JOURNAL OF VIROLOGY, July 1993, p. 4252–4263
0022-538X/93/074252-12$02.00/0

Vol. 67, No. 7

trans Processing of Vaccinia Virus Core Proteins

PEIYU LEE AND DENNIS E. HRUBY*

Center for Gene Research and Biotechnology, Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804

Received 10 December 1992/Accepted 16 April 1993

The three major vaccinia virus (VV) virion proteins (4a, 4b, and 25K) are proteolytically matured from larger precursors (P4a, P4b, and P25K) during virus assembly. Within the precursors, Ala-Gly-X motifs have been noted at the putative processing sites, with cleavage apparently taking place between the Gly and X residues. To identify the sequence and/or structural parameters which are required to define an efficient cleavage site, a *trans*-processing assay system has been developed by tagging the carboxy terminus of the P25K polypeptide (precursor of 25K) with an octapeptide FLAG epitope, which can be specifically recognized by a monoclonal antibody. By using transient expression assays with cells coinfected with VV, the proteolytic processing of the chimeric gene product (P25K:FLAG) was monitored by immunoblotting procedures. The relationship between the P25K:FLAG precursor and the 25K:FLAG cleavage product was established by pulse-chase experiments. The in vivo cleavage of P25K:FLAG was inhibited by the drug rifampin, implying that the reaction was utilizing the same pathway as authentic VV core proteins. Moreover, the 25K:FLAG protein was found in association with mature virions in accord with the notion that cleavage occurs concomitantly with virion assembly. Site-directed mutagenesis of the Ala-Gly-Ala motif at residues 31 to 33 of the P25K:FLAG precursor to Ile-Asp-Ile blocked production of the 25K:FLAG product. The efficiency of 25K:FLAG production (33.71%) is, however, approximately only half of the production of 25K (63.98%) within VV-infected cells transfected with pL4R:FLAG. One explanation for the lower efficiency of 25K:FLAG production was suggested by the observation in the immunofluorescent-staining experiment that 25K:FLAG-related proteins were not specifically localized to the virus assembly factories (virosomes) within VV-infected cells, although virosome localization was prominent for P25K-related polypeptides. Since VV core protein proteolytic processing is believed to take place during virion maturation, only the P25K:FLAG which was assembled into immature virions could undergo proteolytic maturation. Furthermore during these experiments, a potential cleavage intermediate (25K′) of P25K was identified. Amino acid residues 17 to 19 (Ala-Gly-Ser) of the P25K precursor were implicated as the intermediate cleavage site, since no 25K′:FLAG product was produced from a mutant precursor in which the sequence was altered to Ile-Asp-Ile. Taken together, these results provide biochemical and genetic evidence to support the hypothesis that the Ala-Gly-X cleavage motif plays a critical role in VV virion protein proteolytic maturation.

The vaccinia virus (VV) virion is a large complex oval or brick-shaped structure. Five major compartments of the intracellular naked virus particle can be differentiated by the use of chemical and detergent treatments in conjunction with electron-microscopic studies: (i) a biconcave core containing the viral genome and basic viral proteins, (ii) the core wall with bilaminar appearance, (iii) two lateral bodies with unknown functions, (iv) a basement membrane, and (v) a coat layer consisting of both lipids and viral proteins (23). Although the precise sequence of biochemical events which occur during VV morphogenesis is still unclear, the whole process has been divided into a sequence of developmental stages by electron-microscopic analysis (10). The assembly of VV virions is initiated by the production of rigid crescent-shaped bilayer structures covered with spicules on their convex surface. The crescent-shaped structures progress into immature viral particles, which are spheres filled with a uniformly distributed nucleoprotein. With time, the nucleoprotein condenses and the immature particles are converted into maturated infectious intracellular naked virus particles. Virion maturation and proteolytic processing of the major VV structural proteins are coordinately blocked by rifampin treatment (25, 33, 35, 56). Likewise, virion protein processing is blocked at the nonpermissive temperature in several maturation-defective groups of temperature-sensitive mutants (9, 30, 44). These observations suggest that proteolytic cleavage of the VV core proteins occurs in an assembly-dependent manner. In addition to the proteolytic processing of polypeptides to activate different functional domains, as in the RNA viruses (53), proteolytic maturation of viral structural proteins during viral morphogenesis is a common theme which has previously been noted for several different groups of RNA viruses (picornavirus [20], nodavirus [17], retrovirus [2]) and DNA viruses (T4 phage [6] and adenovirus [51]).

Previous studies using tryptic peptide mapping, pulse-chase analyses, monospecific polyclonal antibodies, and N-terminal microsequencing procedures (25, 35, 40, 42, 47, 49, 52, 54, 55) have demonstrated that three major proteins found in VV virions, 4a, 4b and 25K, are the proteolytic cleavage products of higher-molecular-weight precursors, P4a, P4b, and P25K, respectively. Synthesis of viral DNA and polypeptides takes place within the cytoplasm of host cells in a well-regulated temporal fashion (for a review, see reference 34). As might be expected for structural proteins, P4a, P4b, and P25K are all synthesized and proteolytically matured at late times during VV infection. Analysis of the N-terminal sequences of the mature 4b and 25K polypeptides (47, 49, 54) suggests that P4b and P25K are both cleaved at an internal Ala-Gly-Ala motif to remove the

* Corresponding author. Electronic mail address: hrubyd@cgrb.orst.edu.

N-terminal 62 and 31 amino acids, respectively. The identical Ala-Gly-Ala signal is conserved at precisely the same location of the homologous P4b and P25K open reading frames (ORFs) in a distantly related avipoxvirus, fowlpox virus (3, 4). Proteolytic maturation of the VV P4a protein does not follow the same pathway. Rather, at least two smaller nonoverlapping products, 4a and 23K, are derived from the P4a precursor and become virion constituents. N-terminal microsequencing of the 23K polypeptide indicates that it is produced by cleavage of the carboxy-terminal portion of the P4a precursor at the Ala-Gly-Thr site at residues 696 to 698. Peptide mapping experiments suggest that the 4a protein is produced by a second cleavage at the Ala-Gly-Ser site found at residues 613 to 615 of the precursor. The fate of the intervening peptide (residues 615 to 697) is not yet known (47, 49). In any case, analysis of the known cleavage sites of the P4b, P25K, and P4a precursors reveals the consensus sequence, Ala-Gly-X, with cleavage taking place after the Gly residue. No other common sequence or structural elements were evident.

Specific scission of core protein precursors during virion morphogenesis has also been noted for other large DNA-containing viruses such as adenovirus and African swine fever virus. The cleavage of adenovirus core protein precursors is linked to virus maturation and the cleavage sites identified within a conserved Gly-Gly-X motif (32, 51). Likewise, three structural proteins of African swine fever virus, which shares many properties with poxviruses, also are derived from precursors with the proteolytic cleavage taking place between Gly and Ala/Gly in the sequence Gly-Gly-Ala/Gly (32). Obviously, the Ala-Gly-X motif within the VV and fowlpox core protein precursors is quite similar to the Gly-Gly-X sequence, raising the question as to whether these three different viruses may utilize similar proteolytic maturation pathways. Likewise, with particular regard to maturation of the VV core proteins, there are a number of unanswered questions. Is the Ala-Gly-X motif per se sufficient for specifying efficient recognition and cleavage by the proteinase? Are there any other required substrate elements proximal to and/or remote from the Ala-Gly-X motif within the precursor proteins? Is the same proteinase responsible for the processing of all VV major core proteins? Does the cleavage of VV major core proteins occur by an endoproteolytic, exoproteolytic, or combinatorial mechanism? Do the small polypeptides that have been excised from the precursors play any role in VV life cycle? Finally, how many factors, including the proteinase, are involved in the proteolysis machinery for VV core protein proteolytic maturation? Are they virus encoded or provided by the host cells? Addressing these questions requires the establishment of a proteolytic processing assay to facilitate the identification and characterization of the essential *cis* and *trans* factors.

We have previously attempted several different approaches to developing an assay system to study proteolytic processing of VV core proteins: (i) in vitro cleavage assays mixing VV core protein precursors isolated from cells infected with cleavage-deficient temperature-sensitive VV mutants together with extracts from wild-type VV-infected or uninfected cells, (ii) mixing solubilized VV virions with VV core protein precursors made in vivo or in vitro, and (iii) transient expression assays using the hybrid T7/VV system (16) to express various reporter gene constructions containing putative VV core protein cleavage sites. Without exception, no cleavage of the test substrate was observed with any of these systems. This led to our working hypothesis that proteolytic maturation of VV core proteins is contextual, that is, linked directly to virion assembly. The predictions of this hypothesis are that for a VV protein to be cleaved at the Ala-Gly-X motif it must be synthesized late in infection and packaged into the assembling virion and it needs to be associated with the VV core. Thus, any perturbation of the kinetics of synthesis, intracellular targeting, or structure of a VV core protein might be expected to abrogate processing.

Testing this hypothesis requires the development of an assay with which to examine the *cis* and *trans* factors mediating the proteolytic maturation of VV core protein precursors in vivo. To accomplish this goal, several difficulties had to be overcome. First, the genes encoding the major VV core protein precursors are thought to be essential for virus replication. Unfortunately, conditional lethal mutants are presently not available at these loci nor are these genes readily amenable to direct inactivation by gene insertion techniques (45). Second, the VV core proteins are relatively insoluble and difficult to work with in vitro (6a, 23). Third, on the basis of our initial experiments, it appeared that cleavage occurs only within the context of a maturing virion particle. Fourth, the VV core protein precursors are highly expressed at late times during infection, making detection of an exogenously added core protein precursor difficult. To overcome these challenges, a transient expression procedure in which an immunologically marked core protein precursor is expressed at late times within the VV-infected cell has been employed.

In this report, a *trans*-processing assay of VV core protein proteolytic maturation has been developed by tagging VV core protein precursor P25K at the C terminus with an octapeptide epitope, FLAG. The L4R gene was chosen as the target for these studies because it is the smallest of the three major core protein precursor genes, thus facilitating genetic manipulations, and because the L4R gene product, the P25K protein, is relatively soluble and therefore more amenable to biochemical analyses. We describe the expression of the chimeric P25K:FLAG polypeptide by transient expression in VV-infected cells and identification of the proteolytic cleavage products of P25K:FLAG. The effect of rifampin on the proteolytic processing of P25K:FLAG was also applied to validate that the proteolysis of P25K:FLAG is mediated by the same proteolytic machinery as that used in authentic VV core protein processing pathways. The distribution of FLAG-tagged P25K-related polypeptides within VV-infected cells and the ability of the transiently expressed P25K:FLAG-derived products (25K:FLAG), like 25K itself, to be localized into mature VV virions were examined. We also investigated the importance of the cleavage Ala-Gly-X motifs for P25K proteolytic maturation by site-directed mutagenesis. Development of this assay and confirmation that authentic cleavage reactions are occurring should now allow the roles of the Ala-Gly-X motif and the surrounding amino acid sequence in substrate specificity of VV core protein proteolytic processing to be investigated and provide an approach for identifying the responsible proteinase(s).

MATERIALS AND METHODS

Cells and virus. BSC-40 (African green monkey kidney) cells were maintained in modified Eagle's medium (MEM-E; Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker M. A. Bioproducts, Inc., Walkersville, Md.), 2 mM glutamine, and 10 μg of gentamicin per ml. Purification of VV (WR strain)

from infected BSC-40 cells by two cycles of sucrose gradient centrifugation was performed as described previously (22).

Plasmid construction and site-directed mutagenesis. To generate pL4R, an *Aha*III fragment (1.3 kb) containing the L4R promoter region and the entire L4R ORF, which encodes the P25K polypeptide, was isolated from the *Hin*dIII L fragment of the VV genome. Both ends of the fragment were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and cloned into the *Sma*I site of pUC119. Site-directed mutagenesis of pUC119:L4R to generate pL4R:FLAG containing a chimeric ORF encoding the P25K polypeptide with a FLAG epitope (21) was performed as described by Kunkel (28). An oligonucleotide 48-mer, PL30, (5′-AAGTATAGATTAGGCTACTTGTCGT CATCGTCTTTATAATCCTTTGTC-3′) was used to introduce the following seven-amino-acid sequence behind the last amino acid, Asp, of the P25K polypeptide: Tyr-Lys-Asp-Asp-Asp-Asp-Lys. Together, these eight amino acids created the FLAG epitope attached to the carboxy terminus of P25K. A stop codon was engineered to terminate the translation of the chimeric polypeptide immediately after the FLAG epitope. Oligonucleotide PL47, a 33-mer (5′-AAATT TGGATTTAATGTCTATAATAACCATTTG-3′), was synthesized for the mutagenesis of amino acids 31 to 33 of P25K from Ala-Gly-Ala to Ile-Asp-Ile, and oligonucleotide PL32, a 24-mer (5′-CTCAGATATGATATCGATAAAAAA-3′), was synthesized for altering amino acids 17 to 19 from Ala-Gly-Ser to Ile-Asp-Ile. All mutagenesis procedures were confirmed by dideoxynucleotide DNA-sequencing procedures (41).

Transient expression. BSC-40 cells were infected with VV at a multiplicity of infection of 10 infectious particles per cell and transfected with plasmid or salmon sperm DNA at 0 h postinfection (p.i.) by a liposome-mediated transfection protocol (39). Briefly, BSC-40 cells were allowed to grow to about 90% confluency in plates (60 by 15 mm). To prepare the liposome, 1.0 mg of L-α-phosphatidylethanolamine (dioleoyl) (Sigma) and 0.4 mg of dimethyl dioctadecyl ammonium bromide (Sigma) in chloroform were mixed and blown to dryness with a stream of nitrogen gas. The lipids were then resuspended in 1 ml of sterile H_2O and sonicated with Branson sonifier 250 (VWR Scientific, Media, Pa.) (Microprobe; 50 V) on ice for about 5 to 10 min until the solution was almost clear. Before transfection, 1 ml of MEM-E was mixed with 30 μl of liposome and 5 μg of plasmid DNA. The DNA mixtures were incubated at room temperature for 10 min before being added to the VV-infected cells. After 4 h of incubation at 37°C, the DNA-containing medium was replaced with fresh MEM-E containing 5% fetal bovine serum, and the incubation was continued as indicated. When blockage of the proteolytic processing in VV-infected cells was required, 100 μg of rifampin (Sigma) per ml was added to the medium at 0 h p.i.

Immunoblotting. Infected cells were harvested, washed in phosphate-buffered saline (PBS) (pH 7.0), and lysed by freezing and thawing several times. Immunoblot analyses were performed as described by Van Slyke et al. (47). Briefly, cell lysates were heated at 100°C for 5 min in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (50 mM Tris [pH 6.8], 1% [wt/vol] SDS, 0.1% [vol/vol] 2-mercaptoethanol, 1% [vol/vol] glycerol) (29). The proteins were separated on SDS–13% polyacrylamide gels and electrotransferred to nitrocellulose filters in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) (46) for 40 min at 25 V at 4°C. The filters were washed once in Tris-buffered saline (TBS) (20 mM Tris and 500 mM NaCl, pH 7.5) and blocked with 3% (wt/vol) gelatin (Bio-Rad, Richmond, Calif.) in TBS at room temperature for 2 h. The filters were washed three times with TTBS (0.05% Tween-20 in TBS) and then subjected to binding with anti-FLAG antibody M2 (International Biotechnologies, Inc., New Haven, Conn.) (dilution, 1:155) in antibody buffer (1%-gelatin-containing TTBS) at room temperature overnight. After three washes in TTBS buffer followed by one in TBS, the filters were hybridized with an alkaline phosphatase-conjugated goat anti-mouse antibody (dilution, 1:2,000) (Bio-Rad) in antibody buffer for 2 h and washed with TTBS and TBS. The immunoblots were developed in *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Bio-Rad) in carbonate buffer (0.1 M $NaHCO_3$ and 1.0 mM $MgCl_2$, pH 9.8).

Metabolic labeling and immunoprecipitation. VV-infected cells were transfected with plasmid DNA as described above and pulse-labeled with 150 μCi of ^{35}S protein labeling mix (11.04 mCi/ml [1,094.4 Ci/mmol]; New England Nuclear, Wilmington, Del.) for 30 min at 4 h p.i. After 0 or 20 h of "chase" incubation in MEM-E containing 100-fold excess unlabeled methionine, cells were harvested, lysed in 2% SDS solution at 65°C, passed through a 25-gauge needle several times, and immunoprecipitated as described previously (33). Briefly, the cell extracts were diluted 10-fold in a radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% deoxycholate, 1.0% Nonidet P-40, 0.1% SDS) and incubated with 5 μl of anti-25K polyclonal antibody (47a) for 1 h on ice. Two hundred microliters of a 10% (vol/vol) solution of protein A-Sepharose CL-4B beads (Sigma) in the radioimmunoprecipitation assay buffer was then added, and the incubation was continued overnight at 4°C with continuous rocking. The immune complexes were centrifuged for 20 s in the microcentrifuge, and the beads were washed three times with the radioimmunoprecipitation assay buffer. Polypeptides were released from the beads by heating at 100°C for 5 min in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer and resolved on SDS–13% polyacrylamide gels. Gels were then processed for fluorography by using 22.2% (wt/vol) PPO (2,5-diphenyloxazole) in dimethyl sulfoxide, dried, and stored at −70°C while exposed to Kodak XAR-5 X-ray film. Exposures varied from 3 days to 3 weeks. For preparation of continuously ^{35}S-labeled cell extracts, 75 μCi of ^{35}S protein labeling mix per plate was used. To quantitate the radioactivity present in each band, dried gels were scanned and analyzed in the AMBiS radioanalytic imaging system (AMBiS Systems, Inc., San Diego, Calif.).

Immunofluorescent staining. BSC-40 cells were grown on coverslips, infected with VV at a multiplicity of infection of 5 PFU per cell, and transfected with 5 μg of DNA. Cells were washed twice with Dulbecco's PBS (PBS-D), fixed, and permeabilized in cold methanol for 8 min at 5.5 h p.i. The cells were then reacted with P25K specific antiserum (1:200) or with FLAG-specific M2 monoclonal antibody (1:20) diluted in 10% (vol/vol) normal goat serum (Miles Laboratories, Inc., Naperville, Ill.) (in PBS-D) for 30 min. Following two washes with PBS-D, the cells were incubated in a 1:100 dilution of either fluorescein isothiocyanate-conjugated goat anti-rabbit antibody or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Zymed Laboratories, Inc., South San Francisco, Calif.) for 30 min. After incubation with the secondary antibody, the coverslips were washed with PBS-D twice and counterstained with 0.01% Evans blue (Direct Blue 53; Sigma) (in PBS-D) for 1

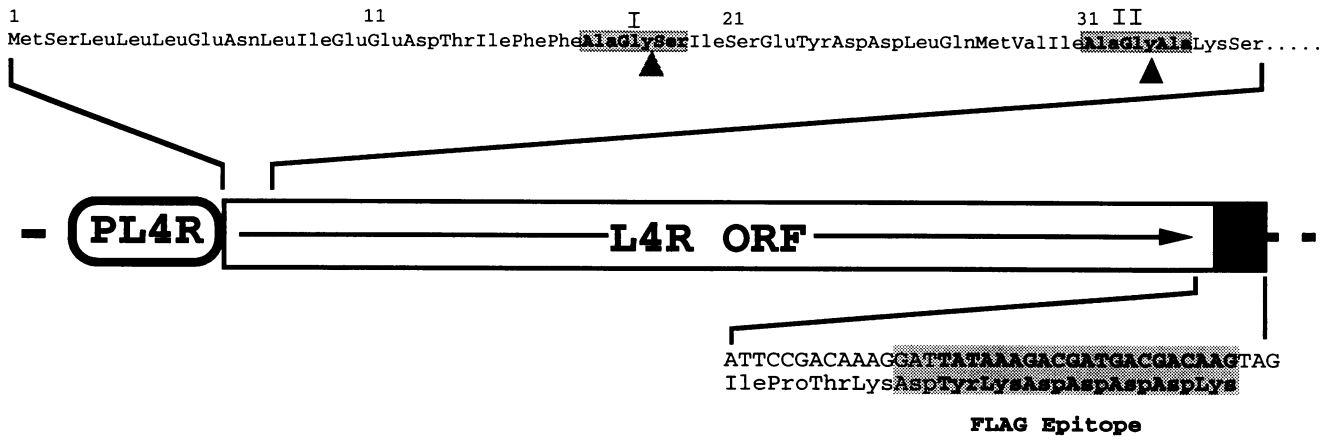

FIG. 1. Structure of the pL4R:FLAG expression vector plasmid. The pL4R:FLAG plasmid containing the VV L4R ORF (encoding the P25K core protein precursor) driven by its cognate promoter (PL4R), with the sequences encoding the FLAG epitope fused in-frame onto the 3′ end, is shown schematically. The N-terminal 35 amino acids of the P25K protein are shown at the top, with the two potential cleavage consensus sites shaded. The known cleavage point between residues 32 and 33 in site II and the potential cleavage point between residues 18 and 19 in site I are indicated (black and gray triangles, respectively). The eight codons encoding the FLAG epitope are shown at the bottom (shaded box). The seven codons shown in boldface were fused by oligonucleotide-directed mutagenesis onto the natural carboxy terminus of the L4R ORF (Asp codon).

min. The coverslips were mounted with 8 μl of DABCO mounting fluid [25% (wt/vol) 1,4-diazabicyclo(2,2,2)octane (DABCO; Sigma), 50% (vol/vol) glycerol, 0.25× PBS, and 0.1% sodium azide, pH 8.6] after two final washes in PBS-D. Observations and photography were performed with a Zeiss fluorescence microscope.

RESULTS

Design and construction of the pL4R:FLAG expression vector plasmid. The pL4R:FLAG expression plasmid that was assembled for these experiments is diagrammed in Fig. 1. The VV L4R gene, encoding the core protein precursor P25K, abutted to its own late promoter element, was cloned into the pUC119 plasmid. This plasmid was used because it contains the bacteriophage M13 replication origin for the synthesis of single-stranded DNA template to facilitate subsequent mutagenesis procedures. Site-directed mutagenesis techniques were then used to create an in-frame fusion at the 3′ end of the L4R ORF such that an octapeptide epitope, designated FLAG (38), would be appended to the carboxy terminus of the P25K protein. This epitope was chosen because its small size would be expected to produce only minimal perturbations of the overall structure and subcellular localization of the P25K precursor protein. Likewise, the epitope was appended to the carboxy terminus to remove it as far from the cleavage site at the amino terminus as possible and to allow both the precursor and the cleaved product to be recognized. The experimental rationale for this construction was that the pL4R:FLAG plasmid could be efficiently introduced into VV-infected cells by using liposome-mediated procedures. During the late phase of VV gene expression, soluble VV RNA polymerase would transcribe the pL4R:FLAG gene product from its natural promoter with normal expression kinetics. The pL4R:FLAG-derived transcript would be translated into a P25K:FLAG precursor, which should be subject to any essential post-translational modifications. Assuming that the tertiary structure and/or targeting of the precursor has not been altered by addition of the FLAG epitope, the P25K:FLAG protein should be incorporated into assembling virions and cleaved by the same proteolytic machinery as authentic P25K during viral assembly. We should be able to distinguish the P25K:FLAG precursor and the 25K:FLAG product from the genomically encoded 25K product by using the M2 monoclonal antibody directed against the FLAG epitope.

***trans* processing of P25K:FLAG in VV-infected cells.** To determine whether P25K:FLAG is expressed and efficiently processed in vivo, the expression vector pL4R:FLAG was introduced into VV-infected BSC-40 cells by liposome-mediated transfection. Twenty hours after transfection, total cell extracts were prepared from the infected cells and subjected to immunoblot analysis using either rabbit anti-25K antiserum or mouse anti-FLAG M2 monoclonal antibody. As a negative control, salmon sperm DNA was transfected into VV-infected cells. As indicated in Fig. 2, two polypeptides (P25K and 25K) were identified by the anti-25K antiserum in VV-infected cells receiving only salmon sperm DNA (lane 2). Two additional polypeptides in VV-infected cells transfected with pL4R:FLAG DNA were observed (lane 1). To demonstrate that the two new polypeptides were derived from the plasmid-encoded chimeric L4R:FLAG gene, anti-FLAG M2 antibody was utilized to analyze a duplicate set of the same extracts. None of the proteins present in the salmon sperm DNA-transfected VV-infected cells reacted with this serum (Fig. 2, lane 3), whereas three polypeptides from pL4R:FLAG-transfected cells (Fig. 2, lane 4) were identified by anti-FLAG M2 antibody. The largest and smallest of the three polypeptides comigrated with the two unique bands identified by the anti-25K antibody in the pL4R:FLAG transfected cell extract (Fig. 2, lane 1). The predicted molecular masses of P25K:FLAG and 25K:FLAG are 29.3 and 25.8 kDa, respectively, agreeing well with the apparent molecular weights of the upper and lower proteins recognized by the M2 antibody. Furthermore, an N terminally truncated P25K:FLAG derivative, when transiently expressed from a construct encoding 25K:FLAG with an extra Met residue for translation initiation at the N terminus, exactly comigrates with the lower protein band (data not shown). We therefore provisionally designated the upper band as the P25K:FLAG precursor and

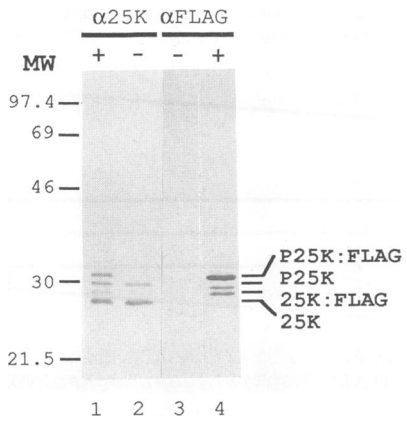

FIG. 2. Proteolytic maturation of the P25K:FLAG precursor protein in infected cells. Monolayers of BSC-40 cells were transfected with sheared salmon sperm DNA (−) or pL4R:FLAG DNA (+) following infection with wild-type VV. After 24 h of incubation, infected cell lysates were prepared and subjected to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

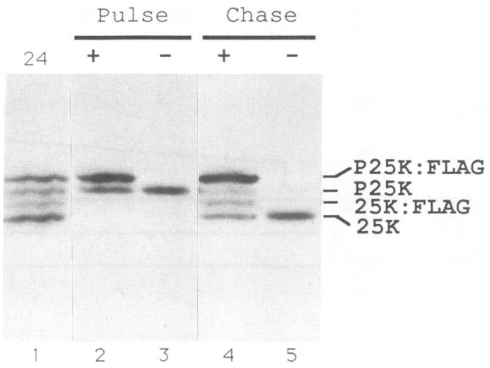

FIG. 3. Pulse-chase analysis of P25K:FLAG protein maturation in vivo. Monolayers of BSC-40 cells were transfected with sheared salmon sperm DNA (−) or pL4R:FLAG DNA (+) following infection with wild-type VV. At 4 h p.i., the cells were incubated for 30 min with a ^{35}S-labeled mixture of methionine and cysteine. Lysates were prepared either immediately (pulse) or after an additional 20 h of incubation in the presence of 100-fold excess unlabeled methionine and cysteine (chase). The continuously labeled cell extract (lane 1) was prepared from cells transfected with pL4R:FLAG DNA after incubation from 4 to 24 h p.i. in ^{35}S-labeled Met + Cys. P25K-related proteins were immunoprecipitated with monospecific anti-25K serum and analyzed by polyacrylamide gel electrophoresis and fluorography. The positions of the 25K and 25K:FLAG precursors and derived products are indicated on the right.

the lower band as the 25K:FLAG cleavage product (Fig. 2, lane 4). N-terminal microsequencing of the 25K:FLAG cleavage product substantiated this conclusion (data not shown). The intermediate-size protein which was detected with the anti-FLAG antibody (Fig. 2, lane 4) but not with anti-25K serum (Fig. 2, lane 1), presumably because of comigration with P25K, was designated 25K′:FLAG. The production of 25K′:FLAG will be investigated in the following sections.

25K:FLAG is a product of P25K:FLAG. To investigate whether a precursor-product relationship exists between P25K:FLAG and 25K:FLAG, VV-infected cells were transfected with pL4R:FLAG DNA, pulse-labeled with ^{35}S labeling mix for 30 min, and incubated in the presence of excess unlabeled methionine for 20 h. FLAG-specific monoclonal antibody did not react well with FLAG-tagged polypeptides in immunoprecipitation experiments; therefore, the immunoprecipitation analysis was performed with 25K-specific antiserum. Cytoplasmic extracts were prepared and incubated with anti-25K antiserum, and the immunoreactive proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography (Fig. 3). In the salmon sperm DNA-transfected cell extracts, P25K was pulse-labeled (Fig. 3, lane 3) and the label was completely chased into 25K (Fig. 3, lane 5) during the incubation, as expected. In the pL4R:FLAG-transfected extracts, in addition to P25K, P25K:FLAG was labeled during the pulse period (Fig. 3, lane 2) and chased into 25K:FLAG during the subsequent incubation (Fig. 3, lane 4). The efficiency of P25K:FLAG-to-25K:FLAG cleavage appeared to be reduced compared with the processing of virus-encoded precursor P25K into 25K (which also did not proceed to completion in the presence of P25K:FLAG expression).

To determine the efficiency of the cleavage of P25K:FLAG to 25K:FLAG, VV-infected cells were labeled with ^{35}S protein labeling mix from 4 to 24 h p.i. during pL4R:FLAG transient expression. Following immunoprecipitation with 25K-specific antiserum and SDS-polyacrylamide gel electrophoresis separation, the radiolabeled polypeptides were visualized by autoradiography (Fig. 3, lane 1). AMBiS radioanalytic imaging system was utilized to measure the radioactivity (counts per minute) of each band with the same gel. The radioactivity of each P25K-derived polypeptide was divided by the total counts of P25K-related polypeptides to calculate the percentage of each polypeptide in its group. Identical calculations were carried out for P25K:FLAG and its derivatives. After 24 h, 63.98% of the P25K synthesized had been proteolytically matured into 25K (Fig. 3, lane 1, and Table 1) in pL4R:FLAG-transfected cells. This could be contrasted with an 83.2% conversion of P25K into 25K in salmon sperm DNA-transfected cells (data not shown). In contrast, only 33.7% of P25K:FLAG was processed into 25K:FLAG. Since the intermediate product 25K′:FLAG, which migrates at a position between P25K and 25K:FLAG in SDS-polyacrylamide gel electrophoresis (as seen in Fig. 2), was not detected by 25K-specific antiserum, the percentage of 25K:FLAG (33.7%) represents an overestimate, since the radioactivity of 25K′:FLAG was not integrated into the analysis. These results suggest that P25K:FLAG is efficiently synthesized during transient expression of pL4R:FLAG in VV-infected cells, but the subsequent conversion to 25K:FLAG proceeds less efficiently than the authentic reaction. However, for the purpose of a proteolytic process-

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

Precursor or product	cpm	%
P25K	25,375	36.02
P25K:FLAG	37,489	66.29
25K	45,072	63.98
25K:FLAG	19,066	33.71

ing assay to use for the dissection of the important elements for defining a specific scissile peptide bond in the polypeptide substrate, this amount of processing product should be more than sufficient. Therefore, we went on to confirm the authenticity of the cleavages in the P25K:FLAG precursor.

P25K:FLAG requires the AG/X motif for cleavage. The appearance of the 25K′:FLAG intermediate noted in Western blot (immunoblot) analysis using FLAG-specific M2 antibody (Fig. 2) was unexpected on the basis of previous studies of P25K processing in vivo (47, 52). Three possible explanations for the appearance of the additional polypeptide were considered. First, the intermediate band could result from translational initiation from an internal ATG codon within the L4R ORF. This possibility was considered unlikely, as the second and third methionines of the L4R ORF are located at residues 27 and 40, respectively, and neither is located in a context likely to facilitate translation (26). Second, premature termination of translation could give rise to the 25K′:FLAG product. This possibility was highly unlikely, as sequence analysis of the pL4R:FLAG insert revealed no mutations. Furthermore, premature termination would result in a protein lacking the FLAG epitope that would not be recognized by the M2 antibody. Third, 25K′:FLAG may represent a proteolytic processing intermediate in which cleavage has occurred at a site other than Ala-Gly-Ala at residues 31 to 33 of P25K. The motifs Ala-Gly-Thr and Ala-Gly-Ser have been suggested as functional cleavage sites which are recognized during maturation of the core protein precursor P4a (49). Examination of the predicted amino acid sequence of the N terminus of P25K revealed that the sequence Ala-Gly-Ser was located between amino acid residues 17 to 19 (site I in Fig. 1). Since cleavage at site I would produce a product with a predicted molecular weight intermediate between those of P25K:FLAG and 25K:FLAG, we considered this to be the most likely possibility.

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

Rifampin inhibits processing of the P25K:FLAG protein. To determine whether maturation of P25K:FLAG into 25K:FLAG uses the same proteolytic machinery that is required for the processing of P25K into 25K, we took advantage of the well-known observation that the proteolytic maturation of VV major core proteins and VV virion assembly can both be blocked by rifampin (35, 36). VV-infected cells which

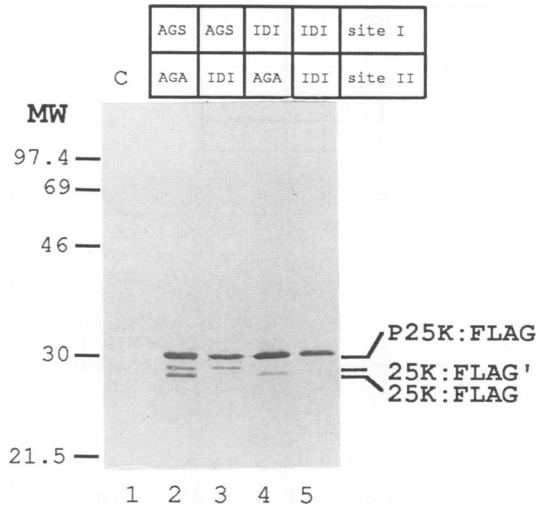

FIG. 4. Genetic inactivation of P25K:FLAG cleavage sites. Site-directed mutagenesis procedures were used to prepare pL4R:FLAG derivatives in which the A-G-S and A-G-A codons corresponding to sites I (residues 17 to 19) and II (residues 31 to 33) were converted to I-D-I. Monolayers of BSC-40 cells were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

were transfected with either salmon sperm DNA or pL4R:FLAG DNA were incubated in the presence or absence of rifampin from 0 to 13 h p.i. As demonstrated in Fig. 5, immunoblot analyses of the transient expression lysates using both 25K-specific antiserum and anti-FLAG M2 antibody indicated that, like P25K and 25K (lane 1), the P25K:FLAG precursor and 25K:FLAG product are both present in the absence of rifampin (lane 4). The production of 25K is significantly reduced in the presence of rifampin for 24 h (Fig. 5, lane 2), and the maturation of the 25K:FLAG product was likewise blocked to a level which was not detectable by Western blot analysis under the same condition (Fig. 5, lane 3). These results suggested that cleavage of site II of the P25K:FLAG precursor to produce 25K:FLAG likely uses the same pathway as maturation of the authentic P25K protein.

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

25K:FLAG localizes in the virion. Sarov and Joklik (42) have previously shown that the mature 25K protein is exclusively found associated with the core of mature VV

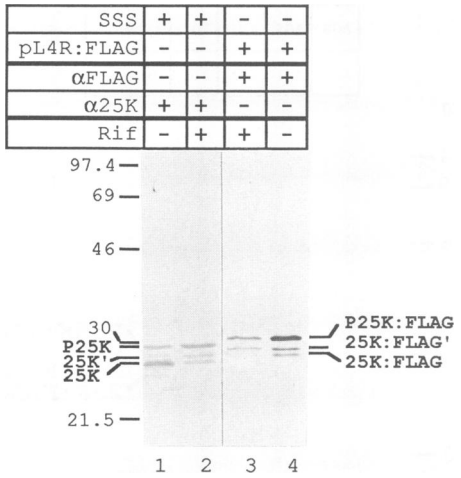

FIG. 5. Effect of rifampin on P25K:FLAG maturation. Monolayers of BSC-40 cells were infected with wild-type VV and transfected with sheared salmon sperm (SSS) DNA or pL4R:FLAG DNA in the presence (+) or absence (−) of rifampin (Rif) (100 μg/ml). Extracts were prepared at 13 h p.i. and subjected to SDS-polyacrylamide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

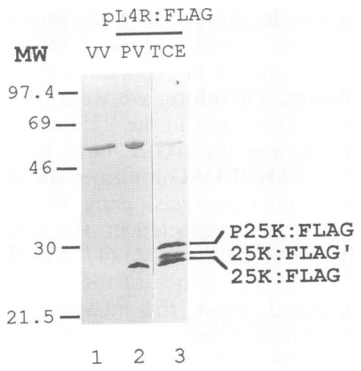

FIG. 6. Virion association of the mature 25K:FLAG protein. Monolayers of BSC-40 cells were infected with wild-type VV and transfected with sheared salmon sperm DNA (VV) or pL4R:FLAG DNA. After 24 h of infection, mature virus particles (PV) were purified from the infected cell lysate (TCE) by differential centrifugation and velocity sedimentation on sucrose gradients (22). The indicated samples were analyzed by polyacrylamide gel electrophoresis and immunoblot analysis using anti-FLAG monoclonal antibody. The positions of the 25K:FLAG precursor and derived products are indicated on the right. The positions and sizes (in thousands) of molecular weight (MW) standards are indicated on the left.

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

Transiently expressed polypeptides are distributed throughout the cytoplasm of VV-infected cells. As noted previously, although 25K:FLAG was cleaved from the P25K:FLAG precursor, the efficiency of the reaction was reduced compared with the authentic reaction. Several explanations for this observation were considered. One hypothesis is that a small degree of disruption in secondary and/or tertiary structure in the P25K polypeptide was introduced by the addition of the eight-amino-acid FLAG epitope at the C terminus, which resulted in a kinetic difference of proteinase-substrate interaction. An alternative explanation is that P25K:FLAG might display a localization pattern different from that of the authentic P25K precursor expressed from viral genomic DNA, which has been shown to be localized specifically in virosomes, where virus assembly takes place. The alteration in localization could result from either structural disruption of the P25K polypeptide discussed above or from distinct sites of gene expression from transfected DNA in VV-infected cells. Consequently, the FLAG-tagged P25K precursor was not efficiently incorporated into immature virions. To explore the latter hypothesis, immunofluorescent staining was used to study the localization of P25K:FLAG and P25K-related proteins.

VV-infected BSC-40 cells were transfected with salmon sperm DNA, pL4R, or pL4R:FLAG. After 5.5 h of infection, the cells were incubated with a primary antibody directed against either 25K or the FLAG epitope, and the incubation was followed by an appropriate fluorescein isothiocyanate-conjugated secondary antibody. In cells transfected with salmon sperm DNA, P25K-related polypeptides localized specifically to the structures in the vicinity of host cell nuclei, most probably virosomes (Fig. 7d). These structures were not stained with preimmune antiserum (Fig. 7a). When pL4R:FLAG-transfected cells were examined, prominent staining was evident throughout the entire cytoplasmic compartment by using FLAG-specific antibody (Fig. 7c) or 25K-specific antiserum (which also highlighted virosomes as described above in all infected cells) (Fig. 7f). No staining was observed by using anti-FLAG antibody in VV-infected cells transfected with pL4R (Fig. 7b), demonstrating the specificity of anti-FLAG antibody recognition of transiently expressed FLAG-tagged polypeptides. Taken together, these results suggest that P25K:FLAG-related proteins are not concentrated around virosomes but rather are distributed throughout the cytoplasm of the infected cells. Since both transiently expressed P25K and P25K:FLAG proteins are localized throughout the cytoplasm of pL4R- and pL4R:FLAG-transfected cells, respectively, as detected by 25K-specific antiserum (Fig. 7e and f), this implies that it is not the FLAG epitope per se which is responsible for inhibiting normal localization of P25K-related proteins. Rather, it appears that when expressed from a transfected plasmid, P25K-related proteins are inefficiently concentrated to the virosome.

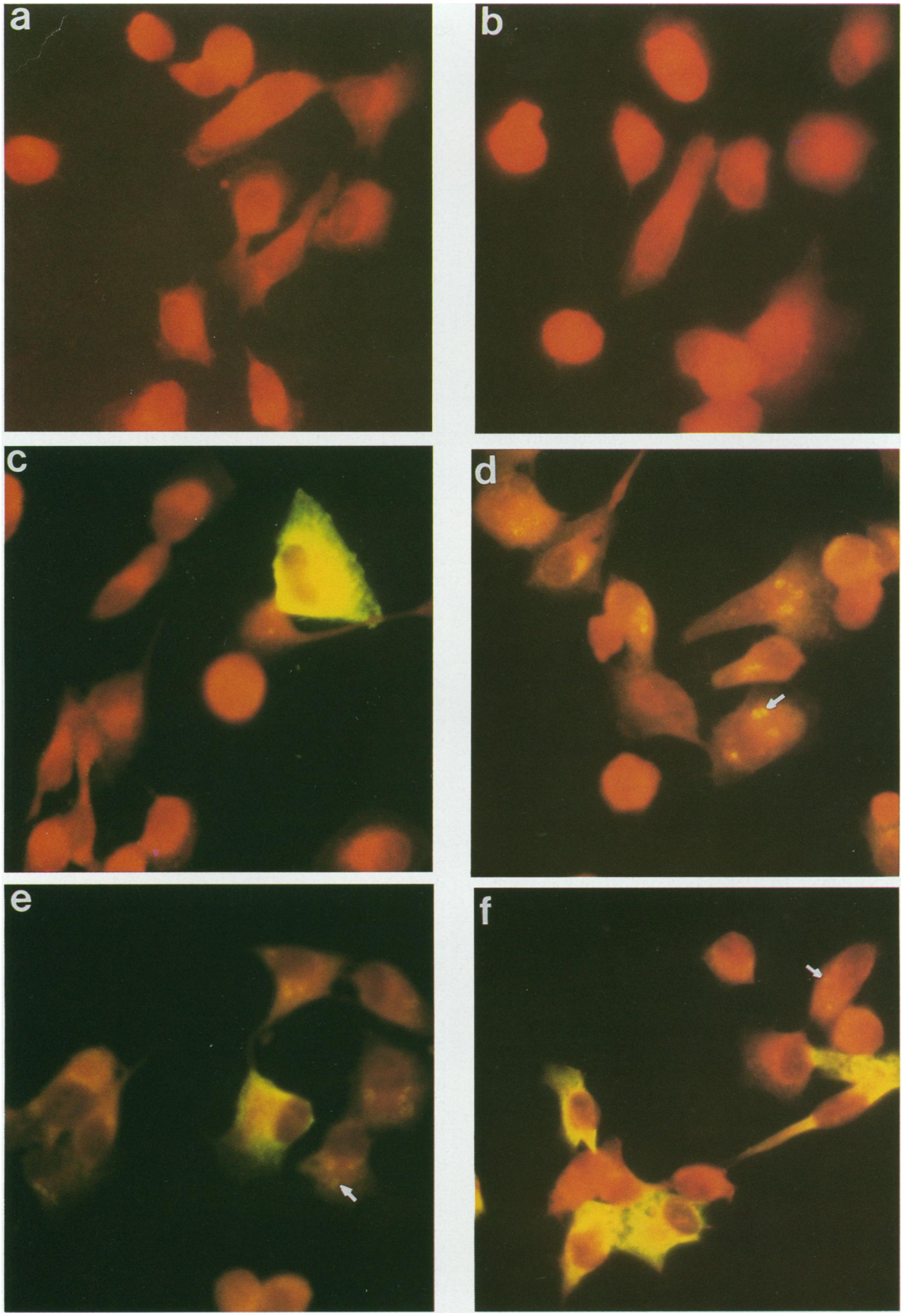

FIG. 7. Immunofluorescent localization of P25K- and P25K:FLAG-related proteins. VV-infected BSC-40 cells were transfected with sheared salmon sperm DNA (a and d), pL4R (b and e), or pL4R:FLAG (c and f) DNA. After 5.5 h of infection, indirect immunofluorescent staining was performed with 25K-specific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

DISCUSSION

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

As shown in Table 1, when pL4R:FLAG was transiently expressed in VV-infected cells, the efficiency of the production of 25K:FLAG (33.71%) from the P25K:FLAG precursor was lower than P25K-to-25K maturation (63.98%). The cytoplasmic distribution of P25K:FLAG and its derivatives shown by fluorescent staining (Fig. 7) and the localization of 25K:FLAG product in mature VV virions (Fig. 6) suggested that only a portion of the P25K:FLAG precursor was assembled into immature virions during VV virion assembly. The subsequent proteolytic cleavage carried out in virions during morphogenesis led to the production of 25K:FLAG. In other words, the P25K:FLAG precursor is likely to be processed authentically into 25K:FLAG only when incorporated into VV immature virions. The P25K:FLAG precursor was not completely processed because only a portion of the precursor protein was targeted to virosomes, where VV virion assembly takes place. We also noted that the proteolytic maturation of 25K (63.98%) from P25K in VV-infected cells receiving pL4R:FLAG was somewhat diminished in comparison with the percentage of P25K cleavage (83.20%) in VV-infected cells receiving salmon sperm DNA. This observation correlates well with the results of the pulse-chase experiment, in which ^{35}S-labeled P25K was completely chased into 25K when P25K:FLAG was transiently expressed (Fig. 3, lane 4) whereas under the same conditions pulse-labeled P25K was completely processed into the 25K product in cells receiving salmon sperm DNA (Fig. 3, lane 5). It is possible that the reduction in 25K production is due to the presence in VV-infected cells of P25K:FLAG, which competes with P25K for incorporation into assembling virions and/or the limited proteolytic machinery for VV core protein proteolysis. On the basis of the inhibition noted above, it may be feasible to design peptide inhibitors which could compete with and therefore block the proteolysis of VV core proteins as well as virus maturation.

The production of the 25K′:FLAG protein from the P25K:FLAG precursor was not anticipated. However, several lines of evidence suggest that it may be a biologically relevant cleavage product. First, the site I motif (Ala-Gly-Ser), which is suspected to give rise to 25K′:FLAG, is implicated as an active cleavage site used during the proteolytic maturation of another VV core protein, P4a (48). Second, mutagenesis of the Ala-Gly-Ser to Ile-Asp-Ile totally abolished the production of the 25K′:FLAG product without affecting either the synthesis of the precursor or processing at site II, located 12 to 14 residues downstream. Third, a polypeptide with a molecular mass intermediate between those of P25K and 25K (Fig. 5, lane 2) is detected within VV-infected cells in the presence of rifampin. This polypeptide was designated 25K′ on the basis of its similar mobility relative to P25K and 25K compared with that of 25K′:FLAG relative to P25K:FLAG and 25K:FLAG, although the identity of 25K′ still needs to be verified. The facts that less 25K′:FLAG is present than 25K:FLAG during transient expression of the pL4R:FLAG plasmid and that the P4a core protein precursor, containing the Ala-Gly-Ser motif, is more slowly processed than is P4b, which contains the Ala-Gly-Ala motif (47), raises the possibility that the two motifs are processed at different rates. This may have functional significance during the viral replication cycle. Differential rates of cleavage of similar, but not identical, cleavage sites have previously been suggested to play a potential regulatory role in several RNA virus systems including potyviruses (12), picornaviruses (31), and alphaviruses (11). Thus, it is possible that 25K and 25K′ are both produced during the course of a normal infection and that they play different roles during the replication cycle.

Previous experiments using N-terminal microsequencing of mature VV core proteins and computer analyses of the predicted amino acid sequences of the core protein precursors have implicated Ala-Gly-Ala and Ala-Gly-Ser/Thr as the conserved sites of cleavage in the P25K (and P4b) and P4a precursors, respectively (47, 49, 54). On the basis of the derived sequences of the processed products, cleavage was predicted to occur between the second and third positions of this motif (AG↓X). However, no evidence was available to address the following questions. Is the AGX motif the actual site of cleavage? Does the cleavage process require exclusively endoproteolytic cuts like many viral systems (27), or, as with neuropeptide maturation (19, 24), are VV core proteins matured by a combination of endo- and exoproteolytic enzymes? Are different permutations of the AGX motif (Ala-Gly-Ala versus Ala-Gly-Ser) recognized by the same or different enzymes? Some of the data reported here have provided some insights into the answers to these questions. First, it was shown that mutagenesis of either site I (Ala-Gly-Ser) or site II (Ala-Gly-Ala) resulted in completely blocking the production of 25K′:FLAG and 25K:FLAG, respectively. This result suggests that the AGX motif is the site of cleavage and that the reaction proceeds by an endoproteolytic mechanism. An alternative explanation of these results is that the introduced mutations disrupted the secondary structure of the precursor protein. We consider this unlikely, as mutations of the two sites behaved independently. Although quantification in Western blot analysis was not feasible, mutation of site I appeared to reduce cleavage at site II only slightly. Likewise, mutation of site II did not interfere with cleavage at site I (Fig. 4). Conclusive proof that maturation proceeds by an endoproteolytic cut at

the AGX motif will require the isolation and identification of the N-terminal peptides which are predicted to be liberated during this reaction. Second, the possibility that the proteolytic reaction at site I is catalyzed by a different enzyme or perhaps within a different compartment within the infected cell was suggested by the following results: (i) the 25K′:FLAG product was not found in the purified mature virions (Fig. 6), (ii) 25K′ was produced in the presence of rifampin, and (iii) the production of 25K′:FLAG was not blocked by rifampin (Fig. 5). Previous studies (50) have demonstrated that a small amount of the P25K precursor was incorporated into immature virion particles but the proteolytic processing was blocked in the presence of rifampin. The inhibition of proteolysis of the P25K precursor to the 25K product at site II in the presence of rifampin may interrupt the incorporation of P25K precursors into the immature particles. Since the synthesis of late gene products is not blocked by rifampin, the concentration of unincorporated P25K would be expected to increase, and this increase could account in part for a less restricted distribution of P25K. This redistribution may result in the inaccessibility of precursors to the virion assembly site as well as the proteolytic processing machinery and hence increase the possibility of the precursor polypeptides interacting with a different proteinase, perhaps one with an increased affinity for Ala-Gly-Ser sites, to produce 25K′. The hypothesis could also be applied to explain the production of 25K′:FLAG during transient expression (with or without rifampin treatment), which may be due to the distribution of P25K:FLAG throughout the cytoplasm, as demonstrated in Fig. 7.

Mutagenesis of the pL4R:FLAG expression vector and transient expression of the derived proteins should allow the sequence and structural requirements within the precursor for efficient cleavage to be delineated. This will undoubtedly involve more than the presence of the AGX motif per se. For example, with regard to the Ala-Gly-Ala motif, examination of the predicted amino acid sequence of the entire VV genome (18) reveals that this sequence occurs in seven different viral proteins, not all of which are subject to proteolytic processing (53a). Likewise, as noted previously, even in the case of the Ala-Gly-Ala motifs, which are known to be cleaved in the P4b and P25K precursors, there is little sequence conservation surrounding the conserved tripeptide. One possible explanation is that maturation of VV core protein precursors occurs via a signal peptidase mechanism similar to that used by both eukaryotic and prokaryotic secretory proteins. As with the VV core protein precursors, little primary sequence homology in the region flanking the cleavage sites of secretory proteins has been identified. However, three major structural motifs have been noted: (i) a basic amino-terminal region, (ii) a hydrophobic core, and (iii) a less hydrophobic cleavage region. At positions −3 and −1 relative to the cleavage site, Ala-X-Ala is the most frequently observed sequence (37, 50). Mutational analysis indicated that the net hydrophobicity determines the kinetics of precursor processing and the translocation of secretory proteins (5, 7). Positive charge at the amino-terminal region of the signal peptides seems to be required for the translocation reaction and apparently determines the rate of translocation (1, 43). On the basis of these studies, it is of interest that a preferential distribution of negatively charged amino acid residues on the amino-terminal side and positively charged residues on the carboxy-terminal side of the Ala-Gly-Ala cleavage motif exists in the P4b and P25K core protein precursors. Furthermore, proline residues can be found only on the carboxy-terminal side of the cleavage motif. One obvious difference between VV core proteins and secretory proteins is that VV core proteins are not secreted from the cytoplasmic membrane of the infected cell. They do, however, become associated with a membranous structure (namely, the previrion), so it is possible that the sequences flanking the Ala-Gly-Ala motif could play some role in packaging of the VV core proteins into the assembling virion. In any case, mutational analysis of site II of the P25K:FLAG protein should reveal the structure-function relationships of these components in VV core protein proteolysis.

The experiments described here have shed little light on the enzyme(s) which is responsible for carrying out the processing of P25K:FLAG. On the basis of the precedents of other viral systems (27) and the fact that processing of VV core proteins occurs efficiently in a wide variety of cells from different tissues and organisms, we would predict that the proteolytic enzyme(s) is encoded by the viral genome. However, it remains an open question whether the processing of the different VV core proteins involves one or several enzymes and whether they are cellular or viral enzymes. Likewise, since core protein maturation seems to be coupled to virion morphogenesis, it is likely that there will be a variety of other VV proteins which influence proteolytic processing. Several groups of VV temperature-sensitive mutants with defects in core protein processing and virion morphogenesis have been isolated (8, 30). Mapping of the lesions encoding the conditional lethal phenotypes has implicated the gene products of the VV D2, D3, D13, and I8 ORFs, in addition to the VV core protein precursors, as being essential for VV morphogenesis (13–15, 33). It is likely that there are a large number of additional viral (and cellular) polypeptides involved in the assembly of the infectious progeny VV virions. It is hoped that utilization of the *trans*-processing assay described in this article will help identify the polypeptide participants and their functions, especially the proteinase, in VV core protein proteolytic processing.

ACKNOWLEDGMENTS

We thank Judy K. Van Slyke for providing the rabbit anti-25K antiserum. We are grateful to William Dougherty for valuable discussions and Reg McParland and his staff at the OSU Center for Gene Research Central Services Laboratory for assistance with oligonucleotide synthesis.

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

REFERENCES

1. **Akita, M., S. Sasaki, S.-I. Matsuyama, and S. Mizushima.** 1990. SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *Escherichia coli*. J. Biol. Chem. **265**:8164–8169.
2. **Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tomasselli, W. G. Tarpley, and B. Moss.** 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. Proc. Natl. Acad. Sci. USA **87**:7472–7476.
3. **Binns, M. M., M. E. G. Boursnell, F. M. Tomley, and J. Campbell.** 1989. Analysis of the fowlpox virus gene encoding the 4b core polypeptide and demonstration that it possesses efficient promoter sequences. Virology **170**:288–291.
4. **Binns, M. M., F. M. Tomley, J. Campbell, and M. E. G. Boursnell.** 1988. Comparison of a conserved region in fowlpox virus and vaccinia virus genomes and the translocation of the fowlpox virus thymidine kinase gene. J. Gen. Virol. **69**:1275–1283.
5. **Bird, P., M.-J. Gething, and J. Sambrook.** 1990. The functional

efficiency of a mammalian signal peptide is directly related to its hydrophobicity. J. Biol. Chem. **265**:8420–8425.
6. **Black, L. W., and M. K. Showe.** 1983. Morphogenesis of the T4 head, p. 219–245. *In* C. K. Matthews, E. M. Kutter, G. Mosig, and P. B. Berget (ed.), Bacteriophage T4. American Society for Microbiology, Washington, D.C.
6a. **Child, S. J., and D. E. Hruby.** Unpublished data.
7. **Chou, M. M., and D. A. Kendall.** 1990. Polymeric sequences reveal a functional interrelationship between hydrophobicity and length of signal peptides. J. Biol. Chem. **265**:2873–2880.
8. **Condit, R. C., A. Motyczka, and G. Spizz.** 1983. Isolation, characterization and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology **128**:429–443.
9. **Dales, S., V. Milovanovitch, B. G. T. Pogo, S. B. Weintraub, T. Huima, S. Wilton, and G. McFadden.** 1978. Biogenesis of vaccinia: isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects. Virology **81**:403–428.
10. **Dales, S., and B. G. T. Pogo.** 1981. Biology of poxviruses. Springer-Verlag, New York.
11. **de Groot, R. J., W. R. Hardy, Y. Shirako, and J. H. Strauss.** 1990. Cleavage-site preference of Sindbis virus polyprotein containing the nonstructural proteinase. Evidence for temporal regulation of polyprotein processing in vivo. EMBO J. **9**:2631–2638.
12. **Dougherty, W. G., S. M. Cary, and T. D. Park.** 1989. Molecular genetic analysis of a plant virus polyprotein cleavage site: a model. Virology **171**:356–364.
13. **Dyster, L. M., and E. G. Niles.** 1991. Genetic and biochemical characterization of vaccinia virus genes D2L and D3R which encode virion structural proteins. Virology **182**:455–467.
14. **Fathi, Z., and R. C. Condit.** 1991. Phenotypic characterization of a vaccinia virus temperature-sensitive complementation group affecting a virion component. Virology **181**:273–276.
15. **Fathi, Z., and R. C. Condit.** 1991. Genetic and molecular biological characterization of a vaccinia virus temperature-sensitive complementation group affecting a virion component. Virology **181**:258–272.
16. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
17. **Gallapher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. **62**:3399–3460.
18. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179**:247–266.
19. **Harper, A. J., and P. M. Keen.** 1986. Methods for the identification of neuropeptide processing products: somatostatin and the tachukinins. Methods Enzymol. **124**:335–348.
20. **Hellen, C. U. T., and E. Wimmer.** 1992. Maturation of poliovirus capsid proteins. Virology **187**:391–397.
21. **Hopp, T. P., K. S. Prickett, V. L. Price, R. T. Libby, C. J. March, D. P. Cerretti, D. L. Urdal, and P. J. Conlon.** 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. Bio/Technology **6**:1204–1210.
22. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. J. Virol. **29**:705–715.
23. **Ichihashi, Y., M. Oie, and T. Tsuruhara.** 1984. Location of DNA-binding proteins and disulfide-linked proteins in vaccinia virus structural elements. J. Virol. **50**:929–938.
24. **Jung, L. J., and R. H. Scheller.** 1991. Peptide processing and targeting in the neuronal secretory pathway. Science **251**:1330–1335.
25. **Katz, E., and B. Moss.** 1970. Formation of a vaccinia virus structural polypeptide from a high-molecular-weight precursor: inhibition by rifampicin. Proc. Natl. Acad. Sci. USA **66**:677–684.
26. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
27. **Kräusslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
28. **Kunkel, T. A.** 1985. Rapid and efficient mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lake, J. R., M. Silver, and S. Dales.** 1979. Biogenesis of vaccinia: complementation and recombination analysis of one group of conditional-lethal mutants defective in envelope self-assembly. Virology **96**:9–20.
31. **Lawson, M. A., and B. L. Semler.** 1990. Picornavirus processing-enzymes, substrates, and genetic regulation. Curr. Top. Microbiol. Immunol. **161**:49–87.
32. **López-Otin, C., C. Simón-Mateo, L. Martinez, and E. Viñuela.** 1989. Gly-Gly-X, a novel consensus sequence for the proteolytic processing of viral and cellular proteins. J. Biol. Chem. **264**:9107–9110.
33. **Miner, J. N., and D. E. Hruby.** 1989. Rifampicin prevents virosome localization of L65, an essential vaccinia virus polypeptide. **170**:227–237.
34. **Moss, B.** 1990. Poxviridae and their replication, p. 2079–2112. *In* B. N. Fields et al. (ed.), Virology. Raven Press, New York.
35. **Moss, B., and E. N. Rosenblum.** 1973. Protein cleavage and poxviral morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J. Mol. Biol. **81**:267–269.
36. **Moss, B., E. N. Rosenblum, and E. Katz.** 1969. Rifampicin: a specific inhibitor of vaccinia virus assembly. Nature (London) **224**:1280–1284.
37. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391–409.
38. **Prickett, K. S., D. C. Amberg, and T. P. Hopp.** 1989. A calcium-dependent antibody for identification and purification of recombinant proteins. BioTechniques **7**:580–589.
39. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10**:520–525.
40. **Rosel, L., and B. Moss.** 1985. Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4B. J. Virol. **56**:830–838.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
42. **Sarov, I., and W. K. Joklik.** 1972. Studies on the nature and location of the capsid polypeptides of vaccinia virions. Virology **50**:579–592.
43. **Sasaki, S., S.-I. Matsuyama, and S. Mizushima.** 1990. In vitro kinetic analysis of the role of the positive charge at the amino-terminal region of signal peptides in translocation of secretory protein across the cytoplasmic membrane in *Escherichia coli*. J. Biol. Chem. **265**:4358–4363.
44. **Stern, W., B. G. T. Pogo, and S. Dales.** 1977. Biogenesis of poxviruses: analysis of the morphogenetic sequence using a conditional-lethal mutant defective in envelope self-assembly. Proc. Natl. Acad. Sci. USA **74**:2162–2166.
45. **Thompson, C. L., and R. C. Condit.** 1986. Marker rescue mapping of vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. Virology **150**:10–20.
46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
47. **Van Slyke, J. K., C. A. Franke, and D. E. Hruby.** 1991. Proteolytic maturation of vaccinia virus core proteins: identification of a conserved motif at the N terminus of the 4b and 25K virion proteins. J. Gen. Virol. **72**:411–416.
47a. **Van Slyke, J. K., and D. E. Hruby.** Unpublished data.
48. **Van Slyke, J. K., P. Lee, E. M. Wilson, and D. E. Hruby.**

Isolation and analysis of vaccinia virus previrions. Virus Genes, in press.
49. **Van Slyke, J. K., S. S. Whitehead, E. M. Wilson, and D. E. Hruby.** 1991. The multiple proteolytic maturation pathway utilized by vaccinia virus P4a protein: a degenerate conserved motif within core proteins. Virology **183**:467–478.
50. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **116**:17–21.
51. **Weber, J.** 1976. Genetic analysis of adenovirus type 2. III. Temperature sensitivity of processing of viral proteins. J. Virol. **17**:462–471.
52. **Weir, J. P., and B. Moss.** 1985. Use of a bacterial expression vector to identify the gene encoding a major core protein of vaccinia virus. J. Virol. **56**:534–540.
53. **Wellink, J., and A. van Kammen.** 1988. Proteases involved in the processing of viral polypeptides. Arch. Virol. **98**:1–26.
53a. **Whitehead, S. S., and D. E. Hruby.** Unpublished data.
54. **Yang, W.-P., and W. R. Bauer.** 1988. Purification and characterization of vaccinia virus structural protein VP8. Virology **187**:578–584.
55. **Yang, W.-P., S.-Y. Kao, and W. R. Bauer.** 1988. Biosynthesis and posttranslational cleavage of vaccinia virus structural protein VP8. Virology **187**:585–590.
56. **Zhang, Y., and B. Moss.** 1992. Immature viral envelope formation is interrupted at the same stage by *lac* operator-mediated repression of the vaccinia virus D13L gene and by the drug rifampicin. Virology **187**:643–653.