Synthesis of Viruslike Particles by Expression of the Putative Capsid Protein of *Leishmania* RNA Virus in a Recombinant Baculovirus Expression System

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The putative capsid open reading frame (ORF2) of the *Leishmania* RNA virus LRV1-4 was expressed in a baculovirus expression system. The expressed protein was identified by Western immunoblot analysis with polyclonal antiserum raised to purified LRV1-4 virus. Electron microscopy and sedimentation analysis indicated that the expressed protein self-assembles into empty viruslike particles of similar size and shape to authentic virus particles, thus confirming that ORF2 encodes the viral capsid. The expressed particles are present exclusively in the cytoplasm of infected SF9 cells and are able to assemble in the absence of LRV1-4 RNA, viral polymerase, or any *Leishmania* host factors.

The genus *Leishmania*, a protozoan parasite, is the causative agent of leishmaniasis. The presence of viruses in protozoan parasites was first described in 1960 (18). Early studies, focusing mainly on amoebas, were primarily detailed electron-microscopic descriptions of virus-like particles and their effect on cell morphology and survival (7). It was not until 1988 that viruses were described in *Leishmania* cells (25). Recent studies have focused on the molecular characterization of these viruses. Much of the motivation for these studies came from the need for a gene transfer system, which, in these organisms, has only recently become available (2, 15). Our principal incentive for identifying and characterizing new viral species is an acknowledgment of the extraordinary role that bacteriophages and viruses have played in our understanding of many unique prokaryotic and eukaryotic molecular mechanisms (19).

Several *Leishmania* strains have been shown to harbor double-stranded RNA (dsRNA) viruses. Viruses have been identified in 12 strains of *Leishmania braziliensis* or *L. braziliensis guyanensis* (10) and are now designated as the genus *Leishmania* RNA virus 1 (LRV1). These viruses are classified as a member of the *Totiviridae* family of viruses (20); other members of the *Totiviridae* include yeast L-A virus and Giardavirus. Infection of *Leishmania* cells by LRV1 is believed to be persistent since no extracellular virus has been observed and since, although electroporation of whole virus achieved a transient infection (1), attempts to stably infect other *Leishmania* strains have failed. The virus appears to be regulated and maintained at a low copy number within the infected cells (5, 6).

The sequence of cDNA copies of viral genomic RNA (22, 23) reveals a coding capacity of at least two proteins. Open reading frame 2 (ORF2) could encode an 82-kDa protein, and ORF3 could encode a 98-kDa protein. The predicted amino acid sequence of ORF3 has motifs characteristic of viral RNA-dependent RNA polymerases (3) and is therefore predicted to encode the viral polymerase. This ORF overlaps the end of ORF2 by 71 nucleotides, suggesting a +1 translational

frameshift to produce a Gag-Pol-type fusion protein (23), as is seen in both yeast L-A virus (8, 11) and Giardavirus (28). ORF2 has been shown by *in vitro* translation (4) to encode a protein of approximately 80 kDa, which is consistent with the size predicted from sequence information (22, 23). ORF2 is presumed, merely by a process of elimination, to encode the major viral coat protein, since there is no sequence homology with any known nucleocapsid protein. Studies of the viral proteins have been greatly inhibited by the low levels of virus within each cell and the relative difficulty of growing *L. braziliensis guyanensis* in culture.

Recently polyclonal antiserum was raised to the peak viral fraction of a sucrose gradient from LRV1-4-infected cells and used in a Western immunoblot analysis to identify viral proteins from various isolates. Consistent with this result, *in vitro* translated protein from cloned RNA corresponding to ORF2 was immunoprecipitated with the same antiserum. The putative capsid at times appeared as a doublet; relative amounts of the two species varied based on method of purification (4).

In this study, we express ORF2 in a baculovirus expression system and show that when expressed it self-assembles into empty viruslike particles, demonstrating that this gene product is indeed the capsid protein.

MATERIALS AND METHODS

Cloning of ORF2 and construction of the transfer vector. Double-stranded RNA from 10⁸ LRV1-4-infected cells (MHOM/BR/75/M4147) was extracted from a 0.7% ethidium bromide-stained agarose gel. The RNA was denatured at 95°C for 5 min and reverse transcribed with a primer 300 bases downstream of the end of ORF2 and Moloney murine leukemia virus reverse transcriptase as specified by the manufacturer (GIBCO-BRL). ORF2 was then amplified by the polymerase chain reaction with a primer at the exact beginning of ORF2 and one 30 bases downstream of the end of ORF2. The primers used for amplification were designed with *Nhe*I restriction enzyme recognition sites on the ends. The amplified fragments were digested with *Nhe*I and ligated into the unique *Nhe*I site of pBluBac (*p*-β-L1), a β-galactosidase-coexpressing vector (Invitrogen). Because of poor expression of capsid protein from recombinant baculovirus when using this transfer

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vector, the ORF2 insert was subcloned into the *Xba*I restriction site of transfer vector pVL1392 (Invitrogen).

Construction of recombinant baculovirus. The generated transfer vector pTCB2 (4 μ g), was transfected into *Spodoptera frugiperda* cells (SF9), along with 1 μ g of linear baculovirus (AcMNPV) DNA, by using cationic liposomes as specified by the manufacturer (Linear AcNPV DNA Transfection Module; Invitrogen). Recombinant plaques were identified visually by the absence of occlusion bodies and purified by three rounds of plaque purification. Expression of recombinant protein was assessed after each round of purification by cross-reaction in Western blot analysis to antiserum generated to LRV1-4 purified from M4147 cells. The plaque-purified recombinant baculovirus was amplified by infection of a monolayer of 5×10^6 SF9 cells on a 100-mm tissue culture dish for 4 days followed by infection of a 100-ml spinner culture of 1.5×10^6 SF9 cells per ml with the supernatant from one 100-mm infected dish. The supernatant of the spinner culture was collected at 4 days postinfection and assayed for viral titer by limiting-dilution assay (24). This viral stock was used for further analysis.

Western blot analysis and Coomassie blue staining of proteins. Protein samples (10 μ l) were mixed with an equal volume of $2 \times$ Laemmli sample buffer, denatured at 95°C for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with either 8% polyacrylamide for Western analysis or 10% polyacrylamide for Coomassie blue staining. Coomassie blue staining of gels was performed by soaking the gels in a 0.25% solution of Coomassie brilliant blue R250 in 45% methanol–10% glacial acetic acid for 2 h and destaining in 45% methanol–10% glacial acetic acid for several hours. Western analysis was performed as described previously (4) with a 1/1,000 dilution of precleared anti-LRV1-4 antiserum.

Purification of virus and synthetic viruslike particles. Virus particles were purified from 1 liter of late-log-phase LRV1-4-infected (MHOM/BR/75/M4147) cells by lysing the cells with Triton X-100 (4, 14) and passing them over a 10 to 40% sucrose gradient as previously described (29). Synthetic viruslike particles were generated by infecting a 100-mm tissue culture dish containing 3×10^6 SF9 cells with recombinant baculovirus at a multiplicity of infection of 10. Other 100-mm plates were infected with wild-type baculovirus or mock infected as controls. The infected cells were harvested at day 4 by scraping the cells from the plate in 0.5 ml of lysis solution (50 mM Trizma base [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40) and analyzed by Coomassie blue staining and Western analysis or loaded onto an 11-ml, 10 to 40% sucrose gradient and centrifuged at 36,000 rpm in a Beckman SW41 rotor for 135 min at 4°C. Sucrose gradients with authentic or synthetic particles were collected in 1-ml fractions and numbered from the bottom of the gradient. The pellet of each gradient was resuspended in 1 ml of gradient buffer (HCN, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM CaCl₂, 150 mM NaCl). A 100-ml spinner culture of SF9 cells were also infected with recombinant baculovirus at a multiplicity of infection of 10. These cells were harvested at day 4, washed with phosphate-buffered saline (PBS), and lysed in 1 ml of lysis solution (above). The lysate was loaded onto an 11-ml 30% CsCl density gradient and centrifuged at 39,000 rpm in a Beckman SW41 rotor for 18 h at 4°C. Fractions were collected as above, and the majority of expressed protein was observed in two large visible bands.

Generation of antiserum to recombinant capsid. CsCl density gradient-purified synthetic LRV1-4 viruslike particles from recombinant baculovirus-infected SF9 cells were used to gen-

erate antiserum. A 1-ml CsCl gradient fraction containing the majority of synthetic particles from a 100-ml spinner culture of infected SF9 cells was dialyzed against PBS (pH 7.4), mixed with an equal volume of Freund's complete adjuvant, and injected intradermally into the back of a female New Zealand White rabbit. Subsequent injections were made with antigen prepared in a similar manner, mixed with an equal volume of Freund's incomplete adjuvant, and injected intramuscularly. Test bleeds were taken at 10 days after each antigen boost. Blood was taken from the central artery of the ear and allowed to clot overnight at 4°C, after which the serum was removed for testing. Western analysis was performed on the second test bleed sample by using the same protocol outlined above.

Electron microscopy. Cell section samples were prepared from spinner cultures at 48 and 72 h postinfection. Cell pellets were washed in PBS and fixed in 3% glutaraldehyde–85 mM sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Samples were stained with 1% osmium tetroxide, dehydrated with ethanol, and embedded in Epon 812 resin by following the standard sample preparation protocol. Sucrose gradient-purified particles were diluted, carbon lifted onto electron microscopy grids, and stained with 1% uranyl formate. Negative staining was done by a miniaturization of the method of Valentine and Green (27).

RESULTS

Expression of ORF2 in *S. frugiperda* cells. The construction of the transfer vector is summarized in Fig. 1. The transfer vector was used to insert ORF2 into the baculovirus genome by homologous recombination, placing ORF2 under control of the baculovirus polyhedrin promoter. The ability of recombinant baculovirus to direct the synthesis of the putative viral coat protein of LRV1-4 was tested in SF9 cells. By Coomassie blue staining of proteins from cell lysates, we observed that recombinant-infected but not mock- or wild-type-infected cells produced a protein (Fig. 2A) of approximately 80 kDa, the size predicted to be generated by ORF2 from in vitro translation studies (4). Confirmation of the viral origin of the protein came from Western analysis on cell lysates by using antiserum generated to purified virus (4). The antiserum recognizes the 80-kDa putative capsid protein from LRV1-4-infected *Leishmania* cell extracts and purified LRV1-4 particles (Fig. 2B, lanes 6 and 7). This antiserum cross-reacted strongly with the 80 kDa protein in recombinant baculovirus-infected SF9 cells but did not cross-react with any protein in mock-infected or wild-type AcMNPV-infected cells (Fig. 2B, lanes 2, 3, and 4).

Sedimentation of the expressed protein. Since many capsid proteins self-assemble, to some degree, when expressed in baculoviruses (9, 12, 13, 16, 17), we believed that assembly into empty particles would be convincing evidence of the function of ORF2 protein. A preliminary test of assembly was comparative sedimentation analysis of expressed protein and authentic particles. We assessed the sedimentation of expressed protein in velocity and density gradients. High levels of expressed protein were obtained by infection of a monolayer (3×10^6) of SF9 cells in 100-mm dishes at a multiplicity of infection of 10. These infected cells were harvested at day 4, loaded onto a 10 to 40% sucrose velocity gradient, centrifuged, and fractionated. The fractions were analyzed by Coomassie blue staining and Western blot analysis. The baculovirus-expressed protein sedimented at a slightly lower rate than that of authentic particles (Fig. 3). Interestingly, a protein of approximately 180 kDa, previously reported in authentic particles (4), also appears to cosediment with recombinant expressed particles.

FIG. 1. Cloning of ORF2 and construction of the baculovirus expression transfer vector. Abbreviations: P_{PH}, polyhedrin promoter; MCS, multiple cloning site; ColE1, plasmid origin of replication; Amp, ampicillin resistance gene.

The amount of protein produced from 3×10^6 SF9 cells was far greater than the amount of authentic capsid produced in 1×10^{10} *Leishmania* cells, since the authentic capsid is produced in amounts below the limit of sensitivity of conventional protein stains (4). On the basis of the sequence information, the virus coat is presumed to be composed primarily of this single protein. It appears from Coomassie blue staining of the proteins from the gradients that the majority of the recombinant protein is assembled into empty capsid.

Antigenicity of the recombinant capsid. Synthetic particles generated from the recombinant baculovirus were purified by CsCl density gradient centrifugation and used to generate antiserum in a New Zealand White rabbit. This antiserum was used for enzyme-linked immunosorbent assay and Western analysis to confirm the antigenicity of the expressed protein. Western analysis (Fig. 4) indicated that authentic particles and synthetic particles generated a similar antigenic response, since

FIG. 2. Expression of ORF2 in SF9 cells. Western blot analysis (B) and Coomassie blue staining (A) of proteins from lysates of 3×10^6 SF9 cells in 100-mm tissue culture dishes mock-infected (lanes 2) or infected with wild-type AcMNPV (lanes 3) or recombinant AcMNPV expressing LRV1-4 ORF2 (lanes 4) at a multiplicity of infection of 10 and harvested at day 4 post infection and of cell extracts from 1×10^8 uninfected *L. braziliensis guyanensis* (MTAM/BR/80/M6244) (lanes 5) and LRV1-4-infected *L. braziliensis guyanensis* (MHOM/BR/75/M4147) (lanes 6) and 1% of the peak fraction of sucrose-purified virus particles from 1×10^{10} M4147 cells (lanes 7). Lanes 1 contain protein molecular mass markers; sizes are indicated in kilodaltons.

FIG. 3. Sedimentation of baculovirus-expressed capsid protein supports the hypothesis of self-assembly. Western blot analysis across fractions from a 10 to 40% sucrose gradient of LRV1-4-infected *Leishmania* cells (A), wild-type AcMNPV-infected SF9 cells (B), and LRV1-4 ORF2-expressing recombinant AcMNPV-infected SF9 cells (C), and Coomassie blue staining of proteins across sucrose gradient fractions of recombinant baculovirus infected SF9 cells (D). Gradient fractions are numbered 1 through 11 from the bottom of the gradient, and the same fractions are shown left to right. P, pellet of the gradient. Molecular masses are indicated in kilodaltons.

the same proteins were recognized by both antisera. It should also be observed that a faint, slightly more slowly migrating species, from the baculovirus-expressed particles, is recognized by the antisera. This could be due to the inclusion of the frameshift region in the expression vector.

Electron microscopy. Electron micrographs of recombinant-infected *S. frugiperda* cells show many small spherical particles in the cytoplasm of the cell at 48 h postinfection (Fig. 5B). By 72 h postinfection these spherical particles nearly fill the cytoplasm of recombinant-infected cells (Fig. 5A). These particles do not appear in wild-type AcMNPV-infected or mock-infected SF9 cells (data not shown) and appear only in the cytoplasm of infected cells (Fig. 5B). This is not unexpected since LRV1-4 exists only in the cytoplasm of infected *Leishmania* cells (25). The recombinant particles purified from sucrose gradients (above) were analyzed by electron micros-

copy (Fig. 6A). The synthetic particles appear almost identical to authentic particles (Fig. 6B). They are the same size and shape but slightly less rigid in appearance. This is not surprising considering the lack of nucleic acids within the particle. Both synthetic and authentic particles were approximately 38 nm in diameter. Purification of the synthetic particles on a CsCl density gradient yielded two visible bands. Western analysis and Coomassie blue staining of proteins from the gradient indicate that the majority of recombinant capsid protein was in the top visible band, with most of the remaining protein in the lower visible band. Electron micrographs of the less dense visible band show that it is composed of free synthetic particles, whereas the lower band contains fewer particles, which appear to be enmeshed in random nucleic acids (data not shown). No visible difference between sucrose- and CsCl-purified particles was observed at this level.

DISCUSSION

Because of the lack of sequence homology to any other known protein, the role of the ORF2 protein was assumed, by a process of elimination, to be that of a capsid. This study confirms the identity of ORF2 protein as the major capsid protein. The protein is expressed in sufficient amounts to enable crystallographic studies as well as other studies that have not been possible because of the small amounts of viral proteins detected in the host. The viral proteins are of low abundance as a result of either instability or low viral copy number.

Since ORF3 has significant sequence homology to other RNA-dependent RNA polymerases, it is presumed to encode the polymerase. Because of the sequence and predicted secondary structure of the ORF2-ORF3 overlap region, ORF3 is predicted to be expressed as a fusion to the capsid protein, since is observed in other members of the *Totiviridae* (11, 28).

FIG. 4. Antigenicity of expressed capsid. Antiserum raised to synthetic particles recognizes viral proteins. Authentic (lanes 2 and 4) and synthetic (lanes 3 and 5) purified particles were electrophoresed on an SDS-PAGE gel (9% polyacrylamide), and Western analysis was performed with antiserum generated to authentic (lanes 1 through 3) or synthetic (lanes 4 and 5) particles. Protein molecular mass markers are shown in lane 1; sizes are indicated in kilodaltons.

FIG. 5. Electron micrographs of recombinant baculovirus-infected cells and purified particles. (A) Cell section of an *S. frugiperda* cell infected with the recombinant virus at 72 h postinfection. Magnification, $\times 14,000$. (B) Viruslike particles in the cytoplasm of the infected cell at 48 h postinfection. Magnification, $\times 70,000$. Arrows point to some recombinant viruslike particles; the nuclear membrane is indicated by N, and wild-type baculovirus particle is indicated by B.

FIG. 6. Electron micrographs of gradient-purified authentic LRV1-4 particles and baculovirus-expressed particles. (A) Authentic particles isolated from M4147 cell extracts. (B) Synthetic particles purified from recombinant baculovirus-infected SF9 cells, as outlined in Materials and Methods. Magnification, $\times 115,000$.

Initial Western analysis (4) indicated a protein of approximately 180 kDa, which was presumed to be the capsid polymerase fusion protein. However, Western analysis of baculovirus-expressed capsid protein also yielded a protein of approximately 180 kDa. This protein appeared to cosediment with the synthetic particles on sucrose gradients (Fig. 3). The appearance and particle cosedimentation of an immuno-cross-reactive protein of 180 kDa from the expressed protein suggests that this 180-kDa protein is an oligomer of capsid protein that was not dissociated under the gel conditions. Although it is very likely that the polymerase is expressed as a fusion protein, it is not clear that this fusion protein has been identified. It also remains possible that the polymerase is translated as a fusion with capsid but is proteolytically processed, making it more difficult to detect by Western analysis. Preliminary data suggest that the NH₂ terminus of the baculovirus-expressed protein is blocked. This may prove relevant since the capsid protein of yeast L-A virus is blocked by acetylation at the amino terminus. This acetylation appears to be required for assembly of the yeast L-A viral capsid (26).

Interestingly, the baculovirus-expressed capsid protein appears as a doublet under some conditions (data not shown), which had been previously observed to occur with the authentic virus (4). In the previous study it was unclear whether the faster-migrating species of the doublet was a product of the capsid protein alone or of the capsid polymerase fusion protein, since a decrease in the amount of the 180-kDa protein corresponded to an increase in the amount of the faster-migrating capsid species and since *in vitro* translation of ORF2 did not yield the same faster-migrating species. This study supports the notion that the faster-migrating species is a product of the capsid protein alone, since the polymerase gene was not expressed; however, it does not rule out the possibility that the faster-migrating species is a product of the frameshift region, presumed to exist between the capsid and polymerase ORFs, since this region was included in the expressed fragment.

We have shown that viruslike particles can be produced from a recombinant baculovirus-expressing LRV1-4 ORF2 protein. Assembly of these particles occurs very efficiently and in the absence of any cofactor such as LRV1-4 RNA or polymerase protein. Previous reports of the native particle have shown a preponderance of empty particles (25, 30), indicating the ability to assemble without encapsidating RNA. To our knowledge, there is no previous example of the use of a baculovirus expression system in the synthesis of protozoan viral or *Leishmania* proteins. However, self-assembly of viral particles from expressed capsid proteins has been shown to occur in many different systems. Roy and colleagues have reported that expression of several combinations of capsid proteins of bluetongue virus, a mammalian dsRNA virus, resulted in self-assembly of viruslike particles of various morphologies (9, 16, 17) within recombinant baculovirus-infected SF9 cells. Another mammalian dsRNA virus, Norwalk virus, has also been shown to self-assemble into viruslike particles within infected SF9 cells when expressed in a recombinant baculovirus system (13). Potyvirus, a single-stranded RNA (ssRNA) virus, has been shown to self-assemble into viruslike particles on expression in either a yeast or *Escherichia coli* cell-free expression system (12). Finally, adeno-associated virus, a ssDNA human parvovirus, has been shown to self-assemble viruslike particles within infected SF9 cells when expressed in a recombinant baculovirus expression system (21).

Finally, it has been observed by hybridization analysis of fractionated *Leishmania* cells that the viral RNA exists entirely in the cytoplasm of infected cells (25). Our report supports this

finding since assembled baculovirus-expressed particles were observed only in the cytoplasm of infected SF9 cells. Since the particles assemble only in the cytoplasm of insect as well as *Leishmania* cells, it is possible that *Leishmania* and insect cells share similar subcellular localization signals or pathways or both. Since little is known about the subcellular localization system in *Leishmania* cells, it may be possible to apply what is known from insect and other higher eukaryotes to elucidate the *Leishmania* pathways.

ACKNOWLEDGMENTS

We gratefully acknowledge expert technical assistance provided by Dierdre Furlong and Bonnie Meek for preparation of the electron micrographs, Margaret Lee for advice and assistance in baculovirus expression, ARCH (Animal Resources, Children's Hospital) for preparation of the antiserum to expressed capsid, and In Chung, Tim Armstrong, Marie Keenan, and Scott Scheffter for helpful discussions. This study was supported by grant AI-28473 from NIH.

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