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Varicella-Zoster Virus Fc Receptor gE Glycoprotein: Serine/Threonine and Tyrosine Phosphorylation of Monomeric and Dimeric Forms

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Varicella-zoster virus (VZV) glycoprotein gE is the predominant viral cell surface molecule; it behaves as an Fc receptor for immunoglobulin G, but its central function may be more closely related to viral egress and cell-to-cell spread. To further analyze the receptor properties of VZV gE, the gE gene (also called open reading frame 68) was expressed by a baculovirus vector in insect cells. The recombinant baculovirus gE product had a molecular mass of 64 kDa, smaller than the previously documented 98 kDa of mature gE expressed in mammalian cells. The major reason for the lowered molecular mass was diminished glycosylation. In addition to the 64-kDa form, a larger (130-kDa) form was observed in insect cells and represented dimerized 64-kDa molecules. Both the monomeric and dimeric gE forms were highly phosphorylated in insect cells. Protein kinase assays conducted in vitro with [γ-^{32}P]ATP and [γ-^{32}P]GTP indicated that endogenous casein kinase II was phosphorylating monomeric gE, while the dimeric gE form was phosphorylated by another kinase which did not utilize [γ-^{32}P]GTP. When immobilized recombinant gE molecules were probed with a monoclonal antibody which specifically recognizes a phosphotyrosine linkage, the gE dimer was found to be tyrosine phosphorylated whereas the monomer was not similarly modified. When recombinant gE produced in HeLa cells was probed with the same antiphosphotyrosine antibody, a dimeric gE form at 130 kDa was detected on the cell surface. These results suggested that VZV gE closely resembled other cell surface receptors, being modified on its various forms by both serine/threonine and tyrosine protein kinases. In this case, tyrosine phosphorylation occurred on a previously unrecognized and underglycosylated VZV gE dimeric product.

Varicella-zoster virus (VZV) is one of the human alphaherpesviruses. VZV is the causative agent of two clinically distinct diseases, chickenpox (varicella) and shingles (herpes zoster). The genome of VZV, the smallest of the human herpesviruses, is 125 kbp and contains approximately 70 open reading frames (ORFs) (3). Six ORFs encode viral glycoproteins which have been designated gE, gB, gH, gI, gC, and gL. Of the six, gE (previously designated gpI or gp98) is the most abundant virion envelope glycoprotein and also the predominant glycosylated VZV cell surface antigen (11, 27). The gE glycoprotein is encoded by ORF 68, which is located within the unique short region of the VZV genome (3). Based on the sequence and structure of VZV gE, it has been designated a typical type I transmembrane glycoprotein which is 623 amino acids in length with a 24-amino-acid cleavable signal sequence. The glycoprotein consists of three regions: a 544-amino-acid hydrophilic extracellular region, a 17-amino-acid hydrophobic transmembrane region, and a 62-amino-acid charged cytoplasmic region tail (3, 11). Examination of the predicted amino acid sequence of VZV gE reveals two cysteine-rich regions, three N-linked glycosylation signals, and putative juxtamembrane domain favoring O-linked glycans (25). In addition, glycoprotein gE contains a consensus site for serine/threonine phosphorylation in the cytoplasmic region. In short, VZV gE displays a combination of structural features commonly observed in some cell surface receptors and adhesion molecules (37, 41, 45).

Previous studies of VZV gE revealed that gE can function as an Fc receptor for nonimmune immunoglobulin G (IgG) (25, 26). In this regard, VZV gE closely resembles its herpes simplex virus type 1 (HSV-1) homolog (38). However, HSV gE binds IgG more efficiently than VZV gE (25). In addition, HSV gI appears to increase IgG binding to HSV gE by forming a gE-gI complex but does not bind Ig directly; similarly, IgG does not attach to VZV gI (18, 25, 26). In an analogous manner, VZV gI (ORF 67; previously designated gpIV) coprecipitates with VZV gE from VZV-infected cells and transfected cells (27, 46). Although either VZV gE or gI can be processed in the endoplasmic reticulum (ER) and Golgi apparatus and transported to the cell surface independently, the cotransfected products are not identical to the individually transfected products, and thus complex formation can alter posttranslational modifications (46, 48).

The functions of gE, in particular, how the Fc receptor activity might protect an alphaherpesvirus-infected cell from lysis by the immune system, remain elusive. More recent results suggest that gE is involved in cell-to-cell spread, with a particular emphasis on the neurotropic properties of an alphaherpesvirus and its ability to travel within neuronal cells (12) or along specific neurological circuits (4, 5, 7). In an attempt to further define the receptor properties of VZV gE which may be involved in these activities, we shuttled the gE gene into a baculovirus expression system in order to increase the yields of recombinant gE product for analysis. Our investigations with the baculovirus-insect cell system have led to the discovery of previously unrecognized forms of gE. Furthermore, the same experiments have greatly expanded our knowledge of serine/threonine phosphorylation of a eukaryotic herpesvirus protein within insect cells and at the same time demonstrated that tyrosine phosphorylation is occurring on a dimeric form of gE.

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Thus, these studies point out potential benefits of studying viral posttranslational events in a baculovirus system; specifically, some modifications which were not apparent in a mammalian cell may be more easily recognized in an insect cell for reasons described herein.

MATERIALS AND METHODS

Viruses and cells and plasmids. The *Autographica californica* nuclear polyhedrosis virus and the linearized baculovirus DNA (Baculogold DNA) were obtained from PharMingen in San Diego, Calif. *Spodoptera frugiperda* clone 9 (Sf9) cells were grown in Grace's medium (Gibco BRL Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS), sodium carbonate, lactalbumin hydrosylate, and yeastolate. High Five cells derived from *Trichoplusia ni* were obtained from Invitrogen (San Diego, Calif.) and grown in Ex Cell 400 medium (JRH Biosciences, Lenexa, Kans.). HeLa cells (ATCC CCL2) were obtained from the American Type Culture Collection, Rockville, Md. HeLa cells were grown in Eagle complete medium supplemented with 10% FBS. Recombinant vaccinia virus (T7-vaccinia virus) and plasmid pTM-1 were obtained from the B. Moss laboratory at the National Institutes of Health (28). Plasmid pTM1-gE containing VZV ORF 68 has been described elsewhere (46). The polyhedrin-based baculovirus transfer vector pVL1393 was obtained from PharMingen. The in vitro coupled transcription-translation expression vector pBluescript was purchased from Stratagene (La Jolla, Calif.).

Construction of the recombinant baculovirus expressing gE. VZV ORF 68 was previously cloned into the eukaryotic pTM1 expression vector as described elsewhere (46). The 2.0-kb fragment containing gE was removed from pTM1 by digestion with restriction endonuclease *Xma*I. The fragment was then subcloned into baculovirus transfer plasmid pVL1393 downstream of the polyhedrin promoter. Sf9 insect cells were cotransfected with linearized baculovirus DNA (Baculogold DNA) and pVL1393-gE plasmid DNA by the method described by PharMingen. Three consecutive plaque purifications were conducted on the recombinant virus. One recombinant virus expressing gE was selected and amplified to obtain a virus titer of 10^7 PFU per ml.

Analysis of infected insect cells by laser scanning confocal microscopy. Sf9 insect cells were prepared by first seeding 2×10^6 cells in a 25-cm^2 culture flask. After 1 h of incubation at 27°C, the cells were infected with gE recombinant baculovirus at a multiplicity of infection of 1. After 48 h of infection, the cells were suspended in the flask. The cell suspension was centrifuged at 800 rpm for 10 min, and the medium was removed from the cells. The cells were fixed with 2% paraformaldehyde in 0.1 M Na_2HPO_4 for 30 min and then washed three times with 0.01 M phosphate in normal saline (pH 7.4; phosphate-buffered saline [PBS]). If the cells were to be permeabilized, 0.05% Triton X-100 (Fisher Biotech) was added to the cells for 30 min; this step was omitted if cell surface expression alone was to be examined. After the Triton X-100 was removed, the cells were washed three times with PBS. The cells were then blocked with 5% normal goat serum in PBS for 1 h. For immunostaining, the primary reagent was murine monoclonal antibody (MAb) 3B3; it recognizes an 11-amino-acid epitope on the ectodomain of gE (13). The secondary antibody was fluorescein-conjugated goat anti-mouse antibody. The cells were viewed with a Bio-Rad MRC 600 laser scanning confocal microscope (6).

Immunoprecipitation of infected insect cells. High Five insect cells were seeded at a concentration of 2.0×10^6 cells in a 25-cm^2 culture flask and incubated for 1 h at 27°C. The cells were infected with recombinant baculovirus at a multiplicity of infection of 1. At 4 h postinfection, cells were labeled with 250 μCi of [4,5-^{3}H]leucine (specific activity, 167 Ci/mmol; Amersham, Arlington Heights, Ill.) per ml and incubated for 48 h at 27°C. The cells were then suspended in the flask and centrifuged at 800 rpm for 10 min. The cells were washed two times with PBS and lysed in radioimmunoprecipitation buffer (10 mM Tris [pH 7.4] containing 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, and 1% sodium dodecyl sulfate [SDS]) with 1 mg of leupeptin per ml, 1 mg of pepstatin A per ml, and 1× phenylmethylsulfonyl fluoride. The cells were sonically disrupted and sedimented ($35,000 \times g$ for 60 min) to remove insoluble macromolecules. For immunoprecipitation, 100 μl of the above lysate and 1 μl (1:100 dilution) of MAb 3B3 were added to 200 μl of lysis buffer and incubated overnight at 4°C. The precipitation of the antigen-antibody complexes with protein A-Sepharose CL-4B beads has been previously described (46). The proteins were eluted from the protein A beads in reducing buffer (125 mM Tris, 6% glycerol, 10% 2-mercaptoethanol) by incubation at 100°C for 5 min. Immunoprecipitated proteins were analyzed in a 10 to 18% gradient polyacrylamide gel containing 0.1% SDS. Gels were prepared for fluorography, dried, and exposed to radiographic film (Kodak).

Analysis of glycosylation. Oligosaccharide structures of the glycoproteins expressed in insect cells were analyzed with endoglycosidase F (endo F), neuraminidase (sialidase), and endo-α-*N*-acetylgalactosaminidase (*O*-glycanase). The method for digestion with each enzyme has been previously described (46). Endo F derived from *Flavobacterium meningosepticum* was purchased from Calbiochem (San Diego, Calif.); neuraminidase derived from *Clostridium perfringens* and *O*-glycanase derived from *Diplococcus pneumoniae* were purchased from Sigma.

In vitro coupled transcription-translation. First, VZV gE was subcloned from pTM1 into the in vitro transcription-translation expression vector pBluescript. Plasmid pTM1-gE was digested with restriction endonucleases *Pst*I and *Bam*HI, and the resulting 2.0-kb fragment encoding gE was inserted into pBluescript behind the T3 promoter. The in vitro transcription-translation reaction was performed by using the TNT coupled reticulocyte lysate system as described by Promega (Madison, Wis.), with the products being labeled with 0.5 mCi of [^{3}H]leucine (Amersham) per ml. The reaction products were immunoprecipitated with MAb 3B3 and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Western blotting of casein kinase II. High Five cells were infected with gE recombinant baculovirus and incubated for 48 h. The cells were washed and lysed as described above. Aliquots were added to reducing buffer and incubated at 100°C for 5 min. After separation by SDS-PAGE, the proteins were transferred to an Immunobilon-P transfer membrane (Millipore, Bedford, Mass.), and immunoblotting was carried out by standard procedures (27). J. Traugh provided both an ovine polyclonal antibody to casein kinase II and a rabbit polyclonal antibody to the β subunit of the same enzyme (30, 34).

Construction of phosphorylation mutant gE by recombination PCR. pTM1-gE was constructed in our laboratory (46). The DNA template required for the mutagenesis consisted of the 2.0-kb DNA fragment containing gE cloned into plasmid pTM1, which is 5.3 kb. Plasmids for PCR and DNA sequencing were prepared by using a Qiagen kit (Qiagen Inc., Chatsworth, Calif.). Site-directed mutagenesis was performed by a recombination PCR methodology (13, 47, 48). Briefly, four oligonucleotide primers were prepared in order to generate two linear fragments containing homologous ends. Two pairs of primers were designed: one pair of mutating primers and one pair of nonmutating primers. The primers were prepared on an Applied Biosystems (Foster City, Calif.) DNA synthesizer at the DNA Core Facility, The University of Iowa. Sequences of the mutating primers were as follows: 5′ GACGCGGAATTCGCAGATGCGGAA GAAGAG 3′ and 5′ CGCATCTGCGAATTCCGCGTCCTCGAAATCGT 3′, with a 21-bp overlap. Sequences of the nonmutating primers located within the ampicillin resistance gene in pTM1 were as follows: 5′ AACAGCGGTAAGAT CCTTGAG 3′ and 5′ AAACTCTCAAGGATCTTAC 3′, with a 14-bp overlap. Plasmid pTM1-gE was first linearized with the restriction endonuclease *Spe*I or *Nco*I. One mutating and one nonmutating primer were used in pairs to generate linear fragments. Amplification of the DNA fragments from the plasmid template was performed by PCR methods previously described (46, 47). The two linear DNA products were combined and transformed into Max competent *Escherichia coli* DH5α cells (BRL, Life Technologies). Recombination occurred between the homologous fragments to produce a plasmid containing the desired mutation, and the mutation was verified by sequencing at the DNA Core Facility, University of Iowa.

Transfection of HeLa cells. HeLa cells were seeded into a six-well plate (Costar) at a concentration of 5×10^5 cells/ml. After overnight incubation at 37°C, the cells were infected with recombinant T7-vaccinia virus and then transfected with 15% Lipofectin (Gibco BRL) containing 4 μg of pTM1-gE construct. The cells were labeled 4 h postinfection with 250 μCi of [^{3}H]leucine per ml and incubated overnight at 37°C. The cells were harvested in radioimmunoassay buffer, and the lysates were immunoprecipitated as described above with MAb 3B3. The immunoprecipitated proteins were analyzed by SDS-PAGE as described above. Lysates required for analysis of tyrosine phosphorylation were concentrated in Centricon 30 concentrators (Amicon, IHC, Beverly, Mass.).

Trypsin analysis of cell surface proteins. HeLa cells were seeded, transfected, and radiolabeled as described above. Cells were washed with cold PBS before incubation with MAb 3B3 (1:2,000 in PBS) at 4°C for 30 min. Then the cells were washed and treated with trypsin (1 mg/ml) for 30 min on ice. The cells were lysed in radioimmunoassay buffer containing soybean trypsin inhibitor (0.5 mg/ml; Sigma). Antigen-antibody complexes were precipitated with protein A as described above and analyzed by SDS-PAGE.

Phosphorylation assays. For analysis of in vivo phosphorylation, Sf9 cells were infected with gE recombinant baculovirus as described above. At 4 h postinfection, Grace's medium was replaced with phosphate-deficient Grace's medium (Gibco BRL Life Technologies) containing 250 μCi of $^{32}P_i$ (specific activity, 10 mCi/ml; Amersham) per ml. The cells were incubated for 48 h at 27°C. The cells were harvested and immunoprecipitation was carried out as described above. In vitro phosphorylation assays have been described previously (47). Briefly, High Five cells were infected with gE recombinant baculovirus and harvested as described above. The lysates (unlabeled) were immunoprecipitated with MAb 3B3 and protein A-Sepharose CL-4B beads. Precipitates were washed five times with PBS wash buffer (10 mM NaCl, 0.05% Nonidet P-40, 0.5% bovine serum albumin, 0.1% SDS, 0.2% NaN_3), washed two times with kinase buffer (50 mM Tris [pH 7.2], 140 mM KCl, 10 mM $MgCl_2$), and then resuspended in 60 μl of kinase buffer or kinase buffer containing 5 μg of heparin per ml. Some precipitates were heat inactivated for 10 min at 60°C. Aliquots of freshly prepared HeLa cell lysate or High Five cell lysate were added to some precipitates as a source of kinase activity (11). Each kinase reaction mixture was incubated with 5 μCi of [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol) or [γ-^{32}P]GTP (specific activity, 5,000 Ci/mmol) for 30 min at 30°C. After incubation, the samples were washed five more times with PBS wash buffer, and the proteins were eluted from protein A beads in reducing buffer as described above. Proteins were analyzed by SDS-PAGE.

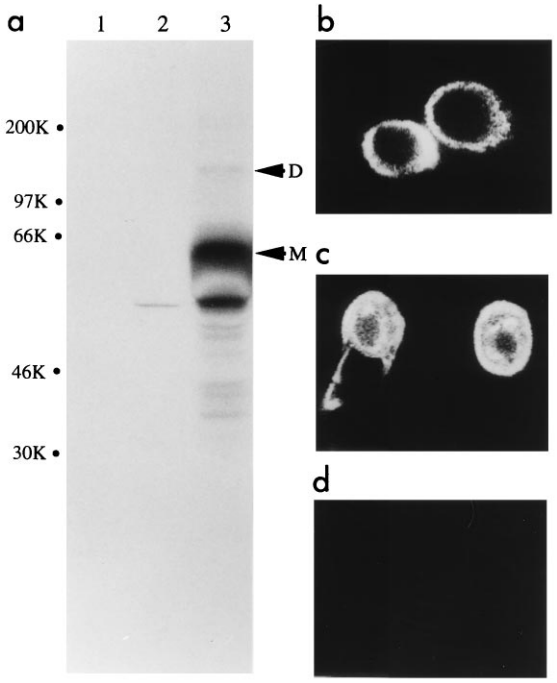

FIG. 1. Expression of gE recombinant baculovirus in insect cells. (a) Lanes 1, 2, and 3 represent immunoprecipitates with MAb 3B3 of a lysate of uninfected High Five cells (lane 1), a lysate of High cells infected with wild-type baculovirus (lane 2), and a lysate of High Five cells infected with gE recombinant baculovirus (lane 3). Arrowheads indicate the gE monomer (M) and the gE dimer (D); positions of molecular mass markers are designated at the left. Panels b to d illustrate Sf9 insect cells infected with recombinant baculovirus and analyzed by laser scanning confocal microscopy. (b) Surface expression of gE in Sf9 insect cells detected by primary MAb 3B3. (c) Cellular distribution of gE in permeabilized Sf9 insect cells detected by primary MAb 3B3. (d) Uninfected Sf9 insect cells incubated with primary MAb 3B3 as a negative control.

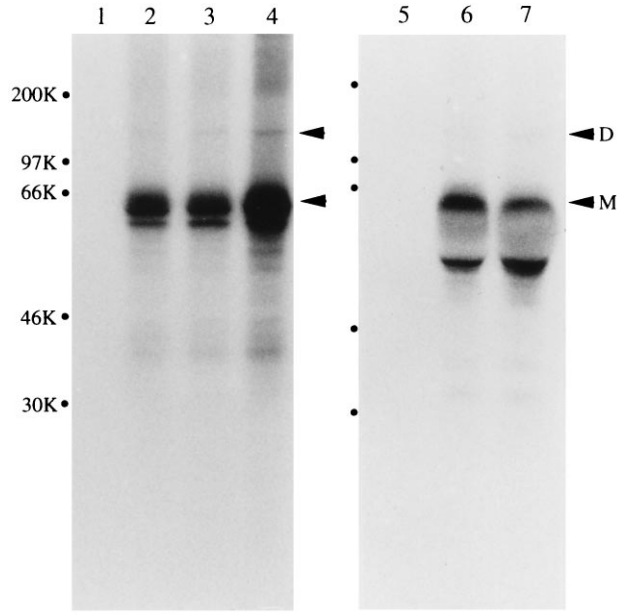

FIG. 2. Glycosylation analyses of gE expressed in insect cells. Lysates were prepared from High Five cells infected with recombinant baculovirus expressing gE and labeled with [^{3}H]leucine for 48 h. The lysates were immunoprecipitated with MAb 3B3. In lanes 1 to 4, the gE precipitates were not treated or were treated with *O*-glycanase and/or with neuraminidase. Lanes: 1, an uninfected High Five cell precipitate not treated; 2, a gE precipitate not treated; 3, a gE precipitate treated with neuraminidase; 4, a gE precipitate treated with neuraminidase and *O*-glycanase. In lanes 5 to 7, the gE precipitates were not treated or were treated with endo F. Lanes: 5, an uninfected High Five cell precipitate not treated; 6, a gE precipitate not treated; 7, a gE precipitate treated with endo F. The gE bands are indicated by the arrowheads; M identifies the gE monomer, and D identifies the gE dimer.

Immunodetection of tyrosine phosphorylation. High Five cells were infected and lysed as described above except that the cells were not radiolabeled. The lysates were immunoprecipitated with MAb 3B3, and the precipitated proteins were separated on a 10 to 18% gradient polyacrylamide gel containing 0.1% SDS. The proteins were transferred to a Hybond-ECL nitrocellulose transfer membrane (Amersham). The membrane was blocked with 5% FBS in TBST buffer (0.01 M Tris [pH 8.0], 0.15 M NaCl, 0.05% Tween 20) for 1 h. After two washes in TBST buffer, the membrane was incubated for 1 h with biotinylated antibody P-tyr-1 (dilution of 1:30,000). After washing, the membrane was incubated with streptavidin-horseradish peroxidase (1:40,000) for 1 h and then washed with TBST buffer. Lastly, the enhanced chemiluminescence (ECL) Western blot solutions were prepared and added to the membrane for 1 min as described by Amersham. The membrane was exposed to film (Kodak). The membrane was stripped by submerging the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) and incubating it at 50°C for 30 min. MAb P-tyr-1 clone was obtained from the American Type Culture Collection (21, 22).

RESULTS

Expression of recombinant VZV-gE baculovirus in insect cells. The recombinant baculovirus containing the VZV gE gene was constructed by first subcloning the 2.0-kb gE fragment into baculovirus transfer plasmid pVL1393 as described in Materials and Methods. To determine the expression of gE in the insect cells, cells were examined with laser scanning confocal microscopy. Sf9 cells were infected for 48 h with the gE recombinant baculovirus. When viewed with a laser scanning microscope, considerable gE was observed on the surface of the Sf9 cells which were not permeabilized (Fig. 1b); likewise, large amounts of gE were seen throughout the cytoplasm of cells which were permeabilized (Fig. 1c). No gE expression was observed in uninfected Sf9 cells (Fig. 1d). Therefore, gE was abundantly expressed in insect cells and furthermore was transported to the cell surface.

To characterize gE expressed in insect cells, immunoprecipitates from [^{3}H]leucine-labeled cells were subjected to SDS-PAGE under reducing conditions. The protein immunoprecipitated from High Five cells infected with recombinant baculovirus expressing gE had a molecular mass of 64 kDa, as shown in Fig. 1a (lane 3); a similar protein was not present in the uninfected (lane 1) or wild-type baculovirus-infected (lane 2) cells. In addition, a less intense band appeared at 130 kDa in the gE recombinant baculovirus lane; this protein band was not present in either of the control lanes. Based on densitometric analysis, the 130-kDa form represented 9% of the total gE produced. Because the 130-kDa form was twice the size of the monomeric form, it was considered to represent a dimeric gE product. The 130-kDa form was not sensitive to reduction (Fig. 1), but it was disrupted by electrophoresis under highly denaturing conditions with 5 M guanidine HCl (data not shown). The band at 55 kDa represented the heavy-chain Ig protein.

Glycosylation of gE expressed in insect cells. VZV gE expressed in insect cells had a considerably reduced M_r compared with gE expressed in mammalian cells. Previous studies showed that gE expressed in mammalian cells is both O-linked and N-linked glycosylated (27, 46). Insect cells generally produce shorter, high-mannose N-linked oligosaccharides because they lack the processing enzymes necessary to convert high-mannose-type oligosaccharides to complex-type glycans (17, 29). When HSV-1 gE was expressed by baculovirus, gE was

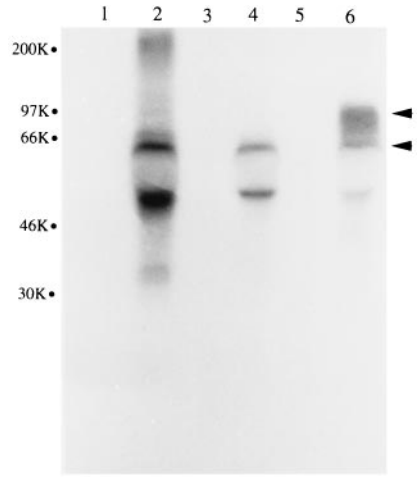

FIG. 3. Determination of the M_r of the gE polypeptide backbone. The gE protein products generated from in vitro coupled transcription-translation, recombinant baculovirus-infected High five cells, or transfected HeLa cells were precipitated with MAb 3B3 and then analyzed by SDS-PAGE. Lanes: 1, product from in vitro transcription-translation with no DNA; 2, product from in vitro transcription-translation with pBluescript-gE; 3, product from uninfected High Five cells; 4, product from High Five cells infected with recombinant baculovirus expressing gE; 5, product from HeLa cells transfected with vector alone; 6, product from transfection with pTM1-gE. The arrowheads beside lane 6 designate the mature 98-kDa form of gE in mammalian cells and an immature gE form in mammalian cells which comigrates with the gE recombinant form in insect cells (lane 4) and with the gE product produced in vitro (lane 2).

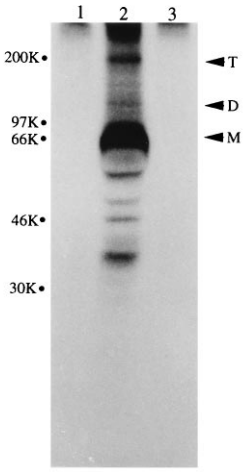

FIG. 4. Phosphorylation of gE expressed in insect cells. Sf9 insect cells were infected with recombinant baculovirus expressing gE and were labeled with ^{32}Pi for 48 h. The lysates were immunoprecipitated with MAb 3B3. Sf9 cells were not infected (lane 1), infected with gE recombinant baculovirus (lane 2), or infected with wild-type baculovirus (lane 3). The three arrowheads designate gE monomers (M), dimers (D), and trimers (T).

shown to be N-linked glycosylated and was transported to the surface of the insect cells (10). O-linked glycosylation appears to be similar but not identical to O-linked glycosylation in mammalian cells (29).

To determine whether VZV gE contained O-linked glycans, gE was subjected to digestion with neuraminidase and *O*-glycanase. As shown in Fig. 2, treatment of gE expressed in insect cells with neuraminidase alone (lane 3) or both neuraminidase and *O*-glycanase (lane 4) showed no difference in M_r compared with untreated gE (lane 2). Similar results were observed with both the gE monomer and the gE dimer. This result indicated that gE expressed in insect cells was not O-linked glycosylated. To determine if gE contained N-linked glycans, gE was subjected to digestion with endo F. As shown in Fig. 2, this digestion (lane 7) led to no reduction in M_r compared to untreated monomer or dimer gE (lane 6). Although representative fluorograms are shown in Fig. 2, these experiments were repeated three times because of the unexpected nature of the results. The fact that gE expressed in insect cells had neither N-linked nor O-linked glycans may explain in part the smaller M_r observed in insect cells.

Examination of the polypeptide backbone of gE. Previous reports estimated that the nonglycosylated gE form had a molecular mass of 73 kDa (27, 46). As shown above, gE was not glycosylated in the insect cells but still migrated at 64 kDa. One explanation for the M_r difference was one or more posttranslational modifications which were not present in the insect cells; e.g., VZV gE is also sulfated, palmytilated, myristylated, acetylated, and phosphorylated (8, 11, 46). To determine the M_r of the polypeptide backbone for gE, in vitro coupled transcription-translation was performed. The 2.0-kb DNA fragment encoding gE was subcloned from pTM1 into phagemid pBluescript by digestion with restriction endonucleases *Bam*HI and *Pst*I. The pBluescript-gE was used for the in vitro coupled transcription-translation reaction, and the products were labeled with [^{3}H]leucine. As shown in Fig. 3, the protein product from the in vitro reaction had a molecular mass of 64 kDa (lane 2). When gE expressed in insect cells was analyzed on the same gel (lane 4), it comigrated with gE obtained from the in vitro transcription-translation reaction. In addition, gE from transfected HeLa cell lysates was analyzed on the same gel. The VZV gE product expressed in HeLa cells had a molecular mass of 98 kDa (lane 6) as previously observed, but a faint band at 64 kDa was also visible. These results demonstrated that the molecular mass of the polypeptide backbone of gE was 64 kDa rather than 73 kDa. The lower value takes into account the fact that the in vitro translation mixture contains numerous protein kinases which could phosphorylate the translated gE product.

In vivo phosphorylation of gE in insect cells. Previous reports suggest that VZV gE is phosphorylated in mammalian cells by the serine/threonine protein kinase casein kinase II (11, 47). VZV gE by itself has no endogenous kinase activity, as is the case for other cell surface receptors with relatively short cytoplasmic tails (27). Insect cells are generally considered to possess a low amount of endogenous protein kinase activity, making the system advantageous for phosphorylation studies. Insect cells do not contain the same kinases as mammalian cells and have not previously been shown with certainty to contain casein kinase II (29). Therefore, the recombinant gE baculovirus represented a useful system for further study of gE phosphorylation in insect cells. To determine the extent of gE phosphorylation, gE was expressed in insect cells in the presence of $^{32}P_i$ and analyzed for phosphate incorporation. Contrary to the expected results, VZV gE was heavily phosphorylated in the insect cells, as shown in Fig. 4 by the protein band at 64 kDa that incorporated $^{32}P_i$ (lane 2). In addition, the dimer at 130 kDa also incorporated phosphate. Yet another phosphorylated protein was detected at 190 kDa; this band may represent a gE trimer but it was not further characterized in this study. Similar bands were not present in uninfected cell precipitates (lane 1) or precipitates from wild-type baculovirus-infected cells (lane 3). Therefore, gE was highly phosphorylated in insect cells.

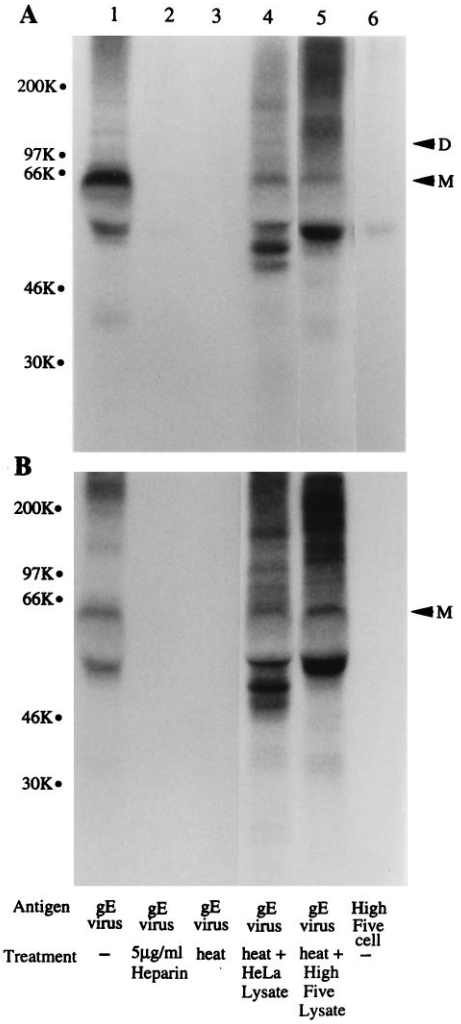

FIG. 5. Analysis of gE phosphorylation in protein kinase assays. The assays are described in Materials and Methods. The gE substrate was precipitated with MAb 3B3. The phosphate source in the assay was either [^{32}P]ATP (A) or [^{32}P]GTP (B). Each assay was performed in duplicate, once with ATP and once with GTP. Lane 1, kinase assay with immunoprecipitate of gE from gE recombinant baculovirus-infected High Five cells; lane 2, kinase assay with immunoprecipitate of gE from gE recombinant baculovirus-infected High Five cells in the presence of heparin; lane 3, kinase assay with immunoprecipitate of gE which was heat inactivated before the assay; lane 4, kinase assay with heat-inactivated immunoprecipitate of gE which was supplemented with fresh HeLa cell lysate added to the reaction mixture; lane 5, kinase assay with heat-inactivated immunoprecipitate of gE which was supplemented with fresh High Five cell lysate added to the reaction mixture; lane 6, kinase assay with immunoprecipitate from uninfected High Five cells. All samples were electrophoresed on the same gel, but some lanes represent longer exposure periods. D, dimer; M, monomer.

Kinase assay with gE expressed in insect cells. To further define the phosphorylation of gE in insect cells, in vitro kinase assays were performed. As mentioned above, gE was shown to be phosphorylated by casein kinase II in mammalian cells. Casein kinase II is a serine/threonine protein kinase with properties shared by few other kinases. First, casein kinase II is capable of utilizing GTP almost as well as ATP as a phosphate donor; second, casein kinase II activity is potently inhibited by heparin (14). These two characteristics were used to determine if gE expressed in insect cells was phosphorylated by casein kinase II present in insect cells. High Five cells infected with recombinant baculovirus expressing gE were lysed, and the lysates were immunoprecipitated with MAb 3B3. Some of the precipitates were heat inactivated at 60°C for 10 min. Next, fresh cell lysates from High Five insect cells or HeLa cells were added to some of the heat-inactivated precipitates. The kinase assay was initiated by the addition of either [γ-^{32}P]ATP or [γ-^{32}P]GTP.

With [γ-^{32}P]ATP, gE was phosphorylated in a manner similar to that demonstrated in vivo, as shown by the 64-kDa monomer as well as the 130-kDa dimer (Fig. 5A, lane 1). These results showed that the kinase coprecipitated with VZV gE; in turn, the same kinase phosphorylated gE during the in vitro assay. When 5 μg of heparin per ml was added to the gE precipitates during the kinase assay, phosphorylation of gE was greatly diminished (lane 2). Thus, the phosphorylation event was inhibited by heparin. When a gE precipitate was heat treated, the associated kinase was inactivated and thus unable to phosphorylate gE (lane 3). However, phosphorylation of gE was restored to the heat-inactivated precipitate when fresh HeLa cell lysate was added to the assay (lane 4). Previous studies demonstrated that large quantities of kinases, including casein kinase II, are present in HeLa cell lysates (11). Next, phosphorylation of gE was restored to the heat-inactivated precipitate when High Five cell lysate was added to the assay (lane 5). Thus, the kinase activity responsible for phosphorylating gE was present in insect cell lysates. No phosphorylation product was observed in insect cells not infected with the gE recombinant baculovirus (lane 6). These results suggested that casein kinase II was phosphorylating gE in insect cells.

To further determine whether casein kinase II was phosphorylating gE in insect cells, the kinase assay was repeated with [γ-^{32}P]GTP as a phosphate donor (Fig. 5B). Monomer gE was once again phosphorylated in a manner similar to that observed with [γ-^{32}P]ATP in the kinase assay, but the dimer at 130 kDa was not phosphorylated (lane 1). The phosphorylation of monomeric gE was inhibited by the presence of 5 μg of heparin per ml (lane 2). In addition, gE was not phosphorylated when the precipitates were heat inactivated prior to the kinase assay (lane 3). Phosphorylation was again restored to the heat-inactivated precipitates by the addition of HeLa cell lysates (lane 4) and High Five cell lysates (lane 5). The fact that a protein kinase in the lysates utilized GTP as a substrate to phosphorylate the gE monomer strongly suggested that insect cells contained endogenous casein kinase II. Mammalian casein kinase II consists of two subunits, α and β, which form an $\alpha_2\beta_2$ structure; the M_r of the α subunit is in the range of 37,000 to 43,000, and that of the β subunit is 24,00 to 28,000 (14, 23). To verify the identity of the protein kinase, a Western blot analysis was performed with two different antisera directed against casein kinase II (30, 34). This analysis confirmed that (i) insect cells contained both the α and β subunits of casein kinase II and (ii) the amount of the enzyme did not increase or decrease substantially in gE recombinant baculovirus-infected insect cells (data not shown).

Casein kinase II consensus sequence in gE. VZV gE contains a sequence within its cytoplasmic tail with a consensus motif for casein kinase II phosphorylation. This amino acid sequence, from residues 590 to 602 in gE (Phe-Glu-Asp-Ser-Glu-Ser-Thr-Asp-Thr-Glu-Glu-Glu-Phe), contains two serine and two threonine residues surrounded by acidic residues. To determine if this sequence was being phosphorylated by casein kinase II in insect cells and to further determine if casein kinase II activity mimicked that seen previously in mammalian cells (47), the sequence was mutated and the phosphorylation of the gE mutant molecule was analyzed. The two serine residues at 593 and 595 were changed to alanine and phenylalanine, respectively, and the two threonine residues at 596 and

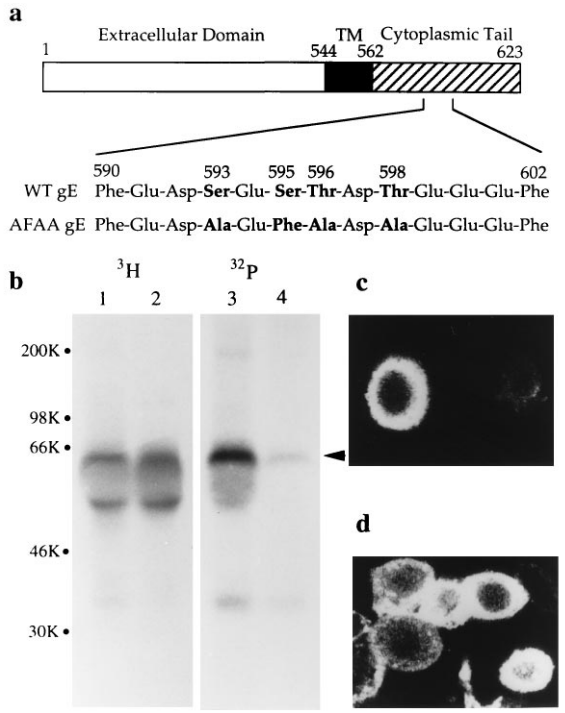

FIG. 6. Casein kinase II phosphorylation consensus sequence in gE. (a) gE contains a sequence for casein kinase II phosphorylation between amino acids 590 and 602. This sequence includes two serines and two threonines which were mutated to three alanines and one phenylalanine in order to disrupt the consensus sequence. The mutated amino acids are designated in boldface. TM, transmembrane region; WT, wild type. (b) Analysis of wild-type and mutant AFAA gE products. High Five cells infected with wild-type gE recombinant baculovirus (lanes 1 and 3) or mutant AFAA gE recombinant baculovirus (lanes 2 and 4) were labeled with either [^{3}H]leucine or $^{32}P_i$ and processed for precipitation with MAb 3B3. The arrowhead designates the monomer. (c and d) Laser scanning confocal microscopy of Sf9 insect cells infected with mutant AFAA gE recombinant baculovirus. Panel c illustrates the surface expression of mutant AFAA gE, and panel d illustrates the intracellular distribution of mutant AFAA gE.

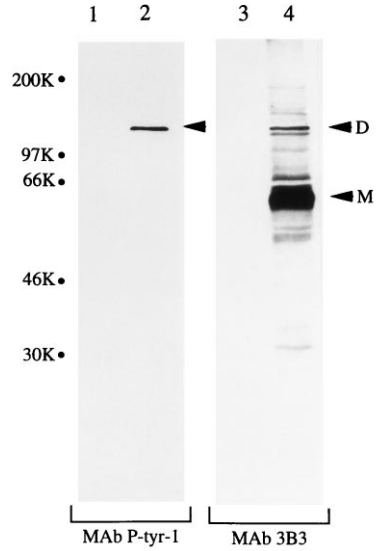

FIG. 7. Tyrosine phosphorylation of gE dimer in insect cells. Immunoblotting with MAb P-tyr-1 was performed to detect phosphotyrosine residues in gE expressed in insect cells. Uninfected High Five cells (25-cm^2 monolayer) or High Five cells infected with recombinant baculovirus expressing gE (25-cm^2 monolayer) were incubated for 48 h and then lysed. The lysates were immunoprecipitated with MAb 3B3 and then separated by SDS-PAGE (10 to 18% gradient gel). The proteins were transferred from the gel to a nitrocellulose membrane. The membrane was incubated with biotinylated MAb P-tyr-1. The membrane was washed and then incubated with streptavidin-horseradish peroxidase. Next, the membrane was treated with ECL Western blot solutions to detect proteins which blotted with MAb P-tyr-1. Lane 1, uninfected High Five cell precipitate; lane 2, gE recombinant baculovirus-infected High Five cell precipitate. The membrane was then stripped and reprobed with MAb 3B3 and peroxidase-conjugated goat anti-mouse antibody. The bands were detected with peroxide and color indicator. Lanes 3 and 4 represent the same samples as those in lanes 1 and lane 2, respectively. The arrowheads indicate the gE bands, with D representing the dimer and M representing the monomer.

598 were both changed to alanines, as shown in Fig. 6a. The mutated gE sequence referred to as AFAA gE was then subcloned into a baculovirus transfer plasmid, pVL1393. The mutant AFAA gE-pVL1393 was cotransfected with linearized baculovirus DNA in Sf9 insect cells. The recombinant baculovirus was plaque purified and amplified to an infectious titer. First, the recombinant baculovirus expressing mutant AFAA gE was analyzed by laser scanning confocal microscopy to determine the extent of expression. To this end, Sf9 insect cells were infected with recombinant baculovirus expressing AFAA gE. The mutant AFAA gE molecule was detected with MAb 3B3 on the cell surface (Fig. 6c) as well as within the cytoplasm of the cell (Fig. 6d). Thus, the AFAA gE protein was distributed in an insect cell in a pattern similar to that of wild type gE (Fig. 1b and c).

Next, the synthesis of mutant AFAA gE was analyzed (Fig. 6b). Infected High Five cells labeled with [^{3}H]leucine were immunoprecipitated with MAb 3B3 and analyzed by SDS-PAGE. Mutant AFAA gE (lane 2) was detected at the same level as wild-type gE (lane 1). Simultaneously, infected High Five cells were labeled with $^{32}P_i$ for 48 h and analyzed. The mutant AFAA gE showed markedly reduced phosphorylation (lane 4) compared to wild-type gE (lane 3). Densitometric analysis of the bands showed a 70% reduction in phosphorylation of mutant AFAA gE. The reduced phosphorylation in the AFAA gE mutant was similar to that observed when a similar gE mutant was expressed in mammalian cells (47). Therefore, the assumption was made that monomer gE expressed in insect cells was phosphorylated primarily by casein kinase II at the consensus site in its cytoplasmic tail.

Tyrosine phosphorylation of gE in insect cells. Since the phosphorylation of the gE dimer was abolished when GTP was the phosphate source, the gE dimer was not primarily phosphorylated by casein kinase II, which can utilize GTP nearly as well as ATP. Knowledge that the amino acid sequence of the cytoplasmic tail of gE contained six tyrosine residues led to an examination for tyrosine phosphorylation. To this end, High Five cells were infected with gE recombinant baculovirus or were left uninfected and incubated for 48 h. Lysates were immunoprecipitated with MAb 3B3. The precipitates were separated by SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. The membrane was incubated for 1 h with biotinylated MAb P-tyr-1, which specifically recognizes only phosphotyrosine linkages (21, 22). After visualization by chemiluminescence, MAb P-tyr-1 detected a single band at 130 kDa which is the gE dimer (Fig. 7, lane 2). In contrast, no bands appeared in the uninfected cell preparation after precipitation with MAb 3B3 and blotting with MAb P-tyr-1 (lane 1). The membrane was then stripped and reprobed with MAb 3B3 by usual immunoblotting methodology without chemiluminescence enhancement. The anti-gE MAb detected the gE dimer at 130 kDa and the gE monomer at 64 kDa (lane 4). However, no bands were detected in the uninfected cell precipitates blotted with MAb 3B3 (lane 3). This experiment

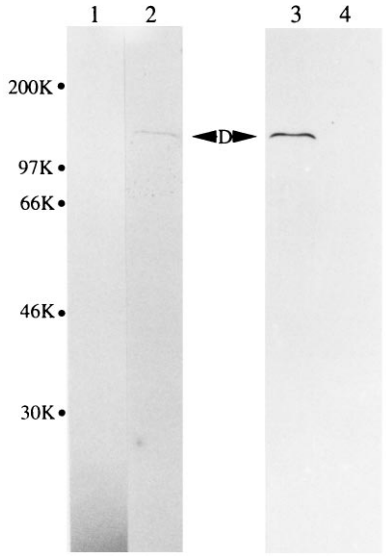

FIG. 8. Tyrosine phosphorylation of gE dimer in HeLa cells. Immunoblotting with MAb P-tyr-1 was performed to detect phosphotyrosine residues in gE expressed in HeLa cells. HeLa cells mock transfected or transfected with gE were lysed. The lysates were concentrated and then immunoprecipitated with MAb 3B3. Immunoblotting was performed as described previously with biotinylated antibody P-tyr-1. Lanes 1 and 2 represent mock-transfected HeLa cells and gE-transfected HeLa cells, respectively, which were concentrated from one 75-cm^2 monolayer. Lanes 3 and 4 represent gE-transfected HeLa cells and mock-transfected HeLa cells, respectively, which were concentrated from a 150-cm^2 monolayer. D, dimer.

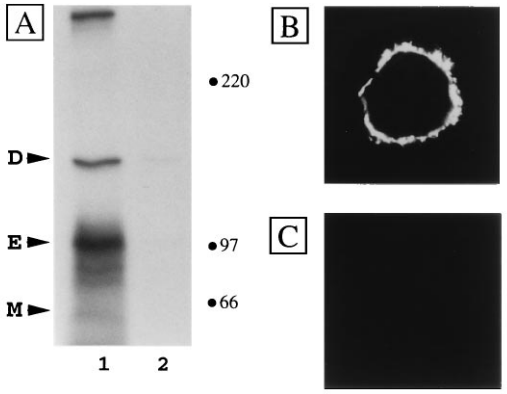

FIG. 9. Surface expression of dimeric gE complex. HeLa cells transfected with gE were radiolabeled and processed as described in Materials and Methods. (A) Viral proteins on the cell surface included dimeric (D) and mature (E) forms of gE, plus a faintly visible underglycosylated monomeric (M) form (lane 1). After trypsin treatment of the cell surface, no gE protein was detected (lane 2). Sizes are indicated in kilodaltons. (B) Confocal microscopy examination of surface of a gE-transfected cell probed with MAb 3B3 prior to trypsin treatment (compare with lane 1 in panel A). (C) Confocal microscopy examination of cells after trypsin treatment, to demonstrate the removal of viral cell surface antigens (compare with lane 2 in panel A).

was repeated four times with the same result. These results showed that a dimeric gE form was tyrosine phosphorylated whereas the gE monomer was not tyrosine phosphorylated.

Tyrosine phosphorylation of gE in mammalian cells. An important remaining question concerned the fact that tyrosine phosphorylation of the gE dimer had not been previously recognized in numerous phosphorylation studies from this laboratory (25, 27, 47). The above data showed that an underglycosylated dimeric form was tyrosine phosphorylated in insect cells. To determine if this same form was present in mammalian cells and to determine whether it was tyrosine phosphorylated in mammalian cells, gE-transfected HeLa cell lysates were immunoblotted with MAb P-tyr-1. Since the transfected HeLa cells do not produce as much gE protein as the baculovirus-infected High Five cells, the gE-transfected HeLa cell lysates were concentrated with Centricon 30 concentrators to obtain sufficient gE for further analysis. The concentrated lysates were immunoprecipitated with MAb 3B3, the precipitates were separated by SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane and probed with MAb P-tyr-1. MAb P-tyr-1 once again detected a phosphotyrosine protein at 130 kDa which corresponded to the underglycosylated gE dimer form, as shown in Fig. 8 (lanes 2 and 3). The immunoprecipitates from cells not transfected with pTM1-gE showed no phosphotyrosine bands (lane 1 and lane 4). Thus, these results confirmed that phosphotyrosine linkages were present in gE dimers found in mammalian cells and, in addition, demonstrated that the underglycosylated gE dimer was a naturally occurring product in mammalian cells. Hence, cloning the VZV gE gene into the baculovirus system allowed the identification of the previously unrecognized underglycosylated dimeric gE product.

Surface expression of the gE dimer. Since many tyrosine-phosphorylated receptors are located on the cell surface, the transfected HeLa cells were analyzed to define the location of the gE dimer. To this end, transfected cells were labeled with [^{35}S]methionine-cysteine mixture. The gE expressed on the cell surface was detected with MAb 3B3 at 4°C. Then one monolayer was harvested while a second monolayer was first treated with trypsin before harvesting. Antigen-antibody complexes were precipitated from each monolayer and subjected to SDS-PAGE (Fig. 9). In the nontreated monolayer, the mature 98-kDa gE molecule found in mammalian cells was the most abundant species, but the 130-kDa dimeric form was easily identified as one of the VZV cell surface constituents; small amounts of the underglycosylated monomer were detected (Fig. 9A, lane 1). No labeled gE was detected in the treated cells (Fig. 9A, lane 2). As an additional control experiment, transfected cells were examined by confocal microscopy before and after trypsin treatment of the outer cell membrane (Fig. 9B and C). Before trypsin treatment, abundant amounts of gE protein were detected on the surface, but no gE remained after trypsin treatment. This last set of experiments demonstrated that the underglycosylated gE dimer was present on the surface of a transfected HeLa cell. At this point, we reviewed our cumulative data on VZV gE collected over the past 12 years (11, 25, 26, 27); the underglycosylated dimeric form of gE was most likely present in some SDS-PAGE analyses but was not recognized as such because our attention was focused on the investigation of posttranslational events associated with biosynthesis of the mature 98-kDa gE monomeric form in mammalian cells (27).

DISCUSSION

When the VZV glycoprotein gE gene was cloned into baculovirus and expressed in insect cells, the gE product produced in the insect cells had a molecular mass of 64 kDa, which was markedly reduced compared to molecular mass of 98 kDa of gE expressed in mammalian cells. The reduced size was mainly a result of gE not being glycosylated in the insect cells. In mammalian cells, gE contains both N-linked and O-linked glycans (46); in contrast, gE expressed in insect cells had little or no oligosaccharides. Previous studies document that insect

cells lack the enzymatic machinery to process complex N-linked glycans, but the cells are usually able to facilitate some high-mannose-type of glycosylation (29). Since gE was detected on the surface of insect cells, a signal other than glycosylation was responsible for gE trafficking to the cell surface. Several reports have directly demonstrated that glycosylation and various posttranslational covalent modifications are not essential for cell surface transport (9). Previous reports also suggest that glycosylation may not be the only mechanism by which proteins exit the ER; instead, ER export of proteins may be regulated by a signal in the protein sequence (35). The signal-dependent membrane protein trafficking may function as a cycling event throughout the lifetime of the protein (42). While the sorting signals for some integral membrane proteins have been located in the cytoplasmic tail, the cytoplasmic domains of other cell surface proteins have been deleted and the proteins were still transported to the cell surface (9). Even similarly mutated proteins can exhibit different trafficking patterns under different transfection conditions; e.g., one report indicated that the VZV gE was unable to reach the *trans*-Golgi network when its cytoplasmic tail was deleted (50), yet another study demonstrated that a different tailless gE mutant molecule travelled to the cell surface of transfected HeLa cells (47).

Proteins expressed by baculovirus which form complexes in insect cells have also been shown to form complexes in their natural host cells (29). The underglycosylated gE dimeric forms were not previously observed when gE was expressed in mammalian cells. This discrepancy can be attributed to the relatively small quantity of protein expressed in mammalian cells; for example, to identify the phosphorylated 130-kDa form in HeLa cells, we concentrated 75 or 150 cm^2 of cell lysate rather than using a single 7-cm^2 monolayer, as is our practice in most transfection studies. In addition, we have initiated studies to evaluate coexpression of gE and gI in insect cells; our preliminary results indicate that gI does participate in complex formation with the 64-kDa form but does not disrupt formation of the 130-kDa gE complex (data not shown).

Structural similarities are commonly observed among cell surface receptors, such as the epidermal growth factor (EGF) receptor and the low-density lipoprotein (LDL) receptor. Figure 10 shows related motifs in VZV gE compared with two receptors, the LDL receptor and the Fc gamma receptor II (FcγRII) (41, 45). A computer-assisted BestFit homology comparison between VZV gE (accession no. X04370) and a human FcγRII isoform (accession no. X17653) showed 44% amino acid similarity and 23% amino acid identity between the two proteins. As a comparison, VZV gE showed 47% amino acid similarity and 27% amino acid identity to HSV-1 gE (25). Another property of cell surface receptors is their propensity to exist as dimers on the cell surface in order to bind the ligand and to become activated (49). The fact that the gE dimers were stable in the presence of reducing agents or heat suggests the possibility of a hydrophobic interaction or a combination of bonding interactions. Of note, dimer interactions have been determined to be among the strongest protein-to-protein interactions in nature (19).

When gE was expressed in insect cells, the glycoprotein was heavily phosphorylated not only in the monomeric form but also in the dimeric form. As in mammalian cells, casein kinase II was determined to be responsible for phosphorylating the gE monomer in insect cells. Casein kinase II is a serine/threonine protein kinase which binds to gE and is coprecipitated with its VZV substrate. This enzyme specifically phosphorylates the serine and threonine residues within the gE amino acid sequence from residues 590 to 602 in the cytoplasmic tail (14, 47). The gE cytoplasmic tail resembles the cytoplasmic tail of

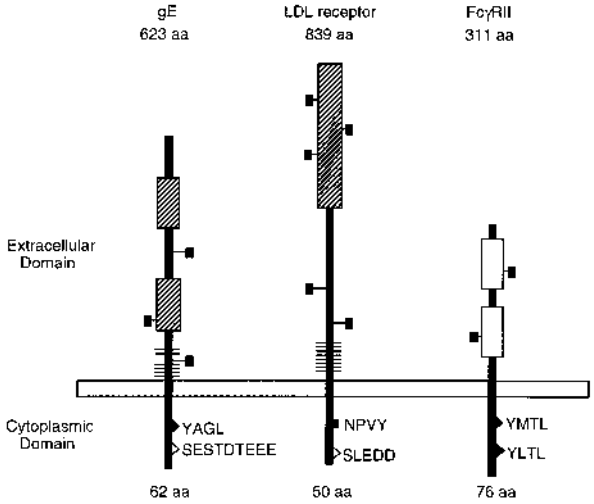

FIG. 10. Comparison of the structures and sequence motifs of VZV gE with two other cell surface receptors, human LDL receptor and human FcγRII. Each receptor spans the membrane one time, and the size in amino acids (aa) of each receptor is indicated below the name. Each receptor contains a carboxy-terminal cytoplasmic domain, with the size indicated below each receptor. The N-terminal regions rich in cysteine residues (LDL receptor and gE) are designated by striped boxes. Members of the Ig gene superfamily contain an Ig-like domain which is designated by open boxes. The potential sites for attachment of N-linked oligosaccharides are indicated by horizontal lines with closed boxes. The potential sites for attachment of O-linked oligosaccharides are indicated by a cluster of horizontal lines. The YXXL sequence which has been designated for tyrosine phosphorylation and receptor endocytosis is indicated by the closed triangles, with the exact sequence identified for each receptor. The casein kinase II phosphorylation sequence is indicated by the open triangles, with the specific sequence identified for each receptor. The receptor-mediated endocytosis sequence identified for the LDL receptor is indicated by the closed box with the designated sequence. Data were derived from references 2, 20, 25, 36, 39, 41, 44, and 46.

the LDL receptor, which has a similar casein kinase II consensus signal, as shown in Fig. 10 (20). The gE casein kinase II site also resembles the acidic sequence within the cytoplasmic domain of the furin endoprotease (44). Serine/threonine phosphorylation has been shown to modulate ligand binding and receptor trafficking within the cell (24, 36, 43, 44).

Protein tyrosine kinases phosphorylate tyrosine residues on proteins in a stable yet reversible modification. Protein tyrosine kinase activity has been detected in all animal cells and has been detected in insect cells as well as even simpler eukaryotic species (16). Recently, MAbs that react specifically with phosphotyrosine for detection of tyrosine-phosphorylated proteins have been produced (33, 40). One such antibody, called P-tyr-1, recognizes the phenyl phosphate moiety in phosphotyrosine and does not attach to phosphoserine or phosphothreonine residues (21, 22). When VZV gE immunoprecipitates were blotted with antibody P-tyr-1, the gE dimer was identified as containing phosphotyrosine residues whereas the gE monomer was not detected. VZV gE contains six tyrosine residues within its cytoplasmic tail; therefore, one of these tyrosine residues could be phosphorylated in the gE dimer. Most interestingly, VZV gE contains a YXXL motif within its cytoplasmic tail. Figure 10 shows the locations of the YXXL sequences in VZV gE and FcγRII. Several members of the Ig gene superfamily, including the Fc receptors, contain a conserved motif termed ITAM (Ig gene tyrosine activation motif) or ARAM (antigen recognition activation motif). The ARAM sequence consists of two YXXL sequences separated by six to eight amino acids (1, 32). The tyrosine residues of the conserved YXXL sequence are required for signal transduction

through the activation of the protein tyrosine kinase pathway. Since these receptors do not possess intrinsic tyrosine kinase activity, they require nonreceptor tyrosine kinases to induce signal transduction. The conserved YXXL sequence in the receptors serves as the putative Src homology 2 domain binding site for the tyrosine kinase (31). Often these receptors are more efficiently phosphorylated upon receptor oligomerization (1, 31). Since VZV gE contains only one YXXL sequence, the gE monomer may not be tyrosine phosphorylated. When gE forms a dimer, however, the YXXL sequences of the two proteins may create a complete ARAM sequence, enabling the YXXL sequences to be tyrosine phosphorylated. Furthermore, the YXXL motif has been shown to be important for receptor endocytosis (36).

Members of the cell surface receptor protein tyrosine kinase (RPTK) family, which includes the EGF receptor and the insulin receptor, possess an intrinsic protein tyrosine kinase activity within their long cytoplasmic tails (43). Evolutionary studies of receptors suggest that the RPTK family evolved from a more ancient receptor which obtained the exons encoding tyrosine kinase by genetic combination of sequences (49). An ancestral receptor for the RPTK family might have resembled the LDL receptor or the VZV gE receptor, both of which display structural similarity with the RPTK family but lack endogenous Src-type tyrosine kinase activity in their cytoplasmic tails. In its dimeric state, the EGF receptor is not only tyrosine phosphorylated but also serine/threonine phosphorylated by several heterologous protein kinases (24, 43). Likewise, the FcγRII is modified by both serine/threonine phosphorylation and tyrosine phosphorylation (15). As summarized in Fig. 10, the phosphorylation modifications of VZV gE support an evolutionary link with other nonviral receptors and, at the same time, suggest a role for gE phosphorylation-dephosphorylation in trafficking events involved in viral egress and cell-to-cell spread (4, 7, 12).

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