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REVIEW

Vaccinia virus proteolysis—a review

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SUMMARY

It is well known that viruses, as obligate intracellular parasites, must use their hosts' metabolic machinery in order to replicate their genomes and form infectious progeny virions. What is less well known are the details of how viruses make sure that once all the necessary proteins are made, that they assume the correct configuration at the proper time in order to catalyse the efficient assembly of infectious virions. One of the methods employed by viruses to regulate this process is the proteolytic cleavage of viral proteins. Over the past several decades, studies in numerous laboratories have demonstrated that morphogenic proteolysis plays a major and essential role during the assembly and maturation of infectious poxvirus virions. In this review we describe the history of vaccinia virus proteolysis as a prototypic viral system. Copyright © 2006 John Wiley & Sons, Ltd.

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GENERAL OVERVIEW OF VIRAL PROTEOLYSIS

The term 'limited proteolysis' was first introduced by Linderstrom-Lang and Ottesen to describe reactions in which the peptide bonds in a polypeptide are selectively hydrolysed, as opposed to protein degradation which involves extensive cleavage of the peptide bonds in the substrate [1]. The enzymes required for the peptide bond cleavage are named proteases which are divided into peptidases and proteinases [2]. Peptidases are exopeptidases which hydrolyse single amino acids from the amino-terminus or the carboxy-terminus of a peptide chain. In contrast, the proteinases (also called proteolytic enzymes or endopeptidases) are capable of selectively recognising and cleaving specific peptide bonds within substrates.

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Abbreviations used
VV, vaccinia virus; IMV, intracellular mature virus; IEV, intracellular enveloped virus; EEV, extracellular enveloped virus; CEV, cell-associated enveloped virus; CPV, cowpox virus; TK, thymidine kinase; vCPP, vaccinia virus core protein proteinase; EMC, encephalomyocarditis virus

Proteinases are further subdivided into four classes based on the identity of their catalytic amino acid residues, whose relative three-dimensional positions are conserved within a group, and the mechanism of catalysis [2]. The four types of proteinases are: serine, cysteine (thiol), aspartic (acid), and metallo. Serine proteinases possess a catalytic triad of aspartic acid, histidine and serine residues, and appear to be the most common and widespread type of proteinase. The serine residue is usually the amino acid that acts as a nucloephile during the reaction by donating an electron to the carbon of the peptide bond to be cleaved. A proton is then donated to the leaving amino group by the histidine residue. The serine is hydrolysed, the product released, and the active site is regenerated [1]. Cysteine proteinases maintain a catalytic diad composed of cysteine and histidine residues in close proximity. In some cases there is a catalytic triad with the addition of an aspartic acid residue which helps in the stability of the active site. The mechanism of action is similar to that of serine proteinases except that the nucleophile is a thiolate ion instead of a hydroxyl group. The sulphhydryl group of the cysteine residue acts as the nucleophile to initiate attack on the carbonyl carbon of the peptide bond to be cleaved. The imidazol ring of the histidine residue removes a proton from the sulphhydryl making it more nucleophilic. Catalysis proceeds through the formation of a

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covalent intermediate. Unlike serine and cysteine proteinases, aspartic and metalloproteinases do not appear to form a covalent enzyme–substrate intermediate [3]. Aspartic proteinases have a catalytic diad of two aspartic acid residues. Catalytic activity occurs as two molecules join together bringing the aspartic acid residues into close proximity of each other. Acid-base catalysis from an activated water molecule leads to the formation of a non-covalent tetrahedral intermediate. For the metalloproteinases, a divalent cation (usually Zn^{2+}) is required together with essential histidine and glutamic acid residues for catalysis. As with aspartic proteinases, the catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the zinc-bound water molecule attacks the carbonyl group of the scissile bond.

Proteinases can be thought of in their most basic form as having a catalytic site, as described above and a substrate binding pocket [4]. The two sites are usually in close proximity. Generally, proteinases are composed of two globular domains, with amino acids involved in catalysis being contributed by each half of the substrate-binding crevice. For most serine, cysteine and aspartic proteinases, the two globular domains are found within the same polypeptide. However in the case of the retroviral proteinases [5], a dimer complex is employed to bring together two individual catalytic centres to form the crevice. Although nearly all substrate-binding crevices achieve a similar three-dimensional structure with respect to the catalytic amino acids for each class of proteinase, the structural conservation does not extend to the substrate binding pocket, which distinguishes a given proteinase from all others. It is this substrate binding region which confers specificity to the proteinase.

It is generally accepted that for the hydrolysis of a specific peptide bond to occur, two requirements must be met. First, the susceptible peptide bonds need to be defined by the nearby amino acid residues with specific side chains which are required for the primary and secondary specificity. According to Polgar [2], the primary specificity has a qualitative feature which targets the selection of the scissile bond, and the secondary specificity conveys a quantitative feature by facilitating the cleavage of the selected bond. Second, the susceptible bond is usually displayed adjacent to the surface of the substrate in a flexible region accessible to the proteinase, and the susceptible peptide must be presented in a three-dimensional conformation which fits the active site pocket of the proteinase. This is referred to as conformational specificity [6].

Many types of post-translational modifications such as phosphorylation, glycosylation, and acylation are required for the acquisition and regulation of protein properties such as enzyme activity, protein–protein interactions and intracellular localisation. Likewise, limited proteolysis is often used to regulate protein activation or assembly by causing changes in the tertiary structure which bring distant functional amino acid residues together. Interestingly, the free energy required for the reconstruction of the hydrolysed peptide bond is high and no biological mechanisms for repairing the broken peptide bond have yet been identified. Thus the changes introduced into substrates by proteolytic cleavage are essentially irreversible. This combination of cleavage specificity and reaction irreversibility have resulted in the common utilisation of the proteolytic processing reaction as a unidirectional mechanism for a wide variety of biological processes including food digestion, signal peptide cleavage, signal transduction, peptide hormone/growth factor production, blood clotting, complement pathway cascade, pathogen elimination, cell migration and reproduction [7–9].

For many plant and animal viruses, a successful infection is dependent on proteolytic processing at one or more stages. In fact, it is the exceptional virus that does not require proteolytic processing during its replication cycle [3]. The required proteolytic enzymes can be provided by either the host cell, the infecting virus, or both. Proteinases provided by the host cell generally contribute to the processing of membrane or envelope proteins that are trafficking through the secretory compartment of the cell. It is within these secretory compartments that viral envelope proteins undergo maturation by cleavage of signal peptides (in addition to acylation and glycosylation), such as the E1 and E2 glycoproteins of Sindbis virus [10]. On the other hand, the proteinases which are responsible for the proteolytic processing of viral proteins are usually encoded by the viruses themselves.

Proteolytic cleavage of viral polypeptides have been categorised as 'formative' or 'morphogenic' proteolysis, depending on the function the reaction serves during the replicative cycle [11].

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Formative proteolysis refers to the processing of viral polyproteins into structural and non-structural protein products. A number of viral formative cleavage proteinases have been identified and are encoded by animal viruses such as picornaviruses, flaviviruses, alphaviruses, retroviruses and coronaviruses [12]. Formative proteolysis provides a mechanism for viruses, such as retroviruses and positive-strand RNA viruses, to utilise a single RNA template for the expression of several viral proteins from a large polyprotein precursor. Morphogenic proteolysis refers to the cleavage of viral structural proteins assembled in previrions during virion maturation. Morphogenic cleavage occurs in conjunction with virion assembly and is often required for the acquisition of infectivity of both DNA and RNA viruses such as picornaviruses, alphaviruses, retroviruses, adenoviruses, and bacteriophage T4 [13]. Although less is known about morphogenic proteolysis, several different functions have been proposed for this process, including: facilitation of correct genomic RNA dimerisation in assembling retroviral particles [14]; unidirectional packaging of bacteriophage T4 DNA [15]; completion of the infectious poliovirus virion in a flexible configuration [16]; and, promotion of proper disassembly of adenovirus particles during the initiation of infection [17].

In addition to formative or morphogenic proteolysis, cleavage reactions can be further described as being either *cis* or *trans*. In *cis* cleavage events one precursor protein contains both the cleavage site and the proteinase activity, which is autocatalytic. In *trans* cleavage events, one protein contains the proteolytic activity and a second contains the substrate cleavage site.

In the most basic case, proteinases are synthesised in their active form, as has been shown through TNT reactions where the enzyme and substrate can be synthesised and activity demonstrated [18–20]. Some proteinases need to be cleaved from a precursor protein in order to become activated while other proteinases require cofactors for catalysis to occur, such as in the adenovirus system where the protease requires both DNA and a specific peptide as cofactors [21,22].

Figure 1 depicts the general requirements for proteolysis including the location of the scissile bond in the protein to be cleaved, the difference between *cis* and *trans* cleavage, and the difference between formative versus morphogenic cleavage.

Regardless of the type of proteolytic maturation employed, it is essential that the activity of the viral proteinase be properly regulated to ensure the efficient production of infectious progeny virions. In general, within biological systems, regulation of proteinases is achieved in several ways including differential compartmentalisation of the enzymes and substrate, presence of specific inhibitors and/or activators, and the proteolytic activation of zymogens. Viruses have adopted similar strategies. For example in the retroviruses, it has been proposed that the acidic extracellular environment triggers the morphogenic cleavage of structural proteins by displacing a portion of the *gag-pol* polyprotein which prevents the active site of the proteinase

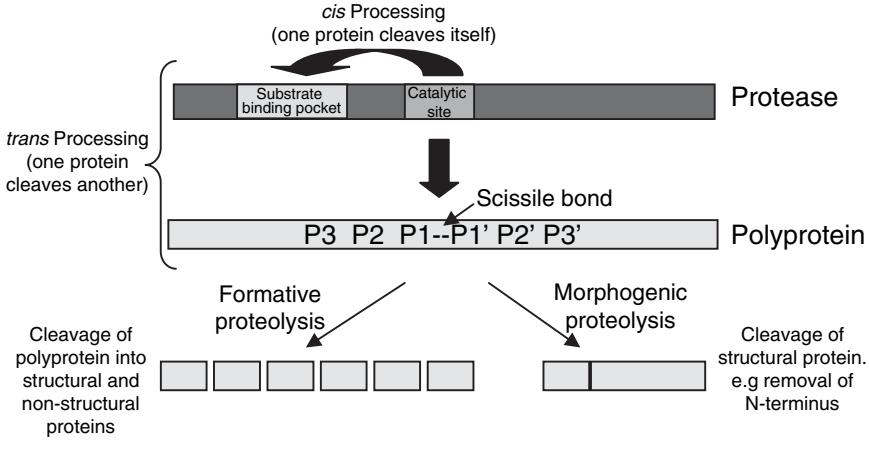

Figure 1. Depiction of the general requirements for proteolysis

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from interacting with its substrate while within the cell [11]. In the case of adenoviruses, it appears that a disulphide-linked peptide produced from the pVI structural protein during the latter stages of replication is required for the activation of the viral proteinase and subsequent virus maturation [21]. Finally, perhaps the most elegant example of regulating viral proteinase activity is provided by the core protein of Sindbis virus which undergoes autoproteolysis to become inactive after assembly of the nucleocapsid. Inactivation of the proteinase activity is accomplished by locating the carboxy terminal region of the protein into the catalytic pocket in concert with the proteolytic cleavage event [23].

VV REPLICATION CYCLE AND POSTTRANSLATIONAL MODIFICATION OF VIRAL GENE PRODUCTS

Given the importance of limited proteolysis as a means to regulate gene expression in biological systems, and the extent and diversity of ways that even simple viral systems apparently employ this regulatory mechanism, it is of interest to consider if and how a complex virus such as vaccinia virus might incorporate this process into its replicative cycle. Vaccinia virus (VV) is the prototype of the *Poxviridae*, a family of DNA viruses distinguished by their unique morphology and cytoplasmic site of replication [24]. The 191 kbp VV DNA genome encodes at least 263 gene products whose expression is regulated in a temporal fashion during the viral replicative cycle that begins with entry of the virus into the host cell and terminates with the assembly of complex macromolecular structures to form an infectious particle (Figure 2). Unlike many other viruses, VV produces a multiplicity of virion forms, all of which appear to be infectious. Although the molecular details of poxvirus assembly and differentiation remain controversial, the most widely accepted scenario of events which transpire is as follows. After (or concurrent with) viral DNA replication, assemblages of progeny DNA molecules, virion enzymes and structural proteins coalesce to form pre-virion particles [25]. These particles then acquire membranes, whether one or two membranes still remains controversial, to become infectious intracellular mature virus (IMV). There are two hypotheses about the origin of the membranes; one holds that the membrane is synthesised *de novo*, while the other hypothesis holds that the membrane is acquired by budding though the intermediate compartment (between the endoplasmic reticulum and the Golgi). A portion of the IMV then become enveloped by additional membranes derived from the trans-Golgi network to form intracellular enveloped virus (IEV). Following migration to the cell surface the outermost IEV membrane fuses with the plasma membrane to give rise to extracellular enveloped virus (EV) [26]. The EV can either remain associated with the cell (cell-associated enveloped virus, CEV) or be released into the external medium as extracellular enveloped virus (EEV) [27]. Some poxviruses, such as cowpox virus (CPV), produce yet another virion form. In CPV-infected cells, large inclusion bodies are produced which are composed primarily of a single 160 kDa viral protein [28]. Within these A-type inclusions are occluded (and infectious) virions. All of the proteolytic reactions that have been characterised for VV to date are involved in IMV formation

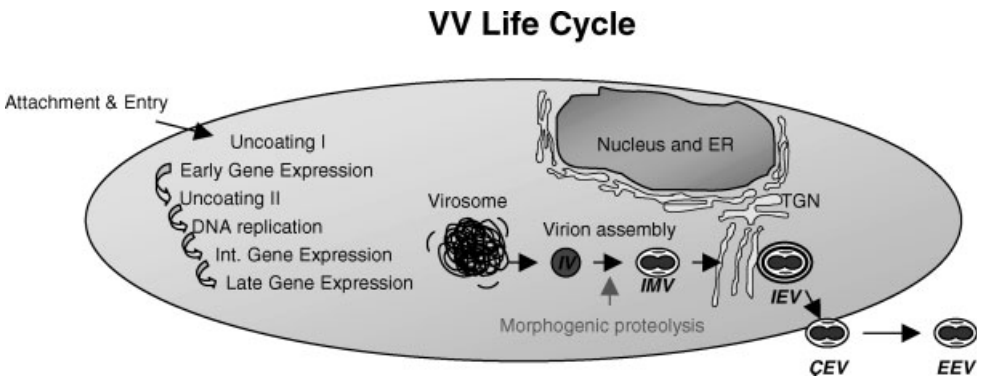

Figure 2. Vaccinia virus replicative cycle

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and not the subsequent maturation of IMV to IEV, EV or CEV.

It is well known that viruses, as obligate intracellular parasites, must use (and in some cases redirect) their hosts' metabolic pathways in order to replicate their genomes and synthesise the constituents needed to form progeny virions. What is perhaps less well appreciated are the logistical problems encountered by viruses during the replicative process. Once viral proteins have been synthesised, how does the virus ensure that these proteins assume active configurations (at the proper time), interact with the correct protein partners and find their way to specific intracellular locations in quantities sufficient to catalyse the efficient assembly of infectious virions? One of the major ways that viruses solve these problems is by taking advantage of protein modification pathways which are normally used to assist with intracellular trafficking of cellular proteins [29–33].

Thus, considering the large number of viral encoded proteins, the multiplicity of VV virion forms and the number of distinct intracellular sites used during the viral assembly and morphogenesis process, one would predict that VV might utilise a number of cellular protein modification and targeting pathways to regulate these complex processes. Indeed, it has been demonstrated that during the course of viral replication, VV proteins are matured by a number of post-translational modifications including myristylation [29], acylation [30], phosphorylation [31], glycosylation [32], ADP-ribosylation [34], and proteolytic processing [33]. Although the details about what role limited proteolytic reactions might play during VV replication were not available when our studies were initiated in 1990, the information that was available in the literature suggested that both formative and morphogenic cleavage pathways might be employed. For example, both the VV growth factor (VGF) [35, 36] and haemagglutinin (HA) proteins [37] appeared to have signal peptides removed via formative proteolysis during their transit through the endoplasmic reticulum and transport to the plasma membrane. Likewise, three of the major structural proteins found in the mature VV virion core, 4a, 4b and 25K, were known to be produced from higher molecular weight precursors at late times during infection, making them candidates for morphogenic cleavages [24, 38]. It was this latter question, namely the nature of the processing reaction by which the major VV core proteins are matured, that the experiments conducted in our laboratory over the last decade have sought to address.

VV PROTEOLYSIS–WHAT WAS KNOWN PRIOR TO 1990

The genes expressed at late times during a VV infection (i.e. those expressed after the initiation of viral DNA synthesis) include most of the structural proteins required for the assembly of progeny virions. The first indication that some of the VV structural proteins might be subject to proteolytic processing occurred when Holowczak and Joklik noted differences in the apparent molecular weights of radioactively-labelled proteins present in VV-infected cells when compared to those found in purified virions [39]. Subsequently, pulse-labelling of VV-infected cells was used to demonstrate that a large precursor protein could be chased into a smaller polypeptide, with concomitant disappearance of the larger sized protein [40]. This conversion could be specifically inhibited by rifampicin with no apparent effect on the synthesis of the precursor. The precursor protein was designated as P4a and the proteolytically processed product called 4a [41]. Additional pulse-chase experiments revealed that several other VV structural polypeptides, in addition to P4a, were apparently cleaved during the late phases of the VV replication cycle [42]. These proteolytically processed proteins, referred to using the Sarov and Joklik designation of virion proteins [43], included 4a, 4b, 8 (referred to as 25K here), 9 and 10. This may in fact represent an underestimate of the number of VV late proteins which are subject to proteolysis, as Pennington [44] reported eleven late proteins disappeared during pulse-chase experiments and seven new proteins appeared. The VV core proteins 4a, 4b and VP8 (25K) are the most abundant proteins in the VV particle, together constituting about 33% of the mass of the virion. Tryptic peptide mapping and immunological reagents have been used to establish the relationships between the P4a, P4b and P25K precursors and their processed products 4a, 4b and 25K [38,45]. Location of the three loci encoding these genes has been mapped and the nucleotide sequence of their open reading frames determined [46, 47]. With the completion of the sequence of the entire genome of the Copenhagen strain of VV, the genes encoding the P4a, P4b and P25K precursors

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received the designations A10L, A3L and L4R, respectively [48]. The proteolytic processing of VV structural proteins appears to be essential for the formation of infectious progeny virions. This conclusion stems from the observation that there are a variety of different drug treatments (e.g. rifampicin and α-amanitin) or conditional-lethal mutations in the genome, which apparently affect proteolysis (and particle maturation) without affecting overall protein synthesis [33].

IDENTIFICATION OF THE AG(X) CLEAVAGE MOTIF

By pulse-labelling VV-infected cells after the onset of viral DNA synthesis and analysing the extracts by SDS:polyacrylamide gel electrophoresis and autoradiography, one observes three major radiolabelled protein species with apparent molecular weights of 97 kDa (P4a), 65 kDa (P4b) and 28 kDa (P25K). Upon addition of media containing an excess of unlabelled amino acids and with continued incubation of the infected cells, these three proteins are chased into 62 kDa (4a), 60 kDa (4b) and 25 kDa (25K) species that co-migrate with the three major virion core proteins. Interestingly, unlike many proteolytic reactions, the conversion process is not rapid with a delay of about 30–45 min observed between the time of precursor synthesis and the appearance of cleavage product. Furthermore, proteolysis of the VV core protein precursors appears to require ongoing *de novo* protein synthesis as addition of cycloheximide at the time of the chase completely inhibits product formation. Both of these observations are in agreement with the hypothesis that cleavage occurs within the context of an assembling virion, a process which requires both time and continual protein synthesis, and can be referred to as 'contextual processing'.

To determine the sites at which the VV core protein precursors were being cleaved, mature 4a, 4b and 25K proteins were isolated, purified and subjected to N-terminal microsequence analysis. The derived sequence was then compared against the predicted amino acid sequences of the P4a, P4b, and P25K ORF's. The amino termini of the 25K and 4b proteins were identified as residue 33 of P25K and residue 62 of P4b, respectively [49]. The predicted decrease in molecular weight due to the loss of 32 and 61 amino acids from the N-terminus of P25K or P4b, respectively, corresponded well with the shift in migration observed in gels following proteolytic processing. Comparison of the predicted amino acid sequences within the P4b and P25K precursors which surround the derived N-termini of the mature 4b and 25K proteins revealed the presence of an identical motif. The N-termini of both cleavage products were found within a conserved Ala-Gly-Ala (AGA) tripeptide, with the predicted cleavage site occurring at the Gly-Ala scissile bond. The importance of the AGA motif as a cleavage site determinant was suggested by two lines of evidence. First, there were no other obvious sequence elements conserved upstream or downstream of the putative P4b and P25K cleavage sites. Second, although the fowlpox virus (FPV) 4b and 25K core protein homologues only share 52 and 33% identity, respectively with the VV proteins [50], both FPV precursors contained an AGA motif at exactly the same location as the VV core protein precursors. Interestingly, although the 4a protein was subjected to similar analyses, no microsequence data was obtained, suggesting that its N-terminus was blocked.

In view of the failure to obtain amino acid sequence from purified 4a protein, it was of interest to note that the predicted amino acid sequence of the P4a precursor did not contain the conserved AGA tripeptide motif utilised in the processing of P4b and P25K. This raised the possibilities that the P4a protein was processed by a different pathway, or that P4a was processed by the same pathway but at sites that were less efficiently cleaved. Support for the latter hypothesis was drawn from the observation that cleavage of the P4a precursor seems to proceed at a slower rate than that of the P4b and P25K precursors *in vivo* [51]. To determine the cleavage site of P4a, immunological reagents which were specific for subregions of the P4a precursor were generated and used in concert with a variety of peptide mapping and protein sequencing procedures. The results obtained demonstrated that the P4a precursor was cleaved at two locations, the AGS site between residues 613 to 615 and the AGT site between residues 697 and 698 [51]. Both the large N-terminal 4a protein (residues 1 to 614) and the C-terminal 23 kDa protein (residues 698 to 891) become major virion core constituents. The location and fate of the small internal peptide predicted to be released (residues 615–697) is not known. However, using transient expression procedures, mutant P4a proteins were

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produced in which either the AGS or AGT sites had been genetically inactivated. Under these conditions it was possible to demonstrate the existence of the 4a-9K or 9K-23K proteins *in vitro*, suggesting that the internal 9 kDa sequence is not inherently unstable. Furthermore, the ability to isolate and microsequence both end products from cleavage at the AGS and AGT sites also strongly suggested that processing was occurring via a single endoproteolytic cleavage event. Although the internal AGS and AGT sites of the P4a precursor were cleaved, two independent lines of peptide mapping data clearly demonstrated that the AGN site at residues 94 to 96 was not processed. Thus, these results suggest that processing of all three major core protein precursors is likely to be coordinately lined and catalysed by the same viral proteinase during viral assembly, with the endoproteolytic cleavage occurring at an internal AGX motif (where X can be A, S, or T, but not N).

CONTEXTUAL PROCESSING

All three of the VV core proteins that were known to be proteolytically processed are cut at AGX motifs. It was therefore of interest to ask within the context of the entire VV genome, how many times an AGX tripeptide is predicted to be present in a VV protein, and to determine the frequency at which the motif is utilised as a cleavage site. Using the complete nucleotide sequence of the VV (Copenhagen) genome [48], the predicted amino acid sequence of each ORF was determined and compiled into a single database. A search of this database for the AGX tripeptide revealed it occurred 82 times among the 198 major ORF's, which is substantially less frequent than the 204 sites expected if AGX occurred randomly. Of these 82 sites, 18 resembled sites that had previously been shown to be actively cleaved, namely AGA of P25K and P4b, and AGT and AGS of P4a.

To explore whether some or all of these AGX motifs serve as cleavage sites during VV replication, we focused our attention on the AGA tripeptide as a test case. The AGA motif occurs seven times. The proteins containing the AGA motif are: P4b and P25K core protein precursors (both of which are cleaved), the A12L and A17L gene products, the palmitylated P37 protein encoded by the F13L ORF which is found in the outer membrane surrounding EEV particles, the VV DNA polymerase (DNAP) encoded by the E9L ORF, and host range (HR) protein encoded by the K1L gene. To determine if these proteins were processed during the course of a VV infection, monospecific antisera were produced or obtained (αDNAP from P. Traktman and αP37K from R. Wittek) for each individual gene product and used in concert with pulse-chase radiolabelling and immunoprecipitation procedures. The results obtained clearly indicated that the DNAP, P37K and HR proteins were *not* proteolytically processed. In contrast, the gene products of the A17L and A12L ORF's were synthesised as 23 kDa and 24 kDa precursors which were processed to 21 kDa and 17 kDa products. The processing reaction was inhibited by rifampicin and the processed products were matured by the same pathway as the P4b and P25K precursors [52]. These results allowed us to propose several rules governing the morphogenic cleavages in VV.

To be processed, a precursor protein must: (1) Contain an AGX motif, (2) Be expressed at late times during infection (DNAP is an early protein, HR is an early-intermediate protein); (3) Be destined for incorporation into the virion core (P37 is a late protein but is located in the membrane not the core). Even with these stringencies, given the number of AGX occurrences plus the large number of proteins found in the complex VV virion, this suggests that as many as 40 to 50 viral proteins may be subject to morphogenic cleavages during virion assembly. Mercer and Traktman [53] have recently shown that the vaccinia virus G7 protein, which plays a role in virion assembly, undergoes cleavage at two AGX sites, AGF (aa182–184) and AGL (aa 237–239), with the processing being important for virus viability. Figure 3 highlights the AGX cleavage sites in several vaccinia virus proteins that are known to undergo cleavage.

VV CORE PROTEIN PROCESSING IS A MORPHOGENIC CLEAVAGE PATHWAY

As an approach to test whether VV core protein proteolysis serves a formative or morphogenic function in the virus life cycle, a method of sucrose log gradient fractionation was developed which allowed the separation and purification of radiollabelled immature and mature VV particles from four distinct peaks based on varying rates of sedimentation [54]. Slower sedimenting fractions were

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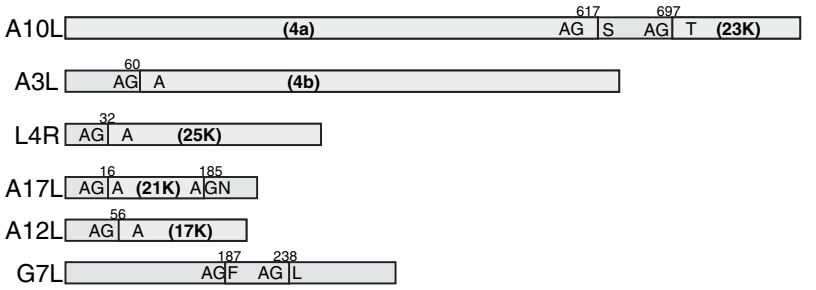

Figure 3. AGX cleavage sites in vaccinia virus proteins

found to move faster through the gradient over time using pulse-chase procedures, indicating precursor-product relationships due to proteolytic maturation. The slowest sedimenting product was found to contain predominantly uncleaved core protein precursors while the fastest moving products were composed almost entirely of the mature core proteins 4a, 4b, 25K and 23K. These results showed that several distinct and separable forms of VV previrions exist, that VV core protein precursor s are associated with previrions prior to cleavage, and that maturation of the core proteins is coordinately linked to the conversion from non-infectious previrions to infectious viral particles. This provides strong support for the hypothesis that VV core protein precursor maturation serves a morphogenic function [55].

In another set of experiments designed to monitor the intracellular and intraviral localisation of the VV core proteins during infection, a collection of monospecific polyclonal antisera were produced that recognised individual core protein precursors and/or their cleaved products. Use of these sera in indirect immunofluorescence analyses of VV-infected cells demonstrated that the VV core protein precursors were not distributed throughout the cytoplasm of infected cells. Rather, the core protein precursors were localised almost exclusively to the 'virosomes or virus factories' where the progeny virions were being assembled. At late times during infection, punctate staining was also evident throughout the cytoplasm which we believed to be individual virus particles. This hypothesis was confirmed by immunoelectron microscopy. The IEM studies also demonstrated that VV core protein precursors were associated with immature VV virions and that as the virions progressed through the maturation cycle the newly-processed core proteins remained associated with the condensing core. [55]

The results of the experiment reported above indicate that the precursor form of the VV core proteins is required for proper localisation to the virosome and assembling previrion. This raises the question of what feature of the core protein precursors is responsible for this behaviour, as not all proteins present in a VV-infected cell are packaged. One can propose that it is the overall structure of the core proteins, the protein partners with whom they interact, or the presence of a specific targeting signal that provides this property. At least in the case of P4b and P25K, one obvious candidate for a potential targeting signal is the N-terminal leader peptide that is removed by proteolysis during viral assembly. To test this hypothesis, a mutant P25K gene was constructed in which the sequence encoding the leader (the 31 N-terminal amino acids) was deleted. The (Δ 31)P25K protein was expressed in the context of a VV-infected cell via transient expression. This protein, which should be functionally equivalent to mature 25K core protein, was not packaged into virions. To determine if the entire leader of the core protein precursor was required to provide this phenotype, a set of deletions in the 61 amino acid leader of the P4b protein were constructed. Deletion of 15, 30, or 44 amino acids had no effect on P4b processing, indicating that the essential information was proximal to the cleavage site. Proof that it was the sequence or structure of the leader which imparted this property was provided by fusing the N-terminal 30 amino acids of the viral thymidine kinase (TK), a soluble early protein, to the 25K protein in a manner which reconstituted the AGA site. This TK:25K fusion protein was neither packaged nor processed. Interestingly, the leaders of the core proteins appeared to be functionally interchangeable as two swap mutants were generated by making P4b leader:25K and P25K leader:4b chimeras. In both cases, the proteins could

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be packaged and processed. This result argues against the overall tertiary structure of the core protein precursors being the primary localisation determinant as the fusion proteins would surely have disrupted structural features. Taken together, these data suggest that the amino terminal peptides of the VV core proteins are to some extent interchangeable and that the residues immediately proximal to the AGA site appear to contribute most directly to the correct intracellular and intraviral localisation. [56]

CHARACTERISATION OF THE CIS SIGNALS RESPONSIBLE FOR P25K CLEAVAGE

Based on precedents in the viral proteinase literature, a number of different approaches to developing an assay system to study the proteolytic processing of VV core proteins were attempted. These included: (i) *in vitro* cleavage assays mixing VV core protein precursors isolated from cells infected with cleavage-deficient temperature-sensitive VV mutants together with extracts from wild-type VV-infected or uninfected cells; (ii) mixing solubilised VV virions with VV core protein precursors made *in vivo* or *in vitro*; (iii) co-translation of core protein precursor mRNA with mixtures of cellular and/or viral mRNA in rabbit reticulocyte lysates, and (iv) transient expression assays using the hybrid T7/VV system [57] to express various reporter gene constructs containing putative VV core protein cleavage sites. Without exception, no cleavage of the test substrate was observed with any of these systems. This led, in part, to the working hypothesis that proteolytic maturation of VV core proteins is contextual, linked directly to virion assembly. The predictions of this hypothesis are that for a VV protein to be cleaved at the AGX motif it must be synthesised late in infection, packaged into the assembling virion and it needs to be associated with the VV core. Any perturbation of the kinetics of synthesis, intracellular targeting or structure of a VV core protein precursor might be expected to abrogate processing.

To test this hypothesis a trans-processing assay was developed [16] to follow VV core protein proteolytic maturation by tagging the P25K VV core protein precursor at the C-terminus with an octapeptide epitope, FLAG [58] (Figure 4). By using transient expression assays in cells co-infected with VV, the proteolysis of the P25K:FLAG fusion gene product could be monitored by immunoblotting and immunoprecipitation procedures using antisera specific for the FLAG epitope or the 25K protein. In tissue culture, the P25K:FLAG precursor was cleaved to smaller products and the precursor-product relationships were established by pulse-chase labelling protocols. The cleavage of the P25K:FLAG precursor was believed to be using the same pathway as authentic VV core protein precursors due to the inhibition of processing by rifampicin. The 25K:FLAG product was found to be associated with mature virions, agreeing with the hypothesis that cleavage occurs in conjunction with virion assembly. Site-directed mutagenic replacement of the AGA site within the

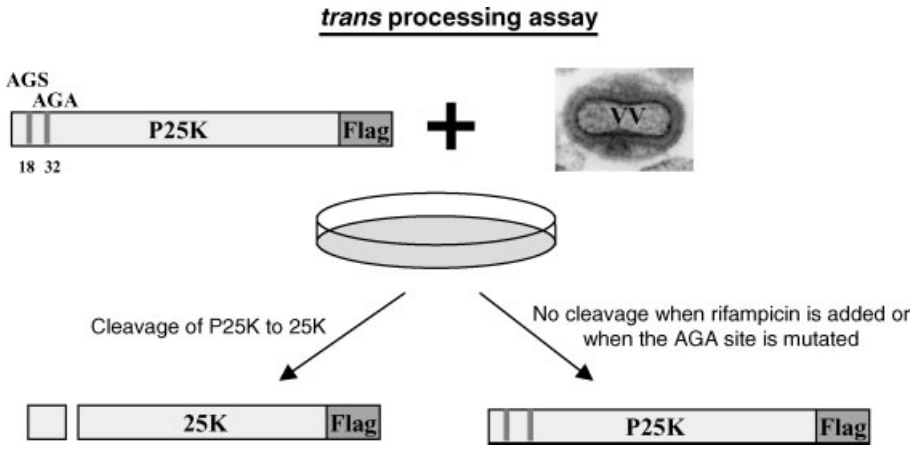

Figure 4. ***trans*** **processing assay. Cells are infected with vaccinia virus and transfected with plasmid borne P25K with a C-terminal Flag tag. Cleavage is monitored by immunoblotting**

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P25K:FLAG precursor with the tripeptide IDI blocked cleavage at the mutated site, indicating that the AGA site was used and recognised independently, and that bonafide proteolysis was occurring. [16]

Although the central importance of the AGX motif in P25K processing is suggested by the failure of the IDI mutant to cleave, there must be additional determinants of cleavage site selection. Not all proteins containing an AGX motif are proteolytically cleaved. One such example is the AGN site of P4a which is not cut, despite the fact that two other downstream AGX sites in the same precursor are. Likewise, as discussed above, the VV DNA polymerase, palmitylated 37K envelope protein and the HR proteins all have AGA motifs which are not cleaved. Therefore, the AGX motif appears to be essential but not sufficient for defining a specific cleavage site. Additional substrate determinants within the precursors must contribute to cleavage site selection. As an approach to identify some of these other determinants, we employed the trans-processing assay described above in concert with site-directed mutagenesis procedures. Specifically, using the P25K:FLAG gene as the template, more than 50 different substitution, insertion and deletion mutants were introduced in and around the AGA site. The phenotype of each individual mutant was analysed by transient expression and immunoblotting procedures [59].

The results are summarised as follows, using the nomenclature of Schechter and Berger [60] in which the positions at the amino- and carboxyl-proximal residues are indicated as P1, P2, etc., and P1′, P2′, etc., respectively. The residue occupancy of the P1′ position was exceedingly permissive with only a proline substitution blocking cleavage. In contrast, for cleavage to occur, the occupancy of the P1 and P2 sites was restricted to just a few amino acids. The presence of additional structural elements is suggested by the observation that insertion or deletion of sequences immediately adjacent (amino- or carboxyl terminal) to the AGA motif completely halted cleavage. [59]. While there is abundant information about the structural requirements surrounding core protein cleavage [49, 51, 55, 56, 59, 52, 61, 42] until recently the proteinase(s) responsible for carrying out these cleavage reactions has remained unknown.

G1L

As mentioned above, a large number of different approaches were attempted to reconstitute VV core protein proteolysis *in vitro*, and all were unsuccessful due to the contextual requirements of this reaction. Therefore, in order to identify the proteinase which is responsible for the cleavage of the VV core protein precursors a tissue culture based mapping procedure was employed, making use of a transcriptionally-controlled *trans*-processing assay. Transcription of VV genes is tightly controlled by a regulatory cascade mechanism [62]. All of the enzymes required for the synthesis and modification of early mRNA are packaged into the infectious virion. Following entry into the cell, early gene expression initiates viral DNA replication which leads to successive expression of intermediate and late viral genes. During a normal viral infection, it has been shown that newly replicated, naked, viral DNA serves as the template for the expression of the late transcription factor genes (A1L, A2L, and G8R) as well as the late genes themselves [63]. Recent studies have demonstrated that transcription from an exogenously supplied late promoter in infected cells, whose DNA replication has been blocked by AraC, can be rescued by co-transfecting plasmid copies of the late transcription factor genes [64]. This finding provided the basis for the development of the transcriptionally-controlled *trans*-processing assay using a plasmid copy of the P25K:FLAG ORF as the reporter gene [61].

Cells infected with VV in the presence of AraC to block late gene expression from the viral genome were transfected with full-length VV DNA, resulting in the expression and cleavage of VV core protein precursors. This indicated that both synthesis and processing could occur under these conditions, and that the proteinase responsible for cleavage was likely the product of a VV late gene. In order to separate substrate expression and proteolysis, VV-infected cells treated with AraC were simultaneously transfected with plasmid copies of the following: (i) late gene transcription factors; (ii) a test substrate; and (iii) a potential source of proteinase. As the test substrate, the P25K:FLAG fusion gene was used. The initial source of proteinase was a set of 6 overlapping cosmid clones of the VV genome supplied by B. Moss [65]. Individually, cosmids 3 and 21 were able to rescue processing of the P25K:FLAG protein at the AGS site

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found at positions 16–18 within the N-terminal leader of the precursor. As these two cosmids overlapped in the HindIII-G region, a cloned copy of the VV HindIII G fragment was tested and also found to direct cleavage of the substrate at the AGS site [61].

Previously, we had used computer analyses to search the predicted amino acid sequences of all of the ORF's in the genomic sequence of VV (Copenhagen) for the presence of motifs that might suggest proteinase activity. The only 'hit' we had was within the G1L ORF, which contained a HXXEH sequence motif, which is a direct inversion of the active site consensus sequence present in metalloproteinases such as thermolysin [66]. G1L is predicted to encode a 68 kDa late protein that is highly conserved amongst the Orthopoxviridae. Although there are examples of viral proteins that require zinc for activity, such as the rubella virus protease [67] and the hepatitis C virus NS5A replicase [68], to date there are no known true viral metalloproteases. Despite this, as well as the observation that the motif was backwards in VV, we nevertheless cloned the G1L ORF, expressed it *in vitro* and tested if for the ability to cleave VV core protein precursors. The plasmid borne G1L was capable of cleaving P25K:FLAG at the AGS, but not AGA site and was unable to cleave transiently expressed P4a or P4b. Mutation of either the conserved HXXEX motif of the G1L ORF or of the AGS site in P25K abrogated cleavage [61]. This implicated G1L as a potential VV metalloproteinase, but not the proteinase responsible for cleavage of each of the major core protein precursors.

While the exact biological function of VV G1L remains unknown, two different recombinant viruses regulating the expression of G1L have shown that G1L is essential for viral morphogenesis [69, 70]. However, in the absence of inducer, no obvious effects on the processing of the major core protein precursors is observed. An electron microscopic examination of the virus in the absence of G1L expression revealed that immature viral particles are formed and there is an initiation of core condensation but the viral particles lack the characteristic oval shape and complete core condensation characteristic of mature viral particles. These results would suggest that G1L activity is likely required at a stage after core protein processing and that more than one VV proteinase is required to produce mature virus.

VACCINIA VIRUS CORE PROTEIN PROTEINASE (vCPP)

As an approach to determining what type of proteinase might be the vaccinia virus core protein proteinase (vCPP), a collection of class-specific proteinase inhibitors were tested to determine their ability to inhibit VV replication in tissue culture cells. To that end BSC40 tissue culture cells were infected with VV in the presence of various concentrations of proteinase inhibitors. Efforts were made to use concentrations of drugs which had minimal effects on the tissue culture cells as judged by morphological appearance and thymidine incorporation. Inhibitors tested included; 1,10-phenanthroline, a metalloproteinase inhibitor (and its non-chelating isomer, 1,7-phenathroline); iodoacetamide, a cysteine proteinase inhibitor; and pepstatin A, an aspartic proteinase inhibitor. Unfortunately, any and all serine proteinase inhibitors tested were acutely toxic to the host cell, perhaps not a surprising result given the ubiquity of this type of proteinase in mammalian cells. Interestingly, VV replication was completely blocked by 10 μM iodoacetamide or 1 μM 1,10-phenanthroline whereas 1,7-phenanthroline or pepstatin A had no effect. These results are consistent with a metalloproteinase and a cysteine proteinase both playing an essential role in the viral replicative cycle.

I7L

Although there are several lines of evidence that suggest that G1L protein may be a metalloproteinase, another candidate proteinase has been identified, the gene product of the I7L open reading frame. This protein was originally identified as a potential protease on the basis of homology to a ubiquitin-like proteinase in yeast [71]. I7L is believed to belong to the SUMO-1- specific family of proteases, which includes the proteases encoded by adenovirus and African swine fever virus (ASFV). Interestingly these last two proteases cleave polyproteins at Gly-Gly-Xaa sites, similar yet distinct from the Ala-Gly-Xaa sites cleaved by I7L. Like G1L, I7L is highly conserved amongst the Orthopoxviruses, with 95–99% identity of the gene between the viruses in this family. It is predicted to be a cysteine proteinase and two potential active sites are evident. Condit and coworkers have isolated a temperature sensitive mutant in the I7L gene [72]. At the non-permissive

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temperature, the core protein precursors P4a, P4b and P25K are synthesized but are not processed. Moreover, viral assembly is halted between immature viral particle formation and conversion to an infectious IMV particle [73]. At the non-permissive temperature no infectious progeny are produced [74].

I7L is predicted to encode a 47 kDa protein that is expressed at late times postinfection. Use of monospecific anti-I7L antisera has demonstrated that the protein is associated with virus factories, immature viral particles and IMV, where it is exclusively located in the core [73].

In order to determine which enzyme is the vaccinia virus core protein proteinase (vCPP), a transient expression assay was used to demonstrate that the I7L gene product and its encoded cysteine proteinase activity is responsible for cleavage of P25K, the product of the L4R open reading frame. Cleavage was demonstrated to occur at the authentic Ala-Gly-Xaa cleavage site and require active enzyme, as mutation of either the cleavage site or the active site residues in I7L abolished this activity. [75]

Having established that I7L was the vCPP, there were several questions that remained to be answered. It was not known whether the entire I7L protein was required for recognition and cleavage of the core precursor proteins, or if just the predicted catalytic domain was required? Was I7L capable of cleaving each of the core protein precursors, and did cleavage occur preferentially at Ala-Gly-Ala versus Ala-Gly-Ser and Ala-Gly-Thr sites? Was there a catalytic triad and were other conserved residues essential for activity? The results obtained from *trans* processing assays showed that intact I7L is necessary and sufficient to direct cleavage of each of the three major core protein precursors and that mutagenesis of either the putative catalytic triad of I7L or of the Ala-Gly-Xaa sites in the precursor proteins abolishes this activity. A series of truncated I7L proteins lost the ability to cleave the core protein precursors. [76]

In order to further characterise I7L, a conditional-lethal recombinant virus was constructed in which the expression of the vaccinia virus I7L gene is under the control of the tetracycline operator/repressor system. In the absence of I7L expression, processing of the major VV core proteins is inhibited and electron microscopy revealed defects in virion morphogenesis subsequent to the formation of immature virion particles but prior to core condensation. Plasmid-borne I7L was capable of rescuing the growth of this virus and rescue was optimal when the I7L gene is expressed using the authentic I7L promoter. [77]. In addition to cleaving the major core protein precursors, I7L has recently been shown to be capable of cleaving membrane proteins as well. Ansarah-Sobrinho and Moss [78] constructed a recombinant virus with I7L regulated by the *E. coli lac* repressor to demonstrate that in the absence of an inducer the A17 membrane protein is not cleaved at the canonical AGX cleavage site and viral morphogenesis is blocked. Taken together, these data suggest that correct temporal expression of the VV I7L cysteine proteinase is required for core protein maturation, virion assembly and production of infectious progeny. [77,78]

Following the identification and characterisation of I7L as an essential proteinase involved in the maturation of VV, it was then of interest to determine whether small molecule inhibitors could be developed to inhibit I7L and halt viral replication, which could then be used to inhibit each virus in this family including the causative agent of smallpox. Using a homology-based bioinformatics approach, a structural model of the vaccinia virus (VV) I7L proteinase was developed. A unique chemical library of ~450 000 compounds was computationally queried to identify potential active site inhibitors. The resulting biased subset of compounds was assayed for both toxicity and the ability to inhibit the growth of VV in tissue culture cells. A family of chemotypically-related compounds were found which exhibited selective activity against orthopoxviruses, inhibiting VV with IC50 values of 3–12 μM. These compounds exhibited no significant cytotoxicity in the four cell lines tested, and did not inhibit the growth of other organisms such as *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa*, adenovirus, or encephalomyocarditis virus (EMC), indicating the selective nature of the compounds. Phenotypic analyses of virus-infected cells were conducted in the presence of active compounds to verify that the correct biochemical step (I7L mediated core protein processing) was being inhibited. An electron microscopic examination of compound-treated VV infected cells indicated a block in morphogenesis with the same phenotype as the temperature

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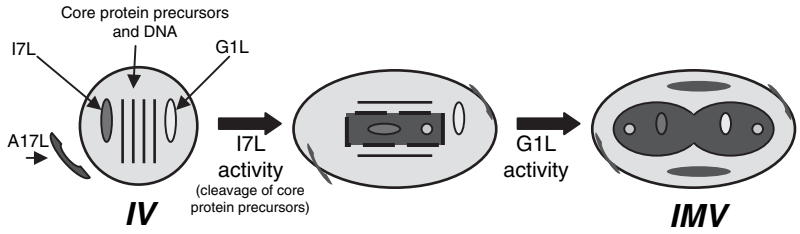

Figure 5. Model of the role of proteolysis in vaccinia virus morphogenesis. During the transition from an IV to an IMV particle there are a series of proteolytic cleavage events including the cleavage of the major core protein precursors by I7L and followed by the activity of G1L to lead to infectious virus particles.

sensitive I7L virus and the I7L conditional lethal virus in the absence of inducer, that is, the formation of immature viral particles but a lack of complete core condensation and no mature virus particles. To further demonstrate that the compound was inhibiting I7L and not another stage in the virus life cycle, compound resistant viruses were generated and resistance was mapped to the I7L ORF. Recombinant VV were then generated in which the wild type I7L ORF was replaced with the mutant I7L from the resistant virus. These recombinant viruses demonstrated resistance to the compound indicating that this was the only gene necessary for resistance and validated I7L as the drug target. This novel class of inhibitors demonstrated the potential for the development of VV proteinase inhibitors an efficient antiviral drug target. [79]

Finally, an *in-vitro* cleavage assay was developed to further characterize the activity of I7L in a cell-free system and show that the proteinase activity observed until now was the result of I7L specifically and not another viral or cellular protein. Using this assay, which is based on producing the major core protein precursors as substrates in a coupled transcription and translation assay and then mixing them with I7L enzyme extracts, I7L was shown to be capable of cleaving each substrate. A time course of activity at various temperatures was performed to determine the optimal temperature for *in-vitro* activity. Antibody pull-down studies showed that I7L specific antiserum could competitively inhibit the cleavage reaction, additionally verifying I7L as the enzyme responsible for activity. I7L was further characterised as a cysteine proteinase due to the inhibitory effects of known cysteine proteinase inhibitors such as NEM and iodoacetic acid, as well as through the use of specific small molecule inhibitors in this *in-vitro* assay. [80]

Taken together, the data presented here, as well as analysis of the VV G1L conditional lethal mutant [69], suggests a morphogenesis model in which these two putative proteases operate sequentially to regulate viral assembly, with I7L functioning to cleave the major core protein precursors to allow them to assume the proper configuration for virion maturation. This activity is followed by the action of G1L to allow for complete core condensation and the progression to the formation of intracellular mature virus particles (Figure 5). If the activity of the I7L proteinase is blocked, viral morphogenesis arrests prior to core condensation. If the activity of G1L is blocked, viral morphogenesis arrests at a stage subsequent to this but still prior to complete core condensation.

There are still several questions that remain unanswered about the activity of I7L including whether it has another role in the viral life cycle aside from cleavage of the major core protein precursors, what activates the enzyme, and how it is regulated so that it acts at a specific time in the viral life cycle. The role and identity of other vaccinia specific proteases has yet to be determined. However, the information provided here has shown that I7L is the first characterised vaccinia virus protease and an attractive and viable target for antiviral drug development. It will be of interest in the future to determine the specific activity of the G1L enzyme to complete the picture of vaccinia virus proteolysis.

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