Short Peptides with Induced β-Turn Inhibit the Interaction between HIV-1 gp120 and CD4

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To identify novel peptides that inhibit the interaction between human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 and CD4, we constructed a targeted phage-displayed peptide library in which phenylalanine and proline were fixed at the fourth and sixth positions, respectively, because Phe43 and the adjacent β-turn of CD4 are critical for interaction with gp120. Two synthetic peptides were selected after three rounds of biopanning against gp120, and one of them, G1 peptide (ARQPSFDLQCGF), exhibited specific inhibition of the interaction between gp120 and CD4 with an IC_{50} of about 50 μM. Structural analysis using NMR demonstrated that G1 peptide forms a compact cyclic structure similar to the CD4 region interacting with gp120. Two derivatives of G1 peptide, a linear hexameric peptide (G1-6) and a cyclic nonameric peptide (G1-c), were synthesized based on the structure of the G1 peptide. Interestingly, they showed higher inhibitory activities than did G1 peptide with IC_{50}'s of 6 and 1 μM, respectively. Thus, this study might provide a new insight into the development of anti-HIV-1 inhibitors.

Introduction

A major limitation of current chemotherapy approaches against human immunodeficiency virus type 1 (HIV-1) is the rapid emergence of resistant mutants.[1,2] The identification of new inhibitory molecules targeted to the novel regions of the virus may improve treatments against AIDS. HIV-1 is an enveloped virus, and its envelope protein which consists of two noncovalently associated polypeptide chains, gp120 and gp41, is critical for viral entry. The initial step of HIV-1 infection is the interaction of gp120 with CD4 through highly conserved regions,[3] and subsequently, gp41 undergoes conformational changes that cause the fusion of the target cell membrane and the viral membrane. Therefore, several therapeutic molecules have been developed to prevent the proliferation of the virus at this initial step. Recombinant soluble CD4 (sCD4) can inhibit the infection of host cells by laboratory-adapted HIV strains.[4] However, there are a number of limitations in the application of CD4 as a therapeutic agent due to its relatively short half-life in vivo and to a reduced affinity to primary isolates. Small peptides corresponding to the third complementarity-determining region (CDR3) of CD4 showed inhibitory effects only with low specificity.[5,6] In addition, peptides corresponding to the second complementarity-determining region (CDR2) of CD4 had little antiviral effect.[6] Thus, it was believed that these peptides could not mimic the gp120 binding motif of CD4. It has been predicted that peptides with unrelated sequences rather than homologous ones might mimic the gp120 binding domain of CD4 and/or show high affinity to the CD4 binding region of gp120.[7,8]

Extensive mutagenesis studies and structural analyses have suggested that CDR2, the major gp120 binding region of CD4, maintains a structural tension that is coordinated by Phe43.[7−11] The mechanical tension imposed on this region may explain why simple replica peptides have failed to inhibit the interaction between gp120 and CD4. The crystal structure of gp120 in complex with CD4 and the Fab of monoclonal antibody 17b further supported the previous mutagenesis studies of gp120 and CD4, re-emphasizing the importance of Phe43 of CD4 in coordinating the interaction with gp120.[12]

Phage-displayed peptide libraries have been used as powerful tools to identify novel ligands.[13,14] Here, we constructed a targeted phage-displayed peptide library in which phenylalanine and proline were fixed to maximize the possibility of finding lead peptides that can inhibit the gp120−CD4 interaction. Based on the structural characteristics of a lead peptide, two smaller derivatives were synthesized and demonstrated to have increased inhibitory activity. This is the first report not only describing the use of a targeted phage-displayed peptide library to identify a lead peptide specifically recognizing gp120 but also showing enhanced inhibitory activity through structural modifications.

Materials and Methods

Construction and Characterization of a Phage-Displayed Peptide Library. A new phage-displayed peptide library was constructed as described[15] with some modifications. Two partially complementary oligonucleotides (Bio-synthesis, Inc.) that encode 10 random and 2 fixed amino acids were synthesized with the sequences 5′-CT TTC TAT GCG GCC CAG CCG GCC ATG $(NNK)_3$ CCG NNK TTT $(NNK)_6$ ATG

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CAT CAC CAC CAT CAT CAC-3′ (α-83) and 5′-CGG CGC ACC AGC GGC CGC $(ACC)_4$ GTG ATG ATG GTG GTG ATG CAT-3′ (*β*-51), in which N is G, A, T, or C; K is G or T. 13.2 *μ*g of α-83 and 8.45 *μ*g of *β*-51 oligonucleotides were mixed in 500 *μ*L, then heated to 95 °C for 10 min, and cooled slowly to room temperature. After annealing, dNTP with the ratio of dGTP/dATP/dTTP/dCTP = 42.4/15.2/20.7/21.7 and Taq polymerase were added and then incubated at 72 and 60 °C alternately 30 times. These double-stranded oligonucleotides were digested with *Sfi*I and *Not*I and ligated into the phagemid pCANTAB 5E (Pharmacia Biotech) which had been also digested with *Sfi*I and *Not*I. The ligated DNA was introduced into *Escherichia coli* XL-1 Blue MRF′ (Promega) by electroporation using Gene Pulser (Bio-Rad). The peptide-displaying phages were recovered from the transformed cells by use of M13KO7 helper phage, and this library was further amplified by re-infecting the phage supernatant to *E. coli* TG1.

The initial diversity of the library was measured by determining the number of ampicillin-resistant colonies formed by a bit of electropulsed cells. Also, a bit of the single-stranded DNA was prepared by phenol extraction from the phage pool, and the sequences of the inserted DNA were determined in mixture using Sequenase (USB) according to the manufacturer's method. A primer with the sequence 5′-TCTGTATGAG-GTTTTGCT-3′ was used for the sequencing process.

Selection of Phages with Affinity to gp120. The affinity selection to gp120 was done as previously reported[16] with some modifications. Candidate phages with affinity to gp120 were selected by biopanning against a well of a 96-well plate (Maxisorp, Nunc) coated with 200 ng of gp120 (SF2) in 50 *μ*L of phosphate-buffered saline (PBS). The gp120 purified from the CHO cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Kathelyn Steimer in Chiron Corp.[17−20] The phages of 3×10^8 cfu diluted in PBS were applied to the gp120-coated well and incubated for 2 h at 37 °C. After washing with PBST (PBS with 0.1% of Tween-20) 40 times and with PBS 35 times at room temperature, the bound phages were recovered by three successive methods and were classified to three groups. First, the bound phages were eluted by incubation with PBS at 37 °C (group T) and the still bound phages were eluted by incubation with excessive gp120 (40 *μ*g/mL, group G). Phages not recovered by these methods were rescued by direct addition of TG1 cell to the well for infective recovery (group C). These three groups of recovered phages were separately amplified using M13KO7, and the obtained phages were bound to three separate gp120-coated wells. In the second round of biopanning, group T phages were recovered by incubation with PBS at 37 °C as before. The group G phages were recovered by incubation with excessive gp120 after pretreatment with PBS at 37 °C and following removal of the eluted phages by this pretreatment. The well in which group G phages had been incubated was serially pretreated with PBS and excessive gp120, and then the detached phages were washed out before the addition of TG1 cell. The three groups of recovered phages from the second round of biopanning were amplified and panned again as the second biopanning round with the same pretreatments.

ELISA Assay. Enzyme-linked immunosorbent assay (ELISA) for phage was carried out as previously descibed.[21] An ELISA plate (Immulon 4, Dynatech) was coated with 100 ng of gp120 (IIIB, ImmunoDiagnostics, Inc.) in PBS by incubation at 4 °C overnight and then blocked with 3% bovine serum albumin (BSA) in PBS. Phages (3×10^8 cfu) diluted in PBS were added to the wells and incubated at 37 °C for 2 h, and then the unbound phages were removed by washing with PBST 40 times and with PBS 35 times. The amount of bound phages was measured by a colorimetric method using horseradish peroxidase-conjugated anti-M13 sheep antibody (Pharmacia Biotech). Peptides of which N-termini were biotinlyated were purchased from Peptidogenic Research & Co, Inc. and Peptron. Synthesized peptides were bound to ELISA plate coated with gp120, and the bound peptides were detected by horseradish peroxidase-conjugated antibiotin goat antibody (Calbiochem).

The inhibitory activities of peptides were measured according to the competitive ELISA as described previously.[22] An ELISA plate was coated with 100 ng of recombinant sCD4 (Biogen) and then blocked with 3% BSA in PBS. Several concentrations of peptides were premixed with 100 ng of gp120 (IIIB) for 2 h at 37 °C, and then these mixtures were added to the CD4-coated plate. After removal of the unbound gp120, the quantities of bound gp120 were measured by anti-gp120 antibody (ImmunoDiagnostics, Inc.). The percent inhibition by each peptide was calculated in comparison to the amount of bound gp120 without any inhibition.

NMR Spectroscopy. NMR sample was prepared by dissolving 10 mg of synthetic G1 peptide into 500 *μ*L of DMSO-d_6 (14 mM). G1 peptide could go into solution in D_2O, H_2O, and F_3CCD_2OD (TFE-d_3) only under a concentration of about 10 mM, and this led us to use deuterated dimethyl sulfoxide (DMSO-d_6). All NMR data were collected on a 400 MHz JEOL Lambda and Varian 300 MHz system at 25 °C using homonuclear and heteronuclear correlation experiments including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY),[23] nuclear Overhauser effect spectroscopy (NOESY), ^{1}H-detected heteronuclear multiple quantum coherence spectroscopy (HMQC),[24] and ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectroscopy (HMBC)[25] techniques. ^{1}H and ^{13}C NMR chemical shifts were referenced to internal DMSO (2.50 ppm, ^{1}H; 39.51 ppm, ^{13}C). Homonuclear correlated spectra were zero-filled to final matrix sizes of 1024 × 1024 real points. The delay time between scans was 1.5 s, and the mixing time was 300 ms. Gaussian line broadening of 2 Hz was used in the t_2 dimension before Fourier transformation. A 90° shifted sine bell squared filtering was used in the t_1 dimension before Fourier transformation. For NMR-based molecular dynamic computations and identification of spin diffusion peaks, NOESY data were collected at five different mixing times of 50, 100, 200, 300, and 500 ms. HMQC and HMBC spectroscopies were performed with a 512 × 2048 data matrix size with 16 scans per t_1 and spectra were zero-filled to 2048 × 2048 real points. Data acquisition and processing were accomplished via JEOL and Varian system software, and paramaters for individual 2D experiments (defined below) were described as follows. COSY: 2 × 256 × 1024 raw data matrix size; 16 scans per t_1 increment; 1.5 s repetition delay; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t_2 and t_1 domains, respectively. Phase-sensitive TOCSY: 2 × 256 × 1024 raw data matrix size; 32 scans per t_1 increment; 1.5 s repetition delay; 65 ms MLEV-16 continuous wave spin−lock period; 6.25 kHz spin−lock field strength, corresponding to 42 *μ*s 90° pulse width; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t_2 and t_1 domains, respectively. Phase-sensitive NOESY: 2 × 256 × 1024 raw data matrix size; 64 scans per t_1 increment; 2.8 s repetition delay period; 10, 50, 100, 300, 500 ms mixing period for NOE buildup profile; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t_2 and t_1 domains, respectively. HMQC: 2 × 128 × 1024 raw data matrix size; 128 scans per t_1 increment; 1.2 s repetition delay; 33 *μ*s 90° ^{13}C pulse widths; 16W RF broad band Walts-16 ^{13}C decoupling during acquisition period; 3.5 ms defocusing and refocusing delay periods; 800 ms "Weft" period; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t_2 and t_1 domains, respectively. HMBC: 2 × 128 × 1024 raw data matrix size; 512 scans per t_1 increment; 2.0 s repetition delay period; 33 *μ*s 90° ^{13}C pulse widths; 3.5 ms delay period for suppression of one-bond signals; 40 ms delay periods for long-range coupling; 15° shifted sine bell filtering in the t_2 domain and no filtering in the t_1 domain.

Determination of Peptide Structures. The NOE restraints were obtained via qualitative assessment of NOE cross-peak volumes in the NOESY spectra. The identification of relay peaks was based on the NOE volume integration for five different mixing times. However, no restraints were included to attempt to fit the spin diffusion cross-peaks. Instead, loose NOE restraints were used for cross-peaks classified as strong, medium, and weak. The ranges of initial NOE restraints were assigned with 2.0−2.5 Å (strong cross-

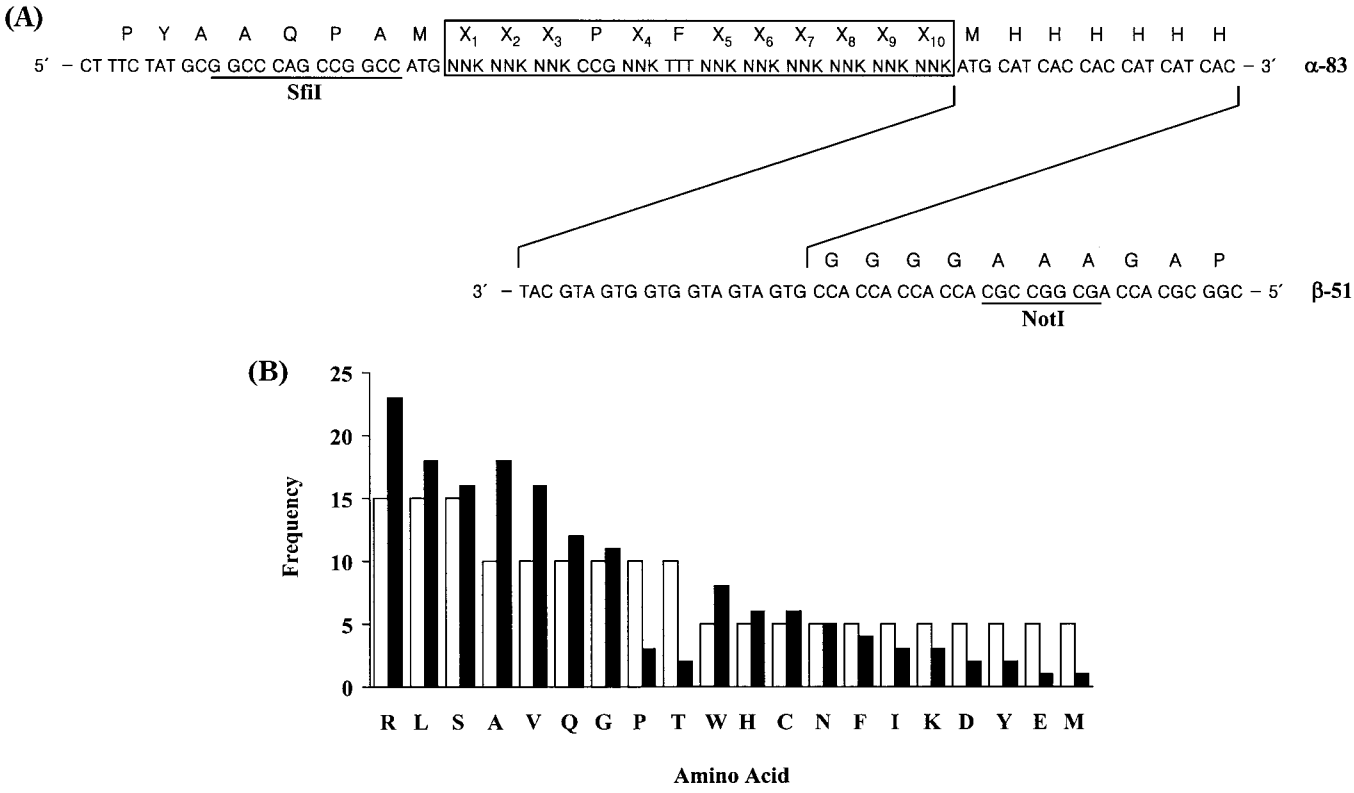

Figure 1. Construction and characterization of a targeted phage-displayed peptide library. (A) Two kinds of oligonucleotides, α-83 and *β*-51, were annealed and filled in for the generation of double-stranded oligonucleotides. After digestion with *Sfi*I and *Not*I, these double-stranded oligonucleotides were ligated into pCANTAB 5E phagemid digested with *Sfi*I and *Not*I. The ligated DNA was introduced into *E. coli*, and phages with peptides of random sequences were recovered by use of M13KO7 helper phage. Note the fixed sequences for a proline and a phenylalanine among the random sequences. N = A, G, C, or T; K = G or T. X denotes a random amino acid encoded by a codon of random sequence, NNK. (B) Analysis of the amino acid composition of the constructed library. The frequencies of amino acids in the random sequence regions from randomly chosen phage clones (closed bars) were compared with those of theoretical values (open bars).

peak intensities), 2.0−3.5 Å (medium cross-peak intensities), and 2.0−4.5 Å (weak cross-peak intensities). Distance geometry (DG) structures were generated and refined by using primary restraints and loose NOE-derived distance restraints. Trial distances generated by selecting random distances between the upper and lower bounds of each element were embedded in 3D space with the metric matrix method and subjected to simulated annealing (SA) and conjugated gradient minimization (CGM).[26] The embedded initial coordinates containing violations of the upper and lower boundary restraints were then refined. After minimization to a moderate target penalty (penalty = squared sum of the covalent and experimental bounds violation, ca. 0.3 Å^2), the initial DG structure was saved, and new DG structures were generated by performing two 10 Å randomizations of atom positions, followed by SA and CGM refinement. Once a low-penalty structure was stored, new starting coordinates were then obtained by performing different embedding, SA, and CGM algorithms. Further refinement was achieved by application of variable velocity simulated annealing (to maximum penalty values of 10−20 Å^2), SHAKE (to penalties of ca. 10 Å^2),[27] and CGM algorithms.

The complete time course for nuclear relaxation was then determined for each refined structure via numerical integration of the Bloch equations. As described previously, this approach accurately accounts for spin diffusion.[28] Generic *Z*-leakage rate constant K_{zl}, accounting for the loss of *Z*-magnetization within a mixing period, was used with 1 s^{-1} (3 s^{-1} for methyl groups in G1 peptide). A cross-relaxation rate constant K_{cr} which governs the cross-relaxation rate was used with 60 s in this NOE back-calculation. K_{cr} was determined via the NOE buildup curve comparisons in advance with well-resolved germinal protons which are structurally known internuclear distances with 1.8 Å. The cross-relaxation rate term includes intramolecular dipolar relaxation, effect of chemical exchange, quadrapolar relaxation, the possible contribution from spin rotation, scalar relaxation, and multiple quantum effects. 2D NOE back-calculation gives a list of normalized auto- and cross-peak intensities for selected increasing mixing times. Profiles of these outputs provide the theoretical NOE buildup curves. The results of back-calculation were then assigned to FELIX (Molecular Simulations, Inc.) to generate 2D NOE back-calculated spectra which can be directly compared with experimental spectra.

Results

Construction and Characterization of a Phage-Displayed Peptide Library. We constructed a linear peptide library with 10 random and 2 fixed amino acids, a proline and a phenylalanine at the fourth and the sixth positions, respectively (Figure 1A). The rationale for fixing two amino acids is based on the involvement of proline in *β*-turn formation as well as the absolute conservation of Phe43 in CD4. These characteristics maximize the imitation of the structural properties of the gp120 binding region of CD4. The initial variants of this library were measured to be approximately 4 × 10^7. The qualities of the library were evaluated by two criteria: the overall expression pattern of the peptides and the existence of potential biological bias. The DNA sequences corresponding to the regions encoding the displayed peptides were determined in mixtures. Separate DNA bands denoting the fixed sequence regions including TTT for phenylalanine and CCG for proline were clearly detected. In addition, NNK sequences were

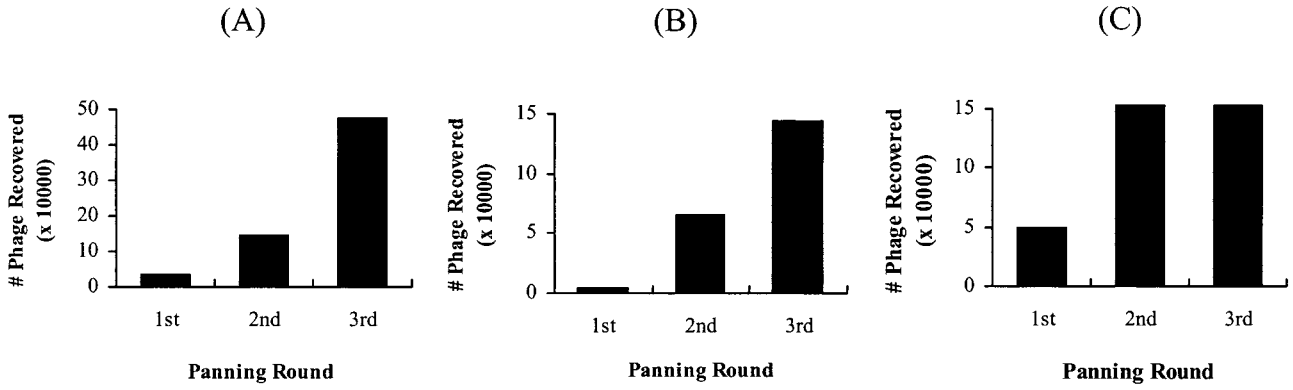

Figure 2. Increase of recovered phages during repeated biopannings. Phages were recovered by three different methods after input of the same number of phages (3×10^8 cfu). The numbers of recovered phages were counted as the numbers of ampicillin-resistant colonies of *E. coli* infected with the recovered phages. (A) Phages were eluted with PBS at 37 °C (group T). (B) Phages were eluted using excess gp120 after being washed with PBS (group G). Elutions in the next biopanning rounds were done also by the addition of gp120 after being washed with PBS. (C) After elution with PBS and gp120, phages still bound were recovered by direct addition of *E. coli* (group C).

Table 1. Peptide Sequences of the Inserts after the Third Round of Biopanning[a]

clones[b]	sequences[c]	frequencies[d]
T1	N-DQFPQFDLSYCL	3/24
T2	N-AFVPQFLVIIDN	3/24
T3	N-QQVPMFMQGRRL	2/24
T4	N-APQPSFKGLRWC	2/24
G1	N-ARQPSFDLQCGF	6/26
G2	N-FQRPYFLVQISL	2/26
C1	N-AAQPFFQFRQSM	3/22

[a] After the third round of biopanning, several tens of phage clones from each group were randomly selected and sequenced. Peptide sequences that emerged more than twice are shown. [b] Phage clones selected by three different panning methods: T clones, by PBS elution at 37 °C; G clones, by gp120 elution; C1 clone, by infective elution. [c] Peptide sequences encoded by the inserted oligonucleotides. Flanking sequences were omitted for clarity. [d] Frequencies of the shown sequences of all the clones sequenced for each method.

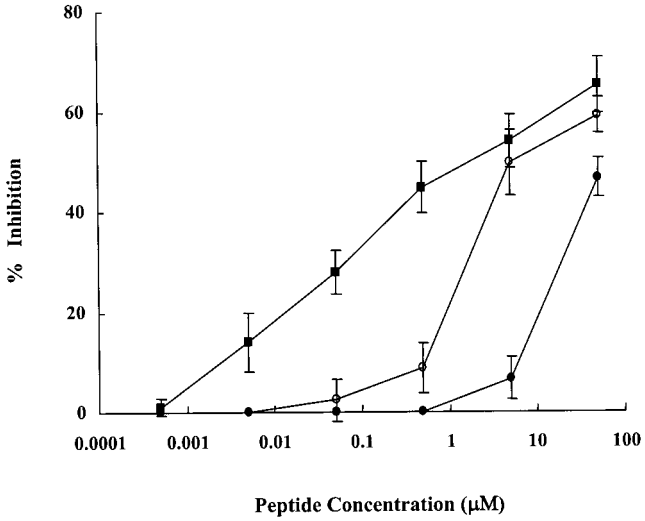

Figure 3. Inhibitions of the interaction between gp120 and CD4 by peptides. An ELISA plate was coated with recombinant sCD4 and then blocked with BSA. Several concentrations of G1 (●) G1-6 (○), and G1-c (■) peptides were premixed with gp120 and added to the CD4-coated plate. After removal of the unbound gp120, the quantities of bound gp120 were measured by anti-gp120 antibody. The percent inhibition was calculated in comparison to the amount of bound gp120 without any inhibition.

obtained in the regions corresponding to the random amino acids (data not shown). Since the biased expression of peptides could be caused by differences in viability and infectivity of phages expressing peptides with different compositions of amino acids,[13,16] we measured frequencies of codon usage in random sequence regions from the randomly chosen phage clones. The frequencies of deduced amino acids from random sequence regions are in accord with the theoretical values (Figure 1B). These results suggest that the peptide library was constructed as designed with acceptable diversity and properties.

Affinity Selection of Phages against gp120. To isolate high-affinity peptides to gp120, we used a biopanning method containing three different elution conditions: temperature shift, ligand (gp120) elution, and direct addition of host cells to the panning well as described in Materials and Methods. The recovered phages were grouped and amplified separately and then used for further biopanning rounds. During this process, the number of recovered phages significantly increased, implying that phages with high affinity to gp120 were selected and amplified by repeated biopanning protocols. After the third biopanning, the numbers of phages recovered by the temperature shift method (group T) and the ligand elution method (group G) were increased about 15- and 20-fold, respectively, compared with those recovered after the first biopanning (Figure 2A,B). However, the number of phages recovered by direct addition of host cells (group C) increased only 3-fold after the third biopanning (Figure 2C). Since unequal amplifications of phages through the biological selection could have occurred,[13,16] we did not proceed with further biopanning rounds. After the third biopanning, about 20−30 phage clones from each group were chosen and sequenced to find any consensus sequence pattern(s). Although there was no obvious intergroup consensus pattern, some peptide sequences were repeated within each group. Four different kinds of repeated sequences were observed in 10 out of 24 clones in group T. In addition, emergent G1 clones numbered 6 out of 26 clones in group G (Table 1).

Binding Specificities and Inhibitory Activities of G1 Peptide and Its Derivatives. Binding affinities

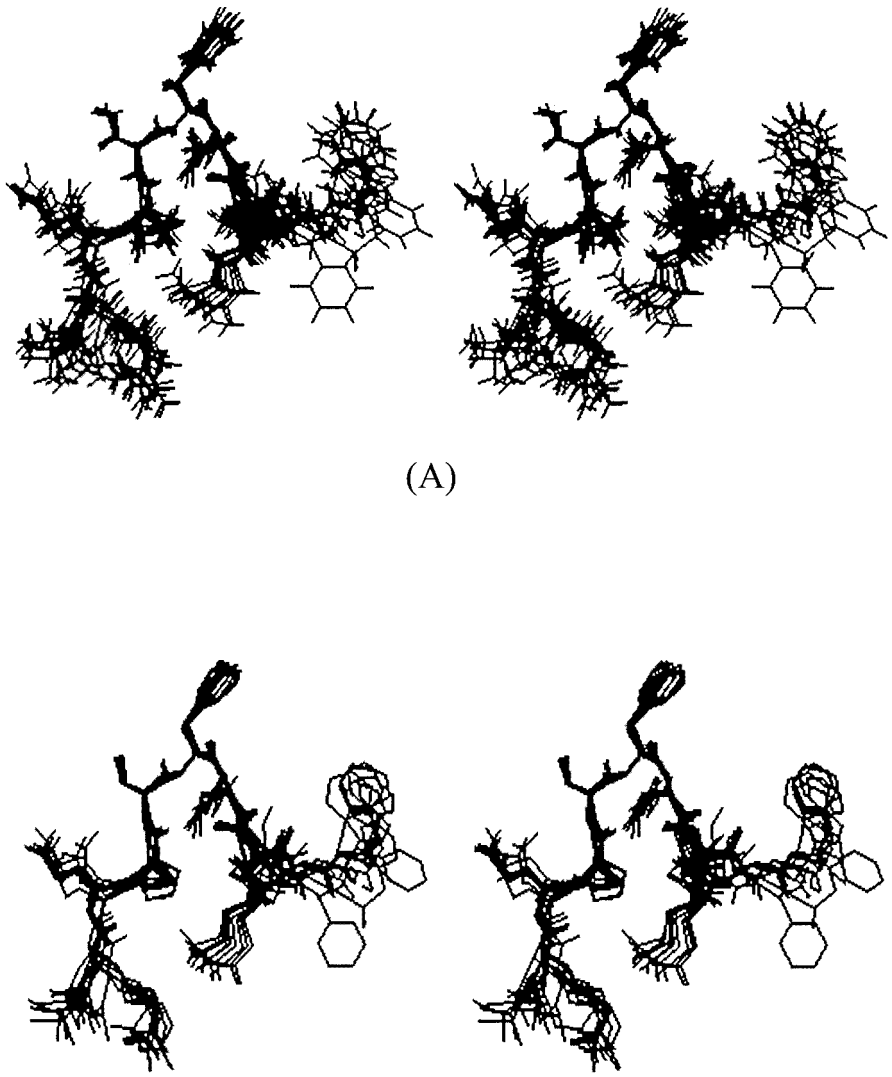

Figure 4. Molecular structures of G1 peptide with a range of 0.2−0.3 rms deviation. The NMR-based solution-state structure of G1 peptide was determined using NOE restraints obtained after iterative back-calculations. Superimposed DG structures are exhibited with (A) or without (B) hydrogen.

of seven different clones (T1−T4, G1, G2, and C1) were determined through a phage ELISA. Compared with control phage (pCANTAB 5E phage), all of these clones showed increased binding affinities to gp120, and in particular, T1 and G1 clones showed higher affinities than others (data not shown). To examine for inhibitory effects on the interaction between gp120 and CD4, two peptides derived from T1 and G1 clones, DQFPQFDLSYCL and ARQPSFDLQCGF, respectively, were synthesized as linear dodecameric peptides in which the N-termini were biotinylated. The T1 and G1 peptides were then demonstrated to specifically bind to gp120 through ELISA (data not shown). However, when these peptides were tested for an inhibitory activity against the interaction between gp120 and CD4 in vitro, only G1 peptide, but not T1 peptide, showed a concentration-dependent, inhibitory activity with an IC_{50} of about 50 μM (Figure 3). T1 peptide likely binds to a region of gp120 which does not interact with CD4.

To elucidate the structural characteristic(s) of G1 as a lead peptide, NMR spectroscopic analysis was performed. At first, G1 peptide was demonstrated to form a globular structure (Figure 4). In addition, the middle region of G1 peptide appeared to be more rigid with less flexibility than the terminal regions, which led us to hypothesize that the 'active core' is located in this middle region. To determine if a shortened peptide containing the active core had an increased activity, a hexameric linear peptide (G1-6) with the sequence PSFDLQ was synthesized and tested for its inhibitory activity using a competitive ELISA. Interestingly, as shown in Figure 3, the hexameric G1-6 peptide showed a lower IC_{50} of about 6 μM, when compared with the dodecameric peptide (G1). Furthermore, we synthesized a nonameric peptide with the sequence CQPSFDLQC (G1-c), containing the active core and cyclized by two terminal cysteines, and tested for its inhibitory activity. This cyclic peptide inhibited the interaction between gp120 and CD4 with an even lower IC_{50} of about 1 μM. The most peculiar property of G1-c peptide, compared with G1 and G1-6, is that its activity does not decrease rapidly with concentration but persists

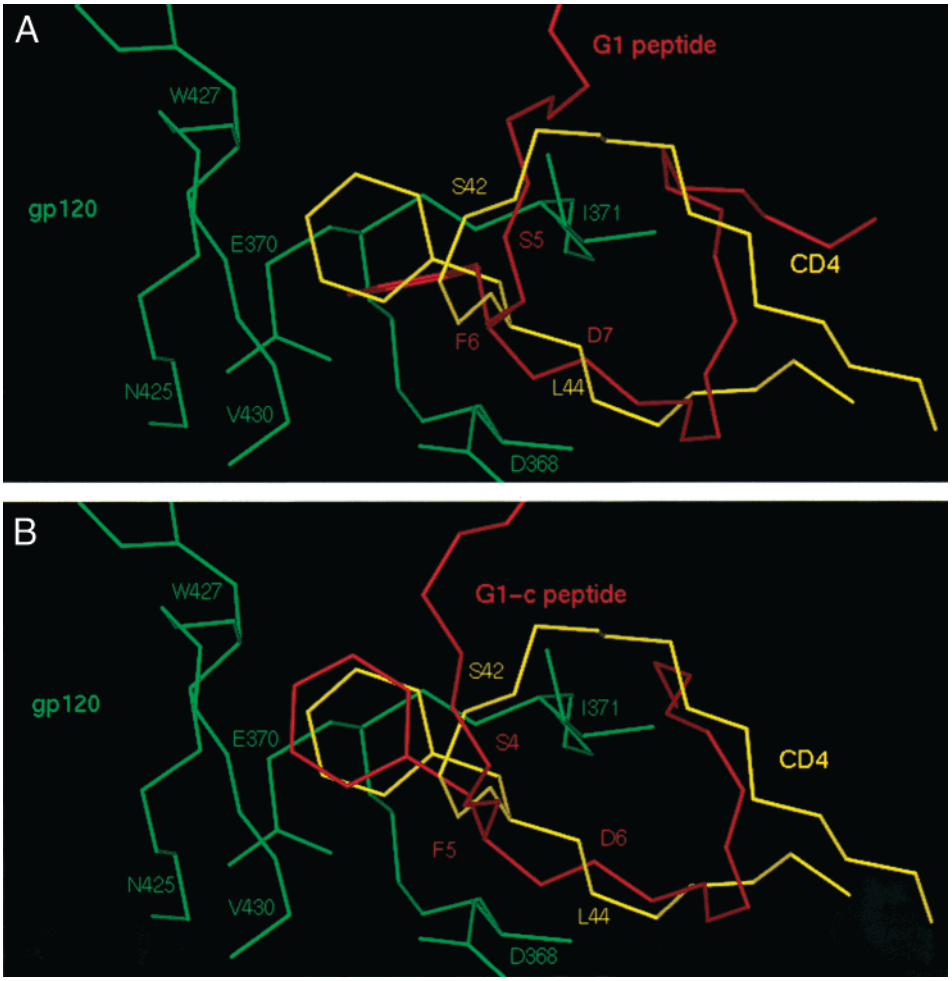

Figure 5. Structure comparisons of CD4 CDR2 with G1 or G1-c peptide. X-ray-determined CD4 CDR2 region (yellow, refs 7, 8) was superimposed with the NMR-determined solution structure of G1 peptide (red, A) or with the molecular dynamics (MD)-derived average conformation of G1-c peptide (red, B). The structure of G1-c peptide was calculated using InsightII 98 (Molecular Simulations Inc.). Energy minimization and molecular dynamics simulations were performed with the CVFF force field implemented in InsightII 98 on Silicon Graphics Indigo2 workstations. Simulations at 300 K were started with a 100 ps equilibration run and followed by 400 ps acquisition run. 1000 structures saved from each MD trajectory in equal time steps were used to calculate average characteristics of MD trajectories. Note that peptide backbones only are shown for clarity except for phenylalanines.

below 0.01 μM. However, a control peptide, STRVTGGTEGRTTNRFVSIF corresponding to the hypervariable region of HCV E2 (hepatitis C virus envelope protein 2),[29] did not show any detectable inhibitory activity (data not shown).

Structural Analysis of G1 Peptide. The structure of G1 peptide was elucidated by NMR spectroscopy and subsequent molecular modeling. The NMR-based solution-state structure of G1 peptide was determined using 16 NOE restraints of interresidues and 52 NOE restraints of intraresidues. Through-space NOE connectivities used in the structure determination are available as Supporting Information. Some interresidue NOEs such as $A1_{\alpha}-P4_{\beta}$, $A1_{\alpha}-R2_{NH}$, $A1_{\beta}-R2_{NH}$, $Q3_{\alpha}-P4_{\delta}$, $P4_{\beta,\gamma}-C10_{NH}$, $D7_{\alpha}-L8_{NH}$, $D7_{\beta}-L8_{NH}$, $D7_{NH}-C10_{\beta}$, $L8_{\delta}-Q9_{NH}$, and $C10_{\alpha}-G11_{\alpha}$ turned out to be important in the peptide backbone structure determination. The iterative 2D NOE back-calculation methods were critical in the refinement of the structure. The back-calculated 2D NOE spectra of a generated NOE-based structure (lowest penalty value, ca. 0.08 Å^2) are consistent with the experimental NOE spectra. Several back-calculated cross-peaks coincide with the experimental spectrum. Comparisons of back-calculated and experimental NOE spectrum of G1 peptide at 300 ms mixing time are also available as Supporting Information. The highly refined structure obtained in the current study (Figure 4) has root-mean-square (rms) values ranging from 0.2 to 0.3 Å^2 for 10 different structures of all the atoms. However, the lack of NOE cross-peaks for N- and C-termini of the peptide gave rise to structural flexibility providing poor local rms values in the 10 superimposed structures. It is also shown that a β-turn structure has been placed in the residue numbers 5−10 as provided from the CD4 structure.[7,8]

Discussion

We have constructed a phage-displayed peptide library expressing 10 random and 2 fixed amino acids and have identified a peptide, G1, that can specifically bind to HIV-1 gp120 and inhibit the interaction between gp120 and CD4. On the basis of the structural analysis of the G1 peptide, we also demonstrated that its derivatives, G1-6 and G1-c, have increased inhibitory activities against gp120−CD4 binding. Importantly, the cyclized peptide, G1-c, was shown to exert inhibitory

activity at much lower concentrations, below 1 μM. Since the linear peptides seem to be more flexible in aqueous conditions possibly due to solvation, it is likely that the tethering of the termini of the linear peptide by a disulfide bridge appears to reduce the flexibility of the peptide, resulting in stable binding to gp120 and, subsequently, enhanced inhibitory activity.[30]

It was recently reported that a phage-displayed peptide library was used for screening peptides that inhibit the interaction between gp120 and CD4.[22] In contrast to our peptides, the peptides in the latter study seemed to exert inhibitory effects by stabilizing the particular conformational states of gp120. In addition, most peptides reported so far have shown reduced activities when shortened.[22,31] In this report, we suggest that the binding site of G1 peptide to gp120 appears to overlap with the CD4 binding site on the basis of the following observations: G1, but not T1, peptide specifically inhibited the interaction between gp120 and CD4. It is likely that the specific structure of G1 peptide, but not its amino acid composition, is critical for its inhibitory activity, since the shortened peptides containing the active core have higher inhibitory activities than the parent peptide. In addition, these peptides did not have any negative effect on the interaction between HCV E2 and anti-E2 antibody, reinforcing the specificity of these peptides (data not shown). Furthermore, the structure of G1 peptide determined by NMR spectroscopy is similar to that of the CDR2 region known as the most important gp120-contacting region of CD4.

It was previously demonstrated that Phe43 of CD4 accounts for 23% of all the interatomic contacts between gp120 and CD4 and that the precise positioning of the phenyl ring of Phe43 in the cavity of gp120 is critical for the interaction with gp120.[12] We performed molecular dynamics and found that there are some differences between the angle of the phenyl ring of phenylalanine in G1 peptide and that of Phe43 of CD4 (Figure 5A). Interestingly, the angular difference of the phenyl ring of phenylalanine in G1-6 was reduced and that of phenylalanine in G1-c was further reduced (Figure 5B), suggesting that the increased activities of G1 derivatives may be due, in part, to the more precise fit of the phenyl ring in the cavity of gp120.

However, we could not detect a significant inhibitory activity for G1, G1-6, and G1-c peptides in tissue culture assays at concentrations of 30, 140, and 50 μM, respectively. It was reported that a peptidomimetic of CD4 with a K_d value of 4−20 μM had an approximately 50% blocking effect on syncytium formation at a concentration over 300 μM in a cell fusion assay.[32] The K_d value of G1-c was estimated by the Cheng−Prusoff equation[33] to be in the sub-micromolar range. This indicates that a concentration of several hundred micromolar for G1-c might be required for detectable inhibitory activity in a tissue culture assay. Unfortunately, we have not been able to synthesize G1-c in a quantity sufficient for tissue culture assays, which might be due to the undefined property of the peptide as well as to the presence of disulfide bonds. Alternatively, it is possible that our peptides had lower binding affinities to trimeric gp120 on the viral surface than those to monomeric gp120, as the binding affinities of ligands to trimeric gp120 were predicted to be different from those to monomeric gp120.[22] However, we do rule out the possibility that our peptides might be readily digested by peptidases in the cell culture medium, since there was no difference in the inhibitory activities of peptides when a competitive ELISA was performed in the presence or absence of cell culture medium (data not shown).

It is worthwhile to note that G1 and G1-c peptides exhibit striking structural homology with the CDR2 region of CD4, even though the origins of these two molecules were totally different. Therefore, our results suggest that the approach using a targeted library might provide new insight into the search for lead peptides that inhibit the interaction between ligand and receptor.

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Supporting Information Available: NMR spectroscopy including NOESY, COSY, TOCSY, HMBC, and HMQC, in addition to through-space NOE connectivities used in structure determination; also comparisons of back-calculated and experimental NOE spectrum of G1 peptide at 300 ms mixing time. This material is available free of charge via the Internet at http://pubs.acs.org.

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