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Biochemistry

Varicella zoster virus glycoprotein gpI is selectively phosphorylated by a virus-induced protein kinase

(protein phosphorylation/viral glycoproteins/glycoprotein processing)

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ABSTRACT **Varicella zoster virus glycoprotein I (VZV gpI; M_r 98,000) was phosphorylated in virus-infected human cell monolayers, while two other major VZV glycoproteins (gpII and gpIII) were not similarly modified. Phosphorylation of VZV gpI was not blocked by inhibitors of glycosylation, nor were the phosphoryl groups enzymatically removed by endoglycosidases. Phosphoamino acid analysis revealed the presence of phosphoserine and phosphothreonine residues on the polypeptide backbone. The selective nature of the phosphorylation event was further demonstrated *in vitro* by a protein kinase (M_r 50,000), which was present in virus-infected cells but absent from uninfected cells or purifed virions. The enzyme catalyzed the transfer of $^{32}P_i$ from [γ-^{32}P]ATP to gpI but not to gpII and gpIII. Like VZV gpI, this virus-induced protein kinase was also a constituent of the plasma membrane of live VZV-infected cells.**

Varicella zoster virus (VZV) is the etiologic agent of chickenpox, a common childhood exanthem. After primary infection, the virus remains latent in the dorsal root ganglia until reactivation later in life results in the disease zoster (shingles). Following chickenpox, a humoral immune response develops that is primarily directed toward the viral glycoproteins (1). These structural products include three major species, which have been recently renamed VZV gpI, gpII, and gpIII (2); all of these viral glycoproteins were identified by panels of murine monoclonal antibodies. Previously, the same glycoproteins were designated in our laboratory by their estimated molecular weights ($\times 10^{-3}$), respectively, as the gp98/gp88/gp62 complex, the disulfide-linked gp140/gp66 complex, and gp118 (3–5). These viral glycoproteins, which are synthesized in the juxtanuclear complex of rough endoplasmic reticulum and Golgi, appear to be incorporated within cytoplasmic vacuoles, which serve as a site for viral envelopment within the infected cell (6, 7).

The predominant glycoprotein constituent of the VZV-infected human cell membrane, whether in the living host or cultured cells, is gpI (8). The mature viral product is a M_r 98,000 species, which contains both N-linked and O-linked glycans on a M_r 73,000 polypeptide backbone (5). By the use of inhibitors of glycosylation, as well as exoglycosidases and endoglycosidases, several intermediates in the biosynthesis of the mature glycoprotein have been identified; these include the high-mannose form (gp81), the O-linked form (gp88), and the desialated penultimate product (gp90). In this communication, we investigated whether phosphorylation can occur as yet another posttranslational modification of VZV glycoproteins. Although earlier reports have described protein kinases induced by other herpesviruses, such as herpes simplex virus type 1 (9), pseudorabies virus (10), and human cytomegalovirus (11), they have not focused on the phosphorylation of virus-specific glycosylated gene products. Herein we describe the selective phosphorylation of a structural herpesviral (VZV) glycoprotein by a virus-induced protein kinase.

MATERIALS AND METHODS

Cells and Virus. Monolayer cultures of human melanoma cells (Mewo strain) were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum/0.002 M glutamine/1% nonessential amino acids/penicillin (100 units/ml)/streptomycin (100 μg/ml). Stock virus for all experiments was the VZV-32 strain (3), which has been passaged <20 times in cell culture.

Intrinsic Radiolabeling. The conditions for VZV infection of human melanoma cell monolayers have been described in detail (3, 7). For *in vivo* radiolabeling experiments, the culture medium in a 25-cm^2 monolayer was replaced by medium containing 1 mCi (1 Ci = 37 GBq) of [^{32}P]orthophosphate at 12 hr postinfection. The infected monolayers were incubated at 32°C for an additional 24 hr and were harvested by scraping the cells into 1 ml of RIPA buffer [0.15 M NaCl/0.1 M Tris·HCl, pH 7.4/1% deoxycholate/1% Nonidet P-40 (NP-40)/0.1% $NaDodSO_4$]. Cells were then sonically disrupted for 1 min and the lysates were further cleared by centrifugation at 85,000 × *g* for 1 hr. The technique for purification of VZV has been described in detail (12). Briefly, infected cell monolayers (75 cm^2) were transferred into 2.5 ml of 0.05 M Tris·HCl/0.15 M sodium chloride, pH 7.4, containing 0.01% gelatin (wt/vol) and 0.001 M EDTA. Purified virions were isolated from sonically disrupted infected cells by centrifugation in linear metrizamide density gradients (12). Radiolabeled infected cell lysates and virion preparations were analyzed by $NaDodSO_4$/PAGE under both reducing and nonreducing conditions (3, 4).

Monoclonal Antibodies to the Viral Glycoproteins and a Protein Kinase. Immunization and screening procedures for production of anti-VZV hybridoma cultures have been described earlier by workers in this laboratory (4, 5). In the above studies, we defined antibodies with reactivities to the viral glycoproteins. In this report, we identified a monoclonal antibody (no. 294) that precipitated a virus-induced polypeptide with protein kinase activity.

Protein Kinase Assay. Immunoprecipitates of antibody-bound protein kinase were washed six times with phosphate-buffered saline (PBS, pH 7.4) containing 0.5% NP-40, 0.1% $NaDodSO_4$, 0.1% bovine serum albumin, and 0.2% sodium azide and twice with kinase buffer (0.05 M magnesium acetate/0.05 M Tris·HCl, pH 8.0/0.001 M dithiothreitol/0.1% NP-40). The pellets were suspended in 50-μl volumes and the phosphorylation assays were initiated by the addition of 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). After

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Abbreviations: VZV, varicella zoster virus; gp, glycoprotein; NP-40, Nonidet P-40; endo F, endo-β-*N*-acetylglucosaminidase F.

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the reaction mixture was incubated at 37°C for 30 min, kinase activity was stopped by the addition of 10 μl of 0.3 M EDTA. The pellets were then washed three times with PBS and proteins were eluted by boiling for 5 min in sample buffer [0.125 M Tris·HCl, pH 6.8/6% $NaDodSO_4$/20% (vol/vol) glycerol] with or without the reducing agent 10% 2-mercaptoethanol. When phosphorylation assays were performed on purified virions, pelleted virions were suspended in 500 μl of kinase buffer, and a 50-μl aliquot was added to immunoprecipitated enzyme. After termination of the kinase assay, antibody–antigen complexes were removed by centrifugation and viral proteins were precipitated from the supernatant with cold 20% trichloroacetic acid. Precipitated proteins were washed three times with cold 50% ethanol/50% ether before suspension in sample buffer.

Digestion of gpI with Endoglycosidases. The method for digestion with endo-β-*N*-acetylglucosaminidase F (endo F) has been described (13). Briefly, gpI immunoprecipitated with specific monoclonal antibody and protein A-Sepharose beads was washed three times with PBS (pH 8.6) containing 0.1% $NaDodSO_4$, 0.1% bovine serum albumin, 0.5% NP-40, and 0.2% sodium azide. The pellets were then suspended in 0.01 M sodium phosphate buffer (pH 6.1) containing 1% 2-mercaptoethanol, 1% NP-40, 0.1% $NaDodSO_4$, 0.05 M EDTA, 0.002 M phenylmethylsulfonyl fluoride, and boiled for 2 min. After the samples were allowed to cool, 2.5 units of endo F (New England Nuclear) or an equivalent amount of water was added to each sample. Reactions were allowed to proceed for 24 hr at 37°C. For digestion with endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), immunoprecipitated gpI pellets were washed three times as described above and then suspended in 100 μl of 0.02 M Tris maleate buffer (pH 6.0) containing 0.1% $NaDodSO_4$, 1% NP-40, 0.01 M D-galactono-γ-lactone, 0.001 M calcium acetate, and 0.002 M phenylmethylsulfonyl fluoride. Neuraminidase (0.1 unit) from *Clostridium perfringens* (Sigma) or an equivalent amount of water was added to samples, which were then incubated at 37°C for 1 hr. After neuraminidase treatment, 5 units of *O*-glycanase (Genzyme, Boston, MA) or an equivalent volume of water was added and the reaction mixtures were incubated at 37°C for 24 hr.

Surface Iodination and Membrane Isolation Techniques. Monolayers (25 cm^2) of 1-day-old virus-infected cells were rinsed three times with PBS (pH 7.4) and once with 0.1 M sodium phosphate buffer (pH 7.0). Thereafter, the cells were suspended in 0.1 M sodium phosphate buffer to which was added two Iodobeads (Pierce). Iodination of surface proteins was initiated by the addition of 1 mCi of carrier-free ^{125}I (Amersham), and the reaction was allowed to proceed for 10 min in an ice bath. After removal of the Iodobeads, the cells were washed six times by repetitive suspension in PBS followed by centrifugation at 500 rpm in a CRU-5000 centrifuge to recover cells. After the sixth wash, the cells were sonically disrupted for 10 sec and suspended in 20 ml of PEG in 0.5 M NaCl. Purified membrane preparations were then prepared by described centrifugation techniques (12).

RESULTS

Phosphorylation of the VZV Glycoproteins. In a previous investigation of the VZV phosphoproteins, we described a phosphorylated p32/p36 complex that is present in both the nuclear matrix of the VZV-infected cell as well as the viral nucleocapsid (14). Because these earlier studies suggested that other viral proteins may be phosphorylated, we examined whether VZV glycoproteins undergo this type of post-translational modification. To this end, detergent-solubilized extracts were prepared from virus-infected cells intrinsically labeled with [^{32}P]orthophosphate for use in immunoprecipitation analyses. The monoclonal antibodies selected for these experiments were reactive with either the gpI, gpII, or gpIII family of VZV glycoproteins (2). As shown in Fig. 1, only monoclonal antibody to one of the glycoprotein groups precipitated ^{32}P-labeled VZV proteins—that reactive antibody bound to the VZV gpI family (lane C). We have previously shown that this monoclonal antibody, designated 3B3, precipitates four fucosylated species of M_r 98,000, 88,000, 62,000, and 45,000 (5). The gp98 and gp88 species represent two different glycosylated forms of the same protein, whereas the relationship to gp62 and gp45 remains unresolved. Only gp98 and gp88 were detected in ^{32}P-labeled immunoprecipitates. Since monoclonal antibodies to gpII and gpIII failed to precipitate any ^{32}P-labeled products, these results suggested that selective phosphorylation of gpI was occurring in VZV-infected cells.

Effect of Inhibitors of Glycosylation and Glycosidase Treatment of gpI. It has recently been shown that lysosomal enzymes undergo mannosyl phosphorylation in the cis Golgi and that this posttranslational modification represents a signal for intracellular trafficking of these products (reviewed in ref. 15). We therefore examined the glycomoieties of gpI for the presence of phosphorylated residues. We first tested whether two inhibitors of glycosylation, tunicamycin and monensin, would interfere with the transfer of phosphate groups onto gpI. Tunicamycin blocks the synthesis of *N*-acetylglucosamine pyrophosphoryldolichol carrier (16) and thereby inhibits all asparagine-linked glycosylation within the rough endoplasmic reticulum (17–19), whereas monensin has been shown to prematurely terminate the processing of asparagine-linked oligosaccharides and the addition of serine/threonine-linked glycans, probably in the trans portion of the Golgi (20, 21). As shown in Fig. 2, treatment of the infected cell cultures with the two different inhibitors of

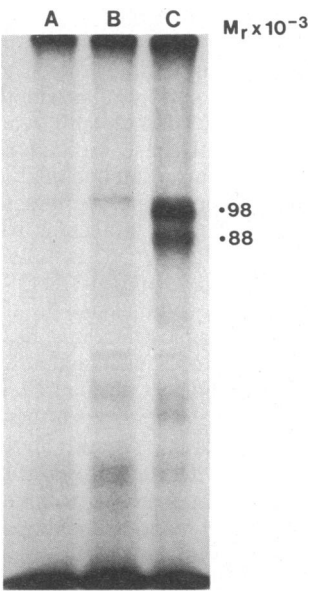

FIG. 1. Immunoprecipitation of ^{32}P-labeled viral antigen with monoclonal antibodies reactive with VZV glycoproteins. VZV-infected cells were grown in medium containing 1 mCi of [^{32}P]orthophosphate per 25-cm^2 monolayer. ^{32}P-labeled virus-infected cell lysates were solubilized and aliquots were incubated with anti-gpIII (lane A), anti-gpII (lane B), or anti-gpI (lane C) monoclonal antibody. The antigen–antibody complexes were precipitated with protein A-Sepharose beads. Eluted antigen was electrophoresed on a $NaDodSO_4$/10% polyacrylamide slab gel, and autoradiography was carried out by exposing the dried gel to Kodak XAR-5 film using a Kronex intensifying screen. The molecular weights of the major viral proteins designated in lane C have been previously determined by this laboratory (3, 12). The faint bands in lanes A and B represent nonspecifically precipitated phosphoproteins.

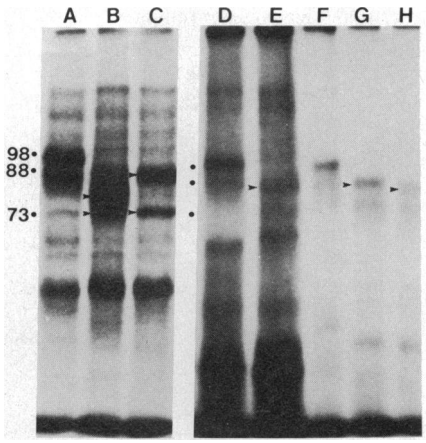

FIG. 2. Effect of glycosylation inhibitors and glycosidases on gpI phosphorylation. VZV-infected cells were grown in medium containing [^{32}P]orthophosphate. Cell lysates were prepared and immunoprecipitation experiments with anti-gpI monoclonal antibody were carried out. Eluted antigen was fractionated by electrophoresis through a NaDodSO$_4$/10% polyacrylamide slab gel and analyzed by autoradiography. Lanes: A, M_r 98,000 gpI synthesized in the absence of inhibitors of glycosylation; B, lower molecular weight precursors synthesized in the presence of 1 μM monensin (arrows); C, M_r 88,000 glycosylated intermediate and M_r 73,000 unglycosylated species synthesized in medium containing tunicamycin (2.5 μg/ml) (arrows); D, phosphorylated gpI prior to digestion with endo F; E, product generated after digestion of gpI with endo F (arrow), showing a lower molecular weight phosphorylated species; F, phosphorylated gpI prior to digestion with endo-α-*N*-acetylgalactosaminidase (*O*-glycanase); G, product generated after digestion of gpI with neuraminidase, showing a phosphorylated species (arrow) of M_r 90,000; H, phosphorylated polypeptide detected after digestion of gpI with neuraminidase and *O*-glycanase (arrow). Samples in lanes A–C and D–H were analyzed in separate slab gels.

glycosylation failed to prevent phosphorylation of gpI. In tunicamycin-treated samples, both the p73 protein, which has been previously shown to represent the unglycosylated precursor to gpI, and the gp88 intermediate form were phosphorylated (lane C). Similarly, the partially processed lower molecular weight glycoproteins in monensin-treated cultures were phosphorylated (lane B).

To confirm and expand on the above results, gpI was digested with two endoglycosidases: endo F, which hydrolyzes both high mannose and complex-type N-linked oligosaccharides (22), and endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), which cleaves O-linked chains from glycoproteins (23). The effects of these two endoglycosidases as well as neuraminidase on the ^{32}P-labeled gpI are shown in Fig. 2 (lanes D–H). The intensity of the glycoprotein bands in the enzyme-treated samples was virtually the same as that in the untreated controls—i.e., deglycosylation had little effect on the degree of phosphorylation. These results, together with the tunicamycin and monensin data, suggested that the phosphoryl groups were attached to the polypeptide backbone of the viral glycoprotein.

Phosphoamino Acid Analysis of gpI. Since gpI was presumably not phosphorylated on its glycans, we carried out an analysis of the modified amino acid residues of this glycoprotein. For this study, ^{32}P-labeled gpI(98) was localized in and excised from NaDodSO$_4$/polyacrylamide gels. After elution and concentration, the proteins were hydrolyzed and subjected to thin-layer chromatography (24). Phosphorylated amino acids were visualized by autoradiography. The results of these experiments (Fig. 3) revealed that gpI was predominantly phosphorylated at serine residues. In addition, a lesser amount of phosphothreonine was detected. However, no phosphorylated tyrosine residues were observed in gpI.

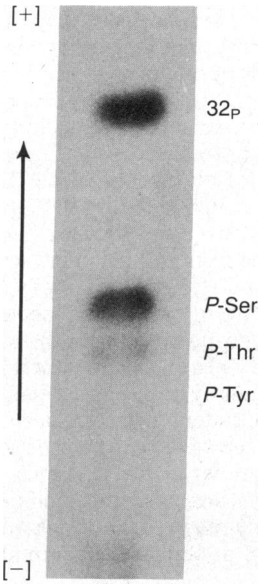

FIG. 3. Phosphoamino acid analysis of gpI. Viral polypeptide gp98 was excised from unfixed NaDodSO$_4$/polyacrylamide gels and eluted in 0.05 M NH_4HCO_3, pH 8.0/0.1% NaDodSO$_4$/5% 2-mercaptoethanol. Eluted protein was precipitated with cold 20% trichloroacetic acid and washed three times with a cold solution of 50% ethanol/50% ether. The proteins were hydrolyzed by resuspending them in a small volume of 6 M HCl at 110°C for 45 min. After the hydrolysates were mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine, they were separated by thin-layer chromatography at pH 3.5 and localized by autoradiography (24). The unlabeled markers were visualized with 0.2% ninhydrin in acetone.

These results are consistent with the phosphoamino acid analyses performed on other phosphoproteins found in herpesvirus-infected culture (11, 25–27).

Phosphorylation of gpI by a VZV-Induced Protein Kinase. Through the use of hybridoma technology, we have recently found a monoclonal antibody (no. 294) that binds to a polypeptide with protein kinase activity (see below). When tested by immunoblot analysis, antibody 294 was reactive with a M_r 50,000 protein, designated p50, found in virus-infected cells (Fig. 4, lane A). In contrast, p50 could not be detected by antibody 294 in either uninfected cells or in purified virion preparations (lanes B and C). We subsequent-

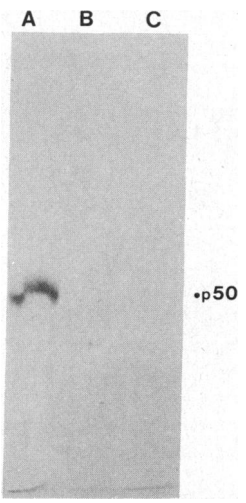

FIG. 4. Immunoblot analyses of VZV-infected cells, uninfected cells, and purified virions. Virus-infected cells, uninfected cells, and purified virions were fractionated by electrophoresis through a NaDodSO$_4$/10% polyacrylamide gel and transferred onto nitrocellulose sheets. The replicas were subsequently incubated with monoclonal antibody 294 (anti-protein kinase) followed by a second incubation with peroxidase-labeled rabbit anti-mouse antibody. After washing, color development was effected by the addition of 4-chloro-1-naphthol and 30% hydrogen peroxide. A M_r 50,000 protein is bound by monoclonal antibody in virus-infected cells (lane A), while monoclonal antibody does not recognize any products found in uninfected cells or purified virions, respectively (lanes B and C). The molecular weight standard proteins used to determine the size of the protein reactive with monoclonal antibody 294 were cytochrome *c* (12,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (93,000), and β-galactosidase (116,000).

ly investigated the ability of p50 to phosphorylate the individual VZV glycoproteins. In a series of immunoprecipitation experiments, each of the viral glycoproteins was isolated alone and then in combination with the virus-induced protein kinase p50. The single and dual precipitates were added to phosphorylation assays to determine whether $^{32}P_i$ was transferred from [γ-^{32}P]ATP to an acceptor glycoprotein. The results from these experiments are shown in Fig. 5; VZV gpI was phosphorylated *in vitro* by the virus-induced kinase (lane F), whereas gpII and gpIII did not incorporate ^{32}P under these experimental conditions (lanes G and H). When antibody to gpI was used alone to precipitate antigen, gpI was not labeled in the protein kinase assay, which also did not contain the p50 kinase protein (lane B). Similarly, gpII and gpIII failed to become phosphorylated when singularly precipitated (lanes C and D). These results were consistent with the *in vivo* experiments, which demonstrated selective phosphorylation of gpI in VZV-infected cultures grown in the presence of [^{32}P]orthophosphate. We have also analyzed the modified amino acids of *in vitro* phosphorylated gpI. Like the *in vivo* product, phosphorylation occurred at serine and threonine residues (results not shown).

Detection of Protein Kinase on Infected Cell Surfaces. To determine the cellular localization of the protein kinase, we first examined the reactivity pattern of monoclonal antibody 294 by immunofluorescence microscopy. Indirect immunofluorescence experiments were carried out in both live VZV-infected and uninfected cells. In the live impermeabilized infected cells, immunofluorescence was detected on the surface of individual cells and syncytia (Fig. 6*A*), whereas the uninfected human melanoma cell culture lacked membrane fluorescence (not shown). To confirm our observation that the kinase protein was exposed on the outer membrane of virus-infected cells, surface iodination was performed on live VZV-infected cells, after which the ^{125}I-labeled cells were solubilized. As shown in Fig. 6 *B* and *C*, a M_r 50,000 protein was precipitated by monoclonal antibody. Taken together, these results demonstrated that p50 was expressed on the surface of virus-infected cells.

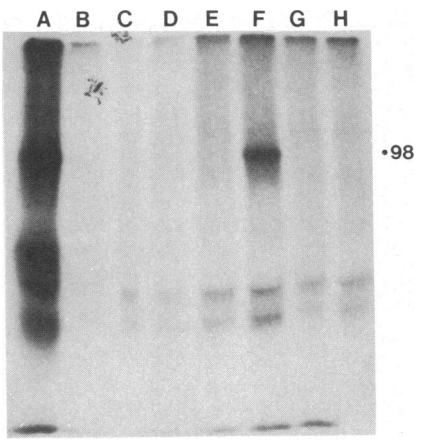

FIG. 5. *In vitro* phosphorylation of gpI. VZV-infected cell lysates were precipitated either singularly, with monoclonal antibody specific for one of the VZV-encoded glycoproteins, or in a dual fashion using viral glycoprotein reactive antibody plus antibody 294 (anti-protein kinase). The washed pellets were subsequently tested for kinase activity by the addition of [γ-^{32}P]ATP. Kinase buffer consisted of 0.05 M magnesium acetate/0.05 M Tris·HCl, pH 8.0/0.001 M dithiothreitol/0.1% NP-40. After a 30-min incubation at 37°C, the pellets were washed, and bound protein was eluted and electrophoresed through a NaDodSO$_4$/10% polyacrylamide gel. Phosphorylated polypeptides were detected by autoradiography on Kodak XAR-5 film. The following antibodies were used in the lanes shown above. Lanes: A, guinea pig polyclonal antisera raised against virus infected cells; B, anti-gpI antibody; C, anti-gpII antibody; D, anti-gpIII antibody; E, antibody 294; F, anti-gpI antibody plus antibody 294; G, anti-gpII antibody plus antibody 294; H, anti-gpIII antibody plus antibody 294. Among the three viral glycoproteins, only gpI was phosphorylated by virus-induced protein kinase (lane F).

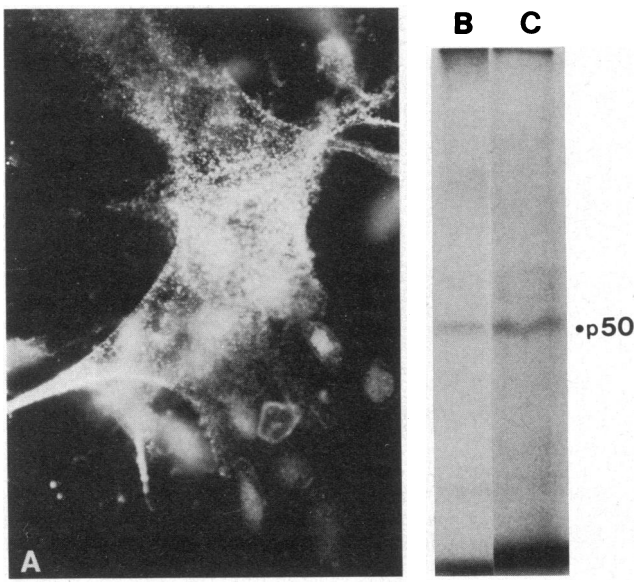

FIG. 6. Surface expression of the protein kinase. VZV-infected cells were dislodged from a culture dish, gently dispersed by repetitive pipetting, sequentially incubated with monoclonal antibody to the protein kinase (no. 294) and goat anti-mouse fluorescein conjugate, and examined by fluorescence microscopy as described (8). The immunostaining pattern in live virus-infected cells demonstrates the surface localization of antigen reactive with antibody 294 (*A*). In addition, purified membranes were isolated from VZV-infected cells that had been surface-labeled with ^{125}I. A detergent-solubilized extract of the membrane preparation was used as antigen for immunoprecipitation experiments with anti-p50 monoclonal antibody. When the immunoprecipitates were analyzed by NaDodSO$_4$/PAGE, it was apparent that a ^{125}I-labeled product was precipitated by specific antibody (*C*). As a control, ^{32}P-labeled p50 was precipitated with the same monoclonal antibody (*B*).

DISCUSSION

In this report, we describe the selective phosphorylation of a human herpesvirus glycoprotein. Specific phosphorylation of VZV gpI can be demonstrated by two distinct methods: (*i*) immunoprecipitation of gpI from ^{32}P-labeled virus-infected cell lysates and (*ii*) *in vitro* transfer of $^{32}P_i$ to gpI by a newly identified virus-induced protein kinase. In both instances, other VZV glycoproteins fail to become phosphorylated. Furthermore, we have taken advantage of our monoclonal antibody reagents to elucidate the phosphorylation pattern of VZV glycoproteins. The results indicate a selective nature of this posttranslational modification. Although two distinct phosphorylated glycoproteins were detected at M_r 98,000 and 88,000, both products were precipitated by the same monoclonal antibody. In a previous study, we have shown that these two glycoproteins represent different glycosylated forms of the same gene product (5). Consistent with other published reports on herpesvirus phosphoproteins, the phosphoryl groups are added at serine and threonine residues (11, 25–27).

Herpes-associated protein kinases have now been defined by workers in several laboratories (9–11, 26–29). These include (*i*) a structural protein found in the tegument of purified virion preparations (26–29) and (*ii*) a virus-induced product whose kinase activity is detectable only in virus-infected cells (9–11). The structural enzyme is insoluble and,

with the exception of equine herpesvirus kinase (27), does not phosphorylate exogenous substrates—e.g., only virus-encoded products are phosphorylated. In addition to the tegument kinase, another kinase, called casein kinase II and presumably of cellular origin, has recently been found in purified pseudorabies virions (29). The latter result lends credence to an early report describing a protein kinase activity in association with the envelope of herpes simplex virus type 1 (30). In contrast to the above products, the virus-induced kinases are soluble and phosphorylate nonviral substrates. However, all reported inducible enzymes among the three members of the herpesvirus family differ with respect to their substrate specificities.

The fact that the VZV-associated p50 cannot be detected in a purified virion preparation leads us to conclude that the kinase is either a virally encoded nonstructural protein or a cellular product whose synthesis is activated by virus infection. Since glycoprotein phosphorylation and cell-surface expression have not been investigated in the other herpesvirus systems, we cannot determine at this time whether any of the previously described inducible enzymes are analogous to the protein kinase described in this report. Nor do we know the functional significance of this modification of VZV gpI, although it is clear that phosphorylation/dephosphorylation reactions represent important regulatory mechanisms, which encompass a broad range of substrates (31, 32). In this respect, a regulatory function has been tentatively assigned to some nonglycosylated herpesvirus phosphoproteins (33). Likewise, the posttranslational addition of phosphoryl groups to glycoproteins of enveloped viruses may represent a general mechanism for coordinating intracellular processes, such as glycoprotein trafficking or virion envelopment and exocytosis.

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