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Intra-nuclear localization of two envelope proteins, gB and gD, of herpes simplex virus

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Summary. The envelopes of herpes simplex virus (HSV) particles are acquired from the inner nuclear membrane (INM) of the infected cell and virus-coded glycoproteins are present in the envelope of mature virions. Our ultrastructural study examined the process of virus envelopment and the targeting of two major viral glycoproteins, gB and gD, to the INM in HSV-infected human embryonic fibroblasts. It was shown that envelopment and transport of virus particles from the nucleus is facilitated by the formation of a dynamic tubulo-reticulum arising from the INM. Capsids were assembled in the nucleus and collected within INM tubules which protruded into the perinuclear space and thence into the cisternae of the endoplasmic reticulum (ER). Envelopment occurred by constriction and fusion of the tubular channel walls, releasing enveloped virions into the ER. Transport to the cell surface took place in membrane-bound compartments and probably followed the normal secretory pathway through the Golgi apparatus. Immunogold probes, tagged with specific monoclonal antibodies, were used to localize gB and gD during the process of virus maturation. Cytoplasmic membranes were not labelled, but probes bound inside the nucleus, mainly at sites of virus assembly. Labelling occurred on the nucleoplasmic side of the INM which surrounded capsids in the process of envelopment, but not on the outside of that membrane, although characteristic gB glycoprotein spikes were labelled on the envelopes of extracellular virus particles and on virions in *trans*-Golgi transport vesicles just prior to their release from the infected cell. gB was not detected on the surface of enveloped virions in the perinuclear space, or the cisternae of the ER or *cis*-Golgi, which suggests that the specific epitope was masked during that stage of intracellular processing. gD probes bound to virion envelopes and also to the tegument region of some particles found in both perinuclear and extracel-

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lular sites. We postulate that precursor core proteins for both gB and gD are transported first to the nucleus, and then, together with maturing capsids, are targeted to the INM, and later inserted into viral envelopes at the site of budding. Post-translational glycosylation of envelope proteins could occur as virus particles exit the nucleus and travel through the ER and Golgi compartments.

Introduction

Herpes simplex virus (HSV) type 1 encodes at least ten glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM). Although their precise functions have not all been defined, three of them, gB, gD and gH, have been shown to play an essential role in virus infectivity [49]. It has been proposed that gB is involved in viral entry and cell fusion [1] and that gD is required for penetration, but not adsorption [11, 23]. In addition, expression of gD in transfected cells is sufficient to render the cells resistant to HSV infection [18, 45], which has led to the proposal that the presence of gD in membranes of infected cells may prevent super-infection of the cell by escaping progeny virions. On extracellular particles, morphologically distinctive glycoprotein spikes project from the virion envelope [50]. In HSV-infected cells, viral glycoproteins are targeted to the inner nuclear membrane (INM) [17], and virus envelopes are acquired from the INM when DNA-containing capsids bud into the perinuclear space [28]. It has been shown that nuclear membranes contain predominantly immature forms of the glycoproteins [5] and that envelope proteins become processed from immature to mature forms during transit to the outside of the cell [53]. The glycosylation pattern of HSV envelope glycoproteins [49] supports the concept that enveloped virions follow the established biosynthetic pathway of secretory proteins [33] and move from the perinuclear space, through the endoplasmic reticulum (ER) and the Golgi apparatus en route to the cell surface.

It is not known how HSV-coded glycoproteins reach the INM. A general assumption, by analogy with other well-characterized membrane-bound viral proteins [20], has been that they are synthesized on ribosomes of the rough endoplasmic reticulum (RER), inserted into these RER membranes and then laterally transported to the outer nuclear membrane. How these glycoproteins may be transferred from the outer to the inner membrane of the nuclear envelope, is more difficult to understand, and traverse of the membranes surrounding the nuclear pores is as yet unproven. Such a pathway would require that the rate of glycoprotein transport kept pace with the rate of proliferation of the INM. Furthermore, unless translation of all integral envelope proteins occurred simultaneously, a complicated sorting process would be needed to ensure that each virion envelope acquired the correct number and type of glycoproteins at the time of budding. We examined the targeting of glycoproteins to HSV virion envelopes by exposing cryosections of infected fibroblasts to immunogold probes specific for either gB or gD. These studies have shown that during envelopment of HSV capsids the INM is restructured into a tubulo-reticulum, and that both gB and gD are present inside the nucleus and on the inside of envelope membranes.

Materials and methods

Preparation of colloidal gold

Homogeneous colloidal gold particles were prepared by the reduction of chloroauric acid with a mixture of sodium citrate and tannic acid according to the method of Slot and Geuze [46]. Different sizes of gold particles (ranging from 3 nm to 9 nm) were prepared by varying the amount of tannic acid in the reduction mixture. Unstabilised gold sols were stored in the dark at 4 °C.

Preparation of colloidal gold labelled monoclonal antibodies

Affinity-purified monoclonal antibodies (Mab), specific for mature and immature forms of HSV glycoproteins gB (Mab 1-59-2) and gD (Mab 1-99-1), characterized by Para et al. [31], and for human cytomegalovirus (HCMV) gp52 (Mab F5), were obtained in lyophilised form in 0.01 M phosphate buffered saline (PBS), pH 7.4. Antibodies were redissolved in distilled water at a concentration of 1.0 mg/ml, then dialysed against 0.2 mM borax buffer pH 9.0 before coupling to colloidal gold suspensions at a pH of approximately 7.4. Details of this preparative procedure have been described previously [50]. Gold-protein complexes were stored at 4 °C in 0.2 M Tris-saline, pH 8.2, containing 0.5% bovine serum albumin (TBSA) and 0.02 M sodium azide.

Virus

The syncytial mutant, (HFEM) syn, of HSV-1 was used for all experiments. This strain has been described previously [50].

Preparation of cells for ultra-thin sections

Human embryonic fibroblast (HEF) monolayer cultures were grown in minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS) until confluent. Cultures in 75 cm^2 flasks were inoculated with 2 ml of HSV-1 at 10^7 p.f.u./ml (an approximate m.o.i. of 5), and maintained in MEM containing 4% FCS for up to 48 h. Cell sheets were harvested at different times after infection. Times of between 4 and 8 h post-infection are described as "early", and 24 to 48 h as "late". Supernatant culture fluids were removed and stored at 4 °C for use in immuno-gold studies of virus suspensions. Infected cell sheets and control uninfected cell layers from the same batch and culture conditions, were rinsed with serum-free culture medium, then with 0.1 M PBS, pH 7.2, and covered with 2% glutaraldehyde in PBS for 2 h at 20 °C. Cells were removed from the flask with a rubber policeman, rinsed by gentle centrifugation in PBS, and kept at 4 °C.

Ultra-thin frozen sections

Infected or uninfected HEF cells, fixed in glutaraldehyde as above, were centrifuged to a pellet (20 min in Microfuge), cut into 1 mm cubes, washed three times in PBS, infiltrated with 2.3 M sucrose in PBS for 1 h, then orientated on stubs and rapidly frozen in liquid nitrogen. Sections (approximately 60–80 nm thick) were cut on a Reichart Ultracut microtome, fitted with the FC4 Cryo attachment, at between −100 °C and −115 °C, using glass knives.

Immunogold labelling of cryosections

Immunolabelling was performed at room temperature according to the method of Tokuyasu and Singer [52] and Griffiths et al. [14] with slight modifications. Briefly, sections were

floated on a blocking solution (0.1 M glycine, 0.5% gelatin, 0.1% Tween 20, 0.5% BSA) for 5 min, washed six times with PBS, then floated on the gold-antibody complex (diluted in TBSA) for 30 min. After eight washes in PBS and three washes in distilled water, the sections were stained for 5 min with 1% uranyl acetate, pH 7.0 (adjusted with oxalic acid) and for 5 min with 1% acidic uranyl acetate. Sections were finally washed three times in distilled water and embedded in a thin layer of 1.5% methyl cellulose containing 1% uranyl acetate.

Ultra-thin sections of resin-embedded cells

Infected HEF cells fixed in 2% glutaraldehyde were post-fixed in osmium tetroxide and embedded in Spurr's resin according to standard procedures. Sections were cut on a Reichart Ultracut microtome and stained with uranyl acetate and lead citrate.

Immunogold labelling of virus suspensions

Virus particles were concentrated from 5 ml of cell culture supernatant fluids by centrifugation at 48 000 **g** for 90 min in a Beckman SW50.1 rotor. Virus pellets were resuspended in 300 µl 0.2 M phosphate buffer pH 7.2 (PB). Approximately 20 µl of each colloidal gold-antibody probe was added, mixed well and left at approximately 18 °C overnight. The volume was then increased to 5 ml with PB and virus particles centrifuged to a pellet at 27 000 **g** for 20 min in a Beckman SW50.1 rotor. Pellets were examined by negative stain electron microscopy.

Electron microscopy

Virus suspensions were negatively stained with 2% phosphotungstic acid, pH 6.2 and examined in an Hitachi 600 electron microscope operating at 75 kV. Ultra-thin frozen sections were examined in an Hitachi-600 electron microscope at 100 kV or in a Phillips 420 electron microscope at 120 kV, and resin-embedded sections were viewed at 75 or 80 kV.

Results

Morphological alterations in HEF cells following infection with HSV-1 were monitored on ultra-thin sections of resin-embedded cells, and used in the interpretation of gold-labelled cryosection images which frequently lack ultrastructural detail. Where appropriate, comparable sections of labelled and unlabelled cells are illustrated side by side.

Extracellular virions were distinctively labelled with immunogold probes specific for gB or gD. gB probes bound to prominent spikes projecting from the virion envelopes (Fig. 1a). These labelled spikes were often present in clusters and on envelope protrusions or "buds". In contrast to gB, gD envelope spikes were short and not clustered (Fig. 1b). The patterns of labelling resembled those previously shown [50] on negatively stained extracellular virions (see also Fig. 5b) and established confidence in the specificity of the probes.

In infected cells, we could find no early cytoplasmic labelling, and probes bound to neither ER nor outer nuclear membrane. To our surprise, however, strong labelling was evident inside the nucleus, especially in areas of nucleocapsid assembly. Capsids were frequently assembled around foci of densely staining material (Fig. 2a) which were always heavily labelled with anti-gD gold

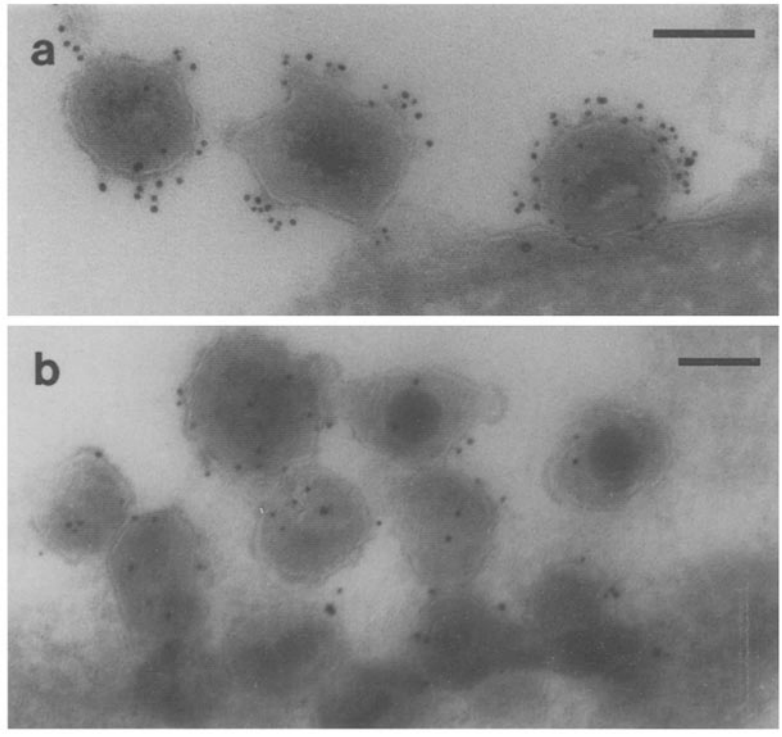

Fig. 1. Extracellular HSV particles adjacent to the cell membrane. **a** gB probes attach to clustered spikes projecting from the envelope of the virion, but **b** gD probes bind close to the virion envelope in a random distribution. Bars: 100 nm

(Fig. 2b) and less strongly with gB probes (Fig. 2c). The capsids were at different stages of maturation – some appeared "empty" and others contained either a central 35 nm core structure or, more rarely, a densely stained centre indicating an apparently full complement of DNA. gB probes bound to the empty nucleocapsids and it was striking that often the attachment occurred on the inner surface of the capsids (Fig. 2c). Similar intra-capsid labelling was seen on capsids that accumulated in closely packed clusters (Fig. 2d). gB probes often bound to a central 35 nm core component (Figs. 2e and 2f) but capsids that were either partially or completely full of DNA were not labelled. Regular arrays of small bodies approximately 35 nm in diameter, that resembled core components, accumulated in the nucleus at late stages of infection. These had a similar affinity for gB probes (not shown). At the nuclear periphery, capsids were aligned alongside the INM. At this site both gB and gD probes bound to poorly resolved structures directly adjacent to the capsids, or interspersed between them in a mesh-like arrangement (Figs. 2g and 2h), but the INM was not labelled.

Characteristic of HSV-infection, the INM underwent extensive proliferation. Earliest stages of this proliferative process had a tubulo-reticular nature. Capsids became enclosed within tubular channels (Fig. 2h and Fig. 3) which spread around the periphery of the nucleus in the perinuclear space (Fig. 3a) and also

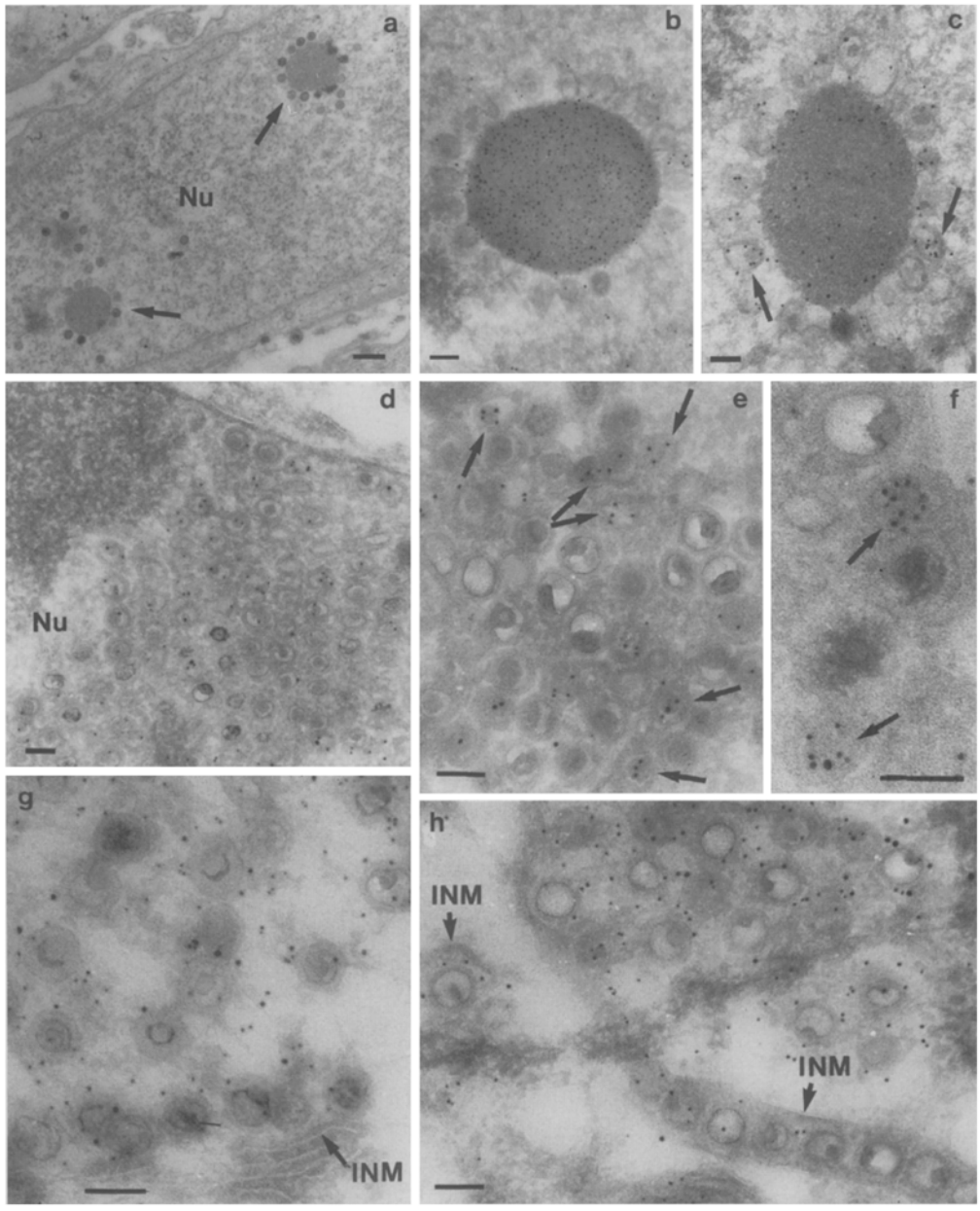

Fig. 2. Inside the nucleus (*Nu*) of HSV-infected HEF cells. **a** Viral nucleocapsids are frequently assembled around globular masses of electron-dense material (arrows). These are heavily labelled with gD probes (**b**). **c** gB probes bind less strongly to the electron dense material but labelling occurs on the internal surface of some of the surrounding capsids (arrows). **d** Maturing capsids accumulate in the nucleus and are also labelled with gB probes at intra-capsid sites. At greater magnification (**e** and **f**) the gB probes are seen to be attached to an internal core component approximately 35 nm in diameter (arrows) seen inside the capsids before they become filled with DNA. **g**, **h** Viral capsids are targeted to the inner nuclear membrane (*INM*) at which stage probes for both gB (small gold) and gD (larger gold) bind close to the capsids and thus on the nucleoplasmic side of the INM. The INM (which will form the virion envelope after budding) is not labelled. Bars: **a** 500 nm, **b**–**h** 100 nm

extended in branching fashion into the cisternae of the ER (Figs. 3b and 3c). The effect of tangential sectioning was to sever the continuity of the tubular structures arising from the INM, thus creating the illusion that isolated virions were lying within the cytoplasm or that capsids were being enveloped at cytoplasmic membranes (Figs. 3c and 3d). Envelopment occurred by constriction and fusion of the tubule walls at sites between capsids, releasing enveloped virions into the ER (Fig. 3d). Branching tubules interconnected in a lattice-like reticulum most clearly seen in sections cut through the peri-nuclear space across the surface of the nucleus (Fig. 3e). Within the tubulo-reticulum, both gB and gD probes bound at sites between capsid and INM, thus on the nucleoplasmic side of the membrane which would eventually become the virion envelope (Fig. 3f).

After envelopment, virions collected within the perinuclear ER cisternae (Fig. 4a). Anti-gD gold bound to these virions at sub-envelope sites, but was also sometimes visible on the envelope surface (Fig. 4b). However, gB spikes were not detectable on envelopes of newly enveloped virions and gB labelling was absent except for a few particles where probes bound to sites between capsid and envelope (Fig. 4c). During transit to the cell surface, virus particles accumulated in smooth membrane-bound compartments located in centralized sites in the cytoplasm (Fig. 4d). From serial sections these compartments were seen to be linked in a tubulo-reticular type network, and in syncytia they appeared to represent collection centres for virions from all the surrounding nuclei. We concluded that the compartments were dilated Golgi sacs. The membranes of the Golgi sacs were never labelled but labelling of the enclosed virions varied between *cis*- and *trans*-cisternae. After exposure to gB probes, virus particles within *cis*-Golgi vesicles (Fig. 4e) were virtually unlabelled (similar to those within the ER). In contrast, gB probes bound strongly to the surface of the envelope of virions within *trans*-Golgi compartments (Fig. 4f) or within transport vesicles near the cell surface (Fig. 4g). The eclipse of labelling within the ER and *cis*-Golgi may represent a stage at which the specific gB epitope is masked. Mature virions were apparently released from the cell by fusion of transport vesicles with the plasma membrane. The envelopes of these virus particles contained both gB and gD, but the plasma membrane itself remained unlabelled. On extracellular virions, gD probes, unlike gB, sometimes also bound at sites between the capsid and the envelope, rather than to the outer surface (Fig. 5), indicating that in some instances gD components remained on the inside of the virion.

At late stages of HSV infection the tubulo-reticular nature of the INM was no longer apparent, and we observed gD labelling on inner nuclear membranes and also on apparent cytoplasmic membranes. However, serial sections of resin-embedded cells revealed that the labelled membranes were in fact not cytoplasmic but nuclear in origin. Following excessive proliferation of the INM, juxtaposed nucleoplasmic surfaces were apparently held together by electron dense material. The "bonded" layers of INM also extruded into the ER (Fig. 6a) or accumulated as multiple layers of folded membrane at the periphery of the nucleus (Fig. 6b). Finally, following the same pathway as the earlier tubulo-

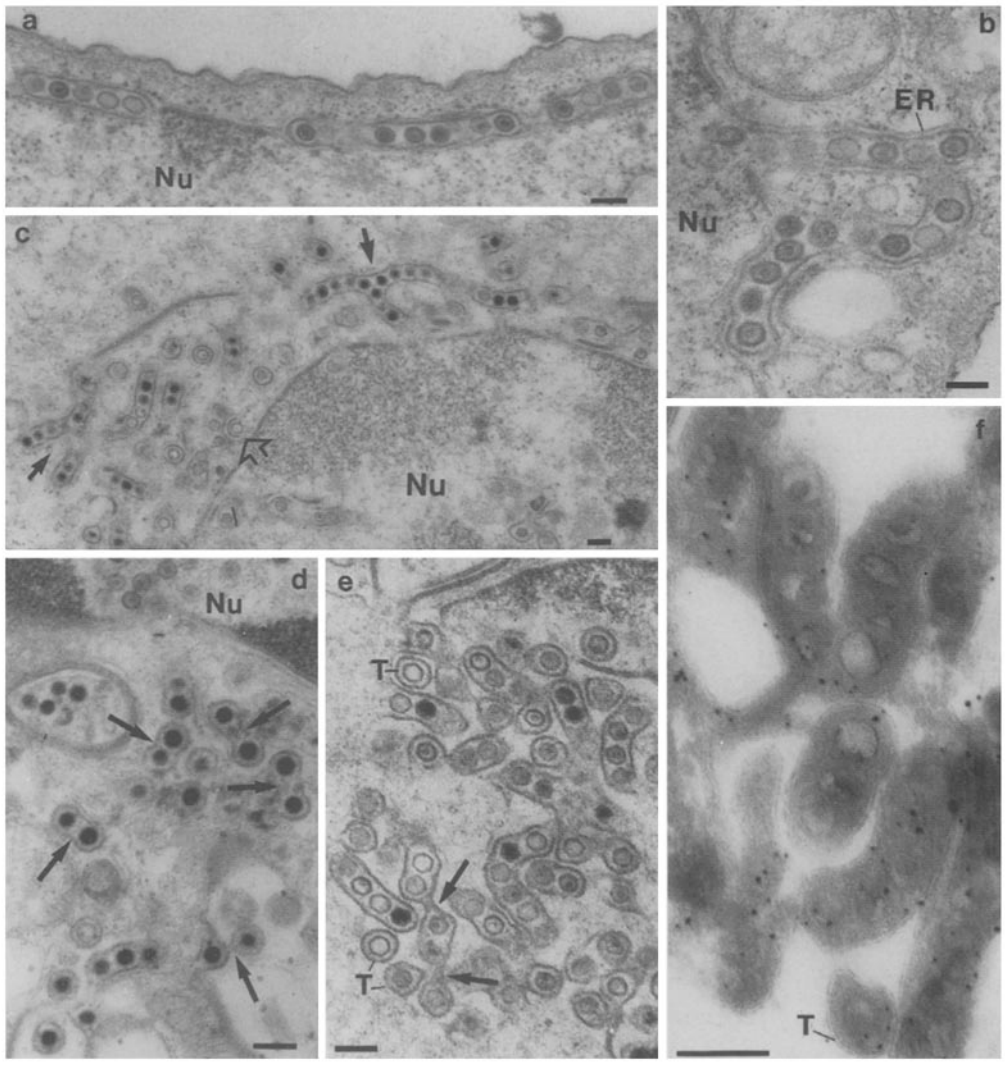

Fig. 3. The INM of HSV-infected HEF proliferates in tubulo-reticular fashion. Viral capsids are encased within tubules that **a** extend into the perinuclear space and thence **b** extrude into the cisternae of the endoplasmic reticulum (*ER*). **c** The INM tubulo-reticulum branches (arrows) and invades the ER. Sectioning severs the continuity of tubular structures and creates the illusion of capsids budding through cytoplasmic membranes (open arrow). **d** Constriction of the tubule walls (arrows) at sites between capsids apparently results in the "pinching-off" of enveloped virions that are then released into the ER cisternae. **e** A section cut across the surface of the nucleus and through the perinuclear space shows how the tubules interconnect in a multi-dimensional reticulum. Arrows indicate sites of constriction which may result in envelopment of the viral capsids, but enveloped virions cannot be distinguished from transverse sections (*T*) through tubular structures. **f** A similar view of a labelled cryosections shows that probes for both gB and gD bind close to the capsids contained within the INM tubulo-reticulum or on the inner (nucleoplasmic) side of the INM. Bars: 200 nm

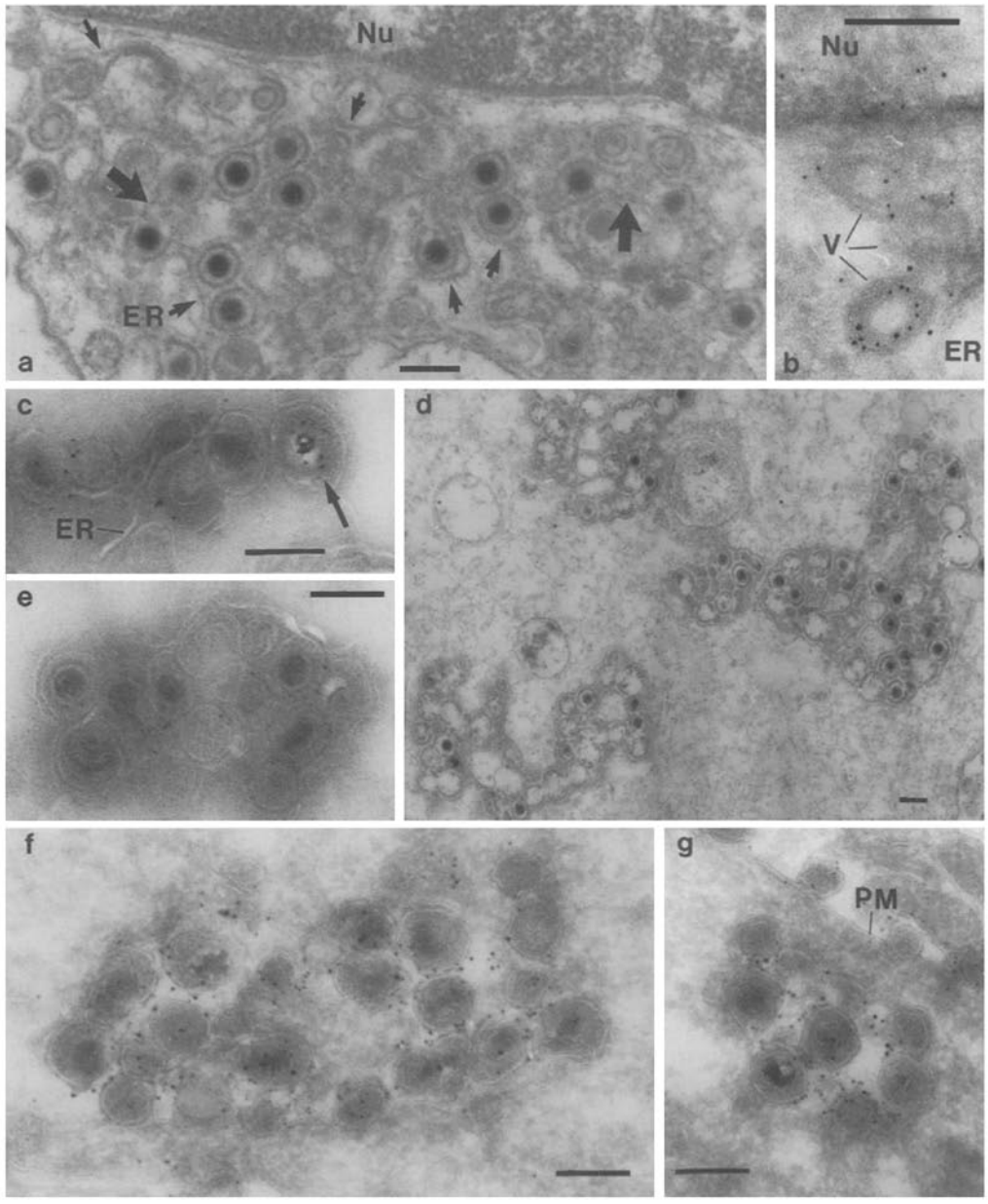

Fig. 4. From the nucleus HSV particles follow the secretory pathway to the cell surface. **a** After envelopment by pinching, virions are present in the endoplasmic reticulum (*ER*). Small arrows indicate ER membranes and large arrows point to sites at which the tubular connection of inner nuclear membrane have not yet been severed. **b** Newly enveloped virions (*V*) within the ER are labelled with gD probes which bind strongly around the capsid inside the envelope, and also to the surface of the virion envelope. **c** Virions within the ER are only sparsely labelled with gB probes, but a few particles are labelled at sites between capsid and envelope (arrow). **d** Virus particles accumulate in tubulo-reticular compartments clustered in centralized sites within the cytoplasm. These were judged to be dilated Golgi compartments. **e** After exposure to gB probes, virus particles within *cis*-Golgi compartments are not labelled, but **f** virions within *trans*-Golgi compartments are labelled with gB probes on the outside of the envelope. **g** gB is labelled on the surface of virions within transport vesicles close to the plasma membrane (*PM*). Bars: 200 nm

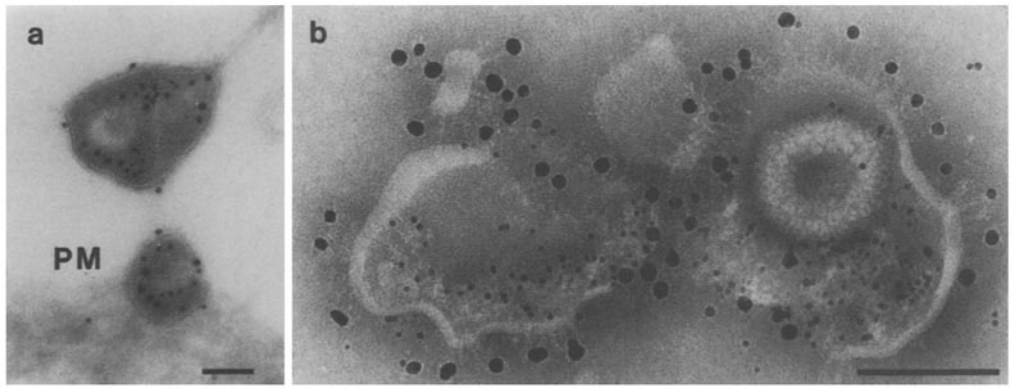

Fig. 5. gD probes bind to the inside of some extracellular virions. **a** gD-labelled cryosection shows virions with a few gold particles attached to the outer surface of the virion but most labelling occurs on the inside of the envelope. **b** Extracellular HSV-1 virions harvested from culture fluids were reacted with immunogold probes for gB and gD and viewed by negative staining. Large gold specific for gB binds to distinctive envelope spikes, and small gold specific for gD binds to amorphous material between capsid and envelope of the virion on the right, and on the inside of the empty envelope lying to its left. Bars: 100 nm. *PM* Plasma membrane

reticulum, they extended throughout the cytoplasm within the ER cisternae (Fig. 6d). Stacks of folded membrane such as seen at the perimeter of the nucleus (Fig. 6b) often appeared to be lying within the cytoplasm (Fig. 6d). Although these late stage "bonded" layers of INM were heavily labelled with gD probes (Figs. 6c and 6e) they were not tagged with gB probes (Fig. 6f). However, occasional clusters of gB spikes were seen on small membranous blebs (Fig. 6g) which budded from the INM into the perinuclear space or ER cisternae.

Immunogold labelling was performed on five different batches of HSV-1-infected HEF cells and results were reproducible:– intranuclear sites were consistently the strongest target for both gB and gD gold probes, and cytoplasmic membranes were not labelled. Cryosections of HSV-1-infected HEF were also exposed to control probes tagged with a Mab to the gB homologue of HCMV, but no labelling was observed. Uninfected HEF cells were not labelled with either the HSV or the HCMV probes.

Immunogold labelling of extracellular virus suspensions

Virions concentrated from the supernatant culture fluids of infected cells were incubated with gold probes for either gB or gD, and examined by negative staining. By using gold particles of different sizes, each coupled to a different antibody, we could localize both glycoprotein determinants simultaneously on single virions. Results were similar to those previously reported [50]; anti-gB binding to long sturdy spikes projecting from the virion envelope, and anti-gD attaching to less well-defined spikes close to the envelope surface. In addition, however, small gold probes specific for gD bound to amorphous material situated on the inner aspect of the envelope of some virions (Fig. 5b).

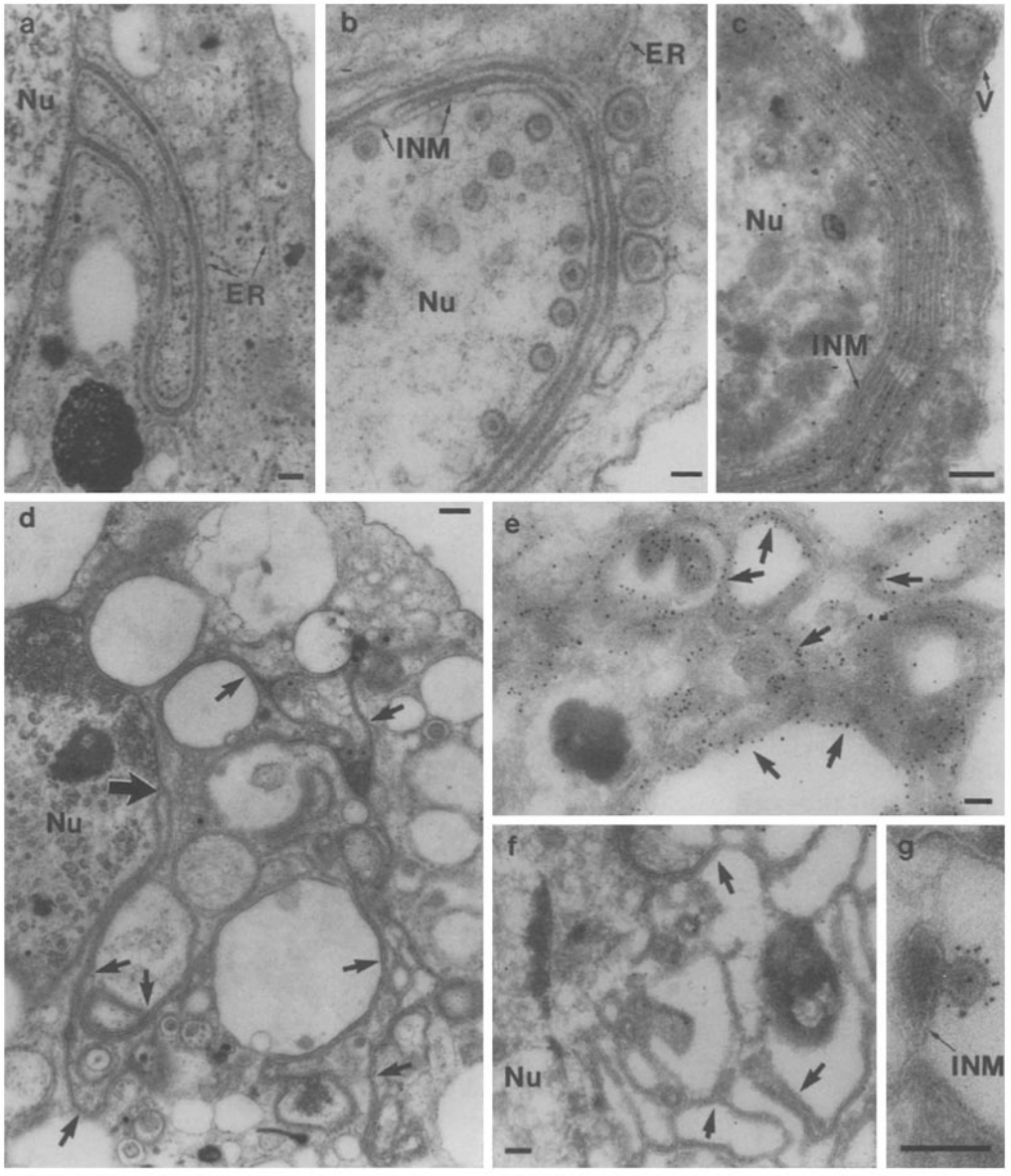

Fig. 6. Late in infection the tubular nature of the proliferated inner nuclear membrane (INM) is no longer apparent. Juxtaposed nucleoplasmic surfaces of INM are held together by electron dense material. These "bonded" layers of INM extend into the ER (**a**), or **b** accumulate in repeated folds at the periphery of the nucleus. **c** gD probes bind to the layers of INM at the edge of the nucleus (*Nu*), but the virion (*V*) just outside the nucleus is labelled inside the envelope. **d** "Bonded" layers of INM leave the nucleus (large arrow) and extend into the ER throughout the cytosol (small arrows). **e** Extruded INM (arrows) is also heavily labelled with gD probes, but **f** not labelled with gB probes. **g** gB probes label clusters of gB spikes that appear to be budding from the INM at late stages of infection. Bars: **d** 500 nm; **a–c**, **e–g** 100 nm

Discussion

The intracellular assembly and transport of HSV has been studied in detail (reviewed in [40]), and opinions differ concerning the manner in which virus particles transit from the nucleus to the cell surface. Based on evidence regarding the sequence of glycosylation of HSV envelope glycoproteins [35] it is believed [49] that virions follow the established secretory pathway and move from the perinuclear space, through the ER and the Golgi apparatus en route to the cell membrane. However, the observation of unenveloped capsids in the cytosol, apparently acquiring envelopes from cytoplasmic membranes, has led to varying hypotheses; either that capsids are sequentially enveloped and de-enveloped at the inner and outer nuclear membranes, and that final envelopment is predominantly a cytoplasmic event [39, 55], or alternatively, that some progeny virions undergo terminal de-envelopment by fusing with cytoplasmic membranes during egress [3]. We favour the model whereby capsids are enveloped only once, at the INM, and then follow the secretory pathway to the cell membrane and, furthermore, that envelopment and transport from the nucleus is facilitated by the formation of a dynamic tubulo-reticulum arising from the INM. HSV-infected cells characteristically have proliferation of the INM, observed late in infection as layers of repeatedly folded and coalesced membranes [25, 28, 40]. We show that initial proliferation gives rise to a network of branching tubules. Viral nucleocapsids collect in the tubular channels which extrude into the perinuclear space and into the cisternae of the ER. When the continuity of the encompassing membranes is interrupted by oblique sectioning, intranuclear capsids may appear to be situated in the cytoplasm, so partially explaining the alternative hypothesis of cytoplasmic envelopment. Envelopment can occur by constriction and fusion of the walls of the tubular channels of INM. This "nipping off" process releases enveloped particles directly into the ER cisternae, so allowing them to be transported, via the Golgi apparatus, to the cell surface.

Tubulo-reticular elements have been shown in reports on herpesvirus morphogenesis [3, 4, 10, 28, 43, 47] with little attention paid to their implications. The tubulo-reticular association with intracellular traffic of many organelles is well recognised [7, 8, 16, 22, 37]. Many such proliferative processes are directed along microtubules, so the role, if any, of cytoskeletal elements in the tubular proliferation of the INM is intriguing. The nuclear laminae, known to be closely related to intermediate filaments, are presumably involved in the re-structuring of the nuclear envelope, although direct microtubule involvement is less likely. Experiments performed with HCMV-infected fibroblasts [36] suggest this infection may trigger the dephosphorylation of lamins A/C and thus cause the "relaxation" of the stabilising lamina meshwork. Future identification of HSV viral gene products that induce tubulo-proliferative changes in nuclear membranes may provide useful insight regarding the control mechanisms of cellular membrane proliferation. Studies with genetically engineered deletion mutants of HSV-1 [2] have shown that deletion of the U_L11 gene results in inefficient

envelopment and transport of virions into the extracellular space, although the level of control is not yet known.

It is known that both gB and gD are embedded in the envelope of extracellular HSV-1 virions where they are morphologically distinguishable from each other and from gC [50]. How these glycoproteins reach the virion envelope during viral morphogenesis remains uncertain. Mechanisms for the transfer of membrane-bound glycoproteins (after translation on ER-bound ribosomes) to the inner layer of the nuclear envelope are complicated by the dynamic virus-induced restructuring of the INM which would reduce direct contact between inner and outer nuclear membranes (as nuclear pore complexes). It is also difficult to understand how the various glycoproteins may be arranged within tubular components of INM so that each budding virion acquired all of the relevant glycoproteins. Our immunolabelling studies, designed to localize viral glycoproteins inside infected cells, yielded unexpected results. Early in infection neither gB nor gD was labelled on ER or nuclear membranes, but labelling with both probes was seen inside the nucleus, at first associated with centres of capsid assembly and later around the nucleocapsids that had been targeted to the INM. Both gB and gD probes were present on the nucleoplasmic side of the enveloping tubulo-reticulum and on the inside of the envelopes of newly enveloped virions in the perinuclear area. Membranes of the secretory organelles were not labelled but virions within *trans*-Golgi vesicles or outside the cell had characteristic glycoprotein spikes on the surface of their envelopes. Neither glycoprotein was detected on the INM itself until very late stages of infection, when gD probes bound strongly to those layers of INM that resulted from excessive proliferation. In summary, the immunocytochemical detection of viral glycoproteins occurred predominantly inside the nucleus; on the nucleoplasmic side of the INM at the site of budding; and on the outside of mature virions when they reached the cell surface.

Our observations pose a number of questions; firstly, why are no glycoproteins detected on ER or nuclear membranes early in infection? The absence of immunocytochemical labelling at these sites may indicate a masking of the epitope, either because of alterations in folding of the protein, or because of its association with other viral or cellular proteins, or because of early glycosylation events. However, the labelling of both gB and gD on the inside of newly enveloped virions in the perinuclear space is not easily explained. Earlier studies [29], using ferritin conjugated to rabbit polyclonal antibodies to HSV, were similarly able to demonstrate tagging of the inner aspect of the envelope of virions seen within the cytoplasm, yet extracellular virions were labelled on the outer surface of the envelope. Other workers [13] used indirect immunogold techniques to locate gp 110, the Epstein-Barr virus glycoprotein homologous to HSV gB, and were also unable to show labelling of the envelope of virions in the peri-nuclear space despite labelling on the nucleoplasmic side of the inner nuclear membrane.

The consistent demonstration of intranuclear gB and gD requires explanation. Intranuclear localization of gB has been observed previously in immuno-

cytochemical studies of Vero cells infected with either HSV-1 or bovine herpes virus type 2 [34]. Those authors propose that gB in the nucleus represents an early form of the mature glycoprotein. In the mammalian cell, active transport to the nucleus requires that proteins contain suitable nuclear localization signals (NLS) [27], the prototype being that of the SV40 T-antigen, where the sequence PKKKRKV is implicated [19]. Homologues of the SV40 NLS are found in many other nuclear proteins [12] and it may be relevant that the gene sequence for HSV-1 gB [32] contains analogous sequences at both the amino and carboxy termini. Future studies with gene constructs could provide confirmation of the karyophilic nature of gB and gD. Transfection with separate genes for gD [17] or gB [1, 48] results in the gene products reaching the cell surface much more quickly than in natural infections, but they are nevertheless transiently detected in nuclear fractions. It has been suggested [17] that the slower intracellular transport of HSV glycoproteins in infected cells may reflect an accumulation time in nuclear membranes, most probably in association with other structural viral proteins. We have shown that association with other structural proteins begins inside the nucleus. Earlier reports indicated that the nuclear fraction of HSV-1-infected cells contains gB, gC and gD, predominantly in the form of partially glycosolated high mannose precursors [5, 17], and it is relevant that incubation of infected cells at a reduced temperature (34 °C) resulted in the accumulation of non-glycosylated precursors to gB and gC in the nuclear fraction [6]. This suggests that high-mannose core sugars may be added to glycoprotein precursors in a post-translational fashion. Blocking of glycosylation with tunicamycin has been shown to lead to an accumulation of core proteins of gB and gD, visible by immuno-fluorescence microscopy at sites at the periphery of the nucleus [30].

Our results indicate that both gB and gD are first transported into the nucleus, in the form of non-membrane-bound proteins, and that within the nucleus they are strongly associated with sites of capsid assembly. Hypothetically, if translocation were then to occur across the INM, glycosylation of these proteins (embedded in the virion envelope) could still follow the prescribed sequence [21] as virus particles travel from the perinuclear space and ER cisternae to the Golgi apparatus. In theory, association between precursor glycoproteins and capsids in the nucleus could ensure that they were simultaneously targeted to the INM, and that each virion envelope acquired the requisite glycoproteins at the time of budding. Translocation of the envelope glycoproteins across the INM (rather than the ER) would obviate the need for multiple sorting signals to carry them first upstream of the default secretory pathway (towards the nuclear membrane) and later downstream (towards the cell surface). A further theoretical advantage of trafficking envelope proteins through the nucleus, would be achieved if single gene products (with the aid of post-translational processing) could be used for multiple functions at sequential stages of infection. The observation of specific tagging of nucleocapsids and centres of viral synthesis within the nuclei suggests that both gB and gD, probably in unglycosylated precursor form, could play a primary functional role

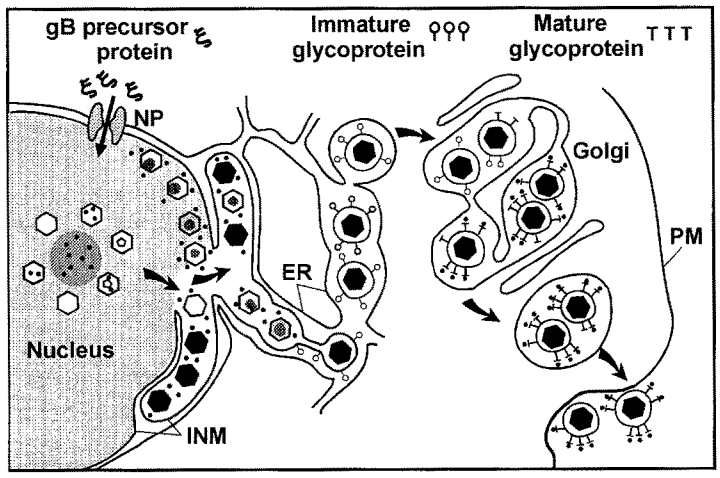

Fig. 7. Diagrammatic representation of the route of egress of progeny HSV particles and the proposed pathway for processing of the gB glycoprotein. The model proposes that viral capsids are assembled inside the nucleus, become filled with viral DNA and are targetted to the INM. The INM proliferates in tubulo-reticular fashion, encasing the maturing capsids, and tubules extend into the perinuclear space and the cisternae of the ER. Viral envelopes are acquired by constriction and fusion of the tubule walls and enveloped virions are transported via the secretory pathway through the ER and Golgi compartments and released to the outside of the cell by fusion of transport vesicles with the plasma membrane (*PM*). Sites of gB labelling are indicated by the small black dots. The most striking features are the presence of strong intra-nuclear labelling and the absence of labelling on any of the cytoplasmic membranes. We postulate that gB precursor proteins enter the nucleus via the nuclear pore (*NP*) complexes and associate with maturing capsids. Together with the capsids they are targetted to the INM and are inserted into the viral envelope at the site of envelopment. After translocation these proteins are glycosylated within the ER and immature glycoproteins (indicated by lollipop symbols) are processed to the mature form of the glycoprotein (T-symbols) as virions pass through the Golgi apparatus. The absence of labelling of envelope glycoproteins on virions within the ER and *cis*-Golgi compartments probably reflects processing events which result in the masking of the specific gB epitope. Characteristic clusters of gB spikes are labelled on extracellular virions. The proposed pathway for gD processing is similar to that for gB with minor differences; viz. gD can be labelled on the virion envelope soon after envelopment, but in a number of instances gD determinants are apparently not inserted into the virus envelope and remain detectable on the inside of the envelope of extracellular virions

in the maturation of infectious nucleocapsids. The strong association of anti-gB-gold with immature viral capsids, and specifically with the internal surface of empty capsids and the primary core of maturing capsids, is particularly intriguing, and could suggest a role in DNA packaging. Figure 7 sketches a proposed pathway of virus egress and postulated route of gB processing.

The subject of protein translocation across lipid bilayers has been a centre of intense interest in reviews [38, 41, 54, 56] published over the past two decades. The hypothetical translocation of HSV envelope proteins at the INM is obviously contrary to the currently accepted concept of how type-I membrane proteins

(including gB and gD) are processed. The universally favoured 'signal hypothesis' involves recognition of a suitable signal sequence on the nascent chain of a polypeptide by a "signal recognition particle" (SRP). After binding of the SRP to the ribosome and its emerging polypeptide, they are directed to the ER and "dock" onto a specific protein receptor. Following dissociation of the SRP from the ribosome-polypeptide complex, translocation is initiated, although the precise molecular mechanism of the translocation process has not been established [9, 15, 24, 44]. SRP probably functions in a manner akin to so-called "chaperone" proteins in that it prevents folding and keeps the nascent polypeptide in a "translocation competent" conformation. An unsolved puzzle has been the lack of any sequence homology between signal sequences on different transmembrane proteins; their only common property being an uninterrupted stretch of at least 6 hydrophobic amino-acids. Analogous signal sequences (and chaperone proteins) apparently direct proteins into the mitochondria, but the nature of these signals, and the mechanism of translocation across the double mitochondrial membrane, is equally poorly understood [26, 42]. There is little information regarding the INM as a site of translocation of proteins, and the unprecedented hypothesis that herpesviruses may utilise this route for the insertion and translocation of envelope proteins requires additional speculation on the existance of mechanisms which selectively direct the appropriate proteins to the INM. Such mechanisms could, for example, involve interactions with other virus-coded proteins in such a way as to encourage polypeptide folding, thus preventing recognition by SRP and conferring or exposing properties on the newly synthesized protein that promote nuclear targeting. If this sorting process were dependent upon the co-expression of certain other viral proteins, it might be impaired by genetic engineering of the viral genome, and would be inoperative in single gene in vitro expression systems. The concept of translocation at the INM is unorthodox, and at present unsubstantiated, but it could provide a novel selection mechanism to ensure that only the relevant HSV glycoproteins are included in the virion envelope.

It is reasonable to assume that some species of HSV-encoded glycoproteins are not incorporated into the virion envelope, and thus their transport to the cell membrane (or other sites of interaction) would not necessitate initial targeting to the INM, but follow the conventional route of processing initiated on ER-bound ribosomes. This assumption is based on immunogold labelling experiments in which we were unable to demonstrate gE on the virion envelopes despite considerable gE-labelling on cell membrane fragments (unpubl. obs.). The converse is true for both gB and gD which are distinctively labelled in virion envelopes but were not detected on the plasma membrane of infected cells (this study). Immunofluorescent (IF) labelling of gB and gD at the surface of infected cells can be accounted for by the observed tendency of virus particles to remain associated with the plasma membrane after their release from the cell. Without recourse to high resolution immuno-labelling, interpretation of IF labelling at the "nuclear periphery" is imprecise. At the resolution of light microscopy, it is not possible to establish whether the fluorescent tags are binding to inner or

outer nuclear membranes; or to components in budding virions in the perinuclear space; or even to proteins lining the inner aspect of the INM. Furthermore, the extent to which the INM invades the cytoplasm (in the ER cisterna) during HSV infection (see Fig. 6d) should be an important consideration in the interpretation of "cytoplasmic" immunolabelling (Fig. 6e) at both light and electron microscopic levels.

It appears likely that factors governing the targeting of envelope glycoproteins to the INM differ according to the specificity of the glycoprotein. Late in infection, gD probes did bind to the INM, but strikingly only to those areas of the membrane which had arisen as the result of virus-induced proliferation, and were not obviously involved in virus envelopment. gB was not detected in the same areas as gD (even in double-labelling experiments) but we did see small pockets of clustered gB spikes budding from the INM into the perinuclear space. These may explain the origin of the small gB-rich membrane vesicles that have been recovered from culture fluids of HSV-infected cells [50]. gB spikes are frequently clustered on the envelope of mature virions ([50, 51], this study) which may reflect mechanisms of intracellular viral morphogenesis. Capping of gB spikes may, for example, play a role in the pinching-off process of envelopment. Unlike gB, where labelling was transferred from sub-envelope sites to the outer surface of the envelope by the time that virions were released from the cell, gD was still detected on the inner surface of numerous extracellular virus particles, both in cryosections of infected cells, and in negatively stained virus suspensions. This shows that some gD determinants accumulate within the virion tegument and are not inserted into the envelope. In summary, this ultrastructural study gives new insight into the processes of HSV envelopment and egress, and provides immunocytochemical evidence of the presence of HSV glycoproteins in unexpected sites.

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