Origin of Unenveloped Capsids in the Cytoplasm of Cells Infected with Herpes Simplex Virus 1

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In cells infected with herpes simplex viruses the capsids acquire an envelope at the nuclear membrane and are usually found in the cytoplasm in structures bound by membranes. Infected cells also accumulate unenveloped capsids alone or juxtaposed to cytoplasmic membranes. The juxtaposed capsids have been variously interpreted as either undergoing terminal deenvelopment resulting from fusion of the envelope with the membrane of the cytoplasmic vesicles or undergoing sequential envelopment and deenvelopment as capsids transit the cytoplasm into the extracellular space. Recent reports have shown that (i) wild-type virus attaches to but does not penetrate cells expressing glycoprotein D (G. Campadelli-Fiume, M. Arsenakis, F. Farabegoli, and B. Roizman, *J. Virol.* 62:159–167, 1988) and that (ii) a mutation in glycoprotein D enables the mutant virus to productively infect cells expressing the wild-type glycoprotein (G. Campadelli-Fiume, S. Qi, E. Avitabile, L. Foà-Tomasi, R. Brandimarti, and B. Roizman, *J. Virol.* 64:6070–6079, 1990). If the unenveloped capsids in the cytoplasm result from fusion of the cytoplasmic membranes with the envelopes of viruses transiting the cytoplasm, cells infected with virus carrying the mutation in glycoprotein D should contain many more unenveloped capsids in the cytoplasm inasmuch as there would be little or no restriction in the fusion of the envelope with cytoplasmic membranes. Comparison of thin sections of baby hamster kidney cells infected with wild-type and mutant viruses indicated that this was the case. Moreover, in contrast to the wild-type parent, the mutant virus was not released efficiently from infected cells. The conclusion that the unenveloped capsids are arrested forms of deenveloped capsids is supported by the observation that the unenveloped capsids were unstable in that they exhibited partially extruded DNA.

The electron micrographs of the assembly, maturation, and release of herpes simplex viruses (HSVs) from infected cells have produced a uniform set of micrographs but divergent interpretations of considerable significance. As reviewed in detail elsewhere (25, 26), there is uniform agreement that viral capsids containing DNA are assembled in the nucleus, that nuclear membranes acquire thickened, curved patches unique to infected cells, and that capsids become enveloped at such patches. However, the cells also exhibit enveloped capsids in structures bound by membranes and unenveloped capsids in the cytoplasm. Some of the unenveloped capsids are juxtaposed to cytoplasmic membrane patches which resemble those seen in nuclear membranes. Although some authors have interpreted the capsids juxtaposed to cytoplasmic membrane patches as virions whose envelopes have fused with cytoplasmic membranes and are frozen in the process of deenvelopment, others have concluded that capsids undergo a series of sequential envelopments and deenvelopments as they make their way from the nucleus to the extracellular space (see, e.g., references 10, 19–21, and 27). Although arguments have been presented to support both sides of the diametrically opposed interpretation of these images, the problem cannot be resolved by examination of static images of sections of infected cells without recourse to genetic or biochemical tools.

Three series of studies are relevant to the resolution of the issues presented in this report. First, evidence emerged that herpes simplex virus 1 (HSV-1)-infected cells express functions which preclude viral progeny of an infected cell or another virus from superinfecting that cell. Thus, in the

process of infection, the virus attaches to receptors on the plasma membrane, the envelope fuses with the plasma membrane, and the capsid containing viral DNA is transported to the nuclear pore (2). At the nuclear pore the DNA is released into the nucleus. The empty capsids have been detected for a few hours but ultimately disappear (2). The observation that a cell infected with a mutant HSV-1 strain accumulated large numbers of empty capsids at nuclear pores late in infection suggested that these cells were becoming reinfected and therefore that cells infected with wild-type virus express a function which precludes cells from being superinfected (33).

The second series of studies led to the conclusion that the function which restricts superinfection of cells is expressed by the viral glycoprotein D (gD). This conclusion was based on the observation that a baby hamster kidney (BHK) cell line expressing the gD (1) of HSV-1 strain F [HSV-1(F)] (9) allowed the wild-type virus to attach, but the virus was internalized by endocytosis and degraded; the synthesis of viral proteins did not ensue (3). Subsequently, similar results were obtained by direct exposure of cells to soluble gD (14) or to noninfectious virions (15). Lastly, genetic and biochemical evidence led to the conclusion that the main target of the restriction to superinfection is gD itself (5). In this instance the approach was to select a mutant [HSV-1(F)U-10] which was capable of infecting BHK cells expressing gD constitutively (e.g., the BJ-1 clonal cell line) and which restricted infection with wild-type virus. Marker rescue experiments mapped the mutation to a fragment which encoded a small number of genes including the gD gene, and sequencing studies predicted that in the mutant virus the Leu-25 amino acid of gD was replaced by proline. The significance of this mutant stems from two observations: (i)

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cell lines expressing the mutated gD were superinfectible by both wild-type and mutant viruses, whereas the BJ-1 cell line expressing wild-type gD was superinfectible only by the mutant virus, and (ii) exposure of the BJ-1 cells to the nonneutralizing monoclonal antibody AP7 (18) which reacts with gD rendered the cells infectible with wild-type virus. Similar treatment of BJ-1 cells with the anti-gD neutralizing monoclonal antibody HD-1 (22) or H170 (7) failed to render BJ-1 cells superinfectible. The AP7 epitope includes Leu-25, and one mutation which conferred resistance to the antibody was mapped at that amino acid (18). These studies led to the conclusion that (i) the target of the restriction is gD, (ii) the functional site involved in the restriction is at or near Leu-25, (iii) the mutant phenotype is dominant, and (iv) the restricting glycoprotein is in the plasma membrane (5).

The significance of these studies with respect to the issues raised in this report centers on the observation that wild-type gD on the virion envelope restricts the virus from penetrating cells expressing on their membranes wild-type gD whereas virion envelopes containing the mutant gD do not exhibit this restriction. One hypothesis that would explain the evolution of such a function for a viral glycoprotein is that superinfection of cells by viral progeny which are in the process of being exported or have egressed that cell may reduce virus yield. A particularly vulnerable population consists of enveloped viruses as they are transported in membrane-delimited structures from the space between the inner and outer lamellae of the nuclear membrane through the cytoplasm to the extracellular space. The membranes which delineate the structures in which the virions are transported originate from the outer nuclear lamella and very probably are embedded with viral glycoproteins (8). In the absence of a blocking function, deenvelopment at the cytoplasmic membranes would occur and capsids would enter the cytoplasm. Entry of capsids into the cytoplasm by fusion of the envelope with cytoplasmic membranes would have the same net effect as the superinfection at the plasma membrane. If one function of gD is to hinder fusion between virion envelopes and the membranes of the transport structures and thereby to interdict the accumulation of capsids in the cytoplasm, it could be expected that cells infected with the HSV-1(F)U-10 mutant would accumulate more cytoplasmic capsids than would cells infected with the wild-type parent and a decrease in the fraction of infectious virus released from cells. The results presented in this report were concordant with these expectations, but they also led to novel findings that have not been previously reported.

For electron-microscopic studies, replicate cultures of BHK cells were infected with 10 PFU of either wild-type HSV-1(F) or mutant HSV-1(F)U-10 per cell. The cells were rinsed with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} , fixed with 2% paraformaldehyde and 2% glutaraldehyde in 100 mM phosphate buffer, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. They were examined under Siemens electron microscopes at

TABLE 1. Number of cells exhibiting aggregates of unenveloped capsids in the cytoplasm of infected cells

Virus	No. of cells examined	% of cells with following no. of cytoplasmic aggregates of unenveloped nucleocapsids:		
		>10	50-100	>100
HSV-1(F)	125	20	12	0
HSV-1(F)U-10	243	52	27	11

the University of Chicago and at the University of Bologna. The results were as follows.

(i) The progression of infection in cells infected with wild-type virus was similar to that in cells infected with mutant virus. In both cultures there were unenveloped capsids in the nucleus and enveloped capsids in the extracellular fluid.

(ii) Both HSV-1(F)-infected (Fig. 1C and D) and HSV-1(F)U-10-infected (Fig. 1A and B) cells exhibited cytoplasmic aggregates of unenveloped capsids intermingled with membranes. Generally, sections of infected cells exhibited one or two such masses per cell. When more than one such mass was present, they were usually located at opposite side of the cell and were separated by the nucleus. In all of the thin sections of infected cells examined, the nuclear membrane of cells containing such masses appeared to be intact. We noted two differences between the HSV-1(F)- and HSV-1(F)U-10-infected BHK cells. First, thin sections of infected cells differed in the size of these masses, and on the basis of this criterion alone coded sections of cells infected with HSV-1(F)U-10 could be differentiated from those infected with HSV-1(F). Some of the masses seen in sections of HSV-1(F)U-10-infected cells contained hundreds of capsids, and therefore the entire volume of the mass spanning many sections could have readily contained thousands of unenveloped capsids (see, e.g., Fig. 1A). The second obvious difference between the two infected cell lines was the number of cells exhibiting aggregates of unenveloped capsids in the cytoplasm. There were significantly more cells exhibiting large cytoplasmic capsid aggregates in cultures infected with HSV-1(F)U-10 than in those infected with wild-type virus (Table 1).

(iii) As noted above, the unenveloped capsids in the cytoplasm were intermingled with membranes. In many instances, the capsids were in apposition to membranes of vesicles which partially surrounded the capsids (Fig. 2C to G). In some instances, the semicircular vesicles appeared to be connected with other deformed vesicles (Fig. 2G).

(iv) Comparison of the morphology of the cytoplasmic capsids with that of capsids accumulating in nuclei and those contained in virions of the same cell suggested that the unenveloped cytoplasmic capsids were partially degraded (Fig. 2C and I). Specifically, in many of the capsids the DNA

FIG. 1. Electron micrographs of thin sections of BHK cells infected with HSV-1(F)U-10 (A and B) and HSV-1(F) (C and D). The cells were fixed 24 h postinfection with 10 PFU per cell. (A) Portion of a cell infected with HSV-1(F)U-10 and containing a large mass of unenveloped capsids. Some of the capsids are juxtaposed to or in the proximity of cytoplasmic membranes. A few capsids are enveloped and contained in structures bound by membranes. (B) Enlargement of a portion of an aggregate of unenveloped capsid from a cell infected with HSV-1(F)U-10. (C and D) Aggregates of enveloped HSV-1(F) capsids contained in a structure bound by cytoplasmic membranes, of unenveloped capsids in the cytoplasm, and of enveloped capsids in vesicles. The enlargement may be calculated from the diameter of the capsids (105 nm). The infected cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde. This procedure differs from the one usually used to display the toroid structure of the viral DNA (11).

FIG. 3. Distribution of HSV-1(F) and HSV-1(F)U-10 progeny viruses in BHK cells and extracellular fluid. The cells in 25-cm² flasks were exposed to 10 PFU of parent or mutant viruses for 90 min and then incubated at 37°C. The cells and extracellular medium were harvested separately and assayed in Vero monolayer cultures. There was less than 10⁶ PFU of either virus in the extracellular fluid immediately after the rinses following the adsorption interval.

appeared to be eccentric or partially extruded from the cytoplasmic capsids. In contrast, the DNA present in the nuclear capsids and in virions of the same cell appeared to be concentric and entirely within the capsid (compare capsids in Fig. 2H with those in the same cell in Fig. 2I).

In the second series of experiments, replicate BHK cell cultures in 25-cm² flasks were exposed to 10 PFU of HSV-1(F) or of HSV-1(F)U-10 per cell for 1.5 h, rinsed twice, and incubated at 37°C. The infected cells and extracellular medium were harvested separately at the end of the exposure interval and at 16, 20, and 26 h after infection. The salient features of the results (Fig. 3) were as follows. There was less than 1 × 10⁶ PFU of extracellular virus at 1.5 h postinfection with HSV-1(F) or with HSV-1(F)U-10. At later intervals the amounts of HSV-1(F) accumulating in extracellular fluid were roughly equivalent to those present in the infected cells. The amounts of virus recovered from

HSV1(F)U-10-infected cells were 5- to 10-fold smaller than those recovered from HSV-1(F)-infected cells, whereas the amounts of virus recovered from extracellular fluid were approximately 50-fold smaller than those recovered from extracellular medium of cultures infected with the wild-type parent.

The significance of the observations reported here stems from two considerations. First, and most important, there are two hypotheses regarding the presence of unenveloped capsids in juxtaposition to the cytoplasmic membranes. These are that (i) the capsids undergo serial envelopment and deenvelopment in transit from the nucleus to the extracellular fluid and (ii) the capsids undergo terminal deenvelopment. Comparison of HSV-1(F) with the mutant virus capable of infecting restricted cells producing wild-type gD suggests that the cytoplasmic capsids undergo terminal deenvelopment rather than serial envelopment. This conclusion is supported by four observations. First, very large masses of cytoplasmic capsids juxtaposed to membranes are characteristic of HSV-1(F)U-10 mutant-infected cells and are rare in cells infected with the wild-type virus. Second, the morphology of the unenveloped cytoplasmic capsids suggests that they are undergoing degradation and therefore are unlikely to contribute to the pool of infectious progeny resulting from viral multiplication in these cells. Third, thin sections showing capsids in the process of envelopment at the nuclear membrane are extremely rare, suggesting that envelopment at the inner lamella of the nuclear membrane takes place very rapidly and is not impeded in either HSV-1(F)- or HSV-1(F)U-10-infected cells. This conclusion is consistent with the notion that nuclear capsids processed for envelopment must have a very high affinity for the patches containing viral glycoproteins in the inner lamella of the nuclear membrane. The accumulation of large numbers of unenveloped capsids juxtaposed to the cytoplasmic membranes suggests that the process of envelopment, if it were to occur, is very slow; i.e., the surfaces of these capsids exhibit an entirely different affinity for the modified membranes. Lastly, we could predict that the transport of fully enveloped, infectious virus to the extracellular fluid would be impaired if (i) accumulation of unenveloped virus in the cytoplasm were deleterious and (ii) the mutation in HSV-1(F)U-10 were to result in an increase in the incidence of fusion of enveloped virus with the cytoplasmic membranes of infected cells. This is in fact the case (Fig. 3).

The second issue concerns the presence of unenveloped capsids in cells infected with wild-type virus. A schematic representation of the envelopment of HSV-1 and its egress from infected cells is shown in Fig. 4. We should note that in cell lines expressing gD, the glycoprotein that is effective in blocking penetration of wild-type virus was shown to be the

FIG. 2. Electron micrographs of thin sections of BHK cells infected with HSV-1(F) (A and B) and HSV-1(F)U-10 (C to I). (A and B) Enveloped capsids in the space between the inner and outer lamellae of the nuclear membrane and in cytoplasmic structures bound by membranes, respectively, in cells infected with HSV-1(F). (C) Higher-magnification micrograph of a thin section of an aggregate of capsids in the cytoplasm of cells infected with HSV-1(F)U-10. 1, 2, Enveloped capsid in structures bound by membranes; 3, 4, capsids with partially protruding DNA; 5, a capsid surrounded by a partial envelope fused at the ends with a thinner cytoplasmic membrane. (D) Higher-magnification micrograph of a capsid partially surrounded by an envelope fused at the ends with a thinner, cytoplasmic membrane in the cytoplasm of an HSV-1(F)U-10-infected cell. (E and F) Higher-magnification micrograph of portions of the cytoplasm of cells infected with HSV-1(F)U-10. Note the capsids juxtaposed to the membrane of a vesicle in panel E and of the semicircular vesicle surrounding the capsid in panel F (arrowhead). (G) Arrowhead points to a cluster of capsids partially surrounded by interconnected double-membrane vesicular structures. (H and I) Micrographs of portions of the same cell. In panel H the enveloped capsids were outside the infected cell; in panel I, the capsids with partially protruding DNA were in the cytoplasm. A single enveloped capsid with nonprotruding DNA is contained inside a vesicle. Magnification for panels A, B, H, and I, ×80,000. The final magnification may be calculated from the diameter of the capsids (105 nm). Fixation and staining were as described in the legend to Fig. 1.

FIG. 4. Schematic representation of the envelopment and egress of HSV-1 from infected cells. Steps and structures: (a) Abutment of a capsid containing DNA to a modified patch in the inner lamella of the nuclear membrane; (b) envelopment of the capsid by the patch in the inner lamella of the nuclear membrane; (c) accumulation of enveloped capsids in the space between the inner and outer lamellae of the nuclear membrane; (d) transit of enveloped capsids through the cytoplasm singly or in groups within structures bound by membranes; (e) release of enveloped capsids into the extracellular space; (f) extracellular enveloped capsids; (The pathway a to f is common to all HSV-1-infected cells. Not shown is the interaction of enveloped nucleocapsids transiting the cytoplasm with the Golgi-processing enzymes.) (g and h) structures that are found in all HSV-1-infected cells but that were particularly numerous in cells infected with the HSV-1(F)U-10 mutant. These structures, for the most part, were capsids with partially protruding DNA that were probably undergoing dissolution (h) and capsids partially juxtaposed to membrane patches (h) similar to those seen in step a but present in cytoplasmic membranes. The structures designated as g could have arisen by fusion of the envelope with membranes of the vesicles in which they were transported to the cytoplasmic membrane.

one that is present on the surface of the infected cells in that it was accessible to monoclonal antibody AP7 (5). As is the case for the other HSV glycoproteins, the gD transported to the plasma membranes is the fully glycosylated form (12, 13) processed by the Golgi enzymes (4, 16, 29, 30; reviewed in references 6 and 32). Inside the cell, a partially processed precursor form (31) is present in the rough endoplasmic reticulum and in pre-Golgi compartments (17, 24, 28). We have no comparative data on the relative efficacy of gD with fully processed and with immature polysaccharide chains in blocking fusion of the envelope with cell membranes; it is conceivable that the unenveloped capsids present in the cytoplasm of cells infected with wild-type virus reflect occasional fusion of envelopes with cytoplasmic membranes containing immature glycoproteins or small areas with a low level of viral glycoproteins.

Lastly, it should be noted that the glycoproteins on the virion envelopes are the mature forms (12, 13, 31), which have been processed by the Golgi enzymes. Processing of the glycoproteins as components of the virion envelope does not in principle require that the viral envelopes fuse with the Golgi membranes. Thus, processing of the virion envelope glycoproteins could occur by (i) fusion of the vesicle carrying the enveloped virus with the Golgi, transit of the enveloped virus through the organelle, and budding of a vesicle containing the virion after processing is complete or (ii)

fusion of vesicles carrying the enveloped virus with vesicles containing Golgi enzymes (23). Examination of the sections failed to show the presence of capsids, enveloped or unenveloped, within characteristic Golgi structures. Although the mechanism by which virions are processed by the Golgi enzymes remains unknown, intact Golgi apparatuses were detected in infected cells, including those that exhibited large numbers of unenveloped capsids juxtaposed to cytoplasmic membranes. The data do not support the hypothesis that the membranes juxtaposed to the unenveloped cytoplasmic capsids are modified Golgi organelles.

In the past several years, a number of herpesviruses infecting diverse cells and species have been reported to undergo sequential envelopment and deenvelopment as the capsid transits from the nucleus to the extracellular space. In most instances, the conclusions were based on electron-microscopic examination. As noted elsewhere (25), conclusions based on electron-microscopic examination are akin to the deduction of the plot of a photoplay based on the examination of a randomized assortment of still photographs.

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