Synthetic Peptides Derived from the Variable Regions of an Anti-CD4 Monoclonal Antibody Bind to CD4 and Inhibit HIV-1 Promoter Activation in Virus-infected Cells*

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The monoclonal antibody (mAb) ST40, specific for the immunoglobulin complementarity-determining region (CDR) 3-like loop in domain 1 of the CD4 molecule, inhibits human immunodeficiency virus type 1 (HIV-1) promoter activity and viral transcription in HIV-infected cells. To design synthetic peptides from the ST40 paratope that could mimic these biological properties, a set of 220 overlapping 12-mer peptides frameshifted by one residue, corresponding to the deduced ST40 amino acid sequence, was synthesized by the Spot method and tested for binding to recombinant soluble CD4 antigen. Several peptides that included in their sequences amino acids from the CDRs of the antibody and framework residues flanking the CDRs were found to bind soluble CD4. Eleven paratope-derived peptides (termed CM1–CM11) were synthesized in a cyclic and soluble form. All the synthetic peptides showed CD4 binding capacity with affinities ranging from 1.6 to 86.4 nM. Moreover, peptides CM2, CM6, CM7, CM9, and CM11 were able to bind a cyclic peptide corresponding to the CDR3-like loop in domain 1 of CD4 (amino acids 81–92 of CD4). Peptide CM9 from the light chain variable region of mAb ST40 and, to a lesser extent, peptides CM2 and CM11 were able to inhibit HIV-1 promoter long terminal repeat-driven β-galactosidase gene expression in the HeLa P4 HIV-1 long terminal repeat β-galactosidase indicator cell line infected with HIV-1. The binding of mAb ST40 to CD4 was also efficiently displaced by peptides CM2, CM9, and CM11. Our results indicate that the information gained from a systematic exploration of the antigen binding capacity of synthetic peptides from immunoglobulin variable sequences can lead to the identification of bioactive paratope-derived peptides of potential pharmacological interest.

The CD4 molecule is a transmembrane glycoprotein (58 kDa) found on thymocytes, mature T-cells, macrophages, monocytes, and Langerhans’ cells (1). This surface protein is required to shape the T-cell repertoire during thymic development (2) and to permit appropriate activation of mature T-cells through adhesion with class II major histocompatibility complex molecules and the T-cell receptor (3). Engaged CD4 subsequently plays a role in signal transduction by association with the protein-tyrosine kinase p56lck (4). Besides its physiological function, the CD4 surface glycoprotein, in association with chemokine receptors, acts as a receptor for HIV-1 entry into cells (5–7). CD4 is a member of the immunoglobulin gene superfamily and consists of four extracellular domains (D1–D4) showing structural homology to immunoglobulin variable regions, a membrane-spanning region, and a cytoplasmic tail (8); in D1, there are three CDR-like regions (9, 10). The CDR2-like loop of D1 has been identified as the primary binding site for the HIV envelope glycoprotein gp120 (11–13), whereas the CDR3-like region represents a CD4 target for inhibition of the class II major histocompatibility complex-restricted immune responses (14–18) and HIV replication (19–24). Previous studies have shown that CDR3-like peptide analogs are strong inhibitors of these functions (14, 16–18, 25–28), probably interfering with CD4 dimerization (29, 30). Similarly, mAbs such as ST40 that bind to the CDR3-like loop in D1 of CD4 inhibit HIV-1 replication in infected cells at a post CD4/gp120 binding step (24).

Antibody paratopes result from the interactions between immunoglobulin variable heavy (VH) and light (VL) chains. The diversity of paratopes is mainly generated by the sequences of the CDRs found in VH and VL, which are exposed hypervariable loop structures. Antigen binding by peptide sequences from selected CDRs of mAbs has been demonstrated to have similarities to those of the original antibody molecule (31–40). Our previous results showed that the systematic exploration of the antigen binding capacity of short peptides derived from an antibody sequence leads to the identification of numerous paratope-derived peptides (PDPs) that display significant affinity for the antigen (40). Therefore, this approach could be useful to identify potentially biologically active peptides from the sequence of a pharmacologically active antibody.

In this study, we have established the nucleotide sequences

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF005354 and AF005355.

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‡ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; D1, domain 1; CDR, complementarity-determining region; mAb, monoclonal antibody; VH, variable region of the heavy chain; VL, variable region of the light chain; PDP, paratope-derived peptide; sCD4, soluble CD4; PBS, phosphate-buffered saline; Fmoc, N-(9-fluorenylethoxycarbonyl; HPLC, high pressure liquid chromatography; LTR, long terminal repeat.
The VL cDNA sequence was determined by double-stranded polymerase chain reaction-amplified DNA product was digested separately with HindIII and XhoI and ligated to pUC19 that was prepared in a similar manner. This digested DNA was ligated to pUC19 that was prepared in a similar manner. The n-mannose-hybridoma cell line that produces mAb ST40 (IgG1,κ) was a kind gift from Dr. D. Carrière (Sanofi Research, Montpellier, France) (43). Total RNA was extracted from 3 x 10^6 hybridoma cells using the TRIzolTM technique (Life Technologies Inc.) and the primer OPP-Soc3' (5'-CGCAGATCTAACACCTTACCTC- GGTGAACT-3') which contains the reverse complement of codons 208–214 of CD4. One μl of first strand cDNA was used as matrix for the polymerase chain reaction to amplify the ST40 V_Cgene using Vent DNA polymerase (New England Biolabs, Hitchin, UK) and the primers OPP-Soc3' and OPP-Sov9 (5'-GA/CT/AAATAAGGCATCAC/CAACA/GTATCCTC-3'). These primers contained restriction sites (underlined) for cloning. The degenerate primer OPP-Sov9 was chosen as the consensus sequence of codons 5–8 in murine Frk Vv. The polymerase chain reaction-amplified DNA product was digested sequence specifically with RflII and ScaI (New England Biolabs) and purified on a 1.5% low melting temperature agarose gel (Life Technologies Inc.). This digested DNA was ligated to pUC19 that was prepared in a similar manner. The Vv cDNA sequence was determined by double-stranded sequencing using the dye chain termination method with the T7 sequencing kit. The numbering of the amino acid sequences of variable regions was that of Kabat (44).

**EXPERIMENTAL PROCEDURES**

**Soluble CD4—**Recombinant purified sCD4, kindly provided by Professor D. Klatzmann (Hopital de La Pitié, Paris), comprised the four external domains of CD4 (42). sCD4 (280 μg in 600 μl of bicarbonate buffer, pH 8.6) was biotinylated using a commercial reagent (Amer sham Pharmacia Biotech, RPN2292) according to the manufacturer’s instructions. Biotinylated sCD4 was stored in PBS at -20°C until use.

Cloning of mAb ST40 Vv and Vv Genes—The murine hybridoma cell line that produces mAb ST40 (IgG1,κ) was a kind gift from Dr. D. Carrière (Sanofi Research, Montpellier, France) (43). Total RNA was extracted from 3 x 10^6 hybridoma cells using the TRIzol™ technique (Life Technologies Inc., Paisley, United Kingdom). The Vv gene of the ST40 antibody was obtained by polymerase chain reaction amplification. Briefly, reverse transcription was performed with 2 μg of total RNA, the reverse transcriptase SuperScript (Life Technologies Inc.), and the primer OPP-Soc3' (5'-CGCAGATCTAACACCTTACCTC- GGTGAACT-3') which contains the reverse complement of codons 208–214 of CD4. One μl of first strand cDNA was used as matrix for the polymerase chain reaction to amplify the ST40 V_v gene using Vent DNA polymerase (New England Biolabs, Hitchin, UK) and the primers OPP-Soc3' and OPP-Sov9 (5'-GA/CT/AAATAAGGCATCAC/CAACA/GTATCCTC-3'). These primers contained restriction sites (underlined) for cloning. The degenerate primer OPP-Sov9 was chosen as the consensus sequence of codons 5–8 in murine Frk Vv. The polymerase chain reaction-amplified DNA product was digested sequence specifically with RflII and ScaI (New England Biolabs) and purified on a 1.5% low melting temperature agarose gel (Life Technologies Inc.). This digested DNA was ligated to pUC19 that was prepared in a similar manner. The Vv cDNA sequence was determined by double-stranded sequencing using the dye chain termination method with the T7 sequencing kit. The numbering of the amino acid sequences of variable regions was that of Kabat (44).

**Peptide Synthesis on Cellulose Membranes**—The general protocol has been described previously (45). Membranes were obtained from Abimed (Langenfeld, Germany). Fmoc amino acids and N-hydroxybenzotriazole were obtained from Novabiochem (Laüelfingen, Switzerland). The ASP222 robot (Abimed) was used for the coupling steps. Two-hundred twenty overlapping dodecapeptides framedhied by one residue repre senting the Vv and Vv sequences of the ST40 antibody were synthesized on cellulose membranes. All peptides were acetylated at their N termini. After the peptide sequences were assembled, the side chain protecting groups were removed by trifluoroacetic acid treatment (46).

**Assay for sCD4 Interaction with Cellulose-bound Peptides—**The technique was performed as described previously for epitope analysis (41) and as adapted to paratope study (40). Briefly, the saturated membranes were incubated with a 1 μg/ml solution of biotinylated sCD4 for 90 min at 37°C. Bound sCD4 was detected by incubation of the membrane at 25°C for 30 min in a 1:3000 dilution of an alkaline phosphatase-streptavidin conjugate (Sigma) and subsequent addition of a phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate and 3,4,5- dimethoxybenzyl-2-yl)-2,5 diphényltetrazolium bromide, Sigma). A blue precipitate on the spots was indicative of binding. After scanning the membrane, NIH software was used to measure the spots’ intensities (45). The membrane was further treated so as to remove precipitated dye and bound CD4 and reused when necessary. Inhibition of sCD4 binding to membrane-bound peptides was evaluated as described below except that biotinylated sCD4 (1 μg/ml) was preincubated with anti-CD4 mAb ST40 (10 μg/ml) for 18 h at 4°C.

**Synthesis of Soluble Peptides and Cyclization—**The 11 dodecapeptides, termed CM1–CM11 (see Table 1), selected by the immunoassay described above, two control peptides (see below), and a CDR3-like peptide (TYICEVEDQKKE) corresponding to CDR3 loop 81–92 in D of Cm3 were prepared by Fmoc-solid-phase synthesis on a AMS422 robot. To improve solubility and to allow cyclization of peptides, Lys-Cys residues were added to both the carboxyl and amino termini of peptides CM1, CM2, CM6, CM7, and CM9–CM11. For peptides CM3–CM5 and CM8 the CDR3-like peptide, the lysine residue was replaced by a tyrosine residue. The peptides were deprotected and released from the resin by trifluoroacetic acid treatment in the presence of appropriate scavengers. They were lyophilized, and their purity was assessed by HPLC. When necessary, the peptides were purified to >90% HPLC homogeneity. The peptides were cyclized by formation of a disulfide bond between the two extra cysteine residues as described by Tam et al. (46): 10 μg of peptide was dissolved in a solution of 20% dimethyl sulfoxide in 50 mM ammonium acetate buffer, pH 7.0, and stirred for 20 min at 37°C to allow the formation of intermolecular disulfide bonds. The efficiency of oxidation was assessed by determination of free sulfhydryl groups in the peptides (47). To this end, peptides (0.5 mg/ml, 10 μl) and 5,5'-dithio-bis(2-nitrobenzoic acid) (0.4 mg/ml, 50 μl) were added to 100 μM Tris, pH 9.0, and the absorbance at 412 nm was determined and compared with the value obtained with the unoxidized peptides. Oxidation efficiency was further assessed by analytical HPLC by the change in the retention time of the oxidized peptide as compared with that of the linear form. The peptides showed >90% intramolecular disulfide bonding at the end of this procedure.

**Enzyme-linked Immunosorbent Assay Monitoring of sCD4 and CDR3-like Peptide Interactions with Cyclic PDPs—**Enzyme immunoassay plates (96-well; Nunc, Paisley) were coated overnight at 4°C with 10-fold serial dilutions of the 11 cyclic PDPs (CM1–CM11) in 100 mM sodium carbonate buffer, pH 9.6. Three replicates were tested for each dilution with an initial peptide concentration of 100 μg/ml. An irrelevant cyclic peptide, 97026c (CKSSQSSLDDSDGKTYLNWC), derived from the heavy chain CDR2 of an anti-p33 antibody was included as a control to verify that binding was sequence-specific. Two cyclic peptides, Digbloc (KLEWIGDYSGGGK) and Dig9c (RCPGDYCYC-LYSACCK), (derived from the heavy chain CDR2 and the light chain CDR3 of anti-digoxin mAb 1C10, respectively) were used as controls to verify the effect on antigen binding of adding Lys-Cys residues to the peptide sequence. After four washes in 160 μl PBS, pH 7.2, containing 0.1% Tween 20 (PBS-T), plates were saturated with a 1% nonfat powdered milk in PBS-T for 30 min at 37°C. Biotinylated sCD4 (1 μg/ml) or biotinylated CDR3-like peptide (1 μg/ml) was added to each well for four washes in PBS-T, and plates were incubated at 37°C for 2 h. Following four washes in PBS-T, 100 μl of an alkaline phosphatase-streptavidin conjugate was added to each well. The conjugate was used at a 1:3000 dilution in PBS-T. The plates were incubated at 37°C for 30 min and then washed four times in PBS-T. Finally, 1 mg/ml 4-nitrophenyl phosphate disodium (Sigma) solution in 1 ml 1 M diethanolamine, pH 9.8, was added for 20 min at 37°C, and the absorbance was measured at 405 nm.

**Real-time Analysis by BLAcore™—**The kinetic parameters (association rate constant (ka) and dissociation rate constant (kd)) were determined by surface plasmon resonance analysis using a BLAcore instrument (BLAcore AB, Uppsala). Using BLAevaluation 3.0 software, ka and kd were determined by the so-called global method (48). The apparent equilibrium constant Kd is the ratio k_d/k_a. All experiments were carried out at 25°C. Free NH_2 from the extraneous lysine residue in CM1, CM2, CM6, CM7, and CM9–CM11 and from the intrasequence lysine residue in CM4 and free COOH from the glutamic acid residue in CM5 were used to chemically immobilize molecules on the sensor chip. Peptides CM3 and CM8 were chemically immobilized by the hydroxyl group of the glutamic acid residue. Peptide 1S (5-carboxyl-1' -carboxyldimiazolo (Sigma-Aldrich). The surface plasmon resonance signal for immobilized peptides was found to be ~30–50 resonance units after completion of the chip regeneration cycle, corresponding to 30–50 pg of peptide/mm^2. The binding kinetics for immobilized peptides were determined by injecting sCD4 (20 μg/ml) in Hepes-buffered saline buffer (running buffer) at a flow rate of 30 μl/min. For the inhibition
FIG. 1. Reactivity of overlapping dodecapeptides derived from the sequence of anti-CD4 mAb ST40 with biotinylated sCD4 (A) and quantitative analysis of the binding (B). The membrane on which the peptides were synthesized was incubated with 1 μg/ml biotinylated sCD4 or with 1 μg/ml biotinylated sCD4 preincubated with 10 μg/ml mAb ST40. In A, CDRs are indicated (H1, H2, and H3 and L1, L2, and L3 correspond to CDR1, CDR2, and CDR3 of the heavy and light chains, respectively), and peptide spots are numbered from 1 to 220. In B, shaded areas indicate the cellulose-bound peptides that reacted with biotinylated sCD4 (cutoff taken at 80 arbitrary units). Boldface amino acids belong to the CDRs. Results correspond to the mean ± S.D. of values obtained from three independent experiments.
study, mAb ST40 (20 μg/ml) and PDP (20 or 200 μg/ml) were co-incubated onto the sensor chip-bound CD4 (30–50 pg/mm²). The kₐ increase was calculated as the ratio of kₐ determined with inhibitor to that obtained without inhibitor.

**HIV-1 Promoter Activation Assay**—The HeLa P4 HIV-1LTR β-galactosidase indicator cell line (49) was provided by O. Schwartz (Institut Pasteur, Paris). HeLa P4 cells, which stably express the β-galactosidase reporter gene cloned downstream of the HIV-1 LTR promoter, were plated in six-well plates at 5 × 10⁴ cells/ml in Dulbecco’s modified Eagle’s medium containing 1% penicillin/streptomycin mixture (Gibco), 1% Glutamax, 1 mg/ml Geneticin (G418), and 10% fetal calf serum. The cells were exposed to 1% of infected HIV-1 producer at 1000 × 50% tissue culture infective dose/ml prepared from the supernatant of chronically infected CEM-T cells, as described previously (50). After incubation for 1 h at 37°C, the cyclic PDPs CM1, CM2, CM6, CM7, and CM9–CM11, at concentrations ranging between 12.5 and 200 μg/ml, were added individually to the cell culture medium. Next, cell cultures were transferred at 37°C in a 5% CO₂ atmosphere to allow infection (note that the HIV-1 infection provides the viral transactivator Tat protein necessary for the HIV promoter in the target cells). After 3 days in culture, cells were lysed, and β-galactosidase activity was determined by incubating 200 μl of total cellular extracts for 1 h at 37°C in 1.5 ml of buffer containing 80 mM Na₂HPO₄, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, and 6 mM o-nitrophenyl β-D-galactopyranoside. β-Galactosidase activity was evaluated by measuring absorbance at 410 nm. Incubation of infected HeLa P4 cells with anti-CD4 mAb ST40 at 20 μg/ml or anti-HLA class II mAb B8-12 (kindly provided by M. Hirn, Immunotech-Coulter, Marseille, France) at 20 μg/ml. No binding was observed with the alkaline phosphatase-streptavidin complex alone (data not shown). Taken together and in accordance with previous observations (40), these results indicate that the binding of sCD4 to immobilized peptides is specific. Eleven peptides (peptides 19, 25, 49, 59, 89, 99, 105, 128, 154, 178, and 220, named CM1–CM11, respectively) showing the highest reactivity with sCD4 (color intensity of the corresponding spots between 119 ± 15 and 165 ± 14) were selected for further study in a soluble form. The selected peptides comprised either exclusively CDR (CM6) or framework (CM1 and CM8) sequences or comprised amino acids from both CDRs and framework sequences (CM2–CM5, CM7, and CM9–CM11).

**CD4 and CDR3-like Loop Specificity of Soluble Cyclic Peptides Derived from the ST40 Antibody Sequence**—The 11 peptides (CM1–CM11), selected from the initial 220 overlapping peptides on the basis of their reactivity with sCD4 in the form of membrane-bound peptides, were synthesized by conventional solid-phase synthesis and N to C terminus-cyclized through cysteine oxidation (Table I). Their binding to whole CD4 and to a CDR3-like loop peptide (corresponding to residues 81–92 in D1 of the CD4 molecule) was assessed by enzyme-linked immunosorbent assay (Fig. 2). Soluble cyclic peptide reacted specifically with sCD4 in a dose-dependent manner, which was not the case for the three irrelevant cyclic peptides 97026c, Dig23c, and Dig97c, the latter two including an extra lysine residue like the CM peptides. Peptides selected from either the V₃ region (Fig. 2A) or the V₅ region (Fig. 2B) displayed CD4 binding activity in a 1–10 μg/ml concentration range. Peptides CM2, CM6, and CM7 (Fig. 2C), derived from the ST40 V₃ region, and peptides CM9 and CM11 (Fig. 2D), derived from the ST40 V₅ region, showed strong reactivity CDR3-like peptide 81–92, whereas other synthetic peptides did not significantly bind this antigen. The linear forms of peptide CM9 and several other PDPs were markedly less reactive than the cyclic form (data not shown), indicating a beneficial effect of preincubating sCD4 with the parental anti-CD4 mAb ST40 (10 μg/ml). No binding was observed with the alkaline phosphatase-streptavidin complex alone (data not shown). Taken together and in accordance with previous observations (40), these results indicate that the binding of sCD4 to immobilized peptides is specific. Eleven peptides (peptides 19, 25, 49, 59, 89, 99, 105, 128, 154, 178, and 220, named CM1–CM11, respectively) showing the highest reactivity with sCD4 (color intensity of the corresponding spots between 119 ± 15 and 165 ± 14) were selected for further study in a soluble form. The selected peptides comprised either exclusively CDR (CM6) or framework (CM1 and CM8) sequences or comprised amino acids from both CDRs and framework sequences (CM2–CM5, CM7, and CM9–CM11).

**RESULTS**

**Sequence of Anti-CD4 mAb ST40**—The nucleotide sequences of the V₃ and V₅ regions from anti-CD4 mAb ST40 were established as described under “Experimental Procedures.” Nucleotide sequences of three individual clones were determined for each chain type and shown to be similar. Comparison of this sequence with other known antibody sequences showed that the V₃ region of mAb ST40 belongs to subgroup IIA according to the classification of Kabat et al. (44) and displays 95.5% identity with the germ line (44) except for a 3-nucleotide difference that is specific. Eleven peptides (peptides 19, 25, 49, 59, 89, 99, 105, 128, 154, 178, and 220, named CM1–CM11, respectively) showing the highest reactivity with sCD4 (color intensity of the corresponding spots between 119 ± 15 and 165 ± 14) were selected for further study in a soluble form. The selected peptides comprised either exclusively CDR (CM6) or framework (CM1 and CM8) sequences or comprised amino acids from both CDRs and framework sequences (CM2–CM5, CM7, and CM9–CM11).
peptides Dig23c and Dig97c showed that the additional cysteine and lysine residues used for cyclization/solubilization are not implicated in the CD4 and CDR3-like binding. Taken together, these results indicate that the selected soluble cyclic peptides derived from mAb ST40 have the capacity to specifically bind the CD4 molecule, but only some of them also demonstrated a specificity for the CDR3-like loop.

The results of the BIAcore study, in which the kinetic parameters $k_a$ and $k_d$ of the interaction between immobilized peptides and soluble CD4 were measured, are summarized in Table I. All 11 peptides exhibited measurable binding to sCD4. No measurable binding was obtained with the irrelevant cyclic peptide. The calculated $K_D$ values ranged from 1.6 to 86.4 nM. Peptides CM2 and CM5–CM7, derived from the CDR1 and CDR3 VH regions of mAb ST40, showed the highest affinity. The $K_D$ values obtained with the peptides showed a 4–8-fold increase in value as compared with the value obtained with the parental ST40 mAb (0.37 nM). This increase is mainly due to a lower dissociation rate of the mAb ($3.3 \times 10^{-4}$ s$^{-1}$) in comparison with that obtained with the PDPs.

Inhibition of HIV-1 Promoter Activation in Virus-infected Cells by PDPs—The ability of the PDPs to inhibit HIV-1 promoter activity was measured in HeLa P4 cells stably transfected with the $\beta$-galactosidase reporter gene under the control of the HIV-1 LTR promoter. Infection of the indicator cell line with HIV-1 LTR strongly stimulated the HIV-1 promoter activity (mean $A_{410\ nm}$ increased from 0.014 to 0.548). As shown in Fig. 3A, no inhibition of the HIV-1 LTR-driven $\beta$-galactosidase gene expression was observed when HIV-1LTR-infected indicator cells were cultured with anti-HLA class II mAb B8-12, whereas 65% inhibition was found following incubation with mAb ST40. Irrelevant linear and cyclic peptides did not affect the $\beta$-galactosidase gene expression. In contrast, treatment with the cyclic PDPs CM2, CM9, and CM11 significantly inhibited the HIV-1 LTR-driven $\beta$-galactosidase gene expression induced by HIV-1_LTR. Several other cyclic PDPs (CM1, CM6, CM7, and CM10) showed no effect. Peptide CM9, corresponding to the sequence $30^{DSYMNYWQKPG41}$ of the CDR1 framework-2 light chain region, was the strongest inhibitor. As shown in Fig. 3B, peptide CM9 inhibited, in a dose-dependent manner, the HIV-1 LTR-driven $\beta$-galactosidase gene expression induced by HIV-1_LTR. At a concentration of 63 µg/ml, peptide CM9 showed $-50\%$ of the effect of the parental antibody used at 20 µg/ml. Taken together, these results indicate that the PDPs CM2, CM9, and CM11, initially selected among all the overlapping dodecapeptides of the VH and VL domains of anti-CD4 mAb ST40, are able to inhibit the HIV-1 promoter, a property previously ascribed to mAb ST40 (24).

Fig. 2. Enzyme-linked immunosorbent assay binding curves of biotinylated sCD4 or CDR3-like peptide 81–92 on adsorbed cyclic peptides derived from the sequence of mAb ST40. Plates were coated with various concentrations of the cyclic peptides synthesized from the $V_H$ sequence (A and C) and from the $V_L$ sequence (B and D). Probing was performed either with biotinylated sCD4 (1 µg/ml) (A and B) or biotinylated CDR3-like peptide (100 µg/ml) (C and D). Irrelevant peptides (Dig23c, Dig97c, and 97026c) were used as negative controls. Each value represents the mean ± S.D. of triplicate determinations.
dose-dependent since a peptide concentration of 20 μg/ml caused only a 30–50-fold increase in the dissociation rate.

**DISCUSSION**

The identification, by using multiple peptide synthesis, of PDPs able to bind antigen was recently described by our group; several of these peptides display a significant fraction of the affinity of the whole antibody (40). Therefore, this approach could conceivably be used to screen peptide ligands mimicking the biological effect of a given antibody. With this perspective in mind, we have studied an anti-CD4 mAb (ST40) that shows interesting pharmacological activities. The ST40 antibody binds to the CDR3-like loop in D1 of CD4 and has been described as a strong inhibitor of HIV promoter activity and provirus transcription (24). We have established the VH and VL amino acid sequences of this antibody and assessed the reactivity of sCD4 with overlapping 12-mer peptides derived from these sequences by the Spot method (40, 41). Eleven peptides were found to react strongly and specifically with the CD4 antigen. We demonstrated that soluble cyclic peptides derived from peptides reactive in the Spot assay were able to recognize the CD4 molecule and a cyclic CDR3-like loop peptide corresponding to region 81–92 of CD4. Among the CDR3-like loop-specific PDPs, three (CM2, CM9, and CM11) were found to block HIV promoter activity and to compete efficiently with the parental mAb for binding to CD4.

An interesting feature was that PDPs showing the strongest reactivity with CD4 in the Spot assay included both residues from the CDRs and residues from the framework flanking the hypervariable regions, extending our previous observations (40). Antibody variable domains comprise a framework of β-sheets surmounted by antigen-binding loops. We can postulate that critical residues, identified in the Spot assay and confirmed by preliminary Alascan analysis (data not shown), located in the β-sheet framework closely underlying the CDRs, probably do not participate in direct interaction with CD4, but could induce a binding conformational state mimicking some of
the structural features of the ST40 paratope. Three points argue in favor of this hypothesis. First, some framework amino acids that modulate the peptide/CD4 interaction (i.e. Tyr\(^{37}\), Trp\(^{47}\), Gly\(^{49}\), and Arg\(^{94}\) in the ST40 V\(_H\) sequence and Tyr\(^{36}\) in the ST40 V\(_V\) segment) belong to the vernier zone, which contains residues that adjust the CDR structure and fine-tune the fitting to the antigen (54). Second, some residues possess an aromatic structure (i.e. Tyr\(^{37}\) and Trp\(^{47}\) in the heavy chain and Tyr\(^{36}\) in the light chain) characterized as protruding into the antigen-binding site surface to stabilize the antigen/antibody interaction (55, 56). Third, framework arginine residues (i.e. Arg\(^{94}\) in V\(_H\) and Arg\(^{18}\) in V\(_L\)) modulate the peptide/CD4 binding, in keeping with previous work demonstrating the critical role of Arg\(^{94}\) in the interaction of a CDR3 V\(_H\) Peptide with phosphatidylserine (36). These six critical residues from the framework regions of the ST40 antibody possess one or several of these characteristics, in agreement with previous results obtained in our laboratory on the interactions of mAb HyHEL-5/lysozyme (40) and mAb Tg10/thyroglobulin and mAb 4D8/angiotensin II.\(^2\)

Based on the observations that CDR3-like synthetic peptides can bind CD4, Langedijk et al. (30) have proposed that the putative dimerization of CD4 involves the CDR3-like loop in D1. Moreover, electrostatic potential contours calculated for a putative CD4 dimerization occurring in D1 predicted that the negative electrostatic potentials of the CDR3-like region were completely compensated for by positive charges on the opposite CD4 molecule in the dimer (30). Recent results (29) suggest that Glu\(^{87}\), Asp\(^{88}\), Glu\(^{91}\), and Glu\(^{92}\) in the CDR3-like loop are positively charged residues, like Arg\(^{100G}\) and Arg\(^{100H}\) found in the sequence of the PDPs CM6 and CM7 from the CDR3 V\(_H\) region, Lys\(^{95}\) belonging to the sequence of peptide CM9 from the CDR1 V\(_I\) domain, and Lys\(^{97}\) in the PDP CM11, could conceivably interact with the negatively charged residues of the ST40 epitope. In agreement with this hypothesis, Arg\(^{100H}\) and Lys\(^{95}\) have been found to be critical amino acids by the Spot method in the peptide/CD4 interactions. Moreover, preliminary results obtained by Alascan analysis of PDPs confirm the contribution of these positively charged residues in CD4 binding (data not shown). However, positively charged amino acids probably reflect only a part of the interaction between ST40 and CD4 since other contributor residues in the CDRs were found by using Alascan analysis.

With regard to the measured binding kinetics of the interaction between soluble linear peptides from the HyHEL-5 paratope and lysozyme (40), a 1-log decrease in the \(k_d\) was observed in the peptide/CD4 binding, whereas association rates were in the same order of magnitude in the two models. In the case of anti-reovirus mAb 87.92.6 (34), it has been reported that the increased conformational stability of cyclic CDR peptides could increase the binding affinity. In addition, other reports (26, 57) suggest that cyclization helps peptides to mimic the CDR conformation. From these observations and from the results obtained with the CM peptide series, it seems that constraining the PDPs improves their affinity for antigen through a decrease in the dissociation rate of the equilibrium reaction between ligands. All the selected PDPs were able to bind sCD4 with \(K_d\) values ranging from \(-2\) to 90 nM, the best values being 4–8-fold higher than those obtained with the parental mAb.

mAb ST40 has been previously shown to inhibit HIV-1 LTR-driven chloramphenicol acetyltransferase gene expression induced by HIV-1 \(\text{tat}\) (24). The PDP CM9, derived from region 30–41 of the ST40 CDR2 V\(_V\) domain, blocks HIV promoter activity through the inhibition of \(\beta\)-galactosidase gene expression in a dose-dependent manner. The biological effect of CM9 was corroborated by further BIACore experiments, in which this peptide was shown to displace the binding of ST40 to CD4 by increasing the rate of the dissociation reaction. Numerous bioactive peptides corresponding to the CDR3-like loop have been used to modulate the T-cell response (14, 17, 18) or to exert anti-HIV activity (26, 28). Disruption of CD4 dimerization by CDR3-like analogs has been proposed as a major mechanism by which cell activation could be inhibited following treatment of CD4-positive cells by CDR3-like analogs (18, 26, 29, 30). Furthermore, negatively charged residues in amino acid region 87–92 of CD4 can potentially be involved in the binding of a CDR3-like analog to CD4 (29). The facts that (i) the PDP CM9 interacts with CDR3-like region 81–92 and inhibits HIV-1 promoter activity and that (ii) residues 87/88 and 91/92 are involved in the epitope of the ST40 antibody, from which peptide CM9 has been designed, suggest that this PDP could act as an inhibitor of CD4 dimerization. Such an effect needs to be confirmed by additional experiments, even though we cannot rule out the fact that other CD4 regions might contribute to the oligomerization. Our results clearly demonstrate that the systematic exploration of sets of short cellulose-bound synthetic overlapping peptides derived from the sequences of immunoglobulin variable regions is a valuable strategy for identifying bioactive PDPs.

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