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Unusual Phosphorylation Sequence in the gpIV (gI) Component of the Varicella-Zoster Virus gpI-gpIV Glycoprotein Complex (VZV gE-gI Complex)

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Varicella-zoster virus (VZV) glycoprotein gpIV, to be renamed VZV gI, forms a heterodimer with glycoprotein gpI (gE) which functions as an Fc receptor in virus-infected cells. Like VZV gpI (gE), this viral glycoprotein is phosphorylated in cell culture during biosynthesis. In this report, we investigated the nature and specificity of the phosphorylation event involving VZV gpIV (gI). Phosphoamino acid analysis indicated that gpIV (gI) was modified mainly on serine residues. To identify the precise location of the phosphorylation site on the 64-kDa protein, a step-by-step mutagenesis procedure was followed. Initially a tailless mutant was generated, and this truncated product was no longer phosphorylated. Thereafter, point mutations were made within the cytoplasmic tail of gpIV (gI) at potential phosphorylation sites. The phosphorylation site was localized to the following sequence: Ser-Pro-Pro (amino acids 343 to 345). Examination of the point mutants established that serine 343 in the cytoplasmic tail was the major phosphoacceptor. In addition, we found that the prolines located immediately to the C terminus of serine 343 were an integral part of the kinase recognition sequence. This site was located immediately N terminal to a predicted β-turn secondary structure. By comparison with known substrate consensus sequences for various protein kinases, these data suggested that the phosphorylation of VZV gpIV (gI) was catalyzed by a proline-directed protein kinase. Computer homology analysis of other alphaherpesviruses demonstrated that a similar potential phosphorylation site was highly conserved in the cytoplasmic tails of herpes simplex virus type 1 gI, equine herpesvirus type 1 gI, and pseudorabies virus gp63.

Varicella-zoster virus (VZV) is the causative agent of two distinct clinical syndromes, chickenpox and shingles. Chickenpox, sometimes called varicella, results from the primary infection with the virus, and herpes zoster occurs upon reactivation of latent VZV infection. The complete nucleotide sequence of the 125-kbp VZV genome has been determined and was shown to contain 70 different open reading frames (ORFs) (4). Six of them encode viral glycoproteins. Those glycoproteins have been designated gpI, gpII, gpIII, gpIV, gpV, and gpVI, which are homologous to herpes simplex virus (HSV) glycoproteins gE, gB, gH, gI, gC, and gL, respectively (3, 4, 7). After a consensus nomenclature meeting at the 1993 International Herpesvirus Workshop, a decision was made to replace prior glycoprotein designations with those of the HSV-1 glycoproteins. Prior to publication of this report, both designations should be utilized. With regard to VZV gpIV, we have described several plasmid constructs in two recent publications, and those were identified as gpIV (33, 34). Since some of the same plasmids were used in this study, for clarity we prefer to use in this report the term gpIV (gI) to describe the VZV homolog of HSV gI.

Of the six VZV glycoproteins, gpI (gE) is the most abundant glycoprotein in the virus-infected cells, while gpIV (gI) is a lesser constituent (5, 10). The glycoprotein gpIV (gI) is encoded by ORF 67, which is located within the unique short region of the viral genome immediately adjacent to gpI (ORF 68). Both the nucleotide sequence of ORF 67 and the secondary structure analysis predict that gpIV (gI) is a typical type I transmembrane glycoprotein. In common with transmembrane glycoproteins, gpIV (gI) contains a 17-amino-acid N-terminal signal sequence, an N-terminal hydrophilic extracellular region of 278 amino acids, a 17-amino-acid hydrophobic membrane-anchoring sequence near the C terminus, and a 59-amino-acid-long C terminus that is intracellular (4). In studies with monoclonal antibodies, gpIV (gI) was composed of a mature 64-kDa form and an intermediary 50-kDa form in VZV-infected cells (11, 23, 33).

VZV gpIV (gI) often coprecipitates with gpI (gE) from VZV-infected cells (23). When coexpressed with gpI (gE) in a transient transfection system, gpIV (gI) was found to form a complex with gpI (gE) (33). VZV gpI (gE) has been shown to function as an Fc receptor for nonimmune immunoglobulin G (19, 20). By forming complexes with gpI (gE), gpIV (gI) is likely to be involved in the Fc receptor activity expressed on the VZV-infected cell surface. The mature gpIV (gI) is subjected to different types of posttranslational modifications, including N-linked and O-linked glycosylation (33). In a previous study, we found that the transfected gene product was also phosphorylated under in vivo labeling conditions (33). In this study, we further characterize the nature and specificity of the phosphorylation event involving VZV gpIV (gI). Specifically, we wanted to identify and characterize the phosphorylation sequence in gpIV (gI). By generating a series of mutations in the cytoplasmic tail of gpIV (gI) and expressing them in transfected HeLa cells, we located the serine phosphoacceptor site in the cytoplasmic tail. In addition, we identified adjacent amino acids that are essential to protein kinase recognition.

MATERIALS AND METHODS

Mutagenesis of gpIV by recombination PCR. The construction of plasmid pTM1-gpIV has been described in detail by

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TABLE 1. Mutating primer sequences of gpIV mutants

Mutant	Primer sequence	
	Sense strand	Antisense strand
Tailless	GTA ATA TAG CTT AAG CGA CGT AGA ATT A	CG TCG CTT AAG CTA TAT TAC AAT AAC AAT AAC C
Ser-296	T ATT GTA ATA GCT GTT AAG CGA CGT AGA A	CT ACG TCG CTT AAC AGC TAT TAC AAT AAC AA
Thr-338, Ser-343	CA GCT ATT CGC GAA GAA GCC CCC CCA CAT T	GGC TTC TTC GCG AAT AGC TGC TAG TTG TGC
Thr-338	T GCA CAA CTA GCA GCT ATT CGC GAA GAA	CG AAT AGC TGC TAG TTG TGC AAT GG
Ser-343	GC GAA GAA GCT CCC CCA CAT TCC GT	A ATG TGG GGG AGC TTC TTC GCG AAT CG
Glu-341, 342	A ACG ATT CGC GCA GCT TCC CCC CCA CATT	G GGG GGA AGC TGC CGA ATC GTT GCT AGT T
Pro-344, 345	GAA GAA TCC GCC GCA CAT TCC GTT GTA A	GGA ATG TGC GGC GGA TTC TTC GCG AAT
Pro-344	GAA GAA TCC GCC CCA CAT TCC GTT GTA A	GGA ATG TGG GGC GGA TTC TTC GCG AAT
Pro-345	GAA GAA TCC CCC GCA CAT TCC GTT GTA A	GGA ATG TGC GGG GGA TTC TTC GCG AAT
His-346	A GAA TCC CCC CCA GCT TCC GTT GTA AAC C	TT TAC AAC GGA AGC TGG GGG GGA TTC TTC GC

Yao et al. (33). The DNA template used for the mutagenesis experiments consisted of a 1.2-kb DNA fragment containing VZV glycoprotein IV (gene 67) target sequence cloned into plasmid pTM1, which is 5.3 kb (25, 33). For the present study, several mutant gpIV constructs were produced. Site-specific mutagenesis was performed by recombination PCR as previously described (15, 16, 35). Briefly, four complementary oligonucleotide primers were prepared in order to generate two linear fragments having homologous ends. To mutate a site, two pairs of primers were needed: one pair of mutating primers as well as a second pair of nonmutating primers. In recombination PCR, one mutating primer plus one nonmutating primer were used in pairs to generate linear products. Plasmids for PCR or DNA sequencing were prepared by using a Qiagen kit (Qiagen Inc., Chatsworth, Calif.). Oligonucleotide primers were prepared on an Applied Biosystems DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the DNA Core Facility, University of Iowa. The sequences of the mutating primers for ORF 67 are shown in Table 1; the nucleotides changes are underlined. The mutating primers overlapped by 21 to 25 nucleotides. The sequences of the nonmutating primers were located within the ampicillin resistance gene in the vector. Their sequences were 5′ AACAGCG GTAAGATCCTTGAG 3′ and 5′ AAACTCAAGGATCTT AC 3′. They overlapped by 15 bp. The DNA templates were linearized with the restriction endonuclease *Sac*I or *Mlu*I and purified by GeneClean (Bio 101, La Jolla, Calif.) before being used for mutagenesis.

PCR amplification was performed with *Taq* polymerase, using a Perkin-Elmer Cetus Thermal Cycler. Amplification of DNA fragments from the plasmid template was achieved by adding 1 ng of linearized template DNA, 200 mM each deoxynucleoside triphosphate, 25 pmol of each primer, and 1.25 U of *Taq* polymerase in a total volume of 50 μl of *Taq* polymerase buffer. These samples were overlaid with 50 μl of mineral oil. Reactants underwent 25 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 2 min), followed by a final extension (72°C, 7 min). Two linear products were generated. After PCR, a 2.5-μl sample of each product was combined and used to directly transform Max-competent *Escherichia coli* DH5α cells (BRL, Life Technologies, Gaithersburg, Md.). The colonies were grown overnight in Circlegrow medium (Bio 101) and screened by a modification of the procedure of Liang and Johnson (18). Briefly, a 100-μl aliquot of each overnight culture was removed and placed in boiling water for 2 min. After centrifugation in a microcentrifuge for 5 s, 2 μl of the sample supernatant, without plasmid purification, underwent 25 cycles of PCR amplification using primers that anneal to the sequence flanking the mutagenesis site. Screening of each clone was accomplished by restriction enzyme digestion of the PCR products. The mutations were verified by sequencing of each plasmid by automated DNA sequence analysis at the DNA Core Facility, University of Iowa.

Conditions for infection and transfection. The conditions for infection of human melanoma cells (Mewo strain) by VZV strain 32 have been described elsewhere (10). Likewise, the conditions for HeLa cell culture and transfection were detailed previously (8, 33). Briefly, HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. For transfection, 10^6 HeLa cells were plated onto 35-mm-diameter dishes 16 to 20 h before infection. Cells were first infected for 30 min at 37°C with a recombinant vaccinia virus encoding T7 polymerase (vTF7) at a multiplicity of infection of 10 to 15 in 0.5 ml of serum-free DMEM. The inoculum was then removed, and the cells were washed two times with serum-free DMEM. DNA (4.0 μg) was transfected by a liposome-mediated method (33). The transfected cells were incubated for 4 h in serum-free DMEM, further incubated in DMEM with 10% fetal calf serum at 37°C for various intervals, and harvested at 16 to 20 h after transfection by being dislodged into radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.4] containing 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate [SDS]) or lysis buffer (50 mM Tris [pH 7.4] containing 150 mM NaCl, 50 mM NaF, 0.1 mM Na_2VO_4, and 0.5% Nonidet P-40). The cells were sonically disrupted and sedimented (85,000 × *g* for 60 min) to remove insoluble macromolecules.

Isotopic labeling and immunoprecipitation. HeLa cells plated in a 35-mm-diameter tissue culture dish were transfected by lipofection as described above. At 4 h after DNA transfection, cell culture medium was replaced with methionine-deficient DMEM (Sigma) and starved for 15 min at 37°C. Thereafter, 1 ml of methionine-deficient medium containing 200 μCi of L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) was added to each dish, and the culture was incubated at 37°C for 12 h. Cell lysates were prepared as described above. For immunoprecipitation, 50 μl of cell lysate and 5 μl of gpIV hybridoma ascites fluid were added to 200 μl of lysis buffer and incubated at room temperature for 60 min. Methods for production of the anti-gpIV monoclonal antibody (clone 6B5) as well as precipitation of the antibody-antigen complexes with protein A-Sepharose beads have been described elsewhere (20, 23). Immunoprecipitates were analyzed on 12% polyacrylamide gels containing 0.1% SDS, and the gels were prepared for fluorography, dried, and exposed to radiographic film (X-Omat AR; Kodak, Rochester, N.Y.).

In vivo labeling with $^{32}P_i$. Cells were transfected with pTM1-gpIV or pTM1 vector DNA as described above. At 4 h posttransfection, 1 ml of DMEM containing 250 μCi of $^{32}P_i$ (370 MBq/ml, 10 mCi/ml; Amersham) was added to each dish, and the culture was incubated for 12 h at 37°C. Prior to immunoprecipitation, cells were washed three times with phosphate-buffered saline, harvested in 0.5 ml of lysis buffer, and prepared as the antigen for immunoprecipitation. Conditions for labeling VZV-infected cells with $^{32}P_i$ have been described elsewhere (12).

Phosphoamino acid analysis. The $^{32}P_i$-labeled gpIV was excised from an unfixed gel and eluted in a Hoefer Scientific gel eluter (Hoefer Scientific, San Francisco, Calif.) in 300 μl of elution buffer (0.1 M Tris [pH 8.3], 0.768 M glycine, 0.4% SDS). The eluted protein was precipitated in cold 20% trichloroacetic acid, washed with a cold solution of 50% ethanol–50% ether, suspended in 6 N HCl, and hydrolyzed at 110°C for 45 min. After hydrolysis, the samples were analyzed by thin-layer electrophoresis on cellulose plates (no. 13255; Eastman Kodak). Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine were placed in an adjacent lane. The unlabeled standards were visualized with 0.2% ninhydrin in isopropanol, while the ^{32}P-labeled phosphoamino acids were localized by autoradiography.

Computer analysis of amino acid sequences. The predicted amino acid sequence of gpIV contains 354 residues (4). Secondary structure predictions for gpIV were determined on either a peptide plot program of the University of Wisconsin Genetics Computer Group sequence analysis software package or a MacDNASIS Pro computer package produced by Hitachi. These programs are based in part on the calculations of Chou and Fasman (1).

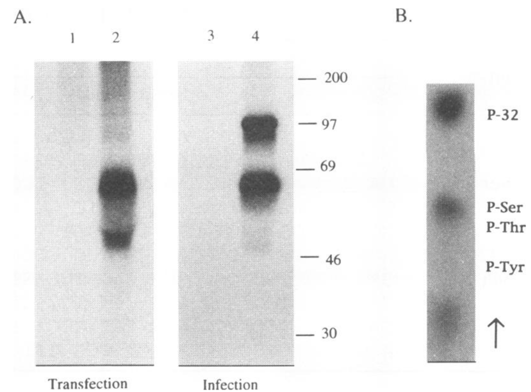


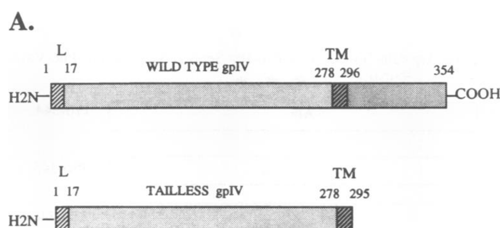
FIG. 1. Phosphorylation of VZV gpIV (gI). (A) Phosphorylation of gpIV in virus-infected Mewo cells and transfected HeLa cells. VZV-infected Mewo cells or transfected HeLa cells were labeled with $^{32}P_i$ and collected as described in Materials and Methods. Following immunoprecipitation, the phosphorylation state of gpIV was assessed by SDS-PAGE (12% gel) and autoradiography. Cells were either mock infected (lane 3) or infected with VZV (lane 4). Alternatively, HeLa cells were transfected by lipofection with pTM1-gpIV (lane 2) or pTM1 vector only as a control (lane 1). The migration of molecular mass marker proteins is indicated in kilodaltons on the right; they included myosin (200 kDa), phosphorylase (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa). The mature 64-kDa gpIV glycoprotein is seen in lanes 2 and 4. In lane 4, note the prominent 98-kDa phosphorylated VZV gpI (gE) which coprecipitates with gpIV (gI) from infected cells. (B) Phosphoamino acid analysis of VZV gpIV. VZV gpIV (64-kDa form) which had been labeled in vivo with $^{32}P_i$ was localized on an unfixed 12% polyacrylamide gel by autoradiography. The $^{32}P_i$-labeled protein was excised from the gel, acid hydrolyzed, and analyzed by thin-layer electrophoresis followed by autoradiography. The direction and relative migration of unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr), as visualized with 0.2% ninhydrin in isopropanol, are indicated at the right.

RESULTS

Phosphorylation and phosphoamino acid analysis of VZV gpIV. To determine whether VZV gpIV from virus-infected cells was phosphorylated, we labeled the virus-infected cells metabolically with $^{32}P_i$. For gpIV derived from transfected cells, HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then transfected by lipofection with plasmid pTM1-gpIV and similarly labeled. Cells were lysed, after which the viral proteins were immunoprecipitated with monoclonal antibody 6B5 and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. As shown in Fig. 1, a prominent M_r-64 protein and a minor 50-kDa protein were constitutively phosphorylated in pTM1-gpIV DNA-transfected cells (lane 2). These two species corresponded to the mature and precursor forms of gpIV in VZV-infected cells (lane 4) (23, 33). Also visible in lane 4 was the prominent 98-kDa phosphorylated VZV gpI (gE) glycoprotein, which has been shown previously to coprecipitate with VZV gpIV (gI) (23, 33). The same phosphoproteins were not observed in immunoprecipitates of cells transfected with the vector alone or in mock-infected cells (lanes 1 and 3). These results suggested that selective phosphorylation of gpIV was occurring in both VZV-infected cells and transfected HeLa cells. To determine which amino acid residues of gpIV were modified, we performed phosphoamino acid analysis. Several samples of $^{32}P_i$-labeled gpIV (64-kDa form) were cut from polyacrylamide gels, and the extracted glycoprotein was subjected to hydrolysis followed by thin-layer electrophoresis (12, 34). By comparison with the migration of the three standards, phosphoserine, phosphothreonine, and phosphotyrosine, we discovered that gpIV was modified predominantly on serine residues (Fig. 1B). The pattern was the same for transfected and VZV-infected gpIV products.

Phosphorylation of tailless gpIV gene product in transfected cells. To determine the general location of the phosphorylation sequence in gpIV, a truncated construct of gpIV was prepared by introducing a stop codon into the gpIV coding region immediately after the triplet encoding the transmembrane sequence at amino acid 295. The deletion mutant lacked all 59 amino acid residues from the C terminus (amino acids 296 to 354; Fig. 2A). This mutant was designated tailless gpIV. The altered gpIV gene was then transfected into HeLa cells. Wild-type and tailless gpIV-transfected cells were metabolically labeled for 12 h with $^{32}P_i$, after which detergent extracts were prepared and subjected to immunoprecipitation. Simultaneously prepared cultures were transfected under identical conditions and labeled with [^{35}S]methionine to monitor the relative amounts of wild-type and tailless gpIV proteins. As shown in Fig. 2B, while the wild-type gpIV was highly phosphorylated (lane 2), the phosphorylation of tailless gpIV was markedly diminished (lane 3). Results in lanes 5 and 6 confirmed that the tailless and wild-type gpIV products were synthesized to roughly comparable levels. These data indicated that the major site of phosphorylation was located in the carboxyl terminus of gpIV.

Localization of the phosphorylation site of gpIV. To confirm the results with tailless gpIV and further define which amino acids were the phosphate acceptors in gpIV, we applied oligonucleotide-directed mutagenesis of the cloned viral DNA



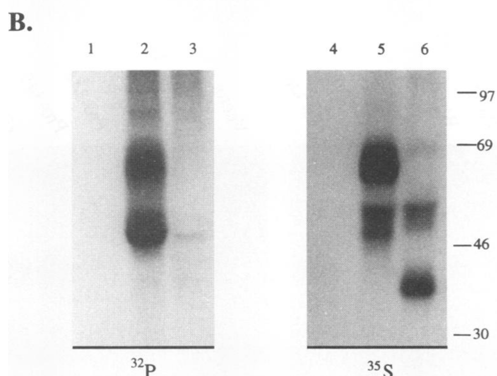


FIG. 2. Phosphorylation analysis of the tailless gpIV mutant. (A) Schematic representation of VZV gpIV and the tailless mutant. The entire 354-amino-acid gpIV protein is shown at the top, with its four major subdivisions. L, leader sequence; TM, transmembrane domain. The length of the tailless gpIV is shown below. (B) Metabolic labeling of tailless and wild-type gpIV in transfected cells. HeLa cells transfected with 4 μg of each tailless gpIV DNA (lanes 3 and 6), wild-type gpIV DNA (lanes 2 and 5) or pTM1 vector only (lanes 1 and 4) were labeled with either 250 μCi of $^{32}P_i$ per ml (lanes 1 to 3) or 200 μCi of [^{35}S]methionine per ml (lanes 4 to 6). Cell lysates were made, and gpIV was immunoprecipitated with monoclonal antibody 6B5 and then analyzed by SDS-PAGE (12% gel). Sizes are indicated in kilodaltons at the right.

to change the triplets encoding serines or threonines in the cytoplasmic tail of gpIV. As shown in Fig. 3, the cytoplasmic region of gpIV contains four serines and four threonines (underlined). We chose three sites for mutagenesis because they conform to potential consensus phosphorylation sequences; e.g., serine 296 has arginines at positions +3 to +5, which constitute a potential protein kinase C phosphorylation site. Alternatively, threonine 338 has a glutamic acid at position +3, which is the potential target for phosphorylation by casein kinase II, while serine 343 has two glutamic acids at positions −1 and −2 a possible target for casein kinase I (30, 31). Serine 343 also has a proline at both positions +1 and +2, which may allow phosphorylation by a proline-directed protein kinase (Fig. 3) (14, 32). Therefore, recombination PCR was applied to generate a total of four single and double gpIV mutants that had altered serine or threonine residues within potential phosphorylation sequences (Table 1). Each residue was replaced with an alanine (Fig. 3). The validity of each mutant construct was confirmed by sequencing across the mutation site. The gpIV mutants were transfected into HeLa cells. Each transfection was carried out in duplicate, one culture being labeled with $^{32}P_i$ and a second being labeled with [^{35}S]methionine.

The relative amount of phosphorylation in each serine and threonine mutant, as compared with wild-type gpIV and tailless gpIV, is illustrated in Fig. 4. The wild-type gpIV

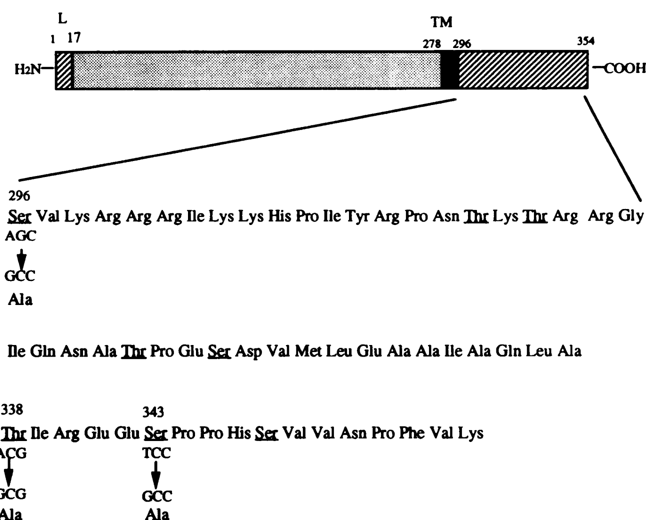


FIG. 3. Amino acid sequence in the cytoplasmic tail of the gpIV and locations of the serine and threonine residues which were replaced. All of the serine and threonine residues in the cytoplasmic tail are underlined. The initial four mutants were generated as described in Materials and Methods. The residues were chosen because they conform to the consensus phosphorylation sequences as described in the text. In mutant Ser-296, serine 296 was replaced by alanine. In mutant Thr-338 or Ser-343, the residue 338 or serine residue 343 was individually changed to alanine. In addition, the double mutant Thr-338, Ser-343 was generated. In this mutant, both residues were simultaneously replaced by alanines. The serines and threonines in the cytoplasmic tail of gpIV were mutagenized as described in Results. The two mutating primers for each reaction are listed in Table 1.

transfection product was phosphorylated under in vivo labeling conditions. Mutation of either serine 296 or threonine 338 did not affect the phosphorylation of gpIV, but mutation of a single serine 343 residue or both threonine 338 and serine 343

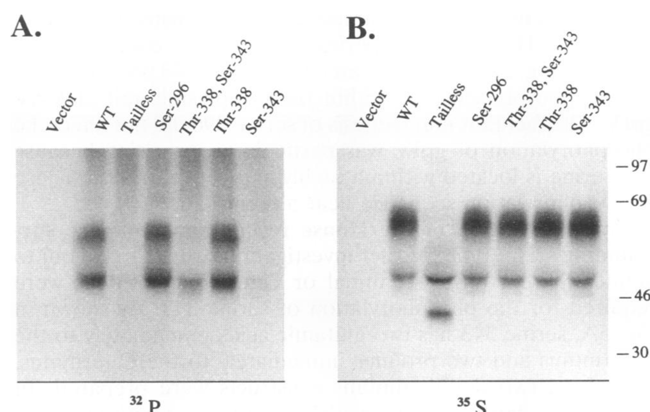


FIG. 4. Metabolic labeling of the mutant gpIV constructs in transfected HeLa cells. HeLa cells were transfected with either wild-type or mutant gpIV constructs as described in the legend to Fig. 2. Cells transfected with vector only served as a control. Four hours after transfection, the cells were labeled with either 200 μCi of [^{35}S]methionine per ml or 250 μCi of $^{32}P_i$ per ml. Cell lysates were made and gpIV was immunoprecipitated with monoclonal antibody 6B5 and then analyzed by SDS-PAGE (12% gel). (A) Phosphorylation profiles; (B) the [^{35}S]methionine-labeled gene products. Positions of the marker proteins are shown in kilodaltons on the right.

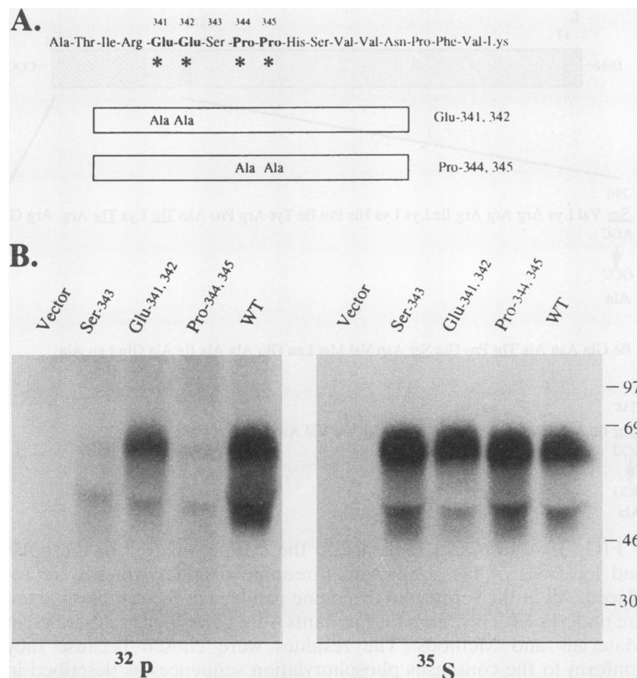


FIG. 5. Phosphorylation analysis of different gpIV mutants expressed in transfected HeLa cells. (A) The two gpIV double mutants are illustrated; the mutated residues were replaced by alanines. (B) Metabolic labeling of mutant gpIV constructs in transfected HeLa cells. HeLa cells were transfected with either wild-type or mutant gpIV constructs. Cells transfected with vector only served as a control. Four hours after transfection, the cells were labeled with either 200 μCi of [^{35}S]methionine per ml or 250 μCi of $^{32}P_i$ per ml. The gene products were immunoprecipitated with monoclonal antibody 6B5 and then analyzed by SDS-PAGE (12% gel).

residues markedly decreased the phosphorylation of gpIV. Results from the [^{35}S]methionine-labeled cultures demonstrated that the wild-type and mutated gene products were synthesized in comparable amounts in the transfection studies (Fig. 4B). Thus, these experiments not only confirmed the results in Fig. 2 but also indicated that serine 343 was the major in vivo phosphoacceptor within the cytoplasmic tail of VZV gpIV. The fact that mutagenesis of serine 296 did not affect the phosphorylation of gpIV was particularly surprising because this serine is located within a highly suggestive protein kinase C phosphorylation sequence near a membrane (30).

Characterization of the kinase recognition sequence surrounding serine 343. Further investigations were carried out to define whether any N-terminal or C-terminal residues were required for the phosphorylation of serine 343. As shown in Fig. 5A, serine 343 has two glutamic acids immediately to the N terminus and two prolines immediately to the C terminus. Therefore, two double-mutant constructs were prepared. In one mutant, both glutamic acids were replaced with alanines, to produce a mutant called Glu-341, 342; in the second, both prolines were replaced with alanines to produce a mutant called Pro-344, 345 (Table 1). Figure 5B illustrates the phosphorylation of these two mutants compared with wild-type gpIV and the serine 343 mutant. When the glutamic acids were changed to alanines, gpIV was still phosphorylated, although there was a moderate decrease in phosphorylation. But when the prolines were changed to alanine residues, phosphorylation of gpIV was drastically decreased. These results established

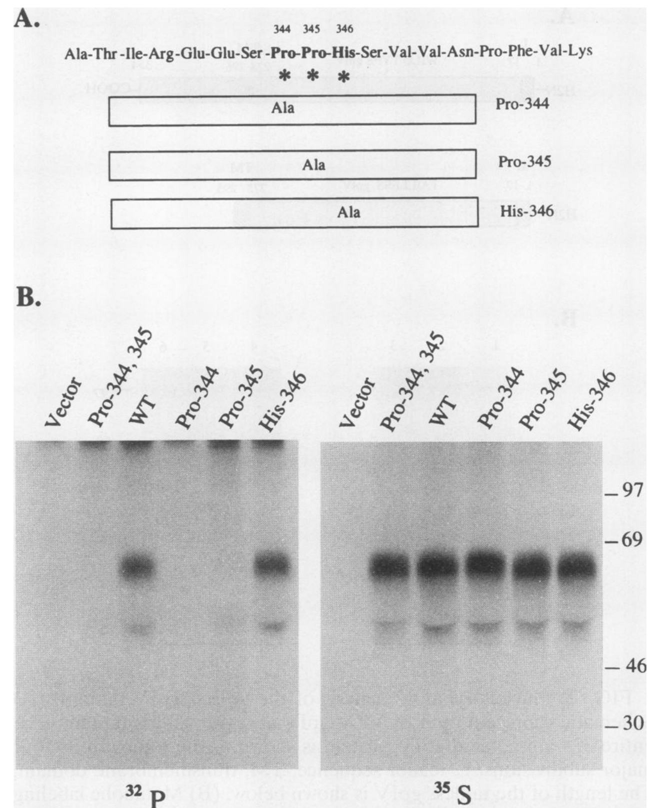


FIG. 6. Phosphorylation analysis of gpIV proline mutants expressed in transfected HeLa cells. (A) An enlargement of amino acids 337 to 354 shows the locations of the mutated residues within the cytoplasmic region. The position of each residue, numbered from the N terminus, is indicated immediately above the line. The three gpIV mutants are shown below the sequence. Each mutated residue was replaced by an alanine. (B) Metabolic labeling of mutant gpIV in transfected HeLa cells. HeLa cells were transfected with either wild-type or mutant gpIV constructs. Cells transfected with vector only served as a control. Subsequent analyses were the same as described in the legend to Fig. 5.

the need for the two proline residues immediately C terminal to the serine 343 residue.

At this point, it was not clear whether one or two prolines were required for the phosphorylation of serine 343 of gpIV. To address this question, single mutations in the proline residues were prepared (Table 1). As shown in Fig. 6A, each proline was changed individually to an alanine. As a control construct, the histidine residue at position 346 was changed to alanine. The in vivo phosphorylation of each of the mutants (designated Pro-344, Pro-345, and His-346) was compared with that of wild-type and double proline mutants (Fig. 6B). Like the double proline mutant, mutation of proline 344 nearly abrogated the phosphorylation of gpIV. Changing proline 345 to alanine also decreased the phosphorylation of gpIV. To adjust for varying band intensities and levels of expression, we quantitated by densitometry the gpIV bands observed in three experiments and calculated the average [^{32}P]phosphate/[^{35}S]methionine ratios for the mutant proteins relative to wild-type gpIV. The level of phosphorylation of the double proline mutant was about 2% that of the wild-type gpIV. For the single proline 344 and 345 mutant forms, levels of phosphorylation were approximately 2 and 8%, respectively. These

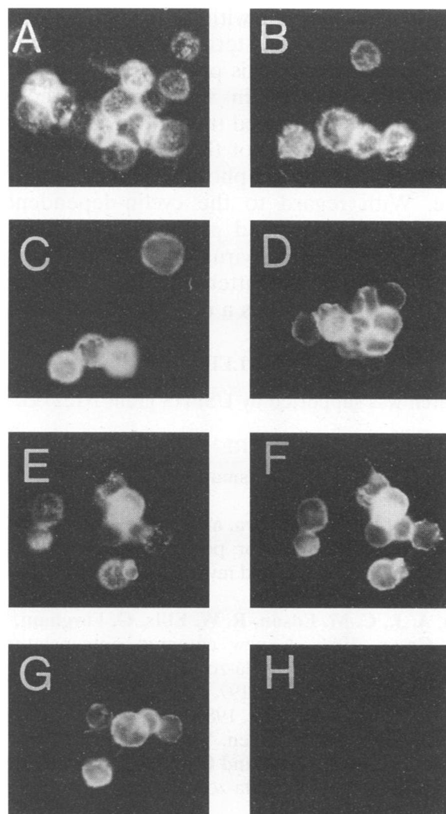


FIG. 7. Immunofluorescence analysis of wild-type and mutant gpIV forms in transfected cells. HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then transfected by lipofection with mutant Ser-343 (A), Pro-344 (B), Pro-345 (C), His-346 (D), or Glu-341, 342 DNA (E), tailless gpIV mutant DNA (F), or wild-type gpIV DNA (G). Cells transfected with pTM1 vector only served as a control (H). At 12 h posttransfection, the live transfected cells were analyzed for localization of the viral glycoproteins at the cell surface by previously described techniques (19, 20). The probe for gpIV was monoclonal antibody 6B5, and the second reagent was fluorescein-conjugated goat anti-mouse antibody.

results suggested that both prolines were important components of the gpIV phosphorylation sequence: proline 344 appeared to be absolutely essential, while proline 345 was a very important determinant.

Intracellular transport of mutant gpIV forms. To investigate whether the phosphorylation-deficient mutants were transported through the cytoplasm and inserted into the outer cell membrane, HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 polymerase and then transfected with either wild-type or mutant pTM1-gpIV DNA. At 12 h posttransfection, cells were harvested and prepared for immunofluorescence analysis without fixation (Fig. 7). When the living cells were probed with anti-gpIV monoclonal antibody 6B5, the results showed that wild-type gpIV as well as the mutant forms of gpIV were transported to and anchored in the plasma membrane. It is interesting that even the tailless form of gpIV was detected in the outer cell membrane.

Amino acid sequence comparison of VZV gpIV and homologs in three other alphaherpesviruses. Finally, we compared the sequence in the cytoplasmic tail of VZV gpIV with

EHV gI amino acid 294-424

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SASPADGDDFKQTNSTSLKARNKIVAMVVIPTACVLMLLLVVVGAIINGAV
RKHLLSCASRRIYRSGQGGASAAERRRLTCGPTLAASSESLADDTTSSPPT
                                               +++
PKPSKKTKLETDPLMEQLNRKLEAIKEES
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HSV gI amino acids 244-390

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PQPPGVNHEPPSNATRATRDSRYALTVTQIIQIAIPASIIALVFLGSCICFI
HRCQRRYRRSRRPIYSPQMPTGISCAVNEAAMARLGAELKSHPSTPPKSRRR
                                            +++
SSRTPMRSLTAIAEESEPAGAAGLPTPPVDPTTPTPTPPLLV
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PRV gp63 amino acids 258-350

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TMVLNASVVSRVLLAAANATAGARGPGKIAMVLGPTIVVLLIFLGGVACAA
RRCARGIASTGRDPGAARRSTRRPRGARPPTPSPGRPSPSPR
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FIG. 8. Comparison of the putative phosphorylation sites in the C-terminal regions of homologous glycoproteins encoded by three alphaherpesviruses. The amino acids sequences of the transmembrane regions and the cytoplasmic tails of EHV-1 gI, HSV-1 gI, and PRV gp63 are shown. The transmembrane regions are over- and underlined. The conserved potential phosphorylation sites in EHV-1 gI, HSV-1 gI, and PRV gp63 are indicated by plus signs below the lines. The SwissProt accession numbers are P09258 for VZV gpIV and P06487 for HSV-1 gI. GenBank and EMBL accession numbers are M14336 for PRV gp63 and M36299 for EHV-1 gI.

its alphaherpesvirus homologous glycoproteins to see whether this phosphorylation site was highly conserved among the homologs. In a previous report, we showed that VZV gpIV closely resembled HSV-1 gI, equine herpes virus-1 (EHV-1) gI, and pseudorabies virus (PRV) gp63 (33). Figure 8 includes the amino acid sequences of the cytoplasmic tail of the different alphaherpesvirus glycoproteins. The sequence similarity ranged from 37 to 46%. The cytoplasmic region of EHV-1 gI had an identical Ser-Pro-Pro site. Interestingly, HSV-1 gI had three similar sites, which were Thr-Pro-Pro (Fig. 8). Although not as apparent, a possible consensus sequence was also found in the cytoplasmic region of PRV gp63; this sequence was Thr-Pro-Ser-Pro.

Secondary structure of the phosphorylation site. The requirement of prolines for the phosphorylation of gpIV and the fact that a single proline can impose a significant constraint on secondary structure prompted an analysis of the residues around the phosphorylation sites of gpIV and its alphaherpesvirus homologs. Since a strong parallelism has been reported between the actual phosphorylation of potential phosphorylation sites and their locations within or adjacent to a predicted β turn (28, 29), we looked specifically for the features of a predicted β turn with a potential (Pt value) greater than 1.00, a value considered significant by Chou and Fasman (1). Indeed, the gpIV phosphorylation site (Ser-Pro-Pro) was located in a predicted β turn, with prolines 344 and 345 being the first and second residues. The Pt value was 3.03. Interestingly, the putative EHV-1, HSV-1, and PRV phosphorylation sites, as shown in Fig. 8, also were located within a predicted β turn. Thus, the current study supports earlier predictions about the importance of β turns to phosphorylation (29).

DISCUSSION

Previous studies of VZV gpI (gE) in virus-infected cells have indicated that the glycoprotein was phosphorylated under in vivo radiolabeling conditions (11, 12). A detailed search for potential kinase consensus phosphorylation sequences revealed one site of special interest. This region was located in the glycoprotein carboxy terminus beginning near amino acid 590: Phe-Glu-Asp-Ser-Glu-Ser-Thr-Asp-Thr-Glu-Glu-Glu-Phe. It contained two threonines and two serines surrounded by acidic amino acids which are important determinants in particular for the phosphorylation by casein kinase II (11, 30). The validity of this analysis was established by site-directed mutagenesis to identify the precise location of the cellular kinase-mediated phosphorylation of gpI (gE) (34). Transfection studies established that mutation of all four serines and threonines in that cluster markedly decreased the phosphorylation of the glycoprotein.

Subsequent in vitro phosphorylation studies were carried out with precipitated wild-type and mutant forms of gpI (gE), together with purified protein kinases (34). The results confirmed the in vivo findings and demonstrated that VZV gpI was an excellent substrate for casein kinase II (34). Because the in vivo and the in vitro phosphorylation data were so similar, we suggested that gpI (gE) was a true physiologic substrate for casein kinase II or a closely related phosphotransferase (12, 34). These conclusions are probably applicable to the other alphaherpesviruses also, because a computer analysis detected similar sequences in the following alphaherpesvirus glycoproteins: HSV gE and PRV gI. A recent report has confirmed that these glycoproteins are phosphorylated in vivo, although the nature of the protein kinase activity was not identified (6).

In this report, we demonstrated that VZV glycoprotein gpIV (gI) undergoes phosphorylation, like its partner in the gpI-gpIV complex. Phosphorylation occurred not only in VZV-infected Mewo cells but also in HeLa cells transfected with VZV gpIV DNA. Thus, the results with whole virus correlated well with the findings obtained with individual gene products. Mutational analysis of ORF 67 indicated that serine 343 was the major phosphoacceptor residue in the cytoplasmic tail, while two prolines immediately C terminal of serine 343 were essential to the kinase recognition site. Subsequent computer analysis predicted that the prolines mediated the formation of a β turn, a type of secondary protein structure composed of four amino acid residues arranged in such a manner as to facilitate chain reversal (1). Since a β turn is often located at an exposed surface of a protein, it is considered to be more easily recognizable and accessible to a phosphotransferase (29). Complex formation between gpI (gE) and gpIV (gI) may also enhance phosphorylation of gpIV (gI) (33).

After an extensive search of the literature, we found that the gpIV (gI) phosphorylation site was most similar to a recognition sequence required by a proline-directed protein kinase (14, 17, 32). The term proline-directed protein kinase was selected to denote a group of kinases united by their requirement for a proline residue situated immediately C terminal to the site of Ser/Thr phosphorylation (26). The enzymes include cyclin-dependent kinases and mitogen-activated protein kinases/extracellular signal-regulated protein kinases (9, 26, 32). Although mitogen-activation protein-related kinases are proline directed (2, 9), the substrate sequence specificity for some of these protein kinases, Pro-Xaa-Ser/Thr-Pro, appears to be different from that found in VZV gpIV(gI) in that a second proline is located two amino acids N terminal of the phosphorylated serine. When the recognition sequence specificity of some cyclin-dependent kinases was assessed, a consensus motif of Ser/Thr-Pro was observed, with an absolute requirement for an adjacent proline on the C-terminal side of the phosphoacceptor site. An example of this protein kinase is found in the complex of $p34^{cdc2}$ and cyclin A (13). Since the gpIV (gI) phosphorylation site resembled the latter consensus sequence, it is possible that a member of this rapidly growing family of protein kinases mediates the phosphorylation of gpIV (gI) in cell culture. With regard to the cyclin-dependent kinases, recent studies have identified a number of candidate substrates, including the simian virus 40 large T antigen (22, 24, 27). It is interesting that the latter antigen is phosphorylated at a site, Thr-Pro-Pro, which has a double proline motif (21).

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