JOURNAL OF VIROLOGY, Aug. 1995, p. 4640–4648 Vol. 69, No. 8
0022-538X/95/$04.00+0

Vaccinia Virus A17L Gene Product Is Essential for an Early Step in Virion Morphogenesis

DOLORES RODRÍGUEZ,[1] MARIANO ESTEBAN,[1,2]* AND JUAN RAMÓN RODRÍGUEZ[1]

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, 28049 Madrid, Spain,[1] *and Department of Biochemistry, State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203*[2]

Received 24 February 1995/Accepted 26 April 1995

Vaccinia virus (VV) A17L gene encodes a 23-kDa protein that is proteolytically cleaved to generate a 21-kDa product that is incorporated into the viral particles. We have previously shown that the 21-kDa protein forms a stable complex with the VV 14-kDa envelope protein and suggested that the 21-kDa protein may serve to anchor the 14-kDa protein to the envelope of the virion (D. Rodríguez, J. R. Rodríguez, and M. Esteban, J. Virol. 67:3435–3440, 1993). To study the role of the 21-kDa protein in virion assembly, in this investigation we generated a VV recombinant, VVindA17L, that contains an inducible A17L gene regulated by the *E. coli* repressor/operator system. In the absence of the inducer, shutoff of the A17L gene was complete, and this shutoff correlated with a reduction in virus yields of about 3 log units. Although early and late viral polypeptides are normally synthesized in the absence of the A17L gene product, proteolytic processing of the major p4a and p4b core proteins was clearly impaired under these conditions. Electron microscopy examination of cells infected in the absence of isopropylthiogalactopyranoside (IPTG) revealed that virion morphogenesis was completely arrested at a very early stage, even prior to the formation of crescent-shaped membranes, which are the first distinguishable viral structures. Only electron-dense structures similar to rifampin bodies, but devoid of membranes, could be observed in the cytoplasm of cells infected with VVindA17L under nonpermissive conditions. Considering the most recent assembly model presented by Sodeik et al. (B. Sodeik, R. W. Doms, M. Ericsson, G. Hiller, C. E. Machamer, W. van't Hof, G. van Meer, B. Moss, and G. Griffiths, J. Cell Biol. 121:521–541, 1993), we propose that this protein is targeted to the intermediate compartment and is involved in the recruitment of these membranes to the viral factories, where it forms the characteristic crescent structures that subsequently result in the formation of virions.

Vaccinia virus (VV), the prototype member of the *Poxviridae* family, has a double-stranded DNA genome with a coding capacity of about 200 polypeptides. Viral transcription, replication, and progeny assembly occur entirely in the cytoplasm of infected cells within discrete areas called viral factories (19). The different stages of VV morphogenesis have been observed in detail by electron microscopy. The first characteristic viral structures formed are crescent-shaped membranes, which have been recently proposed to be derived from the intermediate compartment located between the endoplasmic reticulum and the Golgi apparatus (35). These structures progress into spherical immature virions (IV) enclosing a granular matrix (5, 6, 13), which subsequently mature into oval-shaped structures with an electron-dense core. These virions constitute the first infectious form of the virus, and they are now referred to as intracellular mature virus (IMV). A variable proportion of IMV, depending on the virus strain, acquire an additional double membrane derived from the *trans*-Golgi network (33). These intracellular enveloped virions (IEV) migrate to the cell surface, where the external membrane of the virus fuses with the plasma membrane, releasing the extracellular enveloped virions (EEV) from the cell. Additionally to the proteins incorporated into IMV, 10 virus-encoded polypeptides are exclusively present in EEV (9, 14, 24, 25). EEV play an important role in virus dissemination, both in vitro and in vivo (1, 4, 26, 27).

VV morphogenesis is one of the least characterized steps in the virus life cycle. Very little is known about the mechanism of virion assembly and the protein interactions involved in this complex process. However, through the generation of specific deletion, temperature-sensitive, drug-resistant, and most recently inducer-dependent conditional mutants, considerable progress has been made in the identification of viral polypeptides involved in the formation of both IMV and EEV. Among the proteins affecting IMV formation are the 11-kDa (gene F18R) (44), VP8 (gene L4R) (41), and I7 (gene I7L) (15) core proteins and the P65 (gene D3L) (45) and L1R (28) membrane proteins. Surprisingly, the 14-kDa envelope protein (gene A27L) was found to be dispensable for the formation of infectious IMV but essential for virion enwrapment by Golgi-derived membranes and EEV release (31). Other proteins identified as being required for formation and/or release of EEV are the acylated 37-kDa protein (gene F13L) (2, 34), the gp42 (gene B5R) (10, 42) and gp22-24 (gene A34R) (3, 8) glycoproteins, and a newly identified 43 to 50-kDa protein (gene A36R) (23), all members of the outermost membrane of EEV.

It has been hypothesized that formation and release of EEV may require the interaction between the 14-kDa protein on the surface of IMV and the 37- and/or 22 to 24-kDa proteins of EEV (8, 31, 34). Evidence for such interactions is still lacking. Instead, we have recently shown that the 14-kDa protein interacts with another component of IMV, a 21-kDa protein encoded by the viral A17L gene. Fractionation of virions with detergents and reducing agents showed that the 14-kDa protein is exposed in the outer membrane of IMV and that the 21-kDa protein occupies a more internal position within the virion. This finding, together with the fact that the 21-kDa protein contains two large hydrophobic domains characteristic

* Corresponding author.

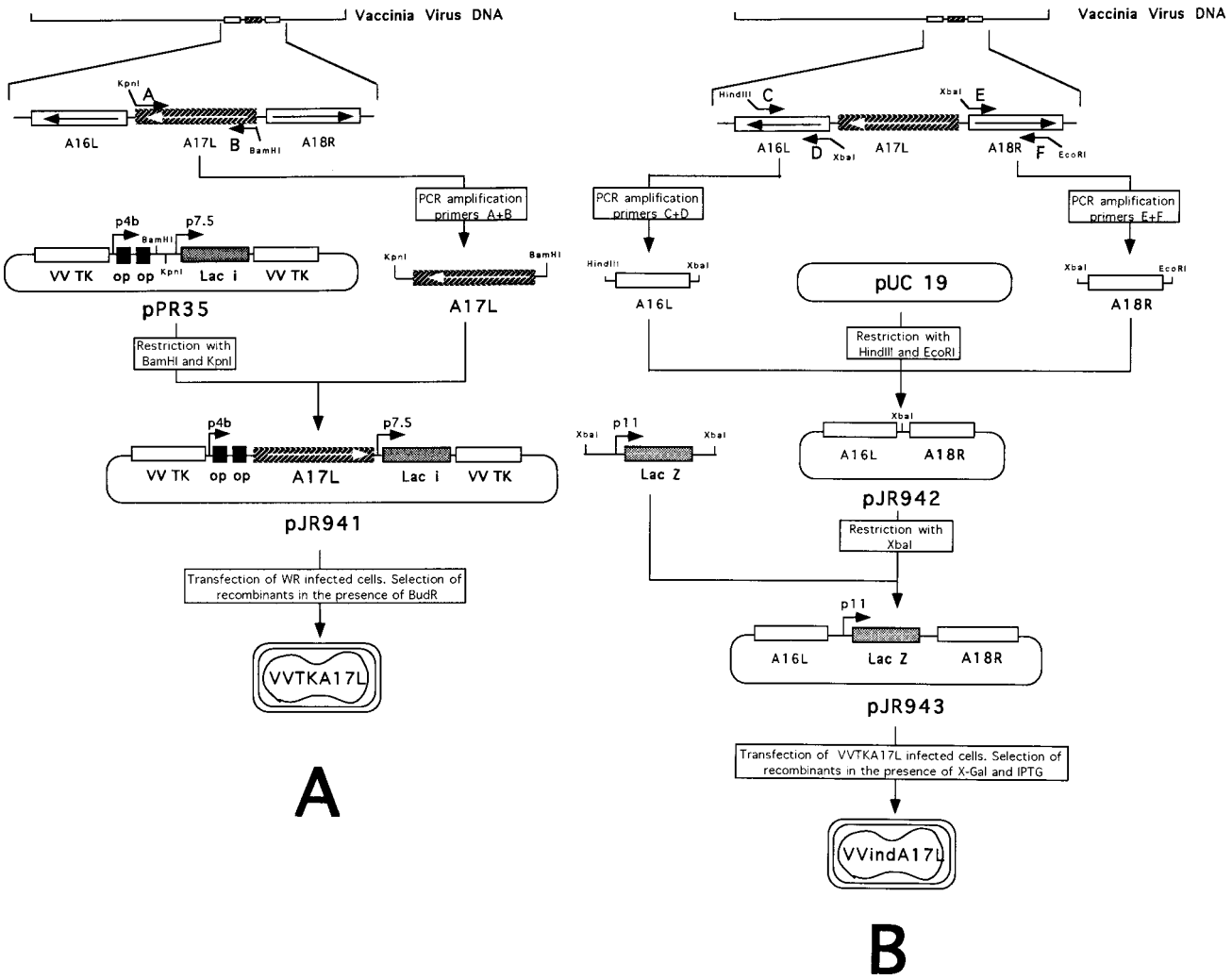

FIG. 1. Strategy used for construction of recombinant virus VVindA17L. (A) Generation of an intermediate VVTKA17L virus containing, in addition to the endogenous A17L gene, an inducible copy of this gene in the TK locus. A DNA fragment corresponding to the complete copy of the A17L gene was synthesized by PCR amplification, using primers A and B (sequences are given in Materials and Methods), and was cloned into plasmid pPR35 downstream of a hybrid inducible promoter consisting of the VV p4b promoter fused to two *lacI* operator (op) units. The resulting plasmid, pJR941, which contains also the *lacI* repressor gene under the control of the VV p7.5 promoter, was used to transfect BSC-40 cells infected with wild-type (WR) virus. TK^- intermediate viruses were selected after infection of TK-143 cells in the presence of bromodeoxyuridine. (B) Deletion from the VVTKA17L genome of the endogenous A17L gene and replacement by the *E. coli lacZ* gene to obtain VVindA17L viruses. Two DNA fragments corresponding to the left and right A17L flanking sequences were amplified by PCR, using primers C plus D and E plus F, respectively (sequences are given in Material and Methods), and were both cloned into pUC19 to generate plasmid pJR942. A DNA fragment containing the *E. coli lacZ* gene fused to the VV p11 promoter was introduced into the *Xba*I site of pJR942, between the two A17L flanking sequences. The resulting plasmid, pJR943, was used to transfect BSC-40 cells infected with VVTKA17L. Recombinant VVindA17L viruses containing only an inducible A17L gene were selected by the blue-plaque phenotype in BSC-40 cells infected in the presence of IPTG, after addition of X-Gal.

of membrane proteins whereas the 14-kDa protein lacks such a region, led us to suggest that the 21-kDa protein may serve to anchor the 14-kDa protein to the envelope of IMV.

To learn more about the role of the 21-kDa protein in virus infection and virion morphogenesis, in this investigation we generated a VV recombinant (VVindA17L) in which expression of the A17L gene product can be regulated by the *Escherichia coli lacI* repressor/operator system (30).

MATERIALS AND METHODS

Cells and viruses. BSC-40 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. TK-143 cells were maintained in DMEM containing 10% fetal calf serum. VV strain WR was propagated and titrated in BSC-40 cells. Recombinant virus VVindA17L was grown in BSC-40 cells in the presence of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Antisera. The rabbit polyclonal anti-14-kDa protein serum has been previously described (7). The rabbit polyclonal anti-23- to 21-kDa protein serum (40) was provided by D. E. Hruby.

Plasmid constructions. A complete copy of the A17L gene flanked by *Bam*HI and *Kpn*I restriction sites at the 5′ and 3′ ends, respectively, was generated by PCR, using VV genomic DNA as the template and oligonucleotide primers A (5′-GGGGTACCCCCATTCTTAATAATCGTCAG-3′) and B (5′-CCGGATCCCCCATGAGTTATTTAAGATATT-3′) (the *Kpn*I and *Bam*HI sites are underlined) as shown in Fig. 1A. The 638-bp PCR product was cloned into plasmid pPR35 (30), previously digested with restriction enzymes *Bam*HI and *Kpn*I, generating the insertion plasmid pJR941 (Fig. 1A). The PCR product was sequenced to confirm its identity to the A17L viral sequence. Two DNA fragments homologous to the 5′ and 3′ flanking sequences of the A17L gene were generated by PCR amplification, using VV DNA as the template and oligonucleotide primers C (5′-GCCCAAGCTTGGGCTAATGCCGTATCCCCAAAAG-3′), D (5′-CGCTCTAGAGCGTGCGGGAAATAAGGTAGATG-3′), E (5′-CGCTCTAGAGCGGCACCCGCAGAGAAGTCGTCA-3′), and F (5′-CGGAATTCCGTCCAATCCGACTGCCTCTACC-3′) (Fig. 1B). Primers C and D, containing *Hin*dIII and *Xba*I sites (underlined), respectively, were used to produce a 786-bp fragment corresponding to the A17L open reading frame. Similarly, oligonucle-

otides E and F, including *Xba*I and *Eco*RI sites (underlined), respectively, were used to amplify a 561-bp DNA fragment homologous to the A18R gene located downstream of the A17L gene (Fig. 1B). The resulting A16L and A18R PCR products were ligated together into *Hin*dIII-*Eco*RI-digested pUC19 to generate pJR942. An *Xba*I fragment containing the *E. coli lacZ* gene under the control of the VV p11 promoter (32) was cloned into the *Xba*I site of pJR942, generating the deletion plasmid pJR943.

Recombinant virus construction. The intermediate recombinant virus VVTKA17L, containing an inducible copy of the A17L gene in the thymidine kinase (TK) locus, was generated by transfecting WR-infected BSC-40 cells with plasmid pJR941. TK^- recombinant viruses were selected and plaque purified twice on TK-143 cells infected in the presence of 25 mg of 5-bromodeoxyuridine per ml. To delete the endogenous A17L gene, BSC-40 cells infected with VVTKA17L in the presence of 5 mM IPTG were transfected with plasmid pJR943. VVindA17L recombinant viruses were selected by blue-plaque phenotype after the addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to the infected monolayer.

Metabolic labeling of viral proteins and immunoprecipitation analysis. BSC-40 cells infected (5 PFU per cell) with WR or VVindA17L in the absence or presence of IPTG (5 mM) were labeled with [^{35}S]methionine from 6 to 24 h postinfection (hpi) by incubation in methionine-free medium supplemented (1:10) with DMEM containing [^{35}S]methionine (10 μCi/ml). After the 18-h labeling period, cells were washed with phosphate-buffered saline (PBS), collected, and resuspended in immunoprecipitation lysis buffer (20 mM Tris [pH 8.0], 80 mM NaCl, 20 mM EDTA, 1% Nonidet P-40, protease inhibitors [bacitracin {2 mg/ml}, trypsin inhibitor {2 mg/ml}, 1 mM phenylmethylsulfonyl fluoride, and leupeptin {10 mg/ml}]). Cell extracts were maintained for 30 min on ice, sonicated, and clarified by centrifugation at 10,000 × *g* for 5 min. The supernatants were immunoprecipitated with specific antibodies as previously described (29). After washing, the immunoprecipitates were resuspended in 2× sample buffer (1.25 M Tris [pH 6.8], 0.2% sodium dodecyl sulfate [SDS], 1% bromophenol blue, 10% 2-β-mercaptoethanol). Samples were boiled for 3 min and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were dried, and the proteins were visualized after autoradiography.

For pulse-chase analysis, cells were infected (5 PFU per cell) with WR or VVindA17L in the presence or absence of 5 mM IPTG; where indicated, cells were also maintained in the presence of rifampin (100 μg/ml) or hydroxyurea (HU) (5 mM). At 6 hpi, cells were washed with methionine-free DMEM and incubated in the same medium for 30 min. Cells were then pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 30 min and chased with a 100-fold excess of unlabeled methionine for the indicated times. Cells were placed on ice, washed three times with ice-cold PBS, collected, and lysed in 1× sample buffer for total protein profile analysis or resuspended in lysis buffer and processed for immunoprecipitation as described above. Immunoprecipitates were resuspended in 2× sample buffer. Proteins were fractionated by SDS-PAGE and visualized after autoradiography of the dried gel.

One-step growth of VVindA17L. Confluent monolayers of BSC-40 cells were infected with strain WR or recombinant virus VVindA17L at a multiplicity of infection (MOI) of 2.5 PFU per cell. The inoculum was removed after 1 h, and the cells were washed with DMEM and overlaid with fresh DMEM supplemented with 2% newborn calf serum and containing or lacking IPTG (5 mM). Cells were harvested at various times postinfection, and progeny viruses were titrated by plaque assay on monolayers of BSC-40 cells in the presence of IPTG.

Electron microscopy. HeLa cells were infected at an MOI of 5 PFU per cell with strain WR in the presence or absence of rifampin (100 μg/ml) or with recombinant virus VVindA17L in the presence or absence of IPTG (5 mM). At 22 hpi, cells were placed on ice, washed twice with ice-cold PBS, and incubated with proteinase K (50 mg/ml) in PBS. After 3 min, cells were collected, pelleted, and processed for electron microscopy as previously described (17). Briefly, cells were fixed in 2% glutaraldehyde and 2% tannic acid in PBS at room temperature for 1 h and washed with PBS three times. Postfixation was carried out with 1% (wt/vol) OsO_4 in PBS at 4°C for 1 h. Samples were dehydrated through a 30 to 100% (vol/vol) ethanol series and embedded in Epon 812 (Electron Microscopy Laboratories, Ltd.).

RESULTS

Generation of recombinant virus VVindA17L. For the construction of a recombinant virus with an inducible A17L gene, we first generated an intermediate recombinant virus containing two copies of the A17L gene, the endogenous copy in its original locus, and a *lacI* operator-controlled gene in the TK region of the viral genome. To obtain this recombinant virus, a complete copy of the of A17L gene obtained by PCR amplification was cloned into plasmid pPR35 (30), downstream of two *lacI* operator units. The resulting plasmid, pJR941, was used to transfect BSC-40 cells infected with wild-type (WR) virus. TK^- viruses (VVTKA17L) were selected after infection of TK-143 cells in the presence of bromodeoxyuridine. The strategy followed to obtain the recombinant virus VVTKA17L is depicted in Fig. 1A.

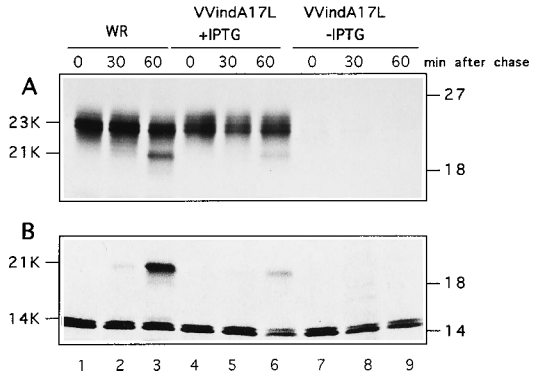

FIG. 2. Kinetics of proteolytic processing of the 23-kDa protein to a 21-kDa protein and of 21-kDa–14-kDa protein complex formation in VVindA17L-infected cells. (A) Immunoprecipitation of the 23-kDa precursor and the 21-kDa cleaved product with anti-21- to 23-kDa protein antibodies. BSC-40 cells were infected (MOI of 5 PFU per cell) with WR (lanes 1 to 3) or VVindA17L in the presence (lanes 4 to 6) or absence (lanes 7 to 9) of IPTG (5 mM). At 6 hpi, cells were pulse-labeled with [^{35}S]methionine for 30 min and chased with a 100-fold excess of unlabeled methionine. Cells were harvested immediately after the 30-min pulse (0) or after 30 or 60 min, and cell extracts were immunoprecipitated with antibodies specific for the 21- to 23-kDa protein. The immunoprecipitated products were separated by SDS-PAGE (12% polyacrylamide gel). The gel was dried, and protein bands were revealed by autoradiography. Chase periods are indicated above the lanes. (B) Coimmunoprecipitation of the 14- and 21-kDa proteins by a polyclonal serum specific for the 14-kDa protein. The extracts used for panel A were subjected to immunoprecipitation with antibodies specific for the 14-kDa protein. The positions of 23-, 21-, and 14-kDa proteins are indicated at the left. Positions of molecular weight markers in kilodaltons are represented at the right.

Next, we deleted the endogenous A17L gene from the VVTKA17L viral genome to obtain recombinant viruses containing only an inducible copy of the gene. For this, two DNA fragments corresponding to left and right A17L flanking sequences were amplified by PCR and cloned into pUC19 to generate plasmid pJR942 (Fig. 1B). Then, a DNA fragment containing the *lacZ* gene from *E. coli* downstream of the VV p11 promoter was introduced into the *Xba*I site of pJR942, between the two A17L flanking sequences. The resulting plasmid, pJR943, was used to transfect BSC-40 cells infected with VVTKA17L. Recombinant VVindA17L viruses were selected by the blue-plaque phenotype in BSC-40 cells infected in the presence of IPTG, after addition of X-Gal (Fig. 1B).

IPTG-dependent expression of the A17L gene in cells infected with recombinant VVindA17L. To confirm that synthesis of the A17L gene product was dependent on the presence of the inducer IPTG in the cells infected with recombinant virus VVindA17L and to investigate if, as occurs in WR-infected cells, the A17L gene product is synthesized as a 23-kDa polypeptide precursor which is subsequently processed into a 21-kDa product (29), we performed a pulse-chase and immunoprecipitation analysis. Cells infected for 6 h with WR or VVindA17L in the absence or presence of IPTG were pulse-labeled with [^{35}S]methionine and chased at different times, and proteins were immunoprecipitated with antibodies specific to the 23-kDa protein. As shown in Fig. 2A (lanes 7 to 9), neither the 23- nor 21-kDa product could be observed when cells were infected in the absence of IPTG, indicating complete shutoff of the A17L gene expression under these conditions. However, in cells infected with VVindA17L under permissive conditions, the pattern of synthesis and processing of the 23-kDa protein was the same as in WR-infected cells (compare lanes 4 to 6 with lanes 1 to 3), although the amounts of both 23- and

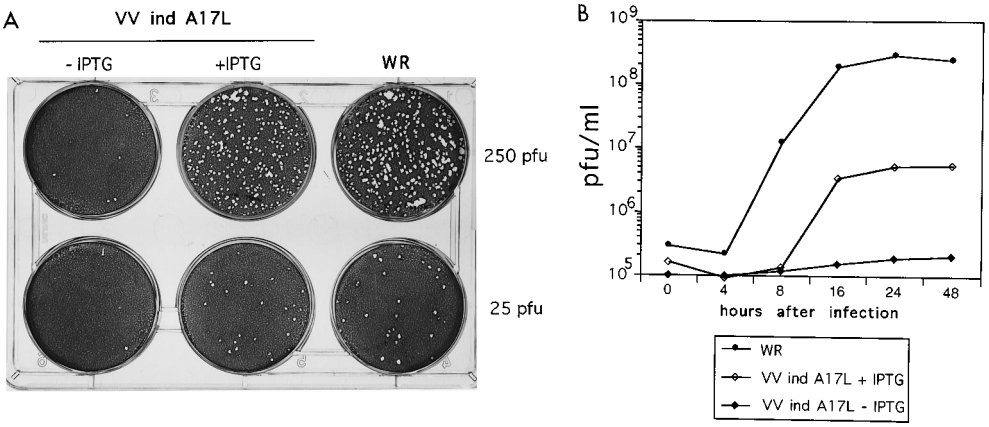

FIG. 3. Effect of inhibition of synthesis of A17L gene product on virus growth. (A) Confluent monolayers of BSC-40 cells were infected with the indicated PFU of either WR or VVindA17L and overlaid with a mixture consisting of DMEM, 0.9% Bacto Agar, and 2% newborn calf serum, containing or lacking 5 mM IPTG. After 48 h, the monolayers were stained with 1% crystal violet. (B) One-step growth curves. BSC-40 cells were infected with WR or VVindA17L in the presence or absence of 5 mM IPTG at an MOI of 2.5 PFU per cell. Cells were collected at the indicated times after infection, and virus yields were determined by titration on BSC-40 cells in the presence of 5 mM IPTG.

21-kDa polypeptides were reduced, most likely because of an incomplete derepression of the gene after IPTG addition (30).

We have also demonstrated that the 21-kDa protein forms a stable complex with the 14-kDa envelope protein (29); thus, to study the interaction between these two proteins, the same cell extracts were immunoprecipitated with antibodies specific for the 14-kDa protein. Between 30 and 60 min of chase, the 21-kDa product coprecipitated together with the 14-kDa product in cells infected with either WR (Fig. 2B, lanes 1 to 3) or VVindA17L (lanes 4 to 6) under permissive conditions. However, the 14-kDa protein was the only product immunoprecipitated from cells infected in the absence of the inducer (lanes 7 to 9).

The results of Fig. 2 demonstrate that expression of the A17L gene can be clearly controlled by the inducible system and that under permissive conditions, expression and processing of the 23-kDa protein encoded by the inducible A17L gene, as well as the interaction between the 21- and 14-kDa proteins, follow the same kinetics as in WR-infected cells.

Virus growth is impaired in cells infected with VVindA17L in the absence of the inducer. Our next approach was to examine the ability of recombinant virus VVindA17L to form plaques in the absence of the inducer IPTG. As shown in Fig. 3A, normal-size plaques, comparable to those of WR, were obtained when cells were infected with VVindA17L in the presence of IPTG. On the other hand, in cells infected with the same virus in the absence of the inducer, a dramatic reduction (40 to 50-fold) in the number of plaques was observed.

To establish if the reduction in plaque number observed in the absence of the inducer correlates with an inhibition in viral replication, one-step virus growth curves were performed. The results represented in Fig. 3B show that in the absence of the inducer, virus titers do not increase over time, and a difference of about 3 log units in virus yields was observed with respect to cells infected with strain WR. Addition of IPTG restores virus yields, but they remained lower than those of strain WR. These results show that the A17L gene product is essential for the generation of infectious virus.

The absence of the A17L gene product results in inhibition of proteolytic processing of the major VV structural proteins. It is possible that the dramatic reduction in the production of infectious progeny viruses under nonpermissive conditions (Fig. 3) correlates with a blockage on the proteolytic cleavage of the major core proteins as a result of the lack of the 21- to 23-kDa protein, as has been shown for other VV structural proteins (28, 44, 45). To test this possibility, we carried out a pulse-chase analysis. Cells infected with WR or VVindA17L in the absence or presence of IPTG were pulse-labeled with [^{35}S]methionine at 6 hpi. After 30 min, cells were chased and harvested immediately or maintained in culture for another 18 h. Cells infected in the presence of HU and pulse-labeled for 30 min were included to test for early protein synthesis. As shown in Fig. 4, the pattern of early protein synthesis in cells infected with VVindA17L with (lane 6) or without (lane 10) IPTG was indistinguishable from that of WR-infected cells (lane 2). Similarly, no differences could be observed in the profiles of late proteins after a 30-min pulse in VVindA17L-infected cells treated (lane 7) or not treated (lane 11) with IPTG and in WR-infected cells (lane 3), except for the appearance of the β-galactosidase protein (120 kDa) in cells infected with the recombinant virus. However, analysis of the 18-h chase samples showed that while in WR-infected cells, the major p4a and p4b precursors had been cleaved to give rise to the 4a and 4b proteins, respectively, these two processed products were completely absent in cells infected with VVindA17L under nonpermissive conditions. An intermediate situation was observed in cells infected with VVindA17L in the presence of the inducer; in these cells, processing of the precursors took place, although the amounts of the mature 4a and 4b products were lower than in WR-infected cells.

From these findings, we conclude that while the A17L gene product is not required for a normal temporal expression of the VV polypeptides, it is essential for the proper proteolytic processing of major core precursors p4a and p4b.

VV morphogenesis is blocked when synthesis of the A17L gene product is repressed. The inability of VVindA17L to establish a productive infection in the absence of the inducer and inhibition of proteolytic processing of the major core precursors in cells infected by VVindA17L indicate that under these conditions, virion morphogenesis should be interrupted at some stage. To investigate this possibility, VV morphogenesis was monitored by electron microscopy. HeLa cells infected for 24 h with either WR or VVindA17L under permissive or nonpermissive conditions were fixed and processed for electron microscopy. In cells infected with VVindA17L in the presence of IPTG, the different stages of virion maturation are observed (Fig. 5B and D); however, in the absence of the

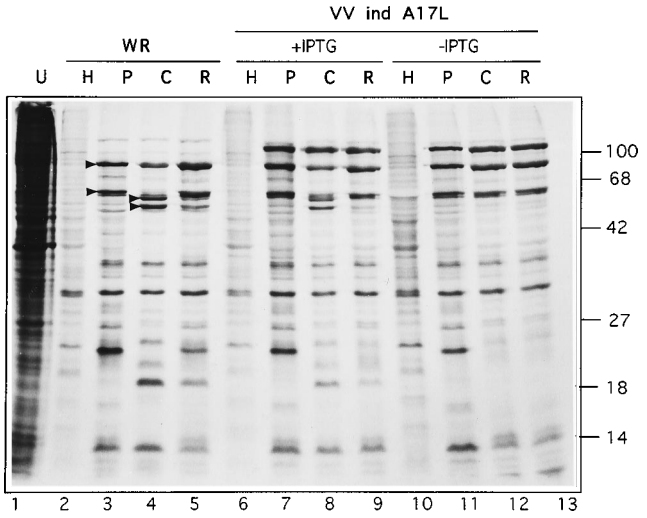

FIG. 4. Inhibition of proteolytic processing of major core proteins p4a and p4b in VVindA17L-infected cells. BSC-40 cells were infected with WR (lanes 2 to 5) or VVindA17L in the presence (lanes 6 to 9) or absence (lanes 10 to 13) of IPTG (5 mM) as indicated at the top. Two cultures of each group of infected cells were treated with either HU (10 mM; H) or rifampin (100 μg/ml; R). At 6 hpi, untreated and treated cells were pulse-labeled with [^{35}S]methionine for 30 min and then chased with unlabeled methionine. The HU-treated cells and one culture of untreated cells from each group (P) were harvested immediately after the chase, while the rifampin-treated cells and the remaining untreated cells (C) were kept in culture for another 18 h. Cells were lysed, and proteins were resolved by SDS-PAGE (12% polyacrylamide gel) and visualized after autoradiography of the dried gel. An extract from uninfected cells (U; lane 1) labeled with [^{35}S]methionine for 30 min was included in the gel. Starting from the top, the arrowheads indicate the positions of the p4a, p4b, 4a, and 4b polypeptides. Positions of molecular weight markers in kilodaltons are represented at the right.

inducer, only discrete electron-dense structures not delimited by a membrane are observed in the cytoplasm of infected cells. Noteworthy in Fig. 5A and C are the absolute absence of either mature particles, IV, or even crescent-shaped membranes, the earliest distinguishable stage in VV morphogenesis.

It is well known that rifampin reversibly blocks VV morphogenesis (20) and that in cells infected with strain WR in the presence of this drug, electron-dense structures surrounded by an irregularly shaped membrane, known as rifampin bodies, accumulate in the cytoplasm (12, 22). As shown in Fig. 6, although the electron-dense structures observed in VVindA17L-infected cells under nonpermissive conditions are devoid of membrane, their morphology clearly resembles that of the rifampin bodies (compare Fig. 6E and F with Fig. 6C and D).

From these results, we conclude that the A17L gene product is required at a very early stage of VV morphogenesis, even before membranes from the intermediate compartment are recruited for the formation of crescent structures.

DISCUSSION

Our knowledge on VV assembly has traditionally been based on observations by conventional electron microscopy. Also, the identification and subviral localization of a number of virion protein components have been possible by fractionation of viral particles through treatment with detergents and reducing agents. However, the development of cryoelectron microscopy techniques, which offer better preservation of the ultrastructure of infected cells and allow for the application of immunolabeling techniques to localize both cellular and viral polypeptides with specific antibodies, is helping to modify and increase our understanding of the VV morphogenesis process. From these studies, it has been proposed that the IMV particles contain two membranes derived from the intermediate compartment (35) and that the membranes surrounding IEV and EEV are acquired from the *trans*-Golgi network (33). In addition, although work with deletion, temperature-sensitive, and drug-resistant mutants has allowed the identification of several virion components that are required for virus assembly, the recent application of the *E. coli lacI* operator-repressor system to generate inducer-dependent conditional mutants (11, 30) has provided a powerful tool with which to investigate the role of specific gene products in VV morphogenesis and/or infectivity. Since the development of this technology, a number of recombinant viruses that can inducibly express different virion proteins have been generated (8, 23, 28, 31, 41, 43–45). From biochemical and ultrastructural studies with all of these mutant viruses, it has been possible to establish that (i) the D13L gene product (p65 membrane protein), which is the target of rifampin (18, 37), is required at an early stage of virion morphogenesis prior to formation of immature viral envelopes (45), (ii) the proteins encoded by F18R (11-kDa core protein) (44) and L1R (myristylated membrane protein) (28) are both needed for transition from IV to IMV, (iii) each of these three proteins is also required for proteolytic cleavage of the major p4a and p4b precursors, a process that has been shown to be coupled to virion maturation (16, 21, 39), (iv) the I7L gene product (47-kDa core protein) is also required at a stage subsequent to the formation of spherical IV particles (15) and thus is likely needed for the processing of p4a and p4b, although this has not been determined, (v) the L4R gene product (VP8 core protein) is required for virus infectivity but not for IMV or EEV formation, although abnormal IV particles are produced when L4R expression is repressed (41), (vi) enwrapment of IMV by Golgi-derived membranes to produce EEV requires the presence of the A27L gene product (14-kDa membrane protein of IMV) (31) and of the proteins present in external membrane of EEV, encoded by the F13L (37-kDa protein) (2, 34), A34R (gp22-24) (8), B5R (gp42) (10, 42), and A36R (43- to 50-kDa protein) (23) genes, and (vii) the A34R gene product is also involved in the release of EEV from the cell membrane (3). These results are summarized in Fig. 7, which shows a schematic representation of the different stages of VV assembly, indicating the proteins that have been demonstrated to participate in the formation of the different stages in viral morphogenesis. Cleavage of virion proteins by a protease(s) appears to occur at a late stage, after IV particles are formed (stage IV in Fig. 7) (38, 39).

Despite recent advances in the identification of all of these essential viral proteins, very little is known about the protein-protein interactions that should mediate the incorporation of the different virion components during the assembly process. In this regard, we have recently identified a 21-kDa virion protein that interacts with the 14-kDa envelope protein. The 21-kDa protein is the processed form of a 23-kDa precursor, which is encoded by the viral A17L gene. To study the role of the 21-kDa protein in VV morphogenesis, in this investigation we constructed a VV recombinant with an IPTG-inducible A17L gene, using the regulatable system mentioned above. The endogenous gene has been completely deleted from the genome and replaced by the *E. coli lacZ* gene (Fig. 1).

Repression of synthesis of the 23-kDa protein was complete in cells infected with VVindA17L in the absence of the inducer. On the other hand, this protein was clearly synthesized when IPTG was added to the infected cells, although the levels of expression were lower than those in WR-infected cells (Fig. 2). This partial induction after IPTG addition is related to the presence in the genome of the recombinant virus of two oper-

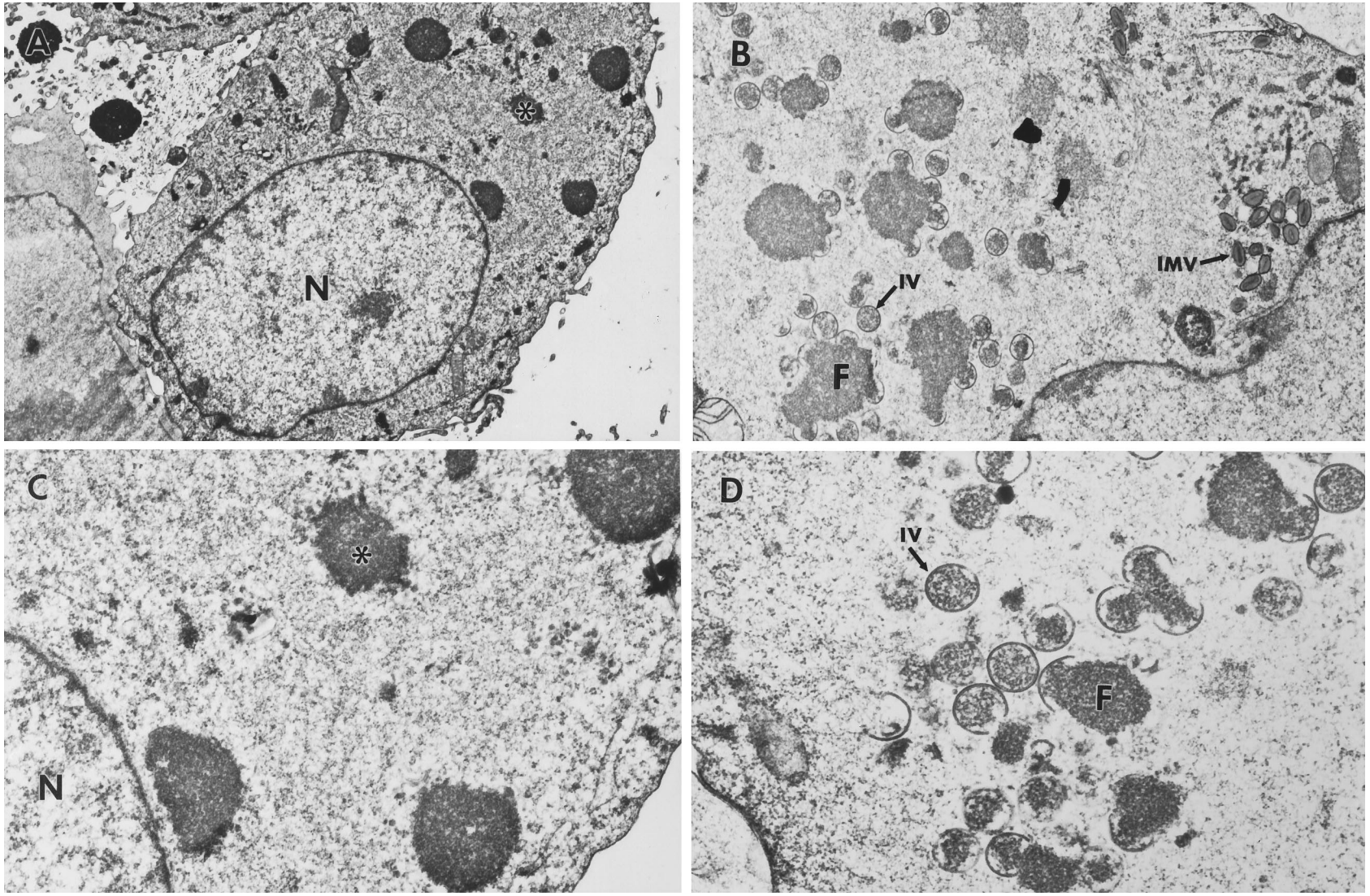

FIG. 5. Electron microscopy analysis of VVindA17L morphogenesis. HeLa cells were infected with VVindA17L (5 PFU per cell) in the absence (A and C) or presence (B and D) of IPTG (5 mM). Samples were prepared for transmission electron microscopy as indicated in Materials and Methods. The different stages of viral morphogenesis are indicated in panels B and D: factories (F), IV, and IMV. In panels A and C, unusual electron-dense structures devoid of membranes (a representative form is indicated by the asterisk) are very abundant, while the characteristic viral forms are completely absent. Magnifications: ×4,000 (A), ×8,000 (B), ×12,000 (C), and ×15,000 (D).

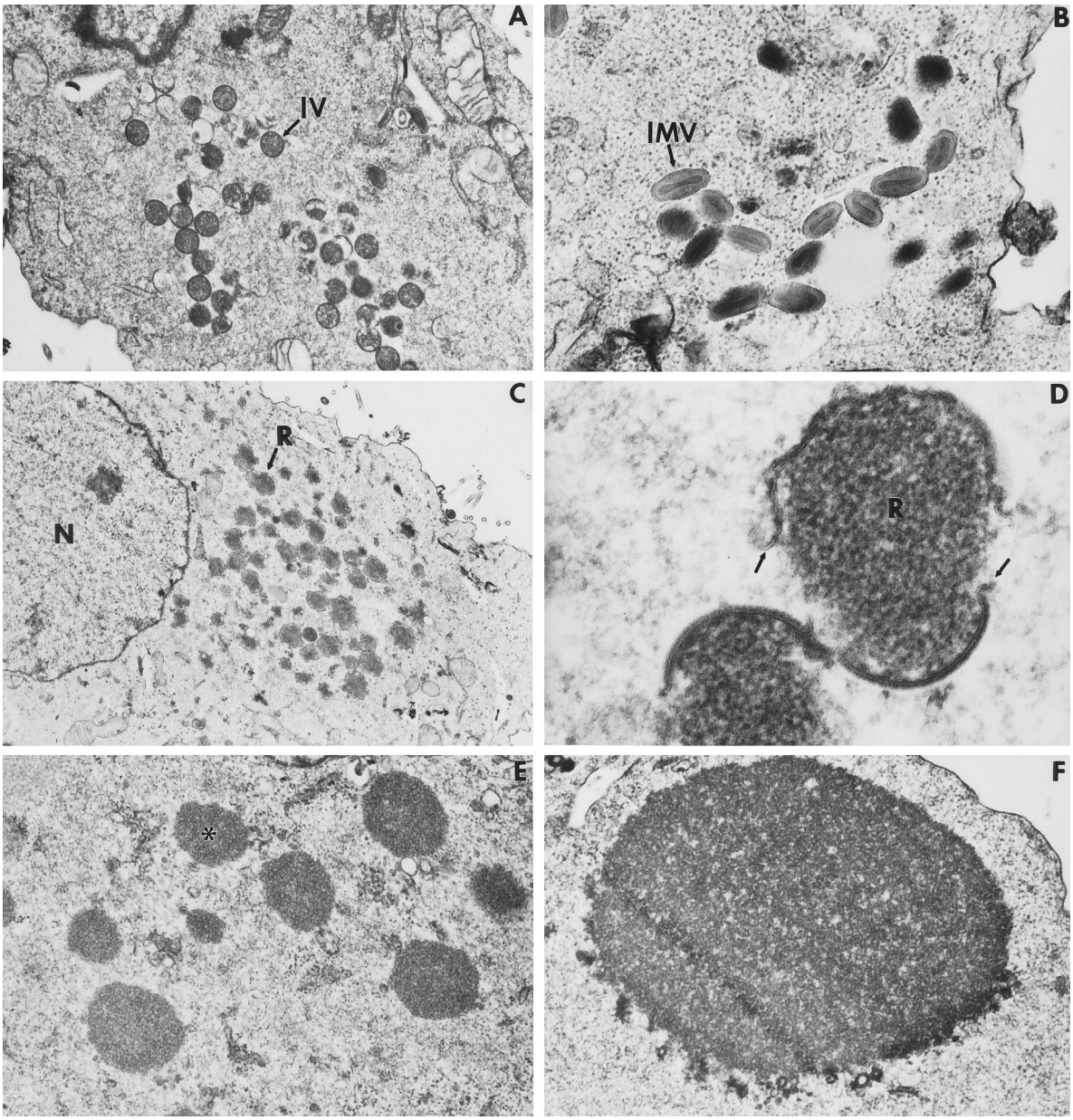

FIG. 6. Comparison by electron microscopy of the morphogenesis blockage induced by inhibition of 21- to 23-kDa protein synthesis and by rifampin treatment. HeLa cells were infected (5 PFU per cell) with WR in medium lacking (A and B) or containing (C and D) rifampin (100 μg/ml) or infected with VVindA17L in the absence of the inducer. At 24 hpi, cells were harvested and processed for electron microscopy. In panels A and B, IV and IMV are the predominant viral forms. In panels C and D, accumulation of rifampin bodies (R) that consist of an electron-dense matrix surrounded by an irregularly shaped membrane (the membranes are indicated by the arrows) can be observed. In panels E and F, the previously described electron-dense structures clearly resemble the rifampcin bodies, although the former apparently are not delimited by membranes (a representative form is marked by an asterisk). Magnifications: ×10,000 (A), ×25,000 (B), ×6,000 (C), ×60,000 (D), ×15,000 (E), and ×15,000 (F).

ator sequences upstream of the A17L gene (Fig. 1A). It has been reported that this type of construct results in tighter (99.9%) inhibition of target gene expression but lower (50%) reversion after addition of the inducer. Although only partial induction of synthesis of the 23-kDa protein was obtained upon IPTG addition (Fig. 2), processing of the 23-kDa precursor to produce the 21-kDa protein, as well as interaction between the 21- and 14-kDa proteins, followed the same kinetics as in WR-infected cells (Fig. 2).

Production of infectious progeny virus was abolished when 21- to 23-kDa protein synthesis was repressed. In the absence of IPTG, very few viral plaques were observed (Fig. 3A). These are normal-size plaques, and they are most likely produced by *lacI* repression-escape mutants, as has been proposed for other conditional-lethal recombinant viruses (41, 45). No other plaques appeared even after 10 days of infection. Moreover, in cells infected with VVindA17L in the absence of IPTG, virus titers were 1,000-fold lower than in WR-infected cells (Fig. 3B). In addition, in the presence of the inducer, VVindA17L yields were restored only up to about 50%. This result is in good agreement with the reduced level of 21- to 23-kDa protein expression observed after IPTG addition. Thus, we conclude

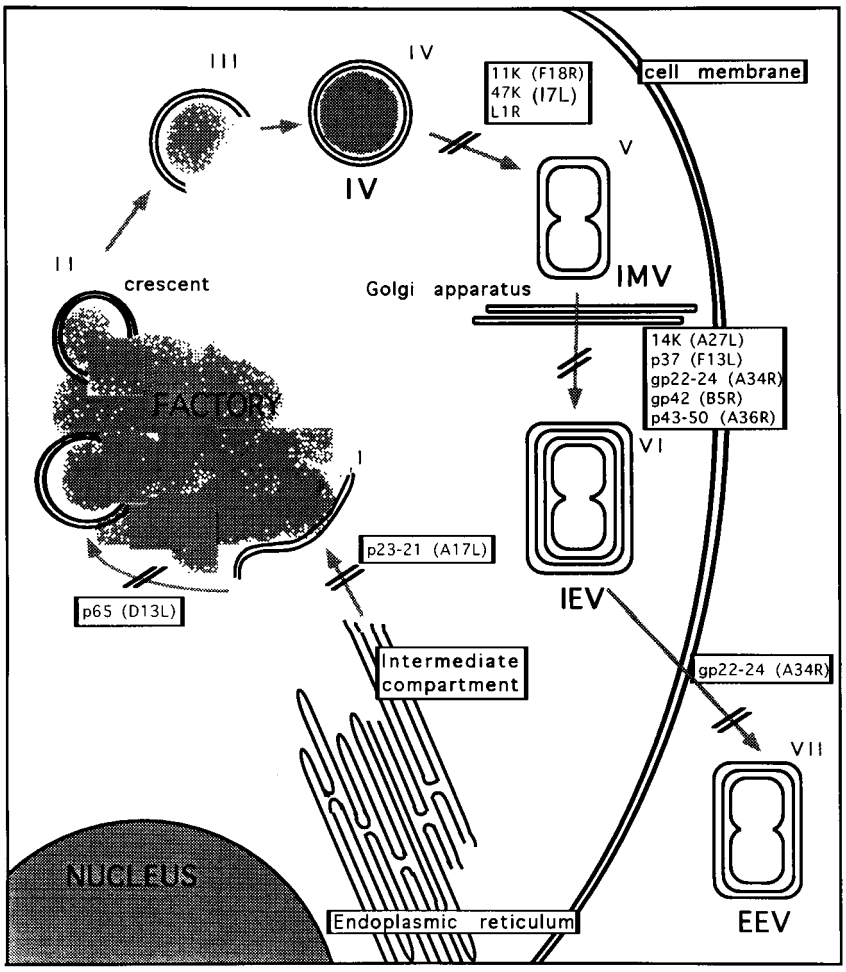

FIG. 7. Scheme of the proposed model for VV morphogenesis. The most recent model in VV assembly (35) proposed that the typical viral crescents (stage II) that are formed in the early stages of VV morphogenesis derived from cellular membranes of the intermediate compartment. Interaction between viral membrane proteins incorporated into the intermediate compartment and core proteins present in the viral factories (stage I) would be required for the formation of the crescent structures. In the present report, we have shown that membranous structures associated with the factories are not observed when the 21- to 23-kDa protein is not synthesized, indicating that this protein is involved in this process. Association of membranes to viral factories occurs in the presence of rifampin; however, acquisition of typical crescent morphology is blocked both by treatment with this drug and by repression of synthesis of the 65-kDa protein, indicating that this latter protein is required for the transition from stage I to stage II. Then, crescent structures separate from the factories (stage III), and IV are formed (stage IV). Maturation to give rise to the first infectious viral particles, IMV (stage V), requires the presence of the 11- and 47-kDa core proteins and the L1R myristylated protein. Envelopment of IMV by Golgi-derived membranes to generate IEV (stage VI) involves the 14-kDa, p37, gp22-24, gp42, and p43-50 proteins. Exit of EEV from the cell occurs after fusion of the most external membrane of IEV with the plasma membrane (stage VII). A proportion of EEV, which varies depending on the virus strain, remains associated to the cell surface, and these retention differences are related to differences in the gp22-24 protein among strains.

that the 21- to 23-kDa protein is essential for virus replication and that it must be required in stoichiometric quantities.

Although viral protein synthesis appears to be normal in the absence of the A17L gene product, pulse-chase experiments revealed that proteolytic cleavage of the p4a and p4b precursors is completely blocked under these conditions (Fig. 4). These results resemble those obtained by inhibition of synthesis of the L65 (D13L) or L1R membrane protein or the 11-kDa core protein.

Electron micrographs of cells infected with VVindA17L showed that in the absence of the 21- to 23-kDa protein, virion morphogenesis was arrested at a very early stage, prior to formation of viral crescents or any other membrane-coated viral structures. Under these conditions, only granular electron-dense structures, similar to rifampin bodies but devoid of membrane, could be observed (Fig. 5A, 5C, 6E, and 6F). These results suggest that the 21- to 23-kDa protein might be involved in the recruitment of membranes derived from the cellular intermediate compartment to the viral factories. Subsequently, and with the participation of the p65 protein, which appears to function as an internal protein scaffold (36), these membranes would give rise to the typical crescent structures which are the precursors of the membranes of IMV. This is consistent with the model proposed by Sodeick et al. (35), in which the 21- to 23-kDa protein would be one of the previously unidentified virus-encoded proteins that are targeted to the membranes of the intermediate compartment and that may interact with the viral core proteins, while L65 would be involved in protein-protein interactions within the membranes. Thus, as is depicted in the assembly model of Fig. 7, the 21- to 23-kDa protein would be the protein known to be required at the earliest stage of the virion assembly process.

Although these results support our previous interpretation that the 21- to 23-kDa protein is a transmembrane protein that serves to anchor the 14-kDa protein to the membrane of IMV, it would be of interest to define the localization of the 14-kDa

protein in infected cells in the absence of the 21- to 23-kDa protein. By immunofluorescence and immunoelectron microscopy, we would be able to determine the fate of the 14-kDa protein and other viral proteins in a context lacking the 21- to 23-kDa protein. Through *trans*-complementation assays as described by Ravanello and Hruby (28), we may be able to determine which domains of the 21- to 23-kDa protein are essential for the interaction with the 14-kDa protein, restoration of morphogenesis, and production of infectious virus.

In summary, in this investigation we demonstrate that 21- to 23-kDa protein is essential for the formation of viral progeny, acting at the earliest stage in the virion assembly process thus far identified. The virus system that we have described will provide the basis for in-depth studies of the early stages of VV morphogenesis.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI32361 and by Comisión Interministerial de Ciencia y Tecnología grant BIO92-0401. D. Rodríguez and J. R. Rodríguez were recipients of contracts from the CSIC-Fundación Ramón Areces and MEC (Spain), respectively.

We thank D. E. Hruby for the generous gift of anti-21- to 23-kDa protein serum. We thank Maria Teresa Rojas for expert assistance with electron microscopy and Victoria Jimenez for skillful technical assistance.

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