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Phosphorylation of Varicella-Zoster Virus Glycoprotein gpI by Mammalian Casein Kinase II and Casein Kinase I

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Varicella-zoster virus (VZV) glycoprotein gpI is the predominant viral glycoprotein within the plasma membranes of infected cells. This viral glycoprotein is phosphorylated on its polypeptide backbone during biosynthesis. In this report, we investigated the protein kinases which participate in the phosphorylation events. Under in vivo conditions, VZV gpI was phosphorylated on its serine and threonine residues by protein kinases present within lysates of either VZV-infected or uninfected cells. Because this activity was diminished by heparin, a known inhibitor of casein kinase II, isolated gpI was incubated with purified casein kinase II and shown to be phosphorylated in an in vitro assay containing [γ-^{32}P]ATP. The same glycoprotein was phosphorylated when [^{32}P]GTP was substituted for [^{32}P]ATP in the protein kinase assay. We also tested whether VZV gpI was phosphorylated by two other ubiquitous mammalian protein kinases—casein kinase I and cyclic AMP-dependent kinase—and found that only casein kinase I modified gpI. When the predicted 623-amino-acid sequence of gpI was examined, two phosphorylation sites known to be optimal for casein kinase II were observed. Immediately upstream from each of the casein kinase II sites was a potential casein kinase I phosphorylation site. In summary, this study showed that VZV gpI was phosphorylated by each of two mammalian protein kinases (casein kinase I and casein kinase II) and that potential serine-threonine phosphorylation sites for each of these two kinases were present in the viral glycoprotein.

Protein kinases have an important regulatory role in the cell, because of their ability to catalyze the transfer of the γ-phosphate of a nucleoside triphosphate to a protein substrate (5, 15; J. A. Traugh, *in* J. J. Mond, J. C. Cambier, and A. Weiss, ed., *Focus on Regulation of Cell Growth and Activation*, in press). Their presence in viruses was first described in 1971 by Strand and August (29), who were studying phosphorylation within cells infected by the Rauscher murine leukemia virus. Within the next year, protein kinase activity was observed in herpes simplex virus, equine herpesvirus, and frog polyhedral cytoplasmic deoxyribovirus (9, 26, 27). In the herpes simplex virus system, protein kinase activity was present in preparations of infectious enveloped virions and absent from purified nucleocapsids (27). Likewise, purified virions of equine herpesvirus were associated with a protein kinase activity, which catalyzed the phosphorylation of some structural viral proteins (26). A third report described a protein kinase activity that was present in enveloped frog polyhedral cytoplasmic deoxyribovirus (9).

An extensively studied viral protein kinase activity is associated with the product of the v-*src* retroviral oncogene, viz., pp60$^{v\text{-}src}$ (15). This protein is made of 526 amino acids, of which tyrosine residue 416 is autophosphorylated by the protein kinase activity of pp60$^{v\text{-}src}$ itself. There are numerous other examples of retroviral oncogenes which possess tyrosine phosphotransferase properties, while a few oncogene products exhibit serine-threonine phosphotransferase activities, e.g., Moloney murine sarcoma virus (17). Interestingly, the earlier investigators of kinases in herpesvirus-infected cultures had observed the phosphorylation of serine or threonine residues rather than tyrosine residues. This finding was supported by more recent studies of alphaherpesviruses which documented diverse serine and threonine phosphotransferase activities (1, 19, 21, 25, 28). In some instances, the activity resembled that of cellular casein kinase II (16). Yet the identity of phosphotransferase activity has rarely been confirmed by in vitro experiments with purified enzymes. Understanding the sources and roles of individual protein kinases in viral systems has become much more complicated because of the recent discovery, based largely on sequence homology data, that some of the human herpesviruses (including VZV) may encode their own protein kinase (7, 22). Thus, the phosphorylation of certain herpes-specific proteins may be catalyzed by viral protein kinases, or mammalian protein kinases, or a combination of viral and nonviral kinases. In this report, we further examine phosphotransferase activities within varicella-zoster virus (VZV)-infected mammalian cells; in particular, we investigate the phosphorylation of VZV glycoprotein gpI(98) by three purified cellular kinases: casein kinase II, casein kinase I, and cyclic AMP-dependent kinase. All three of these serine-threonine kinases are commonly found in mammalian cells.

MATERIALS AND METHODS

Cells and virus. The cell substrate was the Mewo strain of human melanoma cells (HMC) (10). Monolayer cultures were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1.0% nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 μg/ml). The stock virus for all experiments was the VZV-32 strain, which had been passed <20 times in culture (10).

Phosphorylation of gpI by cellular and viral lysates. VZV lysate prepared in RIPA buffer (10 mM Tris [pH 7.4] containing 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate [SDS]) was immunoprecipitated with VZV glycoprotein-specific monoclonal antibody (11) and protein A-Sepharose CL-4B beads (24). The

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immunoprecipitates were washed five times with phosphate-buffered saline wash buffer (10 mM sodium phosphate [pH 7.2] containing 150 mM NaCl, 0.5% Nonidet P-40, 0.5% bovine serum albumin, 0.1% SDS, and 0.2% NaN_3) and washed twice with casein kinase II buffer (50 mM Tris [pH 7.2] containing 140 mM KCl and 10 mM $MgCl_2$) (14). The final pellet was suspended in 70 μl of casein kinase II buffer and heat inactivated at 60°C for 10 min. The mixtures were then cooled on ice, and 10-μl volumes of either VZV-infected or uninfected HMC lysates prepared in casein kinase II buffer were added. Thereafter, 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mM; Amersham Corp., Arlington Heights, Ill.) was added and the mixture was incubated at 30°C for 30 min. Phosphorylation was terminated by washing five times with phosphate-buffered saline wash buffer. Protein A-bound products were eluted by incubation at 100°C for 5 min in 120 μl of sample buffer (125 mM Tris [pH 6.8] containing 6% SDS and 20% glycerol). Phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), after which the gels were dried and exposed to radiographic film. In some experiments, the protein kinase assay was carried out in the presence of various concentrations of heparin. Deoxycholate, Nonidet P-40, and heparin were purchased from Sigma Chemical Co., St. Louis, Mo. Reagents for SDS-PAGE were obtained from Eastman Kodak Co., Rochester, N.Y.

Phosphoamino acid analysis of gpI. VZV gpI, which had been phosphorylated with either VZV-infected or uninfected HMC lysate in the presence of [^{32}P]ATP, was identified on 10% polyacrylamide gels by autoradiography. The ^{32}P-labeled gpI was excised from the unfixed gel and eluted by first boiling the gel for 5 min in 1 ml of elution buffer (50 mM NH_4HCO_3 containing 0.1% SDS and 5% 2-mercaptoethanol) and then incubating it overnight at 37°C in the same buffer. 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.

Protein kinases. Mammalian casein kinase II, casein kinase I, and cyclic AMP-dependent kinase were purified by methods which have been previously described (2, 4, 5, 13; P. T. Tuazon and J. A. Traugh, *in* P. Greengard and G. A. Robinson, ed., *Advances in Second Messenger and Phosphoprotein Research*, in press).

Phosphorylation of immunoprecipitated viral glycoproteins with casein kinase II. VZV-infected HMC monolayers were dislodged into RIPA buffer, sonically disrupted, and ultracentrifuged (85,000 × *g* for 60 min) to remove insoluble macromolecules. Portions of the solubilized lysate were incubated with VZV glycoprotein-specific monoclonal antibody overnight at 4°C, and immune complexes were collected on protein A-Sepharose CL-4B beads. The immunoprecipitates were washed five times with phosphate-buffered saline wash buffer and twice with casein kinase II buffer. The final pellet was suspended in 55 μl of casein kinase II buffer, heat inactivated at 60°C for 10 min, and then cooled on ice. The phosphorylation assay was initiated by adding 18 U of casein kinase II followed immediately by a [γ-^{32}P]ATP-cold ATP mixture (specific activity of 5,000 cpm/pmol) at a final concentration of 0.14 mM ATP (14). The reaction mixture (70 μl) was incubated at 30°C for 30 min. To evaluate the effect of heparin on phosphorylation, heparin was incubated with casein kinase II for 5 min prior to adding [γ-^{32}P]ATP. The protein kinase assay was terminated by washing the immunoprecipitates five times with phosphate-buffered saline wash buffer and once with deionized, distilled H_2O. Bound proteins were eluted either by incubating the immune complexes with 60 μl of 100 mM NaCl, pH 2.0 (adjusted with concentrated HCl), and neutralized with 5 μl of 1.0 N NaOH or by boiling the complexes in 120 μl of sample buffer for 5 min. Sample buffer was added and the proteins were analyzed by SDS-PAGE and autoradiography, as described (23, 24).

Phosphorylation of immunoprecipitated viral glycoproteins with casein kinase I. Immunoprecipitates were prepared and washed as in the previous section. The final pellet was suspended in 55 μl of 50 mM Tris (pH 7.5) containing 10 mM $MgCl_2$ and either 50 mM NaCl or 140 mM KCl. After heat inactivation at 60°C for 10 min, the mixture was cooled on ice and 300 U of casein kinase I was added. After [γ-^{32}]ATP-cold ATP was added, as described with the casein kinase II assay, the mixture was incubated at 30°C for 30 min. The remainder of the procedure was identical to that described for casein kinase II.

Phosphorylation with cAMP-dependent protein kinase. The protein kinase assay was carried out with 20 mM Tris (pH 7.4) containing 10 mM $MgCl_2$ and 30 mM 2-mercaptoethanol. To each reaction, 18 U of cAMP-dependent protein kinase was added. The remainder of the procedure was the same as that described for casein kinase II.

RESULTS

Phosphorylation of VZV gpI by a cellular kinase. In a previous report, we observed that VZV glycoprotein gpI was phosphorylated when $^{32}P_i$ was added into the medium overlying a VZV-infected monolayer (23). To determine whether phosphorylation was being catalyzed by cellular or viral protein kinases, we first determined whether gpI could be phosphorylated by phosphotransferase activities present in uninfected cellular lysates as well as in infected cellular lysates. To this end, uninfected and VZV-infected HMC monolayers were harvested and lysates were prepared in a buffer system known to be optimal for certain cellular kinases. Each of the three major VZV glycoproteins (gpI, gpII, and gpIII) was isolated by immunoprecipitation with a murine monoclonal antibody, as described (11). The immunoprecipitates were subjected to heat treatment (60°C for 10 min) in order to inactivate cellular phosphotransferase activity which may have adhered to the antigen-antibody complex. Subsequently, the heat-inactivated immunoprecipitates were incubated in separate experiments with either cellular or VZV lysate as a source of phosphotransferase activity. In the case of VZV gpI, phosphorylation was observed in both instances (Fig. 1). Similar studies were carried out with immunoprecipitates containing VZV glycoproteins gpII and gpIII. In neither case was phosphorylation observed (Fig. 1). Thus, these in vitro studies confirmed our earlier observation that VZV gpI, but not gpII or gpIII, could be modified in vivo by addition of phosphoryl groups (23). In addition, they expanded the observation by showing that this reaction could be catalyzed by a cellular phosphotransferase activity. The negative results with gpII and gpIII verified the lack of nonspecifically precipitated phosphotransferase activity in the antigen-antibody complex.

Phosphoamino acid analysis of gpI. In order to determine which amino acid residues of gpI were modified, several

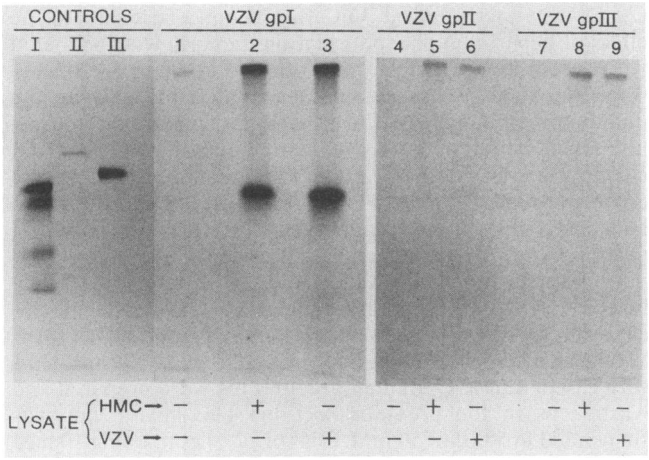

FIG. 1. In vitro phosphorylation of VZV gpI with cellular and viral lysates. Immunoprecipitates of VZV glycoproteins gpI, gpII, and gpIII were first heat inactivated and then added to protein kinase assays containing either HMC or VZV lysate as a source of phosphotransferase activity and [^{32}P]ATP as a phosphate donor. The phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Lanes: 1, 4, and 7, immunoprecipitates added to protein kinase assays with no exogenous source of phosphotransferase activity; 2, 5, and 8, immunoprecipitates added to kinase assays with cellular lysate; 3, 6, and 9, immunoprecipitates added to kinase assays with VZV-infected cell lysate (the source of phosphotransferase activity for each assay is also indicated at the bottom of lanes 1 through 9); I, II, and III, [^{3}H]fucose-labeled immunoprecipitates of VZV glycoproteins gpI, gpII, and gpIII, respectively. The VZV-specific monoclonal antibodies have been described in detail (11, 24).

samples of ^{32}P-labeled gpI were cut from polyacrylamide gels. These included bands from separate kinase experiments, in which either the cellular lysate or the infected cell lysate was used as a source of phosphotransferase activity. The extracted glycoprotein was subjected to hydrolysis followed by thin-layer electrophoresis at pH 3.3, as described (23). By comparison with the migration of the three standards, phosphoserine, phosphothreonine, and phosphotyrosine, we determined that gpI was modified on both serine and threonine residues (Fig. 2). Based on the intensity of the radiolabeling, it was apparent that the cellular and infected lysates exhibited similar phosphoserine and phosphothreonine transferase activities (Fig. 2). Phosphorylation of tyrosine residues was not observed even after a prolonged exposure of the autoradiogram.

Effect of heparin on phosphorylation of gpI. Cellular lysates are known to contain a mixture of several different protein kinases (Traugh, in press). In order to begin characterization of the cellular phosphotransferases modifying VZV gpI, we repeated both in vivo and in vitro ^{32}P labeling experiments in the presence of various amounts of heparin, a sulfated mucopolysaccharide which inhibits one of the ubiquitous serine-threonine kinases, casein kinase II (12). In an in vivo experiment, heparin in concentrations as high as 40 μg/ml was added into the medium overlying VZV-infected monolayers. After a 24-h labeling period with [^{32}P]orthophosphate, the monolayers were harvested and a detergent-solubilized antigen extract was prepared, as described (23, 24). VZV glycoproteins gpI, gpII, and gpIII were precipitated from the extracts and analyzed by SDS-PAGE and autoradiography. Even with high concentrations of heparin in the medium, phosphorylation of gpI was not inhibited, compared with a control-infected monolayer lacking heparin. The results with gpII or gpIII were also unaffected by heparin, i.e., none of the anti-gpII or anti-gpIII precipitates contained detectable bands on the autoradiogram (data not shown). In a second set of experiments, we repeated the in vitro protein kinase assays described in Fig. 1; however, we added heparin into the reaction mixture in addition to the other reagents. Increasing concentrations of heparin markedly decreased the phosphotransferase activity of the cellular lysate on the substrate VZV gpI (Fig. 3). Since most other cellular kinases are not affected by heparin, the in vitro results suggested that casein kinase II was one of the enzymes which catalyzed the phosphorylation of VZV gpI.

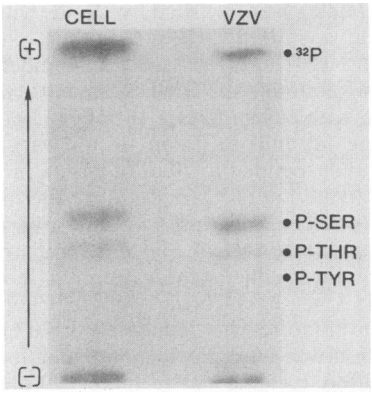

FIG. 2. Phosphoamino acid analyses of VZV gpI. VZV gpI which had been phosphorylated in vitro by either cellular (CELL) or VZV lysate in the presence of [^{32}P]ATP was localized on an unfixed 10% polyacrylamide gel by autoradiography. The ^{32}P-labeled protein was excised from the gel, acid hydrolyzed, and analyzed by thin-layer electrophoresis followed by autoradiography. Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine 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 along the right margin.

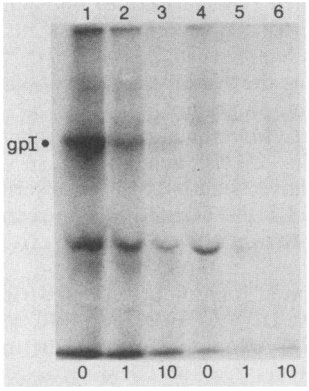

FIG. 3. Effect of heparin on in vitro phosphorylation of VZV gpI by cellular lysate. Immunoprecipitates of gpI were added to protein kinase assays containing cellular lysate as a source of phosphotransferase activity, as described in the legend to Fig. 1. One assay was carried out in the absence of heparin (lane 1), while two other assays included heparin at concentrations of either 1 (lane 2) or 10 (lane 3) μg/ml. Identical kinase assays, but without gpI immunoprecipitates, were included as negative control experiments (lanes 4 through 6). The ^{32}P-labeled samples were subjected to SDS-PAGE followed by autoradiography as shown in this and all subsequent figures. The location of VZV glycoprotein gpI is designated in the left margin, while concentrations (in micrograms per milliliter) of heparin are indicated at the bottom of each lane.

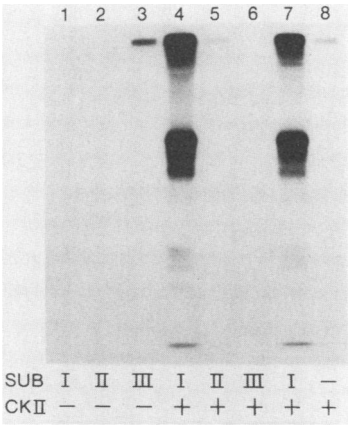

FIG. 4. In vitro phosphorylation of VZV gpI by casein kinase II with ATP as a phosphate donor. Protein kinase assays were performed with immunoprecipitates of VZV glycoproteins gpI (lanes 4 and 7), gpII (lane 5), and gpIII (lane 6) as substrates. In addition, purified casein kinase II and [^{32}P]ATP were added to the assay. Immunoprecipitates of gpI, gpII, and gpIII without casein kinase II (lanes 1 through 3) or casein kinase II alone without an immunoprecipitate (lane 8) were included as negative control experiments. The substrate (SUB) and presence (+) or absence (−) of casein kinase II (CKII) are also indicated at the bottom of each lane.

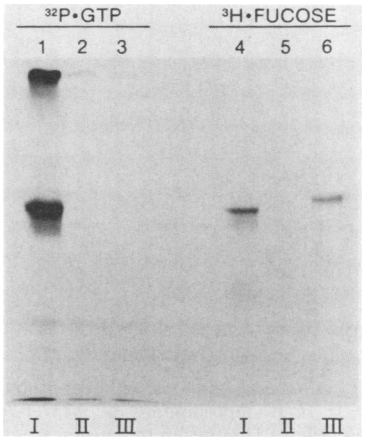

FIG. 5. In vitro phosphorylation of VZV gpI by casein kinase II with GTP as a phosphate donor. Immunoprecipitates of gpI (lane 1), gpII (lane 2), and gpIII (lane 3) were added to protein kinase assays, in addition to casein kinase II and [^{32}P]GTP. The [^{3}H]fucose-labeled immunoprecipitates of VZV gpI, II, and III were subjected to identical conditions of SDS-PAGE (lanes 4 through 6, respectively).

On the other hand, the data in the in vivo experiments suggested that the action of heparin was not mediated by any effect on the cell surface.

Casein kinase II phosphorylation of VZV gpI. The phosphorylation of the VZV glycoproteins was next evaluated in a series of protein kinase assays performed with purified casein kinase II. Because the Michaelis-Menton constant (K_m) of casein kinase II for ATP is 5 to 15 μM (Tuazon and Traugh, in press), the amounts of ATP in the reaction mixture, including both radiolabeled and unlabeled ATP, were adjusted to at least 140 μM to provide a 10-fold excess of nucleotide. Each detergent-solubilized VZV glycoprotein was precipitated with specific monoclonal antibody, washed with casein kinase II buffer, and heat inactivated. The kinase assay was initiated by adding [γ-^{32}P]ATP, unlabeled ATP, and casein kinase II to the immunoprecipitated glycoprotein. The VZV gpI species gp98 and gp88 were selectively phosphorylated by the protein kinase (Fig. 4). In control reactions, gpI was omitted in one case and the enzyme was omitted in the second case; in neither instance was phosphorylation observed. Likewise, VZV gpII and gpIII were not phosphorylated by casein kinase II. In addition to the above-mentioned experiments, we tested whether casein kinase II would catalyze the phosphorylation of VZV gpI when the in vitro reaction was carried out in other buffered solutions, often containing detergents, as described in earlier reports (23). Phosphorylation of gpI was still observed, but the intensity of labeling was markedly diminished (autoradiogram not shown).

Among the cellular serine-threonine kinases, casein kinase II has a distinctive property, i.e., it can utilize GTP as well as ATP as a phosphate donor (19a; Tuazon and Traugh, in press). Although the K_m values for GTP are 15 to 40 μM, about two- to four-fold higher than those for ATP, the values are still relatively low. Therefore, the phosphorylation assays with purified casein kinase II were repeated in the presence of GTP rather than ATP. In this second set of experiments, gpI was equally well phosphorylated with [^{32}P]GTP as the source of phosphate (Fig. 5). Control phosphorylation experiments with other VZV glycoproteins did not demonstrate any transfer of γ-phosphate. The radiolabeled gpI was subjected further to phosphoamino acid analysis by thin-layer electrophoresis, as described above. The modified amino acids included both serine and threonine, with threonine being more prominent than serine. Thus, this set of experiments verified that VZV gpI behaved like a usual substrate of casein kinase II.

Heparin inhibition of casein kinase II activity. In this report, we have previously shown that heparin interfered with the phosphorylation of gpI when the assay was carried out with cellular lysate as a source of phosphotransferase activity. To prove that this inhibition was caused mainly by an effect on casein kinase II, the experiment was repeated with purified enzyme. For these experiments, heparin at concentrations ranging from 1 to 10 μg/ml was added to immunoprecipitated, heat-inactivated gpI and the kinase assay was initiated by adding [γ-^{32}P]ATP. A considerable decrease in phosphorylation of gpI was observed at a heparin concentration of 1.0 μg/ml, while casein kinase II activity was completely abrogated at 10 μg of heparin per ml (Fig. 6). These results clearly illustrated the inhibitory effect of heparin on the phosphorylation of gpI by casein kinase II.

Casein kinase I phosphorylation of VZV gpI. In order to determine whether other common cellular protein kinases catalyze the phosphorylation of the VZV glycoprotein gpI, we first selected casein kinase I for evaluation. Although sharing a similar name with casein kinase II, the enzymes are completely distinct and unrelated species (5; Tuazon and Traugh, in press). In contrast to casein kinase II, casein kinase I is specific for ATP, with K_m values from 7 to 25 μM, and it does not utilize GTP (5; Tuazon and Traugh, in press). In a manner similar to that described above for the casein kinase II experiments, immunoprecipitates of the VZV glycoproteins were placed in a reaction mixture with casein kinase I and [γ-^{32}P]ATP. When the precipitates were subsequently analyzed by SDS-PAGE and autoradiography, gpI was observed to be highly phosphorylated (Fig. 7), while the other VZV glycoproteins lacked any radioactivity. Casein was included as a positive control reagent in the kinase assay (Fig. 7). As an additional control experiment, the reaction

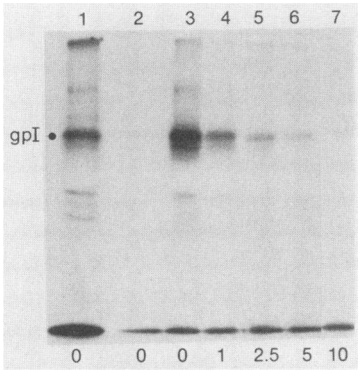

FIG. 6. Effect of heparin on in vitro phosphorylation of VZV gpI by casein kinase II. Immunoprecipitates of gpI were phosphorylated in vitro by casein kinase II either in the absence of heparin (lane 3) or in the presence of increasing concentrations of heparin ranging from 1 to 10 μg/ml. (Heparin concentrations are indicated at the bottom of each lane.) In vitro experiments in which gpI immunoprecipitates were incubated with cellular lysate (lane 1) or without any source of phosphotransferase activity (lane 2) were included as control experiments. The autoradiogram was also analyzed by laser densitometry. Compared with the amount in lane 3, the relative amounts of ^{32}P-labeled VZV gpI in lanes 4 through 7 were 29, 10, 6, and 0%, respectively.

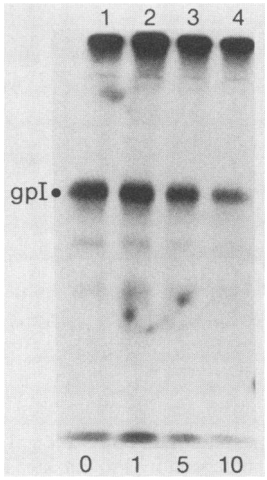

FIG. 8. Effect of heparin on the in vitro phosphorylation of VZV gpI by casein kinase I. Immunoprecipitates of gpI were phosphorylated in vitro by casein kinase I in the absence of heparin (lane 1), as described in the legend to Fig. 7, and with heparin concentrations of 1 (lane 2), 5 (lane 3), and 10 (lane 4) μg/ml. Four exposures of the autoradiogram were analyzed by densitometry. Compared with lane 1, the relative amounts of ^{32}P-labeled VZV gpI in lanes 2, 3, and 4 were 100, 73, and 51%, respectively.

was carried out with only casein kinase I and radiophosphate in order to demonstrate the absence of nonspecifically labeled proteins; under these conditions, no phosphorylation was observed. The effect of different salt concentrations on casein kinase I-mediated phosphorylation was also evaluated. With VZV gpI as a substrate, 50 mM NaCl in the kinase assay was preferable to 140 mM KCl. These results are compatible with the prior observation that the optimal concentration of monovalent cations must be determined for each individual substrate (Tuazon and Traugh, in press).

To further evaluate gpI phosphorylation by casein kinase I, we performed two additional experiments. In the first, the protein kinase assays were repeated in the presence of heparin. The phosphorylation of gpI by casein kinase I was not inhibited by heparin at a concentration of 1 μg/ml (Fig. 8). At the highest concentration of 10 μg/ml, there was a 49% reduction in extent of phosphorylation. In the second experiment, we analyzed the ^{32}P-labeled gpI by thin-layer electrophoresis. As expected with casein kinase I, virtually all of the modified residues were serine and not threonine (autoradiogram not shown). These two experiments confirm that casein kinase I-mediated phosphorylation of VZV gpI is similar to that observed with other substrates (Tuazon and Traugh, in press).

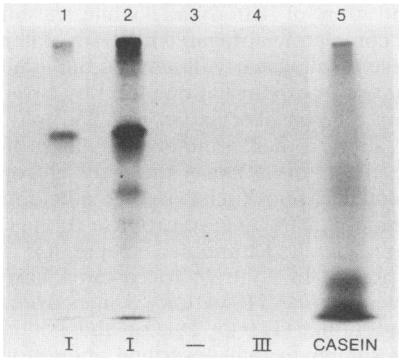

FIG. 7. In vitro phosphorylation of VZV gpI by casein kinase I. Immunoprecipitates of gpI were phosphorylated in vitro by casein kinase I in a buffer containing either 140 mM KCl (lane 1) or 50 mM NaCl (lane 2) and [^{32}P]ATP. Protein kinase assays were also performed without an immunoprecipitate of gpI but with enzyme (lane 3), as well as with an immunoprecipitate of gpIII and casein kinase I (lane 4). In addition, casein was included in a protein kinase assay with casein kinase I as a positive control (lane 5). The substrates are indicated at the bottom of each lane.

Cyclic AMP-dependent protein kinase phosphorylation. As a second alternative cellular kinase, we chose cyclic AMP-dependent protein kinase. This enzyme is normally found in most cell types; it exhibits a strong preference for serine over threonine as a phosphorylation site (5). As described previously, individually precipitated VZV glycoproteins were tested in phosphotransferase assays in which cyclic AMP-dependent protein kinase was added. No phosphorylation of the VZV glycoproteins was demonstrated (Fig. 9). As a control experiment, histones, a substrate of cyclic AMP-dependent kinase, were added to one assay with positive results. Thus, this last experiment demonstrated the selective nature of the phosphorylation of VZV gpI by cellular kinases, that is, the viral glycoprotein was a substrate for phosphorylation catalyzed by two cellular serine-threonine kinases, but it was not a substrate recognized by cyclic AMP-dependent phosphotransferase.

DISCUSSION

In 1986, we first reported that one of the VZV glycoproteins (gpI; gp98) was phosphorylated under in vivo labeling conditions (23). This observation was confirmed by Edson et al. (6), who also showed that herpes simplex virus glycoprotein gE, the homolog of VZV gpI, was similarly phosphorylated. Prior to these reports, phosphorylation had not been recognized as a usual modification of herpesviral glycoproteins and its function remains unknown. In this report, the nature and specificity of the phosphorylation event involving VZV gpI are further characterized, viz., at least two cellular

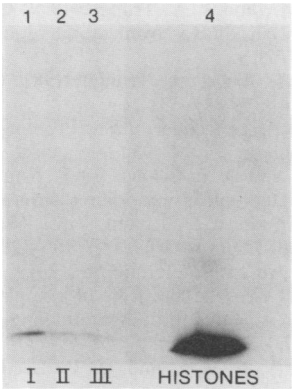

FIG. 9. In vitro phosphorylation with cyclic AMP-dependent protein kinase. Protein kinase assays were performed with immunoprecipitates of gpI (lane 1), gpII (lane 2), and gpIII (lane 3), together with cyclic AMP-dependent protein kinase and [^{32}P]ATP. Histones (lane 4) were included as a substrate in one assay as a positive control. Conditions of SDS-PAGE were the same as previously described; under these conditions, histones migrated near the dye front in lane 4.

enzymes, casein kinases I and II, catalyze the phosphorylation of gpI while a third protein kinase (cyclic AMP-dependent kinase) does not. Of interest, there is another example of a viral protein known to be phosphorylated by both casein kinase I and casein kinase II; that protein is the large T antigen encoded by the papavovirus simian virus 40 (8). The large T antigen is a multifunctional early gene product which has a role in regulation of viral DNA replication. Large T antigen is also intricately involved in two other viral functions: neoplastic transformation of cultured cells and tumor induction in animals. That T antigen is a phosphoprotein has been known for some time; however, the exogenous kinases responsible for the transfer of phosphate residues to the viral protein have been delineated only very recently (8). In brief, when large T antigen is incubated with casein kinase I and [^{32}P]ATP, residues Ser-123, possibly Thr-124, and either Ser-676, Ser-677, or Ser-679 are phosphorylated. In a similar assay with casein kinase II as a source of phosphotransferase activity, Ser-106 and possibly Ser-112 are modified. Taken together, the prior studies of phosphorylation of simian virus 40 T antigen as well as this report suggest that mammalian serine-threonine phosphotransferases contribute to the posttranslational modifications of both structural (gpI) and nonstructural (T antigen) proteins of mammalian viruses.

Both casein kinase I and casein kinase II phosphorylate serine and threonine residues and leave tyrosine residues unaffected. Although both enzymes preferentially phosphorylate serine over threonine, this selection is more pronounced for casein kinase I. In addition to the phosphorylatable amino acids, other residues in the immediate environment help define the specific phosphorylation site for each kinase. In the case of casein kinase II, a cluster of acidic amino acids on the C-terminal side of the serine define such a site (5; Tuazon and Traugh, in press). Of particular importance is a glutamic acid or an aspartic acid in the n+3 position in the acidic cluster on the C-terminal side; for example, when the glutamate residue at this position is substituted with alanine, the site is no longer functional. Although desirable, acidic amino acids at sites n+1 and n+2 appear to be less important than those at the n+3 position. On the N-terminal side, acidic amino acids are not required but may enhance the likelihood of phosphorylation. Taken together, these data suggest that the sequence Ser(Thr)-X-X-Asp(Glu) may represent a typical substrate recognition site for casein kinase II (Traugh, in press; Tuazon and Traugh, in press). Similar criteria have not been as well established for the phosphorylation site of casein kinase I. The latter enzyme seems to prefer serine residues which have a glutamate or glutamine residue on the N-terminal side in the n+2 position (Tuazon and Traugh, in press). Based on the experience with casein kinase II, other restrictions on the phosphorylatable site of casein kinase I probably will be identified. For example, in simian virus 40 large T antigen, Ser-123 and possibly Thr-124, in the sequence Ser-Gln-His-Ser-Thr-Pro, have been identified as phosphorylation sites for casein kinase I under in vitro labeling conditions (8).

Since the sequence of the entire VZV genome has been determined (3), the predicted 623-amino-acid sequence of VZV gpI (gene 68) can be inspected for potential phosphorylation sites by casein kinase I or II. Of great interest is the region near the glycoprotein carboxy terminus beginning at amino acid 590: Phe-Glu-Asp-Ser-Glu-Ser-Thr-Asp-Thr-Glu-Glu-Glu-Phe. Each of the two threonine residues is followed by a glutamate in the n+3 location, which is an ideal construct for phosphorylation by casein kinase II, especially with acidic residues in the immediate vicinity N-terminal to the amino acid to be modified. Another possible phosphorylation site for casein kinase II is serine 175 in the sequence Ser-Val-Glu-Glu. In the latter example, a glutamate is located in the n+3 position following a serine residue. When the gpI sequence is analyzed for potential casein kinase I phosphorylation sites, i.e., serines with either glutamate or glutamine in the N-terminal n+2 position, several examples are easily identified throughout the polypeptide. However, of extraordinary interest, the two best phosphorylation sites for casein kinase I are both located adjacent to the potential casein kinase II sites. They include one site near the C terminus beginning at amino acid 591 (Glu-Asp-Ser) and a second site beginning at amino acid 173 (Glu-Val-Ser).

Based on our results with thin-layer chromatographic analysis of the phosphorylated amino acids of gpI, it appears that more than one threonine and at least one serine residue are modified by casein kinase II in the in vitro experiments. Therefore, all three of the aforementioned potential phosphorylation sites probably are utilized. Furthermore, the approximately equal ratio of phosphoserine and phosphothreonine found on gpI phosphorylated under in vivo conditions shows that at least one protein kinase in addition to casein kinase II is involved, presumably casein kinase I. In this regard, an important concept is that protein kinases may behave as team players, i.e., phosphorylation by one phosphotransferase may require prior phosphorylation by a second or third enzyme (Traugh, in press; Tuazon and Traugh, in press). In particular, the phosphorylation of several substrate proteins by casein kinase II is known to regulate their activity, often by potentiating their subsequent phosphorylation by a second protein kinase (5, 19a). VZV gpI is especially intriguing because of the two domains within this glycoprotein which contain potential phosphorylation sites for both casein kinases I and II. Another kinase, that is, a VZV-induced enzyme, also appears to be involved in the phosphorylation of VZV gpI and, therefore, may constitute a third team player (11, 23). Further clarification of the role of the latter kinase await its purification and characterization, studies which are now in progress (C. Grose, unpublished data). A protein kinase encoded by the porcine pseudorabies virus, another alphaherpesvirus, has been pu-

rified, but its physiological substrates and functions within the infected cell have not yet been identified (20). With regard to VZV gpI, we speculate that phosphorylation plays a role in the intracellular sorting of the viral glycoproteins as they exit the trans Golgi in vacuoles (18). These large cytoplasmic vacuoles, which are found only in infected cells, contain VZV gpI in their outer membrane.

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