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Several vaccinia virus (VV) structural proteins are synthesized as large precursor proteins, subsequently processed into smaller products during the course of virion maturation. During viral replication, it is presumed that the precursor proteins are incorporated into previrion particles and that proteolytic processing is concomitant with and required for virion morphogenesis and maturation. Amino acid alignment of the structural precursor proteins has revealed a conserved Ala-Gly-X motif (AG*X) at the confirmed cleavage sites.

To better understand the regulation of cleavage site selection, VV proteins were surveyed to determine which proteins containing the conserved motif were actually processed. Only those proteins expressed at late times during infection and associated with the virion were candidate substrates for proteolytic processing. These proteins include P4b, P25K, P21K and P17K. Results suggest that temporal

expression and/or substrate presentation in the context of the assembling virion may play a role in regulation of proteolytic processing.

Three potential AG*X cleavage sites have been identified in the P4a precursor protein. Peptide mapping has shown that the AG*N site located proximal to the N-terminus is not proteolytically processed. Cleavage at the previously described AG*S and AG*T sites is predicted to yield both the 4a and 23K products in addition to a yet unidentified, intervening 9K protein. In order to test the stability of the 9K protein, independent mutation of either the AG*S or AG*T site of P4a, followed by transient expression in VV-infected cells, lead to the production of a unique 4a-9K or 9K-23K protein intermediate. The presence of this intermediate implies that neither end of the proposed 9K product is intrinsically destabilizing, although its degradation may be prevented when it remains associated with 4a or 23K. As with the other structural precursor proteins, the amino-terminal propeptide may serve as an intramolecular chaperone which is degraded shortly after its removal.

To map the maturational proteinase activity to a specific VV open reading frame, a transcriptionally-controlled, *trans*-processing assay was developed which allowed for the systematic rescue of P25K processing by co-expression of a variety of VV-derived DNA segments. This lead to the identification of a putative VV proteinase encoded by open reading frame G1L. The predicted amino acid sequence of the protein revealed a conserved HXXEH motif which is a direct inversion of the active site consensus found in thermolysin. Site-directed mutagenesis established the importance of conserved histidine and glutamate residues, implicating the G1L protein as the only, virus-encoded metalloproteinase.

PROTEOLYTIC MATURATION OF VACCINIA VIRUS STRUCTURAL PROTEINS: ENZYME AND SUBSTRATE ANALYSIS

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CHAPTER I
INTRODUCTION

PROTEASE CLASSIFICATION AND STRUCTURE

The enzymes that play a central role in the degradation of proteins by hydrolyzing peptide bonds have been known as proteases. Proteases that act directly on proteins are also referred to as proteinases, while those that act on short polypeptides are referred to as peptidases. Alternatively, proteases may be sub-divided into two classifications according to their site of action on the substrate: exo-proteases and endoproteases. Exoproteases are limited to hydrolysis of terminal peptide linkages and only initiate removal of amino acids from the amino or carboxyl termini of proteins. Endoproteases, also frequently referred to as simply "protein-ases", are capable of cleaving specific peptide linkages located in internal portions of the protein.

Proteinases are further categorized according to their distinct catalytic mechanism. The four types of proteinases are: serine, cysteine (thiol), aspartic (acid), and metallo. Serine proteases appear to be the most numerous group and are extremely widespread. They possess a catalytic triad of amino acids histidine, aspartate, and serine. The reactive serine residue attacks the carbonyl carbon atom of

the substrate, and acting as a nucleophile, forms an acyl-serine tetrahedral intermediate. The intermediate breaks down when a proton is donated by histidine to the departing amino group. The acyl enzyme is then hydrolyzed, the carboxylic acid product is released, and the active site of the proteinase is regenerated (Polgár and Halasz, 1982). The cysteine proteinases maintain a catalytic dyad composed of cysteine and histidine residues in close proximity. The reactive sulfhydryl group of the cysteine residue acts as a nucleophile to initiate attack on the on the carbonyl carbon of the peptide bond to be cleaved. An acyl enzyme is formed, hydrolyzed and subsequent reactions continue in a fashion analogous to serine proteinases (Polgár and Halasz, 1982). The mechanism of action for the aspartic proteinases is less well known. Although enzyme-substrate intermediates do not appear to be formed, it is proposed that water attacks the carbonyl of the scissile peptide bond, with the active site carboxylates mediating the appropriate proton transfers (Fruton, 1976). For the metalloproteinases, a divalent cation, most often Zn^{2+} is coordinated along with water in the active site of the enzyme. Histidine and glutamate residues have been implicated as essential amino acids. Although the mechanism of action differs slightly among different enzymes, it is generally believed that the carboxylate of the conserved glutamate residue acts as a proton shuttle between the attacking water molecule and the nitrogen of the scissile bond, resulting in a tetrahedral intermediate which coordinates the zinc ion. Histidine is thought to stabilize this intermediate which collapses to products with glutamate again acting as proton shuttle between oxygen derived from the nucleophilic water and the new, free amino group (Monzingo and Matthews, 1984).

Proteinases can be thought of in their most basic form as having a catalytic site, as described for each group above, and a substrate binding pocket. Usually these two sites are 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 serine, cysteine, and aspartic proteinases, the two globular domains are found within the same polypeptide, whereas in the retroviral proteinase, a dimer complex would bring together two individual catalytic centers 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, 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.

PROTEOLYTIC PROCESSING IN VIRUS REPLICATION

For many plant and animal viruses, a successful infective cycle 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 (for a review, see Dougherty and Semler, 1993). 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 pathway 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 (Schlesinger and Schlesinger, 1990). On the other

hand, the proteinases which are responsible for the proteolytic processing of viral proteins in the cytoplasm of infected cells are usually encoded by the viruses themselves.

The term formative proteolysis has been proposed to describe processing of viral polyproteins into resulting structural and non-structural proteins. (Hellen and Wimmer, 1992). Retroviruses and positive-strand RNA viruses utilize the production of polyproteins as a mechanism for the expression of several viral proteins from a single RNA template. After proteolytic processing, the protein components become functionally activated and transported to the appropriate compartment of the cell or to the assembling viral particle. A number of viral formative cleavage proteinases have been identified and are encoded by viruses such as picornaviruses, flaviviruses, togaviruses, retroviruses, coronaviruses, potyviruses, comoviruses, nepoviruses, tymoviruses, luteoviruses, and sobemoviruses (for reviews, see Hellen and Wimmer, 1992; Dougherty and Semler, 1993).

Morphogenic proteolysis refers to the cleavage of viral structural proteins assembled in previrions during virion maturation. Appropriate processing is usually required for proper virion assembly, but may be dispensable for the assembly of capsid subunits. Morphogenic cleavage occurs in conjunction with virion maturation and is most often required for the acquisition of infectivity of both DNA and RNA viruses, such as picornaviruses, alphaviruses, retroviruses, adenoviruses, and bacteriophage T4 (for a review, see Kräusslich and Wimmer, 1988). Although less is known about morphogenic proteolysis, different functions have been suggested for the maturation cleavages in several different viruses. A cDNA construct of the retrovirus HIV genome expressing inactivated proteinase molecules failed to produce

infectious virion particles (Navia and McKeever, 1990), suggesting that the cleavage of the capsid protein precursor may be necessary for correct genomic RNA dimerization within premature particles, as proposed for avian retroviruses (Stewart *et al.*, 1990). Similarly, the maturational cleavage of the picornavirus VP0 precursor into VP2 and VP4 appears to be required for the proper aggregation of genomic RNA and capsid components, which is essential for the transfer of RNA into the cytosol during subsequent infection (Lee *et al.*, 1993). In bacteriophage T4, it has been proposed that proteolytic cleavage of structural proteins in the prohead is required during the packaging of DNA. (Hersko and Fry, 1975). However, it has been suggested that the cleavage of adenovirus structural proteins is required more for proper disassembly of the infecting virus, rather than the assembly of new virus particles (Freimuth and Anderson, 1993).

Because maturational processing and virus assembly appear to progress concomitantly, it is crucial that proteolysis be properly regulated to prevent premature cleavage of precursors proteins. Such premature cleavage could conceivably lead to protein misfolding and/or aggregation, such that the product is no longer fit for assembly. Careful regulation of the proteolytic process can theoretically be accomplished in a variety of ways: compartmentalization of enzymes and substrates, requirement for contextual presentation of the substrate, the presence of specific inhibitors or stimulatory co-factors, and proteolytic activation of zymogens. The relatively acidic extracellular environment encountered by retroviral particles upon release from the cellular cytoplasm has been implicated in the activation of the cleavage of structural proteins by displacing a portion of the *gag-pol* polyprotein which may normally prevent the active site of the proteinase from interacting with its

substrates (Partin *et al.*, 1991). In addition, premature activation of the HIV-1 proteinase has been shown to prevent both particle assembly and subsequent infectivity (Kräusslich, 1991). In adenoviruses, a short disulfide-linked peptide derived from the carboxy terminus of structural protein pVI has been shown to be required for the activation of the viral proteinase (Webster *et al.*, 1993). In contrast, the core protein of Sindbis virus, which undergoes a maturational autocatalysis, is inactivated after its assembly into the nucleocapsid. Inactivation of this "suicide" proteinase activity is due to irreversible positioning of its own carboxy terminus into the catalytic site (Choi *et al.*, 1991).

VACCINIA VIRUS STRUCTURE AND MORPHOGENESIS

Vaccinia virus is the most widely studied member of the *orthopoxviridae* and is thus considered the prototype of this viral family. Poxvirus virions appear to be oval or brick shaped structures of about 200 to 400 nm in length. They contain a dense, protein rich core, which contains the viral genome, and are surrounded by a lipoprotein bilayer which appears to be studded by surface tubule elements (Dales, 1963). Also enclosed by this membrane are the lateral bodies. The surface tubule elements and lateral bodies, which are generally observed under the electron microscope in negatively stained or conventionally embedded samples, have been shown to be preparation artifacts and may not naturally exist in the virion particle (Dubochet *et al.*, 1994).

Four forms of infectious virus are produced during the course of a normal VV infection: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV). Electron

microscopy has been extensively used to elucidate the multi-step process of virion morphogenesis, as outlined in Figure I.1. Within the virosome, assembly of virus particles is initiated by the formation of membrane crescents which subsequently evolve into spherical immature virions (Dales and Pogo, 1981). Core condensation of several internal components, including the viral genome soon follows. Some evidence suggests that all of the internal components must be packaged before completion of the viral membrane and subsequent virion maturation (Stern and Dales, 1976). The membrane structures of the IMV as observed in electron microscopy studies have previously been postulated to originate from *de novo* membrane biosynthesis (Stern and Dales, 1974). However, evidence from recent cryosections of VV-infected cells, viewed by electron microscopy, suggest that the viral membrane is derived from the intermediate compartment of the cell, possibly the tubular early endosomal cisternae, between the endoplasmic reticulum and the Golgi stacks (Sodeik *et al.*, 1993; Tooze *et al.*, 1993).

Wrapping of IMV particles by the Golgi membrane cisternae leads to the intracellular, triple-membrane form of virus, IEV. (Hiller and Weber, 1985). The outer membrane of the IEV particle is then fused with the cellular plasma membrane and EEV particles which now possess double membranes are released outside of the cell or remain associated with the cell membrane and are referred to as CEV. It has been proposed that CEV particles are responsible for dissemination of VV to neighboring cells (Blasco and Moss, 1992).

In addition to providing a mechanism for virus dissemination, the virion contains the genomic DNA and a virtual factory for the synthesis of early mRNA. The VV genome consists of a linear, double-stranded DNA molecule which contains

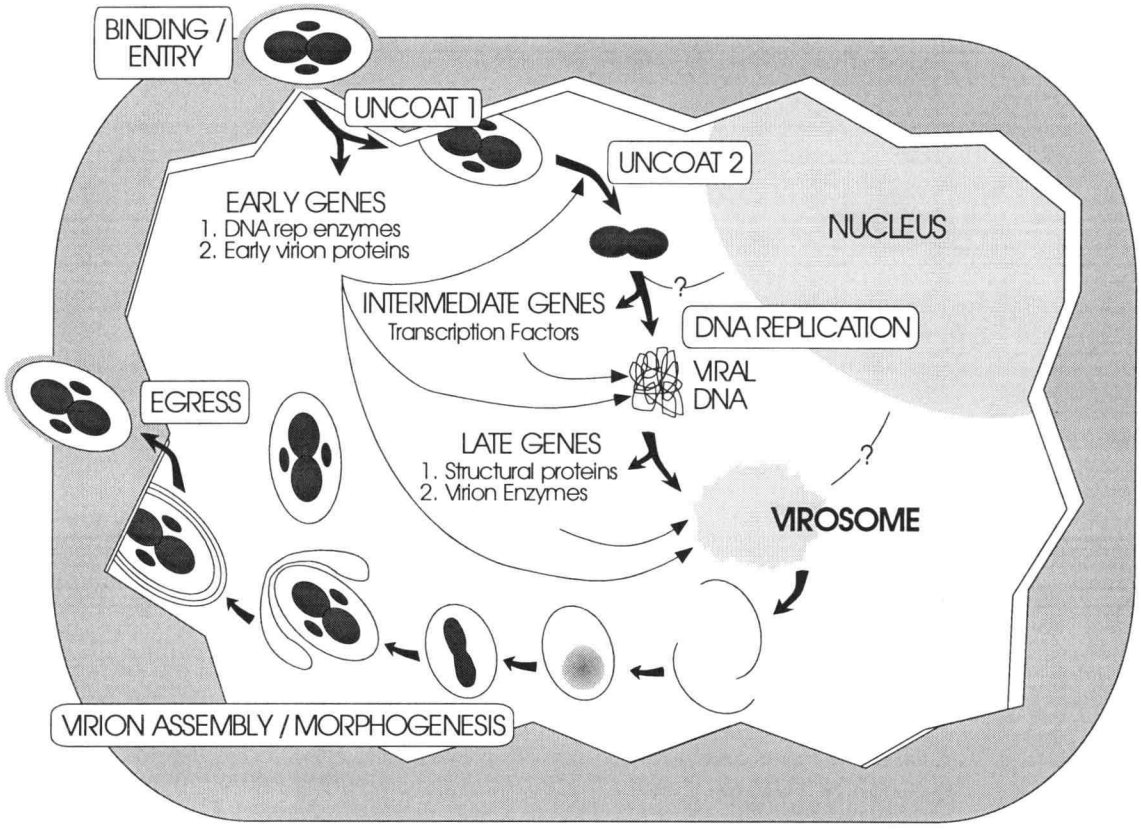


Figure I.1 Vaccinia virus replication cycle

inverted terminal repeats of about 11 Kbp. The recent nucleic acid sequencing of the entire VV (Copenhagen strain) genome (Goebel *et al.*, 1990) has revealed that the 192 Kbp genome is predicted to contain nearly 200 open reading frames (ORFs). Both strands of the DNA molecule are transcribed, and gene expression is regulated in a temporal cascade of early, intermediate, and late transcription (Fig, I.1).

Upon binding and entry into the host cell, the virion begins expression of early genes. The incoming viral particle contains a multi-subunit, virus-encoded, DNA-dependent RNA polymerase; a viral early transcription factor which recognizes early promoters; capping and methylating enzymes; poly(A) polymerase; DNA topoisomerase type 1; and other enzymes involved in mRNA formation (for a review, see Moss, 1990a). The RNA products represent distinct 5′-capped, 3′-polyadenylated mRNAs which are extruded from the virus core and then associate with the cellular ribosomes where early viral proteins, including those required for DNA synthesis, nucleotide metabolism, and transcription of intermediates genes, are made. The parental DNA molecule can not serve as a template for intermediate or late gene expression because of its location in the core or because of specific repressors that block intermediate and late genes. Following the eventual breakdown of the viral core, the DNA molecule is replicated as a catenated molecule within the virosomes (virus factories). The newly replicated DNA provides the template for intermediate gene expression including the three ORFs, A1L, A2L, and G8R which encode a set of late transcription factors (Keck *et al.*, 1990) . In the final phase of transcription, late genes encoding nearly 100 proteins, including virion structural proteins, virion enzymes, and early transcription factors are expressed. Late mRNA contains no distinct 3'-end, but contains a unique 5'-poly(A) leader sequence. Following

expression of the structural proteins and proper post-translational modification, the multi-step process of virion morphogenesis can begin.

STRUCTURAL PROTEINS OF VACCINIA VIRUS

The VV particle is composed of large number of proteins, with more than 100 having been resolved by two dimensional polyacrylamide gel electrophoresis (Essani and Dales, 1979). Although there are many known enzymes within the virus core itself, as discussed in the previous section, they account for only a small portion of the total protein. Four structural proteins, 4a, 4b, 25K and 11K, together account for the majority of the mass of the core. The 11K protein is encoded by the F17R ORF (Zhang and Moss, 1991), and has been identified as a DNA binding protein, and may be involved in the process of DNA packaging and condensation. (Kao *et al.*, 1987). The 25K protein constitutes nearly 8% of the virion mass (Sarov and Joklik, 1972), and also appears to be a major DNA binding protein (Yang and Bauer, 1988). The precursor protein, P25K, is encoded by the L4R ORF and has a predicted molecular mass of 28 kDa. The 4a and 4b core constituents together account for about 25% of the virion mass (Sarov and Joklik, 1972). The 4a protein, with an apparent molecular mass of 62 kDa, is derived by proteolytic processing of P4a, encoded by ORF A10L, and 4b protein, with an apparent molecular mass of 60 kDa, is derived by proteolytic processing of P4b, encoded by ORF A2L (Wittek *et al.*, 1984). An additional structural protein, 23K, is also derived from proteolytic processing of the P4a precursor protein (VanSlyke *et al.*, 1991). Expressed late in infection, D2L and D3R proteins (17 kDa and 27 kDa, respectively) have been demonstrated to be structural core proteins (Dyster and Niles, 1991). Interestingly, *ts*

mutants mapping to these two genes both possess the same phenotype which includes defective processing of P4a (Dyster and Niles, 1991).

In addition to the structural proteins found associated with the virion core, numerous proteins are also found associated with both the viral and extracellular envelopes. Many of the virion-associated proteins are processed by one or more post-translational modifications, including glycosylation, phosphorylation, acylation, ADP-ribosylation, and proteolytic cleavage (for a review, see VanSlyke and Hruby, 1990). However, for the most part the structural organization of the virion associated proteins, the mechanism of virion assembly, and the functions of the proteins and modifications are still poorly understood.

PROTEOLYTIC PROCESSING OF VACCINIA VIRUS PROTEINS

A small subset of vaccinia virus proteins have been shown to be proteolytically processed. Although a few secreted, nonstructural proteins, as well as hemagglutinin have been shown to be proteolytically processed (Stroobant *et al.*, 1985; Kotwal and Moss, 1988; Shida, 1986), at least six virion-associated proteins have been shown to be derived from precursor proteins. These products include 4a, 4b, 25K, 23K, 21K, and 17K (VanSlyke *et al.*, 1991; Whitehead and Hruby, 1994a). When the predicted amino acid sequence of the precursors of these proteins was aligned at the determined cleavage sites, a conserved Ala-Gly-X motif (AG*X) was noted, as shown in Fig. I.2. The predicted cleavage motifs of each of these proteins is conserved in variola major virus, strain India-1967 (Shchelkunov *et al.*, 1993). Likewise, the cleavage motifs of P25K and P4b are conserved in fowlpox protein homologs, FP5 and F4b, respectively (Binns *et al.*, 1988; 1989).

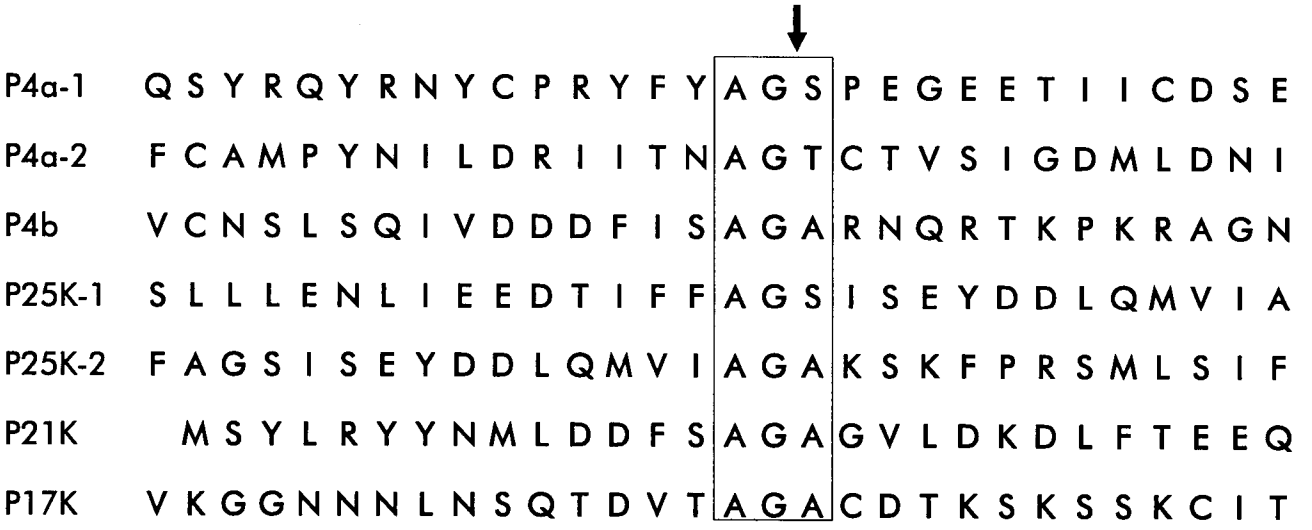


Figure I.2 Alignment of vaccinia virus proteolytic substrates

The proteolytic cleavage of these precursor proteins is believed to be essential for virion maturation, although the exact role of cleavage has not been determined. It has been postulated that cleavage of P25K is important for the proper interaction of 25K with viral DNA or other core proteins after it is assembled into viral cores (Yang *et al.*, 1988). However, this function is most likely limited to P25K since the other processed core proteins have not been implicated in DNA binding. Therefore, similar to the morphogenic proteolytic processing of structural proteins in other viruses, proteolytic cleavage of these precursor proteins may be essential for allowing conformational changes in these proteins that may be required for proper core condensation, virion maturation and infectivity. This model is supported by several observations: (i) Precursor proteins are found exclusively in purified previrion particles (VanSlyke *et al*, 1993). (ii) The appearance of mature cores, observed with the electron microscope, coincided with the time that cleavage commenced after removal of the drug rifampicin (Katz and Moss, 1970a). (iii) A simultaneous block in morphogenesis and proteolytic processing was observed when a set *ts* mutants were incubated at the non-permissive temperature (Stern, *et al.*, 1977; Silver and Dales, 1982).

To better understand the role that proteolytic processing plays in the replication of vaccinia virus, the purpose of this thesis research is to address several questions. What are the participating components, including the proteinase(s)? Are they encoded by the virus, the host cell, or both? Does cleavage occur in an exo- or endoproteolytic reaction? What is the fate of the propeptides removed during proteolytic processing? What VV proteins are proteolytic substrates and what proteins are excluded? The following chapters contain the information gained from my studies which endeavored to answer these questions.

CHAPTER II
DIFFERENTIAL UTILIZATION OF A CONSERVED MOTIF FOR THE PROTEOLYTIC MATURATION OF VACCINIA VIRUS PROTEINS

Authors: Stephen S. Whitehead and Dennis E. Hruby

SUMMARY

Several of the vaccinia virus core proteins are synthesized as large precursor proteins which are subsequently processed to smaller products during the course of viral maturation. Amino acid alignment of these proteins reveals a conserved Ala-Gly-X motif (AG*X) at their confirmed cleavage sites. To better understand the regulation of cleavage site selection, the sequence of the entire vaccinia virus genome was searched for the occurrence of this AG*X motif in predicted open reading frames. Of the 82 sites found, 19 resembled cleavage sites which have previously been shown to be actively processed, namely AG*A of P25K and P4b, and AG*S and AG*T of P4a. To test the universality of the AG*X motif utilization, immunological methods in concert with N-terminal microsequencing procedures have been used to determine which of the subset of predicted proteins containing AG*A sites are utilized *in vivo*. Of the seven AG*A-containing substrates, four were cleaved and three were not. Considering all the known AG*X processing events, it appears that only those proteins expressed at late times during infection and associated with the assembling virion are candidate substrates for proteolytic cleavage. Such proteins include P4a, P4b, P25K, and the newly identified P21K and P17K (derived from genes A17L and A12L, respectively). Although proteins such as DNA polymerase, P37K and a host range protein contain a consensus cleavage site, they are excluded from processing. This proteolytic exclusion presumably occurs because these proteins do not meet both of the above criteria, which suggests that temporal expression or compartmentalization (substrate presentation) in the assembling virion may play a regulatory role in proteolysis.

INTRODUCTION

During the course of virus replication, vaccinia virus (VV) encoded proteins are matured by a number of posttranslational modifications including acylation, phosphorylation, glycosylation, ADP-ribosylation and proteolytic processing (VanSlyke and Hruby, 1990). Of particular interest is proteolysis, since it represents a widely conserved modification among many different viruses while maintaining a high degree of specificity for an individual viral system. Maturational cleavages occur in proteins of both RNA and DNA viruses which infect a wide variety of hosts including animals, plants and yeast. In addition to relying on individual proteases, cleavage specificity and overall regulation of the proteolytic events may result from a combination of several factors. For example, substrate context appears to regulate the cleavage of picornavirus VP0 since capsid preassembly is required for final cleavage (Arnold *et al.,* 1987; Lee *et al.,* 1993). Likewise, specific peptide and DNA cofactors are required for adenovirus core protein processing, which apparently ensures the final cleavage events occur within the immature virion (Mangel *et al.,* 1993). And finally, temporal gene expression has been shown to control the proteolytic pathway of alphaviruses (de Groot *et al.,* 1990). It is highly probable that similar levels of control will regulate the processing of VV proteins.

Although proteolytic processing plays an essential role in the VV life cycle, very little is currently known about VV proteolytic regulation. Cleavage occurs in nonstructural proteins such as hemagglutinin (Shida, 1986), VV growth factor (Stroobant *et al.,* 1985), and a secreted 19 kDa protein (Kotwal and Moss, 1988), as

well as in several of the viral structural proteins. It has been shown that the three major structural core proteins 4a, 4b and 25K are all derived from cleavage of their respective precursor proteins (Katz and Moss, 1970a; Moss and Rosenblum, 1973; Weir and Moss, 1985; VanSlyke *et al.,* 1991). Alignment of the cleavage sites in these proteins reveals a conserved Ala-Gly-X motif (AG*X), suggesting that processing of all three core protein precursors may be regulated and catalyzed by the same protease during viral maturation. Therefore, an analysis of the maturation of other VV proteins containing the AG*X motif should offer clues about the requirements for processing.

Here I report the results of our investigation of the universality of proteolytic processing among a subset of AG*X-containing VV proteins. The occurrence of the AG*A motif was specifically examined because of its prevalence among VV proteins known to be cleaved. Seven VV open reading frames (ORFs) code for proteins carrying the AG*A tripeptide. Proteins expressed at both early and late times of infection are represented in this subset, as well as both virion and non-virion proteins. The results obtained show that all the currently identified VV proteolytic substrates utilizing the AG*X motif are expressed during late times of infection and are associated with the assembling virion particle. In addition, we report the identification of two new virion constituents, P17K and P21K, both of which undergo cleavage at AG*A.

MATERIALS AND METHODS

Viruses and cells. Vaccinia virus (strain WR) was propagated in monolayers of BSC-40 cells and vaccinia virus (strain IHD-J) was propagated in monolayers of RK-13 cells both cultured in MEM-E (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, and 10 μg/ml gentamycin sulfate. Cells were infected and purified VV (WR) was prepared as previously described (Hruby *et al.*, 1979). Extracellular enveloped virus (EEV) was pelleted from the culture medium of VV (IHD-J)-infected cells by centrifugation at 36,000 x *g* for 1 h and further purified by centrifugation through a 36% sucrose cushion at 40,000 x *g* for 2 h. The final pellet was resuspended in 1 m*M* Tris-HCl (pH 8.0).

Rifampicin treatment and radiolabeling of VV-infected cells. Metabolic pulse-chase labeling of VV-infected cells with L-[^{35}S]methionine (Du Pont-NEN) and treatment with rifampicin was as previously described (Hruby *et al.*, 1979; VanSlyke *et al.*, 1991). Infected cells were maintained in media with or without 100 μg/ml of rifampicin and harvested at 16 hpi. For radiolabeling, infected cells were pulse-labeled at various times postinfection as described in the legend of Fig. II.2 and harvested immediately or after a chase period with excess unlabeled methionine. Infected cells were lysed with 0.2% (w/v) sodium dodecyl sulfate (SDS) and treated with 50 U/ml Benzonase endonuclease (EM Science) for 10 min at room temperature, brought to a final concentration of 1% (w/v) SDS, 20 μg/ml phenylmethylsulfonylfluoride (PMSF) and stored at -20°C. In addition, infected cells (1 x 10^8) were pulse-labeled from 6 to 7 hpi with L-[^{35}S]methionine and either lysed as above

or chased with excess unlabeled methionine for 40 h, at which time purified virus was prepared. Virus was fractionated into a Triton X-100-soluble fraction (100 µl) and a core-containing pellet. This pellet was further fractionated into sodium-deoxycholate-soluble and insoluble fractions (100 µl each) (Bauer *et al.*, 1977).

Immunoprecipitation. A 20 µl aliquot of detergent-fractionated virus or a 100-µl sample of lysate prepared from 1.3 x 10^6 radiolabeled cells was diluted to 1 ml in RIPA buffer [1% sodium deoxycholate, 1% Triton X-100, 0.2% SDS, 150 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.4) and 25 U/ml Benzonase endonuclease], precleared by centrifugation and mixed with 5 µl antiserum at 0°C for 4 h. A 100-µl aliquot of 10% (v/v) Protein A-Sepharose (Sigma Chemical Co.) suspension in RIPA buffer was added and the samples were rocked 2 h at 4°C. The Protein A-Sepharose complex was recovered by centrifugation and washed 3 times with RIPA buffer. The final pellet was resuspended in 25 µl of sample loading buffer [3% SDS, 10% glycerol, 5% β-mercaptoethanol, 100 m*M* DTT, and 50 m*M* Tris-HCl (pH 6.8)], heated to 100°C for 3 min and fractionated by discontinuous slab gel electrophoresis on polyacrylamide gels containing SDS. The gels were fluorographed and exposed to Kodak XAR5 film at -70°C.

Cloning of A12L, A17L and K1L ORFs. Polymerase chain reaction (PCR) primers were constructed according to the published VV (Copenhagen) genome sequence (Goebel *et al.*, 1990) and included the initiating Met residue and termination codons for A12L, A17L and K1L ORFs. In addition, each primer for the initiating end of the ORF was constructed to introduce a *Nco*I restriction site at the initiating Met residue codon. This resulted in a Ser to Gly mutation at amino acid 2 of A17L (amino acid 2 of A12L and K1L remain unchanged). PCR was performed

using purified genomic VV DNA (WR strain) as template and the Klenow fragment of *E. coli* DNA polymerase I (Mullis and Faloona, 1987). The PCR-generated fragments were cloned directly into the *Sma*I site of pUC119 (Sambrook *et al.*, 1989).

Fusion protein constructs and overexpression in *E. coli*. Plasmids for the overexpression of P21K, P17K, and host range protein (HR) (encoded by A17L, A12L and K1L, respectively) were created using fragments derived from the pUC119 constructs described above. A 241-bp fragment (*Eco*RI-*Hin*dIII) of A17L coding for P21K amino acids 136-203 was inserted at the *Eco*RI and *Hin*dIII sites of the pATH3 expression vector polylinker (Miner and Hruby, 1989). This construction places codons for the C-terminal portion of P21K downstream of, and in frame with, the bacterial *trp*E ORF. The recombinant plasmid was propagated in *E. coli* JM101 and expression of the 45-kDa TrpE-P21K chimera was induced at 37°C by tryptophan starvation and the addition of indoleacrylic acid as previously described (Miner and Hruby, 1989). Whole cells pelleted by centrifugation were resuspended in 100 m*M* Tris-HCl (pH 6.8) and stored at -20°C until needed.

A 581-bp fragment (*Nco*I-*Sac*I) representing the entire A12L ORF was inserted at the *Nco*I and *Sac*I sites of the pTrc.His7 expression vector polylinker. The pTrc.His7 vector (kindly provided by Dr. William G. Dougherty, Oregon State University) is a derivative of the pTrc99A expression vector (Pharmacia) and contains sequence coding for an initiating methionine and seven histidine residues directly upstream of an *Nco*I cloning site. In-frame insertion of the A12L ORF created a recombinant plasmid expressing a Met-His7-P17K chimera. The recombinant plasmid was propagated in *E. coli* JM101 and expression was induced with

IPTG (Studier *et al.,* 1990). Whole cells pelleted by centrifugation were resuspended in 6 *M* guanidine-HCl, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl (pH 8.0) and stored at -20°C until needed.

A 875-bp fragment (*Nco*I-*Bam*HI) generated by partial digestion of pUC119:-K1L and representing the entire K1L ORF was inserted at the *Nco*I and *Bam*HI sites of expression vector pET3d (Novagen). The recombinant plasmid was propagated in *E. coli* BL21(DE3)pLysS and expression of HR protein was induced with 0.1 m*M* IPTG as described (Studier *et al.,* 1990). Whole cells pelleted by centrifugation were resuspended in 100 m*M* Tris-HCl (pH 6.8) and stored at -20°C until needed.

In order to inducibly express P17K and 17K proteins in *E. coli*, plasmids coding for exactly full-length P17K and product-length 17K were generated. A 596-bp fragment (*Nco*I-*Eco*RI) containing the full-length A12L ORF was inserted into the *Nco*I and *Eco*RI sites of pET3d. Mutagenesis (Kunkel, 1985) of pUC119.A12L was used to introduce an additional *Nco*I and Met codon in place of the codon for Gly at the P1 position of the AG*A cleavage site. The new 414-bp fragment (*Nco*I-*Eco*RI) containing a product-length A12L ORF was inserted into the *Nco*I and *Eco*RI sites of pET3d. Both plasmids were propagated in *E. coli* BL21(DE3)pLysS and expressed as described above in the presence of L-[^{35}S]methionine.

Polyclonal antisera production. Lysates from induced bacteria expressing the TrpE-P21K fusion protein and HR protein were mixed with sample loading buffer and fractionated on 10% polyacrylamide gels (Studier, 1973). Gels were equilibrated in deionized water and proteins were visualized by soaking the gel in 0.3 *M* $CuCl_2$. The induced protein band was excised from the gel, mixed with an equal volume of sterile water, homogenized by forcing through a narrow gauge needle, and emulsified

in complete Freund's adjuvant (GIBCO). The antigen preparation was then used for multiple, subcutaneous inoculations in New Zealand white rabbits after the collection of preimmune serum. Additional fusion protein prepared in the same manner, but emulsified in incomplete Freund's adjuvant (GIBCO), was used for subsequent intramuscular boosts, and immune sera were collected.

The Met-His7-P17K fusion protein was affinity purified over a Ni-NTA-agarose column (QIAGEN) equilibrated in 6 *M* guanidine-HCl, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl (pH 8.0) as described in "The QIA*expressionist* " user bulletin (Henco, 1991). Briefly, total cell lysate of induced bacteria was loaded onto the column followed by exchange of the guanidine-HCl buffer for urea wash buffer [8 *M* urea, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl (pH 8.0)]. Uninduced *E. coli* proteins were washed from the column with urea wash buffer adjusted to pH 6.3, and the Met-His7-P17K fusion protein was eluted from the column with urea wash buffer (pH 5.9). Purity of the elution fractions was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A fraction containing approximately 500 μg of purified protein was emulsified with an equal volume of complete Freund's adjuvant and used for immunization of rabbits as described above.

Polyclonal rabbit antiserum specific for VV DNA polymerase (DNAP) was kindly provided by Dr. Paula Traktman, Cornell University Medical College.

N-terminal microsequencing. Protein substrates for N-terminal microsequencing were processed as previously described (VanSlyke *et al.*, 1991). Virion proteins 21K and 17K were separated from purified VV (WR) by 10% SDS-PAGE and electrophoretically transferred to polyvinylidenedifluoride (PVDF) membranes. P37K was similarly isolated from EEV of VV (IHD-J). Proteins transferred to

PVDF membranes were subjected to automated Edman degradation in a gas-phase cartridge system (ABI Model 475A) and the amino acid derivatives from each cycle were analyzed by reverse phase HPLC.

RESULTS

Universality of the AG*X motif in VV. Since only three VV core proteins have been shown to be proteolytically processed at a AG*X site, we were initially interested in determining if additional VV proteins containing an AG*X cleavage motif might also be cleaved. Using the complete genome sequence of VV (Copenhagen), the predicted amino acid sequence of each ORF was determined and compiled into a single database. A search of this database for the AG*X tripeptide revealed it occurred only 82 times among the 198 predicted ORFs, which is less frequent than the 204 sites expected if AG*X occurred randomly. Of these 82 sites, 18 resembled sites that have previously been shown to be actively cleaved, namely AG*A of P25K and P4b, and AG*S and AG*T of P4a. The AG*A site occurred only 7 times.

Figure II.1 shows the predicted amino acid alignment at the potential cleavage sites of the AG*A-containing proteins. In each case, the sequence is derived from the Copenhagen strain of VV. Although there are slight differences between the predicted amino acid sequence of proteins derived from the Copenhagen and WR strain genomes, the AG*A cleavage motif is conserved between these strains in each of the 7 proteins. For P37K, the AG*A site is also conserved in the IHD-J strain. It should also be noted that each ORF is preceded by a consensus late promotor (TAAAT), with the exception of DNAP, which has been shown to be expressed at early times during infection (McDonald *et al.*, 1992), and host range protein (HR), which is transcribed as an immediate-early gene (Gillard *et al.*, 1989). Although we

Figure II.1. Amino acid alignment of VV proteins containing the AG*A cleavage motif. The sequences are aligned with respect to their putative cleavage sites, and amino acid residues are numbered below the alignment according to their position relative to the scissile bond. The actual position of the P1 glycine residue in the full-length predicted protein is as follows: P25K (32), P4b (61), P17K (56), P21K (16), P37K (10), DNAP (482) and HR (53). Acidic amino acid residues upstream of the cleavage site and basic amino acid residues downstream of the cleavage site are underlined.

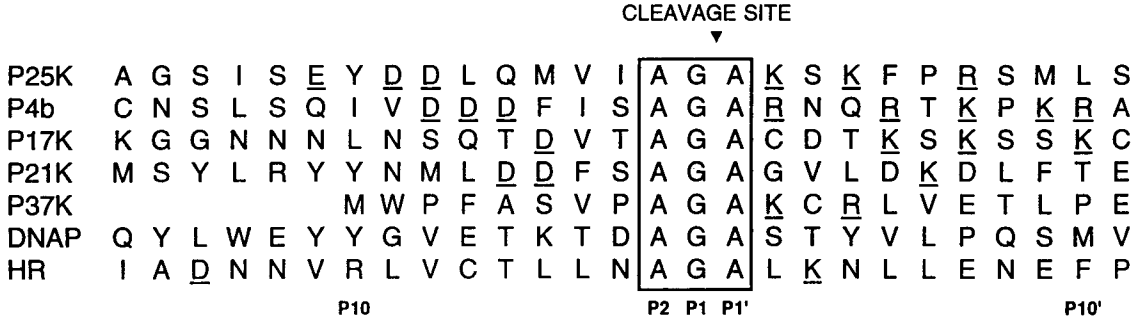


Figure II.1 Amino acid alignment of vaccinia virus proteins containing the AG*A cleavage motif

were hopeful that alignment of these proteins would reveal additional conserved residues surrounding the AG*A motif, examination of the information in Figure II.1 shows this is not the case. Therefore it was of interest to determine which of these 7 proteins were bona fide cleavage substrates *in vivo*.

Proteolytic exclusion of DNAP, HR and P37K. Open reading frame E9L codes for DNAP which has an apparent molecular mass of 116 kDa (1006 amino acids) (Traktman, 1990). If this protein were to be cleaved at its AG*A site, between amino acids 482 and 483, the resulting products would have a predicted molecular mass of 61 and 55 kDa. Using DNAP-specific antiserum, we analyzed pulse-labeled extracts for the existence of these putative cleavage products. Infected cells were radiolabeled with [^{35}S]methionine at 2 to 4 hpi, during which DNAP synthesis is at its peak, and chased with unlabeled methionine for up to 24 h. Figure II.2A shows that only full-length DNAP could be immunoprecipitated from these extracts. Cleavage products could not be detected even after the 24 h chase period or after gross overexposure of the autoradiograph (data not shown), suggesting that the AG*A site of DNAP is excluded from proteolytic processing.

Likewise, only full-length host range (HR) protein could be detected during infection. HR protein, encoded by ORF K1L has a predicted molecular mass of 33 kDa, but tends to migrate as a 30 kDa band on SDS-polyacrylamide gels (Gillard *et al.,* 1989). If HR protein were to be cleaved at its AG*A site, between residues 53 and 54, the resulting products would have a predicted molecular mass of 6 and 26 kDa. Infected cell extracts were radiolabeled from 3 to 3.5 hpi and chased with unlabeled methionine for either 4 or 16 hours. Figure II.2B shows that full-length HR protein was immunoprecipitated with its specific antisera, but no 26-kDa

Figure II.2. Immunoprecipitation analysis of DNAP and HR protein. BSC-40 cells were infected with vaccinia virus (WR, MOI of 10 PFU per cell) and pulse-labeled with L-[^{35}S]methionine. Cell extracts were made at the indicated times, immunoprecipitated with specific antisera and processed for autoradiography as described in Materials and Methods. (A) For DNAP, infected cells were radiolabeled at 2 to 4 hpi, as was a mock infected (MI) control. Labeled proteins were chased with excess methionine for 0 (P), 2, 4, 8 and 24 h, solubilized with 2% SDS and immunoprecipitated with anti-DNAP antiserum. The MI lysate made at 4 hpi was similarly immunoprecipitated. Lane 2 (X) represents labeled cell extract isolated from virus-infected cells at 4 hpi. (B) For HR proteins, infected cells were radiolabeled at 3 to 3.5 hpi. Labeled proteins were chased with excess methionine for 0 (P), 4 and 16 h, solubilized in 2% SDS and immunoprecipitated with anti-HR antiserum. The 4 h chase extract was also immunoprecipitated with preimmune serum (PI). The position of the predicted cleavage products (*) is indicated in each autoradiograph, as are the migration of ^{14}C-labeled molecular weight markers.

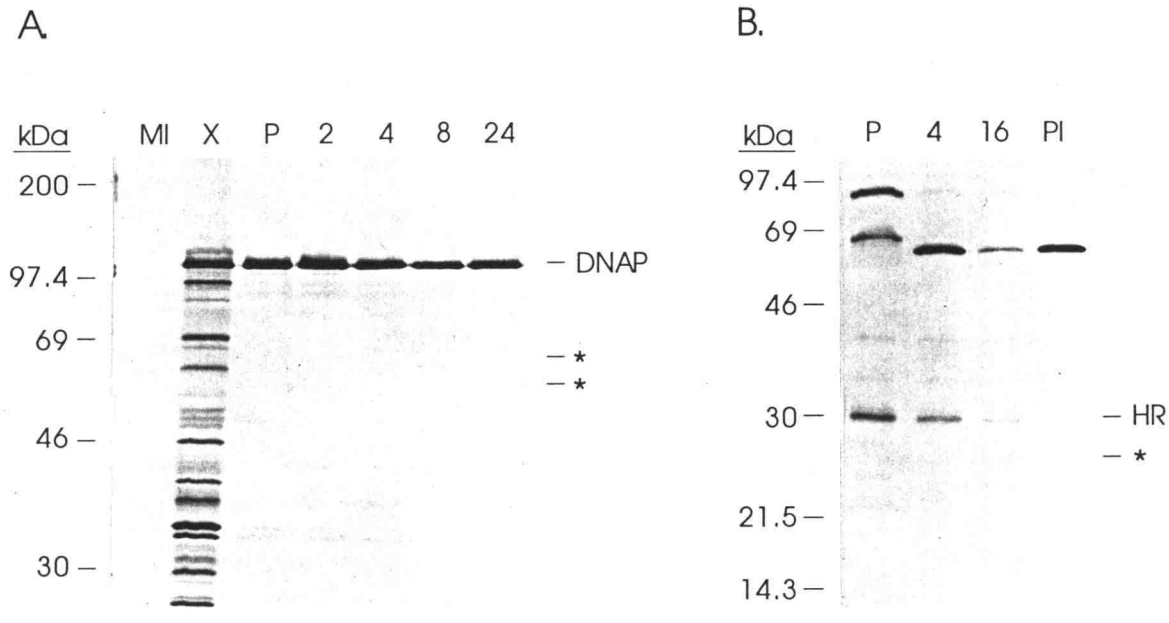


Figure II.2 Immunoprecipitation analysis of DNAP and HR proteins

cleavage product could be detected, suggesting that the AG*A cleavage site of HR protein is also not utilized.

The P37K major extracellular-envelope antigen of VV, encoded by ORF F13L (Hirt *et al.,* 1986), contains an AG*A site near its amino terminal. Cleavage at this site would liberate a peptide with a molecular mass slightly less than 1 kDa. Since the difference in molecular mass of the precursor and the product on SDS-polyacrylamide gels would be slight at best, we approached the question of P37K proteolytic processing by directly microsequencing the N-terminus of the protein. P37K was isolated from VV (IHD-J) by SDS-PAGE and blotted to PVDF membrane. Sequence of an isolated band corresponding to P37K is shown in Fig. II.5. The sequence obtained was unambiguous due to the lack of any secondary sequence, and aligned perfectly with the predicted N-terminal portion of the full-length protein, rather than with the putative cleavage product, indicating that P37K does not undergo proteolytic processing at the AG*A site.

Proteolytic processing of P21K and P17K. Preparation of monospecific, polyclonal antisera for P17K and P21K was essential for our study of the proteolytic processing of these two proteins, since they have previously not been described. The full-length A17L ORF, coding for P21K, was originally cloned in expression vector pET3d. However, we were unable to get adequate overexpression of P12K in several *E. coli* strains, including BL21(DE3)pLysS, possibly due to toxicity of the protein product. The major antigenic portion of the protein, as predicted from its hydrophilicity profile, resides in the C-terminal half of the protein. To circumvent the problem of low expression, this portion of the A17L ORF, codons 136-203, was cloned into the pATH3 expression vector. Induction of this TrpE-P21K protein

chimera yielded sufficient antigen for induction of specific antibodies in rabbits. For overexpression of P17K, copies of the full-length A12L ORF were directly cloned into expression vectors pTrc.His7 and pET3d, and the truncated ORF coding for the predicted 17K product was cloned into pET3d. Expression of full-length and product-length P17K provided molecular weight markers helpful in identifying the authentic precursor and product. The His-tagged P17K protein was easily purified over a Ni-NTA agarose column, and used for immunization of rabbits. Both preimmune and immune antisera were collected and tested for specificity in pulse-chase analyses of detergent-solubilized virus. Preimmune sera failed to recognize any detectable viral proteins (data not shown). As shown in Fig. II.3, P21K-specific antiserum recognized a 23-kDa precursor protein in the pulse-labeled extract, as well as a 21-kDa product which partitions to the detergent-soluble virion fraction. The apparent molecular masses of the precursor and product agree with those predicted from the coding sequence. In contrast, the P17K-specific antiserum recognized a 24-kDa precursor protein in the pulse-labeled extract as well as in the detergent-soluble core fraction. The cleaved 17K product was also detected in the same detergent-soluble core fraction. Although both precursor and product co-migrate exactly with their counterparts expressed in *E. coli*, the apparent molecular masses of each are about 3 kDa larger than those predicted from the coding sequence. Virion fractions were also immunoprecipitated with P25K-specific antiserum for comparison.

Rifampicin has been shown to partially inhibit proteolytic processing of VV proteins, and is useful to demonstrate the precursor-product relationship between VV proteins (Katz and Moss, 1970b; Moss and Rosenblum, 1973). In order to determine if the cleavage of P17K and P21K is sensitive to rifampicin, extracts from VV-

Figure II.3. Pulse-chase analysis of the 17K and 21K proteins. Purified vaccinia virus pulse-labeled with L-[^{35}S]methionine and separated into detergent-soluble virion fractions (SV), detergent-soluble core fractions (SC) and insoluble core fractions (IC) was immunoprecipitated using the indicated antisera. The pulse-labeled cell lysate (P) as well as radiolabeled *E. coli* extracts containing full-length (FL) P17K and product length (PL) 17K were also immunoprecipitated. The recovered immune complexes were fractionated by SDS-PAGE and visualized by fluorography. The migration of ^{14}C-labeled molecular weight markers is shown.

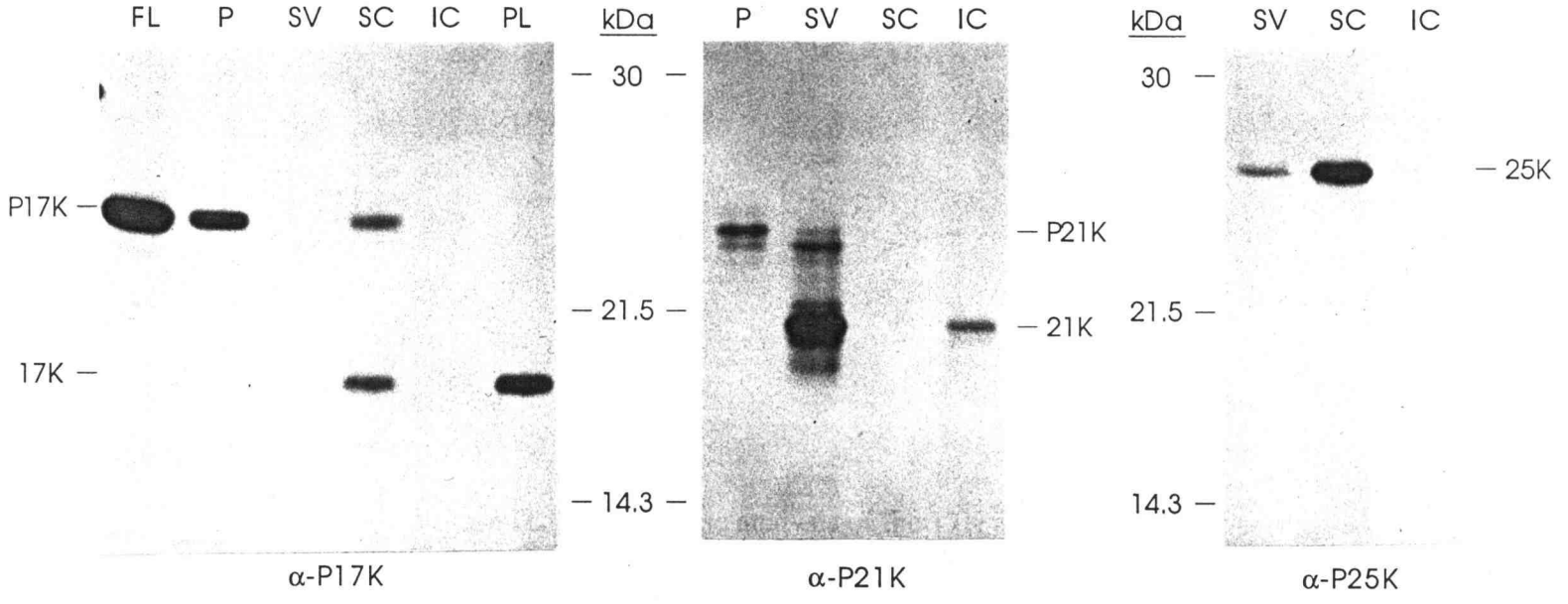


Figure II.3 Pulse-chase analysis of the 17K and 21K proteins

infected cells and rifampicin-treated VV-infected cells were reacted with P21K and P17K antisera in immunoprecipitation assays. P17K antisera reacted with a unique 24-kDa and P21K antisera reacted with a 23-kDa protein in extracts made at late times during VV infection in the presence of rifampicin (Fig. II.4). In these reactions, very little product is visible, indicative of the rifampicin block of proteolytic processing, as shown previously for P4a, P4b, and P25K.

In order to confirm that 21K and 17K are actually products of proteolytic processing, the proteins were purified from virus extracts and subjected to N-terminal microsequence analysis. The results shown in Fig. II.5 confirm that 21K and 17k are in fact cleavage products and that processing occurs at the predicted AG*A site. The N-terminal sequence of 21K was contaminated with sequence corresponding to the P4a-derived 23K protein, and the sequence of 17K was contaminated by a trace amount of an unidentified polypeptide. However, in both cases, assignment of contiguous sequence was unambiguous due to large differences in the molar concentrations of amino acid derivatives for each sequence.

Figure II.4. Rifampicin-sensitivity of P17K and P21K processing. The indicated antisera were used for immunoprecipitation of radiolabeled extracts from VV infected cells cultured in the presence (+) or absence (-) of rifampicin. The recovered immune complexes were fractionated by SDS-PAGE and visualized by fluorography. The migration of molecular weight markers is indicated for each immunoblot.

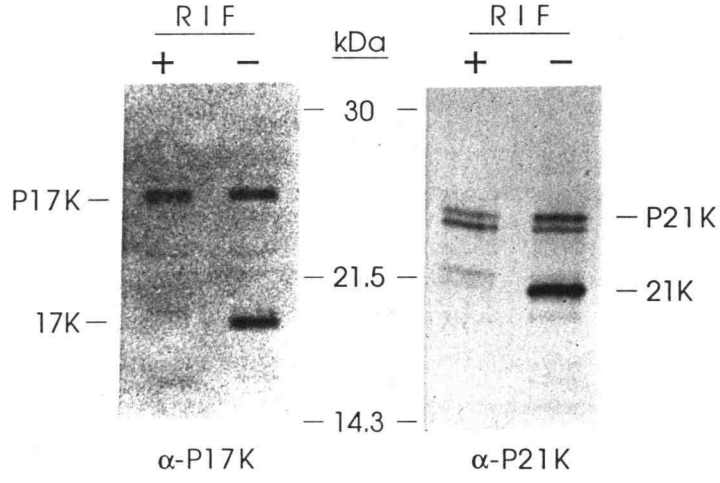


Figure II.4 Rifampicin-sensitivity of P17K and P21K processing

Figure II.5. N-terminal amino acid sequence of the cleavage products of P17K and P21K, as well as P37K. The N-terminal sequence determined by Edman degradation analysis is displayed below the predicted amino acid sequence deduced from the ORF corresponding to each protein. X represents degradation cycles which resulted in undetectable derivatives.

P17K	L	N	S	Q	T	D	V	T	A	G	A	C	D	T	K	S	K	S	S
Sequence											A	X	D	T	K	S	K	S	
P21K	Y	N	M	L	D	D	F	S	A	G	A	G	V	L	D	K	D	L	F
Sequence											A	X	V	L	D	K	D	L	F
P37K	M	W	P	F	A	P	V	P	A	G	A	K	C	R	L	V	E	T	L
Sequence	M	W	P	F	A	P	V	P	A	G	A								

Figure II.5 N-terminal amino acid sequence of P37K and the cleavage products of P17K and P21K.

DISCUSSION

Proteolytic processing has previously been described for the major virion core proteins P4a, P4b and P25K. Together these proteins constitute about 32% of the virion mass (Sarov and Joklik, 1972), indicating that proteolytic processing is responsible for maturing at least one-third of the virion protein mass. Yet surprisingly little is known about VV proteolytic specificity and regulation. Amino acid alignment of cleavage substrates from VV has revealed a conserved AG*X motif at the cleavage site. However, this motif is not limited to only P4a, P4b and P25K, but occurs 82 times among the other proteins predicted to be encoded by the viral genome. By examining the differential utilization of this cleavage motif, we hoped to expand our knowledge of cleavage consensus and regulation.

We limited our initial investigation to the AG*A subset of potential cleavage sites, since this motif occurs with greatest frequency among *bona fide* VV cleavage substrates. Also, the amino acids surrounding the AG*A cleavage site of P4b and P25K show some charge similarities that are not conserved in the AG*S and AG*T regions of P4a, suggesting that AG*A substrates may be processed by the same proteinase. Furthermore, the AG*A subset of proteins represents both early and late proteins, structural and nonstructural proteins, as well as proteins associated with the virion core or extracellular envelope. Amino acid alignment of the AG*A-containing proteins reveals no further primary sequence homology in the region surrounding the AG*A motif. Examination of secondary structure predictions for these regions also failed to show any structural similarities (data not shown). However, distribution of

charged amino acid residues relative to the cleavage site appears to be conserved and may have some predictive value. All four proteins of the AG*A subset which undergo proteolytic cleavage contain both upstream acidic residues and downstream basic residues, whereas P37K, DNAP and HR protein, as well as P4a, do not possess this charge differential.

To directly address the question of which AG*A containing proteins are actually utilized as proteolytic substrates and which proteins are subject to proteolytic exclusion, we employed both immunological methods and N-terminal micro-sequencing. Specific antisera for the nonstructural proteins DNAP and HR protein demonstrated the lack of cleavage products derived from these, as initially predicted by the absence of the cleavage site charge differential. Although it is possible that these antisera are only precursor specific, it seems unlikely since HR- protein-specific antiserum was produced against the full-length protein, and DNAP-specific antiserum is reported to have been produced against a 423 amino acid internal fragment spanning the AG*A site (McDonald *et al.*, 1992). Exclusion of the AG*A site of P37K was determined directly by N-terminal microsequencing. Although previously unobserved, processing of P37K initially seemed probable since P37K is known to migrate on SDS-polyacrylamide gels with an apparent molecular mass slightly less than that predicted (Hirt *et al.*, 1986), and several amino acids downstream of the AG*A site carry basic charges. Nevertheless, N-terminal sequence of a 37 kDa protein band corresponded to only the full-length protein. Even though product and precursor proteins may have been isolated together on the same PVDF strip, no secondary sequence corresponding to a cleavage product could be detected.

Antisera specific for proteins encoded by the A17L and A12L ORFs was used to identify proteins 21K and 17K as virion constituents. In addition, this antisera was used to demonstrate that these proteins are derived by proteolytic processing from AG*A-containing precursor molecules. The fact that 21K is found in the detergent soluble virion fraction is consistent with the view that it serves to anchor the 14-kDa fusion protein in the envelope of virion (Rodriguez *et al*, 1993). It is interesting to note that both precursor and product forms of P17K are localized to the mature virus core. In contrast, very little of the P4a, P4b, and P25K precursors are found in the mature virus. This observation implies that either the processing of P17K is inefficient, or that perhaps the precursor and product are both required for virion maturation.

Proteolytic processing of P17K and P21K is similar to that of P4b and P25K in several aspects. Examination of the primary amino acid sequences of these four proteins shows that the charge differential across the AG*A site is conserved in each, a feature not found at the cleavage sites of P4a. Also, each cleavage event occurs near the N-terminus of the precursor protein and liberates an N-terminal peptide of less that approximately 60 amino acids. This is distinct from P4a which is processed at two sites proximal to the C-terminus of the precursor (VanSlyke *et al.*, 1991). And finally, proteolytic processing of P17K and P21K is sensitive to the drug rifampicin in a way similar to that previously described for VV major core proteins. Taken together, this suggests that proteolysis of these four proteins is similarly regulated and may be catalyzed by the same enzyme. This does not necessarily imply that P4a is processed by a different proteinase, rather that its processing may be regulated differently. Although P4a cleavage consensus differs slightly from these four proteins, its processing is similar in other ways, as described. Therefore,

cleavage site consensus alone is insufficient to explain the apparent regulation of VV proteolytic processing.

To date, the identified AG*X-containing proteins utilized as proteolytic substrates share common features, other than the AG*X motif, which may explain their selection for processing (Table II.1). Only those proteins expressed at late times during viral infection and associated with the assembling virion are candidate substrates. This may suggest several levels of proteolytic regulation. The compartmentalization of precursor proteins within the assembling virion may be a necessary requirement for proteolytic processing. Immunofluorescence studies of 25K and epitope-tagged 25K-related proteins have established a correlation between virosome localization and cleavage efficiency (Lee and Hruby, 1993), which implies a level of regulation beyond simple accessibility of the cleavage site to the proteinase. This localization would allow each protein to be presented in a distinct context, which may permit interaction with the proteinase or specific cofactors, similar to adenovirus proteolytic processing. This also ensures that irreversible maturational cleavages do not occur until the substrates are preassembled into an immature virion, just as preassembly regulates the final cleavage of picornavirus capsid protein VP0. For VV, this dependence on preassembly and substrate context may explain why proteolytic activity is recalcitrant to reconstitution from infected cell extracts *in vitro*. It is also probable that proteolytic processing is temporally regulated. Expression or activation of the proteinase only during late times of infection could protect early proteins from deleterious cleavage. Likewise, temporal regulation similar to that of alphaviruses could also modulate the cleavage efficiency at certain sites. Clearly, additional research on proteolytic regulation in VV, as well

as identification of the responsible proteinase(s), is required for a complete understanding of this important posttranslational process.

Table II.1 Summary of proteolytic cleavage among VV AG*X containing substrates.

ORF	PROTEIN	TEMPORAL CLASS	LOCALE[a]	CLEAVED
A10L	P4a	Late	Core	+
L4R	P25K	Late	Core	+
A3L	P4b	Late	Core	+
A12L	P17K	Late	Core	+
A17L	P21K	Late	Virion	+
F13L	P37K	Late	Envelope	-
E9L	DNAP	Early	Virosome	-
K1L	HR	Imm. Early[b]	Nonstructural[c]	-

[a]Refers to protein localization in the VV-infected cell or mature virion.

[b]Immediate early

[c]Remains soluble in cytoplasm of VV-infected cell.

CHAPTER III
PHYSICAL AND MOLECULAR GENETIC ANALYSIS OF THE MULTISTEP PROTEOLYTIC MATURATION PATHWAY UTILIZED BY VACCINIA VIRUS P4a PROTEIN

Authors: Stephen S. Whitehead, Neil A. Bersani, and Dennis E. Hruby

SUMMARY

During vaccinia virus (VV) assembly, the P4a precursor protein (102.5 kDa) is proteolytically matured into two identifiable products: 4a (62 kDa) and 23K (23 kDa). Three potential AG*X cleavage sites were initially identified in the P4a precursor, AG*N, AG*S, and AG*T. Utilization of the COOH-proximal AG*T site in P4a leads to the release of 23K. It has been proposed that cleavage at the AG*S site alone is responsible for release of 4a. However, 4a migrates on SDS-polyacrylamide gels with an apparent molecular mass about 9 kDa less than that predicted if cleavage occurs at only AG*S. Additional processing at the NH_2-proximal AG*N site would remove about 10.7 kDa, bringing the apparent and predicted molecular masses into closer agreement. Since the 4a protein is recalcitrant to N-terminal microsequencing, peptide mapping in concert with microsequencing was used to show that the NH_2-terminus of 4a is co-terminal with P4a, thus confirming the proteolytic exclusion of the AG*N site. Proteolysis of P4a at AG*S and AG*T to yield 4a and 23K should theoretically also liberate an intervening 9 kDa protein. Efforts to isolate this peptide have been repeatedly unsuccessful. Mutation of the P4a coding sequence at the AG*S site, followed by transient expression in VV-infected cells, lead to the synthesis of a unique 4a-9K chimera. Similar treatment at the AG*T site yielded a unique 9K-23K chimera. This data confirms the proposed multistep P4a maturation pathway and strongly suggests that an internal 9 kDa protein of unknown function is released during this process.

INTRODUCTION

Vaccinia virus (VV) is the prototype member of the orthopoxvirus family and like most other viruses requires the proteolytic maturation of precursor proteins to complete its infectious life cycle. During virion assembly, the major core proteins are proteolytically cleaved from higher molecular weight precursors into the mature proteins that are found in the virion core (Essani and Dales, 1979; Katz and Moss, 1970; Sarov and Joklik, 1972). To date, five virus-associated proteins have been shown to be proteolytically processed, namely, P4a, P4b, P25K, P21K, and P17K (Katz and Moss, 1970; Moss and Rosenblum, 1973; Weir and Moss, 1985; Rosel and Moss, 1985; Yang *et al.*, 1988, Whitehead and Hruby, 1994a). Alignment of the cleavage sites in these precursor proteins has revealed a conserved AG*X cleavage motif. Interestingly, all of these precursors have been shown to be cleaved at a AG*A site, with the exception of P4a, which contains no AGA tripeptide motif. In addition, cleavage of these precursor proteins occurs near the NH_2-terminus, except in the case of P4a, where cleavage appears to occur at two internal sites near the COOH-terminal region of the protein. Three potential cleavage sites have been identified in the P4a predicted sequence, namely, AG*N, AG*S, and AG*T (Fig. III.1).

Processing of the P4a precursor protein at the AG*T site, between amino acids 697 and 698, has previously been confirmed by N-terminal microsequencing of the resulting 23K product (VanSlyke *et al.*, 1991). A similar approach to confirm processing at the AG*S site, between amino acids 614 and 615, has not proven to be

Figure III.1. Proteolytic processing of precursor protein P4a. The location of each of the proposed cleavage sites is shown below the diagram of P4a, as well as the predicted and observed (SDS-PAGE) molecular weights of the resulting products, assuming that cleavage does not occur at the AG*N site. The position of each methionine residue is indicated by hash marks above the diagram.

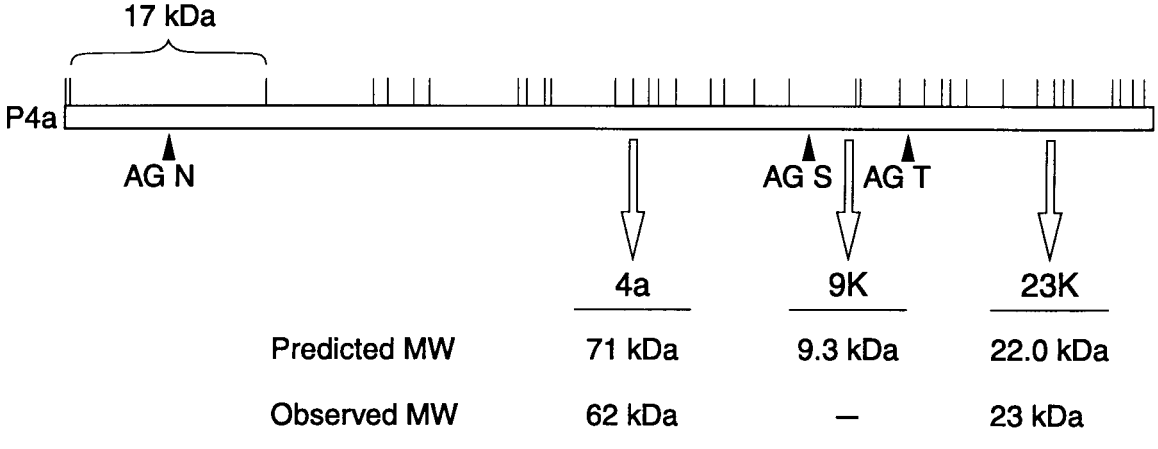


Figure III.1 Proteolytic processing of precursor protein P4a

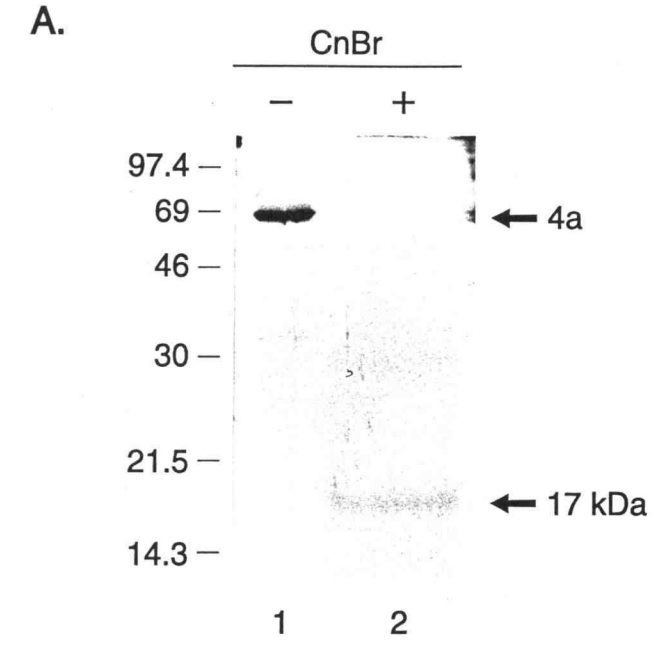
feasible due to our inability to identify and isolate the putative 9K product (amino acids 615 - 697 of P4a). Furthermore, the NH_2-terminus of 4a is blocked to N-terminal microsequencing, making it impossible by direct sequencing to determine if the NH_2-terminus of 4a is derived from cleavage at the AG*N site, between amino acids 95 and 96, or is co-terminal with the P4a precursor. However, direct, physical evidence using chemical cleavage with *N*-chlorosuccinimide and peptide mapping suggests that P4a is processed at the AG*S site but not at the AG*N site (VanSlyke *et al.*, 1991). Such a cleavage scheme is predicted to yield a 71 kDa product, although the apparent molecular mass of the 4a product following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is 62 kDa. Several possible explanations may account for this disparity; anomalous migration of the 4a product, another post translational modification, or additional proteolytic cleavage events. Earlier work has shown that full-length P4a protein as well as COOH-terminal truncated derivatives synthesized in rabbit reticulocyte lysates migrate faster than predicted in SDS-PAGE (VanSlyke *et al.*, 1991).

RESULTS AND DISCUSSION

To confirm that processing is not occurring at the AG*N site, our approach was to chemically cleave purified 4a protein with cyanogen bromide (CnBr) and subject the resulting, 17-kDa, NH_2-terminal fragment to N-terminal microsequencing. The location of each predicted methionine residue in P4a is shown in Figure III.1. The P4a precursor protein initiates with two tandem Met residues, which should both remain associated with the polypeptide in accordance with the N-terminal rules for methionine aminopeptidase processing (Bachmair *et al.*, 1986, Moerschell *et al.*, 1990). It was therefore hypothesized that the NH_2-terminal Met residue carried a modifying group that blocked N-terminal microsequencing, and that removal of this residue with CnBr would allow the fragment to be sequenced.

Disrupted, radiolabeled VV was fractionated by electrophoresis on SDS-containing Prosieve agarose (FMC, Rockland, ME), the corresponding 4a band was excised from the gel, and 4a protein was separated from the gel matrix (Fig III.2A, lane 1). This purified protein was reacted with CnBr and again fractionated by SDS-PAGE (Fig III.2A, lane 2). The 17-kDa fragment was blotted onto PVDF membrane and subjected to N-terminal microsequencing. As shown in Figure III.2B, the actual amino acid sequence of the 17-kDa CnBr fragment aligns perfectly with the predicted NH_2-terminal sequence of P4a, thus confirming that proteolytic processing does not occur at the AG*N site of P4a. It is likely that proteolytic exclusion of this site is due more to an overall unfavorable context or inaccessibility of the site, rather than to a deviation of the local primary sequence from the consensus motif, since the

Figure III.2. Peptide mapping and N-terminal sequence of 4a protein. (A) Insoluble core protein fraction (Bauer *et al.*, 1977) was prepared from purified VV labeled with ^{35}S-cysteine and electrophoresed through Prosieve derivatized agarose. A Coomassie-stained, 4a protein band was excised from the gel, melted at 75°C in 7 volumes of extract buffer (50 mM Tris [pH 7.8], 10% w/v SDS, and 1 mM EDTA), frozen in dry ice, and thawed at room temperature. Following microcentrifugation, the protein-containing supernatant was removed and concentrated by acetone precipitation. The pellet was resuspended in 70% formic acid and reacted with CnBr (Walker, 1988). An aliquot of purified protein (lane 1) and CnBr fragments (lane 2) were fractionated by SDS-PAGE and processed for autoradiography. The migration of ^{14}C-labeled molecular weight markers is shown. (B) Following CnBr treatment, the reaction mix was dried to remove formic acid and again electrophoresed through Prosieve agarose. The 17-kDa fragment was purified as described above and concentrated onto a PVDF membrane by centrifugation in an Ultrafree MC cartridge (Millipore, Bedford, MA). The PVDF membrane was removed, allowed to air dry, rinsed with 20% methanol, and subjected to automated Edman degradation in a gas-phase cartridge system (ABI, model 475A). The amino acid derivatives from each cycle were analyzed by reverse-phase HPLC. The N-terminal sequence is displayed below the predicted P4a sequence.



B.

Predicted P4a	M	M	P	I	K	S	I	V	T	L	D	Q
Sequenced 4a			P	I	K	S	I	V	T	L	D	Q

Figure III.2 Peptide mapping and N-terminal sequence of 4a protein

amino acids flanking the site are similar in charge and hydrophobicity to those surrounding the downstream AG*T site. In addition, it has been shown that mutation of the wild-type AG*A site to AG*N in the VV P25K precursor protein does not abolish cleavage (Lee and Hruby, 1994). Therefore it remains unknown why this site is not utilized, and why 4a protein migrates faster than predicted during SDS-PAGE.

A question that has yet to be answered is the fate of the 9K protein predicted to be liberated following cleavage at the AG*S and AG*T sites of P4a. Since both the 4a and 23K products are found associated with the virion core, it seemed likely that the 9K product would be found in a similar location (VanSlyke and Hruby, 1994; Whitehead and Hruby, 1994a). However, no corresponding protein has been detected in purified virus preparations or in immunoblot analysis of infected cell extracts or culture media. It is possible that targeted degradation is responsible for the absence of this product. However, N-end rules for ubiquitin-mediated degradation predict serine to be a stabilizing NH_2-terminal residue (Bachmair *et al.*, 1986), and since cleavage at AG*S should result in a 9K protein with a NH_2-terminal serine residue, it seems unlikely that 9K is selectively degraded by this mechanism. It is possible that 9K may be targeted for degradation by COOH-terminal determinants, similar to those identified in *E. coli* (Parsell *et al.*, 1990), or by some other uncharacterized pathway.

In order to verify that cleavage is occurring at the AG*S site of P4a and to determine the relative stability of the 9K protein, mutations were introduced at the AG*S and AG*T sites of P4a with the expectation of creating the chimeric proteins 4a-9K (71 kDa) and 9K-23K (32 kDa). The A10L open reading frame of VV, along with its flanking promoter region was cloned into vector pUC119. Site-directed

mutagenesis (Kunkel, 1985) was used to independently mutate the AG*S or AG*T motif to an unprocessed ARS site, thereby creating plasmids pA10LΔAGS and pA10LΔAGT. To transiently express mutant P4a protein precursor, VV-infected cells were transfected with these constructs. Radiolabeled cell extracts were processed for immunoprecipitation and reacted with antiserum P4a-2 which is specific for amino acids 362 - 698 of P4a (VanSlyke *et al.*, 1991). Expression of pA10LΔAGS resulted in the appearance of a unique 71-kDa product derived from P4a processing at only the AG*T site (Fig III.3, lane 1). Similarly, expression of pA10LΔAGT resulted in a unique 32-kDa product derived from P4a processing at only the AG*S site (Fig. III.3 lane 2). It should be noted that in this and previous experiments, P4a-2 antiserum was shown to react well with P4a and 23K, but not with 4a. This would seem to indicate that the epitope recognized in the 4a-9K fusion product is located within the 9K region, providing evidence that the 9K protein is not immunologically silent, and therefore should have been detectable, if not degraded, in previous assays.

It is impossible to determine the relative efficiency of processing for the transiently expressed P4a mutant proteins in such an assay, since endogenous and mutant P4a are indistinguishable. Likewise, it is impossible to estimate the half-life of the 9K product. However, the mere presence of the 4a-9K and 9K-23K fusion proteins suggests that neither the NH_2- nor COOH-terminus of 9K is responsible for signaling its degradation while fused to another protein. Association of 9K with either 4a or 23K, both of which are viral core constituents, may isolate the protein in the assembling particle, thus protecting it from degradation by cellular proteases. Also, the presence of both chimeric proteins implies that processing of P4a results

Figure III.3. Transient expression and immunoprecipitation of P4a mutants. An *Eco*RI - *Hin*dIII fragment of plasmid pJV-1 (VanSlyke *et al.*, 1991), containing the P4a coding sequence and promoter region, was subcloned into pUC119. Site-directed mutagenesis (Kunkel, 1985) was used to change the AG*S and AG*T sites of P4a each into A-R-S amino acids, creating plasmids pA10LΔAGS and pA10L-ΔAGT. Each plasmid was introduced into VV-infected cells by liposome-mediated transfection (Lee and Hruby, 1993). At 4 h post-infection, the liposome-containing medium was replaced with medium containing ^{35}S-methionine/cysteine (du Pont-NEN, Wilmington, DE). After 20 h of infection, cell lysates were prepared, immunoprecipitated with P4a-2 antiserum, electrophoresed on SDS-polyacrylamide gels, and processed for autoradiography (Whitehead and Hruby, 1994b). Mock-transfected cells (lane 3) were transfected with pUC119 vector alone. Mock-infected cells (lane 4) were similarly treated, but received no VV inoculum or plasmid DNA. The migration of ^{14}C-labeled molecular weight markers is shown.

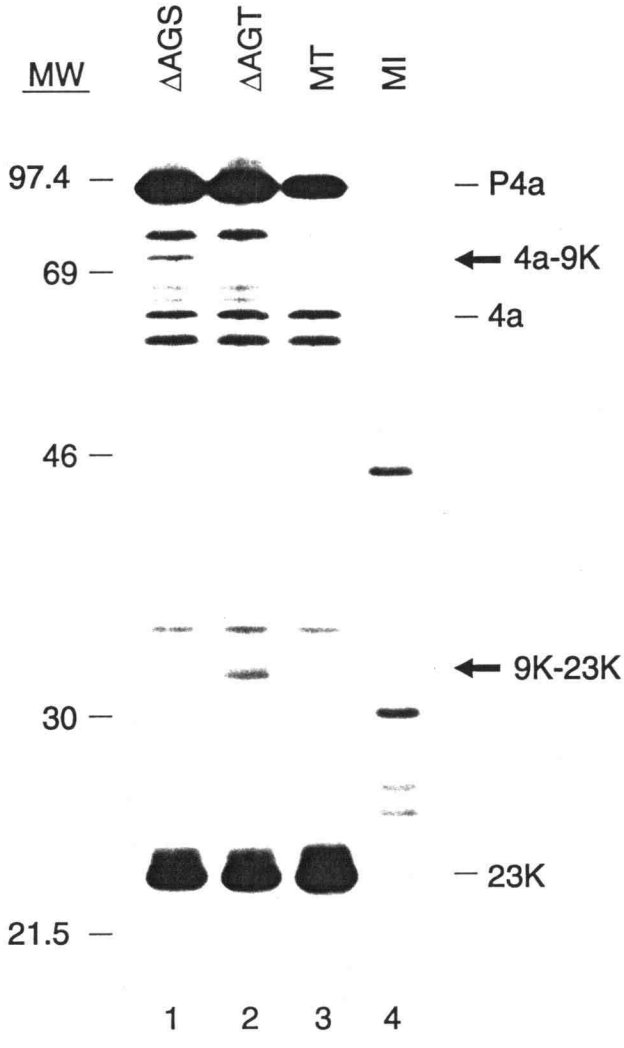


Figure III.3 Transient expression and immunoprecipitation of P4a mutants

from two independent endoproteolytic events, rather than a single endoproteolytic event followed by exoproteolysis to a second site. The fact that a trace amount of 71-kDa product is found in mock-transfected cells (Fig. III.3 lane 3), while the 32-kDa product is found exclusively in cells transfected with pA10LΔAGT, may signify that the 71-kDa product is a bona fide processing intermediate, and that cleavage occurs first at the AG*T site followed by processing at AG*S.

Although the models presented above imply that the 9K protein is rapidly degraded in the course of a normal infection, the protein may have a function. Core protein precursors P4b, P25K, and P17K, as well as P21K are all processed at sites near the NH_2-terminus, resulting in the loss of less than 7 kDa. These NH_2-terminal peptides may act as intramolecular chaperones and direct proper folding of the products they are attached to, similar to the 77-residue propeptide attached to subtilisin (Ikemura and Inouye, 1988). The proposed function of the intramolecular chaperone is to aid self-assembly of polypeptides by inhibiting incorrect folding or aggregation. In the case of VV, these NH_2-terminal peptides may prevent premature folding until the precursor protein is localized to the assembling virion, where it is proteolytic processed and folded into its final conformation. This model is supported in part by the finding that product-length 25K, transiently expressed from a plasmid construct lacking the NH_2-terminal peptide coding region, is inefficiently incorporated into mature virus particles (Lee and Hruby, 1994).

CHAPTER IV
A TRANSCRIPTIONALLY-CONTROLLED *TRANS*-PROCESSING ASSAY: IDENTIFICATION OF A VACCINIA VIRUS-ENCODED PROTEINASE WHICH CLEAVES PRECURSOR PROTEIN P25K

Authors: Stephen S. Whitehead and Dennis E. Hruby

SUMMARY

Proteolytic maturation of at least 5 vaccinia virus proteins has been shown to occur at a conserved AG*X motif. Both viral assembly and subsequent maturation into infectious particles appears to be dependent on these cleavage events. It has been hypothesized that processing of these substrates is dependent on their presentation in or association with the previrion particle. The restrictions imposed by this model may explain previous difficulties in identifying the responsible proteinase. To circumvent this contextual requirement, a transcriptionally-controlled trans-processing assay was developed to study cleavage at the AG*S site of the P25K core protein precursor. Processing at this AG*S site in transiently-expressed P25K does not appear to be contextually regulated. Therefore, the use of this assay in concert with an AraC block of late transcription in VV-infected cells has allowed the systematic rescue of proteolytic processing by co-transfection of a variety of vaccinia virus derived genes. This lead to the identification of a putative VV proteinase encoded by open reading frame G1L. The predicted 68 kDa protein was found to contain a HXXEH sequence which is a direct inversion of the active site consensus sequence present in thermolysin and other metalloendopeptidases. To determine if this region represents the active site of the proteinase, the glutamate and histidine residues were mutated. In the *trans*-processing assay, such mutants showed a total loss of activity compared to the wild-type G1L protein. These findings implicate the G1L protein as a novel, virus-encoded metalloendoproteinase.

INTRODUCTION

Vaccinia virus (VV) represents the most widely studied member of the Orthopoxvirus family. During its replication in the cell cytoplasm, a number of virus-encoded proteins are matured by proteolytic processing, an event that is essential for proper virion maturation. Previous studies have established the connection between virion morphogenesis and proteolytic processing. These include demonstration that several of the VV major core proteins remain unprocessed when virus maturation is blocked by the addition of the drug rifampicin (Katz and Moss, 1970). A similar limitation of core protein processing and virus maturation has been observed in cells infected with maturation-defective *ts* mutants at the non-permissive temperatures (Stern *et al.*, 1977; Dales *et al.*, 1979; Silver and Dales, 1982). More recently, direct evidence was provided by the observation that core proteins are found primarily as precursors in immature viral particles. (VanSlyke *et al.*, 1993). Although proteolysis is required during the final steps of virion maturation, it now appears that assembly of the precursor proteins into immature particles may be a necessary requirement for proteolytic processing. It is this later assumption which may explain the difficulties that have been encountered in demonstrating proteolytic activity of extracts made from either VV-infected cells or disrupted virions used in conjunction with soluble substrates.

To date, five VV-encoded, virion-associated proteins have been shown to be proteolytically processed (Whitehead and Hruby, 1994). Alignment of the cleavage sites in these precursor proteins has revealed a conserved AG*X cleavage motif. The

presence of this novel cleavage motif, in addition to the fact that proteolysis is comparably blocked in at least four of these proteins by rifampicin, would seem to indicate that proteolysis of each substrate may be catalyzed by the same proteinase. It also seems likely that the proteinase is virus encoded, rather than of cellular origin since processing of the major core proteins appears to be identical across a wide variety of host cells.

During development of a *trans*-processing assay for the study of cis-acting sequence elements on AG*X cleavage site definition, a new AG*S cleavage site was identified 14 amino acid residues upstream of the previously identified AG*A site of P25K (Lee and Hruby, 1993). Although the product (25K′) resulting from cleavage at this site is found only in limited quantities in extracts made from normal VV-infected cells, it is seen in much greater quantity among infected cells transiently expressing the P25K precursor protein. Interestingly, little if any 25K′ is assembled in mature virus, and cleavage at this AG*S site does not appear to be blocked by the drug rifampicin (Lee and Hruby, 1993). If proteolysis at this site is catalyzed by the same proteinase responsible for processing of the other core protein sites, it may represent a fortuitous site not restricted by assembly context. Employment of such a cleavage site would be useful in developing an assay to identify the proteinase. Since all known AG*X cleavage substrates are expressed during late times of viral infection, it seemed reasonable to assume that the proteinase would likewise be a late-gene product. In order to systematically screen viral gene products for proteolytic activity, a method was needed to selectively transcribe viral late genes following transfection into infected cells.

Transcription of VV genes is tightly controlled by a cascade mechanism (Moss, 1990b). All the enzymes required for the synthesis and modification of early mRNA are packaged into the infectious virion. Following entry into the cell cytoplasm, early gene expression initiates DNA replication which leads to successive expression of intermediate and late genes. During a normal viral infection, it has been shown that newly-replicated, naked, viral DNA serves as template for expression of the late transcription factor genes A1L, A2L, and G8R, as well as late genes (Keck *et al.*, 1990). 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 cotransfecting plasmid copies of the late transcription factor genes (Keck *et al.*, 1990). This finding became the framework for development of the transcriptionally-controlled trans-processing assay used in this study to selectively rescue processing of the AG*S site of P25K.

Here we report the identification of a novel proteinase encoded by the G1L open reading frame of VV. Amino acid alignment of the predicted G1L polypeptide with known proteinase sequences revealed a conserved HXXEH motif common to a newly described group of metalloendopeptidases (Becker and Roth, 1992). Members of this group display an HXXEH motif at their catalytic site which is a direct inversion of the HEXXH active-site consensus sequence common to other metallo-endopeptidases such as thermolysin (Vallee and Auld, 1990). Site-directed mutagenesis of the putative active site has been used to confirm the G1L protein as a member of the metalloendoproteinase family, a family heretofore not represented by a virus-encoded member.

MATERIALS AND METHODS

Viruses and cells. BSC-40 cells were maintained in modified Eagle's medium (MEM-E; Sigma Chemical Co., St. Louis, MO.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY), 2 mM glutamine, and 10 μg/ml gentamicin. Vaccinia virus (VVTF7-3 and WR) was purified from infected cells as previously described (Hruby *et al.*, 1979).

Viral DNA, cosmids, and plasmid constructions. Vaccinia virus genomic DNA was isolated from purified virus (WR) as previously described (Ausubel *et al.*, 1993). Briefly, purified virus was diluted in lysis buffer (0.5% sodium dodecyl sulfate (SDS), 6% sucrose, 50 mM Tris-HCl [pH 7.8], and 0.002% proteinase K) and incubated at 55°C for 1 h. Following extraction with water saturated phenol and 1:1 phenol/chloroform, DNA was precipitated with 0.05 volume 3 M NaCl and 2.5 volumes of 100% ethanol, microcentrifuged, washed with 70% ethanol, and stored at 4°C in sterile water.

A complete library of overlapping cosmid clones comprising the entire vaccinia virus genome was provided by Dr. Bernard Moss (Baldick and Moss, 1987). Individual plasmid clones containing the three VV late transcription factor genes (pTM.A1L, pTM.A2L, and pTM.G8R) were also provided by Dr. Bernard Moss. Gene expression from these plasmids is controlled by the T7 promoter sequence.

To generate pG1L, a *Eco*RI - *Kpn*I fragment (3.75 kb) containing the G1L promoter region and the entire G1L ORF was isolated from the *Hin*dIII G fragment of the VV genome and cloned into pTZ19U. Site-directed mutagenesis of pG1L was

performed as described by Kunkel (Kunkel, 1985). To generate pG1L.ΔHXXEH, an oligonucleotide 29-mer, SW30, (5′-GGGAATTGCAAGCTTGTTGGAAGATCTAC-3′) was used to mutate His-41 to Ser and His-45 to Asp. To generate pG1L.E44A, an oligonucleotide 30-mer, SW32, (5′-GCTCACTTGCTAGCACATCTACT-TATATCC-3′) was used to mutate Glu-44 to Ala. To generate pG1L.E112A, an oligonucleotide 27-mer, SW33, (5′-CACATTAAAGAGCTAGCAAACGAATAC-3′) was used to mutate Glu-112 to Ala. To generate pG1L.E114K, an oligonucleotide 26-mer, SW34, (5′-GAATTAGAAAACAAGTACTATTTTAG-3′) was used to mutate Glu-114 to Lys. An oligonucleotide 26-mer, SW31, (5′-CTCCTTAGTCCATGG-TGGTCTTACCG-3′) was used to introduce an *Nco*I site at the initiating Met codon of pG1L, which also resulted in the mutation of Ile-2 to Val.

The pUC119-based plasmid pL4R, containing the L4R promoter region and the entire L4R ORF, which encodes the P25K polypeptide was provided by Dr. Peiyu Lee. To generate pL4R.AGS:IDI, an oligonucleotide 33-mer, PL47, (5′-AAATTTGGATTTAATGTCTATAATAACCATTTG-3′) was used to mutate amino acids 31 to 33 of P25K from Ala-Gly-Ala to Ile-Asp-Ile. Likewise, in order to generate pL4R.IDI:AGA, an oligonucleotide 24-mer, PL32, (5′-CTCAGATA-TGATATCGATAAAAAA-3′) was used to mutate amino acids 17 to 19 from Ala-Gly-Ser to Ile-Asp-Ile. All mutagenic events were confirmed by dideoxynucleotide DNA-sequencing (Sanger *et al.*, 1977).

Transcriptionally-controlled processing assay. Confluent BSC-40 cells in 35 mm diameter wells were infected with VVTF7-3 at a multiplicity of infection of 10. At 0 h postinfection, cells were transfected using cationic liposomes as previously described (Lee and Hruby, 1993). Cells in each well were transfected in

the presence of 40 μg/ml AraC (CALBIOCHEM, San Diego, CA) with 0.2 μg each of plasmids pTM.A1L, pTM.A2L, and pTM.G8R, (vLTFs) as well as 2.5 μg of plasmid coding for the substrate protein and 2.5 μg of test DNA. After 4 h of incubation at 37°C, the DNA-containing medium was replaced with MEM-E containing 10% dialyzed fetal calf serum, 40 μg/ml AraC, and 90 μCi/ml ^{35}S protein labeling mix (11.0 mCi/ml [1,175 Ci/mmol]; du Pont-NEN, Wilmington, DE). At 20 h postinfection the cells were lysed for immunoprecipitation by removing the labeling medium and adding 150 μl of radioimmunoprecipitation buffer (RIPA)(150 mM NaCl, 1.0% deoxycholic acid, 1.0% Triton X-100, 0.2% SDS, 50 mM Tris-HCl [pH 7.4], and 100 U/ml Benzonase endonuclease, [EM Science, Gibbstown, NJ]). Cell extract volumes were brought up to 1 ml by the addition of RIPA buffer containing 0.4% SDS, precleared by microcentrifugation, and mixed with 3 μl P25K antiserum overnight at 4°C (VanSlyke and Hruby, 1994) . A 100-μl aliquot of 20% (v/v) Protein A-sepharose (Sigma Chemical Co.) suspension in RIPA buffer was added and the samples were rocked 2 h at 4°C. The protein A-sepharose complex was recovered by centrifugation and washed 3 times with RIPA buffer. The final pellet was resuspended in 25 μl of sample loading buffer (3% SDS, 10% glycerol, 5% β-mercaptoethanol, 100 mM DTT, and 50 mM Tris-HCl [pH 6.8]), heated to 100°C for 3 min and fractionated by discontinuous slab gel electrophoresis on 11% polyacrylamide gels containing SDS (Studier, 1973). The gels were processed for fluorography by soaking in 1 M salicylic acid, dried, and exposed to Kodak XAR5 film at -70°C.

In vitro protein expression. Expression vectors pET3a.G1L and pET3d.G1LΔHXXEH were constructed by cloning a *Nco*I - *Bam*HI or *Nde*I - *Bam*HI

(3.25 kb) fragment from the appropriate pG1L mutant into either pET3a or pET3d (Studier *et al.*, 1990). A 50 µl transcription reaction (80 mM HEPES-KOH, [pH 7.5], 12 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol [DTT], 3 mM each of ATP, CTP, GTP, UTP, 20 U RNasin RNase inhibitor [Promega, Madison, WI], 250 mU inorganic pyrophosphatase [Sigma Chemical Co.], 40 U T7 RNA polymerase [Boehringer Mannheim Corp., Indianapolis, IN], and 5 µg plasmid template) was incubated at 37°C for 1 h, followed by extraction with 1:1 phenol/chloroform and precipitation with 0.5 volume of 7.5 M ammonium acetate (pH 7.5) and 2.5 volumes of 100% ethanol. RNA produced in the transcription reaction was used to program an activated, rabbit reticulocyte lysate (Hruby and Ball, 1981). For radiolabeling of protein, translation reactions were performed in the presence of 0.8 mCi/ml ^{35}S protein labeling mix.

RESULTS

Processing of P4a and P4b occurs in AraC blocked cells transfected with viral DNA. At the onset of this project it was crucial to determine if full-length viral DNA was sufficient to rescue late transcription and proteolytic processing of core proteins in DNA-replication deficient cells. VV-infected cells were treated with the drug AraC at 0 h post-infection and transfected with full-length VV DNA. Immunoprecipitation analysis of radiolabeled cell extracts with antisera specific for P4a and P4b is shown in Fig. IV.1. As expected, AraC completely blocked the synthesis of P4a and P4b, since these are expressed from late genes, whose transcription is dependent on viral DNA replication. Transfection with viral DNA provided template for expression of the late-gene transcription factors, as well as template for P4a and P4b transcription. As shown, not only were P4a and P4b expressed, but they were also processed. Although it is impossible to separate gene expression and proteolysis in such an assay, both processes seemed operational in replication deficient cells.

Expression of ORF G1L is sufficient to rescue processing of P25K. In order to separate substrate expression and proteolysis into two controllable steps, VV-infected cells treated with AraC were simultaneously transfected with genes copies of the following: (i) late transcription factors A1L, A2L and G8R (vLTFs), (ii) substrate, and (iii) test proteinase. Initially, each of six overlapping VV cosmids was assayed as a source for the proteinase gene using P4b as substrate. In spite of ample expression of P4b, processed product was repeatedly not visible following

Figure IV.1. Rescue of expression and processing of P4a and P4b in AraC blocked cells. Monolayers of BSC-40 cells were infected with wild-type VV, treated immediately with AraC, and transfected by liposomes with either control vector pTZ18U, lanes 1 - 3, or full-length VV DNA, lanes 4 - 6. At 4 h post-infection, the liposome-containing medium was replaced with medium containing ^{35}S-methionine/cysteine and AraC. After 20 h of infection, cells lysates were prepared and immunoprecipitated with the indicated antisera and processed for autoradiography. Antiserum 4a1, generated to the NH_2-terminal portion of P4a, recognizes P4a and 4a, while antiserum 4a2, generated to the COOH-terminal portion of P4a, recognizes P4a and 23K (VanSlyke *et al.*, 1991). The migration of ^{14}C-labeled molecular weight markers is shown.

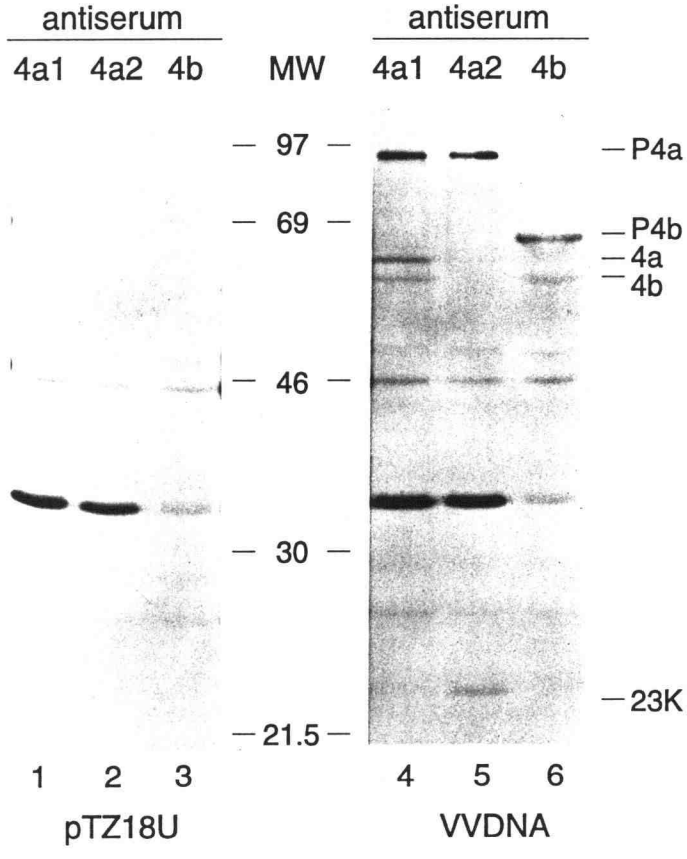


Figure IV.1 Rescue of expression and processing of P4a and P4b in AraC blocked cells

immunoprecipitation (data not shown). In subsequent assays, expression of P25K served as the source for substrate. Proteolytic activity which was apparently specific for the AG*S site of P25K (resulting in the appearance of 25K') was mapped to cosmids 3 and 21 as shown in Fig. IV.2. Because these two cosmids overlap primarily in the *Hin*dIII G region of the VV genome, it was not surprising that a plasmid clone of the *Hin*dIII G region (pBR322.H3G) was also able to rescue processing (Fig. IV.2 lane 7). Primary sequence analysis of the potential ORFs of the *Hin*dIII G region revealed that ORF G1L contained several possible proteinase active-site signatures. Expression of G1L alone (plasmid pG1L) was sufficient to rescue processing (lane 8), indicating that the G1L protein may function as a proteinase.

G1L protein mediates cleavage of P25K at the AG*S site. Full-length P25K has a predicted molecular mass of 28.5 kDa, and is processed during a normal infection into 25K, which has a predicted molecular mass of 24.9 kDa. As shown previously, transiently expressed P25K is processed into 25K, as well as a 26.4 kDa product (designated 25K'). The appearance of 25K' as the sole proteolytic product resulting from co-expression of P25K and G1L protein was unexpected on the basis of previous studies of P25K processing *in vitro* (Lee and Hruby, 1993). In Fig. IV.2, appearance of the 26.4 kDa product correlates with expression of the G1L ORF, however, a protein band migrating at approximately 25 kDa appears in each assay. This lower band may represent a product derived from cleavage at the AG*A site, which would signify autoproteolysis of P25K, since its appearance is independent of transfected test DNA. Alternatively, the lower band could result from translational initiation from an internal ATG codon within the coding sequence. This seemed

Figure IV.2. (A) Rescue of P25K processing in a transcriptionally-controlled assay. Monolayers of BCS-40 cells were infected with VVTF7-3, treated immediately with AraC and transfected by liposomes with the indicated DNA molecules. At 4 h post infection, the liposome-containing medium was replaced with medium containing ^{35}S-methionine/cysteine and AraC. After 20 h of infection, cells lysates were prepared and immunoprecipitated with αP25K antiserum and processed for autoradiography. An arrow indicates the position of the 25K′ product. The migration of ^{14}C-labeled molecular weight markers is shown. (B) Schematic representation of the VV genome and cosmid segments. The upper line shows the *Hin*dIII restriction map of the full-length genome. Below this line the approximate location of each cloned DNA segment is depicted.

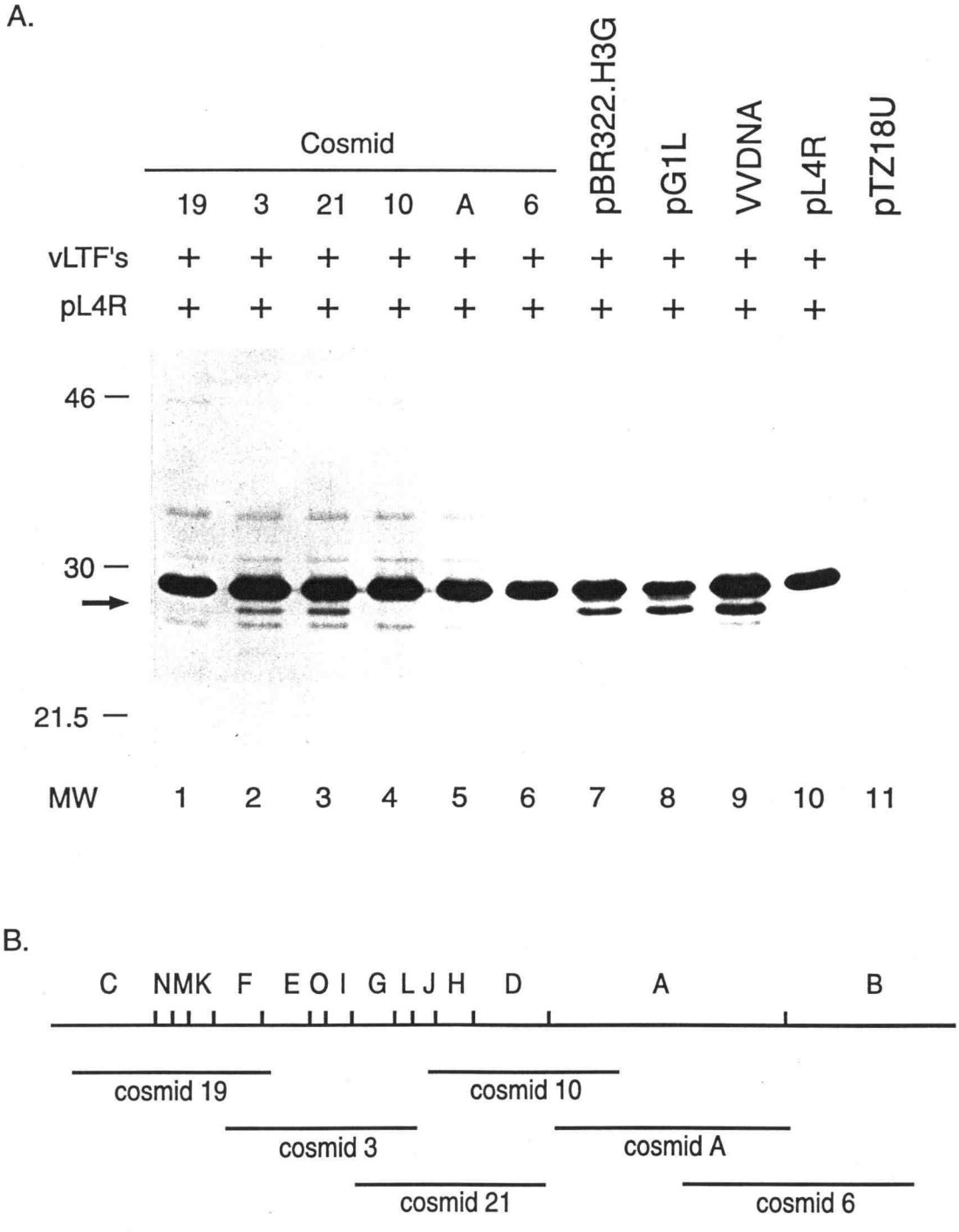


Figure IV.2 Rescue of P25K processing in a transcriptionally-controlled assay

plausible since a methionine residue is located just five residues upstream of the AG*A scissile bond.

In order to verify that cleavage was occurring at the AG*S site, and to determine if the faster migrating protein was derived from cleavage at AG*A, the amino acids at these sites were altered by site-directed mutagenesis. The AG*S and AG*A tripeptide motifs were independently mutagenized to an IDI tripeptide. The ability of the mutants to be processed during co-expression of G1L is shown in Fig. IV.3. Mutation of the AG*S site results in no production of the 25K' product (lane 2), indicating that cleavage of P25K due to expression of the G1L protein is occurring at the AG*S site. On the other hand, mutation of the AG*A site did not abrogate the appearance of the lower band (lane 3). This would suggest that in the current trans-processing assay, P25K is only processed at the AG*S, and that a low level of translational initiation is occurring at an internal ATG codon.

G1L proteinase contains a conserved metalloendopeptidase active-site motif. Amino acid alignment of the predicted G1L protein with known viral proteinases revealed no significant primary or secondary structure homology. However, careful examination of the sequence revealed a motif common to a subset of metalloproteinases (Table IV.1). The mammalian and bacterial insulin-degrading metalloendopeptidases contain the active site sequence HXXEH, embedded in the extended signature, UBUHUUEHZUU (where U signifies uncharged residues, B signifies hydrophobic residues, and Z represents a residue that can be either charged or uncharged)(Becker and Roth, 1992), which is an inversion of the active site of most zinc-dependent metalloendopeptidases. An asparagine-glutamate pair (NE) downstream of the active site is conserved in many metalloproteinases, such as

Figure IV.3. Inactivation of the P25K cleavage sites. Site-directed mutagenesis was used to prepare pL4R derivatives in which the AGS and AGA codons corresponding to residues 17 - 19 and 31 - 33, respectively, were changed to IDI. Expression of P25K from the resulting plasmids pL4R.IDI:AGA and pL4R.AGS:IDI provided test substrate for co-expression with G1L proteinase in a transcriptionally controlled assay. Monolayers of BCS-40 cells were infected with VVTF7-3, treated immediately with AraC and cotransfected by liposomes with pTM.A1L, pTM.A2L, pTM.G8R, pG1L and the indicated pL4R mutants. At 4 h post infection, the liposome-containing medium was replaced with medium containing ^{35}S-methionine-/cysteine and AraC. After 20 h of infection, cells lysates were prepared and immunoprecipitated with αP25K antisera and processed for autoradiography. Core protein markers in lane 4 were prepared by immunoprecipitation using a cocktail of 4a, 4b, and 25K antisera and a radiolabeled, VV-infected cell extract made at 20 h post-infection. An arrow indicates the position of the 25K′product. The migration of ^{14}C-labeled molecular weight markers is shown.

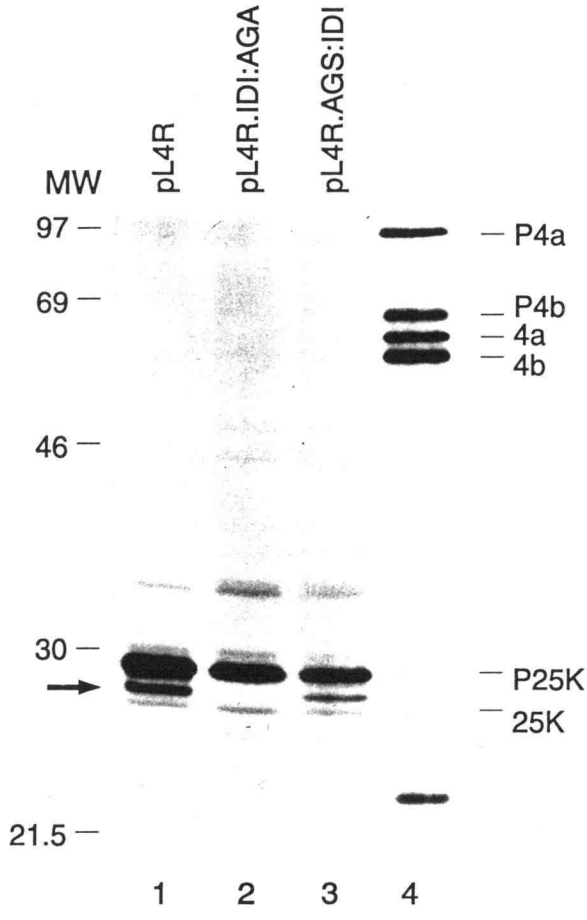


Figure IV.3 Inactivation of the P25K cleavage sites

Table IV.1. Alignment of the proposed active site residues of G1L proteinase and several insulin degrading enzymes and other zinc metalloproteinases.

Enzyme	Sequence	Reference
	* * * * *	
G1L	G I A **H** L L **E** **H** L L I - 64 - E N E	
Protease III	G L A **H** Y L **E** **H** M L F - 38 - E N D	Finch, 1986
dIDE	G L A **H** F C **E** **H** M L F - 8 - E N G	Kuo, 1990
hIDE	G L S **H** F C **E** **H** M L F - 8 - E N E	Affholter, 1988
Thermolysin	V V A **H** **E** L T **H** A V T - 14 - I N E	Kubo, 1988
Aminopeptidase N	V I A **H** **E** L A **H** Q W F - 13 - L N E	Olsen, 1988
Collagenase	V A A **H** **E** L G **H** S L G	Goldberg, 1986

The mutated G1L residues are marked with an asterisk. Collagenase enzyme does not contain a downstream Asn-Glu pair

thermolysin and serves to contribute to zinc binding (Vallee and Auld, 1990). Again, in insulin-degrading enzymes this conserved pair is inverted. As shown in Table 1, this NE pair is conserved in the G1L protein in both the forward and reverse orientation.

Mutagenesis of the G1L proteinase active site. To determine the importance of the HXXEH motif and NE pair in the activity of the G1L proteinase, site-directed mutagenesis of the G1L ORF (pG1L) was used to alter these regions. The HLLEH pentapeptide region (amino acids 41 through 45) was mutated to SLLED, and expression of the mutated protein from vector pG1L.ΔHXXEH showed a total loss of activity in our assay (Fig. IV.4, lane 2), suggesting that this region plays a functional role in the protein, similar to that of metalloproteinases. To verify that full-length protein was synthesized by the wild-type and ΔHXXEH mutant ORFs, these ORFs were respectively subcloned into pET3a and pET3d expression vectors, transcribed *in vitro* using T7 RNA polymerase, and translated in rabbit reticulocyte lysates. Fractionation by SDS-PAGE indicated that both the wild-type and mutant proteins migrated at the appropriate molecular mass (data not shown). It is possible, however, that either of the histidine residues in this region may actually participate in a serine- or cysteine-proteinase catalytic triad. To test this alternate hypothesis, glutamate at residue 44 of wild-type G1L protein was mutated to alanine and the expression of the resulting clone, pG1L.E44A was assayed for its ability to rescue processing. Again, Fig. IV.4, lane 3 shows that this mutant was nonfunctional, which implies that the complete HXXEH domain played a central role in the activity of the proteinase.

Figure IV.4. Genetic inactivation of G1L proteinase. As described in the Materials and Methods section, site-directed mutagenesis was used to prepare the following pG1L mutants: pG1L.ΔHXXEH, pG1L.E44A, pG1L.E112A, and pG1L.E114K. Proteolytic activity of the G1L protein expressed from each mutant was tested in a transcriptionally-controlled assay expressing wild-type P25K as substrate. Monolayers of BCS-40 cells were infected with VVTF7-3, treated immediately with AraC and cotransfected by liposomes with pTM.A1L, pTM.A2L, pTM.G8R, pL4R, and the indicated pG1L mutants. At 4 h post infection, the liposome-containing medium was replaced with medium containing ^{35}S-methionine/-cysteine and AraC. After 20 h of infection, cells lysates were prepared and immuno-precipitated with αP25K antisera and processed for autoradiography. An arrow indicates the position of the 25K′ product. The migration of ^{14}C-labeled molecular weight markers is shown.

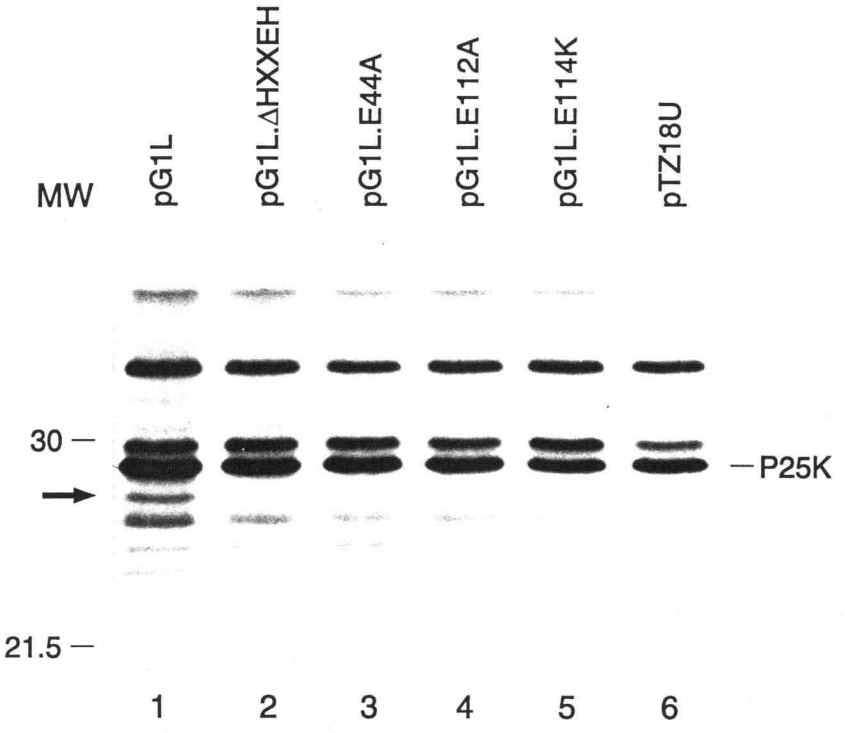


Figure IV.4 Genetic inactivation of G1L proteinase

To ascertain the significance of the NE pair downstream of the putative active site, glutamate residues 112 and 114 of wild-type G1L protein were independently mutated to alanine and aspartate, respectively. Mutants pG1L.E112A and pG1L-.E114D were assayed for expression of functional proteinase as shown in lanes 4 and 5 of Fig. IV.4. Neither the E112A nor the E114D mutant showed activity, implying that both glutamate residues are crucial for proteolytic activity.

DISCUSSION

It has long been recognized that several of the VV late proteins that are incorporated into the virion are derived from larger precursor proteins. A great amount of effort has been expended to elucidate many aspects of this process. The nature of the precursor-product relationship has been established through the use of peptide mapping (Moss and Rosenblum, 1973), monospecific, polyclonal antibodies (Weir and Moss, 1985), and N-terminal microsequencing (VanSlyke *et al.*, 1991). The *cis*-acting sequence elements surrounding the scissile bond have been defined by the use of site-directed mutagenesis (Lee and Hruby, 1993). Differential utilization of the common cleavage motif among known VV proteins has been studied to determine levels of contextual and temporal regulation on proteolytic processing of VV substrates (Whitehead and Hruby, 1994). The drug rifampicin has been used to establish the relationship between virion maturation and proteolytic processing of the major core proteins (Moss *et al.*, 1969a; Moss *et al.*, 1969b; Katz and Moss, 1970; Moss and Rosenblum, 1973). Immunofluorescent studies have indicated that core precursors and products are localized to the virosome of infected cells, and immuno-electron microscopy has further shown that the core proteins are found in the dense centers of both immature and mature virus particles (VanSlyke and Hruby, 1994). Conditional-lethal and ts-mutant viruses deficient in processing have been isolated (Lake *et al.*, 1979; Condit *et al.*, 1983; Miner and Hruby, 1989; Dyster and Niles, 1991; Fathi and Condit 1991a&b). However, little progress has been made towards identifying the necessary proteinase or proteolytic activity required for maturation of

these proteins. Although an alkaline protease has been reported to be associated with purified virions (Arzoglou *et al.*, 1979), it is unclear if it is encoded by the cell or virus and whether it plays a role in proteolytic processing of the core proteins.

Earlier studies have established that although the proteolytic activity responsible for cleavage of P4a and P4b is present within the infected cell, it is not functional or is absent in infected cell extracts or disrupted virions, it is unable to diffuse to a neighboring cell during cell-cell fusions, and it is apparently short-lived (Silver and Dales, 1982). Also, translation of VV late mRNA in rabbit reticulocyte lysate results in the production of full-length precursors proteins with no discernible processing (Cooper and Moss, 1979). Several hypotheses can be presented to explain these results: (i) the proteolytic activity is sequestered to virosomes, which may be tightly associated with cytoskeletal elements of the cell, (ii) substrate precursors must assume a suitable configuration or be preassembled in the maturing particle prior to proteolysis, (iii) the precursor proteins are autoproteolytic, or (iv) the proteinase is simply not present or inactive. A combination of these hypotheses is also possible. Autoproteolysis seems unlikely since no significant homology exists between the precursor proteins, in spite of the fact that they are each processed at a very similar AG*X site. Based on the assumption that the proteinase does exist, that it (or a regulatory cofactor) is encoded by the virus, and that proteolysis is dependent on contextual elements, it became necessary to design an assay that could be used to map the proteolytic activity to a specific viral gene while either preserving or circumventing these essential contextual requirements.

Previous studies by Keck *et al.* have demonstrated that VV-infected cells could be specifically programmed to exclusively express transfected genes driven by

a VV late promoter. This is accomplished by blocking viral genome replication with the drug AraC and providing plasmid copies of the required late transcription factor genes *in trans*, since these genes are transcribed solely from newly synthesized viral DNA. Using this type of transcriptionally-controlled expression, we were able to consistently express precursor protein P25K by transfecting with plasmid carrying the entire L4R ORF preceded by its own authentic, late promoter sequence (Fig IV.2A). Promoter recognition in this system is almost entirely limited to the plasmid copy of the gene, with very little background expression from the endogenous viral genome. (Fig. IV.2A, lane 11).

Proteolytic processing of transiently expressed P25K was initially rescued by cotransfection with full length viral DNA (Fig. IV.2A). This established that proteolysis was not only controlled by a viral gene (or genes), but that the responsible gene is expressed from a late promoter. To rapidly locate the region of the VV genome coding for this activity, an overlapping cosmid library was used for cotransfection. Two of the six cosmids, 3 and 21 rescued processing, which localized the gene to the *Hin*dIII G region of the genome, since this region is overlapped by both cosmids. The predicted amino acid sequences of the ten ORFs in this region were examined for the existence of conserved active site sequences common to known proteinases. No convincing active site sequences were found in common with the serine, cysteine, or aspartyl proteinases. However, the previously uncharacterized G1L ORF contained an active site motif common to a newly described class of metalloendopeptidases, and cotransfection of the G1L ORF was sufficient to rescue processing of the P25K precursor.

Although expression of G1L protein is sufficient for proteolytic processing of P25K, it was repeatedly unable to mediate cleavage of transiently expressed P4a or P4b in our transcriptionally-controlled assay (data not shown). Nevertheless, when these substrates were expressed from full-length viral DNA, both precursor and product species were present (Fig. IV.1). VV infected, AraC blocked cells transfected with full-length viral DNA should theoretically contain a full complement of early, intermediate, and late proteins. The competency of these cells for proteolytic processing compared to cells similarly treated but expressing only G1L protein and P4a/P4b may be explained in several ways. It is possible that processing of these proteins requires an essential cofactor, similar to the short peptide required for activity of the adenovirus proteinase (Mangel *et al.*, 1993). Absence of such a cofactor gene would certainly explain why expression of only G1L protein and P4a is devoid of cleavage products. To test this idea, VV infected, AraC blocked cells were cotransfected with both cosmids 21 and A in combination with each of the other VV cosmids followed by immunoprecipitation with antisera specific for 4a and 4b. The cosmid 21-cosmid A combination provided coding sequence for each of the three late transcription factors, P4a, P4b, as well as the putative G1L proteinase. Although the P4a and P4b precursors were expressed well, processing could not be rescued, even with simultaneous expression of all six cosmids (data not shown). This would suggest that the intact genome molecule itself is required for protein processing in our assay. The presence of this DNA molecule may allow for some degree of previrion assembly, which in turn would place the core structural proteins into a context favorable for cleavage by the G1L proteinase, supporting the notion that VV cleavage maturation is context specific.

In light of the apparent contextual regulation of proteolytic processing during virion replication, the discovery of an AG*X cleavage site excluded from such regulation within a core protein precursor was paramount to the development of a processing assay in which viral assembly was not preserved. In the course of a normal infection, P25K is primarily processed into 25K, which is found assembled into mature virions. Lee *et al.* has shown that transient expression of the L4R ORF in VV-infected cells yields P25K precursor, 25K, as well as a slightly larger product, 25K′, derived from cleavage at a unique AG*S site. In our transcriptionally-controlled processing assay, cleavage at only this alternate AG*S site could be rescued by co-expression of the G1L proteinase. This was verified by the loss of the 25K′ product when the AG*S cleavage site was mutated. Since 25K' is found in only trace amounts during a normal infection, it is unlikely that it plays a role in virus maturation. The absence of this product in virions may reflect a decrease in cleavage efficiency at the AG*S site relative to the AG*A site, thereby masking the presence of any 25K′ intermediates. Since virus assembly, and subsequent processing of the AG*A site is presumed to be blocked in our assay, 25K′ emerges as the only processed product.

The results of site-directed mutagenesis suggest that the G1L proteinase can be classified as a metalloproteinase. Although additional studies are required to determine the role of the G1L proteinase in maturation of the VV structural proteins, this is the first report of a virus encoded proteinase having similarity to any member of the metalloproteinase family. A recent study by Hijikata *et al.* identified an apparent zinc-dependent proteinase activity in Hepatitis C virus that is inhibited by EDTA (Hijikata *et al.*, 1993). Site-directed mutagenesis established the importance

of a conserved histidine and cysteine residue, although the primary protein sequence bears no reported similarity or motif common to any known metalloproteinase. The regions of similarity found between G1L proteinase and other metalloproteinases are shown in Table 1. The conserved HEXXH domain has been identified in many metalloproteinases, most of which show little overall sequence identity to thermolysin outside of this specific domain. (Vallee and Auld, 1990). However, the HEXXH motif of thermolysin is inverted in the G1L proteinase, as well as in a small group of metalloendopeptidases which possess insulin-degrading activity, although the relevance of this activity to the actual physiological role remains unknown. In the case of G1L proteinase, site-directed mutagenesis has established the importance of His-41 and His-45, as well as Glu-44, -112, and -114 in the overall activity of the proteinase. It is on the basis of these findings that we propose to classify G1L protein as a metalloproteinase. Due to the nature of the assay required to demonstrate activity of the G1L proteinase, we are unable to employ inhibitors such as EDTA or 1,10 phenanthroline.

The role that G1L proteinase plays in core protein processing remains unknown. The relevance of its activity to proteolytic maturation is based on the fact that it processes P25K at a consensus site known to be utilized in five viral precursor proteins. There has previously been no function or homology identified with the product of the G1L ORF, even though a homologous ORF containing the exact HXXEH and ENE motifs is conserved in VV strain Copenhagen (Goebel *et al.*, 1990) as well as variola major virus strains Bangladesh (R. Massung, personal communication) and India-1967 (Shchelkunov *et al.*, 1993). Consistent with the precedents of other viral systems (Kräusslich and Wimmer, 1988; Dougherty and

Semler, 1993), and in light of the fact that the processing of VV core proteins occurs among a wide range of host cells derived from different tissues and organisms, one would predict that the responsible proteinase is encoded by the viral genome. Owing to the apparent complex regulation of processing and the identification of VV conditional-lethal mutants defective in core protein processing and virion morphogenesis, it seems likely that a variety of proteins may influence proteolytic processing. Although additional research will be required to determine the part that G1L proteinase plays in VV replication, the current study suggests that VV encodes its own proteolytic enzyme, which appears to belong to the metalloproteinase family.

CHAPTER V
CONCLUSIONS

Analysis of a variety of vaccinia virus proteolytic substrates has allowed us to study the regulation of processing at the consensus AG*X site and has lead to the identification of two new viral substrates. A survey of AG*A-containing vaccinia proteins identified both P21K and P17K as potential precursor proteins whose processing was confirmed by N-terminal microsequencing. The AG*X-containing precursors utilized as proteolytic substrates share several common features which may explain their selection for processing. It appears that both expression during late times of infection and association with the assembling virion are requirements for proper proteolytic processing. Expression or activation of the proteinase only during late times of infection could serve to protect early proteins from deleterious cleavage. Additionally, contextual presentation of the structural precursor proteins during virion assembly may insure that maturational cleavages do not occur until the substrates are preassembled into an immature virion, at which time their respective cleavage sites are made accessible to the proteinase. This is supported by the observation that in cells where previrion assembly is blocked, co-expression of the G1L proteinase and either P4a, P4b or P25K does not yield products processed at any of the relevant maturation cleavage sites.

It should be noted that although expression during late times of infection and association with the previrion are requirements for proteolytic processing, these

criteria are not sufficient to guarantee processing at an AG*X site. The P4a precursor protein meets these requirements and is processed at both an AG*S and AG*T site, but is not processed at an AG*N site, even though the flanking amino acids around this site are similar to those found at other processed sites, and the AG*N motif, when substituted into P25K has been proven to be utilized. This is not, however, the only enigma presented by P4a. Cleavage at AG*S and AG*T should theoretically yield 3 proteins: 4a, 9K, and 23K. Both 4a and 23K are readily detected in purified virions and infected cell extracts, yet the intervening 9K protein has thus far eluded detection. Individual mutation of the AG*S and AG*T sites, followed by transient expression in VV-infected cells, has produced processing intermediates of the appropriate size. Both 4a-9K and 9K-23K intermediates appear to be stable in VV-infected cells, indicating that neither end of the putative 9K product is intrinsically destabilizing, and that fusion of the 9K product to another protein does not target the chimera for degradation. Based on the assumption that the 9K protein is normally turned over rapidly, association with either 4a or 23K as a protein chimera may isolate the 9K in the assembling virion particle, thus protecting it from degradation by cellular proteases. The 9K protein, along with the other small peptides cleaved from P4b, P25K, P21K, and P17K may function as intramolecular chaperones prior to cleavage. These regions may aid in the assembly of precursor proteins, preventing premature folding until the precursor protein is localized to the assembling previrion, where it is proteolytically processed and folded into its final conformation.

Development of a transcriptionally-controlled, *trans*-processing assay has lead to the identification of a proteinase activity encoded by VV open reading frame G1L.

Examination of the predicted amino acid sequence revealed the existence of a novel metalloproteinase active site similar to that found in other known metalloendoproteinases which carry an inverted active site relative to that found in thermolysin. Mutational analysis of the putative active site of the G1L proteinase has established the importance of conserved histidine and glutamate residues, and is the basis for suggesting the enzyme is a metalloproteinase. The G1L proteinase has been shown to cleave at a novel AG*S site found in the P25K precursor protein. Demonstration of its activity at maturational sites found in other core-protein precursors has not been possible due to the strict contextual requirements of these proteins for processing, which may not be maintained in this assay. Therefore, the role that the G1L proteinase plays in core-protein processing remains unknown. Nevertheless, G1L proteinase is the first example of a virus-encoded proteinase with similarity to a member of the metalloendoproteinase family.

In summary, significant progress has been made towards defining the regulation of proteolytic processing in vaccinia virus, as well as identifying a putative maturational proteinase. This body of information, together with further characterization of the G1L proteinase will eventually lead towards a better understanding of the mechanism, regulation, and biological role of proteolytic processing in VV structural protein maturation.

CHAPTER VI
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