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Evidence for a Phosphorylation Site in Cytomegalovirus Glycoprotein gB

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As part of our vaccine program, we have purified a recombinant form of human cytomegalovirus glycoprotein B that is able to induce high titers of virus-neutralizing antibodies. The isolated protein was found to be phosphorylated at a serine residue in position −7 from the C terminus of the protein. The corresponding synthetic peptide, HLKDSDEEENV, was an efficient in vitro substrate of casein kinase II.

Human cytomegalovirus (CMV), a member of the herpesvirus family, is a major cause of morbidity and mortality following congenital infection (22). In addition, the virus causes significant life- and sight-threatening diseases in immunocompromised individuals (for a review, see reference 23). However, despite recognition of the seriousness of CMV infections, a vaccine for this disease has not yet been developed (1, 22). R. Spaete (for a review, see reference 18) and, more recently, our group have been pursuing the goal of developing a subunit vaccine containing recombinant CMV proteins that are able to induce virus-neutralizing antibodies in humans. The best CMV candidate protein appears to be glycoprotein B (gB), a protein constituent of the virion envelope which has been recognized as a major target of the immune response to CMV infection (17) and the predominant target of neutralizing antibodies (6). The gB molecule also plays a central role in CMV infectivity by promoting virion entry into cells and cell-to-cell-spread of the virus (12).

As part of our vaccine program effort, we have recently purified and characterized a recombinant derivative of human CMV gB strain Towne, designated gBΔTM (results to be published in detail elsewhere). The protein, which is efficiently expressed as a secreted, glycosylated molecule from Chinese hamster ovary cells, contains the N-terminal 676 amino acid residues of the intact CMV gB extracellular domain fused to the last 131 amino acid residues of the cytoplasmic domain with a complete deletion of the transmembrane domain (for sequence information and numbering, see reference 19). In addition, the gene has been mutated at amino acid residues 457, 459, and 460 to block proteolytic cleavage (18). Upon characterizing tryptic peptides derived from CMV gBΔTM, we found that the purified protein is phosphorylated. The present report demonstrates that the phosphorylated site is located at the serine residue in position −7 from the C terminus of the recombinant protein. Phosphorylation studies with the synthetic peptide HLKDSDEEENV (residues 897 to 907 of CMV gB) demonstrate that this peptide is a substrate of casein kinase II.

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Identification of the phosphorylated serine. For characterization studies, we have sequenced, using N-terminal Edman degradation and mass spectrometry, a large number of peptides generated by tryptic cleavage of gBΔTM. We focused on the identification and characterization of peptides containing the putative carboxy terminus of the purified protein because this sequence is both a direct test of the accuracy of the DNA-deduced amino acid sequence and of the intactness of the protein. Ten milligrams of purified CMV gBΔTM was dissolved in 750 μl of 6 M guanidine-HCl–3 mM EDTA in 200 mM Tris-HCl (pH 8.5), and dithiothreitol was added to a final

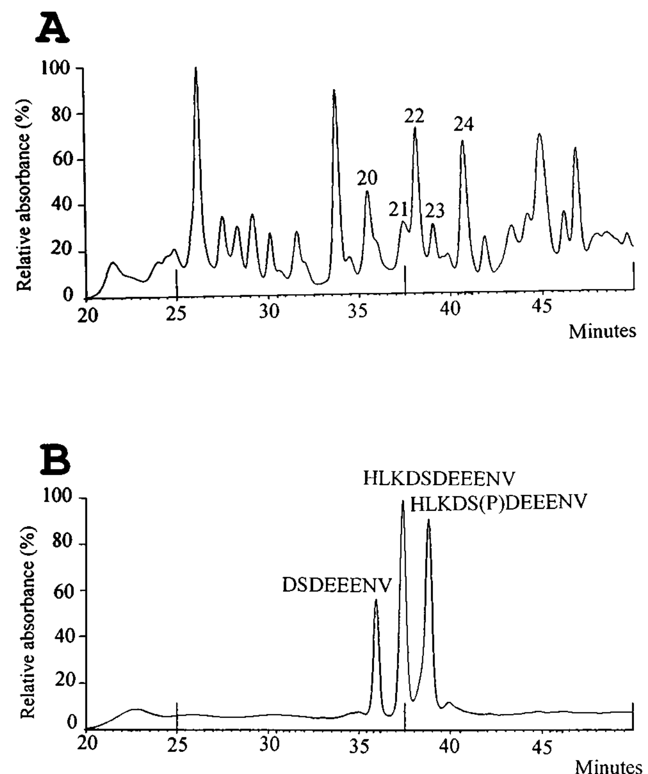

FIG. 1. Reversed-phase HPLC chromatograms of tryptic peptides of CMV gBΔTM (A) and of the synthetic peptides DSDEEENV, HLKDSDEEENV, and HLKDS(P)DEEENV (B). Chromatographic conditions were as described in the text. Only the elution from 20 to 50 min is shown. Chromatogram A was generated with 10 mg of trypsin-digested CMV gBΔTM. Peaks were numbered according to the order of elution, but numbers are shown only for peaks eluting in the region of the synthetic peptides. Only the numbered peaks were analyzed by ESMS. Chromatogram B was generated with 5 nmol each of the synthetic peptides DSDEEENV, HLKDSDEEENV, and HLKDS(P)DEEENV.

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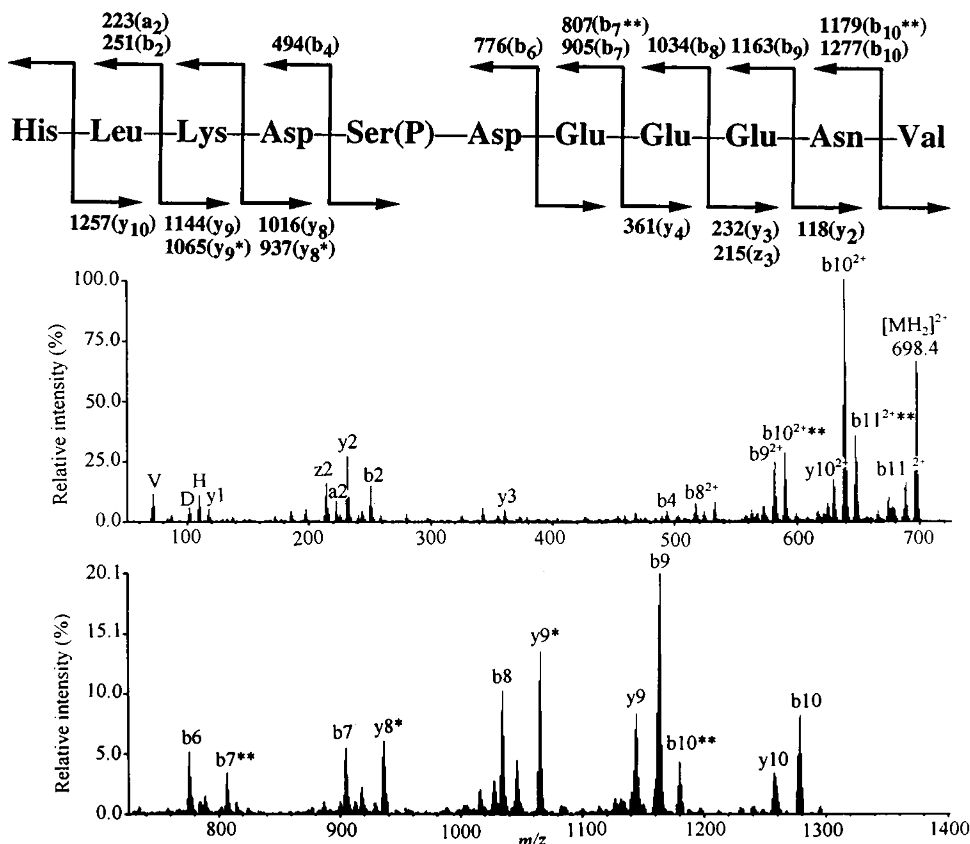

FIG. 2. Low-energy CID-MS/MS spectrum for molecular ion $[MH_2]^{2+}$ of *m/z* 698.4 in fraction 22. Significant ions indicating the sequence are labeled. The ions labeled bn^{2+} and yn^{2+} denote a doubly charged product ion. The ions labeled yn* were formed by the loss of PO_3^- from the corresponding yn ions. The ions labeled bn** were formed by the loss of $H_2PO_4^-$ from the corresponding bn ions. A scheme of the fragmentation is given at the top of the figure.

concentration of 42 mM. The solution was purged with argon. The sample was then incubated for 1 h at 60°C. The sulfhydryl groups formed upon reduction were modified with 79 mM iodoacetic acid. The solution was again purged with argon, and the reaction was allowed to proceed at 22°C. After 30 min, the sample was desalted by gel filtration on a GH 25 column (2 by 18 cm; Amicon, Danvers, Mass.), equilibrated with 1% acetic acid, and dried by vacuum centrifugation. The dried, modified CMV gBΔTM was dissolved in 0.2 ml of 50 mM ammonium bicarbonate (pH 8.5). Trypsin (diphenylcarbamyl chloride treated; Sigma Chemical Co., St. Louis, Mo.) was then added at a 100:1 (wt/wt) ratio of CMV gBΔTM to trypsin, and digestion was allowed to proceed for 18 h at 37°C. The reaction was stopped by freezing. The tryptic digest was fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a 5μ Vydac C_{18} column (4.6 by 250 mm). Buffers and gradient were as described in reference 20. The HPLC chromatogram of the tryptic peptides (Fig. 1A) was compared with the HPLC profile of three synthetic peptides corresponding to sequences that potentially could be released as tryptic fragments containing the C terminus of CMV gB (Fig. 1B). These peptides, synthesized by Chiron Mimotopes (Clayton, Australia) are DSDEEENV, which corresponds to the C-terminal peptide of residues 900 to 907 of gB; HLKDSDEEENV, corresponding to residues 897 to 907 of gB; and HLKDS(P) DEEENV, the phosphorylated form of the same peptide. The identification of the putative tryptic carboxy terminal peptide of CMV gB is of particular interest because an inspection of the amino acid sequence of this protein shows that the carboxy-terminal end of the molecule contains the amino acid sequence Ser-Asp-Glu-Glu. This sequence is a typical recognition sequence for casein kinase II phosphorylation since it contains a serine residue followed by a cluster of acidic amino acid residues in which an acidic residue (Asp or Glu) is located 3 residues on the C-terminal side of the phosphate acceptor, Ser or Thr (4, 10, 14). No peptide corresponding to the sequence DS(P)DEEENV was synthesized because it seemed unlikely that this peptide would be generated by trypsin (11). Major peptides eluting in the region of the synthetic peptide standards (Fig. 1A, peaks 20 to 24) were selected for electrospray ionization mass spectrometry (ESMS) analysis (7). Peaks 20 to 24 contained a total of 10 peptides, and all of them were identified by sequencing by low-energy collision-induced dissociation mass spectrometry/mass spectrometry (CID-MS/MS) (8) and/or Edman degradation. Eight of the 10 peptide sequences identified were found to be unrelated to the C terminus of gBΔTM. These sequences corresponded to the gB peptides of residues 89 to 92, 226 to 230, 291 to 297, 493 to 497, 686 to 691, 790 to 804, and 884 to 887 and to the N-glycosylated peptide of residues 861 to 878. The C-terminal peptides were found in fractions 21 and 22. Fraction 21 contained the peptide HLKDSDEEENV, corresponding to the C-terminal peptide. The retention time of the authentic peptide was identical to that of the corresponding synthetic peptide used as a standard (Fig. 1B). The observed mass (1,314.3 Da) was consistent with the calculated mass. Furthermore, the above sequence was verified by CID-MS/MS and Edman degradation (results not shown). From the mass spectrum of fraction 22, a peptide with

a molecular mass of 1,393.8 ± 0.8 Da was identified from the ions of *m/z* 698.0 ($[MH_2]^{2+}$) and 1,394.6 ($[MH]^{+}$). The mass was consistent with the phosphorylated form of the peptide HLKDSDEEENV. The presence of a phosphate group was confirmed by CID-MS/MS in the negative (results not shown) and positive ion modes. The CID-MS/MS spectrum of the molecular ion $[MH_2]^{2+}$ of *m/z* 698.4, obtained in the positive ion mode, is shown in Fig. 2. The data confirmed the sequence HLKDS(P)DEEENV. Significant ions arising from the fragmentation of the peptide are indicated by standard nomenclature (5). The amino acid sequences of the peptides present in fractions 21 and 22 were also confirmed by Edman degradation (data not shown). The peptides DSDEEENV and DS(P)DEE ENV were not found among the peptides generated by the digestion of gBΔTM with trypsin, suggesting, as expected, that the peptide bond between Lys-899 and Asp-900 was not cleaved by trypsin (2, 11). From all of the above data, we can conclude that gBΔTM exists in both phosphorylated and dephosphorylated forms. However, an accurate ratio of the forms cannot be established with certainty because the recovery of peptides by reversed-phase HPLC is known to be sequence dependent (13).

In vitro phosphorylation of the peptide HLKDSDEEENV. Since the peptide sequence HLKDSDEEENV, present at the C terminus of CMV gB, is characteristic of a typical recognition sequence by casein kinase II (10), a corresponding synthetic peptide was tested as a potential substrate of the enzyme. The reactions were carried out at 30°C in a reaction mixture containing 100 μM ATP, 10 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5). Casein kinase II, from Sea Star (Upstate Biotechnology Inc., New York, N.Y.), and the synthetic peptide were used at 2 μg/ml and 50 μM, respectively. The final volume was 50 μl. The reaction was started by the addition of the enzyme, and at various times, 5 μl of the reaction mixture was diluted in 20 μl of 1 N HCl. The samples were analyzed directly by ESMS. The results obtained during the course of the reaction are shown in Fig. 3. After 2 min of incubation (Fig. 3A), only one peptide can be identified from the ions of *m/z* 439.0 ($[MH_3]^{3+}$) and 658.0 ($[MH_2]^{2+}$). The mass obtained (1,314.0 Da) was consistent with the theoretical mass of the initial peptide substrate. At 40 min (Fig. 3B), two additional ions, of *m/z* 465.6 and 697.9, are observed. These ratios are characteristic of a single peptide under two different states of protonation ($[MH_3]^{3+}$ and $[MH_2]^{2+}$, respectively). The deduced mass (1,393.8 Da) was consistent with the mass of the expected phosphopeptide product HLKDS(P)DEEENV. This peptide constitutes the major product observed in the spectrum obtained after 200 min of reaction (Fig. 3C). By using the conventional protein kinase assay with [γ-^{32}P]ATP, the CMV gB carboxy-terminal peptide has been found to be an excellent substrate of casein kinase II. The synthetic peptide RHLKDSDEEENV (sequence of residues 797 to 807 of CMV gBΔTM, plus an extra Arg residue at the amino terminus) exhibited a K_m of 0.57 mM, which compares well with the apparent K_m of 0.23 mM of the model peptide substrate RRREEETEEE (3). Although the C termini of human and murine CMV gBs have a low degree of homology, the sequence RLPAEDSDFEY (residues 918 to 928 of murine CMV gB [Fig. 5 in reference 15]) also contains the typical SXXE casein kinase II consensus sequence. As expected, the synthetic peptide RRLPAEDSDFEY was found, by mass spectrometry analysis, to be a substrate of casein kinase II (results not shown). The C-terminal amino acid sequences of gB of the infectious laryngotracheitis virus (9) and of Marek's disease virus (16) are also predicted to be phosphorylated since they also contain the characteristic sequence of substrates of casein kinase II. Although the recombinant form of CMV gB used in the present study is phosphorylated in mammalian cells in the absence of any other herpesvirus products, we suggest that CMV gB is an in vivo substrate for a casein kinase II-related phosphotransferase. In this respect, it is interesting to note the recent work of Tugizov et al. (21), who have demonstrated phosphorylation at the carboxy terminus of CMV gB. It remains to be determined whether the phosphorylation of Ser-801, described herein, is related to the role of the carboxy-terminal region of CMV gB in syncytium formation (21) or has any other physiological relevance.

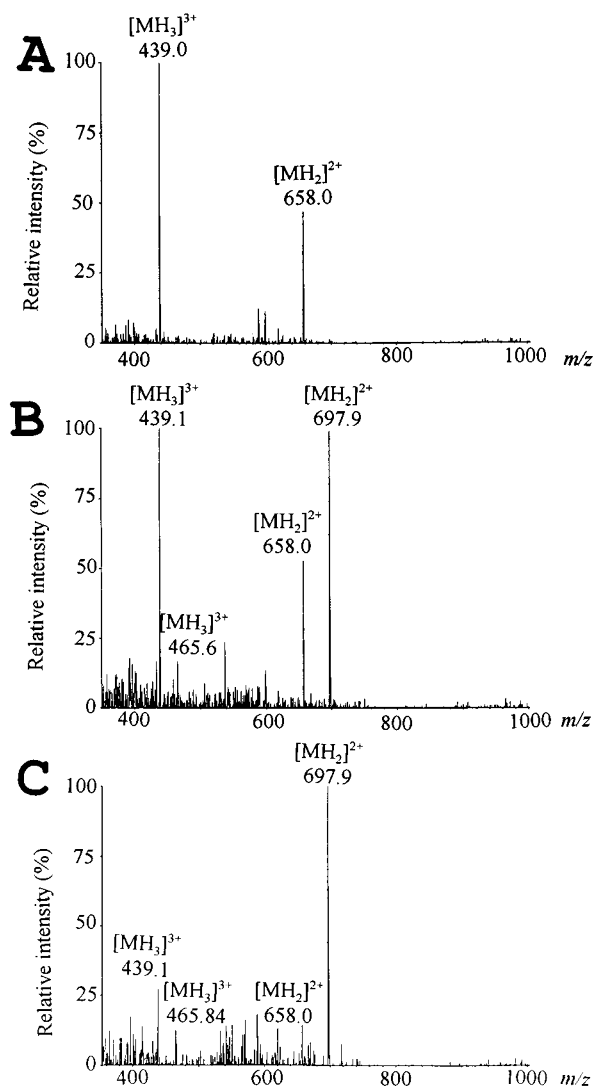

FIG. 3. ESMS spectrum of the product of the phosphorylation reaction of the synthetic peptide HLKDSDEEENV and casein kinase II. For experimental details see the text. The ESMS spectra shown in panels A, B, and C were obtained after 2, 40, and 200 min of reaction, respectively.

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