Intracellular Transport of Newly Synthesized Varicella-Zoster Virus: Final Envelopment in the *trans*-Golgi Network

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The maturation and envelopment of varicella-zoster virus (VZV) was studied in infected human embryonic lung fibroblasts. Transmission electron microscopy confirmed that nucleocapsids acquire an envelope from the inner nuclear membrane as they enter the perinuclear-cisterna-rough endoplasmic reticulum (RER). Tegument is not detectable in these virions; moreover, in contrast to the mature VZV envelope, the envelope of VZV in the RER is not radioautographically labeled in pulse-chase experiments with [³H]mannose, and it lacks gpl immunoreactivity and complex oligosaccharides. This primary envelope fuses with the RER membrane (detected in cells incubated at 20°C), thereby releasing nucleocapsids to the cytosol. Viral glycoproteins, traced by transmission electron microscopy radioautography in pulse-chase experiments with [³H]mannose, are transported to the *trans*-Golgi network (TGN) by a pathway that runs from the RER through an intermediate compartment and the Golgi stack. At later chase intervals, [³H]mannose labeling becomes associated with enveloped virions in post-Golgi locations (prelysosomes and plasma membrane). Nucleocapsids appear to be enveloped by wrapping in specialized cisternae, identified as the TGN with specific markers. Tegument-like material adheres to the cytosolic face of the concave surface of TGN sacs; nucleocapsids adhere to this protein, which is thus trapped between the nucleocapsid and the TGN-derived membrane that wraps around it. Experiments with brefeldin A suggest that tegument may bind to the cytosolic tails of viral glycoproteins. Fusion and fission convert the TGN-derived wrapping sacs into an inner enveloped virion and an outer transport vesicle that carries newly enveloped virions to cytoplasmic vacuoles. These vacuoles are acidic and were identified as prelysosomes. It is postulated that secreted virions are partially degraded by their exposure to the prelysosomal internal milieu and rendered noninfectious. This process explains the cell-associated nature of VZV in vitro; however, the mechanism by which the virus escapes diversion from the secretory pathway to the lysosomal pathway in vivo remains to be determined.

Varicella-zoster virus (VZV) is highly cell associated when propagated in cultured cells (36). Infection in vitro is spread by fusion of adjacent cells; tissue culture medium is not infectious. Viral particles are present in the medium; however, their pleomorphic morphology suggests that they have been degraded (9). Assembly of newly synthesized VZV, like that of other alphaherpesviruses, begins in the nucleus, where viral DNA and capsid proteins are incorporated into complete nucleocapsids (3). Nucleocapsids are enveloped as they move through the inner membrane of the nuclear envelope (3, 9). This process delivers enveloped virions to the perinuclear cisterna and the rough endoplasmic reticulum (RER), with which the perinuclear cisterna is continuous. The subsequent route of intracellular transport and reasons for the apparent degradation of enveloped virions prior to exodus from cells remain unknown.

Although there is general agreement that enveloped virions accumulate within large cytoplasmic vacuoles (3, 9, 18), the identity of these vacuoles and of the process responsible for delivering virions to them is controversial. One hypothesis is that the vacuoles are sites of viral envelopment (18). Accordingly, nucleocapsids are transported through the cytosol to the vacuoles and, while entering them by budding, acquire their envelope from the membrane of the vacuole. This hypothesis

does not specify how nonenveloped nucleocapsids move from the nucleoplasm to the cytosol, how viral tegument gains access to the space between the nucleocapsid and the viral envelope, or why enveloped viral particles are observed in the perinuclear cisterna. An alternative hypothesis is that the route of viral transport is similar to that of lysosomal enzymes (9). According to this idea, enveloped virions would move from the perinuclear cisterna through the RER, the Golgi complex, and the *trans*-Golgi network (TGN), where they are targeted to prelysosomes. Accumulation in prelysosomes, a compartment in which the pathway by which material that enters cells by endocytosis is delivered to lysosomes converges with the pathway by which newly synthesized lysosomal enzymes from the TGN reach lysosomes (12), would explain the degradation of VZV that appears to precede its release from cells. The observation that the cytoplasmic vacuoles in which VZV accumulates exhibit properties of prelysosomes provides support for this second route of viral egress (8); thus, the VZV-containing vacuoles have been found to contain a lysosomal enzyme (acid phosphatase) (9) and to be accessible within 1 h to a fluid phase endocytic tracer (horseradish peroxidase) (8). The tracer is cleared from the VZV-containing vacuoles within 24 h and is delivered to lysosomes, which do not contain virions. Sorting to prelysosomes occurs in the TGN when proteins, such as lysosomal enzymes, express the mannose 6-phosphate (Man 6-P) recognition signal (4). Binding of Man 6-P-containing proteins to the small cation-dependent and/or the large cation-independent (MPR^{cl}) Man 6-P receptor causes these proteins to be withdrawn from the

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secretory pathway (4, 37). There are five envelope glycoproteins of VZV, gpI to -V (5). They contain complex phosphorylated oligosaccharides, which when hydrolyzed, yield residues that migrate on paper chromatograms as Man 6-P (8). It is thus possible that envelope glycoproteins of intact virions are processed during transport through the cisternal compartment of infected cells and that viral particles are routed to prelysosomes because they interact with one or both MPRs. If virions were rendered noninfectious in prelysosomes, it could account for the cell-associated properties of VZV *in vitro* and for the pleomorphic appearance of secreted virions.

Recently, a very different route of intracellular transport has been reported for pseudorabies virus (38). This virus is initially enveloped as its nucleocapsid passes through the inner membrane of the nuclear envelope by a process similar to that which applies to VZV. After reaching the cisternal space, however, the initial envelope is thought to fuse with the membrane of the RER to deliver the nucleocapsids to the cytosol of infected cells. The nucleocapsids are then encapsulated a second and final time in the Golgi region. A membranous sac wraps around the nucleocapsid, which thus becomes surrounded by a vacuole with a double membrane. Fusion and fission of the membrane of the membranous sac then provides both the viral envelope (derived from the domain of the membrane of the original sac that is in contact with the nucleocapsid) and a transport vesicle (derived from the opposite membrane of the original sac). Tegument, synthesized in the cytosol, would have to bind to the nucleocapsids and/or the membrane that surrounds them so as to become incorporated into the enveloped virion.

Experiments were carried out to identify the route of egress followed by VZV in infected cells *in vitro*. Predictions made by the three hypotheses outlined above were tested. The pathway followed by VZV glycoproteins was traced by quantitative electron-microscopic (EM) radioautography of cells incubated with [³H]mannose and chased for various periods in the presence of an excess of nonradioactive mannose. VZV gpI and MPR^{ci}s were located by EM immunocytochemistry, and the ability of the viral glycoproteins to bind wheat germ agglutinin (WGA), a marker for the presence of complex oligosaccharides (40), was also assessed. Intracellular compartments were identified, either by their characteristic morphology or with specific markers. For this purpose, a prelysosome was defined as an acidic compartment on the itinerary of the MPR^{ci} (MPR^{ci} immunoreactive) that was accessible to endocytic tracers (12, 14, 27). Acidity was assessed immunocytochemically by following the trapping of the weak base DAMP [*N*-(3-((2,4-dinitrophenyl)amino)propyl)-*N*-(3-aminopropyl)methylamine dihydrochloride] with monoclonal antibodies to dinitrophenol (1). The TGN was identified as a tubulovesicular compartment adjacent to the most *trans* element of the Golgi stack (13) that concentrated the lipid *N*-(ε-7-nitrobenz-2-oxa-1,3-diazo-4-yl-aminocaproyl)-D-*erythro*-sphingosine (C6-NBD-Cer) (29) and which also exhibited at least some MPR^{ci} immunoreactivity (4, 13, 14). The most *trans* element of the Golgi stack was identified by its nucleoside diphosphatase (thiamine pyrophosphatase) activity (7, 28). Finally, several experiments in which the egress of VZV was studied under conditions in which transport of material through the Golgi apparatus was inhibited were carried out. Antagonism of transport beyond the Golgi was achieved by incubating cells at 20°C (14) or in the presence of brefeldin A (6, 20, 25).

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MATERIALS AND METHODS

Growth of cells and preparation of cell-free VZV. A wild-type strain (Ellen strain) of VZV was propagated in human embryonic lung fibroblasts (HELFL) (Whittaker M. A., Walkersville, Md.) as previously described (8). For EM, confluent HELFL monolayers were grown on Aclar discs (Allied Fibers and Plastics, Pottsville, Pa.). The cells were fixed on the discs for 1 h before further processing. In experiments in which interference with intracellular transport was to be studied, cells grown on Aclar discs were exposed for various periods to a reduced temperature (20°C) or to brefeldin A (1 to 5 µg/ml) (Epicentre Technologies, Madison, Wis.). Brefeldin A was dissolved in ethanol and freshly diluted prior to use. Cell-free virus was obtained as previously described (8, 9). Cells were infected with cell-free virus (≈1,000 PFU/ml; multiplicity of infection, ≈1:100) and analyzed after 48 to 72 h.

EM and immunocytochemistry. For routine EM, cells were fixed with 2.5% glutaraldehyde and postfixed in 1% OsO₄ containing 1% potassium ferrocyanide as described previously (8). When immunocytochemistry was to be carried out, the fixative mixture contained 4% formaldehyde (from paraformaldehyde) and 0.05 or 0.1% glutaraldehyde in 100 mM sodium phosphate buffer containing 3% sucrose. In most experiments, fixed tissues were washed in buffer and residual aldehyde groups were quenched with 50 mM ammonium chloride in the same buffer. Tissues were then washed, dehydrated, and embedded in a hydrophilic embedding medium (LR White or LR Gold; Ernest Fullam, Schenectady, N.Y.) for immunocytochemistry. Embedding was carried out in a well between two layers of Aclar. LR White was then polymerized under N₂ at 52°C. More recently, the procedure was modified in order to enhance preservation of membranes. Fixed tissue was briefly washed and mordanted with 0.25% tannic acid in 100 mM sodium phosphate buffer containing 3.5% sucrose for 1 h at 4°C. Specimens were then quenched with 50 mM ammonium chloride in the same buffer, washed extensively with 100 mM sodium maleate buffer containing 4% sucrose (pH 6.2), and stained en bloc with maleate-buffered 2% uranyl acetate. The stained tissues were finally dehydrated in the cold. Once the specimens were in 70% ethanol, the temperature was lowered to -20°C to complete dehydration, clearing, and embedding in LR Gold. LR Gold was polymerized by exposure to UV light at -20°C. Thin sections were cut and picked up on nickel grids that had been layered first with Formvar and then with carbon. The sections were treated on the grids with a blocking solution containing 1.0% bovine serum albumin (BSA), 0.5% cold-water fish gelatin, and 0.1% Triton X-100 in Tris-buffered saline for 60 min at room temperature. The sections were then incubated with a monoclonal antibody to VZV gpI (from Paul Keller of Merck and Co.) (19) and/or rabbit polyclonal antibody to the MPR^{ci} (8) diluted 1:100 with blocking solution in which the BSA concentration was reduced to 0.3%. Grids were subsequently washed and incubated for 3 h at room temperature with goat anti-rabbit or anti-mouse secondary antibodies coupled to 5- or 10-nm particles of colloidal gold (diluted 1:20 with blocking solution) (Amersham Corp., Arlington Heights, Ill.). In experiments in which lectins were employed to investigate oligosaccharides on the envelope glycoproteins of VZV, tissue was processed identically as for immunocytochemistry through the step of picking up sections of LR White-embedded material on nickel grids. For these sections, however, Tween-20 (0.2%) in phosphate-buffered saline (PBS) was used to suppress nonspecific binding. The blocked sections were then incubated for 60 min at room temperature with WGA (1.4 µg/ml) (EY Laboratories, San Mateo, Calif.) coupled to 10-nm

FIG. 1. VZV acquires an envelope from the inner nuclear membrane as the virion exists from the nucleus. (A) A viral particle has been captured in the process of envelopment, which is incomplete (arrow). The partially enveloped particle has evidently moved through the nuclear lamin. Note the thickening and increase in osmiophilia that has occurred in the inner nuclear membrane, which has wrapped halfway around the exiting nucleocapsid. This change in the appearance of the membrane suggests that the domain that envelops the viral particle has been modified by the concentration within it of viral proteins. The budding virion evaginates into the perinuclear cisterna.

particles of colloidal gold diluted with Tween-PBS. The sections were then washed. The grids, treated either for immunocytochemistry or lectin cytochemistry, were washed; postfixed with 2.5% glutaraldehyde in water for 10 min; stained with aqueous 2% OsO_4 , uranyl acetate, and lead citrate; and examined in a JEOL 1200 EX electron microscope.

Radioautographic demonstration of glycoproteins labeled by [^3H]mannose. In order to monitor the intracellular transport and processing of N-linked glycoproteins, cells were washed twice with glucose-free growth medium (GIBCO) and then incubated with D-[^3H]mannose (100 $\mu\text{Ci}/\text{ml}$, 15 Ci/mmol) (American Radiolabeled Chemicals Inc., St. Louis, Mo.) in this medium for 15 min. Following the 15-min labeling pulse, the cells were washed in growth medium supplemented with a 100-fold excess of nonradioactive D-mannose (0.7 mM) and chased in this medium for 1, 2, 4, 8, 16, or 24 h. After the chase period, cells were fixed for examination by EM as described above. For radioautography, silver sections were cut and picked up on collodion-coated glass slides (10). The slides were then shadowed with a thin carbon layer. Melted Ilford L4 nuclear track emulsion was subsequently applied to the carbon-coated sections on the slides. The thickness of the emulsion was regulated by noting the interference color (purple) of the coated slides. The emulsion-coated sections on the slides were exposed in a light-tight box for 7 to 14 days at room temperature under an atmosphere of dry 100% CO_2 . Kodak Microdol-X (3 min at 20°C) was used for development, following which the slides were fixed and washed. The collodion layer carrying the sections and the emulsion was floated off the glass slides. The sections were picked up on 200-mesh copper grids. The collodion was dissolved from the grids with absolute ethanol for 5 min, following which the sections on grids were taken to water through a descending series of alcohols and stained with uranyl acetate and lead citrate for examination by EM. Quantitative analysis of EM radioautographs was carried out by the method of Williams (39). Briefly, random pictures of cells fixed and processed after each period of chase were taken at a constant magnification. A regular grid of circles and a regular array of points were superimposed over the micrographs. The diameter of the circles was 1.5 times the half distance, the distance from a radioactive line within which 50% of silver grains will lie (32). Cellular components lying within each circle (circle distribution) and underlying each point (point distribution) were identified and scored. Similar circles were centered over each radioautographic silver grain. For the circles, cellular components were classified as single (circle entirely within the component), junctional (circle contacting more than one component), or compound (diameter of the component less than that of the circle). The grid of circles was used to determine the effective area of each single, junctional, or compound item. The frequency with which cellular components were included in the circles centered over silver grains (grain distribution) was compared with that of the same components included in the regular grid of circles (circle distribution). The grain distribution was compared with the

A second, unenveloped nucleocapsid can be seen in the nucleoplasm near the nuclear membrane. (B) A viral particle (arrow) has completed its exodus from the nucleus and now lies in the perinuclear cisterna, where it is continuous with the RER. Note the relatively osmiophilic viral envelope, which resembles the modified domain of the inner nuclear membrane that could be seen enveloping a virion in panel A. No tegument-like material can be identified either in the partially enveloped virion or in the enveloped virion that has exited from the nucleus and lies in the perinuclear cisterna-RER. Bars, 1.0 μm .

FIG. 2. Localization of gpI immunoreactivity in infected cells. (A) Enveloped virions (thin arrow) which have just exited from the nucleus (N) and lie in the perinuclear cisterna-RER do not display gpI immunoreactivity. In contrast, gpI immunoreactivity is demonstrable in cisternae of the RER (thick arrow). (B) Enveloped virions and membranes within the cytoplasmic vacuoles in which VZV accumulates are gpI immunoreactive. (C) Extracellular virions adherent to the plasma membrane are gpI immunoreactive. (D) Membranes of sacs *trans* to the *trans*-most element of the Golgi stack are gpI immunoreactive. Note that these sacs are curvilinear and that dense material is adherent to the cytosolic face of the membrane of the concave side of the sac. gpI immunoreactivity was visualized with 5-nm particles of colloidal gold. Bars, 1.0 μm.

TABLE 1. Relative specific activity as a function of time following exposure to [^3H]mannose

Chase (h)	Sp act ^a							
	RER	Nuclear envelope	Intermediate compartment	Golgi	Prelysosome	Plasma membrane	Lysosome	VZV-plasma membrane
0	15	10	10	9	0	0	0	0
1	10	9	0	38	2	33	1	0
2	8	6	0	91	6	46	32	0
4	10	27	1	16	74	20	13	121
8	8	12	0	10	16	10	139	81
16	2	19	0	13	41	36	38	95
24	5	3	2	10	22	45	99	67

^a Percentage of grains/percentage of circles. At least 200 radioautographic silver grains and 1,000 circles were analyzed at each time point (see Materials and Methods).

circle distribution by means of a chi-square analysis, in which the circle distribution served as the expected distribution of radioactivity had the labeling been random. After an initial comparison was made, the individual components of junctional items were reassigned on the basis of their size, which was determined by point count planimetry (point distribution) and the relative specific activity (percentage of grains/percentage of circles) of each component occurring as a single item. It was assumed that the relative specific activity of any cellular component that was part of a junctional item would not be different from that of the same component as a single item.

Demonstration of acidic compartments. The weak base DAMP (Molecular Probes, Eugene, Oreg.) was used as a probe to analyze the relative acidity of intracellular compartments. This compound is distributed across membranes in response to H^+ gradients, concentrating in acidic compartments (1, 2). Infected and control HELF, in glass prescription bottles, were incubated with 200 μM DAMP for 60 min at 37°C in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered minimal essential medium. The weak base chloroquine (50 μM), which alkalinizes intracellular compartments, was used as a control. A fixative mixture, containing 4% formaldehyde (from paraformaldehyde) and 1.0% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), was then added to the cells at 37°C. Fixation was allowed to proceed for 60 min at room temperature. The fixed cells were washed in buffer at 4°C, and excess aldehydes were quenched with 50 mM NH_4Cl in buffer for 30 min. The cells again were washed, scraped from flasks with a rubber policeman, pelleted, and then dehydrated and embedded in LR White. Thin sections of DAMP-treated cells were incubated for 3 h at room temperature with a murine monoclonal antibody (Oxford Biomedical Research, Oxford, Mich.) to dinitrophenol (50 $\mu\text{g}/\text{ml}$) that cross-reacts with DAMP diluted in blocking solution buffered to pH 8.0. The sites where DAMP was concentrated were visualized with a goat anti-mouse secondary antibody coupled to 5-nm particles of colloidal gold (diluted 1:20) (Amersham). In some sections VZV was located simultaneously by using a human convalescent-phase zoster serum (8). Bound antibodies to VZV were located in the sections with biotinylated goat anti-human secondary antibodies (diluted 1:200) (Kirkegaard and Perry, Gaithersburg, Md.) and streptavidin coupled to 15-nm particles of

colloidal gold (diluted 1:20) (Amersham). Sections were further processed for electron microscopy as described above.

Location of the TGN and the *trans*-Golgi cisterna. The TGN marker C6-NBD-Cer (Molecular Probes) was used essentially as described by Pagano et al. (29). C6-NBD-Cer (4.0 μM ; diluted from a dimethyl sulfoxide stock solution) was added to infected and control HELF cultures for 10 min. After 30 min of chase at 37°C to allow for transport to the Golgi apparatus, the cells were fixed for 30 min at 4°C with 1% glutaraldehyde (buffered with 0.1 M sodium cacodylate at pH 7.4). Cells were then washed with 0.1 M Tris buffer (pH 7.6) and exposed to a solution containing 0.9 ml of a fresh solution of 1.5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) per ml of 0.1 M Tris buffer at pH 7.6. After 10 min, a small region of the culture dish was irradiated with light from a Nikon inverted fluorescence microscope (excitation λ , 450 to 490 nm; 25 $\times$ objective; numerical aperture, 0.5). After irradiation, the cells were washed again in 0.1 M Tris buffer (pH 7.6) and observed by light microscopy for the presence of a DAB reaction product in the irradiated area. When evidence of a successful reaction was obtained, the cells were rinsed with 0.1 M cacodylate buffer (pH 7.4), postfixed with cacodylate-buffered 1% OsO_4 containing 1% potassium ferrocyanide for 60 min at room temperature, dehydrated, embedded in Epon 812, and processed for EM as described above. As a control, C6-NBD-Cer was omitted.

In order to demonstrate nucleoside diphosphatase (23, 28), glutaraldehyde-fixed control or VZV-infected cells were incubated overnight at 4°C in 100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)]-buffered (pH 7.0) sucrose (10%). Cells were rinsed with 80 mM Tris-maleate buffer (pH 7.2) and incubated for 90 min at 70°C in a solution containing newly prepared 4 mM $\text{Pb}(\text{NO}_3)_2$, 5 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.5 mM cocarboxylase, 80 mM Tris maleate (pH 7.2), and 146 mM sucrose. This solution was centrifuged at 15,000 $\times g$ before use; cells were rinsed with 80 mM Tris maleate (pH 7.2) and finally incubated for 2 min at room temperature with 1% ammonium sulfide.

RESULTS

VZV receives a primary envelope from the inner nuclear membrane. As has been described in an earlier article (8), EM observations of infected HELF confirmed that VZV nucleo-

FIG. 3. Localization of WGA-binding sites in infected cells. (A) Enveloped virions which have just exited from the nucleus and lie in the perinuclear cisterna-RER do not bind WGA-gold. (B) The membranes of the sacs *trans* to the *trans*-most element of the Golgi stack that were found to be gpI immunoreactive (Fig. 2) also bind WGA-gold. (C) Enveloped virions and membranes within the cytoplasmic vacuoles in which VZV accumulates bind WGA-gold. (D) Extracellular virions adherent to the plasma membrane bind WGA-gold. Note that membrane structure is not visible in this tissue, which has not been osmicated and which has been embedded in LR White. WGA-binding sites are visualized with WGA coupled to 10-nm particles of colloidal gold. Bars, 0.2 μm .

TABLE 2. Distribution of radioactivity among organelles

Chase (h)	% ^a							
	RER	Nuclear envelope	Intermediate compartment	Golgi	Prelysosome	Plasma membrane	Lysosome	VZV-plasma membrane
0	34	23	23	20	0	0	0	0
1	11	10	0	41	2	35	1	0
2	4	3	0	48	3	25	17	0
4	3	10	0	6	26	7	5	43
8	3	4	0	4	6	4	50	29
16	1	7	0	5	14	27	13	33
24	2	1	1	4	9	18	39	26

^a The percentages in each row add up to 100.

capsids are assembled in the nucleus and acquire an envelope as the virions pass through the inner nuclear membrane (Fig. 1A). This sequence is indicated by the frequent observation of partially enveloped virions within nuclear buds that evaginate into the perinuclear space. The membrane that becomes associated with the virus can be distinguished from the remainder of the inner nuclear membrane because the virus-associated domain is more osmiophilic and is rounded to enclose the nucleocapsid. Fully enveloped virions, therefore, are observed within the perinuclear space and elements of the RER with which the perinuclear space is continuous (Fig. 1B). The appearance of enveloped virions within the perinuclear space and RER, however, is not identical to that of enveloped virions in cytoplasmic vacuoles or adherent to the external face of the plasma membrane. The envelope of the virions in the perinuclear space and RER is thinner than that of the virions in other locations, and the amorphous osmiophilic mass of tegument that normally occupies the space between the nucleocapsid and the viral envelope cannot be discerned (Fig. 1B). These observations suggest that tegument may be missing from virions entering the cisternal space from the nucleus and that the viral envelope may be modified or replaced during its cytoplasmic transport and processing. It is difficult to envision a mechanism that would allow tegument proteins to pass through a viral envelope to reach their correct location; therefore, the hypothesis that the envelope surrounding virions in the perinuclear space and RER is replaced was tested.

Experiments were carried out to determine if the envelopes of virions in the perinuclear space and RER contain a glycoprotein known to be present in the final envelope of VZV. For this purpose, gpI was located by EM immunocytochemistry. Several different antibodies, each of which reacts with the peptide backbone of gpI and not with an oligosaccharide, were employed. Virions in the perinuclear space and RER were not gpI immunoreactive (Fig. 2A), although gpI immunoreactivity was demonstrable in the RER itself. In contrast, enveloped

virions within cytoplasmic vacuoles (Fig. 2B) and those adherent to the external face of the plasma membrane displayed gpI immunoreactivity (Fig. 2C). The immunoreactivity of gpI was also seen in membranes that are located near the *trans* face of the Golgi apparatus and which are not associated with enveloped virions (Fig. 2D). These membranes frequently formed flattened curvilinear sacs, the concave and convex surfaces of which differed in appearance. Osmiophilic material was adherent to the cytosolic face of the concave surface of each gpI-immunoreactive sac. These observations are consistent with the idea that the envelope applied to nucleocapsids as they emerge from the nucleus lacks at least one glycoprotein of the final envelope of VZV. Further support for this idea was obtained from studies with WGA-gold. WGA binds to the complex oligosaccharides of the final membrane of VZV. WGA-gold did not bind to virions in the perinuclear space or RER (Fig. 3A); however, WGA-gold was found to bind to those enveloped virions that were within cytoplasmic vacuoles (Fig. 3C) or adherent to the external face of the plasma membrane (Fig. 3D). WGA-gold also reacted with the membranous sacs near the *trans* face of the Golgi apparatus that were previously found to be labeled by antibodies to gpI (Fig. 3B). These data indicate that the enveloped virions in the perinuclear space and RER do not contain complex oligosaccharides, and thus, if the viral envelope at this stage contains glycoproteins, they have not passed through the Golgi apparatus. In contrast, enveloped virions in other locations, as well as the membranous sacs near the *trans* face of the Golgi apparatus, do contain complex oligosaccharides; therefore, the glycoproteins in all of these membranes can be considered to have traversed the Golgi apparatus. Further experiments were undertaken to determine whether the initial forms of enveloped VZV in the nuclear envelope and RER contain glycoproteins that become modified as enveloped virions pass through the cisternal space or whether the final envelope of

TABLE 3. Distribution of radioactivity across time

Chase (h)	% ^a							
	RER	Nuclear envelope	Intermediate compartment	Golgi	Prelysosome	Plasma membrane	Lysosome	VZV-plasma membrane
0	26	12	77	5	0	0	0	0
1	17	10	0	20	1	14	0	0
2	14	7	0	49	4	20	10	0
4	17	31	8	9	46	9	4	33
8	14	14	0	5	10	4	43	22
16	3	22	0	7	25	33	12	26
24	9	4	15	5	14	20	31	19

^a The percentages in each column add up to 100.

VZV is synthesized and processed independently of the enveloped virions.

Radioautography after incubation of cells with [3 H]mannose. Pulse-chase experiments were carried out with [3 H]mannose. N-linked oligosaccharides labeled with the probe were located in cells by EM radioautography. At each time of chase (0, 1, 2, 4, 8, 16, and 24 h) the micrographs were analyzed statistically to determine whether the distribution of label was random (see Materials and Methods for details) and to identify components of the cells that were labeled. At each time, the distribution of silver grains over the tissue differed significantly ($P < 0.001$) from that expected for a random distribution. The relative specific activity (percentage of silver grains/percentage of random circles) of several organelles found statistically to be labeled was monitored as a function of time in chase media in order to identify the route of intracellular transport of [3 H]mannose-labeled glycoproteins (Table 1). In addition to the actual value of the relative specific activity (Table 1), the radioactivity of each organelle relative to those of other organelles at the same time of chase (Table 2) and the time-related change in the specific activity of each organelle (Table 3) were analyzed. Immediately after the 15-min labeling period (0 h of chase), four cellular components were found to be labeled. These included the RER, the nuclear envelope (the outer component of which is part of the RER), and an intermediate compartment that corresponds in its morphology to that shown by Hobman et al. (16), on the basis of studies with rubella virus, to be intercalated between the RER and the Golgi apparatus (Fig. 4A). The Golgi apparatus was also labeled; however, the labeling of the Golgi apparatus increased with increasing time in chase medium, and its labeling at time zero was slight in comparison with the degree of labeling of the Golgi apparatus at later intervals of chase. After 1 h of chase the relative specific activity of the RER declined, but the intermediate compartment was no longer labeled. In contrast, the labeling of the Golgi apparatus (Fig. 5B) increased substantially (Table 3) and exhibited the highest relative specific activity in the cells (Table 2). The relative specific activity of the Golgi apparatus peaked at 2 h and then declined rapidly after longer intervals of chase (Table 3). The cytoplasmic compartments (prelysosomes; for identification, see below) in which enveloped VZV accumulates (Fig. 4C) were labeled significantly for the first time at 2 h, but the relative specific activity of these structures reached a peak later than that of the Golgi apparatus, at 4 h of chase (Tables 2 and 3). Lysosomes (for identification, see below) (Fig. 4D) began to be labeled and peaked in radioactivity later than prelysosomes, at 8 h (Tables 2 and 3); moreover, although their relative specific activity was very high after 8 h of chase (Table 1), no enveloped virions were ever detected in lysosomes. The absence of enveloped virions from labeled lysosomes stands in marked contrast to labeled prelysosomes, in which virions were evident. Radioactive material in lysosomes, therefore, was not associated with particles recognizable as VZV related. The plasma membrane and/or extracellular virions which were adherent to its outer surface also became labeled following incubation with [3 H]mannose. For purposes of analysis, the plasma membrane was divided into two regions, domains that were (Fig. 4E) or were not (Fig. 4F) in contact with enveloped VZV. The VZV-plasma membrane domain did not become labeled significantly until 4 h of chase had elapsed (Tables 1 to 3). Once labeled, however, the relative specific activity of the VZV-plasma membrane unit remained high through 24 h of chase (Tables 2 and 3). Interestingly, the relative specific activity of domains of the plasma membrane that were not associated with enveloped virions displayed two peaks (Tables

2 and 3). Significant labeling of the plasma membrane distant from particles of VZV was detectable as early as 1 h, peaked at 2 h, and declined through 8 h of chase. A later peak in relative specific activity appeared after 16 h in chase medium, and the radioactivity of the plasma membrane remained elevated at 24 h of chase. If it is assumed that the specific activity of the VZV-plasma membrane unit reflects primarily the radioactivity of viral particles, these data would suggest that newly enveloped VZV virions begin to emerge from cells in which they are synthesized 4 h after the biosynthesis of the glycoproteins of the viral envelope. It is important to draw attention to the fact that neither after the pulse-labeling period nor at any time of incubation in chase media was radioactivity found to be associated with enveloped virions in the perinuclear cisterna or in the RER (although the RER was itself labeled). The radioautographic observations, taken as a whole, are consistent with the hypotheses that the initial viral envelope, acquired as nucleocapsids pass through the inner nuclear membrane, does not contain N-linked oligosaccharides and that the glycoproteins of the final membrane of the VZV envelope are transported and processed separately from the initially enveloped virions. The intracellular route of transport of viral glycoproteins probably reflects that of [3 H]mannose-labeled proteins and is likely to be RER→intermediate compartment→Golgi apparatus (including the TGN)→prelysosomes→plasma membrane. Viral glycoproteins not associated with enveloped virions may also reach the plasma membrane by a faster route and, if not incorporated into the envelope of VZV, be delivered to lysosomes.

The cytoplasmic vacuoles in which enveloped VZV accumulates are prelysosomes. The earlier observations that the cytoplasmic vacuoles in which VZV accumulates contain acid phosphatase (9) and that a fluid phase marker, taken up by endocytosis, passes through these vacuoles before reaching lysosomes (8) suggested that the VZV-containing vacuoles are prelysosomes. Further confirmation of this identification was sought. If the VZV-containing vacuoles are prelysosomes, then their interior would be predicted to be acidic. The ability of the vacuoles to trap the weak base DAMP was therefore investigated. Cells were incubated with 200 μ M DAMP in the presence (control) or absence of 50 μ M chloroquine, fixed, and prepared for the immunocytochemical localization of DAMP. In cells that were infected with VZV, DAMP was found to accumulate in cytoplasmic vacuoles that contained enveloped virions (Fig. 5). Gold particles, indicative of DAMP immunoreactivity, tended to be spread nonuniformly within labeled vacuoles; the particles often clustered near the periphery of the organelles. DAMP-labeled vacuoles often contained amorphous osmiophilic material as well as enveloped VZV. In order to confirm that the particles in the vacuoles that appeared morphologically to be virions actually were virions, VZV immunoreactivity was located simultaneously with that of DAMP by using a human antiserum derived from a patient convalescing from zoster. The human serum labeled the particles that appeared to be virions in the acidic compartments revealed by the accumulation of DAMP (Fig. 5B). In addition to the DAMP-labeled vacuoles that contained VZV, infected cells contained vacuoles in which DAMP immunoreactivity was concentrated but which contained no viral material (not shown). These vacuoles were probably lysosomes, because they were more heavily labeled by DAMP than were the vacuoles that contained virions or any other cellular compartment. No accumulation of DAMP in any intracellular vacuole was observed in cells infected with VZV when chloroquine was present with DAMP in the incubation medium (data not shown). No structures that had the appearance of virions or

FIG. 4. Distribution of radioautographic silver grains over sections of infected cells that were incubated with [^3H]mannose and chased for various periods prior to fixation. (A) Immediately after the pulse (15 min), labeling is found over the RER and an intermediate compartment (inset). (B) At 2 h the Golgi apparatus is labeled (labeling is also significant at 1 h). (C) At 4 h prelysosomes are labeled. Note that enveloped virions have accumulated in these structures. No labeling of the intermediate compartment, which is visible at the lower edge of the field, can be found. (D) At 8 h lysosomes are labeled; however, in contrast to prelysosomes, no recognizable viral particles can be seen in lysosomes. (E and F) After 16 h, labeling is evident at the plasma membrane. Two domains were recognized: that associated with enveloped virions (E) and that where no virions could be discerned (F). Many grains could be found over enveloped extracellular virions at a considerable distance from plasma membranes (inset in panel E). Bars, 1.0 μm .

which were labeled by the VZV antiserum were found in DAMP-labeled vacuoles in control cells.

A second marker that was investigated was the MPR^{ci}. In most cells, this receptor is known to be present in the TGN, prelysosomes, and the plasma membrane (4, 26, 27, 37). It is

not targeted to lysosomes themselves (12, 14). If the VZV-containing vacuoles are prelysosomes, therefore, they should contain the MPR^{ci}. Double-label immunocytochemistry was utilized to determine if the cytoplasmic vacuoles in which enveloped VZV accumulates contain MPR^{ci}. The immunoreactivity of gpI was employed as a marker to distinguish the final viral envelope from that acquired from the inner nuclear membrane, which, as noted above, is not gpI immunoreactive. Virions attached to the cell surface (Fig. 6A) as well as those in the cytoplasmic vacuoles (Fig. 6B) were observed to be gpI immunoreactive. MPR^{ci} immunoreactivity was also seen in both of these locations (Fig. 6A and B) and was often located very close to gpI-immunoreactive virions. Double labeling by antibodies to gpI and the MPR^{ci} was observed at one additional site in infected cells. This doubly labeled structure was the array of curving membranous sacs, described above, which is located near the *trans* face of the Golgi apparatus and to which osmiophilic material that resembles tegument is adherent (Fig. 6C). These observations confirm that the cytoplasmic vacuoles within which VZV accumulates are prelysosomes. Remaining to be shown are where VZV acquires its definitive envelope and how virions gain access to prelysosomes.

Enveloped VZV can fuse with the RER. Although profiles of enveloped virions entering the RER from the perinuclear cisterna are frequently encountered in infected cells (Fig. 1), enveloped virions are not often seen in the bulk of the RER and they have not been observed within the flattened cisternae of the Golgi stack. The paucity of virions in the RER and Golgi cisternae could be explained if virions were transported through these rapidly and diffusely, with the result that the particles are rare in thin sections. Alternatively, virions may leave the RER soon after they enter it and not pass through the entire organelle. An attempt was made, therefore, to cause virions to accumulate in cisternae of the RER. Cells were incubated at 20°C in order to slow intracellular transport and prevent movement of material beyond the Golgi apparatus (14). When this was done, enveloped virions were observed within the RER in large numbers; however, many profiles in which the viral envelope fused with the membrane of the RER were also seen (Fig. 7A). The viral envelope was more osmiophilic than that of the RER; therefore, the viral envelope could be recognized, even after fusion with the RER. The fused envelope also maintained its curvature after fusion and invaginated into the lumen of the RER. As a result of the fusion of the viral envelope with the RER, the viral nucleocapsid gained access to the cytosol. Indeed, even in cells incubated at 37°C, many nonenveloped nucleocapsids can be observed in the cytosol of cells (not shown). The block at 20°C did not prevent transport of newly synthesized protein from the RER to the Golgi apparatus. When cells were incubated with [^3H]mannose for 15 min (as described previously) and chased for 4 h at 20°C, both the RER and the Golgi apparatus were radioautographically labeled (Fig. 7B). Even after 4 h of chase at 20°C, however, the enveloped virions in the RER and perinuclear cisterna were not labeled by [^3H]mannose. Again,

FIG. 5. The cytoplasmic vacuoles in which enveloped VZV accumulates are acidic. Trapping of the weak base DAMP was investigated. Antibodies to dinitrophenol and secondary antibodies coupled to 5-nm particles of colloidal gold were utilized to visualize DAMP in tissues. (A) Concentration of DAMP (arrows) can be detected with cytoplasmic vacuoles that contain recognizable viral particles. (B) Double-label immunocytochemistry is used to show simultaneously the immunoreactivity of DAMP (5-nm gold particles) (arrow) and that of VZV (15-nm gold particles) (arrowheads). VZV immunoreactivity was demonstrated with a polyclonal serum obtained from a patient convalescing from zoster. Bars, 0.1 μm .

the enveloped virions accumulating within the RER at 20°C failed to demonstrate gpI immunoreactivity (Fig. 7C), even though virions in prelysosomes in the same material were readily immunostained by the antibodies to gpI (Fig. 7D).

The final envelope of VZV is acquired in the TGN. Membranes that resemble the envelope of VZV are always encountered in the Golgi region of infected cells (Fig. 8A). These membranes, discussed earlier, form thin curving sacs, display gpI immunoreactivity (Fig. 6C), and bind WGA (Fig. 3D). Finely granular, but otherwise amorphous, osmiophilic material with the density of viral tegument was adherent to the cytosolic face of the concave surface of these sacs. Nonenveloped nucleocapsids could be seen to associate with the concave surface of the sacs (Fig. 8B), which often appeared to wrap around and ultimately to surround a single nucleocapsid (Fig. 8C and D). Opposing tips of the membranous sacs appeared to fuse, so that the inner, formerly concave, surface of the membranous sac became a viral envelope and the outer, formerly convex, surface of the sac became a vesicle in which a now-enveloped virion was suspended. The tegument-like material that was adherent to the concave surface of the original membrane was trapped by this process between the nucleocapsid and the newly acquired envelope. Newly synthesized glycoproteins evidently are delivered to these wrapping structures. When cells were incubated with [³H]mannose for 15 min, the membranes surrounding the cytosolic nucleocapsids became labeled after 1 to 2 h of chase (Fig. 8E). Selective staining was used to try to identify the origin of the membranes in which nucleocapsids became wrapped to form enveloped virions. Nucleoside diphosphatase (formerly thiamine pyrophosphatase), a marker for the *trans*-most element of the Golgi stack, was demonstrated. The membranes in which VZV became enveloped did not display nucleoside diphosphatase activity; however, the structures were found to be situated just *trans* to the element of the Golgi stack that did exhibit nucleoside diphosphatase activity (Fig. 9A and B). Cells were incubated with a marker for the TGN, C6-NBD-Cer (29), which was then demonstrated for EM by photoconversion in the presence of DAB. The membranes that wrapped VZV were labeled by C6-NBD-Cer (Fig. 9C), which thus also labeled the envelopes of enveloped virions (Fig. 9D). The suggestion of these data that the final viral envelope of VZV is acquired from the TGN was supported by the observation that the membranes that wrap nucleocapsids and provide the viral envelope also exhibit immunoreactivity to MPR^{ci} (Fig. 6C), another TGN marker.

Nucleocapsids adhere to tegument-like material in the cytosol of infected cells. Brefeldin A was used to disrupt RER to Golgi transport. When cells were exposed to brefeldin A, the Golgi apparatus disappeared and enveloped virions could be seen within dilated cisternae of the RER. Most strikingly, however, massive accumulations of tegument-like material appeared in the cytosol (Fig. 10A). This material was ringed at the cytosolic interface by many nucleocapsids, each of which appeared to indent the tegument-like substance. In most sections, the large accumulations of tegument-like material seemed to be surrounded by cytosol; however, occasionally, the

plane of section included cisternae of the RER to which the cytosolic accumulations of tegument-like material could be seen to adhere (Fig. 10B). Ribosomes were missing from the membrane of the RER at its junction with the tegument-like protein; however, ribosomes were present on the RER membrane across the lumen from the membrane in contact with tegument-like material. The tegument-like protein was immunostained by human polyclonal antibodies to VZV, confirming its viral origin (Fig. 10C). The tegument-like protein was not immunostained with antibodies to gpI, however, suggesting that the tegument-like material itself does not contain envelope glycoproteins. On the other hand, the periphery of the tegument-like accumulations often displayed gpI immunoreactivity (Fig. 10D), suggesting that the membrane to which the tegument-like protein adhered was enriched in gpI.

DISCUSSION

The data obtained in this study suggest that the intracellular pathway of maturation and transport of VZV in infected HELF (Fig. 11) is analogous to that followed by pseudorabies virus (38). A similar pathway of assembly, involving de-envelopment and re-envelopment, has also been described for Epstein-Barr virus (11). Assembled nucleocapsids bud through the inner nuclear membrane (Fig. 11A) and thus acquire an envelope. This envelope, however, is a temporary one that may lack N-linked glycoproteins and surrounds an immature virion that contains no tegument. As a result, the enveloped virions in the perinuclear cisterna and RER are not labeled with [³H]mannose, do not display the immunoreactivity of gpI, and are not bound by WGA. The primary envelope fuses with the RER soon after the particles enter this organelle, thereby delivering nucleocapsids to the cytosol (Fig. 11A). Because this phenomenon is rapid, virions are not seen in the RER (exclusive of the perinuclear cisterna) when cells are grown at 37°C. In contrast, fusion of virions with the RER becomes apparent when cells are incubated at 20°C, either because the process of fusion is slowed or because the block in intracellular transport causes virions to accumulate in the RER. In addition, when infected cells are incubated at 20°C, the viral envelope maintains its integrity long enough so that the sites where enveloped virions have fused with the RER remain visible and can be recognized even after the nucleocapsids have escaped from them.

The integral membrane proteins of the final envelope of VZV must be synthesized in the RER, but they are probably transported to the Golgi apparatus independently of assembled virions (Fig. 11B). Since cells infected with an alphaherpesvirus synthesize primarily viral proteins (31), [³H]mannose is a good probe with which to investigate the intracellular transport of viral glycoproteins and their assembly into the final viral envelope. [³H]mannose is a better probe for this purpose than is [³H]fucose, which has previously been employed (18), because [³H]fucose is added to complex oligosaccharides in the *trans* Golgi and, thus, would not reveal pre-Golgi stages in the transport of glycoproteins. The radioautographic analysis of [³H]mannose-labeled proteins of the

FIG. 6. Structures in infected cells that display the immunoreactivity of gpI (labeled with 5-nm particles of colloidal gold) also display the immunoreactivity of MPR^{ci} (labeled with 10-nm gold particles). (A) The envelopes of VZV at the plasma membrane are gpI immunoreactive (arrows). Labeled virions are also located close to sites of MPR^{ci} immunoreactivity (arrowheads). (B) A prelysosome, located close to the plasma membrane, contains recognizable VZV and is both gpI immunoreactive (arrow) and MPR^{ci} immunoreactive (arrowhead). (C) The VZV-associated membranous sacs *trans* to the Golgi apparatus are gpI immunoreactive (arrows). MPR^{ci} (arrowheads) is also located near the ends of these sacs. Tissues were embedded in LR White (A and C) or in LR Gold (B). Bars, 0.2 μ m.

FIG. 7. The envelope obtained by VZV from the inner nuclear membrane is a temporary one. (A) Fusion of intracisternal enveloped virions with the membranes of the RER can be detected when cells are incubated at 20°C. This process allows nucleocapsids to escape to the cytosol. The osmiophilic envelope remains fused to the RER. (B) Infected cells were incubated with [3 H]mannose for 15 min and chased with an excess of nonradioactive mannose for 4 h at 20°C. Radioautographic labeling is apparent over the RER and the Golgi apparatus. No labeling of VZV emerging from the nucleus and entering the perinuclear cisterna-RER occurs (arrow). (C) Virions in the perinuclear cisterna-RER do not display gpI immunoreactivity. (D) Virions in prelysosomes in the same sections are labeled with 5-nm particles of colloidal gold and thus are gpI immunoreactive. Bars, 0.5 μ m.

FIG. 8. The final envelope of VZV is acquired from the VZV-associated sacs *trans* to the Golgi apparatus. (A) These sacs contain dense, tegument-like material adherent to the cytosolic face of their concave surface. Note their close proximity to the Golgi stack (on the right). (B) Cytosolic nucleocapsids adhere to the concave surface of the VZV-associated sac. (C) The sac appears to wrap around the nucleocapsid, trapping the tegument-like material between the nucleocapsid and what had been the concave surface of the sac. The ends of the sac seem to fuse. (D) An almost completely wrapped nucleocapsid now appears to lie within a vesicle with a double membrane. The inner membrane is adherent to tegument and now seems to be associated with the viral particle, while the outer membrane encloses the apparently newly enveloped virion. (E) The VZV-associated sacs that appear to wrap around nucleocapsids and thus provide them with an envelope are radioautographically labeled 1 to 2 h after infected cells were pulsed with [^3H]mannose. Bars, 0.2 μm .

FIG. 9. The VZV-associated sacs that appear to provide a final envelope for VZV are elements of the TGN. (A and B) The sacs that newly envelop VZV (arrows) lie *trans* to the *trans*-most cisterna of the Golgi stack and do not display nucleoside diphosphatase activity, as does the *trans* cisterna of the Golgi stack itself. In panel A the section has not been stained in order to make more obvious the histochemical reaction product. (C) The sacs that wrap around VZV nucleocapsids are labeled by the TGN marker C6-NBD-Cer (photoconverted to an osmiophilic reaction product). (D) The final envelopes of the newly wrapped virions are labeled by C6-NBD-Cer. Bars, 0.2 μ m.

FIG. 10. Tegument-like material to which the VZV nucleocapsid evidently binds accumulates in the cytosol when cells are incubated in the presence of brefeldin A. (A) An accumulation of tegument-like material is seen in an infected cell that was cultured for 2 h in the presence of brefeldin A. Note that the cytosolic boundary of the accumulated tegument-like material is studded with nucleocapsids, which appear to indent it. (B) The tegument-like material that accumulates in cells exposed to brefeldin A is adherent to the cytosolic face of the RER. Where a free surface is exposed to the cytosol, rather than the membrane, nucleocapsids appear to stick to it (arrows). (C) The tegument-like material that accumulates in infected cells exposed to brefeldin A is labeled by polyclonal antibodies to VZV. (D) The tegument-like material is not itself labeled by monoclonal antibody to gpI; however, surfaces of the accumulations (where they are presumably in contact with the RER) (arrow) exhibit gpI immunoreactivity. Note that in this LR White (nonosmicated material) membranes are not visible. Bars, 0.5 μ m.

FIG. 11. Putative route of intracellular transport and maturation of VZV. The structures are not drawn to scale. (A) Nucleocapsids assemble in the nuclei of infected cells and bud through the nuclear envelope to reach the perinuclear cisterna-RER. A temporary, primary envelope is acquired from the inner nuclear membrane. This envelope fuses with the RER, delivering unenveloped nucleocapsids to the cytosol. The nucleocapsids move through the cytosol to reach the TGN. (B) The glycoproteins of the viral envelope are synthesized in the RER independently of the nucleocapsids and are integral membrane proteins. As is true of other such proteins, their luminal domain is glycosylated by the addition of high-mannose sugars to asparagine residues. Tegument proteins are probably synthesized by free ribosomes. A hypothesis, not yet verified, is that they bind to the cytoplasmic tails of viral glycoproteins. The glycoproteins are transported through an intermediate compartment (IC) to reach the Golgi stack, where the original high-mannose sugars are modified to become complex oligosaccharides. During transport the sidedness of the glycoproteins is maintained; sugar groups are always found within the lumen of whatever cisternal compartment the glycoproteins are in. Tegument proteins could passively follow the glycoproteins by remaining bound to their cytoplasmic tails or be transported independently. The glycoproteins finally are transported to the TGN, where they accumulate on the concave surface of a TGN-derived sac. Tegument adheres to the cytosolic face of the concave surface. The TGN-derived sac contains MPR^{ci}. The concave surface of the TGN-derived sac becomes the definitive viral envelope, while the convex surface becomes a transport vesicle, which diverts the virions from the secretory pathway to the lysosomal pathway, delivering the virions to acidic structures, identified as prelysosomes.

viral envelope suggests that the route from the RER passes rapidly through an intermediate compartment to reach the Golgi apparatus within 1 to 2 h. The [³H]mannose-labeled glycoproteins then appear in cytoplasmic vacuoles, within which enveloped virions can be discerned. These vacuoles are identified as prelysosomes because they accumulate the weak base DAMP (1) and they exhibit the immunoreactivity of the marker MPR^{ci} (4, 37); moreover, the vacuoles have previously been shown to contain acid phosphatase (9) and to be accessible within 1 h to a fluid phase tracer taken up by endocytosis (8). The endocytic tracer is cleared from the vacuoles to

lysosomes within 24 h. Viral [³H]mannose-labeled glycoproteins reach lysosomes after 8 h of chase; however, enveloped virions cannot be observed in lysosomes. It seems likely, therefore, that the viral glycoproteins that eventually reach lysosomes represent material that has not been incorporated into viral envelopes. In contrast to the envelopes of virions in the perinuclear cisterna and the RER, the envelopes of virions in prelysosomes are gpI immunoreactive and are decorated by WGA. The glycoproteins of these envelopes, therefore, are probably the complex oligosaccharide-containing glycoproteins of the final viral envelope. This conclusion is strongly sup-

ported by a radioautographic study that showed that the virions in the cytoplasmic vacuoles (now identified as prelysosomes) are labeled by [^3H]fucose (18), which is added to oligosaccharides in the cisternae of the Golgi stack (15). The envelope glycoproteins of the virions in prelysosomes, therefore, must have passed through the Golgi apparatus before entering these organelles (Fig. 11B). Processing of the oligosaccharides of the envelope glycoproteins, therefore, must occur during their transport through cisternal compartments before final viral envelopment.

Observations suggest that the final envelopment of VZV occurs by a wrapping process in the TGN (Fig. 11B). Vaccinia virus has also been shown recently to undergo a second wrapping phase of development in the TGN (33). Distinctive curving membranous sacs are present near the Golgi stack in infected cells. These sacs must contain envelope glycoproteins because they are labeled with antibodies to gpI. Since the sacs are also bound by WGA-gold, the oligosaccharides are complex (40) and thus post-Golgi (21). The convex surface of each sac is smooth; however, tegument-like material adheres to the cytosolic face of the concave surface. Nonenveloped nucleocapsids accumulate in the cytosol near these sacs and associate with the concave surface. Intermediate stages suggest that the sacs wrap around the nucleocapsids and fuse to envelop virions. The inner membrane of the fused sac, to which tegument-like material is adherent, becomes the viral envelope. The outer membrane becomes a transport vesicle. The derivation of the wrapping sacs from the TGN can be inferred from three observations: (i) the sacs are MPR^{ci} immunoreactive and thus display a TGN marker (4, 14, 17, 22), (ii) the sacs and enveloped intracellular virions are labeled by another TGN marker, C6-NBD-Cer (29), and (iii) the sacs do not exhibit the *trans*-Golgi marker nucleoside diphosphatase activity but are located just *trans* to it (7, 28). Note that the proposed mechanism assumes that the viral glycoproteins concentrate in the concave TGN membrane, which is associated with tegument. Viral nucleocapsids may be attracted selectively to this site because they adhere to tegument. MPR^{ci}, on the other hand, is probably concentrated in the convex surface of the wrapping sacs, because it is not incorporated into the viral envelope. MPR^{ci}, therefore, becomes associated with the transport vesicle. The presence of the MPR^{ci} in the transport vesicle membrane could target the delivery of the newly enveloped virions to prelysosomes because prelysosomes are on the itinerary of this receptor (4, 14, 22, 26, 27). Mature viral glycoproteins have been shown to contain oligosaccharides, which, when hydrolyzed, yield residues that migrate as Man 6-P (8). The presence of these phosphorylated sugars in the glycoproteins of the viral envelope could be the cause of the accumulation of the MPR^{ci} in the elements of the TGN within which viral glycoproteins are concentrated.

Tegument proteins are probably produced by free polyribosomes in the cytosol. Tegument-like material accumulated in the cytosol of cells exposed to brefeldin A. Brefeldin A interferes with coat proteins of Golgi elements, causing the Golgi apparatus to fuse with the RER (6, 20, 25) and also affects other membranous compartments (24, 30). The ability of brefeldin A to affect a protein of the cytosol could be explained if the cytosolic protein were to bind to the cytosolic tail of an integral membrane protein. Conceivably, tegument proteins adhere to the cytosolic tails of VZV glycoproteins (Fig. 11B). Such a phenomenon would explain the accumulation of VZV-immunoreactive, tegument-like material on the cytosolic face of the TGN, to which the glycoproteins are targeted. It would also account for the accumulation in brefeldin A-treated cells of tegument-like protein adherent to the

RER near domains that are gpI immunoreactive. This phenomenon could thus be secondary to the accumulation of newly synthesized glycoproteins in the RER that is forced by the prevention by brefeldin A of transport from the RER to the Golgi. Nonenveloped nucleocapsids are evidently attracted to the accumulated tegument-like material and they adhere to it, just as nucleocapsids adhere to tegument-like material attached to the cytosolic face of the membrane of the concave surface of TGN sacs. It is possible that tegument proteins, which are synthesized in the cytosol, normally bind to the cytoplasmic tails of viral glycoproteins and are cotransported with them; nevertheless, there is no evidence, as yet, that binding of tegument protein to glycoproteins in the RER occurs in the absence of brefeldin A. The following model is proposed to account for envelopment of VZV. Envelope glycoproteins are transported from the RER to the Golgi apparatus. The oligosaccharide chains of the glycoproteins are then trimmed, phosphorylated, and converted to complex sugars within the lumen of the compartments of the Golgi apparatus. The glycoproteins are ultimately delivered to the TGN, where they accumulate in specialized sacs. Tegument is transported to these sacs, either with glycoproteins or independently. One side of the TGN (concave) contains VZV glycoproteins and concentrates bound tegument in the immediately adjacent cytoplasm. These TGN-derived sacs then wrap around nucleocapsids, which have been attracted to the adherent tegument. The process of envelopment then proceeds, trapping tegument between the TGN-derived envelope and the nucleocapsid.

The transport of enveloped virions from the TGN to prelysosomes may account for the cell-associated nature of VZV *in vitro* (34). The acidic interior of these structures and the presence within them of lysosomal enzymes may render virions noninfectious. Degradation in prelysosomes would explain the pleomorphic, partially digested appearance of virions in the prelysosomes and at the plasma membrane. Newly enveloped virions within the TGN, which have not yet been exposed to the acidic environment in prelysosomes, never exhibit this morphology.

When cells are pulse-labeled with [^3H]mannose, radioactive virions cannot be detected at the cell surface until after radioactivity has appeared in prelysosomes. Even before radioactive virions reach the plasma membrane, however, domains of the plasma membrane that are not associated with enveloped VZV become labeled by [^3H]mannose. The observations suggest that radioactive glycoproteins reach the plasma membrane within 1 h of their biosynthesis. This material may represent the incorporation into the plasma membrane of virally encoded glycoproteins that do not become components of assembled virions. It is well known that viral glycoproteins are expressed on the surface of infected cells independently of enveloped viral particles. A later peak of radioactive glycoproteins, appearing at the plasma membrane after 16 h of chase, may represent the discharge of viral glycoproteins that were not incorporated into virions and that had earlier been targeted to lysosomes.

The mechanism of viral envelopment, for which supporting evidence is presented in this study, does not explain how infectious virions emerge from infected cells *in vivo*. Clearly, diversion of newly synthesized virions from the secretory pathway to the lysosomal pathway is not compatible with the production of large numbers of infectious particles. The virus must escape this diversion within its target organ in infected patients. The target organ for VZV in infected patients is the epidermal layer of the skin (35). The fluid in the cutaneous vesicles of such individuals contains a high titer of infectious

cell-free virus. It seems likely, therefore, that cells of the epidermis do not process VZV in exactly the same manner as do infected HELF cells in vitro. In order to understand the pathogenesis of varicella and zoster, therefore, it will be important to learn how maturation of VZV is affected by the cellular biology of maturing keratinocytes.

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