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13. ABSTRACT (Maximum 200 Words)
Using a homology-based bioinformatics approach, a structural model of the vaccinia virus (VV) 17L proteinase was developed. A unique chemical library of ~51,000 compounds was computationally queried to identify potential active site inhibitors. The resulting biased subset of compounds was assayed for both toxicity and the ability to inhibit the growth of VV in tissue culture cells. A family of chemotypically-related compounds were found which exhibit selective activity against orthopoxviruses, inhibiting VV with IC50 values of 3-12 μM. These compounds exhibited no significant cytotoxicity in the four cell lines tested, and did not inhibit the growth of other organisms such as Saccharomyces cerevisiae, Pseudomonas aeruginosa, adenovirus, or encephalomyocarditis virus (EMC). Compound resistant viruses were generated and resistance was mapped to the 17L ORF. A medicinal chemistry program is under way to further develop this novel class of inhibitors. The final goal of this project will be the development of a potent antiviral against various pox viruses ready for preclinical development.

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Introduction
Smallpox virus is considered one of the most significant threats for use as a biowarfare agent. Due to complications from vaccination, mass immunization of the populace is contra-indicated. Our current research seeks to develop effective anti-poxvirus drug(s). Using vaccinia virus (VV) as a model system, the goal of our currently funded work is to determine whether the I7L cysteine proteinase or the G1L metalloproteinase encoded by VV is the poxvirus core protein proteinase (vCPP) that is essential for viral maturation and production of infectious progeny. We have recently demonstrated that the I7L cysteine proteinase is the vCPP (Byrd et al., 2002) and will continue to study the role of this enzyme during virus growth. Given this information however, we are also positioned to launch a concerted effort to identify and develop I7L inhibitors as candidate antiviral drugs. The specific goals of the experiments outlined in this report are to: 1) Over-express and purify enzymatically-active I7L proteins; 2) Develop both biochemical and tissue culture assays to measure I7L activity; 3) Utilize a combination of rational drug design and high throughput screening procedures to identify potential I7L inhibitors; and 4) To test candidate inhibitors for their ability to inhibit poxvirus replication in infected cells and appropriate animal models. Successful completion of these experiments will identify I7L inhibitors that can be advanced into pre-clinical and clinical development as antiviral drugs. Such drugs will be an essential addition to our pharmaceutical armamentarium against the deliberate or accidental introduction of a pathogenic poxvirus into our environment in order to protect members of the armed forces or the general populace.

Body
1) Expression and purification of vCPP (I7L). SIGA has utilized many different systems to try to express enzymatically active I7L protein including numerous E. coli based expression systems, SIGA’s proprietary SPEX and PLEX systems, mammalian cell expression, Baculovirus, and yeast. Some success has been seen with the Baculovirus system so efforts are on-going with various baculovirus recombinant constructs. The yeast system has just been started, so it is not apparent yet if that expression system will be successful. At the same time SIGA is testing various mutants and truncated derivatives in all of the expression systems listed above. We continue to try to scale up and purify and test for activity.

2) In vivo screening for inhibitor activity. TTP and SIGA have chosen a lead compound series and TTP has been doing medicinal chemistry to improve the over-all drug characteristics. TTP continues to synthesize new iterations to decrease toxicity and improve activity in vitro. To date, TTP has synthesized ~300 analogs that SIGA has tested. We have lead molecules with activity around 0.5 uM and TI's ranging from 50-200. We are continuing to do SAR (structure activity relationships) around the compounds and trying to reduce toxicity by adding war heads.
3) Generation of resistant mutant virus. As shown in Fig. 2 we passaged vaccinia virus in the presence of lead compounds to generate resistant mutants. The titer starts out high and then drops in the presence of inhibitor and then as resistance is generated the titer comes back up. Sequencing of the I7L open reading frame has produced two sets of mutations (aa104, aa324) and (aa56, aa104). The first set of mutations is shown in figure 2 below. We are doing marker rescue studies with the mutant I7L genes. Work on the first set of mutations (104, 324) has been published in the November issue of the Journal of Virology (see attached). This data proves that the lead compounds are targeting I7L.
WT 101-HSIYDVFELP 321-NQLLESEGGM
v17 101-HSICDVFELP 321-NQLMESEGGM

Figure 2. Graphical representation of the Vaccinia virus titer during successive passage with I7L inhibitor. Below the graph is the amino acid mutations generated in the resistance virus and their location within the I7L protein sequence.

4) Animal efficacy studies. SIGA has set up the vaccinia virus (Western Reserve and IHDJ) mouse intranasal challenge animal model in 4 week and 6 week old BALB/c mice and validated it using the known inhibitors: cidofovir and ribavirin. SIGA has also set up the vaccinia virus (Copenhagen) mouse tail vein model (Neyts et al) in 4 week CD1 mice and validated it using the known inhibitor cidofovir. SIGA is starting to evaluate the pharmacokinetics of selected compounds to see if they are orally bioavailable and what the serum levels and half life are. Once appropriate drug candidates are chosen they will be tested in the animal models set up and validated at SIGA.

Key Research Accomplishments

☐ Discovery of a series of compounds that target the I7L protease and are effective against various pox viruses in cell culture.
☐ Successful iterative chemistry program making I7L inhibitors with increased activity against pox viruses in cell culture.
☐ Vaccinia virus murine intranasal challenge model set up and validated at SIGA
☐ Vaccinia virus murine tail vein model set up and validated at SIGA

Reportable Outcomes (manuscripts, abstracts, presentations, patents, etc)

• Manuscripts


- Abstracts & Presentations


- Patents
Screening Method for Orthopoxvirus Antivirals, (US & PCT applications)

Conclusions
By using homology-based computational modeling, a structural model of the VV I7L cysteine proteinase, which is responsible for essential morphogenic cleavage reactions during viral maturation, was developed. This model was used in concert with in silico drug docking procedures to query a combinatorial
chemical library. A biased subset of compounds that were predicted to bind to the I7L catalytic site were directly tested for their ability to inhibit VV replication in vivo. A number of chemically related effective inhibitors were identified with the prototype being compound TTP-6171. Investigation of the mechanism of lead compound mediated inhibition of VV replication indicated that although early stages of VV replication were unaffected, cleavage of the major core protein precursors and subsequent maturation of the immature viral intermediates into infectious intracellular mature virions was blocked. This data was consistent with I7L catalytic activity being the target of TTP-compound inhibition. To confirm this hypothesis, drug resistant mutants were selected and mutations within the I7L gene were shown to be responsible for the resistant phenotypes.

The TTP compounds represent very promising poxvirus antiviral drug candidates. When compared to cidofovir, which is the best-known poxvirus antiviral drug, our lead compounds appear to have superior characteristics in tissue culture. Whereas, cidofovir has a TC50 of 280 μM, IC50 of 33 μM and a therapeutic index of 8 against VV (5), under similar conditions TTP-6171 has an IC50 of 12 μM and limited toxicity against a variety of tissue culture cells from a number of species, including monkey (BSC40), human (HeLa and 293), and mouse (L929). The lead compounds appear to exhibit an excellent specificity in that they do not inhibit growth of any of the other organisms tested, which include RNA viruses (EMV and MHV), yeast, bacteria or adenovirus. The latter result is particularly noteworthy because adenovirus has been shown to have a 90 amino acid region with homology to the cysteine proteinase of I7L (1,4). It appears that virtually all poxviruses require I7L-mediated essential morphogenic maturation of their core proteins as all contain an AGX motif at the same location within the P4b precursor. Within the Orthopoxviridae, sequence identity of the I7L gene is between 95-99% (including variola and Monkeypox virus) and the residues flanking the catalytic site are completely conserved. Furthermore, the sequence conservation of the I7L gene remains considerable in other poxvirus genera, especially around the catalytic site, suggesting that these compounds may have promise as an antiviral drug to treat more exotic poxvirus diseases as well as those of current concern, such as smallpox and monkeypox.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Catalytic triad sequence</th>
<th>% identity to VV77L</th>
<th>P4b (AGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV I7L</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 301- nqllsesegm</td>
<td>100% yes</td>
<td></td>
</tr>
<tr>
<td>Cowpox</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>96% yes</td>
<td></td>
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<tr>
<td>Camelpox</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>99% yes</td>
<td></td>
</tr>
<tr>
<td>Variola major</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>99% yes</td>
<td></td>
</tr>
<tr>
<td>Variola minor</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>99% yes</td>
<td></td>
</tr>
<tr>
<td>Monkeypox</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>99% yes</td>
<td></td>
</tr>
<tr>
<td>Ectromelia</td>
<td>(Orthopoxivirus)</td>
<td>241- hwkcviyddk qclvsfydag 321- nqllsesegm</td>
<td>95% yes</td>
<td></td>
</tr>
<tr>
<td>Sheepx</td>
<td>(Carppoxivirus)</td>
<td>251- hwkcvifdke klvvcfydag 321- nqllsesegm</td>
<td>63% yes</td>
<td></td>
</tr>
<tr>
<td>Lumpy skin</td>
<td>(Carppoxivirus)</td>
<td>251- hwkcvifdke klvvcfydag 321- nqllsesegm</td>
<td>66% yes</td>
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<tr>
<td>Yaba-like</td>
<td>(Yatapoxivirus)</td>
<td>246- hwkcvilke klfvafydag 321- nqllsesegm</td>
<td>69% yes</td>
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<tr>
<td>Swinepox</td>
<td>(Suipoxivirus)</td>
<td>249- hwkcvifdke htvfcvdfydag 330- nqllsesegm</td>
<td>68% yes</td>
<td></td>
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<tr>
<td>Rabbit fibroma</td>
<td>(Leporipoxivirus)</td>
<td>245- hwkcvifdke kqisacvfydag 326- nqllsesegm</td>
<td>66% AGV</td>
<td></td>
</tr>
<tr>
<td>Myxoma virus</td>
<td>(Leporipoxivirus)</td>
<td>245- hwkcvifdke kqicvfcvfydag 326- nqllsesegm</td>
<td>66% AGV</td>
<td></td>
</tr>
<tr>
<td>Molluscum contagiosum- (Molluscipox)</td>
<td>242- hwkalsvdfdr qlfvafydag 322- nqllsesegm</td>
<td>62% yes</td>
<td></td>
<td></td>
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<tr>
<td>Fowlpox virus</td>
<td>(Avipoxivirus)</td>
<td>242- hwkcaiydkn rdficfydag 322- nqllsesegm</td>
<td>58% yes</td>
<td></td>
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<tr>
<td>Canarypox virus</td>
<td>(Avipoxivirus)</td>
<td>242- hwkcnildydr ndfvcvfydag 323- nqllsesegm</td>
<td>55% NP</td>
<td></td>
</tr>
<tr>
<td>Anasacta moorei</td>
<td>(Entomopoxivirus)</td>
<td>254- hftsvkgk rkicvlfns 336- igydspdegm</td>
<td>25% NP</td>
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</table>
Based on the results reported here, the chemical compound family represented by TTP-6171 represents a promising avenue towards developing an effective antiviral drug that can be used to prevent or treat diseases caused by orthopoxviruses, such as smallpox. We have initiated a iterative chemistry program in collaboration with Transtech to optimize this chemical series with the final goal being development of a lead antiviral candidate ready for pre-clinical development.

References

Molecular Dissection of the Vaccinia Virus I7L Core Protein Proteinase

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The vaccinia virus I7L gene product is predicted to be a cysteine proteinase and is demonstrated in this study to be responsible for cleavage of each of the three major core protein precursors (P4a, P4b, and P25K) in vivo. Mutagenesis of the putative catalytic triad of I7L or of the cleavage sites in the core protein precursors inhibits processing. A truncated protein lost the ability to cleave the core protein precursors.

Vaccinia virus (VV) is a large double-stranded-DNA virus with a cytoplasmic site of replication. It has over 200 open reading frames (ORFs) and has been extensively used as a eukaryotic cloning and expression vector and for vaccine research. VV is closely related to variola virus, the causative agent of smallpox, and therefore is of interest as a surrogate target in the development of antiviral drugs and vaccines. It is therefore of interest that the gene product of the I7L ORF of VV, which is predicted to be the core protein proteinase (2), shares 99% identity with the homologous K7L gene of variola major virus.

Most viruses, including poliovirus, human immunodeficiency virus, and adenovirus, use posttranslational proteolytic processing as an essential step in their replication cycles (8). Therefore, it was not surprising to discover that proteolytic maturation of orthopoxvirus core proteins appears to be required for infectious progeny to be produced (6). Three of the major structural proteins found within the mature VV virion core are 4a, 4b, and 25K, known to be produced from higher-molecular-weight precursors at late times during infection (12). VanSlyke et al. (13, 14) demonstrated that a large number of VV core proteins, including 4a, 4b, and 25K, appear to be processed via a common morphologic cleavage pathway that is intimately linked with virion assembly and maturation. Cleavage of the precursors occurs only within the context of the maturing virion. All of the precursor proteins appear to be cleaved at a novel Ala-Gly-Xaa motif. This motif is distinct from that utilized in any other viral system, although some of the cysteine proteinases identified in other systems cleave polyproteins at Gly-Gly-Xaa sites, as demonstrated by the yeast cysteine proteinase (9, 10), the adenosine proteinase (15, 3, 5), and the African swine fever virus (ASFV) proteinase (1).

The gene product of the I7L ORF of VV was originally identified as a putative proteinase due to its homology to an ubiquitin-like proteinase in yeast (9) and was recently shown to be one of the proteinases responsible for cleavage of the VV core proteins (2). While there is a relatively detailed understanding of the cis-signals (sequences and protein structure characteristics) that direct the cleavage of the core protein precursors, relatively little is known about the enzyme that carries out these reactions. It is not known whether the entire I7L protein is required for recognition and cleavage of the core precursor proteins or if just the predicted catalytic domain is required. Is I7L capable of cleaving each of the core protein precursors, and does cleavage occur preferentially at Ala-Gly-Ala versus Ala-Gly-Ser and Ala-Gly-Thr sites? Is there a catalytic triad and are other conserved residues essential for activity? The results obtained show that intact I7L is necessary and sufficient to direct cleavage of each of the three major core protein precursors and that mutagenesis of either the putative catalytic triad of I7L or of the Ala-Gly-Xaa sites in the precursor proteins abolishes this activity.

The VV I7L ORF is predicted to encode a 423-amino acid protein with the catalytic domain located towards the carboxy terminus of the protein. Figure 1 shows a predicted hydropathy plot of the I7L protein made by using the Kyte-Doolittle program. The residues above the zero line are hydrophobic, and those beneath are hydrophilic. There are several hydrophobic domains near the amino terminus and the carboxy terminus of the protein. The positions of the residues of the putative catalytic triad (H, D, and C) are indicated, as are the positions of four other highly conserved amino acids (W, D, O, and G). Also shown is the position of the t16 mutation, where a proline was altered to a leucine (7), creating a temperature-sensitive virus capable of growth at 31°C but not at 41°C. The t16 mutation was originally isolated by Condit et al. (4). Shown below the hydropathy profile are representations of the variola virus, camelpox, and monkeypox enzymes with positions of variance from VV I7L indicated with bars, which show that these enzymes are virtually identical to VV I7L and that the residues of the putative catalytic triad are conserved. The region within the I7L ORF with homology to the ASFV core proteinase is near the C terminus and overlaps the location of the putative catalytic triad. To determine if the N-terminal portion of the protein is required for activity, a truncated I7L was created, cutting off the N-terminal region up to amino acid residue 228. This process was done to remove both the N-terminal hydrophobic region and the region of the protein that was previously determined to have similarity to a topoisomerase. Li and Hochstrasser (9) and Andres et al. (1) have iden-
Hydropobicity Profile of Vaccinia virus I7L protein

![Graph showing hydrophobicity profile of Vaccinia virus I7L protein]

- **Varicella virus I7L**
- **Camelpox virus**
- **Monkeypox virus I7L**

**17L-truncate**

**Homology to ASFV protease**

**344 ts16 mutation P-9L**

**I7L**

- 423 amino acids

FIG. 1. Characterization of I7L and predicted hydrophobicity plot of the I7L protein made with the Kyte-Doolittle program. The positions of seven highly conserved amino acids, including the putative catalytic triad, and the position of the ts16 mutation are indicated by arrows. The positions of the amino acids are indicated on the x axis. Sequence similarity to the corresponding gene in varicella virus, camelpox virus, and monkeypox virus is indicated by rectangles underneath the plot, with differing amino acids indicated at the correct positions by black bars. Sequence similarity of the conserved catalytic domain between VV I7L, the ASFV protease, adenovirus protease (A2E2), and a yeast cysteine protease (Ulp1) is indicated at the bottom of the figure, with arrows pointing at the highly conserved amino acids.

In previous work, we have shown that I7L is capable of cleaving P25K at the AGA and AGS sites. However, it was not known whether this cleavage reaction was specific to the P25K substrate or whether I7L was capable of cleaving the other core protein precursors. To determine whether I7L is responsible for cleavage of each of the three major core protein precursors, an in vivo trans-processing assay was utilized, where cells were infected with ts16 at the nonpermissive temperature and cotransfected with plasmid-borne substrate and enzyme. Both the substrate proteins and I7L protease were constitutively expressed in vivo by using a synthetic early-late promoter. Each core protein precursor-expressing plasmid was designed to express a Flag epitope on the C terminus for detection by Western blotting and differentiation from the analogous gene product expressed from the viral genome. Figure 2 is a map of the three major core protein precursors, P4a, P4b, and P25K, which are products of the A10L, A3L, and L4R ORFs, respectively, with the previously determined cleavage sites indicated on them. These cleavage sites have all been mapped to an Ala-Gly-Xaa motif (14, 16). P4a is the largest precursor protein, with a molecular weight of 98 kDa, and contains both an Ala-Gly-Ser and an Ala-Gly-Thr cleavage site in the C-terminal region of the protein. P4b is a 71-kDa polyprotein with an N-terminal Ala-Gly-Ala site, and P25K is a 28-kDa polyprotein with both Ala-Gly-Ser and Ala-Gly-Ala cleavage sites in the N-terminal region of the protein. Also indicated are the relative sizes of the plasmid-borne I7L protein (pI7L) and the truncated I7L protein, which was truncated at amino acid 228,
leaving residues 229 through 423, with a reengineered start site.

Cleavage of P25K by I7L has been shown previously (2), but here we demonstrate that I7L is capable of directly cleavage of the other core protein precursors as well. BSC40 cells (11) were infected with VV ts16 at a multiplicity of infection of five and transfected with 10 μg of plasmid DNA containing either I7L, P4b, P4a, or a mixture of these via a liposome-mediated transfection protocol. The cells were harvested 24 h postinfection, and the extracts were analyzed by Western blotting with anti-Flag antisera. Figure 3A indicates that I7L cleaves P4b from its precursor form to the mature processed form (Fig. 3A, lane 2) but that when the histidine residue 241 (a member of the putative catalytic triad) of I7L is mutated to an alanine, this cleavage is no longer observed (Fig. 3A, lane 3). Lanes 1 and 4 are controls showing P4b and P4bID1 expressed alone. When the AGA site of P4b is mutated to IDI residues, no cleavage by I7L is observed (Fig. 3A, lane 5). Lane 6 is a final control showing that with mutant P4b and mutant I7L no cleavage products are observed, indicating that other proteases in the virus or cells are not causing the cleavage reactions. This experiment was repeated with the P4a polyprotein, as shown in Fig. 3B. When P4a is expressed alone (Fig. 3B, lane 1), it runs at its mature size of 98 kDa, but when I7L is transfected in with

FIG. 3. Proteolytic processing of the core protein precursors. BSC40 cells were infected with VV ts16 and transfected with plasmids containing either I7L, P4a, P4b, or a mixture of these. Cells were harvested 24 h postinfection, and the extracts were analyzed by Western blot with anti-Flag antisera. (A) Processing of P4b. In each lane, cells are infected with ts16 and then transfected with either substrate alone or with substrate plus enzyme. The substrate is pP4b or pP4bID1 (where the AGA site is mutated to an IDI), and the enzyme is either pI7L or pH241A (pI7L with His 241 mutated to Ala). (B) Processing of P4b. In each lane, cells are infected with ts16 and then transfected with either substrate alone or with substrate plus enzyme. In this case, the substrate is either pP4a, pP4aID1696 (P4a with the AGT site mutated to an IDI), or pP4aID1613 (P4a with the AGS site mutated to an IDI).
P4a, two cleavage products are observed at around 22 kDa and 32 kDa (Fig. 3B, lane 2), indicating that cleavage is occurring at both the AGS and AGT sites. Mutation of 17L abolishes this cleavage (Fig. 3B, lane 3). When the AGS site of P4a is mutated to an ID1 and the transfection is carried out with 17L, only one band at around 22 kDa is observed exhibiting cleavage at only the AGT site (Fig. 3B, lane 8). A similar result is obtained when the AGT site is mutated to an ID1 and transfected with 17L (Fig. 3B, lane 5), where a band at around 30 kDa is observed, showing that cleavage is blocked at the ID1 site but still occurring at the AGS site. This finding shows that the catalytic activity of 17L, as well as the presence of the authentic cleavage sites, is necessary for cleavage to occur.

To further characterize whether the catalytic domain of the 17L protein was necessary and sufficient for recognition and cleavage of the core protein precursors, a truncated 17L was created with the N terminus removed up to amino acid 228. This truncated 17L was cloned into a plasmid behind the synthetic early-late promoter. The trans-processing assay was repeated with this 17L truncate. The 17L truncate was unable to cleave either P4a, P4b, or P25K, indicating that this region is essential for activity (data not shown).

To determine which of the seven previously indicated conserved amino acids is necessary for catalytic activity of 17L, site-directed mutagenesis was performed on each in turn to mutate the residue of interest to an alanine. Transient-expression assays were performed to test the activity of the mutant proteins on each of the core protein precursors. Briefly, cells were infected with VV ts16 at a multiplicity of infection of five and transfected with 10 μg of plasmid DNA by using DMRIE-C liposome-mediated reagent. Virus-infected cells were harvested 24 hours postinfection and centrifuged, and the resuspended pellet was subjected to three cycles of freeze-thaw to release the virus from the cell. The supernatant was used for analysis by polyacrylamide gel electrophoresis. Western blotting was performed with anti-17L serum to test for expression of the enzyme and with Flag monoclonal antibodies to check for processing of the precursor proteins. Each of the mutant 17L enzymes was expressed equally well (data not shown). Figure 4 presents the results of each core protein precursor transiently expressed along with 17L and each mutant 17L. The top panel shows results with P25K, the middle panel shows results with P4b, and the bottom panel shows results with P4a. As shown on Fig. 4, full-length 17L is capable of cleaving each precursor protein, but when H 241, W 242, D 248, Q 322, C 328, or G 329 is mutated to an alanine, this cleavage is lost. The only mutant 17L that was still capable of cleavage was D 258 mutated to an alanine, signifying that this mutant might not be a member of the catalytic triad. Cotransfection with PD258A showed that this protein was still capable of cleaving P25K and P4b, although cleavage of P4a was not seen.

To test whether 17L is capable of rescuing the growth and proteolytic processing activity of the ts16 virus, the virus was either grown alone, in the presence of transfected full-length 17L, in the presence of transfected mutant 17L, or with truncated 17L at the nonpermissive temperature. After 24 hours of infection, the virus-infected cells were harvested, and then the titers of the virus were determined to determine rescue. As shown in Table 1, full-length 17L was capable of rescuing the ts16 growth of ts16, indicating that 17L is indeed the gene product that is mutated in ts16. Neither the truncated 17L nor any of the mutant 17L enzymes was capable of rescuing growth of ts16 except for PD248A and PD258A.

The identity of the protein responsible for cleavage of the VV core protein precursors has recently been identified as the gene product of the 17L ORF (2). In this report, we further characterized the properties of this protein. The data reported here utilizing an in vivo trans-processing assay with an epitope-tagged substrate and plasmid-borne enzyme indicates that 17L is capable of driving the cleavage reaction and further verifies that it is the viral core proteinase. Mutational analysis has shown that for this reaction to occur, catalytic activity of 17L is required and the authentic cleavage site has to be present in the substrate. This appears to be a global effect in...
that 17L is able to cut at the authentic Ala-Gly-Ala sites of P4b and P25K as well as the Ala-Gly-Ser and Ala-Gly-Thr sites within P4a, although it appears that the cleavage of P4a and P4b is less efficient than that of P25K. Whether this finding reflects natural cleavage kinetics or fast versus slow cleavage sites or whether it is a consequence of the transient expression system remains to be determined.

In this study, we utilized an in vivo assay to look at the proteolytic processing of core protein precursors. This assay does not enable the identification of potential cofactors or the biochemical parameters of the cleavage reaction. While an in vitro transcription-translation system would be helpful to show if other viral proteins or induced cellular proteins are required for this processing, we have not yet succeeded in establishing this assay, which may be due to our incomplete understanding of the reaction or the hydrophobic nature of the 17L gene product.

The 17L protein is characterized as a cysteine protease because mutation of the histidine, cysteine, and aspartic acid residues eliminates proteolytic activity. In addition, the other highly conserved residues in the catalytic core domain (W 242, Q 322, C 328, and G 329) are all necessary for proteolysis to occur. Of the conserved amino acids mutated, the only residue that was not found to be essential for proteolysis was D 258. Truncation of the protein at amino acid 228 results in loss of processing of the core proteins, indicating that the amino terminal portion of the protein is necessary for either recognition or catalytic activity. It is not clear if the truncated protein is inactive because of the loss of essential activities inherent in this region or if this inactivity is due to an indirect effect on protein structure that disrupts essential folding needed by the catalytic domain. A series of site-specific mutants and truncations will be required to address this issue. One attractive and testable hypothesis might be that the N-terminal region of the protein has DNA-binding activity necessary to ensure virion packaging of the proteinase activity.

Regardless of the type of proteolytic maturation utilized by the virus during maturation, it is essential that the activity of the viral proteases be regulated to ensure efficient production of infectious progeny virions. It will be of interest to discover the trigger that signals the activation of the VV 17L protease and how it is regulated to carry out its activity at a distinct point in the virus life cycle.

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REFERENCES


