Intracellular Transport of Varicella-Zoster Glycoproteins

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Previous observations have established that varicella-zoster virus (VZV) is enveloped in the trans-Golgi network (TGN) in cultures infected with VZV and that the glycoprotein gE is targeted to the TGN by a signal sequence (AYRV) and an acidic TGN signal patch in its cytosolic domain. Neither sequence is present in other VZV glycoproteins. Like gE, gI was targeted to the TGN when it was expressed in transfected cells, suggesting that gI also contains TGN targeting information (colocalized with gE and the AP-1 adaptin complex). In contrast, gB, gC, gH, and gL immunoreactivities were not detected in the TGN when they were expressed individually in transfected cells. In VZV-infected cells, gE, gI, gH, and gL were all concentrated in the TGN. Since VZV glycoproteins that lack targeting sequences (gB, gC, gH, and gL) concentrated in the TGN of infected cells, it is proposed that gE and gI, which have such sequences, serve as navigator glycoproteins, forming complexes that direct the signal-deficient glycoproteins to the TGN.

Varicella-zoster virus (VZV) is an α-herpesvirus that causes chickenpox and, following the reactivation of latent virus in partially immune hosts, zoster (shingles) [1]. VZV is highly infectious in vivo, but the virus spreads only by direct cell-to-cell contact when it is propagated in culture [1]. At least 6 glycoproteins of VZV have been described, gE, gB, gH, gL, gC, and gL [2]. These glycoproteins are synthesized in the rough endoplasmic reticulum (RER), cotranslationally N-glycosylated, and transported independently of VZV nucleocapsids to the trans-Golgi network (TGN) [3]. Since there is evidence that VZV acquires its final envelope in the TGN [3], we have proposed that the targeting of glycoproteins to the TGN is essential for virus envelopment. We have previously determined that gE, the major glycoprotein of VZV, is targeted to the TGN [4, 5] because of the presence in the cytosolic domain of the molecule of a TGN targeting signal with the amino acid sequence AYRV and a second TGN targeting patch that is rich in acidic amino acids [6]. In the current study, we examined the location of individual VZV glycoproteins in cells infected with VZV as well as the location of gH, gI, and gL following their expression in transfected cells.

Materials and Methods

Propagation of VZV. The virus was propagated in human embryonic lung fibroblasts (HEL), as previously described [3].

Polymerase chain reaction cloning. DNA encoding individual VZV glycoproteins was cloned from VZV genomic DNA. In brief, reaction mixtures were initially incubated for 3 min at 94°C and then subjected to 35 cycles of 1 min at 94°C, 1 min at 58°C, and 3 min at 72°C. The amplified DNA was digested with appropriate restriction enzymes and gel-purified. The resultant polymerase chain reaction fragments were cloned into the multiple cloning sites of the eukaryotic expression vector pSVK3 (Pharmacia, Piscataway, NJ.). Primers used to clone gI were: 5'-CCCGAATTCTCATTATATATCGCGATGTT-3' and 5'-CCCTCTAGATATAAACTATTTAGTTCTATTTAAC-3'. Primers used to clone gB were: 5'-CCCCGAATTCTCATTATTTTCTAGTTGTGT-3' and 5'-CCCTCCTAGACCCCGTGGCATTTTAC-3'. Primers used to clone gH were: 5'-CCCCGGTACCAAGCGACTATGTTGCGCT-3' and 5'-CCCTCCTAGATCAATGTTTTTTATGTCA-3'. Primers used to clone gL were: 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3' and 5'-CCCTCCTAGATCAATGTTTTTTATGTCA-3'. Primers used to clone gc were: 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3' and 5'-CCCTTGACGAGCGGCGTTGGTGTG-3'. Primers used to clone gC were: 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3' and 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3'. Primers used to clone gL were: 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3' and 5'-CCCCGTACCAAGCGACTATGTTGCGCT-3'.

Transfection of cells. Cos-7 cells were grown in Dulbecco’s modified Eagle medium containing heat-inactivated 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. Cultures were maintained at 37°C with 5% CO₂. Transfection was performed with Lipofectin (Life Technologies Gibco BRL, Gaithersburg, MD), according to the manufacturer’s directions. After 12 h, the media were changed and incubation was continued for another 48 h at 37°C with 5% CO₂.

Immunocytochemistry. Transfected Cos-7 cells, growing on cover slides in a two- or eight-well tissue culture dish, were fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) for 2 h at room temperature. The cells were washed with PBS and, except when cell surfaces were to be examined, permeabilized with 0.1% Triton X-100 in PBS containing 2.0 mg/mL bovine serum albumin. The cells were then exposed to dilutions of primary antibodies for 2 h at room temperature. Monoclonal antibodies to gB, gE, gH, and gL were purchased from Viro Research (Rockford, IL). In addition, polyclonal antibodies to these glycoproteins and to gC and gL, were raised as described below. The immunocytochemical localization of the adaptin protein, AP-1 (monoclonal anti-γ-adaptin clone 100/3; Sigma, St. Louis), was used as a marker for the TGN [5, 7]. After being rinsed with PBS, the cultures were exposed to fluorescein- or Cy3-labeled affinity-puri-
Figure 1. Specificities of antibodies to VZV glycoproteins (gE, gI, gH, gB) were verified by Western blot analysis. VZV-infected (in) and uninfected control (c) human embryonic lung fibroblast lysates were analyzed by SDS-PAGE on 7.5% gels, and proteins were subsequently transferred electrophoretically onto nitrocellulose membranes. Membranes were probed with indicated antibodies at dilution of 1:1000. VZV proteins recognized by antibodies are identified at top of figure. Molecular mass (MW) of prestained high-molecular-mass markers (Life Technologies Gibco BRL, Gaithersburg, MD) are indicated.

Preparation of antibodies. Regions from specified VZV glycoprotein open-reading frames were selected for cloning on the basis of predicted hydrophobic, solubility, and near neutral isoelectric point, as previously described [8]. Sequences were amplified from VZV genomic DNA, using Vent polymerase (New England Biolabs, Beverly, MA), and the resulting products were cloned in a dual Escherichia coli expression vector (pAlex) to produce fusion proteins in which the viral sequence was flanked by glutathione-S-transferase (GST) and 6-histidine (His6) [8]. Fusion proteins were purified by chromatography on columns of GST beads, washed, eluted, and tested for purity by SDS-PAGE. The dual GST-His6 fusion proteins facilitated determining whether the desired sequences were inserted in-frame and thus correctly translated. The purified fusion proteins were used to immunize rabbits. Sera collected after 6 and 12 weeks were purified by adsorption with E. coli extracts, purified GST, and uninfected HELF. Sera were then tested for reactivity against the appropriate protein by Western blot analysis (figure 1) [8, 9].

Results

The location of glycoproteins was first examined in cells that were infected with VZV. The TGN was identified by demonstrating the location of the adaptin protein complex AP-1 [5, 7] and by comparing the localization of gB, gH, and gI to that of gE, which has previously been demonstrated to be specifically located in the TGN [4–6]. The immunoreactivities of gB, gE, gI, and gH were all concentrated in the TGN in infected HELF (figure 2).

To examine the targeting of individual glycoproteins, COS-7 cells were transfected with DNA encoding gB, gC, gE, gI, gH, or gL. Again, the location of gE and the AP-1 adaptin complex served as TGN markers. Of these, only gE and gI were found to be targeted to the TGN in the transfected cells. No difference was observed in the localization of gI (figure 3A) and gE (figure 3B), and the two proteins were colocalized when they were expressed by cells that were cotransfected with each (figure 3C, D). Both gE and gI (figure 3E, F) recruited the AP-1 adaptin complex to the TGN, and thus there was a coincident localization of each with AP-1 in the TGN (illustrated for gI in figure 3E, F). In contrast to gE and gI, no glycoprotein immunoreactivity could be detected by immunocytochemistry in the TGN of cells expressing gB, gC, gH, or gL. In fact, no immunoreactivity could be seen in cells individually transfected with gH or gL; nevertheless, when cells were cotransfected with both gH and gL, gH immunoreactivity could be detected in the RER and on cell surfaces (figure 4A). In cells that were stably transfected only with gH, the protein could be detected in scattered intracellular vesicles (figure 4C). Even when expressed on cell surfaces or in vesicles, there was still no concentration of gH immunoreactivity in the TGN, so their appearance was very different from that of cells that express gI (figure 4B).

Discussion

When propagated in cell cultures, VZV is highly cell-associated. The ambient medium is noninfectious, and the virus spreads only by direct cell contact. We have previously demonstrated that VZV becomes localized in acidic vesicles within infected cells [3]. These vesicles were identified as late endosomes (prolysosomes) because they are accessible within minutes to a fluid-phase marker (horseradish peroxidase) that was added to the medium. In contrast, when horseradish peroxidase was added 24 h prior to infection, it became concentrated in lysosomes and did not colocalize with virions. A quantitative
Figure 2. VZV glycoproteins (gB, gE, gH, gI) are targeted to trans-Golgi network (TGN) in cells infected with VZV. Human embryonic lung fibroblast cells were infected with cell-free VZV and immunostained with antibodies to gE (A), gB (B), gH (C), and gI (D). All 4 glycoproteins are identically concentrated in TGN (arrows). Same cell is shown in E (illuminated with fluorescein isothiocyanate to visualize gE) and F (illuminated with Cy3 to visualize gI). Note colocalization of gE and gI in TGN (arrows). Bars = 10.0 μm.
Glycoprotein gI is targeted to trans-Golgi network (TGN) when expressed in transfected cells. Cos-7 cells were transfected with DNA encoding gI or gE. A, gI immunoreactivity was demonstrated with monoclonal antibody and visualized with secondary antibodies labeled with Cy3. Note that gI appears at cell surface as well as in TGN (arrow). B, gE immunoreactivity was demonstrated with monoclonal antibody and visualized with secondary antibodies labeled with fluorescein isothiocyanate (FITC). Note concentration in TGN (arrow). Some immunoreactivity is retained at cell surface, and there is more surface immunoreactivity in cells transfected with gI than with gE. C, D, Double-label immunocytochemistry with monoclonal antibodies to gI (C) and polyclonal antibodies to gE (D). Same cell is illustrated in both panels, but it is illuminated to reveal Cy3 (C) or FITC (D). Immuno-fluorescence is concentrated in TGN (arrows). E, F, Double-label immunocytochemistry with polyclonal antibodies to gI and monoclonal antibodies to γ-adaptin (for AP-1). Same cell is illustrated in both panels, but it is illuminated to reveal Cy3 (AP-1) or FITC (gI) fluorescence. Immuno-fluorescence is concentrated in TGN (arrows). Bar = 10.0 μm.
Figure 4. A, Glycoproteins gH and gL can be detected on cell surfaces and in rough endoplasmic reticulum (arrows) when expressed in cells cotransfected with DNA encoding both of these glycoproteins. B, Localization of gH and gL is not like that of gl in cells transfected with DNA encoding that protein, which is illustrated for comparison. gl is located in trans-Golgi network (TGN; indicated by arrows; also see figure 3). C, gH could be detected in scattered intracellular vesicles in cells stably transfected with gH and gL. TGN was not immunofluorescent in either transiently or stably transfected cells. Bars = 10.0 μm.

Pulse-chase radioautographic investigation of the transport of viral proteins labeled cotranslationally with [3H]mannose revealed that virions are delivered to late endosomes prior to their release from cells by exocytosis. Extracellular virions and those contained within the late endosomes are pleomorphic and appear to have been degraded. We therefore postulated that the cell association of VZV is due to its inactivation in endosomes, either as a result of its exposure to an acidic environment or to lysosomal enzymes or both. Newly synthesized lysosomal enzymes are targeted to late endosomes from the TGN. We thus examined the possibility that this pathway might also be utilized to divert VZV to late endosomes.

Morphologic observations and radioautographic studies of the transport of [3H]mannose-labeled VZV glycoproteins suggested that the final envelope of the virus is acquired in the TGN [3, 4, 6]. Nucleocapsids assemble in the nucleus of infected cells and acquire a temporary envelope from the inner nuclear membrane. This envelope lacks gE immunoreactivity and is not labeled by [3H]mannose. When cells are incubated with brefeldin A or at 20°C, enveloped virions can be detected in the RER. These virions fuse with RER membranes, releasing naked nucleocapsids into the cytosol.

These nucleocapsids can later be seen to become “wrapped” by flattened membranous sacs in the TGN. These sacs are curvilinear with concave and convex surfaces. Tegument adheres to the cytosolic face of the concave surface, which contains gE immunoreactivity. Nucleocapsids adhere to the tegument. In contrast, neither tegument nor nucleocapsids associate with the smooth convex surface. This surface contains mannose 6–phosphate receptors (MPRs) but does not contain gE. As the wrapping proceeds, the original MPR-containing convex surface becomes a transport vesicle, while the concave surface becomes the viral envelope. The presence of MPRs in the transport vesicle may account for the subsequent delivery of virions to endosomes. For the envelopment of virions to take place in the TGN, it is necessary for all of the glycoproteins to be present in the membrane of the concave surface of the TGN wrapping structure, because this membrane becomes the viral envelope. Radioautographic experiments with [3H]mannose established that the glycoproteins are transported to the Golgi apparatus independently of nucleocapsids.

We have shown that gE, the major glycoprotein of VZV, has a TGN targeting sequence (AYRV) and a TGN targeting patch in the cytoplasmic domain of the molecule [6]. Consequently, gE is targeted to the TGN when it is expressed in transfected cells [4–6]. Targeting of gE to the TGN is prevented by deletion of the entire cytosolic domain of gE, and it is inhibited by deletion of the AYRV sequence or the targeting patch [4, 6].
Integral membrane fusion proteins that contain the AYRV sequence or the acidic patch in their cytosolic domains are targeted to the TGN. The presence of signaling sequences or patches in the cytosolic domains of viral glycoproteins is thus one possible mechanism to account for their essential targeting to the TGN. None of the other VZV glycoproteins, however, contain sequences that resemble the AYRV or the acidic sequences that account for the targeting of gE.

In the current study, gI (as well as gE) was demonstrated to be targeted to the TGN when it was expressed by itself in transfected cells and when it was cotransfected with gE. This observation implies that gI, like gE, contains targeting information in its primary structure. Since the signals that direct gE to the TGN are not present in the sequence of gI, this observation also establishes that one or more additional TGN targeting signal(s) exist. Both gE and gI were able to recruit the AP-1 adaptin protein to the TGN and colocalize with it. We were able to analyze the intracellular localization of expressed gB, gC, gI, gH, and gL because of the availability of good commercial monoclonal antibodies and highly specific rabbit polyclonal antibodies against these glycoproteins [9].

No targeting to the TGN was found for any of the other glycoproteins (gB, gC, gH, or gL) when they were expressed in transfected cells. It can thus be concluded that these glycoproteins lack the requisite targeting information. When gH and gL were cotransfected, gH appeared on the plasma membranes of the transfected cells. When expressed by stably transfected cells, gH could also be detected in intracellular vesicles. It is not clear why the vesicles could be detected in stably transfected cells, although the surface and RER localization in transiently transfected cells might have obscured the vesicles and prevented their visualization. gH and gL have been shown to form a complex, which is necessary for their transport out of the RER [10]. Concentration in the TGN, however, occurs as the result of the selective retrieval of glycoproteins from the plasma membrane [6]; therefore, exit from the RER is necessary for TGN targeting, although it alone is not sufficient to achieve targeting.

Since only two of the glycoproteins of VZV, gE and gI, are independently targeted to the TGN, the other glycoproteins cannot get there on their own. We therefore propose that gE or gI (or both) form complexes of glycoproteins that enable the glycoproteins that lack TGN targeting signal sequences to reach the TGN. gE or gI (or both) may thus act as navigator glycoproteins that bring complexes of glycoproteins to the TGN. This navigation and complex formation is essential because all of the glycoproteins must aggregate in the TGN in order to enable the final envelopment of VZV to take place.

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References