Design and characterization of HIV-1 gp120 mimetic peptides
by Alex Charles Johnson

A thesis submitted in partial fulfillment of the requirements for the degree of' Master of Science in Biochemistry
Montana State University

Abstract:
The goal of this research project is to design constrained peptides which mimic the CD4 binding site of HIV-1 gpl20 for use as vaccine antigens. Using the recently elucidated crystal structure of gpl20 in complex with CD4, two peptides were designed using molecular modeling software. The first peptide, Peptide A, is an 18-mer mimicking the gpl20 β20/β21 hairpin loop (from residues 420-437) in which amino acids 420 and 437 have been replaced by cysteine residues and oxidized to form a disulfide bond. The second peptide, Peptide B, is a 27-mer representative of a discontinuous epitope of gpl20 and consists of residues 365-373 and 422-436 joined by three glycine residues. Residues 422 and 436 have been replaced with cysteines and oxidized to form a disulfide bond.

Peptide A was synthesized on an automated peptide synthesizer using standard F-moc chemistry, then N-acetylated, air-oxidized to form the intramolecular disulfide bond, and purified by preparative HPLC. Peptide B was produced by GeneMed Synthesis, Inc. using similar methods. Both peptides were further characterized by MALDI-TOF mass spectrometry, analytical HPLC, and circular dichroism. Peptide A was tested for binding to CD4 via a competitive ELISA assay with gpl20. Peptide B was tested for binding to a CD4-IgG2 chimera via a competitive ELISA assay with gpl20 and tested for HIV neutralization activity using a focal infectivity assay with CD4+ HeLa cells.

It was found that Peptide A had 47% β strand and 48% random coil by CD, indicating that it was likely mimicking the intended gpl20 structure of the β20/β21 hairpin loop. However, it did not bind CD4 in ELISA assays with any greater affinity than an irrelevant peptide. This may be due to insufficient binding interactions between Peptide A and CD4.

Peptide B was found to have 47% β strand and 48% random coil by CD, consistent with the intended portion of gp120's CD4 binding site. This peptide did not inhibit binding of CD4-IgG2 to ELISA plates coated with gpl20, but instead appeared to bind specifically to the D1/D2 domains of CD4-IgG2, resulting in non-specific binding of CD4-IgG2 to the ELISA plate wells, whether or not gpl20 was present. An assay for the HIV-1 p24 protein on HeLa CD4+ cells used in the focal infectivity assay suggested that Peptide B blocked HTV infection at micromolar concentrations.

DESIGN AND CHARACTERIZATION OF HIV-1 GP120 MIMETIC PEPTIDES

by

Alex Charles Johnson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

August 1999

APPROVAL

of a thesis submitted by

Alex Charles Johnson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Martin Teintze _____ (Signature) 7/29/99 Date

Approved for the Department of Chemistry and Biochemistry

Paul Grieco _____ (Signature) 7-27-99 Date

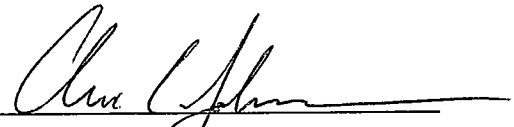
Approved for the College of Graduate Studies

Bruce McLeod _____ (Signature) 7-30-99 Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature 

Date 7/29/99

ACKNOWLEDGMENTS

There are several people to whom I wish to give thanks concerning the completion of this work. First, I would like to thank my advisor, Martin Teintze, for his guidance throughout the project and his unwavering support of my goals. I would also like to thank my other committee members, Ed Dratz and Jean Starkey, for their insightful comments and continual support.

During the course of this project, I have had the courtesy of working with others in the Chemistry and Biochemistry department and the Microbiology department. I would like to thank Steve Arbogast for his help during peptide synthesis, HPLC, and MALDI-TOF; Hua Fang for his guidance through the ELISA techniques; John Mills for his help with HPLC; Tami Peters for taking me under her wing and starting me off in the right direction; and Dave Wooster, Paul Kraft, Brian Bailey, and John Jacobs whose brains I picked endlessly for suggestions and knowledge throughout the project.

Finally, a fond thanks also goes to Dallas and Ann Johnson who have given me a home away from home. I would especially like to thank my wife, Stephanie, for the pillar of support, patience, and encouragement she has given me over the course of this study.

TABLE OF CONTENTS

Page

1. AN INTRODUCTION TO AIDS, HIV, AND VACCINE DEVELOPMENT ... 1

Discovery of AIDS ... 1
Overall HIV Structure and Method of Infection ... 2
Gp120 Structure ... 6
Gp120 Interaction with CD4 ... 8
Vaccine Development ... 10
Statement of Problem ... 12
References Cited ... 14

2. PEPTIDE DESIGN, PURIFICATION, AND ANALYSIS ... 16

Previous Attempts to Create Peptide Vaccines ... 16
Peptide Design ... 21
Circular Dichroism ... 28
Materials and Methods ... 29
Molecular Modeling ... 29
Peptide Synthesis ... 31
MALDI-TOF Mass Spectrometry ... 32
HPLC Purification ... 33
Ellman Assay ... 35
Circular Dichroism ... 35
Results and Discussion ... 36
Peptide A ... 36
Peptide B ... 36
References Cited ... 44

3. PEPTIDE ACTIVITY DETERMINATION BY ELISA AND FOCAL INFECTIVITY ASSAY ... 47

ELISA ... 47
Focal Infectivity Assay ... 48
Materials and Methods ... 49
Peptide A ELISA ... 49
Peptide B ELISA ... 51
Focal Infectivity Assay ... 54

TABLE OF CONTENTS (continued)

Page

Results and Discussion 56
Peptide A 56
Peptide B 57
References Cited 60

APPENDIX – Input File for Discover Generated by Insight 62

LIST OF TABLES

Table Page

1. Acetonitrile gradient used for Peptide A. 34
2. Acetonitrile gradient used for Peptide B 34
3. The binding energies of gp120 and Peptide B to CD4. 41
4. Peptide A ELISA at 90 min. 56
5. Peptide B ELISA #1 at 90 min. 57
6. Peptide B ELISA #2 at 60 min. 58
7. Peptide B p24 Assay 59

LIST OF FIGURES

Figure Page

1. An HIV retrovirus 4
2. The proposed process of infection of a T-cell by HIV 5
3. The amino acid sequence of gp120 7
4. Ribbon diagram of the gp120 core x-ray crystal structure 9
5. Schematic of synthetic, branched, 47-mer peptide 20
6. The crystal structure of gp120's CD4 binding cavity 22
7. Schematic representation of Peptide A 24
8. A molecular modeled structure of Peptide A 25
9. Schematic representation of Peptide B 26
10. A molecular modeled structure of Peptide B bound to CD4 27
11. MALDI-TOF of Peptide A 37
12. CD Spectrum of Peptide A 38
13. MALDI-TOF of Peptide B 42
14. CD Spectrum of Peptide B 43

ABSTRACT

The goal of this research project is to design constrained peptides which mimic the CD4 binding site of HIV-1 gp120 for use as vaccine antigens. Using the recently elucidated crystal structure of gp120 in complex with CD4, two peptides were designed using molecular modeling software. The first peptide, Peptide A, is an 18-mer mimicking the gp120 β20/β21 hairpin loop (from residues 420-437) in which amino acids 420 and 437 have been replaced by cysteine residues and oxidized to form a disulfide bond. The second peptide, Peptide B, is a 27-mer representative of a discontinuous epitope of gp120 and consists of residues 365-373 and 422-436 joined by three glycine residues. Residues 422 and 436 have been replaced with cysteines and oxidized to form a disulfide bond.

Peptide A was synthesized on an automated peptide synthesizer using standard F-moc chemistry, then N-acetylated, air-oxidized to form the intramolecular disulfide bond, and purified by preparative HPLC. Peptide B was produced by GeneMed Synthesis, Inc. using similar methods. Both peptides were further characterized by MALDI-TOF mass spectrometry, analytical HPLC, and circular dichroism. Peptide A was tested for binding to CD4 via a competitive ELISA assay with gp120. Peptide B was tested for binding to a CD4-IgG2 chimera via a competitive ELISA assay with gp120 and tested for HIV neutralization activity using a focal infectivity assay with $CD4^+$ HeLa cells.

It was found that Peptide A had 47% β strand and 48% random coil by CD, indicating that it was likely mimicking the intended gp120 structure of the β20/β21 hairpin loop. However, it did not bind CD4 in ELISA assays with any greater affinity than an irrelevant peptide. This may be due to insufficient binding interactions between Peptide A and CD4.

Peptide B was found to have 47% β strand and 48% random coil by CD, consistent with the intended portion of gp120's CD4 binding site. This peptide did not inhibit binding of CD4-IgG2 to ELISA plates coated with gp120, but instead appeared to bind specifically to the D1/D2 domains of CD4-IgG2, resulting in non-specific binding of CD4-IgG2 to the ELISA plate wells, whether or not gp120 was present. An assay for the HIV-1 p24 protein on HeLa $CD4^+$ cells used in the focal infectivity assay suggested that Peptide B blocked HIV infection at micromolar concentrations.

CHAPTER ONE

AN INTRODUCTION TO AIDS, HIV, AND VACCINE DEVELOPMENT

Discovery of AIDS

In 1981, a rare form of pneumonia named *Pneumocystis carinii* began to appear primarily in the homosexual male population of California. This infection is an oddity in healthy individuals, but patients with immune deficiencies, commonly caused by lymphatic cancers, become susceptible to the disease. In addition to pneumonia, other opportunistic infections caused by various bacteria, viruses, fungi, and protozoa also began to take hold in the same population, and a rare form of skin cancer named *Kaposi's sarcoma* was also concomitant with many of these cases. This set of symptoms became the basis for a new syndrome called *Acquired Immune Deficiency Syndrome* (AIDS), so named for its destructive impact on the immune system which allows the development of the above listed symptoms (1). Humoral and cell-mediated responses to the virus fail to halt progression of the disease. With the exception of a few relatively rare "long term non-progressors," some of which lack proteins integral to the viral infection mechanism(2), to date the mortality rate of AIDS patients without effective treatment is 100%.

Soon after, the infectious pathway for AIDS was found to be blood-linked as well as transmissible by certain bodily fluids, most notably by semen. By 1983, the pathogenic factor was determined to be a previously uncharacterized retrovirus which, after some

debate, was named the *human immunodeficiency virus* (HIV) in 1986 (2).

HIV's complexity and high mutation rate have hindered research efforts towards effective treatment of AIDS, as well as hindered vaccine development. AIDS has since spread into the heterosexual population, mainly through needle sharing of intravenous drug users, and as of 1993, it was thought that 1 of every 250 persons in the United States was infected with HIV (2). Today, there are between 40,000 to 60,000 new cases of HIV infection in the United States each year, and the number of individuals who die from AIDS is 35,000 to 40,000 annually (1). The United States, however, has suffered relatively lightly compared to the remainder of the world. Worldwide, AIDS now ranks as the third leading cause of death after malaria and tuberculosis (1). There are an estimated 13 million HIV positive individuals, and this number is expected to rise to 30 to 100 million HIV infected persons in the first decade of the next century (1, 2). This exponential growth combined with the lethality of the virus make finding a vaccine for HIV critical.

Overall HIV Structure and Method of Infection

There are two major types of HIV: HIV-1 and HIV-2. Although HIV-2 differs from HIV-1 by more than 55% and is actually closer in primary sequence to *simian immunodeficiency virus* (SIV), it also causes AIDS and appears to infect the host by a similar mechanism as HIV-1 (2). Since HIV-1 is responsible for the vast majority of AIDS infected persons worldwide and was used as the template for these peptide studies, it will be the focus of this discussion.

HIV-1 is a member of one of the five major primate lineages of the lentivirus

family of retroviruses (3). It has a cone-shaped core, composed of the viral p24 Gag capsid protein and the p6 Gag nucleocapsid protein, which contains two identical RNA strands closely associated with reverse transcriptase (RT) and the nucleocapsid Gag protein p7 (Figure 1). A myristolated p17 core Gag protein provides structure for the viral matrix. The RNA strands code for *gag* (Gag capsids proteins), *pol* (RT, protease, and integrase), and *env* (gp160) proteins as well as the unique accessory genes *tat*, *rev*, *nef*, *vif*, *vpu*, and *vpr* (2). The surface of the protein consists of a lipid bilayer containing up to 72 knobs of trimers of the envelope glycoprotein (2, 4-6). This envelope structure is derived from gp160, a glycosylated 160-kDa precursor, which is cleaved inside the infected cell's Golgi apparatus into a gp120 external surface envelope protein and a gp41 transmembrane protein (6). The virion gp120, which is located on the virus surface and is held to the transmembrane gp41 by noncovalent interactions, contains the binding sites for cellular receptors and the major antibody neutralizing domains (7).

To initiate a new infection, HIV must encounter a potential target cell that expresses the appropriate proteins (Figure 2). In HIV's case, viral entry begins with the recognition of CD4 on T-cells by the viral gp120 envelope protein (8). CD4 is also expressed, although in smaller quantities, on monocytes, dendritic cells, and brain microglia, and these cells are also subject to HIV-1 infection (6). The CD4 binding site on gp120 is transiently occluded by gp120's V1 and V2 loops (see loop descriptions below) (8). Although this may act to help foil the host's immune system, gp120 can still bind to CD4, since the binding site probably fluctuates between an open and closed conformation (8). Following docking to the CD4 receptor, a conformational change in the viral

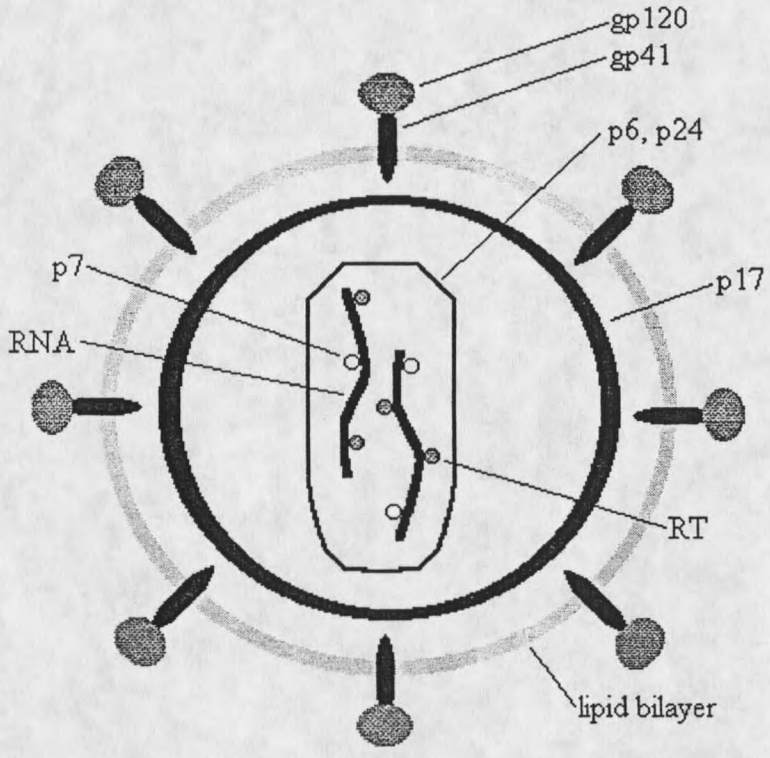


Figure 1. An HIV retrovirus with the structural and other viral proteins identified. Gp120 is shown as a monomer for simplification.

envelope protein's V3 loop allows binding to an obligate second coreceptor, CXCR-4 or CCR-5 (for T-cells and macrophages respectively) (2, 9, 10). Coreceptor binding, in turn, causes a chain of events which allows viral entry into the cell through fusion of the viral membrane with the host cell membrane, a process mediated by gp41, permitting the viral RNA and related machinery to invade the host cell (2, 8). Interestingly, a strain of HIV-2 can fuse efficiently in a CD4-independent, coreceptor-dependent manner (5). This route of entry, however, is quite limited compared with the process mediated by CD4. It is possible that CD4 merely enhances the interaction of gp120 with a coreceptor and this method of viral entry is less efficient in $CD4^-$ cells (2).

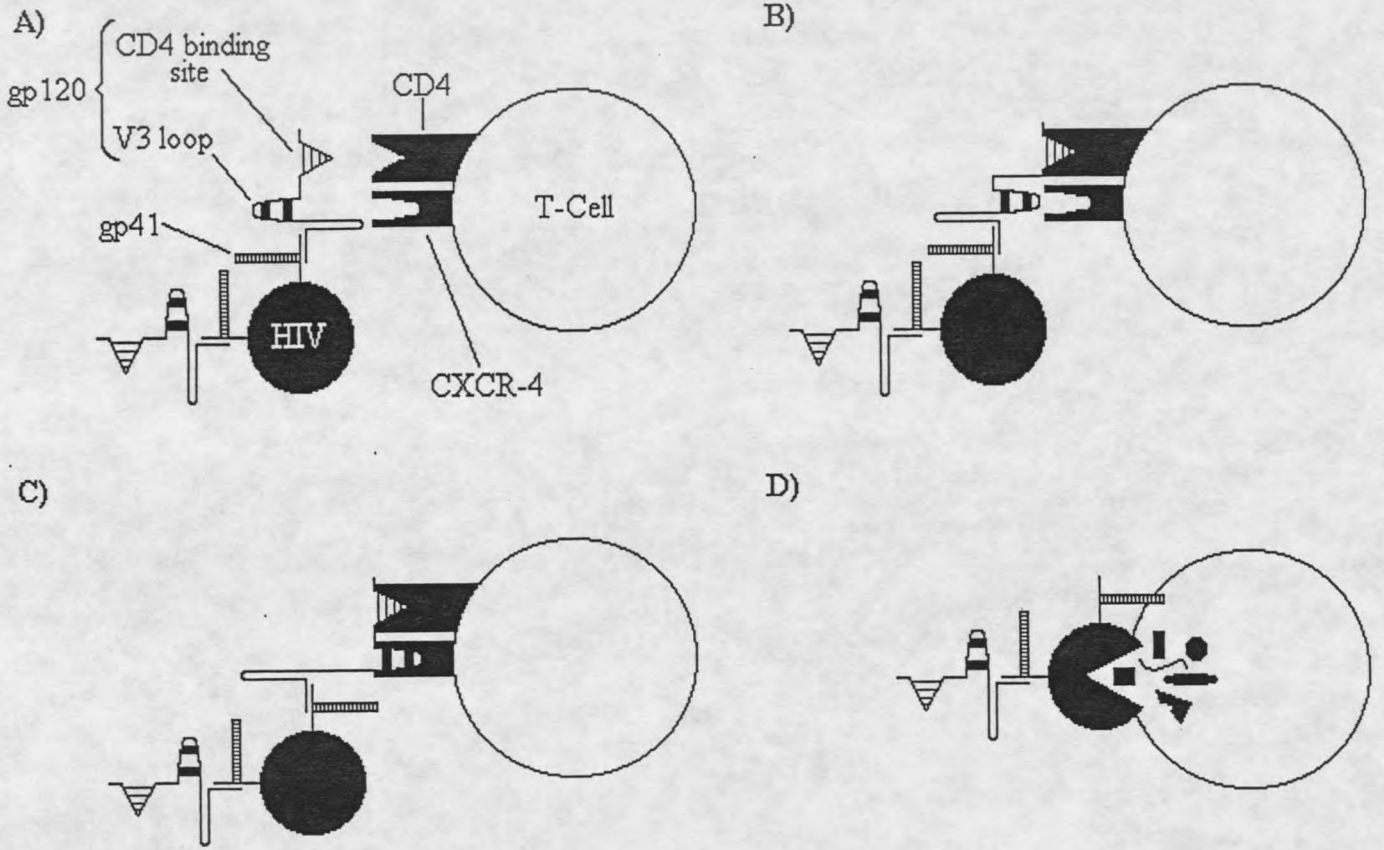


Figure 2. The proposed process of infection of a T-cell by HIV. (A) HIV approaches the cell, and the gp120 protein on the HIV surface interacts with the CD4 receptor on the target cell. (B) Upon gp120 binding to CD4, a conformational change in the viral V3 loop allows binding to the coreceptor CXCR-4. (C, D) Coreceptor binding, in turn, causes a chain of events which allows viral entry into the cell via a gp41 mediated process.

Gp120 Structure

The major protein on the outer surface of HIV-1 is the envelope glycoprotein gp120 (Figure 3). gp120 is a 120-kDa glycoprotein which is non-covalently bound to the viral transmembrane glycoprotein, gp41. There are 20-26 glycosylation sites on gp120 which account for over 50% of gp120's mass. Importantly, the inner domain surface and the receptor binding region are devoid of glycosylation (4). The protein consists of 492 amino acid residues and contains five hypervariable regions (V1-V5) and five constant regions (C1-C5) (6, 8, 9). The five variable regions form loops on the surface of the protein that contain disulfide bonds at their bases, whereas the conserved regions form a central core important for gp41 interaction and interaction with the viral receptors on the target cell (8, 9). Upon initial infection, the V3 region is the major antigenic site. However, due to its high variability (up to 50% difference in this loop alone between HIV isolates) the elicited neutralizing antibody is only specific to one particular strain. Furthermore, variation is manifested at several additional levels: within individuals, between isolates from individuals infected with HIV-1 within a localized geographical area, and between isolates identified in distinct areas of the world (11). Regardless of variability, HIV isolates can be grouped into a small number of classes, or clades, based upon their few conserved residues, and specifically upon a somewhat conserved region within the V3 loop called the crown sequence (11). The major clade found in the United States and Europe is the B clade, from which the crystal structure of gp120 was solved (9, 11). It is important to note that for the crystallization studies, loops V1-V3 were deleted

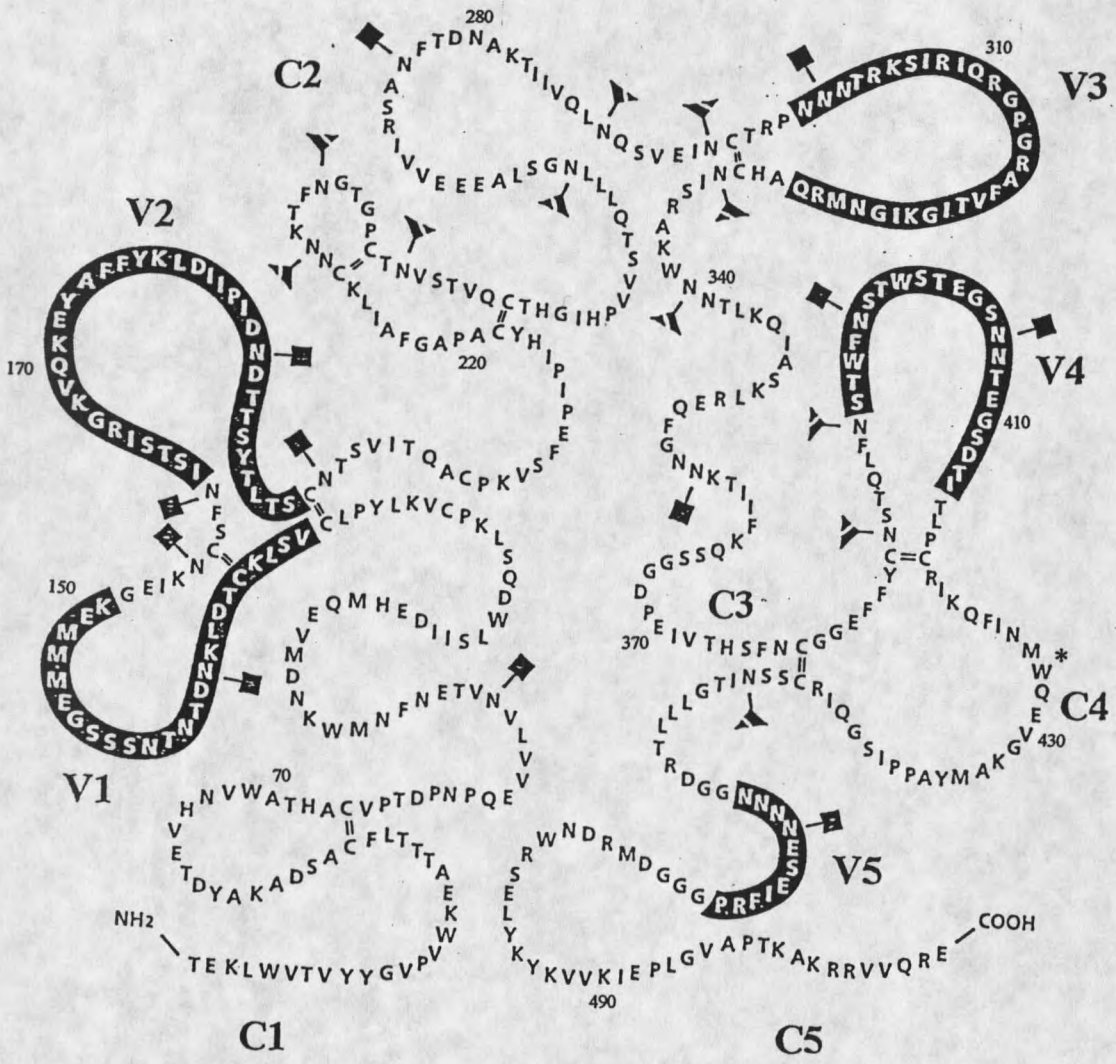


Figure 3. The primary amino acid sequence of gp120. The five hypervariable regions are shown in black (V1-V5) and the five constant regions of the gp120 core are shown in white (C1-C5). Glycosylation sites are shown by black squares (high manose/hybrid-type oligosaccharides) and broken triangles (complex-type oligosaccharides). Naturally occurring disulfide bonds are shown between cysteine residues by a double lines. (Note: this schematic does not include the leader sequence. For reference, Trp427 is indicated by an asterisk.

due to their conformational instability (8, 9); 90% of the attached carbohydrate was also removed (8, 9). Thus, the crystal structure, albeit a major breakthrough in the study of HIV, is not the *exact* structure of native gp120. However, the "core gp120" used for the x-ray structure was still able to bind CD4 with high affinity (8, 9).

Gp120 Interaction with CD4

HIV's first step in gaining access to T-cells is attachment through recognition of CD4 by gp120 (2). The residues on CD4 in contact with gp120 are concentrated in the span from 25 to 64 and are located on a protuberant ridge along one face of CD4's D1 domain. Two key amino acid residues, Phe43 and Arg59, make multiple contacts with residues Asp368, Glu370, and Trp427 in the C4 region of gp120, which are all conserved among primate immunodeficiency viruses (2, 4, 9).

The x-ray crystal structure at 2.5 Å resolution of a HIV-1 gp120 core complexed with a two-domain fragment of the human CD4 and an antigen-binding fragment of a neutralizing antibody revealed much about the CD4 binding surface. A relatively acidic gp120 surface was shown to complement a basic patch on CD4, and two major pockets were shown to be associated with the CD4 binding surface: a hydrophobic cavity (~150 $Å^3$) and a water-filled cavity (~280 $Å^3$) (8, 9). The overall binding surface is ~800 $Å^2$ and is a discontinuous epitope consisting of six segments of gp120: one residue from the V1/V2 stem, loop ζD, the β15-α3 excursion, the β20-β21 hairpin, strand β23, and the β24-α5 connection (Figure 4) (8, 9). Residues within the hydrophobic cavity, which extends roughly 10 Å into the interior of gp120 and is plugged by Phe43 of CD4, are

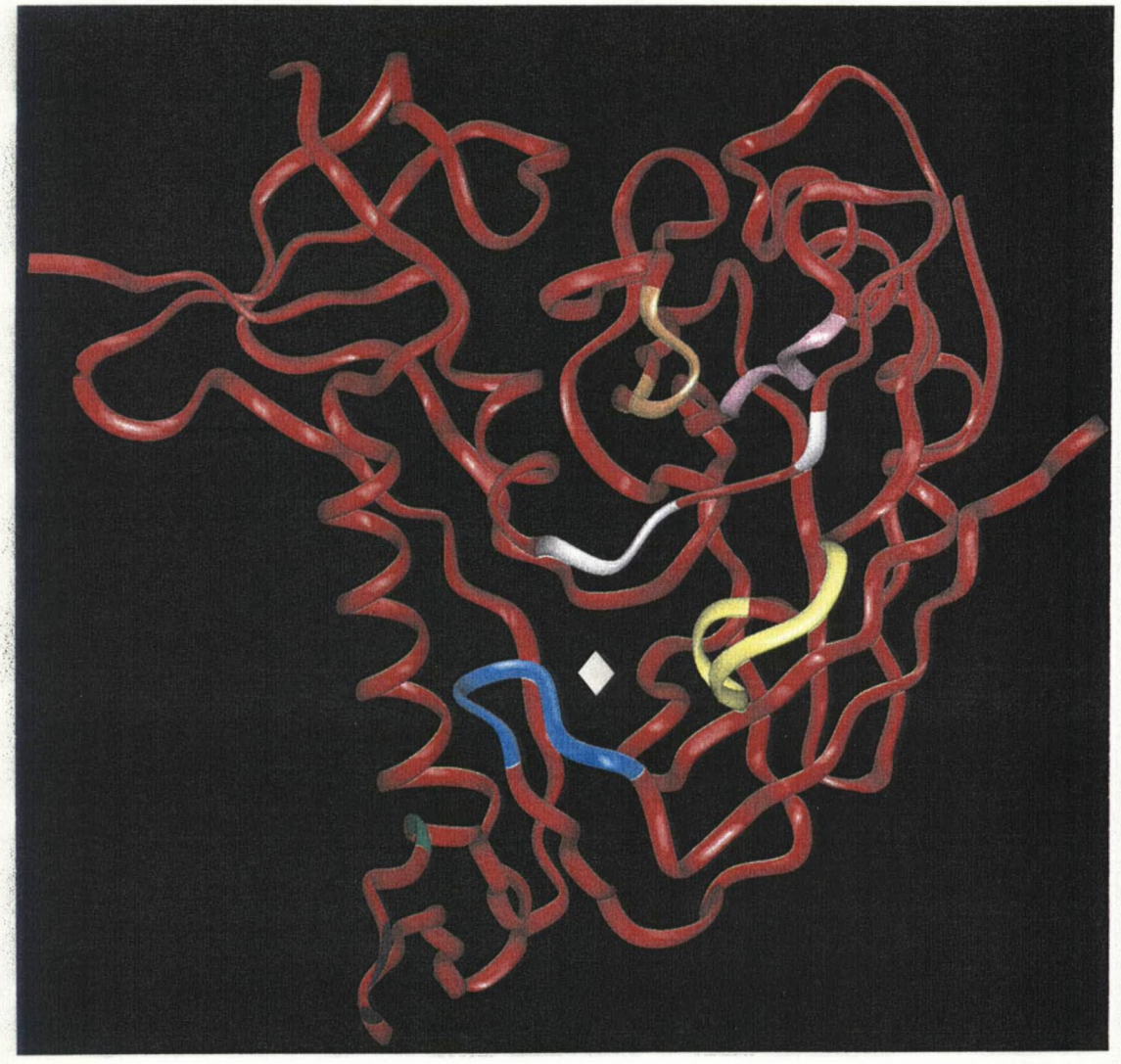


Figure 4. Ribbon diagram of the x-ray crystal structure at 2.5 Å resolution of a HIV-1 gp120 core from the perspective of CD4. The gp120 binding surface is a discontinuous epitope consisting of six segments of gp120 with the important residues highlighted: one residue from the V1/V2 stem (green), loop ζD (orange), the β15-α3 excursion (yellow), the β20-β21 hairpin (light blue), strand β23 (pink), and the β24-α5 connection (white) (9). The hydrophobic cavity is plugged by Phe43 of CD4 at the position of the diamond (4).

highly conserved between gp120 molecules of different clades, permitting gp120 from different strains to recognize the same region of CD4 (8, 6, 9, 11). The recessed nature of this cavity may delay the generation of high-affinity antibodies against CD4 (4).

The water-filled cavity, in contrast to the hydrophobic cavity's predominant contact with a single residue (Phe43 of CD4), is bounded equally by both proteins (4, 8). Although CD4 binding site epitopes are uniformly disrupted by changes in Asp368, Glu370, as well as other key residues of gp120 which surround the opening of the hydrophobic cavity, the water filled cavity has high variability among HIV-1 strains. This variability indicates the water-filled cavity is not directly involved in any sequence-specific connections with CD4. In fact, the only known interactions within the water-filled cavity are backbone-backbone hydrogen bonds between two β-strands (one from gp120, one from CD4) lining the cavity. Therefore, it is likely that variability in the water-filled cavity is tolerated, allowing the generation of escape mutants (i.e. HIV molecules with alternate primary sequences that still bind CD4, but permit immune system evasion) (4, 9).

Vaccine Development

The principal mechanisms that allow HIV to evade the host immune response involve its extensive glycosylation and the use of hypervariable loops which can support significant structural polymorphism while occluding regions critical for viral function (8, 12). Since most carbohydrate moieties appear as self to the immune system, the extensive glycosylation of the viral surface may render the virus less visible to immune surveillance (4). Upon infection, neutralizing antibodies *are* produced which recognize the functional

envelope glycoprotein complex (which is probably a function of gp120/gp41 quaternary structure), recognizing either conserved or variable regions (2, 4, 11). The fact that neutralizing antibodies generated during the course of HIV-1 infection do not provide an effective antiviral response may in part be due to the generation of neutralization escape virus variants and to the general decline in the host immune system associated with pathogenesis (2, 12, 13).

Highly purified viral proteins can be produced relatively inexpensively by expression of viral genes in tumor cell lines, yeast, or insect cells. A recombinant protein made in this manner is used in the successful hepatitis B vaccine (13). Attempts to use gp120 as a vaccine, however, have so far been unsuccessful. This may be due to the fact that most antibodies generated by such immunizations are able to neutralize only a limited number of HIV strains, including the laboratory strains from which they were derived, but are unable to neutralize many primary isolates. Many of the neutralizing antibodies that arise early in HIV infections are directed at the principle neutralizing domain (PND) found in the crown of theV3 loop (amino acids ~308 to 322) and neutralize only a limited number of HIV strains (2, 14, 15). Mutations arising in this region of the sequence are responsible for generating escape variant viruses that are no longer neutralized by such antibodies.

Late in the course of HIV-1 infection, antibodies with broader specificity for different HIV-1 isolates appear (14). Most of these recognize discontinuous epitopes, including the CD4 binding site, and probably block CD4-gp120 interaction (2, 4, 12, 14, 15). Regardless of these secondary antibodies, the HIV-1 virus is still pathogenic. This

may be because the CD4 binding site contains clusters of residues that do not contact CD4 and, as with the V3 loop, are subject to mutation over time (i.e. the water filled cavity) (8). HIV's continued pathogenicity is also may be due to the lack of CD4+ cells present late in the course of infection. Furthermore, free gp120 may not necessarily have exactly the same structure as the gp120/gp41 complex, and antibodies to free gp120 will not always neutralize the virus in its combined gp120/gp41 form (13).

Statement of Problem

Although the current generation of anti-viral drugs represents a major breakthrough in the battle against AIDS, most HIV-1 individuals will never benefit from these therapeutic agents. High cost, poor toleration of the drugs, and the emergence of resistant strains of HIV make these drugs a far cry from the panacea desired by the medical community. What is truly desired is not a form of HIV treatment, but a vaccine which prevents HIV infection.

A successful AIDS vaccine must elicit an immune state that will prevent the establishment of persistent infection following introduction of the virus into the host. Furthermore, if a decrease in viral load were to be achieved by a vaccine, the hope is that transmission would be equally reduced (2). A great challenge to building such a vaccine is HIV's heterogeneity. Currently, vaccines can only protect against homologous strains of HIV. Inoculation with anti-V3 antibodies in chimpanzees affords protection *in vivo* against HIV-1 infection with the strain containing that particular gp120 V3 loop sequence (16). In addition, severe combined immunodeficiency (SCID) mice, which lack

the capability of an immune response, have been used in similar studies. These mice have been surgically grafted with human thymus, lymph, and liver cells, which allows maturation of human B and T cells because of the mouse's lack of immune response(17). These model mice with human immune systems (SCID-hu mice) have also been protected from HIV infection by IgGs raised against the strain used to elicit the immune response (2). Unfortunately, these antibodies are worthless in the face of a rapidly mutating virus.

The rationale put forth is that the most effective antigen for an HIV vaccine with broad strain specificity will be one that elicits antibodies to the conformation-dependent epitopes in the structurally conserved regions of the viral envelope. The CD4 binding site on gp120, which is devoid of carbohydrate, represents a good candidate for such an antigen (4, 9). The crystallized gp120 was from the HXBc2 strain of HIV-1 (9). It has deletions of 52 and 19 residues from the N and C termini, respectively. It was also stripped of loops V1-V3 and over 90% of the surface carbohydrate. Remarkably, its capacity to interact with CD4 and relevant antibodies is preserved at or near wild type levels (9). Using this structure as a template, this project aims at the rational design of synthetic peptide antigens which can elicit broadly specific HIV-neutralizing antibodies.

Linear peptides are flexible and generally have very few conformational constraints in solution. As a result, the peptides spend relatively little time in a conformation capable of a specific binding interaction and therefore have relatively unfavorable entropies of binding and relatively low affinities compared to the proteins from whose sequence they are derived. The object is to design molecules that are constrained to assume the structural features of the bound peptides in the unbound state for use as vaccine antigens.

These constraints, if properly executed, should make the entropy of binding more favorable and greatly increase the affinity. They would also increase the *in vivo* half-life of the peptide by rendering it less susceptible to proteolytic degradation. One way to do this is to introduce cysteine residues at two positions in the peptide chain that are not involved in interaction with gp120, but are close enough to each other in the bound structure that formation of an intramolecular disulfide bond would constrain the peptide in a way that favors the desired conformation. Once such peptides have been synthesized, the long term goal is to identify candidate vaccine antigens that can be tested for their ability to elicit antibodies which neutralize a broad range of HIV strains, like F105 and I5e, (12, 14, 15).

References Cited

1. Fan, H., Conner, R.F., and Villarreal, L.P. (1998) *AIDS, Science and Society, Second Edition*, Jones and Bartlett Publishers, Boston.

2. Levy, J.A. (1998) *HIV and the Pathogenesis of AIDS, Second Edition*, ASM Press, Washington D.C.

3. Emerman, M. and Malim, M.H. (1998) HIV-1 Regulatory/Accessory Genes: Keys to Unraveling Viral and Host Cell Biology, *Science* **280**, 1880-1884.

4. Wyatt, R., Kwong, P.D., Desjardins, E., Sweet, R.W., Robinson, J., Hendrickson, W.A., and Sodroski, J.G. (1998) The Antigenic Structure of the HIV gp120 Envelope Glycoprotein, *Nature* **393**, 705-711.

5. Binley, J., Moore, J.P. (1997) The Viral Mousetrap, *Nature* **387**, 346-348.

6. Wyatt, R. and Sodroski, J. (1998) The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens, *Science* **280**, 1884-1888.

7. Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W., and Sodroski, J. (1990) Identification of Individual Human Immunodeficiency Virus Type 1 gp120 Amino Acids Important for DC4 Receptor Binding, *J. Virology* **64**, 5701-5707.

8. Sattentau, Q.J. (1998) HIV gp120: Double Lock Strategy Foils Host Defenses, *Structure* **6**, 945-949.

9. Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., and Hendrickson, W.A. (1998) Structure of an HIV gp120 Envelope Glycoprotein in Complex With the CD4 Receptor and a Neutralizing Human Antibody, *Nature* **393**, 648-659.

10. Sakaida, H., Hori, T., Yonezawa, A., Sato, A., Isaka, Y., Yoshie, O., Hattori, T., and Uchiyama, T. (1998) T-Tropic Human Immunodeficiency Virus Type 1 (HIV-1)-Derived V3 Loop Peptides Directly Bind to CXCR-4 and Inhibit T-Tropic HIV-1 Infection, *J. Virology* **72**, 976-9770.

11. Moore, J.P., McCutchan, F.E., Poon, S., Mascola, J., Liu, J., Cao, Y., and Ho, D.D. (1994) Exploration of Antigenic Variation in gp120 from Clades A through F of Human Immunodeficiency Virus Type 1 by Using Monoclonal Antibodies, *J. Virology* **68**, 8350-8364.

12. Thali, M., Furman, C., Ho, D.D., Robinson, J., Tilley, S., Pinter, A., and Sodroski, J. (1992) Discontinuous, Conserved Neutralization Epitopes Overlapping the CD4-Binding Region of Human Immunodeficiency Virus Type 1 gp120 Envelope Glycoprotein, *J. Virology* **66**, 5635-5641.

13. Letvin, N.L. (1998) Progress in the Development of an HIV-1 Vaccine, *Science* **280**, 1875-1880.

14. Posner, M.R., Hideshima, T., Cannon, T., Mukherjee, M., Mayer, K.H., and Byrn, R.A. (1991) An IgG Human Monoclonal Antibody That Reacts With HIV-1/gp120, Inhibits Virus Binding to Cells, and Neutralizes Infection, *J. Immunology* **146**, 4325-4332.

15. Ho, D.D., McKeating, J.A., Li, X.L., Moudggil, T., Daar, E.S., Sun, N., and Robinson, J.E. (1991) Conformational Epitope on gp120 Important in CD4 Binding and Human Immunodeficiency Virus Type 1 Neutralization Identified by a Human Monoclonal Antibody, *J. Virology* **65**, 489-493.

16. Emini, E.A., Schleif, W.A., Nunberg, J.H., Conley, A.J., Eda, Y., Tokiyoshi, S., Putney, S.D., Matsushita, S., Cobb, K.E., Jett, C.M., Eichberg, J.W., and Murthy, K.K. (1992) Prevention of HIV-1 Infection in Chimpanzees by gp120 V3 Domain-Specific Monoclonal Antibody, *Nature* **355**, 728-730.

17. McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M., and Weissman, I.L. (1988) The SCID-hu Mouse: Murine Model for the Analysis of Human Hematolymphoid Differentiation and Function, *Science* **241**, 1632-1639.

CHAPTER TWO

PEPTIDE DESIGN, PURIFICATION, AND ANALYSIS

Previous Attempts to Create Peptide Vaccines

There have been several attempts at creating peptide vaccines to HIV-1. The basis behind these attempts has been the elicitation of virus neutralizing antibodies, since antibodies generally are considered an essential component of the repertoire of protective immune responses against virus infections (1). Proteins with similar primary structures, in this case gp120 and peptide mimics, can be expected to show functional or topographic similarities, such as specific epitopes, which may cross-react with antibodies derived from the immunization of animals with other members of the same protein family (2). However, in order to elicit a strong immune response a peptide must theoretically adopt a specific conformation in solution, a requirement which is not easily met (3).

A vaccine trial has demonstrated that a partially protective immune response against SIV can be induced in macaques using a peptide subunit mimicking the principle neutralizing domain (PND) of SIV as an immunogen (4). However, this immune response only defends against the same strain of SIV as the strain from which the peptide was derived (the homologous strain). Other successful passive immunizations with antibodies against the PND of homologous retroviruses have prevented HIV-1, HIV-2, and SIV infections, suggesting that humoral immune response alone is sufficient to prevent

HIV/SIV infection (5). However, these PND antibodies cannot be considered therapeutic, because antibodies to the V3 loop are predominantly type specific and cannot be expected to protect against the heterogeneity of HIV isolates (1).

The best efforts to elicit antibodies against large numbers of heterogeneous strains of HIV-1 to date have focused on conserved sequences. Although phage display combinatorial peptide libraries may overcome some of the limitations imposed by a rapidly mutating virus by using random peptide sequence generation to narrow in on a conserved peptide sequence which can be exploited as a mimetic peptide, the failure of such libraries to produce a consensus sequence for the CD4 binding site on gp120 indicates that the need still exists for synthetic peptides which can induce antibodies that efficiently cross-react with viral components (2). To this end, several techniques have been applied to peptide design: free peptides, disulfide-constrained peptides, and peptides representing discontinuous epitopes.

In theory, free mimetic peptides can fold into the proper three dimensional structure and elicit an immune response towards the native protein. Based on this assumption, a free peptide containing part of the conserved C4 region has been synthesized, consisting of residues 378-385 disulfide bonded to residues 418-445 by terminal cysteine residues at both ends of each sequence (1). Although this structure included residues important for CD4 binding (418-445), it is now known that residues 378-385 are not required for proper tertiary structure and, in fact, probably impeded the proper folding of this mimetic (1). Regardless, this peptide elicited antibodies recognizing gp120, but not of enough quantity or affinity to neutralize HIV-1 infectivity (1). A related

unconstrained peptide of the C4 region, ranging from residues 414-434 of the C4 domain, did not mimic gp120 and did not make cross-reactive antibodies (2). Failure in both cases can probably be ascribed to insufficient mimicry.

Disulfide-constrained circularized peptides have mostly focused on HIV-2. The amino acid sequence of HIV-2 is related more closely to SIVmac, a virus causing an AIDS-like disease in macaque monkeys, than to HIV-1 and is also less pathogenic (5). However, HIV-2 and HIV-1 share structural, genetic, and biological properties, and are believed to cause $CD4^+$ cell depletion by similar mechanisms (6). Linear and cyclic peptides of gp125 (HIV-2's analog of the gp120 envelope protein) have elicited antibodies against native gp125. In this regard, anti-peptide antibodies are known to often exhibit low binding reactivity to the native protein (6). This is usually explained by assuming that short peptides in solution assume a variety of random conformations and that only the rare conformations which approximate that found in the native protein will produce antibodies which bind the native protein (6). Circularization with disulfide bridges mimicking HIV's own disulfide bridges, therefore, enhances the rate of success for elicitation of antibodies that recognize the native protein because a larger fraction of its conformations will approximate that found in the native protein (6). In keeping with this theory, disulfide cyclization significantly increased the level of anti-peptide antibodies reacting with the intact antigen HIV-2 protein (6). Importantly, a cyclic peptide from HIV-2 containing parts of the C4 region elicited antibodies against gp125 (6). Unfortunately, none of the peptides in these trials induced virus-neutralizing antibodies. It is plausible that the failure of synthetic gp125 peptides to elicit virus-neutralizing antibodies could be in part due to

insufficient mimicry and therefore insufficient affinity, since none of the peptides designed took into account the discontinuous epitope of the gp125's CD4 binding site.

The greatest success to date has been with peptides designed to mimic discontinuous epitopes of the C4 domain. The five amino acids thought to be most important for the high-affinity binding of gp120 to CD4 are Thr257 (C2 region), Asp368 and Glu370 (C3 region), and Trp427 and Asp457 (C4 region) (7). A rationally designed synthetic discontinuous peptide epitope of the gp120 CD4 binding site containing residues 363-378 with a disulfide linkage to residues 445-459 has been shown to induce antibodies that cross-react with the native protein from which it was derived (8). It is thought that this peptide can adopt a stable conformation which binds to CD4 and thus represents part of gp120's CD4 binding site. Although this trial was verified by a competitive ELISA assay, the affinity was extremely low (10^5-fold lower than gp120) (8). This poor affinity is most likely due to the peptide containing only part of the CD4 binding site as well as to the peptide's lack of cyclization.

In an effort to include more potential binding residues, the authors then attached an additional arm to the peptide (7). The resultant 44-mer three-armed peptide contained four of the five residues needed for CD4 binding as opposed to the previous peptide's three (Figure 5). It is known that cross-linking of cell surface CD4 by the interaction between HIV-1 gp120 and anti-gp120 antibodies with $CD4^+$ T-cells induces apoptosis both in vitro and in vivo; this 44-mer competitively inhibited this gp120-induced T-lymphocyte apoptosis (7). Additionally, polyclonal sera raised to the peptide in mice indicated that the peptide contains epitopes specifically recognized by B- and T-

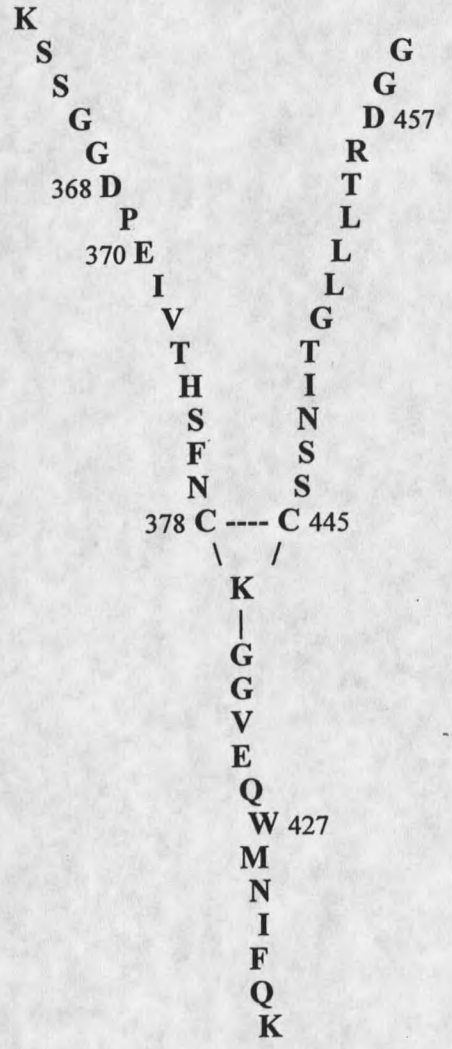


Figure 5. Schematic representation of a synthetic, branched, 47-mer peptide. Asp368, Glu370, Trp427, and Asp457 are critical for CD4 binding.

lymphocytes, since purified IgG antibodies from these mice recognized this peptide with a greater affinity than its lower affinity precursor (7). This same peptide also inhibited the binding to CD4 of Q4120, a monoclonal antibody which binds the D1 domain of CD4 and inhibits gp120 binding (9). It is likely that this peptide somewhat mimics the structure and function of gp120 (7). However, the peptide is only 12.6% as efficient in binding CD4 than recombinant gp120 (9). Since the percentage of peptide molecules folded in any one

particular configuration will be relatively small, this is not surprising, especially considering the lack of any inherent secondary structure constraints like a disulfide bond (9).

Peptide Design

Regardless of the above successes, no peptide has yet elicited neutralizing antibodies to heterogeneous strains of HIV-1; therefore, the need for rational peptide design still exists. In building on results of previous AIDS peptide vaccine research, peptide designs should incorporate the discontinuous CD4 binding site and should be cyclized by disulfide bonds or some other method to restrict conformational fluidity. These constraints, if properly executed, should make the entropy of binding more favorable and greatly increase the affinity as well as the *in vivo* half-life of the peptide. Unfortunately, there exists very little information as to how primary sequence variation affects the tertiary structure of gp120 or its antigenicity (10). Nevertheless, by examining the 3D crystal structure of gp120 bound to CD4, examining mutagenic studies, and conducting computer modeling trials, a reasonable approximation of the final binding energy of a peptide may be obtained.

Certain residues play a key role in the CD4 binding cavity. The spans of 365-371 and 425-430 of gp120 contribute 57% of the predicted total binding energy to CD4 based on analysis of the x-ray crystal structure (11). Residues 365-371 are part of the β15 strand which forms an antiparallel β-sheet with C" of CD4 (11). Residues 425-430 form part of the β20-β21 loop (Figure 6). Both of these spans make direct contact with CD4's

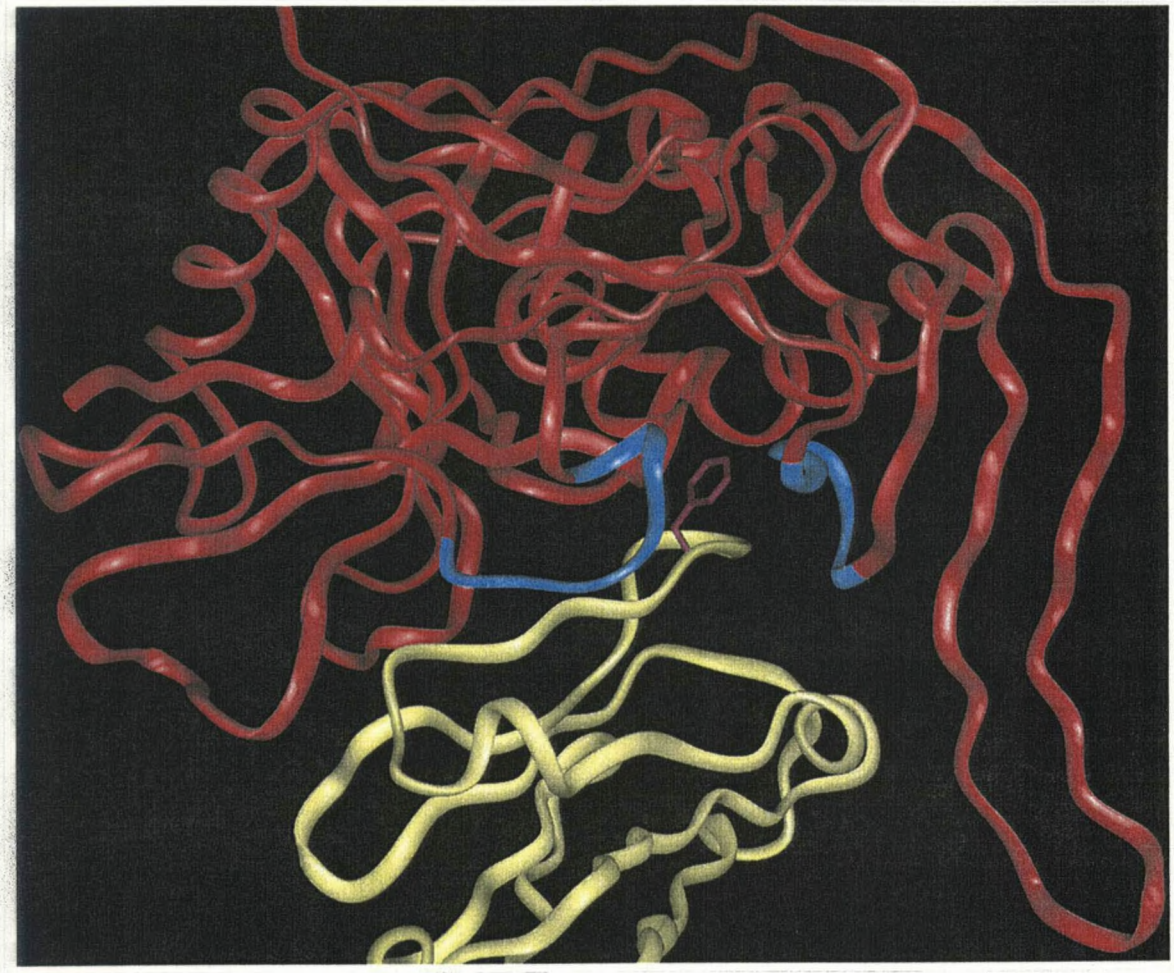


Figure 6. The crystal structure of gp120's CD4 binding cavity (gp120 is shown in red; CD4 yellow). The β15 strand and β20-β21 loop (blue) contribute to 57% of the predicted total CD4 binding energy. The β15 strand at left forms an antiparallel β-sheet with C" of CD4. Both of these gp120 spans make direct contact with CD4's Phe43 (violet).

Phe43. Phe43 specifically interacts with residues Asp368, Glu370, Ile371, Asn425, Met426, Trp427, and Gly473. Contacts to residues 425-427 and 473 involve the backbone, whereas the Ile371 sidechain contributes a hydrophobic interaction (11). The residues that line the Phe43 cavity are primarily hydrophobic and highly conserved (Trp112, Val255, Thr257, Glu370, Phe382, Tyr384, Trp427, and Met475) (11).

Mutagenic studies of Trp427 have shown that a single substitution at this site can abrogate CD4 binding and infection (12, 13, 14). This mutation either directly eliminates an important part of the CD4 binding site on gp120 or causes local conformational changes that interfere with CD4 recognition (12). Since this single amino acid change has such a tremendous impact on CD4 binding, it was decided to attempt construction of a disulfide-constrained peptide centered on this amino acid. The crystal structure of gp120 shows Trp427 to be included in the β20-β21 sheet, and this sheet represents nearly one half of the hydrophobic pocket for Phe43 of CD4. This strong involvement of the β20-β21 sheet in the binding pocket was considered sufficiently important to design a β20-β21 sheet peptide which included Trp427 (Peptide A). Peptide A was designed to maximize structural features as found in the β20-β21 region of the crystal structure. These included the general fold, proper orientation of the Trp427 residue, and retention of four known hydrogen bonds in the β20-β21 loop. To restrict conformational entropy in solution and to promote this proper tertiary structure formation, Cys residues were placed at the ends of the peptide and oxidized to form a disulfide linkage. Various residues were examined for appropriate Cys replacement by computer modeling as described in the "Materials and Methods" section. Using this method to derive a peptide in solution which most closely

resembled the gp120 β20-β21 sheet crystal structure resulted in a final sequence of an 18-mer, CKQIINMWQKVGKAMYAC, representing residues 421-436 of gp120 from the HXBc2 HIV-1 variant (Figures 7 & 8).

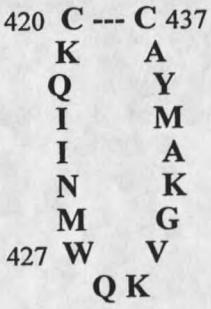


Figure 7. Schematic representation of Peptide A. Trp427 is critical for CD4 binding.

A second peptide (Peptide B) was designed to incorporate a more thorough representation of the binding site. It was noted that Asp368 and Glu370 mutations cause significant reduction in CD4 binding and that Asp368 and Glu370 are invariant in all HIV (13). Asp368 of gp120 also forms an important salt bridge with Arg59 of CD4 (15). Furthermore, the CD4 region important for gp120 binding contains several basic residues that might require neutralization by acidic amino acids on gp120 to stabilize the gp120-CD4 interaction (13). Residues 365-371, which contain Asp368 and Glu370, are likely to be important for this neutralization. The crystal structure of the gp120-CD4 complex was accomplished on the HXBc2 strain of virus, which actually has a sequence that seems to interfere with this acid/base stabilization by having two positively charged Lys residues in the β20-β21 sheet. This was confirmed through computer modeling which indicated that the binding affinity of peptide mimetics would be increased by substitution of these

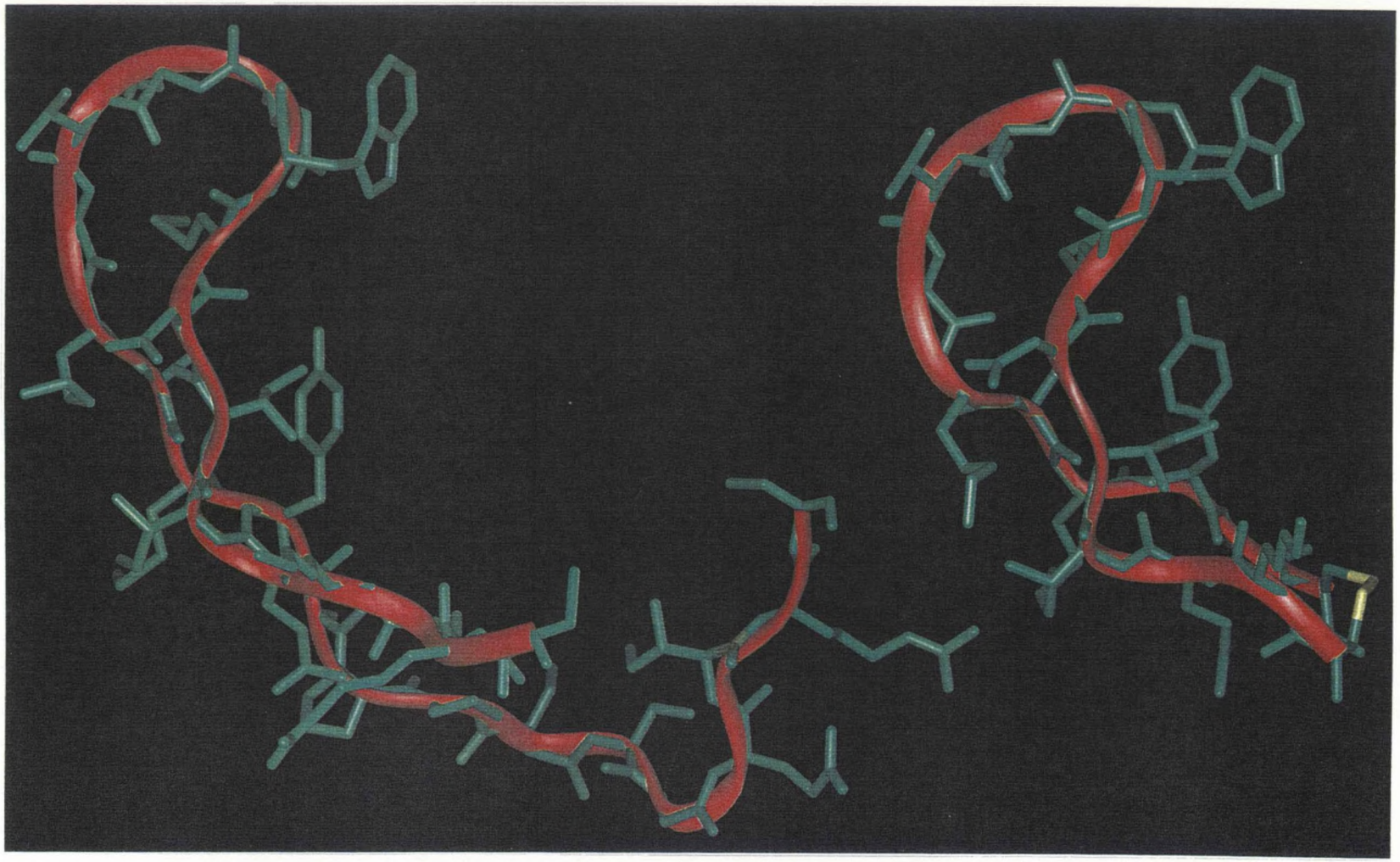


Figure 8. The simulated structure of Peptide A is seen at right. For comparison, the simulated structure of the β20-β21 sheet as seen in the crystal structure is shown at left. Peptide A's disulfide bond is shown in yellow.

positive residues with neutrally or negatively charged residues. Based on the importance of this data and the importance of the β20-β21 sheet, Peptide B was designed to compensate for a potentially poor electrostatic interaction with CD4 by substituting Glu for Lys429 and Gln for Lys432. Glu was considered an appropriate substitution for Lys429, since Lys appears in this position in only 21 of 214 strains examined whereas Glu appears in 84 (16). Furthermore, Glu429 is the predominate amino acid in the B clade of HIV-1. Although Lys432 is predominantly in the B clade, Gln was considered an appropriate substitution for this residue, since it is found frequently in other clades (36 of 214 strains) (16). Asp368 and Glu370 were incorporated as part of the β15 strand, which was joined to the β20-β21 region by a three-Gly linkage, allowing enough flexibility for the two regions of the peptide to fold into conformations similar to the native state. The sequence of the peptide was the 27-mer SGGDPEIVTGGGCIINMWQKVGKAMYC, representing residues 365-373 and 423-435 of gp120. In keeping with the constrained peptide requirements, the β20-β21 sheet was stabilized with a disulfide bond similar to the first peptide with Cys substitutions for residues 422 and 436 (Figures 9 & 10).

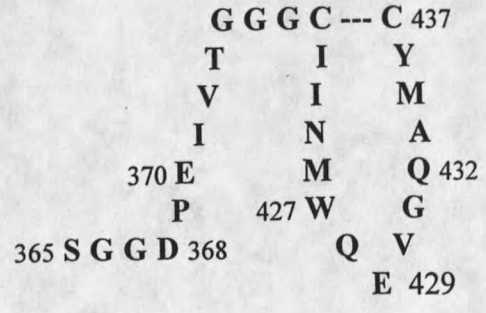


Figure 9. Schematic representation of Peptide B. Asp368, Glu370, and Trp427 are critical for CD4 binding. Residue substitutions K429E and K432Q are shown.

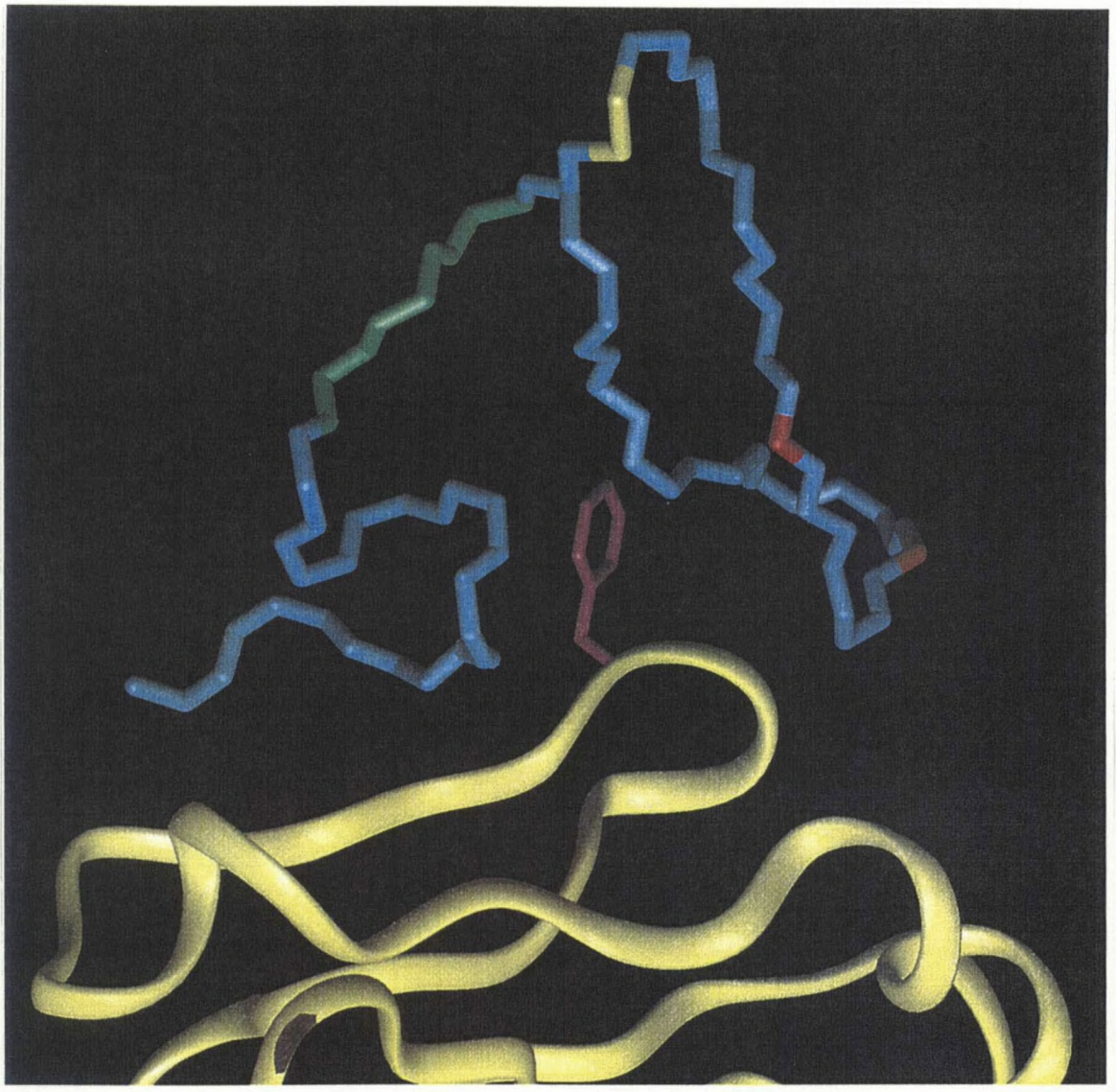


Figure 10. A simulation of Peptide B bound to CD4 (Peptide B is shown in blue; CD4 yellow). For clarity, only the backbone structure of Peptide B is shown. Peptide B's disulfide linkage is shown in yellow, the K429E and K432N substitutions are shown in red, and the three-Gly linkage is shown in green. CD4's Phe43 is shown in violet.

Circular Dichroism

Circular dichroism (CD) is an excellent method for determining the secondary structure of polypeptides in solution. CD is a phenomenon that results when the L-amino acids in peptides absorb left- and right-circularly polarized light differently (17, 18). This absorbance is very sensitive to the conformation of the amide peptide backbone in the far-UV region (below 250nm) and can be used to determine the amount of α-helix, β-sheet, and random coil in the sample. Consequently, an estimation of the relative amounts of each secondary structure within a peptide can be obtained. However, the agreement between circular dichroism (CD) spectra and secondary structure analysis can be affected by side chains with aromatic rings. Caution must therefore be used in interpreting these spectra.

Useful estimates of protein conformation can be obtained with data from between 200nm and 240nm, which allows proteins to be examined in physiological buffers (18). The data points received can be fed into various programs for analysis. The program used in this study, K2D, takes advantage of neural network analysis, which detects patterns and correlations in data based upon CD data of 18 different proteins of known crystal structure. The K2D program gives a correlation coefficient of 0.95±0.09 for α-helix and 0.77±0.10 for β-sheet when compared to these crystal structures (18). Although the β-sheet correlation coefficient may appear low, K2D still gives the best estimates of β-sheet when only data with a limited wavelength are available and works well with polypeptides (18).

Materials and Methods

Molecular Modeling

Molecular modeling was performed using Molecular Simulations Inc. Insight II, Release 97.0. Starting with the crystal structure of gp120 obtained from the Protein Data Bank (accession code 1gc1), peptides were designed according to the criteria stated above by deletion of unnecessary residues and addition of the appropriate amino acids and bonds. Peptides were then run through an algorithm which minimized their conformational energy while bound to CD4, and then their binding affinity to CD4 was determined (see Appendix). Unfortunately, the hydrophobic effect is not easily simulated because the entropy of the water molecules around the peptide-protein complex is not explicitly paramaterized in the Insight II program (19, 20). Therefore, the entropy of binding cannot be determined accurately in these modeling calculations, which were done in a vacuum with a dielectric constant of 1.00 to simulate the water. Regardless, the remaining interaction calculations allow comparison of relative binding energies of similar peptides and serve as a good guide for model analysis. Each peptide was positioned close to CD4 at the approximate position of analogous residues in the gp120–CD4 crystal structure. Two algorithms were then run on each peptide using Insight II's Discover program: 300 iterations using a steepest descent algorithm and two times 1000 iterations using a conjugate descent algorithm.

The general minimization process is done in two steps (21). First, an equation or "target function" describing the energy of the system, in this case a peptide adjacent to

CD4, as a function of its coordinates must be defined and evaluated for a given conformation. Second, the conformation of the given structure is changed to reduce the value of the target function. By successive iterations of these two steps, a minimum energy conformation can be found. This may take only one step, or it could take thousands.

In the simplest model, the steepest descent approach defines a target function and then searches linearly along a vector for a lower conformational energy along a downhill gradient towards the energy minimum. This linear search takes approximately 8 target function evaluations and consistently overshoots the minimum energy along the given vector. The search is then redirected perpendicularly towards the theoretical energy minimum along the downhill gradient and this process is repeated until the lowest minimum is met. The steepest descent approach is highly efficient for systems with large initial gradients where the configurations are far from minimum. However, at energies close to the actual minimum, the efficiency of this method plummets, because perpendicular redirection of a linear search tends to reverse progress made in the pervious iteration during the overshoot. At this point, the conjugate descent approach is applied.

The conjugate method is advantageous at more defined minimization energies, because it takes into account the overshooting of steepest descent gradients. This means using an algorithm that uses a complete basis set of mutually conjugate directions so that each step redefines the direction toward the minimum. Thus instead of directing the subsequent linear search orthogonally to the previous vector, the subsequent search is directed more towards the energy minimum. This can only be done by performing a

number of function evaluations at the end of each linear search, thus consuming considerable amounts of time in comparison to the steepest descent approach. However, the resultant minimum energy is more accurate than the steepest descent. Once minimized, binding energies were computed using Insight97's docking module, which measures the interaction energy between two molecules by summing the energy contributions between all atoms of the two molecules. These binding energies were compared for each theoretical peptide and its individual amino acids to determine the best design.

Peptide Synthesis

Peptide A was synthesized using a MilliGen Model 9050 Plus continuous flow automated peptide synthesizer using Fmoc protected amino acids (Advanced ChemTech). Each residue was double-coupled using 0-(7-azabenzotriazol-1-yl)-l, 1, 3, 3-tetramethyluronium hexafluorophosphate (HATU – PerSeptive Biosystems). Pepsyn-KB resin (MilliGen/Bioresearch) was used to initiate the synthesis. N-terminal acetylation was performed on half of the peptide (the remainder was frozen for later use) while still attached to the resin by stirring for ~2hrs. at room temp with a solution of 0.5g N–acetyl-imidazole in 10ml N,N-dimethylformamide (DMF). N-acetylated peptide resin was collected by vacuum filtration, rinsing once with 10ml dichloromethane (DCM)/55mg 4-dimethylamino/pyridine (DMAP), and lyophilizing the remaining solid overnight. The completed peptide was then cleaved from the resin using a 95% trifluoroacetic acid (TFA)/3% thioanisole/2% ethane dithiol cleavage solution with stirring for five hours at

room temp. Prior to stirring, argon gas was bubbled through the cleavage solution for two minutes to ensure cysteine residues remained reduced. The crude peptide was isolated by filtration and diethyl ether precipitation of the filtrate, followed by centrifugation (5000g for 10 min.) and collection of the precipitate. The precipitate was frozen and lyophilized overnight. The peptide was dissolved in 20ml of ammonium bicarbonate(10mM). Half was immediately frozen for later use. In order to form intramolecular disulfide bonds, the other half was diluted to 100ml with ammonium bicarbonate(10mM, pH 7.5) and bubbled with air for ~2hrs. at room temperature. The solution was then frozen in an ethanol/dry ice bath and lyophilized overnight. It was then purified by reverse phase high performance liquid chromatography (HPLC) and analyzed with matrix-assisted laser desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry, HPLC, and the Ellman assay to determine the purity.

Peptide B was ordered from Genemed Synthesis Inc. at 80% purity. MALDI-TOF mass spectrometry and analytical HPLC were performed on the peptide to validate proper synthesis.

MALDI-TOF Mass Spectrometry

Sample preparation was accomplished by mixing a spec (≤1mg) of peptide with 5µl 50% acetonitrile in water. α-cyano-4-hydroxy-cynnamic acid (CHCA: Sigma, St. Louis) matrix was prepared by making a solution of 450µl 50% acetonitrile/50µl 0.1% TFA and adding 5mg CHCA to supersaturate. The solution was vortexed and spun down at 10,000g for 3min. 5µl of the supernatant was removed and mixed with 1µl of prepared

sample and 1µl at 1mg/ml of bradykinin, the molecular standard (MW. 1061Da.).

The MALDI plate was prepared by washing with soapy water followed by washing with acetone and washing with methanol. The acetone and methanol washes were accompanied by 2 min. of sonication. The plate was wrapped in Kim-Wipes to dry. Once dry, 1µl of sample was spotted onto a well of the MALDI plate and was allowed to dry before MALDI-TOF analysis.

Mass Spectrometry was performed on a VoyagerRP BioSpectrometry Workstation (PerSeptive Biosystems) in linear scan function mode. The method which gave best results was obtained using the following settings: laser power:300, accelerating voltage:30000, grid voltage:70.000%, and guide wire voltage:0.150%. Standards used were sodium and bradykinin for Peptide A, and bradykinin and another peptide of known mass (Ac-ILGNQSFLTKGPSKLNDRADSRR; MW. 2614.83) for Peptide B.

HPLC Purification

Dry peptide was dissolved in 0.5ml 10% acetonitrile/90% water/~0.1% TFA. The sample was vortexed, spun down at 10,000g for 5 min., and the supernatant was drawn off for the HPLC column. Peptide A was purified on a Gilson HPLC using a 250x25mmID VyDac 218TP reverse phase column packed with 10 micron C18 silica. Samples were run with a 5% to 95% gradient of HPLC grade acetonitrile in 5.1mM HCl over 50 min.

Both Peptide A and B were analyzed on a Hitachi L-6200 Intelligent Pump HPLC with a L-4200 UV-VIS Detector and F-1050 Fluorescence Spectrophotometer using a

VyDac pH stable RP C8 Mini-Prep column. A 5% to 90% or 85% acetonitrile/0.1% TFA step gradient in water/0.1% TFA was run as shown in Table 1 for Peptide A and Table 2 for Peptide B. Absorbance at 280nm and fluorescence of Trp at 360nm were measured. Peptide B was purified on the VyDac pH stable RP C8 Mini-Prep column as well.

Table 1. Acetonitrile gradient used for Peptide A.

Time (min)	% Water/0.1%TFA	% CH3CN/0.1%TFA
0	95	5
2	85	15
27	5	42
32	10	90
37	10	90
45	100	0
50	100	0

Table 2. Acetonitrile gradient used for Peptide B.

Time (min)	% Water/0.1%TFA	% CH3CN/0.1%TFA
0	95	5
2	60	40
20	65	35
30	15	85
37	15	85
45	85	15
50	85	15

Ellman Assay

A stock solution of 1.5mM cysteine was made immediately before use by dissolving 6.59mg of cysteinehydrochloride monohydrate in 25ml 0.1M sodium phosphate, pH 8.0. A standard cysteine curve was created by preparing 2.5ml solutions of 5, 25, 50, 100, and 200μM cysteine in 0.1M sodium phosphate, pH 8. 50μl Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)) was then added to each standard and the solutions were allowed to sit for 15 min. Absorbance was read at 412nm on an Ultrospec 2000 UV/VIS Spectrophotometer (Pharmacia Biotech).

Concomitant with the cysteine standard solutions, a 20μM solution of peptide sample was made from HPLC purified sample (Peptide A) or the commercially prepared peptide (Peptide B) in 2.5ml of 0.1M sodium phosphate solution, pH 8. 50ul Ellman's reagent was added to this sample and the solution was allowed to incubate for 15 min. Absorbance was read at 412nm.

A plot of cysteine concentration vs. absorbance was plotted for the five standard cysteine concentrations and was fit with the best line by the LabWorks II, Version 4.0 computer program (SCI Technologies). The absorbance value for the unknown was compared to the standard curve to obtain a quantitative determination of free sulfhydryl groups.

Circular Dichrosim

The CD experiments were performed on a JASCO J-710/720 circular dichrometer. 40μl of the ~500μM peptide solution was diluted in water to100μl (~200μM) and

aqueous NaOH was added until a pH of 7 was reached. Eight averages of spectra were run from 200 to 240nm using a Hellma 0.1mm pathlength cylindrical QS cell at room temperature. Data in the form of 41 CD values ranging from 200nm to 240nm (given in degrees cm^2dmol^{-1} x 0.001) was submitted to the K2D server for analysis (22, 23, 24).

Results and Discussion

Peptide A

Peptide A was synthesized and purified by HPLC. MALDI-TOF mass spectroscopy of HPLC purification peak samples resulted in one peak with a resultant mass of 2152.33kDa with corresponding Na^+ and K^+ adduct peaks to its right (Figure 11). This weight corresponds well to the theoretical mass of 2155.7kDa and this sample was considered of sufficient purity for ELISA. No dimers of higher molecular weight were observed, and Ellman assay indicated a lack of free sulfhydryl groups, indicating intramolecular disulfide bond formation had occurred. The CD spectrum of Peptide A (Figure 12), as submitted to the K2D server, gave 5% α-helix, 47% β-sheet, and 48% random coil, which correlates well with the intended β-sheet conformation.

Peptide B

Peptide B was designed with more input from modeling experiments than Peptide A. After running the crystal structure of gp120 obtained from the Protein Data Bank through 300 iterations using a steepest descent algorithm and 2000 iterations using a

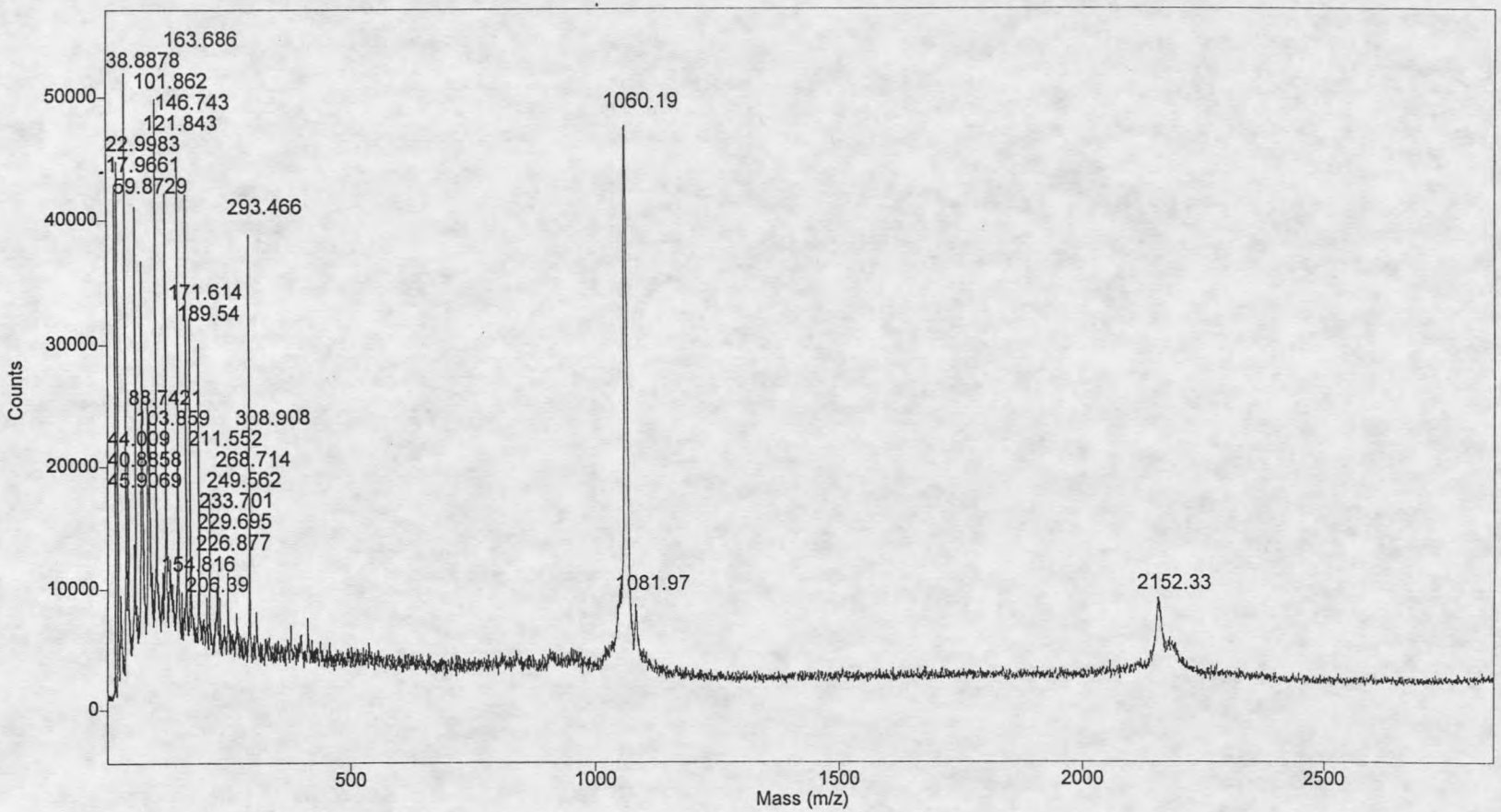


Figure 11. MALDI-TOF of Peptide A. Bradykinin was used as an internal standard and is seen at 1060.19kDa and Peptide A shows a mass of 2152.33kDa.

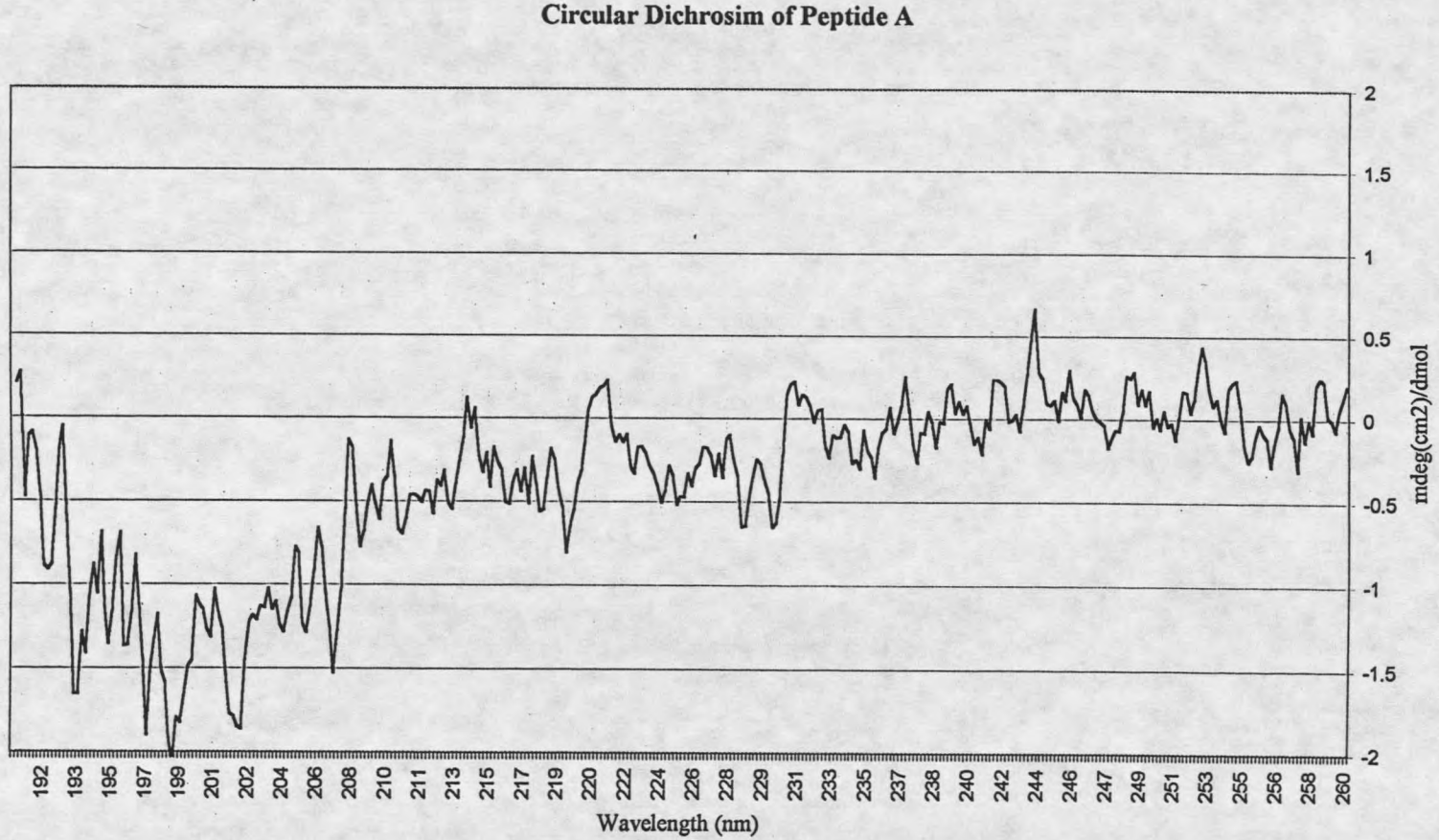


Figure 12. CD spectrum of Peptide A in water at pH 7.0. Peptide concentration was ~100μM; cell pathlength was 0.1 mm.

conjugate descent algorithm on Insight97's Discover program, the Docking Module computed the binding energy of the crystalized form of HIV-1 bound to CD4 at pH 7 to be –199.7kJ/mol. Therefore, binding energies of synthetic peptides bound to CD4 should be equal to or less than –199.7kJ/mol to effectively block the HIV-CD4 interaction. Various linkages of the β20-β21 sheet to the β15 strand were tried out. The 3-Gly linkage appeared to offer the correct number of residues to allow a similar conformation of the peptide to the crystalized HIV-1 structure, without altering the peptide's overall hydrophobic and electrostatic properties.

In the original HIV-1 crystal structure, the residues which conform to the sequence of the synthetic peptide have a binding energy of -199.7kJ/mol after 2000 conjugate gradient iterations. The K429E replacement lowers this number to -271kJ/mol within 1000 iterations, with the majority of the added binding energy coming from the decrease in positive charge. The K432Q replacement, with K429E, lowers the binding energy to –383.3kJ/mol within 1000 conjugate gradient iterations and -449.2kJ/mol after 2000 iterations. During the first 1000 iterations, the reduction in energy is mostly due to the replacement of the Lys positive charges with the Glu negative charge and the uncharged Gln residue. Model analysis confirmed that the Lys429 residue's binding energy of 39.1kJ/mol converted to -58.4kJ/mol upon Glu substitution (a difference of 97.5kJ/mol) and Lys432's energy of 76.9kJ/mol converted to 2.3kJ/mol for Gln432 (a difference of 74.6kJ/mol). These differences in energy for these two residues alone account for the 71.3kJ/mol difference between the two overall binding energies. The improvement in binding energy shown for the second 1000 iterations is primarily due to a more favorable

conformation of the peptide rather than a reduction in charge. This is confirmed upon examining the same two residues, now Glu429 and Gln432, to find an overall binding energy change of only -14.9kJ/mol during the second 1000 iterations (these residues gave a combined energy change of -172.1kJ/mol for the first1000). The much larger overall change of 65.9kJ/mol between the first and second 1000 conjugate iterations occurs instead as a result of a more favorable conformation for the remaining residues, most notably by Glu370, which drops from -90.4 to -149.9kJ/mol during this time (Table 3).

Receipt of Peptide B was accompanied by an HPLC trace and MALDI-TOF mass spectrometry (Figure 13). Attempts at verification of the given MALDI-TOF spectrum gave a similar broad peak as the one seen in the GeneMed-provided spectrum, but did not generate a sharp peak at the desired molecular weight. HPLC verified a purity of ~86% by absorbance. However, fluorescence indicated a purity of only ~24%. MALDI-TOF mass spectrometry of isolated HPLC samples also gave a broad peak with no sharp peak at the desired molecular weight. It is clear that the isolated HPLC sample contained the vast majority of peptide, but the lack of MALDI-TOF flight may be inherent in the physical properties of Peptide B. The Ellman assay showed ~87% of the free sulfhydryls had oxidized, indicating the majority of Cys residues had formed disulfide bonds. Circular dichroism of purified Peptide B (Figure 14), as submitted to the K2D server, gave 5% α-helix, 47% β-sheet, and 48% random coil, which correlates well with the intended β-sheet conformation.

Table 3. The binding energies of gp120 and Peptide B to CD4. All energies have been computed by Insight97's Docking Module. All energies are in kJ/mol.

Residue Number	gp120 in the Minimized Crystal Structure, pH7	Peptide w/ K429E Substitution	Peptide w/ K429E and K432Q Substitution	Peptide w/ K429E and K432Q Substitution
Ser365	-3.5	7.4	6.8	5.9
Gly366	-9.7	-9.2	-7.9	-1.5
Gly367	-4.8	-4.9	-10	-10.2
Asp368	-160.9	-163.3	-159.5	-152.1
Pro369	-0.3	0.4	0	-1.6
Glu370	-73.5	-73.1	-90.4	-149.9
Ile371	-2.8	-1.1	-0.2	0.2
Val372	0.6	0.6	-0.3	-1.2
Thr373	-0.7	1.5	1.6	2.3
Gly		-0.4	-0.5	-0.5
Gly		0.6	1.1	1.23
Gly		-0.3	-0.4	-0.5
Cys		-0.3	-0.4	-0.43
Ile423	-0.3	0.31	0.3	0.9
Ile424	0.2	0.7	1.4	0.73
Asn425	4.4	-0.6	-15.2	-13.9
Met426	-7.5	-7.7	-8.4	-6.9
Trp427	-5.6	-10.3	-9.1	-5.4
Gln428	-3.9	-4.1	-4.1	-6
Lys429	39.1	-58.4	-64	-70.4
Val430	-4.8	0.56	1.6	-5.4
Gly431	-3.3	-4.6	-6.6	-13.5
Lys432	76.9	75.4	2.3	5.2
Ala433	-1.5	-0.2	-0.9	-1.2
Met434	-0.4	0.1	0	0.2
Tyr435	-1.1	-0.4	-0.7	-1.1
Cys		-1.1	-0.3	-1.1
300 Steep/ 1000 Conjugate	-79.28	-271	-383.3	
300 Steep/ 2000 Conjugate	-199.7			-449.2

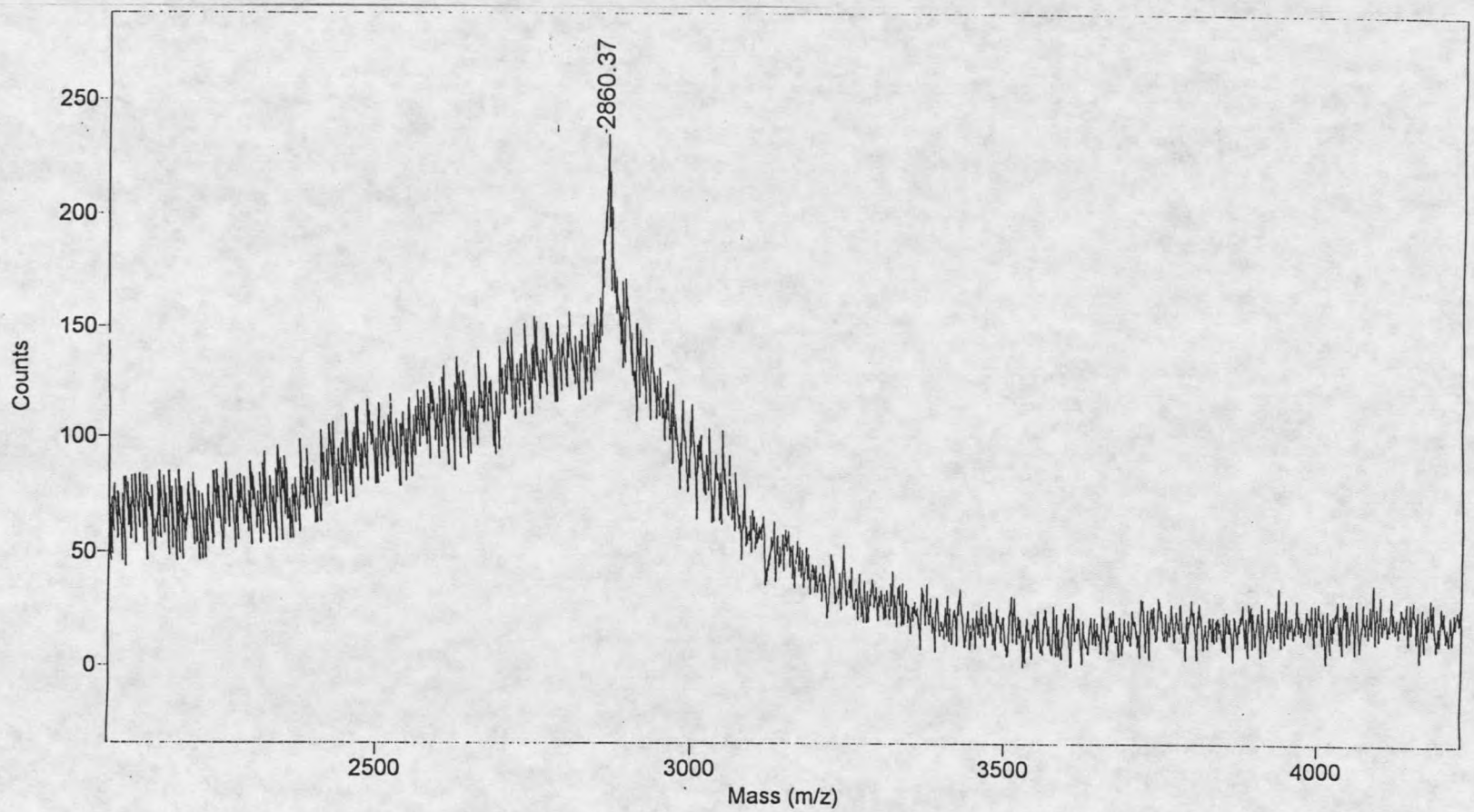


Figure 13. MALDI-TOF of Peptide B provided by GeneMed. Peptide B shows a mass of 2860.37kDa.

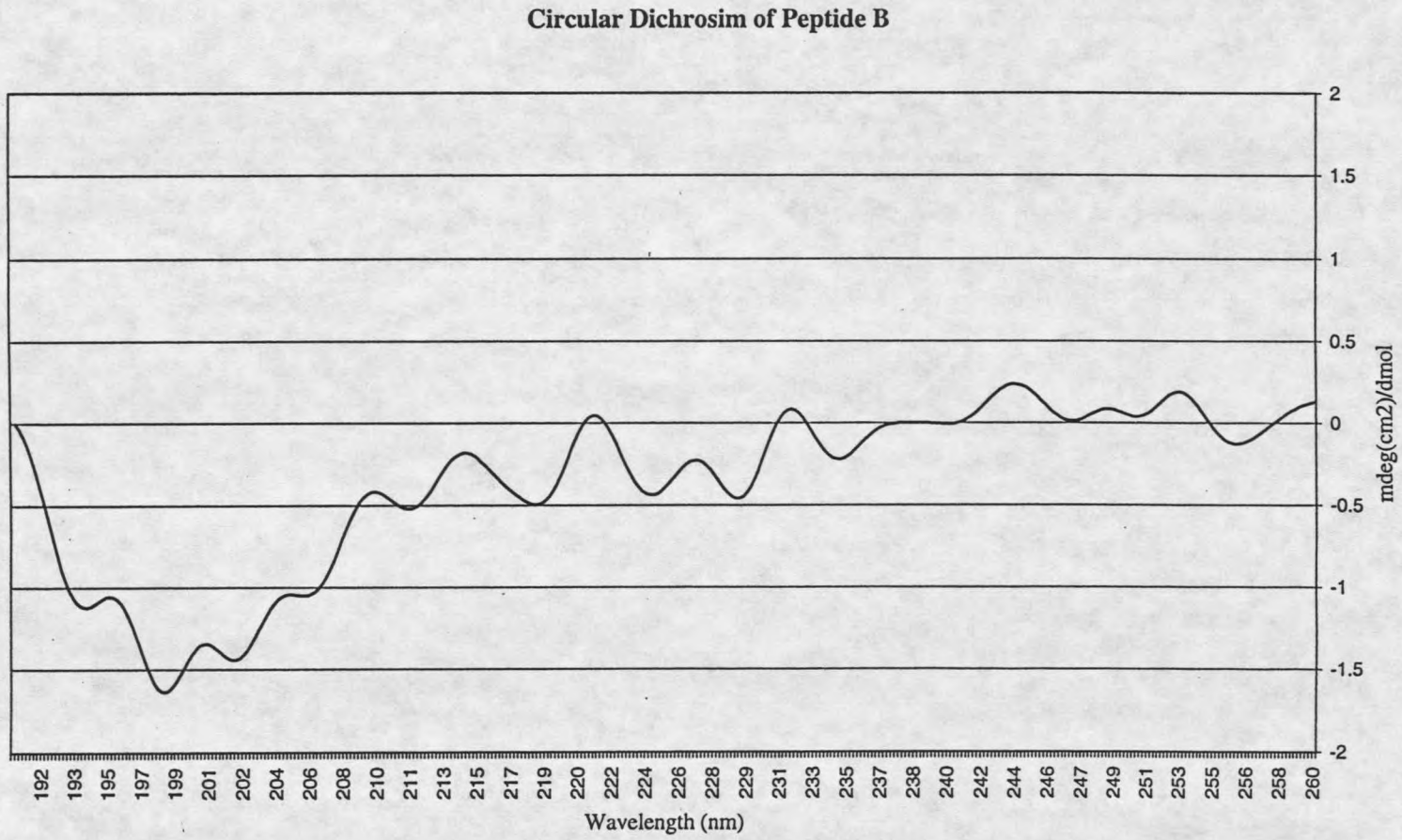


Figure 14. CD spectrum of Peptide B in 0.01M NH_4OH adjusted to pH 7.4. Peptide concentration was ~60μM; cell pathlength was 0.1 mm.

References Cited

1. Neurath, A.R., Strick, N., Fields, R., and Jiang, S. (1991) Peptides Mimicking Selected Disulfide Loops in HIV-1 gp120, Other Than V3, Do Not Elicit Virus-Neutralizing Antibodies, *AIDS Research and Human Retroviruses* **7**, 657-662.

2. Krsmanovic, V., Biquard, J.M., Sikorska-Walker, M., Cosic, I., Desgranges, C., Trabaud, M.A., Whictfield, J.F., Durkin, J.P., Achour, A., and Hearn, M.T.W. (1998) Investigations Into the Cross-reactivity of Rabbit Antibodies Raised Against Nonhomologous Pairs of Synthetic Peptides Derived From HIV-1 gp120 Proteins, *J. Peptide Res.* **52**, 410-420.

3. Vu, H.M., Myers, D., DeLorimier, R., Matthews, T.J., Moody, M.A., Heinly, C., Torres, J.V., Haynes, B.F., and Spicer, L. (1999) Nuclear Magnetic Resonance Analysis of Solution Conformations in C4-V3 Hybrid Peptides Derived from Human Immunodeficiency Virus (HIV) Type 1 gp120: Relation to Specificity of Peptide-Induced Anti-HIV Neutralizing Antibodies, *J. Virology* **73**, 746-750.

4. Shafferman, A., Jahrling, P.B., Benveniste, R.E., Lewis, M.G., Phipps, T.J., Eden-McCutchan, F., Sadoff, J., Eddy, G.A., and Burke, D.S. (1991) Protection of Macaques With a Simian Immunodeficiency Virus Envelope Peptide Vaccine Based on Conserved Human Immunodeficiency Virus Type 1 Sequences, *Proc. Natl. Acad. Sci. USA* **88**, 7126-7130.

5. Jrad, B.B. and Bajraoui, E. (1999) Antigenicity of Linear and Cyclic Peptides Mimicking the Disulfide Loops in HIV-2 Envelope Glycoprotein: Synthesis, Reoxidation and Purification, *J. Peptide Res.* **51**, 370-385.

6. Jrad, B.B. and Bajraoui, E. (1997) Linear and Cyclic Peptides Mimicking the Disulfide Loops in HIV-2 Envelope Glycoprotein Induced Antibodies With Different Specificity, *Molecular Immunology* **34**, 1177-1189.

7. Howie, S.E.M., Cotton, G.J., Heslop, I., Martin, N.J., J.A., Harrison, D.J., and Ramage, R. (1998) Synthetic Peptides Representing Discontinuous CD4 Binding Epitopes of HIV-1 gp120 That Induce T Cell Apoptosis and Block Cell Death Induced by gp120, *The FASEB J.* **12**, 991-998.

8. Cotton, G.J., Howie, S.E.M., Heslop, I., Ross, J.A., Harrison, D.J., and Ramage, R. (1996) Design and Synthesis of a Highly Immunogenic, Discontinuous Epitope of HIV-1 gp120 Which Binds to CD4+ve Transfected Cells, *Molecular Immunology* **33**, 171-178.

9. Howie, S.E.M., Fernandes, M.L., Heslop, I., Hewson, T.J., Cotton, G.J., Moore, M.J.,

Innes, D., Ramage, R., and Harrison, D.J. (1999) A Functional, Discontinuous HIV-1 gp120 C3/C4 Domain-Derived, Branched, Synthetic Peptide That Binds to CD4 and Inhibits MIP-1α Chemokine Binding, *The FASEB J.* **13**, 503-511.

10. Moore, J.P., McCutchan, F.E., Poon, S., Mascola, J., Liu, J., Cao, Y., and Ho, D.D. (1994) Exploration of Antigenic Variation in gp120 from Clades A through F of Human Immunodeficiency Virus Type 1 by Using Monoclonal Antibodies, *J. Virology* **68**, 8350-8364.

11. Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., and Hendirckson, W.A. (1998) Structure of an HIV gp120 Envelope Glycoprotein in Complex With the CD4 Receptor and a Neutralizing Human Antibody, *Nature* **393**, 648-659.

12. Thali, M., Furman, C., Ho, D.D., Robinson, J., Tilley, S., Pinter, A., and Sodroski, J. (1992) Discontinuous, Conserved Neutralization Epitopes Overlapping the CD4-Binding Region of Human Immunodeficiency Virus Type 1 gp120 Envelope Glycoprotein, *J. Virology* **66**, 5635-5641.

13. Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W., and Sodroski, J. (1990) Identification of Individual Human Immunodeficiency Virus Type 1 gp120 Amino Acids Important for DC4 Receptor Binding, *J. Virology* **64**, 5701-5707.

14. Cordonnier, A., Montagnier, L., and Emerman, M. (1989) Single Amino-Acid Changes in HIV Envelope Affect Viral Tropism and Receptor Binding, *Nature* **340**, 571-574.

15. Wyatt, R. and Sodroski, J. (1998) The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens, *Science* **280**, 1884-1888.
u. Fultz, P.N., Nara, P., Barre-Sinoussi, F., Chaput, A., Greenberg, M.L., Muchmore, E., Kieny, M., Girard, M. (1992) *Science* **256**, 1687-1690.

16. Alamos, N.M., Korber B., Hahn B., Foley B., Mellors J.W., Leitner T., Myers G., McCutchan, F., and Kuiken C.L., (1999) Human Retroviruses and AIDS 1999: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM. In HIV Sequence Database, http://hiv-web/lanl.gov.

17. Creighton, T.E. (1993) *Proteins, Structures and Molecular Properties, Second Edition*, W.H. Freeman and Co., New York.

18. Greenfield, N.J. (1996) Methods to Estimate the Conformation of Proteins and Polypeptides from Circular Dichroism Data, *Analytical Biochemistry* **235**, 1-10.

19. Molecular Simulations Inc. (1998) Forcefield-Based Simulations, Release 97-2, http://www.msi.com/doc/insight980/forcefields/FFBSim/FF_SimulTOC.doc.html.

20. Shaulsky, J. Senior Scientist, Scientific Support at Molecular Simulations, Inc. Personal Correspondence.

21. *Discover User Guide*, versions 2.9.5, San Diego: Biosym Technologies, 1994.

22. Andrade, M.A. (1998) K2D Homepage, http://columbia.ebi.ac.uk:1765/~andrade/k2d.html.

23. Andrade, M.A., Chacon, P., Merelo, J.J. and Moran, F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network, *Prot. Eng.* **6**, 383-390.

24. Merelo, J.J., Andrade, M.A., Preto, A., and Moran, F. (1994) Proteinotopic feature maps, *Neurocomputing* **6**, 443-454.

CHAPTER 3

PEPTIDE ACTIVITY DETERMINATION BY ELISA AND FOCAL INFECTIVITY ASSAY

ELISA

Following peptide synthesis, ELISA (enzyme linked immunosorbant assay) was conducted to determine the CD4 binding affinities of Peptides A and B. In particular, ELISA has been a popular technique for determining HIV antibody reactivity to peptides (1, 2, 3, 4), affinity of small peptides for CD4 (2), and inhibitory effects of peptide-elicited antibodies towards gp120 (5).

The most sensitive and specific ELISA assay is the competitive assay, because the distinction between positive and negative results is clearer than other commonly used methods (6). Therefore, a competitive ELISA was performed which utilized an indirect method of antibody detection (6). Briefly, the gp120 antigen was bound to 96-well microtitre plates through the interaction of gp120's oligosaccharide coat with Concanavalin A (ConA). After washing off unbound gp120, CD4 is introduced to the plate. Washing the plate to remove unbound CD4 is followed by the addition of primary and secondary antibodies. The primary antibody is a rabbit anti-CD4 antibody. The secondary antibody is an alkaline phosphatase(AP)-labeled goat anti-rabbit globulin which has affinity for the primary antibody. AP's reaction with the substrate p-nitrophenyl phosphate (PNPP) will produce p-nitrophenol (yellow color) on any fully completed

ELISA "sandwich" which can be quantitated spectrophotometrically.

To determine the effect of mimetic peptides on the CD4-gp120 interaction, ELISAs were performed in which synthetic peptide is mixed with CD4 prior to assay addition. In theory, the peptide will bind CD4 at the D1 domain and prevent it rom binding gp120. Washing the plate will then remove the CD4-peptide complex, resulting in fewer completed ELISA "sandwiches," and a weaker signal than with CD4 alone.

The drawbacks to the ELISA method are that it relies entirely on the physical interactions of the proteins involved. The adherence of an antigen to the solid phase may render the appropriate binding site unavailable for binding, resulting in a weak signal. Furthermore, non-specific interactions do occur. Peptides may be "sticky" and bind to CD4, gp120, or other molecules found in ELISA solutions non-specifically, and antibodies used in the indirect detection method may also exhibit promiscuity (6). Stringent controls must be analyzed congruently with test samples to account for these problems and to allow proper data analysis.

Focal Infectivity Assay

It is important to realize that binding assays, such as the ELISA assay, do not readily predict virus neutralization (7). The focal infectivity assay (FIA) is an extremely sensitive quantitative method that may be used to detect infectious virions of HIV and is capable of detecting one HIV-infected cell in 10^6 uninfected cells (8). In general, a tissue culture plate is seeded with a monolayer of HeLa cells that have been genetically altered to display the HIV receptor CD4 and its coreceptors CCR5 and CXCR4. The mimetic

peptide is then transferred onto the monolayer in an attempt to block the binding site on CD4. In theory, by specifically interacting with the CD4 receptor, a mimetic peptide will interfere with the binding of gp120, and thus, the viral infection mechanism. The cells are then infected with HIV, and any cells which have been protected by the mimetic peptide will remain HIV free. Following culture the cells are fixed with ethanol, stained with an human anti-HIV antibody which does not recognize the CD4 binding site, followed by a horse radish peroxidase (HRP)-conjugated anti-human immunoglobulin. When developed with an aminoethyl carbazol solution, the peroxidase will give characteristic red staining of the cytoplasm, with white nuclei, of any infected cells syncytia (8). In verification of the focal infectivity assay, an ELISA assay is performed to detect viral p24 produced upon HIV infection.

Materials and Methods

Peptide A ELISA

Peptide A was dissolved in 200µl water and its concentration was determined by absorbance at 280nm on a Hewlett Packard Ultra Violet(UV) Vis Spectrophotometer to be ~500µM. 50µl dilutions were made in ELISA blocking buffer (EBB: 1x phosphate buffer solution (PBS)/1% bovine serum albumin (BSA)/ 0.01% thimerosal) to 200, 60, 20, 6, and 2µM. When added to 50µl CD4 solutions, these dilutions resulted in the desired peptide concentrations of 100, 30, 10, 3, and 1µM.

To ensure peptides were specifically binding CD4, an irrelevant peptide was used as a control. The irrelevant peptide was N-acetylated, and had the following sequence:

Ac-ILGNQSFLTKGPSKLNDRADSRR-NH_3^+. This peptide is normally part of CD4 but is missing a key Gly and is therefore unable to bind gp120. The irrelevant peptide was dissolved in 200µl water and the concentration was determined to be ~20mM by UV spectrophotometry. 50µl dilutions were made in EBB to 200, 60, 20, 6, and 2µM. When added to 50µl CD4 solutions, final irrelevant peptide concentrations were 100, 30, 10, 3, and 1µM.

A DYNEX Immucon 2HB 96-well microtitre plate was rinsed with water, once with distilled water, and once with sterile 1xPBS buffer. Wells were coated with 50µg/ml ConA in 1xPBS (100µl/well) and incubated at room temp. for 3 hrs. The plate was then emptied and rinsed once with ELISA wash buffer (EWB: 0.1% Tween 20/0.1M PBS). 0.5µg/ml gp120 (Chiron SF2, #830.1) in 1xPBS was added in 100µl aliquots to each well and the plate was incubated overnight at 4°C. The plate was then emptied and sites unbound by gp120 were blocked by rinsing twice with 1xPBS containing 10% fetal calf serum (FCS) and 0.01% thimerosal. The second rinse was left at room temperature for four hours until ready for the competitive assay.

Recombinant soluble CD4-183, which contains the aminoterminal D1 and D2 domains (S0824A.3, Pharmacia & UpJohn Inc.), was made from stock at 2µg/ml in EBB so that when added to peptide dilutions the final concentration resulted in 1µg/ml CD4. 50µl of 2µg/ml CD4 were mixed with each of the 50µl Peptide A and irrelevant peptide dilutions described above and incubated overnight at 4°C. Controls for 100, 30, and 10µM peptide dilutions were done for nonspecific peptide-gp120 interactions by excluding CD4 from the binding process. Positive controls for the CD4-gp120 interaction were

done at 1μg/ml CD4 with no peptide. All were incubated overnight at 4°C.

Peptides-CD4 mixtures were then brought to room temp for 2 hrs. Excess blocking solution was flicked out of the plate, and the plate was rinsed once with EWB. The CD4/peptide mixtures were added in 100μl aliquots and incubated overnight at 4°C.

The plate was then emptied and rinsed six times in EWB and pounded face down on a paper towel to dry. 100μl of rabbit anti-CD4 polyclonal serum (#1315, American Bio-Technologies, Inc.) at 0.33μg/ml in EBB was added to each well and incubated for three hours at room temp. The plate was then emptied and rinsed six times in EWB and pounded face down on a paper towel to dry. 100μl of AP-conjugated goat anti-rabbit IgG (H + L) (Zymed, #1192) at 1:1000 dilution in EBB was added to each well and incubated at room temp. for 3 hrs.

The plate was emptied of excess, washed six times with EWB, and pounded face down on a paper towel to dry. One tablet of PNPP (5mg) was dissolved in 10ml of 9.8% diethanolamine/0.5mM $MgCl_2$, pH 9.8, and 100μl of the PNPP solution was added to each well. Absorbance was measured at 405nm by an EL320 Microplate Reader (Bio-Tek Instruments) every 15 min. for 90 min.

Peptide B ELISA

For Peptide B, a different assay was used than for Peptide A. The reason behind this is twofold. First, attempts at using Peptide B in the above described assay were problematic in that the secondary antibody bound directly to the peptide. Secondly, in the six month time period between the Peptide A and B assays, the CD4 used in the first

ELISA lost its specificity for gp120. Therefore, the ELISA for Peptide B was redesigned to account for these differences. Instead of using CD4, CD4-immunoglobulin G2 (CD4-IgG2, Progenics Pharmaceuticals, Inc.) was used. CD4-IgG2 is a heterotetramer fusion protein composed of two chains of CD4-human IgG2 heavy-chain fusion protein and two chains of a CD4-human kappa light-chain fusion protein (9, 10). In both heavy and light chain cases, the Fv portions of the IgG2 have been removed and been replaced with the D1 and D2 domains of human CD4. The protein is specific for purified gp120 from both laboratory-adapted strains, as well as primary isolates of HIV-1, with nanomolar affinity, which is comparable to the binding affinity of CD4 (9). Additionally, to prevent peptide stickiness, blotto (5% wt/vol nonfat dry milk/0.02% sodium azide in 1x PBS) was used as a blocking agent both before and after peptide addition.

A DYNEX Immucon 2HB 96-well microtitre plate was rinsed with tap water, distilled water, and once with sterile 1xPBS buffer. Wells were coated with 50µg/ml ConA in 1xPBS (100µl/well) and incubated at room temp. for 3 hrs. The plate was then emptied and rinsed once with EWB. 2µg/ml gp120 (Chiron SF2, #830.1) in 1xPBS was added in 100µl aliquots to each well and the plate was incubated overnight at 4°C. The plate was then emptied and sites unbound by gp120 were blocked by rinsing twice with 200µl blotto, leaving the second rinse in the wells at room temp. for two hours until ready for competitive assay.

Peptide B was dissolved in 10µl of 1M NH_4OH and diluted to 0.01M NH_4OH with distilled water. Dilutions were made in 1xPBS, as described above for Peptide A, so that the final concentration of peptides was 100, 30, 10, 3, and 1µM with 2µg/ml CD4-IgG2.

These solutions were incubated at room temp. for one hour prior to being added to the gp120-coated plate. The plate was emptied, rinsed once with EWB, and 100µl of each peptide/CD4-IgG2 dilution was added to each well and allowed to sit at room temp. for four hours

The plate was then emptied, rinsed six times in EWB, pounded face down on a paper towel to dry, and 100µl of 1µg/ml AP-conjugated goat anti-human IgG + A + M (H + L) (#823, Zymed) was added to each well and left at room temp. for two hours. The plate was then emptied of excess, washed six times with EWB, and pounded face down on a paper towel to dry. One tablet of PNPP (5mg) was dissolved in 10ml of 9.8% diethanolamine/0.5mM $MgCl_2$, pH 9.8, and 100µl of the PNPP solution was added to each well. Absorbance was measured at 405nm every 10 min. for 90 min.

To ensure specificity of the Peptide B interaction, two irrelevant peptides were used at 100µM concentration. Irrelevant peptide #1 (IR1) had the sequence of Ac-IRENLKDDGLF and irrelevant peptide #2 (IR2) had the sequence of Ac-AEAAAAEAAAAEAAAA. These peptides were chosen on the rationale that a previously observed interaction of Peptide B (which has a net negative charge) with CD4-IgG2 (which has a positive charge in the gp120-binding domain) might be electrostatic in nature (results not shown). Both irrelevant peptides have a negative charge, with IR1 at -1 and IR2 at -3, which should serve as a control for an electrostatic interaction of Peptide B with CD4-IgG2.

Two irrelevant IgGs were chosen to serve as controls against nonspecific binding of the peptide to the IgG2 portion of CD4-IgG2. The first control was IgG isolated from

human serum (Chemical Credentials). Theoretically, the IgG2 content of serum isolated IgG is 22.2% (11), ensuring a control against IgG2 peptide specificity. The second control was isolated human IgG4 (G4-H41-17, obtained from Carol Horgan), which controls against the peptide's binding to other irrelevant antibodies.

Focal Infectivity Assay

The FIA was accomplished by coating a monolayer of HeLa $CD4^+$ cells (H1-JC.53, David Kabat at Oregon Health Sciences University) in RPMI 1640 media (Gibco BRL) completed with 1xKeiko's solution (0.080g/l insulin, 0.055g/l pyruvate, and 0.132g/l oxaloacetic acid) in 10% FCS onto a 96-well flat-bottomed microtitre plate (Corning) at $4x10^4$cells/ml, 200µl/well, and incubating overnight at 37°C in a 5%CO_2 incubator (Fisher Scientific).

50µl/well of 100µM peptide was then added for 30min at 37°C in a 5%CO_2 incubator. The plate was then emptied and rinsed three times with100µl serum-free RPMI. The plate was then infected with HIV infected cells (H9/NL4-3, National Institutes of Health AIDS Reagent Program (NIH)) at 120, 60, 30, and 15 cells/well and incubated for four hours at 37°C in a 5%CO_2 incubator. The plate was rinsed with twice with RPMI, then 200µl/well complete RPMI was added, and the cells were allowed to grow for three days at 37°C in a 5%CO_2 incubator.

The cells were harvested by emptying the plate of medium and fixing the monolayer with 95% ethanol for 10min. The ethanol was discarded and the plate was rinsed twice with 200 µl/well EBB, leaving the second rinse in the wells to block for

30min. The blocking buffer was emptied and the plate was rinsed twice with EWB. The primary antibody, anti-HIVIg serum (#743b, NIH, 1:2000 in EBB, 50µl/well), was added and incubated on a rocker for one hour and room temp. The plate was then washed twice with EWB and the secondary antibody, goat anti-human HRP conjugate 1:800 (in EBB, 50µl/well), was added and incubated on a rocker for one hour. The plate was then washed four times in EWB. Substrate (0.52ml 4mg/ml aminoethylcarbazol (AEC)/DMF and 4.14µl 30%H_2O_2 in 10ml 0.05M sodium acetate, pH 5.0) was then added (300µl/well) and the plate was wrapped in aluminum foil to keep out light and incubated at room temp. for 25 min. The plate was rinsed with running water, dried, and foci were counted under an inverted microscope.

p24 detection was accomplished by coating plates (96 well Immulon2, Dynex Technologies, Inc.) with 30µg/ml of antibody 183-H12-5c (#1351,NIH) in 1xPBS overnight at 4°C. Unbound reagent was then flicked out and the plate was rinsed once with EWB, and blocked with 200µl/well of blotto for 2 hours at room temp. Blotto was then emptied from the plate and antigen from the FIA assay, either p24 control (#991, NIH) or p24 produced by HIV^+ cells, was added. Plates were then incubated at room temp. for four hrs. They were then washed six times with EWB and an anti-HIV antibody conjugated to biotin 1:20 (#743b, New York Blood Center) at 1:2000 in blotto was added at 100µl/well and incubated overnight at 4°C. Wells were washed six times in EWB and steptavidin-HRP (#1505, Amdex) at 1:10,000 dilution in blotto was added at 100µl/well for 45 min. Wells were then washed six times in EWB and pounded dry on a paper towel. Tetramethylbenzidine (TMB) substrate (1ml sodium acetate, pH 5.0/30µl TMB at 4mg/ml

in DMSO/0.5µl of 30% H_2O_2) was added at 100µl/well and incubated for 20 min. at room temperature. 50µl/well H_2SO_4 was added to stop the reaction and the absorbance levels were read at 450nm.

Results and Discussion

Peptide A

Peptide A showed little affinity for CD4 by the above described assay. Unfortunately, due to an error in protocol, the positive control of CD4 without peptide was not tested in this assay. However, since the completed ELISA "sandwich" gave similar absorbance levels with Peptide A and with the irrelevant peptide, without regard to peptide concentration, it can be reasonably concluded that the affinity of Peptide A for CD4 is negligible, and it does not block the gp120-CD4 interaction (Table 3).

Table 4. Peptide A ELISA at 90 min.

Peptide Concentration	Peptide A	Irrelevant Peptide
100µM w/ 1µg/ml CD4	0.87 ± 0.02	0.93 ± 0.02
30µM w/ 1µg/ml CD4	0.89 ± 0.07	0.76 ± 0.02
10µM w/ 1µg/ml CD4	0.87 ± 0.11	0.83 ± 0.01
3µM w/ 1µg/ml CD4	0.88 ± 0.04	0.70 ± 0.02
1µM w/ 1µg/ml CD4	0.94 ± 0.00	0.72 ± 0.06
Blank	0.09 ± 0.00	0.09 ± 0.01
100µM w/o 1µg/ml CD4	0.11 ± 0.00	0.07 ± 0.00
30µM w/o 1µg/ml CD4	0.09 ± 0.00	0.08 ± 0.00
10µM w/o 1µg/ml CD4	0.09 ± 0.01	0.09 ± 0.02

Peptide B

Peptide B caused CD4-IgG2 to exhibit a general stickiness. This increased binding of CD4-IgG2 in the presence of peptide was evident in binding to both ConA-fixed gp120 as well as blotto-blocked ConA alone, increasing the binding affinity of CD4-IgG2 by 60% and 337% respectively (Table 4). This stickiness was not observed when Peptide B was combined with non-CD4 bearing IgGs, indicating that Peptide B is interacting specifically with the D1 and D2 domains of CD4 on CD4-IgG2. The nature of this interaction may be

Table 5. Peptide B ELISA #1 at 90 min.

Assay	Absorbance
gp120 + CD4-IgG2 (positive control)	1.66 ± 0.01
gp120 + Serum IgG	0.32 ± 0.08
gp120 + IgG4	0.02 ± 0.01
gp120 + CD4-IgG2/100μM Peptide B	2.66 ± 0.08
gp120 + Serum IgG/100μM Peptide B	0.30 ± 0.02
gp120 + IgG4/100μM Peptide B	0.02 ± 0.00
CD4-IgG2/100μM Peptide B w/o gp120	2.02 ± 0.11
Serum IgG/100μM Peptide B w/o gp120	0.33 ± 0.02
IgG4/100μM Peptide B w/o gp120	0.02 ± 0.00
gp120 alone	0.01 ± 0.00
gp120 + CD4-IgG2/100μM IR1	1.27 ± 0.14
gp120 + CD4-IgG2/100μM IR2	1.11 ± 0.12
CD4-IgG2 w/o gp120	0.06 ± 0.02

purely electrostatic; therefore, two similarly charged irrelevant peptides were also used with CD4-IgG2. Neither IR1 or IR2 caused an increase in binding of CD4-IgG2 to ConA-fixed gp120 or to blotto-blocked ConA. If anything, both IR1 and IR2 appeared to slightly interfere with CD4-IgG2 binding to gp120, since they actually lowered absorbance levels of both trials compared to the positive control.

In order to determine if the increased binding of Peptide B bound CD4-IgG2 could be duplicated in a Peptide B concentration dependent manner, dilutions of Peptide B at 100, 30, 10, 3, and 1μM with 2μg/ml CD4-IgG2 were run through the same assay as above (Table 5). Results showed that there is a direct correlation between peptide concentration and binding of the peptide/CD4-IgG2 complex to blotto blocked ConA. On the other hand, there was no significant change in binding of to ConA-fixed gp120 as the concentration of Peptide B was increased. However, since the ratio of gp120 to CD4-IgG2 is already optimized for maximum signal strength in the positive control, a further increase in absorbance would not be expected.

Table 6. Peptide B ELISA #2 at 60 min.

Assay	Peptide B Absorbance w/ gp120	Peptide B Absorbance w/o gp120
100μM w/ 2μg/ml CD4-IgG2	1.66 ± 0.07	1.19 ± 0.28
30μM w/ 2μg/ml CD4-IgG2	1.71 ± 0.10	1.10 ± 0.04
10μM w/ 2μg/ml CD4-IgG2	1.75 ± 0.02	0.83 ± 0.00
3μM w/ 2μg/ml CD4-IgG2	1.62 ± 0.09	0.26 ± 0.02
1μM w/ 2μg/ml CD4-IgG2	1.53 ± 0.09	0.17 ± 0.02
0μM w/ 2μg/ml CD4-IgG2	1.58 ± 0.08	0.06 ± 0.02

The focal infectivity assay was inconclusive, because the number of foci was insufficient to obtain relevant data. The cause of this effect is believed to be incubation with PBS instead of serum-free media. Perhaps the PBS did not allow for the full growth potential of the cells or caused cells to slough off the bottom of the wells. The assay is currently being repeated with peptide dissolved in serum-free media.

The p24 assay, on the other hand, showed that p24 production was inhibited in those cells to which Peptide B was introduced (Table 6). This inhibition is readily seen at micromolar concentrations of peptide. The best results are seen in the row with 30 cells in which p24 production was inhibited in a peptide concentration-dependant manner for the 100, 30, and 10μM peptide concentrations. Experiments are currently underway to substantiate these results.

Table 7. Peptide B p24 Assay.

HIV Cells w/	PBS only	100μM Peptide B	30μM Peptide B	10μM Peptide B	1μM Peptide B
120 cells	0.43 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.01
60 cells	0.12 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
30 cells	0.20 ± 0.01	0.02 ± 0.01	0.05 ± 0.04	0.12 ± 0.02	0.02 ± 0.02
15 cells	0.11 ± 0.01	0.01 ± 0.00	0.07 ± 0.05	0.09 ± 0.07	0.07 ± 0.03

To verify peptide binding affinity, future experiments will include measuring binding of Peptide B to CD4 directly using Peptide B either iodinated with ^{125}I on its tyrosine residue or Peptide B biotinylated at its N- or C-terminal. To further substantiate circular dichroism data, two-dimensional total correlation spectroscopy (TOCSY) and

nuclear Overhauser effect (NOESY) nuclear magnetic resonance (NMR) experiments can be performed.

References Cited

1. Krsmanovic, V., Biquard, J.M., Sikorska-Walker, M., Cosic, I., Desgranges, C., Trabaud, M.A., Whictfield, J.F., Durkin, J.P., Achour, A., and Hearn, M.T.W. (1998) Investigations Into the Cross-reactivity of Rabbit Antibodies Raised Against Nonhomologous Pairs of Synthetic Peptides Derived From HIV-1 gp120 Proteins, *J. Peptide Res.* **52**, 410-420.

2. Cotton, G.J., Howie, S.E.M., Heslop, I., Ross, J.A., Harrison, D.J., and Ramage, R. (1996) Design and Synthesis of a Highly immunogenic, Discontinuous Epitope of HIV-1 gp120 Which Binds to CD4+ve Transfected Cells, *Molecular Immunology* **33**, 171-178.

3. Howie, S.E.M., Fernandes, M.L., Heslop, I., Hewson, T.J., Cotton, G.J., Moore, M.J., Innes, D., Ramage, R., and Harrison, D.J. (1999) A Functional, Discontinuous HIV-1 gp120 C3/C4 Domain-Derived, Branched, Synthetic Peptide That Binds to CD4 and Inhibits MIP-1α Chemokine Binding, *The FASEB J.* **13**, 503-511.

4. Jrad, B.B. and Bajraoui, E. (1999) Antigenicity of Linear and Cyclic Peptides Mimicking the Disulfide Loops in HIV-2 Envelope Glycoprotein: Synthesis, Reoxidation and Purification, *J. Peptide Res.* **51**, 370-385.

5. Neurath, A.R., Strick, N., Fields, R., and Jiang, S. (1991) Peptides Mimicking Selected Disulfide Loops in HIV-1 gp120, Other Than V3, Do Not Elicit Virus-Neutralizing Antibodies, *AIDS Research and Human Retroviruses* **7**, 657-662.

6. Wood, H.C. and Wreghitt, T.G. (1990) Techniques. In: *ELISA in the Clinical Microbiology Laboratory*, pp.7-21. Eds. T.G. Wreghitt and P. Morgan-Capner. Public Health Laboratory Services, London.

7. Moore, J.P., McCutchan, F.E., Poon, S., Mascola, J., Liu, J., Cao, Y., and Ho, D.D. (1994) Exploration of Antigenic Variation in gp120 from Clades A through F of Human Immunodeficiency Virus Type 1 by Using Monoclonal Antibodies, *J. Virology* **68**, 8350-8364.

8. Pincus, S.H., Wehrly, K., and Chesebro, B. (1991) Use of a Focal Infectivity Assay for Testing Susceptibility of HIV to Antiviral Agents, *BioTechniques* **10**, 336-342.

9. Gauduin, M.C., Allaway, G.P, Olson, W.C., Weir, R., Maddon, P.J., and Koup, R.A. (1995) Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates, *AIDS Res Hum Retroviruses* **11**, 533-539.

10. Allaway, G.P, Davis-Bruno, K.L., Beaudry, G.A., Garcia, E.B., Wong, E.L., Ryder, A.M., Hasel, K.W., Gauduin, M.C., Koup, R.A., and McDougal J.S. (1998) CD4-Immunoglobulin G2 Protects Hu-PBL-SCID Mice against Challenge by Primary Human Immunodeficiency Virus Type 1, *Journal of Virology* **72**, 3475-3478.

11. Abbas, A.K., Lichtman, A.H., and Pober, J.S. (1997) Antibodies and Antigens. In: *Cellular and Molecular Immunology, Third Ed.*, pp.37-65. W.B. Saunders Co., Philadelphia.

APPENDIX

Input File for Discover Generated by Insight

```
      overlap = 0.01
      begin simulation
*         add-automatic bond torsion valence out-of-plane
      reduce
!
      set dielectric  = 1.000000
!
      intermolecular interaction is on between
*   molecule 1 and 2

!
      Fixed atom list generation
*         add main
*         molecule 1 residue GLNn C40
      Fixed atom list generation
*         add main
*         molecule 1 residue SER C60 to GLU C91
*         add main
*         molecule 1 residue PRO C48 to ARG C58

!
      Minimize
*         no cross terms
*         no morse
*         add charges
*         for 1000 iterations
*         using conjugate gradient
*         until the maximum derivative is lass than 0.001000000 kcal/A

      archive as file number ifnum
      ifnum = ifnum + 1
!
!
      end
```