

AN ABSTRACT OF THE THESIS OF

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Vaccinia virus (VV) is a large DNA virus belonging to the *Orthopoxvirus* family. The viral replicative life cycle takes place solely within the cytoplasm of a mammalian host cell. The VV genome contains 196 open reading frames which are expressed in a highly regulated and temporal fashion in order to bring about the production of a mature virion. In the process of viral replication many VV proteins are synthesized that require posttranslational modifications to become functional. A few of these modifications include, glycosylation, ADP-ribosylation, phosphorylation, fatty acid acylation, and proteolytic processing. This last modification is especially important with regard to the structural proteins of the virus in that they undergo proteolysis for an infectious virus particle to be formed, a common theme in viral systems. In order to understand these events in more detail, three abundant virion protein constituents 4a, 4b, and 25K were chosen as models for study. The three main questions we wanted to answer were: Is there a cleavage

consensus site within the precursors, what protease(s) and/or factors are necessary for the process, and how are the events regulated *in vivo*? Our approach included development of specific immunological reagents to identify cleavage products as well as to show where these core proteins are located during virion assembly. We have subsequently identified cleavage products by N-terminal microsequence from each of the three structural proteins and this information has elucidated a putative cleavage consensus site of Ala-Gly-X, where cleavage is proposed to take place between the Gly and X and X is usually an aliphatic residue. The immunological reagents were used in conjunction with immunofluorescent and immunogold labeling analyses to identify the location of these core proteins during virion assembly. Core proteins were localized to the virosomes in VV infected cells, to the viroplasm of immature virus particles, and to the center of mature virions. Precursor specific antiserum indicated that the larger molecular weight precursors of core proteins are within immature virions as well. From these results the following conclusions can be made. Identification of a putative cleavage consensus site suggests that proteolytic processing is an endoproteolytic event. The observation that precursor structural proteins were found within immature particles indicates that the proteinase responsible for cleavage is also present. The fact that assembly has to occur before proteolytic processing of VV structural proteins suggests that the cleavage events are dependent upon a specific core protein conformation. However the nature of this conformational requirement is not known. Further research is underway to develop a full understanding of the proteolytic events during virion morphogenesis.

PROTEOLYTIC MATURATION OF VACCINIA VIRUS
STRUCTURAL PROTEINS

by

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PREFACE

In chapter two of this thesis, Chris Franke is a co-author of this work. She generated the results shown in Fig. II.2B and was involved in the development of the preparation of proteins for N-terminal microsequencing.

In chapter three of this thesis, the co-authors include Stephen S. Whitehead and Elizabeth M. Wilson. Their contributions to this publication are as follows: Stephen Whitehead performed the N-chlorosuccinimide cleavage of P4a and generated the results shown in Fig. III.5 and discussed in the text. Elizabeth Wilson constructed the clones of the P25K and P4a genes that were later utilized for making fusion protein constructions and generating truncated P4a derivatives shown in Fig. III.4. These colleagues along with Peiyu Lee contributed in many useful discussions while this work was in progress.

PROTEOLYTIC MATURATION OF VACCINIA VIRUS STRUCTURAL PROTEINS

CHAPTER I

INTRODUCTION

Vaccinia Life Cycle

The major features of the replication cycle of vaccinia virus (VV) are outlined in Fig. I.1 (Moss, 1974). For the sake of this discussion, note that expression of the viral genetic program can be subdivided into two distinct major phases, early and late, which are delineated by replication of the viral DNA molecule. Prior to DNA synthesis, early VV genes representing about one-half of the viral genetic potential, are transcribed by enzymes and factors present within the incoming virion. The products represent distinct 5'-capped/3'-polyadenylated mRNAs which encode a variety of enzymatic activities including those involved in nucleotide metabolism and DNA replication. Once the VV early genes have been expressed, the core particle disintegrates, liberating the viral DNA which is then replicated within large cytoplasmic inclusion bodies known as "virosomes" or "virus factories". The virosome, which consists of large aggregates of viral protein and catenated VV DNA, is also the site of subsequent assembly of immature viral particles. Concomitant with the onset of viral DNA

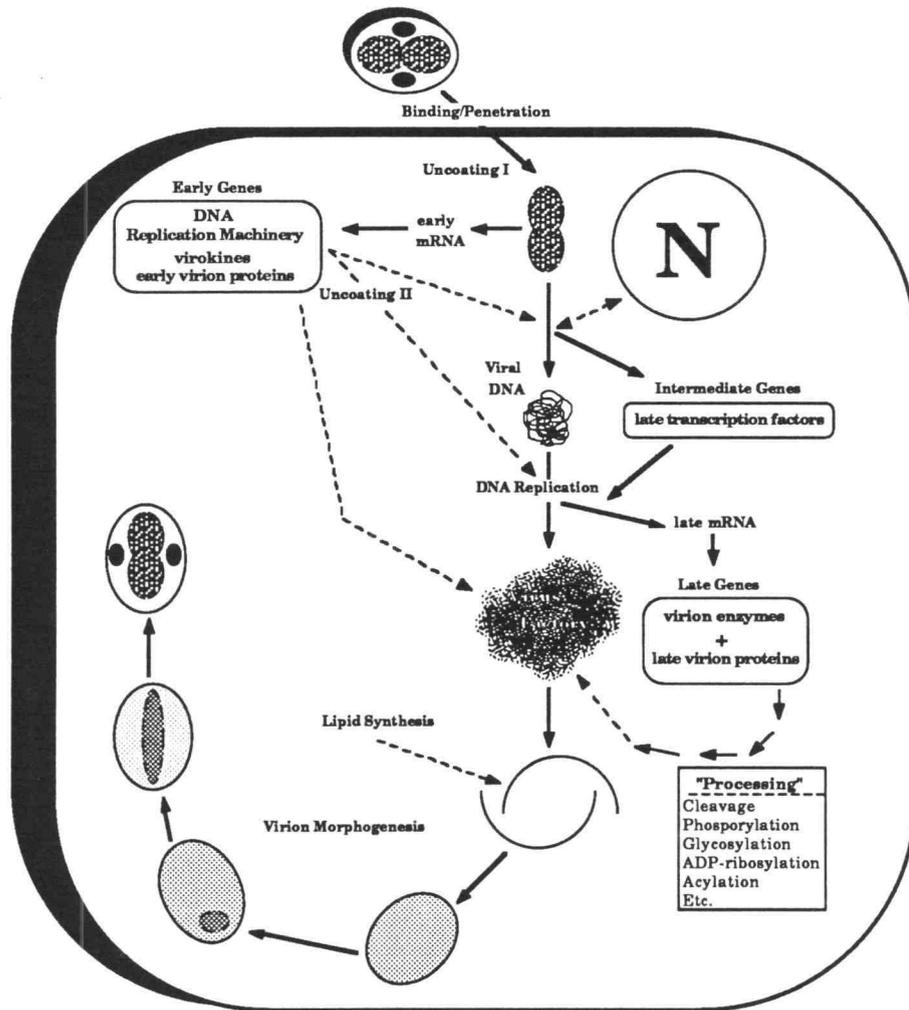


Figure I.1. Vaccinia virus replication strategy. This diagram represents the events that take place during infection of a host cell that lead to production of mature vaccinia virions.

replication, the transcription of early genes is attenuated and the expression of VV late genes is initiated (Rohrmann *et al.*, 1986). VV late RNAs have two unique structural features, namely no distinct 3'-ends and the presence of a short (30 nt) 5'-poly(A) leader (Schwer *et al.*, 1987; Weir and Moss, 1984). The mechanisms which are responsible for the transcriptional specificity of VV early and late genes, i.e., the cis- and trans-acting elements, is the subject of intense research interest (Moss, 1990; Wilson *et al.*, 1988). Suffice it to say, the information available at present indicates that poxvirus transcriptional regulatory mechanisms are substantially different from those of other previously characterized eukaryotic systems (Hanggi *et al.*, 1986). The VV late RNA is translated into approximately 100 different polypeptides including enzymes, as well as a number of structural proteins that are apparently processed by posttranslational modification prior to participating in the multi-step process of virion morphogenesis. Although the general features of the VV life cycle are evident, a molecular understanding of how this highly regulated complex process occurs *in vivo* remains to be accomplished.

VV as a Model System

In many respects vaccinia virus (VV) represents an ideal model eukaryotic system with which to investigate a variety of questions pertaining to development, assembly of macromolecular structures, virus-host cell interactions, DNA replication, and the regulation of gene expression. In particular, a study of the basic mechanisms used to regulate the expression of a complex genetic program would appear to be most appropriate to the VV system since this virus apparently encodes more than 200 viral genes whose expression is tightly regulated in a

temporal fashion during the viral replicative cycle (Wittek, 1983; Essani and Dales, 1979; Moss, 1985). As an experimental system, VV provides a number of significant advantages for this type of study. First, the linear 185 kbp DNA genome of the virus contains approximately 90% unique sequences (Baroudy *et al.*, 1982). This renders the molecule functionally haploid and makes it a convenient substrate for both classical and directed genetic approaches (Bertholet *et al.*, 1986; Condit and Motyczka, 1981). Second, the genomic DNA has been cloned and is available in a variety of plasmid and bacteriophage libraries. At the time when this work was initiated, about 60% of the VV genome has been sequenced (VII International Poxvirus/Iridovirus Workshop). By 1990, Goebel *et al.* had sequenced the entire genome of the Copenhagen strain of VV. Having the sequence of most of the viral genome available obviously facilitates the identification, excision, and manipulation of individual genes. Third, the methodologies of marker transfer (Nakano *et al.*, 1982) and transient expression (Cochran, 1985) have been developed. This allows reverse genetics to be used to specifically alter the nucleotide sequence of viral genes (or the amino acid sequence of their encoded gene products). The biological activity of the mutated genes can then be assessed by recombination back into the viral genome (marker transfer) or expressed within the context of the infected cell (transient expression). Finally, unlike other DNA-containing animal viruses (e.g., adenovirus or herpesvirus), VV does not replicate within the nucleus of infected cells (Pennington and Follet, 1974). Since VV apparently completes most, if not all, of its replicative cycle within the cytoplasmic compartment of infected cells, this necessitates that VV contain or encode many of the enzymes and regulatory factors required to replicate the viral genome, as well as to express and modify viral gene

products, since cognate cellular enzymes are primarily located in the nuclear compartment and/or do not interact with viral substrates (Moss, 1985). Thus, in theory, using VV as a model system, one should have ready access to viral genes encoding essential enzymatic and structural functions as well as the factors which regulate or modulate their expression *in vivo*.

Regulation of Gene Expression

In eukaryotic organisms, regulation of gene expression can be exerted at a number of different levels including: chromatin structure; DNA methylation; gene copy number; transcriptional initiation; RNA processing, transport, and stability; translation; posttranslational modifications; and assembly of individual monomers into active macromolecular structures (Fig. I.2, Creighton, 1984). Since VV expresses its genetic program within the confines of a host cell, one would presume that many of these same mechanisms will be employed by the virus. One VV gene which has been examined in detail with regard to these questions is the VV thymidine kinase gene (Hruby and Ball, 1981; Hruby *et al.*, 1983). As predicted, evidence has been obtained to indicate that the expression of thymidine kinase activity in VV-infected cells is complex, being regulated at the levels of transcription, translation, RNA turnover, and feedback inhibition (Hruby, 1985; Wilson *et al.*, 1989). Most of the current research efforts into understanding the important elements of VV gene regulation are focused on the process of transcription (Yuen *et al.*, 1987; Miner and Hruby, 1989; Mars and Beaud, 1987; Rosel *et al.*, 1986; Broyles *et al.*, 1988). However, it is likely that host cell factors, protein-protein interactions, and posttranslational modifications may prove to be equally important features of the overall regulatory scheme employed by VV.

Postranslational Modification of Proteins

The spectrum of the different types of potential posttranslational modifications which can occur is virtually endless. Protein modifications in general can occur before, during, or after polymerization of amino acids into peptide chains. Moreover, modifications can occur on tRNA-associated amino acids (amino acylated-tRNAs), on amino acids associated with ribosomes in a growing amino acid polymer, or on the polymerized chain after translation has been completed (Wold, 1983). This introduction will mainly to consider those posttranslational modifications which occur after polymerization, which is the more accepted definition of this term anyway. Some of the more common themes in protein modification are proteolytic processing, modification of the amino or carboxyl termini, glycosylation, hydroxylation of prolines or lysines, iodination, addition of non-amino acid groups covalently, phosphorylation, methylation, ADP-ribosylation and disulfide linkages (Creighton, 1984).

A variety of posttranslational modifications of VV polypeptides have already been reported, including proteolytic cleavage (Moss and Rosenblum, 1973), glycosylation and phosphorylation (Garon and Moss, 1971; Moss *et al.*, 1971; Rosemond and Moss, 1973), acylation (Franke *et al.*, 1989), and most recently, ADP-ribosylation (Child *et al.* 1988). It has become evident that posttranslational modifications can play an important regulatory role in biological systems. For example, with regard to ADP-ribosylation, the addition and/or removal of this prosthetic group from substrate proteins is known to be involved in a large number of essential reactions including the activation-inactivation of enzymes such as adenylate cyclase (Lefkowitz *et al.*, 1983) and transducin (Stryer, 1983) which participate in signal transduction pathways, the switch on-switch off

of DNA topoisomerase I (Ferro and Olivera, 1984), and the regulation of gene expression in general (Rastl and Swetly, 1978). However, in eukaryotic systems our mechanistic knowledge of posttranslational modifications is limited with regard to understanding the manner in which the correct sites of modification are selected, the enzymology of the reactions, how the substrate/enzyme interaction is regulated, and the effect of the modification on the structure and function of the substrate protein. This then provides two strong justifications for a detailed molecular genetic examination of modified VV proteins. First, based on the biological precedents, such as those described above, these reactions are likely to play an important role in the viral life cycle. Second, it is possible to carry out defined and detailed structure studies of VV substrate proteins, and perhaps the enzymes which modify them, and thereby address questions which are of general interest and importance in all biological systems.

Proteolytic Processing

Many proteins are synthesized initially in a precursor or inactive form, referred to as the "pro-protein", and subsequently cleaved to their active form. This cleavage event can be a single-step or multi-step process and can involve both endo- and exoproteolytic enzymes. Proteolysis is responsible for the posttranslational removal of the initiating methionine from most proteins as well as clipping the leader sequences that serve as a signal for membrane translocation of membrane-bound or secreted proteins. Furthermore, proteolytic processing of polyproteins is intimately involved in a number of other important pathways such as the production of functional neuropeptides or the genomic expression of poliovirus (Creighton, 1984). Along this latter line, proteolytic maturation of virion

structural proteins from larger molecular weight precursor polypeptides during virion morphogenesis is a common theme in many viral life cycles (Krausslich and Wimmer, 1988; Wellink and van Kammen, 1988). In addition, most of the responsible proteases are virally-encoded and are themselves synthesized as inactive precursors until a cleavage event activates their proteolytic activity (Creighton, 1984).

The genes expressed at late times during a VV infection (after DNA synthesis) include most of the structural proteins required for assembly of progeny virions. The first indication that VV structural proteins might be subject to proteolytic processing occurred when Holowczak and Joklik, (1967) noted differences in the apparent molecular weights of the radioactively-labeled proteins present in VV-infected cells as compared to those found in purified virions. Following this initial observation, pulse-labeling of VV-infected cells was used to demonstrate that a precursor protein with an estimated molecular weight of 125 kDa could be chased to a smaller 76 kDa VV core polypeptide, with the concomitant disappearance of the larger sized protein (Katz and Moss, 1970a). This phenomenon could be specifically inhibited by rifampicin with no apparent effect on the synthesis of the precursor. This precursor protein was subsequently designated as P4a and the proteolytically processed product called 4a (Katz and Moss, 1970b). Additional pulse-chase experiments revealed that several other VV structural polypeptides, in addition to P4a, were apparently subject to cleavage during the late phases of the VV replication cycle (Moss and Rosenblum, 1973). These proteolytically processed proteins, referred to by the Sarov and Joklik (1972) designation of virion proteins, include 4a, 4b, 8, 9, and 10. This may in fact represent a conservative estimate of the number of VV late proteins which are produced by cleavage as Pennington (1974)

reported that eleven proteins synthesized late in infection disappeared during pulse-chase experiments and seven new proteins appeared. 4a and 4b are the most abundant proteins in the VV particle, together constituting about 25% of the mass of the virion. Tryptic peptide mapping of P4a, 4a, P4b, and 4b has confirmed the nature of the precursor to product relationships (Moss and Rosenblum, 1973). The more recent analyses of the VV major structural proteins have centered around mapping and sequencing the VV genes that encode these precursor core polypeptides (Wittek *et al.*, 1984; Rosel and Moss, 1985; Van Meir and Wittek, 1989). The precursor proteins P4a and P4b exhibit approximate molecular weights of 95 and 65 kDa, respectively, when subjected to denaturing gel electrophoresis, whereas the proteolytically processed products (4a and 4b) are estimated to be 62 and 60 kDa in size. The third major core protein, VP8, makes up approximately 7% of the VV virion and is undoubtedly encoded by the VV late gene mapped and sequenced by Weir and Moss (1985) that apparently encodes a 28 kDa precursor. This protein is cleaved to a 25 kDa product which is found in purified virions.

The proteolytic processing of VV structural proteins is believed to be absolutely essential for the formation of infectious progeny virions. This conclusion is based on the fact that there are variety of different drug treatments (e.g., rifampicin, α -amanitin) and conditional-lethal mutations in the VV genome which apparently inhibit proteolysis and subsequent virion assembly without affecting overall viral protein synthesis. (Silver and Dales, 1982; Moss and Rosenblum, 1973). In contrast to the situation with VV late proteins, little is currently known concerning the status of proteolytic processing during the early phases of VV replication.

Several non-structural VV proteins secreted from the infected host

cell also undergo proteolytic processing prior to release into the surrounding medium. VV growth factor, a nonessential protein made early in vaccinia replication, is synthesized, glycosylated, and integrated into the plasma membrane, where it is proteolytically processed and released. This cleavage process results in the loss of a signal sequence located near the N-terminus of the protein and loss of a proposed transmembrane spanning region near the carboxy terminus. The proteases responsible for these reactions may be viral or cellular in origin (Stroobant *et al.*, 1985; Chang *et al.*, 1987). Another secreted protein with an apparent molecular weight of 19 kDa was recently identified and shown to be missing 19 amino acid residues from the predicted N-terminus, suggesting that proteolytic cleavage was involved in the removal of a signal sequence (Kotwal and Moss, 1988). Hemagglutinin is a viral-encoded glycoprotein located in the extracellular envelope of VV particles and on the plasma membrane of infected cells (Shida and Dales, 1981). As with many integral membrane proteins, HA contains a putative leader sequence that is likely cleaved after insertion into the phospholipid bilayer (von Heijne, 1983). This hypothesis is supported by the discrepancy between the molecular weights of HA synthesized in a cell-free system and that expressed in an *in vivo* system. A protein with a slower mobility was synthesized in the reticulocyte lysate presumably due to the presence of the signal sequence (Shida, 1986). Finally, an enzyme isolated from viral cores which has a DNA-dependent nicking-joining activity appears to be activated by proteolytic processing. This 50 kDa pronuclease is proposed undergo proteolysis to become an active 44 kDa form, possibly after penetration of infected cells (Reddy and Bauer, 1989).

Evidence for a protease packaged within VV virions was reported by Arzoglou *et al.*, (1979). When virus particles were disrupted under alkaline conditions, a proteolytic activity was detected, but no further information is available concerning the identity or function of this enzyme. A trypsin-like protease isolated from infected cells was able to uncoat VV *in vitro* (Pedley and Cooper, 1987). This protease activity was purified to a protein with a molecular weight of approximately 23 kDa, but its origin is unknown.

Other than these preliminary reports, little is known concerning the protease(s) involved in proteolytic maturation of VV encoded polypeptides. Based on the precedents from other viral systems, one would predict that at least some of these activities will prove to be specified by the virus. Obviously a great deal remains to be learned concerning the proteolytic pathways, what enzymes are carrying out the reactions, and the kinetics and subcellular localization of these reactions.

Conclusions

Vaccinia virus replicates in the cytoplasm of infected host cells in a very highly controlled manner, regulating the expression of its numerous genes at the level of genomic organization, transcription, translation, and posttranslational modifications. For the virus to successfully complete a replication cycle, certain gene products must appear at specific times and in specific quantities during infection. The ability to carry out the necessary replicative events in the same cellular compartment undoubtedly requires the use of many regulatory mechanisms. The posttranslational modifications reported thus far in the vaccinia literature presumably help accomplish this requirement. Proteolytic processing, glycosylation, phosphorylation, ADP-

ribosylation, acylation, and disulfide crosslinking are involved in the expression of VV structural and non-structural proteins. The viral core and membrane polypeptides appear to be most affected by these modifications, which is not surprising when one considers that the virion is thought to contain approximately 100 proteins (Essani and Dales, 1979), accounting for about 50% of the viral-encoded genes. As more of the viral proteins, whatever their function, are studied and detailed information becomes available, it is likely that many of these factors will be subject to posttranslational events and novel forms of modifications.

In summary, our understanding of this aspect of regulation of gene expression is still fairly underdeveloped. The information presented here is probably a small portion of the posttranslational modifications that exist *in vivo*. Also limited is our understanding of the extent to which they are involved in controlling the appearance of functional polypeptides. Because VV is estimated to encode approximately 200 proteins, it is not surprising that the virus employs this form of regulation for effective viral replication. Proteolytic processing of VV structural proteins was selected to be the focus of an in depth study of posttranslational modifications, centering on the mechanism itself and how it regulates gene expression. Three structural proteins (4a, 4b, and 25K) were chosen as models for study because they are major virion constituents, their open reading frames had already been reported, and they are the products of proteolytic maturation during virus particle formation. The questions outlined in the initial approach were:

Where within the proteolytic substrates does cleavage take place?

What factor(s) are responsible for this event?

How are the substrate-proteolytic enzyme(s) interactions regulated within the infected host cell?

The following chapters contain the knowledge gained from the my endeavor to answer these questions.

CHAPTER II

**PROTEOLYTIC MATURATION OF VACCINIA VIRUS
CORE PROTEINS: IDENTIFICATION OF A CONSERVED MOTIF AT
THE N-TERMINI OF THE 4B AND 25K VIRION PROTEINS**

Authors: Judy K. VanSlyke, Christine A. Franke, and Dennis E. Hruby

SUMMARY

Three structural proteins (4a, 4b and 25K) located within the virion core of vaccinia virus are cleavage products of precursor polypeptides (P4a, P4b, and P25K) synthesized late in viral infection. Pulse-chase labeling experiments revealed that cleavage of the core proteins lags considerably behind precursor synthesis and processing requires continuous protein synthesis. The N-terminal sequences of 4b and 25K, but not 4a, were determined by microsequencing core proteins isolated from purified virions. Comparison of this data with the predicted amino acid sequence of P4b and P25K revealed a conserved Ala-Gly-Ala motif flanking the apparent N-termini of both proteins, as well as several additional sequence similarities between the P4b and P25K precursors both upstream and downstream of the putative cleavage site. The Ala-Gly-Ala tripeptide signal was also found in the same region of amino acid sequence of the homologous proteins of fowlpox virus.

INTRODUCTION

Vaccinia virus (VV) is a large DNA-containing animal virus that replicates within the cytoplasm of infected host cells. It is the prototype member of the orthopoxvirus family and the approximate 185 kbp VV DNA molecule encodes on the order of 200 different viral proteins (Essani & Dales, 1979). As with other viruses which require the cleavage of precursor proteins (or polyproteins) to complete their infectious life cycle, e.g. members of the *Retroviridae* and *Picornaviridae* families (McCune *et al.*, 1988; Palmenberg, 1973), proteolytic maturation of at least three major structural proteins (4a, 4b, and 25K) is essential for the formation of mature infectious VV progeny and they constitute 14, 11, and 7%, respectively, of the virion by mass (Sarov & Joklik, 1972). The nascent P4a, P4b, and P25K precursor proteins are highly expressed at late times during VV infection (after DNA replication), thereby facilitating the use of reverse genetic procedures to map the parental genes. Hybrid-selected and hybrid-arrested cell free translation methods have been previously employed to map the genes encoding P4a and P4b precursors to the left hand portion of the *HindIII* A fragment of the VV genome as shown in Fig. II.1 (Wittek, *et al.*, 1984; Rosel & Moss, 1985). The two open reading frames (ORFs) were sequenced and are predicted to encode polypeptides of 891 (P4a) and 643 (P4b) amino acids in length (Rosel & Moss, 1985; Van Meir & Wittek, 1988). The predicted molecular weights of the P4a and P4b precursors (102.3 kDa and 72.5 kDa, respectively) are slightly larger than the observed molecular weights (95 kDa and 65 kDa, respectively) of SDS-PAGE analyzed proteins. The reason for this apparent discrepancy is not known. The P25K gene was mapped within the *HindIII* L fragment, sequenced, and shown to encode a

251 amino acid polypeptide with a predicted molecular weight of 28 kDa (Fig. II.1, Weir & Moss, 1985). Unlike P4a and P4b, the apparent size of the P25K protein is in close agreement with the predicted molecular weight. The relationships between the core proteins and their precursors has previously been established by tryptic peptide mapping, pulse-chase analysis and immunological assays (Katz & Moss, 1970*a,b*; Moss & Rosenblum, 1973; Rosel & Moss, 1985; Sarov & Joklik, 1972; Weir & Moss, 1985; Yang *et al.*, 1988). The cleavage of the P4a, P4b and P25K precursor proteins during VV morphogenesis is apparently an obligatory reaction based on the observation that drugs which inhibit cleavage, either directly or indirectly [e.g., rifampicin (Katz & Moss, 1970*b*; Moss & Rosenblum, 1973; Miner & Hruby, 1989), α -amanitin (Villarreal *et al.*, 1984), nicotinamide (Child *et al.*, 1988), or isatin- β -thiosemicarbazone (Katz *et al.*, 1973)], block virus replication, suggesting that proteolytic processing plays a key role in poxvirus development. Very little is known concerning the pathways, biochemistry or mechanisms by which the VV core protein precursors P4a, P4b, and P25K undergo proteolytic maturation during infection.

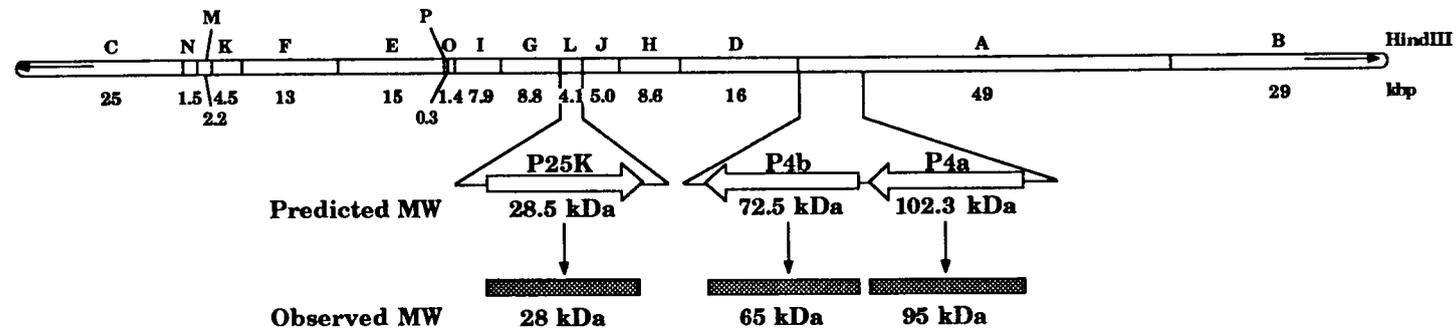


Figure II.1. Position of the three core proteins' genes within the VV genome. The location and orientation of the open reading frames encoding the P4a, P4b, and P25K precursor proteins (indicated by open arrows) are shown with respect to the VV DNA *HindIII* restriction map. The letters above the map refer to the fragment designation. The numbers below the map indicate the approximate sizes (in kbp) of each fragment. The molecular weight predicted for each precursor is shown below each arrow whereas the molecular weight determined by migration on SDS-polyacrylamide gels is shown below the shaded boxes which represent the primary translation products.

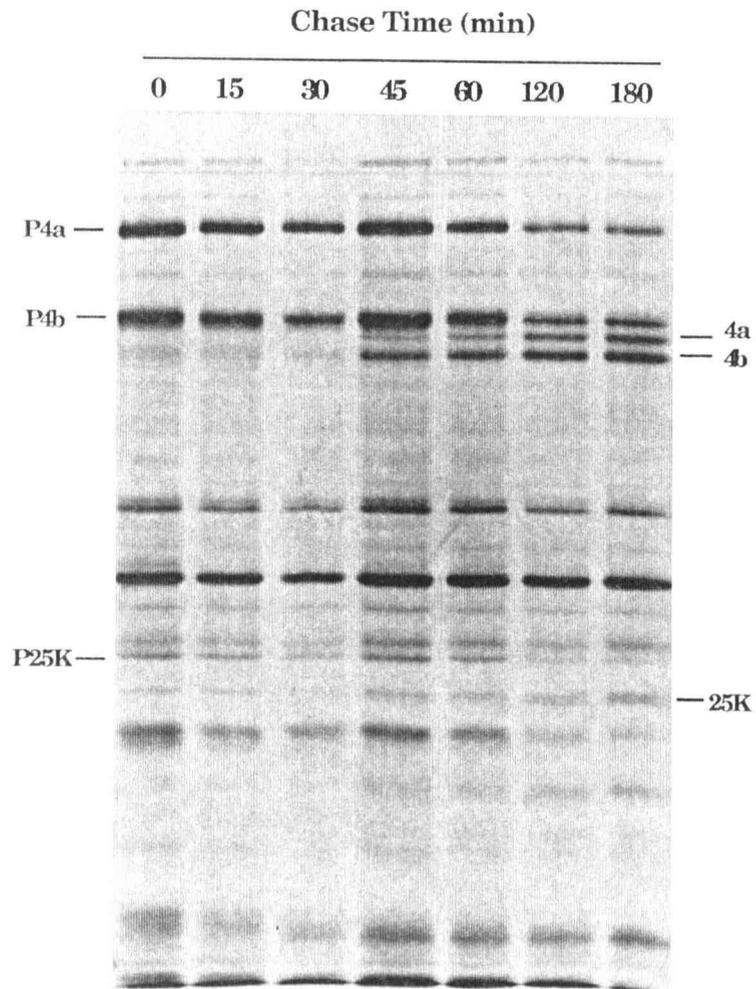
RESULTS AND DISCUSSION

To define the kinetics of proteolytic processing of P4a, P4b, and P25K, BSC₄₀ cells were infected at a multiplicity of infection of 10, pulse-labeled for 30 minutes with ³⁵S-methionine at 4 hours post-infection, and chased with a 100-fold excess of cold methionine for brief time intervals (Hruby, *et al.*, 1979). A fluorograph (Bonner & Laskey, 1974) of the labeled proteins present at each chase time, separated on a 10% acrylamide-SDS gel (Studier, 1973), revealed that proteolytic maturation to 4a, 4b, and 25K does not occur rapidly (Fig. II.2A). Rather, there is a delay of almost 30-45 minutes after synthesis of the precursors before any appreciable proteolysis occurs. To determine whether the rate of proteolysis might vary during infection, the experimental protocol was repeated with the period of pulse-labeling being from 6-6.5 hours post-infection, followed by chase periods of brief duration. No appreciable differences in the rate of virion core maturation between 4 and 6 hours post-infection were observed. Similar experiments carried out at even later times in VV infection have provided the same results (data not shown).

To determine whether proteolytic processing of the VV core proteins could proceed in the absence of *de novo* protein synthesis, VV-infected cells were pulsed-labeled at 8 hours post-infection, chased for periods up to 24 hours in the presence or absence of cycloheximide (a protein synthesis inhibitor) and the pattern of labeled polypeptides was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. II.2B). As expected during normal pulse-chase labeling, P4a, P4b, and P25K are present in the pulse extract but disappear in the chase extracts coincident with the appearance of 4a, 4b and 25K. However, if cycloheximide is added to the medium at the

Figure II.2 (A) Kinetics of VV core protein maturation. Four hours after initiating a synchronous VV infection, BSC40 cells were pulse-labeled with L-[³⁵S]methionine for 30 min, followed by the indicated length of chase with a 100-fold excess of unlabeled methionine. The labeled infected cells were harvested and cytoplasmic extracts prepared in buffer containing 20% SDS plus 20 µg/ml PMSF (phenylmethylsulfonylfluoride). The extracts were analyzed on a 10% SDS-polyacrylamide gel that was subjected to fluorography and exposed to X-ray film. Molecular weight markers are on the left side of the fluorographs and precursors and products are labeled on the right side. (B) Effect of protein synthesis inhibition on VV core protein processing. Eight hours after initiating a synchronous VV infection, BSC40 cells were pulse-chase labeled the same as in part (a), except 100 µg/ml cycloheximide (CHX) was added to the chase medium where indicated. The extracts were analyzed on a 10% SDS-polyacrylamide gel that was subjected to fluorography and exposed to X-ray film. The precursors and products are indicated on the right hand side of the fluorograph and molecular weight markers are labeled on the right.

A.



B.

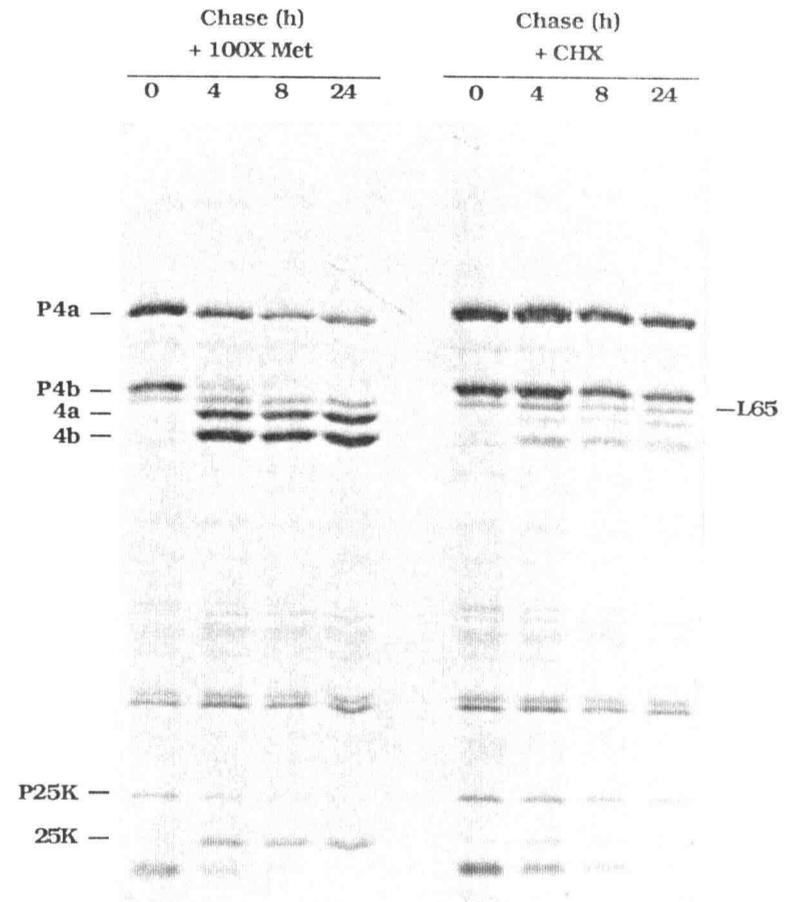


Figure II.2.

beginning of the chase period, proteolytic processing of all three precursor proteins is inhibited throughout the 24 hour time span. At late times during viral infection host protein synthesis is severely reduced by the VV-infection. Therefore the requirement for ongoing protein synthesis suggests a need for the expression of a viral encoded protein (or proteins). It was also of interest to note that most of the VV late proteins labeled under these conditions were stable for 24 hours either in the presence or absence of cycloheximide. However, as can be seen in Fig. II.2B, there is a 20 kDa protein which is an exception to this rule. This protein disappears rapidly regardless of whether protein synthesis is ongoing. The reason for the selective breakdown of this protein is not apparent at the present time.

The considerable lag between the synthesis and processing of the VV core protein precursors is somewhat surprising considering that proteolytic processing in many other viral systems occurs quite rapidly. For example, in the case of poliovirus, the nascent polyprotein is cleaved between the P1 and P2 junctions even before synthesis of the polyprotein is completed (Kräusslich and Wimmer, 1988). There are several hypotheses to explain the delay in processing of the VV core protein precursors. First, it is possible that the VV core proteins are synthesized prior to the synthesis or activation of a viral-encoded proteinase. However, the fact that the lag in processing is observed regardless of whether pulse-chase labeling is conducted at 4 or 6 hours post infection argues against this possibility. Secondly, the nascent VV core protein precursors may not be competent substrates for processing, requiring either interaction with a chaperonin for proper folding (Rothman, 1989) or perhaps one or more posttranslational modifications to achieve their active conformation. In support of this latter notion is the work of Child *et al.* (1988), which provided

evidence that the VV core proteins are apparently substrates for ADP-ribosylation reactions. Finally, it is possible that the individual VV core protein precursors by themselves are not suitable substrates for the cleavage to occur. Perhaps assembly into an immature virion is required to achieve the correct conformation or to bring the proteinase and substrates together into the correct context. There are several lines of experimental evidence supporting the hypothesis of "contextual processing". It is well-recognized that a variety of conditions which interfere with virion maturation block core protein cleavage, including a variety of drugs (e.g. rifampicin, Katz & Moss, 1970b), physical treatments (e.g., enucleation, Hruby *et al.*, 1979), and conditional-lethal mutants (Thompson & Condit, 1986; VanSlyke & Hruby, unpublished results). Because the reversal reaction occurs quite rapidly, it can be argued that the core protein precursors must have existed within the immature particles in an uncleaved form prior to drug reversal. The second line of evidence comes from the observation that VV core protein precursors produced in cell-free systems are not processed by enzymes either present in the cell-free system, cotranslated from other late VV messages, or exogenously added in extracts from VV-infected cells which would be expected to contain fully functional proteinases (Silver & Dales, 1982; Hruby, unpublished data).

Since the predicted amino acid sequences of the P4a, P4b, and P25K precursor proteins were available, it was of interest to locate the potential sites of precursor protein cleavage as an initial step towards a molecular genetic examination of the proteolytic maturation of the VV core proteins. Therefore, the mature 4a, 4b and 25K proteins were isolated from purified virions and subjected to N-terminal microsequence analysis. The procedures reported by Yuen, *et al.*, 1986 and Yuen, *et al.*, 1988, were

followed except the acrylamide gels were prerun and run at 6mA and 14mA, respectively, and they were equilibrated for 30 to 60 minutes in electroblotting buffer prior to transfer to membranes. Proteins were visualized by staining with 1% Ponceau S (Salinovich & Montelaro, 1986), excised from the membrane, and subjected to automated Edman degradation in a gas phase cartridge system. The results obtained are shown in Fig. II.3A, identifying the amino terminus of 25K as residue 33 of the P25K sequence and the amino terminus of 4b as residue 62 of the P4b sequence. The decrease in approximate molecular weight due to the loss of 32 or 61 amino acid residues from the N-terminus of P25K or P4b, respectively, corresponds with the shift in migration following proteolytic processing observed by SDS-polyacrylamide gel analysis. The fate and potential biological function of the small proteins released is not yet known. Our identification of the N-terminus of 25K is in agreement with the recent work of Yang *et al.* (1988), identifying the N-terminus of VV VP8 (proposed to be the same protein as 25K). Despite several efforts using different sequencing strategies, N-terminal sequence data for 4a were not obtained, suggesting that the N-terminus of this protein may be blocked, although further analysis is needed to confirm this result.

Comparison of the predicted amino acid sequences within the P4b and P25K precursors which surround the derived N-termini of the mature 4b and 25K proteins reveals the presence of a highly similar motif (Fig. II.3B). The N-termini of both cleavage products are found within a conserved Ala-Gly-Ala tripeptide with the predicted cleavage site occurring at the Gly-Ala bond. Other identical residues are found upstream at positions -8, -12 and -14, a region that is usually important in substrate recognition by a proteinase (Wellink & van Kammen, 1988), suggesting that

Figure II.3 (A) Protein microsequence analysis of the VV 4b and 25K core proteins. The N-terminal residues of purified 4b and 25K proteins were subjected to protein microsequencing procedures and the derived sequence aligned to the predicted amino acid sequence of the P4b and P25K precursor proteins. The indicated regions of each respective open reading frame are expanded to show the predicted amino acid sequences. The residue numbers are relative to the N-terminus of the precursor with the initiator methionine as the first position.

(B) The amino acid sequences within the P4b and P25K precursor proteins which flank the derived N-termini 4b and 25K are aligned for comparison. Shared conserved residues are in bold type. Similar amino acids are bracketed and identified as large nonpolar (LN), small nonpolar (SN), large polar (LP), and small polar (SP). The numbers above the amino acids indicate their position with respect to the potential cleavage site.

(C) Conservation of potential VV core protein cleavage motif. The sequence of predicted amino acid residues surrounding potential cleavage sites within the VV core protein precursors P25K and P4b are aligned with the predicted amino acid sequence from the same region of homologous genes from fowlpox virus (FPV). The Ala-Gly-Ala motif, conserved among all four sequences is indicated by the box. Residues shared by two or more sequences are shaded. The numbers above the amino acids are described in part (B).

it may be part of a potential cleavage recognition site. Furthermore, many of the residues occupying other positions (-17, -16, -15, -13, -11, -5, -4, +2, +3, +4 and +7) within this region of the two sequences are related structurally in size and polarity, suggesting that this region might adopt a similar conformation in each precursor. In view of the failure to obtain amino acid sequence from purified 4a protein, it is of interest to note that the predicted amino acid sequence of the P4a precursor did not contain the conserved Ala-Gly-Ala tripeptide motif.

As an approach to determine which of the residues within the conserved P4b/P25K motif identified in Fig. II.3B were likely to be biologically relevant, the predicted amino acid sequences from this region of the homologous P4b and P25K genes from a distantly related avipoxvirus, fowlpox virus (FPV), were compared to that of the orthopoxvirus VV. Binns *et al.* (1989) reported the sequence of F4b, a fowlpox core polypeptide that has a 52% homology at the amino acid level with VV P4b. Interestingly, the 657 amino acid fowlpox protein (versus 644 for VV P4b) contains a single Ala-Gly-Ala sequence spanning residues number 60-62 relative to the N-terminus. Likewise, FP5 was shown by Binns *et al.* (1988) to have 33% homology with VV-encoded F5 protein, a polypeptide previously identified as P25K (Weir & Moss, 1985). FP5 (labeled in Fig. II.3C as FPV P25K) is a 253 amino acid protein (versus 251 for VV P25K) with an Ala-Gly-Ala motif located at residues number 30-32. The alignment of the regions of predicted amino acid sequence flanking the Ala-Gly-Ala tripeptide from all four precursor polypeptides is shown in Fig. II.3C. The striking feature is the absolute conservation of the Ala-Gly-Ala signal which occurs at precisely the same position in both the P4b and P25K homologs of VV and FPV, suggesting that these residues are critical and essential for cleavage to

occur. In contrast, positions -8, -12 and -14, which are conserved between the VV P4b and P25K proteins, are not conserved in the FPV proteins suggesting that they are of less importance in determining cleavage site selection. There are no other strict conservations between all four sequences in the region shown, but there are some similarities. The residues at positions +2 and +7 are always basic amino acids (Arg or Lys). This suggests that other residues beyond the Ala-Gly-Ala motif may be involved at some level in determining the secondary and tertiary structure of the precursor proteins necessary for authentic cleavage to occur. It remains, of course, to be experimentally verified whether the endpoints determined in this study represent endoproteolytic cleavage points or whether these termini have been generated by a combination of endo- and exoproteolytic reactions such as those that occur during the maturation of neuropeptide precursors (Douglass *et al.*, 1984). A detailed, directed genetic approach, such as that which has been elegantly employed to study the processing of the tobacco etch virus polyprotein (Dougherty *et al.*, 1989), will be required to identify which of the amino acid residues within and without the proposed cleavage site contribute to the specificity and efficiency of cleavage reactions *in vivo*.

When the predicted amino acid sequences of all VV genes present in the data banks were searched for the presence of an Ala-Gly-Ala motif, three occurrences were noted: the 32.5 kDa host range factor (Gillard *et al.*, 1986), the DNA polymerase gene (Earl *et al.*, 1986), and the palmitylated 37 kDa protein associated with extracellular enveloped virions (Hiller & Weber, 1985; Hirt *et al.*, 1986). To date, none of these three proteins have been reported to undergo proteolytic processing during infection. Since the 32.5 kDa and DNA polymerase proteins are both early proteins, they might

not be expected to be processed by a viral proteinase proposed to be expressed at late times. This does raise the possibility that these proteins could be cleaved at late times as part of a potential regulatory mechanism.

Interestingly, the 37 kDa protein is expressed late in VV infection.

However, the Ala-Gly-Ala signal is located at positions 8-10 within the predicted open reading frame, so that if the N-terminal 9 amino acids were clipped off it probably would not be evident on the basis of migration in a polyacrylamide gel.

Given the coordinate synthesis, similar location, and apparently similar function of the three major core proteins, it was most surprising to discover that the 4a protein could not be sequenced under the same conditions as 4b and 25K (suggesting that it may be blocked in some manner) and that the P4a precursor lacks any apparent Ala-Gly-Ala signals. There would seem to be two possible explanations for this data. First, the P4a protein may be processed by a different mechanism (than are 4b and 25K) that utilizes different proteinase(s). A second possibility is that P4a is processed by the same pathway as P4b and P25K but at sites which are less efficiently cleaved. Support for this hypothesis can be drawn from the fact that cleavage of the P4a precursor appears to proceed at a slower rate than the P4b and P25K precursors *in vivo* (Fig. II.2A). With that in mind, the predicted amino acid sequence of the P4a precursor was searched for the sequence Ala-Gly-X and three signals were found, Ala-Gly-Asn (residues 94-96), Ala-Gly-Ser (residues 613-615) and Ala-Gly-Thr (residues 696-698). If cleavage were to occur at all three sites within the P4a precursor, an internal polypeptide of approximately the correct size would be released. Immunological reagents corresponding to various portions of

the P4a precursor are currently being generated to prove or disprove this hypothesis.

The nature and identity of the proteinases which are responsible for the processing of the VV core proteins remains an open question. The possibilities include cellular enzymes, viral encoded enzymes, autocatalytic mechanisms, or a combination of all three. None of the data presented here address this issue. Also left unanswered is the question of what cleavage events take place within P4a during proteolytic processing. Further investigation of proteolytic maturation of VV structural proteins is needed to answer these questions.

CHAPTER III

**THE MULTISTEP PROTEOLYTIC MATURATION PATHWAY
UTILIZED BY VACCINIA VIRUS P4a PROTEIN:
A DEGENERATE CONSERVED CLEAVAGE MOTIF
WITHIN CORE PROTEINS**

Authors: Judy K. VanSlyke, Stephen S. Whitehead, Elizabeth M. Wilson,
and Dennis E. Hruby

SUMMARY

The most abundant vaccinia virus (VV) core protein found within the virion is protein 4a which represents approximately 14% of the particle's dry weight. The 4a protein is synthesized as an 102.5 kDa precursor which is proteolytically processed to a 62 kDa product concomitant with virion assembly. To identify the pathway by which P4a is converted into 4a, immunological reagents which are specific for subregions of the P4a precursor were developed and used in concert with peptide mapping and protein sequencing procedures. The results suggested that the 891 amino acid P4a precursor is cleaved at two locations, between residues 614-615 and 697-698. Both the large amino terminal 4a protein (residues 1-614) and the carboxy terminal derived 23 kDa protein (residues 698-891) become major virion constituents. The location and fate of the small internal peptide (residues 615-697) is not known. Interestingly, an analysis of the predicted amino acid sequences at the sites of cleavage within the P4a precursor indicated the presence of an Ala-Gly↓Thr motif flanking the 697-698 site and an Ala-Gly↓Ser motif flanking the 614-615 site. Since both of these signals are quite similar to the Ala-Gly↓Ala signal previously identified as the cleavage point within the VV P4b and P25K core protein precursors (VanSlyke *et al.*, 1991), this suggests that processing of all three core protein precursors may be coordinately linked and/or catalyzed by the same proteinase during viral assembly.

INTRODUCTION

As our understanding of different viruses and their diverse life cycles increases, certain common themes have become obvious. For example, proteolytic processing of viral structural proteins is a common event during the replication of many viruses (poliovirus, human immunodeficiency virus, adenovirus, etc.) (Kräusslich and Wimmer, 1988). Likewise, during the assembly of infectious vaccinia virus (VV) virions, the major structural proteins of the virus are proteolytically cleaved from higher molecular weight precursors into the mature proteins that are found in the virion core (Essani and Dales, 1979; Katz and Moss, 1970; Sarov and Joklik, 1972). Thus far, very little is known about the biochemistry or regulation of proteolysis which occurs during VV morphogenesis.

The major VV virion protein precursors (P4a, P4b, and P25K) are synthesized at late times in the viral infection and are subsequently cleaved to smaller forms (4a, 4b, and 25K) found in the virion (Fig. III.1). The genes encoding these three structural proteins have been previously mapped to the VV genome and the nucleotide sequences of the open reading frames determined (Wittek *et al.*, 1984; Rosel and Moss, 1985; Weir and Moss, 1985; Van Meir and Wittek, 1988). By comparison of the predicted amino acid sequence for the P4b and P25K precursors with the sequence of residues determined by N-terminal microsequencing of 4b and 25K virion proteins, a potential concensus cleavage recognition site (Ala-Gly↓Ala) was identified. The cleavage site was located near the N-termini of both precursors suggesting that proteolytic maturation of the 4b and 25K proteins required a single endoproteolytic cleavage event. Interestingly, similar attempts to determine the N-terminal sequence of the 4a virion

Figure III.1. Genomic location of the P4a gene and *in vivo* processing of the P4a precursor protein. (A) *Hind*III restriction map of the VV DNA genome. Fragments (A-P) are labeled in order of their lengths, which are indicated in Kbp. The genomic location and orientation of the genes encoding the three major VV core proteins are indicated by open arrows. The predicted molecular weights of each ORF are indicated below the arrows. The encoded precursor polypeptides and the mature cleavage products are shown as shaded bars with their observed molecular weights. (B) *In vivo* processing of VV core proteins. Radiolabeled extracts from VV infected cells which had been pulse-labeled (P) followed by a four hour chase period (C), or from purified VV virions were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. Precursor proteins are labeled on the left and cleavage products are labeled on the right. The positions and sizes of molecular weight standards included in this analysis are indicated.

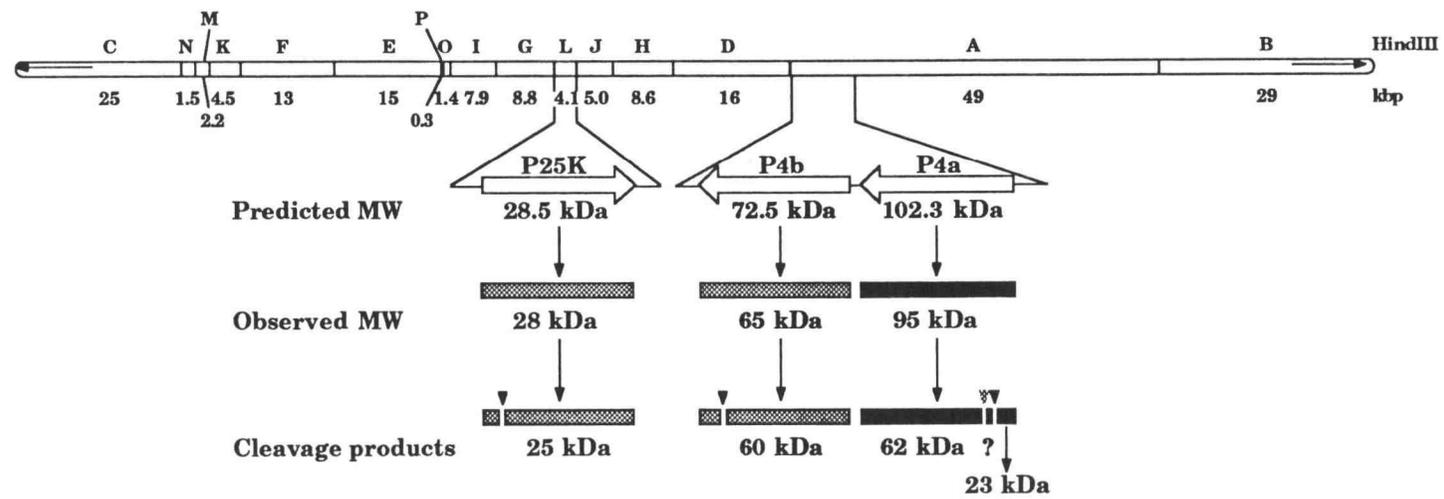


Figure III.1A

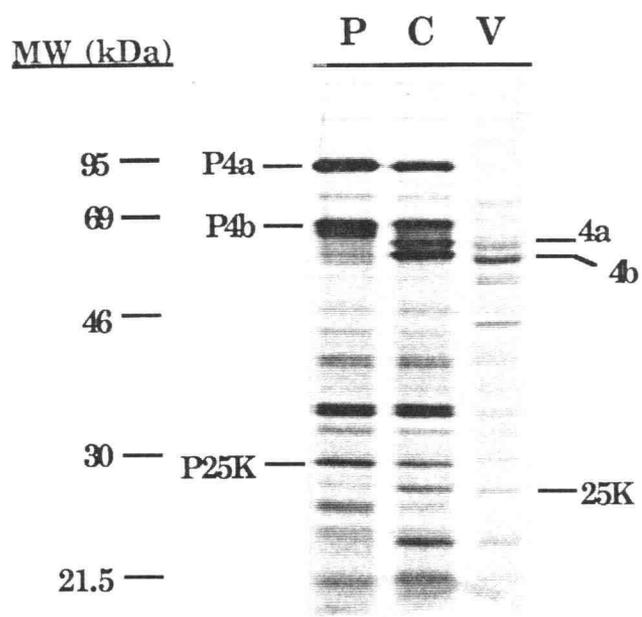


Figure III.1B

protein were unsuccessful. Furthermore, a search of the predicted amino acid sequence of the P4a precursor revealed no Ala-Gly-Ala sequences, suggesting that the P4a precursor might be processed in somewhat different manner than are the P4b and P25K precursors (VanSlyke *et al.*, 1991).

Here we report the results of our investigation of the proteolytic processing of P4a. Antisera specific for various portions of the P4a precursor were used to demonstrate that two virion proteins, a 62 kDa (4a) and 23 kDa species, were derived from the P4a precursor. Protein microsequencing and peptide mapping procedures were employed to demonstrate that the P4a precursor is cleaved at least at two C-terminal proximal sites. The apparent cleavage sites were Ala-Gly↓Thr and Ala-Gly↓Ser, which are distinct from but related to the Ala-Gly↓Ala motif present in the P4b and P25K core protein precursors.

MATERIALS AND METHODS

Virus and cells

Vaccinia virus (strain WR) was propagated in monolayers of BSC-40 cells (African green monkey kidney) cultured in Eagle's minimal essential medium with Earle salts (MEM-E) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 10 µg/ml gentamycin sulfate. Cells were infected and purified virus was prepared as previously described (Hruby *et al.*, 1979).

Radioactive labeling and rifampicin treatment

Pulse-chase labeling of VV-infected BSC-40 cells with L-[³⁵S]-methionine was as previously described (Hruby *et al.*, 1979, VanSlyke *et al.*, 1991). Infected cells were pulse-labeled for 30 min at 7 hpi and harvested by centrifugation immediately, or after a 2 h chase with 100X excess unlabeled methionine. Infected cells were lysed in a buffer containing 2% (w/v) sodium dodecyl sulfate (SDS) and 20 µg/ml phenylmethylsulfonylfluoride (PMSF) which had been heated to 65°C. Nuclear DNA was sheared by passage through a 25 ga. needle several times and the extracts were stored at -20°C until analyzed. Rifampicin treated VV-infected cells were manipulated in the same manner except that 100 µg/ml of rifampicin (Sigma Chemical Corp.) was maintained in the medium (where indicated) throughout infection.

Purification of radiolabeled virus was carried out as previously described (Hruby *et al.*, 1979). VV was labeled by incubating infected cells in the presence of methionine-deficient MEM-E containing L-[³⁵S]-methionine or normal MEM-E plus L-[³⁵S]-cysteine. L-[³⁵S]-methionine

(1163 Ci/mmol) and L-[³⁵S]-cysteine (1016.3 Ci/mmol) were obtained from New England Nuclear Corp. and added at a concentration of 8 µCi per ml of medium.

Construction of a plasmid for *in vitro* expression of P4a

All plasmid manipulations were performed essentially by the methods outlined in Sambrook *et al.* (1989). Initially, a 16 kbp piece of VV genomic DNA that contained the leftmost region of the *Hind*III A fragment was isolated by digestion with *Hind*III and *Pvu*II and cloned into pBR322 at the *Hind*III and *Pvu*II sites. This plasmid was designated pJV-1 and used in subsequent subclonings of the P4a open reading frame. To construct a T7 expression plasmid, portions of the P4a gene were subcloned into two different plasmids and then religated. The 3' end of the P4a open reading frame (ORF) was excised from pJV-1 with *Bam*HI and cloned into pUC118. Site-directed mutagenesis was used to generate a *Hind*III site immediately 3' to the termination codon of the P4a ORF. The 5' end of the P4a gene was amplified by PCR using a chimeric 5' primer containing the essential nucleotides of the T7 promoter abutted to sequences corresponding to the 5' end of the the P4a ORF. The 5' end of the P4a ORF, now abutted to a T7 promoter, was cloned into the *Sma*I site of pUC119. This plasmid was then restricted with *Bam*HI (located within the P4a gene and used to create pUC118:P4a-3') and *Hind*III (located in the pUC119 polycloning region) and the *Bam*HI - *Hind*III fragment isolated from pUC118:P4a-3' was inserted to generate a full length P4a ORF under the direction of a T7 promoter.

Cesium chloride purified plasmid DNA was restricted with *Hind*III which cuts immediately proximal to the 3' end of the P4a ORF, or other restriction endonucleases with sites located within the P4a ORF, and

transcribed *in vitro* with T7 RNA polymerase as previously described (Tabor and Richardson, 1985). The P4a transcripts (1 µg/reaction) were translated in a wheat germ extract system (Promega) according to the supplier's technical information. The ³⁵S-methionine labeled translation products were incubated with 10 µg of ribonuclease A at 37°C for 30 minutes, mixed with SDS-PAGE loading buffer, and analyzed by SDS-polyacrylamide gel electrophoresis.

pATH derived fusion proteins and antiserum

The pATH vectors (Miner and Hruby, 1989) contain the 5' portion of the *E. coli trp* operon including the amino terminal portion of the *trpE* gene followed by a polycloning region that allows for the insertion of foreign ORFs in-frame and the indoleacrylic acid (IAA) induced expression of *trpE* hybrid proteins (37 kDa *trpE* plus the foreign protein). The pT7:P4a plasmid was the source of P4a DNA used in these constructions. A 586 bp fragment (*Bgl*II fragment) containing the portion of the P4a open reading frame that corresponds to amino acid residues 166-362 was inserted into the *Bam*HI site located in the polycloning region of the pATH-2 vector. Likewise, *Bgl*II and *Hind*III were used to excise a 1609 bp fragment from the P4a gene that encodes amino acids 362-891. The *Hind*III site is located downstream of the P4a translational stop codon. Therefore, when this fragment is ligated into the *Bam*HI and *Hind*III sites of pATH-3, the resulting fusion protein will be expected to use the P4a termination signal.

The recombinant plasmids were grown and fusion proteins induced as described by Miner and Hruby (1989), except for the following changes. Plasmids were maintained and expressed in a JM101 strain of *E. coli* and induced at 37°C. After recombinant plasmids were screened for the

induction of a fusion proteins of the predicted size, large scale lysates were prepared, the proteins were separated on 10% polyacrylamide-SDS gels, stained and destained briefly, and the bands corresponding to the fusion proteins were excised from the gel. Gel slices were homogenized with adjuvant and injected into New Zealand white rabbits as described previously (Miner and Hruby, 1989) except that immunization was performed subcutaneously for the first two injections and intramuscularly for the third. Rabbits were test bled 10 days after the last injection and checked for immunoreactivity by immunoblot analysis.

Gel electrophoresis and Western blot analysis

Infected cell extracts or *in vitro* translation products were diluted with sample loading buffer [50 mM Tris-HCl (pH 6.8), 1% SDS, 1% glycerol, 0.1% β -mercaptoethanol] and heated to 100°C for 3 min. Purified virions were pelleted by centrifugation in a microfuge at 15,000 rpm for 5 min, resuspended in sample loading buffer and heated. The denatured proteins were loaded onto discontinuous 4.5/10% polyacrylamide mini-gels containing SDS (unless noted otherwise) and subjected to electrophoresis at 150 V for 30 min to 1 h (Studier, 1973). Gels were subsequently either treated for fluorography and exposed to X-ray film (Bonner and Laskey, 1974), transferred to PVDF membranes as described below, or transferred to nitrocellulose for Western blot analysis.

Electrophoretic transfer to nitrocellulose was carried out essentially by the method published by Towbin *et al.* (1979) at 25 mA for 40 min in Towbin Transfer Buffer [25mM Tris, 192mM glycine, 20%(v/v) methanol]. Nitrocellulose membranes containing proteins from VV-infected cell extracts (+/- rifampicin), uninfected cell extracts, and purified virions were

analyzed with polyclonal antiserum in the following manner. Filters were first washed in Tris Buffered Saline (TBS; 20mM Tris, 500mM NaCl, pH adjusted to 7.5 with HCl), incubated with blocking solution [3% (w/v) gelatin (Bio-Rad EIA purity reagent) in TBS] for 4 h, and then washed with 0.05% (v/v) Tween-20 in TBS (TTBS). The filters were immunoblotted with a 1:500 dilution of pre-immune, α -trpE:P4a(166-362), or α -trpE:P4a(362-891) serum in antibody solution [1% (w/v) gelatin in TTBS] overnight and then washed with TTBS. Immunoreactive protein bands were detected by incubation with goat- α -rabbit alkaline phosphatase conjugate (Bio-Rad) for 1 h, washing with TTBS and TBS, and reacting with p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) in carbonate buffer (Bio-Rad detection kit).

Amino terminal microsequencing

Virion proteins were separated on 10% SDS-PAGE mini-slab gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes by the methods described in ABI's User Bulletins, Issue No. 25 and 36 (Yuen *et al.*, 1986, 1988, respectively), except for the minor variations reported in VanSlyke *et al.* (1991). The membranes were stained with 0.1% Ponceau S in 0.1% aqueous acetic acid, similar to the protocol reported by Salinovich and Montelaro (1986). Membranes (first rinsed with double distilled water) were stained for no longer than 60 s and quickly destained (1 to 2 min) in 1% acetic acid. The regions containing the proteins of interest were immediately excised with a clean razor blade and placed in a 1.5 ml microfuge tube. The slices were neutralized and further destained with 200 μ M NaOH for 1 to 2 min and then washed 4 times with double distilled water. The strips were completely dried under vacuum and immediately

placed at -70°C for storage prior to analysis. The virion proteins captured on the membrane slices were subjected to N-terminal microsequencing by automated Edman degradation in a gas phase cartridge system. Each polypeptide was subjected to nine degradative cycles and the converted cleavage products from each cycle were analyzed by reverse-phase HPLC.

Purification of the 4a virion protein

L-[^{35}S]-methionine and L-[^{35}S]-cysteine labeled protein 4a was isolated from purified VV by electrophoresis on ProsieveTM agarose (FMC BioProducts) by a method similar to that reported in FMC Newsletter Vol. 6 No. 1 (1990). Following protein staining with Coomassie blue (10% ethanol, 5% acetic acid, 0.08% Coomassie brilliant blue R250), the 4a protein band was excised and the agarose was melted at 80°C in 9 volumes of extract buffer [50 mM Tris-HCl (pH7.8), 1 mM EDTA, 1% SDS]. After complete mixing of the gel in buffer, the mixture was frozen at -70°C , thawed, vortexed, and centrifuged in a microfuge at 13,000 rpm for 15 min at 4°C . The supernatant was removed and protein was extracted from the gel again by repeating the freeze-thaw cycle. The protein in the combined supernatants was precipitated in 5 volumes of cold acetone, pelleted by centrifugation as described above, washed in 90% acetone, and dried under vacuum.

Cleavage of 4a at the tryptophan residue

Purified 4a was digested with *N*-chlorosuccinimide (NCS, Sigma Chemical Corp.) resulting in partial cleavage at the C-terminal side of tryptophan residues (Lischwe and Sung, 1977). Each 150 μl reaction contained ^{35}S -methionine or ^{35}S -cysteine labeled 4a (12 and 25 kcpm,

respectively) in 5 mM Tris-HCl (pH7.8), 75 mg urea, 50% glacial acetic acid, and 30 mM NCS. The reactions were incubated at room temperature for 40 min. The peptide products were precipitated by the addition of 5 volumes of cold acetone and placed at -20°C for 30 min. The precipitate was recovered in the same manner that 4a was recovered above. The vacuum dried pellet was resuspended in 10 µl of sample loading buffer and peptides were fractionated by 12% polyacrylamide-SDS gel electrophoresis (Studier, 1973). The gels were fluorographed and exposed to Kodak X-OMAT AR film for 2-7 days at -70°C (Bonner and Laskey, 1974).

RESULTS

Proteolytic processing of VV core proteins P4b and P25K.

Our initial approach to determine the site(s) of proteolytic cleavage within the VV core protein precursors was to isolate the mature forms of the structural proteins from the virion and carry out N-terminal microsequencing procedures. Towards that end, SDS-polyacrylamide gel electrophoresis was used to separate the proteins present in purified virions. After transfer to membranes, the portions of the filter containing 4a, 4b, and 25K proteins were excised and subjected to Edman degradation procedures. As previously reported, whereas the N-terminal sequences of 4b and 25K sequence were readily determined, repeated parallel attempts to obtain the N-terminal amino acid sequence of 4a proved uniformly unsuccessful (VanSlyke *et al.*, 1991). Cleavage of P4b and P25K removed 61 and 32 amino acids, respectively, from the N-termini of the precursors to generate the mature 4b and 25K proteins. The residues flanking the deduced N-termini of 4b and 25K revealed a conserved tripeptide motif (Ala-Gly-Ala) where the last alanine residue is the N-terminus of the processed products. The conserved Ala-Gly-Ala motif was not present within the predicted amino acid sequence of P4a, nor was it present elsewhere within the P4b or P25K precursors. Considering (i) that the 4a and 4b proteins are similar in apparent molecular weight (62 and 60 kDa, respectively) and were isolated in the same manner for N-terminal microsequencing, yet no sequence was obtained for 4a (VanSlyke *et al.*, 1991) and (ii) that there are no Ala-Gly-Ala sites within the P4a precursor (Van Meir and Wittek, 1988), the proteolytic maturation of P4a appeared to proceed via an alternative pathway to the one utilized by the P4b and P25K precursors. As an

alternative approach to examine P4a proteolysis, monospecific antisera were generated to facilitate the identification of P4a cleavage products and/or intermediates.

Identification of P4a cleavage products

Polyclonal antisera were developed against specific regions of the P4a precursor by using bacterial expression plasmids to produce *trpE* fusion proteins to use as immunogens in rabbits (Miner and Hruby, 1989). As diagrammed in Fig. III.2, two plasmid constructions were assembled; one which resulted in a hybrid protein with amino acid residues 166-362 from the P4a ORF fused in frame to the 37 kDa amino terminus of the *trpE* protein; and another construction in which amino acid residues 362-891 from the P4a ORF were inserted in the same context. The two derived antisera were tested for their immunoreactivity in an immunoblot of infected cell extracts (Fig. 2). Four extracts were tested: an extract from uninfected cells, an extract from VV-infected cells (which would be expected to contain both P4a precursor and processed products), and an extract from rifampicin-treated VV-infected cells (which would be expected to contain mainly P4a precursor due to the rifampicin-mediated partial inhibition of cleavage), and a virion extract. The *trpE*:P4a(166-362) antiserum reacted with 95 kDa (P4a) and 62 kDa (4a) proteins, as well as a 35 kDa species. This latter protein is apparently a nonspecific cross-reacting band as all sera, including the pre-immune control, detect this protein in extracts made late in VV infection in the presence or absence of rifampicin (Fig. III.2, VanSlyke and Hruby, 1990). The *trpE*:P4a(362-891) antiserum recognized the 95 kDa P4a precursor as well as a second protein with an approximate molecular weight of 23 kDa. Since the 23 kDa protein

Figure III.2. Generation of polyclonal antisera directed against specific portions of the P4a precursor protein. DNA sequences encoding portions of the P4a ORF were inserted in-frame with the bacterial *trpE* gene in order to generate hybrid proteins containing the N-terminus of the *trpE* protein (open bar) fused to the indicated regions of the P4a precursor (shaded bars). The fusion proteins were purified and used as immunogens to produce polyclonal antisera in rabbits. The derived antisera were designated according to the amino acid boundaries of the P4a fragment contained in the fusion protein. Shown below the diagram are immunoblots carried out using pre-immune, α -*trpE*:P4a(#166-362), or α -*trpE*:P4a(362-891) antisera to react with mock infected cell extracts (M), extracts of cells infected with VV in the presence (+R) or absence (-R) of rifampicin, and extracts prepared from purified virions (V). The positions and sizes of molecular weight standards included in this analysis are indicated at the extreme left of the blots.

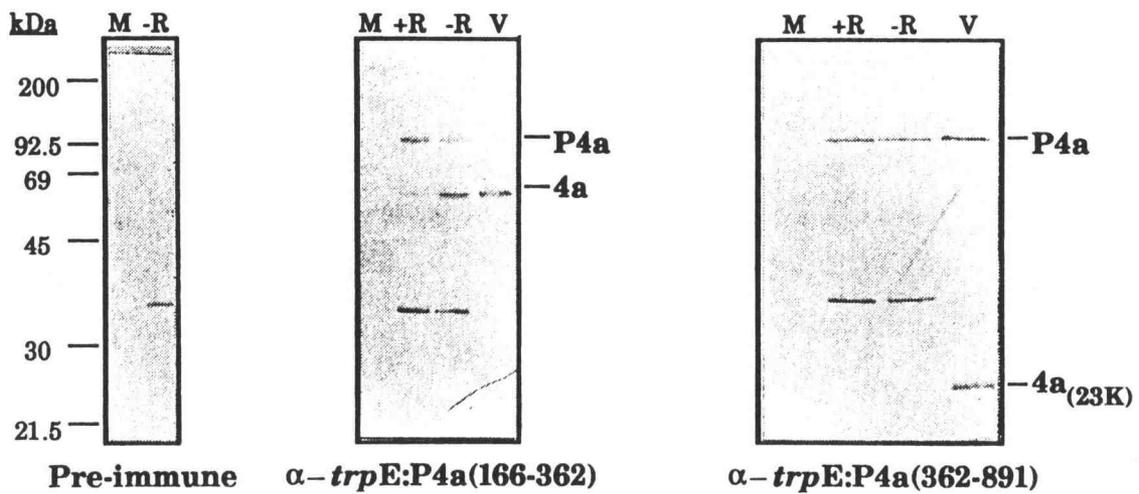
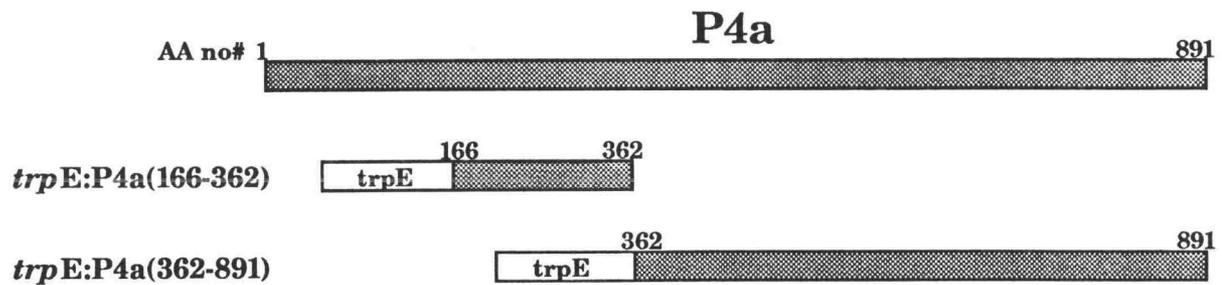


Figure III.2

was detected only in the absence of rifampicin (and in purified virions) this suggests that it is derived by proteolytic cleavage from the P4a precursor. (The small amount of P4a that is processed in the presence of rifampicin probably results in too little 23 kDa product to be detected by this western analysis.) Somewhat surprisingly, the *trpE*:P4a(362-891) did not react with the mature 4a protein. This may suggest that the epitopes found in the middle of the P4a precursor are relatively silent.

In order to verify that the 23 kDa protein was derived from the P4a precursor, the 23 kDa protein was purified from virion extracts and subjected to N-terminal microsequence analysis. The results shown in Fig. III.3 confirm the P4a origin of the 23 kDa protein and suggest that cleavage occurs between amino acid residues 697-698 of the precursor. Cleavage at this point would liberate the carboxy terminal 194 amino acids which would correspond to a protein with a predicted molecular weight of 22 kDa. However, the remaining N-terminal portion of P4a would then have a predicted weight of 80 kDa which is significantly larger than the apparent molecular weight of 62 kDa for virion-derived 4a, estimated from its migration on SDS-polyacrylamide gels. Likewise, it should be noted that the predicted molecular weight of the intact P4a precursor is 102.5 kDa, whereas its apparent molecular weight on gels is 95 kDa. One explanation for these discrepancies might be that some unusual amino acid sequences found within the first 697 residues of P4a cause the polypeptides harboring this region to migrate anomalously on gels. If so, the prediction would be that the difference in predicted and observed molecular weights should become increasingly noticeable as more and more of the C-terminal P4a sequences are removed. To test this hypothesis, an *in vitro* transcription and translation system was used to produce full length and carboxy

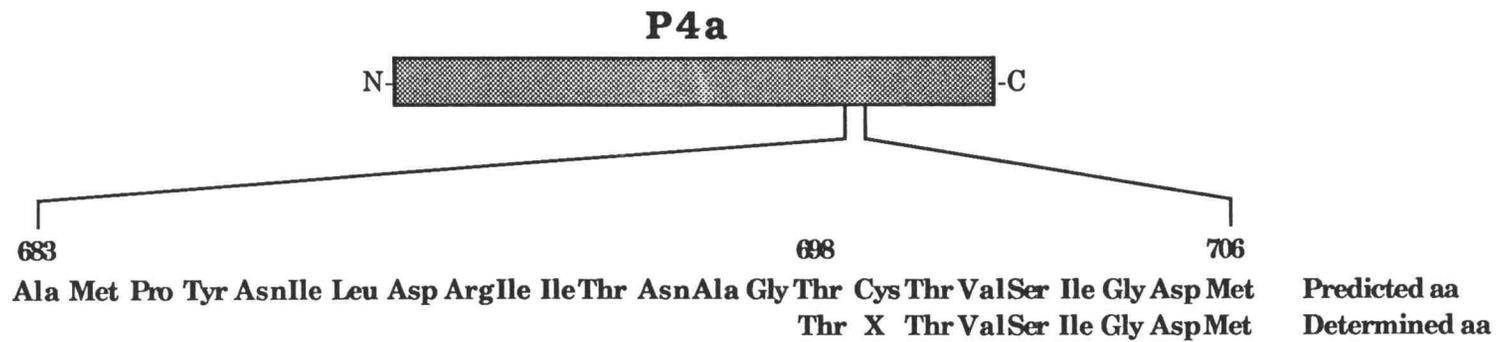


Figure III.3. N-terminal amino acid sequence of P4a derived 23 kDa cleavage product. The N-terminal sequence of the VV 23 kDa virion protein determined by Edman degradation analysis is displayed below the predicted amino acid residues (683-706) of the P4a ORF.

terminal truncated P4a polypeptides (Fig. III.4). When the predicted molecular weight for each polypeptide was compared to that deduced from gel migration, the predicted was always larger than the observed molecular weight except in the comparison of the *in vitro* and *in vivo* generated P4a polypeptides. The *in vitro* generated P4a migrates slightly faster than the P4a from VV-infected cell extracts. This could be due to a posttranslational modification that occurs on P4a *in vivo* but is absent in a cell free translation system. The rest of the truncated proteins all migrate anomalously suggesting that something within the P4a sequence may cause aberrant migration on a SDS-PAGE gel but more precise electrophoretic analysis is needed to determine the cause of this observation. However the truncation that results in a polypeptide 697 amino acids in length yields an observed molecular weight of 68 kDa. Comparing the 4a protein identified in lane P with the protein on the gel in lane 3 shows that a single cleavage event at the Ala-Gly-Thr site would result in a polypeptide larger than the 4a core protein. Therefore a single cleavage event and aberrant migration does not explain the discrepancy in size between 4a and the 80 kDa polypeptide. These results suggest a more likely explanation for the origin of the 62 kDa 4a protein is that the P4a precursor is subject to additional proteolytic maturation steps besides the cleavage between amino acid residues 697-698.

Peptide mapping of the 4a protein

The amino acid sequence within the P4a precursor which flanks the derived N-terminus of the 23 kDa protein is Ala-Gly↓Thr (Fig. III.3), a sequence which is distinct from but related to the Ala-Gly↓Ala motif identified at the cleavage sites within the P4b and P25K core protein

Figure III.4. Comparison of observed vs. predicted molecular weights of P4a protein and truncated derivatives. The number of amino acids and predicted molecular weights of the P4a precursor and the two proteins produced by cleavage between residues 697-698 are depicted by shaded bars at the top of the figure. Below this are schematic representations of a series of 5' co-terminal truncations of the P4a ORF which were made by cutting a parental P4a gene abutted to the bacteriophage T7 promoter with *HindIII* (1), *ClaI* (2), *AflIII* (3), *BstNI* (4), *Pst I* (5), or *NdeI* (6) restriction endonucleases. The parental and truncated templates were transcribed and translated *in vitro*, treated with RNase, and the expressed protein products were analyzed by discontinuous gel electrophoresis and fluorography. At the right are listed the molecular weights predicted and observed (from the fluorograph below) for each P4a protein product by comparison of the gel migration to that of known molecular weight standards. The lanes are labeled 1 - 6 to correspond with each truncated protein on the figure above or P for a 2 h continuous pulse-labeled extract from VV-infected cells starting at 6 hpi.

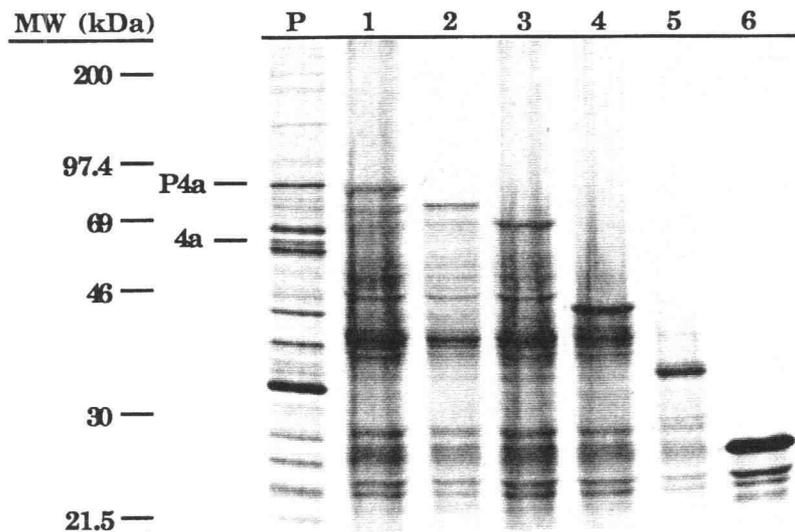
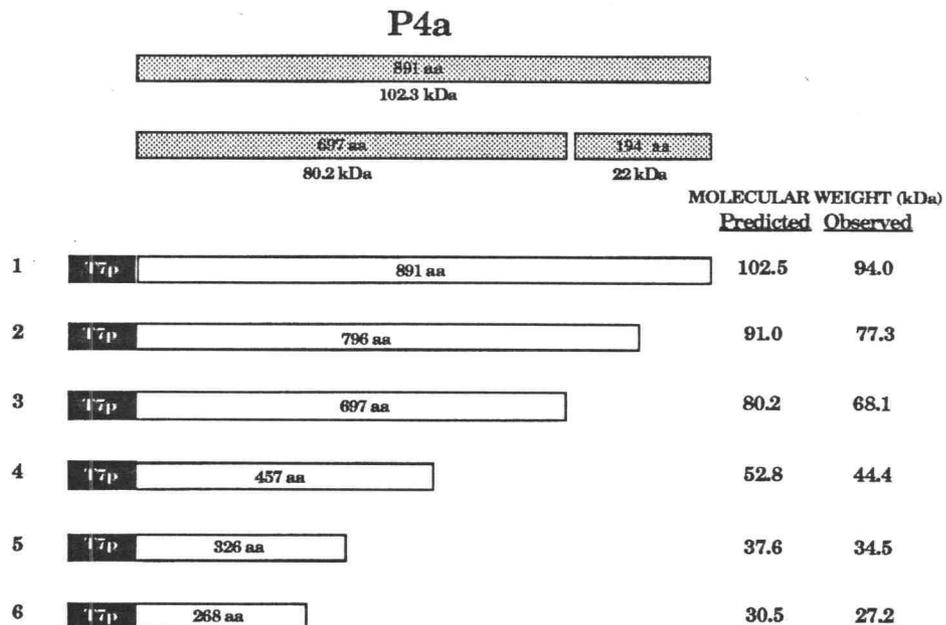


Figure III.4

precursors (VanSlyke *et al.*, 1991). This observation raised the possibility that the degenerate motif Ala-Gly-X was the signal for proteolytic cleavage. The predicted amino acid sequence of the P4a ORF was scanned, and three occurrences of an Ala-Gly-X sequence were noted (Fig. III.5A): Ala-Gly-Asn at residues 94-96 which would remove a 10.7 kDa peptide from the N-terminus; Ala-Gly-Ser at residues 613-615 which would result in 71 kDa and 31.3 kDa products; and the Ala-Gly-Thr at residues 696-698 which accounts for removal of the carboxy terminal 22 kDa. If cleavage occurred at all three Ala-Gly-X sites, then the predicted products would be 60, 22, 10.7, and 9.3 kDa in size. Since threonine and serine residues are closely related in structure, size, and charge (Creighton 1984), it is highly likely that the Ala-Gly-Ser site is recognized and cleaved. An asparagine residue however, differs significantly, making it difficult to predict whether the Ala-Gly-Asn site is efficiently processed.

In order to address this question, peptide mapping procedures were employed. The predicted amino acid sequence of the P4a ORF contains a single, asymmetrically located, tryptophan residue at position 373 (Fig. III.5A). Chemical cleavage at this position should give four distinct patterns of cleavage products depending on which of the Ala-Gly-X sites are utilized to produce the mature 62 kDa 4a protein. If processing occurs only at the Ala-Gly-Thr site, 43 and 37 kDa proteins would be produced by tryptophan cleavage. Processing at the Ala-Gly-Ser site (in addition to the Ala-Gly-Thr site) would generate a protein substrate that is cleaved into two peptides that should migrate on gels as 43 and 28 kDa species. Cleavage at the Ala-Gly-Asn and Ala-Gly-Thr signals would predict a protein that subsequently generates 32 and 37 kDa peptide fragments. Finally, if all Ala-Gly-X sites were utilized, protein products of 32 and 28 kDa would be

Figure III.5. Asymmetric chemical cleavage of the P4a precursor protein. (A) The position of the only tryptophan (residue 373) contained within the P4a precursor is indicated by an arrow head as are the potential Ala-Gly-X cleavage sites. The potential cleavage site sequence (and number of the G residue in each) as well as the location of cysteine residues are shown above the bar. The predicted molecular weights (kDa) of the portions of P4a located between all the sites designated by arrow heads are indicated below. The thick lines beneath the P4a map illustrate of all possible combinations of Ala-Gly-X cleavage site(s) utilization along with the predicted sizes (kDa) of the products which would be generated by chemical cleavage at the tryptophan residue. (B) Radiolabeled (^{35}S -methionine or ^{35}S -cysteine) 4a proteins were isolated from purified virions and analyzed by discontinuous gel electrophoresis and fluorography before (-) or after (+) treatment with *N*-chlorosuccinimide under conditions which specifically cleave proteins at tryptophan residues. The lanes labeled VV, (met and cys) depict purified virions labeled with the indicated isotopic metabolite that were the source of the purified 4a protein used in this experiment. The positions and sizes (kDa) of molecular weight standards (M) included in this analysis are indicated at the left.

generated by tryptophan cleavage. Mature 4a protein was isolated from ^{35}S -methionine labeled virions and treated with *N*-chlorosuccinimide under conditions which are specific for cleavage at tryptophan residues. As the results in Fig. III.5B indicate, the two major cleavage products migrate as 43 and 28 kDa species on SDS-polyacrylamide gels. This result would suggest that the Ala-Gly-Ser and Ala-Gly-Thr sites are cleaved, whereas the Ala-Gly-Asn site is not.

Additional evidence that virion-derived 4a protein contains the N-terminus of P4a was obtained by carrying out a similar experiment using ^{35}S -cysteine labeled 4a substrate. As indicated in Fig. III.5A, there are only two cysteine residues located between the N-terminus and the tryptophan residue at position 373 of the P4a precursor, both of which are within the first 95 amino acids which would be removed if cleavage occurred at the Ala-Gly-Asn site. There are five cysteine residues between tryptophan 373 and the Ala-Gly-Ser site at residues 613-615. If the N-terminus of P4a is present in 4a, the 43 kDa tryptophan cleavage product should be labeled with ^{35}S -cysteine. As the data in Fig. III.5B demonstrate, the patterns of ^{35}S -cysteine and ^{35}S -methionine labeled products produced by treatment of 4a protein with *N*-chlorosuccinimide are indistinguishable, suggesting that the first 95 amino acids of P4a are present in the virion 4a protein. Densitometric scanning of the film revealed that the 28 and 43 kDa ^{35}S -cysteine labeled species were present in a 2 to 1 ratio, a value closer to the 5:2 ratio predicted (from the number of cysteine residues located in each peptide fragment) if the Ala-Gly-Ser site is utilized than the 7:2 ratio expected if the site is not cleaved.

Alignment of cleavage consensus sites in P4a, P4b, and P25K

It was of interest to compare the two deduced cleavage sites within the P4a precursor to those previously identified within the P4b and P25K precursor proteins (VanSlyke *et al.*, 1991). These four sequences have been aligned in Fig. III.6. Interestingly, only the alanine and glycine residues at positions -2 and -1 relative to the predicted cleavage point are strictly conserved in all four precursors. When the amino acid sequences upstream and downstream from the Ala-Gly-Ala site in the P4b and P25K precursors were aligned with the same regions of the homologous proteins from fowlpox virus, only the Ala-Gly-Ala motif was conserved among all four proteins (Binns *et al.*, 1988, 1989; VanSlyke *et al.*, 1991). However, at positions +2 and +7 relative to the predicted cleavage site, all four sequences contained either arginine or lysine residues. In contrast, the residues found in the P4a at positions +2 and +7 are not basic amino acids. Taken together, this analysis would suggest that the signal Ala-Gly-X specifies cleavage within the VV virion precursors, with the alanine and glycine residues being invariant. There appear to be some restrictions on which residue occupies the third position, as both serine and threonine constitute a functional cleavage site whereas asparagine apparently does not. The amino acids found upstream (or in the negative positions) of a cleavage site are usually important in determining a cleavage consensus site (Wellink and van Kammen, 1988). Since few similarities are present in this region of the VV core protein precursors, it will be of interest to determine which residues in or around the Ala-Gly-X motif are responsible for specifying cleavage site selection and processing efficiency.

DISCUSSION

Based on our initial investigation of proteolytic processing of VV core proteins we were able to generate information about the cleavage events that occur within the P4b and P25K precursors (VanSlyke *et al.*, 1991). An Ala-Gly-Ala motif surrounding the N-termini of 25K and 4b proteins was conserved at a unique location within each respective precursor sequence (as well as in the homologous proteins of fowlpox virus), suggesting that this signal was a consensus cleavage site. The location of the Ala-Gly-Ala site in the P4b and P25K precursors was consistent with the production of mature proteins by a single endoproteolytic event removing the N-terminal 62 and 31 amino acids respectively. The results obtained in the present work indicate that the proteolytic maturation of the P4a precursor is distinctly different with respect to the location, sequence, and number of cleavage sites which are processed during the conversion of P4a into mature 4a protein.

We previously generated a polyclonal antiserum directed against 4a and P4a proteins by using 4a protein isolated from purified virus particles as the source of antigen. The antiserum which was obtained reacted poorly with P4a and 4a, but very strongly with P4b and 4b (VanSlyke and Hruby, 1990) which agrees with data previously reported by others using a similar approach (Wittek *et al.*, 1984). This result is likely due to small amounts of 60 kDa 4b protein contaminating the 62 kDa 4a antigen and suggests that the 4b protein is a more effective immunogen. We therefore utilized the *trpE* fusion proteins as an alternative approach for obtaining P4a specific antigen. This method of isolating protein for antibody induction has the advantage of purification away from other viral proteins as well as the

presentation of distinct portions of P4a for antiserum generation that wouldn't necessarily be found in 4a. Two sera were produced, α -*trpE*:P4a(166-362) which reacts with P4a and 4a in Western analysis and immunoprecipitations (data not shown), and α -*trpE*:P4a(362-891) which reacts with the P4a precursor and a 23 kDa virion protein in similar immunoassays. Noting the sequences used to generate the α -*trpE*:P4a(362-891) serum suggested that the C-terminal portion of P4a was cleaved to generate the 23 kDa protein found in the virion. This hypothesis was confirmed by microsequence analysis of the virion-derived 23 kDa protein.

Removal of the carboxy terminal 23 kDa of P4a precursor leaves an approximately 80 kDa product, yet 4a migrates as a 62 kDa protein on polyacrylamide gels. Two possible explanations were considered: anomalous migration or additional proteolytic cleavage events. To address the first possibility, the gel migration of a series of amino co-terminal P4a truncated proteins were analyzed and discrepancies between the predicted and observed molecular weights were noted. Synthesis of the full length P4a polypeptide in a cell free system was almost identical to the authentic P4a synthesized in infected tissue culture cells. The slight difference might be due to a posttranslational modification that only occurs *in vivo*, such as ADP-ribosylation (Child *et al.*, 1988). The truncated P4a products all migrate faster than predicted, but an extensive analysis of these products on gradient SDS-PAGE gels would be necessary to determine more precisely the variation in size. Regardless of the size anomaly, the truncated form that represents P4a from amino acid 1 through 697 and the cleavage product after a single proteolytic event still migrates more slowly than the authentic 4a protein. This evidence along with the similarities in size of the full length P4a molecules supports the multiple cleavage event

hypothesis. The second possibility was investigated by noting the presence of three potential degenerate cleavage signals (Ala-Gly-X) within the P4a precursor. N-terminal microsequencing and peptide mapping procedures were employed to verify that the Ala-Gly-Thr site (at 696-698) is utilized, the Ala-Gly-Ser site (at 613-615) is putatively used, but the Ala-Gly-Asn site (at 94-96) is not utilized during the maturation of the 4a core protein.

One question that has yet to be answered is the fate of the 9 kDa protein that would be liberated by cleavage at the Ala-Gly-Ser (613-615) and Ala-Gly-Thr (696-698) sites. Since both the N- and C-terminal portions of P4a are found within the virion, one might expect to detect the 9 kDa protein in a similar location. However, when extracts of viral infected cells or purified virions were separated on 15 or 20% polyacrylamide-SDS gels and subjected to immunoblot analysis with α -*trpE*:P4a(362-891) serum, no protein was detected, even though the sequences of the 9 kDa should have been present in the fusion protein used to prepare this antiserum. It remains to be established whether this protein is unstable, or perhaps immunologically silent in this context. Along these lines it is also of interest to note that the α -*trpE*:P4a(362-891) serum also fails to react with mature 4a protein despite the presence of a 252 amino acid long region (residues 362-614) which was present in the fusion protein. Our current working hypothesis is that sequences present within the 23 kDa protein are "immunodominant" and prevent recognition of the internal epitopes. Peptide specific antisera are currently being generated to address these questions.

In a recent review of proteolytic processing of viral polyproteins it was suggested that residues located in the negative positions (N-terminal to a cleavage site) are usually more important in determining specificity

(Kräusslich and Wimmer, 1988). This could explain the strict Ala-Gly conservation found at the -2 and -1 positions, respectively, of the P4a, P4b and P25K cleavage sites. Kräusslich and Wimmer (1988) also suggest that residues on the carboxyl side of the cleavage point (except for +1) may be of less importance when defining a cleavage site. The presence of alanine, threonine, or serine at the +1 position of VV core protein cleavage sites could represent the amount of variability allowed at this cleavage consensus site. Furthermore, the residue located at this position may play a role in determining the rate of proteolysis. When measured in pulse-chase labeling experiments, the cleavage of P4a lags behind proteolytic processing of P4b and P25K (VanSlyke *et al.*, 1991). Since the alanine residue is present at the +1 position in both of the two precursor polypeptides that undergo proteolysis first, Ala-Gly-Ala might be the motif that is recognized and utilized most efficiently. When a serine or threonine residue is situated at the +1 position, then cleavage might occur at a slower rate. The potential biological consequences of differential processing rates remain to be investigated.

Wellink and van Kammen (1988) noted that proteolytic processing of viral polyproteins usually results in the production of polypeptides with stabilizing amino acids located at the N-terminus. Accordingly, all the detectable proteins (4a, 23 kDa, 4b, and 25K) produced by proteolysis of VV core protein precursors contain N-terminal amino acids (alanine, threonine, serine, and methionine) that are proposed to be stabilizing by the N-end rule (Bachmair and Varshavsky, 1989). The amino terminal fragments removed from P25K and P4b would contain serine and glutamine, respectively, at the N-terminus with the latter residue being destabilizing. The 9 kDa peptide liberated by cleavage at Ala-Gly-Ser (613-

615) and Ala-Gly-Thr (696-698) would have a stabilizing serine residue at its N-terminus, suggesting that it should be found in infected cells.

The proposed cleavage VV core protein consensus cleavage site, Ala-Gly↓X, is strikingly similar to the cleavage site reported for the virion proteins of adenovirus. A proteinase activity required for the production of mature virus particles was identified by using a *ts* mutant, defective in proteolytic processing of precursor virion proteins at nonpermissive temperatures. Comparisons of the amino acid sequence of the precursor proteins led to a proposal that cleavage was occurring between Gly-Ala residues. Webster *et al.* (1989) used synthetic peptides to analyze the amino acid determinants required for cleavage site recognition by the proteinase. The peptides (Met,Leu)-X-Gly-X↓Gly or (Met,Leu)-X-Gly-Gly↓X were cleaved in assays using disrupted particles as the source of proteinase. Apparently it is important that the glycine is located in either the -1 or +1 position. Alphaviruses also exhibit similar amino acid sequences at cleavage sites (Kräusslich and Wimmer, 1988). These sites are located in the polyprotein sequence between the nonstructural proteins and indicate there is a strong requirement for a Gly in the -2 position and a residue with a small side chain in the -1 and +1 positions. In some alphavirus cleavage sites these positions are occupied by Ala and Gly residues. The utility of these comparisons are that they may provide the basis for definition of the proteinase required for the maturation of the VV core proteins.

In conclusion, the information reported here concerning the proteolytic cleavage of P4a suggests that cleavage consensus sites (Ala-Gly↓X) exist within the three VV core polypeptides that are utilized to produce their mature virion forms. Questions which now must be addressed include determination of which other residues within the

precursors contribute to cleavage site selection, destination of the cleavage products, identification of the proteolytic activity which is responsible for these reactions, elucidation of where in the infected cell proteolysis occurs, and regulation of the proteolytic reaction during the course of VV infection.

CHAPTER IV

PROTEIN TRAFFICKING: TARGETING AND LOCALIZATION OF VACCINIA VIRUS STRUCTURAL PROTEINS DURING VIRION FORMATION

Authors: Judy K. VanSlyke and Dennis E. Hruby

SUMMARY

Proteolytic processing of vaccinia virus core proteins is an essential part of mature virion formation and occurs during virion morphogenesis. In order to better understand how these structural proteins are assembled into virus particles during maturation, immunological reagents generated against VV core proteins 4a, 4b, and 25K and their precursor molecules P4a, P4b, and P25K were used in conjunction with immunofluorescent and immunogold labeling of infected tissue culture cells. The results indicated that all three precursors and their cleavage products were localized to virosomes. As the infection progressed, punctate staining with these sera in immunofluorescent assays appeared to become distributed throughout the cytoplasm which suggested that individual virion particles were immunologically tagged. Immunoelectron microscopy showed that the core proteins were localized to the center of immature and mature virus particles. In contrast, L65, a protein previously shown to be involved with virion assembly, was located along the inner side of the immature virion membrane and remained there even as the viroplasm began to condense away from the membrane. A synthetic peptide strategy was used to generate antiserum that recognized only P4a in immunoprecipitation reactions. This reagent directed fluorescein isothiocyanate conjugated antibodies to produce a speckled staining pattern throughout the cytoplasm. Immunogold labeling indicated that P4a was found in the viroplasm of immature particles and in low levels in the mature virion. Intracellular localization of core and L65 proteins during virion morphogenesis will be discussed.

INTRODUCTION

The successful completion of replication in a viral life cycle is the formation of a infectious virus particle. For vaccinia virus (VV), a large dsDNA virus that is the prototypic member of the orthopoxvirus family, replication involves coordination of the temporal expression of its 196 open reading frames (Moss, 1990; Goebel *et al.*, 1990) solely within the cytoplasm of a mammalian host cell. At late times in the VV life cycle, virion assembly and maturation takes place and is referred to as virion morphogenesis. Visually the process has been well characterized by electron microscopic analysis of VV infected cells (Dales and Pogo, 1981; Moss, 1990). Shortly after DNA replication begins (about 3.5 hours post infection), electron dense areas form within the cytoplasm around the nucleus usually, but not exclusively. These areas are referred to as virus factories or virosomes and this is where DNA replication and virus assembly takes place. At the edges of virosomes, crescent-shaped structures form that eventually appear to completely engulf a portion of the dark staining material. This immature particle undergoes distinct and well documented morphological changes as the center (viroplasm) condenses into the typical dumbbell-shaped core seen in mature virions. During this process, what appears to be two electron dense staining structures form on either side of the core and have been labeled lateral bodies. This process results in what is known as intracellular naked virus (INV) that is fully infectious. An extracellular form of virion is also formed in a VV infection that appears to have extra membrane around it and therefore is referred to as an extracellular enveloped virus (EEV, Hiller *et al.*, 1981). EEV has been implicated as playing a key role in viral spread within a host organism (Payne, 1980). Although the morphogenic forms in

VV maturation are well established, little is known about the molecular events that take place.

For many viral systems, proteolytic processing of virion proteins is essential and closely linked to the process of virus maturation (reviewed by Hellen and Wimmer, 1992). Viruses that express their structural polypeptides as a polyprotein require proteolysis before virion replication can occur (e.g. poliovirus). However, many viruses assemble their structural proteins and virion constituents together in a specific conformation before proteolytic cleavage takes place and mature infectious particles are formed (e.g. adenovirus, T4 bacteriophage). This mechanism of virion formation appears to be employed by vaccinia virus. Three proteins that are major virion constituents or core proteins (4a, 4b, 25K) are cleavage products of larger molecular weight precursors P4a, P4b, and P25K, gene products of the A10L, A3L, and L4R open reading frames, respectively (Rosel and Moss, 1985; Van Meir and Wittek, 1988; Weir and Moss, 1985; Goebel *et al.*, 1990). Previous reports show that anything that disrupts proteolytic processing of these three proteins, inhibits the formation of infectious virus particles (Katz and Moss, 1970; Moss and Rosenblum, 1973, Villarreal *et al.*, 1984; Child *et al.*, 1988; Katz *et al.*, 1973). Rifampicin, one of the drugs that established this inhibition phenomenon, also causes aberrant immature virus particle formation (Moss *et al.*, 1971). The target of this drug appears to be L65 (product of the D13L orf), a protein essential for virion morphogenesis and the normal formation of immature virus particles (Miner and Hruby, 1989; Zhang and Moss, 1992). It is therefore evident that proteolytic cleavage of VV structural proteins is closely coordinated to the process of virion morphogenesis, but what this means at the molecular level is not known.

Investigation of proteolytic processing of VV core proteins has yielded information about the intramolecular events that occur during proteolysis (Yang *et al.*, 1988; VanSlyke *et al.*, 1991a,b), but how these proteins achieve their biological function is still a mystery. For instance, where are these structural proteins during virion morphogenesis? Isolation of different forms of virus particles by sucrose log gradient sedimentation indicated that precursors P4a, P4b, and P25K are associated with immature virus particles, but this method of analysis could lead to exclusion of unstable virus particles (VanSlyke *et al.*, 1992). In this report, various immunological reagents generated to 4a, 4b, 25K, their precursors, and L65 (product of the D13L open reading frame) were utilized for *in situ* labeling of the proteins in VV infected cells. A synthetic peptide approach was used to generate antiserum that recognizes the precursor P4a protein. Both immunofluorescent and immunoelectron microscopy were used to determine the cytolocalization of these proteins during virion morphogenesis. The results of labeling with these antibodies are discussed.

MATERIALS AND METHOD

Virus, cells, and radiolabeling

Monolayers of BSC-40 cells (African green monkey kidney cells adapted to 40°C) were maintained in supplemented Eagle's minimal essential medium with Earle salts (MEM-E) in a 37°C CO₂ incubator, as previously reported (VanSlyke *et al.*, 1991b). Purified virus (WR) was prepared as described by Hruby *et al.* (1979). Pulse and chase labeled infected cell extracts were prepared as described in VanSlyke *et al.* (1991a) except that the labeling medium was placed on infected cells at 6 hours post-infection, the chase period was a total of 120 minutes (unless otherwise noted), and L-[³⁵S]-methionine from Amersham ([³⁵S]-protein labeling mix, NEG-072, 1100 Ci/mmole) was used as the labeling reagent. Extracts were prepared in 2% SDS heated to 65°C after which they were stored at -20°C until further analysis.

Antiserum production

Polyclonal antiserum against P4a/4a and P25K/25K was generated using *trpE* fusion proteins. A fusion protein designated as *trpE*:P4a(166-362) was used to immunize rabbits as previously reported (VanSlyke *et al.*, 1991b). The open reading frame encoding P25K (L4R) was amplified by using Taq polymerase in a polymerase chain reaction and subsequently cloned into the *Sma*I site of pUC118 so that the gene was oriented 5' to 3' against the *tac* promoter. A unique *Bgl*II site within the P25K open reading frame and a *Hind*III site within the vector were then utilized to excise a fragment that could be ligated into the *Bam*HI and *Hind*III sites of the pATH3 plasmid. This construction resulted in a *trpE* fusion protein containing amino acid

residues number 40 through 251 of the P25K protein, and this protein was used as an immunogen to make polyclonal antiserum. Preparation of fusion proteins for immunization, inoculation routes, and collection of the antisera was performed as previously described (VanSlyke *et al.*, 1991b). Polyclonal antiserum to P4b/4b was made by isolation of the mature 4b protein from purified virions on 10% acrylamide-SDS preparative gels and injecting rabbits with a gel slice immulsified with SDS and adjuvant (complete for the first injection, incomplete for all the subsequent injections). Antiserum that recognizes the L65 protein was prepared by Jeff Miner (Miner and Hruby, 1989).

A synthetic multiple antigenic peptide (MAP) was made by the Central Services Laboratory at the Center for Gene Research and Biotechnology by using fMOC chemistry (McLean *et al.*, 1991; Posnett *et al.*, 1988; Tam, 1988). Amino acids corresponding to residues number 649 to 667 of the P4a protein were chosen for the MAP synthesis. After the structure was cleaved from the support resin and lyophilized, an aqueous solution of the peptide was subjected to dialysis against water, aliquoted into individual samples of approximately 150 μg , and placed at -20°C until used for immunization of a rabbit. Before an injection was needed, 150 μg of the MAP was emulsified with adjuvant and the same immunization schedule referenced above was followed.

Immunoprecipitations

Pulse and chase radiolabeled extracts were thawed and centrifuged at 15,000 rpm in a microfuge for one minute to pellet insoluble particulates. One hundred μl of the supernatant was diluted in 900 μl RIPA buffer [1% sodium deoxycholate (w/v) 1% Triton X-100 (v/v), 0.1% SDS (w/v), 150 mM

NaCl, 50 mM Tris·HCl (pH 7.4)] in a 1.5 ml microfuge tube. Five μ l of antiserum that had also been precleared of insoluble portions was added to the buffer and the mixture was inverted to mix and then placed on ice for two hours. Protein A-Sepharose beads that had been hydrated overnight in RIPA buffer were washed in the same buffer three times by centrifuging in a Beckman GC-6R at 2000 rpm for five minutes, discarding the supernatant and resuspending gently in RIPA buffer. After the last spin, the beads were diluted to a 10% suspension and 200 μ l of this suspension was added to each immunoprecipitation reaction and allowed to rock gently overnight at 4°C. The following day the beads were washed three times by pelleting at 15,000 rpm in a microfuge for 20 seconds, removing the supernatant, and resuspending in one ml of RIPA buffer. The last time it was resuspended it was placed in a new tube before pelleting. The beads were then resuspended in 50 μ l of sample loading buffer (Studier, 1973), heated to 100°C for three minutes, pelleted for 20 seconds, and the supernatant was loaded on a discontinuous SDS-polyacrylamide gel.

Gel electrophoresis

The gel system used in all analyses was described by Studier (1973) and is similar to what was reported earlier (VanSlyke *et al.*, 1991b) except that large slab gels were used at a 0.75mm width for analysis of immunoprecipitation results or *trpE* fusion protein constructions and a 1.5mm width was used when preparing proteins for immunization of rabbits. Gels that were used to analyze radioactive polypeptides were fluorographed before exposure to X-ray film (Bonner and Laskey, 1974).

Immunofluorescence

Analysis by immunofluorescence was performed as described by Miner and Hruby (1989) with the following modifications. DNA was specifically stained before fixing the cells by incubating them in a 50 μ M solution of bisbenzamide (Hoescht 33342, Sigma) in phosphate buffered saline (PBS) for 30 minutes at 37°C in a CO₂ incubator. The cells were then washed with PBS and fixed with HPLC grade methanol. The primary antibody was diluted 1:100 in a 10% normal goat serum in PBS solution and the secondary antibody (goat anti-rabbit FITC conjugate from Zymed Laboratories, Inc.) was diluted 1:100 in PBS. After incubation with antisera, cells were stained with 0.1% Evans blue for 1 minute, washed with PBS, and allowed to dry in the dark. A standard Zeiss microscope with a UV light source was used to visualize both stains and a 340 nm filter (FT400-LP420) was used for Hoescht stain.

Immunological electron microscopy

Fixing and embedding VV infected BSC-40 cells was carried by the method developed and described by Russell and Rohrmann (1990), with the following alterations. Approximately 2×10^7 cells were infected at an m.o.i. of five and harvested at 24 hours post-infection by pelleting at 2000 rpm in a Beckman GS-6R centrifuge at 4°C, washed in PBS, and pelleted again. Cells were fixed with 1% glutaraldehyde (HPLC grade, Sigma) and equilibrated in L. R. White resin (medium grade, Ted Pella, Inc.) before embedded in resin using BEEM embedding capsules. The samples were allowed to polymerize in a vacuum oven at 52 to 54°C for two days. Blocks were then sliced for immunocytochemistry with a diamond knife by personnel at the OSU Electronmicroscopy Laboratory and placed on uncoated Pelco nickel grids, 200 mesh. These grids were then subjected immunogold labeling as described

by Erickson *et al.* (1987) using a 1:1000 fold dilution of the primary antibody and a 1:20 dilution of the goat anti-rabbit IgG 10nm gold conjugate. Samples were stained with uranyl acetate and lead citrate (Russell and Rohrmann, 1990; Venable and Coggeshall, 1965) and gold particles and infected structures were visualized on a Phillips EM 300 electron microscope.

RESULTS

Immunological reagents

Immunolabeling of structural proteins at late times within VV infected cells was used to determine where the core proteins were located during virion morphogenesis. Antisera that recognized P4a/4a, P4b/4b, P25K/25K, or L65 were used in the following experiments and the mechanisms for generating these immunological reagents are described below and in the Materials and Methods. *TrpE* fusion proteins containing either residues number 362 through 891 of the P4a polypeptide or number 40 through 251 of the P25K protein (Fig. IV.1) were generated from pATH vectors and used to immunize rabbits. The mature virion form of 4b was isolated from purified virus and used to make polyclonal antiserum. L65 antiserum was made to a *trpE* fusion protein prepared by Jeff Miner (Miner and Hruby, 1989). Specificity of the antibodies was analyzed by using immunoprecipitation reactions containing pulse and chase labeled infected cell extracts. The results are shown in Fig. IV.2. As we expected, the core protein antiserum recognized precursor and product for P4a/4a, P4b/4b, or P25K/25K because the immunogens used for each antiserum contains portions of the core proteins present in both forms of these proteins. As shown before, L65 doesn't undergo proteolytic processing and the antibody is monospecific for the protein. With the specificity of these immunological reagents identified, they could then be useful tools for *in vivo* labeling of these important virion maturation proteins.

Figure IV.1. Production of antiserum against the VV core proteins. Shaded bars labeled as P4a, P25K, and P4b represent the precursor polypeptides and below each of them are bars that represent the corresponding portion of the precursor used to immunize rabbits. Residues number 166 through 362 of P4a and number 40 through 251 of P25K were fused in frame to the *trpE* protein (represented by a black bar) and injected into rabbits. The mature cleavage product 4b, beginning with residue number 62 of the precursor, was used as an immunogen. The arrowheads above the shaded bars indicate where cleavage occurs within the precursor polypeptides during proteolytic processing.

Figure IV.1

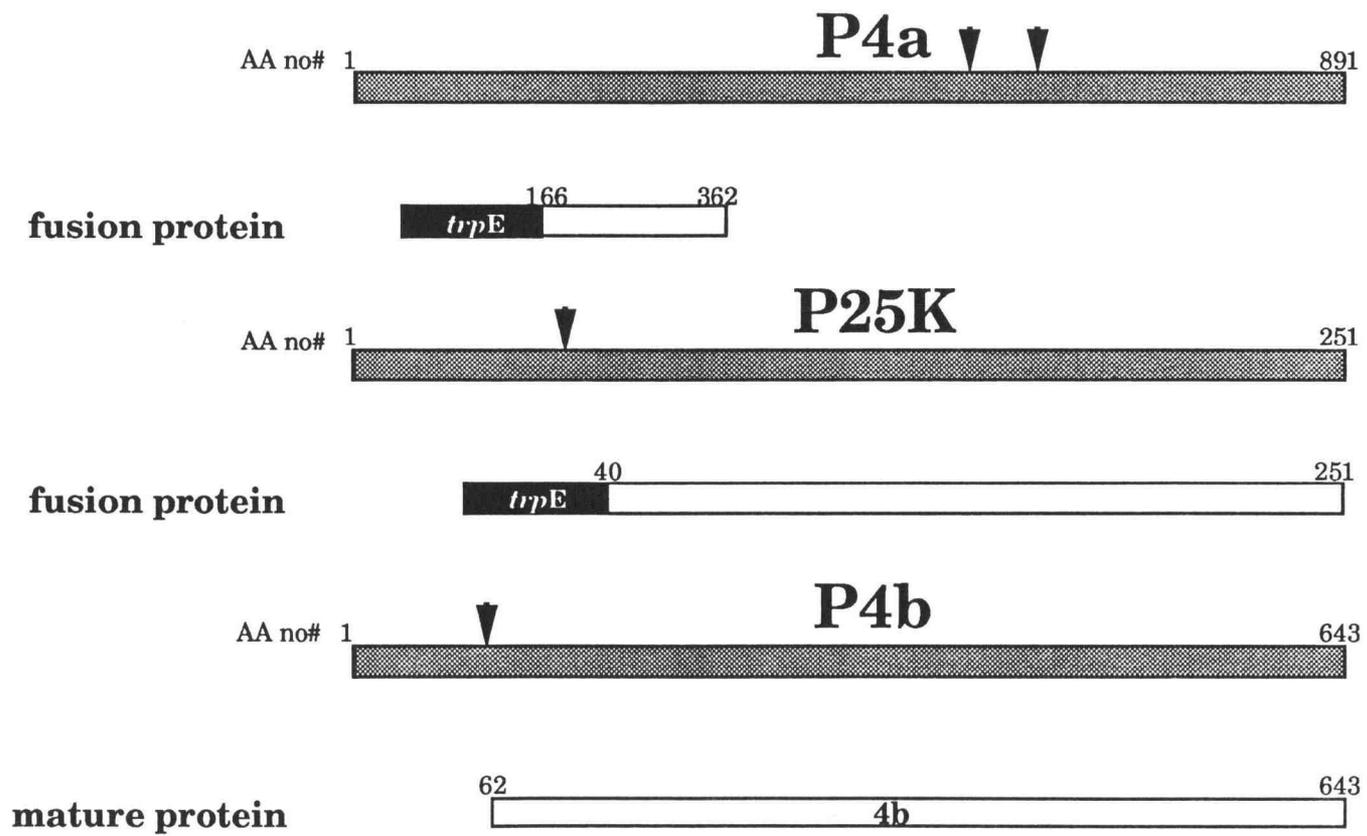


Figure IV. 2. Characterization of core protein antisera by immunoprecipitation analysis. Infected cell extracts pulse (P) labeled with ^{35}S -protein labeling mix at 6 hours post infection for 30 minutes and then chased (C) for 2 hours with excess cold methionine were harvested and lysed in 2% SDS. One hundred μl of either extract was diluted in RIPA buffer and subjected to an immunoprecipitation reaction using core protein or L65 antisera (indicated as labeled above each pair of lanes). The results were analyzed on a discontinuous 10% polyacrylamide-SDS gel, which was treated for fluorography, and exposed to X-ray film. The location of precursor proteins (P4a, P4b, and P25K) are shown by arrows on the left side of the figure and the cleavage products (4a, 4b, and 25K) and L65 are indicated on the right side.

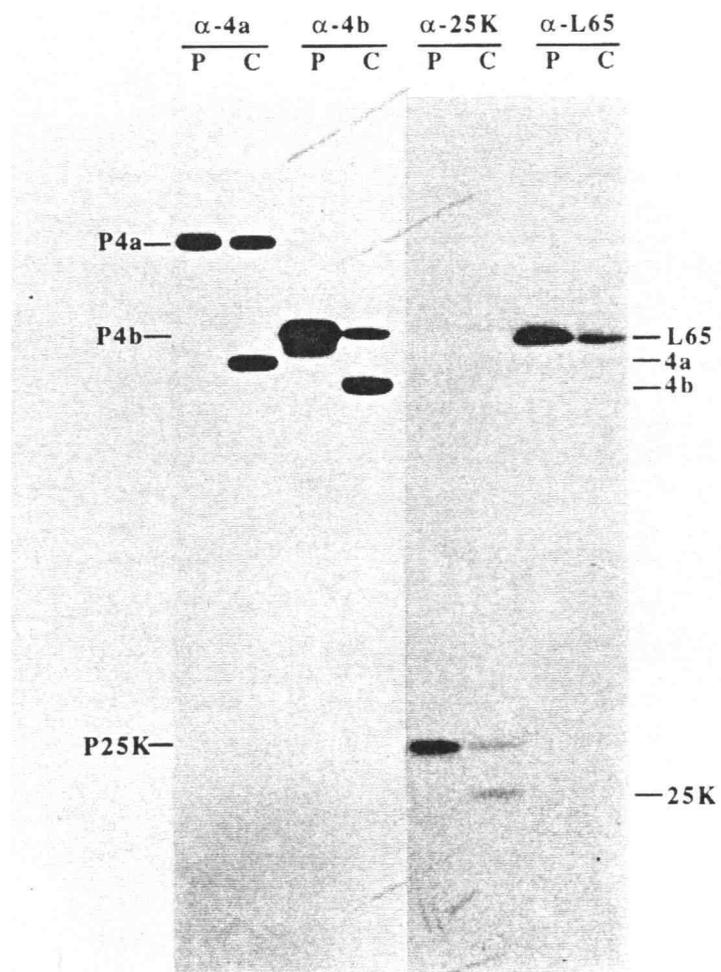


Figure IV.2

Immunofluorescence

Localization of these three core proteins during the vaccinia virus life cycle was investigated by using immunofluorescence. The results obtained with cells fixed at 7.5 hours post infection are shown in Fig. IV.3(A-F). The fluorescein-tagged antibodies were localized to cytoplasmic masses usually surrounding the nuclei of the cells (shown in the left side of each pair of photos). This was proposed to be the virosome or virus factory that forms at late times in VV infection and where DNA replication and virion assembly begin to take place. These were the same structures identified as virosomes by Miner and Hruby (1989) with the L65 antiserum and are also shown in Fig. IV.3F as a point of reference. The control that is labeled as mock infected cells (Fig. IV.3A) was performed using 4b antiserum, but the lack of fluorescence seen is a representation of what was seen when the other antibodies were used. Another important control involved infected cells assayed with preimmune serum and the results are shown in Fig. IV.3B. The fact that mock infected cells with immune serum or infected cells with preimmune serum directed no localization of the FITC conjugate antibodies indicated that P4a/4a, P4b/4b, and P25K/25K were specifically localized to putative virosome structures in the cytoplasm of infected cells. To further determine whether these structures surrounding the nucleus were virus factories, the Hoescht stain was included to see if they contained DNA. As shown in Fig. IV.3 (right side of each pair of photos), the same structures specifically stained by FITC labeled antibodies were fluorescing blue under UV light, confirming that they contain DNA.

In the process of investigating core protein cytolocalization an interesting phenomenon was observed when cells were fixed at successively later times in VV infection. The localized fluorescence seen at earlier times in

Figure IV.3. Immunofluorescent analysis of infected BSC-40 cells. The results of immunofluorescent assays using various antisera are shown in pairs, with the photo on the left representing what is visualized under UV light when the filters specific for fluorescein stain are used and the photo on the right representing the same field of view only when the filters specific for Hoescht stain are used. BSC-40 cells grown on coverslips were infected with VV (B through F) or mock infected (A), stained with Hoescht dye 33342 at 7 hours post infection for 30 minutes, harvested, and fixed to the coverslips with cold methanol. Cells were then incubated in the presence of preimmune (B), P4a/4a (A,C), P4b/4b (D), P25K/25K (E), or L65 (F) antisera followed by goat anti-rabbit fluorescein conjugate and finally counterstained with Evans blue. Fixed and stained cells were overlayed with mounting medium and a coverslip and visualized under oil immersion with a 63X Neofluar objective. (Magnification was 157.5X.)

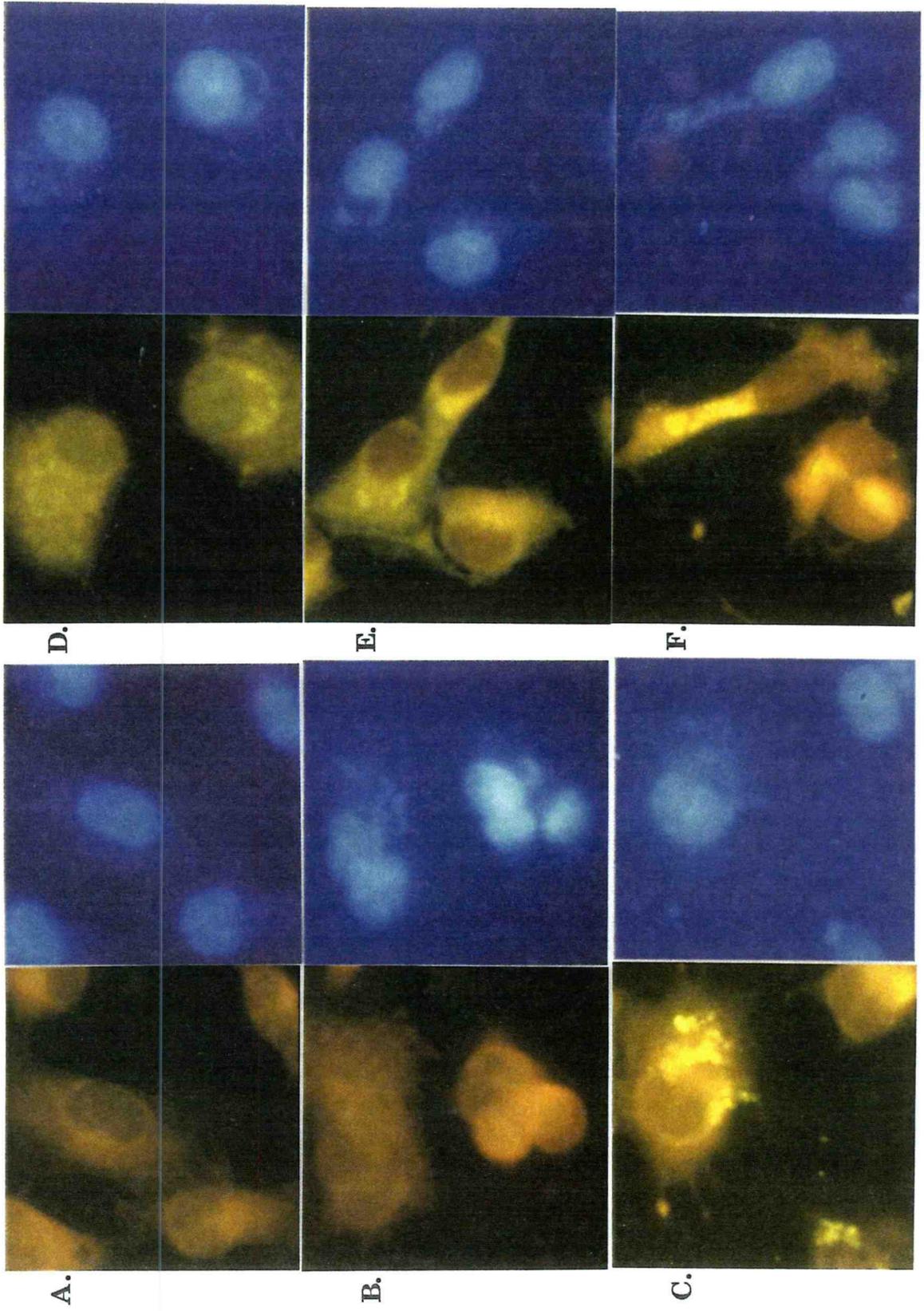


Figure IV. 3

infections appeared to dissipate in the cells as time elapsed. As shown in Fig. IV.4, immunofluorescence derived from the P4a/4a antibody is shown in infected cells at 12, 18, and 24 hours post-infection. The fluorescent antibodies appear localized to the virosome-like structures at 7 hpi, but were spread throughout the cell at 10 and 12 hpi. This observation could be due to the migration of immature virions away from the virosome as virion morphogenesis takes place, a process that has been previously well documented by electron microscopy of VV infected cells (Dales and Pogo, 1981). It could be possible that the speckled immunofluorescent appearance seen at progressively later times in VV infected cells was due to recognition of the 4a core protein in individual virions as they move away from the virus factory. The pattern was seen with the other core protein antisera but it was most distinct when P4a/4a antibody was used.

Immunogold electronmicroscopy

The results of immunofluorescent studies indicated where the core proteins were localized within the VV infected cell on a gross level. The magnification achieved on a light microscope allows for the identification of structures like nucleus and virosomes, but beyond that a different level of magnification was needed. To this end, immunogold labeling and electron microscopy was employed to distinguish the fine structure of individual virions while searching for the location of the core proteins. BSC-40 cells were infected with VV at an m.o.i. that would predict to deliver at least one virus per cell (5) and after 24 hours were harvested and fixed with glutaraldehyde. Cells were then embedded in resin as described in the Materials and Methods and selected for indirect immunogold staining. Goat anti-rabbit gold conjugate (with 10 nm gold particles) was used to localize the

Figure IV.4. Immunofluorescent results using P4a/4a antiserum versus elapsed time during a VV infection. Preparation of infected BSC-40 cells for immunofluorescent analysis was the same as in the figure legend for Fig. IV.3. except that cells were not treated with Hoescht stain. Coverslips with infected cells were fixed at 5 (A), 10 (B), or 12 (C) hours post infection and incubated with P4a/4a antibodies and FITC-tagged goat anti-rabbit antiserum. Fluorescein staining was visualized as described previously except that a 100X oil immersion objective was used. (Magnification was 250X).

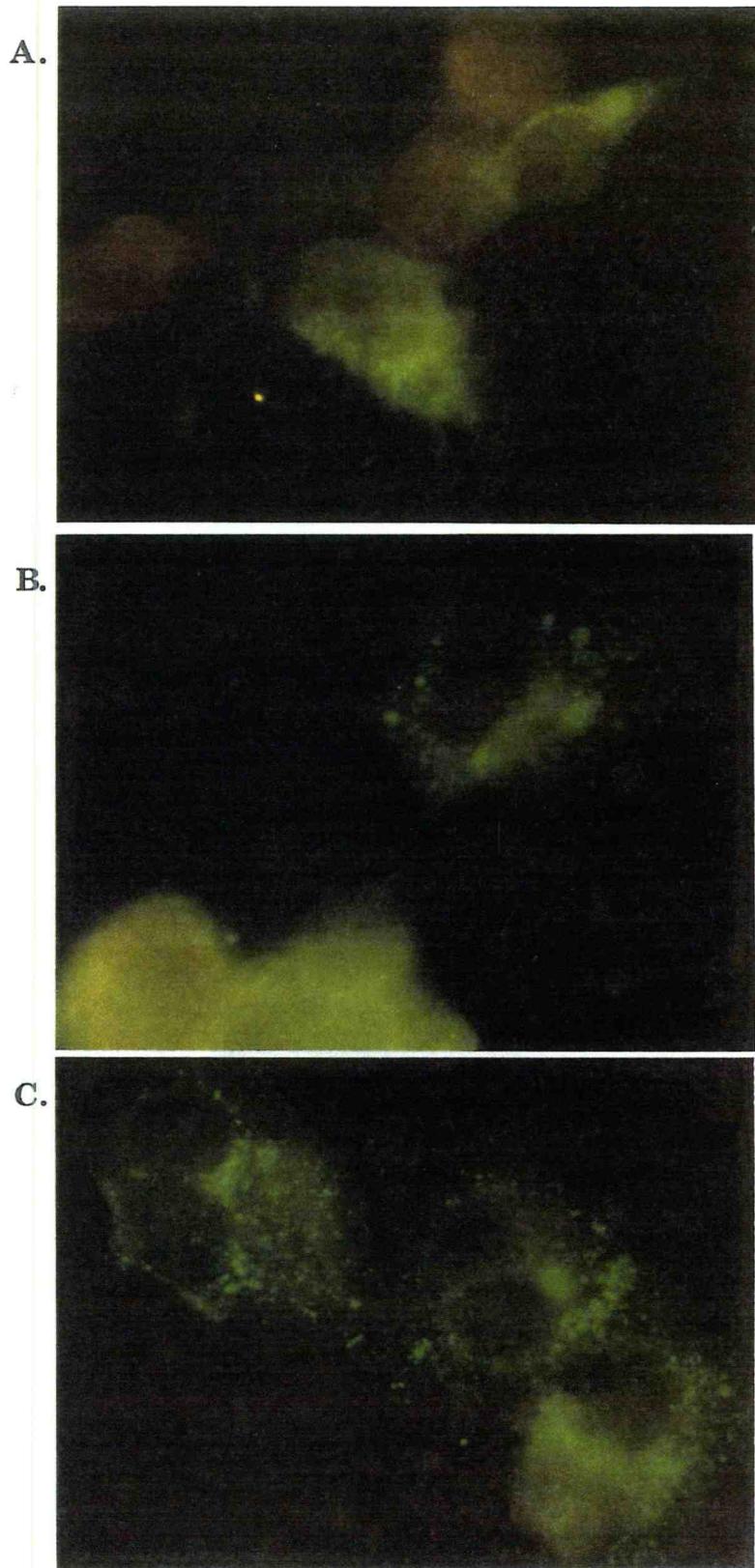


Figure IV. 4

various primary antibodies. The results shown in Fig. IV.5(C-E) show areas containing mostly immature or mature virus particles (left photo and right photo of each pair, respectively) for each of the core protein antisera. The results for all three reagents appeared to be the same; the gold particles were localized to the viroplasm (or center) of immature particles and in general to the center of mature virions (P25K/25K appeared more strictly localized to the virion core as opposed to P4a/4a and P4b/4b spread throughout).

Labeling at or near the core structure in mature virus particles was not surprising because proteins 4a, 4b, and 25K have already been biochemically classified by their association with the virion core. The results when either no primary antibody or preimmune serum (Fig. IV.5A and B, respectively) was used showed that cross reactivity was not a problem in these experiments.

L65 antiserum was included because it was known that L65 was involved in immature virus particle formation (Miner and Hraby, 1989; Zhang and Moss, 1992). Jeff Miner showed that L65 was found in mature virions to a very low extent by immunoblot analysis. The results shown in Fig. IV.5F (right side of pair) agreed with this information when mature virions were analyzed, but the antiserum directed a very precise pattern of staining when immature particles were visualized. Gold particles appeared localized around the inner side of the thick membrane surrounding the viroplasm of immature virions, even after the central core begins to condense away from the membrane (Fig.5F and inset). In contrast, but not surprising, core proteins appeared to stay with the core as condensation took place (data not shown). The localization pattern of L65 was interesting in light of what was already known about this protein's function and shows that differences in localization of proteins in virus particles can be determined by immunogold

Figure IV.5. Immunogold labeling and electron microscopy of infected BSC-40 cells. VV infected cells harvested and fixed for embedding at 24 hours post infection were prepared for immunoelectron microscopy as described in Material and Methods. Slices from the embedded cells were incubated in the presence of no primary antibody (A), preimmune serum (B), α -P4a/4a (C), α -P4b/4b (D), α -P25K/25K (E), or α -L65 (F including inset photo) followed by a second incubation with goat anti-rabbit gold conjugate (10 nm particles). Localization of gold particles are shown for each condition in pairs with the photo on the left showing an area with immature particles present and the photo on the right showing mainly mature virions. Magnification was 75,000X for all photos except for A (left side) and F (left side inset) were 60,000X and 100,000X, respectively.

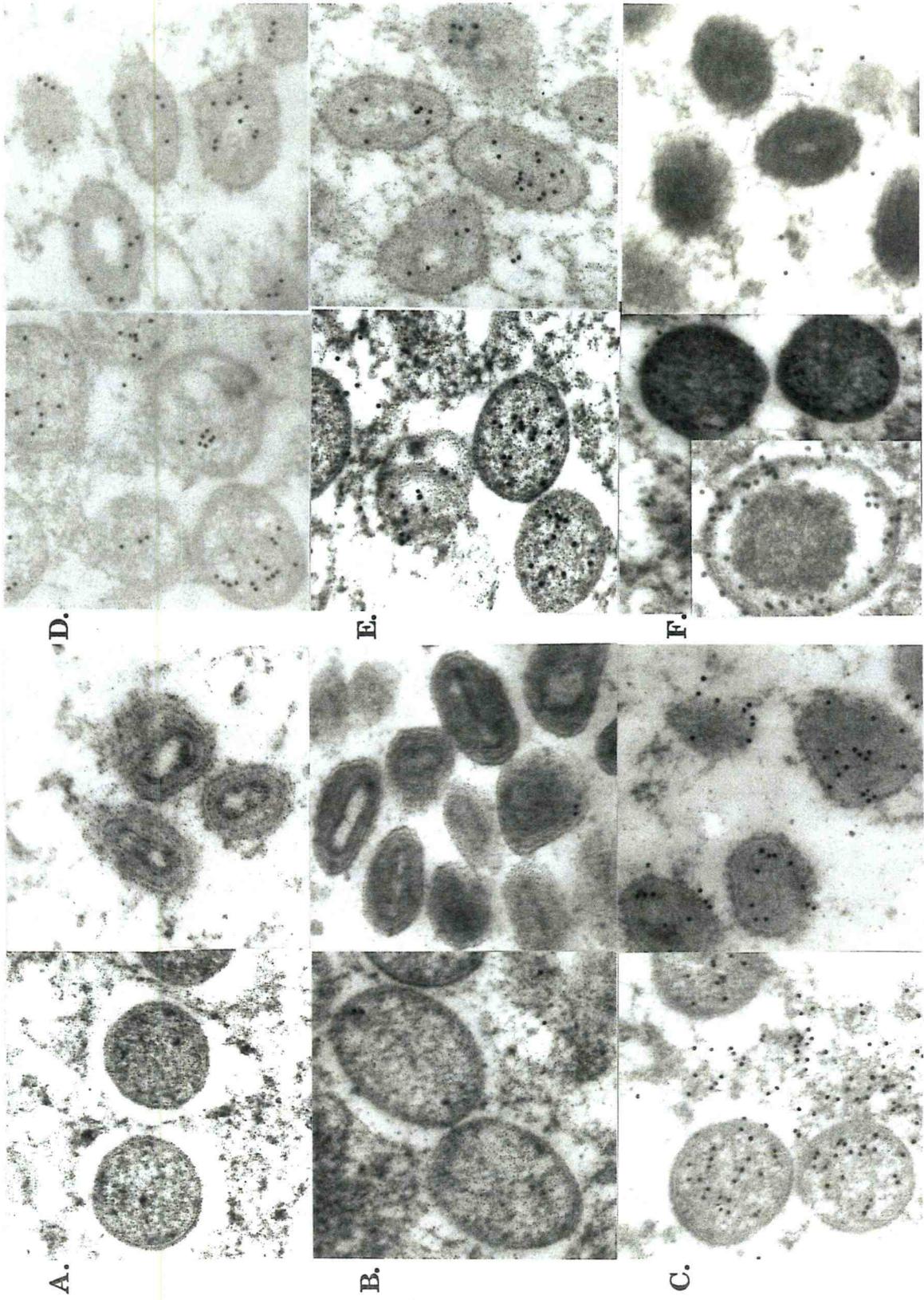


Figure IV.5

staining results.

However, the limitation of information yielded from the results presented so far was due to the fact that the core protein antisera recognize precursors and products. The inability to distinguish these two forms of each protein left us unable to show definitively when proteolytic processing occurs with respect to virion morphogenesis. It was obvious that more specific immunological reagents were needed to answer these questions more completely.

MAP antiserum

In an effort to develop an immunological reagent that distinguished between precursor and product, a strategy using a synthetic peptide corresponding to the portion of the precursor polypeptide removed during proteolytic processing of P4a was employed. A new technology for making synthetic peptides referred to as multiple antigenic peptide (MAP) became available. This technology utilized the two amino groups present on a lysine residue multiple times to build a branched structure (Fig. IV.6A) that ultimately allows for the addition of eight identical peptides. A small polyglycine region was added to the lysine residues before synthesis of the selected peptides as a spacer. When this branched structure was cleaved from the starting resin, it behaved as a small polypeptide (predicted molecular weight of about 20,363 Daltons) and dialysis against water was all that was necessary to prepare it for immunizing rabbits. The portion of P4a that was selected for designing a synthetic peptide was chosen because it lies within the 9 kDa portion of P4a proposed to be proteolytically removed (VanSlyke *et al.*, 1991b) and predicted to have hydrophilic properties. The MAP molecule containing these residues was emulsified with adjuvant and

Figure IV.6. Production of precursor specific polyclonal antiserum against the P4a protein. (A) A synthetic peptide that corresponds to residues number 649 through 667 of the P4a polypeptide followed by four glycine residues serving as spacers was used in conjunction with multiple antigenic peptide technology to generate the molecule represented by the drawing. The molecule was emulsified with adjuvant and injected into rabbits. (B) The polyclonal antiserum generated to the MAP was analyzed for specificity by using an immunoprecipitation analysis. Infected cell extracts pulse labeled for 30 minutes and chased for various time periods were utilized and the resulting samples were separated by 15% SDS-PAGE, treated for fluorography, and visualized after exposure to X-ray film. The protein molecular weight standards and their respective sizes are shown at the left and the chase time periods are displayed above the individual lanes. The pulse and 240 minute chase time extracts shown on the left hand side of the fluorograph were included for orientation of immunoprecipitation results.

A.



B.

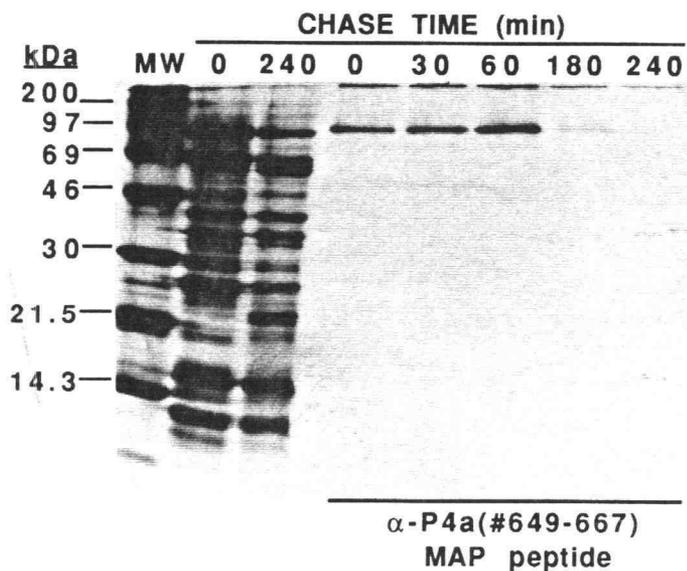


Figure IV.6

injected into rabbits. The serum showed specificity in immunoprecipitation reactions for P4a, but not 4a, as was anticipated (Fig IV.6B). It was also interesting that the precursor polypeptide recognized by this antiserum disappeared as extracts from longer chase times were assayed and as more of the initially radiolabeled P4a protein proteolytically matures. The 9 kDa portion putatively removed from P4a has not been identified by this antiserum and the fate of this peptide is still under investigation.

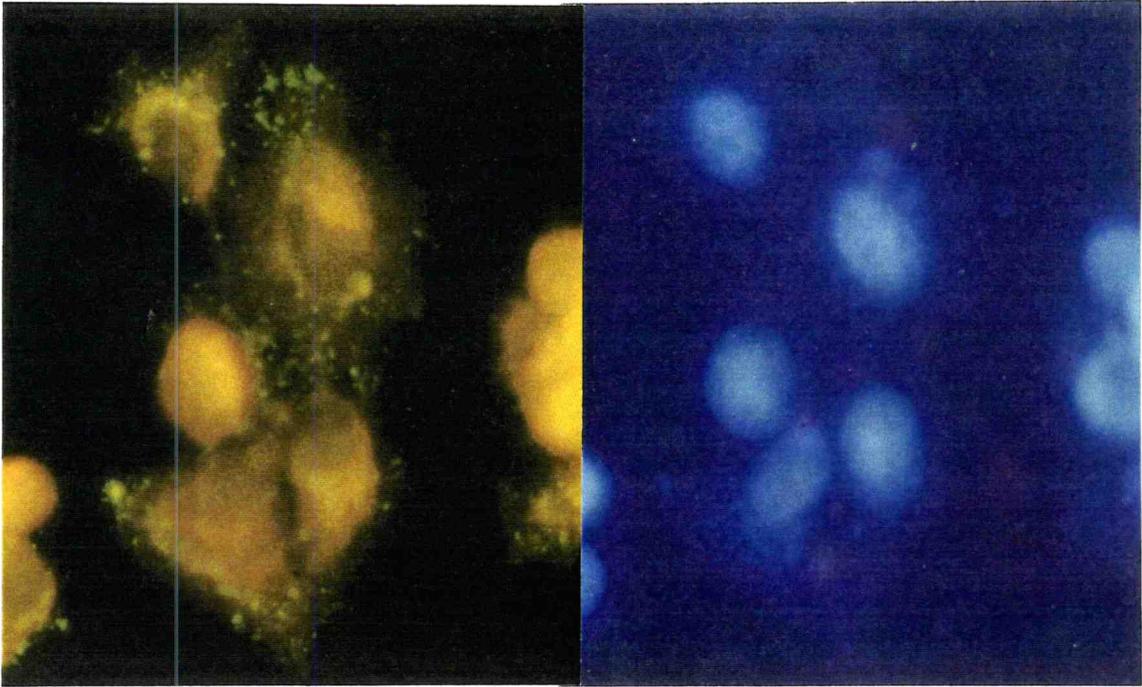
Immunofluorescent staining of VV infected cells showed a slightly varied pattern as compared to the results with other antisera. The FITC conjugate wasn't strictly localized to virosomes, although some localization was apparent. There was a lot of particulate staining throughout the cytoplasm, especially at the edge of plasma membrane. Although the Hoescht staining in these areas was faint, it was visible, indicating that DNA was present. Whether these fluorescent bodies represent virus factories or individual particles (immature or mature) is not known, but the possibilities will be addressed in the Discussion section.

Immunogold labeling with the MAP generated antibody showed gold particles localized to the viroplasm of immature virus particles and very few within the mature virion (Fig. IV.7B, left side and right side of photo pair, respectively). As previously mentioned with the L65 protein, a small amount of P4a can be found in mature virus particles when purified virus is analyzed by western blot analysis and P4a/4a antiserum (VanSlyke *et al.*, 1991b). These results indicate that the precursor form of P4a is engulfed in an immature virion and that proteolytic processing must take place after that event. When that occurs with respect to morphogenic changes like condensation of the core cannot yet be determined.

Figure IV.7. Immunofluorescence and immunoelectron microscopy using the P4a/9K antiserum. The results of immunofluorescent analysis (A) and immunogold labeling with electron microscopy (B) are shown when P4a/9K antiserum was used as the primary antibody. The method for each analysis is the same as that described in Fig. IV.3 and IV.5 legends, respectively.

Again the results are shown in pairs of photos, with the results for fluorescein staining on the left and Hoescht staining on the right (A) and the results for immunogold particle localization in immature virus particles on the left and in mature virions on the right (B). Magnification in A was 157.5X and in B was 75,000X.

A.



B.

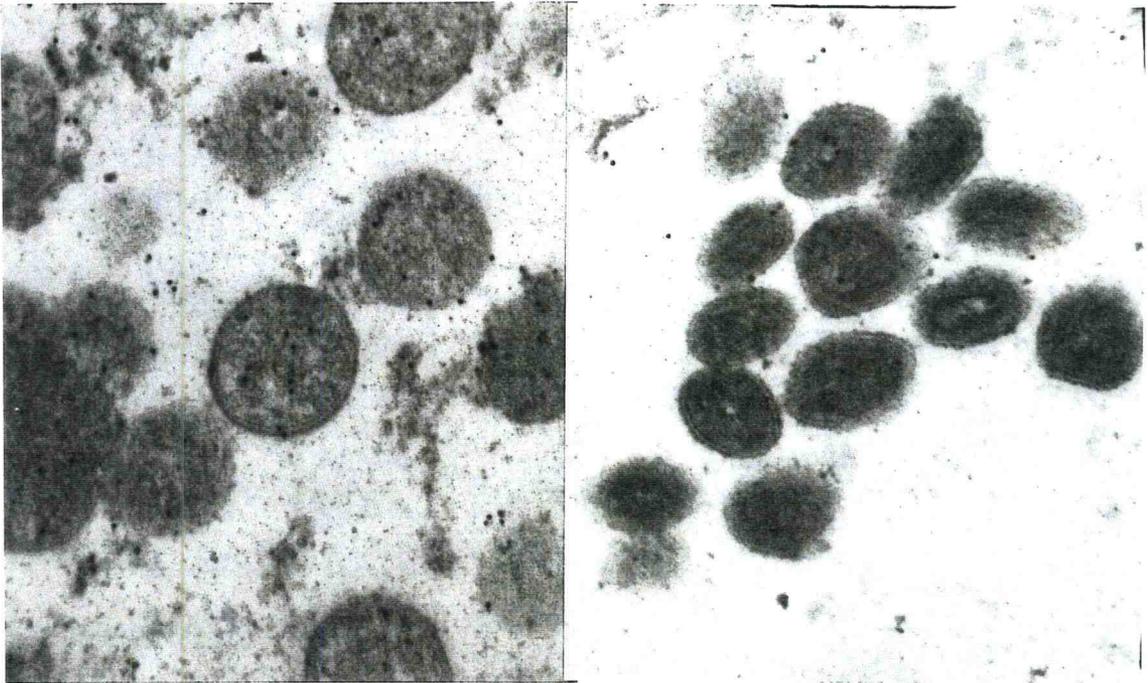


Figure IV.7

DISCUSSION

Virion morphogenesis is a complex set of events that take place in vaccinia virus infected cells to bring about the production of mature virus particles. Proteolytic processing of vaccinia virus structural proteins is also an essential event that takes place late in viral infection and appears to be closely associated with virion morphogenesis. Biochemically, the process of proteolytic maturation of core proteins has been fairly well characterized, but molecularly what is known about these polypeptides and their cleavage events is limited. Using the various immunological reagents previously generated to the vaccinia core proteins P4a/4a, P4b/4b, and P25K/25K, localization of these molecules was investigated during a VV infection.

Initially cytolocalization of core proteins was determined by using immunofluorescent analysis of infected tissue culture cells fixed to coverslips at late times post infection. The results of fluorescent staining in controls using mock infected cells and P4a/4a antiserum or infected cells with preimmune serum indicated that background and nonspecific staining was not a problem in these assays. All three antisera showed that the proteins were found mainly localized to large structures in the cytoplasm, usually surrounding the nucleus, of infected cells. These structures also stained with Hoescht stain, indicating that they are the virosomes which are typically formed in VV infected cells. This method of identifying virosomes has been previously used by other investigators (Estaban, 1977, Hiller and Weber, 1985) and the absence of these structures in mock infected cells agreed with this assumption. Localization of core proteins to the virus factory was not surprising since this is the site of virion assembly as seen by earlier electron microscopy work.

It was interesting that the fluorescent staining of the core proteins and L65 appeared to have a granular appearance to it. This might be due to staining of individual virus particles that have formed at the edge of the virosome. This possibility is feasible because the vaccinia virion is large and these core proteins are the most abundant proteins in the virion so that virus particles would represent concentrated areas of P4a/4a, P4b/4b, and P25K/25K and similar results have been seen with antiserum to other virion proteins (Hiller and Weber, 1985). This idea was strengthened by the results of immunofluorescence analysis at progressively later times post infection. As shown in Fig. IV.4, the speckled or punctate staining was localized to virosomes around 5 hours post infection (or 7.5 hours in Fig. IV.3), but at 10 or 12 hours post infection, the fluorescent speckled stain was spread throughout the cytoplasm. Electron microscopic analysis of VV infected cells has already shown that after immature virus particles form at the outer edge of virus factories, they migrate away from the virosome and are seen throughout the cytoplasm (Dales and Pogo, 1981). The difference seen with punctate staining at later times in infection was most apparent with P4a/4a antiserum, another indication that these represent individual particles since 4a is the most abundant protein constituent of the virion.

The cytolocalization results visualized using immunofluorescent assays were interesting but limited in that only gross features could be identified with this form of analysis. To look at core protein localization in finer detail, immunoelectron microscopy was employed and the results are shown in Fig. IV.5. Infected cells harvested at 24 hours post infection were embedded and sliced for immunogold labeling. Again the controls using either no primary antibody or preimmune antiserum showed that nonspecific labeling was not a problem. The results with core antiserum again all yielded similar results:

P4a/4a, P4b/4b, P25K/25K proteins are localized to the center of immature virus particles (viroplasm) and mature virions. The fact that these proteins are found in or near the core mature virus particles is not surprising, for this had been biochemically determined by other researchers (Sarov and Joklik, 1972; Ichihashi, *et al.*, 1974). As already noted, P25K/25K appeared localized more strictly to the virion core whereas P4b/4b and especially P4a/4a were found more throughout (at least more so than P25K/25K). This agrees with the report by Ichihashi *et al.* (1984) that identified by using various detergent treatments of purified virions the location 4b (presumably the 57K protein) to the inner core wall and 4a (61K protein) to the outer core wall, while 25K (presumed to be 27K) was found to be surrounding the DNA and classified as a nucleoprotein. This also agrees with the DNA binding properties reported by Yang *et al.* (1988) for 25K (referred to as VP8).

The localization of these proteins to the viroplasm as opposed to membranes of immature virions gives the impression that there is no specific internal organization of these proteins in immature virus particles. But the complex set of events that will occur during virion morphogenesis, such as these three proteins undergoing proteolytic processing (and many other events) as well as condensation of the core and genomic DNA, argue that correct protein-protein interactions and precise molecular architecture must be present within a newly assembled virion for the process to proceed normally. The impression of an immature virus particle simply being a bag of proteins and DNA would be a naive assumption and expansion of our limited knowledge of these viral entities molecularly would greatly enhance our understanding of virion morphogenesis.

Determination that L65 was localized to the inner side of the immature particle's membrane and at a low level within mature virions does indicate

that specific structural organization is present during virion assembly. This was interesting information in light of the previous observation that normal expression of the L65 protein was necessary for the formation of the typical crescent structures that envelop viroplasm to form immature particles (Moss *et al.*, 1969; Nagayama *et al.*, 1970; Pennington *et al.*, 1970; Moss *et al.*, 1971; Zhang and Moss, 1992) as well as for the proteolytic maturation of virions (Katz and Moss, 1970; Rosenblum, 1973). L65's apparent role in maintaining rigid crescent-shaped membrane structures and its specific location in immature particles bring us one step closer to understanding how this protein mechanistically performs its biological function. Zhang and Moss (1992) proposed that L65 normally interacts with viral membrane precursors and the results presented here are in agreement with that hypothesis.

The fact that the core protein sera recognizes precursors and products, determination of the point at which proteolytic processing actually takes place with respect to distinct morphogenic events was not possible. In an attempt to bypass this limitation, an immunogen was designed that could yield antiserum that recognized the precursor P4a and/or the 9K internal cleavage fragment removed during proteolytic maturation. A synthetic peptide strategy was employed that negated the use of carrier molecules and the resulting polyclonal antiserum showed recognition to P4a. So far the 9K fragment has not been identified with this serum, which could be explained many ways: the cleavage product doesn't maintain the same epitope as the one recognized in P4a, the peptide is unstable, or the fragment is inaccessible to antibody in an immunoprecipitation reaction. Nevertheless, this putative precursor specific antiserum was utilized in immunofluorescence assays and the results shown in Fig. IV.7 were surprising. The fluorescent staining appeared to be somewhat localized to virosomes near the nucleus, but more

notably the fluorescence was speckled throughout the cytoplasm. Some of the more intense fluorescently tagged spots located near the plasma membrane, especially in the protrusions that extend out in a finger-like manner, also stained faintly with Hoescht, but it is doubtful whether these represent virus factories. The overall pattern was similar to the dissemination results shown in Fig. IV.4 and the possibility that individual virion particles might be identified by this assay could explain this result, either by immunologically tagging P4a in immature virus particles or at low levels in mature virions. It also could represent the location of the 9K fragment, even though it was not seen in immunoprecipitation assays, possibly in mature virions or scattered throughout the infected cell. Extensive searches for this peptide in VV infected cells argue against the latter possibility, but does not rule it out (VanSlyke and Hruby, unpublished data). The conditions under which serum was allowed to interact with proteins harvested in 2% SDS and diluted in RIPA buffer versus proteins in an infected cell fixed to a coverslip were different and may account for the difference in epitope recognition.

Immunogold electron microscopy with the P4a/9K antiserum indicated that the precursor P4a was located in the viroplasm of immature virus particles and again in small amounts in the mature virions. The level of immunogold staining overall (compared to the core protein sera) was low, potentially a result of the harsh nature of the fixation treatment destroying the few epitopes the primary antiserum can recognize. This antiserum was generated to a peptide, limiting the number of immunogenic sites available, as opposed to the fusion or virion proteins used to immunize rabbits when core protein antibodies were made. The possibility that available epitopes could be limited makes it impossible to interpret results where there is no specific localization of gold particles. The immunoelectron microscopy results

did not reveal any specific structures besides virion particles in the cytoplasm stained with gold particles.

All this information together indicates that the spherical immature virus particles form with the precursor forms of the core proteins enveloped inside while L65 is located along the internal side of the immature viral membrane. As the viroplasm condenses into its typical dumbbell shaped core, the core proteins and/or their precursors stay localized to it and L65 remains associated with the viral membrane. There is some evidence that 25K ends up located closest to the viral genome with respect to 4a and 4b. However, exactly when proteolytic processing occurs with respect to these morphological changes cannot be determined. Development of precursor specific antiserum provided some insight, but now the limitations lie in the method used to preserve infected cells for immunogold labeling and other procedures are currently being pursued.

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