Varicella-Zoster Virus Glycoprotein gpI/gpIV Receptor: Expression, Complex Formation, and Antigenicity within the Vaccinia Virus-T7 RNA Polymerase Transfection System

ZHENGBIN YAO, WALLEN JACKSON, BAGHER FORGHANI, AND CHARLES GROSE*

Departments of Microbiology and Pediatrics, University of Iowa College of Medicine, 2501 JCP, University Hospital, Iowa City, Iowa 52242-1083, and Viral and Rickettsial Disease Laboratory, Division of Laboratories, California Department of Health Services, Berkeley, California 94704

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The unique short region of the varicella-zoster virus (VZV) genome contains two open reading frames which encode glycoproteins designated gpI and gpIV (herpes simplex virus homologs E and Gl, respectively). Like its herpesviral counterpart gE, the VZV gpI gene product functions as a cell surface receptor (V. Litwin, W. Jackson, and C. Grose, J. Virol. 66:3643-3651, 1992). To evaluate the biosynthesis of the two VZV glycoproteins and further explore their relationship to one another, the two glycoprotein genes were individually cloned into a pTiM1 vector under control of the T7 promoter. Transfection of the cloned gpI or gpIV construct into HeLa cells previously infected with vaccinia recombinant virus expressing bacteriophage T7 polymerase resulted in a much higher level expression of each VZV glycoprotein than previously achieved. Synthesis of both gpI and gpIV included intermediary partially glycosylated forms and mature N- and O-linked final product. Transfections in the presence of 32P, demonstrated that the mature forms of both gpI and gpIV were phosphorylated, while similar experiments with [35S]sulfate showed that only the mature gpI was sulfated. When gpI and gpIV were coexpressed in the same cell, the two glycoproteins were complexed to each other, as both proteins could be immunoprecipitated by antibodies against either gpI or gpIV. Coimmunoprecipitation did not occur as a result of a shared epitope, because gpI expressed alone was not precipitated by antibody to gpI, and gpIV expressed alone was not precipitated by antibody to gpIV. Pulse-chase analysis demonstrated that the gpI-gpIV association occurred early in processing; furthermore, this complex formation interfered with posttranslational modifications and thereby reduced the Mts of the mature forms of both gpI and gpIV. Similarly, the molecular masses of the cotransfected gene products corresponded with those of the infected cell glycoproteins, a result which suggested that authentic gpI and gpIV were ordinarily found within a complex. Thus, the adjacent open reading frames 67 and 68 code for two glycoproteins which in turn form a distinctive sulfated and phosphorylated cell surface complex with receptor properties.

Varicella-zoster virus (VZV), one of the human alphaherpesviruses, is the etiological agent of two clinically distinct syndromes: (i) chicken pox (varicella) and (ii) shingles (herpes zoster). The genome of VZV, the smallest of the human herpesviruses, is 80 MDA and potentially contains approximately 70 open reading frames, five of which code for glycoproteins (5). The VZV glycoproteins have been designated gpI, gpII, gpIII, gpIV, and gpV and have various degrees of amino acid homology to herpes simplex virus (HSV) glycoproteins gE, gB, gH, gL, and gC, respectively (5, 14). Of the five VZV glycoproteins, gpI is the predominant virion envelope glycoprotein and also the major glycosylated VZV cell surface antigen. This type I integral membrane glycoprotein is known to undergo extensive co- and posttranslational modifications, including both N-linked and O-linked glycosylation, phosphorylation, and acylation (8, 9, 14-16, 27). Characterization of gpIV, the smallest of the five VZV glycoproteins, has been far less complete because the glycoprotein has been difficult to isolate from infected cultured cells (6).

The gpI and gpIV glycoproteins are encoded by VZV genes 68 and 67, respectively, which are located within the unique short (Us) region of the VZV genome (5). Their HSV homologs, gE and gl, are much less prominent species, yet they function as a receptor for the Fc portion of immunoglobulin G (IgG) (33). Similarly, VZV gpI can behave as an Fc receptor for nonimmune IgG but much less efficiently than HSV gE (21). HSV gI appears to increase IgG binding to gE but does not bind immunoglobulin directly; likewise, IgG does not attach to VZV gpIV (20, 21). There has been considerable speculation about the relationship between the two Us glycoproteins of HSV or VZV described above. HSV gE and gl are often coprecipitated (19, 20); likewise, numerous monoclonal antibodies which immunoprecipitate both VZV gpI and gpIV have been produced (9, 12, 25, 27). In addition, one monoclonal antibody which precipitated the primary translation products of both gpI and gpIV in VZV-infected cells has been reported (36). On the basis of computer-assisted sequence analysis, which found small similar fragments within gpI and gpIV, investigators proposed that the homologous regions harbor an epitope shared by VZV gpI and gpIV (35, 36), but earlier evidence was inconsistent with this result: (i) when the sodium dodecyl sulfate (SDS) concentration in an immunoprecipitate was increased from 0.1 to 0.5%, the gpI specific antibodies no longer coprecipitated gpIV, and (ii) antibodies to gpI precipitated but failed to immunoblot gpIV (12, 25). Rather than a shared epitope, these results suggested that gpI and gpIV associated with each other by protein-protein interactions during their biosynthesis. However, no kinetic studies have documented the rate and site of these interactions in either an HSV or a VZV cell system.

* Corresponding author.
To address the issues mentioned above, we have subcloned VZV genes 67 and 68 into the pTM1 vector under control of the T7 promoter (26). Transfection of the VZV gpI or gpIV construct into HeLa cells led to a much higher level of expression than was found in either VZV-infected cultured cells or any other transfection system tested previously by our laboratory (21). Therefore, we have been able to investigate in greater detail than was previously possible the processing and posttranslational modifications of each glycoprotein, the formation of a gpI-gpIV glycoprotein complex within the rough endoplasmic reticulum-Golgi apparatus, and the antigenicities of the individual and complexed glycoproteins of this cell surface viral receptor. Altogether, this study further illustrates that the two VZV U5 glycoproteins display several properties which distinguish them from the three major VZV unique long glycoproteins.

MATERIALS AND METHODS

Construction of plasmids. The plasmid pTM1 was obtained from B. Moss, National Institutes of Health, Bethesda, Md. (26), and the VZV genomic library was obtained from R. Hyman, Palo Alto, Calif. The strategy for construction of the pTM1-gpI and pTM1-gpIV recombinant plasmids is shown in Fig. 1. The genes encoding glycoproteins gpI and gpIV are located within the C fragment of the HindIII library, which has been cloned into the pBR322 vector (7). The regions encoding full-length gpI and gpIV were amplified by polymerase chain reaction (PCR) using the pBR322-HindIII C plasmid as template DNA. The template DNA was subjected to 10 cycles of PCR amplification with the 5' oligonucleotide primer 5'-CGAGCCGGGGAGCTCCATGGGACAGTTAATAAAACC 3' and the downstream 3' primer 5'-CGCTCTAGAATCTTCCTCGGCGGATTTGAATCG 3'. The PCR oligonucleotides generated a SacI site upstream of the first AUG and a SpeI site in the 3' untranslated sequence. The resulting PCR products were digested with SacI and SpeI and cloned into the polylinker sites of the expression vector pTM1, which was linearized with SacI and SpeI. For cloning of gpIV, two primers were required to add a SacI site upstream of the gpIV coding region and a SpeI site downstream of the stop codon. The primers were 5'-GGCCGGGGGAAGTTTCTGCTTGGTGGGGATTCGAATGTG 3' and 5'-AGAAGACTTTTCTTAATCTTCCTCCCTCAT 3'. The resulting fragment was similarly cleaved and cloned into the pTM1 vector.

Conditions for cell culture and transfection. HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. For transfection, 10^6 HeLa cells were plated onto 35-mm dishes 16 to 20 h before infection. Cells were first infected with a recombinant vaccinia virus encoding T7 polymerase (vTF7) (13) at a multiplicity of infection of 10 to 15 in 0.5 ml of serum-free DMEM for 30 min at 37°C. The inoculum was then removed, and the cells were washed two times with serum-free DMEM. DNA (4.0 μg) was transfected by a liposome-mediated method (10). The vTF7-encoded bacteriophage T7 RNA polymerase transcribes the cytoplasmic plasmid DNA. For cotransfection, 4.0-μg samples of each DNA were mixed together before transfection. Transfected cells were incubated for 4 h in serum-free DMEM, further incubated in DMEM with 10% fetal calf serum at 37°C for various times, and harvested at 16 to 20 h after transfection by being dislodged into radioimmunoprecipitation buffer (10 mM Tris [pH 7.4] containing 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, and 0.1% SDS). The cells were sonically disrupted and sedimented (35,000 × g for 60 min) to remove insoluble macromolecules.

Isotopic labeling and immunoprecipitation. HeLa cells plated in a 35-mm tissue culture dish were transfected by lipofection as described above. At 4 h post-DNA transfection, cell culture medium was replaced with methionine-deficient DMEM (Sigma) and cells were starved for 15 min at 37°C. Thereafter, 1 ml of methionine-deficient medium containing 100 μCi of L-[^35]S)methionine (Amersham, Arlington Heights, Ill.) was added to each petri dish, and the culture was incubated at 37°C for 2 h. For pulse-chase analysis, transfected cells were similarly starved with methionine-deficient medium. Subsequently, cells were pulse-labeled with 100 μCi of[^35]S)methionine for 15 min and chased with DMEM for various periods. Cell lysates were prepared as described above. For immunoprecipitation, 50 μl of cell lysate and 5 μl of gpI or gpIV hybridoma ascites were added to 200 μl of lysis buffer and incubated at room temperature for 60 min. Production of monoclonal antibodies and pre-
citation of antibody-antigen complexes with protein A-Sepharose beads have been described elsewhere (12, 14, 25).
Immunoprecipitates were analyzed on 8, 10, or 12% polyacrylamide gels containing 0.1% SDS with or without 2-mercaptoethanol (24, 25). Gels were prepared for fluorography, dried, and exposed to radiographic film (Kodak). Usual exposure times were 12 to 72 h.

In vivo labeling with ³²P or ³⁵Sulfate. Cells were transfected singly or dually with pTM1-gpl or pTM1-gplIV as described above. At 4 h posttransfection, 1 ml of DMEM containing 250 μCi of ³²P (370 MBq/ml; 10 mCi/ml; Amersham) was added to each petri dish, and the culture was incubated for 12 h at 37°C. Prior to immunoprecipitation, cells were washed three times with phosphate-buffered saline (PBS), harvested in 0.5 ml of lysis buffer, and prepared as antigen for immunoprecipitation. To study sulfation, the conditions were identical except for the radiolabel: 300 μCi of ³⁵Sulfate (0.9 to 1.5 TBq; 25 to 40 Ci/mg; Amersham) per petri dish.

Indirect immunofluorescence. HeLa cells were seeded onto coverslips, transfected with the appropriate plasmid, and incubated for 12 to 16 h. Then the coverslips were washed three times with 0.01 M PBS (pH 7.4) and fixed in cold acetone at 4°C for 10 min. For surface staining, transfected cells were removed by incubation in 25 mM EDTA in 0.01 M PBS at ambient temperature for 5 min. Cells were washed three times with PBS containing 2% fetal calf serum and 0.1% NaN₃ and incubated with glycoprotein-specific monoclonal ascites (dilution, 1:20) for 1 h at room temperature. Cells were washed again prior to incubation with goat anti-mouse fluorescein conjugate (Boehringer Mannheim) at room temperature for 1 h. The cells were viewed with a Leitz fluorescence microscope, and photographs were taken with Kodak 400 ASA film.

Analysis of glycoproteins. Oligosaccharide structure of each viral glycoprotein was investigated with the following enzymes: endoglycosidase H (endo H), endo F, neuraminidase (sialidase), and endo-α-N-acetylglactosaminidase (O-glycanase). The method for digestion with each enzyme has been previously described (24, 25, 29, 34). In addition to studies using the enzymes listed above, studies were carried out with tunicamycin, which inhibits the addition of N-linked oligosaccharides to glycoproteins. Reagents were purchased from Sigma and Genzyme.

RESULTS

Expression of gpl and gplIV in transfected HeLa cells and their reactivities to monoclonal antibodies. HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then transfected by lipofection with pTM1-gpl, pTM1-gplIV, or pTM1 vector only. At 14 h posttransfection, cells were analyzed for synthesis and intracellular localization of the VZV gene products. The probe for gpl was monoclonal antibody 3B3, which recognizes an as-yet-undefined determinant in the N terminus of VZV gpl (25). The probe for VZV gplIV was monoclonal antibody 6B5, which also recognizes an undefined antigenic site located within the N terminus of this type 1 glycoprotein (22). Expression of gpl and gplIV was easily detectable in the cytoplasts of the acetone-fixed transfected cells (Fig. 2A and E). In a series of three experiments, the percentage of positive cells ranged from 80 to 95. Neither antibody reacted with cells transfected with vector only (Fig. 2C and G). In unfixed cells, immunofluorescence was detected exclusively in the membranes of gpl- or gplIV-transfected cells, as shown in Fig. 2B and F. To determine whether monoclonal antibody 3B3 or 6B5 cross-reacted with individually expressed viral glycoproteins, we stained the gpl-transfected cells with antibody 6B5 and the gplIV-transfected cells with antibody 3B3. As shown in Fig. 3, antibody 3B3 reacted specifically with fixed and unfixed gpl-transfected cells. Similarly, antibody 6B5 specifically bound to gplIV-transfected cells. From this experiment, it was concluded that neither monoclonal antibody detected an epitope shared between glycoproteins gpl and gplIV. Even though antibody 3B3 precipitates both gpl and gplIV in the presence of a low concentration of SDS, it obviously recognized an epitope confined to gpl. This experiment further confirmed the specificity of antibody 6B5, which was first tested against VZV-infected cells as part of a flow cytometric analysis of viral glycoproteins detectable on the cell surface (22).

Specificities of other anti-gpl and -gplIV monoclonal antibodies. Because of the reports of a shared epitope between gpl and gplIV (35, 36), several additional monoclonal antibodies which precipitated either gpl or gplIV or both gpl and gplIV were tested by immunofluorescence analyses. In these
were transfected with or goat to either transfected cells infected cells and subjected Table 1

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<th>MAb</th>
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<td>3B8</td>
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* MAb, monoclonal antibody; BF, B. Forghani’s laboratory; CG, C. Grose’s laboratory.

with pTM1 vector alone served as a control. Lysates of transfected cells prepared as described in Materials and Methods were immunoprecipitated with monoclonal antibody 3B3 and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The results in Fig. 4A show both an 81- and a 100-kDa species in pTM1-gpI-transfected cells (lane 2) but not in pTM1-transfected cells (lane 1). The two proteins corresponded to previously described partially and fully glycosylated gpI forms in VZV-infected cells (25, 27). In the presence of tunicamycin, which inhibits the addition of N-linked but not O-linked oligosaccharides to the native protein, two proteins of approximately 73 and 88 kDa were detected; a result which suggested that the 73-kDa form was the nonglycosylated backbone of gpI, while the 86-kDa form was an O-linked product (lane 3).

To study glycosylation in more detail, endoglycosidase digestions were performed. Figure 4A shows the digestion of immunoprecipitates with endo H, an enzyme which predominantly cleaves between the two proximal N-acetylglucosamine residues of high-mannose oligosaccharides but does not affect complex oligosaccharides (29, 34). Radiolabeled gpI immunoprecipitates were incubated in the presence of either endo H or an equivalent amount of water, and then the proteins were precipitated with cold acetone and subjected to electrophoretic separation. As described above, antibody 3B3 precipitated two gpI species (gp100 and gp81) (Fig. 4A, lane 4). Endo H treatment reduced the Mr of gp81 to 73 kDa, whereas the relative migration of gp100 was unchanged (Fig. 4A, lane 5). This result indicated that gp81 contained high-mannose oligosaccharides, while the oligosaccharides on gp100 were already fully processed to complex moieties (endo H resistant). To determine whether gpI contained O-linked oligosaccharides, the cell lysates from gpI-transfected cells were subjected to serial neuraminidase and O-glycanase digestions. As shown in Fig. 4A, removal of N-acetylneuraminic acid residues generated a protein with a molecular weight of 90 kDa (lane 7), while subsequent treatment with endo-α-N-acetylgalactosaminidase (O-glycanase) caused a further shift of gpI from 90 to 88 kDa (lane 8). This result demonstrated that gpI in transfected
cells contained O-linked glycans. This analysis also confirmed the similarity in biosynthesis of gpI in transfected cells and VZV-infected cells (25).

**Analysis of glycosylation of gpI.** Only limited biochemical studies of the gpIV glycoprotein gene products. HeLa cells were transfected with pTM1-gpl or pTM1-gpIV and labeled with [35S]methionine for 2 h. Cell lysates were immunoprecipitated with monoclonal antibody 3B3 for gpI or 6B5 for gpIV and analyzed by SDS-PAGE. (A) Analysis of gpI. Lanes: 1, cells transfected with pTM1 vector only; 2, cells transfected with pTM1-gpI; 3, transfection in the presence of 10 μg of tunicamycin (TM) per ml; 4 and 5, gpI not treated (lane 4) or digested with endo H (lane 5); 6 to 8, gpI not treated (lane 6) or treated with neuraminidase (NEU) alone (lane 7) or both neuraminidase and O-glycanase (OGL) (lane 8). Lanes 1 to 3, 4 and 5, and 6 to 8 represent separate gels. (B) Analysis of gpIV. Lanes: 1, cells transfected with pTM1 vector only; 2, cells transfected with pTM1-gpIV; 3, transfection in the presence of 10 μg of tunicamycin (TM) per ml; 4 and 5, gpIV before (lane 4) and after (lane 5) treatment with endo H; 6 to 8, gpIV before (lane 6) or after treatment of gpIV with neuraminidase alone (lane 7) or both neuraminidase and O-glycanase (lane 8). Lanes 1 to 3, 4 and 5, and 6 to 8 represent separate gels. The molecular mass marker proteins included myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

**Complex formation by gpI and gpIV.** As mentioned above, the relationship of the two glycoproteins gpI and gpIV has not yet been resolved. Because of the high expression of gpIV in the vaccinia virus-pTM system, we were able for the first time to investigate the fate of the individual gene products. To determine whether gpI and gpIV formed a complex when both proteins were expressed together, we singly and dually transfected with pTM1-gpI and pTM1-gpIV. HeLa cells expressing gpI, gpIV, or both proteins were metabolically labeled with [35S]methionine for 2 h. Transfected cell cultures were detergent solubilized, and the lysates were immunoprecipitated with antibodies to gpI or gpIV. The results were the same, regardless of whether SDS-PAGE was performed under reducing or nonreducing conditions (former gel not shown). On the basis of the immunoprecipitation data given above, we concluded that gpI and gpIV formed complexes when coexpressed in the same cells.

**Kinetics of VZV glycoprotein complex formation.** The ability to detect an interaction between gpI and gpIV by metabolic labeling in HeLa cells also permitted the study of the rate and site of complex formation in the cell. Transfected cells expressing both gpI and gpIV were pulsed labeled with [35S]methionine for 15 min and then either lysed immediately or incubated in chase medium containing unlabeled methionine for 10, 30, 60, and 90 min prior to lysis. Immunoprecipitates formed with both antibodies were then analyzed by SDS-PAGE to assess coprecipitation of gpI and gpIV. The results were the same in lanes 4 and 8. The results were the same, regardless of whether SDS-PAGE was performed under reducing or nonreducing conditions (former gel not shown). On the basis of the immunoprecipitation data given above, we concluded that gpI and gpIV formed complexes when coexpressed in the same cells.

by O-linked glycosylation (45 kDa). The mature 64-kDa protein was resistant to endo H digestion, presumably because it contained fully processed N-linked glycans (as well as O-linked glycans), while an intermediate 50-kDa protein was sensitive, a result which indicated that it contained high-mannose glycans (Fig. 4B, lanes 4 and 5).

To confirm that gpIV contained O-linked oligosaccharides, the cell lysates from gpIV-transfected cells were subjected to neuraminidase and O-glycanase digestions. As shown in Fig. 4B, removal of N-acetylgalactosamine residues from the 64-kDa protein generated a protein with a molecular mass of 57 kDa (lane 7), while treatment with endo-α-N-acetylgalactosaminidase (O-glycanase) further reduced the molecular mass to 54 kDa (lane 8). In addition, we radiolabeled a gpIV transfection with [14C]glucosamine and repeated the neuraminidase and O-glycanase digestions. The profiles were similar to those shown in Fig. 4B (gel not shown). These results, together with the tunicamycin data, indicated that gpIV made in transfected cells contained O-linked oligosaccharides. The variable amount of gpIV intermediary forms visible in the protein profiles depended on the radiolabeling intervals: lesser amounts were detectable with labeling overnight (lanes 6 through 8) than with labeling for 2 h (lanes 1 through 5).
excised from the gel, and their radioactivities were counted by scintillation to quantitate the association of gpI with gpIV. The result indicated that the association of gpI and IV increased over time, from 18% during the pulse period (lane 1) to 68% after 90 min of chase time (lane 9). Analysis of the association of gpIV with gpI showed that 8% of gpIV coprecipitated with gpI during the pulse period (lane 2) and 35% coprecipitated after 90 min of chase (lane 10). An alternative explanation for the apparently rapid association between gpI and IV was that the event occurred during the preparation of the cell lysate and not during actual biosynthesis of the glycoproteins in the transfected cell. To examine this possibility, we attempted to reconstitute the interaction by expressing the proteins separately and mixing the solubilized cell lysates. Even after prolonged incubation of the two cell lysates, we were not able to detect gpI-gpIV complex formation (data not shown). This result indicated that gpI-gpIV complex formation was not an artifact of detergent lysis.

**Effect of complex formation on processing of gpI and gpIV.** The association of VZV gpI and IV during the 15-min pulse indicated that the event occurred early in the exocytic pathway. Furthermore, a reexamination of Fig. 5 suggested that the $M_r$ of either gpI or gpIV was reduced during a cotransfection compared with a single transfection (compare lanes 3 and 4 with lanes 7 and 8). To confirm this observation, the experiment was repeated under conditions of SDS-PAGE designed to further separate the individual constituents of gpI. As seen in Fig. 7, the $M_r$ of the mature gpI was 100 kDa when cells were transfected with gpI alone (lane 1), while the $M_r$ shifted to 98 kDa as a result of cotransfection with gpIV (lane 2). The $M_r$ of a previously described 81-kDa high-mannose form of gpI was not changed, regardless of whether gpI was transfected alone or with the gpIV gene. In earlier reports from this laboratory, the $M_r$ of gpI from an infected cell culture was estimated to be 98 kDa (25). To verify that the mass of gpI from an infected culture resembled that from a cotransfection, gpI was isolated under three conditions: (i) from infected cells, (ii) from cotransfected cells, and (iii) from cells transfected with gpI gene alone. As shown in Fig. 7, the $M_r$ of gpI from infected cells was similar to that of gpI from cotransfected
cells (lanes 4 and 5), while gpI from singly transfected cells had a higher $M_r$ (lane 3). Similarly, the data for gpIV were compared in the three circumstances listed above. Again, the singly transfected culture had a more heterogeneous gpIV product (lane 8) with a higher $M_r$, while gpIV recovered from either infected cells or a gpI-gpIV cotransfected culture had a lower $M_r$ (Fig. 7, lanes 6 and 7).

We considered the possibility that the differences in $M_r$ were related to the switch in cell substrates, i.e., human melanoma cells for infection and HeLa cells for infection-transfection. To this end, we repeated the infection-transfection experiments in human melanoma cells. Although transfection was not as efficient as in HeLa cells, the results were similar to those described above; i.e., the singly transfected glycoprotein had a higher $M_r$ than the glycoprotein from a cotransfected culture (data not shown). The most reasonable explanation for these events is an alteration in the Golgi apparatus. In an attempt to define differences in N- and O-linked glycoproteins before and after complex formation, the gpI-gpIV complex was subjected to analysis with endo F, neuraminidase, and O-glycanase, but no marked changes in sensitivity were noted. However, a comparative analysis of complexed versus noncomplexed glycoproteins was complicated by the fact that both gpI and gpIV have three or more potential N-linked sites and perhaps as many O-linked sites. If gpI-gpIV complex formation impaired oligosaccharide processing of only one chain, an event which could lower the $M_r$ by 2,000, we would have difficulty detecting this alteration by the methods described above.

Sulfation of VZV gpI and gpIV. In the sections above, we demonstrated that the $M_r$ of the each complexed glycoprotein was reduced compared with the $M_r$ of the individually transfected VZV glycoprotein. The $M_r$ of a glycoprotein can be greatly altered by two late posttranslational modifications: sialation and sulfation. Prior data in Fig. 7 suggested that complex formation interferes with processing of the mature forms and thereby may decrease sialation. To determine whether oligosaccharide sulfation was also involved with the mobility changes, we investigated whether this posttranslational modification was seen in the individually transfected VZV gene products and, if so, whether this modification was altered under conditions of cotransfection with the two glycoprotein genes. To this end, we added $^35$SO$_4$ to culture medium overlying VZV pTM1-gpI- or pTM1-gpIV-transfected cells and analyzed the cell lysates by immunoprecipitation. As shown in Fig. 8, there were no sulfated proteins precipitated from cells transfected with pTM1 vector only (lane 1), nor was any sulfated protein detected in precipitates of the pTM1-gpIV-transfected cell (lane 2). In pTM1-gpI-transfected cells, however, the mature VZV gpI form was highly sulfated (lane 3). Cotransfection of gpI and gpIV did not abolish sulfation of gpI (lane 4). Figure 8 again illustrated the effect of this protein-protein association in lowering the $M_r$ of VZV gpI.

Phosphorylation of VZV gpI and gpIV. The VZV gpI product is characterized by phosphorylation of its polypeptide backbone both in cell culture and in vitro by cellular kinases (15). The precursor form of gpIV in VZV-infected cells was also shown to be phosphorylated during in vivo labeling (36). Yet, investigations to further define gpIV phosphorylation have been inhibited by the small yields of this glycoprotein within either the infected cell or the transfected cell. Furthermore, we questioned whether phosphorylation of the viral glycoproteins was altered by gpI-gpIV complex formation. Because of the high expression of gpI and, in particular, gpIV in the pTM1 transfection system, we reexamined the in vivo phosphorylation of VZV gene products 67 and 68. Transfected cells were metabolically labeled for 12 h with $^32$P$_i$, after which detergent extracts were prepared and subjected to immunoprecipitation. As shown in Fig. 9, $^32$P-labeled intermediate and mature forms were easily detected in the SDS-PAGE profile of gpI-transfected cells (lane 2). These phosphoproteins were not observed in cells transfected with the vector alone (lane 1). Comparison of gpI in lanes 2 and 3 again demonstrated the lowered $M_r$, as a result of cotransfection with pTM1-gpIV. Similar analyses of gpIV-transfected cells led to the detection of two phosphorylated species (50 and 64 kDa; lane 5). These results indicated that both intermediary and mature forms of gpIV were phosphorylated. When gpIV was precipitated from cultures cotransfected with pTM1-gpI, there was a more heavily $^32$P-labeled gpIV (lane 6). This result was reproducible in three repeated experiments.

Reduction in gpI-gpIV complex formation. To further analyze gpI-gpIV complex formation, we produced a gpI mutant with a deletion in a highly conserved region of gpI (21, 28). On the basis of the VZV DNA sequence published by Davison and Scott (5), two Ndel restriction sites were localized in the exocytosomal portion of gpI. After excision of the intervening nucleotides from the pTM1-gpI construct, a truncated form of gpI with a deletion of 105 amino acid residues (amino acid residues 342 to 446) was constructed (Fig. 10A). This deletion mutant lacked 6 cysteine residues from a total of 10 cysteines in the ectodomain of gpI. When HeLa cells were transfected with the mutant construct, an immunofluorescence study showed that the gene product could be detected on the surfaces of living cells (Fig. 10B); this result indicated not only that the truncated glycoprotein was processed in the Golgi complex and transported to the outer cell membrane but also that the epitope for monoclonal antibody 3B3 was located toward the N terminus of gpI. Dual transfections were performed with both pTM1-gpI and the truncated gpI gene. As illustrated in Fig. 10C, analysis of the gene products by immunoprecipitation with antibodies to gpI and gpIV found that wild-type gpI was capable of binding to gpIV (lanes 3 and 4) while truncated gpI showed a reduction in the degree of coprecipitation (lanes 1 and 2). It is likely that the six cysteines will
be involved in intramolecular disulfide bond formation, a process which occurs in the endoplasmic reticulum, the site of gpI-gpIV complex formation (Fig. 6). Thus, a substantial deletion of cysteine residues could alter the conformation of the gpI molecule and thereby impair its protein-protein interaction with gpIV in the endoplasmic reticulum.

DISCUSSION

The relationship of the two VZV glycoproteins gpI and gpIV has been a subject of speculation since the initial observations that they were frequently co precipitated by murine monoclonal antibodies. In this paper, we readdressed this issue in a multistep process. First, an improved transfection system was required. In an earlier study, transfection had been carried out by a more-traditional method (21). Under prior conditions, between 5 and 20% of cells reacted positively with gpI- or gpIV-specific monoclonal antibodies. Cells transfected with the gpI gene produced an Fc receptor which bound nonimmune human IgG but not IgA or IgM, while cells transfected with the gpIV gene failed to bind any nonimmune immunoglobulin. Although VZV-specific activity was detectable on the transfected cells, the relatively small percentage of VZV-positive cells proscribed a more-detailed study of gpI-gpIV interactions. As part of a new approach, we selected a method developed in the B. Moss laboratory (13, 26) which combines infection with a recombinant vaccinia virus expressing T7 RNA polymerase and transfection with genes under control of the T7 promoter. This infection-transfection system is ideally suited for analysis of protein-protein interactions because of the high levels of coexpression of each gene product. For example, the T7 polymerase-T7 promoter transfection system has been successfully

FIG. 9. Phosphorylation of transfected VZV gene products. Transfected cells were metabolically labeled for 12 h with 32P, after which detergent extracts were prepared and subjected to immunoprecipitation as described in the text. Antibodies (Ab) are indicated at the bottom of the gel. The immunoprecipitates were analyzed by 10% SDS-PAGE. Cells were transfected with pTM1 vector only (lane 1), pTM1-gpI (lane 2), pTM1-gpI and pTM1-gpIV (lane 3), pTM1 vector only (lane 4), pTM1-gpIV (lane 5), or pTM1-gpI and pTM1-gpIV (lane 6). Molecular mass markers are indicated (in kilodaltons).

FIG. 10. Reduced association of gpI deletion mutant with gpIV in transfected cells. (A) Schematic diagram of the VZV gpI polypeptide and location of deletion. A line drawing was derived from the predicted secondary structure of VZV gpI (not to scale). The thickened line indicates the deletion. (B) Expression of the gpI deletion mutant on the transfected cell surface. HeLa cells were transfected as described in the text. At 14 h posttransfection, unfixed transfected cells were stained with monoclonal antibody 3B3 and fluorescein conjugate. (C) Coexpression of VZV gpI deletion mutant with gpIV. After HeLa cells were transfected with pTM1-gpI deletion mutant and pTM1-gpIV, cells were labeled with [35S]methionine for 2 h. Cell lysates were prepared and immunoprecipitated with monoclonal antibody 3B3 or 6B5. Cells were cotransfected with gpI deletion mutant and gpIV and precipitated with antibody 6B5 (lane 1) or antibody 3B3 (lane 2), or cells were transfected with wild-type gpI and gpIV and precipitated with antibody 6B5 (lane 3) or antibody 3B3 (lane 4). Molecular mass markers are indicated on the right in kilodaltons.
applied to the study of CD4 interactions with human immunodeficiency virus gp160 (4, 18) and p56ck tyrosine kinase (30).

Under conditions of transfection within the vaccinia virus-T7 RNA polymerase system, the yields of the two VZV glycoprotein gene products were estimated to be as much as 10-fold greater than previously achieved by transient transfection. This approach appeared to be superior to having recombinant vaccinia virus themselves express herpesviral glycoprotein genes (1) in that we were able to mimic or exceed the gpI-gpIV levels found in VZV-infected cells. Indeed, for VZV gpIV, the levels were much higher as gauged by degree of immunostaining and lengths of exposure of fluorograms: 1 day for infection-transfection versus up to 4 months for infection (6). Thus, we have been able to perform studies of gpI-gpIV interactions not possible under previously described conditions of transfection or infection of cultured cells. The stoichiometry of the gpI-gpIV complex remains to be resolved. Based on the data presented here as well as on prior results with infected cells (6, 8, 25, 27), a plausible explanation is that the complex in infected cells consists of more molecules of gpI than of gpIV.

In our studies, monoclonal antibodies prepared against native gpI did not immunoprecipitate gpIV from cell extracts transfected with gpIV gene alone, and monoclonal antibodies against native gpIV did not immunoprecipitate the transfected gpI product. Vafai et al. (36) reported a monoclonal antibody designated 79.0 which precipitated the in vitro translation products of open reading frames 67 and 68 as well as the mature, fully glycosylated gpI. The binding site was further localized to a 14-mer (residues 109 to 123) on gpI. By comparison of the predicted amino acid sequences of gpI and gpIV, they found that 14 amino acids of gpI (residues 107 and 121) exhibited a 36% similarity to two regions of gpIV (residues 55 to 69 and 245 to 259). In a subsequent report, Vafai et al. (35) prepared a rabbit antibody to a synthetic peptide comprising the 14 amino acids. The rabbit antibody recognized the primary translation products of gpI and gpIV but not the mature forms of either glycoprotein. Therefore, this epitope on the polypeptide backbone of gpI is no longer present or detectable after glycosylation and processing have occurred. Attempts to block binding of antibody 79.0 to gpI by addition of the 14-mer peptide to the reaction mixture were unsuccessful (35).

Our phosphorylation experiments demonstrated that both intermediary and mature forms of gpI were phosphorylated in vivo. An earlier report suggested that only the precursor forms of gpI were phosphorylated in VZV-infected cells (36). An explanation for this discrepancy is the low level of gpIV expression in virus-infected cells. Other interesting aspects of gpIV phosphorylation were apparent in the transfection system; for example, the mature form of gpIV appeared more highly phosphorylated during cotransfection than when singly transfected. Thus, complex formation may make a phosphorylation site more accessible to the protein kinase, or alternatively, highly phosphorylated gpIV is better able to form protein-protein complexes with gpI. To further investigate this issue, we have devised a protocol by which to mutagenize the putative phosphorylation sites in the cytoplasmic tails of the glycoproteins (37). Further progress has awaited completion of the improved transfection system described in this report.

In addition to phosphorylation, the property of oligosaccharide sulfation was also addressed with VZV gpI and gpIV. A major sulfated HSV glycoprotein is gE (VZV gpI homolog) (17). In this study, we found that VZV gpI was heavily sulfated, while gpIV was not. The fact that there must be specificity to oligosaccharide sulfation is indicated by similarities between HSV and VZV homologs. While gpI is the predominant VZV glycoprotein synthesized in infected cells, gE is a relatively obscure HSV glycoprotein, yet both are heavily sulfated. We have examined the amino acid sequence of gpI for the tripeptide recognition marker ProXaaArg/Lys utilized by GaINAc-transferase when the trimer is located six to nine residues amino terminal to an N-linked glycosylation site (11), but we did not find it. Therefore, oligosaccharide sulfation of gpI does not appear to be similar to the process recently described for the sulfated glycoprotein lutropin.

Finally, we performed a computer-assisted sequence analysis of VZV gpIV and its alphaherpesviral homologous glycoproteins. In previous reports (21, 28), we and others showed that VZV gpI closely resembled pseudorabies virus (PRV) gpI and HSV gE not only in the exoplasmic portions but also in the cytoplasmic portions of the respective molecules. In Table 2, we compare VZV gpIV with HSV gI, PRV gp63, and gI of equine herpesvirus 1, a third alphaherpesvirus. The sequence identity reached as high as 39% in one domain; i.e., an extracellular region of VZV gpIV between amino acids 50 and 150 contains three cysteine residues which were highly conserved across the four viruses. These results closely resemble those previously described for VZV gpI and its homologs; viz., the regions of highest homology were the cysteine-rich portions of the exoplasmic molecule (21, 28). Presumably, intramolecular disulfide binding contributes to the tertiary structure of each of the two glycoproteins and this native conformation facilitates intermolecular complex formation. Since the homology extends through the lengthy cytoplasmic portions of the two alphaherpesviral glycoproteins, the Fc receptor complex probably has similar functions in each virus. Recent investigations of the PRV counterparts indicate that the viral glycoproteins behave as adhesion molecules and provide specificity for attachment to neuronal cells (2, 3, 23). Thus, these two U5 glycoproteins may influence the pronounced neuropathism and neurovirulence of the Alpha herpesvirinae.

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### Table 2. Amino acid sequence comparison of homologous glycoproteins

<table>
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<th>Residue no. in gpIV</th>
<th>% Identity</th>
<th>% Similarity</th>
<th>Ratio</th>
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<td>79.0</td>
<td></td>
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<td>1-390</td>
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<tr>
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</table>

* Data were generated by using the University of Wisconsin Genetics Computer Group Bestfit program (32).

* SwissProt accession numbers are P09258 for VZV gpI and P06487 for HSV gI. GenBank and EMBL accession numbers are M14336 for PRV gp63 and M36299 for equine herpesvirus (EHV) gI.

* Ratio is equal to the quality score divided by the number of residues in the shorter of the two compared segments.
REFERENCES


