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NOTES

Structural Properties and Reactivity of N-Terminal Synthetic Peptides of Herpes Simplex Virus Type 1 Glycoprotein D by Using Antipeptide Antibodies and Group VII Monoclonal Antibodies

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To investigate the contribution of individual amino acids to the antigenicity of the N-terminal region of herpes simplex virus type 1 glycoprotein D, a series of 14 overlapping synthetic peptides within residues 1 to 30 were examined for their reactivity with monoclonal antibody LP14 (a group VII monoclonal antibody; in herpes simplex virus mutants resistant to LP14, arginine 16 is substituted by histidine) and two antipeptide antisera (antipeptide 9-21 and antipeptide 1-23). Maximal binding was achieved with peptides 9-21, 10-30, 9-30, and 8-30 and the chymotryptic fragment 9-17 of peptide 9-21, suggesting that a major antigenic site is located within residues 10 through 17. Lysine 10 was shown to be essential for high reactivity, either by binding directly to the antibody molecule or by stabilizing an ordered structure of the peptide. The importance of ordered structure was demonstrated by a decrease in reactivity after sodium dodecyl sulfate treatment of peptides 9-21 and 8-30.

Glycoprotein D (gD) is a structural component of the envelope of herpes simplex virus (HSV) (26, 27). gD appears to be one of the major targets for the immune response to HSV, since immunity to gD, obtained either by transfer of monoclonal antibodies against gD (2, 25) or by immunization with gD (4, 7, 15, 16, 21, 24), interferes with the infection process. gD is likely to be involved in the initial phases of infection, such as adsorption of virus to the target cells (12, 20, 22), although many aspects of its precise role in this event are still unclear.

Monoclonal antibodies to gD were used to localize eight separate antigenic domains. These monoclonal antibodies can be grouped according to their reactivity with gD into eight groups (10). Groups II, V, and VII are reactive with native and denatured gD, whereas groups I, III, IV, and VI only react with gD under nondenaturing conditions. Studies with overlapping synthetic peptides in the N-terminal region of gD showed that group VII antibodies react with a continuous epitope within residues 11 through 19 (5, 8, 18). Similar studies showed that groups II and V react within the regions 268 through 287 and 340 through 356, respectively (11). The remaining groups of monoclonal antibodies, I, III, IV, and VI, are directed against discontinuous epitopes. Reactivity of monoclonal antibodies with fragments of gD, obtained either by proteolysis of native gD or by recombinant DNA methods, suggests that epitopes of groups III, IV, and VI are located within the region 1 through 182 of gD (6). Monoclonal antibodies of group VIII are only reactive with gD of HSV type 2 (HSV-2) (10). From studies with truncated forms of gD, it was concluded that group I antibodies react with an epitope that is partly located between residues 233 and 256 (6).

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Monoclonal antibody LP14, which is directed to HSV, reacted with synthetic peptide 7-23 and HSV-1 equally well, and to a lesser extent with peptide 11-32. Monoclonal antibody LP14 has been classified in group VII. Viral infectivity is neutralized by LP14, and the antibodies suppress cell fusion (18). Furthermore, it was demonstrated that in HSV mutants resistant to LP14 arginine 16 was substituted by histidine (18).

Binding properties of synthetic peptides. To study the N-terminal boundary of the antigenic region recognized by antibody LP14 in more detail, we determined the reactivity of peptides 1-13, 9-21, and 18-30 with this monoclonal antibody LP14. (LP14 [18] was a gift from A. C. Minson, Division of Virology, Department of Pathology, University of Cambridge, United Kingdom.) Of these, only peptide 9-21 reacted with LP14. This was not entirely unexpected, since it is known that in HSV mutants resistant to LP14, arginine 16 is substituted by histidine (18). It is possible that residue 16 lies within or very close to the antibody binding site of LP14. To study the contribution of individual amino acids to the antigenicity, we then synthesized a series of overlapping peptides by elongating peptide 18-30, which did not react with LP14, toward the N terminus and subsequently determined their reactivity with LP14 and two anti-peptide antisera.

Synthesis of peptides 8-30, 9-30, 10-30, 11-30, 12-30, 13-30, 14-30, 15-30, 16-30, 17-30, and 18-30 followed the stepwise solid-phase strategy developed by Barany and Merrifield (3, 17) and was performed with a Labortec SP640 semiautomatic peptide synthesizer (Labortec AG, Bubendorf, Switzerland). The *tert*-butyloxycarbonyl group was used for temporary amino-terminal protection. Side-chain protection was as follows: benzylether for threonine, benzylester for aspartic acid, *p*-methoxybenzenesulfonyl for arginine, and benzyloxycarbonyl for lysine. To avoid side-chain reactions,

methionine was substituted by norleucine, which has the same overall shape (3). After synthesis the protective groups were removed, and the peptides were cleaved from the resin with 1 M trifluoromethanesulfonic acid-thioanisole in trifluoroacetic acid (31). The peptides were purified by gel filtration on Sephadex G-10 in 0.1 M acetic acid. Peptides were characterized by amino acid analysis, determination of the N-terminal amino acid by dansylation, and reversed-phase high-performance liquid chromatography. Amino acid analyses of the peptides were performed on a Kontron Liquimat III analyzer (28). Peptide concentrations used in the experiments were based on amino acid analyses. Determination of the N-terminal amino acid by dansylation with identification of dansyl amino acids by thin-layer chromatography on polyamide sheets (5 by 5 cm) was done by the method of Hartley (14).

The reactivity of these overlapping peptides with antibody LP14 and with antipeptide antisera was studied in an enzyme-linked immunosorbent assay (ELISA). The antiserum directed to residue 1-23 (8, 9) of mature gD of HSV-1 was a gift from G. H. Cohen and R. J. Eisenberg (Department of Microbiology and the Center for Oral Health Research, Philadelphia, Pa.). Antisera to peptides 9-21 and 18-30 were obtained by immunization of rabbits with peptide-carrier protein conjugates (13, 23). All three antipeptide sera neutralize viral infectivity (8, 9; unpublished results). To determine the reactivity between the peptides and the antisera, ELISA plates (Dynatech immulon M-129B, Nutacon, The Netherlands) were coated with 2 μg of peptide in 100 μl of 50 mM sodium carbonate (pH 9.6) per well. The amount of 2 μg per well resulted in an optimal reaction with the polyclonal and monoclonal sera. Antisera and peroxydase-labeled anti-mouse or anti-rabbit immunoglobulin G were diluted in phosphate-buffered saline supplemented with 0.5 M NaCl–0.3% Tween 20. To visualize the enzyme reaction, 0.2 mg of orthophenylene diamine dihydrochloride (Eastman Kodak Co., Rochester, N.Y.), per ml–0.006% H_2O_2 in 50 mM sodium phosphate (pH 5.6) was used. The titer was expressed as the reciprocal serum dilution with an optical density at 490 nm higher than 0.2.

The binding of synthetic peptides 8-30, 9-30, 10-30, 11-30, 12-30, 13-30, 14-30, 15-30, 16-30, 17-30, and 18-30 with monoclonal antibody LP14, antipeptide 9-21 antiserum, and antipeptide 1-23 antiserum was determined. Figure 1 shows the reactions of these 11 overlapping peptides with the antisera. All peptides lacking residues 11, 10, 9, and 8 did not react with LP14. Extension of peptide 12-30 with one amino acid (norleucine) resulted in a minor reactivity with LP14; a titer of 3,200 was measured. However, the addition of lysine 10 resulted in a strong reaction of peptide 10-30 with LP14 (titer, 204,800). Further extension of peptide 10-30 with leucine 9 and serine 8 did not increase the binding activity. This means that not only arginine 16 is important for binding of LP14 to gD, but also other amino acids must be present to obtain optimal binding with LP14. Lysine 10 is necessary to get an optimal reaction with LP14 (Fig. 1). Figure 1 also shows the reaction of two polyvalent sera, antipeptide 9-21 and antipeptide 1-23 antisera, with the series of overlapping peptides. Peptides 18-30, 17-30, 16-30, 15-30, and 14-30 did not react with antipeptide 9-21 antiserum. Peptide 13-30 showed a minor reaction with antipeptide 9-21 antiserum (titer, 800), and extension of this peptide resulted in a gradual increase of binding between peptides and polyclonal sera, reaching a maximum at peptide 11-30 with a titer of 6,400, which is 16 times less than the reaction with LP14. In addition, we investigated the binding of antipeptide 1-23 antiserum with all peptides. The reactivity of this antiserum started to increase with peptide 15-30. The reactivity gradually increased and finally reached the same maximum as antipeptide 9-21 antiserum at peptide 11-30 with a titer of 6,400. The fact that these antisera recognized shorter peptides as compared with LP14 is probably due to the fact that these sera are polyclonal; they consist of many species of antibodies with multiple specificities that may be directed against various antigenic sites of the peptide. In contrast to this, LP14 consists of only one species of antibody directed against one specific epitope. The importance of lysine 10 was further demonstrated in two experiments: a competition binding assay of citraconylated peptide 9-21 and digestion of this peptide with chymotrypsin.

Citraconylation of peptide 9-21. Peptide 9-21 shows the same reactivity with LP14 as peptides 8-30, 9-30, and 10-30 and was used in modification studies. The lysines at positions 10 and 20 and the α-NH_2 of the N-terminal leucine of peptide 9-21 were modified by citraconylation to confirm the importance of the lysines in the reaction of peptide 9-21 with LP14. Peptide 9-21 was modified with citraconic anhydride essentially as described by Weng et al. (32). Peptide 9-21 (0.5 mg) was dissolved in 200 μl of 0.2 M *N*-ethylmorpholine acetate (pH 8.5). A 275-fold molar excess of citraconic anhydride (30 μl) over the calculated number of free amino groups present was added under rapid stirring in 5-μl portions at 5-min intervals to the peptide solution. The pH was maintained between 8 and 9 by dropwise addition of 6 M NaOH. After the last addition of citraconic anhydride, the reaction mixture was stirred for 1 h at room temperature. Excess reagent was removed by gel filtration on Sephadex G-10 in 0.1 M ammonium bicarbonate (pH 8.5). The extent of citraconylation was monitored by reaction of residual α- and ε-NH_2 groups with dansylchloride. Despite the presence of sufficient peptide material as was shown by examination of a parallel sample hydrolyzed before dansylation, no dansylated amino acids could be detected, indicating that all α- and ε-NH_2 amino acid groups were modified.

The reaction of peptide 9-21 and citraconylated peptide 9-21 with LP14 was compared in a competition ELISA experiment. ELISA trays were coated with peptide 9-21 (2

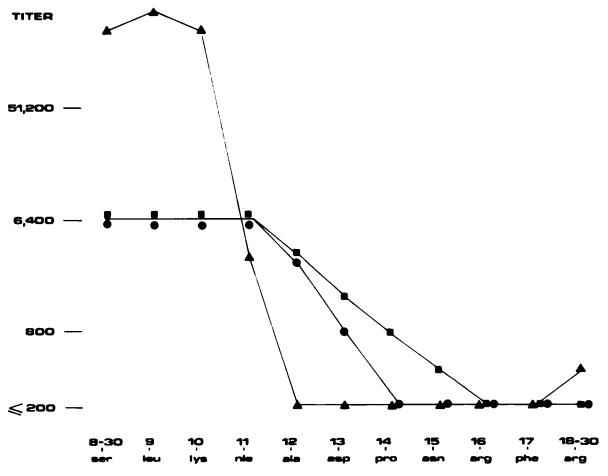

FIG. 1. Reactivity of peptides 8-30, 9-30, 10-30, 11-30, 12-30, 13-30, 14-30, 15-30, 16-30, 17-30, and 18-30 with monoclonal antibody LP14 (▲), antipeptide 1-23 antiserum (■), and antipeptide 9-21 antiserum (●). The titers are expressed as the reciprocal serum dilution with an optical density at 490 nm higher than 0.2. (Nle is norleucine.)

μg per well). An optimal dilution (1:6,400) of monoclonal antibody was preincubated for 1 h at room temperature with different amounts of peptide 9-21 or modified peptide 9-21. To determine residual binding activity of LP14 after preincubation, the reaction mixture was added to the wells coated with peptide 9-21, and the ELISA was continued as described above.

The results of the competition binding assay are shown in Fig. 2. Preincubation of 3.26×10^{-5} mol of peptide 9-21 with a 6,400-fold dilution of antibody LP14 completely inhibited the homologous reaction with immobilized peptide 9-21. A 16-fold excess of citraconylated peptide 9-21 (5.22×10^{-4} mol) was needed to show inhibition to the same extent as the unmodified peptide. Thus, one or both lysines at positions 10 and 20 are involved in the antigenic reaction with LP14. This experiment does not exclude the influence of lysine 20, which is also modified by citraconylation. To eliminate the contribution of lysine 20, we digested peptide 9-21 with chymotrypsin to obtain a smaller fragment.

Digestion with chymotrypsin. To determine whether lysine 20, in addition to lysine 10, is important in the antigenic reaction of peptide 9-21 with LP14, a smaller fragment that did not contain lysine 20 was generated by digestion with chymotrypsin. Fragments of peptide 9-21 were obtained by incubation of 0.5 mg of peptide with chymotrypsin in 1 ml of 0.1 M ammonium bicarbonate (pH 8.0) at 37°C for 4 h at an enzyme/substrate ratio of 1:250 (wt/wt). After lyophilization, 0.5 ml of 0.1% trifluoroacetic acid was added, and the fragments were separated by reversed-phase high-performance liquid chromatography on Nucleosil 10 C18 (250 by 4.6 mm) with a 30-min linear gradient of 30 to 70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Amino acid analysis of the major peaks and determination of the N-terminal amino acid by dansylation showed that the peak that was eluted at 23 min contains a peptide corresponding to residue 9-17 and that the peak that was eluted at 6 min contains the smaller peptide 18-21. Peptide 9-17 was tested for reactivity with antibody LP14 in an ELISA. Despite the absence of lysine 20, the reactivity of peptide 9-17 with LP14 was identical to that of peptide 9-21 (titer, 409,600). This suggests that lysine 20 is not involved in the reaction with LP14. This allows the conclusion that lysine 10 indeed is important for antigenicity of N-terminal synthetic peptides

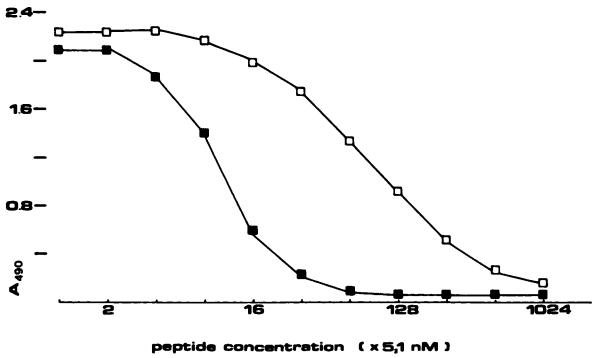

FIG. 2. Competition binding experiment by ELISA with monoclonal antibody LP14, peptide 9-21, and citraconylated peptide 9-21. A 6,400-fold dilution of monoclonal antibody LP14 was preincubated with different concentrations of peptide 9-21 and citraconylated peptide 9-21. The residual binding activity between peptide 9-21 and LP14, after preincubation with peptide 9-21 (■) and citraconylated peptide 9-21 (□), respectively, is expressed as absorbance at 490 nm.

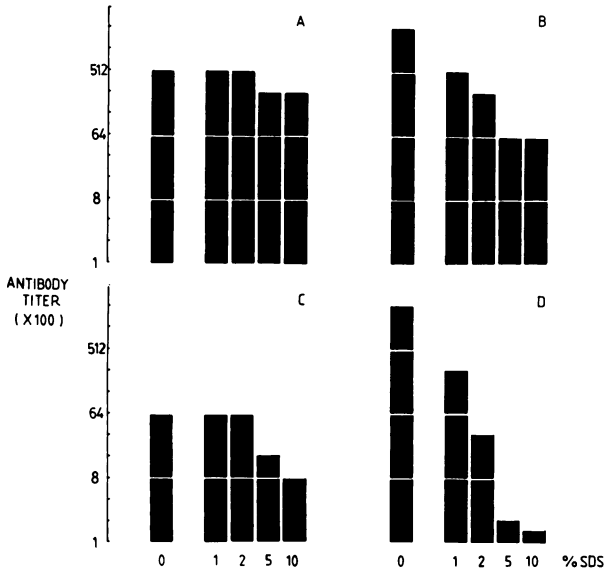

FIG. 3. Effect of SDS treatment on the binding between peptides 9-21 and 8-30 and monoclonal antibody LP14 and antipeptide 9-21 serum, respectively. Peptides 9-21 and 8-30 were boiled in the presence of the indicated concentrations of SDS. The peptides were diluted to 20 μg/ml and coated for ELISA determination. The reactivity between peptide 9-21 and SDS-treated peptide 9-21 and antipeptide 9-21 serum (A) and monoclonal antibody LP14 (B) and between peptide 8-30 and SDS-treated peptide 8-30 and antipeptide 9-21 serum (C) and monoclonal antibody LP14 (D) are shown. The reactivity is expressed in antibody titers.

of gD. Studies with another group VII monoclonal antibody, 170, showed that in that case lysine 10 was not involved in the antigenicity of the N-terminal epitope (5, 8). It is possible that lysine 10 is one of the contact residues within the binding site of antibody LP14 or, alternatively, that it stabilizes a certain structural conformation that mimics the native conformation of gD.

Denaturation. It is generally supposed that the antigenic activity of peptides is related to their secondary structure (1), although it is not clear to what extent such structure is present in solution (19). To obtain information on the importance of ordered structure of the synthetic peptides in the reaction with the antisera, peptides 9-21 and 8-30 were heated in boiling water in the presence of various concentrations of sodium dodecyl sulfate (SDS) and tested in ELISA with LP14 and antipeptide 9-21 antiserum. To denature peptide 9-21 and 8-30, SDS was added to the solution of peptides (2 mg/ml) in a final concentration of 1, 2, 5, or 10% (wt/vol). Subsequently, the samples were kept in a boiling water bath for 5 min. The SDS-treated peptides were diluted to a concentration of 20 μg/ml in 50 mM sodium carbonate buffer (pH 9.6) and used to coat ELISA plates. The SDS concentration did not exceed 0.1% (wt/vol) and did not affect the coating of the peptides. Figures 3A and B show the reaction of peptide 9-21 with antipeptide 9-21 serum and LP14, respectively. SDS treatment hardly had any effect on the reaction of peptide 9-21 with antipeptide 9-21 antiserum. In contrast to this, denaturation of peptide 9-21 markedly reduced the reaction with LP14; 5 and 10% SDS-treated peptide 9-21 (Fig. 3B) with LP14 resulted in a titer of 6,400, whereas untreated peptide 9-21 (Fig. 3B, 0%) showed a strong reaction with LP14 (titer, 409,600). Figures 3C and D show the reaction of peptide 8-30 with antipeptide 9-21

antiserum and LP14, respectively. Untreated peptide 8-30 did react with antipeptide 9-21 antiserum (Fig. 3C), and some decrease of antigenic activity was observed when SDS-treated peptide 8-30 was used (Fig. 3C). After treatment of peptide 8-30 with 5 or 10% SDS, the antigenic reaction with LP14 almost completely disappeared (Fig. 3D).

This confirms the assumption that LP14 is directed against a continuous determinant which requires a certain conformation. Denaturation had more effect on the reaction of peptides 9-21 and 8-30 with the monoclonal antibody than on the reaction of antipeptide 9-21 antiserum with these peptides. From these results, it may be concluded that the polyclonal antipeptide antibodies are directed against a less ordered structure than LP14. Alternatively, reaction of the antipeptide antibodies may affect to some extent the secondary structure of the peptide. A comparison of the two peptides 9-21 and 8-30 with regard to their reactivity with antibodies after denaturation shows that the binding capacity of the longer peptide is more drastically reduced by SDS treatment than that of the smaller peptide. It is possible that peptide 8-30 has a more pronounced ordered structure and therefore is more sensitive to SDS treatment. Recent nuclear magnetic resonance studies (29, 30) revealed interesting aspects about the relation of ordered structure and amino acid composition of synthetic peptides. Studies of peptide 7-23 of HSV gD showed two tight turns around residues 14 and 15 and 18 and 19. In peptide 11-32, secondary structure was present as a turn at residues 14 and 15, as a helical structure at residues 20 through 24, and a turn was present as a minor component at residues 18 and 19. Since peptide 11-32 does not include lysine 10, it may be possible that lysine stabilizes a secondary structure in which turns at positions 14 and 15 and 18 and 19 are preferred. Because monoclonal antibody LP14 reacted optimally with peptides comprising residues 10 through 30 or longer toward the N terminus, we think that lysine 10 is a crucial amino acid in the antigenicity of the N-terminal epitope reacting with monoclonal antibody LP14.

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