JOURNAL OF VIROLOGY, Nov. 1994, p. 7603–7608
0022-538X/94/$04.00+0

Vol. 68, No. 11

A Transcriptionally Controlled *trans*-Processing Assay: Putative Identification of a Vaccinia Virus-Encoded Proteinase Which Cleaves Precursor Protein P25K

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Received 31 March 1994/Accepted 5 August 1994

Vaccinia virus maturation into infectious particles appears to be dependent on the proteolytic processing of at least five viral proteins, each containing a conserved AG*X cleavage motif and each requiring proper association with the previrion particle. To identify the responsible proteinase, a transcriptionally controlled *trans*-processing assay was developed to monitor cleavage at the permissive AG*S site of the P25K core protein precursor. This assay led to the putative identification of a VV proteinase encoded by open reading frame G1L. The predicted protein contains an HXXEH sequence which is a direct inversion of the active site consensus sequence present in thermolysin and other metalloendopeptidases. Site-directed mutation of this consensus sequence suggests that the G1L protein may be a novel, virus-encoded metalloendoproteinase, although confirmation of this activity must await the development of a suitable cell-free processing assay.

During replication of vaccinia virus (VV) in the host cell cytoplasm, a number of virus-encoded proteins are matured by proteolytic processing, an event that is essential for proper virion maturation. Previous studies have established the relationship between virion morphogenesis and proteolytic processing. These include demonstration that several of the VV major core proteins remain unprocessed when virus maturation is blocked by the addition of the drug rifampin (13, 22). A similar limitation of core protein processing and virus maturation has been observed in cells infected with maturation-defective temperature-sensitive mutants at the nonpermissive temperatures (5, 7, 26, 27), and more recently, direct evidence was provided by the observation that core proteins are found primarily as precursors in immature viral particles (32). Although proteolysis is required during the final steps of virion maturation, it now appears that assembly of the precursor proteins into immature particles may be a necessary requirement for proteolytic processing. It is this later assumption which may explain the difficulties that have been encountered in demonstration of proteolytic activity in extracts made from either VV-infected cells or disrupted virions.

To date, five VV-encoded, virion-associated proteins have been shown to be proteolytically processed (34). Alignment of the cleavage sites in these precursor proteins has revealed a conserved AG*X cleavage motif (30). The presence of this novel cleavage motif would seem to indicate that proteolysis of each substrate may be catalyzed by the same proteinase. It also seems likely that the proteinase is virus encoded, rather than of cellular origin, since processing of the major core proteins appears to be identical across a wide variety of host cells.

During development of a *trans*-processing assay for the study of *cis*-acting sequence elements on AG*X cleavage site definition, a new AG*S cleavage site was identified 14 amino acid residues upstream of the previously identified AG*A site of P25K (20). Although the product (designated 25K′) resulting from cleavage at this site is found only in limited quantities in extracts made from normal VV-infected cells, it is seen in much greater quantity among infected cells transiently expressing the P25K precursor protein. Interestingly, little if any 25K′ is assembled in mature virus, and cleavage at this AG*S site does not appear to be blocked by the drug rifampin (20). If proteolysis at this site is catalyzed by the same proteinase responsible for processing of the other core protein sites, it may represent a fortuitous site not restricted by assembly context. Employment of such a cleavage site would be useful in developing an assay to identify the proteinase. Since all known AG*X cleavage substrates are expressed during late times of viral infection, it seemed reasonable to assume that the proteinase would likewise be a late-gene product. In order to systematically screen viral gene products for proteolytic activity, a method for selective transcription of viral late genes following transfection into infected cells was required.

For a normal VV infection, it has been shown that newly replicated, naked, viral DNA serves as template for expression of the late-transcription-factor genes A1L, A2L, and G8R, as well as late genes (14, 15). Previous studies by Keck et al. have demonstrated that VV-infected cells could be specifically programmed to exclusively express transfected genes driven by a VV late promoter (14). This is accomplished by blockage of viral genome replication with the drug 1-β-D-arabinofuranosylcytosine (Ara-C) and provision of plasmid copies of the required late-transcription-factor genes in *trans*. This finding became the framework for development of the transcriptionally controlled *trans*-processing assay used in this study to selectively rescue processing of the AG*S site of P25K.

At the onset of this project, it was crucial to determine if full-length viral DNA was sufficient to rescue late transcription and proteolytic processing of core proteins in DNA replication-deficient cells. VV-infected cells were treated with the drug Ara-C at 0 h postinfection and transfected with full-length purified VV DNA (3). Immunoprecipitation analysis of radiolabeled cell extracts with antisera specific for P4a and P4b is shown in Fig. 1. In cells that were not transfected, Ara-C completely blocked the synthesis of P4a and P4b, since these proteins are expressed from late genes, whose transcription is

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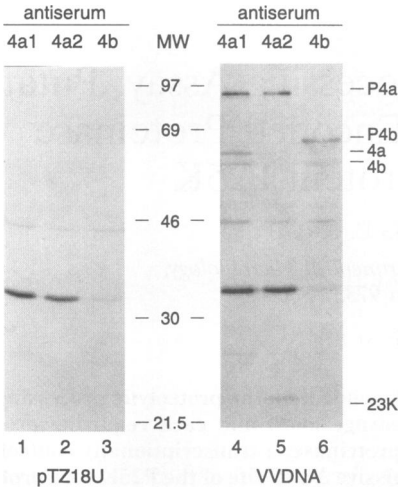

FIG. 1. Rescue of expression and processing of P4a and P4b in Ara-C-blocked cells. Monolayers of BSC-40 cells were infected with wild-type VV, treated immediately with Ara-C, and transfected by liposomes with either control vector pTZ18U (lanes 1 to 3) or full-length VV DNA (lanes 4 to 6). At 4 h postinfection, the liposome-containing medium was replaced with medium containing [^{35}S]methionine-cysteine and Ara-C. After 20 h of infection, cell lysates were prepared, immunoprecipitated with the indicated antisera, and processed for autoradiography. Antiserum 4a1, generated to the NH_2-terminal portion of P4a, recognizes P4a and 4a, while antiserum 4a2, generated to the COOH-terminal portion of P4a, recognizes P4a and 23K (33). The migration of ^{14}C-labeled molecular weight markers is shown (in thousands).

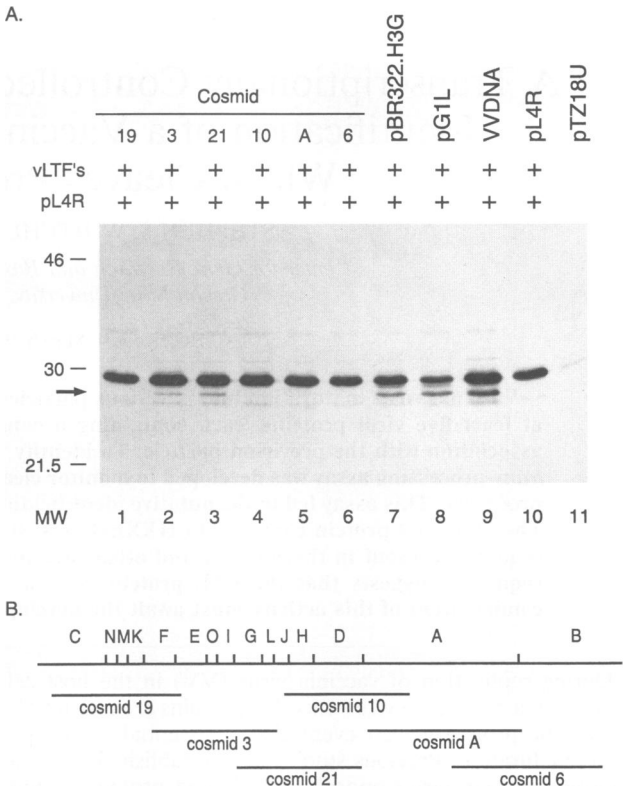

FIG. 2. (A) Rescue of P25K processing in a transcriptionally controlled assay. Monolayers of BCS-40 cells were infected with VVTF7-3, treated immediately with Ara-C, and transfected by liposomes with the indicated DNA molecules. At 4 h postinfection, the liposome-containing medium was replaced with medium containing [^{35}S]methionine-cysteine and Ara-C. After 20 h of infection, cell lysates were prepared and immunoprecipitated with P25K antiserum and processed for autoradiography. To generate pG1L, an *Eco*RI-*Kpn*I fragment (3.75 kb) containing the G1L promoter region and the entire G1L ORF was isolated from the *Hin*dIII G fragment of the VV genome and cloned into pTZ19U. The pUC119-based plasmid pL4R, containing the L4R promoter region and the entire L4R ORF, which encodes the P25K polypeptide, was provided by Peiyu Lee. An arrow indicates the position of the 25K′ product. The migration of ^{14}C-labeled molecular weight markers is shown (in thousands). (B) Schematic representation of the VV genome and cosmid segments. The upper line shows the *Hin*dIII restriction map of the full-length genome. Below this line, the approximate location of each cloned DNA segment is depicted.

dependent on viral DNA replication. However, transfection with viral DNA provided template for expression of the late-gene transcription factors, as well as template for P4a and P4b transcription. As shown, not only were P4a and P4b expressed, but they were also processed. This not only established that proteolysis was controlled by a viral gene (or genes) but that the responsible gene is expressed at late times during infection. Although it is impossible to separate gene expression and proteolysis in such an assay, both processes seemed operational in DNA replication-deficient cells.

In order to separate substrate expression and proteolysis into two controllable steps, VV-infected cells treated with Ara-C were simultaneously transfected with gene copies of the following: (i) late-transcription factors A1L, A2L, and G8R, (ii) substrate, and (iii) test proteinase. In the basic transcriptionally controlled processing assay, confluent BSC-40 cells in 35-mm-diameter wells were infected with VVTF7-3 at a multiplicity of infection of 10. At 0 h postinfection, cells were transfected by use of liposomes as previously described (20). Cells in each well were transfected in the presence of 40 μg of Ara-C with 0.2 μg each of plasmids pTM.A1L, pTM.A2L, and pTM.G8R (provided by Bernard Moss), as well as 2.5 μg of plasmid coding for the substrate protein and 2.5 μg of test DNA. After 4 h of incubation at 37°C, the DNA-containing medium was replaced with minimal essential medium E containing 40 μg of Ara-C per ml and 90 μCi of ^{35}S protein-labeling mix per ml (11.0 mCi/ml [1,175 Ci/mmol]; du Pont-NEN, Wilmington, Del.). At 20 h postinfection, the cells were lysed for immunoprecipitation by removal of the labeling medium and addition of 150 μl of radioimmunoprecipitation assay buffer (RIPA) (20) containing 100 U of Benzonase endonuclease per ml (EM Science, Gibbstown, N.J.). Cell extracts were precleared by microcentrifugation and mixed overnight at 4°C with 3 μl of specific antiserum (31). A 100-μl aliquot of 20% (vol/vol) protein A-Sepharose (Sigma Chemical Co.) suspension in RIPA buffer was added, and the samples were rocked for 2 h at 4°C. The protein A-Sepharose complex was recovered by centrifugation, washed with RIPA buffer, and fractionated by electrophoresis on 11% polyacrylamide gels containing sodium dodecyl sulfate (28).

Initially, each of six overlapping VV cosmids (provided by Bernard Moss) was assayed as a source for the proteinase gene by use of P4b as substrate. In spite of ample expression of P4b, processed product was repeatedly not visible following immunoprecipitation (data not shown). In subsequent assays, expression of P25K served as the source for substrate. Proteolytic activity which was apparently specific for the AG*S site of P25K (resulting in the appearance of 25K′) was mapped to cosmids 3 and 21 as shown in Fig. 2. Because these two cosmids

overlap primarily in the *Hin*dIII G region of the VV genome, it was not surprising that a plasmid clone of the *Hin*dIII G region (pBR322.H3G) was also able to rescue processing (Fig. 2, lane 7). The predicted amino acid sequences of the ten open reading frames (ORFs) in this region were examined for the existence of conserved active site sequences common to known proteinases. No obvious active-site sequences were found in common with serine, cysteine, or aspartyl proteinases. Expression of G1L alone (plasmid pG1L) was sufficient to rescue processing (Fig. 2, lane 8), indicating that the G1L protein may function as a proteinase. Using this type of transcriptionally controlled expression, we were able to consistently express precursor protein P25K by transfecting with plasmid carrying the entire L4R ORF preceded by its own authentic, late-promoter sequence. Promoter recognition in this system was almost entirely limited to the plasmid copy of the gene, with very little background expression from the endogenous viral genome (Fig. 2A, lane 11).

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

In the course of a normal infection, P25K, with a predicted molecular mass of 28.5 kDa, is primarily processed into 25K, which is found in mature virions. Lee and Hruby have shown that transient expression of the L4R ORF in VV-infected cells yields P25K precursor and 25K product, as well as a slightly larger product, 25K′, derived from cleavage at a unique AG*S site (20). The appearance of 25K′ as the sole proteolytic product resulting from coexpression of P25K and G1L protein was unexpected on the basis of this previous study. In Fig. 2, appearance of the 26.4-kDa product correlates with expression of the G1L ORF. However, a protein band migrating at

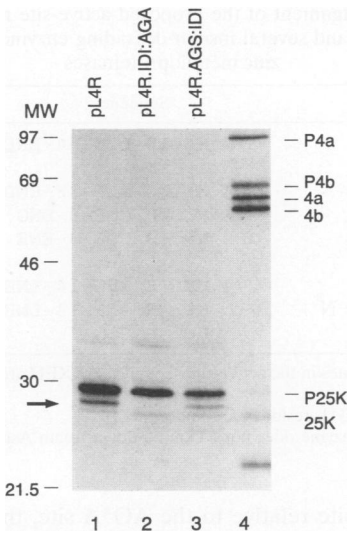

FIG. 3. Inactivation of the P25K cleavage sites. Site-directed mutagenesis was used to prepare pL4R derivatives in which the AGS and AGA codons corresponding to residues 17 to 19 and 31 to 33, respectively, were changed to IDI. Expression of P25K from the resulting pL4R.AGA:IDI and pL4R.AGS:IDI plasmids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

approximately 25 kDa appears in each assay. It is possible that this lower band represents a product derived from cleavage at the AG*A site, which could result from proteolysis mediated by an early protein or from autoproteolysis of P25K, since its appearance is independent of transfected test DNA. Alternatively, the lower band could result from translational initiation from an internal ATG codon within the coding sequence. This seemed plausible since a methionine residue is located just 5 residues upstream of the AG*A scissile bond.

In order to verify that cleavage was occurring at the AG*S site and to determine if the faster-migrating protein was derived from cleavage at AG*A, the amino acids at these sites were altered by site-directed mutagenesis (18). The AG*S and AG*A tripeptide motifs of P25K were independently mutagenized to an IDI tripeptide. The ability of the mutants to be processed during coexpression of G1L is shown in Fig. 3. Mutation of the AG*S site results in no production of the 25K′ product (lane 2), indicating that cleavage of P25K during expression of G1L protein is occurring at this site. On the other hand, mutation of the AG*A site did not abrogate the appearance of the lower band (Fig. 3, lane 3). This would suggest that in the current *trans*-processing assay, P25K is only processed at the AG*S and a low level of translational initiation may be occurring at an internal ATG codon. Since 25K′ is found only in trace amounts during a normal infection, it is unlikely that it plays a role in virus maturation. The absence of this product in mature virions may be due to a decrease in cleavage efficiency

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

Enzyme	Sequence[a]	Reference
G1L[b]	GIA **H**LL**EH** LLI-64-ENE	
Protease III	GLA **H**YL**EH** MLF-38-END	9
dIDE	GLA **H**FC**EH** MLF-8-ENG	19
hIDE	GLS **H**FC**EH** MLF-8-ENE	1
Thermolysin	VVA **HE**LT**H** AVT-14-INE	17
Aminopeptidase N	VIA **HE**LA**H** QWF-13-LNE	24
Collagenase[c]	VAA **HE**LG**H** SLG	11

[a] Constant residues in the active-site sequence HXXEH are shown in boldface type.
[b] The mutated G1L residues are underlined.
[c] Collagenase enzyme does not contain a downstream Asn-Glu pair.

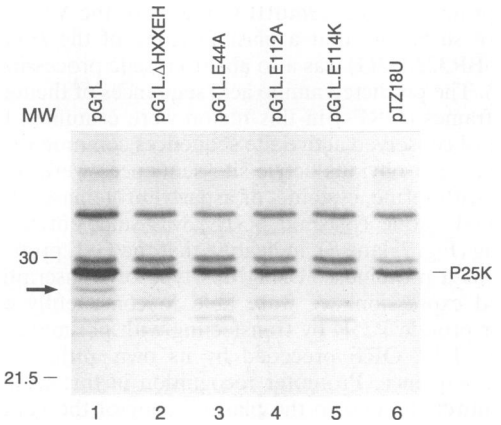

FIG. 4. Genetic inactivation of G1L proteinase. Site-directed mutagenesis was used to prepare the following pG1L mutants: pG1L.ΔHXXEH, pG1L.E44A, pG1L.E112A, and pG1L.E114K. Proteolytic activity of the G1L protein expressed from each mutant was tested in a transcriptionally controlled assay expressing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

at the AG*S site relative to the AG*A site, thereby masking the presence of any 25K′ intermediates. Since virus assembly and subsequent processing of the AG*A site are presumed to be blocked in our assay, 25K′ emerges as the only processed product.

Amino acid alignment of the predicted G1L protein with known viral proteinases revealed no significant primary or secondary structure homology. However, careful examination of the sequence revealed a motif common to a subset of metalloproteinases (Table 1). The mammalian and bacterial insulin-degrading metalloendopeptidases contain the active-site sequence HXXEH, embedded in the extended signature UBUHUUEHZUU (where U signifies uncharged residues, B signifies hydrophobic residues, and Z represents a residue that can be either charged or uncharged) (4), which is an inversion of the active site of other zinc-dependent metalloendopeptidases. An asparagine-glutamate pair (NE) downstream of the active site is conserved in many metalloproteinases such as thermolysin and serves to contribute to zinc binding (29). Likewise, in insulin-degrading enzymes this conserved NE pair is also inverted. As shown in Table 1, this NE pair is conserved in G1L protein in both the forward and reverse orientations.

To determine the importance of the HXXEH motif and NE pair in the activity of the G1L proteinase, site-directed mutagenesis of the G1L ORF was used to alter these regions. The HLLEH pentapeptide region (amino acids 41 through 45) was mutated to SLLED, and expression of the mutated protein from vector pG1L.ΔHXXEH showed a total loss of activity in our assay (Fig. 4, lane 2), suggesting that this region plays a functional role in the protein, similar to that of other metalloproteinases. It is possible, however, that either of the histidine residues in this domain may actually participate in a serine- or cysteine-proteinase catalytic triad. To test this alternate hypothesis, glutamate at residue 44 of wild-type G1L protein was mutated to alanine and expression of the resulting pG1L.E44A clone was assayed for its ability to rescue processing. Again, Fig. 4, lane 3 shows that this mutant was nonfunctional, which implies that the complete HXXEH domain plays a central role in the activity of the proteinase.

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

The results of site-directed mutagenesis suggest that the G1L protein may be classified as a metalloproteinase. Although additional studies are required to determine the role of the G1L proteinase in maturation of the VV structural proteins, this is the first report of a virus-encoded proteinase having similarity to any member of the metalloproteinase family. A recent study by Hijikata et al. identified an apparent zinc-dependent proteinase activity in hepatitis C virus that is inhibited by EDTA (12). Site-directed mutagenesis established the importance of a conserved histidine and cysteine residue, although the primary protein sequence bears no reported similarity or motif common to any known metalloproteinase. The regions of similarity found between G1L protein and other metalloproteinases are shown in Table 1. The conserved HEXXH domain has been identified in many metalloproteinases, most of which show little overall sequence identity to thermolysin outside of this specific domain (29). In the case of G1L protein, site-directed mutagenesis has established the importance of His-41 and His-45, as well as Glu-44, Glu-112, and Glu-114, in the overall activity of the proteinase. It is on the basis of these findings that we propose to class G1L protein as a metalloproteinase. Because of the nature of the assay required to demonstrate activity of the G1L proteinase, we are unable to employ inhibitors such as EDTA or 1,10-phenanthroline.

It has long been recognized that several of the VV late proteins that are incorporated into the virion are derived from larger precursor proteins. A great amount of effort has been expended to elucidate many aspects of this process. However, little progress has been made towards identifying the necessary proteinase or proteolytic activity required for maturation of these proteins. Although an alkaline protease has been reported to be associated with purified virions (2), it is unclear whether it is encoded by the cell or virus and whether it plays a role in proteolytic processing of the core proteins.

Earlier studies have established that although the proteolytic activity responsible for cleavage of P4a and P4b is present within the infected cell, it is not functional or is absent in infected cell extracts or disrupted virions, it is unable to diffuse to a neighboring cell during cell-cell fusions, and it is apparently short-lived (26). Also, translation of VV late mRNA in rabbit reticulocyte lysate results in the production of full-length precursor proteins with no discernible processing (6). Several hypotheses can be presented to explain these results: (i) the proteolytic activity is sequestered to virosomes, which may be tightly associated with cytoskeletal elements of the cell, (ii) substrate precursors must assume a suitable configuration or be preassembled in the maturing particle prior to proteolysis, (iii) the precursor proteins are autoproteolytic, and (iv) the proteinase is simply not present or is inactive. A combination of these hypotheses is also possible. Autoproteolysis seems unlikely, since no significant homology exists between the precursor proteins, in spite of the fact that they are each processed at a very similar AG*X site. On the basis of the assumption that the proteinase does exist, that it (or a regulatory cofactor) is encoded by the virus, and that proteolysis is dependent on contextual elements, it was necessary to design an assay that could be used to map the proteolytic activity to a specific viral gene while either preserving or circumventing these essential contextual requirements.

The role that G1L proteinase plays in core protein processing remains unknown. The relevance of its activity to proteolytic maturation is based on the fact that it processes P25K at a consensus site known to be utilized in five viral precursor proteins. There has previously been no function or homology identified with the product of the G1L ORF, even though a homologous ORF containing exactly the same HXXEH and ENE motifs is conserved in VV strain Copenhagen (10) as well as variola major virus strains Bangladesh (22) and India-1967 (25). Consistent with the precedents of other viral systems (8, 16) and in light of the fact that processing of VV core proteins occurs among a wide range of host cells derived from different tissues and organisms, one would predict that the responsible proteinase is encoded by the viral genome. Because of the apparent complex regulation of processing and the identification of VV conditional-lethal mutants defective in core protein processing and virion morphogenesis, it seems likely that a variety of proteins may influence proteolytic processing. Although additional research will be required to determine the part that G1L proteinase plays in VV replication, the current study suggests that VV may encode its own proteolytic enzyme, which appears to belong to the metalloproteinase family.

We thank Michael Merchlinsky and Bernard Moss for providing late-transcription-factor clones and the VV DNA cosmid library; Judy VanSlyke for providing the polyclonal rabbit antiserum to P4a, P4b, and 25K; and Peiyu Lee for providing plasmid pL4R. We are especially grateful to James Keck and William Dougherty for valuable discussions and to the Central Service Laboratory of the Center for Gene Research and Biotechnology (Oregon State University) for assistance with DNA sequencing and oligonucleotide synthesis.

This work was supported by a research grant (AI-29367) from the National Institutes of Health.

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