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Disulfide Bonds of Herpes Simplex Virus Type 2 Glycoprotein gB

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Glycoprotein B (gB) is the most highly conserved envelope glycoprotein of herpesviruses. The gB protein is required for virus infectivity and cell penetration. Recombinant forms of gB being used for the development of subunit vaccines are able to induce virus-neutralizing antibodies and protective efficacy in animal models. To gain structural information about the protein, we have determined the location of the disulfide bonds of a 696-amino-acid residue truncated, recombinant form of herpes simplex virus type 2 glycoprotein gB (HSV gB2t) produced by expression in Chinese hamster ovary cells. The purified protein, which contains virtually the entire extracellular domain of herpes simplex virus type 2 gB, was digested with trypsin under nonreducing conditions, and peptides were isolated by reversed-phase high-performance liquid chromatography (HPLC). The peptides were characterized by using mass spectrometry and amino acid sequence analysis. The conditions of cleavage (4 M urea, pH 7) induced partial carbamylation of the N termini of the peptides, and each disulfide peptide was found with two or three different HPLC retention times (peptides with and without carbamylation of either one or both N termini). The 10 cysteines of the molecule were found to be involved in disulfide bridges. These bonds were located between Cys-89 (C1) and Cys-548 (C8), Cys-106 (C2) and Cys-504 (C7), Cys-180 (C3) and Cys-244 (C4), Cys-337 (C5) and Cys-385 (C6), and Cys-571 (C9) and Cys-608 (C10). These disulfide bonds are anticipated to be similar in the corresponding gBs from other herpesviruses because the 10 cysteines listed above are always conserved in the corresponding protein sequences.

Herpes simplex virus (HSV) has at least 12 membrane-associated glycoproteins. Of these, glycoprotein B (gB) is the most abundant. The gB protein plays an essential, multifunctional role in virus infection (for a review, see reference 30). The gB molecule is involved in the fusion of the viral and cellular membranes to facilitate virus entry. This process occurs at neutral pH and is likely to be mediated by a multiprotein complex involving glycoproteins H, L, and K in addition to gB. The protein may have a similar role in viral egress since monoclonal antibodies to gB can block cell to cell spread, syncytium formation, and virus entry, thus neutralizing virus infection. Immune responses directed to gB are likely to play a major role in protection. In addition to being potent virus-neutralizing antibodies, gB-specific monoclonal antibodies also initiate antibody-mediated cellular cytotoxicity and antibody-dependent, complement-mediated cellular cytotoxicity (26). The gB molecule contains also epitopes recognized by $CD4^+$ T helper cells as well as cytotoxic $CD8^+$ T cells (21, 47). Monoclonal antibodies to gB provide passive protection against lethal viral challenge, and gB constitutes an effective vaccine in animal models of HSV infection when delivered as a subunit vaccine or in a live virus vector (reviewed in reference 4). On the basis of the encouraging results for animal models, large-scale human clinical trials are currently in progress with an HSV type 2 subunit vaccine incorporating gB as a key component. Because of its central role in virus infection and the generation of a protective immune response, a better understanding of the structure of gB could contribute to the generation of effective antiviral agents and vaccines.

The first sequenced herpesvirus gB gene was that encoded by HSV type 1 (6). A year later, in 1985, Pellett et al. (29) determined the gB sequence of the Epstein-Barr virus genome and noticed the high degree of similarity between the two sequences. In particular, all 10 cysteine residues located in the extracellular domain of both proteins were conserved. To date, more than 20 complete amino acid sequences of gB from diverse members of the herpesvirus family have been deduced from nucleotide sequences. One of the most remarkable features of these sequences is the conservation and location of 10 cysteine residues. Indeed, the alignment of gBs of different viruses of the herpesvirus family can be achieved by the alignment of these cysteine residues. The alignable sequences include those of gB from HSV type 1 (6), Epstein-Barr virus (29), varicella-zoster virus (20), human cytomegalovirus (CMV) (10), HSV type 2 (5), pseudorabies virus gII (36), bovine herpesvirus 2 (28), equine herpesvirus 1 (50), equine herpesvirus 4 (34), Marek's disease virus (38), herpesvirus saimiri (1), simian herpesvirus SA8 (11), laryngotracheitis virus (22), murine CMV (33), human herpesvirus 6 (13), two platyrrhine monkey alphaherpesviruses (12), murine gammaherpesvirus 68 (43), and simian varicella virus (31).

The DNA sequence of HSV type 2 gB (gB2) reveals an N-terminal 22-amino-acid hydrophobic signal sequence, an extracellular domain of 700 residues containing 10 cysteine residues and 8 potential N-linked glycosylation sites, a large hydrophobic 78-residue-membrane-spanning domain from amino acids 702 to 776, and a 106-residue cytoplasmic domain (45). Although there is no published evidence about the redox status of the 10 cysteine residues of HSV gB2, it is assumed that, as is the case for other viral membrane glycoproteins (human immunodeficiency virus gp120 [24] and HSV gD2 [25]), all cysteine residues present in HSV gB2 are disulfide bonded. Disulfide bonds between cysteine residues are of particular importance, since these bridges are a key protein structural element that stabilize functional native conformations. In this report, we describe our results on the determination of the location of disulfide bonds of a truncated, recombinant form of HSV gB2 (HSV gB2t) by using mass spectrometry (MS) and amino acid sequence analysis. All 10 cysteines of this protein were found to be involved in disulfide bridges. These are lo-

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cated between Cys-89 (C1) and Cys-548 (C8), Cys-106 (C2) and Cys-504 (C7), Cys-180 (C3) and Cys-244 (C4), Cys-337 (C5) and Cys-385 (C6), and Cys-571 (C9) and Cys-608 (C10).

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MATERIALS AND METHODS

Isolation of HSV gB2t. HSV gB2t was engineered for expression and secretion in Chinese hamster ovary cells. The expression vector contained a truncated form of HSV gB2 ending in Thr-696 of the predicted amino acid sequence (45) plus the sequence Phe-Asp-Leu-Asp-Lys added by an in-frame fusion at the C terminus of the truncated gene with a polylinker in the expression vector. The mature secreted protein, which contains virtually the entire 702-amino-acid residue extracellular domain of HSV gB2, was purified by a procedure which consisted of chromatographic steps on S-Sepharose and Fractogel EMD/DEAE, followed by precipitation with polyethylene glycol and redissolution of the protein in a pH 6.0 buffer containing 10 mM sodium citrate, 160 mM NaCl, and 2% glycine. The final product had a purity of about 95% (details on the purification and characterization of HSV gB2t will be published elsewhere). The estimated purity is based on densitometric analysis of sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie blue and Western blot (immunoblot) analysis using a rabbit anti-HSV type 2 polyclonal antibody. Under reducing conditions, HSV gB2t shows three protein bands at molecular masses of 97, 72, and 31 kDa. The major band (70 to 80%), migrating at 97 kDa, corresponds to intact HSV gB2t, while the two other bands are the products of a specific proteolytic cleavage on the C-terminal side of proline 456 (results not shown; sequences are shown in Fig. 1). The cleavage between proline 456 and leucine 457 has no effect in the determination of the disulfide bond locations described herein.

Determination of total free sulfhydryl groups in HSV gB2t. The total free sulfhydryls were determined under denaturing conditions by reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in the presence of 2% SDS as described by Habeeb (16). Aldolase from rabbit muscle (Sigma Chemical Co., St. Louis, Mo.) was used as a positive control.

MALDI-TOF MS. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectra were acquired in the linear mode on a Bruker (Billerica, Mass.) Reflex instrument equipped with a nitrogen laser (337 nm, 4-ns pulse). Samples were prepared by adding an aliquot of the protein in 1% acetic acid to a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid) (1/5 [vol/vol]). Approximately 1 pmol of protein in sinapinic acid was loaded onto the sample target for MALDI-TOF MS. The air-dried sample-matrix mixture was introduced into the mass spectrometer by means of a vacuum lock. Spectra were recorded by using an accelerating voltage of 28.5 kV. Spectra were calibrated by the analysis of a bovine serum albumin dimer (molecular weight, 132,861), which was used as an external calibrant.

Digestion with trypsin and separation of peptides. Purified HSV gB2t was extensively dialyzed against 5% acetic acid and dried in a Speed Vac concentrator (Savant Instruments, Farmingdale, N.Y.). The dried protein (8 nmol) was dissolved in 0.1 M Tris-HCl (pH 7) containing 8 M urea and incubated for 16 h at 37°C. After incubation, the solution was diluted twofold with 0.1 M Tris-HCl (pH 7), and $CaCl_2$ was added to a final concentration of 1 mM. Trypsin (1-1-*p*-tosylamido-2-phenylethyl chloromethyl ketone treated; Sigma) was added at a 40:1 (wt/wt) ratio of HSV gB2t to trypsin, and digestion was allowed to proceed for 6 h at 37°C. An additional 2.5% weight of trypsin was added, and digestion continued for 18 h at 37°C. Digestion was stopped by freezing. The tryptic digest was fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a 5-μm Vydac (Hesperia, Calif.) C_{18} column (4.6 by 250 mm). Buffer A was 0.06% trifluoroacetic acid in H_2O, and buffer B was 0.056% trifluoroacetic acid in 80% acetonitrile in H_2O (44). The gradient was 5 min at 2% buffer B, from 2 to 63% buffer B in 63 min, and from 63 to 75% buffer B in 32 min. Absorbance was measured at 214 nm.

Digestion with endoproteinase Glu-C. Tryptic peptides (ca. 250 pmol) purified by reversed-phase HPLC as described above were dried and reconstituted in 25 μl of 50 mM 3-[*N*-morpholino]propanesulfonic acid (pH 7). Endoproteinase Glu-C (Sigma) was added at a 200:1 (wt/wt) ratio of peptide to protease. Digestion was for 24 h at 37°C, and the reaction was stopped by freezing.

Reduction of isolated peptides and modification with VP. Reversed-phase HPLC isolated peptides were dried by vacuum centrifugation and dissolved in 8.8 μl of 2.5 M 1,1,3,3-tetramethylurea in 50 mM *N*-ethyl morpholine-acetate buffer (pH 8.5), and β-mercaptoethanol (β-ME) was added to a final concentration of 14.2 mM. The tube headspace was purged with argon. The sample was incubated for 1 h at 60°C. The sulfhydryl groups formed upon reduction were modified with 93 mM 4-vinylpyridine (VP). The headspace was again purged with argon, and the reaction was allowed to proceed at 22°C. After 3 h, 25 μl of H_2O was added and the sample was dried by vacuum centrifugation. To remove all reagents, 50 μl of 50 mM *N*-ethyl morpholine-acetate (pH 8.5) was added and the sample was dried again.

Peptide identification. Peptides collected after reversed-phase HPLC were identified by MS and/or Edman sequence analysis. Electrospray ionization mass spectrometry (ESMS) spectra were acquired on a Sciex API-III triple-quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) interfaced to a Michrom HPLC system (Michrom BioResources Inc., Auburn, Calif.), using a 100-μm-inside-diameter fused silica tubing. The instrument was calibrated by using a propylene glycol (Aldrich, Milwaukee, Wis.) mixture (3.3×10^{-5} M propylene glycol 425, 10^{-4} M propylene glycol 1000, 2×10^{-4} M propylene glycol 2000) in a 50/50/0.1 (vol/vol/vol) H_2O-methanol-formic acid mixture and 2 mM ammonium acetate. Spectra were acquired with isotopic resolution at *m/z* 2000. Samples (ca. 5 to 20 pmol) were introduced into the mass spectrometer source in the flow injection mode, in a 50/50/1 (vol/vol/vol) H_2O-acetonitrile-acetic acid mixture at a flow rate of 20 μl/min, and spectra were recorded by scanning a mass range of *m/z* 300 to 1,800 at 8 s per scan, using a step

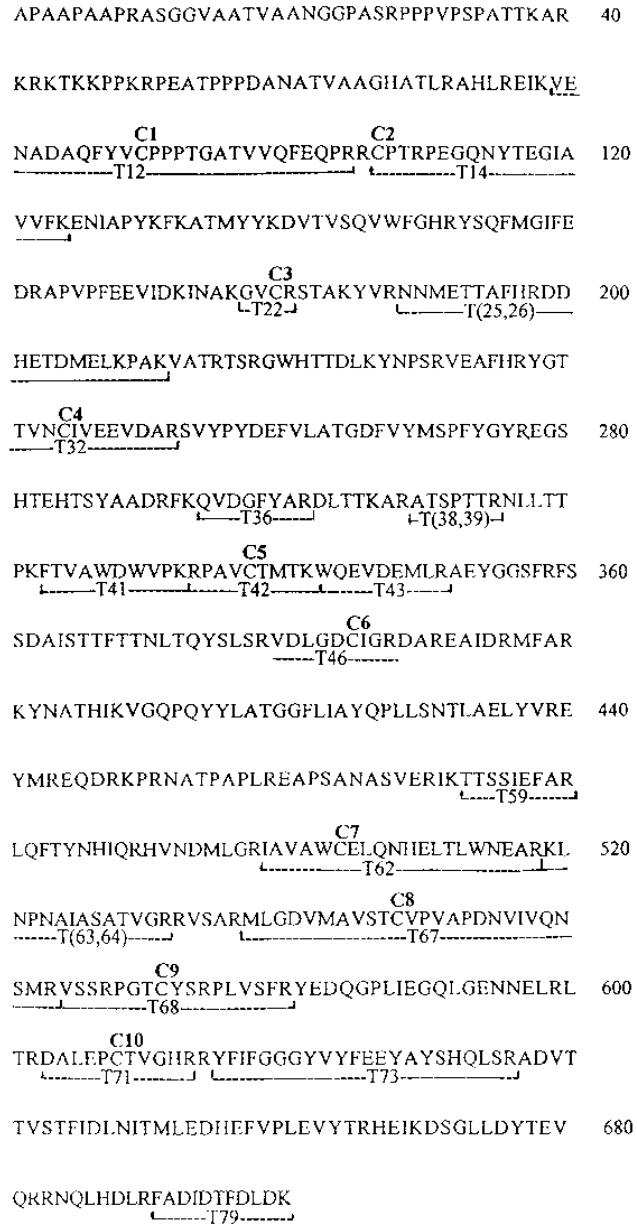

FIG. 1. Amino acid sequence of HSV gB2t. Numbering of amino acids begins at the N terminus of the mature recombinant protein. The underlined sequences represent tryptic peptides described in the text (for peptide nomenclature, see Materials and Methods). The amino acids indicated above the sequence (C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10) correspond to the cysteines of HSV gB2t. These are located in positions 89, 106, 180, 244, 337, 385, 504, 548, 571, and 608, respectively. The same cysteine residues and locations occur in the native gB of HSV type 2.

size of 0.1 Da. In some cases, samples were purified on-line by using a peptide trap cartridge (Michrom BioResources) which replaced the normal loop in the HPLC system. The sample was injected onto the trap cartridge, and the cartridge was washed with 50 μl of 99/1/1 (vol/vol/vol) H_2O-acetonitrile-acetic acid with the injector valve in the off-line position. The valve was then switched to the on-line position, and the sample was eluted directly into the mass spectrometer source with the buffer described above. Using this technique, the fused silica line going into the mass spectrometer source was kept free of low-molecular-weight hydrophilic contaminants. Low-energy collisionally induced dissociation (CID)-MS/MS spectra were acquired in the positive ion mode by using an upgraded high-pressure collision cell. Samples were introduced into the mass spectrometer source as described above, using a flow rate of 2 to 5 μl/min. The second quadrupole was scanned over a range of *m/z* 50 to 2,300 at 10 s per scan, using a step size of 1 Da. The orifice potential was set at 35 V. The collision energy was 60 eV, and the collision gas (99.999% argon) thickness (collision gas thickness) was approximately 3.5×10^{14} atoms per cm^3.

Automated sequence analysis of peptides was performed on an Applied Biosystems 470A gas-phase sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer (model 120A) or on an Applied Biosystems liquid pulse sequencer (model 473A).

Peptide nomenclature. In this report, amino acid residues are numbered according to their positions in the corresponding sequence from its N terminus. Cysteines are designated by their sequence location, e.g., Cys-89, Cys-106, Cys-180, etc., and also as C1, C2, C3, etc., the order in which they appear in the amino acid sequence. Tryptic peptides are designated by a T followed by an arabic numeral indicating their sequential location in the polypeptide chain. For example, T12 is the peptide comprising Val-79 through the first Arg in the sequence that is susceptible to cleavage by trypsin. In this nomenclature, the carboxyl side of Arg and Lys is considered a putative trypsin site except those in which a Pro follows Arg or Lys. Peptides which contained tryptic sites but were not hydrolyzed are designated by two T numbers separated by a comma [for example, T(38,39)]. Peptides containing a disulfide bridge are designated by two T numbers separated by a plus sign (for example, T22+T32). Peptides containing pyridylethylated cysteine are indicated by a T number followed by the abbreviation PEC (for example, T32-PEC). Peptides found to be carbamylated at their N termini are indicated by a T number followed by the abbreviation CB (for example, T36-CB).

RESULTS

The amino acid sequence of HSV gB2t, the purified protein used in this study, is shown in Fig. 1. The protein contains the N-terminal 696 of a total of 882 amino acids of intact HSV gB2, lacks both the transmembrane and the cytoplasmic domain (45), but contains a 5-amino-acid residue carboxy-terminal extension (residues 697 to 701). The amino acid sequence of purified HSV gB2t was confirmed by N-terminal amino acid sequence analysis using automated Edman degradation, by C-terminal sequence analysis using carboxypeptidase Y, and by sequencing a large number of tryptic peptides derived from native and reduced sulfhydryl-modified HSV gB2t. The mass of the intact, nonreduced HSV gB2t molecule determined by MALDI-TOF MS was 94,245 kDa, indicating a monomeric glycoprotein structure (Fig. 2). The mass was determined from the *m/z* ratio corresponding to the singly protonated molecular ion ($[MH]^+$). The low proportion of dimer observed in the mass spectrum was originated from the MALDI-TOF MS process (14). The MALDI-TOF MS gives the minimal molecular weight of the disulfide-bonded form. A molecular mass of about 100 kDa is also observed after SDS-polyacrylamide gel electrophoresis of HSV gB2t samples heated in the absence of β-ME (data not shown). These results are in agreement with those obtained with HSV gB extracted from either virions or infected cells (8, 9, 39). All 10 cysteine residues of HSV gB2t are involved in disulfide bonding, since no free cysteines are detected by titration of the protein with DTNB in the presence of 2% SDS. Thus, the HSV gB2t minimal subunit appears to be a monomer containing five intrachain disulfide bonds.

However, higher-molecular-weight aggregates are observed in solution. In non-SDS solutions, HSV gB2t appears to be polymeric. A minimal of 600,000 Da is observed in gel filtration experiments on Sephacryl S-500, but aggregates of molecular mass higher than 2,000,000 Da predominate (27). Assembly into oligomers appears to be a characteristic of gB homologs of the *Herpesviridae* family (9, 30). The formation of homodimers and perhaps larger oligomers is essential for gB function and virus infectivity, as determined by analysis of temperature-sensitive mutations in HSV gB (17) and the existence of domain-negative mutations in gB (7).

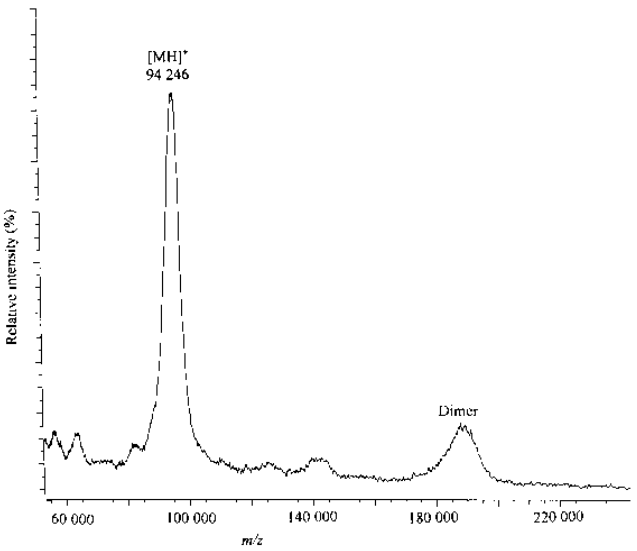

FIG. 2. MALDI-TOF MS spectrum of HSV gB2t. The spectrum was acquired as described in Materials and Methods. The peak corresponding to the singly charged monomer ($[MH]^+$) is also designated with its *m/z* ratio.

General strategy for disulfide bond identification. The strategy used for the identification of the disulfide linkage pattern of HSV gB2t used tryptic cleavage of the purified protein under conditions which left the disulfide bonds intact, the separation of the peptide fragments by reversed-phase HPLC (Fig. 3), and their analysis by ESMS (14). Nearly every HPLC peak of Fig. 3 was analyzed by ESMS. When an observed mass agreed with the sum of two theoretical masses of peptides containing cysteine, the peptide was reanalyzed by ESMS after disulfide bond reduction and modification of cysteines with 4-VP; the N-terminal amino acid sequence of the selected peptides was also determined. As described below, the locations of all five disulfide bonds of HSV gB2t have been established.

Disulfide bridge Cys-180 (C3)–Cys-244 (C4). The ESMS spectrum (Fig. 4A) of fraction 50 in Fig. 3 provided evidence for the presence of a peptide containing the disulfide bond between Cys-180 (C3)–Cys-244 (C4). The disulfide-bonded peptide was identified from the ion series of *m/z* 525.8, 701.0, and 1,050.8, which corresponded to the peptide in three different states of protonation ($[MH_4]^{4+}$, $[MH_3]^{3+}$, and $[MH_2]^{2+}$, respectively) (15). The mass was identified as 2,099.6 ± 0.4 Da and was consistent with the theoretical mass of peptide T22 linked by a disulfide bridge to peptide T32 (2,100.4 Da), containing Cys-180 (C3) and Cys-244 (C4), respectively:

peptide T22 $G^{178}VCR^{181}$
|
peptide T32 $Y^{238}GTTVNCIVEEVDAR^{252}$

To confirm the existence of this disulfide bridge, fraction 50 was reduced with β-ME and then treated with VP. The spectrum obtained after both reactions is presented in Fig. 4B. The

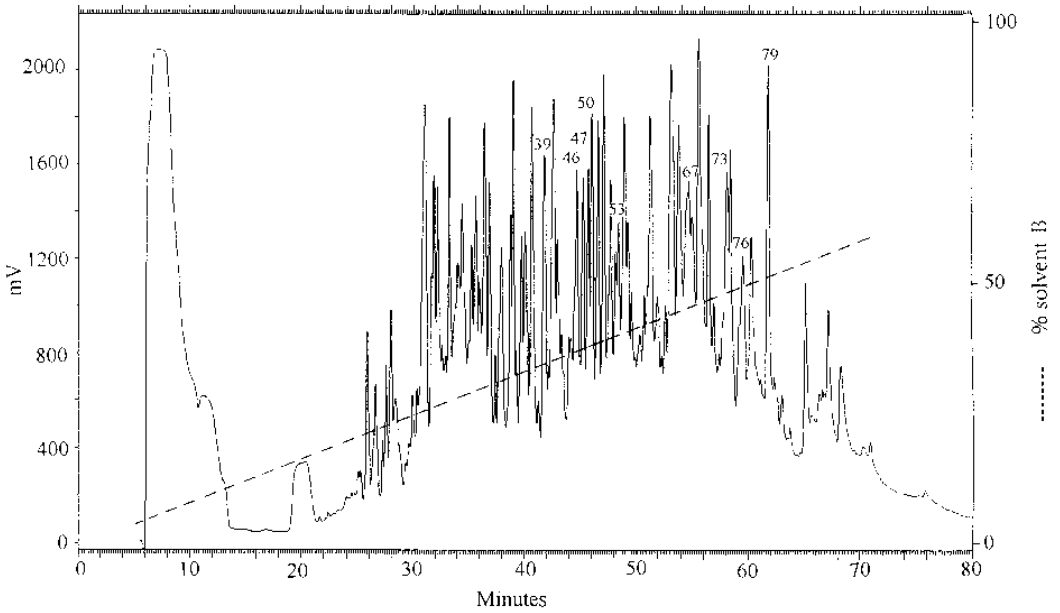

FIG. 3. Fractionation by reversed-phase HPLC of tryptic peptides of HSV gB2t. This chromatogram was generated with 8 nmol of trypsin-digested HSV gB2t. Chromatographic conditions were as described in Materials and Methods. Peaks were collected and identified by ESMS, and in some cases, identity was confirmed by N-terminal analysis. Peaks were numbered on the basis of their order of elution, but only the numbers of selected fractions are shown.

peptide with a mass of 1,773.9 ± 0.3 Da {*m/z* 592.4 ($[MH_3]^{3+}$) and 887.8 ($[MH_2]^{2+}$)} corresponded to peptide T32 with an observed increase in mass of 105.1 Da as a result of the addition of VP to Cys-244 (C4) (theoretical increase, 105.1 Da). No signal corresponding to peptide T22 with Cys-180 (C3) modified with VP was detected by ESMS. Figure 4A also shows an ion series of *m/z* 603.4 ($[MH_2]^{2+}$) and 1,205.6 ($[MH]^{+}$), which identified a molecular mass of 1,204.7 ± 0.1 Da, consistent with peptide T43 (1,204.6 Da). The sequence analysis of fraction 50 by Edman degradation (results not shown) confirmed the presence of the three peptides described above (peptides T22, T32, and T43). The conditions used for reduction with β-ME and the treatment of the peptides with VP increased the mass of peptide T43 by 12.3 Da (Fig. 4B). CID-MS/MS fragmentation of the peptide indicated that the modification was on the N-terminal tryptophan of the peptide; however, we have not identified the nature of this modification. Independent confirmation of a disulfide bond between Cys-180 (C3) and Cys-244 (C4) was obtained by analysis of HPLC fraction 53 (results not shown). In this fraction, peptide T22 was found linked to peptide T32 (disulfide bridge Cys-180 [C3]–Cys-244 [C4]), but the mass was 43 Da higher than predicted. The higher mass corresponds to the presence of a carbamyl group. The analysis of fraction 53 by ESMS, after reduction and pyridylethylation, showed that the carbamylation was associated with peptide T22 (Cys-180 [C3]). Analysis of the modified peptide by CID-MS/MS indicated that cyanate had reacted with the N terminus of peptide T22.

Disulfide bridge Cys-571 (C9)–Cys-608 (C10). Evidence for a disulfide bridge between Cys-571 (C9) and Cys-608 (C10) was found in HPLC fraction 46 (Fig. 3). The ESMS spectrum of fraction 46 is shown in Fig. 5A. The major ion series of *m/z* 518.8 ($[MH_6]^{6+}$), 622.4 ($[MH_5]^{5+}$), 777.8 ($[MH_4]^{4+}$), and 1,036.8 ($[MH_3]^{3+}$) identified a molecular mass of 3,107.1 ± 0.3 Da, which was consistent with the mass of tryptic peptide T68 linked to peptide T71 (3,107.5 Da) containing Cys-571 (C9) and Cys-608 (C10), respectively:

peptide T68 $V^{564}SSRPGTCYSRPLVSFR^{580}$
|
peptide T71 $D^{603}ALEPCTVGHR^{613}$

The mass spectrum after reduction of fraction 46 with β-ME and modification with VP is shown in Fig. 5B. The ion series of *m/z* 505.0 ($[MH_4]^{4+}$), 673.2 ($[MH_3]^{3+}$), and 1,009.4 ($[MH_2]^{2+}$) identified a peptide with a mass of 2,016.5 ± 0.5 Da. This mass corresponded to peptide T68 containing Cys-571 (C9) modified with VP (2,017.2 Da). The ion series of *m/z* 435.0 and 652.0 identified a peptide mass of 1,302.0 ± 0.1 Da, consistent with peptide T71 containing Cys-608 (C10) modified with VP (1,302.3 Da). Edman degradation sequence analysis of fraction 46 showed the sequences predicted for tryptic peptides T68 and T71 (results not shown). Since no other sequences were observed, it was concluded that other peptides observed in fraction 46 must be blocked by N-terminal carbamylation. Indeed, ions of *m/z* 502.8 ($[MH_2]^{2+}$) and 728.0 ($[MH_2]^{2+}$) (Fig. 5) corresponded to the N-terminally carbamylated peptides T(38,39) (959.52 Da) and T(63,64) (1,410.8 Da), respectively. The peptide containing a disulfide bridge between Cys-571 (C9) and Cys-608 (C10) was also present in fraction 48, but the molecular mass identified was 43.2 Da higher than predicted. The analysis of this peptide after reduction and modification with VP showed the mass increment corresponded to carbamylation in peptide T68 containing Cys-571 (C9) (data not shown).

Disulfide bridge Cys-337 (C5)–Cys-385 (C6). The ESMS spectrum of fraction 39 (Fig. 3) is shown in Fig. 6A. The data provide evidence for the presence of a peptide with the disulfide bond between Cys-337 (C5) and Cys-385 (C6). The presence of the disulfide-bonded peptide was identified from the minor ion series of *m/z* 488.6 ($[MH_4]^{4+}$), 651.4 ($[MH_3]^{3+}$), and 976.6 ($[MH_2]^{2+}$). The mass identified was 1,950.9 ± 0.5 Da, consistent with the theoretical mass of the peptide T42 linked, by a disulfide bridge, to peptide T46 (1,951.3 Da) containing Cys-337 (C5) and Cys-385 (C6), respectively:

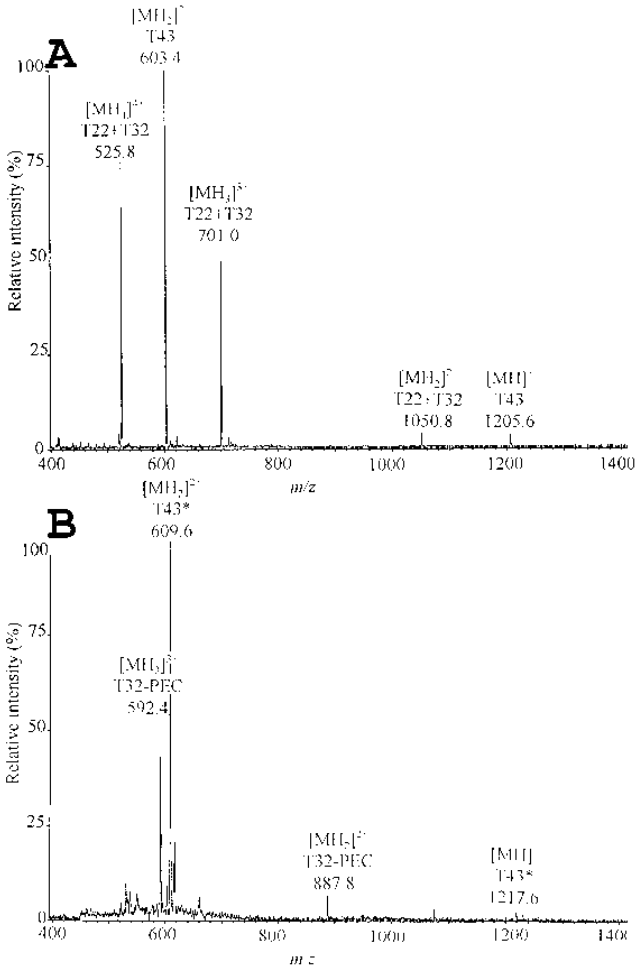

FIG. 4. ESMS spectra of fraction 50 (Fig. 3) before (A) and after (B) treatment with β-ME and VP. For details on peptide nomenclature and identification by MS, see Materials and Methods. Each peak is labeled with an *m/z* ratio and a charge state designation. An asterisk indicates an unknown modification.

peptide T42 $R^{333}PAVCTMTK^{341}$

peptide T46 $V^{380}DLGDCIGR^{388}$

An attempt was made to confirm this disulfide bond assignment by ESMS of the fraction after reduction and modification of the peptides with VP (Fig. 6B). None of the ions expected for peptide T42 linked to peptide T46 were detected, indicating a modification of the linked peptides by the treatment, but neither peptide T42, nor peptide T46, modified with VP was observed in the spectrum. However, the presence of peptides T42 and T46 was confirmed by Edman degradation sequence analysis (results not shown). The disulfide bond between Cys-337 (C5) and Cys-385 (C6) was also found in fraction 47; in this fraction, both peptides T42 and T46 were found to have their N termini carbamylated. Other peptides present in fraction 39 (Fig. 6) were the N-terminally carbamylated peptides T36 {*m/z* 999.6 ($[MH]^+$)}, T59 {*m/z* 528.0 ($[MH_2]^{2+}$) and 1,054.6 ($[MH]^+$)}, and T(25,26) {*m/z* 555.8 ($[MH_5]^{5+}$), 694.6 ($[MH_4]^{4+}$), and 925.8 ($[MH_3]^{3+}$)}. The sequences of these peptides were confirmed by CID-MS/MS (results not shown). Figure 6 also shows a minor component of *m/z* 577.6 ($[MH_2]^{2+}$) corresponding to a peptide with a molecular mass of 1,152.7 ± 0.1 Da. The sequence established from Edman degradation analysis as well as by CID-MS/MS was SSGT SYPDVLK, which corresponds to residues 132 to 142 of bovine trypsin (1,152.6 Da).

Disulfide bridge Cys-89 (C1)–Cys-548 (C8). The peptide containing a disulfide bridge between Cys-89 (C1) and Cys-548 (C8) was found in several fractions in various forms. For instance, fraction 67 (Fig. 3) contained a peptide with a mass of 5,610.3 ± 1.3 Da, identified from the series of *m/z* 1,122.8 ($[MH_5]^{5+}$), 1,403.8 ($[MH_4]^{4+}$), and 1,871.2 ($[MH_3]^{3+}$) (Fig. 7A). The peptide was identified as T12 linked by a disulfide bond to peptide T67 (5,609.5 Da), containing Cys-89 (C1) and Cys-548 (C8), respectively:

peptide T12 $V^{79}ENADAQFYVCPPPTGATVVQFEQPR^{104}$

peptide T67 $M^{538}LGDVMAVSTCVPVAPDNVIVQNSMR^{563}$

As expected, the ESMS analysis of fraction 67 after reduction and reaction with VP reveals both peptides T12 and T67, containing Cys-89 (C1) and Cys-548 (C8), respectively, modified with VP (Fig. 7B). The ion series of *m/z* 743.4 ($[MH_4]^{4+}$) and 990.7 ($[MH_3]^{3+}$) identified a mass of 2,969.4 ± 0.3 Da, which was consistent with the predicted mass of peptide T12, containing pyridylethylated Cys-89 (C1). The peptide with a mass of 2,851.8 Da {*m/z* 951.6 ($[MH_3]^{3+}$)} corresponded to

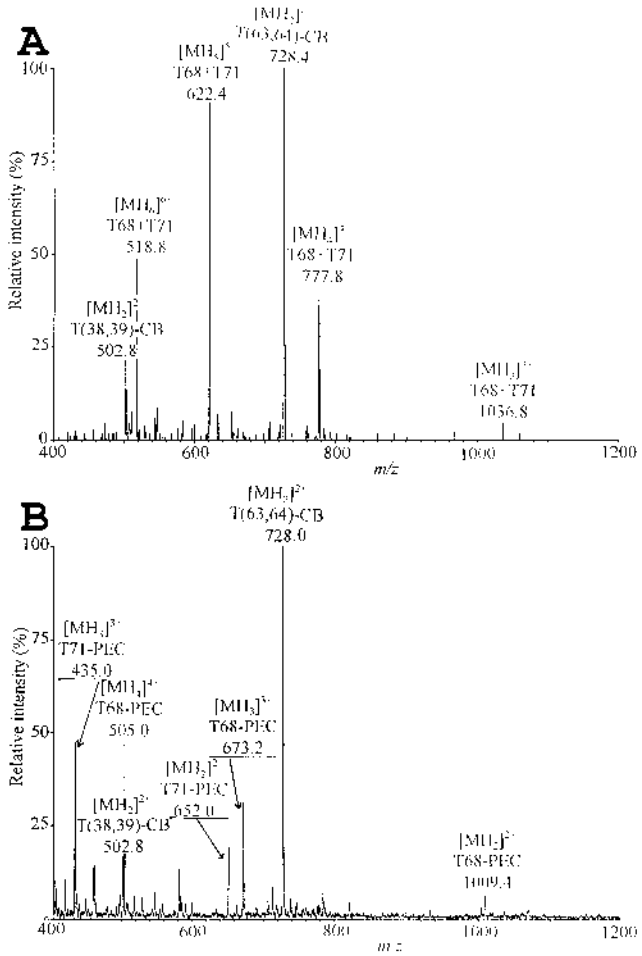

FIG. 5. ESMS spectra of fraction 46 (Fig. 3) before (A) and after (B) treatment with β-ME and VP. For details on peptide nomenclature and identification by MS, see Materials and Methods. Each peak is labeled with an *m/z* ratio and a charge state designation.

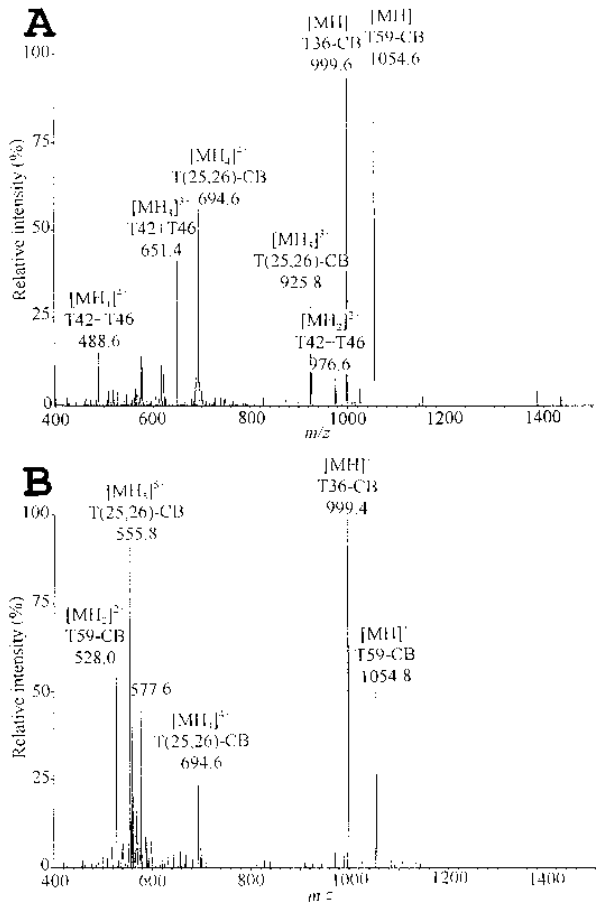

FIG. 6. ESMS spectra of fraction 39 (Fig. 3) before (A) and after (B) treatment with β-ME and VP. For details on peptide nomenclature and identification by MS, see Materials and Methods. Each peak is labeled with an *m/z* ratio and a charge state designation.

peptide T67 (2,852.5 Da) containing the VP-modified Cys-548 (C8). Sequence analysis by Edman degradation confirmed the presence of peptides T12 and T67 (results not shown). Peptide T12 linked to peptide T67 was also found in two other fractions. For instance, peptide T12 was found with N-terminal carbamylation in fraction 73; in fraction 76, both peptides T12 and T67 showed N-terminal carbamylation (results not shown). Figure 7A also reveals a peptide with a mass of 1,342.3 ± 0.3 Da {*m/z* 672.0 ($[MH_2]^{2+}$) and 1,343.6 ($[MH]^{+}$)}. The mass of this peptide did not change upon reduction and modification with VP (Fig. 7B), indicating that it contained no cysteine residues. The peptide mass was consistent with peptide T79 with carbamylation of its N terminus (1,341.6 Da). Automated Edman degradation did not yield a sequence, indicating that peptide T79 indeed was N-terminally blocked.

Disulfide bridge Cys-106 (C2)–Cys-504 (C7). The work described above accounts for eight cysteine residues involved in the formation of four disulfide bonds. Since HSV gB2t contains no free cysteines and all of the disulfide bridges are intrachain, it is possible that the ionization of the disulfide-linked peptide containing Cys-106 (C2) and Cys-504 (C7) was suppressed during ESMS. This 39-residue disulfide-linked peptide was expected to be present in one of the fractions eluting late in the chromatogram (Fig. 3). For this reason, the peptides of the four major fractions eluting after 60 min in the chromatogram shown in Fig. 3 were reduced with β-ME and modified with VP. Evidence for the presence of a disulfide bridge was found by ESMS analysis of fraction 79. Indeed, the ion of *m/z* 849.2 ($[MH_3]^{3+}$) identified a peptide mass of 2,544.6 Da, corresponding to peptide T62 (2,544.7 Da) containing the VP-modified Cys-504 (C7) and a carbamyl group, probably associated with the N terminus of the peptide (Fig. 8). Fraction 79 also contained two additional peptides, but these did not contain cysteine residues. They were characterized as the N-terminally carbamylated peptide T41 {*m/z* 646.6 ($[MH_2]^{2+}$) and 1,291.6 ($[MH]^{+}$)} and peptide T73 {*m/z* 899.8 ($[MH_3]^{3+}$) in Fig. 8}. Figure 9 shows ions in the *m/z* range 50 to 500, using an orifice potential of 140 V, for HPLC fraction 79. The CID at the orifice results in the fragmentation of glycopeptides (18), and the generation of the characteristic carbohydrate oxonium ions, e.g., *m/z* 204.0 and 366.2 (from *N*-acetylhexosamine and from *N*-acetylhexosamine plus hexose, respectively). The presence of ions of *m/z* 204.0 and 366.2 in Fig. 9 indicated the presence of a glycopeptide in fraction 79. This finding led us to believe that peptide T62, containing Cys-504 (C7), could be disulfide linked to peptide T14, which contained Cys-106 (C2) and the consensus sequence for glycosylation at Asn-114:

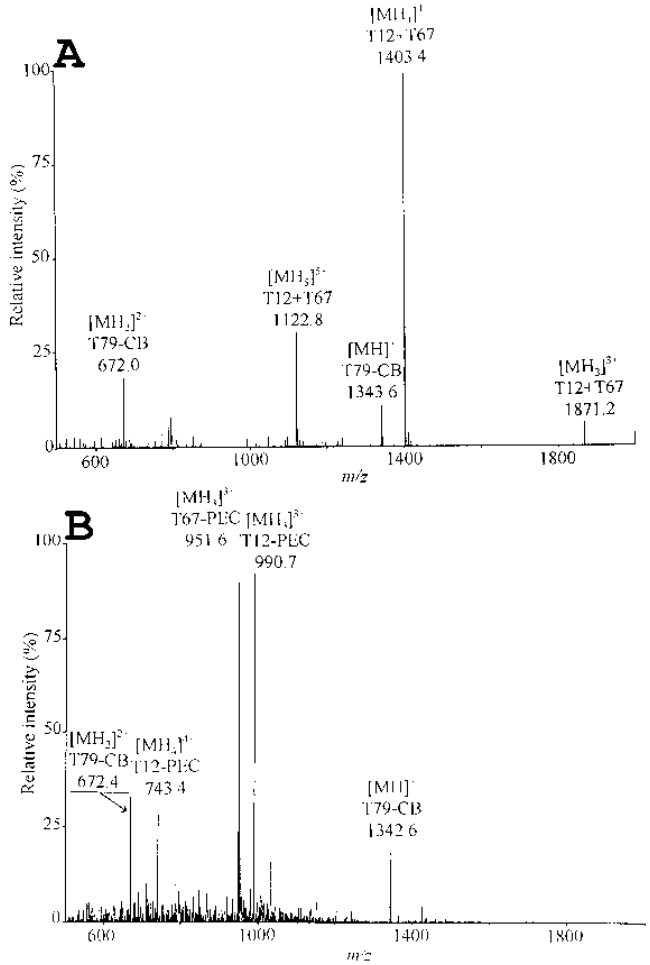

FIG. 7. ESMS spectra of fraction 67 (Fig. 3) before (A) and after (B) treatment with β-ME and VP. For details on peptide nomenclature and identification by MS, see Materials and Methods. Each peak is labeled with an *m/z* ratio and a charge state designation.

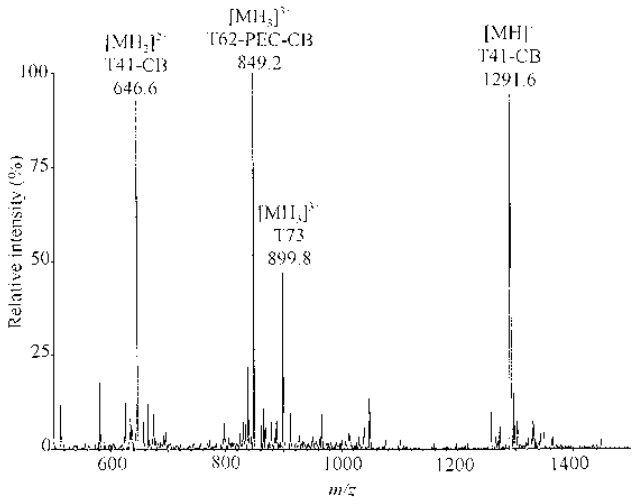

FIG. 8. ESMS spectrum of fraction 79 (Fig. 2) after treatment with β-ME and VP. For details on peptide nomenclature and identification by MS, see Materials and Methods. Each peak is labeled with an *m/z* ratio and a charge state designation.

(carbohydrate)

peptide T14 C^{106}PTRPEGQNYTEGIAVVFK^{124}

peptide T62 I^{499}AVAWCELQNHELTLWNEAR^{518}

To ascertain whether T14 was present, the peptides contained in fraction 79 were cleaved with endoproteinase Glu-C and the subfragments generated from the digestion were analyzed by ESMS. Figure 10 shows the ESMS spectrum after endoprotease Glu-C treatment and shows the ion of *m/z* 788.5 ($[MH_2]^{2+}$), which identified peptide Cys-106–Glu-111 (701.3 Da), containing Cys-106 (C2), linked by a disulfide bridge to the peptide Ile-499–Glu-505 (790.4 Da), containing Cys-504 (C7). The mass was 85.3 Da higher than predicted, which is consistent with carbamylation of the amino termini of both peptides. The results demonstrated the existence of a disulfide bond between Cys-102 (C2) and Cys-504 (C7). Additional data (not shown) indicated that fractions 69 and 73 also contained peptides with this disulfide linkage. The disulfide linkage was confirmed by CID-MS/MS. This technique is useful in identifying the amino acid sequence of a peptide or a disulfide-linked peptide. The peptide of interest is fragmented by collision with an inert gas in the mass spectrometer, and the fragment ions are analyzed. Peptides essentially fragment with cleavage at bonds along the peptide backbone. Fragment ions containing the amino terminus are termed a-, b-, and c-type ions, depending on the site of cleavage in the backbone (2, 37). Similarly, fragment ions containing the carboxy terminus are termed x-, y-, and z-type ions. Internal acyl ions involve the loss of both the amino and the carboxy terminus. The disulfide linkage between Cys-102 (C2) and Cys-504 (C7) was confirmed by fragmentation of the molecular ion of *m/z* 788.5 ($[MH_2]^{2+}$), which generated the mass spectrum shown in Fig. 11. The fragment ions shown in the mass spectrum are consistent with the structure shown in Fig. 11, where specific fragments are identified. In addition, the ions of *m/z* 874.5, 1,102.5, and 1,105.6 are consistent with internal acyl ions for the structure shown in Fig. 11.

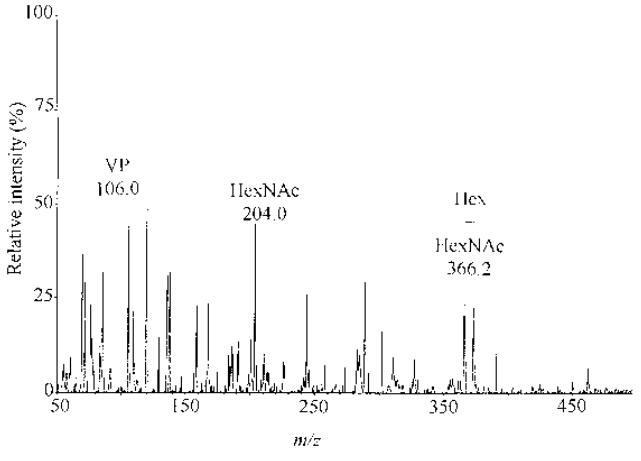

FIG. 9. Partial ESMS spectrum of fraction 79 (Fig. 3) after treatment with β-ME and VP. The spectrum of ions of *m/z* lower than 500 was obtained with a high orifice potential of 140 V as described in Materials and Methods. The ions of *m/z* of 204 and 366.2 correspond to N-acetylhexosamine (HexNAc) and *N*-acetylhexosamine plus hexose (Hex), respectively. The ion of *m/z* 106 was generated by the loss of VP from pyridylethylated cysteine.

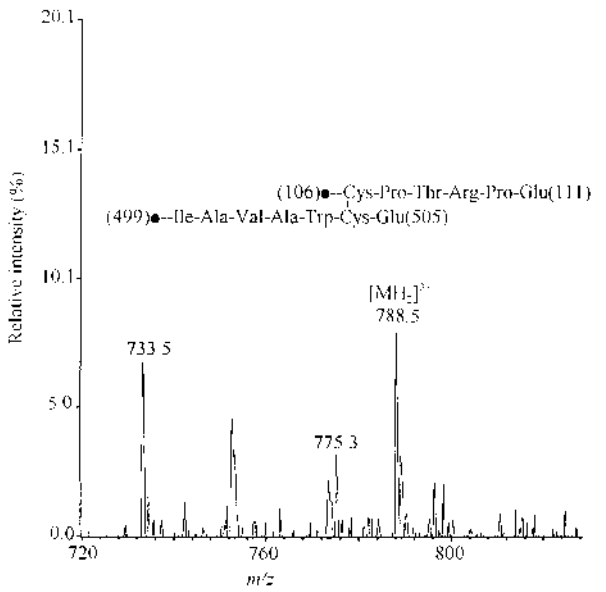

FIG. 10. Partial ESMS spectrum of the peptides of fraction 79 treated with endoproteinase Glu-C. The peptides present in fraction 79 were hydrolyzed by endoproteinase Glu-C as described in Materials and Methods. The products of the digestion were analyzed by ESMS without any purification step. The ion of *m/z* 788.5 ($[MH_2]^{2+}$) corresponds to peptides T14 and T62 linked by a disulfide bridge between Cys-106 (C2) and Cys-504 (C7). The numbers in parentheses indicate the positions of the amino acids in HSV gB2t (Fig. 1). ●– – indicates a carbamylated N terminus. Ions of *m/z* 733.5 and 775.3 (both $[MH]^+$) correspond to the peptides ^{118}GIVVFK^{124} and ^{511}LTLWNE^{516}, respectively, generated by the digestion of peptide T14+T62.

DISCUSSION

The disulfide bonds of HSV gB2t have been determined from analyses by ESMS and Edman degradation of peptides generated by tryptic cleavage of the protein under nonreducing conditions. Trypsin was used for digestion of HSV gB2t because of its high degree of specificity and its activity at pH 7 in the presence of 4 M urea (35). Neutral pH was used to avoid disulfide interchange. Under alkaline conditions, disulfide bonds are slowly reduced and disulfide interchange can occur

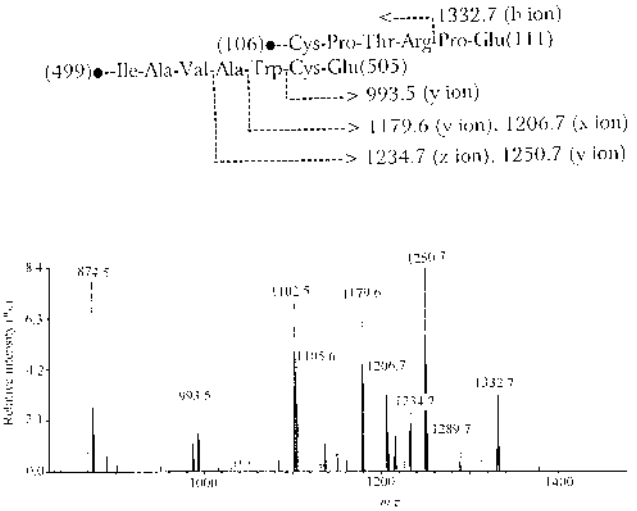

FIG. 11. Partial CID-MS/MS spectrum for molecular ion $[MH_2]^{2+}$ 788.5 (Fig. 10). The CID-MS/MS spectrum is shown at the bottom; only fragments with the intact disulfide bridge Cys-106 (C2)–Cys-504 (C7) are shown. A scheme of the fragmentations is given at the top. All peaks seen in the spectrum are illustrated except for the ions of *m/z* 874.5, 1,102.5, and 1,105.6, which are generated by a double fragmentation as described in the text, and the ion of *m/z* 1,289.7, which corresponds to the ion of *m/z* 1,332.7 plus the loss of the carbamyl group from the peptide containing Cys-106. The numbers in parentheses indicate the positions of the amino acids in the HSV gB2t sequence (Fig. 1). ●– – indicates a carbamylated N terminus.

(40). In the present work, every Cys residue was only found in a unique disulfide bond, indicating that no disulfide bond rearrangement had occurred. At pH 7, however, the cyanate ions present in urea solution react extensively with the α-amino group of peptides (19, 42). The carbamylation reaction is partially responsible for the complexity of the chromatogram obtained in the separation of the tryptic peptides (Fig. 3). It appears, however, that the carbamylation reaction is peptide dependent. For example, peptides T22, T42, T46, T62, T67, and T88, containing Cys-180 (C3), Cys-377 (C5), Cys-385 (C6), Cys-504 (C7), Cys-548 (C8), and Cys-571 (C9), respectively, were found either with or without the modification. Peptides T12, T32, and T71, containing Cys-89 (C1), Cys-244 (C4), and Cys-608 (C10), respectively, were not found to be carbamylated. Despite these challenges, the present work has established the complete location of the disulfide bonds of HSV gB2t. All 10 cysteines of the protein were found to be in disulfide bridges. These were located between Cys-89 (C1) and Cys-548 (C8), Cys-106 (C2) and Cys-504 (C7), Cys-180 (C3) and Cys-244 (C4), Cys-337 (C5) and Cys-385 (C6), and Cys-571 (C9) and Cys-608 (C10). A schematic representation of the disulfide bond pattern of HSV gB2t is shown in Fig. 12. The pattern indicates the presence of five disulfide loops, with a rather large range of residues (38 to 460) per loop. However, three of these loops (C3-C4, C5-C6, and C9-C10) are formed between near neighbor cysteines, in agreement with the notion that cysteines that are close together in sequence preferentially

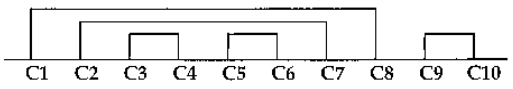

FIG. 12. Schematic representation of the proposed disulfide bridge pattern of HSV gB2t. Cysteines are designated C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10, corresponding to cysteines 89, 106, 180, 244, 337, 385, 504, 548, 571, and 608, respectively.

form disulfide bridges (46). The smallest of all the gB loops, the C9-C10 loop, can be considered a local disulfide since the half-cysteine separation is less than 45 residues (46). This region includes the immunodominant domain of human CMV gB (49), which is critical for the immunoreactivity of the protein. It is also of interest to note that the C9-C10 loops of all gBs of the herpesvirus family contain the most conserved linear sequence of the entire protein. This always conserved gB sequence, GQLGXXNE/D (residues 590 to 597 in HSV gB2t), might have a functional role.

The disulfide bond between C5 and C6 also merits some discussions because the integrity of the C5-C6 disulfide bond appears to be critical for maintaining proper folding of the molecule. Site-specific mutations of either of these cysteine residues profoundly alters the structure of the full-length molecule, with the resultant loss of the presentation of a number of epitopes mapping proximal to and more distal to this region (32).

Although HSV gB2 is not cleaved in vivo like the CMV gB homolog, we have observed that in vitro, 5 to 30% of HSV gB2t is proteolytically cleaved between Pro-456 and Leu-457 (27), a location that exactly corresponds to the CMV gB cleavage site. The fact that the two resulting HSV gB2t fragments are held together under nonreducing conditions is explained by the presence of intrachain disulfide bonds between C1-C8 and C2-C7. The existence of intrachain disulfide bonds between C1-C8 and C2-C7 in all gBs would also explain why the in vivo proteolytic cleavage of gBs into two fragments, which occurs for many members of the herpesvirus family, including human CMV and varicella-zoster virus (for a review, see reference 26), does not result in the separation of the two parts unless the disulfide bonds are reduced. The location of the proteolytic processing site in a peptide bond located between C6 and C7 has been established for the varicella-zoster virus gpII analog (20), CMV gB Towne strain (41), human herpesvirus 6 gB (13), and gB of Marek's disease virus (51). Proteolysis of CMV gB appears to be mediated by the endoprotease furin (48), but cleavage does not appear to be essential for gB's role in initiating infection for bovine herpesvirus or pseudorabies virus (3, 23).

As noted in the introduction, all 10 cysteines of HSV gB2 are conserved in all gBs of the herpesvirus family, and all of these sequences can be aligned by using the cysteine residues. The alignment of the predicted amino acid sequences of the herpesvirus saimiri gB analog with the corresponding glycoproteins of human herpesviruses (1) led these authors to suggest that all gB analogs of the herpesviruses have a highly conserved secondary structure. We would now like to extend this suggestion by stating that the disulfide bond patterns in all gBs of herpesviruses are identical to the one reported herein; that is, intrachain disulfide bonds exist between cysteines in positions equivalent to C1-C8, C2-C7, C3-C4, C5-C6, and C9-C10.

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REFERENCES

1. **Albrecht, J. C., and B. Fleckenstein.** 1990. Structural organization of the conserved gene block of herpesvirus saimiri coding for DNA polymerase, glycoprotein B, and major DNA binding protein. Virology **174:**533–542.
2. **Biemann, K.** 1990. Nomenclature for peptide fragment ions (positive ions). Methods Enzymol. **193:**886–887.
3. **Blewett, E. L., and V. Misra.** 1991. Cleavage of the bovine herpesvirus glycoprotein B is not essential for its function. J. Gen. Virol. **72:**2083–2090.
4. **Burke, R. L.** 1993. Current developments in herpes simplex virus vaccines. Semin. Virol. **4:**187–197.

5. **Bzik, D. J., C. Debroy, B. A. Fox, N. E. Pederson, and S. Person.** 1986. The nucleotide sequence of the gB glycoprotein gene f HSV-2 and comparison with the corresponding gene of HSV-1. Virology **155:**322–333.
6. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133:**301–314.
7. **Cai, W., S. Person, C. DebRoy, and B. Gu.** 1988. Functional region and structural features of gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. J. Mol. Biol. **201:**575–588.
8. **Chapsal, J. M., and L. Pereira.** 1988. Characterization of epitopes on native and denatured forms of herpes simplex virus glycoprotein B. Virology **16:** 427–434.
9. **Claesson-Welsh, L., and P. G. Spear.** 1986. Oligomerization of herpes simplex virus glycoprotein B. J. Virol. **60:**803–806.
10. **Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith.** 1986. Identification of the human cytomegalovirus glycoprotein b gene and induction of the neutralizing antibodies via its expression in the recombinant vaccinia virus. EMBO J. **5:**3057–3063.
11. **Eberle, R., and D. Black.** 1991. The simian herpesvirus SA8 homologue of the herpes simplex virus gB gene: mapping, sequencing, and comparison to the HSV gB. Arch. Virol. **118:**67–82.
12. **Eberle, R., and D. Black.** 1993. Sequence analysis of herpes simplex virus gB gene homologs of two platyrrhine monkey alpha-herpesviruses. Arch. Virol. **129:**167–182.
13. **Ellinger, K., F. Neipel, L. Foa-Tomasi, G. Campadelli-Fiume, and B. Fleckenstein.** 1993. The glycoprotein B homologue of human herpesvirus 6. J. Gen. Virol. **74:**495–500.
14. **Farmer, T. B., and R. M. Caprioli.** 1991. Assessing the multimeric states of proteins: studies using laser desorption mass spectrometry. Biol. Mass Spectrom. **20:**769–800.
15. **Fenn, J. M., M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse.** 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science **246:**64–71.
16. **Habeeb, A. F. S. A.** 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol. **25B:**457–464.
17. **Haffey, M. L., and P. G. Spear.** 1980. Alterations in glycoprotein gB specified by mutants and their partial revertants in herpes simplex virus type 1 and relationship to other mutant phenotypes. J. Virol. **35:**114–128.
18. **Huddleston, M. J., M. F. Bean, and S. A. Carr.** 1993. Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests. Anal. Chem. **65:**877–884.
19. **Johnock, P., G. Kohnstam, and D. Speight.** 1958. The decomposition of ionised cyanates in aqueous solution. Part II. Kinetic studies on the isomerisation of alkylammonium cyanates. J. Chem. Soc. (London) **1958:**2544–2551.
20. **Keller, P. M., A. J. Davison, R. S. Lowe, C. D. Bennett, and R. W. Ellis.** 1986. Identification and structure of the gene encoding gpII, a major glycoprotein of varicella-zoster virus. Virology **15:**181–191.
21. **Koelle, D. M., L. Corey, R. L. Burke, R. J. Eisenberg, G. H. Cohen, R. Pichyangkura, and S. J. Triezenberg.** 1994. Antigenic specificities of human $CD4^+$ T-cell clones recovered from recurrent genital herpes simplex virus type 2 lesions. J. Virol. **68:**2803–2810.
22. **Kongsuwan, K., C. T. Prideaux, M. A. Johnson, M. Sheppard, and K. J. Fahey.** 1991. Nucleotide sequence of the gene encoding infectious laryngotracheitis virus glycoprotein B. Virology **184:**404–410.
23. **Kopp, A., E. Blewett, V. Misra, and T. C. Mettenleiter.** 1994. Proteolytic cleavage of bovine herpesvirus 1 (BHV-1) glycoprotein gB is not necessary for its function in BHV-1 or pseudorabies virus. J. Virol. **68:**1667–1674.
24. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory.** 1990. Assignment of the intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373–10382.
25. **Long, D., W. C. Wilcox, W. R. Abrams, G. H. Cohen, and R. J. Eisenberg.** 1992. Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2. J. Virol. **66:**6668–6685.
26. **Lopez, C., A. M. Arvin, and R. Ashley.** 1993. Immunity to herpesvirus infections in humans, p. 397–425. *In* B. Roizman, R. J. Whitley, and C. Lopez (ed.), The human herpesviruses. Raven Press Ltd., New York.
27. **Marcus, F., and J. Hall.** Personal communication.
28. **Misra, V., R. Nelson, and M. Smith.** 1988. Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. Virology **166:**542–549.
29. **Pellett, P. E., M. D. Biggin, B. Barrell, and B. Roizman.** 1985. Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. J. Virol. **56:**807–813.
30. **Pereira, L.** 1994. Function of glycoprotein B homologues of the family Herpesviridae. Infect. Agents Dis. **3:**9–28.
31. **Pumphrey, C. Y., and W. L. Gray.** 1994. DNA sequence and transcriptional analysis of the simian varicella virus glycoprotein B gene. J. Gen. Virol. **75:**3219–3227.
32. **Qadri, I., C. Gimeno, D. Navarro, and L. Pereira.** 1991. Mutations in conformation-dependent domains of herpes simplex virus 1 glycoprotein B affect the antigenic properties, dimerization, and transport of the molecule. Virology **180:**135–152.
33. **Rapp, M., M. Messerle, B. Buhler, M. Tannheimer, G. M. Keil, and U. H. Koszinowski.** 1992. Identification of the murine cytomegalovirus glycoprotein B and its expression by recombinant vaccinia virus. J. Virol. **66:**4399–4406.
34. **Riggio, M. P., A. A. Cullinane, and D. E. Onions.** 1989. Identification and nucleotide sequence of the glycoprotein gB gene of equine herpesvirus 4. J. Virol. **63:**1123–1133.
35. **Riviere, L. R., M. Fleming, C. Elicone, and P. Tempst.** 1991. Study and applications of the effects of detergents and chaotropes on enzymatic proteolysis, p. 171–179. *In* J. J. Villafranca (ed.), Techniques in protein chemistry II. Academic Press, Orlando, Fla.
36. **Robbins, A. K., D. J. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. C. Glorioso, and L. W. Enquist.** 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein gene of the herpes simplex virus. J. Virol. **61:**2691–2701.
37. **Roepstroff, P., and M. J. Fohlman.** 1984. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed. Mass Spectrom. **11:**601.
38. **Ross, L. J. N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne.** 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. **70:**1789–1804.
39. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. **29:**1149–1158.
40. **Spackman, D. H., W. H. Stein, and S. Moore.** 1960. The disulfide bonds of ribonuclease. J. Biol. Chem. **235:**648–659.
41. **Spaete, R. R., R. M. Thayer, W. S. Probert, F. R. Masiarz, S. H. Chamberlain, L. Rasmussen, T. C. Mergan, and C. Pachl.** 1988. Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage. Virology **167:**207–225.
42. **Stark, G. R.** 1965. Reactions of cyanate with functional groups of proteins. III. Reactions with amino and carboxyl group. Biochemistry **4:**1030–1036.
43. **Stewart, J. P., N. J. Janjua, N. P. Sunil-Chandra, A. A. Nash, and J. R. Arrand.** 1994. Characterization of murine gammaherpesvirus 68 glycoprotein B (gB) homolog: similarity to Epstein-Barr virus gB (gp110). J. Virol. **68:**6496–6504.
44. **Stone, K. L., M. B. LoPresti, N. D. Williams, J. M. Crawford, R. DeAngelis, and K. R. Williams.** 1989. Enzymatic digestion of proteins and HPLC peptide isolation in the sub-nanomole range, p. 377–391. *In* T. E. Hugli (ed.), Techniques in Protein Chemistry. Academic Press, Orlando, Fla.
45. **Stuve, L. L., S. Brown-Shimer, C. Pachl, R. Najarian, D. Dina, and R. L. Burke.** 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. J. Virol. **61:**326–335.
46. **Thornton, J. M.** 1981. Disulfide bridges in globular proteins. J. Mol. Biol. **151:**261–287.
47. **Tigges, M. A., D. Koelle, K. Hartog, R. E. Sekulovich, L. Corey, and R. L. Burke.** 1992. Human $CD8^+$ herpes simplex virus-specific cytotoxic T-lymphocyte clones recognize diverse virion protein antigens. J. Virol. **66:**1622–1634.
48. **Vey, M., W. Schafer, B. Reis, R. Ohuchi, W. Britt, W. Garten, H. D. Klenk, and K. Radsak.** 1995. Proteolytic processing of human cytomegalovirus glycoprotein B (gpUL55) is mediated by the human endoprotease furin. Virology **206:**746–749.
49. **Wagner, B., B. Kropff, H. Kalbacher, W. Britt, V. A. Sundqvist, L. Ostberg, and M. Mach.** 1992. A continuous sequence of more than 70 amino acids is essential for antibody binding to the dominant antigenic site of glycoprotein gp58 of human cytomegalovirus. J. Virol. **66:**5290–5297.
50. **Whalley, J. M., G. R. Robertson, N. A. Scott, G. C. Hudson, C. W. Bell, and L. M. Woodworth.** 1989. Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. J. Gen. Virol. **70:**383–394.
51. **Yoshida, S., L. F. Lee, N. Yanagida, and K. Nazerian.** 1994. Mutational analysis of the proteolytic site of glycoprotein B (gB) of Marek's disease virus. Gene **150:**103–306.