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Mass Spectrometric Characterization of a Discontinuous Epitope of the HIV Envelope Protein HIV-gp120 Recognized by the Human Monoclonal Antibody 1331A

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The characterization of a discontinuous epitope in the C5 region of the HIV envelope protein HIV-gp120, recognized by 1331A, a human mAb, is reported. Regions involved in affinity binding in the HIV-gp120 molecule were identified by epitope excision/extraction methods followed by matrix assisted laser desorption-time of flight mass spectrometry. In epitope excision, the protein is bound in its native conformation to an immobilized Ab and then digested with proteolytic enzymes. In epitope extraction, the protein is first digested and subsequently allowed to react with the Ab. A series of proteolytic digestions of the 1331A/HIV-gp120 complex allowed the identification of protected amino acids in two noncontiguous regions of the C5 region of HIV-gp120. Interaction of the Ab with amino acids 1487 and E507 of HIV-gp120 is essential for efficient binding. This is the first application of this approach for the identification and characterization of a discontinuous epitope. The results are consistent with molecular modeling results, indicating that these amino acids are located on opposite sides of a hydrophobic pocket. This pocket is thought to be of importance for the interaction of HIV-gp120 with the transmembrane protein HIV-gp41.

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Primary isolates, which may have been cultured in PBMC but have not been passaged in cell lines, are closely related to the viruses occurring during natural infection. Several human mAbs specific for various regions of HIV-gp120 have been reported to have potent neutralizing activity against primary isolates (9, 10). Passive immunization of SCID-human mice with human mAbs provided protection against infection with primary isolates (11). However, escape variants of the virus have been isolated, which emphasizes the need for combinations of Abs with different specificities (12).

Elucidation of the epitopes that these Abs recognize can be an important part of the development of HIV vaccines. Furthermore, it can provide insights into the tertiary structure of HIV-gp120 (13), because x-ray analysis of only a truncated HIV-gp120 molecule is available (5). Several methods for the characterization of epitopes have been described, such as random phage epitope library screening, x-ray analysis, proteolytic footprinting, and epitope excision/extraction techniques. Random phage epitope library screening was used for the identification of peptide sequences, which were capable of interacting with anti-HIV-gp120 Abs (14, 15). X-ray analysis was used successfully for the elucidation of epitopes (16). However, this method is time consuming, because of difficulties associated with protein crystallization.

Proteolytic footprinting methods are based on the protection of residues in the Ag that are involved in affinity binding against proteolysis or chemical modification and the high resistance of the Ab to proteolytic digestion (17, 18). In epitope excision methods, the protein of interest is complexed with the Ab and then digested enzymatically. Only fragments that are protected by the Ab remain affinity bound and can be identified. Because the Ag is bound to the Ab in its native conformation under physiological conditions, this approach allows the identification and characterization of linear and discontinuous epitopes. In epitope extraction, the Ag of interest is first digested enzymatically and subsequently passed over immobilized Ab beads. Recently, epitope excision/extraction techniques have been used in combination with matrix-assisted
laser desorption (MALDI)-time of flight (TOF) mass spectrometry for the characterization of linear epitopes of the HIV proteins HIV-p24 and HIV-gp120 (19, 20).

We report here the elucidation of a discontinuous epitope in the HIV envelope protein HIV-gp120, recognized by the mAb 1331A by epitope excision/extraction techniques followed by MALDI-TOF mass spectrometry. 1331A is a human mAb specific to the C5 region of HIV-gp120, which has been produced from PBMC of an HIV-infected patient, as described previously (21, 22). Binding studies of intact virus particles of HIV revealed a high affinity of 1331A to laboratory-adapted strains and primary isolates (23). These studies also demonstrated that the epitope recognized by 1331A Ab is highly conserved and well exposed on HIV.

Materials and Methods
Materials
Recombinant HIV-gp120 from HIV-1 strain SF-2 was obtained from Austral Biologicals (San Ramon, CA). It was produced by secretion from Chinese hamster ovary cells, purified by ion exchange chromatography followed by gel filtration chromatography, dissolved in PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2), 1 mM EDTA, and 1 mM EGTA, pH 7.4, and stored at −20°C. The human mAb 1331A (subclass IgG3) was produced as described previously (21, 22). The Ab solution was stored at −20°C. The synthetic peptides were received from Genosys Biotechnologies (The Woodlands, TX). Endoproteases Glu-C and Asp-N were purchased from Sigma (St. Louis, MO), trypsin-(tosyl-amido-2-phenyl)ethylchloromethyl ketone-treated (TPCK) was obtained from Worthington Biochemical (Freehold, NJ), and endoproteinase Lys-C and Asp-N were purchased from Wako Chemical (Dallas, TX). Aminopeptidase M and carboxypeptidase Y were obtained from Sigma.

MALDI-TOF mass spectrometry
MALDI-TOF mass spectra were acquired on a Voyager-RII MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA), equipped with a nitrogen laser (λ = 337 nm). The matrix used was α-cyano-4-hydroxy-cinnamic acid (Aldrich, Milwaukee, WI). It was recrystallized in hot methanol, stored in the dark, and before sample analysis a fresh saturated solution of the matrix in water/ethanol/formic acid 45/45/10 (v/v) was prepared. One-half microliter of the sample was mixed with 0.5 μl of the matrix solution and dried at room temperature. One hundred twenty-five laser shots were summed per sample spectrum. Two standard proteins were used for external calibration in the desired mass range.

Immobilization procedure
Cyanogen bromide (CNBr)-activated Sepharose beads (0.1 g) were suspended in 5 ml of 1 mM HCl and incubated for 15 min. After washing the beads with 1 M NaHCO3, coupling buffer, the Ab (20 μg) was added to the beads and incubated for 2 h at room temperature with slow rotation. The beads with the now covalently attached Ab were subsequently washed with 0.1 M Tris-HCl, pH 8.0. Unreacted groups on the CNBr Sepharose beads were blocked by incubation with 0.1 M Tris-HCl, pH 8.0, for 2 h. The beads were washed successively with 0.1 M NaNO2Ac, pH 4.0, buffer and a 0.1 M Tris-HCl, pH 8.0, buffer. The beads were equilibrated in PBS and incubated for 2 h at room temperature with and without HIV-gp120. After washing the beads with PBS, an aliquot of the suspension was removed for MALDI-TOF analysis. Epitope excision and extraction experiments were performed as previously described (19). For epitope excision, the beads were resuspended in 50 mM NH4 CO3, pH 7.8, and digested with Asp-N (1:20 enzyme:substrate ratio) at 37°C for 12 h. The beads were then washed with the digestion buffer and an aliquot was removed for MALDI-TOF analysis. The fragment that remained affinity bound was further digested with the exopeptidases aminopeptidase M and carboxypeptidase Y. For aminopeptidase digestion, the beads with the affinity-bound peptides were equilibrated in 100 mM phosphate buffer, pH 7.2. The enzyme was added in an enzyme:substrate ratio of 1:10 and incubated at 37°C with gentle rotation for 12–72 h. For digestion with carboxypeptidase Y, the beads with the affinity-bound peptide were incubated with carboxypeptidase (1:20 enzyme:substrate ratio) in 50 mM N-methylmorpholine buffer, pH 7.2, at 37°C for 14 h with slow rotation. After washing three times with the digestion buffer, an aliquot of the beads was removed for MALDI-TOF analysis.

Results
Binding of HIV-gp120SF2 to immobilized 1331A Ab
To define the epitope that the 1331A Ab recognizes in the intact HIV-gp120 molecule, the Ab was first immobilized on CNBr Sepharose beads and was incubated with HIV-gp120 from HIV-1 strain SF-2. The beads were subsequently analyzed directly by MALDI-TOF mass spectrometry. The MALDI-TOF mass spectrum of HIV-gp120 affinity bound to immobilized 1331A Ab on CNBr Sepharose beads is shown in Fig. 1. No affinity-bound proteins were observed in a control experiment without previous incubation with HIV-gp120. The molecular mass of affinity-bound HIV-gp120 was found to be approximately 100 kDa. A theoretical average molecular mass of 57,496 Da can be calculated from the amino acid sequence of HIV-gp120 (24).

FIGURE 1. MALDI-TOF mass spectrum (mass range 10,000–120,000 Da) of HIV-gp120 affinity bound to 1331A Ab. The singly, doubly, and triply charged molecular ions are marked.

FIGURE 2. Amino acid sequence of the HIV envelope protein HIV-gp120 (amino acids 1–509) from strain SF-2 according to Sanchez-Pescador et al. (24). The numbering of the sequence includes the leader sequence.
are due to the high glycosylation of HIV-gp120. These data are consistent with the molecular mass previously determined by MALDI-TOF mass spectrometry and SDS-PAGE (20, 25). As observed previously, this HIV-gp120 preparation lacks the terminal two amino acids, K-508 and R-509 (20). Furthermore, the C terminus is heterogeneous with truncation residues E507, R506, Q505-V504, and V503 having been observed (20).

Endo- and exoproteolytic digestion of affinity-bound HIV-gp120

To define the epitope recognized by the 1331A mAb, affinity-bound HIV-gp120 was then digested with endoproteases. After treatment of the Ab-Ag complex with endoproteinase Asp-N, one fragment of m/z 3989 was found to remain affinity bound (Fig. 3). The molecular mass of this fragment is in good agreement with the theoretical average molecular mass of the C-terminal peptide 475–507 of HIV-gp120: DNWRSELYKYKVIKIEPLGIAPTKAKRRVVQRE (3986 Da), as calculated from its amino acid sequence. Interestingly, no truncated peptides remained affinity bound to the 1331A Ab. Peptide 475–507, affinity bound to 1331A Ab, was digested further with endoproteinase Glu-C. Fig. 4 shows the MALDI spectrum of the peptide of m/z 3200, which remained affinity bound to the immobilized 1331A Ab after Glu-C digestion. This corresponds to the theoretical average molecular mass of the peptide 481–507 of HIV-gp120: LYKKYKVIKIEPLGIAPTKAKRRVVQRE (3198 Da). Attempts to characterize the epitope further by digestion for 6 h with endoproteinase Lys-C, which has five potential cleavage sites in this fragment, led to complete loss of affinity binding. In the supernatant of the digest, two peptides with m/z 1038 and m/z 942 were identified, corresponding to peptides IEPLGIAPT (amino acids 489–498, 1038 Da) and RRVQRE (amino acids 501–507, 942 Da) of HIV-gp120 (data not shown).

To further characterize the epitope, the affinity-bound fragment 481–507 was treated with carboxypeptidase P to remove non-affinity-bound C-terminal amino acids. After digestion for 14 h, no loss of C-terminal amino acids was observed. Another digestion was performed with aminopeptidase M, which cleaves N-terminal amino acids except for amino acids N-terminal of proline. Digestion for 24 h revealed digestion up to V486 (Fig. 5). After prolonged digestion (48–72 h) with additional enzyme, further cleavage was not observed.

Epitope extraction experiment

To test if peptide 481–507 shows different behavior when extracted from solution, HIV-gp120 was digested 36 h with endoproteinase Glu-C and subsequently passed over immobilized 1331A beads. Only one peptide was observed to remain affinity bound (Fig. 6) after epitope extraction of the Glu-C digest of HIV-gp120. This peptide, with a mass of m/z 3200, corresponded to peptide 481–507 of HIV-gp120. As observed in the epitope excision experiments, no truncated peptides bound to the Ab from solution. Digestion of the affinity-bound peptide with exoproteinase aminopeptidase M led again to digestion up to V486. Subsequent Lys-C digestion for 6 h resulted again in loss of affinity binding (data not shown).

To obtain additional information about the amino acids involved in binding to the Ab, the affinity binding from solution of three peptides was tested: 1) peptide, amino acids 487–507, IKIEPLGIAPT (amino acids 487–507, 2403 Da); 2) peptide, amino acids 488–507, IKIEPLGIAPT (amino acids 488–507, 2290 Da); and 3) peptide, amino acids 489–507, IEPLGIAPT (amino acids 489–507, 2162 Da).
Only the first peptide (amino acids 487–507) was found to bind to the Ab (Fig. 7).

**Discussion**

The C5 region is one of the most immunogenic regions of the HIV envelope (26), giving rise to high levels of anti-C5 Abs in HIV-infected individuals. Abs to the C5 region have been best characterized through the study of human anti-C5 mAbs, of which eight have been reported (22, 27). The core epitopes of these Abs have been mapped, using peptides, to two adjacent regions of HIV-gp120. The core epitope of four of the anti-C5 mAbs occurs at amino acids 503–509 (PTKAKR) while that of the other four (including 1331A) occurs at amino acids 510–516 (VVQREKR).

Mapping of the core epitopes with linear peptides gives a limited picture of the binding site recognized by the mAbs, as previously demonstrated by affinity studies that suggest that the core epitope of a mAb may contribute only 10% of the highest binding energy (28). The mAb 1331A studied here has the highest affinity of the eight anti-C5 mAbs studied to date and was found to be particularly useful because of its affinity in immunofluorescent studies of HIV-gp120 binding to CD4 and CXCR4 (22). Although Abs specific to the C5 region do not display significant neutralization, they do bind to the surface of infected cells (29) and to intact primary HIV virions (23). Some anti-C5 Abs were able to mediate Ab-dependent cellular cytotoxicity (27). Similarly, the ability of anti-C5 Abs to bind to virions would suggest that they may play a role in virus clearance. Both of these functions could be of biologic value in clearing an established infection. The combination of human anti-C5 Abs and Abs specific to the CD4 binding region of HIV-gp120 may be of therapeutic value, because synergistic neutralization of HIV isolates by combinations of Abs with these specificities was observed (30).

In this study, we describe the mapping of the epitope recognized by the 1331A Ab, using proteolytic digestions of the HIV-gp120/1331A complex followed by MALDI-TOF mass spectrometry. Digestions with endoprotease Glu-C of HIV-gp120 affinity bound to immobilized 1331A Ab resulted in one affinity-bound peptide, LYKYKVIKIEPLGIAPTKAKRRVVQRE (amino acids 481–507), which contains the strongly immunogenic region APTKAKRRVVQRE. Because E507 cannot be cleaved with carboxypeptidase Y, and HIV-gp120 with deleted E507 does not bind to the Ab, E507 must be involved in the affinity binding with the 1331A Ab. Assuming that the epitope is linear, Lys-C digestion, which has five potential cleavage sites in this fragment, should result in fragments YKVIKIEPLGIAPTKAKRRVVQRE (481–507), VIKIEPLGIAPTKAKRRVVQRE (481–508, 3069 Da), IEPLGIAPTKAKRRVVQRE (481–509, 2913 Da), AKRRVVQRE (481–510, 2685 Da), or RRVQRE (501–507, 2586 Da), depending on the length of the epitope. However, Lys-C digestion results in loss of binding to the Ab. These data indicate that one or more of the lysine residues within the peptide 481–507 are not protected by the Ab. When this (or these) sites are cleaved by endoproteinase Lys-C, affinity is lost. These results are consistent with a discontinuous and/or conformational epitope. Cleavage with aminopeptidase M was observed only up to V486, which is different from the results of a previous study of a polyclonal goat Ab preparation specific to the C5 region of HIV-gp120, where cleavage was observed up to E-490 (20). An epitope extraction experiment was performed to determine whether any peptides can be extracted from a digest of HIV-gp120 with endoproteinase Lys-C, affinity bound to immobilized 1331A Ab. The data indicate that one or more of the lysine residues within the peptide 481–507 are not protected by the Ab. When this (or these) sites are cleaved by endoproteinase Lys-C, affinity is lost. These results are consistent with a discontinuous and/or conformational epitope. Cleavage with aminopeptidase M was observed only up to V486, which is different from the results of a previous study of a polyclonal goat Ab preparation specific to the C5 region of HIV-gp120, where cleavage was observed up to E-490 (20).
binding of the peptides. We conclude from these experiments that amino acids of two noncontinuous regions are involved in binding to 1331A Ab, E-507 and I-487.

Interestingly, I-487 and E-507 are located on opposite sides of a hydrophobic pocket (Fig. 8), which was postulated from computer modeling and sequence comparisons (31). It has been proposed that this pocket is important for interaction with the 5-aa cysteine-linked loop of HIV-gp120 (31, 32). Mutations of amino acids in the sequence IEPLGVAPT (amino acids 489–497) resulted in a nearly complete loss of the affinity of HIV-gp120 for HIV-gp41 (33). Although x-ray analysis of HIV-gp120 in complex with the CD4 receptor and a mAb has been determined, structural information from x-ray analysis about the extreme C terminus is still missing, because a truncated HIV-gp120 preparation was used in this study (5).

Recently, the mouse mAb 803–15.6 that is specific to the amino acids APTKAKR (amino acids 495–501) of HIV-gp120 was described. The affinity of this Ab to the native HIV-gp120 and synthetic peptides was evaluated to obtain further structural information about the tertiary structure of this region. HIV-gp120 and the synthetic peptides had comparable affinity for the Ab. Because the peptides had a low percentage of α helices in solution, a disordered conformation of the same sequence in the native HIV-gp120 molecule was proposed (13). However, the mAb used by Ferrer et al. is of murine origin induced by immunization with recombinant HIV-1 gp120, and therefore the structural information obtained may not reflect the conformation of HIV-gp120 in the intact virus particle. In the present study, we used a human mAb, and observed a discontinuous epitope involving amino acids I-487 and E-507 of HIV-gp120. These data support the loop structure predicted by the molecular modeling studies. Similar to 1331A, the mouse mAb M38 was mapped to two noncontinuous stretches of amino acids flanking both sides of the loop: KYK (amino acids 483–485) and KAKR (amino acids 498–501) (34).

Several methods for the characterization of epitopes have been described. However, only a few of these methods are applicable to discontinuous epitopes. X-ray analysis is probably the most precise method for the characterization of an epitope structure. However, this method is time consuming and therefore may not be preferable for epitope mapping studies (16). Other rather time consuming approaches are random phage epitope library screening and site-directed mutagenesis (14, 15, 35). Epitope mapping studies using overlapping peptides in combination with competitive ELISA are widely used for the rapid characterization of linear epitopes. However, the application of this method to discontinuous epitopes was successful in only a few cases (36). Another approach for the characterization of discontinuous epitopes is differential chemical modification of amino acids in the immune complex and in the free Ag. Differences in the reactivity of the same amino acid in the Ab-Ag complex and the free Ag can provide information about the amino acids involved in affinity binding (37).

MALDI-TOF mass spectrometry has been used for the rapid characterization of linear epitopes. Proteolytic digestion of the Ag was followed by immunoprecipitation with the Ab and mass spectrometric characterization of the antigenic peptides (38). Epitope excision of the affinity-bound Ag in combination with MALDI-TOF mass spectrometry was used for the characterization of linear epitopes (19, 20). We report here the first application of this method to a discontinuous epitope. Advantages of epitope excision/extraction methods in combination with MALDI-TOF mass spectrometry are their low time consumption and that the enzymatic digestions are performed with the native protein, which allows the identification of discontinuous epitopes. This method can provide information impossible to obtain from mapping studies with peptide ELISAs. While such ELISA methods can sometimes demonstrate reactivity with peptides of 6–8 aa, increased affinity is observed when the epitope is determined using the complete molecule. For example, the identification of additional regions, which are involved in affinity binding, can explain the differences observed between the affinities of a synthetic peptide and native HIV-gp120 for the human mAb 694/98-D (28). Furthermore, the data obtained in this study may explain why two anti-C5 human mAbs, 858-D and 989-D, by ELISA map to the same core epitope, but by immunofluorescence on HIV-infected cells show different clade specificities (29).

Elucidation of epitopes in HIV-gp120 recognized by the human immune system is crucial for a better understanding of the antigenic nature of HIV. Mass spectrometric characterization has provided further knowledge about the tertiary structure of the C5 region of HIV-gp120, and this information may be useful for vaccine development.

References


