Varicella-Zoster Virus Glycoproteins E and I Expressed in Insect Cells Form a Heterodimer That Requires the N-Terminal Domain of Glycoprotein I

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Varicella-zoster virus (VZV) glycoproteins E and I (gE and gI), which are major components of the virion envelope, form a noncovalently linked complex. To understand their properties and functions, we expressed and purified soluble forms of gE and gI in the baculovirus system. Extracellular domains of gE and gI were cloned into baculoviruses, using either native or insect-derived signal peptides. Each recombinant virus yielded soluble protein in culture medium although a higher level of secretion was achieved with insect-derived signal peptides in recombinant gE baculoviruses. A soluble gE–gI complex was formed by co-infecting insect cells with recombinant gE and gI baculoviruses and detected by immunoprecipitation followed by Western blotting analyses. By gel filtration and cross-linking studies, we showed that the VZV gE–gI complex expressed in insect cells is a heterodimer. Interestingly, two recombinant gI proteins in which signal peptides were replaced with insect-derived signal peptides did not associate with gE. Amino-terminal sequencing and site-specific mutational studies showed that the replacement of only the signal peptides did not prevent complex formation but alterations in the processed amino-terminus of gI abrogated its ability to complex with gE. These findings indicate that the mature amino-terminus of gI is required for gE–gI complex formation by the external domains of VZV gE and gI.

INTRODUCTION

Varicella-zoster virus (VZV), a member of alphaherpesvirus subfamily, is the causative agent of chicken pox (varicella) and shingles (herpes zoster). The VZV genome contains 69 unique open reading frames, 6 of which encode glycoproteins termed gB, gC, gE, gH, gL, and gM by analogy to their counterparts in herpes simplex virus type 1 (HSV-1) (Cohen and Straus, 1996). VZV gE, a product of open reading frame 68, is a major component of the virion membrane and the most abundant glycoprotein detected in infected cells (Montalvo et al., 1985). In infected cells, VZV gE forms a noncovalently linked complex with VZV gI, the product of open reading frame 67 (Yao et al., 1993). Both gE and gI serve as targets for neutralizing antibodies and induce cellular immunity in animal models (Arvin et al., 1987; Forghani et al., 1990; Haumont et al., 1996; Huang et al., 1992; Vafai and Yang, 1991).

VZV gE can serve as an Fc receptor for human IgG but much less efficiently than does its HSV-1 gE homolog (Litwin et al., 1992). The HSV gE also has been implicated in facilitating viral spread from neuron to neuron (Dingwell et al., 1995). Recent investigations of pseudorabies virus (PRV) indicate that its gE, another homolog of VZV gE, provides specificity for attachment to neuronal cells (Card et al., 1992; Mulder et al., 1996). It is possible that VZV gE also confers specificity for cellular tropism and viral spread; however, no cellular ligands for any of the gE homologs have been defined, and the roles of VZV gI and its homologs are also not known.

To understand the properties and functions of gE, gI, and the complex they form, and to provide reagents for the studies of their immunogenicity and receptor interactions, we sought to express and purify them using the baculovirus expression system that had provided high-level expression of secreted forms of other viral glycoproteins, including HSV-1 gC and gD (Sisk et al., 1994; Tal-Singer et al., 1995). The extracellular domains of VZV gE and gI were cloned into baculoviruses, using either native VZV or insect-derived signal peptides. Each recombinant virus yielded a soluble protein that was secreted into the culture medium. We could purify some forms of gE and gI by introduction of polyhistidine tags and metal affinity chromatography. To produce the gE–gI complex, insect cells were co-infected with recombinant gE and gI baculoviruses. Through these and other methods that we describe, it was possible to characterize the structure and properties of gE, gI, and their complex and to discern a specific region of gI that is required for complex formation.

MATERIALS AND METHODS

Cell culture and virus

Spodoptera frugiperda (Sf9) cells, which were kindly provided from Dr. T. L. Leto (Laboratory of Host Defenses,
The VZV gene encoding gE was cloned from cosmid VZV MstIIA (Cohen and Seidel, 1993) into a transfer vector (pBacPAK8; Clontech) downstream of the polyhedrin promoter (Kitts and Possee, 1993). To eliminate its transmembrane and cytoplasmic domains, VZV gE was truncated at amino acid 537 by site-specific, oligonucleotide-directed mutagenesis. To enhance expression and secretion, this extracellular domain of gE was also cloned into a second transfer vector, pAcGP67A (Invitrogen), which contained the baculovirus gp67 signal peptide following the polyhedrin promoter. To achieve this construction, the DNA fragment corresponding to amino acids 23 to 537 of VZV gE was amplified by PCR using a 5’ oligonucleotide primer (TTTGGATCCCCGTATAACGAA-TCGGGCT; primer A) that contained a BamHI site (underlined) and a 3’ oligonucleotide primer (TTTCTG-CATTATCGTAGAAGTGGTACG; primer B) that contained a stop codon and a PsiI site (underlined). The fragment was digested with BamHI and PsiI and ligated into pAcGP67A. A third transfer vector, pVT-Bac (a gift from Drs. G. Cohen and R. Eizenberg, University of Pennsylvania, Philadelphia, PA), was also used in the hope of enhancing secretion (Sisk et al., 1994; Tessier et al., 1991). This vector contained the honeybee melittin signal peptide. The same gE fragment that had been inserted into pAcGP67A was cloned into pVT-Bac. It was amplified by PCR using 5’ oligonucleotide primer A and a 3’ oligonucleotide primer (TTTCTG-CAGTTAATGATGATGATGATGATGATGATG; primer C) that introduced a stop codon, a PsiI site (underlined), and histidine hexapeptide tag sequences (double-underlined).

A similar strategy was used to clone a series of VZV gl constructs. The gene encoding VZV gl was cloned from plasmid pGVEcORIA (Reinhold et al., 1988) into pBacPAK8 following the polyhedrin promoter. The coding sequences of gl was truncated at amino acid 271 by site-specific, oligonucleotide-directed mutagenesis. In an attempt to enhance expression and secretion, the extracellular domain of gl was also cloned into pAcGP67 downstream of its polyhedrin promoter. The DNA fragment encoding amino acids 24 to 271 of gl was amplified by PCR using a 5’ oligonucleotide primer (TTTGGATCC-CAAGGGCGACCACGTAG; primer D) that contained a BamHI site (underlined) and a 3’ oligonucleotide primer (TTTCTG-CAATTATCGTAGAAGTGGTACG; primer E) that introduced a stop codon and a PsiI site (underlined) and then cloned into pAcGP67A. The same fragment was cloned into pVT-Bac. It was amplified by PCR using 5’ oligonucleotide primer D and a 3’ oligonucleotide primer (TTTCTGCAGTTAATGATGATGATGATGATGATGATG; primer F) that introduced a stop codon, a PsiI site (underlined), and histidine hexapeptide tag sequences (double-underlined). All gE and gl constructs were confirmed by DNA sequencing.

BamHI site (underlined) and a 3’ oligonucleotide primer (TTTCTGCAATTATCGTAGAAGTGGTACG; primer E) that introduced a stop codon and a PsiI site (underlined) and then cloned into pAcGP67A. The same fragment was cloned into pVT-Bac. It was amplified by PCR using 5’ oligonucleotide primer D and a 3’ oligonucleotide primer (TTTCTGCAGTTAATGATGATGATGATGATGATG; primer F) that introduced a stop codon, a PsiI site (underlined), and histidine hexapeptide tag sequences (double-underlined). All gE and gl constructs were confirmed by DNA sequencing.

These gE- or gl-containing plasmids were co-transfected onto Sf9 cells via lipofection with Bsu361-digested BacPAK6 DNA (Clontech), a derivative of AcMNPV (Kitts and Possee, 1993). All recombinant viruses were harvested after 3 days, plaque-purified three times, and amplified in Sf9 cells. The resultant viruses were designated Bac – gEt, Bac – gEt – gp67, and Bac – gEt – his for the recombinant gE baculoviruses and Bac – glt, Bac – glt – gp67, and Bac – glt – his for the recombinant gl baculoviruses. The recombinant proteins expressed by these viruses had the native VZV signal peptides (truncated proteins designated gEt and glt), the baculovirus gp67 signal peptides (designated gEt-gp67 and glt-gp67), or the honeybee melittin signal peptides (gEt-his and glt-his). Figure 1 shows a schematic representation of each of these six recombinant proteins.
Construction of mutant gI recombinant baculoviruses

An additional family of baculoviruses encoding mutated gI proteins was derived from the Bac–glt virus. One gI mutant in which the viral signal peptide was replaced by the melittin signal peptide before amino acid 21 was constructed using site-specific, oligonucleotide-directed mutagenesis. The resulting recombinant protein was designated glt–MSP. A second gI mutant replaced amino acids 21–23 from Leu-Ile-Phe with Asp-Pro by site-directed mutagenesis. This yielded recombinant protein glt–DP. Four other mutant baculoviruses were made by inserting linkers at unique restriction sites of the gI gene. EcoRI linkers (New England Biolabs) were inserted in the Pml site (base pair 81; numbering begins at the start codon of open reading frame 67), the HpaI site (base pair 96), the NdeI site (base pair 647), or the StyI site (base pair 688). The recombinant proteins expressed by these mutant viruses had insertions of 4 amino acids after amino acids 27, 32, 216, and 231 of VZV gI and were designated glt–Pml, glt–Hpa, glt–Nde, and glt–Sty, respectively.

Expression of glycoproteins from recombinant baculoviruses

Each recombinant baculovirus was used to infect 1.5 × 10^6 Sf9 cells in a 35-mm dish at a multiplicity of infection (m.o.i.) of 4. After incubating for 3 days at 27°C, the medium was collected and filtered through a 0.45-μm filter (Millipore). Then, 30 μl of the medium was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in the presence of β-mercaptoethanol. For Western blotting, proteins were transferred onto nitrocellulose membranes (Protran; Schleicher & Schuell) and reacted with either anti-VZV gE monoclonal antibody (3G8; a gift of Dr. B. Forghani, California Department of Health Services, Berkeley, CA) or anti-VZV gI rabbit serum (a gift of Dr. P. R. Kinchington, University of Pittsburgh, Pittsburgh, PA) (Forghani et al., 1990). The blots were incubated with peroxidase-conjugated anti-mouse antibody or anti-rabbit antibody as required and then developed using enhanced chemiluminescence (ECL kit; Amersham).

Larger scale protein synthesis and purification

Suspension cultures of Sf9 cells were infected with either Bac–gEt–his or Bac–glt–his at an m.o.i. of 4 and incubated at 27°C for 3 days. Cells were pelleted by centrifugation and the supernatants were collected, supplemented with 1/10 vol of 1 M NaCl and 200 mM Tris–HCl, pH 8.1, and incubated with 1/20 vol of metal affinity resin (TALON; Clontech) for 1 hr at 4°C. The resin was washed four times with washing buffer (100 mM NaCl and 20 mM Tris–HCl, pH 8.0) and then packed into a 10-ml polypropylene column. The column was washed again with washing buffer and eluted with 100 mM imidazole/100 mM NaCl/20 mM Tris–HCl, pH 8.0. The eluted proteins were concentrated by ultrafiltration (centricon 30 or centriplus 100, Amicon) and dialyzed overnight against PBS.

To express and purify gE–gI complexes, suspension culture of Sf9 cells were co-infected with Bac–gEt–his at an m.o.i. of 1 and Bac–glt at an m.o.i. of 3 and incubated at 27°C for 3 days. Purification by metal affinity chromatography was performed as described above. The gE–gI complex was further purified by gel filtration using a Sephacryl S-200 column (Pharmacia Biotech). The protein solutions were loaded onto columns equilibrated with PBS at a flow rate of 0.1 ml/min. Fractions (1 ml each) were collected, subjected to SDS–PAGE, and analyzed by Western blotting.

Determination of the molecular mass of purified proteins was also done by Sephacryl S-200. Purified gE–gI complex, gEt–his, or gIt–his was loaded on the column, and elution was monitored by its absorbance at 280 nm using a UV lamp and absorbance detector (Monitor UV-MII; Pharmacia Biotech). The molecular mass of each protein was then calculated using a calibration curve obtained with four protein standards: catalase (250 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa).

N-terminal amino acid sequencing

Ten micrograms of each purified protein was electrophoresed and blotted onto a polyvinylidene difluoride membrane (Millipore) for microsequencing. Bands were stained with Ponceau S (Sigma), cut from the membrane, and then eluted. N-terminal amino acid sequence analysis was performed for us by Dr. J. Coligan (Laboratory of Molecular Structure, National Institute of Allergy and Infectious Diseases, Rockville, MD).

Immunoprecipitation of proteins followed by Western blotting

Recombinant VZV gE and gI baculoviruses, each at an m.o.i. of 2, were used to co-infect 1.5 × 10^6 Sf9 cells in 35-mm dishes. After 3 days of incubation, the medium was collected, filtered (0.45 μm), and centrifuged at 13,000 g for 20 min to remove insoluble materials. Supernatant proteins were added to equal volumes of immunoprecipitation buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4, 0.5% Tween 20, and 0.1% NP-40) and incubated with either the 3B3 VZV gE monoclonal antibody or the 6B5 VZV gI monoclonal antibody (both gifts of Dr. C. Grose, University of Iowa, Iowa City, IA) for 1 hr at 4°C (Litwin et al., 1992). Antibody–antigen complexes were precipitated with Protein A–agarose beads (Pierce) and subjected to 10% SDS–PAGE in the presence of β-
mercaptoethanol. Gels were transferred onto nitrocellulose membranes and incubated with either anti-VZV gE monoclonal antibody (3G8) or anti-VZV gl rabbit serum. Blots were incubated and developed using the ECL kit, as described above.

Analysis of carbohydrates by enzyme digestion

Each purified protein (0.2 μg) was digested at 37°C with either 3 mU of endo-β-N-acetylglucosaminidase H (endo H; Boeringer Mannheim), 150 mU of endoglycosidase F/N-glycosidase F (endo F; Boeringer Mannheim), or 1 mU of endo-α-N-acetylgalactosaminidase (O-glycanase; Genzyme) for 18 hr, or with 45 μU of type VIII neuraminidase from Clostridium perfringens (Sigma) for 1 hr. The digestion products were analyzed by 8 or 10% SDS-PAGE followed by Western blotting.

Cross-linking of glycoproteins

Dithiobis [succinimidyl-propionate] (DSP, Pierce) is a water-insoluble, homobifunctional N-hydroxysuccinimide ester, and 3,3′-dithiobis [sulfo succinimidyl-propionate] (DTSSP, Pierce) is its water-soluble analog. Both moieties are thiol-cleavable molecules that have medium-length spacer arms and are suitable for intermolecular cross-linking (Hordern et al., 1979; Waugh et al., 1989). Each purified glycoprotein was incubated with a 50-fold molar excess of either DSP or DTSSP for 2 hr at 4°C. The reactions were stopped with 2 M Tris - HCl, pH 7.4, at a final concentration of 40 mM. Cross-linked glycoproteins were subjected to 8% SDS-PAGE with or without β-mercaptoethanol. Gels were stained with silver nitrate or analyzed by Western blotting.

RESULTS

Expression and secretion of VZV gE and gl from recombinant baculovirus

The extracellular domain of VZV gE was cloned into baculoviruses to express truncated gE bearing the native VZV signal peptide, the baculovirus gp67 signal peptide, or the melittin signal peptide. The resulting recombinant baculoviruses Bac-gEt, Bac-gEt-gp67, and Bac-gEt-his were used to infect SF9 cells, and 30 μl aliquots of the infected cell medium were analyzed by SDS-PAGE. Western blotting showed that cells infected with all three recombinant viruses secreted proteins of approximately 61 kDa into the medium (Fig. 2A). These three proteins reacted with monoclonal anti-gE antibody and migrated as being slightly larger than the predicted size of the core of truncated gE (59 kDa). Western blotting also showed that smaller proteins of approximately 50 kDa, which apparently result from proteolytic degradation of the intact 61-kDa protein as determined from N-terminal sequencing of the purified 50-kDa species (data not shown), were also faintly detected in the same medium.
be obtained per liter of culture (Fig. 2C). The N-terminal amino acid sequence of the purified protein was DPK-
GDVSLQV, corresponding to the cleaved N-terminus of VZV and including an inserted Asp-Pro dipeptide.

Co-infection with gE and gI baculoviruses and complex formation

VZV gE and gI form a noncovalently linked complex in VZV infected cells (Yao et al., 1993). We wanted to determine if gE and gI expressed in the baculovirus system could also form a noncovalent complex. To do so, recombinant gE and gI baculoviruses were used to co-infect SF9 cells. Secreted proteins were immunoprecipitated with either anti-gE or anti-gI antibody and followed by Western blotting with anti-gE antibody (Fig. 3A). Complex formation was detected when the precipitate of the anti-gI antibody was positive for Western blotting with the anti-gE antibody. Insect cells were co-infected with nine combinations of recombinant gE and gI baculoviruses. Recombinant gE protein was secreted from each of the cultures, but complex formation was found only in co-infections of gEt-his with gI, gEt with gI, and gEt-gp67 with gI. Interestingly, of three different gI proteins, only gI could associate with gE. The same membrane was analyzed by Western blotting with the anti-gI antibody (Fig. 3B). Co-immunoprecipitation of both proteins was found in the same combinations of infecting baculoviruses.

Purification of the gE–gI complex

Since the gI protein could participate in complex formation and gEt-his has a polyhistidine tag at its C-terminal end, the combination of these proteins was selected for larger scale protein synthesis and purification. Insect cells were co-infected with Bac–gEt–his and Bac–gI. The gI, which does not have a polyhistidine tag, could be co-purified with gEt–his by metal affinity chromatography (Fig. 4A). On the other hand, when gEt–his and gI were expressed separately and mixed together, gI could not

FIG. 3. Formation of gE–gI complexes during co-infection with gE and gI baculoviruses. All nine combinations of recombinant gE- and gI-expressing baculoviruses were used in co-infections of SF9 cells. The secreted proteins from each culture were co-immunoprecipitated with anti-gE (a-gE) or anti-gI (a-gI) antibody and detected by Western blotting. (A) Western blotting with the anti-gE antibody. (B) Western blotting with the anti-gI antibody. It is noted that both gE and gI co-immunoprecipitate as a complex only in lanes displaying supernatants of cells co-infected with gEt–his and gI, gEt and gI, and gEt–gp67 and gI. MW, molecular weight marker.

FIG. 4. Purification of the gE–gI complex. (A) SF9 cells were co-infected with Bac–gEt–his and Bac–gI or infected with these viruses individually. Secreted proteins were purified by metal affinity chromatography, subjected to SDS–PAGE, and silver-stained. Lane 1, protein secreted from cells infected alone with Bac–gEt–his; lane 2, protein secreted from cells infected alone with Bac–gI; lane 3, protein secreted from cells co-infected with Bac–gEt–his and Bac–gI. The gI protein, which does not possess a polyhistidine tag, was co-purified here with the gEt–his. Lane 4, a mixture of separately expressed gEt–
his and gI. (B) Suspension cultures of SF9 cells were co-infected with Bac–gEt–his and Bac–gI. Secreted proteins were purified by metal affinity chromatography, analyzed by SDS–PAGE, and silver-stained. Lane 1, the supernatant of co-infected cells; lane 2, the flow through; lanes 3–5, imidazole-eluted fractions. The 61-kDa gEt–his and 39-kDa gI proteins are apparent. MW, molecular weight marker.
be co-purified, indicating that mixture of gEt – his and gIt does not make a complex efficiently (Fig. 4A, lane 4). In larger cultures we co-expressed them by co-infection and purified several milligrams of the gE – gI complex (Fig. 4B). The purified gE – gI complex was co-immunoprecipitated with either anti-gE or anti-gI followed by Western blotting. Co-immunoprecipitation was found in the purified protein solution, as expected. It was possible, however, that the protein concentrates contained both the gE – gI complex and free gE monomers which also could bind to the metal affinity resin. To remove the gE monomers and purify the gE – gI complex further, we used gel filtration through a Sephacryl S-200 column. Using gel filtration, the gE – gI complex, which is larger than the gE monomers, could be separated from the monomers into two distinct peaks. When each fraction was analyzed by Western blotting, both gE and gI were detected in the first peak which contained the gE – gI complex. On the other hand, only gE was detected in the second peak (data not shown). The gE – gI complex was finally purified by pooling fractions in the first peak.

Characterization of the purified gE – gI complex

We estimated the molecular size of purified gEt – his, gIt – his, and the complex by two independent methods.

Using gel filtration, gIt – his was estimated to be 39 kDa, which nearly equals its size in SDS – PAGE, while gEt – his was 78 kDa, larger than observed in SDS – PAGE. The gE – gI complex was estimated to be 128 kDa on this column, suggesting that the gE – gI complex might be a heterodimer. To confirm the stoichiometry of the complex, the purified gE – gI complex was cross-linked with either DSP or DTSSP and analyzed by SDS – PAGE. Cross-linking by either DSP or DTSSP yielded a 100-kDa band (the data for the DTSSP reaction are shown in Fig. 5A). Western blotting showed that the band contained both gE and gI (Figs. 5B and 5C). A similar band could not be detected in samples not cross-linked (Fig. 4B). Based on these results, the gE – gI complex is a heterodimer consisting of one gE polypeptide bound to one gI polypeptide.

Many posttranslational modifications typical of eukaryotic cells occur in insect cells; however, glycosylation in insect cells is not identical to that seen in mammalian cells (O'Reilly et al., 1994). To determine how the gE – gI complex expressed in insect cells is glycosylated, we treated purified gEt – his, gIt – his, and the complex with various glycosidases or neuraminidase. Endo H treatment increased the electrophoretic mobility of each protein, but endo F treatment increased the mobility more, indicating that each of the recombinant proteins had N-linked carbohydrates and that most of them were in processed forms (Fig. 6). Endo F treatment decreased the sizes of gE and gI by an estimated 3 and 9 kDa, which correspond to one to two and three to four N-glycosylation sites, respectively. These numbers correspond closely to the numbers of predicted N-glycosylation sites in each protein (Fig. 1). All of the purified proteins were resistant to
neuraminidase and O-glycanase, indicating that these recombinant proteins have few, if any, sialic acid residues and O-linked carbohydrates. We did not detect any differences in glycosylation between the gE-gI complex and the individually expressed monomers.

Analysis of the N-terminal region of gI and its role in complex formation

As mentioned above, when insect cells were co-infected with nine combinations of recombinant gE and gI baculoviruses, complex formation resulted only from co-infections of gEt-his with gIt, gEt with gIt, and gEt–gp67 with gIt (Fig. 3). These results indicate that each of the three recombinant gE proteins could form a complex with recombinant gI protein, but only gIt bearing its native N-terminal sequence could participate in complex formation. gIt–gp67 and gIt–his, which contain altered N-terminal sequences, could not complex with gE. To analyze the amino acid sequence of the N-terminus region of gIt, we used the purified gE–gI complex that contains the gI protein. Microsequencing showed that the mature N-terminus of co-purified gIt was LIFKGDHVSLQV, indicating that the original gI signal peptide was cleaved before Leu-21 (Fig. 7A). When compared with gIt, both gIt–gp67 and gIt–his have not only the replacement of the VZV gI signal peptide but also the substitution of Asp-Pro for Leu-Ile-Phe at their mature N-termini. These findings suggest that some part of the N-terminal sequences of gI is important for gE–gI complex formation, since both substitutions are in this region.

To test whether it is the signal peptide or the mature N-terminus of gI that determines complex formation, we constructed a family of mutant gI recombinant baculoviruses by either site-directed mutagenesis or linker insertion. Six mutant gI recombinant viruses were used to co-infect Sf9 cells with Bac–gEt-his. The secreted proteins were co-immunoprecipitated with anti-gE or anti-gI antibody and were detected by Western blotting. A schematic representation of mutant gI proteins and their abilities to participate in complex formation is shown in Fig. 7B. gIt–MSP, in which the viral signal peptide was replaced by the melittin signal peptide but retained the original mature N-terminus, could associate with VZV gE. On the other hand, gIt–DP, which has the native signal peptide and Asp-Pro at its mature N-terminus instead of Leu-Ile-Phe, could not complex with gE. These results indicated that the substitution of Leu-Ile-Phe with Asp-Pro abolished the abilities of gIt–gp67 and gIt–his to bind efficiently to gE. Data obtained with the linker insertion mutants also showed that the disruption of the mature N-terminal sequence abrogated complex formation (gIt-Pml and gIt–Hpa in Fig. 7B). However, when the C-terminal regions were disrupted, the resulting mutant gIs could still complex with gE (gIt–Nde and gIt–Sty in Fig. 7B). These results indicate that the mature N-terminus of gI is important for the formation or stability of the gE–gI complex. We also constructed seven other C-terminal truncation and linker insertion mutants of gI. Of these, however, only one C-terminal truncated protein, spanning 87% of the extracellular domain of gI, could be secreted in the medium. The other mutants failed to yield detectable secreted proteins. These later mutations, therefore, appear to have affected the biosynthesis and/or stability of gI (data not shown).

FIG. 7. Analysis of the N-terminal region of gI and its role in complex formation. (A) The predicted N- and C-terminal amino acid sequences of each truncated recombinant protein and their abilities to form the gE–gI complex are shown. The cloning of gE and gI coding regions into pVT-Bac and pAGP67A introduced not only insect signal peptides but also the insertion of Asp and Pro dipeptides. Each of the recombinant gE proteins could form a complex with a recombinant gI protein, but only the gI protein bearing the authentic VZV N-terminal sequence could participate in complex formation. The predicted N-terminal sequences of gEt–his, gIt, and gIt–his were confirmed by microsequencing. (B) Six recombinant viruses expressing mutant gI proteins were constructed by site-directed mutagenesis or linker insertion. The schematic organizations of these mutant proteins and their abilities to complex with gE are shown. gIt-MSP contains the melittin signal peptide instead of the VZV signal peptide but retains the original mature N-terminus. gIt-DP has the VZV signal peptide but amino acids Leu-Ile-Phe are replaced with Asp-Pro at the mature N-terminus. Each of the other mutants has the VZV signal peptides and amino acids insertions in either the N-terminal region or the C-terminal region. Experiments showed that replacement or addition of N-terminal amino acids abrogated complex formation with VZV gE. aa, amino acid; SP, signal peptide. “Balloons” indicate the predicted N-linked glycosylation sites.

**DISCUSSION**

In this report, we describe the expression and subsequent purification of VZV gE and gI using the baculovirus system. Higher protein yields were achieved by using insect signal peptides, especially in the recombinant gE constructs. gE and gI formed noncovalent complexes,
similar to those reported in VZV-infected cells, by co-infecting insect cells with baculoviruses expressing each of them alone. Gel filtration and in vitro cross-linking studies determined that gE and gI assemble into a heterodimeric complex. Mutational analyses showed that this complex is formed only if appropriate (native) amino acid sequences are retained at the mature N-terminus of gI. It is our expectation that the purified gE, gI, and gE–gI complex will prove functional in studies of their receptor interactions and immunogenicity.

The function of the VZV gE–gI heterodimer in the VZV life cycle is unclear, although gE is shown to bind to the Fc portion of IgG with low affinity (Litwin et al., 1992). The role of VZV gI is even less well understood. VZV deleted for gI can still replicate and grow in cell culture (personal communication from Dr. J. I. Cohen, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Some light is shed on the importance of gI and its relationship to gE by recent studies with other alphaherpesviruses. For the feline herpesvirus a gE–gI interaction is required to allow egress of gE from the endoplasmic reticulum (Mijnes et al., 1996). In PRV, export of gE and gI from the endoplasmic reticulum is also inefficient unless both proteins are present (Whealy et al., 1993). In both HSV and VZV, however, gE and gI are readily exported from the endoplasmic reticulum to the plasma membrane when they are individually expressed in mammalian cells (Bell et al., 1990; Dubin et al., 1990; Litwin et al., 1992; Yao et al., 1993). Certainly, we noted that carboxy-truncated VZV gE and gI expressed individually in insect cells were efficiently secreted. These results suggest that the role of gI may vary in different alphaherpesviruses. On the other hand, it is known that both HSV-1 gE monomers and gE–gI complexes can act as Fc receptors but they differ in their binding preferences for aggregated versus monomeric IgG, suggesting that gI alters the binding affinity of gE (Dubin et al., 1990). Moreover, HSV-1 gE has two regions involved in Fc binding activity, one that interacts with gI, leading to an alteration of the binding affinity of gE, and a second region that binds IgG (Basu et al., 1995). Whatever role the gE–gI complexes play in VZV and other related viruses, their evolutionary conservation indicates that their organization and structure are important.

In the present study, we determined that the mature N-terminus of gI was important for complex formation. Obviously, neither the intracellular nor the transmembrane domains were required for the gE–gI complex formation, since these protein regions were absent in the panel of recombinant proteins investigated in this study. Mutational studies showed that alterations of the processed N-terminus of gI abrogated its ability to form a complex with gE. Replacement of the N-terminal Leu-1-Phe by Asp-Pro would, theoretically, alter the region from a hydrophobic to hydrophilic one, add a negative charge in the region, and might greatly influence protein folding. N-terminal sequencing of the expressed protein indicated that the original signal peptide of VZV gI is cleaved before the leucine at position 21 (Leu-21). It is not known whether this hydrophobic N-terminus is exposed to solvent or is buried within the core of the protein. It is of interest, though, that in both HSV-1 and HSV-2 the Leu-21 is conserved and the 22nd amino acid is valine instead of the isoleucine seen in VZV gI (McGeoch et al., 1985, 1987). Our analyses also showed that selected disruptions of the C-terminal region of gI did not influence the complex formation. These findings together raise the possibility that the N-terminal region of gI is required for intermolecular binding.

There are few published data concerning the stoichiometry of the gE–gI complex of VZV or of other alphaherpesvirus homologs. Whealy et al. suggested that the PRV gE–gI complex is a heterodimer using pulse–chase labeling followed by sucrose gradient sedimentation (Whealy et al., 1993). By gel filtration and cross-linking studies, we showed that the VZV gE–gI complex expressed in insect cells was a heterodimer. A recent investigation showed that full-length VZV gE expressed in the absence of gI in insect cells could form a homodimer which was resistant to reduction and detergent disruption, although most of the expressed gE was monomeric (Olson et al., 1997). We did not find a significant level of dimeric gE. When gE expressed in the absence of gI was overloaded on SDS–PAGE, however, a larger form of gE, which was nearly double in size, could be detected in addition to the main band of monomer size (data not shown). A similar band was also detected in gels overloaded with purified gI. Although such a dimeric form of gE (and possibly gI) may have a different function from that of monomers (Olson et al., 1997), the vast majority of expressed gE proved to be monomeric. Since insect cells are eukaryotic, they can confer similar posttranslational modifications to proteins as are seen in mammalian cells. Proper cleavage of signal peptides is also observed in baculovirus-infected cells (O’Reilly et al., 1994). Here, the native signal peptide of VZV gI was cleaved between Ala-20 and Leu-21. The alanine at the −1 position relative to the cleavage site of this signal peptide is the most frequently found amino acid at such a position in eukaryotic signal peptides (von Heijne, 1986). In addition, Thr-18 of the signal peptide is also frequently seen at the −3 position from the cleavage site in eukaryotic signal peptides. These findings suggest that the VZV gI signal peptide was cleaved properly in insect cells, although the cleavage site of the signal peptide has not been verified in VZV-infected mammalian cells.

In infected mammalian cells, VZV gE and gI contain both N- and O-linked sugars and are heavily sialated (Grose, 1990). Glycoproteins synthesized in insect cells contain N- and O-linked carbohydrates but they generally lack sialic acid (O’Reilly et al., 1994). The resistance of
the baculovirus-expressed gE and gI to neuraminidase that we noted indicated a lack of sialic acid residues in gE and gI. The results of glycosidase treatments established that recombinant proteins had N-linked carbohydrates but few if any O-linked sugars. Admittedly, a small number of sialic acid or short O-linked carbohydrate residues might be present and not be detected by our methods. The efficient formation of the gE–gI complex by co-infection of insect cells with the recombinant baculoviruses, though, indicates that complex formation does not require sialic acid or extensive O-linked glycosylation.

Recent investigations showed that VZV gE contains trans-Golgi network targeting signal sequences in its intracellular domain (Alconada et al., 1996; Zhu et al., 1996). Our gE recombinant proteins lack these intracellular domains. Therefore, the deletion of these sequences might have limited further the extent and type of posttranslational modifications. It was reported that gE–gI complex formation in itself interferes with posttranslational modifications and thereby reduces the relative molecular masses of both gE and gI when compared with gE and gI monomers (Mijnes et al., 1996; Yao et al., 1993). We did not, however, detect any differences in glycosylation between the gE–gI complex and the individual monomers. Clearly, as currently examined, the cytoplasmic sequences of VZV gE and gI, their retention in the trans-Golgi network, and their complete glycosylation were not required for efficient formation of the gE–gI complex.

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