Rview Article
The HIV-1 passage from cytoplasm to nucleus: the process involving a complex exchange between the components of HIV-1 and cellular machinery to access nucleus and successful integration

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Abstract: The human immunodeficiency virus 1 (HIV-1) synthesizes its genomic DNA in cytoplasm as soon as it enters the cell. The newly synthesized DNA remains associated with viral/cellular proteins as a high molecular weight pre-integration complex (PIC), which precludes passive diffusion across intact nuclear membrane. However, HIV-1 successfully overcomes nuclear membrane barrier by actively delivering its DNA into nucleus with the help of host nuclear import machinery. Such ability allows HIV-1 to productively infect non-dividing cells as well as dividing cells at interphase. Further, HIV-1 nuclear import is also found important for the proper integration of viral DNA. Thus, nuclear import plays a crucial role in establishment of infection and disease progression. While several viral components, including matrix, viral protein R, integrase, capsid, and central DNA flap are implicated in HIV-1 nuclear import, their molecular mechanism remains poorly understood. In this review, we will elaborate the role of individual viral factors and some of current insights on their molecular mechanism(s) associated with HIV-1 nuclear import. In addition, we will discuss the importance of nuclear import for subsequent step of viral DNA integration. Hereby we aim to further our understanding on molecular mechanism of HIV-1 nuclear import and its potential usefulness for anti-HIV-1 strategies.

Keywords: HIV-1, nuclear import, integrase, matrix, viral protein R, capsid, importin

Introduction

The human immunodeficiency virus-1 (HIV-1) is a positive sense, single stranded RNA virus in the family Retroviridae, genus *Lentivirus*. HIV-1 infects cells of immune system by targeting surface receptors, CD4 [1] and co-receptors CCR5 or CXCR4 [2]. Soon after entry into the cytoplasm, the viral core undergoes "uncoating", during which most of capsid (CA) sheds off, while nucleocapsid (NC), viral protein R (Vpr), integrase (IN), and a small portion of matrix (MA) are still associated [3-5] (**Figure 1A, B**). As uncoating progresses, the viral genomic RNA reverse transcribes into complementary DNA (cDNA), and newly synthesised viral cDNA remains associated with viral and cellular proteins as a high molecular weight nucleoprotein complex called Pre-integration complex (PIC) (**Figure**

1C). The cDNA as a component of PIC reaches the nucleus through nuclear pore complexes (NPCs) and stably integrates into genomic DNA. The NPCs are the specialized channels of nuclear membrane made up of a group of proteins called nucleophorins (Nups). The NPCs allow movement of various water soluble molecules across nuclear membrane. Depending on the size, the molecules are allowed to pass through the NPC by either simple passive diffusion or active transportation. As the passive diffusion of macromolecules across intact nuclear membrane is not possible, the PIC enters nucleus by active mechanism with the help of host nuclear import machinery [6-11] The nuclear import of proteins in eukaryotic cells is carried out by two distinct pathways, namely the classical and non-classical nuclear import pathways. The classical nuclear import pathway involves the initial rec-
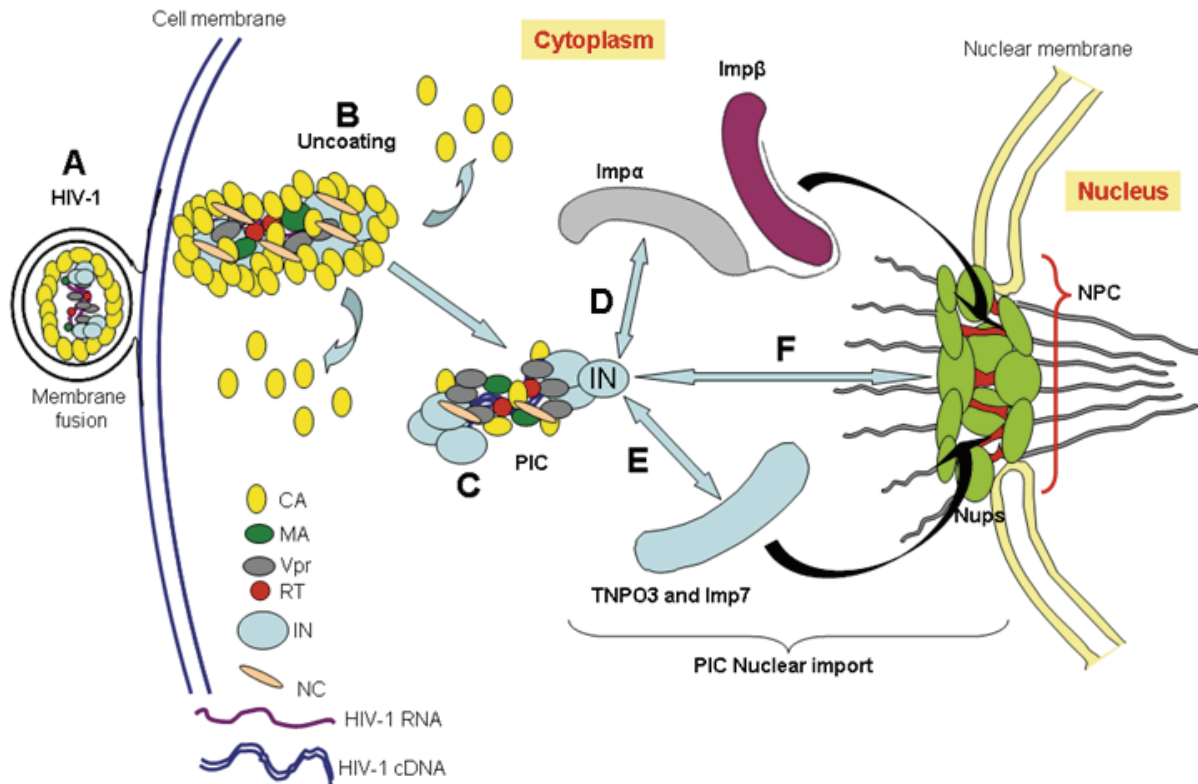
Figure 1. The HIV-1 nuclear import machanisms mediated by IN: (A) Virus entry: Receptor mediated membrane fusion of mature virus particle. (B) Uncoating: Disengagement of CA protein molecules from reversetranscription complex in cytoplasm. (C). Pre-integration complex (PIC): The mature HIV-1 nucleoprotein complex waiting for nuclear import. (D). Impα/β mediated nuclear import: The propsed recrutitment of Impα to PIC by IN and subsequent nuclear import by Impα/β mediated classical nuclear import pathway. (E). TNPO3 or Imp7 mediated nuclear import: The propsed re-crutitment of TNPO3 or Imp7 by IN and subsequent nuclear import by non-classical nuclear import pathway. (F). Di-rect interaction with Nups: The proposed nuclear translocation of PIC by direct interaction with Nups without the re-quirement of soluble factors.

ognition of nuclear localization signal (NLS) con-taining cargo proteins by an adapter protein called Importin (Imp)α, followed by the cargo protein bound Impα is recognized by a soluble import receptor called Impβ. Subsequently, Impβ docks the trimeric complex to NPC via in-teraction with Nups and facilitates the protein nuclear import [12]. In case of non-classical nuclear import, the cargo proteins are subjected to nuclear import by direct interaction with im-portins belongs to Impβ family of import recep-tors without the requirement of Impα adapter proteins. Although underlying mechanism is so far inconclusive, bulk of the studies have impli-cated viral nucleophilic proteins such as IN [13-18], Vpr [19-23] and MA [24] in HIV-1 nuclear import. In addition, the CA protein, HIV-1 central

DNA flap, and cellular tRNA are also suggested for nuclear import [25-30]

The ability to overcome nuclear membrane bar-rier allows HIV-1 to successfully replicate in cells rendered non-dividing in lab conditions by cycle arrest [11] or metabolically active terminally differentiated cells such as macrophages [31-34] and dendritic cells [35, 36]. Conversely, the oncoretroviruses such as murine leukemia virus (MLV) that lacks nuclear import ability depends solely on nuclear membrane dissolution during mitosis to access host cell genomic DNA [37, 38]. Thus, MLV is only able to infect dividing cells, which is again evident from the inefficient transduction of non-dividing cells by onco-retrovirus based vectors [39, 40]. In contrast to

metabolically active terminally differentiated cells, the metabolically inactive non-dividing monocytes and quiescent T lymphocytes are largely refractory for HIV-1 replication [41-43]. The HIV-1 restriction in metabolically inactive non-dividing cells is observed at various levels including reverse transcription, nuclear import, and integration [43-46]. The studies have showed that quiescent T cells have very low level of nucleotides in their cytoplasm, which would be attributed to lower de novo reverse transcription of viral RNA [45] and elongation of newly synthesised viral cDNA [43, 47]. Rarely, small amount of complete viral cDNA is indeed formed in quiescent T cells, but cDNA nuclear import and integration are defective [46]. Although exact reason for defective nuclear import or integration of viral DNA in quiescent T cells is not known, either improper reverse transcription or limited availability of certain cellular co-factors may contribute to this phenotype.

It was initially assumed that HIV-1 nuclear import is only required for non-dividing cell infection, but recent reports showed that HIV-1 nuclear import also plays an important role in proliferating cell infection [48-50]. As only small proportion of T-lymphocytes in the body are actively proliferating at any given point of time, the ability to enter cell nucleus during interphase would greatly benefit viral replication and possibly contribute to high replication rate observed in infected individuals [51, 52]. Moreover, recent studies have made another interesting observation, the nuclear import coupled integration. The nuclear import is not simply a way to access nuclear comportment but rather plays distinct role in viral cDNA integration [53, 54]. Thus, the nuclear import plays a crucial role in HIV-1 infection of non-dividing as well as dividing cells. The ability of HIV-1 to successfully infect non-dividing cells and tremendous replication potential in dividing cells plays crucial role in establishment of infection, disease progression, and AIDS pathogenesis [55-57] In the following section, we will summarize and discuss recent progresses made in molecular biology of HIV-1 nuclear import and its significance for viral replication.

Passage of HIV-1 PIC to perinuclear compartment

The cytoplasm is not simply a dilute aqueous environment but it is rather viscous and densely packed with numerous cellular components. The movement of macromolecules by passive diffusion is highly restricted in cytoplasm due to steric hindrance and unexpected interactions with cytoplasmic components [58]. For instance, the passive diffusion of molecules as small as a DNA fragment of 2000bp is over 100 times slower in cytoplasm compared to in aqueous water [59]. Considering the fact that the HIV-1 PIC is a high molecular weight nucleoprotein complex with an average diameter of 56 nm, the movement of PIC in cytoplasm by passive diffusion is highly unlikely. Moreover, as passive diffusion is characterized by random movement, the localization of PIC near NPC at perinuclear compartment may not be always attained by simple passive diffusion. On the contrary, the cytoskeleton dependent active intracytoplasmic movement would greatly facilitate HIV-1 translocation to perinuclear compartment. In fact, such mechanism is commonly observed in many viruses such as herpes simplex virus (HSV)-1[60], adenovirus [61], vaccinia virus [62, 63], and canine parvo virus [64]. The cytoskeleton is a dynamic three dimensional structure found in most of eukaryotic cells, and it is made up of heterogeneous filaments subdivided into actin, microtubule, and intermediate filaments. While intermediate filaments give mechanical stability to the cell, both microtubule and actin filaments are involved in intracellular trafficking. The actin and microtubule selectively transport endogenous cargos from cell periphery towards nucleus with the help of specialized motor proteins called Myosin VI and Dynein respectively (**Figure 2D** and **E**). Several viruses have been shown to target these motor proteins and undergo cytoskeleton dependent retrograde transportation in cytoplasm. The actin is extensively found beneath the cell membrane and at cytoplasmic face of nuclear membrane (**Figure 2A**), where as microtubule covers vast space between cell membrane and nuclear periphery (**Figure 2B**). The microtubule consists of long filamentous structures radiating from a region within perinuclear compartment called microtubule organizing center (MTOC) towards cell periphery. The filaments are formed by polymerization of α and β- tubulin subunits, and assembly or disassembly of these subunits from the filaments at cell periphery maintains growing and shrinking phases of microtubule. The polymerization and depolymerization of microtubules is strictly regulated by a group of proteins called microtubule associated proteins (MAPs). In an elegant study,
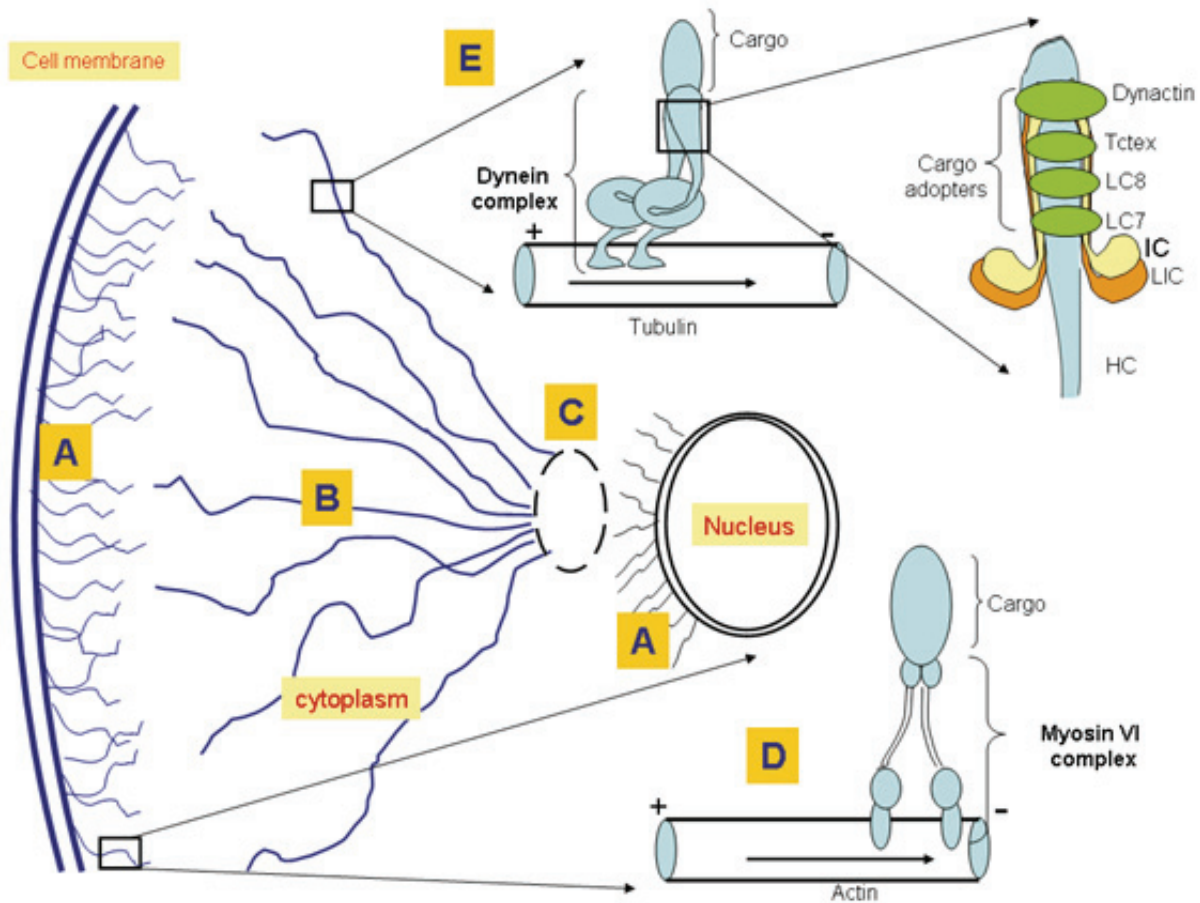
Figure 2. The cellular cytoskeleton and associated components: (A). The actin filaments covering narrow space beneath cytoplasmic membrane and around nucleus. (B). The tubulin filaments covering vast space in cytoplasm between actin filaments. (C). Microtubue organizing center (MTOC): A region in perinuclear compartment marked by microtubule nucleation. (D). Myosin VI: The motor protein complex that mediates retrograde movement of cargos along actin filaments. (E) Dynein: The motor protein complex that mediates retrograde movement of cargos along tubulin filaments.

Donald et al., demonstrated the specific co-localization of HIV-1 PIC with cytoplasmic microtubules and subsequent concentration at MTOC in live cells [65]. Furthermore, microinjection of anti-dynein intermediate chain (DIC) antibodies into infected cells abolished PIC localization to MTOC, providing strong evidence in favor of dynein mediated transportation of PIC. However, the significance of microtubule for early stage HIV-1 replication is so far elusive. In the beginning, Bukrinskaya et al reported that the replication of luciferase reporter HIV-1 virus was only reduced slightly when infection was carried out in presence microtubule depolymerising agent, nocodazole [66]. Recently, another study showed that HIV-1 replication in resting and activated CD+ T lymphocytes is unaffected in the presence of different microtubule depolymerising agents [67]. Intriguingly, in contrast to Donald et al, these latter studies were carried out in cell lines or T lymphocytes that consist of narrow cytoplasmic space and scanty microtubules. As cortical actin filaments can support short distance transport of viral particles in cytoplasm [68], the actin dependent movement would provide alternative means of transport for HIV-1, and this would be sufficient enough to traverse narrow cytoplasmic space in T lymphocytes. Alternatively, microtubule dependent movement may be important in cells having vast cytoplasm such as primary macrophages or dendritic cells. The existence of such redundant mechanisms

becomes more obvious as movement of HIV-1 PIC in cytoplasm was completely blocked in the presence both nocodazole and latrunculin B, the latter does actin depolymerisation [65]. Despite the fact that cytoskeleton plays a crucial role in delivering of HIV-1 PIC to perinuclear compartment, little is known about how HIV-1 targets cytoskeleton. The dynein complex cargo adopter proteins such as Dynein light chain1 (DYNLL1), Tctex1 and Dynactin have been shown to be associated with microtubule dependent retrograde transportation of several viruses [60, 62, 64, 65]. Although mechanism is not quite clear, these proteins possibly mediate the recruitment of viruses on to dynein complex. However, whether any of these dynein complex cargo adopter proteins also mediate recruitment of PIC to dynein complex and facilitate retrograde movement of HIV-1 remains elusive. Further, as PIC availability at NPC is one of pre-requisites for nuclear import, whether and how microtubule dependent retrograde movement of PIC influences HIV-1 nuclear import deserves investigation. Understanding the role of cytoskeleton in early stage of HIV-1 replication and its molecular mechanism would help to design novel anti-HIV-1 strategies.

The translocation of HIV-1 PIC into cell nucleus

The transportation of HIV-1 PIC into cell nucleus has been subjected to extensive investigation during past two decades. After the initial report that HIV-1 infects non-dividing cells [31], the energy dependent active uptake of HIV-1 PIC into cell nucleus was demonstrated [9], which led to the speculation that HIV-1 taps host nuclear import machinery and delivers its cDNA into nucleus for successful integration. Thereafter, several viral and/or cellular factors have been proposed for HIV-1 nuclear import. In the following section, we will discuss some of the key factors and their contribution to HIV-1 nuclear import.

HIV-1 Matrix (MA)

In the beginning, Burkinsky et al showed that the HIV-1 Matrix (MA) protein contains canonical NLS, which was upon conjugated with heterologous protein, induced its nuclear localization [24, 69]. The PICs isolated from the nuclear fraction of HIV-1 infected cells found incorporated MA protein [24]. Subsequently, a study showed that nuclear import of HIV-1 PIC is inhibited in the presence of excess NLS peptide of

simian virus (SV) 40 large T antigen that closely resembles NLS of MA. Notably, MA protein is subjected to phosphorylation by cellular kinases belong to the family of serine-threonine kinase [70, 71] and kinases of MAPK pathway [72, 73]. Some of these kinases are also incorporated into viral particles [72, 73]. Interestingly, phosphorylation of C-terminal tyrosine (Y132) residue of MA by serine-threonine kinase was shown to mediate MA incorporation into the progeny virus and infection of non-dividing cells [70, 74, 75]. The phosphorylation of MA^{Y132} residue, in addition to facilitating the incorporation MA into virion core, is also required for interaction with IN and nuclear import [75]. These results support the notion that MA protein due to its association with HIV-1 PIC and karyophilic nature mediates viral cDNA nuclear import. However, a conflicting picture emerged during the follow up studies on role of MA in HIV-1 nuclear import. Interestingly, both phosphorylation of Y132 residue or presence of NLS in MA was not absolutely essential for HIV-1 nuclear import and/or replication in non-dividing cells [15, 76-78]. Although a novel NLS has been recently reported in MA [79], it is still difficult to reconcile these latter contradictory findings. Moreover, even after deletion of entire MA protein except N-terminal myristoylation signal from HIV-1, the virus still retained a low level of replication in both dividing and non-dividing cells [80]. Taken together, although these findings fail to rule out the contribution of MA entirely, they refrain from suggesting MA protein as the only factor involved in HIV-1 nuclear import.

Viral protein R (Vpr)

The Vpr is another viral protein implicated in HIV-1 nuclear import. The Vpr is a virion associated HIV-1 accessory protein [81, 82] with a molecular weight of 14 kDa and 96 amino acids in length. It consists of hydrophobic core with three alpha helices (αH1, αH2 and αH3), a negatively charged flexible N-terminal domain, and an arginine rich positively charged C-terminal domain [83]. The Vpr localizes to the nucleus in transfected cells when expressed alone or as fusion protein with β-galactosidase, bovine serum albumin (BSA) or green fluorescent protein (GFP) [21, 23, 84-86]. Unlike MA, Vpr lacks any known classical NLSs, but two non-conventional nuclear targeting signals, each in N and C- terminal of Vpr, have been identified [20, 23]. Consistent with these findings, the nuclear localization of MA but not Vpr is inhib-

ited in the presence of peptide corresponding to SV40 Large T antigen NLS [87]. However, either the role of Vpr or specific pathway(s) used by Vpr during HIV-1 nuclear import is not clear. Nevertheless, various explanations have been put forward. The Vpr has been shown to interact with soluble nuclear import protein Impα [87], and nuclear localization of Vpr in *in vitro* transport assay is enhanced in the presence of Impα [88]. Further, the Impα1 interaction defective Vpr mutant, "αLA/N17C7", has failed to localize into nucleus [89]. These reports support the widely accepted hypothesis that Vpr is imported to the nucleus through typical classical nuclear import pathway. However, conflicting results do exists. The presence of Vpr has been shown to enhance the interaction of classical NLSs with Impα, including NLSs of MA [90]. Interestingly, another study showed that Impα dependent nuclear localization of Vpr does not require Impβ [89], instead the presence of excess of Impβ affects Vpr nuclear localization, suggesting Vpr could potentially act as Impβ. Indeed, binding of Impβ to Impα enhances cargo protein/Impα interaction by relieving the autoinhibitory control (Reviewed in [12]). Interestingly, the studies also showed that Vpr interacts with various Nups [91-94] and localizes to nuclear envelope [91, 94]. Thus, it is possible that Vpr only act as accessory factor by enhancing overall nuclear import of HIV-1 PIC [95]. Consistently, studies have showed that the HIV-1 having either specific mutations or deletion of entire Vpr is only reduced but not completely abolished its replication in primary macrophages [19, 94-96]. Further, Vpr deleted HIV-1 replication is almost unaffected in CD4^{+} T lymphocytes [94, 96]. Also, some additional explanations but not so popular do exist. The Vpr has been shown to induce transient herniation of nuclear membrane and lead to mixing of the nuclear and cytoplasmic contents [10], which would allow PIC to directly access the nucleus. However, underlying mechanism of Vpr induced local bursting of nuclear membrane or whether PIC is able to access nucleus by this mechanism is not clarified. Further study on some of these aspects would potentially determine the role of Vpr in HIV-1 nuclear import.

Integrase (IN)

The IN has been suggested for HIV-1 nuclear import by various studies lately [15, 16, 97, 98] The work by Gallay et al provided first convinc-ing evidence that IN plays a crucial role in HIV-1 nuclear import. Using single-round infectivity assay, authors showed that the mutant HIV-1 (MA ₐₙₗₛΔVpr) that lacks both NLSs of MA and entire Vpr protein is equally infectious as wild type virus in γ irradiated P4 cells. In contrast, triple mutant HIV-1 that lacks IN protein in addition to MA NLA and Vpr has failed to enter nucleus [15], suggesting the importance of IN for HIV-1 nuclear import. The IN is a 288 amino acid protein with a molecular weight of 32 kDa. It consists of three functionally distinct domains; N-terminal domain (NTD, residues 1-50), catalytic core domain (CCD, residues 51-212) and C-terminal domain (CTD, residues 213-288). The NTD contains a highly conserved zinc finger like motif, which is implicated in providing stability to IN structure and enhancing the catalytic activity [99]. The CCD harbours an important component called DDE motif, which catalyzes the integration reaction. The CTD is relatively less conserved region of IN, the CTD along with CCD has been implicated in viral cDNA nuclear import [17, 18, 100, 101]. The IN is a virion associated protein and found tightly associated with PIC throughout the course of nuclear import and integration [3]. In transfected cells, IN is exclusively localizes to nucleus even when it is expressed as fusion protein with GFP [18, 102], FLAG [103], pyruvate kinase [50], and glutathione S-transferase (GST) [15], and this strong nucleophilic property of IN has been linked to HIV-1 cDNA nuclear import [18]. However, a small number of studies attributed IN nuclear localization to passive diffusion followed by nuclear retention due to non-specific DNA binding property [104, 105], but these arguments have failed to stand against energy and temperature dependent rapid nuclear accumulation of IN [14].

Interestingly, IN contains several basic amino acid rich putative NLSs ([186]KRK, [211]KELQKQITK, [236]KGPAKLLWK and [262]RRKAK), some of them have been implicated in HIV-1 nuclear import. From the initial report that IN mutants, K186Q and Q214/216L, have lost their nuclear localization and interaction with Impa1 *in vitro* [15], a non-conventional bi-partite NLS ([186]KRK and [215]KELQKQITK) was proposed. However, subsequent studies argued that these IN mutants (K186Q and Q214/216L) affect reverse transcription, integration or unknown postnuclear entry steps rather than nuclear import [100, 103, 106]. A later study by Bouyac et al., pro-

posed a atypical NLS (161IIGQVRDQAEHLK173) in CCD of IN, and showed that it is required for IN nuclear localization and viral cDNA nuclear import [50]. The peptide containing 161-173 amino acids of IN was found interacting with Impα1 and able to mediate nuclear localization of Bovine serum albumin (BSA) when covalently attached. However, follow-up studies, in an attempt to reassess these findings, have failed to arrive at conclusion [27, 107].

Recently, IN has been shown to interact with several additional nuclear import receptors such as Imp7 [17], Tranportin (TNPO)3 [108] and Impα3 . The Imp7 was initially shown to facilitate nuclear import of purified reverse transcription complexes *in vitro* [109]. Subsequently, the study from our lab demonstrated the Imp7 interaction with IN, and the IN having specific mutations (INKK240, 4AA/INRK263, 4AA) in [236]KGPAKLLWK and [262]RRKAK region were failed to interact with Imp7 [17]. However, the HIV-1 having Imp7 interaction defective IN mutations had only moderately reduced HIV-1 cDNA nuclear import [17]. Furthermore, Imp7 knockdown (KD) had only modest effect on HIV-1 replication, but infectivity of progeny viruses produced from Imp7 KD cells showed a greater reduction, suggesting possible involvement of Imp7 in late stage HIV-1 replication. Meanwhile, another study by Zielske et al did not reveal the requirement of Imp7 for HIV-1 nuclear import in primary macrophages when Imp7 KD macrophages were infected with HIV-1 [110]. Interestingly, a recent study showed that, although Imp7 is dispensable for HIV-1 nuclear import, the presence of Imp7 enhances over all nuclear import of viral cDNA [111].

The importance of TNPO3 for HIV-1 replication is only appreciated in a recent study involving large scale gene knockdown analysis [112]. Subsequently, in a study by Christ et al., demonstrated the TNPO3 interaction with IN and its involvement in HIV-1 nuclear import [108]. The TNPO3, a member of Impβ family of import receptors, recognizes cargo proteins through serine-arginine (SR) rich repeats [113, 114]. Since IN does not contain any SR repeats, the mechanism of IN/TNPO3 interaction is quite intriguing. Meanwhile, a recent study by Krishnan et al., argued that CA protein but not IN determines TNPO3 requirement [25]. Nevertheless, an isolated study showed that the peptide optamer ("WQCLTLTHRGFVLLTITVL") that interacts with

IN [115] and disrupts IN/TNPO3 interaction is also affected HIV-1 nuclear import [116]. Moreover, expression of either TNPO3Δcargo binding domain or cargo binding domain mutants (F918A/F922A or LL967-8AA) in TNPO3 knockdown cells was unable to rescue HIV-1 replication, these findings further underscore the involvement of TNPO3 in HIV-1 nuclear import [117].

The Impα1 and Impα3 are members of Impα family of adopter proteins for classical nuclear import pathway. In total, classical nuclear import pathway includes six Impα subtypes in human. Although Impα subtypes show high level of similarity at amino acid level (50-80%) [118], they often differ in their substrate recognition. Indeed, classical nuclear import pathway serve as a single most common route for protein nuclear import in eukaryotic cells (reviewed in [119]). Initially, Gally et al., proposed the requirement of IN/Impα1 interaction for HIV-1 nuclear import, but subsequent studies have contradicted these former findings. In order to fully understand the importance of classical nuclear import pathway for HIV-1 replication, we carried out gene knockdown (KD) analysis for individual Impα subtypes. Among all the subtypes we have studied, Impα3 KD showed maximum effect on HIV-1 replication, while Impα1 KD affected only moderately[48]. Further, Impα3 was found interacting with IN, and CTD of IN was indeed required for this interaction[48]. In the follow-up study, by mutagenic analysis, we have identified a non-conventional bi-partite NLS ([211]KELQKQITK and [262]RRKAK) within IN required for Impα3 interaction, and the HIV-1 having specific mutations in this NLS showed defective nuclear import and/or replication [13]. However, even though human cells express multiple Impα subtypes [120, 121], only few of them play a leading role in HIV-1 nuclear import [15, 48]. Although it is premature to speculate, the Impα subtype requirement may be determined in a cell specific manner. The Impα subtype is known to show cell type or cell differentiation specific expression patterns [120, 121]. In addition, a study showed that the nuclear import efficiencies of Impα subtypes for a particular cargo-protein can be modulated in the presence of other cellular proteins [118]. Taken together, the requirement of particular Impα subtype would be decided by the abundance of particular Impα subtype or intra-cellular environment of particular cell type or both.

Apart from known importins, several studies indicate that IN is able to directly interact with Nups. Recently, Woodward et al demonstrated the IN interaction with Nup153 *in vitro* [122], and their study also showed that overexpression of Nup153 c-terminal domain disrupted IN/Nup153 interaction and affected HIV-1 cDNA nuclear import. The requirement of Nup153 for HIV-1 replication has been confirmed in recent studies through gene knockdown approach [123, 124]. In addition, an earlier study, by using an inhibitor protein (vesicular stomatitis virus matrix protein) that binds and masks the availability of phenylalanine-glycine (FG) repeat region of Nup98 for cargo protein interaction, demonstrated the involvement of Nup98 in HIV-1 nuclear import [125]. However, whether Nup98 is directly involved in PIC nuclear translocation or acting as intermediate docking site during the process of nuclear import still remain to be defined. Moreover, as FG repeat region is commonly present in many Nups, the above observations due to general effect cannot be ruled out. On contrary, a recent study contradicted the requirement of Nup98 for HIV-1 nuclear import as Nup98 KD is only affected HIV-1 integration but not the nuclear import [54]. Even though the role of Nups in HIV-1 nuclear import is yet to be studied in detail, these preliminary findings suggest the possible existence of yet another alternative pathway for HIV-1 nuclear import, and argue in favor of earlier suggestion that IN nuclear import is independent of soluble cytoplasmic factors [16]. Clearly, among all the known viral factors, so far IN remained as a leading partner for HIV-1 nuclear import. While studies have revealed several different nuclear import mechanisms associated with IN (**Figure 1D**, **1E**, and **1F**), the biggest challenge remains to understand how these redundant pathways influence actual infection and whether manipulation at this level would benefit the development of new anti-HIV-1 strategies.

Capsid (CA)

Interestingly, some of recent studies have promoted CA protein as a decisive partner for HIV-1 nuclear import. The MLV/HIV-1 chimera virus where HIV-1 CA is replaced with MLV CA showed a greatly reduced replication in cell cycle arrested (aphidicolin treated) cells, and this replication defect was mainly attributed to impaired nuclear import of PIC; these finding prompted authors to conclude that CA plays decisive role

in HIV-1 nuclear import [26]. Further, the MLV/HIV-1 chimera virus replication was found insensitive to TNPO3 knockdown [25], which led to speculation that CA determines the TNPO3 requirement. However, these arguments should be considered with caution as none of these studies clarify how exactly CA mediates HIV-1 nuclear import. Unlike HIV-1, the PIC of MLV contains higher amount of CA protein indicating delayed uncoating [126-128], this has been suggested for the differences between HIV-1 and MLV nuclear import. Moreover, neither HIV-1 nor MLV CA has been shown to interact with any of the known nuclear import receptors or components of nuclear import machinery. However, it is highly possible that proper uncoating may allow timely exposure of viral proteins in PIC for targeting nuclear import machinery, which is in a way indirectly influencing subsequent nuclear import step.

Contemporary factors (HIV-1 central DNA flap, cellular tRNA)

The mechanism of HIV-1 nuclear import is further confounded by additional factors that have been shown to influence nuclear import by some poorly understood mechanisms. In the newly synthesized HIV-1 cDNA, a small stretch of overlapping plus stand DNA at central polypurin tract (cPPT) forms a triple stranded DNA structure called central DNA flap [129]. Large number of studies showed that either deletion or mutations of cPPT that abrogates the central DNA flap formation also affected HIV-1 replication to a various extent [28, 30, 130-134], and this defective viral replication is largely attributed to reduced entry of viral cDNA into the nucleus [28, 29, 130]. Although the mechanism is not clear, a recent study showed that the absence of cPPT affects viral uncoating and leaves cDNA trapped in viral coat making it impossible to enter nucleus[135]. These results suggest that cPPT, by affecting steps prior to nuclear import, would indirectly influences HIV-1 nuclear import. Hence, the precise role of central DNA flap in HIV-1 nuclear import is currently a matter of strong debate. Nevertheless, due to its ability to influence nuclear import and enhance the transduction, the cPPT is now commonly used in lentiviral vectors for gene therapy [136-139].

In one of the recent studies, an unusual link between cellular tRNA and HIV-1 nuclear import is also reported [140]. Authors showed that a

fraction of cytosolic extract that lacks most of soluble proteins was still able to support HIV-1 reverse transcript complex (RTC) nuclear import *in vitro*. Interestingly, this particular fraction was found enriched with tRNAs, but most of them were having defective 3'CCA ends. Moreover, the tRNAs that supported HIV-1 nuclear import were found incorporated into viral particles. Although these results could convince that tRNAs can promote HIV-1 nuclear import, the mechanistic details of whether and how tRNAs are indeed involved in HIV-1 nuclear import so far remains elusive. Taken together, the mechanism of HIV-1 nuclear import is much more complex and much more elusive than it was thought earlier. While bulk of studies put forward IN as key mediator of HIV-1 nuclear import, the contribution of other known viral and/or cellular factors cannot be ruled out but needs careful consideration.

Nuclear import coupled integration

The concept of functional link between HIV-1 nuclear import and integration is one of the most fascinating ideas proposed during recent years [54]. It is now evident that HIV-1 nuclear import is not only required for non-dividing cell infection, but also important for dividing cell infection [54, 108, 112]. Interestingly, the lentiviral integration in cycling cells has been shown to occur at "S" phase of cell cycle prior to mitosis [141, 142]. Furthermore, Katz et al made an interesting observation that when G1 synchronized HeLa cells were infected with single cycle replicating HIV-1 at very low MOI (<0.05 to 0.1) that statistically proven to generate just one integration per cell, the proviral DNA was invariably found in both daughter cells after cell division. The authors argued that if in this condition the cells were infected at the time of mitosis (DNA segregation), only one of the daughter cell should have integrated HIV-1 DNA. As both of daughter cells were found to contain proviral DNA, the HIV-1 integration is predicted to have occurred prior to mitosis. Furthermore, authors showed that when cells were challenged just prior to mitosis, the HIV-1 integration was delayed until the following interphase [49]. Taken together, although this study does not demonstrate any direct link between HIV-1 nuclear import and integration, it provided convincing evidence that HIV-1 integration and nuclear import may be functionally linked as integration only happens during interphase where nuclear

membrane is intact. Incidentally, a recent study made another interesting observation that HIV-1 integration in TNPO3 and RanBP2 depleted cells is favored towards less gene dense regions, where as in control cells the integration was observed in the region of chromosomes having G/C rich and highly expressing genes [53], this would also suggest that the proper trafficking through the NPC might allow PIC to hijack some unknown cellular cofactors that are required for proper integration of viral DNA.

Since nucleoporins (Nups) constitute major components of NPC that acts as channel for selective translocation of macromolecules, the HIV-1 could frequently encounter with Nups during nuclear import. Interestingly, studies in yeast showed that the Nups can bind to transcriptionally active genes in chromatin [143, 144], and several nucleoporins including Nup62, Nup50, Nup98, Nup153 are mobile and able to migrate freely in the nucleoplasm [145-150]. Thus, Nups could serve as favourable intermediate factors that drive PIC to transcriptionally active genes in chromatin. Interestingly, in our recent study, when we probed the potential interaction between IN and several different Nups, the IN was specifically interacted with Nup62. Further, the IN/Nup62 interaction was specifically observed in chromatin-bound fraction, and both Nup62 and IN were associated with chromatin. Consistently, the knockdown of Nup62 significantly reduced IN chromatin association and HIV-1 integration[151]. These findings along with other reports strongly suggest a functional link between HIV-1 nuclear import and integration. However, as discussed in the earlier section, HIV-1 nuclear import involves redundant pathways including Impα/Impβ, TNPO3, and direct interaction with Nups. Whether HIV-1 nuclear import involving these individual pathways has any distinct outcome on subsequent integration is not known. In such situation, do any of these individual pathways play cell cycle or cell type dependent role in HIV-1 integration and how this would influence establishment of infection or overall disease progression deserves further investigation.

Conclusions

The nuclear import is one of the most complex and fascinating areas of HIV-1 research. Over the past decade, considerable progress has been made in uncovering some of the key as-

pects, but research community is still struggling to reach consensus on many important issues surrounding HIV-1 nuclear import. One of the major hurdles encountered in this field of study is the involvement of large number of viral/cellular factors and associated pathways. Nevertheless, upon considerable success, it is now possible to envisage the probable mechanism of HIV-1 nuclear import. The HIV-1 Vpr could act as facilitator of HIV-1 nuclear import either by mediating the docking of PIC to NPC or by enhancing kinetics of nuclear translocation of PIC. The IN due to its strong karyophilic nature and ability to interact with components of nuclear import machinery would serve as key viral factor that taps host nuclear import machinery and mediate active translocation of PIC into nucleus. However, the importance of redundant pathways or distinct mechanisms associated with IN may need further investigation. The cPPT and CA on the other hand may influence HIV-1 nuclear import probably by maintaining proper orientation or architecture of PIC and proper uncoating of virus respectively. While the MA also contains NLS and interacts with Impα, its importance for HIV-1 nuclear import still remains elusive after recent contradictory findings. Unless the role of MA is properly defined, its significance for HIV-1 nuclear import still remains elusive. With the advent of recent findings that nuclear import influences subsequent integration step, the HIV-1 nuclear import may gather much wider attention by the research community due its possible implications on establishment of infection and disease progression. Taken together, while important progress has been made on overall mechanism of HIV-1 nuclear import, the detailed understanding on some of the key issues still remain to be clarified. The proper understanding of HIV-1 nuclear import would serve promising target for novel anti-HIV-1 strategy.

Acknowledgements

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