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A Role for Bovine Herpesvirus 1 (BHV-1) Glycoprotein E (gE) Tyrosine Phosphorylation in Replication of BHV-1 Wild-Type Virus but Not BHV-1 gE Deletion Mutant Virus

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Bovine herpesvirus 1 (BHV-1), an alphaherpesvirus, is a major pathogen that causes respiratory and reproductive infections. We observed tyrosine phosphorylation of a 95-kDa viral protein and dephosphorylation of 55- and 103-kDa cellular proteins during the course of BHV-1 infection. We demonstrated BHV-1 glycoprotein E (gE) to be the tyrosine phosphorylated viral protein by immunoprecipitation. Inhibition of phosphorylation of BHV-1 gE by tyrosine kinase inhibitors genistein and tyrphostin AG1478 substantially lowered the viral titer in Madin-Darby bovine kidney cells. The decrease in viral titer was directly proportional to the decrease in phosphorylation of the BHV-1 gE. Interestingly, these kinase inhibitors did not inhibit the replication of the BHV-1 gE deletion mutant virion (BHV-1gEΔ3.1). Our findings suggest that the wild-type BHV-1, with a functional gE protein, uses a different pathway of signaling events than the BHV-1 gE deletion mutant in replication. Our results indicate that the tyrosine phosphorylation of the cytoplasmic tail of BHV-1 gE is an important post-translational modification of the functional protein. An application of this study may be the use of tyrosine kinase inhibitors in controlling the BHV-1 infection.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), an alphaherpesvirus (Roizman *et al.,* 1992), causes respiratory and reproductive disease in cattle (Gibbs and Rweyemamu, 1977). BHV-1 infections involve a well-orchestrated interaction of the virus with its host cell machinery. Phosphorylation cycles form a major part of such virus–host interaction. A phosphorylation cycle (Hardie, 1995) includes highly regulated phosphorylation events (catalyzed by protein kinases) and dephosphorylation events (catalyzed by protein phosphatases). Several of the viral proteins are phosphorylated. Phosphorylation of viral (Morrison *et al.,* 1998) and host cellular proteins (Qie *et al.,* 1999) is an important event during the course of the viral infection. In particular, tyrosine phosphorylation is critical to the regulation of cell proliferation and differentiation (Schlessinger and Ullrich, 1992). This regulation of cellular function makes tyrosine phosphorylation essential for productive viral infection, as viruses have adapted to replicate in cells at certain stages of cell cycle and/or differentiation. For example, tyrosine phosphorylation of cellular proteins occurs as a result of human immunodeficiency virus type-1 infection (Cohen *et al.,* 1992). Tyrosine phosphorylation of cellular proteins by Src-like kinases during the course of coxsackie B3 virus infection is necessary for effective replication (Huber *et al.,* 1999). An active role of tyrosine phosphorylation has been suggested in the entry of cytomegalovirus, another herpesvirus (Keay and Baldwin, 1996).

Tyrosine kinase inhibitors are used to study the role of tyrosine phosphorylation and/or related signaling pathways in cellular and viral processes. In this study, we used both genistein (Mera *et al.,* 1999) and tyrphostin (Fuortes *et al.,* 1999), which are potent tyrosine kinase inhibitors. Genistein and tyrphostin inhibit Herpes-Simplex-Virus (HSV-1) infection by lowering the phosphorylation of tyrosine phosphoproteins (Yura *et al.,* 1993, 1997). Genistein and tyrphostin inhibit the tyrosine phosphorylation signaling pathway used by the pseudorabies virus (Favoreel *et al.,* 1999).

Herpesviral glycoproteins play a major role in the infection process. Glycoproteins specified by herpesviruses are involved in the host–cell interactions essential for a productive infection (Schwyzer and Ackerman, 1996). Alphaherpesvirus glycoprotein E (gE) homologues share common protein motifs for tyrosine phosphorylation and sorting signals (Alconada *et al.,* 1999). gE is functionally conserved among the alphaherpesviruses (Knapp and Enquist, 1997). BHV-1 gE plays a major role in cell-to-cell spread and in virulence (Reberdosa *et al.,* 1996). gE enhances morbidity and mortality rates in infected animals (Yang *et al.,* 1999). Previous studies revealed that BHV-1 gE and glycoprotein I (gI) form a noncovalent complex soon after their synthesis and function as one unit (Whitbeck *et al.,* 1996). BHV-1 gE–gI complex is present in the cell plasma membrane, virus envelope, and all the internal cell membranes (except

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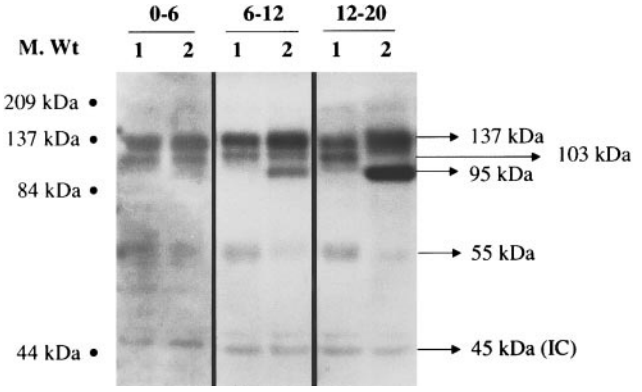

FIG. 1. The effect of BHV-1 infection on tyrosine phosphorylation. This was studied using uninfected cell lysates (lanes 1) and BHV-1-infected cell lysates (lanes 2). The tyrosine phosphorylation was analyzed using the PY20 antibody at 6, 12, and 20 h p.i. The tyrosine phosphorylated proteins that changed pattern during BHV-1 infection are shown on the right side, and the molecular weights are indicated on the left side. The 45-kDa host cellular tyrosine phosphorylated protein is the internal control (IC).

the mitochondrial membranes) and plays an important role in neurotropism of the alphaherpesviruses (Jacobs, 1994). The BHV-1 gE deletion mutant virus (BHV-1 gEΔ3.1) and the tyrosine kinase inhibitors genistein and tyrphostin AG1478 were used to study the biological significance of BHV-1 gE tyrosine phosphorylation. We provide evidence that tyrosine phosphorylation of BHV-1 gE is an important post-translational modification of the functional protein.

RESULTS

BHV-1 infection and tyrosine phosphorylation

Tyrosine phosphorylation of proteins was identified using a mouse monoclonal antibody to phosphotyrosine (PY20) in a chemiluminescent assay. The specificity of the PY20 antibody to the phosphotyrosine residues was established using protein tyrosine phosphatase (PTPase). The PY20 antibody failed to detect the PTPase-treated tyrosine phosphorylated proteins in the cell lysates (data not shown). The two major changes observed in the levels of tyrosine phosphorylation during BHV-1 infection in Madin-Darby bovine kidney (MDBK) cells (Fig. 1) were the phosphorylation of a 95-kDa viral protein (VP) and the dephosphorylation of two cellular proteins (CPs) with molecular weights of 55 and 103 kDa. The relationship of the level of phosphorylation among these three proteins is shown in Table 1. The 95-kDa tyrosine phosphorylated protein was present only in BHV-1-infected cells. The protein was not a cellular stress protein as it could not be induced by thermal shock (42°C for 15 min), cold shock (+4°C for 30 min), chemical treatment (70% ethanol for 5 s), or infection with an unrelated virus (vesicular stomatitis virus for 12 h) (data not shown). This 95-kDa VP was detected as early as 4 h postinfection (p.i.) and was dependent on the multiplicity of infection (m.o.i.) (data not shown). The phosphorylation of this protein increased with time and reached a peak by 20 h p.i. (Fig. 1, lane 2, at 20 h p.i., and Table 1).

The 55- and 103-kDa CPs are expressed in MDBK cells as tyrosine phosphorylated proteins. There was a 50–60% (average of 55%, $n = 3$ experiments) decrease in phosphorylation of the 55-kDa CP observed from 12 h p.i. through 20 h p.i. (Fig. 1, lane 2, at 6–12 and 12–20 h p.i., and Table 1). The level of phosphorylation of the 103-kDa CP was reduced by almost 100% by 20 h p.i. (Fig. 1, lane 2, at 12–20 h p.i., and Table 1). The levels of 55 and 103 kDa were determined by comparison of chemiluminescent signal of each protein in virally infected cells with the chemiluminescent signal of each protein in uninfected cells at each time point (Fig. 1). In addition to these changes, we also observed a 46–50% (average of 48%, $n = 3$ experiments) increase in the phosphorylation of a 137-kDa cellular tyrosine phosphorylated protein at 20 h p.i. (Fig. 1, lane 2, at 12–20 h p.i.) compared with the uninfected lane (Fig. 1, lane 1, at 12–20 h p.i.).

TABLE 1

Relationship between the Tyrosine Phosphorylation of BHV-1 gE and the CPs

	Time							
	0–6 h p.i.		6–12 h p.i.		12–20 h p.i.			
Protein	C	B	C	B	C	B	G	T
103 kDa (CP)	2.5^{a}	2.6^{a}	5.2^{a}	5.0^{a}	5.1^{a}	0^{b}	4.0^{c}	$4.8^{a,c}$
gE (95 kDa VP)	0^{a}	2.0^{b}	0^{a}	7.5^{b}	0^{a}	13.2^{b}	8.5^{c}	5.0^{d}
55 kDa (CP)	5.0^{a}	4.7^{a}	5.0^{a}	2.8^{b}	4.9^{a}	2.5^{b}	4.5^{a}	4.9^{a}

Note. The kinetics of the phosphoprotein expression are represented by the relative phosphorylation (RTP) units. Cell lysates that were uninfected (C), BHV-1 infected (B), infected in the presence of genistein (G), and infected in the presence of tyrphostin (T) were used in this study. Values within the same row and treatment time with different superscripts are statistically significant ($P < 0.05$) by least significance difference as calculated with the SAS program. The results are from an average of three experiments.

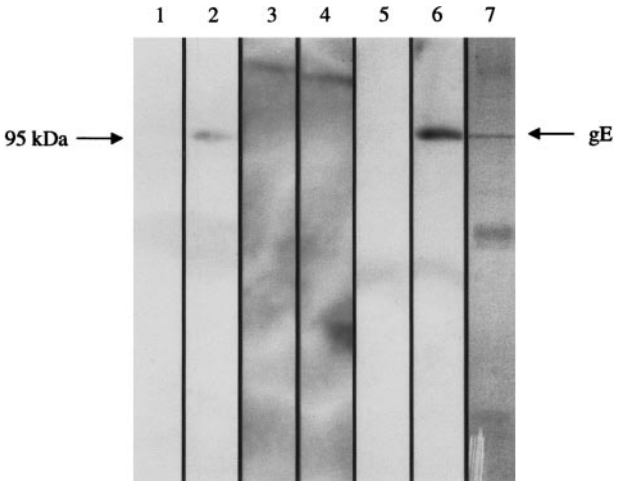

FIG. 2. Identifying the 95-kDa viral protein by immunoprecipitation. Lanes 1–6 were visualized by the chemiluminescent detection system after treatment with the PY20 antibody. Lanes 1 and 2, immunoprecipitate of uninfected cell lysate and BHV-1-infected cell lysate with goat polyclonal anti-BHV-1 sera, respectively; lane 3, immunoprecipitate of BHV-1-infected cell lysate with murine monoclonal antibody to BHV-1 VP8; lane 4, immunoprecipitate of BHV-1-infected cell lysate with rabbit polyclonal antisera to BHV-1 BICP0; lanes 5 and 6, immunoprecipitate of uninfected cell lysate and BHV-1-infected cell lysates with rabbit polyclonal antisera to BHV-1 gE, respectively; and lane 7, immunoprecipitate of BHV-1-infected cell lysate with rabbit polyclonal antisera to BHV-1 gE visualized by Coomassie blue staining. The molecular weight of the protein is indicated on the left side.

Identifying the 95-kDa viral tyrosine phosphorylated protein

The 95-kDa protein was immunoprecipitated from BHV-1-infected cell lysate using a polyclonal goat anti-BHV-1 sera, treated with PY20 antibody, and demonstrated by chemiluminescence to be a BHV-1 tyrosine phosphorylated protein (Fig. 2: lane 2). Three viral BHV-1 viral proteins, VP8, BICP0, and gE, were candidate phosphoproteins based on the molecular weight of 95 kDa. To verify the identify of this BHV-1 tyrosine phosphorylated protein, similar immunoprecipitation and chemiluminescence studies (like those described) using anti-VP8 and anti-BICP0, or anti-gE antibodies and PY20 antibody, were conducted. Our results indicated that VP8 and BICP0 proteins were not tyrosine phosphorylated (Fig. 2, lanes 3 and 4). However, BHV-1 gE was a tyrosine phosphorylated protein (Fig. 2, lane 6). Uninfected cell lysates were used as controls in these assays (Fig. 2, lanes 1 and 5). To further confirm the identity of the gE protein, the membrane strip in lane 6 was stained with Coomassie blue, and a band at 95 kDa was identified (Fig. 2, lane 7).

Optimization of dose for the tyrosine kinase inhibitors

Nontoxic levels for genistein and tyrphostin in MDBK cells were determined by use of the trypan blue exclusion test and lactate dehydrogenase release (LDH) assay. Genistein at 25 μM and tyrphostin at 12.5 μM were determined to be the optimal nontoxic doses based on greater than 95% viability of cells by trypan blue (data not shown) and less than 5% release of LDH (Fig. 3) after 24 h of treatment. DMSO, the solvent for both genistein and tyrphostin, had no cytotoxic effect at the dose used (Fig. 3).

The relationship between phosphorylation of gE and the 55- and 103-kDa CP

The levels of tyrosine phosphorylation were measured using PY20 antibody and chemiluminescence on BHV-1-infected and tyrosine kinase inhibitor-treated BHV-1-infected cell lysates at 20 h p.i. Genistein (Fig. 4, lane 3, and Table 1) and tyrphostin (Fig. 4, lane 4, and Table 1) decreased the phosphorylation of the gE protein while increasing the phosphorylation of the 55- and 103-kDa tyrosine phosphorylated CPs compared with untreated BHV-1-infected controls (Fig. 4, lane 2, and Table 1).

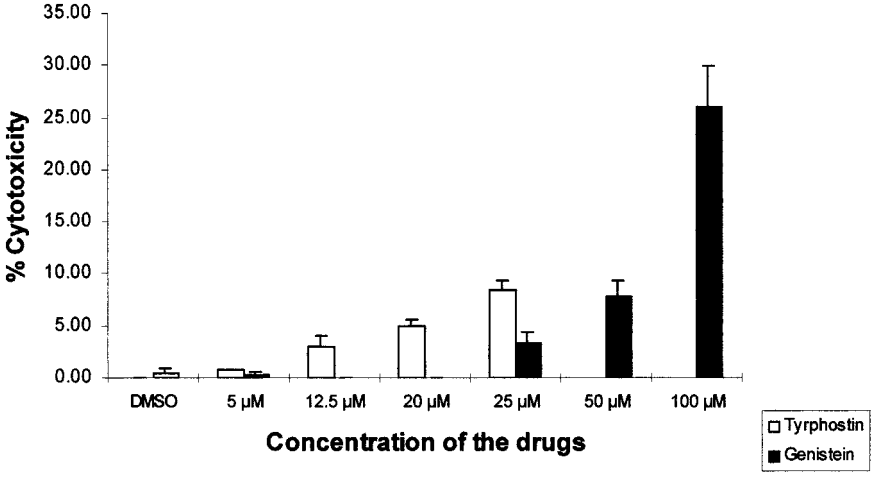

FIG. 3. LDH release after treatment of MDBK cells with genistein and tyrphostin. The results are an average of triplicate trials.

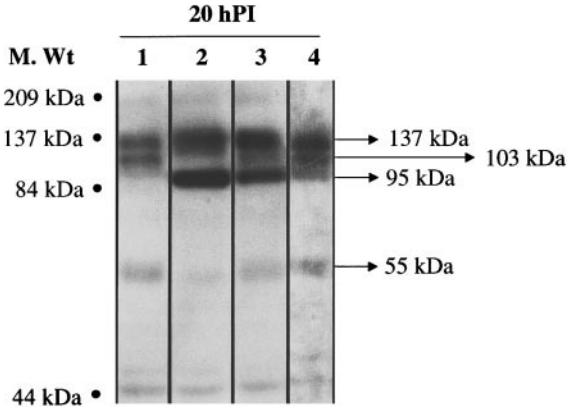

FIG. 4. The effect of tyrosine kinase inhibitors on tyrosine phosphorylation during BHV-1 infection. MDBK cells were uninfected (lane 1) or infected with BHV-1 (lane 2) or BHV-1 in the presence of genistein (lane 3) and tyrphostin (lane 4) respectively. The cell lysates were prepared at 20 h p.i., resolved by SDS–PAGE, blotted onto a membrane, and analyzed using PY20 antibody for tyrosine phosphorylation.

Genistein and tyrphostin reduced the phosphorylation of gE by an average of 36% and 62%, respectively (Fig. 4, lanes 3 and 4, and Table 1). The phosphorylation of the 55-kDa CP returned, in the presence of genistein and tyrphostin, to levels identical to uninfected cell lysates (Fig. 4, lane 1, and Table 1). No phosphorylated 103-kDa protein could be detected in BHV-1-infected cells at 20 h p.i. (Fig. 4, lane 2, and Table 1). However, the presence of genistein or tyrphostin in BHV-1-infected cells maintained the phosphorylation of the 103-kDa CP at 80% or 95%, respectively (Fig. 4, lanes 3 and 4, and Table 1), of the phosphorylation level of the uninfected cell lysate (Fig. 4, lane 1, and Table 1). An additional observation was that a 137-kDa cellular tyrosine phosphorylated protein, which increased in phosphorylation during the course of infection, was unaffected by either of the inhibitors (Fig. 4, lanes 3 and 4).

The effect of tyrosine kinase inhibitors on BHV-1 and BHV-1gEΔ3.1 replication

The tyrosine kinase inhibitors decreased the titer of wild-type BHV-1 in MDBK cells (Fig. 5). Tyrphostin had a greater negative impact on the phosphorylation of gE and thus lowered the viral titer to a greater extent than did genistein. The inhibitors had no effect on the BHV-1gEΔ3.1 titers at 24 h p.i. in MDBK cells (Fig. 5).

DISCUSSION

Although the complete genome of BHV-1 has been described, little work has been done in understanding the signaling pathway used by the BHV-1 during infection. The initial approach was to determine whether phosphorylation inhibitors regulated BHV-1 replication, so the effects of several compounds known to affect phosphorylation were screened. The compounds tested included protein phosphatase inhibitors (sodium vanadate, sodium fluoride, cypermethrin, dephostatin, okadaic acid, and tautomycin), serine/threonine kinase inhibitors (bisindolylmaleimide I, H-89, KN-93, KT 5823, and KT 5926), and tyrosine kinase inhibitors (genistein and tyrphostin AG1478) for their effects on the replication of BHV-1. Of all of the compounds screened, we found genistein and tyrphostin, tyrosine kinase inhibitors, to be the most effective inhibitors of BHV-1 replication. Once tyrosine phosphorylation was identified as an important step of BHV-1 replication, we expected to identify changes in the tyrosine phosphorylation of the viral and the host cellular proteins after treatment with these inhibitors.

BHV-1 infection in MDBK cells produced two major changes in tyrosine phosphorylation of the host cellular proteins: the dephosphorylation of 55- and 103-kDa CPs that occurred as an increase in phosphorylation of gE was observed. The relationship between these phosphorylated proteins was studied by the use of tyrosine kinase inhibitors genistein and tyrphostin. We chose two tyrosine kinase inhibitors in our study for the following reasons: (1) to confirm the specificity of the inhibition to tyrosine kinase and (2) to determine whether the level of inhibition was affected by mechanism of action because the two tyrosine kinase inhibitors act by different mech-

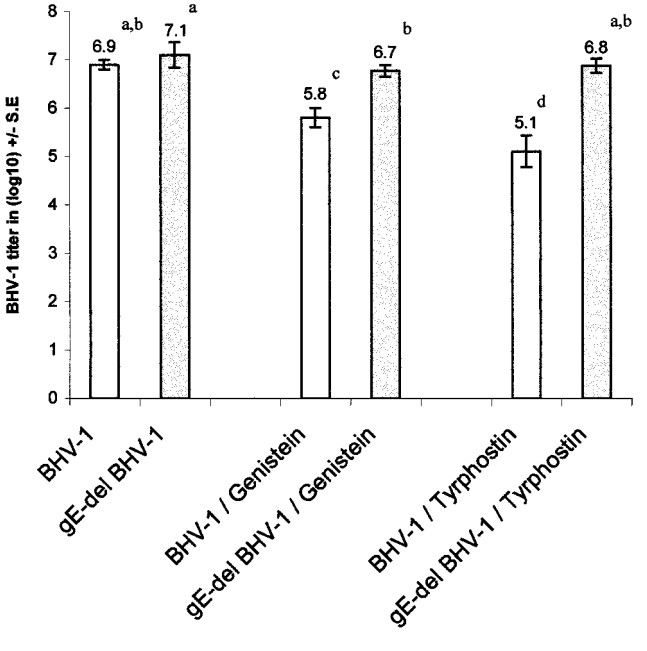

FIG. 5. The effect of genistein and tyrphostin on BHV-1 and BHV-1 gEΔ3.1 replication. The six columns termed BHV-1, gE-del BHV-1, BHV-1/genistein, gE-del BHV-1/genistein, BHV-1/tyrphostin, and gE-del BHV-1/tyrphostin denote titers of BHV-1, BHV-1 gEΔ3.1, BHV-1, and gE-del BHV-1 in the presence of 25 μM genistein and BHV-1 and gE-del BHV-1 in the presence of 12.5 μM tyrphostin at 24 h p.i. in MDBKs. The average titer is indicated above the bar. The results are from the average of triplicate trials. Mean values on the columns with different superscripts are statistically significant ($P < 0.05$) by least significance difference calculated using the SAS program.

anisms. Genistein is an isoflavone that has multiple activities besides the inhibition of tyrosine kinase. As a tyrosine kinase inhibitor, genistein acts by binding to the ATP binding site of the kinase (Akiyama *et al.,* 1987), whereas tyrphostins inhibit the tyrosine kinases by binding to the substrate binding site (Gazit *et al.,* 1991). Our results indicated that tyrosine phosphorylation of the gE was inhibited by both compounds. However, tyrphostin was a better inhibitor of gE phosphorylation than genistein (Table 1). This confirms the earlier reports that tyrphostins have higher inhibitory activity against tyrosine kinases than genistein (Gazit *et al.,* 1989). The inhibitors used in this study decreased the phosphorylation of gE while increasing phosphorylation of the 55- and 103-kDa CPs in a "dynamic balance relationship." This model supports the activation of a cellular PTPase by BHV-1 infection that in turn dephosphorylates the 55- and 103-kDa CPs. The significance of the increased phosphorylation of the 137-kDa tyrosine phosphorylated cellular protein during the infection remains unclear. We did not analyze the relationship between phosphorylation and quantity of the 55-, 103-, and 137-kDa CPs during BHV-1 infection. Hence, it would be premature to conclude that the levels of phosphorylation of 137-kDa CP and dephosphorylation of 55- and 103-kDa CPs are independent of the relative quantity of the respective proteins expressed during infection. The failure of these inhibitors to affect cellular proteins may indicate a high rate of turnover of the CPs and the cellular tyrosine kinases. This could also indicate that viral protein phosphorylation was more sensitive to the kinase inhibitors.

BHV-1 gE was tyrosine phosphorylated during the course of infection. BHV-1 gE is a viral envelope protein classified as a γ2 protein (Fields *et al.,* 1996). The tyrosine phosphorylated BHV-1 gE is homologous to gE described in other alphaherpesviruses. The level of amino acid identity between the ectodomain of BHV-1 gE (accession no. AJ004801.1) and varicella-zoster virus (VZV) gE (accession no. X04370.1) or pseudorabies virus (PRV) gE (accession no. M14336.1) or HSV-1 gE (accession no. X02138.1) was 29.66%, 31.48% and 31.96%, respectively, as analyzed by Protein Information Resource (PIR) database/software (Barker *et al.,* 1999). The level of amino acid identity between the cytoplasmic tail of BHV-1 gE (accession no. AJ004801.1) and VZV gE (accession no. X04370.1) or PRV gE (accession no. M14336.1) or HSV-1 gE (accession no. X02138.1) was 20.00%, 34.59%, and 20.41%, respectively, as analyzed using the PIR software. A slightly higher amino acid identity between the cytoplasmic tail of BHV-1 gE and PRV gE is probably the reason for the ability of BHV-1gE to complement the virulence defect of PRV lacking its own gE (Knapp and Enquist, 1997). Phosphorylation of envelope glycoproteins is a rare post-translational modification in the neurotropic alphaherpesviruses (Jacobs, 1994). Analysis of the 575-amino-acid-long BHV-1 gE sequence (accession no. AJ004801.1) using PSORT program (Nakai, 1991) designated it as a type Ia transmembrane protein (Fig. 6). The predicted structure of the protein is composed of a hydrophilic extracellular domain spanning amino acid residues 1–423, a hydrophobic transmembrane region of residues 424–440, and a fairly long cytoplasmic tail of residues 441–575. The predicted cytoplasmic tail of the BHV-1 gE contains a conserved antigen recognition activation motif (ARAM) that includes at least two YXXL sequences separated by six amino acids (amino acids 457–470). Tyrosine residues in these ARAMs are phosphorylated and play a major role in signal transduction via the activation of protein tyrosine kinase pathway (Park and Scheiber, 1995). These ARAMs are present in the cytoplasmic tail of several other transmembrane proteins that function as tyrosine phosphorylated receptors, such as latent membrane protein 2A of Epstein–Barr virus (Rowe *et al.,* 1990), bovine leukemia virus gp30, and several components of the T and B cell receptors (Chan *et al.,* 1994). Alteration of the tyrosine residues (Letourneur and Klausner, 1992) or deletion of the C-terminal leucine residue (Irving *et al.,* 1994) within the ARAM resulted in a markedly reduced receptor function or the loss of receptor function. Interestingly, this ARAM region is one of the most highly conserved domains within the gE homologues of BHV-1, simian varicella virus, feline herpesvirus 1, equid herpesvirus 1, PRV, VZV, and HSV-1 (Alconada *et al.,* 1999). This domain is essential for the intracellular sorting of the protein (Zhu *et al.,* 1996; Alconada *et al.,* 1996; and Alconada *et al.,* 1999) and in activation of tyrosine phosphorylation signal transduction pathway (Favoreel *et al.,* 1999). Recently, a single YXXL sequence in the cytoplasmic tail of the VZV gE amino acid sequence has been implicated in tyrosine phosphorylation. In this case, dimerization of VZV gE receptor molecule has been hypothesized to create a perfect ARAM by the YXXL sequences in the two proteins that serve as a binding site for the Src homology 2 domain of tyrosine kinase (Olson *et al.,* 1997). The BHV-1 gE lacks any tyrosine kinase activity, and the kinases coded by the BHV-1 genome (accession: AJ004801) have been identified to be only of the serine/threonine ki-

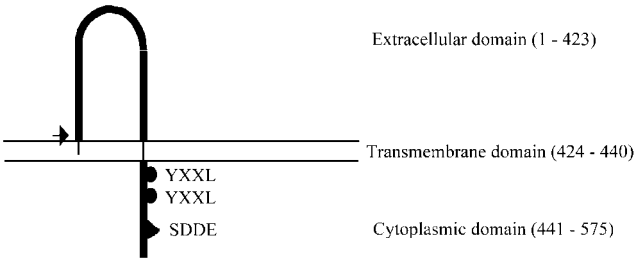

FIG. 6. Membrane topology: type 1a. The cytoplasmic domain contains a tyrosine phosphorylation motif (paired YXXL sequence) and a putative casein kinase II phosphorylation site (SDDE). The arrow indicates the possible cleavable signal peptide (amino acids 1–20).

TABLE 2

Comparison of Tyrosine Phosphorylation Events during BHV-1 and BHV-1gEΔ3.1 Infection

Properties	BHV-1	BHV-1gEΔ3.1
Replication in MDBKs	++	++
Tyrosine phosphorylation of gE	+	−
Effect of tyrosine kinase inhibitors on viral replication	↓	++
Effect of tyrosine kinase inhibitors on gE phosphorylation	↓	−

Note. + Indicates presence; ++, unrestricted replication of the virus; −, absence; and ↓, negative regulation.

nases. Therefore, we hypothesize that BHV-1 gE is phosphorylated on its tyrosine residue by the host cellular (non-receptor) tyrosine kinases to start a signaling cascade. Thus BHV-1 gE is the latest addition to the small family of VZV gE, HSV-1 gE, and PRV gE that are phospho-gE proteins of alphaherpesviruses. The cytoplasmic domain of BHV-1 gE amino acid sequence contains a potential casein kinase II phosphorylation motif, SDDE (amino acids 483–486). This motif is shared by other substrate proteins, such as heat shock protein 90, simian virus 40 large T antigen, and ornithine decarboxylase, that undergo phosphorylation on serine residue by casein kinase II (Yao *et al.,* 1993). Although we did not demonstrate the phosphorylation of BHV-1 gE on its serine residue, it carries a potential motif making it identical to all of the other phosphorylated gEs of alphaherpesviruses.

The gE protein is essential for the cell-to-cell spread of the virus, especially in epithelial and nerve cells (Yoshitake *et al.,* 1997). Our results indicate that inhibition of phosphorylation of gE using tyrosine kinase inhibitors lowers the viral titer in MDBK cells, an epithelial cell type. Tyrphostin decreased phosphorylation of gE and lowered the viral titer more than genistein. The specificity of these inhibitors for the inhibition of tyrosine phosphorylation of BHV-1 gE was confirmed using the BHV-1gEΔ3.1. However, results using the BHV-1gEΔ3.1 clearly indicate that gE is dispensable for *in vitro* replication, as documented earlier (Mettenleiter *et al.,* 1985; Chowdhury *et al.,* 1999). An extension of our findings (Table 2) is that the wild-type BHV-1, with an intact and functional gE protein, uses a pathway of signaling events different from the BHV-1 gE deletion mutant in replication. We are currently involved in studying the signaling pathway used by the BHV-1gEΔ3.1 and the importance of the dephosphorylation of CPs.

Taken together, we conclude that tyrosine phosphorylation of gE is one of the major signaling events in wild-type BHV-1 infections. Inhibition of phosphorylation of gE on its tyrosine residue impairs the function of the protein. The inhibition of tyrosine phosphorylation of gE attenuates the viral titer, which suggests that this event is important in BHV-1 replication and may be affecting the intracellular transmission of a lethal signal by some other route yet to be determined. Finally, an application of this study is the possible use of tyrosine kinase inhibitors in control of the BHV-1 infection. Work using these inhibitors to prevent gE phosphorylation may be of use in preventing latency and infection by BHV-1.

MATERIALS AND METHODS

Cells

MDBK cells (American Type Culture Collection, Rockville, MD) were used in this study. The cells were grown in minimum essential media (GIBCO, Grand Island, NY) containing 10% equine serum (Sigma Chemical Co., St. Louis, MO).

Virus

Plaque-purified Cooper (Colorado-1) strain of BHV-1 (American Type Culture Collection) and the recombinant glycoprotein gE gene-deleted BHV-1, BHV-1gEΔ3.1 (kindly provided by S. I. Chowdhury, Kansas State University), were used in this study. The BHV-1gEΔ3.1 contains a chimeric β-gal gene regulated by a human cytomegalovirus immediate-early promoter in the place of most of the deleted gE coding sequence (encoding the first 372 of 575 amino acids) that has been previously described (Chowdhury *et al.,* 1999). BHV-1 and BHV-1gEΔ3.1 were propagated and titrated in MDBK cells as previously described (Chase *et al.,* 1989).

Antibodies and reagents

Murine monoclonal antibody to BHV-1 VP8 (a gift from Vikram Misra, University of Saskatchewan), rabbit polyclonal antisera to BHV-1 BICP0 (a gift from Martin Schwyzer, University of Zurich), rabbit polyclonal antisera to BHV-1 gE (a gift from Lynn Enquist, Princeton University), and a goat polyclonal anti-BHV-1 sera (VMRD, Pullman, WA), were used to immunoprecipitate the BHV-1 protein. A mouse monoclonal antibody to phosphotyrosine (PY20; Calbiochem, La Jolla, CA) conjugated to alkaline phosphatase was used to detect tyrosine phosphorylated proteins.

Protein tyrosine phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was used to dephosphorylate the phosphotyrosine residue to test the specificity of the PY20 antibody according to the manufacturer's recommendations.

Detection of tyrosine phosphorylated proteins

Actively dividing (70–75% confluent) MDBK cells were infected at 0.5 m.o.i. of BHV-1 in minimum essential media (MEM) containing 2% equine serum. The mock-infected cells were treated with MEM containing

2% equine serum and no virus. The cells were incubated at 37°C for 6, 12, and 20 h p.i. At the end of each time point, the cells were washed once in cold NET buffer (Marshall *et al.,* 1986) and lysed on ice using lysis buffer (NET buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 U/ml aprotinin, 0.2 mM sodium vanadate, 1 mM PMSF, and 2 μg/ml leupeptin). Equal amounts of protein were loaded and resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a PVDF membrane (Millipore, Bedford, MA) using a TE42 transfer tank (Hoefer Scientific Instruments, San Francisco, CA). The membrane was probed with PY20 antibody, treated with the Western-Light chemiluminescent detection system (Tropix, Bedford, MA), and exposed to X-ray film (Kodak X-OMAT; Sigma Chemical Co.), and the image was digitalized and analyzed with Molecular Analyst Software, version 2.1 (Bio-Rad, Hercules, CA). The level of the tyrosine phosphorylated proteins was studied using the relative tyrosine phosphorylation (RTP) units. The RTP units are the ratio of each of the tyrosine phosphorylated protein (55- and 103-kDa CPs and 95-kDa VP) compared with the internal control (45-kDa tyrosine phosphorylated host cellular protein).

Immunoprecipitation

Actively dividing MDBK cells were infected at 0.5 m.o.i. of BHV-1. Mock infection was used as a control. The cells were incubated at 37°C. The cells were washed once with NET buffer at the end of 12 h p.i. and lysed as described. Immunoprecipitation was performed using immobilized protein G cross-linked to 6% agarose beads (Pierce Chemical, Rockford, IL) as previously described (Van Drunen Littel-Van Den Hurk *et al.,* 1995). The immunoprecipitated proteins were resolved by 10% SDS–PAGE and transferred to PVDF membrane. The proteins were visualized by Coomassie blue staining or by a chemiluminescent detection system using the PY20 antibody as described earlier.

Dose optimization assays for the tyrosine kinase inhibitors

The trypan blue exclusion test (Lappalainen *et al.,* 1994) and LDH assay were performed to measure the cytotoxicity of genistein (Calbiochem) and tyrphostin (Calbiochem) on MDBK cells. Genistein (5, 25, 50, and 100 μM) and tyrphostin (5, 12.5, 20, and 25 μM) were tested. DMSO control, the solvent for both genistein and tyrphostin, was tested at 0.025% for its cytotoxic effect on the MDBKs. The LDH assay was performed using CytoTox 96 (Promega, Madison, WI) according to the manufacturer's instruction.

Determination of the effect of tyrosine kinase inhibitors on the viral replication

The effect of the inhibitors on the viral replication was assessed in actively growing MDBK cells that were supplemented with MEM containing 2% equine serum. The cells were infected at 0.5 m.o.i. with BHV-1 or BHV-1gEΔ3.1 and treated with a predetermined optimal dosage of respective inhibitors at 37°C in a humidified atmosphere of 5% CO_2. After 24 h p.i., the cells were scrapped and frozen with the viral supernatant at −70°C. Before viral titration, the cell lysate was centrifuged at 1000 × *g* for 7 min to clarify, and the supernatant was used. The virus yield was titrated on MDBK cells, and the $TCID_{50}$ was calculated using the Reed and Muench formula (Reed and Muench, 1938).

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