Chapter 2

Materials and Methods
**Materials:** AZT, 3’-azido-3’-deoxythymidine (Sigma chemicals), T-20 (Virchow labs) Calcein AM and Calcein blue AM (Molecular probes, USA), RPMI-1640 (Invitrogen), DMEM (Invitrogen), FBS (Invitrogen, Carlsbad, CA), C-18 Reverse phase column (Restek), SNA matrix (EY laboratories), RITC (Himedia), Histopaque (Sigma)

**Cells Lines:** The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA: Chinese hamster ovary (CHO) JRL, clone A 9 (J. Arthos); HL 2/3 cells (Dr. B. Felbar and G. Pavlakis) expressing IIIB HIV-1 Env, CD4+ SupT1 cells (Dr. J. Hoxie), TZM-BL cells

**Antibodies:** The various epitope specific gp120 Monoclonal antibodies were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA:

<table>
<thead>
<tr>
<th>gp120 epitope specific monoclonal antibodies</th>
<th>Contributors</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2 specific: 697-30D, V3 epitope: 257-DIV (cf 257D) C5 region: 670-30D</td>
<td>Dr. S. Zolla-Pazner</td>
</tr>
<tr>
<td>V3 loop: V3-21, SVEINCTRPNNNTRKSI, 298-315</td>
<td>Dr. J. Laman</td>
</tr>
<tr>
<td>V3 loop: F425 B 4a.1 (cf F425) and gp41: F240</td>
<td>Dr. M. Posner and Dr. L. Cavacini</td>
</tr>
<tr>
<td>CD4 Mab: SIM4</td>
<td>Dr. J. E. K. Hildreth</td>
</tr>
<tr>
<td>C1 reactive: B2-FNMW, 94-97; C2: B13-TQLLLN, 257-262; C3: B32-FFY, 382-384 and V4 domain : B15</td>
<td>Dr. G. W. Lewis</td>
</tr>
</tbody>
</table>

**Viruses:** The following HIV-1 strain is procured form NIH-AIDS Research and reference reagent program, USA:
HIV-1\textsubscript{93IN101} is of biotype-NSI (X5) (Dr. R. Bollinger), isolated from a seropositive individual in India.

HIV-1 \textsubscript{93RW024}. The UNAIDS Network for HIV Isolation and Characterization, and the DAIDS, NIAID.

HIV-1 \textsubscript{94UG103}. The UNAIDS Network for HIV Isolation and Characterization, and the DAIDS, NIAID.

**Methods**

**Isolation of native Epap-1**

Placental tissue (MTP-Medically Terminated Pregnancy) of Human I Trimester is washed with PBS pH-7.3 (Sterile and Chilled) to completely remove the blood. Processing is done to remove the connective tissue with clean and autoclaved forceps and scissors. Homogenization was done in PBS. Centrifuge at 16000 \texttimes g for 20 min at 4\textdegree C. The protein in the supernatant is precipitated at 0-60\% Ammonium Sulphate saturation. Centrifuge at 14000 \texttimes g for 15min. Protein in the supernatant was reprecipitated with 60-80\% Ammonium Sulphate, pellet was collected at 800 \texttimes g for 20min at 4\textdegree C. The pellet is suspended in minimum volume of 1 X PBS. Dialyse extensively against 1X PBS. Dialysate is Centrifuged at 16000 \texttimes g for 20min at 4\textdegree C. Supernatent containing protein was eluted and estimated by Bradford method.
Purification of Protein by SNA Affinity Chromatography

10mg of Protein was loaded onto SNA column (Sambrucus nigra Agarose Lectin). Flow through was adjusted to 1ml/5min and flow through was stored at -20\(^\circ\)C. Unbound protein was washed with 10 times the bed volume, with 1X PBS-pH-7.3 until the A\(_{280}\) is < 0.05. Bound protein was eluted with buffer containing 100mM Galactose in 1X PBS pH-7.3. 1ml Aliquots of protein were collected in 1.5ml eppendorfs and stored at -80\(^\circ\)C.

Isolation of rec Epap-1

E. coli BL21 cells were grown in LB medium containing 100 mg/ml ampicillin until OD\(_{595}\) of 0.6 is reached. Protein expression was induced using 1 mM IPTG for 4 hours. Bacterial pellet was lysed in presence of lysozyme followed by sonication. recEpap-1 (Recombinant Epap-1) was purified using Ni-NTA column (Roda Rani et al., 2006) and the protein eluted with 200 mM imadazole was estimated using Bradford method (Bradford, 1976). Protein is stored at -80\(^\circ\)C until use.

Partial Tryptic digestion of Epap-1

Epap-1 was digested by Trypsin (1:50 ratio) by using the following protocol. 70\(\mu\)l of Epap-1 (0.75mg/ml ) was mixed with 1 \(\mu\)l Trypsin (1mg/ml) in 50 mM pH 7.0 Tris-HCl buffer. The reaction was carried out by incubation for at 37\(^\circ\)C for 1 hour.
**Purification of digested peptides by HPLC**

The digested fragments of Epap-1 were purified by HPLC (Waters e2695) using c-18 (reverse phase) column through the following gradient program. Here, 2 solvents A, B were used. Solvent A is water + 0.1% TFA and the solvent B is Acetonitrile + 0.1% TFA. The Flow rate was adjusted to 1ml/min and the pressure was adjusted to 5000 psi. Initially washing was done with Buffer B for 10min followed by equilibration with Buffer A for 10min. HPLC gradient program: Step 1 - A – 100%, B – 0% (upto 3 min); Step 2 - A - 0%, B - 100% (upto 55min); step 3 - A – 100%, B - 0% (upto 60 min). Finally washing was done with 100% Acetonitrile after reversing the column for 10min. Two major peaks were obtained at the retention time 22 min (F-1) and 35 min (F-2) along with the unbound peak. These were collected and concentrated by lyophilisation. Now these three fractions were kept for antiviral assay.

**SDS- PAGE**

Polyacrylamide gel electrophoresis was performed according to the method of Lammelli (1970) in 0.1% SDS on a vertical slab gel system. The gels contained 10% acrylamide with 30:1 ratio of acrylamide to N, N, N’, N’- methylene-bis-acrylamide. Samples were boiled at 100°C for 10 min in the presence of loading dye to dissociate proteins into their individual polypeptide chains. The loading dye contained: SDS (1% w/v), 2-mercaptoethanol (5% v/v) in 0.063 M Tris.HCl, pH 6.8. The protein mixture was subjected to electrophoresis on 4.5% (w/v) polyacrylamide stacking gel in 0.125 M Tris-HCl, pH 6.8 and 10% (w/v)
resolving gel in 0.375M Tris. HCl, pH 8.8 at 100 volts till the dye front reaches the end of the gel. The electrode buffer (pH 8.5) contained 0.025M Tris buffer, 0.192M glycine and 0.1% SDS.

**Validation of Epap-1 model**

Structural modeling of Epap-1 was done earlier in our lab using Threading technique by WURST server and the energy minimization was done by GROMACS. This model was further validated by L-ALIGN and EMBOSS to proceed for docking.

**Molecular docking**

Molecular docking was carried out between Epap-1 and gp120 trimer (obtained from PDB) using HEX 5.6 software (Ritchie, 2008; Ritchie et al., 2010). Based on the docking conformation, potential gp120 interacting peptides of Epap-1 were selected for analysis of their interaction with gp120.

**Analysis of Cytotoxicity of peptides**

Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) by active mitochondria was chosen as a cell viability measurement optimal endpoint. SupT-1 cells (0.2x10⁶) in RPMI 1640, 10 %FCS were seeded in 96 well plates. Increasing concentrations of compounds were added to the cells and incubated at 37°C for 14 hrs in a CO₂ Incubator with 5% CO₂. The media was replaced with a fresh growth medium along with 20μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). After
incubation for 4 hours in a humidified atmosphere, the media was removed and 200 ul of 0.1 N acidic isopropyl alcohol was added to the wells to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm immediately after the development of purple colour. Each experiment was conducted in triplicate and the data was represented as an average with standard deviation.

**Isolation of PBMCs from human blood**

Ten milliliters of blood was collected from healthy volunteers and diluted it to 1:1 ratio with saline. Diluted 20ml blood was layered on 10 ml of histopaque in a 50 ml round bottomed centrifuge tube. The sample was subjected to centrifugation at 400 x g for 20 minutes in a bucket centrifuge (Heareus) without applying brakes. The clear white band of cells is seen at the histopaque gradient. The cells were carefully pipetted out without disturbing the contents in the tube and resuspended in saline (50ml). This was again centrifuged at 150 x g for 10 minutes. The supernatant is discarded and the pellet was washed 3 times in saline. The cells were seeded at density of 1x 10^6 cells /ml in RPMI 1640 containing 10% FCS and 2μg of PHA. The cells were cultured at 37°C and 5% CO₂.

**Anti-viral activity in Sup-T1 cells**

One million of SupT1 cells with 100% viability were seeded with RPMI 1640, 0.1% FBS in 12-well plates. Increasing concentration of peptides (50, 100, 150 μg/ml) were added to the cells and they were infected with HIV-193IN101 at a final concentration of virus equivalent to 2 ng of p24 per ml. The infected cells were incubated at 37°C in 5% CO₂ incubator for 2 h. After 2 h, the cells were pelleted
at 350x g for 10 min, supernatant was discarded and cells were washed with RPMI 1640 containing 10% FBS. The cells were resuspended in the same medium and incubated for 96 h. The supernate were collected after 96 h and analyzed using p24 antigen capture assay kit (ABL, USA). The infection in the absence of Epap-1 was considered to be 0% inhibition. Azidothymidine (AZT) and T20 were taken as positive controls. The result given was an average of three independent experiments.

**Conjugation of Peptides with RITC and binding to gp120 expressing HL2/3 cells**

Rodmine isothiocynate (30µl, 1mg/ml PBS) was added to 50 μg of Peptide and kept for overnight incubation at 4 °C. The conjugated peptide was dialysed for 1hr against PBS. The RITC conjugated peptides were incubated with HL 2/3 cells for 1hr. The cells were washed with PBS and monitored for their Peptide binding using Flow Cytometer (Partec) and confocal microscope (Leica)

**Dye transfer assay to examine cell fusion**

**Calcein AM labeling (ex/em 496/517):** HL2/3 cells expressing HIV-1IIIB gp120 on surface were incubated with 0.5 µM of calcein AM for 1 h at 37 °C, washed, incubated in fresh medium for 30 min at 37 °C, washed and then resuspended in complete medium at 1 million cells/ml.

**Calcein blue loading (ex/em 354/469):** SupT1 cells were loaded with 20 µM of calcein blue for 1 h at 37 °C, washed, incubated in fresh medium for 30 min at 37 °C, washed and then resuspended in complete medium at 1 million cells/ml.
Fluorescently labeled gp120–41 expressing (HL2/3) cells and CD4+, CXCR4+ (SupT1) cells were co-cultured at 1:1 ratio for 2 h at 37°C. Cell fusion was monitored using a dye redistribution assay. The fusion inhibition was monitored in the presence of peptides. The cell fusion in the absence of peptide was considered to be control cell fusion.

**Reporter based assay in TZM-BL cells**

Action of Peptides on virus infection is further confirmed by using a reporter cell line, TZM-BI which upon HIV infection leads to Tat induced LTR –associated β-gal expression. In the control HIV infection, the cells express β-gal which reduces the substrate, X-gal when added. The reduced X-gal fluoresces in the presence of Azo dye (fast red violet). 1 million of TZM-BL cells with 100% viability were seeded with DMEM, 0.1% FBS in 12-well plates. Increasing concentrations of peptides were added with T20. The cells were incubated at 37°C in 5% CO2 incubator for 48 h. After this X-gal with azo dye was added to count for the number of fluorescent cells. No fluorescence was observed in the presence of T20 which was used as positive control.

**Peptide interaction with gp160 in the presence of receptor and coreceptor**

Mouse monoclonal anti-human gp160 antibodies spanning different regions of HIV-1 gp160 were added into wells of 96-well RIA plate at 10 ng per well in PBS, the plates were incubated overnight. Following day the wells were blocked with 3% BSA for 2 h at 37°C, binary complexes containing gp160–peptides were formed by incubation of gp160 in PBS with increasing concentrations of peptides.
at 37 °C for 1 h. Binary complexes were captured with gp160 monoclonal antibody pre-coated wells and incubated for 1 h at 37 °C. The unbound complexes were removed by washing thrice with wash buffer. Captured binary complexes were probed for the peptides using 10 ng of affinity purified rabbit polyclonal anti-human Epap-1 antibody by incubating for 1 h at 37 °C and wells were washed thrice with wash buffer. Bound rabbit polyclonal was probed with 1:2000 dilution of goat–anti-rabbit IgG-peroxidase antibody by incubating at 37 °C for 30 min, the wells were washed thrice with wash buffer and developed with TMB substrate system. The reaction was stopped after 30 min with 1N HCl and plates were read at 450 nm. Each experiment was done in triplicates and average, standard deviations were calculated. The same experiment was repeated in the presence of receptor and coreceptor.

**Peptide interaction with virus surface gp120**

HIV-1 virus was incubated in the presence of different concentrations of peptides. Bound complex of HIV-1 and peptide were captured by mouse monoclonal gp160 antibodies spanning different regions of gp160 as mentioned in the previous protocol. The captured complex was estimated for HIV-1 bound in terms of p24 released with 1% of Triton X-100 using ABL kit.