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CD4(81–92)-BASED PEPTIDE DERIVATIVES

STRUCTURAL REQUIREMENTS FOR BLOCKADE OF HIV INFECTION, BLOCKADE OF HIV-INDUCED SYNCYTIUM FORMATION, AND VIROSTATIC ACTIVITY *IN VITRO*

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Abstract—CD4(81–92) peptides block human immunodeficiency virus (HIV) infection, virus-induced cell fusion, and antigen production by HIV-1-infected cells when derivatized on specific amino acid residues. An extensive series of structural variants of 1,4,5-tribenzyl-10-acetyl-CD4(81–92) were tested as anti-viral agents in an attempt to define the sequence and derivatization requirements for antiviral activity, and to maximize potency and stability for use as potential therapeutic agents. Alteration of the primary amino acid sequence of the stem compound 1,4,5-tribenzyl-CD4(81–92) diminished or abolished in parallel all three indices of anti-viral activity in a series of altered sequence compounds. Replacement of *d*- for *l*-amino acid residues at positions 1, 2, 3, 4, 5, or 6 but not position 10 decreased anti-viral potency, again with parallel effects on infection, synctium formation, and virostatic activity. Omission of the glutamine residue at position 9 did not affect anti-viral potency, while removal of the glutamic acids at positions 11 and 12 resulted in virtually complete loss of biological activity. Changes in the derivatization pattern of the CD4(81–92) peptide backbone also affected anti-viral potency and efficacy. Optimal activity was obtained with benzyl residues at positions 1, 4, and 5, whereas the 1,4,7-tribenzyl-CD4(81–92) compound was without activity in all assays tested. Replacement of one of the benzyl groups with an acetamidomethyl moiety resulted in complete loss of biological activity. The previously reported (Nara *et al.*, *Proc Natl Acad Sci USA* **86**: 7139–7143, 1989) virostatic activity of 1,4,5-tribenzyl-10-acetyl-CD4(81–92) (peptide #18) is apparently due to acetylation, since the des-acetyl stem compound shows much less virostatic activity while still possessing full anti-infective and anti-syncytial activity, and acetylation of the N-terminus rather than the lysine of 1,4,5-tribenzyl-CD4(81–92) yields a virostatic compound equipotent to peptide #18. Cyclization of the tribenzyl peptide to further conformationally restrict the molecule resulted in a compound with anti-infection, anti-syncytial, and virostatic activity at submicromolar concentrations.

The human immunodeficiency virus (HIV) infects lymphocytes, macrophages, and brain microglial cells through an initial attachment to the CD4 surface antigen via the external viral envelope glycoprotein gp120 [1–6]. At least two regions of the CD4 molecule have been implicated in high-affinity binding of gp120 to CD4, amino acids 40–53 and 74–92, which contain the CDR2- and CDR3-like domains of the CD4 molecule, respectively. Studies implicating these regions of CD4 in gp120 binding include *in vitro* site-directed mutagenesis [7–13], antibody neutralization [2, 4], and peptide blocking experiments [14–19].

Benzylated peptide derivatives corresponding to CD4(81–92) block HIV infection, HIV-induced cell fusion, and HIV binding to CD4 at micromolar concentrations [14, 15, 17, 18]. Certain derivatized peptides also demonstrate virostatic activity, or the ability to block viral protein production and cellular infectivity (or transmission) in previously infected cells [14]. We wished to correlate the biological activity of these compounds with respect to three parameters relevant to potential anti-HIV activity, i.e. inhibition of HIV infection, inhibition of HIV-induced cell fusion, and virostatic activity. These studies were done to determine whether systematic structural alterations could yield compounds with improved potency, stability, or specific anti-infective, anti-cytopathic, or virostatic activities.

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METHODS

Peptide synthesis

The peptide derivatives employed in this study were synthesized by standard Fmoc solid-phase methods [14, 15], and their structures and sequences were verified by amino acid analysis and Edman degradative sequencing [20]. All compounds were greater than 90% pure, i.e. migrated as a single symmetrical peak on C18 reversed-phase high pressure liquid chromatography. A cyclic derivative (W3) of the peptide $T_bYIC_bE_bVEDQKEE$ was synthesized using 3,3′,4,4′-benzophenone-tetracarboxylic dianhydride to bridge the amino groups of the amino terminal threonine and the epsilon amino group of the lysine at position 10. This was used without further purification in the biological assays for anti-viral activity described below.

Cell lines

H9 cells, H9 cells chronically infected with HIV-1_{HXB2} [21], VB cells [22], CEM-SS cells [23], and CEM-174 cells [24] were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) as described previously.

Virus stocks

Aliquots of viral stocks were stored at −70°, and thawed only once immediately prior to use. HIV-$1_{HTLV-IIIB}$ and HIV-1_{RF-II} virus stocks were generated from infected H9 cells and titered on CEM-SS cells. Inocula used ranged from 100 to 300 infectious units per well in a 96-well dish seeded with 50,000 CEM-SS cells ([14] and references therein).

$SIV_{SMM-B670}$ virus stocks were generated from infected CEM-174 cells and titered on CEM-174 cells to give a peak of reverse transcriptase activity ($>10^6$ cpm RT activity/mL) 5–7 days after infection of DEAE-dextran-treated cells.

Cell fusion assay: Co-cultivation of chronically HIV-infected cells with uninfected CD4-positive cells

Peptides were tested for their ability to inhibit CD4-dependent, HIV envelope glycoprotein-induced cell fusion in a co-cultivation assay as described previously [15, 25]. Briefly, H9 cells chronically infected with HIV-1_{HXB2} and expressing cell surface HIV envelope glycoproteins were mixed with uninfected CD4-positive VB indicator cells in the presence of various concentrations of peptide. Cultures were scored for the appearance of characteristic syncytia 24 hr after initiation of co-cultivation. The lowest concentration of peptide that resulted in complete prevention of cell fusion was defined as the IC_{100} value for that peptide.

Inhibition of HIV in a syncytial-forming microassay

A syncytial-forming microassay [23, 26] was employed to assess three types of anti-viral activity during distinct kinetic phases of the assay as previously described [14] by evaluation of syncytium formation, p24 production, and cell-associated infectivity following HIV-infection and exposure to peptides. The assay discriminates between effects operative at the level of inhibition of acute viral infection, inhibition of virus-induced cell fusion, and inhibition of viral transmission from infected to uninfected cells.

Assessment of anti-infective activity. CEM-SS cells were plated onto poly-(L)-lysine coated 96-well tissue culture dishes at 50,000 cells/well. Peptides were dissolved in phosphate-buffered saline (PBS) or 140 mM $NaHCO_3$ and diluted with medium (RPMI 1640/10% FBS) to the desired concentrations. Medium alone or peptide solutions were mixed with cell-free virus and added to duplicate wells of cells. HIV-$1_{HTLV-IIIB}$ was used in all experiments except where indicated HIV-1_{RF-II}. Cells were incubated with virus or virus plus peptide for 60 min at 37°; then virus was removed and replaced with the appropriate concentration of peptide in medium, or medium alone. At the end of 48 hr, medium was again removed and replaced with fresh medium. At this time infection is complete but no syncytia have formed [14]. Thus, the presence of test compounds only during the first 48 hr of culture ("infection phase" of assay) allows evaluation for effects on HIV infection.

The number of syncytia per well was counted on an inverted microscope 72 hr later, or 120 hr post-inoculation. After scoring for syncytia, supernatant from each well was harvested for HIV p24 quantitation by a commercial ELISA assay (DuPont, Wilmington, DE). Thus, compounds that block infection decrease syncytia formation and p24 production.

Assessment of anti-syncytial-forming activity. Inoculation of CEM-SS cells was carried out with virus in medium, and after 1 hr at 37° virus was removed and replaced with medium alone. At the end of 48 hr, medium was removed from the wells and replaced with peptide in medium or medium alone. At the end of a further 72 hr (120 hr post-inoculation), syncytia were counted in each well. The presence of test compounds during the last 72 hr of culture ("cell fusion phase" of the assay) allows for evaluation of effects on HIV-induced syncytia formation independent of any effects on viral attachment or the early stages of infection. Culture supernatants were harvested from each well 120 hr post-inoculation for determination of HIV p24 content by ELISA.

Assessment of virostatic activity. An infectious cell center assay and production of p24 antigen were employed for confirmation of anti-infection activity and assessment of virostatic activity, i.e. anti-viral effects of post-infection treatment. After scoring for syncytia in the infection and fusion phases of the primary syncytial-forming microassay, cells were harvested from duplicate wells and washed one time with medium; then approximately 10,000 infected cells were plated onto a monolayer of fresh, uninfected CEM-SS cells in the absence of added peptide. The resulting syncytia in this infectious cell center co-culture were scored after 48 hr, i.e. following fusion of input cells with fresh indicator cells but prior to formation of secondary syncytia from newly infected cells. This assay therefore provides a measure of the cell-associated infectivity of the cells exposed to virus in the initial syncytial-forming microassay, and constitutes the third phase ("infectious cell center" phase) of the modified syncytial-forming microassay. In addition, quantitation of the p24 production and cell-associated infectivity in cultures of cells infected and subsequently treated with anti-viral agent only during the cell fusion stage of the assay (48–120 hr post-infection) gives an index of virostatic activity, or the ability of the anti-viral agent to inhibit progression of viral replication in infected cells.

$SIV_{SMM-B670}$ infection assay

CEM-174 cells were treated with DEAE-Dextran (25 μg/mL) for 30 min at 37°. Cells were washed and resuspended with 200 μL of virus ($SIV_{SMM-B670}$ [27] from titred viral stocks stored in liquid nitrogen) or virus plus peptide at appropriate concentrations. After 1 hr at 37°, medium or medium plus peptide was added to the cells, which were then plated in triplicate into 48-well tissue culture dishes. Culture supernatants were harvested every 3–4 days and

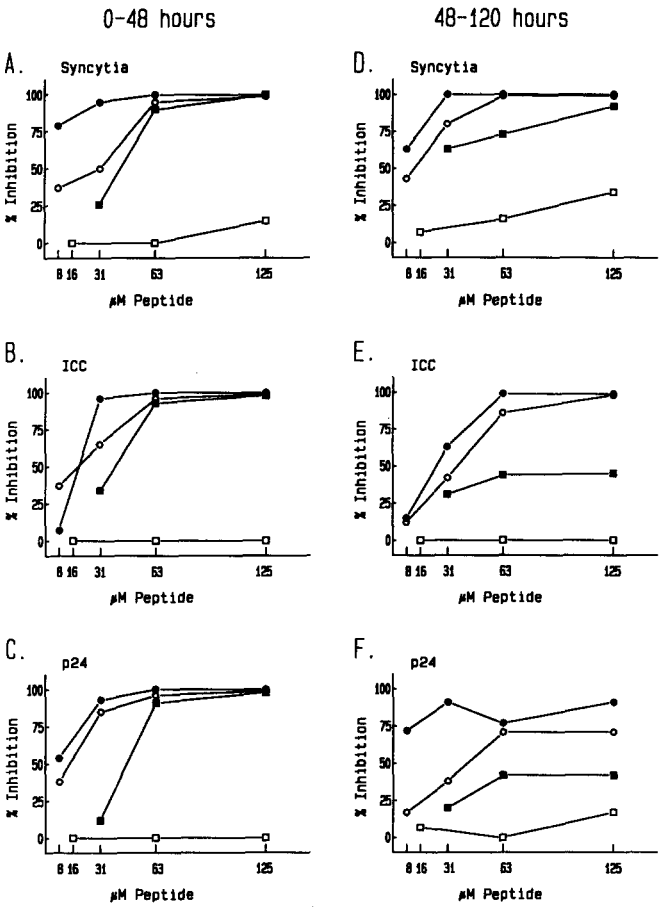

Fig. 1. Concentration–response curves for anti-viral effects of the CD4(81–92) peptides #30, #18, tribenzyl, and an inactive control, in the three phases of the syncytial-forming microassay, along with the associated p24 levels. The syncytial-forming microassay was performed as described in Methods. Data are presented as percent inhibition of the indicated parameters for peptide-treated cells compared to infected cells without peptide. (A) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 0–48 hr of the assay. (B) Number of syncytia in duplicate wells of HIV-infected cells from part A following replating in the infectious cell center assay. (C) Culture supernatant HIV p24 content from duplicate wells of cells from part A. (D) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 48–120 hr of the assay. (E) Number of syncytia in duplicate wells of HIV-infected cells from part D following replating in the infectious cell center assay. (F) Culture supernatant HIV p24 content in duplicate wells of cells from part D. Each graph is an average of 3–6 experiments, with each data point representing an average of the results of 2–6 separate assays. Key: (●) #30; (○) #18; (■) tribenzyl; and (□) #9, an inactive control identical to tribenzyl but benzylated at position 7 instead of 5.

assayed for reverse transcriptase activity as an index of viral replication. Parallel infection of CEM-174 cells with HIV-$1_{HTLV\text{-}IIIB}$ served as a positive control in this assay.

RESULTS

Effects of derivatization of the core peptide $T_1C_4E_5$-tribenzyl-CD4(81–92) on anti-infective, anti-syncytial, and virostatic potency

Figure 1 compares the anti-infection, anti-fusion, and virostatic efficacy of the peptides T_1,C_4,E_5-tribenzyl-CD4(81–92) (tribenzyl), T_1,C_4,E_5-tribenzyl-10-acetyl-CD4(81–92) (#18), and the further derivatized *N*-acetyl-T_1,C_4,E_5-tribenzyl-10-acetyl-CD4(81–92)-amide (#30). The peptides showed similar concentration–response curves in both the infection (Fig. 1, A, B and C) and the fusion phases of the assay (Fig. 1D). The IC_{50} values for the three peptides in the infection assay were 0.5–5 μM for #30, 31 μM for # 18, and 31–63 μM for tribenzyl. The IC_{50} values for the peptides in the fusion assay were 0.5–5 μM for #30, 16 μM for # 18, and 16–31 μM for tribenzyl. Inhibition of p24 production (Fig. 1C) when the peptides were present during the infection phase of the assay closely paralleled the extent of inhibition of syncytium formation (Fig. 1A) with all three peptides across the range of concentrations tested.

The peptides differed more significantly in their virostatic activity, as measured by inhibition of p24 production 120 hr post-infection (Fig. 1F), and in

Table 1. Anti-infection and anti-fusion activity of CD4(81–92) peptides derivatized at various amino acid residues

	Peptide	Infection	Fusion (SFA)	Fusion (co-cultivation)
			IC_{90} (μM)	
(native)	TYICEVEDQKEE	>500	>500	>500*
(MB)	$TYIC_bEVEDQKEE$	>500	>500	>500*
(DB)	$TYIC_bE_bVEDQKEE$	250	>250	125*
Tribenzyl CD4(81–92) series:				
(TB)	$T_bYIC_bE_bVEDQKEE$	50*	125	63*
(21)	$TY_bIC_bE_bVEDQKEE$	12.5–63	100–250	63
(7)	$T_bY_bIC_bEVEDQKEE$	125–250	63–125	63*
(9)	$T_bYIC_bEVE_bDQKEE$	>250*	125–250	>250*
(29)	$T_bYIC_{acm}E_bVEDQKEE$	>250	100–250	>250*
(8)	$T_bYIC_bE_bVEDQK$	>250	>250	>250
#18 CD4(81–92) series:				
(18)	$T_bYIC_bE_bVEDQK_{ac}EE$	5–50*	5–50*	32*
(33)	$T_bYIC_bE_bVEDQK_{cb\ z}EE$	5–50	5–50	
(19)	$T_bY_bIC_{acm}EVEDQK_{ac}EE$	>250	>250	
(34)	$_{ac}T_bYIC_bE_bVEDQKEE\text{-}NH_2$	12.5–25	>12.5	
(32)	$T_bYIC_bE_bVEDQK_{ac}EE\text{-}NH_2$	5–50	5–50	
(K216)	$YIC_bE_bVEDQK_{ac}EE$	>125	>125	>250
#30 CD4(81–92) series:				
(30)	$_{ac}T_bYIC_bE_bVEDQK_{ac}EE\text{-}NH_2$	5–50	5–50	16*
(277)	$_{ac}T_bYIC_bEVE_bDQK_{ac}EE\text{-}NH_2$	>250	>250	
(695)	$_{ac}T_bYIC_bEVEDQK_{ac}EE\text{-}NH_2$	>250	>250	

Specificity of derivatized residues to block infection and fusion is shown as the concentration of peptide (μM) required to give ≥90% inhibition in the indicated assay. Results indicated by an asterisk were published previously [14, 15, 17, 18, 28]. Peptide #30, #18, and tribenzyl were tested in more than 15 experiments; all other peptides were tested in 1–4 experiments.

the infectious cell center assay (Fig. 1E), when the peptides were present only 48–120 hr post-infection. The IC_{50} values for virostatic activity of the peptides were 8–16 μM for #30, 31–63 μM for #18, and >255 μM for tribenzyl.

Effects of derivatization pattern of CD4(81–92) on anti-viral activity

The requirement for a specific derivatization pattern of CD4(81–92) to yield anti-viral activity was studied using a series of peptides in which the core CD4(81–92) peptide was synthesized with derivatization of side chains of different amino acids (Table 1). As described previously, multiple derivatization by benzylation of the CD4(81–92) sequence was required for anti-syncytial activity. This was also the case for anti-infective and virostatic activity. Non-derivatized CD4(81–92) and peptide derivatized only at C4 within the CD4(81–92) sequence were without activity (Table 1, and [15]). Derivatization of the CD4(81–92) peptide with benzyl groups at two residues, C_4,E_5 in the native series (Table 1, peptide DB) and T_1,C_4 in the #30 series (Table 1, peptide 695), resulted in a peptide with minimal potency or no potency, respectively (see also [15]). Within the series of compounds containing three benzyl-derivatized residues, there were also clear restrictions with respect to the derivatization patterns which maintained anti-viral activity.

Some alterations in the pattern of side chain derivatizations of amino acids within the N-terminal region of the peptide resulted in minimal loss of activity, e.g. T_1 versus Y_2 as well as Y_2 versus E_5 (Table 1, peptide TB compared to peptides #21 and #7). Benzylation at E_7 instead of at E_5 essentially eliminated anti-viral activity in both the tribenzyl as well as the #30 peptide series (Table 1, peptide TB compared to peptide #9, and #30 compared to peptide #277). Similarly, substituting an acetamidomethyl group for the benzyl derivatization on C_4 in both the tribenzyl series and the #18 series also abolished anti-viral activity (Table 1, TB compared to #29, and peptide #18 compared to #19), while derivatization of the epsilon amino group of K_{10} with a carboxybenzyl group instead of the acetyl group did not decrease anti-viral potency (peptide #18 compared to #33, Table 1). Truncating the peptide at the C terminus by removing E_{11} and E_{12} in the tribenzyl series (Table 1, peptide #8) or removing the N-terminal threonine in the #18 series (Table 1, peptide K216) eliminated all anti-viral and anti-fusion activity.

Effects of primary sequence alteration of CD4(81–92) on anti-viral activity

Primary amino acid sequence requirements for maintenance of anti-viral activity were examined by testing a series of peptides with scrambled amino acid sequences, in which the residues benzylated (T, C, and E) were constant. Peptides studied included those with unmodified termini (Table 2, tribenzyl series, #16, #17, K29, K30), and those in which the NH_2 and COOH termini had been acetylated and amidated, respectively (Table 2, #30 series, peptides #149, #019, #292, #404). Concentrations of peptide

Table 2. Effect of altered peptide sequence on anti-infection and anti-fusion specificity of CD4-derived peptides

	Peptide	Infection	Fusion (SFA)	Fusion (co-cultivation)
			IC_{90} (μM)	
Tribenzyl CD4(81–92) series:				
(TB)	$T_bYIC_bE_bVEDQKEE$	50*	125	63*
(16)	$T_bYIC_bE_bKVQDEEE$	31–63	>250	63–132*
(17)	$T_bEYEIKC_bQE_bDVE$	125–250	63–250	125–250
(K29)	$T_bEVE_bIKC_bQEDYE$	250	125–250	>250*
(K30)	$KEEIC_bE_bVEDQT_bY$	>250*	>250	>250*
#30 CD4(81–92) series:				
(30)	$_{ac}T_bYIC_bE_bVEDQK_{ac}EE\text{-}NH_2$	5–50	5–50	16*
(149)	$_{ac}T_bYIC_bE_bVEDK_{ac}EE\text{-}NH_2$	5–50	5–50	16
(019)	$_{ac}T_bK_{ac}EEIC_bE_bVEDQY\text{-}NH_2$	>250	>250	>250
(292)	$_{ac}EVE_bIK_{ac}C_bQEDYET_b\text{-}NH_2$	250	250	>250
(404)	$_{ac}K_{ac}EEIC_bE_bVEDQT_bY\text{-}NH_2$	>125	>125	

Altered peptide sequence specificity to inhibit infection or fusion is shown as the concentration of peptide (μM) which gave ≥90% inhibition of the indicated assay. Results indicated by an asterisk were published previously [17, 18]. All peptides were tested in 1–6 experiments.

needed to inhibit either HIV infection or HIV-induced cell fusion were determined (Table 2). Keeping the N-terminal sequence and the T_1,C_4,E_5 derivatization pattern constant, but scrambling the C-terminal 7 amino acids did not decrease significantly the activity of the peptide (Table 2, #16 compared to TB). Switching only the N-terminal T_bY to the C-terminus and the C-terminal KEE to the N-terminus resulted in a completely inactive peptide (Table 2, K30 compared to TB). Other scrambled permutations of the peptide gave decreased potency. Removal of the glutamine at position 10 did not decrease potency of the #30 peptide to block infection or fusion (Table 2, #149 compared to #30), but this peptide had slightly less virostatic activity, reflected in the p24 level following peptide treatment during the fusion phase of the syncytial-forming microassay (data not shown).

d-Amino acid substitutions

Peptides synthesized with *d*-amino acid substitutions, in both the tribenzyl and #30 series, were tested for anti-viral activity in comparison to the reference compounds (Table 3). In the tribenzyl series, replacing the N-terminal *l*-thronine with *d*-threonine did not alter the potency of the peptide. Replacing the tyrosine, isoleucine, cysteine, or glutamic acid with the respective *d*-amino acids moderately decreased potency to inhibit both infection and fusion. In the #30 series, when the *l*-valine was substituted with a *d*-valine both anti-infection and anti-fusion potencies were decreased. When *d*-lysine was substituted for *l*-lysine in peptide #30, no change in potency was apparent. The same substitution made in the tribenzyl series resulted in a compound with increased potency to inhibit infection and fusion, but not virostatic activity (data not shown). The differential effects of optical isomers of single amino acids on the potency of the peptides indicate relatively stringent conformational requirements for effective blockade of HIV infection and HIV-induced cell fusion. Replacement of *d*- for *l*-amino acids, like alterations in the primary amino acid sequence were less well-tolerated in the N-terminal than in the C-terminal region of CD4(81–92).

Cyclization of CD4(81–92)

Modification of the tribenzylated compound by use of a bifunctional derivatizing reagent to bridge the amino groups at the N-terminus and the lysine at position 10 resulted in a cyclized peptide, W3, which showed anti-infection and anti-fusion activity at submicromolar concentrations (Fig. 2). This increased potency was also apparent in the virostatic phase of the assay, reflected in the inhibition of viral transmission by infected cells.

Specificity of peptides for other viral isolates

The CD4(81–92)-derived peptides #18, #30, and W3 blocked infection with the HIV-$1_{RF\text{-}II}$ isolate at concentrations similar to those required for blockade of HIV-$1_{HTLV\text{-}IIIB}$ (Fig. 3). These observations are particularly noteworthy given the extensive sequence divergence between the envelope glycoproteins of the HIV-$1_{HTLV\text{-}IIIB}$ and HIV-$1_{RF\text{-}II}$ isolates [29]. In addition, these peptides were able to block infection of the CD4-positive cell line, CEM-174, by the B670 isolate of SIV_{SMM}, a simian immunodeficiency virus which also uses the CD4 receptor for viral entry but whose viral envelope is quite divergent from that of HIV-1 [30] (Table 4).

DISCUSSION

The core peptide TYICEVEDQKEE corresponding to CD4(81–92) was active to inhibit syncytia in a fusion assay at a potency of 125 μM when derivatized by benzylation at amino acids C_4 and E_5 [14], and at 63 μM when derivatized at T_1, C_4, and E_5 [15] (see Table 1). This increase in activity was reflected in the concentration of peptide needed to inhibit infection of CD4 positive cells as well as HIV gp120 binding of CD4 [17, 18]. Further modification of the T_1,C_4,E_5 peptide (tribenzyl) intended to increase its stability *in vivo* not only increased its anti-viral potency, but also conferred virostatic activity on the peptides. These modifications included acetylation of the lysine at position 10 (peptide #18), and acetylation of the amino terminal threonine as well as the lysine, plus amidation of the C-terminal glutamic acid (peptide #30).

Table 3. Effect of *d*-amino acid substitutions on activity of CD4-derived peptides to block HIV-1_{IIIB} infection and fusion

	Peptide	Infection	Fusion (SFA)	Fusion (co-cultivation)
			IC_{90} (μM)	
Tribenzyl CD4(81–92) series:				
(TB)	$T_bYIC_bE_bVEDQKEE$	50*	125	63*
(24)	$_dT_bYIC_bE_bVEDQKEE$	63–125	125	63
(25)	$T_{bd}YIC_bE_bVEDQKEE$	125–250	125–250	250*
(27)	$T_bY_dIC_bE_bVEDQKEE$	250	125–250	125–250
(D4)	$T_bYI_dC_bE_bVEDQKEE$	>250	63–250	
(D5)	$T_bYIC_{bd}E_bVEDQKEE$	63–250	63–250	
(26)	$T_bYIC_bE_bVEDQ_dKEE$	7.8–63	<25	32
#30 CD4(81–92) series:				
(30)	$_{ac}T_bYIC_bE_bVEDQK_{ac}EE\text{-}NH_2$	5–50	5–50	16*
(971)	$_{ac}T_bYIC_bE_{bd}VEDQK_{ac}EE\text{-}NH_2$	63–125	63–125	250
(31)	$_{ac}T_bYIC_bE_bVEDQ_dK_{ac}EE\text{-}NH_2$	5–50	5–50	16*

Specificity of peptides with *d*-amino acid substitutions is shown as the concentration of peptide (μM) which resulted in ≥90% inhibition of the indicated assay. Results indicated by asterisks have been published previously [17, 18]. All peptides were tested in 2–6 experiments.

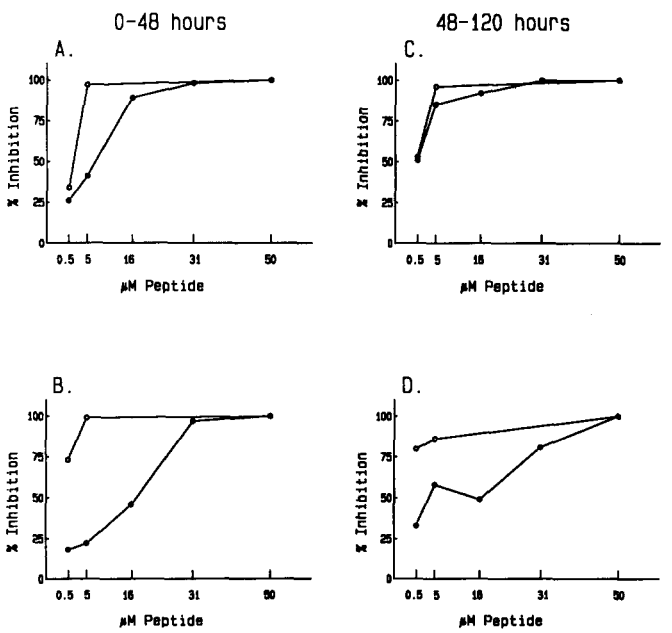

Fig. 2. Comparison of concentration–response curves for anti-viral effects of peptides #30 and W3 in the three phases of the syncytial-forming microassay. The assay was performed as described in Methods. Data are presented as percent inhibition of the indicated parameters for peptide-treated cells compared to infected cells without peptide. (A) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 0–48 hr of the assay. (B) Number of syncytia in duplicate wells of HIV-infected cells from part A following replating in the infectious cell center assay. (C) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 48–120 hr of the assay. (D) Number of syncytia in duplicate wells of HIV-infected cells from part C following replating in the infectious cell center assay. Each graph is an average of 2 experiments, with each data point representing an average of the results of 2–3 separate measurements. Key: (●) #30; (○) W3.

Stringent requirements for sequence specificity, pattern, and type of residue derivatization for anti-infective, anti-syncytial, and virostatic activity suggest that CD4(81–92) peptide conformation is important for anti-viral activity. Sequence and other structural modifications of the peptide in the C-terminus were more readily tolerated than alterations in the N-terminus with respect to potency to inhibit both infection and fusion. However, extending the peptide in the N-terminal direction did not increase the anti-viral potency (Rausch DM, Padgett MP, Lifson JD and Eiden LE, unpublished observations), and truncating the peptide from the C-terminus decreased potency when initially assayed as a crude

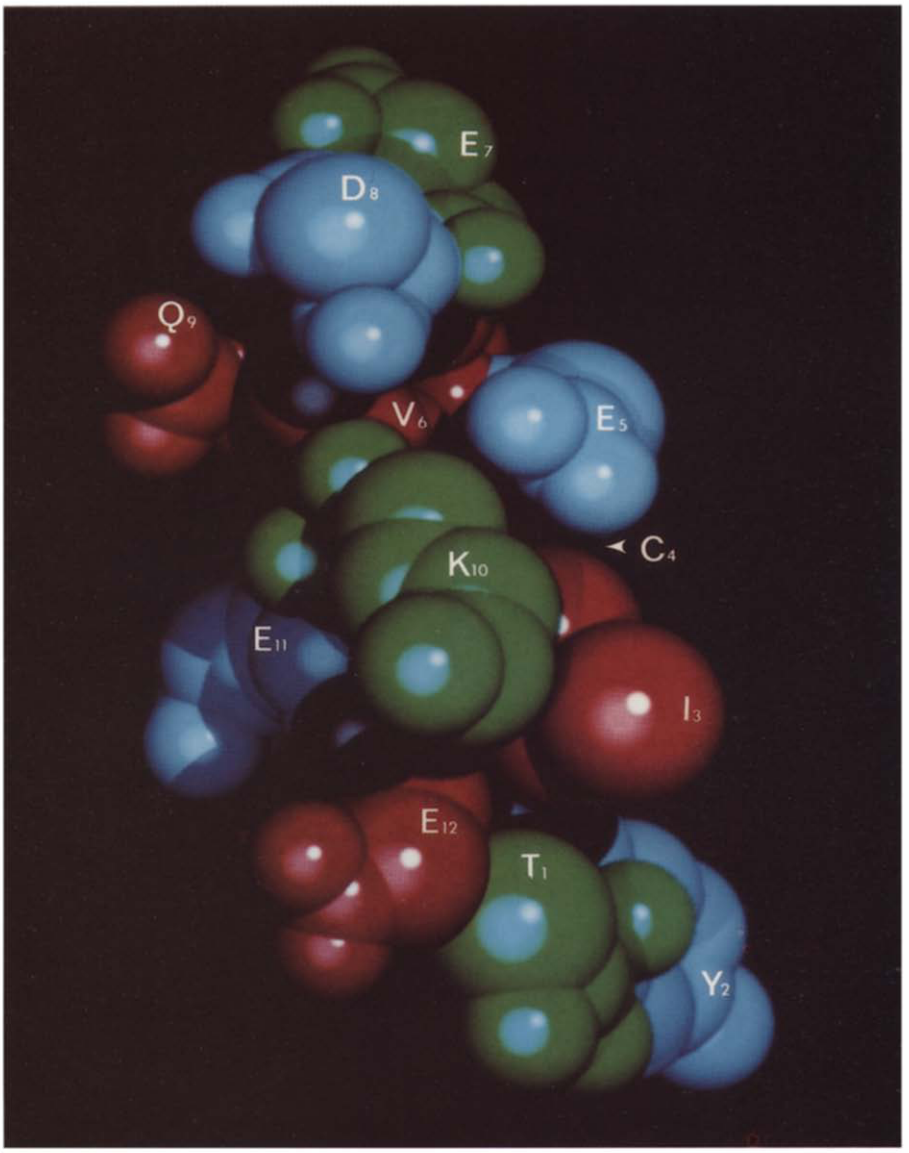

Fig. 4. A three-dimensional view of the CD4(81–92) region of the CD4 molecule based on the X-ray crystal structure reported for soluble CD4. The amino acids are identified in sequence.

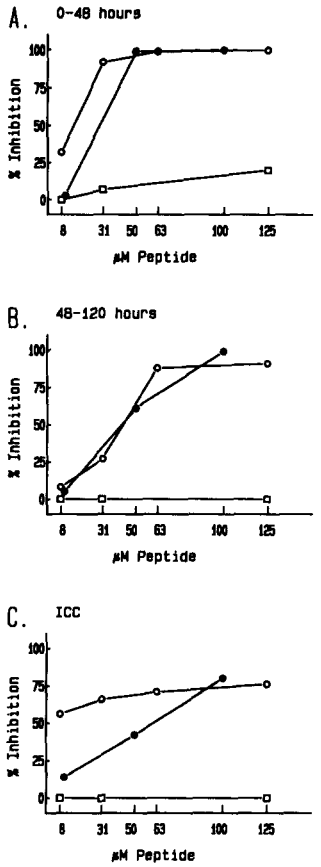

Fig. 3. Concentration–response curves for anti-viral effects in the three phases of the syncytial-forming microassay along with the associated culture supernatant HIV p24 content for HIV-1_{RF-II} infected cultures treated with the CD4(81–92) peptides #18, #30, and an inactive control. The assay was performed according to the protocol described in Methods. Data are presented as percent inhibition of the indicated parameters for peptide-treated cells compared to infected cells without peptide treatment. (A) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 0–48 hr of the assay. (B) Number of syncytia in duplicate wells of HIV-infected cells 120 hr after infection, cultured in the presence of peptide at the indicated dilutions from 48–120 hr of the assay. (C) HIV p24 concentrations in duplicate wells of cells from part B. Each graph is an average of 3–4 experiments, wich each data point representing an average of the results of 2–4 separate assays. Key: (●) #30; (○) #18; and (□) #9, an inactive control.

Table 4. Inhibition of HIV-$1_{HTLV-IIIB}$ and $SIV_{SMM-B670}$ infection of CEM-174 cells by peptides #18, #30, and W3

Peptide	$HTLV_{IIB}$	SIV_{B670}
	Peptide concentration (μM)	
#30	50	100–200
#18	50	250
W3	ND	100

Each value is the concentration of peptide (μM) which completely blocked infection by the indicated virus measured by RT assay of cell culture supernatant 11 or 12 days post-inoculation as described in Methods.

preparation, but eliminated anti-viral activity when the peptide was purified and then assayed [15], suggesting that there is a critical number of amino acids necessary to assume the appropriate structure for anti-viral potency. Side chain benzylation of glutamic acid 7 in CD4(81–92) instead of glutamic acid 5 (corresponding to Glu87 and Glu85, respectively, in the holomolecule) essentially eliminated anti-viral efficacy.

Figure 4 shows a three-dimensional view of the CD4(81–92) region as it resides in the CD4 molecule, based on the X-ray crystal structure reported for a soluble fragment of CD4 containing the V1 and V2 domains [31–33]. This sequence of amino acids forms a hairpin-like loop with a bend at amino acids D8 and Q9. Amino acids T_1, I_3, E_5, E_7, D_8, K_{10} and E_{12} are primarily exposed on one face, and Y_2, C_4, V_6, Q_9 and E_{11} are primarily exposed on the other. Amino acids C_4 and V_6 are not exposed at the surface of the CD4 molecule. The specific constraints on both amino acid sequence and side chain derivatization pattern requirements for anti-viral activity of CD4(81–92) peptides reported here may reflect a requirement for maintenance of the loop structure for interaction with HIV gp120.

Specific side chain derivatization could impose conformational constraints on the peptides favorable for interaction with gp120, and also provide an element of hydrophobic character to stabilize relatively low-affinity binding interactions. Conformational restriction through derivatization may also account for the increased activity of the W3 peptide if cyclization results in a more rigid molecule which preferentially assumes a conformation favoring interaction with gp120 (Fig. 2 and Table 4).

In addition to blocking HIV infection and HIV-induced cell fusion, some CD4(81–92) peptides exhibit a virostatic effect inhibiting the production of p24 in infected cells, and decreasing the cell-associated infectious activity after peptide removal ([14], Figs. 1 and 2). The mechanism for this virostatic activity is not known, but may involve binding of the peptide to gp120 both on the surface and within infected cells resulting in the neutralization of CD4/gp120 interactions required for infection and cell fusion. Acetylation of the lysine at position 10 (position 90 in the holomolecule) significantly increased virostatic potency (panels E and F of Fig. 1). The virostatic activity of peptide #18 previously reported [14], as well as #30 shown here (Figs. 1 and 2), is apparently due to acetylation, since the des-acetyl stem compounds showed much less virostatic activity relative to their anti-infective and anti-syncytial activity. Cellular entry is likely to be necessary for the virostatic effect of CD4(81–92) peptides, since a potent neutralizing anti-CD4 antibody did not show this effect when present in the fusion phase of the syncytial-forming microassay [14]. If so, acetylation may result in a peptide which is more readily taken up by the cell or is more stable within the cell to interfere with gp120/CD4 binding.

Substitution of *d*- for *l*-amino acids increases the *in vivo* stability of synthetic peptides [34, 35]. Therefore, single *d*-amino acids were substituted for *l*-amino acids in both the tribenzyl and the #30 series of CD4(81–92) peptides, and the resulting peptides

tested for their ability to block HIV infection and HIV-induced cell fusion (Table 3). Substitution of *d*- for *l*-amino acids decreased the anti-viral efficacy of these peptides to varying degrees, depending on the residue involved. Similar to the observations with sequence and derivatized residue permuted peptides, *d*-amino acid substitutions in the C-terminus decreased potency to a lesser degree than alterations in the N-terminus. In addition, substituting a *d*-lysine for *l*-lysine at position 10 in the tribenzyl series increased the potency of that peptide to block infection and fusion similar to #18, which has an acetyl group at position 10. This modification, however, did not, increase the virostatic activity of this peptide.

Several studies using mutational analysis of the CD4 molecule have shown that CD4-gp120 binding was decreased when amino acid substitutions were made in the CDR2-like region of CD4 (amino acids 40–53) [8–12, 36]. These data have been interpreted as indicating that the CDR2 region of CD4 is the high-affinity binding site for HIV gp120. Recent data with CD4 peptide fragments conjugated to a carrier protein provide further support for an important role for the CDR2-like region of CD4 in binding interactions with gp120 [37]. However, data obtained with synthetic peptides [14–19, 38] also implicate the CDR3-like domain of CD4 in gp120 binding. Additional data supporting a role for the CDR3-like domain in CD4/gp120 interaction derive from site-directed mutagenesis studies carefully controlled for conformational effects of mutations in the mutated CD4 molecules [13], and blockade of HIV infection and syncytia formation by anti-CD4 antibodies directed to the CDR3-like domain of CD4 [39, 40]. Together, these data are most consistent with a model in which both the CDR2-like and CDR3-like domains of CD4 participate in interactions with HIV envelope glycoproteins. Given the apparent conformational flexibility of both the CD4 molecule and gp120/gp41 HIV envelope glycoprotein complexes [31], and seeming requirement for binding associated conformational changes in the initiation of HIV-induced membrane fusion events [12, 28, 38, 41], multipoint interactions and sequential or processive binding interactions between gp120 and quite widely separated regions of the CD4 surface [32, 33] can be envisaged.

Vaccine approaches based on antibodies directed to the HIV envelope glycoprotein have been complicated by the heterogeneity of HIV envelope sequences and the ability of the virus to mutate, altering the ability of these antibodies to block infection [42]. Therefore, immunization strategies might be better directed toward a specific region of the CD4 molecule that is essential for interaction with the gp120 envelope glycoprotein. The epitopes on the CD4 molecule important in MHC class II function are separable from the putative CDR2-like gp120 binding site on CD4 [36, 43]. However, there is overlap between the gp120-binding site in the CDR2 region of CD4 and sites in the CDR2 region of CD4 necessary for MHC class II recognition and function [36, 44]. CD4(81–92) peptides do not block CD4-dependent immune functions [17], raising the possibility that antibodies reactive with the appropriate epitope within this domain may be effective in blocking HIV infection as well as HIV-induced cell fusion, without interfering with important CD4-mediated immune interactions.

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