AN ABSTRACT OF THE THESIS

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Title: Proteolytic Maturation of Vaccinia Virus Virion-Associated Proteins: Analysis of Substrate Determinants.

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Several vaccinia virus (VV) structural proteins are the proteolytic products of larger molecular-weight precursors. During virus replication, it is believed that the precursors are incorporated into previrions and a limited proteolytic cleavage is coordinated with and required for the virion morphogenesis. Analysis of the amino acid sequences flanking the identified N-termini of the products has revealed a consensus -A-G*X- cleavage motif in the precursors, with cleavage taking place after the glycine residue. In most cases, cleavage takes place at sites located close to the N-termini of the precursors and liberates terminal peptides which possess low pI values.

In order to investigate the substrate requirements for the proteinase recognition and cleavage, a transient expression system in VV-infected cells was established by using an epitope-tagged P25K substrate which allows recognition of the chimeric protein by an epitope-specific monoclonal antibody. Results of the pulse-chase, directed genetics, and rifampin treatment, as well as virion localization experiments confirmed that cleavage takes place in the tagged P25K at the -A-G*A- cleavage motif. An additional cleavage product, 25K', of P25K

was identified which was apparently derived from the utilization of an -A-G*S- cleavage motif. The biological function, if any, of the 25K' protein still remains to be identified.

Using the *trans* processing assay, mutational analyses of the -A-G*A- site in P25K were carried out to define the primary specificity determinants. The results agreed with the -A-G*X- motif proposed previously. With the exception of proline, a variety of residues can be accepted at the P1' position. In contrast, the occupancy of both the P1 and P2 positions is restricted to small residues and jointly may constitute a combinatorial determinant for the cleavage. Involvement of residues beyond the tripeptide motif was suggested by the analyses of deletion or insertion mutants. Mutational analysis concluded that cleavage requires a hydrophobic residue at position P4.

To investigate the potential role of the N-terminal peptides of the precursors in virion localization and/or cleavage substrate specificity, several P25K-derivatives in which the terminal peptide was either deleted or replaced by a heterologous terminal peptide were generated. Overall, the results suggested that the N-terminal peptides of the protein precursors are inter-changeable. The terminal peptide are not required for localization of the precursors to VV previrion, whereas proteolytic removal of the P25K terminal peptide is likely to be crucial for the incorporation of the 25K product into mature VV virions.

In summary, a *trans* processing assay was established and utilized to examine the substrate requirements for the cleavage of VV structural protein precursors. The data suggested the presence of substrate requirements in the terminal peptide region which is likely to be interchangeable among different precursors. The proteolytic cleavage of the precursors apparently requires a hydrophobic residue at P4 and small residues at both the P2 and P1 positions.

PROTEOLYTIC MATURATION OF VACCINIA VIRUS VIRION-ASSOCIATED PROTEINS: ANALYSIS OF SUBSTRATE DETERMINANTS

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INTRODUCTION

Limited Proteolytic Processing

The term "limited proteolysis" was first introduced by Linderstrøm-Lang and Ottesen (1949) to describe reactions in which the peptide bonds in a polypeptide are selectively hydrolyzed, as opposed to protein degradations which involve extensive cleavage of the peptide bonds in the substrate. The enzymes required for the peptide-bond cleavage are named proteases which are divided into peptidases and proteinases (Polgár, 1989). Peptidases are exopeptidases which hydrolyze single amino acids or dipeptide units either from the amino-terminus or the carboxy-terminus of a peptide chain. The proteinases, also called proteolytic enzymes or endopeptidases are capable of selectively recognizing and cleaving specific peptide bonds in the substrates.

The proteinases for limited proteolysis have been divided into four classes according to the participating amino acid residue(s), whose relative three-dimension positions are conserved in each group, and the mechanism of action. The four types of proteinases are (1) serine proteinases which possess a catalytic triad consisting of aspartic acid, histidine, and serine resides; (2)

cysteine proteinases which have cysteine and histidine residues in their catalytic dyad; (3) aspartic proteinases whose catalytic dyad involves two aspartic acid residues; and (4) metalloproteinases which require a metal ion as well as histidine and glutamic acid residues for the catalytic process (for review see Polgár, 1989).

On the basis of the specificity studies of a variety of proteinases, it is generally accepted that for the hydrolysis of the specific peptide bond to take place, two requirements must be met. First, the susceptible peptide bond needs to be defined by the nearby amino acid residues with specific side chains which are required for the primary and secondary specificity. According to Polgár (1987), the primary specificity has a qualitative feature which targets the selection of the scissile bond, and the secondary specificity conveys a quantitative feature by facilitating the cleavage of the selected bond. Some proteinases possess strong primary specificity and apparently require secondary specificity only to a lesser extent. An example is trypsin which cleaves exclusively after basic amino acid residues. In contrast, pancreatic elastase recognizes a 4- to 5-residue peptide backbone instead of one specific amino acid residue. Second, the susceptible peptide bond is usually displayed adjacent to the surface of the substrate in a flexible region accessible to the proteinase, and the susceptible peptide should be presented in a three-dimensional conformation which fits the active site pocket of the proteinase. This is referred to as the conformational specificity (Wright, 1977).

Many types of post-translational modifications, such as phosphorylation, glycosylation, and acylation, are known to be indispensable in the regulation of protein properties including enzyme activity, protein-protein interaction and localization. Likewise, limited proteolytic processing is often used to regulate protein activation or assembly by causing changes in the tertiary structure

which ultimately bring the distant functional amino acid residues together. Interestingly, because the free energy required for the reconstruction of the hydrolyzed peptide bond is high and no biological mechanisms for repairing the broken peptide bond have been identified thus far, the changes introduced into the substrates by the proteolytic cleavage are essentially irreversible. The specificity and irreversibility together render the common utilization of the proteolytic processing reaction a unidirectional regulatory mechanism of a wide variety of biological processes, including food digestion, signal peptide cleavage, signal transduction, peptide hormone/growth factor production, blood clotting, complement pathway, pathogen elimination, cell migration, and reproduction (Neurath, 1989; Hörl and Heidland, 1982; Reich *et al.*, 1975).

Limited Proteolytic Processing in Virus Replication

For many plant and animal viruses, a successful infection cycle is dependent on the limited proteolytic processing at one or more stages. The proteolytic activities can be provided by either the host cells, the viruses *per se*, or both. Proteinases provided by the host cells in general contribute to the processing of membrane/envelope proteins transiting the secretory pathways in cells. These proteins are either retained within specific membrane compartments (such as endoplasmic reticulum and Golgi apparatus) for functions such as virus assembly (Petterson, 1991), or secreted from virus-infected cells. On the other hand, the proteinases which are responsible for the proteolytic processing of viral proteins in the cytoplasm of virus-infected cells are usually encoded by the viruses themselves.

Proteolytic processing of viral polypeptides have been categorized as formative or morphogenic proteolysis, according to the functions these reactions serve during virus replication cycles (Hellen and Wimmer, 1992b). In

order to utilize the production of polyproteins as a strategy for the expression of several viral proteins from single RNA template (e.g., retroviruses and positive-strand RNA viruses), viruses have adopted formative proteolysis as a means to separate structural from nonstructural polypeptides, as well as a mechanism to generate both non-structural and structural proteins from the same polyprotein precursor. After proteolytic cleavage, the viral components either become functionally activated and/or are transported to different compartments of the infected cells.

A number of the viral formative cleavage proteinases encoded by viruses such as picornaviruses, togaviruses, flaviviruses, retroviruses, nodaviruses, comoviruses, nepoviruses, potyviruses, and tymoviruses, have been identified and well characterized (reviewed by Kay and Dunn, 1990; Wellink and Kammen, 1988; Hellen *et al.*, 1989; Hellen and Wimmer, 1992a). The X-ray crystallographic and mutational studies have revealed that the retroviral proteinase is a member of the aspartic proteinases. The two aspartic acid residues required for the catalytic dyad are provided by the dimerization of two polypeptides with individual catalytic centers (Loeb *et al.*, 1989; Blundell, *et al.*, 1990). Another example is the well-characterized trypsin- or chymotrypsin-like cysteine proteinases encoded by several virus groups including picornaviruses, potyviruses, comoviruses, and nepoviruses. On the basis of the active-site geometry, theses enzymes are similar to trypsin- or chymotrypsin-like serine proteinases, although a cysteine residue was found to replace the serine residue as their active site component (Bazan and Fletterick, 1990; Brenner, 1988).

Morphogenic proteolysis, on the other hand, refers to the cleavage of viral structural proteins assembled in previrions during virion maturation. Morphogenic cleavages are usually required for proper virion assembly, but

are dispensable for the assembly of capsid subunits. Morphogenic cleavages occur concomitantly with virion maturation and are usually essential for the acquisition of infectivity of both RNA and DNA viruses, such as picornaviruses, alphavirusis, retroviruses, nodaviruses, adenoviruses, and bacteriophage T4 (for references see Kräusslich and Wimmer, 1988; Hellen and Wimmer, 1992b). Compared to formative proteolysis, far less is known about the morphogenic proteolysis in terms of the responsible proteinase, the determinants of cleavage site recognition, and the mechanism and regulation of cleavage.

Different functions have been proposed for the maturation cleavages in different viruses. Examples include: 1) The maturational cleavage of VP0 into VP2 and VP4 appears to be required for the proper aggregation of picornaviral RNA and capsid components which is essential for transferring packaged RNA into the cytosol and the establishment of infection (Lee *et al.*, 1993); 2) In bacteriophage T4, the precise function of the morphogenic cleavage is unknown. However, it has been proposed that proteolytic cleavage of precursors of bacteriophage T4 structural proteins in the prohead is likely to be required at the DNA packaging step (Hershko and Fry, 1975); 3) In contrast, it has been suggested that the cleavage of adenovirus structural proteins is required for the productive disassembly of virions after entry into host cells (Freimuth and Anderson, 1993), and 4) Morphogenic cleavage of the retrovirus capsid protein precursor appears to be necessary for correct genomic RNA dimerization within premature particles (Stewart,*et al.*, 1990). This conclusion was supported by the observation that a cDNA construct of retrovirus genome expressing inactivated proteinase molecules failed to produce infectious virion particles (Navia and McKeever, 1990).

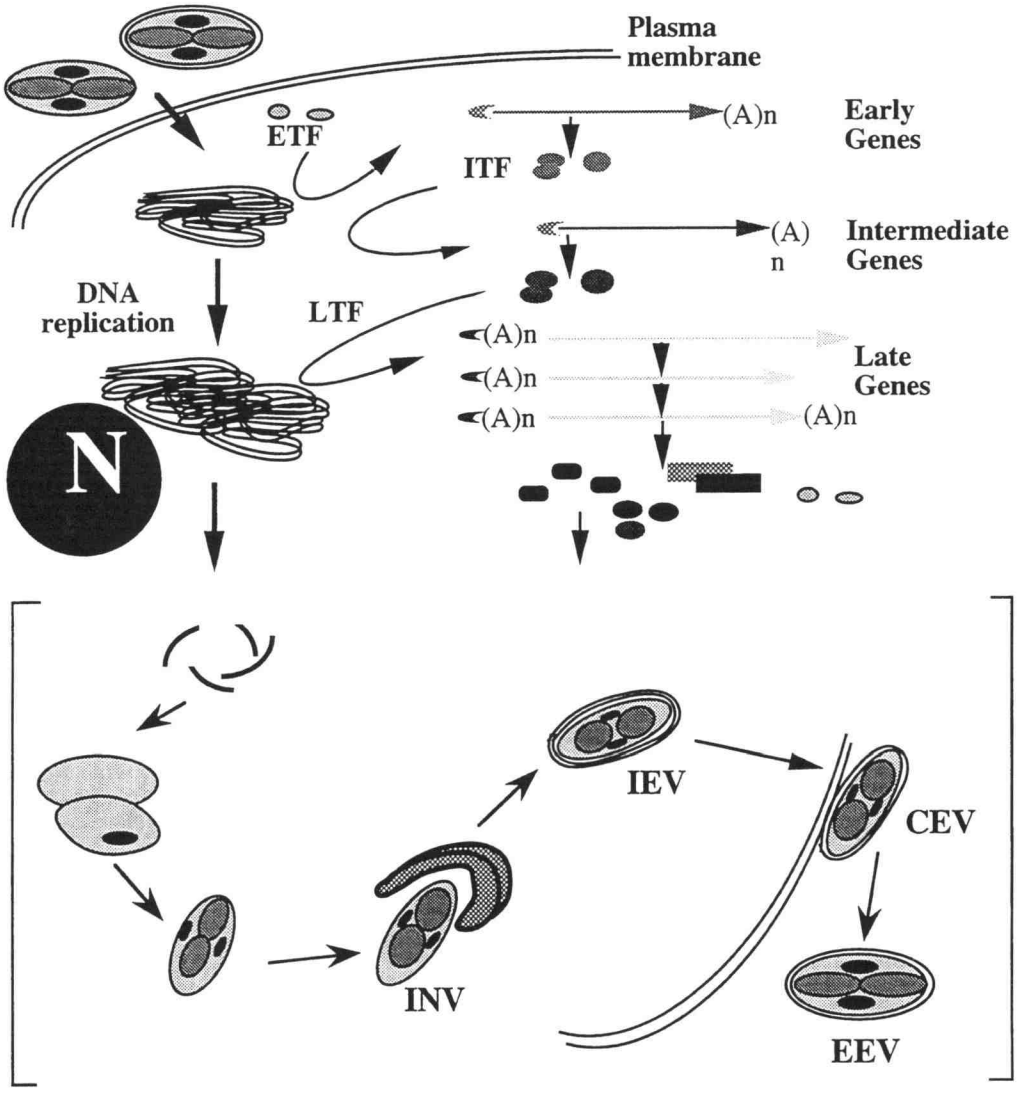
The proteolytic machinery for adenovirus structural proteins which occurs during morphogenesis is perhaps the best characterized among the morphogenic proteinases. The responsible 23-kDa proteinase has been identified on the basis of *ts* mutant studies and proposed to be a trypsin-like cysteine proteinase which requires DNA and a peptide as its cofactors (Bhatti and Weber, 1978; 1979; Webster *et al.*, 1993; Mangal *et al.*, 1993). A consensus -(M/L/I)-X-G-(G/X)*(X/G)- cleavage motif for proteinase recognition and cleavage has been defined using precursor proteins or synthetic peptides (Freimuth and Anderson, 1993; Webster *et al.*, 1989a; 1989b). In bacteriophage T4, the proteinase is known to be encoded by gene 21 and synthesized as a zymogen which is activated by autoproteolytic processing (Showe *et al.*, 1976). In the picornaviruses, the mechanism of the proteolytic cleavage within VP0 at the -N-S- dipeptide for the production of VP4 and VP2 during assembly is still unclear. On the basis of its structural similarity to the active site found in serine proteases, the serine 10 residue of VP2 of human rhinovirus 14 has been suggested to be the catalytic residue for this proteolytic activity. However, the hypothesis was not supported by two mutational analyses in which cleavage of VP2 was still observed when serine 10 is replaced with a different residue (Harber *et al.*, 1991; Lee *et al.*, 1993).

Regardless of the function and operative mechanism, it is essential that the activation of proteinases must be properly regulated to prevent premature cleavage of precursors before assembly. In general, the regulation of proteinases is carried out in a number of different ways, including compartmentalization of the enzymes and substrates, the presence of specific inhibitors, and proteolytic activation of zymogens (Webster *et al.*, 1993). In retroviruses, the relatively acidic environment outside cells after virus particles are released from the cytoplasm has been proposed to activate the morphogenic

cleavage of structural polypeptides by displacing a portion of the *gag-pol* polyprotein which prevents the active site of the proteinase from interacting with its substrates (see Hellen and Wimmer, 1992b for references). Activation of HIV-1 proteinase prematurely was shown to prevent virus assembly and viral infectivity (Kräusslich, 1991). In the adenoviruses, a disulfide-linked peptide derived from one of the structural proteins, pVI, has been suggested to be required for the activation of viral proteinase and hence virus maturation (Webster *et al.*, 1993). The importance of proper inactivation of viral proteinase is best demonstrated by the core protein of Sindbis virus which undergoes auto proteolysis to become inactive after the assembly of the nucleocapsid (Choi *et al.*, 1991). Inactivation of the proteinase activity is accomplished by locating the carboxy terminal region of the protein into the catalytic pocket in concert with the proteolytic cleavage event.

Vaccinia Virus Morphogenesis

Vaccinia virus (VV) is the prototype of the largest and most complex virus family, the Poxviridae, which replicates in virus factories found in the cytoplasm of infected host cells (Fig. I.1) (Moss, 1990). The VV genome consists of a linear double-stranded DNA molecule which contains inverted terminal repetitions of about 11.6 Kbp (Wittek *et al.*, 1978). DNA sequencing of VV (Copenhagen strain) genome has been completed (Goebel *et al.*, 1990). VV genes appear to be closely packed in the 191,636-bp genome which is predicted to encode 263 potential genes, including 198 "major" and 65 overlapping "minor" open reading frames. Both strands of the DNA are transcribed and interspersed with early, intermediate, and late genes which are expressed in a regulated temporal cascade (Fig I.1).



Vaccinia Virus Virion Morphogenesis

Vaccinia virus replication cycle
Figure I.1

Four forms of infectious virus are produced during the course of a "typical" VV infection. They are the intracellular naked virus (INV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and the extracellular enveloped virus (EEV). On the basis of electromicroscopic studies, VV virion morphogenesis is thought to proceed through a complex multi-step process (Fig. I.1) (Dales and Pogo, 1981). Within the virus factories, assembly of virus particles is initiated by the formation of crescent-shaped membranes which subsequently evolve into spherical immature virions with the machinery for early-gene transcription enclosed. Core condensation of different internal components, including the electron-dense materials which are thought to be the viral DNA genome follows. Evidence suggests that packaging of the internal components before the completion of the envelope is critical for the formation of mature virions (Stern and Dales, 1976). Surprisingly, the formation of the membrane structure and enclosed immature particles does not require viral DNA synthesis (Pogo and Dales, 1971). According to the model proposed by Sodeik *et al* (1993), certain viral transmembrane protein(s) are present in the trans-Golgi membranes which facilitate the incorporation of viral core proteins into the immature virions. The membrane structures observed by electron microscopy have previously been postulated to originate from *de novo* membrane biosynthesis (see review by Moss, 1990). However, evidence from recent immunoelectron-microscopic studies on cryosections of VV-infected cells suggested that membrane cisternae derived from the intermediate compartment, possibly the tubular early endosomal cisternae, between the ER and the Golgi stacks are utilized by VV for enwrapping virus DNA and polypeptides (Sodeik *et al.*, 1993; Tooze *et al.*, 1993). The morphological maturation of INV particles is completed by the adoption of the typical brick-shaped, infectious form.

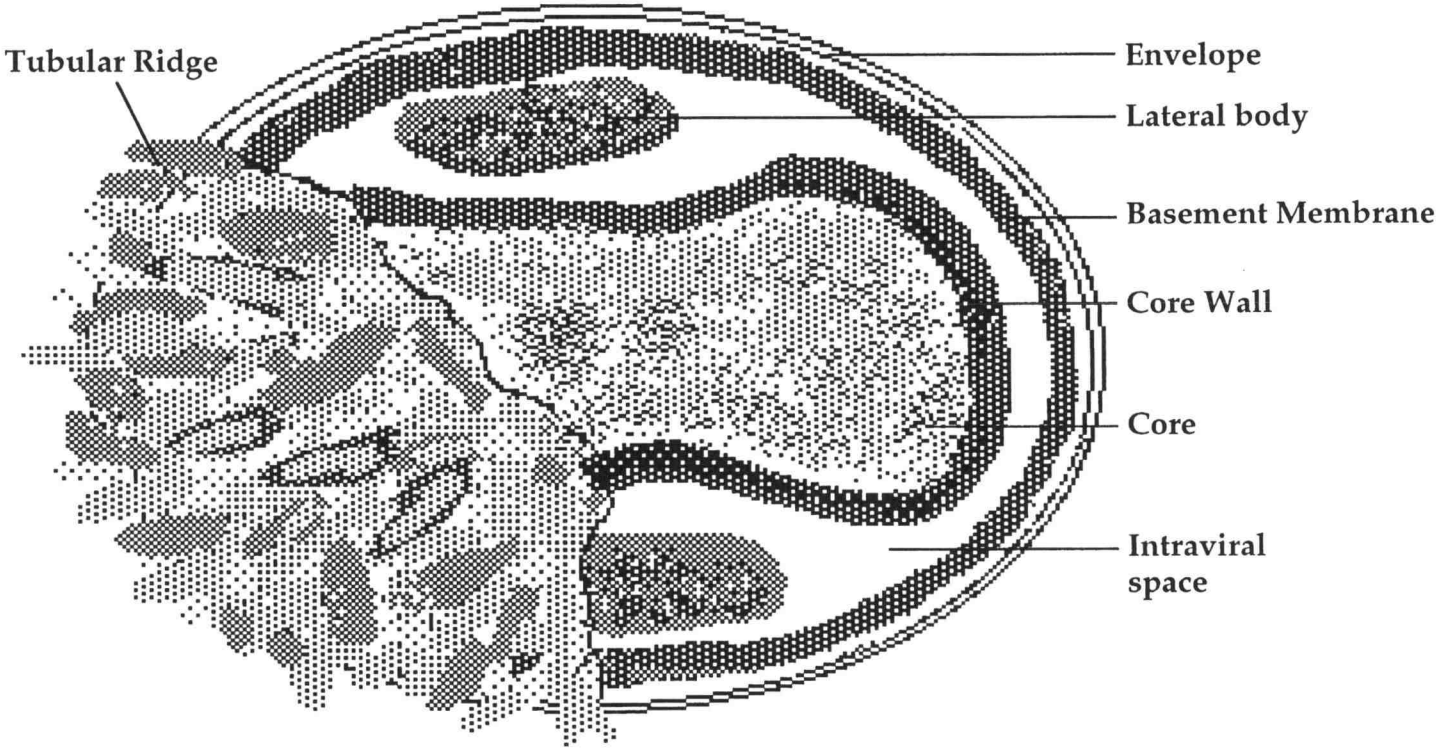
Enwrapment of INV by the Golgi membrane cisternae leads to the triple-membrane form of virus termed intracellular enveloped virus (IEV) (Ichihashi *et al.*, 1971; Hiller and Weber, 1985). This step is shown to be inhibited by glycosylation inhibitors (Payne and Kristensson, 1982). The outer membrane of IEV is then fused with the plasma membrane and EEV particles which possesses double membranes are released outside of the cells (Fenner *et al.*, 1989). The importance of EEV is believed to be for the long-range virus dissemination both in tissue culture and in animals (Payne, 1980; Payne and Kristensson, 1985). A portion of the IEV virus particles are found associated with host cell plasma membrane and can be released after trypsin treatment of infected cells. These CEV particles are thought to mediate spread of VV to neighboring cells (Blasco and Moss, 1992).

The morphogenesis of VV virus particles is reversibly inhibited by the drug rifampin, however VV DNA synthesis, late-gene transcription and translation appear to be unaffected (Moss *et. al.*, 1969; Esteban, 1977). Rifampin-resistant mutants have been isolated and mapped to the D13L ORF which encodes a 65-kDa protein expressed at late times during virus infection (Tartaglia and Paoletti, 1985; Baldick and Moss, 1987, McNulty-Kowalczyk and Paoletti, 1993). An apparent requirement of the D13L protein for an early stage in virion morphogenesis is indicated by the block of both VV maturation at the immature-particle stage as well as the proteolytic processing of major core proteins when a conditional lethal mutant of D13L is grown under non-permissive conditions (Zhang and Moss, 1992). Rifampin is believed to block the translocation of the D13L protein from its cytoplasmic pool to the virus factories (Miner and Hruby, 1989). The finding that wild-type D13L gene is identified in two rifampin-hypersensitive mutants suggested that other viral

functions may also be affected by rifampin (McNulty-Kowalczyk and Paoletti, 1993).

Vaccinia Virus Structural Proteins

On the basis of two-dimensional gel electrophoresis studies, approximately 100 different polypeptides are present within VV (IHD-W strain) virion particles (Essani and Dales, 1979; Oie and Ichihashi, 1981). Several virion-associated proteins are enzymes involved in nucleic acid metabolism, including RNA polymerase, Poly(A) polymerase, RNA triphosphatase, RNA guanylyltransferase, adenosine triphosphatase, DNA topoisomerase, and deoxyribonuclease (see Moss, 1991 for references). Several strategies, including freeze-etching, *in situ* labeling with iodine and fluorescein isothiocyanate, and solubilization with different detergents and/or reducing agents have been used to resolve different virus compartments within the VV virion (Medzon and Bauer, 1970; Sarov and Joklik, 1972; Ichihachi *et al.*; 1984; Sososki and Holowczak, 1981). For example, supercoiled nucleoprotein complexes consisting of DNA and four polypeptides of 90-, 68-, 58, and 10-kDa molecular mass can be released after treatment of vaccinia virus with 1% SDS in the absence of reducing agents (Soloski and Holowczak, 1981). Five major structural compartments can be delineated from these studies: a biconcave core with a tightly coiled DNA genome, a core wall, two lateral bodies, a basement membrane and a coat layer (Fig. I.2). Virion-associated proteins can then be categorized on the basis of their subviral localization. Characterization of virion-associated structural proteins identified so far will be discussed in the next section. The nomenclature of the open reading frames by Geobel *et al.* (1990) on the basis of the DNA sequence of VV Copenhagen strain will be employed.



Vaccinia virus virion structure
Figure I.2

EEV Membrane proteins

Nine out of the ten proteins localized on the EEV outer envelope have been shown to be glycosylated (Payne, 1978). Three of the EEV polypeptides, namely 37-, 22 to 24-, and 42-kDa polypeptides, have been demonstrated by genetic studies to be essential for the envelopment of INV to become EEV (Blasco and Moss, 1991; Schmutz *et al.*, 1991, Duncan and Smith, 1992; Engelstad *et al.*, 1992). The 37-kDa palmitylated protein, encoded by F13L ORF, is a late gene product. Although not glycosylated, it has been found also in Golgi-derived membranes (Dales and Pogo, 1981).

The 42-kDa glycoprotein is encoded by the B5R ORF and possesses amino acid sequence homology to complement control factors (Kotwal and Moss, 1988). The N-terminal 19 amino acids are removed as the signal peptide to form the mature 42-kDa protein (Isaacs *et al.*, 1992). Deletion mutants lacking the B5R ORF produce normal yields of INV which does not become enwrapped by the Golgi-membrane for the formation of EEV (Engelstad and Smith, 1993).

The 22 to 24- kDa glycoprotein is encoded by the A24R ORF and expressed during the late stage of VV infection. Similar to the situation with B5R, without the expression of the 22- to 24-kDa glycoprotein, INV apparently is not enveloped by Golgi-derived membrane. Consequently, the egress of EEV is blocked (Duncan and Smith, 1992).

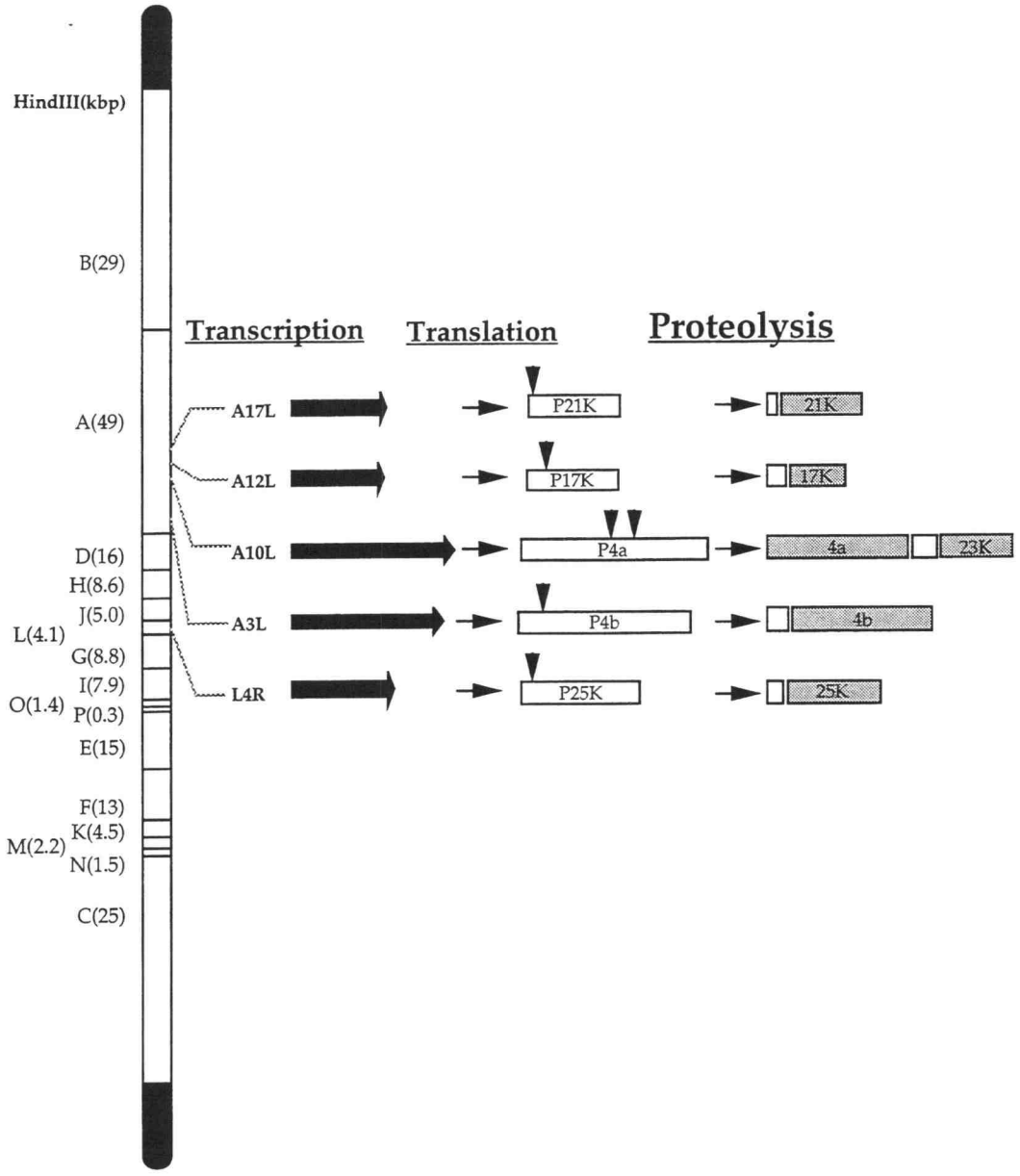
The molecular mass of VV hemagglutinin (HA), encoded by A56R (Shida, 1986), is predicted to be approximately 32 kDa. However, it migrates in SDS-polyacrylamide gels as a 58-kDa protein (Weintraub and Dales, 1974). Removal of a signal peptide during formation of the glycoprotein has been suggested on the basis of the difference observed between the protein synthesized in vitro and the one made *in vivo* in the presence of glycosylation inhibitor (Shida and Dales, 1982).

INV membrane proteins

A 58-kDa protein (p58) was found to be a constituent of the surface tubules structures of INV (Stern and Dales, 1976). Like other proteins found at or near the surface, p58 integrates into maturing virus in the late stage of VV morphogenesis. Antiserum against the tubule protein appeared to neutralize virus infectivity and inhibit cell-cell fusion.

The 14-kDa fusion protein which is encoded by the A27L ORF is present in INV envelope as a complex of disulfide-linked trimer (Rodriguez *et al.*, 1987). Like some of the EEV membrane-specific proteins, inducible expression of A27L from a IPTG-dependent VV recombinant has demonstrated that the 14-kDa protein is non-essential for INV production, but plays a role in enwrapment of INV by Golgi membrane and egress of EEV (Rodriguez and Smith, 1990). The N-terminal region of the 14-kDa protein has been shown to be responsible for the fusion activity of the VV-infected cell membrane and possibly virus penetration (Gong *et al.*, 1990). Furthermore, the 14-kDa protein has been suggested to be anchored to INV by forming a complex with a virion-associated protein of approximately 21 kDa (Rodriguez and Esteban, 1987; Rodriguez *et al.*, 1993). The 21-kDa protein, which is a putative transmembrane protein and could be released from VV virions after detergent treatment, has been demonstrated by N-terminal microsequencing to be the proteolytic processing product of A17L ORF product. The P21K precursor is expressed at late times as a 23 kDa precursor (Fig I.3) (Whitehead and Hruby, in press).

Whitehead and Hruby (in press) have also shown that a 17-kDa protein is the proteolytic product of a precursor polypeptide encoded by the A12L ORF (Fig. I.3). The 24-kDa precursor protein is expressed at late times in



Genome locations and expression of vaccinia virus virion protein precursor genes
Figure I.3

infection. The 17-kDa protein can be solubilized from purified virions by treatment with mild detergent.

Another integral component of the INV envelope is designated Ag35, encoded by the H5R ORF (Gordon at al., 1988). Unlike most of the structural proteins, Ag35 is expressed in the presence of DNA-synthesis inhibitors, indicating that it is an early viral protein. It has been proposed that A35 is required for assembly of VV envelopes for the formation of immature virions.

D8L has been shown to encode a 32-kDa INV transmembrane protein with a major extraviral domain which has been suggested to play a role in cell surface binding (Niles and Setto, 1988, Maa *et al.*, 1990). The gene product is expressed at late times in infection, and does not appear to be glycosylated. Amino acid sequence homologies to the attachment glycoprotein VP7 of rotavirus and of transmembrane proteins were found in the C-terminal region of the 32-kDa protein, whereas a N-terminus domain has sequence similar to carbonic anhydrases. The functional significance of this later observation is unknown.

Finally, two myristylated proteins, 25- and 35- kDa, have been identified as INV-associated proteins. Not much is known about the 35-kDa protein. The 25-kDa protein is encoded by the L1R ORF and expressed at late times during virus infection (Franke *et al.*, 1989). It was shown that the 25-kDa protein is located on the outside of INV membrane. Furthermore, the N-terminus 12 amino acid sequence is sufficient for myristylation and myristylation is required for localization of a fusion protein to the INV membrane (Ravanello *et al.*, 1993).

Core proteins

VP11 is a late gene product and has been shown to be phosphorylated at two serine residues (Kao and Bauer, 1987). The gene coding for VP11 has been

mapped to F17R ORF (F18L in WR strain, Zhang and Moss, 1991). Furthermore, VP11 has been identified as a DNA binding protein with a preferential binding affinity to superhelical DNA. The similarity of VP11 to histone H1 protein suggested VP11 may be involved in the process of DNA packaging, specifically the condensation of viral DNA (Kao *et al.*, 1981; Kao and Bauer, 1987). The production of aberrant core structures was observed when infections with a VV recombinant inducibly expressing the 11-kDa proteins were carried out in the absence of the inducer (Zhong and Moss, 1991).

Expressed late in infection, D2L and D3R proteins (16.9 and 27 kDa, respectively) have been demonstrated as two detergent-insoluble core structural proteins in immunoblot assay of subvirion fractions (Dyster and Niles, 1991). Several *ts* mutants mapped to these two genes possess the same phenotype which includes defect in the proteolytic processing of the major core protein precursor, P4a (Condit *et al.*, 1983; Ensinger, 1982; Dyster and Niles, 1991). Studies of these mutants have suggested that both proteins are inserted into an immature core structure which leads to significantly increase of the stability of both proteins.

Two of the major core proteins, 4a and 4b, constitute approximately 14 and 11%, respectively, of the virion by mass (Sarov and Joklik, 1972). Results from pulse-chase experiments, tryptic peptide analysis, and inhibition by the drug rifampin (Moss and Rosenblum, 1973, Pennington, 1973; Pennington, 1974; Katz and Moss, 1970b) suggested that both 4a and 4b are derived from the larger molecular-weight precursors P4a and P4b. The genes encoding P4a and P4b have been mapped to A10L and A3L ORFs, respectively (Fig I.3) (Wittek *et al*, 1984; Rosel and Moss, 1985, Van Meir and Wittek, 1988). A 50% conversion of P4a to 4a requires about 1 to 2 hours (Katz and Moss, 1970a). A 30- to 45-min lag was observed between the synthesis of P4b and the detection of appreciable

level of 4b product (VanSlyke *et al.*, 1991a). The P4a precursor also is processed at a second site near the C-terminal region to produce another major core protein designated 23-kDa protein (VanSlyke *et al.*, 1991b).

The other major core protein, 25K, is also called VP8 according to Sarov and Joklik's assignment (1972). The 25K protein constitutes 8% of VV virion (Sarov and Joklik, 1972) and appears to be a major DNA binding protein (Yang and Bauer, 1988). The precursor P25K, encoded by L4R ORF (Fig I.3) (Weir and Moss, 1985), has a molecular mass predicted to be of 28 kDa and is synthesized as a viral late protein. Similar to 4b, production of 25K from P25K requires a 30- to 45-min delay (VanSlyke *et al.*, 1991a).

Overall, less than one fifth of the estimated 100 polypeptides constituting VV virions have been identified so far. All of the VV virion-associated proteins identified so far, with the exception of Ag35, are translated late in infection. Many of them are processed by different types of post translational modification, such as glycosylation, phosphorylation, myristylation, palmitylation, and proteolytic cleavage. However, the structural organization of VV virion proteins, the mechanism of virion assembly, the function of the proteins *per se* as well as their post translational modifications are still poorly understood.

Proteolytic Processing of Vaccinia Virus Virion Proteins

Six of the VV virion proteins identified thus far, namely 4a, 4b, 25K, 23K, 21K, and 17K, are derived from larger molecular-weight precursors (VanSlyke *et al.*, 1991a, 1991b, Whitehead and Hruby, in press). The proteolytic cleavage of these virion-associated proteins is believed to be essential for the virus replication, although the roles of these reactions still remain an enigma to us. Proteolytic cleavage of P25K to remove the amino-terminal 32 amino acid

residues has been suggested to be 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, the function can not be applied to the other proteolytically matured proteins which have not been shown to interact with viral DNA. Therefore, similar to the morphogenic proteolytic processing of structural proteins in other viruses, proteolytic cleavage of these precursor proteins may be essential for inducing specific conformational changes to these proteins which are required for proper rearrangement of the immature virion into a mature infectious virion with the appearance of condensed core and for the acquisition of infectivity. This hypothesis is supported by the following observations. (i) The products, such as 4a, were detected exclusively in a particulate form during virus infection (Katz and Moss, 1970a; Pennington, 1973). The synthesis of precursor proteins is believed to take place elsewhere in the cytoplasm on the basis of the observation that polyribosomes are not localized around virosomes (Morgan, 1976). (ii) Mature VV cores could be visualized by electron microscopy at the time the cleavage products are detected after the removal of rifampin (Katz and Moss, 1970a). (iii) Reversible interruption of morphogenesis caused by the antibiotic rifampin was observed more than two decades ago (Moss *et al.*, 1969; Pennington *et al.*, 1970b). In the presence of rifampin, synthesis of core protein precursors appears to be normal whereas cleavage products can not be detected (Katz and Moss, 1970b). (iv) Likewise, concomitant block of VV morphogenesis and proteolytic processing was observed when several *ts* mutants were incubated at the non permissive temperature (Stern *et al.*, 1977; Silver and Dales, 1982). (v) Aberrant core structures were formed in the absence of the major core protein processing when the synthesis of VP11 was suppressed from a inducible VV recombinant (Zhong and Moss, 1991). (vi) A P4a precursor-specific antiserum strongly

reacted with virosome with immature particles but weakly with the mature virions (VanSlyke and Hruby, in press)

When the alignment of the predicted amino acid sequence of the P4a, P4b, P25K, P21K, and P15K precursor proteins at the regions surrounding the amino terminal sequence of the cleavage products are examined, a conserved -Ala-Gly*Ala/Ser/Thr- motif was noted (Fig I.4). It is therefore possible that a single proteinase activity is responsible for the cleavage in all precursor proteins, on the basis of the consensus motif in precursors as well as the virion localization of the products. One contradictory observation was made by Dyster and Niles (1991) stating that proteolytic processing of P25K was unaffected, while P4a and to a lesser extent P4b is blocked, at the nonpermissive temperature during the infection of *ts* mutants that map in genes D2L and D3R. Moreover, the proteinase factor(s) has been suggested to be an indiffusable component on the basis of the result that proteolytic maturation of VV structural proteins takes place when cells were coinfected with wild-type and cleavage defective *ts* mutant but not after fusion between cells singlely infected with wild-type or cleavage defective *ts* mutant virus (Silver and Dales, 1982). The result that proteolytic processing of P4a, P4b and P25K is blocked in the presence of cycloheximide suggested that virus-encoded proteins, possibly the proteinase(s), are responsible for the cleavage (VanSlyke *et al.*, 1991a).

Conclusions

Taken together, organization and function of the components in the complex VV virions are still poorly characterized. Besides precise regulation of gene expression at the transcription and translation levels, the structural proteins have to be properly assembled and modified for the virus to

successfully accomplish a replication cycle. Different post-translational mechanisms are apparently employed by the virus for regulating the proper assembly of various components at the right time and right place.

Proteolytic cleavage of VV structural polypeptides is essential for virion maturation. In order to obtain a better understanding of the role of proteolytic processing of VV virion proteins during the virus life cycle, the following questions need to be addressed. At which stage during virion maturation are the precursor proteins cleaved? What are the participating components, including the proteinase factor(s) and additional substrates? Are they encoded by VV, host cells, or both? What is the mechanism of the proteolytic cleavage? Is it an endo- or exoproteolytic reaction, or both? How is the proteolytic machinery regulated? Where within the precursor proteins are they cleaved? What are the determinants residing in the substrates for defining the cleavage site specificity? How do the participating components interact with each other? Are the smaller peptide produced from the substrates after proteolytic cleavage only desposable fragments? If not, what are their roles?

Within this context, the purpose of this thesis is to focus on the establishment of a proteolytic processing assay to enable the definition of the substrate determinants for the proteolytic cleavage of VV virion-associated proteins. Specifically, it involves the identification and detailed dissection of the specificity determinants around the cleavage motif within the substrates for proteinase recognition and cleavage. In addition, the possible roles of the terminal peptide fragments of the substrate proteins in proteolytic processing and/or virion localization will be investigated.

Chapter II

TRANS PROCESSING OF VACCINIA VIRUS CORE PROTEINS

Authors: Peiyu Lee and Dennis E. Hruby

Summary

The three major vaccinia virus (VV) virion proteins (4a, 4b and 25K) are proteolytically matured from larger precursors (P4a, P4b and P25K) during virus assembly. Within the precursors, Ala-Gly-X motifs have been noted at the putative processing sites, with cleavage apparently taking place between the Gly and X residues. To identify the sequence and/or structural parameters which are required to define an efficient cleavage site, a *trans* processing assay system has been developed by tagging the carboxy-terminus of the P25K polypeptide (precursor of 25K) with an octapeptide "FLAG" epitope which can be specifically recognized by a monoclonal antibody. By using transient expression assays with cells coinfected with VV, the proteolytic processing of the chimeric gene product (P25K:FLAG) was monitored by immunoblotting procedures. The relationship between the P25K:FLAG precursor and the 25K:FLAG cleavage product was established by pulse-chase experiments. The *in vivo* cleavage of P25K:FLAG was inhibited by the drug rifampin, implying that the reaction was utilizing the same pathway as authentic VV core proteins. Moreover, the 25K:FLAG protein was found in association with mature virions in accord with the notion that cleavage occurs concomitantly with virion assembly. Site-directed mutagenesis of the Ala-Gly-Ala motif at residues 31 to 33 of the P25K:FLAG precursor to Ile-Asp-Ile blocked production of the 25K:FLAG

product. The efficiency of 25K:FLAG production (33.71%) is, however, approximately only half of the production of 25K (63.98%) within VV-infected cells transfected with pL4R:FLAG. One explanation for the lower efficiency of 25K:FLAG production was suggested by the observation made in the immunofluorescent staining experiment that P25K:FLAG related proteins were not specifically localized to the virus assembly factories (virosomes) within VV-infected cells, although virosome localization was prominent for P25K related polypeptides. Since VV core protein proteolytic processing is believed to take place during virion maturation, only the P25K:FLAG which was assembled into immature virions could undergo proteolytic maturation. Furthermore during these experiments, a potential cleavage intermediate (25K') of P25K was identified. Amino acid residues 17 to 19 (Ala-Gly-Ser) of the P25K precursor were implicated as the intermediate cleavage site, since no 25K':FLAG product was produced from a mutant precursor in which the sequence was altered to Ile-Asp-Ile. Taken together, these results provide biochemical and genetic evidence to support the hypothesis that the Ala-Gly-X cleavage motif plays a critical role in VV virion protein proteolytic maturation.

Introduction

The vaccinia virus (VV) virion is a large complex oval or brick-shaped structure. Five major compartments of the intracellular naked virus (INV) particle can be differentiated by the use of chemical and detergent treatments in conjunction with electron microscopic studies: (i) a biconcave-shaped core containing the viral genome and basic viral proteins, (ii) the core wall with bilaminar appearance, (iii) two lateral bodies with unknown functions, (iv) a basement membrane, and (v) a coat layer consisting of both lipids and viral proteins (Ichihashi et al., 1984). Although the precise sequence of biochemical events which occur during VV morphogenesis are still unclear, the whole process has been divided into a sequence of developmental stages by electron microscopic analysis (Dales and Pogo, 1981). The assembly of VV virions is initiated by the production of rigid crescent-shaped bilayer structures covered with spicules on their convex surface. The crescent-shaped structures progress into immature viral particles, which are spheres filled with a uniformly distributed nucleoprotein. With time, the nucleoprotein condenses and the immature particles are converted into maturated infectious intracellular naked virus particles. Virion maturation and proteolytic processing of the major VV structural proteins are coordinately blocked by rifampin treatment (Katz and Moss, 1970b; Miner and Hruby, 1989; Moss and Rosenblum, 1973; Zhang and Moss, 1992). Likewise, virion protein processing is blocked at the nonpermissive temperature in several maturation-defective groups of temperature-sensitive mutants (Dales et al., 1978; Lake et al., 1979; Stern et al., 1977). These observations suggest that proteolytic cleavage of the VV

core proteins occurs in an assembly-dependent manner. In addition to the proteolytic processing of polypeptides to activate different functional domains, as in the RNA viruses (Wellink and van Kammen, 1988), proteolytic maturation of viral structural proteins during viral morphogenesis is a common theme which has previously been noted for several different groups of RNA viruses (picornavirus [Hellen and Wimmer, 1992b], nodavirus [Gallapher and Rueckert, 1988], retrovirus [Ashorn et al., 1990]), and DNA viruses (T4 phage [Black and Showe, 1983], and adenovirus [Weber, 1976]).

Previous studies using tryptic peptide mapping, pulse-chase analyses, monospecific polyclonal antibodies, and N-terminal microsequencing procedures (Katz and Moss, 1970a; Moss and Rosenblum, 1973; Rosel and Moss, 1985; Sarov and Joklik, 1972; VanSlyke et al., 1991a; 1991b; Weir and Moss, 1985; Yang and Bauer, 1988; Yang et al., 1988) have demonstrated that three major proteins found in VV virions, 4a, 4b and 25K, are the proteolytic cleavage products of higher molecular weight precursors, P4a, P4b, and P25K, respectively. Synthesis of viral DNA and polypeptides takes place within the cytoplasm of host cells in a well-regulated temporal fashion (for a review, see Moss, 1990). As might be expected for structural proteins P4a, P4b and P25K are all synthesized and proteolytically matured at late times during VV infection. Analysis of the amino-terminal sequences of the mature 4b and 25K product polypeptides (VanSlyke et al., 1991a; 1991b; Yang and Bauer, 1988) suggests that the P4b and P25K precursors are both cleaved at an internal Ala-Gly-Ala tripeptide motif to remove the amino-terminal 62 and 31 amino acids, respectively. The identical Ala-Gly-Ala signal is conserved at precisely the same location of the homologous P4b and P25K open reading frames (ORFs), in a distantly related avipoxvirus,

fowlpox virus (Binns et al., 1989; 1988). Proteolytic maturation of the VV P4a protein does not follow the same pathway. Rather, at least two smaller nonoverlapping products, 4a and 23K, are derived from the P4a precursor and become virion constituents. N-terminal microsequencing of the 23K polypeptide indicates that it is produced by cleavage of the carboxy terminal portion of the P4a precursor at the Ala-Gly-Thr site at residues 696 to 698. Peptide mapping experiments suggest that the 4a protein is produced by a second cleavage at the Ala-Gly-Ser site found at residues 613 to 615 of the precursor. The fate of the intervening peptide (residues 615-697) is not yet known (VanSlyke et al., 1991a, VanSlyke et al., 1991b). In any case, analysis of the known cleavage sites of the P4b, P25K, and P4a precursors reveals the consensus sequence, Ala-Gly-X, with cleavage taking place after the Gly residue. No other common sequence or structural elements were evident.

Specific scission of core protein precursors during virion morphogenesis has also been noted for other large DNA-containing viruses such as adenovirus and African swine fever virus. The cleavage of adenovirus core protein precursors is linked to virus maturation and the cleavage sites identified within a conserved Gly-Gly-X motif (López-Otin et al., 1989; Weber, 1976). Likewise, three structural proteins of African swine fever virus, which shares many properties with poxviruses, also are derived from precursors with the proteolytic cleavage taking place between Gly and Ala/Gly in the sequence Gly-Gly-Ala/Gly (López-Otin et al., 1989). Obviously, the Ala-Gly-X motif within the VV and fowlpox core protein precursors is quite similar to the Gly-Gly-X sequence, raising the question as to whether these three different viruses may utilize similar proteolytic maturation pathways. Likewise, with particular regard to maturation of the VV core proteins, there are a number of unanswered questions. Is the Ala-

Gly-X motif *per se* sufficient for specifying efficient recognition and cleavage by the proteinase? Are there any other required substrate elements proximal to and/or remote from the Ala-Gly-X motif within the precursor proteins? Is the same proteinase responsible for the processing of all VV major core proteins? Does the cleavage of VV major core proteins occur by an endoproteolytic, exoproteolytic, or combinatorial mechanism? Do the small polypeptides that have been excised from the precursors play any role in VV life cycle? Finally, how many factors, including the proteinase, are involved in the proteolysis machinery for VV core protein proteolytic maturation? Are they virus-encoded or provided by the host cells? Addressing these questions requires the establishment of a proteolytic processing assay to facilitate the identification and characterization of the essential *cis* and *trans* factors.

We have previously attempted several different approaches to developing an assay system to study proteolytic processing of VV core proteins: (i) *in vitro* cleavage assays mixing VV core protein precursors isolated from cells infected with cleavage-deficient temperature sensitive VV mutants together with extracts from wild-type VV-infected or uninfected cells; (ii) mixing solubilized VV virions with VV core protein precursors made *in vivo* or *in vitro*, and (iii) transient expression assays using the hybrid T7/VV system (Fuerst et al., 1986) to express various reporter gene constructions containing putative VV core protein cleavage sites. Without exception, no cleavage of the test substrate was observed with any of these systems. This led to our working hypothesis that proteolytic maturation of VV core proteins is contextual, that is, linked directly to virion assembly. The predictions of this hypothesis are that for a VV protein to be cleaved at the Ala-Gly-X motif it must be synthesized late

in infection and packaged into the assembling virion and it needs to be associated with the VV core. Thus, any perturbation of the kinetics of synthesis, intracellular targeting, or structure of a VV core protein might be expected to abrogate processing.

Testing this hypothesis requires the development of an assay with which to examine the *cis* and *trans* factors mediating the proteolytic maturation of VV core protein precursors *in vivo*. To accomplish this goal, several difficulties had to be overcome. First, the genes encoding the major VV core protein precursors are thought to be essential for virus replication. Unfortunately, conditional lethal mutants are presently not available at these loci nor are these genes readily amenable to direct inactivation by gene insertion techniques (Thompson and Condit, 1986). Second, the VV core proteins are relatively insoluble and difficult to work with *in vitro* (Child and Hruby, unpublished data; Ichihashi et al., 1984,). Third, on the basis of our initial experiments, it appeared that cleavage occurs only within the context of a maturing virion particle. Fourth, the VV core protein precursors are highly expressed at late times during infection, making detection of an exogenously added core protein precursor difficult. To overcome these challenges, a transient-expression procedure in which an immunologically marked core protein precursor is expressed at late times within the VV-infected cell has been employed.

In this report, a *trans*-processing assay of VV core protein proteolytic maturation has been developed by tagging VV core protein precursor P25K at the C-terminus with an octapeptide epitope, FLAG. The L4R gene was chosen as the target for these studies because it is the smallest of the three major core protein precursor genes, thus facilitating genetic manipulations, and because the L4R gene product, the P25K protein, is relatively soluble

and therefore more amenable to biochemical analyses. We describe the expression of the chimeric P25K:FLAG polypeptide by transient expression in VV-infected cells and identification of the proteolytic cleavage products of P25K:FLAG. The effect of rifampin on the proteolytic processing of P25K:FLAG was also applied to validate that the proteolysis of P25K:FLAG is mediated by the same proteolytic machinery as that used in authentic VV core protein processing pathways. The distribution of FLAG-tagged P25K-related polypeptides within VV-infected cells and the ability of the transiently expressed P25K:FLAG-derived products (25K:FLAG), like 25K itself, to be localized into mature VV were examined. We also investigated the importance of the cleavage Ala-Gly-X motifs for P25K proteolytic maturation by site-directed mutagenesis. Developement of this assay and confirmation that authentic cleavage reactions are occurring should now allow the roles of the Ala-Gly-X motif and the surrounding amino acid sequence in substrate specificity of VV core protein proteolytic processing to be investigated and provide an approach for identifying the responsible proteinase(s).

Materials and Methods

Cells and virus.

BSC-40 (African green monkey kidney) cells were maintained in MEM Eagle's medium (MEM-E; Sigma Chemical Co., St. Louis, Mo.] supplemented with 10% heat-inactivated fetal bovine serum (Whittaker M. A., Bioproducts, Inc., Walkersville, Md.), 2 mM glutamine, and 10 mg of gentamicin per ml. Purification of VV (WR strain) from infected BSC-40 cells by two cycles of sucrose gradient centrifugation was performed as described previously (Hruby et al., 1979).

Plasmid construction and site-directed mutagenesis.

To generate pL4R, an *Aha*III fragment (1.3 Kb) containing the L4R promoter region and the entire L4R ORF, which encodes the P25K polypeptide, was isolated from the *Hind*III L fragment of the VV genome. Both ends of the fragment were filled in with the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and cloned into the *Sma*I site of pUC119. Site-directed mutagenesis of pUC119:L4R to generate pL4R:FLAG containing a chimeric ORF encoding the P25K polypeptide with a FLAG epitope (Hopp et al., 1988) was performed as described by Kunkel (1985). An oligonucleotide 48-mer, PL30, (5'-AAGTATAGATTAGGCTACTTGTCGTCATCGTCTTTATAATCCT-TTGTC-3') was used to introduce the following seven-amino-acid sequence behind the last amino acid, Asp, of the P25K polypeptide: Tyr-Lys-Asp-Asp-Asp-Asp-Lys. Together, these eight amino acids created the FLAG epitope attached to the carboxy-terminus of P25K. A stop codon was engineered to

terminate the translation of the chimeric polypeptide immediately after the FLAG epitope. Oligonucleotide PL47, a 33-mer (5'-AAATTTGGATTTAAT-GTCTATAATAACCATTTG-3'), was synthesized for the mutagenesis of amino acids 31 to 33 of P25K from Ala-Gly-Ala to Ile-Asp-Ale, and oligonucleotide PL32, a 24-mer, (5'-CTCAGATATGATATCGATAAAAAA-3') was synthesized for altering amino acids 17 to 19 from Ala-Gly-Ser to Ile-Asp-Ile. All mutagenesis procedures were confirmed by dideoxynucleotide DNA sequencing procedures (Sanger et al., 1977).

Transient expression.

BSC-40 cells were infected with VV at a multiplicity of infection of 10 infectious particles per cell and transfected with plasmid or salmon sperm DNA at 0 h postinfection (p.i.) by a liposome-mediated transfection protocol (Rose et al., 1991). Briefly, BSC-40 cells were allowed to grow to about 90% confluency in plates (60 by 15 mm). To prepare the liposome, 1.0 mg of L-a-phosphatidylethanolamine (dioleoyl) (Sigma) and 0.4 mg of dimethyl dioctadecyl ammonium bromide (Sigma) in chloroform were mixed and blown to dryness with a stream of nitrogen gas. The lipids were then resuspended in 1 ml of sterile H_2O and sonicated with Branson sonifier 250 (VWR Scientific, Media, Pa.) (Microprobe, 50 V setting) on ice for about 5 to 10 min until the solution was almost clear. Before transfection, 1 ml of MEM-E was mixed with 30 μl of liposome and 5 μg of plasmid DNA. The DNA mixtures were incubated at room temperature for 10 min before being added to the VV-infected cells. After 4 h of incubation at 37°C, the DNA-containing medium was replaced with fresh MEM-E containing 5% fetal bovine serum, and the incubation was continued as indicated. When

blockage of the proteolytic processing in VV-infected cells was required, 100 μg of rifampin (Sigma) per ml was added to the medium at 0 h p.i..

Immunoblotting.

Infected cells were harvested, washed in phosphate-buffered saline (PBS) (pH 7.0) and lysed by freezing and thawing several times. Immunoblot analyses were performed as described by Van Slyke et al. (1991a). Briefly, cell lysates were heated at 100°C for 5 min in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (50 mM Tris [pH 6.8], 1% [wt/vol] SDS, 0.1% [vol/vol] 2-mercaptoethanol, 1% [vol/vol] glycerol) (Laemmli, 1970). The proteins were separated on 13% SDS-polyacrylamide gels, and electrotransferred to nitrocellulose filters in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol] (Towbin et al., 1979) for 40 min at 25 V at 4°C. The filters were washed once in Tris-buffered saline (TBS) (20 mM Tris and 500 mM NaCl, pH 7.5) and blocked with 3% (wt/vol) gelatin (Bio-Rad, Richmond, Calif.) in TBS at room temperature for 2 h. The filters were washed three times with TTBS (0.05% Tween-20 in TBS) and then subjected to binding with anti-FLAG antibody M2 (International Biotechnologies, Inc., New Haven, Conn.) (dilution 1:155) in antibody buffer (1% gelatin-containing TTBS) at room temperature overnight. After three washes in TTBS buffer followed by one in TBS, the filters were hybridized with an alkaline phosphatase-conjugated goat anti-mouse antibody (dilution 1:2,000; Bio-Rad) in antibody buffer for 2 h and washed with TTBS and TBS. The immunoblots were developed in ρ-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate r-toluidine salt (Bio-Rad) in carbonate buffer (0.1 M $NaHCO_3$ and 1.0 mM $MgCl_2$, pH 9.8).

Metabolic labeling and immunoprecipitation.

VV-infected cells were transfected with plasmid DNA as described above and pulse-labeled with 150 μCi of ^{35}S protein labeling mix (11.04 mCi/ml [1094.4 Ci/mmol]; New England Nuclear, Wilmington, Del.) for 30 min at 4 h. p.i. After 0 or 20 h of "chase" incubation in MEM-E containing 100-fold excess unlabeled methionine, cells were harvested, lysed in 2% SDS solution at 65°C, passed through a 25-gauge needle several times, and immunoprecipitated as described previously (Miner and Hruby, 1989). Briefly, the cell extracts were diluted 10-fold in a radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% deoxycholate, 1.0% Nonidet P-40, 0.1% SDS) and incubated with 5 μl of anti-25K polyclonal antibody (VanSlyke and Hruby, unpublished data) for 1 h on ice. Two hundred microliters of a 10% (vol/vol) solution of protein A-Sepharose CL-4B beads (Sigma) in the radioimmunoprecipitation buffer was then added, and the incubation was continued overnight at 4°C with continuous rocking. The immune complexes were centrifuged for 20 s in the microcentrifuge, and the beads were washed three times with the radioimmunoprecipitation buffer. Polypeptides were released from the beads by heating at 100°C for 5 min in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer and resolved on 13% SDS-polyacrylamide gels. Gels were then processed for fluorography by using 22.2% (wt/vol) PPO (2,5-diphenyloxazol) in dimethyl sulfoxide, dried and stored at -70°C while exposed to Kodak XAR-5 X-ray film. Exposures varied from 3 days to 3 weeks. For preparation of continuously ^{35}S-labeled cell extracts, 75 μCi of ^{35}S-protein labeling mix per plate was used. To quantitate the radioactivity

present in each band, dried gels were scanned and analyzed in the AMBiS radioanalytic imaging system (AMBiS Systems, Inc., San Diego, Calif.).

Immunofluorescent staining.

BSC-40 cells were grown on coverslips, infected with VV at a multiplicity of infection of 5 PFU per cell, and transfected with 5 μg of DNA. Cells were washed twice with PBS-D, fixed, and permeablized in cold methanol for 8 min at 5.5 h p. i.. The cells were then reacted with P25K specific antiserum (1:200) or with FLAG-specific M2 monoclonal antibody (1:20) diluted in 10% (vol/vol) normal goat serum (Miles Laboratories, Inc., Naperville, Ill.; in PBS-D) for 30 min. Following two washes with PBS-D, the cells were incubated in a 1:100 dilution of either fluorescein isothiocyanate-conjugated goat anti-rabbit anitbody or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Zymed Laboratories, Inc., South San Francisco, Calif.) for 30 min. After incubation with the secondary antibody, the coverslips were washed with PBS-D twice, and counter-stained with 0.01% Evans blue (Direct Blue 53; Sigma) (in PBS-D) for 1 min. The coverslips were mounted with 8 μl of DABCO mounting fluid (25% [wt/vol] 1,4-diazabicyclo (2,2,2,) octane [DABCO; Sigma], 50% (vol/vol) glycerol, 0.25X PBS, and 0.1% sodium azide, pH 8.6) after two final washes in PBS-D. Observations and photography were performed with a Zeiss fluorescence microscope.

Results

Design and construction of the pL4R:FLAG expression vector plasmid.

The pL4R:FLAG expression plasmid that was assembled for these experiments is diagrammed in Figure II.1. The VV L4R gene, encoding the core protein precursor P25K, abutted to its own late promoter element was cloned into the pUC119 plasmid. This plasmid was used because it contains the bacteriophage M13 replication origin for the synthesis of single-stranded DNA template to facilitate subsequent mutagenesis procedures. Site-directed mutagenesis techniques were then used to create an in-frame fusion at the 3'-end of the L4R ORF such that an octapeptide epitope, designated FLAG (Prickett et al., 1989), would be appended to the carboxy-terminus of the P25K protein. This epitope was chosen because its small size would be expected to produce only minimal perturbations of the overall structure and subcellular localization of the P25K precursor protein. Likewise, the epitope was appended to the carboxy terminus to remove it as far from the cleavage site at the amino-terminus as possible and to allow both the precursor and the cleaved product to be recognized. The experimental rationale for this construction was that the pL4R:FLAG plasmid could be efficiently introduced into VV-infected cells by using liposome mediated procedures. During the late phase of VV gene expression, soluble VV RNA polymerase would transcribe the pL4R:FLAG gene product from its natural promoter with normal expression kinetics. The pL4R:FLAG-derived transcript would be translated into a P25K:FLAG precursor, which should be subject to any essential posttranslational modifications. Assuming that the tertiary structure and/or targeting of the

Figure II.1. Structure of the pL4R:FLAG expression vector plasmid. The pL4R:FLAG plasmid containing the VV L4R ORF (encoding the P25K core protein precursor) driven by its cognate promoter (PL4R), with the sequences encoding the FLAG epitope fused in-frame onto the 3' end is shown schematically. The N-terminal 37 amino acids of the P25K protein are shown at the top, with the two potential cleavage consensus sites shaded. The known cleavage point between residues 32 and 33 in site II and the potential cleavage point between residues 18 and 19 in site I are indicated (black and gray triangles, respectively). The eight codons encoding the FLAG epitope shown at the bottom (shaded box). The seven codons shown in bold face were fused by oligonucleotide-directed mutagenesis onto the natural carboxy terminus of the L4R ORF (Asp codon).

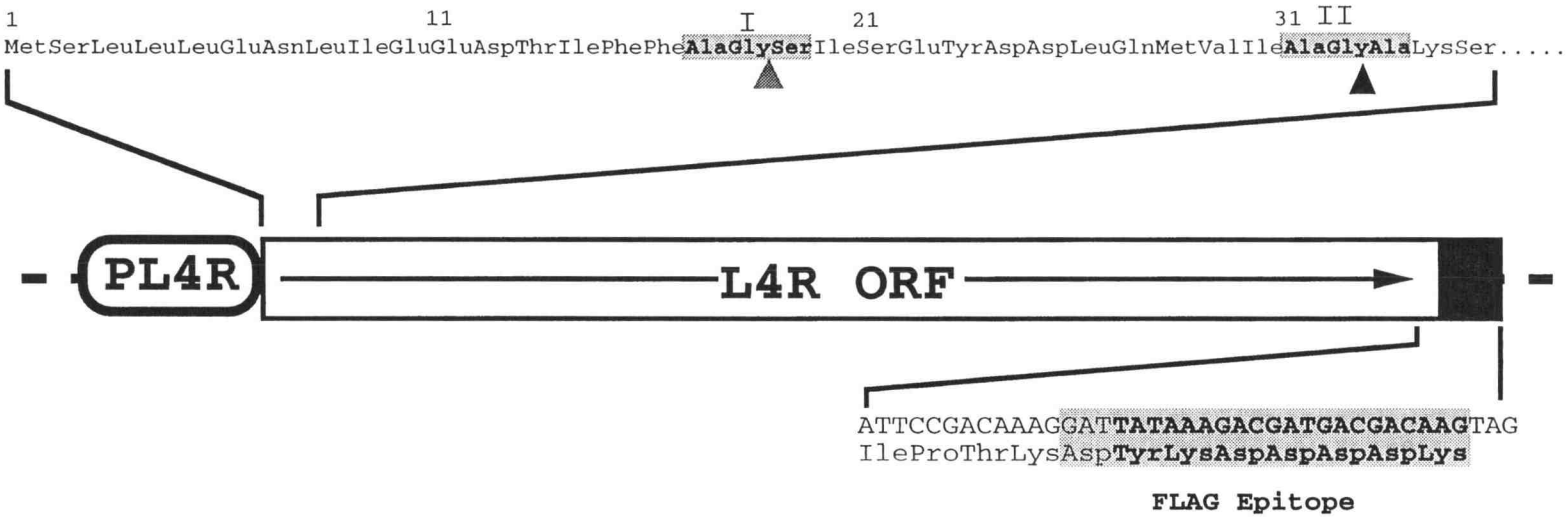

1
11
I
21
31
II
MetSerLeuLeuLeuGluAsnLeuIleGluGluAspThrIlePhePheAlaGlySerIleSerGluTyrAspAspLeuGlnMetValIleAlaGlyAlaLysSer.....
PL4R
L4R ORF
ATTCCGACAAAGGATTATAAAGACGATGACGACAAGTAG
IleProThrLysAspTyrLysAspAspAspAspLys
FLAG Epitope

Figure II.1

precursor has not been altered by addition of the FLAG epitope, the P25K:FLAG protein should be incorporated into assembling virions and cleaved by the same proteolytic machinery as authentic P25K during viral assembly. We should be able to distinguish the P25K:FLAG precursor and the 25K:FLAG product from the genomically encoded 25K product by using the M2 monoclonal antibody directed against the FLAG epitope.

***Trans* processing of P25K:FLAG in VV-infected cells.**

To determine whether P25K:FLAG is expressed and efficiently processed *in vivo*, the expression vector pL4R:FLAG was introduced into VV-infected BSC-40 cells by liposome-mediated transfection. Twenty hours after transfection, total cell extracts were prepared from the infected cells and subjected to immunoblot analysis using either rabbit anti-25K antiserum or mouse anti-FLAG M2 monoclonal antibody. As a negative control, salmon sperm DNA was transfected into VV-infected cells. As indicated in Figure II.2, two polypeptides (P25K and 25K) were identified by the anti-25K antiserum in VV-infected cells receiving only salmon sperm DNA (lane 2). Two additional polypeptides in VV-infected cells transfected with pL4R:FLAG DNA were observed (lane 1). To demonstrate that the two new polypeptides were derived from the plasmid-encoded chimeric L4R:FLAG gene, anti-FLAG M2 antibody was utilized to analyze a duplicate set of the same extracts. None of the proteins present in the salmon-sperm DNA-transfected VV-infected cells reacted with this serum (Figure II.2, lane 3) whereas three polypeptides from pL4R:FLAG-transfected cells (Figure II.2, lane 4) were identified by anti-FLAG M2 antibody. The largest and smallest of the three polypeptides comigrated with the two unique bands identified by the anti-25K antibody in the pL4R:FLAG transfected cell extract (Figure

Figure II.2 Proteolytic maturation of the P25K:FLAG precursor protein in infected cells. Monolayers of BSC-40 cells were transfected with sheared salmon sperm DNA (-) or pL4R:FLAG DNA (+) following infection with wild-type VV. After 24 hours of incubation, infected cell lysates were prepared and subjected to polyacrylamide gel electrophoresis and immunoblot analysis with monospecific antiserum directed against the 25K protein (α25K) or a mouse monoclonal antibody which recognizes the FLAG epitope (αFLAG). The positions of the 25K and 25K:FLAG precursors and derived products are indicated on the right. The positions and sizes (in thousands) of molecular weight (MW) standards are indicated on the left.

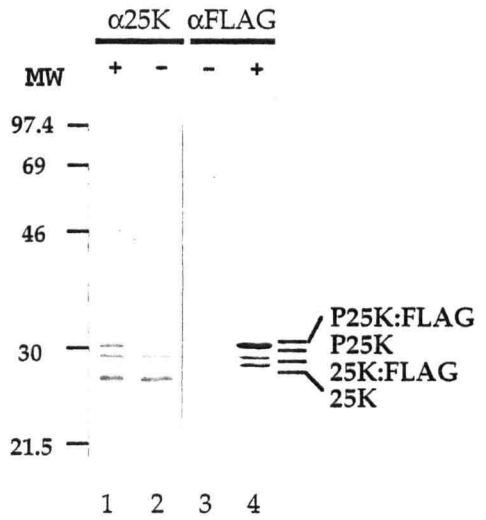


Figure II.2

II.2, lane 1). The predicted molecular masses of P25K:FLAG and 25K:FLAG are 29.3 and 25.8 kDa, respectively, agreeing well with the apparent molecular weights of the upper and lower proteins recognized by the M2 antibody. Furthermore, an N terminally truncated P25K:FLAG derivative, when transiently expressed from a construct encoding 25K:FLAG with an extra Met residue for translation initiation at the N terminus, exactly comigrates with the lower protein band (data not shown). We therefore provisionally designated the upper band as the P25K:FLAG precursor and the lower band as the 25K:FLAG cleavage product (Figure II.2, lane 4). N terminal microsequencing of the 25K:FLAG cleavage product substantiated this conclusion (data not shown). The intermediate-sized protein which was detected with the anti-FLAG antibody (Figure II.2, lane 4) but not with anti-25K serum (Figure II.2, lane 1), presumably because of comigration with P25K, was designated 25K':FLAG. The production of 25K':FLAG will be investigated in the following sections.

25K:FLAG is a product of P25K:FLAG.

To investigate whether a precursor-product relationship exists between P25K:FLAG and 25K:FLAG, VV-infected cells were transfected with pL4R:FLAG DNA, pulse-labeled with ^{35}S-labeling mix for 30 min, and incubated in the presence of excess unlabeled methionine for 20 h. FLAG-specific monoclonal antibody did not react well with FLAG-tagged polypeptides in immunoprecipitation experiments, therefore, the immunoprecipitation analysis was performed with 25K-specific antiserum. Cytoplasmic extracts were prepared, and incubated with anti-25K antiserum, and the immunoreactive proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography (Figure II.3). In the salmon sperm

DNA transfected cell extracts, P25K was pulsed-labeled (Figure II.3, lane 3) and the label was completely chased into 25K (Figure II.3, lane 5) during the incubation, as expected. In the pL4R:FLAG transfected extracts, in addition to P25K, P25K:FLAG was labeled during the pulse period (Figure II.3, lane 2) and chased into 25K:FLAG during the subsequent incubation (Figure II.3, lane 4). The efficiency of P25K:FLAG to 25K:FLAG cleavage appeared to be reduced compared with the processing of virus-encoded precursor P25K into 25K (which also did not proceed to completion in the presence of P25K:FLAG expression).

To determine the efficiency of the cleavage of P25K:FLAG to 25K:FLAG, VV-infected cells were labeled with ^{35}S-protein labeling mix from 4 to 24 h p.i. during pL4R:FLAG transient expression. Following immunoprecipitation with 25K-specific antiserum and SDS-polyacrylamide gel electrophoresis separation, the radiolabeled polypeptides were visualized by autoradiography (Figure II.3, lane 1). AMBiS radioanalytic imaging system was utilized to measure the radioactivity (counts per minute) of each band with the same gel. The radioactivity of each P25K derived polypeptide was divided by the total counts of P25K related polypeptides to calculate the percentage of each polypeptide in its group. Identical calculations were carried out for P25K:FLAG and its derivatives. After 24 h, 63.98% of the P25K synthesized had been proteolytically matured into 25K (Figure II.3, lane 1, and Table II.I) in pL4R:FLAG transfected cells. This could be contrasted with an 83.2% conversion of P25K into 25K in salmon sperm DNA-transfected cells (data not shown). In contrast, only 33.7% of P25K:FLAG was processed into 25K:FLAG. Since the intermediate product 25K':FLAG, which migrates at a position between P25K and 25K:FLAG in SDS-polyacrylamide gel electrophoresis (as seen in Fig. 1), was not detected by 25K-specific

Figure II.3 Pulse-chase analysis of P25K:FLAG protein maturation in vivo. Monolayers of BSC-40 cells were transfected with sheared salmon sperm DNA (-) or pL4R:FLAG DNA (+) following infection with wild-type VV. At 4 h p. i. the cells were incubated for 30 min with a ^{35}S-labeled mixture of methionine and cysteine. Lysates were prepared either immediately (pulse) or after an additional 20 h of incubation in the presence of 100-fold excess unlabeled methionine and cysteine (chase). P25K-related proteins were immunoprecipitated with monospecific anti-25K serum and analyzed by polyacrylamide gel electrophoresis and fluorography. The positions of the 25K and 25K:FLAG precursors and derived products are indicated on the right.

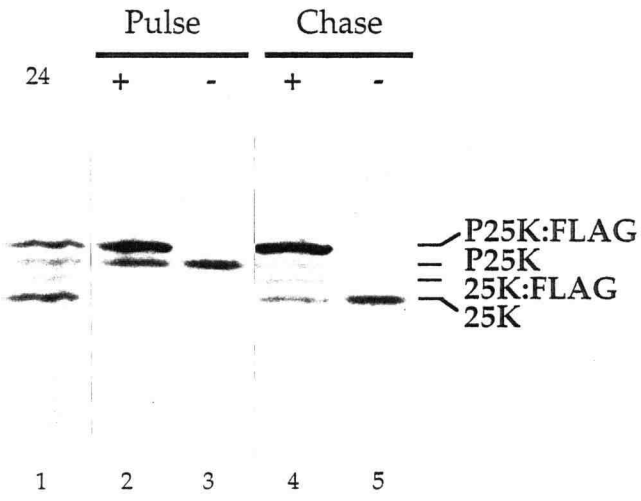


Figure II.3

Precursor or product	cpm	%
P25K	25,375	36.02
P25K:FLAG	37,489	66.29
25K	45,072	63.98
25K:FLAG	19066	33.71

Table II.1 Analysis of the efficiency of P25K:FLAG and P25K proteolytic processing

antiserum, the percentage of 25K:FLAG (33.7%) represents an overestimate, since the radioactivity of 25K':FLAG was not integrated into the analysis. These results suggest that P25K:FLAG is efficiently synthesized during transient expression of pL4R:FLAG in VV-infected cells, but the subsequent conversion to 25K:FLAG proceeds less efficiently than the authentic reaction. However, for the purpose of a proteolytic processing assay to use for the dissection of the important elements for defining a specific scissile peptide bond in the polypeptide substrate, this amount of processing product should be more than sufficient. Therefore, we went on to confirm the authenticity of the cleavages in the P25K:FLAG precursor.

P25K:FLAG requires the AG/X motif for cleavage.

The appearance of the 25K':FLAG intermediate noted in immunoblot analysis using FLAG-specific M2 antibody (Figure II.2) was unexpected, on the basis of previous studies of P25K processing *in vivo* (VanSlyke et al., 1991a; Weir and Moss, 1985). Three possible explanations for the appearance of the additional polypeptide were considered. First, the intermediate band could result from translational initiation from an internal ATG codon within the L4R ORF. This possibility was considered unlikely, as the second and third methionines of the L4R ORF are located at residues 27 and 40 respectively, and neither is located in a context likely to facilitate translation (Kozak, 1989). Second, premature termination of translation could give rise to the 25K':FLAG product. This possibility was highly unlikely, as sequence analysis of the pL4R:FLAG insert revealed no mutations. Furthermore, premature termination would result in a protein lacking the FLAG epitope that would not be recognized by the M2 antibody. Third, 25K':FLAG may represent a proteolytic processing intermediate in which cleavage has

occurred at a site other than Ala-Gly-Ala at residues 31 to 33 of P25K. The motifs Ala-Gly-Thr and Ala-Gly-Ser have been suggested as functional cleavage sites which are recognized during maturation of the core protein precursor P4a (VanSlyke et al., 1991b). Examination of the predicted amino acid sequence of the N-terminus of P25K revealed that the sequence Ala-Gly-Ser was located between amino acid residues 17 to 19 (site I in Figure II.1). Since cleavage at site I would produce a product with a predicted molecular weight intermediate between those of P25K:FLAG and 25K:FLAG, we considered this to be the most likely possibility.

In order to test whether the site I and site II motifs of the P25K:FLAG precursor were required for production of 25K':FLAG and 25K:FLAG, respectively, the amino acids present at these locations were altered by site-directed mutagenesis. Since little was known concerning which positions within the AG/X motif are essential and what the substitution constraints at each position are, amino acid substitutions were introduced into all three positions to increase the likelihood of inhibiting processing if sites I and II are the points of cleavage. Amino acid sequences at site I (residues 17 to 19 [Ala-Gly-Ser]) and site II (residues 31 to 33 [Ala-Gly-Ala]) were both mutated to Ile-Asp-Ile. The ability of the mutant proteins to be processed was analyzed by transient expression in VV-infected cells. As demonstrated by the immunoblot using FLAG-specific M2 antibody, mutation of site I results in no production of the 25K':FLAG product (Figure II.4, lane 4), whereas mutation of site II inhibits production of the 25K:FLAG protein (Figure II.4, lane 3). Transient expression of a site I-site II double mutant produced only precursor protein with no cleavage products (Figure II.4, lane 5). Thus, these results would suggest that both AG/X motifs present at sites I and II are subject to proteolytic cleavage, at least during transient expression.

Figure II.4 Genetic inactivation of P25K:FLAG cleavage sites. Site-directed mutagenesis procedures were used to prepare pL4R:FLAG derivatives in which the A-G-S and A-G-A codons corresponding to sites I (residues 17 to 19) and II (residues 31 to 33) were converted to I-D-I. Monolayers of BSC-40 cells were infected with wild-type VV and transfected with sheared salmon sperm DNA (C) or pL4R:FLAG plasmid derivatives (lanes 2 to 5). After 24 hours of infection, lysates were prepared and subjected to polyacrylamide gel electrophoresis and immunoblot analysis using anti-FLAG monoclonal antibody. The positions of the 25K:FLAG precursor and derived products are indicated on the right. The positions and sizes (in thousands) of molecular weight (MW) standards are indicated on the left.

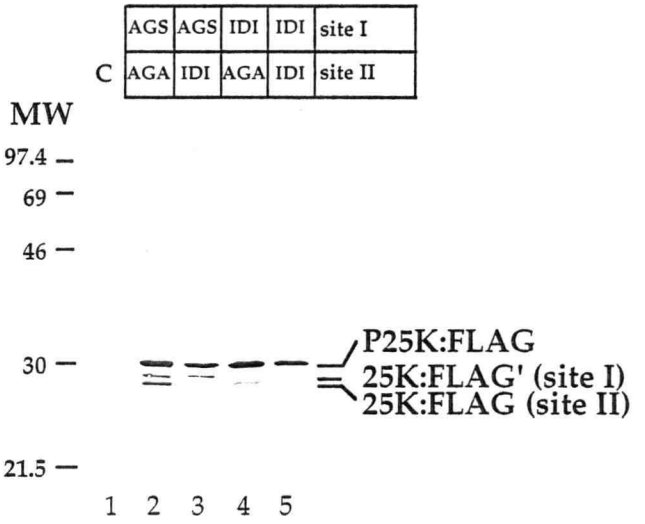

AGS AGS IDI IDI site I
C AGA IDI AGA IDI site II
MW
97.4
69
46
30
21.5
P25K:FLAG
25K:FLAG' (site I)
25K:FLAG (site II)
1 2 3 4 5

Figure II.4

Figure II.5 Effect of rifampin on P25K:FLAG maturation. Monolayers of BSC-40 cells were infected with wild-type VV and transfected with sheared salmon sperm (SSS) DNA or pL4R:FLAG DNA in the presence (+) or absence (-) of rifampin (Rif) (100 μg/ml). Extracts were prepared at 13 h p. i. and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis using P25K-specific antiserum (α25K) or anti-FLAG (αFLAG) monoclonal antibody. The positions of the precursors P25K and its derived products are indicated on the left, and those of P25K:FLAG and its derivatives are indicated on the right. The sizes of molecular weight standards are indicated in thousands.

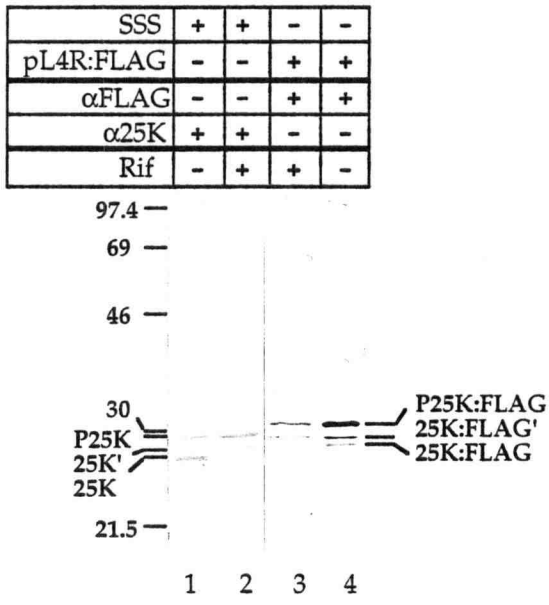

SSS + + - -
pL4R:FLAG - - + +
αFLAG - - + +
α25K + + - -
Rif - + + -
97.4
69
46
30
P25K
25K'
25K
21.5
P25K:FLAG
25K:FLAG'
25K:FLAG
1 2 3 4

Figure II.5

Rifampin inhibits processing of the P25K:FLAG protein.

To determine whether maturation of P25K:FLAG into 25K:FLAG uses the same proteolytic machinery that is required for the processing of P25K into 25K, we took advantage of the well-known observation that the proteolytic maturation of VV major core proteins and VV virion assembly can both be blocked by rifampin (Moss and Rosenblum, 1973; Moss et al., 1969). VV-infected cells which were transfected with either salmon sperm DNA or pL4R:FLAG DNA were incubated in the presence or absence of rifampin from 0 to 13 h p.i. As demonstrated in Figure II.5, immunoblot analyses of the transient expression lysates using both 25K-specific antiserum and anti-FLAG M2 antibody indicated that, like P25K and 25K (lane 1), the P25K:FLAG precursor and 25K:FLAG product are both present in the absence of rifampin (lane 4). The production of 25K is significantly reduced in the presence of rifampicin for 24 h (Figure II.5, lane 2) and the maturation of 25K:FLAG product was likewise blocked to a level which was not detectable by immunoblott analysis under the same condition (Figure II.5, lane 3). These results suggested that cleavage of site II of the P25K:FLAG precursor to produce 25K:FLAG likely uses the same pathway as maturation of the authentic P25K protein.

Surprisingly, unlike 25K:FLAG, the production of 25K':FLAG was not affected by rifampin (Figure II.5, lane 3). Furthermore, an unexpected polypeptide with an molecular mass intermediate between those of P25K and 25K was detected in cell extracts harvested from VV-infected cells in the presence of rifampin (Figure II.5, lane 2) but was totally absent in cells not treated with the drug (Figure II.5, lane 1). The mobility of this polypeptide relative to P25K and 25K appeared identical to that of 25K':FLAG relative to P25K:FLAG and 25K:FLAG, we therefore postulate it to be the authentic

equivalent (namely, 25K') of 25K':FLAG. Thus, it appears that rifampin does not block the processing of 25K':FLAG from P25K:FLAG.

25K:FLAG localizes in the virion.

Sarov and Joklik (1972) have previously shown that the mature 25K protein is exclusively found associated with the core of mature VV virions whereas only a trace of the P25K precursor was evident in the viral particle. Furthermore, the work of Katz and Moss (Katz and Moss, 1970a) suggested that proteolytic processing of VV core proteins is a late event in the virus maturation pathway. Thus, if the P25K:FLAG to 25K:FLAG processing reaction is occurring via authentic mechanisms, one would predict that the 25K:FLAG product would be found associated with the virion while the precursor would be detected primarily in the cytoplasm of infected cells. To test this prediction, progeny virions were purified from cells in which the pL4R:FLAG plasmid had been transiently expressed. The data shown in Figure II.6 demonstrates that although P25K:FLAG, 25K':FLAG, and 25K:FLAG proteins were present within the cytoplasm of infected cells (lane 3), only 25K:FLAG was detected within the mature VV virions (lane 2). The fact that 25K':FLAG intermediate was not detected in the mature virions suggested that the protein produced by processing at site I does not function *per se* as a structural protein in the virion.

Transiently expressed polypeptides are distributed throughout the cytoplasm of VV-infected cells.

As noted previously, although 25K:FLAG was cleaved from the P25K:FLAG precursor, the efficiency of the reaction was reduced compared with the authentic reaction. Several explanations for this observation were

Figure II.6 Virion association of the mature 25K:FLAG protein. Monolayers of BSC-40 cells were infected with wild-type VV and transfected with sheared salmon sperm DNA (VV) or pL4R:FLAG DNA. After 24 h of infection, mature virus particles (PV) were purified from the infected cell lysate (TCE) by differential centrifugation and velocity sedimentation on sucrose gradients (Hruby et al., 1979). The indicated samples were analyzed by polyacrylamide gel electrophoresis and immunoblot analysis using anti-FLAG monoclonal antibody. The positions of the 25K:FLAG precursor and derived products are indicated on the right. The positions and sizes (in thousands) of molecular weight (MW) standards are indicated on the left.

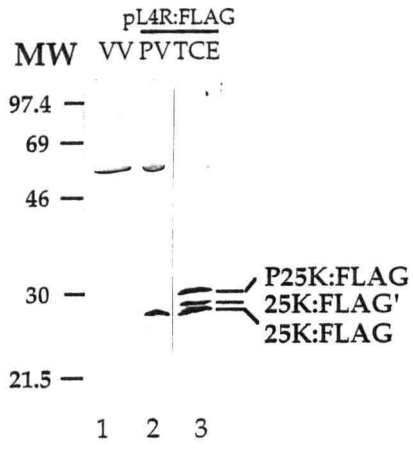

pL4R:FLAG
MW
VV
PVTCE
97.4
69
46
30
21.5
P25K:FLAG
25K:FLAG'
25K:FLAG
1
2
3

Figure II.6

considered. One hypothesis is that a small degree of disruption in secondary and/or tertiary structure in the P25K polypeptide was introduced by the addition of the eitht-amino-acid FLAG epitope at the C-terminus, which resulted in a kinetic difference of proteinase-substrate interaction. An alternative explanation is that P25K:FLAG might display a localization pattern different from tthat of he authentic P25K precursor expressed from viral genomic DNA, which has been shown to be localized specifically in virosomes, where virus assembly takes place. The alteration in localization could result from either structural disruption of the P25K polypeptide discussed above or from distinct sites of gene expression from transfected DNA in VV-infected cells. Consequently, the FLAG-tagged P25K precursor was not efficiently incorporated into immature virions. To explore the latter hypothesis, immunofluorescent staining was used to study the localization of P25K:FLAG and P25K related proteins.VV-infected BSC-40 cells were transfected with salmon sperm DNA, pL4R, or pL4R:FLAG. After 5.5 h of infection, the cells were incubated with a primary antibody directed against either 25K or the FLAG-epitope and the incubation was followed by an appropriate fluorescein isothiocyanate-conjugated secondary antibody. In cells transfected with salmon sperm DNA, P25K related polypeptides localized specifically to the structures in the vicinity of host cell nuclei, most probably virosomes (Figure II.7d). These structures were not stained with preimmune antiserum (Figure II.7a). When pL4R:FLAG transfected cells were examined, prominent staining was evident throughout the entire cytoplasmic compartment by using FLAG-specific antibody (Figure II.7c) or 25K-specific antiserum (which also highlighted virosomes as described above in all infected cells) (Figure II.7f). No staining was observed by using anti-FLAG antibody in VV-infected cells

transfected with pL4R (Figure II.7b), demonstrating the specificity of anti-FLAG antibody recognition of transiently expressed FLAG-tagged polypeptides. Taken together, these results suggest that P25K:FLAG related proteins are not concentrated around virosomes but rather are distributed throughout the cytoplasm of the infected cells. Since both transiently expressed P25K and P25K:FLAG proteins are localized throughout the cytoplasm of pL4R- and pL4R:FLAG-transfected cells, respectively, as detected by 25K-specific antiserum (Figure II.7e and f), this implies that it is not the FLAG epitope *per se* which is responsible for inhibiting normal localization of P25K-related proteins. Rather, it appears that when expressed from a transfected plasmid, P25K-related proteins are inefficiently concentrated around the virosome.

Figure II.7 Immunofluorescent localization of P25K- and P25K:FLAG-related proteins. VV-infected BSC-40 cells were transfected with sheared salmon sperm DNA (a and d), pL4R (b and e), or pL4R:FLAG (c and f) DNA. After 5.5 h of infection, indirect immunofluorescent staining was performed with 25K-specific antiserum (d, e, and f), anti-FLAG monoclonal antibody (b and c), or rabbit preimmune antiserum (a). Following a incubation with a secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit [a, d, e, and f] or goat anti-mouse [b and c] immunoglobulin), cells were counterstained with Evans blue. Virosomes are indicated (arrows).

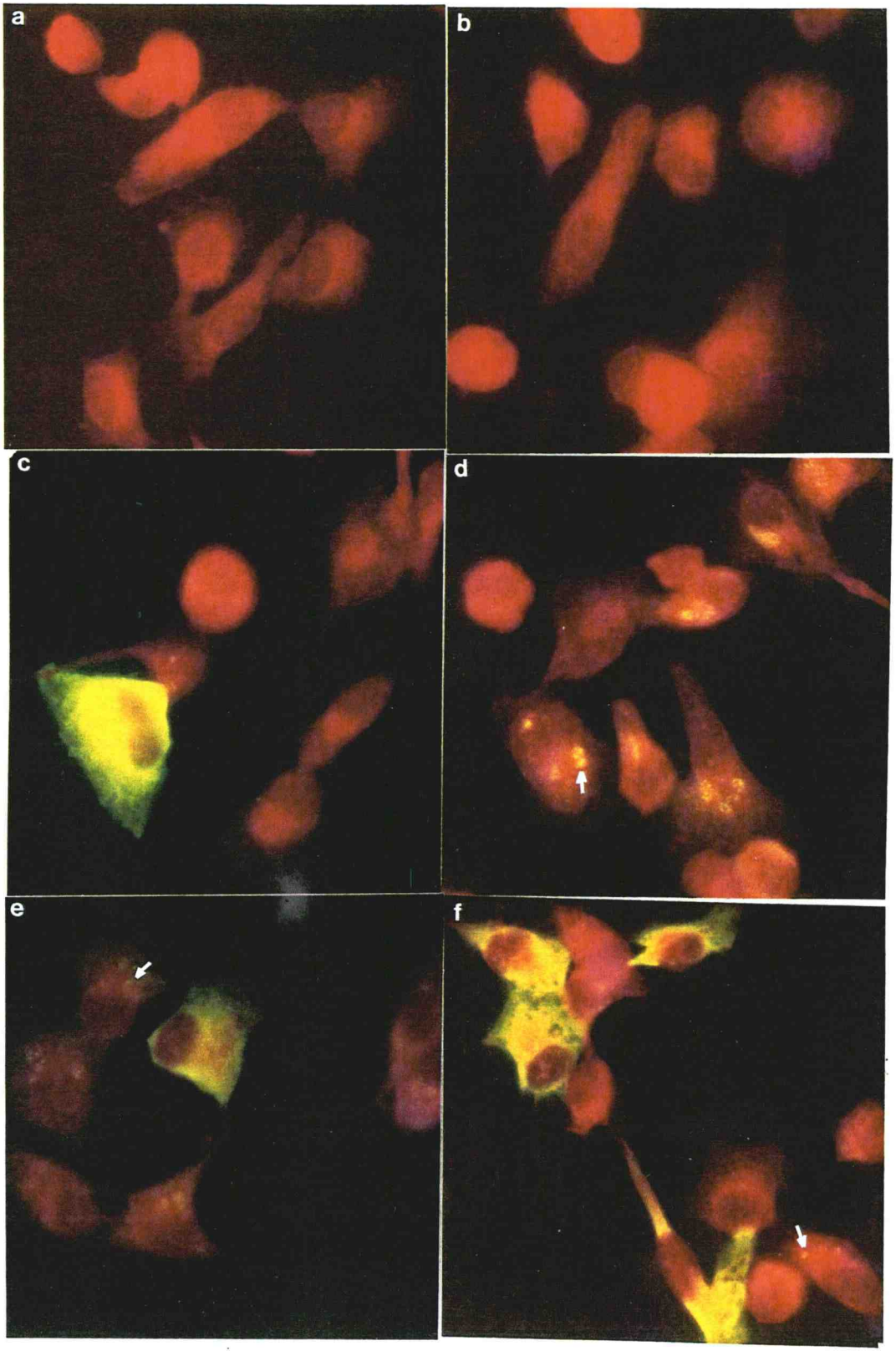


Figure II.7

Discussion

The experiments reported in this article have described the construction and analysis of the pL4R:FLAG expression vector. When transfected into VV-infected cells, this plasmid directs the synthesis of a chimeric reporter gene product consisting of the P25K VV core protein precursor abutted to an immunologically distinguishable epitope tag (FLAG). In VV infected cells, two sites within the P25K:FLAG precursor were apparently processed to produce the 25K':FLAG and 25K:FLAG products. The production of 25K:FLAG proceeded with a lower efficiency than that of 25K. Nevertheless, by all of the criteria tested, 25K:FLAG appears to represent cleavage at the authentic Ala-Gly-Ala motif, and therefore may serve as a tagged substrate for the study of VV core protein proteolytic processing in VV-transfected cells. These criteria included a demonstration by pulse-chase labeling that the P25K:FLAG precursor is synthesized first, with 25K:FLAG appearing subsequently, concurrently with the reduction in the amount of precursor (Figure II.3). This maturation process was blocked by rifampin (Figure II.5). Furthermore, the cleavage reaction appeared to occur only in the context of the maturing virion, as the mature 25K:FLAG protein but not the P25K:FLAG precursor was found associated with virus particles (Figure II.6). Finally, a directed genetic (Figure II.4) approach was used to show that the conversion of P25K:FLAG to 25K:FLAG proceeds via cleavage at the conserved Ala-Gly-Ala motif within the precursor. Immunofluorescent staining experiments (Figure II.7) indicated that, unlike P25K and its derivatives, which display distinct

localization to virosomes in VV-infected cells, FLAG-tagged P25K related proteins are distributed throughout the cytoplasm.

As shown in Table II.I, when pL4R:FLAG was transiently expressed in VV-infected cells, the efficiency of the production of 25K:FLAG (33.71%) from the P25K:FLAG precursor was lower than P25K to 25K maturation (63.98%). The cytoplasmic distribution of P25K:FLAG and its derivatives shown by fluorescent staining (Figure II.7) and the localization of 25K:FLAG product in mature VV virions (Figure II.6) suggested that only a portion of the P25K:FLAG precursor was assembled into immature virions during VV virion assembly. The subsequent proteolytic cleavage carried out in virions during morphogenesis led to the production of 25K:FLAG. In other words, the P25K:FLAG precursor is likely to be processed authentically into 25K:FLAG only when incorporated into VV immature virions. The P25K:FLAG precursor was not completely processed because only a portion of the precursor protein was targeted to virosomes, where VV virion assembly takes place. We also noted that the proteolytic maturation of 25K (63.98%) from P25K in VV-infected cells receiving pL4R:FLAG was somewhat diminished in comparison with the percentage of P25K cleavage (83.20%) in VV-infected cells receiving salmon sperm DNA. This observation correlates well with the results of the pulse-chase experiment, in which ^{35}S-labeled P25K was completely chased into 25K when P25K:FLAG was transiently expressed (Figure II.3, lane 4) whereas under the same conditions pulse-labeled P25K was completely processed into the 25K product in cells receiving salmon sperm DNA (Figure II.3, lane 5). It is possible that the reduction in 25K production is due to the presence in VV-infected cells of P25K:FLAG, which competes with P25K for incorporation into assembling virions and/or the limited proteolytic machinery for VV

core protein proteolysis. On the basis of the inhibition noted above, it may be feasible to design peptide inhibitors which could compete with and therefore block the proteolysis of VV core proteins as well as virus maturation.

The production of the 25K':FLAG protein from the P25K:FLAG precursor was not anticipated. However, several lines of evidence suggest that it may be a biologically relevant cleavage product. First, the site I motif (Ala-Gly-Ser), which is suspected to give rise to 25K':FLAG, is implicated as an active cleavage site used during the proteolytic maturation of another VV core protein, P4a (VanSlyke et al., 1993). Second, mutagenesis of the Ala-Gly-Ser to Ile-Asp-Ile totally abolished the production of the 25K':FLAG product without affecting either the synthesis of the precursor or processing at site II, located 12 to 14 residues downstream. Third, a polypeptide with molecular intermediate mass between those of P25K and 25K (Figure II.6, lane 2) is detected within VV-infected cells in the presence of rifampin. This polypeptide was designated 25K' on the basis of its similar mobility relative to P25K and 25K compared with that of 25K':FLAG relative to P25K:FLAG and 25K:FLAG, although the identity of 25K' still needs to be verified. The facts that less 25K':FLAG is present than 25K:FLAG during transient expression of the pL4R:FLAG plasmid and that the P4a core protein precursor, containing the Ala-Gly-Ser motif, is more slowly processed than is P4b, which contains the Ala-Gly-Ala motif (VanSlyke *et al.*, 1991a), raises the possibility that the two motifs are processed at different rates. This may have functional significance during the viral replication cycle. Differential rates of cleavage of similar, but not identical, cleavage sites have previously been suggested to play a potential regulatory role in several RNA virus systems including potyviruses (Dougherty *et al.*, 1989), picornaviruses

(Lawson and Semler, 1990), and alphaviruses (de Groot *et al.*, 1990). Thus it is possible that 25K and 25K' are both produced during the course of a normal infection and that they play different roles during the replication cycle.

Previous experiments using N-terminal microsequencing of mature VV core proteins and computer analyses of the predicted amino acid sequences of the core protein precursors have implicated Ala-Gly-Ala and Ala-Gly-Ser/Thr as the conserved sites of cleavage in the P25K (and P4b) and P4a precursors, respectively (VanSlyke *et al.*, 1991a; 1991b; Yang and Bauer, 1988). On the basis of the derived sequences of the processed products, cleavage was predicted to occur between the second and third positions of this motif (AGX). However, no evidence was available to address the following questions. Is the AGX motif the actual site of cleavage? Does the cleavage process require exclusively endoproteolytic cuts like many viral systems (Kräusslich and Wimmer, 1988), or as with neuropeptide maturation (Harper and Keen, 1986; Jung and Scheller, 1991), are VV core proteins matured by a combination of endo- and exoproteolytic enzymes? Are different permutations of the AGX motif (Ala-Gly-Ala versus Ala-Gly-Ser) recognized by the same or different enzymes? Some of the data reported here have provided some insights into the answers to these questions. First, it was shown that mutagenesis of either site I (Ala-Gly-Ser) or site II (Ala-Gly-Ala) resulted in completely blocking the production of 25K':FLAG and 25K:FLAG, respectively. This result suggests that the AGX motif is the site of cleavage and that the reaction proceeds by an endoproteolytic mechanism. An alternative explanation of these results is that the introduced mutations disrupted the secondary structure of the precursor protein. We consider this unlikely, as mutations of the two sites

behaved independently. Although quantification in immunoblot analysis was not feasible, mutation of site I appeared to reduce cleavage at site II only slightly. Likewise, mutation of site II did not interfere with cleavage at site I (Figure II.4). Conclusive proof that maturation proceeds by an endoproteolytic cut at the AGX motif will require the isolation and identification of the N-terminal peptides which are predicted to be liberated during this reaction. Second, the possibility that the proteolytic reaction at site I is catalyzed by a different enzyme or perhaps within a different compartment within the infected cell was suggested by the following results: (i) the 25K':FLAG product was not found in the purified mature virions (Figure II.6), (ii) 25K' was produced in the presence of rifampin, and (iii) the production of 25K':FLAG was not blocked by rifampin (Figure II.5). Previous studies (von Heijne, 1983) have demonstrated that a small amount of the P25K precursor was incorporated into immature virion particles but the proteolytic processing was blocked in the presence of rifampin. The inhibition of proteolysis of the P25K precursor to the 25K product at site II in the presence of rifampin may interrupt the incorporation of P25K precursors into the immature particles. Since the synthesis of late gene products is not blocked by rifampin, the concentration of unincorporated P25K would be expected to increase and this increase could account in part for a less restricted distribution of P25K. This redistribution may result in the inaccessibility of precursors to the virion assembly site as well as the proteolytic processing machinery and hence increase the possibility of the precursor polypeptides interacting with a different proteinase, perhaps one with an increased affinity for Ala-Gly-Ser sites, to produce 25K'. The hypothesis could also be applied to explain the production of 25K':FLAG during transient expression (with or without

rifampin treatment), which may be due to the distribution of P25K:FLAG throughout the cytoplasm, as demonstrated in Figure II.7.

Mutagenesis of the pL4R:FLAG expression vector and transient expression of the derived proteins should allow the sequence and structural requirements within the precursor for efficient cleavage to be delineated. This will undoubtedly involve more than the presence of the AGX motif *per se*. For example, with regard to the Ala-Gly-Ala motif, examination of the predicted amino acid sequence of the entire VV genome (Goebel *et al.*, 1990) reveals that this sequence occurs in seven different viral proteins, not all of which are subject to proteolytic processing (Whitehead and Hruby, in press). Likewise, as noted previously, even in the case of the Ala-Gly-Ala motifs, which are known to be cleaved in the P4b and P25K precursors, there is little sequence conservation surrounding the conserved tripeptide. One possible explanation is that maturation of the VV core protein precursors occurs via a signal peptidase mechanism similar to that used by both eukaryotic and prokaryotic secretory proteins. As with the VV core protein precursors, little primary sequence homology in the region flanking the cleavage sites of secretory proteins has been identified. However, three major structural motifs have been noted: (i) a basic amino-terminal region, (ii) a hydrophobic core, and (iii) a less hydrophobic cleavage region. At positions -3 and -1 relative to the cleavage site, Ala-X-Ala is the most frequently observed sequence (Perlman and Halvorson, 1983; von Heijne, 1983). Mutational analysis indicated that the net hydrophobicity determines the kinetics of precursor processing and the translocation of secretory proteins (Bird *et al.*, 1990, Chou and Kendall, 1990). Positive charge at the amino-terminal region of the signal peptides seems to be required for the translocation reaction and apparently determines the rate of translocation

(Akita *et al.*, 1990, Sasaki *et al.*, 1990). On the basis of these studies, it is of interest that a preferential distribution of negatively charged amino acid residues on the amino-terminal side and positively charged residues on the carboxy-terminal side of the Ala-Gly-Ala cleavage motif exists in the P4b and P25K core protein precursors. Furthermore, proline residues can be found only on the carboxy-terminal side of the cleavage motif. One obvious difference between VV core proteins and secretory proteins is that VV core proteins are not secreted from the cytoplasmic membrane of the infected cell. They do, however, become associated with a membranous structure (namely, the previrion) so it is possible that the sequences flanking the Ala-Gly-Ala motif could play some role in packaging of the VV core proteins into the assembling virion. In any case, mutational analysis of site II of the P25K:FLAG protein should reveal the structure-function relationships of these components in VV core protein proteolysis.

The experiments described here have shed little light on the enzyme(s) which are responsible for carrying out the processing of P25K:FLAG. On the basis of the precedents of other viral systems (Kräusslich and Wimmer, 1988) and the fact that processing of VV core proteins occurs efficiently in a wide variety of cells from different tissues and organisms, we would predict that the proteolytic enzyme(s) is encoded by the viral genome. However, it remains an open question whether the processing of the different VV core proteins involves one or several enzymes and if they are cellular or viral enzymes. Likewise, since core protein maturation seems to be coupled to virion morphogenesis, it is likely that there will be a variety of other VV proteins which influence proteolytic processing. Several groups of VV temperature-sensitive mutants with defects in core protein processing and virion morphogenesis have been

isolated (Condit *et al.*, 1983; Lake *et al.*, 1979). Mapping of the lesions encoding the conditional-lethal phenotypes has implicated the gene products of the VV D2, D3, D13, and I8 ORFs, in addition to the VV core protein precursors, as being essential for VV morphogenesis (Dyster and Niles, 1991; Fathi and Condit, 1991a; 1991b). It is likely that there are a large number of additional viral (and cellular) polypeptides involved in the assembly of the infectious progeny VV virions. It is hoped that utilization of the *trans* processing assay described in this article will help identify the polypeptide participants and their functions, especially the proteinase, in VV core protein proteolytic processing.

Chapter III

PROTEOLYTIC CLEAVAGE OF VACCINIA VIRUS VIRION PROTEINS: MUTATIONAL ANALYSIS OF THE SPECIFICITY DETERMINANTS

Authors: Peiyu Lee and Dennis E. Hruby

Summary

Previous studies have suggested that cleavage of vaccinia virus core protein precursors occurs within the consensus tripeptide motif -A-G*X-. As an approach to delineate the sequence and structural features of the precursor polypeptides which are responsible for directing site-specific scission within this element, site-directed mutagenesis procedures were employed in concert with an *in vivotrans* processing assay of the P25K:FLAG reporter plasmid. The results obtained suggest that residue occupancy at the P1' site [Following the nomenclature of Schechter and Berger (*Biochem. Biophys. Res. Comm.* 27:157-162), the positions at the amino- and the carboxy-proximal residues are indicated as P1, P2, etc., and P1', P2', etc., respectively.] was extremely permissive, with only a proline substitution blocking cleavage. In contrast, the permissible occupancy of the P1 (serine or alanine) and P2 (cysteine, serine or asparagine) sites was extremely restricted. Analysis of P1/P2 double mutants supported this conclusion and suggested additional levels of combinatorial stringency. Insertion or deletion of sequences immediately adjacent (amino or carboxyl) to the AGX motif completely abrogated cleavage, suggesting the presence of additional important structural determinants. Mutation of the conserved proline or basic amino acid residues in these regions had no effect on cleavage, whereas it appeared that the presence of a hydrophobic residue in the P4 site was required.

Introduction

Several vaccinia virus (VV) virion proteins, including 4a, 23K, 4b, 25K (VP8), 21K, and 17K have been identified to be proteolytic products of larger molecular weight precursors (Yang and Bauer, 1988; VanSlyke *et al.*, 1991a; 1991b; Whitehead and Hruby, in press). Several lines of evidence led us to the hypothesis that the proteolytic processing of VV virion proteins is a critical step interconnected to virion maturation. First of all, the proteolytic processing of VV major core proteins is found to be arrested when virus maturation is prevented by the addition of the drug rifampin (Katz and Moss, 1970b). Likewise, similar concurrent blockage of core protein proteolysis and virus maturation were observed in cells infected with one subset of *ts* mutants at the non-permissive temperature (Silver and Dales, 1982). Finally, direct evidence was provided by the discovery that core proteins are found primarily as precursors in immature particles isolated on sucrose log-gradients (VanSlyke *et al.*, 1993).

Although the proteolytic activity responsible is yet to be identified, the cleavage sites within the P4a, P4b, and P25K precursors have been identified. A consensus -A-G*A- motif with the cleavage taking place after the Gly residues was identified in VV P4b, P25K, P21K and P17K precursors (Fig. 1) (Yang and Bauer, 1988; VanSlyke *et al.*, 1991a, Whitehead and Hruby, in press). The same sequences are also located at the same location in fowlpox virus (FPV) FP4b and FP5, the homologues of VV P4b and P25K, respectively (Binns *et al.*, 1989; Binns *et al.*, 1988). Interestingly, all of the processed -A-G*A- sequences are positioned near the amino-termini of the precursor proteins. Furthermore, two cleavage sites, namely -A-G*S- and -A-G*T- are used for the production of 4a

Figure III.1. Alignment of the predicted amino acid sequences surrounding the cleavage sites of vaccinia virus and fowl pox virus proteins. *: N-terminal amino acid sequences of the products have been confirmed by N-terminal micro sequencing. ❋: Cleavage sites are suggested in homologous polypeptides (FPV/FP4b and FP5) or by peptide mapping (VV/P4a). Basic amino acid (bold type), proline (underlined) residues and the hydrophobic residues at position P4 (shaded) are indicated.

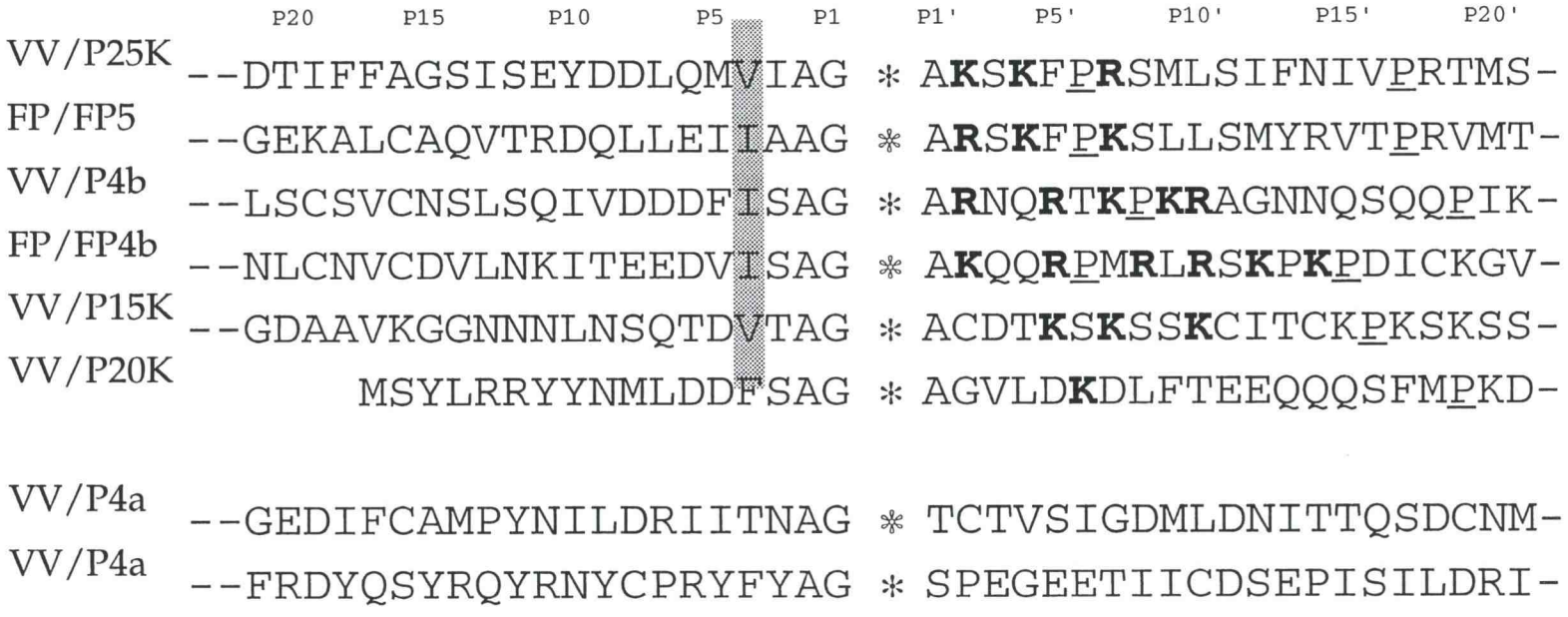

P20 P15 P10 P5 P1 P1' P5' P10' P15' P20'
VV/P25K --DTIFFAGSISEYDDLQMVIAG * AKSKFPRSMLSIFNIVPRTMS-
FP/FP5 --GEKALCAQVTRDQLLEIIAAG * ARSKFPKSLLSMYRVTPRVMT-
VV/P4b --LSCSVCNSLSQIVDDDFISAG * ARNQRTKPKRAGNNQSQQPIK-
FP/FP4b --NLCNVCDVLNKITEEDVISAG * AKQQRPMRLRSKPKPDICKGV-
VV/P15K --GDAAVKGGNNNLNSQTDVTAG * ACDTKSKSSKCITCKPKSKSS-
VV/P20K MSYLRRYYNMLDDFSAG * AGVLDKDLFTEEQQQSFMPKD-
VV/P4a --GEDIFCAMPYNILDRIITNAG * TCTVSIGDMLDNITTQSDCNM-
VV/P4a --FRDYQSYRQYRNYCPRYFYAG * SPEGEETIICDSEPISILDRI-

Figure III.1

and 23K from the P4a precursor, and an -A-G*S- sequence has been implicated in the production of a second product, 25K', from the P25K precursor (VanSlyke *et al.*, 1991a, Lee and Hruby *et al.*, 1993). Therefore, a consensus -A-G*X- sequence has been proposed in the precursor substrates as the site of proteinase recognition and hydrolysis.

The importance of the primary -A-G*X- sequence in P25K proteolytic cleavage is supported by the observation that no cleavage product was detected when the -A-G*A- sequence (residues 31-33) was altered to -I-D*I- (Lee and Hruby, 1993). However, not all VV proteins containing a -A-G*X- tripeptide motif are proteolytically cleaved. One such example is the -A-G-N- site (residues 94-96) found in P4a does not appear to function as a cleavage site even though this precursor is cleaved at two downstream sites (VanSlyke *et al.*, 1991b). In addition, three other virus-encoded polypeptides, P37K, DNAP, and HR have been demonstrated not to undergo proteolytic processing at the sequence -A-G-A- sites found within the polypeptides (Whitehead and Hruby, in press). Therefore, the -A-G*X motif appears to be essential but not sufficient for defining a specific cleavage site, and additional substrate determinants within the precursors must contribute to cleavage site selection.

In order to dissect the substrate determinants, we have employed the previously-established *trans* processing assay (Lee and Hruby, 1993). The P25K:FLAG substrate consists of the authentic P25K precursor plus an eight amino-acid epitope (FLAG) tagged to its carboxyl-terminus for the detection of transiently expressed P25K derivatives as opposed to the genomic P25K derivatives. Using this assay, an additional cleavage product, 25K', derived from cleavage at the -A-G*S- motif (residues 17-19, site I) in P25K polypeptides, was detected. This chapter details the results of our investigation to identify and characterize the substrate determinants for the production of 25K product

via cleavage at the -A-G*A- sequence (site II) in P25K precursor, since the authentic 25K product is the major core component in mature VV particles whereas the significance, if any, of 25K' product is unknown. The substrate requirements at positions P2, P1 and P1' and in the flanking regions of the -A-G*A- sequence for proteinase cleavage were dissected by analyzing the effect of amino acid substitutions, insertions and deletions on the production of 25K product.

Materials and Methods

Cells and virus.

Infection and purification of vaccinia virus (VV, WR strain) were performed as previously described (10). BSC-40 (African green monkey kidney) cells were maintained in MEM Eagle's medium ([MEM-E], Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker Bioproducts, Inc., Walkersville, MD), 2 mM glutamine, and 10 μg/ml of gentamycin.

Plasmid construction and site-directed mutagenesis.

The construction of pL4R:FLAG which contains the coding region for P25K polypeptide with the eight-amino-acid FLAG epitope fused to its carboxy terminus was previously described (Lee and Hruby, 1993). Oligonucleotide site-directed mutagenesis utilizing the E. coli strains CJ236 (*dut-1, ung-1, thi-1, relA-1*/pCJ105(Cm^r) and MV1190 (*Δ(lacproAB), thi, supE, (srl-recA)306*::Tn*10* (tet^r) [F':*traD36, proAB, lacI*^{q}*Z* ΔM15]) together with M13KO7 helper phage was performed according to the method of Kunkel (Kunkel, 1985). Oligonucleotides for the introduction of substitution, insertion and deletion mutations were synthesized on Applied Biosystems DNA synthesizer. All of the mutants were constructed by using single-stranded wild-type pL4R:FLAG template except mutant KKR/EED which employs mutant K39D as the template. Each mutant was confirmed by subjecting plasmid DNA to dideoxynucleotide DNA sequencing procedures (Sanger *et al.*, 1977). Mutants with single amino-acid substitution are designated with the wild type amino acid residue in one-letter

code and its position in the P25K precursor polypeptide followed by the substituting amino acid residue.

Transient expression.

Monolayers of BSC-40 cells were infected with VV at a multiplicity of infection of 5 infectious particles per cell and transfected with plasmid or sheared salmon sperm DNA at 0 h p.i. using a liposome-mediated transfection protocol previously described (Lee and Hruby, 1993).

Immunoblotting.

The immunoblot analyses were performed as previously described (Lee and Hruby, 1993). Briefly, cell extracts were prepared, resolved in sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis, electrotransfered to nitrocellulose filter membranes and subjected to binding with the FLAG-specific M2 monoclonal antibody (MAb) (1:1000 dilution) (International Biotechnologies, Inc., New Haven, CT). The filters were incubated with the alkaline phosphatase-conjugated goat-anti-mouse antibody (1:2000 dilution) and probed with the alkaline phosphatase assay kit (Bio-Rad).

Results

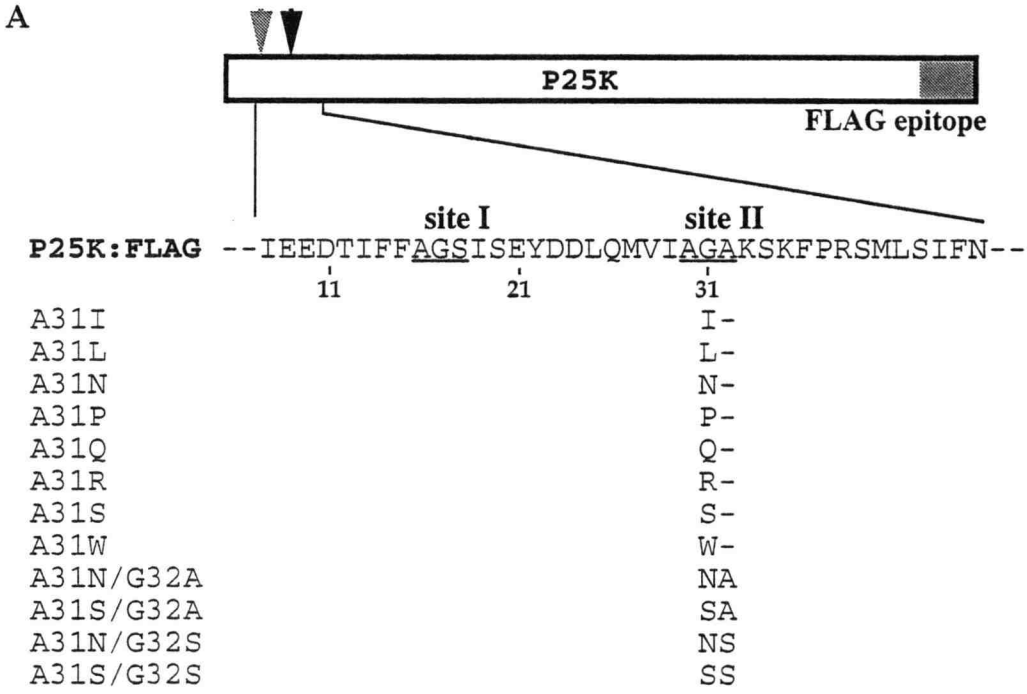
Amino acid sequence analysis

When the aligned amino acid sequence of the precursors of VV and FPV virion proteins are examined, in addition to the obvious conservation of an Ala at P2 and a Gly at P1 position, two of the three previously suggested characteristics in the regions surrounding the -G*A- pairs in P4b and P25K (Lee and Hruby, 1993) are also found in P21K and P17K (Figure III.1). Namely, basic residues are found to reside on the carboxyl side of the cleavage sites, and at least one proline residue is found near the P' region of the scissile bond. An additional conserved feature noted here is that all of the cleavage sites contain a hydrophobic residue (Val, Ile, or Phe) at position P4, except in the -G*T- cleavage sites in P4a where a Thr residue is found.

Expression and proteolytic cleavage of P25K:FLAG mutants in VV-infected cells.

In order to investigate the substrate requirements for correct proteinase-substrate interaction, mutants of P25K:FLAG with amino acid substitutions were created by site-directed mutagenesis. Following transient expression in VV-infected cells overnight, cell extracts were analyzed by immunoblot analysis using the FLAG-specific M2 MAb. Since the M2 MAb immunoprecipitates poorly, at best, immunoblot analysis were carried out and the results were categorized as "-" for no detection and "+" for detection of the products from cleavage at site II. An example of typical results are shown in Figure III.2B, with structure of the mutants illustrated in Figure III.2A.

Figure III.2. Effect of amino acid substitutions on the proteolytic cleavage of P25K:FLAG precursor. (A) Schematic representation of P25K:FLAG precursor with cleavage sites I and II marked above (shaded arrow and solid arrow, respectively). The predicted amino acid sequences flanking the two cleavage sites are displayed underneath in the single-letter amino acid code with both cleavage motifs (underline). The relative positions of the amino acid residues within the precursors are designated by the numbers beneath the amino acid sequence. The amino acid substitutions in the mutants analyzed in Fig 2B and 2C are shown below. Amino acid residues identical to the wild type P25K:FLAG are indicted as a dash. (B) Mutants with single-amino-acid substitutions at position P2. (C) Mutants with double mutations at both positions P1 and P2. Cells extracts were prepared following overnight transient expression of the wild type pL4R:FLAG plasmid DNA(+), sheared salmon sperm DNA (-), or mutant plasmid DNA in VV-infected cells, and analyzed by immunoblot with a FLAG-specific M2 monoclonal antibody. The P25K:FLAG precursor polypeptide and the 25K':FLAG cleavage product from proteolytic cleavage at -A-G*S- (site I) are indicated (arrowhead and square dot, respectively). Finally, 25K:FLAG products derived from -A-G*A- (site II) are the fastest moving bands without labels. The positions and sizes (kDa) of molecular weight (MW) standards are indicated on the left.


A
P25K
FLAG epitope
site I
site II
P25K:FLAG --IEEDTIFFAGSISEYDDLQMVIAGAKSKFPRSMLSIFN--
11
21
31
A31I I-
A31L L-
A31N N-
A31P P-
A31Q Q-
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A31N/G32S NS
A31S/G32S SS

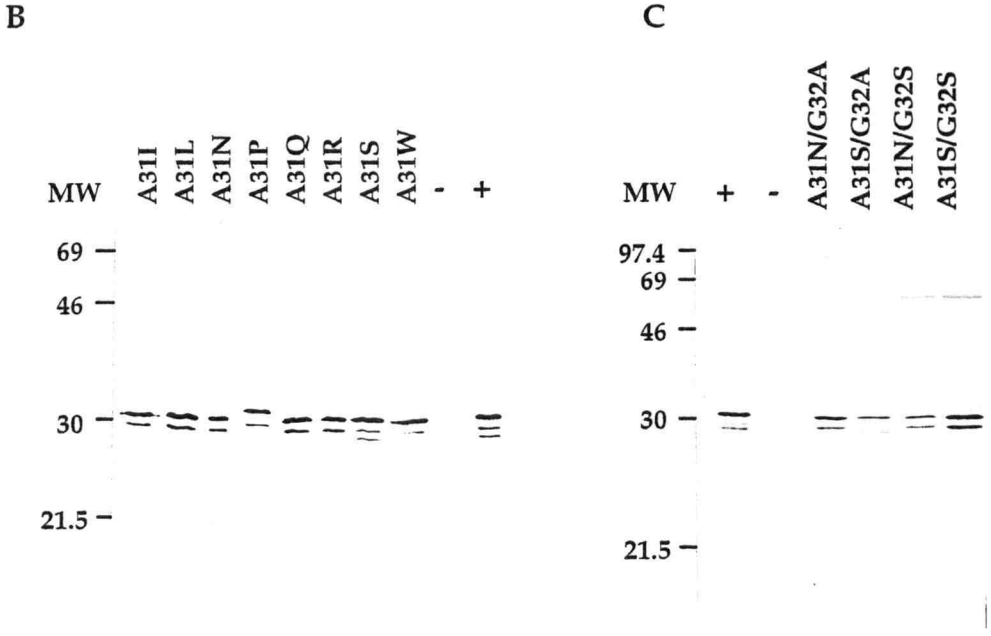

B
MW
A31I
A31L
A31N
A31P
A31Q
A31R
A31S
A31W
-
+
69
46
30
21.5
C
MW
+
-
A31N/G32A
A31S/G32A
A31N/G32S
A31S/G32S
97.4
69
46
30
21.5

Figure III.2

Substitution of the Ala residue at position P2 with Ile (A31I), Leu (A31L), Pro (A31P), Gln (A31Q), Arg (A31R), or Trp (A31W) (Figure III.2B) totally abolished the production of the 25K:FLAG product. In contrast, substitutions such as Ser (A31S) or Asn (A31N) (Figure III.2B) allowed cleavage at site II to proceed albeit to different extents. Also, it was noted that the electrophoretic mobility of the P25K:FLAG precursor and the 25K:FLAG' product were both altered in the A31P mutant (Figure III.2B). Nucleotide sequencing of the A31P mutant verified that no other alterations in the sequence of the P25K:FLAG polypeptide were present (data not shown).

Substitution at Position P1' with proline but not other amino acids blocks cleavage.

The immunoblot analyses of the proteolytic processing assay of all the mutants with amino acid substitutions at P1' are summarized in Table III.I (A33 mutants). Replacement of the wild-type Ala residue by Asp (A33D), Glu (A33E), Phe (A33F), His (A33H), Leu (A33L), Asn (A33N), Arg (A33R), Ser (A33S), Thr (A33T), Val (A33V), or Tyr (A33Y) did not block cleavage reaction, whereas replacement with a Pro residue (A33P) completely blocked cleavage. It is possible that the Pro residue, which is known to introduce β-turn conformation in a polypeptide, when introduced at P1' position may disrupt the correct presentation of other proteinase recognition signals. Therefore, the P25K:FLAG substrate-proteinase interaction apparently does not have any specific occupancy requirements at position P1'.

Proteolytic cleavage requires amino acids with small side chains at positions P1 and P2.

Substrate	Amino acid sequence[b]	Cleavage[c]
P25K:FLAG	-GSISEYDDLQMVIAG * AKSKFPRSMLSIFNI-	+
A31C	C- * -	+
A31D	D- * -	-
A31F	F- * -	-
A31I	I- * -	-
A31L	L- * -	-
A31N	N- * -	+
A31P	P- * -	-
A31Q	Q- * -	-
A31R	R- * -	-
A31S	S- * -	+
A31T	T- * -	-
A31G	V- * -	-
A31W	W- * -	-
G32A	-A * -	+
G32C	-C * -	-
G32E	-E * -	-
G32I	-I * -	-
G32L	-L * -	-
G32N	-N * -	-
G32P	-P * -	-
G32R	-R * -	-
G32S	-S * -	+
G32T	-T * -	-
G32V	-V * -	-
G32Y	-Y * -	-
A33D	-- * D	+
A33E	-- * E	+
A33F	-- * F	+
A33H	-- * H	+
A33L	-- * L	+
A33N	-- * N	+
A33P	-- * P	-
A33R	-- * R	+
A33S	-- * S	+
A33T	-- * T	+
A33V	-- * V	+
A33Y	-- * Y	+
A31S/G32S	SS * -	-
A31S/G32A	SA * -	+
A31N/G32S	NS * -	-
A31N/G32A	NA * -	-

a. Mutaions at each position were introduced by site-directed mutagenesis and assayed by transient expression in VV-infected cells as described in the legent to Fig. 2.

b. The amino acid sequence flanking P25K cleavage site II (-A-G*A-, residues 31-33) is listed in the wild type (P25K:FLAG) panel. For the mutants, only the substituting residues were designated in the panels below and the dashes represent residues identical to wild type.

c. The results were classified as "+" for detection and "-" for no detection of cleavage products in the immunoblot assays using anti-FLAG M2 monoclonal antibody.

Table III.1 Effect of amino acid substitutions at the P2, P1, and P1' positions on the cleavage of site II in P25K:FLAG in VV-infected cells[a]

The results of all the substitutions made at P2 and P1 positions are summarized in Table III.I. Hydrolysis at the -G*A- bond did not take place when the conserved Ala residue at position P2 was replaced by Asp (A31D), Phe (A31F), Ile (A31I), Leu (A31L), Pro (A31P), Gln (A31Q), Arg (A31R), Thr (A31T), Val (A31V), or Trp (A31W). However, in addition to Ser (A31S) and Asn (A31N) (Figure III.2B) mentioned above, a substitution by Cys (A31C) allows hydrolysis of the -G*A- scissile bond. Examination of the chemical characteristics of the wild type (Ala) and the acceptable substituting residues reveals that they all display uncharged side chains, and their relatively small size readily distinguishes them from the other uncharged amino-acid residues such as Gln and Thr.

Likewise, the replacement of the Gly at position P1 by Ser (G32S) or Ala (G32A) residue, in which the scissile bond becomes -S*A- or -A*A- respectively, allows the cleavage of the peptide bonds to proceed. Whereas, mutants with P1 substituted with Cys (G32C), Glu (G32E), Ile (G32I), Leu (G32L), Asn (G32N), Pro (G32P), Arg (G32R), Thr (G32T), Val (G32V), or Tyr (G32Y) residues produced no detectable amount of the 25K:FLAG product. The results would suggest that the side chains at both P2 and P1 positions in the P25K:FLAG substrates must be small and uncharged for proteinase recognition.

Specific combination of amino acid residues at positions P1 and P2 is required for cleavage at site II in P25K.

To address whether the combination of the side chains at P1 and P2 positions places any additional stringency on the scissile bond hydrolysis, mutations at both P1 (either Asn or Ser) and P2 (either Ser or Ala) positions were introduced (Figure III.2A). Of the four different combinations, namely A31S/G32S, A31S/G32A, A31N/G32S, and A31N/G32A, only the A31S/G32A

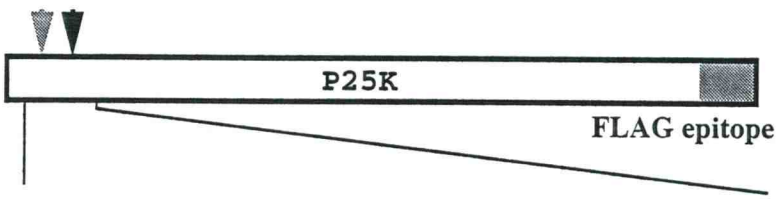
mutant produced a detectable level of cleavage product from site II (Figure III.2C), suggesting that cooperation between the two side chains at P1 and P2 may also be an essential determinants for substrate recognition. Taken together, the results support the hypothesis that site occupancy limitations are present at both the P1 and P2 positions for proteinase recognition.

-A-G*A- sequence requires correct context for proteolytic cleavage

To determine whether other specificity features reside in the regions flanking the scissile bond, mutants which acquired either a ten-Leu insertion or a ten-amino-acid deletion upstream or downstream of the -A-G*A- motif, designated (L)10AGA, AGA(L10), AGAΔ10, and Δ10AGA, were constructed (Figure III.3A). The predicted molecular masses of the precursors was 30.7, 30.7, 28.0 and 28.0 kDa, respectively. The insertion of ten leucine residues next to the cleavage site should isolate the -A-G*A- motif from the other substrate determinants in the flanking region. Likewise, deletion of amino-acid sequence would remove any pertinent substrate determinants. Immunoblot analysis indicates that only precursors migrating at the expected molecular weight could be detected in (L)10AGA, AGA(L10), and AGAΔ10 mutants (Figure III.3B). Cleavage products resulting from either site I or II could not be detected. However, the precursor and a single product (marked with an asterisk) are produced from the Δ10AGA construct (Figure III.3B). Since the predicted molecular masses for 25K:FLAG' and 25K:FLAG (26.0 and 25.8 kDa, respectively) are very similar, it is difficult to determine whether the product is derived from site I or II. In any event, the results suggest that the proteinase has recognition requirements in addition to the -A-G*X- tripeptide. Concurrent arrest of cleavage at site I with the manipulation of amino-acid segments downstream of site II in these three mutants suggests either that the additional

Figure III.3 Effect of insertion or deletion of ten-amino-acid residues next to the -A-G*A- cleavage motif on cleavage of P25K:FLAG. (A) Schematic representation of P25K:FLAG precursor with the two cleavage sites is as described in Figure III.2. The relative positions of the amino acid residues within the precursors are designated by the numbers above the amino acid sequence. Wild type P25K:FLAG and mutants with ten-leucine residues inserted to either the amino- ((L)10AGA) or carboxy-side (AGA(L)10), or with ten-amino-acid residues deleted either upstream (Δ10AGA) or downstream (AGAΔ10) of the -A-G*A- sequence are listed beneath with their amino acid sequences flanking the cleavage site shown in the single-letter amino acid code. (B). Wild type pL4R:FLAG DNA (+), sheared salmon sperm DNA (-), or mutant DNA was transfected into VV-infected cells. Cell extracts prepared and analyzed by immunoblot with the FLAG-specific M2 monoclonal antibody. The P25K:FLAG precursor polypeptides and 25K:FLAG' cleavage products from proteolytic cleavage at -A-G*S- (site I) are indicated (arrow heads and square dots, respectively). The fastest moving bands, which are the 25K:FLAG products derived from the -A-G*A- sequence (site II), are not labeled. The positions and sizes (kDa) of molecular weight (MW) standards are indicated on the left.

A




```
                  11          21          31
P25K:FLAG     -EDTIFFAGSISEYDDLQMVIAGAKSKFPRSMLSIFN-
(L)10AGA      -EDTIFFAGSISEYDDLQMVI(L)10AGAKSKFPRSMLSIFN-
AGA(L)10      -EDTIFFAGSISEYDDLQMVIAGA(L)10KSKFPRSMLSIFN-
AGAΔ10        -EDTIFFAGSISEYDDLQMVIAGA~~~~~~~~~~IFN-
Δ10AGA        -EDTIFFAGSI~~~~~~~~~~AGAKSKFPRSMLSIFN-
```

B

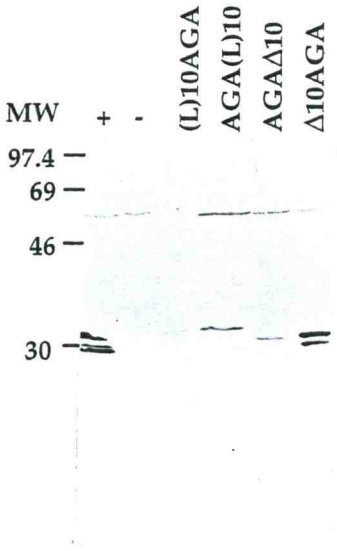


Figure III.3

Figure III.4 Immunoblot analysis of P25K:FLAG mutants with a second -A-G-A- downstream. (A) The position and flanking sequences of two engineered AGA sites are indicated below the schematic representation of P25K:FLAG. Cell extracts harvested and analyzed by immunoblot as before except SDS-15% polyacrylamide gels were used. The P25K:FLAG precursor and 25K:FLAG' product are indicated (arrowheads and square dots, respectively) and 25K:FLAG product is not labeled. The positions and sizes (kDa) of molecular weight (MW) standards are indicated on the left.

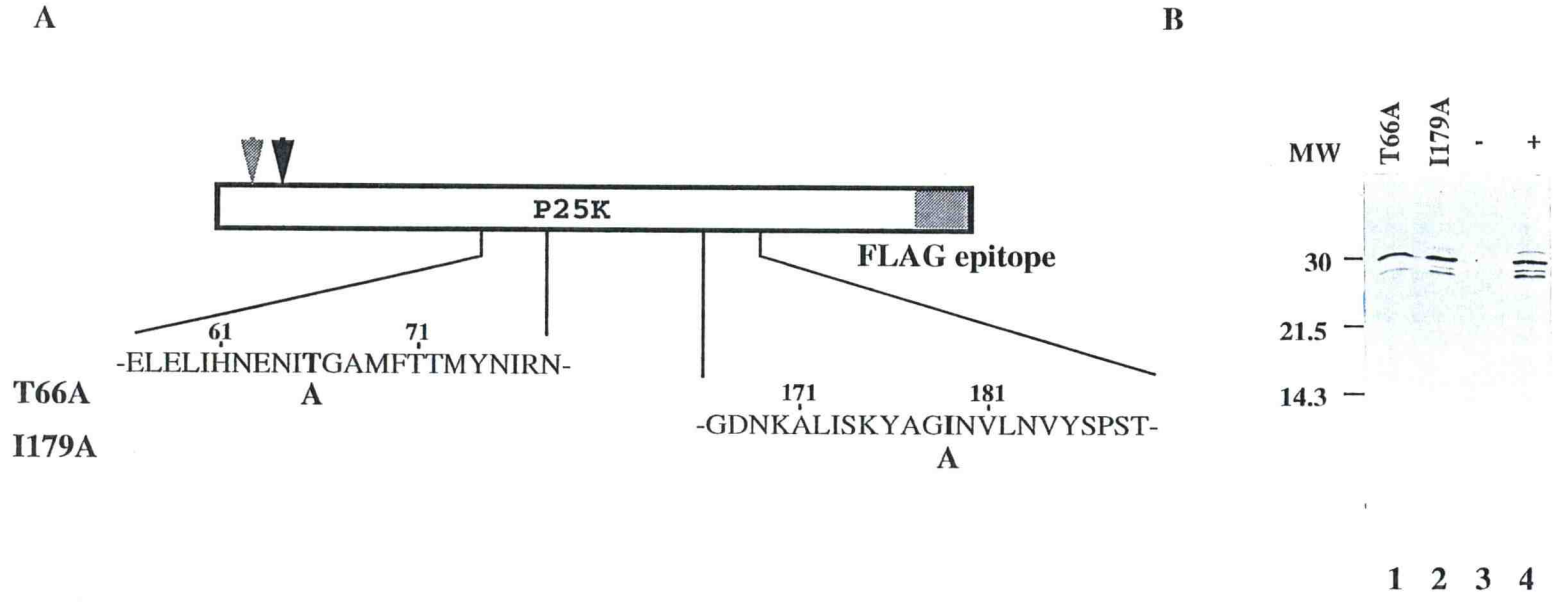

A
B
P25K
FLAG epitope
61
71
-ELELIHNENITGAMFTTMYNIRN-
A
T66A
I179A
171
181
-GDNKALISKYAGINVLNVYSPST-
A
MW
T66A
I179A
-
+
30
21.5
14.3
1 2 3 4

Figure III.4

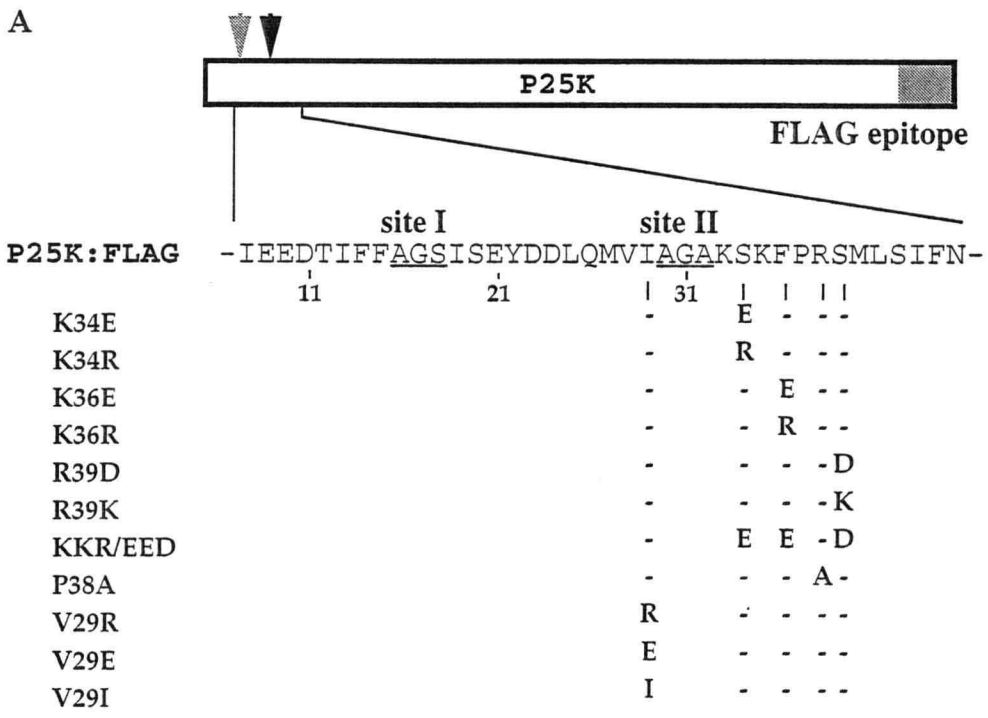
determinants may reside as far as 15-24 residues downstream from the -A-G*S- cleavage motifs or that the deletion and insertion may alter the conformation at both cleavage motifs essential for proteinase recognition.

To verify the importance of protein context for cleavage, a second -A-G-A- sequence was created in P25K:FLAG polypeptide by altering the Thr residue at position 66 (T66A) or the Ile residue at position 179 (I179A) to an Ala residue (Figure III.4A). The molecular masses of the cleavage products from the new sites III and IV are predicted to be 21.7 and 9.1 kDa, respectively. No cleavage products other than 25K:FLAG and 25K:FLAG' were detected from either mutant (Figure III.4B). The proteinase appeared not to recognize the -A-G-A- sequences engineered downstream in P25K polypeptide even though the precursor has been brought into the proximity with the proteinase as indicated by the detection of both 25K:FLAG and 25K:FLAG' products. Taken together, the results support the hypothesis that the -A-G-X- tripeptide motif alone is not sufficient for the proteinase recognition. It was therefore of interest to investigate the importance of several conserved features of all known VV structural protein cleavage sites, namely, clustering of positive charges and a proline residue in the P' region, and a hydrophobic residue at the P4 position.

Mutations of the basic amino acids at positions P2', P4' and P7' do not affect cleavage.

To determine whether the individual basic residues downstream of site II are required for proteolytic cleavage, the amino acids at P2', P4' or P7' in P25K were each mutated to either similar positive charges (K34R, K36R and R39K) or the opposite charges (K34E, K36E, and R39D) (Figure III.5A). The P25K:FLAG precursor and both 25K:FLAG' and 25K:FLAG cleavage products were detected in all cases (Figure III.5B). Furthermore, the KKR/EED mutant

Figure III.5 Effect of amino acid substitutions in the flanking regions of the -A-G*A- motif on the proteolytic cleavage of P25K:FLAG. (A) Schematic representation of P25K:FLAG precursor is shown together with mutants with amino acid substitutions at P4, P2', P4', P6' and P7' positions. Amino acid residues identical to the wild type P25K:FLAG are indicated (dash). The parental pL4R:FLAG DNA (+), sheared salmon sperm DNA (-), as well as (B) plasmid DNA of mutants with single or triple amino acid substitutions of the basic residues at P2', P4' and P7' positions, or (C) plasmid DNA of mutants with a Pro residue at position P6' and a Val residue at P4 were analyzed by transient expression and immunoblot. Reaction of the FLAG-specific M2 monoclonal antibody to precursor P25K:FLAG are indicated (arrow head). The products 25K:FLAG obtained from cleavage at the -A-G*A- motif are the smaller products of the two. The larger products 25K:FLAG' are indicated by square dots. The positions and sizes (kDa) of molecular weight (MW) standards are indicated on the left.


A
P25K
FLAG epitope
site I
site II
P25K:FLAG -IEEDTIFFAGSISEYDDLQMVIAGAKSKFPRSMLSIFN-
11
21
31
K34E
K34R
K36E
K36R
R39D
R39K
KKR/EED
P38A
V29R
V29E
V29I

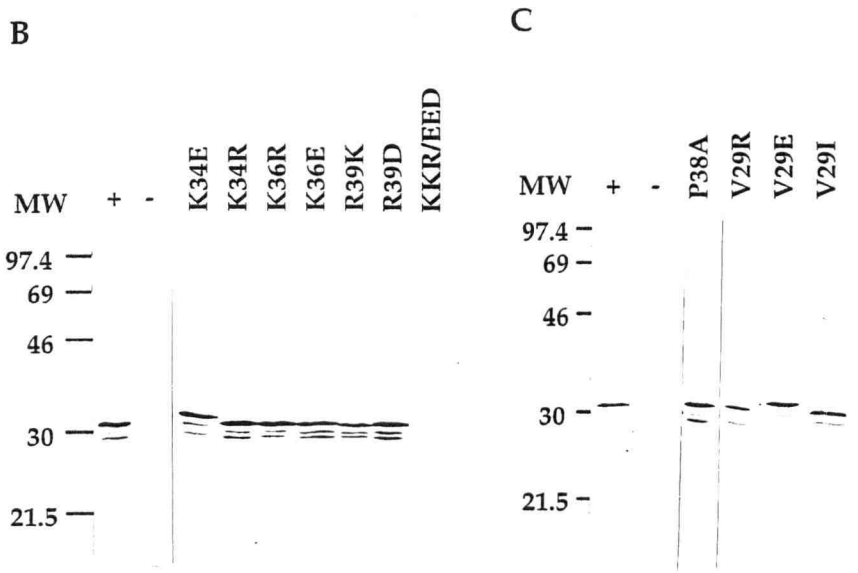

B
MW + -
K34E
K34R
K36R
K36E
R39K
R39D
KKR/EED
97.4
69
46
30
21.5
C
MW + -
P38A
V29R
V29E
V29I
97.4
69
46
30
21.5

Figure III.5

with all three positions substituted with acidic residues was constructed. Only one polypeptide band is detected (Figure III.5B) which is presumably the unprocessed precursor migrating slower than the wild-type P25K:FLAG due to the structural alteration introduced by the mutations, suggesting that these three side chains may contribute certain overall conformational or chemical stringency to the cleavage site selection in the P25K precursor.

Proline at position P6' is not essential for P25K cleavage.

To determine whether the Pro residue at position P6' is required for cleavage at site II in P25K, a mutant with an Ala substitution (P38A) at this position was constructed (Figure III.5A). The presence of the 25K:FLAG product suggests that the proline residue downstream of the cleavage sites is not essential for the recognition by the proteinase.

Position P4 requires amino acids with hydrophobic side chains.

To determine whether a hydrophobic residue at position P4 is required for the proteolytic cleavage, mutants with charged residues or alternative hydrophobic residues at this position were constructed (Figure III.5A). Replacement of the wild-type Val residue by the Arg (V29R) or Glu (V29E) residue diminishes the production of 25K:FLAG product (Figure III.5C), whereas the product was detected when the hydrophobicity was maintained by the substituting Ile (V29I) (Figure III.5C). The results support the hypothesis that the hydrophobic side chains at position P4 are essential for cleavage site recognition and/or hydrolysis.

Discussion

The first experimental evidence demonstrating the importance of the -A-G*A- motif in P25K cleavage was provided by the observation that mutation of this sequence to -I-D-I- blocked maturation of the 25K protein (Lee and Hruby, 1993). The main objectives of this study were to identify and characterize the specificity determinants for the proteolytic processing of VV virion proteins by determining the effects of amino-acid substitutions in the tripeptide motif and its flanking regions on subsequent peptide bond hydrolysis *in vivo* using the P25K:FLAG reporter plasmid.

Interestingly, different migration rates have been observed in mutants A31P (Figure III.2B), K34E (Figure III.5B), V29R and V29E (Figure III.5C). Presumably, this is due to the alteration in charges or conformation of the polypeptides caused by the amino acid substitutions which include Pro, acidic or basic residues. Similar migration shifts have also been noted when similar amino acid substitutions or insertions were introduced in poliovirus polyprotein substrates (Ypma-Wang and Semler, 1987; Ypma-Wang, *et al.*, 1988). Likewise, there is the example of a Pro-rich fragment from VV Ag35 polypeptide which apparently migrates at a position about 12.5 kDa larger than the predicted molecular mass (Gordon *et al.*, 1991). Both 25K:FLAG' and 25K:FLAG bands were detected from mutants with the methionine residue at residues 28 or 41 of P25K:FLAG precursor individually substituted with a different amino acid residue (data not shown). This result apparently rules out the possibility that the polypeptides detected by FLAG-specific MAb originate from an internal translation initiation.

The origin of the slowest migrating protein species detected by the M2 MAb, shown most prominently in Figure III.5C, is unknown. It is produced at low levels and can be detected only when excess cell extracts or less dilute M2 MAb were used in immunoblot analysis. Because it displays similar levels of migration shift to the other FLAG-tagged protein bands, it is likely valid to designate the second slowest migrating band in V29E and V29R (Figure III.5C, marked with arrowheads) as the full-length precursor. The identity of the slowest migrating band remains to be determined.

Taken together, the effects of the single substitutions at P2, P1 or P1' position (Figure III.2 and Table III.I) on proteolytic processing of P25K:FLAG precursor support the previously proposed -A-G*X- cleavage motif, although with additional cleavage tripeptides (-(A, C, N, S)-G*X-, -A-(C, A, S)*X-, and -S-A*X-) having been identified. The conclusion that additional specificity determinants in the flanking regions are required for hydrolysis of the -G*A- scissile bond was reached by the results from the insertion and deletion mutants (Figure III.3) as well as the T66A and I179A mutants (Figure III.4). This finding correlates well with the observation that the -A-G-N- sequence which is cleaved when presented at site II of P25K (A33N mutant, Table III.I) is not utilized as a cleavage site within P4a (VanSlyke *et al.*, 1991b).

Although, the single positive-charged residues at P2', P4' and P7' apparently are not required for proper proteinase-substrate interaction (Figure III.5B; K34E, K36E, R39D), the three positions altogether are likely essential for the specific cleavage at the -G*A- scissile bond in P25K (Figure III.5B, KKR/EED). The amino acids at these positions may contribute either the overall positive charges or a specific conformation which may not be modified by the replacement with acidic residues one at a time. The latter hypothesis is favored on the basis that (i) aberrant migration rate of the KKR/EED

precursors was observed in SDS-polyacrylamide gels, and (ii) no basic amino-acid residues are found in the P' region of the -A-G*(S, T) sites within P4a polypeptides. Although computer analysis for predicted secondary structure of the regions flanking the -A-G*X- motifs has not revealed any significant consensus, precedents are plentiful for the involvement of conformational determinants in defining cleavage sites in other viral systems, such as picornaviruses, flaviviruses, and retroviruses (Ypma-Wang and Semler, 1987; Ypma-Wang, *et al.*, 1988; Blair *et al.*, 1990; Dewalt *et al.*, 1989; Trich *et al.*, 1991; Zhang and Padmanabhan, 1993). Verification of either hypothesis will require further experiments.

Substitution analysis of the Val residue at position P4 (Figure III.5C) suggested that a hydrophobic residue at this position is one of the substrate determinants. This prediction is further supported by the occupancy of a Phe residue at position P4 of P25K cleavage site I (Figure III.2A). This finding expands the consensus cleavage motif to the -P4--P2-P1*X- region, which would accordingly support the hypothesis that all the -A-G*X- motifs are processed by the same proteinase which has been suggested on the basis of the following common characteristics shared by 4a, 4b, 25K, 21K and 17K: (i) Synthesis and proteolytic processing of the precursor proteins are detected concurrently at late stages during virus infection. (ii) They all localize to mature virions, and (iii) Cleavage of all of the precursors can be blocked by the drug rifampin. In the same light, we have previously observed that the appearance of 4a appears to be slower than that of 4b and 25K (VanSlyke *et al.*, 1991a). The presence of a Thr residue (Fig. 1), rather than a hydrophobic residue, at position P4 of the -A-G*T- motif within P4a precursor might imply that amino-acid side chains at position P4 may be involved in rate determination for -G*X- peptide bond hydrolysis. Temporal regulation of proteolytic cleavage of viral proteins

by cleavage-site preference defined by the amino-acid residues occupying a specific position proximal to the cleavage sites has been suggested as a level of gene-expression regulation in several other viral systems including the potyviruses (Dougherty and Park, 1989) and alphaviruses (de Groot *et al.*, 1990).

Our results were surprising in that the actual identity of amino acid residue at position P1' (Ala, Ser, and Thr) in the identified cleavage sites (Fig. 1) appears to be far more restricted than what was determined by the *trans*-processing assay. Likewise, although the Ala at P2 and Gly at P1 appear to be strictly conserved in virion proteins undergoing cleavage, these positions can be replaced by a number of residues with similar characteristics in our assay. Two different hypotheses may explain the preference in the natural cleavage sites. First, cleavage may take place at sites in other viral proteins and has yet to be identified. Second, the amino-acid identities at these three positions may be essential for the kinetics of peptide bond hydrolysis which in turn is critically coordinated with the correct virion maturation.

Comparison of the substrate requirements for VV cleavage with other viral systems reveals that The -A-G*X- tripeptide displays similarity to the -G-G*X- and -G-(G/A)*X- motifs used for polyprotein processing in African swine fever virus (López-Otín *et al.*, 1989) and Sindbis virus (Anderson, 1990), respectively, as well as the -G-(G/X)*(X/G)- in the structural protein precursors for adenovirus proteinase (Webster *et al.*, 1989a). Furthermore, the stringent requirements at P4, P2 and P1 positions for VV -A-G*X- cleavage are similar to the those determined so far for several viral proteinases. The preference of Ala at P4, Gly at P1 and Gln at P1' for poliovirus $3CP^{ro}/3CDP^{ro}$ have been demonstrated (Pallai *et al.*, 1989; Blair and Semler, 1991). Adenovirus protease also displays a substrate specificity of a hydrophobic residue at P4 position

(Anderson, 1990; Webster *et al.*, 1989a). The proteinases encoded by Sindbis virus is classified as a serine proteinase whereas the poliovirus 3C proteinase and adenovirus proteinase are both serine-like cysteine proteinases (Kay and Dunn, 1990). Therefore, the recognition and hydrolysis of VV proteins might conceivably be carried out in a similar mechanism.

Different ratios of cleavage products produced from sites I and II were noticed in different mutants. However, the potential relationship between the cleavage at sites I and II was not investigated because of the difficulty in quantifying immunoblots. Further characterization of the substrate specificity will await identification of the proteinase and detailed cleavage-kinetic analysis using either synthetic peptide substrates or mutant substrates generated by directed genetics. Overall, on the basis of the data provided in this report we propose that the hydrolysis of the -G*X- peptide bonds in VV core protein precursors is likely carried out by a proteolytic activity requiring (i) conformational features in the flanking regions for proper presentation of the scissile bonds, (ii) a hydrophobic residue at position P4, (iii) small and uncharged residues at both P1 and P2 positions for the specific interaction with the proteinase recognition and/or active sites.

Chapter IV

BIOLOGICAL FUNCTION OF THE AMINO-TERMINAL PEPTIDE OF VACCINIA VIRUS STRUCTURAL PROTEIN PRECURSORS

Authors: Peiyu Lee and Dennis E. Hruby

Summary

Several VV structural proteins are produced by the removal of amino-terminal peptides from their cognate precursors. Directed genetic approaches were undertaken to investigate the possible role of these terminal peptides in VV life cycle. As a model system, the P25K precursor which was abutted to an epitope tag was used to prepare constructs in which the P25K terminal peptide was removed or replaced by heterologous sequences, while the -A-G*-A cleavage motif was retained. Similar levels of 25K product were generated from P4b:25KF, which consists of P4b terminal peptide, and from P4b:25KF with 15, 30 or 44 residues of the amino terminus were deleted. In contrast, no 25K was produced from the TK:25KF, which contains the amino-terminal 30 residues of VV thymidine kinase. Only a small amount of P25KF(Δ31), derived from the leader-less construct, was found within the mature virions, implying that proteolytic processing is necessary for the incorporation of 25K product into mature virions. Precursors with or without terminal peptides were detected in DNA-containing immature particles, suggesting that the terminal peptides are not required for targeting the precursors to previrions. Taken together, these data suggest that the amino-terminal peptides are interchangeable and essential for proteolytic cleavage of VV structural protein precursors to occur during virus maturation.

Introduction

Previous studies revealed that approximately 100 species of polypeptides can be found in the relatively large and complex vaccinia virus (VV) virion (Essani and Dales, 1979). Production of infectious VV progeny within the cytoplasm of VV-infected cells apparently requires expression, correct post-translational modification, and appropriate subcellular localization of various structural and non-structural polypeptides (for review see Moss, 1990).

Thus far, several VV virion-associated proteins, including 4a, 4b, 25K, 23K, 21K, and 17K, are known to be originally produced as higher molecular-weight precursors, P4a, P4b, P25K, P21K, and P17K, respectively, at late times during infection (Katz and Moss, 1970a; Moss and Rosenblum, 1973; VanSlyke *et al.*, 1991a, 1991b, Yang *et al.*, 1988, Whitehead and Hruby, in press). On the basis of studies which employed different *ts* mutants, drug treatments, and purified previrions, it is believed that the proteolytic reactions take place after the precursors are assembled into VV previrions, and that these proteolytic cleavages are required for VV virion maturation as well as the acquisition of infectivity (Katz and Moss, 1970b, Stern *et al.*, 1977, Silver and Dales, 1982, VanSlyke *et al.*, 1993). Immunogold labeling using precursor-specific antiserum demonstrated that the P4a precursor could be found primarily in the immature particles (VanSlyke and Hruby, in press).

The coding sequences for the precursors P4a, P4b, P25K, P21K and P17K have been mapped and determined (Rosel and Moss, 1985; Wittek *et al.*, 1984; Van Meir and Wittek, 1988; Whitehead and Hruby, submitted;

Weir and Moss, 1985). Alignment of the predicted amino acid sequences of the precursors at the regions flanking the amino termini of the cleavage products has revealed several conserved characteristics. First, a putative -A-G*X- cleavage motif, which was noticed in all precursors with cleavage taking place after the glycine residue. Utilization of this motif was verified by a mutational study of the -A-G*A- site in P25K precursor (VanSlyke *et al.*, 1991a; Whitehead and Hruby, in press; Lee and Hruby, submitted). Second, the proteolytic cleavage motifs are located proximal to the N-termini of P4b, P25K, P21K, and P17K precursors (Figure IV.1). Cleavage releases N-terminal peptides with predicted molecular mass of 6.5, 3.6, 1.9, and 5.9 kDa, respectively. Proteolytic processing at the two cleavage sites within P4a, leads to the liberation of a 9-kDa internal peptide. Third, the terminal peptides of P4b, P25K, P21K and P17K as well as the internal peptide of P4a generated after the cleavage are predicted to be acidic under the physiological conditions. The predicted pI values are 3.94, 3.14, 4.11, 6.67, and 3.89, respectively.

Removal of terminal peptides by proteolytic processing has been noted as a common theme for the structural proteins in other DNA viruses, such as adenoviruses, herpesviruses, and bacteriophage T4 during virus maturation (Hellen and Wimmer, 1992; Hershko and Fry, 1975). Several small polypeptides identified in the bacteriophage T4 virions are likely to be the peptides released from structural protein precursors through proteolytic processing (Showe and Black, 1973). Very little is known about the function of these peptides. In adenovirus, a 13-residue peptide, which is generated from the carboxy end of core protein pVI by proteolytic processing, appears to be involved in viral proteinase activation (Webster *et al.*, 1993, Mangel *et al.*, 1993).

Figure IV.1 Alignment of the predicted amino acid sequences at the amino-termini of VV virion-associated protein precursors. The positions of the scissile peptide bonds (*) in the precursor polypeptides, namely P25K, P4b, P17K and P21K, are indicated above each amino acid sequence. The length of each polypeptide is indicated on the right.

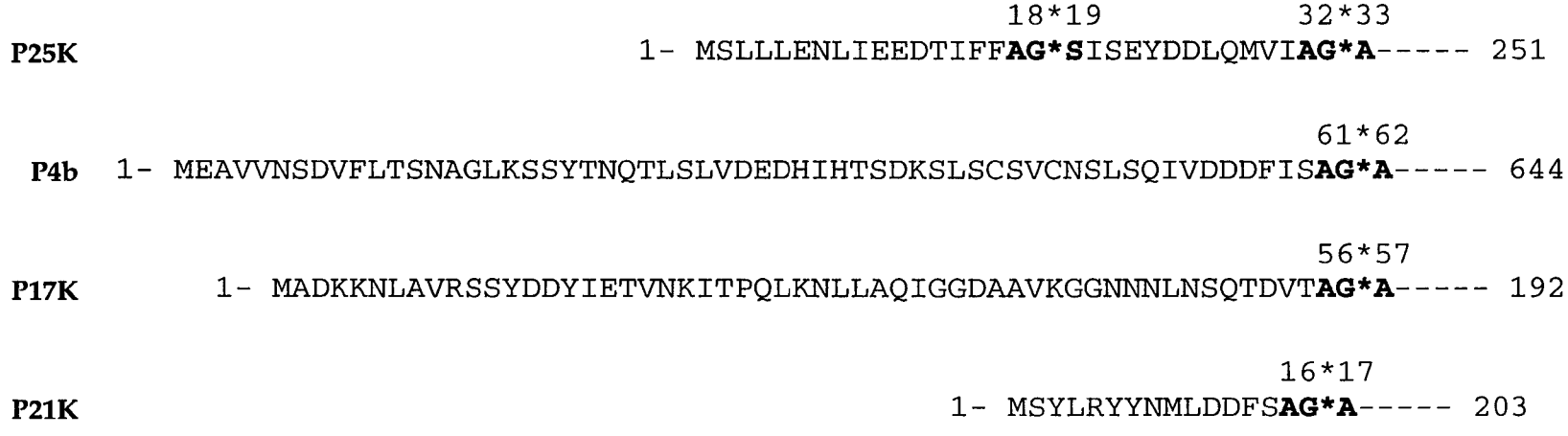

18*19
32*33
P25K
1- MSLLLENLIEEDTIFFAG*SISEYDDLQMVIAG*A----- 251
61*62
P4b
1- MEAVVNSDVFLTSNAGLKSSYTNQTLSLVDEDHIHTSDKSLSCSVCNSLSQIVDDDFISAG*A----- 644
56*57
P17K
1- MADKKNLAVRSSYDDYIETVNKITPQLKNLLAQIGGDAAVKGGNNNLNSQTDVTAG*A----- 192
16*17
P21K
1- MSYLRYYNMLDDFSAG*A----- 203

Figure IV.1

It is of interest to consider what roles the terminal/internal peptides in VV structural protein precursors play during the VV life cycle. Previously, it was proposed that the peptides may be required for virion localization of the precursor polypeptides before the proteolytic cleavage takes place (Lee and Hruby, 1993). Furthermore, the terminal peptides may be essential for the proteolytic processing of VV virion-associated protein precursors. Following the removal of the peptides from the precursors by proteolytic cleavage, the maturated virion-associated protein may adopt different structural conformations which are needed for proper virion maturation. This mechanism is also known to be used by a variety of viruses, including picornaviruses, retroviruses, and adenoviruses (see Hellen and Wimmer, 1992b for references). Finally, although the presence of the free peptides in VV-infected cells is yet to be demonstrated, the released acidic peptides themselves may serve important structural or non-structural roles in VV replication cycle.

In this report, experiments were carried out to investigate the possible role of the amino-terminal peptides in virion localization and/or proper proteolytic cleavage of the precursor polypeptides. Expression vectors for several chimeric and truncated forms of the FLAG-tagged P25K were constructed. The effect of different terminal peptides in the generation of the proteolytic cleavage product, 25KF, from the -A-G*A- cleavage motif and subcellular localization of the precursor polypeptides in VV-infected cells were examined.

Materials and Methods

Cells and virus.

Purified vaccinia virus (WR strain) of a known titer was used for cell infections. BSC-40 (African green monkey kidney) cells were maintained in MEM Eagle's medium [(MEM-E), Sigma Chemical Co., St. Louis, MO] supplemented with 10% heat-inactivated fetal bovine serum (FBS, Whittaker Bioproducts, Inc., Walkersville, MD), 2 mM glutamine, and 10 μg of gentamycin per ml.

Plasmid construction and site-directed mutagenesis.

The open reading frames encoding P25K and P4b are designated as L4R and A3L, respectively, according to the nomenclature by Goebel *et al* (1990). The construction of pL4R:FLAG which contains the promoter and coding regions for P25K polypeptide with the eight-amino-acid FLAG epitope fused to its carboxy-terminus was described previously (Lee and Hruby, 1993). The FLAG epitope was engineered for the detection of the transiently expressed polypeptides but not the endogenous polypeptides expressed from the viral genome. The construction of plasmid pP4b:25KF, which contains the P4b amino-terminal peptide abutted in frame to the amino terminus of 25K:FLAG, is diagrammed and described in Figure IV. 2A. Restriction and modified enzymes were purchased from Boeringer & Mannheim.

Plasmids pP25KF(Δ31), pTK:25KF and various deletion mutants of pP4b:25KF were generated by using oligonucleotide-directed mutagenesis according to the method of Kunkel (1985). Starting with a single-stranded pL4R:FLAG DNA template, pP25KF(Δ31) was generated and used as the initial

Figure IV.2 Construction of the expression vectors for the fusion 25K polypeptides with different amino-terminal peptides. **A**. The strategies for the construction of the chimeric P4b:25KF by polymerase chain reaction (PCR). The 227-bp A3L promoter region together with the 861-bp coding region for the N-terminal 61 amino acid residues of P4b was fused in frame to the 5' end of the coding sequence for residues 33-258 of the P25KF precursor. Using plasmids pA3L and pL4R:FLAG as the templates, oligonucleotides 1, 2, 3, and 4 were utilized for PCR. The ends of PCR fragments were blunted with DNA POL I and kinased with T4 polynucleotide kinase, cleaved with SacI and inserted into pUC118 which has been digested with SmaI and SacI. **B**. Schematic depiction of vectors expressing FLAG-tagged 25K polypeptides with various amino terminal peptides fused to its amino terminus, namely pL4R:FLAG, pP25KF(Δ31), pP4b:25KF, pP4b:25KF(Δ15), pP4b:25KF(Δ30), pP4b:25KF(Δ44) and pTK:25KF. The amino acid sequences at the joining region are indicated within the polypeptides. The sequence of the N-terminal 30 amino acid residues of TK protein is indicated below the pTK:25KF construct.

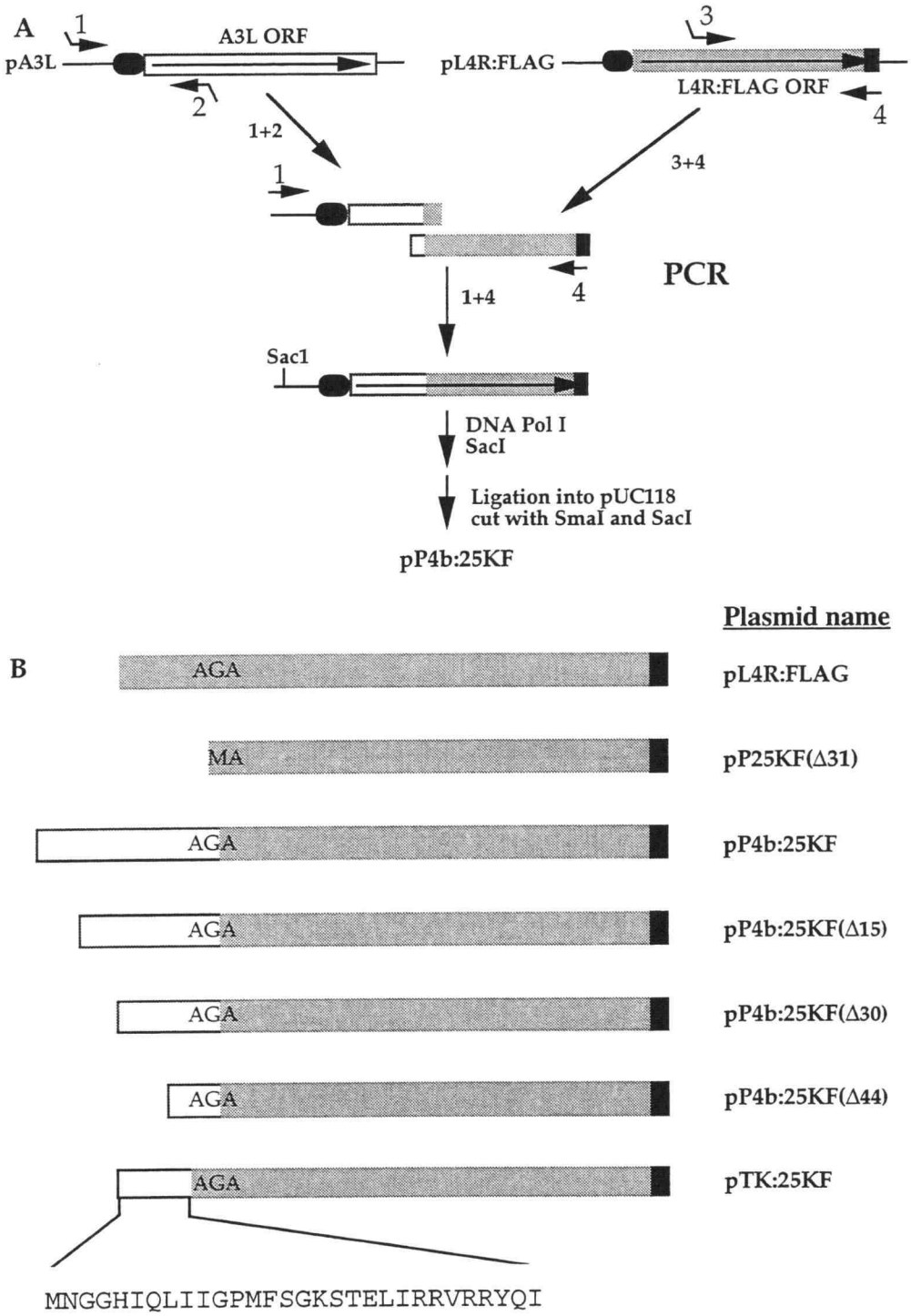

A
1
A3L ORF
pA3L
2
1+2
3
pL4R:FLAG
L4R:FLAG ORF
4
3+4
1
4
PCR
1+4
Sac1
DNA Pol I
SacI
Ligation into pUC118
cut with SmaI and SacI
pP4b:25KF
Plasmid name
B
AGA
pL4R:FLAG
MA
pP25KF(Δ31)
AGA
pP4b:25KF
AGA
pP4b:25KF(Δ15)
AGA
pP4b:25KF(Δ30)
AGA
pP4b:25KF(Δ44)
AGA
pTK:25KF
MNGGHIQLIIGPMFSGKSTELIRRVRRYQI

Figure IV.2

template for constructing pTK:25KF (Figure IV.2B) through three consecutive rounds of mutagenesis. The pP4b:25KF was used as the template to create constructs for expressing P4b:25KF polypeptide with deletions of amino acid residues 2-16 (Δ15), 2-31 (Δ30), and 2-45 (Δ44), respectively (Figure IV.2B). Each mutagenesis procedure was confirmed by subjecting plasmid DNA to dideoxynucleotide DNA sequencing procedures (Sanger *et al.*, 1977). Oligonucleotides were synthesized on Applied Biosystem DNA synthesizer.

Transient expression and metabolic labeling.

Virus infection and liposome-mediated DNA transfection were performed as described previously (Lee and Hruby, 1993). For labeling of VV viral DNA in the presence of rifampin, virus infection and DNA transfection were carried out as described above in medium supplemented with rifampin (100 μg/ml). Following the removal of the DNA-containing medium after 3 hours of incubation, medium supplemented with [methyl-^{3}H] thymidine (85 Ci/mmol; Amersham) and rifampin was added to the cells and the incubation continue overnight at 37°C.

Gradient sedimentation and analysis.

Purification of mature VV particles via sedimentation through a 36% sucrose cushion and a 25-40% linear sucrose gradient was performed as previously described (Hruby *et al.*, 1979). Isolation and analysis of the ^{3}H-labeled fraction from rifampin treated cells is based on the protocol described by VanSlyke *et al* (1993) with some modifications. Briefly, after infected cells were harvested, lysed, homogenized and centrifuged at low speed, the supernatant was overlayed on a 10 ml 20-45% linear sucrose gradient. The gradients were subsequently centrifuged at 20,000 rpm at 4°C for 20 hr in a

Beckman SW41Ti rotor. The gradients were fractionated and twenty 0.6 ml fractions were collected. Gradient fractions containing the [methyl-^{3}H] thymidine labeled polynucleotides were determined by subjecting 10 µl of each fraction to ice-cold tricholroacetic acid precipitation and scintillation counting. The refractive index of each fraction was determined in a refractometer. The refractive index was subsequently converted to density on the basis of the measurements of standard sucrose solutions.

Immunoblotting.

Infected cells were harvested, washed in phosphate-buffered saline (PBS, pH 7.0) and resuspended in hypotonic buffer (10 mM Tris-Cl, pH 8.0, 10 mM ethyldiaminetetraacetic acid, and 25 mM KCl). Sodium dodecyl sulfate (SDS)-12%-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Lee and Hruby, 1993). The filters were incubated with the FLAG-specific M2 monoclonal antibody (MAb) (1:500 dilution) or rabbit anti-P4b/4b antiserum (1:10,000 dilution) (VanSlyke and Hruby, in press), probed with the alkaline phosphatase-conjugated goat anti-mouse or -rabbit antibody (1:2,000 dilution, Bio-Rad), and developed using the alkaline phosphatase assay kit (Bio-Rad).

Results

Mature-virion localization of 25K product requires terminal peptide of P25K precursor.

In order to investigate whether proteolytic processing reaction is essential for virion localization of the 25K protein, amino acid residues 2-32 of the P25KF precursor (previously designated P25K:FLAG [Lee and Hruby, 1993]) were deleted to generate pP25KF(Δ31) which encodes the equivalent of 25KF product (previously designated 25K:FLAG [Lee and Hruby, 1993]) with an extra methionine residue at the N-terminus for translational initiation (Figure IV.2B). Following transient expression in VV-infected cells, immunoblot analyses of the whole cell lysates demonstrated that the production of P25KF and cleavage product 25KF from pL4R:FLAG (Figure IV.3A). Likewise, the P25KF(Δ31) protein, which comigrated with 25KF in SDS-polyacrylamide gels, was expressed (Figure IV.3A). Following the isolation of VV virions, equivalent numbers of virion particles were subjected to the immunoblot analysis. Although P25KF & P25KF(Δ31) were expressed at similar levels in infected cell lysates (Figure IV.3A), there was significantly more 25KF than P25KF(Δ31) product found in mature virions. This result suggests the N-terminal 32 residues of the P25KF precursor enhances in some manner the virion association of the 25KF cleavage product.

Terminal peptides of P4b and P25K precursor polypeptides are interchangeable.

To investigate whether the terminal peptides of the structural protein precursors are functionally interchangeable for the proteolytic processing

Figure IV.3 Immunoblot analysis of P25KF(Δ31) expression and virion localization in VV-infected cells. Whole cell lysates (A) and purified virion particles (B) were prepared following overnight transfection of sheared salmon sperm (SSS), pL4R:FLAG, or pP25KF(Δ31) plasmid DNA in VV-infected cells. Equivalent amounts of purified viral particles determined on the basis of OD_{260} measurement or 10 µl of the whole cell extracts were resolved in 12% SDS-polyacrylamide gels followed by immunoblot detection with the FLAG-specific M2 MAb. The precursor proteins (open circle) and the proteolytic cleavage products (square dots) are indicated on the right of each lane. Sizes of the molecular mass standards in kilodaltons (kDa) are shown on the left.

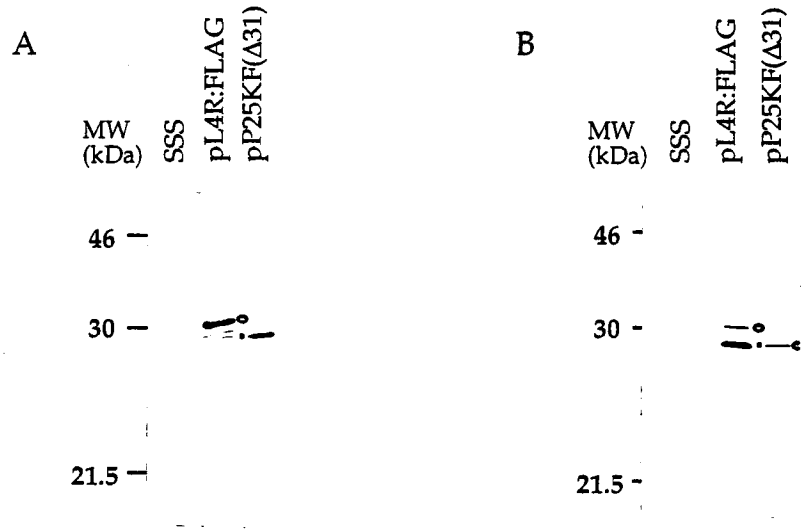

A
MW (kDa)
SSS
pL4R:FLAG
pP25KF(Δ31)
46
30
21.5
B
MW (kDa)
SSS
pL4R:FLAG
pP25KF(Δ31)
46
30
21.5

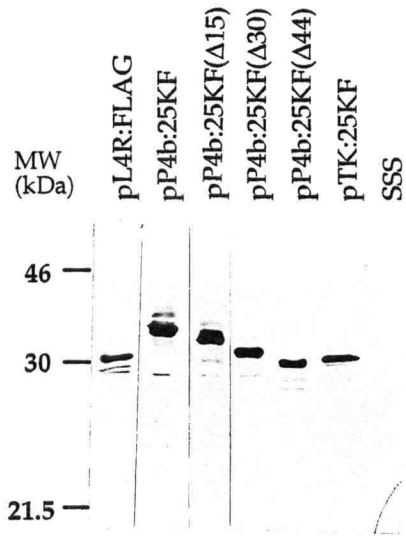
Figure IV. 3

and/or targeting, pP4b:25KF was constructed by replacing the terminal peptide of P25KF precursor with the 61-amino-acid terminal peptide of P4b precursor using PCR methodology (Figure IV.2A and 2B). A full length P4b:25KF protein of molecular mass estimated about 35 kDa and a trace amount of a polypeptide comigrating with the 25KF product were detected in the whole cell lysates with the FLAG-specific M2 MAb (Figure IV.4A). In order to detect the 25KF product, relatively large amounts of whole cell lysates had to be loaded onto the gels, which resulted in the detection of several cross-reactive bands. To verify that the 25KF-comigrating band is actually 25KF, purified virions were prepared and analyzed with M2 MAb. Only the P4b:25KF precursor and the 25KF-comigrating protein were detected in purified VV virions (Figure IV.4B). Furthermore, like the 25KF product which has been demonstrated above to be the primary P25KF-derived component in the purified virions, the level of the 25KF-comigrating polypeptide intensified significantly in the purified VV virions, whereas low level of P4b:25KF was detected, suggesting that the 25KF-comigrating protein is indeed 25KF product derived from the chimeric P4b:25KF precursor. The proteolytic cleavage reaction appeared to be relatively inefficient comparing to bonafide P25KF. The reduced ratio between the product and the precursor implied that the leader peptides of P4b and P25K are compatible only to a limited extent and both amino- and carboxy-terminal fragments of the precursors must work in concert for optimal cleavage efficacy at the -G*A- scissile bond.

To determine whether the sequence or structure of the N-terminal region was important for cleavage to occur, the plasmid pTK:25KF which was generated by swapping the coding sequence for the 30 residues at the N-terminus of P25KF with the corresponding sequence of VV TK protein, a uncleaved non-virion protein (Figure IV.2B). Although the -A-G-A- tripeptide

Figure IV.4 Immunoblot analysis of the expression, proteolytic cleavage, and virion localization of chimeric 25K proteins with different amino terminal peptides. Following overnight transfection of sheared salmon sperm (SSS), pL4R:FLAG, pP4b:25KF, pP4b:25KF(Δ15), pP4b:25KF(Δ30), pP4b:25KF(Δ44) and pTK:25KF in VV-infected BSC40 cells, whole cell lysates (A) or purified virion particles (B) were prepared and resolved by 12% SDS-polyacrylamide gel electrophoresis. Equivalent amounts of purified viral particles determined on the basis of OD_{260} measurement were loaded. The FLAG-tagged proteins were electrotransfered to nitrocellulose membranes and detected by immunoblot analysis with FLAG-specific M2 MAb. The precursor proteins (open circle) and the proteolytic cleavage products (square dots) are indicated on the right of each lane. Sizes of the molecular mass standards in kilodaltons (kDa) are shown on the left.

A



B

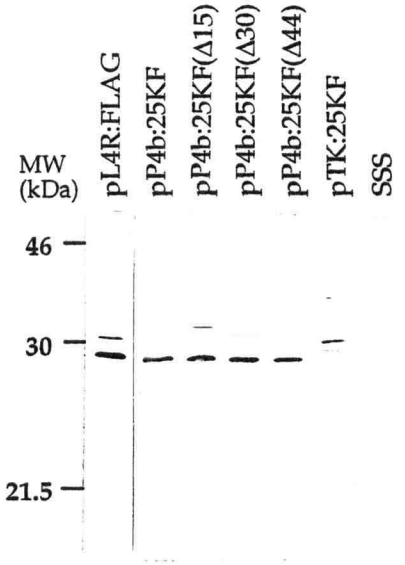


Figure IV. 4

(residues 31-33) is retained, 25KF polypeptide was not detected in the whole cell lysates or in purified VV virion particles prepared from cells transfected with pTK:25KF (Figure IV.4A and 4B). This result suggests that the specific determinants for the proteolytic cleavage of VV structural protein precursors are present in their terminal peptide region.

Effect of deletion mutation of terminal peptide on proteolytic cleavage.

The question addressed next is whether the entire P4b terminal peptide is required for the proteolytic cleavage of the chimeric P4b:25KF precursor. Amino residues 2 to 16, 2 to 31, or 2 to 45 were deleted from P4b:25KF to generate P4b:25KF(Δ15), P4b:25KF(Δ30), or P4b:25KF(Δ44), respectively, and the constructs were expressed in VV-infected cells overnight. The full-length P4b:25KF(Δ15), P4b:25KF(Δ30), and P4b:25KF(Δ44) proteins of expected molecular mass and a protein comigrating with 25KF were detected in all cases with the FLAG-specific MAb (Figure IV.4A). The levels of the 25KF product from all deletion mutants in purified virion particles were relatively similar to that of 25KF produced from P25KF and P4b:25KF (Figure IV.4B).

Terminal peptide is dispensable for localization of VV virion-associated protein precursors to the immature particle.

To investigate whether the N-terminal peptides are involved in targeting of the precursor polypeptides to VV previrions, transfection of pL4R:FLAG, pP25KF(Δ31), pP4b:25KF, and pTK:25KF constructs were carried out in the presence of rifampin and ^{3}H-thymidine. Rifampin is known to reversibly block VV morphogenesis and proteolytic cleavage of structural protein precursors (Katz and Moss, 1970b). Fractions were collected after sucrose gradient sedimentation. The incorporation of ^{3}H-thymidine was assayed by cold TCA

precipitation and plotted against density of the fractions derived from their refractory index (Figure IV.5A). The five fractions flanking each peak fraction (Figure IV.5A, closed symbols) were combined and subjected to immunoblot analysis using FLAG-specific M2 MAb. The results indicated that the full-length proteins, P25KF, P25KF(Δ31), P4b:25KF, and TK:25KF copurified with VV DNA-associated particles (Figure IV.4B). The presence of P4b precursor expressed from viral genome in the same fractions was demonstrated by immunoblot analysis with P4b/4b-specific antiserum (Figure IV.5C). As observed previously (VanSlyke *et al.*, 1993), the block of proteolytic processing by rifampin appeared to be incomplete since relatively low levels of 4b were also detected.

Figure IV.5 Immunoblot analysis of precursor proteins in VV DNA-associated particles isolated after rifampin treatment. Cell lysates were prepared following transfection of pL4R:FLAG (diamond), pP25KF(Δ31) (circle), pP4b:25KF (triangle), and pTK:25KF (square) in VV-infected cells in the presence of rifampicin for 18 hours. The medium was supplemented with [methyl-^{3}H] thymidine (85 Ci/mmol) from 3 hr p. i. until the time of harvest. Fractions were collected after the lysates were subjected to sedimentation in 20-45% sucrose linear gradients and the level of the incorporated [methyl-^{3}H] thymidine was counted after ice-cold TCA precipitation (A). The level of ^{3}H incorporation was plotted against the density of each fraction which was calculated on the basis of the refractive index determined by using a refractometer. The peak fraction and its flanking 4 fractions (closed symbols) were combined and resolved in 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer to nitrocellulose membranes and detection by (B) FLAG-specific M2 Mab or (C) P4b/4b-specific rabbit antiserum. Sizes of the molecular mass standards in kilodaltons (kDa) are shown on the left.

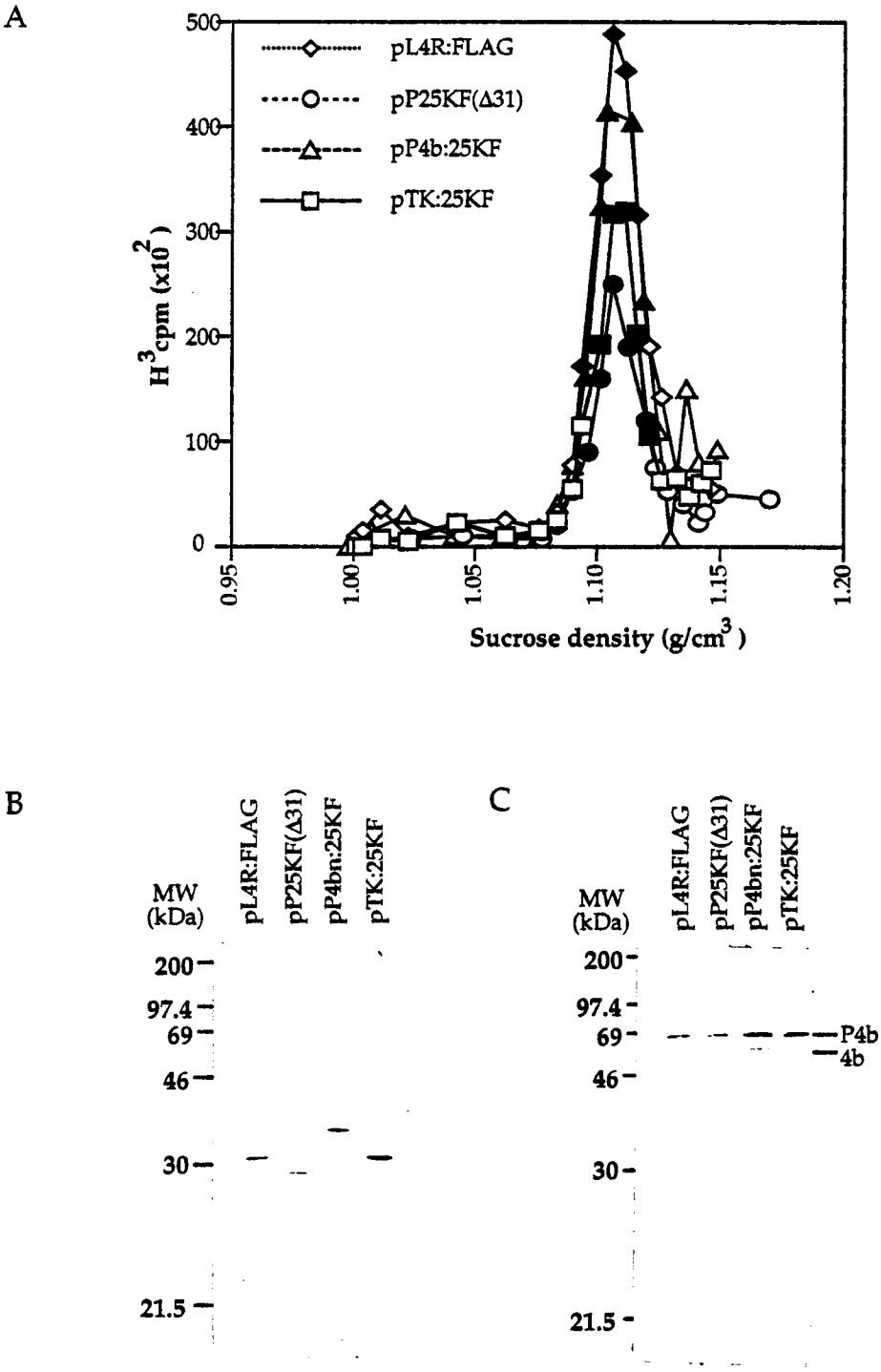

A
pL4R:FLAG
pP25KF(Δ31)
pP4b:25KF
pTK:25KF
H3cpm (x10 2)
Sucrose density (g/cm3)
B
MW (kDa)
pL4R:FLAG
pP25KF(Δ31)
pP4bn:25KF
pTK:25KF
200
97.4
69
46
30
21.5
C
MW (kDa)
pL4R:FLAG
pP25KF(Δ31)
pP4bn:25KF
pTK:25KF
P4b
4b

Figure IV. 5

Discussion

In this study, expression vectors for the VV 25K core protein precursor with its N-terminal peptide deleted or replaced by heterogeneous terminal peptides (Figure IV.2) were used to study the function of the N-terminal peptides of VV structural protein precursors in VV-infected cells. The 25K protein has been shown to compose 7% of VV cores proteins (Sarov and Joklik, 1972), however only a trace amount of P25K precursor could be detected. The detection of a relatively low level of P25KF(Δ31) in mature virion preparation (Figure IV.3B) indicated that 25KF fragment produced without proteolytic processing apparently did not concentrate into mature virions and rather behaved similarly to the P25KF precursor which is present at low level in mature particles (Figure IV.3B). Therefore, the proteolytic processing step appears indispensable for the condensation of 25K product protein into mature virions. The difference in distribution of the 25KF and P25KF(Δ31) polypeptides could be due to either the extra methionine at the N-terminus of P25KF(Δ31), or the chemical and/or structural transition from P25KF precursor to 25KF product. The latter hypothesis is more likely because two possible changes could be introduced by the dissociation of the terminal peptides from the precursors. First, elimination of the acidic peptides would render the products become more positively charged than the precursors. Second, the product could adopt different structural conformation. This hypothesis, if proved to be true, together with the different levels of 25KF and P25KF(Δ31) detected in the purified virions would suggest that the structural transition also cause the 25K product to translocate into a different compartment within VV virions. The level of the 25KF product increases because of binding and processing of more

precursors into virus particles, whereas P25KF(Δ31) stays at the primary localization site since it can not be translocated into a different compartment.

Although the proteolytic activity responsible for VV virion-associated protein processing has yet to be identified, the relative interchangeability of the terminal peptides between P4b and P25K precursor polypeptides supports the hypothesis that the proteolytic processing of different VV virion-associated proteins are carried out by the same processing pathway. To our surprise, deletion of up to 44 amino acid residues from P4b terminal peptides, which leaves a 17-amino-acid terminal peptide in the P4b:25KF polypeptide, still allowed cleavage to take place (Figure IV.4). Previous studies indicated that proteolytic processing at the -A-G*A- cleavage motif could be abrogated by the deletion of ten residues (residues 21-30) on the carboxy side of the P25KF terminal peptide (Lee and Hruby, submitted). Taken together, the results support the notion that at least some determinants for substrate specificity in addition to the -A-G*X- cleavage motif are present in the carboxy region of the amino terminal peptide.

Studies by Morgan (1976) suggested that within VV-infected cells, synthesis of VV structural protein precursors takes place at a site remote from the VV virus factory which led to the question how VV structural protein precursors traffic within cytoplasm and target into premature virions. Myristylation of one VV membrane protein encoded by L1R ORF has been suggested to be required for virion membrane targeting (Ravanello *et al.*, 1993). Since TK polypeptide is not a structural protein and does not undergo proteolytic processing, the detection in VV DNA-associated particles of the precursor proteins TK:25KF or P25KF(Δ31) (Figure IV.5) suggested that terminal peptides are not required for localization of the precursors to premature particle. Therefore, if the previrion-targeting signals for the P25K

precursor do exist, they are likely to reside within the 25K fragment. Alternatively, other types of posttranslational modification and/or association of the precursors with specific carrier proteins which contain the localization signal, may be employed for targeting structural protein precursors to the previrions. Carrier proteins are known to be required for transport of polypeptides cotranslationally to the surface of rough endoplasmic reticulum (Walter and Lingappa, 1986).

The results in this study suggested that the terminal peptides of VV virion-associated protein precursors are interchangeable and substrate determinants for proteolytic cleavage are not present in the amino region of the P4b terminal peptide. The terminal peptides are not likely to participate in targeting of the precursors to VV previrion particles. Furthermore, the importance of the proteolytic removal of the terminal peptide for the incorporation of matured structural proteins into mature virion particles was supported by our findings. The presence of the peptides released from VV virion-associated protein precursors in VV-infected cells or virion particles remains to be demonstrated and the free terminal peptides may serve additional functions yet to be elucidated.

Chapter V

CONCLUSIONS

In this thesis, the *trans* processing asssay system in VV-infected cells in concert with the mutational manipulation of the substrates have allowed us to study the specificity determinants required for defining the cleavage sites residing in VV structural protein precursors, although other participating components, including the proteinase factor(s) and additional substrates still remain to be identified. The expression of the FLAG-tagged P25K precursor polypeptide and its proteolytic processing at the -A-G*A- cleavage motif in VV-infected cells have been confirmed by pulse-chase, rifampin treatment, and reverse genetics experiments. An additional cleavage product, namely 25K', was also identified.

The substrate requirements for hydrophobic residues at P4 position, as well as the small and uncharged residues at P2 and P1 positions at the -A-G*A- motif of P25K were suggested by the mutation studies. The results obtained from substrates with the chimeric terminal peptides suggested that the transition from the precursor to the product by removing the terminal/internal peptides through the limited proteolysis step is essential for the proper assembly of the 25K product into the mature VV virions. The results did not support the hypothesis that the terminal peptides may serve as the leader peptides for structural-protein previrion localization.

Because the FLAG-specific monoclonal antibody immunoprecipitates poorly, studies in the kinetics of the proteolytic reactions in the *trans* processing assays were not performed. Therefore, the conclusions were reached on the basis of a total disruption of the proteolytic cleavage, i.e., the absence of the cleavage product. Among the amino-acid substitutions which allowed cleavage to proceed, the examination of the differences in the degrees of cleavage efficiency was not attempted because of the difficulty in measuring relative band intensity in immunoblots. Ideally, an *in vitro* reconstitution assay in which the concentrations of the different constituents can be manipulated and tested individually would help unravel the different aspects of the proteinase activities. Several approaches were attempted at the beginning of this study to develop an *in vitro* reconstitution assay by preparing the precursor substrates from VV-infected cells incubated at non-permissive conditions for the proteolytic processing, such as non-permissive temperature or in the presence of drug treatments. The precursors were later combined with different potential sources of the proteolytic activity including VV-infected cell extracts, and intact or disrupted virion particles purified from VV-infected cells. No proteolytic processing of the precursors were detected. The results implied that the structural context of the precursor within the previrions is likely to be critical for the proteolytic processing to take place.

In conclusion, I believe I have made significant progress in elucidating the biological importance and specificity determinants of the proteolytic maturation of VV structural protein precursors. This information, together with the future identification and characterization of the responsible proteinase(s), will eventually lead us towards a better understanding of the mechanism, regulation and biological relevance of the proteinase-substrate interaction in VV structural protein maturation.

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