The Vaccinia Virus G1L Putative Metalloproteinase Is Essential for Viral Replication In Vivo

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The function of the putative metalloproteinase encoded by the vaccinia virus GIL gene is unknown. To address this question, we have generated a vaccinia virus strain in which expression of the GIL gene is dependent on the addition of tetracycline (TET) when infection proceeds in a cell line expressing the tetracycline repressor. The vv tetOG1L virus replicated similarly to wild-type Western Reserve (WR) virus in these cells when TET was present but was arrested at a late stage in viral maturation in the absence of TET. This arrest resulted in the accumulation of 98.5% round immature virus particles compared to 6.9% at a similar time point when TET was present. Likewise, the titer of infectious virus progeny decreased by 98.9% ± 0.97% when the vv tetOG1L virus was propagated in the absence of TET. Mutant virus replication was partially rescued by plasmid-encoded GIL, but not by GIL containing an HXXEH motif mutated to RXXQR. Modeling of GIL revealed a predicted structural similarity to the α-subunit of Saccharomyces cerevisiae mitochondrial processing peptidase (α-MPP). The HXXEH motif of GIL perfectly overlaps the HXXDR motif of α-MPP in this model. These results demonstrate that GIL is essential for virus maturation and suggest that GIL is a metalloproteinase with structural homology to α-MPP. However, no obvious effects of the expression and processing of the vaccinia virus major core proteins were observed in the GIL conditional mutant in the absence of TET compared to results for the TET and wild-type WR controls, suggesting that GIL activity is required after this step in viral morphogenesis.

Proteolytic cleavage of precursor proteins to produce functional protein products is a central theme in most biological systems. To that end, it is now well recognized that many viruses encode one or more proteases that catalyze the unique formative and morphogenic proteolytic reactions required for the production of infectious progeny. Therefore, it was of interest to note that poxviruses, such as vaccinia virus, are predicted to encode at least two candidate proteinases: I7L and GIL (22, 32).

The first candidate proteinase is encoded by the I7L open reading frame (ORF). This ORF was identified on the basis of homology to the African swine fever virus proteinase and a ubiquitin-like proteinase in Saccharomyces cerevisiae (2). It was predicted to encode a cysteine proteinase and is highly conserved among the orthopoxviruses (3). The 47-kDa I7L protein is expressed at late times postinfection. Use of monospecific anti-I7L antisera has demonstrated that the protein is associated with virus factories, immature viral particles, and intracellular mature virus (IMV), where it is exclusively located in the core (22). Interestingly, Condit and coworkers have isolated a conditional-lethal mutation in the I7L gene (6). At the nonpermissive temperature, the core protein precursors P4a, P4b, and P25K are synthesized but are not processed. Moreover, viral assembly is halted between immature viral particle formation and conversion to an infectious IMV particle (9). At the nonpermissive temperature, no infectious progeny are produced. Recently, we have used a trans-processing assay to demonstrate that the I7L cysteine proteinase is responsible for the major viral core protein processing reactions and identified the essential catalytic residues of the enzyme (3, 4).

The second candidate protease is the putative metalloproteinase encoded by the GIL ORF, which contains an HXXEH sequence motif, a direct inversion of the active site consensus sequence present in metalloproteinases such as thermolysin (32). GIL is predicted to encode a 68-kDa late protein that is also highly conserved among pathogenic orthopoxviruses. Previously, a transcriptionally controlled trans-processing assay using a reporter plasmid expressing P25K-FLAG was employed to demonstrate that GIL activity was required to cleave a cryptic AG+S site within the precursor protein (32). However, given the recent discovery that the I7L cysteine proteinase appears to catalyze most, if not all, of the core protein maturation cleavages, the question became whether GIL is an essential gene product and, if so, in what stage of the viral replicative cycle is its activity required?

In the experiments reported here, we have taken advantage of a newly developed conditional expression system to demonstrate that enzymatic activity of the GIL metalloproteinase is required for viral replication (14). Furthermore, phenotypic analysis of virus grown in the absence of GIL suggests that the block in replication is at a site distinct from that of I7L, namely, subsequent to core protein cleavage but prior to core condensation and acquisition of infectivity. It remains to be determined whether GIL is responsible for a subset of core protein cleavages or if it catalyzes cleavage of an as yet unidentified substrate. In either case, it would appear that both proteinases are required for a proteolytic cascade that occurs during poxvirus assembly.
MATERIALS AND METHODS

Cells and virus. BSC40 cells (18) and T-REx-HeLa cells (Invitrogen, Carlsbad, Calif.) were grown in minimum essential medium with Earle’s salts (E-MEM; Invitrogen) containing 10% Tet system approved fetal bovine serum (BD Biosciences Clontech, Palo Alto, Calif.), 2 mM Glutamax (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). T-REx-293 cells (Invitrogen) were grown in Dulbecco’s modified eagle medium (Invitrogen) containing the same supplements as for E-MEM. Cells lines containing the pcDNA6/TR plasmid expressing TetR were grown in the presence of 5 μg of blasticidin (Invitrogen)/ml for selection of the plasmid. All cells were cultured at 37°C in a humidified atmosphere of 95% humidity and 5% CO2. Vaccinia virus strain Western Reserve (WR) and vvetOG1L were purified from BSC40 cells (18) and used for infection experiments.

Construction of vvetOG1L virus. A plasmid to be used for targeted mutagenesis was made by extracting the neomycin resistance gene driven by the vaccinia virus 7.5 early/late promoter from pVV:NEO (11) by PCR using primers 1 (5’ CATAACCACCAAGCCCGGCG 3’) and 2 (5’ TCAGAAGAATCTGGTCAA GAAG 3’). The PCR product was then cloned into pCR2.1 with the TA cloning kit (Invitrogen), resulting in the p7.5:Neo plasmid.

To PCR amplify a targeting region, starting from the WR genomic DNA (gDNA), were used to place tetO between the promoter and the coding sequence of the GIL gene as previously described (30). First, a PCR fragment consisting of the 233-bp 3’ end of the GIL ORF and a downstream partial tetO (italized) was generated by using primers 3 (5’ GGATCCGAGATGAAAGCCGAG 3’) and 4 (5’ TCTATACGTATAGGGCTATCTATACCGG 3’). In parallel, a PCR fragment spanning GIL ORF with an upstream partial tetO (italized) was amplified by using primers 5 (5’ CCTATAGCTATAGGAGATATGTTGTCATTCC 3’) and 6 (5’ AACGCTTTACAAACTTACGTC 3’). Second, the products of these two PCR products were used as templates in an overlap PCR (15) using primers 3 and 6. The final PCR product was first TA cloned into pCR2.1 (Invitrogen) and then subcloned into p7.5:Neo by using BamHI and HindIII restriction sites (underlined) that were incorporated into primers 3 and 6, respectively.

The resulting p7.5tetOG1L:Neo plasmid was used in the transient dominant selection method (10) to generate vvetOG1L. BSC40 cells were infected with vvetR (30) at a multiplicity of infection (MOI) of 0.1 PFU/cell and transfected with 10 μg of p7.5tetOG1L:Neo plasmid (Neo+) by using DMRIE-C reagent (Invitrogen). To select for Neo+ isolates, 1 mg of Geneticin G418 sulfate (Invitrogen) and 1 μg of tetracycline (TET; Sigma-Aldrich, St. Louis, Mo.) were added to the cultures at 20 h postinfection (hpi). Cells were harvested at 48 hpi by a 10-min centrifugation (700 × g), resuspended in 100 μl of phosphate-buffered saline (PBS) supplemented by 1 mM MgCl2, and subjected to three cycles of freeze/thaw in a liquid nitrogen bath to disrupt the cells and recover the virus. Plaque purifications were performed by first incubating virus-infected cells with a thin layer of 0.6% SeaPlaque low-melting-temperature agarose (Cambrex Bio Science Rockland, Inc.) in selective media for 48 h and then adding a second layer containing 1% low-melting-temperature agarose and 0.02% neutral red (Sigma-Aldrich) in PBS. Visualized individual plaques were either used to infect new cells for propagation of virus and purification of G1L DNA or transferred to 100 μl of 1 mM Tris-CHI (pH 8) as a virus stock. Purified gDNA or 10 μl of the Tris-CHI virus stock was used as template for PCR. After three rounds of plaque purification in the presence of G418 sulfate, viral isolates were screened for the presence of the tetO and neo genes by using primer pair 6 and 7 (5’ GGATCCCTATGCTTTGAAAAG 3’), 8 (5’ CTCTGGAGGGCTATCTGGTTGTC 3’), and 9 (5’ GATTATGAGATGAAATCTGGTCAA GAAG 3’). The final PCR product was then cloned into pCR2.1 with the TA cloning kit (Invitrogen) and then subcloned into p7.5:Neo by using BamHI and HindIII restriction sites (underlined) that were incorporated into primers 6 and 7, respectively.

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Transmission electron microscopy. T-REx-HeLa cells were infected at a MOI of 1 PFU/cell with vvetOG1L, harvested as described above at 24 hpi, fixed with 2% glutaraldehyde and 1.25% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3), postfixed in osmium tetroxide, dehydrated, and embedded in Spurr’s resin (28). Ultrathin sections were stained by the double-lead stain technique (8). Analysis was performed on a Philips CM-12 scanning transmission electron microscope (FEI, Hillsboro, Oreg.). Images were enhanced with Photoshop (Adobe, San Jose, Calif.).

RESULTS

Previous attempts to generate GIL loss-of-function mutants by insertional mutagenesis of the GIL gene in our laboratory have been unsuccessful, suggesting but not proving that the presence of GIL is essential for the replication of vaccinia virus. To investigate GIL function, we set out to engineer a vaccinia virus strain in which the expression of the GIL gene could be regulated by the presence or absence of TET. This conditional system utilizes the components of the bacterial TET operon (12, 16, 21) and has successfully been used to regulate the vaccinia virus A14L gene (30). We constructed a plasmid that contained the genomic DNA sequence from 233 bp upstream of the G3L stop codon to the stop codon of the GIL gene, including a tetO placed upstream of the GIL ORF (Fig. 1) in order to regulate GIL expression with TET in the presence of a TET repressor (TetR). This plasmid was used as...
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A tetO was placed between the putative promoter and coding sequence of GIL. The G3L gene overlaps with the GIL ORF. Four additional bases were added upstream of the tetO in order to restore the 3′ end of the G3L gene when the tetO was introduced.

FIG. 1. Design of the vv tetOG1L mutant. A tetO was placed between the putative promoter and coding sequence of GIL. The G3L gene overlaps with the GIL ORF. Four additional bases were added upstream of the tetO in order to restore the 3′ end of the G3L gene when the tetO was introduced.

FIG. 2. Virus titers of vv tetOG1L in TET-expressing 293 cells. T-REx-293 cells were infected with vv tetOG1L in the absence (−) or presence of TET. The amount of infectious virus was measured from the diluted cell lysates by titration on BSC40 cells. Infections proceeding from 25 to 51 h did not alter the titer ratio between infections in the absence or presence of TET.

GIL is essential for complete virus maturation. To test whether GIL was essential for vaccinia virus growth, we infected T-REx-293 and T-REx-HeLa cells with vv tetOG1L virus in the presence or absence of TET and monitored virus replication by plaque assay. Cell lysates from virus-infected T-REx-293 and T-REx-HeLa (data not shown) cells were titrated on BSC40 cells in order to analyze the amount of infectious virus produced in the presence or absence of TET. The BSC40 cells we used did not express any TetR, allowing us to titrate the virus in the absence of TET. An average reduction of 98.9% ± 0.97% of infectious virus particles was observed in 11 separate experiments using T-REx-293 cells. The same result was observed when experiments were performed by using T-REx-HeLa cells instead of T-REx-293 cells. However, because of the mixture of floating and attached cells in the T-REx-HeLa cell line, the remainder of the reported experiments were carried out with the T-REx-293 cell line, which maintained an adherent monolayer during the early stages of viral infection.

We next investigated if changing parameters during the infection had an effect on vv tetOG1L virus titers in T-REx-293 cells. First, we examined if decreasing the time of infection would have an effect on the virus titers. Varying the time between 25 and 51 h did not markedly change the reduction of the virus titers (Fig. 2). Second, we tested if decreasing the MOI would have an effect on virus titers. Infections at MOIs of 1, 0.1, and 0.01 PFU/cell did not alter the ratio between the titers in the absence or presence of TET (data not shown).

Third, we investigated if varying the TET concentration would have an effect on virus titers of vv tetOG1L as well as of wild-type WR virus. A TET concentration as 0.1 μg/ml was sufficient to rescue the vv tetOG1L titers (data not shown). Increasing the TET concentration from 0.1 to 10 μg/ml did not change the virus titers appreciably. However, TET concentrations higher than 50 μg/ml decreased the titers of both vv tetOG1L and WR.

Viral protein expression and processing of the major core proteins appear normal in the vv tetOG1L mutant in the absence of TET. To kinetically examine viral gene expression, we labeled newly synthesized proteins with radioactive amino acids at various time points postinfection in the absence or presence of TET (Fig. 3A). Infection with wild-type WR in the absence of TET was used as a control. The typical shift in protein expression patterns from cellular proteins to early, intermediate, and late viral proteins was seen during the infection. No obvious difference was seen after infection with vv tetOG1L in the absence or presence of TET. Although some effect on virus titers was seen at higher concentrations of TET (Fig. 2C), levels of protein expression in the TET and WR controls looked essentially the same.

The proteolytic processing of the major vaccinia virus core proteins is an essential step in virus maturation (9, 23, 31). To study the processing of the major viral core proteins in the vv tetOG1L mutant, we labeled proteins at 5 hpi for 45 min; this was followed by a chase with medium containing unlabeled amino acids. The cells were then harvested at 0 and 18 h postlabeling. As shown in Fig. 3B, processing of the core proteins (P4a, P4b, and P25K) appeared the same for the vv tetOG1L mutant and also for the wild-type WR virus in the absence and presence of TET. No differences were obvious for the other labeled proteins. Since core protein cleavage is intimately associated with virion morphogenesis, this result suggested that virion assembly should also be unaffected in the absence of GIL expression. To test this hypothesis, we directly
analyzed the virus assembly by electron microscopy. *vv tetOG1L* was able to infect both cell lines and develop into mature virus particles when TET was present (Fig. 4). By counting virus in several well-separated sections, we estimated that an average of 93% of the virus particles per cell in both cell lines had developed into mature virions (Table 1). In contrast, only an average of 1.5% of virions per cell had developed into mature particles in the absence of TET. In contrast, 98.5% of the virus particles remained round in the absence of TET. However, it was noted that many of the immature particles had at least partially condensed DNA within them. This would be consistent with virion core condensation being initiated along with concomitant cleavage of the major core proteins, but with morphogenesis being aborted at a later stage of development.

**The HXXEH motif is necessary for rescue of the vv tetOG1L mutant in the absence of TET.** To see if the *vv tetOG1L* mutant phenotype in the absence of TET could be rescued by reintroduction of G1L, we transfected T-REx-293 cells with the p-229-G1L-FLAG plasmid, which expresses G1L-FLAG from the native G1L promoter, and then infected the cells with *vv tetOG1L*. The cells were then harvested and analyzed by electron microscopy. The titer of infectious progeny was also determined by plaque assay. As controls we used mock-transfected cells in the absence or presence of TET and cells transfected with the plasmid p-ELP-GFP, which expresses GFP under the control of the synthetic early/late promoter (5). A partial rescue of the defective phenotype could be seen by electron microscopy after transfection of the p-229-G1L-FLAG plasmid (Fig. 5A). On average, one-half of the virus particles per cell were mature in the absence of TET compared to 5 and 10% in the p-ELP-GFP and mock-transfected controls without TET, respectively (Table 2). These were approximately 5- and 10-
fold increases compared to the GFP and mock-transfected controls without TET, respectively. Similarly, the relative titers increased from 0.24 and 0.83% in the p-ELP-GFP and mock-transfected controls without TET, respectively, to 14.4% in the cells transfected with the p-229-G1L-FLAG plasmid (Fig. 5B). These were 60- and 17-fold increases, respectively.

This result suggested that G1L was responsible for facilitating late-stage assembly. To determine if this was likely due to the predicted metalloproteinase activity of the protein or some other unrecognized feature, the putative HXXEH active site in G1L was mutated to RLLQR using the p-229-G1L-FLAG plasmid. As seen in Fig. 5A, this p-229-G1L-RLLQR-FLAG plasmid was not able to rescue the vv tetOG1L mutant phenotype in the absence of TET. On average less than 8% of the virus particles per cell were mature after transfection with the p-229-G1L-RLLQR-FLAG plasmid (Table 2). In the titer experiment, the titers from the p-229-G1L-RLLQR-FLAG-transfected cells were comparable to those from the p-ELP-GFP-transfected control without TET (Fig. 5B).

**G1L is structurally similar to the mitochondrial processing peptidase.** Although we have previously shown similarities in the regions of the HXXEH motif of G1L with eukaryotic metalloproteinases (32), the overall amino acid identity between these proteins and G1L was too low to generate a hit with the full G1L amino acid sequence and National Center for Biotechnology Information protein-protein BLAST (blastp) program (1). The vaccinia virus G1L protein is highly conserved among poxviruses and has high overall identity with proteins from poxviruses ranging from cowpox (99%), variola, and monkeypox (98%) virus to molluscum contagiosum (47%) and fowlpox (42%) virus. The closest nonpoxvirus protein that...
we found using blastp was the ymxG hypothetical zinc protease from *Bacillus subtilis* (SWISSPROT accession no. Q04805) (24), which only has 28% identity to G1L over a 101-amino-acid stretch. Interestingly, the putative active site of the *B. subtilis* protease has residues HFLEH and the amino acids ENE are located 62 amino acids downstream of the HXXEH motif. This is very similar to G1L, in which the ENE sequence is located 67 amino acids downstream of the HLLEH motif (32). Since the overall identity to G1L was low on the amino acid level outside the family of *Poxviridae*, we decided to search for related proteins on the 3D structure level.

From the mGenTHREADER program, four structures were flagged as probable hits, PDB accession codes 1hr6A (29), 1be3A, 1be3B (20), and 1ezvA (19). The PDB accession code 1hr6A is the α-mitochondrial processing peptidase (α-MPP) from yeast, while 1be3A/B and 1ezvA are the core proteins from the cytochrome bc₁ complex of bovine heart mitochondria and yeast mitochondria, respectively. Homology models were generated from each of these structures with the SWISS-MODEL program. Superpositions between the model and the original structure were performed in InsightII with all backbone atoms for equivalent residues from the SWISS-MODEL sequence alignment. The 1ezvA-based model showed the lowest root mean square deviation for backbone atoms at 3.2Å, followed by the 1hr6A-based model at 4.3Å, the 1be3B-based model at 5.4Å, and the 1be3A-based model at 6.6Å. The two “best” models by this criterion are shown in Fig. 6 with their base structures overlaid (Fig. 6B and C). Interestingly, the HILDR zinc-binding motif from 1hr6A aligns quite closely with the putative zinc-binding HILLEH motif from G1L (Fig. 6D), supporting the notion that G1L is in fact a metalloprotease.

**DISCUSSION**

To determine if the vaccinia virus G1L protein is required for viral growth and, if so, to get some insights into its potential role during the replication cycle, we have developed a conditional-lethal mutant, vv  

replication was *GIL*, with a FLAG epitope fused to the carboxy terminus of the GIL protein. This addition apparently does not disrupt the enzymatic activity of the GIL protein. This is an important observation, as the GIL protein is apparently expressed in vivo at exceedingly low concentrations and repeated attempts to produce antisera against this protein have been unsuccessful. Having a functional fusion protein available should make future experiments designed to purify the enzyme and/or monitor its subcellular location feasible.

Mutation of the predicted catalytic site of the GIL protein leads to a loss of ability to catalyze infectious progeny production in a marker rescue experiment. This observation suggests that GIL is a metalloprotease. The predicted structural similarity of GIL and α-MPP strengthens this hypothesis. Analysis of the *B. subtilis* hypothetical zinc protease included the same probable hits as those for GIL using the mGenTHREADER program and the ymxG HILLEH motif aligned with the yeast α-MPP HILDR motif (data not shown). The *B. subtilis* protease has higher identity to the α-MPP than GIL and could provide a link between the two proteins. The fact that the structure is conserved between the three species could reveal a practical structural solution for an important function. It is possible that the three amino acids of the HILLEH motif that were mutated to RLLQR could affect the 3D structure of the
protein and thereby inhibit its function. However, the substitutions were chosen to minimize this effect and were confirmed not to affect the theoretical 3D model of G1L (data not shown). Furthermore, a search with the mutated protein using the mGenTHREADER program generated the same hits and alignments as the native G1L.

If G1L is a metalloprotease, the question will then become what its native substrates and biochemical functions are during viral replication. There are at least two working hypotheses. The first is that, while the 7L cysteine protease is responsible for the major core protein cleavage reactions within P4a, P4b, and P25K, perhaps G1L is responsible for a subset of cleavage reactions which occur at atypical AG(X) motifs of structural proteins. This would be in agreement with the previous observation that G1L mediates cleavage of the P25K precursor at a cryptic AGS site in vivo (32). A second possibility is that G1L recognizes and cleaves a completely different motif in an as yet unidentified substrate. In either case, the evidence to date suggests that the I7L and G1L proteinases act in concert to facilitate a productive cascade during viral assembly. The activity of both seems to be required but I7L appears to act early in the morphogenesis process whereas G1L seems to be required at a later stage after DNA condensation has occurred.

Armed with the knowledge that G1L is an essential gene and that the encoded proteolytic activity is required, it will now be of interest to identify the protein’s cleavage substrate(s) and biochemical function. Furthermore, given the high degree of conservation of the GIL gene within the orthopoxvirus group and the lack of mammalian homologues, this enzyme may provide an appropriate and attractive target for antiviral drug development.

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