IDENTIFICATION AND CHARACTERIZATION OF TOPOISOMERASE II ALPHA AND BETA KINASE ACTIVITY IN HIV-1 INFECTED CELLS AND VIRUS PARTICLES

A thesis submitted for the degree of

Doctor of Philosophy

By

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DECLARATION

I here by declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Dr. Anand K. Kondapi. I further declare that this has not been submitted before for the award of degree of diploma from any institute or university.

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CERTIFICATE

This is to certify that this thesis entitled "**Identification and characterization of topoisomerase II alpha and beta kinase activity in HIV-1 infected cells and virus particles** " submitted to the University of Hyderabad by Ms Gade Padmaja for the degree of Doctor of Philosophy, is based on the studies carried out by her under my supervision. This work has not been submitted before for the award of degree or diploma from any University or Institution.

Dr. Anand K. Kondapi
Supervisor

Head, Department of Biochemistry

Dean, School of Life Sciences

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ABBREVIATIONS:

HIV-1:	Human immunodeficiency virus type-1
PKC:	protein kinase C
CK-II:	casein kinase II
ATP:	Adenosyl triphosphate
dNTP:	deoxynucleotide triphosphates
ddNTP:	dideoxynucleotide triphosphates
p.i:	HIV-1 post infection period
PBS:	phosphate buffered saline
TBS:	Tris buffer saline
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
NBT:	Nitro blue tetrazoleum
BCIP:	5-Bromo, 4-chloro 3-indolyl phosphate
UCS:	Uninfected cell supernatant
PMSF:	Poly-methyl Sulphonyl Fluoride
BSA:	Bovine Serum Albumin
DTT:	Dithrio thrietol
PVDF:	Polyvinylidene fluoride
PEG-8000:	Poly Ethylene Glycol
RIPA buffer:	Radioimmunoprecipitation
CIP buffer:	Calf Intestinal Alkaline Phosphatase buffer
EDTA:	Ethylene di-amine tetra-acetate
DNA:	Deoxyribo Nucleic Acid
RNA:	Ribo Nucleic Acid
TCA:	Tri chloro acetic acid
PPO:	2,5 diphenyl oxazole
POPOP:	1,4 bis-(5 phenyl oxazol 2 yl) benzene
LTR:	Long Terminal Repeat
gag:	HIV-1 group antigen gene

RPMI :	Rosewell Park Memorial Institute
FBS:	Fetal Bovine Serum
LB:	Luria-Bertani
TAE:	Tris-acetic EDTA
TMB:	3,3’,5,5’-tetramethylbenzidine

Contents

CHAPTER 1

Introduction

Human T cell leukemia viruses (HTLV) belong to the family of Retroviruses. HTLV is present in 3 types, Type I and Type II are frequently associated with cancers such as leukemia, (Poiesz *et al.,*1980; Kalyanaramin *et al.,*1982), while Type III (Gallo *et al.,*1984) is frequently associated with immunodeficiency syndrome in humans. Hence this has been referred as human immunodeficiency virus type I (HIV-1). Consequently similar virus has been reported in monkeys (Simian immunodeficiency virus), cat (feline immunodeficiency virus). Though it is well accepted that HIV-1 is frequently associated with immunodeficiency syndrome, HIV-1 as sole cause of Acquired immunodeficiency syndrome is subject of discussion by many research groups.

STRUCTURE OF HIV PARTICLE: HIV-1 is an enveloped virus with spiked envelope glycoprotein (Fig 1). The virus contains an icasohedral nuclear capsid comprised of two molecules of viral RNA and other viral coded and cellular proteins.

A) **The Viral Envelope**: The diameter of HIV is of 1/10,000 of a millimeter and is spherical in shape. The viral envelope is outer coat of the virus; it is composed of two layers of lipids, taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell, as well as 72 copies (on average) of a complex HIV protein that protrudes from the envelope surface. This protein, known as Env, consists of a cap made of three or four molecules called glycoprotein (gp) 120, and a stem consisting of three or four gp41 molecules that anchor the

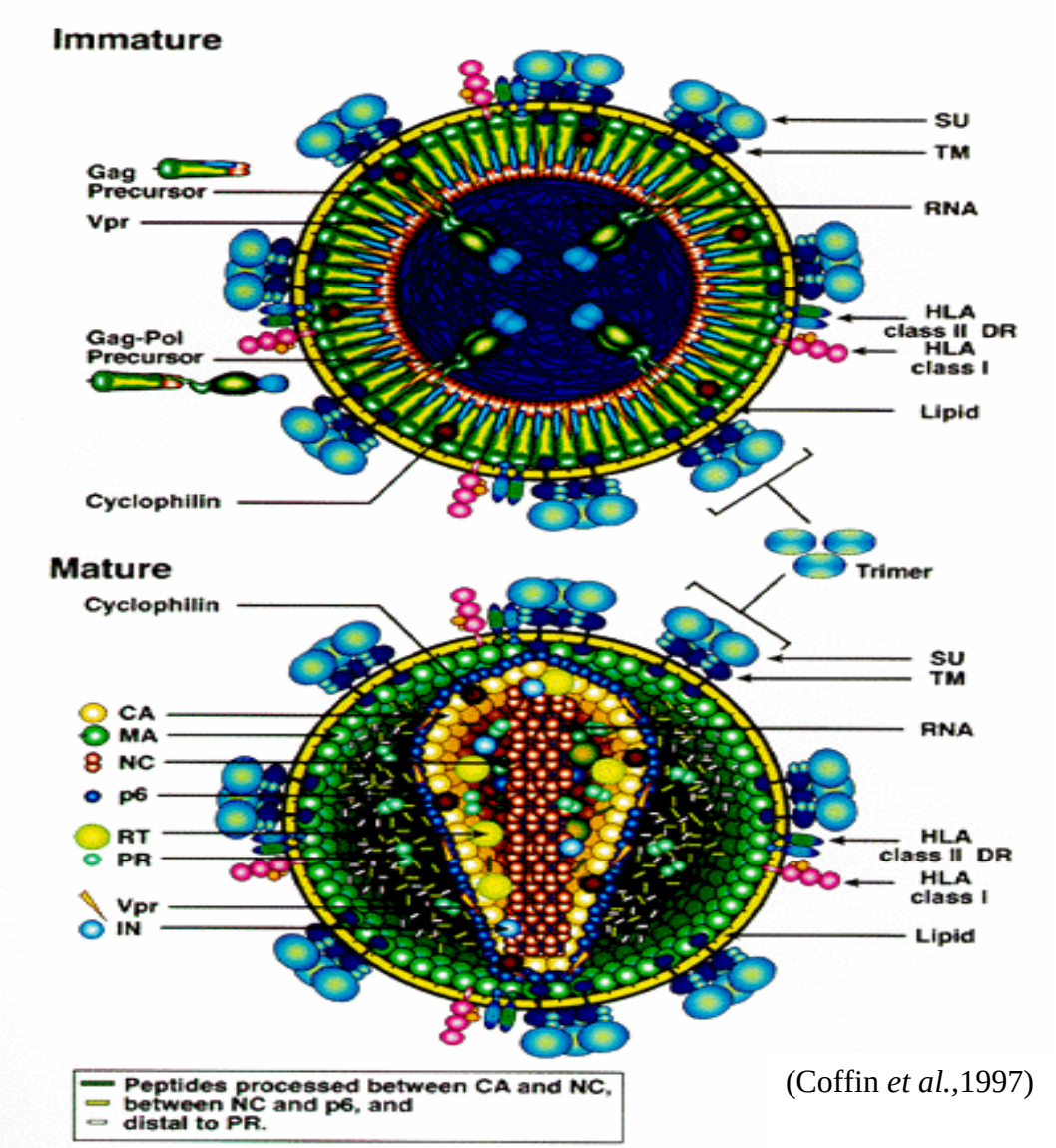
structure in the viral envelope. Much of the research is focused on these envelope proteins to develop a vaccine against HIV.

B) The Viral Core: Icosahedral-shaped core or capsid is within the envelope of a mature HIV particle and is made of 2000 copies of another viral protein, p24. The capsid surrounds two single strands of HIV RNA, each of which has a copy of the virus's nine genes. The dimeric nature of the retroviral genome (Bender and Davidson, 1976) is responsible for a high rate of recombination during infection, since portions of both molecules can be copied during reverse transcription into viral DNA. In addition to the viral genes, the genomic RNA contains number of *cis*-acting sequences that are important in the viral life cycle. The core of HIV also includes a protein called p7, the HIV nucleocapsid protein; and three enzymes reverse transcriptase, integrase and protease that carry out later steps in the virus's life cycle. Another HIV protein called p17, or the HIV matrix protein, lies between the viral core and the viral envelope.

The organization of protein domains in Gag is a key feature that sets up the structure of the virions. The shells of protein from outside to inside match the amino- to carboxy-terminal order of MA, CA, and NC and probably also define the relationship of RT and IN (Bolognesi *et al.,* 1978).

FIGURE 1

Models for HIV-1 structure.



(Coffin *et al.*,1997)

(HIV-1 Models from Lou Henderson.) (Top) The immature HIV-1 virion. The Gag and Gag-Pol proteins are shown with different colors to suggest the domains corresponding to the mature proteins formed from these precursors. The SU and TM components of Env are shown jutting out from the lipid membrane, as are HLA host proteins selectively incorporated into HIV particles. Cyclophilin also is a host protein specific for HIV-1virions. See text for details. (Bottom) The mature HIV-1 virion. The major Gag and Pol proteins and the cone-shaped core characteristic of viruses of this genus are shown. Vpr is an HIV accessory protein.

Genomic organization and gene products:

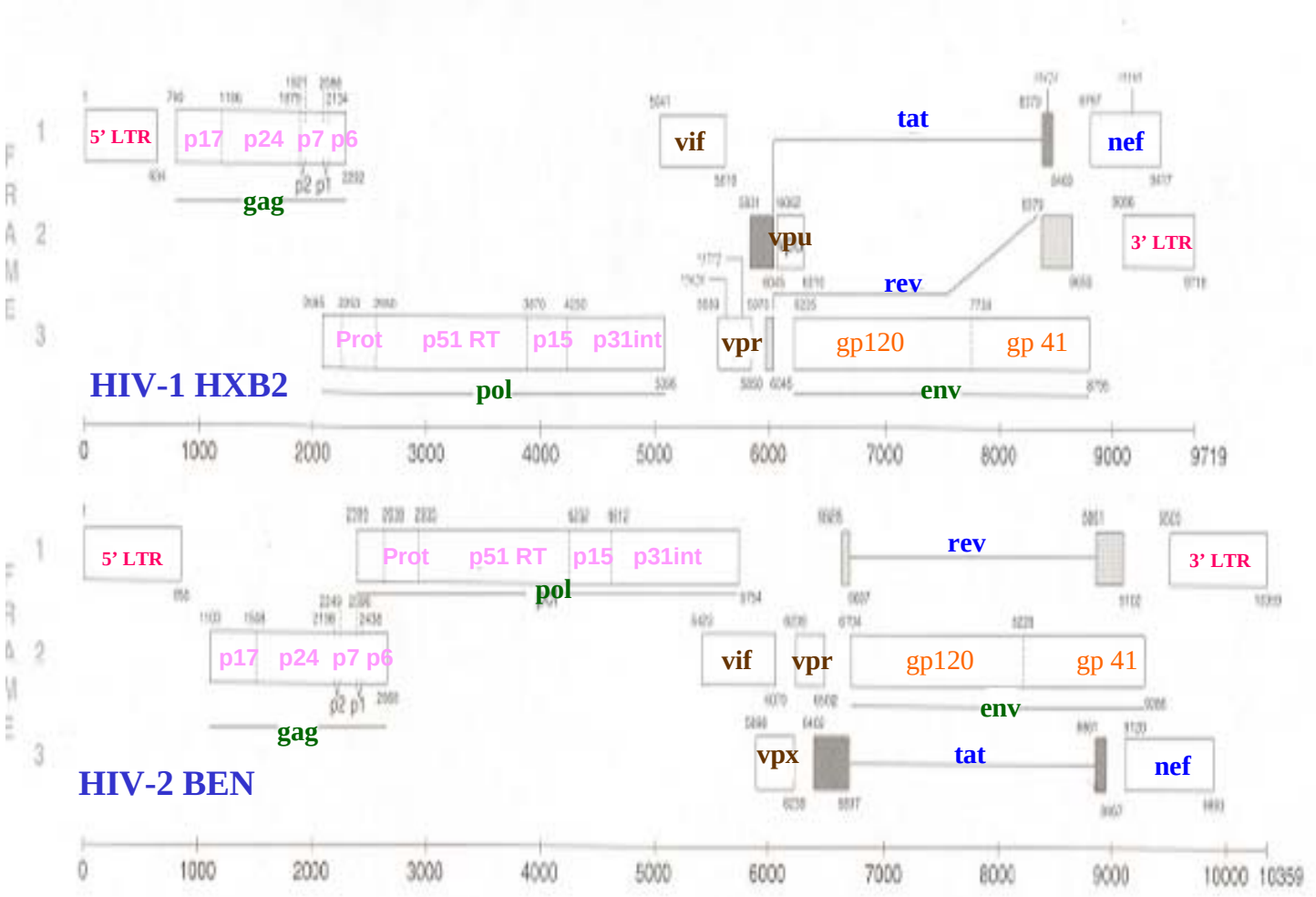
The genome of HIV-1 (Fig 2) contains three major genes flanked by long terminal repeats (LTR) that are essential for the replication mechanism (Sodroski *et al.,* 1985). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell.

Three polypeptides viz *gag*, *pol* and *env*, contain information needed to make structural proteins for new virus particles.

1. *gag* gene encodes the precursor p55, which is further cleaved by the viral protease to the structural proteins p24,p17,p7 and p6.
2. The pol gene codes for a precursor protein, which after proteolytic cleavage results in three viral enzymes: p11 protease, p66/51 RT, and p32 integrase.
3. The *env* gene, codes for a protein called gp160 that is broken down by endoproteases to form gp120 and gp41, the components of Env.

FIGURE 2

GENETIC ORGANIZATION OF HIV-1 AND HIV-2



The gene start, indicated by the small number in the upper left comer of each rectangle normally records the position of the **a** in the **atg** start codon for that gene while the number in the lower right records the last position of the stop codon. For ***pol***, the start is taken to be the first **t** in the sequence **t t t t t t ag** which forms part of the stem loop that potentiates ribosomal slippage on the RNA and a resulting - **I frame shift** and the translation of the **gag-pol** polyprotein. The ***tat*** and ***rev*** spliced exons are shown as shaded rectangles. In HXB2, *5628 and *5772 mark positions of frame shifts in the vpr gene; **!6062** indicates a defective **acg** start codon in *vpu;* ***t8424,*** and ***t9168*** mark premature stop codons in ***tat*** and ***nef***

(Model proposed by Korber *et al.*, 1998).

TABLE 1

HIV PROTEINS

NAME	SIZE	FUNCTION	LOCALIZATION
Gag MA	p^{17}	Membrane anchoring; env interaction; nuclear transport of viral core. (Myristylated protein)	Virion
CA	p^{24}	Core capsid	Virion
NC	p^{7}	Nucleocapsid, binds RNA	Virion
	p^{6}	Binds Vpr	Virion
Protease (PR)	p^{15}	gag/pol cleavage and maturation	Virion
Reversetranscriptase (RT), RNase H	p^{66} p^{51}	Reverse transcription, RNase H activity	Virion
Integrase (IN)		DNA provirus integration	Virion
Env	gp^{120} gp^{41}	External viral glycoproteins bind to CD4 and secondary receptors	Plasma membrane virion envelope
Tat	p^{16}/p^{14}	Viral transcriptional transactivator	Primarily in nucleolus/nucleus
Rev	p^{19}	RNA transport, stability and utilization factor (phosphoprotein)	Primarily in nucleolus/nucleus Shuttling between nucleolus and Cytoplasm
Vif	p^{23}	Promotes virion maturation and infectivity	Cytoplasm (cytosol, membranes) virion
Vpr	p^{10-15}	Promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	Virion, nucleus (nuclear membrane?)
Vpu	p^{16}	Promotes extra cellular release viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-I and SIVcpz)	Integral membrane protein
Nef	p^{27}-p^{25}	CD4 and class I down regulation (Myristylated protein)	Plasma membrane, cytoplasm (virion?)
Vpx	p^{12-16}	Vpr homology (not in HIV-I, only in HIV-2 and SIV)	Virion (nucleus?)

Three regulatory genes, *tat, rev* and *nef*, and three auxiliary genes, *vif*, *vpr* and *vpu,* contain information necessary for the production of proteins that control the ability of HIV to infect a cell, produce new copies of virus or cause disease.

Genetic variation:

There are two types of HIV **(HIV-1 and HIV-2)** and several subtypes.

HIV-1 can be divided into two groups, based on the genomic analysis of viral sequences from *env* and *gag* genes called: **M (major) and O (outlier**) (Korber *et al.,* 1997). A third group **N (novel)** was presented later after the analysis of an HIV1 variant from a Cameroonian individual (Simon *et al.,* 1998). **Group M** has 8 subtypes, designated A to H, and accounts for the vast majority of HIV-1 infections. Two additional new subtypes (I and J) were further described (Kostrikis *et al.,* 1995: Leitner *et al.,* 1995). **Group O** contains several very heterogeneous viruses sharing 55-70% amino acid homology with other HIV-1 subtypes. Group O is endemic to western and central Africa. **HIV-2**, which is primarily confined to West Africa, appears to be less virulent and less contagious than HIV-1, but is spreading rapidly.

The DNA sequence diversity seen in HIV is generated during reverse transcription by RT enzyme, which has been shown to be extremely error-prone and thus gives rise to nucleotide substitutions, insertions, deletions, repetitions and recombinations.

Viral phenotypes:

According to distinct infectivity of virus *invitro* the primary HIV-1 isolates have been classified into different phenotypic groups.

HIV-1 isolates have been classified on the basis of virus replication rate, host sensitivity, syncytium-induction and ability to infect CD4+ cell lines.

A) Slow/low non-syncytium inducing (**NSI**) macrophage-tropic

B) Rapid/high syncytium inducing (**SI)** T-cell tropic viruses

(Asjo *et al.,* 1986: Fenyo *et al.,* 1988: Koot *et al.,* 1992; Schwartz *et al.,* 1989).

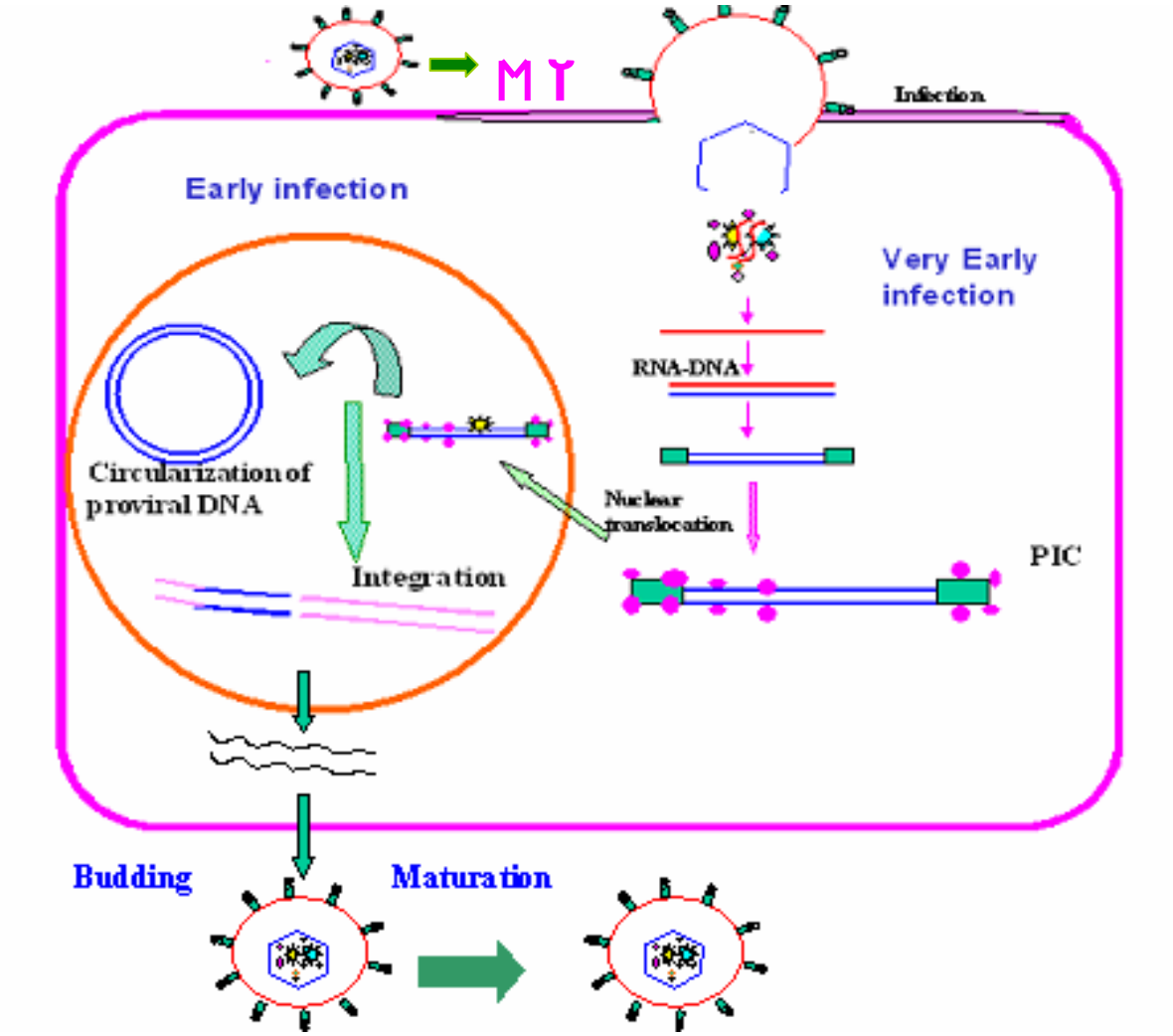
The finding that chemokine receptors plays a critical role in the cellular entry of viruses has led to a new classification for HIV-1 according to co-receptor usage (Berger *et al.,* 1998). Isolates with the ability to enter cells using CxCR4 as co-receptor correspond to rapid/high S1 isolates (renamed X4). Isolates, which uses CCR5 comprise the slow/low NSI isolates (renamed R5).

Replication cycle:

HIV begins its infection of a susceptible host cell by binding to the CD4 receptor of the host cell. CD4 is present on the surface of many lymphocytes, which plays a critical part of the body's immune system. Infection of a susceptible host cell is initiated by binding of the viral envelope protein gp120 to the CD4 receptor on the surface of the cell membrane (Dalgleish *et al.,*1984; Klatzmann *et al.,*1984). Subsequently the gp120-CD4 interaction causes conformational changes in the viral protein gp120 that contribute to the exposure of the binding sites for the cellular co-receptors (Brand *et al.,*1995).

FIGURE 3

HIV REPLICATION CYCLE



These co-receptors are the members of the chemokine receptor family and are necessary for inducing fusion between the viral and cellular membranes (Deng *et al.,*1996; Doranz *et al.,*1996; Dragic *et al.,*1996; Feng *et al.,*1996). These alterations of the gp120 molecule allow the fusogenic domain of gp41 to be exposed to the cell surface, which, in turn, leads to the fusion of, viral and host cell membranes (Freed *et al.,*1990; Weissenhorn *et al.,*1997) and thus the entry of virus into the host cell. After fusing with the target cell, the viral core is released into the cytosol. Whereas reverse transcription can initiate within the core, its disassembly appears to be essential for the progress of reverse transcription. Disassembly of the core is characterized by dissociation of the CA p24 protein (Bukrinsky *et al.,*1993; Fassati, and Goff, 2001), which is an important step in viral uncoating. Uncoating results in formation of a reverse transcription complex (RTC) comprised of HIV-1 RNA and associated viral proteins critical for reverse transcription (reverse transcriptase [RT]), nuclear targeting (matrix protein [MA], viral protein R [Vpr], and integrase [IN] along with cellular protein(s)), and integration is promoted by integrase and cellular protein(s). Reverse transcription occurs during the HIV-1 RTC transport through the cytoplasm and is mostly completed by the time the complex reaches the nucleus. Conversion of the viral genomic RNA into DNA likely leads to the dissociation of reverse transcriptase, thus further reducing the size of the complex in preparation for translocation through the nuclear pore. Upon completion of reverse transcription, the complex becomes integration-competent (Miller and Bushman, 1997) and is termed as pre-integration complex (PIC). Studies imply that a component or components of the complex contain targeting signals engage the cellular transport proteins, which direct the PIC through the nuclear pore. The integration of reverse-transcribed viral cDNA into a host chromosome is an obligatory early step in the HIV-1 life cycle in order to obtain a productive infection. The key protein player in the retroviral

integration is the 32 KDa viral integrase (IN), which enters the cell as part of the virion. The viral IN, encoded by the *pol* gene of the virus, is translated as part of a large Gag-Pol polyprotein and is processed into its mature form by the viral protease (Maele and Debyser, 2005). The viral DNA integrated into the host genome is identical in structure to the unintegrated linear molecule, except for the absence of the 2 bp from each LTR terminus at the sites of joining to host DNA (Varamus, 1982). It has been demonstrated that the linear viral DNA synthesized in the cytoplasm is the precursor to the circular DNA forms found in the nucleus (Shank and Varamus, 1978). Atleast two forms of circular viral DNA are also found in the nucleus. The most abundant form contains a single copy of the LTR, while a smaller number of circles contain two LTRs (Shank *et al.,*1978; Shank and Varamus, 1978). Studies in a number of retroviral systems have provided convincing evidence that the linear unintegrated form of viral DNA is the direct precursor to the integrated provirus (Brown *et al.,*1989; Fujiwara and Mizuuchi 1988). Once the viral DNA is integrated into the genetic material of the host, it is possible that HIV may persist in a latent state for many years. This ability of HIV to persist in certain latently infected cells is the major barrier to eradication or cure of HIV. Activation of the host cells results in the transcription of viral DNA into messenger RNA (mRNA), which is then translated into viral proteins. The new viral RNA forms the genetic material of the next generation of viruses. The viral RNA and viral proteins assemble at the cell membrane into a new virus.

HIV protease is required to process other HIV proteins into their functional forms. Following assembly at the cell surface, the virus then buds from the cell and is released to infect another cell.

Unless the HIV lifecycle is interrupted by treatment, the virus infection spreads throughout the body and results in the destruction of the body's

immune system. With current anti-viral medications, such as reverse transcriptase inhibitors and protease inhibitors, HIV infection can be contained. However, a great deal more must be achieved before AIDS epidemic is brought under control. The rapid increase of HIV infection, heterogeneity of virus phenotypes and emergence of resistance to virus against various inhibitors warranted the studies for identification of novel viral or cellular targets for control of HIV infection.

HIV-1 and cellular proteins:

Recent studies indicate that virion-bound host molecules are still functional and are affecting the biological properties of HIV-1. Therefore, these nonviral components should be considered as factors putatively affecting the pathogenesis of HIV-1 infection.

A number of studies indicate that cellular proteins aid integration of HIV-1 DNA into host cell. These cellular proteins can play a role during different steps of the integration process, including nuclear import, integrase catalysis, integration site selection and DNA gap repair. One could postulate that different DNA repair proteins may be involved in a redundant manner and in a cell-specific way during the early stage that comprises the circularization of viral cDNA and/or the final gap repair following strand transfer (Van Maele and Debyser, 2005). Acquisition of host cellular constituents is also a property of HIV-1. In fact, in addition to its virally encoded structures, HIV-1 can incorporate a vast array of host proteins while budding out from infected cells (Tremblay *et al.,* 1998).

Host range and cell cycle regulation of HIV-1:

Human immunodeficiency virus type 1 (HIV-1) replicates efficiently in nonproliferation monocytes and macrophages but not in resting primary T lymphocyte (Li *et al.,* 1993). HIV reproducibly establishes a latent infection after acute infection of T cells *in vitro*. The presence of latent reservoirs has prevented the eradication of human immunodeficiency virus (HIV) from infected patients successfully treated with anti-retroviral therapy. HIV latency occurred reproducibly, albeit with low frequency, during an acute infection (Jordan *et al.,* 2003; Hermankova *et al.,* 2003). The influence of mitosis on the kinetics of human immunodeficiency virus type 1 integration in T cells was studied. Single-round infection of cells arrested in G1b or allowed to synchronously proceed through division showing mitosis can delay virus integration until 18-24 h post infection, whereas integration reaches maximum levels by 15 h in G1b-arrested cells (Mannioui *et al.,*2004). Human immunodeficiency virus type 1 DNA nuclear import and integration are mitosis independent in cycling cells. If cycling cells are infected before S phase, viral DNA can be integrated prior to passage of the host DNA replication fork through the integration site, as indicated by stable inheritance in both daughter cells (Katz *et al.,* 2003). Diminished human immunodeficiency virus type 1 reverse transcription and nuclear transport was observed in primary macrophages arrested in early G (1) phase of the cell cycle. In primary macrophages, the intracellular nucleotide pools and other cellular factors that coincide with late G (1) phase of the cell cycle may contribute to efficient reverse transcription and nuclear localization (Kootstra *et al.,* 2000).

Cell stimulation and propagation of HIV-1 infection:

Several steps of the human immunodeficiency virus type 1 (HIV-1) virus life cycle depend on cellular activation by mitogenic stimuli (McCune, 1995). HIV-1 cannot replicate in quiescent T cells, which comprise the majority of circulating T cells in vivo (McCune, 1995; Zack *et al.,* 1990). Virus replication is blocked due to incomplete reverse transcription and lack of proviral DNA integration (Zack et, al., 1990). Upon stimulation with mitogens, such as phytohemagglutinin (PHA) or interleukin 2, reverse transcription proceeds to completion and allows integration and virus production to occur (Zack *et al.,* 1992). Mitogenic stimulation can also activate viral gene expression in cells that are latently infected and harbor integrated proviral DNA (Garcia-Blanco *et al.,* 1991, McCune 1995). In contrast to the inability to replicate in quiescent T cells, HIV-1 can replicate in non-dividing terminally differentiated macrophages (Cobb, and Goldsmith, 1996; Gallay *et al.,* 1995; Heinziger *et al.,* 1994, von Schwedler *et al.,* 1994). Thus, mitogenic stimulation is not required to allow HIV-1 replication in all contexts.

The mitogen-activated protein (MAP) kinases ERK1 and ERK2 (also known as p44/42 MAPK and hereafter referred to as MAPK) are central components of signal transduction pathways activated by diverse extra cellular stimuli. These serine and threonine kinases are present in all cell types and play a critical role in the regulation of cell proliferation and differentiation in response to mitogens and a wide variety of growth factors and cytokines (Blenis, 1993; Davis, 1993; Seger and Krebs, 1995.). Upon activation, these closely related MAPK isoforms phosphorylate a large number of substrates, including transcription factors (e.g., c-Myc, c-Jun, NF-

IL6, ATF-2, AP-1, and Elk-1), the epidermal growth factor (EGF) receptor, phospholipase A2, protein tyrosine phosphatase 2C, and cytoskeletal proteins (Blenis, 1993; Davis, 1993; Seger and Krebs, 1995).

MAPK itself is activated by phosphorylation on threonine and tyrosine residues by the MAPK kinase (also known as MEK). The best understood mechanism for activation of MAPK is via activation of Ras by growth factor receptors or tyrosine kinases. Activation of Ras induces Raf-1 targeting to the membrane, leading to activation of Raf, which then phosphorylates and activates MEK (Cobb and Goldsmith, 1996; Davis, 1993; Seger and Krebs, 1995). Ras-independent mechanisms have also been implicated in activation of MAPK (Cobb and Goldsmith, 1996). Activation of MAPK occurs during the G0/G1 transition and may be required for progression through the cell cycle (Lavoie *et al.,* 1996; Meloche, 1995; Pages, 1993; Seger and Krebs, 1995). Thus, MAPK serves to link stimuli from the cell surface to cellular events involved in proliferation and differentiation, including the cell cycle, generation of phospholipid messengers, transcription, and translation. Other MAP kinases in mammalian cells are JNK/SAPK and p38/HOG, which are activated by stress stimuli and inflammatory cytokines. Mitogens and other extra cellular stimuli that activate MAPK have been shown to activate HIV-1 replication (Fairhurst *et al.,* 1993, Garcia-Blanco and Cullen, 1991, McCune, 1995, Perkins *et al.,* 1993, Winston *et al.,* 1995).

Activation of ERK1 and ERK2 MAPK by the Ras/Raf/MEK signaling pathway plays a role in HIV-1 replication by enhancing the infectivity of HIV-1 virions. MAPK can enhance HIV-1 infectivity by phosphorylating Vif (Yang and

Gabuzda, 1998) thus suggesting the involvement of Vif-dependent as well as Vif-independent mechanisms. MAPK in the producer cell plays a role in regulation of HIV-1 replication by enhancing virion infectivity. This mechanism may contribute to the activation of HIV-1 replication when T cells are activated by mitogens and other extra cellular stimuli.

HIV-1 and Kinases:

Phosphorylation of viral proteins plays an important role in regulation of viral cycle (Bukrinskaya, 1996). Several HIV-1 proteins, including structural and accessory proteins, have been shown to be phosphorylated.

- **Vif** is phosphorylated *in vitro* and *in vivo* at 5 serine residues, 2 of these are phosphorylated by MAPK and the 3 others by another unidentified protein kinase. The phosphorylation of at least two of these sites seems to be essential for **Vif** function and HIV-1 replication (Yang *et al.,* (1996), Yang and Gabuzda (1998)). **Vif** is phosphorylated and regulated by MAPK and other yet unknown kinases (Yang *et al.,* 1996).
- The cytoplasmic domain of **Vpu** is phosphorylated by casein kinase 2 at 2 serine residues, and this phosphorylation is necessary for the degradation of CD4 in endoplasmic reticulum (Friborg *et al.,* 1995; Paul *et al.,* 1997; Schubert and Strebel, 1994; Tiganos *et al.,* 1997).
- **Nef** is phosphorylated by the protein kinase C *in vitro* (Guy *et al.,* 1987; Coates and Harris 1995) and *in vivo* (Coats *et al.,* 1997), and this phosphorylation leads to an increased down-regulation of CD4 from the cell surface (Luo *et al.,* 1997).

- **Rev** is phosphorylated *in vitro* by casein kinase 2 and mitogen-activated protein kinase (MAPK) (Hauber *et al.,* 1988, Cochrane *et al.,* 1989, Meggio *et al.,* 1996), promoting rapid formation of an efficient RNA binding state (Fouts *et al.,* 1997).
- Two forms of **Tat** are synthesized during HIV-1 infection, and only the two-exon form (Tat 86) can be phosphorylated by the preactivated double-stranded RNA-dependent kinase. Whereas the functional significance of this phosphorylation is still unclear, it has been suggested that it could promote the release of the protein from the substrate-binding region of the preactivated double-stranded RNA-dependent kinase (McMillan *et al.,* 1995).
- In mature virions, the **p55Gag precursor** is cleaved by the viral protease to form the p17Gag, p24Gag, p7Gag, and p6Gag proteins. Structural proteins, **CAp24** and **MAp17**, have shown to be phosphorylated. Phosphorylation of **MAp17** is well documented. Indeed, **MAp17** is phosphorylated on serine (Veronese *et al.,* 1988, Mervis *et al.,* 1988) and tyrosine residues (Gallay *et al.,* 1995). It has been shown that the phosphorylation of serine residues of **MAp17** would be essential to the association of the protein with the preintegration complex (Bukrinskaya *et al.,* 1996). Phosphorylation of the COOH-terminal Tyr residue (Tyr-131) of **MAp17** would be implicated in the nuclear translocation of the preintegration complex and, thus, would be essential for the infection of non dividing cells (Gallay *et al.,* 1995, 1996). However, participation of **MAp17** during the infection of non-dividing cells and the putative role of the phosphorylation of Tyr-131 in this process has been discussed by other authors (Fouchier *et al.,* 1997, Freed *et al.,* 1997, Trono and Gallay 1997, Reil *et al.,* 1998). **MAp17** has been identified as a substrate

of protein kinase C (Burnette 1993), and this result is consistent with the identification of a highly conserved consensus protein kinase C phosphorylation motif in the HIV-1 Gag protein. Recently, the ERK/MAPK has been proposed to phosphorylate **MAp17**(Jacqué *et al.,* 1998). Compared with phosphorylation of **MAp17**, phosphorylation of **CAp24** has not been studied in detail. It has only been shown that **CAp24** is phosphorylated on serine residues (Veronese *et al.,* 1988, Mervis *et al.,* 1988). However, the phosphorylation sites of **CAp24** are still unknown.

- Previous studies have shown that the HIV-1 **p17Gag, p24Gag, Vif, Vpu, Rev**, and **Nef** proteins are phosphorylated by cellular kinases in vitro and in vivo (Bandres *et al.,* 1994, Bodéus *et al.,* 1995, Burnette *et al.,* 1993, Cochrane *et al.,* 1989, Hauber *et al.,* 1988, Luo *et al.,* 1997, Veronese *et al.,* 1988, Yang 1996). **p17Gag, Nef, and Rev** are phosphorylated by protein kinase C (PKC) (Burnette *et al.,* 1993, Hauber *et al.,* 1988), and **Vpu** is phosphorylated by casein kinase II (Schubert *et al.,* 1994). **Gag MA** is phosphorylated by HIV-1 virion associated serine/threonine kinase (Bukrinskya *et al.,* 1996).

Many kinases that phosphorylate and regulate the functions of HIV-1 proteins have not yet been identified. Understanding signal transduction pathways that regulate HIV-1 replication upon mitogenic stimulation is likely to provide important insights into mechanisms of virus replication and pathogenesis.

The efficiency of HIV-1 replication depends on the infectivity of virus and the sensitivity of the host cell type. Both viral factors and host proteins together cooperatively involved in successful proviral DNA synthesis, integration of proviral DNA and production of newly replicated viruses. The infection of virus depends on various virus-coded proteins and virus associated as well as host proteins. Some reports have shown that in addition to viral protein, virus can encapsudate host specific immunogens, kinases etc. The sensitivity of host depends on the presence of appropriate receptor(s) for virus entry, successful proviral DNA-synthesis, formation of preintegrated complex, translocation to the nuclei and integration with help of active viral proteins and various unknown host proteins would help in completion of HIV-1 early replication cycle.

Topoisomerase II:

Topoisomerase II is an enzyme that plays a crucial role in DNA replication (Yang *et al.,* 1987; Snapka, 1988; Uemura *et al.,* 1987, Richter and Strausfeld, 1988) and is found to be present in high amounts in rapidly proliferating cells (Heck and Earn shah, 1986; Hsiang *et al.,* 1988, Hwang *et al.,* 1989).

Virtually every facet of nucleic acid physiology is influenced by the topological state of DNA (Wang, 1985). Three types of reactions are catalyzed by the topoisomerases. Relaxation of supercoiled DNA, interconversions between single stranded DNA rings, linking of single stranded rings of complimentary sequences. Clearly the cell's ability to regulate the topological state of DNA is imperative for its viability.

Two classes of ubiquitous enzymes the type 1 and type II topoisomerases modulate DNA topology.

a) **Topoisomerase I** can relieve torsional constraints in DNA by passing a single strand of DNA through a transient nick made in the complementary strand (Wang, 1985; Osheroff, 1989). Although type I enzyme is not required for cell survival (Uemura and Yanagida, 1984), it plays important roles in DNA replication (Goto and Wang, 1985) and transcription (Muller *et al.,* 1985; Gilmour *et al.,* 1986; Garg *et al.,* 1987)

b) **Topoisomerase II** can relieve both torsional and interlocking constraints in double stranded nucleic acids by passing an intact helix through a transient double stranded break made in a second helix (Wang, 1985; Osheroff, 1989). Topoisomerase II is essential to the Eukaryotic cell (Uemura and Yanagida, 1984; Holm *et al.,* 1985). It is required for chromosome

segregation (Uemura and Yanagida, 1984; Holm *et al.,* 1985) and maintenance of chromosome structure (Earnshaw *et al.,* 1985) and plays role in DNA replication and recombination (Wang, 1985). DNA Gyrase is a prokaryotic Topoisomerase II and its catalytic activity is same as that of eukaryotic enzyme.

Human Topoisomerase II:

Topoisomerase II is uniformly distributed along the chromosomes (Heck *et al.,* 1988; Hsiang *et al.,* 1988) and is uniquely required for segregation of completely replicated daughter molecules during mitosis (Yang *et al.,* 1987, Snapka, 1988; Uemura *et al.,* 1987; Ritcher and Straufeild, 1988) and meosis (DeVardo *et al.,* 1984; Holm *et al.,* 1985, Rose and Holm, 1993; Uemura and Yanagida, 1986).

In mammals topoisomerase II is found to be present as 170 KDa, α and 180 KDa, β isoforms (Woessner *et al.,* 1990, Coutts *et al.,* 1993). Both the isoforms show structural similarity but are genetically, immunologically and biochemicallly distinct. They show distinct cellular localization and cell cycle expression profiles. Topoisomerase II α activity is shown to be highest during the G_2/ M phase of the cell cycle (Heck *et al.,* 1988). Where as Topoisomerase II β is constant throughout the cell cycle (Woessner *et al.,* 1991). Topoisomerase II α activity is distributed in the nucleoplasm (Woessner *et al.,* 1990) in contrast to Topoisomerase II β, which is localized in the nucleolus during interphase, and in the cytoplasm during mitosis (Negri *et al.,* 1992). Both the isoforms show distinct patterns of tissue distribution.

Topoisomerase II α is shown to be higher in testes, spleen, bone marrow and liver, in which replicative cells are predominantly present.

Both the isoforms of Topoisomerase II show similar catalytic activities. But the exact roles shared by these enzymes are unknown. Topoisomerase IIβ protein differs from Topoisomerase II α in many important aspects. The genes coding for the Topoisomerase II α and β proteins map to chromosomes 17q21-22 and 3p24 and are clearly distinct. Topoisomerase II β protein is less sensitive to inhibition by intercalating agents and epipodophillotoxins than the α protein.

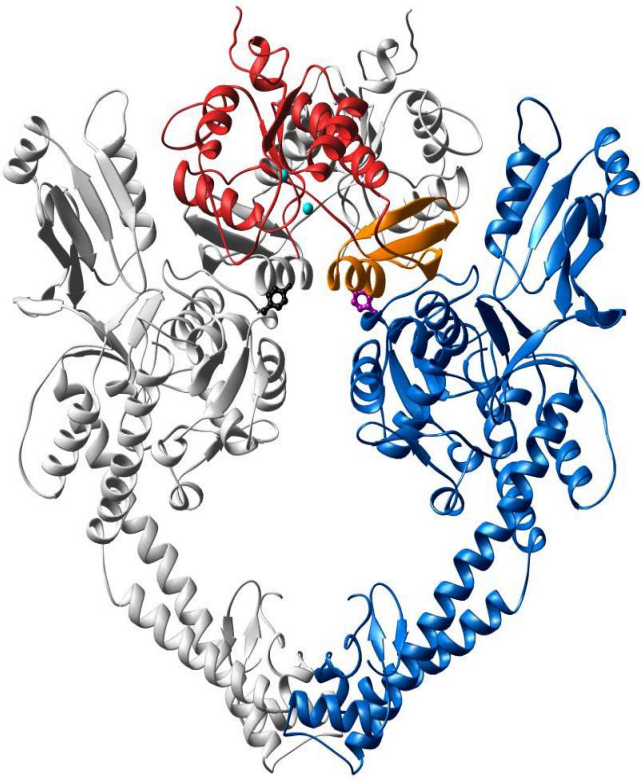
Topoisomerase II isoforms are functionally distinct. The functional aspects of Topoisomerase II β remain unknown. Significant activity of this enzyme was found in isolated nuclei from post mitotic neuronal cells (Kondapi *et al.,*2004). Topoisomerase II β is also shown to be expressed in other non-proliferative and fully differentiated tissues (Capranico *et al.,* 1992). Analysis of this enzyme during cell cycle did not show any significant alteration. Topoisomerase II β is not essential for cell proliferation and survival *in vitro* since in some cell types this enzyme is not expressed at all. These observations suggest that Topoisomerase II β is not required for maintenance of general cellular activities but involved in more specific processes in growth and development *in vivo*.

Structure of topoisomerase II:

Berger et al. reported the crystal structure of topoisomerase II in detail, in 1996(Fig 4). This study shows topoisomerase II in its active form is a heart shaped homodimer with a large central hole. The monomer is a flat crescent shaped fragment, which can be distinguished into three discrete domains. The first is the binding domain in the N-terminal region ($B^{/}$ region). It has a consensus sequence for ATP binding and has the capacity to hydrolyze ATP. This domain dimerizes with the other monomer upon binding of ATP, and imposes a conformational change all over the enzyme, required for catalytic activity. The second is the DNA binding domain or the DNA breakage/reunion domain, present in the $A^{/}$ region. The active site tyrosine's, which associate with the broken ends of DNA during the catalytic cycle, are present in this domain. The third is the primary dimer interface in the C-terminal region, which forms the dimer interface of the enzyme by associating with the other monomer. Apart from forming the dimer interface, this region is also implicated in regulation of enzyme activity and nuclear localization.

FIGURE 4

STRUCTURE OF TOPOISOMERASE II



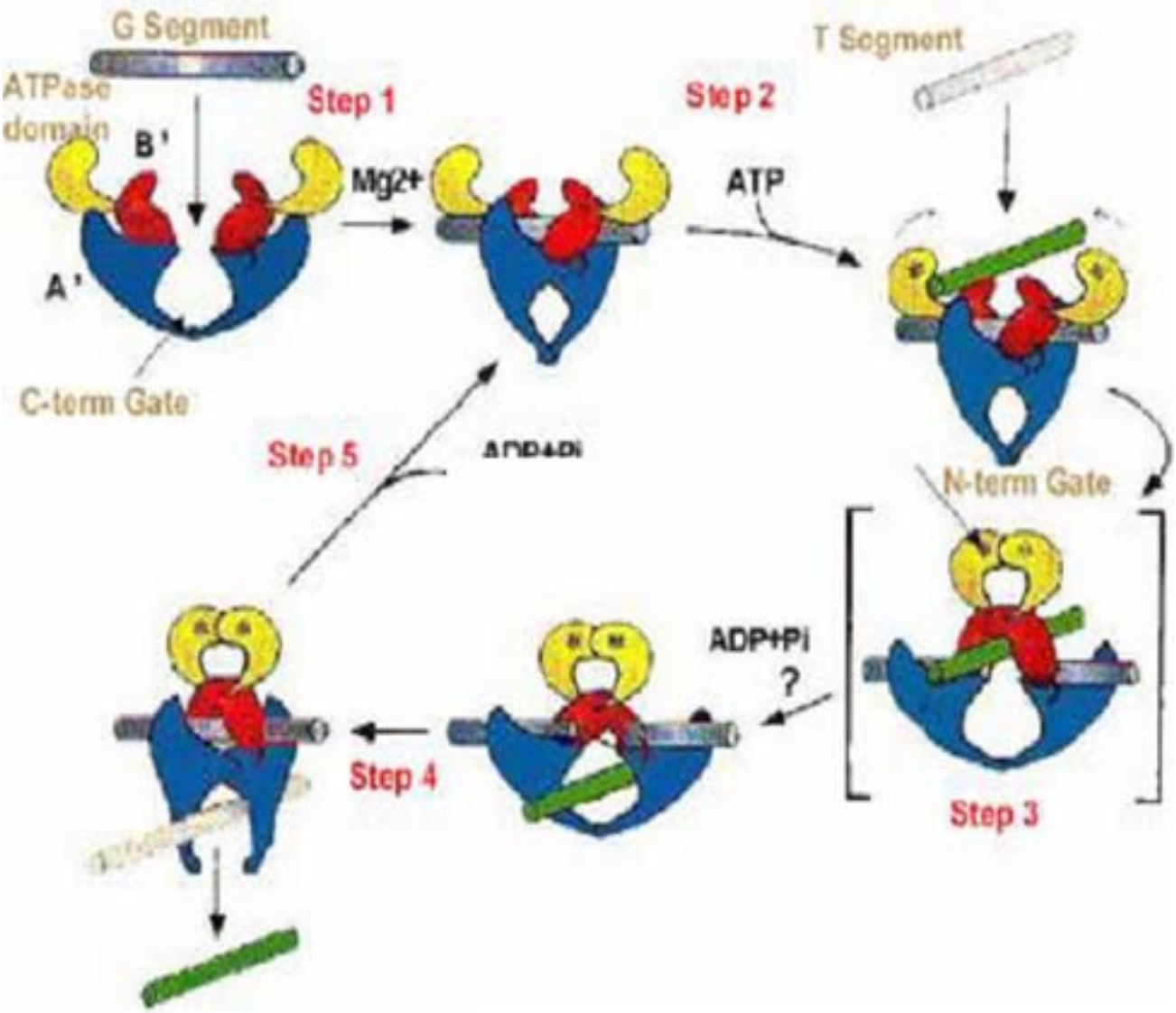
Berger *et al.*,1996

Catalytic Activity of Topoisomerase II:

In its catalytic cycle (Berger *et al.,* 1996) (Fig 5), the topoisomerase II dimer first binds to a duplex DNA segment termed as the 'G' (gated) segment and undergoes a conformational change. The enzyme binds to ATP through its ATP binding domain and also binds to a second DNA segment called the 'T' (transported) segment. This binding causes a series of conformational changes in the enzyme, which causes the $A^{/}$ regions to be pulled apart from each other, leading to cleavage of the G-segment in both the strands, four base pairs apart. The active site tyrosine in the DNA binding domains then form covalent bonds with the nicked DNA strands through a trans-esterification reaction between the phenolic hydroxyl groups of the tyrosine and the $5^{/}$-phosphoryl ends of the nicked DNA. Concomitantly, the ATP domains dimerize and the T-segment is transported through the gate formed by the nicked DNA into the central hole. Following this transport, the G-segment is rejoined by a second trans-esterification reaction and the T-segment is transported out of the enzyme through the opening formed in the dimer interface. The monomers immediately dimerize at the interface and the ATP is hydrolyzed and released. This regenerates the starting state and the enzyme is ready to begin a fresh catalytic cycle. The DNA cleavage/religation reactions do not require energy from a high-energy co-factor (like ATP) because the phosphate bond energy is conserved in the two successive trans-esterification reactions (Roca, 1995). The ATP binding and hydrolysis is only involved in introducing conformational changes in the enzyme for carrying out its catalytic functions and not for DNA nicking and resealing.

FIGURE 5

CATALYTIC CYCLE OF TOPOISOMERASE II



Model proposed by Berger *et al.*, 1996

Regulation of Topoisomerase II activity:

Topoisomerase II is found to be a major **mitotic phosphoprotein** in chromosomes. Eukaryotic Topoisomerase II cleaves at alternating purine-pyrimidine repeats. When bound to DNA, Topoisomerase II aggregation helps in mediating chromosomal condensation, presumably **via phosphorylation and dephosphorylation** of Topoisomerase II (Hirano and Mitchison, 1993).

The specific activity of Topoisomerase II increases 2-3 fold as a result of phosphorylation thus enhancing the rate of ATP hydrolysis (Corbett *et al.,* 1992, 1993b). C-terminal region is the major target for the regulatory phosphorylation of Topoisomerase II. Additional phosphorylation sites are also found on the N-terminal region. Serine1212 of topoisomerase II alpha is phosphorylated specifically during mitosis, and suggest that the serine1212-**phosphorylated topoisomerase II alpha** acts in resolving topological constraint progressively from the chromosome arm to the centromere during metaphase chromosome condensation. (Ishida *et al.,* 1994).

Functions of Topoisomerase II:

Topoisomerase II enzyme has an ability to promote topological interconversions of DNA. It plays an important role in various cellular processes such as chromosome segregation, chromosome condensation, replication, transcription, maintaining the genomic integrity and recombination.

Cell cycle regulation of phosphorylation of Topoisomerase II:

Phosphorylation of topoisomerase II is tightly regulated throughout all phases of the cell cycle and cellular growth state in eukaryotes. Cellular topoisomerase II levels are high in rapidly proliferating cells but they decrease, when cell growth is arrested or when cells are induced to differentiate. Topoisomerase II alpha phosphorylation is higher in mitotic cells than in G1 cells. Topoisomerase II alpha expression and phosphorylation is maximal during the G2/M phase of cell cycle. Topoisomerase II beta is uniformly expressed through out the cell cycle. Topoisomerase II is over expressed in cancer cells (Hsiang *et al.,* 1988; Tricoli *et al.,* 1985; Bodley *et al.,* 1987).

Role of Topoisomerase II in viral replication:

- **Herpes simplex virus** recruits topoisomerase II alpha for post-DNA synthesis expression of viral genes suggests that topoisomerase II alpha is required for untangling concatemeric DNA progeny for optimal transcription of late genes (Advani *et al.,* 2003). Inhibition of topoisomerase II by ICRF-193 prevents efficient replication of **herpes simplex virus type 1** (Hammarsten *et al.,* 1996).
- Topoisomerase II plays an essential role as a swivelase in the late stage of **SV40** chromosome replication in vitro. (Ishimi *et al.,* 1992).
- A DNA gyrase-binding site at the center of the **bacteriophage Mu** genome is required for efficient replicative transposition**.**
  (Pato *et al.,* 1990)
- DNA gyrase subunits have a role in synthesis of **bacteriophage phi X174** viral DNA. (Hamatake *et al.,* 1981).

- Topoisomerase II from **Chlorella virus** PBCV-1 is the smallest known type II topoisomerase (Lavrukhin *et al.,* 2000). It has an exceptionally high DNA cleavage activity (Fortune *et al.,* 2001).

Kinases involved in phosphorylation of Topoisomerase II:

- Ser-1106 is identified as a major phosphorylation site in the catalytic domain of topo II alpha (Chikamori *et al.,* 2003). This site lies within the consensus sequence for the acidotrophic kinases, casein kinase I and casein kinase II. Mutation of serine 1106 to alanine (S1106A) abrogates **phosphorylation of phosphopeptides** that were found to be hypophosphorylated in resistant HL-60 cells or sensitive cells treated with BAPTA-AM. Further, Chikamori *et al.,* demonstrated that Ca (2+)-regulated phosphorylation of Ser-1106 in the catalytic domain of topo II alpha modulates its enzymatic activity and sensitivity to topo II-targeting drugs.
- **Serine 1524** is a major site of phosphorylation on human topoisomerase II alpha protein in vivo and is a substrate for **casein kinase II** in vitro (Wells *et al.,* 1994). DNA topoisomerase II co-purifies with and is phosphorylated by protein kinase CKII (Redwood *et al.,* 1998, Bojanowski *et al.,* 1993, Cardenas *et al.,* 1993).
- Topoisomerase II beta residues 1099-1263 and topoisomerase II alpha residues 1078-1182 mediate the interaction with the **CKII** beta subunit, providing evidence that the leucine zipper motif and the major CKII-dependent phosphorylation sites of topoisomerase II are unnecessary for its physical binding to CKII beta. Furthermore, an analysis of DNA

relaxation activity in presence of CKII demonstrated that the CKII subunit enhances topoisomerase II activity by physical interaction with topoisomerase II. (Park *et al.,* 2001)

- An ATR-dependent G(2) checkpoint responds to inhibition of topoisomerase II and delays entry into mitosis by sustaining nuclear exclusion of cyclin B1-Cdk1 complexes. ATR-dependent inhibition of Plk1 kinase activity may be one mechanism to regulate **cyclin B1 phosphorylation** and sustain nuclear exclusion during the G (2) checkpoint response to topoisomerase II inhibition. (Deming *et al.,* 2002a).
- **Casein kinase II** also catalyzes a mitotic phosphorylation on threonine 1342 of human DNA topoisomerase II alpha, which is recognized by the 3F3/2 a mitotic specific-phosphoepitope antibody (Daum and Gorbsky 1998).
- Protein kinase **CK2** identified as a new **MPM-2** kinase able to phosphorylate an important mitotic protein, topoisomerase II alpha, on Ser-1469 (Escargueil *et al.,* 2000). **Casein kinase II**-mediated phosphorylation of topoisomerase II enhances the ATP hydrolysis of the enzyme (Corbett *et al.,* 1992).
- **Cdc2 kinase** forms stable molecular complexes with the nuclear enzyme DNA topoisomerase II, which is associated with marked stimulation of both DNA binding and catalytic activity of topoisomerase II, albeit in a phosphorylation-independent manner. The recruitment of cdc2 kinase by topoisomerase II is coupled to chromatin remodeling. (Escargueil *et al.,* 2001)

- Studies of topoisomerase II phosphorylation in HeLa cells show that heterodimers are phosphorylated in vivo to a significantly lower level compared to homodimeric alpha enzymes, but in contrast to the latter neither heterodimers nor topoisomerase II beta homodimers co-precipitate together with a kinase activity that is able to mediate their **phosphorylation**. However, both enzymes can still be phosphorylated by exogenously added **casein kinase II**. The differential phosphorylation of topoisomerase II heterodimers suggests an alternative regulation of this topoisomerase II subclass compared to the homodimeric topoisomerase II alpha counterparts (Gromova *et al.,* 1998).
- **Beta II PKC** plays a role in modulating the VP-16-induced DNA binding activity of topoisomerase II in resistant K/VP.5 cells through a mechanism linked to phosphorylation of topoisomerase II (Ritke *et al.,* 1995).
- **Protein kinase C** modulates the catalytic activity of topoisomerase II by enhancing the rate of ATP hydrolysis. The site(s) of protein kinase C modification was (were) localized to the 350 amino acid C-terminal regulatory domain of topoisomerase II within approximately 50 amino acids of the site(s) phosphorylated by casein kinase (Corbett *et al.,* 1993).
- The effect of protein tyrosine kinase inhibitors with different modes of action on topoisomerase activity and cell death is studied in CTLL-2 cells, whose growth is IL-2-dependent. The **Flavonoids genistein, biochanin A,** and **apigenin** inhibited topoisomerase II to the same

extent as **etoposide**, a specific inhibitor of the enzyme. (Azuma *et al.,* 1995)

- **Methyl 2,5-dihydroxycinnamate (2,5-MeC)** also inhibited topoisomerase II, but was less potent than **genistein**. **Herbimycin A** and **staurosporine** did not inhibit topoisomerase II. None of the inhibitors of **protein tyrosine kinases** examined inhibited topoisomerase I activity. All the inhibitors induced cell death with internucleosomal DNA fragmentation in the presence of IL-2. **Genistein, biochanin A,** and **apigenin** induced DNA fragmentation and cell death early in the incubation period and did not alter the profiles of phosphotyrosine proteins in either the lysate or pelleted fractions, indicating that the early cell death was induced by the inhibition of topoisomerase II activity rather than by the inhibition of protein tyrosine kinase activity. **2,5-MeC** similarly induced early cell death and DNA fragmentation, but to a lesser extent than **genistein** presumably due to the inhibition of topoisomerase II activity. **Herbimycin A** induced a slow increase in DNA fragmentation and cell death, accompanied by a decrease in phosphotyrosine proteins in the pelleted fraction, suggesting that the inhibition of protein tyrosine phosphorylation, presumably of the nuclear proteins, is related to cell death and DNA fragmentation**. Staurosporine**-induced DNA fragmentation appeared to be due to mechanism(s) other than the inhibition of topoisomerases and protein tyrosine kinases, since it neither altered the profiles of phosphotyrosine proteins nor inhibited topoisomerase activity.

- One or more **proline-directed kinases** are involved in the cell-cycle-dependent regulation of topoisomerase II alpha enzyme activity in human cells (Wells and Hickson 1995).
- The mitogen-activated protein (MAP) kinases, extra cellular signal-related kinase (ERK1 and ERK2) regulate cellular responses by mediating extra cellular growth signals toward cytoplasmic and nuclear targets. A potential target for ERK is topoisomerase II alpha, which becomes highly phosphorylated during mitosis and is required for several aspects of nucleic acid metabolism, including chromosome condensation and daughter chromosome separation. ERK2 regulates topoisomerase II alpha in vitro and in vivo, suggesting a potential target for the MKK/ERK pathway in the modulation of chromatin reorganization events during mitosis and in other phases of the cell cycle (Shapiro *et al.,* 1999).

Topoisomerase II and HIV:

- The genomic mapping experiments (Howard and Griffith 1993) for the Topoisomerase II cleavage sites in human genome suggests **strong Topoisomerase II binding and cleavage sites** are located at 5' LTR and gag coding regions of HIV genome. But the role of these cleavage sites in the vicinity of HIV integration sites requires a thorough investigation.
- DNA topoisomerase II may act as a new target for agents, which can augment the predisposition of individuals to HIV infection (Petmitr *et al.,* 2000). Topoisomerase sites were mapped in the 5'LTR of HIV –1 DNA at transcription initiation region and TATA box thus consistent with possible role

cleavage sites were located approximately 850bp upstream from integration site and it is a complex repeating element constituting alternate purine /pyrimidine sequences (Howard and Griffith, 1993).

- **RNA Antisense** mediated under-expression of Topoisomerase II decreased 4 to 6 fold lead to impairment in HIV-1 replication. By using PCR it has been found that no of integration events has decreased in cells although viral DNA synthesis and circularization are equivalent to that of parental ones (Bouille *et al.,* 1999).
- The two naturally occurring **lignanolides arctigenin** and **trachelogenin** inhibit replication of HIV-1 specific mRNA from a predominance of singly and multiply spliced mRNA to the production of full length HIV-1 RNA. Sodium butyrate causes inhibition of cellular histone deacetylase, which results in disruption of nucleosomes and also effects blockage by **Novobiocin** an inhibitor of Topoisomerase II (Schroder *et al.,* 1990). Infection of H9 cells with HIV-1 found to decrease the phosphorylation of DNA topoisomerase II during initial phase of infection. Inhibition of **Protein kinase C** mediated phosphorylation of DNA topoisomerase II results in an inhibition of HIV-1 production. There are two isoforms of **Protein kinase C**; the data suggests that the inhibitors, which inhibit these two forms, are also potential candidates for anti HIV therapy (Matthes *et al.,* 1990).

Against this background, the characterization and isolation of the HIV-1 associated isoform specific Topoisomerase II phosphorylating kinase activity and the study of inhibitors against these kinase activities would be very useful in development of novel anti-HIV agents.

The rationale:

The rationale of present investigation is to find the levels of Topoisomerase II alpha and beta phosphorylation immediately after virus infection and to identify the factors that promote the phosphorylation of Topoisomerase II isoforms during the course of HIV-1 infection. Such information would help in identifying the pathways that regulate activation of Topoisomerase II isoforms through phosphorylation and HIV-1 replication.

Chapter 2

Experimental Materials

EXPERIMENTAL MATERIALS

HIV-1 isolates: HIV-1_{MN}, HIV-$1_{93IN101}$ (Sub type C/ NSI&R5) was an Indian isolate obtained from the NIH-AIDS Research and Reference Reagent program, USA contributors; HIV-1_{CRI50}, an Indian subtype C isolate was isolated, propagated, and maintained at the Cancer Research Institute by

Dr Robin (unpublished information).

Mouse anti-human Topoisomerase II alpha (Clone: 31) and Topoisomerase II beta (Clone: 40) monoclonal antibodies were from BD Biosciences, USA, these antibodies showed specific reactivity to alpha and beta isoforms, we have not detected any cross reactivity of these antibodies to antigens as per the western blot analysis.

Monoclonal anti-protein kinase C (clone MC5) and anti-casein-kinase 2 α, human (clone 1AD9), ATP, PMSF, BSA, and Protein A agarose, Triton X 100, Ficoll, DTT, were from Sigma.

PVDF membrane was obtained from PALL Life Sciences, USA.

PEG 8000 from Gibco,

Butanol and di-isopropyl ether from s.d.fine-chem limited.

Mono S, Mono Q and phenyl sepharose are from Amersham Bioscience.

All other reagents and chemicals are of standard grade.

Methods

- Protein estimation was done according to the calorimetric method described by Bradford (1976).
- SDS-PAGE electrophoresis was carried out according to the procedure of Laemmli (1970).
- Western blotting was done following the procedure of Towbin *et al.,* (1979).

Buffers

10 x kinase buffer: 0.5 M Tris pH 7.5, 50% glycerol, 0.1 M $MgCl_2$ and 50mM DTT;

RIPA buffer : 50 mM Tris Cl pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA pH 7.0, 150 mM NaCl, 1 mM PMSF, 1% aprotonin 1%, pepstatin 10 µg/ml, 1 mM Na_3VO_4, 50 mM NaF;

10 x CIP buffer : 10mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM, Tris-HCl pH 8.3;

Extraction buffer: 20 mM TrisHCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol,0.1% Triton X- 100, 0.5 mM KCl, 0.5 mM PMSF and 1µg/ µl pepstatin and leupeptin;

Lysis buffer : 10 mM Tris HCl pH 7.4, 1% Triton X 100,

Relaxation buffer: 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 µg/ ml BSA, 1 mM ATP.

Buffers used in the purification of Topoisomerase II.

Buffer A : 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl, 0.34 M sucrose and 0.1 mM PMSF.

Lysis Buffer : 5 mM potassium phosphate (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol and 0.5 mM PMSF.

PR buffer : 20mM potassium phosphate (pH 7.5), 10 Mm $NaHSO_3$, 10% glycerol, 10 mM 2-mercaptoethanol and 0.5 mM PMSF.

Storage buffer : 30 mM potassium phosphate (pH 7.5), 50% glycerol, 0.1mM EDTA and o.5 mM DTT.

Buffers used in the purification of plasmid

Lysis buffer : 50 mM glucose, 25 mM tris-HCl, pH 8.0, 10 mM EDTA and 5 mg/ml lysozyme.

Alkaline solution : 0.2 N NaOH and 1% SDS, 3M Sodium Acetate solution

Tris-EDTA : 10 mM tris-HCl, pH 7.5 and 1mM EDTA.

Tris buffer saturated Phenol: Phenol was mixed with 8-hydroxyquinolin and dialysed extensively with Tris EDTA pH 8.0.

Sodium acetate Buffer : 50 mM Tris, 100mM Sodium acetate (freshly prepared)

Chapter 3

Analysis of Topoisomerase II phosphorylation during the course of HIV-1 infection

Topoisomerase II, an enzyme involved in resolving the topological constraints of DNA during its replication and recombination events. In eukaryotes Topoisomerase II is present in two isoforms α and β. The α isoform is strongly associated with DNA replication during cell division, while the exact cellular activities associated with β isoform are unknown. Studies implicate the role of Topoisomerase II in replication of both DNA and RNA viruses namely Herpes simplex virus (Advani *et al.,* 2003), SV40 (Ishimi *et al.,* 1992), bacteriophage phi X174 (Hamatake *et al.,* 1981), Chlorella virus (Lavrukhin *et al.,* 2000). In this investigation we have used HIV-1 as a model system and analysed whether the topoisomerase II activity is regulated during the course of HIV-1 infection.

The work incorporated in this chapter addresses the kinetics of appearance of active, phosphorylated Topoisomerase II α and β during the progress of HIV-1 replication. Further, analysis was carried out to monitor if observed phosphorylated topoisomerase II was associated with corresponding Topoisomerase II α and β kinase activity.

EXPERIMENTAL METHODS

In situ* phosphorylation assay*:

5 x10^7 $CD4^+$ T cells (SupT1) were incubated with ^{32}P phosphoric acid for two hours in RPMI-1640 complete medium at 37°C in a CO_2 incubator (Forma) with 5 % (±0.1) CO_2. The cells were incubated with and without HIV-$1_{93IN101}$ (10 ηg of p24 equivalent). Infection was stopped at indicated time points after infection and the cells (5 x 10^6) were centrifuged at 400-x g. The cell pellet was washed twice with the cold PBS. The washed cells were lysed with PBS containing 1% Triton X100 followed by sonication at 3x10 second pulses. Topoisomerase II α or Topoisomerase II β were immunoprecipitated in separate aliquots by addition of monoclonal antibodies and the immunoprecipitates collected by addition of 6 % protein A agarose. The protein A beads were incubated at 4°C for 15 minutes, and the supernatants were discarded. The beads were washed twice with PBS containing 0.5 % Triton X-100. Bound Topoisomerase II was eluted by 20 μl 5% TCA. The eluate was spotted on Whatman No.1 filter paper discs and dried. ^{32}P on filters was measured in scintillation fluid (0.5 gm POPOP, 5 gm PPO in 1 l of toluene) using a Wallac 1400 DSA scintillation counter. Each experiment was performed in triplicate and all data points represent an average of results from the triplicate experiments. For autoradiograms, 30μl of elute was resolved on 7.5% SDS-PAGE gels. Gels were dried and exposed to X-ray film (Indu) at –70° C overnight and developed.

Infection assay: Infection was done as described in (Kondapi *et al.,* 2002). 10^8 $CD4^+$ T-cells (SupT1) were infected with 10 ηg p24 equivalent of HIV-1 (HIV-

$1_{93IN101}$) and incubated with RPMI 1640 containing 10% FBS at 37°C in a CO_2 incubator (Forma) with 5% (±0.1) CO_2. 10^6 infected cells were aliquoted at different time intervals. The same number of cells without HIV-1 infection was used as a control. Aliquoted cells were pelleted at 400xg and washed twice with cold PBS, pH 7.4.

Cell lysate preparation: The cells were lysed in Lysis buffer, incubated on ice for 30 min, and sonicated with 3 x 10 sec pulses. The sonicated lysate was centrifuged at 10000 rpm for 10 min. The supernatants were collected and protein estimated using Bradford's method (Bradford, 1976). Topoisomerase II in lysates was dephosphorylated by incubation of lysates containing 75 μg of total protein with 1 unit of alkaline phosphatase in CIP buffer at 37°C for 30 min. The reaction was stopped by addition of phosphate to the final concentration of 10 mM. The dephosphorylated topoisomerase II was analyzed using the i*n vitro* phosphorylation assay.

Western blot analysis of topoisomerase II in cell lysates: Time course experiments were conducted as explained above for infection assays. Protein was solubilized in RIPA buffer. Lysates containing 75 μg of total protein were resolved on 7.5% SDS PAGE mini gels and transferred to PVDF membranes, then immunoblotted with a 1:1000 dilution of mouse anti-human topoisomerase II α or β or Actin antibodies and incubated with alkaline phosphatase-conjugated anti-mouse IgG antibody (1:2000 dilution in TBS) for 60 min at room temperature and then with TBS containing 0.15 % Tween. The blots were developed using

NBT- BCIP substrates in TBS and documented using an UVI-Tech gel documentation system.

Analysis of Topoisomerase II α and β mRNA: Time course experiments were conducted as discussed above for infection assays. Total RNA was isolated by standard methods. Twenty μg of total RNA was taken at each time point for further analysis. Probes corresponding to the sequence of topoisomerase II αand topoisomerase II β (Austin *et al.,* 1993) were used to screen the total RNA at different time points.

The probes were dephosphorylated and 5'- end labeled with ^{32}P, incubated with RNA in buffer containing 10 μM NaCl and 5 μM EDTA at 95°C for 5 min and at 55°C for 15 min in a Robocycler (Stratagene). RNA: DNA hybrids were isolated by addition of 5 μl of 30% (v/v) chloroform and 5μl of 5 x loading dye and the aqueous phase was loaded on 10% TBE polyacrylamide gel. The gel was dried and exposed to X-Ray film (Indu) for over night at -70^0 C and developed subsequently.

***In vitro* kinase assay:** The reaction mixture containing dephosphorylated topoisomerase II was incubated at 37°C with 10 μl of ATP (100 μM ATP) and 5 μCi of γ[^{32}P]-ATP in 1x kinase buffer for 30 minutes. Three μl (3 ηg) of topoisomerase II α or topoisomerase II β antibodies were added to the lysate. After incubation at 37°C for 30 minutes, 6% protein A agarose beads were added. The lysates were incubated at 4° C for 15 minutes with intermittent shaking, and then centrifuged at 2000 rpm for 5 minutes. The sediment was washed twice with PBS and eluted with 40 μl 5 % trichloroacetic acid (TCA). Ten

µl of elute was spotted on filter paper discs and dried and ^{32}P on filters was measured by liquid scintillation. Each experiment was performed in triplicate and all data points represent an average of results from the triplicate experiments. For autoradiograms, 30µl of elute was resolved on 7.5% SDS-PAGE gels. Gels were dried and exposed to X-ray film (Indu) at –70°C overnight and developed. In all the experiments Casein kinase II (CK-II) a cell cycle specific kinase that phosphorylate both Topoisomerase II isoforms was used as a positive control.

RESULTS

Measurement of phosphorylated topoisomerase II during HIV-1 infection: The levels of active forms of Topoisomerase II in terms of its phosphorylated form were monitored during the course of HIV-1 post infection. CD4+ T cells (Sup T1) cells were pre-incubated with ^{32}P phosphoric acid and then challenged with the virus. The infected cells were harvested during the time course of infection and Topoisomerase II isoforms present in the cell lysate was immunoprecipitated. The immunoprecipitate was extensively washed and the amount of ^{32}P incorporation in topoisomerase II isoform was estimated. The data Fig 6A shows that topoisomerase II α phosphorylation levels peak at 8 hours of post infection and also at 32 hours of post infection (p.i). In contrast, topoisomerase II β phosphorylation increases significantly early after HIV-1 infection and peaks at 4 hr post infection followed by another peak at 64 hr of post infection (Fig 6B). A control experiment of uninfected cells showed that phosphorylated Topoisomerase II α and β remained low compared to the infected one during these time points, in addition the phosphorylated Topoisomerase II isoforms in uninfected cells continuously increased and reached a limiting value (Fig 6A and B). These findings suggest that HIV-1 infection stimulates topoisomerase II α and β kinase activity early as well as late stages of infection (Fig 6B). Phosphorylation of HIV-1 coat protein p24 was estimated as a positive control (Fig 6C). To know the observed differences in Topoisomerase II phosphorylation is not due to the change in protein level, a Western blot analyses of the protein levels was carried out in these lysates using isoform-specific monoclonal antibodies, the results shown in Fig 6D suggest that the increased topoisomerase II alpha and beta

phosphorylation in HIV infected cells is not due to changes in topoisomerase II protein levels. To study the mRNA levels during the course of HIV infection northern analysis was carried out and the results shown in Fig 6E show that the levels of Topoisomerase II alpha and beta mRNA is unchanged during the course of HIV-1 infection. Through we see phosphorylation of Topoisomerase II alpha at 72 hours of post infection in auto-radiograph in Fig. 6A, but it was not confirmed in an independent immunoprecipitated experiment (showed as line plot in Fig 6A), hence we have not considered the phosphorylation of Topoisomerase II alpha is significant at 72 hours. Observed changes in the phosphorylated topoisomerase II during the course of HIV-1 infection is due to alterations in either a kinase activity or a phosphatase activity. In this investigation we examined whether it was the kinase activity that is changing during the course of HIV-1 infection. Hence Topoisomerase II alpha and beta kinase activity during the course of HIV-1 infection was analyzed using the In *vitro* kinase assay.

HIV-1 infection induces topoisomerase II α and β activity at different times of post infection: $CD4^+$ T cells (Sup T-1) were infected with $HIV\text{-}1_{93IN101}$ in RPMI complete medium and the infected cells were harvested at different time points of post infection. Topoisomerase II α and β isoforms in the lysate was immunoprecipitated separately, dephosphorylated and re-phosphorylated *in vitro* by γ^{32}P-ATP in the presence of endogenous kinase. Topoisomerase II α kinase activity increases from 8 hour of post infection and remained high till 32 hours of post infection (Fig 7 A), while topoisomerase II β kinase activity reached a peak at 4 hours post-infection followed by a minor peak at 64 hour of post infection (Fig 7 B).

DISCUSSION

Topoisomerase II α and β isoforms that originate from different loci may have distinct functions in the cellular processes. The localization of Topoisomerase II α and β in subcellular organells suggest that Topoisomerase II α strongly associated with the nucleus whereas β isoform moves away from nucleus and exhibits a reticular pattern (Woessner *et al.,* 1990). These observations suggest that α may be associated with regulation of topological changes in the nucleus, while β in the cytoplasm and other organells. The presence of Topoisomerase II binding and cleavage sites in the HIV-1 genome at its LTR and gag regions (Howard and Griffith 1993) implicates the possible interaction of Topoisomerase II α and β at these regions during certain events of HIV-1 replication. It has been shown that topoisomerase II α is present in phosphorylated form in the infected cells. In this investigation the results show that topoisomerase II phosphorylation indeed increased upon HIV-1 infection. During the course of HIV-1 infection, the increase in activities of Topoisomerase II α and β were distinctly regulated, where the activity of α isoform is involved after 8 hr of p.i while activity of β isoform is involved in prior to 8 hr of p.i. Interestingly there is an isoform specific kinase activity associated with this phosphorylation activity. Topoisomerase II beta kinase activity reached a maximum during the initial post infection period, reaching a peak at four hours, while topoisomerase II alpha kinase activity increased from eight hours of post-infection. These results suggest that HIV-1 infection promotes the phosphorylation of both isoforms. Proviral DNA is formed between 2-4 hrs after the initiation of infection at a rate of 150-180 bases/min (Karageorgos *et al.,* 1995). Linear proviral DNA is synthesized initially, followed

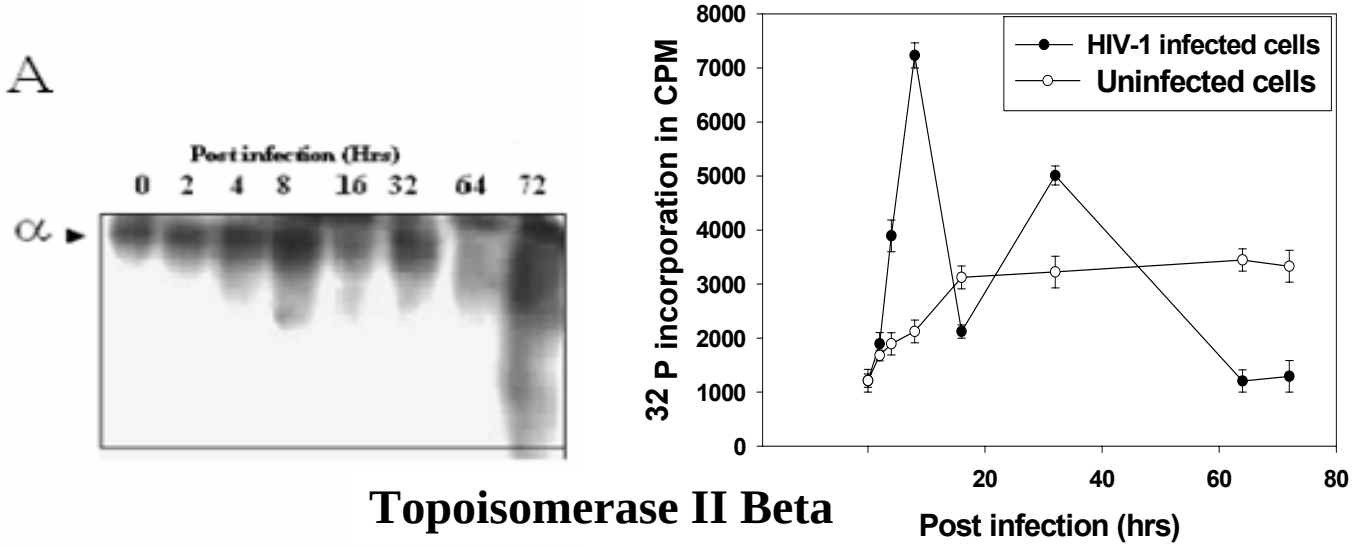
by the formation of two types of circular proviral DNA (with one or two LTRs) (Barbosa *et al.,* 1994) that are not functional for integration. Integration of the linear viral DNA starts one hour after the first appearance of proviral DNA (Vandergraff *et al.,* 2001) and is completed by 72 hrs after infection (Kok *et al.,* 2001). Interestingly, arresting the cycling of donor and acceptor cells in cell-to-cell infection at G0/G1 phases of cell cycle inhibits circularization of proviral DNA (Li, and Burrell, 1992; Kootstra *et al.,* 2000). This suggests that there may be a factor in the late G1 phase that is involved in circularization of proviral DNA. DNA synthesis is virtually completed by twelve hours after infection, while integration occurs after twelve hrs. Hence, we propose that Topoisomerase II alpha is involved in topological changes of cellular and viral DNA during viral DNA integration. In contrast, topoisomerase II β may be involved in promoting viral DNA rearrangements during viral DNA synthesis, preintegration complex formation and nuclear translocation of preintegration complexes. The enhancement of phosphorylation of Topoisomerase II isoforms at late infection periods viz. alpha at 32 hrs p.i, beta at 64 p.i, may be due to their participation in nucleic acid topological changes during chromosomal DNA re-organization and viral nucleic packaging as it has been shown that RNA molecules can also serve as substrates for Topoisomerase II beta (Wang *et al.,* 1999).

Fig. 6: Analysis of phosphorylated topoisomerase II α, topoisomerase II β and p24 in situ during time course of HIV-1 infection. The Cells were challenged with the virus in presence of ^{32}P labeled phosphoric acid. The infection was stopped at the indicated times (on X-axis) and topoisomerase II α (Panel A), topoisomerase II β (Panel B) and p24 (Panel C) was immunoprecipitated. The immunoprecipitate was washed, Tris-glycine eluted and resolved by SDS PAGE, autoradiographed, scanned and shown on left panel. An independent immunoprecipitation experiment was carried out and the P-32 in Topoisomerase II isoform was TCA eluted, counted and shown as line graph on the right panel that depicts ^{32}P incorporation (in CPM) into immunoprecipitated topoisomerase II α, topoisomerase β and p24 at different post infection time points with and without virus infection. Panel D shows a Western blot analysis of the protein levels of Topoisomerase II α and β in the lysates, Actin is shown as protein loading control. Panel E shows northern analysis of the RNA levels of Topoisomerase II α and β in the lysates.

Figure 6

Levels of phosphorylated topoisomerase II α, topoisomerase II β and p24 in situ during time course of HIV-1 post-infection

Topoisomerase II Alpha



Topoisomerase II Beta

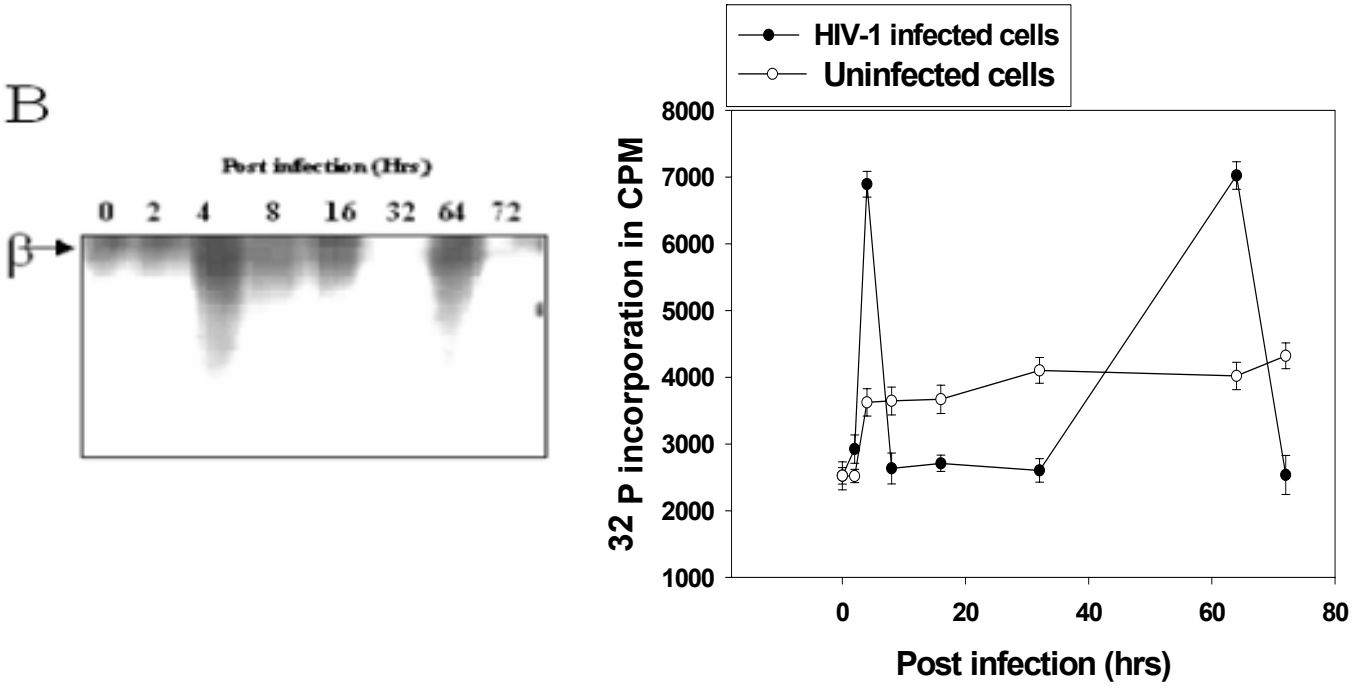


Figure 6 contd

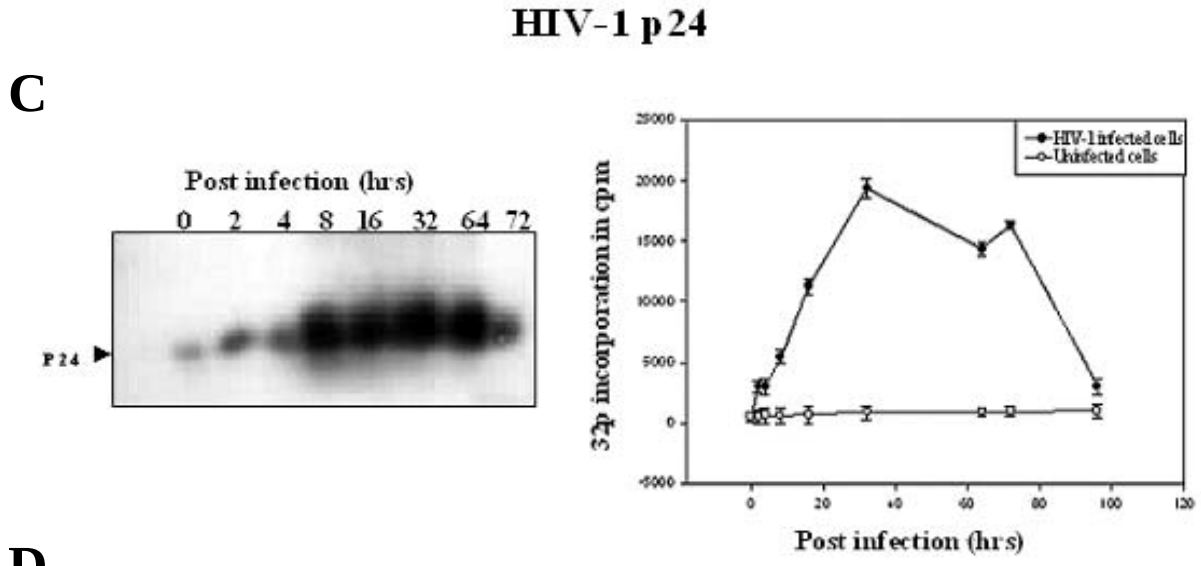
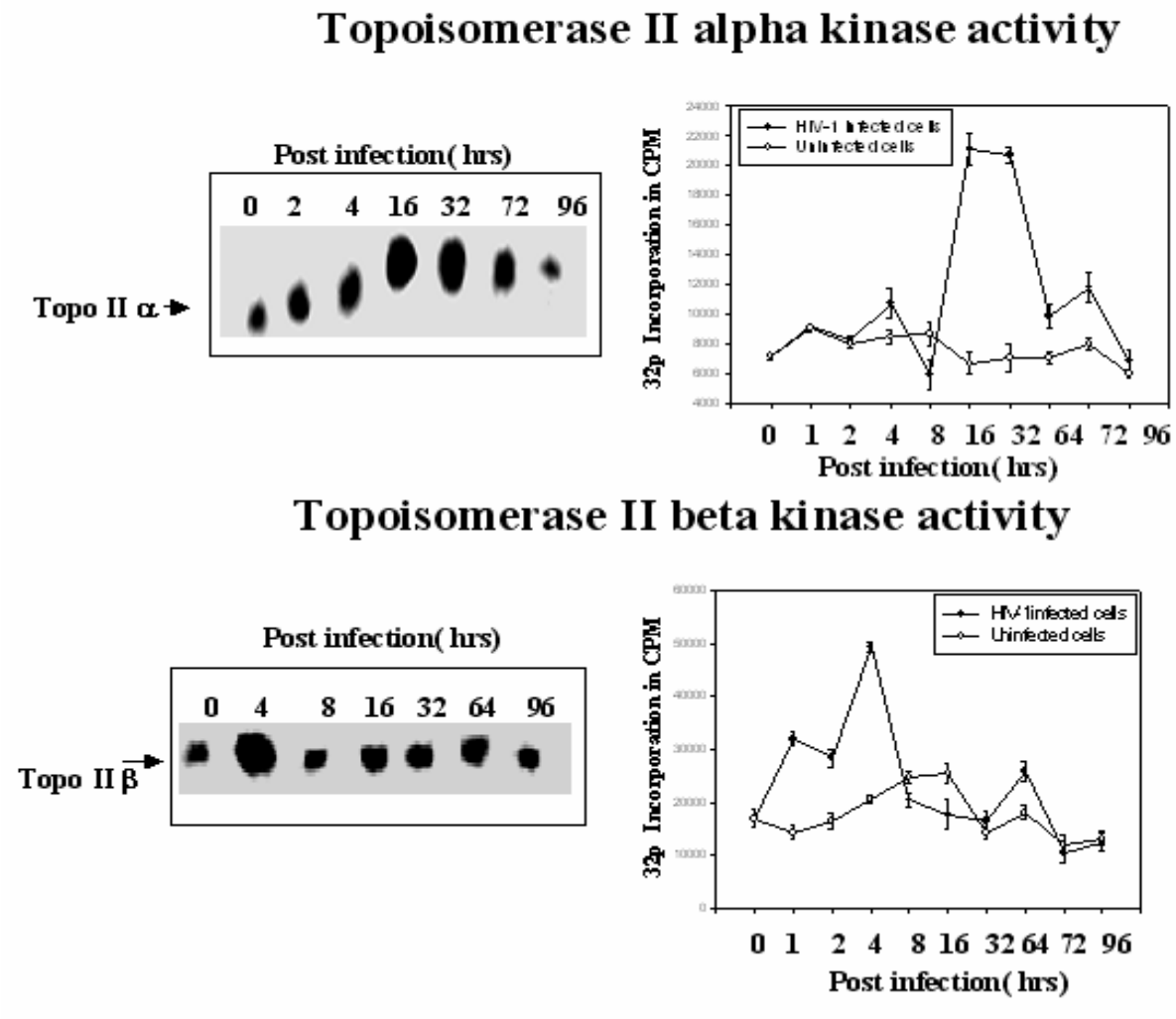


Fig. 7. Topoisomerase II α and β kinase activity during the course of HIV-1 post infection: Cells were challenged with virus and infection stopped at various time points during post infection. The kinase activities in whole cell lysates were analyzed by *in vitro* phosphorylation assay. Left panel shows an autoradiogram of an SDS-PAGE gel analyzed for kinase catalysed incorporation of ^{32}P into topoisomerase II α and β at the indicated time points during post infection. An independent immunoprecipitation experiment was carried out and the kinase catalysed P-32 incorporation of Topoisomerase II isoforms were TCA eluted, counted and shown as line graph on the right panel.

Figure 7

Topoisomerase II α and β kinase activity during the course of HIV-1 post infection



Chapter 4

Identification and Isolation of Topoisomerase II alpha and beta Kinase activities

The results in the earlier chapter showed that Topoisomerase II α and β are distinctly phosphorylated during the course of HIV-1 p.i. The changes in the phosphorylated forms concomitantly associated with corresponding isoform specific kinase activity. Such a regulation of the topoisomerase II phosphorylation during the course of HIV-1 replication may occur due to the presence of a kinase activity. The present aim is to identify the origin of the kinase activity that phosphorylated Topoisomerase II α and β during HIV-1 infection. It has been reported that the activity of Topoisomerase II during various cellular processes was strongly regulated by Casein Kinase II and Protein Kinase C (Ackerman *et al.,* 1985, Sahyoun ***et al.,*** 1986). To verify whether Topoisomerase II α and β phosphorylation in infected ells is also regulated by these Kinases, we have immunoprecipitated both the kinases from infected cell lysates and measured the Topoisomerase II kinase activity. The results in Fig 8 showed that infected cell lysate retains the Topoisomerase II phosphorylation activity even in the absence of Casein Kinase II and Protein Kinase C, Thus suggesting that there may be distinct kinase activities that may be associated with viral infection. Since HIV-1 particles known to posses both viral coded and cellular proteins, in this chapter the purified HIV-1 viral particles were analysed for Topoisomerase II α and β kinase activities.

The objectives addressed were:

1. Analysis of purified virus for the Topoisomerase II α and β kinase activity.
2. Isolation of the Topoisomerase II α and β kinase activities from virus preparation.

EXPERIMENTAL METHODS

Purification of topoisomerase II α: Topoisomerase II α was purified as described by Galande and Muniyappa (Galande, S., and Muniyappa K., 1996) from rat liver or testis tissues, which are abundant in Topoisomerase alpha isoform. In brief, the procedure involves the isolation of enriched nuclei for minimization of protease action. Topoisomerase II was isolated from the nuclei by polymin P precipitation of chromatin followed by salt extraction of proteins and ammonium sulfate precipitation. The ammonium sulphate fraction was purified by hydroxyapatite column chromatography. All steps were carried out in a cold room at 4^0 C. The protein-containing fraction was extensively dialyzed in PBS (pH 7.3) and used as source of topoisomerase IIα.

Procedure:

Isolation of enriched nuclei:

- 400 gm liver from 2 month old rats was washed twice using ice cold saline, minced thoroughly and homogenized in 2.5 liters of buffer A. The homogenate is incubated at 4°C for 1 hr. Then the homogenate is centrifuged at 5,000 rpm for 10 min. The pellet is suspended in 700 ml of buffer A containing 2.2 M sucrose and the supernatant was discarded. Enriched nuclei were subjected to ultra centrifugation of the reconstituted pellet at 28,000 rpm for 1hour in a Beckman Ti-70 rotor. The nuclear pellet was washed once at 15,000 rpm with 200ml of buffer A containing 1M sucrose then by 200ml of buffer A containing 1 M sucrose followed by 200ml of buffer A with 0.1% triton X-100.

- **Lysis of Nuclei:**

  The nuclear pellet was resuspended in lysis buffer and subjected to lysis in an MSE sonicator with a macroprobe for 4 times, 30sec. pulses with two min intervals.

- **Polymin P precipitation:**

  10% Polymin P (pH 7.8) was added slowly to the lysate, while stirring to a final concentration of 0.35% during a period of 15 min. The precipitate was pelleted at 6000 rpm for 10 min. The pellet was resuspended in 200 ml of PR buffer. Proteins were extracted from the chromatin-Polymin P complex with 0.55M NaCl, while stirring for 30 min. Nucleic acid precipitate was removed by centrifugation and the supernatant was filtered with glass wool.

- **Ammonium sulfate precipitation and dialysis:**

  The clarified supernatant was subjected to ammonium sulfate (60%) precipitation with continuous stirring for 1 hour. The precipitate was collected by centrifugation at 12,000 rpm for 20 min. The pellet was resuspended in 100 ml of PR buffer and dialyzed against 3 X 1 litres of the same buffer over a period of 15 hours. A precipitate formed during the dialysis, was removed by centrifugation at 26,000 rpm for 20 min.

  The ammonium sulfate fraction was purified by hydroxyapatite column chromatography. All steps were carried out in a cold room at 4^0 C. The protein-containing fraction was extensively dialyzed in PBS (pH 7.3) and used as source of topoisomerase IIα.

Purification of Topoisomerase II β: Topoisomerase II β was isolated from young rat cerebellum (<10 days), this source contains highest Topoisomerase II beta isoform (Kondapi, *et al.,* 2004). In brief, the cerebellum tissue was homogenized in extraction buffer, incubated at 4^{o}C for an hour and centrifuged at 100,000-x g for an hour in an ultracentrifuge. The supernatant containing the cytosolic and nuclear proteins was precipitated with ammonium sulphate. The ammonium sulfate precipitate was purified by hydroxyapatite chromatography. Topoisomerase II β containing elute was pooled and dephosphorylated with alkaline phosphatase and dephosphorylated Topoisomerase II isoforms were used in all subsequent experiments.

Dephosphorylation of Topoisomerase II: Since Topoisomerase II isoforms are present as phosphoproteins; the topoisomerase II isoforms present in the sample was brought to a basal phosphorylation level by dephosphorylation using calf intestinal alkaline phosphatase. One µg of the enzyme (topoisomerase II α and β) was dephosphorylated by adding 0.02 units of alkaline phosphatase in 4 μl of 10x CIP buffer and incubated at 37^{0} C for 30 min. The reaction was terminated with 2μl of PBS (pH 7.3) and orthovanedate. The dephosphorylated topoisomerase II was used for all phosphorylation studies.

Purification of pRYG negatively supercoiled plasmid DNA:

The negatively supercoiled pRYG plasmid DNA was purified from the E.coli HB101 strain containing the plasmid, using the alkaline lysis procedure of Wang and Rossman (1994). The procedure described is for a 1litre culture,

which can be scaled up to 4 liters. An overnight culture of the plasmid containing bacteria (grown in the presence of 100 μg/ml ampicillin) was used for purification of the plasmid.

Procedure:

- **Bacterial cell growth and harvesting:**

25 ml of LB broth was inoculated with a single bacterial colony containing the plasmid. The culture was grown in a shaking incubator for 8 h at 37^0C. This culture was used for inoculating 1 litre of LB broth. The 1-liter culture was grown overnight (12 -14 h) at 37 ^{0}C in a shaker incubator. The purification procedures were carried out at 4 ^{0}C. Cells were harvested by centrifugation at 5000 rpm for 10 min. The cells were lysed with 40 ml of lysis buffer by constant stirring over a period of 15 min.

- **Alkaline lysis:**

80 ml of freshly prepared alkaline solution was added and the constituents were mixed by swirling in a bottle. The mixture was placed on ice for 10 min. 50 ml of freshly prepared saturated ammonium acetate solution was added gently against the walls of the bottle. The bottle was placed on ice for 10 min. The precipitated proteins were removed by centrifugation at 12,000 rpm. The supernatant was clarified by filtering it through glass wool. Ice-cold isopropanol (0.7 volume) was added to the supernatant and placed on ice for 20 min.

- **Phenol-chloroform extraction of DNA:**

The precipitated DNA was pelleted at 12,000 rpm. The supernatant was removed and the pellet allowed for drying. This pellet was dissolved in 40 ml of sodium acetate buffer. After 5 min on ice, an equal volume of Tris saturated phenol and chloroform was added and vortex mixed for 2 min in 50 ml tubes. The tubes were centrifuged at 12,000 rpm for 10 min. The aqueous phase was taken in an autoclaved conical flask and the phenol phase was removed.

- **Precipitation and dissolution of DNA:**

The aqueous phase containing the DNA was treated with 0.7 volume of ice-cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 4.2) and placed on ice for 20 min. The DNA was pelleted and washed twice with ice-cold ethanol (70%). The pellet was dissolved in a proper volume of Tris-EDTA buffer.

Topoisomerase II activity assay to determine the activity of isolated topoisomerase II: This assay was performed following the procedure of Osheroff et al. (1983). The reaction mixture (50 μl) contained relaxation buffer (50 mM Tris-HCl pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 Mm DTT, 10mM MgCl2, 30 μg/ml BSA, 1mM ATP), 0.6 μg of negatively supercoiled pRYG plasmid DNA. The reaction was initiated by adding immunoprecipitated Topoisomerase II and incubated at 37^{o}C for 30 min. The reaction was stopped by adding 2 μl of 10% SDS. To this 3μl of loading dye (0.5% bromo-phenol blue, 0.5% Xylene cyanol, 60% sucrose, 10 mM Tris-HCl, pH 8.0) was added

and the products were separated on a 1% agarose gel in 0.5XTAE buffer (20mM tris-acetate, 0.5 mM EDTA) at 50V for 8h. The gel was stained with ethidium bromide, visualized using photodyne UV transilluminator and photographed. The control assay carried out using purified Topo II α and β is shown in Fig 9.

Purification of virions by ultra centrifugation: Culture supernatants of HIV-1_{CRI50} infected cells were filtered through 0.22-micron filters and virus pelleted in glycerol by ultra centrifugation at 30,000 rpm for 1 hour 30 minutes. The virus pellet was lysed with PBS containing 1% Triton-X100.

Analysis of Topoisomerase II Kinase activity in HIV-1 viral lysates: One μg of dephosphorylated topoisomerase II α or β was incubated with increasing concentrations of HIV-1 viral lysate and the extent of phosphorylation studied using the i*n vitro* kinase assay.

Glycerol gradient fractionation of HIV-1 viral lysates: 50 μg of protein from HIV-1 viral lysate was layered on a 10 ml 10-50% (v/v) glycerol gradient and centrifuged in a Hitachi swinging bucket rotor (No T40ST1488) using Sorvall ultracentrifuge at 40,000 rpm for 6 hours. 250 μl fractions were collected from the top and topoisomerase II α kinase activity was measured. Fractions with high levels of activity were collected and labeled as pool 1 and pool 2.

Virus isolation for partial purification: We have used PEG precipitated virus for partial purification studies. Virus supernatant was filtered through 0.22 μm filter and was PEG precipitated with 30% of PEG 8000 overnight at 4°C. The precipitate was pelleted at 800-x g for 20 min at 4°C.The pellet was resuspended in PBS containing 1% Triton X100, and dialyzed extensively using dialysis tubing with 12,000 Da molecular weight cutoff. The dialysate was centrifuged at 10,000 rpm and the protein was estimated using the method of Bradford (Bradford, 1976). This protein (viral protein, VP) was delipidated and used for partial purification of kinase. Uninfected cell supernatant (labeled as UCSP) processed same as above and was used as negative control in all the experiments.

Delipidation of viral proteins: 100 μg of the above viral protein was delipidated to remove the lipids associated with the protein. One volume of viral protein was added to two volumes of a mixture of butanol/di-isopropyl ether (40/60, v/v) (Cham, and Knowles, 1976). The vials were tightly closed and fastened on a mechanical rotator providing end-over-end rotation at about 30 rpm for 30 min at 4°C. After extraction, the mixture is centrifuged at low speed (2000 rpm) for 2 min to separate the aqueous and organic phase. The aqueous phase containing the delipidated proteins was removed by careful suction with a syringe.

Mono S column chromatography: Column containing 1 ml of Mono S (Amersham) was washed and then equilibrated according to supplier instructions. 100 μg of delipidated viral protein was taken and loaded onto the

pre-equilibrated Mono S Column. The Column was washed with 50 bed volumes of equilibration buffer containing 100 mM phosphate buffer pH 7. Bound protein was eluted with increasing concentrations of NaCl (10 mM to 1 M). Elutes were dialyzed extensively against PBS and the activity of fractions was determined using *in vitro* kinase assay. Active fractions were pooled and pooled fractions were stored at -70°C.

Mono Q column chromatography: Column containing 1 ml of Mono Q (Amersham) was washed and then equilibrated according to the supplier instructions. 100 μg of delipidated viral protein was loaded on to the pre-equilibrated Mono Q Column. Column was washed with 50 bed volumes of equilibration buffer containing 100mM Tris pH 7. Bound protein was eluted with increasing concentrations of NaCl (10mM to 1M). Elutes were dialyzed extensively against PBS and the activity of the fractions was determined using *in vitro* kinase assay. Active fractions were pooled and pooled fractions were stored at -70°C.

Phenyl sepharose chromatography: 100 μg of delipidated viral protein was loaded onto phenyl sepharose **c**olumn. The Column was washed with 50 bed volumes of PBS pH 7and eluted with increasing concentrations of isopropanol (v/v %). Elutes were dialyzed extensively against PBS and the activity of the fractions was determined using *in vitro* kinase assay. Active fractions were pooled and pooled fractions were stored at -70°C.

Mono S phenyl sepharose column chromatography: The Mono S bound protein eluted as above using 400mM NaCl was dialyzed extensively against PBS and subjected to phenyl sepharose column chromatography. Phenyl sepharose bound proteins were eluted with increasing percentages of Isopropanol (v/v). Elutes were dialyzed extensively against PBS and the activity of the fractions was determined using *in vitro* kinase assay.

Mono Q phenyl sepharose column chromatography: The Mono Q bound protein eluted at 800mM was extensively dialyzed against PBS and subjected to phenyl sepharose column chromatography. Phenyl sepharose bound proteins were eluted with increasing percentages of Isopropanol (v/v). Elutes were dialyzed extensively against PBS and the activity of the fractions was determined using *in vitro* kinase assay.

DNA relaxation assay: Dephosphorylated topoisomerase II isoforms were treated by incubation with viral lysates / purified viral protein fraction in kinase buffer at 30^{o}C for 30 min. The re-phosphorylated Topoisomerase II isoforms were immunoprecipitated by addition of the corresponding isoform-specific monoclonal antibodies. The immunoprecipitates were washed twice with PBS containing 0.1% Tween 20. Enzyme activity in immunoprecipitates was measured as described in Kondapi et. al (2004). Briefly, immunoprecipitates were incubated with 0.6 μg of negatively supercoiled pRYG plasmid DNA in relaxation buffer and incubated at 37^{o}C for 30 min. The reaction was stopped with 2 μl of 10% SDS. The enzymatic activity was measured by the extent of conversion of supercoiled DNA into the relaxed form. The DNA bands were

resolved on 1% agarose gel, the gel was ethidium bromide stained, and documented under ultraviolet light with a UVI tech gel documentation system. Supercoiled DNA was quantified and the results were plotted to determine enzyme activity.

RESULTS

Purified HIV-1 viral lysates possess topoisomerase II kinase activity: Dephosphorylated Topoisomerase II α and β were incubated with increasing concentrations of viral protein prepared from HIV-1 virus that was purified by ultra centrifugation. The results in Fig 10A show that topoisomerase II α phosphorylation increased with the increasing concentrations of viral protein, while Topoisomerase II β phosphorylation was marginally increased (Fig 10 B). These results suggest that topoisomerase II kinase activity is present in HIV-1 virions. The lower beta kinase activity observed in these experiments could be due to the presence another substrate for the beta kinase activity that is present in the virus lysate that may be competitively affecting beta phosphorylation if whole virus lysate is used. To resolve this problem, we have fractionated the virus lysate using a gradient centrifugation.

Glycerol fractionation of HIV-1 viral lysates separates distinctly different Topoisomerase II kinase activities: To determine whether HIV-1 virions contain single or multiple species of topoisomerase II kinase activity, the proteins in the viral lysates were fractionated on 10-50% glycerol gradients and fractions were analyzed for topoisomerase II kinase activity using the *in vitro* kinase assay (Fig.11 A). We found two distinct peaks of topoisomerase II kinase activity centered on fractions 6 and 19 (Fig 11 B and C). These fractions were pooled separately into pool 1 (fractions 6-9) and pool 2 (fractions 19-21) and assayed. Both pool 1 and pool 2 have catalyzed phosphorylation of Topoisomerase II α, while pool 2 is predominantly catalyzed phosphorylation Topoisomerase II β (Fig12). These results suggest that HIV-1 virions indeed contain two distinct topoisomerase II isoform-specific kinase activities.

Isolation of Topoisomerase II α and β kinase activities: The results of the analysis of gradient fractionation of proteins in virus lysate pointed out the presence of two distinct Topoisomerase II isoform specific activities in HIV-1 viral lysate. To validate these observations, we have carried out the isolation of Topoisomerase II α and β kinase activities from the PEG precipitated HIV-$1_{93IN101.}$ Since the virus particle carries lipids and to reduce the interference of the lipids in the chromatographic separations as well as to avoid their non-specific action, the virus lysate was delipidated using the procedure of Cham and Knowles (1976). A control experiment of comparative analysis of the phosphorylation activity of viral proteins with and without delipidation shows that the method of delipidation does not affect topoisomerase II phosphorylating kinase activity (Fig 13). Hence we used delipidated viral protein for all chromatographic separation experiments. The results of cationic and anionic exchange chromatographic separation of delipidated viral protein shows a single Topoisomerase II α kinase species can be eluted from Mono Q chromatography (Fig 14 A), while the purification using Mono S ion exchange chromatography shows the elution of both Topoisomerase II α and β kinase activities (Fig 15A). Further, Topoisomerase II α kinase activity also possesses affinity to hydrophobic phenyl-sepharose (Fig 16A). When the isolation of Topoisomerase II kinase activity was performed using two-steps, first, using an ion exchange (Mono S/Mono Q), followed by a phenyl-sepharose chromatography, the results in Figs 14B and 15B show that Topoisomerase II α and Topoisomerase II β kinase activities can be isolated using the two-step method. The Mono S bound kinase activity was eluted at higher isopropanol concentration compared to Mono Q bound kinase activity

suggesting that HIV-1 virion possess two kinase activities with distinct hydrophobic character that can phosphorylate Topoisomerase II α and Topoisomerase II β. The kinase activity purified by cation exchange chromatography possesses higher hydrophobicity with significant Topoisomerase II β kinase activity (Fig 15B) than the anionic exchange chromatography (Fig 16A). These results strongly prove that HIV-1 virus carries two distinctly different Topoisomerase II isoform-specific kinase activities.

Catalytic activity and phosphorylation of Topoisomerase II α and β in presence of virus-associated kinases were correlated: To examine whether phosphorylation of topoisomerase II α and β promoted by virus associated kinases indeed modify the catalytic activity of the enzymes. The phosphorylation of Topoisomerase II was done in the presence of isolated kinases from virus lysate (Fig 16B and 16C). The phosphorylated enzyme was used to monitor the catalytic activity of topoisomerase II α and β using supercoiled DNA as substrate. The disappearance of supercoiled DNA was measured for the catalytic activity. The results in Fig 17 show that enzymatic activity was highest with topoisomerase II α phosphorylated with kinase isolated form Mono Q chromatography compared to that isolated using Mono S chromatography (Fig 14 A & B). In contrast, the enzymatic activity of topoisomerase II β phosphorylated with kinase isolated from Mono S and Mono S-phenyl sepharose chromatographic methods was the highest compared to that isolated from Mono Q chromatography (Fig. 15 A & B).

DISCUSSION

HIV-1 viral capsid is composed of RNA and viral proteins (Korber et aI., 1998). The viruses were also reported to contain cellular proteins (Tremblay, *et al.,* 1998). Since infected cells posses distinct Topoisomerase II kinase than CKII and PKC HIV-1 viral particles were purified and analysed for Topoisomerase II kinase activity using purified Topoisomerase II α and β. The results of the experiments using purified viral lysate showed that HIV-1 virus itself contain α and β kinase activities. To determine whether viral lysates contain a single molecular species of topoisomerase II kinase activity, the HIV-1 viral lysates were fractionated on isopycnic glycerol gradients. Analysis of these fractions showed that viral lysates contain two distinct molecular species of topoisomerase II kinase activity, one at a lower density that phosphorylated topoisomerase II alpha, and the other at higher density that predominantly phosphorylated topoisomerase II beta. Hence, HIV-1 virions may contain two distinct topoisomerase II kinase activities, and these may activate topoisomerase II during HIV-1 infection. The presence of two distinct Topoisomerase II α and β kinase activities in the virion was further confirmed by the isolation of the kinase activities using ion exchange and phenyl sepharose chromatography.

The one isolated from cation exchange chromatography of higher hydrophobic activity possess both Topoisomerase II alpha and beta kinase activities. On the other hand, the second one isolated from anionic exchange chromatography of lower hydrophobicity showed higher phosphorylation of alpha isoform compared to beta isoform. Furthermore, the distinct

Topoisomerase II α and β phosphorylation by the isolated viral kinases was correlated with the catalytic activities of the enzyme, wherein Topoisomerase II α catalytic activities was maximal when phosphorylated with Mono Q bound kinase activity, while Topoisomerase II β activity was maximum when phosphorylated with Mono S isolated kinase activity. These results suggest that Topoisomerase II alpha and beta kinase activities that were observed in HIV-1 infected cells was present in purified virus, and it was of different molecular species, with distinct sedimentation rate or molecular weight.

Fig 8: Analysis of the phosphorylation activity of Topoisomerase II in the absence of CKII and PKC: HIV-1 infected and uninfected cell lysates were analyzed for Topoisomerase II phosphorylation activity in the absence of CKII, PKC and both the kinases as represented.

Figure 8

Analysis of the phosphorylation activity of Topoisomerase II in the absence of CKII and PKC

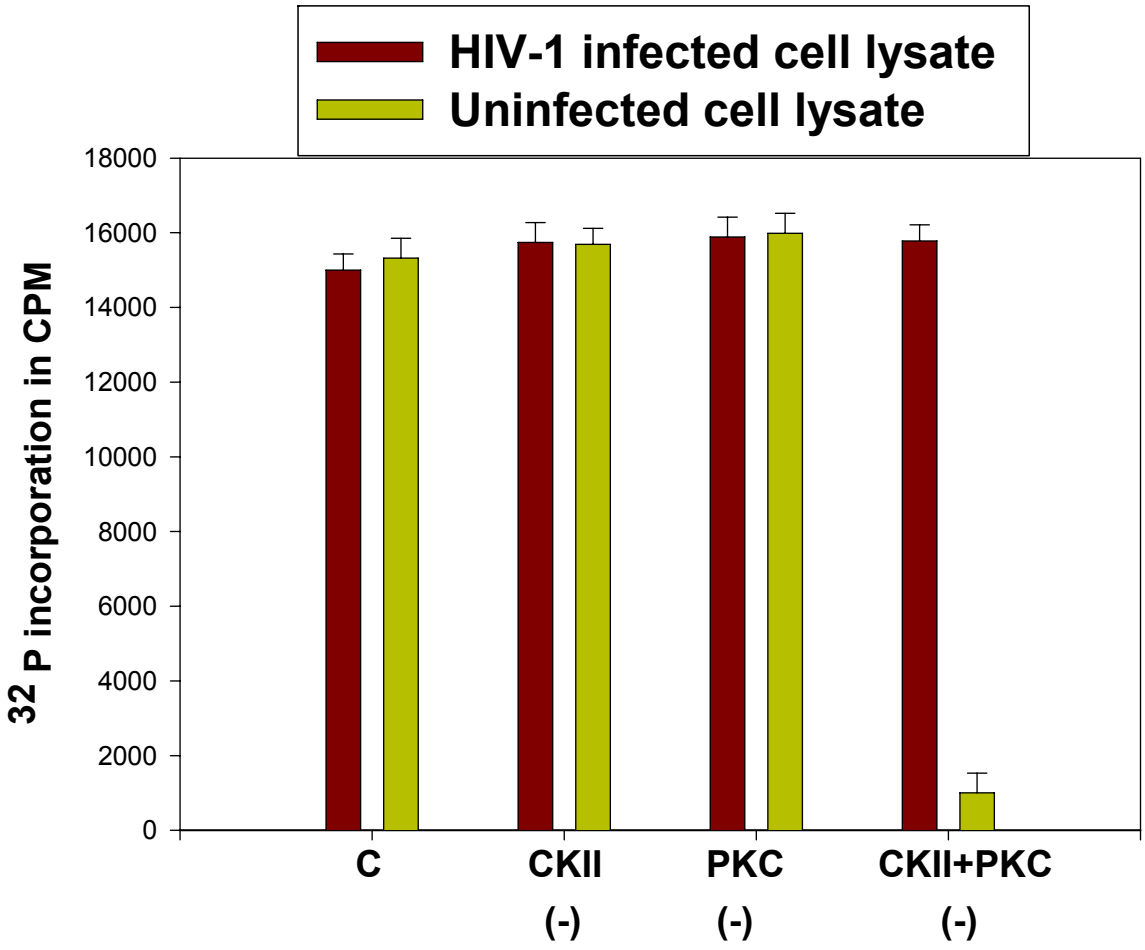
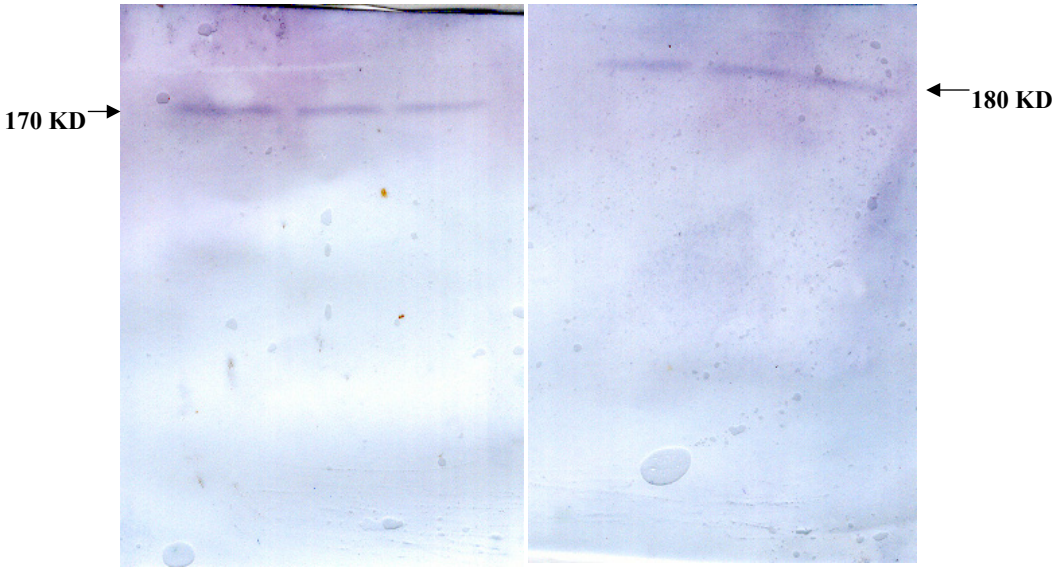


Fig 9: Isolated Topoisomerase II: Panel A shows western analysis for the isolated topoisomerase II alpha and beta as detected by the isoform specific antibodies. Panel B shows enzyme catalysed relaxation assay in the presence and in the absence of purified topoisomerase II alpha and beta as indicated.

Figure 9

A

Isolated topoisomerase II alpha and beta as detected by Isoform specific antibody



B

Activity of Isolated topoisomerase II alpha and beta

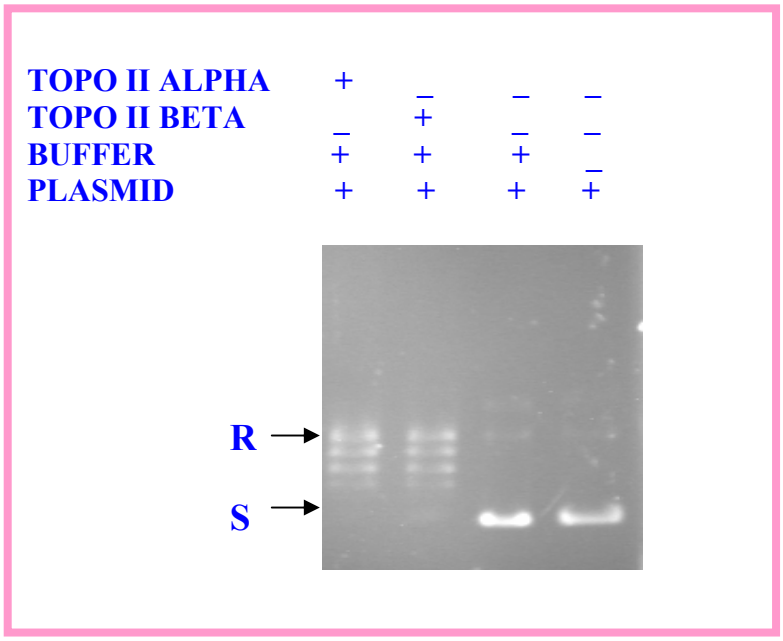
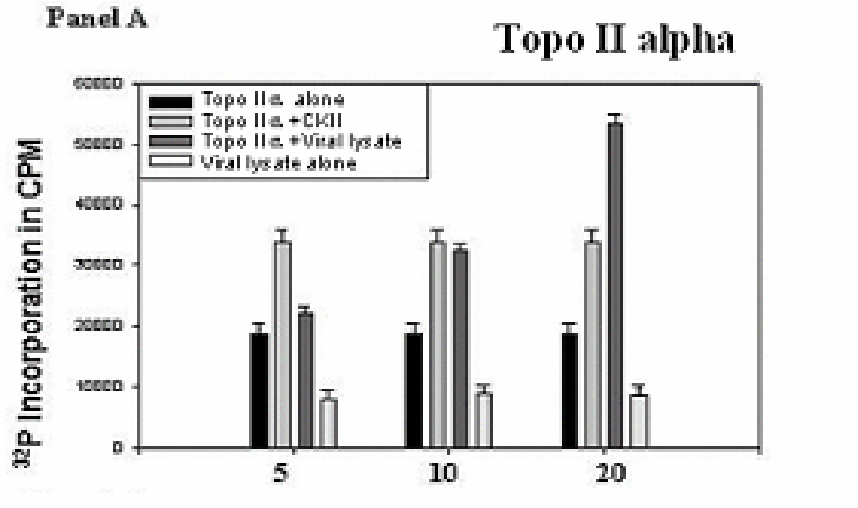


Fig 10: Phosphorylation profiles of topoisomerase II α and β in the presence of viral lysatẽs: Topoisomerase II α and β incubated with increasing concentrations of HIV-1 viral lysate and the kinase activity was analysed. Panel A shows the phosphorylation profiles for topoisomerase II α . Panel B shows the phosphorylation profiles for topoisomerase II β.

Figure 10

Topoisomerase II phosphorylation with increasing concentrations of viral protein



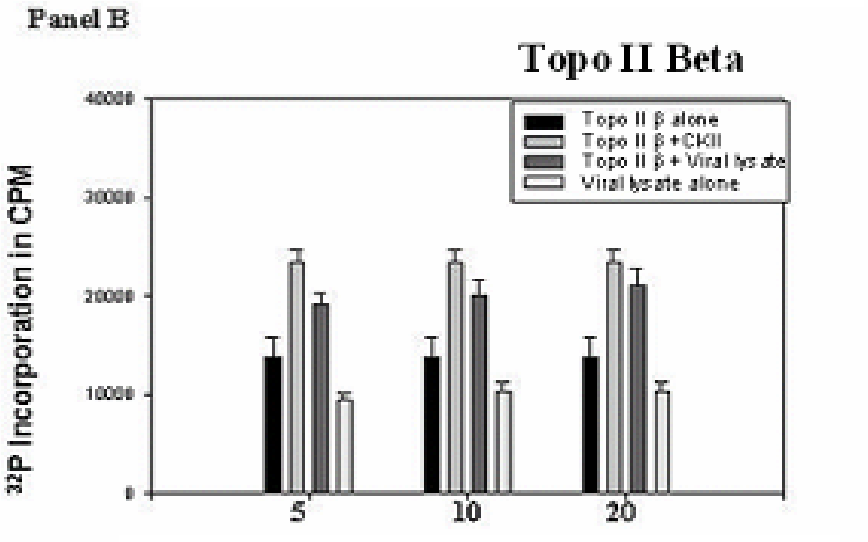


Fig 11: Phosphorylation of topoisomerase II α by glycerol gradient fractions of HIV-1 lysate. HIV-1 isopycnic glycerol gradient fractions were incubated with topoisomerase II α and assayed for phosphorylation. Panel A and B shows ^{32}P incorporation (in CPM) into immunoprecipitated Topoisomerase II α on the (Y axis) in the presence of fractions from 10-50% glycerol gradients of HIV-1 viral lysates. Samples Nos 1-11 contained topoisomerase II alone (C), topoisomerase II + Casein kinase II (CKII), topoisomerase II + viral lysate (V), or topoisomerase II + fractions 5, 6, 9, 19, 21, respectively, from the glycerol gradient. Panel C shows an autoradiogram of an SDS-PAGE gel containing topoisomerase II α labeled with ^{32}P: topoisomerase II alone(C), topoisomerase II +ckII (CKII), topoisomerase II + viral lysate (V), and topoisomerase II + fractions 5, 6, 9, 19, and 21, respectively, from the glycerol gradient.

Figure 11

Glycerol gradient fractionation of viral proteins

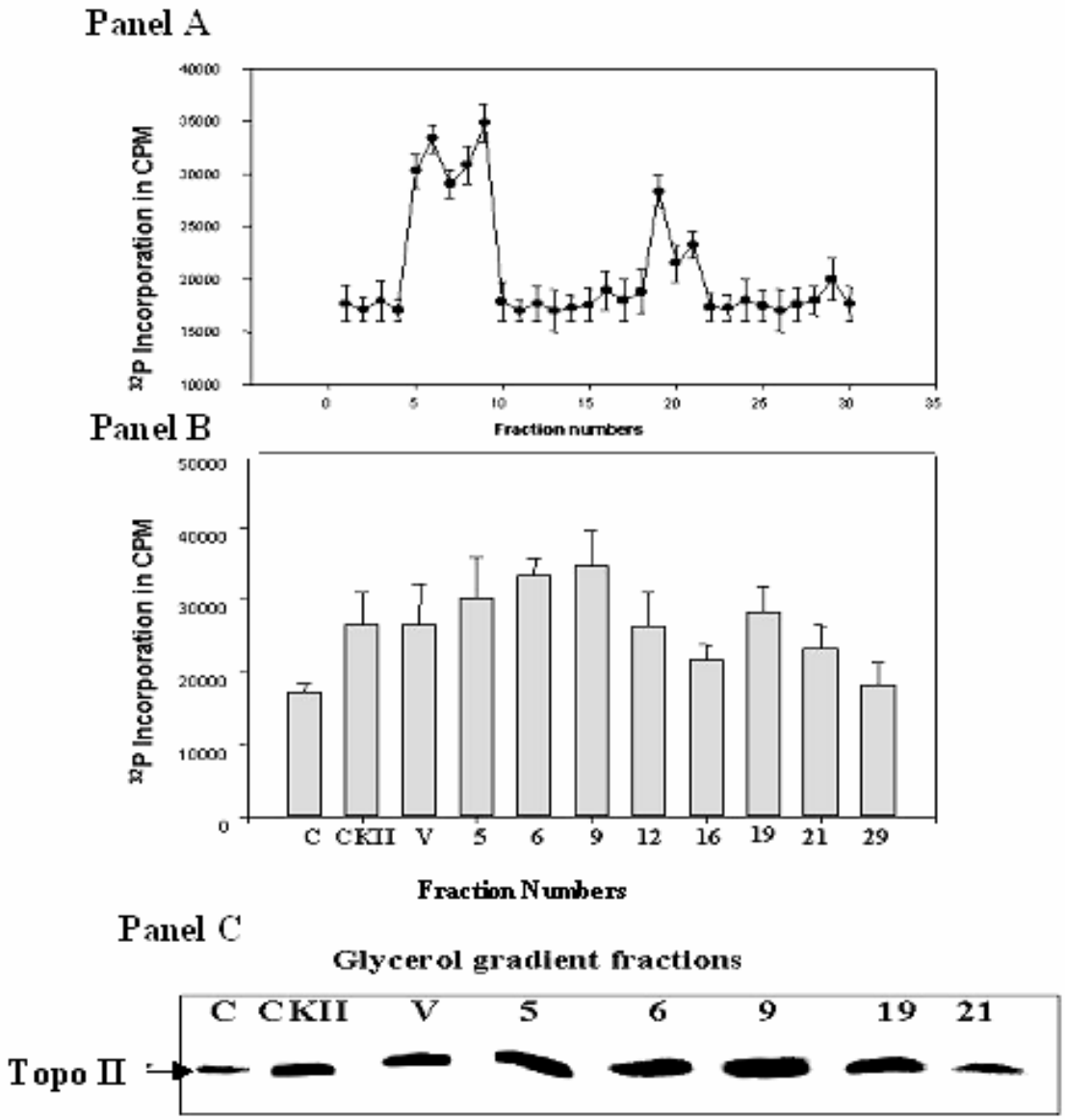
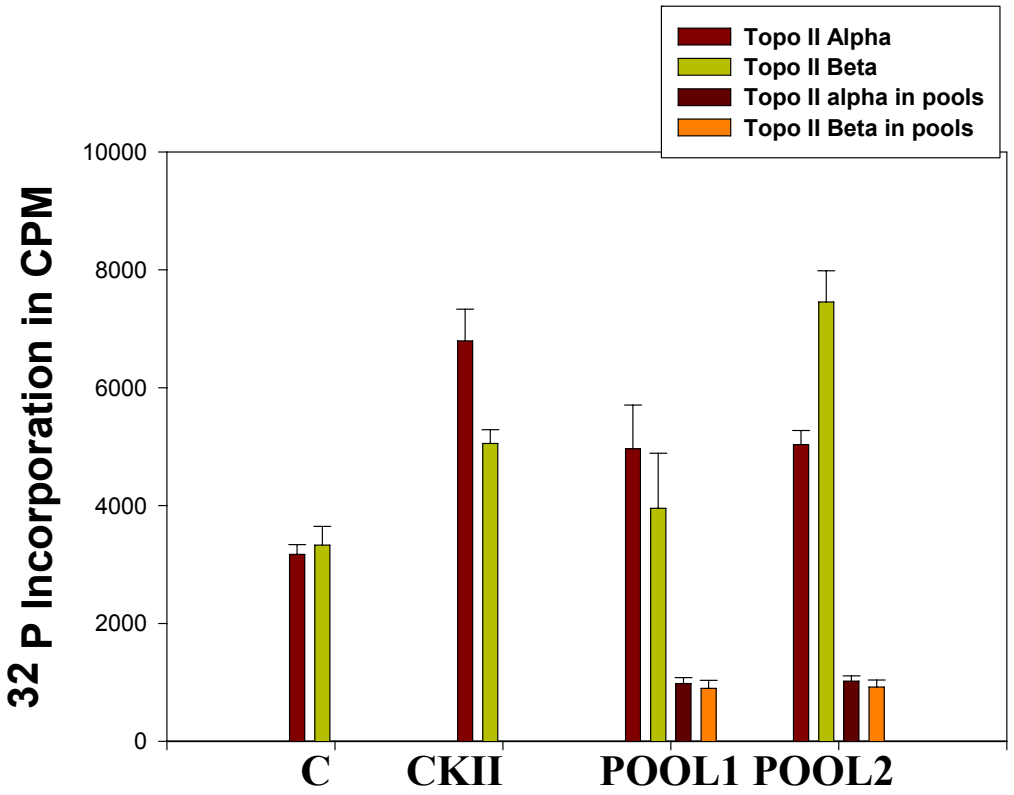


Fig 12: Differential phosphorylation of topoisomerase II α and β by pool 1 and pool 2 of glycerol gradient fractions: Active fractions were pooled and Topoisomerase II α and β were incubated with these pools and assayed for phosphorylation. Panel D shows ^{32}P incorporation into colon cell Topoisomerase II α and β by pool 1 and pool 2 of the glycerol gradient fractions. (C), topoisomerase II alone; (CK2), topoisomerase II +CkII; (Pool 1), topoisomerase II + pool 1; (Pool 2), topoisomerase II + pool 2. Panel E shows an autoradiogram of an SDS-PAGE gel of the same samples.

Figure 12

Analysis of phosphorylation of Pool 1 and Pool 2 on topoisomerase II

A



B

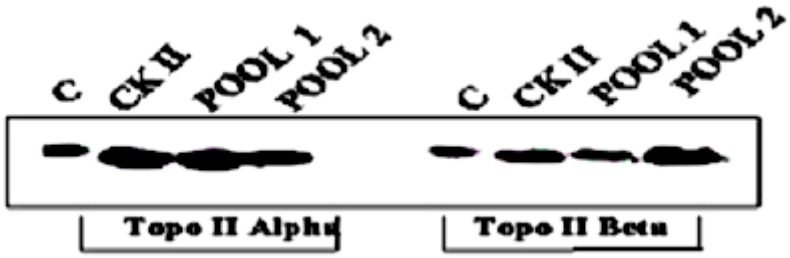


Fig 13: Comparative analysis of the phosphorylation activity of viral proteins with and without Delipidation: Topoisomerase II α and β were incubated with the delipidated viral lysate protein and undelipidated viral lysate protein i.e viral lysate protein as such and assayed for kinase activity.

Figure 13

Comparative analysis of the phosphorylation activity of viral proteins with and without Delipidation

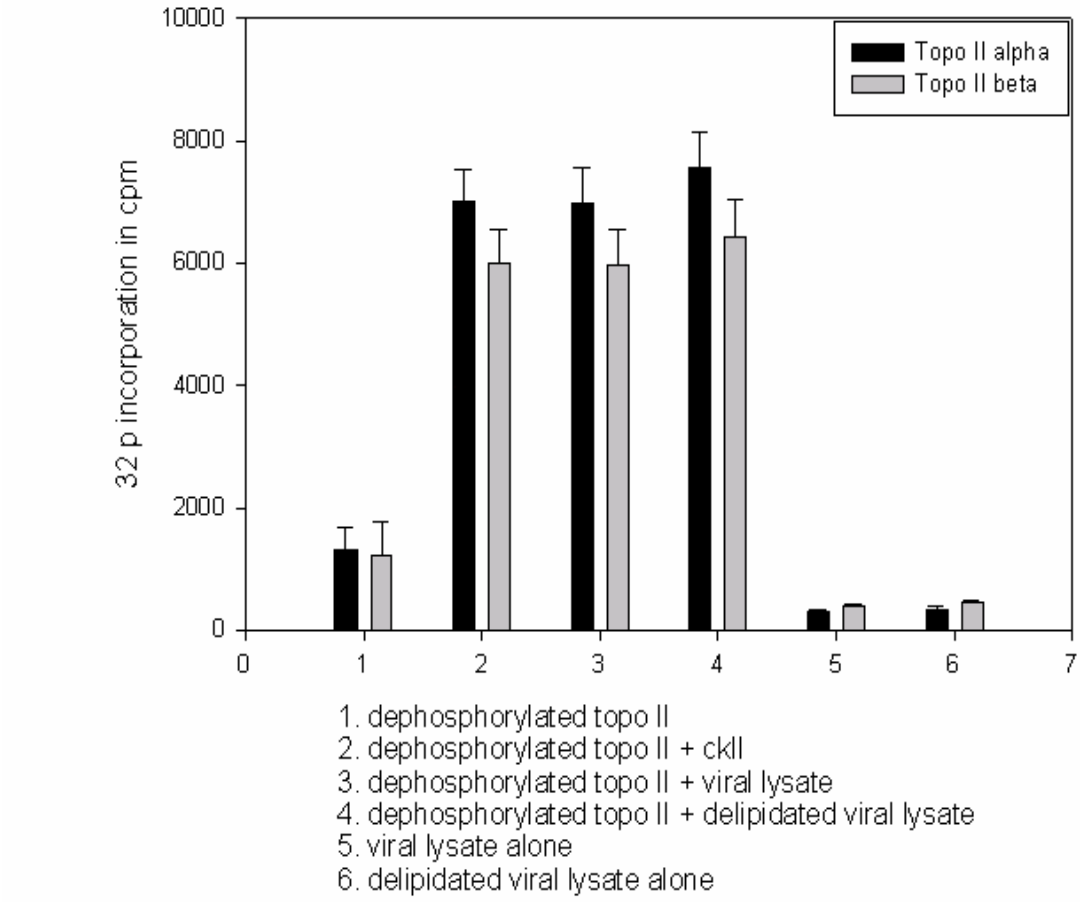


Fig 14. Isolation of Topoisomerase II α and β kinase activities from virus lysates by Mono S Chromatography:

Panel A: Delipidated viral protein was loaded on to Mono S agarose. The column was washed and bound protein was step eluted with indicated concentration of NaCl. The elutes were dialyzed and analysed for Topoisomerase II α and β kinase activities.

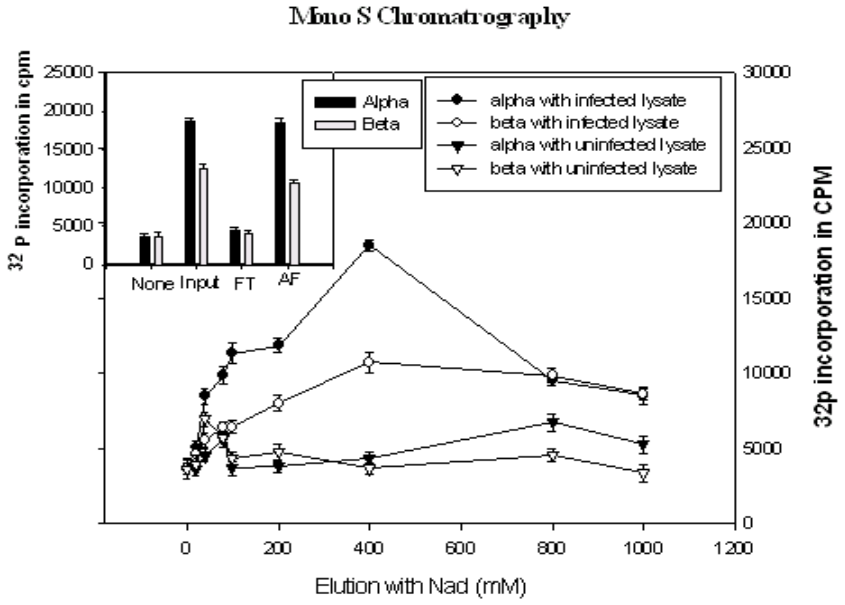
Panel B: The active Mono S elute was dialysed and loaded on to Phenyl Sepharose. Phenyl sepharose bound protein was step eluted with indicated concentrations of isoproponal (W/V), elutes were dialyzed and analysed for Topoisomerase II α and β kinase activity.

In panels A,B the activity in controls was given inset bar diagram. FT: Flow through, AF: Active fraction.

Figure 14

PURIFICATION OF DELIPIDATED HIV-1 VIRAL PROTEINS BY MONO S CHROMATOGRAPHY

A



B

Mono S (400 mM NaCl) elute on phenyl sepharose cromatrography.

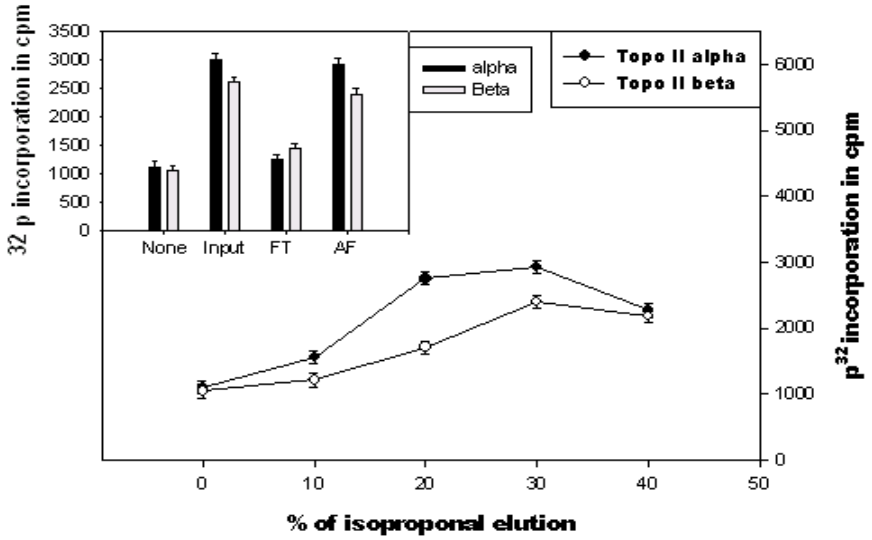


Fig 15. Isolation of Topoisomerase II α and β kinase activities from virus lysates by Mono Q Chromatography:

Panel A: Delipidated viral protein was loaded on to Mono Q. The column was washed and bound protein was step eluted with indicated concentrations of NaCl, elutes were dialyzed and analysed for Topoisomerase II α and β kinase activities.

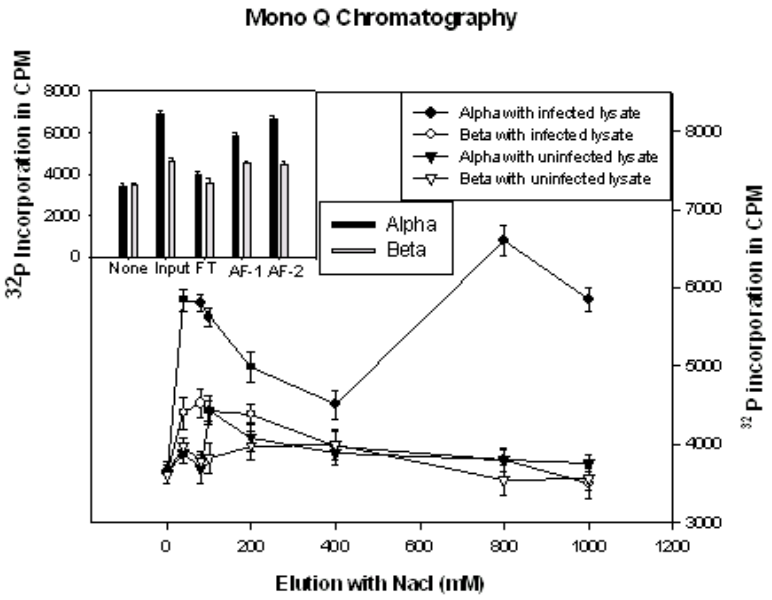
Panel B: The active Mono Q elute was dialysed and loaded on to Phenyl Sepharose. Phenyl sepharose bound protein was step eluted with indicated concentrations of isoproponal (W/V), elutes were dialyzed and analysed for Topoisomerase II α and β kinase activity.

In panels A, B the activity in controls was given inset bar diagram. FT: Flow through, AF: Active fraction

Figure 15

PURIFICATION OF DELIPIDATED HIV-1 VIRAL PROTEINS BY MONO Q CHROMATOGRAPHY

A



B

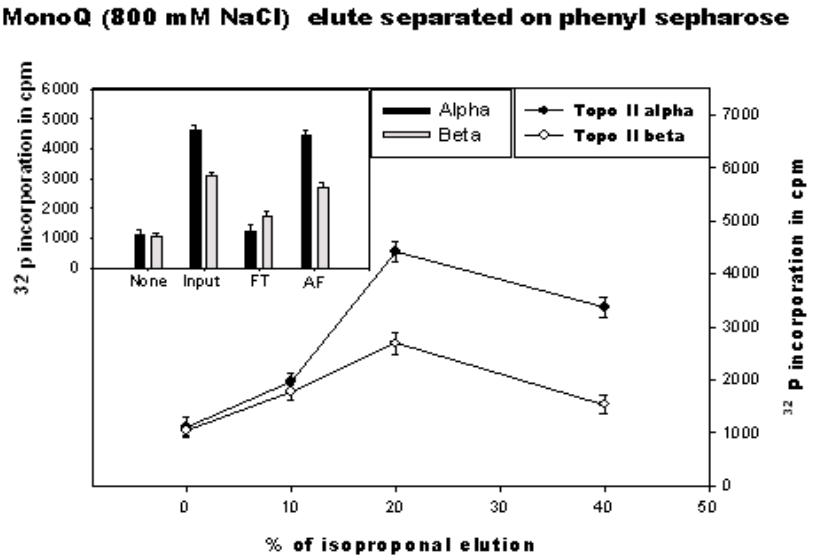


Fig 16. Isolation of Topoisomerase II α and β kinase activities from virus lysates by Phenyl Sepharose Chromatography and summary of Topoisomerase II α and β phosphorylation:

Panel A: Delipidated viral protein was loaded on to Phenyl Sepharose. The column was washed and bound protein was eluted with increasing concentration of (v/v) isopropanol, elutes were dialyzed and analysed for Topoisomerase II α and β kinase activities.

In panel E, the activity in controls was given inset bar diagram. FT: Flow through, AF: Active fraction.

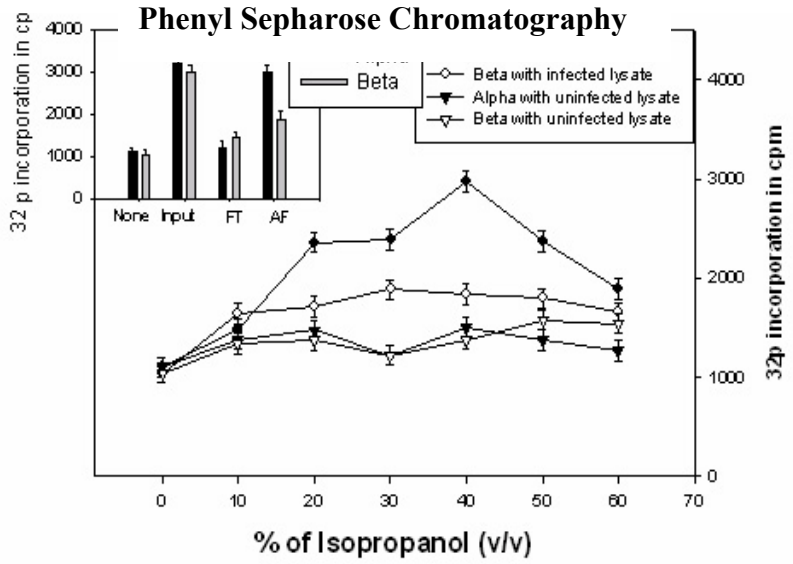
Panel B: Summary of Topoisomerase II alpha phosphorylation promoted by kinase isolated from delipidated viral lysate was shown on Panel F and G respectively. C-1: with CK-II. Virus lysate without delipidation with (C-2) and without (C-3) Topoisomerase II.

Panel C: Summary of Topoisomerase II beta Phosphorylation promoted by kinase isolated from delipidated viral lysate was shown on Panel F and G respectively. C-1: with CK-II. Virus lysate without delipidation with (C-2) and without (C-3) Topoisomerase II.

Figure 13 contd 2

PURIFICATION OF DELIPIDATED HIV-1 VIRAL PROTEINS

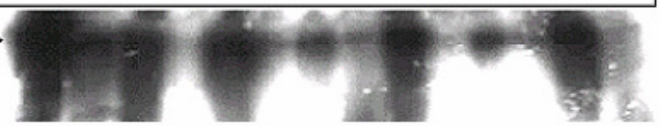
A



B

MonoS PheSep	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Mono Q	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Phe S	-	-	-	-	-	-	-	-	-	-	+	+	-	-
MonoQ PheS	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Delip Vir	C1	-	C2	C3	-	-	-	-	-	-	-	-	+	+
Topo II	+	+	+	-	+	-	+	-	+	-	+	-	+	-

Topo II alpha →



G

MonoS PheSep	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Mono Q	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Phe S	-	-	-	-	-	-	-	-	-	-	+	+	-	-
MonoQ PheS	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Delip Vir	-	C1	C2	C3	-	-	-	-	-	-	-	-	+	+
Topo II	+	+	+	-	+	-	+	-	+	-	+	-	+	-

Topo II beta →

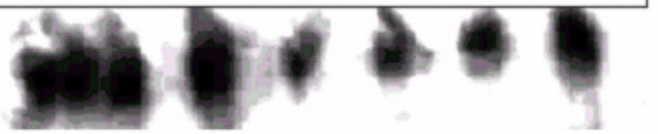


Fig.17: Catalytic activity of phosphorylated Topoisomerase II α and β in the presence of isolated viral kinase activities: Topoisomerase II α (**Panel A & B**) and β (**Panel C & D**) were phosphorylated in presence of isolated kinases from delipidated virus as indicated. The activity of phosphorylated Topoisomerase II isoforms were analysed by DNA relaxation assay. Relaxed and supercoiled forms were indicated as R and S respectively.

Lane1: DNA alone,

Lane 2: DNA with Topo II phosphorylated by CKII,

Lane 3:DNA with Topo II,

Lane 4: DNA with dephosphorylated Topo II,

Lane 5: DNA with Topo II phosphorylated by undelipidated viral lysate,

Lane 6: DNA with Topo II phosphorylated by kinase isolated from Mono S followed by phenyl sepharose,

Lane 7: DNA with Topo II phosphorylated by kinase isolated from Mono S alone, **Lane 8**: DNA with Topo II phosphorylated by kinase isolated from delipidated virus lysate from Mono Q followed by phenyl sepharose,

Lane 9: DNA with Topo II phosphorylated by kinase isolated from delipidated virus using Mono Q alone,

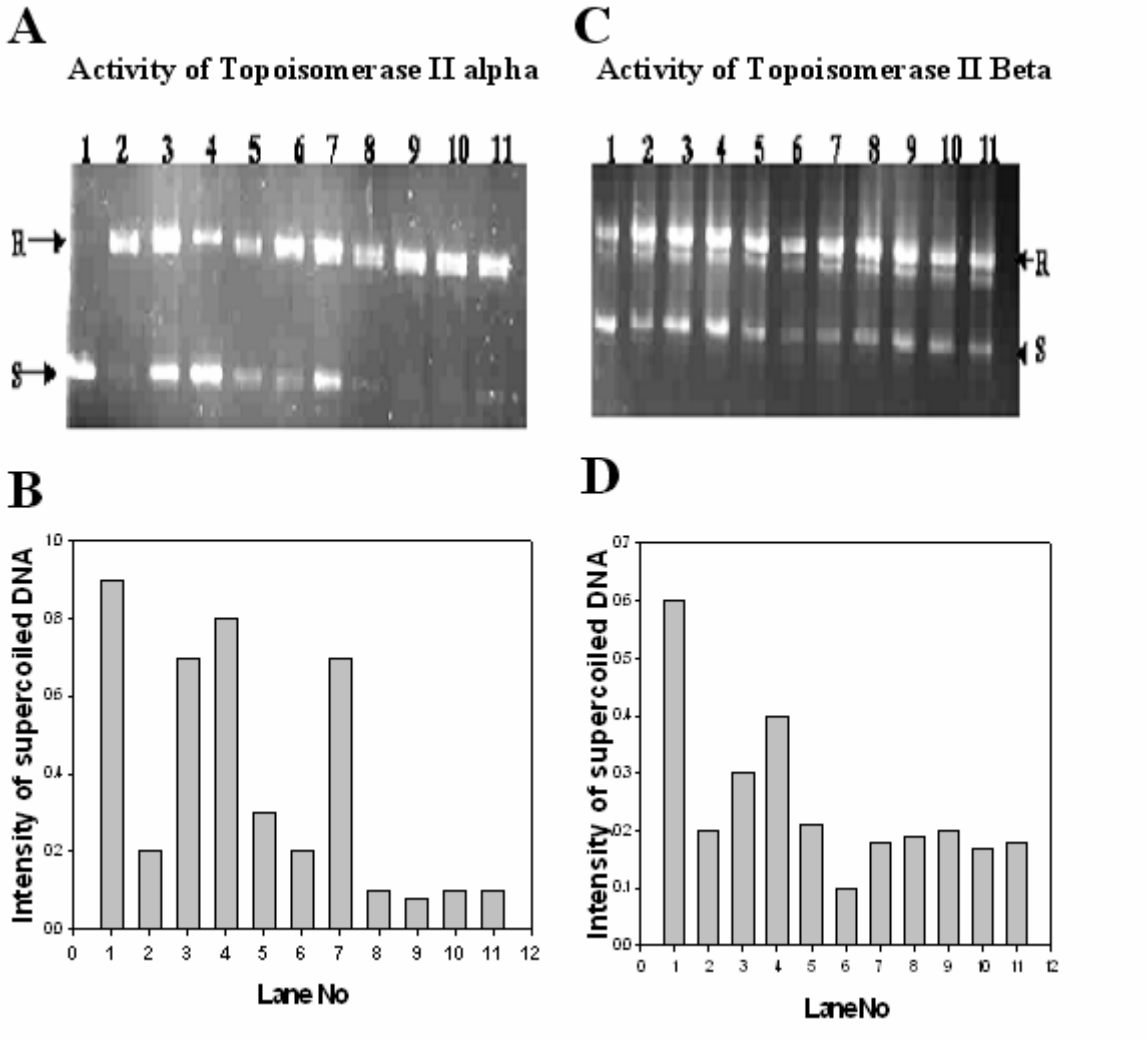
Lane 10: DNA with Topo II phosphorylated by kinase isolated from virus lysate using phenyl sepharose chromatography,

Lane11: DNA with Topo II phosphorylated by delipidated virus.

S represents supercoiled DNA and **R** represents relaxed DNA. The supercoiled DNA in data of Panel A and Panel C were quantified and plotted on Panel B and Panel D respectively.

Figure 17

Catalytic activity of phosphorylated Topoisomerase II in presence of isolated viral kinase activities



Chapter 5

Characterization of

Topoisomerase II alpha and beta

Kinase activities

The results incorporated in the previous chapter demonstrated the presence of two molecular species of kinases in the purified virus, one that can phosphorylate both topoisomerase II α and β referred as (TopoII α,β K_{HIV}) a Mono S isolated kinase and the other that can phosphorylate topoisomerase II α alone referred as (TopoIIα K_{HIV})a Mono Q isolated kinase. These virus associated kinase activities exhibited distinct hydrophobic and surface electrostatic properties, which was utilized for the isolation from the lysates made from purified virus lysates. These kinases were analysed for their ability in phosphorylation of serine /tyrosine residues of topoisomerase II. Isolated distinct kinase activities (TopoIIα,β K_{HIV} , TopoIIα K_{HIV}) were characterized for their ability in utilization of other cofactors in preference to ATP. Further, the kinase activities were characterized in terms of physicochemical properties, viz. temperature and pH.

The following objectives were addressed in this chapter:

1. Analysis of site of phosphorylation of topoisomerase II alpha and beta promoted by HIV-1 isolated kinase.
2. Effect of co-factors on Topoisomerase II α, β phosphorylation by HIV-1 associated Kinases.
   a) dNTP's
   b) ddNTP's
3. Influence of physiochemical parameters on the kinase activity.
   a) pH
   b) Temperature.

Table 3

Kinase	Optimum pH	Optimum temp	Reference
A cyclic nucleotide-independent degrees protein kinase C; in *Leishmania donovani*	7	37°C.	(Das *et al.,* 1986)
Novel *Arabidopsis* Protein Kinase	7.5	30°C.	(Deming Gong *et al.,* 2002b)
Human cytomegalovirus induced protein kinase	6 and 6.5	----	**(**Michelson *et al.,* 1984)
Protein kinase activity in Purified Foot- and-mouth Disease virus.	7 or greater	------	(Grubman *et al.,* 1994)
Pkn2, a protein Ser/Thr kinase	7.5	35°C.	(Udo *et al.,* 1997)
Muscle isoforms of creatine kinase	6.2	30°C.	(Winnard *et al.,* 2003)

Table 4

Kinase	Co Factors	References
HMG-CoA reductase	ATP>dATP>GTP>ITP, UTP, TTP and CTP did not replace ATP.	(Harwood *et al.,* 1984).
	GTP can replace ATP GMP and GDP cannot replace MP.	(Ferrer *et al.,* 1987).
Human Deoxycytidine kinase (dCK)	Adenosine-2'-triphosphates and three adenosine-3'-triphosphates were compared with ATP as potential donors	(Krawiec *et al.,* 2003).
CKII	GTP	

EXPERIMENTAL METHODS

Western blot analysis of phosphorylation at serine and tyrosine of topoisomerase II alpha and beta in presence of isolated kinases: 25 μg of Topoisomerase II alpha/ beta are incubated in presence of 100 nanograsms of isolated kinase from Mono S, Mono Q. The phosphorylated Topoisomerase II alpha/ beta was resolved on 7.5% SDS PAGE mini gels and transferred to PVDF membranes, then immunoblotted with biotinylated mouse anti-phosphotyrosine and mouse anti-phosphoserine and incubated with peroxidase-conjugated strapatavidin IgG antibody (1:2000 dilution in TBS) for 60 min at room temperature and then with TBS containing 0.15 % Tween. The blots were developed using TMB/H_2O_2 substrate for localization and documented using a UVI-Tech gel documentation system. A Control analysis for Topoisomerase II protein was done using a 1:1000 dilution of mouse anti-human topoisomerase II α or β followed by anti-mouse IgG conjugated with peroxidase and developed as described above.

Competition of unlabelled dNTPs /ddNTPS with γ^{32} P labeled ATP:

HIV-1 virus lysate was preincubated with increasing concentration of dNTPs /ddNTPS for 10 min at 30°C. Then dephosphorylated topo II α or β was added and kinase assay was performed in vitro.

Effect of temperature on topoisomerase II alpha and beta phosphorylation by HIV-1 Kinase: 10 ηg of isolated kinase was added to 1μg of dephosphorylated topo II α or β and kinase assay was performed in vitro at increasing temperatures.

Effect of pH on topoisomerase II alpha and beta phosphorylation by HIV-1 Kinase: 10 ηg of isolated kinase was added to 1μg of dephosphorylated topo II α or β and kinase assay was performed in vitro at increasing pH.

RESULTS

Both Topoisomerase II α and β kinases are serine kinases:

To monitor the site of phosphorylation of Topo II α and β catalyzed by virus associated kinases topoisomerase α and β phosphorylation was carried out in presence of Mono S and Mono Q isolated kinase. The phosphorylation of Serine and Tyrosine residues were carried out through western blot analysis using monoclonal antibodies to phosphorylated serine and tyrosine. The results in Fig 18 shows that both the Kinases phosphorylated on serine residues of Topo II α and β suggesting that both the Topoisomerase II α and β kinases associated with HIV-1 particles are serine kinases.

Topoisomerase II alpha and beta phosphorylation by HIV-1 Kinase is competed by unlabelled dNTPs /ddNTPS with γ^{32} P labeled ATP: The results in Fig 19 show that Topoisomerase II alpha phosphorylation by TopoIIα K_{HIV} is highly competed by dTTP and ddGTP. However there is no significant effect of these cofactors on topoisomerase II beta phosphorylation by TopoII β K_{HIV}. The results in Fig 20 reconfirm the effect on Topoisomerase II alpha phosphorylation in the presence of dTTP and ddGTP.

Topo II alpha and beta kinase activity exhibit distinct optimum Temperature and pH: The effect of temperature and pH of Topoisomerase II alpha and beta kinase activity was studied, the results in Fig 21 and 22 shows TopoIIα K_{HIV} exhibit optimum activity at pH 5 and 37°C, while TopoIIβ K_{HIV} optimum activity was observed at pH 8 and 35°C.

DISCUSSION

Topoisomerase II α and β has been shown to be phosphorylated by the serine kinase. Analysis of Topoisomerase II α and β phosphorylation by the HIV-1 associated kinases suggested that both the kinases are serine kinases. The virus promotes the phosphorylation of Topoisomerase II α and β at its serine residues thus activating the enzyme. These two kinases show distinct optimum conditions of pH and temperature, suggesting that they may be localized at distinct sites in the infected cells. Human cytomegalovirus induced protein kinase indeed posses optimum pH of 6, (Michelson *et al.,* 1984) (Table 3) While protein kinase activity in Purified Foot-and-Mouth disease virus optimum pH is greater than 7 (Grubman *et al.,* 1994). Novel *Arabidopsis* protein kinase posses optimum temperature of 30^0C whereas Pkn2, muscle isoforms of creatine kinase posses optimum temperatures 35^0C and 30^0C respectively, suggesting that the viral associated kinase have distinct physiochemical properties, the significance of such properties in HIV-1 infection would be more interesting to study. Further α kinase activity (TopoIIα K_{HIV}) prefer to dGTP and ddTTP in phosphyl transfer, while β kinase activity (TopoIIβ K_{HIV}) use ATP as sole co-factor. These observations clearly demonstrate that α and β kinase activity associated with HIV-1 is distinctly different in terms of enzymatic and thermodynamic properties.

Figure 18: Analysis of phosphorylation of Serine and Tyrosine residues of Topo II α and β: 25μg of Topoisomerase II α and β were phosphorylated with 100ηg of kinase isolated from Mono S and Mono Q. The phosphorylated Topo II α and β were western analyzed using monoclonal antibodies specific to Phospho-serine and Phospho-tyrosine. The Topo II α and β monoclonal antibodies were used as positive control and shown in the bottom panel. Casein Kinase II was used as a serine kinase control.

Figure 18

Analysis of phosphorylation of Serine and Tyrosine residues of Topo II α and β

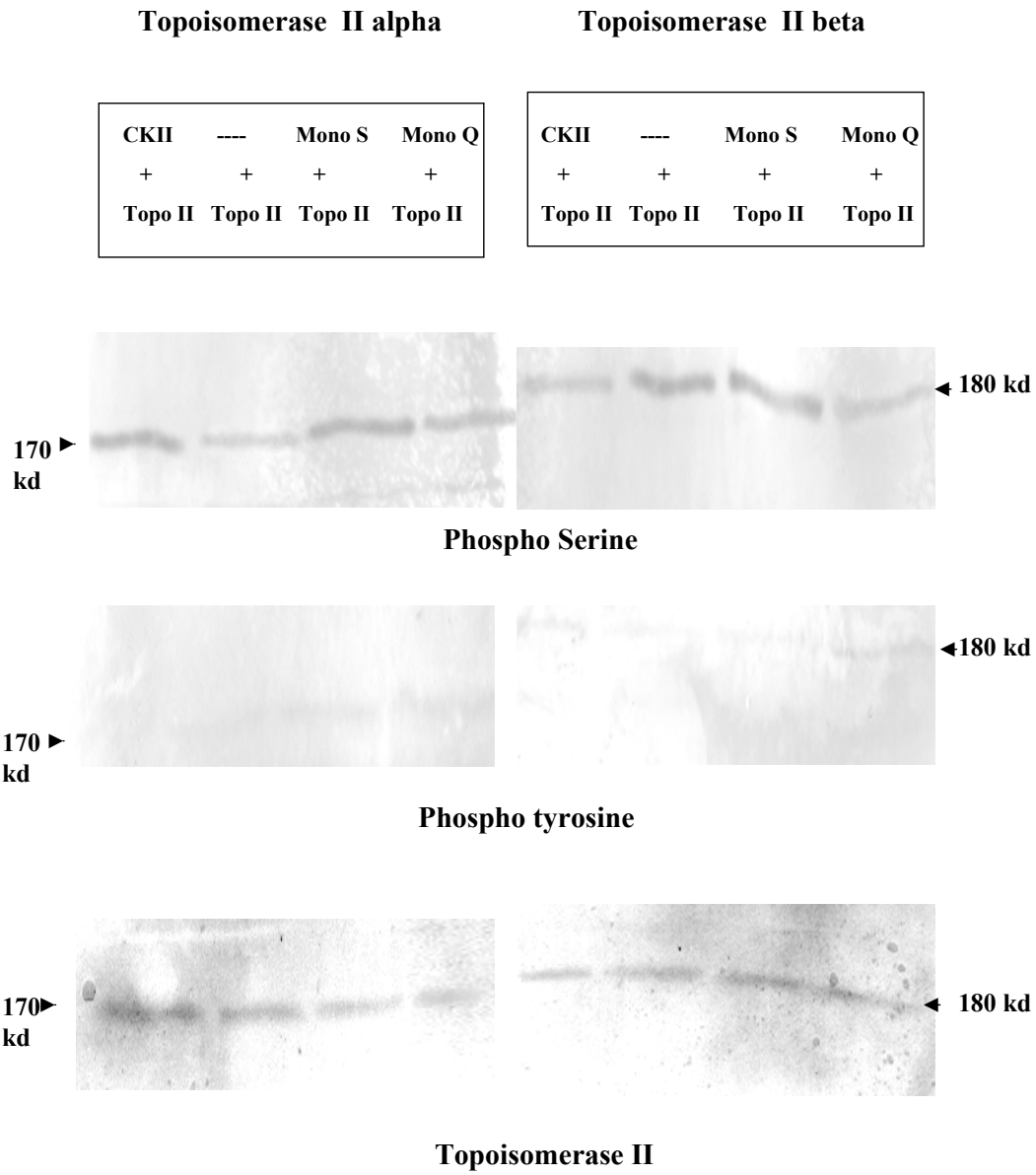


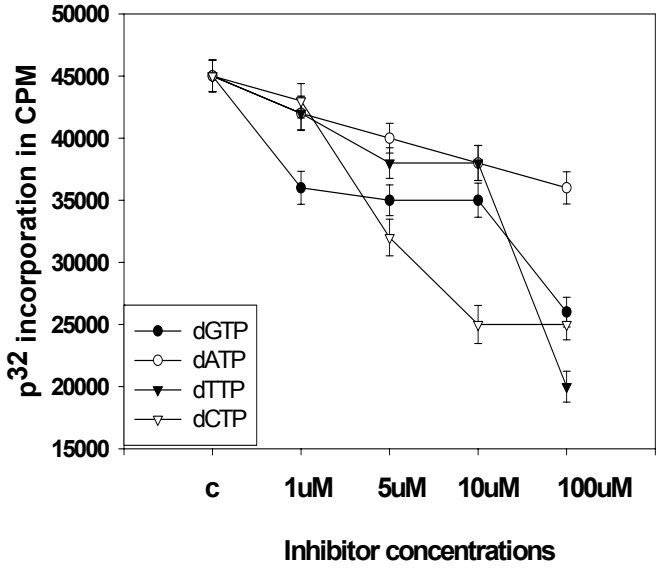
Fig 19: Competition of unlabelled dNTPs /ddNTPS with γ^{32} P labeled ATP:

HIV-1 virus lysate was preincubated with increasing concentration of dNTPs /ddNTPS for 10 min at 30°C. Then dephosphorylated topo II α or β was added and kinase assay was performed in vitro. X-axis depicts dNTPs /ddNTPS concentrations and Y-axis reads incorporated ^{32} P into Topoisomerase II α or β.

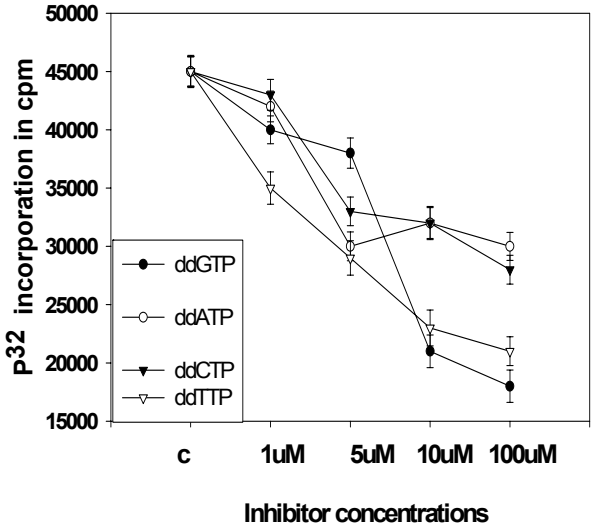
Figure 19

Competition of dNTP's and ddNTP's against ATP in Topo II alpha and beta phosphorylation with HIV-1 associated kinase

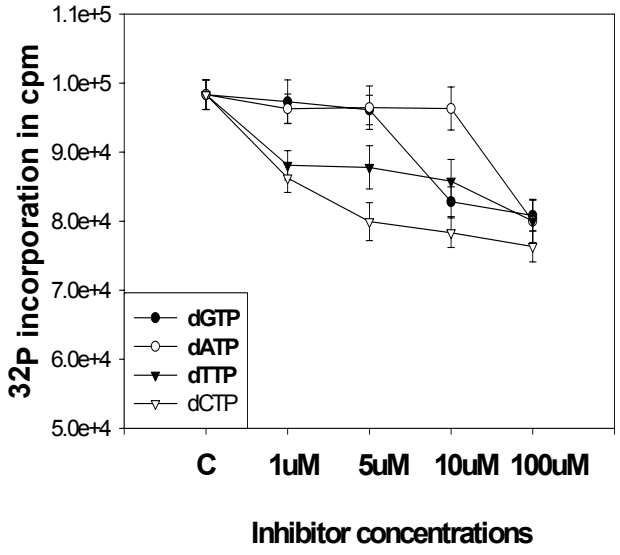
Topo II α with TopoIIα K_{HIV}



Topo II α with TopoIIα K_{HIV}



Topo II β with TopoIIβ K_{HIV}



Topo II β with TopoIIβ K_{HIV}

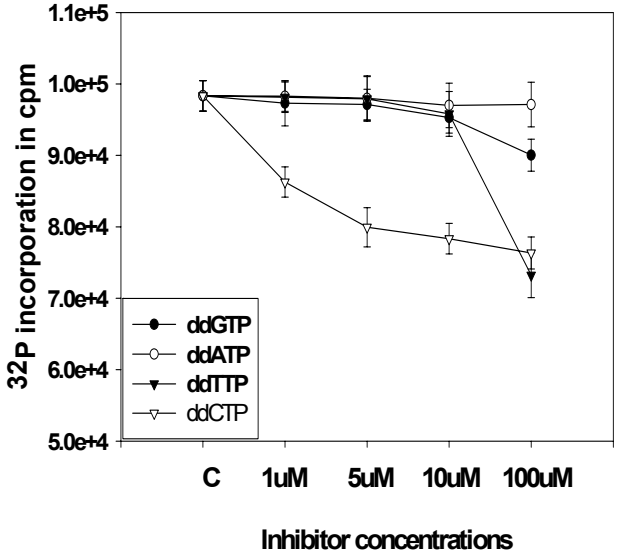
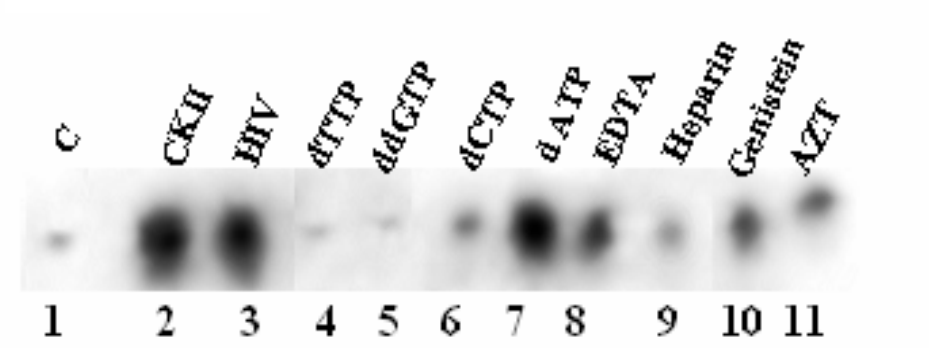


Fig 20: Reconfirmation of Topoisomerase II alpha phosphorylation by HIV-1 Kinases: HIV-1 virus lysate was preincubated with 100 μM of indicated of dNTPs /ddNTPS for 10 min at 30°C. Then dephosphorylated topo II α was added and kinase assay was performed in vitro. Panel A is an autoradiographic analysis of the topoisomerase II α phosphorylation in competition of γ^{32} P ATP with indicated unlabelled dNTPs /ddNTPS. P-32 topoisomerase II α in the same samples were quantified by the immunoprecipitation experiments.

Figure 20

Reconfirmation of competition of dTTP and ddGTP on Topoisomerase II alpha phosphorylation by Topo II α with Topo II α K_{HIV}

Panel A



Panel B

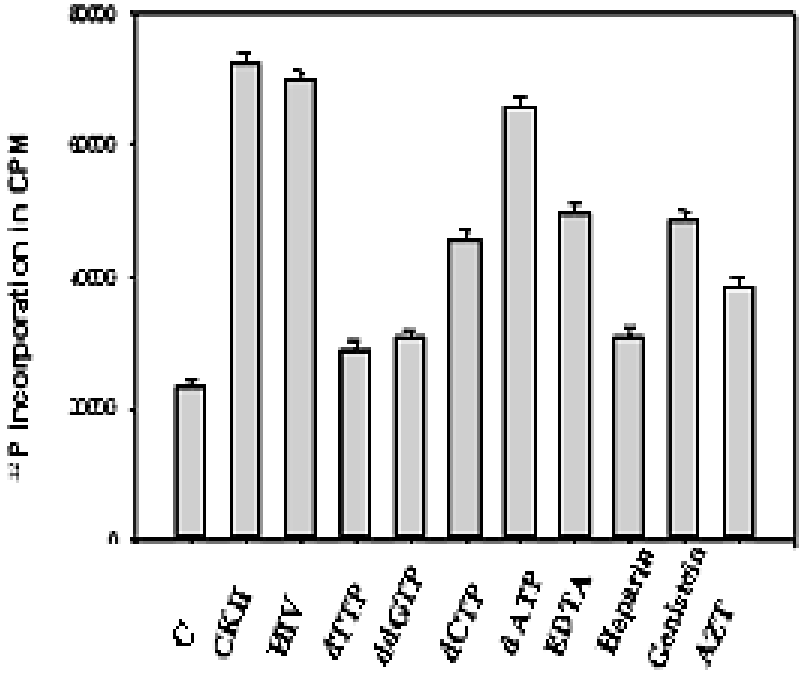


Fig 21: **Topo II alpha and beta kinase activity exhibit distinct optimum pH**: 10 ηg of isolated kinase was added to 1μg of dephosphorylated topo II α or β and kinase assay was performed *in vitro* at increasing pH. X-axis depicts pH range whereas Y-axis reads incorporated ^{32} P into Topoisomerase II α or β.

Figure 21

Effect of pH on Topoisomerase II alpha and beta kinase activity

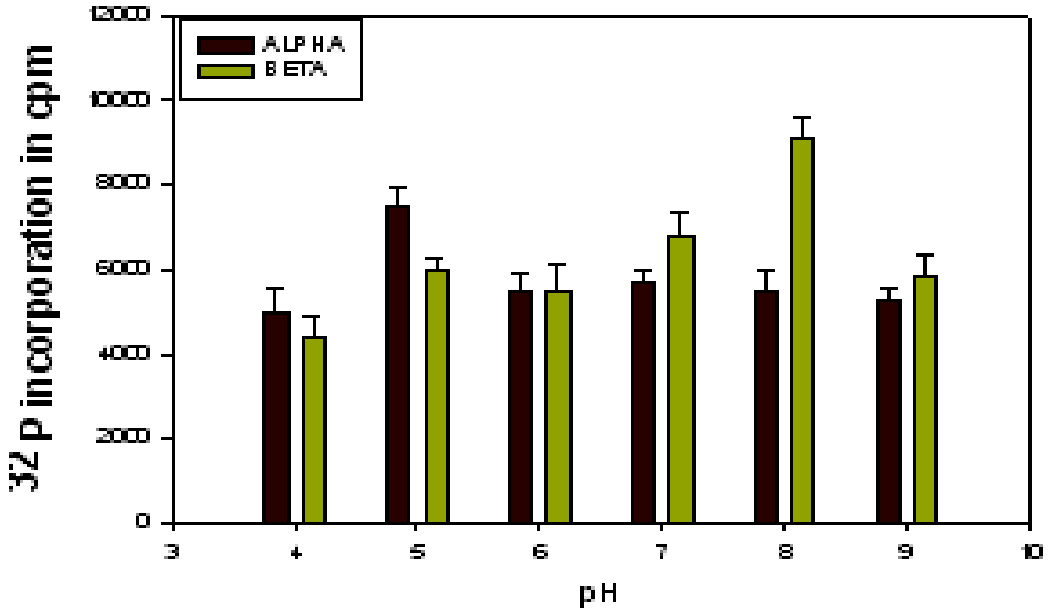
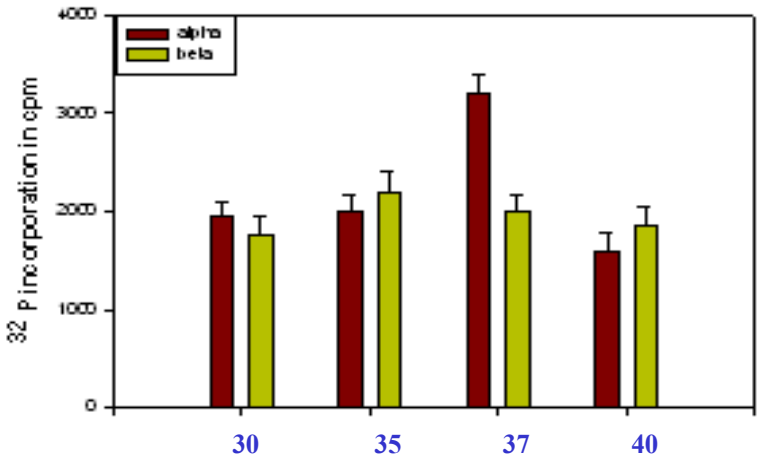


Fig 22: **Topo II alpha and beta kinase activity exhibit distinct optimum Temperature**: 10 ηg of isolated kinase was added to 1μg of dephosphorylated topo II α or β and kinase assay was performed in vitro at increasing temperature. Mono S isolated kinase was used in this assay as it possesses both alpha and beta kinase activities. X-axis depicts temperature range whereas Y-axis reads incorporated ^{32} p into Topoisomerase II α or β.

Figure 22

Effect of temperature on topoisomerase II alpha and beta Kinase activity



Chapter 6

Action of Inhibitors on

The Isolated Kinase activities

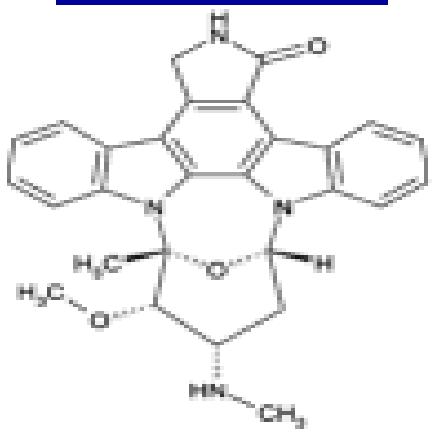
The characterization of Topoisomerase II α and β Kinase activities suggested that both TopoIIα K_{HIV} and TopoII β K_{HIV} are serine kinases posses distinct properties in terms of their optimum temperature, pH and interaction with dNTPs and ddNTPs. To further understand the molecular feature of their catalytic site, we have studied the interaction of two known kinase inhibitors, namely staurosporin and PD98059 (Fig 23) against these kinases. Staurosporin is a protein kinase inhibitor with broad specificity, whereas PD98059 is specific inhibitor of MAPKinase Kinase pathway kinases (Gibellini *et al.,* 1998).

In addition we present the data on the inhibition action of 18 small molecules with distinct structural features (Table 5) against Topoisomerase II α and β kinase activity. The results of these studies were embodied in this chapter.

FIGURE 23

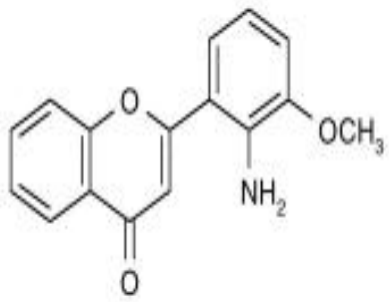
KINASE INHIBITORS

Staurosporin



Staurosporin, isolated from Streptomyces staurosporeus, is a cell permeable general inhibitor of protein kinases IC_{50} values for PKC=2.7-5.5nM, PKA=6nM, PKG=8.5nM, CAMKII =20nM, MLKC=21nM, c-Src=10nM.

PD 98059



PD 98059 is a potent, selective and cell-permeable inhibitor of MAP kinase kinase (also known as MAPK/ERK kinase or MEK). It inhibits phosphorylation of MAP kinase by MAP kinase kinase. PD 98059 does not inhibit MAP kinase itself. The IC_{50} values for PD 98059-induced effects are in the 1-20 µM range for many assays.

(Gibellini *et al.*, 1998)

TABLE 5

Studied small molecules for analysis of their action of

Topollα K_{HIV} and Topoll β K_{HIV}

1. Phenolphthalein
2. 2,2'-Bipyridine-dibyridyl
3. L-Ascorbic acid
4. Diphenylamine
5. 2,4-Dinitrophenol
6. Guanine
7. 1,10-Phenanthroline monohydrate
8. Salicylaldehyde
9. Hexamethylene diamene
10. Nicotylonyl hydrazide
11. Pyridine 3-sulphonic acid
12. Nicotinic acid hydrazide
13. 3-Amino pyridine
14. 3-Acetyl pyridine
15. Pyridine-3-carboxaldehyde
16. 3-Hydroxy pyridine
17. 3-Hydroxy methyl pyridine
18. Nicotinamide

EXPERIMENTAL METHODS

Analysis of action of kinase inhibitors on topoisomerase II kinase activity of pool 1 and pool 2 fractions of HIV-1 viral lysate: Pooled virus lysate (500 ηg protein), Mono S (10 ηg protein) and MONO Q (10 ηg protein) fractions were pre-incubated with the indicated concentrations of staurosporin (a PKC inhibitor), and PD98059 (a MAPK/ERK kinase inhibitor) for 10 minutes. 1 μg of dephosphorylated topoisomerase II was added and assayed by i*n vitro* kinase assay.

Development of molecules with potential inhibitory action against isolated kinases: TopoII α K_{HIV} or TopoII β K_{HIV} {10ng) is pre incubated with indicated concentrations of molecules for 10 minutes at 37ºC. Topo II alpha or beta is immunoprecipitated and added to this. Phosphorylation assay was performed invitro as described earlier. Molecules that showed inhibition were selected and reanalyzed for dose dependency in coded forms. Simultaneously these coded molecules were also analyzed for inhibition of catalytic activity of Topoisomerase II. The molecules, which showed dose dependency and that, did not inhibit catalytic activity of topoisomerase II were further analyzed with invitro kinase assay. All the molecules were randomly coded and then activity was measured then decoded to confirm the results.

Catalytic activity of topoisomerase II in the presence of molecules:

Relaxation assay of topoisomerase II α was performed as described earlier in the presence of indicated concentrations of the molecules. The catalytic activity was measured by the extent of enzyme catalyzed the conversion of supercoiled DNA form to relaxed DNA form. The molecules that inhibited catalytic activity of Topoisomerase II α and β were eliminated from the further kinase autoradiography.

RESULTS

Topoisomerase II isoform-specific kinase activities show distinctly different sensitivity against staurosporin and PD98059: To examine the characteristics of Topoisomerase II alpha and beta phosphorylation by virus associated serine kinase activity, we have studied the effect of known inhibitors staurosporin (a protein kinase C inhibitor) and PD98059 (a MAP kinase inhibitor) on Topoisomerase II kinase activity of pool 1 and 2 fractions. The phosphorylation of Topoisomerase II alpha and beta by pool 1 and 2 was not significantly inhibited by both the inhibitors (Fig.24). However, Topoisomerase II alpha and beta kinase activity present in pool 1 fraction is more sensitive to staurosporin and PD 98059 compared to that of pool 2 (Fig24). These findings suggest that the β kinase activity present in pool 2 is distinctly different from that of pool 1.

Further analysis was carried out to find the sensitivity of Topoisomerase II α and β kinases isolated from Mono S and Mono Q against Staurosporin and PD98059. The results in Fig 25 show that, the α kinase isolated from Mono S possesses highest sensitivity to staurosporin compared to that of PD98059, while the α kinase isolated from Mono Q show only sensitivity to PD98059. These results suggest that α kinase may share certain catalytic features common from PKC and MAPK kinase pathway kinases. The analysis of sensitivity of β kinase activity to these inhibitors shows β kinase is less sensitive to both the inhibitors. Both Mono S and Mono Q isolated beta kinase show low sensitivity to staurosporin while PD98059 did not exhibit significant inhibitory action against the kinase activity.

These results suggest that the active site of Topoisomerase II α and β serine kinases is distinctly different. The α kinase activity share common catalytic features with PKC and MAPK Kinase pathway kinases, where as β kinase may be a distinctly different kinase.

Development of new molecules with potential inhibitory activity against Topoisomerase II alpha and beta kinase activities: The interaction of Topoisomerase II alpha and beta kinases against small molecules was studied. The results in Figs 27-32 show that the molecules Hexamethylene diamene, 3-Acetyl pyridine, 3-amino pyridine Inhibited the activity of TopoII α K_{HIV} and the molecules Diphenylamine and 3-Hydroxy pyridine inhibited the activity of TopoII β K_{HIV}. Thus the alpha kinase activity and beta kinase activity was inhibited by entirely different molecules reconfirming the distinct isoform specific Topoisomerase II kinase activity associated with HIV-1 virus. Since 2,4-dinitrophenol, 1,10-pheneanthrolene and phenolphthalein showed inhibition of enzymatic activity of Topo II (Fig 33), they have been eliminated. The inhibitory action of these inhibitors has been re-confirmed through autoradiographic analysis (Fig 34). These results suggest that α and β kinase may share distinct catalytic features.

DISCUSSION

Protein Kinases are the enzymes that promote the phosphorylation of its substrate proteins. The activity of a protein kinase is highly substrate specific. A structural class of molecules that can competitively or non-competitively binds the active sites and can cause inhibition. Such an inhibition is very specific to that particular Kinase. Study of interaction of inhibitor at the active site of enzyme in terms of their inhibition of activity of the enzyme would delineate the molecular differences existing in the active site of the enzyme. Hence, in this chapter an analysis of inhibitory action of known kinase inhibitor staurosporin, a broad-spectrum kinase inhibitor and PD98059, a MAPKinase kinase pathway inhibitor was studied. The results showed that both staurosporin and PD98059 could interact with Topoisomerase II α kinase activity. Between the two inhibitors PD98059 show strongest interaction to α kinase isolated from Mono S. Whereas the α kinase isolated from Mono Q shows moderate sensitivity to these inhibitors suggesting that α kinase bound to Mono S may be different from that of Mono Q. The higher sensitivity of alpha kinase present in Pool-1 fraction as well as Mono S isolated kinase against both Staurosporin and PD98059 suggest that Topoisomerase II alpha kinase share common catalytic features of Protein kinase C and MAPK kinase pathway kinase as reported to be associated with HIV-1 virions (Cartier, *et al.,* 1997). The studies of interaction of β kinase activity isolated from both Mono S and Mono Q as well as Pool-2 fraction against both the inhibitors showed that β kinases posses no sensitivity to either of the inhibitors suggesting that they posses distinctly different catalytic properties than the known kinases.

In an effort to distinguish the structural properties of two kinases, 18 small molecules of distinct structural classes were randomly screened for their action on the catalytic activity of α and β kinases, the results show that three inhibitors namely Hexamethylene diamene, 3-Acetyl pyridine, 3-Amino pyridine can selectively interact with the α kinase activity (TopoII α K_{HIV}) and two inhibitors namely Diphenyl amine and 3-Hydroxy pyridine with β kinase activity (TopoII β K_{HIV}). Hence, the α and β kinases are distinctly different and these kinases can be inhibited by a distinct structural group of molecules.

Fig 24: Action of staurosporin, and PD98059 on kinase activity of Topoisomerase II α and β by pool 1 and pool 2 fractions: Panel A shows the effect of staurosporin on Topoisomerase II α and β phosphorylation by pool 1 and pool 2. Panel B shows effect of PD98059 on Topoisomerase II α and β. phosphorylation by pool 1 and pool 2 fractions. The amount of radioactive P-32 incorporation in Topoisomerase II α and β were plotted on Y-axis and inhibitor concentrations were plotted on X-axis.

Figure 24

Action of inhibitors on Topoisomerase II phosphorylation promoted by pool I and pool II

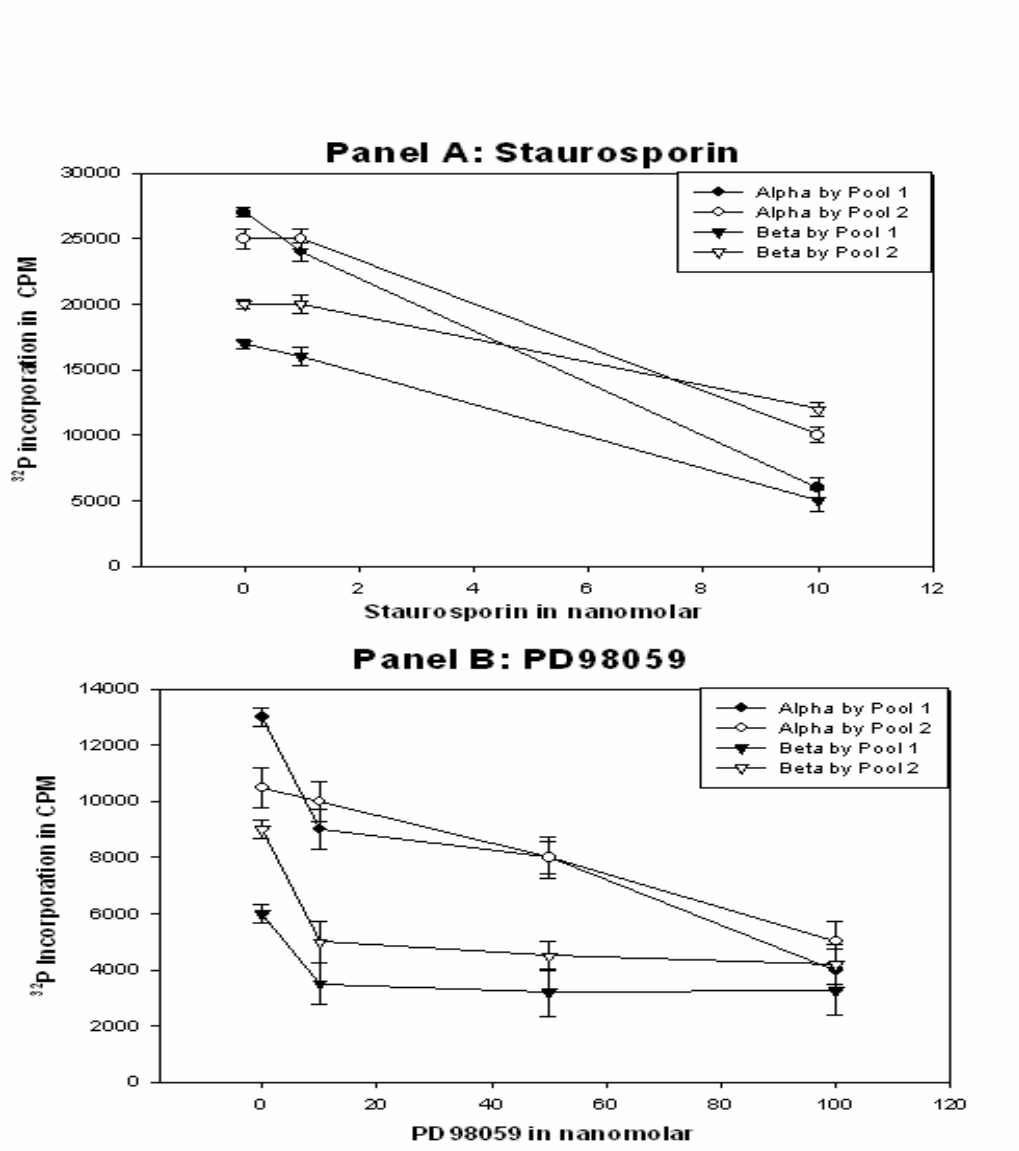
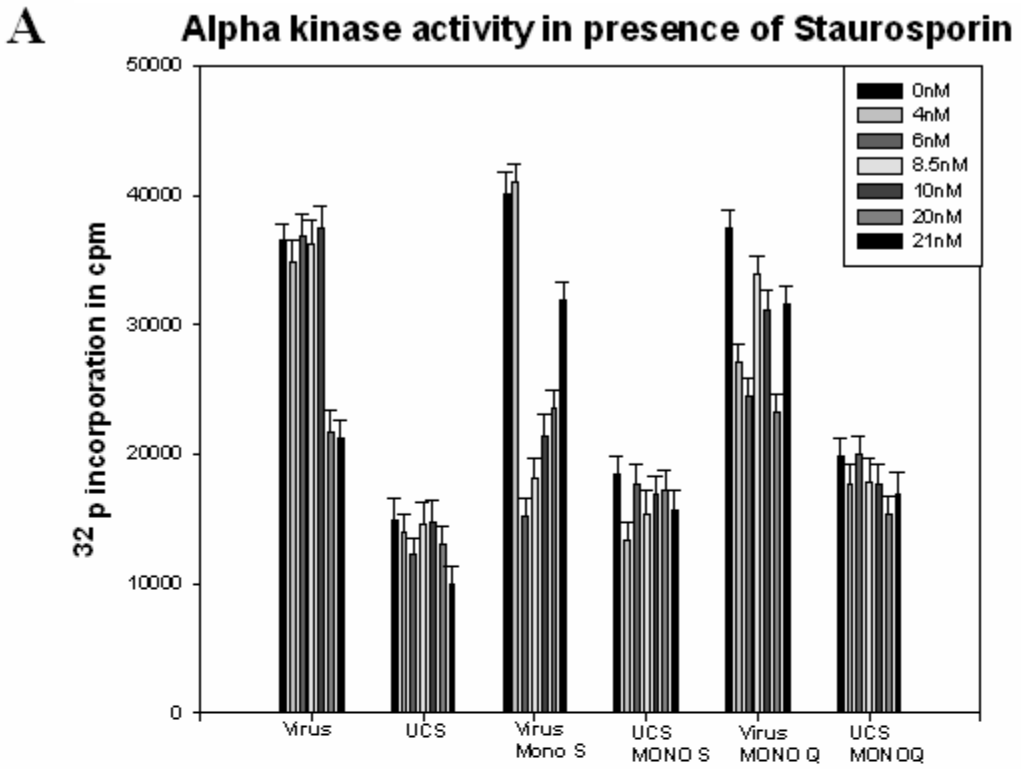


Fig 25: The differential sensitivity of Mono S and Mono Q isolated alpha and beta kinase activities against inhibitors: Topo II α (Panel A) and β (Panel B) kinase activity isolated from Mono S and Mono Q chromatography was analysed at the indicated concentrations of staurosporin (panel A, B). The inhibition concentrations were plotted on X-axis as a negative control we used uninfected cell supernatant that was pelleted as done with virus.

Figure 25

Action of Staurosporin on Topoisomerase II phosphorylation promoted by Mono S/Mono Q purified kinase



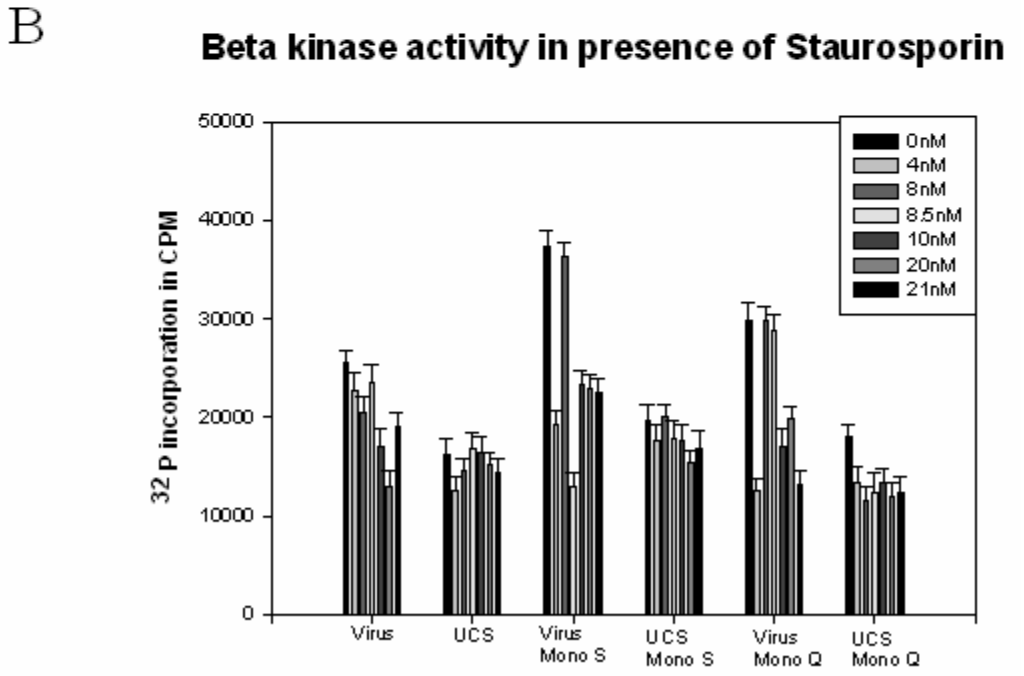
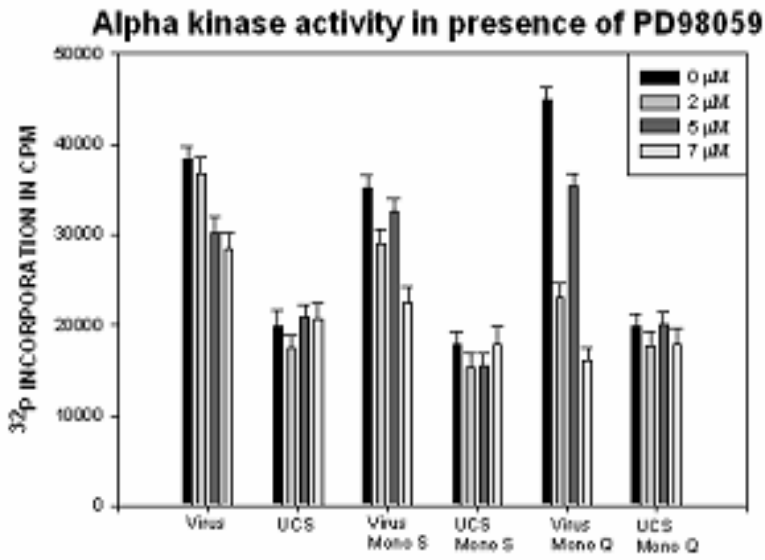


Fig 26: The differential sensitivity of Mono S and Mono Q isolated alpha and beta kinase activities against inhibitors: Topo II α (PanelA) and β (Panel B) kinase activity isolated from Mono S and Mono Q chromatography was analysed in the indicated concentrations of PD98059 (panel A,B). The inhibition concentrations were plotted on X-axis. Uninfected cell supernatant was pelleted as per the process of infected cell lysate virus and was used as negative control.

Figure 26

Action of PD98059 on Topoisomerase II phosphorylation promoted by Mono S/Mono Q purified kinase

A



B

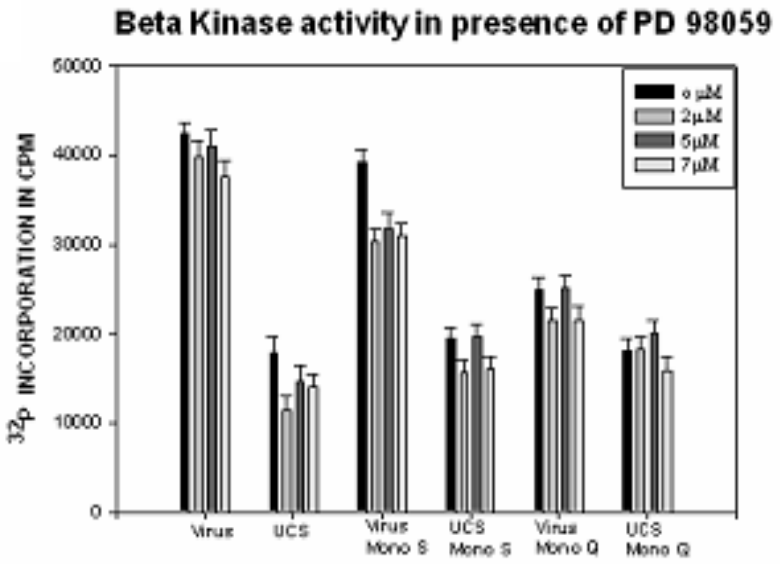


Fig 27: Phosphorylation of Topoisomerase II alpha by purified viral protein in the presence of specified molecules: The activity of TopoIIα K_{HIV} was analysed in the presence of indicated concentrations of specified molecules. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II α was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 27

Phosphorylation of Topoisomerase II alpha by TopoIIα K_{HIV} in the presence of following molecules

A

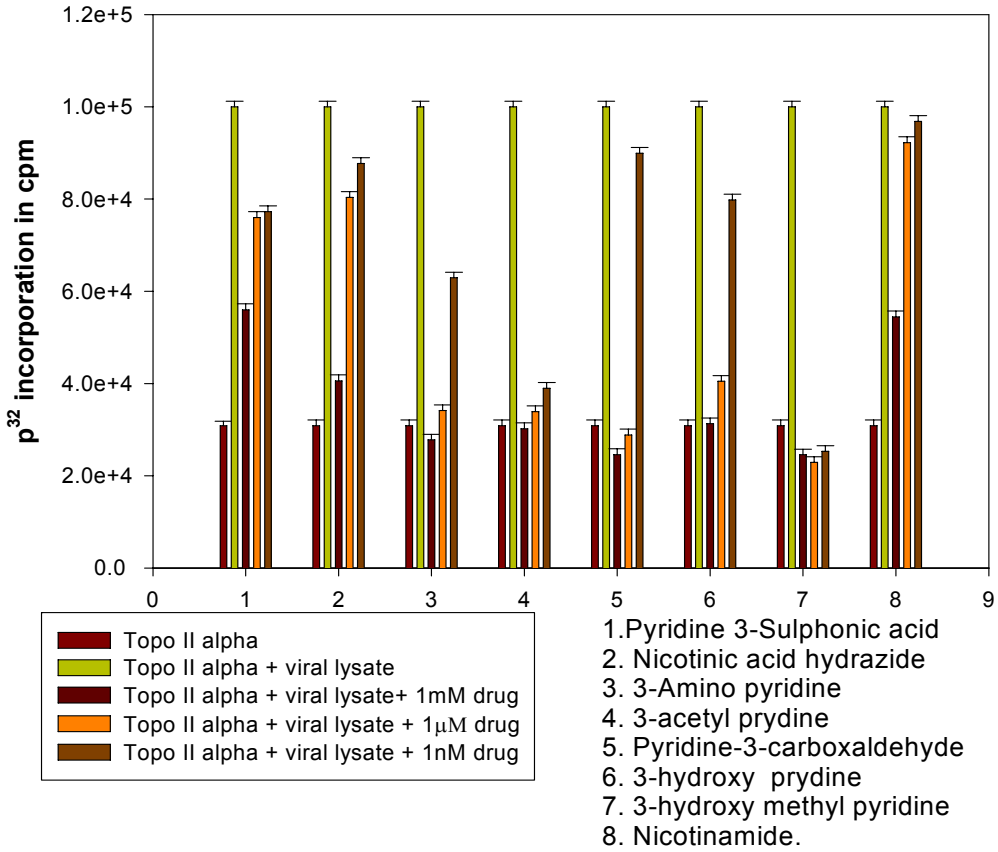


Fig 28: Phosphorylation of Topoisomerase II alpha by purified viral protein in the presence of specified molecules: The activity of TopoIIα K_{HIV} was analysed in the presence of indicated concentrations of specified molecules. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II α was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 28

Phosphorylation of Topoisomerase II alpha by TopoIIα K_{HIV} in the presence of following molecules

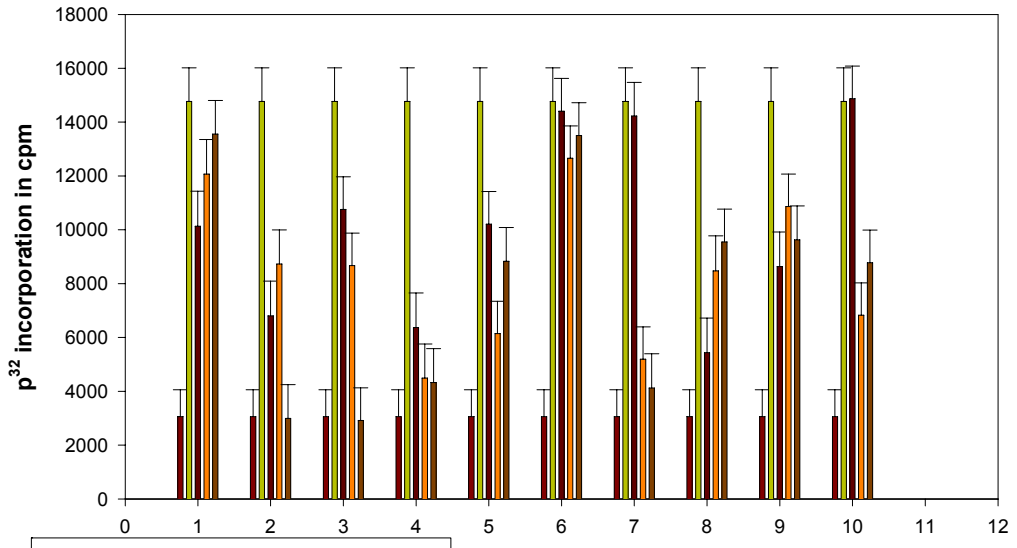


Fig 29: Phosphorylation of Topoisomerase II alpha by TopoIIα K_{HIV} in the presence of coded molecules: Molecules that showed inhibition were selected and reanalyzed for dose dependency in coded forms. Topoisomerase II α kinase activity of purified HIV-1 was analysed in the presence of indicated concentrations of coded molecules to reconfirm the results. The activity of TopoIIα K_{HIV} was analysed in the presence of indicated concentrations of specified molecules. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II α was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 29

Phosphorylation of Topoisomerase II alpha by TopoIIα K_{HIV} in the presence of coded molecules

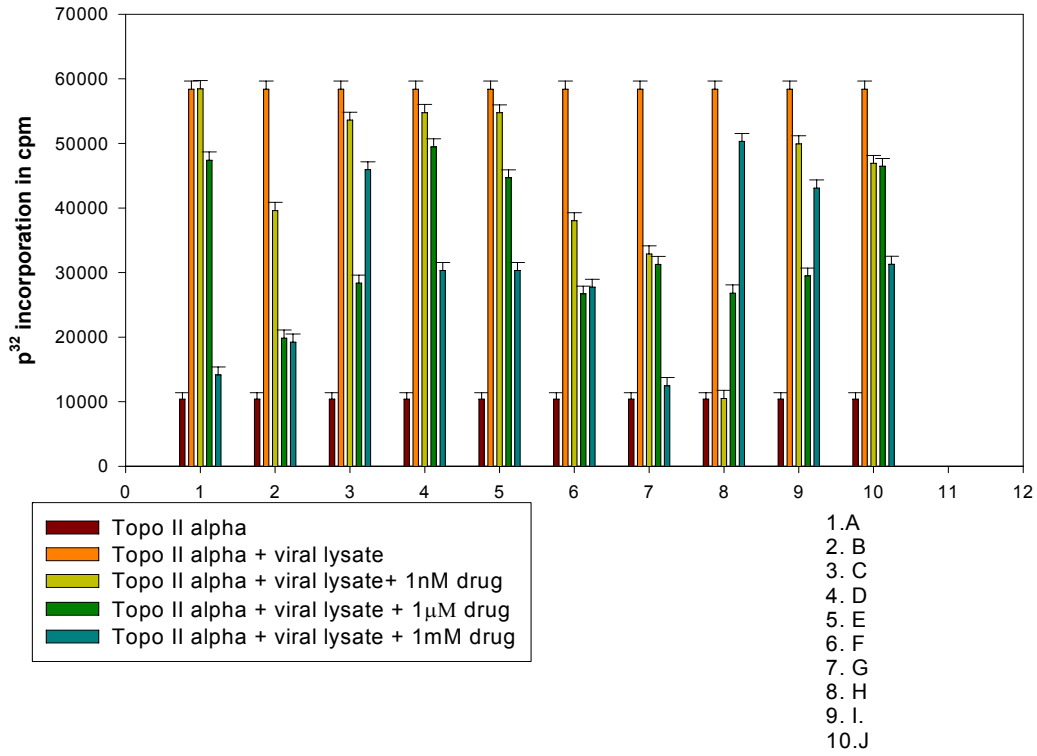


Fig 30: Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} in the presence of specified molecules Activity of TopoIIβ K_{HIV} was analysed in the presence of indicated concentrations of specified molecules. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II β was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 30

Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} in the presence of following molecules

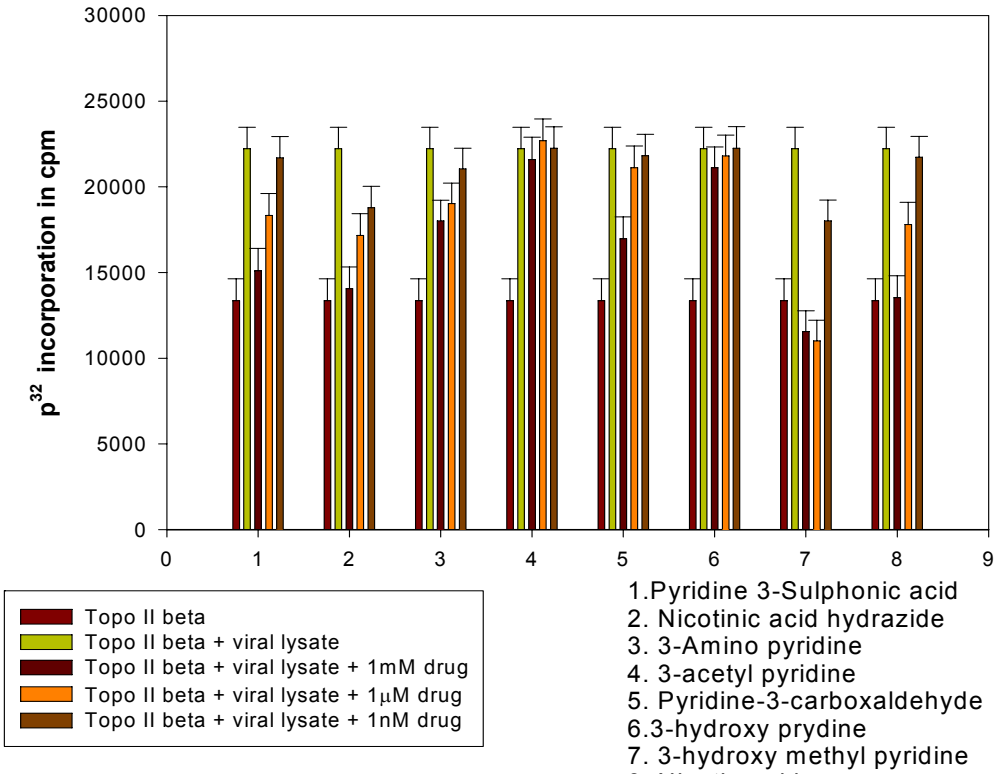


Fig 31: Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} the presence of specified molecules: The activity of TopoIIβ K_{HIV} purified HIV-1 was analysed in the presence of indicated concentrations of specified molecules. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II β was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 31

Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} in the presence of following molecules

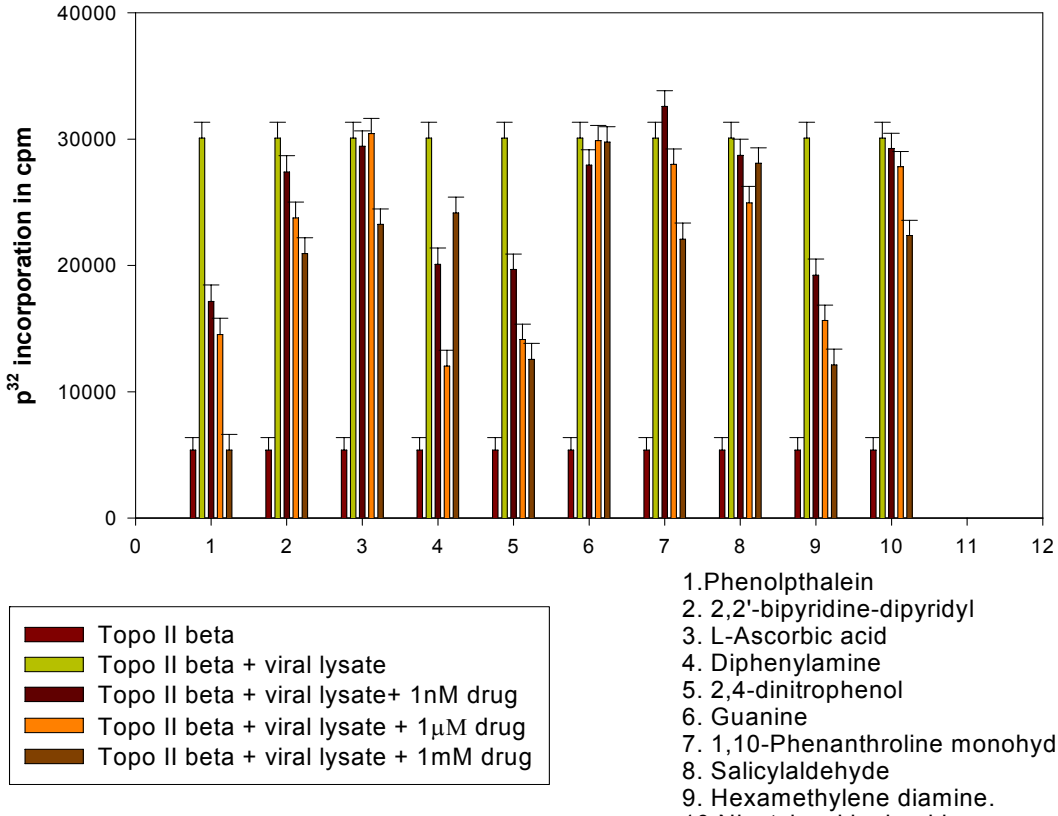


Fig32: Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} in the presence of coded molecules: Molecules that showed inhibition were selected and reanalyzed for dose dependency in coded forms. The activity of TopoIIβ K_{HIV} was analysed in the presence of indicated concentrations of coded molecules to reconfirm the results. The increasing concentrations of inhibitors were shown on X-axis. The kinase activity of P-32 incorporation of Topoisomerase II α was represented on Y-axis. Uninfected cell supernatant that was processed like infected one and was used as negative control.

Figure 32

Phosphorylation of Topoisomerase II beta by TopoIIβ K_{HIV} in the presence of coded molecules

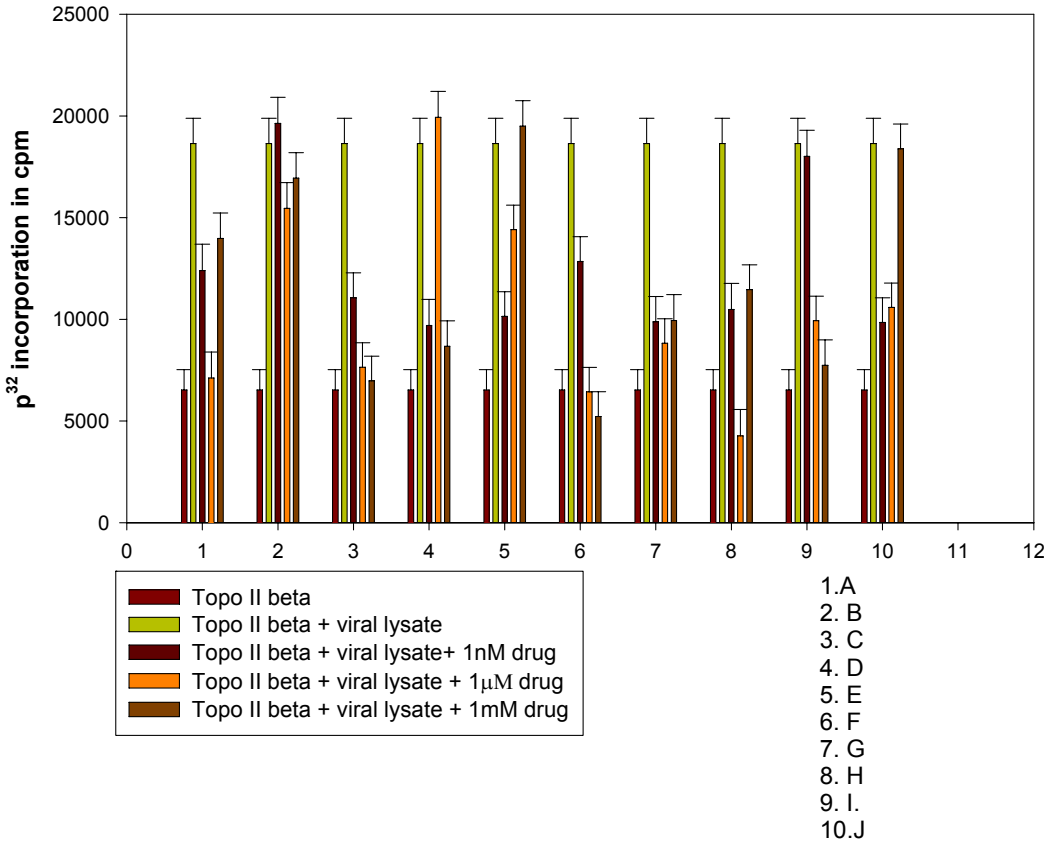
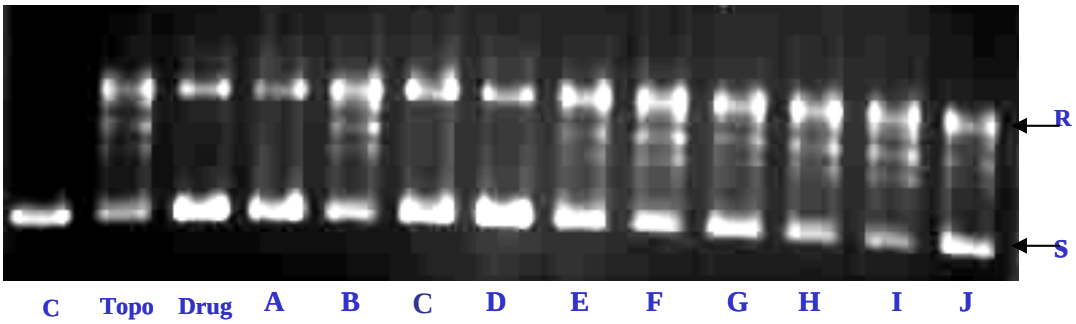


Fig 33: Catalytic activity of topoisomerase II in the presence of molecules: Relaxation assay of topoisomerase II was performed as in the presence of indicated concentrations of the molecules. C-plasmid alone, Topo-plasmid+Topoisomerase II, Drug- plasmid +Topoisomerase II + drug, remaining lanes contain same as drug specific molecules. The molecules that inhibited catalytic activity of Topoisomerase II α and β were eliminated from the further study.

Figure 33

Catalytic activity of Topoisomerase II α in the presence of molecules



A. 2,4-dinitrophenol
B. Hexamethylene diamene
C. 1,10-pheneanthroline
D. Phenolpthalein
E. L-ascorbic acid
F. diphenyl amine
G. 3-Acetyl pyridine
H. 3-hydroxy methyl pyridine
I. 3hydroxy pyridine
J. Amino pyridine

Fig 34: Phosphorylation of Topoisomerase II alpha and beta by purified viral kinase in the presence molecules: Molecules that showed dose dependent inhibition were reanalyzed for confirmation. The panel below shows autoradiogram of SDS-PAGE gels analyzed for ^{32}P incorporated into topoisomerase II α and β in the presence of molecules. C -dephosphorylated Topoisomerase II alone; V -dephosphorylated Topoisomerase II + Viral protein; CKII - dephosphorylated Topoisomerase II + CKII; A1,A2,A3,B1,B2 designates dephosphorylated Topoisomerase II + Viral protein + respective molecules.

Figure 34

Topoisomerase II alpha by TopoIIα K_{HIV}

Topoisomerase II beta by TopoII β K_{HIV}

***In vitro* phosphorylation**

A1) Hexamethylene diamene
A2) 3-Acetyl pyridine
A3) 3-Amino pyridine

B1) Diphenyl amine
B2) 3-Hydroxy pyridine

Conclusions

The following conclusions were derived from the work incorporated in the thesis.

- Analysis of Topoisomerase II α and β phosphorylation and isoform specific kinase activity during the course of HIV-1 infection suggest that HIV-1 infection stimulates both Topoisomerase II alpha and beta kinase activity at early as well as late stages of viral infection.

- Analysis of purified virus for the possible association of Topoisomerase II α and β kinase activity suggest that Topoisomerase II alpha and beta kinase activities are present in the purified viral particles. These kinases can be fractionated from isopycnic gradients.

- Isolation of kinase activity using ion exchange and hydrophobic chromatography suggests that Alpha kinase is –vely charged with low electrostatic and hydrophobic nature. Beta kinase is +vely charged with high electrostatic and moderate hydrophobic nature.

- Analysis of site of phosphorylation of topoisomerase II α and β promoted by isolated kinases shows that the Topoisomerase II α and β kinases contained in HIV-1 virus were serine kinases.

- Analysis of other nucleotide Co-factors used by the kinase in preference to ATP shows that Topoisomerase II alpha Kinase activity prefer dTTP and ddGTP, while topoisomerase II beta kinase use ATP alone.

- Studies of effect of pH and Temperature on the kinases suggest that Topoisomerase II alpha kinase activity was optimum at pH 5 and 37°C, whereas Topo II beta activity was optimum at pH 8 and 35°C.

- Analysis of sensitivity of α and β kinase to staurosporin and PD 98059 showed that α kinase is sensitive to both inhibitors, while no significant sensitivity is observed for β kinase activity. Hence the α kinase may share certain catalytic features common from PKC and MAPK kinase pathway kinases, while β kinase is distinctly different which need to be identified.

- Analysis of action of 18 distinct structural small molecules on topoisomerase II alpha and beta kinase activity have yielded 3 molecules with inhibitory action against topoisomerase II alpha kinase and 2 molecules against beta kinase.

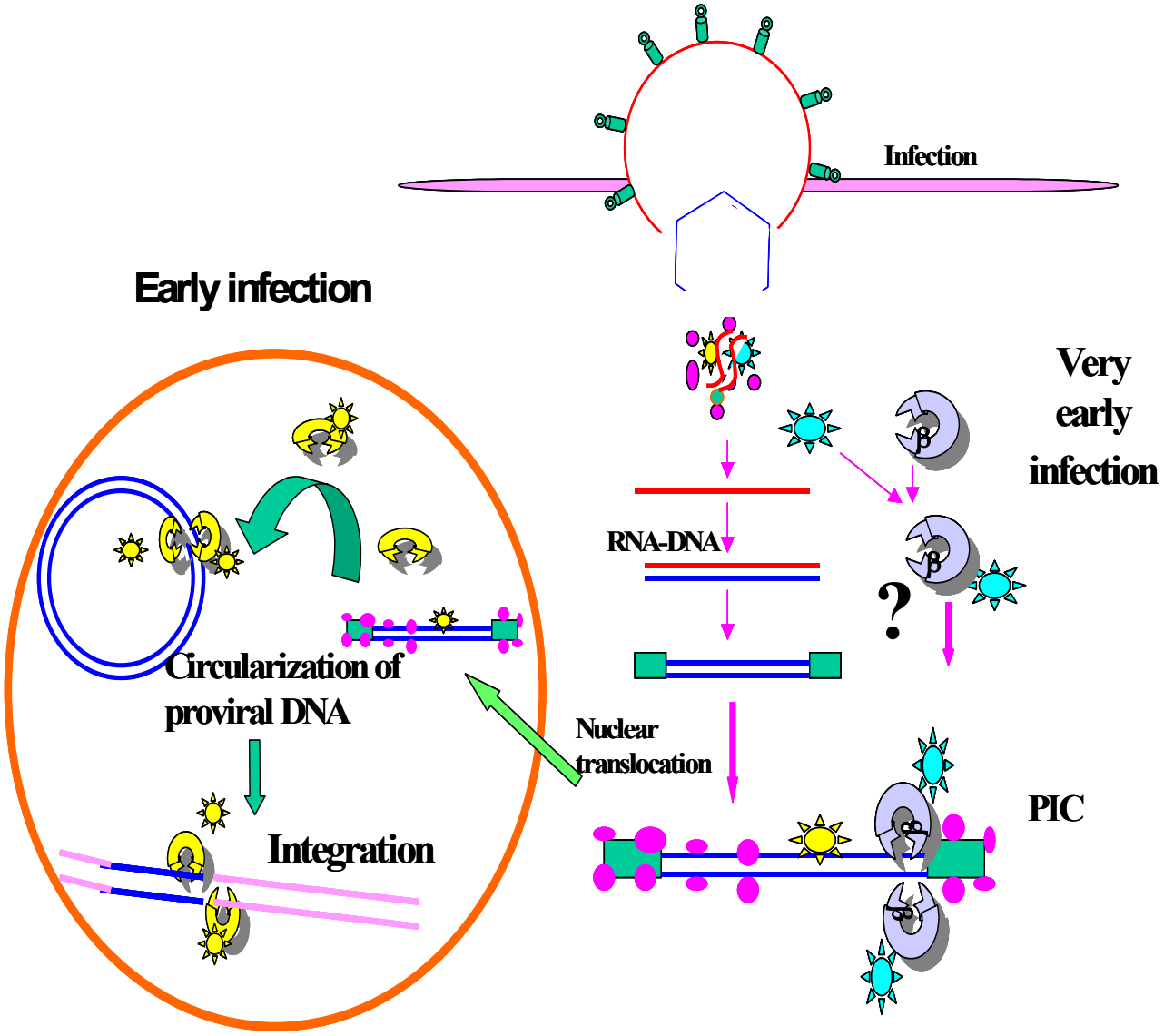
In summary HIV-1 viral particle is associated with a Topoisomerase II α and β specific serine kinases. These kinases are distinctly different serine kinases. The activities of Topoisomerase II α and β may have a role in early as well as late stages of HIV-1 replication.

A PROPABLE HYPOTHETICAL MODEL FOR THE ACTION OF HIV-1 ASSOCIATED TOPOISOMERASE II ALPHA AND BETA KINASE ACTIVITIES

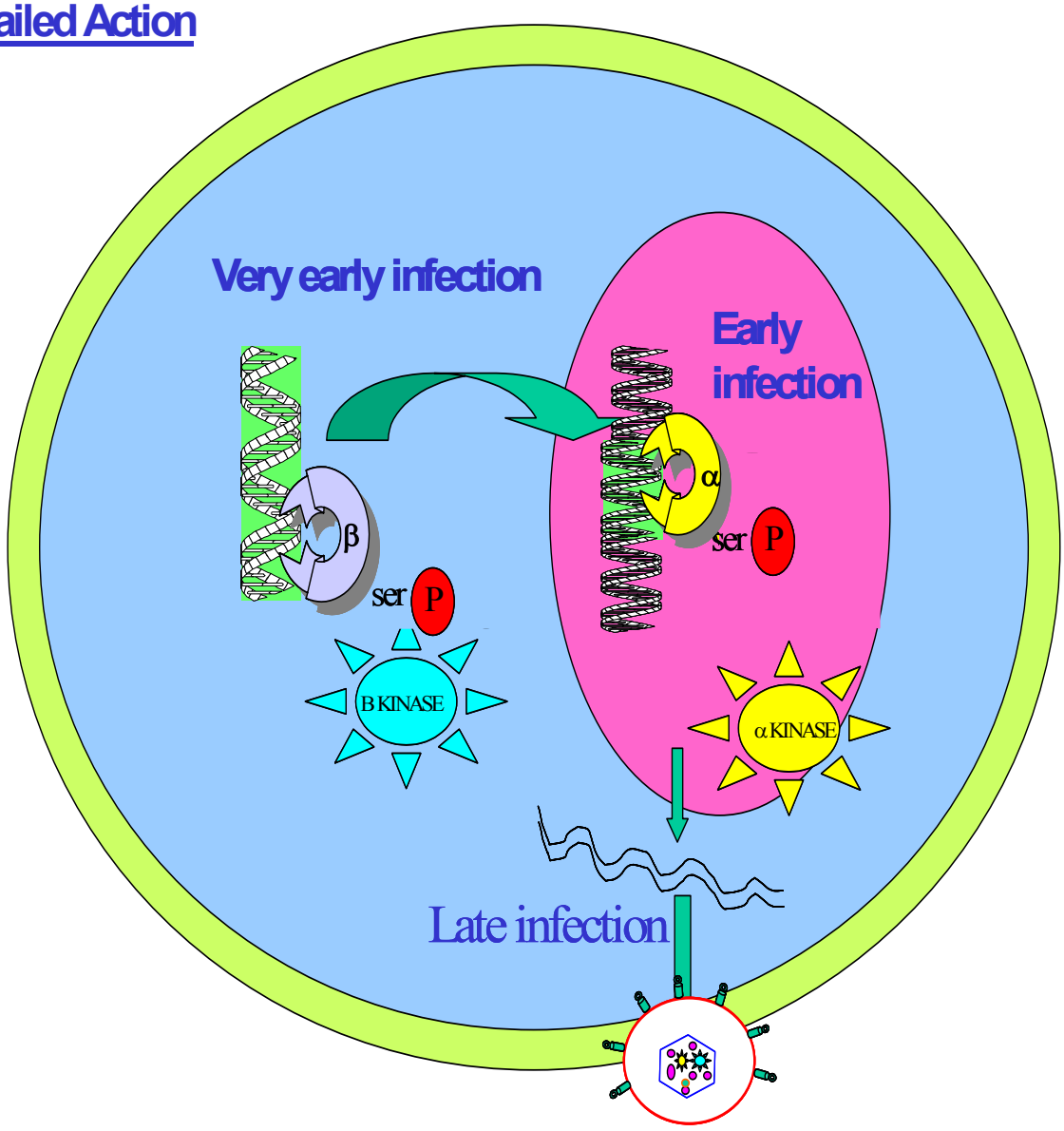
Based on the results incorporated in the thesis we propose the following hypothetical model. In this model the α and β specific kinases are present in the virus capsid. After uncoating of viral protein the capsid, the β kinase may be released and remain in the cytosol, while α kinase may be translocated to the nucleus alone or along with the preintegration complexes. The cytosolic β kinase activity released from the virus capsid may activate the topoisomerase II β isoform; the activated β isoform may participate in catalytic changes in the viral nucleic acids viz. both RNA and proviral DNA or proviral DNA alone and in the formation of preintegration complexes.

The α kinase independently or in association with pre-integration complexes may translocate to nucleus, where it catalyses the phosphorylation of Topoisomerase II α. The phosphorylated Topoisomerase II α may promote rearrangements of proviral DNA essential for the circularization of proviral DNA and integration into the host chromosome.

HYPOTHETICAL MODEL



Detailed Action



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