SYNOPSIS OF THE THESIS

TITLE: PROTEIN MINIMIZATION OF HUMAN CD4 AND DESIGN OF gp120-CD4 SINGLE CHAIN IMMUNOGENS

By: Deepak Kumar Sharma

Thesis Supervisor: Prof. Raghavan Varadarajan
Department: Molecular Biophysics Unit, IISc, Bangalore, India

Synopsis:
There have been several attempts to generate an effective treatment against HIV, the causative organism for AIDS. However till to date no drug or vaccine is able to neutralize a broad spectrum of HIV-1 primary isolates. Entry of the virus into the host cell is mediated by the interaction of the envelope (env) glycoprotein subunit gp120 expressed on the viral surface and host T-cell receptor CD4. The interaction between gp120 and CD4, which is predominantly hydrophobic in nature, leads to conformational changes in gp120 (Sattentau and Weiss 1988; Sattentau and Moore 1991; Bachelder et al. 1995) causing the previously buried cryptic epitopes (CD4i) in gp120 to get exposed. This promote binding of gp120 to its co-receptor CCR5 (Trkola et al. 1996; Wu et al. 1996). Further conformational changes in the gp41 subunit of env lead to fusion of the viral and target cell membranes and subsequent entry of the viral genome into the host cell (Blacklow et al. 1995; Lu et al. 1995; Moore et al. 1997; Eckert and Kim 2001b). There have been numerous attempts to develop an inhibitor or a vaccine that could be effective against diverse isolates of HIV. The main reasons for the failure in the development of such an agent is because of the high mutability of HIV, extensive glycosylation of gp120, shielding of neutralizing epitopes by variable loops and significant alteration in the tertiary structure of envelope protein after interaction with CD4. The crystal structures of unliganded form of the gp120 core (Chen et al. 2005) and the ternary complex of gp120 core with its primary receptor CD4 and the neutralizing antibody 17b Fab fragment (gp120-CD4-17b) are known (Kwong et al. 1998; Kwong et al. 2000).
The long term aim of the work reported in this thesis is to develop suitable immunogens and inhibitors that are able to result in neutralization of different primary isolates of HIV. These approaches are based upon the available crystal structure of gp120-CD4-17b.

**Abbreviations**: HIV, Human immunodeficiency virus; CD, circular dichroism; GdnCl, Guanidinium chloride; CD4D12, two N-terminal domains of human CD4 (amino acids 1-183); CD4D1, first N-terminal domain of human CD4 (amino acids 1-99); CD4D1(S-S), CD4D1 with an additional disulfide; CD4PEP1, region 21 to 64 of human CD4.

**Chapter 1** discusses general aspects about the structural organization of HIV, its life cycle and the mechanism of entry into the target cell. It describes the various approaches adopted to neutralize the virus and the main hindrances to develop an effective vaccine or drug against it.

**Chapter 2** describes the expression, purification and thermodynamic characterization of several analogs of human CD4. Equilibrium unfolding of these derivatives by Guanidinium chloride (GdnCl) was monitored by a combination of fluorescence and circular dichroism spectroscopy. CD4D12 shows a multistep folding pathway and revealed the existence of partially folded intermediate states in the unfolding by GdnCl. Thermal denaturation of CD4D12 was found to be irreversible and resulted in the formation of a visible precipitate. CD4D12, CD4D1, CD4D1(S-S) show a non-cooperative transition for chemical denaturation. These derivatives of CD4 have high affinity for gp120 and are able to expose the neutralizing epitope 17b of gp120. This study provides a framework to generate CD4 analogs with improved stability.

**Chapter 3** describes the design and structural characterization of truncated versions of human CD4. Analysis of the gp120-CD4-17b crystal structure showed that most of the residues important for binding to gp120 lie in region 21-64 of domain D1 of CD4. Based upon the a computational analysis of the crystal structure, a mutational strategy was designed to express CD4D1 and region 21-64 of CD4 (CD4PEP1) in E.coli. The binding
affinity of each CD4 derivative and the previously designed M33 (Martin et al. 2003) for gp120 was measured using surface plasmon resonance. Measured \( K_D \)'s were 15nM, 40nM, 340nM and 26\( \mu \)M for CD4D12, CD4D1, M33 and CD4PEP1 respectively. The aggregation behavior of all the constructs was characterized. CD4D12 alone was found to form amyloid like fibrils at neutral pH within 5-6 days at room temperature. The Effect of osmolytes on the structure and stability of CD4D12, CD4D1 and CD4D1(S-S) and CD4PEP1 was studied using CD, fluorescence emission spectroscopy and protease digestion. Both CD4D1 and CD4PEP1 were partially structured and showed enhanced structure in the presence of the osmolyte sarcosine. These CD4 derivatives should be useful tools in HIV vaccine design and entry inhibition studies.

Chapter 4 describes the design and characterization of a trimeric version of CD4M9. M9 is a 27 residue CD4 analog based on a scyllatoxin scaffold (Vita et al. 1999). Monomeric CD4M9 is known to bind to gp120 with binding affinity 100 fold weaker than CD4D12 (Vita et al. 1999). The GCN4 derived peptide (Eckert and Kim 2001a) has been shown to form a trimeric coiled coil. To further increase the thermodynamic stability of the coiled coil, disulfides were introduced at either the N-terminus (CCIZ) or C-terminus (IZCC) of the peptide. Mass spectrometry and DTNB assay confirmed the formation of the disulfides. Introduction of disulfides resulted in an increase in stability of the coiled coil against chemical as well as thermal denaturation. Denaturation of IZ and IZCC were found to be >95% reversible for chemical as well as thermal denaturation. Chemical (GdnCl) denaturation of CCIZ was > 95% reversible but thermal denaturation was completely irreversible. IZCC was chosen as the trimeric motif for the trimerization of CD4M9 because of its well defined helical structure, higher thermodynamic stability than IZm and reversibility against both thermal as well as chemical denaturation. Moreover the C-terminal of CD4M9 points away from gp120 and therefore addition of IZCC at the C-terminus of CD4M9 would not interfere with binding of CD4M9 to gp120. CD spectra showed that CD4M9-IZCC was predominantly helical. Size exclusion chromatography indicated the presence of trimeric species. Mass spectrometry confirmed the formation of trimeric as well as dimeric species connected by intersubunit disulfides. The Activity of M9-IZCC was confirmed using SPR and
molecule was found to be able to expose 17b epitope of gp120. Future studies will use the trimeric M9-IZCC as an inhibitor for HIV entry into host cells.

**Chapter 5** describes the construction and immunological characterization of single chain derivatives of JRFL gp120 linked to the first two domains of human CD4 and to the CD4 miniprotein analog, CD4M9. Binding affinities of the single chain derivatives to 17b was determined using SPR. gp120-CD4D12 and gp120-M9 bound to the antibody 17b with K_d's of 0.8 nM and 25 nM respectively at pH 7.0 while gp120 alone did not bind. M9 component of gp120-M9 was found to be competed out for its binding to gp120 by CD4D12. Immunological characterization of gp120 and its single chain derivatives in guinea pigs showed that all three molecules were highly immunogenic. Broadly neutralizing responses were observed only from sera generated against gp120-CD4D12, however the sera contained high titer of anti-CD4 antibodies. The antisera, when depleted for anti-CD4D12 antibodies, showed a loss of broadly neutralizing activity. Sera that were affinity purified over a column containing immobilized gp120-M9 also lacked such neutralizing activity. This suggests that the observed broadly neutralizing response is exclusively due to anti-CD4 antibodies. Only antisera generated against gp120-CD4D12 competed with the CD4, antibody 17b and this activity was unaffected by depletion of anti-CD4 antibodies. The data indicate that although antibodies targeting the CD4_i epitope were generated by the gp120-CD4D12 immunogen, these antibodies were non-neutralizing.

**Chapter 6** discusses the attempts to introduce disulfide connectivity between gp120 and CD4 in non-covalent complex as well as the single chain derivatives of gp120. The locations to introduce Cys residues to form putative disulfide were predicted using the program MODIP (Sowdhamini et al. 1989). Several different constructs were designed. All the constructs were expressed by transient transfection into 293 cell lines and purified using affinity chromatography using Lentil-Lectin Sepharose 4B. Purified protein was labeled with thiol specific probe MPB to detect the presence of free thiols. gp120-CD4D12 (430-59) derivative in which residue V430 in gp120 and R59 in CD4D12 is replaced with Cys residue did not bind to MPB indicating the absence of any
free thiols and the presence of a gp120-CD4 intersubunit disulfide. None of the gp120-M9 derivative was found to contain intersubunit disulfide. Activity of all the mutants was confirmed by their ability to interact with 17b using SPR. All of the gp120-CD4D12 single chain derivative were found to interact with gp120 though with relatively weaker affinity as compared to wt gp120-CD4D12. gp120-CD4D12 (430-59) had higher activity than the other mutants. gp120-M9 mutants showed weaker affinity than wt gp120-M9. In order to minimize CD4 specific immune responses the single chain derivative of gp120 was constructed with domain D1 of CD4D12 and characterized for its ability to bind with 17b.