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Title: Structure-Function Analysis of the Vaccinia Virus I7L Proteinase

Abstract approved:

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Dennis E. Hruby

Vaccinia virus is the prototypic member of the *Orthopoxvirus* genus. It undergoes a complex replication process where a key step in the transition from immature virion to intracellular mature virion is the cleavage of the major core protein precursors. The product of the I7L open reading frame (ORF) is a protein with an apparent molecular weight of 47kDa and has been shown to be responsible for these cleavages. However, relatively little is known about the biochemistry of the cleavage reaction, the structural features which allow I7L to direct regulated catalysis, or mechanisms by which I7L activity is regulated.

As one approach to answering these questions, a phenotypic analysis was conducted on a collection of six conditional-lethal mutants in which the

mutation had been mapped to the I7L locus. Genomic sequencing showed all the mutants have single amino acid substitutions within the I7L ORF. The mutations fall into two groups: changes at three positions at the N-terminus between amino acids 29 and 37 and two different substitutions at amino acid 344, near the catalytic cysteine. Two of the mutants had the exact same change. Regardless of where the mutation occurred, mutants at the non-permissive temperature failed to cleave core protein precursors and had their development arrested after immature virion assembly but prior to core condensation. Thus, we propose that the two clusters of mutations may affect two different functional domains required for proteinase activity.

In a second approach, by using a combination of immunoprecipitation, immunoblotting and mass spectrometry, we showed that I7L can form a homodimer and be cleaved into a product with a molecular weight of approximately 40kDa. Mass spectrometry analysis of proteins that co-immunoprecipitated with I7L failed to provide leads on potential I7L cofactors. I7L proteins with mutations matching those in the temperature-sensitive mutants described above were capable of forming dimers and being cleaved. Thus the mechanism by which the N-terminal mutants obtain their conditional-lethal phenotype remains unknown. Taken together, this suggests several possible new models for the regulation of I7L.

Structure-Function Analysis of the Vaccinia Virus I7L Proteinase

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Megan J. Moerdyk, Author

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CONTRIBUTION OF AUTHORS

For Chapter 2, Megan J. Moerdyk conducted the experiments and wrote the manuscript. Dr. Chelsea M. Byrd assisted with the sequencing and edited the manuscript. Dr. Dennis E. Hruby conceived the study, coordinated the research efforts and edited the manuscript.

For Chapter 3, Megan J. Moerdyk conducted the experiments, except as otherwise indicated, and wrote the manuscript. Dr. Chelsea M. Byrd conducted the experiments to identify the 40kDa band and edited the manuscript. Dr. Dennis E. Hruby conceived the study, coordinated the research efforts and edited the manuscript.

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GENERAL INTRODUCTION

Background

Vaccinia virus is a large, double stranded DNA virus with a genome of about 200 kbp which encodes more than 200 proteins. It is a part of the *Poxviridae* family of viruses, which is divided into two subfamilies; the *Entomopoxvirnae* infect insect hosts while the *Chordopoxvirnae*, which includes vaccinia, infect vertebrate hosts. The *Chordopoxvirnae* contains eight genera of which vaccinia virus is considered the prototypic member of the *Orthopoxvirus* genus. Other orthopoxviruses include variola, monkeypox, camelpox, cowpox, ectromelia, raccoonpox, skunkpox, and volepox. (Moss, 2001)

Vaccinia is perhaps most commonly known as the virus used to vaccinate against its close relatives variola major and variola minor, the causative agents of smallpox. Vaccinia and variola share greater than 90% overall identity at the amino acid level (Massung *et al.*, 1994) a strong enough similarity to allow for immunological cross-reactivity and protection. Immunization with vaccinia has also been shown to provide protection against infection with other orthopoxviruses such as monkeypox and cowpox (Shchelkunov *et al.*, 2005). It is believed that this high degree of similarity will also make antivirals developed against vaccinia effective against a wide range

of orthopoxviruses, particularly when the drug target is a highly conserved protein.

Variola major and minor are some of the world's most feared pathogens, killing approximately 30% and 1% of individuals infected respectively, and leaving many survivors with permanent scars (Fenner *et al.*, 1988). At its peak, smallpox is estimated to have been responsible for 10% of all deaths in Europe and Asia (Fenner *et al.*, 1988). Although smallpox has been eradicated through an intensive worldwide vaccination effort, the last naturally occurring case was in Somalia in 1977, orthopoxviruses remain of medical interest. While variola virus has been removed from the natural environment, two known viral stocks in the United States and Russia and possibly others remain, raising concerns over the potential accidental or intentional reintroduction of the virus. As routine vaccination for smallpox was discontinued in the U.S. in 1972 and worldwide in 1984, the effect of such a release on a mostly immunologically naïve population would be devastating (Fenner *et al.*, 1988). A more immediate health concern is the monkeypox virus. Monkeypox is primarily a zoonotic disease as it is poorly transmitted between humans but periodic outbreaks have been reported in portions of central and west Africa (Nalca *et al.*, 2005). In the summer of 2003, there were over 70 suspected and confirmed cases of monkeypox in the midwestern United States when people were infected with monkeypox after coming into contact with infected pet prairie dogs (CDC, 2003).

While vaccinia continues to be a model organism for investigation of orthopoxvirus biology, poxviruses, including vaccinia, are also of interest in the world of biotechnology. Poxviruses are being evaluated as potential expression vectors for the creation of recombinant vaccines and as delivery vehicles for recombinant gene therapy. The large genome size and flexible packaging requirements of poxviruses make them attractive as they can be used to express large genes or even multiple genes. These recombinants are also quite stable. Since the replication of poxviruses occurs entirely in the cytoplasm, they are unlikely to be useful in permanent gene replacement therapies, but in applications where only temporary expression is required this feature reduces the chances of insertional mutagenesis (Moroziewicz and Kaufman, 2005; Vanderplasschen and Pastoret, 2003; Perkus *et al.*, 1995).

Vaccinia Virus Replication Cycle

Vaccinia virus has a complex replication cycle (Figure 1) which is not yet completely understood. Following attachment to an unknown receptor or receptors, the virus membrane fuses with the host cell membrane, delivering the core and lateral bodies into the cytoplasm of the cell. Once in the cell, the virus partially uncoats and, with its own transcriptional apparatus which is contained within the viral core, initiates transcription of early genes. mRNAs pass through holes in the partially opened core and are translated by the host cell machinery. Among the early genes are transcription factors for
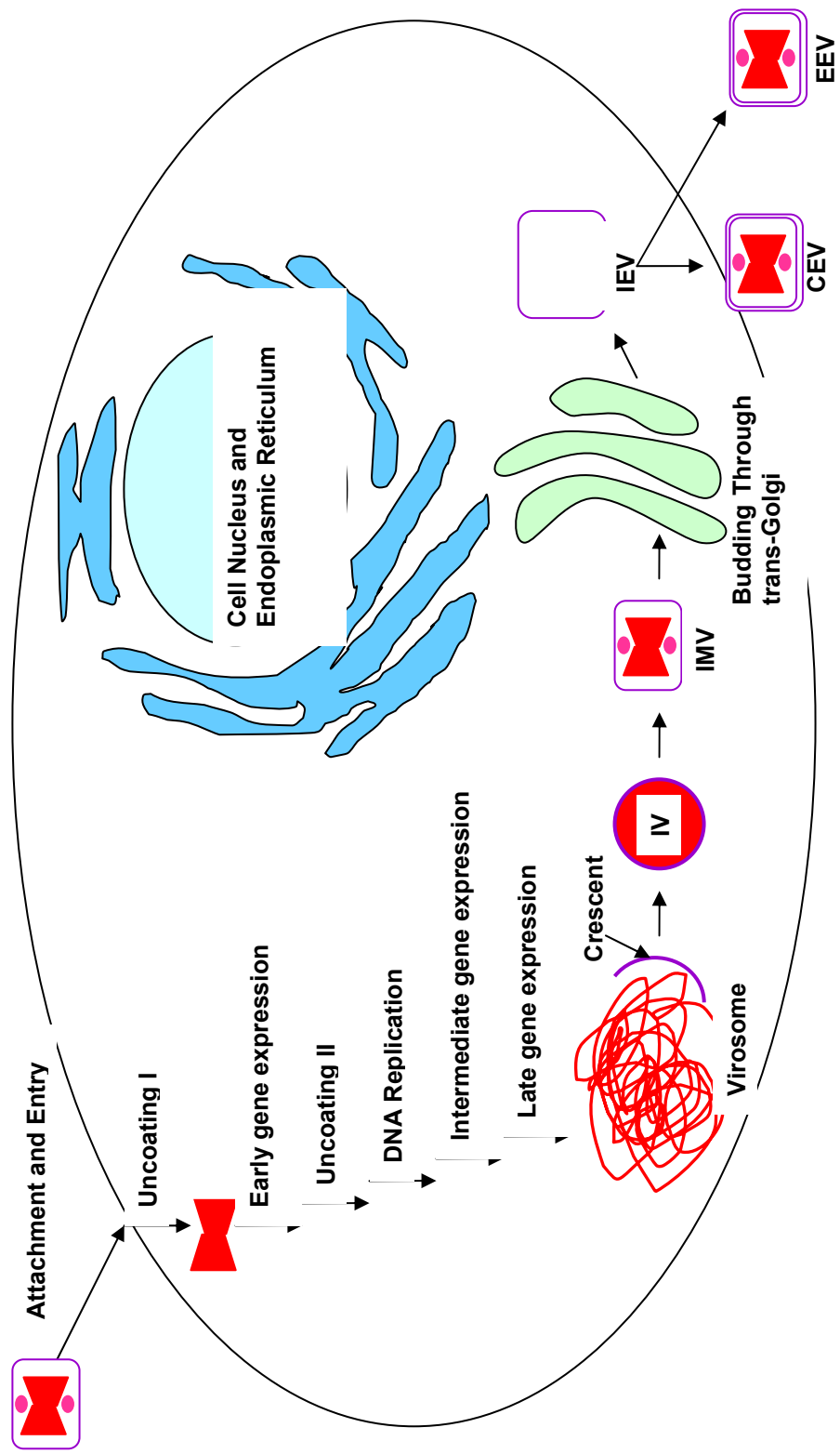
Figure 1. Vaccinia virus replication cycle. IV, immature virion; IMV, intracellular mature virion; IEV, intracellular enveloped virion; CEV, cell-associated enveloped virion; EEV, extracellular enveloped virion.

transcription of intermediate genes which in turn encode the transcription factors for late gene transcription, creating a regulatory cascade. Other early gene products are involved in DNA synthesis which takes place late during the expression of early genes but prior to the transcription of intermediate genes. Vaccinia encodes the proteins necessary for the replication of its genome, which takes place in cytoplasm of infected cells. The sites of vaccinia DNA synthesis form electron dense regions known as virus factories or virosomes. Portions of this DNA and newly synthesized proteins, together referred to as viroplasm, are enveloped by crescent shaped membranes. These membranes are most commonly thought to originate from the endoplasmic reticulum and Golgi network but have also been proposed to be formed by *de novo* membrane synthesis in the cytoplasm. This forms immature virions (IV) which then undergo a series of complex morphological changes, including core condensation, to become intracellular mature virions (IMV), the first infectious form. These brick-shaped virions possess the characteristic biconcave core and are bounded on either side by structures referred to as lateral bodies. IMV can also undergo further modification to become one of several additional infectious forms. IMV can acquire two additional membranes from the trans-Golgi or early endosomal network to become intracellular enveloped viruses (IEV). The outermost membrane can fuse with the cellular membrane and the virion can either remain attached to the cell as a cell-associated enveloped virion (CEV) or be released into the surrounding

environment as an extracellular enveloped virion (EEV). (Moss, 2001; Condit *et al.*, 2006; Moss, 2006).

The Role of Proteolysis in Regulating Viral Replication

Regardless of their genomic complexity, all viruses face the challenge of regulating the synthesis and assembly of viral components. One strategy commonly used by viruses is proteolysis. The term protease refers to enzymes that catalyze the hydrolysis of an amide bond between two amino acids. Proteases can be placed into two categories based on the type of peptide bond that they cleave. Exopeptidases remove single amino acids from the N or C-terminus of a polypeptide while endopeptidases, also known as proteinases, cleave internal bonds within the peptide chain (Polgar, 1989). Proteases are also classified by their mechanism of action as either serine, cysteine, aspartic acid, threonine, glutamic acid or metallo- proteases (Polgar, 1989; Seemuller *et al.*, 1995; Jedrzejas, 2002). For serine, cysteine and threonine proteinases, the indicated active site amino acid directly initiates the nucleophilic attack on the carbonyl group of the peptide bond. Similarly, aspartic acid and glutamic acid, as well as the metal ion bound by metalloproteases, position water molecules for nucleophilic attack on the peptide bond (Polgar, 1989; Seemuller *et al.*, 1995; Jedrzejas, 2002).

Helen and Wimmer (1992) have suggested that in regards to viral replication, proteolytic events can be classified as either morphogenic or formative. Formative proteolysis is used to separate individual proteins from a

large polyprotein precursor, made from either a genomic or a subgenomic mRNA. This strategy is used by a variety of positive strand RNA viruses including picornaviruses, flaviviruses, potyviruses and togaviruses and all retroviruses (Krausslich and Wimmer, 1988). In contrast, morphogenic cleavages are not required for initial virion assembly but are necessary for the production of infectious viral particles. This strategy is employed by a wide range of viruses including T4 bacteriophage, retroviruses and adenoviruses (Hellen and Wimmer, 1992) and is an essential step in the transition of vaccinia virus IV into IMV. Cleavages for both formative and morphogenic proteolysis may be autoproteolytic and/or carried out by a separate proteinase. (Helen and Wimmer, 1992)

One of the earliest known and best studied examples of formative proteolysis comes from poliovirus, a picornavirus. All poliovirus proteins are derived from single polyprotein translated from the genome. The initial cleavage occurs just prior to the 2A protein to separate the structural and non-structural proteins and is an autocatalytic event directed by 2A itself while it is still a part of the polyprotein. The remaining cleavages, except for that of VP0, are directed by the 3C protein which acts in an autocatalytic manner to liberate itself as well as to separate the remaining structural and non-structural proteins (Palmenberg, 1990). Poliovirus also utilizes a morphogenic cleavage. The capsid originally forms as an assemblage of 60 promoters composed of one molecule each of the structural proteins VP0, VP1, and VP3. However,

the particles become infectious only after most of the VP0 has been cleaved into VP2 and VP4 (Helen and Wimmer, 1991).

Vaccinia Virus Proteins Undergo Morphogenic Proteolysis

The cleavage of vaccinia virus proteins was first observed by Holowczak and Joklik (1967) who noted size differences between polypeptides found in purified virions and those found in the cytoplasm of infected cells from which the whole virions had been removed. This suggested that some vaccinia virus proteins might be synthesized as larger polypeptides than those found in the virions. Later pulse-chase experiments also documented the possible cleavage of vaccinia virus proteins. While examining late protein synthesis, Pennington noted the disappearance or diminished intensity of 11 protein bands and the appearance of seven new bands (1974), while Stern and Dales noted five polypeptides that were absent at the end of the pulse but appeared in the chase sample (1976). These same five potential cleavage products failed to appear when the temperature sensitive mutant ts1085, which is defective in the assembly of crescents and the envelopment of DNA and proteins to form IV, was incubated at the non-permissive temperature. Upon being shifted back to the permissive temperature, both the appearance of these products and the completion of virion morphogenesis depended upon continuing protein synthesis (Stern *et al.*, 1977). Along with experiments showing that the drug rifampicin blocked both morphogenesis and cleavage

(Moss *et al.*, 1969; Katz and Moss, 1970), this provided early evidence for an association between precursor cleavage and virus morphogenesis.

The first specific product/precursor relationship to be documented was that of P4a being cleaved into 4a (Katz and Moss, 1970). P4a is the product of the A10L open reading frame (ORF) and has an apparent molecular weight of 95kDa while 4a has an apparent molecular weight of 60kDa. In addition to the P4a to 4a cleavage, a precursor product relationship was also shown to exist between a 65kDa band given the designation P4b and a 60kDa band designated 4b (Moss and Rosenblum, 1973), as well as a 28kDa precursor designated P25K and its 25kDa product, 25K (Weir and Moss, 1985). P4b and P25K are products of the A3L and L4R ORFs. Together P4a, P4b and P25K are referred to as the major core protein precursors and account for approximately 14, 11, and 7% of the virion mass respectively (Sarov and Joklik, 1972). Separation of the various immature virion forms through the use of a sucrose log gradient was used to show that the major core protein precursors are associated with the early forms. Cleavage of the precursors is associated with the transition to a later form of immature virion with a more defined core (VanSlyke *et al.*, 1993). Similarly, immunogold labeling with an antibody specific to the P4a precursor, showed precursor to be present in the viroplasm of IV while very few gold particles labeled the IMV, suggesting limited presence of the precursor in mature cores (VanSlyke and Hruby, 1994).

N-terminal sequencing of the cleavage products 4b and 25K showed that both are cut at Ala-Gly-Ala (AG*A) sites with cleavage occurring after the glycine residue prior to residues 62 and 33 respectively (Yang *et al.*, 1988; VanSlyke *et al.*, 1991a). It has also been shown that P25K can be cleaved prior to amino acid (AA) 19 at an AG*S site to create a higher molecular weight cleavage product designated 25K'. However, this does not appear to be a stable intermediate as it has not been detected in the core or mature virions (Lee and Hruby, 1993; Takahashi *et. al.*, 1994). Peptide specific antiserum revealed that in addition to 4a, P4a is also processed into a 23kDa polypeptide which is incorporated into the virion. N-terminal sequencing demonstrated that this 23kDa product was created by cleavage at an AG*T site with cleavage occurring prior to the T at amino acid 698. Alternative peptide mapping procedures showed 4a to be created by a second cleavage prior to amino acid 614 at an AG*S site but that cleavage did not occur at an AG*N site (VanSlyke *et al.*, 1991b). The fate of the intervening 9kDa polypeptide from P4a as well as the N-terminal leader peptides to 4b and 25K remains unknown.

This data suggested AG*X (with * indicating the site of cleavage) as the cleavage motif being utilized by vaccinia virus. A search of the 198 predicted ORFs of the vaccinia virus Copenhagen genome revealed that the AGX motif occurs 82 times, far less that the 204 times that this sequence would be expected to appear if it occurred randomly, which supports the potential

functional significance of this sequence (Whitehead and Hruby, 1994a). Based on this information, Whitehead and Hruby undertook an analysis of all the AGA sites in the vaccinia virus genome as a subsample of all potential AG*X cleavage sites. In addition to P4b and P25K, there are five proteins that contain an AGA tripeptide in both the Copenhagen and Western Reserve (WR) strains of vaccinia virus. These are A12L, A17L, F13L, vaccinia virus DNA polymerase (DNAP, the product of the E9L ORF) and vaccinia virus host range protein (HR, the product of the K1L ORF). F13L, DNAP and HR were all shown not to be cleaved (Whitehead and Hruby, 1994a). On the other hand, polyclonal antiserum to A17L detected a 23kDa precursor and a 21kDa cleavage product while polyclonal antiserum to A12L showed a 24kDa precursor and a 17kDa cleavage product (Whitehead and Hruby, 1994a). In both cases, cleavage at the AG*A site was confirmed with N-terminal sequencing (Whitehead and Hruby, 1994a). The pattern of cleavage/non-cleavage indicates that context is important in determining protein cleavage and suggests several rules governing that process. Timing of protein synthesis appears to be important as all the proteins which were cleaved are late proteins while DNAP (McDonald *et al.*, 1992) and HR (Gillard *et al.*, 1989) are expressed at early times in infection. While F13L is a late protein, it is found only in the membranes of enveloped virions (Husain *et al.*, 2003). In contrast, P4b, P25K and A12L are core proteins (Whitehead and Hruby, 1994a), while A17L is found in the membranes of IMV and IV (Wolffe *et al.*,

1996).   This suggests that protein location is an additional important determinant in whether or not cleavage occurs.

A sequence comparison of the four proteins cleaved at AG*A sites revealed that they all have acidic upstream residues and basic downstream residues but lack other sequence or structural similarities (Whitehead and Hruby, 1994a).   The P4a AG*X sites lack this property although the intervening 9kDa peptide is acidic (Lee and Hruby, 1995).   Interestingly, P4a processing occurs more slowly than P4b and P25K processing, suggesting less efficient cleavage (VanSlyke *et al.*, 1991a).   This indicates that sequence may influence the rate of cleavage and might be used by the virus as a regulatory mechanism.

To be able to further study the sequence determinants of cleavage, a mutagenesis study was carried out on P25K.   A *trans*-processing assay was developed where plasmid constructs encoding a P25K-FLAG fusion protein were tranfected into vaccinia virus infected cells (Lee and Hruby, 1993).   Using the nomenclature where -1 denotes the amino acid immediately prior to the cleavage site and +1 indicates the amino acids immediately after the cleavage site, the +1 position was found to be extremely permissive to substitution with any amino acid but proline allowing retention of substrate cleavage (Lee and Hruby, 1994).   This supports the definition of the consensus cleavage site as AG*X.   The -1 and -2 positions were more restrictive as only amino acids with small, uncharged side chains could be substituted.   Of the amino acids that

could be individually substituted, only a limited number of combinations still allowed cleavage to occur. The -4 position required a hydrophobic residue, although this is not a characteristic shared by all VV proteins cleaved at an AG*X site. Individual conversions of the downstream acidic residues to basic residues did not lead to a loss of cleavage but mutating all three did indicating that overall charge and/or conformation is important. Further lending support to this idea, insertion or deletion of 10 amino acids either before or after the AG*X site prevented cleavage (Lee and Hruby, 1994). In the absence of the N-terminal leader sequence, P25K was not incorporated into virions suggesting that it may play a role in proper localization of the protein in addition to providing structural determinants for cleavage (Lee and Hruby, 1995). However, leaving as few as 17 amino acids immediately prior to the cleavage site allowed for both cleavage and incorporation into the virion indicating that the essential features are located immediately adjacent to the cleavage site. Substitution of the P25K leader sequence with the leader sequence from P4b but not the N-terminal portion of the thymidine kinase (TK) protein (a protein which is not cleaved), allowed for cleavage to occur and led to more efficient incorporation into the virion than the TK sequence. This suggests that while the P25K and P4b leader sequences largely lack sequence similarity, there are some functional similarities (Lee and Hruby, 1995).

Takahashi and colleagues (1994) conducted an N-terminal sequencing analysis of all the major structural polypeptides found in purified mature virions. In doing so they identified eight AG*X cleavage sites. In addition, to the cleavages already mentioned, A12L was found to also be cleaved prior to AA 155 at an AG*K site while a second cleavage site, this time an AG*N, site was found in A17L prior to AA 186. G7L, whose cleavage was not previously known, was found to be cleaved at an AG*L site prior to AA 239. Consistent with what has been observed for the other proteins cleaved at an AG*X site, G7L is a late protein that is a component of a virion core. While one role G7L plays is in IV assembly, it appears that it is not cleaved until the IV to IMV transition (Szajner *et al.*, 2004; Mercer and Traktman, 2005). A summary of all known AG*X cleavage sites is given in Table 1.

Table 1. Known proteolytic events occurring at AG*X sites. AG*X motifs are indicated in bold.

ORF	Amino Acids	Sequence
L4R	25-39	L-Q-M-V-I-**A-G-A**-K-S-L-F-P
A3L	54-68	D-D-F-I-S-**A-G-A**-R-N-Q-R-T
A10L	607-621	P-R-Y-F-Y-**A-G-S**-P-E-G-E-E
A10L	690-704	R-I-I-T-N-**A-G-T**-C-T-V-S-I
A17L	9-23	L-D-D-F-S-**A-G-A**-G-V-L-D-K
A17L	179-191	N-K-P-Y-T-**A-G-N**-K-V-D-V-D
A12L	49-63	Q-T-D-V-T-**A-G-A**-C-D-T-K-S
G7L	180-194	E-P-I-I-V-**A-G-F**-S-G-K-E-P
G7L	231-245	I-A-E-Y-I-**A-G-L**-K-I-E-E-I

G1L is a Putative Metalloproteinase

The product of the G1L ORF is expressed late in infection, has an apparent molecular weight of 68kDa and is associated with the virion core (Ansarah-Sobrinho and Moss, 2004a). An early experiment indicated that G1L might be responsible for cleavage of the core protein precursors (Whitehead and Hruby, 1994b). Cells were treated with Ara-C to prevent genome replication and hence late protein synthesis and virion assembly. The cells were then co-transfected with a plasmid containing a test substrate and a cosmid (or plasmid) construct covering a portion of the vaccinia virus genome. Plasmids encoding the late transcription factors (A1L, A2L, G8R) were also transfected to allow for the transcription of these DNAs. Constructs containing G1L were able to direct cleavage of P25K at the AG*S site to give 25K'. However, none of the constructs were able to direct P4a or P4b cleavage or cleavage of P25K at the natural AG*A site (Whitehead and Hruby, 1994b), so definitive identification of the core protein proteinase remained elusive.

Since then, inducible mutants in which the expression of G1L is under the control of either the *Escherichia coli lac* repressor system or the bacterial TET repressor system have shown that G1L is not responsible for core protein precursor processing (Ansarah-Sobrinho and Moss, 2004a; Hedengren-Olcott *et al.*, 2004). In the absence of induction of G1L, core protein precursors as well as A17L and G7L are cleaved normally. However, G1L is required for the production IMV. In its absence, core and DNA condensation are initiated but

not completed and particles remain rounded, indicating that G1L is involved in a morphogenic step subsequent to major core protein cleavage (Ansarah-Sobrinho and Moss, 2004a; Hedengren-Olcott *et al.*, 2004). However, while no substrates have yet been identified, G1L may still act as a proteinase.

Several lines of evidence argue for G1L being a zinc dependent metalloendopeptidase. G1L contains an HXXEH motif which, along with its flanking residues, is highly conserved among orthopoxviruses (Ansarah-Sobrinho and Moss, 2004a). This sequence is an inversion of the HEXXH active site motif of most metalloproteinases but is characteristic of a subset of those enzymes (Becker and Roth, 1992). Within these enzymes, coordination of the zinc ion is believed to be by the two histidines in the HXXEH motif and a downstream glutamic acid (E). The downstream E is typically found as part of a cluster of conserved glutamic acids and poxviruses contain such a conserved stretch (ELENEX$_5$E, residues 110 to 120) (Honeychurch *et al.*, 2006). The glutamic acid in the HXXEH motif is believed to participate in the hydrolysis of the substrate peptide bond (Kitada, *et al.*, 1998).

In the absence of the induction of G1L, replication of the conditional lethal viruses can be rescued by plasmid borne wild-type G1L. However, if either histidine or the glutamic acid in the HXXEH motif are mutated, either together or individually, rescue does not occur (Ansarah-Sobrinho and Moss, 2004a; Hedengren-Olcott *et al.,* 2004; Honeychurch *et al.*, 2006). Furthermore, of the downstream glutamic acids, only E120 can be mutated

and still rescue replication of the conditional lethal mutant (Honeychurch *et al.*, 2006). This indicates that the putative active site residues are essential to G1L function. Immunoblot analysis has also shown that G1L may be cleaved into 40 and 28kDa products (Ansarah-Sobrinho and Moss, 2004a). Loss of rescue has also been associated with a loss of cleavage of G1L. Thus, G1L cleavage may be a necessary event for viral replication (Honeychurch *et al.*, 2006).

G1L also has structural homology with several proteins: α-mitochondrial processing peptidase from yeast (1hr6A), core proteins from the cytochrome bc1 complex in bovine heart mitochondria (1be3A and 1be3B), and a core protein from the cytochrome bc1 complex of yeast mitochondria (1ezvA). These were used to make theoretical models of G1L including the putative HLLEH zinc-binding motif which showed good alignment with that of the other proteins supporting the identification of G1L as a metalloproteinase (Hedengren-Olcott *et al.*, 2004).

I7L is the Vaccinia Viral Core Protein Proteinase

I7L was originally identified as a putative cysteine proteinase based on its homology to an ubiquitin-like proteinase, Ulp1, in *Saccharomyces cerevisiae* (Li and Hochstrasser, 1999). The catalytic domain is conserved amongst these two proteins, the African Swine Fever Virus (ASFV) core protease and the adenovirus protease (Li and Hochstrasser, 1999; Andres *et al.*, 2001). Within the active sites of these four proteinases, there are seven

conserved residues (Li and Hochstrasser, 1999). These include the histidine, cysteine and aspartate that make up the putative catalytic triads and the glutamine just upstream of the catalytic cysteine that is believed to form the oxyanion hole (Kim *et al.*, 2000). Interestingly, these proteases all utilize a cleavage motif similar to the AG*X in vaccinia. Ulp1 (Li and Hochstrasser, 1999) and the ASFV protease (Andres et al. 2001) cleave at GG*X sites, while the adenovirus protease cleaves at GG*X or GX*G motifs (Weber, 1995).

Early insights into the role of I7L came from characterization of the temperature sensitive mutant Cts-16 (also referred to as ts16) (Condit and Motyczka, 1981). Marker rescue was used to identify Cts-16 as a mutant in the I7L ORF with the specific mutation being a proline to leucine change at amino acid 344 (Kane and Shuman, 1993). This ORF encodes a 423 amino acid, 47kDa product. Consistent with the protein being expressed late in infection, the ORF is immediately preceded by the canonical late gene promoter sequence TAAATG, where the ATG is also the start codon (Schmitt and Stunnenberg, 1988).

Cts-16 was originally classified by Condit as having a wild type pattern of protein synthesis (Condit and Motyczka, 1981), although it was later shown to be defective in core protein precursor cleavage (Ericsson *et al.*, 1995) and in the cleavage of the A17L membrane protein (Ansarah-Sobrinho and Moss, 2004b). Consistent with this, a defect in virion morphogenesis was also observed. When Cts-16 infected cells incubated at the non-permissive were

viewed using electron microscopy, wild-type IV but not IMV were seen along with a large number of abnormal IV. While still spherical, these particles were more electron dense than true IV. Nucleoids were observed in some of these particles while in others the viroplasm was asymmetrically distributed within the particle. This was sometimes accompanied by a collapse of the virion membrane on the side opposite of the viroplasm concentration (Kane and Shuman, 1993; Ericsson *et al.*, 1995). Also consistent with previous observations, the arrest in virion morphogenesis is irreversible. Following a temperature shift to the permissive temperature, and in the absence of any new protein synthesis, no IMV were seen and the core protein precursors remained uncleaved (Ericsson *et al.*, 1995). The I7L produced by Cts-16 at the non-permissive temperature is made late in infection and appears to be stable (Kane and Shuman, 1993). Thus, the observed phenotype is due to loss of I7L function and not lack of the protein itself.

Wild type I7L has been shown to be present in the virosomes and in the viroplasm of crescents and IV and to be ultimately incorporated into the virion core (Kane and Shuman, 1993; Ericsson et al., 1995). Mutant I7L produced by Cts-16 at the non-permissive temperature is also found in the virosomes and while the evidence is less clear, it appears to be present in the viroplasm of the crescents and IV. The major core proteins localized normally at the non-permissive temperature (Ericsson *et al.*, 1995).

Cts-16 was used to develop a new *in vivo trans*-processing assay to test for proteinase activity (Byrd *et al.*, 2002). Cts-16 infected cells transfected with a FLAG-tagged test substrate were incubated at the non-permissive temperature to create a situation were cleavage of native core protein precursors and the test substrate did not occur but all potential viral and cellular cofactors were present. Candidate proteinases were added to the system by cotransfection with the test substrate. Transfection with wild-type I7L but not wild-type G1L led to cleavage of the P25K-FLAG fusion protein at both the AG*A and the AG*S site (Byrd *et al.*, 2002). Furthermore, wild-type I7L was able to direct cleavage of a P4a-FLAG substrate at both the AG*S and AG*T sites and of a P4b-FLAG substrate at the AG*A site (Byrd *et al.*, 2003). Mutation of the AG*X sites prevented the production of cleavage products, indicating the I7L directed cleavage was occurring at the authentic cleavage sites. Mutation of the histidine residue in the putative active site also prevented cleavage, suggesting that I7L's proteinase activity is required for these cleavage reactions to occur (Byrd *et al.*, 2002; Byrd *et al.*, 2003).

The role of I7L as the VV core protein proteinase was confirmed by the creation of inducible mutants where I7L expression was under the control of either the *E. coli lac* repressor system or the bacterial TET repressor system (Ansarah-Sobrinho and Moss, 2004b; Byrd and Hruby, 2005a). In the absence of induction, the three major core proteins and A17L were not cleaved. I7L's role in the cleavage of A17L shows that its activity is not strictly

limited to the virion core. As would be expected, without I7L expression, IV but not IMV were formed and defective particles similar to those seen in Cts-16 at the non-permissive temperature were observed. Additionally, particles with poorly formed cores were noted. With induction, IMV were produced normally. Transfection with a plasmid containing wild-type I7L but not I7L with either the putative histidine or cysteine active site residues mutated, was able to restore both protein processing activity and viral replication (Ansarah-Sobrinho and Moss, 2004b; Byrd and Hruby, 2005a). The role of I7L in the processing of the other two proteins known to be cleavaged at AG*X sites, A12L and G7L, has yet to be determined.

Structural and Biochemical Features of I7L

While I7L has been clearly identified as the vaccinia viral core protein proteinase, its biochemistry and regulatory mechanisms remain less well understood. I7L is highly conserved amongst all orthopoxviruses and to a lesser extent among the other poxviruses including the sequenced entomopoxviruses (Gubser *et al.*, 2004). While I7L lacks overall homology with any protein outside of the poxviruses, individual regions show homology with other proteins. As mentioned previously, I7L shares active site homology with Ulp-1, adenovirus protease, and ASFV core protease including seven conserved residues. In I7L these are H241, W242, D248, D258, Q322, C328, G329 with H241, D248, and C328 making up the proposed catalytic triad. These residues were individually mutated in plasmid borne I7L (Byrd *et al.*,

2003). Only the mutation to D258 allowed retention of cleavage activity and rescue of viral replication when transfected into Cts-16 infected cells held at the non-permissive temperature. A mutation in D248 allowed minimal rescue of replication (Byrd *et al.*, 2003). This suggests that aspartic acid residue 248 is not absolutely essential despite being a predicted part of the catalytic triad. This is not too surprising as not all cysteine proteinases utilize aspartic acid as part of their active site (Polgar, 1989). In addition, the N-terminal portion of I7L from residues 30 to 246 shows homology to a portion of the type II DNA topoisomerase (TOP2) of *Saccharomyces cerevisiae* with 20% amino acid identity (Kane and Shuman, 1993), indicating that the protein may also have nucleic acid binding activity though this has never been demonstrated.

A threading and homology model of the I7L catalytic site has been constructed based on the C-terminal domain of the Ulp1 protease (Byrd *et al.*, 2004). These proteins share 22% sequence identity at the amino acid level within this region. This model was used to conduct *in silico* screening for small molecule inhibitors of the I7L active site which yielded several compounds capable of inhibiting vaccinia virus replication in tissue culture. While not all of these compounds targeted I7L proteinase activity, a class of compounds typified by TTP-6171 appeared to do so as resistance mutations mapped to I7L. In the presence of the drug, P4b was produced but not cleaved and virion morphogenesis was arrested during the transition from IV to IMV as was seen in Cts-16 and the inducible mutants (Byrd *et al.*, 2004). However, the current

working model is incomplete as it only represents the catalytic domain and does not include the 130 N-terminal amino acids of I7L. While the first 70 amino acids of I7L are highly conserved, it lacks a predicted structure. This highly conserved region is followed by a hypervariable region that likely represents a flexible loop. Thus even if the structure of the N-terminus could be determined it would be difficult to determine its position relative to the catalytic domain (Vsevolod Katritch, personal communications). In their most basic form, proteinases consist only of a catalytic domain and a substrate binding domain. In the case of I7L, removal of the N-terminus up through amino acid 228 eliminated its ability to rescue replication of Cts-16 and to cleave core protein precursors in a *trans*-processing assay despite leaving the catalytic region intact (Byrd *et al.*, 2003). Thus the N-terminal portion of I7L appears to have an essential structural and/or functional role.

Development of an *in vitro* assay system utilizing lysates from infected/transfected cells has allowed for limited biochemical analysis of I7L (Byrd and Hruby, 2005). In this assay, core protein precursors were produced by *in vitro* transcription and translation for use as substrates while the lysate provides the enzyme activity. Extracts from Cts-16 infected cells incubated at the non-permissive temperature did not drive cleavage unless also transfected with wild-type I7L. Transfection with I7L with a mutated active site did not allow for cleavage. Removal of I7L by immunoprecipitation with I7L specific antibodies also prevented substrate cleavage, while immunoprecipitation with

G1L specific antibodies had no effect on cleavage. Thus, cleavage in this system is dependent on I7L proteinase activity. In the *in vitro* system, optimal cleavage occurs at 25C. Only minimal cleavage is observed at 37C, the optimal temperature for vaccinia replication. This assay was also used to determine the effect of various proteinase inhibitors on cleavage activity. Ethylenediaminetetraacetic acid (EDTA, a metalloproteinase inhibitor), pepstatin (an aspartic proteinase inhibitor), and phenylmethanesulfonyl (PMSF, a serine proteinase inhibitor) had no effect on cleavage. The cysteine proteinase inhibitors iodoacetic acid (IA) and N-ethylmaleimide (NEM) inhibited cleavage while E-64 and EST inhibited cleavage only at high concentrations. This is consistent for what has been seen with the related proteins, adenovirus protease and ASFV protease (Webster *et al.*, 1989; Tihanyi *et al.*, 1993; Rubio *et al.*, 2003). The cysteine proteinase inhibitor leupeptin did not inhibit cleavage activity which was also seen with adenovirus (Webster *et al.* 1989). This indicates this group of cysteine proteinases may have different sensitivities to the standard cysteine proteinase inhibitors. Additionally, small molecule inhibitors (TTP-6171 and TTP-1021) shown to target I7L by resistance mapping, prevented substrate cleavage (Byrd and Hruby, 2005).

While I7L in the context of infected cell extracts is capable of cleaving exogenously added substrates, I7L made using *in vitro* transcription and translation or a variety of bacterial and mammalian expression systems does

not have cleavage activity (Byrd and Hruby, 2005). This suggests that I7L may require a cofactor(s) or biochemical activation. In addition to its potential nucleic acid binding activity mentioned above, I7L contains several hydrophobic regions in both the N and C-terminus (Byrd *et al.*, 2003). Such hydrophobic patches are common at sites of protein-protein interaction. Adenovirus protease, an I7L active site homolog, has been shown to require both a DNA and a viral peptide cofactor for maximal activity (Mangel *et al.*, 1993).

Mechanisms of Proteinase Regulation

The use of proteinases is one strategy for controlling viral assembly and maturation, but the activity of the proteinases themselves must also be regulated. Numerous means for regulating proteinase activity have been documented in both viral and non-viral systems. Examples of some of the most common mechanisms are given below.

One of the most obvious ways to regulate proteinase activity is by having its activity limited to specific substrates. As mentioned previously, most proteinases cleave at specific sequences such as the AG*X site recognized by I7L. This is called the primary specificity of the proteinase (Polgar, 1989). Secondary specificity refers to the effect of the chemistry and structure of the amino acids surrounding the potential cleavage sites on substrate-enzyme interactions (Polgar, 1989). A final level is conformational specificity, the effect that the overall shape of the protein has on access to and recognition of a

cleavage site (Polgar, 1989). For example, a site that might normally be cleaved may be protected by being buried in the interior of a protein (Polgar, 1989). These factors not only influence whether or not a particular protein is cleaved but at what rate a protein is cleaved as they can alter a proteinase's affinity for a particular site. The importance of the amino acid sequence in determining cleavage rate has been well documented by Dougherty and collegues (1989) for the tobacco etch virus (TEV), a potyvirus. The TEV polyprotein is cleavaged at five sites, each of which is defined by the consensus sequence EXXYXQ*S/G. It has been shown experimentally using site-directed mutagenesis that almost any amino acid may be substituted into the -2, -4, and -5 without preventing cleavage. However, the rate at which the polypeptide substrate was cleaved varied greatly based upon which amino acids occupied those positions. Thus it appears that the natural variation occuring in the cleavage site sequences of the TEV polyprotein may be used to regulate the rate at which the protein products are released.

The context in which a particular protein is found can also influence whether or not it is cleaved. A protein must be expressed at a time and in a place that allow it to come into contact with the proteinase. This is been shown to be important for vaccinia virus as mentioned above. Since I7L is a late protein, found first in the viroplasm and then in the core, only proteins also expressed at late times during infection and present in the same locations are potentially subject to its activity.

Regulation can also occur at the level of the proteinase itself. Many proteinases are produced in an inactive form, referred to as zymogens, and are only activated upon cleavage (Polgar, 1989). Two well known examples of zymogens occur in higher organisms. The first is the blood clotting cascade. Upon injury, factor VII is cleaved to produce its active form factor VIIa, a serine proteinase (Davie *et al.*, 1991). Factor VIIa then cleaves the next member of the cascade to convert it into an active proteinase and so on until the cleavage of fibrinogen to fibrin, the clot forming molecule (Davie *et al.*, 1991). Utilization of such a cascade has several advantages in that it allows for multiple points of regulation and for temporal control of proteinase activity as the activation of a particular zymogen within the cascade is dependent on the activation of those that precede it.

Zymogen activation can occur as a result of cleavage by a separate species of proteinase, as is seen in the blood clotting cascade, or a proteinase can have itself as a substrate as proposed in the induced proximity model for procaspase-8 activation as described by Hengartner (2000). The cleavage site for the activation of procaspase-8 is the same as that recognized by procaspase-8 and even as a zymogen, procaspase-8 has a low level of proteolytic activity. Therefore this model proposes that multimerization of procaspase-8 molecules following activation of the death receptor pathway brings them close enough together to increase the chances of them acting on each other. Once one procaspase-8 is activated to caspase-8 it rapidly acts

on and activates the other procaspases. Self-cleavage can either be in *trans* as seen for the caspases or in *cis* as is the case for the poliovirus 2C proteinase (Palmenberg, 1990).

In addition to activating proteinases, cleavage can also inactivate proteinases. The Sindbis core protein acts autocatalytically to release itself from the p130 polyprotein but becomes inactive afterwards as a result of the newly created C-terminus binding to and blocking the active site (Choi *et al.*, 1991). In another example, Kaposi's sarcoma-associated herpesvirus (KSHV) protease is active as a dimer. However, it can cleave itself at a site within the dimer interface, thus preventing dimer formation and leading to a loss of proteolytic activity (Pray *et al.*, 1999).

The KSHV protease is not alone in acting as a dimer. Homodimers have been identified as the active form of many viral proteinases, though the mechanistic reasons for doing so vary. All retroviral proteases form homodimers in order to construct single, complete aspartic protease active site (Katoh *et al.*, 1989). In contrast, each subunit of the human cytomegalovirus protease contains a complete active site (Batra *et al.*, 2001). Instead, the conformational change caused by dimerization is proposed to be necessary for stabilization of the oxyanion hole (Batra *et al.*, 2001). Similarly, dimerization is believed to be necessary for the severe acute respiratory syndrome (SARS) coronavirus 3C-like proteinase to achieve the correct conformation (Chen *et al.*, 2006). Interestingly, only one subunit of the dimer

has the correct conformation, and therefore is proteolytically active, at any one time (Chen *et al.*, 2006). Other viral proteinases known to form dimers include those of potyviruses (Plisson, 2003) and Norwalk virus (Zeitler, 2006).

Many proteinases do not act alone but require cofactors. As mentioned above, adenovirus requires both a viral peptide and DNA to have maximal activity. It has been proposed that the peptide binding requirement is for temporal regulation while the DNA binding helps properly localize the protein (McGrath *et al.*, 2001; Mangel *et al.*, 1993). In another example, the NS2 protease of bovine viral diarrhea virus, a pestivirus, has been shown to require the cellular protein Jiv in order to autocatalytically separate itself from NS3 (Lackner *et al.*, 2005). The amount of Jiv present in the cell limits the extent to which this cleavage, and in turn RNA replication, occurs. In this case the cofactor is believed to be required for proper orientation of the protease and the cleavage site (Lackner *et al.*, 2006). Such interactions can also have a negative regulatory effect. For example, the poliovirus 2C polypeptide is believed to inhibit the activity of the 3C protease (Banerjee *et al.*, 2004).

Finally, proteinases may be regulated by biochemical modifications of the enzyme. Davis and co-workers (2003) have shown in several retroviral proteases that oxidation or glutathionylation of sulfur-containing amino acids at the dimer interface can prevent dimerization and thus protease activity. These modifications can be reversed to restore activity.

Potential Means of Regulating I7L

Both the timing of a protein's expression and its context within the maturing virion have been shown to be important in determining whether a vaccinia virus protein will be cleaved by I7L (Whitehead and Hruby, 1994a). Additionally, the cleavage motif utilized by I7L, along with some of the necessary structural and biochemical determinants, have been established (Lee and Hruby, 1994; Lee and Hruby, 1995). However, much remains unknown about the regulation of I7L. While it is suspected to require a cofactor(s), none have yet been identified. Furthermore, since the active form of I7L has not been isolated, it is uncertain whether it acts as a monomer or as a multimer or if cleavage plays a role in either its activation or inactivation. As vaccinia virus contains a second putative proteinase, G1L, which is involved at a later step in morphogenesis it is also possible that vaccinia virus utilizes a proteolytic cascade to control its maturation.

The research that follows attempts to address some of these questions about I7L in several ways. First, six I7L temperature sensitive mutants, including the previously described Cts-16, were characterized to identify regions of I7L important to its activity and to look for possible additional functions of I7L. Second, attempts were made to isolate and identify the components of the active form of I7L using a combination of serology, protein purification techniques, and mass spectrometry.

Analysis of vaccinia virus temperature-sensitive I7L mutants reveals two potential functional domains

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ANALYSIS OF VACCINIA VIRUS TEMPERATURE-SENSITIVE I7L MUTANTS REVEALS TWO POTENTIAL FUNCTIONAL DOMAINS

Abstract

As an approach to initiating a structure-function analysis of the vaccinia virus I7L core protein proteinase, a collection of conditional-lethal mutants in which the mutation had been mapped to the I7L locus were subjected to genomic sequencing and phenotypic analyses. Mutations in six vaccinia virus I7L temperature sensitive mutants fall into two groups: changes at three positions at the N-terminal end between amino acids 29 and 37 and two different substitutions at amino acid 344, near the catalytic domain. Regardless of the position of the mutation, mutants at the non-permissive temperature failed to cleave core protein precursors and had their development arrested prior to core condensation. Thus it appears that the two clusters of mutations may affect two different functional domains required for proteinase activity.

Introduction

Vaccinia virus (VV) is the prototypic member of the orthopoxviruses, a genus of large, double-stranded DNA viruses which includes the human pathogens variola virus and monkeypox virus. VV has a complex replication cycle where, as in many other viruses, proteolysis plays a key role in the maturation process. The initial step in virion assembly is envelopment of

viroplasm by crescent shaped membranes to form immature virions (IV). The IVs must then undergo a series of morphological changes, including cleavage of a number of core protein precursors, to become intracellular mature virions (IMV), the first of several different infectious forms.

The product of the VV I7L open reading frame (ORF) has been shown to be the viral core protein proteinase responsible for cleavage of the major core protein precursors P4a (A10L), P4b (A3L), and P25K(L4R) (Byrd *et al.*, 2002; Byrd *et al.*, 2003). It is a cysteine proteinase, with a catalytic triad consisting of a histidine, an aspartate and a cysteine residue (Byrd *et al.*, 2003) and cleaves its substrates at conserved AG*X motifs (VanSlyke *et al.*, 1991a; VanSlyke *et al.*, 1991b; Whitehead and Hruby, 1994a). In addition to the major core protein precursors, I7L has been shown to cleave the membrane protein A17L (Ansarah-Sobrinho and Moss, 2004b) and may also be responsible for the cleavage of other viral proteins containing the AG*X motif such as A12L and G7L whose cleavage has been documented but not attributed to a particular proteinase (Whitehead and Hruby, 1994a; Szajner *et al.*, 2003).

In the absence of functional I7L, virion morphogenesis is irreversibly arrested after the formation of IV but prior to the formation of IMV (Ansarah-Sobrinho and Moss, 2004b; Byrd and Hruby, 2005a, Kane and Shuman, 1993). Despite the potential importance of this enzyme, relatively little is known about the biochemistry of the cleavage reaction or the structural

features which allow I7L to direct regulated catalysis. Up to this point, all attempts to produce purified, functional I7L have failed, thereby limiting progress in this area. An alternative approach for studying the I7L protein is an analysis of the existing collections of temperature-sensitive (ts) mutants. Six ts mutants from the Dales and Condit collections have been identified as I7L mutants using complementation analysis (Lackner *et al.*, 2003; and S. Kato, T. Bainbridge, N. Moussatche, and R. Condit, personal communications). Using the classification system proposed by Lackner *et al.* (with the original Dales designations in parenthesis), these are: Cts-16, Cts-34. Dts-4 (260), Dts-8 (991), Dts-35 (5804), and Dts-93 (9281). Though both collections were created by chemical mutagenesis, the Condit mutants were derived from the commonly used strain Western Reserve (WR) (Condit and Motyczka, 1981; Condit *et al.*, 1983), while the Dales mutants were derived from the strain IHD-W, an IHD-J subtype (Dales *et al.*, 1978).

Of the six mutants, Cts-16 has been the best studied and most frequently used, primarily as a means to establish a viral infection in the absence of functional I7L. Originally it was classified as having a wild type pattern of protein synthesis (Condit and Motyczka, 1981), although it was later shown that while the major core protein precursors are synthesized, they are not cleaved at the non-permissive temperature (Ericsson *et al.*, 1995). In Cts-16, I7L has also been shown to be stably produced at the non-permissive temperature (Kane and Shuman, 1993) and is probably included in the core.

The core protein precursors also localize normally at the non-permissive temperature (Ericsson *et al.,* 1995).

Dales grouped his mutants into categories based on the apparent level of development attained as determined by electron microscopy. He classified Dts-8 as a category L mutant ("immature particles with nucleoids and defective membranes with spicules") and Dts-35 as category O ("immature normal particles and mature particles with aberrant cores") (Dales *et al.,* 1978). Using his classification system, Cts-16 best fits category K ("granular foci and immature particles with nucleoids but lacking internal dense material") or category L. Dales did not assign Dts-93 to a category while Dts-4 was not included in the original publication. Cts-34 has also not been described other than as an I7L mutant.

Results and Discussion

In order to further characterize these ts viruses and to determine the exact location of the mutation or mutations within the I7L ORF of each virus, genomic DNA was extracted from each virus type. The I7L ORF was PCR-amplified using the primers CB26 and CB90 (Byrd *et al.,* 2004), and the same primers used to sequence the purified PCR products. Multiple copies of the sequence of the WR I7L ORF have been deposited with GenBank [GenBank:AY49736, GenBank:AY243312, and GenBank:J03399] and were obtained for this analysis.

Sequencing of the parental strain IHD-W revealed two differences as compared to the I7L ORF of WR with arginine instead of lysine at amino acid (aa) 287 and glutamine instead of histidine at aa376 (Figure 2). WR is reported to have either aspartate or asparagine at aa420 while IHD-W has asparagine. The I7L sequence from IHD-W was identical to that of Dts-97, a mutant in the E9 ORF (S. Kato, T. Bainbridge, N. Moussatche, and R. Condit, personal communications). When these polymorphisms are taken into account, all the I7L ts mutants contain a single amino acid change. Cts-16, as previously reported and reconfirmed in our stock, has a proline to leucine change at aa344 (Kane and Shuman, 1993). Cts-34 has glycine to glutamate at aa29, Dts-4 has serine to phenylalanine at aa37, Dts-8 has proline to serine at aa344, and both Dts-35 and Dts-93 have aspartate to asparagine at aa35. Interestingly, the mutations seem to form two clusters with Cts-34, Dts-4, Dts-35, and Dts-93 containing three different mutations in a stretch of nine amino acids at the N-terminal end and Cts-16 and Dts-8 representing two different mutations in a single amino acid located toward the C-terminus and just downstream of the catalytic cysteine. The possible significance of these groupings is discussed below.
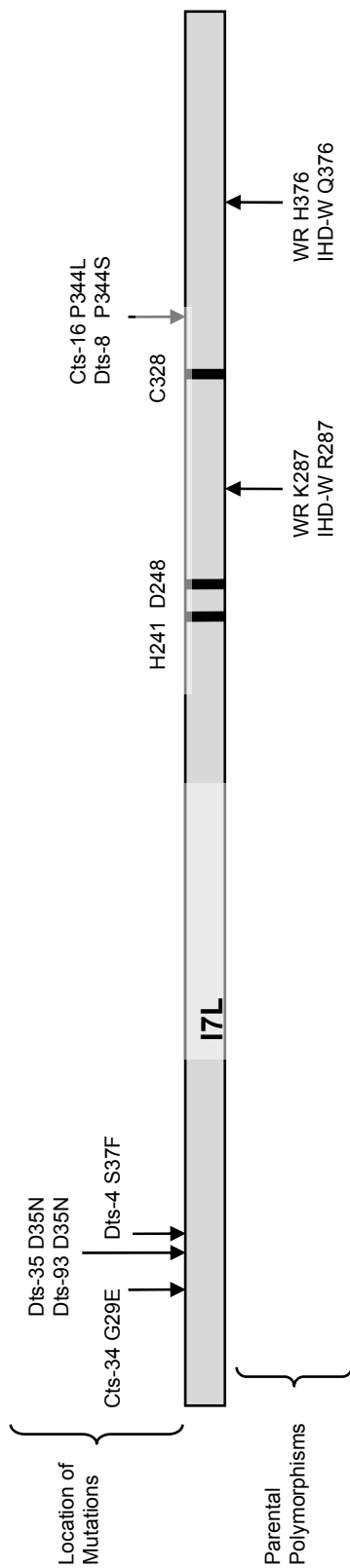
Figure 2. Schematic diagram of the I7L open reading frame. The amino acid changes found in the temperature-sensitive mutants are represented above while the parental polymorphisms are given below. Black bars represent the putative catalytic triad.

Since the mutants were created by chemical mutagenesis, there is the possibility of second-site mutations that contribute to the observed phenotype. To check for this, we attempted to rescue the replication of each virus with plasmid borne I7L. Using DMRIE-C (Invitrogen), BSC_{40} cells were transfected with 2μg of either the empty vector pRb21 (Blasco and Moss, 1995) or pI7L (Byrd and Hruby, 2005a), infected at a multiplicity of infection (MOI) of 2, and incubated at the non-permissive temperature of 41C. pI7L contains the I7L ORF under the control of its native promoter and has been shown to give more efficient rescue than I7L under the control of a synthetic early/late promoter (Byrd and Hruby, 2005a). Mock transfected cells were also infected at an MOI of 2 and incubated at either 41C or the permissive temperature of 31C. The cells were harvest at 24 hours post infection (hpi), resuspended in 100μl PBS and subjected to three freeze-thaw cycles. These lysates were titered onto confluent BSC_{40} cells in a series of 10-fold dilutions. After 48 hours of incubation at 31C, plaques were visualized by staining with 0.1% crystal violet. Cells transfected in the same manner but infected at an MOI of 10 were examined by electron microscopy after 24 hours of incubation at 41C.

All the ts mutants were rescued by the plasmid containing I7L, while transfection with an empty plasmid caused no increase in viral titer (Figure 3). This indicates that for each virus the mutation within the I7L ORF is the primary, if not only, cause of their temperature sensitive phenotype.
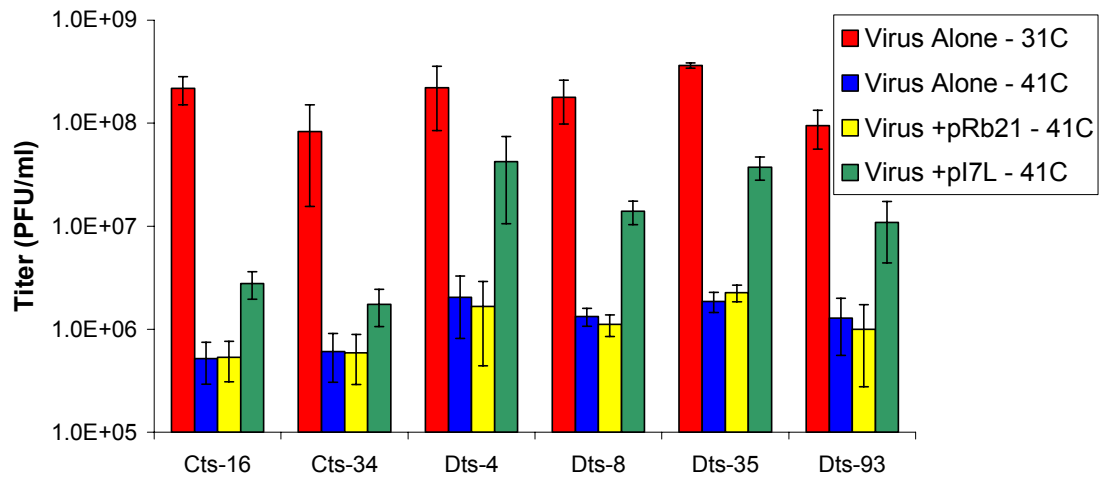
	Virus Alone-31C			Virus Alone-41C			Virus +pRb21-41C			Virus +pI7L-41C		
	Average	%	Fold Increase	Average	%	Fold Increase	Average	%	Fold Increase	Average	%	Fold Increase
Cts-16	2.2E+08	100%	418	5.2E+05	0.2%	1.0	5.3E+05	0.2%	1.0	2.8E+06	1.3%	5.3
Cts-34	8.3E+07	100%	137	6.1E+05	0.7%	1.0	5.9E+05	0.7%	1.0	1.7E+06	2.1%	2.9
Dts-4	2.2E+08	100%	108	2.0E+06	0.9%	1.0	1.7E+06	0.8%	0.8	4.2E+07	19.1%	20.7
Dts-8	1.8E+08	100%	134	1.3E+06	0.7%	1.0	1.1E+06	0.6%	0.8	1.4E+07	7.8%	10.5
Dts-35	3.6E+08	100%	195	1.9E+06	0.5%	1.0	2.3E+06	0.6%	1.2	3.7E+07	10.3%	20.1
Dts-93	9.5E+07	100%	74	1.3E+06	1.4%	1.0	1.0E+06	1.1%	0.8	1.1E+07	11.5%	8.5

Figure 3. Rescue of replication by plasmid borne I7L. (A) BSC_{40} cells were infected/ transfected as indicated and incubated at the permissive (31C) or non-permissive (41C) temperature. At 24 hours after infection, the cells were harvested and the viral titer of the diluted cell lysate was determined. Fold increase was determined by dividing the titer by the titer of virus alone at 41C. % is the percentage of the viral titer at 31C. Bars=+/-1 standard error. Electron micrographs of BSC_{40} cells transfected with pI7L and infected with Dts-4 (B) Dts-8 (C) and Dts-35 (D) at an MOI of 10 showed the presence of mature viral particles within some cells after incubation at 41C for 24 hours. Bars represent 200nm.

Transfection with pI7L resulted in a 2.9 to 20.7 fold increase in viral titer over virus alone at the non-permissive temperature, causing the viruses to reach between 1.3 and 19.1% of their permissive temperature titer. Cts-34 showed the weakest rescue with a fold increase in titer only about half that of the next lowest value. However, as discussed below, its electron microscopic appearance and cleavage activity were identical to those of the other mutants, indicating that even if a second-site mutation exists, the affected protein acts with or after I7L. The degree of leakiness was low for all the mutants with the non-permissive temperature titer being 1.4% or less than that of the permissive temperature titer. As such, leakiness is not expected to have significantly affected the experiments.

Since I7L has been implicated as the core protein proteinase, it was of interest to see if the mutants were all defective in core protein precursor cleavage. Cleavage of the core protein precursors P4a, P4b, and P25K, products of the A10L, A3L and L4R ORF's respectively, was initially assessed by western blot. BSC$_{40}$ cells were infected at an MOI of 5, incubated at the appropriate temperature and harvested at 24hpi. 100µg/ml rifampicin (Boehringer-Manheim) and 8mM hydroxyurea (applied one hour prior to infection) were used where needed. Cell pellets were resuspended in 50µl of buffer and subjected to three freeze/thaw cycles. Aliquots of lysate were boiled with sample buffer and separated on 4-12% SDS PAGE gradient gels for P4a and P4b detection and 12% SDS PAGE gels for P25K detection. Membranes

were incubated with a 1:1000 dilution of the appropriate polyclonal antibody, followed by a 1:2000 dilution of an anti-rabbit-HRP secondary antibody (Promega). Bands were visualized using the Opti-4CN detection system (BioRad). For all mutants, cleavage of P4a and P4b occurred at the permissive temperature but was absent or strongly reduced at the non-permissive temperature (Figure 4A). Cleavage of P25K at the AG*A site to produce 25K did not occur at the non-permissive temperature, while a higher molecular weight band corresponding to the product created by cleavage at an AG*S site was present (Lee and Hruby, 1993). The banding patterns at the non-permissive temperature were similar to those seen in cells treated with rifampicin, a drug known to inhibit cleavage of core proteins (Katz and Moss, 1970). Core protein precursor processing in both parental strains proceeded normally at 41C (data not shown).

The absence of cleavage at the non-permissive temperature was confirmed for P4a using pulse-chase immunoprecipitation. 100mm plates of BSC_{40} cells were infected with virus at an MOI of 10 and incubated at 31 or 41C, as appropriate. At 8hpi, the cells were labeled with 100µCi of [^{35}S]-methionine and [^{35}S]-cysteine (EasyTag EXPRE^{35}S^{35}S; PerkinElmer) in methionine and cysteine free media. Rifampicin, where needed, was added at 100µg/ml. After a 45 minute incubation, pulse wells were harvested, while chase wells were washed and treated with media containing a 100 fold excess of unlabeled methionine and cysteine and rifampicin if necessary. These were

harvested at 24hpi. Cell pellets were resuspended in 600µl of RIPA buffer and subjected to three freeze/thaw cycles and sonication. Samples were centrifuged to remove debris and the lysate was incubated overnight with polyclonal antibodies against P4a followed by a second incubation after the addition of Protein A Sepharose beads (Amersham BioSciences). The washed beads were boiled in 20µl of sample buffer and subjected to electrophoresis on a 4-12% SDS PAGE gel.

In all virus containing pulse samples, a strong band corresponding to P4a was clearly visible (Figure 4B). For IHD-W and Dts-4 at 31C, a representative example of the behavior of the viruses at the permissive temperature, the precursor containing band was strongly diminished after the chase period while a lower molecular weight band representing the cleaved product 4a appeared. At the non-permissive temperature, there was no change in the intensity of the precursor and no or little cleavage product appeared. The pattern seen at the non-permissive temperature was similar to that of IHD-W infected cells treated with rifampicin.
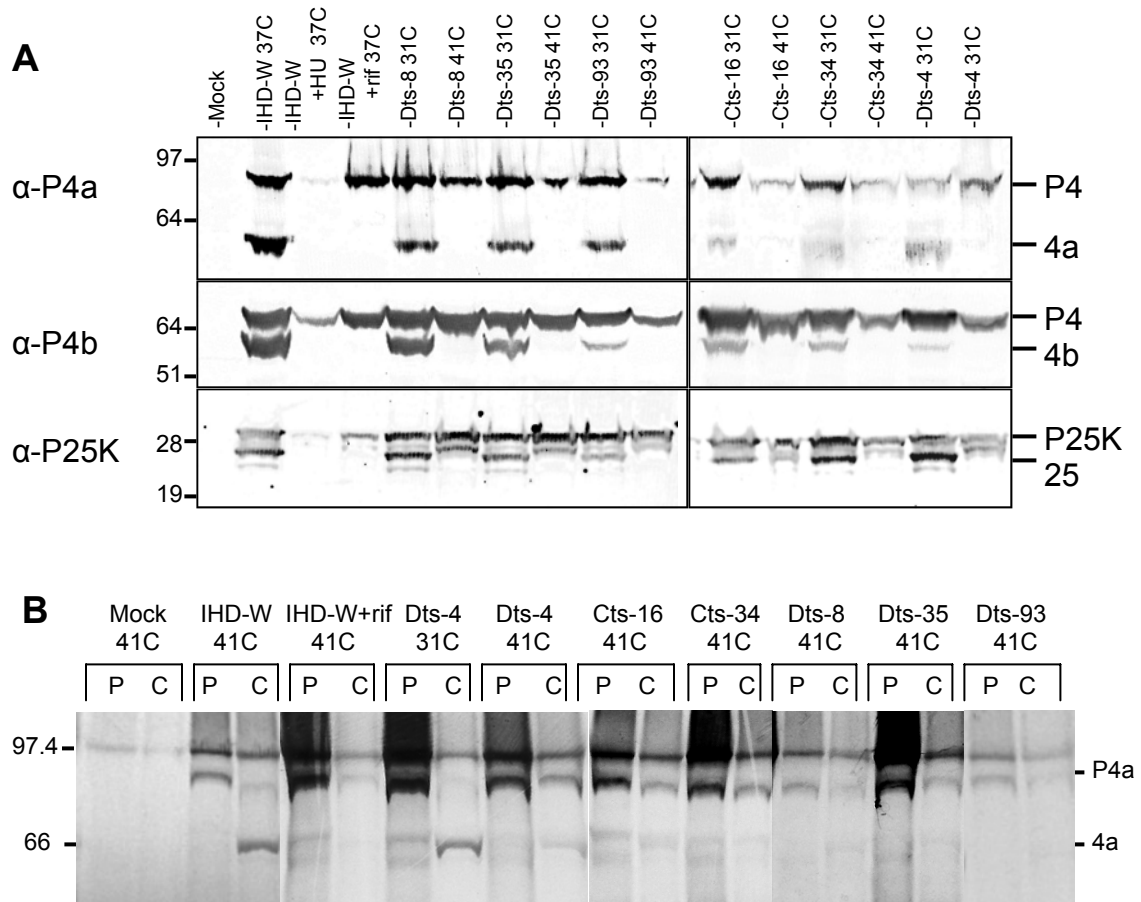
Figure 4. Analysis of core protein precursor processing at the permissive (31C) and non-permissive (41C) temperatures. (A) Infected BSC$_{40}$ cells were incubated at the indicated temperature and harvested 24 hours after infection. Lysates were analyzed by Western blot using the antisera against the indicated protein. Rifampicin (rif) and hydroxyurea (HU) were used at final concentrations of 100µg/ml and 8mM respectively. (B) Infected BSC$_{40}$ cells were labeled with [^{35}S]-methionine and [^{35}S]-cysteine for 45 minutes at 8 hours after infection. Cells were harvested after the pulse (P) and or after being chased (C) with unlabeled methionine and cysteine until 24 hours after infection. Immunoprecipitated samples were separated on a 4-12% SDS PAGE gradient gel.

The overall morphology of all six mutants was examined using electron microscopy. BSC_{40} cells were infected at an MOI of 10, and after a one hour absorption period, incubated at 31 or 41C. Infected cells were collected at 24hpi, fixed, embedded and stained. Dts-4 grown at 31C was examined as a representative of the ts mutants at the permissive temperature and was wild-type in its appearance. Both mature, brick-shaped particles with characteristic biconcave cores and spherical IV containing electron dense viroplasm were seen (Figure 5A-C). At the non-permissive temperature, all the ts mutants were similar in their microscopic appearance (Figure 5D-I). Normal crescent shaped membranes and IV were seen along with large numbers of defective IV. Many of the particles had asymmetrical condensation of the viroplasm, with the membrane sometimes collapsing on the empty side. Others formed dark, electron dense nucleoids. The appearance of these mutants is similar to what has previously been reported for Cts-16 (Kane and Shuman, 1993; Ericsson *et al.*, 1995), Dts-8 (Dales *et al.*, 1978), and I7L conditional-lethals where I7L expression was inhibited by an operator/repressor system (Ansarah-Sobrinho and Moss, 2004b; Byrd and Hruby, 2005a). The appearance of Dts-35 differed from that reported by Dales (1978), as particles with defective cores were not seen. However, Ansarah-Sobrinho and Moss also reported poorly formed cores in some of their I7L null mutants (2004b). It seems then, that a deficiency in I7L can manifest itself in two different ways,
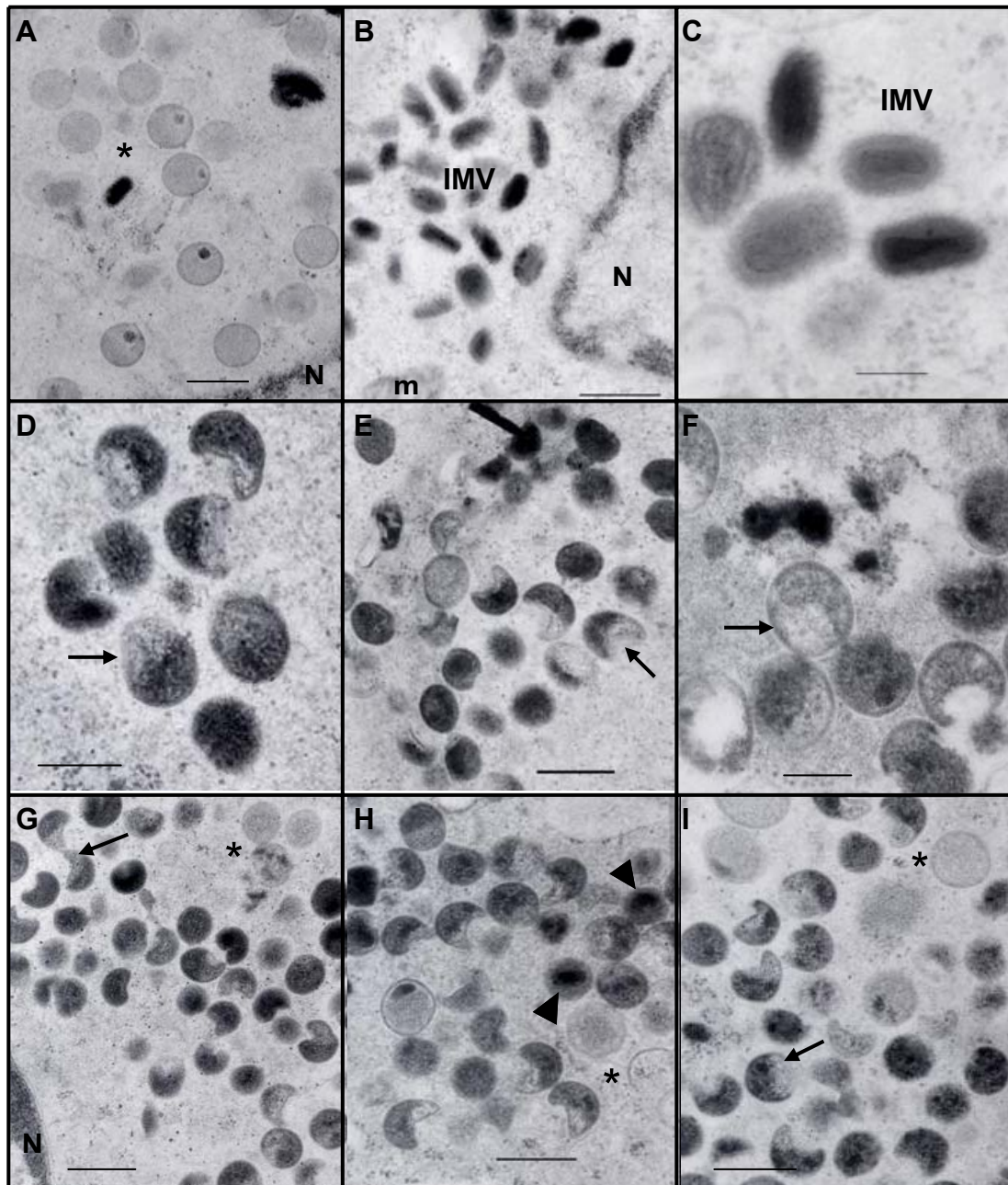
Figure 5. Electron micrographs of virus infected BSC$_{40}$ cells. MOI=10 and cells were harvested and fixed 24 hours after infection. Dts-4 at the permissive temperature of 31C (A-C). Dts-4 (D), Cts-16 (E), Cts-34 (F), Dts-8 (G), Dts-35 (H) and Dts-93 (I) at the non-permissive temperature of 41C. Bars represent 400nm except in C, D and F (bar=200nm). N, nucleus; m, mitochondria; IMV, intracellular mature virion; asterisk, immature viral particle; arrow, representative particles with asymmetrical viroplasm condensation; arrow head, nucleoids

with the virion morphology described here having been the most frequently observed.

Since the mutations in the I7L ts mutants fall into two distinct groups it is tempting to speculate that they might affect two different functions of I7L. Our results indicate that this is not the case, at least at the level of the virion formation, as all mutants were defective in the cleavage of core protein precursors and had their development arrested at a similar stage. Yet the possibility remains that the mutations affect two different elements required for proteinase function. The mutation in Cts-16 (and now Dts-8) at aa344 has been suspected, without proof, to inhibit protein cleavage by disrupting the arrangement of the catalytic triad due to its proximity to the cysteine residue at aa328. It is possible that the other mutants, with amino acid changes at the N-terminus of the protein between residues 29 and 37, may also sufficiently alter the structure or stability of the catalytic site to prevent proteolysis. However, because of their position this seems less likely. Instead, we suggest that the mutations occur within a region that constitutes a separate domain of unknown function that is necessary for I7L proteinase activity. Unfortunately the existing threading and homology model of I7L does not include the 130 N-terminal most amino acids as this region does not fit any known structural domain (Vsevolod Katritch, personal communications).

Nevertheless, the properties of this region suggest several potential functions. One possibility is that the mutations disrupt the binding site of an

unidentified co-factor(s) that I7L is believed to require, as I7L produced in a cell-free translation system lacks cleavage activity (Byrd and Hruby, 2005b). The affected stretch of amino acids lies within a hydrophobic region (Byrd *et al.*, 2003), a common characteristic of sites of protein-protein interaction. The mutations also lie within a region that shows weak homology to the type II DNA topoisomerase of *Saccharomyces cerevisiae* (Kane and Shuman, 1993), raising the possibility of a nucleic acid binding site. Adenovirus proteinase, an I7L homolog, requires both a peptide and a DNA cofactor for full activity (Mangel *et al.*, 1993). Alternatively, the mutations may interfere with a potential regulatory cleavage as I7L contains two AGX motifs at its N-terminal end and third at its C-terminal end. One of these AGX sites is directly disrupted by the mutation in Cts-34 (AGL to AEL)

It is important to note that until more detailed structural and biochemical information about I7L is available, any conclusions about the processes disrupted by the mutations within these ts mutants are tentative. However, their location provides a starting point in the search for regions of I7L important to its activity.

The Vaccinia Virus I7L Proteinase Can Form a Homodimer and Be Cleaved to Give a 40kDa Product

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THE VACCINIA VIRUS I7L PROTEINASE CAN FORM A HOMODIMER AND BE CLEAVED TO GIVE A 40KDA PRODUCT

Abstract

Cleavage of the vaccinia virus major core protein precursors has been shown to be an essential event in the production of mature, infectious progeny virions. These cleavages are performed by the product of the I7L open reading frame, a cysteine proteinase with an apparent molecular weight of 47kDa, but relatively little is known about the regulation of this enzyme. The functional form of I7L has not yet been purified. Using a combination of immunoblots, immunoprecipitation and mass spectrometry, we have shown that I7L is capable of forming a homodimer and of being cleaved to give a 40kDa product. While the biological significance of these forms has not been determined, their existence suggests several new models for I7L regulation. The known I7L temperature-sensitive mutations were also shown not to affect cleavage or dimerization of I7L.

Introduction

While viruses are generally regarded as simple, their assembly and maturation, and the regulation thereof, is still relatively complex. Proteolysis is a common strategy employed by viruses as a means to control these processes. It has been proposed that proteolytic events in viruses can be classified as either formative or morphogenic (Helen and Wimmer, 1992). Formative proteolysis is used to separate individual proteins from a large

polyprotein precursor. This strategy is used by a large number of viruses including most positive-strand RNA viruses, such as picornaviruses, flaviviruses, potyviruses and togaviruses, and retroviruses (Krausslich and Wimmer, 1988). Morphogenic proteolysis is required following the assembly of an immature virion to produce a mature, infectious particle. This strategy is also employed by a wide range of viruses including picornaviruses, T4 bacteriophage, retroviruses, adenoviruses (Helen and Wimmer, 1992) and poxviruses.

Vaccinia virus (VV) is the prototypic member of the *Orthopoxvirus* genus, a part of the *Poxviridae* family of viruses. It is a large, double stranded DNA virus with a genome of about 200kbp which encodes more than 200 proteins. Proteolytic cleavage of the vaccinia virus major core protein precursors P4a, P4b and P25K (products of the A10L, A3L and L4R open reading frames [ORFs]) has been shown to be a critical event in initiating core condensation and the transition of VV immature virions (IV) to intracellular mature virion (IMV) (Byrd and Hruby, 2005a; Ansarah-Sobrihno and Moss, 2004b) . These proteins are cleaved at conserved AG*X motifs by the product of the vaccinia virus I7L ORF (Byrd *et al.*, 2002; Byrd *et al.*, 2003; Ansarah-Sobrihno and Moss, 2004b; Byrd and Hruby, 2005a). The product of the ORF is a 47kDa protein expressed late in infection and has been identified as a cysteine proteinase (Byrd *et al.*, 2003). The core proteins G7L (Szajner *et al.*, 2003) and A12L (Whitehead and Hruby, 1994a) and the membrane protein

A17L (Whitehead and Hruby, 1994a; Ansarah-Sobrihno and Moss, 2004b) have also been shown to be cleaved at one or more AG*X motifs. While I7L has also been shown to be responsible for the cleavage of A17L (Ansarah-Sobrihno and Moss, 2004b), the proteinase responsible for the cleavage of G7L and A12L has not yet been determined.

Although proteolysis is an important and powerful tool in the regulation of virus assembly and maturation, proteolysis itself must be regulated. Most proteinases are limited to recognizing and cleaving specific amino acid sequences, although some recognize three-dimensional structures. However, the accessibility of these sequences and the effect of the surrounding amino acids on the ability of the proteinase to bind the substrate, influence whether or not and at what rate these sequences are cleaved (Polgar, 1989) Furthermore, if a potential substrate is separated from the proteinase by time and/or space proteolysis will not occur.

In addition to containing AG*X motifs, all known I7L substrates are produced late in infection (Whitehead and Hruby, 1994a, Szajner *et al.*, 2004) indicating that when a VV protein is present is an important determinant in whether or not it is cleaved. Furthermore, while A17L is a membrane protein (Wolffe *et al.*, 1996), all of the remaining I7L substrates are components of the virion core, as is I7L, indicating that location is also a key in determining what proteins are cleaved. Interestingly, unlike the other proteins, A17L is cleaved prior to core condensation and its cleavage is apparently not affected by the

drug rifampicin (Ansarah-Sobrihno and Moss, 2004b) suggesting that while A17L, like the major core proteins, is cleaved by I7L the regulation of this event may be somewhat different.

Regulation of proteinase activity may also involve the proteinase itself. Some proteinases, such as those of the blood clotting cascade (Davie *et al.*, 1991) are produced as zymogens and require cleavage for activation while others, such as the Sindbis core protein (Choi *et al.*, 1991) and the Kaposi's sarcoma-associated herpesvirus (KSHV) protease (Pray *et al.*, 1999), are inactivated as a result of being cleaved. Many proteinases require one or more cofactors. Adenovirus, a catalytic domain homolog of I7L, requires both a viral peptide and a DNA cofactor for full activity (Mangel *et al.*, 1993). Similarly, proteinases may only be in their active form when complexed with other proteins or by forming a homomultimer with additional molecules of itself. The proteinases of retroviruses (Katoh *et al.*, 1989), potyviruses (Plisson, 2003), herpesviruses (Shimba *et al.*, 2004), at least some coronaviruses (Fan *et al.*, 2004) and Norwalk virus (Zeitler, 2006) have all been shown to be active as homodimers. Finally, biochemical modifications may determine whether or not a proteinase is active. In several retroviruses, oxidation or glutathionylation of sulfur-containing amino acids at the dimer interface can prevent dimerization and thus protease activity (Davis *et al.*, 2003).

While I7L has clearly been shown to be the proteinase responsible for at least some of the morphogenic cleavage events in vaccinia virus, the

mechanisms regulating its activity remain largely unknown. I7L appears to require a cofactor(s) as I7L produced by *in vitro* transcription and translation does not show proteolytic activity (Byrd and Hruby, 2005b), but this cofactor has not yet been identified. Furthermore, it is not known if I7L functions as a monomer or as a multimer and if any proteolytic events are involved in its regulation. Using a combination of immunoblots, immunoprecipitation and mass spectrometry, we were able to identify both an I7L homodimer and a cleaved form of I7L with an apparent molecular weight of approximately 40kDa.

Methods and Materials

Cells and viruses

BSC$_{40}$ cells were maintained in mimimum essential media with Earle's salts (MEM-E; Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 25µg/ml gentamicin reagent solution (Gibco) and incubated at 37C in a humidified atmosphere with 5% CO_2. Viral infections were carried out in MEM-E containing 5% FBS and 25µg/ml gentamicin. Infected cultures were incubated at 37C for wild type viruses or 31C (permissive temperature) or 40C (non-permissive temperature) for temperature sensitive mutants. Viruses were purified as described previously (Hruby *et al.*, 1979) with VV strains IHD-W and Western Reserve (WR) being grown on BSC$_{40}$ cells at 37C while ts mutants were propagated at 31C.

Immunoblots

Samples were mixed with 4x NuPAGE LDS sample buffer (Invitrogen) and boiled for 10 minutes. For reducing gels, 10x NuPAGE sample reducing agent (Invitrogen) was added prior to boiling. Samples were then separated on 10% or 4-12% NuPAGE Bis/Tris SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes (Millipore). The membranes were blocked with 3% gelatin in TBS [20mM Tris, 0.5M NaCl] before overnight incubation with the primary antibody in antibody buffer (1% gelatin in TTBS [20mM Tris, 0.5M NaCl, 0.05% Tween 20]). α-I7L-654 (Anaspec), a polyclonal antibody against the synthetic I7L peptide ELKTRYHSIYDVFEL (amino acids 95-109), was used at a 1:500 dilution while anti-FLAG M2-peroxidase conjugate (α-FLAG-HRP; Sigma) was used at a 1:1000 dilution. For blots with α-I7L-654, anti-rabbit IgG HRP conjugate (Zymed) was used as the secondary antibody at a 1:2000 dilution in antibody buffer. Blots were visualized using either Opti-4CN (BioRad) or SuperSignal West Pico Chemiluminescent Substrate (Pierce). For the preparation of cellular lysates, two 100mm plates of BSC40 cells were harvested at 24 to 72 hours post infection (hpi) and resuspended in 100µl RIPA buffer [150mM NaCl, 50mM Tris-HCl (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1.0% deoxycholate, 5mM EDTA, 100µM sodium orthovanadate, complete mini protease inhibitor cocktail (Roche)]. These were then subjected to three freeze-thaw cycles followed by a low speed spin to remove nuclei and

other cellular debris. Preparation of the other sample types is described elsewhere in this paper.

Transient Expression

Transfections were performed using the transfection reagent DMRIE-C (Invitrogen) in accordance with manufacturer directions. A mixture consisting of 2ml Opti-MEM (Gibco), 16µl DMRIE-C (Invitrogen) and 4µg plasmid was used for each 100mm plate of BSC_{40} cells. An additional 3ml of Opti-MEM were added to the plates following addition of the transfection mixture. For expression at 37C, transfection was simultaneous with infection with IHD-W or WR at a multiplicity of infection (MOI) of one. For expression at 40C, plates were held at 37C for four hours prior to being infected with IHD-W at an MOI of one and transferred to 40C. The plasmid pRb21:I7L has been previously described (Byrd *et al.*, 2002). pRb21:I7L-FLAG was created by PCR amplification of the I7L ORF using the primers CB52 (5'-CCCAAGCCTTCTACTTGTCGTCATCGTCTTTATAATCTTCATCGTCGTCTAC-3') and CB105 (5'-AACTGCAGATGGAAAGATATACAGATTTAG-3'). The PCR product was cloned into pRb21 (Blasco and Moss, 1995) using the newly created HindIII and PstI restriction sites. Site directed mutagenesis was carried out on this plasmid using the QuikChange II site-directed mutagenesis kit (Stratagene) to create pRb21:I7L-FLAG-C16, pRb21:I7L-FLAG-C34, pRb21:I7L-FLAG-D4, pRb21:I7L-FLAG-D8, and pRb21:I7L-FLAG-D35. These recreated the mutations found in the I7L ORF of the temperature sensitive

mutants Cts-16, Cts-34, Dts-4, Dts-8 and Dts-35. A list of the primers used is

found in Table 2.

Table 2. Primers used for site-directed mutatgenesis of pRb21:I7L-FLAG

Primer Name	Primer Sequence (5' to 3')	Plasmid Created
MM1	GTGTACTAGGACACCACTTAAAAGTTTTAAATCTTTG	pRb21:I7L-FLAG-C16
MM2	CAAAGATTTAAAACTTTTAAGTGGTGTCCTAGTACAC	pRb21:I7L-FLAG-C16
MM3	GTCATATATATTCACTAGCTGAATTATGTAGCAATATAGATG	pRb21:I7L-FLAG-C34
MM4	CATCTATATTGCTACATAATTCAGCTAGTGAATATATATGAC	pRb21:I7L-FLAG-C34
MM5	GCAATATAGATGTATTTAAATTTTTAACAAATTGTAACGG	pRb21:I7L-FLAG-D4
MM6	CCGTTACAATTTGTTAAAAATTTAAATACATCTATATTGC	pRb21:I7L-FLAG-D4
MM7	GTGTACTAGGACACCATCTAAAAGTTTTAAATCTTTG	pRb21:I7L-FLAG-D8
MM8	CAAAGATTTAAAACTTTTAGATGGTGTCCTAGTACAC	pRb21:I7L-FLAG-D8
MM9	GTAGCAATATAAATGTATCTAAATTTTTAACAAATTGTAACGG	pRb21:I7L-FLAG-D35
MM10	CCGTTACAATTTGTTAAAAATTTAGATACATTTATATTGCTAC	pRb21:I7L-FLAG-D35

Immunoprecipitation

Immunoprecipitation (IP) of the I7L-FLAG fusion protein was carried out

using the FLAG tagged protein immunoprecipitation kit (Sigma) in accordance

with manufacturer instructions. Pellets from one to ten 100mm plates of

infected BSC_{40} cells transfected with pRb21:I7L-FLAG were lysed in lysis

buffer. Lysates were incubated overnight with resin bound antibodies.

Proteins were eluted from washed resin by boiling with 2x sample buffer or by

incubating with 3x FLAG peptide in wash buffer [50mM Tris (pH 7.4), 150mM

NaCl]. Arginine buffer [0.5M L-arginine, 20mM Tris (pH 7.6), 150mM NaCl]

was substituted for wash buffer where noted.

Size Exclusion Chromatography

200-500µl of FLAG IP extract (eluted with 3x FLAG peptide) was separated on BioSep SEC-S 3000 column (7 x 300mm, Phenomenex). The column was run with a Waters 1525 Binary HPLC pump at a constant flow rate of 1ml/minute. Protein was detected using a Waters 2487 Dual λ Absorbance Detector which recorded absorbance at 214 and 254nm. The column was equilibrated with 100mM KPO_4 (pH 6.8) until constant baseline absorbencies were obtained. 100mM KPO_4 was also used as the mobile phase. Aqueous SEC 1 column performance check standards (Phenomenex) were used to create a standard elution profile. Fractions were collected by peak. Each fraction was concentrated to a volume of approximately 100µl using a speed vac (Savant) and then dialyzed against 20mM Tris-HCl (pH 8.0) using D-tube Dialyzer Minis (Novagen). 20µl aliquots were analyzed via immunoblot using α-I7L-HRP. Fractions were also analyzed by mass spectrometry.

Glycerol Gradient Centrifugation

Solutions were made containing 10, 15, 20, 25, 30, and 35% glycerol. The solutions also contained either 20mM HEPES, pH 7.4 or 0.5M L-arginine, 20mM Tris-HCl (pH 7.6) and 150mM NaCl. 875µl of each solution was added to a 13 x 51mm Quick-Seal centrifuge tube (Beckman) by underlaying the solutions, starting with the 10% glycerol solution and finishing with the 35% solution. This discontinuous gradient was allowed to become continuous by a three hour incubation at room temperature. 150-200µl of FLAG IP extract

(eluted with 3x FLAG peptide) concentrated approximately two-fold using a speed vac, was mixed with 30µg carbonic anhydrase (CA), 100µg bovine serum albumin (BSA) and 100µg alcohol dehydrogenase (AD) (Gel filtration molecular weight markers, Sigma) and layered on top of the continuous gradient. The tube was sealed and centrifuged at 66,000rpm and 4C for 3.5hrs in a Beckman VTi80 rotor. Three drop fractions (~200µl) were collected by puncturing the bottom of the tube with a 23 gauge needle. 20µl aliquots of each fraction were subjected to immunoblot analysis using α-FLAG-HRP.

Mass spectrometry

For analysis of specific protein bands, FLAG immunoprecipitation samples boiled in loading buffer were separated on 10% NuPAGE Bis/Tris SDS-PAGE gels (Invitrogen). Gels were stained with coomassie brilliant blue [0.1% coomassie brilliant blue R-250, 40% methanol, 10% acetic acid] and destained overnight using 45% methanol and 10% acetic acid. Bands of interest were cut out and subjected to in-gel trypsin digest. Gel pieces were placed in 0.5ml Eppendorf tubes and dehydrated with acetonitrile (AcN). After vortexing for 10 minutes, slices were rehydrated with 50mM ammonium bicarbonate and vortexed for 10 minutes. This was repeated twice before a final dehydration with AcN. After vortexing, all remaining liquid was removed and the gel slices were dried with a speed vac for 5 minutes. A sufficient volume of digestion buffer [20µg/ml trypsin (Promega) in 40mM Tris-HCl, pH 8.0] was added to each tube to completely cover the gel slice and the tubes

were incubated on ice for 35-40 minutes. After incubation, all excess digestion buffer was removed and enough 10mM Tris-HCl (pH 8.0) was added to completely cover the slice. Samples were then incubated at 37C overnight. Any non-absorbed solution was placed in a new tube, while the peptides were extracted from the gel by adding 30-70μl of a 50%AcN/5% formic acid solution and vortexing for 10 minutes. The liquid portion was removed and combined with the previously collected non-absorbed solution. The volume was then reduced to 10-15μl using a speed vac.

For fractions collected from size exclusion chromatography, following dialyzation against 20mM Tris-HCl (pH 8.0) the volume of the samples was reduced to 10-15μl using a speed vac. Trypsin was then added to a final concentration of approximately 0.2μg/μl and the samples were incubated overnight at 37C.

Sample analysis was conducted using a quadrupole-time of flight mass spectrometer with HPLC and electrospray ionization interfaces (LC-ESI-Q-TOF MS) as previously described (Yoder *et al.*, 2006). Peptide identification was made using the Mascot software (Matrix Science). Mass spectral data were searched against the MSDB database and an in-house database of vaccinia virus proteins based on the complete vaccinia virus DNA sequence. The Mascot program assigns peptide matches an ion score based on the probability of the match being correct (Score= $-10*Log_{10}(P)$, where P is the probability that the observed match is a random event). For the purposes of

this study a score of 20 (P=0.01) was considered sufficient to establish identity when utilizing the vaccinia database while a score of 50 (P=0.00001) was required to establish identity when using the MSDB database. Identified peptides not of viral or mammalian origin were not included in the analysis along with trypsin, which was added in the digestion step.

Analysis of Mutant I7L

100mm plates transfected with wild-type pRb21:I7L-FLAG or one of its mutant derivatives and infected with IHD-W at an MOI of one, were harvested at 24hpi and immunoprecipitated using the FLAG IP kit. The washed resin was boiled in sample buffer. An immunoblot was then carried out using α-FLAG-HRP

Results

I7L Forms a Dimer

The I7L ORF encodes a protein with a predicted molecular weight of 49kDa and an apparent molecular weight of 47kDa. Immunoblots performed under non-reducing conditions using lysates from IHD-W cells tranfected with pRb21:I7L-FLAG showed the presence of a virus specific band at approximately 94kDa. Upon addition of a reducing agent, this 94kDa band disappeared and there was an increase in the intensity of the band at 47kDa representing full-length, monomeric I7L. This was seen using both α-FLAG and α-I7L-654 antibodies (Figure 6 and data not shown). This suggested that I7L might be forming a homodimer. To confirm that the 94kDa band was I7L,

it was cut out and subjected to analysis by mass spectrometry. In each of two analyses, I7L was the only vaccinia virus protein present with a significant peptide match. The only significant cellular match was keratin, an abundant cellular protein likely to be a contaminant. This not only shows that I7L is present in the 94kDa species but that it is likely an I7L homodimer. A sample spectrum and the peptides identified are shown in Figure 7.
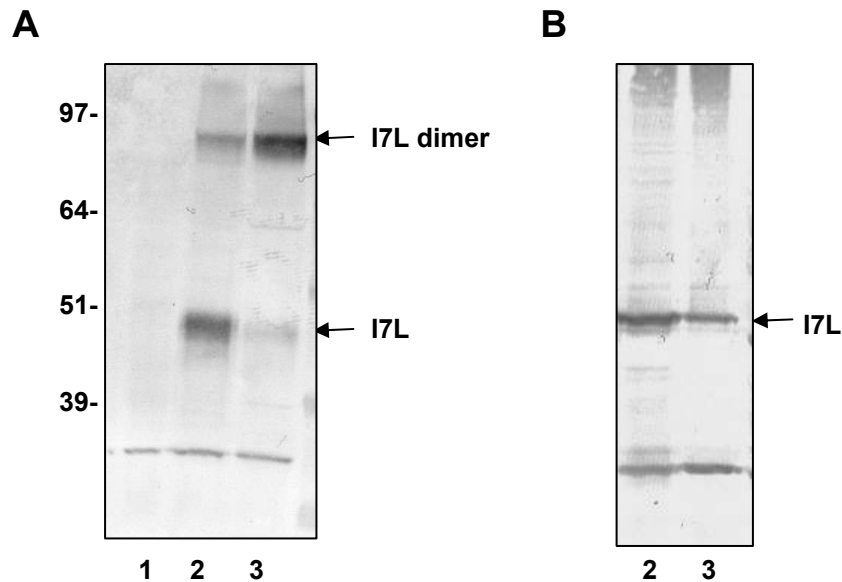
Figure 6. I7L forms a dimer. Cells were infected with IHD-W and transfected with pRb21:I7L-FLAG and the resulting lysates were separated on 10% SDS PAGE gels. The presence of I7L was detected in an immunoblot using α-I7L-654 under non-reducing (A) and reducing (B) conditions. The locations of I7L and its dimer are indicated. Lane 1, cells alone; lane 2, IHD-W + pRb21:I7L-FLAG, 50 hours post-infection (hpi); lane 3, IHD-W + pRb21:I7L-FLAG, 72 hpi.

A

```
MERYTDLVISKIPELGFTNLLCHIY    SLAGLCSNIDVSKFLTNCNGYVVEK
YDKSTTAGKVSCIPIGMMLELVESG    HLSRPNSSDELDQKKELTDELKTRY
HSIYDVFELPTSIPLAYFFKPRLRE    KVSKAIDFSQMDLKIDDLSRKGIHT
GENPKVVKMKIEPERGAWMSNRSIK    NLVSQFAYGSEVDYIGQFDMRFLNS
LAIHEKFDAFMNKHILSYILKDKIK    SSTSRFVMFGFCYLSHWKCVIYDKK
QCLVSFYDSGGNIPTEFHHYNNFYF    YSFSDGFNTNHKHSVLDNTNCDIDV
LFRFFECTFGAKIGCINVEVNQLLE    SECGMFISLFMILCTRTPPKSFKSL
KKVYTFFKFLADKKMTLFKSILFNL    HDLSLDITETDNAGLKEYKRMEKWT
KKSINVICDKLTTKLNRIVNDDE
```

B
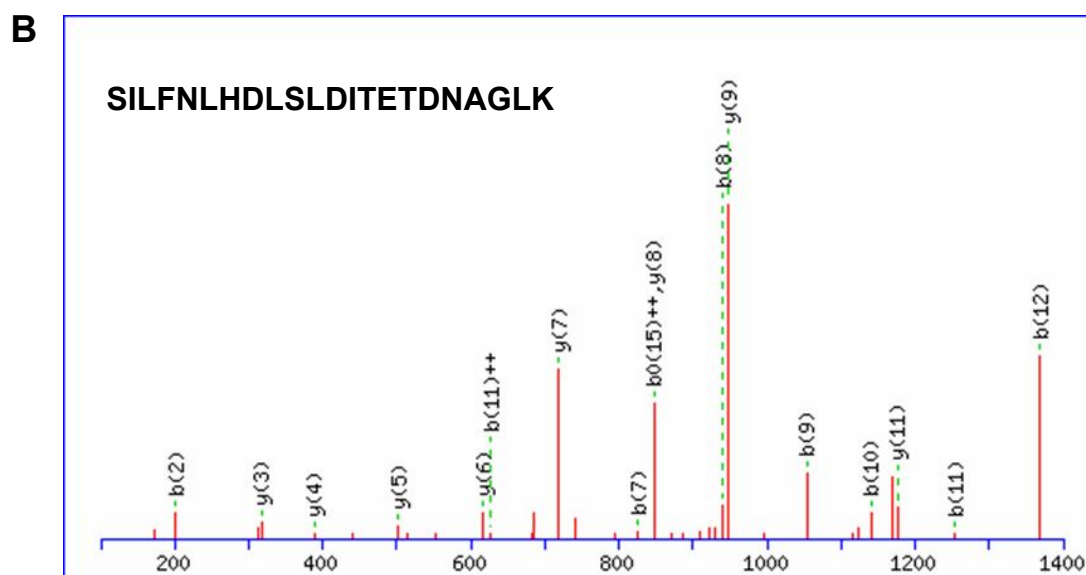
Figure 7. Peptides identified in putative I7L homodimer using mass spectrometry. Proteins immunoprecipitated by an α-FLAG mAb were separated on a 10% SDS PAGE gel and the band corresponding to the putative I7L dimer was subjected to in-gel trypsin digest and analysis by mass spectrometry. The process was repeated twice. A. Peptides found in both analyses are shown in bold italics while peptides found only once are in bold. B. Sample spectrum for the peptide SILFNLHDLSLDITETDNAGLK. The X-axis represents ion size while the Y-axis represents the signal intensity. A letter-number combination above a line indicates a match with an ion fragment from this peptide.

I7L is Cleaved to Form a 40kDa Product

Immunoblots of infected cells using α-I7L-654 show a virus specific band with an apparent molecular weight of 40kDa. A band of the same molecular weight, presumably representing the same protein, is seen using α-FLAG-HRP in immunoblots of infected cells transfected with pRb21:I7L-FLAG (Figure 8). Using mass spectrometry, this band was identified as containing I7L (data not shown). Based on its size, it appears to be a cleavage product of I7L. However, as N-terminal sequencing found the N-terminus of this polypeptide to be blocked, the site of this potential cleavage is unknown (data not shown).

Hrs. p. i.   24   48   72
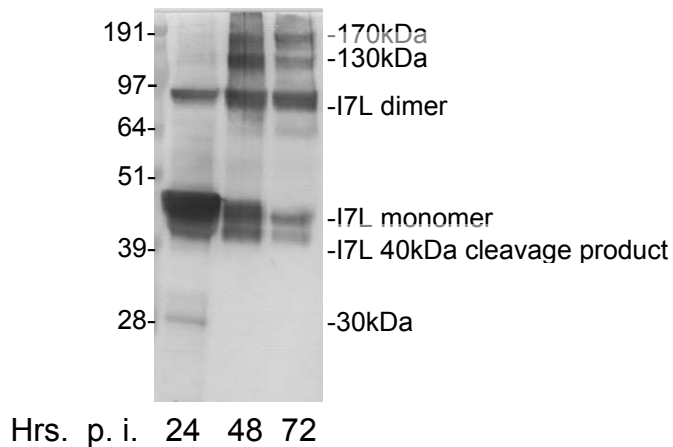
Figure 8.   α-FLAG reactive species in immunoprecipitation extracts. Lysates from infected cells transfected with pRb21:I7L-FLAG and harvested at various time post infection (p.i.) were immunoprecipitated using resin-bound α-FLAG antibodies and eluted in wash buffer using a 3x FLAG peptide. Eluted proteins were separated on a 10% SDS PAGE gel and detected with α-FLAG-HRP. The identity of the I7L dimer, the full-length I7L monomer and the 40kDa cleavage product have been confirmed using mass spectrometry (MS). The composition of the 30, 130 and 170kDa bands is unknown.

Size Exclusion Chromatography

Since I7L was determined to exist in both a monomeric and a dimeric form, it was of interest to separate the two species and determine which has proteolytic activity. The sample utilized was eluted from a FLAG immunoprecipitation. An immunoblot with α-FLAG-HRP showed that all three identified forms of I7L were present in this extract as well as bands with apparent molecular weights of approximately 30kDa, 130kDa and 170kDa (Figure 8). The identity of the protein constituents in these bands has not been determined. All of these bands are specific to transfection with I7L as they did not appear in cells transfected with untagged I7L (data not shown).

Separation by size exclusion chromatography was attempted twice. Both attempts gave an identical absorption profile with six distinct regions of increased absorbance (Figure 9A), but during the second attempt the elution time for each peak was delayed by about five minutes, suggesting that something in the preparation was clogging the column. Thus, use of this method of separation was discontinued. Upon analysis of peak fractions with immunoblot using α-FLAG-HRP, all of the major bands were in a single fraction, fraction 4 (Figure 9B). This included both monomeric and dimeric I7L and the two high molecular weight bands. There was no significant recognition by α-FLAG of any of the proteins in the other fractions. During the first separation, the elution time of this peak corresponded to that of the ovalbumin molecular weight standard (43.0kDa).
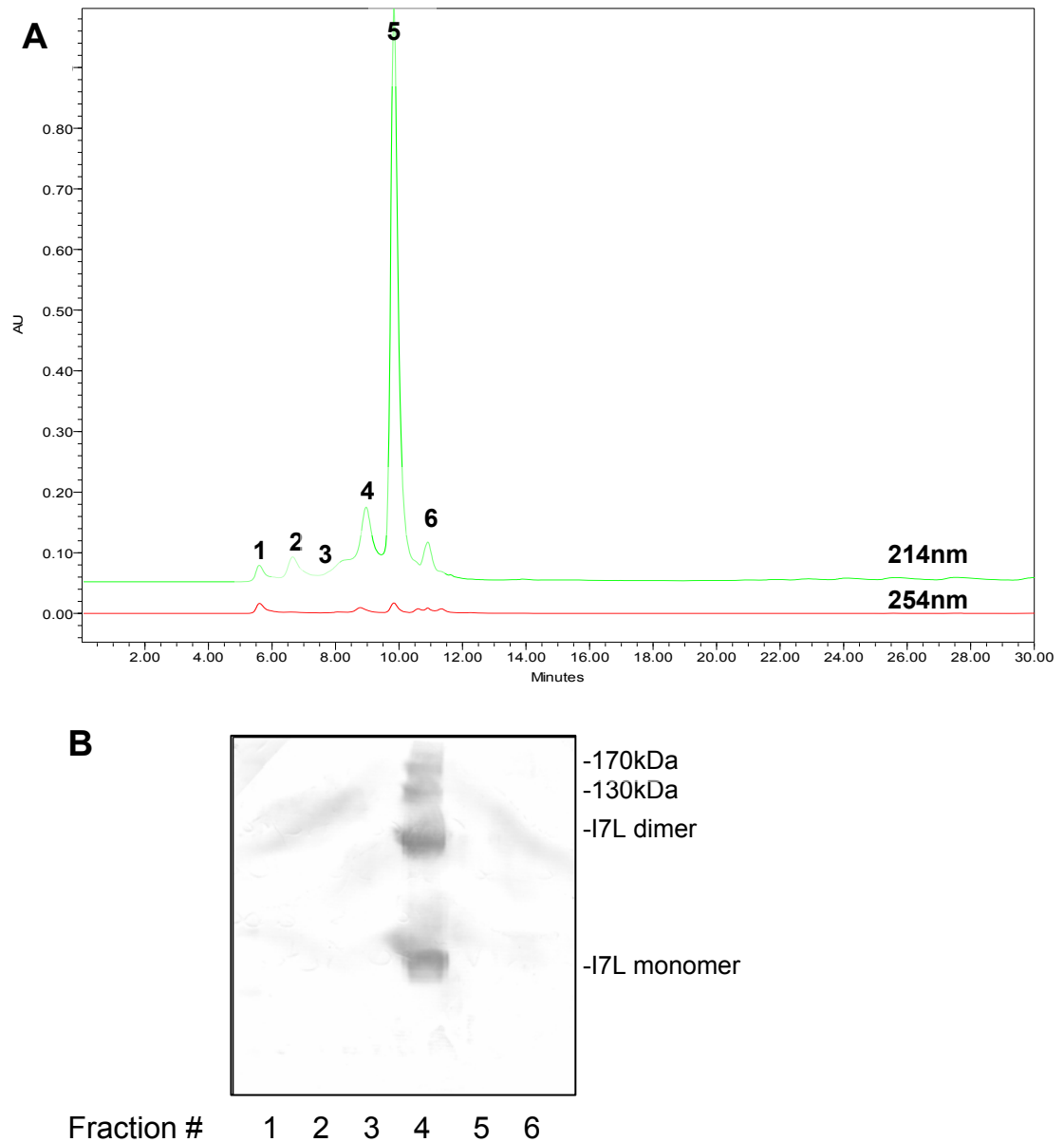
Figure 9. Separation of IP extracts using size exclusion chromatography. Lysates from infected cells transfected with pRb21:I7L-FLAG were immunoprecipitated using resin-bound α-FLAG antibodies and eluted in wash buffer using a 3x FLAG peptide. The proteins in this extract were separated using size exclusion chromatography. A. Absorbance of the flow through was measured at 214 and 254nm and fractions were collected by absorbance peak. B. Fractions were concentrated and dialyzed and aliquots

were separated on a 10% SDS PAGE gel and immunoblotted using α-FLAG-HRP. Fraction numbers correspond to numbered peaks in A.

Mass spectrometry showed I7L to be present in fractions number 1, 3, 4, 5 and 6. The largest number of I7L peptides was found in fraction 4. This suggests that I7L was most abundant in that fraction in agreement with the immunoblot. The remaining identified peptides were primarily from abundant cellular or viral proteins not expected to be recognized by α-FLAG. A summary of the proteins detected can be found in Table 3.

Table 3. Summary of mammalian and vaccinia virus peptides found in size exclusion chromatography fractions using mass spectrometry. Immunoprecipitation extracts from IHD-W infected cells transfected with pRb21:I7L-FLAG were separated using size exclusion chromatography. Fractions were collected by peak and analyzed for protein content using mass spectrometry. Probabilities of P=0.01 and P=0.00001 were required for viral and mammalian peptide matches respectively to be considered significant. Proteins are listed in order of the strength of the peptide(s) match. Proteins with more than one subtype (e.g. actin, 70kDa heat shock proteins) are grouped together. Viral proteins are indicated in bold.

Fraction	Protein	Molecular Weight (kDa)	Comments
1	Keratin	54-66	
	P25K	28.4	Major core protein
	Actin	41	
	Nuclease sensitive element-binding protein 1	35.7	
	Nucleolin	76.4	
	Histones H1a, H1b, H1c and H1d	21-23	
	I7L	49.0	
	60S ribosomal proteins L27, L6, L10E, L18, P2 and L7	12-35.0	
2	Transitional endoplasmic reticulum ATPase	89.1	
	Actin	102-104	
	Tropomyosin alpha-3 and alpha-4 chains	28-33	
	Cell division control protein 48 homolog A	89	
	A-type inclusion protein (A25L)	84.2	

(Table 3, continued)

| 3 | Immunoglobulin heavy chain binding protein (BiP) | 72 | |
	Transitional endoplasmic reticulum ATPase	89.1	
	Heat shock 70kDa proteins	70-71	
	Tropomyosin 1 alpha or beta chain	33	
	Calreticulin precursor	48.1	
	Prothymosin alpha	12	
	Fructose-bisphosphate aldolase A	39.3	
	I7L	49.0	
	D13L	61.8	Scaffold protein (Szajner *et al.*, 2005)
4	Actin	36-43	
	I7L	49.0	
	Keratin	48-62	
	Alpha-enolase	47	
	Calmodulin	16-17	
	Transitional endoplasmic reticulum ATPase	89.1	
	E3L	21.4	Inhibitor of host interferon-induced double-stranded RNA-dependent protein kinase (Chang *et al.*, 1992)
	Transgelin-2	22.3	
	Heat shock 70kDa protein	70.1	
	Peroxiredoxin	21.5	
5	Actin	41-42	
	Keratin	65-66	
	E3L	21.4	
	I7L	49.0	
	B7R precursor	21.3	Cleavage product localizes to endoplasmic reticulum (Price *et al.*, 2000)
6	**I7L**	49.0	
	P25K	28.4	

Glycerol Gradient Centrifugation

An attempt was also made to separate the I7L monomers from the dimers using glycerol gradient centrifugation and a more concentrated form of the IP extract used in size exclusion chromatography. When using a gradient

made with HEPES buffer, the majority of the protein detected by α-FLAG (the species shown in Figure 8) was found apparently stuck to the side of the tube (data not shown). When using a vertical rotor, this is effectively the bottom of the gradient, indicating that high molecular weight protein aggregates had formed. Arginine buffer has been shown to be effective in preventing non-specific protein-protein interactions (Tsumoto *et al.*, 2005). When arginine buffer was substituted for HEPES there was a gradual accumulation of the forms starting with the I7L monomer and then the I7L dimer followed by the 130kDa band and the 170kDa band (Figure 10A). Using this method the I7L monomer but not the dimer was achieved in isolation. The apparent weight of the highest molecular weight species in a fraction was in general agreement with the weights of the molecular weight standards.

When arginine buffer was used to elute the bound proteins as well as in the glycerol gradient, the I7L monomer and the 30kDa band dominated both the original sample and the collected fractions (Figure 10B). In this instance, the I7L monomer migrated much lower in the gradient. The greatest amount of I7L monomer was found in the fractions also containing alcohol dehydrogenase, a 150kDa protein.   In all cases, the proteolytic activity of the fractions was not assessed.
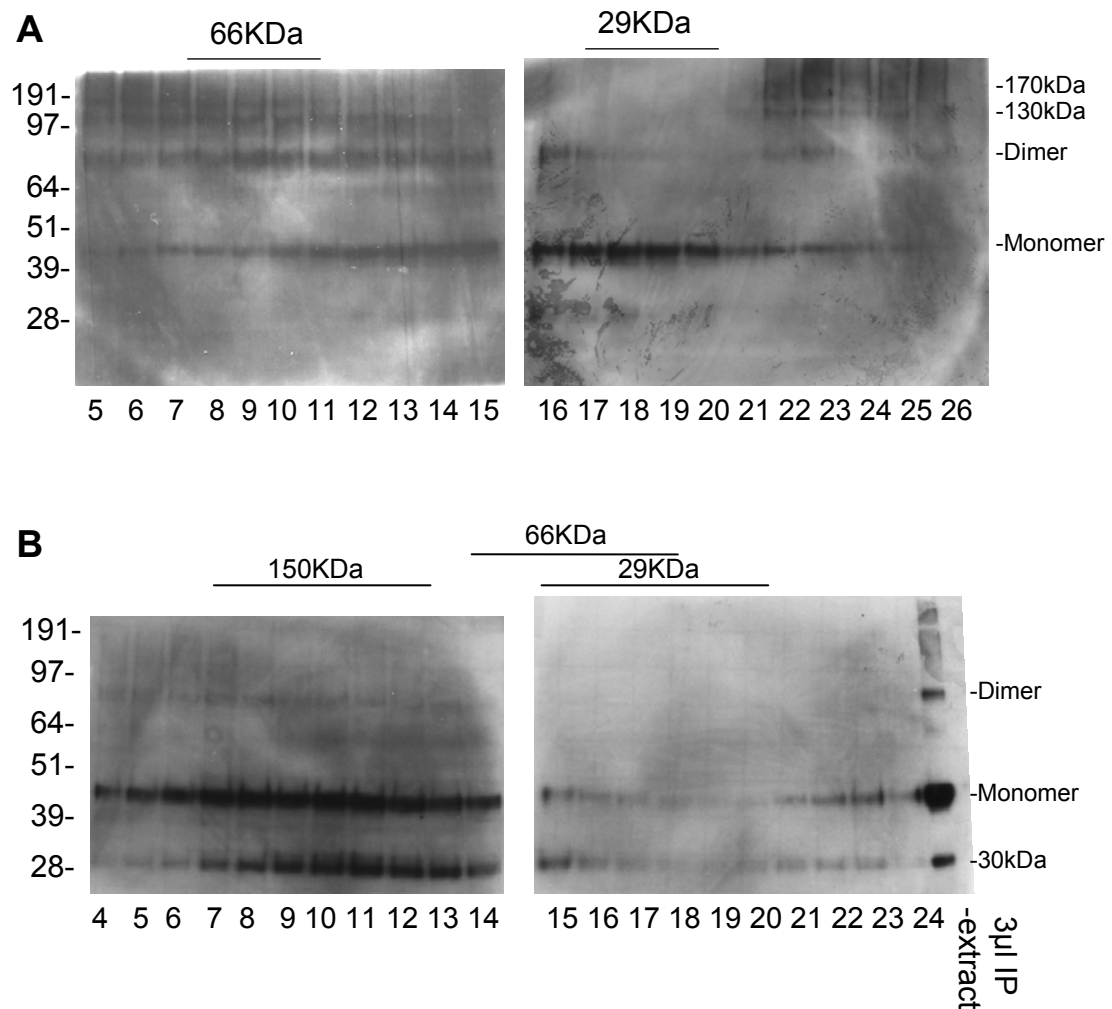
Figure 10. Separation of IP extracts using glycerol gradient. Lysates from infected cells transfected with pRb21:I7L-FLAG were immunoprecipitated using resin-bound α-FLAG antibodies and eluted in wash buffer (A) or arginine buffer (B) using a 3x FLAG peptide. The proteins in these extracts were separated in a 10-35% continuous glycerol gradient. Fractions were collected from the bottom of the tube and analyzed by immunoblot using 4-12% SDS PAGE gels and α-FLAG-HRP. The location of the molecular weight standards are indicated by bars above the corresponding fractions. Lane numbers indicate fraction number.

Dimerization and Cleavage of Mutant I7L

There are six known I7L temperature-sensitive mutants, representing a total of five distinct amino acid substitutions (Chapter 2). It was of interest to determine if the I7L produced by these mutants was capable of forming dimers and/or if it was being cleaved. In order to obtain sufficient amounts of protein, the expression of the mutant constructs was driven by infection with IHD-W, resulting in the presence of a small amount of wild-type I7L. The mutant I7L was distinguishable from the wild-type in an immunoblot by the presence of a C-terminal FLAG tag. Under these conditions, all of the mutant proteins were incorporated into dimers and produced what is believed to be the 40kDa cleavage product (Figure 11). Thus, it does not appear that the lack of functional I7L is due to a defect in either of these functions. While difficult to quantify due to the divergent protein levels, it appears that the N-terminal mutants (Cts-34, Dts-4, and Dts-35) have a higher ratio of 40kDa I7L:47kDa I7L than the C-terminal mutants or wild-type I7L.
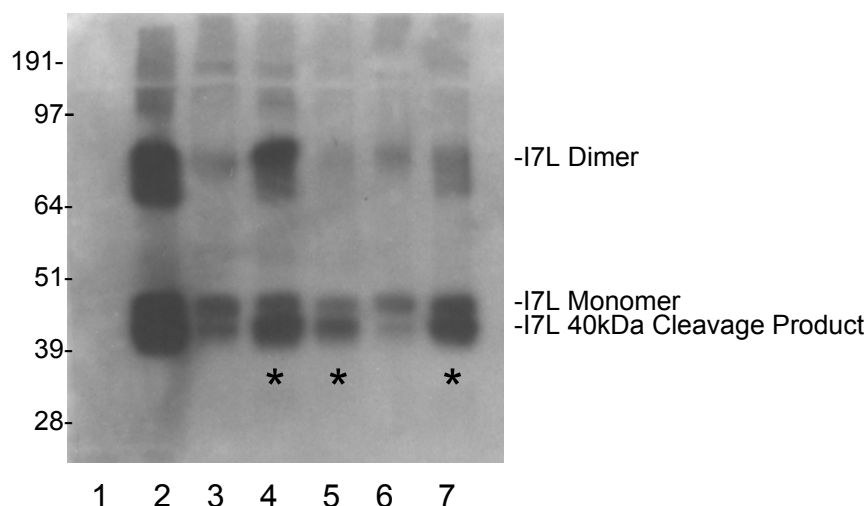
Figure 11. Dimerization and cleavage of mutant I7L. Lysates from IHD-W infected cells transfected with pRb21:I7L-FLAG or one of its mutant derivatives and incubated at the non-permissive temperature (40C) were immunoprecipitated using resin-bound α-FLAG antibodies. The resulting proteins were analyzed by an immunoblot using α-FLAG-HRP after separation on a 4-12% SDS PAGE gel. Lane 1, IHD-W + pRb21:I7L; lane 2, IHD-W + pRb21:I7L-FLAG; lane 3, IHD-W + pRb21:I7L-FLAG-C16; lane 4, IHD-W + pRb21:I7L-FLAG-C34; lane 5, IHD-W + pRb21:I7L-FLAG-D4; lane 6, IHD-W + pRb21:I7L-FLAG-D8; lane 7, IHD-W + pRb21:I7L-FLAG-D35. * indicates I7L with an N-terminal mutation.

Discussion

In this study we have shown that I7L is capable of forming a dimer and that it can be cleaved into a protein with an apparent molecular weight of approximately 40kDa. However, the functional significance, if any, of these forms remains to be determined. Our inability to separate monomeric and dimeric I7L leaves open the question of which of these forms possesses catalytic activity. Additionally, it is uncertain if the cleavage of I7L is performed

by I7L or if it is mediated by a separate proteinase, and the location of the cleavage site is also unknown.

Yet the very existence of these two forms suggests several possible models for I7L regulation. It is possible that the dimer is an artifact created by the unnaturally high levels of I7L present in the transfected cells or, if natural, an inconsequential biproduct. Then the monomer would be the active form possibly with cleavage causing inactivation of the protein, or the full-length monomer may be a zymogen with the active 40kDa product being released upon cleavage.

On the other hand, the dimer may be important. Examples of viral proteinases forming dimers are abundant although the mechanistic reasons for doing so are diverse. For example, dimerization of the human cytomegalovirus protease is believed to stabilize the oxyanion hole (Batra *et al.*, 2001), while dimerization appears to be necessary for the severe acute respiratory syndrome (SARS) coronavirus 3C-like proteinase active site to achieve the correct conformation (Chen *et al.*, 2006). It is possible that dimerization occurs after I7L is cleaved, in which case cleavage would be activating. Alternatively, the dimer could be made up of full-length I7L with cleavage disrupting dimer formation and inactivating the enzyme. This seems more likely as the apparent molecular weight is consistent with the dimer being made up of two molecules of full-length I7L. However, apparent molecular weight can be deceiving so in the absence of N-terminal sequencing data this

observation is not definitive. The latter model may be used by KSHV. Its protease protomers are capable of cleaving each other at a site in the dimer interface, preventing dimerization, and thus activation, of the cleaved molecules (Pray *et al.*, 1999).

As vaccinia virus has a second putative proteinase encoded by the G1L ORF, it is tempting to speculate that these proteinases may act together as part of a proteolytic cascade. The substrates of G1L have not yet been identified but it has been shown to be essential in a late stage of virion morphogenesis subsequent to the cleavage of the major core protein precursors by I7L and it also appears to be cleaved (Ansarah-Sobrinho and Moss, 2004a; Hedengren-Olcott *et al.*, 2004). Thus in this model, cleavage of I7L activates the enzyme leading to cleavage of its substrates and partial condensation of the virion core. At a later step, G1L is also cleaved and activated and then acts on its substrates to aid the completion of virion morphogenesis.

Our data shed little light on which of these models are correct. However, this remains a key question both in terms of understanding virion morphogenesis and for drug development. Traditional proteinase inhibitors target the active sites of these proteins. However, for proteinases that form dimers both small molecule and peptide inhibitors of dimerization have been shown to inhibit proteinase activity in several viruses including SARS

coronavirus (Wei *et al.*, 2006), KSHV (Shimba *et al.*,2004) and HIV-1 (Bannwarth *et al.*, 2006).

It is of interest that it was difficult to separate the FLAG reactive components of the IP extracts. One explanation is that the high concentrations of proteins found in these extracts caused non-specific protein-protein interactions and that once formed these aggregates are difficult to disrupt. Another explanation is that the observed bands represent different protein complexes and that these complexes are in equilibrium with each other and their monomers. In this case, the forms would not be expected to be separable and the apparent molecular weight as determined by size exclusion chromatography or glycerol gradient centrifugation would be intermediate to that of the complexes and the monomers. It is also possible that the I7L dimer is only stable as part of a larger protein complex and therefore will only be present in conjunction with the other proteins. If this is the case, the stand-alone I7L dimers detectable in immunoblots are the result of the denaturing conditions disrupting the larger complex.

It is notable that when arginine buffer was used for elution as well as in the glycerol gradient, the 30kDa band became much more prominent and the majority of the I7L was in monomeric form in both the original sample as well as in the gradient. It is possible that under these conditions the complexes dissociated almost completely into their monomers. In which case, the 130 and 170kDa bands likely represent the I7L dimer plus either one or two

molecules of the 30kDa proteins (although it is also possible, due to inaccuracies in molecular weight predictions, that these are I7L trimers and tetramers). If this is the case and is a natural interaction, the 30kDa protein may be an I7L cofactor. Efforts are currently underway to identify the composition of the 30kDa, 130kDa and 170kDa bands. If successful, their identification should help clarify the situation.

It is also curious that all of these bands reacted with α-FLAG antiserum as, unless they represent even more forms of I7L or are part of an I7L containing complex, they would not contain the FLAG octapeptide. Furthermore, if they non-specifically reacted with α-FLAG antibodies they would be expected appear in IPs of virus alone or virus tranfected with untagged I7L which is not the case. This raises yet another possibility, that these proteins are interacting with the FLAG octapeptide. This interaction with the tagged I7L would allow them to be immunoprecipitated and binding to the 3x FLAG peptide used in the elution step would allow the protein-peptide complex to be recognized by the α-FLAG antibodies.

While all the FLAG reactive proteins eluted in a single fraction during size exclusion chromatography, mass spectrometry was carried out on all the fractions to determine what proteins has co-precipitated with I7L. Most of the detected peptides are from abundant cellular or viral proteins, suggesting that they are contaminants rather than proteins that specifically interact with I7L. However, this does not rule out I7L having a functional interaction with one of

these proteins or even one of these proteins being a necessary cofactor. Importantly, no antibody components were detected in any of the fractions indicating that residual antibodies from the IP are not responsible for the additional bands or for the formation of the protein aggregates.

The six known I7L temperature-sensitive mutants have all been shown to be defective in core protein precursor cleavage and production of IMV (Chapter 2). Two of the identified mutations lie in the C-terminus and are believed to disrupt the catalytic site due to their proximity to the catalytic cysteine. The remaining three mutations all lie in the N-terminus of I7L. Since the N-terminus is distant from the catalytic triad, at least in terms of primary sequence, it has been proposed that the N-terminus of I7L represents a domain of unknown function distinct from the catalytic domain. As such it was of interest to determine if the I7L produced by these mutants was capable of forming dimers and/or if it was being cleaved.

In the presence of wild-type I7L, distinguishable from the mutant I7L by the lack of a FLAG tag, all mutant forms were incorporated into dimers, indicating that at most one wild-type protein is necessary for a dimer to form. All of the mutant I7L proteins were also cleaved to form the 40kDa product. Whether wild-type I7L is necessary for this cleavage to actually take place is uncertain, but none of the mutant proteins are intrinsically uncleavable. However, it is possible that the cleavage, which may be the result of I7L acting on itself, is being carried out by the wild-type protein and would not be

observed if only mutant I7L was present. Thus, definite conclusions cannot be drawn until the behavior of these proteins is analyzed in the absence of wild-type proteinase. Interestingly, cleavage of the N-terminal mutants seemed to occur more frequently, perhaps because the mutations cause a structural alteration that makes the cleavage site more accessible.

It is particularly notable that the Cts-34 form of I7L is still cleaved as its mutation converts an N-terminal AGL site into an AEL site, thus eliminating one of the three AGX sites within I7L that could be used if I7L is acting on itself. Furthermore, the fact that this product can be visualized using α-FLAG antibodies indicates that the C-terminal AGL site is also not being utilized. This leaves the AGK site at amino acids 57 to 59 as the remaining possible AG*X site, and the size of the product resulting from a cleavage at this site would be in agreement with that observed using SDS-PAGE. Of course it is also possible that I7L is cleaved by a different protein, in which case a different cleavage motif might be utilized. Both the cleavage site and the responsible proteinase remain a subject for further research.

Conclusion

We have shown that the I7L VV proteinase can form a homodimer and that it can be cleaved to form a product with an apparent molecular weight of 40kDa. The existence of these forms has greatly expanded the number of possible models for I7L regulation. However, difficulty in separating the different forms of I7L have hampered efforts to elucidate which of these

models is correct.   As the answer to this question is important to both understanding poxvirus biology and antiviral development, further research needs to be conducted in this area. We have also noted that all of the known I7L temperature-sensitive mutants do not appear to be defective in either dimerization or formation of the 40kDa cleavage product.

CONCLUSIONS

Vaccinia virus has been and remains an important model system, both for understanding the etiology of viral disease and for gaining an understanding of basic biological mechanisms. Past studies of the vaccinia virus I7L proteinase have been conducted with both of these goals in mind. Cleavage of the major core protein precursors, catalyzed by I7L, has been shown to be an essential event in the development of mature, infectious vaccinia virus. Yet while the proteolytic activity of I7L has been well characterized, many questions about its structure, the composition of the functional enzyme and its regulation remain. In this thesis, we have attempted to begin addressing some of those questions.

Genomic sequencing of six I7L temperature-sensitive mutants surprisingly showed that three of the five amino acid changes were at the N-terminus despite the catalytic triad being located in the C-terminal third of the protein. Since the homology based model of I7L does not include the 130 N-terminal amino acids, it is difficult to predict what effect these N-terminal mutations might have on the protein. However, based on their distance from the catalytic triad in terms of primary amino acid sequence, it seems unlikely that they are impacting the structure of the catalytic triad and more likely that they are affecting a separate domain also required for I7L proteolytic activity. That they impact proteolysis, either directly or indirectly, is clear from the fact that all six mutants were defective in the cleavage of the major core protein

precursors at the non-permissive temperature. Later analysis also showed the mutant forms of I7L produced by these mutants to be capable of forming homodimers and of being cleaved, at least when small amounts of wild-type I7L are present. This leaves open the possibility that the N-terminal mutations are affecting yet another necessary function, perhaps cofactor binding, or that the tertiary structure of the protein is such that they are in fact influencing the catalytic site structure.

By showing that I7L is capable of forming a homodimer and of being cleaved to a 40kDa product, a number of additional models for the regulation of I7L activity can be postulated. It is possible that I7L may be active as a homodimer as has already been demonstrated for several other viral proteinases. Furthermore, whether I7L acts as a monomer or a dimer, the observed cleavage may either activate or inactivate the proteinase. Should the cleavage be shown to be activating, it would lend further credence to a hypothesis that I7L may act in conjuction with the putative vaccinia virus metalloproteinase G1L and other proteinases to form a proteolytic cascade, where first I7L and then G1L act on the core proteins to aid core condensation. However, as we were unsuccessful in our attempts to separate the I7L monomer from the I7L homodimer, we could not assess the proteolytic activity of these forms. Thus, which model is most likely to be correct remains unknown.

Additional Research Needed

As identifying the composition of the active I7L proteinase and its means of regulation has both scientific and medical implications, it is important that research in this area continue. As we were unsuccessful in separating the I7L monomers from the dimers using two common techniques, size exclusion chromatography and glycerol gradient centrifugation, alternative methods may need to be employed to assess the activity of the I7L monomer, dimer and cleavage product. Identification of the composition of the 30, 130 and 170kDa FLAG-reactive bands that fractionate with I7L may also help shed light on the problem. Furthermore, the identity of the cofactor(s) that I7L is believed to require remains unknown. Their discovery will be important to reconstitute the active I7L proteinase *in vitro*, something that would be a useful tool for both drug development and studying the enzyme's biochemical properties. Finally, a better understanding of G1L and its substrates will be required to determine its possible relationship to I7L and to give a more complete picture of the role of proteolysis in the maturation of vaccinia virus.

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