RAPID COMMUNICATION

Enveloped Virus Is the Major Virus Form Produced during Productive Infection with the Modified Vaccinia Virus Ankara Strain

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Modified vaccinia virus Ankara (MVA) is a highly attenuated virus strain that may be useful as a vaccine vector. Ultrastructural examination of purified MVA showed that most of the viral particles are enveloped in contrast to the Copenhagen strain (COP). In CsCl gradients, the majority of the MVA particles displayed a light buoyant density characteristic of the enveloped form. Consistent with these results, MVA particles were poorly labeled with antibodies against the surface of intracellular mature virus but strongly labeled with antibodies against an envelope antigen. Furthermore, MVA was more resistant than the COP strain to neutralization by mouse anti-COP antibodies. These results suggest that the MVA strain may be particularly suitable for the engineering of envelope proteins and that MVA may be able to resist the humoral immunity displayed by previously vaccinated individuals. © 2000 Academic Press

Introduction. Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus (VV) obtained by passing a VV isolate from Ankara, Turkey, more than 500 times in chick embryo fibroblasts (1). The host range of MVA is considerably restricted compared to other VV strains since it fails to multiply efficiently in most mammalian cells with the exception of hamster BHK 21 cells (1–3). The host range defects have been mapped and found to be the consequence of deletions in several regions of the viral genome (4). Determination of the complete sequence of the MVA genome and its comparison to that of the VV Copenhagen strain (COP) has allowed the precise identification of seven deletions and numerous mutations leading to fragmented ORFs (5). Because of its high degree of attenuation, MVA has appeared as an attractive alternative to standard VV strains for the development of viral vectors to be used in vaccination or immunotherapy. One strategy designed to take full advantage of the viral life cycle in the construction of recombinant VV relies on the finding that intracellular mature virus (IMV) may be enveloped in a series of steps to give rise to extracellular enveloped virus (EEV). In a first step, IMVs acquire a double-layer envelope by wrapping with VV modified Golgi vesicles. The outer envelope of these particles, designated intracellular enveloped virus (IEV), is lost through fusion with the cell membrane resulting in the release at the cell surface of cell-associated enveloped virus (CEV). In a final step, the CEV may detach from the cell to become truly EEV. The envelopes of both the CEV and EEV contain virus-encoded membrane proteins whose genes can be fused with foreign sequences in such a way that the proteins they encode are displayed on the virus surface (6). This feature has been exploited to generate recombinant EEVs to immunize against foreign antigens (7) or as a means of targeting VV to specific cell types (8). As a prerequisite to employing such a strategy with MVA and since little knowledge is currently available concerning the ability of MVA to give rise to enveloped virions, we have examined the formation of enveloped virus upon MVA infection.

Results. Purified MVA particles are surrounded by an envelope. MVA and the COP strain of VV were purified from combined infected CEF lysates and culture medium using an identical sucrose gradient procedure. The purified stocks were then observed under an electron microscope after negative staining. MVA particles were often clumped together (Fig. 1C) in contrast to Cop particles (Fig. 1A). Furthermore, MVA particles displayed a smooth surface (Fig. 1D) unlike the COP particles (Fig. 1B), which usually had a rough appearance referred to as the mulberry form. The majority of the MVA particles also displayed a surrounding envelope (Fig. 1D) that was not apparent in particles of the COP strain (Fig. 1B). The rough surface of purified VV particles has been considered evidence for the presence of tubules on the IMV surface. However, cryoelectron microscopic observa-
tions have indicated that the tubules are artifacts which appear during the staining and dehydration procedures required for observation by standard EM methods (9). The presence of an additional envelope surrounding MVA particles could prevent the occurrence and/or visualization of such artifacts. Further evidence for an extra envelope surrounding MVA particles was provided by examination of unstained virus using cryoelectron microscopy (results not shown).

Enveloped virus predominates in intracellular and extracellular fractions of MVA-infected cells. The additional envelope surrounding MVA particles could appear through the process known to give rise to IEV, CEV, and EEV. To investigate the relative production of extracellular virus released into the medium as well as cell-associated virus, we titrated both fractions produced after infection of chick embryo fibroblasts. Over a 2-day period, the MVA and COP strains produced a similar amount of cell-associated virus; however, MVA produced a 20- to 40-fold larger amount of extracellular virus than the COP strain (not shown). To quantify the ratio of enveloped virus versus unenveloped virus in the medium and cell lysates we submitted the two fractions to density gradient analysis (Fig. 2). The COP particles produced were essentially cell associated (78%) and sedimented at 1.28 g/ml, a buoyant density characteristic of IMV (Fig. 2A). MVA particles, on the other hand, were found in both intracellular and extracellular fractions (Figs. 2B and 2D). The cell-associated MVA was composed of IMV (26%) and to an even larger extent enveloped virus (63%) displaying a lighter buoyant density (1.23 g/ml). Whereas very little extracellular virus (2%) was detectable in the CsCl gradients after infection with the COP strain (Fig. 2C), MVA infection led to the accumulation of a larger amount of extracellular virus (11%) with a buoyant density characteristic of EEV (Fig. 2D). Analysis of the ability of the original Ankara strain to give rise to the various virus forms showed that it behaved like the COP strain with respect to the formation of both intra- and extracellular enveloped virus (results not shown).

MVA particles are not labeled with antibody against a surface IMV protein but are labeled with antibody against an EEV protein. The surface of IMV is covered by a 14-kDa protein encoded by the A27L ORF (10). In an
attempts to distinguish the MVA surface from the COP surface, purified viral particles from both strains were incubated with antibodies against the A27L protein followed by an anti-species antibody coupled with colloidal gold and silver enhancement. Under these conditions, the COP surface was heavily labeled (Fig. 3A) whereas the MVA surface was mostly unlabeled (Fig. 3B). Where envelope damage was apparent, the MVA surface was labeled (arrows in Fig. 3B) suggesting the presence of an underlying A27L protein. Furthermore, extensive ultrasonic treatment enhanced the degree of labeling with the A27L antibody indicating that this procedure damaged the envelope (not shown). To confirm that the origin of the envelope surrounding MVA particles is the viral envelope found on the IEVs and EEVs we incubated purified viral particles with antibodies against EEVs proteins encoded either by the B5R (11, 12) or the F13L ORFs (13). A relatively strong background labeling was apparent with the anti-B5R serum in both virus samples (Figs. 3C and 3D) which could be due either to some free B5R proteins in the virus stocks or to unrelated antibodies present in the rabbit serum. Despite this background it could be clearly noticed that the antibody against the B5R protein strongly labeled MVA (Fig. 3D) but did not significantly label the COP particles (Figs. 3C). This suggested that the MVA envelope mainly corresponds to the single envelope that surrounds CEV and EEV and not to the double envelope that surrounds IEV since the bulk of the B5R protein in the latter lies between the two envelopes of the IEV (11, 14) and would not be readily reached by an antibody without prior permeabilization. In agreement with this result, antibody against the F13L protein, which is exposed on the outside surface of IEV and on the inside of EEV (14, 15), failed to label the COP strain (Fig. 3E) and the MVA strain (Fig. 3F) but strongly labeled the MVA envelope when it was damaged (Fig. 3G).

MVA is not efficiently neutralized by antibodies against the COP strain. We wondered whether the distinct surface properties of MVA may affect its ability to be neutralized by anti-VV sera. To investigate this, mice were inoculated twice intramuscularly with COP and the sera obtained were tested in a neutralization assay with COP and MVA. As illustrated in Fig. 4, COP was efficiently neutralized by sera taken from the animals over a 60-day period whereas MVA largely resisted neutralization. Furthermore, as expected from the immunolabeling experiment in Fig. 3, MVA was also more resistant to neutralization by an anti-A27L antibody than the COP strain (results not shown).

Discussion. These studies have demonstrated that most of the virus produced during a productive MVA infection is enveloped whether the virus is amplified on CEF or BHK21 cells (not shown). Quantification by CsCl gradient analysis indicated that as much as 74% of MVA was enveloped in contrast to only 22% for the COP strain. The relative proportion of double-enveloped and single-enveloped, cell-associated virus was not precisely determined although the latter appeared to predominate in the purified MVA stocks examined by electron microscopy. It is likely that during purification the outer envelope of the IEVs may be removed to some extent to generate EEV-like particles. Work on other VV strains, such as IHD-J which produces a large amount of EEV, has shown that the enveloped form of VV is very fragile (16). Although some 22% of COP particles were apparently enveloped according to CsCl gradient analysis, enveloped virus was rarely detected after standard purification of the virus. Thus it is particularly surprising that much of the virus produced after purification of MVA remained enveloped despite the harsh treatment associated with the procedure. This suggests that the envelope surrounding MVA may be particularly resistant due to a tighter association with the IMV.

Sequencing of the MVA genome (5) has shown that all of the known genes encoding VV envelope proteins are intact, with the exception of the A36R ORF (MVA 147R) which has two small internal deletions. Thus it is possible that alterations of this protein in the MVA strain may
have some consequence on enveloped virus production and stability. However, a number of other genes encoding proteins of unknown function are deleted or altered in MVA and any one of these or combinations of mutations may be responsible for the particular phenotype of MVA with respect to EEV formation. In any case, during the course of cell culture passaging the original Ankara strain acquired mutations leading to the enveloped phenotype of MVA since Ankara behaved more like the COP strain. One notable feature associated with MVA infection of permissive cells is the reduced cytopathic effect (CPE) compared to the parental Ankara strain as well as the COP strain used in this work. A reduced CPE could account for better preservation of a functional Golgi apparatus required for the generation of IEV and EEV (17). Interestingly, Carroll and Moss (2) also noticed extensive membrane wrapping of immature particles in MVA-infected HeLa cells but found no difference at the ultrastructural level between MVA and its parental Ankara strain in permissive CEF or BHK-21 cells. However, the fact that different laboratories are working with slightly different MVA isolates following cell passaging or cloning cannot be excluded. In this regard it is interesting to note that a recombinant MVA virus obtained from Gerd Sutter in Munich, amplified and purified by us, also contained a majority of enveloped virus.

The production of large amounts of enveloped virus by MVA has a number of consequences. First, as shown in this study, MVA infectivity is poorly neutralized by antibodies against the COP strain and against the A27L protein. The fact that Czerny et al. (18) were able to neutralize virus infectivity and immunolabel MVA with
antibodies against the A27L protein is probably related to their use of virus prepared by precipitation with Frigen 113 (trichlorotrifluoroethane) as well as extensive sonication, procedures which both damage the envelope (16). Our finding that MVA resisted neutralization by mouse antibodies against the COP strain is in agreement with previous data showing that enveloped virus is resistant not only to neutralization by antibodies against envelope proteins in view of targeting infection or raising immune responses against antigens expressed on the EEV surface.

Materials and Methods. Cells and virus. Primary chick embryo fibroblasts (CEF) were established from 11-day-old embryos and grown in Eagle's basal medium supplemented with tryptose phosphate broth and 10% fetal calf serum. BHK21 cells were grown in BHK21 medium (Glasgow MEM) supplemented with tryptose phosphate broth and 10% fetal calf serum. A lyophilized vaccine preparation of MVA (II/85) was obtained from Anton Mayr and diluted in 1 ml of PBS. The virus was plaque purified twice on CEF and plaques were screened by PCR for deletion II (primer 1 OTG7475: cttcttagcaagatgcatattag-gcggatg; primer 2 OTG7476: tggccattaactaactattag-gtgccggtga) and deletion III (primer 1 OTG7473: ggtttgtag-ggatggtgagtcatgcatctgta and primer 2 OTG7474: gaat-gcacactaataagtaccggcatcctagcga). One viral clone designated MVATGN33.1. was chosen for further studies and is referred to hereafter as MVA. A clonal isolate of the VV Copenhagen strain was also used whereas the vaccinia virus Ankara strain (CVA) obtained from Gerd Sutter was employed without prior plaque purification. Titration of virus was performed on CEF under low melting temperature agarose and plaques were stained using neutral red. Virus was purified from infected CEF or BHK21 cells by the standard sucrose gradient procedure. No difference in the rate of migration on sucrose gradients was observed between the MVA and COP strains although the virus band in the former was slightly broader.

Electron microscopy and immunolabeling. For negative staining of purified virus, formvar carbon-coated nickel grids were made hydrophilic by submitting them to a glow discharge. Virus was deposited on the grids for 1 min and the grids were stained for 20 s with 2% uranyl acetate or 2% phosphotungstic acid. For immunolabeling of virus, grids prepared as above were first preincubated with blocking buffer (PBS containing 5% BSA, 0.1% cold water fish skin gelatin, 1% normal goat serum) for 30 min. About 10^7 PFU of purified virus (10 μl) was then deposited on the grids for a minimum of 2 h. The grids were washed several times with PBS containing 0.1% acetylated BSA (PBS–BSA, Aurion, The Netherlands) and antibodies were added at a 1/100 dilution. After 2 to 3 h of incubation the grids were washed twice for 5 min in PBS–BSA and then goat anti-rabbit Fab or goat antimouse Fab, both coupled to ultrasmall colloidal gold particles (Aurion) and used at a 1/40 dilution, was added on the grids for a minimum of 2 h. The grids were then washed twice with PBS–BSA, twice with PBS, and fixed for 10 min with 2.5% glutaraldehyde in PBS. The grids were washed five times with distilled water and the ultrasmall gold particles enhanced with silver for 30 min using the SE-EM kit from Aurion. Finally, the grids were washed with water to stop enhancement and negative staining was performed with 2% uranyl acetate in water and the grids were left to dry. Electron microscopic observations were carried out using a Philips CM120 Biotwin electron microscope at 120 kV. Electron micrographs of gold-labeled particles were underexposed to facilitate visualization of the silver-enhanced gold particles.

Antibodies and neutralization assays. To prepare antibodies against the A27L protein the corresponding cod-
ing region was amplified from MVA genomic DNA by PCR using the primers OTG 12707 (5’-ggggggatcatgggagaacctttc-3’) and OTG12708 (5’-ggggggattcttcattcatgaggcgc-3’). The gene fragment was then inserted into the BamHI and EcoRI sites of the pGEX2T vector (Pharmacia, Uppsala, Sweden) to generate a plasmid designated pTG14367. Expression of the fusion gene GST-A27L was performed in the BL21 strain of Escherichia coli at 20°C for 4 h in the presence of 1 mM IPTG in order to obtain a soluble fusion protein. Purification of native A27L from the GST-fusion was performed after thrombin cleavage according the procedure described by Pharmacia. Mice were immunized intraperitoneally (IP) with 10 μg of A27L in complete Freund’s adjuvant and boosted 14 days later with 10 μg of A27L injected IP in incomplete Freund’s adjuvant. Sera were collected 10 days after the boost and pooled. To prepare sera against the VV Copenhagen strain, 8-week-old C57Bl6 mice (Charles River, Rouen, France) were vaccinated with 10^5 PFU of VV containing predominantly IMV particles. Animals were inoculated twice by the intramuscular route and sera were obtained at days 10, 31, 46, and 60 after the boost. Sera from several mice for each time point after virus inoculation were pooled and tested for neutralizing antibodies.

For neutralization assays, 10^5 PFU of crude virus preparations were diluted in 1 ml PBS containing cations and 1% fetal calf serum. Mouse serum was added at a 1/20 dilution and incubation was performed for 4 h at room temperature. Virus was then titrated on CEF monolayers under low melting agarose and plaques were stained with neutral red.

Density gradient analysis. The buoyant densities of extracellular virus and intracellular virus were determined as described previously (20). Briefly, CEF (about 20 × 10^6 cells) were infected with 0.1 PFU per cell. [3H]Thymidine (10μCi/ml) was added after the first hour and left for 30 h until the end of the infection. At this point the culture medium was removed and centrifuged at 4500 rpm for 10 min and the extracellular virus in the supernatant was recovered. Infected cells were removed from the plates by scraping, resuspended in 10 mM Tris–HCl buffer (pH 8.8) for 10 min, and lysed by dounce homogenization. The nuclei were pelleted by centrifugation at 1500 rpm for 5 min and the supernatants further cleared by centrifugation for 10 min at 4500 rpm. The cell-associated virus in these supernatants as well as the extracellular virus was concentrated by pelleting through a 36% sucrose cushion in a SW 28 Beckman rotor for 2 h at 12000 rpm. The pellets were then resuspended in 1 ml and overlaid onto discontinuous CsCl gradients (2.5 ml at 1.3 g/ml, 3.5 ml at 1.25 g/ml, 4.5 ml at 1.2 g/ml). The gradients were spun for 95 min at 2500 rpm and 20°C in a Beckman SW41 rotor. Gradient fractions were then collected from the bottom of the tubes and refractive indexes were determined. The samples were then precipitated with 5% trichloroacetic acid, the precipitates were recovered onto glass fiber filters (Whatman GF/A), and the radioactivity was counted.

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