The Vaccinia Virus 14-Kilodalton Fusion Protein Forms a Stable Complex with the Processed Protein Encoded by the Vaccinia Virus A17L Gene†

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Vaccinia virus (VV) is a large-DNA animal virus which replicates entirely in the cell cytoplasm (22). The double-stranded DNA genome has the potential to code for about 200 different polypeptides (10), of which about 100 are found in the virus particle (9, 25). Quite unprecedented for animal viruses, in the course of VV infection, two forms of infectious virus are produced: the intracellular naked virus (INV), which consists of a nucleocapsid surrounded by a membrane, and the extracellular enveloped virus (EEV), which represents the INV form surrounded by an additional envelope (13, 14, 21). The EEV form is considered to play an important role in virus dissemination in cells in culture and in the spread of virus infection in experimental animals (26, 27). VV morphogenesis is a complex multistep process in which a large number of polypeptides must be assembled in precise order to form a virus particle. Although much progress has been made in the identification of viral genes encoding polypeptides that are incorporated in the virion, very little is known about the structural organization of the protein components in the virion.

One protein component of INV that plays an important role in the biology of VV is the 14-kDa fusion protein. This protein is highly conserved in members of the Orthopoxvirus genus (31) and is present in the envelope of INV, where it forms disulfide-linked trimers (32). The 14-kDa protein is involved in the fusion of the viral envelope with the cell plasma membrane (5, 32), indicating a role in virus penetration, and it is also involved in cell-to-cell fusion late in virus infection (12, 31, 32). The 14-kDa protein induces neutralizing antibodies (18, 19, 31) and confers immunity to VV in experimental animals (4, 19). In addition, the 14-kDa protein plays an important role in virus dissemination, both in cells in culture and in infected animals (2, 3, 11, 29). In this regard, it has been shown that the 14-kDa protein is required for the envelopment of INV that leads to the formation of EEV (33). Two other viral polypeptides have been identified with roles in the formation of infectious EEV: the 37-kDa acylated protein (1, 34) and the 22- to 24-kDa glycoprotein with homology to C-type animal lectins (7). It has been speculated that the 14-kDa protein may interact with either the 37- or the 22- to 24-kDa protein in order to form EEV (7, 33). Because the 14-kDa protein lacks a membrane signal sequence or a large hydrophobic domain (30), it has been difficult to explain how this protein finds its way to the envelope of INV. In this investigation, we provide evidence that the 14-kDa protein interacts with another virion-associated protein of about 21 kDa. We have identified the VV gene encoding the 21-kDa protein and defined a number of structural properties of the 14-kDa–21-kDa protein complex. Our findings are consistent with the view that the 21-kDa protein serves to anchor the 14-kDa protein in the envelope of INV.

MATERIALS AND METHODS

Virus and cells. The WR strain of VV was propagated and titrated in African green monkey kidney (BSC-40) cells and purified as previously described (15). BSC-40 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% newborn calf serum.

Antisera. The monoclonal antibodies (MAbs) C3 and B11, specific for the 14-kDa protein, have been previously described (31). The rabbit polyclonal anti-14-kDa, anti-R7, and anti-R8 sera were produced by immunization of rabbits with the purified 14-kDa protein (19), the synthetic peptide R7...
Immunoprecipitation analysis. BSC-40 cells were infected with VV at 10 PFU per cell. At 6 h postinfection (p.i.), the medium was removed and replaced by methionine-free DMEM supplemented (1:10) with regular DMEM and \[^{35}S\]methionine (10 \(\mu\)Ci/ml). After 18 h, cells were collected, washed with phosphate-buffered saline (PBS), 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 (bacitracin [2 \(\mu\)g/ml], trypsin inhibitor [2 \(\mu\)g/ml], 1 mM phenylmethylsulfonyl fluoride, leupeptin [10 \(\mu\)g/ml]). After 30 min on ice, cell extracts were sonicated and clarified by centrifugation at 10,000 \(\times\) g for 5 min. These extracts were incubated with preimmune serum coupled to protein A-Sepharose beads (Pharmacia) for 2 h at room temperature. After centrifugation, supernatants were immunoprecipitated by incubation overnight at 4°C with protein A-Sepharose beads coated with specific antibodies against the 14-kDa protein. The immunoprecipitates were washed three times with lysis buffer and three times with PBS and resuspended in 2 \(\times\) 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 (PAGE) (12% polyacrylamide gel). The gels were dried, and the proteins were visualized after autoradiography.

Protein microsequence analysis. Extracts of VV-infected cells were immunoprecipitated with the MAb C3. The immunoprecipitated products were resolved on an SDS–12% polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Problott; Applied Biosystems, Inc.). Proteins electrolubblotted onto the Problott membrane were stained with Ponceau S, and the band corresponding to the 21-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 phosphothiohydantoin amino acid analyzer (Applied Biosystems). The sequence corresponding to the first 10 amino-terminal residues was searched for homology in the EMBL protein data bank, using the PC gene program (Intelligenetics).

Pulse-chase analysis. BSC-40 cells were infected with VV at 10 PFU per cell. At 6 h p.i., cells were washed with methionine-free DMEM and incubated in the same medium. After 30 min, cells were pulse-labeled with \[^{35}S\]methionine (100 \(\mu\)Ci/ml) for 30 min and then chased with a 100-fold excess of unlabeled methionine for the indicated times. Cells were then pelleted on ice, washed three times with ice-cold PBS, and collected. Cells were lysed and immunoprecipitated with MAb C3 as described above.

Fractionation of \(^{35}S\)-labeled virions. \(^{35}S\)-labeled virions were purified from BSC-40 cells infected with VV and metabolically labeled with \[^{35}S\]methionine (10 \(\mu\)Ci/ml) from 6 to 24 h p.i. Viral envelopes were released from virions by treatment with 0.5% NP-40 in lysis buffer, in the absence or presence of the reducing agent dithiothreitol (DTT) (1 and 10 mM), for 30 min at room temperature. Envelopes were separated from cores by centrifugation at 10,000 \(\times\) g for 5 min. The DTT was removed from soluble envelopes by dialysis at 4°C against PBS.

RESULTS

The 14-kDa protein interacts with a 21-kDa protein in VV-infected cells. To test the possibility that the 14-kDa protein interacts with some other protein(s) during VV infection, we performed immunoprecipitation analysis of VV-infected cells, using five different antibodies specific for the 14-kDa protein. BSC-40 cells infected with VV (10 PFU per cell) were labeled at 6 h p.i. with \[^{35}S\]methionine. After 18 h, cells were collected, cell extracts were immunoprecipitated with the various anti-14-kDa antibodies, and the immunoprecipitated products were analyzed by SDS-PAGE. As shown in Fig. 1, a protein with an apparent molecular mass of 21 kDa coprecipitated with the 14-kDa protein with the five different anti-14-kDa polyclonal and monoclonal antibodies. In addition, an 11-kDa protein corresponding to a processed form of the 14-kDa protein (18) was immunoprecipitated by these antibodies. These proteins were not immunoprecipitated by a rabbit preimmune serum (lane F) or by rabbit antisera raised against the VV core p4a and 39-kDa proteins (data not shown).

The 21-kDa protein is encoded by the VV A17L gene. To determine the genome localization of the 21-kDa protein, we carried out microsequencing analysis. Extracts from VV-infected cells were immunoprecipitated with MAb C3, the immunoprecipitated products were fractionated by SDS-PAGE (12% polyacrylamide gel), and proteins were transferred to a Problott membrane. The band corresponding to the 21-kDa protein was excised and processed for microsequencing. The first 10 amino acid residues at the N terminus of the 21-kDa protein were determined. A search of the EMBL protein data bank showed that this amino acid sequence is 100% identical to amino acids 17 to 26 from a VV protein with a predicted molecular mass of 23 kDa, which is encoded by the VV A17L gene of the Copenhagen strain (10) (Fig. 2A). This result indicates that the 21-kDa protein represents a processed product of the 23-kDa precursor that is generated by removal of the first 16 amino acids of the N terminus. The hydropathy plot of the 23-kDa protein (Fig. 2B) revealed the presence of two large hydrophobic domains characteristic of membrane proteins, as previously noted (10).

The 14-kDa protein forms a stable complex with the 21-kDa protein soon after synthesis. Since synthesis of the 14-kDa protein begins at about 6 h p.i. (32), it was of interest to determine how soon after its synthesis the 14-kDa protein interacts with the 21-kDa protein. Cells labeled with \[^{35}S\]me-
with [35S]methionine, by microsequence analysis as described in Materials and Methods. The 10 N-terminal amino acid residues of the 21-kDa protein were identified (boxed in panel A). The hydrophathy plot was determined as described by Kyte and Doolittle (17). Hydrophilic regions are above the axis; hydrophobic regions are below it.

VACCINIA VIRUS 14-kDa–21-kDa PROTEIN COMPLEX

FIG. 2. Protein microsequence analysis of the 21-kDa protein. (A) Deduced amino acid sequence; (B) hydrophathy plot of the 23-kDa protein. Extracts of BSC-40 cells infected with VV (10 PFU per cell) were immunoprecipitated with MAb C3. The immunoprecipitated products were fractionated by SDS-PAGE (12% polyacrylamide gel) and electroblotted to a Problott membrane. The 21-kDa band was identified after Ponceau S staining, cut out, and subjected to microsequence analysis as described in Materials and Methods. The 10 N-terminal amino acid residues of the 21-kDa protein were identified (boxed in panel A). The hydrophathy plot was determined as described by Kyte and Doolittle (17). Hydrophilic regions are above the axis; hydrophobic regions are below it.

conditions the 14- and 21-kDa proteins are the main products immunoprecipitated with MAb C3 (Fig. 5a, lane B), under nonreducing conditions, the protein pattern changes and new products with apparent molecular masses of 26, 30, 40, and 52 kDa appear (Fig. 5a, lane C). To determine whether these products represent oligomers of the 14-kDa protein and 14-kDa–21-kDa heterodimers, we performed two-dimensional SDS-PAGE analysis, in which the first dimension was run under nonreducing conditions and the second dimension was run under reducing conditions. It is clear from Fig. 5b that the 26-, 40-, and 52-kDa aggregates are derived from the 14-kDa protein and correspond, respectively, to the dimer, trimer, and tetramer forms of the 14-kDa protein. The 30-kDa aggregate (indicated by a star) is resolved as a 21-kDa protein, indicating that the 30-kDa aggregate is a dimer of the 21-kDa protein. In addition, we observed another product of about 18 kDa that could represent a processed form of the 21-kDa protein. There was no evidence for a 14-kDa–21-kDa protein complex, indicating that the linkage between these two proteins is not mediated by disulfide bonds and that the complex is disrupted under the conditions used for running the first dimension on SDS-PAGE.

Compartmentalization of the 14-kDa–21-kDa protein complex in the virion. Since the 14-kDa protein is a component of the envelope of INV, and since we have shown that the interaction between the 14- and 21-kDa proteins is stable throughout infection, these two proteins might be present in the virus particle as a complex. To test this possibility, purified 35S-labeled virions were disrupted by treatment with the nonionic detergent NP-40, in the absence or presence of...
the reducing agent DTT. The soluble envelopes were removed from viral cores by centrifugation and dialyzed against PBS. The proteins were immunoprecipitated by MAb C3 and fractionated by SDS-PAGE. As shown in Fig. 6A, the 14-kDa protein was the only product immunoprecipitated by MAb C3 when envelopes were prepared from detergent-treated virions (lane 4). However, when envelopes were extracted with detergent plus the reducing agent DTT, the 21-kDa protein coprecipitated with the 14-kDa protein (lanes 5 and 6). In addition, the 11-kDa protein, a cleavage product of the 14-kDa protein (18), was released from virions by this treatment and was immunoprecipitated by MAb C3 (lanes 5 and 6). In agreement with previous observations (8), the amount and number of polypeptides released from virions by detergent increased in the presence of a reducing agent (Fig. 6A; compare lane 1 with lanes 2 and 3). When the same immunoprecipitated products were analyzed under nonreducing conditions (Fig. 6B), the 14-kDa protein appeared mostly as a trimer (lanes 2 to 4), as previously described (32). In addition, the dimer of the 21-kDa protein (denoted by a star) was observed (lanes 2 and 3). We conclude that the 14-kDa–21-kDa protein complex in the virion consists mostly of trimers of the 14-kDa protein and of dimers of the 21-kDa protein.

**DISCUSSION**

The morphogenesis of VV is a complex process that results in the formation of two infectious forms of virions: INV, which is the most abundant form within the cell, and EEV, which consists of INV wrapped in a double membrane acquired from the Golgi apparatus (13, 14, 21). Since purified virions contain about 100 different polypeptides (9, 25), it has been difficult to assign the role of distinct proteins in virus assembly. Three virus proteins of 14 kDa (33), 37 kDa (1, 34), and 22 to 24 kDa (7) have been proposed to be required for EEV formation and virus egress. Unlike the 37-kDa acylated protein and the 22- to 24-kDa protein, which are present in the outer membrane of EEV, the 14-kDa protein is a component of the envelope of INV.

In this investigation, we demonstrate that the 14-kDa protein forms a complex with a 21-kDa protein. The 14-kDa–21-kDa protein complex was immunoprecipitated from infected cells by different antibodies directed against the 14-kDa protein (Fig. 1), but neither of these two envelopes was immunoprecipitated by preimmune serum or by antibodies against the core p4a and 39-kDa proteins (data not shown), thus establishing the specificity of the 14-kDa–21-kDa protein interaction. The first 10 amino acids at the N terminus of the 21-kDa protein were determined by microsequencing analysis, and it was found that the 21-kDa protein corresponded to the processed form of a predicted 23-kDa viral polypeptide. This polypeptide is encoded by the VV A17L gene of the Copenhagen strain (Fig. 2) that is in close proximity to A27L, the gene that encodes the 14-kDa protein. The N terminus of the 21-kDa protein lies within the tripeptide sequence Ala-Gly-Ala, previously identified as a consensus motif for proteolytic processing of some structural proteins of VV and fowlpox virus (35). The cleavage site within the A17L gene product has also been identified (36). Analysis of the 23-kDa protein sequence revealed the presence of two large hydrophobic domains in the central region of the protein, which could provide sites for membrane anchoring.

Formation of the 14-kDa–21-kDa protein complex was a rapid process but with a lag period. Within 1 h after synthesis, the 14-kDa protein associated with the 21-kDa protein, and this association remained stable throughout infection (Fig. 3). Significantly, the 14-kDa protein interacted only with the processed 21-kDa product and not with the 23-kDa precursor, although both the 23- and 21-kDa
proteins were immunoprecipitated from extracts of infected cells with a polyclonal antibody reactive against these two proteins (kindly provided by D. E. Hruby) (data not shown). Moreover, processing and complex formation were not affected by rifampin, a drug that blocks virus morphogenesis at an early step by blocking proteolytic processing of several virion core proteins (16, 23, 24, 28). The fact that the 14-kDa–21-kDa protein complex was immunoprecipitated from extracts of cells infected in the presence of rifampin (Fig. 4) indicated that the proteolytic cleavage of the 23-kDa precursor is independent of the drug and that the 14-kDa–21-kDa protein complex formation occurred prior to virus maturation. This finding is in agreement with the rapid interaction seen for both proteins by pulse-chase analysis (Fig. 3).

By two-dimensional SDS-PAGE analysis of the MAb C3-immunoprecipitated products in which the first dimension was run under nonreducing conditions and the second dimension was run under reducing conditions, we did not observe a protein aggregate consisting of both 14- and 21-kDa proteins (Fig. 3). This result implies that the interaction between 14- and 21-kDa proteins is not maintained by disulfide bonds and can be disrupted by the conditions used during the electrophoresis. In fact, we have observed that the 14-kDa–21-kDa protein complex is dissociated by 0.5% SDS (data not shown). While the 21-kDa protein formed trimers linked by disulfide bonds, there was a discrepancy between the predicted (42 kDa) and the observed (30 kDa) molecular masses of the 21-kDa dimer. This discrepancy may be related to folding of the dimer. In addition, we observed that the 14-kDa protein forms not only dimers and trimers but also tetramers. Formation of 14-kDa dimers and trimers has been previously described (18, 32), but the presence of 14-kDa tetramers in the course of virus infection has not been reported before.

The 21-kDa protein does not contain a signal sequence or a large hydrophobic domain characteristic of membrane proteins (30). Because of these properties, it has been difficult to explain how this protein is anchored in the outer membrane of INV. From our findings, it appears likely that the 21-kDa protein serves to anchor the 14-kDa protein on the envelope of INV. In support of this notion, we have observed that stripping envelopes of INV with the nonionic detergent NP-40 results in the release of the 14-kDa protein but not of the 21-kDa protein; however, the 14-kDa–21-kDa protein complex is released when virions are treated with both a detergent and a reducing agent (Fig. 6A). These findings suggest that the 21-kDa protein is localized in a more internal position within the virion than is the 14-kDa protein. Moreover, when the products immunoprecipitated from soluble envelopes were analyzed by SDS-PAGE under nonreducing conditions, the 14-kDa trimer and the 21-kDa dimer were found to be the most abundant products (Fig. 6B), providing evidence that these two oligomeric structures are involved in the 14-kDa–21-kDa protein complex. The interaction between trimers of the 14-kDa protein and dimers of the 21-kDa protein could be similar to the interaction observed between the viral matrix and the envelope glycoprotein of vesicular stomatitis virus (6, 20).

Since the 14-kDa protein is one of the three proteins identified as being involved in the envelopment of INV by a Golgi-derived membrane (33), and this protein is required for the formation of EEV, it has been proposed that an interaction might exist between 14-kDa protein and either the 37- or 22- to 24-kDa protein of EEV (7, 33). Although the association between the 14- and 21-kDa proteins was revealed by immunoprecipitation analysis with antibodies against the 14-kDa protein and also by cosedimentation on sucrose gradients of NP-40-treated extracts from virus-infected cells (data not shown), interaction of the 14-kDa protein with either the 37- or 22- to 24-kDa protein was not observed. However, treatment of both virions and extracts from virus-infected cells with the cross-linker agent disuccinimidyl suberate, using conditions previously described (18), results in the formation of a large protein complex which is recognized by antibodies to the 14-kDa protein, indicating the interaction of the 14-kDa protein with other protein(s) (data not shown).

Several lines of evidence indicate that the trimeric form of the 14-kDa protein is exposed on the outside of the envelope of INV. First, MAb C3 neutralizes the infectivity of INV and also prevents virus-induced cell fusion (12, 31, 32); second, removal of the 14-kDa protein from INV is obtained with low concentrations of a nonionic detergent (Fig. 6) (31), a condition that preserves the structure of the nucleocapsid; third, electron microscopy of immunogold-labeled INV, based on reactivity with MAb C3 and with goat anti-mouse immunoglobulin G conjugated with gold, reveals exclusive labeling around the viral envelope (unpublished results). These observations suggest that trimers of the 14-kDa protein are projected from outside the viral envelope. Since the N terminus of the 14-kDa protein has been proposed to be involved in virus-cell fusion (12), the N-terminal portion of the protein could be exposed on the outside of the envelope, while on the inside, the C terminus of the 14-kDa protein could interact with the 21-kDa protein. Further study of these two proteins should provide better understanding of the structure-function relationship of membrane fusion proteins.

Whether the 21-kDa protein is essential for virus infection is not yet known. The generation of VV recombinants with an inducible A17L gene will reveal more insights about the role of the 21-kDa protein in virus infection and virion morphogenesis.

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