Plasma Membrane Budding as an Alternative Release Mechanism of the Extracellular Enveloped Form of Vaccinia Virus from HeLa Cells

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In HeLa cells the assembly of modified vaccinia virus Ankara (MVA), an attenuated vaccinia virus (VV) strain, is blocked. No intracellular mature viruses (IMVs) are made and instead, immature viruses accumulate, some of which undergo condensation and are released from the cell. The condensed particles may undergo wrapping by membranes of the trans-Golgi network and fusion with the plasma membrane prior to their release (M. W. Carroll and B. Moss, *Virology* 238:198–211, 1997). The present study shows by electron microscopy (EM), however, that the dense particles made in HeLa cells are also released by a budding process at the plasma membrane. By labeling the plasma membrane with antibodies to B5R, a membrane protein of the extracellular enveloped virus, we show that budding occurs at sites that concentrate this protein. EM quantitation revealed that the cell surface around a budding profile was as strongly labeled with anti-B5R antibody as were the extracellular particles, whereas the remainder of the plasma membrane was significantly less labeled. To test whether budding was a characteristic of MVA infection, HeLa cells were infected with the replication competent VV strains Western Reserve strain (WR) and International Health Department strain-J (IHD-J) and also prepared for EM. EM analyses, surprisingly, revealed for both virus strains IMVs that evidently budded at the cell surface at sites that were significantly labeled with anti-B5R. EM also indicated that budding of MVA dense particles was more efficient than budding of IMVs from WR- or IHD-J-infected cells. This was confirmed by semipurifying [^{35}S]methionine-labeled dense particles or extracellular enveloped virus (EEVs) from the culture supernatant of MVA- or IHD-J-infected HeLa cells, respectively, showing that threefold more labeled dense particles were secreted than EEVs. Finally, although the released MVA dense particles contain some DNA, they are not infectious, as assessed by plaque assays.

Vaccinia virus (VV) is the prototype member of the *Poxviridae*, a family of large DNA viruses. The VV life cycle is characterized by distinct steps that occur in the cytoplasm of the infected host cell. These steps include entry, early transcription, DNA replication, and virion assembly (14). Assembly is initiated by the formation of typical crescent-shaped membranes containing viral membrane proteins. The crescents develop into the immature virus (IV), a spherical particle that encloses an electron-dense inner part containing the viral core proteins. Upon DNA uptake the IVs mature into the first infectious form of the virus: the intracellular mature virus (IMV). This process is accompanied by the cleavage of several core proteins, such as the gene products of A3L and A10L, as well as the formation of a morphological and biochemical distinct brick-shaped core. The IMVs are generally thought to remain intracellularly and to leave the cell only upon cell lysis (see below). A certain percentage of the IMVs acquire a double membrane derived from the trans-Golgi network (TGN) to form the intracellular enveloped virus (IEV). The latter is released by active transport along microtubules and fusion with the plasma membrane. Upon fusion the IEV loses the outermost of its membranes at the cell surface and forms the extracellular enveloped virus (EEV). EEVs may be released in the

extracellular medium or remain attached to the plasma membrane as cell-associated enveloped virus (CEV). The outer IEV membrane that fuses with the plasma membrane upon EEV or CEV formation can induce the formation of actin tails at the cell surface. These tails form long plasma membrane-derived filopodia with a CEV attached to their tip, a process that is thought to promote cell-to-cell spread of the virus (reviewed in reference 15).

The extent of EEV production is known to depend on the cell type and virus strain (16, 17). HeLa cells, for instance, produce little EEV, whereas RK-13 cells efficiently release the latter viral form. Furthermore, the IHD-J strain of VV is more efficient in EEV production than is WR (16, 17). Studies conducted about 20 years ago suggested that in FL cells a subset of the IMVs can bypass the TGN-wrapping process and are instead released from the cell by budding at the plasma membrane (27, 28). Such a mechanism of virus release may also be the major way in which the avipox fowlpox virus exits infected chicken embryo fibroblasts (CEFs) (3).

Modified VV Ankara (MVA) was derived from VV strain Ankara. It was attenuated by more than 570 passages on CEFs and has lost its ability to grow efficiently on most mammalian cells (4, 7). Compared to VV, strain Copenhagen, about one-third of the MVA genes are deleted or inactive, genes that encode in particular for host range and immune evasion factors and are located on the left- and right-hand sides of the genome (1). When tested as a putative vaccine against smallpox, the

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MVA caused none of the side effects observed with replication-competent strains while eliciting an immunoresponse (12, 24). More recently, MVA has become an important candidate for use as a live vaccine against other pathogens and in the treatment of certain forms of cancer (reviewed in reference 10).

MVA grows efficiently only in CEFs and BHK cells. The stage at which its life cycle is interrupted in other mammalian cells depends on the cell type. In HeLa cells, late protein synthesis occurs, and IVs have been shown to accumulate (25). In a recent study, we characterized the assembly block of MVA in HeLa cells in detail and compared MVA to the replication-competent VV strain WR (20). The early stages of MVA infection, entry, and DNA replication were entirely comparable to those of WR in these cells. The main difference occurred at ca. 5 h postinfection; whereas under permissive conditions the major IMV membrane protein p16 (A14L) was efficiently targeted to the DNA replication sites at this time of infection, in MVA-infected cells this targeting was inefficient. Moreover, IVs that normally colocalize with the replication sites did not colocalize to the same extent with the viral DNA upon MVA infection and consequently fewer IVs acquired a viral genome, as quantified by electron microscopy (EM). Whereas under permissive conditions 30 to 35% of all IVs contained a nucleoid at 8 h postinfection, this percentage was only 5% in MVA-infected HeLa cells (20). In a study by Carroll and Moss (4), it was also observed that at later times of infection a subset of the IVs that accumulated in HeLa cells became more electron dense, and these particles were apparently able to leave the cell (4).

In the present study, MVA assembly in HeLa cells late in infection and, in particular, the release of these dense particles was studied in detail. The data show that their release is more efficient than EEV release upon IHD-J or WR infection in HeLa cells. Although a subset of these particles can take up the viral genome, we show that they are not infectious. Surprisingly, although some of these secreted particles undergo TGN wrapping prior to their release, most may be secreted via a budding process at the plasma membrane. Upon comparing MVA infection to infection with the replication-competent strains WR and IHD-J, we found that in HeLa cells these two viruses also release EEVs via the budding of IMVs at the plasma membrane.

MATERIALS AND METHODS

Cells, viruses, and antibodies. HeLa (CCL-3), BSC-40, and BHK cells were prepared and grown as described previously (23). CEFs were prepared and grown as described by Meiser et al. (13). Semipurified stocks of the WR and IHD-J strains of VV were prepared from infected HeLa cells as described previously (19). MVA, clone F6, kindly obtained from Gerd Sutter (11), was grown on BHK cells and semipurified as described for WR and IHD-J. Virus stocks were titrated on BSC-40 (WR and IHD-J) or BHK (MVA) as described previously (13). The rat monoclonal antibody to B5R was a kind gift of Gerhard Hiller (21). Rabbit anti-rat was from Cappel (Organon Teknika Corp., Durham, N.C.).

EM and preembedding labeling. HeLa cells grown in 6-cm dishes were infected at a multiplicity of infection (MOI) of 10 and fixed at the indicated times postinfection (16 to 24 h postinfection). For Epon embedding, cells were fixed with 1% glutaraldehyde in 200 mM cacodylic acid (pH 7.4) and embedded into Epon as described previously (9). For cryosections, cells were fixed and prepared as described by van der Meer et al. (30). For preembedding labeling, infected cells were fixed at the indicated time postinfection by adding an equal volume of

8% paraformaldehyde in PHEM buffer straight to the medium. After 10 min at room temperature, the fixing mixture was removed, and the cells were extensively washed with phosphate-buffered saline (PBS)–10 mM glycine. The cells were then blocked with 5% fetal calf serum–10 mM glycine in PBS, followed by incubation in the blocking solution for 2 h at 4°C with anti-B5R antibody. After an extensive washing, the cells were incubated with rabbit anti-rat antibody (Cappel), followed by the addition of protein A-gold. After another extensive washing, the cells were postfixed with 1% glutaraldehyde in PBS and then embedded into Epon.

Stereology and quantitation of different viral profiles. Quantification of the different viral forms was done as described previously (13). Briefly, after overnight infection the cells were fixed and embedded in Epon. IVs, IMVs, IEVs, and CEVs were counted in 50 cell profiles of sections of infected CEFs and HeLa cells. For the quantification of the B5R labeling, random pictures were taken of cells after preembedding labeling. Gold particles were counted on 30 randomly chosen budding events, extracellular virus particles, and on the plasma membrane. The number of gold particles/membrane length in microns was estimated by relating them to the number of intersections of a series of test lines, as described previously (9).

Metabolic labeling. HeLa cells were infected at an MOI of 10 with IHD-J and MVA. At 6 h postinfection, the medium was replaced by a one-to-one mixture of Dulbecco modified Eagle medium–5% fetal calf serum and methionine-free minimal essential medium (Sigma) containing 10 μ Ci of [35 S]methionine and of [3 H]thymidine (both from Amersham)/ml. At 24 h postinfection, the culture supernatant was harvested and then spun through a 36% sucrose cushion in 10 mM Tris-Cl (pH 9) for 30 min at 150,000 $\times$ g. The pellet was resuspended and partly precipitated with trichloroacetic acid before liquid scintillation counting. The pelleted material was separated on a 15% polyacrylamide gel that was prepared for autoradiography and then exposed.

RESULTS

Upon overnight infection, MVA-infected HeLa cells secreted dense immature particles. Our previous study on MVA in HeLa cells was performed at between 0 and 8 h postinfection (20). MVA assembly was known to be aborted in HeLa cells at a late stage of infection, but the goal of that study was to investigate whether any of the stages of the VV life cycle preceding virion assembly were also affected and if so to what extent. We found, however, that compared to the replication-competent VV strain WR the processes of entry and DNA replication were both quantitatively and qualitatively the same. The main difference occurred at ca. 5 h postinfection, one of the earliest time points at which viral late membrane proteins can be detected by immunofluorescence when an MOI of 10 is used. While at this time point viral late membrane proteins clearly colocalized with the replication sites in WR infection, this putative targeting of viral membrane precursors to the viral DNA sites was inefficient in MVA. Accordingly, IVs accumulated mostly outside the replication sites, and no IMVs were seen at 8 h postinfection (20).

Since some of these IVs did acquire a nucleoid, we now sought to determine whether the failure to detect IMVs at 8 h postinfection was simply due to a delay in virion assembly. HeLa cells were therefore infected at an MOI of 10, fixed at 16 h postinfection, and prepared for thin-section EM. We observed, in sections of Epon-embedded samples, many IVs and spherical particles that were more dense and appeared to be slightly smaller than IVs, but we noted no IMVs (Fig. 1A). The dense spherical particles were apparently able to leave the cell since they were also observed at the cell surface (Fig. 1B), where some of these were attached to a distinct membrane stalk (Fig. 1D and 2E). Intracellularly, the dense particles were seen to undergo a wrapping process close to the Golgi complex (Fig. 2A to C). This apparent TGN wrapping process was also

FIG. 1. Upon overnight infection of HeLa cells with MVA, dense spherical particles are produced. In panels A through D cells were infected with MVA at an MOI of 10 and then fixed at 16 h postinfection, and samples were prepared for Epon embedding. Panel A is an overview image showing the accumulation of many IVs (small arrowheads) and electron-dense spherical particles (large arrowheads). Panel B shows that some of these dense particles (small arrowheads) can also be found at the extracellular side of the plasma membrane. Panel C shows a dense particle beneath the cell surface, and panel D shows a dense particle attached to the plasma membrane via a membrane stalk (arrow). PM, plasma membrane. Bars: A, 1 μ m; B to D, 200 nm.

FIG. 2. Dense particles undergo TGN wrapping and are released from the cell. HeLa cells were infected as described in Fig. 1 and either embedded in Epon (A and B) or prepared for cryosectioning and labeled with anti-B5R (C to F). Panels A and B show that dense particles may undergo wrapping by membranes (large arrowheads) that locate close to the Golgi complex ("G" in panel A). Panel C shows a Golgi complex (G) that is labeled on one side with anti-B5R (small arrows). Dense particles undergo wrapping (large arrowheads) close to the Golgi with membranes that are also labeled with anti-B5R (small arrows; 10-nm gold). Panels D to F show a panel of extracellular dense particles that are abundantly labeled with anti-B5R on their surface. In panel E the extracellular particle is attached to the plasma membrane (PM) via a membrane stalk. Bars, 200 nm.

seen in cryosections labeled with an antibody to B5R, an EEV-specific membrane protein. Dense particles underwent wrapping by membranes that labeled positive for B5R close to one side of the Golgi complex, presumably the TGN (Fig. 2C). Importantly, all extracellular particles were abundantly labeled on their surface with B5R (Fig. 2D and F), indicating that only particles that had acquired EEV-specific membranes were released. In cryosections we again frequently observed particles that were attached to the plasma membrane via some membrane stalk (Fig. 2E).

Thus, in HeLa cells infected with MVA, no IMVs could be detected at later times postinfection. Instead, a subset of the IVs that accumulated became more electron dense, became wrapped by TGN membranes, and were released from the cell, a finding consistent with the results of Carroll and Moss (4).

Secretion of the dense particles also occurs via a budding process at the plasma membrane. In addition to TGN wrapping and fusion of MVA dense particles, many EM images also showed these particles beneath the intracellular side of the plasma membrane in an apparent process of budding at the cell surface (Fig. 3A and C). Budding of IMVs at the plasma membrane has been described before in FL cells (27, 28), and we therefore decided to study this process in more detail. In analogy to the budding of some enveloped viruses that exit cells at the cell surface, we suggested that a putative budding event occurred at specialized sites of the plasma membrane that concentrate EEV membrane proteins (since all extracellular particles were B5R positive [8]). This was tested and quantified by a preembedding labeling technique; the cell surface of cells fixed after overnight infection, was labeled with antibodies to B5R before they were embedded into Epon. We expected the budding profiles to be significantly more labeled with anti-B5R than the rest of plasma membrane.

Sections of such preembedded samples showed extracellular dense particles that were significantly labeled with anti-B5R on their surface (Fig. 3B). Furthermore, many intracellular particles were readily observed that were apparently leaving the cell at the plasma membrane via a budding process (Fig. 3A, C, and D). Importantly, the cell surface surrounding these budding particles was significantly labeled with anti-B5R. A stereological approach was then used to quantify the anti-B5R labeling. For this, the anti-B5R labeling density of extracellular particles and of the plasma membrane around budding particles, as well as of other parts the plasma membrane, was estimated. If particles were released by budding, we expected the labeling density per unit length of membrane to be the same in the extracellular particles and in the budding profiles but significantly lower in random pieces of the cell surface compared to budding profiles. The quantification revealed, as expected, that the B5R labeling densities were similar on extracellular particles and on the plasma membrane around the budding profiles (Fig. 4A). Importantly, the labeling density of other parts of the plasma membrane not involved in a budding event was ~6-fold lower than for both the budding profiles and the extracellular particles.

These data show, consistent with our prediction, that the EEV-specific membrane protein B5R was concentrated at the plasma membrane around the budding profiles. These results therefore strongly suggested that a subset of the dense MVA

particles were leaving the cell via budding at the plasma membrane.

The WR and IHD-J strains of VV may also be released by budding at the plasma membrane in HeLa cells. Having shown that some of the dense MVA particles were released from HeLa cells via budding at the plasma membrane, we wondered whether this was MVA or cell type specific. It is well established that HeLa cells infected with the common laboratory strain WR or IHD-J produce very little EEV (16). Although not studied in detail, we expected that in these cells the release of EEV was mediated by TGN wrapping of the IMV and fusion of the IEV with the plasma membrane.

To study the mechanism of EEV release in HeLa cells in more detail, cells infected with IHD-J and WR were subjected to the same preembedding technique as described above for MVA. For both WR and IHD-J the most obvious and surprising observation made in such sections was the accumulation of IMVs beneath the cell surface that were apparently leaving the cell via budding at the plasma membrane (Fig. 5). In addition, very few IMV particles were seen to undergo TGN wrapping (see below). More detailed EM analyses revealed, however, that the putative IMV budding profiles seen in WR- and IHD-J-infected HeLa cells differed from the profiles observed upon infection with MVA in a number of subtle ways. First, budding profiles were rather prominently seen upon MVA infection in almost every infected cell. Most WR- or IHD-J-infected cell profiles, however, revealed no IMVs beneath the plasma membrane (or extracellular CEVs or EEVs) at all, whereas some profiles showed many putative budding particles. Apparently budding was a relatively active process in some cells, while in others budding did not occur. Second, in MVA-infected cells the budding profiles were often seen inside a plasma membrane bud that extended away from the cell surface, as expected of budding (see for examples Fig. 3A, C, and D). Although some exceptions were seen (see Fig. 5B and E) in WR and IHD-J, the IMVs often appeared to accumulate beneath the cell surface without obviously being inside a bud (Fig. 5A, C, and D). Instead, the IMVs that accumulated at the cell surface were mostly seen to lie under a plasma membrane patch that appeared slightly elevated (Fig. 5A to D). Third, as shown above, in MVA the labeling densities over budding sites and the extracellular particles was the same (Fig. 4A). In WR- and IHD-J-infected cells the same quantitation revealed that the labeling density at a budding site was ~3 times higher than over the rest of the cell surface but ~2-fold lower than in CEVs and EEVs (Fig. 4A). Finally, the B5R labeling at the plasma membrane of both WR- or IHD-J-infected cells was lower overall than in MVA-infected HeLa cells. In random pieces of plasma membrane the average labeling density was ~2-fold lower and in the budding profiles the density was ~3-fold lower versus MVA (Fig. 4A). This suggested that this EEV membrane protein was not as efficiently targeted to the plasma membrane upon WR and IHD-J infection compared to MVA (see Discussion).

Thus, upon WR and IHD-J infection, IMVs were apparently also leaving the cell via budding. The data also showed that the budding profiles seen upon WR and IHD-J infection were very similar, while those seen upon MVA infection were different from both WR and IHD-J. Moreover, these differences collectively suggested to us that perhaps some of the WR or IHD-J

FIG. 3. Dense particles may also be released via budding at the plasma membrane. In all images HeLa cells were infected as in Fig. 1, except for panel F, which is from an infection with WR. After infection the cells were fixed, and the cell monolayer was labeled with anti-B5R, rabbit anti-rat antibody, and 10-nm protein A-gold. The cells were then postfixed with 1% glutaraldehyde and embedded in Epon. Panels A, C, and D show dense particles that are in the process of leaving the cell via budding at the plasma membrane (large arrows). The plasma membrane (P) around the buds is significantly labeled with anti-B5R (small arrows). Panel B shows extracellular particles (large arrowheads) that are abundantly labeled with anti-B5R (small arrows). Panels E and F show putative fusion profiles of MVA (E) and WR (F). The images show a dense particle in panel E or a CEV in panel F that lies in a plasma membrane (P) cavity that has a different electron density (small arrowheads) than the surrounding cell surface. Bars, 200 nm.

IMVs were not in the process of budding at all or that the putative budding process was less efficient than upon MVA infection (see Discussion and below).

In HeLa cells virions may be released predominantly via budding, while in CEFs TGN wrapping and fusion predomi-

nates. In the next set of experiments we sought to quantify budding versus fusion events (this is TGN wrapping followed by fusion) at the plasma membrane of infected HeLa cells. Moreover, we compared our observations made in HeLa cells to infected CEFs. We decided to use CEFs for comparison

FIG. 4. Quantification of B5R labeling over budding profiles, extracellular particles, and the plasma membrane; the production of the different viral forms in HeLa cells and CEFs; and the quantification of budding and fusion events. (A) HeLa cells were infected with WR, IHD-J, and MVA, and the cells were fixed, labeled with anti-B5R, and embedded as described for Fig. 3. The gold particles over extracellular particles ("free" particles), the cell surface around budding particles, and random pieces of plasma membrane were counted for 30 randomly chosen electron micrographs showing budding profiles and extracellular particles. The values are expressed as number of gold particles per micron of membrane and represent the average and standard deviations. (B) HeLa cells or CEFs were infected at an MOI of 10 and fixed at 16 h postinfection. The total amount of IVs (white bars), IMVs (dense particles in HeLa cells infected with MVA; black bars), IEVs or TGN wrapping profiles (dark gray bars), and CEVs (dense extracellular particles near HeLa cells infected with MVA; light gray bars) were counted in 50 profiles of infected cells and are expressed as a percentage of the total particles counted. (C) HeLa cells were infected and embedded as described for panel A with MVA (black bars), WR (gray bars), and IHD-J (white bars). Fusion and budding profiles, defined as

because this is one of the two cell lines that are permissive for MVA infection. Furthermore, we recently made a detailed comparison of the formation of the TGN-wrapped forms in CEFs infected with MVA, WR, and IHD-J (13). In that study we did not observe the budding profiles we observed here in HeLa cells.

We first quantified and compared by EM the extent of wrapping and IEV and CEV formation in CEFs and HeLa cells infected with the three virus strains. Cells were infected overnight at an MOI of 10, fixed, and embedded in Epon. We have previously shown that in such sections IMVs, IEVs, and CEVs can readily be distinguished based on their morphology, in particular the number of membranes surrounding the core (13). The numbers of IVs, IMVs, IEVs, TGN-wrapping events, and CEVs were subsequently counted in 50 cell profiles of HeLa cells or CEFs infected with the three virus strains.

The data showed that in HeLa cells the bulk of the particles were IMVs or IVs (WR and IHD-J) or IVs or dense particles (MVA), and very few IEVs or CEVs were counted (Fig. 4B). This finding was in contrast to that noted with CEFs, in which all three virus strains underwent significant amounts of TGN wrapping and IEV and CEV formation (Fig. 4B). In HeLa cells, MVA resulted in the highest percentage of extracellular particles at the plasma membrane, suggesting that this virus was most efficient in the secretion of extracellular virions (see also below).

In a next set of experiments we sought to quantify budding versus fusion. We first reexamined Epon sections of infected CEFs. Neither budding profiles similar to those seen in HeLa cells nor IMVs that accumulated beneath the cell surface were seen with any of the three viruses, a finding consistent with our previous results (13; results not shown). Similar results were obtained in RK-13 cells infected with WR and IHD-J (in RK-13 cells MVA fails to synthesize late proteins, and consequently no IVs and IMVs are made [26]), indicating that in these two cell lines budding may not occur or may be a very rare event (not shown).

In HeLa cells the budding events were rather obvious because IMVs or dense particles were readily observed beneath the plasma membrane in a process of leaving the cell at sites that were significantly labeled with anti-B5R (for examples, see Fig. 3A, C, and D and Fig. 5). Fusion was, however, much less obvious, in particular because with all three viruses IEVs or TGN-wrapped dense particles were never observed to accumulate beneath the plasma membrane in an apparent process of fusion. Based on recent observations made in infected CEFs, we therefore decided to define fusion in the following way. In CEFs infected with MVA, WR, and IHD-J, CEVs tended to lie in a small plasma membrane cavity. Moreover, the cell surface underneath such a CEV showed a different electron density compared to the rest of the cytoplasm (for

described in the text, at the plasma membrane were counted in 30 randomly chosen electron micrographs and are expressed as events per micron of plasma membrane. Note that the standard deviations are high. This reflects the fact (as described in Results) that in some cells budding is very abundant, whereas in other cells no budding occurs at all. Also note that fusion is barely detectable upon IHD-J infection, which probably reflects the way fusion was defined (see the text).

FIG. 5. IHD-J and WR are also released via budding at the plasma membrane in HeLa cells. HeLa cells were infected at an MOI of 10 with either WR (A, B, and F) or IHD-J (C to E) and then fixed at 16 h postinfection. The cell surface was labeled with anti-B5R as described in Fig. 3, and cells were embedded in Epon. Panels A and B show the plasma membrane (P) with a collection of IMVs (large arrows) in the process of budding. The sites of budding are labeled with anti-B5R (small arrows). Panels C through E show similar images with IHD-J. Large arrows indicate IMVs that are budding at sites labeled with anti-B5R (small arrows). Although most putative budding profiles lie beneath the cell surface that is slightly elevated, some IMVs appear to be in a "real" plasma membrane bud (B and F) similar to those seen upon MVA infection (see Fig. 3A, C, and D). Panel F shows an EEV that has just left the cell and is attached to a membrane stalk. The surface of this particle is abundantly labeled with anti-B5R. Bars, 200 nm.

example, see Fig. 3E and F), and we have proposed this membrane to represent the outermost portion of the IEV membrane that fuses with the plasma membrane upon virus release (13). Such profiles were also seen in HeLa cells, which we defined as fusion profiles (see also the Discussion). Import-

tantly, based on this definition, fusion was readily distinguishable from budding profiles. This was true in particular because the "fused" CEVs were lying in a plasma membrane cavity, whereas budding profiles were budding out and extending away from the cell surface.

Using these criteria for budding and fusion, we then estimated by EM the budding and fusion events per unit length of membrane. As expected from the EM images described above, budding was especially prominent upon MVA infection, whereas the lowest number of budding profiles were counted upon WR infection (Fig. 4C). Upon WR infection the number of fusion profiles almost equaled the number of budding events, whereas in MVA threefold-more budding than fusion was observed. Fusion was a particularly rare event upon IHD-J infection, and almost all of the virions at the plasma membrane seemed to be engaged in budding with this virus strain (see Discussion).

Thus, considering the limitation of defining fusion (see Discussion), it seemed that all three virus strains preferentially, but not exclusively, released extracellular particles via budding. This was also consistent with the fact that, compared to CEFs, IMVs or dense particles underwent relatively little TGN wrapping and only a few IEVs were formed.

MVA particle release may be more efficient than EEV release by IHD-J. The combined EM results suggested that MVA was most efficient in the release of dense particles via budding compared to the release of EEV upon WR or IHD-J infection. To confirm this, the release of dense MVA particles was compared to the release of EEV upon IHD-J infection by metabolic labeling. MVA- or IHD-J-infected cells were labeled with both [35 S]methionine and [3 H]thymidine to label viral proteins and DNA, respectively. The culture supernatant was collected at 24 h postinfection and pelleted through a 36% sucrose cushion, and the radioactivity was determined. Threefold-higher 35 S counts were obtained from the culture supernatant of MVA-infected cells, suggesting that threefold more MVA particles were being released than IHD-J and EEVs (Fig. 6A). By analyzing the protein pattern contained in the pelleted fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, a number of viral proteins were readily discernible (Fig. 6B). The most prominent bands in the IHD-J pellet was a doublet of 65 kDa of the cleaved forms of A3L and A10L, the two major core proteins. In the MVA pellet these same proteins were apparent, but both were in their uncleaved precursor form, a finding consistent with the fact that the dense particles lacked a typical brick-shaped core structure (see the introduction). Negative staining EM showed that the pellets contained B5R-positive EEVs in the case of IHD-J or round particles labeled on their surface with the same antibody in the case of MVA (results not shown).

The total thymidine-labeled counts was the same for both the MVA particles and the IHD-J EEVs. Since about threefold more 35 S-labeled particles were secreted than EEVs, it appeared that the MVA particles were threefold less labeled with [3 H]thymidine (Fig. 6A). At present we do not know whether these results imply that all particles acquire some DNA or whether only a subset of the particles contain DNA. Our previous EM images suggest the latter option, since such images showed that at 8 h postinfection sixfold fewer IVs had acquired a nucleoid compared to permissive VV infection conditions (see the introduction above) (20).

The released dense particles are not infectious. Since the dense particles did acquire some DNA, an obvious question was whether they were infectious. To address this question,

FIG. 6. The secretion of dense particles may be more efficient than the secretion of EEV. (A) HeLa cells were infected at an MOI of 10 with IHD-J or MVA and metabolically labeled from 6 to 24 h postinfection with [35 S]methionine (35S) and [3 H]thymidine (3H). The culture supernatant was harvested and spun through a 36% sucrose cushion, and the pellets were resuspended in an equal volume. The counts per minute contained in the pellet were determined by liquid scintillation counting. The values are the average and standard deviations of triplicate samples. (B) Similar counts from pelleted material obtained as described for panel A were run on a 15% polyacrylamide gel, and the gel was processed for autoradiography. The asterisks indicate the positions of the core proteins A3L and A10L. In the case of IHD-J, these proteins run as a doublet of the cleaved forms of 65 kDa. In MVA the uncleaved precursor forms are seen. On the right, the position of the molecular weight marker proteins (M) is indicated.

monolayers of HeLa cells were infected with MVA, WR, or IHD-J, and the culture supernatant and cells were harvested at 0, 12, 24, and 48 h postinfection; the samples were then titrated by plaque assay. Figure 7 shows that upon WR and IHD-J infection both the intracellular and extracellular titers increased over time. No such increase in infectivity was measured upon MVA infection, indicating that the particles are not infectious.

DISCUSSION

The present study investigates the secretion of dense particles made upon MVA infection in HeLa cells in greater detail. Since these particles are not infectious, they may represent interesting vehicles for the secretion of antigens exposed on their surface to elicit a humoral immune response. Upon EM

FIG. 7. The dense particles are not infectious. HeLa cells were infected at an MOI of 10 with MVA (◆), WR (■), and IHD-J (▲). The culture supernatant (A) and the cells (B) were harvested at the indicated times in hours postinfection (hpi), and the infectivity (i.e., the titer in PFU/milliliter) was determined by plaque assay. The values are the averages of triplicate samples titrated in duplicate.

analyses we found that a substantial subset of these particles were apparently released via budding rather than the more common TGN wrapping and fusion processes. Because this could be specific for MVA, we then extended our analyses to the replication-competent strains WR and IHD-J and found that these strains also secreted EEVs via budding.

The IMV budding profiles are different from the budding of the MVA dense particles. The budding profiles of the dense MVA particles seen by EM were rather striking; dense particles could be seen beneath the cell surface in obvious buds. The labeling density of the EEV membrane protein B5R was as high over the budding profiles as over extracellular particles, providing strong support for the idea that the budding process was driven by the concentration of EEV proteins at the plasma membrane, in analogy to the budding of other enveloped viruses. The IMV budding profiles seen upon WR and IHD-J infection revealed a number of striking differences compared to the MVA dense particle budding. The IMVs were often not obviously inside a plasma membrane bud, and the labeling density of B5R in the WR and IHD-J buds was significantly lower than in the EEVs and CEVs. These observations together may suggest that some of the IMVs of putative budding

profiles seen upon WR and IHD-J infection perhaps collect beneath the cell surface but are actually not in the process of leaving the cell. The budding process seemed to require the same EEV proteins as are involved in the TGN wrapping process, as the concentration of the EEV-specific B5R membrane protein around the budding profiles suggested. We also found that, in addition to B5R, the peripheral membrane protein F13L, a protein known to be essential for TGN wrapping, localized to the budding sites at the plasma membrane (not shown). Consequently, it can be expected that drugs that inhibit TGN wrapping, such as *N*₁-isonicotinoyl-*N*₂-3-methyl-4-chlorobenzoylhydrazine (IMCBH) and brefeldin A (BFA) also inhibit the budding process. IMCBH that targets the F13L protein (18, 22) could simply block this same protein at the plasma membrane rather than at the TGN and, as a consequence, block budding. BFA, a drug known to target the exchange factor of the small GTPase ARF (6), blocks the exit of proteins from the endoplasmic reticulum to the Golgi complex (5) and thus prevents cellular and viral membrane proteins from reaching the TGN and the plasma membrane. Failure of EEV membranes proteins to reach the TGN in the presence of BFA blocks TGN wrapping (29) and consequently can be expected to block budding at the plasma membrane as well. Thus, together with the notion that EEV formation is a very inefficient process in HeLa cells (16), these drugs did not allow us to discriminate, biochemically or by EM, whether and to what extent EEVs are released via budding rather than fusion from infected HeLa cells (data not shown).

Another important distinction between MVA and WR/IHD-J was that, in MVA infection, approximately two- to threefold more B5R protein accumulated at the plasma membrane. Comparison of the sequence of the EEV proteins of MVA to both WR and IHD-J revealed a number of differences consisting of single point mutations and in-frame deletions of short sequences (1). These differences could result in an efficient targeting of EEV membrane proteins to the plasma membrane or to a decreased internalization from the cell surface. Decreased internalization and/or increased targeting could lead to more of the B5R protein (as well as other EEV membrane proteins) at the cell surface and result in a more efficient budding of MVA particles compared to both WR and IHD-J.

Budding and TGN wrapping are processes that coexist. The study by Tsutsui (27) was the first to describe the budding of IMVs at the plasma membrane in FL cells. These cells of human origin were thought to be derived from normal amnion. However, the FL clone (CCL-62) available from the American Type Culture Collection (ATCC [http://www.atcc.org]) may be contaminated with HeLa cells. It is not clear, however, whether the FL cells used approximately 20 years ago by Tsutsui and colleagues correspond to the CCL-62 FL clone that can be purchased from the ATCC, and thus whether their observations were based on FL cell or on HeLa cell contaminants. In that study it was noted that viruses were also released via a second process, one the author called "reversed phagocytosis" and which most likely represents the fusion of IEVs with the plasma membrane. Interestingly, these profiles described by Tsutsui resembled in two important ways the profiles that we propose here to define as fusion: CEVs released after fusion tended to lie in a small plasma membrane cavity, while the

plasma membrane of that cavity showed a different electron density compared to the rest of the cell surface (13, 27). It thus seems that whereas in cells such as FL and HeLa cells a significant subset of the IMVs may be released via budding at the plasma membrane, EEVs are also released via the more common TGN wrapping and fusion process and that two mechanisms may therefore exist in parallel in these cells. It is conceivable that the opposite is true as well and that in cells that obviously release the bulk of their EEVs via wrapping and fusion some may exit cells via budding. The fact that we did not observe any budding profile in infected CEFs and RK-13 cells does not necessarily contradict this assumption. It could simply indicate that in these cells budding is a particularly rare event.

Upon quantifying events at the plasma membrane, fusion was less frequently seen than budding in HeLa cells, in particular with MVA, indicating that the latter process may predominate. This quantification should, however, be interpreted with caution. Fusion events could simply be seen less often than budding. Fusion could be considerably faster than budding, and the release of CEVs from the plasma membrane that have left the cell via fusion could be very efficient. The latter notion is consistent with the fact that upon infection with IHD-J, a VV strain in which CEVs are known to be efficiently released from the plasma membrane (2), few such fusion profiles were observed. That fusion may be a fast process is also consistent with the fact that we never saw IEVs beneath the plasma membrane, whereas IMVs were readily observed to accumulate at this site. We nevertheless speculate that in HeLa cells budding is the process that predominates, particularly in the release of the dense MVA particles. This assumption is consistent with the fact that IMVs may undergo very little TGN wrapping and IEV formation in HeLa cells compared to, for instance, CEFs.

A question that arises from the present study is whether EEVs released from infected cells via budding or fusion are the same. Our study shows that the EEVs released via budding contain at least two EEV-specific proteins: the gene products of B5R and F13L. We consider it therefore likely that they contain all other proteins known to be required for IEV or EEV formation. Since the lipid composition of the plasma membrane may be different from the TGN, budding of EEVs at the cell surface could result in particles with different lipids. The impact of such a difference on, for instance, the infectivity of plasma membrane-derived EEVs remains to be investigated. Another important difference of particle budding at the plasma membrane is that such particles may not be able to form actin-tipped filopodia at the cell surface, since the proteins and membrane (the outer IEV membrane and associated proteins; see the introduction) may not be available for the formation of such actin tails. The failure to form actin tails could have implications for the efficiency with which EEVs that bud from the plasma membrane are able to spread from cell to cell (see introduction).

Budding in HeLa cells: implication for the transport of IMVs? It is not clear why EEVs in some cells are released predominantly via TGN wrapping and plasma membrane fusion, whereas in other cell budding may predominate. Perhaps budding predominates in cells in which the Golgi complex is particularly small and thus few TGN membranes are available or in cells in which EEV proteins accumulate preferentially at the plasma membrane and to a lesser extent in the TGN. In the

present study we did not investigate either of these two options. What argues against cell type-dependent differences are observations made by Boulanger et al. (3) in CEFs. We found that in CEFs infected with MVA, WR, and IHD-J, EEVs are predominantly released via wrapping and fusion and that budding profiles were not observed (13). Instead, the avipox fowlpox virus seems to release its EEVs from these same cells predominantly via budding, suggesting that the preferred mechanism of EEV release may also depend on the virus strain.

EEV release is considered to be an active process; TGN-wrapped IEVs rapidly move along microtubules in a kinesin-dependent fashion toward the cell's periphery where fusion occurs. CEVs are then pushed on the tip of a cell surface filopodia that actively polymerizes actin tails (15). Some of these studies are, in fact, based on infected HeLa cells. We consider it likely that the transport of IMVs or the MVA dense particles to the cell surface is also microtubule mediated. Further work is, however, required to answer these questions in detail.

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