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

Céline Monnet, Daniel Laune, Jeanny Laroche-Traineau, Martine Biard-Piechaczyk, Laurence Briant, Cédric Bès, Martine Pugnière, Jean-Claude Mani, Bernard Pau, Martine Cerutti, et al.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 6, Issue of February 5, pp. 3789–3796, 1999

Printed in U.S.A.

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*

(Received for publication, July 6, 1998, and in revised form, October 15, 1998)

Céline Monnet‡, Daniel Laune‡, Jeanny Laroche-Traineau‡, Martine Biard-Piechaczyk‡§, Laurence Briant§, Cédric Bès‡, Martine Pugnière‡, Jean-Claude Mani‡, Bernard Pau‡, Martine Cerutti¶, Gérard Devauchelle¶, Christian Devaux§, Claude Granier‡, and Thierry Chardès¶‖

From ‡CNRS UMR 9921, Faculté de Pharmacie, 15 Avenue Charles Flahault and §CNRS UPR 1086, CRBM-Laboratoire Infections Rétrovirales et Signalisation Cellulaire, 34060 Montpellier, France and ¶INRA-CNRS URA 2209, Laboratoire de Pathologie Comparée, 30380 Saint-Christol-Lez-Alès, France

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 $p56^{lck}$ (4). Besides its physiological function, the CD4 surface glycoprotein, in association with chemokine receptors, acts as a receptor for HIV-1 [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 (V_H) and light (V_L) chains. The diversity of paratopes is mainly generated by the sequences of the CDRs found in V_H and V_L, which are exposed hypervariable loop structures. Antigen binding by peptide sequences from selected CDRs of mAbs has been demonstrated to have specificities similar 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

* This work was supported by institutional funds from CNRS and by grants from ELF and from the Biotechnology Program of the Ministère de l'Enseignement Supérieur et de la Recherche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ005354 and AJ005355.

‖ To whom correspondence should be addressed. Tel.: 33-4-67-54-86-04; Fax 33-4-67-54-86-10; E-mail: chardes@pharma.univ-montp1.fr.

[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; D1, domain 1; CDR, complementarity-determining region; mAb, monoclonal antibody; V_H, variable region of the heavy chain; V_L, variable region of the light chain; PDP, paratope-derived peptide; sCD4, soluble CD4; PBS, phosphate-buffered saline; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; LTR, long terminal repeat.

of the V_H and V_L regions of mAb ST40. A set of immobilized overlapping dodecapeptides covering the deduced amino acid sequences of mAb ST40 variable regions was prepared by the Spot method (40, 41). The ability of biotinylated soluble CD4 (sCD4) to bind these peptides was then investigated and led to the selection of peptides with CD4 binding activity. All the selected PDPs prepared in a soluble cyclic form showed CD4 binding capacity, and three of them blocked HIV-1 promoter activity and efficiently competed with mAb ST40 for binding to CD4.

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 (Amersham Pharmacia Biotech RPN2202) according to the manufacturer's instructions. Biotinylated sCD4 was stored in PBS at −20 °C until use.

Cloning of mAb ST40 V_H and V_L Genes—The murine hybridoma cell line that produces mAb ST40/F142-63 (IgG1,κ) was a kind gift from Dr. D. Carrière (Sanofi Recherche, Montpellier, France) (43). Total RNA was extracted from 3×10^8 hybridoma cells using the TRIzol™ technique (Life Technologies Inc., Paisley, United Kingdom). The V_L 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-SoCκ3′ (5′-CGCGCAGATCTAACACTCATTCCTGTTGAAGC-3′), which contains the reverse complement of codons 208–214 of Cκ. One μl of first strand cDNA was used as matrix for the polymerase chain reaction to amplify the ST40 V_L/C_L genes using Vent DNA polymerase (New England Biolabs, Hitchin, UK) and the primers OPP-SoCκ3′ and OPP-SoVκ5′ (5′-GA(C/T/A)ATTGAGCTCAC(C/A)CAG(T/A)CTCCA-3′). These primers contained restriction sites (underlined) for cloning. The degenerate primer OPP-SoVκ5′ was chosen as the consensus sequence of codons 5–8 in murine FR1 Vκ. The polymerase chain reaction-amplified DNA product was digested sequentially with *Bgl*II and *Sac*I (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 V_L cDNA sequence was determined by double-stranded sequencing using the dideoxy chain termination method with the T7 sequencing kit (Amersham Pharmacia Biotech, Uppsala). The V_H gene of the ST40 antibody was isolated from a cDNA library. Briefly, poly(A)$^+$ RNAs were magnetically separated from total RNAs by hybridization with a biotinylated oligo(dT) primer and then captured by streptavidin coupled to paramagnetic beads as described by the manufacturer (Polytract™, Promega, Madison, WI). A cDNA library was constructed from 10 μg of ST40 poly(A)$^+$ RNA in the pSPORT1 vector using the Superscript™ plasmid system (Life Technologies Inc.). This library was screened by plaque hybridization with ^{32}P-labeled primer Muγ_1CH1 (5′-GAAATAGCCCTTGACCAGGCA-3′). This primer contains sequence information for the reverse complement of the murine γ_1 constant region gene, which codes for amino acids 142–148. The dideoxy chain termination sequencing of the V_H cDNA from selected clones was carried out on both strands using the T7 sequencing kit. The numbering of the amino acid sequences of variable regions was that of Kabat *et al.* (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 (Läufelfingen, Switzerland). The ASP222 robot (Abimed) was used for the coupling steps. Two-hundred twenty overlapping dodecapeptides frameshifted by one residue representing the V_H and V_L 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 (41).

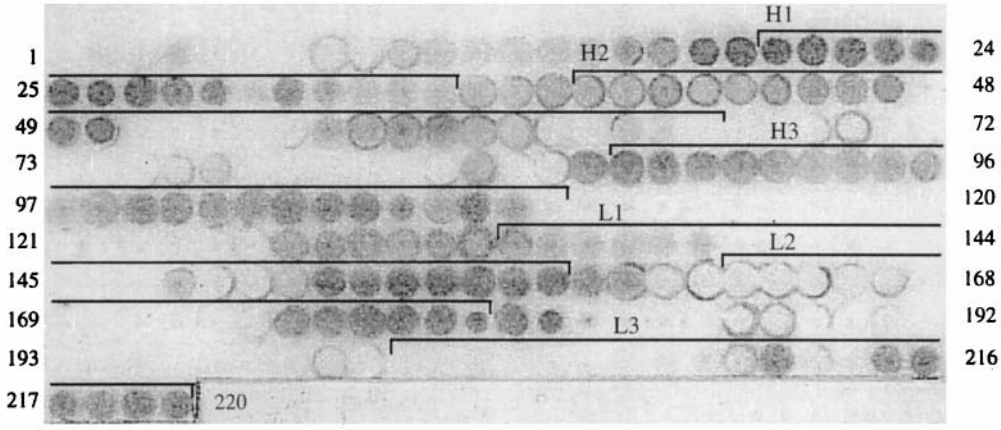
Assay for sCD4 Interaction with Cellulose-bound Peptides—The technique was performed as described previously for epitope analysis (41) and as adapted to paratope study (40). Briefly, the saturated membranes were incubated with a 1 μg/ml solution of biotinylated sCD4 for 90 min at 37 °C. Bound sCD4 was 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-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium 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 above, 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 I), selected by the immunoassay described above, two control peptides (see below), and a CDR3-like peptide (TYICEVEDQKEE) corresponding to CDR3 loop 81–92 in D1 of CD4 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 and 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 mg of peptide was dissolved in a solution of 20% dimethyl sulfoxide in 50 mM ammonium acetate buffer, pH 7.0, and stirred for 24 h at 20 °C. Peptide concentration was adjusted to 0.5 mg/ml to avoid 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′-dithiobis(2-nitrobenzoic acid) (0.4 mg/ml, 50 μl) were added to 100 mM 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 (CKSSQSLLDSDGKTYLNWC), derived from the heavy chain CDR2 of an anti-p53 antibody was included as a control to verify that binding was sequence-specific. Two cyclic peptides, Dig23c (KCLEWIGDIYSGGGCK) and Dig97c (KCFGDYYCLQYASSCK), (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 mM 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 (100 μg/ml) was added after 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, a 1 mg/ml 4-nitrophenyl phosphate disodium (Sigma) solution in 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 BIAcore™—The kinetic parameters (association rate constant (k_a) and dissociation rate constant (k_d)) were determined by surface plasmon resonance analysis using a BIAcore instrument (BIAcore AB, Uppsala). Using BIAevaluation 3.0 software, k_a and k_d were determined by the so-called global method (48). The apparent equilibrium constant K_D is the ratio k_d/k_a. All experiments were carried out at 25 °C. Free NH_2 from the extrasequence 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 groups of threonine and serine, respectively, after activation by 1,1′-carbonyldiimidazole (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

A



B

	Peptide	Sequence	Color intensity of the spot (± SD)	
			Without preincubation	Preincubation with anti-CD4 mAb
ST40 V_H sequence	7	^{7}SGPELKKPGETV^{18}	6 ± 2	4
	15	^{15}GETVRISCKASG^{26}	66 ± 13	22
	16	^{16}ETVRISCKASGY^{27}	116 ± 24	31
	19 (CM1)	^{19}RISCKASGYGFT^{30}	164 ± 14	42
	25 (CM2)	^{25}SGYGFT**NAGMQ**W^{36}	162 ± 17	69
	29	^{29}FT**NAGMQ**WVQKM^{40}	101 ± 17	35
	30	^{30}**TNAGMQ**WVQKMP^{41}	35 ± 5	14
	37	^{37}VQKMPGKGLRWI^{48}	77 ± 12	22
	38	^{38}QKMPGKGLRWIG^{49}	98 ± 14	26
	47	^{47}WIG**WINTHSGVP**^{57}	92 ± 7	32
	48	^{48}IG**WINTHSGVPT**^{58}	11 ± 6	7
	49 (CM3)	^{49}G**WINTHSGVPTY**^{59}	131 ± 20	39
	50	^{50}**WINTHSGVPTYA**^{60}	109 ± 9	27
	51	^{51}**INTHSGVPTYAE**^{61}	17 ± 5	10
	55	^{54}**SGVPTYAEDFKG**^{65}	15 ± 2	4
	56	^{55}**GVPTYAEDFKG**R^{66}	80 ± 16	13
	59 (CM4)	^{58}**TYAEDFKG**RFAF^{69}	125 ± 10	47
	60	^{59}**YAEDFKG**RFAFS^{70}	107 ± 27	28
	61	^{60}**AEDFKG**RFAFSL^{71}	72 ± 18	29
	86	^{82C}LKNEDTATYFCA^{93}	28 ± 5	18
	87	^{83}KNEDTATYFCAR^{94}	124 ± 20	31
	89 (CM5)	^{85}EDTATYFCAR**GG**^{96}	139 ± 14	46
	99 (CM6)	^{95}**GGVLWSRRGDFD**^{101}	119 ± 15	57
	105 (CM7)	^{100c}**RRGDFDY**WGQGT^{107}	145 ± 25	60
	106	^{100d}**RGDFDY**WGQGTA^{108}	127 ± 23	43
	107	^{100f}**GDFDY**WGQGTAL^{109}	79 ± 8	30
	108	^{100f}**DFDY**WGQGTALT^{110}	124 ± 1	38
ST40 V_L sequence	121	^{1}DIELTQSPASLA^{12}	9 ± 4	4
	126	^{6}QSPASLAVSLGQ^{17}	28 ± 6	5
	127	^{7}SPASLAVSLGQR^{18}	93 ± 25	22
	128 (CM8)	^{8}PASLAVSLGQRA^{19}	122 ± 30	48
	133	^{13}VSLGQRATISC**K**^{24}	97 ± 5	25
	134	^{14}SLGQRATISC**KA**^{25}	77 ± 5	27
	148	^{27}**QSVDYDGDSYMN**^{34}	6 ± 1	4
	151	^{27b}**YDGDSYMN**WYQQ^{38}	50 ± 9	19
	152	^{28}**DGDSYMN**WYQQK^{39}	145 ± 29	86
	154 (CM9)	^{30}**DSYMN**WYQQKPG^{41}	162 ± 24	96
	160	^{36}YQQKPGQPPKLL^{47}	100 ± 24	31
	161	^{37}QQKPGQPPKLLI^{48}	38 ± 10	12
	174	^{50}**AASNLES**GIPAR^{61}	19 ± 7	10
	175	^{51}**ASNLES**GIPARF^{62}	119 ± 23	45
	178 (CM10)	^{54}**LES**GIPARFSGS^{65}	165 ± 14	80
	182	^{58}IPARFSGSGSGT^{69}	150 ± 16	67
	183	^{59}PARFSGSGSGTD^{70}	47 ± 9	28
	211	^{87}YC**QQSNEDPWTF**^{98}	33 ± 8	13
	212	^{88}C**QQSNEDPWT**FG^{99}	85 ± 16	37
	213	^{89}**QQSNEDPWT**FGG^{100}	14 ± 4	14
	214	^{90}**QSNEDPWT**FGGG^{101}	17 ± 4	14
	215	^{91}**SNEDPWT**FGGGT^{102}	97 ± 16	49
	220 (CM11)	^{96}**WT**FGGGTYLEIK^{107}	143 ± 24	70

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-injected onto the sensor chip-bound CD4 (30–50 pg/mm^2). The k_d increase was calculated as the ratio of k_d determined with inhibitor to that obtained without inhibitor.

HIV-1 Promoter Activation Assay—The HeLa P4 HIV-1 LTR 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 5×10^5 cells/ml in Dulbecco's modified Eagle's medium containing a 1% penicillin/streptomycin mixture (Gibco), 1% Glutamax, 1 mg/ml Geneticin (G418), and 10% fetal calf serum. The cells were exposed to 1 ml of infectious HIV-1_{Lai} 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 4 °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% CO_2 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 Na_2HPO_4, 10 mM $MgCl_2$, 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 served as positive and negative controls, respectively. Additional controls consisted of linear Lyso-3 peptide (biotinyl-YKKSGTSPKRWIYDT), derived from the light chain CDR2 of anti-lysozyme mAb HyHEL-5 (40), and the cyclized 97026c peptide described above.

RESULTS

Sequence of Anti-CD4 mAb ST40—The nucleotide sequences of the V_H and V_L 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 V_H region of mAb ST40 belongs to subgroup IIA according to the classification of Kabat *et al*. (44) and displays 95.5% homology to the closest *VGK2* germ line gene (51) from the V-Gam 3.8 family. mAb ST40 used a member of the *DSP2* DH gene segment family, and the JH gene segment is homologous to the *JH2* germ line (44) except for a 3-nucleotide difference probably accounted for by somatic mutation. Sequence analysis suggests that the ST40 V_L region results from the rearrangement of a Vκ subgroup III gene with the *Jκ1* gene segment (44). More precisely, the ST40 V_L region shows 88% homology to the closest *Vκ21G* germ line gene (52) from the *Vκ21* family. Computer-assisted comparisons of these variable regions with other sequenced genes from anti-CD4 mAbs indicated that the ST40 V_L region shows strong homology to the V_L region of anti-CD4 mAb L71 (53). No significant homology to the anti-CD4 heavy chain has been found for the V_H sequence of mAb ST40.

Systematic Evaluation of the Reactivity of Overlapping Peptides from the ST40 Antibody Sequence with Biotinylated sCD4—Two-hundred twenty overlapping 12-mer peptides frameshifted by one residue, corresponding to the deduced amino acid sequences of V_H and V_L from mAb ST40, were synthesized according to the Spot method (40). These membrane-bound peptides were then probed with biotinylated sCD4. The results are described qualitatively in Fig. 1*A*, in which peptide spots showing at least one CDR residue are highlighted, and quantitatively in Fig. 1*B*. Biotinylated sCD4 bound mainly to peptides including amino acid(s) from the six CDRs of mAb ST40 (peptides 20–29, 31, 39–47, 49–50, 56–60, 88–106, 108, 133, 152–158, 175–180, 212, and 215–220). The majority of peptides containing only framework residues did not display any binding activities. However, several peptides (peptides 16–19, 38, 87, 127–132, 159–160, and 181–182) that contained only framework residues, mainly flanking the CDRs, were able to bind the sCD4 antigen. Little or no reactivity with sCD4 was observed with peptides comprising amino acids from the middle of the CDR sequence (peptides 30, 32–35, 48, 51–55, 61–66, 107, 134–151, 163–174, 202–211, and 213–214). As shown in Fig. 1*B*, this binding pattern was strongly affected by 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).

TABLE I
BIAcore determination of the binding kinetics of the interaction between sensor chip-bound peptides derived from the V_H and V_L sequences of ST40 and biotinylated CD4

Boldface amino acids belong to the CDRs of mAb ST40.

PDP	Peptide sequence[a]	k_a	k_d	K_D
		$10^4\ s^{-1}\ M^{-1}$	$10^{-4}\ s^{-1}$	nM
CM1	KCRISCKASGYGFTCK	9.4	3.0	3.2
CM2	KCSGYGFT**NAGMQ**WCK	13.7	2.2	1.6
CM3	YCG**WINTHSGVPTY**CY	7.7	16.9	22.0
CM4	YC**TYAEDFKG**RFAFCY	3.0	8.2	27.0
CM5	YCEDTATYFCAR**GG**CY	10.3	3.5	3.4
CM6	KC**GGVLWSRRGDFD**CK	21.9	3.6	1.6
CM7	KC**RRGDFDY**WGQGTCK	22.4	7.2	3.2
CM8	YCPASLAVSLGQRACY	2.4	20.4	86.4
CM9	KC**DSYMN**WYQQKPGCK	8.5	9.1	10.6
CM10	KC**LES**GIPARFSGSCK	8.2	4.5	5.5
CM11	KC**WT**FGGGTYLEIKCK	8.1	3.7	4.6
97026c	CKSSQSLLDSDGKTYLNWC	NM[b]	NM	NM

[a] All peptides were cyclized through N- to C-terminal disulfide bridging.
[b] Not measurable.

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 peptides 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 V_H region (Fig. 2*A*) or the V_L region (Fig. 2*B*) displayed CD4 binding activity in a 1–100 μg/ml concentration range. Peptides CM2, CM6, and CM7 (Fig. 2*C*), derived from the ST40 V_H region, and peptides CM9 and CM11 (Fig. 2*D*), derived from the ST40 V_L region, strongly recognized 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 N- to C-terminal cyclization on binding properties. Furthermore, the absence of reactivity of the 12-mer Lys-Cys-cyclized

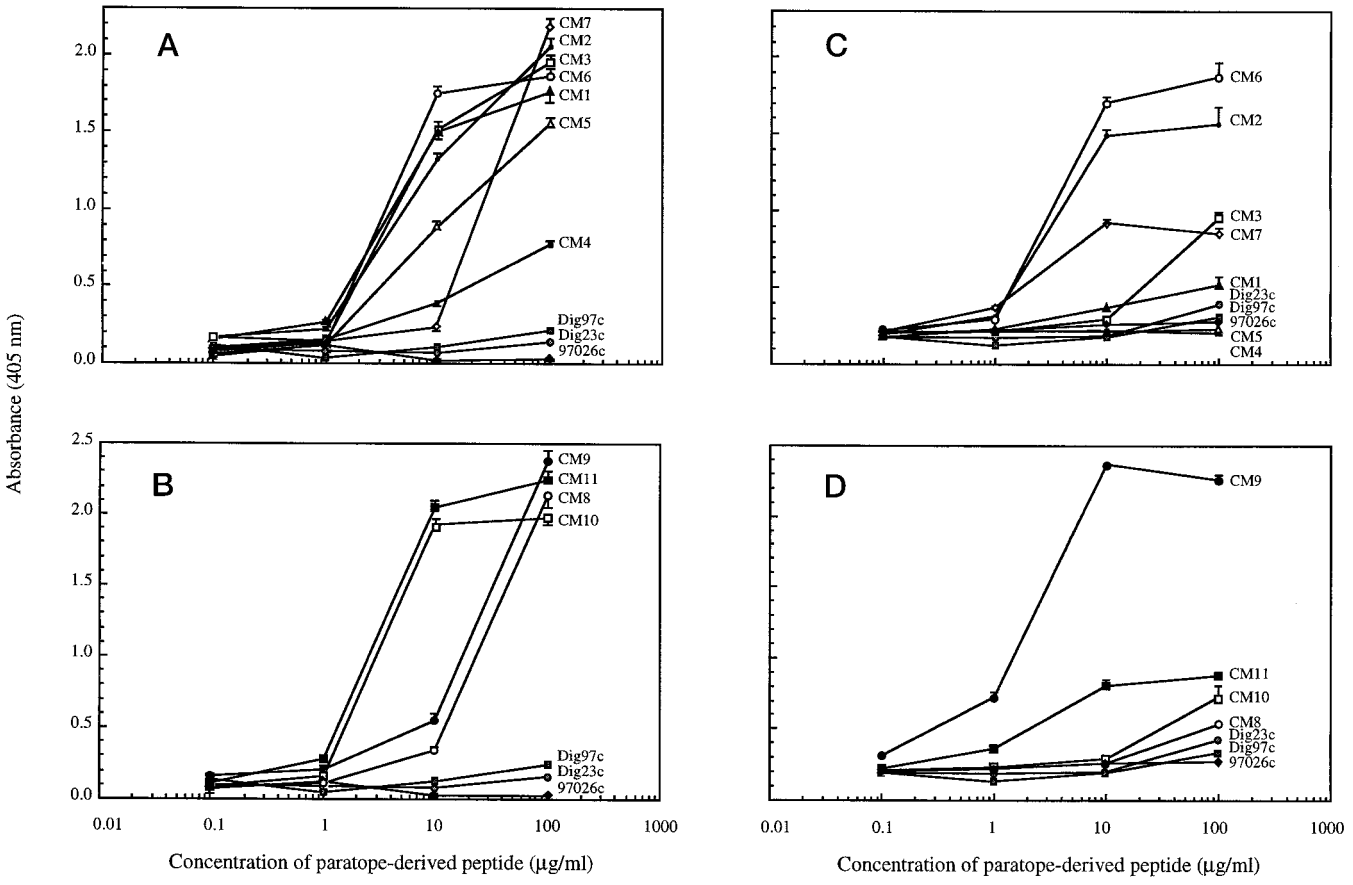


FIG. 2. **Enzyme-linked immunosorbent assay binding curves of biotinylated sCD4 or CDR3-like peptide 81–92 on adsorbed cyclic peptides derived from the sequence of mAb ST40.** Plates were coated with various concentrations of the cyclic peptides synthesized from the V_H sequence (*A* and *C*) and from 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.

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 V_H 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 (0.33×10^{-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 β-galactosidase reporter gene under the control of the HIV-1_{Lai} LTR promoter. Infection of the indicator cell line with HIV-1_{Lai} strongly stimulated the HIV-1 promoter activity (mean $A_{410\ nm}$ increased from 0.014 to 0.548). As shown in Fig. 3*A*, no inhibition of the HIV-1 LTR-driven β-galactosidase gene expression was observed when HIV-1_{Lai}-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 β-galactosidase gene expression. In contrast, treatment with the cyclic PDPs CM2, CM9, and CM11 significantly inhibited the HIV-1 LTR-driven β-galactosidase gene expression induced by HIV-1_{Lai}. Several other cyclic PDPs (CM1, CM6, CM7, and CM10) showed no effect. Peptide CM9, corresponding to the sequence ^{30}DSYMNWYQQKPG^{41} of the CDR1 framework-2 light chain region, was the strongest inhibitor. As shown in Fig. 3*B*, peptide CM9 inhibited, in a dose-dependent manner, the HIV-1 LTR-driven β-galactosidase gene expression induced by HIV-1_{Lai}. 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 V_H and V_L domains of anti-CD4 mAb ST40, are able to inhibit the HIV-1 promoter, a property previously ascribed to mAb ST40 (24).

Inhibition of ST40 Binding to CD4 by Three Paratope-derived Peptides—Competitive binding assays were performed to examine the ability of peptides CM2, CM9, and CM11 to block the binding of the parental ST40 mAb to sensor chip-bound CD4 (Table II). The three PDPs competed with the anti-CD4 antibody for binding to sensor chip-bound CD4, as determined by BIAcore analysis. This competition led to the enhancement of the dissociation rate of the antibody to the CD4 molecule. A 1000–2000-fold k_d increase was obtained when peptides were used at a concentration of 200 μg/ml. This inhibitory effect was

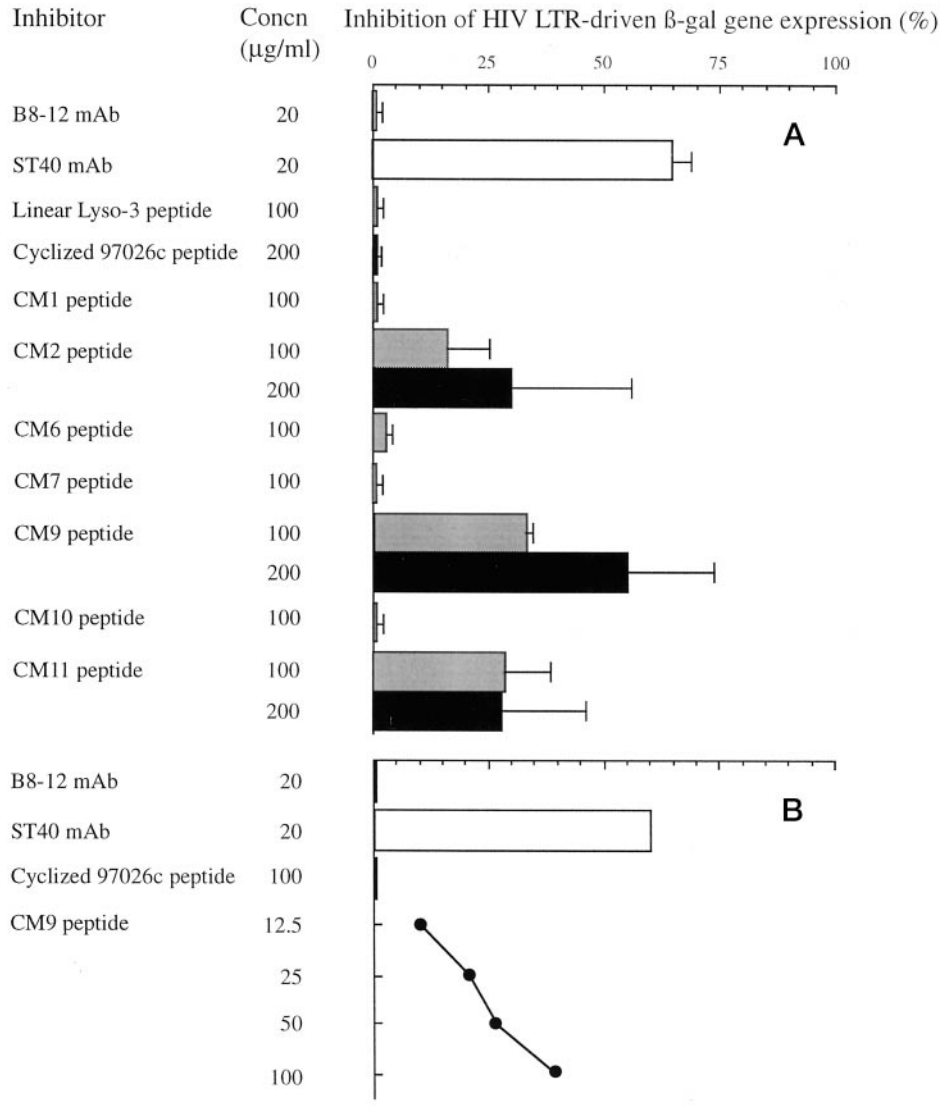


FIG. 3. **Inhibition of HIV LTR-driven β-galactosidase gene expression induced by HIV-1_{Lai} following incubation with cyclic PDPs synthesized from the ST40 sequence (*A*) and with different concentrations of peptide CM9 (*B*).** Irrelevant linear Lyso-3 and cyclic 97026c peptides were used as negative controls. Results correspond to the mean ± S.D. of inhibition values obtained from at least four independent experiments. Mean absorbances at 410 nm varied from 0.014 for uninfected indicator cells to 0.548 for HIV-1_{Lai}-infected indicator cells. *β-gal*, β-galactosidase.

TABLE II
Increase in the dissociation rate of the binding between sensor chip-bound CD4 and the anti-CD4 mAb co-injected with PDPs derived from the sequence of the antibody

Inhibitor	Conc	CD4/anti-CD4 interaction	
		k_d	-Fold increase
	μg/ml	$s^{-1}\ M^{-1}$	
None		3.3×10^{-5}	1
PDP			
CM2	20	1.2×10^{-3}	37
	200	2.6×10^{-2}	787
CM9	20	1.6×10^{-3}	48
	200	3.5×10^{-2}	1060
CM11	20	8.9×10^{-4}	27
	200	6.1×10^{-2}	1848

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 V_H and V_L 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^{27}, Trp^{47}, Gly^{49}, and Arg^{94} in the ST40 V_H sequence and Tyr^{36} in the ST40 V_L 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^{27} 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 essential for CD4 dimerization and that these four negatively charged amino acids are involved in the ST40 epitope. These observations may have important implications for understanding how mAb ST40 interacts with CD4. We can speculate that positively charged residues from the CDRs of mAb ST40 could participate in the paratope. The cyclic peptides CM2, CM6, and CM7 from the V_H region and CM9 and CM11 from the V_L domain have been demonstrated to bind strongly to the CDR3-like loop of CD4 domain 1, and Lys-Cys residues added for cyclization/solubilization are not implicated in this binding. 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^{39} belonging to the sequence of peptide CM9 from the CDR1 V_L domain, and Lys^{107} 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^{39} 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 $HIV\text{-}1_{Lai}$ (24). The PDP CM9, derived from region 30–41 of the ST40 CDR2 V_L domain, blocks HIV promoter activity through the inhibition of β-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.

Acknowledgments—The skillful assistance of N. Domergue in the synthesis and purification of peptides is acknowledged. We thank Dr. S. L. Salhi for editorial revision of the manuscript. We also gratefully acknowledge Professor D. Klatzmann and Drs. D. Carrière and O. Schwartz for providing reagents and cells.

REFERENCES

1. Littman, D. R. (1987) *Annu. Rev. Immunol.* **5,** 561–584
2. Kruisbeek, A. M., Mond, J. J., Fowlkes, B. J., Carmen, J. A., Bridges, S., and Longo, D. L. (1985) *J. Exp. Med.* **161,** 1029–1047
3. Lamarre, D., Capon, D. J., Karp, D. R., Gregory, T., Long, E. O., and Sekaly, R. P. (1989) *EMBO J* **8,** 3271–3277
4. Rudd, C. E., Trevilliyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5190–5194
5. Klatzmann, D., Barré-Sinoussi, F., Nugeyre, M. T., Dauguet, C., Vilmer, E., Griscelli, C., Brun-Vezinet, F., Rouzioux, C., Gluckman, J. C., Chermann, J.-C., and Montagnier, L. (1984) *Science* **225,** 59–63
6. Dalgleish, A. G., Beverley, P. C. L., Clapham, P. R., Crawford, D. H., Greaves, M. R., and Weiss, R. A. (1984) *Nature* **312,** 763–767
7. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) *Science* **272,** 872–877
8. Leahy, D. J. (1995) *FASEB J.* **9,** 17–25
9. Wang, J., Yan, Y., Garret, T. P. J., Liu, J., Rodgers, D. W., Garlick, R. L., Tarr, G. E., Husain, Y., Reinherz, E. L., and Harrison, S. C. (1990) *Nature* **348,** 411–418
10. Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X., Xuong, N. H., Axel, R., Sweet, R. W., and Hendrickson, W. A. (1990) *Nature* **348,** 419–426
11. Arthos, J., Deen, K. C., Chaikin, M. A., Fornwald, J. A., Sathe, G., Sattentau, Q. J., Clapham, P. R., Weiss, R. A., McDougal, J. S., Pietropaolo, C., Axel, R., Truneh, A., Maddon, P. J., and Sweet, R. W. (1989) *Cell* **57,** 469–481
12. Clayton, L. K., Sieh, M., Pious, D. A., and Reinherz, E. L. (1989) *Nature* **339,** 548–551
13. Mizukami, T., Fuerst, T. R., Berger, E. A., and Moss, B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9273–9277
14. McDonnell, J. M., Blank, K. J., Rao, P. E., and Jameson, B. A. (1992) *J. Immunol.* **149,** 1626–1631
15. McDonnell, J. M., Varnum, J. M., Mayo, K. H., and Jameson, B. A. (1992) *Immunomethods* **1,** 33–37
16. Jameson, B. A., McDonnell, J. M., Marini, J. C., and Korngold, R. (1994) *Nature* **368,** 744–746

[2] D. Laune, F. Molina, G. Ferrières, J.-C. Mani, P. Cohen, D. Simon, T. Bernardi, M. Piechaczyk, B. Pau, and C. Granier, unpublished data.

17. Zhang, X., Piatier-Tonneau, D., Auffray, C., Murali, R., Mahapatra, A., Zhang, F., Maier, C. C., Saragovi, H., and Greene, M. I. (1996) *Nature Biotechnol.* **14,** 472–475
18. Koch, U., and Korngold, R. (1997) *Blood* **89,** 2880–2890
19. Batinic, D., and Robey, F. A. (1992) *J. Biol. Chem.* **267,** 6664–6671
20. Camerini, D., and Seed, B. (1990) *Cell* **60,** 747–754
21. Kalyanaraman, V. S., Rausch, D. M., Osborne, J., Padgett, M., Hwang, K. M., Lifson, J. D., and Eiden, L. E. (1990) *J. Immunol.* **145,** 4072–4078
22. Lifson, J. D., Hwang, K. M., Nara, P. L., Fraser, B., Padgett, M., Dunlop, N. M., and Eiden, L. E. (1988) *Science* **241,** 712–716
23. Truneh, A., Buck, D., Cassat, D. R., Juszczak, R., Kassis, S., Ryu, S. E., Healey, D., Sweet, R., and Sattentau, Q. (1991) *J. Biol. Chem.* **266,** 5942–5948
24. Benkirane, M., Hirn, M., Carrière, D., and Devaux, C. (1995) *J. Virol.* **69,** 6898–6903
25. Smith, D. H., Byrn, R. A., Marsters, S. A., Gregory, T., Groopman, J. E., and Capon, D. J. (1987) *Science* **238,** 1704–1707
26. Zhang, X., Gaubin, M., Briant, L., Srikantan, V., Murali, R., Saragovi, U., Weiner, D., Devaux, C., Autiero, M., Piatier-Tonneau, D., and Greene, M. I. (1997) *Nature Biotechnol.* **15,** 150–154
27. Nara, P. L., Hwang, K. M., Rausch, D. M., Lifson, J. D., and Eiden, L. E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7139–7143
28. Lasarte, J. J., Sarobe, P., Golvano, J., Prieto, J., Civeira, M. P., Gullon, A., Sarin, P. S., and Borras-Cuestas, F. J. (1994) *J. Acquired Immune Defic. Syndr.* **7,** 129–134
29. Briant, L., Signoret, N., Gaubin, M., Robert-Hebmann, V., Zhang, X., Murali, R., Greene, M. I., Piatier-Tonneau, D., and Devaux, C. (1997) *J. Biol. Chem.* **272,** 19441–19450
30. Langedijk, J. P., Puijk, W. C., van Hoorn, W., and Meloen, R. H. (1993) *J. Biol. Chem.* **268,** 16875–16878
31. Kang, C. Y., Brunck, T. K., Kieber-Emmons, T., Blalock, J. E., and Kohler, H. (1988) *Science* **240,** 1034–1036
32. Taub, R., Gould, R. J., Garsky, V. M., Ciccarone, T. M., Hoxie, J., Friedman, P. A., and Shattil, S. J. (1989) *J. Biol. Chem.* **264,** 259–265
33. Williams, W. V., Moss, D. A., Kieber-Emmons, T., Cohen, J. A., Myers, J. N., Weiner, D. B., and Greene, M. I. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5537–5541
34. Williams, W. V., Kieber-Emmons, T., VonFeldt, J., Greene, M. I., and Weiner, D. B. (1991) *J. Biol. Chem.* **266,** 5182–5190
35. Welling, G. W., Van Gorkum, J., Damhof, R. A., and Wouter Drijfhout, J. (1991) *J. Chromatogr.* **548,** 235–242
36. Igarashi, K., Asai, K., Kaneda, M., Umeda, M., and Inoue, K. (1995) *J. Biochem.* (*Tokyo*) **117,** 452–457
37. Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., III (1994) *J. Biol. Chem.* **269,** 32788–32795
38. Jarrin, A., Andrieux, A., Chaoel, A., Buchou, T., and Marguerie, G. (1994) *FEBS Lett.* **354,** 169–172
39. Levi, M., Sällberg, M., Rudén, U., Herlyn, D., Maruyama, H., Wigzell, H., Marks, J., and Wahren, B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4374–4378
40. Laune, D., Molina, F., Ferrières, G., Mani, J.-C., Cohen, P., Simon, D., Bernardi, T., Piechaczyk, M., Pau, B., and Granier, C. (1997) *J. Biol. Chem.* **272,** 30937–30944
41. Frank, R. (1992) *Tetrahedron* **48,** 9217–9232
42. Idziorek, T., and Klatzmann, D. (1991) *Biochim. Biophys. Acta* **1062,** 39–45
43. Carrière, D., Fontaine, C., Berthier, A. M., Rouquette, A. M., Carayon, P., Laprade, M., Juillard, R., Jansen, A., Paoli, P., Paolucci, F., Gros, P., and Pau, B. (1994) *Clin. Chem.* **40,** 31–37
44. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) *Sequences of Proteins of Immunological Interest,* 5th Ed., U. S. Department of Health and Human Services, Washington, D. C.
45. Molina, F., Laune, D., Gougat, C., Pau, B., and Granier, C. (1996) *Pept. Res.* **9,** 151–155
46. Tam, J. P., Wu, C. R., Liu, W., and Zhang, J. W. (1991) *J. Am. Chem. Soc.* **113,** 6657–6662
47. Habeeb, F. F. (1973) *Anal. Biochem.* **56,** 60–65
48. Karlsson, R., Roos, H., Fägerstam, L., and Persson, B. (1994) *Methods* (*Orlando*) **6,** 99–110
49. Dragic, T., Charneau, P., Clavel, F., and Alizon, M. (1992) *J. Virol.* **66,** 4794–4802
50. Corbeau, P., Devaux, C., Kourilsky, F., and Chermann, J.-C. (1990) *J. Virol.* **64,** 1459–1464
51. Press, J. L., and Giorgetti, C. A. (1993) *J. Immunol.* **151,** 1998–2013
52. Alanen, A., and Weiss, S. (1989) *Eur. J. Immunol.* **19,** 1961–1963
53. Lohman, K. L., Attanassio, R., Buck, D., Carillo, M. A., Allan, J. S., and Kennedy, R. C. (1992) *J. Immunol.* **149,** 3247–3253
54. Foote, J., and Winter, G. (1992) *J. Mol. Biol.* **224,** 487–499
55. Dougall, W. C., Peterson, N. C., and Greene, M. I. (1994) *Trends Biotechnol.* **12,** 372–379
56. Bhat, T. N., Bentley, G., Boulot, G., Greene, M. I., Souchon, H., Schwartz, F., Mariuzza, R., and Poljak, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1089–1093
57. Saragovi, T. N., Fitzpatrick, D., Raktabuhr, A., Nakanishi, H., Kahn, M., and Greene, M. I. (1991) *Science* **253,** 792–795