ELSEVIER

Antiviral Research 54 (2002) 47–57

Antiviral Research

www.elsevier.com/locate/antiviral

Anti-HIV activity of a glycoprotein from first trimester placental tissue

Anand K. Kondapi *, M.A. Hafiz, T. Sivaram

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

Received 17 April 2001; accepted 4 October 2001

Abstract

Human Immunodeficiency Virus-1 (HIV-1) transmission from a mother to the offspring during pregnancy is highly complex and a variable process in part due to the presence of several anti-HIV factors. In this study, we report an early pregnancy-associated protein (early pregnancy associated protein-1, Epap-1) with an apparent molecular weight of 90 kDa in placental tissue of pregnant women during the first trimester. Epap-1 was isolated and purified using *Sambucus nigra* agarose lectin affinity chromatography. Epap-1 strongly inhibits HIV-1_{MN}, HIV-$1_{91US056}$, HIV-1_{VB-7} replication in vitro while it exhibits low activity with HIV-1_{VB-66} strain. The molecular analysis of action of Epap-1 shows that it affects HIV–CD4 binding through inhibition of CD4–gp120 interaction.

Keywords: Early pregnancy associated protein; Anti-HIV activity; Pregnancy; gp120–CD4 interaction

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the causative agent of AIDS, a chronic immunosuppressive disease leading to severe opportunistic infections. The problem of transmission of HIV-1 from the mother to the fetus has been receiving increasing attention in recent years. The transmission of HIV from mother to offspring may occur at an early gestation period (Soeiro et al., 1992) or during late stage of pregnancy or during delivery (Ehrnst et al., 1991). Some reports suggest that HIV titers are low in fetus due to the action of interferon-alpha and female hormones produced by placental trophoblast cells (Bourinbaiar and Nagomy, 1992; Bourinbaiar and Sylvia,

Abbreviations: AZT, 3′-azido-3′-deoxythymidine; ConA, concanavalin A affinity chromatography; Epap-1, early pregnancy associated protein-1; gp160, HIV-1 envelope glycoprotein 160; gp120, HIV-1 envelope glycoprotein 120; HIV-1, human immunodeficiency virus-1; HPLC, high pressure liquid chromatography; MTP, medically terminated pregnancy; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue); PBS, phosphate buffer saline; PM-1, HUT 78 derived CD4+ T-cell clone; SNA, Sambucus nigra-agarose affinity chromatography; SDS-PAGE, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UhCG, human urinary chorionic gonadotropin.

* Corresponding author. Tel.: +91-40-3010500x4571; fax: +91-40-3010120 or +91-40-3010135.

E-mail address: akksl@uohyd.ernet.in (A.K. Kondapi).

0166-3542/02/$ - see front matter
PII: S0166-3542(01)00212-1

1995). HIV is known to be sensitive to HIV-specific antibodies against gp120 in maternal serum and their presence is correlated with the uninfected status of the children born to seropositive mothers (Devash et al., 1990; Rossi et al., 1989). These reports indicate the presence of anti-HIV factor(s) in circulation of pregnant women. Studies using commercial preparation of human urinary chorionic gonadotropin (UhCG) and HPLC fractionated low molecular weight proteins from urine of pregnant women, showed the presence of some factor(s) exhibiting anti-HIV and anti-Kaposi sarcoma activity (Lunardi-Iskandar et al., 1998). Recently Patterson et al. (2001) has shown that a 58 kDa leukemia inhibitory factor exhibits anti-HIV activity. Interestingly, Sharma et al. (1998) have demonstrated the presence of a 50 kDa protein fraction that exhibits anti-HIV activity using HIV-1_{MN} in cultured placental stromal cell supernatants of chronic villus biopsy samples from HIV negative subjects. In the present study, we have detected a glycoprotein of apparent molecular weight of 90 kDa in placental tissue of HIV negative women, who underwent medically terminated pregnancy (MTP) during the first trimester. The purified glycoprotein exhibits significant anti-HIV activity and affects HIV-1 infection through inhibition of CD4–gp120 binding.

2. Materials and methods

2.1. Materials

The following two HIV-1 strains were procured from NIH-AIDS Research and reference reagent program, USA:

HIV-I_{MN}, Biotype-SI (X4). This strain exhibits the same cytopathic effects on H9 cells as HTLV-1-IIIB.

HIV-$1_{91US056}$, Bio-type-NSI (R5). Isolated from seropositive infant in the United States in-utero for the DAIDS, NIAIDS and the UNAIDS Network for HIV isolation and characterization.

The following two Indian strains were procured from National AIDS Research Institute, Pune, India:

HIV-1_{VB-7}: this is an Indian strain and HIV-1 isolation was carried out from the blood sample obtained from a six and half-year-old female child. Risk factor for HIV infection was by the vertical route from the mother. Biotyping data was not available.

HIV-1_{VB-66}: this is an Indian strain and HIV isolate was obtained from a 3-year old male child of a HIV seropositive mother. Biotyping data was not available. PM1 cell line (Clonal derivative of HUT 78. Cells are permissive for growth of macrophage and T cell tropic viruses.), vaccinia-expressed LAI gp160 were procured from Smith-Kline Beecham Biologicals and other monoclonals used were as described in Table 1. Ten kiloDalton protein ladder from GIBCO–BRL and SNA matrix from EY Laboratories, Inc (California), Anti-mouse IgG conjugated with horse radish peroxidase (H + L specific), ConA, D(+) galactose, α-methyl mannoside and other reagents were from SIGMA Chemical Co, USA.

2.2. Purification of Epap-1

Subjects: HIV negative women who underwent MTP during their first trimester were taken for this study.

Placental extract: placental tissue collected from medically terminated pregnancies during first trimester was trimmed and the connective tissue was removed in sterile conditions. The tissue was washed thoroughly with phosphate buffered saline, pH 7.3 (PBS) until it is devoid of blood.

Table 1
Sequence of monoclonals raised against gp160 regions

Monoclonal antibody	Epitope	Region in gp160
C1-reactive (B2)[a]	FNMW	94–97
C2 reactive (B13)[a]	TQLLLN	257–262
V3 loop (III-V3-13)[b]	SVEINCTRPNNNT RKSI	298–315
V3 domain (III-V3-21)[b]	IRIQRGPGR	V3-peptide
gp41-reactive (C8)[a]	PDRPEG	727–732

[a] Provided by Professor George Lewis, IHV, Baltimore, USA.

[b] Provided by NIH AIDS Research and Reference Reagent Program, contributed by Dr Jon Laman.

Homogenization: the tissue was homogenized in PBS (pH 7.3) buffer containing 137 mM NaCl, 27 mM KCl, 43 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 and centrifuged at 8000 × *g* for 20 min at 4 °C.

Ammonium sulfate precipitation: the proteins in homogenate were fractionated with ammonium sulfate at 40% saturation. The pellet was discarded and proteins present in the supernatant were re-fractionated with ammonium sulfate at 80% saturation. About 40–80%-fractionated proteins were dissolved in minimum volume of PBS and dialyzed extensively against PBS. The dialyzed fraction was used for further purification studies. The dialysate was centrifuged at 8000 × *g* for 10 min at 4 °C. The protein in the supernatant was estimated using Bradford colorimetric method (Bradford, 1976).

Early pregnancy associated protein-1 (Epap-1) purification from first trimester medically terminated human placenta by Sambucus nigra affinity chromatography, 10 mg of total protein from 40 to 80% ammonium sulfate precipitated fraction was loaded on to a 5 ml immobilized Sambucus nigra lectin-agarose affinity column (Broekaert et al., 1984). Flow through was collected and the column was washed with 50 bed volumes of PBS until OD_{280} of the flow through reaches 0.01. The protein was eluted with PBS, pH 7.3 containing 50 mM D(+) galactose in 1 ml fractions. The eluted protein was dialyzed extensively and analyzed immediately using 10% SDS-PAGE and silver stained. A 10 kDa protein ladder was used as protein standard (this method filed for Indian patent).

Epap-1 purification from first trimester medically terminated human placenta by ConA and followed by SNA affinity chromatography, 10 mg of total protein from 40 to 80% ammonium sulfate precipitated fraction was loaded onto 5 ml immobilized ConA affinity column. Flow through was collected and the column was washed with 50 bed volumes of PBS until OD_{280} of the flow through reaches 0.01. The bound proteins were eluted with 50 mM α-methyl mannoside in PBS. The eluted protein was analyzed immediately using 10% SDS-PAGE and silver stained. A 10 kDa protein ladder was used as protein standard. The SDS-PAGE analysis showed the presence of two proteins (10 ng/μl) of high molecular weight, which got eluted by the above-mentioned conditions. The protein containing fractions were pooled and dialyzed extensively against PBS and were further loaded onto the SNA affinity matrix. The bound proteins were eluted with 50 mM D(+) galactose. The eluate was analyzed on 10% SDS-PAGE and silver stained.

2.3. Characterization of Epap-1

The following separate and distinct studies were conducted to characterize whether Epap-1 is a protein: (i) treatment with Trypsin (1 mM) at 37 °C for 5 and 30 min and reaction stopped with 1 mM soyabean trypsin inhibitor; (ii) treatment with chaotropic salt guanidinium thiocyanate (1 mM) for 3 h at 37 °C; (iii) treatment with anionic detergent, SDS (1 mM) for 3 h at 4 °C; (iv) treatment with urea (1.5, 3 and 6M) for 3 h at 4 °C; v) denatured at 94 °C for 2 min. The total sample after respective treatment was dialyzed extensively against PBS and was tested for its anti-HIV activity.

Chemical deglycosylation of Epap-1 was conducted with 48% hydrofluoric acid for 3 h at 4 °C followed by extensive dialysis against PBS. The dialysate was tested for its anti-HIV activity.

2.4. HIV-1-PM-1 infection assay

One million per ml of PM1 cells or 2-day PHA-stimulated peripheral blood mononuclear cells with 100% viability in RPMI 1640, 0.5% FBS were seeded in four different 12 well plates. Increasing concentration of Epap-1 were added to the cells and were infected with HIV-1_{MN}, HIV-$1_{91US056}$, HIV-1_{VB-7} and HIV-1_{VB-66} each at a final concentration of virus equivalent to 2 ng of p24 per ml. The infected cells were incubated at 37 °C, in a CO_2 incubator (Forma) with 5% (±0.1) CO_2 for 2 h. After 2 h, the cells were pelleted at 500 × *g* for 5 min, supernatant was discarded and cells were washed with RPMI 1640 containing 10% FBS. The cells were resuspended in the same medium with increasing concentrations of Epap-1 and were incubated for 96 h. The supernatants were collected after 96 h and analyzed using p24

antigen capture assay kit (SAIC Frederic). The infection in absence of Epap-1 was considered to be 0% inhibition and the % inhibition of HIV replication was calculated based on this control. Control has produced HIV equivalent to 10.70 ng/ml of p24 inHIV-1_{MN}, 12.02 ng/ml of p24 in HIV-1_{VB-7}, 8.29 ng/ml of p24 in HIV-$1_{191US056}$ and 7.6 ng/ml of p24 in HIV-1_{VB-66}. In the infection conducted with PBMC's, 10 U per ml of IL2 is added. The data given is an average of three independent experiments and a maximum deviation of 10% observed in the triplicates. Cell viability did not change in presence and absence of Epap-1 after 96 h of infection as assessed by a formazan dye cell viability assay.

2.5. Anti-HIV activity assay with Epap-1

ELISA plate (Costar) wells were coated with 10 ng of Dupont's recombinant soluble CD4 in 1 × PBS, pH 7.3 for overnight at 4 °C. The next day, wells were blocked with 3% BSA for 2 h in PBS.

Treatment: about 1 ng of HIV-1_{IIIB} (Table 4a) or 10 ng gp160 (Table 4b) was incubated in presence of increasing concentrations of Epap-1 in a 96-well microtiter plate at 37 °C for 1 h.

Capture: the treated HIV-1_{IIIB} (Table 4a) or gp160 (Table 4b) was transferred to CD4 (Table 4a) precoated plate and incubated at 37 °C for 1 h. After incubation, plate washed three times with wash buffer containing 0.15% Tween-20 in PBS.

Probing: the captured virus (Table 4a) or gp160 (Table 4b) were incubated for 1 h with 1:100 dilution of mouse anti-gp160 monoclonals (Abacioglu et al., 1994; Lifson et al., 1986), C1 reactive, C2 reactive, gp41-reactive, V3 loop reactive, V3 domain reactive. After incubation, the wells were washed three times with wash buffer.

Detection; mouse IgG was probed with 1:1000 dilution of peroxidase conjugated anti-mouse IgG (H + L) for 1 h at 37 °C. The peroxidase labeled protein was quantified by incubation with TMB substrate (Sigma) at 37 °C for 30 min. The reaction was stopped with 4 N H_2SO_4 and plate was read at 450 nm on ELISA reader (Molecular Devices). All anti-body dilutions were done with 1 mg/ml BSA in PBS. Each data point was an average of three determinations and a maximum of 10% deviation was observed among the triplicates.

3. Results

3.1. Isolation of glycoprotein (Epap-1)

A glycoprotein having O-linked sugars such as GalNAc and Sialic acid was isolated using SNA matrix. Incidentally, human leutinizing hormone and β-hCG that are present in high amounts in early pregnancy also contain O-linked sugars-NeuNAc, Gal, GalNAc (Bahl et al., 1979). To isolate glycoproteins having O-linked sugars, two lectins are being used. One is Limulus polyphemus (horseshoe crab) which has high affinity to *N*-acetyl D-Galactosamine/*N*-acetyl neuraminic acid and the other is Sambucus nigra (Elderberry bark lectin) which has high affinity to *N*-acetyl D-galactosamine/D(+)galactose/α-lactose, NeuNAc-Gal-GalNAc and Neu5Ac (α2-6) Gal/Gal/GalNAc (Shibuya et al., 1987). Initially, we have used Limulus polyphemus lectin (LPL) to purify O-linked proteins that has affinity to this lectin. When LPL bound proteins were eluted with NeuNAc/NAcGal, it was found that more than two proteins have been eluted (data not shown). Hence Sambucus nigra lectin was used. About 40–80% ammonium sulfate fraction of first trimester placental tissue was loaded onto Sambucus nigra agarose matrix (SNA). The proteins that bound to SNA were eluted using 50 mM D(+) galactose. Interestingly we found that, there is a single protein (Fig. 1; lane no. 3) eluted under these conditions. The protein exhibits an apparent molecular weight of 90 kDa on silver stained SDS-PAGE. This 90 kDa protein will be referred as Epap-1. Dialyzed Epap-1 is tested for its anti-HIV activity using HIV-1_{MN} in PM-1 cell line and PBMC's with IC_{50} value of 19.4 ± 0.4 ng/ml. It is found that the protein exhibits a significant anti-HIV activity in both the cells suggesting the presence of an anti-HIV glycoprotein in the first trimester placental tissue homogenate. The results of specific activity of purified fraction (Table 2) show that we could purify Epap-1 with significantly high specific activity suggesting homogene-

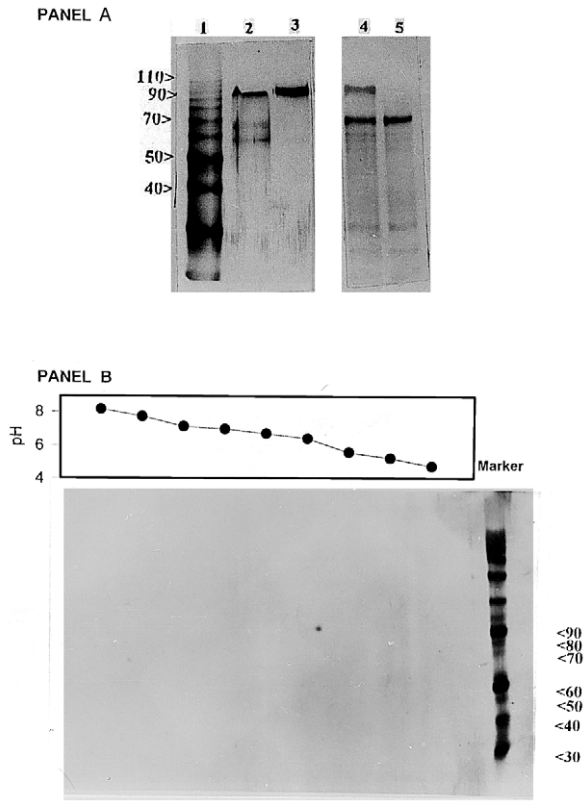

Fig. 1. Purification of early pregnancy associated protein using SNA affinity chromatography. Panel A, Lanes 1–5, protein analyzed on 10% SDS-PAGE immediately after purification on SNA matrix. SNA bound proteins that are eluted in 1 ml fractions with 50 mM D(+) galactose. Lane 1, 10 kDa Protein ladder as standard; lane 2, PO_4 buffer wash, pH 6.0; lane 3, Epap-1 (90 kDa); lane 4, 40–80% ammonium sulfate precipitated protein fraction; lane 5, unbound protein fraction from SNA. Panel B, The Epap-1 (purified from SNA affinity chromatography) was isoelectric focused using pH 3–10 ampholytes in 7 M urea. The focusing was done at 50 V for 1 h, 100 V for 2 h, 250 V for 2 h. After separation, the first dimension tube gel was aligned onto a 10% SDS-PAGE for the second dimension run. The gel was run at 100 V for 2 h and silver stained. The 2-D gel analysis of Epap-1 shows, that it has only one species of protein that migrates at 90 kDa with pI 6.2.

ity of protein. To verify whether the above anti-HIV activity is due to the non-specific cytotoxicity of Epap-1, we have incubated PM-1 cells and PBMC's with increasing concentration of Epap-1 for 24 h and quantitated the cell viability. The results show that Epap-1 does not possess any cytotoxic activity rather it shows a marginal stimulation of cellular proliferation (Fig. 2). When lactose was used instead of D(+) galactose, it was found that hCG and other proteins were eluted along with 90 kDa Epap-1 suggesting that many proteins containing O-linked sugars have affinity to SNA while, only 90 kDa Epap-1 was eluted with 50 mM galactose.

Further, to examine whether the Epap-1 also contains N-linked carbohydrates such as mannose, we have chosen a widely used lectin that has affinity to N-linked mannose sugars, ConA (Gunther et al., 1973). The 40–80% ammonium sulfate fraction of first trimester placental tissue was loaded on ConA sepharose column and the bound proteins were eluted using 50 mM α-methyl mannoside. Two proteins with apparent molecular weight 80 and 90 kDa (Fig. 3; lane no. 5) were found when this elute was analyzed on SDS-PAGE. As this 90 kDa protein resembles Epap-1, this protein fraction was dialyzed extensively against PBS and the dialyzed fraction was further loaded on SNA matrix. But these two proteins were observed in the flow through as they failed to bind to the SNA matrix (Fig. 3; lane no. 6). When this flow through fraction was analyzed for its anti-HIV activity-using HIV-1_{MN} in PM1 cells, it is found that this fraction possesses anti-HIV activity with IC_{50} value of 17.5 ± 0.1 ng/ml.

The experiments with term placenta showed that Epap-1 was absent in term placenta, but is predominantly present in first trimester MTP placental tissue (Fig. 4).

Table 2
Activity profile of Epap-1

	Specific activity in U per mg[a]
MTP placental homogenate	800 ± 24[b]
40–80% ammonium sulfate fraction	1176 ± 12[b]
SNA unbound protein fraction	235 ± 21[b]
SNA bound protein fraction	$20\,000 \pm 19$[b]

[a] one unit is equivalent to the amount of protein required for 50% inhibition of HIV-1_{MN} replication in PM-1 cells.

[b] S.D. calculated based on the determinations using three independent MTP tissue experiments.

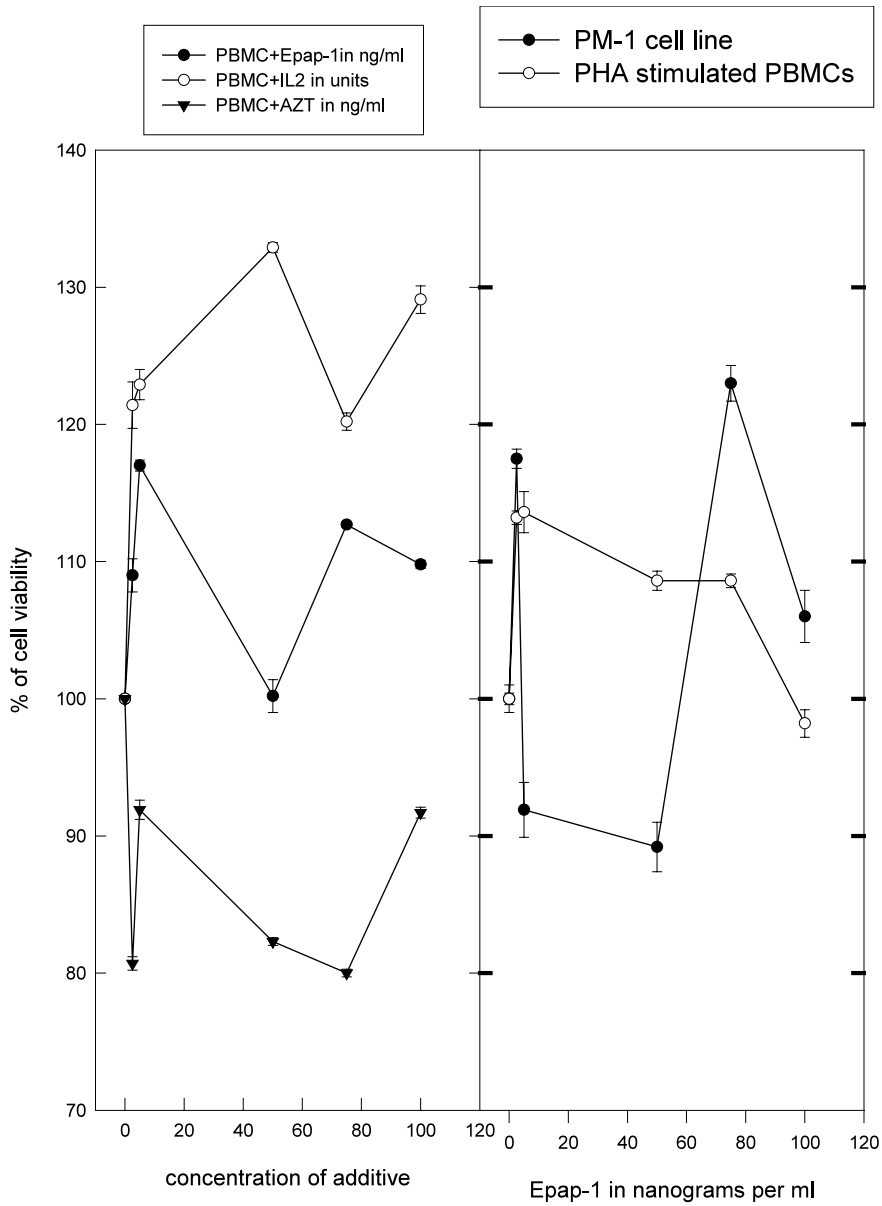

Fig. 2. Epap-1 cytotoxicity assay: cytotoxicity assay was done in PM-1 cell line, PHA stimulated (24 h) and freshly isolated PBMCs in presence of increasing concentration Epap-1 which was incubated for 24 h. Twenty microliter of MTT (5 mg/ml) was added and incubated for 4 h followed by acidic isopropanol addition, which was read at 450 nm. Each data point is an average three independent determinations, a maximum deviation 5% observed in the data. AZT is in ng scale.

The Epap-1 purified by using SNA agarose affinity chromatography was isoelectric focused using pH 3–10 ampholytes in 7 M urea. The focusing was done at 50 V for 1 h, 100 V for 2 h, 250 V for 2 h. After separation, the first dimension tube gel was aligned onto a 10% SDS-PAGE for the second dimension run. The gel was run at 100 V for 2 h and silver stained. The 2-D gel analysis of Epap-1 shows, that it has only one species of protein that migrates at 90 kDa and is focused at

pH 6 (Fig. 1Panel B). It is also observed that the migration of Epap-1 does not match with hCG heterodimer or β-hCG (data not shown).

3.2. The characterization of Epap-1

The characterization of Epap-1 was done by treating Epap-1 under various conditions as described and the treated Epap-1 was tested for anti-HIV activity using HIV-1_{MN} in concentration range 5–100 ng/ml. The following results are derived from the data as representatives of these results. We have given the data at 20 ng/ml of Epap-1 ($\sim IC_{50}$ of native protein) in Table 3. To verify whether Epap-1 is a protein, we have incubated Epap-1 with trypsin at 37 °C for 5 and 30 min. The results show that the trypsin digestion lead to 50% decrease in activity of Epap-1 compared with native protein (Table 3). Thirty minutes incubation with trypsin could further decline the activity, suggesting the proteolytic destruction of Epap-1 may affect its anti-HIV activity. The incubation in presence of 1.5, 3 and 6 M urea has marginally affected the activity of Epap-1 (Table 3) thus suggesting that Epap-1 may be resistant to denaturation in presence of urea under these conditions. Also, heat denatured and mild treatment with SDS has no significant affect on activity of Epap-1 while the treatment with chemically inactivating agent guanidinium thiocyanate decreases the activity of Epap-1 by 50% (Table 3).

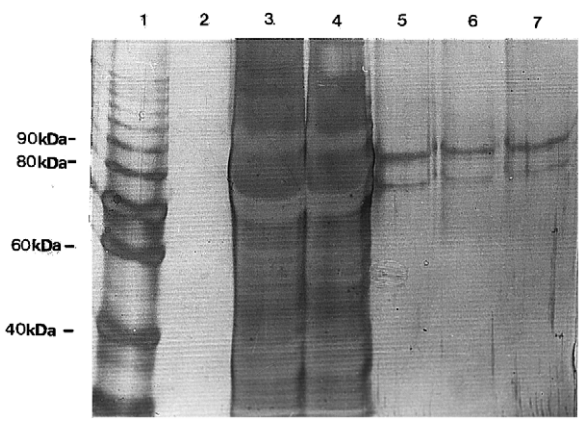

Fig. 3. Epap-1 purification from first trimester medically terminated human placenta by ConA and SNA affinity chromatography: Lane 1, 10 kDa protein ladder as standard; lane 2, no addition; lane 3, 40–80% ammonium sulfate precipitated protein fraction; lane 4, unbound proteins from ConA; lane 5, proteins bound to ConA; lane 6, unbound proteins from SNA matrix.

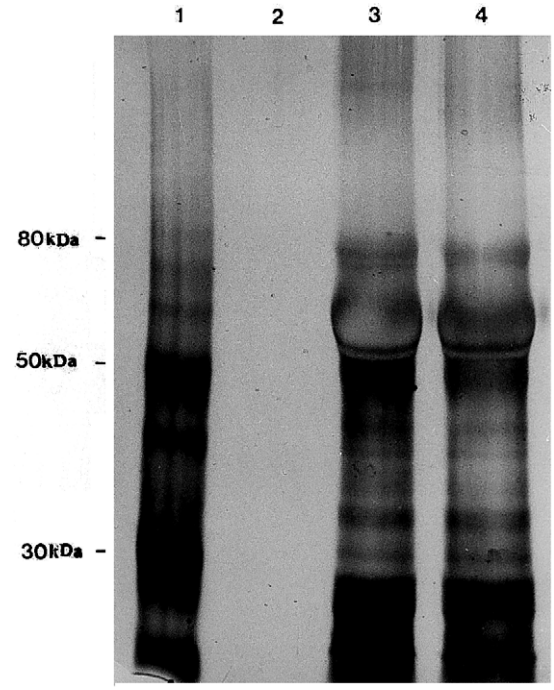

Fig. 4. SDS-PAGE analysis of 40–80% ammonium sulfate precipitated protein of term placenta: Lane 1, 10 kDa protein ladder as standard; lane 2, no addition; lane 3, 40–80% ammonium sulfate precipitated protein fraction of term placenta; lane 4, unbound proteins from SNA.

Further to verify whether the glycosylation is essential for the anti-HIV activity of Epap-1, we have partially deglycosylated Epap-1 in presence of 48% hydrofluoric acid for 3 h at 4 °C and when tested for the anti-HIV activity of deglycosylated Epap-1, the results show that its anti-HIV activity is decreased by 50% (Table 3) suggesting that Epap-1 requires glycosylation groups for its anti-HIV activity. These studies suggest that Epap-1 is a protein and the glycosylation is required for its anti-HIV activity.

3.3. Activity of Epap-1 on HIV-1 isolates implicated in vertical transmission

To understand the efficiency of Epap-1 in neutralization of HIV-1 that may be involved in

vertical transmission, we have conducted HIV-1-PM-1 infection assay in presence of increasing concentration of Epap-1 using HIV-1 strains that may be involved in vertical transmission viz; HIV-1_{MN}, HIV-$1_{91US056}$, HIV-1_{VB-7} and HIV-1_{VB-66}. The results suggest (Fig. 5) that Epap-1 inhibits the replication of HIV-1_{MN}, HIV-$1_{91US056}$, HIV-1_{VB-7} in PM1 cells, while Epap-1 stimulates HIV-1_{VB-66} replication at low concentration and shows a marginal inhibition of HIV-1_{VB-66} replication at above 50 ng/ml. Hence it is evident that the Epap-1 exhibits anti-HIV activity with few of the HIV strains used for this study. The reason for the marginal anti-HIV activity at higher concentrations of Epap-1 on HIV-1_{VB-66} strain and stimulation of HIV-1_{VB-66} at lower concentrations of Epap-1 is unclear. We assume that this difference could be due to the molecular variation of HIV-1_{VB-66} in comparison with other three strains. As the results show that Epap-1 can inhibit both SI (X4) HIV-1_{MN} and NSI (R5) HIV-$1_{91US056}$, the difference in biotype alone may not be a cause for lack of its activity against HIV-1_{VB-66}, further studies are warranted using various HIV isolates to understand the molecular specificity of Epap-1 in affect to HIV-1 infection.

Table 3
Characterization of Epap-1

	% Inhibition of HIV-1_{MN} replication in presence of 20 ng/ml of Epap-1
Trypsin (1mM)	
5 min/37 °C	35.3 ± 0.7
30 min/37 °C	21.9 ± 0.6
Urea	
6M, 3 h/4 °C	66.3 ± 0.6
3M, 3 h/4 °C	58.6 ± 0.4
1.5 M 3 h/4 °C	55.8 ± 0.2
Other conditions	
Heat denatured, (94 °C/2 min)	60.2 ± 0.0
SDS, 3 h/4 °C	62.3 ± 1.0
Inactivation by GTC, 3 h/4 °C	25.2 ± 0.2
Deglycosylation	
BSA+HF	53.1 ± 0.6
HF	27.8 ± 0.6
Native Epap-1	50.2 ± 0.2
AZT	61.3 ± 1.0
Indinavir	72.9 ± 0.6

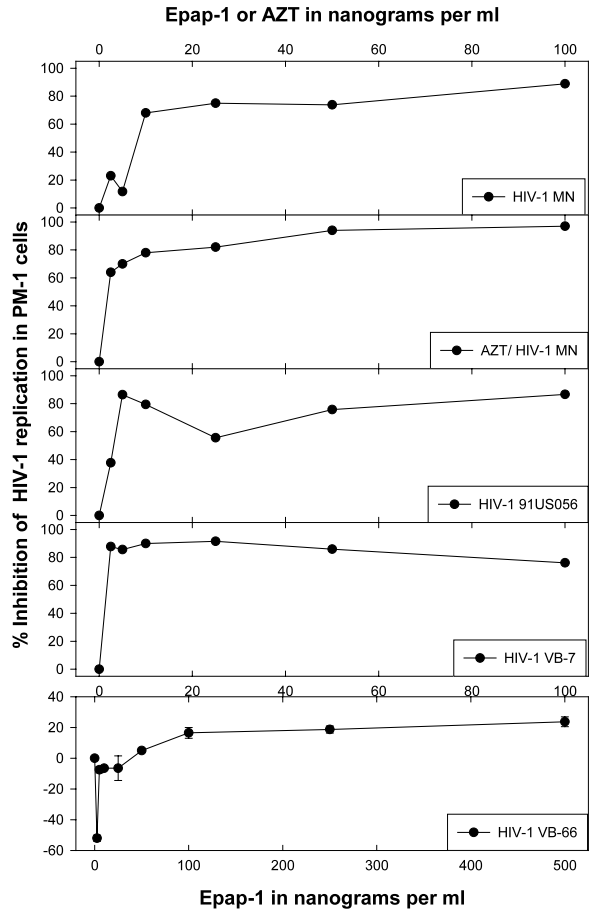

Fig. 5. HIV-1 PM1 infection: HIV-1-PM1 infection assay conducted in presence of increasing concentration of Epap-1 using HIV-1 strains viz HIV-1_{MN}, HIV-$1_{91US056}$, HIV-1_{VB-7}, HIV-1_{VB-66}. The HIV-1 replicated in 96 h was quantified using p24 antigen capture assay. The % of HIV replication was calculated taking p24 values and (100 − % of HIV replicated) gives the % of inhibition. AZT was used as a positive control. Each data is an average of three independent determinants.

3.4. Mechanism of action of Epap-1

To examine the role of Epap-1 on the viral entry, we have studied the action of Epap-1 on HIV-1–CD4 binding using monoclonal antibodies against gp160. About 1 ng of p24 equivalent of HIV-1_{IIIB} was incubated in presence of increasing concentra-

tion of Epap-1 at 37 °C for 1 h and the treated virus was captured with CD4 precoated ELISA plate. The bound HIV was probed with various anti-gp160 monoclonals (Laman et al., 1992). It was observed that the recognition of gp120 V3 loop reactive directed monoclonal was maximum inhibited (Table 4a), thus suggesting that Epap-1 affects the HIV-1_{IIIB}–CD4 interaction through its action on viral envelope protein gp120.

Further, we have analyzed the action of Epap-1 on CD4–gp160 binding, a primary and vital step in HIV-1–CD4 interaction.10 ng of gp160 was treated with increasing concentrations of Epap-1 at 37 °C for 1 h (referred to as treated gp160) and the treated gp160 was captured on CD4 precoated ELISA plate. The CD4 bound gp160 was probed using mouse anti-HIV-1 gp160 monoclonal antibodies, which can recognize epitopes in C1, C2, V3-loop, V3-domain and gp41 region of gp160. The results clearly show that Epap-1 can inhibit gp160–CD4 interaction (Table 4b). To test whether Epap-1 interacts with CD4 thereby inhibiting gp160 interaction, we have incubated precoated CD4 ELISA plate with increasing concentrations of Epap-1 for 1 h at 37 °C and CD4–gp160 binding was monitored using monoclonal antibodies against gp160 and the results show that Epap-1 has no affect on CD4 (data not shown). We have also repeated the above experiment with soluble CD4 using gp160 precoated ELISA plate; the result of this experiment also confirms that Epap-1 does not affect CD4 (data not shown).

4. Discussions

Understanding the barriers involved in the meternofetal transmission of HIV-1 has been the goal of many scientists working in pediatric AIDS research. Some studies reported the involvement of urinary hCG preparation and peptides (Lunardi-Iskandar et al., 1998). The present study is focused on the isolation of anti-HIV active glycoprotein from placental tissue collected from the first trimester MTP. A 90 kDa glycoprotein was purified from the early placental tissue, which is named as Epap-1. The binding characteristics of Epap-1 to lectins ConA and SNA suggest that Epap-1 is a glycoprotein with both N-linked and O-linked glycosyalation. The specificity of Epap-1 in binding to SNA and elution with 50 mM D(+) galactose suggests that, it may have O-linked carbohydrates that are rich in galactose. The failure of ConA elute in binding to SNA matrix is intriguing. Since the pI of Epap-1 is near neutral i.e 6.2; the possibility of sialic acid residue in O-linked sugar may be less.

In terms of anti-HIV activity of Epap-1, the results suggest that it inhibits three strains namely

Table 4
Action of Epap-1 on HIV-1 entry

	% Inhibition of binding				
Epap-1 (ng/ml)	C1-reactive	C2-reactive	V3-loop	V3-domain	gp41-reactive
HIV-1_{IIIB}–CD4 binding					
0	0 ± 0.5	0 ± 0.4	0 ± 1	0 ± 0.2	0 ± 0.1
25	14 ± 1	15 ± 0.7	10 ± 0.8	11 ± 0.7	15 ± 0.8
125	20 ± 1.1	15 ± 0.3	21 ± 0.1	19 ± 0.4	21 ± 0.7
250	27 ± 1.3	10 ± 0	20 ± 1.2	18 ± 0.5	15 ± 0.8
500	44 ± 1.7	12 ± 0.4	28 ± 1.1	23 ± 0.6	19 ± 0.3
gp160–CD4 binding					
0	0 ± 0.3	0 ± 0.2	0 ± 0.1	0 ± 3	0 ± 0.2
25	22 ± 0	13 ± 0.3	20.5 ± 9	1 ± 1	1 ± 0.4
125	52 ± 0.8	30 ± 0.4	51.7 ± 0.7	3.6 ± 0.5	54 ± 0.5
250	51 ± 1.5	33 ± 0.2	43.32 ± 2.7	9.9 ± 8	48 ± 0.8
500	52 ± 0.4	45 ± 0.9	33.98 ± 7.5	10.2 ± 4	59 ± 0.4

HIV-1_{MN}, HIV-$1_{91US056}$, and HIV-1_{VB-7} amongst four strains tested. The reason for marginal inhibition of HIV-1_{VB-66} replication by Epap-1 is unclear to us. Also, the route through which Epap-1 (at low concentrations lesser than 50 ng/ml) stimulates HIV-1_{VB-66} replication is not clear. This differential activity on HIV-1_{VB-66} may be due to selectivity of HIV-1_{VB-66} in PM-1 infection compared with other three strains. As we do not have biotyping data for this strain, we could not test this possibility. Since Epap-1 does not exhibit cytotoxicity to PBMCs and PM1 cells, there is no possibility of Epap-1 (90 kDa) being the same as LIF (58 kDa). The observation that Epap-1 is present only in first trimester placental tissue but not in term placental tissue may invite certain questions of its role in early pregnancy, during which time large amount of hCG is reported to be produced by trophoblastic cells. Since the placental tissue is collected only in the first trimester, the extent of fetal components may be negligible. Hence, Epap-1 is of maternal origin. Also, we need to understand the status of this protein in the later stages of pregnancy.

The studies on CD4 localization on human placenta (Laimore et al., 1993) suggest that it is expressed in placental villus tissue leukocytes (Dalgleish et al., 1984). The CD4 present in this tissue may be involved in HIV transmission to foetus (Blanche et al., 1989). But the observations on the transmission of HIV from mother to offspring show that only 20–50% transmit the virus (Abacioglu et al., 1994) and hence there is a need to understand the mechanism of selective transmission of the virus through placental barrier. Since CD4 present in placental villus tissue may play a role in HIV-1 vertical transmission, we have studied the molecular action of Epap-1 in inhibiting HIV-1–CD4 interactions. The results suggest that the anti-HIV activity of Epap-1 is through inhibition of HIV-1–CD4 interaction, which would lead to blocking of virus entry. The observations of Sharma et al. (1998) on the presence of an anti-HIV protein fraction below 50 kDa (PF) from first trimester placental stromal cells suggest the possibility of similarity of Epap-1 activity with that of PF. As the molecular weight of PF fraction is below 50 kDa, Epap-1 may be a different protein.

In summary, we have purified an anti-HIV early pregnancy associated protein (Epap-1) from first trimester placental tissue that inhibits CD4–HIV-1 interaction.

Acknowledgements

A.K. Kondapi is grateful to Professor R.C. Gallo and Professor M.S. Reitz of Institute of Human Virology, Baltimore for their support, encouragement and providing initial thoughts on anti-HIV activity in commercial urinary hCG preparations. HIV-1_{VB-7} and HIV-1_{VB-66} were obtained from National AIDS Research Institute, Pune, India. We thank Dr A. DeVico and Professor G.K. Lewis of IHV for their advises and generous help in providing HIV-1 envelope proteins and antibodies. The following reagents were obtained through NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH: HIV-1 V3 monoclonal antibodies IIIB V3-21 and III_B V3-13 from Dr Jon Laman. Dr Robert Gallo contributed HIV-1_{MN} strain and Dr D. Ho contributed HIV-$1_{91US056}$ strain. We acknowledge Durgabai Deshmukh hospital and Govt. Maternity hospital, Koti, Hyderabad for providing tissue. This work in part was funded by the Department of Biotechnology, India through the research grant BT/PRO577/Med/9/109/97.

References

Abacioglu, Y.H., Timothy, R.F., Jon, D.L., Eric, C., Seth, H.P., John, P.M., Corol, A.R., Roberta, K.-L., George, K.L., 1994. Epitope mapping and topology of baculovirus-expressed HIV-1 gp160 determinant with a panel of murine monoclonal antibodies. AIDS Res. Hum. Retroviruses 10, 372–381.

Bahl, O.P., Michael, J.K., Takashi, M., Rajendra, D.G., 1979. Structure and location of the O-Glycosidic carbohydrate units of hCG. J. Biol. Chem. 254, 7901–7908.

Blanche, S., Rouzioux, C., Giuhard-Moscato, M., Mayaux, F.V., Jocomet, C., 1989. A prospective study of infants born to women seropositive for human immunodeficiency virus type I. New Engl. J. Med. 320, 1643–1644.

Bourinbaiar, A.S., Nagomy, R., 1992. Pregnancy hormones, estrogen and progesterone, prevent HIV-1 synthesis in monocytes but not in lymphocytes. FEBS Lett. 302, 206–208.

Bourinbaiar, A.S., Sylvia, L.-H., 1995. Anti-HIV effect of beta subunit of human chorionic gonadotropin (β-hCG). Immunol. Lett. 44, 13–18.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B., Peuman, W.J., 1984. A Lectin from elder (*Sambucus nigra* L.) bark. Biochem. J. 221, 163–169.

Dalgleish, A.G., Peter, C.L.B., Paul, R.C., Dorothy, H.C., Melvyn, F.G., Robin, A.W., 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) 312, 763–776.

Devash, Y., Calvelli, T.A., Wood, D.G., Reagan, K.J., Rubinstein, A., 1990. Vertical transmission of human immunodeficiency virus I correlated with the absence of high affinity/avidity maternal antibodies to gp120 principal neutralizing domain. Proc. Natl. Acad. Sci. USA 87, 3445–3445.

Ehrnst, E., Lindgern, S., Dictor, M., Johanson, B., Sonnerborg, A., Czajkowsky, J., Sudin, G., Bohlin, A.B., 1991. HIV in pregnant women and their offspring: evidence for late transmission. Lancet, 338, 201–206.

Gunther, G.R., Wang, J.L., Yahara, I., Cunningham, B.A., Edalman, G.M., 1973. Concanavalin A derivatives with altered biological activities. Proc. Natl. Acad. Sci. USA 70, 1012–1016.

Laimore, M.D., Peggy, S.C., Lori, L.U., Claudia, J.M., Charlene, S.D., Clark, L.A., Daniel, D.S., 1993. Cellular localization of CD4 in the human placenta. J. Immunol. 151, 1673–1681.

Laman, J.D., Schellekens, M.M., Abacioglu, Y.H., Lewis, G.K., Tersmette, M., Fouchier, R.A., Langedijk, J.P., Claassen, E., Boersma, W.J., 1992. Variant-specific monoclonal and group-specific polyclonal human immunodeficiency virus type 1 neutralizing antibodies raised with synthetic peptides from the gp120 third variable domain, J. Virol. 66, 1823–1831.

Lifson, J., Coutre, S., Huang, E., Engleman, E., 1986. Role of envelope glycoprotein carbohydrate in human immunodeficiency virus (HIV) infectivity and virus-induced cell fusion. J. Exp. Med. 1986 Dec 1;164(6):2101–6.

Lunardi-Iskandar, Y., Joseph, L.B., William, A.B., Chia, L.H., Louis, F., Parkash, G., Philippe, H., Steven, B., Robert, C.G., 1998. Effects of a urinary factor from women in early pregnancy on HIV-1, SIV and associated disease. Nat. Med. 4, 428–443.

Patterson, B.K., Behbahani, H., Kabat, W.J., Sullivan, Y., O'Gorman, M.R., Landay, A., Flener, Z., Khan, N., Yogev, R., Andersson, J., 2001. Leukemia inhibitory factor inhibits HIV-1 replication and is upregulated in placenta from nontransmitting women. J. Clin. Invest. 107 (3), 287–294.

Rossi, P., Moschese, V., Broliden, P.A., Fundaro, C., Quinti, I., Plebani, A., Giaquinto, C., Tovo, P.A., Ljunggren, K., Rosen, J., Wigzell, H., Jondal, M., Wahren, B., 1989. Presence of maternal antibodies to human immunodeficiency virus 1 envelope glycoprotein gp120 epitopes correlates with the uninfected status of children born to seropositive mother. Proc. Natl. Acad. Sci. USA 86, 8055–8058.

Sharma, U.K., Trujillo, J., song, H.F., Saitta, F.P., Laeyendecker, O.B., Castillo, R., Arango-Jaramillo, S., Sridharan, G., Dettenhofer, M., Blakemore, K., Yu, X.F., Schwartz, D.H., 1998. A novel factor produced by placental cells with activity against HIV-1. J. Immunol. 161 (11), 6406–6412.

Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsinba-Lubaki, M., Peeters, B., Peumans, W.J., 1987. The Elderberry (*Sambucus nigra*) bark lectin recognizes the Neu5Ac(a2-6)Gal/GalNAc sequence. J. Biol. Chem. 262, 1596–1601.

Soeiro, R., Rubinstein, A., Rashbaum, W.K., Lyman, W.D., 1992. Meternofetal transmission of AIDS frequency of human immunodeficiency virus type 1 nucleic acid sequences in human fetal DNA. J. Infect. Dis. 166, 699–703.