View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

VIROLOGY 213, 19–27 (1995)

Biochemical Analysis of the Major Vaccinia Virus Envelope Antigen

CAROLINE SCHMUTZ, LORENZ RINDISBACHER, MARIE C. GALMICHE, and RICCARDO WITTEK[1]

Institut de Biologie Animale, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne, Switzerland

Received February 14, 1995; accepted August 7, 1995

The major envelope antigen of vaccinia virus is an acylated protein of M_r 37,000 (p37K) which is required for the formation of extracellular enveloped virions (EEV). Despite its important role in the wrapping process, p37K has not been studied in much detail. In order to better characterize this protein we have undertaken a detailed biochemical analysis. Sodium carbonate treatment showed that p37K is tightly bound to the viral envelope. Its resistance to proteinase K digestion indicates that it is not exposed on the surface of EEV but lines the inner side of the envelope. Since p37K does not contain a signal peptide characteristic of most membrane proteins, we examined the possibility that the protein acquires its membrane affinity through the addition of fatty acids. Indeed, Triton *X*-114 phase partitioning experiments demonstrated that p37K is hydrophobic when acylated, but hydrophilic in the absence of fatty acids. Three other viral proteins have been shown to be required for virus envelopment and release from the host cell and we therefore tested whether p37K interacts with viral proteins. In EEV and in absence of reducing agents, an 80-kDa complex reacting with an anti-37K antiserum was found. Analysis of this complex showed that it most likely consists of a p37K homodimer. Interestingly, only a small amount of p37K occurs as a complex, most of it is present in the viral envelope as monomers. © 1995 Academic Press, Inc.

INTRODUCTION

Vaccinia virus, the representative member of the Poxviridae, has a complex morphology and replicates in the cytoplasm of infected cells. Among the four distinct forms of vaccinia virions generated during the replication cycle, intracellular mature virions (IMV) and extracellular enveloped virions (EEV) are the best characterized. IMV contain two lipoprotein membranes of which the inner encloses the complex core structure containing the viral genome while the outer represents the IMV external membrane. The EEV are surrounded by a third membrane. The mechanism by which IMV are enveloped is poorly understood. The process starts with the association of fully infectious IMV with the trans-Golgi network to produce the four-membraned structure of intracellular enveloped virus (IEV) (Schmelz *et al.*, 1994). These particles migrate along actin-containing microfilaments to the cell surface (Hiller *et al.*, 1981b) where they lose their outermost membrane by fusion with the plasma membrane. The final separation between the virions and the host cell results in the release of EEV which are composed of the original IMV enclosed by one trans-Golgi network-derived membrane. The envelope of EEV contains 10 proteins, 9 glycoproteins and 1 nonglycosylated acylated protein which has been designated p37K (Payne, 1978; Hiller and Weber, 1985). Several studies have recently shown that the 42K and 22–24K envelope proteins, as well as the 14K IMV protein, are essential for virus envelopment and egress of EEV (Rodriguez and Smith, 1990a; Duncan and Smith, 1992; Engelstad and Smith, 1993; Wolffe *et al.*, 1993). The 37K protein also plays a crucial role in this process (Blasco and Moss, 1991; Schmutz *et al.*, 1991), but has been poorly characterized so far. This protein is encoded by the F13L open reading frame (Goebel *et al.*, 1990) that is expressed during the late phase, i.e., 6–8 hr postinfection (Hirt *et al.*, 1986) and is the major viral envelope-specific protein representing 5–7% of the total particle proteins (Hiller *et al.*, 1981a). The p37K is acylated by palmitic and oleic acids (Hiller and Weber, 1985; Payne 1992) and is important for local cell-to-cell transmission of the virus and in the virus-induced formation of microvilli (Blasco and Moss, 1991).

In view of its important role in the formation of enveloped virions, we have studied some biochemical characteristics of p37K and analyzed its potential interaction with other viral proteins.

MATERIALS AND METHODS

Cells and viruses

The rabbit kidney cell line RK13 (ATCC No. CCL 37) was cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS) and containing 100 U/ml and 100 μg/ml penicillin and streptomycin, respectively. The IHD-J strain of vaccinia virus, obtained from L. G. Payne, was used in this study.

Antisera

The monoclonal antibodies anti-42K (19C2; Schmelz *et al.*, 1994) and anti-hemagglutinin (B2D10; Oie *et al.*,

[1] To whom correspondence and reprint requests should be addressed. Fax: (+41 21) 692 41 05.

0042-6822/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

1990) were kindly provided by G. Griffiths, EMBL, Heidelberg, Germany, and Y. Ichihashi, Niigata University, Asahimachi, Japan, respectively. The anti-37K antiserum was prepared by immunizing rabbits with the purified protein. The gene was cloned and expressed using the QIAexpressionist system (Qiagen). A 1716-bp *Eco*RI fragment containing the F13L gene coding and flanking sequences of the IHD-J strain (Schmutz *et al.*, 1991) was cleaved with *Ssp*I, and the fragment containing almost the entire F13L open reading frame was subcloned into the pQE-18 vector (Qiagen) linearized with *Bgl*II and blunt-ended with the Klenow fragment of DNA polymerase I. This introduced the amino acid sequence MIFRS-$(H)_6$stop after the K_{356}, 16 amino acids upstream of the natural stop codon of the F13L gene. The engineered F13L gene was excised using the *Kpn*I site located 15 nucleotides downstream of the natural start codon and the *Bgl*I site 3′ of the stop codon in the vector DNA. This fragment, together with a synthetic oligonucleotide reconstituting the 15 5′-terminal nucleotides, was ligated into the expression vector pQE-5 at the sites *Bam*HI (blunt) and *Bgl*I. The truncated p37K was overexpressed in *Escherichia coli* SC 13′009 and purified by Ni^{2+} chelate affinity chromatography (Qiagen) under denaturing conditions as described by the supplier. The protein was further purified by SDS–PAGE.

Antisera were raised in rabbits by four subcutaneous injections of recombinant protein at monthly intervals. The first dose contained 500 μg of protein in complete Freund's adjuvant and the following three 250 μg of protein in incomplete Freund's adjuvant. Antibody titers were monitored by Western blotting and immunoprecipitation.

Phase partitioning experiments

The hydrophobic or amphiphilic nature of p37K was determined by phase separation experiments (Bordier, 1981). Briefly, infected cells or virus were suspended in 1% (final concentration) ice-cold Triton X-114, kept on ice for 10 min with occasional shaking, and then centrifuged at 10,000 *g*. The supernatant was incubated at 37° for 5 min and then again centrifuged for 5 min at 10,000 *g*. After centrifugation the detergent phase was found at the bottom of the tube and the aqueous phase at the top. The two phases were separated, reconstituted with the counterpart fractions originating from a blank, and reextracted as before. The aqueous and detergent phases were adjusted to conditions for immunoprecipitation or the proteins were precipitated with methanol.

Hydroxylamine treatment

Infected cells were harvested in buffer H (390 m*M* Tris–HCl, pH 8.8, 0.1% NP-40), kept on ice for 10 min, homogenized by 30 passages through a $26\frac{3}{8}$ gauge needle, and the lysates were clarified by a 5-min centrifugation at 700 *g*. Equal volumes of 2 *M* hydroxylamine, pH 7.0, or buffer H as a control were added to the extracts and the samples were incubated at room temperature for 2 hr and then lyophilized overnight. The dry pellets were resuspended in PBS and subjected to phase partitioning experiments.

Sodium carbonate treatment

EEV preparations were diluted 50- to 1000-fold with ice-cold 100 m*M* sodium carbonate, pH 11.5, kept on ice for 30 min, and centrifuged at 4° for 1 hr at 230,000 *g* as described previously (Fujiki *et al.*, 1982). Proteins in the supernatant were TCA precipitated, and the pellet was washed with distilled water and centrifuged as before. Pellets were resuspended either in 2× sample buffer (62.5 m*M* Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 0.003% bromophenol blue) for SDS–PAGE or lysis buffer [10 m*M* Tris–HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate (NaDOC), 0.1% SDS, 150 m*M* NaCl, 1 m*M* EDTA] for immunoprecipitation.

Proteinase K digestion on EEV

EEV were recovered from the culture medium of infected cells (175-cm^2 flask) grown in DMEM without FCS. The medium was clarified by low-speed centrifugation and the EEV were concentrated by a 30-min centrifugation at 100,000 *g* and 4° and were resuspended in 50 μl of PBS.

EEV (4 μl) were digested for 1 hr or overnight with 0.57 μl of proteinase K (3 mg/ml) in 9 μl final volume, in presence or absence of 3% Triton X-100. The proteins were precipitated with TCA and immediately boiled in 2× sample buffer.

Immunoprecipitation

Confluent monolayers of RK13 cells were infected at a multiplicity of infection of 10 and labeled, for the periods of time indicated, with either 20 μCi/ml [^{35}S]methionine or 80 μCi/ml [^{3}H]palmitic acid. After 24 hr, EEV were prepared as described above. The total cell extracts were prepared by resuspending the cells in the indicated lysis buffer (see below) after washing with PBS. After 30 min on ice, cell extracts were sonicated (water bath) and clarified by centrifugation for 5 min at 10,000 *g*. The amount of extract equivalent to 10^6 cpm was mixed with anti-37K rabbit antiserum at room temperature for 90 min. Then, 20 μl of a 50% slurry of protein A–Sepharose beads in PBS was added and incubation was continued for 90 min. The immunoprecipitates were washed three times with the corresponding lysis buffer and three times with PBS and resuspended in 2× sample buffer without reducing agents. Samples were boiled for 3 min and split into two aliquots. The proteins of one aliquot were reduced by the addition of 150 m*M* DTT and 1.5% β-mercaptoethanol for 30 min before being boiled again. The different samples were resolved on SDS–PAGE. The

gels were dried, and the proteins were visualized by autoradiography.

Lysis buffers:

I. 50 m*M* Tris–HCl, pH 8.0, 1% Triton X-100, 0.5% NaDOC, 0.1% SDS, 150 m*M* NaCl, 1 m*M* EDTA.

II. 50 m*M* Tris–HCl, pH 8.0, 1% Triton X-100, 0.5% NaDOC, 0.1% SDS, 1 *M* NaCl, 1 m*M* EDTA.

III. 50 m*M* Tris–HCl, pH 8.0, 0.2% Triton X-100, 0.5% NaDOC, 150 m*M* NaCl.

IV. 50 m*M* Tris–HCl, pH 8.0, 0.2% Triton X-100, 0.5% NaDOC, 1 *M* NaCl.

EEV lysis, alkylation, and cross-linking

EEV prepared as above were washed with and resuspended in PBS at about 1.5×10^8 PFU/ml and stored as 50-μl aliquots at −20°. Samples of 7.5 μl of this suspension were lysed in a total volume of 60 μl of sample buffer without reducing agent containing 1, 0.1, or 0.01% SDS and were either boiled or heated to 37° for 5 min, as indicated in the figure legends. In order to block unbound SH-groups by alkylation, the lysis reactions contained 1% SDS and either 40 m*M* *N*-ethyl-maleimide (NEM) or 100 m*M* 2-iodoacetamide (both from SIGMA) and were incubated at 25° for 30 min before being boiled for 5 min. For cross-linking studies, 7.5 μl of EEV suspension was incubated in a total volume of 50 μl of PBS containing 0.2% Triton X-100 and 0–0.1% glutaraldehyde (see figure legends) for 30 min at 25°. SDS–PAGE samples were then prepared by adding 14 μl of 5× sample buffer containing reducing agent.

For SDS–PAGE and Western blotting using anti-37K rabbit antiserum, 10 μl of these lysates was loaded per lane on 10% gels.

Western blotting

Proteins were fractionated in 10 or 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. After transfer, the membranes were soaked in blocking buffer made out of TBS–Tween (25 m*M* Tris–HCl, pH 7.5, 137 m*M* NaCl, 3 m*M* KCl, 0.05% Tween 20) containing 5% nonfat dry milk. The membranes were then incubated with anti-37K rabbit antiserum (diluted 1:100,000), anti-42K monoclonal antibody (diluted 1:500), or anti-hemagglutinin monoclonal antibody (diluted 1:10) in blocking buffer for 1 hr at room temperature. After four 10-min washes with TBS–Tween, the membranes were incubated for 1 hr at room temperature with rabbit or mouse anti-IgG antibody (diluted 1:2000) conjugated with horseradish peroxidase in blocking buffer. After four washes with TBS–Tween, bound antibodies were detected using the enhanced chemiluminescence kit from Amersham.

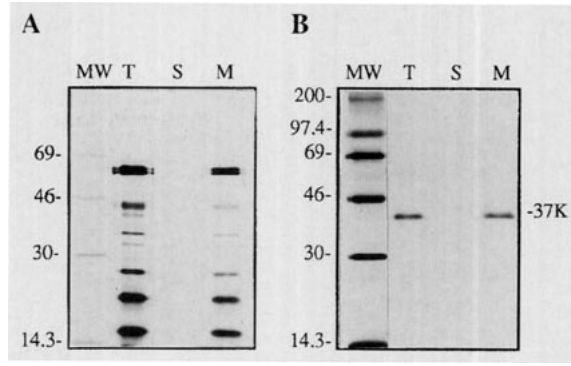

FIG. 1. Sodium carbonate treatment of labeled EEV. EEV labeled with [^{35}S]methionine in the late phase of infection were subjected to sodium carbonate treatment and the fractions containing the total (T), soluble (S), and membrane (M) proteins were analyzed (A) as such or (B) after immunoprecipitation with anti-37K antiserum. The molecular weights of marker proteins (MW) are indicated in kilodaltons.

RESULTS

The p37K is tightly bound to the viral envelope

Treatment with sodium carbonate at pH 11.5 is used to separate soluble proteins and proteins loosely attached to the membrane from tightly bound and integral membrane proteins (Fujiki *et al.,* 1982). This procedure was applied to partially purified [^{35}S]methionine-labeled EEV in order to characterize the attachment of p37K to the envelope.

All proteins present in the starting material were found in the pellet fraction after sodium carbonate treatment (Fig. 1A). Western blotting confirmed that this is also true for p37K (Fig. 1B), and we therefore conclude that this protein is tightly bound to the membrane. In contrast, when similar experiments were performed with IMV, some proteins were found in the supernatant fraction (data not shown). This indicates that loosely bound proteins can indeed be removed by this technique, validating the results obtained with EEV.

The hydrophobic nature of p37K is due to the presence of fatty acids

The 37K protein is located in the viral envelope, although the hydropathic index does not reveal hydrophobic regions characteristic of a signal peptide and a membrane-spanning anchor sequence. Nevertheless it has been suggested that p37K is relatively hydrophobic (Hiller and Weber, 1985) and indeed there are two hydrophobic regions close to the center of the polypeptide (Hirt *et al.,* 1986). Phase partitioning using Triton X-114 allows a fast determination of the relative hydrophobicity of a protein. RK13 cells were infected and cell extracts as well as EEV particles were treated with Triton X-114 and analyzed on SDS–PAGE. Western blotting of cell extracts showed an enrichment of the p37K in the detergent phase (Fig. 2A). In contrast, EEV particles were only

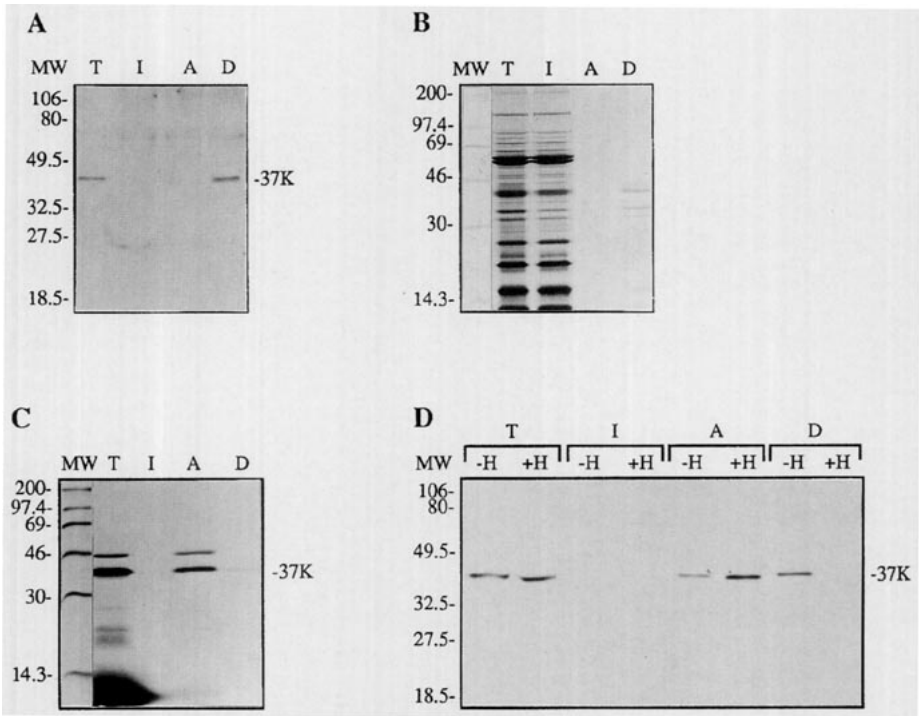

FIG. 2. Triton X-114 phase partitioning analysis. (A) Infected cell extracts, (B) labeled EEV, (C) *in vitro*-translated p37K, and (D) infected cell extracts treated (+H) or nontreated (−H) with hydroxylamine were subjected to phase partitioning. The total (T), insoluble (I), aqueous (A), and detergent (D) fractions were analyzed by autoradiography (B, C) or Western blotting using anti-37K antiserum (A, D). The molecular weights of marker proteins (MW) are indicated in kilodaltons.

weakly solubilized by this treatment and most of the proteins were present as insoluble material. After phase separation some proteins were nevertheless visible in the detergent phase, the largest one most likely representing p37K (Fig. 2B). This overall hydrophobic nature of p37K could be due either to the two hydrophobic amino acid streches or to the presence of fatty acids. To distinguish between these possibilities a phase partitioning experiment was performed on p37K translated *in vitro* in rabbit reticulocyte lysates, which presumably do not add fatty acids to the translation products. Figure 2C shows an enrichment of the protein in the aqueous phase, and only a very weak signal is observed in the detergent phase. This result indicates that the hydrophobic behavior of the p37K is mainly due to its acylation and that the two hydrophobic regions play only a minor role in this respect. To confirm this result we made use of the fact that palmitate−protein bonds are highly labile and susceptible to cleavage by hydroxylamine (Magee *et al.*, 1984). Indeed the p37K−palmitate bond has previously been shown to be sensitive to this treatment (Franke *et al.*, 1989). A phase separation experiment on hydroxylamine-treated cell extacts is shown in Fig. 2D. Upon depalmitation p37K exclusively partitions in the aqueous phase. This result confirmed that the palmitic acid moiety confers the hydrophobicity to p37K. The small amount of p37K observed in the aqueous phase of the nontreated extracts might be explained by the lability of the palmitate bond.

Location of p37K within the envelope

To investigate the topology of p37K in the envelope we performed protease shaving experiments. When viral particles are treated with a nonspecific protease such as proteinase K, proteins or parts of proteins exposed to the outside of the virions will be cleaved and progressively digested by the protease. The separation of virions and shaved protein fragments can be achieved by centrifugation and both fractions can be analyzed using specific antibodies. Depending on the size of the proteins or protein fragments and the fraction where these are found, their position with respect to the membrane can be determined.

Partially purified EEV were treated with proteinase K for 1 hr or overnight. As a control for protease action, the same starting material was exposed to the enzyme overnight in presence of Triton X-100. The detergent disrupts the envelope and therefore should render p37K accessible to the action of the protease. By means of the anti-37K antiserum, intact p37K was detected in the viral pellets which had or had not been treated with protease (Fig. 3A). However, overnight digestion in presence of Triton X-100 completely degraded the protein. This indicates that the protein is not exposed to the outside of the EEV but probably lines the inner surface of the envelope. Similar analyses of the same fractions using anti-42K (Fig. 3B) and anti-hemagglutinin (data not shown) monoclonal antibodies indicated that these enve-

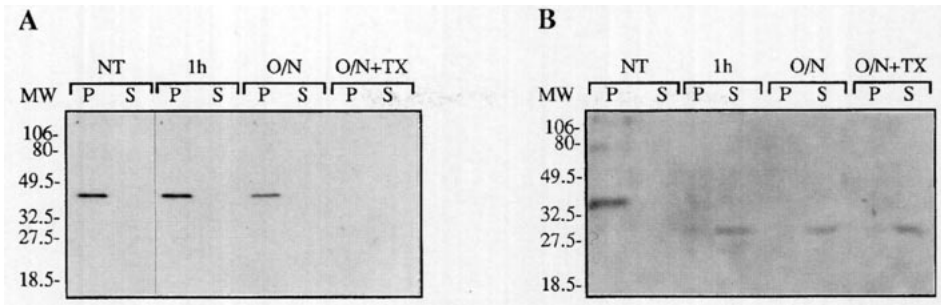

FIG. 3. Protease shaving experiments on EEV. EEV were nontreated (NT), treated for 1 hr (1h) or overnight (O/N) with proteinase K, or digested overnight in presence of Triton X-100 (O/N+TX). After centrifugation, the pellet (P) and supernatant (S) fractions were analyzed by Western blotting with (A) anti-37K antiserum or (B) anti-42K monoclonal antibody. The molecular weights of marker proteins (MW) are indicated in kilodaltons.

lope proteins are exposed to the outside of EEV since single fragments of reduced size were detected in the supernatant fraction. The unexpected resistance of these shaved fragments to complete proteolysis might be explained by a heavily glycosylated globular structure which may protect regions of the exposed fragments of the proteins.

Protein interactions

The 37K, 42K, and 22–24K envelope proteins as well as the 14K protein present on the surface of the IMV have been shown to be required for the formation or release of enveloped particles (Rodriguez *et al.*, 1985; Rodriguez and Smith, 1990b; Blasco and Moss, 1991; Duncan and Smith, 1992; Engelstad and Smith, 1993; Wolffe *et al.*, 1993). Interactions between these proteins might therefore be needed during the complex process leading to the formation and release of enveloped virions.

In order to detect potential interactions of these proteins with p37K, [^{35}S]methionine-labeled infected cells or EEV were lysed with different buffers. One of these has previously allowed the identification of protein–protein interactions in vaccinia virus (Rodriguez *et al.*, 1993). After immunoprecipitation, the proteins were solubilized in sample buffer under either reducing or nonreducing conditions before being analyzed by SDS–PAGE. Despite various conditions tested (lysis buffers, infection times, presence or absence of reducing agents), we were not able to detect any protein interactions in infected cell lysates (data not shown). However, these experiments revealed a difference in the migration of the immunoprecipitated p37K depending on the presence of reducing agents (data not shown), suggesting the presence of intramolecular disulfide bonds.

In contrast, immunoprecipitation of p37K from lysed EEV revealed a high molecular weight complex of about 80 kDa which was present in all lysis conditions used but only in the absence of reducing agents (Figs. 4A and 4B). This indicates that the proteins in the complex are covalently linked by intermolecular disulfide bonds which are already present in EEV released only 10 hr postinfection (Figs. 4C and 4D).

The fact that some p37K is present in a complex containing disulfide bonds is surprising in view of the reducing environment of the cytosol. However, such bonds apparently form as a consequence of the envelopment process since the complex was only found in EEV and not in cell extracts. It is therefore conceivable that these bonds are only present in p37K contained in the inner trans-Golgi network-derived membrane which remains associated with EEV, and not in p37K of the outer membrane of the intracellular four-membraned particles. Nevertheless, to rule out the possibility that the disulfide bonds are formed as an artifact after EEV lysis, purified virions were lysed in the presence of alkylating agents which block free SH groups. After EEV lysis, the proteins were separated by SDS–PAGE under nonreducing conditions and p37K was detected by Western blotting (Fig. 5A). Neither NEM nor iodoacetamide had any effect on the amount of the 80-kDa complex detected. In the presence of the alkylating agents, both p37K and the 80-kDa complex had a slightly reduced electrophoretic mobility, suggesting that the compounds had indeed bound to the SH groups, thus increasing the apparent M_r. This experiment therefore strongly suggests that the complex is present in the envelope prior to EEV lysis.

Since p37K contained in the 80-kDa complex represents a minor fraction with respect to monomeric p37K, we tested the possibility that some complex was lost as a result of too harsh lysis conditions. EEV particles were therefore lysed at lower SDS concentration and at reduced temperature. These milder conditions did not alter the ratio of complexed to monomeric p37K (Fig. 5B). As an alternative approach to detect more p37K in complexed form, we performed cross-linking experiments. Purified EEV were lysed with increasing concentrations of glutaraldehyde, and the proteins were separated by SDS–PAGE under reducing conditions and analyzed by Western blotting. Significantly, with 0.001 and 0.01% glutaraldehyde a protein complex in the 80-kDa range was observed, as well as a higher M_r complex (Fig. 5C). With higher concentrations of the cross-linking agent, these complexes as well as the signals for monomeric p37K were no longer detectable. The reason for this is unclear,

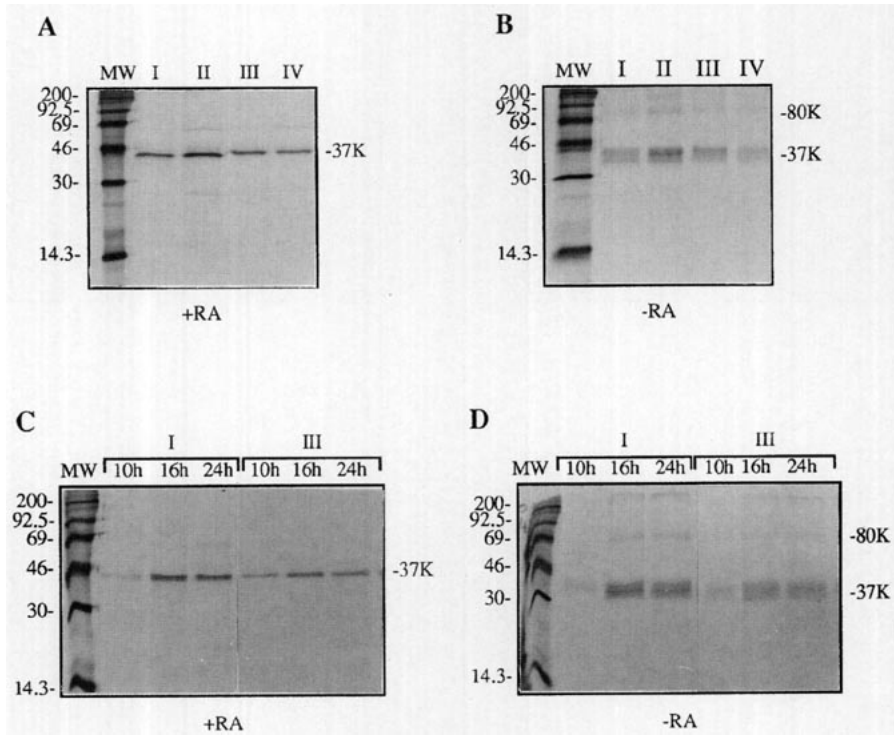

FIG. 4. Immunoprecipitation of EEV lysates. [^{35}S]Methionine-labeled EEV were harvested (A, B) after 20 hr or (C, D) at the indicated times and lysed with stringent (I, II) or nonstringent (III, IV) lysis buffers (see Materials and Methods). p37K was immunoprecipitated in the corresponding lysis buffers in the absence of reducing agents. The proteins were (+RA) or were not (−RA) reduced before SDS–PAGE. The molecular weights of marker proteins (MW) are indicated in kilodaltons.

but one possibility is that extensive cross-linking renders the epitopes inaccessible to antibody binding. Taken together, the results obtained with alkylating agents, different lysis conditions, and cross-linking agents strongly suggest that some p37K is present in the viral envelope as a complex, but that the majority of p37K occurs as monomers.

As a first step to identify the partners constituting the complex, nonreduced lysed EEV were run on a 10% SDS gel and analyzed with an anti-37K rabbit antiserum and an anti-42K antibody to test whether these two proteins are present in the 80-kDa complex.

As expected, anti-37K rabbit antiserum detected the band for monomeric p37K and also reacted with the 80-kDa complex (Fig. 6B1). In addition to the 42K monomer, the anti-42K antibody bound a complex migrating slightly above the 80-kDa band containing p37K (Fig. 6D1). To confirm that the two complexes are different, the two membranes were stripped and reprobed with the other antibody (Figs. 6B2 and 6D2). Comparison of the positions of the bands obtained on each membrane with the two antibodies clearly indicated that the two complexes are different. Their sizes suggested that each complex is a homodimer of either the 37K or the 42K protein.

The p37K-containing complex was further analyzed using a different approach (Fig. 7). An immunoprecipitation

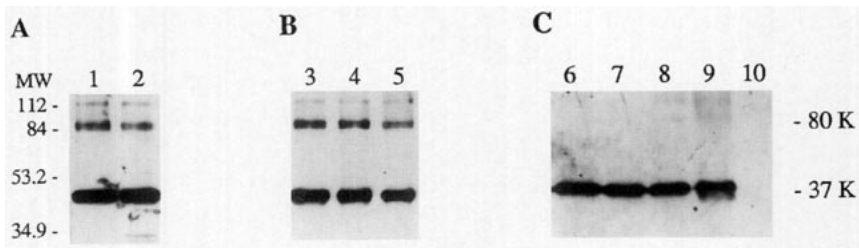

FIG. 5. Effect of alkylating agents and different lysis conditions on the 80-kDa complex. (A) EEV were incubated in sample buffer containing 40 m*M* NEM (lane 1) or 100 m*M* iodoacetamide (lane 2) or (B) EEV were lysed in sample buffer containing 1% SDS at 100° (lane 3) or containing 0.1 or 0.01% SDS at 37° (lanes 4 and 5, respectively) prior to SDS–PAGE under nonreducing conditions. (C) Alternatively, EEV were lysed in the absence (lane 6) or in the presence of glutaraldehyde at concentrations of 0.0001, 0.001, 0.01, or 0.1% (lanes 7–10, respectively) and then subjected to SDS–PAGE under reducing conditions.

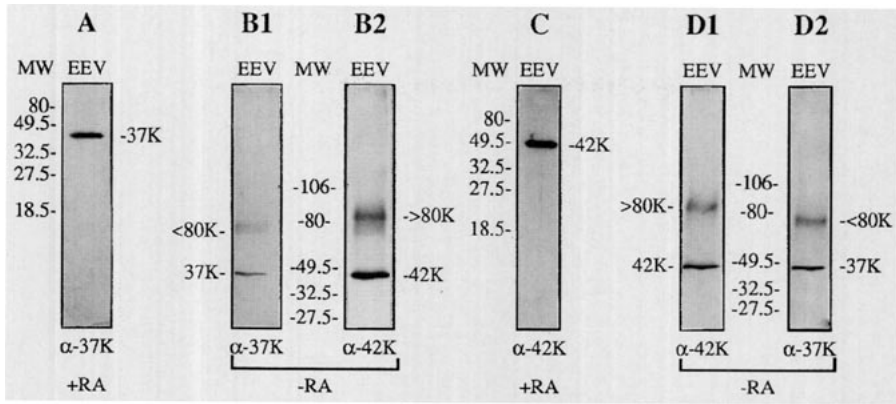

FIG. 6. Detection of 37K and 42K proteins in higher M_r complexes. EEV resuspended in sample buffer with (+RA) or without (−RA) reducing agents were loaded on (A, C) 15% or (B, D) 10% SDS gels. The proteins were transferred to nitrocellulose membranes and visualized with (A, B1) anti-37K antiserum or (C, D1) anti-42K monoclonal antibody. The membranes were stripped and reciprocally reprobed with (B2) anti-42K monoclonal antibody or (D2) anti-37K antiserum. The molecular weights of marker proteins (MW) are indicated in kilodaltons.

was performed on EEV lysates in presence or absence of reducing agents. After SDS–PAGE the regions corresponding to the p37K and 80-kDa complex were excised from the gel and subjected to a second SDS–PAGE under reducing conditions. The 80-kDa complex was again present only under nonreducing conditions during immunoprecipitation (Fig. 7, lanes 80K), and, upon reduction, was resolved into a single band migrating with an apparent molecular weight of 37K (Fig. 7, lanes 37K). This further supports the idea that the 80-kDa complex is composed of a p37K homodimer.

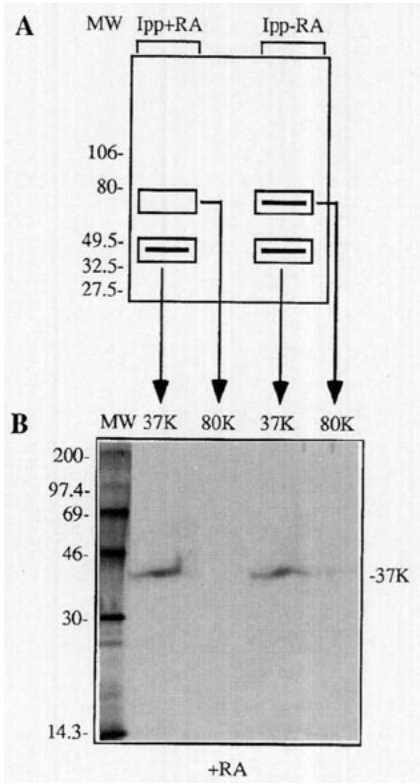

FIG. 7. Composition of the 80-kDa protein complex. The proteins of labeled EEV were immunoprecipitated with anti-37K antiserum in the absence of reducing agents. The immunoprecipitate was split into two aliquots, which were reduced (Ipp + RA) or nonreduced (Ipp − RA), respectively, and loaded on a 10% SDS gel. Gel slices corresponding to the positions of p37K and the 80-kDa complex were excised. The proteins in these gel slices were reduced and separated on a 15% SDS gel. The molecular weights of marker proteins (MW) are indicated in kilodaltons.

DISCUSSION

Even though p37K is the major envelope protein and is required for the formation of enveloped virus, it has been only poorly characterized so far. As a first step in understanding the role of the envelope proteins in the wrapping process, we studied p37K and its potential interaction with other viral proteins.

Although p37K cofractionates with the envelope upon Brij 58 extraction (Payne, 1978), this experiment did not provide any information about the nature and strength of attachment to the envelope. Sodium carbonate treatment provides such information and the results of this analysis showed that p37K is tightly bound to the envelope. Despite its primary structure which is not typical of transmembrane proteins (Hirt *et al.*, 1986), Triton X-114 phase partitioning confirmed the hydrophobic nature of p37K (Hiller and Weber, 1985). Furthermore, such experiments on p37K synthesized in rabbit reticulocyte lysates as well as on hydroxylamine-depalmitated proteins showed that the hydrophobic behavior of the p37K is due to its fatty acid content rather than to the two small central hydrophobic domains.

Knowledge about the location of p37K within the EEV could also contribute to the understanding of the process of envelopment. To determine whether the protein is totally, partially, or not at all exposed on the EEV surface, proteinase K shaving experiments were performed. The complete resistance of the protein within an intact virus envelope indicated that p37K is not exposed on the surface but seems to line the inner side of the envelope. In contrast, the hemagglutinin and the 42K proteins, two

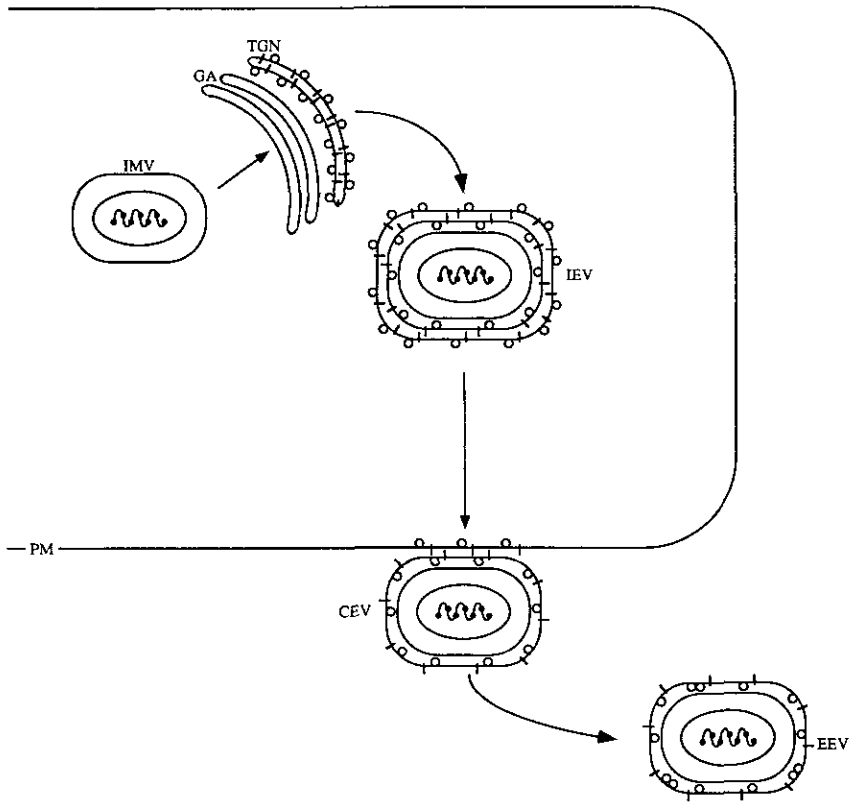

FIG. 8. Model for vaccinia virus envelopment. IMV particles are wrapped by TGN membranes, and the resulting enveloped virus particles migrate to the cell surface, where their outer membrane fuses with plasma membrane releasing EEV. Cellular structures: GA, Golgi apparatus; PM, plasma membrane; TGN, trans-Golgi network. Virus: IMV, intracellular mature virus; IEV, intracellular enveloped virus; CEV, cell-associated enveloped virus; EEV, extracellular enveloped virus. O, p37K; OO, 80-kDa protein complex; —, envelope glycoproteins.

glycosylated envelope proteins, were sensitive to protease treatment, indicating that both contain a portion which is oriented to the outside of EEV. These results are consistent with the location of potential transmembrane domains predicted from the sequences of these proteins (Shida, 1986; Smith *et al.*, 1991). The topology of p37K suggests that it might interact both with the 14K protein on the surface of the IMV and with the inner portions of the glycoproteins of other envelope antigens. EEV contains a high molecular weight protein complex of about 80 kDa containing p37K. The analysis of this complex did not reveal any other envelope proteins and seems to be composed of a p37K homodimer. Alternatively, the complex may be composed of p37K and an unlabeled host protein of similar size. We have not investigated this possibility further. Interestingly, only a small portion of p37K appears to be present in the viral envelope in complexed form, the majority occuring as monomers. It is unlikely that this is due to a loss of complex upon EEV lysis, since cross-linking experiments and the use of mild lysis conditions did not allow us to detect more p37K as dimers.

We were unable to detect the complex in immunoprecipitates from infected cell lysates at any of the tested time points postinfection. This can best be explained by the reducing environment of the cytosol which is not compatible with the formation of disulfide bonds. Thus, the complex is apparently only formed upon EEV release.

Our results suggest the model of vaccinia virus envelopment presented in Fig. 8. Initially, p37K is located on the outer surface of the trans-Golgi network (TGN). When the IMV are surrounded by these membranes, p37K is found on the cytoplasmic side of IEV but also inside the IEV, that is, between the IMV particle and the TGN membrane. Upon fusion of IEV with the plasma membrane, p37K remains located on the cytoplasmic side of the cell membrane and on the inner side of the envelope in the released EEV. Within the enveloped viruses, p37K exists in both monomeric and dimeric forms. This model is consistent with the immunohistochemical observations, where p37K has been detected on the TGN, on both sides of IEV envelopes, on the plasma membrane, and in EEV (Schmelz *et al.*, 1994).

Vaccinia virions are enveloped and released by a mechanism that seems quite different from the budding process used by other enveloped animal viruses, nevertheless there are also several analogies. The envelopes of all these viruses are composed of lipid membranes originating from the plasma or Golgi membrane of the host cell and contain glycosylated and nonglycosylated

proteins. The glycoproteins play a role in specific adsorption to the cell surface, they have biological properties such as hemagglutinin or neuraminidase activity, and they induce neutralizing antibodies. The biochemical characteristics and the topology of p37K show several analogies to the matrix proteins of other enveloped animal viruses. These proteins have molecular weights ranging from 25 to 45 kDa, they line the inner surface of the envelope, and although they contain a small hydrophobic domain in their center, they are not transmembrane proteins. Futhermore, mutations in or deletions of the envelope protein genes inhibit assembly and budding of enveloped virus in analogy to what is observed in vaccinia virus where deletion of the F13L gene blocks envelopment and release of EEV (Blasco and Moss, 1991). All these analogies strongly suggest that virus budding and vaccinia virus envelopment are related mechanisms and that p37K may act as a matrix protein. A similar role has also been proposed for the vaccinia p65 protein, which is involved in formation of the viral crescents at the very beginning of the assembly process (Sodeik *et al.*, 1994), and for the 14K protein (Rodriguez *et al.*, 1993).

ACKNOWLEDGMENTS

We thank Grigorios Krey for helpful discussions and critical reading of the manuscript. We also thank Béatrice Kunz and Jacqueline Piffaretti for their excellent technical assistance and G. Griffith and Y. Ichihashi for gifts of antibodies. This work was supported by the Swiss National Science Foundation, Grant 31-33748.92, by the Swiss Biotechnology Priority Program, Grant 5002-034603, and by "Recherche Suisse Contre le Cancer" (Akt 312).

REFERENCES

Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope protein. *J. Virol.* **65,** 5910–5920.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256,** 1604–1607.

Duncan, S. A., and Smith, G. L. (1992). Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. *J. Virol.* **66,** 1610–1621.

Engelstad, M., and Smith, G. L. (1993). The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* **194,** 627–637.

Franke, C. A., Reynolds, P. L., and Hruby, D. E. (1989). Fatty acid acylation of vaccinia virus proteins. *J. Virol.* **63,** 485–491.

Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *J. Cell Biol.* **93,** 97–102.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179,** 247–266.

Hiller, G., and Weber, K. (1985). Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *J. Virol.* **55,** 651–659.

Hiller, G., Eibl, H., and Weber, K. (1981a). Characterization of intracellular and extracellular vaccinia virus variants: N_1-Isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine interferes with cytoplasmic virus dissemination and release. *J. Virol.* **39,** 903–913.

Hiller, G., Jungwirth, C., and Weber, K. (1981b). Fluorescence microscopical analysis of the life cycle of vaccinia virus in chick embryo fibroblasts. *Exp. Cell Res.* **132,** 81–87.

Hirt, P., Hiller, G., and Wittek, R. (1986). Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. *J. Virol.* **58,** 757–764.

Magee, A. I., Koyama, A. H., Malfer, C., Wen, D., and Schlesinger, M. J. (1984). Release of fatty acids from virus glycoproteins by hydroxylamine. *Biochim. Biophys. Acta* **798,** 156–166.

Oie, M., Shida, H., and Ichihashi, Y. (1990). The function of the vaccinia hemagglutinin in the proteolytic activation of infectivity. *Virology* **176,** 494–504.

Payne, L. G. (1978). Polypeptide composition of extracellular enveloped vaccinia virus. *J. Virol.* **27,** 28–37.

Payne, L. G. (1992). Characterization of vaccinia virus glycoproteins by monoclonal antibody precipitation. *Virology* **187,** 251–260.

Rodriguez, D., Rodriguez, J.-R., and Esteban, M. (1993). The vaccinia virus 14-kilodalton fusion protein forms a stable complex with the processed protein encoded by the vaccinia virus A17L gene. *J. Virol.* **67,** 3435–3440.

Rodriguez, J. F., and Smith, G. L. (1990a). Inducible gene expression from vaccinia virus vectors. *Virology* **177,** 239–250.

Rodriguez, J. F., and Smith, G. L. (1990b). IPTG-dependent vaccinia virus: Identification of a virus protein enabling virion envelopment by Golgi membrane and egress. *Nucleic Acids Res.* **18,** 5347–5351.

Rodriguez, J. F., Janeczko, R., and Esteban, M. (1985). Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *J. Virol.* **56,** 482–488.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G., and Griffiths, G. (1994). Assembly of vaccinia virus: The second wrapping cisterna is derived from the trans Golgi network. *J. Virol.* **68,** 130–147.

Schmutz, C., Payne, L. G., Gubser, J., and Wittek, R. (1991). A mutation in the gene encoding the vaccinia virus 37,000-M_r protein confers resistance to an inhibitor of virus envelopment and release. *J. Virol.* **65,** 3435–3442.

Shida, H. (1986). Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* **150,** 451–462.

Smith, G. L., Sang Chan, Y., and Howard, S. T. (1991). Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. *J. Gen. Virol.* **72,** 1349–1376.

Sodeik, B., Griffiths, G., Ericsson, M., Moss, B., and Doms, R. W. (1994). Assembly of vaccinia virus: Effects of rifampin on the intracellular distribution of viral protein p65. *J. Virol.* **68,** 1103–1114.

Wolffe, E. J., Isaacs, S. N., and Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* **67,** 4732–4741.