Chapter 3

Anti-HIV potential of proteolysed Epap-1
Introduction

Epap-1 is a 90 KDa protein expressed in placental tissue with significant affinity to gp120 (Kondapi et al, 2002). The bacterial expressed Epap-1 showed significant affinity to gp120 and anti-HIV activity but stoichiometrically the recEpap-1 binds to Epap-1 at 1:1 ratio while the native Epap-1 binds to gp120 at 1:2 ratio. It would be interesting to analyze the molecular region of Epap-1 associated with high affinity to gp120. Hence rec Epap-1 was partially digested with Trypsin. The cleaved fragments were separated on HPLC to analyze if the anti-HIV-1 activity resides in the smaller fragments of Epap-1.

Results

Native and recEpap-1 possess similar surface peptide backbone

Native Epap-1 was purified from MTP placental tissue using Sambucus nigra agarose matrix. Bound protein was eluted with galactose. Rec Epap-1 was expressed in BL21 cells and protein production was induced by IPTG. Expressed protein was isolated using Ni-NTA column. Partial Tryptic digestion was carried out by incubating Epap-1 with Trypsin(1mg/ml) in Tris-Hcl, pH 7.4 for 1 hour (Fig 3.1). Peptide profile is analyzed by MALDI-TOFF. The MALDI analysis revealed an identical digestion pattern for the both native and recEpap-1 suggested similar surface organization of Epap-1 polypeptide chain that is accessible to Trypsin (Fig 3.2).
**Protease digest of Epap-1 retain anti-HIV-1 activity**

The digested Epap-1 yielded proteins ranging from 72KDa to small peptides. The protease digest was tested for anti-HIV activity in Sup-T1 cells. In this assay, 1 million cells were infected with virus equivalent to 2ng of p24 per ml in the presence and absence of rec Epap-1 and digested pool of Epap-1. AZT was used as positive control. After 96h, the supernatants were analyzed for the amount of HIV-1 p24 protein equivalence of the virus replicated. Both Epap-1 and its proteolytic digest showed significant inhibition of HIV-1 replication with 60% inhibition at 1µg protein concentration (Fig 3.3). This suggested that the entire tertiary structure of Epap-1 is not necessary to exert its anti-viral activity. Thus peptides in the digest were separated by HPLC using C-18 reverse phase column and two major fractions at 22min (Fraction-1) and 35min (Fraction-2) respectively (Fig 3.4) were obtained. These fractions were tested for their anti-HIV activity. Fraction 1 results showed 40% inhibition of HIV replication whereas the fraction 2 possesses only 20% inhibition of HIV-1 replication while small peptides could not be isolated (Fig 3.5). The binding affinity of peptides in these two fractions to gp120 expressed on HL2/3 cells and their inhibitory activity in cell fusion assay was analyzed. In the former assay, the peptides in fractions were coupled with RITC and incubated with HL2/3 cells (Fig 3.6). The cells bound with RITC-peptide(s) bound were counted to monitor whether the peptides possess affinity to gp120. Peptides in fraction 1 did bound to cells whereas the peptides in fraction 2 do not possess affinity to gp120. This was confirmed by the inhibitory action of peptides of fraction 1 on the fusion mediated dye transfer
between calcein Blue loaded SupT1 and calcein AM loaded HL 2/3 cells where in peptides of the fraction 2 do not possess any activity while peptide(s) in fraction -1 blocked cell fusion (Fig 3.7).

Discussion

The results of these studies showed that the entire tertiary structure is not essential for the anti-HIV activity of Epap-1. Since the Polypeptide component of Epap-1 showed partial activity, identification of the important residues that were involved in binding to gp120 remains a challenging task. It is difficult to analyze the residues which were anti-HIV because the random digestion of peptides results not only in the loss of crucial tertiary conformations but also in the loss of small peptides. Isolation and characterization of small peptides which contribute to the residual anti-HIV is complex because their separation by HPLC becomes increasingly difficult in the selection of an ideal column which can separate the protein fragments ranging from several Kilo Daltons to small peptides. Even though the peptides in the fractions could show affinity to gp120 on HL 2/3 cells and inhibited dye transfer between Sup-T1 and HL 2/3 cells at the present isolation of small individual peptides was not possible. Thus, prediction of the structure of Epap-1 and interaction contributed by the peptide backbone with gp120 would help in identification of exact regions of Epap-1 that can exhibit potential interaction with gp120.
Fig 3.1

SDS-PAGE analysis of native, rec and digested Epap-1

Native, recombinant Epap-1 and the digested Epap-1 were separated by SDS-PAGE and stained by silver staining according to the mentioned protocol. Both forms were found to be of 90KDa molecular weight whereas the digested forms resulted in the fragments ranging from 72KDa to smaller peptides.
Both native and recombinant proteins were subjected to extensive digestion of Trypsin as per protocol and given for MALDI analysis. MALDI analysis reveals identical digestion pattern of both native and recEpap-1. There are six major identical peaks in both proteins. This confirms the similar folding of both the proteins.
Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. 10 µg/ml of nativeEpap-1 and recEpap-1 were used. 1 µM AZT is used as control.
HPLC profile of recEpap-1 tryptic digest: Tryptic digested protein was loaded on C-18 column and washed with water containing 0.1% TFA. Bound peptides were eluted with a gradient of Water-acetonitrile.
Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. 20 µg/ml of Epap-1 was used for recEpap-1 (recE-1), Tryptic digested recEpap-1 (DigestE-1), 60 µg/ml of Fraction-1 (F-1) and Fraction-2 (F-2) were used. 1 µM AZT and 2 µg/ml of T-20 were used as controls.
HL 2/3 cells were incubated with Fraction-1 conjugated with RITC for 2 hours at 37°C. Cells were washed extensively and visualized using Confocal microscope. Red colour on the surface of the cells shows the binding of peptide – RITC conjugate to the cells. Here Epap-1 – RITC conjugate is used as a positive control for the assay.
HL 2/3 cells were incubated with Fraction-1 for 2 hours at 37°C. The Sup T-1 cells were added and incubation continued 2 more hours. Cells were visualized using Confocal microscope. Transfer of dye takes place in the absence of any inhibitor. Panel A: Control fusion in the absence of drug or peptides, Panel B: Fusion inhibition in the presence of Fraction-1. Panel C: Fusion inhibition in the presence of Epap-1.