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Regulation of Vaccinia Virus Morphogenesis: Phosphorylation of the A14L and A17L Membrane Proteins and C-Terminal Truncation of the A17L Protein Are Dependent on the F10L Kinase

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This study focused on three vaccinia virus-encoded proteins that participate in early steps of virion morphogenesis: the A17L and A14L membrane proteins and the F10L protein kinase. We found that (i) the A17L protein was cleaved at or near an AGX consensus motif at amino acid 185, thereby removing its acidic C terminus; (ii) the nontruncated form was associated with immature virions, but only the C-terminal truncated protein was present in mature virions; (iii) the nontruncated form of the A17L protein was phosphorylated on serine, threonine, and tyrosine residues, whereas the truncated form was unphosphorylated; (iv) nontruncated and truncated forms of the A17L protein existed in a complex with the A14L membrane protein; (v) C-terminal cleavage of the A17L protein and phosphorylation of the A17L and A14L proteins failed to occur in cells infected with a F10L kinase mutant at the nonpermissive temperature; and (vi) the F10L kinase was the only viral late protein that was necessary for phosphorylation of the A17L protein, whereas additional proteins were needed for C-terminal cleavage. We suggest that phosphorylation of the A17L and A14L proteins is mediated by the F10L kinase and is required to form the membranes associated with immature virions. Removal of phosphates and the A17L acidic C-terminal peptide occur during the transition to mature virions.

The initial steps in vaccinia virus morphogenesis are poorly understood. The first viral structures are crescent-shaped membranes that appear to form de novo in specialized factory regions of the cytoplasm which are largely devoid of cellular organelles (6, 10, 24). Griffiths and coworkers (36, 42) have proposed that the viral membranes are derived from the cellular intermediate compartment by a wrapping mechanism. Regardless of their origin, the crescents develop into spherical, immature virions (IV) containing the double-stranded DNA genome and subsequently into dense, brick-shaped, infectious intracellular mature virions (IMV). Some of the IMV escape from the assembly regions and are wrapped by membrane cisternae, derived from the trans-Golgi or early endosomal network, to form the intracellular enveloped virions (IEV) (13, 15, 23, 38, 47). A subset of IEV are propelled through the cytoplasm via actin tails and form the tips of specialized microvilli that protrude from the cell surface and mediate efficient cell-to-cell virus spread (5, 12, 14, 35, 37, 44, 55, 57). IEV without actin tails also reach the periphery (55), where they fuse with the plasma membrane to form cell-associated enveloped virions and released extracellular enveloped virions (3, 28).

At least 11 virus-encoded proteins are associated with IMV membranes (16, 45). Studies with conditional lethal mutants have shown that three of these (the A17L, D13L, and A14L proteins) and one additional protein encoded by the F10L gene are required for formation of viral crescents. Under nonpermissive conditions, F10L mutants make no membrane structures (48, 51), A17L mutants make small vesicles (34, 56), D13L mutants make irregular membranes without spicules (58) that resemble structures formed in the presence of the drug rifampin (10, 26, 27), and A14L mutants make aberrant crescents (34). The product of the F10L gene is a serine/threonine protein kinase whose protein targets are unidentified (21). The A17L product undergoes proteolytic processing near the N terminus at an AGX cleavage site consensus motif (34, 45, 54) and is cotranslationally inserted into membranes and exposed on the concave surface of IV (18, 30, 56). Rifampin-resistant mutants have been mapped to the D13L gene (1, 46), and the protein has been localized to the concave surface of crescents and IV (43). The A14L product is a phosphorylated component of the IMV membrane that forms a complex with the A17L and A27L proteins (34, 36). Although associated with IMV membranes, the A27L protein has a role in the formation of IEV rather than IMV (32, 33).

In the present study, we investigated the posttranslational modifications of the A17L protein and the role of these in virion assembly. Proteolytic processing was shown to occur near the C terminus of the A17L protein, as well as at the previously described N-terminal site. In addition, the A17L protein with an intact C terminus was found to be phosphorylated. Both of these modifications, as well as phosphorylation of the A14L protein, depended on the F10L protein kinase, providing insights into the role of the enzyme in the early steps of morphogenesis.

MATERIALS AND METHODS

Cells and viruses. BS-C-1 (ATCC CCL26) cells were grown in Eagle minimum essential medium (EMEM; Quality Biologicals) containing 2.5% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. Recombinant vaccinia virus vA17LΔ5 in which the original A17L open reading frame (ORF) was replaced by an isopropyl-β-D-galactopyranoside (IPTG)-inducible copy (56), and recombinant vaccinia virus vTF7-3 which expresses the bacteriophage T7 RNA polymerase gene (9) were propagated in HeLa cells as described previously (8). Tempera-

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ture-sensitive (*ts*) F10L mutants of vaccinia virus, *ts*15 and *ts*28 (4) were grown at 32°C.

Antibodies. Rabbit antiserum to the C-terminal peptide of the A17L ORF was previously described (56). Rabbit antisera were produced to the peptide corresponding to the sequence TEEQQQSFMPKD of the A17L ORF (Fig. 1A) and to the C-terminal peptide CAPHRVSGVIHTN of the A14L ORF conjugated to keyhole limpet hemocyanin. Polyclonal antibodies to phosphorylated amino acids were from Zymed Laboratories (San Francisco, Calif.).

Plasmid construction. Plasmid pVOTE.1-A17L, containing the A17L ORF, was previously described under the name pDMA17L.2 (56). A copy of the F10L gene, with an *Nde*I site as the initiation codon and *Bam*HI site after the TAA termination signal, was obtained by PCR using vaccinia virus genomic DNA as the template and oligonucleotide primers TB90 (GGGGGGCATATGGGTGT TGCCAATGATTCATCC) and TB91 (GGGGGGGGATCCTTAGTTTCCGC CATTTATCC). The T at position −510 was changed to C to eliminate an internal *Nde*I restriction site by PCR using primers TB92 (CTCTGTATAAAC GGGTTCTTCACATGTTGCTATTATTGATAC) and TB93 (GTATCAATAA TAGCAACATGTGAAGAACCCGTTTATACAGAG). The PCR product was cut with *Nde*I and *Bam*HI and inserted into plasmid pVOTE.2 (52) to generate pVOTE.2-F10L. Plasmid DNA was purified using the Wizard Plus SV Minipreps DNA purification system (Promega).

Metabolic labeling. Confluent BS-C-1 cells were infected with 10 PFU of vaccinia virus strain WR per cell in the presence or absence of rifampin (100 μg/ml) at 37°C or with vaccinia virus *ts*15 or *ts*28 at 32 or 39.5°C. At 6 h after infection, the medium was removed and the cells were overlaid with methionine-free EMEM containing [^{35}S]methionine (50 μCi/ml). After a 30-min incubation, the cells were washed once and overlaid with EMEM containing 2.5% fetal bovine serum. Cells in individual wells were lysed in extraction buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.5], 1 mM phenylmethylsulfonyl fluoride) immediately after labeling (time zero) or at later times. After 10 min on ice, the lysates were cleared by microcentrifugation for 1 min, and the supernatants were used for immunoprecipitations.

For metabolic labeling with $^{32}P_i$, infected BS-C-1 cells were incubated overnight with 50 μCi/ml and lysed in the presence of the phosphatase inhibitors sodium fluoride and sodium metavanadate as described elsewhere (39).

Immunoprecipitation. Clarified lysates (100 to 200 μl) were mixed with 2 μl of antiserum and 500 μl of phosphate-buffered saline (PBS) and rotated overnight at 4°C. Sodium fluoride and metavanadate were included for analysis of phosphoproteins (39). Protein A-Sepharose (Pharmacia) beads (150 μl of a 10% suspension in PBS) were added to each sample. After 2 h, the beads were washed five times with PBS, resuspended in electrophoresis sample buffer. Sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) was performed as described elsewhere (19), and the gels were immersed in fixative (10 ml of acetic acid, 35 ml of 2-propanol, 55 ml of water). The gels were dried and exposed to BioMax MS film (Kodak) at −70°C. The molecular masses of viral proteins were estimated by comparison with standard protein markers (Amersham).

Western blotting. After fractionation by SDS-PAGE, the protein were electrophoretically transferred to a nitrocellulose membrane. The blot was incubated in 5% nonfat dry milk in PBS overnight and then for 1 h with A17L C or N antibody. The membrane was washed several times with PBS and incubated with secondary antibody coupled to alkaline phosphatase (Sigma). The blot was washed several time with PBS and visualized using the 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium phosphatase substrate system (Kirkegaard & Perry Laboratories).

Transfection experiments. BS-C-1 cells in six-well plates were infected with vTF7-3 (10 PFU/cell) in OPTIMEM (GIBCO) medium containing 40 μg of cytosine arabinoside (AraC) per ml at 37°C. After 1 h, the infected cells were transfected with 2 μg of pVOTE.1-A17L or pVOTE.2-F10L or both in DOTAP (Boehringer Mannheim). After 4 h, the cells were washed and overlaid with either 2 ml of phosphate-free EMEM with 50 μCi of $^{32}P_i$ or 2 ml of methionine-free MEM with 50 μCi of [^{35}S]methionine. After an additional 20 h, the cells were washed with PBS, lysed, and analyzed as described above.

Phosphoamino acid analysis. BS-C-1 cells were infected with vA17LΔ5 virus in the presence or absence of IPTG and metabolically labeled with $^{32}P_i$ as described above. After immunoprecipitation with antibodies and SDS-PAGE, the phosphoproteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The band of interest was excised, and the protein was hydrolyzed in 6 M HCl for 60 min at 110°C. After drying, the material was resuspended and analyzed by two-dimensional thin-layer electrophoresis (40).

Electron microscopy. Infected BS-C-1 cells were fixed with paraformaldehyde and cryosectioned as previously described (55). Thawed cryosections were incubated with rabbit serum containing A17L N or A17L C antibodies, washed, incubated with 10-nm-diameter gold particles conjugated to protein A (Department of Cell Biology, Utrecht University School of Medicine, Utrecht, The Netherlands), and viewed with a Philips CM100 electron microscope.

RESULTS

C-terminal truncation of the A17L protein. The A17L ORF encodes a 203-amino-acid protein of 23 kDa (Fig. 1A). Pulse-chase experiments and N-terminal sequencing studies, however, indicated the formation of a 21-kDa species that started at amino acid 17, consistent with proteolytic processing at an AGX consensus motif (31, 34, 45, 54). Based on the electrophoretic mobility of the processed protein, Takahashi et al. (45) suggested that cleavage also occurred at another AGX motif near the C terminus. To investigate this, Western blots were prepared from cytoplasmic extracts of cells infected with vaccinia virus and then probed with an antibody to the peptide corresponding to amino acids 26 to 37, which would be conserved in processed forms of the A17L protein, or with an antibody to the peptide comprising amino acids 192 to 203, which would be removed by C-terminal processing (Fig. 1A). The A17L N antibody reacted with a band that was usually resolved as a doublet of 23- and 25-kDa species and a faster-migrating band of 21 kDa (Fig. 1B). In contrast, the A17L C antibody failed to react with the 21-kDa protein (Fig. 1B). These data indicated that the 21-kDa species lacked epitopes formed by amino acids 192 to 203 and supported the C-terminal proteolytic processing of the A17L protein. Presumably, the 25- and 23-kDa proteins represent full-length and N-terminally cleaved species, respectively. Evidence for the latter was obtained by experiments in which a mutated gene encoding an N-terminally truncated form of the A17L protein was transfected into cells and detected by reactivity with C antibody (unpublished data). Whether an intermediate C-terminally truncated species with an intact N terminus exists could not be determined.

The C-terminal truncated form of the A17L protein is in mature virions. Western blots were prepared from sucrose gradient-purified vaccinia virions. In this case, only the 21-kDa protein was detected with the N antibody and the C antibody was nonreactive (Fig. 1C).

Previous immunoelectron microscopic studies had shown that the C antibody labeled the concave surface of crescents and IV as well as immature membrane precursors that accumulated in the presence of the drug rifampin but did not label IMV (56). We repeated these studies using the newly available N antibody in addition to the C antibody. Whereas the C antibody labeled only membranes of immature virus forms, the N antibody also labeled mature forms (Fig. 2). These results were consistent with the biochemical analysis and indicated that C-terminal truncation occurred during the transition from IV to IMV.

Relationship of C-terminal cleavage and morphogenesis. The antibiotic rifampin blocks the formation of viral crescents, leading to the accumulation of immature membrane precursors lacking a spicule coat (10, 26, 27), and prevents the proteolytic processing of some core proteins (17, 25). To determine the relationship between C-terminal cleavage of the A17L protein and morphogenesis, [^{35}S]methionine pulse-chase experiments were carried out in the absence or presence of rifampin. In the absence of rifampin, a pulse-labeled band of 23 to 25 kDa was detected with C or N antibody (Fig. 3A). The intensity of this band was relatively constant for an additional hour but then diminished. A 21-kDa species was detected after a 1-h chase and only with N antibody (Fig. 3A). The 21-kDa C-terminal truncated species also formed in the presence of rifampin, indicating that processing of the A17L protein does not require viral crescent formation (Fig. 3B). Rifampin was shown to block the cleavage of the precursor to the 4B core protein, indicating that the drug was working effectively (Fig. 3).

To block morphogenesis prior to the rifampin-sensitive stage, we acquired the conditional lethal F10L mutants *ts*15 and *ts*28 (4). Under nonpermissive conditions, viral protein

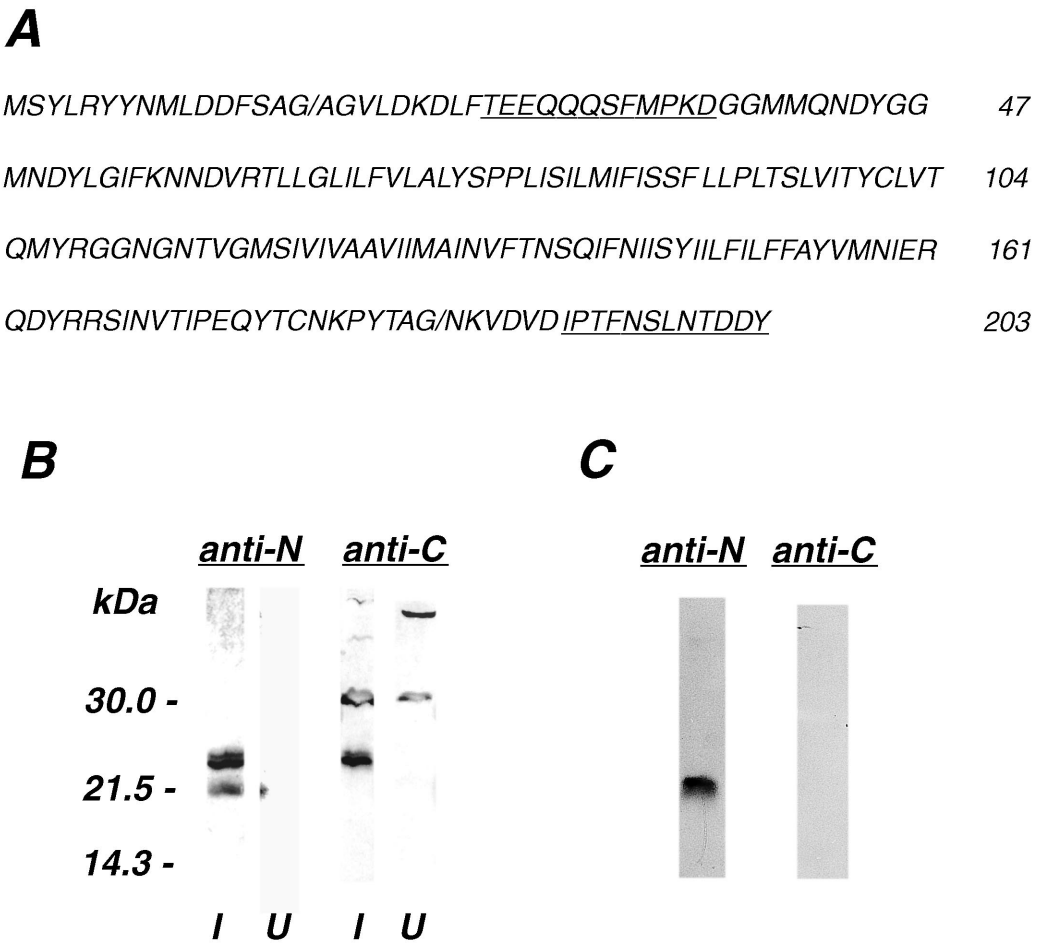

FIG. 1. C-terminal cleavage of the A17L protein. (A) The A17L ORF is shown with the sequences used to generate synthetic peptides for immunization underlined and a slash at the known AGA and predicted AGN cleavage motifs. The antibodies induced by peptides TEEQQQSFMPKD and IPTFNSLNTDDY are referred to as A17L N antibody and A17L C antibody, respectively. (B) Western blot of extract from uninfected cells (U) and cells infected with vaccinia virus (I) and probed with A17L N antibody (anti-N) and A17L C-antibody (anti-C). The masses and positions of marker proteins are indicated on the left. Close inspection reveals that the 23- to 25-kDa bands from infected cells detected with N and C antibodies are doublets. The C antibody cross-reacted with more slowly migrating bands from uninfected and infected cells. (C) Western blot of proteins from 11 μg of sucrose gradient-purified vaccinia virions probed with A17L N and C antibodies.

synthesis occurs normally but formation of crescents or other viral membranes and processing of core proteins is largely blocked (48, 51). Pulse-chase experiments and immunoprecipitation with N and C antibodies indicated that C-terminal cleavage of the A17L protein occurred in cells infected with *ts*15 at the permissive temperature of 32°C but not at the nonpermissive temperature of 39.5°C (Fig. 4), suggesting a direct or indirect role of the F10L kinase in proteolytic processing. Similar results were obtained for *ts*28 (data not shown). However, processing of the A17L protein occurred in cells infected with wild-type vaccinia virus at 39.5°C (data not shown).

Association of the A17L and A14L proteins. Rodriguez et al. (31, 34) found that antibody to the A27L IMV membrane protein precipitated a complex containing the 21-kDa form of the A17L protein, the 15-kDa A14L protein, and the A27L protein. In contrast, neither the A14L nor the A27L protein coprecipitated with the A17L protein when the A17L N or C antibody was used (Fig. 3A). Proteins corresponding in size to those derived from A17L did coprecipitate with the A14L protein, however, when antibody to the C-terminal peptide of the latter was used (see below). To be certain that the coprecipitating proteins were derived from A17L, we repeated the experiment using the A17L-inducible conditional lethal mutant vA17LΔ5 (56). Previous studies had shown that the absence of IPTG, synthesis of the A17L protein was undetectable but synthesis of other proteins appeared unaffected (56). Under these conditions, small vesicles assembled around areas of dense viroplasm without formation of the characteristic viral membranes and processing of core proteins (56). Pulse-chase experiments were carried out in the presence and absence of IPTG, and the labeled proteins were immunoprecipitated with A17L N antibody (Fig. 5A) or A14L C antibody (Fig. 5B). As shown in Fig. 5A, the A17L protein was not pulse-labeled in the absence of IPTG. In the presence of IPTG, synthesis and processing of the A17L protein was similar to that observed with wild-type vaccinia virus (compare Fig. 3A and 5A). No evidence of coprecipitation of the 15-kDa A14L protein was obtained with A17L N antibody. Nevertheless, the pulse-labeled 25- and 23-kDa A17L proteins coprecipitated with the 15-kDa A14L protein using antibody to the latter (Fig. 5B). After a 1-h chase, the 21-kDa A17L protein also coprecipitated with the 15-kDa A14L protein (Fig. 5B). As expected, no proteins of 21, 23, or 25 kDa were coprecipitated when A17L expression was not induced. The amount of labeled A14L

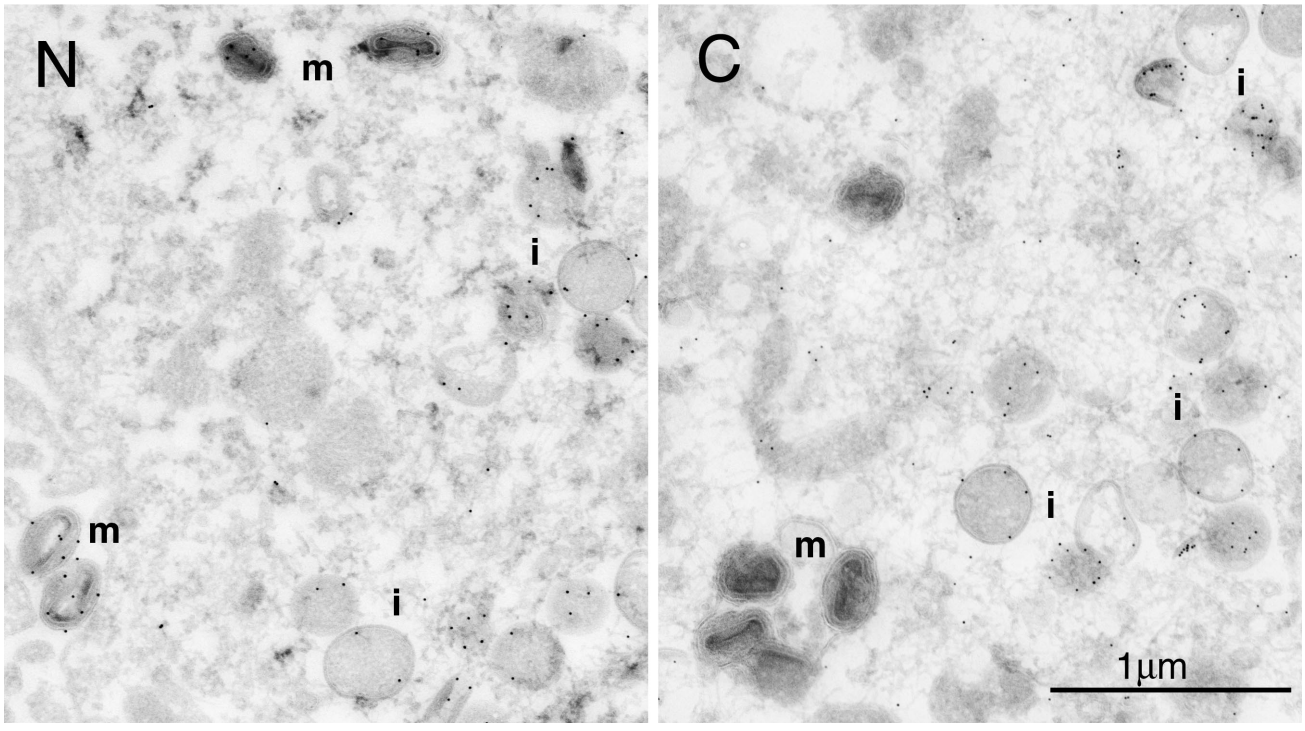

FIG. 2. Immunogold labeling of viral membranes with antibodies to the A17L protein. BS-C-1 cells that had been infected with vaccinia virus for 24 h were fixed in paraformaldehyde, cryosectioned, and incubated with A17L N antibody (N) or A17L C antibody (C) and then with 10-nm-diameter gold particles conjugated to protein A. Electron microscopic images are shown with a 1-μm marker. m, mature particles; i, immature particles.

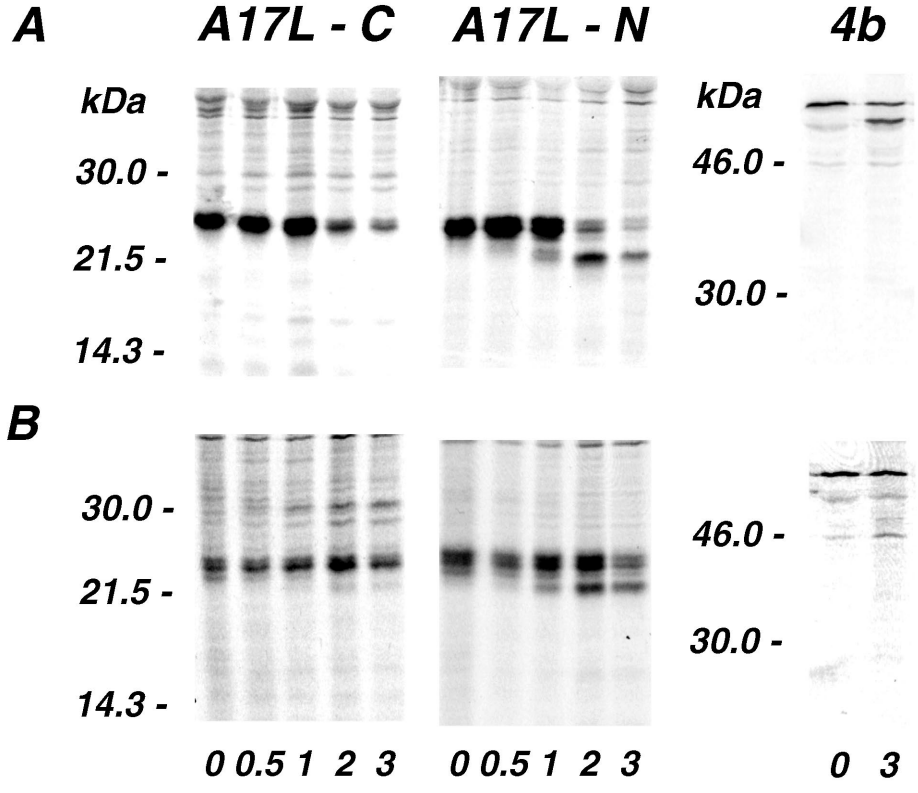

FIG. 3. C-terminal truncation of the A17L protein in the presence of rifampin. Replicate wells containing BS-C-1 cells were infected with vaccinia virus and incubated at 37°C in the absence (A) or continued presence (B) of rifampin. At 6 h after infection, the cells were incubated for 30 min with [^{35}S]methionine. The cells were then washed and incubated with medium containing unlabeled amino acids. Cells were harvested after the 30-min pulse (time zero) and after 0.5, 1, 2, and 3 h of chase as indicated, lysed, and incubated with A17L C antibody, A17L N antibody, or antibody to the 4b core protein. The bound proteins were analyzed by SDS-PAGE and autoradiography. The positions and masses of marker proteins are indicated.

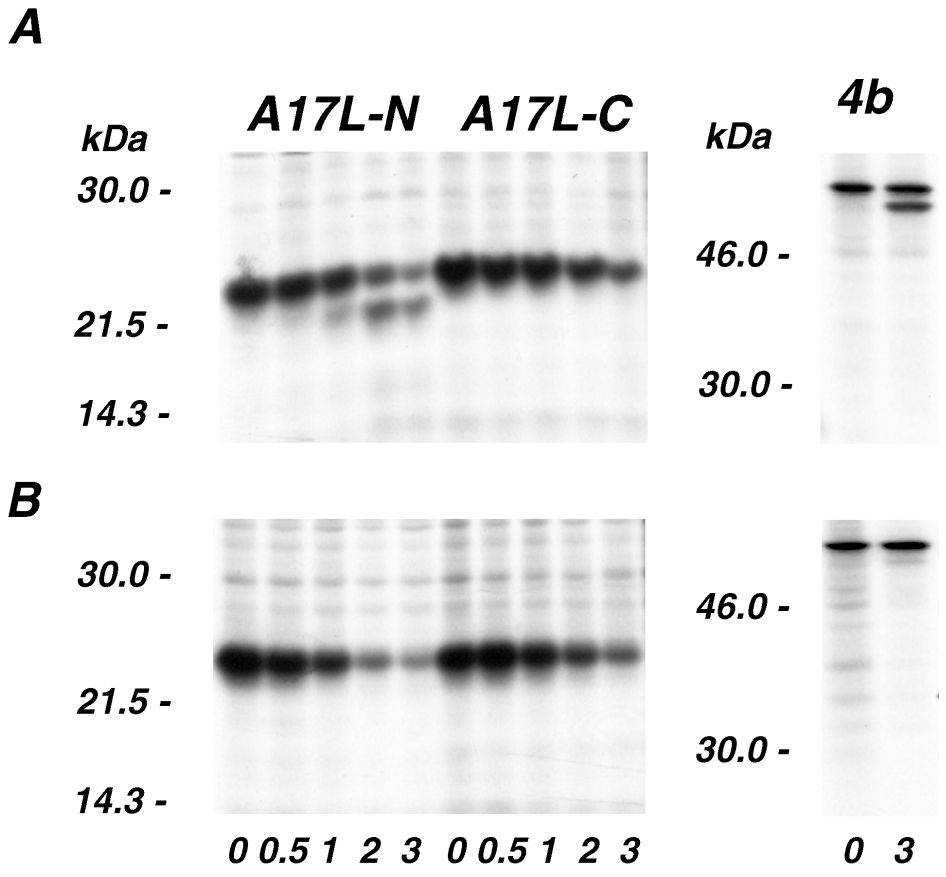

FIG. 4. C-terminal truncation of the A17L protein failed to occur in cells infected at the nonpermissive temperature with an F10L mutant. Replicate BS-C-1 cells were infected with *ts*15 at 32°C (A) or 39.5°C (B). At 6 h after infection, the cells were incubated for 30 min with [^{35}S]methionine. The cells were then washed and incubated with medium containing unlabeled amino acids. Care was taken to maintain the temperature at 32 or 39.5°C during the labeling and chase periods. Cells were harvested after the 30-min pulse (time zero) and after 0.5, 1, 2, and 3 h of chase as indicated, lysed, and incubated with A17L C antibody or A17L N antibody. The bound proteins were analyzed by SDS-PAGE and autoradiography. The positions and masses of marker proteins are indicated.

protein was diminished in the absence of IPTG (Fig. 5B), suggesting that the A17L protein exerted a positive effect on the synthesis or stability of the A14L protein.

Phosphorylation of the A17L protein. Since the A14L protein is phosphorylated (34), we carried out experiments to determine whether the A17L protein is also a phosphoprotein. Initial $^{32}P_i$ labeling experiments were carried out with wild-type vaccinia virus. These studies indicated that a phosphorylated protein was immunoprecipitated with A17L N antibody (data not shown). To confirm the specificity of the immunoprecipitation, we again used the inducible mutant vA17LΔ5 (56).

Cells were infected with vA17LΔ5 in the presence or absence of IPTG and labeled with [^{35}S]methionine to analyze polypeptide synthesis or $^{32}P_i$ to detect phosphorylation. In the absence of IPTG, no labeled products of 21 to 25 kDa were immunoprecipitated with A17L N antibody (Fig. 6), consistent with repression of A17L expression. In the presence of IPTG, however, a ^{32}P-labeled polypeptide of 25 kDa was immunoprecipitated with A17L N antibody (Fig. 6B), whereas [^{35}S]methionine-labeled polypeptides of 25 and 23 kDa as well as a faint one of 21 kDa were immunoprecipitated (Fig. 6A). The comigration of the ^{32}P-labeled band with the upper band of the [^{35}S]methionine-labeled doublet was confirmed by their electrophoresis in the same gel (data not shown). The inability to detect a phosphorylated 21-kDa A17L species in numerous experiments could indicate that the phosphate was removed by a phosphatase or by proteolytic processing during morphogenesis. An additional ^{32}P-labeled band was present near the bottom of most lanes regardless of which antibody was used (Fig. 6B). Neither the identity nor the significance of this material was determined.

The A14L antibody was also used to immunoprecipitate the labeled proteins in extracts of cells infected with vA17LΔ5. Phosphorylation of the 15-kDa A14L protein was independent of A17L induction (Fig. 6B), even though the amount of the [^{35}S]methionine-labeled A14L protein was decreased in the absence of IPTG. In the presence of inducer, C-terminal processed and unprocessed [^{35}S]methionine-labeled A17L protein species (Fig. 6A) and a single ^{32}P-labeled 25-kDa species (Fig. 6B) coprecipitated with the A14L protein.

Role of the F10L kinase in phosphorylation of A17L and A14L proteins. Because the F10L kinase mutants exhibited a defect in processing of the A17L protein at 39.5°C (Fig. 4B), we investigated the phosphorylation of the A17L and A14L proteins at the nonpermissive temperature. Control experiments with wild-type vaccinia virus demonstrated that ^{32}P-labeling of the A17L and A14L proteins was unaffected by elevation of the temperature to 39.5°C (data not shown). However, in cells infected with *ts*15 or *ts*28, ^{32}P-labeling of the A17L

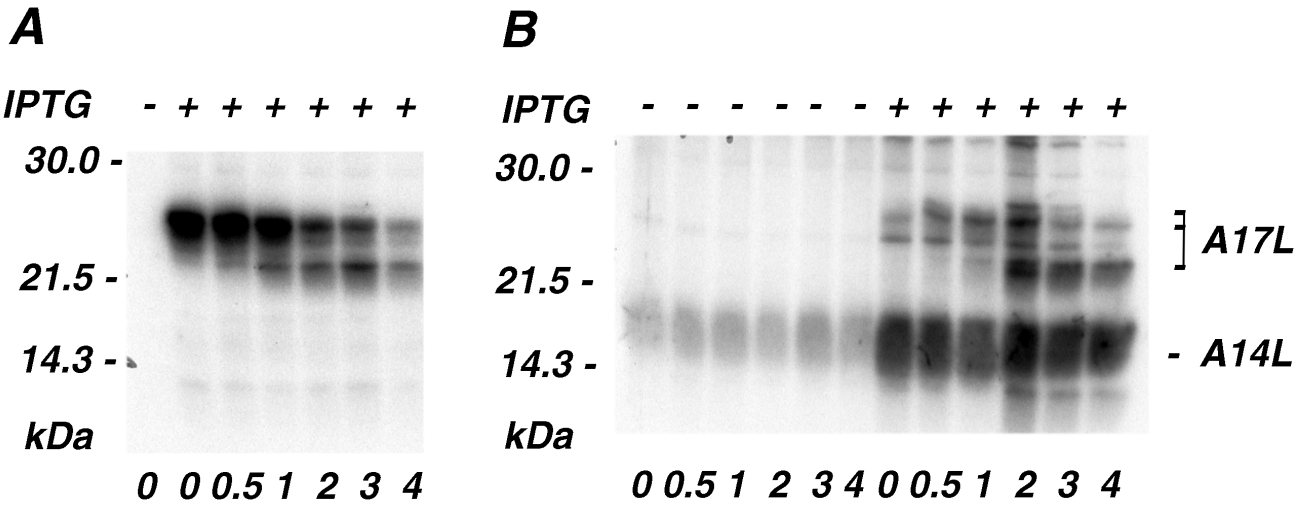

FIG. 5. Coimmunoprecipitation of A17L and A14L proteins. Pulse-chase [^{35}S]methionine-labeling experiments were carried out as described in the legend to Fig. 4 except that cells were infected with vA17LΔ5 virus in the absence (−) or presence (+) of IPTG at 37°C and the chase was continued for 4 h. Lysates were immunoprecipitated with A17L N antibody (A) or A14L C antibody (B) and analyzed by SDS-PAGE and autoradiography. In panel A, no pulse-labeled protein was detected in the absence of IPTG, and so only the chase samples in the presence of IPTG are shown. The positions and masses of marker proteins are indicated.

and the A14L proteins was undetectable or greatly reduced at the elevated temperature (Fig. 6B). This correlated with a block in formation of the 21-kDa [^{35}S]methionine-labeled A17L species (Fig. 6A). In addition, the 23-kDa [^{35}S]methionine-labeled A17L protein did not coprecipitate with the A14L protein at 39.5°C (Fig. 6A). These data suggested that the F10L kinase was required for phosphorylation of the A17L and A14L proteins as well as for their physical association.

To further study the role of the F10L kinase, we devised conditions in which the A17L and F10L proteins could be

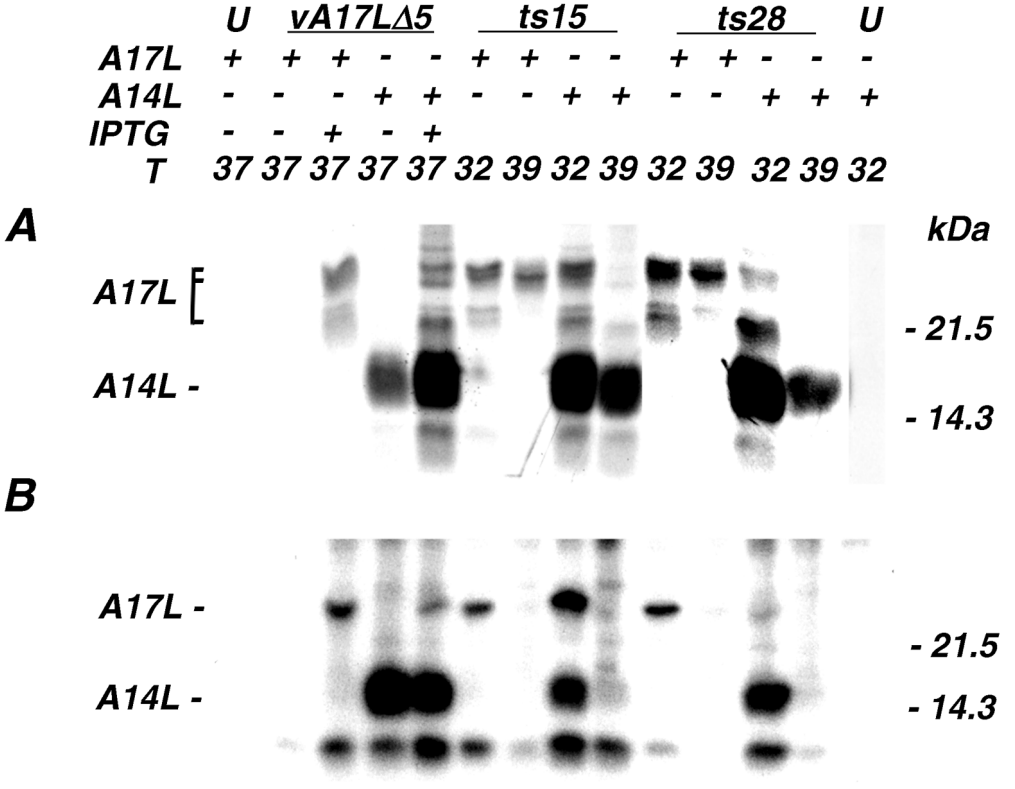

FIG. 6. Phosphorylation of the A17L and the A14L proteins. BS-C-1 cells were infected with vaccinia virus vA17LΔ5 at 37°C in the presence (+) or absence (−) of IPTG or with vaccinia virus *ts*15 virus at the permissive (32°C) or nonpermissive (39.5°C) temperature (T) and then labeled overnight with [^{35}S]methionine (A) or with $^{32}P_i$ (B). Immunoprecipitation was performed with A17L N antibody (A17L) or with A14L C antibody (A14L) as indicated. The bound proteins were analyzed by SDS-PAGE and autoradiography. U, uninfected cells. Positions and masses of marker proteins are shown on the right.

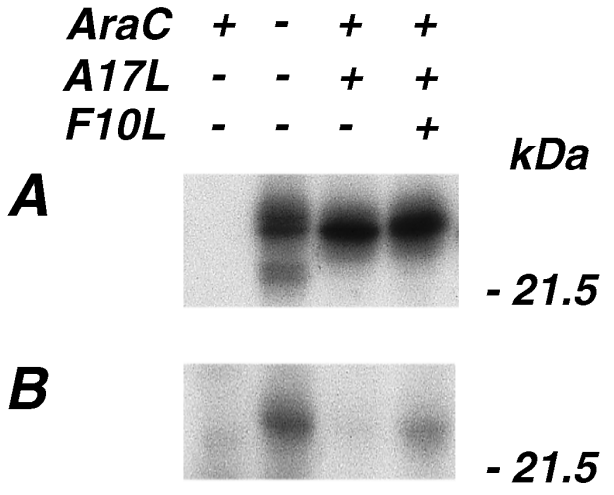

FIG. 7. Phosphorylation of the A17L protein in cells transfected with plasmids expressing the A17L and F10L genes. BS-C-1 cells were infected with vTF7-3 in the absence or presence of AraC and transfected with pVOTE.1-A17L (A17L) alone or together with.pVOTE.2-F10L (F10L). The cells were metabolically labeled with [^{35}S]methionine (A) or $^{32}P_i$ (B), and the lysates were immunoprecipitated with A17L N antibody and analyzed by SDS-PAGE and autoradiography.

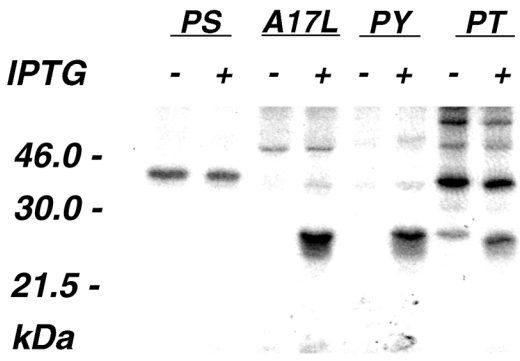

FIG. 8. SDS-PAGE analysis of proteins immunoprecipitated with antibodies to phosphorylated amino acids. BS-C-1 cells were infected with vA17LΔ5 virus in the absence (−) or presence (+) of IPTG and labeled with $^{32}P_i$. Lysates were immunoprecipitated with antibody to phosphoserine (PS), phosphotyrosine (PY), or phosphothreonine (PT) or with A17L N-antibody (A17L) and analyzed by SDS-PAGE. The positions and masses in kilodaltons of marker proteins are shown on the left.

selectively expressed. This was accomplished with plasmids pVOTE.1-A17L and pVOTE.2-F10L, in which the A17L and F10L ORFs are regulated by a bacteriophage T7 promoter, and recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase. BS-C-1 cells were infected with vTF7-3 in the presence of AraC to prevent replication of the viral genome and expression of intermediate and late genes. The cells were transfected with pVOTE.1-A17L alone or together with pVOTE.2-F10L and then metabolically labeled with $^{32}P_i$ or [^{35}S]methionine. Although synthesis of the A17L protein occurred independently of F10L kinase (Fig. 7A), it was phosphorylated only when F10L kinase was coexpressed (Fig. 7B). These results suggested that the F10L kinase was the only viral late protein required for phosphorylation of the A17L protein. However, the 21-kDa species of the A17L protein was not detected suggesting that synthesis of other viral late proteins was required for C-terminal truncation. Attempts to carry out similar experiments by cotransfection of a plasmid containing the A14L gene, either with or without plasmids expressing A17L and F10L, were not interpretable because expression of the A14L protein could not be demonstrated (2).

Analysis of phosphorylated amino acids. We used two methods to identify the amino acids in the A17L and A14L proteins that were phosphorylated. The first depended on the specificity of antibodies for phosphoserine, phosphothreonine, and phosphotyrosine and the ability to selectively induce expression of the A17L protein. Lysates were prepared from cells infected with vA17LΔ5 in the presence or absence of IPTG and labeled with $^{32}P_i$. An IPTG-induced protein of 25 kDa was immunoprecipitated with the antibody to phosphotyrosine (Fig. 8). Although in the absence of IPTG a protein that gave a faint 25-kDa band was immunoprecipitated with antibody to phosphothreonine, a stronger band appeared with IPTG (Fig. 8). No band of the correct size was detected using antibody to phosphoserine. Thus, these results suggested that the A17L protein was phosphorylated on tyrosine and threonine residues.

In the second approach, cells were infected with vA17LΔ5 in the presence or absence of IPTG and then labeled with $^{32}P_i$. The lysates were immunoprecipitated with A17L N or C antibody, A14L antibody, or antiphosphotyrosine antibody. The proteins were then resolved by SDS-PAGE and transferred to a PVDF membrane. The polypeptides were located by autoradiography, and the segments corresponding to the induced 25-kDa protein were excised and hydrolyzed with HCl. The absence of radioactive 25-kDa bands from noninduced cells supported the specificity of the immunoprecipitation (data not shown). After acid hydrolysis, the samples were analyzed by two-dimensional thin-layer electrophoresis with phosphoamino acid standards. The latter were visualized with ninhydrin, and the plate was then autoradiographed. Phosphoserine was identified in the hydrolysate of the A14L protein (Fig. 9, anti-A14L), and both phosphothreonine and phosphoserine were identified in the hydrolysate of the A17L protein isolated either with A17L N antibody (Fig. 9, anti-A17L) or A17L C antibody (not shown). No phosphotyrosine was detected in these hydrolysates. Nevertheless, phosphotyrosine, phosphothreonine, and phosphoserine were detected in the hydrolysate of the IPTG-induced 25-kDa protein that was immunoprecipitated with phosphotyrosine antibody (Fig. 9, Anti-PY). Presumably, the latter procedure provided an enrichment for phosphotyrosine-containing A17L protein species of low abundance.

DISCUSSION

Viral membrane formation is the initial step in poxvirus morphogenesis. We examined the A17L protein primarily because it is an integral membrane component (18, 31) and repression of A17L gene expression results in a very early block in morphogenesis, prior to the formation of viral membrane crescents (29, 30, 56). In addition, the A17L protein is posttranslationally modified by cleavage at a consensus motif, AGX, near the N terminus (34, 45, 54), and it forms a complex with the A14L and A27L membrane proteins (31, 34). New information provided by the present study is that (i) the A17L protein was cleaved at or near an AGX motif at amino acid 185, thereby removing its acidic C terminus; (ii) only the C-terminal truncated form was present in mature virus particles; (iii) the A17L precursor protein was phosphorylated whereas the C-terminal truncated form was not; (iv) nontruncated and C-terminal truncated forms of the A17L protein associated in a complex with the A14L protein; (v) C-terminal cleavage of

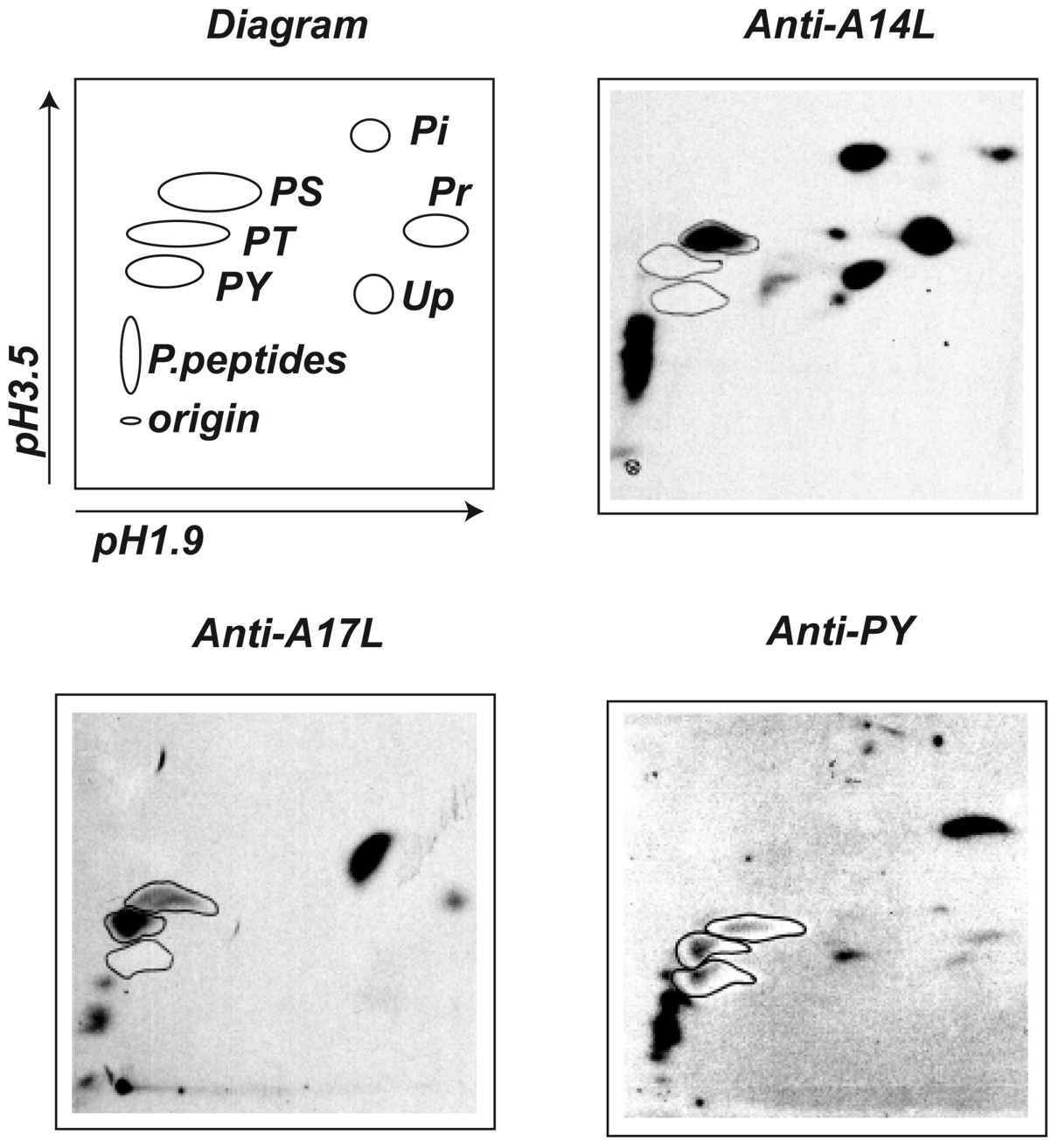

FIG. 9. Phosphoamino acid analysis. BS-C-1 cells were infected with vaccinia virus vA17LΔ5 in the presence of IPTG and labeled overnight with $^{32}P_i$. After lysis and immunoprecipitation with A14L antibody, A17L N antibody, or antibody to phosphotyrosine (anti-PY), the proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. The radioactively labeled bands were excised, hydrolyzed with HCl, and analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5. The standards amino acids were visualized with ninhydrin, and the plates were autoradiographed. The spots corresponding to phosphoserine (PS), phosphothreonine (PT), phosphotyrosine (PY), P_i, phosphoribose (Pr), and phosphouridine (Up) are identified. P.peptides, phosphorylated peptides.

the A17L protein and phosphorylation of the A17L and A14L proteins failed to occur in cells infected with F10L kinase mutants at the nonpermissive temperature; and (vi) the F10L kinase was the only viral late stage protein that was necessary for phosphorylation of the A17L protein, whereas other intermediate- or late-stage proteins may be required for C-terminal cleavage. Taken together, the data provided a model in which phosphorylation and proteolytic processing are key events in vaccinia virus morphogenesis that are regulated by the F10L kinase.

Proteolytic processing of vaccinia virus structural proteins occurs in conjunction with viral morphogenesis (17, 25). Hruby and coworkers (49, 50, 54) defined the cleavage site as the AGX motif. An AGX motif in which X is A was previously identified as an N-terminal cleavage site in the A17L protein (34, 45, 54). We confirmed the presence of a second cleavage at or near the AGN sequence at amino acid 186. Although a natural cleavage site in which X is N had not previously been demonstrated, N as well as many other amino acids can function in a *trans*-processing assay (20). Proof that cleavage occurs

precisely at the A17L AGN site, however, will require C-terminal sequencing of the 21-kDa fragment or isolation and N-terminal sequencing of the putative 19-amino-acid fragment.

Cleavage of vaccinia virus structural proteins is generally blocked by the antibiotic rifampin, indicating that proteolysis occurs after the attachment of the D13L protein and formation of viral crescents (17, 58). Removal of the C terminus of the A17L protein was detected by 60 to 90 min after labeling but was unaffected by rifampin. This result agreed with data of Rodriguez et al. (31) and suggested that proteolysis of the A17L protein occurred at a very early stage of virus assembly or was independent of morphogenesis. Evidence supporting the former was obtained using F10L *ts* mutants that interrupt morphogenesis prior to the detection of viral membranes (48, 51). Importantly, the C-terminal cleavage of the A17L protein did not occur at the nonpermissive temperature. Therefore, viral membrane association appeared to be necessary for processing. Although membrane association has not yet been shown to be required for N-terminal processing of the A17L protein, it may be significant that both the N and C termini of the A17L protein are oriented on the concave surface of the IMV membrane (18, 56) where they could be accessed by a viral protease. Whitehead and Hruby (53) proposed that the vaccinia virus G1L ORF encodes a protease that is responsible for processing of vaccinia virus structural proteins. However, the absence of a conditional lethal G1L mutant has precluded the testing of whether this protein has a role in processing of the A17L protein.

Our studies also revealed that the A17L protein was phosphorylated. Interestingly, only the A17L protein with an intact C terminus was ^{32}P labeled. The absence of label in the 21-kDa processed form could have several explanations. One is that the kinase sites are in the C or the N termini and are removed by cleavage. Another is that the processed form becomes accessible to a phosphatase. In this respect, vaccinia virus encodes a dual-specificity tyrosine/serine phosphatase that is packaged in virus particles (11, 22). Chemical analysis of the 25-kDa species immunoprecipitated with antibody to the A17L protein revealed phosphorylated serine and threonine residues. Although we could not detect other phosphoamino acids in the protein immunoprecipitated with A17L N or C antibody, phosphotyrosine, phosphoserine, and phosphothreonine were detected in the 25-kDa protein induced by IPTG and immunoprecipitated with antibody to phosphotyrosine. Both the size of the protein and its specific induction support the idea that the phosphotyrosine was present in the A17L protein and not a contaminant. Although there are several possible explanations for the apparent discrepancy, the most likely one is that the phosphotyrosine antibody provided an enrichment for a small population of A17L protein molecules with phosphotyrosine. We also determined the phosphoamino acid composition of the A14L membrane protein because this had not previously been reported. Only phosphoserine was detected.

The presence of phosphothreonine and phosphoserine in A17L and A14L proteins provided an explanation for the morphogenesis defect of conditional lethal F10L kinase mutants. We found that neither the A17L nor the A14L protein was phosphorylated in cells infected with F10L *ts* mutants at a nonpermissive temperature. Similar results regarding phosphorylation of the A17L protein were independently observed by Derrien et al. (7). Since F10L is a serine/threonine kinase and is packaged in virus particles (21), the A17L and A14L proteins are probably substrates. Support for this hypothesis came from cotransfection experiments which demonstrated that the F10L kinase is the only vaccinia virus late gene product required for phosphorylation of the A17L protein. Another explanation, however, is that the F10L kinase activates a cellular kinase that is responsible for phosphorylation of the A14L and A17L proteins. A cellular kinase could explain the occurrence of tyrosine phosphorylation, as this specificity has not yet been described for the F10L kinase.

Previous studies demonstrated that antibody to the A27L IMV membrane protein bound a complex that contained the A14L and 21-kDa processed form of the A17L protein (34). We extended this observation by demonstrating coprecipitation of the C-terminal truncated and the nontruncated forms of the A17L protein with antibody to the C terminus of the A14L protein. This result indicated that the A14L and A17L proteins associate with each other before C-terminal cleavage of the latter or their interaction with the A27L protein. This sequence of events is consistent with immunoelectron microscopic studies which showed that the A27L protein does not associate with crescents but attaches to IV at a later stage of morphogenesis (41). Under conditions in which phosphorylation of the A14L and A17L proteins was blocked, coprecipitation of the two could not be demonstrated, suggesting that this modification is required for their interaction. However, under these conditions, as well as when expression of the A17L protein was repressed, the amount of A14L protein recovered after a 30-min pulse with [^{35}S]methionine was reduced. Shorter pulses might help to discriminate between the effect of the A17L protein on the synthesis and stability of the A14L protein.

In summary, we suggest that phosphorylation of the A17L and A14L proteins is mediated by the F10L kinase and is required to form the viral membranes associated with immature virions. Removal of phosphates and the acidic C-terminal peptide of the A17L protein occur during the transition to mature virions.

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