Role of host-encoded proteins in restriction of retroviral integration

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INTRODUCTION

After entry into a target host cell, retroviruses carry out reverse transcription for the synthesis of double-stranded DNA from their RNA genome in a complex derived from the viral core. Subsequently, the newly synthesized viral DNA remains associated with viral and cellular proteins and forms a high-ordered nucleoprotein complex called the preintegration complex (PIC). The PIC guides nuclear entry and the subsequent integration process of the viral DNA genome (Suzuki and Craigie, 2007). The viral DNA that is integrated into chromosomal DNA, termed the provirus, acts as a template for the efficient transcription of viral RNA and ensures the equal segregation of the genetic material to daughter cells upon division. Once established, proviruses cannot be distinguished or excised from the cellular genomic DNA of infected cells. Therefore, integration occupies an important place not only in viral replication but also in cellular antiviral defense strategies called intrinsic immunity, which has evolved to well-studied antiviral cellular proteins such as APOBEC and TRIM family proteins, and is a hallmark of retroviral infections (Lewinski and Bushman, 2005). The viral enzyme catalyzing the integration is integrase (IN). As shall be shown, the chemical mechanism of the integration reaction has been elucidated by biochemical studies using oligonucleotide DNA substrates and purified IN protein produced by the Escherichia coli IN gene, several host cellular proteins have also been implicated as key factors involved in the integration step during viral replication. Although studies on integration cofactors have mostly emphasized factors that aid the integration process either through direct or indirect association with IN, it has become apparent that host cells may also harbor proteins that act as inhibitors of retroviral integration. Intriguingly, some of these inhibitory proteins appear to hamper the integration process via posttranslational modifications of the components of the preintegration complex including IN. A better understanding of the molecular mechanisms leading to the inhibition of integration will provide us with clues for the development of new strategies for treating retroviral infections. In this review, we draw attention to recent insights regarding potential host cellular factors that restrict integration, and illustrate how these inhibitory effects are achieved.

Keywords: retrovirus, integration, host factors, RAD51, KAP1, VRK, posttranslational modifications

In retroviral infections, a copy of the viral DNA is first synthesized from genomic RNA by reverse transcription and subsequently integrated into host chromatin. This integration step, executed by the viral enzyme integrase (IN), is one of the hallmarks of retroviral infection. Although an obligate role for IN in retroviral integration has been clearly defined by numerous biochemical analysis of its recombinant protein and genetic analysis of the viral IN gene, several host cellular proteins have also been implicated as key factors involved in the integration step during viral replication. Although studies on integration cofactors have mostly emphasized factors that aid the integration process either through direct or indirect association with IN, it has become apparent that host cells may also harbor proteins that act as inhibitors of retroviral integration. Intriguingly, some of these inhibitory proteins appear to hamper the integration process via posttranslational modifications of the components of the preintegration complex including IN. A better understanding of the molecular mechanisms leading to the inhibition of integration will provide us with clues for the development of new strategies for treating retroviral infections. In this review, we draw attention to recent insights regarding potential host cellular factors that restrict integration, and illustrate how these inhibitory effects are achieved.
BIOCHEMICAL FEATURES OF THE INTEGRATION REACTION

Retroviral IN is expressed and incorporated into virions as the C-terminal part of the Gag-Pol polyprotein precursor during the late phase of retroviral infection. IN comprises three structurally and functionally distinct domains, termed the N-terminal domain (NTD), the catalytic core domain (CCD), and the C-terminal domain (CTD) (Figure 1A; Lewinski and Bushman, 2003). The NTD of IN is relatively well conserved amongst retroviruses especially with the respect to a motif consisting of two histidines and two cysteines (HHHC motif). This HHHC motif makes up the zinc-binding site with an overall helix-turn-helix fold commonly found in IN of all retroviruses and retrotransposons (Craigie, 2001). Further studies utilizing mutants containing amino acid mutations in the HHHC motif also elucidated the importance of the NTD in the key steps of integration as well as its involvement in the multimerization of IN (Zheng et al., 1996; Lee et al., 1997; Craigie, 2001). The CCD is composed of a mixture of α-helices and β-sheets folds that bring a triad of critical acidic residues called the DDE motif into close proximity (Lewinski and Bushman, 2005). Crystal structure studies of HIV-1 and avian sarcoma virus (ASV) IN have shown this motif to be important for the binding of divalent metal cations such as Mg$^{2+}$ (Craigie, 2001). Additionally, the CCD was also shown to possess viral DNA recognition and binding ability (Lewinski and Bushman, 2005). Compared to the NTD and CCD of IN, the CTD displays the greatest degree of variation across the retrovirus family. Despite this, studies on the CTD region of HIV-1 have revealed that it has strong DNA-binding activity and also potentially has multimerization capabilities (Woerner et al., 1991; Engelman et al., 1994; Lutzke and Plasterk, 1998; Gao et al., 2001).

The retrovirus-mediated integration reaction consists of three biochemically distinct steps (Figure 1B; Craigie, 2001). In the first step, IN specifically recognizes the viral attachment (att) sites on the 5′ and 3′ long terminal repeats (LTRs) of newly synthesized viral DNA ends. This interaction between IN and DNA allows for the 3′ processing of viral DNA, where water serves as the nucleophile for the cleavage of viral DNA. In this 3′ processing step, most retroviral IN such as HIV-1 IN catalyze the removal of two nucleotides adjacent to the highly conserved CA dinucleotide from the 3′ end of LTR regions. This chemically activates the viral DNA through the formation of 3′ hydroxyl radicals at the terminal ends of viral DNA, allowing it to participate in the next reaction.

The second step of integration involves the insertion of 3′ processed, activated viral DNA strand into the target DNA through a single transesterification reaction, which is called the strand transfer step (Engelman et al., 1991). IN brings the viral DNA ends into close proximity with the target DNA allowing a nucleophilic attack by the 3′ hydroxyl radical on the target DNA. Subsequently, IN ligates both the 3′ hydroxyl radical terminal of the viral DNA to the 5′ phosphoribyl ends of the target DNA and establishes new phosphodiester bond between them. This forms intermediate DNA products with unpaired gaps between the non-ligated 5′ ends of viral DNA as well as the 3′ ends of target DNA. Additionally, the unpaired gaps result in the short duplication of target DNA sequences flanking both strand ends, followed by the formation of imperfect inverted repeats upon the sealing of the nick. The length of this inverted repeat sequences can vary from 4 to 6 base pairs (bp) and is highly characteristic of the retrovirus species from which the IN protein is derived. It should be noted that the 3′ processing and strand transfer steps have been shown to be catalyzed by IN proteins.
through various in vitro studies conducted with E. coli-expressed IN and DNA substrates harboring viral LTR sequence (Turlure et al., 2004).

Lastly, after the ligation reaction, the gaps in the intermediatedata products are repaired to yield fully functional integrated proviruses (gap repair step). The previous step generates an intermediate product in which viral DNA is joined to the target DNA solely by the 3' ends of both strands. As such, DNA repair is needed to seal the nick between the 5' end of viral DNA and the 3' end of the target DNA to complete the provirus formation. Unlike the earlier steps which are catalyzed by IN, this step is probably mediated by some cellular enzymes involved in a variety of DNA repair pathways including the non-homologous end joining (NHEJ) repair pathway. However, whether or not the NHEJ machinery is involved is controversial, and identification of the specific enzymes conducting this gap repair step is anticipated in future studies (Turlure et al., 2004; Smith and Daniel, 2006; Yoder et al., 2006).

**HOST PROTEINS ASSOCIATED WITH THE RETROVIRAL INTEGRATION COMPLEX**

The PIC, the key nucleoprotein complex responsible for the integration, is formed in the cytoplasm following the reverse transcription of viral DNA from the RNA genome (Figure 2). Although the full composition of this nucleoprotein complex has yet to be determined, most of the viral protein components of the PIC are thought to be derived from the core of the infecting virus, which includes not only IN but also reverse transcriptase (RT), matrix (MA), capsid (CA), and some HIV-1 accessory proteins (Suzuki and Craigie, 2007). With respect to the cellular components of the PIC, significant efforts have been made to identify them through surveys of IN-interacting proteins using yeast two-hybrid screenings and/or co-immunoprecipitation analysis (Figure 2, Turlure et al., 2004).

Integrase interactor 1 (INI1), also known as hSNF5, was the first binding partner of HIV-1 IN identified by yeast two-hybrid assay (Kalpana et al., 1994). This nuclear protein is the human homolog of the yeast SNF5 protein, a transcription activator and part of the SWI/SNF complex in mammalian systems (Wang et al., 1996). Interaction with the HIV-1 IN requires a direct imperfect repeat region of IN1 termed Rpt1 (Yang et al., 2001). The function of IN1 in HIV-1 integration was demonstrated in an in vitro integration assay where it stimulated the strand transfer activity of recombinant IN (Kalpana et al., 1994). However, the role of IN1 during virus replication is still a matter of debate. Co-transfection of the minimal IN-binding domain of IN1, including the Rpt1 region, with HIV-1 proviral DNA in HEK293 cells resulted in a nearly 10,000-fold reduction in virus production from the transfected cells (Yang et al., 2001). Furthermore, subsequent studies showed that IN1 is specifically incorporated into HIV-1 virions during virus production (Yang et al., 2004), suggesting a possible role of IN1 in the late stage of HIV-1 replication rather than in the integration step. Interestingly, it was revealed that IN and IN1 selectively recruit the components of the Sin3a-HDAC1 complex into HIV-1 virions, whereas it is required for the reverse transcription step following the infection of subsequent target cells (Sotin et al., 2009). Thus, IN1 does not seem to be involved in the integration step in spite of its interaction with IN.

Lens epithelium-derived growth factor (LEDGF) is the first cellular protein to have been demonstrated as a bona fide cofactor for HIV-1 integration (Engelman and Cherepanov, 2008). LEDGF is a transcriptional regulatory protein with a molecular mass of 76 kDa from the hepatoma-derived growth factor (HDGF)-related protein (HRP) family, and was found to be strongly associated with chromatin throughout the cell cycle. This protein was identified as an interaction partner of HIV-1 IN through a co-immunoprecipitation study in human cells (Cherepanov et al., 2003) and has been shown to stimulate in vitro integration activity of IN (Cherepanov et al., 2004). LEDGF comprises several functional domains that have been implicated in the integration process. The N-terminal of LEDGF contains the PWPF (proline–tryptophan–tryptophan–proline) domain that is highly conserved amongst the members of the HRP family and was revealed to be crucial for protein–protein interaction and/or DNA-binding. Furthermore, the N-terminal also contains a putative nuclear localization signal (NLS) and dual copies of the AT-hook DNA-binding motif (Engelman and Cherepanov, 2008). In addition, a limited proteolysis analysis of recombinant LEDGF has shown that an evolutionally conserved domain, termed the integrase-binding domain (IBD), is included in its C-terminal and as its name suggests, this domain was found to be crucial for the specificity of the interaction with HIV-1 IN (Engelman and Cherepanov, 2008).

LEDGF is likely to play a critical but not strictly essential role in HIV-1 integration and replication. A study performed using intensified RNA interference demonstrated a significant reduction of HIV-1 replication in human CEM+T cells in which there was a complete knockdown of endogenous LEDGF (Llano et al., 2006). Moreover, a knockout study done in mouse embryonic fibroblasts (MEFs) cell lines reported a reduction of 90% in HIV-1 infectivity upon the depletion of LEDGF/p75 and that this depletion was ablated upon re-expression of LEDGF (Shun et al., 2007). In these studies, it was found that the block in HIV-1 infection occurs specifically at the integration step, and that both the FNVF and IBD domains are of critical importance for HIV-1 integration and replication (Llano et al., 2006; Shun et al., 2007). Based on these observations, a simple mechanism model is proposed for LEDGF's function in HIV-1 integration: LEDGF serves as a molecular adapter that tethers HIV-1 IN to the target DNA (i.e., chromatin). Since LEDGF is a transcriptional coactivator, this tethering activity is thought to be responsible for targeting the integration site of HIV-1 into transcriptionally active regions (Engelman and Cherepanov, 2008).

After the PIC is formed in cytoplasm, it needs to be shuttled into the nucleus to allow the integration process to take place. One of the striking features of HIV-1 is its ability to get across the intact nuclear envelope easily even in non-dividing cells. This phenomenon is believed to be strongly associated with the karyophilic properties of the HIV-1 PIC (Suzuki and Craigie, 2007). Although the main component causing the PICs to be actively imported into the nucleus remains undetermined, some host-encoded proteins that interact with IN have been reported to facilitate the nuclear import of HIV-1 PICs. To date, importin 7 and TNPO3, have been
FIGURE 2 | Possible roles of IN- and PIC-associated cellular proteins in retrovirus replication. After penetrating the cell membrane, the viral nucleoprotein core containing genomic RNA is released into the cytoplasm, after which reverse transcription of the viral RNA takes place. The synthesis of full-length viral DNA produces the PIC, in which cellular as well as viral proteins including IN are associated.

3′ processing of the viral DNA by IN occurs in the cytoplasmic PIC. Once the PIC reaches the nuclear periphery it must gain entry into the nucleus for integration. Some retroviruses seem to take advantage of nuclear-envelope disassembly during cell division to access chromosomal DNA, whereas the PICs of lentiviruses, including HIV-1, are believed to be capable of crossing the intact nuclear-envelope during interphase. In the nucleus, the PIC gains access to chromosomal DNA and mediates viral DNA integration to produce a provirus, which is acted upon by viral and/or cellular transcription factors to express viral RNAs.

To date, a number of host proteins have been reported to interact with the retroviral IN and PIC (representative proteins are listed on the right). Many of them are likely to assist in the integration step, and some other cofactors appear to have functional roles in other steps including reverse transcription, nuclear entry, gene expression, and virion production during virus infection. In addition, recent studies demonstrate that several cellular interactors of IN may negatively regulate retrovirus replication (highlighted in red).

reported as IN-interacting importins that direct the HIV-1 PICs to the nucleus (Fasatti et al., 2003; Ao et al., 2007; Christ et al., 2008). Both proteins are members of the importin β family, which act as import receptors for cargo molecules through association with nucleoporin (NUP) of the nuclear pore complex (Suzuki and Craigie, 2007). While a confocal microscopy analysis of digitonin-permeabilized human cells showed the involvement of importin 7 in the nuclear accumulation of HIV-1 integration complexes, the functional role of this protein in virus replication is still unresolved (Fasatti et al., 2003; Zaitseva et al., 2009). TNPO3, an HIV-1 IN-binding protein identified by yeast two-hybrid screening, was found to promote viral nuclear import as well (Christ et al., 2008).

Although subsequent studies have confirmed the requirement of TNPO3 for efficient replication of HIV-1, the TNPO3–CA interaction, rather than the TNPO3–IN interaction, is likely to be required for the nuclear import of PIC in infected cells (Krishnan et al., 2010; De Iaco and Luban, 2011). By the same token, another cellular protein regulating nucleocytoplasmic trafficking, NUP153, was also shown to interact with HIV-1 IN by a pull-down assay (Woodward et al., 2009), but again, viral CA appears to be the viral determinant for the NUP153-mediated nuclear entry of the PIC (Matreyek and Engelman, 2011).

Intriguingly, a certain host protein called Gemini2 may be of importance to the reverse transcription process of HIV-1 via...
its physical association with IN. Gemin2 is a component of the survival of motor neurons (SMN) complex that is essential for the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) and was identified as a novel interactor of HIV-1 IN by a yeast two-hybrid screening (Hamamoto et al., 2006). Although this cellular protein is found in the HIV-1 PIC, depletion of endogenous Gemin2 by siRNA in HIV-1-infected primary monocye-derived macrophages resulted in impairment of the early reverse transcription step rather than the integration step (Hamamoto et al., 2006). Furthermore, a recent study showed that viral DNA synthesis by HIV-1 RT in vitro is enhanced by the concerted action of Gemin2 and IN (Nishitsuji et al., 2009). This augmentation of RT activity by an IN interaction appears to be reasonable because functional interaction between HIV-1 RT and IN has been revealed (Wilkinson et al., 2009). Hence, it would be assumed that Gemin2 serves as cofactor that stimulates and/or stabilizes the formation of the reverse transcription complex to initiate DNA synthesis through its interaction with IN (Masuda, 2011).

Several cellular proteins have been also identified as components of HIV-1 and Moloney murine leukemia virus (MoMLV) PIC by the in vitro reconstitution analysis and immunoprecipitation assays of PICs isolated from virus-infected cells. These include HMG proteins, barrier-to-autointegration factor (BAF, discussed below), Ku, and LEM proteins (Figure 2). Although there are some controversies as to whether these PIC-associated factors are indispensable to retrovirus infection, they may be indirectly involved in the integration process by stabilizing the nucleoprotein complex, promoting the nuclear retention of the PIC, or protecting host cells from viral DNA termini-induced apoptosis (Turluire et al., 2004; Van Marele et al., 2006; Suzuki and Craigie, 2007). Besides the host proteins listed above, more extensive discussions about the other cellular factors interacting with the IN/PIC and their roles in retrovirus replication are presented in previous comprehensive reviews (Turluire et al., 2004; Al-Mawsawi and Neamati, 2007; Goff, 2007; Studamire and Goff, 2010).

**POTENTIAL CELLULAR INHIBITORS AGAINST INTEGRATION**

In addition to the cellular enhancers for retroviral integration, the existence of potential antiviral proteins controlling the integration activity of IN and PIC has been revealed by several recent studies. Understanding the molecular details by which these factors hamper the integration reaction may potentially lead to future therapeutic inventions against retroviral infectious diseases. Recent findings on the potential restriction factors for integration process are summarized in this section, and their molecular inhibitory mechanisms are discussed.

**RAD51**

Although retroviral integration is completed by the gap repair step, which is believed to be mediated by the cellular DNA repair enzymes (Daniel et al., 1999; Lewinski and Bushman, 2005), one of the homologous recombination (HR) proteins, RAD51, has been shown to suppress HIV-1 integration (Desfarges et al., 2006; Cosnefroy et al., 2012). The downregulation of integration by RAD51 was first observed in a unique integration assay using a budding yeast strain in which DNA substrates containing zeocin-resistance gene flanked by HIV-1 U3 and U5 LTR ends can be integrated into genomic DNA by HIV-1 IN expressed by the yeast (Desfarges et al., 2006). When a haphazard yeast strain deficient for the RAD51 encoding gene was examined using the integration assay, higher numbers of zeocin-resistant clones were obtained, indicating an increase in integration events in the yeast cells lacking RAD51. This result suggests that RAD51 negatively regulates the HIV-1 IN-catalyzed integration reaction in yeast. In accordance with the yeast integration assay, additional in vitro analyses using purified recombinant proteins showed that human RAD51 (hRAD51) inhibited integration by IN. Interestingly, HIV-1 IN binds hRAD51 in vitro, suggesting that the inhibition of the integration reaction may be mediated through the physical interaction between IN and RAD51 (Desfarges et al., 2006).

Human RAD51 belongs to the RAD52 epistasis group, and members of the RAD52 epistasis group are highly conserved in yeast and humans, which mediate mitotic HR events as well as chromosome segregation during meiosis (San Filippo et al., 2008). RAD51 is also an ortholog of the E. coli RecA (Figure 3A). The RecA family proteins possess a conserved, ~230 amino acid core domain that is involved in the binding and hydrolysis of ATP through Walker A and B motifs, with the human RAD51 also bearing a long N-terminal and a short CTDs (Figure 3A; Lin et al., 2006; Wang et al., 2008). The RAD51 protein exists as a homo-octamer in solution, and binds to single-stranded (or double-stranded) DNA to form a right-handed helical presynaptic nucleoprotein. In the presence of ATP, RAD51 can polymerize on the DNA to form a nucleofilament that expands for thousands of bases (or base pairs, Figure 3B). This nucleoprotein filament serves as the catalytic center for DNA strand-exchange reactions during HR (San Filippo et al., 2008).

The formation of the nucleofilament is also likely to be important for the downregulation of HIV-1 IN activity by RAD51. In a recent report, Cosnefroy et al. (2012) set up an in vitro integration assay for IN in which active nucleofilament formation of RAD51 on substrate DNA was allowed to take place. They revealed that strong inhibition of HIV-1 IN activity was only observed with wild-type hRAD51 in the presence of ATP, but not in the reactions without ATP or with an hRAD51 mutant containing a single amino acid substitution in the Walker A motif (K133A) that ablated hRAD51–ATP-binding and therefore prevented hRAD51 polymerization (Cosnefroy et al., 2012). An additional in vitro experiment using a substrate DNA containing LTR sequences complexed with HIV-1 IN showed that even after the IN–viral DNA complexes were formed, subsequent incubation with hRAD51-induced dissociation of IN from its substrate viral DNA. The dissociation effect was not observed with hRAD51 in the absence of ATP or with the K133A mutant (Cosnefroy et al., 2012). Given that RAD51 can polymerize on DNA in an ATP-dependent manner, these observations suggest that the formation of the nucleofilament on viral DNA is responsible for the RAD51-induced restriction of HIV-1 integration via displacement of IN (Figure 3C). Furthermore, when an allosteric effector of RAD51, RS-1 (RAD51-stimulatory compound 1), which promotes formation of the active RAD51 nucleofilament, was used, HIV-1 integration and replication was significantly impaired in CD4+ cells. This was accompanied in an increase in 2-LTR circles,
FIGURE 3 | RAD51-mediated inhibition of HIV-1 integration. (A) Domain structure of human RAD51. RAD51 and its bacterial counterpart, RecA, share a highly conserved ∼230 amino acid domain that contains two putative ATP-binding domains, Walker A and Walker B motifs. DNA-binding activity of hRAD51 is attributed to its N-terminal domain, which includes a modified HhH motif, while RecA binds to DNA through its long C-terminal domain. (B) Nucleofilament formation of RAD51. Upon assembly on DNA, RAD51 forms a right-handed helical polymer on the DNA in presence of ATP, and the RAD51-DNA nucleoprotein filament expands to thousands of bases. (C) Disruption of HIV-1 integration complex by the hRAD51 nucleofilament. It is suggested that formation of an active nuclear filament following polymerization of hRAD51 on viral DNA eventually displaces IN, thereby inducing dysfunction of the HIV-1 integration complex prior to the integration reaction. Recruitment of hRAD51 onto viral DNA may be facilitated by its interaction with IN.

which is a byproduct from the failure to integrate (Cosnefroy et al., 2012). This in vivo experiment supports the proposed model in which hRAD51 recruited by HIV-1 IN is able to release the IN from viral DNA via formation of the nucleofilament, resulting in dysfunction of the PIC (Figure 3C). Although it is still unclear whether the hRAD51 in a natural setting could restrict HIV-1 integration, this study provides the possibility of using a specific stimulator of RAD51 such as RS-1 for a new antiviral approach against HIV infection.

KAP1

Posttranslational modifications (PTMs) including phosphorylation, ubiquitination, and acetylation is the chemical alternation of a protein’s primary structure after its translation, a process which is well known to have a great impact on various protein functions such as enzymatic activity, protein–protein interaction, and subcellular localization (Walsh et al., 2005). Although the association between PTMs and the retroviral integration process has not been fully addressed, it was demonstrated that the enzymatic activity of HIV-1 IN is positively regulated by histone acetyltransferases (HATs; Ceresteto et al., 2005; Terreni et al., 2010). HATs are the cellular enzymes that transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-aminogroup of certain basic lysine residues of histone’s N-terminal, which modifies the accessibility of DNA to other proteins (Roth et al., 2001). p300, the first HAT protein that was reported to catalyze the acetylation of HIV-1 IN, is a ∼300 kDa nuclear phosphoprotein originally isolated as an interaction partner of adenovirus E1A (Sterner and Berger, 2000). This HAT is also a paralog of the CREB-binding protein (CBP), and the p300/CBP family proteins contain several identical domains necessary for their function as HATs, which include three cysteine-histidine rich domains (CH1, CH2, CH3), a KIX domain, a bromodomain, and a HAT domain. (Figure 4A). p300 directly binds to and acetylates three specific lysine residues, Lys-264, Lys-266, and Lys-273, of the C-terminal region of HIV-1 IN (Figure 4C), which is likely to enhance the binding affinity to LTR DNA as well as strand transfer activity of IN in vitro (Ceresteto et al., 2005). Importantly, an HIV-1 strain
lysine residues on IN exhibited a significant replication defect in cells, and the replication defect occurred at the integration step, indicating the importance of C-terminal acetylation of IN in HIV-1 infection (Cereseto et al., 2009). An another HAT, GCN5 (Figure 4A), was also found to mediate the acetylation of HIV-1 IN at the same C-terminal lysines, and HIV-1 integration was shown to be impaired in GCN5 knockdown cells (Terroni et al., 2010).

In the case of histones, acetylation of the internal lysines affects several aspects of nucleosome function and generates new protein–protein interactions. However, it is a reversible process, and transcriptional activation of the nucleosome is repressed by deacetylation mechanisms, which are mediated by histone deacetylases (HDACs; Shabanaxian and Gurestein, 2007). It is likely that deacetylation of HIV-1 IN also takes place. To find a cellular partner that selectively recognizes the acetylated IN, Cereseto and colleagues developed a unique yeast two-hybrid screening assay, in which acetylated HIV-1 IN was constitutively expressed as the bait by fusion of the p300 HAT domain to IN (Allouch et al., 2011). From the screening of a human lymphocyte cDNA library, they identified that a cellular protein, KAP1 was able to interact with acetylated IN (Figure 4B; Allouch et al., 2011). KAP1 was first identified as a transcriptional corepressor that is recruited to its target genes via interaction with the Keap1-associating box-domain-containing (KRAB) zinc-finger DNA proteins (Friedman et al., 1996; Peng et al., 2000). Interestingly, KAP1 is a member of the human TRIM family, and hence contains a RING (really interesting new gene) finger, two B-box zinc fingers and a coiled coil domain at the N-terminal, which is a characteristic feature of the TRIM family protein domains (Figure 4B; Iyengar and Farrarham, 2011). Additionally, KAP1, also called TRIM28 and transcription intermediary factor (TIF) 1β, is composed of a plant homodomain (PHD) and bromodomain at the C-terminus, and contains a central TIF signature sequence (TSS) domain and a heterochromatin protein 1 (HP1)-binding domain, which are found in other TIF1 subfamily members (Iyengar and Farrarham, 2011). In accordance with the yeast two-hybrid screen, preferential binding of KAP1 to acetylated HIV-1 IN was confirmed by in vitro and in vivo analysis. Importantly, knockdown of KAP1 increased the level of integration in HIV-1-infected cells, which was accompanied by a decrease in 2-LTR circles (Allouch et al., 2011). When the infection experiments were performed with HIV-1 carrying mutations in the C-terminal lysines (Lys-264, Lys-266, Lys-273) of IN, knockdown of KAP1 did not significantly increase the levels of integrated DNA, indicating that KAP1-mediated inhibition was related to the C-terminal acetylation of IN. Consistent with this, the level of acetylated IN was shown to be decreased by expression of KAP1 in cells (Allouch et al., 2011). KAP1 has been reported to form complexes with HDAC to regulate transcription at specific target sites on the genome via modification of histone structure (Iyengar and Farrarham, 2011). Together with the observation that KAP1 has a higher affinity to acetylated IN, the evidence raises a possibility that KAP1 may serve as a scaffolding mediator that recruits HDAC to acetylated HIV-1 IN (Figure 4C). Indeed, HIV-1 IN formed a complex with KAP1 and HDAC1, and HIV-1 integration was enhanced by treatment with the HDAC inhibitors (Allouch et al., 2011). These evidences therefore support the proposed model that KAP1 recruits the HDAC to acetylated IN allowing for the HDAC1-mediated deacetylation of IN to take place, which results in the subsequent reduction of integration efficiency in HIV-1 infection (Figure 4C).
A different inhibitory mechanism of KAP1 has been also demonstrated in other retroviruses and endogenous retroviruses, which involves the transcriptional repression of the provirus in embryonic carcinoma and embryonic stem cells (Wolf and Goff, 2007, 2009; Wolf et al., 2008; Matsuji et al., 2010; Rowe et al., 2010). The KAP1-mediated inhibition of viral transcription appeared to be largely attributable to the recruitment of KAP1 to certain types of primer binding sites (PBS) near the 5′-end of the provirus containing PBS sequences complementary to tRNA^Tyr^ (MoMLV and human T-lymphotropic virus type I [HTLV-I]) and tRNA^A^-1,2 (spumavirus and Mason–Pfeizer monkey virus; Wolf et al., 2008; Wolf and Goff, 2009). In contrast, HIV-1 is not susceptible to the transcriptional silencing induced by KAP1 as its PBS is complementary to neither tRNA^Tyr^ nor tRNA^A^-1,2 (Wolf et al., 2008). Since the study by Ceres et al. and colleagues showed that MoMLV integration was not affected by depletion of KAP1 (Allouch et al., 2011), the KAP1-mediated retroviral integration block should only occur in HIV-1 replication due to the specific interaction between KAP1 and HIV-1 IN. Therefore, it can be concluded that KAP1 is an intrinsic restriction factor against a broad range of retroviruses, which inhibits either the integration or post-integration step. However, despite the ubiquitous expression of KAP1 in many cell types, substantial levels of HIV-1 integration can still be detected, indicating KAP1 may not be a very powerful integration inhibitor. Yet, it would shed light on a potentially novel KAP1-based strategy for the treatment of retrovirus infections.

**REGULATION OF INTEGRASE STABILITY BY PHOSPHORYLATION: IMPLICATION OF UNKNOWN CELLULAR INHIBITORY FACTOR INVOLVED IN DEGRADATION OF UNPHOSPHORYLATED INTEGRASE**

Phosphorylation is another type of PTMs that is also likely to influence in HIV-1 integration. Recently, phosphorylation of HIV-1 IN by c-Jun NH2-terminal kinase (JNK) was reported (Manganaro et al., 2010). JNK is one of the major groups of mitogen-activated protein kinases (MAPKs), a family of well-studied serine/threonine kinases involved in signal transduction from extracellular stimuli such as growth factors, cytokines, infection, and stress (Raman et al., 2007; Cargnello and Roux, 2011). JNK consists of three variants, JNK1, JNK2, and JNK5, which are generated through alternative splicing but exhibit distinct expressions and functions (Sabapathy et al., 2004). The MAPK signaling pathway can also be activated by the interaction of T cell receptors with antigen-major histocompatibility complex (MHC) class II molecules, resulting in T cell activation (Weiss and Littman, 1994). In HIV infection, the requirement for the activation states of CD4^+ T cells for efficient initiation and maintenance of replication has been well established (Stevenson et al., 1995; Pope and Haase, 2003; Stevenson, 2003; Chu et al., 2005). In particular, it has been demonstrated that HIV-1 infection is restricted at the post-entry stages in resting T cells, following reverse transcription and prior to integration (Burkinsky et al., 1992; Pierson et al., 2002; Coiras et al., 2009).

Manganaro et al. (2010) reported that although expression levels of JNK was low in the resting CD4^+ T cells, it became activated upon stimulation, and that even in activated CD4^+ T cells, HIV-1 infection was impaired by treatment with a specific JNK inhibitor, SP600125. Furthermore, decreased amounts of integrated DNA was detected in the SP600125-treated activated CD4^+ T cells, indicating JNK contributes to the efficient infection and integration of HIV-1. Importantly, IN was found to be phosphorylated, and a conserved serine residue in the CCD, Ser-57, was identified as the target site of phosphorylation. Since the phosphorylated IN could not be detected in cells treated with SP600125, JNK appeared to be the kinase responsible for the phosphorylation of IN (Manganaro et al., 2010).

Next question to be asked was how the IN phosphorylation augmented the efficiency of HIV-1 integration. In vitro integration activity assays using recombinant IN showed that an alanine substitution at the Ser-57 residue did not affect the strand transfer activity of HIV-1 IN, suggesting that this serine residue itself is not involved in the enzymatic activity of IN (Manganaro et al., 2010). Meanwhile, Manganaro et al. (2010) found that the Ser-57 and a flanking Pro-58 are conserved amongst most of the HIV-1 clades and subtypes, and that the phosphorylated of the serine/proline motif can potentially be recognized by peptidyl-prolyl cis-trans isomerase (PPIase), Pin1 (Lu and Zhou, 2007). Pin1 is a regulator of phosphorylation signaling which specifically recognizes phosphorylated serine or threonine residues preceding a proline. Pin1 then catalyzes the structural rearrangement of a target molecule through cis-trans isomerization of its specific proline (Lu and Zhou, 2007). Pin1 binds wild-type HIV-1 IN, but not the IN mutant with a single amino-acid mutant on the phosphorylation site (SS7A). As expected, treatment of IN-expressing cells with a Pin1 inhibitor, Pih, decreased the stability of HIV-1 IN. Moreover, in vitro incubation of recombinant IN with Pin1 increased IN resistance against a protease, subtilisin, suggesting that Pin1 induced a conformational change in phosphorylated IN, thereby rendering it less sensitive to protein degradation (Manganaro et al., 2010). Supporting this notion, treatment of cells with a Pin1 inhibitor or the depletion of endogenous Pin1 by siRNA reduced the level of integrated DNA in HIV-1-infected cells without any impairment of the reverse transcription step (Manganaro et al., 2010). More important, whereas HIV-1 carrying the Ser-57A IN mutant exhibited decreased integration activity, treatment with the proteasome inhibitor MG132 restored integration activity (Manganaro et al., 2010), demonstrating that JNK-mediated phosphorylation of HIV-1 IN at Ser-57 and subsequent prolyl cis-trans isomerization by Pin1 increased the stability of IN and thereby enhanced integration efficiency in infected CD4^+ T cells. Taken together, resting CD4^+ T cells could have lower expression/activation levels of JNK, leading to decreased IN phosphorylation and consequently decreased IN stability, resulting in inefficient HIV-1 replication. Also, one could speculate that extracellular stimulations, including cytokines and chemokines, may enhance integration in resting T cells via activation of the JNK pathway (Cameron et al., 2010; Gao et al., 2011).

Of particular interest is that the study by Manganaro et al. (2010) also implies yet-to-be identified host factor(s) participating the degradation of IN, particularly unphosphorylated form of IN, in the resting T cells. HIV-1 IN is well known to undergo proteasome-mediated degradation via ubiquitination (Devroe et al., 2003). A previous study has reported that the von...
Barrier-to-autointegration factor is involved in the organization of nuclear architecture through interaction with lamin A and lamina-associated polypeptide 2-emerin-MAN1 (LEM) family proteins, and therefore predominantly localizes in the nucleus during mitosis (Margalit et al., 2007). On the other hand, in the mitotic phase, BAF is diffusely localized and subsequently assembles on the chromatid core region at anaphase to form a scaffold for the gathering of lamin A and LEM proteins to reconstitute the nuclear envelope (Segura-Totten and Wilson, 2004; Margalit et al., 2007). Intriguingly, these cell cycle-dependent actions of BAF appear to be regulated by the phosphorylation (Margalit et al., 2007). So far, vaccinia-related kinases (VRKs) have been identified as the cellular kinases that participate in the BAF phosphorylation (Figure 5B, Nichols et al., 2006). VRKs are a family of serine/threonine kinases, and three alternative splicing isoforms, VRK1, VRK2, and VRK3, are found in human and murine cells (Nichols et al., 2006; Kleber et al., 2009). They share several domains comprised of ATP-binding motifs (Walker A and Walker B), catalytic domain (subdomain VI), active site (subdomain VII), and substrate recognition domain (subdomain VIII). Figure 5B, Boyle and T raktman, 2004). VRK was first identified as cellular homolog of the poxvirus (vaccinia virus) B1 kinase that plays an essential role in viral DNA replication in the early stage of vaccinia virus infections (Figure 5B, Nichols et al., 2006). Importantly, VRK-mediated phosphorylation of BAF causes the loss of its DNA-binding ability (Nichols et al., 2006). Depletion of VRK1 in Camarohabditis elegans results in several mitotic defects and BAF delocalization (Cortezajaz et al., 2007), suggesting that the release of BAF from chromatin during mitosis is regulated by the VRK family proteins in a phosphorylation-dependent manner.

VACCINIA-RELATED KINASES

Integration reactions mediated by PICs can be categorized into two pathways: (i) intermolecular integration into a target DNA and (ii) intramolecular integration into the viral DNA, which is also called autointegration (Lee and Craige, 1994). In order to establish productive infection, the retrovirus (or retroviral PIC) needs to avoid the suicidal autointegration pathway. Hence, a strong preference for intermolecular integration is one of the key features of the retroviral PIC (Lee and Craige, 1994). BAF was identified as a host factor responsible for blocking the autointegration of MoMLV PICs (Lee and Craige, 1998). BAF is a small dimeric protein (Margalit et al., 2007) and shown as a cellular component of HIV-1 and MoMLV PICs (Suzuki and Craige, 2002; Lin and Engelman, 2003). Crystal structure analysis has revealed that BAF binds double-stranded DNA through its helix-hairpin-helix (HHH) motif (Bradley et al., 2005; Figure 5A). Importantly, the dimerization of BAF appears to cross-bundle DNA, thereby preventing the autointegration of retroviral PICs by compacting viral DNA into rigid structure (Lee and Craige, 1998; Suzuki and Craige, 2002; Bradley et al., 2005).

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Since the DNA-binding property of BAF is crucial to the authentic integration activity of the retroviral PIC, down-modulation of the BAF activity by phosphorylation may disrupt the integration function of the PIC through dissociation of BAF from viral DNA. Indeed, our recent study revealed that BAF could be removed from the PIC following treatment with recombinant VRK1 in vitro (Suzuki et al., 2010). More important, the intermolecular integration activity of the PIC assay was strongly inhibited by the VRK1 treatment, and this was accompanied by an increase in autointegration activity (Lee and Craigie, 1994; Suzuki et al., 2010). Little inhibition of the PIC activity was observed in following treatment with mutant VRK1 harboring alanine substitutions in the catalytic subdomain VI, and also in PICs treated with wild-type VRK1 in the presence of a well-known kinase inhibitor, staurosporine, indicating that disruption of PIC function is attributed to the kinase activity of VRK1 (Suzuki et al., 2010). Based on these findings, a potential inhibitory mechanism by which VRK1 restricts PIC integration activity can be proposed: phosphorylation by VRK1 dissociates BAF from the PIC, resulting in destructive autointegration of viral DNA (Figure 5A).

When other members of the VRK family of proteins were assessed for their ability to inhibit the intermolecular integration activity of PICs, VRK2, but not VRK3, was found to abrogate the function of PICs in a similar manner with VRK1 (Suzuki et al., 2010). This raised the question of why VRK3 was unable to inhibit PIC activity. Phosphorylation of BAF exhibits biphasic phases (hypophosphorylation and hyperphosphorylation), with the phosphorylation taking place on the Thr-2, Thr-3, and Ser-4 located on the N-terminus of BAF (Figure 5A). VRK1 and VRK2 are able to catalyze the hyperphosphorylation (Nichols et al., 2006). However, the BAF product phosphorylated by an in vitro kinase assay using BAF mutants, in which Thr-2, Thr-3, and/or Ser-4 had been substituted with alanine, revealed that Ser-4 is the sole phosphorylation site by VRK1 and VRK2 (Nichols et al., 2006) and our preliminary results of an in vitro kinase assay using BAF mutants showed that subsequent phosphorylation likely takes place on Thr-3 (Suzuki and Suzuki, unpublished data). These results suggest that Ser-4 is the primary target of all VRK family proteins, but that Thr-3 is an additional preferred target of VRK1 and VRK2. The hyperphosphorylation of BAF at both the N-terminal Thr-3 and Ser-4 should therefore be a prerequisite for the functional disruption of PIC function. Although the findings of our in vitro studies propose that VRK is a cellular kinase harboring potentially restrictive activity against retroviral integration, delineating the precise role of the VRK family of proteins in retrovirus infection and integration in vivo requires further experimentation.

CONCLUSION

As discussed in this review, the processes leading up to integration are assisted by a number of host proteins. Furthermore, it has become evident that there are some cellular proteins that act as potential restriction factors. Research into intracellular effectors and inhibitors of retroviral integration therefore provides an important insight for the development of novel classes of anti-HIV drugs that can act synergistically with existing drugs targeting the active sites of viral enzymes or the binding pockets of cellular receptors.

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