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The Vaccinia Virus 39-kDa Protein Forms a Stable Complex with the p4a/4a Major Core Protein Early in Morphogenesis

Cristina Risco,* Juan Ramón Rodríguez,† Walter Demkowicz,[1] Ritva Heljasvaara,[2] José L. Carrascosa,* Mariano Esteban,†,[3] and Dolores Rodríguez†

*Department of Macromolecular Structure and †Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, Madrid 28049, Spain

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The vaccinia virus (VV) 39-kDa protein, the product of the *A4L* gene, is a highly antigenic protein of the viral core. Pulse-chase and immunoprecipitation experiments have shown that the 39-kDa protein interacts with p4a (encoded by the *A10L* gene), the precursor of the most abundant virion protein. This interaction is maintained with the processed 4a form that arises during virion maturation. The controlled disruption of mature viral particles showed that the 39-kDa and 4a proteins are tightly bound within the virion. Immunoelectron microscopy showed that both proteins first localize within the cytoplasm and later accumulate inside the viral factories, reaching these locations via a mechanism apparently unrelated to cellular membranes. Double labeling experiments showed a colocalization of both proteins in all virus-induced structures.

INTRODUCTION

Vaccinia virus (VV) is a large double-stranded DNA virus and the best studied member of the Poxviridae family, a group of animal viruses whose life cycle takes place entirely in the cytoplasm of the infected cells (reviewed by Moss, 1990). At late times during this life cycle, a series of events lead to the formation of the intracellular mature virion (IMV), through a process whose complexity could be compared with the biogenesis of cellular organelles (Wang and Shuman, 1995). The first viral structures that can be visualized in the cytoplasm of infected cells are crescent-shaped membranes, whose origin remains controversial. In the original model proposed by Dales *et al.* (1961, 1968) viral membranes would be synthesized *de novo* by an unknown mechanism. More recently, Sodeik *et al.* (1993) proposed that the IMV membrane is of cellular origin and derived from the endoplasmic reticulum–Golgi intermediate compartment (ERGIC), one of the components of the constitutive secretory pathway of the cell. In accordance with this model, several viral proteins have been shown to be associated with membranes of this tubulovesicular compartment (Krijnse-Locker *et al.,* 1996; Rodríguez *et al.,* 1997; Salmons *et al.,* 1997). These virus-modified membranes, which would then consist of two lipid bilayers, would be later recruited to the periphery of the viral factories to form the characteristic crescent-shaped membranes (Krijnse-Locker *et al.,* 1996; Rodríguez *et al.,* 1997). However, this model has lately been disputed by Hollinshead *et al.* (1999), who claimed that there is no continuity between cellular and viral membranes and that the latter are made of only one lipid bilayer and invoke again the *de novo* synthesis for the origin of IMV membrane. Regardless of the origin of the viral membrane, the next step in VV morphogenesis is the formation of immature virus (IV), a process during which electron-dense material from the viral factory becomes engulfed by the viral membrane (Ichihashi *et al.,* 1971; Silver and Dales, 1982). The IV undergoes a major structural transformation to produce the IMV, and this appears to involve the proteolytic processing of certain polypeptide precursors (Lee and Hruby, 1994; Moss and Rosenblum, 1973; Silver and Dales, 1982; Vanslyke *et al.,* 1991) and the intervention of some particular proteins (Hu *et al.,* 1996; Kane and Shuman, 1993; Klemperer *et al.,* 1997; Ravanello and Hruby, 1994; Zhang and Moss, 1991), one of which has been recently reported to be required for DNA packaging (Cassetti *et al.,* 1998). A fraction of the IMVs is later wrapped by a membrane cisternae derived from the trans-Golgi network, to be later released from the cell via fusion with the plasma membrane (Dales and Pogo, 1981; Payne and Kristenson, 1979; Schmelz *et al.,* 1994), with the participation of VV proteins that play specific roles in these final morphogenetic steps (Blasco and Moss, 1991; Duncan and Smith,

[1] Present address: Department of Medicine, University of Massachusetts Medical Center, Worcester, MA 01655.
[2] Present address: Department of Medical Biochemistry, University of Oulu, Finland.
[3] To whom reprint requests should be addressed. Fax: 34-1-585-4506. E-mail: mesteban@cnb.uam.es.

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1992; Engelstad and Smith, 1993; Parkinson and Smith, 1994; Rodríguez and Smith, 1990). Recent studies have started to provide information about the complex molecular events involved in the early morphogenetic process for which the formation of complexes of VV proteins must be crucial (Rodríguez *et al.,* 1993, 1995, 1996, 1997). In this respect, a protein complex constituted by VV 21-kDa (*A17L*), 15-kDa (*A14L*), and 14-kDa (*A27L*) envelope proteins has been recently described (Rodríguez *et al.,* 1997). The 21-kDa and the 15-kDa proteins play essential roles in the biogenesis of the first viral membranes, the viral crescents (Rodríguez *et al.,* 1995, 1996, 1998; Wolffe *et al.,* 1996), whereas the 14-kDa protein is required for the envelopment of IMVs with Golgi-derived membranes (Rodríguez and Smith, 1990). The 65-kDa protein, the product of the *D13L* gene and the target of the drug rifampin (Miner and Hruby, 1989; Tartaglia *et al.,* 1986; Zhang and Moss, 1992), probably acts as a scaffolding protein, providing the curvature typical of the crescents (Sodeik *et al.,* 1994). In addition, temperature-sensitive (ts) mutants with lesions in the F10 protein kinase showed that F10 and/or its associated kinase activity plays a crucial role in the initiation of virion assembly (Traktman *et al.,* 1995; Wang and Shuman, 1995).

Poorly characterized aspects of the sequence of VV assembly are how the viral core proteins are transported to the viral factory and how the dense content of the factory becomes associated with the membranes during the formation of the viral crescents and immature virions. It has been suggested that viral core proteins could bind to an unidentified viral transmembrane protein or proteins, resulting in the formation of the crescents and, subsequently, the immature virions (Sodeik *et al.,* 1993). Coimmunoprecipitation experiments have suggested that the 21-kDa envelope protein could interact with the 39-kDa core protein (Cudmore *et al.,* 1996). The 39-kDa protein, which is the product of the *A4L* gene, is an immunodominant protein first described by Maa and Esteban (1987), who demonstrated that this acidic polypeptide is a product of the late class of genes and is present in the VV core. The 39-kDa protein elicits strong humoral immune responses in humans and animals, being the strongest antigenic domain contained within the C-terminal 103 amino acids (Demkowicz *et al.,* 1992). This protein localizes in the viroplasm of the viral factories and rifampin bodies (Cudmore *et al.,* 1996) and in the core of the IMV, where it has been proposed to act as a matrix protein, connecting the surface of the core with the innermost membrane of the IMV particle (Ross *et al.,* 1996). Moreover, it has been recently shown that this protein is essential for VV growth, being required for the progression of IV to IMV particles (Williams *et al.,* 1999). Equivalent proteins to VV 39 kDa, showing variable degrees of homology, are found in other members of the poxvirus family: fowlpox (Boulanger *et al.,* 1998), *Molluscum contagiosum* (Senkevich *et al.,* 1996), and variola (Bangladesh 1975) (Massung *et al.,* 1994) viruses. The fowlpox 39-kDa homolog is also an immunodominant protein and shows the same localization in viral factories and mature virions as the VV 39-kDa protein. In the present study, we identified and characterized the formation of a stable complex between the VV 39-kDa protein and another immunodominant core protein, p4a/4a, which is the product of the *A10L* gene and represents the most abundant protein of the virion (Sarov and Joklik, 1972). This major core antigen, which could represent the common nucleoprotein agent found in most members of the poxvirus family (Woodroofe and Fenner, 1962), is an important determinant in the immune response mounted against VV. We have found that the association 39-kDa–p4a/4a is established early in morphogenesis and maintained after their incorporation into virions and the proteolytic processing of p4a precursor, which points to a key role for that interaction in virion architecture. In addition, the localization of both proteins within the viral factory and some other virus-induced structures raises some questions about the internal organization and origin of these centers of assembly.

RESULTS

The VV 39-kDa protein interacts with the precursor and the processed form of the VV 4a major core protein

In previous studies, we have characterized through immunoprecipitation analysis the interaction between several VV membrane proteins that may be relevant for the formation of the mature virion envelope (Rodríguez *et al.,* 1993, 1995, 1996, 1997). Protein–protein interactions are also likely to occur between the proteins involved in the maturation of the viral core. With the aim of identifying such interactions, we performed immunoprecipitation experiments using extracts from VV-infected cells and antibodies against the 39-kDa core protein (Demkowicz *et al.,* 1992; Maa and Esteban, 1987). For this, BSC40 cells infected with Western reserve (WR) VV (10 PFU/cell) were metabolically labeled with [^{35}S]methionine from 6 to 24 h postinfection (p.i.). Cells were lysed by incubation at 37°C during 30 min in lysis buffer. Cell extracts were cleared by centrifugation and incubated with Sepharose beads coated with polyclonal anti-39-kDa antibodies, and the immunoprecipitated proteins were analyzed by SDS–PAGE. As shown in Fig. 1A, lane 1, in addition to the 39-kDa protein, the anti-39-kDa antibody coprecipitated two proteins with apparent molecular masses of ~100 and ~60 kDa from extracts of infected cells. The upper of these two bands (denoted by one asterisk) was the predominant product coprecipitated with the 39-kDa protein from extracts of cells infected in the presence of rifampin (lane 2), a drug that reversibly blocks VV morphogenesis at a step previous

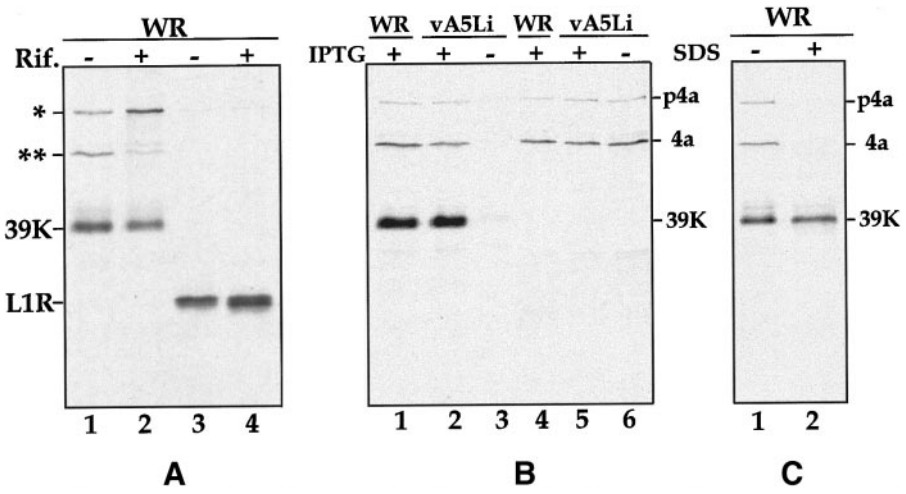

FIG. 1. VV 39-kDa protein interacts with the major p4a and 4a proteins. (A) Immunoprecipitation with anti-39K antibodies. BSC40 cells infected with VV (10 PFU/cell) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of rifampin (100 μg/ml) were metabolically labeled with [^{35}S]methionine from 6 to 24 h p.i. Cells were harvested and treated with lysis buffer for 30 min at 37°C. Proteins were immunoprecipitated with antibodies against the 39-kDa (lanes 1 and 2) or L1R (lanes 3 and 4), fractionated by SDS–PAGE, and detected by autoradiography. Two proteins coimmunoprecipitated with the 39-kDa protein are indicated on the left side by one or two asterisks. The positions of the 39-kDa and L1R proteins are also indicated. (B) Specific coprecipitation of p4a and 4a with the 39-kDa protein. BSC40 cells were infected (10 PFU/cell) with WR (lanes 1 and 4) or with the mutant vA5Li in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of 50 μM IPTG and metabolically labeled as described in A. Proteins were immunoprecipitated with antibodies specific for the 39-kDa (lanes 1–3) or p4a/4a (lanes 4–6) proteins. (C) The 39 kDa–p4a/4a interaction is disrupted by SDS treatment. Cells infected (10 PFU/cell) with WR were treated with lysis buffer containing (lane 2) or lacking (lane 1) 0.2% SDS. After 30 min at 37°C, proteins were immunoprecipitated with anti-39-kDa antibodies. The positions of p4a, 4a, and 39-kDa proteins are indicated on the right side of B and C.

to the cleavage of the core precursors p4a and p4b (Moss *et al.,* 1971). None of these two proteins were observed when immunoprecipitation was performed with antibodies against the VV L1R protein (lanes 3 and 4), which excludes the possibility that p4a and 4a coprecipitation could simply be due to aggregation or nonspecific binding of these proteins to the Sepharose beads during the incubations. These results suggested that the 39-kDa protein associates with a polypeptide that is proteolytically cleaved during virion assembly. After this hypothesis and in consideration of the molecular sizes of the two coprecipitating proteins, the most likely candidates were the p4a precursor and its 4a cleaved product, whose predicted molecular weights are 102 and 62 kDa, respectively (Vanslyke *et al.,* 1991). To investigate this possibility, similar cell lysates were immunoprecipitated with antibodies raised against the 4a product or with anti-39-kDa antibodies, and the immunoprecipitated proteins were run in parallel in SDS–PAGE. As shown in Fig. 1B, the anti-p4a/4a antibodies clearly immunoprecipitated both the p4a precursor and the 4a protein (lane 4), and these proteins comigrated with the two products coprecipitated by anti-39-kDa antibodies (lane 1). To guarantee that the coprecipitation of p4a and 4a was not due to nonspecific cross-reactivity of anti-39K antibodies with these two proteins in this experiment, we performed a similar immunoprecipitation with lysates from cells infected with a mutant virus, vA5Li, that inducibly expresses the 39-kDa protein (Williams *et al.,* 1999). Immunoprecipitation of p4a and 4a with anti-39K antibodies was observed when cells were infected with the mutant in the presence of the inducer (lane 2), conditions under which 39-kDa protein is produced but not when expression of 39-kDa was repressed [−isopropyl-β-D-thiogalactoside (IPTG)] (lane 3), although these two products could be clearly immunoprecipitated by the specific anti-p4a/4a antiserum (lane 6) to the same extent as from WR-infected cells (lane 4) or from cells infected with the mutant in the presence of the inducer (lane 5). These results clearly demonstrated that immunoprecipitation of p4a and 4a products with anti-39-kDa antibodies is due to their specific interaction with the 39-kDa protein.

To further characterize the nature of this 39-kDa–p4a/4a complex cell lysates were treated with the anionic detergent SDS before immunoprecipitation. As shown in Fig. 1C, after treatment of the cells with SDS, the anti-39-kDa antibodies immunoprecipitated only the 39-kDa protein (lane 2), indicating that the 39-kDa–p4a/4a interaction is disrupted by this treatment.

The identity of the coprecipitated proteins as VV p4a and 4a polypeptides was also confirmed by reacting in Western blotting the immunoprecipitated products with antibodies against the 4a protein (not shown); however, we could not observe coprecipitation of the 39-kDa protein with anti-p4a/4a antibodies Fig. 1B, (lanes 5 and 6).

Taken together, these results suggest that the 39-kDa protein interacts with the p4a and 4a proteins. The 39-kDa and 4a proteins are tightly bound within the virion,

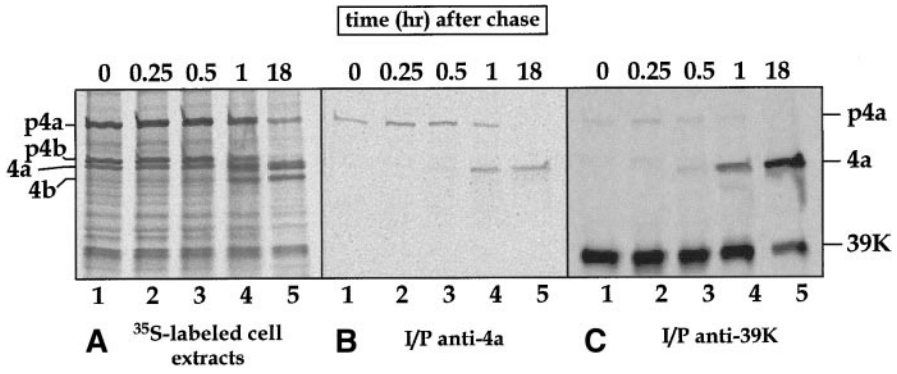

FIG. 2. Kinetics of the 39-kDa–p4a/4a protein complex formation in WR VV-infected cells. (A) Profile of radiolabeled protein from BSC40 cells infected with WR VV (10 PFU/cell), which were pulse-labeled with [^{35}S]methionine at 6 h p.i. After 10-min pulse, cells were chased with a 100-fold excess of unlabeled methionine. Cells were harvested immediately after the pulse (lane 1) or after 0.25-, 0.5-, 1-, and 18-h chase periods (lanes 2–5, respectively) and resuspended in lysis buffer, and an aliquot of each sample was mixed with 2× sample buffer and analyzed by SDS–PAGE (10%). The gel was dried, and protein bands were visualized after autoradiography. (B) Kinetics of p4a proteolytic processing analyzed by pulse-chase and immunoprecipitation with antibodies against p4a/4a proteins. (C) Kinetics of the 39-kDa–p4a/4a complex formation analyzed by immunoprecipitation with antibodies against the 39-kDa protein. Immunoprecipitated proteins were separated by SDS–PAGE and visualized by autoradiography. The positions of precursors p4a and p4b and the cleaved products 4a and 4b are indicated on the left side of A. Migration of p4a, 4a, and 39-kDa proteins is also indicated in C.

and this interaction is broken by treatment with anionic detergents.

Time course of the p4a proteolytic processing and its interaction with the 39-kDa protein

To study in more detail the kinetics of the 39-kDa–p4a/4a interaction, we performed a pulse-chase analysis. Thus, cells were pulse-labeled for 10 min with [^{35}S]methionine at 6 h p.i. Immediately thereafter, a 100-fold excess of unlabeled methionine was added to the cells, and one culture was placed on ice and washed with PBS (time 0). The other cultures were further incubated at 37°C and harvested at 0.25, 0.5, 1, and 18 h after the chase. In Fig. 2A, which shows the profile of [^{35}S]labeled proteins at different times after the chase, the disappearance of the p4a and p4b major core precursors with time and the corresponding increase in the amount of both 4a and 4b mature products can be observed. The kinetics of p4a proteolytic processing can be more clearly observed in Fig. 2B after immunoprecipitation of the cell extracts with the anti-p4a/4a antiserum. A clear decrease in the amount of p4a precursor was observed with the time of chase that was concomitant with the appearance of the 4a mature product (lanes 4 and 5). A similar picture was obtained when immunoprecipitation was carried out with anti-39-kDa antibodies, although in this case the 39-kDa protein was present at all times after chase (Fig. 2C). These results indicate that soon after its synthesis, the 39-kDa protein interacts with the p4a core precursor, and this interaction is maintained with the processed 4a protein that arises after virion maturation.

Localization of the 39-kDa and 4a proteins within the virion

Both 39-kDa and 4a have been defined as core proteins. Recently, it has been proposed that the 39-kDa protein is a membrane-associated protein that localizes on the outside of the viral core (Cudmore *et al.,* 1996). To establish the relative position of 39-kDa and 4a proteins within the viral particle, we treated purified virions with different detergents and a reducing agent to selectively solubilize their proteins and fractionate their components. The 4a protein was only partially solubilized after the treatment of virions with SDS and DOC (Fig. 3A, lane 2), and ~50% of the protein remained in the insoluble fraction after this treatment (Fig. 3A, lane 1). In contrast, the majority of the 39-kDa protein was solubilized by treatment with SDS and DOC (Fig. 3B, lane 2), although a considerable amount of it was already released by treatment with DTT in combination with Nonidet P-40 (NP-40) (Fig. 3B, lane 3), and a small amount was solubilized by only NP-40 (Fig. 3B, lane 4). However, as discussed earlier, a portion of the protein remained insoluble after the different extraction treatments (Fig. 3B, lane 1). A comparison with the distribution obtained when localizing a membrane protein, the 15-kDa protein, is shown in Fig. 3C. This envelope protein was almost totally extracted by the sequential treatment with NP-40 and NP-40 plus DTT (Fig. 3C, lanes 4 and 3, respectively).

This result indicated that the 4a protein is more confined within the core structure, whereas, in agreement with previous reports (Cudmore *et al.,* 1996; Ross *et al.,* 1996), the 39-kDa protein might be localized on the surface of the viral core.

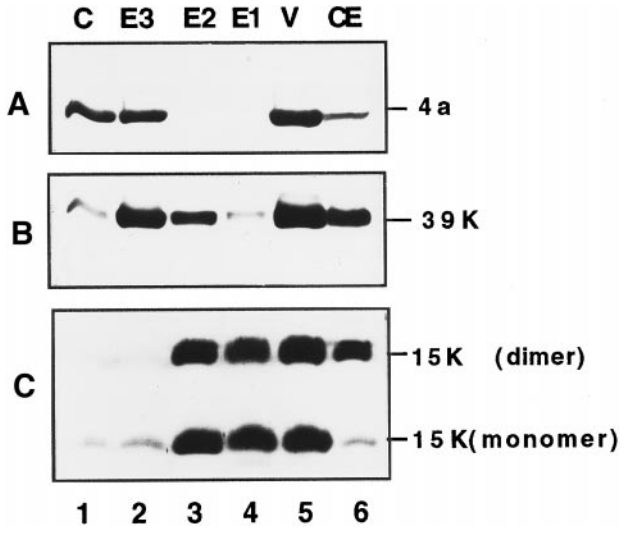

FIG. 3. Localization of 39-kDa and 4a proteins within the virion. Purified IMV virions were disrupted by treatment with the nonionic detergent NP-40 (1%) in Tris buffer (50 mM Tris–HCl, pH 8.5, containing 10 mM $MgCl_2$). This and subsequent treatments were performed at 37°C for 30 min. The soluble envelopes (E1) were removed by centrifugation, and the insoluble fraction was resuspended in Tris buffer containing 1% NP-40 plus 50 mM DTT. The soluble proteins (E2) were collected, and the pellet was treated with the same buffer containing 1% NP-40, 0.5% DOC, and 0.1% SDS. After centrifugation, soluble proteins (E3) were separated from cores (C). Extracts from VV-infected cells (CE), unfractionated virions (V), and the collected fractions were resolved by SDS–PAGE under reducing conditions and analyzed by Western blotting using antibodies against the p4a/4a (A), the 39-kDa (B), or the 15-kDa (C) proteins.

The 39-kDa and p4a VV core proteins colocalize inside viral factories

Ultrathin sections of infected cells embedded in Lowicryl K4M were used in a first step of this study to follow the intracellular distribution of the viral proteins during VV assembly. The cells were subjected to a mild fixation and embedded at a low temperature to facilitate the preservation of the epitopes and to obtain strong immunolabeling signals.

The previously characterized conditional mutant VVindA17L (Rodríguez *et al.,* 1995, 1996, 1997), which inducibly expresses the 21- to 23-kDa envelope protein, was shown to provide a synchronized system to follow the incorporation of viral proteins during morphogenesis. In cells infected with VVindA17L under nonpermissive conditions, virion morphogenesis is arrested, and electron-dense masses, similar to viral factories, are the only viral structures observed at late times postinfection. After induction of the *A17L* gene expression, characteristic viral factories and the different viral forms can be observed in the cytoplasm of infected cells. Small viral factories are visible in perinuclear areas of low electron density shortly after addition of the inducer (2 h). These factories increase in number and size at longer postinduction times (4–18 h).

Immunogold detection indicated that from the beginning of assembly, the 39-kDa and p4a/4a proteins accumulate in the same intracellular localizations (Fig. 4). The signal obtained with both antisera at different postinduction times shows that these proteins are incorporated into the viral factories, probably after having been synthesized in free polysomes of the infected cell, as suggested by the scattered labeling observed in the cytoplasm at short postinduction times (Fig. 4A). At 2 h after the addition of IPTG to the cultures, small electron-dense cytoplasmic masses, as well as small viral factories, are detectable, and according to the strong labeling signals observed, these structures contain both 39-kDa and p4a/4a proteins (Figs. 4A and 4B). At 18 h postinduction, strong signals associated to both proteins are present inside large factories (Figs. 4C and 4D).

The distribution of the labeling associated to 39-kDa and p4a proteins inside the viral factories does not seem to correspond to a random pattern, because the organization in domains, frequently with linear arrays of gold particles, is very clear (arrows in Figs. 4B–4D).

Double-labeling experiments show that both p4a/4a and 39-kDa proteins colocalize inside small and large factories (Figs. 4E and 4F). The small cytoplasmic electron-dense spots that form at short postinduction times already contain labeling associated to both proteins, as well as rifampin bodies and the large dense masses formed when infecting with the VVindA17L virus in the absence of the inducer (not shown).

Incorporation of 39-kDa and p4a/4a proteins into viral factories and viral particles does not seem to require the participation of membranes

Immunolocalization of the core proteins was repeated on ultrathin sections of cells embedded in an Epon-type resin (Fig. 5). In these sections, the immunolabeling signal is reduced, but as an important advantage, there is a very good definition of the membranous systems, poorly defined in conventional Lowicryl sections. The 39-kDa protein did not significantly associate to the tubulovesicular membranes recruited on the periphery of the factories when infection with the VVindA17L occurs under permissive conditions (Fig. 5A). These membranous elements are known to be related to the ERGIC (Rodríguez *et al.,* 1997; Sodeik *et al.,* 1993). A similar result was obtained when labeling was done with the anti-p4a/4a antiserum (not shown).

The electron-dense masses that organize in HeLa cells infected with the recombinant virus VVindA17L under nonpermissive conditions accumulate both 39-kDa and p4a proteins (Figs. 5B and 5C). These results suggest that both in the presence and absence of viral assembly, the 39-kDa and p4a/4a core proteins are efficiently incorporated into virus-induced structures (the viral factories or their precursors) via a mechanism ap-

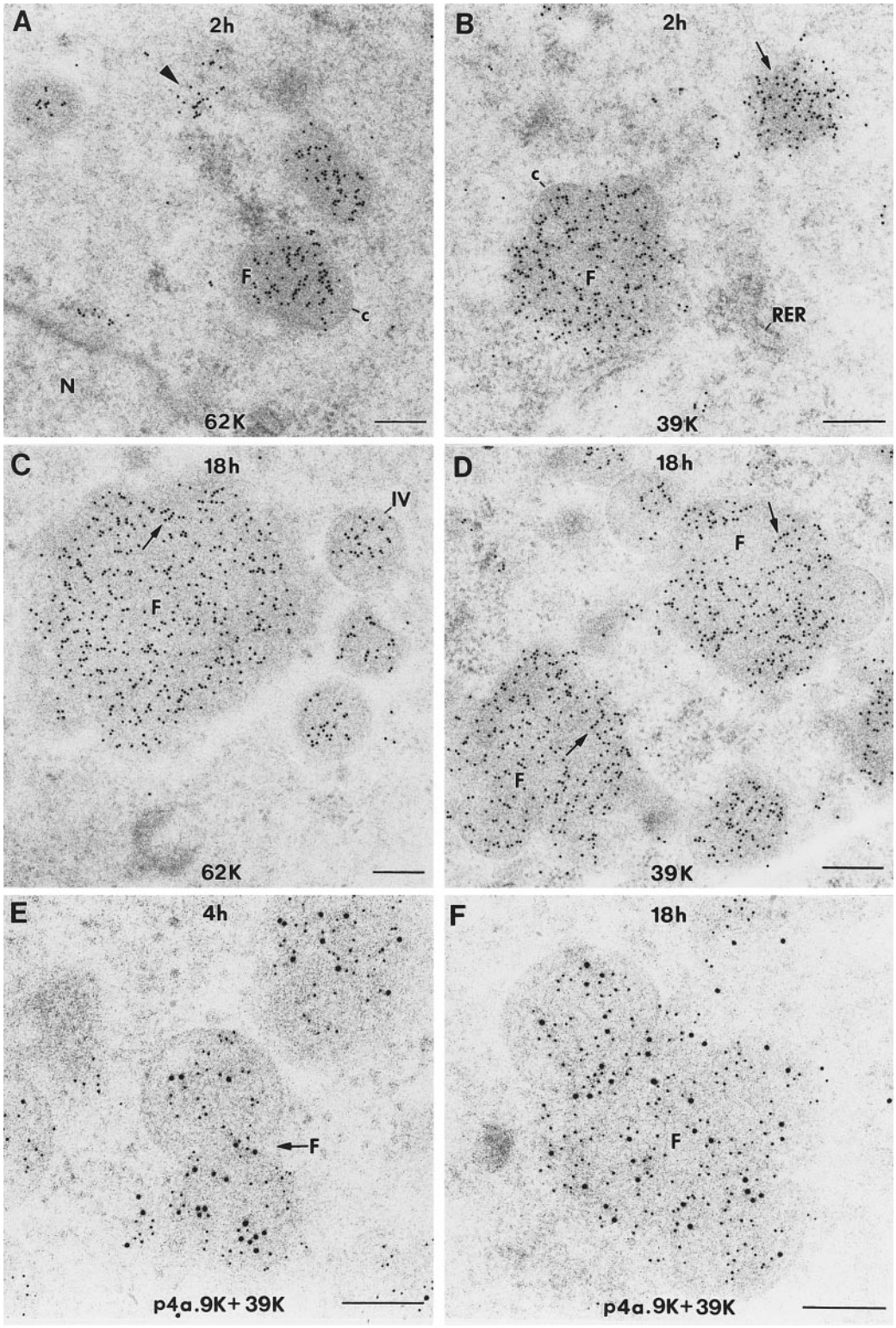

FIG. 4. Colocalization of p4a/4a (62k) and p39-kDa (39 k) in HeLa cells infected with the recombinant virus V VindA17L at different postinduction times. Lowicryl K4M sections were used in all experiments. (A–D) Single labeling experiments with 10-nm gold particles. Two hours after the addition of the inducer (IPTG) to the cell cultures, labeling associated to 62-kDa (A) and 39-kDa (B) V V proteins is detected in the cytoplasm (arrowhead in A) as well as inside small electron-dense masses (arrow in B) and viral factories (F). (C and D) At 18 h postinduction, large viral factories (F) exhibit a strong internal labeling associated to both 62-kDa (C) and 39-kDa (D) proteins. Gold particles frequently organize forming linear arrays (arrows). (E and F) Double labeling experiments, where larger gold particles (10 nm) are associated to p4a/4a protein, and small gold particles (5 nm) are detecting 39-kDa protein molecules. Both proteins specifically colocalize inside small viral factories formed at 4 h postinduction (E) and large viral factories from cultures at 18 h postinduction (F). N, cell nucleus; c, viral crescent; RER, rough endoplasmic reticulum. Bars represent 250 nm in A–D and 200 nm in E and F.

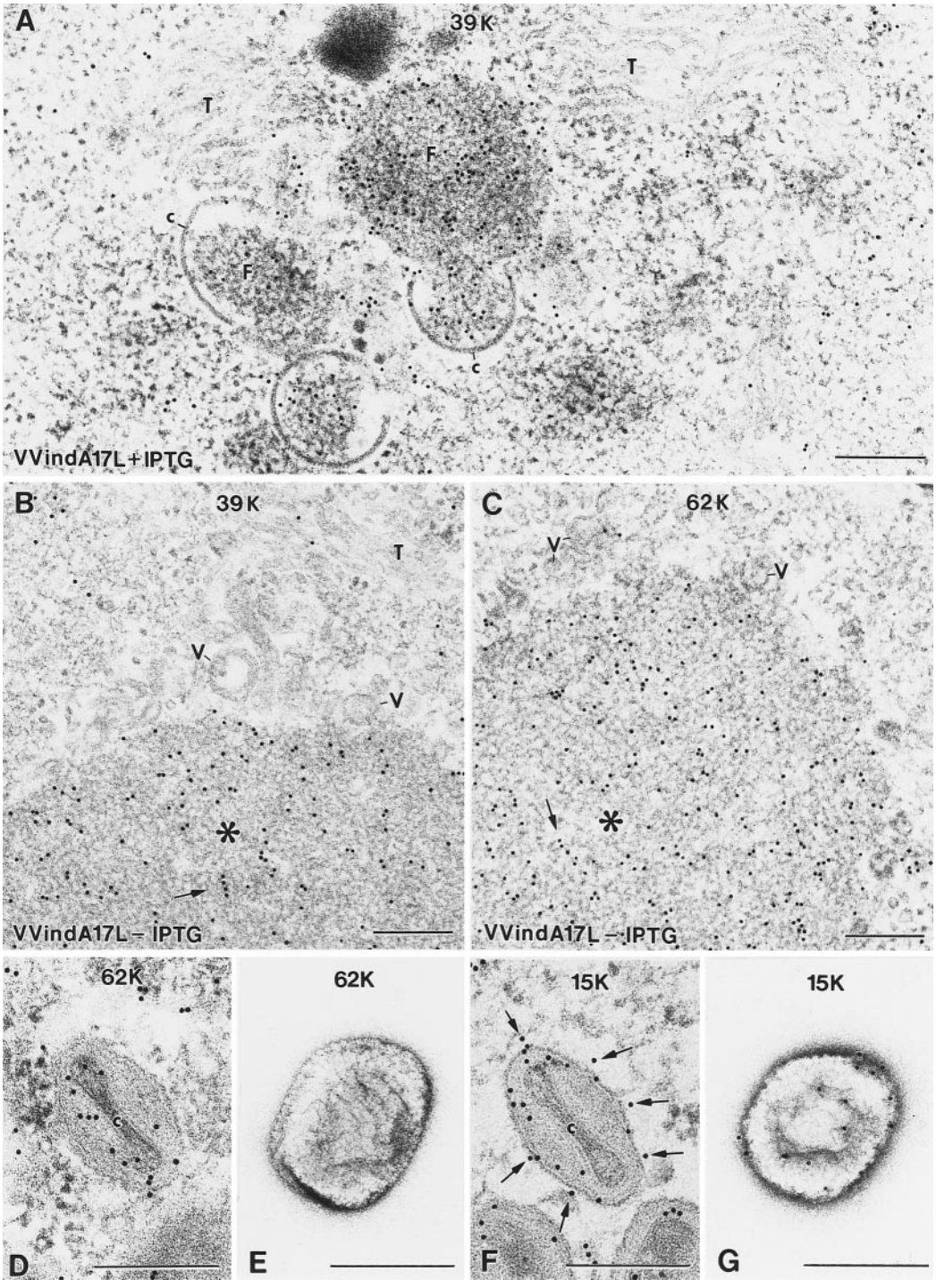

FIG. 5. 39-kDa and 62-kDa (p4a/4a) VV proteins do not associate *in situ* to cellular or viral membranes. EML-812 sections of infected HeLa cells at 24 h p.i. (A–D and F) or suspensions of purified VV IMVs (E and G) were used. (A) In HeLa cells infected with the recombinant virus VVindA17L in the presence of IPTG, the immunogold signal associated to 39-kDa protein localizes inside the viral factories (F). The tubular membranous elements (T) recruited on the periphery of the factories or the viral crescents (c) are devoid of labeling. In the absence of viral assembly (in HeLa cells infected with the recombinant virus VVindA17L in the absence of IPTG), both 39-kDa (B) and 62-kDa (C) VV proteins accumulate inside the perinuclear electron-dense masses (asterisks). The numerous tubular (T) and vesicular (V) membranous elements that cover the periphery of the masses are devoid of labeling. Arrows in B and C point to linear arrays of gold particles. Filamentous elements are clearly distinguished inside the mass shown in C. (D) In assembled IMV particles, 62-kDa protein is localized associated to the internal viral core (c), and no signal was obtained on the surface of virions (E). (F and G) Equivalent situation for a VV envelope protein (15-kDa) that localizes on the envelope of sectioned IMVs (F) and on the surface of purified virions (G). The 10-nm gold particles were used in all immunolabeling assays. Bars, 250 nm.

parently independent of cellular membranes, clearly different from the pathway used by VV membrane proteins such as 21- to 23-kDa or 15-kDa proteins, that reach the viral structures in ERGIC-related cellular membranes (Rodríguez *et al.,* 1997).

When viral assembly progresses, the 39-kDa and p4a/4a core proteins incorporate inside viral particles. The labeling pattern obtained clearly differs from the signal obtained when localizing a typical VV membrane protein such as the 15-kDa protein (Rodríguez *et al.,* 1997) (Figs. 5D–5G). No exposure of 4a (Fig. 5E) or 39-kDa (not shown) epitopes was detected on the surface of the IMV particles, according to immunogold labeling of purified IMVs, whereas a moderate exposure of the peptides detected by the anti-15-kDa antibodies was observed (Fig. 5G).

DISCUSSION

The morphogenesis of VV has been the subject of a number of reports that revealed the great complexity of the assembly process and its clear interest from the cell biology point of view. Recent studies on the identification of new VV structural proteins and their localization in the infected cell are providing molecular data on this complex process, in which several cellular compartments are involved. Our studies had first focused on the interaction between several VV membrane proteins and their potential relevance in the formation of the mature virion envelope. In these studies, a protein complex constituted by VV 21-, 15-, and 14-kDa envelope proteins was characterized. Two of these proteins, the 21- and 15-kDa proteins, were assigned to the VV membrane biogenesis (Rodríguez and Smith, 1990; Rodríguez *et al.,* 1995, 1996, 1997, 1998). The formation of complexes of viral proteins are also likely to play an important role in the maturation of the viral core. In the present report, we show through immunoprecipitation studies that two VV core proteins, the 39-kDa protein (Maa and Esteban, 1987) and the p4a/4a, the most abundant protein of the virion (Sarov and Joklik, 1972), form a stable complex soon after their synthesis. The 39-kDa and 4a proteins remain associated within the virion after the proteolytic processing of the p4a precursor that accompanies IV maturation. By using antibodies against the 39-kDa protein, we were able to coprecipitate the p4a precursor from rifampin-treated infected cultures and both p4a and 4a proteins from cells infected under conditions where virion assembly is allowed to proceed normally. The identity of the coprecipitated products as the VV p4a and 4a proteins was unequivocally established by their reactivity with an anti-p4a/4a serum on Western blotting. Neither of these two proteins was observed in parallel immunoprecipitations with antibodies against a different VV polypeptide, the L1R envelope protein, indicating the specificity of the 39-kDa–p4a/4a coprecipitation. However, it was still possible that the immunoprecipitation of p4a and 4a proteins was due to cross-reactivity of the anti-39-kDa antibodies with these two proteins. We have ruled out this possibility by using a mutant virus that inducibly expresses the 39-kDa protein and showing that neither of these two products was immunoprecipitated by the anti-39-kDa antibodies when expression of the 39-kDa protein was repressed, conditions under which they could be immunoprecipitated by an anti-p4a/4a antibody.

We studied the kinetics of this interaction by pulse-chase and immunoprecipitation and observed that soon after its synthesis, the 39-kDa protein interacts with the precursor p4a. Coprecipitation of 4a protein was clearly observed 1 h after the chase, which coincides with the time at which this product was first detected with the specific anti p4a/4a antibody. Interestingly, at the latest time point, the amount of the 4a product coprecipitated was higher than the amount of the 39-kDa protein itself. This might indicate that within the virion, the interaction is not at 1:1 molar ratio. In support of this hypothesis is the fact that the 62-kDa protein is the most abundant protein of the virion, representing 14% of the particle's dry weight (Sarov and Joklik, 1972). In addition, a differential representation of the two proteins within the complex could provide an explanation for the inability to detect 39-kDa protein coprecipitating when we use antibodies against the 4a protein. However, a different situation is observed in the interaction between 39-kDa protein and the p4a precursor, where only a fraction of p4a synthesized can be coprecipitated by the 39-kDa antibodies, and this would suggest that the stoichiometry of the interaction varies during the assembly process.

Another question that arises from the immunoprecipitation experiments is why antibodies to 39-kDa protein are more effective in immunoprecipitating the 4a protein than the anti-p4a/4a antibodies. In this regard, the gradual disruption of virions showed that 4a protein occupied a more internal location, whereas 39-kDa protein locates more externally and that would be compatible with the latter being a matrix-like protein, acting as a link between the core and the surrounding membranes, as previously proposed (Cudmore *et al.,* 1996; Ross *et al.,* 1996). Then, after treatment of cell extracts with NP-40, DOC, and DTT, the envelope proteins could be removed from virions, and the 39-kDa protein would be exposed and accessible to be immunoprecipitated by the anti-39-kDa-specific antibodies and, in turn, would bring down the 4a protein. On the other hand, 4a protein would be buried within the core structure and hidden from its own antibodies.

It has been suggested that the 39-kDa protein may interact with the 21-kDa (A17L gene product) envelope protein (Cudmore *et al.,* 1996). However, in our immunoprecipitation experiments, we have not observed coprecipitation of the 21-kDa protein with the 39-kDa protein. This apparent discrepancy may be due to the different

antibodies and immunoprecipitation conditions used in both studies.

Before being incorporated into the IMV structure, 39-kDa and p4a proteins together with the remainder of VV core proteins are part of the viroplasm, a poorly defined structure that includes many of the structural proteins of the IMV (Vanslyke and Hruby, 1994; Wang and Shuman, 1995). This dense material fills the immature virions and, before them, the viral factories. In this regard, we have observed that the viral factories, rifampin bodies, and electron-dense masses exhibit internal structures that suggest a network-like organization (see Fig. 5). Modified membranes, proteinaceous filaments, or even DNA fibers covered by proteins could participate in this organization. The viral factories, and the other assemblies that relate to them (rifampin bodies and electron-dense masses), are occasionally found in the extracellular environment, keeping their size and shape and frequently also their viral crescents (Risco *et al.,* unpublished results). Regardless of the origin of these extracellular structures (probably released by cell lysis), they must be rather compact and organized structures. Another undefined process is how 39-kDa and p4a proteins reach the viral factories after being apparently synthesized by free ribosomes. This transport does not seem to be related to ERGIC membranous elements, as in the case of VV 21 or 15-kDa envelope proteins (Krijnse-Locker *et al.,* 1996; Rodríguez *et al.,* 1997), which suggests that either the core proteins are transported to the factories in association with some as-yet-unidentified cellular elements or the factories could organize around cytoplasmic accumulations of 39-kDa and p4a protein-containing viral elements. No ribosomes were clearly seen inside the factories, as previously indicated by Cudmore *et al.* (1996), although their presence cannot be discarded until a more detailed analysis of the ultrastructure and composition of the viral factory is completed. According to double-labeling experiments, both proteins colocalize in all areas where an accumulation of gold particles is observed. Although weak, scattered labeling in cytoplasmic areas is constituted by separated signals, in all spots with significant accumulations of gold particles, both 5- and 10-nm signals coexist.

Because the association between the core proteins 39-kDa and p4a/4a takes place early in morphogenesis and occurs even before the proteolytic processing of the p4a precursor, it could be one of the protein complexes fundamental for the construction of the mature infectious virus. Finally, a detailed characterization of the internal structure of the viral factories and the dynamics of its formation, presently under way, might provide important data about the cellular elements that participate in their biogenesis, the mechanism of incorporation of the core proteins, and an still unsolved aspect, the encapsidation of the viral DNA.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney cells (BSC40) and HeLa cells were grown in DMEM supplemented with 10% newborn calf serum. VV strain WR was propagated and titrated in BSC40 cells. The VV mutant vA5Li that inducibly expresses the 39-kDa protein was kindly provided by Dr. Merchlinsky and was grown and titrated as previously described (Williams *et al.,* 1999). Recombinant virus VVindA17L (Rodríguez *et al.,* 1995) was grown in BSC40 cells in the presence of 2 mM IPTG. IMVs were purified according to standard procedures previously described (Joklik, 1962).

Antisera

The rabbit polyclonal anti-39-kDa and anti-p4a/4a antisera have been previously described (Demkowicz *et al.,* 1992; Maa and Esteban, 1987). The anti-39-kDa antiserum was obtained through immunization with the whole protein. The rabbit polyclonal anti-p4a/4a serum was raised through immunization with the 4a protein (also referred to as the 62-kDa protein). A murine polyclonal anti-p4a serum (anti-p4a.9K) was generated by immunizing mice with a 9-kDa fragment encompassing amino acids 615–697 from p4a, which was expressed in *Escherichia coli* as histidine-tagged fusion protein. The 15-kDa VV membrane protein was localized with a rabbit antiserum obtained against two synthetic peptides spanning amino acids 35–44 and 80–90 (Rodríguez *et al.,* 1997). The rabbit polyclonal serum against L1R envelope protein was kindly provided by Dr. Hruby (Oregon State University).

Metabolic labeling and immunoprecipitation analysis

For continuous labeling, BSC40 cells infected with VV (10 PFU/cell) in the absence or presence of rifampin (100 μg/ml) or with the mutant vA5Li in the absence or presence of 50 μM IPTG were incubated with [^{35}S]methionine (10 μCi/ml) (Amersham) from 6 to 24 h p.i. At the end of the labeling period, the cells were collected, washed with PBS, and resuspended in lysis buffer (20 mM Tris, pH 8.0, containing 80 mM NaCl, 20 mM EDTA, 1% NP-40, 0.5% DOC, and 50 mM DTT) in the presence of protease inhibitors (2 μg/ml bacitracin, 2 μg/ml trypsin inhibitor, 1 mM PMSF, and 10 μg/ml leupeptin). When indicated, 0.2% SDS was also included in the lysis buffer. After 30 min at 37°C, cell extracts were sonicated and cleared by centrifugation at 1500 rpm for 5 min. Before immunoprecipitation with specific antibodies, cell 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 39-kDa, L1R, or p4a/4a proteins. The immunoprecipitates were washed three times with lysis buffer and three times with PBS and resuspended in 2× sample buffer (125 mM Tris, pH 6.8, containing 4% SDS, 0.25% bromophenol blue, and 10% 2-mercaptoethanol). Samples were boiled for 3 min and resolved by 10% SDS–PAGE. The gels were dried, and the proteins were visualized after autoradiography.

For pulse-chase analysis, cells were infected with WR VV. At 6 h p.i., cells were washed with methionine-free DMEM and incubated in the same medium for 30 min at 37°C. Cells were then pulse-labeled with [^{35}S]methionine (150 μCi/ml) for 10 min and chased with a 100 fold-excess of unlabeled methionine for different times. At the end of the chase periods cells, were placed on ice, washed 3 times with ice-cold PBS, collected, and resuspended in lysis buffer. For total protein profile analysis, an aliquot of each sample was mixed with an equal volume of 2× sample buffer. Immunoprecipitation of cell lysates was performed as described above. Immunoprecipitates were resuspended in 2× sample buffer. Total cell extracts and immunoprecipitated proteins were fractionated by SDS–PAGE and visualized after autoradiography of the dried gel.

Electron microscopy

Processing of cell cultures. Cultures of HeLa cells were infected at a multiplicity of infection of 5 PFU/cell with strain WR in the presence or absence of rifampin (100 μg/ml) or with recombinant virus VVindA17L in the absence of IPTG. At 6 h p.i., IPTG (2 mM) was added to the cultures for 2, 4, or 18 h. At the times indicated, the supernatant was removed from the cultures, and the cell monolayers were submitted to a mild fixation with a solution of 4% paraformaldehyde containing 0.1% glutaraldehyde in PBS. Fixed cells were then removed from the dishes and processed for low-temperature embedding in Lowicryl K4M, as previously described in detail (Risco *et al.,* 1995b). Equivalent cell cultures were submitted to a stronger fixation with a solution of 2% glutaraldehyde containing 2% tannic acid in PBS and processed for embedding in the epoxy-resin EML-812 as previously described (Rodríguez *et al.,* 1995). Ultrathin sections (20–30 nm) of the samples were collected in gold grids covered with Formvar and carbon and were either stained or processed for immunogold labeling, according to general principles previously established (Risco *et al.,* 1995a; Rodríguez *et al.,* 1996).

Immunogold labeling of ultrathin sections. Immunogold localization on sections of infected cells was performed by placing the sections on drops of different solutions. After a 30-min incubation with Tris buffer gelatin (TBG) (30 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 0.1% BSA, and 1% gelatin) to block nonspecific binding of the antibodies to the samples, sections were floated for 90 min on a drop of the specific primary antiserum, diluted in TBG (1:100 for all the antisera described above). After jet-washing with PBS, grids were floated on 4 drops of TBG and incubated 10 min on the last drop before a 45-min incubation with the secondary antibody, a goat anti-rabbit immunoglobulin G conjugated with colloidal gold of 10 nm that was purchased from BioCell (Cardiff, UK). Washing was repeated as before, and grids were then floated on several drops of distilled water before staining with a solution of saturated uranyl acetate for 20 min (for Lowicryl sections) or 25 min with saturated uranyl acetate followed by 1 min with lead citrate for EML 812 sections.

Double labeling experiments were performed according to the principles previously described (Risco *et al.,* 1995b). For the simultaneous detection of p4a/4a and 39 kDa proteins, we used specific primary antibodies raised in two different hosts (the mouse anti-P4a-9K and the rabbit anti-39-kDa antisera described above) and the corresponding secondary antibodies conjugated with gold particles of two different sizes (a 10-nm goat anti-mouse gold conjugate and a 5-nm goat anti-rabbit gold complex). Saturation, incubation times with different reagents, and washing steps were performed as described for single labeling experiments. The double labeling procedure was done in two or four steps, labeling first one of the proteins with the corresponding primary antibody and gold conjugate (four steps) or mixing the primary antibodies and later both gold conjugates (two steps).

Samples were studied in a JEOL 1200EX II electron microscope.

Immunogold labeling of isolated virions for detection of VV proteins exposed on the surface of IMVs. Purified IMVs were attached to electron microscopy gold grids covered with Formvar and carbon that were previously made hydrophilic by glow discharge. These grids were placed on drops of a concentrated IMV suspension, and virions were adsorbed for 5 min. Adsorbed virions were submitted to immunogold labeling as previously described (Risco *et al.,* 1996; Rodríguez *et al.,* 1997) and negatively stained with a 2% solution of uranyl acetate. Samples were finally studied by electron microscopy.

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