Concerted action of cellular JNK and Pin-1 restricts HIV-1 genome integration to activated CD4$^+$ T lymphocytes

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a mamma, papà

e Paolo

The only means of strengthening one's intellect is to make up one's mind about nothing, to let the mind be a thoroughfare for all thoughts.

L'unico modo per rafforzare l'intelletto è quello di non decidere niente riguardo a nulla, di lasciare che la mente sia una strada percorribile da tutti i pensieri.

John Keats

Introduction

1. The Human Immunodeficiency Virus 1 (HIV-1)

1.1 HIV-1 Life Cycle and host factors functions

Human Immunodeficiency virus (HIV) is a retrovirus, classified into the Lentivirus genus, which is the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). A hallmark of all members of the Retroviridae family is to produce DNA from an RNA genome via the enzyme reverse transcriptase. The DNA is incorporated into the host genome by the integrase enzyme (IN), then it replicates as part of the host cell DNA. In 1983 Dr. Montagnier isolated from lymphonodes of an asymptomatic individual, who presented a generalized lymphadenopatia, an agent containing a reverse transcriptase activity that was highly cytopathic in human peripheral blood mononuclear cells (Barré-Sinoussi et al., 1983).

In the same period, Dr. Gallo and Dr. Levy isolated a retrovirus from both immunodeficiency patients and healthy individuals from the various risk groups (Gallo et al., 1984; Popovic et al., 1984). This new retrovirus was originally named LAV by the French group and HTLV-III by the US group, and later called Human Immunodeficiency virus (HIV); the new disease was named Acquired Immunodeficiency Syndrome (AIDS). In 1986 a related virus was isolated from African individuals and was named HIV-2, which is less pathogenic than HIV-1 (Clavel et al., 1986). In the last 25 years therapeutic strategies have been successful to control viral spread, however AIDS is still considered a devastating and incurable disease. There are several reasons for the impossibility to cure HIV-1 infection, that relate to both the HIV-1 cellular targets and its life cycle. First HIV-1 mainly infects $CD4^+$ T cells and macrophages causing catastrophic effects on the immune system such as $CD4^+$ T cell depletion and destruction of lymphoid organs. Moreover the emergence of viral strains that are resistant to

currently available drugs is a crucial feature of the infection. One of the major reasons for the emergence of resistant strains is that both RNA Polymearse II and in particular Reverse Transcriptase, the two enzymes that synthesize viral genomes during the infection cycle are an error prone enzymes, lacking exonucleolytic proof-reading activity. In addition, during reverse transcription template switching occurs, causing deletions, insertions and mutations; these variations allow the virus to evade the immune response. Besides immune escape, a major reason why HIV-1 infection cannot be eradicated by the current therapies is due to the remarkable property of the virus to establish latent infection in resting $CD4^+$ T cells: since these cells do not express viral proteins, they remain completely unseen by the immune system and untouched by current antiretroviral therapies (Chun et al., 1995; Lassen et al., 2004; Williams and Greene 2007).

The HIV-1 genome is composed of two copies of positive single-stranded RNA that codes for the viral genes (Alizon et al., 1984). These two copies of the viral genome are enclosed by a conical capsid composed of 2,000 copies of the viral protein p24; single-stranded RNA is bound to nucleocapsid proteins. The virion contains two enzymes needed for the early steps of infection: Reverse Transcriptase (RT) and Integrase (IN), and Protease (PR), which ia important for virion maturation. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope, which is composed by phospholipid bilayer derived from the cell membrane when a newly formed viral particle buds from it. Two viral proteins that derive from the Env gene are embedded in the viral envelope: the glycoprotein 120 (gp120) and the glycoprotein 41 (gp41). These molecules are responsible for the recognition, attachment and fusion with the target cell.

The RNA genome consists of at least nine genes (*gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*) encoding 19 proteins. Two of these genes, *gag* and *env*, contain informations needed to make the structural proteins for new viral particles. *Env* codes for a polyprotein called gp160 that is cleaved by a viral enzyme to form gp120 and gp41 and *gag* is translated into a p55 polyprotein that is cleaved to form p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC) and p6. *Pol* codifies for RT, IN and PR, while the six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* are regulatory genes (Figure 1) (Martin et al., 2000). The Tat protein (p16 and p14) is a transcriptional transactivator for the LTR promoter acting by binding the TAR RNA element (Berkhout et al., 1989). The Rev protein (p19) is involved in shuttling RNAs from the nucleus to the cytoplasm by binding to the RRE RNA element (Rev Nekhai and Jeang 2006). A role for Vif (p23) has only recently been uncovered. This protein is necessary for an efficient infection of certain cells types, while other cells support infection in the absence of it (Sakai et al., 1991). Now it has been demonstrated that Vif prevents the action of the restriction factor APOBEC3G (Stopak et al., 2003; Sheehy et al., 2003). The Vpr protein (p14) seems to play a role in the translocation of the PIC from cytoplasm to nucleus and has been shown to arrest cell division at G2/M phase when transcription from LTR promoter is more efficient (He et al., 1995). The Nef protein (p27) downregulates both CD4 and MHC molecules probably through the recruitment of lck (leukocyte-specific protein tyrosine kinase) and PACS-1 (Phosphofurin acidic cluster sorting protein 1) (Piguet et al., 2000; Salghetti et al., 1995). A number of studies suggests that Nef may control the activation status of infected cells and their survival responses as it interacts with p21-activated kinase, Src-family kinases (PAK), Phosphoinositide 3-kinases (PI3-Kinase) and apoptosis signal-regulating kinase (ASK) (Nunn et al., 1996; Wolf et al., 2001; Geleziunas et al., 2001; Graziani et al 1996; Blagoveshchenskaya et al., 2002). However its role in signal transduction pathways still re mains

controversial (Marsh, 1999). The Vpu protein (p16) is an integral membrane phosphoprotein. Early studies have demonstrated that it is necessary for an efficient release of the viral particles (Klimkait et al., 1990). The mechanisms by which Vpu influences particle release was disclosed only recently by two different groups. Biesniasz and colleagues discovered a new cellular factor (Tetherin) that blocks viral particle release and is antagonized by Vpu (Neil et al., 2008) and Freed laboratory found that Calcium-modulating cyclophilin ligand (CAML), which restricts HIV-1 release, is also a Vpu-interacting factor (Varthakavi et al., 2008).

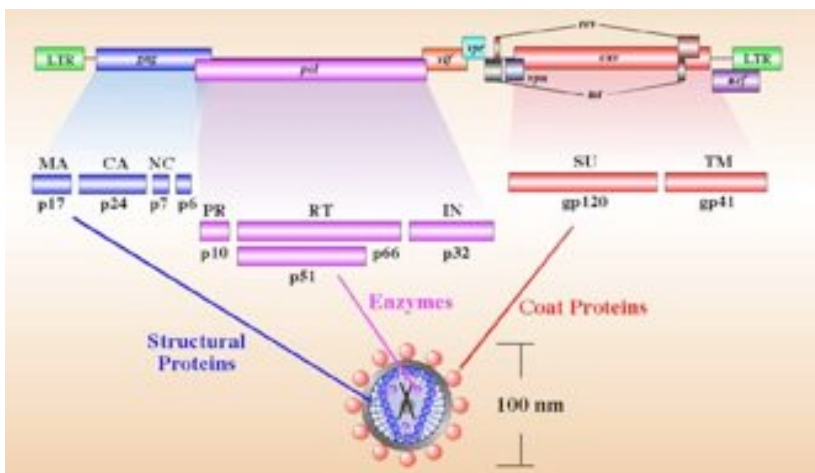


Figure 1. Schematic representation of the HIV-1 genome. (Standford University Web Site).

1.1.1 Cellular proteins regulating HIV-1 infection

HIV-1 is a very sophisticated virus despite its simple genomic structure. Virtually all steps of its viral replication cycle involve cellular factors, suggesting a very complex dynamic relationship between the virus and the infected cell. Cellular proteins seem to have either pro-viral or anti-viral functions. Over the past several years, many cellular proteins involved in HIV-1 infection have been discovered, nevertheless the complexity of HIV-1 viral cycle suggests that presence of several other cellular partners. In 2008 three different large scale RNA interference screenings were performed to identify these partners (Brass et al., 2008; König et al., 2008; Zhou et al., 2008). Hundreds of proteins have been found to be involved in HIV-1 infection unlocking a broad range of possible future investigations.

Cellular Factor	**Viral Phase**	**Activity**	**Reference**
CD4	Entry	Binding with Env	Dalgleish et al., 1984
CXCR4	Entry	Binding with Env	Endres et al 1996
CCR5	Entry	Binding with Env	Choe et al., 1996
DC-SIGN	Entry	Virion Internalization in DC cells	Geijtenbeek et al., 2000
Dynamins	Entry	Necessary for virions endocytosis	Miyauchi et al., 2009
Trim5 alpha	Early Phase	*Interferes with the uncoating process* RESTRICTION FACTOR	Hatziioannou et al., 2004 Keckesova et al., 2004
Cyclophilin A	Early Phase	*Protects HIV-1 from an unknown antiviral factor*	Luban et al., 1993 Braaten and Luban 1996
APOBEC3	Reverse Transcription	Induces mutations in viral cDNA RESTRICTION FACTOR	Sheeny et al 2002 Harris et al., 2003
Uracil-DNA	Reverse	Controls dUTP	Bouhamdan et al.,

glycosylase	Transcription	misincorporation in viral cDNA	1996 Priet et al., 2005
Cofilin	Post-Entry Migration	Actin depolymerizing factor	Yoder et al., 2008
Transportin-SR2 (TRN-SR2)	Nuclear Translocation	PIC entry	Chirst et al., 2008
Importin7	Nuclear Translocation	PIC entry	Fassati et al., 2003
BAF	Integration	Prevents autointegration	Lee and Craigie, 1994
INI1/hSNF5	Integration	IN interactor involved in virion production	Kalpana et al., 1994 Sorin et al., 2006
LEDGF	Integration	Tethering factor	Cherepanov et al., 2003 Maartens et al., 2003
Emerin	Integration	Bridges together chromatin and viral cDNA	Jacque and Stevenson 2006 Shun et al., 2007
p300/CBP	Integration/ Transcription	Positively modulates integration and transcription	Cereseto et al., 2005 Marzio et al., 1998 Benkirane et al., 1998
Cyclin T1	Transcription	Regulation of HIV-1 transcription	Fujinaga et al., 1998 Bieniasz et al., 1999
NAP-1	Transcription	Regulation of HIV-1 transcription	Vardabasso et al., 2007
CRM-1	Nuclear export	Nuclear export receptor for unspliced viral RNA	Askjaer et al., 1998
TSG 101	Assembly and Budding	Interaction with ESCRT machinery	Garrus et al., 2001
Tetherin	Assembly and Budding	Inhibits Budding RESTRICTION FACTOR	Neil et al., 2008
Calcium-modulating cyclophilin ligand	Assembly and Budding	Inhibits Budding RESTRICTION FACTOR	Varthakavi et al., 2008

(CAML)			
PACS-1	Host-Virus Interaction	Down-regulation of MHC	Piguet et al., 2000
lck	Host-Virus interaction	Down-regulation of CD4	Salghetti et al., 1995
PAK	Host-Virus interaction	Anti-apoptotic signals	Nunn et al., 1996 Wolf et al., 2001
ASK	Host-Virus interaction	Anti-apoptotic signals	Geleziunas et al., 2001
PI3-Kinase	Host-Virus interaction	Anti-apoptotic signals/ Down-regulation of MHC	Graziani et al., 1996 Wolf et al., 2001 Blagoveshchenskaya et al., 2002

Table I. Some of the most relevant and validated cellular partners for HIV-1 infection; restriction factors are indicated in red.

1.1.2 Regulation of HIV-1 infection early phase by cellular proteins: a balance between restriction and permissivity factors

ADSORPTION AND INTERNALIZATION

HIV-1 is an enveloped virus. The envelope, formed during budding, is a lipid bilayer carrying phospholipids and both viral and cellular proteins. Cellular components, which constitute a significant fraction of the envelope, represent particular areas of the plasma membrane from which budding occurred. The HIV-1 envelope is rich in cholesterol, indicating its preferential derivation from lipid rafts, as well as in MHC molecules. Two viral proteins are present in the envelope: gp120 or the surface env subunit (SU) and gp41, the transmembrane subunit (TM); these proteins are products of proteolytic cleavage of the gp160 precursor .

The sequence of gp120 is highly variable and heavily glicosylated: the most variable domains, termed hypervariable loops, are exposed at the surface of the virion while the conserved domains are folded to form the core of the protein (Fennie et al., 1989). gp120 interacts with specific cellular

receptors, the CD4 molecule and two different chemokine receptors (Dalgleish et al., 1984; Klatzmann et al., 1984; Alkhatib et al., 1996; Choe et al., 1996; Endres et al., 1996; Deng et al., 1996, Feng et al., 1996). The CCR5 protein functions as a receptor for chemokines belonging to the CC group, including RANTES, MIP-1α and MIP-1β. This receptor is predominantly expressed on T cells, macrophages, dendritic cells and microglia. The CXCR4 molecule, also called fusin, is an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1), a molecule possessing a potent chemotactic activity for lymphocytes. Both CCR5 and CXCR4 belong to the family of G-protein-coupled chemokine receptors and share a seven transmembrane-spanning alpha-helix structure that mediates signal propagation from biological membranes (rev: Lodowski et al., 2009). Based on its ability to bind these chemokine receptors, HIV-1 can be divided into different strains: CCR5 (R5 strain) or CXCR4R (X4 strains) or both (R5X4 strains). R5 strains can be isolated throughout the natural course of human infection and reflects the capability of HIV-1 to infect macrophages and monocytes. X4 and R5X4 strains appear in the late stages of infection, when the immune system is impaired; appearance of strains possessing this receptor specificity is an aggravating factor because it reflects the ability of HIV-1 to infect a larger spectrum of cells including resting T lymphocytes. The adhesion of HIV-1 to cells is mediated by the aspecific interaction between gp120 and charged groups on the cell surface. These interactions are important for the subsequent binding between gp120 and its specific receptors (Brelot and Alizon, 2001). HIV-1 has long been assumed to fuse directly with the plasma membrane (Stein et al., 1987; McClure et al 1988; Melikyan 2008). This fusion occurs after the induction of a conformational change in TM (Chan and Kim, 1998). Recent work entailing live cell imaging demonstrates that HIV-1 entry also occurs after virion endocytosis and shows that the cellular protein dynamin plays a pivotal role in this process (Figure 2) (Miyauchi 2009).

Adhesion of HIV-1 to the cell surface is particularly important for the mucosal transmission of the infection. It has been shown that dendritic cells present in the skin and mucosae can uptake virions thorough the mannose-binding C-type lectin domain of a type II membrane protein named DC-SIGN (Geijtenbeek et al., 2000). The virions are retained in dendritic cells for extended periods of time in an infectious state and are presented to permissive T cells. The sites of contact and HIV-1 presentation between DC and $CD4^+$ T cells, called immunological synapses or virological synapses, were visualized by high resolution microspcopy. The authors showed that, in these contact sites, the viral receptors cluster, thus allowing highly efficient infection (Stoll at al., 2002; McDonald et al., 2003).

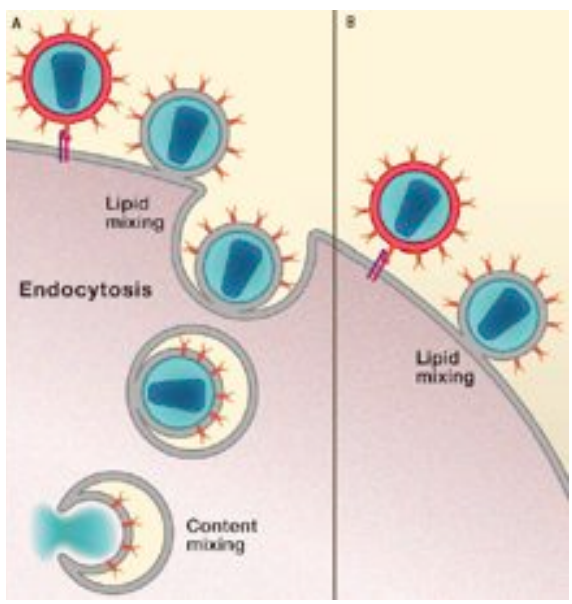


Figure 2. The new model of HIV-1 entry. Fusion events that occur at the plasma membrane and proceed at least to the stage of hemifusion and subsequent endocytosis (A). Fusion events at the plasma membrane that do not result in any subsequent content mixing (B) (Uchil and Mothes 2009).

UNCOATING

Following entry, the uncoating steps take place; removal of the CA and release of the viral genome into the cytoplasm are necessary for the initiation of reverse transcription.

The early steps of viral replication seems to be the main targets for host proteins that elicit an innate antiviral response. These factors are generally termed as "Restriction Factors". The existence of retroviral restriction factors was discovered by Lilly and colleagues in 1967. In particular these authors found a protein that confers resistance to the Friend's murine leukemia virus in murine cells. This factor was named Fv1. This molecule probably exerts its restriction activity after the initiation of reverse transcription but before viral integration (DesGroseillers and Jolicoeur 1983). Interestingly it was found that restriction can be blocked by saturation of target cells with non infectious viral particles and that resistance was abrogated by a single mutation in the CA protein. In human cells, the factor that conferred resistance to N-MLV at the early post-entry stages of viral infection was termed restriction factor 1 (Ref1) while, in primate cells, a group of factors restricting lentiviral infection was termed lentivirus susceptibility factor 1 (Lv1) (Towers et al., 2002; Hatziioannou et al., 2003). Lv1 was found to induce resistance to HIV-1 and numerous other lentiviruses in Old World and New World monkeys and to act in a dominant manner (Münk et al., 2002). A few years later, Tripartite motif protein 5 alpha (Trim5 α) was identified through a screening of a rhesus cDNA library, and was shown to confer resistance to HIV-1 infection in HeLa cells (Stremlau et al., 2004). Both Ref1 and Lv1 were shown to belong to the Trim5α family (Hatziioannou et al., 2004; Keckesova et al., 2004). The antiviral mechanism of Trim5α action has not been completely elucidated, and seems to involve blockade of HIV-1 infection at several steps. The protein possesses three well defined domains, a RING finger, a B-box and a coiled coil domain, common for all the TRIM family members,

as well as an additional SPRY domain which is necessary for its binding with viral CA. Several proteins having a RING finger domain possess a E3 ubiquitin ligase activity, thus it was initially hypothesized that TRIM5α might acta as an antiviral factor by inducing the degradation of the core proteins of the virions; despite that TRIM5α mutants in the RING finger domain still possess an antiviral activity, albeit lower than the wild type (see reviews Luban, 2007; Towers, 2007). Moreover, proteosomal inhibition induces accumulation of viral DNA in the cytoplasm, but the nuclear import remained impaired (Perez-Caballero et al., 2005; Javanbakht et al., 2005). Therefore it was proposed that TRIM5α acts through blocking the nuclear import of PICs (Wu et al., 2006). Interestingly, in New World owl monkeys, which are the only New World primates resistant to HIV-1 infection, Trim5α action was found to be dependent on Cyclophilin A (CypA), a cytoplasmatic peptidyl-prolyl isomerase that belongs to the family of Cyclosporin A (CsA)-binding cyclophilins. In particular the authors found that, in owl monkeys, three different short hairpin RNAs (shRNAs) against CypA were able to eliminate resistance; however reintroduction of CypA did not restore the antiviral activity. Only after a screen of owl monkey cDNAs, the existence of a mRNA coding a fusion protein between CypA and Trim5α was revealed. Hence, in these primates, the antiviral activity is determined by the presence of a fusion protein composed by 299 N terminal aminoacids of Trim5α and CypA (Sayah et al., 2004). The fact that CypA was required for Trim5α restiction against HIV-1 seems paradoxical, since it has been previously demonstrated that human CypA is required for the early steps of HIV-1 infection exerting its function on CA, the same protein targeted by Trim5α (Luban eal., 1993). CypA binds to CA protein in the producer cell and it is incorporated into the virions. Therefore, it was then proposed that CypA might play a role in viral assembly (Franke et al., 1994; Thali et al., 1994). However, knockdown of CypA does not affect viral particle

formation and release. Additionally in the same period it was demonstrated that CypA was necessary for the early steps of viral life cycle (Braaten et al 1996; Braaten and Luban 2001). In paricular, isomerization of CA by CypA seemed to protect the virion from TRIM5α antiviral activity. In closing, it should be emphasized that other studies have demonstrated that CypA protects HIV-1 from an unknown antiviral factor that is independent from TRIM5α (Keckesova et al., 2006; Sokolskaja et al., 2006).

REVERSE TRANSCRIPTION

Anotother mechanism that can suppress HIV-1 replication is mediated by the apolipoprotein B mRNA editing enzyme family (APOBEC3), also known as CEM-15, which most probably exerts its antiviral function during the step of reverse transcription. The first member of APOBEC to be isolated as a restriction factor was APOBEC3G (Sheehy et al., 2002). It was known that certain cells are not permissive to HIV-1 strains harbouring mutation in the *vif* gene, while other cell types remain permissive to HIV-1 lacking a functional Vif protein (Gabuzda et al., 1992). Through subtractive cloning between non-permissive and permissive cells it was discovered that the inhibitory factor for HIV-1 replication in non permissive cells was APOBEC3G. Later, its close relatives APOBEC3F, and to a lesser degree APOBEC3B, were found to posses similar antiviral activities (Zheng et al., 2004). APOBEC3G from a producer cell is incorporated into new virions. Both virion-associated and cellular APOBEC3G play an antiviral function in the newly infected cell. Vif counteracts this antiviral activity recruiting a E3 ubiquitin ligase complex that target APOBEC3G to proteosomal degradation (Stopak et al., 2003; Sheehy et al., 2003). APOBEC3G belongs to the family of polynucleotide cytosine deaminase (CDAs), that catalyzes the deamination of cytosine to uracil in DNAand/or RNA strands (Teng et al

1993). Hypermutation of viral genomes clearly is deleterious for the spread of HIV-1 infection by causing replication defects at multiple steps. Nonetheless there is still an ongoing discussion whether editing of the viral genome can explain completely the antiviral effect of APOBEC. In particular, it was shown that a catalytic inactive mutant of APOBEC still possessed antiviral effect. These authors observed that overexpression of inactive APOBEC3G reduced the accumulation of reverse transcripts similar to the wild type protein, and proposed that APOBEC3G interferes with the removal of tRNA primer and thus exerted antiviral effects independent from its enzymatic activity (Guo et al., 2006). Recent work however demonstrate that an enxymatically inactive APOBEC3G display less efficient resctriction activity, thus calling again into question the relevance of deamination (Miyagi et al., 2007; Browne et al., 2008; Aguiar and Peterlin, 2008).

One possible mechanism explaining the antiviral activity of APOBEC is the degradation of uracilated viral cDNA through the activity of cellular DNA glycosylases, e.g. uracil-N-glycosidase (UNG) and Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1). During reverse transcription, APOBEC proteins introduce C to U mutations in the newly synthetized minus strand viral cDNA. Since uracils are not tolerated in the DNA, they are removed by a cellular enzyme named (UNG) and the nicked DNA is further degraded in the cytoplasm or, once integrated, might lead to the production of aberrant mRNA and protein products (Lecossier et al., 2003; Mangeat et al., 2003; Harris et al., 2003). Again, the interplay between APOBEC and UNG enzymes is still not clear. One report descirbed that the nuclear form of UNG (UNG2) is packaged into HIV-1 virions through an interaction with Vpr to modulate viral mutation rate (Mansky et al., 2000). In contrast, another study concluded that Vpr induces UNG and SMUG proteasomal degradation thus reducing their packaging into virions (Schrofelbauer et al., 2005). A third study did not observe any effect of Vpr

on UNG packaging (Kaiser et al., 2006). Consistent with this latter study, a fourth investigation reported that UNG packaging was, indeed, Vpr-independent and instead involved an interaction with the HIV-1 IN (Willets et al., 1999). Thus, the mode of UNG packaging remains under discussion; however, most studies agree on the presence of UNG2 in HIV-1 virions (Rev Goila-Gaur and Strebel 2008). An additional interesting role of APOBEC3G is the regulation of the permissivity of $CD4^+$ resting T cells to HIV-1 infection. Resting $CD4^+$ T cells are highly resistant to HIV infection and, as demonstrated recently, in these cells APOBEC3G is associated with a low molecular weight complex in which it is enzymatically active and thus restricts HIV-1 infection. In activated T cells, APOBEC3G becomes associated with a high molecular mass complex (HMM) and its enzymatic activity is inhibited (Chiu et al., 2005).

1.1.3 Interactions between cellular and viral proteins during HIV-1 integration and transcription

NUCLEAR TRANSPORT OF VIRAL cDNA

A hallmark of all retroviruses is the integration of the their genomes into the host DNA, however only lentiviruses have the capability to integrate into the genome of non-dividing cells. In the case of HIV-1, this feature is very important since non-dividing macrophages are fundamental reservoirs of the virus in infected individuals (Yamashita et al., 2006). Products of reverse transcription are transported through the cytoplasm and into the nucleus as a structure termed Pre-integration complex (PIC), which contains cDNA and viral proteins as well as some cellular proteins. The

mechanism by which PICs are transported through the nuclear pores into the nucleus is still poorly understood, and depends on both host and viral factors. Once in the nucleus, full length linear copies of reverse transcripts are integrated in to the host genome by the viral enzyme IN. Although each virion contains two RNA molecules, only one copy ends up integrated into the host cell genome (Suzuki et al., 2007). PICs consist of double stranded viral cDNA and both viral (IN, NC, MA, RT and Vpr) and cellular proteins (BAF, HMGs, LAP2a, Ku, LEDGF/p75). Nuclear pores (NP) allow the active transport of complexes and macromolecules, they have diameter of 25 nm while the diameter of the PIC is about 56 nm, thus suggesting that HIV-1 PICs use active transport to reach the nucleus (Bukrinsky et al., 2004). Studies using fluorescently labelled PICs indicate that they co-localize with microtubules organizing centres (MTOC), which are located in cytoplasm close to the nuclear membrane (McDonald et al., 2002). The molecular processes of PIC entry into the nucleus are still poorly understood but it is possible that the virus uses a redundancy of mechanisms. In general, all the PIC components play a role in nuclear translocation.

MA was the first viral protein to be implicated in nuclear translocation of the PIC (Bukrinsky et al., 1993). Early studies discovered that MA, although itself unable to localize in the nucleus, contains a nuclear localization signal (NLS). This peptide induced nuclear translocation of other proteins when fused to them (Depienne et al., 2000). Phosphorylation of Tyr 132 in the MA protein was also proposed to be important for nuclear entry, however in the absence of functional MA HIV-1 can still efficiently infect non dividing cells such as macrophages (Gallay et al., 1995 ; Reil et al., 1998). The accessory protein Vpr was also suggested to be involved in the nuclear import of the PIC since it possesses karyophilic sequences and localizes to the nucleus. The role of Vpr also remained unclear since some groups

reported that it is important for HIV-1 replication in resting macrophages (but not in cycling T cells), while others showed that it is not necessary for nuclear import of the PIC in growth arrested cell lines (Sherman and Greene 2002). An intriguing hypothesis regarding the function of Vpr was made by Greene and colleagues, who demonstrated that Vpr alters nuclear structure by causing the formation of herniations. These ruptures in the nuclear envelope provide an access for the PICs (de Noronha et al., 2001).

The particular structure present in the viral DNA, named DNA-Flap, seems to participate in PICs nuclear import. DNA-Flap is a triple stranded intermediate created during reverse transcription and mutations in this sequence greatly impairs nuclear import (Zennou et al., 2000). However, the mechanism by which this DNA sequence functions as a nuclear localization signals remains unclear.

Although PIC entry into the nucleus remains still poorly understood, it is clear that IN plays a leading role in the process. IN accumulates in the nucleus and contains several putative NLS, the most important of which seems to be the sequence present in the catalytic domain. When this sequence is mutated, the nuclear import is greatly impaired albeit IN catalytic activity remains intact (Bouyac-Bertoia et al., 2001). Some more recent studies propose that, even in the absence of a transferable NLS, IN still localizes in the nucleus due to its interaction with LEDGF/p75 (Maertens et al., 2003; Vanegas et al., 2005).

In general, the import of PICs into the nucleus also involves cellular proteins. Several components of the PIC interact with the family of importin alpha while Vpr also interacts with nucleoporins (Suzuki and Craigie 2007). A yeast two-hybrid screen recently revealed a new IN partner, named transportin-SR2 (TRN-SR2) involved in regulation of the nuclear import. By using fluorescently labeled HIV-1 particles, the authors demonstrated that,

under conditions of TRN-SR2 knockdown, nuclear import was impaired and that HIV-1 replication in macrophages was blocked (Christ et al., 2008).

INTEGRATION

Once in the nucleus, viral DNA stably interacts with chromosomal DNA. LEDGF/p75 seems to be the key factor that contributes to stable tethering of IN to chromatin (Maertens et al., 2003; Emiliani et al., 2005; Hombrouck et al., 2007). A role in tethering HIV-1 to chromatin has also been attributed to emerin, a component of the nuclear envelope. In particular, this protein was demonstrated to regulate HIV-1 integration in macrophages (Jacque and Stevenson 2006). However, subsequent studies failed to confirm the involvement of this protein in tethering HIV-1 to chromatin (Shun et al., 2007).

HIV-1 integration is not site-specific and in vitro studies revealed that different primary DNA sequences can function as acceptor sites for viral integration (Bor et al., 1996). Early in vitro studies demonstrated that the presence of proteins bound to the acceptor DNA inhibits the integration reaction by steric hindrance (Bor et al., 1995). However, incorporation of histones does not inhibit integration but rather distorts the target DNA, thus creating hot spots that often favour integration (Pryciak and Varmus 1992).

Retroviral integration does not occur at random in the host DNA and a number of different studies suggest a role for chromatin in the site selection of HIV-1. It has been shown that centromeric heterochromatin is an unfavourable site for HIV-1 integration probably due to the poor accessibility of these regions (Carteau et al., 1998). Nevertheless, integrations into heterochromatic regions do occur and are proposed to be

tightly connected with a phenomenon of post-integration latency (Jordan et al., 2001 ; Jordan et al., 2003 ; Lewinski et al., 2005). Sequencing of more that 500 sites of HIV-1 integration in SupT1 T cell line revealed that gene regions were preferentially chosen by HIV-1 for its integration (Schroder et al., 2002). Moreover, comparative analysis of integration sites of HIV-1, MLV and ASLV showed that these retroviruses have different preferences for integration. In particular, MLV preferentially integrates into the so-called DNase I hypersensitivity regions, CpG island and promoters, whereas HIV-1 preferentially integrates into the reading frames of active genes (often intronic regions). At the same time, high rate of transcription seems to inhibit ASLV integration (Vijaya et al., 1986; Bushman et al., 2005; Lewinski et al., 2006).

In achieving efficient integration into the genome of infected cell, viral IN is assisted by cellular proteins, or co-factors. The barrier-to-autointegration factor (BAF) has been identified as a part of the PIC that binds IN and was suggested to have a role in preventing autointegration. Its ability to bridge DNA and the finding that the nuclear lamina-associated polypeptide-2alpha interacts with BAF suggest a role in nuclear structure organization (Lin et al., 2003). IN interactor 1 (INI1) was found to directly interact with HIV-1 IN and to activate its DNA-joining activity, and the high mobility group chromosomal protein A1 (HMGA1) might approximate both long terminal repeat (LTR) ends and facilitate IN binding by unwinding the LTR termini (rev Van Maele et al., 2006).

Recently, we shown that HIV-1 IN interacts and is acetylated by p300, a transcriptional co-activator and histone acetyl-transferase (HAT) that facilitates the access of the transcription machinery to DNA by acetylating histones. p300mediated acetylation increased the affinity of IN for its DNA template and suggested that this protein may also function as a tethering factor for an open chromatin structure (Cereseto et al., 2005).

TRANSCRIPTION

Upon integration into the cellular DNA, the HIV-1 provirus adopts a chromatinized conformation with two nucleosomes that are precisely positioned in the 5' long terminal repeat (LTR). These two nucleosomes overlap binding sites for several transcription factors in the transcriptional initiation site; the modification status of these nucleosomes is a key modulator of HIV-1 transcriptional activity (Williams and Greene 2007). Transcription of the integrated viral DNA is critical for the establishment of efficient infection. This process is essentially regulated by a variety of host cell factors that act in concert with the viral protein Tat. Tat is a small nuclear protein of 86 to 101 amino acids (depending on the viral strain) and is encoded from two separate exons. Tat binds to an RNA sequence named transactivation-responsive region (TAR) that is located downstream of the initiation site for transcription. TAR RNA sequence forms a highly stable stem – loop structure (Berkhout et al., 1989). Mutations that destabilize the TAR stem - loop structure impair Tat – stimulated transcription. The interaction of Tat with TAR permits activation of HIV-1 transcription by promoting the assembly of transcriptionally active complexes at the LTR by multiple protein–protein interactions (Marcello et al., 2001; Marcello et al., 2004).

HIV-1 transcription is highly dependent on host proteins and consequently is influenced by the cellular activation status. The expression of viral genes is a complex balance that involves both trancriptional activators and repressors and it is also influenced by external stimuli. HIV transcription can be induced by a wide range of stimuli, including T-cell receptor ligation by anti-CD3 antibodies, cytokines, including IL-1β, IL-2 and TNF-α, and mitogens. Such signalling ultimately drives HIV-1 transcription through the

induction of activating cellular transcription factors, including NF-κB, NFAT and AP-1. The LTR contains also several additional transcription factors binding domains that recruit various cellular transcription factors, including Sp1, LEF-1, COUP-TF, YY1, Ets-1 and USF (Rohr et al., 2003). In addition to binding of different factors to the promoter/enhancer region in the LTR, different histone acetyltransferases were shown to be recruited to the viral promoter. These HATs (p300/CBP, P/CAF and GCN5) are recruited by the viral transactivator Tat protein and were shown to acetylate Tat and to induce changes in the histone hyperacetylation and remodeling of nuc-1 at the LTR (Marzio et al., 1998; Benkirane et al., 1998; Lusic et al., 2003). Recently in our lab, through a proteomic screening, we found that the human Nucleosome Assembly Protein-1 (hNAP-1), an histone chaperone that shuttles histones H2A/H2B into the nucleus, assembles nucleosomes and promotes chromatin fluidity, interacts and cooperates with Tat during trancription of the viral genes (Vardabasso et al., 2008).

After initiation of transcription, expression of full-length HIV-1 transcripts requires the concerted action of several cellular proteins. Tat is necessary for an efficient elongation of the transcripts by RNA pol II. In particular, Tat is known to interact with the cyclin component of the cellular transcriptional kinase P-TEFb. This kinase is a heterodimer composed of CDK9 and CylinT1 (CycT1). P-TEFb phosphorylates two serines in the eptad repeats present in the C-terminal domain (CTD) of the largest subunit of RNA polymerase II thus increasing its elongation activity. Chemical inhibition of CDK9 with DRB of flavopiridol or genetic inhibition with CDK9 dominant negative mutants strongly impairs Tat-mediated activation of HIV gene expression (Wei et al., 1998; Fujinaga et al., 1998; Bieniasz et al., 1998). It has been proposed, in some models of latency, that the limited expression of CycT1 in resting $CD4^+$ T cells might be the responsible for the block in HIV-1 expression in these cells (Liou et al., 2002).

It has been hypothesized that chromosomal location of integrated provirus may affect the transcription of HIV-1. In particular it has been proposed that latently infected cells harbour proviruses in disfavoured regions. Taking advantage of T cell line models of latency it has been found that, in latently infected cells, integration occurs in three different chromatin regions: centromeric heterochromatin, gene deserts and surprisingly highly transcribed genes. It is possible that a high rate of transcription of host genes might inhibit viral transcription (Lewinski et al., 2005). These findings suggest that chromatin status and environment can influence both HIV-1 integration and transcription.

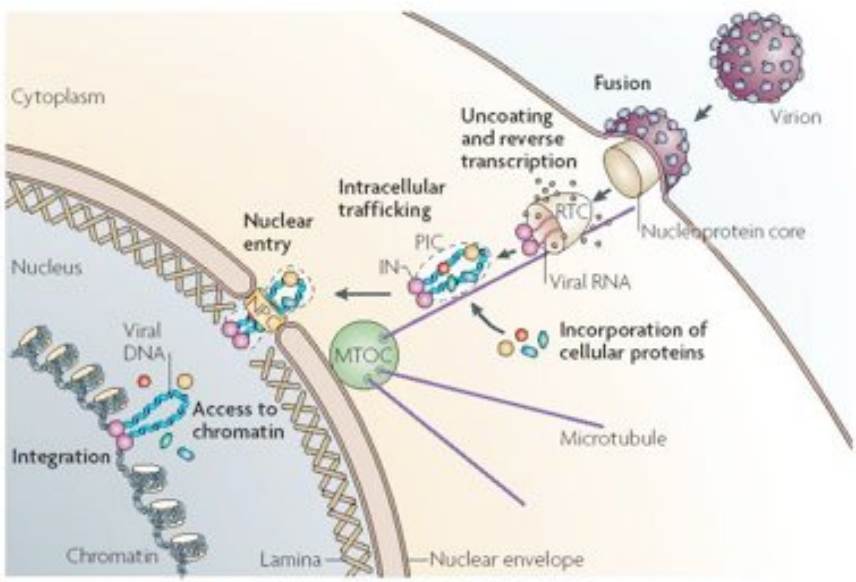


Figure 3. From cell entry to DNA integration. The virus enters the target cell by fusion between the cellular and viral membranes. The nucleoprotein core containing the genomic RNA is delivered into the cytoplasm where reverse transcription takes place. The viral cDNA with viral and cellular proteins form the pre-integration complex (PIC), that reaches the nuclear envelope by active transport along microtubules and then cross the intact nuclear envelope, presumably through the nuclear pore complex (NPC). The PIC gains access to chromatin and the viral protein IN catalyzes the integration reaction of the viral DNA into the host genome (Suzuki et al., 2007).

HIV-1 encodes at least nine genes which must be expressed during viral life cycle in the correct temporal order. Early in the viral infection, small, multiply spliced transcripts (2 kb) encoding Tat, Rev and Nef predominate in the cytoplasm. During the late phase of HIV infection, genomic (unspliced, 9kb) RNAs and singly spliced RNAs (4 kb) become leading species in the infected cell. A key factor controlling late phase transition is the viral protein Rev. Rev is a small, positively charged RNA-binding protein that is approximately 116 amino acids in size. It is encoded from two exons which are joined by splicing to produce a monocystronic transcript early in the viral replication cycle. Rev contains both nuclear localization sequence (NLS) and nuclear export signal (NES). Hence, Rev is a shuttling protein. Rev permits export of unspliced/partially spliced transcripts from the nucleus to the cytoplasm, where they serve as templates for translation of the gag-pol open reading frame or as the full-length genomic RNA (Zapp and Green, 1989). Rev specifically recognizes an RNA element located within the coding sequence for Env. This sequence, called RRE (Rev-responsive element), is present within the env-coding region and is about 200 nucleotides in size and forms secondary RNA structure. Rev exports the intron-containing HIV-1 mRNA via a CRM1 shuttling system (Askjaer et al., 1998). CRM1 is a member of the karyopherin family of nucleocytoplasmic-transport factors and, like others karyopherins involved in nuclear export, binds its cargo in the nucleus in the presence of the GTP-bound form of the Ran GTPase. After nuclear export, hydrolysis of the bound GTP to GDP causes a conformational shift that induces cargo release in the cytoplasm, thus providing the directionality of this export pathway. CRM1 also interacts with components of the nuclear pore complex (NPC) and this interaction is essential for nuclear RNA export. In the nucleus, Ran-GTP bound CRM1 binds the NES domain of Rev, which in turn is bound to RRE-containing HIV-1 transcripts. This interaction enables CRM1 to export the resulting RNA/protein complex into the cytoplasm. In the

cytoplasm, conversion from Ran-GTP to Ran-GDP releases the Rev/RNA cargo. Rev returns to the nucleus by binding to importin-β and Ran-GDP for subsequent rounds of export (Suhasini and Reddy 2009).

Although it is now clear that the primarily effect of Rev is in promoting the nuclear export of RRE- containing HIV RNAs the mechanism of Rev function is not fully understood. This protein functions through several cellular posttranscriptional mechanisms, such as mRNA splicing, RNA stability, nucleocytoplasmic transport, and translation. Nevertheless, numerous studies have provided insights on several posttranscriptional steps in viral gene expression regulated by Rev. Studies of Cochrane and co-workers indicate that Rev acts early in HIV biogenesis (Iacampo and Cochrane 1996). In addition to influencing the fate of viral mRNA within the nucleus, Rev has been proposed to affect utilization of viral mRNA for translation. Rev efficiently loads RRE-containing mRNAs onto polysomes leading to high levels of structural proteins translation (D'Agostino et al., 1992). There are many lines of evidence which suggest the involvement of Rev in stabilization of unspliced HIV transcripts either by disassembly of spliceosomes or by overcoming the destabilization effect of cis-acting elements present in HIV RNAs.

1.1.4 Cellular proteins involved in the late phases of HIV-1 infection

The late phases of HIV-1 life cycle are characterized by the assembly of new viral particles, their release from the plasma membrane and their maturation. These steps are also highly dependent on cellular proteins.

ASSEMBLY, BUDDING AND MATURATION

Several groups have shown that HIV-1 recruits the cellular endocytosis machinery for assembly and budding (Martin-Serrano et al., 2003; von Schwedler et al., 2003; Pornillos et al., 2002). The HIV-1 envelope contains a high percentage of cholesterol suggesting that HIV may bud from specific membrane microdomains named lipid rafts (Campbell et al., 2001). The viral particles assemble at the plasma membrane, then are wrapped with the host membrane and bud out from the cell surface. The gag polyprotein, which is composed by the matrix domain (MA), the capsid domain (CA) and the nucleocapsid domain (NC), plays a pivotal role in driving all these processes. The MA domain targets gag to the site of assembly, where CA plays a role in gag multimerization and NC domain packages viral genomic RNA during assembly. In addition to these domains, the p6 domain positioned at the carboxyl terminus of gag is necessary for particle release from the host cell.

The mature virion is generated during viral release upon cleavage of the gag precursor by the viral protease (PR). Different studies have suggested that HIV-1 viral particles recruit the high molecular weight endosomal sorting complexes (ESCRTI, ESCRTII, ESCRTIII) (Rev Demirov and Freed, 2004). The ESCRT machineries are usually involved in the sorting of cargo proteins, such as activated receptors, to Multi Vesicular Bodies (MVB) before their degradation in the lysosomes (Fujii et al., 2007).

Independent studies have shown that p6 interacts with Tsg101, a component of ESCRTI machinery, and this interaction is necessary for viral particle release. Depletion of endogenous Tsg101 or disruption of Tsg101-p6 interaction inhibits virus release (Garrus et al., 2001; VerPlank et al., 2001). Another minor player in HIV-1 budding is the apoptosis-linked-gene 2 interacting protein (Alix) protein. Strack and colleagues demonstrated that Alix was involved in EIAV (Equine Infectious Anemia Virus) release (Strack et al., 2003). However many observation suggest that Alix can also be used by HIV-1 as an alternative route when Tsg101 is not available (Fisher et al., 2007; Usami et al., 2007). All these findings suggest that, under particular circumstances, retrovirus assembly and budding can take place in the MVB. Additional electromicroscopy experiments describe virus positive intracellular compartments that also display MVB specific markers. After budding of the virus into the MVB lumen, particle release occurs via the endosomal pathway (Figure 4) (Sherer et al., 2003; Ono and Freed, 2004).

In certains types of human cells the absence of Vpu leads to inefficient HIV particle release due to the failure to detach from the plasma membrane and accumulation in large numbers at the cell surface (Klimkait et al., 1990). Interestingly, in simian cells, Vpu is dispensable for efficient HIV release (Varthakavi et al 2003). Neil and colleagues found a new protein (Tetherin) that, in the absence of Vpu, inhibits viral particle release; this factor is induced by IFN-alpha. The mechanism by which Tetherin inhibits viral release is still unknown, but it has been shown that this factor also antagonizes the release of MLV and that its overexpression, induced by IFN alpha, blocks infection, thus suggesting that Tetherin could be a generic defence against enveloped viruses (Neil et al., 2008).

A second release restriction factor is represented by calcium-modulating cyclophilin ligand (CAML-1), an integral membrane protein involved in T cell

development and regulation (Tran et al., 2004). In 2008 it was shown that CAML is a Vpu-sensitive human host restriction factor to HIV-1 release. Indeed, expression of human CAML rendered simian cells restrictive for viral release in the absence of Vpu, suggesting that CAML is a human host restriction factor blocking viral paricle release or budding, the action of which is counteracted by Vpu (Varthakavi et al 2008).

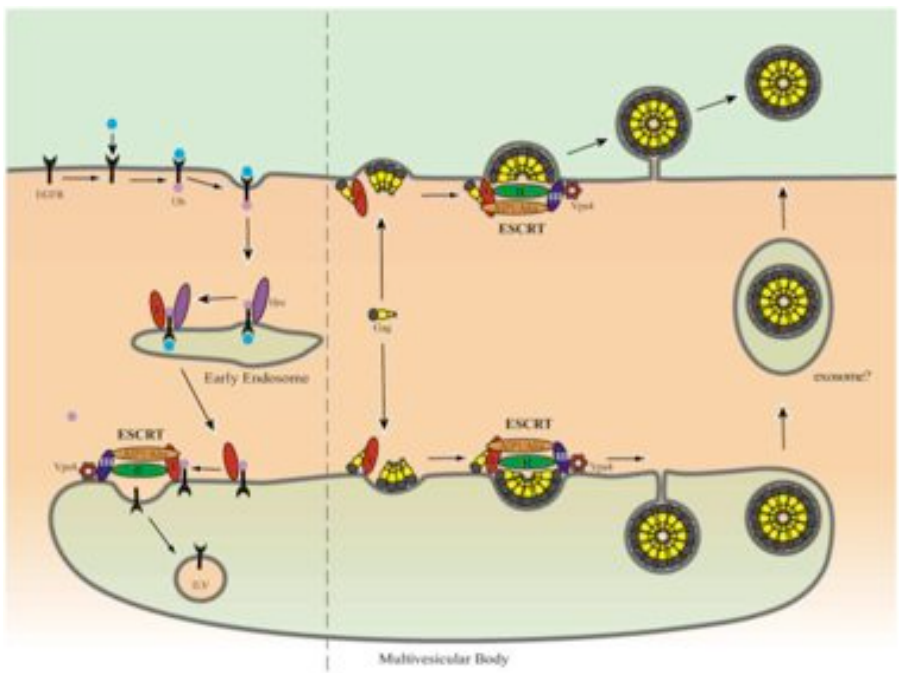


Figure 4. Model for retrovirus release. On the left is a schematic representation of endocytosis and MVB sorting of an activated growth factor receptor. An Hrs- containing complex (dark purple) recognizes and sequesters ubiquitylated cargo (Ub, light purple) at clathrin-rich regions of the early endosomal membrane. On the right is depicted the hijacking of MVB sorting machinery for virus release. Virus particles are shown to assemble and bud at the plasma or to be released (e.g., from macrophages) through the exosome pathway following assembly in the MVB (Demirov and Freed, 2004).

1.1.5 Cellular MicroRNAs: novel partner for HIV-1

In addition to cellular proteins that regulate HIV-1 infection, there is mounting interest in the regulatory roles served by non-coding RNAs (NcRNA) and by the RNAi cellular machinery. The best known subtype of NcRNAs are the small, 21–23 nucleotides long, single-stranded microRNA (miRNA) molecules, which are key modulators of eukaryotic gene expression and are involved in many cellular processes such as development oncogenesis, cell cycle control and immunity (Calin and Croce 2006; Scaria and Jadhav 2007). Pri-miRNAs are encoded in introns, intergenic regions, and specific transcription units in both sense and antisense orientations and transcribed by RNA polymerase II. The pri-miRNA is processed in the nucleus by the Drosha-DGCR8 complex (RNase III endonuclease complex), into a shorter 70 nucleotide RNA (pre-miRNA) containing a stem-loop structure (Han et al., 2006). The pre-miRNA is then transported into the cytoplasm where it is further processed by a second RNase III endonuclease complex, Dicer-TRBP, to generate a 21–23 nucleotide imperfectly duplexed mature miRNA (Chendrimada et al., 2005; Haase et al., 2005; rev Bushati and Cohen 2007). A mature miRNA is assembled into an RNA-induced silencing complex (RISC). The miRNA-RISC (miRISC) complex recognizes the target mRNA via imperfect base pairing at the 3' -UTR region (Grimson et al., 2007). Since the base pairing is "imperfect" a single miRNA could potentially recognize and downregulate up to 100 mRNAs (Brennecke et al., 2005).

It has been shown that plants and lower eukaryotes use de novo synthesized virus-derived small interfering RNAs to regulate infecting viruses (Ding and Voinnet 2007). Although it remains unclear if mammals conserve an RNAi-based antiviral strategy, recent evidence indicate that this might be the case (Berkhout and Jeang 2007; Yeung et al., 2005). The

first finding of a cellular miRNA involvement in antiviral immunity describes a cellular miRNA (miR-32) as a inhibitor of primate foamy virus type 1 (PFV-1) infection (Lecellier et al., 2005).

Recently, it has been shown that cellular miRNAs are involved in the regulation of HIV-1 latency. In particular, Huang and colleagues found that the 3' end of HIV-1 mRNA is targeted by miR-28, miR-125b, miR-150, miR-223, miR-382, which are more abundant in resting primary $CD4^+$ T cells, and that inhibition of these miRNA induces HIV-1 expression in latently infected cells (Huang et al., 2007). Moreover, it has been recently reported that another miRNA (miR-198) functions to restrict HIV-1 replication in monocytes, by repressing Cyclin T1 expression (Sung et al., 2009).

This restrictive role of microRNAs on HIV infection in part explains the reason why HIV-1 has evolved ways to affect the cellular RNAi machinery. First of all, it seems that HIV-1 can reshape the infected cell miRNA expression profile (Yeung et al., 2005; Houzet et al., 2008). Moreover it was demonstrated that HIV-1 actively suppressed the expression of the polycistronic miRNA cluster miR-17/92 and that this suppression is required for efficient viral replication (Triboulet et al., 2007).

Since HIV-1 produces a large number of small viral RNA hairpins, it is probable that it evolved a strategy to avoid viral RNA processing by the RNAi machinery (Bennasser et al., 2006; Bennasser et al., 2006; Klase et al., 2007; Ouellet et al., 2008). However, miRNAs and RNAi are conserved factors/processes whose complete suppression is incompatible with cellular viability (Muljo et al., 2005). Most likely HIV-1 avoids RNAi antiviral mechanisms by mutating viral RNA-sequences to alter base-complementarity with cellular miRNAs. Indeed, there is evidence that selective and evasive nucleotide changes in HIV-1 sequences can be

elicited rapidly by siRNA/shRNA induced RNAi (Dash et al., 2004; Westerhout et al., 2005).

Finally, a large number of viral miRNAs (vmiRNAs) have been described to be encoded by viral genomes, in particular herpes and polyoma viruses (SV40) (Pfeffer et al., 2005; Sullivan et al., 2005). In the specific case of HIV-1, a vmiRNA encoded by the nef region of HIV-1 termed miRN367 was physically identified and isolated by Omoto and colleagues (Omoto et al., 2005). The role of this vmiRNA in HIV-1 infection remains unclear.

1.2 Lymphocytes activation status and HIV-1 infection

HIV-1 replication is greatly influenced by the activation status of the target cell. Activated $CD4^+$ T lymphocytes are permissive to HIV-1 infection, whereas in resting T cells, despite the efficient entry of HIV-1, no viral progeny is produced. Since the early 1990s, different hypothesis have been made to explain this block. Initially, Zack and colleagues demonstrated that quiescent T cells can be infected by HIV-1 and that viral cDNA synthesis initiates at levels comparable with those of activated T cells. However, the viral genome remains incompletely reverse transcribed and may persist in an inactive state until subsequent mitogenic stimulation (Zack et al., 1990). In the same year, Stevenson and colleagues suggested that the block in resting T cells occurs at the level of integration. They found that, in resting T cells, viral cDNA was unable to integrate into the host cell genome and was maintained extrachromosomally for several weeks. Subsequent T cell activation allowed integration of extrachromosomal DNA that seemed to be transcriptionally active (Stevenson et al., 1990). However studies from different groups performed later on, suggested that, in resting T cells, a block to HIV-1 infection occurs at the level of reverse transcription and that a progression through the G1b phase was needed to achieve efficient

reverse transcription (Korin and Zack, 1998). Latest findings from Greene and colleagues better elucidated this mechanism of resting T cell resistance to HIV-1 infection: they demonstrated that APOBEC3G, in a form of a low molecular mass ribonucleoprotein complex present exclusively in resting $CD4^+$ T lymphocytes, blocks formation of late products of reverse transcription via the RNA binding ability of APOBEC3G. In activated T cells, the APOBEC-containing low molecular mass complex forms a larger complex that looses the capacity to restrict the viral infection thus allowing fully efficient reverse transcription (Chiu et al., 2005). Understanding the fate of HIV-1 in resting T cells is particularly important since the majority of T lymphocytes are in resting state (Tang et al., 1995). Siliciano and colleagues monitored the kinetics of HIV-1 decay in resting $CD4^+$ T cells and found that slow kinetics of reverse transcription and blocks at subsequent steps limit HIV-1 infection in these cells. They also showed that the reservoir of unintegrated HIV-1 in recently infected resting $CD4^+$ T cells is highly labile. By examining the decay of integration-competent HIV-1 DNA, they found that this form of fully retrotranscribed HIV-1 has a half-life of 1 day in resting T cells. They proposed that degradation of either viral DNA and viral proteins that constitute the preintegration complex would lead to a functional decay of the virus (Zhou et al., 2005). Moreover, several studies suggest that inhibition of the proteasome increases the production of proviral DNA by blocking the degradation of the preintegration complexes (Butler et al., 2002; Schwartz et al., 1998). In 2007, a work performed using immunofluorescence experiments on CA viral protein and FISH analysis on viral DNA in resting infected cells demonstrated that full length reverse transcribed HIV-1 cDNA together with CA localizes at the centrosomes 4 days post infection (Zamborlini et al., 2007). Collectively, these data suggest that, in resting $CD4^+$ T cells, different blocks that impair viral replication may exist at multiple steps of

the early infection. It seems that reverse transcription is the most affected step as it resulted to be both impaired and delayed. However, when the full length transcripts accumulate in the infected cells, they are anyhow unable to reach the nucleus and stably integrate into the host genome.

In addition to this pre-integration latency mechanisms, another typical characteristic of HIV-1 infection, that correlates with T cell activation status, is the establishment of a latent reservoir of infected cells (post-integration latency). The major obstacle to HIV-1 eradication is the establishment of a latent infection. The formation of this latent reservoir is a natural consequence of the fact that the virus replicates in activated $CD4^+$ T cells (Persaud et al., 2003), while the vast majority of $CD4^+$ T lymphocytes is in a resting G_0 state. In adults, about half of the resting cells are naïve, having yet to encounter an appropriate antigen (Ag) and the other half is represented by memory cells, that have previously responded to an Ag. Ag-driven responses involve a burst of cellular proliferation and differentiation, giving rise to effector cells, most of which die quickly; a surviving subset reverts to the resting G_0 state as memory. These cells are characterized by long-term survival and rapid responses to the Ag in the future (review ref. Kaech et al., 2002). The virus replicates preferentially in activated $CD4^+$ T cells with cytopathic effects (Ho et al., 1995). Because it takes a few weeks for effector cells to revert to a resting state, most infected $CD4^+$ lymphoblasts die before becoming memory cells. Nevertheless, the presence of cells harbouring integrated provirus in patients under Highly Active Antiretroviral Therapy (HAART) suggests that some activated cells, after infection, can revert to a resting state. Otherwise it could be possible that HIV-1 might infect some of these cells in a state in which they are still permissive for early steps in the virus life cycle (up to integration), but not for virus gene expression (Stevenson 1997).

HIV-1 gene expression has been clearly shown to be dependent on inducible host transcription factors that are transiently activated following exposure to Ag, and thus viral gene expression is automatically extinguished as cells return to a resting state (Tong-Starksen et al., 1987; Nabel and Baltimore 1987). The result is a stably integrated but transcriptionally silent form of HIV-1. The direct infection of resting cells does not generally proceed to integration (Zhou et al., 2005). However, resting $CD4^+$ T cells with integrated HIV-1 DNA can be detected in vivo and their phenotype suggests that they arise from infected $CD4^+$ T lymphoblasts that have reverted to a resting memory state (Chun et al., 1995). Jordan and colleagues have suggested, as mechanism of latency, an integration into centromeric heterochromatin, that is known to be repressive (Jordan et al., 2003). However subsequent studies on $CD4^+$ isolatated from patients on HAART therapy hae demonstrated that, in the cell population carring latent HIV-1, integration into centromeric regions does not occur (Han et al., 2004).

An additional potential explanation for latency is transcriptional interference (TI). TI is a cis-acting suppressive effect that is observed when transcriptional activity initiated from an upstream promoter suppresses the transcription from a downstream promoter (Greger at al., 1998). In activated $CD4^+$ T cells, HIV-1 gene expression might be efficient because the concentration of crucial host transcription factors is high enough to overcome TI, while in resting T cells these factors are less abundant and transcription from an unpstream promoter might interfere with the downstream promoter (Hogan et al., 2003; Weil et al., 2004). Furthermore, it has been shown that transcription factors and coactivators necessary for HIV-1 transcription are less abundant in resting T cells. For example, two of the key host transcription factors involved in HIV-1 expression (NFAT and NF-kB) are sequestered in the cytoplasm of resting T cells. Both factors are

only recruited to the nucleus following cellular activation. As a consequence, HIV-1 gene expression is stricktly dependent on to the activation state of the host cells. Tat-associated proteins could be one of the limiting factors for processive transcription in resting T cells: low levels of P-TEFb kinase activity (CDK9 and Cyclin T1), that have been observed in resting T cells, are increased in response to activating stimuli (Ghose et al., 2001). Tat itself could be the main limiting factor being subject to tight post-translational modifications (Bres et al., 2002; Bres et al., 2003).

Finally, RNAi-mediated pathways of transcriptional silencing have also been shown to be involved in the latency mechanism. As described above, cellular microRNAs (miRNAs) have been shown to potently inhibit HIV-1 production in resting primary $CD4^+$ T cells (Huang et al., 2007).

The major obstacle to HIV-1 eradication is the establishment of a latent infection. Viral reservoirs established early during the infection remain unaffected by anti-retroviral therapy for a long time and are able to restore infection upon interruption of HAART, thus therapeutic targeting of viral latency is one of the most important goal in HIV-1 research (Lassen et al., 2004; Persaud et al., 2003; Marcello 2006).

2. The IN protein

2.1 IN structure and domains

Integration into the host genome is a defining feature of all retroviruses. Once integrated, the viral DNA is replicated together with cellular DNA during the cell cycle. The viral enzyme that carries out this reaction is the viral protein IN.

IN is a 32 kDa protein that is present inside the mature virion, and is encoded by the *pol* gene, which also encodes for viral protease (PR) and reverse transcriptase (RT). IN is translated as a part of the large polyprotein Gag-Pol and is processed by viral protease during virion maturation. The enzyme is supposed to work as a tetramer.

IN is composed of three domains: the N-terminal domain (residues 1-50), the Core domain (residues 51-212) and the C-terminal domain (residues 213-288) (Figure 5). The division of IN into these three domains is based on functional and protelolytic studies (Engelman and Craigie, 1992; Engelman et al., 1993; van Gent et al., 1993).

The N-terminal domain is a bundle of three alpha-helices and it characterized by a two His and two Cys motifs named HHCC domain, and a SH3 fold domain (Craigie, 2001). This region is important for the activity of IN both in vitro and in vivo (Khan et al., 1991; Schauer and Billich, 1992). Mutations in these conserved residues block the integration mechanism (Cannon et al., 1994). This domain binds to a zinc ion and the conserved HHCC motif seems to be important for this interaction. N-terminal domain is important for the multimerization of IN; in the absence of zinc this domain has a disordered structure while in the presence of this ion it adopts an ordered secondary structure and the tetramerization occurs more rapidly (Zheng et al., 1996).

The Core domain is responsible for the catalytic activity of IN and is the most conserved among all retroviral INs (Kulkosky et al., 1992). It consists of a central five-stranded β-sheet with six surrounding helices. The catalytic region contains the invariant triad of acidic residues, the D,D-35E motif. Mutagenesis of these aminoacids greatly impairs IN enzymatic activity and viral replication as a consequence (LaFemina et al., 1992; Shin et al., 1994; Taddeo et al., 1994). The structure of the Core domain is very similar to the Rnase H domain of RT enzyme, the RuvC protein of E.Coli and the bacteriophage Mu transposase, all enzymes that catalyses substitution reactions involving phosphodiester bonds (Hostomska et al., 1991; Ariyoshi et al., 1994; Rice and Mizuuchi, 1995). In addition to its catalytic activity, the core domain has also been proposed to be involved in DNA binding (Drelich et al., 1993).

The C-terminal domain is the most variable region among retroviral INs (Lutzke et al., 1994). This domain binds DNA in a non specific manner, and since integration into host DNA has been demonstrated to be non specific, the C-terminal domain was suggested to interact with the target DNA. However studies performed with chimeric INs demonstrated that the Core domain is responsible for targeted DNA binding (Katzman and Sudol, 1995). It appears more likely that the C-terminal domain binds to the very ends of the viral DNA (Esposito and Craigie, 1998; Jenkins et al., 1997).

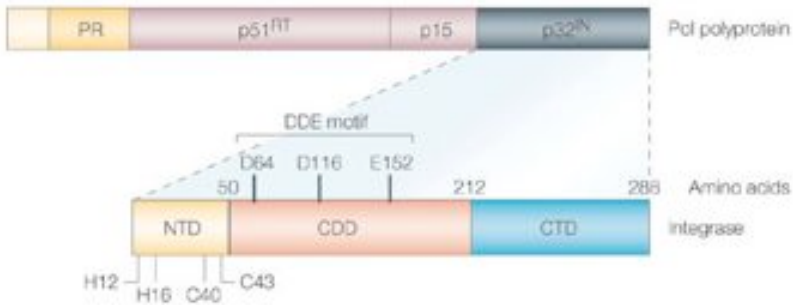


Figure 5. Schematic rapresentation of the Pol gene and the IN domains. IN (p32IN) is encoded at the 3'-end of the pol gene. HIV-1 IN is composed by three domains. The amino-terminal domain (NTD) that coordinate one zinc atom through H12, H16, C40 and C43. The catalytic core domain (CCD) containing the DDE motif: D64, D116 and E152. The carboxy-terminal domain (CTD) is involved in DNA binding (Pommier et al., 2005).

2.2 IN enzymatic activity

Integration is an essential step for all retroviruses and mutations that interfere with this process block their replication.

The integration mechanism can be divided into two distinct steps: 3' processing and strand transfer.

The 3' processing step occurs in the cytoplasm within the pre-integration complex. During this step, IN removes a GT dinucleotide from the 3' end of each viral LTR. This dinucleotide is always adjacent to a highly conserved CA dinucleotide, and is crucial for recognition of viral DNA by IN. Mutations in this site cause severe defects in integration.

Following the nuclear entry of the PIC, IN catalyses the strand transfer reaction that consists in a nucleophilic attack by the 3'-hydroxyl residues of the viral ends on phosphodiester bridges located on either side of the major groove in the host DNA. Then IN catalyses a transesterification reaction, the processed CA-3'-OH viral DNA ends are ligated to the 5'-O-phosphate ends of the host DNA. At this point viral DNA is ligated to the cellular DNA by only one strand at each end. Through an unknown process, the gaps flanking the viral DNA are filled in by extending the free 3' end of the target DNA, the mismatched viral 5' end is trimmed and the resulting ends are ligated.

The identity of the protein that performs the gap repair remains unknown;, it is possible that viral proteins direct cellular enzymes to the viral DNA or even act directly to repair the gap (Coffin J.M., Hughes S.H., Varmus H.E. Retroviruses).

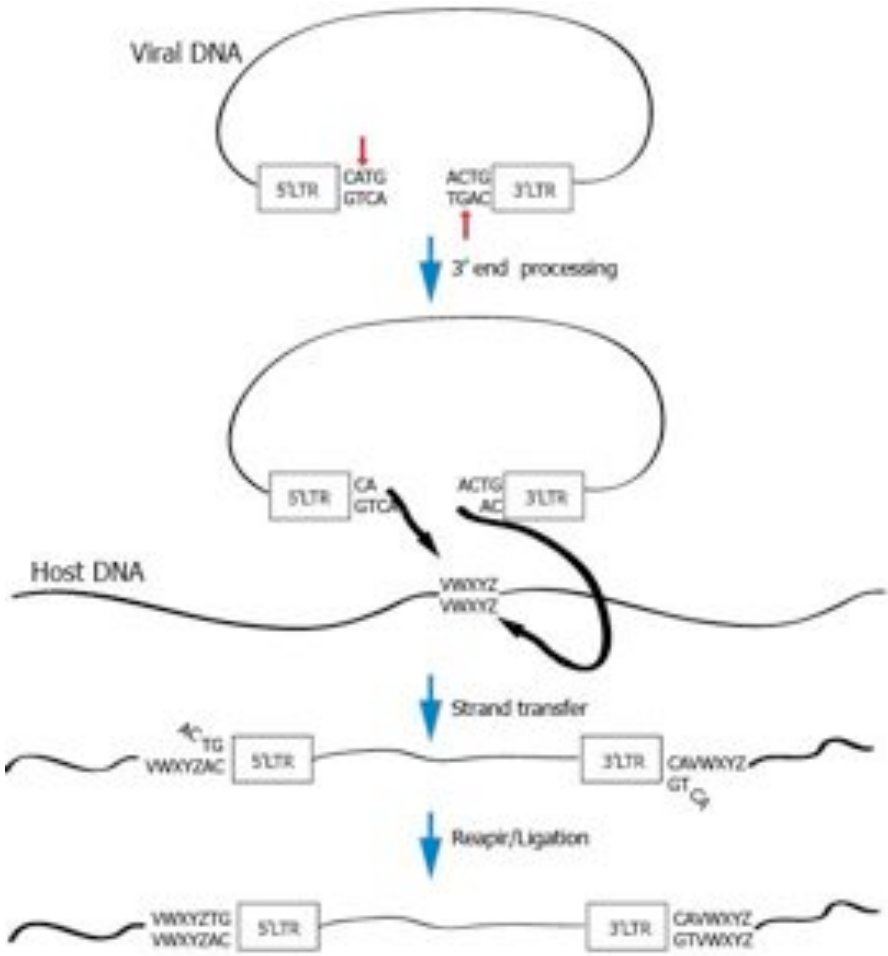


Figure 6. Schematic representation of the integration reaction. (Modified from Field's Virology).

In addition to integrated viral DNA, three classes of extrachromosomal viral DNA are present in the nucleus of acutely infected cells.

All these forms are circular and are divided in 1-LTR circles, 2-LTR circles, and autointegration products. The 1-LTR circles are formed by homologous recombination between the two LTRs of a linear viral DNA. The 2-LTR circles derive from the ligation of the two ends of the linear precursor and the last product derives from an intramolecular integration (Figure 7). All these forms are a hallmark of inefficient viral integration.

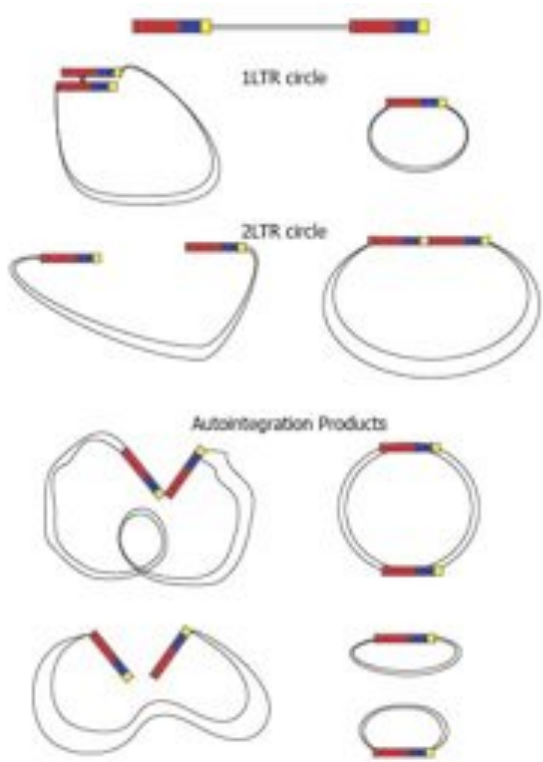


Figure 7. The different products of aberrant integration reactions

2.3 IN interactors

Purified recombinant IN is sufficient to carry out both 3' processing and strand transfer. Yet, a variety of cellular proteins that take part in HIV-1 integration have been identified over the last years by yeast two hybrid screening, co-immunoprecipitation or reconstitution of enzymatic activity of salt-stripped PICs.

IN interactor 1 (INI1) was the first cellular protein to be identified as an IN partner. This protein is the human homologue of yeast SNF5, a component of the chromatin remodelling SWI/SNF complex. INI1 was found through a two hybrid screening in 1994 (Kalpana et al., 1994). It was originally found that INI1 stimulates IN activity in vitro, however, subsequently, Yung and colleagues found that overexpression of the minimal IN-interaction domain of INI1 (S6) inhibits the late steps of HIV-1 infection (Yung et al., 2001). Moreover, INI1 was also involved in the production of virions, since it was demonstrated that the overexpression of this factor in MON cells, that lack this protein, increases virion production (Sorin et al., 2006). Recently, it has been reported that viral clones harbouring mutation in IN that impairs IN-INI1 interaction replicates more efficiently (Maroun et al., 2006). Thus, the actual molecular mechanism by which INI1 influences a variety of viral steps remains contradictive and poorly understood.

In 1994 Lee and Craigie demonstrated that the PIC of MoMLV contained a cellular protein that prevents suicidal autointegration; for that reason, this protein was named barrier-to-autointegration factor (BAF) (Lee and Craigie, 1994). BAF was later found to be involved in restoring the salt-inactivated activity of HIV-1 PICs (Chen and Engelman, 1998). BAF does not influence IN enzymatic activity and the mechanism by which it prevents autointegration is still unknown, however the finding that BAF interacts with a protein associated with nuclear lamina and its ability to bridge DNA suggest a role in nuclear structure organization.

More recently, Jacque and Stevenson reported that emerin, an inner nuclear-envelope protein, was necessary for HIV-1 integration and that BAF contributed to the ability of emerin to support viral infection (Jacque and Stevenson, 2006). However, recent work by Engelman and colleagues have disputed the conclusion that both BAF and emerin are dispensable for HIV-1 infection (Shun et al., 2007).

Another protein that was found to restore PIC activity after salt stripping was the high mobility group chromosomal protein A1 (HMGA1), a protein that binds DNA and takes part in chromatin regulation and transcription (Farnet and Bushman, 1997). It has been suggested that HMGA1 could approximate both LTRs and facilitate IN binding by unwinding the LTR termini, however chicken cells that lack HMGA1 still sustain retroviral integration (Hindmarsh et al., 1999; Beitzel and Bushman, 2003). Henderson and colleagues proposed that HMGA1 could play a role in HIV-1 transcription: it facilitates the binding of ATF3, which seems to be responsible for the recruitment of the SWI/SNF complex to the HIV-1 promoter (Henderson et al., 2000; Henderson et al., 2004).

Recently a new interactor of HIV-1 IN was identified: a protein of 76 kDa, containing a canonical NLS, named lens epithelium derived growth factor (LEDGF/p75) (Cherepanov et al., 2003). This protein is a member of the hepatoma-derived growth factor family and is involved in cell growth and protection against apoptosis via transcriptional activation of anti-apoptotic proteins. LEDGF/p75 is necessary for HIV-1 replication as shown by knock down experiments; moreover, an HIV-1 clone containing a mutation in IN (Q168A) that abolishes the interaction with LEDGF is defective for replication remaining blocked at the integration step (Emiliani et al., 2005). LEDGF has been suggested to tether IN to chromosomes since its knock down completely abolishes IN nuclear localization and its association with chromosomes (Maertens et al., 2003; Ciuffi et al., 2005). In addition, IN

degradation, that occurrs trough the ubiquitin-proteasome proteolytic system, also involves LEDGEF/p75 protein. Namely, LEDGF acts to protect IN from proteosomal degradation and addition of proteosome inhibitors to cells defective for LEDGF restores IN nuclear localization (Llano et al., 2004; Emiliani et al., 2005). In conclusion, LEDGEF/p75 exerts multiple roles in controlling IN by tethering it to the actively transcribed genes, by increasing the affinity of IN for DNA and by protecting it from proteosomal degradation in the cytoplasm (Van Maele et al., 2006).

The integration reaction has been suggested to be targeted by cyclin-dependent kinase inhibitor (CKI) p21Waf1/Cip1/Sdi1 (p21), as a new restriction factor. Namely, Zhang and colleagues suggested that the regulator of stem cell pool size, p21 restricts HIV-1 infection of primitive hematopoietic cells by complexing with HIV-1 IN and aborting chromosomal integration (Zhang et al., 2007).

Post-translational modifications of proteins, widely studied in the last decade, have been described to be involved in regulating HIV-1 IN as well. As previuosly mentioned IN is degraded through the ubiquitin-proteasome proteolytic system. It has been shown that the IN N-terminal phenylalanine is recognized as a degradation signal by a ubiquitin-proteasome proteolytic system known as the N-end rule pathway (Mulder et al., 2000). More recently Emiliani and colleagues identified von Hippel–Lindau binding protein 1 (VBP1), a subunit of the prefoldin chaperone, as an IN cellular binding protein. They demonstrated that VBP1 and the Cul2/VHL ligase cooperate in the efficient polyubiquitylation of IN and its subsequent proteasome-mediated degradation (Mousinier et al., 2007). In addition we found that IN is post-translationally modified by acetylation through the action of cellular acetyltransferase p300 that acetylates IN at three lysines (K264, K266, K273) localized in the C-terminal domain of the protein. This modification increases both the strand transfer activity of IN and its affinity

for DNA. Moreover, inhibition of p300 enzymatic activity greatly impairs the integration of HIV-1 virus (Cereseto et al., 2005).

3. The Jun N-terminal Kinase (JNK)

3.1 JNK structure and function

Protein phosphorylation regulates almost all cellular processes such as cell cycle, apoptosis, movement, survival and metabolism. Protein kinases are grouped in 20 families based on their sequence similarities (Bogoyevitch and Kobe, 2006). The c-Jun N-terminal Kinases (JNKs), together with p38 kinase and extracellular signal-regulated kinases (ERKs), belong to the family of the mitogen activated protein kinases (MAPK) which comprises of cyclin-dependent kinases (CDKs), glycogen syntase kinase 3 (GSK3) and casein 2-related protein kinases as well. MAPK are proline directed kinases, meaning that they phosphorylate serine or threonin residues followed by a proline.

These evolutionary conserved enzymes connect stimuli from the external environment to intracellular functions, and are thus activated by a variety of external agents such as growth factors, nutrients, cytokines, mechanical stress and changes in pH or osmolarity. In particular, external stimuli that lead to potent activation of JNK kinase activity are tumour necrosis factor alpha (TNF-α), and interleukin 1. JNKs were originally identified as a stress activated protein kinases and subsequent studies revealed their capacity to phosphorylate and activate the transcription factor c-Jun (Kyriakis and Avruch, 1990; Derijard et al., 1994; Minden et al., 1994).

The mammalian JNKs are encoded by three different genes (*Jnk1, Jnk2, Jnk3*) whereas ten different isoforms are generated by splicing processes. *Jnk1* and *Jnk2* gene each encodes for four splice variants while *Jnk3* encodes for two different proteins. Transcripts derived from all three genes encode both 54 kDa and 46 kDa proteins. The alternative splicing products of *Jnk1* and *Jnk2* were suggested to have different substrate specificity; however, the analysis of *Jnk* gene disruption in mice showed extensive

complementation between the *Jnk* genes. JNK1 and JNK2 are expressed in a variety of tissues while JNK3 is mainly expressed in brain, heart and testes (Figure 8).

The structure of JNK1 and JNK3 is similar to other MAPK. They have the typical protein kinase fold, which consists in N-terminus rich in β-structure and C-terminus rich in α-helices. These two domains are connected by two segments where substrates and ATP are expected to bind. The non-phosphorylated inactive form of JNK has the N- and C- terminal domains not correctly aligned. The kinase activity of JNK is regulated by phosphorylation of the two residues (Thr-Pro-Tyr) in the activation loop and is mediated by the MAPK kinases MKK4 and MKK7. Upon phosphorylation of these residues, JNK changes its conformation becoming enzymatically active (Bogoyevitch and Kobe, 2006).

JNK substrates, as revealed by initial studies performed on c-jun, are characterized by the presence of a specific domain, named JNK-binding domain (JBD) or d-domain that mediates the interaction with the kinase. However, the site of JNK phosphorylation does not always reside within this domain (Adler et al., 1994; Dai et al., 1995).

The precise role of JNK is still unclear and it seems to be dependent on the cellular context. Initial studies demonstrated that JNK plays a role in promoting apoptosis especially in neurons (Yang et al., 1997). In fact, JNK-mediated phosphorylation of p53 induces its stabilization and subsequent activation of proapoptotic genes (Fuchs et al., 1998; Buschmann et al., 2001). Yet the p53 mediated apoptotic pathway seems not to be the only apoptotic pathway that involves JNK. More recently, JNK was shown to be necessary for the stress induced release of mitochondrial cytocrome C suggesting a role of JNK in the apoptotic process involving mitochondria (Chen and Tan, 2000; Tournier et al., 2000).

Despite the established role of JNK in programmed cell death most stimuli inducing JNK activation do not cause apoptosis. It has been proposed that, in dependence of the time course of activation, JNK can have either pro-apoptotic or pro-survival effects. Many cytokines and different forms of environmental stress cause a transient activation of JNK that cells interpret as a pro-survival signal while sustained activation may lead to apoptosis (Davis, 2000).

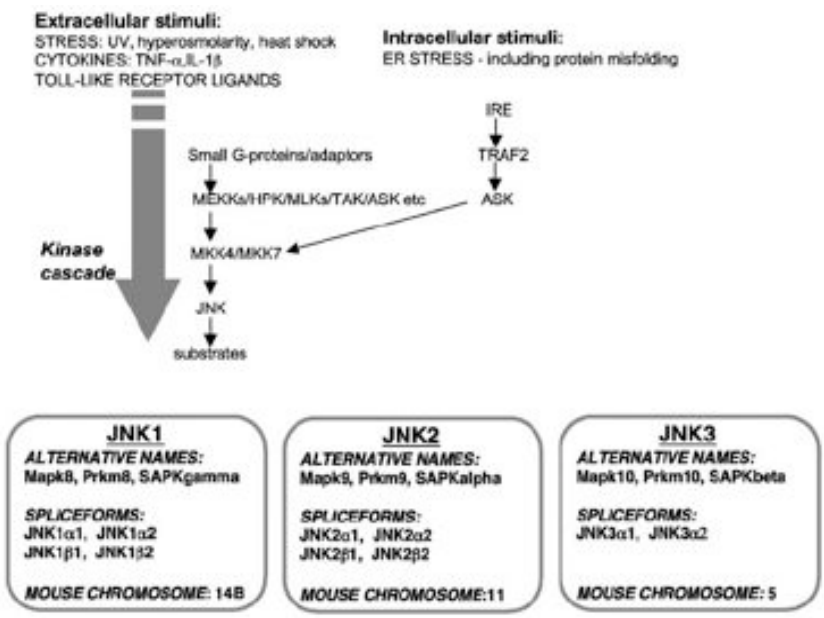


Figure 8. Overview of the JNK pathway. The classical JNK pathway was considered to be activated following the exposure of cells to extracellular stresses. Subsequently, JNK activation was also demonstrated following the exposure of cells to some proinflammatory cytokines, including TNF-a and interleukin-1 (IL-1), as well as following the activation of Toll-like receptors. This cascade is mediated by a large number of MAPK including MKK4 and MKK7 that activate JNK (left panel). ER stress can also activate JNKs (right panel). In mammalian cells JNK proteins are encoded by threee genes (jnk1, jnk2, and jnk3). These genes are subjected to alternative splicing process that leads to the formation of different isoforms of JNK (lower panel) (Bogoyevitch and Kobe, 2006).

3.2 JNK substrates

Among different JNK substrates, transcription factors and nuclear hormone receptors are of special interest since their phosphorylation represents a direct link between external stimuli and gene expression.
JNK phosphorylation increases the transcriptional activity of all the proteins that form the AP-1 (activator protein 1) complex, such as jun and fos family members as well as ATF2, that is also activated by this post translational modification (Leppa and Bohmann, 1999; Gupta et al., 1995).

JNK also phosphorylates different transcription factors that are not involved in AP-1 complex formation. Elk1 is phosphorylated by JNK on Ser 383 and Ser 389 and this modification increases its transcriptional activity (Cavigelli et al., 1995). The tumour suppressor p53 is stabilized and activated by JNK and the absence of JNK greatly impairs p53 ability to elicit apoptosis in response to specific stimuli (Fuchs et al., 1998; Buschmann et al., 2001).
It is known since many years that JNK plays a key role in regulating several functions of the immune system. In fact, many transcription factors that are involved in lymphocyte signalling are JNK substrates. Among them, two of the major transducers of cytokine signals, STAT1 and 3, are phosphorylated by JNK (Zhang et al., 2001; Zhao et al., 2005). Moreover JNK increases transcriptional activity of TCFb1, a key regulator during lymphocyte activation (Kasibhatla et al., 1999).
Many cellular proteins that are not related to transcriptional events are also known to be regulated by JNK. For example JNK modulates the activity, stability and the localization of tau protein, synaptotagmin 4 (membrane trafficking protein) and AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor). JNK, especially isoform 2 has been shown to phosphorylate tau protein thus greatly impairing its ability to promote microtubule assembly (Yoshida et al., 2004). Modification of synaptotagmin 4 by JNK mediates its translocation from immature to

mature secretory vesicles (Mori et al., 2008). Finally, JNK phosphorylation of AMPA receptors controls their reinsertion to the cell surface after NMDA treatment (Thomas et al., 2008).

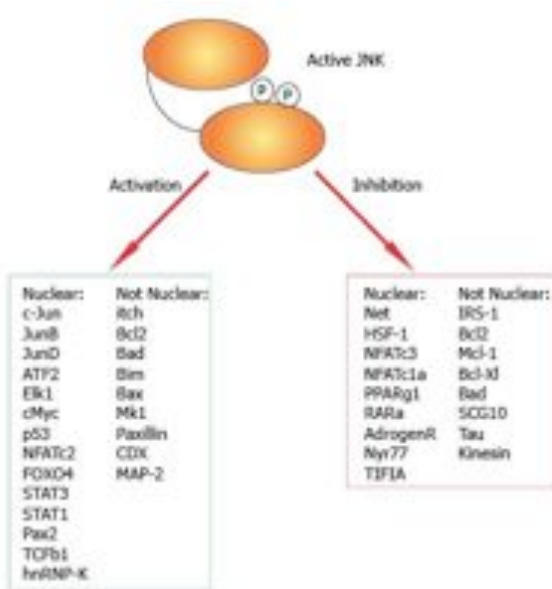


Figure 9. Summary of the substrates of JNKs.

To be active, JNK requires double phosphorylation on a specific threonine and tyrosine within its activation loop. JNK can phosphorylate a range of substrates. Phosphorylation can modulate the substrate protein activity in a positive or negative fashion. Modified from (Bogoyevitch and Kobe, 2006).

3.3 JNK function in the immune system

MAP kinases are very conserved proteins as they are involved in many important cellular processes, such as mediating signals triggered by growth factors, environmental stress and cytokines. As mentioned above, MAPK kinases can be divided into three families: the p38 family, the Erk family and the JNK family. All of these three groups are important in regulating the immune system starting from its early development. In particular, mice lacking JNK1 or JNK2 are not immuno-competent due to severe defects in T cell lineage.

JNK has been implicated in T cell activation since it is rapidly activated in response to phorbol-12 myristate 12 acetate (PMA) or anti CD3 antibodies in Jurkat cells. Being AP-1 a trancription factor regulated by JNK phosphorylation, JNK is also involved in the regulation of IL-2 expression (Su et al., 1994; Matsuda et al., 1998). However, mice deficient for either *jnk1* or *jnk2* produce normal levels of IL-2 (Dong et al., 1998).

In addition both JNK expression and enzymatic activity peak after 30-60 hours of primary T-cell activation thus indicating that JNK is probably not involved in the early phases of T-cell activation but rather modulates in T-cell function. Beside the phosphorylation of JNK mediated by MKK4 and MKK7, that control its activity, the levels of JNK expression are known to be tightly regulated exclusively in T lymphocytes (Weiss et al., 2000). It is possible that JNK is strictly regulated in T lymphocytes in order to avoid production of effector cytokines by naïve cells. In fact, it has been demonstrated that TCR signals are sufficient to induce JNK expression, but CD28 signals are required to activate the kinase (Weiss et al., 2000). On the basis of these findings an interesting question was raised: can JNK be involved in the selection of thymocytes. The development of T cells occurs in the thymus where the early precursors, that do not express either $CD4^+$

or $CD8^+$ molecules and are named double negative cells (DN), differentiate in double positive cells (DP) and then in single positive (SP) $CD4^+$ or $CD8^+$. Before completing the differentiation from DP to SP state, thymocytes undergo positive and negative selection. Positive selection is characterized by recognition of self major histocompatibility complex (MHC) by a T cell receptor and rescue from apoptosis. Recognition of self antigens that leads to programmed cell death is a hallmark of negative selection. JNK is highly expressed in double positive thymocytes ($CD4^+$ and $CD8^+$) and its activation leads to depletion of autoreactive T cells thus underlining the role of JNK in negative selection of thymocytes (Rincon et al., 1998).

However, the mechanism by which JNK controls negative selection of DP cells is not completely understood. JNK has been demonstrated to phosphorylate the NFAT family of transcription factors, which are involved in the regulation of several cytokines genes (IL-2, IL-4, TNF, GM-CSF) and antiapoptotic proteins (Bcl-2). JNK mediated phosphorylation of NFAT-4, a transcription factor mainly expressed in the thymus, exerts a negative effect on its activity by increasing its nuclear export or cytoplasmic retention (Chow et al., 1997). Moreover, NFAT deficient mice show increased rate of thymocyte apoptosis, probably due to the inhibition of Bcl-2 expression. Thus, it has been hypothesized that during negative selection JNK prevents nuclear localization of NFAT, impedes its transcriptional activity and increases cell death susceptibility (Rincon et al., 1998).

Another possible role of JNK in the regulation of immune system is its involvement in the control of Th1/Th2 differentiation (Rincon, 2001). Studies involving mice deficient for JNK2 show that these animals had normal amounts of $CD4^+$ and $CD8^+$ cells and normal production of IL-2. However IFN-γ production was very low compared with wild type animals

and, consequently, Th1 differentiation was impaired (Yang et al., 1998). Interestingly, Th2 cells deriving from JNK1-knock out mice produce normal levels of IFN-γ but high amounts IL-4 and IL-5 compared to wild type, thus these animals display an exaggerated Th2 response (Dong et al., 1998; Constant et al., 2000). These results suggest a distinct role of JNK1 and JNK2 during Th1/Th2 differentiation (Figure 10).

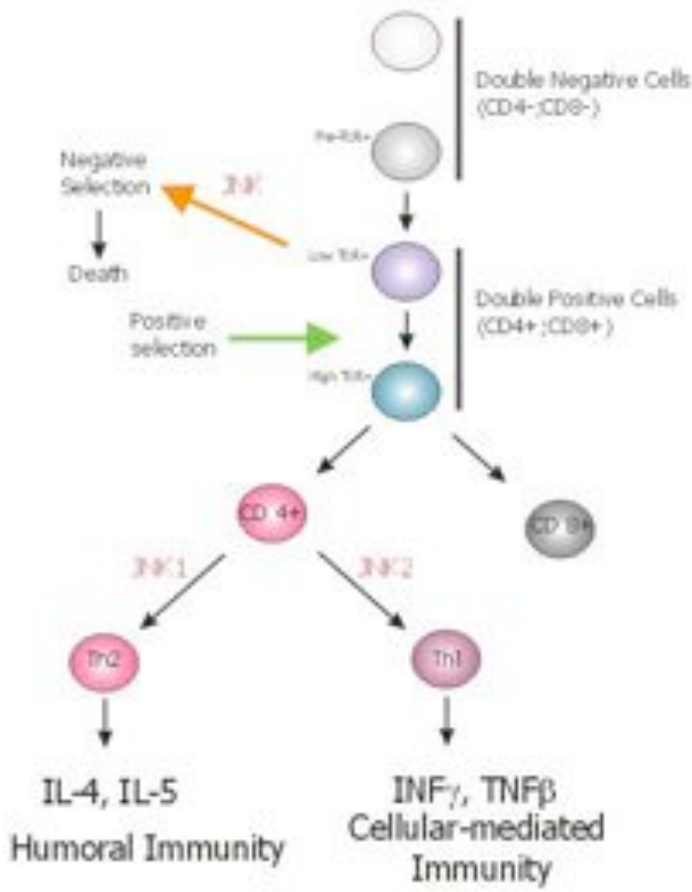


Figure 10. A schematic representation of JNK role in the Immune system development and regulation.

4. The Prolyl Isomerase Pin1

4.1 Pin1 structure and enzymatic activity

Pin1 is a member of the peptidyl-prolyl isomerase family of proteins and was discovered in a two-hybrid screening as an interactor of NIMA (never in mitosis gene A) in Apergillus nidulans (Lu et al., 1996). The peptidyl-prolyl isomerases (PPIases) are ubiquitous proteins expressed in both eukaryotic and prokaryotic cells. These enzymes catalyze the cis/trans isomerization of peptidyl-prolyl peptide bonds.

On the basis of drug sensitivity, the PPIases are divided into three different groups: Cyclophilins that bind Cyclosporin A, FK506 binding proteins, and parvulins that do not bind immunosupressants.

Pin1 belongs to the parvulin group of isomerases and it is the only PPIase that specifically recognizes phosphorylated Ser/Thr Pro sequences.

Serine or Threonine residues that precede a Proline are the major regulatory phosphorylated motifs in cells. The kinases that specifically phosphorylate these motifs belong to the family of Proline-directed Kinases, which include Cyclin-dependent Kinases (CDKs), stress activated kinases/c-Jun-N-terminal kinases (SAPKs/JNKs), extracellular signal-regulated kinases (Erks), glycogen syntase kinase-3 (GSK3) and Polo-like Kinase (PLKs). These kinases are involved in almost all cellular processes such as cell cycle, apoptosis, differentiation and survival. The peculiar structure of Proline permits two different conformational states: the cis conformation or the trans conformation. The interconversion between these two states can occur naturally but is rather slow; Pin1 can increase the rate of interconversion by as much as 1000 fold (Lu et al., 2002) (Figure 11).

Pro-directed kinases and phosphatases are conformation-specific and recognize substrates only in trans conformation. Moreover, phosphorylation decreases the rate of natural isomerization and renders the phosphopeptide more resistant to other PPIases; these characteristics

render Pin1 especially important in regulating the conformation of phosphorylated proteins.

Pin1 is composed by two domains, an aminoterminal WW domain (1-39) and a carboxyterminal PPIase domain (45-163); the two domains are linked by a short flexible linker region. The WW domain (named after two conserved Tryptophan residues) binds specifically the pSer/Thr-Pro region on Pin1 substrates, while the PPIase domain isomerizes the target protein (Yeh and Means, 2007).

Many targets of Pin1 contain only one pSer/Thr-Pro motif, suggesting that the WW domain and, subsequently, the PPIase domain acts on the same region. This implies that the WW domain has to dissociate from its consensus site and then the catalytic domain isomerizes the bond, but the detailed dynamic of the reaction remains unclear (Lu and Zhou, 2007).

Pin1 primarily localizes in the nucleus of cells in culture but is detected also in the cytoplasm in many dividing cells and in normal or cancerous tissues (Lufei and Cao, 2009). Pin1 does not have a defined nuclear localization signal and, given it is a small protein, its distribution could be driven by the localization of its targets.

In contrast to other PPIases, Pin1 function is tightly regulated inside the cell. In neurons, for example, Pin1 is induced upon differentiation (Hamdane et al., 2006); in other cell types, Pin1 is correlated with the proliferative status (Atchison et al., 2003). Moreover Pin1 expression is deregulated in many cancer tissues and transformed cell lines (Wulf et al., 2003; Bao et al., 2004).

Pin1 expression is dependent on E2F-mediated transcriptional regulation and thus is upregulated when cells progress from G0 to S phase of the cell cycle. Pin1 transcription is suppressed by BRCA1 (Lu and Zhou, 2007).

Finally, Pin1 is also regulated at post-translational level in a cell cycle dependent manner (Eckerdt et al., 2005). It is known that phosphorylation on Ser16 in the Pin1 WW domain abolishes the capability of Pin1 to bind its

substrates while phosphorylation on Ser 65 by Polo-like Kinase (PLK) increases its stability (Eckerdt et al., 2005). Finally Pin1 enzymatic activity is inhibited by oxidative modifications (Sultana et al., 2006).

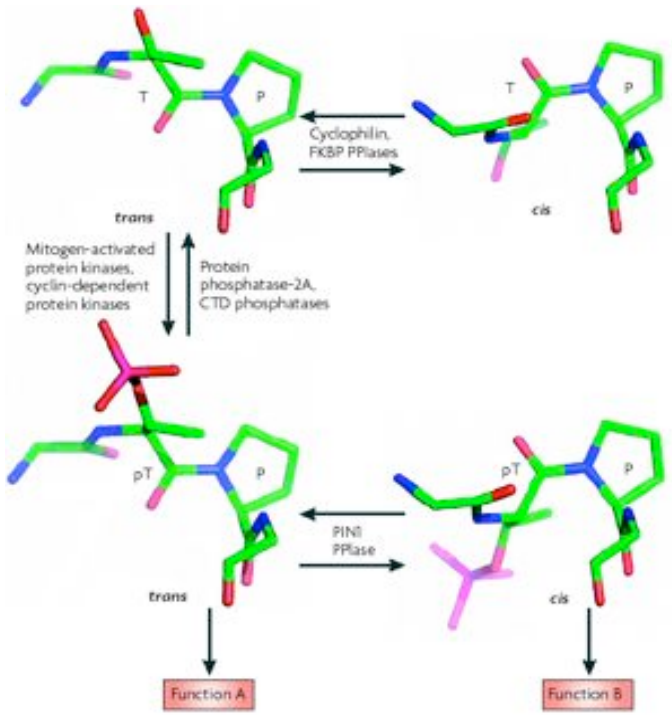


Figure 11. Proline can adopt either the cis or trans state of the backbone torsion angle, due to its five-membered ring in the peptide backbone. Uncatalysed isomerization is a rather slow process but can be greatly accelerated by peptidyl-prolyl cis/trans isomerases (PPIases). Pin1 is the only PPIase that requires the phosphorylation of the Ser or Thr residue preceeding the Pro (Lu and Zhou, 2007).

4.2 Pin1 in the cell cycle

Pin1 was originally discovered by its ability to bind a mitotic kinase (NIMA) involved in the mitosis of Aspergillus nidulans. Pin1 blocks the ability of NIMA to cause mitotic catastrophe in budding yeast.

Pin1 has a crucial role in mitosis in Xenopus Laevis as well as in mammalian cells. Inhibition of Pin1 activity induces mitotic arrest and apoptosis in both budding yeast and tumour cell lines; moreover depletion of Pin1 affects DNA replication, mitotic checkpoint and the G2-M transition in Xenopus Laevis (Shen et al., 1998).

Progression through the cell cycle is governed by activation and inactivation of different cyclin dependent kinases that belong to the group of Proline-directed kinases. In particular, in S. pombe, Pin1 regulates the activity of the protein phosphatase Cdc25C and the kinase WEE1, that activates and inhibits respectively CDC2 kinase (Stukenberg and Kirschner, 2001).

Several studies suggest that Pin1 acts mainly during G0/G1-S transition. Pin1 induces the increase of transcription and stability of cyclin D1 (a key regulator of G1-S transition) and affects the transcription of c-Myc and cyclin E, which are also important during G1-S progress in the cell cycle (rev in Boonstra 2003 and Obaya et al., 1999).

Regulation of cyclin D1 by Pin1 is an interesting example highlighting how this isomerase is involved in multiple steps of protein function. c-Jun, a component of AP-1 transcriptional complex, is phosphorylated by JNK in response to various signals becoming a target for Pin1 (Wulf et al., 2001). Pin1 action on phosphorylated c-Jun increases its transcriptional activity towards cyclin D1. In addition, Pin1 interaction with β-catenin (another transcriptional factor that induces cyclin D1 expression) prevents its binding to APC (Anaphase Promoting Complex) that triggers β-catenin to

degradation (Ryo et al., 2001). Moreover Pin1 can bind directly to cyclin D1 increasing its stability and nuclear accumulation.

A recent study indicates that Pin1 has a role in the coordination of centrosome duplication and DNA synthesis during the cell cycle (Suizu et al., 2006). In addition, it has been demonstrated that Pin1 plays a role in controlling DNA damage response. In response to DNA damage, both p53 and p73 are phosphorylated and become targets for Pin1; this results in their stabilization and nuclear accumulation that in turn increases the rate of apoptosis (Zacchi et al., 2002; Mantovani et al., 2004; Wulf et al., 2002).

Finally, it has been proposed that Pin1 acts as a molecular timer during the cell cycle. Indeed, mouse embryo fibroblast (MEFs), deriving from Pin1 knock out mice, show slower asynchronous growth than wild-type MEFs.

A. nidulans, that has reduced expression of Pin1 homologous, needs a longer period to complete nuclear division than the wild type (Lu et al., 1996).

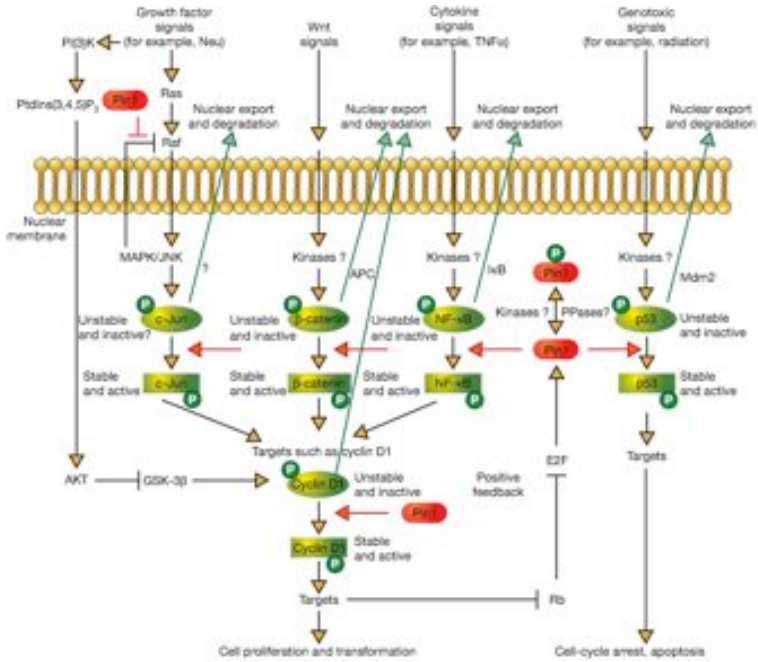


Figure 12. Pin1 plays an important role both pro-proliferative and pro-apoptotic pathways. Phosphorylation of proteins on certain Ser/ Thr-Pro motifs is an essential signalling mechanism in cell proliferation, differentiation and transformation, and genotoxic response. By binding and isomerizing these residues, Pin1 regulates signalling following phosphorylation. Pin1 activity is usually tightly regulated by multiple mechanisms. This isomerase is found to be both overexpressed and activated in many human cancer tissues and cells. Overexpression of Pin1 can activate multiple oncogenic pathways at different levels. For example, Pin1 can stabilize cyclin D1 and increase its transcription by acting in three different pathways. Pin1 can increase c-Jun transcriptional activity. Pin1 can inhibit the degradation of both β-catenin and NF-kB thus increasing their nuclear accumulation. Furthermore, Pin1 transcription is activated following oncogenic activation suggesting the existence of a positive feedback loop. Finally, Pin1 prevents Raf kinase from being inactivated after growth stimulation. All these Pin1 effects promote proliferative and oncogenic signalling. However, following genotoxic insult, Pin1 can increase the nuclear localization and protein half-life of p53 thus increasing its activity on cell-cycle arrest and apoptotic genes. It remains to be determined how Pin1 coordinates pro-proliferative and pro-apoptotic signal pathways (Wulf et al., 2005).

4.3 Pin1 in the immune system

Recent studies have shown that Pin1 is involved in the regulation of GM-CSF (granulocyte-macrophage colony-stimulating factor) contributing to the onset of allergic asthma (Shen et al., 2005). In patients suffering from allergic asthma, eosinophils migrate to the lung parenchyma and pulmonary airways and secrete GM-CSF, which is an essential survival and maturation factor. Pin1 is a component of a multi protein complex that stabilizes the GM-CSF mRNA. This mRNA is indeed very unstable in resting eosinophils because AUF1 proteins (RNA-binding proteins that regulate target mRNA molecules by enhancing their decay) bind to it and induce its degradation. Upon stimulation with matrix proteoglycans, resting eosinophils are activated and otherwise inactive Pin1 is dephosphorylated and than catalytically active. The same signal also induces the phosphorylation of AUF1 proteins that become Pin1 targets. Isomerization of AUF1 proteins causes their release from GM-CSF mRNA, which is in turn bound by hnRNP C and stabilized (Shen et al., 2005; Esnault et al., 2006).

New evidence suggest that Pin1 may be also involved in the innate immune response against viral infections by influencing the Interferon-β (IFNβ) pathway.

IFNβ controls the transcription of many genes that protect the cell from viral infection. IFNβ is induced by IRF3 (interferon-regulatory-factor 3), which is activated after the stimulation of TLR3 (Toll-Like receptor 3) by double strand viral RNA. As reported recently, Pin1 binds IRF3 inducing its degradation, and Pin1-/- macrophages secrete three times more IFNβ in response to viral RNA than the wild type ones (Saitoh et al., 2006).

In addition it can be speculated that Pin1 might be involved in other aspects of the immune system since many of its targets control the immune response.

In general, lymphocytes are stimulated through engagement of MHC molecules and CD3, CD28 and CD40 receptors. These signals trigger a variety of phosphorylation events and involve many kinases such as MAPKs and CDKs. These signal transduction pathways induce the transcription of many genes and eventually cell division. Interleukin-2 (IL-2) is a critical signalling cytokine that controls mammalian immune system. The expression of IL-2 is governed by at least four families of transcription factors AP-1, NF-AT, NF-κB and Oct. Pin1 is known to be involved in the regulation of NF-AT, NF-κB, AP-1 by different mechanism. As mentioned in the previous chapter c-Jun (a component of AP-1) is positively regulated by Pin1. On the other hand it has been reported that overexpression of Pin1 inhibits transcription of genes responsive to NF-AT by preventing its activation (Liu et al., 2001). Moreover, in response to cytokines, Pin1 regulate both the activity and the stability of NF-kB, a well known factor involved in the regulation of immune system. Pin1 isomerises the phosphorylated form of p65, prevents its binding to IkB (inhibitor of NF-kB function) thus leading to an increase in NF-kB activity. In addition, Pin1 inhibits NF-kB degradation by SOCS1 (suppressor of cytokine signalling-1) (Ryo et al., 2003).

Given the importance of all themase factors in the immune system it is probable that Pin1 regulates many other aspects of the immune response. (Esnault et al., 2008).

Materials and Methods

1. In vitro binding assays and immunoprecipitation

Binding of GST-IN and its truncated variants to ^{35}S-JNK1 was performed as previously described (Marzio et al., 1998). In brief 1μg of recombinant proteins were incubated with 400 cpm of in vitro translated jnk in a solution containing 0,2 mg/ml ethidium bromide, following 6 washes the reaction mixture was resolved by SDS-PAGE electrophoresis and analyzed by PhosphoImager.

For IN and JNK co-immunoprecipitation experiment, HEK 293T cells overexpressing IN-Flag were collected in RIPA buffer containing 100 mM NaF and 1 mM sodium orthovanadate and Protease inhibitor cocktail EDTA free (Roche).

GST pull down assays with recombinant Pin1 were performed as previously described (Zacchi et al., 2002) briefly cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% NP-40, 100 mM NaF 1 mM sodium orthovanadate and Protease inhibitor cocktail EDTA free (Roche) and incubated with recombinants GST-Pin1 proteins. In the case of CIP treatment 40U/ml of CIP (New England Biolabs) were added to the extracts and the reaction was continued for 30 min at 30°C. In the case of λ-PPase treatment 5μl of λ-PPase were added to the extracts and the reaction was continued fot 30 min at 30°C. Flag-IN and endogenous Pin1 co-immunoprecipitation was performed as previously described (Zacchi et al., 2002) in brief HEK 293T cells overexpressig Flag-IN were lysed in PBS (pH 8.3), 0.1% Tween 20, 10 mM EDTA and inhibitors as above. Immunoprecipitation was performed with Anti-Pin1 antibody (Calbiochem).

2. Subtilisin Proteolysis

^{35}S-his-IN was produced using TNT kit (Invitrogen) purified on nickel column, and incubated with 100 ng of either GST, GST-Pin1 wt and GST-Pin1(C113A) in a buffer containing 50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothritol and phosphatase inhibitors for 20 min at 20°C, subtilisin (200 ng) was added for 20 min at 20°C and reaction was stopped by adding sample buffer containing 6 M UREA, the proteolitic samples were resolved by SDS-PAGE electrophoresis and analyzed by PhosphoImager.

3. Kinase Assay

his-IN wt and his-IN(S57A) mutant were produced in BL-21 and purified as follows: bacterial pellet was resuspended in a buffer containing 20 mM Tris-HCl pH7.9, 5 mM imidazole, 1 M NaCl, bacterial suspension was centrifuged and Ni-NTA Quiagen Resin was added to the supernatant.

his-IN fusion proteins were eluted in Elution buffer (1 M imidazole, 1 M NaCl, 20 mM Tris-HCl pH 7.9). Concentration was determined by SDS-PAGE and Coomassie staining.

500 ng of either his-IN and his-IN(S57A) were incubated with recombinant JNK1 (Millipore) for 1 hour and 30 min at 30°C in JNK buffer (50 mM Tris-HCl pH 7.5, 0,1 mM EGTA, 0,1% β-mercaptoethanol, 1 mM sodium orthovanadate, 5 mM NaF and 10 mM β-glycerolphosphate), 500 mM ATP and 25 mM $MgCl_2$ was added or not to the reaction as a control.

Proteins were resolved by SDS-PAGE electrophoresis and revealed by Western Blot using an affinity purified anti-phospho IN polyclonal antibody and an Antiserum anti IN (NIH).

4. Cell culture and treatments

Primary lymphocytes are isolated from healthy donors obtained buffy coats by density gradient centrifugation (Ficoll Hystopaque Sigma), briefly the ficoll gradient was overlayed with diluted buffy coats and centrifuged for 15 min at 950 g with the break off, the PBMC at the interface were collected and washed with RPMI.

The PBMC suspension was layed onto hyper-osmotic Percoll solution (48,5% Percoll, 160 mM NaCl) and centrifuged 15 min at 580 g. The monocytes fraction at the interface was discarted and the pellet of lymphocytes was resuspended in RPMI and kept in culture.

$CD4^+$ positive T cells were isolated by negative selection with a $CD4^+$ T cell isolation kit (Miltenyi). Cells were maintained in RPMI 1640 (Gibco) supplemented with 10% heat inactivated fetal bovine serum and gentamycine. The cells were activated with Phyto hemo agglutinin (PHA-P) (5 mg/ml) and Interleukin-2 (IL-2) (40 ng/ml) for 48 hours.

PHA-P and human IL-2 were purchased by Sigma. SupT1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and gentamycine. Both HEK 293T and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and gentamycine at 37°C.

For cyclohexamide experiment HeLa cells were transfected with either Flag-INwt or Flag-IN S57A mut and treated with Parvulin inhibitor (PiB, Calbiochem) 3μM for 18 hours, then the culture medium was supplemented CHX (Sigma) 30g/ml and cells were collected in RIPA at the indicated time points. All the inhibitors (PD98059, SB203580, SP600125) were used at a concentration of 40μM. Phorbol-myristate acetate (PMA) was purchased by Sigma and used at a concentration of 10 mg/ml.

5. Plasmids, SIRNA and Antibodies

The pGEX-IN, pFlag-IN and pINSD-His mutated in S57 were constructed using recombinant PCR starting from each original vector. pFlag-IN codon-optimized was kindly provided by Dr. A. Engelman. The HIV-1 mut viral clone was prepared by subcloning IN from the pGEX-IN mutated vector. All constructs were verified by DNA sequencing. pGEX-Pin1 wild type and its mutants were kindly provided by Dr. G. Del Sal, p-Cs2-JNK was kindly provided by Dr L. Collavin.

SiRNA against Pin1 (5'-GCCAUUUGAAGACGCCUCG-3') and RISC-Free control SiRNA were purchased by Dharmacom.

Anti phosphorylated IN rabbit polyclonal antibody was raised against the IN phosphoS57 peptide (AMHGQVDCphosphoSPGIWQLDC) and purified with Immunopure igG Purification kit (Pierce). Purified IgG fraction was depleted from anti-total IN.

The following primary antibodies were used: mouse monoclonal antibody anti-Flag (Stratagene), M2 mouse monoclonal anti-Flag beads (Sigma), rabbit polyclonal anti JNK-fl (Santa Cruz), rabbit polyclonal anti IN (purchased by NIH), rat anti-HA (Roche), 16b4 mouse monoclonal anti phospho serine-proline/anti phospho serine-lysine and anti-Pin1 antibody (Calbiochem), anti p38, anti-phospho-p38, anti Erk1/2, anti-phospho-Erk1/2 ad anti-phospho-JNK were all purchased by Cell Signalling.

6. HIV-1 RT Assay

To measure RT activity in HIV-1 infected cells, 10 μl of cell culture supernatants are incubated with 25 μl of RT Buffer (60 mM Tris-HCl pH 8, 75 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EDTA, 40 mM DTT), 5 mg/ml PolyA-oligo dT (Boeringher) and 10 μCi/ml deoxythymidine 5'-[^{32}P] triphosphate (Amersham). The reactions are incubated for 2 hours at 37°C and 15 μl are spotted onto a DEAE filter, washed tree times with SSC-Buffer (150 mM NaCl, 15 mM Sodium Citrate) and once in ethanol. The filter is dried and quantified using PhosphoImager.

7. Dot Blot

Serial dilutions of IN-S57 and IN-phS57 peptides were blotted on a Protran BA79 membrane 0.1μM pore size. The membrane was air dried, blocked in 5% BSA/TBS Tween 0.1% and incubated over night with the affinity purified a-ph-IN. the same membrane was stripped and incubated with IgG anti-ph-IN 2 hours at room temperature.

8. Strand Transfer

For the strand transfer reaction 5 pmol of His-IN wild and His-IN (S57A) were used. Recombinant proteins were incubated with 1pmol of radiolabeled oligonucleotide (5'-GTGTGGAAAATCTCTAGCA-3') and (5'ACTGCTAGAGATTTTCCACAC-3') for 1hour at 37°C in Strand transfer buffer (20 mM Hepes pH 7.5, 7.5 mM $MnCl_2$, 0.05% NP-40, 10 mM DTT). The reaction mixture was analyzed on a 0.3 mm denaturing gel (15% polycarylamide, 6 M urea), and visualized by phosphoimaging (Cyclone).

9. Virus Production, Infection and Alu–PCR

Viral stocks were prepared by the standard calcium-phosphate method of transfection, of the viral clones HIV-1_{BRU} (Petit et al, 1999) and HIV-$1_{BRU(S57A)}$ in HEK 293T cells. The supernatant containing virions were collected 48 hours after transfection, centrifuged 5 minutes at 1500 rpm and filtered with 45 μm Millipore filter. Before infection viral stocks were treated with DnaseI (Invitrogen) 40U/ml for 1 hour at room temperature, cells were infected for 4 hours in the presence of polybren.

Genomic DNA of infected cells was extracted with Dnaeasy Tissue Kit (Quiagen) and quantified by spectrophotometric analysis.

The integrated proviral DNA was evaluated by Alu-PCR as previously described (Tan *et al*, 2006) with minor modifications. Alu-LTR sequences were amplified from 300ng of genomic DNA in the first round PCR. In the second round Real Time PCR one fifth of the first round PCR was used as a template together with the l-specific primer λT , the internal LTR primer LR and the probe ZXF-P.

Real time PCR amplifications were performed on an AbiPrism 7000 machine, using the TaqMan technology (Applied Biosystem). The levels of Late transcripts were analyzed by Real time PCR using 250ng of genomic DNA as a template. Results were normalized by the amount of cellular DNA quantified by Real Time PCR of the lamin B2 gene.

Primer	**Sequence**	**Position**
LM667	TGGCTAACTAGGGAACCCACTGC	40-62
LR	TCCACACTGACTAAAAGGGCTTGA	145-168

ZXF-P probe	TGTGACTCTGGTAACTAGAGATCCCTCAGACCC	120-152
Alu1	TCCCAGCTACTGGGGAGGCTGAGG	
λ-primer	ATGCCACGTAAGCGAAACT	
MH 531	TGTGTGCCCGTCTGTTGTGT	103-122
MH532	GAGTCCTGCGTCGAGAGAGC	226-246
LRT probe	CAGTGGCGCCCGAACAGGGA	179-198
MH 535	AACTAGGGAACCCACTGCTTAAG	8888
MH 536	TCCACAGATCAAGGATATCTTGTC	8888
2LTR probe	ACACTACTTGAAGCACTCAAGGCAAGCTTT	9888
ERT fw (Nuc1B177)	CGTCTGTTGTGTGACTCTGGTAACT	111
ERT rev (Nuc1B177)	CACTGCTAGACATTTTCCACACTGA	158
ERT probe	ATCCCTCAGACCCTTT	140

Results

1. Cellular JNK activity is required for efficient HIV-1 infection and integration in primary human T-lymphocytes

Activation of T cells is essential for an efficient HIV-1 infection (Zack et al., 1990; Korin et al., 1998). Primary human $CD4^+$ T cells were purified from peripheral blood of three normal donors and either left untreated (>98% resting cells) (Tang et al., 1995) or stimulated with PHA/IL-2 under standard conditions (Terai et al., 1991). At 1, 18 or 48 hours after stimulation, cells were infected with a VSV-G-pNL4-Luc virus (vesicular stomatitis virus G psuedotyped NL4-3 expressing a luciferase reporter gene in lieu of Nef). We used this virus in order to selectively study the events following entry of the virions into the cells and avoiding the possibility of re-infection. Similar to wt HIV-1, viral infection, as assessed by luciferase activity at 72 hours after infection, was remarkably dependent on cellular activation (>80 fold increase in luciferase activity in the PHA/IL-2-stimulated $CD4^+$ cells at 48 hours compared to unstimulated controls) (**Figure 1a**)**.** Under the same experimental conditions, the levels of expression and phosphorylation of the ERK1/2, p38 and JNK groups of MAP kinases (MAPKs) were assessed by Western Blotting both before and after 48 hours PHA/IL-2 stimulation. As shown for a representative sample in **Figure 1b**, both ERK1/2 and p38 were constitutively expressed in resting conditions, and their overall levels remain unchanged at 48 hours. In contrast the amounts of the two JNK splicing isoforms (p52 and p46) significantly increased at the latter time point. All three MAPKs were found phosphorylated at 48 hours however not in the absence of stimulation.

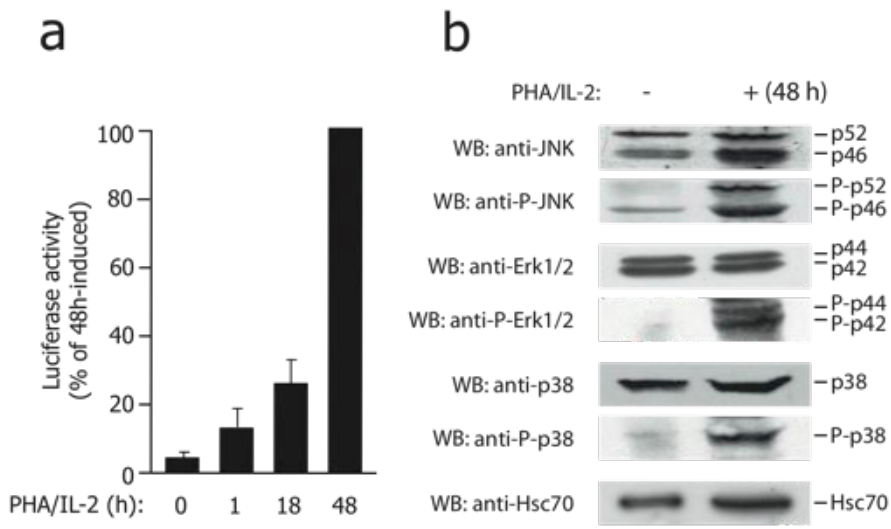


Figure 1 a,b.

(a) Efficient HIV-1 infection of primary $CD4^+$ T lymphocytes requires stimulation with PHA/IL-2. Primary human $CD4^+$ T cells were stimulated or not for 1, 18 or 48 hours with PHA/IL-2 and then infected with the VSV-G-Luc virus. Relative luciferase activity of each sample was represented as a percentage of the value obtained for the sample stimulated for 48 hours prior to infection (mean ± sem of at least three experiments) **(b)** Treatment of primary human $CD4^+$ T lymphocytes with PHA/IL-2 activates all MAPKs. Cell lysates from primary $CD4^+$ T cells stimulated with PHA/IL-2 for 48 hours were analyzed by Western-Blot with the indicated antibodies; Hsc70 protein was used as a control for total protein levels.

To start investigating whether the activation of these MAPKs might impact on the early phases of HIV-1 infection, we infected primary $CD4^+$ T cells with VSV-G-pNL4-Luc in the presence of the chemical inhibitors SP600125, PD98059 and SB203580, which selectively block the activity of JNK, ERK/21 and p38 respectively (Bennett et al., 2001; Alessi et al., 1995; Cuenda et al., 1995). Cell treatment with 40 μM SP600125, a compound specifically

inhibiting JNK, selectively impaired viral infection, while inhibitors of ERK1/2 and p38, used at the same concentration, had no observable effect (**Figure 2a**). Next we wanted to investigate which step of the viral life cycle, after viral internalization and including proviral transcription, might be affected by the JNK inhibitor SP600125. For this purpose, primary peripheral blood lymphocytes (PBLs) from healthy donors, either in resting conditions or after PHA/IL-2 activation, were infected with the HIV-1_{BRU}-Flag-IN viral clone (Petit et al., 1999; Cereseto et al., 2005) after treatment with the SP600125 inhibitor and the efficiency of reverse transcription was monitored by assessing the levels of the early and late HIV-1 reverse transcripts by a real-time PCR-based quantitative assay (Butler et al., 2001). Reverse transcription is catalyzed by the reverse transcriptase (RT) enzyme and is mainly carried out in the cytoplasm soon after viral penetration into the cell; the process is generally completed in 8 to 12 hours (Iordanskiy et al., 2006). In order to specifically amplify the early reverse transcripts, we collected PBLs 5 hours post infection and amplified the strong stop HIV-1 cDNA using specific primers (Iordanskiy et al., 2006). For the late reverse transcripts analysis, cells were collected 8 hours after infection and the viral cDNA was amplified with specific primers annealing between the U5 region and the gag gene (Butler et al., 2001). As shown in **Figure 2b** and consistent with previous observations, formation of the late, but not of the early, reverse transcripts was impaired in the absence of PHA/IL-2 stimulation (Zhou et al., 2005). In the activated cells, accumulation of both early and late transcripts were not affected by cell treatment with the JNK inhibitor SP500125.

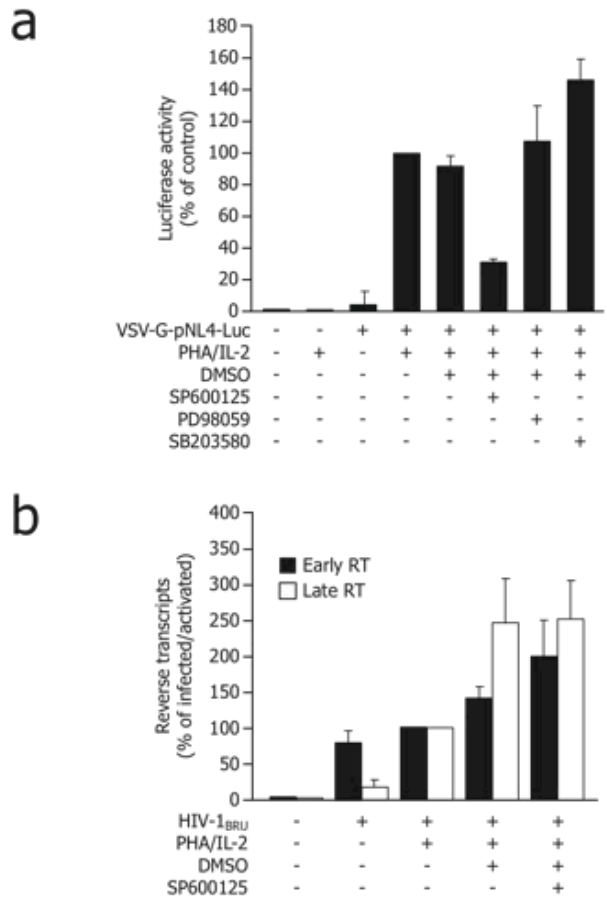


Figure 2 a,b.

(a) Inhibition of JNK leads to decreased HIV-1 infectivity. $CD4^+$ lymphocytes, stimulated with PHA/IL-2 and treated for 15 hours either with chemical inhibitors of MAPK activity (SP600125 for JNK1/2, PD98059 for Erk, and SB203580 for p38, all dissolved in DMSO) or with DMSO alone, and infected with VSV-G-Luc, were assayed for luciferase activity 72 hours post infection. Results are presented as a percent of the sample stimulated for 48 hours prior to infection. **(b)** JNK inhibition does not affect the levels of early or late reverse transcripts in Primary Blood Lymphocytes (PBLs). PBLs, stimulated as above and treated with the inhibitor of JNK activity (SP600125) for 15 hours prior to infection with the $HIV\text{-}1_{BRU}$ clone, were analyzed for the relative levels of Early Reverse Transcripts (Early RT, black

bars) and Late Reverse Transcripts (Late RT, white bars) by Real time PCR. Each sample, normalized for the amount of total genomic DNA, represents a mean value of at least three experiments ± sem. All the values were calculated with respect to the sample stimulated with PHA/IL-2 for 48 hours prior to infection with the HIV-1_{BRU} clone.

In contrast, a major effect of the drug was noticed when the levels of integrated viral DNA were assessed using a real-time PCR integration assay based on Alu-LTR amplification (Tan et al., 2004). As expected, the extent of viral integration was highly dependent on cellular activation with PHA/IL-2 (>80 fold increase of integrated DNA in the PHA/IL-2 stimulated cells compared to untreated controls). In activated cells treated with the JNK inhibitor, the levels of integrated provirus were markedly reduced (>6 fold compared to the untreated controls) (**Figure 3a**).

Proviral integration as well as transport of viral cDNA into the nucleus are mediated by the functions of HIV-1 IN. Impaired enzymatic activity of this protein still allows nuclear import of the viral cDNA, however determines accumulation of 2LTR circles (Wiskerchen et al., 1995). Compared to activated PBLs, the levels of 2LTR circles were significantly reduced upon treatment with SP600125 (>3 fold compared to untreated controls), consistent with the conclusion that, preceding integration, nuclear transport of viral cDNA was also affected by JNK inhibition (**Figure 3b**).

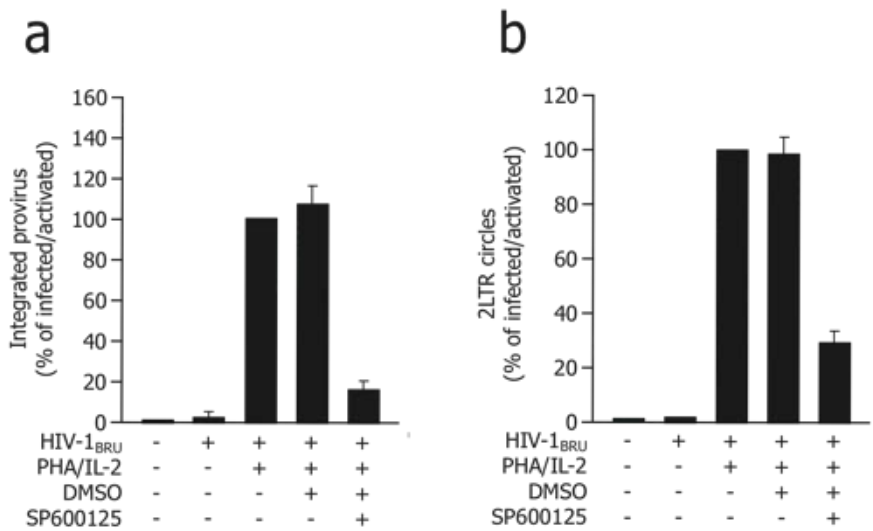


Figure 3a,b

(a) Inhibition of JNK in PBLs decreases HIV-1 integration. PBLs were treated and infected as previously described. Relative levels of integrated viral DNA were measured 24 hours post infection by Real Time PCR. **(b)** JNK inhibition affects HIV-1 nuclear entry, as measured from the formation of 2LTR circles. PBLs treated and infected as previously described, were analyzed by Real Time PCR with primers specific for the detection of 2LTR circles 24 hours post infection. Values are mean±sem of at least three different experiments.

The strong inhibitory effect of SP600125 on HIV-1 proviral integration was further confirmed by the dose-dependent decrease in the levels of integrated viral DNA observed upon HIV-1 infection of purified $CD4^+$ T cells treated with increasing amounts of the inhibitor (**Figure 4a**).

Taken together, these results indicate that the activity of JNK is essential to allow efficient viral infection acting at the level of viral cDNA integration, also involving nuclear transport of viral cDNA. Analogous findings were also detected by infecting PBLs with the VSV-G-Luc virus (**Figure 4b**).

To definitely support the notion that JNK plays a key role in the early steps of HIV-1 infection, a time-course experiment was performed by infecting primary $CD4^+$ T lymphocytes either in resting conditions or at different times (1, 18 and 48 hours) after activation with PHA/IL-2, followed by the measurement of the levels of integrated viral DNA after additional 24 hours post-infection. Efficient viral integration required 48 hours of PHA/IL-2 stimulation, while it was highly ineffective (>10-fold less) upon shorter stimulation, **Figure 4c upper part**. Of notice, the levels of JNK were barely appreciable in unstimulated cells or in cells stimulated for 1 or 18 hours, while the protein was clearly detectable at 48 hours (**Figure 4c lower part**). Thus, HIV-1 provirus integration into the genome of activated $CD4^+$ T cells positively correlated with the induction of JNK.

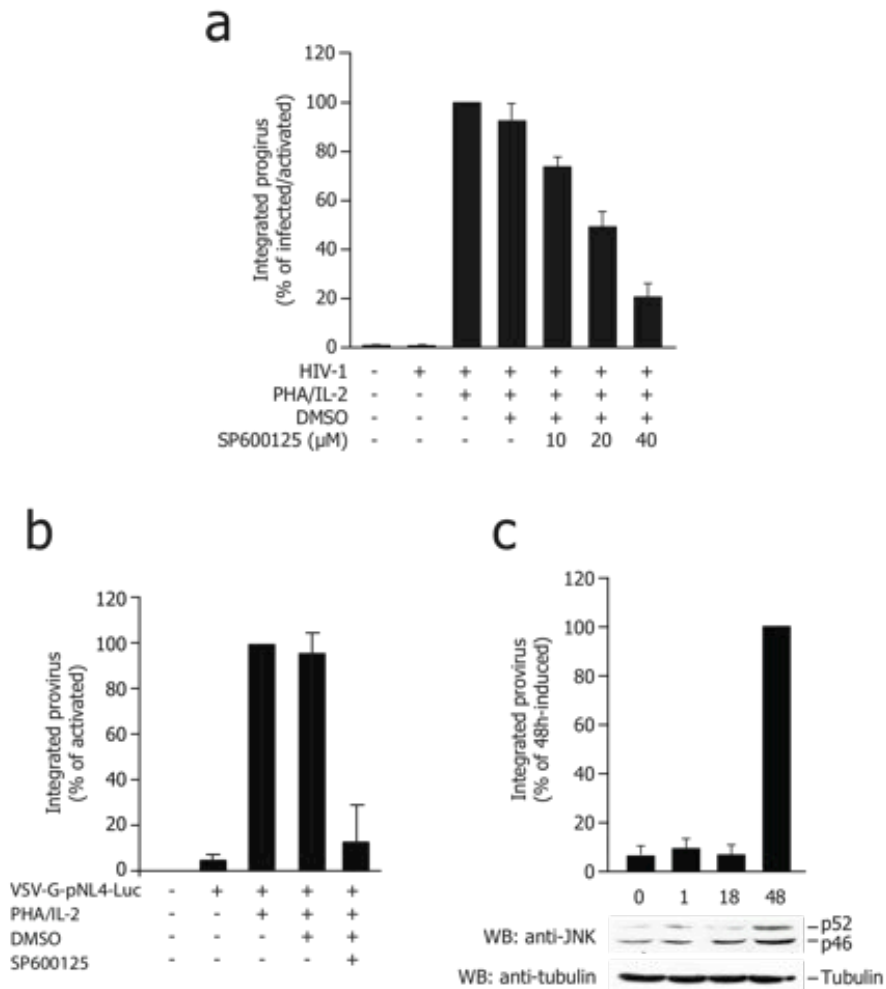


Figure 4a,b,c.

(a) JNK inhibition blocks HIV-1 integration in a dose dependent manner. Primary human $CD4^+$ T cells, stimulated as previously described, were treated with indicated concentrations of SP600125 for 15 hours prior to infection with $HIV\text{-}1_{BRU}$ and assayed for relative levels of integrated viral DNA. **(b)** JNK requirement for efficient integration is not dependent on viral entry. PBLs, stimulated with PHA/IL-2, were infected with VSV-G-Luc virus and the relative levels of integrated viral DNA were determined 24 hours post infection by Real-Time PCR. **(c)** Levels of JNK kinases and levels of

HIV integration increase significantly at 48 hours post stimulation. $CD4^+$ T lymphocytes stimulated with PHA/IL-2 for the indicated time points were infected with HIV-1_{BRU} and the relative levels of integrated viral DNA were determined 24 hours post infection by Real-Time PCR analysis (upper panel). Values are mean±sem of at least three different experiments. Lysates from these cells were probed by Western Blot with either anti-JNK or anti-Tubulin antibody (lower panel).

A schematic representation of the primers positions within the HIV-1 genome is shown in **Figure 5.**

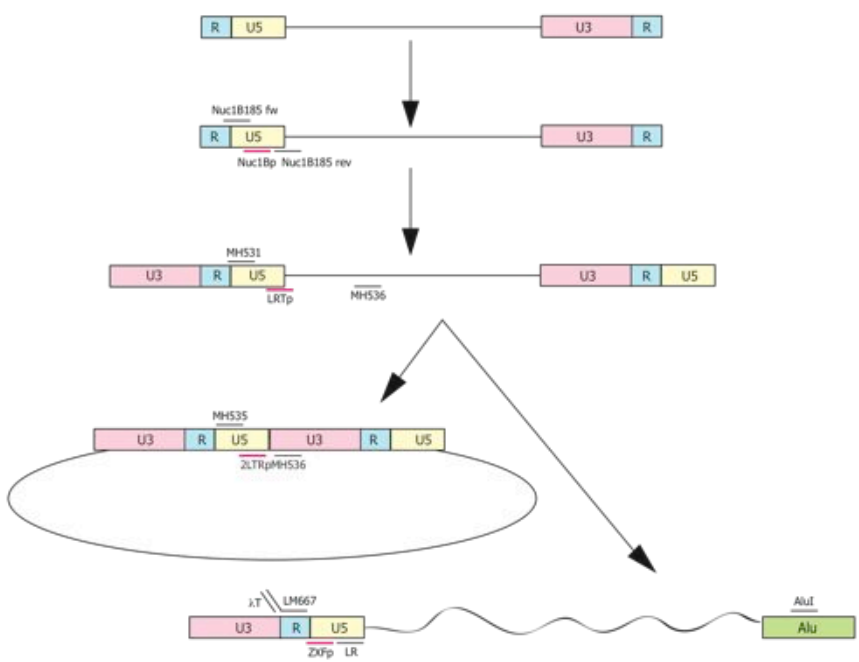


Figure 5. Schematic representation of the PCR primers employed (modified from Butler et al., 2001).

2. HIV-1 IN is phosphorylated on Ser 57 by cellular JNK

Our results indicated that JNK plays a major role in HIV-1 infection by acting at the levels of either PIC nuclear entry or provirus integration. Since both steps are known to be mediated by viral IN, we wondered whether IN itself was a substrate for JNK. HIV-1 IN is 32 kDa viral protein encoded by the Pol gene and translated as a part of Gag-Pol. It is composed by three domains: a N-terminal domain (aa 1-50) that binds to zinc ions, a core domain possessing catalytic activity (aa 51-212) and a C-terminal domain (aa 213-288) involved in DNA binding (Van Maele et al., 2006) **Figure 6**.

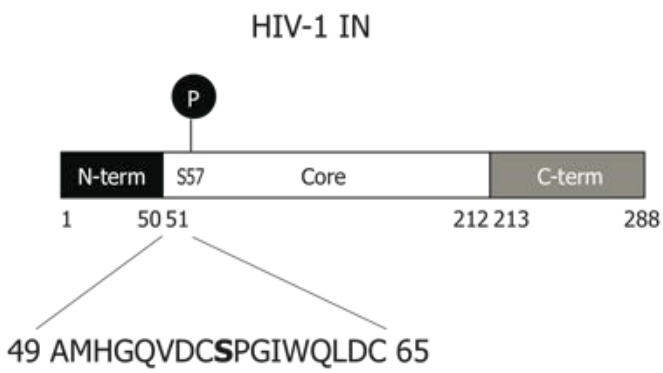


Figure 6

Schematic representation of HIV-1 IN Domains. HIV-1 IN is composed of three domains: an N-terminal domain, which is involved in the multimerization process and participates in specific recognition of DNA ends, a catalytic Core domain, and a C-terminal domain, which participates in non-specific DNA binding.

JNK belongs to a family of proline-directed kinases, which only phosphorylate serine or threonine residues followed by a proline (Bogoyevitch et al., 2006). A thorough analysis of the IN sequence revealed only one possible consensus site for JNK phosphorylation, a serine at position 57 (S57) in the core domain of the protein. To verify whether S57 might effectively constitute a target for phosphorylation, HEK 293T cells were transfected with Flag-tagged wild type IN (Flag-IN) or with a

mutant in which S57 was substituted with an alanine IN(S57A) or with Flag-Luciferase (Flag-Luc) as a control and, after immunoprecipitation with anti-Flag beads, phosphorylation was revealed using a commercial antibody recognizing phospho-serine followed by either a proline or a lysine (S-P or S-K; antibody 16b4) (Cheung et al., 2008; LaFevre-Bernt et al., 2003); since no S-K sequence is present in IN, this antibody should only recognize IN phosphorylated on S57. A band corresponding to phosphorylated IN was clearly detected, while IN(S57A) scored negative for phosphorylation (**Figure 7**).

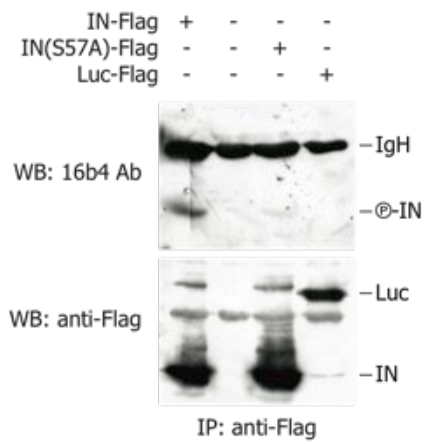


Figure 7

HIV-1 IN is phosphorylated on Ser57. HEK 293T cells were transfected with Flag-IN or Flag-IN(S57A) and Flag-Luc; cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Immunoprecipitates were immunoblotted with a commercial anti Phospho-Serine antibody (16b4) (upper panel). The same membrane was reprobed with anti-Flag antibody (lower panel).

On the basis of the above described result we raised a specific antibody that recognises IN phosphorylated on serine 57. To this aim we synthesized a peptide corresponding to IN amino acids 49-65 carrying phosphorylated S57, which was then coupled to KHL (Keyhole Limpet Hemocyanin). We used the coupled peptide to immunize rabbits. Total IgG was purified from rabbit antiserum with Immunopure IgG Purification. In order to deplete total IgGs from antibodies that recognize unmodified IN, we coupled an unmodified IN-S57 peptide (AMHGQVDCSPGIWQLDC) to SulfoLink Coupling Gel and the purified IgG fraction in PBS was loaded into the column. The flow through represented the total IgG anti-IN depleted. In order to further purify by affinity the total IgG anti-IN depleted, the phosphorylated IN-S57 peptide (AMHGQVDCphosphoSPGIWQLDC) was coupled to SulfoLink Coupling Gel and total IgG anti-IN depleted was loaded into this affinity column and specific antibodies (anti-P-IN) were eluted with degassed IgG elution buffer. To test the specificity of the anti-P-IN antibody, a dot-blot experiment was performed using serial dilutions of IN-S57 and IN-phS57 peptides. These peptides were blotted on a Protran BA79 membrane and the membrane was incubated with affinity purified anti-P-IN. The same membrane was reprobed with anti phospho-IN antiserum. As it is shown in **Figure 8** after affinity purification, the anti-P-IN antibody specifically recognizes the phosphorylated, but not the unmodified, peptide.

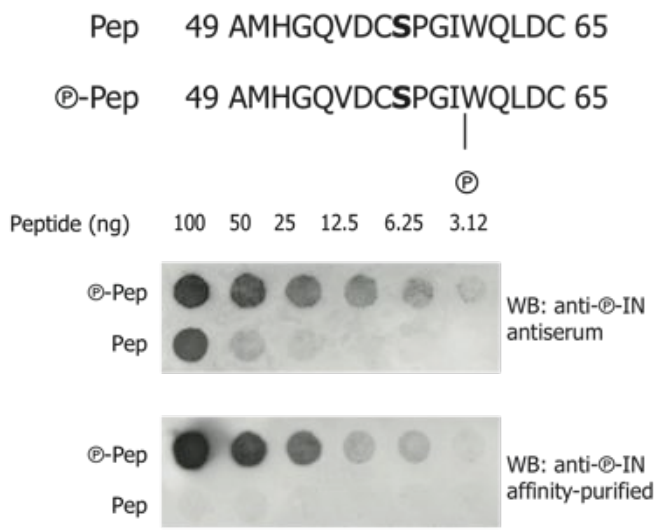


Figure 8

Sequence of the peptide corresponding to HIV-1 IN aminoacids 49-65 either phosphorylated on not on serine 57 and Dot Blot experiment showing the specificity of anti-P-IN antibody. Serial dilutions of IN-S57 and IN-phS57 peptides blotted on a Protran BA79 membrane were probed with anti Phospho-IN antiserum (upper panel) and with affinity purified anti-P-IN antibody (lower panel).

To further test the specificity of the anti-P-IN antibody, HEK 293T cells were transfected with Flag-IN and the lysate was either left untreated or incubated with λ-phosphatase (λ-PPase), prior to immunoprecipitation with anti-Flag beads and immunoblotting using anti-P-IN antibody. The antibody revealed the presence of a signal corresponding to phosphorylated IN, which almost disappeared upon phosphatase treatment (**Figure 9a**). Cell stimulation with phorbol 12-myristate 13-acetate (PMA), which is known to induce the MAPK pathways (Whitehurst et al., 1992; Jiang et al., 2003),

increased IN phosphorylation, whereas subsequent treatment with λ-PPase removed the signal. Mutant IN(S57A) was not recognized by the anti-P-IN antibody even after cell treatment with PMA. The results so far reported provide a clear indication that JNK phosphorylates IN at position S57. To provide further support to this notion, we tested whether IN could also be phosphorylated in vitro by JNK. Functionally active histidine-tagged IN (His-IN) (Cereseto er al., 2005) was incubated with commercial recombinant JNK1 and then probed for phosphorylation using the anti-P-IN antibody. Wild type IN scored clearly positive in this in vitro kinase assay, while no phosphorylation could be detected for the IN(S57A) mutant. Phosphorylation was strictly dependent on the addition of ATP and Mg^{2+} to the kinase incubation buffer, thus providing further support to the specificity of the reaction (**Figure 9b**).

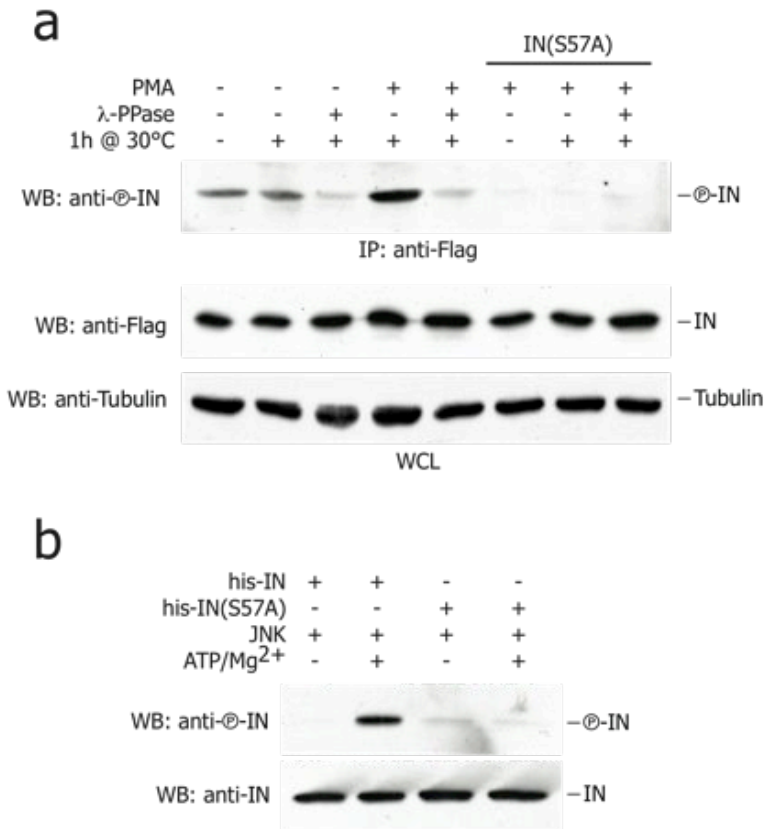


Figure 9a,b

(a) Our affinity purified anti-P-IN antibody detects wild type but not mutated S57A IN. Specificity of the antibody was confirmed upon Phorbol myristate acetate (PMA) induction and/or λ-phosphatase treatment of HEK 293T cells transfected with Flag-IN (upper panel). The levels of transfected IN were assayed by anti-Flag immunoblot (medium panel), while total protein levels were analyzed with anti-Tubulin Western Blot (lower panel). **(b)** wt IN and S57A mutant, carrying a histidine tag, were incubated with recombinant JNK1 with or without ATP and Mg^{2+}. The reaction mixture was resolved by SDS-PAGE and analyzed by Western-Blotting using a polyclonal anti-phosphorylated IN antibody (upper panel). The same membrane was analyzed with anti-IN antiserum for total IN amounts (lower panel).

To further explore IN phosphorylation by JNK in vivo, cells transfected with wild type IN were treated with the different MAPK inhibitors PD98059 (ERK1/2), SP600125 (JNK) and SB203580 (p38), and IN phosphorylation was then tested using the anti-P-IN antibody on immunoprecipitated IN. In the same cell extracts, the total levels of IN were also measured by direct Western Blotting. Phosphorylation of IN was found to be almost completely abolished using the JNK inhibitor, while was even increased upon p38 inhibition (~1.5 fold); (cfr. **Figure 10a** for a representative blot and **Figure 10b** for quantification of the results of three different experiments). Of notice, the JNK and p38 inhibitors also exerted a significant effect on the total levels of IN in the cells, as quantified in **Figure 10c**. In particular, the total levels of IN were reduced of ~5 folds in cells treated with the JNK inhibitor, while they were even slightly increased after inhibition of p38. Taken together, these results provide evidence that HIV-1 is phosphorylated inside the cells on S57 and that JNK is a cellular kinase responsible for this modification.

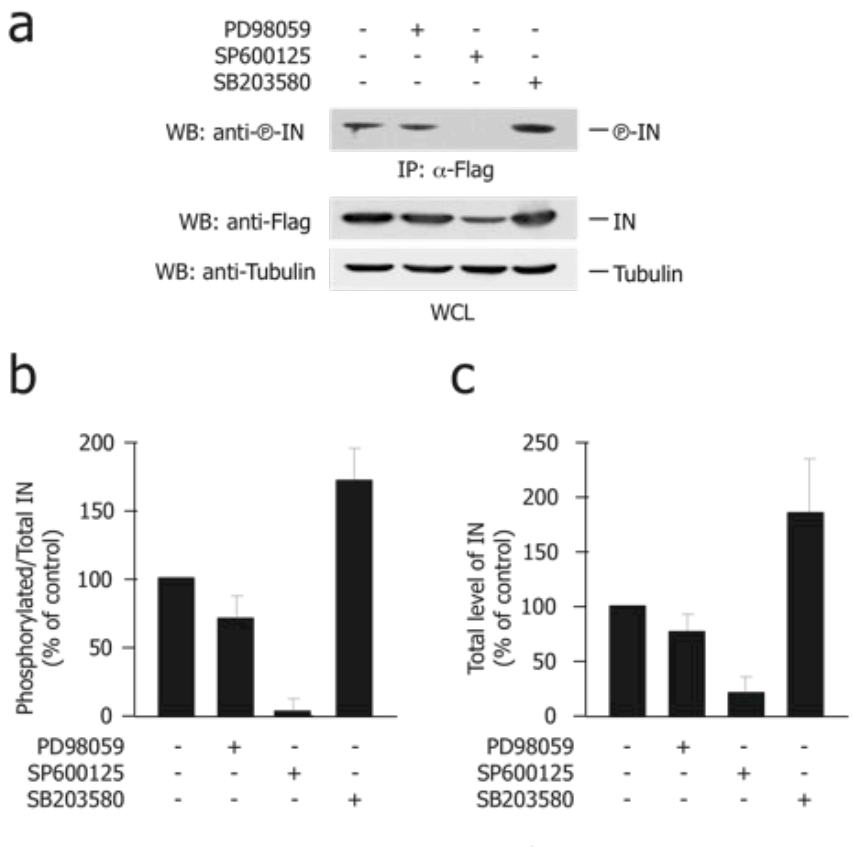


Figure 10a,b,c

(a) Inhibition of JNK kinase activity completely abolishes IN phosphorylation. Transfected Flag-IN was immunoprecipitated with anti-Flag antibody from HEK 293T cells, treated with PD98059 (Erk1/2 inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor) and followed by anti-P-IN immunoblot. Levels of Flag-IN in the whole cell lysate where checked by immunoblot with anti-Flag antibody or with anti-tubulin antibody for total protein levels. **(b)** The graph shows the percentage of phosphorylated IN protein remaining after the cell treatment with the MAPK inhibitors. Samples are calculated with respect to control treated with DMSO only (mean ±sd of at least three experiments). **(c)** Graph shows the percentage of total IN protein respect to DMSO treated cells (n=3).

Since it is known that JNK interacts with its substrates through its interaction domain, we decided to explore whether IN might bind endogenous JNK. To this aim we transfected HEK 293T cells with a Flag-IN and a Flag-Luc as a control. The protein lysates were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblot anti JNK1. As shown in **Figure 11,** we found that IN interacts with both the p46 and p52 isoforms of JNK1.

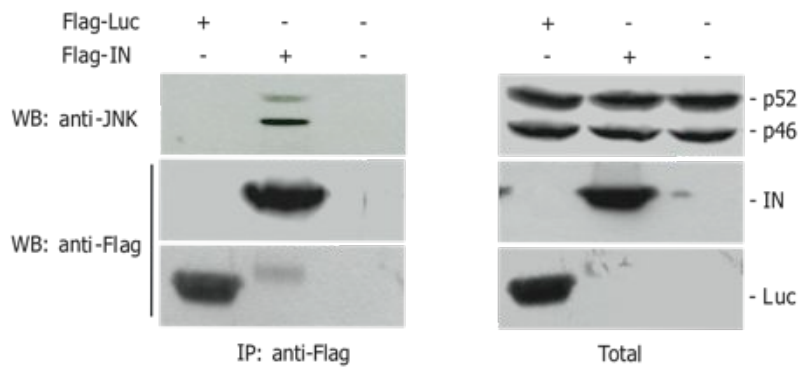


Figure 11

IN interacts with JNK *in vivo*. Extracts from HEK 293T cells transfected with Flag-IN or Flag-Luc as a control were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-JNK1 and anti-Flag antibodies (left panel). The same lysates were run on an SDS-PAGE gel and immunoblotted with either anti-JNK or anti-Flag antibodies (right panel).

To map the JNK interacting domain of IN, we purified from bacteria the single domains of IN and the full-length protein as GST fusion proteins. The single domains were incubated with an *in vitro* translated ^{35}S JNK1 p46.

We found that GST-IN interacts with JNK also in vitro and that this interaction is mediated by the core domain of IN (**Figure 12**).

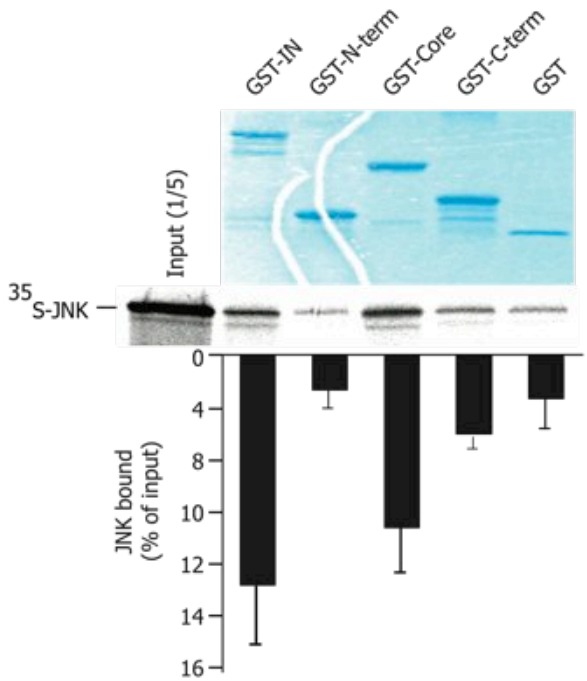


Figure 12

IN interacts with JNK1 in vitro through its Core domain. Radiolabeled ^{35}S-JNK p46 was incubated with IN full length, N-terminal domain, Core domain and C-terminal domain. The gel was exposed to Cyclone screen. Coomassie blue staining of purified GST proteins is shown. The graphs show the amounts of bound proteins as percentages of the input, radiolabeled protein. The quantification shows mean±sd of three independent binding experiments.

We previously reported that HIV-1 IN interacts with the human acetylatransferase p300 and is acetylated on three lysines (positions 264, 266, 273) in the C-terminal domain (Cereseto et al., 2005). We therefore investigated the possibility that these two modifications may crosstalk. To

explore this hypothesis, we first performed a co-immunoprecipitation experiment to verify whether the mutation at position 57 might affect IN binding to p300. As it shown in **Figure 13a,** the IN (S57A) mutant interacted with p300 as well as the wild type protein. Moreover this mutant was still acetylated and the levels of acetylation increase when p300 was overexpressed **Figure 13b**. We finally asked whether acetylation of lysines 264, 266 and 273 was necessary for the phosphorylation of serine 57. To answer this question, we checked the levels of phosphorylation of the mutant IN(K3R), in which lysines 264, 266, 273 were substituted with arginines using the anti-P-IN antibody. The Western Blot analysis in **Figure 13c** clearly shows that the IN(K3R) mutant was phosphorylated at the same extent as the wild type protein. Taken together, these experiments indicate that there is no evident crosstalk between the two post-translational modifications.

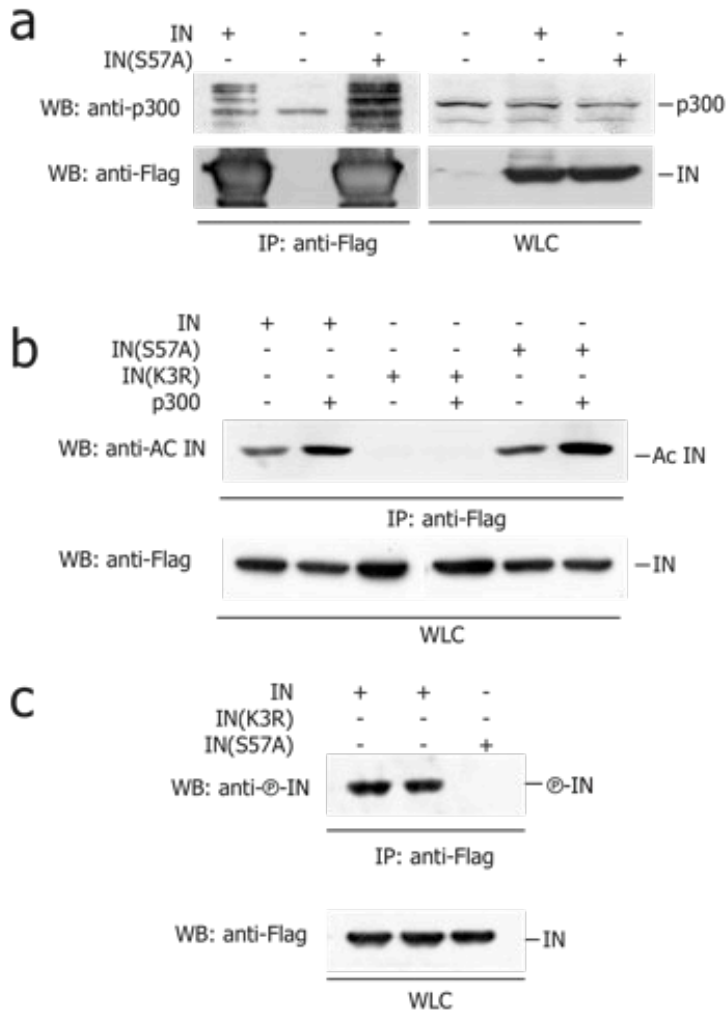


Figure 13a,b,c

(a) IN (S57A) mutant interacts with p300 *in vivo*. Extracts from HEK 293T cells transfected with Flag-IN, Flag-IN (S57A) or with an empty vector as a control were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-p300 and anti-Flag antibodies (left panel). The same lysates were run on an SDS-PAGE gel and immunoblotted with either an anti-p300 or an anti-Flag antibody (right panel). **(b)** IN(S57A) is still acetylated on lysines 264, 266, and 273. HEK 293T cells were transfected with Flag-IN, Flag-IN(K3R), Flag-IN (S57A) and with or without p300. The whole cell lysates were immunoprecipitated with anti-Flag antibody and

immunoblotted with anti-acetylated IN. The same lysates were run on SDS-Page and immunoblotted with anti-Flag antibody to verify protein expression levels. **(c)** Extracts prepared from HEK 293T cells transfected with Flag-IN, Flag-IN(K3R) mutant and Flag-IN (S57A) mutant were subjected to immunoprecipitation with anti-Flag antibody and immunoblotting with anti-P-IN. Protein expression levels were verified by western-blot on total cell lysates with anti-Flag antibody.

3. The cellular prolyl-isomerase Pin1 interacts with HIV-1 IN depending on JNK-mediated phosphorylation

Next we wondered whether mutation of S57, the residue essential for IN phosphorylation, might affect the catalytic activity of the protein. This was assessed by the strand-transfer assay, which evaluates the capacity of IN to produce DNA fragments of different sizes as a result of multiple integration events, when a short oligonucleotide is used as a substrate. However we found that the recombinant IN(S57A) mutant was enzymatically as active as its wt counterpart (**Figure 14**).

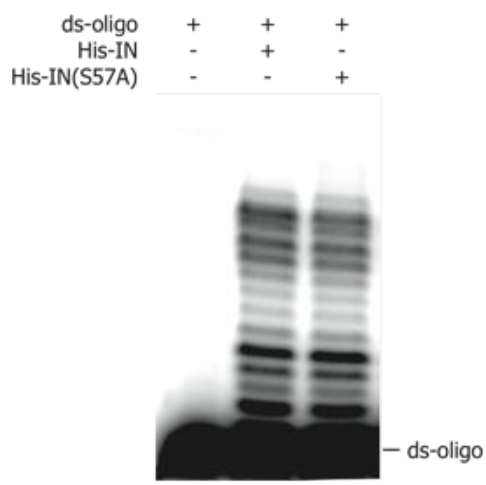


Figure 14

In vitro strand transfer activity of His-IN wt or His-IN (S57A). In the first lane the substrate without His-IN was loaded as a control.

Proteins that are phosphorylated on Ser-Pro motif represent potential targets for the prolyl-isomerase Pin1, an enzyme that acts to change the conformation of its substrates through the isomerization of the peptidyl-prolyl bound (Lu et al., 2007; Lu et al., 2002). Phosphorylated serine 57 of

IN, followed by a proline, represents a potential target for Pin1-mediated isomerization. The possibility that IN and Pin1 might interact was explored by a modified pull-down assay in which IN, produced and phosphorylated in HEK 293T cells, was incubated with GST-Pin1. IN was readily detected bound to Pin1 but not to the control beads. Of interest, binding was dependent on IN phosphorylation, since incubation of the IN-containing cell lysate with calf intestinal phosphatase (CIP) completely abrogated binding (**Figure 15a**). To further demonstrate that phosphorylation of IN by JNK is a prerequisite for Pin1 binding, cell lysates obtained from cells expressing IN and treated with the JNK inhibitor SP600125 were subjected to Pin1 pull-down. As shown in **Figure 15b**, this treatment greatly impaired binding of IN to GST-Pin1 beads. The specificity of this interaction was further confirmed by incubating GST-Pin1 with the mutant IN(S75A), which scored negative for binding (**Figure 15c**). Next we probed binding of IN to two Pin1 mutants, namely Pin1(Y23A), harbouring a substitution of tyrosine 23 to alanine in the WW domain of the protein, that impairs binding to its phoshorylated targets, and Pin1(C113A), in which cysteine 113 in the catalytic domain is converted to alanine; the latter mutant still recognizes its substrates, but its enzymatic activity is abolished (Lu et al., 1999; Zhou et al., 2000). Pin1(Y23A) resulted incapable of binding to IN, while binding was still preserved with mutant Pin1(C113A) (**Figure 15d**).

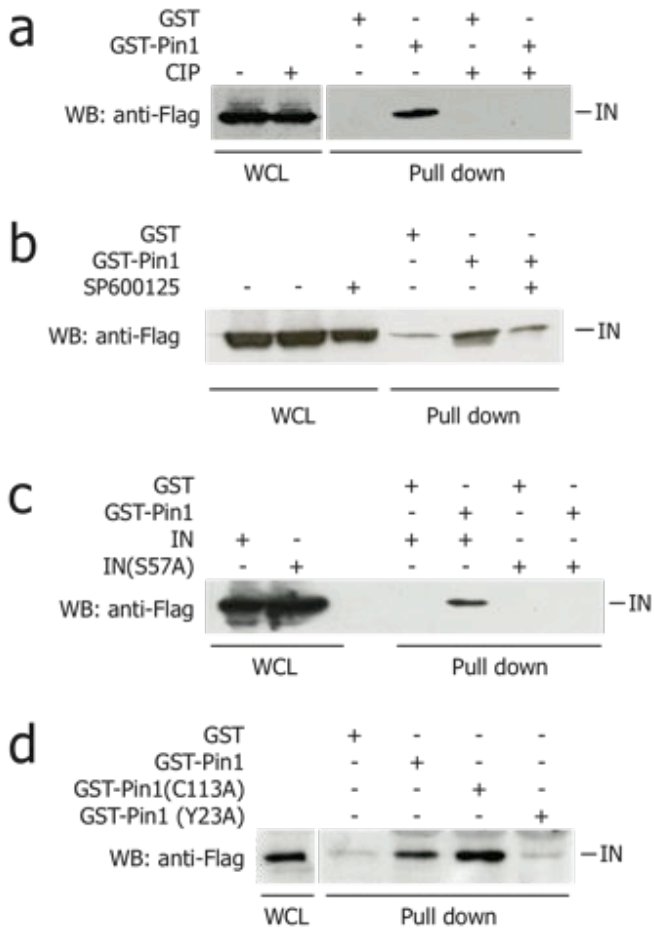


Figure 15a,b,c,d

(a) Pin1 binds HIV-1 IN in a phosphorylation-dependent manner. Cell extracts of HEK 293T cells transfected with Flag-IN and treated or not with Calf Intestinal Phosphatase (CIP) were incubated with GST or GST-Pin1. **(b)** Binding of Pin1 to IN depends on the catalytic action of JNK. Lysates of HEK 293T cells transfected with Flag-IN were treated or not with SP600125 and subjected to GST or GST-Pin1 pull down followed by immunoblot analysis with anti-Flag antibody. **(c)** Integrity of S57 is essential for binding of IN to Pin1. Lysates of HEK 293T cells expressing either Flag-IN or Flag-IN(S57A) were probed for binding to GST or GST-Pin1. **(d)** A wild type WW domain of Pin1 is required for its binding to IN. Lysates of HEK 293T cells

overexpressing Flag-IN were probed for binding to GST, GST-Pin1 wt, GST-Pin1(Y23A) mutant and GST-Pin1(C113A) mutant.

To prove that IN also interacts with endogenous Pin1 *in vivo*, extracts from IN-expressing cells were immunoprecipitated with an anti-Pin1 antibody; in the immunoprecipitate, wild type IN was readily detected. In contrast, binding was almost abolished when using the IN(S57A) mutant, further emphasizing the importance of this phosphorylated residue for the interaction (**Figure 16a**). Finally, we wanted to investigate whether the interaction of IN to Pin1 might determine a conformational change of IN. For this purpose, ^{35}S-labeled, histidine-tagged IN, purified after *in vitro* translation from a reticulocyte lysate using a nickel column was incubated with recombinant Pin1 or with the catalytically inactive mutant Pin1(C113A) prior to the addition of subtilisin, a protease specifically sensitive to substrate conformation (Stukenberg et al., 2001). As already demonstrated for other Pin1 substrates (Zita Moretto et al., 2007; Mantovani et al., 2004; Zacchi et al., 2002), Pin1 significantly protected IN from proteolytic degradation, while the Pin1(C113A), despite binding IN, was completely ineffective (**Figure 16b**). Collectively, these results indicate that IN, phosphorylated on S57 by JNK, is a substrate for cellular Pin1 binding and enzymatic activity.

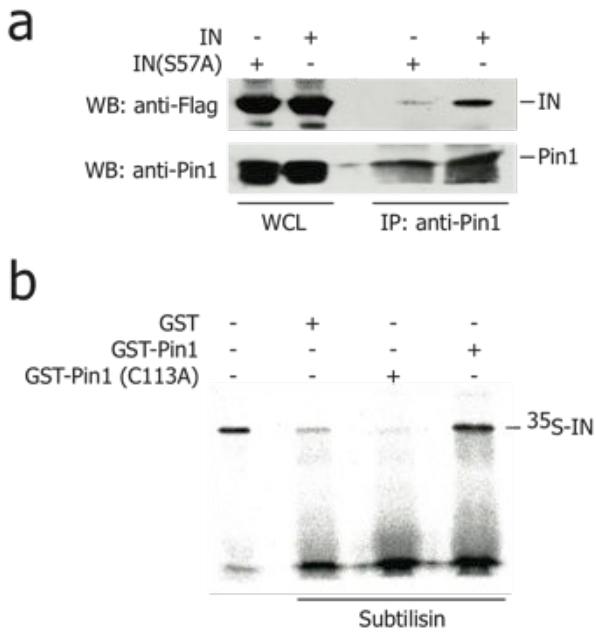


Figure 16a,b

(a) Pin1 binds to HIV-1 IN *in vivo*. Lysates of HEK 293T cells transiently expressing Flag-IN or Flag-IN(S57A), were immunoprecipitated with anti-Pin1 antibody and analyzed by Western Blot with anti-Flag antibody. The same extracts were analyzed for proteins expression levels either with anti-Pin1 and anti-Flag antibody (lower panel). **(b)** Wild type Pin1 can isomerize HIV-1 IN. ^{35}S- histidine IN was purified on a nickel column and incubated with GST, GST-Pin1 and GST- Pin1 (C113A), followed by incubation with subtilisin. The gel was resolved by SDS-PAGE and visualized on a PhosphoImager.

4. Pin1 regulates HIV-1 IN stability

It has been demonstrated that the conformational changes catalysed by Pin1 affect the stability of several of its substrates (see refs. Ryo et al., 2003; Ryo et al., 200 and citations therein). To understand whether this might also be the case for IN, HeLa cells expressing Flag-IN were treated with cyclohexamide (CHX) to block protein synthesis either in the presence or absence of PPIase-parvulin inhibitor (Pib), a compound specifically inhibiting the enzymatic activity of Pin1 (Rustighi et al., 2009; Uchida et al., 2003). In the absence of treatment the half-life of IN was <80 min (**Figure 17a**, gels in the left panel and quantification in the right panel). Remarkably, cell treatment with Pib determined a marked decrease in the total levels of IN at time 0 of CHX treatment (<4 fold of the wt) and in the stability of the protein afterwords. In sharp contrast, the levels of the IN(S75A) mutant were already highly reduced in the absence of CHX and remained unmodified after treatment with Pib (**Figure 17b**).

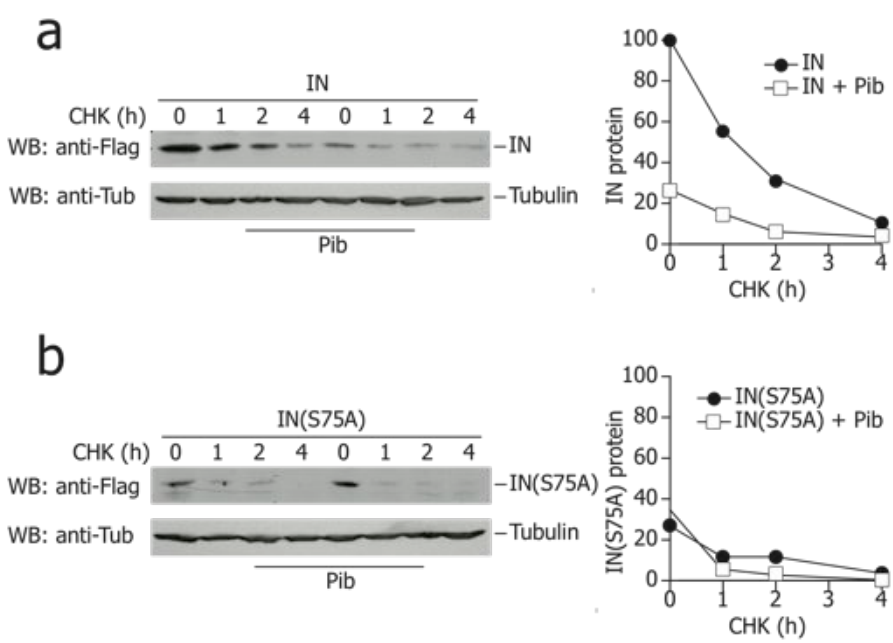


Figure 17a,b

(a) Pin1 inhibition affects IN stability, but has no effect on IN(S57A) mutant. HeLa cells were transfected with Flag-IN or Flag IN(S57A) **(b)** and treated with Pin1 inhibitor (3 μM) or left untreated, after 17 hours CHX was added to block protein synthesis, cells were harvested at the indicated time points. The amount of Flag-IN was analyzed by Western Blot with anti-Flag antibody, while total protein levels were controlled by immunoblot with anti-Tubulin antibody. The results are represented in the graphs (right panels a,b) where the amount of IN at time 0 was set as 100%.

Stability of HIV-1 IN is known to be regulated by the ubiquitin-proteasome pathway (Llano et al., 2004; Mousnier et al., 2007; Emiliani et al, 2005). Since the results so far obtained suggested that either mutation of S57 or cell treatment with Pib affected stability of the protein, we assessed the levels of wild type IN and IN(S57A), after treatment with CHX for 2 hours, in the presence of MG132, a well known inhibitor of the proteasome. As in the previous experiments, the IN(S57A) mutant proved to be less abundant and less stable than the wild type protein (**Figure 18a**), while cell treatment with MG132 determined a marked increase of both wt IN and IN(S57A) and, most notably, no significant difference in the overall levels of the two proteins. These observations clearly indicate that the IN(S57A) mutant is degraded to a higher extent than the wild type protein.

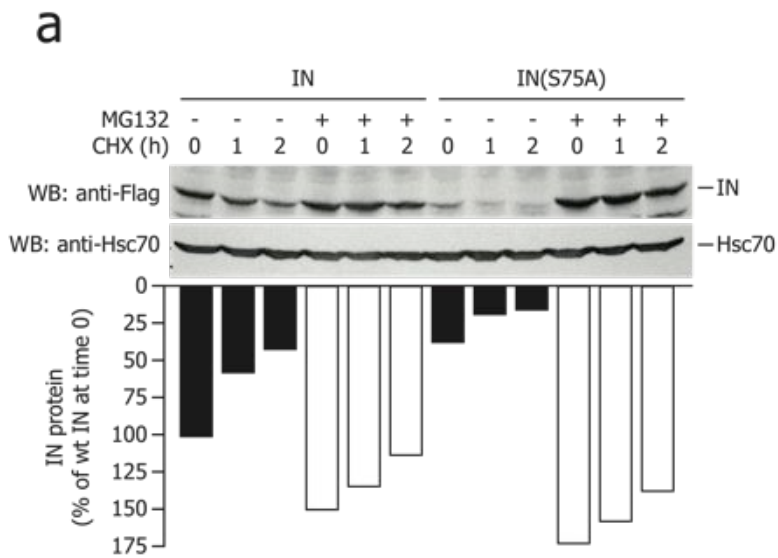


Figure 18a

(a) IN(S57A) is less stable than the wild type protein. HeLa cells ectopically expressing Flag-IN and Flag-IN(S57A) were treated or not with the proteosome inhibitor MG132 and assayed for stability with CHX. Cell lysates were analyzed by Western Blot with anti-Flag or anti-Tubulin antibodies and the results were quantified densitometrically. The graph below shows samples treated or not with MG132 (white and black bars respectively): protein levels were calculated with respect to the amount of IN wt at time 0.

The differential degradation of wt IN and IN(S57A) was further explored by analyzing the ubiquitination levels of the two proteins. For this purpose, HeLa cells were transfected with plasmids expressing either of the two proteins together with increasing amounts of a plasmid coding for HA-ubiquitin, followed by recovery of the IN ubiquitinated forms on anti-Flag beads and immunoblotting with an anti-HA antibody; total protein levels were controlled with anti-Flag antibody. Consistent with the observation that the IN(S57A) mutant is less stable than the wild type protein, it also proved to be more ubiquitinated (gels in **Figure 19a** and quantification of

the ubiquitinated forms of IN in **Figure 19b**). Collectively, these results indicate that the ultimate effect of JNK-phosphorylation followed by Pin1 isomerization of IN is to substantially increase the stability of the protein by preventing its proteasome-mediated degradation.

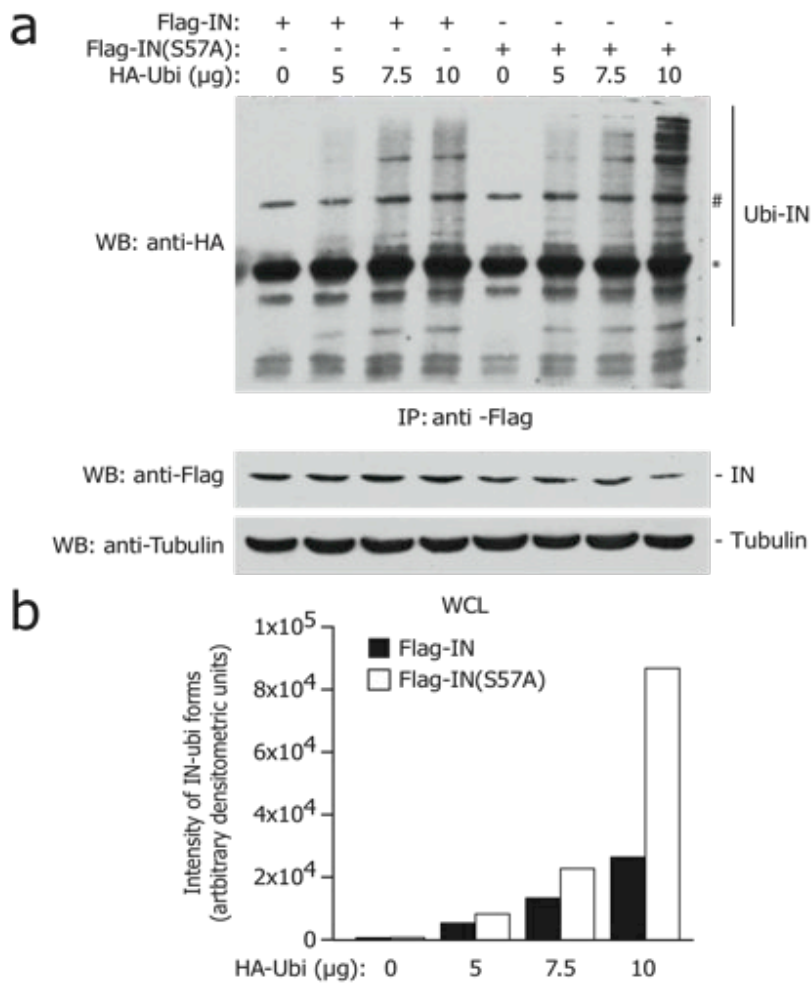


Figure 19a,b

(a) The IN(S57A) mutant is more ubiquitinated than IN wt. HeLa cells were transfected with Flag-IN or Flag-IN(S57A) and HA-Ubi (5, 7.5 and 10 μg), and treated with MG132 or DMSO prior to lysis. Equal amounts of cell lysates were immunoprecipitated using anti-Flag antibody. The levels of

ubiquitination were assayed using anti-HA antibody, cell lysates were tested for IN levels with anti-Flag antibody and for total protein levels with anti-tubulin antibody (*IgGH, #IgG). **(b)** The histogram shows the amounts of ubiquitinated wt IN (black bars) and mutant IN(S57A) (white bars).

5. Inhibition of Pin1 activity and mutation of IN serine 57 impair HIV-1 integration

Finally, we wanted to ascertain what the biological significance of the observed Pin1 modification might be in the context of HIV-1 infection. A first experiment was performed by infecting SupT1 T-cells with HIV-1_{BRU}-Flag-IN viral clone after treatment with the Pin1 inhibitor. Under these conditions, we found that the levels of integrated HIV-1 DNA detected in the cells at 24 hours after infection by quantitative Alu-PCR was remarkably diminished (<10% of control infected cells; **Figure 20a**). Additionally, we also found that silencing of Pin1, with a Pin1-specific siRNA, in HOS $CCR5^+$ $CD4^+$ cells markedly impaired cell infection with a HIV-1_{BAL}pNL4-luciferase virus while transfection with a RISC-free siRNA control has no effect on Luc activity (**Figure 20b**).

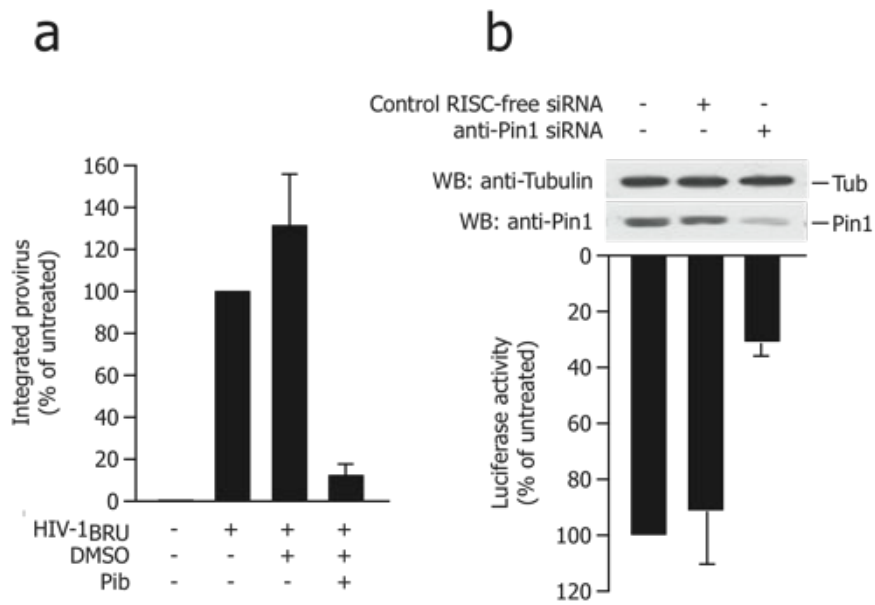


Figure 20a,b

(a) Pin1 activity is necessary for HIV-1 integration. SupT1 cells were treated with 1μM Pin Inhibitor (Pib) 15 hours prior to infection with HIV-1_{BRU} virus; real time Alu-PCR was performed on total genomic DNA extracted 24 hours post infection. The graph summarizes the results of three independent experiments. **(b)** Silencing of Pin1 negatively affects HIV-1 infectivity. Anti-Pin1 siRNA or control RISC-free siRNA were transfected into HOS $CCR5^+$ $CD4^+$ cells, that were subsequently infected with HIV-1_{BAL}pNL4-luciferase viral clone, and luciferase activity (arbitrary units) was measured 72 hours late. Values are mean±sem of at least three different experiments. Pin1 silencing was detected by Western Blotting analysis with anti-Pin1 antibody; total protein levels were verified with anti-Tubulin antibody.

The relevance of the serine 57 for JNK mediated phosphorylation and subsequent Pin-1 mediate isomerization for viral infectivity was assessed by obtaining an HIV-1_{BRU} mutant clone bearing the IN(S57A) substitution. Production and maturation of the mutated virus was as efficient as wt HIV-1_{BRU}-Flag-IN, as concluded by the levels of IN and matrix proteins in the

virions (representative experiment shown in **Figure 21a**). However, we found that the HIV-1_{BRU} IN(S57A) virus was remarkably less infectious than wild type HIV-1_{BRU}, as detected by RT activity assays on the supernatants of SupT1 T cells infected with the same amount of each virus and monitored over a 9 day period (**Figure 21b**).

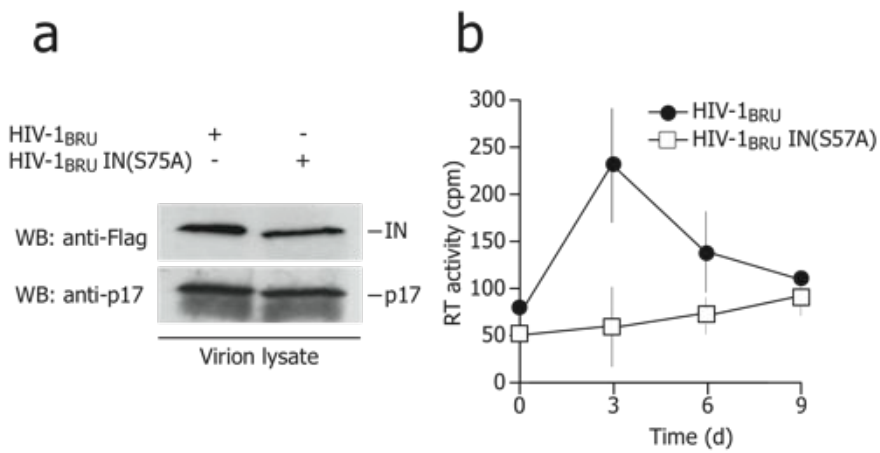


Figure 21a,b

(a) HIV-1_{BRU} wt and HIV-1_{BRU} IN(S57A) mutant produce comparable amounts of viral proteins. Supernatants of HEK 293T cells transfected with HIV-1_{BRU} wt or HIV-1_{BRU} IN(S57A) mutant, containing viral particles, were assayed by WB using either anti-Flag (upper panel) or an anti-MA antibody (lower panel). **(b)** Integrity of Ser57 in HIV-1 IN is indispensable for efficient HIV-1 infectivity. SupT1 cells were infected with the same amounts of wild type HIV-1_{BRU} and HIV-1_{BRU} IN(S57A) viruses. Replication kinetics were monitored by RT activity analysis at the indicated time points after infection.

Of notice, when genomic DNA was extracted from the infected cells at 1 day after infection, the levels integrated DNA, as detected by quantitative Alu-PCR, were markedly decreased (>5 fold) in cells infected with HIV-1_{BRU} IN(S57A); no integration by this assay was detected when using an VSVG-pseudotyped HIV-1_{BRU} mutant, not expressing functional IN (HIV-1_{BRU} ΔIN),

as expected (**Figure 22a**). Finally, we also monitored the extent of viral replication of the wt virus and of the HIV-1_{BRU} IN(S57A) mutant in primary $CD4^+$ T cells, purified from healthy donors and stimulated with PHA/IL-2 prior to infection, over a period of 15 days. As shown in the graph presented in **Figure 22b**, replication of the wt virus peaked at day 3 after infection, while replication of the mutant remained severely impaired throughout the whole observation period. Taken together, these results are consistent with the conclusion that the modification introduced by Pin1 on phosphorylated IN is essential for efficient HIV-1 replication.

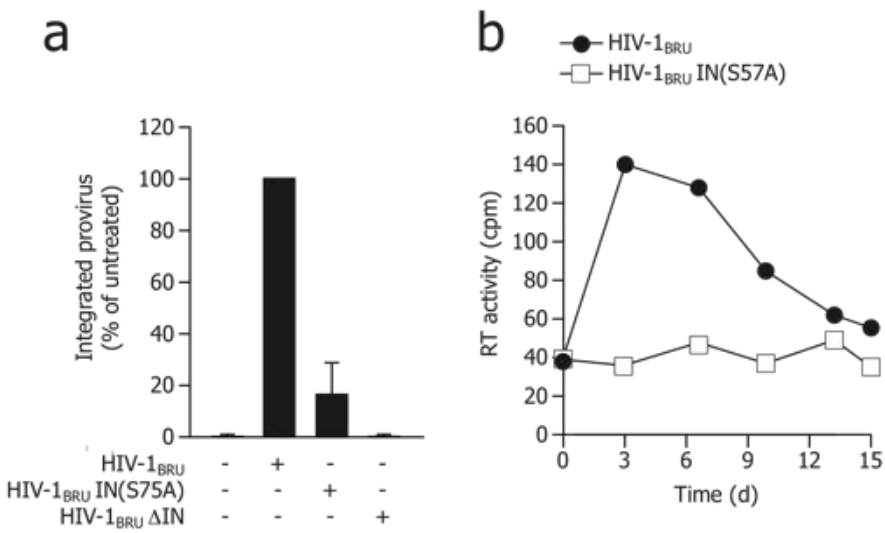


Figure 22a,b

(a) Integration of an HIV-1 clone containing mutant IN(S57A) is impaired. SupT1 cells infected with equal amounts of HIV-1_{BRU} wt, HIV-1_{BRU} IN(S57A) or with VSVG-pseudotyped HIV-1 ΔIN virus as a control, were assayed for proviral integration by Real Time Alu PCR on total genomic DNA extracted 24 hours post infection. The graph summarizes the results of three independent experiments. **(b)** Ser57 in HIV-1 IN is indispensable for efficient infectivity of primary $CD4^+$ T cells. Replication kinetics of wild type HIV-1_{Bru} and HIV-1_{BRU} IN(S57A) mutant in stimulated primary $CD4^+$ T cells was assessed by measuring RT activity of the supernatants in a 2-week periods.

Discussion

One of the peculiar characteristics of the HIV-1 life cycle is that viral replication is efficient in activated peripheral blood T cells, while resting cells are resistant to infection. In these cells, the predominant form of the HIV-1 DNA is full-length, linear, and, most remarkably, unintegrated (Bukrinsky et al., 1991; Chun et al., 1997). It is known that a potent block of reverse transcription in resting $CD4^+$ T cells exists, however the Siliciano and Malim groups demonstrated that RT in resting $CD4^+$ T cells was completed despite a delayed kinetics (Zhou et al., 2005). These findings suggest the presence, in resting T lymphocytes, of further blocks in HIV-1 infection.

In this thesis we demonstrate that efficient integration of the HIV-1 provirus in primary $CD4^+$ T cells requires the concerted functions of the cellular kinase JNK and the prolyl-isomerase Pin1 and that these functions essentially restrict viral infection to activated cells. More specifically, we show that the HIV-1 IN is phosphorylated by JNK and that this modification is a prerequisite for its isomerization by Pin1. Both post-translational modifications occur sequentially on IN S57. These concerted modifications increase IN stability and are a requisite for efficient integration and infection. JNK protein kinases are essential for normal T-cell function, and mouse resting T cells do not express JNK unless stimulated with anti-CD3 and anti-CD28 antibodies or by prolonged (at least 24 hours) stimulation with PMA and ionomycin (Weiss et al., 2000). we confirm that, also in primary human $CD4^+$ T cells, JNKs are expressed only after prolonged stimulation with IL-2 and PHA and correlate this kinetics with the susceptibility of T-cells to HIV-1 infection. In essence, we found that one of the essential blocks that restricts viral integration, and thus replication, in resting T cells is the absence of JNK activity and the consequent lack of

Pin-1-mediated IN isomerization, followed by IN degradation and inability of the viral cDNA to become integrated.

A role for JNK in determining permissivity to HIV-1 infection

HIV-1 replication is greatly influenced by the activation status of the target cell. While activated $CD4^+$ T lymphocytes are permissive to HIV-1 infection, resting T cells are highly resistant. HIV-1 can efficiently enter into resting cells, however no progeny is produced. A sustained stimulation with PHA, IL-2 or ionomycin is required for an efficient infection (Oswald-Richter et al., 2004). To further confirm this notion we performed a time course stimulation of primary $CD4^+$ T cells with PHA/IL-2 followed by infection with a VSVG-Luc virus. We found that a 48 hour-stimulation was required for efficient expression of the Luc gene. Shorter times of stimulation were not sufficient for the establishment of a productive infection (Figure 1a).

All of the three groups of MAP Kinases, p38 family, Erk family and JNK family, are important in regulating the immune system from its early development and participate in the cascade that triggers lymphocyte activation (Dong et al., 2002). In particular, JNK has been implicated in T-cell activation and IL-2 expression as it was rapidly activated in response to PMA or anti CD3 antibodies in Jurkat cells (Su et al., 1994). JNK protein kinases are essential for normal function of T cells and mice deficient in either JNK1 or JNK2 exhibit severe defects in T cell mediated response (Dong al., 1998; Dong et al., 2000). The cascade of signaling events that JNK kinases trigger need to be strictly controlled to ensure accurate cellular response to specific stimuli (Dong et al., 2001). It has been demonstrated that JNK is implicated in both negative selection of thymocytes, and in T helper cell differentiation (Rincon, et al. 2001; Rincon and Pedraza-Alva 2003). Additionally, JNK expression by $CD4^+$ T cells increases after prolonged (at least 24 hours) stimulation with PMA and ionomycin (Weiss

et al., 2000). By measuring the levels of phosphorylated proteins, we confirmed that all the three groups of MAPK were activated in human primary $CD4^+$ T cells after prolonged stimulation with PHA/IL-2; interestingly, we found that the levels of total JNK - but not of the other kinases - were selectively increased upon prolonged stimulation of $CD4^+$ T cells. The regulation of JNK expression represents an additional mechanism that controls its activity besides phosphorylation by MKK4 and MKK7, and it seems specific for T lymphocytes. Thus, it appears plausible that JNK is not necessary for the rapid activation of T lymphocytes, which requires minutes, however the kinase is most likely induced and activated after T cell stimulation and required for subsequent regulation of T cell function.

Taking into consideration that a prolonged stimulation of $CD4^+$ T cells is required for an efficient infection by HIV-1 and that a sustained stimulation results in an increase of JNK expression besides its activation, we investigated the possibility that there might be a crosstalk between HIV-1 susceptibility and the levels and activity of JNK in $CD4^+$ T lymphocytes. To this aim we purified primary $CD4^+$ T cells and treated these lymphocytes with the three specific inhibitors of JNK, Erk and p38 before infection with a VSVG-Luc virus. We noticed that unstimulated cells were not efficiently infected, as previously reported; more interestingly, by inhibiting the JNK enzymatic activity, we observed a significant decrease in HIV infection.

We then asked which step of HIV-1 infection was affected by JNK activity inhibition. Analysis of the early steps in viral infection led us to conclude that neither viral entry nor reverse transcription were affected. Of notice, we found that, in unstimulated $CD4^+$ T cells, the formation of early reverse transcripts was normal while the formation of late reverse transcripts was highly inefficient as previously reported (Zhou et al., 2005). In 1990, Zack and colleagues proposed that, in resting T cells, reverse transcription is blocked, thus precluding the establishment of productive HIV-1 infection.

Subsequently, the same authors suggested that the low concentration of dNTPs was the limiting factor in a productive infection of resting T lymphocytes (Korin et al., 1999). However, addition of dNTPs, allowing the completion of reverse transcription, did not result in successful HIV-1 infection, suggesting the presence of additional blocks in resting $CD4^+$ T cells. In particular, transition of the cell cycle from G1a to G1b seemed to be necessary for the completion of reverse transcription (Korin and Zack 1998). The Greene's group further confirmed that reverse transcription is a main block of the viral life cycle. In particular, these authors proposed that the inability of HIV-1 to successfully complete reverse transcription in resting $CD4^+$ cells circulating in blood was due to the activity of the restriction factor APOBEC3G. More precisely, in stimulated $CD4^+$ T cells, APOBEC3G exists in a high molecular mass ribonuclear complex that is enzymatically inactive, while a low molecular mass (LMM) form of APOBEC3G present in resting $CD4^+$ T lymphocytes functions as a highly active post-entry restriction factor for HIV (Chiu et al., 2005).

To better understand the fate of HIV-1 genome inside resting T lymphocytes, Siliciano and colleagues monitored the kinetics of HIV-1 decay in these cells. They again found that resistance of $CD4^+$ T cells to HIV-1 infection was due to a slow kinetics of reverse transcription and blocks at subsequent steps (Zhou, et al. 2005). In their work, they also monitored the decay of integration-competent DNA and, interestingly, found that this form of fully reverse transcribed HIV-1 has a half-life of 1 day in resting T cells. The authors proposed that degradation of either viral DNA and/or viral proteins, that form the preintegration, complex would lead to a functional decay of the virus (Zhou, et al. 2005). Additional work performed in 2007 by immunofluorescence experiments aimed at detecting localization of the CA protein of HIV-1 and by FISH analysis on viral cDNA in resting infected cells showed that full length reverse transcribed viral

genome, together with CA, localizes at the centrosomes 4 days post infection (Zamborlini et al., 2007).

Taken together, all the above mentioned findings suggest that unstimulated T lymphocytes may restrict HIV-1 infection at different steps. Reverse Transcription seems to be the most affected step; however a molecular explanation is still lacking why the full length cDNAs that are formed in resting T-cell despite the reverse transcription slow down do not get eventually integrated into the genome. In this respect, in 1990 Stevenson and colleagues showed and that resting T cells showed a block at the level of integration and that viral DNA was maintained extra chromosomally (Stevenson et al., 1990). Consistent with this observation, we investigated the integration step the conditions of JNK inhibition and observed a decrease in the levels of integrated provirus.

In the context of viral replication cycle HIV-1 IN regulates both nuclear entry of the PIC and integration of the viral cDNA into the host genome (Suzuki and Craigie 2007). It is known that, besides efficient integration, into the host genome, viral cDNA can undergo aberrant modifications in the nucleus of infected cells, with 2LTRs circles being the most common products (Coffin et al., 1997). IN mutations that affect enzymatic activity but not PIC entry into the nucleus are kwnown to cause an increase in the formation of these aberrant products (Cereseto at al., 2005; Maroun et al., 2006; Svarovskaia et al., 2004). Real Time analysis of the formation of 2LTR circles in cells in which JNK was inhibited revealed that the formation of this particular species of viral cDNA was affected, thus indirectly indicating that the integration defect could at least in part be ascribed to the diminished nuclear import of PICs. Of notice, in unstimualated cells, 2LTR circles were not detected at all. This finding is in agreement with findings published by Wu and Marsh in 2008, in which resting T cells are reported to show an impairment in PIC nuclear entry. The authors of this

work ascribe this block to an effect on the static cortical actin of quiescent T lymphocytes and propose that signaling downstream of CXCR4 activates cofilin, a cellular factor critical for actin dynamics and viral nuclear migration. In the absence of CXCR4 activation, there is no downstream signaling and HIV-1 is unable to reach the nucleus (Yoder et al., 2008). Interestingly, JNK activity is also triggered after CXCR4 binding to its targets (Tokiwa et al., 1996).

HIV-1 IN is a substrate of JNK activity

Here we demonstrated for the first time that IN is also modified by phosphorylation on serine 57 in the catalytic/core domain of the protein and that JNK is the kinase responsible for this modification. These data were further confirmed by using the MAPK inhibitors (SP600125, PD98059 and SB203580). Chemical inhibition of Erk had no effect on IN phosphorylation and inhibition of p38 caused a slight increase, while block of JNK activity completely abolished IN phosphorylation. Interestingly, when JNK was inhibited the total levels of IN decreased (Figure 10a middle panel), thus suggesting a role for JNK in regulating IN stability.

It has been previously reported that JNK mediated phosphorylation plays a role in regulating the half life of crucial JNK targets, such as p53, ATF2 and SP1, by preventing their ubiquitination and subsequent proteasomal degradation (Fuchs et al., 1998; Fuchs 2000; Chuang et al., 2008). JNK is able to bind its targets through a so called JNK binding domain or d-domain (Adler et al., 1994; Dai et al., 1995). We therefore investigated whether JNK also binds IN. A co-immunoprecipitation assay confirmed that IN was indeed able to interact with endogenous JNK in vivo. Moreover, we discovered that the core domain of IN was the region involved in the interaction with JNK.

We have previously reported that IN was also acetylated in lysines 264, 266, 273 by p300 and that these two proteins interacted (Cereseto et al., 2005). A large number of proteins, such as CDC6, STAT1 and histone H3, are post-translationally modified at multiple residues by kinases and acetyltransferases; in several instances, these modifications are interconnected (Paolinelli et al., 2009; Kramer et al., 2009; Edmondson et al., 2002). We then wondered whether there is a crosstalk between phosphorylation and acetylation of IN. Initially, we investigated the possibility that IN phosphorylation might affect its binding to p300, however we found that IN(S57A) bound p300 as well as the wt protein. Next we tried to correlate these two modifications of IN by investigating the acetylation status of the S57A mutant, however we found that this mutant was acetylated to the similar extent as the wt protein. In addtion, we also demonstrated that the triple mutant, in which lysines 264, 266 and 273 were substituted with arginines, was still phosphorylated in vivo. These findings suggested that, in the case of IN, acetylation and phosphorylation are two independent, apparently not correlated post-translational modification. How to reconcile these two modifications? One possibility is that they occur in two different cellular compartments such as the cytosol and the nucleus. Another other possibility is that these two events occur at two different time points of infection and that the first modification is not required for the second to occur. In this respect, it might be hypothesized that, after infection, the phosphorylated form of IN is stabilized by isomerization and can reach the nucleus where it becomes acetylated by p300, thus increasing its DNA affinity and enzymatic activity. The IN mutant in Ser 57 is rapidly degraded, as discussed below, and does not reach the nucleus efficienctly. Thus, acetylation is not dependent on phosphorylation, however, in the context of viral replication, the IN(S57A) mutant is not acetylated because it does not reach the right compartment for acetylation. This view predicts that most of the acetylated IN is also

phosphorylated on Ser 57, a prediction that might be experimentally assessed by mass spectrometry experiments.

We also analyzed whether the mutation of Ser 57 might affect the strand transfer activity of IN and found that the enzymatic activity of IN(S57A) was similar to the wt protein. This result was in agreement with the finding that SP600125 caused a reduction, rather than an increase, in 2LTR circles formation, suggesting that JNK inhibition does not affect IN enzymatic activity, but most likely affects a step of IN regulation prior to nuclear entry or impact on the overall levels of IN in the cells after infection.

A careful analysis of the IN proteins revealed that the sequence S-P at positions 57-58 is highly conserved in the various subtypes of HIV-1, as it is in the IN of the Simian Immonudeficiency Virus (SIV) and Equine Immonudeficiency Anemia Virus (EIAV). The IN of the Feline Immonudeficiency Virus (FIV) conserves the proline at position 58 while IN from HIV-2 does contain neither Ser 57 nor Pro 58 (Table I). The observation that these residues are very conserved among HIV-1 subtypes and that they are also present in other lentiviruses further indicates their relevance.

Table I Conservation of the serine-proline sequence (position 57-58 in HIV-1 IN, in red) in the IN proteins of other Lentiviruses

HGQVDCSPGIWQLD	HIV-1 Subtype A
HGQVDCSPGIWQLD	HIV-1 Subtype B
HGQVDCSPGIWQLD	HIV-1 Subtype C
HGQVDCSPGIWQLD	HIV-1 Subtype D
HGQVDCSPGIWQLD	HIV-1 Subtype F
HGQVDCSPGIWQLD	HIV-1 Subtype G
HGQVDCSPGIWQLD	HIV-1 Subtype H
HGQVDCSPGIWQLD	HIV-1 Subtype 0
HGCVNAELGTWQMD	HIV-2 Integrase
HGQVDASPGTWQMD	SIV Integrase
AGCVMRSPNHWQAD	EIAV Integrase
GGQLKIGPGIWQMD	FIV Integrase

A role for the prolyl-isomerase Pin1 in HIV-1 infection

JNKs, like other MAPKs, are Proline directed Ser/Thr protein kinases and the consensus sequence (Pro)-X-Ser/Thr-Pro [(P)-X-S/T-P] is present in c-Jun as the archetypical substrate of JNKs (Bogoyevitch and Kobe 2006). In recent years, several studies on phosphorylated proteins revealed that a key component of signal transduction pathways is the prolyl-isomerase Pin1. This isomerase specifically binds phosphorylated serines/threonines followed by a proline and isomerizes the peptidyl bond between the phosphorylated aminoacid and the proline residue. Conformational changes catalyzed by Pin1 greatly influence the stability, localization and enzymatic function of the protein targets (Lu and Zhou 2007). In our study we present evidence that IN phosphorylated at serine 57 is recognized and isomerized by Pin1. We found that IN and Pin1 interact in a GST pull-down assay and that this interaction was dependent on phosphorylation. We

discovered that JNK inhibition greatly impairs IN binding to Pin1, suggesting that JNK phosphorylation is upstream Pin1 recognition of IN. Moreover, the IN(S57A) mutant does not interact with the prolyl-isomerase, further confirming the importance of the residue at position 57. Finally, we validated the binding between Pin1 and IN by using the Pin1 mutants.

Pin1 consists of two domains: a WW domain, which recognizes the Pin1 targets, and a catalytic domain. IN is not recognized by the Pin1(Y23A) mutant, which contains a mutation in the WW domain, while it is still able to bind the catalytically inactive mutant of Pin1 (C113A) which possesses a wt WW domain. Co-immunoprecipitation experiments corroborate the conclusion that Pin1 and IN also interact also in vivo. It has been previously published by different groups that Pin1-mediated isomerization protects proteins from subtilisin digestion, as a mean to demonstrate that the enzyme induces a conformational change in its targets (Zacchi et al., 2002; Mantovani et al., 2004; Zita et al., 2007). Indeed, we proved that IN was protected by subtilisin activity by co-incubation with Pin1, while IN was degraded when co-incubated with that catalytically inactive mutant Pin1(C113A).

Conformational changes may affect many aspects of protein regulation. Taking into consideration that JNK inhibition decreases the total levels of IN, we investigated the possibility that Pin1, being downstream of JNK action, was also involved in controlling IN stability. We confirmed that Pin1 plays a role in IN stability by using a chemical compound that specifically inhibits Pin1 catalalytic activity (Pib) (Uchida et al., 2003; Rustighi et al., 2009). We found that Pin1 inhibition impaired wt IN stability, while having no significant effect on the IN(S57A), which however was itself intrinsically less stable.

Pin1 is already known to also regulate stability of other proteins, determining stabilization of several of its targets, including NF-kB, p73, Emi1 and b-catenin (Ryo et al., 2003; Mantovani et al., 2004, Bernis et al., 2007; Ryo et al., 2001). Interestingly, it has been recently shown that Pin1 also stabilizes the human T-cell leukemia virus type 1 (HTLV-1) protein Tax (Jeong et al., 2009). Since cellular proteins are mainly degraded by the proteasome, we investigated the possibility that IN was degraded by this pathway. To this aim, we blocked the proteasome system by treating cells with the proteasome inhibitor MG132 and checked for the recovery of IN levels. We found that MG132 treatment led to an increase in the levels of both wt IN and IN(S57A). Polyubiquitination is a well known signal that targets proteins to degradation. Therefore, we investigated the possibility that Ser 57 might be a crucial residue for this modification (Hunter 2007). We found that the IN(S57A) mutant, which is not phosphorylated by JNK and thus cannot be recognized by Pin1, was also more ubiquitinated in vivo, suggesting that phosphorylation and consequential isomerization might prevent IN ubiquitination and subsequent proteasomal degradation. It has been demonstrated that IN is degraded by the ubiquitin-proteasome pathway probably via interaction with the Cul2-based VHL ubiquitin ligase (Mulder and Muesing 2000; Mousnier et al., 2007). Moreover it has been proposed that LEDGF/p75, besides its role in tethering IN to chromatin, prevents its proteasomal degradation (Llano et al., 2004). However the mechanisms upstream IN degradation are still unclear. Since Pin1 affects on the stability of other targets by preventing or enhancing their interaction with ubiquitin ligase complexes (Ryo et al., 2001; Saitoh et al., 2006), it is plausible that Pin1-mediated isomerisation might either affect binding with Cul2-based VHL ubiquitin ligase or could increase IN affinity for LEDGF/p75.

In the context of viral replication, Pin1 inhibition led to a decrease in HIV-1 integration and infection. We further confirmed the importance of Ser 57 by mutating this residue in the HIV-1_{BRU} viral clone. The mutated virus, despite containing normal levels of IN inside its virions, had impaired capacity of integration in the SupT1 T cell line. This impairment in integration determined the inability to establish a productive infection in primary $CD4^+$ T cells over a period of 15 days.

Several studies have suggested that inhibition of the proteasome increases the production of proviral DNA by blocking the degradation of the pre-integration complexes (Butler et al., 2002; Schwartz et al., 1998). In addition a recent paper by Naldini and colleagues showed that the proteasome also limits lentiviral gene transfer in stem cell (Santoni de Sio et al., 2008). The observation that Pin1 inhibition reduced HIV-1 integration and that substitution of IN Ser57 with Ala in the context of and HIV-1 viral clone greatly impaired viral DNA integration and infection is fully consistent with this notion. This is not the first evidence of Pin1 involvement in regulating the function of viral proteins. It has been demonstrated that Pin1 regulates HTLV-1 Tax half-life by suppressing its ubiquitination and subsequent degradation as well as regulates Tax-induced NF-kB activation (Bernis et al., 2008; Peloponese et al., 2009). A role for Pin1 has also been proposed in the context of HBV-related hepatocellular carcinoma, where Pin1 increases the stability of the oncogenic HBV protein HBx and enhances HBx-mediated transactivation (Pang et al., 2007). Recent work from the Jeang group showed that Pin1 is also indirectly involved in HIV-1 infection. In particular, these authors proposed that Pin1 affects the expression levels of APOBEC3G, which is a well known restriction factor for HIV-1 infection. They demonstrated that Pin1 reduces APOBEC3G expression and its incorporation into new formed virions, thus limiting its antiviral function. In the same paper, these authors also showed that HIV-1 infection can

positively modulate Pin1 activity hence enhancing its pro-viral action (Watashi et al., 2008).

Pin1 activity in stimulated PBMC, which are more susceptible to HIV-1 infection, is known to be increased (Esnault et al., 2006). Intriguingly, in 2006 a paper was published in which Pin1 was correlated to the stability of interferon-regulatory factor 3 (IRF3) and the consequent production of IFNb and establishment of innate immunity in response to either viral or bacterial infection. In particular, the Yamaoka group demonstrated that Pin1 affects IRF3 stability and that exogenous expression of Pin1 increased the production of infectious vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) in the culture supernatants of infected cells (Saitoh et al., 2006). Although no immunological phenotype was described for Pin1-/- mice (Fujimori et al., 1999; Liou et al., 2002), it would be interesting to investigate the involvement of Pin1 in the general suceptibilty to viral infection (Esnault et al., 2008).

Finally, it is worth discussing that, in the past years, it has already been demonstrated that HIV-1 takes advantage of another cellular isomerase, Cyclophylin A (CypA). This peptidyl-prolyl isomerase was found in a yeast two-hybrid screen for HIV-1 CA interactors by Luban and colleagues (Luban et al., 1993). It has been shown that CypA is able to isomerize CA and promotes an early step in HIV-1 infection (Braaten et al., 1996; Braaten et al., 2001; Bosco et al., 2002). The role of CypA catalytic activity on CA is still unknown. Interestingly in non-human primates CypA promotes an anti-HIV-1 restriction activity (Towers et al., 2003; Sayah et al., 2004).

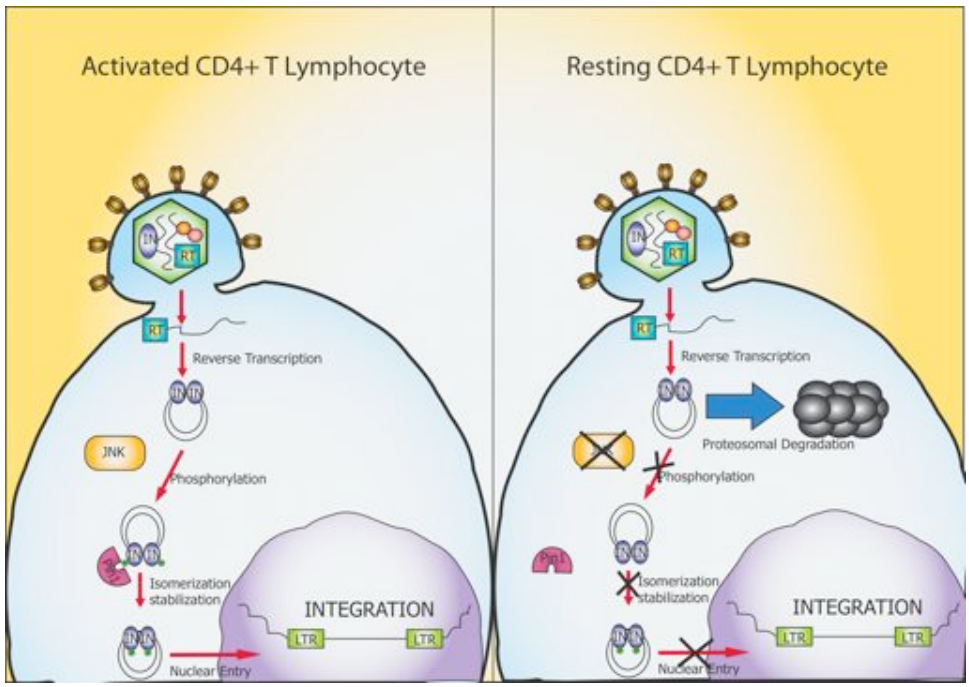
The detected post-translational modifications that regulate IN stability after infection are likely to be essentially involved in the phenomenon known as pre-integration latency, a form of non-productive infection due to the persistence of unintegrated viral genomes. In this respect, it has been

shown a complete activation of T cells is not necessary for the establishment of infection, and that signaling through the CD4 or CXCR4 might be sufficient for infection to be established (Stevenson 2003). Consistent with this notion, recent work by Wu and colleagues has shown that CXCR4 signaling is necessary for the infection of quiescent T cells; if signaling is block, PIC transport to the nucleus is selectively impaired (Yoder et al., 2008) This effect can be ascribed to cofilin, which cannot in turn depolimerize actin. Interestingly, it has also been found that signaling from CXCR4 also leads to JNK activation (Tokiwa et al., 1996), thus suggesting the intriguing possibility that JNK might play a dual role in permitting infection of $CD4^+$ T lymphocytes, on one side by acting on the cofilin/actin pathway while, on the other side, increasing IN stability.

It has been recently demonstrated that, in contrast to $CD4^+$ T cells from peripheral blood, HIV-1 can infect resting $CD4^+$ T cells residing in lymphoid tissues (Eckstein et al., 2001; Kreisberg et al., 2006). Kreisberg and colleagues proposed that the peculiar microenviroment in which these cells reside, guarantees a status of partial activation that allowing efficient infection. In particular, these authors found that endogenous IL-2 and IL-15 played a key role in rendering resident naive $CD4^+$ T cells susceptible to HIV-1 infection. It will be interesting to investigate also JNK status in resident resting T cells from lymphoid tissues, since it is well possible that cytokines produced by stromal cells and other resident cells may be sufficient to increase JNK expression and trigger its activation, thus facilitating HIV-1 infection.

A model to explain the poor susceptibility of resting $CD4^+$ T cells to HIV-1 infection

Taking our data and those from the literature together, we wish to propose a new model explaining the poor susceptibility of resting $CD4^+$ T cells to HIV-1 infection. In resting T cells, reverse transcription proceeds at a slower rate with respect to activated lymphocytes, requiring 2-3 days to reach the peak levels, whereas in activated T cells only 8 hours are needed (Chiu et al. 2005; Zhou et al. 2005). In resting cells, HIV-1 fails to progress quickly to integration even after the completion of fully reverse transcribed viral cDNA (Swiggart et al.,2004). We propose that the viral cDNAs that are formed in resting T cells fail to eventually become integrated due to the lack of IN phosphorylation and the subsequent degradation of this enzyme. In activated $CD4^+$ T cells, JNK is both highly expressed and active, thus allowing an efficient IN phosphorylation. Phosphorylated IN becomes in turn a substrate for Pin1, which binds this viral protein and changes its conformation, thus increasing its half-life and allowing efficient PIC nuclear entry and integration of the HIV-1 cDNA into the host genome. In contrast, in resting T cells, the absence of JNK kinases and the levels of of Pin1 render IN more susceptible to ubiquitination and to consequent proteosomal degradation. The increase rate of IN degradation impairs both nuclear entry of the complete full-length viral cDNA and its integration into the cellular genome. Our findings disclose a new mechanism that could help in understanding the cellular barriers to viral infection and the means that the virus found to avoid them. Moreover, our data might in part explain the resistance of lentiviral gene transfer in resting cells such as non replicating stem cells and the possibility to increase the efficiency of transduction of these cells by inhibiting proteosomal activity or by enhancing JNK function.


Activated CD4+ T Lymphocyte
Resting CD4+ T Lymphocyte
IN
RT
Reverse Transcription
JNK
Phosphorylation
Isomerization
stabilization
INTEGRATION
LTR
Nuclear Entry
Proteosomal Degradation
PIN1

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Publications

Cereseto A, Manganaro L, Gutierrez MI, Terreni M, Fittipaldi A, Lusic M, Marcello A, Giacca M. Acetylation of HIV-1 integrase by p300 regulates viral integration. EMBO J. 2005;24(17):3070-3081 - Paper attached

Vardabasso C, Manganaro L, Lusic M, Marcello A, Giacca M. The histone chaperone protein Nucleosome Assembly Protein-1 (hNAP-1) binds HIV-1 Tat and promotes viral transcription. Retrovirology. 2008;5(1):8 - Paper attached

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