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Review

The role of the *trans*-Golgi network in varicella zoster virus biology

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Abstract. The task of assembling nascent virions presents a formidable challenge to large, enveloped DNA viruses such as varicella zoster virus (VZV). After parasitising the host cell's compartmentalised biosynthetic machinery, viral constituents must be brought together in appropriate proportions for packaging and export. Recent evidence places the *trans*-Golgi network (TGN) in an orchestrating role with respect to the assembly, envelopment and egress of herpesviruses. This role accords with known functions of the TGN in the uninfected cell. The targeting of viral glycoproteins to the TGN appears to provide a crucial platform for viral assembly. Tegument proteins, interacting with the cytoplasmic domains of glycoproteins, in turn recruit nucleocapsids to the developing supramolecular array. Molecular studies are continually refining understanding of these processes, building upon elegant electron microscopic data. Knowledge of VZV's use of endogenous trafficking pathways from the TGN sheds light on important aspects of viral behaviour in vitro and in vivo.

Key words. Herpesvirus; VZV; envelopment; TGN; mannose 6-phosphate receptor.

Varicella zoster virus in human disease

Varicella zoster virus (VZV) is a remarkably successful pathogen. It is so contagious and ubiquitous that, in the absence of vaccination, few individuals escape childhood infection [1]. Primary infection (varicella; chickenpox), importantly, is not often lethal, and its aftermath is lifelong latent infection of sensory ganglia with VZV. Long-term immunity protects the host from subsequent primary infections, which are rare [2], but provides incomplete protection against zoster, the disease that results when VZV reactivates from latency [1]. Reactivations return VZV to the skin of the host, now in the dermatomal distribution of the ganglion in which latent VZV has reactivated. The virus is thus provided with two opportunities to spread to additional hosts: first, during primary infection, when VZV generates infectious enveloped particles in the epidermis, and second, during reactivation, when the virus is transported via nerves back to the epidermis and once again generates infectious virions [3–6]. Despite the widespread immunity that arises as an epidemic sweeps through paediatric populations, therefore, the sporadic occurrence of zoster in adults continues to provide a source of VZV to infect new generations of susceptible hosts.

Occasionally, the paediatric targets of VZV cannot survive the initial infection, sometimes, but not always, because they are immunocompromised [7]. Immunosuppression may lead to early, prolonged and/or severe zoster. VZV can also be problematic when primary infection is postponed until adult life [8] or when a victim of zoster (usually an adult) is left with post-herpetic neuralgia [9–11] as a residuum of infection. The introduction of the varicella vaccine has mitigated these problems, but

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VZV still causes significant disease [12, 13]. The varicella vaccine is not in widespread use outside the US, and even if it were to be, the presence of latent wild-type VZV in an adult population subject to zoster assures that VZV will not disappear anytime soon [9]. Understanding the pathobiology of VZV thus continues to be important from a medical point of view and offers the hope that this knowledge can be turned against the VZV for therapeutic advantage.

VZV is delivered to late endosomes when propagated in vitro

Current understanding of VZV envelopment in the TGN grew from early morphological observations of the virus in tissue culture. It is a paradox that an agent as highly transmissible as VZV should be notoriously slow growing and cell-associated when propagated in vitro [14]. Viral spread in this context is believed to involve the fusion of adjacent cells with transfer of unenveloped infectious particles that do not emerge into the extracellular space. Cook and Stevens [15] first suggested that the cell association of VZV might be explained by the intracellular degradation of the viral 'coat' or envelope before virions are released from infected cells. This suggestion was supported by their observation that virions in cultured cells were present in vacuoles that they thought were lysosomes, and by the 'pleomorphic' morphology of extracellular virions in vitro, which they believed suggested that virions were released with an envelope that had been digested prior to exocytosis. In the study of Cook and Stevens, Herpes simplex virus (HSV), which is far less cell-associated than VZV and served as a standard of comparison, was not pleomorphic when secreted in vitro.

The idea that cultured cells divert newly synthesised VZV to the endosomal pathway received further support when the lysosomal enzyme, acid phosphatase, was found to be present in the intracellular vacuoles within which virions accumulate [16]. These vacuoles, however, were eventually identified not as lysosomes, but as late endosomes (prelysosomes) based on their accessibility within 1 h to a fluid phase marker that enters cells by endocytosis [17]. Late endosomes are branch points in the endosomal pathway where mannose 6-phosphate receptor (MPR)-containing vesicles, bearing a cargo of newly synthesized lysosomal enzymes, fuse with endosomes carrying internalized material from the cell surface [18–23] (fig. 1). The environment within VZV-containing late endosomes was demonstrated to be acidic by showing that they trap weak bases, such as 3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine (DAMP), which can be fixed in endosomes and subsequently visualized by electron microscopic immunocytochemistry [24]. The acidic environment of the interior of late endosomes normally causes lysosomal enzymes to dissociate from MPRs [22]. The MPRs recycle to the cell surface and the TGN, while the lysosomal enzymes, and most of the endocytic cargo, are delivered to lysosomes [18, 19]. VZV, however, is not delivered to lysosomes but instead is released by exocytosis [17, 25]. The released viral particles are degraded and they are also not infectious [15], presumably reflecting the effects of the acidic late endosomal milieu.

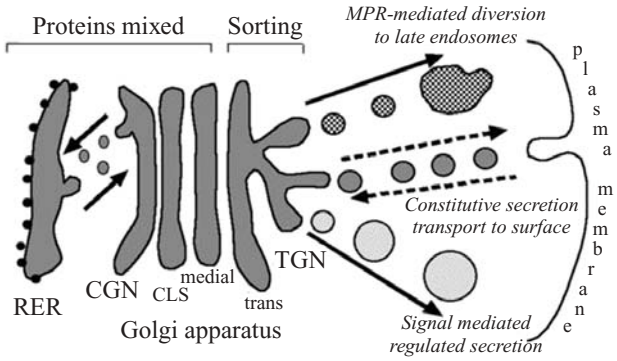

Figure 1. Protein sorting from the TGN. Proteins synthesised in the endoplasmic reticulum traverse the Golgi apparatus to reach the TGN. Their subsequent fate depends on the molecular recognition of specific sorting signals. The default pathway is constitutive secretion, i.e. direct export to the cell surface. Specific signals mediate diversion of some molecules to the regulated secretory pathway. Alternatively, interaction with the mannose-6-phosphate receptor (MPR) enables routing of certain glycosylated proteins to the late endosomal-prelysosomal compartment. MPRs recycle to the plasma membrane and thence back to the TGN, leaving behind ligands such as lysosomal enzymes which dissociate at the acidic late endosomal pH.

The observation that newly assembled VZV in cultured cells is transported to late endosomes provides an explanation for the cell association of VZV but appears counterproductive for the propagation of the virus. Host-to-host transmission of VZV is assumed to require the formation of infectious enveloped virions, which are highly abundant within the cutaneous lesions of patients with chickenpox or shingles [26, 27]. In some cells of an infected subject, therefore, VZV must escape diversion to late endosomes. Within other cells of such a host, however, degradation in endosomes might well be advantageous to VZV from an evolutionary point of view. Clearly, overwhelming a host deprives a virus of a home and is incompatible with evolution as a successful parasite. If infectious virions cannot be released from most infected cells (because virions move from their point of assembly in the TGN to late endosomes), then VZV is forced to spread within a host by cell-to-cell contact (fusion of adjacent cells). In that case, dissemination could be expected to occur slowly, perhaps helping to explain the 2-week-long incubation period of chickenpox. This postponement of the emergence of infectious viral particles may have been selected in evolution because it gives

time for the engagement of adaptive mechanisms of host defence. The eventual release of host-threatening virions in the epidermis also has the effect of causing minimal damage to the host whilst allowing efficient contagion during primary infection and, we will argue, the establishment of latency (with the possibility of subsequent reactivation). We suggest that the slowing of dissemination by diversion to late endosomes might thus be considered a fail-safe modification that saves VZV from what would otherwise be the catastrophic consequences of its own virulence.

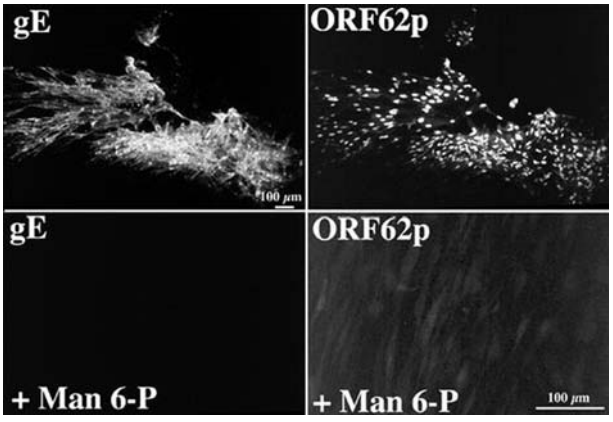

Figure 2. Mannose 6-Phosphate inhibits infection of HELF cells by cell-free VZV. Monolayers of cultured HELF cells (human embryonic lung fibroblasts) were exposed to cell-free VZV in the absence (upper panels) or the presence (lower panels) of Man 6-P (20 μM) After several days and the appearance of cytopathic effects in the control condition, cells were fixed, permeabilised and stained for dual-colour immunofluorescence microscopy with antibodies against VZV ORF68 (gE, left panels) and ORF62 (right panels). In the upper panels, a plaque of VZV-infected cells is seen to stain with both antibodies, whereas cells exposed to VZV in the presence of Man 6-P failed to show any evidence of infection (lower panels).

VZV glycoproteins interact with MPRs: virus entry

In routing from late endosomes to the cell surface, VZV follows the itinerary of MPRs (fig. 1). It was therefore natural to investigate the possibility that an interaction of VZV with these receptors is causally related to the diversion of VZV to late endosomes in a manner analogous to that which diverts Man 6-P-bearing lysosomal enzymes to late endosomes [18, 19]. This possibility was supported by the observation that at least four viral glycoproteins (gB, gE, gH and gI) contain N-linked complex oligosaccharides in which a core mannose residue is Man 6-P [17]. Strong circumstantial evidence exists for the interaction of viral glycoproteins with cell surface MPRs. Man 6-P protects cells from infection by cell-free (enveloped) VZV in a concentration-dependent manner [17], suggesting that Man 6-P competes with VZV for access to cellular receptors (fig. 2). That these receptors are MPRs is suggested by the observation that the efficacy of a series of phosphorylated sugars in protecting cells from VZV infection parallels the affinities of these sugars for MPRs. Infection of cells by VZV is also blocked when the transport of MPRs to the plasma membrane is prevented by exposure of cells to a weak base, such as chloroquine [17]. Additional support for a role of cell surface MPRs in VZV uptake has come from experiments with transfected cells that express mutant forms of gI, one of the glycoproteins of the VZV envelope that have been demonstrated to contain Man 6-P as a core mannose residue [17]. When a construct consisting of only the ectodomain of gI (lacking both transmembrane and endodomains) is expressed by transfected cells, the ectodomain is glycosylated, secreted and taken up by non-transfected cells of the same cultures [28]. Uptake of the gI ectodomain, like that of secreted lysosomal enzymes, is blocked by Man 6-P, and thus is probably due to MPR-mediated endocytosis [28]. In a further, recent study of the interaction of VZV with MPRs, five stable human cell lines deficient in cation-independent MPRs (MPR^{ci}) were generated from parental human melanoma cells by expression of antisense complementary DNA (cDNA) or siRNA-like transcripts [J. Chen et al., unpublished observations presented at the 29th International Herpesvirus Workshop in Reno, NV, 2004]. In contrast to the parental cells, which are readily infected by cell-free VZV, all of these MPR^{ci}-deficient lines were found to be resistant to infection by cell-free, but not cell-associated, VZV. These observations suggest that VZV entry is MPR-dependent.

Like gI, the HSV glycoprotein gD acquires Man 6-P and binds to MPRs [29]. MPRs may thus facilitate the entry of HSV into target cells, but their role in viral entry is minor at best, and MPRs seem to be far less critical for HSV than VZV infection [30, 31]. Despite the putative role of MPRs in VZV entry, however, Man 6-P does not prevent the adherence of VZV to cell surfaces [32]. Instead, as with HSV [33, 34], VZV adherence is blocked by heparin or by inhibiting the production of heparan sulphate proteoglycan (HSPG) [32]. VZV gB appears to be important in the interaction of VZV with HSPG [35]. VZV entry is thus likely to be a two-stage process in which initial binding to HSPG stabilizes VZV on the plasmalemma of target cells, enabling subsequent viral entry in an MPR-dependent manner.

The evidence that VZV can interact with the MPR and follows the receptor's itinerary from late endosomes to the plasma membrane is compelling. An attractive hypothesis is that MPRs are also responsible for delivering VZV to the late endosomal compartment from the TGN. Such an MPR-mediated delivery system would be entirely compatible with the physiological role of MPRs with respect to lysosomal enzymes, which are diverted

from the TGN to late endosomes because they bind to MPRs [24]. Topologically, this implies prior envelopment of VZV derived from the TGN or an interrelated membrane-bound compartment that contains MPRs. The glycoproteins of the VZV envelope must be placed in the correct orientation with respect to MPRs; the ectodomain of the MPR would have to be apposed to the ectodomains of the viral glycoproteins. If this relationship were to be attained then, like lysosomal enzymes, newly enveloped virions would be packaged within transport vesicles. The ectodomain of the MPR would project into the VZV-containing vesicular lumen, while the endodomain of the MPR would project into the cytosol, where it would be exposed to adaptor molecules in the cytoskeleton that mediate transport to late endosomes.

This hypothesis is supported by further experiments using the MPR^{ci} -deficient cell lines described above. These not only resist infection by cell-free VZV, but once infected with cell-associated VZV, they secrete infectious virions to the ambient medium with an efficiency that is more than 10-fold greater than that of the parental melanoma cells [J. Chen et al., unpublished observations presented at the 29th International Herpesvirus Workshop in Reno, NV, 2004]. The diversion of newly enveloped VZV to late endosomes thus appears to be mediated by intracellular MPRs, while plasmalemmal MPRs are necessary for infection of cells by cell-free VZV. Biopsies from VZV-infected human skin confirm that maturation of squames is accompanied by the loss of expression of MPR^{ci} [J. Chen et al., unpublished observations presented at the 29th International Herpesvirus Workshop in Reno, NV, 2004], whereas these are otherwise ubiquitously expressed [36]. Loss of MPR expression may be the mechanism whereby superficial epidermal cells uniquely fail to divert VZV to endosomes and thus constitutively release infectious VZV.

VZV acquires its mature envelope from the TGN

The hypothesis that VZV is enveloped in the TGN is similar to that originally proposed for pseudorabies virus (PRV) and later extended to HSV and other herpes viruses [37–39]. The nucleocapsids of HSV [40, 41], PRV [38] and VZV [24] are all assembled in the nuclei of infected cells. The nucleocapsids then acquire an envelope as they pass through the inner nuclear membrane to enter the perinuclear cisterna, which is continuous with the rough endoplasmic reticulum (RER). This much is accepted for all of the α-herpesviruses [37]. Subsequent steps in viral egress have been controversial. If the primary envelope that is acquired by VZV budding into the perinuclear cisternae were to become the final envelope of the virus, then the glycoproteins that are embedded in the viral envelope would have to be transported from the RER where they are synthesized to the inner nuclear membrane. Such a transport process, occurring by lateral diffusion within the plane of the membrane, would require envelope glycoproteins to travel from the outer nuclear membrane, which is continuous with the RER, to the inner nuclear membrane. This route would require the viral glycoproteins to go through nuclear pore complexes. There is no evidence that viral glycoproteins do so. In fact, virions in the perinuclear cisterna/RER are not radioautographically labeled by ^{3}H-mannose in pulse-chase experiments [24], in contrast to mature virions. Because N-linked sugars are added to proteins co-translationally, the failure of ^{3}H-mannose, at any time of chase, to label the primary envelope acquired by VZV from the inner nuclear membrane clearly implies that the primary envelope cannot be the definitive envelope of VZV. The N-linked oligosaccharide-containing membrane thus has to envelop VZV after the primary envelope has been lost. If the final envelope is to be acquired not from the inner nuclear membrane, but from the TGN instead, then the particles of VZV that have initially been enveloped at the inner nuclear membrane would have to shed that membrane in order to acquire another from the TGN. The number of mechanisms by which an enveloped particle within the cisternal space can shed a membrane are limited. One mechanism, and one that the intracisternal virions counterintuitively appear to utilise, is to fuse their nuclear membrane-derived envelope with membranes of the RER [24]. The primary envelope thus is lost and merges with RER membrane while viral nucleocapsids move from the cisternal space to the cytosol. VZV thus undergoes de-envelopment and re-envelopment.

Unenveloped nucleocapsids are frequently seen in the cytosol of cells infected with any of the α-herpesviruses, although their presence was, at one time, dismissed as inconsequential or lacking in physiological significance [37]. The nuclear membrane-derived envelopes of PRV [39] and VZV [24] thus are temporary. A consideration of tegument proteins, which lack signal sequences and thus are synthesised in the cytosol, may explain why the curious process of de-envelopment and re-envelopment evolved. Tegument protein cannot be recognised in the space between the nucleocapsid and the viral envelope within the temporarily enveloped virions that lie in the perinuclear cisternae (fig. 3, panels A, B). These virions and the inner nuclear membrane also lack the gE immunoreactivity of mature virions. The de-envelopment and subsequent re-envelopment of VZV has been proposed to be a mechanism that enables tegument to be incorporated into mature viral particles (as seen in fig. 3, panels C, D). Naked cytosolic nucleocapsids are presumably the form in which cell-associated viral transfer occurs when an infected cell fuses with its neighbour. The morphology of TGN cisternae in infected cells, apparently enveloping tegument and a nucleocapsid in a virion,

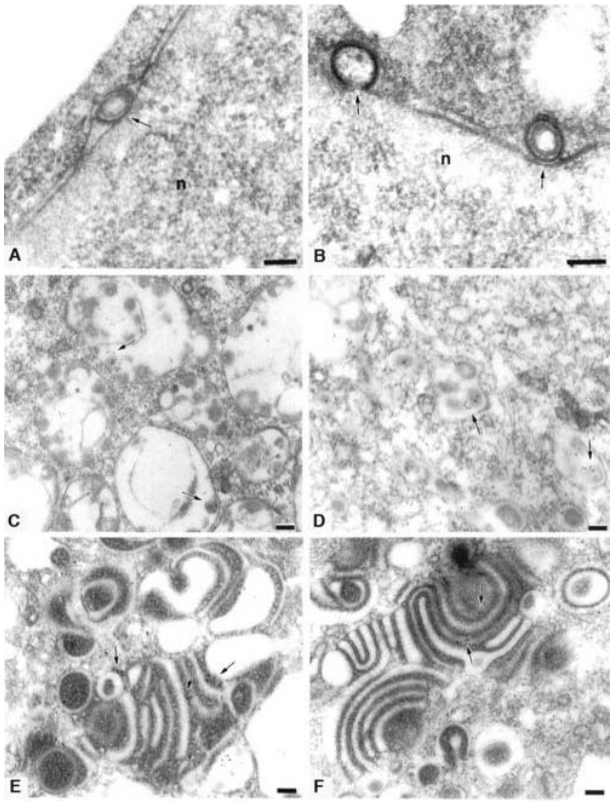

Figure 3. Immunogold detection of tegument protein (ORF10p), visualised by transmission electron microscopy of cells infected with wild-type (*A–B*, *C–D*) or gI mutant (*E–F*) VZV. *A*, *B*: Primary enveloped virions, adjacent to the nucleus (n) and within the perinuclear cisterna (arrows), lack ORF10p immunoreactivity. *C*, *D*: Pleomorphic virions and ORF10p reactivity (arrows) accumulate within late endosomes of cells infected with wild-type VZV. *E*, *F*: In cells infected with gI mutant virus, ORF10p reactivity (arrows) is found within electron dense material adhering to bizarrely shaped stacks of TGN membranes. Bars, 150 nm.

is consistent with the idea that the TGN is the site of final VZV envelopment.

Recent studies have suggested that HSV, like VZV and PRV, could be subject to de-envelopment in the RER and subsequent re-envelopment in or near the Golgi apparatus. HSV was first postulated to be transported entirely within the cisternal compartment [via the constitutive pathway (fig. 1)] to the plasma membrane where release would occur by exocytosis [41–43]. The lipid composition of the HSV envelope, however, is similar to that of the Golgi, and not to that of the nuclear envelope [44]; moreover, a mutated HSV gH, which is retained in the RER, is not incorporated into the envelopes of secreted HSV [45]. HSV virions and HSV gD reach endosomes, either by endocytosis of extracellular virions or by routing from the TGN [31]. (There is no analogue of HSV gD in the VZV genome [46].) There is also evidence that HSV is de-enveloped in infected neurons and subjected to anterograde transport without an envelope, to be enveloped prior to secretion within the nerve terminals [47].

Conceivably, there may be two methods of enveloping α-herpesviruses: one, exemplified by the mechanism originally thought to be utilised by HSV, in which the envelope is derived from the inner nuclear membrane, and another, in which the first envelope is lost by fusion with the RER and the final envelope is derived from the TGN. It seems more likely, however, that a common envelopment-de-envelopment-re-envelopment model is likely to be utilized by all of the α-herpesviruses. A testable prediction of this model is that all of the glycoproteins of the viral envelope must be delivered to the TGN. In the case of VZV, this requirement is close to having been satisfied.

Envelope glycoproteins are delivered to the TGN

Quantitative electron microscope (EM) radioautographic pulse-chase experiments in VZV-infected cells reveal that ^{3}H-mannose-containing viral glycoproteins are transported from the RER to the Golgi (including the TGN) and subsequently appear in the enveloped virions of late endosomes [24]. In infected cells, all of the glycoproteins that have been examined thus far (gB, gC, gE, gH, gI, gL; gK has not yet been investigated) are sorted selectively to the TGN [48]. Experiments in which viral glycoproteins are expressed individually in transfected cells have demonstrated 'autonomous' targeting of both gE [49, 50] and gI [28] to the TGN. The endodomain of gB contains a sequence that targets gB at least as far as the Golgi apparatus [51].

gE reaches the TGN as a result of signals encoded in its endodomain [52]. In order to determine the location of the TGN-targeting sequence, cells were first transfected with cDNA encoding either gE wild type (gE_{wt}) or a truncated mutant (gE_{trc}), lacking transmembrane and cytosolic domains. gE_{wt} accumulated in the TGN and reached the plasmalemma, but none was secreted. In contrast, gE_{trc} was retained and probably degraded in the RER; none was found on cell surfaces, but some was secreted. The distribution of gE_{trc} was not affected by deletion of potential glycosylation sites. Thus, TGN localisation was not intrinsic to the ectodomain of gE. Subsequent experiments were facilitated by the study of chimeric proteins in which the ectodomain of gE was replaced by the marker ectodomain, tac. These experiments linked TGN targeting activity to the endodomain of gE, which was necessary and sufficient to route chimeric proteins containing the tac ectodomain to the TGN.

Similar techniques enabled the identification of a targeting sequence (AYRV) and a second, acidic amino acid-rich region of the gE endodomain (putative signal patch). Each of these sequences was sufficient to cause expressed protein to co-localise with TGN markers [49]. Point mutagenesis showed limited tolerance to conservative amino acid substitution within the AYRV sequence.

Exposure of live transfected cells to antibodies to the tac ectodomain revealed that the TGN targeting of expressed tac-gE chimeric proteins occurred as a result of selective retrieval from the plasmalemma by endocytosis. Antibodies do not cross the plasma membranes of living cells; they enter cells by endocytosis and, barring an intervention, would be routed to endosomes. Non-transfected control cells that do not express gE take up antibodies to gE poorly and route them only to endosomes. The fact that antibodies to gE are concentrated in the TGN when living cells that express gE are exposed to anti-gE establishes that the antibodies bind to gE on the plasma membrane and are carried to the TGN when gE is retrieved by endocytosis. The dependence of this process on the same sequences that target gE to the TGN indicates that gE targeting involves plasmalemmal transport, endocytosis and the selective TGN targeting of gE-containing endosomes.

gI does not have the AYRV or putative signal patch that have been identified in gE; nevertheless, gI is targeted to the TGN when expressed in transfected Cos-7 cells, [28], suggesting that gI also contains TGN targeting information. Surface labelling of singly transfected cells revealed that gI is not retrieved to the TGN from the plasma membrane, again in contrast to gE. TGN targeting of gI depends on the T^{338} of its endodomain and was lost when T^{338} was deleted or mutated to A, S or D [28]. The endodomain of gI was sufficient, if it contained T^{338}, to target to the TGN a fusion protein with the ectodomain of tac.

The glycoproteins gE and gI have long been known to interact to form a complex [53, 54], with the result that either full-length protein can route the ectodomain of the other to the TGN [55]. The physiological consequence of this interaction is not yet understood. Following co-transfection, both gI and gE were retrieved to the TGN from the plasma membrane in 26.7% of cells, neither gI nor gE was internalized in 18.3%, and gE was retrieved to the TGN while gI remained at the plasma membrane in 55% [55]. Although gE and gI can interact, therefore, they either do not always do so or the complex can be broken.

Interactions with the TGN-targeted glycoproteins may be important for gC, gH and gL, immunoreactivities of which were not detected in the TGN when expressed individually in transfected Cos-7 cells [48]. Indeed, when transiently transfected individually, neither gH nor gL immunoreactivities could be detected. When expressed together, however, gH and gL were each found in a RER pattern and on cell surfaces. These observations support the reported idea that gL acts as a chaperone to enable its gH partner to exit the RER [56–59]. When cells that were stably transfected with cDNA encoding gH were co-transfected with cDNA encoding gL, the gH:gL complex was found in cytoplasmic vesicles thought to be endosomes, a pattern that differed from that seen after transient transfection, perhaps because trafficking of these proteins is affected by the degree to which each is expressed (fig. 4A). Again, no matter how cells were transfected, none of the immunoreactivity either of gH or gL was located in the TGN. In VZV-infected cells, however, gE, gB, gC, gH and gL are all concentrated in the TGN [48]. Because those VZV glycoproteins that lack targeting sequences (gC, gH and gL) concentrate in the TGN of infected cells, it has been proposed that gE and gI, which have such sequences, serve as navigators, forming complexes that direct the signal-deficient glycoproteins to the TGN. Accordingly, when cells stably expressing gH were co-transfected with cDNAs encoding gE and gL, all three glycoproteins localised to the TGN (fig. 4B). Duus and Grose have also reported that gE can interact with gH, although they did not investigate the intracellular localisation of the resulting gE:gH complex [56]. Preliminary observations have suggested that gI can also redirect the gH:gL complex to the TGN [28].

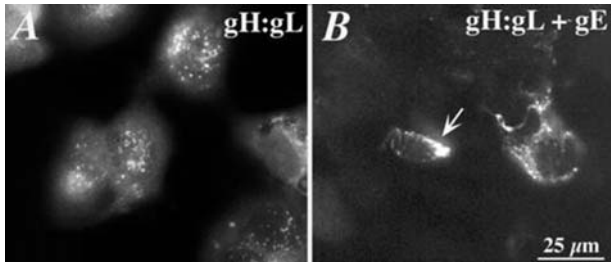

Figure 4. gE can target co-expressed gH:gL to the TGN. Fluorescence microscopy of transfected tissue culture cells. (*A*) In the absence of gE, gH and gL colocalise to cytoplasmic vesicles (see text) (*B*) On cotransfection with gE, the immunoreactivities of all three glycoproteins are found in the TGN.

Clues to the mechanism of viral assembly

Mechanisms thus exist to deliver VZV glycoproteins to the TGN for incorporation into the viral envelope. What of the other crucial constituents of the virion, the nucleocapsid and tegument proteins? The fusion of primary enveloped virions with the RER releases them from the cisternal space into the cytosol, where tegument proteins are also synthesized on free ribosomes. As alluded to above, EM studies appear to show the envelopment of cytosolic nucleocapsids by invagination into the TGN, acquiring as they do so an intervening layer of tegument. Recent studies suggest a critical role for VZV glycoproteins, particularly gI, in this process.

In infected cells, EM immunocytochemistry reveals that the tegument protein ORF10p coats the cytosolic surfaces of the putative enveloping TGN membranes. Several pieces of evidence indicate that tegument may be binding

to the cytosolic domains of VZV glycoproteins. First, in transfected cells, co-expression with gI is able to target the tegument protein, ORF10p, to the TGN [28]. In the absence of gI, ORF10p is diffusely cytosolic. Second, when VZV-infected cells are exposed to brefeldin A, which causes the RER and Golgi apparatus to fuse and thus disrupts trafficking through the cisternal space [60, 61], great masses of tegument accumulate in the cytosol. These masses are adherent to membranes of the RER, in which VZV glycoproteins are concentrated [24]. Nucleocapsids adhere to and embed themselves in the tegument mass. The adherence of nucleocapsids to tegument and tegument's adherence to glycoprotein endodomains may enable nucleocapsids and tegument to be incorporated into enveloping virions in the TGN.

The sacs of the TGN flatten and curve in infected cells to acquire the shape of the letter C. The immunoreactivities of viral (gE, gI) and cellular (MPRs) proteins are found on opposing concave and convex faces of the C-shaped TGN cisternae that wrap around and enclose tegument and a nucleocapsid [24]. This segregation of viral glycoproteins and MPRs may serve to organise viral assembly and egress and, possibly, to permit cellular MPRs to interact with glycoproteins of the VZV envelope (fig. 5). Tegument coats the concave face of the C-shaped enveloping cisternae of the TGN. A nucleocapsid attaches to the tegument-coated face, and both tegument and nucleocapsid become enveloped by the encroaching arms of the TGN sac (fig. 5B, C). The membranes of the concave and convex faces then split, so that the original VZV glycoprotein-containing concave face becomes the viral envelope, while that of the MPR-containing convex face becomes a transport vesicle for the newly enveloped virion (fig. 5D). Because it contains MPRs, the vesicle follows their itinerary to late endosomes, delivering virions to the endosomal lumen [62–64]. Multiple virions thus accumulate in late endosomes, which function as a sink to which individually enveloped virions are transported in the MPR-containing transport vesicles.

In support of the model of viral envelopment described above, deletion of gI, or its C-terminal domain, prevents the segregation of cellular and viral proteins in TGN cisternae [28] (fig. 6). As a result, glycoprotein immunoreactivity spreads around the entire circumferences of TGN cisternae, which fail to indent, and their cytosolic surfaces become completely coated with material that has the appearance of tegument and displays ORF 10p immunoreactivity (fig. 3 panels E, F; fig. 6). The adjacent tegument-lined sacs adhere to one another, forming stacks of cisternae and, eventually, turn the TGN into a bizarre multi-chambered 'honeycomb'. When this occurs, envelopment of VZV in the TGN is blocked. No enveloped viruses can be found in post-TGN late endosomes, and none are secreted. The blockade of envelopment that occurs when gI or its endodomain is deleted is not lethal to VZV in vitro where infection spreads by cell-to-cell contact. The deletion of gI or its C-terminal region, however, does prevent the spread of infection in human skin maintained as a transplant in humanised severe combined immunodeficient (SCID-Hu) mice [65]. The spread of infection in human skin, at least after transplantation to SCID-Hu mice, thus may depend on the formation of enveloped virions.

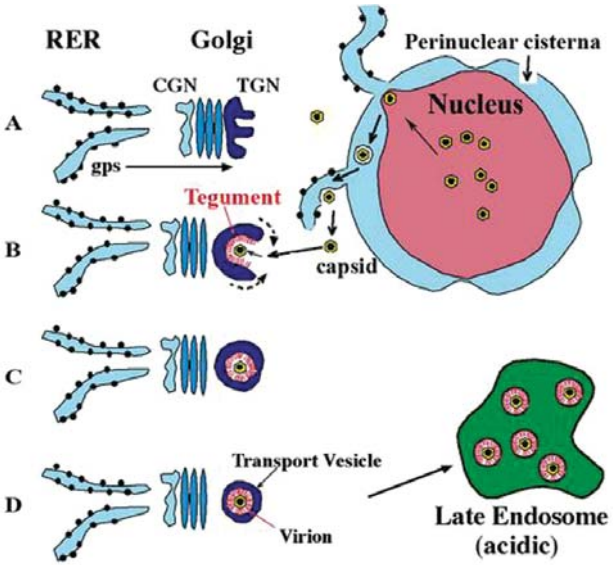

Figure 5. Proposed egress of VZV. Nucleocapsids assemble in nuclei and bud into the perinuclear cisterna/rough endoplasmic reticulum (RER), acquiring a primary envelope; this fuses with the RER, delivering capsids to the cytosol. Glycoproteins (gps) are synthesised on RER and delivered to the TGN independent of nucleocapsids and tegument (*A*). Note that some gps (notably gE) take a detour via the plasma membrane en route to the TGN. The TGN forms C-shaped cisternae, each of which envelops a VZV nucleocapsid (*B*); viral glycoproteins are segregated to the concave face of these cisternae, while MPRs and other cellular proteins are restricted to the convex face of the same C-shaped cisternae. The encroaching arms of the TGN fuse (*C*) with the creation of a double membrane-bound compartment (*D*). The concave, inner face becomes the gp-containing VZV envelope, while the convex, outer face functions as a transport vesicle, resulting in MPR-dependent delivery of VZV to late endosomes in most cells.

Future directions

The work described above has provided a tentative outline of the pathobiology of VZV, but large areas of uncertainty remain. One issue of great clinical importance is the mechanism by which VZV establishes latency. New work suggests that latency may be dependent on the production of infectious virions [66], which emerge in the skin [J. Chen et al., unpublished observations presented at the 29th International Herpesvirus Workshop in Reno, NV, 2004]. As noted earlier, MPRs appear to be necessary, both for the ability of cell-free VZV to enter naive

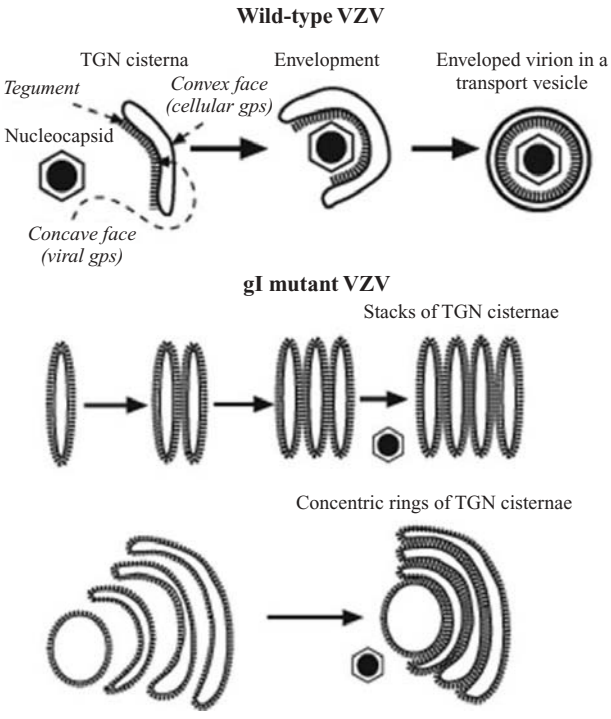

Figure 6. Envelopment of wild-type VZV in the TGN and effects on envelopment of gI mutations. Top: Wild-type VZV is enveloped in the TGN (see also legend to fig. 5). Cellular proteins are segregated to the convex face and viral gps to the concave face of TGN 'wrapping' cisternae. Tegument adheres to the concave face (probably via viral gp endodomains), and nucleocapsids adhere to tegument. Thus an intervening layer of tegument is trapped between the new VZV envelope and the nucleocapsid. Bottom: In the absence of gI, viral proteins are not segregated within the TGN; tegument circumferentially coats 'wrapping' cisternae, which adhere, forming stacks and rings. VZV is not enveloped.

cells, and for the diversion of VZV to late endosomes during intracellular transport within infected cells. The diversion of VZV to late endosomes, which leads to the inactivation of varicella virions before they are released from infected cells, would account for the cell-association of VZV in vitro, and probably also for the slow spread of VZV within an infected host. This seemingly paradoxical inactivation of varicella virions before they are released from most cells of the body was probably selected for during evolution, because it prevents this highly infectious agent from overwhelming its host (which would be counterproductive for a successful parasite). Expression of the MPR^{ci} is downregulated in the superficial epidermis, which permits infectious varicella virions to emerge in this location (and only in this location) to infect new hosts. Sensory nerve fibers enter the epidermis, and the exposure of their terminals to cell-free VZV there could well account for the predilection of VZV to establish latency in sensory ganglia.

In support of the hypothesis that cell-free virions are important in the establishment of latency, VZV has recently been found to infect neurons in ganglia isolated from the guinea pig intestine, which has emerged as a valuable model of VZV pathogenesis [66]. When enteric ganglia, in the absence of fibroblasts, are exposed to cell-free VZV, latent infection results. Neurons survive indefinitely in vitro, despite their expression of up to six viral proteins (ORFs 4, 21, 29, 40, 62, 63). All of these proteins, however, are cytosolic. In contrast, when fibroblasts are present at the time of infection, lytic infection is induced. Neurons now die within 48–72 h, all VZV proteins are expressed and critical immediate early proteins, ORFs 29 and 62, translocate into the nucleus. To cause infection to be lytic, the fibroblasts must be present at the time of infection; adding them to latently infected neurons does not cause latent infection to become lytic. Cell-free VZV was demonstrated to be able to infect fibroblasts, which by fusing with neurons induced lytic infection. Fusion of infected fibroblasts allows all VZV proteins to enter targeted neurons, while infection by a varicella virion introduces only structural proteins (those present in the virion). The hypothesis that forcing expression of a non-structural protein reactivates VZV from latency in neurons was thus tested. This hypothesis was confirmed, with VZV ORF 61p and with its HSV orthologue, IPC0. These studies, now extended to enteric ganglia of mice, imply that only cell-free VZV can induce latency in neurons. That implication fits well with the observation that infectious cell-free VZV is released in the epidermis where it comes into contact with the cutaneous branches of sensory neurons where VZV establishes latency.

Future therapies aimed at preventing the release of infectious virions in the skin could, if the dependence of the establishment of latency on cell-free VZV is correct, be expected to reduce later zoster as well as prevent transmission. In order to design such therapies we need to know more about how VZV assembly occurs, and how virions escape the endosomal pathway in keratinocytes. Such knowledge may also help us to develop safer and more efficacious vaccines.

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