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The Carboxy-Terminal 41 Amino Acids of Herpes Simplex Virus Type 1 Glycoprotein B Are Not Essential for Production of Infectious Virus Particles

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Glycoprotein B (gB) is a virally encoded protein that is found in the envelope of herpes simplex virus type 1 and membranes of cells infected with herpes simplex virus type 1. It is essential for the production of infectious virus particles. An amber mutation was introduced into the gB gene by oligonucleotide-directed mutagenesis at the codon for amino acid 863 of the protein. Virus carrying this mutation should synthesize gB molecules lacking the last 41 amino acids of the cytoplasmic domain. Immunoprecipitation of infected cell extracts demonstrated the synthesis of appropriately truncated gB molecules. Characterization of the mutant virus indicated that the loss of the carboxy-terminal 41 amino acids has little effect on gB function.

Glycoprotein B (gB) is one of several virally encoded proteins that is found in the envelope of the herpes simplex virus type 1 (HSV-1) virus particle and in HSV-1-infected cell membranes. The gB gene has been cloned and sequenced (1, 2, 19, 21) and has been predicted to encode a polypeptide of 904 amino acids with a molecular weight of 100,500. Mature forms of the protein range in size from 110 to 120 kilodaltons because of glycosylation (23). Prediction of protein secondary structure, based on the amino acid sequence inferred from the DNA sequence, has suggested the localization of domains in gB. These analyses predict that the protein has a 30-amino-acid signal sequence, a 697-amino-acid external domain, a 68-amino-acid transmembrane domain, and a 109-amino-acid cytoplasmic domain at the carboxy terminus (1, 2, 19, 21).

gB is essential for the production of infectious virus particles. Various HSV-1 gB mutants display a range of phenotypes, including an altered rate of viral entry into the host cell, failure to enter cells, temperature sensitivity for infectious virus production, altered immunoreactivity to monoclonal antibodies, altered carbohydrate processing, and the ability to form syncytial plaques (4, 4a, 7, 10, 11, 13–15, 22, 23; S. L. Highlander, D. J. Dorney, P. J. Gage, T. C. Holland, W. Cai, S. Person, M. Levine, and J. C. Glorioso, submitted for publication). Analyses of these mutant phenotypes have led to the hypothesis that gB is involved in the penetration of virions into the host cell and that it can act as a fusion protein. Marker rescue experiments and DNA sequence analysis of gB mutants have mapped monoclonal antibody resistance, rate of entry, and temperature sensitivity mutations to the external domain of gB (3, 6, 11; Highlander et al., submitted). Syncytium-inducing (*syn*) mutations have been mapped to the cytoplasmic domain (3, 4, 7). Using a complementation test, Cai et al. (4) have shown that gB is essential for virus-induced cell fusion.

To examine further the function of the cytoplasmic domain, an amber mutation was introduced by oligonucleotide-directed mutagenesis into the gB gene at the codon for amino acid 863. An 18-nucleotide fragment, 5′-GCCCTTTTTC TAGGCCTT-3′, was synthesized. This oligonucleotide was complementary to the sense strand of the gB gene, except for two base mismatches at nucleotides 3379 and 3384 (Fig. 1). Oligonucleotide-directed mutagenesis was carried out by modifications of the procedures described by Norris et al. (18). M13mp9-110, an M13mp9 recombinant plasmid containing the 1,060-base-pair (bp) *Sma*I fragment of the sense strand of the gB gene, was used as the target DNA (Fig. 2). This fragment encodes the cytoplasmic domain of the gB gene. A 19-base oligonucleotide M13 universal sequencing primer (P-L Biochemicals, Inc., Milwaukee, Wis.) and the mutagenizing oligonucleotide were annealed to the single-stranded M13mp9-110 DNA at 37°C for 45 min so that they could act as primers for the synthesis of a second DNA strand by Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) by the protocol described by the manufacturer. The mismatch at nucleotide 3379 simultaneously created an in-frame amber (TAG) codon and a *Stu*I restriction site (Fig. 1). The mutation resulting from the second mismatch at nucleotide 3384 did not alter the amino acid, but to facilitate the identification of mutant DNA, it was introduced to use the mutagenizing oligomer as a hybridization probe for the screening of M13 plaques. Use of this method was not necessary, since the mutant plasmid was readily identified by restriction fragment analysis.

Following extension from the two primers, the mutagenized 310-bp *Sph*I-*Sst*I restriction fragment was removed from the M13mp9-110 hybrid and replaced the 310-bp *Sph*I-*Sst*I fragment in pTO4 (Fig. 2). Plasmid pTO4 contains the 3,320-bp *Xho*I-*Kpn*I fragment from HSV-1 (KOS) DNA cloned into the *Hin*dIII and *Ava*I restriction sites of pBR322. The ligation products were transfected into *Escherichia coli* DH-1 (8). A plasmid containing a novel *Stu*I site was isolated

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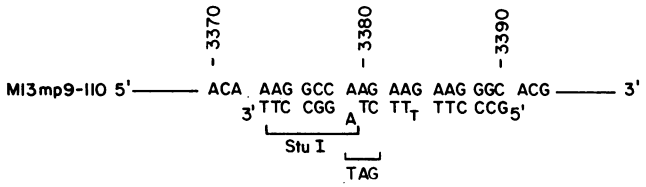

FIG. 1. Annealing of the mutagenizing oligonucleotide (lower sequence) to the target site between nucleotides 3370 and 3390 in the sense strand of M13mp9-110. The mismatch at nucleotide 3379 creates an amber codon (TAG) and a *Stu*I recognition site. The point mutation resulting from the mismatch at nucleotide 3384 is silent.

from *E. coli* and was designated pTO4-*ambB*1 (Fig. 2). DNA sequencing (17) of the 310-bp *Sph*I-*Sst*I fragment and sequences around it verified the presence of the two point mutations and the fact that no other mutations were introduced during the mutagenesis procedures.

To introduce the mutations into the HSV-1 genome, D-6 cells were cotransfected (12) with pTO4-*ambB*1 DNA and that of the HSV-1 mutant KΔT. D-6 is a Vero cell line that expresses gB on HSV-1 infection or HSV-1 DNA transfection and can complement gB-defective viruses (5). D6 cells were used because it was possible that the induced mutation would be lethal for virus production, since the gB function is known to be essential. HSV-1 KΔT (Fig. 2) is a mutant that has the 969-bp *Bst*II E fragment of the gB gene deleted (5). Viruses from the cotransfection experiment carrying the whole gB gene sequence were identified by the viral plaque hybridization procedure of Homa et al. (12) by using the radiolabeled pTO4 *Bst*II E fragment DNA (Fig. 2) as a probe. One of these was designated *ambB*1.

To verify that the mutation in pTO4-*ambB*1 was incorporated into the viral genome of *ambB*1, the viral DNA was analyzed by Southern hybridization for the presence of a new *Stu*I restriction site generated by the mutation (Fig. 3A). Neither the 2,600-bp *Sal*I fragment from wild-type KOS DNA (Fig. 3A, lane e) nor the 1,630-bp *Sal*I band from the gB-deficient KΔT DNA (Fig. 3A, lane f) was cleaved by *Stu*I (Fig. 3A, lanes b and c). The 2,600-bp *Sal*I fragment from *ambB*1 DNA, however, did contain a *Stu*I site (Fig. 3A, lane a). The fragments seen are the sizes predicted if a *Stu*I site was introduced into *ambB*1 DNA at nucleotide 3374 (Fig. 3B). We conclude that the oligonucleotide-directed mutation creating an amber nonsense codon was introduced into the gB gene of this virus.

As a result of the amber mutation at nucleotide 3379, the mutant *ambB*1 gB gene is predicted to encode a protein of 863 amino acids, a loss of 41 amino acid residues from the carboxy terminus of the cytoplasmic domain. A search for the truncated protein was initiated, and the effect of the truncation on the phenotype of HSV-1 mutant *ambB*1 was investigated.

To demonstrate truncated gB, Vero cell cultures were infected separately with wild-type KOS and *ambB*1 virus at multiplicities of infection of 10. At 6 h postinfection, the cultures were pulse-labeled with [^{35}S]methionine for 20 min, and samples were then chased for 2.5 h. The pulse-labeled and pulse-chased samples were then lysed, and extracts were subjected to immunoprecipitation with a pool of anti-gB monoclonal antibodies (B3, B4, B6, and B9 [16]). A portion of each immunoprecipitate was treated with endo-β-*N*-acetylglucosaminidase H (20, 24) to remove N-linked sugars. Figure 4 shows the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the immunoprecipitates. The faster-migrating precursor form of gB, pgB, was seen in all the infections immediately after pulse-labeling. Approximately half of the pgB was converted to the mature form of the protein after the 2.5-h chase. The glycosylated forms of the *ambB*1 protein migrated faster than the wild-

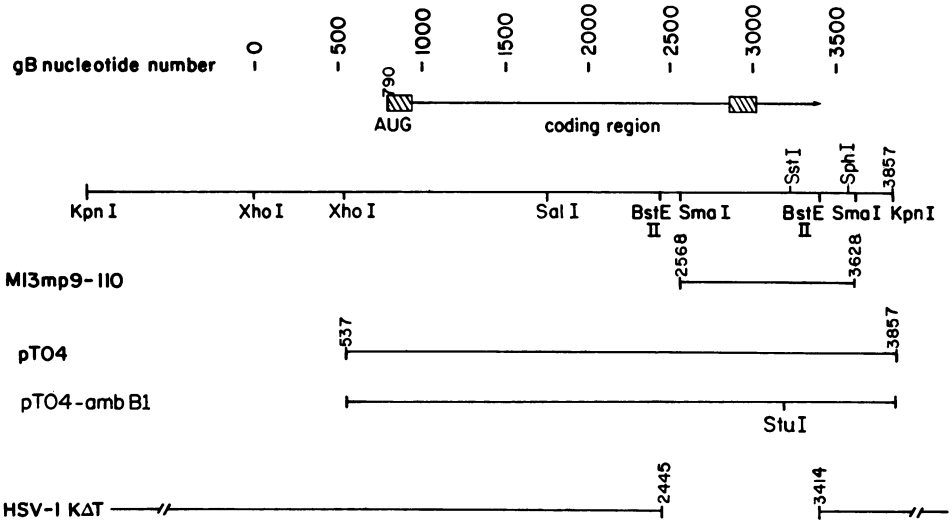

FIG. 2. Viral and cloned gB sequences used in the generation and isolation of the *ambB*1 mutant virus. The 4,900-bp *Kpn*I fragment (HSV-1 genome coordinates 0.347 to 0.378) is shown, with the relevant restriction enzyme sites indicated. The gB gene nucleotides are numbered as described by Bzik et al. (2), and numbering starts from the leftmost *Xho*I site. Numbering for amino acids starts at the initiating methionine codon. The boxes indicate the positions of the signal domain-coding (left) and the transmembrane domain-coding (right) sequences. The *Sma*I fragment in M13mp9-110 contains the target for mutagenesis. The mutated 310-bp *Sst*I-*Sph*I fragment (identified by the restriction sites above the line of the *Kpn*I fragment) was moved from M13mp9-110 into pTO4 to generate pTO4-*ambB*1. The mutated sequence was introduced into the HSV-1 genome by cotransfection of pTO4-*ambB*1 DNA with the DNA of HSV-1 KΔT, a deletion mutant lacking the 969-bp between the two *Bst*II-E sites. In addition to the *Sal*I site shown, there is a second *Sal*I site to the right of nucleotide 3857 (the *Kpn*I site) which was used to generate the *Sal*I fragments described in the legend to Fig. 3.

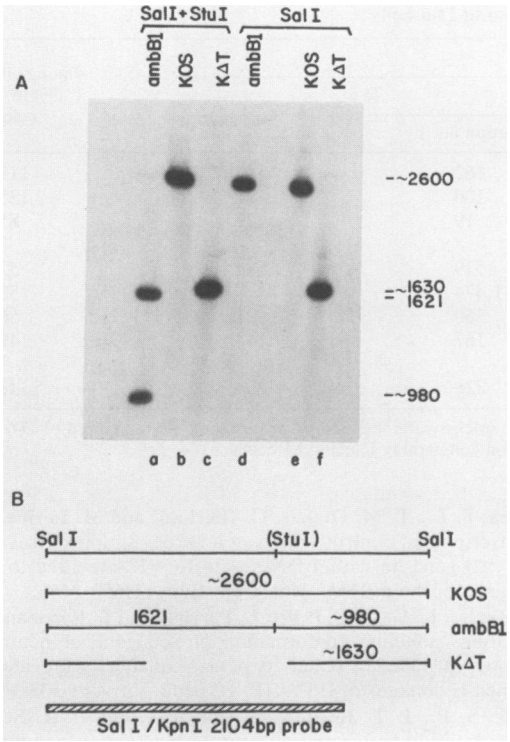

FIG. 3. Southern analysis of viral DNA. (A) *ambB*1, KOS, and KΔT DNA *Sal*I restriction fragments were hybridized to the radiolabeled 2,104-bp *Sal*I-*Kpn*I gB fragment. The *Sal*I site on the right is the same as the second *Sal*I site to the right of nucleotide 3857 (the *Kpn*I site) mentioned in the legend to Fig. 2. *ambB*1 DNA contains a *Stu*I recognition site that is not present in KOS or KΔT DNA. (B) Schematic diagram of hybridizing fragments.

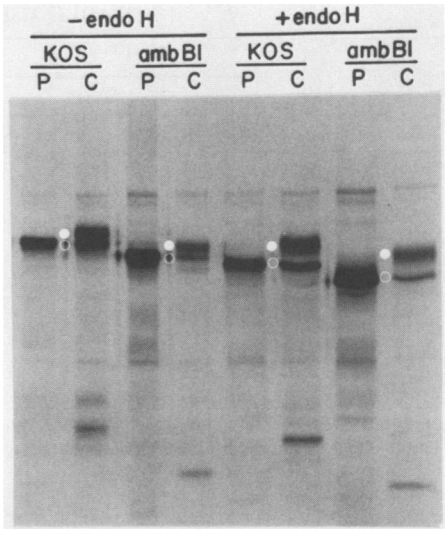

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated [^{35}S]methionine pulse-labeled (P) or pulse-chased (C) gB protein from KOS and *ambB*1 HSV-1 infections. Lanes 1 to 4, No treatment with endo-β-*N*-acetylglucosaminidase H; lanes 4 to 8, treatment with endo-β-*N*-acetylglucosaminidase H. Symbols: ○, positions in the gels of the precursor forms of gB; ●, positions of the mature gB.

type gB protein. Similar size differences between wild-type KOS and *ambB*1 gB were observed after glycosidase H treatment, demonstrating that the differences were caused by truncation of the backbone of the protein rather than an alteration in posttranslational glycosylation.

Somewhat surprisingly, the alteration in the *ambB*1 gB gene did not result in a conditionally lethal mutation. *ambB*1 virions produced infectious progeny and formed phenotypically normal plaques on Vero cells, albeit at a 50% reduction in plating efficiency as compared with that on the gB-complementing cell line D-6 (Table 1). *ambB*1 virus that was propagated in Vero or D-6 cells displayed similar plating efficiencies and yields (data not shown). Thus, *ambB*1 does not have an altered host range. Furthermore, the *ambB*1 plaque morphology was normal on Vero and D-6 cells.

gB has been implicated in the penetration of virions into the host cell. Since mutants of gB showed altered rates of entry, a comparison of the rate at which wild-type and *ambB*1 virus entered Vero and D-6 cells was undertaken by using the citrate buffer inactivation procedure described by Highlander et al. (9). The gB truncation had no effect on the rate of entry kinetics. Both viruses, whether propagated in Vero or D-6 cells and whether they infected either cell line, showed similar rates of entry kinetics (data not shown).

The data presented here lead to the conclusion that the carboxy-terminal 41 amino acids of the HSV-1 gB are not essential for the production of infectious virus particles. Despite the fact that gB has been shown to be required for infectious particle formation, the *ambB*1 virus, which produced gB molecules that were truncated at amino acid 863, showed near normal plating efficiencies and progeny yields on infection of Vero cells, a noncomplementing cell line. In addition, the *ambB*1 virus penetrated cells with normal kinetics, as might be expected, since the rate of entry domain of gB resides in the extracellular domain of the protein. This domain is intact in the truncated gB produced in *ambB*1 infections. The *ambB*1 virus also produced morphologically normal plaques. An amino acid substitution at residue 855 (3) and the addition of two amino acids between residues 816 and 817 (4) resulted in mutant virus which gave rise to syncytial plaques. Both sites are also in the cytoplasmic domain of gB. Our data suggest that amino acid residues that are carboxy terminal to residue 863 are not part of the site for the syncytial phenotype on the gB molecule.

Cai and colleagues (4, 4a) have isolated a set of *Hpa*I linker insertion mutations in plasmids containing the gB-coding sequences. These include addition, deletion, and nonsense mutations. Transfection-infection assays were developed to test the ability of these mutant gB plasmids to complement gB-null virions for virus production and for the syncytial phenotype in Vero cells. A strict correlation was observed between the ability of a mutant plasmid to complement for virus production and for fusion activity. All mutant plasmids that were complementation negative for virus production did not cause any detectable cell fusion, while those plasmids that were completely or partially complementation positive also caused complete or partial cell fusion. A plasmid carrying the *ambB*1 mutant gB gene gave both complementation for virus formation and fusion in the transient expression assays. Other plasmids carrying nonsense mutations that resulted in truncations of gB between amino acid residues 779 and 851 gave neither complementation for virus production nor measurable cell fusion. The boundary between that part of the cytoplasmic domain that is essential for the normal function of gB lies in the 12 amino acids between residues 863 and 851. These data support the conclusion that the terminal 38% of the cytoplasmic domain of gB is dispensable for the function of the protein.

TABLE 1. Plating of virus on Vero and D-6 cells[a]

Virus	No. of plaques				Plating efficiency (%) in Vero cells[b]
	Vero		D-6		
	Infection no. 1	Infection no. 2	Infection no. 1	Infection no. 2	
KOS	189	173	162	162	112
	107		100	71	125
	17	18	19	22	85
*ambB*1 from D-6	318	269	539	584	52
	790	932	1,474		58
	219		360	421	56
	71	82	167	144	49
*ambB*1 from Vero	118	95	226	268	43

[a] Cell monolayers were infected in duplicate with different dilutions of virus. The *ambB*1 stocks were obtained by propagating them on Vero or D-6 cells.
[b] Plaquing efficiency on Vero cells is expressed as a percentage of the plaques observed on comparably infected D-6 cells.

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