AN ABSTRACT OF THE DISSERTATION OF

Chelsea M. Byrd for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on April 8, 2005.

Title: Vaccinia Virus I7L Core Protein Proteinase

Abstract approved: Redacted for privacy

Dr. Dennis E. Hruby

Vaccinia virus (VV) is a large double-stranded DNA virus that is a prototypic member of the orthopoxvirus family. Previous works has showed that three of the major structural proteins found within the mature VV virion core 4a, 4b, and 25K are produced from higher molecular weight precursors at late times during infection and processed via a common morphogenic cleavage pathway that is intimately linked with virion assembly and maturation. The enzyme that carries out these cleavage reactions is unknown.

A transient expression assay was used to demonstrate that the I7L gene product and its encoded cysteine proteinase activity is responsible for cleavage of each of the three major core protein precursors. Cleavage was demonstrated to occur at the authentic Ala-Gly-Xaa cleavage sites and require active enzyme. A truncated I7L protein lost the ability to cleave the core protein precursors.

A conditional-lethal recombinant virus was constructed in which the expression of the I7L gene is under the control of the tetracycline operator/repressor system. In the absence of I7L expression, processing of the major VV core proteins is inhibited and

electron microscopy revealed defects in virion morphogenesis prior to complete core condensation. Plasmid-borne I7L is capable of rescuing the growth of this virus.

A structural model of I7L was developed and a unique chemical library was assayed for both cell toxicity and the ability to inhibit the growth of VV in tissue culture cells. A novel class of inhibitors was discovered that is capable of inhibiting VV.

An *in-vitro* cleavage assay was developed to further characterize the activity of I7L. This assay is based on producing the major core protein precursors in a coupled transcription and translation assay and then mixing them with I7L enzyme extracts. Using this assay, I7L is shown to be capable of cleavage of each substrate. I7L is further characterized as a cysteine proteinase due to the inhibitory effects of known cysteine proteinase inhibitors such as NEM and iodoacetic acid, as well as through the use of specific small molecule inhibitors in this *in-vitro* assay.

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Vaccinia Virus I7L Core Protein Proteinase

by

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Chelsea M. Byrd, Author

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CONTRIBUTION OF AUTHORS

In chapter two and three of this thesis, Tove' Bolken is a co-author of the work. She helped in the design of the experiments and edited the manuscripts.

In chapter five of this thesis, the co-authors include Tove' Bolken, Adnan Mjalli, Murty Arimilli, Robert Andrews, Robert Rothlein, Tariq Andrea, Mohan Roa, and Katrina Owens. Their contributions to this publication are as follows: Tove' Bolken helped screen the initial 4000 compounds and assisted with the generation of the results in figure 4. Adnan Mjalli, CEO of TransTech Pharma. Murty Arimilli, Associate Director of Chemistry at TransTech Pharma. Robert Andrews, vice president of chemistry at TransTech Pharma. Robert Rothlein, vice president of biology at TransTech Pharma. Dr's Arimilli, Andrews, and Rothlein served in a supervisiory capacity. Tariq Andrea and Mohan Roa developed the three-dimensional model of I7L depicted in figure 1. Katrina Owens provided technical support to verify the results. Mike Nesson performed the thin-sectioning and prepared the samples for the electron microscopy in figure 6.

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VACCINIA VIRUS I7L CORE PROTEIN PROTEINASE

CHAPTER 1

INTRODUCTION

Vaccinia Virus Life Cycle

Poxviruses, such as vaccinia virus (VV), are amongst the largest and most complex of the eukaryotic DNA viruses and are distinguished by replicating exclusively within the cytoplasmic compartment of infected cells. VV regulates the expression of its' more than 250 gene products in a temporal fashion during the viral replicative cycle that begins with entry of the virus into the host cell and terminates with the assembly of complex macromolecular structures to form an infectious particle. Figure 1.1 highlights the stages of the VV life cycle. Entry begins with attachment, fusion, penetration and the first phase of uncoating. There are three phases of gene expression, early, intermediate, and late. Early gene expression occurs through the action of enzymes and factors that are present within the incoming viral particle. After the early genes are expressed the viral core is further uncoated releasing the viral DNA which is replicated in the cytoplasm in an area termed the virosome or viroplasm (Buller & Palumbo, 1991). The virosome is a large inclusion body and also the site of later assembly of immature viral particles.

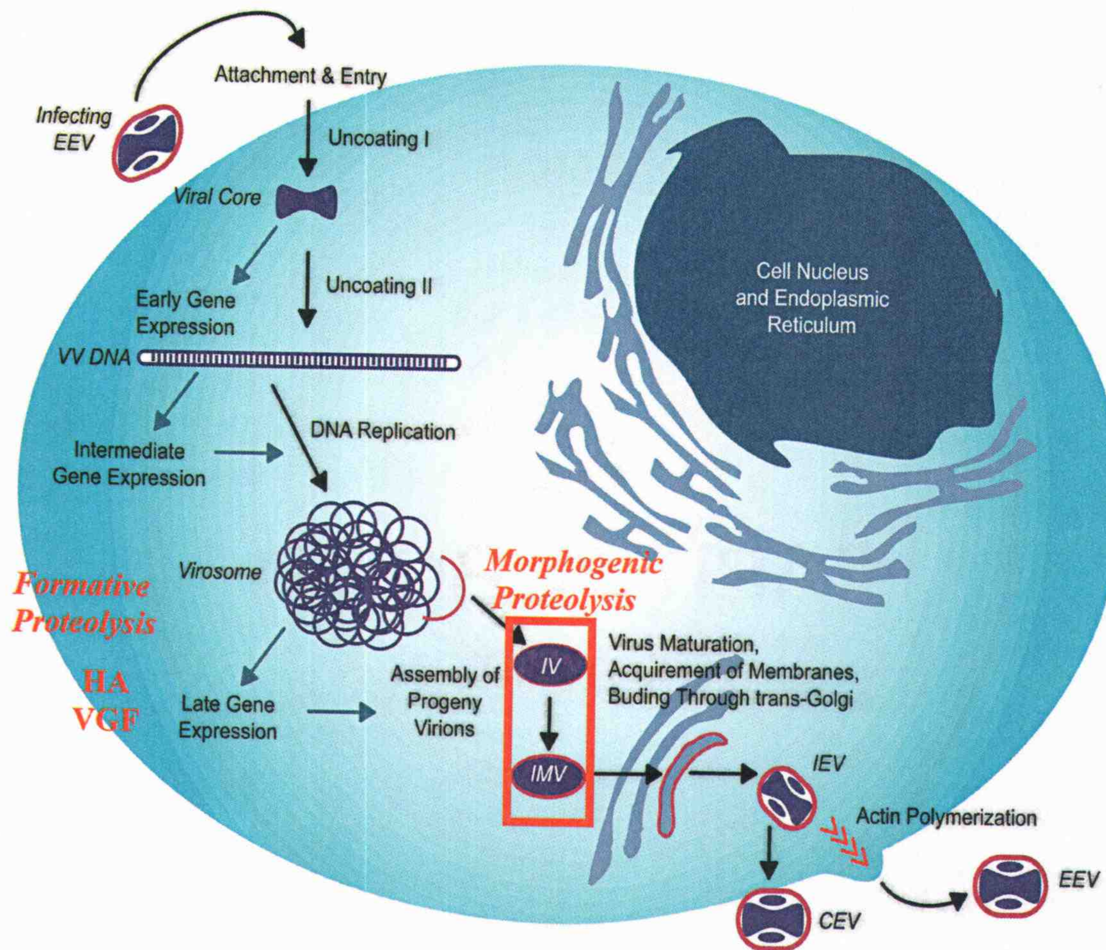

Figure1.1. Vaccinia virus life cycle

The initiation of late gene expression occurs as the transcription of early genes is attenuated. The late genes are transcribed and translated into polypeptides including enzymes and structural proteins, some of which are processed during virion morphogenesis. Virion assembly begins with the formation of crescent shaped particles that are assembled into immature particles. Core condensation, along with the associated enzymatic activities, occurs during this phase as the spherical immature particle matures into a brick-shaped intracellular mature virus (IMV).

Unlike many other viruses, VV produces multiple virion forms, all of which appear to be infectious. The four types of infectious virus produced are; intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV). IEV are formed as the IMV buds through the trans-Golgi network acquiring an additional membrane. The IEV can then move along microtubules toward the surface of the cell where it can either remain attached to the surface as CEV or initiate actin polymerization which releases the virus from the cell to produce the EEV.

It is well known that viruses, as obligate intracellular parasites, must use (and in some cases redirect) their hosts' metabolic pathways in order to replicate their genomes and synthesize the constituents needed to form progeny virions. What is perhaps less well appreciated are the logistical problems encountered by viruses during the replicative process. Once viral proteins have been synthesized, how does the virus ensure that these proteins assume active configurations (at the proper time), interact with the correct protein partners and find their way to specific intracellular locations in quantities sufficient to catalyze the efficient assembly of infectious virions? One of the major ways

that viruses solve these problems is by taking advantage of protein modification pathways (such as phosphorylation, glycosylation, acylation, and proteolytic cleavage) which are normally used to assist with intracellular trafficking of cellular proteins (Franke *et al.*, 1989; Rosemond & Moss, 1973; Garon & Moss, 1971; Hruby & VanSlyke, 1990).

The involvement of a proteinase activity in the infection cycle of vaccinia virus was first indicated with Holowczak & Joklik (1967), surveying the structural proteins of VV, noted differences between the apparent molecular masses of radiolabeled proteins present in VV-infected cells and those found in purified virions. It has since become appreciated that proteolytic processing is a crucial event in VV particle maturation. Indeed, the three most abundant proteins in the VV particle, 4a, 4b, and 25K, have all been demonstrated to be derived from higher molecular mass precursors (Moss & Rosenblum, 1973; Silver & Dales, 1982; Yang *et al.*, 1988).

Proteolysis

Proteases are enzymes that catalyze the hydrolysis of peptide bonds (Barrett, 1986). Proteases (or peptidases) can be subdivided into two categories; exoproteases and endoproteases. Exoproteases cut amino acids from the amino or carboxy termini of proteins, while endoproteases (or proteinases) cut specific peptide bonds between amino acids in the internal part of the substrate (Dougherty & Semler, 1993). There are two basic parts to a protease, a substrate-binding site that recognizes the protein, and a catalytic site nearby that carries out the cleavage reaction. Proteinases are further classified into four groups based on the identity of the amino acids in their catalytic site. The four groups or classes are serine, cysteine, aspartic, and metalloproteinases.

Serine proteinases have a catalytic triad composed of His, Asp, and Ser. The Ser residue is usually the amino acid that acts as a nucleophile during the reaction by donating an electron to the carbon of the peptide bond to be cleaved. A proton is then donated to the leaving amino group by the His residue. The Ser is hydrolyzed, the product released, and the active site is regenerated (Dougherty & Semler, 1993). Cysteine proteinases have a catalytic dyad composed of Cys and His residues. In some cases there is a catalytic triad with the addition of an Asp residue that helps in stability of the active site. The mechanism of action is similar to that of serine proteinases except that the nucleophile is a thiolate ion instead of a hydroxyl group. The sulfhydryl group of the cysteine residue acts as the nucleophile to initiate attack on the carbonyl carbon of the peptide bond to be cleaved. The imidazole ring of the His residue removes a proton from the sulfhydryl making it more nucleophilic. Catalysis proceeds through the formation of a covalent intermediate.

Unlike serine and cysteine proteinases, aspartic and metalloproteinases do not appear to form a covalent enzyme-substrate intermediate (Dougherty & Semler, 1993). Aspartic proteinases have a catalytic dyad composed of two Asp residues. These enzymes function as two molecules join together with the aspartic acid residues close together. Acid-base catalysis from an activated water molecule leads to the formation of a non-covalent tetrahedral intermediate. Metalloproteinases work through the action of a bound divalent cation, frequently Zn^{2+}, which is catalytically active. There is a conserved motif containing His-Xaa-Xaa-Glu-His or the inverse of this (where Xaa is any amino acid). The histidine and glutamic acid residues bind the zinc. As with aspartic proteinases, the

catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the zinc-bound water molecule attacks the carbonyl group of the scissile bond.

While the catalytic triad (or dyad) spacing is conserved among the same class of proteinases, the substrate binding pocket is unique in each case and is what distinguishes one proteinase from any other.

Proteolytic processing in viruses

There are two types of cleavage reactions, *cis* or *trans*. In *cis* cleavage events one precursor protein contains both the cleavage site and the proteinase, which is autocatalytic. In *trans* cleavage events, one protein contains the proteolytic activity and a second protein contains the substrate cleavage site. In the most basic case, proteinases are made in their active form, as has been shown through TNT reactions where the enzyme and substrate can be synthesized and activity demonstrated (Pelham, 1978; McGrath *et al.,* 1996; Andres *et al.,* 2001). Some proteinases need to be cleaved from a precursor protein in order to become activated while other proteinases require cofactors for catalysis to occur, such as in the adenovirus system where the protease requires both DNA and a specific peptide as cofactors (Webster *et al.,* 1993; Mangel *et al.,* 1993).

There are two types of proteolytic processing, formative and morphogenic. Formative proteolysis refers to the processing of viral polyproteins into structural and non-structural protein products. An example of formative proteolysis is the cleavage that occurs with the poliovirus polyprotein to produce each of the viral structural proteins. Morphogenic proteolysis, on the other hand, refers to the cleavage of viral structural proteins assembled in previrions during virion assembly. An example of morphogenic

proteolysis is the cleavage of some of the HIV precursor proteins into their mature form. Vaccinia virus uses both formative and morphogenic cleavage pathways. Formative cleavage is demonstrated by the removal of a signal peptide from both VV hemagglutinin (HA) encoded by A56R (Shida & Dales, 1982), and the glycoprotein encoded by B5R (Isaacs *et al.*, 1992). Morphogenic cleavage has been demonstrated with three of the major structural proteins found in the mature VV virion 4a, 4b, and 25K through pulse chase experiments. Figure 1.2 (VanSlyke & Hruby, 1994) is a pulse-chase immunoprecipitation with specific antisera that shows the three major core protein precursors (labeled on the left) being cleaved into their mature processed forms (labeled on the right). The late protein, L65, is also shown on the gel as an example of a protein that is not cleaved.

Most viruses use proteolytic processing at some stage in their life cycle. Posttranslational proteolytic processing is one of the ways that certain viruses regulate gene expression, as opposed to regulation at the transcriptional level. HIV-1, a retrovirus, encodes an aspartic proteinase (Pro) as part of a polyprotein precursor that must be cleaved out. The enzyme is active as a dimer complex in acidic environments where two catalytic centers are brought together at the dimer interface (Dougherty & Semler, 1993). Expression of the retrovirus proteinases occurs as the result of either suppression of an in-frame stop codon, or by ribosomal frameshifting. N-terminal modification (such as addition of myristic acid) may also influence retroviral proteinase activity (Henderson, *et al.*, 1983). The HIV protease is responsible for cleavage of each of the junctions of the viral polyprotein initially formed by translation of the viral mRNA.

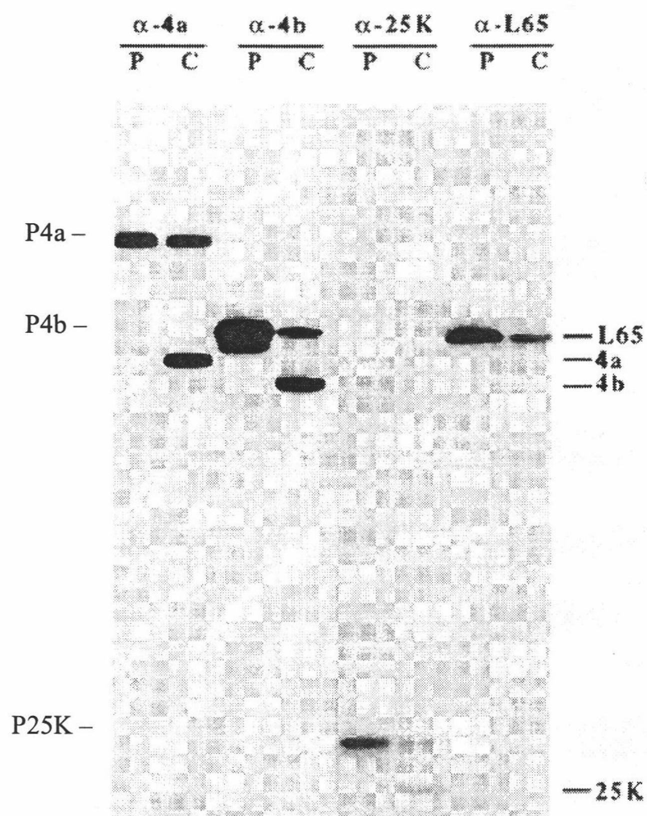

Figure 1.2. Morphogenic cleavage demonstrated with 3 major VV core protein precursors: P4a (A10L), P4b (A3L), and P25K (L4R)

VanSlyke & Hruby 1994

Hepatitis C virus (HCV), a flavivirus, produces a major translation product of about 3000 amino acids that is subject to both co- and post-translational proteolytic processing to generate all of the structural and nonstructural proteins. HCV encodes a serine proteinase (NS3) as part of this polyprotein from a single genome size mRNA. The NS3 proteinase is autocatalytic and forms a complex with NS4A, a viral membrane protein that acts as an activator of the proteinase activity. NS3 cleaves the HCV polyprotein at all of the junctions downstream of itself (Lohmann *et al.,* 1996). The proteinase also has a zinc-binding site formed by three cysteine and one histidine residue that has a role in maintaining the structural stability of the proteinase domain (De Fransesco *et al.,* 1998).

Adenovirus encodes a 23 kDa cysteine proteinase, the product of the L3 ORF that requires both DNA and a specific peptide as cofactors (Krausslich & Wimmer, 1988; Weber, 1990; Webster *et al.,* 1993). The adenovirus proteinase cleaves proteins at a conserved (Met, Ile, Leu)-Xaa-Gly-Gly-Xaa site, with cleavage occurring after the second glycine residue (Webster *et al.,* 1989; Anderson, 1990). Virus infectivity is dependent on the activity of the proteinase.

African swine fever virus (ASFV) encodes a cysteine protease encoded by the S237R gene that cleaves two polyproteins at Gly-Gly-Xaa sites into six major structural components of the core of the mature virus (Andres *et al.*, 2001). The catalytic domain of pS237R is similar to that of the adenovirus L3 protease, the UlpI endopeptidase from *Saccharomyces cerevisiae*, and the I7L protease of vaccinia virus.

Regardless of the function and the type of proteinase in the virus, it is essential that the activation of the proteinase be regulated in some fashion to prevent cleavage of

precursor proteins at the incorrect time. This regulation can be carried out in several ways including the requirement for proteolytic activation by zymogens (pro-enzymes that are expressed as an inactive precursor that is activated by catalysis), separation of the enzyme and substrate until the necessary time for cleavage, and the presence of specific inhibitors. This regulation is demonstrated in several ways. Adenoviruses require a peptide from one of the structural proteins, pVI, to activate the viral proteinase and lead to viral maturation (Webster *et al.*, 1991). In the case of HIV-1, premature activation of the proteinase has been shown to prevent virus assembly (Krausslich, 1991). Dimerization of the protease is essential to form a functional enzyme and may provide a mechanism to control the timing of processing of the polyprotein. With the ASFV proteinase, co-localization of the protease and polyproteins in viral structures within the virus factories is required for processing to occur, and repression of processing leads to the generation of empty particles lacking the nucleoid and core (Andres *et al.*, 2001). Activation of the HCV NS3 proteinase requires the presence of NS4A, a polyprotein cleavage product, to form a stable complex (Bartenschlager, 1999). In addition to positive regulation, the NS3 proteinase activity can also be inhibited by the cleavage products of several substrate peptides, including those from the NS4A/NS4B and NS4B/NS5A cleavage sites (De Francesco *et al.,* 1998).

Proteolytic processing in VV

Vaccinia virus uses morphogenic proteolytic processing during virion maturation as a number of structural proteins that are present in immature virions are cleaved into their mature form during assembly and maturation into intracellular mature virions.

Interference with this processing, either through mutation or drug treatment, prevents the assembly of mature virus particles.

Cleavage of three core protein precursors has been studied in detail. The core protein precursors, P4a, P4b, and P25K, products of the A10L, A3L, and L4R open reading frames respectively, are cleaved within a conserved Ala-Gly-Xaa motif, with cleavage occurring after the glycine residue (VanSlyke *et al*, 1991 a&b, VanSlyke & Hruby, 1994; Lee & Hruby, 1994&1995; Whitehead, *et al.,* 1995). Figure 1.3 is a representation of the core protein precursors with their associated cleavage sites identified. Cleavage of P25K is important for the correct interaction of this protein with viral DNA or other core proteins once assembled into viral cores (Yang *et al.*, 1988). Cleavage of P4a and P4b may be required for the proper rearrangement of the immature virion and concurrent core condensation necessary for the production of mature infectious virions. This hypothesis is supported first; by the observation that virion assembly is blocked in the absence of expression of P4a and that the cleavage product of P4a, 4a, is required for the correct assembly of the nucleoprotein complex within immature viral particles (Heljasvarra *et al.,* 2001), and second; by the observation that in temperature sensitive mutants mapping to the A3L gene, there is a defect in the transition from immature virions to intracellular mature virus and a subsequent lack of transcriptionally active virion particles (Kato *et al.,* 2004). Other vaccinia proteins have been shown to be cleaved at this conserved Ala-Gly-Xaa motif as well, including the gene products of the A17L and A12L open reading frames (Whitehead & Hruby, 1994b) as well as G7L (Takahashi *et al.,* 1994). Analysis of the core proteins has revealed several requirements for processing. Using a *trans* processing assay Lee & Hruby (1995)

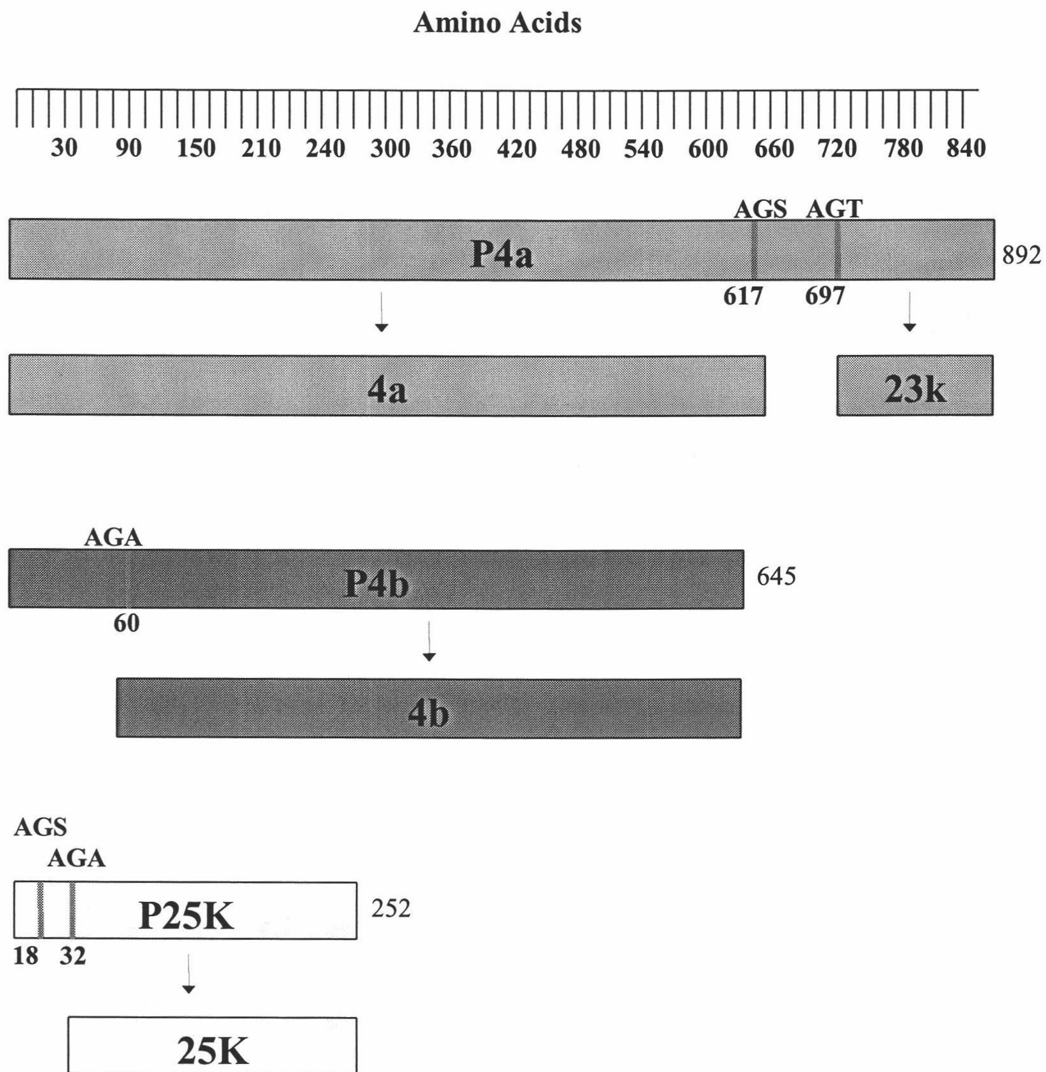

Figure 1.3. Vaccinia virus core protein precursor cleavage sites.

showed that the amino-terminal peptides of the VV core proteins are interchangeable and that a hydrophobic residue at position P4 of the cleavage site is required for processing of P25K. Insertion or deletion of sequences adjacent to the cleavage site interfered with cleavage suggesting other structural determinants (Lee & Hruby, 1994). The Ala-Gly-Xaa motif alone is not sufficient for proteolysis to occur. Rather, only those proteins that are expressed at late times during infection and are associated with the assembling virion are potential substrates for proteolytic cleavage (Whitehead & Hruby, 1994; VanSlyke *et al.,* 1993).

While there is abundant information about the structural requirements surrounding core protein cleavage (VanSlyke *et al.*, 1991 a&b; VanSlyke & Hruby, 1994; Lee & Hruby, 1994&1995; Whitehead, *et al.,* 1995; Moss & Rosenblum, 1973) until now the proteinase(s) responsible for carrying out these cleavage reactions has remained unknown.

As an approach to determining what type of proteinase might be the vaccinia virus core protein proteinase (vCPP), a collection of class-specific proteinase inhibitors were tested to determine their ability to inhibit VV replication in tissue culture cells. To that end BSC40 tissue culture cells were infected with VV in the presence of various concentrations of proteinase inhibitors. Efforts were made to use concentrations of drugs which had minimal effects on the tissue culture cells as judged by morphological appearance and thymidine incorporation. Inhibitors tested included; 1,10-phenanthroline, a metalloproteinase inhibitor (and it's non-chelating isomer, 1,7-phenathroline); iodoacetamide, a cysteine proteinase inhibitor; and pepstatin A, an aspartic proteinase inhibitor. Unfortunately, any and all serine proteinase inhibitors tested were acutely toxic

to the host cell, perhaps not a surprising result given the ubiquity of this type of proteinase in mammalian cells. Interestingly, VV replication was completely blocked by 10 μM iodoacetamide or 1 μM 1,10-phenanthroline whereas 1,7-phenanthroline or pepstatin A had no effect. These results are consistent with a metalloproteinase and a cysteine proteinase both playing an essential role in the viral replicative cycle.

There are several lines of evidence that implicate the gene product of the I7L open reading frame as the vaccinia virus core protein proteinase. This protein was originally identified as a putative proteinase on the basis of its homology to a ubiquitin-like proteinase in yeast (Li & Hochstrasser, 1999). It is predicted to be a cysteine proteinase and two potential active sites are evident. Condit and co-workers have isolated a temperature sensitive mutant in the I7L gene (Condit *et al.,* 1983). At the non-permissive 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 (Kane & Shuman, 1993). At the non-permissive temperature no infectious progeny are produced (Ericcson *et al.*, 1995).

I7L is highly conserved amongst the orthopoxviridae and is predicted to encode a 47 kDa protein that is expressed at late times post infection. Use of monospecific anti-I7L antisera has demonstrated that the protein is associated with virus factories, immature viral particles and IMV, where it is exclusively located in the core (Kane & Shuman, 1993).

Conclusions

The purpose of this thesis research is to discover and characterize the enzyme responsible to the cleavage of the core protein precursors of vaccinia virus. Further, it is hoped that once this enzyme has been characterized, a compound can be found that will specifically block the activity of this enzyme, inhibiting the replication of the virus and other viruses in this family.

CHAPTER 2

THE VACCINIA VIRUS I7L GENE PRODUCT IS THE CORE PROTEIN PROTEINASE

Authors: Chelsea M. Byrd, Tove' C. Bolken, and Dennis E. Hruby

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SUMMARY

Maturation of vaccinia virus (VV) core proteins is required for the production of infectious virions. The VV G1L and I7L gene products are the leading candidates for the viral core protein proteinase (vCPP). Using transient expression assays, data was obtained to demonstrate that the I7L gene product and its encoded cysteine proteinase activity are responsible for vCPP activity.

INTRODUCTION

Traditional antiviral compounds have focused on viral nucleic acid synthesizing enzymes, but since viruses are obligate intracellular parasites which utilize many of the host cell enzymes during their replication it has proved difficult to identify compounds that specifically block viral enzymes. Fortunately the emerging realization that most viruses use proteolysis catalyzed by viral-encoded proteinases as a key step in their developmental cycle has opened up a new class of targets for antiviral drug development. Recently proteinase inhibitors have been developed that specifically target HIV, rhinovirus, and influenza enzymes, and have proven very effective at preventing disease in the human host. Based on the fact that conditional lethal mutants and metabolic inhibitors of late protein synthesis such as α-amanitin result in assembly of immature particles but no proteolytic maturation and no infectivity, it appears that proteolytic maturation of orthopoxvirus core proteins is required for infectious progeny to be produced (Hruby *et al.,* 1979).

There are two types of proteolytic processing that occur during viral replication, formative and morphogenic (Hellen & Wimmer, 1992), both of which are used by poxviruses such as vaccinia virus (VV). Obligatory morphogenic cleavage has been demonstrated for three of the major structural proteins found in the mature VV virion, 4a, 4b, and 25K (VanSlyke *et al.,* 1991a), thereby providing a viable target for poxvirus antiviral drug development, the orthopoxvirus core protein proteinase (vCPP). The goal of the experiments reported here is to identify the vaccinia virus (VV) gene that encodes

the viral core protein proteinase (vCPP). Currently there are two putative VV proteinases, the products of the G1L and I7L open reading frame (ORF).

The VV G1L ORF encodes a 67-kDa late protein suspected to be a metalloproteinase by virtue its homology to the insulin degrading enzyme family of metalloproteinases. In common with this family, the G1L protein contains both the inverted H-X-X-E-H active site and a downstream E-N-E metal binding site. Furthermore, the G1L protein was previously demonstrated to direct the *in vivo* endoproteolytic cleavage of the VV P25K core protein precursor, albeit at a cryptic cleavage motif. This cleavage activity was inhibited if either the active site or metal binding domains were mutated, suggesting that G1L-mediated catalysis was required (Whitehead & Hruby, 1994).

The VV I7L ORF encodes an approximately 47-kDa late protein believed to be a cysteine proteinase due to its homology to the African Swine Fever virus (ASFV) proteinase and the adenovirus proteinase, both of which are known to process viral core proteins in their respective systems. The I7L protein, like these other enzymes, contains putative catalytic diad residues, histidine and cysteine, imbedded in a conserved region containing an aspartic acid. These enzymes, including I7L, also contain an invariant glutamine (Q) residue just upstream of the cysteine residue, which is predicted to form the oxyanion hole in the active site (Kim *et al.,* 2000). The I7L gene is known to be essential for viral replication because a conditional-lethal mutant, *ts16*, has been mapped to this locus (Ericsson *et al.,* 1995). Interestingly, at the non-permissive temperature *ts16* displays a defective late phenotype in which immature particles are assembled containing

uncleaved core protein precursors, consistent with I7L having a role in core protein processing.

RESULTS AND DISCUSSION

In order to determine whether G1L or I7L were the vCPP, it was necessary to develop an *in vivo* trans-processing assay as all previous attempts to demonstrate vCPP activity in cell-free extracts have failed. Earlier studies conducted in our lab have shown that the vCPP substrates include P4a, P4b, P25K and P17K (Lee & Hruby, 1994; Lee & Hruby, 1993; VanSlyke *et al.,* 1991a; Whitehead & Hruby, 1994) which are all proteolytically processed during viral assembly. Alignment of the cleavage sites in these precursors revealed a conserved AG*X cleavage motif (Whitehead *et al.,* 1995). For the current study P25K was used as the reporter substrate. P25K is the product of the L4R gene and was chosen because it is the smallest of the major core protein precursors and is relatively soluble. P25K contains two putative cleavage sites, a cryptic AG*S site at amino acids 17-19 and the AG*A site at amino acids 31-33 which is the authentic cleavage site (Fig. 2.1). The P25K precursor was tagged at the C-terminus with an octapeptide epitope, FLAG (Lee & Hruby, 1994), in order to monitor proteolytic cleavage of the substrate and distinguish it from the L4R gene product encoded within the viral genome. To further characterize the cleavage site, two mutations were made in the P25K open reading frame altering the amino acids at the two cleavage sites by site-directed mutagenesis. The first mutant has amino acids 17-19 changed from AGS to IDI and the second mutant has amino acids 31-33 changed from AGA to RDP (Fig. 2.1).

Substrate

(p25K-FLAG) p25K flag

site I site II

--DTIFFAGSISEYDDLQMVIAGAKSK--

12 24

IDI RDP

Enzymes

(pRB21:17L) I7L

344 ts16 mutation P → L

catalytic triad: HWKCVIYD QLLESEC

241 248 328

(pRB21:G1L) G1L

HLLEH ENE

40 112

(active site) (potential active site)

Figure 2.1. Structure of the p25K:FLAG, I7L, and G1L expression vector plasmids.

This assay system and these mutations were previously developed to allow a mutagenic analysis of the *cis*-signals required for P25K processing (Lee & Hruby, 1993).

Both the I7L and G1L gene products were expressed from the plasmid pRB21 that was constructed with a 6-His tag fused to the C-terminus and have a synthetic early/late vaccinia virus promoter. The 6-His tag was included to facilitate subsequent purification of the enzymes in the event they demonstrated activity in the assay. In Figure 2.1, the proposed catalytic diad of I7L is shown along with the conserved aspartic acid (His241, Asp248, Cys328), which are common to this family of cysteine proteinases. Also shown is the single amino acid that was found to be mutated in *ts16* (Pro to Leu at position 344) that conferred a temperature sensitive phenotype on the enzyme. The active sites of G1L are similarly indicated, both a HLLEH motif at amino acid 40-44 which is inversely related to the conserved HXXEX active sites of metalloproteinases, similar to insulin-degrading enzymes (Becker & Roth, 1992), and the ENE metal-binding site at position 112-114.

In order to test if either of these enzymes is the vCPP required for cleavage of the core protein precursor P25K, a transient expression assay was utilized in which cells were infected with VV and then transfected with plasmids encoding the P25K:FLAG reporter in the presence or absence of pRB21:I7L or pRB21:G1L Under these conditions, the virus supplies both RNA polymerase and trans-acting factors necessary to drive expression of the substrate and enzyme in the cytoplasm of the infected cells. The initial experiments were carried out using *ts16* as the source of super-infecting virus so that viral assembly would be blocked, perhaps providing essential co-factors to the processing reaction. Total cell extracts were prepared from the infected cells 24 hours after

transfection and subjected to immunoblot analysis using either rabbit anti-I7L polyclonal antiserum (data not shown), anti-G1L antiserum (data not shown) or mouse anti-FLAG M2 monoclonal antibody (Figure 2.2). As expected in uninfected cells, *ts16*-infected cells, or *ts16*-infected cells transfected with pRB21:I7L or pRB21:G1L, there is no specific substrate signal (Fig. 2.2, lanes 1-4). In the absence of a source of exogenous proteinase, p25K:FLAG appears as an unprocessed precursor protein with an apparent molecular weight of 28-kDa (Fig. 2.2, lane 5). When pRB21:I7L was co-transfected with P25K:FLAG, the P25K:FLAG substrate was completely cleaved to a 25-kDa species consistent with processing at the AG*A site (Fig. 2.2, lane 6). In contrast, co-transfection of pRB21:G1L with P25K:FLAG resulted in no demonstrable cleavage of the P25K precursor. This result strongly suggests that at least in regard to P25K, I7L appears to be the vCPP. Furthermore, cleavage of P4b was also rescued by I7L in *ts16* infected cells (data not shown).

We next sought to determine whether the apparent I7L processing of the P25K:FLAG precursor could be observed in cells infected with wild-type VV instead of *ts16* (Figure 2.3). This experiment was performed to determine if the reaction would proceed while viral maturation was occurring and to ensure that the *ts16* result was not the result of an unmapped second site mutation. As can be seen in lane 4, partial conversion of P25K:FLAG to 25K:FLAG is observed in the absence of plasmid-derived I7L. Co-expression of pRB21:I7L with P25K:FLAG drove processing to completion and produced a product with the same apparent molecular weight (Fig. 2.3, lane 5). Analysis of this blot with anti-I7L antisera demonstrated the presence of the wild-type VV I7L as a 47-kDa band in lane 4, with an increased signal in lane 5 (data not shown), consistent

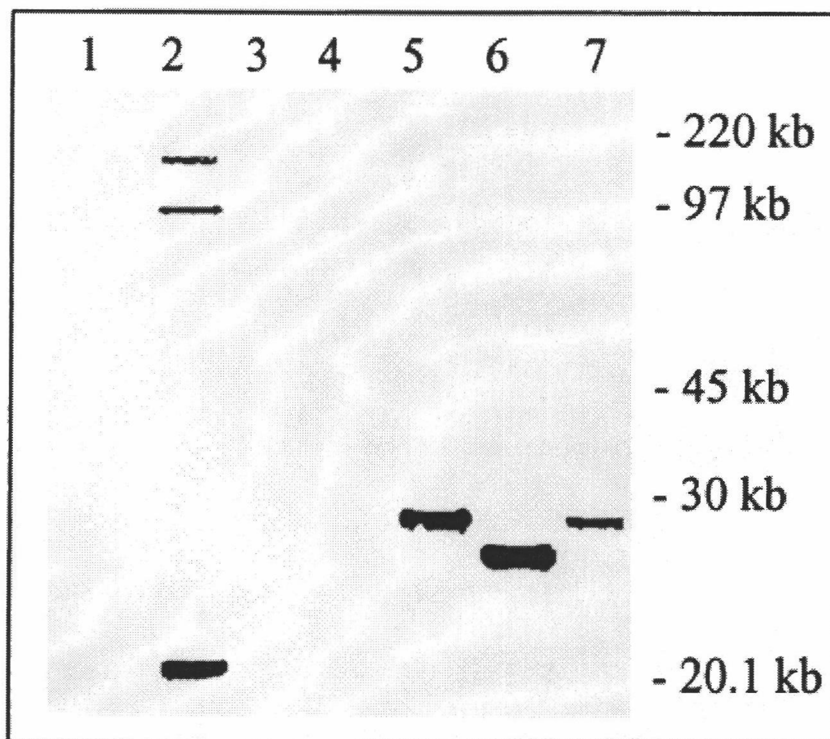

Figure 2.2. Trans-complementation of P25K:FLAG processing in *ts16*-infected cells. BSC_{40} cells were infected with a temperature sensitive mutant of vaccinia virus (*ts16*) and transfected with either pRB21:17L, pRB21:G1L, P25K:FLAG, or a combination of these and cleavage of the P25K:FLAG substrate was determined by Western blotting using anti-FLAG mAb. Lane 1 are uninfected cells as a negative control, lane 2 is *ts16* virus alone, lane 3 is *ts16* with I7L, lane 4 is *ts16* with G1L, lane 5 is *ts16* with the P25K:FLAG substrate, lane 6 is *ts16* with I7L and p25K:FLAG, and lane 7 is *ts16* with G1L and P25K:FLAG.

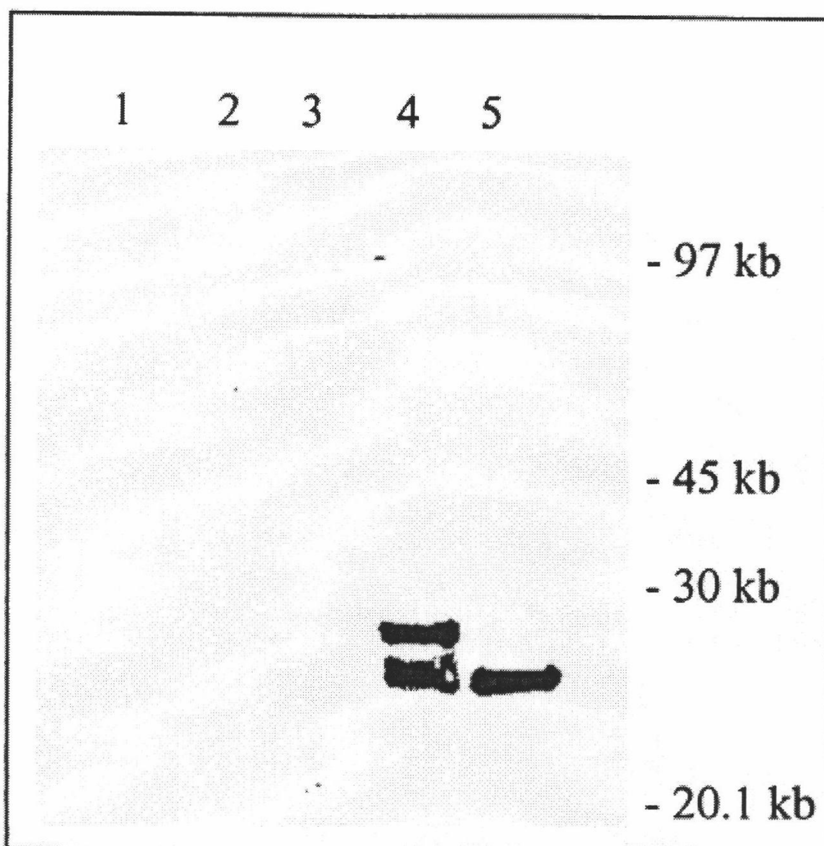

Figure 2.3. Trans-complementation of P25K:FLAG processing in wild-type VV-infected cells. Cells were infected with wild type vaccinia virus and transfected with either pRB21:17L, P25K:FLAG, or a combination of these and cleavage of the P25K:FLAG substrate was determined by Western blotting using anti-FLAG mAb. Lane 1 is cells alone, lane 2 is wt VV, lane 3 is VV with I7L, lane 4 is VV with p25K:FLAG, and lane 5 is VV with I7L and p25K:FLAG.

with the extent of processing being dictated by the amount of I7L protein that was present.

In order to demonstrate that the putative proteinase activity of I7L was directly involved in the processing of the P25K:FLAG precursor and that processing was occurring at the authentic A-G-A site, we utilized a site-specific mutagenesis approach in combination with the trans-complementation assay in *ts16*-infected cells incubated at the non-permissive temperature (Figure 2.4). As can be seen in lanes 1-3, no anti-FLAG reactive proteins were detected in control cells, *ts16*-infected cells or *ts16*-infected cells transfected with pRB21:I7L alone. Lane 4 demonstrates that a 28-kDa immunoreactive band is present when P25K:FLAG is transfected into infected cells and that this precursor is quantitatively converted to a 25-kDa species when pRB21:I7L is co-transfected (lane 5). Processing of the P25K:FLAG precursor was not abrogated when the cryptic A-G-S was mutated to I-D-I (lane 6), a mutation previously shown to inhibit cleavage by the G1L gene product (Whitehead & Hruby, 1994). In contrast, when the authentic A-G-A site was mutated to R-D-P, there was only minimal processing and the size of the product is consistent with cleavage at the upstream A-G-S site (lane 7). This cleavage activity could either be mediated by the I7L gene product, or more likely, by the G1L gene product as previously described. Also, as previously described, it should be noted that insertion of the proline residue results in slightly faster migration of the P25K:FLAG precursor, but this doesn't affect access to the A-G-S site (Lee & Hruby, 1993). Finally, the effect of mutating the putative active site of the I7L protein was investigated. As shown in lane 8, Figure 2.4, mutation of the histidine residue at position 240 in I7L to an alanine completely blocked I7L-mediated cleavage of the P25K:FLAG precursor. Taken

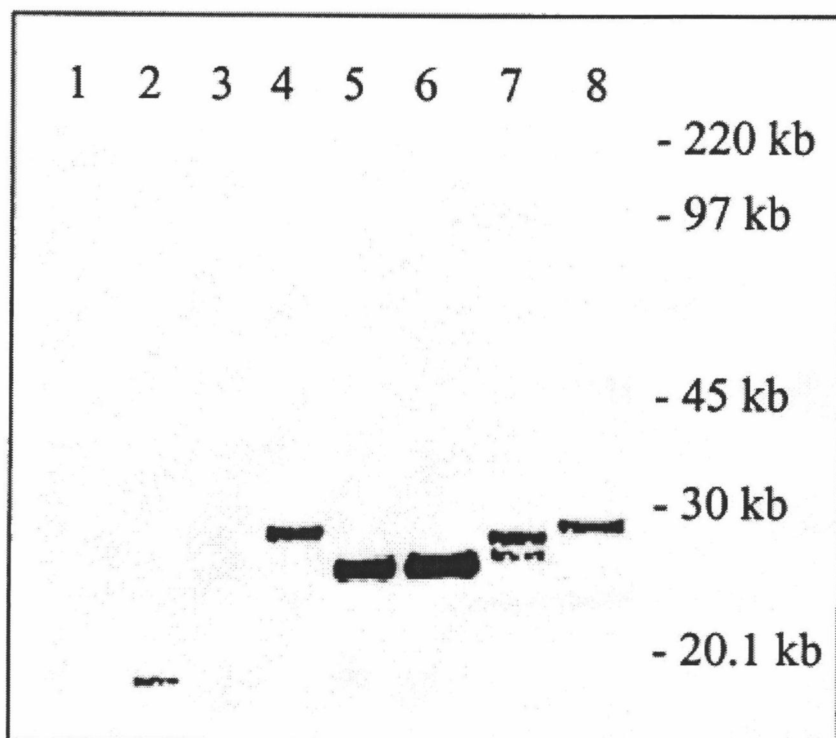

Figure 2.4. Mutational analysis of the enzyme and substrate requirements in the trans-complementation of P25K:FLAG processing. Cells were infected with *ts16* virus and transfected with either pRB21:17L, P25K:FLAG, P25K:FLAG:IDI, P25K:FLAG:RDP, pRB21:I7L:mut, or a combination of these and cleavage of the P25K:FLAG substrate was determined by Western blotting using anti-FLAG mAb. Lane 1 is cells alone, lane 2 is *ts16* alone, lane 3 is *ts16* with I7L, lane 4 is *ts16* with p25K:FLAG, lane 5 is *ts16* with I7L and p25K:FLAG, lane 6 is *ts16* with I7L and the p25K:IDI mutant, lane 7 is *ts16* with I7L and the p25K:RDP mutant, and lane 8 is *ts16* with the I7L:mutant and p25K:FLAG.

together, these data support the conclusion that the I7L gene product is cleaving the P25K:FLAG precursor at the authentic A-G-A site and that this reaction requires the I7L gene product to be catalytically active. This conclusion was supported by results of an experiment in which the replication of *ts16* was rescued 15-fold by a plasmid copy of I7L whereas the mutant I7L (mutated in the active site) was incapable of rescuing replication (data not shown).

Previous studies in our laboratory have identified the unique *cis* signals required to direct endoproteolytic cleavage of core protein precursors (Lee & Hruby, 1993), established the contextual requirements of core protein maturation (Lee & Hruby, 1995), and suggested strongly that the proteinase which carries out this essential reaction is viral-encoded. The transfection experiments have demonstrated that the gene product encoded by the I7L ORF is likely to be the proteinase that recognizes and cleaves the canonical A-G-A motif found in several of the major core protein precursors (Figures 2.2 - 2.4). This conclusion is supported by the studies of Condit and Shuman, working with the *ts16* mutant, who demonstrated that I7L was an essential late gene product (Condit *et al.,* 1983; Kane & Shuman, 1993). At the non-permissive temperature the core protein precursors are synthesized but not processed and no infectious progeny are produced, which is consistent with a proteinase-minus phenotype (Ericsson *et al.,* 1995).

Assuming that the I7L gene product is the major vCPP involved in VV core protein maturation, the issue is raised of what the function of GIL-encoded metalloproteinase is, and what its *in vivo* substrates are. Previous experiments demonstrated that the GIL gene product has proteolytic activity *in vivo* on an A-G-S site and the present work conclusively shows that the I7L gene product recognizes and cuts at

an A-G-A site. These data give rise to the following hypothesis: that both enzymes are involved in VV core protein maturation, with I7L recognizing the A-G-A motifs found in P25K and P4b, and G1L recognizing the A-G-S, A-G-T and A-G-K motifs found in P4a and P21K. There are several lines of evidence supporting the idea that there are functionally two different classes of A-G-X motif: 1) A-G-A motifs are found near the N-termini of precursor proteins whereas A-G-S/T/K motifs are found within the interior of the precursor proteins; 2) A-G-A sites are cleaved more rapidly than are A-G-S and A-G-T sites (VanSlyke *et al.,* 1991a); and 3) Mutagenesis studies have shown that A-G-A sites are flanked by conserved features [upstream by a V or I residues at the –4 position, relative to the scissile bond, and downstream by a number of basic residues (R or K)] which are essential for efficient cleavage (Lee & Hruby, 1994). A-G-S and A-G-T sites lack these features but still cleaved efficiently. Taken together this suggests that there may be two subclasses of A-G-X sites within VV core protein precursors that are recognized by two separate and distinct proteinases. The potential biological relevance of this hypothesis during virion assembly remains to be examined, but it may well play a regulatory role ordering processing reactions such as that proposed for the fast and slow cleavage sites within the potyvirus polyprotein (Dougherty & Parks, 1989).

Having two poxvirus proteinases apparently involved in virion maturation provides two targets for poxvirus antiviral drug development. This is highly advantageous for several reasons. First, not all targets are equally "drugable" due to inherent structural features of the protein. Second, for any given target there can be specificity issues. Along that line, I7L shares sequences with the ASFV proteinase that processes proteins at G-G-X sites (Andres *et al.,* 2001) and with the adenovirus protease

that cleaves at G-G-A sites (Orth *et al.,* 2000), and it shares the critical residues that surround the catalytic triad of a proteinase found in the ubiquitin pathway of protein degradation, SUMO-1 (Andres *et al.,* 2001), as well as the YopJ proteinase of *Yersinia pestis* (Orth *et al.,* 2000). Thus, I7L inhibitors could be broad spectrum anti-infectives, or could lack the specificity required to be effective drugs. Finally, due to inherently high mutation rates, viruses have the ability to rapidly acquire resistance when exposed to drug selection. Having inhibitors directed at two targets, one a cysteine proteinase (I7L) and the other a metalloproteinase (G1L) will provide the opportunity to use both in combination to achieve synergistic inhibition, or to reserve one family of inhibitors as a drug of last resort. Given the current concerns regarding smallpox as an agent of bioterrorism, it is essential that effective poxvirus antiviral drugs are developed and available in our pharmaceutical repertoire to complement the existing vaccine. Furthermore, such drugs should be effective in the event that other orthopoxvirus pathogens find their way into the human population. Fortunately, the orthopoxviruses are highly related at the DNA level (e.g. 90% between variola and vaccinia) making it likely that any antiviral agent developed would inhibit the replication of this entire group of viruses (Esposito & Knight, 1985).

CHAPTER 3

MOLECULAR DISSECTION OF THE VACCINIA VIRUS I7L CORE PROTEIN PROTEINASE

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SUMMARY

The vaccinia virus (VV) I7L gene product is predicted to be a cysteine proteinase and is demonstrated here 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.

INTRODUCTION

Vaccinia virus (VV) is a large double-stranded DNA virus with a cytoplasmic site of replication. It encodes over 200 open reading frames (ORFs) and has been extensively used as a eukaryotic cloning and expression vector, and for vaccine research. Vaccinia virus 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 to note that the gene product of the I7L open reading frame in VV, which is predicted to be the core protein protease (Byrd *et al.,* 2002), shares 99% identity with the homologous K7L gene in variola major virus.

Most viruses, including poliovirus, human immunodeficiency virus, and adenovirus, use post-translational proteolytic processing as an essential step in their replication cycles (Krausslich & Wimmer, 1988). Therefore it was not surprising to discover that proteolytic maturation of orthopoxvirus core proteins appears to be required for infectious progeny to be produced (Hruby *et al.,* 1979b). Three of the major structural proteins found within the mature VV virion core 4a, 4b, and 25K were known to be produced from higher molecular weight precursors at late times during infection (Silver & Dales, 1982). VanSlyke *et al* (1991a,b) demonstrated that a large number of VV core proteins, including 4a, 4b, and 25K, appear to be processed via a common morphogenic 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 protease (Li & Hochstrasser, 1999, 2000), the adenovirus protease (Webster *et al.,* 1993; Chen *et al.,* 1993; Freimuth & Anderson, 1993), and the African Swine Fever Virus (ASFV) protease (Andres *et al.,* 2001).

The gene product of the I7L open reading frame in VV was originally identified as a putative proteinase due to its homology to an ubiquitin-like proteinase in yeast (Li & Hochstrasser, 1999), and was recently shown to be one of the proteinases responsible for cleavage of the vaccinia virus core proteins (Byrd *et al.,* 2002). 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 vaccinia virus 17L open reading frame is predicted to encode a 423 amino acid protein with the catalytic domain located towards the carboxy terminus of the protein. Figure 3.1 shows a predicted hydrophobicity plot of the I7L protein using the Kyte-Doolittle program. The residues above the zero line are hydrophobic, those beneath

are hydrophilic. There are several hydrophobic domains near the amino terminus and again near the carboxy terminus of the protein. The positions of the residues of the putative catalytic triad (H, D, and C) are indicated as well as the positions of four other highly conserved amino acids (W, D, Q, G). Also shown is the position of the *ts16* mutation where a proline was altered to a leucine (Kane & Shuman, 1993) creating a temperature sensitive virus capable of growth at 31°C but not at 41°C. The *ts16* virus was originally isolated by Condit *et al* (1983). Shown below the hydrophobicity profile are the variola virus, camelpox, and monkeypox enzymes with positions of variance from VV I7L indicated with bars showing 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 open reading frame 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 was done to remove both the N-terminal hydrophobic region as well as to remove the region of the protein that was previously determined to have similarity to a topoisomerase. This is depicted relative to full length I7L. Finally, Li and Hochstrasser (1999) and Andres *et al* (2001) have identified a conserved catalytic core domain between VV I7L, the ASFV protease, the adenovirus protease, and the *Saccharomyces cerevisiae* protease with several highly conserved amino acids. This is indicated in the bottom of Figure 3.1 with arrows pointing at the conserved amino acids.

Figure 3.1. Characterization of I7L. Predicted hydrophobicity plot of the I7L protein using Kyte-Doolittle program. Positions of seven highly conserved amino acids including the putative catalytic triad are indicated with arrows as well as the position of the *ts16* mutation. The positions of the amino acids are indicated on the x-axis. Sequence similarity to the corresponding gene in Variola virus, Camelpox virus, and Monkeypox virus are indicated underneath by rectangles with positions of differing amino acids indicated at the correct position by a black bar. Sequence similarity of the conserved catalytic domain between VV I7L, the ASFV protease, adenovirus protease (ADE2), and a yeast cysteine protease (Ulp-1) is indicated in the bottom of the figure with arrows pointing at the highly conserved amino acids.

RESULTS AND DISCUSSION

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 non-permissive temperature and co-transfected with plasmid-born- substrate and enzyme. Both the substrate proteins and I7L protease are constitutively expressed *in-vivo* 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 blot and differentiation from the analogous gene product expressed from the viral genome. Figure 3.2 is a map of the 3 major core protein precursors P4a, P4b, and P25K which are products of the A10L, A3L, and L4R open reading frames respectively, with the previously determined cleavage sites indicated on them. These cleavage sites have all been mapped to an Ala-Gly-Xaa motif (VanSlyke *et al.,* 1991a; Whitehead & Hruby, 1994). P4a is the largest precursor protein with a molecular weight of 98 kDa and contains both an Ala-Gly-Ser and 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

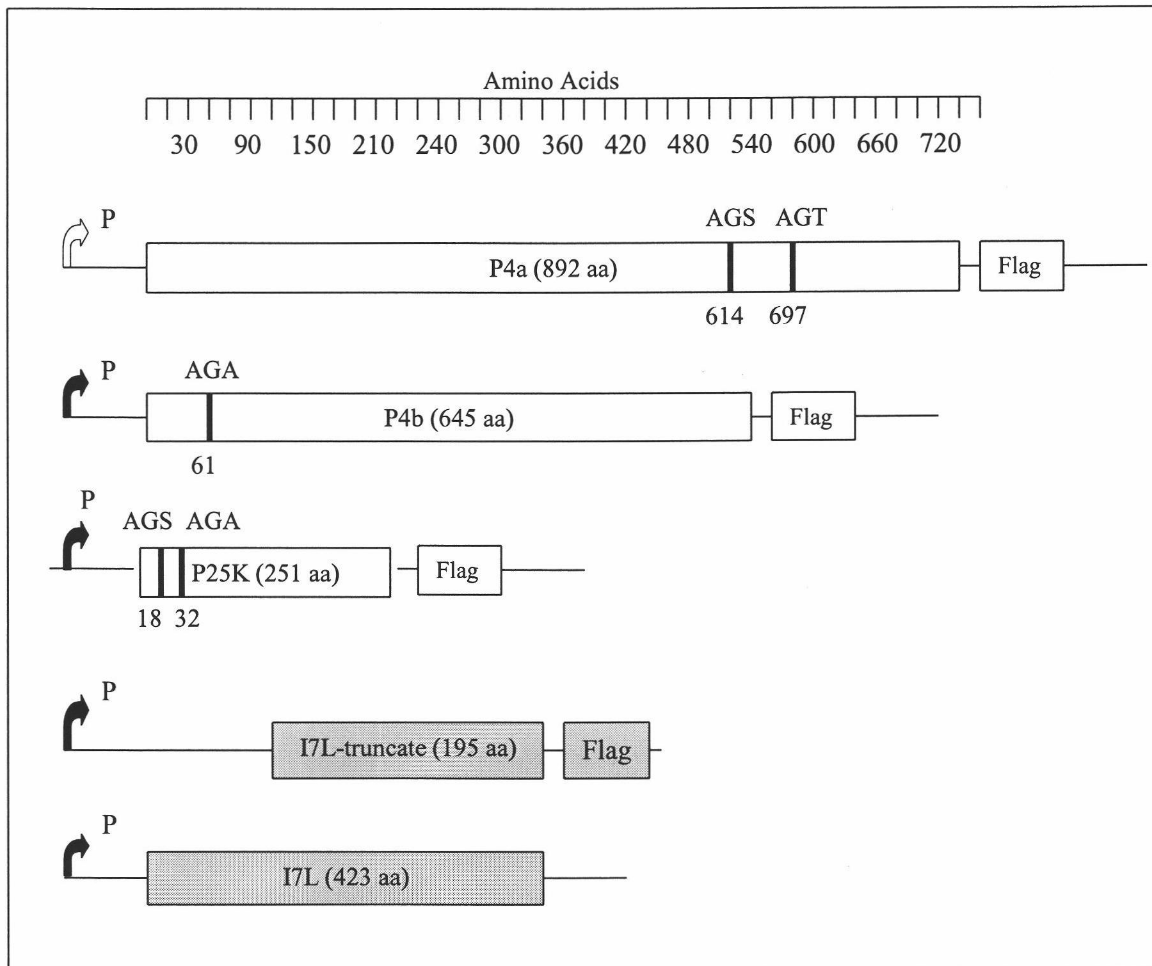

Figure 3.2. VV Core Protein Cleavage Sites. Schematic representation of the three major core protein precursors (P4a, P4b, and P25K) along with full length and truncated I7L. The positions of the AGX cleavage sites are indicated with the amino acid number of the glycine.

(pI7L-T), which was truncated at amino acid 228 leaving residues 229 through 423, with a re-engineered start site.

Cleavage of P25K by I7L has been shown previously (Byrd *et al.,* 2002), but here we demonstrate that I7L is capable of directing cleavage of the other core protein precursors as well. BSC_{40} cells (Raczynski & Condit, 1983) were infected with *ts16* VV at a multiplicity of infection (moi) of 5 and transfected with 10 μg of plasmid DNA containing either I7L, P4b, P4a or a mixture of these via a liposome-mediated transfection protocol. Cells were harvested 24 h post-infection and the extracts analyzed by Western blot with anti-Flag antisera. Figure 3.3A indicates that I7L cleaves P4b from its precursor form to the mature processed form (3.3A, 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 (3.3A, lane 3). Lanes 1 and 4 are controls showing P4b and P4bIDI expressed alone. When the AGA site of P4b is mutated to IDI residues, no cleavage by I7L is observed (3.3A, 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 Figure 3.3B. When P4a is expressed alone (3.3B, lane 1) it runs at its mature size of 98 kDa but when I7L is transfected in with P4a, two cleavage products are observed around 22 kDa and 32 kDa (3.3B, lane 2) indicating that cleavage is occurring at both the AGS and AGT sites. Mutation of I7L abolishes this cleavage (3.3B, lane 3). When the AGS site of P4a is mutated to an IDI, and the transfection is carried out with I7L, only one band around 22

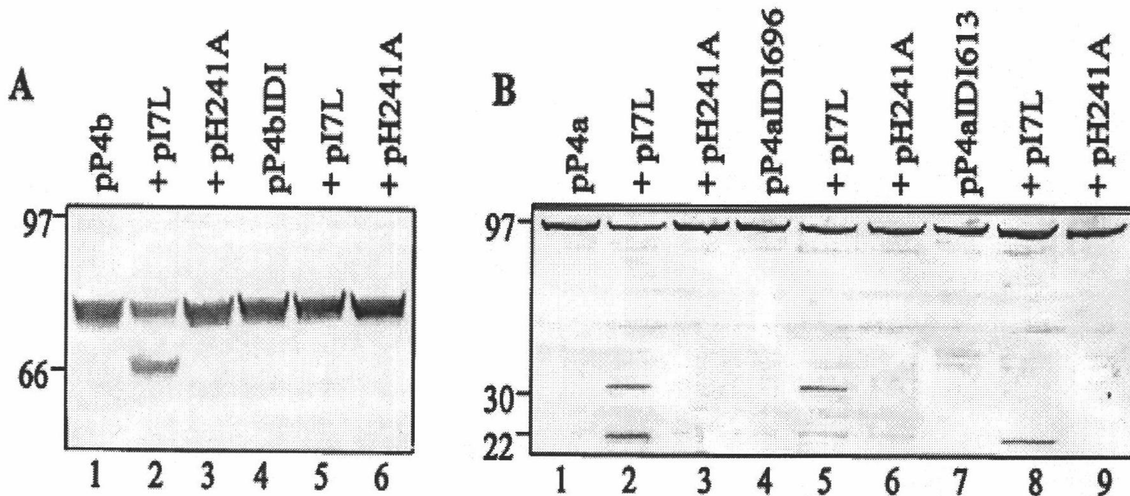

Figure 3.3. Proteolytic Processing of the Core Protein Precursors. BSC40 cells were infected with *ts16* VV and transfected with plasmids containing either I7L, P4a, P4b or a mixture of these. Cells were harvested 24 h post-infection and the extracts analyzed by Western blot with anti-Flag antisera. (A) Processing of P4b: In each lane cells are infected with *ts16* and then transfected with either substrate alone or substrate plus enzyme. The substrate is pP4b or pP4bIDI (where the AGA site is mutated to an IDI) while 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 substrate plus enzyme. In this case the substrate is either pP4a, pP4aIDI696 (P4a with the AGT site mutated to an IDI), or pP4aIDI613 (P4a with the AGS site mutated to an IDI).

kDa is observed exhibiting cleavage at only the AGT site (3.3B, lane 8). A similar result is obtained when the AGT site is mutated to an IDI and transfected with I7L (3.3B, lane 5) where a band around 30 kDa is observed showing that cleavage is blocked at the IDI site but is still occurring at the AGS site. This shows that the catalytic activity of I7L is necessary for cleavage to occur as well as the presence of the authentic cleavage sites.

To further characterize whether the catalytic domain of the I7L protein was necessary and sufficient for recognition and cleavage of the core protein precursors, a truncated I7L was created with the N-terminus removed up to amino acid 228. This truncated I7L was cloned into a plasmid behind the synthetic early/late promoter. The *trans*-processing assay was repeated with this I7L truncate. The I7L 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 I7L, 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 *ts16* VV at a moi of 5 and transfected with 10 μg plasmid DNA using DMRIE-C liposome-mediated reagent. Virus infected cells were harvested 24 hpi, centrifuged, and the resuspended pellet subjected to three cycles of freeze-thaw to release the virus from the cell. The supernatant was used for PAGE analysis. Western blots were performed using anti-I7L serum to test for expression of the enzyme as well as with FLAG monoclonal antisera to check for processing of the precursor proteins. Each of the mutant I7L enzymes was expressed equally well (data not

shown). Figure 3.4 shows each core protein precursor transiently expressed along with I7L and each mutant I7L. The top immunoblot is with P25K, the middle is with P4b, and the bottom immunoblot is with P4a. Figure 3.4 indicates that full length I7L 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 I7L that was still capable of cleavage was when D 258 was mutated to an alanine signifying that this might not be a member of the catalytic triad. Co-transfection with pD258A showed that this protein was still capable of cleaving P25K and P4b, although cleavage of P4a was not seen.

To test whether I7L 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 I7L, in the presence of transfected mutant I7L or with truncated I7L at the non-permissive temperature. After 24 hr of infection the virus infected cells were harvested and then titered to determine rescue. As shown in Table 3.1 full length I7L was capable of rescuing the growth of *ts16* indicating that I7L is indeed the gene product that is mutated in *ts16*. Neither the truncated I7L, nor any of the mutant I7L enzymes were 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 I7L open reading frame (Byrd *et al.,* 2002). 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 has indicated that I7L is capable of driving the cleavage reaction and further verify that it is the viral core protein proteinase. Mutational

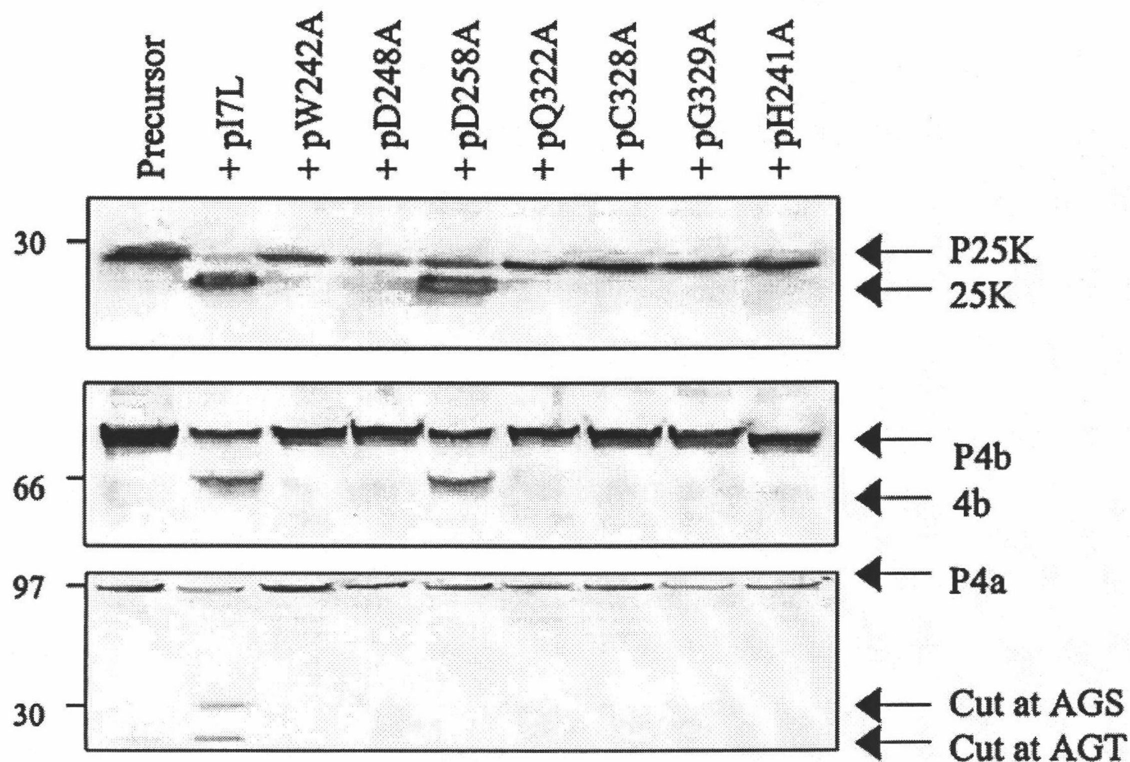

Figure 3.4. Ability of Mutant I7L Enzymes to Cleave the Core Protein Precursors. BSC_{40} cells were infected with *ts16* VV and transfected with plasmids containing either P4a, P4b, or P25K and cotransfected with either pI7L, or one of the seven mutant I7L plasmids. Cells were harvested 24 h post-infection and the extracts analyzed by Western blot with anti-Flag antisera to determine cleavage of the precursor protein. Top is P25K transfected with each mutant I7L plasmid, middle is P4b transfected with each mutant I7L plasmid, and bottom is P4a transfected with each mutant enzyme.

Table 3.1. Rescue of the growth and proteolytic processing activity of vaccinia virus *ts16* by I7L and I7L mutants[a]

Plasmids	Amt of VV *ts*16 (titer)	Amt of folding rescued
*ts*16	1.2×10^6	1
pI7L	9.2×10^6	7.7
pI7L-T[b]	1.4×10^6	1.2
pH241A	1.2×10^6	1.0
pW242A	1.3×10^6	1.1
pD248A	2.4×10^6	2.0
pD258A	4.3×10^6	3.6
pQ322A	1.3×10^6	1.1
pC328A	1.2×10^6	1.0
pG329A	1.3×10^6	1.1

[a] BSC_{40} cells were infected with *ts16* VV and transfected with plasmids containing either full length I7L, truncated I7L, or one of the mutant I7L, at the nonpermissive temperature. Cells were harvested 24 h post-infection and tittered in 6-well plates to determine the ability to rescue viral replication. The fold rescue was determined by dividing the titer by the titer of *ts16*.

analysis has shown that for this reaction to occur, catalytic activity of I7L is required and the authentic cleavage site has to be present in the substrate. This appears to be a global effect in that I7L 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 would appear that the cleavage of P4a and P4b is less efficient than that of P25K. Whether this reflects natural cleavage kinetics, fast versus slow cleavage sites, or if it is a consequence of the transient expression system remains to be determined.

In this study we have utilized an *in-vivo* assay to look at the proteolytic processing of core protein precursors. This assay does not enable the identification of potential co-factors 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. This may be due to our incomplete understanding of the reaction or the hydrophobic nature of the I7L gene product.

The I7L 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, 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 a 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 whether this 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 proteinases 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 I7L protease and how it is regulated to carry out its activity in a distinct point in the virus life cycle.

CHAPTER 4

A CONDITIONAL-LETHAL VACCINIA VIRUS MUTANT DEMONSTRATES THAT THE I7L GENE PRODUCT IS REQUIRED FOR VIRION MORPHOGENESIS

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Virology Journal

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SUMMARY

A conditional-lethal recombinant virus was constructed in which the expression of the vaccinia virus I7L gene is under the control of the tetracycline operator/repressor system. In the absence of I7L expression, processing of the major VV core proteins is inhibited and electron microscopy reveals defects in virion morphogenesis subsequent to the formation of immature virion particles but prior to core condensation. Plasmid-borne I7L is capable of rescuing the growth of this virus and rescue is optimal when the I7L gene is expressed using the authentic I7L promoter. Taken together, these data suggest that correct temporal expression of the VV I7L cysteine proteinase is required for core protein maturation, virion assembly and production of infectious progeny.

INTRODUCTION

Proteolytic cleavage of precursor proteins is an essential process in the life cycle of many viruses, including vaccinia virus (VV). The cysteine proteinase encoded by the VV I7L gene, was originally identified based on a sequence comparison with the African Swine Fever virus proteinase and an ubiquitin-like proteinase in yeast (Andres *et al.*,2001; Li & Hochstrasster, 1999). We have previously shown through *trans* processing assays that the I7L gene product is capable of cleaving the core protein precursors p4a, p4b, and p25K at conserved AG/X sites and have used reverse genetics to identify active site residues (Byrd *et al.,* 2002; Byrd *et al.,*2003). To determine the role that the I7L proteinase plays in the VV replication cycle, we report here the construction and *in-vivo* analysis of a VV mutant in which the expression of the I7L gene can be conditionally regulated.

While this work was in progress, Ansarah-Sobrinho and Moss (2004) published a report demonstrating that the I7L proteinase, in an inducible mutant virus regulated by the *lac* operator and driven off of the T7 promoter, was responsible for cleaving the A17L membrane protein as well as the L4R core protein precursor. In this work, we show that I7L proteinase, in a different inducible mutant virus, this one regulated by the tetracycline (TET) operator/repressor system and driven off of the I7L native promoter, is responsible for cleaving the other core protein precursors (p4a and p4b). We also demonstrate that expression of the I7L gene from its native promoter appears to be important for optimal viral assembly and replication.

RESULTS AND DISCUSSION

To investigate the role of the I7L proteinase in the viral life cycle, an inducible mutant virus was constructed in which the expression of the I7L gene could be regulated by the presence or absence of TET using the components of the bacterial tetracycline operon (Hillen *et al.,* 1982; Jorgensen & Reznikoff 1979). This system has been shown to be successful in the regulation of the vaccinia virus G1L (Hedengren-Olcott & Hruby 2004; Hedengren-Olcott *et al.,* 2004) and A14L (Traktman *et al.,* 2000) genes. A plasmid was constructed containing the tetO just upstream of the I7L open reading frame (ORF) in order to regulate expression of I7L proteinase with TET in the presence of a tetracycline repressor (TetR). Also included was the genomic DNA sequence from 250 bp upstream of the I7L ORF, to include the native promoter, and to aid in homologous recombination. This plasmid was used to create the recombinant virus vtetOI7L using the transient dominant selection method (Falkner & Moss, 1990). A commercially available cell line, T-Rex-293 (Invitrogen), expressing the TetR was used to regulate the expression of the I7L gene from the infecting recombinant virus. This conditional-lethal expression system has recently been used to show that the enzymatic activity of the VV G1L metalloproteinase is essential for viral replication (Hedengren-Olcott *et al.,* 2004).

The conditional-lethal phenotype of the recombinant virus was shown by plaque assay (Fig. 4.1), in which the formation of plaques from vtetOI7L is dependent on the presence of TET, while the wild-type virus is unaffected by either the presence or absence of TET. To determine the optimum TET concentration required for replication of vtetOI7L, TREx-293 cells were infected with vtetOI7L in the presence of varying

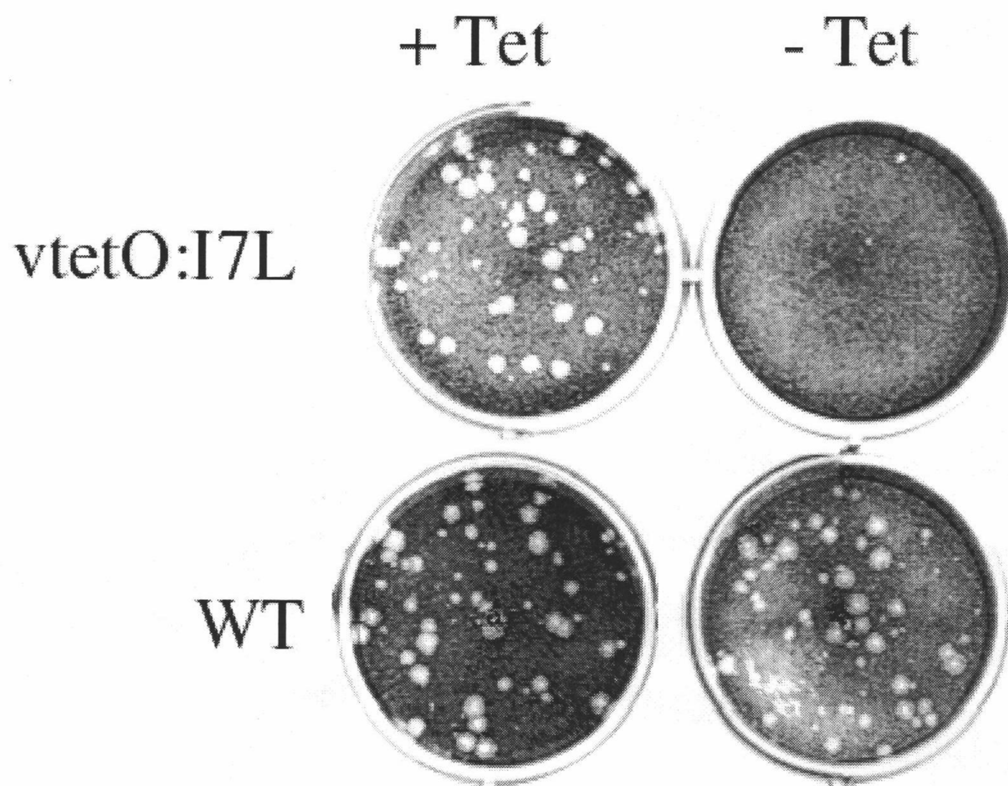
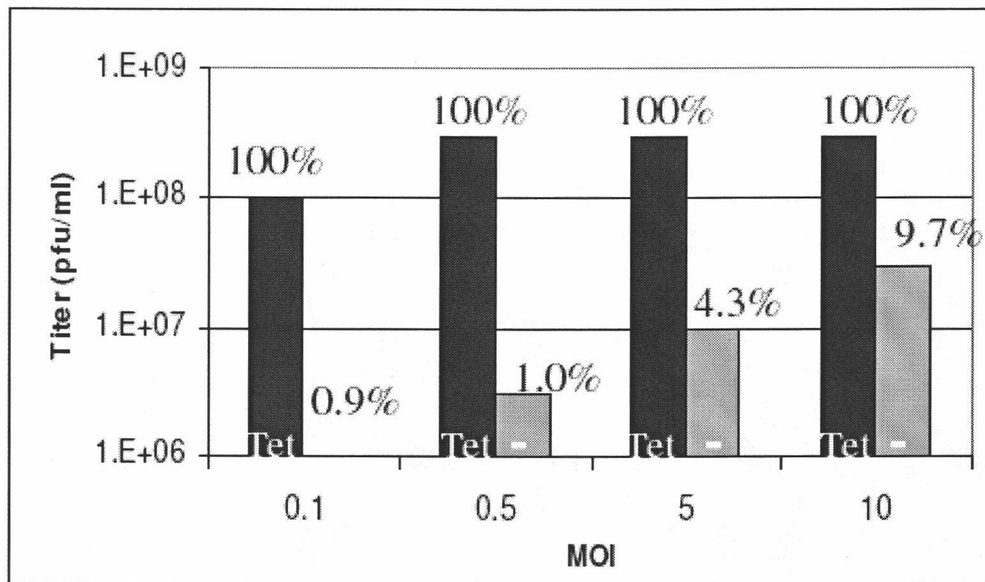


Figure 4.1. Effect of TET on plaque formation. TREx-293 cells were infected with vtetOI7L or wild-type virus in the presence or absence of 1 μg/ml TET and harvested 24hpi. BSC_{40} cells were then infected and stained with crystal violet 48 hpi.

concentrations of TET, harvested 24 h later, and the titer determined on BSC_{40} cells (Hruby *et al.,* 1979b). A 2-log increase in viral yield was observed with 1 μg/ml TET (data not shown). To confirm that expression of the I7L gene was essential for viral replication, TREx-293 cells were infected with vtetOI7L at a multiplicity of infection (MOI) of 0.1, 0.5, 5, or 10 in the presence or absence of TET, harvested 24 h later, and the titer of the virus infected cell lysates determined on BSC_{40} cells. At an MOI of 0.1 or 0.5 there was an average reduction of 99.1% of infectious virus particles (Fig. 4.2). At an MOI of 5 there was an average reduction of 95.7%, and at an MOI of 10 there was an average reduction of 90.3% (Fig. 4.2). This multiplicity-dependent breakthrough of viral replication is likely due to gene copy overwhelming the amount of TetR being expressed by the TREx-293 cell line.

To test whether the insertion of the TET operator just upstream of the I7L ORF had an effect on the viral growth kinetics, a one-step growth curve was conducted. TREx-293 cells were infected with wild type virus or vtetOI7L in the presence or absence of TET and infected cell lysates were harvested at the indicated times and the titer determined on BSC_{40} cells (Fig. 4.3A). In the presence of TET, the recombinant virus grew to the same yield and with the same kinetics as wild type virus while in the absence of TET the production of infectious virus was much lower indicating that the presence of the TET operator did not have an effect on the growth kinetics of the inducible mutant virus.

To demonstrate that the replication defect of the vtetOI7L mutant virus in the absence of TET was due to the I7L gene we tested whether viral replication could be



MOI	Tet	Pfu/ml	%	% reduction
0.1	- +	1.3E+06 1.4E+08	0.9 100	99.1
0.5	- +	3.2E+06 3.1E+08	1.0 100	99.0
5	- +	1.4E+07 3.2E+08	4.3 100	95.7
10	- +	3.0E+07 3.1E+08	9.7 100	90.3

Figure 4.2. Effect of TET on viral replication and rescue of the vtetOI7L mutant. TREx-293 cells were infected with vtetOI7L in the absence (-) or presence of 1 μg/ml TET at an MOI of 0.1, 0.5, 5, or 10. Infected cells were harvested 24hpi and titrated on BSC_{40} cells.

rescued by the introduction of a plasmid-borne I7L gene. TREx-293 cells in 6-well plates were transfected with 1.8 μg of plasmid DNA (containing either no insert, a wild type I7L gene under the control of the synthetic early-late promoter, a I7L gene with the catalytic His241 mutated to Ala, or the I7L gene under the control of its native promoter) and infected with vtetOI7L at an MOI of 0.2 plaque-forming units per cell in the absence of TET. Cells were harvested 24 hours post infection (hpi) and the titer determined on BSC_{40} cells. As an additional control, TREx-293 cells were mock transfected and infected with vtetOI7L in the presence of 1 μg/ml TET to compare growth conditions. A partial rescue of viral replication was observed when cells were transfected with the I7L gene under the control of the synthetic early/late promoter, but not when cells were transfected with plasmid alone or with a mutant I7L gene (Fig. 4.3B). This was an approximate 5-fold increase in virus replication compared to the pRB21 or pI7LH241A transfected controls. When the I7L gene was driven off of its own promoter in pCB26 and transfected in, there was a much higher level of rescue (Fig. 4.3B), suggesting that the timing and amount of I7L gene expression has important implications for the viral life cycle.

We have previously shown through transient expression assays that the I7L proteinase is capable of cleaving the p4b, p4a, and p25k core protein precursors (Byrd *et al.*, 2002; Byrd *et al.*, 2003) which are products of the A3L, A10L, and L4R open reading frames respectively. Here we were interested to see whether the I7L proteinase in the conditional lethal mutant system was also capable of cleaving these proteins in the presence but not the absence of TET. First, to see whether I7L protein was expressed at the same time from the mutant virus as from the wild type virus, TREx-293

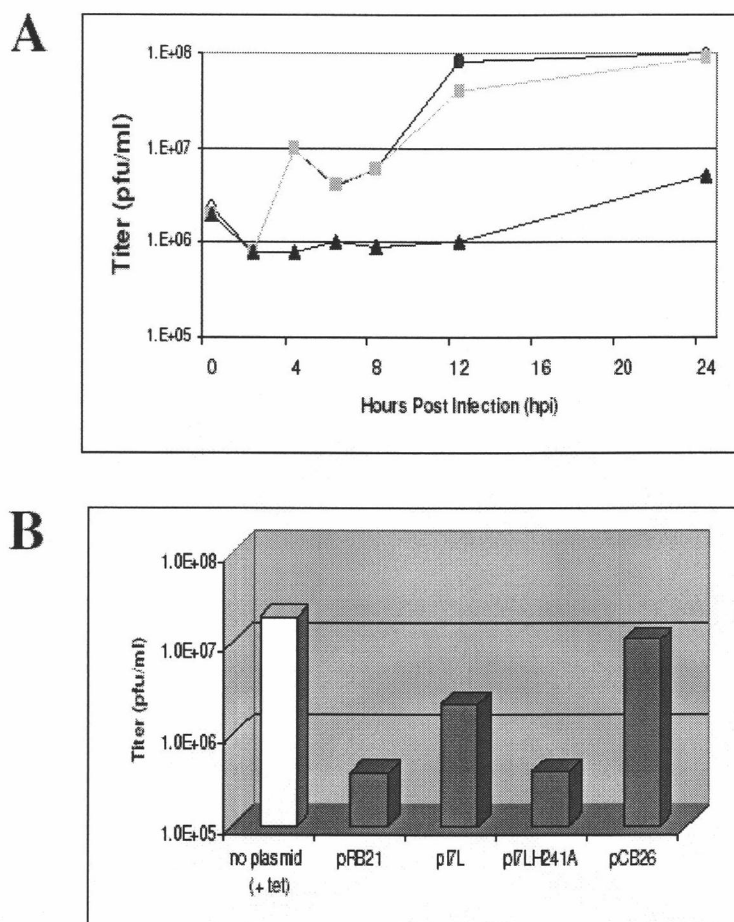


Figure 4.3. (A) One step growth curve. TREx-293 cells were infected with wild-type virus (•) or vtetOI7L in the presence (¦) or absence (?) of 1 μg/ml TET. Infected cells were harvested at the indicated times and the titer determined on BSC_{40} cells. (B) Rescue of replication. TREx-293 cells were infected with vtetOI7L and transfected with either vector alone (pRB21), plasmid with wild-type I7L driven off of a synthetic early/late promoter (pI7L), plasmid with mutant I7L, mutated in the putative active site, driven off of a synthetic early/late promoter (pI7LH241A), or wild-type I7L driven off of its native promoter (pCB26) in the absence of TET. Infected cells were harvested 24 hpi and the titer determined on BSC_{40} cells. Transfection of plasmid borne wild-type I7L but not of mutant I7L or vector alone partially rescued the replication of vtetOI7L.

cells were infected in the presence of TET and cells harvested at various time points. Proteins in the crude cell extracts were separated by SDS-PAGE and detected by Western blot with anti-I7L antisera. I7L enzyme from both viruses appeared at late times after infection, around 8 hpi and increased as time progressed (data not shown). To determine the effect of TET on I7L protein expression, cells were infected and treated with 0 to 5 μg/ml TET. After 6 h, the infected cells were labeled with 60 μCi/ml ^{35}S-met and harvested after 24 h. Extracts were immunoprecipitated with I7L antisera and protein detected by autoradiography. With wild type virus, I7L protein was expressed at each TET concentration (data not shown). However, in the mutant virus, expression of I7L enzyme was repressed in the absence of TET and increased with the addition of TET.

To determine the effect of TET concentration on p4b core protein precursor processing, cells were infected in the presence of 0 to 5 μg/ml TET, harvested 24 hpi, and the extracts immunoblotted with anti-4b antisera. With wild type virus p4b was processed at each TET concentration as expected, however with the mutant virus, p4b processing was repressed in the absence of TET (data not shown). The slight processing in the absence of TET is likely due to slight leak-through of I7L gene expression in this system. The same results were seen for the processing of p4a, with processing in each of the wild type virus lanes, repressed processing with the mutant in the absence of TET and increased processing in the presence of TET (data not shown). Kane and Shuman (1993) have previously shown that I7L protein is located in the virus core. To verify that the I7L protein from the inducible mutant was localized correctly, purified virions were treated with DTT and NP-40 to separate the envelope fraction from the core fraction and protein from each sample was separated by SDS-PAGE and detected by Western blot with anti-

I7L antisera. As expected, the I7L enzyme from the inducible mutant was detected in the core sample, as was the wild type virus (data not shown).

The morphogenesis of vtetOI7L under nonpermissive conditions was analyzed via electron microscopy. TREx-293 cells were infected with vtetOI7L at an MOI of 1 in the presence or absence of TET and harvested 24 h later. In the presence of TET, cells contained a variety of both immature and mature forms of the virus (Fig. 4.4, panels A-C), which were indistinguishable from cells infected with wild type virus (not shown). However, in the absence of TET, no mature virions were observed in any of the infected cells observed. There appeared to be an accumulation of immature viral particles, some with nucleoids, as well as the appearance of crescent shaped particles (Fig. 4.4, panels D-F), similar to those observed by Ansarah-Sobrinho *et al* (2004). Also observed were numerous dense virus particles. Virion morphogenesis appears to arrest at a stage prior to core condensation. The observation that there is still some processing of p4b in the absence of TET and yet the morphology of the mutant virus in the absence of TET shows only immature virus particles suggests the hypothesis that there is a requirement for the processing threshold of the core protein precursors to be achieved before morphogenesis can proceed.

Taken together, the data we have presented here, as well as analysis of the VV G1L conditional lethal mutant (Hedengren-Olcott *et al.,* 2004), suggests a morphogenesis model in which these two putative proteases operate sequentially to regulate assembly. According to this model, if we assume that both I7L and G1L are associated with the immature virus along with the accompanying DNA and other viral proteins, then activation of I7L leads to the process of core protein precursor cleavage and the initiation

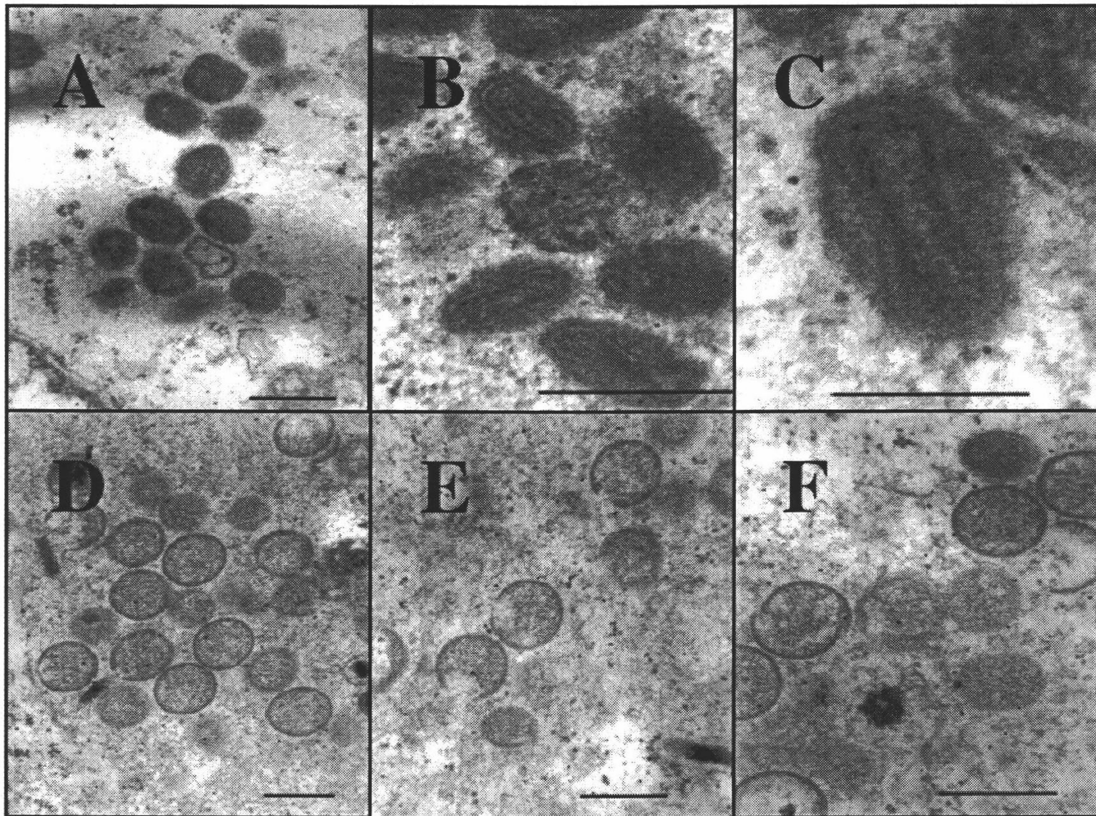


Figure 4.4. Electron microscopy of cells infected with vtetOI7L. TREx-293 cells were infected with vtetOI7L at an MOI of 1 in the presence (panels A, B, and C) of 10 μg/ml TET or in the absence (panels D, E, and F) of TET. Cells were harvested at 24 hpi, immediately fixed and prepared for transmission electron microscopy. The bar in panels A, B, D, E, and F represents 400 nm. The bar in panel C represents 200 nm.

of core condensation. Following this activity, the activation of G1L completes core condensation and allows progression to the formation of intracellular mature virus. If the activity of the I7L proteinase is blocked, viral morphogenesis arrests prior to core condensation. If the activity of G1L proteinase is blocked, viral morphogenesis arrests at a stage subsequent to this but still prior to complete core condensation. To test this model, it will be of interest to isolate biochemically active I7L and G1L enzymes and determine the series of events that lead to their activation.

CHAPTER 5

NEW CLASS OF ORTHOPOXVIRUS ANTIVIRAL DRUGS THAT BLOCK VIRAL MATURATION

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SUMMARY

By using a homology-based bioinformatics approach, a structural model of the vaccinia virus (VV) I7L 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 was found which exhibits selective activity against orthopoxviruses, inhibiting VV with 50% inhibitory values of 3 to 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). Phenotypic analyses of virus-infected cells were conducted in the presence of active compounds to verify that the correct biochemical step (I7L mediated core protein processing) was being inhibited. Electron microscopy of compound-treated VV infected cells indicated a block in morphogenesis. Compound resistant viruses were generated and resistance was mapped to the I7L open reading frame. Transient expression with the mutant I7L gene rescued the ability of wild type virus to replicate in the presence of compound, indicating that this is the only gene necessary for resistance. This novel class of inhibitors has potential for development as an efficient antiviral drug against pathogenic orthopoxviruses, including smallpox.

INTRODUCTION

The recent deliberate dispersal of anthrax spores into our environment has raised awareness and concern over our ability to effectively prevent or treat infections caused by infectious agents. Of particular concern is the list of CDC Category A agents, which includes variola virus, the causative agent of smallpox. A smallpox vaccine is available, but due to the small but significant risk of serious complications that can include encephalitis (Miravalle & Roos, 2003), myopericarditis, hemolytic anemia and even death (Fulginiti *et al.,* 2003), the vaccine has only been administered to a select population (mainly laboratory personnel working with orthopoxviruses, military personnel and some first-responders). While smallpox was eradicated from the natural biosphere in the 1970's, the subsequent 30 years have produced a population that is immunologically naïve and susceptible to infection. In the advent of a release of smallpox into the environment, the vaccine would need to be administered within three days of exposure to be effective, but there is a period of 10-14 days when a person is infected but before obvious clinical symptoms become evident. In addition to smallpox concerns, there is the recent spate of monkeypox virus infections in North America. Likewise, there are health concerns due to complications to vaccination against smallpox in immunocompromised individuals, such as progressive vaccinia. Thus there is a pressing need for effective anti-orthopoxvirus therapeutics to address these problems and concerns.

No antiviral drug has been proven to be effective in the treatment of human smallpox. The only antiviral agent currently approved for use against orthopoxviruses is

cidofovir, which has been shown to be useful against Molluscum contagiosum and Orf in humans (De Clercq, 2002). However, cidofovir has low oral bioavailability and must be administered intravenously (Cundy, 1999), which limits its usefulness. Cidofovir has also been shown to cause nephrotoxicity affecting renal cells and must be co-administered with oral probenecid to reduce the toxic effects (Lalezari & Kuppermann, 1997).

Development of an effective antiviral drug requires the identification of a specific interaction or activity whose disruption will be lethal to the virus and relatively benign to the host. Since viruses, such as orthopoxviruses, are obligate intracellular parasites which utilize many of the host cell's enzymes and metabolic pathways during their replication, this task is often quite difficult and this fact is chiefly responsible for the relative paucity of successful antiviral drugs. The drugs that have proven effective, such as acyclovir, are typically directed against nucleotide metabolizing or biosynthetic enzymes. Since many of the orthopoxvirus-encoded enzymes involved in nucleic acid biosynthesis are highly similar to their mammalian counterparts (for example, vaccinia virus thymidine kinase shares more than 90% identity with the human enzyme), it may prove difficult, if not impossible, to identify compounds that specifically block these viral enzymes.

Fortunately it has recently been discovered that proteolysis catalyzed by viral-encoded proteinases is a necessary step in the developmental cycle of most viruses. This realization has created a new class of target for antiviral drug development. Proteinase inhibitors have proven to be effective antiviral drugs that target HIV (Roberts *et al.*, 1990), influenza (Hayden *et al.*, 2004), hepatitis C (Tsantrizos, 2004), and rhinovirus (Binford *et al.*, 2005) enzymes and which ameliorate disease in the human host. Small molecule inhibitors of the NS3 protease in hepatitis C virus have recently been shown to

be effective both in animal and human trials (Lamarre *et al.,* 2003). Thus, it is of particular interest to note that proteolytic maturation of orthopoxvirus core proteins appears to be required for infectious progeny to be produced (Hruby *et al.,* 1979b). Studies in our laboratory over the past ten years have identified the unique *cis* signals required to direct endoproteolytic cleavage of core protein precursors, established the contextual requirements of core protein maturation, and in recent work, we have identified the poxvirus gene (I7L) that encodes the viral core protein proteinase.

Vaccinia virus is a large double-stranded DNA virus that is a prototypic member of the orthopoxvirus family. It shares around 90% similarity with variola virus, the causative agent of smallpox, and it is believed that a compound that inhibits VV will also inhibit variola virus. Poxviruses are unique in that they replicate entirely in the cytoplasm of infected cells, encoding most of the genes necessary for their own replication. Briefly, the virus attaches to the outside of the cells, enters, uncoats, undergoes early gene expression, intermediate gene expression, DNA synthesis, late gene expression, virion formation, and virus maturation before egress from the cell. Morphogenic proteolysis occurs in the stage between the formation of the infectious intracellular mature virus (IMV) from the non-infectious intracellular virus (IV). The gene product of the I7L open reading frame has been shown to be the cysteine proteinase responsible for the cleavage of the core protein precursors that occurs in this stage of the viral life cycle (Byrd *et al.,* 2002; Byrd *et al.,* 2003).

The long-range goal of the experiments reported here is to discover and develop compounds capable of inhibiting the growth of pathogenic orthopoxviruses (such as smallpox) and disease-associated pathology in the human host. In this study, we describe

the identification of a novel class of small molecule inhibitors that were modeled to fit into the predicted active site pocket of I7L. The inhibitory phenotype and genetic analysis are consistent with a defect in morphogenesis. Since these compounds are specific for orthopoxviruses and appear to have minimal toxicity to cells, they would appear to warrant further development as smallpox antiviral drug candidates.

MATERIALS AND METHODS

Chemical compounds

Compound stocks were prepared at a concentration of 10 mM in 100% dimethyl sulfoxide (DMSO).

Computational Modeling

TransTech Pharma's Translational Technology was designed and developed for rapid lead generation and optimization into preclinical drug candidates. It consists of two sub-technologies: TTProbes and TTPredict. TTProbes is a set of >51,000 high information density and pharmacophorically diverse molecules. TTPredict on the other hand, is a state-of-the-art computer-based technology that automates high throughput 3D target-model building, binding site identification and conformational analysis. It is used to dock, score, and rank members of TTProbes set into targets' binding sites.

Cells and Viruses

Table 5.1 provides a list of each cell line and virus strain used. BSC_{40} cells (Raczynski & Condit, 1969), HeLa cells, 293 cells, and L929 cells were grown in Eagle's minimal essential medium (MEM-E; Gibco-BRL, Rockville, MD) containing 10% fetal calf serum (FCS) (Gibco-BRL), 2 mM glutamine, and 15 μg/ml gentamicin sulfate in a 37°C incubator with 5% CO_2. Vaccinia virus, cowpox virus (CPV), adenovirus, and encephalomyocarditis virus infections were carried out in MEM containing 5% FCS, 2 mM glutamine, and 15 μg/ml gentamicin sulfate in a 37°C incubator with 5% CO_2.

Table 5.1. Plasmids, oligonucleotides, cells, and strains used in this study[a]

Plasmid or Oligo	Description	Source
Plasmid		
pCR2.1	Km^r, Amp^r	Invitrogen
pCB26-23	pCR2.1 with I7L behind its native promoter	This work
pCB26-23-17	pCB26-23 with aa# 104 mutated Y→C and aa# 324 mutated L→M	This work
pRB21	puc derived with F13L flanks, MCS downstream of synthetic early/late promoter	Blasco
pI7L	pRB21 plasmid with full length I7L	Byrd
pI7L-17	pI7L with aa# 104 mutated Y→C and aa# 324 mutated L→M	This work
pI7L-78/104	pI7L with aa# 78 mutated to create an XbaI site, aa# 104 mutated Y→C	This work
pI7L-78/324	pI7L with aa# 78 mutated to create an XbaI site, and aa# 324 mutated L→M	This work
pI7L-78/104/324	pI7L with aa# 78 mutated to create an XbaI site, aa# 104 Y→C, aa# 324 L→M	This work
Oligonucleotides		
CB26	5' - GAG CTC GTT TTC CTA GTG ATG GAG GAG -3'	This work
CB23	5' - AAG CTT TTA TTC ATC GTC GTC TAC -3'	This work
CB84	5' - GAG TCG GGG CAC CTG TCT AGA CCC AAT AGT AGC G -3'	This work
CB85	5' - CGC TAC TAT TGG GTC TAG ACA GGT GCC CCG ACT C -3'	This work
CB86	5' - CGC GTT ACC ATT CTA TAT GTG ATG TTT TTG AGT TAC C -3'	This work
CB87	5' - GGT AAC TCA AAA ACA TCA CAT ATA GAA TGG TAA CGC G -3'	This work
CB88	5' - GTG GAA GTT AAT CAG CTG ATG GAA TCT GAA TGC GGG -3'	This work
CB89	5' - CCC GCA TTC AGA TTC CAT CAG CTG ATT AAC TTC CAC -3'	This work
Cell line		
BSC40	BSC1 African Green Monkey Kidney cells adapted to grow at 40°C	Raczynski
293	Human embryonic kidney cells	Invitrogen
HeLa	Human cervical carcinoma cells	ATCC
L929	Mouse fibroblast cells. ATCC #CCL-1	ATCC
Virus		
VV WR	Western reserve strain of Vaccinia virus	ATCC
VV COP	Copenhagen strain of Vaccinia virus	ATCC
VV IHDJ	IHDJ strain of Vaccinia virus	ATCC
Cowpox	Brighton Red strain	Duke University
Adenovirus	Mastadenovirus, human adenovirus C	ATCC
MHV-A59	Mouse hepatitis virus	ATCC
EMC	Encephalomyocarditis virus	ATCC
vvGFP	Western reserve Vaccinia virus with GFP in the TK locus	This work
v17-3	vvGFP resistant to 176171 with mutations in I7L aa# 104 and 324	This work
vCB	Recombinant vvGFP with the I7L gene replaced with the mutant I7L gene	This work
ts16	Temperature sensitive VV with mutation mapped to I7L	Condit
Bacteria		
E. coli		
INVαF'	F' *endA*1 *recA*1 *hsdR*17(rk-, mk+) *supE*44 *thi*-1 *gyrA*96*rel* A1O80 *lacZ*AM15 A(*lacZYA-arg*-F)U16	Invitrogen
Pseudomonas aeruginosa	Broad spectrum of resistance to various commercial germicides	ATCC
Yeast		
Saccharomyces cerevisiae	Budding yeast- derived from X2180	ATCC

[a] aa, amino acid; TK, thymidine kinase

Purified vaccinia virus was prepared as described (Hruby *et al.,* 1979b). *Escherichia coli* strains were grown in Luria-Bertani broth or on Luria-Bertani medium containing 1.5% agar and ampicillin at 50 μg/ml. Recombinant VV expressing the green fluorescent protein was constructed by inserting the GFP gene into the TK locus in the Western Reserve (WR) strain of VV. *Saccharomyces cerevisiae* was grown in 1245 YEPD media (ATCC, Manassas, VA), and *Pseudomonas aeruginosa* was grown in nutrient broth (Difco, Detroit, MI).

Cytotoxicity assay

Confluent monolayers of BSC_{40} cells were grown up in 96-well black-sided view plates (Packard, Meriden, CT). Compounds were diluted to the desired concentration in MEM and applied to the cell monolayers in doubling dilutions. Controls included cells treated without compound, cells treated with hydroxyurea, rifampicin, or cytosine arabinoside (AraC). The cells were incubated for 24 hrs at 37°C. Background fluorescence was measured using a Wallac Victor^2 V multilabel HTC counter (Perkin Elmer, Turku, Finland) with an excitation wavelength of 485 nm and read at 535 nm. Cells were visualized under a light microscope for cytopathic effect as well as treated with alamar blue (BioSource Intl, Inc., Camarillo, CA) to determine toxicity of the compounds.

Fluorescence assay

Confluent monolayers of BSC_{40} cells in 96-well black-sided view plates (Packard) were infected with vvGFP a multiplicity of infection (moi) of 0.1 for 30 min at 37°C

before addition of compounds. Controls included uninfected cells, vvGFP infected cells treated with AraC, hydroxyurea, rifampicin, or no compound individually. Compounds were applied to the infected cells in doubling dilutions. Monolayers were incubated at 37°C for 24 hrs. Plates were washed with 1 x PBS and fluorescence measured using a Wallac Victor^2 V multilabel HTC counter (Perkin Elmer, Turku, Finland) with an excitation wavelength of 485 nm and read at 535 nm. Wells showing a reduction in fluorescence over the untreated vvGFP infected control lane were visualized under a light microscope to verify loss of virus replication versus removal of cell monolayer from a productive and concomitant cytopathic effect (CPE).

Determination of therapeutic index (TI)

IC_{50} values (where IC_{50} is the 50% inhibitory concentration) were determined by CPE inhibition as seen by fluorescence using vvGFP and plaque reduction assays using crystal violet staining or neutral red uptake. TC_{50} values (where TC_{50} is the 50% cell toxicity concentration) were determined as the concentration of compound that caused 50% of the cells to round up and show signs of toxicity both visibly and through the indication of Alamar blue dye. The therapeutic index (TI) was calculated as the value for the TC_{50} divided by the IC_{50}.

Western Blot Analysis

Confluent monolayers of BSC_{40} cells in 6-well plates were infected with vvGFP at a moi of 1 for 30 min prior to addition of compound. One well of cells was pretreated

with hydroxyurea for 1 hr prior to infection. Compounds were added to the infected cell monolayers at a concentration of 100 μM. Monolayers were incubated at 37°C for 24hr. Virus infected cell extracts were harvested by scraping and the total cell extract collected in 1.5 ml microfuge tubes. The total cell extract was centrifuged for 15 min at 4°C and the pellet resuspended in 100 μl of 1 x PBS. This was subjected to three cycles of freeze-thaw to release the virus from the cell debris. 15 μl of the whole cell extract was run on SDS-polyacrylamide gels and transferred to PVDF (Pall, AnnArbor, MI) membranes. The membranes were incubated with a 1:1000 dilution of anti-4b antisera (VanSlyke & Hruby, 1994) and then with a 1:2000 dilution of goat anti-rabbit AP antisera (Bio-Rad, Hercules, CA). The proteins were detected using the AP development system (Bio-Rad, Hercules, CA) following the manufacturer's instructions.

Isolation of compound resistant vvGFP isolates

BSC_{40} cells in 6-well plates were infected with vvGFP at a moi of 0.05 for 30 min at 37°C prior to treatment with compound. Compound was added at the 50% inhibitory concentration and monolayers incubated at 37°C for 24 hr. Total cell extracts were harvested as above and 10 μl of crude cell extract used to infect fresh monolayers of BSC_{40} cells in the presence of compound. This process was repeated 9 times with the titer of each passage determined. After the 9th passage the titer had returned to a level similar to untreated virus. Compound resistant virus was plaque purified to isolate individual resistant viruses and then amplified in 6-well plates.

Genomic DNA preparation

Confluent monolayers of BSC_{40} cells in 6-well plates were infected with compound resistant virus, treated with the same compound at the 50% inhibitory concentration, and incubated at 37°C until about 90% cytopathic effect (CPE) was observed. Total cell extracts were prepared by scraping and placed into a 1.5 ml centrifuge tube, centrifuged for 10 min at 4°C, and the pellet resuspended in 1 ml 1x PBS. This was subjected to three cycles of freeze-thaw. 100 μl aliquots were removed for storage and the remaining 900 μl treated with 10 mM Tris-hydrochloride (pH 8), 1 mM EDTA, 5 mM β-mercaptoethanol, 150 μg/ml proteinase K, 200 mM NaCl, 1%SDS, and the DNA extracted with Tris-EDTA buffer saturated with phenol-chloroform-isoamly alcohol (25:24:1) and ethanol precipitated. The resulting DNA was used for PCR amplification of the vv I7L gene and for cloning.

Plasmids and Site-directed mutagenesis

See Table 5.1 for a description of all plasmids, oligonucleotides, and strains used. The VV I7L gene was amplified from Western Reserve strain genomic DNA and cloned into pRB21 (Blasco & Moss, 1995) with *PstI* and *HindIII* flanking yielding pI7L. Mutant I7L was amplified from the TTP-6171 compound resistant virus DNA with primers CB26 and CB23 and cloned into pCR2.1- Topo Vector (Invitrogen, Carlsbad, CA) with *PstI* and *HindIII* flanking yielding pCR2.1:I7L. Site directed mutagenesis of the I7L gene was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) following the manufacturer's indications and using primers CB84-85, CB86-87, and CB88-89 to mutate aa#78, aa#104, and aa#324 respectively, using pI7L as template,

yielding pI7L-78/104, pI7L-78/324, and pI7L-78/104/324. I7L aa#78 was mutated to create an *XbaI* site via a silent mutation to facilitate screening for recombinant viruses. I7L aa# 104 was mutated from a Y →C, and I7L aa# 324 was mutated from a L→M.

Transient expression

Confluent monolayers of BSC_{40} cells in 6-well plates were infected with vvGFP at a multiplicity of infection of 0.01 plaque-forming units per cell and transfected with 2 μg of plasmid DNA using DMRIE-C (Invitrogen, Carlsbad, CA) following the manufacturer's indications. Plasmids contained either no insert, the wt I7L ORF, or the mutant I7L ORF with each single mutation or the double mutation. Cells were treated with or without compound at the 50% inhibitory concentration and incubated at 37°C. Cells were harvested at 24 h post-infection by pipetting up and down to lift the cells from the surface. The crude extract was centrifuged at 15000 rpm for 10 min, the supernatant aspirated off, and the pellet resuspended in 1 ml of 1 x PBS. This was freeze-thawed three times and then centrifuged at 2500 rpm for 3 min to sediment cellular debris. The supernatant was tittered to determine viral yield.

Electron microscopy

Confluent monolayers of BSC_{40} cells in 6-well plates were infected with vvGFP at a multiplicity of infection of 3 plaque-forming units per cell for 30 min prior to treatment with compound. Compound was added at a concentration of 100 μM and the cells incubated at 37°C for 24 hr. Cells were harvested as above and fixed with 2.5% glutaraldehyde and 1.2% paraformaldehyde in 0.1M sodium cacodylate (pH 7.3),

postfixed in osmium tetroxide, dehydrated, and embedded in Spurrs' resin (Spurr, 1969). Ultrathin sections were stained by using the double lead stain technique of Daddow (Daddow, 1986), with lead citrate (Venable & Coggeshall, 1965), and uranyl acetate. Sections were viewed with a Philips CM12 transmission electron microscope, operated at 60 KeV.

One-step time course of viral replication

Confluent monolayers of BSC_{40} cells in six-well plates were infected with either vvGFP or v17 at an MOI of 1. One hour after infection, the virus-infected cells were treated with TTP-6171 at a final concentration of either 0,10,25, or 60 μM. Cells were harvested at 2, 12, 24, and 48 h postinfection and subjected to three freeze-thaw cycles to release virus, and viral titers were determined in BSC_{40} cells.

Transfection and isolation of mutant I7L recombinant virus

Confluent monolayers of BSC40 cells in six-well plates were infected with vvGFP at an MOI of 0.05 at 37°C. At 3 h postinfection, 1.5 μg of DNA was transfected into the cells by using DMRIE-C reagent (Invitrogen). Infected cells were harvested 24 h postinfection and the virus was released by three freeze-thaw cycles. After two round of plaque purification in the presence of TTP-6171 to select for recombinant virus (vCB), incorporation of the mutated I7L gene into the viral isolates was confirmed by PCR, digest with XbaI, and sequencing.

RESULTS

Identification of a new class of orthopoxvirus antiviral compounds.

TTPredict was used to construct threading and homology models for I7L (Fig. 5.1A). A sequence comparison to proteins with experimentally determined three-dimensional structures showed that the highest sequence identity with vaccinia virus I7L was to the C-terminal domain of the UlP1 protease. The latter consists of 221 amino acids and has a 22% sequence identity with I7L. UlP1 protease served as a template for building the 3-dimensional structure of I7L. TTPredict site search algorithms readily identified the catalytic site of I7L (Fig. 5.1B), based on the location of the active site residues, H241, D248, and C328, which are essential for activity (Byrd *et al.*,2003). TTProbes were docked into this site. The fit of every docked TTProbe was computed using several scoring functions. High scoring probes were identified and the highest-ranking TTProbes were submitted for *in vivo* screening.

In order to screen these compounds for their ability to inhibit VV replication , an *in vivo* tissue culture-based screening assay was developed. Though more cumbersome than a biochemical assay, this approach had the advantage of providing information regarding compound toxicity and cellular uptake, along with the antiviral activity read out. For this purpose, a fluorescence assay was developed utilizing recombinant VV expressing the green-fluorescent protein (vvGFP) as the readout in a 96-well plate format. vvGFP infected BSC_{40} cells in 96-well plates were treated with compound and

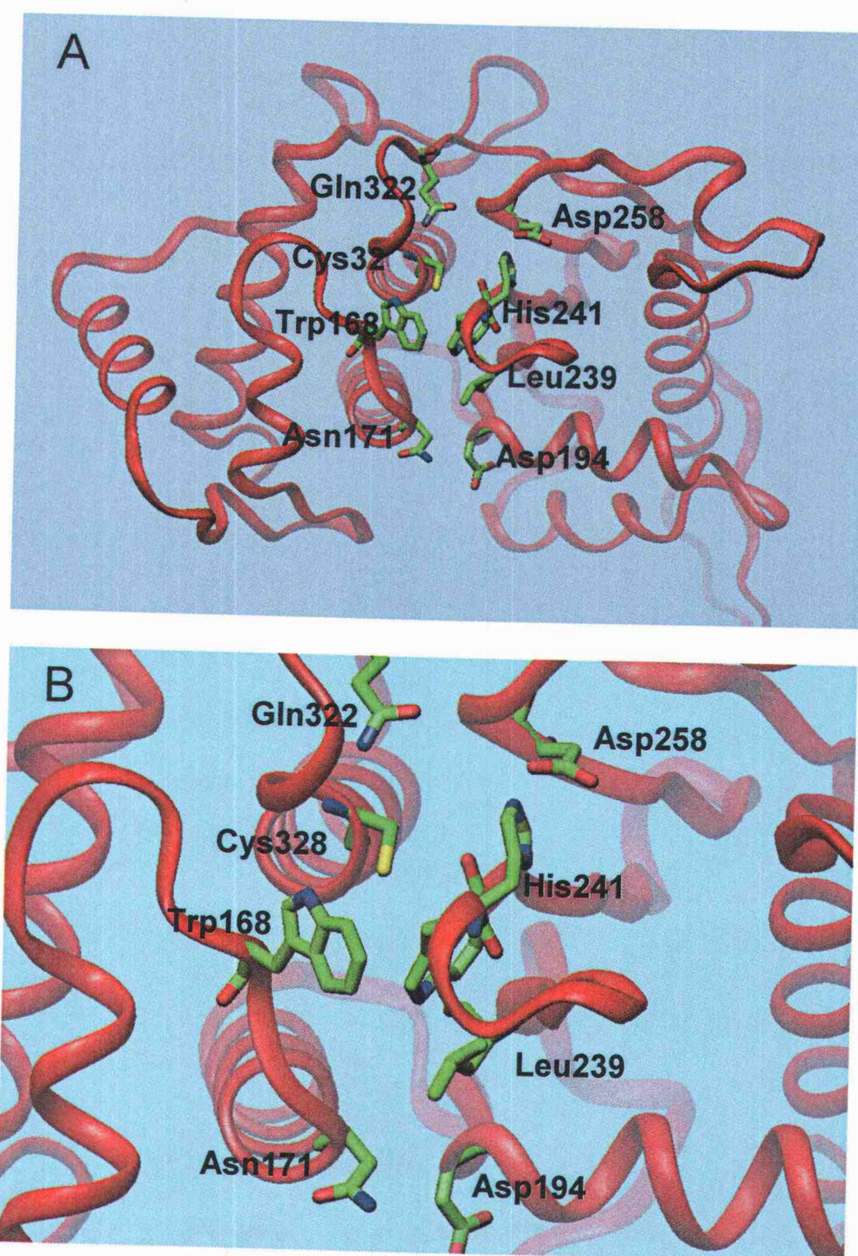


Figure 5.1. TTPredict three-dimensional model of the VV I7L cysteine proteinase modeled using the structure of the C-terminal ULP1 protease. **(A)** Critical binding site residues are shown in colored tubes. **(B)** Depicts a close-up view of the I7L ligand binding/catalytic domain.

analyzed 24 hr later for relative fluorescence. Considerable effort was placed on determining the appropriate cell density, multiplicity of infection and incubation time needed to provide a validated assay capable of providing reproducible results (data not shown). Controls included uninfected cells which have a low fluorescence background (Fig. 5.2), and vvGFP infected cells treated with either rifampicin, an inhibitor of viral assembly (Heller *et al.,* 1969; Moss *et al.,* 1969), hydroxyurea, which blocks DNA replication, (Rosenkranz *et al.,* 1966), or AraC, an inhibitor of DNA synthesis in VV (Herrmann 1968; Berger *et al.,* 1978). These control treatments display low levels of fluorescence regardless of whether they inhibit late or early in the replication cycle. As a negative control, cells were infected with vvGFP and not treated with any compound. As the data in Figure 5.2 demonstrates, a robust signal of approximately 25-fold over background was observed in vvGFP-infected cells versus uninfected cells.

A total of 3460 compounds were identified as potential I7L inhibitors by *in silico* screening. This biased library was evaluated using the vvGFP fluorescence assay to identify compounds which were non-toxic to cells and which inhibited virus growth. Of the compounds that were initially found to be toxic to the cells at a concentration of 100 μM, serial dilutions were performed until a concentration was found that inhibited virus replication but was no longer toxic. Of the 3,460 compounds, 136 were found to inhibit viral replication without being toxic. These compounds were then assessed to determine which stage of the virus life cycle they inhibit, looking for morphogenesis inhibitors, as well as looking at the relative IC_{50}, which narrowed the lead compound list down to 19. Therapeutic index was then taken into consideration and the list reduced to TTP-6171 and several other chemically related compounds (Table 5.2). During initial screening,

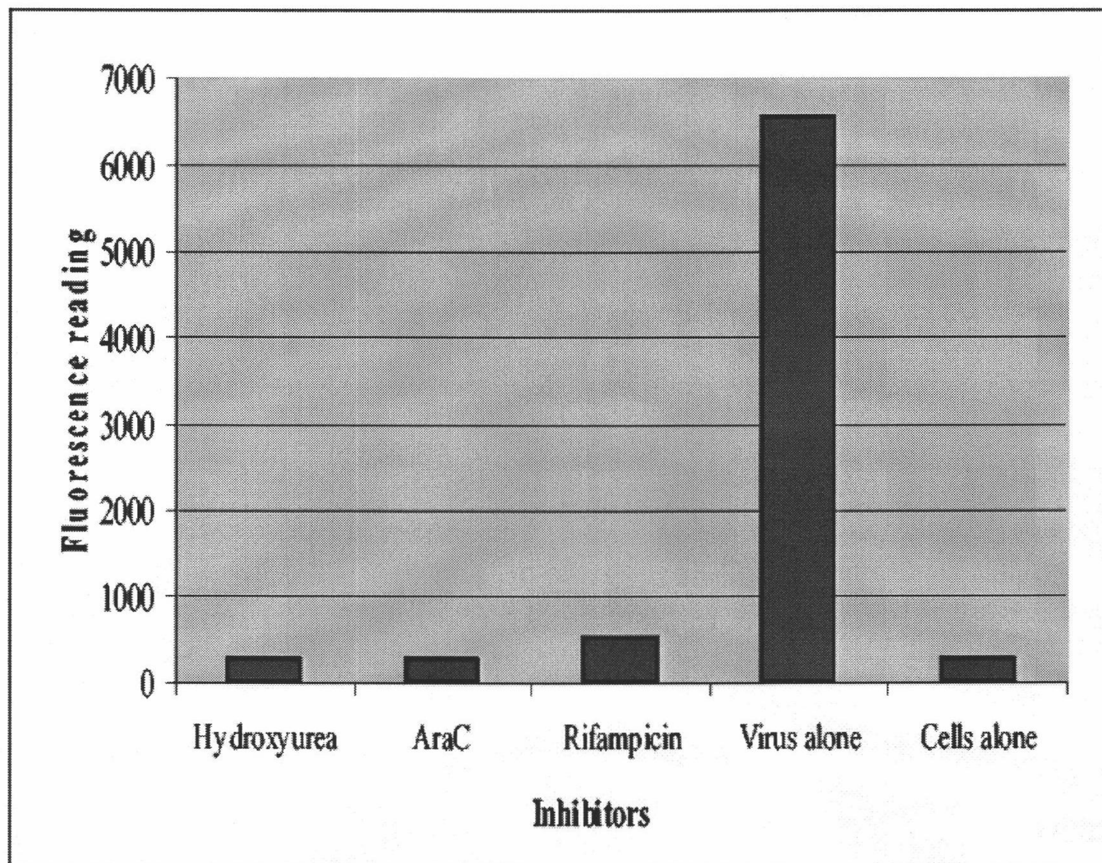


Figure 5.2. vvGFP assay. Graphical representation of the relative fluorescence units from 96-well plate of vvGFP infected BSC-40 cells treated with various compounds with an excitation wavelength of 485 nm and read at 535 nm. AraC is cytosine arabinoside.

Table 5.2. TI values of selected compounds

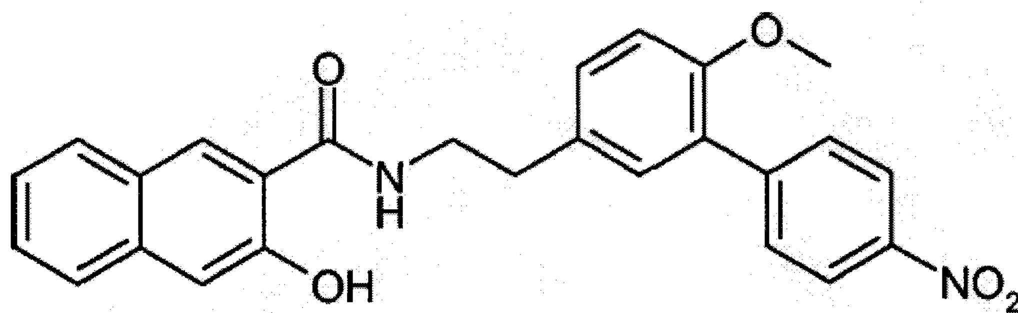
Compound	TC_{50} (μM)	IC_{50} (μM)	TI
6171	900	12	75
201018	600	200	3
200480	55	14	4
174878	400	50	8
130961	150	10	15
176510	400	56	7
123045	200	50	4

TTP-6171 demonstrated an IC_{50} of 12, a TC_{50} of 900 with a calculated therapeutic index of 75. For the rest of the experiments reported here, only TTP-6171 was tested as the prototype member of this compound family. Figure 5.3 shows the chemical structure of TTP-6171.

Figure 5.4 illustrates the screening data obtained with TTP-6171. The top left panel shows light microscopy of vvGFP infected BSC_{40} cells with characteristic rounding up and cytopathic effect. The top right panel is the same field viewed with fluorescence microscopy to demonstrate a vigorous viral infection as demonstrated by GFP expression. In contrast, the bottom panels show the result of similar analyses carried out in the presence of TTP-6171. No cytopathic effect (or toxicity) is evident, nor is there any demonstrable vvGFP replication. At high concentrations and upon prolonged incubation (48 to 72 h), TTP-6171 has toxic effects on cells, and plaque assays cannot be carried out in the presence of inhibitory concentrations of the drug.

TTP-6171 is specific for orthopoxviruses.

To determine the target specificity of TTP-6171, we tested the compound against a variety of organisms including those that contain a cysteine protease with partial similarity to I7L, such as adenovirus (Andres, *et al.,* 2001; Li & Hochstrasser, 1999). It was found that TTP-6171 inhibited the growth of various strains of VV including Western Reserve, Copenhagen, and IHDJ, as well as cowpox virus at a concentration of 25 μM. In contrast no inhibition was observed in the growth of *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, or mouse hepatitis virus, each of which express a cysteine protease. Likewise, TTP-6171 did not inhibit the replication of Encephlomyocarditis



TTP - 6171

Figure 5.3. Chemical structure of TTP-6171

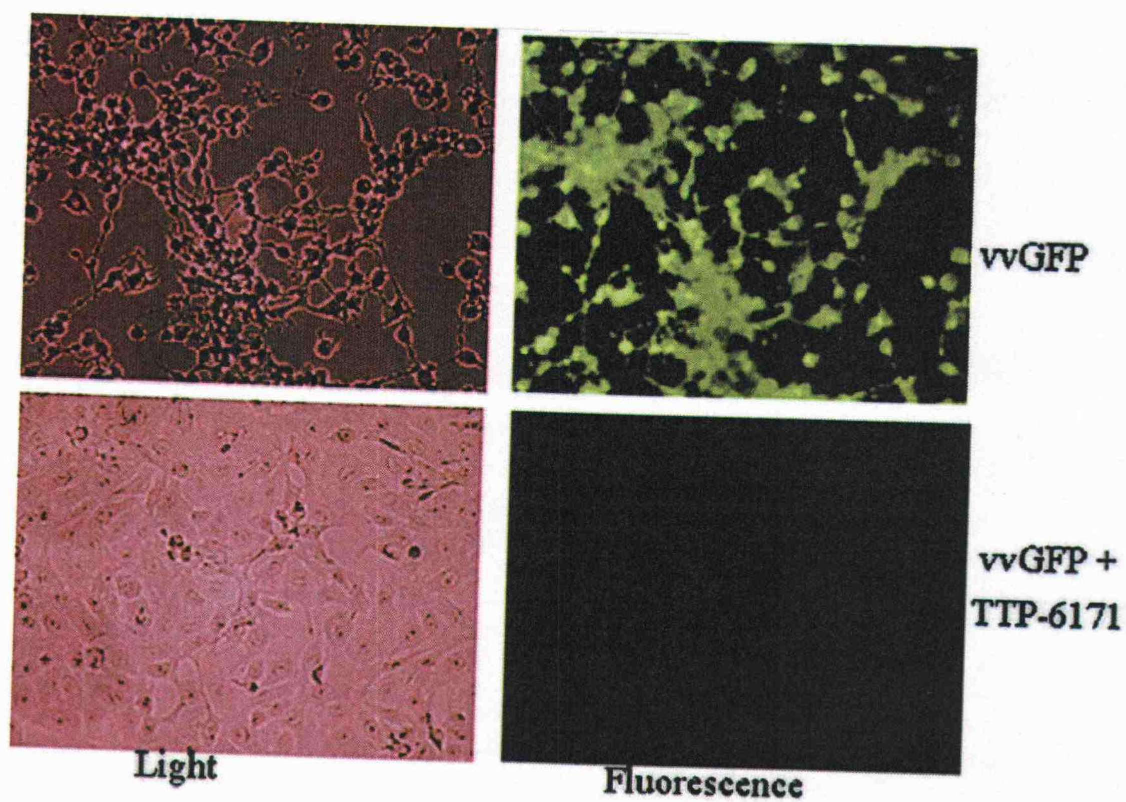


Figure 5.4. Light and fluorescent images of vvGFP infected cells with and without compound TTP-6171.

virus, or Adenovirus at a concentration of 100 μM (data not shown). These results suggest that TTP-6171-mediated inhibition appears to be specific for members of the *Orthopoxviridae*.

TTP-6171 inhibits a late stage in the viral life cycle.

To investigate which stage of the viral life cycle TTP-6171 was inhibiting, immunoblot analyses of extracts from cells infected with vvGFP (+/- candidate drugs) were conducted using a monospecific antiserum against the VV A3L protein. The gene product of the A3L ORF, P4b, is a 72-kDa late protein that is cleaved by I7L to form the mature core protein 4b during a late stage of morphogenesis (Byrd *et al.,* 2003). As A3L is a late gene, no P4b signal on the immunoblot would indicate that TTP-6171 inhibited a early step in viral replication (binding, penetration, early or delayed-early transcription, uncoating, or DNA replication). If both the P4b precursor and 4b product were observed, this would suggest that TTP-6171 acted at a very late stage of viral assembly or egress. In contrast, expression of P4b with no, or reduced, processing to the 4b product would be the phenotype expected if TTP-6171 was an I7L inhibitor. In control experiments the expected phenotypes were observed. Cells infected with vvGFP in the absence of compound express both P4b precursor (apparent MW 66 kDa) and the processed 4b product (apparent MW 62 kDa, Fig. 5.5, lane 2). When vvGFP infected cells are treated with rifampicin, a morphogenesis inhibitor, P4b is made but not processed (Fig. 5.5, lane 3). When vvGFP infected cells are treated with hydroxyurea, an inhibitor of viral DNA replication, no P4b is observed (Fig. 5.5, lane 4). Lanes 5-8 show representative results

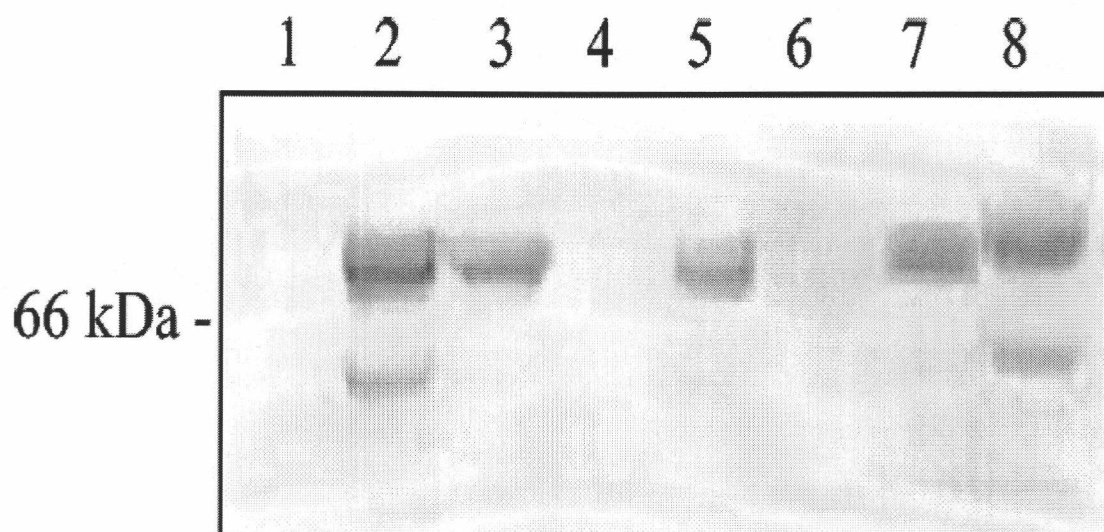


Figure 5.5. Processing of P4b core protein precursor. Western blot of virus-infected whole cell extracts probed with P4b antisera. Molecular weights in kDa indicated on the left side of the blot. In each lane cells are infected with vvGFP in the presence of 100 μM compound. Lane 1) mock infected; lane 2) vvGFP alone; lane 3) rifampicin at 100μg/ml; lane 4) hydroxyurea at 5 mM ; lane 5) TTP-6171; lane 6) TTP-130961; lane 7) TTP-176510; lane 8) TTP-123045.

with a number of the drug candidates tested. In the presence of the drug tested in lane 8, P4b is still being processed to 4b indicating inhibition of virus replication is occurring at some stage after morphogenic proteolysis and therefore, the drug is likely not targeting I7L. Likewise the drug tested in lane 6 inhibits the production of P4b entirely and therefore blocks some early stage of replication prior to I7L expression. In contrast, the compounds tested in lanes 5 and 7 appear to be hitting the correct stage of the viral lifecycle since P4b is made but not processed. The compound tested in lane 5 is TTP-6171.

Phenotypic analysis via electron microscopy.

Ts16 is a temperature sensitive mutant VV in which the responsible mutation has been mapped to the I7L gene (Condit *et al.,* 1983; Kane & Shuman, 1993). At the permissive temperature, a variety of typical poxvirus assembly intermediates are observed including dense oval and brick-shaped virions that are consistent with infectious intracellular mature virions. At the non-permissive temperature, assembly is aborted with the accumulation of immature particles containing enveloped viroplasm but with little or no subsequent nucleoid condensation. An electron microscopic examination of v17- or vvGFP-infected cells in the absence or presence of TTP-6171 is shown in Figure 5.6, panels A to P. As seen in panels A through H of Figure 5.6, the phenotypes of v17 observed in the presence or absence of TTP-6171 are identical to wild-type virus, with both immature virus and intracellular mature virus particles present. The phenotype of the parental virus vvGFP treated with TTP-6171 shows that no intracellular mature virus is observed in any of the cells. There was an accumulation of immature particles with

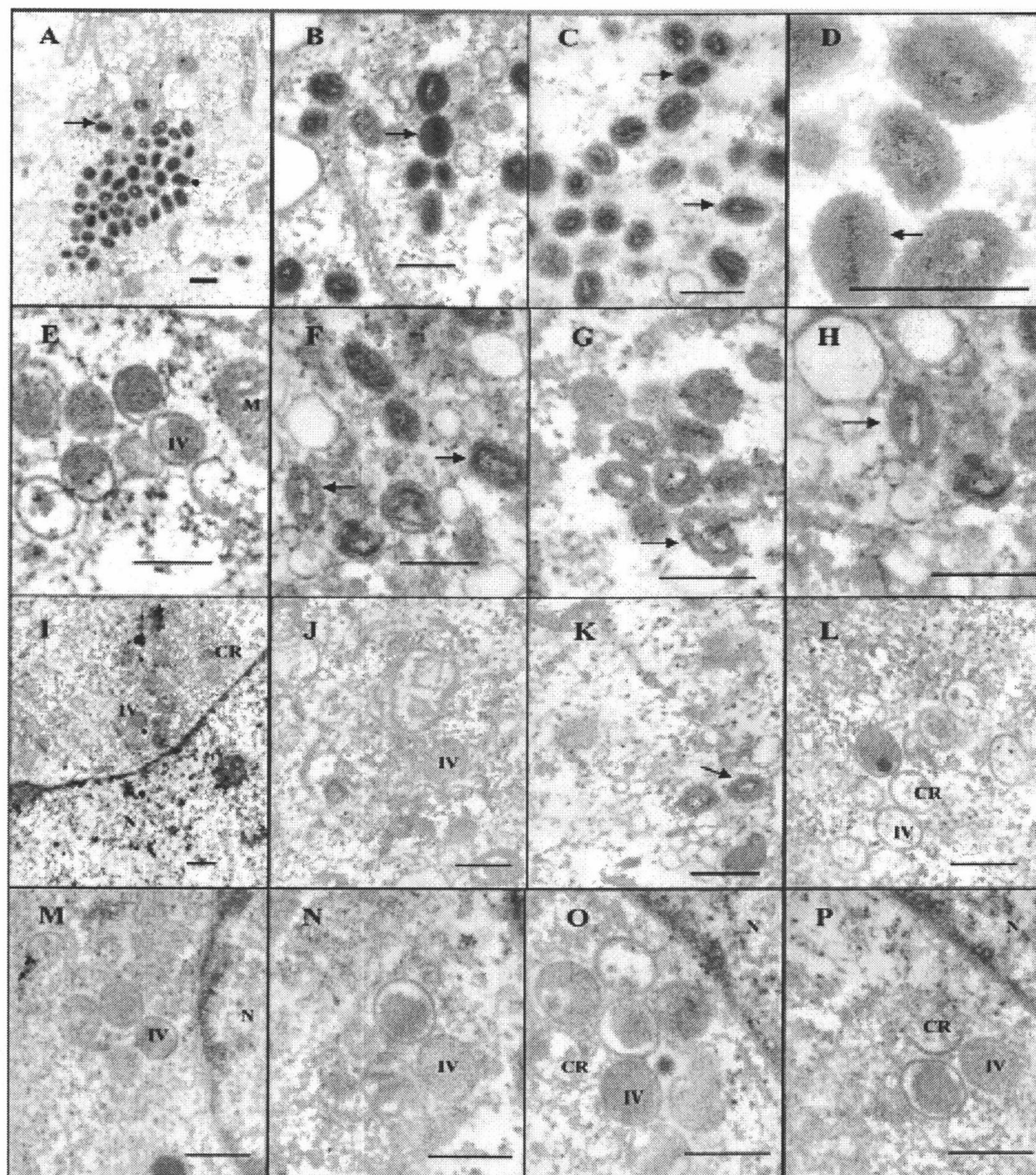


Figure 5.6. Electron micrographs of BSC_{40} cells infected with virus at an moi of 3. Cell were harvested 24 h after infection and fixed, and ultrathin sections were prepared for transmission electron microscopy. Bar represents 400 nm. Arrows indicate intracellular mature virus. IV, intracellular virus; N, nucleus; CR, crescent-shaped viral particles. Panels A to D show v17 in the absence of compound, panels E to H show v17 treated with TTP-6171 (100 μM), and panels I to P show vvGFP treated with TTP-6171 (100 μM).

numerous crescent-shaped particles present. This result supports the hypothesis that TTP-6171 is an inhibitor of I7L activity.

Isolation of drug resistant virus.

In order to directly demonstrate that the target of TTP-6171 mediated inhibition was the I7L gene product, vvGFP was subjected to numerous passages in the presence of TTP-6171 to generate drug-resistant viral mutants. Cells were infected with vvGFP at a moi of 0.1 in the presence of the IC_{50} concentration of compound for 24 hr prior to being harvested. The titer of the virus-infected cellular cytoplasmic extract was determined, and a portion of this extract was used to infect fresh BSC_{40} cells. As the data in Figure 5.7A shows, the titer of infectious progeny dropped 7 logs from passage 0 to 4. Starting with passage 5 the progeny titer began to rise in the presence of the drug until a 4 log increase was observed by passage 7, presumably due to the emergence of a drug-resistant mutant population. After passage 9, individual resistant viral plaques were subjected to several rounds of plaque purification, the genomic DNA extracted, and the I7L gene was amplified by PCR and sequenced. All the resistant viruses, such as v17, were found to have mutations in positions 104 and 324 within the I7L open reading frame with a Y to C mutation at 104 and an L to M mutation at position 324 (Fig. 5.7B). Because of the manner in which they were isolated, it is likely that the drug-resistant mutant viruses are siblings of each other and that these common mutations do not necessarily represent a common point for mutation. Interestingly, the latter mutation is in close proximity to the C328 residue that is part of the catalytic triad, so that it might be expected to influence active site binding specificity. Once purified, drug-resistant v17 grows with the same

kinetics and produces the same yield as wild-type virus in the absence of drug (data not shown) indicating the mutations did not have deleterious effects on viral replication *per se*. To compare the growth of the parental virus vvGFP with the drug-resistant virus v17, a one-step growth experiment was performed where cells were infected with either vvGFP or v17 at an MOI of 1, treated with various concentrations of TTP-6171 1 h postinfection. Figure 5.7C shows that there is no difference in viral replication between the two viruses at any drug concentration at 2 h postinfection but that at 12, 24, and 48 h postinfection, v17 replicates to a higher titer at all drug concentrations. An immunoblot analysis of extracts from cells infected with v17 (with or without TTP-6171) and using antiserum against the VV A3L protein indicates that P4b to 4b processing is restored in the resistant mutant, although no to the same level as in the absence of compound (data not shown). Pulse-label and pulse-chase analyses were carried out and support the results of the immunoblotting (data not shown). However, at late times TTP-6171 has general inhibitory effects on late protein synthesis, which, in conjunction with toxicity at high concentrations makes interpretation of processing difficult. It is possible that this compound inhibits another aspect of viral replication in addition to proteolytic processing.

Transient expression.

To confirm the hypothesis that the TTP-6171 resistant phenotype of v17 was due to the observed mutations in I7L and not another second site mutation and to determine whether one or both of the observed mutations contributed to resistance, transient expression and marker rescue procedures were employed. The mutant I7L gene from v17

was cloned into a plasmid vector, pRB21 (Blasco & Moss, 1995), resulting in plasmid pI7L-17, such that its expression would be driven by a strong synthetic early-late VV promoter. Using this plasmid (pI7L-17) as a substrate, site-directed mutagenesis was used to create I7L genes containing only one of the observed mutations, both of them, or neither (wild-type). First these plasmids were tested for their ability to rescue the replication of vvGFP in the presence of TTP-6171. The data in Figure 5.8 shows that as expected, over-expression of wild-type I7L provides some rescue suggesting that TTP-6171 is a competitive inhibitor of I7L. Expression of the v17 I7L gene product provided 10-fold higher rescue, consistent with the hypothesis that it is responsible for the TTP-6171 resistance of v17. Neither of the single mutations in v17 provided enhanced rescue when compared to wild-type I7L or the double mutant (vI7 I7L). This result suggests that both mutations contribute to the full resistance phenotype of v17. As a final biological proof, the mutant alleles were recombined into the vvGFP genome, replacing the genomic I7L locus. In agreement with the transient expression results, both single mutants, v104 and v324, provide some degree of resistance to TTP-6171 inhibition, whereas the double mutant, v104-324, has the full resistance phenotype of v17 (Fig. 5.8). Furthermore, since the mutations were selected for by passage in the presence of TTP-6171, this strongly suggests that the I7L cysteine proteinase is the target of this drug.

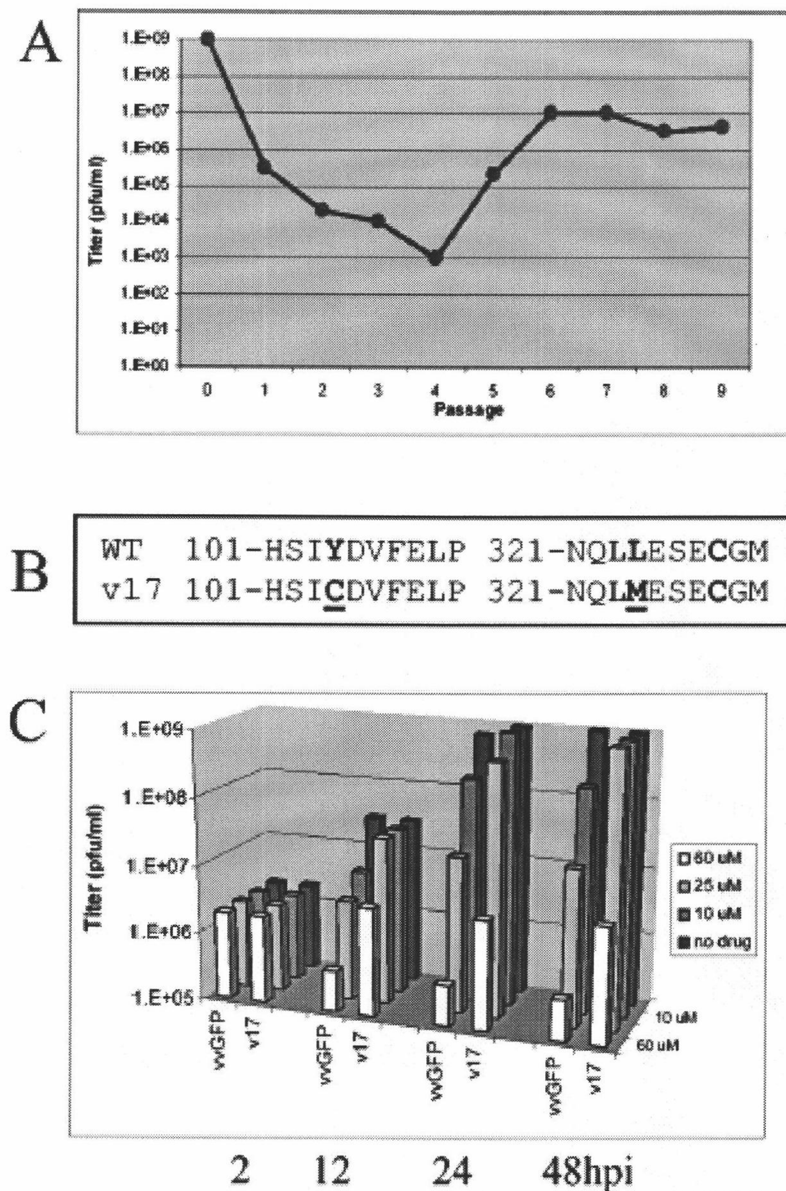


Figure 5.7. Passaging for drug resistance. (A) Viral titer of vvGFP passaged in the presence of TTP-6171. (B) Locations of mutations within the I7L open reading frame found in the TTP-6171 resistant virus isolate. WT is wild type virus; v17 is a TTP-6171 resistant isolate. (C) One-step growth curve of vvGFP and v17. Cells were infected at an MOI of 1 and treated with either 0, 10, 25, or 60 μM TTP-6171; cells were harvested at 2, 12, 24, or 48 h postinfection, and the viral titers were determined.

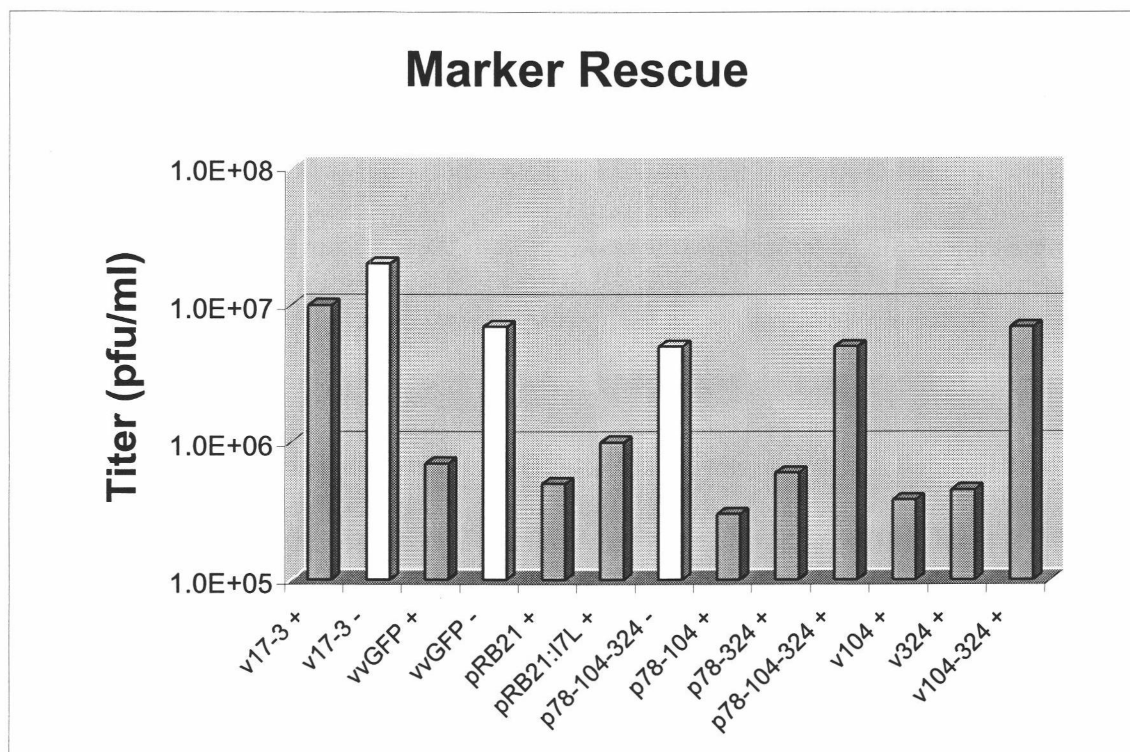


Figure 5.8. Transient expression. Wild type or mutant viruses were grown in BSC_{40} cells and transfected with either plasmids containing no insert, wild-type I7L, or mutant I7L. The graph shows viral titers obtained in the presence or absence of TTP-6171. Transient expression with each plasmid is with wild type virus. Light bars indicate no drug treatment (-) while dark bars indicate treatment with 25 μM TTP-6171 (+).

DISCUSSION

The classic approach to antiviral drug discovery involves high-throughput screening of large libraries of chemical compounds to look for inhibitors of viral replication. While effective, this approach has proven to be slow and tedious. An alternative approach, which we have utilized here, involves modeling of the target structure and computational docking of potential inhibitor molecules, followed by in vitro screening to discover candidate drugs. Drugs that do not hit the target will also be discovered by this approach, and further investigation into their mode of action will be necessary to determine their utility.

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 (Figure 5.1). 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* (Figures 5.2 and 5.3). A number of chemically related effective inhibitors were identified with the prototype being compound TTP-6171 (Table 5.2). Investigation of the mechanism of TTP-6171 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 (Figures 5.5 and 5.6). These data were consistent with the idea that I7L catalytic activity is the target of TTP-6171 inhibition. To confirm this hypothesis, TTP-6171 resistant

mutants were selected and mutations within the I7L gene were shown to be responsible for the resistant phenotypes (Figures 5.7 and 5.8).

TTP-6171 represents a very promising poxvirus antiviral drug candidate. When compared to cidofovir, which is the best-known poxvirus antiviral drug, TTP-6171 appears to have superior characteristics in tissue culture. Whereas, cidofovir has a TC_{50} of 280 μM, IC_{50} of 33 μM and a therapeutic index of 8 against VV (Kern, 2003), under similar conditions TTP-6171 has an IC_{50} of 12 μM and almost undetectable toxicity against a variety of tissue culture cells from a number of species, including monkey (BSC40), human (HeLa and 293), and mouse (L929). TTP-6171 appears to exhibit an excellent specificity in that it does 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 (Andres *et al.,* 2001; Li & Hochstrasser, 1999). Although in this study, TTP-6171 was only tested against several strains of VV and cowpox virus, it is highly likely that it will be broadly effective against other orthopoxviruses. As shown in Table 5.3, 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 TTP-6171

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.

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. Although the development of effective antiviral drugs has proved challenging for some viruses, the 10-14 day incubation period between exposure to infectious smallpox and the development of obvious serious disease symptoms may provide a therapeutic window. It is envisioned that an effective smallpox antiviral drug will have several utilities including prophylaxis of individuals at risk to exposure, treatment of individuals already exposed, and as a adjunct to vaccination in immunocompromised patients. In any case, TTP-6171 represents an appropriate launch point for initiating hit-to-lead chemical optimization to improve drug activity as a prequel to initiation of animal efficacy studies in appropriate surrogate poxvirus animal challenge models.

ACKNOWLEDGEMENTS: We would like to thank Brita Hanson, Stephen Ireland and Guoxiang Huang for technical assistance. We would like to thank Mike Nisson for assistance with the electron microscopy. We would also like to thank R.C. Condit for *ts16* and D. Pickup for CPV. This work was funded by NIH grant AI-48486 and ARMY contract DAMD-17-080C-0040.

Table 5. 3. Sequence identity of catalytic region of I7L among various poxviruses

Virus	Family	Catalytic triad sequence	% Identity with vvI7L ORF	P4b (AGA)
VV I7L	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	100	Yes
Cowpox	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	96	Yes
Camelpox	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	99	Yes
Variola major	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	99	Yes
Variola minor	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	99	Yes
Monkeypox	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	99	Yes
Ectromelia	Orthopoxvirus	241- HWKCVIYDKK QCLVSFYDSG 321- NQLLESECGM	95	Yes
Sheeppox	Capripoxvirus	251- HWKCVIFDKE KLVVCFYDSG 332- NQLLESECGM	63	Yes
Lumpy skin	Capripoxvirus	251- HWKCVIFDKE KLVVCFYDSG 332- NQLLESECGM	66	Yes
Yaba-like	Yatapoxvirus	246- HWKCVIINKE KLFVAFYDSG 327- NQLLESECGM	69	Yes
Swinepox	Suipoxvirus	249- HWKCVIFDKE HHIVCFYDSG 330- NQLLESECGM	68	Yes
Rabbit fibroma	Leporipoxvirus	245- HWKCVIFDKE KQIACFYDSG 321- NQLLESECGM	66	AGV
Myxoma virus	Leporipoxvirus	245- HWKCVIFDKE KQIVCFYDSG 326- NQLLESECGM	66	AGV
Molluscum contagiosum	Molluscipox	242- HWKSLVFDRR QRLVAFYDSG 322- NQLLESECGM	62	Yes
Fowlpox virus	Avipoxvirus	242- HWKCAIYDKN RDFICFYDSG 322- NQLLESECGM	58	Yes
Canarypox virus	Avipoxvirus	242- HWKCLIYDRE NDFVCFYDSG 323- NQLMESECGM	55	NP
Amsacta moorei	Entomopoxvirus	254- HFTSAVIDKK RKICYLFNSS 336- IQYDSPDCGM	25	NP

CHAPTER 6

DEVELOPMENT OF AN *IN VITRO* CLEAVAGE ASSAY SYSTEM TO EXAMINE VACCINIA VIRUS I7L CYSTEINE PROTEINASE ACTIVITY

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SUMMARY

Through the use of transient expression assays and directed genetics, the vaccinia virus (VV) I7L gene product has been implicated as the major maturational proteinase required for viral core protein cleavage to occur during virion assembly. To confirm this hypothesis and to enable a biochemical examination of the I7L cysteine proteinase, an *in vitro* cleavage assay was developed. Using extracts of VV infected cells as the source of enzyme, reaction conditions were developed which allowed accurate and efficient cleavage of exogenously added core protein precursors (P4a, P4b and P25K). The cleavage reaction proceeded in a time-dependent manner and was optimal when incubated at 25°C. I7L-mediated cleavage was not affected by selected inhibitors of metalloproteinases, aspartic acid proteinases or serine proteinases (EDTA, pepstatin, and PMSF, respectively), but was sensitive to several general cysteine proteinase inhibitors (E-64, EST, Iodoacetic acid, and NEM) as well as the I7L active site inhibitor TTP-6171 [C. Byrd *et al.*, J. Virol. 78:12147-12156 (2004)]. Finally, in antibody pull down experiments, it could be demonstrated that monospecific αI7L serum depleted the enzyme activity whereas control sera including αG1L, directed against the VV metalloproteinase, did not. Taken together, these data provide biochemical evidence that I7L is a cysteine proteinase which is directly involved in VV core protein cleavage. Furthermore, establishment of this I7L-mediated *in vitro* cleavage assay should enable future studies into the enzymology and co-factor requirements of the proteolysis reaction, and facilitate antiviral drug development against this essential target.

INTRODUCTION

The *Orthopoxviridae* include vaccinia virus, camelpox, cowpox, ectromelia, monkeypox, raccoonpox, skunkpox, taterapox, volepox, and variola. Viruses in this family are the cause of numerous diseases including smallpox (variola), and recent human outbreaks of monkeypox. Orthopoxviruses are large double-stranded DNA viruses that are unique amongst DNA viruses in that they replicate exclusively within the cytoplasm of infected cells. Vaccinia virus (VV) is the most extensively studied virus in this group and is the prototypic member. The genome of VV is predicted to encode over 200 open reading frames. VV expresses its genetic information in three stages, as early, intermediate, and late genes. The early genes, which account for approximately half of the genome and are transcribed prior to DNA replication, encode many of the proteins involved in viral DNA replication and intermediate gene expression. The intermediate genes, of which only a handful have been identified, are expressed after DNA replication, and encode proteins that are activators of late gene expression. The late genes encode many proteins required for the transcription of early genes, the viral structural proteins and the enzymes necessary to process these proteins into their mature form.

Many viruses use proteolytic processing as a key step in their developmental cycle. RNA viruses and retroviruses commonly undergo formative proteolysis in which large polyproteins are cleaved by viral encoded proteinases to produce the structural and nonstructural proteins required for morphogenesis. DNA viruses such as poxviruses and adenoviruses commonly use another type of proteolysis, called morphogenic proteolysis where precursor proteins are first synthesized and then cleaved by viral proteinases to

produce the mature form of the protein. The mature protein then plays an essential role in virion formation. During VV assembly, as the spherical immature virions (IVs) are maturing into the first infectious form of vaccinia virus, intracellular mature virus (IMV), a series of events takes place including proteolytic processing of viral core proteins (Li & Hochstrasser, 1999; Silver & Dales, 1982; VanSlyke *et al.*, 1991a & b).

Our lab has worked to identify and characterize the proteinases of VV in order to understand their regulation, function, and biochemistry, with a long term goal of developing inhibitors of these enzymes as antiviral drugs. The gene product of the I7L open reading frame has recently been suggested to be the core protein proteinase of VV through the use of an *in vivo trans* processing assay (Byrd *et al.*, 2002; Byrd *et al.*, 2003). I7L is an essential late gene, as shown through temperature sensitive mutant viruses (Ericsson *et al.*, 1995; Kane & Shuman, 1993) and conditional lethal mutant viruses (Ansarah-Sobrinho & Moss, 2004; Byrd & Hruby, 2005) where under non-permissive conditions, viral morphogenesis is blocked prior to the formation of IMV. I7L is predicted to be a 47 kDa cysteine proteinase that cleaves the major core protein precursors P4a, P4b, and P25K, products of the A10L, A3L, and L4R open reading frames respectively, at a novel Ala-Gly-Xaa cleavage site with cleavage occurring after the glycine residue (Byrd *et al.*, 2002; Byrd *et al.*, 2003). I7L also is likely to be responsible for cleavage of the A17 membrane protein, at an Ala-Gly-Ala site (Ansarah-Sobrinho & Moss, 2004). This consensus Ala-Gly-Xaa cleavage site of vaccinia is similar to that used for both the adenovirus and African swine fever virus proteinases which cleave after the second glycine in a Gly-Gly-Xaa motif (Webster *et al.*, 1989; Andres *et al.*, 2001).

Comparative sequence analysis has suggested that the VV I7L proteinase is related to the ASFV and adenovirus cysteine proteinases and may form a new family of SUMO-1 related enzymes (Li & Hochstrasser, 1999; Andres *et al.,* 2001). The nucleophilic cysteine is responsible for cleavage and is activated by the imidazol group of the catalytic histidine residue. Substrate specificity is determined by the substrate binding pocket and is unique for each proteinase. Several critical residues have been identified as being necessary for enzymatic activity of I7L including the catalytic triad residues (Byrd *et al.*, 2003). Based on the identification of the catalytic residues and the predicted structure of the I7L proteinase, a new class of small molecule inhibitors was developed that are capable of inhibiting the replication of VV, and were found to specifically target I7L through the generation of drug resistant mutant viruses with the mutations mapping to I7L (Byrd *et al.*, 2004).

To date, direct studies on the enzymology of I7L-mediated proteolysis have not been possible due to the absence of a suitable biochemical assay. In the experiments reported here, we describe the development of an *in vitro* I7L-mediated cleavage assay. We have used this system to obtain both biochemical and immunological data to prove that I7L is directly involved in cleavage of the major VV core protein precursors. Having this assay available will now facilitate biochemistry of the I7L enzyme and identification of all the required reaction components to be undertaken.

MATERIALS AND METHODS

Cells and Viruses

BSC_{40} cells (Raczynski & Condit, 1983) were grown in Eagle's minimal essential medium containing 5% fetal calf serum (FCS) (Sigma, St. Louis, MO), 2 mM glutamine (Invitrogen, Carlsbad, CA), and 15 µg/ml gentamicin sulfate (Invitrogen) in a 37°C incubator with 5% CO_2. Purified *ts16* vaccinia virus was prepared as described (Hruby *et al.*, 1979). *Escherichia coli* strains were grown in Luria-Bertani broth or on Luria-Bertani medium containing 1.5% agar and ampicillin at 50µg/ml.

Plasmids

The A10L (P4a) gene was amplified by polymerase chain reaction using oligonucleotides KH10 (5' CATGCCATGGATGATGCCTATTAAGTCAATAGTTACT CTT-3') and KH11 (5'-CCGCTCGAGTTATTCATCATCAAAAGAGACAGAGTC-3'), digested with NcoI and XhoI, and cloned into the pTM1 vector, yielding pTM-P4a which utilizes a T7 promoter for expression. The A3L (P4b) gene was amplified using oligonucleotides KH08 (5'-CATGCCATGGATGGAAGCCGTGGTCAATAG-3') and KH09 (5'-TCCCCCGGGCTAAAAATAGTTCTGTAATATGTCTAGCGCT-3'), digested with NcoI and SmaI, and cloned into the pTM1 vector to yield pTM-P4b. The L4R (P25K) gene was amplified using oligonucleotides DN51 (5'-CATGCCATG GATGAGTCTACTGCTAGAAAAC-3') and KH07 (5'- CCGCTCGAGTCAATCCTTT GTCG-3'), digested with NcoI and XhoI, and cloned into the pTM1 vector to yield pTM-P25K. The pI7L and pI7LH241A plasmids were described in Byrd *et al.,* 2002.

Preparation of polyprotein or proteinase-containing extracts

Confluent monolayers of BSC_{40} cells in 6-well plates were infected with *ts16* VV at a multiplicity of infection of 2 plaque-forming units per cell and transfected with 2 μg of plasmid DNA using DMRIE-C (Invitrogen) following the manufacturer's indications. Infected cells were incubated either at the permissive temperature of 31.5°C or the non-permissive temperature of 39°C. Cells were harvested at 24 h post-infection by pipetting up and down to lift the cells from the surface. The infected cells were centrifuged at 10,000 x g for 10 min, the supernatant was aspirated off, and the pellet was resuspended in 500 μL homogenization buffer containing 20 mM HEPES (pH 7.4), 0.28 M sucrose, 2 mM EDTA. This was passed through a 25-gauge syringe 15 times. The homogenate was centrifuged at 700 x g for 5 min to separate the nuclei and unbroken cells from the supernatant. The supernatant was centrifuged at 100,000 x g for 30 min at 4°C to separate the membrane/particulate material from the supernatant. The supernatant was used as the source of enzyme.

Coupled TNT reactions with T7 RNA polymerase were performed according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin) as a source of substrate. Briefly, the TNT reactions were performed at 30°C in a final volume of 25 μL with 1 μg of plasmid DNA, using the non-radioactive Transcend label (biotinylated lysine residues are incorporated in the protein) provided with the kit for detection of protein.

<u>In vitro cleavage assay</u>

Reactions were performed at the indicated temperature in a final volume of 20 μL containing 1 μL of substrate, 13 μL of enzyme extract, and 6 μL of HEPES buffer, pH 7.4. After the indicated times, the reaction was stopped by the addition of SDS sample buffer, and the samples were subjected to SDS-polyacrylamide gel electrophoresis. The results were analyzed by immunoblotting following the instructions provided by the TNT kit.

<u>Inhibitor studies</u>

For inhibitor studies, the reactions described above were incubated for 6 hr in the presence or absence of the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma), 10 μM Pepstatin A (Sigma), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma), 10 μM or 100 μM N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) (Sigma), 1 mM iodoacetic acid (Sigma), 10 μM or 100 μM Leupeptin (Roche, Indianapolis, IN), 2.5 mM *N*-ethylmaleimide (NEM) (Sigma). For I7L specific inhibition studies, the reactions described above were incubated for 6 hr in the presence or absence of TTP-6171, TTP-1021, or TTP-0961 (Byrd *et al.,* 2004) at 5 μM or 20 μM final concentrations.

<u>Antibody competition studies</u>

For the antibody competition studies, 25 μl of I7L or G1L specific antiserum was added to 25 μL of enzyme extract on a rotating shaker overnight at 4°C. ProteinA:

Sepharose beads (Amersham Biosciences, Uppsla, Sweden) were added for 3 hrs and the antibody complex was centrifuged to pull down the I7L enzyme. The supernatant was used as the source of extract in the *in vitro* assay described above. As a control, enzyme extract was mixed with buffer instead of antibody and treated with beads in a similar manner.

RESULTS

To date, all studies of VV I7L activity have been carried out indirectly in transfected/infected tissue culture cells. Although this approach has provided some important insights into I7L biology, it is limited with respect to the study of I7L enzymology and identification of all the *cis* and *trans* factors required for substrate identification and catalysis. In order to approach these questions, we have sought to develop an *in vitro* cleavage assay for I7L. Thus far, the obvious approaches of expressing and purifying I7L from prokaryotic and eukaryotic expression vectors and combining with peptides or proteins containing a canonical A-G-X cleavage site have not been successful (data not shown), perhaps due to either the lack of essential co-factors or inappropriate assay conditions. As an alternative approach, we sought to develop a cleavage assay using infected cell extracts as the source of I7L activity and labeled core protein precursors made *in vitro* as the substrate. If successful, this system would provide the starting point for a dissection of the essential reaction components.

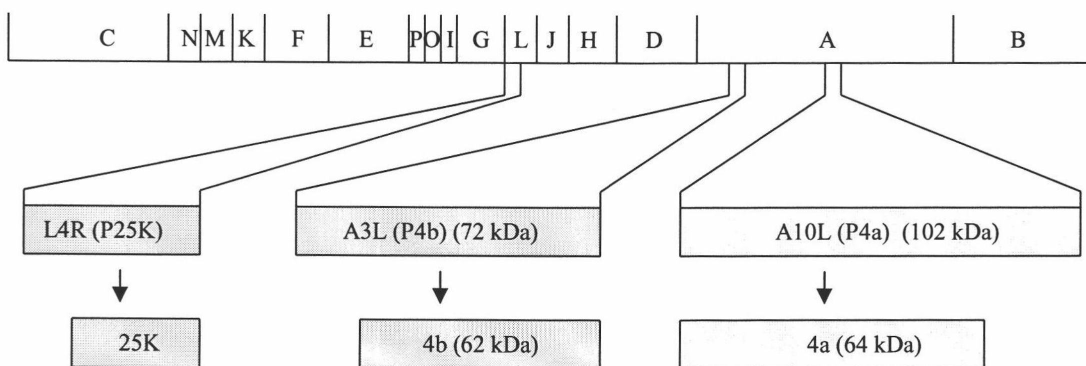

Figure 6.1. Schematic representation of the major core protein precursor cleavage products. The vaccinia virus genome is represented depicting three of the major core protein precursors, the gene products of the L4R, A10L, and A3L open reading frames, P25K, P4a, and P4b respectively. The precursors are shown being cleaved into their mature form. Molecular mass is indicated.

In-vitro Processing of Core Protein Precursors

The three major core protein precursors P4a (A10L), P4b (A3L), and P25K (L4R) which are known to be cleaved to a mature form (Figure 6.1) were cloned into plasmid vectors driven off of a T7 promoter to be used as a source of substrate for the assay. To investigate the ability of I7L to cleave the P4a, P4b, and P25K substrates *in vitro*, we have used a system where the substrates are produced from an *in vitro* transcription and translation assay using rabbit reticulocyte lysates and then mixed with I7L expressed from virus infected cells. BSC40 cells are infected with *ts16*, a temperature sensitive mutant virus in which the responsible mutation maps to I7L. The virus infected cells are incubated at the non-permissive temperature and transfected with plasmids expressing either wild-type I7L (pI7L) or I7L with the catalytic histidine residue mutated to an alanine (pI7LH241A). The extracts are prepared as described in the Materials and Methods. The extracts are mixed and incubated with the substrates for 3 hrs and then analyzed through SDS-PAGE and chemiluminescent detection. As shown in Figure 6.2, a specific band corresponding to unprocessed P4a (top panel), P4b (middle panel), or P25K (bottom panel) is produced when the substrate is run alone. When mixed with cellular extracts, or extracts from cells infected with *ts16* at the non-permissive temperature and transfected with mutant I7L, no cleavage products are observed. However, when mixed with extracts from either cells infected with *ts16* at the permissive temperature or cells infected with *ts16* at the non-permissive temperature transfected with wild-type I7L, the cleaved products 4a, 4b, and 25K are observed. Substrates with mutated A-G-X sites were not cleaved indicating that cleavage was occurring at the correct sites (data not

shown). For the rest of the reported studies, P25K was used as the source of substrate since it gave the best cleavage profile.

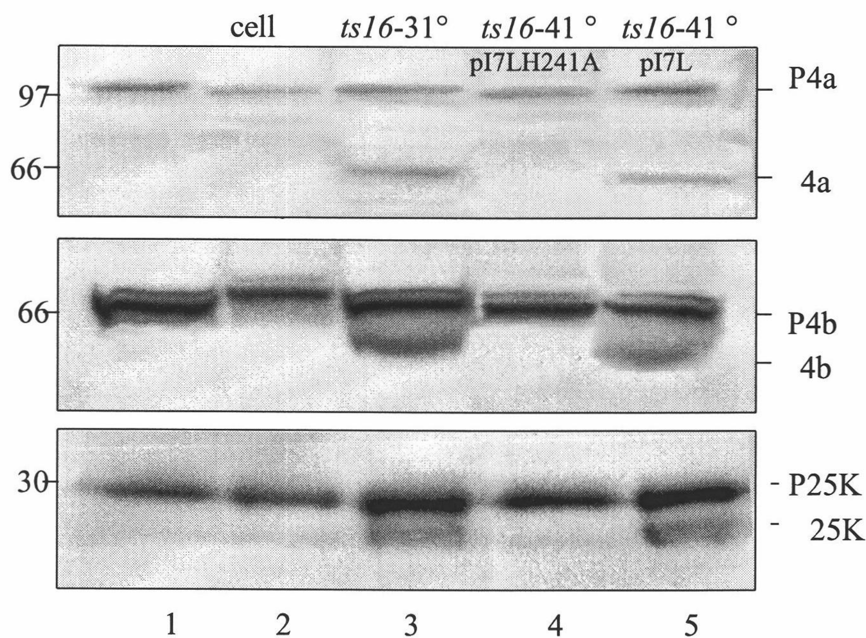

Figure 6.2. *In-vitro* proteolytic processing of P4a, P4b, and P25K. 1 μl of TNT produced substrate either P4a (A), P4b (B), or P25K (C) was mixed with 5 μl of Hepes buffer and 14 μl of enzyme extracts, either from uninfected cells, or cells infected with *ts16* at the permissive or non-permissive termperature. At the non-permissive temperature plasmid borne I7L, either wild-type (pI7L) or mutant I7L (pI7LH241A) was transfected in as the source of enzyme. The reaction was incubated at 29°C for 6 hrs before being stopped by the addition of SDS sample buffer. Molecular weight is indicated on the left and the core protein precursor and product on the right.

Processing Kinetics of Core Protein Precursors

To determine the optimal temperature and kinetics of processing of the core protein precursors in the *in vitro* cleavage assay, a time course of I7L-mediated processing at various temperatures was performed. As shown in Figure 6.3A, at 0°C, no processing was observed during the 20 hr time period. At 25°C, a gradual increase in the amount of P25K cleavage product was observed starting at 15 min and increasing throughout the 20 hr incubation period (Fig. 6.3B). Compared with the rate of cleavage at 25°C, cleavage was slower at 30°C (Fig. 6.3C), starting around 30 min and increasing through the 20 hr period, but never to the same level as at 25°C. Processing is greatly reduced at 37°C with only a faint processed band ever appearing (Fig 6.3D).

Influence of Thiol Reagents on the Protease Activity

Based on its sequence similarity to the adenovirus protease, the African swine fever virus protease, and an ubiquitin-degrading enzyme in yeast, as well as the identity of a catalytic triad composed of histidine, cysteine, and aspartic acid, I7L has been classified as a cysteine proteinase. The thiol reagents dithiothreitol (DTT) and cysteine have been shown to enhance the cleavage activity of the adenovirus protease in an *in vitro* peptide cleavage assay (Webster *et al.*, 1993). To determine whether these agents have a similar effect on the activity of I7L, they were added to the *in vitro* assay in a final concentration from 0-10mM. However, no increase in cleavage activity was observed with the addition of either DTT or cysteine (data not shown). It is possible that once purified recombinant enzyme is produced these thiol reagents may increase its activity.

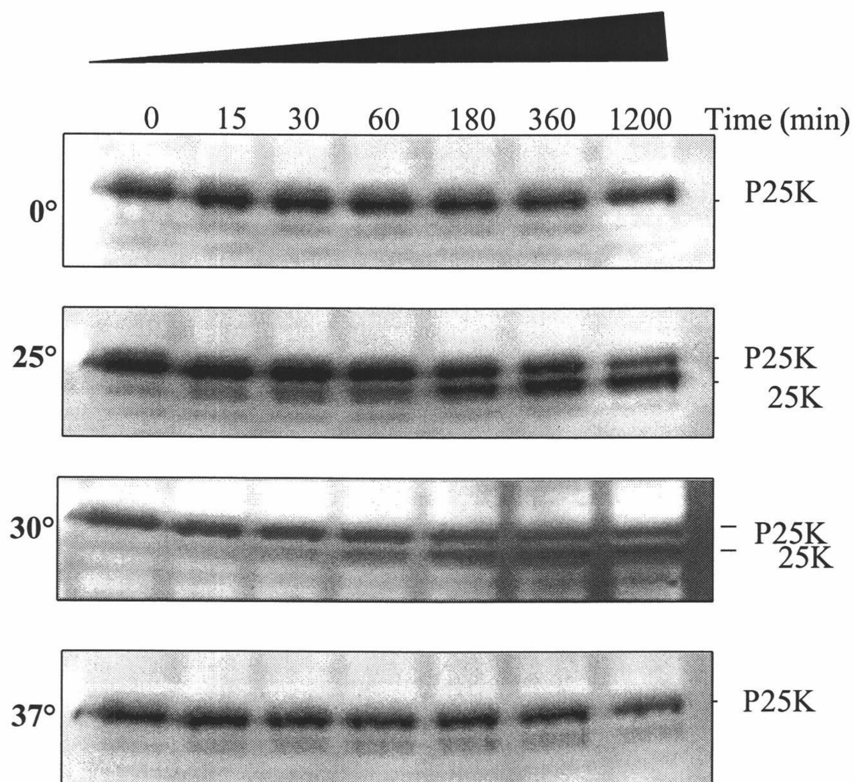

Figure 6.3. Processing kinetics of P25K. Samples were incubated at either 0°C (A), 25°C (B), 30°C (C), or 37°C (D) for up to 20 hrs, harvested at the indicated times and the reaction stopped by the addition of SDS sample buffer. Incubation temperature is indicated on the left and P25K precursor and 25K mature product are indicated on the right.

Effect of Inhibitors on Protease Activity and Characterization as a Cysteine Proteinase

The *in vitro* assay allowed us to test the effects of various protease inhibitors, as well as specific small molecule inhibitors on the activity of I7L. As shown in Figure 6.4, the metalloproteinase inhibitor ethylenediaminetetraacetic acid (EDTA), the aspartic proteinase inhibitor pepstatin, and the serine proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) had no detectable effect on cleavage activity. The cysteine proteinase inhibitors iodoacetic acid (IA) and *N*-ethylmaleimide (NEM) efficiently blocked I7L mediated proteolysis of P25K. The cysteine proteinase inhibitors E-64 and EST were shown to inhibit protease activity at a relatively high concentration, but not at the lower concentration tested. This is consistent with what has been observed for both the adenovirus protease (Webster *et al.*, 1989b), and the African swine fever virus protease (Rubio *et al.*, 2003). The failure of E-64 to inhibit protease activity at the lower concentration tested, and the location of the active site residues may suggest that each of these enzymes are not conventional papain-like enzymes, but may be a new family of cysteine proteinases. Somewhat surprisingly, the cysteine protease inhibitor leupeptin also failed to inhibit protease activity, although this lack of inhibition was also observed with the adenovirus proteinase (Webster *et al.*, 1989b).

Next we wanted to determine if the small molecule I7L inhibitors previously developed as antiviral drug candidates (Byrd *et al.*, 2004) could be shown to specifically inhibit the activity of I7L in the *in vitro* assay. The compound TTP-6171 has been shown to inhibit viral replication in tissue culture, with drug resistant virus mutations mapping to I7L (Byrd *et al.*, 2004). Here we see that this compound along with TTP-1021, which was also found to inhibit I7L in tissue culture, inhibits the processing of P25K *in vitro*.

However the compound TTP-0961, which was not found to generate resistant mutants in the I7L gene (data not shown), does not inhibit cleavage. These results demonstrate that this assay can be used for the screening of specific I7L inhibitors and confirms that this class of molecules targets I7L.

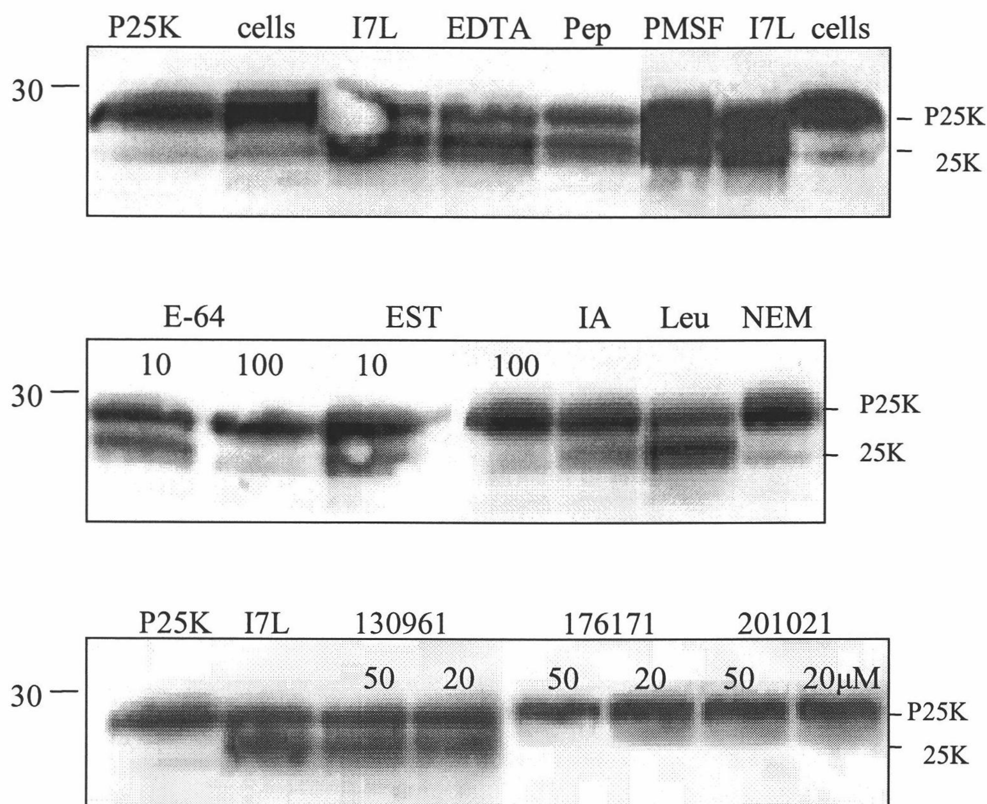

Figure 6.4. Effect of inhibitors on *in-vitro* processing. Various concentrations of protease inhibitors were added to the *in-vitro* processing assay for 6hr at 29 deg. The first lane is P25K expressed alone with no extract added. The second lane is P25K mixed with cellular extracts and the third lane is P25K mixed with I7L enzyme extracts. Each of the remaining lanes has P25K mixed with I7L enzyme extracts plus inhibitor. Ethylenediaminetetraacetic acid (EDTA) was used at 1mM. Pepstatin A, Pep, was used at 10 μM. Phenlymethanesulfonyl fluoride (PMSF) was used at 1 mM. N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) and a related product EST, were used at 10 μM and 100 μM concentrations. Iodoacetic acid (IA) was used at 1 mM. Leupeptin (Leu) was used at 1 mM, and N-ethlymaleimide (NEM) was used at 2.5 mM. The concentrations of TTP-6171, TTP-1021, and TTP-0961 are indicated.

Table 6.1: Effect of Protease Inhibitors on *in-vitro* Processing

Inhibitor	Concentration	Inhibit Cleavage
Metalloproteinase inhibitor		
EDTA	1 mM	-
Aspartic proteinase inhibitor		
Pepstatin	10 μM	-
Serine proteinase inhibitor		
PMSF	1 mM	-
Cysteine proteinase inhibitor		
E-64	10 μM	-
E-64	100 μM	+
EST	10 μM	-
EST	100 μM	+
Iodoacetic acid	1 mM	+
Leupeptin	1 mM	-
NEM	2.5 mM	+
TTP inhibitors		
176171	50 μM	+
	20 μM	+
201021	50 μM	+
	20 μM	+
130961	50 μM	-
	20 μM	-

Effects of I7L antibody competition on cleavage

To directly demonstrate that the cleavage observed in the *in vitro* assay requires the presence of I7L, increasing concentrations of I7L specific antiserum were added to the enzyme extracts overnight, and then the complex was precipitated with Protein A sepharose beads to deplete the extract of I7L and any associated co-factors. As shown in Figure 6.5, both of the I7L antisera tested inhibited cleavage of P25K while an antiserum targeting a different VV gene product, G1L, did not inhibit cleavage.

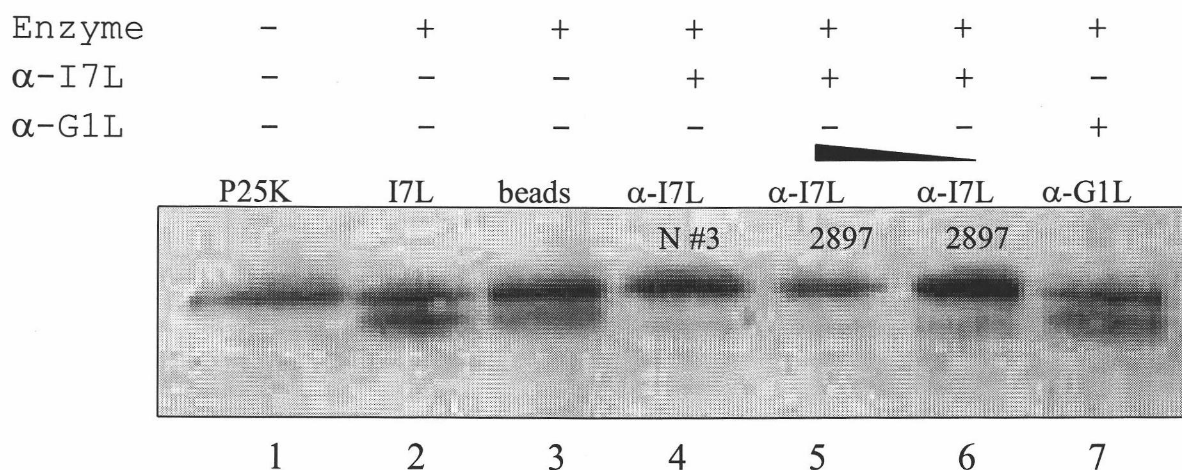

Figure 6.5. Effect of antibody competition on *in-vitro* processing. The first lane is P25K expressed alone. Lane 2 is P25K mixed with I7L enzyme extracts. Lane 3 is P25K mixed with I7L extracts that have been diluted with Hepes buffer and treated with Sepharose beads. Lanes 4, 5, and 6 are P25K mixed with I7L extracts that have been incubated overnight with different I7L antiserum (indicated on each lane), treated with Sepharose beads and the antibody complex removed by centrifugation. Lane 7 is P25K mixed with I7L extracts incubated with G1L antiserum as above.

DISCUSSION

In this report, a cell-free transcription and translation system was used to develop an *in vitro* cleavage assay for the VV cysteine proteinase I7L. Proteolytic activity was obtained by co-expression of I7L in *ts16* infected cells at the non-permissive temperature. Each of the major core protein precursors, P4a, P4b, and P25K, were shown to be cleaved to their mature products by I7L using the *in vitro* assay. Evidence that this cleavage is specific to I7L was shown through the fact that expressing a mutant form of I7L resulted in the inability to cleave the core protein precursors. Antibody pull down experiments with αI7L supported the conclusion that I7L plays a direct role in the proteolytic reaction.

A time course of processing at various temperatures indicated that for this particular assay, the optimal temperature for the reaction to be carried out at is 25°C with processing beginning as soon as 15 minutes after addition of enzyme and increasing as time progresses. The cleavage reaction was never driven to completion and this may be due to a lack of replenishing co-factors or the enzyme may have been used up in the reaction. It was surprising that the optimal reaction temperature was 25°C instead of 37°C which is the optimal growth temperature for VV in cell culture. One possible explanation is that I7L is present at high concentrations in the extract and one can measure marginal activity at low temperature, whereas at higher temperatures other proteinases are activated which degrade the I7L enzyme.

Known cysteine protease inhibitors such as E-64, iodoacetic acid, and NEM were shown to inhibit the *in-vitro* cleavage reaction while the metalloproteinase inhibitor EDTA, the aspartic acid protease inhibitor pepstatin, and the serine protease inhibitor

PMSF all failed to inhibit the cleavage reaction indicating that the enzyme responsible for cleavage is a cysteine protease. Interestingly the cysteine protease inhibitors leupeptin, and low concentrations of E-64 did not inhibit the reaction. These cysteine protease inhibitors were also not shown to be effective against either the African Swine Fever Virus protease (Rubio *et al.,* 2003) or the adenovirus protease (Webster *et al.,* 1989b), further providing support for the theory that these enzymes may form a new family of cysteine proteases that differ from papain-like cysteine proteases.

Of particular interest, the small molecule inhibitors designed to fit into the active site pocket of I7L and previously shown to inhibit viral replication (Byrd *et al.*, 2004), were found to be active in inhibiting the *in vitro* cleavage reaction described here. A related compound (TTP-0961) that was not found to map to I7L was not able to abolish cleavage. This indicated that this assay may be useful for high-throughput screening of compounds to identify those that have specific activity for I7L.

Until this point, all work demonstrating that I7L is the core protein proteinase has been done through transient-expression assays and the use of conditional lethal viruses in tissue culture (Ansarah-Sobrinho & Moss, 2004; Byrd *et al.*, 2002; Byrd *et al.*, 2003; Byrd & Hruby, 2005). While the data obtained has indicated that I7L is essential for these processing activities, it did not rule out the possibility that some other factor or enzyme was also required for this activity to occur. Through the use of an *in vitro* assay we have shown that I7L is capable of cleaving the core protein precursors but that an additional co-factor is required for this activity to occur since expression of the enzyme through cell-free translation produced inactive enzyme. The co-factor(s) necessary for cleavage have yet to be determined. However, having the assay described in this report available

will now enable a reductive analysis to be conducted to identify all the essential components of the reaction and to study their individual biochemical characteristics.

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CHAPTER 7

CONCLUSIONS

In many ways vaccinia virus represents an ideal model system in which to study a variety of questions having to do with development, DNA replication, assembly of complex macromolecular structures, and regulation of gene expression. The investigation of the regulation of a complex genetic system is particularly suited to vaccinia since the virus encodes over 200 genes whose expression is regulated in a temporal fashion. The entire genome has been sequenced which facilitates the identification and manipulation of individual genes. Some of the advantages of using vaccinia include the fact that unlike most other DNA viruses, VV replicates exclusively within the cytoplasm of host cells, requiring the virus to encode many of the enzymes and factors necessary to replicate its genome. The ability to use marker transfer (mutating particular genes and then investigating the results by recombination back into the viral genome), transient expression (expressing tagged, mutated, or wild type genes from a plasmid within infected cells), or creating recombinant viruses facilitates the study of the genes and processes occurring in the virus.

Regulation of gene expression can occur in different stages including at the level of genome organization, transcription and processing, translation, post-translational modification, and assembly. Proteolytic cleavage of proteins is one method of post

translational modification that we have chosen to look at through the studies presented in this thesis. Prior to this work, it was known that a number of viral proteins are cleaved during the course of the viral life cycle and that the processing of some of these proteins, especially the major core protein precursors is essential for infectious progeny to be produced, however the enzyme responsible for these cleavage reactions was unknown.

In this thesis, a *trans* processing assay in VV-infected cells with epitope tagged substrate and enzyme has allowed us to determine that the core protein proteinase of vaccinia (vCPP) is encoded by the I7L open reading frame. Mutation of any of the active site residues of I7L or of the Ala-Gly-Xaa cleavage site in the substrates abolishes this cleavage. I7L was shown to cleave each of the major core protein precursors including P4a, P4b, and P25K. A conditional-lethal virus in which I7L was placed under the control of the Tet operator/repressor system showed that I7L is essential for virion morphogenesis, and that in the absence of I7L expression, morphogenesis arrests at a stage prior to complete core condensation. Using homology-based computational modeling, a structural model of I7L was developed and used along with *in silico* drug docking to search a chemical library for compounds predicted to fit into the active site pocket of the enzyme. A novel class of small molecule inhibitors was shown to specifically inhibit the activity of I7L, thereby blocking viral replication. It is hoped that with further development, these compounds and others will be shown to be able to inhibit the replication of each virus in the orthopoxvirus family, including the causative agent of smallpox. To verify conclusively that it is the action of I7L and not another cellular or viral protease that is responsible for cleavage of the core protein precursors, an *in vitro* assay was developed where I7L enzyme was shown to be able to cleave each of the core

protein precursors P4a, P4b, and P25K produced through a transcription and translation assay. Known cysteine protease inhibitors as well as the specific small molecule compounds developed in this thesis were able to inhibit the activity of I7L in this *in vitro* assay whereas known metallo, aspartic acid, or serine protease inhibitors had no effect on the enzymatic activity of I7L. This assay may be useful for high-throughput screening of compounds to identify those that have specific activity for I7L. Purified recombinant I7L enzyme has yet to be produced and used in this assay to further characterize the enzyme activity.

Taken together, the data we have presented here, as well as analysis of the VV G1L conditional lethal mutant (Hedengren-Olcott *et al.,* 2004), suggests a morphogenesis model (Figure 7.1) in which these two putative proteases operate sequentially to regulate assembly, with I7L functioning to cleave the major core protein precursors to allow them to assume the proper configuration for virion maturation. This activity is followed by the activity of G1L to allow for complete core condensation and the progression to the formation of intracellular mature virus particles. If the activity of the I7L proteinase is blocked, viral morphogenesis arrests prior to core condensation. If the activity of G1L proteinase is blocked, viral morphogenesis arrests at a stage subsequent to this but still prior to complete core condensation. It will be of interest in the future to determine the specific activity of the G1L enzyme to complete the picture of vaccinia virus proteolysis.

Due to the sequence similarity between VV I7L, the adenovirus protease, and the African Swine Fever virus protease, as well as a similar profile of repression through protease inhibitors, these enzymes may form a new family of cysteine proteases. It will be interesting to discover more about the function of each of these enzymes.

In conclusion, I have shown through the experiments described in this thesis that the gene product of the I7L open reading frame is the vaccinia virus core protein proteinase. This is the first characterization and identification of a VV proteinase. There are still several questions that remain unanswered about the activity of I7L including whether it has another role in the viral life cycle aside from cleavage of the major core protein precursors, what activates the enzyme, and how it is regulated so that it acts at a specific time in the viral life cycle. Regulation is particularly important since the protease needs to be active at the right time and correct place in the viral life cycle to carry out the enzymatic reactions necessary but not cleave for too long or at the wrong time. The regulation of proteinases could occur through different compartmentalization of the enzyme and substrate, presence of specific inhibitors and/or activators, or through the proteolytic activation of zymogens. Different viruses utilize different methods to regulate the activity of their proteinases. In the case of retroviruses, the acidic extracellular environment causes a conformational change in the gag-pol polyprotein activating the proteinase. With adenovirus, a disulfide-linked peptide as well as viral DNA is required for activation of the proteinase. In the case of VV, proteolysis of the major core proteins by I7L is not a rapid process, requiring at least 30-45 minutes, and also requiring ongoing protein synthesis, which leads to the hypothesis that cleavage only occurs within the context of an assembling virion. I7L demonstrates a degree of specificity since to date it has only been shown to cleave late proteins associated with the core at a specific Ala-Gly-Xaa motif unique to VV. However, the factors necessary to activate I7L have yet to be determined.

The specificity of I7L, the essential nature of the enzyme, the conservation among poxviruses, and the fact that this proteinase is very different from cellular enzymes makes I7L an attractive and viable target for antiviral drug development. We have shown the proof of concept that compounds can be designed to specifically target I7L, inhibiting its ability to cleave the core protein precursors and therefore blocking viral replication without being harmful to the host cell. Given the current concerns regarding smallpox as an agent of bioterrorism, it is essential that effective poxvirus antiviral drugs are developed and available in our pharmaceutical repertoire to complement the existing vaccine. Furthermore, such drugs should be effective in the event that other orthopoxvirus pathogens find their way into the human population. Fortunately, the orthopoxviruses are highly related at the DNA level (e.g. 90% between variola and vaccinia) making it likely that any antiviral agent developed would inhibit the replication of this entire group of viruses.

In the future it will be of interest to further explore the possibility that there are other proteinases encoded within the VV genome, specifically to determine whether the gene product of the G1L ORF may encode a metalloproteinase, and to further characterize the activity of I7L.

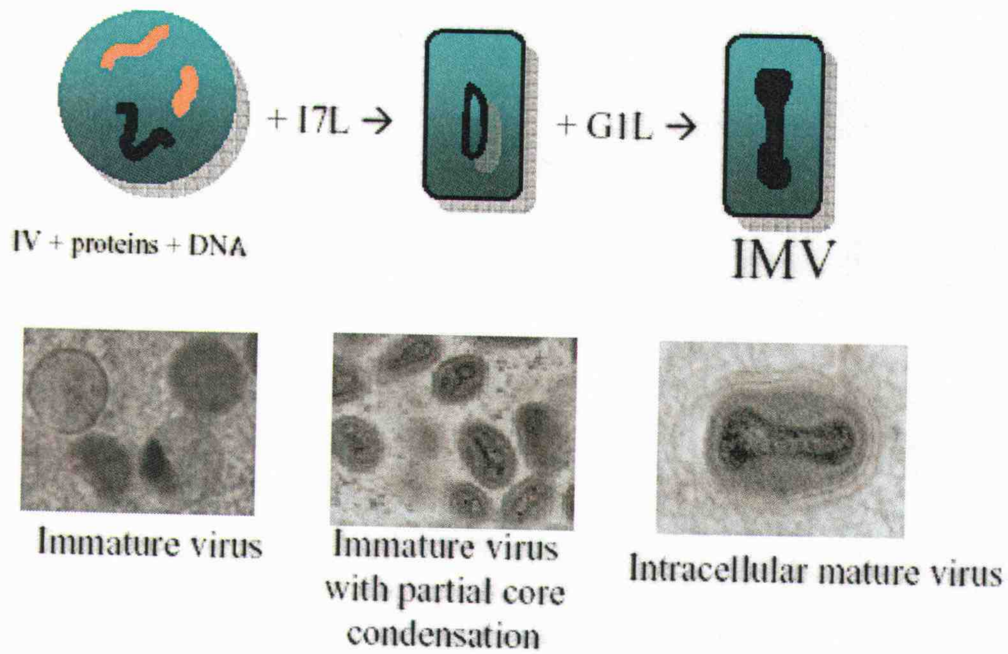

Figure 7.1. Model of the role of proteolysis in vaccinia virus morphogenesis

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