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Characterization of Early Stages in Vaccinia Virus Membrane Biogenesis: Implications of the 21-Kilodalton Protein and a Newly Identified 15-Kilodalton Envelope Protein

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Vaccinia virus (VV) membrane biogenesis is a poorly understood process. It has been proposed that cellular membranes derived from the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) are incorporated in the early stages of virion assembly. We have recently shown that the VV 21-kDa (A17L gene) envelope protein is essential for the formation of viral membranes. In the present work, we identify a 15-kDa VV membrane protein encoded by the A14L gene. This protein is phosphorylated and myristylated during infection and is incorporated into the virion envelope. Both the 21- and 15-kDa proteins are found associated with cellular tubulovesicular elements related to the ERGIC, suggesting that these proteins are transported in these membranes to the nascent viral factories. When synthesis of the 21-kDa protein is repressed, organized membranes are not formed but numerous ERGIC-derived tubulovesicular structures containing the 15-kDa protein accumulate in the boundaries of the precursors of the viral factories. These data suggest that the 21-kDa protein is involved in organizing the recruited viral membranes, while the 15-kDa protein appears to be one of the viral elements participating in the membrane recruitment process from the ERGIC, to initiate virus formation.

Vaccinia virus (VV) is a large DNA animal virus that belongs to the *Poxviridae* family. The 191-kb DNA genome (Copenhagen strain) has been sequenced and found to contain about 200 potential genes (11). A distinctive characteristic of poxviruses is that transcription, DNA replication, and virion assembly all occur within the cytoplasm of the infected cells, in particular areas designated viral factories (reviewed in reference 25). Traditionally, the description of VV assembly after visualization of infected cells by electron microscopy starts with the appearance of characteristic crescent-shaped membranes in the periphery of the electron-dense viral factories. Part of the electron-dense material is engulfed by this rigid membrane, giving rise to the formation of immature virions (IV) (5, 6, 15). Through a poorly understood mechanism that appears to involve the proteolytic cleavage of certain VV polypeptides (18, 27, 51), these spherical virions develop into characteristic brick-shaped particles, referred to as intracellular mature virions (IMV). A small proportion of the IMVs became wrapped by membrane cisternae derived from the *trans*-Golgi network (14, 42). These four-membrane intracellular enveloped virions (IEV) are released from the cell by fusion with the plasma membrane, a process in which they lose the outermost membrane. The resulting extracellular enveloped virions (EEV) which expose in their external surface proteins not present in IEVs are largely responsible for virus spread both in tissue culture and in experimental animals (3, 30).

VV morphogenesis has become the focus of many recent studies. Considerable progress has been made in the identification of viral proteins essential for wrapping of IMVs with the Golgi-derived cisternae. Involved in this process are the 37-kDa (F13L gene) (2, 43), the 22- to 24-kDa (A34R gene) (7), the 42-kDa (B5R gene) (8, 53), and the 43- to 50-kDa (A36R gene) (29) EEV-specific proteins and the 14-kDa (A27L gene) (38) IMV envelope protein. Proteins required at the transition stage between IV and IMV have also been identified: the 11-kDa (F18R) (56) and the 47-kDa (I7L) (17) core proteins and the 25-kDa myristylated envelope protein (L1R gene) (31). In addition, the 65-kDa protein (D13L gene) was shown to be required for the acquisition of the characteristic convex shape of the viral crescents. This protein is the target of rifampin (24, 49), a drug that reversibly arrests VV assembly (26, 28). Inhibition of 65-kDa protein synthesis (57) and treatment with rifampin both result in the formation of electron-dense structures surrounded by irregularly shaped membranes termed rifampin bodies (RBs) (12). The 65-kDa protein has been suggested to have a scaffold function over the preformed membranes (46). However, very little is known about the VV proteins involved in the early stages of IMV membrane biogenesis. From classical electron microscopy studies, it was postulated that the assembly process initiated with de novo formation of viral membranes that are arranged to produce the characteristic crescent-shaped membranes (47, 48). The most recent model of assembly proposed that viral crescents are composed of a double membrane derived from the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (45). Thus, according to this later model, a viral integral membrane protein(s) would be targeted to the IC for the generation of virus-induced membranes. How these viral proteins are targeted to the IC and how they interact during the initial steps of morphogenesis to promote the formation of the nascent viral membranes are still open questions, which will be resolved as viral proteins essential for this process are identified. In this regard, we have previously identified a VV 21-kDa membrane protein (A17L gene) that forms a stable complex with the 14-kDa envelope protein (34) and have shown that repression of 21-kDa protein synthesis in cells infected with the conditional mutant VVindA17L results in the interruption of viral

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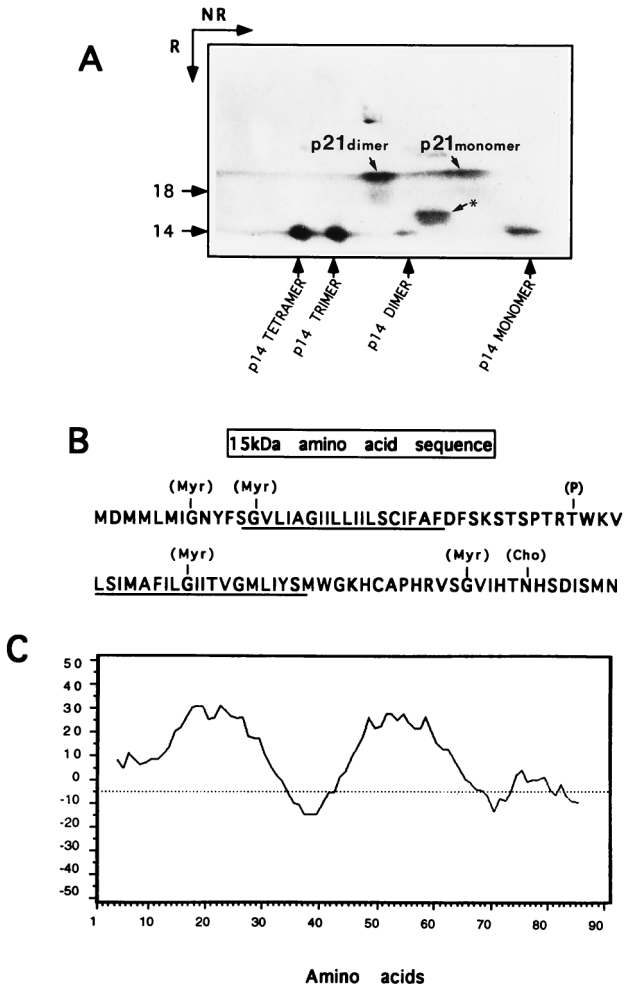


FIG. 1. VV 14-, 15-, and 21-kDa proteins are associated in vivo. (A) Protein complex immunoprecipitated by MAbC3. BSC-40 cells infected with VV (10 PFU/cell) were metabolically labelled with [^{35}S]methionine from 6 to 24 h p.i. Cells were collected, and cell extracts were immunoprecipitated with MAbC3. The immunoprecipitated products were analyzed by two-dimensional SDS-PAGE, in which the first dimension was run in a 12% polyacrylamide gel under nonreducing (NR) conditions. The lane of the polyacrylamide containing the fractionated proteins was cut from the gel and incubated for 30 min in sample buffer containing 10% 2-mercaptoethanol, and the gel was placed horizontally on top of a 12% preparative polyacrylamide gel. The proteins were then fractionated in the second dimension by electrophoresis under reducing (R) conditions. The proteins were visualized after autoradiography of the dried gel. Molecular mass markers (in kilodaltons) are represented to the left. The 15-kDa protein is denoted by an asterisk. (B) Deduced amino acid sequence of the 15-kDa protein. The amino acids corresponding to two α-helical putative membrane-spanning domains are underlined. Potential sites for myristylation (Myr), phosphorylation (P), and N glycosylation (Cho) are indicated. (C) Hydropathy plot of the predicted A14L gene product. The hydropathy plot was determined as described by Kyte and Doolittle (20). Hydrophobic regions are above the dotted line; hydrophilic regions are below it.

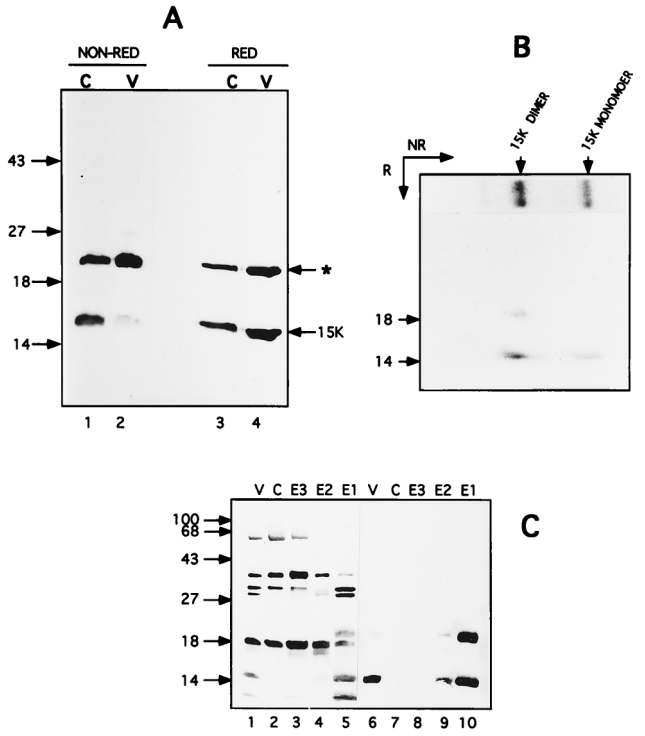


FIG. 2. The 15-kDa protein is a component of the virion membrane and forms dimers. (A) Western blot analysis of extracts from BSC-40 cells infected (10 PFU/cell) with WR for 24 h (C) (lanes 1 and 3), and of purified virions (V) (lanes 2 and 4). The proteins were fractionated by SDS-PAGE under nonreducing (NON-RED) (lanes 1 and 2) or reducing (RED) (lanes 3 and 4) conditions, transferred to nitrocellulose paper, and reacted with an anti-15-kDa polyclonal antiserum. Antibody reactivity was detected by immunoperoxidase staining by previously described procedures (37). The 22-kDa protein is denoted by an asterisk. (B) Two-dimensional gel electrophoresis of the products immunoprecipitated by the anti-15-kDa serum. BSC-40-infected cells were labeled, immunoprecipitated with anti-15-kDa antibodies, and analyzed by two-dimensional SDS-PAGE as described in the legend to Fig. 1. Proteins were visualized after autoradiography of the dried gel. Migration of the 15-kDa dimer and monomer identified after one-dimensional SDS-PAGE analysis is indicated at the top of the Fig. NR, nonreducing conditions; R, reducing conditions. (C) Localization of the 15-kDa protein within the virion. Purified virions were disrupted by treatment with the nonionic detergent NP-40 (1%) in Tris buffer [50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$]. The soluble envelopes (E1) were removed by centrifugation, and the insoluble fraction was resuspended in Tris buffer containing NP-40 (1%) plus dithiothreitol (50 mM). The soluble proteins (E2) were collected, and the pellet was treated with the same buffer containing NP-40 (1%), deoxycholate (0.5%), and SDS (0.1%). After centrifugation, soluble proteins (E3) were separated from cores (C). The proteins in the collected fractions were resolved by SDS-PAGE under reducing conditions and analyzed by Western blotting with the anti-15-kDa serum (lanes 6 to 10) or a rabbit polyclonal serum against live VV (lanes 1 to 5). Molecular mass markers (in kilodaltons) are represented to the left of each panel.

morphogenesis at a stage prior to the formation of the viral crescents (35). These studies led us to suggest that the 21-kDa protein might be involved in the recruitment of membranes derived from the cellular ERGIC to the nascent viral factories. However, in this report, we demonstrate that in cells infected with VVindA17L virus in the absence of the 21-kDa protein, numerous cellular tubulovesicular membranes related to the ERGIC are actually recruited to the periphery of the dense structures, indicating that other viral factors must be involved in the early recruitment of membranes.

It has been suggested that protein phosphorylation may be an essential function during these initial steps of virion assembly (50, 52). In this regard, in this investigation we have identified and characterized a VV membrane protein which is phosphorylated and myristylated during infection. This protein, with an apparent molecular mass of 15 kDa, is encoded by the A14L gene. It interacts with the 14-kDa–21-kDa protein complex in the virion envelope. The intracellular transport of the 15-kDa protein defined here by immunoelectron microscopy (rough endoplasmic reticulum to the ERGIC to the viral crescent) suggests that this protein may be one of the viral elements that participate in the membrane recruitment process.

The findings provided in this investigation contribute to the

understanding of the initial sequence of events in the formation of VV membranes and ascribe two viral proteins (21 and 15 kDa) to this membrane assembly process.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (BSC-40) and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. VV strain WR (Western Reserve) was propagated and subjected to titer determination in BSC-40 cells. Recombinant virus VVindA17L (35) was grown in BSC-40 cells in the presence of 2 mM isopropyl-β-D-thiogalactoside (IPTG). IMVs were purified by previously described standard procedures (16).

Antisera. The monoclonal antibody anti-14-kDa MAbC3 (37) and the rabbit polyclonal antiserum anti-21-kDa protein (36) have been previously described. A rabbit polyclonal antiserum raised against two synthetic peptides spanning amino acids 35 to 44 and 80 to 90 was produced by immunization of a rabbit with the purified peptides coupled to keyhole limpet hemocyanin. The mouse monoclonal antibody MAb G1/93 against human ERGIC-53 was kindly provided by H. P. Hauri (Biozentrum of the University of Basel). The gold-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Biocell (Cardiff, United Kingdom).

Metabolic labeling and immunoprecipitation analysis. BSC-40 cells infected with VV (10 PFU/cell) were metabolically labeled with [^{35}S]methionine (10 μCi/ml; Amersham) or [^{3}H]myristic acid (200 μCi/ml; Amersham) from 6 to 24 h postinfection (p.i.). At the end of the labeling period, cells were collected, washed with phosphate-buffered saline, and resuspended in lysis buffer (20 mM Tris [pH 8.0], 80 mM NaCl, 20 mM EDTA, 1% Nonidet P-40 [NP-40]) in the presence of protease inhibitors (2 μg of bacitracin per ml, 2 μg of trypsin inhibitor per ml, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml). After 30 min on ice, cell extracts were sonicated and cleared by centrifugation at 10,000 × *g* for 5 min. For radiolabeling with [^{32}P]orthophosphate, BSC-40 cells infected with VV for 6 h were starved for 30 min with DMEM without phosphate salts. [^{32}P]orthophosphate (200 μCi/ml; Amersham) was then added to the medium, and the cells were incubated for 3 h. After this period, cells were collected and cell lysates were prepared as described above.

Before immunoprecipitation with specific antibodies, cell extracts were incubated for 2 h at room temperature with preimmune serum coupled to protein A-Sepharose beads (Pharmacia). After centrifugation, supernatants were immunoprecipitated by incubation overnight at 4°C with protein A-Sepharose beads coated with MAbC3 or with specific antibodies against the 15-kDa protein. The immunoprecipitates were washed three times with lysis buffer and three times with phosphate-buffered saline and resuspended in 2× sample buffer (1.25 M Tris [pH 6.8], 0.2% sodium dodecyl sulfate [SDS], 0.5% bromophenol blue) with (reducing) or without (nonreducing) 10% 2-mercaptoethanol. Samples were boiled for 3 min and resolved by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) (PAGE). The gels were dried, and the proteins were visualized after autoradiography.

Protein microsequence analysis. Extracts of [^{35}S]methionine-labeled infected cells were immunoprecipitated with MAbC3. The immunoprecipitated products were resolved by two-dimensional gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Problott; Applied Biosystems, Inc.). Proteins electroblotted onto the Problott membrane were detected after autoradiography, and the spot corresponding to the 15-kDa protein was cut out with a razor. The N-terminal sequencing was performed on a model 470A gas-phase protein sequencer connected on line to a microbore high-pressure liquid chromatography PTH amino acid analyzer (Applied Biosystems Inc.). The sequence corresponding to the first 10 amino-terminal residues was used in a search for homology in the EMBL protein data bank, using the PC gene program (IntelliGenetics).

Electron microscopy. HeLa cells infected under different conditions were processed for embedding in Lowicryl K4M at low temperature or in the epoxy resin EML 812 (EM Laboratories Ltd.) as previously described in detail (32, 35). Ultrathin sections of the samples were collected in copper or gold grids covered with Formvar and carbon and were either stained or processed for immunogold labeling by previously established procedures (32, 33).

Immunogold localization of the 15- and 21-kDa VV proteins on ultrathin sections of infected cells was carried out with specific polyclonal rabbit antisera and a 10-nm goat anti-rabbit gold conjugate. For detection of the 15-kDa protein on the surface of IMVs, immunogold labeling of purified virions was performed as previously described (36). Labeling with MAb G1/93, a well-characterized marker for the IC at the endoplasmic reticulum-Golgi interface (41), was completed with a 10-nm goat anti-mouse gold conjugate. After immunolabeling, staining was performed for 15 min with saturated uranyl acetate for Lowicryl sections, 25 min with saturated uranyl acetate followed by 1 min with lead citrate for EML 812 sections, and 30 s with a 2% solution of uranyl acetate for negative staining of purified IMVs. Samples were studied in a JEOL 1200EX II electron microscope.

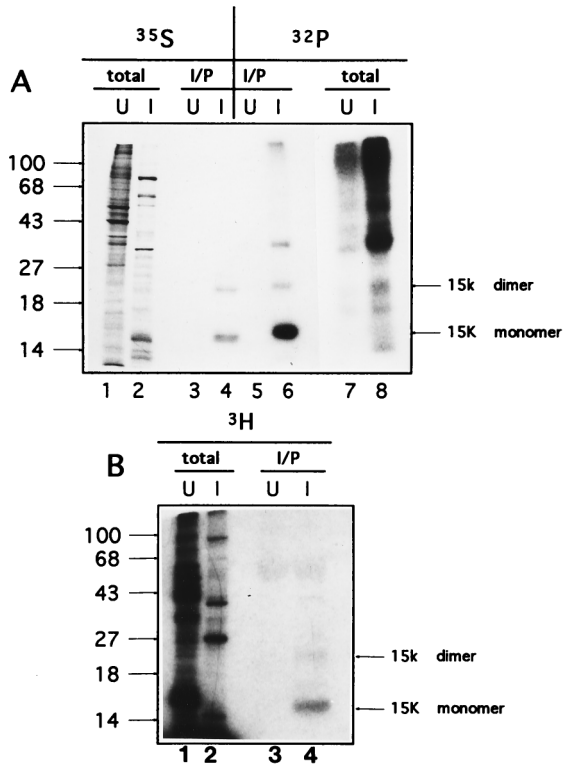
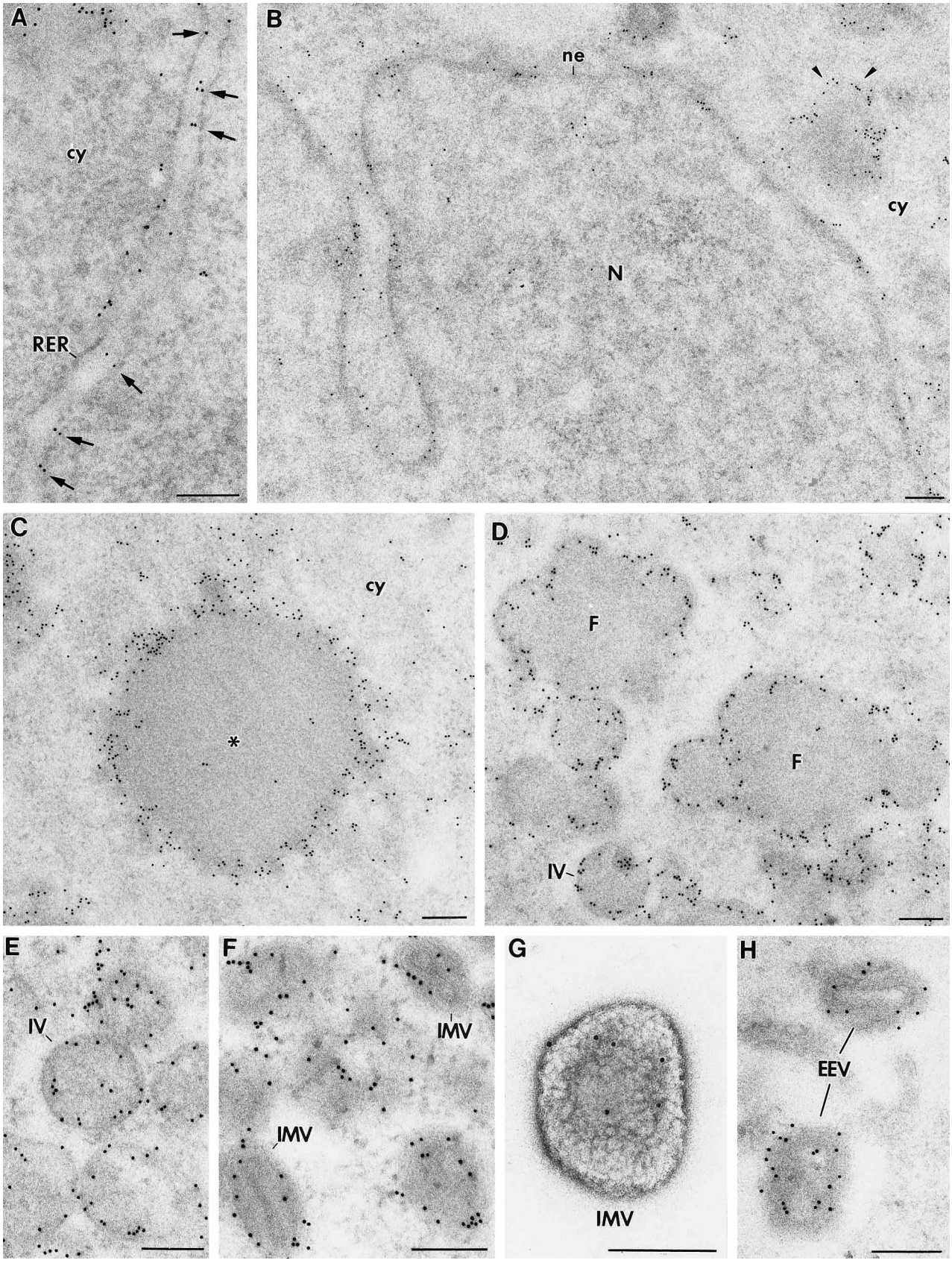


FIG. 3. The 15-kDa protein is phosphorylated and myristylated. (A) Uninfected (U) and VV-infected (10 PFU/cell) BSC-40 cells (I) were metabolically labelled with either [^{32}P]orthophosphate (200 μCi/ml) from 6 to 9 h p.i. (lanes 5 to 8) or [^{35}S]methionine (10 μCi/ml) from 6 to 24 h p.i. (lanes 1 to 4) (A) or [^{3}H]myristic acid (200 μCi/ml) from 6 to 24 h p.i. (lanes 1 to 4) (B). Cell extracts were immunoprecipitated with anti-15-kDa antibodies, and the total labelled proteins (total) and the immunoprecipitated products (I/P) were analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography. To visualize ^{3}H-radiolabeled proteins before autoradiography, the gel was subjected to fluorography. Molecular mass markers (in kilodaltons) are represented to the left.

RESULTS

A 15-kDa VV protein is associated with the 14-kDa–21-kDa protein complex. We have previously reported that MAbC3 immunoprecipitates a VV membrane protein complex consisting of the 14-kDa envelope protein (gene A27L), and the 21-kDa protein (gene A17L) (34). A detailed analysis of this immunoprecipitated complex reveals the presence of an additional polypeptide with an apparent molecular mass of 15 kDa. Figure 1A shows the protein pattern obtained when the MAbC3-immunoprecipitated proteins were analyzed by two-dimensional SDS-PAGE in which the first dimension was run under nonreducing conditions and the second dimension was run under reducing conditions. In addition to the 14-kDa protein (which can be observed in monomeric and different oligomeric forms) and the 21-kDa protein (monomer and dimer), a novel protein with a relative molecular mass of 15 kDa is detected.

The 15-kDa protein is encoded by the VV A14L gene. To identify the viral gene coding for the 15-kDa protein, we performed microsequencing analysis. Thus, the products immunoprecipitated with MAbC3 were separated by two-dimensional SDS-PAGE as described above and transferred to a Problott membrane. The spot corresponding to the 15-kDa protein was excised and processed for microsequencing. The first 10 amino acid residues at the N terminus of the 15-kDa protein were determined. A search of the EMBL protein data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bank showed that this amino acid sequence is identical to the predicted N terminus of the A14L gene product (Copenhagen strain). This gene codes for a polypeptide of 90 amino acids with a predicted molecular mass of 10 kDa (Fig. 1B). Analysis of the deduced amino acid sequence shows the presence of four putative sites for myristylation, one potential site for phosphorylation, and one possible site for N glycosylation. The hydropathy plot of the protein (Fig. 1C) reveals the presence of two large hydrophobic domains characteristic of membrane proteins. Moreover, analysis of the 15-kDa protein sequence with a secondary-structure prediction program (39) predicts an α-helical structure, characteristic of transmembrane domains, for each of these two hydrophobic stretches (from amino acids 13 to 31 and 45 to 64). This suggests that the A14L gene product is an integral membrane protein with two membrane-spanning domains (Fig. 1B).

Identification of the 15-kDa protein in infected cells and in purified virions. To identify the 15-kDa protein, a polyclonal antiserum was raised against two synthetic peptides corresponding to amino acids 35 to 44 and 80 to 90. Using this antiserum, we performed a Western blot analysis of protein extracts from VV-infected cells and purified virions. The proteins were fractionated by SDS-PAGE under reducing or nonreducing conditions, transferred to nitrocellulose paper, and reacted with the anti-15-kDa polyclonal antiserum. As shown in Fig. 2A, under nonreducing conditions (lanes 1 and 2), in addition to the 15-kDa protein, the antibodies specifically recognize an abundant product of about 22 kDa in both cell extracts and purified virions. Upon reduction (lanes 3 and 4), a clear increase in the reactivity of the 15-kDa product concomitant with a reduction in the amount of the 22-kDa product was observed, and this is particularly evident in the virions, where the 15-kDa protein was almost undetectable under nonreducing conditions (lane 2). This result indicates that the 22-kDa product represents a dimer of the 15-kDa protein. To test this possibility, the 15-kDa protein was immunoprecipitated from ^{35}S-labeled, VV-infected cells with the anti-15-kDa serum and analyzed by two-dimensional SDS-PAGE as described above. As shown in Fig. 2B, after the second dimension, which was run under reducing conditions, the 22-kDa product was resolved as a 15-kDa protein, confirming that the 22-kDa protein is a dimer of the 15-kDa protein. Neither of these two products was observed when cells were infected in the presence of the DNA replication inhibitor AraC, indicating that the protein is produced late during infection (data not shown).

Since the 15-kDa protein coprecipitates with the 14-kDa–21-kDa protein complex, located in the virion membrane, it is likely that the 15-kDa protein also localizes to the same viral structure. Therefore, we studied the localization of the 15-kDa protein within the virion. To do this, purified virions were sequentially stripped by treatments with different detergents and the reducing agent dithiothreitol. After each treatment, the solubilized proteins were separated from the insoluble fraction by centrifugation, and the collected fractions were subjected to SDS-PAGE under reducing conditions and analyzed by Western blotting with either the anti-15-kDa or an anti-VV polyclonal antiserum. As shown in Fig. 2C (lane 10), after reactivity with anti-15-kDa-specific antisera, the 15-kDa protein and its dimeric form (22 kDa) are present almost exclusively in fraction E1, extracted with the non-ionic detergent NP-40 alone. The distribution of virion proteins is shown in Fig. 2C (lanes 1 to 5) after reactivity with the polyclonal anti-VV serum.

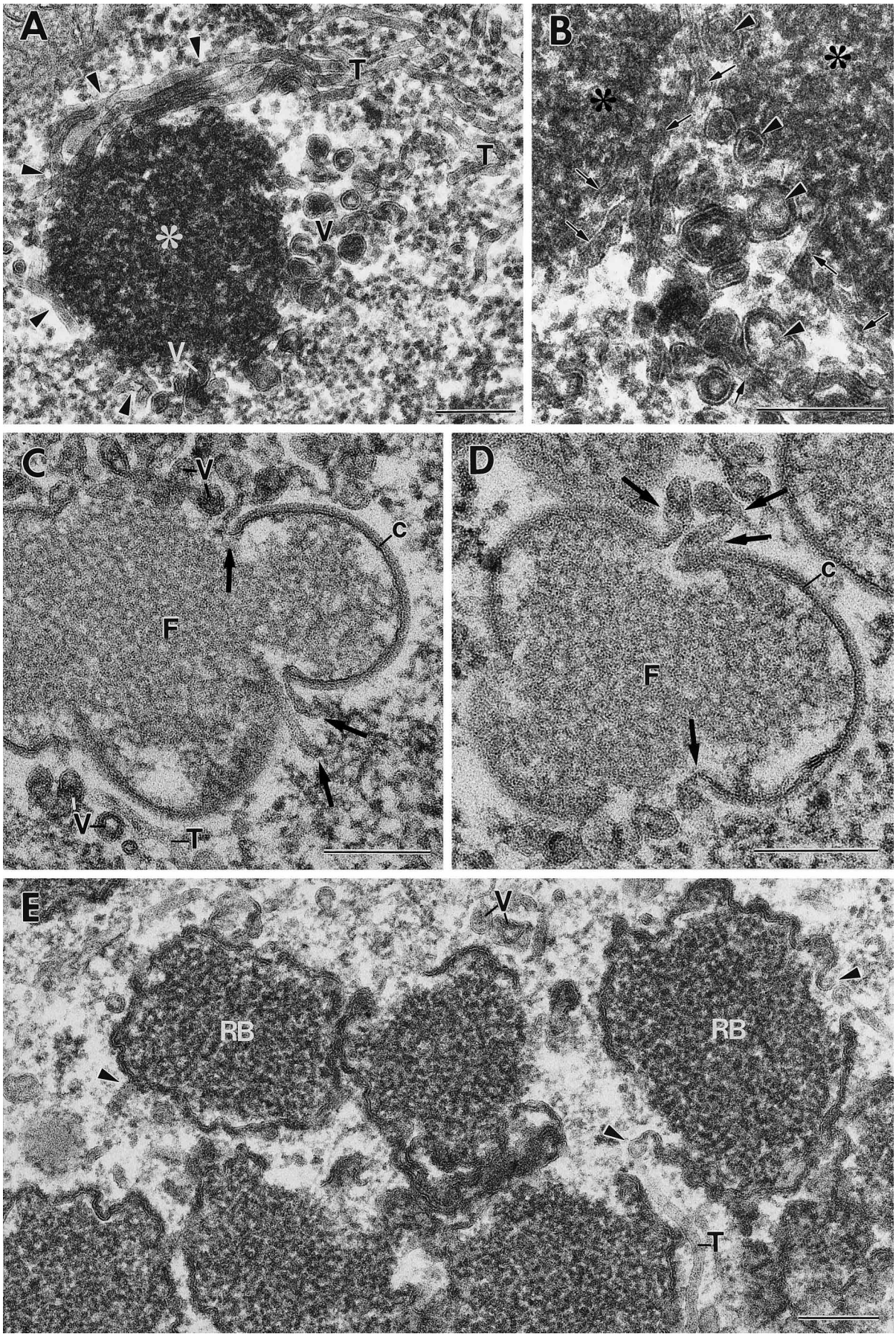
Taken together, these results show that in infected cells, the A14L gene product is detected as two immunoreactive polypeptides, the 15-kDa protein, corresponding to the monomer, and the 22-kDa protein, representing the dimer. The protein is also present in the membrane of the viral particles, where it appears mostly in a dimeric form.

Modifications of the 15-kDa protein. As mentioned above, analysis of the 15-kDa protein sequence reveals the presence of putative sites for phosphorylation, myristylation, and glycosylation. It has been recently suggested that phosphorylation might be an essential function during virion morphogenesis (50, 52). Also, additions of N-linked carbohydrates or fatty acids are protein modifications characteristic of membrane targeting. It was therefore of interest to examine if the 15-kDa protein undergoes any of these co- or posttranslational modifications.

To determine if the 15-kDa protein is phosphorylated during infection, we performed immunoprecipitation analysis with anti-15-kDa antibodies by using extracts of VV-infected cells metabolically labeled with [^{32}P]orthophosphate or with [^{35}S]methionine. The immunoprecipitated products were resolved by SDS-PAGE under reducing conditions. As shown in Fig. 3A, both the 15-kDa protein and its 22-kDa dimeric form can be observed in the immunoprecipitated fraction from infected cells radiolabeled with either [^{35}S]methionine (lane 4) or [^{32}P]orthophosphate (lane 6). An additional phosphoprotein with an apparent molecular mass of about 35 kDa is also observed in the immunoprecipitated fraction (lane 6), which appears to correspond to the major phosphorylated protein observed in the total-cell extract (lane 8). None of these proteins was observed in the immunoprecipitated fraction from ^{35}S- and ^{32}P-labeled uninfected cells (lanes 3 and 5, respectively).

Similarly, to investigate if the 15-kDa protein was also modified by myristate acylation, VV-infected cells were metabolically labeled with [^{3}H]myristic acid from 6 to 24 h pi and cell extracts were subjected to immunoprecipitation analysis. As previously described, in these extracts (Fig. 3B, lane 2) the major ^{3}H-labeled proteins were a 25-kDa polypeptide that probably corresponds to the L1R virion protein (10) and a 35-kDa polypeptide that was previously designated M35 (9). Three other radiolabeled proteins with relative molecular masses of about 15, 50, and 85 kDa were also observed. After immunoprecipitation with the anti-15-kDa antibody (Fig. 3B, lane 4), two myristylated products with sizes identical to those of proteins immunoprecipitated from ^{35}S- and ^{32}P-labeled in-

FIG. 4. Immunogold localization of the 15-kDa protein in Lowicryl K4M sections of infected cells and in virions at the electron microscopy level. HeLa cells were infected (5 PFU/cell) with either WR virus or VVindA17L. With the latter, cells were either maintained in the absence of IPTG for 6 h and then treated with IPTG (2 mM) or maintained uninduced for the whole infection period. At 24 h p.i., cells were fixed and processed for immunogold labelling with anti-15-kDa antiserum. (A) Cells infected for 24 h with WR virus contain this protein (arrows) in the membranes of the rough endoplasmic reticulum (RER). (B) The membranes of the nuclear envelope (ne) also accumulate the 15-kDa protein in these cells, which contain cytoplasmic areas where the protein exhibits a linear or circular pattern (arrowheads). (C) In cells infected with VVindA17L in the absence of the inducer IPTG, the 15-kDa protein accumulates on the periphery of characteristic electron-dense structures similar to RBs (asterisk). (D) After induction of the A17L gene by incubation with IPTG (4 h), the 15-kDa protein is localized in the crescent-shaped membranes that organize on the periphery of the viral factories (F). (E and F) Both IVs (E) and IMVs (F) present this protein localized in their membranes. (G) The 15-kDa protein is moderately exposed on the surface of purified IMVs according to immunogold labeling before negative staining. (H) EEVs also exhibit the 15-kDa protein localized in their membranes. cy, cytoplasm; N, nucleus. Bars, 200 nm.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fected cells (Fig. 3A, lanes 4 and 6, respectively) were observed. Again, neither of the two polypeptides was observed in the lane corresponding to uninfected cells (Fig. 3B, lane 3). Regarding the possibility of N-linked glycosylation, we did not observe a shift in the mobility of either the 15-kDa or the 22-kDa form after treatment of infected cells with tunycamicin, suggesting that the protein is not glycosylated (data not shown).

The above results show that in infected cells, the 15-kDa protein is modified by phosphorylation and fatty acid acylation.

Localization of the 15-kDa protein by immunoelectron microscopy. To investigate in detail the intracellular localization of the 15-kDa protein, we performed an immunoelectron microscopy study of HeLa cells infected with either wild-type WR virus or the conditional lethal mutant VVindA17L. In WR-infected cells, the 15-kDa protein is detected in the membranes of the rough endoplasmic reticulum cisternae (Fig. 4A) and in the membranes of the nuclear envelope (Fig. 4B). It also has an apparent cytoplasmic localization in particular areas, where the protein is arranged in a linear or circular pattern, which most probably corresponds to the association of protein molecules with smooth-walled membranes, poorly defined in conventional Lowicryl sections (Fig. 4B, arrowheads).

We have recently described that in cells infected with the IPTG-inducible conditional mutant VVindA17L, in which the expression of the 21-kDa protein is repressed, virion morphogenesis is arrested at an early stage, prior to the formation of the characteristic crescent-shaped membranes (35). Therefore, it was of interest to study the presence and localization of the 15-kDa protein in cells infected with the VVindA17L virus under nonpermissive conditions (24 h in the absence of IPTG). In the absence of the 21-kDa protein, the 15-kDa protein accumulates in large amounts, preferentially on the periphery of the electron-dense structures similar to RBs observed in these cells (Fig. 4C).

Once the A17L gene was induced by incubation with IPTG and the assembly of virions was triggered, the 15-kDa protein was detected in association with viral membranes in all stages of virion formation. At 4 h postinduction, it was associated with the crescent-shaped membranes of the viral factories (Fig. 4D) and localized in the membranes of IVs (Fig. 4E), IMVs (Fig. 4F), and EEVs (Fig. 4H). According to the signal obtained by immunolabeling of purified virions, one or both peptides recognized by the anti-15-kDa antibodies were moderately exposed on the surface of IMVs (Fig. 4G).

Considering the early as well as the late association of the 15-kDa protein to cellular and viral membranes, a strong labeling associated with this protein on the periphery of the electron-dense structures formed when VV morphogenesis was arrested, suggests the existence of some kind of membranous elements in those locations. These are probably difficult to visualize, since they were not detected in our Lowicryl sections or in a previous study with sections of EML 812 epoxy resin (35). Thus, very thin sections (30 to 40 nm) of infected cells embedded in EML 812 resin were used to carefully analyze fine structural details of the viral assemblies formed under different conditions. In this study, the electron-dense masses that formed in the cytoplasm of cells infected with VVindA17L in the absence of IPTG had numerous membranous structures of two types: tubules and vesicles (Fig. 5A and B). All of the electron-dense masses observed had most of their periphery occupied by these tubulovesicular membranes, which also accumulated in clear areas of cytoplasm around the masses. The tubular elements had a diameter of ~30 nm, while the vesicles ranged from 40 to 80 nm. When assembly started under permissive conditions, tubulovesicular elements, indistinguishable from those that accumulate around the masses in the absence of viral assembly, were also found around the factories (Fig. 5C and D). Some of these tubulovesicular membranes were seen in continuity with the crescents (arrows in Fig. 5C and D). In cells infected with WR virus in the presence of rifampin, similar tubulovesicular elements (Fig. 5E) were observed around the RBs. Some of them are clearly connected or in continuity with the ruffled dense membranes that occupy the periphery of the RBs. These tubulovesicular elements are apparently similar to the characteristic membranous structures described for the ERGIC (40, 41, 44) and are also similar to cellular membranes detected in continuity with the crescents of the VV factories in infected cells (45). In this previous study, the use of antibodies against several markers for different subcellular compartments confirmed that the viral crescents derive from elements of the ERGIC. We have used one of the markers employed in the previous study to confirm the origin of the tubulovesicular elements recruited on the periphery of the electron-dense masses. MAb G1/93 recognizes the protein ERGIC-53, a nonglycosylated transmembrane protein that concentrates in the intermediate compartment at the ER-Golgi interface (40, 44). This antibody specifically labels the vesicles and tubules attached to the periphery of the VV-related electron-dense masses (Fig. 6A and B), as well as membranous elements seen in continuity with the viral crescents of the viral factories (Fig. 6C). Even a few immature viruses present some labeling associated with their membranes (Fig. 6D), confirming the relationship of these tubulovesicular structures to elements of the ERGIC. The tubulovesicular structures visualized around the RBs also presented specific labeling associated with the ERGIC marker ERGIC-53 protein (data not shown).

From the results shown in Fig. 5 and 6, we can conclude that in the absence of the 21-kDa protein there is a significant recruitment and accumulation of ERGIC-53-positive cellular membranes to the virus-related electron-dense masses. Immunogold labeling with an antiserum against the 15-kDa protein showed that it accumulates in large amounts in these tubulovesicular membranes (Fig. 7A and B). As seen in the Lowicryl sections (Fig. 4C), labeling is restricted to the periphery of the masses, and it is clearly associated with the tubulovesicular

FIG. 5. VV-related structures as visualized in EML 812 ultrathin sections of infected cells. Cultures of HeLa cells were infected at a multiplicity of infection of 5 PFU/cell with either WR virus in the presence of rifampin (100 μg/ml) or VVindA17L in the absence or presence of IPTG (2 mM). Cells were fixed at 24 h p.i. (A) Ultrastructure of the electron-dense masses (asterisk) characteristic of cells infected with VVindA17L in the absence of the inducer, conditions that represent the absence of the 21-kDa protein and the arrest of viral morphogenesis at a very early stage (35). The periphery of all these masses contains numerous tubulovesicular membranous structures. The tubules (T) have a diameter of ~30 nm, while the vesicles (V) are of variable sizes (40 to 80 nm). Arrowheads point to the tubules attached or in close proximity to the periphery of the electron-dense masses. (B) Higher-magnification field showing tubular (arrows) and vesicular (arrowheads) membranous structures located on the periphery of two electron-dense masses (asterisks). (C and D) Ultrastructure of viral factories (F) that organize in cells infected with VVindA17L in the presence of the inducer. The periphery of the viral factories contains tubular (T) and vesicular (V) elements, indistinguishable from the tubulovesicular structures that accumulate around the electron-dense structures formed in the absence of IPTG. Both tubules and vesicles are connected (arrows) to the membranes of the viral crescents (c). (E) Ultrathin sections of a cell infected with WR in the presence of rifampin. Tubules (T) and vesicles (V), similar to those described above, are found around the RBs. Some of these tubulovesicular membranes are seen in contact with the RB surface or in continuity with the ruffled membranes of their periphery (arrowheads). Bars, 200 nm.

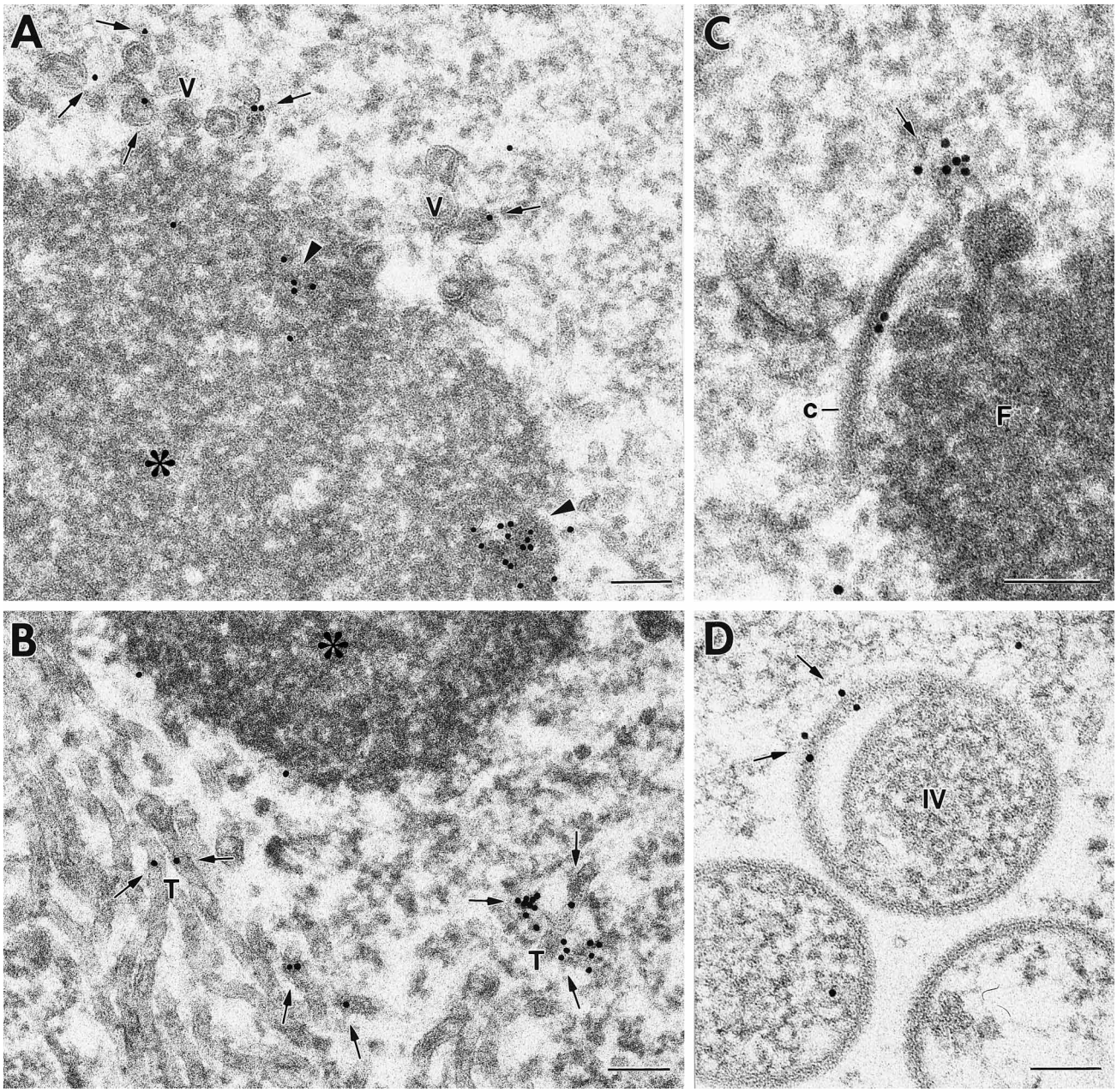

FIG. 6. Immunogold labeling with a MAb specific for ERGIC-53, a marker of the intermediate compartment at the endoplasmic reticulum-Golgi interphase. EML 812 ultrathin sections of HeLa cells infected with VVindA17L in the absence (A and B) or presence (C and D) of IPTG were used. (A) The antibody specifically labels vesicular elements (arrows) on the periphery of the electron-dense masses (asterisk). Labeling also associates with peripheral spots of the masses where membranes are not clearly distinguished (arrowheads). (B) Tubular membranes around the masses (asterisk) also show specific labeling (arrows). (C) When assembly takes place in the presence of IPTG, ERGIC-53-positive membranous elements (arrow) can be seen in continuity with viral crescents (c) that start to organize at the viral factories (F). (D) A few immature viruses (IV) show some labeling associated with ERGIC-53 in their membranes (arrows). Bars, 200 nm.

membranes (Fig. 7B). When we used an antiserum against the 21-kDa protein, absent in these conditions, we found that the tubulovesicular elements around the masses were completely devoided of labeling (Fig. 7C), which confirms that there are no minute detectable amounts of the 21-kDa protein incorporated in those locations. However, when viral assembly started after induction of the A17L gene, both 15- and 21-kDa proteins are detected in tubulovesicular elements around the viral factories, as well as in the viral crescents (Fig. 7D and E). These VV proteins were also detected in tubulovesicular structures seen around the RBs in cells infected with WR virus in the presence of rifampin, as well as in the ruffled dense membranes attached to the surface of the bodies (Fig. 7F and G). These results strongly support the common origin of the tubulovesicular membranes associated with the VV-related structures in cells infected under the different conditions described and its implication in the assembly of viral membranes early in morphogenesis. VV 15- and 21-kDa proteins are transported in these membranes of the ERGIC to the locations of viral assembly. The 15-kDa protein can be one of the viral factors

involved in the recruitment of ERGIC membranes to the forming VV factories.

A general model for VV assembly, illustrating the initial stages of viral membrane formation proposed in this report, is shown in Fig. 8. The proteins known to be involved in the different stages are indicated.

DISCUSSION

VV morphogenesis is a complex process in which numerous viral elements participate in the ordered incorporation of structures required to assemble the virus particle (Fig. 8). The definition of the first steps of this process has been the subject of recent studies. The results obtained supported the cellular origin of the membranes of IVs and IMVs (45). However, the key viral factors involved in the early recruitment of the IC membranes to the locations of viral assembly have not been identified. The 21-kDa protein has been a candidate for such a function, since its absence induces the arrest of VV morphogenesis in a very early stage before the formation of organized viral membranes (35, 36).

In this investigation, we have identified and characterized a 15-kDa VV membrane protein that interacts with the 14-kDa–21-kDa protein complex (Fig. 1A) (34, 35). We have established that this protein is encoded by the A14L gene. The analysis of the deduced amino acid sequence predicts two stretches of α-helical structure (18 and 19 amino acids long) corresponding to the two hydrophobic regions, indicating that the A14L gene product is an integral membrane protein with two transmembrane domains (Fig. 1B and C). To characterize the protein during infection, we produced a polyclonal serum against two peptides located within the two short hydrophilic regions. This antibody recognized two polypeptides with molecular masses of about 15 and 22 kDa (Fig. 2A). To test the possibility that the 22-kDa product represents a dimer of the 15-kDa protein, it was immunoprecipitated with the anti-15-kDa serum and analyzed by two-dimensional SDS-PAGE. After reduction, all the 22-kDa product migrated to the 15-kDa position, showing that the former is a homodimer of the 15-kDa polypeptide (Fig. 2B). Since the protein contains two cysteine residues, the disappearance of the 22-kDa polypeptide upon reduction suggests that the dimerization is mediated by disulfide linkage. It has been suggested that oligomerization might be important for the stability of structural VV proteins, especially those present in the external envelope (54), although it might also be relevant for their function. That the 15-kDa protein is a component of the virion membrane is suggested not only by its sequence but also from the fact that it interacts with the 14-kDa–21-kDa membrane complex. Moreover, the 15-kDa protein was readily extracted from purified IMVs by treatment with NP-40 alone (Fig. 2C), behaving in this respect as the 14-kDa (37), the 32-kDa (24), and the L1R (9) membrane proteins.

The predicted size of the A14L gene product is 10 kDa. Although the apparent molecular mass of the dimer (22 kDa) agrees with its predicted size, there is a discrepancy in the relative molecular mass of the monomer. This anomalous migration may be due to the positively charged histidine-rich C-terminal region of the protein (Fig. 1B). Alternatively, since the analysis of the 15-kDa protein sequence predicts putative sites for phosphorylation, fatty acid acylation, and glycosylation, it is possible that the discrepancy between the predicted and observed sizes of the protein results from co- or posttranslational modifications. After radiolabeling with [^{32}P]orthophosphate and immunoprecipitation with the anti-15-kDa antibodies, the 15-kDa protein was shown to be highly phosphorylated, while the signal associated with the 22-kDa dimer was weaker (Fig. 3A), suggesting that phosphorylation is hindered in the dimer. Another protein with an apparent molecular mass of 35 kDa was coprecipitated with this antibody, and it comigrated with the major phosphorylated species in the total-cell extract. This protein might correspond to the H5R phosphoprotein (1). Whether this is a specific coprecipitation remains to be elucidated. That phosphorylation may be an essential function during VV assembly has been recently suggested. It has been shown that temperature-sensitive mutations in the F10 gene, encoding a protein kinase, cause a morphogenesis block at an early stage (50, 52). Moreover, Liu et al. (22) have also shown that the A14L product is a phosphoprotein and a substrate of the recently identified VV H1 phosphatase (13). Phosphorylation of the 15-kDa protein might be required for targeting this protein to the ERGIC or for establishing protein-protein interactions with other membrane proteins, i.e., with the 14-kDa–21-kDa protein complex to initiate the formation of viral membranes or to hold the two membranes of the cisternae more tightly together. Phosphorylation of the 15-kDa protein is predicted to occur at residue Thr^{42}, which is located within the short hydrophilic loop in the central region of the protein. Then, generation of specific conditional lethal mutant viruses with mutations in the gene for the Thr^{42} of the A14L product can provide the means to test this hypothesis.

Our results of labeling with [^{3}H]myristic acid indicate that the 15-kDa protein is also modified by fatty acid addition (Fig. 3B). Several VV proteins, including the L1R major myristylated protein, are acylated (9, 10). Like the 15-kDa protein, the L1R protein is a membrane-associated virion protein. It has been shown that inhibition of L1R protein synthesis or lack of myristic acid results in the interruption of virion assembly at a stage subsequent to the formation of immature virions (31). Since both proteins are localized in the membrane of the IMV, it is tempting to speculate that they may interact through their acidic domains. The myristate moiety may also be involved in anchoring the 15-kDa protein to the membrane, although such a function may not be essential, given that the protein appears to contain two large transmembrane domains. Treatment of infected cells with tunycamicin did not affect the mobility of either the 15-kDa or the 22-kDa form of the protein, suggesting that the protein is not glycosylated.

Despite the strong reactivity of the anti-15-kDa antibody and the localization of the protein in the virion membrane, this antibody did not neutralize virus infectivity. This is in agreement with the poor surface localization of the protein by immunolabeling of purified virions (Fig. 4G). However, since we are using an antibody raised against only two peptides in these two experiments, we cannot exclude the possibility that antibodies to the full protein or to other domains would localize the protein in the surface of the virion and/or neutralize the virus.

A detailed study of the intracellular localization of the 15-kDa protein by immunoelectron microscopy showed that the protein is associated with the membranes of the ER and the nuclear envelope (Fig. 4A and B). Moreover, the 15-kDa protein appears to be associated with the viral membranes in all stages of virion assembly (viral crescents, IV, IMV, and EEV). A similar distribution was described for the VV 21-kDa protein in a previous study (36), which suggests that both proteins follow the same intracellular transport pathway. Interestingly, the 15-kDa protein appeared at the periphery of the electron-dense structures that are produced in cells infected with the conditional-lethal mutant VVindA17L in the absence of the inducer (35) (Fig. 4C). In a previous work, where we first

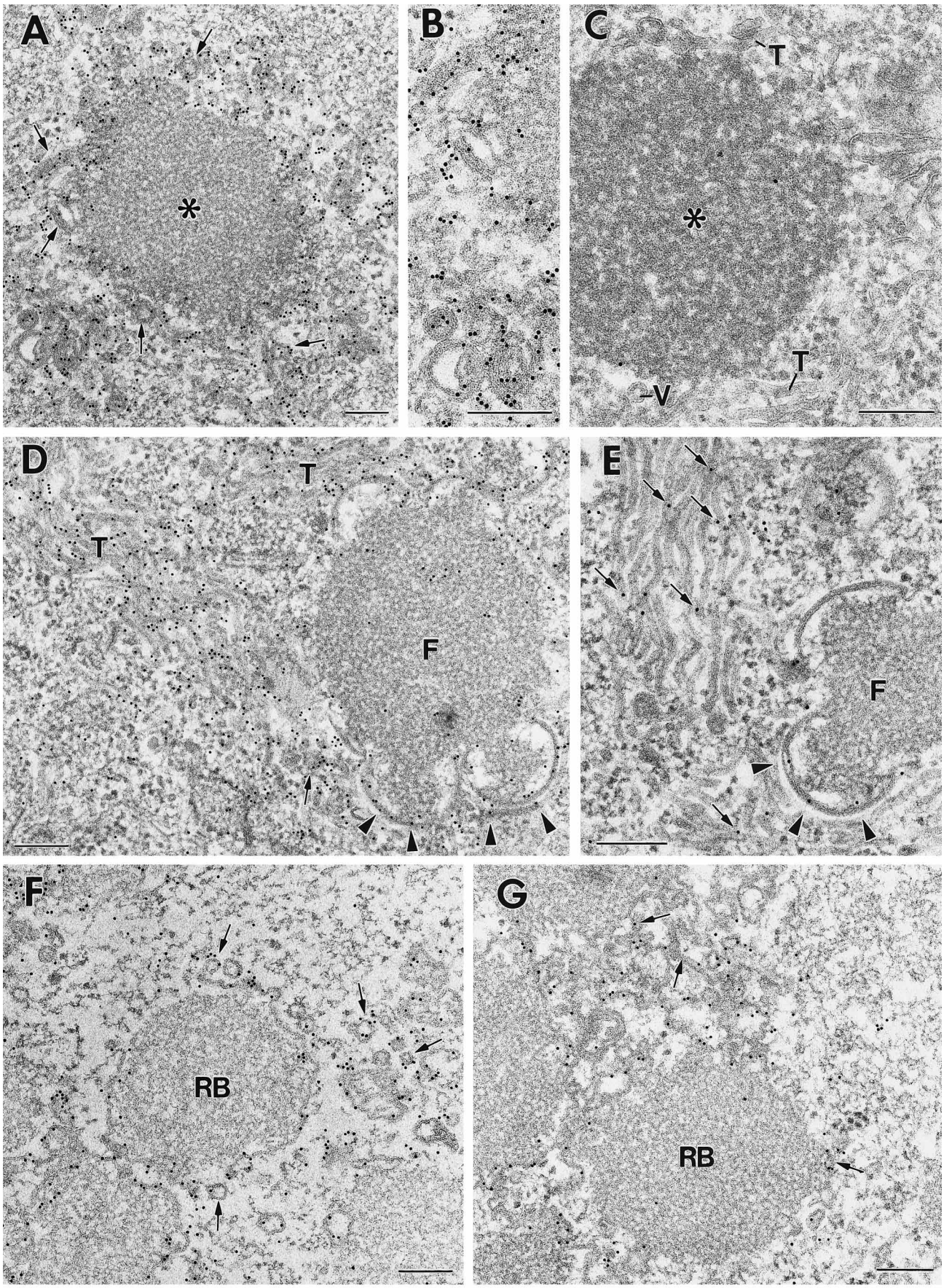
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FIG. 7. Localization of the 15- and 21-kDa VV proteins by immunogold labeling on EML 812 ultrathin sections of infected cells. Ultrathin sections of HeLa cells infected as described in the legend to Fig. 5 were processed for immunogold labeling with anti-15-kDa and anti-21-kDa antisera. (A) The 15-kDa protein is localized in the numerous tubulovesicular elements (arrows) that accumulate on the periphery of the electron-dense masses (asterisk) characteristic of cells infected with VVindA17L in the absence of IPTG. (B) High-magnification field of the left-hand area in panel A. Labeling on vesicles and tubules can be clearly seen. (C) Tubules (T) and vesicles (V) on the periphery of the electron-dense masses are free of labeling when an antibody specific for the 21-kDa protein is used; this represents a cytochemical control. (D and E) Localization of the 15-kDa (D) and 21-kDa (E) proteins in ultrathin sections of cells infected with VVindA17L in the presence of IPTG. (D) The 15-kDa protein is localized in tubules (T) and vesicles (arrow) close to the viral factories (F) as well as in membranes that organize as crescents (arrowheads) on the periphery of the viral factories. (E) The 21-kDa protein localizes in the same structures as the 15-kDa protein, as specific labeling is detected in tubulovesicular elements (arrows) and viral crescents (arrowheads) of the viral factories (F). (F and G) Localization of the 15-kDa (F) and 21-kDa (G) proteins in ultrathin sections of cells infected with WR virus in the presence of rifampin. (F) The 15-kDa protein localizes in the ruffled membranes that occupy the periphery of the RBs as well as in membranous elements around them (arrows). (G) The 21-kDa protein exhibits the same distribution as the 15-kDa protein, being specifically located in the peripheral ruffled membranes of the RBs and in the tubulovesicular membranes around the RBs (arrows). Bars, 200 nm.

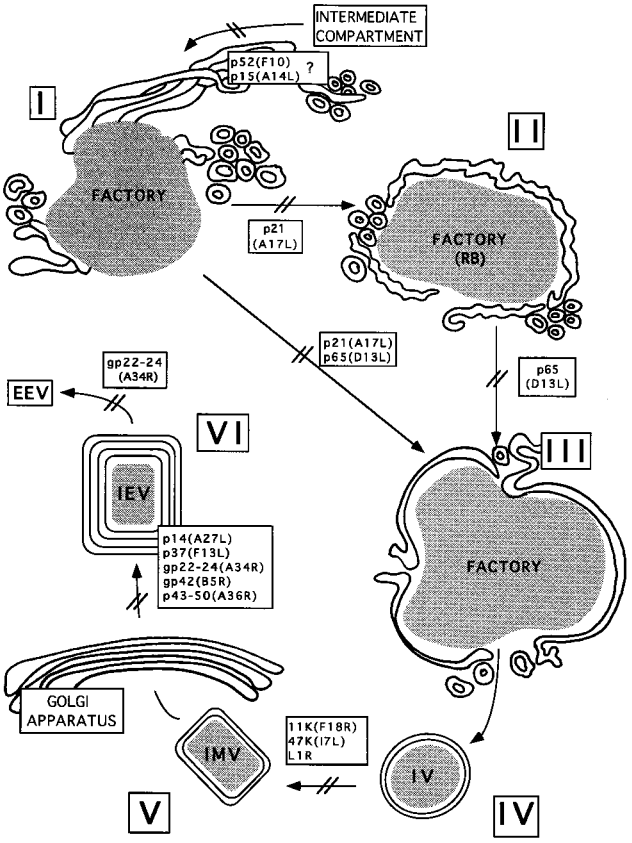

FIG. 8. In a WR infection, the first distinguishable sign of VV assembly is the formation of crescent-shaped membranes in the periphery of the electron-dense viral factories (stage III), which have been recently proposed to be derived from the IC (45). The formation of viral crescents can be prevented by treatment with rifampin or by repression of synthesis of the 65-kDa protein. Under these conditions, irregularly shaped membranes, known as RBs, are formed around the precursors of the viral factories (stage II). We have recently shown that inhibition of 21-kDa protein synthesis blocks viral assembly at a stage prior to the formation of the characteristic membranes around the precursor of the viral factories (35). In this study, we showed that tubules and vesicles derived from the ERGIC readily accumulate around the electron-dense bodies formed under these conditions (stage I). In light of this result, we now propose that the 21-kDa protein is required to join these membranous elements to produce continuous membrane structures. Since the tubulovesicular elements are strongly labeled with antibodies to the newly identified 15-kDa protein, it appears that this protein may be required to gather the virally modified cellular membranes around the factories. Two recent reports have shown that mutations in the F10 gene encoding the 52-kDa protein kinase also block virion assembly at an early step (50, 52), suggesting that phosphorylation might be an essential function early in morphogenesis. After the formation of the viral crescents, spherical IVs (stage IV) arise that will maturate to produce the first IMVrs (stage V), a process that can be impaired by interfering with the expression of the 11- or 47-kDa core proteins or the L1R membrane protein. Wrapping of IMVs by Golgi-derived membranes to generate IEVs (stage VI) requires p14, p37, gp22-24, gp42 and p43-50. EEV exits the cell by fusion of the most external membrane of IEV with the plasma membrane. A proportion of EEV, which varies depending on the virus strain, remains associated with the cell surface, and these retention differences are related to differences in the gp22-24 protein among strains.

observed these structures on ultrathin sections of Epon-embedded, VVindA17L-infected cells, we could not distinguish any apparent membrane in their periphery. Moreover, a recent report by Wolffe et al. (55) shows similar cytoplasmic electron-dense masses produced by a different A17L inducible mutant that are also apparently devoid of membranous structures. However, given the localization of the 15-kDa protein in both cellular and viral membranes, the presence of strong labeling associated with this protein in the periphery of the electron-dense masses suggests the existence of some kind of membranous elements around them. Therefore, in this investigation, we examined very thin sections from VVindA17L-infected cells to obtain a better resolution of membranes. Here we demonstrate that in cells infected with VVindA17L in the absence of the 21-kDa protein, numerous tubulovesicular membranes are actually recruited to the periphery of the dense structures (Fig. 5). The ERGIC origin of these tubulovesicular structures is supported by immunolabeling with a monoclonal antibody specific for the ERGIC-53 protein (Fig. 6), a characterized marker of this compartment (40, 44). According to these results, the 21-kDa protein does not seem to be a key viral factor involved in the early recruitment of the cellular membranes that will originate the viral crescents, as previously proposed (35, 36), although it is strictly required for the formation of crescents. Thus, some other VV protein(s) must be involved in such recruitment of membranes. In this regard, the 15-kDa protein is found associated with the tubulovesicular elements that accumulate around the electron-dense structures formed in the absence of the 21-kDa protein (Fig. 7A and B). The intracellular transport of the 15-kDa protein defined here (RER to ERGIC to viral crescent) suggests that this protein might be one of the viral elements that participate in the membrane recruitment process. When the 21-kDa protein is synthesized, it is also transported to the viral factories in ERGIC-related elements and then is incorporated into viral crescents (Fig. 7E). A similar observation has been recently reported by Krijnse-Locker et al. (19). These data suggest a possible role for the 21-kDa protein in the organization of the viral membranes recruited in the electron-dense masses.

In summary, our results contribute to the elucidation of some of the initial events in the generation of VV membranes and to the assignation of two specific viral proteins to these stages. In support of the model proposed by Sodeik et al. (45), we have found that in addition to the 21-kDa protein, the 15-kDa protein is incorporated into tubulovesicular elements derived from the cellular ERGIC. In cells infected in the absence of the 21-kDa protein, these viral membrane precursors accumulate in the proximity of the arrested viral factories and do not progress to form the crescent-shaped membranes. These data indicate that the 21-kDa protein is not responsible for the recruitment of membranes from the ERGIC to the viral factories but instead appears to be instrumental for the organization of viral membranes, in particular in the changes lead-

ing to the transition from vesicles and tubules to dense structures like those attached to the RBs. The absence of the 65-kDa protein in RBs appears to be related to the lack of curvature and rigidity characteristic of the viral crescents that have incorporated both the 21- and 65-kDa proteins.

The association of the 15-kDa protein with the tubulovesicular membrane precursors occurs irrespective of the 21-kDa protein. This has several implications: (i) targeting of the 15-kDa protein to the membranes of the ERGIC is not mediated through its interaction with the 21-kDa protein, (ii) it occurs at a stage before the assembly arrest caused by inhibition of the 21-kDa protein synthesis, and (iii) the 15-kDa protein could then be involved in bringing the ERGIC membranes to the factories.

The study of the cellular elements that participate in VV assembly has shown that membranes of the *trans*-Golgi network are involved in the wrapping event that transforms the IMVs into IEVs, while elements from the ERGIC participate in earlier steps, to generate the membranes of the immature virions (42, 45). The so-called ERGIC or "*trans*-endoplasmic reticulum network" has been described as a tubulovesicular system connected with the endoplasmic reticulum, which seems to function in protein sorting and regulation of membrane traffic in the early part of the exocytic pathway. The characterization of this compartment was initiated through the study of the accumulation and movement of viral proteins (40). Although the specific signals that regulate the communication between the endoplasmic reticulum and the Golgi complex and the intracellular trafficking of proteins have been extensively studied during the last few years (4, 21), many aspects of the functions and dynamics of this complex membranous system remain to be elucidated. The inducible VV mutant used in this investigation to synchronize morphogenesis is a valuable tool not only for analyzing the complex assembly of VV but also in the search for signals involved in the regulation of intracellular membrane traffic and for the analysis of functional domains in the boundary between the endoplasmic reticulum and Golgi complex. A more detailed analysis of the intracellular trafficking of VV membrane proteins, such as the 15- and 21-kDa proteins and p65, will probably provide new data about the signals participating in the displacement of membranous elements, that usually move along microtubular arrays, to be recruited in different cellular locations, where they are deeply modified through the constitution of macromolecular complexes of viral proteins.

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