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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 *G1L* gene is unknown. To address this question, we have generated a vaccinia virus strain in which expression of the *G1L* 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 G1L, but not by G1L containing an HXXEH motif mutated to RXXQR. Modeling of G1L revealed a predicted structural similarity to the α-subunit of *Saccharomyces cerevisiae* mitochondrial processing peptidase (α-MPP). The HXXEH motif of G1L perfectly overlaps the HXXDR motif of α-MPP in this model. These results demonstrate that *G1L* is essential for virus maturation and suggest that G1L is a metalloproteinase with structural homology to α-MPP. However, no obvious effects on the expression and processing of the vaccinia virus major core proteins were observed in the *G1L* conditional mutant in the absence of TET compared to results for the TET and wild-type WR controls, suggesting that G1L 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 proteinases 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 G1L (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 proteinase is the putative metalloproteinase encoded by the *G1L* ORF, which contains an HXXEH sequence motif, a direct inversion of the active site consensus sequence present in metalloproteinases such as thermolysin (32). G1L 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 G1L 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 G1L 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 G1L metalloproteinase is required for viral replication (14). Furthermore, phenotypic analysis of virus grown in the absence of G1L 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 G1L 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.

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MATERIALS AND METHODS

Cells and virus. BSC_{40} 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. Cell 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 cultivated at 37°C in a humidified atmosphere of 95% humidity and 5% CO_2. Vaccinia virus strain Western Reserve (WR) and vv*tetOG1L* were purified from BSC_{40} cells (18) and used for infection experiments.

Construction of vv*tetOG1L* 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′ CTAATTCCAAACCCACCCGC 3′) and 2 (5′ TCAGAAGAACTCGTCAA GAAG 3′). The PCR product was then cloned into pCR2.1 with the TA cloning kit (Invitrogen), resulting in the p7.5:Neo plasmid.

Two PCR amplification steps, originating from WR genomic DNA (gDNA), were used to place *tetO* between the promoter and the coding sequence of the *G1L* gene as previously described (30). First, a PCR fragment consisting of the 233-bp 3′ end of the *G3L* ORF and a downstream partial *tetO* (italicized) was generated by using primers 3 (5′ GGATCCCAGATAGAGAAAACGC 3′) and 4 (5′ *TCTATCACTGATAGGGA*TCATTTACTAAGG 3′). In parallel, a PCR fragment consisting of the *G1L* ORF with an upstream partial *tetO* (italicized) was amplified by using primers 5 (5′ CC*TATCAGTGATAGAGA*ATGATTGTC TTACCG 3′) and 6 (5′ AAGCTTTCAAACTCTAATGACC 3′). Second, the products of these two PCRs 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 the BamHI and HindIII restriction sites (underlined) that were incorporated into primers 3 and 6, respectively.

The resulting p7.5:*tetOG1L*:Neo plasmid was used in the transient-dominant selection method (10) to generate vv*tetOG1L*. BSC_{40} cells were infected with v*tetR* (30) at a multiplicity of infection (MOI) of 0.1 PFU/cell and transfected with 10 μg of p7.5:*tetOG1L*:Neo plasmid (Neo^r) by using DMRIE-C reagent (Invitrogen). To select for Neo^r isolates, 1 mg of Geneticin G418 sulfate (Invitrogen)/ml and 1 μg of tetracycline (TET; Sigma-Aldrich, St. Louis, Mo.)/ml 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 $MgCl_2$, and subjected to three cycles of freeze/thaw in a liquid N_2-37°C water bath to lyse 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 gDNA or transferred to 100 μl of 1 mM Tris-HCl (pH 8) as a virus stock. Purified gDNA or 10 μl of the Tris-HCl 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′GGGTCCCTATCAGTGATAGAGA3′), 8 (5′ CCGTCGCGAGTGCGCA AATACACC 3′) and 9 (5′ GAGTATATTGAAGATCTACATCTTCG 3′), or 1 and 2 in PCR. The G418 selection was then removed to allow for a second recombination event to occur, resulting in either the production of virus that had reverted back to the wild type or the recombinant vv*tetOG1L* virus. Pure vv*tetOG1L* was generated by several plaque purifications and PCR screening cycles as described above.

Titration experiments. Cells were grown to approximately 60 to 80% confluence and infected in selective media containing 5% fetal bovine serum in the presence or absence of 0.1 to 100 μg of TET/ml. A MOI in the range of 0.01 to 1 PFU/cell was used. Fresh selective medium was added at 24 hpi. Cells were harvested at 48 hpi by centrifugation (750 × *g*) at 4°C for 5 min. The cell pellet was resuspended in PBS and subjected to three cycles of freeze/thaw in a liquid N_2-37°C water bath. Each sample was titered for virus on BSC_{40} cells and stained with 0.1% crystal violet (Sigma) in 30% ethanol (18).

Transmission electron microscopy. T-REx-293 and T-REx-HeLa cells were infected at a MOI of 1 PFU/cell with vv*tetOG1L*, 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.).

Rescue experiments. For rescue experiments, 60 to 80% confluent T-REx-293 cells were transfected with 1.6 μg of plasmid p-229-G1L-FLAG, p-229-G1L-RLLQR-FLAG, or p-ELP-GFP by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Two mock transfections were added as controls. The p-229-G1L-FLAG plasmid includes the *G1L* ORF with the coding sequence for a carboxy-terminal FLAG epitope (17) and the 229-bp upstream sequence of the *G1L* ORF (14). Site-directed mutagenesis of the HLLEH motif to RLLQR was performed on the p-229-G1L-FLAG plasmid using the QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, Calif.). The mutated plasmid was dubbed p-229-G1L-RLLQR-FLAG. The p-ELP-GFP plasmid expresses the red-shifted green fluorescent protein (GFP) variant GFPmut 1 (7) by regulation of the vaccinia synthetic early/late promoter (5) and is used as a control plasmid. The next day, cells were first infected from a master mixture of vv*tetOG1L* diluted for a MOI of 1 PFU/cell. Second, medium with or without TET (10 μg/ml) was added. At 3 hpi, the medium was replaced with fresh medium with or without TET (10 μg/ml). At 42 hpi, the cells were harvested by centrifugation (350 × *g*) for 5 min at 4°C. The first set of infected cells was used for electron microscopy analysis as described above. The second set was titered as described above.

Protein-labeling experiments. In general, T-REx-293 cells were grown to 60 to 80% confluence and then infected at a MOI of 25. The virus was added to the cells for 30 min and then replaced by fresh medium in an attempt to synchronize the infection. At specific time points postinfection, newly synthesized proteins were labeled with the EasyTag EXPRE^{35}S^{35}S protein labeling mixture (Perkin-Elmer Life Sciences, Boston, Mass.). The infections were performed in the absence or presence of TET. Uninfected cells and cells infected with WR were used as controls. Cells were harvested by centrifugation (750 × *g*) for 5 min at 4°C. The cell pellet was washed twice in PBS, frozen, dissolved in sodium dodecyl sulfate (SDS)-containing sample buffer, passed 10 times through a 21-gauge needle, and boiled for 5 min before loading onto a SDS–12% polyacrylamide gel electrophoresis gel. Labeled proteins were visualized by exposure of the dried gel to Biomax MR film (Kodak, Rochester, N.Y.).

Specifically, for pulse-labeling experiments, proteins were labeled for 20 min at 0, 1.5, 3, 5, and 8 hpi with10 μCi of labeling mixture. Pulse-labeling experiments were performed in the absence or presence of TET (50 μg/ml). For pulse-chase experiments, proteins were labeled at 5 hpi for 45 min with 100 μCi of labeling mixture; this was followed by a chase with medium containing unlabeled amino acids, and cells were harvested at 0 and 18 h postlabeling. Pulse-chase experiments were performed in the absence or presence of TET (100 μg/ml). Uninfected cells and cells infected with WR were used as controls.

Bioinformatics. The National Center for Biotechnology Information protein-protein BLAST (blastp) program (1) was used to search for G1L-related proteins. Theoretical three-dimensional (3D) structure prediction modeling of G1L was achieved by using the mGenTHREADER program at the PSIPRED Protein Structure Prediction Server (25) and the SWISS-MODEL alignment interface (13, 26, 27). Analysis of the predicted 3D model was performed using the Deep View, spdbv 3.7 (13), and InsightII (Accelrys, San Diego, Calif.) programs.

RESULTS

Previous attempts to generate *G1L* loss-of-function mutants by insertional mutagenesis of the *G1L* gene in our laboratory have been unsuccessful, suggesting but not proving that the presence of G1L is essential for the replication of vaccinia virus. To investigate G1L function, we set out to engineer a vaccinia virus strain in which the expression of the *G1L* 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 *G1L* gene, including a *tetO* placed upstream of the *G1L* ORF (Fig. 1) in order to regulate G1L expression with TET in the presence of a TET repressor (TetR). This plasmid was used as

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G3L 3' end...AGTAA-----------------------ATGA
G1L 5' end                               ATGATT...
WR         ...AGTAA-----------------------ATGATT...
vvtetOG1L ...AGTAAATGATCCCTATCAGTGATAGAGAATGATT...
tetO                  TCCCTATCAGTGATAGAGA
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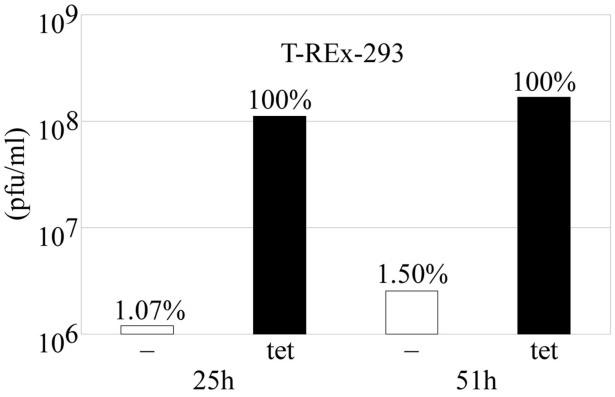
FIG. 1. Design of the vv*tetOG1L* mutant. A *tetO* was placed between the putative promoter and coding sequence of *G1L*. The *G3L* gene overlaps with the *G1L* ORF. Four additional bases were added upstream of the *tetO* in order to restore the 3′ end of the *G3L* gene when the *tetO* was introduced.

a template for creating a vv*tetOG1L* virus by the transient-dominant selection method (10). The virus to be mutated contained the *tetR* gene driven by the early/late vaccinia virus 7.5 promoter inserted into the thymidine kinase gene (30). Although we succeeded in mutating the *G1L* gene in the virus, we were not able to isolate any virus containing both the *tetO* upstream of the *G1L* gene and an intact *tetR* gene due to recombination events in the region of the *tetR* gene (data not shown). The *tetR* gene and the 7.5 early/late promoter were rearranged such that the promoter was located downstream of the *tetR* gene and in an opposite direction. This resulted in an inactive *tetR* gene and hence no production of viral TetR (data not shown). Since TetR is crucial for the system, we investigated the possibility of expressing the TetR from the infected cells instead of from the virus and were successful in regulating the *tetO*-containing *G1L* promoter by TetR expressed from the T-REx-293 and T-REx-HeLa cell lines (14).

G1L is essential for complete virus maturation. To test whether G1L 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 titered on BSC_{40} cells in order to analyze the amount of infectious virus produced in the presence or absence of TET. The BSC_{40} cells we used did not express any TetR, allowing us to titer 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 hpi 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



Time	tet	pfu/ml*	%	% reduction
25h	-	1.2E+06	1.1	98.9
25h	+	1.1E+08	100.0	0.0
51h	-	2.5E+06	1.5	98.5
51h	+	1.7E+08	100.0	0.0
*pfu/ml in diluted cell lysate				

FIG. 2. Virus titers of vv*tetOG1L* in TetR-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 BSC_{40} cells. Infections proceeding from 25 to 51 h did not alter the titer ratio between infections in the absence or presence of TET.

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 G1L expression. To test this hypothesis, we directly

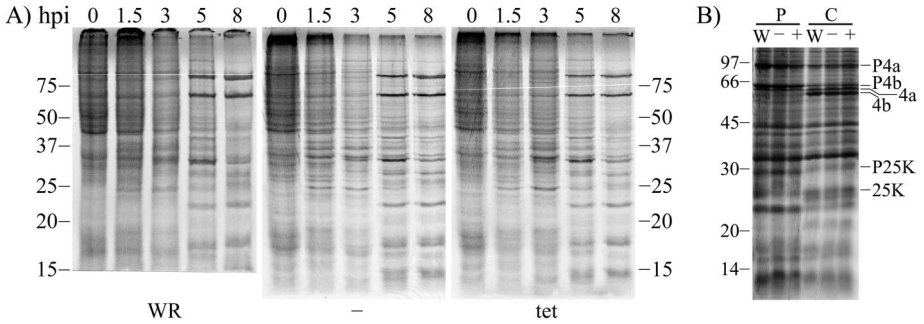


FIG. 3. Expression and processing of viral proteins. T-REx-293 cells were infected with vv*tetOG1L* in the absence or presence of TET. Newly synthesized proteins were ^{35}S labeled at various times postinfection. Infections with wild-type WR were used as the reference. (A) Pulse-labeling of cellular and viral proteins at various times postinfection. The major protein expression shifted from mainly cellular proteins (0 hpi) to late viral proteins (5 hpi). No difference of the protein patterns between the vv*tetOG1L* and wild-type WR infections in the absence or presence of TET were seen. (B) Pulse-chase of viral proteins. The cells were labeled for 45 min at 5 hpi and either harvested directly (P) or 18 h postlabeling (C). The major viral core proteins P4a, P4b, and P25K were processed to 4a, 4b, and 25K, respectively, in all samples.

analyzed the virus assembly by electron microcopy. 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 express 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-

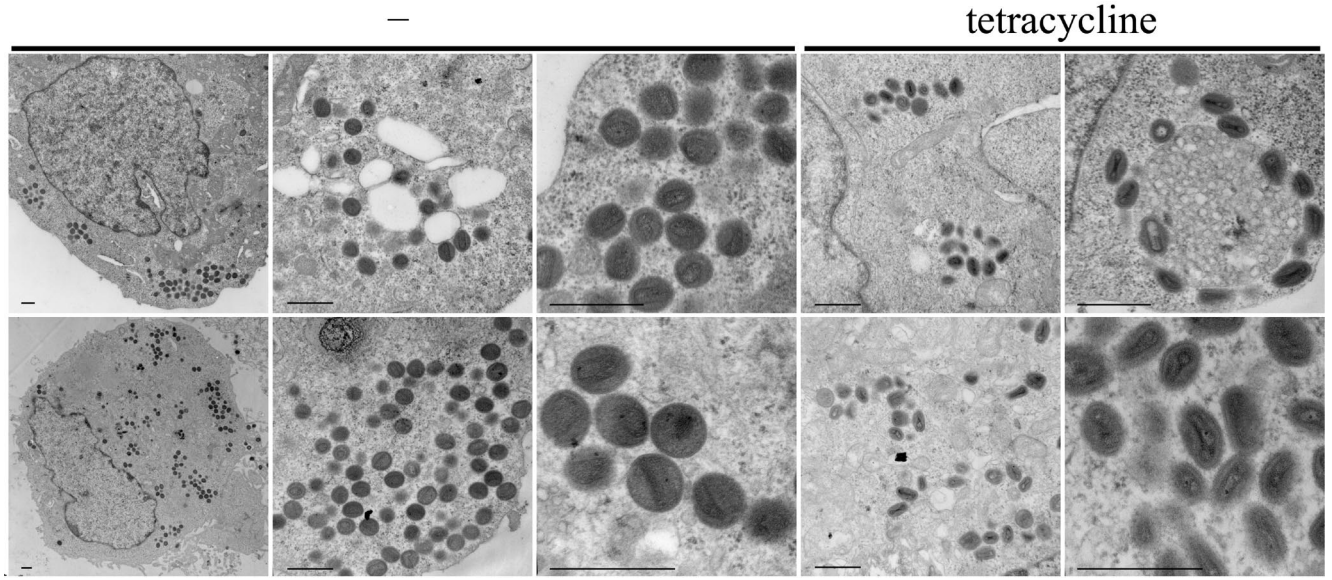


FIG. 4. Morphology of the vv*tetOG1L* mutant. T-REx-293 (top) and T-REx-HeLa (bottom) cells were infected with vv*tetOG1L* at a MOI of 1 PFU/cell in the presence or absence of TET, harvested at 24 hpi, immediately fixed, and prepared for transmission electron microscopy. Mostly mature virus particles were seen in the presence of TET in both cell lines. In the absence of TET, mostly round virus particles, many containing condensed DNA, were seen. Scale bars, 600 nm.

TABLE 1. Electron microscopy count

Cell line	TET[a]	% of virus particles that were:		SD (±)
		Round	Mature	
T-REx-293	−	97.7	2.3	3.8
T-REx-293	+	8.3	91.7	5.5
T-REx-HeLa	−	99.3	0.7	1.5
T-REx-HeLa	+	5.6	94.4	1.9
Total	−	98.5	1.5	
Total	+	6.9	93.1	

[a] −, absent; +, present.

TABLE 2. Electron microscopy count rescue

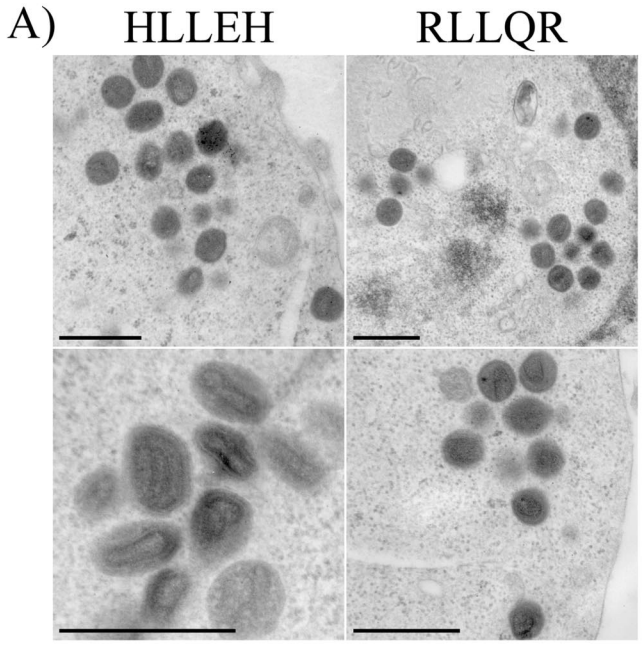
Plasmid[a]	TET[b]	% of virus particles that were:		SD (±)
		Round	Mature	
None	−	90.5	9.5	25.4
HLLEH	−	50.1	49.9	31.0
RLLQR	−	92.3	7.7	10.0
GFP	−	94.9	5.1	8.7
None	+	8.4	91.6	7.5

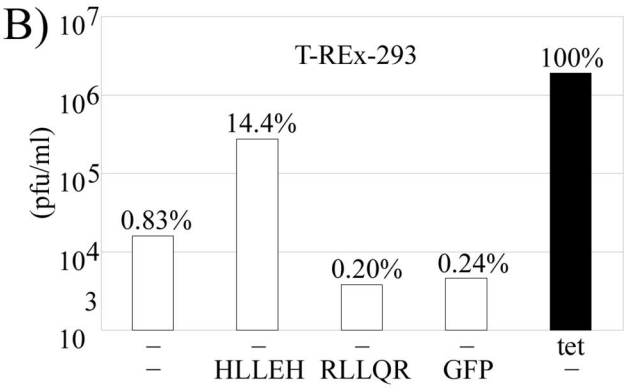
[a] HLLEH, p-229-G1L-FLAG; RLLQR, p-229-G1L-RLLQR-FLAG; GFP, p-ELP-GFP.
[b] −, absent; +, present.

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





Plasmid	tet	pfu/ml*	%	% reduction
-	-	1.58E+04	0.8	99.2
HLLEH	-	2.74E+05	14.4	85.6
RLLQR	-	3.78E+03	0.2	99.8
GFP	-	4.63E+03	0.2	99.8
-	+	1.90E+06	100.0	0.0
*pfu/ml in diluted cell lysate				

FIG. 5. Virus morphology and titers after rescue of the vv*tetOG1L* mutant in T-REx-293 cells. T-REx-293 cells were either mock transfected or transfected with plasmid p-229-G1L-FLAG, p-229-G1L-RLLQR-FLAG, or p-ELP-GFP and infected with vv*tetOG1L* in the absence or presence of TET. One set of infected cells were assayed for virus morphology by electron microscopy (A), and the other set were assayed for infectious virus particles by titration on BSC_{40} cells (B). (A) Transfection of the p-229-G1L-FLAG plasmid (HLLEH), but not transfection of the p-229-G1L-RLLQR-FLAG plasmid (RLLQR), partially rescued the vv*tetOG1L* mutant phenotype in the absence of TET. Scale bars, 600 nm. (B) Transfection of the p-229-G1L-FLAG plasmid (HLLEH), but not transfection of the p-229-G1L-RLLQR-FLAG plasmid (RLLQR) or the p-ELP-GFP plasmid (GFP), partially rescued the vv*tetOG1L* mutant phenotype in the absence of TET.

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 bc_1 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 HLLEH motif from G1L (Fig. 6D), supporting the notion that G1L is in fact a metalloproteinase.

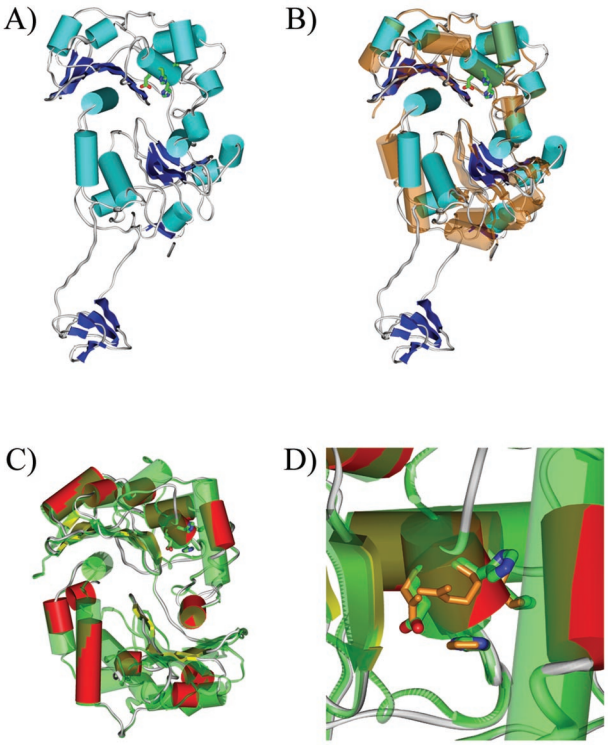


FIG. 6. Models of G1L. (A) Model of G1L based on the structure of 1ezvA (core protein 1 from the yeast cytochrome bc_1 complex). The HLLEH motif is rendered with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue. (B) G1L model with 1ezvA (orange, transparent) overlaid. (C) Model of G1L based on the structure of 1hr6A (yeast α-MPP), with 1hr6A overlaid (green, transparent). (D) Closeup of putative zinc binding residues of G1L (HLLEH) rendered in atomic colors with zinc-binding HILDR motif of α-MPP residues in orange.

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*tetOG1L*. During infection of cell lines which express the TET repressor, expression of the *G1L* gene can be turned on or off by the presence or absence of TET in the growth medium. The data presented here establish that *G1L* is an essential gene whose activity is required for the production of infectious progeny. In the absence of *G1L* expression, production of infectious progeny is inhibited by >98% despite the fact that the pattern of early and late viral protein expression is unaffected. Electron-microscopic analysis revealed that, in the absence of G1L, virion morphogenesis was aborted at a late stage somewhere after DNA condensation commences but prior to infectious oval virion formation. This stage appears to be subsequent to the cleavage of the major core protein precursors mediated by the viral I7L proteinase since processing occurs normally in the absence of G1L.

Marker rescue of virus replication with a transfected copy of the *G1L* gene demonstrated that the observed phenotype was due to the mutant *G1L* gene and not a silent secondary mutation. Furthermore, mutation of the putative active site of the transfected *G1L* gene inhibited its ability to rescue, suggesting that it was the proteolytic activity of the G1L protein that was required for its function. Note that the gene which rescued replication was *G1L*, with a FLAG epitope fused to the carboxy terminus of the G1L protein. This addition apparently does not disrupt the enzymatic activity of the G1L protein. This is an important observation, as the G1L 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 G1L protein leads to a loss of ability to catalyze infectious progeny production in a marker rescue experiment. This observation suggests that G1L is a metalloproteinase. The predicted structural similarity of G1L and α-MPP strengthens this hypothesis. Analysis of the *B. subtilis* hypothetical zinc protease included the same probable hits as those for G1L using the mGenTHREADER program and the ymxG HFLEH motif aligned with the yeast α-MPP HILDR motif (data not shown). The *B. subtilis* protease has higher identity to the α-MPP than G1L 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 HLLEH 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 metalloproteinase, 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 I7L cysteine proteinase 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 a regulated proteolytic 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 *G1L* 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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