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Identification of the Phosphorylation Sequence in the Cytoplasmic Tail of the Varicella-Zoster Virus Fc Receptor Glycoprotein gpI

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Varicella-zoster virus (VZV) glycoprotein gpI, the homolog of herpes simplex virus gE, functions as a receptor for the Fc portion of immunoglobulin G. Like other cell surface receptors, this viral receptor is highly phosphorylated in cell culture. To identify the precise location of the cellular kinase-mediated phosphorylation, we generated a tailless deletion mutant and several point mutants which had altered serine and threonine residues within the cytoplasmic domain of gpI. The mutated and wild-type genes of gpI were transfected and expressed within a vaccinia virus-T7 polymerase transfection system in order to determine what effect these mutations had on the phosphorylation state of the protein in vivo and in vitro. Truncation of the cytoplasmic domain of gpI diminished the phosphorylation of gpI in vivo. Examination of the point mutants established that the major phosphorylation sequence of gpI was located between amino acids 593 and 598, a site which included four phosphorylatable serine and threonine residues. Phosphorylation analyses of the mutant and wild-type glycoproteins confirmed that gpI was a substrate for casein kinase II, with threonines 596 and 598 being critical residues. Although the mutant glycoproteins were phosphorylated by casein kinase I, protease V8 partial digestion profiles suggested that casein kinase II exerted the major effect. Thus, these mutagenesis studies demonstrated that the gpI cytoplasmic sequence Ser-Glu-Ser-Thr-Asp-Thr was phosphorylated in mammalian cells in the absence of any other herpesvirus products. Since the region defined by transfection was consistent with results obtained with in vitro phosphorylation by casein kinase II, we propose that VZV gpI is a physiologic substrate for casein kinase II. Immunofluorescence and pulse-chase experiments demonstrated that the mutant glycoproteins were processed and transported to the outer cell membrane.

Varicella-zoster virus (VZV) is one of the human herpesviruses. The enveloped and double-stranded DNA virus specifies at least five major glycoproteins designated gpI, gpII, gpIII, gpIV, and gpV (4, 5, 7, 12). Of the five VZV glycoproteins, the herpes simplex virus gE homolog called gpI is the predominant virion envelope glycoprotein and also the major glycosylated VZV cell surface antigen (12, 23). Nucleotide sequencing of the VZV gpI gene and secondary structure analysis predict that gpI is a typical type I transmembrane glycoprotein, 623 amino acids in length with a 24-amino-acid cleavable signal sequence. The glycoprotein comprises three regions: an amino-terminal hydrophilic extracellular region of 544 amino acids, a hydrophobic transmembrane domain of 17 amino acids, and a charged carboxy terminus of 62 amino acids that is intracellular (4, 5). Examination of the predicted amino acid sequence of VZV gpI reveals that it has a combination of structural features unusual among viral surface glycoproteins. This glycoprotein contains two cysteine-rich regions, three N-linked glycosylation signals, as well as putative membrane proximal O linkages (19). The viral glycoprotein displays features also seen in some cell surface receptors and adhesion molecules (27). Indeed, like its herpes simplex virus homolog, VZV gpI was found to function as a receptor for the Fc portion of immunoglobulin G (19, 20).

In addition to the above-mentioned features, initial studies of VZV gpI from VZV-infected cells indicated that gpI was phosphorylated under in vivo labeling conditions. Phosphoamino acid analysis demonstrated that gpI was phosphorylated on both serine and threonine residues but not on tyrosine residues (22). When the nature and specificity of the phosphorylation event involving VZV gpI were further characterized, it was found that at least two cellular enzymes, casein kinases I and II, could catalyze the phosphorylation of gpI in vitro while a third protein kinase (cyclic AMP-dependent kinase) did not (12, 13). However, the actual amino acid residues within the domain that are phosphorylated remain unknown. A computer search for potential casein kinase I and casein kinase II consensus phosphorylation sequences within gpI revealed several potential sites in the molecule. Of greatest interest is a region near the glycoprotein carboxy terminus beginning at amino acid 590: Phe-Glu-Asp-Ser-Glu-Ser-Thr-Asp-Thr-Glu-Glu-Glu-Phe. This cluster of threonines and serines is an ideal construct for phosphorylation by casein kinase II (13).

In an attempt to obtain a better understanding of the mechanism of host cell-mediated phosphorylation of gpI and its role, we have used recombination polymerase chain reaction (PCR) to alter possible phosphate acceptor serines and threonines in the cytoplasmic domain of the viral receptor. The phosphorylation state of the wild-type and mutant gpI was studied in transfected HeLa cells by both in vivo labeling and in vitro phosphorylation with purified mammalian casein kinase II and casein kinase I. Our results demonstrate that the VZV gpI can be phosphorylated in vivo in the absence of any other VZV gene products and confirm the location of the major phosphorylation consensus sequence within the cytoplasmic tail, a location similar to that of other nonviral mammalian receptors.

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MATERIALS AND METHODS

Construction of a truncated pTM1-gpI plasmid DNA. The gpI gene was cloned into the pGEM-Blue vector as described previously (19). To construct a tailless gpI, the pGEM-Blue gpI DNA was digested with *Ssp*I and *Pst*I to remove the intervening nucleotides. The DNA termini were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase and then religated. The truncated gpI DNA was then excised from the pGEM-Blue gpI plasmid by digestion with *Bst*EII and *Bam*HI and ligated into a pTM1-gpI plasmid that has been linearized with these two restriction enzymes. The construct was followed by multiple TAA stop codons in the polylinker region of pTM1 (24, 32).

Mutagenesis of gpI by recombination PCR. The construction of pCMV5-gpI has been described in detail previously (19). Plasmids for PCR or DNA sequencing were prepared by using a Circleprep kit (Bio 101). Oligonucleotide primers were prepared on an Applied Biosystems DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.) at the DNA Core Facility, University of Iowa. Site-specific mutagenesis was performed by recombination PCR as described previously (15, 33). Briefly, four complementary oligonucleotide primers were prepared in order to generate two linear fragments having homologous ends. These fragments were combined and used to transform *E. coli* cells. Recombination in vivo of the linear products effected the generation of circular plasmids with the desired mutations. 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 template DNA, 200 μM 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 of 3.8 and 3.3 kb were generated. A 2.5-μl sample of each product was combined and used to directly transform Max competent *E. coli* DH5α cells (BRL, Life Technologies, Gaithersburg, Md.). The mutated gpI tail was then subcloned into a pTM1-gpI construct under the control of T7 promoter (24, 32). Mutations were verified by sequencing of the plasmids by the dideoxy method with Sequenase (United States Biochemical, Cleveland, Ohio) according to the manufacturer's protocol.

Conditions for cell culture and transfection. The conditions for HeLa cell culture and transfection were detailed previously (10, 32). 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 (10) 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 (9). 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]). 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 100 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 2 h. For pulse-chase analysis, transfected cells were similarly starved with methionine-deficient medium. Subsequently, cells were pulse-labeled with 100 μCi of [^{35}S]methionine for 15 min and chased with DMEM for various intervals. Cell lysates were prepared as described above. For immunoprecipitation, 50 μl of cell lysate and 5 μl of gpI hybridoma ascites fluid were added to 200 μl of lysis buffer and incubated at room temperature for 60 min. Production of monoclonal antibodies and precipitation of the antibody-antigen complexes with protein A-Sepharose beads have been described elsewhere (23). Immunoprecipitates were analyzed on 10% polyacrylamide gels containing 0.1% SDS, and the gels were prepared for fluorography, dried, and exposed to radiographic film (X-Omat AR; Kodak).

In vivo labeling with $^{32}P_i$. Cells were transfected with pTM1-gpI 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 (PBS), harvested in 0.5 ml of lysis buffer, and prepared as antigen for immunoprecipitation.

Phosphoamino acid analysis of mutant and wild-type gpI. The $^{32}P_i$-labeled gpI 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% ninhyrin in isopropanol, while the ^{32}P-labeled phosphoamino acids were localized by autoradiography.

In vitro phosphorylation. In vitro phosphorylation assays with purified preparations of mammalian casein kinase I and casein kinase II have been described in detail elsewhere (13). Briefly, cell lysate prepared in radioimmunoprecipitation assay buffer was immunoprecipitated with a VZV glycoprotein-specific monoclonal antibody and protein A-Sepharose CL-4B beads. Cell precipitates were then washed five times in PBS wash buffer (10 mM NaCl, 0.5% Nonidet P-40, 0.5% bovine serum albumin, 0.1% SDS, 0.2% NaN_3), twice with appropriate kinase buffer (for casein kinase I, 50 mM Tris [pH 7.5]–10 mM $MgCl_2$–50 mM NaCl; for casein kinase II, 50 mM Tris [pH 7.2]–140 mM KCl–10 mM $MgCl_2$) and then resuspended in 55 μl of kinase buffer. After heat inactivation for 10 min at 60°C, the pellets were cooled on ice. Then 10 μl of purified kinase was added along with 5 μl of [γ-^{32}P]ATP and incubated at 30°C for 30 min. Following five washes in PBS wash buffer, the glycoprotein was eluted from the protein A beads in 2× sample buffer (125 mM Tris, 6% SDS,

20% glycerol) by boiling at 100°C for 5 min. Phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), after which the gels were dried and exposed to radiographic film.

RESULTS

Phosphorylation of the gpI gene product in transfected cells. In previous studies, it was observed that VZV glycoprotein gpI from virus-infected cells was phosphorylated when $^{32}P_i$ was added into the medium, and it was also found that gpI from virus-infected cells could be phosphorylated in vitro by mammalian casein kinase II and casein kinase I (8, 13, 22). To determine whether VZV gpI was similarly phosphorylated in transfected HeLa cells, HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then transfected by lipofection with pTM1-gpI or pTM1 vector alone. The cells were metabolically labeled with either [^{35}S]methionine or $^{32}P_i$, and gpI was immunoprecipitated with monoclonal antibody 3B3 (23). Two ^{35}S-labeled species were present at approximately 98 and 81 kDa, corresponding to the previously described complex and high-mannose forms of gpI in VZV-infected cells (Fig. 1A, lane 2) (23). The absence of these two species in cells transfected with vector only confirmed the specificity of immunoprecipitation (lane 1). Lanes 3 and 4 show the companion set of immunoprecipitations from cultures labeled with $^{32}P_i$. As can be seen, phosphate was incorporated into both the mature 98-kDa product and the high-mannose form of gpI (lane 4). These phosphoproteins were not observed in immunoprecipitates of cells transfected with the vector alone (lane 3). The results indicated that both precursor and mature forms of gpI in transfected cells were phosphorylated under in vivo labeling conditions in the absence of any other VZV gene products.

To investigate whether gpI in transfected cells could be phosphorylated by casein kinase I and casein kinase II in vitro, cells were transfected with pTM1-gpI, and gpI was isolated by immunoprecipitation. The immune complexes were incubated with protein kinase and [γ-^{32}P]ATP, and the reaction products were analyzed by SDS-PAGE (10% gel). As shown, gpI was found to be phosphorylated by both casein kinase I and casein kinase II in vitro (lanes 5 to 8). As observed with gpI isolated from virus-infected cells (13), the phosphorylation of gpI by casein kinase II was more pronounced.

To determine whether the regions of gpI phosphorylated in transfected cultures corresponded to those phosphorylated in vitro by casein kinase II or casein kinase I, one-dimensional phosphopeptide maps of gpI were compared. $^{32}P_i$-labeled gpI or in vitro-phosphorylated gpI was excised from gels and subjected to *Staphylococcus aureus* V8 digestion. The profiles are shown in Fig. 1B. When in vivo ^{32}P-labeled gpI was partially digested with *S. aureus* V8 protease, two major phosphopeptides of 33 and 27 kDa were identified by SDS-PAGE (15% gel) (Fig. 1B, lane 1). The same two major phosphopeptides were observed after partial digestion of the in vitro casein kinase II-phosphorylated gpI protein (lane 2). In contrast to these results, four major peptides were observed after partial digestion of casein kinase I-phosphorylated gpI (lane 3). In addition to the 33- and 27-kDa species observed in the in vivo $^{32}P_i$-labeled gpI, two faster-migrating phosphopeptides at 21 and 17 kDa were observed. The latter result suggested that conditions of the in vitro casein kinase I reaction allowed modification of more gpI sites than those normally phosphorylated in vivo.

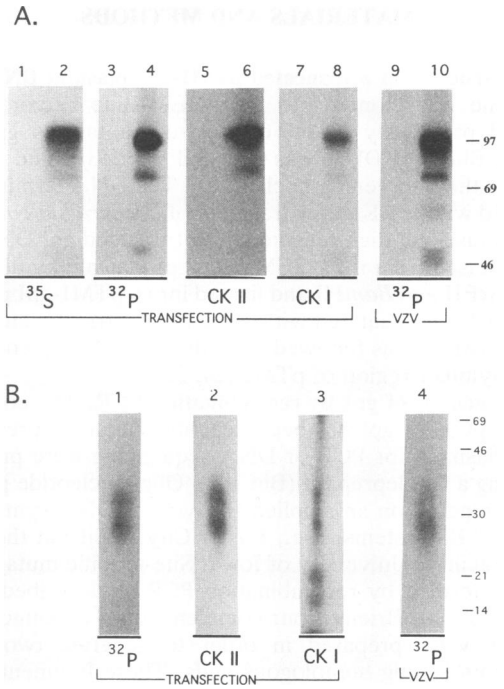

FIG. 1. Expression and phosphorylation of VZV gpI in transfected HeLa cells. (A) HeLa cells transfected by lipofection with pTM1-gpI (lanes 2, 4, 6, and 8) or pTM1 vector only (lanes 1, 3, 5, and 7) were labeled with either 100 μCi of [^{35}S]methionine per ml (lanes 1 and 2) or 250 μCi of $^{32}P_i$ per ml (lanes 3 and 4). Alternatively, transfected cell lysates were precipitated with monoclonal antibody 3B3 and used as substrates in protein kinase assays containing either purified casein kinase (CK) II (lanes 5 and 6) or casein kinase I (lanes 7 and 8). For controls, immunoprecipitates from mock-infected cells (lane 9) and VZV-infected cells (lane 10), both labeled with 250 μCi of $^{32}P_i$ per ml, were included. (B) Digestion of gpI with *S. aureus* V8 protease. In vivo-$^{32}P_i$-labeled gpI (lane 1), gpI phosphorylated in vitro with casein kinase II (lane 2) or casein kinase I (lane 3), or in vivo-$^{32}P_i$-labeled gpI from VZV-infected cells (lane 4) was immunoprecipitated from transfected HeLa cells as described in the text. The precipitates were analyzed by SDS-PAGE (10% gel), and gel slices containing the mature forms of gpI were reconstituted in vitro by using Cleveland buffer, inserted into a second 15% gel, and overlaid with 1 μg of V8 protease. The migration of molecular weight marker proteins is indicated in kilodaltons on the right.

Phosphorylation of the tailless gpI gene product in transfected cells. Having established a system with which to analyze the phosphorylation of transfected gpI, we next sought to determine the general location of the phosphorylation consensus sequence in gpI. Because the optimal serine/threonine sequence was located in the cytoplasmic region (13, 29), a truncated construct was prepared by restriction enzyme digestions of plasmid pGEM-gpI. The deletion mutant lacked all 62 amino acid residues from the C terminus and 3 amino acids from the transmembrane domain of gpI (Fig. 2A). This mutant was designated tailless gpI. The altered gpI gene was subcloned into a pTM1 vector and then transfected into HeLa cells. Wild-type and tailless gpI-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

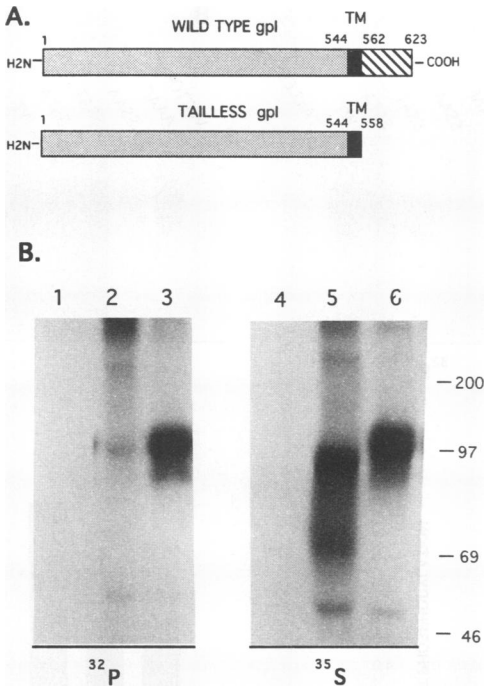

FIG. 2. Phosphorylation analysis of tailless gpI expressed in transfected HeLa cells. (A) Schematic representation of VZV gpI and the tailless mutant. The entire 623-amino-acid gpI protein, with its three major subdivisions, is shown at the top. The length of the tailless gpI is shown below. TM, transmembrane domain. (B) Metabolic labeling of tailless and wild-type gpI in transfected cells. HeLa cells transfected with tailless gpI (lanes 2 and 5), wild-type gpI (lanes 3 and 6), 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 100 μCi of [^{35}S]methionine per ml (lanes 4 to 6). Sizes are indicated in kilodaltons.

of wild-type and tailless gpI proteins. As shown in Fig. 2B, while the wild-type gpI was highly phosphorylated (lane 3), tailless gpI contained very little if any phosphate (lane 2). Results in lanes 5 and 6 confirmed that the tailless and wild-type gpI products were synthesized to about comparable levels. These results suggested that phosphorylation of gpI occurred mainly in the cytoplasmic tail.

Identification of the major phosphorylation sequence. To confirm the foregoing results and further define which amino acid(s) was the phosphate acceptor, we applied oligonucleotide-directed mutagenesis of the cloned viral DNA to change the triplets encoding the serines or threonines in the cytoplasmic tail of gpI. As previously noted, the cytoplasmic region of gpI contains a cluster of two serines and two threonines surrounded by several acidic amino acid residues (amino acid residues 593 to 598), which are potential targets for phosphorylation by both casein kinase II and casein kinase I (Fig. 3A) (4, 13). By recombination PCR, we generated single, double, and quadruple mutants that have altered threonines or serines within the phosphorylation consensus sequence (Fig. 3B). The validity of each mutant construct was confirmed by sequencing across the mutation site. Each mutated fragment was subcloned into a pTM1 vector. Each transfection was carried out in duplicate, one culture being labeled with $^{32}P_i$ and a second being labeled with [^{35}S]methionine.

Figure 3C illustrates the amount of phosphorylation in the

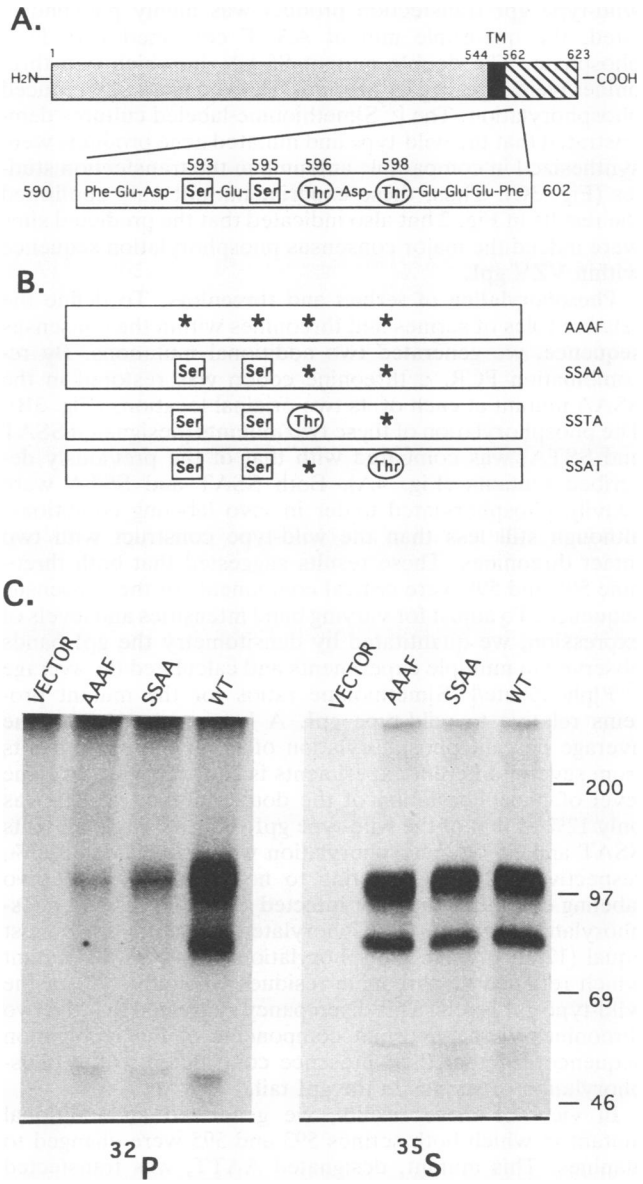

FIG. 3. Phosphorylation analysis of different gpI mutants expressed in transfected HeLa cells. (A) Schematic representation of the 623-amino-acid VZV gpI. An enlargement of amino acids 590 to 602 shows the location of the four serines and threonines within the cytoplasmic region. Serines or threonines fitting the criteria of casein kinase II or casein kinase I recognition sites are indicated by a Ser inside a box or a Thr in a circle. The location of each of these residues, numbered from the N terminus, is indicated immediately above. TM, transmembrane domain. (B) Diagrams of the different gpI mutants, with each mutated serine or threonine represented by an asterisk. Except in mutant AAAF, in which threonine 598 was changed to phenylalanine, all mutated serines and threonines were replaced by alanines. (C) Metabolic labeling of mutant gpI in transfected HeLa cells. HeLa cells were transfected with either wild-type (WT) or mutant gpI. Cells transfected with vector only served as a control. Four hours after transfection, the cells were labeled with either 100 μCi of [^{35}S]methionine per ml or 250 μCi of $^{32}P_i$ per ml. gpI was immunoprecipitated with monoclonal antibody 3B3 and then analyzed by SDS-PAGE (10% gel). Sizes are indicated in kilodaltons.

two most drastic mutants, AAAF and SSAA. While the wild-type gpI transfection product was highly phosphorylated, the quadruple mutant AAAF contained very little phosphate. The double mutant SSAA, in which two threonines were replaced by alanines, showed markedly reduced phosphorylation. 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. 3C). Thus, these experiments not only confirmed the results in Fig. 2 but also indicated that the predicted sites were indeed the major consensus phosphorylation sequence within VZV gpI.

Phosphorylation of serines and threonines. To define the relative roles of serines and threonines within the consensus sequence, we generated two additional mutations. By recombination PCR, a threonine codon was restored in the SSAA mutant at each of its two original locations (Fig. 3B). The phosphorylation of these two mutants (designated SSAT and SSTA) was compared with that of the previously described mutants (Fig. 4A). Both SSAT and SSTA were heavily phosphorylated under in vivo labeling conditions, although still less than the wild-type construct with two intact threonines. These results suggested that both threonine 596 and 598 were critical components of the consensus sequence. To adjust for varying band intensities and levels of expression, we quantitated by densitometry the gpI bands observed in multiple experiments and calculated the average [^{32}P]phosphate/[^{35}S]methionine ratios for the mutant proteins relative to wild-type gpI. A bar graph showing the average percent phosphorylation of all of the gpI mutants from several different experiments is shown in Fig. 4C. The level of phosphorylation of the double mutant SSAA was only 12% of that of the wild-type gpI. For the single mutants SSAT and SSTA, phosphorylation was about 85 and 76%, respectively. It is interesting to note that under in vivo labeling conditions in VZV-infected cells, the ratio of phosphorylated serine and phosphorylated threonine was almost equal (12, 13), while phosphorylation of the SSAA mutant which retained no threonine residues was only 12% of the wild-type gpI levels. This discrepancy suggested that the two threonines were important components of the recognition sequence and that their presence contributed to the phosphorylation of serines in the gpI tail.

In view of these results, we generated an additional mutant in which both serines 593 and 595 were changed to alanines. This mutant, designated AATT, was transfected into HeLa cells. The phosphorylation of this mutant was compared with that of mutant AAAF and wild-type gpI. As shown in Fig. 5, in vivo labeling with $^{32}P_i$ demonstrated that this mutant was still phosphorylated although to a lesser extent than wild-type gpI. When quantitated by densitometry, the phosphorylation of AATT was estimated to be 40% of that of wild-type gpI, while the phosphorylation of AAAF was only 5% of that of wild-type gpI. This result confirmed our previous observation that threonines 596 and 598 were critical residues within the gpI phosphorylation sequence. The fact that phosphorylation of AATT was decreased suggested that serines 593 and 595 were components of the gpI phosphorylation sequence.

Phosphoamino acid analysis of mutant and wild-type gpI. To determine whether threonines 596 and 598 influenced the phosphorylation of the serine residues, we analyzed the ratio of phosphorylated serine and threonine in mutants SSAT and SSTA and wild-type gpI. Several samples of $^{32}P_i$-labeled gpI were cut from polyacrylamide gels, and the extracted glycoprotein was subjected to hydrolysis followed by thin-

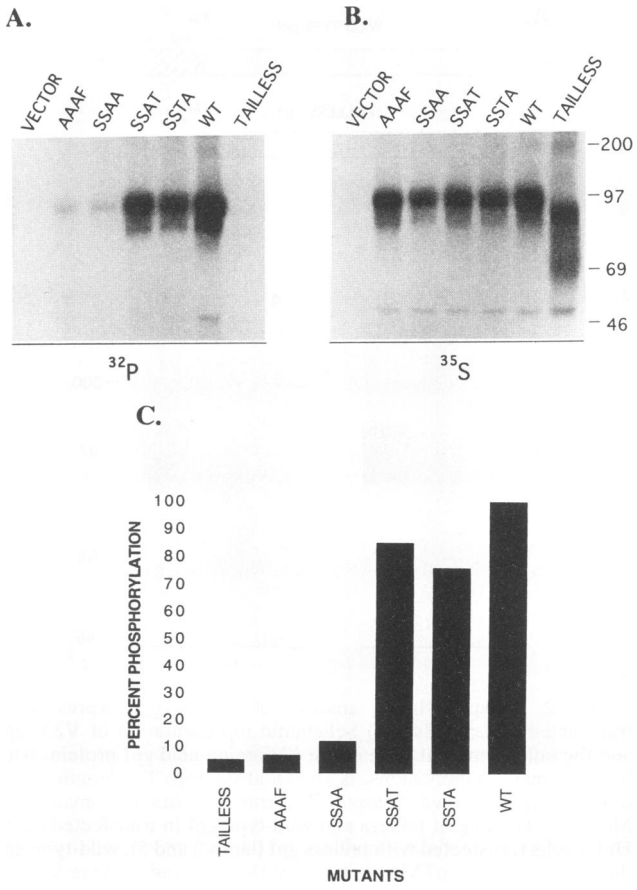

FIG. 4. Phosphorylation of serines and threonines. (A and B) Metabolic labeling of mutant gpI in transfected HeLa cells. HeLa cells were transfected with either wild-type (WT) or mutant gpI. Cells transfected with vector only served as a control. Four hours after transfection, the cells were labeled with either 100 μCi of [^{35}S]methionine per ml or 250 μCi of $^{32}P_i$ per ml. gpI was immunoprecipitated with monoclonal antibody 3B3 and then analyzed by SDS-PAGE (10% gel). Sizes are indicated in kilodaltons. (C) Percent phosphorylation of mutant gpI proteins. Autoradiographs from several experiments were scanned with a densitometer. The $^{32}P_i$/[^{35}S]methionine ratios in the mutants from three experiments were calculated, and an average value was obtained. For this graph, phosphorylation of the wild-type (WT) gpI protein was considered to be 100%.

layer electrophoresis (13, 22). By comparison with the migration of the three standards phosphoserine, phosphothreonine, and phosphotyrosine, we determined that wild-type gpI was modified on both serine and threonine residues but not on the tyrosine residues (Fig. 6A). For mutant SSAA, upon a prolonged exposure of the gel, phosphorylated serine residues were faintly visible (Fig. 6B). Interestingly, for mutants SSAT and SSTA, the major phosphorylated amino acid residues were serines, although threonines were also modified. Since the only difference between the SSAT or SSTA mutant and the SSAA mutant was the absence of a threonine in the SSAA construct, the results suggested that one of the two threonines had to be present for the optimal phosphorylation of serines.

Phosphorylation of mutant gpI proteins by casein kinase II. Because the defined recognition sequence closely resembled a casein kinase II phosphorylation site, the various gpI

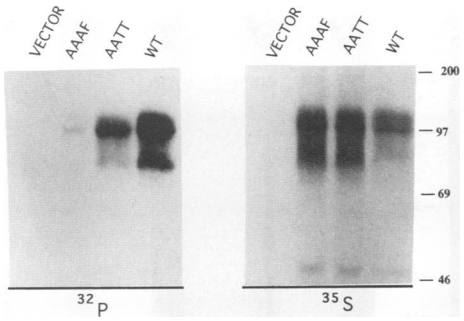

FIG. 5. Phosphorylation of a serine mutant gpI. HeLa cells were transfected with either wild-type (WT) gpI or a serine mutant gpI designated AATT. Cells transfected with vector only or mutant AAAF served as controls. Four hours after transfection, the cells were labeled with either 100 μCi of [^{35}S]methionine per ml or 250 μCi of $^{32}P_i$ per ml. gpI was immunoprecipitated with monoclonal antibody 3B3 and then analyzed by SDS-PAGE (10% gel). Sizes are indicated in kilodaltons.

proteins were next evaluated in a series of protein kinase assays performed with purified casein kinase II. The gpI gene was transfected into HeLa cells, and cell lysates were made as previously described. After quantitation of gpI by immunoblot analysis, lysates containing equal amounts of mutant or wild-type gpI protein were precipitated with specific monoclonal antibody, washed with casein kinase II buffer, and heat inactivated. The protein kinase assay was initiated by adding [γ-^{32}P]ATP and casein kinase II to the immunoprecipitated glycoprotein. The gp98 and gp88 species of wild-type gpI were highly phosphorylated, whereas the tailless gpI, AAAF, and SSAA mutants were markedly less phosphorylated (Fig. 7). These same constructs have diminished phosphorylation under in vivo conditions (Fig. 4). The two mutant forms of gpI (SSAT and SSTA) that were

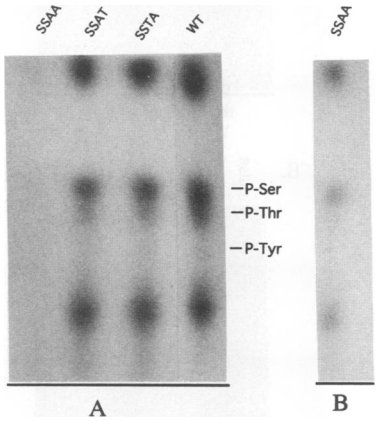

FIG. 6. Phosphoamino acid analysis of mutant and wild-type gpI. Mutant or wild-type (WT) gpI which had been labeled in vivo with $^{32}P_i$ was localized on an unfixed 10% polyacrylamide gel by autoradiography. The $^{32}P_i$-labeled protein was excised from the gel, acid hydrolyzed, and analyzed by thin-layer electrophoresis followed by autoradiography. Unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were mixed and run in an adjacent lane. The relative migration of the unlabeled standards, as visualized with 0.2% ninhydrin in isopropanol, is indicated at the right. All lanes were taken from the same gel. Exposure times were 48 h for panel A and 14 days for panel B.

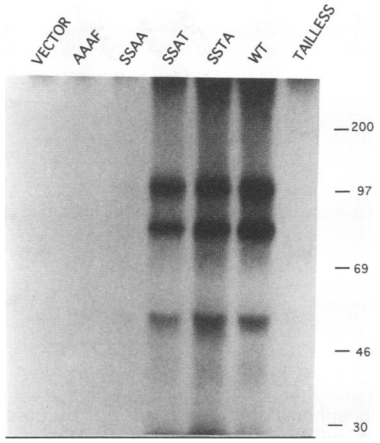

FIG. 7. Phosphorylation of mutant gpI by casein kinase II. HeLa cells were transfected with either mutant or wild-type (WT) gpI DNA, as indicated above each lane. Cell lysates were made and immunoprecipitated with monoclonal antibody 3B3 and then phosphorylated by casein kinase II in the presence of [γ-^{32}P]ATP. Protein kinase assays were performed with immunoprecipitates of mutant and wild-type gpI. Immunoprecipitates from cells transfected with pTM1 vector alone were included as a negative control. Sizes are indicated in kilodaltons.

previously shown to be phosphorylated in vivo were also phosphorylated in vitro by casein kinase II (Fig. 7). These results suggested that the same serine and threonine residues were modified in vitro by casein kinase II as under in vivo conditions.

Phosphorylation of mutant gpI by casein kinase I. Since gpI can be phosphorylated by casein kinase I in vitro, we next evaluated the phosphorylation of wild-type and mutant gpI proteins by this mammalian kinase. In a manner similar to that described above, immunoprecipitates of the transfected VZV glycoproteins were placed in a reaction mixture with casein kinase I and [γ-^{32}P]ATP. The precipitates were subsequently analyzed by SDS-PAGE and autoradiography. As shown in Fig. 8A, wild-type gpI was observed to be highly phosphorylated. Mutants AAAF and SSAA were also phosphorylated in vitro by casein kinase I to about the same level as the wild type (Fig. 8A). This result was unexpected because in mutant AAAF, the predicted casein kinase I consensus phosphorylation sequence was disrupted (13, 29). Either this sequence did not represent the actual phosphorylation sites for casein kinase I or, alternatively, there were other sites in gpI that became highly phosphorylated only under in vitro conditions. To distinguish these possibilities, we phosphorylated the wild-type and mutant gpI products with casein kinase I in vitro and performed a partial digestion with *S. aureus* V8. The results shown in Fig. 8B indicated that the protease V8 partial digestion profiles of in vitro-phosphorylated wild-type and SSAA mutant gpI protein have four major phosphorylated peptides, while the mutant AAAF has a diminished profile. Since the 33- and 27-kDa peptides that were phosphorylated in vivo were absent in mutant AAAF, these data suggested that the 33- and 27-kDa phosphorylated peptides represented the SSTT domain within the cytoplasmic tail. Although this domain can be phosphorylated in vitro by casein kinase I, the same enzyme also phosphorylated a second site distinguished by the two faster-migrating phosphopeptides. To ensure that the change in the *S. aureus* V8 protease map was not due to a mutated cleavage site in either the AAAF or the SSAA protein, we

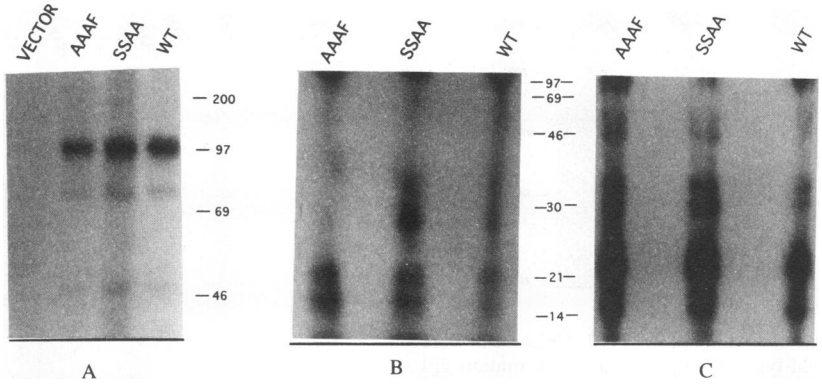

FIG. 8. Phosphorylation of mutant gpI by casein kinase I and analysis by protease digestion. (A) HeLa cells transfected with mutant AAAF, SSAA, wild-type (WT) gpI, or pTM1 vector only. Cell lysates were made and immunoprecipitated with monoclonal antibody 3B3 and then phosphorylated by casein kinase I in the presence of [γ-^{32}P]ATP. (B) *S. aureus* V8 protease digestion of in vitro casein kinase I-phosphorylated mutant AAAF, mutant SSAA, and wild-type (WT) gpI. (C) *S. aureus* V8 protease digestion of [^{35}S]methionine-labeled mutant AAAF, mutant SSAA, and wild-type (WT) gpI. Sizes are indicated in kilodaltons.

labeled transfected cells with [^{35}S]methionine and similarly analyzed the peptide digestion products. As shown in Fig. 8C, an identical pattern of phosphopeptides was obtained with both mutant and wild-type gpI proteins. Thus, the amino acid mutations in the gpI tail did not alter the sensitivity to cleavage by V8 protease.

Intracellular transport and membrane anchoring of wild-type and mutant gpI in transfected cells. Conversion of the gpI precursor (containing high-mannose glycosylation) to the mature form (containing complex-type glycosylation) is accompanied by a distinct shift in electrophoretic mobility (from 81 to 98 kDa). Since this conversion requires transport from the endoplasmic reticulum to the correct compartment within the Golgi apparatus, an analysis of glycoprotein processing serves as a measure of the efficiency of gpI transport. It has been speculated that phosphorylation may play a role in gpI membrane insertion and targeting of glycoproteins (13, 16). Therefore we investigated intracellular transport of the phosphorylation mutants by both pulse-chase analyses and cell surface immunofluorescence. Transfected cells were pulse-labeled with [^{35}S]methionine for 15 min and either harvested immediately or chased for various intervals. Both the wild-type and mutant gpI forms were processed from a faster-migrating precursor form (81 kDa) to the more slowly migrating product form (98 kDa); in each case, processing began within 20 to 30 min of chase (Fig. 9). To quantitate the data, we analyzed the bands by densitometry. The rate of processing of wild-type and mutant gpI proteins was calculated as [gpI (98 kDa)/gpI (81 kDa) + gpI (98 kDa)] $\times$ 100. It was found that 36% of AAAF was processed to mature form after 30 min of chase, while only 21% of wild-type gpI was processed to mature form at this time point. After 60 min of chase, 78% of mutant AAAF was processed to mature form, while only 61% of the wild-type gpI was processed at this time point. These results, reproducible in three repeated experiments, indicated that the processing of mutant AAAF was not inhibited. To determine whether the phosphorylation-deficient mutants were inserted into the outer cell membrane, we transfected HeLa cells with either mutant or wild-type gpI DNA. When the unfixed cells were stained with monoclonal antibody to gpI, the pattern of cell surface immunofluorescence showed that wild-type as well as mutant gpI products were transported to and anchored in the plasma membrane (Fig. 10).

DISCUSSION

Prior studies of VZV gpI in virus-infected cells have established that the glycoprotein is phosphorylated under conditions of radiolabeling a cell culture with $^{32}P_i$. Examination of the published amino acid sequence of gpI uncovered potential phosphorylation consensus sequences for casein kinases II and I in the cytoplasmic region (amino

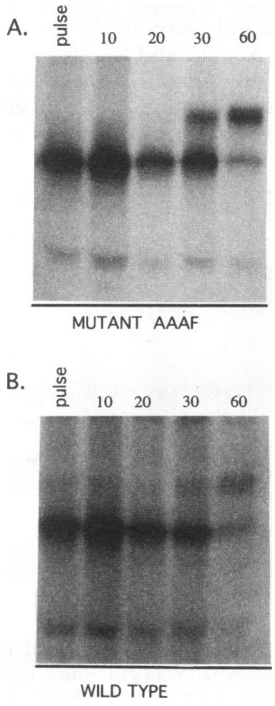

FIG. 9. Pulse-chase labeling of mutant and wild-type gpI. Transfected cells expressing either mutant AAAF (A) or wild-type gpI (B) were pulsed-labeled with [^{35}S]methionine for 15 min and then either lysed immediately or incubated in chase medium containing unlabeled methionine for 10, 20, 30, and 60 min, as indicated above the lanes. Cell lysates were precipitated with anti-gpI monoclonal antibody, and the immunoprecipitates were analyzed by SDS-PAGE (10% gel).

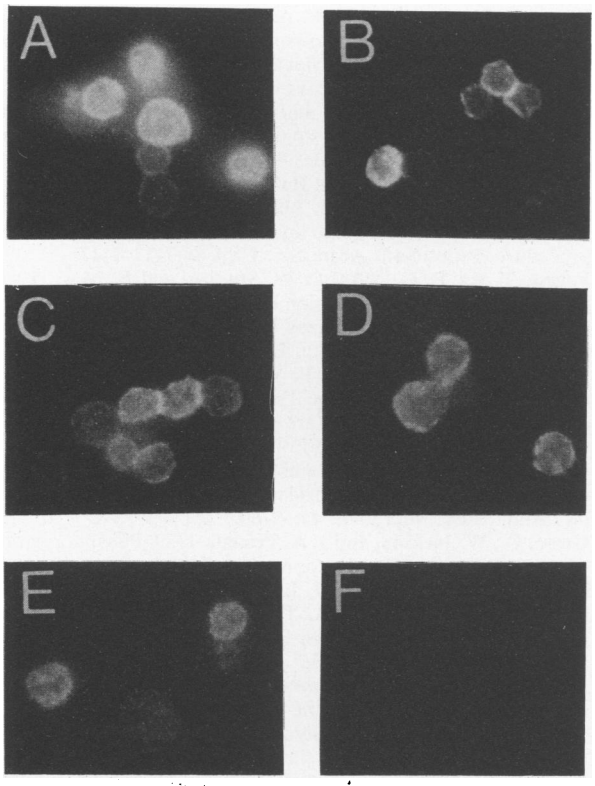

FIG. 10. Immunofluorescence analysis of wild-type and mutant gpI forms in transfected cells. HeLa cells were infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase and then transfected by lipofection with pTM1-gpI AAAF (A), SSAA (B), or SSAT (C), tailless gpI mutant DNA (D), or wild-type gpI DNA (E). Cells transfected with pTM1 vector only served as a control (F). At 14 h posttransfection, the live transfected cells were analyzed for localization of the viral glycoproteins at the cell surface by published techniques (32). Since mutant SSTA appeared identical to mutant SSAT (C), the photograph of mutant SSTA is not shown. The probe for gpI was monoclonal antibody 3B3 (23).

acids 591 to 601). We therefore sought to investigate the in vivo relevance of this observation by examining the phosphorylation of wild-type and mutated gpI in transfected cells. Truncation of the cytoplasmic tail or mutation of all four serines and threonines in one cluster decreased phosphorylation by more than 95%, a result which indicated that this domain was the major phosphorylation site of the gpI protein. Studies of additional mutants determined that threonines 596 and 598 in the cytoplasmic tail were critical for gpI phosphorylation. The similarity in phosphopeptide maps after in vivo labeling and in vitro phosphorylation of these mutants by casein kinase II strongly suggested that VZV gpI was a physiological substrate for casein kinase II.

Casein kinase II, a cyclic AMP-independent kinase, phosphorylates serine and threonine residues and leaves tyrosine residues unaffected. The specificity of casein kinase II has been well defined by studies using both protein and synthetic peptide substrates (6, 14, 28, 29). Sequences phosphorylated by casein kinase II contain serines or threonines that are followed by acidic residues (Table 1). While the most critical determinant is an acidic residue three positions to the carboxy terminus, other nearby acidic residues can improve the kinetic constants. Thus, sequences containing a Ser or

TABLE 1. Examples of sites phosphorylated by casein kinase II[a]

No.	Substrate protein	Sequence
1	Heat shock protein 90 (Ser)	KEVSDDEAEE
2	Heat shock protein 90 (Ser)	DVGSDEEDDS
3	Simian virus 40 large T antigen (Ser)	MPSSDDEATA
4	Glycogen synthase (Ser)	PHQSEDEEPR
5	Ornithine decarboxylase (Ser)	QPGSDDEDES
6	Cyclic AMP-dependent kinase RII (Ser, Ser)	DSESEDEEDL
7	Myosin light chain (Thr)	DRFTDEEVDE
8	VZV gpI (Thr, Thr)	STDTEEEFGN

[a] Examples 1 to 7 are from references 28 and 29.

Thr located within a cluster of acidic residues represent an ideal casein kinase II phosphorylation site. An examination of the amino acid sequence around threonines 596 and 598 (EDSESTDTEEE) reveals that both threonines are within an optimal casein kinase II consensus sequence ([S/T]XX[D/E]). An additional acidic residue at the N +1 position has been found to improve the phosphorylation kinetics of casein kinase II. Both threonines 596 and 598 have an Asp or Glu at the N +1 position as well as the N +3 position. Taken together, these results strongly suggest that cellular casein kinase II phosphorylates gpI at positions 596 and 598. The fact that single mutants SSAT and SSTA were phosphorylated to a lesser extent than wild-type gpI suggested that both threonines were phosphorylated in vivo.

Earlier publications suggested that casein kinase I phosphorylates serines with either glutamine or glutamate in the immediate N-terminal position (28). Recent evidence indicates that a phosphoserine in the N −3 position is an even better determinant, while a phosphoserine at N −2 may also be beneficial (30). Analysis of the consensus phosphorylation sequence suggests that serine 595 may be a phosphate acceptor for casein kinase I if serine 593 is first phosphorylated by casein kinase II. Alternatively, casein kinase I in vitro may phosphorylate a serine located outside of the consensus sequence noted above. The reason for the difference between the phosphorylation that occurs in vivo and that seen in vitro is not always clear, but such differences are often observed in other phosphorylated proteins and may represent superphosphorylation (28, 29, 31). Because the phosphopeptide maps of casein kinase I modified gpI differ from those after in vivo labeling, there is no compelling argument for or against the physiological role of casein kinase I in the phosphorylation of gpI. By the same reasoning, gpI is most likely an authentic substrate of casein kinase II because its phosphopeptide maps are the same as those following in vivo labeling. Among the true substrates of casein kinase II, this phosphorylation sequence is unusual in that two threonines are modified (29).

Gabel et al. reported that VZV glycoprotein gpI contains phosphorylated oligosaccharides (11). In that study, glycopeptides generated by pronase digestion of whole VZV glycoprotein gpI were separated on concanavalin A-Sepharose columns into three fractions corresponding to triantennary complex-type units, biantennary complex-type units, and high-mannose-type units. Each of the fractions was subsequently degraded by acid hydrolysis, and the constituent monosaccharides were separated by descending paper chromatography. The identity of the monosaccharides was determined by comparing their migration with that of three standards: mannose, mannose-6-phosphate, and fucose. When the triantennary complex-type fraction was subjected to the procedures described above, mannose-6-

phosphate was identified as one of the constituents. In our study, the tailless gpI and mutant AAAF glycoproteins were phosphorylated to about 5% of the wild-type gpI level. We considered the possibility that this small amount of phosphorylation may be due to phosphorylated oligosaccharides. To test this hypothesis, we treated the in vivo $^{32}P_i$-labeled mutant ÀAAF with *N*-glycosidase, which removes all N-linked oligosaccharides from glycoprotein. Although there was a clear shift in the molecular mass of the AAAF mutant protein after *N*-glycosidase digestion, a small amount of phosphophate was still associated with this mutant (data not shown). Thus, under our conditions of transfection and glycoprotein analysis, phosphorylated oligosaccharides were, at most, a minimal contributor to the phosphorylation profile of gpI.

Phosphorylation is known to be an important posttranslational modification of plasma membrane receptors. For these receptors, phosphorylation has been shown to be involved in endocycling and transcytosis (3, 17, 21, 25, 26). Many different receptors are phosphorylated at cytoplasmic serine or threonine residues by protein kinase C, which can be activated by phorbol esters. Receptor hyperphosphorylation induced by phorbol esters is often temporally correlated with changes in receptor distribution (2, 18), suggesting that phosphorylation can be involved in regulating receptor trafficking. For example, in the polymeric immunoglobulin receptor, serine 664, the single site of phosphorylation, is required for efficient transcytosis (1). With regard to VZV gpI, phosphorylation may play a role in the intracellular sorting of the viral glycoproteins as they exit the *trans* Golgi in vacuoles (16). Since all of the mutant forms of gpI, including the tailless gpI, are still transported to the cell surface, it is clear that phosphorylation is not essential for the process. However, phosphorylation of gpI may play a role in some aspect of the trafficking because the phosphorylation-deficient mutant AAAF appeared to be processed faster than the wild-type gpI. Another possibility is that phosphorylation plays a role in envelopment of the virion. The mechanisms by which the glycoprotein-enriched envelope is transported and attached to the nucleocapsid remain a subject of considerable dispute.

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