Truncation of the Carboxy-Terminal 28 Amino Acids of Glycoprotein B Specified by Herpes Simplex Virus Type 1 Mutant *amb1511-7* Causes Extensive Cell Fusion†

ABOLGHASEM BAGHIAN, LI HUANG, SUSAN NEWMAN, SUKHANYA JAYACHANDRA,
AND KONSTANTIN G. KOUSOULAS*

*Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine,
Louisiana State University, Baton Rouge, Louisiana 70803-8416*

Received 3 November 1992/Accepted 12 January 1993

Three amber mutations were introduced proximal to the *syn3* locus of the herpes simplex virus type 1 glycoprotein B (gB) gene specifying gB derivatives lacking the carboxy-terminal 28, 49, or 64 amino acids. A complementation system that utilized gBs expressed in COS cells to complement gB-null virus KΔT was established. The 49- or 64-amino-acid-truncated gBs failed to complement gB-null virus KΔT, while the 28-amino-acid-truncated gB complemented KΔT efficiently. Mutant herpes simplex virus type 1 KOS (*amb1511-7*) specifying the 28-amino-acid-truncated gB fused Vero cells extensively.

Herpes simplex virus type 1 (HSV-1) glycoprotein B (gB) is virally encoded and embedded in the viral envelope and in cell membranes of infected cells. The gB gene was sequenced, and the primary and secondary structures of the gB protein were predicted (3, 17). gB is essential for the production of infectious virus particles (6, 16). Analyses of several mutant viruses showed that gB is essential in the penetration of virions into host cells and virus-induced membrane fusion of adjacent cells (3, 6, 9, 13, 15, 16, 21-23). A mutation in gB causing extensive syncytium (*syn*) formation was mapped to the carboxy-terminal portion of gB (2, 13) which was predicted to lie in the cytoplasm (8, 17). This mutation (*syn3*) is most probably an Arg-to-His change at amino acid position 857 in the published HSV-1(KOS) protein sequence (2), which is amino acid position 828 in the HSV-1(KOS) and HSV-1(F) mature proteins after cleavage of the signal peptide (17). This amino acid change is located near the carboxy terminus of a predicted 31-amino-acid α -helical peptide segment (Fig. 1B). A second site in the cytoplasmic portion of gB that when altered causes cell fusion was identified between residues 816 and 817 in the HSV-1(KOS) protein (4), which are residues 786 (glutamine) and 787 (leucine) in the HSV-1(KOS) and HSV-1(F) mature proteins (17). An elegant complementation system which utilizes gB-null viruses and transfected gB genes was used to map functional domains of gB (4-6). The carboxy-terminal 41-amino-acid segment of HSV-1 gB was shown to be nonessential for production of infectious virus particles, glycoprotein maturation, and virus-induced cell fusion (11). We report here the development of a system which utilizes simian virus 40-based expression vectors to complement gB-null viruses and to create recombinant viruses specifying altered gBs. We used this system to delineate carboxy-terminal domains of gB involved in the production of infectious virus and cell fusion.

Construction of gB genes that specify carboxy-terminal truncations. The *syn3* mutation is located at the end of a

predicted 31-amino-acid α helix (Fig. 1B). To examine the role of this predicted α -helical domain of gB in virus-induced cell fusion and virus growth, we constructed three amber mutations in gB genes. Plasmid pRB2080 contains a 3,353-bp HSV-1(F) DNA including the entire coding region of gB, 150 bp from the 5'-flanking sequence, and 493 bp from the 3'-flanking sequence (13). A *SalI*-*Bam*HI DNA fragment from pRB2080 containing the gB sequence was cloned into a pUC19 plasmid (24) that was previously modified to carry a *Bgl*II site within its polylinker, creating plasmid p2080. Subsequently, gB was excised from p2080 with *Bgl*II and *Bam*HI and cloned into the *Bgl*II site of P90123, placing the 5' end of the gB gene proximal to the adenovirus late promoter in P90123 (26) as explained for a similar construct previously (18). Plasmid constructs p1511, p1528, and p1513 were constructed by insertion of the synthetic oligonucleotide linker *Spe*I* (CTAGACTAGTCTAG) that carries stop codons in all three reading frames and the recognition sequence for restriction enzyme *Spe*I into unique restriction sites *Bst*EII (p1511), *Nco*I (p1528), and *Nhe*I (p1513) of p2080. Plasmid p1537 was created by an in-frame fusion of clones pRB2024 and pRB2107 (13) resulting in an internal truncation from amino acids 130 to 697. These modified gB genes were then cloned into P90123 to produce plasmids P9-1511, P9-1528, P9-1513, and P9-1537 as explained above for p2080. The DNA sequence of each construct containing the site of the *Spe*I* linker insertion was determined by polymerase chain reaction (PCR)-assisted DNA sequencing as described previously (12). Briefly, a 297-bp DNA fragment of gB was amplified by PCR using primers gB1 (5'-CTAACCACCAAGGAGCTCAAGAA-3') and gB2 (5'-AGGTCGTCCTCGTCGGCGTCA-3'). PCRs were performed with 2.5 U of *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, Md.) in 100- μ l reaction mixtures containing 0.01% bovine serum albumin and 0.1% Tween 20 in 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 2 mM MgCl₂, 200 μ M (each) deoxynucleoside triphosphates, and each primer at 0.2 μ M. The samples were overlaid with mineral oil and subjected to 22 cycles of 2 s at 98°C, 1 min at 66°C, and 2 min at 72°C in a programmable DNA thermal cycler (Eppendorf, Fremont, Calif.). Final extension was at 72°C for 5 min. Single-stranded DNA of the amplified gB DNA fragment was

* Corresponding author.

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FIG. 1. (A) DNA and predicted amino acid sequences of a portion of the HSV-1(F) gB gene, *amb*1511-7, and plasmids P9-1511, P9-1513, and P9-1528 from amino acids 804 to 848 (17). The corresponding portion of the published HSV-1(KOS) gB sequence is from amino acids 834 to 878 (3). The *Spe*I* insertions in *amb*1511-7, P9-1511, P9-1513, and P9-1528 are shown underneath the gB sequence, and the in-frame stop codons are indicated. (B) A schematic representation of the predicted secondary structure of the cytoplasmic portion of glycoprotein B is shown as described previously (17). The gB truncations in *amb*1 and *amb*1511-7 mutant viruses, P9-1513, P9-1528, and the *syn*3 mutation are indicated by arrows. The relevant predicted α helices are denoted as H17a and H17b and marked by dashed lines.

FIG. 2. COS cells were transfected with plasmids P9-2080, P9-1511, P9-1528, P9-1513, and P9-1537 and labeled with ^{35}S -methionine from 40 to 46 h posttransfection. COS cell lysates were immunoprecipitated with anti-gB MAb HSB1. Immunoprecipitates were electrophoresed in an SDS-10% polyacrylamide gel and visualized by autoradiography. Lanes: 1, P9-2080 gB; 2, P9-1511 gB; 3, P9-1528 gB; 4, P9-1513 gB; 5, P9-1537 gB. Molecular mass standards were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). The positions of the molecular mass standards were marked with radioactive ink after the gel was stained with Coomassie blue.

produced by PCR using 10 μl of the amplified gB DNA and primer gB2 at 0.2 μM . PCR parameters were 25 cycles of 3 s at 98°C, 1 min at 66°C, and 1.5 min at 72°C with a final incubation at 72°C for 5 min. The best results were obtained by using 5 to 10 pmol of primer gB1 per reaction for dideoxy DNA sequencing reactions (20).

The gB DNA sequence specifying the 45-amino-acid peptide segment from amino acids 804 to 848 of the HSV-1(F) protein that contains the *SpeI** linker insertions is shown in Fig. 1A. The predicted secondary structure of the carboxy-terminal portion of gB showing the locations of the truncations specified by P9-1511, P9-1513, and P9-1528 as well as the locations of other relevant mutations is shown in Fig. 1B. P9-1511, P9-1513, and P9-1528 are predicted to specify carboxy-terminal truncations of 28, 49, and 64 amino acids, respectively. In P9-1511, the insertion of the *SpeI** linker produced an in-frame stop codon. Insertion of the *SpeI** linker in P9-1513 added two amino acids (Leu and Asp) before encountering a stop codon. Similarly, an additional Ile was added in P9-1528 (Fig. 1A).

Expression of gB genes in COS cells. COS cells were transfected with gB gene constructs cloned into P90123 by the calcium phosphate method (7, 10). Transfected cells

FIG. 3. Photograph of an ethidium bromide-stained agarose gel in which each PCR DNA fragment obtained with primers gB1 and gB2 was digested with *SpeI* and electrophoretically separated. Lane 1, λ phage DNA 1-kbp ladder (BRL); lanes 2, 3, 4, 5, and 6, PCR DNA fragments restricted with *SpeI* from KOS and *amb1511*-4, -7, -9, and -19 viral DNAs, respectively.

were labeled with ^{35}S -methionine from 40 to 46 h posttransfection. Proteins were extracted from transfected cells with 1% Nonidet P-40 and 0.5% sodium deoxycholate in extraction buffer (50 mM Tris buffer, 50 mM NaCl, 100 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of aprotinin per ml, pH 7.5) and immunoprecipitated as described previously (18). Samples were electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gels (14, 19). Monoclonal antibody (MAb) HSB1 was produced in this laboratory by following standard procedures (1). HSB1 precipitated gB from cellular extracts of HSV-1(KOS)-infected Vero cells and COS cells in a manner similar to that of MAb H126 (18, 19). A comparison of the reactivities of MAb HSB1 and H126 is shown in Fig. 5. Because of the availability of larger amounts of MAb HSB1, it was used throughout these experiments. Radioimmunoprecipitates of gB expressed in COS cells are shown in Fig. 2. P9-2080 specified gB migrating with an apparent molecular mass of approximately 115 kDa, determined by comparison with the molecular mass standards described in the legend to Fig. 2. P9-1511, P9-1528, and P9-1513 expressed two forms of gB. The faster-migrating gB form produced by each gB construct represented most probably the high-mannose precursor form of gB, since it comigrated with gB radiolabeled during a 30-min labeling pulse (not shown). As expected, gB was not detected in P9-1537-transfected COS cells.

Isolation of virus recombinants. To isolate viruses that specify the truncated gB derivatives, COS cells were cotransfected with intact plasmids P9-1511, P9-1513, P9-1528, and P9-2080 (15 μg each per 25-cm² flask) and intact wild-type HSV-1(KOS) viral DNA (1 μg per flask). Viral DNA was prepared by centrifugation of infected-cell lysates in NaI density gradients (25). Progeny viruses were harvested 72 h postinfection (hpi) and used to infect confluent monolayers of Vero cells. Individual, well-separated viral plaques were examined by phase-contrast microscopy at 24 to 48 hpi. With the exception of virus stocks from cotransfections with P9-1511, all other cotransfections produced virus plaques that were indistinguishable from wild-type KOS plaques. Viruses obtained from cotransfections of wild-type KOS viral DNA with P9-1511 reproducibly re-

FIG. 4. Phase-contrast photomicrographs (magnification, $\times 125$) of KOS (A), *tsB5* (B) and *amb1511-7* (C) virus-infected Vero cells observed at 24 hpi.

sulted in approximately 5% syncytial plaques in Vero cells but not in HEP-2 cells (data not shown).

Several syncytial plaques from cotransfections of intact KOS viral DNA and P9-1511 were isolated and plaque purified three times. Viral DNAs from each virus plaque were subjected to PCR analysis using primers gB1 and gB2, and PCR products were digested with restriction endonuclease *SpeI*. The resultant DNA fragments were visualized after electrophoresis in a 1.5% agarose gel followed by ethidium bromide staining (Fig. 3). Digestion of the 297-bp DNA fragments obtained from syncytial *amb4*, *amb7*, *amb9*, and *amb19* mutants with *SpeI* resulted in faster-migrating DNA species, indicating that these DNA fragments contained an *SpeI* site. In contrast, digestion with *SpeI* of the 297-bp fragment derived from KOS viral DNA did not result in faster-migrating DNA species (Fig. 3). The presence of the *SpeI** linker was confirmed by DNA sequencing of the PCR-amplified 297-bp DNA fragments. No other mutation was found within the PCR-amplified 297-bp DNA fragments. A gB DNA segment containing the *SpeI** linker from mutant *amb1511-7* virus is shown in Fig. 1A. Additional DNA sequencing of approximately the terminal one-third of the entire gB gene of the *amb1511-7* virus revealed no other mutations.

Cell fusion caused by *amb1511-7*. Confluent monolayers of Vero cells were infected with the KOS, *tsB5*, and *amb1511-7* strains at a multiplicity of infection of 5, and the cell cultures were examined by phase-contrast microscopy and photographed at 24 hpi. The *amb1511-7* strain fused Vero cells extensively (Fig. 4) but failed to fuse HEP-2 cells (not shown).

Synthesis of gB specified by mutant *amb1511-7*. Vero cell monolayers were infected with HSV-1(KOS), the *tsB5* strain, or the *amb1511-7* strain at a multiplicity of infection of 5 and labeled with 50 μ Ci of 35 S-methionine per ml from 6 to 24 hpi. Infected cells were harvested by scraping with a rubber policeman and were washed two times in phosphate-buffered saline. Immunoprecipitates from KOS, *tsB5*, and *amb1511-7* strain-infected cellular extracts with MAb HSB1 or H126 revealed the presence of gB migrating with an apparent molecular mass of approximately 115 kDa (Fig. 5). In addition, two gB-related peptides of 49 and 44 kDa were precipitated. Cell surface expression of gB was tested by immunofluorescence assay and fluorescence-activated cell sorting analysis. *amb1511-7* strain-infected cells expressed gB that was transported to the cell surface in amounts similar to the amount of gB synthesized in KOS-infected cells (not shown).

Complementation of gB-null virus KAT by COS cells transfected with gB genes. gB-null virus KAT replicates in D6 cells but not Vero cells. To ascertain whether gB constructs cloned into expression vector P90123 under the control of the adenovirus late promoter were able to complement KAT, COS cells were transfected with the gB constructs and at 16 h posttransfection were infected with gB-null virus KAT. Infected cells were collected at 24 hpi, and viral stocks were prepared and their titers were determined on both Vero and D6 cells. The results of these experiments are shown in Table 1. Incorporation of a functional gB molecule (provided by the transfected gB gene) into the KAT virion envelope resulted in infectious virus particles that successfully infected D6 and Vero cells (complementation). Viral plaques formed on D6 cells because these cells provided gB for subsequent rounds of infectious virus particle production required for plaque formation. Viruses that formed plaques on Vero cells probably represent KAT rescued virus in

FIG. 5. Vero cells were infected with KOS, *tsB5*, and *amb1511-7* virus strains and labeled with 35 S-methionine from 6 to 24 hpi. Cell lysates were reacted with anti-gB MAb HSB1 or H126, and the precipitates were electrophoretically separated in an SDS-10% polyacrylamide gel. Lanes: M, molecular mass standards as described in the legend to Fig. 2; 1 and 2, HSV-1(KOS); 3 and 4, *tsB5* virus; 5 and 6, *amb1511-7* virus. Odd-numbered lanes, reactions with MAb H126; even-numbered lanes, reactions with MAb HSB1.

which the truncation in the gB gene of KAT virus was repaired through recombination with the transfected gB gene. P9-1537 served as the negative control for these experiments. This plasmid carries a large deletion in the gB gene and cannot complement or rescue the KAT virus. Virus stocks prepared from COS cells transfected with P9-1537 and superinfected with virus KAT did not produce any viral plaques on either D6 or Vero cells. Virus stocks from cultures transfected with P9-2080 and later superinfected with virus KAT produced 5.2×10^3 virus plaques on D6 cells and 4 plaques on Vero cells, indicating that gB specified by P9-2080 complemented virus KAT. The small number of viral plaques produced on Vero cells probably represented KAT rescued viruses. Plasmid P9-1511 complemented KAT virus as efficiently as P9-2080. In contrast, plasmids P9-1513,

TABLE 1. Complementation of HSV-1(KAT) by COS cells transfected with gB genes

Transfected plasmid	Growth (PFU/ml) ^a in:	
	D6 cells	Vero cells
P9-2080	5.2×10^3	4
P9-1511	2.4×10^3	8 (6 <i>syn</i> ⁺ + 2 <i>syn</i>)

^a COS cells were transfected with gB genes and infected with mutant KAT 16 h later. Virus stocks were prepared at 24 hpi. There was no growth in either D6 or Vero cells transfected with P9-1513, P9-1528, or P9-1537.

P9-1528, and P9-1537 failed to complement KAT (Table 1). Virus stocks from P9-1511 transfections produced *syn* and *syn*⁺ wild-type virus plaques on Vero cells. These viral plaques were collected, and viral DNA was extracted and tested for the presence of an *SpeI** linker by restriction with *SpeI* and DNA sequencing as explained earlier. Viral DNA from *syn* viral isolates contained an *SpeI** linker site within the PCR-amplified gB DNA fragments. Viral DNAs from *syn*⁺ viruses did not contain *SpeI** linkers.

Conclusions. Transient expression vectors such as P90123 used in this study replicate to high numbers in COS cells, allowing the expression and characterization of modified gB proteins in the absence of any other viral proteins. We show here for the first time that modified gB genes cloned into P90123 can be used to efficiently complement gB-null virus KAT and to isolate recombinant viruses.

It was reported that mutant *amb1* virus specifying a 41-amino-acid-truncated gB derivative replicated normally in Vero cells, yielding titers similar to those yielded by the wild-type virus strain HSV-1(KOS) (11). Similarly, we found that the truncation of 28 amino acids of gB specified by mutant *amb1511-7* did not affect the ability of this virus to replicate in cell culture. The 41-amino-acid gB truncation specified by the *amb1* virus leaves the α -helical domain H17a intact (Fig. 1B). We found that truncated gB derivatives of 49 and 64 amino acids failed to complement gB-null virus KAT, and recombinant viruses specifying these truncations could not be isolated. These truncations disrupt the α -helical domain H17a (Fig. 1B). Therefore, domain H17a may be important for virus replication.

The *amb1* virus did not fuse Vero cells, although it produced smaller plaques (11). We show here that the 28-amino-acid gB truncation specified by *amb1511-7* caused extensive cell fusion. This truncation eliminates half of a predicted 14-amino-acid α helix (H17b) located immediately after the 31-amino-acid α helix H17a (Fig. 1B) which may be directly involved in the production of cell fusion. Alternatively, we favor the hypothesis that the 28-amino-acid truncation induces certain conformational changes in the cytoplasmic portion of gB that result in cell fusion. This implies that the conformation of the cytoplasmic portion of gB may be important in virus-induced cell fusion.

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