGE, and visualized by autoradiography (right). Positions of the fusion-size protein (asterisk) and the two major cleavage products (arrows) are indicated. Positions of size markers are shown on the right.

FIG. 5. Cleavage activity does not copurify with virus particles isolated from infected parasites. M4147 cell lysates were fractionated over a 10 to 40% sucrose density gradient as previously described. Individual fractions (1 ml) were collected and examined for the presence of virus particles by Western blot analysis (A) or in vitro polymerase assays (B). SDS-PAGE and Western blot analysis with capsid-specific antisera were performed as described in the text. Molecular masses and positions of the capsid monomer and dimer (arrows) are indicated. In vitro RDRP assays were performed in the presence of radiolabeled nucleotide substrate (see Materials and Methods). Polymerase reaction products were resolved on a 6% polyacrylamide-7 M urea gel and visualized by autoradiography. FT, full-length transcript; ST, short transcript. The results show that virus particles are concentrated in fractions 1 to 3, derived from the bottom of the sucrose gradient. Proteolytic cleavage assays (C) were performed on individual gradient fractions, and the reaction products were examined by SDS-PAGE and autoradiography as described for Fig. 2. The position of uncleaved 180-kDa

In vitro cleavage of a synthetic LRV1-4 capsid-polymerase fusion protein. Based upon similarities to the yeast L-A virus (7) and *Giardia* virus (37), it has been predicted that LRV produces its RDRP as a Cap-Pol type of fusion protein. Consistent with this possibility, the 71-nucleotide gene overlap in LRV1-4 encodes a putative slippery translation site and a potential RNA pseudoknot structures required for ribosomal frameshifting in yeast L-A virus, the closest known relative of LRVs (7). Moreover, Lee et al. (21) have recently demonstrated synthesis of an LRV1-4 capsid-RDRP fusion protein by +1 translational frameshifting during in vitro translation of viral transcripts. It is also noteworthy in this regard that ORF3 lacks a consensus translation initiation sequence near its 5' end (17, 18).

The conflicting in vitro (21) and in vivo (Fig. 1) data are reconciled by using a model that predicts proteolytic processing of the fusion protein in vivo. We have developed an in vitro cleavage assay to test the possibility that a Cap-Pol fusion protein is subject to proteolytic processing in vivo. The assay system consists of incubating a synthetic (radiolabeled) Cap-Pol fusion protein, produced by in vitro transcription-translation using rabbit reticulocyte lysates, with extracts from uninfected or virally infected parasites. Coincubation of the fusion substrate with M4147 cell lysates resulted in loss of the 180-kDa fusion protein and the appearances of at least two distinct products absent from control incubations lacking cell lysate (Fig. 2A). A control protein (luciferase) was not cleaved in this assay system (Fig. 2B), showing that cleavage is specific. Interestingly, a similar cleavage activity exists in extracts prepared from uninfected M6244 cells.

Cleavage time course studies. Time course experiments were performed to demonstrate a correlation between the full-length fusion protein and the putative cleavage products detected in the in vitro assay system. Cleavage reactions were performed essentially as described for Fig. 2 except that equal volume aliquots were removed at the indicated time points. The results document gradual disappearance of the full-length fusion protein and concomitant appearance of the two smaller products (Fig. 3). The cleavage reaction products are similar in size to those predicted from translation of the individual capsid (82 kDa) and polymerase (95 kDa) genes, suggesting that cleavage may occur at or near the junction of viral capsid and polymerase domains to yield the individual gene products.

Immunoprecipitation of in vitro-produced cleavage products. To identify the in vitro cleavage products as representing the individual capsid and polymerase gene products, an immunoprecipitation analysis was performed with specific antisera (Fig. 4). The RDRP-specific antiserum recognized the 95-kDa cleavage product but not the 82-kDa product. Thus, the 95-kDa peptide derives from the RDRP domain of the fusion protein, while the 82-kDa peptide lacks recognizable RDRP determinants. Conversely, the 82-kDa peptide was recognized by a capsid-specific antiserum, showing that it originates from the capsid domain of the fusion protein. As expected, both antisera recognized the 180-kDa fusion protein. Somewhat unexpectedly, an antiserum raised against the translation product of the capsid gene reacted specifically, albeit very weakly, with the 95-kDa cleavage product, suggesting either the presence of capsid sequences or the existence of shared epitopes.

fusion substrate (asterisk) and the two major cleavage products (arrows) are indicated. A control digest was performed in the absence of cell lysate (-). The results show that cleavage activity is concentrated in fractions 8 to 11 and apparently absent from virus-containing fractions.

FIG. 6. Cysteine protease inhibitors specifically block cleavage of the fusion protein in the in vitro assay system. A panel of protease inhibitors was examined for cleavage-blocking activity in the in vitro cleavage assay. Protease inhibitor stock solutions were prepared in distilled deionized H_2O at 5 mg/ml except for EDTA (10 mg/ml), Pefabloc SC (20 mg/ml), and soybean trypsin inhibitor (50 mg/ml). Cleavage assays were performed essentially as before except that the M4147 cell lysate (Fig. 5, fraction 9) was preincubated with the indicated protease inhibitor for 20 min at room temperature prior to addition of the cleavage substrate. Control incubations were performed in the absence of extract or inhibitor (—), as indicated. The position of the uncleaved fusion substrate (asterisk) and the two major cleavage products (arrows) are indicated. Positions of ^{14}C -labeled molecular mass markers (lane M; Amersham) are indicated on the right. The results show that inhibitors of cysteine-like proteases specifically block cleavage of the fusion protein.

Proteolytic cleavage activity does not copurify with assembled virus particles isolated from virus-infected parasites. Virus-infected M4147 cells were fractionated by sucrose gradient centrifugation as described above. Individual gradient fractions were characterized for the presence of virus particles by SDS-PAGE and Western blot analysis with capsid-specific antisera (Fig. 5A) or in vitro RDRP assays using a radiolabeled nucleotide substrate (Fig. 5B). The results show that virus particles are concentrated in high-density sucrose fractions. Also, in RDRP assays, a short transcript, a cleavage product from a full-length transcript by the assembled capsid proteins described (22), was generated as a reaction product, showing that the virus particles are intact. Viral antigens and biological activities were absent from low-density sucrose fractions recovered from the top of the gradient. The 42-kDa antigen detected in low-density fractions is also present in uninfected M6244 cells (data not shown) and is therefore a nonviral protein. In contrast to the distribution of virus in high-density gradient fractions, detectable cleavage activity localized in low-density fractions. The finding that cleavage activity is not associated with virus particles in infected parasites is consistent with data documenting cleavage activity in uninfected M6244 cells.

A host cysteine-like protease is responsible for in vitro cleavage of the synthetic Cap-Pol fusion protein. A series of commercially available protease inhibitors were titrated into the cleavage assay reaction mixtures and tested for the ability to inhibit cleavage of the Cap-Pol fusion protein in vitro. For these studies, 40 μ g of cell lysate (Fig. 5C, gradient fraction 9) was preincubated with the indicated protease inhibitor prior to

addition of the Cap-Pol fusion protein substrate. The results (Fig. 6) show that the metalloprotease inhibitors EDTA and phosphoramidon failed to inhibit in vitro cleavage of the fusion protein, as did the serine protease inhibitors aprotinin and Pefabloc SC. In contrast, antipain dihydrochloride, leupeptin, and soybean trypsin inhibitor prevented cleavage in this assay system. We could not test the effect of 1,10-phenanthroline or phenylmethylsulfonyl fluoride on the cleavage activity because ethanol or isopropanol, respectively, used to dissolve the inhibitor interfered the cleavage assay reaction (data not shown). In another set of experiments, lysates were preincubated with serial 10-fold dilutions of selected inhibitors before being added to the cleavage assay reaction mixture (Fig. 7). The results show that antipain dihydrochloride completely inhibited the cleavage activity at 5 μ g/ml (final concentration). Leupeptin, an inhibitor of both cysteine and serine-type proteases, also abolished the cleavage activity at concentrations of less than 1 μ g/ml. Soybean trypsin inhibitor slightly inhibited the cleavage activity at a high concentration (5 mg/ml). In contrast, aprotinin did not prevent cleavage even at a concentration (0.5 mg/ml) 250-fold higher than that recommended by the manufacturer (Boehringer Mannheim). In subsequent studies, the reducing agents dithiothreitol and iodoacetamide were tested for activity in the cleavage assay. Further evidence of the involvement of cysteine residues in cleavage was obtained by showing that a low concentration of dithiothreitol (1 to 2 mM) enhanced cleavage activity whereas covalent modification by iodoacetic acid (2 mM) strongly inhibited cleavage activity (data not shown).

DISCUSSION

Existence of a capsid-polymerase fusion protein produced via -1 translational frameshifting has been reported for other members of the *Totiviridae* family of dsRNA viruses, including the L-A virus of *Saccharomyces cerevisiae* (7) and *Giardia* virus (37). Both viruses encode overlapping capsid and polymerase genes which contain a slippery translation sequence and a predicted RNA pseudoknot. In contrast, the totivirus *Helminthosporium victoriae* 190S virus (Hv190S), which lacks known structural features required for translational frameshifting, instead expresses its polymerase gene by internal translation initiation (13).

LRV is similar in genomic organization to yeast L-A virus and *Giardia* virus (7, 10, 37). In LRV1-1 and LRV1-4, the 71-nucleotide overlapping region between ORF2 (capsid) and ORF3 (polymerase) encodes both a slippery translation site and a potential RNA pseudoknot similar to those described for L-A virus. However, like many retroviruses (14, 15), LRV1 require a $+1$ translational frameshift to synthesize a Cap-Pol fusion protein. Early studies using antisera directed either against purified viral particles (5) or baculovirus-expressed capsid protein (4) detected an 180-kDa fusion-size antigen associated with sucrose-purified virus particles, but subsequent studies identified an immunologically reactive 180-kDa form of the LRV capsid antigen, presumed to be a capsid dimer, in Sf9 cells expressing the LRV capsid gene only. It was not clear from these studies whether a Cap-Pol fusion protein exists in vivo.

Here we addressed the existence of a Cap-Pol fusion protein in LRV by using an antiserum raised against baculovirus-expressed LRV1-4 RDRP protein. The antiserum failed to recognize a Cap-Pol fusion-size antigen in concentrated LRV1-4 particles. The result confirms previous finding (4) that the 180-kDa protein detected by capsid-specific antisera is derived from the capsid gene and extends those studies by showing the

FIG. 7. Inhibitor titration studies. Cleavage assays were performed essentially as described for Fig. 6 except that cell lysates were preincubated with a 10-fold dilution series of the indicated inhibitor. Final inhibitor concentrations are shown. Arrows indicate positions of the uncleaved fusion substrate and the two major cleavage products. The results show that antipain dihydrochloride, leupeptin, and soybean trypsin inhibitor, which inhibit cysteine-like proteases, prevent cleavage of the fusion protein at low concentrations. Aprotinin, a serine protease inhibitor, failed to prevent cleavage at the highest concentration examined (250-fold greater than the concentration recommended by the manufacturer).

absence of polymerase epitopes in a fusion-size antigen. Moreover, the present studies document the existence of a 95-kDa RDRP antigen, the size predicted for expression of the RDRP gene alone, as well as two smaller RDRP-related proteins (56 and 41 kDa). These immunological studies raise doubts regarding synthesis of a Cap-Pol fusion protein by LRV and are seemingly consistent with a notion that the LRV RDRP gene is expressed independent of the capsid gene, possibly via internal translation initiation as shown for Hv190S (13). However, LRV is more closely related to the yeast dsRNA virus L-A (7) and *Giardia* virus (37), both of which express their RDRP genes as a Cap-Pol fusion protein via translational frameshifting. Like these two closely related viruses, LRV encodes both a potential RNA pseudoknot and a slippery translation site within the overlapping capsid and RDRP gene sequences. These elements are essential to translational frameshifting in L-A virus. In addition, frameshifting has been shown to occur during *in vitro* translation of LRV1-4 transcripts on eukaryotic ribosomes (21). Synthesis of the viral polymerase as a gene fusion product is also consistent with

the notable absence of a traditional translation initiation consensus sequence near the 5' end of the RDRP gene (17, 18).

The considerable indirect evidence to support a fusion protein by LRV prompted us to explore a possibility that such a fusion product is proteolytically processed *in vivo*, thereby obscuring its detection in these and earlier studies. Consistent with this possibility, we have shown that a synthetic 180-kDa LRV Cap-Pol fusion protein is processed to yield two major cleavage products in an *in vitro* cleavage assay system. The sizes of the two cleavage products (82 and 95 kDa) are identical with those of the capsid and RDRP antigens detected *in vivo*. Immunoprecipitation identified the *in vitro*-produced peptides as derived from the capsid and polymerase domains of the fusion protein. Thus, a protease-sensitive site exists at the junction of the two functional domains in an LRV Cap-Pol fusion protein. Weak recognition of the 95-kDa RDRP peptide by an antisera raised against the virus capsid protein is consistent with the results of Western blot analysis (Fig. 1B, lane 4) showing that these same antisera weakly recognized GST-

RDRP protein expressed in Sf9 cells and imply the existence of shared epitopes.

Several lines of evidence support the view that the proteolytic activity defined in the in vitro cleavage assay is host rather than virus encoded. Foremost, a similar activity is present in the uninfected parasite line M6244. Second, cleavage assays using sucrose gradient fractions (Fig. 5C) clearly show that the detected proteolytic activity does not copurify with virus particles in virus-infected cells. The possibility that the protease activity dissociated from the virus during preparation is inconsistent with the results of in vitro polymerase assays demonstrating that the virus particles recovered from high-density gradient fractions are intact. There was no evidence of auto-proteolytic activity in these studies, and sequence analysis (29) has failed to identify any homology to known proteases. The simplest interpretation of these data is that the viral fusion protein is processed by a host-encoded protease present in virus-infected cells; however, it remains possible that the crude cell fractions used in the present studies contribute an essential cofactor to a virally encoded protease.

There are numerous reports that proteolytic processing of viral proteins is important to the life cycles of many viruses (see references 8 and 19 for reviews). Autoprocessing of a viral Gag-Pol polyprotein is essential in the life cycles of RNA viruses and retroviruses (16, 32), where specific cleavages are required to release the structural and functional proteins necessary for viral maturation. In these cases, the cleavages are mediated by virally encoded proteinases. Conversely, maturation of the glycoprotein components of many myxovirus and coronaviruses is mediated by cellular proteases (see reference 19 for a review). Another example of host proteases involved in virus maturation comes from human immunodeficiency virus type 1, where a cellular protease cleaves the envelope glycoprotein precursor (gp160) into the external glycoprotein gp120 and the transmembrane glycoprotein gp41 components necessary for activation of the virus (9, 24). Also, the E2 glycoprotein of Sindbis virus is cleaved from its precursor, PE2, by a cellular protease at a late stage of virus maturation (11).

Here we show that leupeptin, antipain dihydrochloride, and, to a lesser extent, soybean trypsin inhibitor specifically block cleavage of the fusion protein in in vitro assays, suggesting the involvement of a host cysteine protease in the cleavage reaction. Cysteine proteases exist in a variety of organisms (1, 31) and have been shown to play critical roles in the pathogenesis of some parasitic protozoal infections (see reference 23 for a review). Cysteine proteases have been identified in all species of *Leishmania* studied thus far, including *L. mexicana*, *L. pifanoi*, *L. amazonensis*, *L. donovani* (2), and *L. major* (28). Two of the cysteine proteases purified from *L. maxicana* amastigotes are homologous to the mammalian cathepsin B-like cysteine proteases (27). *L. major* also has at least two cysteine proteases; one is structurally similar to the cathepsin L family, and another is similar to the cathepsin B family of cysteine proteases (28).

While cysteine proteases play critical roles in the pathogenesis of parasitic protozoal infections (23), we do not yet know if the cysteine protease characterized in the present studies is involved in maturation of LRV in vivo. It is noteworthy that a host-encoded cysteine protease is apparently involved in the posttranslational processing and maturation of the *Giardia* virus capsid (39). In the totivirus Hv190S, the nonphosphorylated capsid component, p78, is derived from its precursor, p88, via C-terminal proteolytic cleavage (33).

In summary, we have for the first time identified polymerase antigens in LRV. A major 95-kDa RDRP antigen is consistent with the size predicted for expression of the pol domain alone.

On the surface, the findings here appear to conflict with the results of other studies demonstrating production of an LRV Cap-Pol fusion protein in vitro. However, we have clearly documented both the existence of a protease-sensitive site at or near the junction of the capsid and polymerase domains of an LRV fusion protein as well as a host-encoded cysteine-like protease which specifically cleaves at that site in an in vitro assay system. Preliminary studies aimed at mapping the cleavage site (6) localize it to a sequence 32 amino acids upstream from the first methionine codon in ORF3 (29). Site-directed mutagenesis studies are under way to map the site precisely. A possibility that proteolytic cleavage is important to virus assembly or maturation will be addressed via mutational analysis using bacterial or Sf9 cell expression systems.

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