Assembly of Vaccinia Virus: Effects of Rifampin on the Intracellular Distribution of Viral Protein p65

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The cytoplasmic assembly of vaccinia virus is reversibly blocked by the antibiotic rifampin, leading to the accumulation of partially membrane-delineated rifampin bodies in infected cells. Rifampin-resistant vaccinia virus mutants have point mutations in the D13L gene, which is controlled by a late promoter and expresses a 65-kDa protein, designated p65. To further characterize the mechanism of rifampin inhibition and the function of p65 in virus assembly, we raised antibodies to this protein. Immunoreactive p65 was expressed at late times of infection, and neither its expression nor its turnover was affected by rifampin. Virus-associated p65 could be extracted only with denaturing detergents from purified virions, suggesting that it is an integral viral component. Immunofluorescence studies showed that p65 is localized to the sites of virus assembly. Also, immunoelectron microscopy showed p65 to be associated with viral crescents as well as spherical, immature virions, in both cases predominantly on the inner or concave surface. In the presence of rifampin, p65 was found in large, cytoplasmic inclusion bodies that were distinct from rifampin bodies. The rifampin bodies themselves were labeled with p65 antibodies only after reversal of the rifampin block, predominantly on the viral crescents which rapidly formed following removal of the drug. We propose that p65 functions as an internal scaffold in the formation of viral crescents and immature virions, analogously to the matrix proteins of other viruses.

Vaccinia virus is the best-studied member of the family *Poxviridae*, the largest and most complex of the animal viruses (for reviews, see references 6, 9, and 17). Its double-stranded linear DNA genome of nearly 200 kbp has been sequenced, and approximately 200 potential genes have been identified (10). Unlike other DNA viruses, both vaccinia virus replication and assembly occur in discrete cytoplasmic foci that are termed viral factories, with two structurally distinct, infectious forms of the virus being produced. The first characteristic virus structures are crescent-shaped membranes on the convex surface of which protrude spicules (5). It was originally proposed that these membranes were synthesized de novo within the viral factories by virally encoded enzymes (23, 29, 30). However, by using immunoelectron microscopy with antibodies specific for cellular marker proteins as well as lipid analysis of purified virions, it now appears that the viral crescents are derived from a membrane cisterna of the intermediate compartment, which is located between the endoplasmic reticulum and the Golgi apparatus (27). As a consequence, the crescents contain two tightly apposed membranes.

The viral crescents eventually form spherical, immature virions which undergo additional maturational events, including the cleavage of viral core proteins (19, 32, 33), to give rise to the first infectious form of vaccinia virus, commonly referred to as intracellular naked virus. However, since this form of the virus is surrounded by two membranes, we recently proposed

that intracellular mature virus (IMV) would be a more-appropriate designation (27). The IMV form of vaccinia virus is the best-characterized form and the one most commonly used in the laboratory. However, a fraction of IMV becomes enwrapped by an additional membrane cisterna derived from the *trans* Golgi network (26). The outermost membrane of this intracellular enveloped virus fuses with the plasma membrane, thereby releasing the extracellular enveloped virus (EEV) into the medium (25). Thus, EEV contains an additional membrane relative to IMV as well as several virally encoded glycoproteins. With some strains of vaccinia virus, most of the EEV remains attached to the outside of the plasma membrane and has been designated cell-associated enveloped virus (3). The cell-associated and released EEVs are largely responsible for cell-to-cell transmission in vitro and widespread dissemination, respectively (1, 3, 4, 24).

The assembly of vaccinia virus is reversibly blocked at a discrete step by the macrolide antibiotic rifampin (21). At inhibiting concentrations, the membranes associated with the viral factories are irregularly shaped, and crescents and spherical, immature particles do not form (14, 22). These altered structures have been referred to as rifampin bodies, a term which we will use in this article. The effects of rifampin are rapidly reversible, and within minutes after its removal, the membranes around the rifampin bodies acquire their usual convex shape and normal infectious IMVs are formed (14, 22). Reversal of the rifampin inhibition does not require macromolecular synthesis (18), and the drug has been shown to have no significant effect on viral DNA replication or protein synthesis (21).

The target of rifampin is a 65-kDa, late structural viral protein, referred to as p65, encoded by the vaccinia virus gene

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Anti-peptide antibodies made to L65

FIG. 1. Preparation of anti-peptide antibodies against the vaccinia virus protein p65. Four anti-peptide antibodies were produced by using peptides B1, B2, B3, and B4 as immunogens. The positions of these peptides within the protein p65 are shown. The diagram also indicates point mutations at amino acid positions 27 (from Gln to Lys) and 480 (from Asn to Asp) which have been demonstrated to confer rifampin resistance (2, 31).

D13L. Rifampin-resistant vaccinia virus mutants have single point mutations in this gene (Fig. 1) (2, 31), and repression of p65 synthesis blocks virus assembly at the same stage as rifampin (35). Using subcellular fractionation and immunofluorescence, Miner and Hruby have shown that in the presence of rifampin, p65 is not present in viral factories (16). The possibility that p65 forms the spicule coat which is conspicuously absent in the presence of rifampin has been proposed (2, 35).

To further define the role that this protein might play in vaccinia virus assembly, we raised antibodies against p65 and studied its biosynthesis, subcellular localization, and incorporation into the IMV. Our results show that p65 localizes to the crescent-shaped membranes, predominantly on the inner surface of the membrane cisternae. In the presence of rifampin, p65 is concentrated in inclusion bodies throughout the cytoplasm. Its localization suggests that it may be required for the formation of the characteristic crescent-shaped membranes during the early steps of vaccinia virus assembly and may play a role analogous to that of the matrix proteins of other viruses.

MATERIALS AND METHODS

Materials. Tissue culture reagents were obtained from GIBCO-BRL (Gaithersburg, Md.). HeLa and BSC-1 cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (heat inactivated) and nonessential amino acids. BHK-21 cells were grown in Glasgow's modified Eagle's medium supplemented with 5% fetal calf serum and 10% tryptose phosphate broth. All media contained 2 mM glutamine, 100 U of penicillin per ml, and 10 mg of streptomycin per ml, and all cell lines were grown as adherent cultures in a 5% CO₂ incubator at 37°C. Virus propagation and titration using the vaccinia virus strain WR or a conditional-lethal recombinant vaccinia virus, vRO65K, which expresses p65 only in the presence of isopropyl-β-thiogalactoside (35), were performed as previously described (8). Antibodies against each of four peptides from different regions of the p65 protein were raised (Fig. 1). Peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and were injected into rabbits as described by Kreis (15).

Virus infections and drug treatments. Cells were grown for 2 days to approximately 80% confluency, washed once in phosphate-buffered saline (PBS), and then infected in serum-

free Eagle's minimal essential medium with 10 to 20 PFU per cell. After 1 h at 37°C with intermittent agitation, the inoculum was aspirated and the cells were washed with PBS and then placed in normal growth medium at 37°C with or without rifampin (final concentration, 100 µg/ml; Sigma, St. Louis, Mo.). Rifampin was stored as a 1,000-fold stock solution in dimethyl sulfoxide at -20°C. For chase experiments, the cells were washed three times with ice-cold PBS at the indicated times and were then further incubated with prewarmed, normal drug-free medium at 37°C.

Purification of IMV. Vaccinia virus-infected BSC-1 cells were scraped into the medium at 48 h postinfection, pelleted, resuspended in 10 mM Tris (pH 9.0), and lysed by Dounce homogenization. After low-speed centrifugation to remove nuclei, the virus was sedimented through a 36% sucrose cushion (10 mM Tris, pH 9.0) in an SW28 rotor (Beckman Instruments, Inc.) at 24,000 rpm for 30 min. The virus pellet was resuspended in 10 mM Tris (pH 9.0), and the IMV was purified from the EEV by equilibrium gradient sedimentation as previously described (7). Virus bands were collected, and the virus particles were concentrated by centrifugation.

Immunoprecipitation, SDS-PAGE, and Western blotting (immunoblotting). For immunoprecipitations, samples were centrifuged at high speed in a microcentrifuge (Eppendorf) for 15 min at 4°C to clear the samples of intact virus particles. The supernatant was then incubated with the appropriate primary antibody for 1 h at 4°C, followed by protein A-Sepharose for 1 h. Samples were washed three times with PBS containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) prior to SDS-PAGE (polyacrylamide gel electrophoresis). Samples for SDS-PAGE were incubated in Laemmli buffer with 1% SDS and 0.5% β-mercaptoethanol. For Western blotting, the proteins were transferred to nitrocellulose membranes. The nitrocellulose was incubated in PBS with 0.2% Tween 20 and 1.0% bovine serum albumin for 1 h prior to incubation with the indicated antibody, generally at a 1:1,000 dilution. Antibody was detected with ¹²⁵I-labeled protein A (New England Nuclear).

Light microscopy. All incubations for immunofluorescence labeling were carried out in PBS, pH 7.4, at room temperature. The samples were fixed after the indicated times with 3% paraformaldehyde for 20 min. After washing with PBS, the remaining fixative was quenched with 50 mM NH₄Cl for 10 min. The cells were permeabilized with 0.2% Triton X-100 for 4 min and were transferred to 0.2% gelatin. They were then labeled with the first antibody in 0.2% gelatin for 20 min, washed extensively, and incubated with fluorescently labeled secondary antibodies in 0.2% gelatin for 20 min. Nuclear and viral DNAs were visualized by staining with 5 µg of bis-benzimide per ml of PBS (Hoechst no. 33258; Sigma) for 5 min. The coverslips were mounted on glass slides using Moviol as mounting medium, examined with an Axiophot fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with the appropriate filters, and photographed with Kodak film TMAX (ASA 400).

Electron microscopy. Infected BHK or HeLa cells were processed for Epon embedding and for postembedding immunolabeling as previously described (27). The cells were fixed for 60 min in 1% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, stained with 1% OsO₄ with 1.5% K₂[Fe(CN)₆] and 1% magnesium uranyl acetate for 60 min, respectively, and processed for conventional Epon embedding. All incubations were carried out at room temperature. For postembedding immunolabeling, infected cells were removed from the tissue culture dish by treatment with proteinase K (25 µg/ml for HeLa cells and 50 µg/ml for BHK cells; Merck, Darmstadt, Germany) on

FIG. 2. Characterization of antisera. (A) Vaccinia virus-infected cells were lysed with Triton X-100 24 h after infection, and aliquots were analyzed by SDS-PAGE and Western blotting with antisera B1, B2, B3, and B4 as indicated; (B) vaccinia virus-infected cells were labeled with [³⁵S]methionine overnight and lysed with Triton X-100, and aliquots were immunoprecipitated with the different antisera. Lane -, noninfected cells immunoprecipitated with B1. In both panels, only antisera B1 and B2 recognized p65.

ice for 2 to 3 min. The cells were then prefixed with 1.5% paraformaldehyde in 0.25 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, spun at 800 × *g*, and fixed in 8% paraformaldehyde in 0.25 M HEPES, pH 7.4, overnight. The cell pellets were infiltrated with 2.1 M sucrose as a cryoprotectant in PBS, frozen, and then stored in liquid nitrogen. Ultrathin sections were cut at -90°C and transferred to Formvar-coated grids. The immunolabeling, as well as the drying and contrasting of the sections, was performed as described elsewhere (11).

For pre-embedding immunolabeling, plates of infected HeLa cells were set on ice, and the plasma membrane was permeabilized with the bacterial toxin streptolysin O (Wellcome Diagnostics) as previously described (27). Subsequently, the cells were fixed lightly with 4% paraformaldehyde for 5 min on ice, washed for 60 min in incubation buffer (150 mM sucrose, 50 mM magnesium acetate, 25 mM HEPES, 5 mM potassium acetate, 1% fish skin gelatin [pH 7.4]), and then incubated with or without the B1 antibody in the same buffer at 4°C with agitation overnight. The cells were washed again for 120 min in buffer, incubated with protein A-gold for 60 min, washed again for 120 min, fixed in 1% glutaraldehyde in 200 mM cacodylate buffer, and contrasted and embedded in Epon (27).

RESULTS

Characterization of the antisera. To analyze the role of the vaccinia virus protein p65 in viral assembly, we raised rabbit antibodies against four synthetic peptides derived from the sequence of its open reading frame, D13L (Fig. 1). To determine whether the antipeptide antisera recognized p65, BSC-1 cells were infected with vaccinia virus for 24 h, after which they were lysed with Triton X-100. Portions of the infected cell lysate were subjected to SDS-PAGE and Western blotting with the four antisera. Antisera B1 and B2 recognized a single 65-kDa band in infected (Fig. 2a) but not in uninfected cells (data not shown). In addition, the 65-kDa band was greatly reduced in cells infected with VV-vRO65K in the absence of isopropyl-β-thiogalactoside when the synthesis of p65 was greatly reduced (35, 36). Antiserum B4 did not recognize p65, while antiserum B3 reacted with a 92-kDa protein (data not

shown). Since one mechanism to account for the effects of rifampin is binding of the drug to p65, we included rifampin (100 μg/ml) during the antibody incubations and washes to determine whether binding of one or more of the antibodies was blocked. Inclusion of rifampin had no effect on antibody binding (data not shown).

The abilities of the various antibodies to immunoprecipitate p65 were also determined. Vaccinia virus-infected cells were labeled with [³⁵S]methionine overnight, after which they were lysed with Triton X-100. The lysates were then subjected to immunoprecipitation with the antisera. Both antisera B1 and B2 were capable of immunoprecipitating p65 (Fig. 2b). Again, inclusion of rifampin during the antibody incubations and washes did not affect binding (data not shown). Thus, the antisera B1 and B2 recognized the D13L gene product p65 by Western blotting and by immunoprecipitation in the absence and in the presence of rifampin.

Time course of p65 expression and its incorporation into IMV. The vaccinia virus gene D13L is regulated by a strong late viral promoter (34). To determine the time course during which p65 is synthesized, cells were lysed at various times after infection, and the amount of p65 was analyzed by Western blotting with antibody B1. p65 was first detected approximately 6 h after infection and accumulated over the course of a 24-h infection (data not shown). When rifampin was present, the level of p65 was slightly decreased, consistent with previous observations that have shown a small effect of rifampin on total viral protein synthesis (18). Expression of p65 was also analyzed by pulse-labeling experiments. Cells were incubated with [³⁵S]methionine for 15 min at different times after infection, lysed, and immunoprecipitated with antibody B1. In agreement with the results obtained by Western blotting and previous studies (16), expression of p65 was detected only at late times after infection (data not shown).

To ascertain whether p65 is incorporated into IMV, cells were labeled with [³⁵S]methionine between 6 and 12 h postinfection. The cells were then placed in normal growth medium for an additional 12 h, after which virus was purified by sucrose gradient centrifugation. In agreement with Miner and Hruby (16), we found that significant levels of p65 could be recovered from purified virions (data not shown). In addition, we found that p65 remained virus associated under a variety of conditions including incubation with the nonionic detergents Nonidet P-40, Triton X-100, or Brij 58, high concentrations of salt and urea, and reducing agents. However, p65 could be extracted when the entire virus structure was disrupted by SDS and sodium deoxycholate, suggesting that p65 is an integral component of the mature virus.

Viral structures induced in the presence of rifampin. We examined the ultrastructure of vaccinia virus-infected cells at 8 to 12 h postinfection in the presence of rifampin using conventional Epon-embedded samples. In the presence of rifampin, the membranes surrounding portions of the electron-dense viral factories were irregularly shaped (Fig. 3, arrowheads), and membrane crescents and immature virions did not form. These viral structures, termed rifampin bodies, are easily identified by phase-contrast microscopy and have been described by electron microscopy as well (13, 14, 22, 27). As with normal viral membranes, those delimiting the rifampin bodies can be labeled with antibodies against cellular marker proteins of the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (27). Some rifampin bodies show a membrane cisterna close to the first membrane (triple arrowheads in Fig. 3a). The membranes do not completely enclose the rifampin bodies and often appear to extend away from them (Fig. 3a to c). Occasionally, there are membrane

tubules in continuity with the membranes surrounding the rifampin bodies (Fig. 3c, arrows).

In addition to the rifampin bodies, two additional viral structures were readily seen in rifampin-treated cells. Very electron dense, irregularly shaped crystalloids containing DNA are seen (D in Fig. 3a; see Fig. 5b). That these structures in fact contain viral DNA has been shown by autoradiography experiments using [^3H]thymidine (14) as well as using anti-DNA antibodies (27a). A second, previously undescribed structure was also seen. We refer to these structures, which were slightly more electron dense than the cytoplasm, as inclusion bodies (IB in Fig. 3a). As will be seen below, the inclusion bodies contain high amounts of p65. While both DNA crystalloids as well as inclusion bodies were occasionally seen in infected cells not treated with rifampin, their numbers and sizes were greatly increased with rifampin treatment.

Rifampin affects the subcellular localization of p65. To determine the intracellular localization of p65 in vaccinia virus-infected cells we carried out immunofluorescence experiments. Both of the antibodies against p65, B1 and B2, gave identical results and at 8 h postinfection strongly labeled most of the viral factories (Fig. 4b) which were defined by DNA staining (Fig. 4a) (27), as well as some punctate cytoplasmic structures, most likely inclusion bodies (arrows in Fig. 4b). However, in the presence of rifampin, p65 did not colocalize with the viral DNA (Fig. 4c and d). Instead, a number of intensely labeled inclusion bodies were observed throughout the cytoplasm (Fig. 4d). The localization of p65 changed dramatically and very quickly after the removal of rifampin since within 5 to 15 min after removal of the drug most of the p65 again colocalized with the factories (Fig. 4e and f). These experiments show that p65 localization to the viral factories is reversibly prevented by rifampin.

Anti-p65 labels viral crescents and immature virions. We next localized p65 at the electron microscopic level. To synchronize assembly, BHK and HeLa cells were infected in the presence or absence of rifampin followed by different chase times in drug-free medium prior to fixation. Postembedding immunolabeling was performed on thawed cryosections (11). In the presence of rifampin, there was little p65 antibody labeling over the rifampin bodies (R in Fig. 5a and b). Instead, electron-dense structures most likely corresponding to the inclusion bodies observed in the Epon-embedded samples (Fig. 4a) were heavily labeled (IB in Fig. 5).

We next monitored the distribution of p65 following removal of rifampin. After a 10-min chase in drug-free medium, p65 was clearly detected on the viral crescent-shaped membranes which had formed from the rifampin bodies (Fig. 6). While the spicule-containing membranes forming the viral particles were labeled extensively, the irregular shaped membrane cisternae which are in direct continuity with the viral buds, as well as the viroplasm (R in Fig. 6), were not strongly labeled (Fig. 6). After longer times of chase in rifampin-free medium, we had the impression that the sizes of the p65-positive inclusion bodies decreased, whereas the numbers of immature virions which were strongly labeled increased.

The pattern of labeling seen in Fig. 6 was similar to that seen in infected cells not treated with rifampin, with the membrane crescents and immature virions being labeled to a similar extent (Fig. 7a and b). Under all conditions, the labeling was mostly but not exclusively associated with the concave, inner side of the assembling virions (Fig. 6 and 7). In addition, the viral forms which are intermediate between the spherical immature virions and the IMV (27) were strongly labeled for p65 (Fig. 8a and b). However, IMV and the intracellular enveloped virus (26) were poorly labeled under these conditions (Fig. 8c). It seems likely that the epitopes seen by the antibodies are not accessible at these later steps of morphogenesis or that the amount of p65 is greatly diminished even though some p65 could be recovered from purified IMV.

Anti-p65 labels the inner membrane of crescents and immature virions. Using cryosections for immunoelectron microscopy, we were not able to determine unequivocally whether p65 was exclusively located on the inner side of the viral crescents and immature virions. As is evident in Fig. 6 to 8, a small but significant amount of label could always be found on the outer side of the crescents and the immature virions. With the postembedding labeling technique, we could not determine whether this was due to a real dual localization of p65 or simply due to insufficient resolution. Thus, we carried out pre-embedding labeling experiments in which cells were permeabilized using the bacterial toxin streptolysin O (27) and subsequently exposed to the antibody B1 followed by protein A-gold prior to their embedding in Epon. We have previously shown that a large fraction of the immature virions are still accessible to cytoplasmic markers under these experimental conditions (27). Thus, antibody B1 should have access to p65 whether it is located on the inner and/or outer membrane surface. As shown in Fig. 9, the majority of the label was in fact located on the inner viral surface, as the postembedding experiments already suggested. It is important to note that virions which contain viral DNA and are presumably completely sealed did not acquire any gold label, providing further support that p65 is located on only one surface of the membrane cisternae.

DISCUSSION

In a recent study, we showed that the crescent and the immature forms of vaccinia virus possess two tightly apposed membranes which are derived from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (27). How virus assembly is initiated at the intermediate compartment and how the viral genome and core proteins are enclosed by the intermediate compartment-derived crescent-shaped membranes are not known. Presumably, there are viral proteins that mediate these complex events. The identity of one such protein has been suggested by studies on rifampin. It has long been known that rifampin reversibly prevents the formation of the viral crescents (14, 21, 22). Rifampin-resistant vaccinia virus mutants, which arose spontaneously, have mutations in their D13L gene coding for a late protein, designated

FIG. 3. Rifampin-induced structures in vaccinia virus-infected cells. Epon sections of rifampin-treated, vaccinia virus-infected BHK (a and c) and HeLa (b) cells at 8 h postinfection. The cells contain three different types of viral structures induced by rifampin treatment: rifampin bodies (R), DNA crystalloids (D), and inclusion bodies (IB). The rifampin bodies are surrounded by one or two membranes with irregular shapes (arrowheads in all panels). Note that these membranes do not enclose the rifampin bodies completely. Membrane cisternae may also be closely associated with the membrane around the rifampin bodies (three arrowheads in panel a). Some of the rifampin bodies are located in close proximity to the Golgi apparatus (G in panels a and b). Occasionally, there are membrane tubules in continuity with the membranes which surround the rifampin bodies (arrows in panel c). Similar tubules are seen in continuity with the crescents that form during a normal infection without rifampin (27). ER, endoplasmic reticulum; M, mitochondrion; bars, 250 (a and b) and 200 (c) nm.

FIG. 4. Localization of the vaccinia virus protein p65 by immunofluorescence. Vaccinia virus-infected HeLa cells were examined 8 h postinfection in the absence (a and b) or the presence (c and d) of rifampin or after treatment with rifampin followed by a 15-min chase in drug-free medium (e and f). The cells were labeled with anti-p65 (B1) and then with rhodamine-conjugated goat anti-rabbit antibody (b, d, and f) or with a fluorescent DNA dye (a, c, and e). Anti-p65 (b) labeling overlaps significantly with the viral factories as defined by DNA staining (arrowheads in panel a) as well as some cytoplasmic aggregates (arrows in panel b). Note that there is some cytoplasmic DNA staining that does not colocalize with the p65 labeling (white circle in panel b). In the presence of rifampin, p65 (d) does not colocalize with the viral factories (arrowheads in panel c) but is found in a punctate, cytoplasmic distribution (arrows in panel d). When rifampin is removed for 15 min, a significant amount of the label for p65 (f) again colocalizes with the viral factories (arrowheads in panel e). N, nucleus.

p65 (2, 31). The amino acid sequence of p65 lacks an obvious membrane anchor or signal sequence, suggesting that it is a cytosolic protein. Further support for this topology is provided by the fact that p65 is not glycosylated despite the presence of 10 potential N-linked sites (2, 16, 31). Given its probable

cytosolic localization, p65 could potentially influence both crescent formation and subsequent interactions with the viral core. To investigate these possibilities, we raised antibodies against p65 to study its biosynthesis and intracellular localization at both the light and electron microscopic levels.

FIG. 5. Immunolocalization of p65 in the presence of rifampin. Cryosections of vaccinia virus-infected, rifampin-treated HeLa cells at 8 h postinfection were labeled with anti-p65 and then with 9-nm protein A-gold. Three types of virally induced structures are seen: inclusion bodies (IB), rifampin bodies (R) and electron-dense DNA crystalloids (D). Anti-p65 strongly labels the inclusion bodies but only weakly labels the rifampin bodies and the cytoplasm. Note that the inclusion bodies can be continuous with the rifampin bodies (arrows in panels a and b). PM, plasma membrane; bars, 200 nm.

FIG. 6. p65 is localized on the viral crescents after removal of rifampin. Cryosections were made of vaccinia virus-infected BHK cells 8 h after infection in the presence of rifampin after a 15-min chase in drug-free medium. Sections were labeled with anti-p65 and then with 9-nm protein A-gold. Viral crescents (*) arising from the rifampin bodies (R) were heavily labeled by anti-p65 (arrows). While the bulk of the label is associated with the inner side of the crescents, a small but significant number of gold particles were associated with the outer side. The resolution of the labeling was not sufficient to determine for certain whether some of the antigen is present on the outer side. Note that the crescents contain two membranes (arrowheads) and are continuous with cellular membranes. Bar, 200 nm.

In agreement with Miner and Hruby (16), we found that p65 is synthesized at late times after infection, is stable, is not glycosylated, and is incorporated into virions. In addition, we found that p65 is an integral component of the virus particle since it could be extracted from purified IMV only by denaturing detergents. We also examined the immunolocalization of p65 during the course of infection and confirmed that p65 was localized in the viral factories, as judged by colocalization with a stain for viral DNA. At the electron microscopic level, antibodies to p65 specifically and strongly labeled the virus crescents, preferentially on their concave surface. The membranes of fully formed immature virions were also heavily labeled. However, little labeling was observed on mature virions. Given that p65 can be easily detected in purified virus preparations, we think it most likely that the epitopes recog-

nized by our antibodies become sequestered during the course of virus maturation. However, the possibility of a decrease in the amount of p65 during maturation cannot be ruled out.

The distribution of p65 in virus-infected cells is reversibly affected by rifampin. In the continuous presence of the drug, p65 no longer colocalized with the viral DNA. Instead, p65 was distributed in a punctate pattern throughout the cytoplasm. Electron microscopy showed that in the presence of rifampin, p65 was present in previously undescribed structures which we refer to as p65 inclusion bodies. By contrast, the rifampin bodies, which contain the precursor membranes of the viral crescents, showed little p65 labeling. Since the rifampin block is readily reversible and does not require new protein synthesis (20), it might be possible that rifampin causes p65 to aggregate. After removal of the drug, p65 might dissociate and move

FIG. 7. Localization of p65 in the absence of rifampin. Cryosections of vaccinia virus-infected HeLa cells 9 h postinfection were labeled with anti-p65 and then with 9-nm protein A-gold. As in Fig. 6, the bulk of the p65 labeling appeared to be on the inner side of the viral crescents (CR). Strong labeling was also found on inclusion bodies (IB) and immature virions (IV in panels b and c). Bars, 200 nm. Arrowheads show two membranes associated with immature virions, while small arrows show labeling of membranes in association with viral crescents.

FIG. 8. p65 labels immature but not mature virus particles. Cryosections of vaccinia virus-infected HeLa cells 9 h postinfection were labeled with anti-p65 and then with 9-nm protein A-gold. As seen in earlier figures, p65 antibodies labeled the inner side of the viral crescents (CR) and the immature virions (IV). The viral forms that are intermediate between the spherical IV and the IMV were also labeled (arrowheads in panels a and b), whereas the IMV and the intracellular enveloped virus (IEV; also, the four arrows in panel c) were only weakly labeled (IMV and IEV in panel c). PM, plasma membrane; N, nucleus; bars, 200 nm.

FIG. 9. p55 labels the inner membrane of the viral crescents and the immature virions. A cryosection (a) and Epon sections (b to d) of vaccinia virus-infected HeLa cells 9 h postinfection were taken. The samples were labeled after embedding with anti-p55 and then with 9-nm protein A-gold (postembedding [panel a]), or after permeabilization with streptolysin O the cells were lightly fixed and incubated with anti-p55 and then with protein A-gold (6 nm) prior to embedding in Epon (preembedding [panels b to d]). By postembedding labeling (a), the labeling for p55 was predominantly associated with the inner side of the viral crescents (arrows; cf. also Fig. 7 to 9). When the cells were incubated with the antibody prior to embedding, the labeling was even more strongly localized to the inner site of the viral crescents and the immature virions (arrows in panels b to d). Note the continuity between a viral crescent and a membrane cisterna (arrowheads in panel c). Bars, 200 nm.

quickly from the inclusion bodies to the membranes of the rifampin bodies. In this scenario, the inclusion bodies would consist largely of aggregated p55. However, careful examination of the electron micrographs reveals the presence of irregularly shaped membranes within the inclusion bodies. Thus, the inclusion bodies may contain more than aggregated p55.

The immunolocalization of p55 in the presence and absence of rifampin suggests a role that it might play during vaccinia virus assembly. Although it is a putative cytosolic protein (2, 31), it is targeted to the membranes of the viral crescents. More specifically, pre-embedding labeling experiments showed

that most and perhaps all of the p55 is localized to the inner or concave surface of the viral crescent. Since the crescents do not form either when p55 synthesis is repressed (35) or when its localization is disrupted by rifampin, p55 appears to be required for the transition of an intermediate compartment membrane cisterna into a typical viral crescent. Because p55 is presumably located at the inner of these two membranes, it might function as an internal protein scaffold. In fact, we have found that p55 is easily cross-linked into higher-order homooligomers by a number of chemical cross-linking reagents (data not shown). Baldick and Moss (2) noted that mutations conferring rifampin resistance occur near both ends of p55

(Fig. 1) and suggested the possibility of head-to-tail interactions that are modulated by rifampin. That p65 may self-assemble in a highly cooperative manner is also supported by studies in which p65 expression was suppressed by approximately 95%. Nevertheless, the remaining low level of p65 expression was sufficient to form occasional viral crescents (35). Cooperativity is also suggested by images of intermediate structures formed within a few minutes of removing rifampin (14). By forming a higher-order structure, p65 might thereby effectively cross-link a putative p65 receptor and orient the inner crescent membrane into its typical spherical shape. This hypothesis is analogous to ideas about the function of viral matrix proteins during the budding at the plasma membrane of vesicular stomatitis virus or influenza virus (12, 28).

It is unlikely that p65 by itself is responsible for targeting vaccinia virus assembly to intermediate compartment membranes. Rather, given its rapid redistribution in response to rifampin, we suspect that p65 binds to a not yet identified virally encoded membrane receptor(s) which is targeted to the intermediate compartment. Thus, identifying additional vaccinia virus proteins involved in assembly may be useful in identifying mechanisms by which proteins in general are targeted to the intermediate compartment.

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