Mutations in the conserved carboxy-terminal hydrophobic region of glycoprotein gB affect infectivity of herpes simplex virus

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Glycoprotein gB is the most highly conserved glycoprotein in the herpesvirus family and plays a critical role in virus entry and fusion. Glycoprotein gB of herpes simplex virus type 1 contains a hydrophobic stretch of 69 aa near the carboxy terminus that is essential for its biological activity. To determine the role(s) of specific amino acids in the carboxy-terminal hydrophobic region, a number of amino acids were mutagenized that are highly conserved in this region within the gB homologues of the family Herpesviridae. Three conserved residues in the membrane anchor domain, namely A786, A790 and A791, as well as amino acids G743, G746, G766, G770 and P774, that are non-variant in Herpesviridae, were mutagenized. The ability of the mutant proteins to rescue the infectivity of the gB-null virus, K082, in trans was measured by a complementation assay. All of the mutant proteins formed dimers and were incorporated in virion particles produced in the complementation assay. Mutants G746N, G766N, F770S and P774L showed negligible complementation of K082, whereas mutant G743R showed a reduced activity. Virion particles containing these four mutant glycoproteins also showed a markedly reduced rate of entry compared to the wild-type. The results suggest that non-variant residues in the carboxy-terminal hydrophobic region of the gB protein may be important in virus infectivity.

Introduction

Herpes simplex virus type 1 (HSV-1) is an enveloped virus whose genomic DNA encodes at least 11 glycoproteins (Roizman & Sears, 1996). Four of these glycoproteins, namely gB, gD, gH and gL, are essential for virus replication and are required for virus entry and cell fusion (Cai et al., 1988a; Ligas & Johnson, 1988; Roop et al., 1993). Glycoprotein gB is the most highly conserved glycoprotein in the family Herpesviridae (Pereira, 1994; Roizman & Sears, 1996). Homologues of gB have been identified in all subfamilies of the Herpesviridae and are essential for virus replication. Studies involving HSV-1 gB glycoprotein (gB-1) have indicated essential roles of gB in virus penetration, membrane fusion and cell-to-cell spreading (Cai et al., 1988a; Little et al., 1981; Pereira, 1994; Spear, 1993). The deduced sequence of gB-1 predicts the presence of a carboxy-terminal hydrophobic stretch of 69 aa which could serve as a membrane anchor domain, a large cytoplasmic domain of 109 aa, an ectodomain of 696 residues containing N-linked oligosaccharides and a cleavable signal sequence of 30 aa (Bzik et al., 1984). The carboxy-terminal hydrophobic region was proposed to have three segments containing 20–21 aa corresponding to residues 727–746 (segment 1), 752–772 (segment 2) and 775–795 (segment 3) which can traverse the membrane three times (Pellett et al., 1985). Studies from our laboratory using mutants with deletions in the predicted membrane-anchoring sequence of the 69 aa residues in gB-1 protein showed that segment 3, encompassing residues 775–795, specified the membrane-anchoring domain and segments 1 and 2 may be peripherally associated with the membrane (Rasile et al., 1993). However, deletion of any of the three hydrophobic segments individually or in combination affected the intracellular transport and processing of the mutant gB-1 proteins. The mutant glycoproteins were localized in the nuclear envelope but failed to complete the gB-null virus K082, indicating that the carboxy-terminal hydrophobic region contains essential structural determinants of biologically functional gB glycoprotein. Recent studies with gB glyco-

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proteins of human cytomegalovirus (HCMV) (Bold et al., 1996; Reschke et al., 1995; Tugizov et al., 1995; Zheng et al., 1996) and bovine herpesvirus-1 (BHV-1) (Li et al., 1997) also identified that the most hydrophobic segment 3 functions as the membrane anchor. However, truncation of any of the hydrophobic segments affected intracellular transport and processing. Segment 2 of BHV-1 gB protein, which includes residues 785–771, and hydrophobic domain 1 of HCMV gB protein, which includes residues 714–750, were also proposed as fusogenic domains (Bold et al., 1996; Reschke et al., 1995).

Analyses of the amino acid sequences of gB homologues of herpesviruses show that the carboxy-terminal hydrophobic region is conserved and a number of residues are non-variant within the Herpesviridae. The gB glycoproteins of the Alphaherpesvirinae, however, show a very high degree of homology in this region and the membrane-anchoring segment 3 shows the highest number of conserved amino acids (Pereira, 1994). Complementation experiments showed that the gB glycoprotein of HSV-1 could be substituted with the corresponding homologue from either BHV-1 (Misra & Blewett, 1991) or pseudorabies virus (PrV) (Mettenleiter & Spear, 1994). Also, gB-null PrV could be complemented by gB protein of BHV-1 (Kopp & Mettenleiter, 1992; Rauh et al., 1991). It was thus suggested that homologues of gB may possess common structural and functional domains. The highly conserved residues in the carboxy-terminal hydrophobic region of gB may, therefore, be essential for the biological activity of herpesviruses.

In this report, we have attempted to determine the roles of amino acids that are highly conserved within the carboxy-terminal hydrophobic region of the gB homologues of herpesviruses in virus infectivity by mutagenesis of these residues. Conserved alanines in the membrane-anchoring segment 3 of Alphaherpesvirinae were substituted by a number of neutral polar residues. Two glycines in segment 1 as well as glycine, phenylalanine and proline residues in segment 2, all of which are non-variant in the gB homologues of Herpesviridae, were also mutated. The mutant proteins were localized in the nuclear envelope. Complementation with a gB null-virus showed that mutants G746N, G766N, F770S and P774L were also mutated. The mutant proteins were localized in the nuclear envelope. Complementation with a gB null-virus showed that mutants G746N, G766N, F770S and P774L were also mutated. The mutant proteins were localized in the nuclear envelope.

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**Methods**

**Construction of mutant gB proteins.** The gene encoding gB protein of HSV-1 was cloned into the high efficiency eukaryotic expression vector pXM (Yang et al., 1986) to produce the plasmid pXMgB as described previously (Gilbert et al., 1994). The 2.1 kb SalI–EcoRI fragment of pXMgB was cloned into M13 mp18 and was mutagenized following the procedure of Kunkel et al. (1987). The oligonucleotides used for mutagenesis were GCCCGGCTGGCGGCA-AGCTTCTTGGGCTT, GGGCGTGTCTCGAGCAGTCCACCC- CTT, CTTCGAGGAGTGAAGATCTGGGCCCAGGCT, GAT- GGGCCACCTGAATCGGCCGTGCGAA, GTATCGGCGTGC- TCAAGGCTTCTCCTCTCCTCTCA and CCTTCTAGTCAAACC- TCCTTTGGCCTGCT for constructing mutants A791S, F770S, G743R, G746N, G766N and P774L, respectively. Primers containing mixed sequence oligonucleotides were synthesized by using a mixture of two nucleotides at the specific mutagenesis position (Derbyshire et al., 1986). The heterogeneous population of oligonucleotides was used in the mutagenesis reaction to generate multiple substitution of specific residues. The primers GCCCGGCTGGCGGCTC(C)(A)GGCTTCTTGGGCTT and CTGTGTTCTCTGA(T)A(C)GGCTTCTTGGGCGCTT were used for mutagenesis of A790 and A786 to produce A790S or A790Q and A786S, A786T, A786Y or A786N, respectively. The mutagenic oligonucleotide was annealed to the template DNA and second-strand synthesis was performed with Klenow DNA polymerase. The reaction mixtures were used to transform competent Escherichia coli JM109 cells. The plaques were screened by nucleotide sequence analysis. The replicative-form DNA from the mutant bacteriophage was digested with Sall and EcoRI, and the fragment was ligated to the 6.8 kb fragment obtained from EcoRI and Sall digestion of pXMgB DNA (pXM containing the full-length gB-1 gene), thus regenerating pXMgB harbouring the desired mutation in the gB gene. The mutants were also cloned into pkBXX (Cai et al., 1986b) with SnaBI and KpnI in order to perform the complementation studies. The pkBXX plasmid was obtained from S. Person (Johns Hopkins University, School of Medicine, Baltimore, MD, USA).

**Transfection, labelling and characterization of mutant protein.** Subconfluent monolayers of COS-1 cells were transfected by the CaPO₄ method, as described previously (Gilbert et al., 1994). The transfected cells were radiolabelled at 24 h post-transfection and the mutant proteins were immunoprecipitated with a polyclonal anti-gB antibody. Proteins were analysed by SDS–PAGE.

**Intracellular transport and localization.** Transport of the mutant proteins from ER to the Golgi apparatus was determined by the acquisition of endoglycosidase H (Endo H) resistance (Gilbert et al., 1994). Transfected COS-1 cells were fixed with 2% paraformaldehyde for cell surface immunofluorescence. For internal immunofluorescence, cells were stained with FITC-conjugated goat anti-rabbit IgG (Gilbert et al., 1994). The heterogeneous population of oligonucleotides was used in the mutagenesis reaction to generate multiple substitution of specific residues. The primers GCCCGGCTGGCGGCTC(C)(A)GGCTTCTTGGGCTT and CTGTGTTCTCTGA(T)A(C)GGCTTCTTGGGCGCTT were used for mutagenesis of A790 and A786 to produce A790S or A790Q and A786S, A786T, A786Y or A786N, respectively. The mutagenic oligonucleotide was annealed to the template DNA and second-strand synthesis was performed with Klenow DNA polymerase. The reaction mixtures were used to transform competent Escherichia coli JM109 cells. The plaques were screened by nucleotide sequence analysis. The replicative-form DNA from the mutant bacteriophage was digested with Sall and EcoRI, and the fragment was ligated to the 6.8 kb fragment obtained from EcoRI and Sall digestion of pXMgB DNA (pXM containing the full-length gB-1 gene), thus regenerating pXMgB harbouring the desired mutation in the gB gene. The mutants were also cloned into pkBXX (Cai et al., 1986b) with SnaBI and KpnI in order to perform the complementation studies. The pkBXX plasmid was obtained from S. Person (Johns Hopkins University, School of Medicine, Baltimore, MD, USA).

**Oligomerization assay.** The oligomeric state of wild-type and mutant gB-1 proteins was determined by SDS–PAGE analysis of the heat-dissociable, detergent-stable oligomers (Claxton-Wells & Spear, 1986). Oligomeric forms of gB-1 were also detected by immuno- precipitation with a dimer-specific monoclonal antibody DL16 (Laquerre et al., 1998).

**Complementation assay.** Complementation of the gB-null HSV-1, KO82, was carried out in Vero cells as described previously (Cai et al., 1986b; Rasile et al., 1993). Briefly, 0.5–3.0 µg pkBXX plasmid encoding the gB mutants was mixed with DEAE-dextran in a serum-free growth medium. This mixture was added to 8 × 10⁸ Vero cells and incubated at 37 °C for 2–4 h. After removal of DNA and washing, the cells were incubated at 37 °C in regular growth medium. At 16–18 h post-transfection, the cells were infected with 2 × 10⁶ p.f.u. of KO82 virus and incubated for 2 h at 37 °C. Virions remaining outside the cell were removed by treatment with a glycine buffer (0.1 M glycine, pH 3.0). The cells were incubated at 37 °C for 24 h and then harvested. Virus stocks
were prepared and titres of the progeny virus particles were determined on the gB-1-expressing VB38 cell line and Vero cells. The VB38 cells (kindly donated by D. Johnson, Oregon Health Sciences University, Portland, OR, USA) were constructed by integrating the HSV-1 gB gene under the control of its own promoter into Vero cells using histidinol as a selection marker.

- **Radiolabelling of progeny virus particles.** Vero cells were transfected with pKBXX plasmids followed by infection with KO82 virus as described for the complementation assay. Cells were labelled with [35S]methionine for 24 h and the virus was harvested. The labelled virus stock was clarified by centrifugation at 3000 r.p.m. for 5 min. Virus particles were pelleted by centrifugation and washed with a cushion of 30% sucrose solution by centrifugation in a Beckman SW41 Ti rotor at 4 °C for 2 h at 4 °C. The pelleted virus was suspended in lysis buffer; half of the recovered radioactivity was immunoprecipitated with dimer-specific anti-gB antibody DL16 and analysed by SDS–PAGE.

- **Virus penetration assay.** The rate of virus penetration was determined by measuring the rate at which viruses adsorbed to cells become resistant to inactivation by exposure to a low pH buffer (Highlander et al., 1989). Confluent VB38 cells were incubated at 4 °C with 250 p.f.u. progeny virus generated from the complementation experiment. The cells were washed three times to remove unadsorbed virus and incubated at 37 °C in the presence of complete medium when the adsorbed viruses entered the cell. At various times after being shifted, viruses that had not entered the cells were inactivated by exposure to 2 ml glycine buffer (pH 3–0) for 1 min while the control cells were treated with Tris-buffered saline (TBS) (pH 7–4). The cells were washed and overlaid with medium containing 0.5% methyl cellulose and incubated at 37 °C. After 36–48 h, cells were fixed and stained and the plaques were counted. The percentage of penetrated virus was calculated from the p.f.u. obtained with the sample treated with low pH buffer versus the total plaque number (100% entry) as obtained from the non-buffer-treated sample.

**Results**

**Rationale for mutagenesis in the carboxy-terminal hydrophobic region of gB-1 glycoprotein**

The amino acid sequence of the carboxy-terminal hydrophobic stretch encompassing residues 727–795 of HSV-1 glycoprotein gB-1 is shown in Fig. 1. Hydrophobic segments of 20–21 aa corresponding to residues 727–746, 752–772 and 775–795 have been designated segments 1, 2 and 3, respectively (Rasile et al., 1993). A comparison of the sequence of gB homologues of herpesviruses belonging to the *Alphaherpesvirinae* shows a high degree of conservation of amino acids in this region (Pereira, 1994). It may also be noted that a number of residues, such as G743, G746, V764, G766, F770, N773, P774 and F775, are non-variant in the gB homologues of all herpesviruses. Maximum conservation is, however, observed within segment 3 and within the *Alphaherpesvirinae*. This segment is almost totally conserved suggesting a common functional role. To determine whether these conserved residues in segments 1, 2 and 3 of the hydrophobic region play a role in the biological function of gB-1 glycoprotein, 13 amino acid substitution mutants were constructed by site-directed mutagenesis (Fig. 1). The rationale behind the amino acid substitutions was to retain the hydrophobic character of the particular region after mutagenesis. Thus, substitution of alanine residues with serine, threonine or tyrosine did not change the hydrophobicity markedly. Substitution of glycine with asparagine or phenylalanine with serine reduced the hydrophobicity slightly. All of these residues, however, are present within the transmembrane anchor sequence of membrane proteins. In segment 1, the two non-variant glycines at

![Fig. 1. Mutations in the hydrophobic carboxy-terminal regions of the HSV-1 glycoprotein B. Amino acids 725–798 are shown. Boxed residues indicate the hydrophobic sequences corresponding to segment 1 (aa 727–746), segment 2 (aa 752–772), and segment 3 (aa 775–795) (Rasile et al., 1993). Amino acids conserved in alphaherpesviruses are underlined. Residues marked with an asterisk (*) are non-variant in gB glycoproteins of alpha-, beta- and gammaherpesviruses. Arrows indicate the particular amino acid replacements with oligonucleotide-directed base changes. Mutants are designated according to the corresponding wild-type residue, amino acid position and amino acid replacement, as indicated in the text.](image-url)
Fig. 3. Cell surface and intracellular localization of mutant gB proteins by immunofluorescence. COS-1 cells transfected with mutant gB constructs were fixed with paraformaldehyde and processed for cell surface or internal immunofluorescence. For internal immunofluorescence, cells were fixed with paraformaldehyde and then treated with 1% Triton to permeabilize the cells.
positions 743 and 746 were changed to arginine and asparagine to produce mutants G743R and G746N, respectively. Since these glycine residues are located at the carboxy terminus of segment 1, substitution with a charged polar residue or an uncharged polar residue may affect the structural organization of segments 1 and 2. In segment 2, the non-variant G766 and F770 were changed to uncharged polar residues asparagine and serine to generate mutants G766N and F770S, respectively. The non-variant proline-774 is located at the junction of segments 2 and 3 and was substituted by a non-polar residue, leucine. Within segment 3, A786 was mutated to serine, threonine, asparagine or tyrosine to produce mutants A786S, A786T, A786N or A786Y, whereas A790 was changed to serine and glutamine to produce mutants A790S and A790Q. Mutant A791S was obtained by changing A791 to serine. A double mutant A786S/L788M was unexpectedly generated during mutagenesis of A786, and was also used in this study. It may be further noted that, in the case of mutants in segment 3, a non-polar residue was replaced with an uncharged polar residue.

The hydrophobicities of each of the mutated segments were determined by the Kyte–Doolittle algorithm (Kyte & Doolittle, 1982) and the results show that the point mutations did not significantly alter the hydrophobicities of the segments. Determination of predicted secondary structures of the substitution mutants in segment 3 using the Chou and Fasman algorithm (Chou & Fasman, 1978) also did not show any significant change in the α-helical nature of this segment.

Expression, intracellular transport and oligomerization of mutant gB proteins

COS-1 cells transfected with pXM vector encoding the mutant or the wild-type gB protein were metabolically labelled with [35S]methionine, and proteins immunoprecipitated with anti-gB antiserum were analysed by PAGE. Fig. 2 shows that all of the mutants of gB proteins were expressed and co-migrated with wild-type gB which is 110–120 kDa. The oligomeric states of the mutant gB glycoproteins were examined by analyses of the heat-dissociable, detergent-stable protein on SDS–polyacrylamide gels (Rasile et al., 1993). All of the mutants similar to the wild-type gB showed the presence of dimers which could be dissociated into monomers by heating (data not presented). These results are in agreement with the findings that the oligomerization domains of HSV-1 gB protein are localized in the ectodomain and not in the carboxy-terminal hydrophobic region (Laquerre et al., 1996).

Intracellular localization of mutant glycoprotein by immunofluorescence staining

The gB glycoprotein has been shown to be localized in the ER, Golgi complex, cell surface and the nuclear envelope by indirect immunofluorescence or immunoelectron microscopy (Gilbert & Ghosh, 1993; Gilbert et al., 1994; Torrisi et al., 1992). The intracellular localization of the mutant proteins was determined by indirect immunofluorescence staining of COS-1 cells transfected with wild-type or mutant gB gene. In agreement with an earlier report, wild-type gB was localized in the nuclear envelope and in the perinuclear structures representing Golgi complex and ER (Gilbert et al., 1994). All 13 mutant gB proteins showed similar distinct nuclear rim staining (Fig. 3). The mutants also showed perinuclear labelling in addition to the nuclear rim staining indicating ER and Golgi localization. Examination for cell surface immunofluorescence showed staining of the plasma membrane of cells expressing all mutants except P774L. The mutants G743R and F770S showed a much weaker staining at the cell surface. The rate of intracellular transport was also determined by acquisition of Endo H resistance. Results of Endo H digestion showed that mutant P774L was completely sensitive to Endo H digestion even after a chase of 2 h while mutants G743R and F770S showed partial resistance. All of the other mutants showed Endo H resistance pattern similar to wild-type gB protein (data not shown).

Complementation of a gB-null virus by the gB-1 substitution mutants in the hydrophobic region

A trans complementation assay using wild-type or mutant glycoproteins such as gB, gD or gH expressed from a transfected plasmid and defective HSV virions lacking the corresponding glycoprotein providing all other viral proteins, has been extensively used to determine regions of the glycoprotein that are essential for infectivity (Cai et al., 1988a; Chiang et al., 1994; Wilson et al., 1994). The gB-null virus K082 has been used for complementation of mutant gB proteins to delineate functional regions (Cai et al., 1988a; Rasile et al., 1993). Functional complementation between gB proteins of different herpesviruses using K082 virus as a complementing virus have also been reported to determine their functional homology (Mettenleiter & Spear, 1994). The abilities of the mutant gB-1 proteins constructed by substitution of conserved residues in the hydrophobic region to rescue the infectivity of the gB-null virus, K082, were determined by the complementation assay. Vero cells were transfected with plasmid pKBXX containing the wild-type gB-1 or the mutant constructs and subsequently infected with K082 virus, which can grow only on a gB-producing cell line such as D6 (Cai et al., 1988a) or VB38. The titres of progeny virus stock obtained from transfected Vero cells after infection with K082 virus were determined on both Vero and VB38 cells. The complementation efficiency was calculated as a percentage of plaquing efficiency of the progeny virus obtained from mutant gB transfected cells compared to the plaquing efficiency of the progeny virus produced by transfection with wild-type gB-containing plasmid. Plaquing efficiency of complemented virus
Fig. 4. Complementation of K082 virus by transfection with plasmids containing mutant gB constructs. Vero cells were transfected with pKBXX plasmid containing the wild-type gB or mutant gB constructs. After 24 h, transfected cells were infected with K082 virus at an m.o.i. of 2. Virus stocks were isolated 24 h later and titered on duplicate on both Vero and VB38 cells. Virus produced by complementation of K082 with wild-type gB showed titres of \( \left(5 \times 10^2 \right) - \left(1 \times 10^3\right) \) p.f.u. per ml in Vero and VB38 cells, respectively. The titres for virus produced in non-transfected Vero cells infected with K082 virus were \( \left(2 \times 10^2\right) - \left(6 \times 10^3\right) \) p.f.u. per ml in Vero and VB38 cells, respectively. Titres are the average of three separate complementation experiments. The titre on the non-complementing Vero cells could be due to a low level contamination of wild-type virus in the K082 stock or from recombination between transfected plasmid and K082 virus. Plaquing efficiency of complemented virus was determined as the ratio of the titre on VB38 cells to the titre on Vero cells. Complementation efficiency is expressed as the percentage of plaquing efficiency of virus produced by transfection with wild-type gB plasmid pKBXX. The error bars show standard deviation for the three experiments.

Fig. 5. Incorporation of mutant gB proteins into the virion. Vero cells transfected with plasmids containing wild-type or mutant gB genes were infected with K082 virus and labelled with \[^{35}\text{S}\]methionine for 24 h. Radioactive virus was isolated by pelleting through a 30% sucrose cushion. Radiolabelled viruses were suspended in lysis buffer, immunoprecipitated and analysed by SDS–PAGE. Results shown in \((a)\) and \((b)\) are from two separate experiments. WTgB, virus particles recovered from cells transfected with wild-type gB gene. Virus particles recovered from cells transfected with the gB mutants are indicated.

G766N, F770S and P774L showed negligible complementation of K082 virus indicating that these non-variant residues may be important for virus infectivity.

**Incorporation of mutant gB proteins into virions**

The negligibly low complementation efficiency of the mutants G746N, G776N, F770S and P774L could be due to the fact that although the mutants formed dimers and associated with the nuclear envelope, they could not be incorporated into the virion particles, suggesting that these conserved residues may be required for the assembly of gB in the viral envelope. Alternatively, the mutants were incorporated in the virion particles, which were, however, non-infectious, suggesting the possibility that particles containing mutant gB molecules were incompetent for virus entry. The presence of mutant gB molecules in the virion particles was, therefore, determined by immunoprecipitation of the labelled proteins with a gB antibody specific for dimer. Similar amounts of radioactivities were recovered from pelleted virion particles generated from the complementation experiments. SDS–PAGE analyses of the same amount of the virion particles showed that the polypeptide profiles for the wild-type and the mutant virions were very similar (data not presented). Results presented in Fig. 5.
show that virion particles obtained from the complementation experiments contained proteins which were immuno-precipitated with anti-gB antibody and co-migrated with gB protein, indicating that mutant gB proteins were incorporated in the recovered virion particles. Virion particles obtained from Vero cells infected with K082 virus in the absence of any transfecting plasmid did not show the presence of any protein band corresponding to the gB band.

**Effect of substitution of conserved residues in the hydrophobic region of gB protein on virus penetration**

The entry of HSV-1 into the host cell depends on initial binding of the virus particles to the cell surface followed by fusion of the viral envelope with the cell membrane. Since gB-1 was shown not to be essential for virus attachment (Cai et al., 1988a; Herold et al., 1991) the observed lack of complementation by the mutant gB-1 proteins could not be due to lack of binding of the virus to the host cell plasma membrane. Thus, the substitution of conserved residues in the hydrophobic region of gB-1 could affect its entry. We, therefore, measured the rate of virus entry by determining the rate at which the virus attached to the cell surface becomes resistant to treatment with low pH buffer as a result of penetration of the virus into the cell as compared to controls not exposed to acidic pH (Highlander et al., 1989). The rate of entry of the progeny virion particles was determined by using the gB-producing VB38 cell line, which allows replication of the penetrated virion particles. Analogous studies to assess the rate of entry of virus particles containing mutant gB or gH protein using gB- or gH-producing cell lines have been described (Desai et al., 1994; Wilson et al., 1994). The results presented in Fig. 6 show that mutants A786S, A786T, A790S and A786S/L788M, which were complementation-competent, showed rates of entry that were similar to that of the wild-type gB. Mutants A791S and A790Q, which showed diminished complementation of about 50 and 25%, respectively, also showed kinetics of virus entry similar to that of the wild-type. The rate of entry of the remainder of the mutants which showed reduced or negligible complementation activities was, however, markedly reduced compared to the wild-type (Fig. 6). The mutant P774L, which failed to show any complementation and was also compromised in its transport to the cell surface, was extremely slow in entering the cells. In this case, 50% of virus particles containing P774L were resistant to acid-treatment after 150 min compared to 20 min required for 50% survival of virus particles containing wild-type gB-1 protein.

**Discussion**

In the present report, we have investigated the role(s) played by a number of highly conserved amino acids present in the carboxy-terminal hydrophobic region of gB, which is essential for its infectivity (Rasile et al., 1993). A complementation assay measuring the ability of mutant proteins to rescue the infectivity of gB-null virus, K082, in trans showed that the mutants could be grouped under three categories: (i) mutants that were fully infectious as the wild-type gB included A786S, A786T, A790S and A786S/L788M; (ii) mutants that showed reduced infectivity of 50–20% included A791S, A786Y, A786N, A790Q and G743R; and (iii) mutants that showed negligible infectivity of less than 10% included G746N, G766N, F770S and P774L. The mutation sites in the first two groups, except for G743R, are present in the membrane anchor region of gB. The mutation sites of the last group are located in segments 1 and 2 of gB and represent residues that are non-variant in the family Herpesviridae. Except for A790Q and A791S, mutants which showed reduced infectivity also showed decreased rate of entry. Mutants which were negligibly infectious also showed drastically reduced rate of entry. Mutants that showed reduced or negligible infectivity contained mutant gB proteins in the envelope of the virions in amounts comparable to the amounts of mutant proteins present in progeny virions that were fully infectious. Thus, the observed loss of infectivity due to substitution of the non-variant residues in segments 1 and 2 of gB protein may not be due to reduced incorporation in the virion envelope. Mutant G743R which exhibited reduced infectivity also showed a slower intracellular transport suggesting that replacement of glycine with arginine affected the folding pattern. Substitution
of the non-variant residues in segment 2, namely P770 and P774, also affected their intracellular transport and hence folding pattern. Proline-774 is a non-variant residue which is located between segment 2 and the membrane-anchoring segment 3 and thus may separate the two domains. Replacement of this residue with hydrophobic leucine could thus induce a drastic conformational change as evidenced by its defective intracellular transport. The observed loss of the complementation ability and 7-fold reduction in its rate of entry could, therefore, be due to this conformational change. This is in agreement with the observation that replacement of the tripeptide KNP containing non-variant residues N749 and P750 present in segment 2 of HCMV gB protein with a hydrophobic tripeptide VAI caused misfolding as shown by blocked cell surface expression and arrested transport in the endoplasmic reticulum (Zheng et al., 1996). However, the mutant protein formed oligomers and was recognized by conformational-specific monoclonal antibodies. The biological activity of this mutant was not tested.

Significant reduction in the rate of entry of viruses containing mutations in segment 1 and 2 suggests the involvement of non-variant residues in virus penetration. In the case of gB glycoprotein of HCMV (Bold et al., 1996; Tugizov et al., 1995) or BHV-1 (Li et al., 1997), putative fusogenic domains spanning segments 1 and 2 or segment 2 have been proposed. However, in the case of HSV-1 gB protein, no fusogenic domain has yet been established. Since the non-variant residues in gB protein that affect the entry of HSV-1 are also conserved in the fusogenic domains of HCMV or BHV-1 gB protein it may be hypothesized that hydrophobic segment 2 either alone or in combination with segment 1 may serve as an internal fusogenic domain of HSV-1 gB. Segments 1 and 2 are not embedded in the viral envelope but peripherally associated with the membrane and thus available for insertion into a target membrane. The presence of internal fusion peptide in viral fusion protein such as vesicular stomatitis virus G protein (Zhang & Ghosh, 1994) or Semliki Forest virus E1 glycoprotein (Levy-Mintz & Kielian, 1992) has already been established. Since glycine residues present in the fusion peptides of a number of viral fusion proteins (Hernandez et al., 1996), namely influenza virus HA (Skehel et al., 1995), Semliki Forest virus E1 (Levy-Mintz & Kielian, 1992), vesicular stomatitis virus G (Zhang & Ghosh, 1994), human immunodeficiency virus gp 41 (Freed & Martin, 1995) and paramyxovirus F1 (Sergel-Germans et al., 1994), were shown to be required for fusogenic activity, the non-variant glycines in segments 1 and 2 of the hydrophobic domain of gB protein may be important for membrane fusion. Proline and phenylalanine are also present in fusion peptides and have been implicated in fusogenic activity (Hernandez et al., 1996). Studies involving site-specific monoclonal antibodies of HSV-1 (Highlander et al., 1988; Navarro et al., 1992) and HCMV (Zheng et al., 1996) gB protein suggest that the mid-region of the ectodomains, for example, residues 241–441 of HSV-1 gB, may be required for virus penetration and cell fusion. It was further shown that the cytoplasmic region of HSV-1 and HCMV gB protein also contained important determinants for virus entry and membrane fusion (syncytia induction) (Gage et al., 1993; Highlander et al., 1988; Zheng et al., 1996). It was suggested that transmembrane region of gB protein may participate in transduction of signals from fusogenic domains in the cytoplasmic tail to the fusogenic regions in the ectodomain. Thus, the substitution of the non-variant residues within the hydrophobic region can affect the transduction of signals involved in fusogenic activity.

The entry of herpesviruses requires the involvement of four transmembrane glycoproteins gB, gD, gH and gL (Cai et al., 1997; Davis-Poynter et al., 1994; Ligas & Johnson, 1988; Roop et al., 1993). It is suggested that in the viral envelope, they may form hetero-oligomers (Handler et al., 1996a, b; Zhu & Courtney, 1988) which are involved in virus entry. The multi-subunit complex containing gB protein involved in virus entry and cell fusion could further interact with cellular coreceptors (Montgomery et al., 1996; Terry-Allison et al., 1998) or with specific cellular proteins which would induce conformational change of the fusogenic region. Changes in conformation from a non-fusogenic to fusogenic state by exposure to low pH (Gaudin et al., 1995; Hernandez et al., 1996) or binding to coreceptors (Berger, 1997) have been documented for other viral fusion proteins. Thus, one can hypothesize that, in the case of herpesviruses, the multi-subunit complex involved in entry and fusion may be formed by interactions via the transmembrane segment as well as the ecto- and cytoplasmic domains of gB protein. Recent studies have indeed shown that interactions between the transmembrane segments within lipid bilayers are essential for functional assembly of integral membrane proteins (Casson & Bonfaccino, 1992; Shai, 1995). Disruption of the specific amino acid sequence or local peptide structure of gB protein within the viral membrane may thus affect the biological activity. Recently a system using gB, gD, gH and gL proteins of HSV-1 has been used to demonstrate in vitro fusion of cells (Turner et al., 1998). Using this system, it may be possible to test if mutations of non-variant residues present in the hydrophobic region of gB-1 protein also affect fusion. Alternately, one can introduce these specific mutations in the fusogenic domains of gB protein of BHV-1 (Li et al., 1997) or HCMV (Bold et al., 1996; Tugizov et al., 1995) and determine the fusogenic activity of the mutant proteins.

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References

Mutations in HSV gB affect infectivity


