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The role of Vif oligomerization and RNA chaperone activity in HIV-1 replication

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ABSTRACT

The viral infectivity factor (Vif) is essential for the productive infection and dissemination of HIV-1 in non-permissive cells that involve most natural HIV-1 target cells. Vif counteracts the packaging of two cellular cytidine deaminases named APOBEC3G (A3G) and A3F by diverse mechanisms including the recruitment of an E3 ubiquitin ligase complex and the proteasomal degradation of A3G/A3F, the inhibition of A3G mRNA translation or by a direct competition mechanism. In addition, Vif appears to be an active partner of the late steps of viral replication by participating in virus assembly and Gag processing, thus regulating the final stage of virion formation notably genomic RNA dimerization and by inhibiting the initiation of reverse transcription. Vif is a small pleiotropic protein with multiple domains, and recent studies highlighted the importance of Vif conformation and flexibility in counteracting A3G and in binding RNA. In this review, we will focus on the oligomerization and RNA chaperone properties of Vif and show that the intrinsic disordered nature of some Vif domains could play an important role in virus assembly and replication. Experimental evidence demonstrating the RNA chaperone activity of Vif will be presented.

1. Introduction

Lentiviruses differ from simple retroviruses such as alpha and gamma retroviruses by the presence of regulatory genes in their genome. In addition to the structural and enzymatic proteins required for viral replication, HIV-1 encodes for several additional proteins. These proteins can be subdivided into two groups: the so-called essential proteins Tat and Rev and the auxiliary proteins, namely Nef, Vpr, Vpu and Vif. Although the latter proteins are not required for viral replication in certain tissue cultures, they are key factors for virus dissemination *in vivo* and pathogenesis.

The viral infectivity factor (Vif) is a highly basic 23 kDa protein that is essential for HIV-1 replication in non-permissive cells including lymphocytes, macrophages and a few T-cell lines (Fisher et al., 1987; Gabuzda et al., 1992; Madani and Kabat, 1998; Simon et al., 1998; Strebel et al., 1987; Zou and Luciw, 1996). Ten years ago, the group of M. Malim demonstrated that Vif neutralizes a potent intracellular innate defense that protects mammals from retrovirus infection and retro-elements that tend to persistently invade their genome (Malim and Emerman, 2008; Sheehy et al., 2002). This component of innate immunity, called APOBEC3G (apolipoprotein B mRNA-editing enzyme-catalytic, polypeptide-like 3G or A3G) is closely related to APOBEC1, a cytidine deaminase that causes a specific cytosine to uracil change in the apolipoprotein B mRNA (Teng et al., 1993). Other members of this family include APOBEC2, A3A to A3H, and the AID enzyme that causes hypermutation of immunoglobulin genes (Harris et al., 2002). Since then, A3G was found to be a potent cytidine deaminase that causes lethal hypermutations of HIV-1 and other retroviruses during cDNA synthesis by reverse transcriptase (RT) (Henriet et al., 2009; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003). Beside its crucial counteracting activity against cellular antiviral factors, early studies revealed that Vif was also playing a role in virion assembly, favoring conformational rearrangements of the viral core (Borman et al., 1995; Hoglund et al., 1994; von Schwedler et al., 1993). It was proposed that Vif acts as a temporal regulator of viral assembly (Henriet et al., 2007). Although a large amount of data clearly showed the important role of Vif in viral pathogenesis, antiviral strategies aimed at inhibiting Vif in infected cells are slowed down due to the lack of structural information on Vif

Abbreviations: HIV-1, human immunodeficiency virus type 1; SIV, Simian immunodeficiency virus; RSV, Rous sarcoma virus; MuLV, murine leukemia virus; Vif, virion infectivity factor; APOBEC3G, apolipoprotein B mRNA-editing enzyme-catalytic, polypeptide-like 3G; SOCS, suppressor of cytokine signaling; NC, nucleocapsid protein; Tat, transcription activator protein; DLS, dimer linkage structure; PBS, primer binding site; PPT, polypurine tract.

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(Barraud et al., 2008). However recent biochemical studies revealed the importance of Vif oligomerization, conformation and flexibility in counteracting A3G and in binding RNA (Bergeron et al., 2010; Bernacchi et al., 2011; Marcsisin et al., 2011; Reingewertz et al., 2010; Wolfe et al., 2010). This prompted us to review the oligomerization and RNA chaperone activities of Vif involved in HIV-1 viral assembly and replication. Firstly we present an overview of the general characteristics and structural organization of Vif, and secondly we show that the intrinsically disordered domains of Vif could play an important role in Vif multimerization and RNA chaperone activity. Results demonstrating this latter activity are presented suggesting that this property may help Vif to achieve its pleiotropic functions during virus infection.

2. HIV-1 Vif: general characteristics and biological functions

2.1. Vif function in the infected cell

Vif is encoded for by all lentiviruses except for the Equine Infectious Anemia Virus. In HIV-1, the gene product is a 23 kDa basic protein that is produced in a Rev-dependent manner (Mandal et al., 2009) during the late phase of the replication cycle. This protein has been named Vif because its deletion has been associated with a reduction or a complete loss of viral infectivity (100–1000-fold) (Strebel et al., 1987). Interestingly, this phenotype is cell-type specific since Vif was shown to be required in cells termed non-permissive such as lymphocytes, PBMC, or macrophages (Borman et al., 1995; Courcoul et al., 1995; Fan and Peden, 1992; Gabuzda et al., 1992; Sova and Volsky, 1993; von Schwedler et al., 1993). By contrast, production of infectious particles does not require a functional Vif in permissive cell lines such as non-hematopoietic cells (HeLa, 293T, Cos7) and some leukemic T-cells (Jurkat, SupT1, C8166) (Gabuzda et al., 1992; Sakai et al., 1993). The observation that some, but not all cell lines require Vif for the production of infectious virus, could be explained by the fact that either permissive cells express a positive factor that substitutes for the absence of Vif, or else non-permissive cells express a negative factor that is counteracted by Vif. The fusion of these two cell types, giving rise to heterokaryons, exhibited a non-permissive phenotype indicating the presence of an intrinsic antiviral factor in non-permissive cells (Madani and Kabat, 1998; Simon et al., 1998). By using subtractive cDNA cloning analyses (permissive cells *versus* non-permissive cells), Sheehy and collaborators identified the cellular factor APOBEC3G, or A3G (Sheehy et al., 2002). When expressed in permissive cells, this factor renders these cells non-permissive for the replication of HIV-1Δ*vif* viruses (by reducing their infectivity) and northern blot analysis showed that A3G mRNAs were exclusively expressed in non-permissive cells (Sheehy et al., 2002). Altogether, these results showed that A3G is necessary and sufficient to confer the non-permissive phenotype to cells targeted by HIV-1.

2.2. The APOBEC3 family and its innate antiviral function

A3G is an enzyme member of the APOBEC family of cytidine deaminases, which includes (i) APOBEC1 and activation induced deaminase (AID) encoded for by genes arranged in tandem on chromosome 12, (ii) APOBEC2 located on chromosome 6 and (iii) A3A to A3H arranged in tandem on chromosome 22 (Jarmuz et al., 2002; Kitamura et al., 2011). A3G and A3F, the most closely related A3 family members, are expressed primarily in lymphoid and myeloid cell lineages (Jarmuz et al., 2002; Liddament et al., 2004; Wiegand et al., 2004). A3F constitutes the second highly potent anti-HIV host factor that specifically inhibits HIV-1Δvif infectivity and is sensitive to Vif (Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). Although a consensus concerning anti-HIV activities of other A3 proteins has not currently been reached, it is now generally admitted that A3G, A3F, A3DE and A3H (under certain conditions) are sensitive to Vif, while A3A, A3B and A3C are probably not the most relevant factors for HIV-1 restriction *in vivo* (for a recent review, see Albin and Harris, 2010), except for A3A in monocytic cells (Berger et al., 2011).

A3 proteins contain one (A3A, A3C and A3H) or a duplication (A3B, A3DE, A3F, and A3G) of the catalytic site, which contains a Cys-His Zn^{2+} coordination motif characteristic of cytidine deaminases (Jarmuz et al., 2002). Concerning A3G, the second active site was shown to be the effective one (Shindo et al., 2003). In non-permissive cells, A3G and A3F are recruited into viral particles, leading to a ~1000-fold reduction in infectivity of HIV-1Δ*vif* (Liddament et al., 2004; Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Wiegand et al., 2004). This restriction results from an intense cytidine deamination of the newly made (−) strand viral DNA during reverse transcription (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003), causing G to A transitions within the (+) strand DNA (Lecossier et al., 2003). These transitions were found all over the genome, but with a graded frequency in the 5′ to 3′ direction suggesting that modifications occur preferentially in regions ((−) strand) that become and stay transiently single-stranded during the reverse transcription (regions 3′ to the PPTs and to the PBS) (Suspene et al., 2006; Yu et al., 2004a). The target sequence preference for A3 proteins has been extensively studied (Harris et al., 2003; Yu et al., 2004a; Zhang et al., 2003). A3G, for instance, preferentially mutates cytidine residues that are preceded by two cytidines (5′-CCCA-3′ in (−) strand DNA, the underlined nucleotide being targeted) (for review see Henriet et al., 2009). While G to A hypermutations resulting in lethal mutagenesis was initially believed to be the only mechanism of viral inhibition, it later appeared that these restriction factors can also inhibit viral replication independently of their enzymatic activity and interfere with primer $tRNA^{lys,3}$ annealing, (−) and (+) strand DNA transfer, viral DNA synthesis and integration (Anderson and Hope, 2008; Bishop et al., 2006, 2008; Guo et al., 2007; Holmes et al., 2007; Iwatani et al., 2007; Li et al., 2007; Luo et al., 2007; Mbisa et al., 2007; Miyagi et al., 2007; Newman et al., 2005; Yang et al., 2007).

2.3. Vif-mediated neutralization of APOBEC3G

The different mechanisms used by Vif to prevent the antiviral activity of A3G/3F have been extensively studied (Conticello et al., 2003; Liu et al., 2005; Marin et al., 2003; Mehle et al., 2004b; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). The main mechanisms are briefly reviewed here (Fig. 1A), and readers can consult recent reviews for more detailed explanations (Albin and Harris, 2010; Henriet et al., 2009; Smith et al., 2009). Firstly, the inhibition of A3G packaging by Vif is associated with a strong reduction of its intracellular level, which has been attributed to the poly-ubiquitination and proteasomal degradation of A3G/3F by Vif (Conticello et al., 2003; Liu et al., 2005; Marin et al., 2003; Mehle et al., 2004b; Sheehy et al., 2003; Stopak et al., 2003; Yu et al., 2003). Indeed, Vif is able to bind A3G/3F through various domains (Fig. 2C), and to recruit an E3 ubiquitin ligase complex composed of Elongin B, C, Cullin 5, and Rbx2 leading to the poly-ubiquitination and degradation of A3G/3F (Luo et al., 2005; Mehle et al., 2004a, 2006; Yu et al., 2003) (Fig. 1A, pathway 2). Very recently, the transcription cofactor CBF-β (core binding factor β) has been shown to be an integral component of this ubiquitin ligase complex and to be required for Vif-mediated degradation of A3G (Hultquist et al., 2011b, 2012; Jager et al., 2011; Zhang et al., 2011). Besides, Vif has also been shown to counteract A3G by inhibiting its translation

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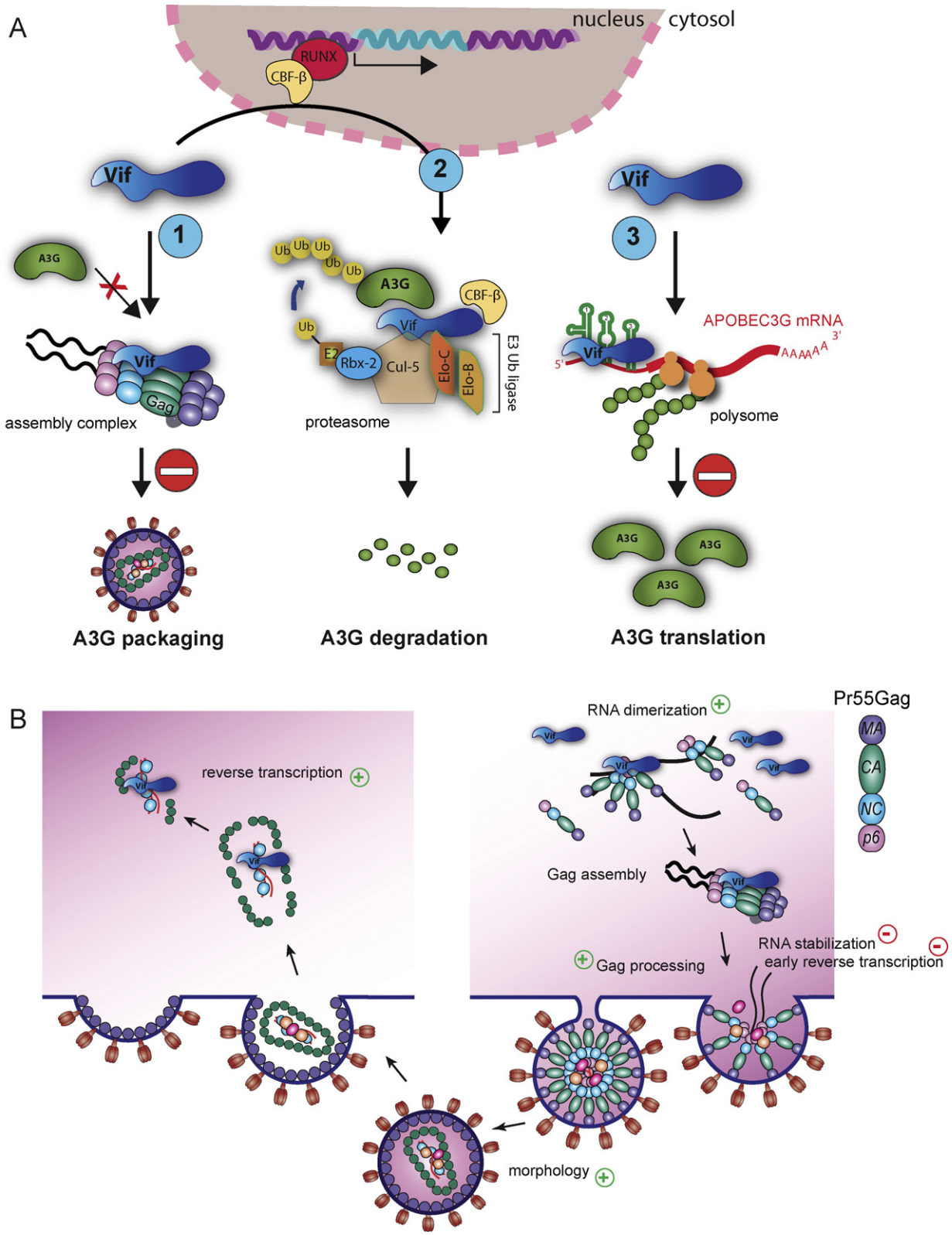

Fig. 1. Schematic representation of the functions of Vif and cellular A3G in the course of HIV-1 replication. (A) During viral particle assembly, Vif is found in the cytoplasm of infected cells and is packaged at a low copy number into virions. Vif neutralizes A3G/A3F in virus-producing cells by different mechanisms. (1) Vif competes with A3G for binding to viral components like the nucleocapsid domain of Gag and/or the genomic RNA. (2) Vif binding to A3G recruits an E3 ubiquitin ligase that mediates the poly-ubiquitynation of A3G and its degradation. (3) Vif impairs the translation of A3G mRNA through an mRNA-binding mechanism. Taken together, these three different actions of Vif on translation, degradation and packaging not only deplete A3G from virus-producing cells but also prevent A3G from being incorporated in virions. (B) Intracellular Vif may also influence viral assembly by regulating in a timely fashion genomic RNA dimerization and protease (PR)-mediated cleavage of the Gag precursor molecules. Due to its chaperone activities, Vif could facilitate late events such as Gag precursor maturation, RNA dimer maturation and initiation of reverse transcription during and after viral budding. Finally, Vif might participate in virion morphology and in reverse transcription after viral entry. The green "plus" and red "minus" signs indicate the positive and negative effects of Vif, respectively.

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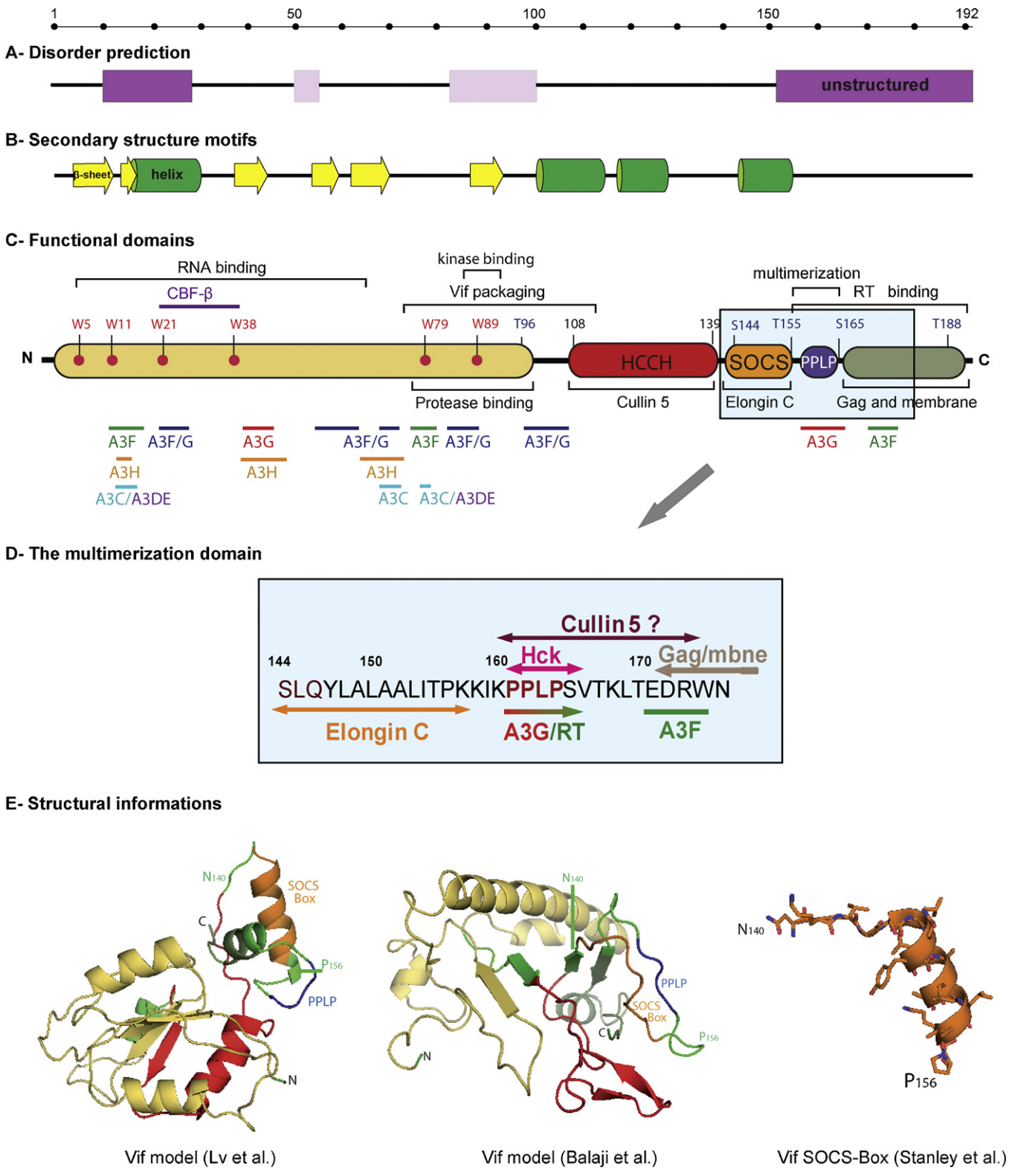

Fig. 2. Vif Sequence, structure and domains. (A) Vif disordered domain prediction according to Predictor of Naturally Disordered Regions (PONDR®): dark and light purple boxes correspond to highly disordered domains (PONDR score > 0.5), and slightly disordered domains (PONDR score between 0 and 0.2), respectively. (B) Vif secondary structure prediction showing strand in yellow arrows and helices in green was performed using PsiPred (McGuffin et al., 2000). (C) Schematic representation of Vif functional domains described in the text together with key residues and the interaction map with A3G, A3F, A3H, A3C, A3DE, and other Vif partners. (D) Vif multimerization domain, including the PPLP motif has been enlarged for a detailed description. (E) 3D structure of Vif. Left panel: structural model of Lv and collaborators (Lv et al., 2007), colored according to Vif functional domains in C; central panel: structural model (PDB: 1VZF) from Balaji and collaborators (Balaji et al., 2006); right panel: tridimensional structure of the Vif SOCS box (PDB: 3DCG – residues N_{140}–P_{156} – Stanley et al., 2008) with the same orientation as the left model.

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(Fig. 1A, pathway 3) (Kao et al., 2003; Mariani et al., 2003; Stopak et al., 2003). Yet this process is not clearly understood, but our last study suggests that a steric hindrance mechanism may be considered as Vif was shown to specifically bind to the 5′-untranslated region (UTR) of A3G mRNA, that is required for the translational repression (Mercenne et al., 2010; Guerrero and Paillart, unpublished results). Lastly, the simplest way to prevent A3G activity is probably to prevent its packaging into virions (Sheehy et al., 2003), and indeed reports showed that Vif was able to impede packaging and counteract the antiviral activity of a degradation-resistant A3G variant (Fig. 1A, pathway 1) (Mariani et al., 2003; Opi et al., 2007). Interestingly, during virus assembly, A3G interacts with the nucleocapsid (NC) domain of $Pr55^{Gag}$ and the genomic RNA, while the same partners are involved in the packaging of Vif (Burnett and Spearman, 2007; Khan et al., 2005; Luo et al., 2004; Schafer et al., 2004; Svarovskaia et al., 2004; Zennou et al., 2004), suggesting that Vif may prevent A3G packaging by means of a competition mechanism.

2.4. Functions of Vif in virus assembly

Beside its action against the APOBEC3 restriction factors, Vif was found to be involved in the late steps of virus replication (Fig. 1B). Indeed Vif interacts with the $Pr55^{Gag}$ polyprotein precursor, that drives virus assembly, and the $Pr160^{Gag\text{-}Pol}$ precursor, possibly allowing its transient association with Gag assembly intermediates in the cytoplasm (Akari et al., 2004; Bardy et al., 2001; Bouyac et al., 1997; Lee et al., 1999). Accordingly Vif is able to counteract the effect of a betulinic acid derivative that inhibits HIV-1 Gag assembly (Dafonseca et al., 2008).

In addition, Vif, as an RNA-binding protein, can bind to the HIV-1 genomic RNA *in vitro* (Bernacchi et al., 2007; Henriet et al., 2005) and in infected cells (Dettenhofer et al., 2000; Zhang et al., 2000). This interaction seems to be required for Vif packaging, since inhibiting RNA packaging by mutating NC or the Psi packaging signal led to a complete absence of Vif in virions (Khan et al., 2001). This RNA-binding ability of Vif seems to be governed by its N-terminal domain (Fig. 2C) (Khan et al., 2001; Zhang et al., 2000).

Vif was also reported to be required for normal virion morphology and optimal core stability (Borman et al., 1995; Ohagen and Gabuzda, 2000; Sakai et al., 1993). As this defect has been exclusively observed in Δ*vif* virions produced from non-permissive cells, it is likely a (direct or indirect) consequence of the expression of the antiretroviral factors A3G/3F in these cells, even though the involvement of another cellular factor cannot be totally excluded. In fact, it is known that APOBEC3G inhibits some of the reverse transcription steps that require the RNA chaperone activities of NC (Guo et al., 2007; Iwatani et al., 2007; Yang et al., 2007). These are precisely the steps that Vif is able to activate (Henriet et al., 2007). As NC is a more potent RNA chaperone activity than Vif (Henriet et al., 2007), Vif would not be required in the absence of A3G (*i.e.* in permissive cells), however, it would be required in restrictive cells, because NC chaperone activities are inhibited by APOBEC3G in these cells.

Moreover, NC and RT were less stably associated with the viral core in HIV-1Δ*vif* particles (Hoglund et al., 1994), and $Pr55^{Gag}$ processing by the viral protease (PR) was altered at the MA-CA and CA-NC junctions (Akari et al., 2004; Bardy et al., 2001; Kotler et al., 1997). Even though Vif is dispensable for HIV-1 replication in permissive cells, it appears to be part of the HIV-1 replication machinery during early reverse transcription and acts as an helper factor to promote reverse transcription and viral infectivity (Carr et al., 2006, 2008; Henriet et al., 2007; Kataropoulou et al., 2009). Indeed, HIV-1Δ*vif* replicated normally in permissive cells under optimal *in vitro* replication conditions; however, HIV-1Δ*vif* replication was impaired at the level of reverse transcription when cells were treated with a thymidylate synthase inhibitor that altered the cellular dNTP levels, suggesting that preventing the A3G anti-viral action is not the only biological function of Vif.

Vif functions in virus assembly could be viewed as controversial (Henriet et al., 2009) because Vif modulates PR activity but does not prevent maturation of newly made virions. Along this line little Vif is found in cell-free virions (between 10 and 100 molecules, with a Vif/Gag ratio of 1/47 to 1/89) while it is found in large quantity in cells (Vif/gag ratio 1:1.1 to 1:1.3) (Fouchier et al., 1996; Simon et al., 1999). One hypothesis could be that Vif acts as a temporal regulator of $Pr55^{Gag}$ assembly and processing and should thus be considered as a Janus factor with both protein and RNA chaperone activities (Henriet et al., 2007; Kovacs et al., 2009; Tompa and Kovacs, 2010) (see Section 5). In accordance with this notion, Vif might prevent premature Gag processing in the cytoplasm of infected cells, and/or modulate PR activity, thus inhibiting premature reverse transcription (Akari et al., 2000; Bardy et al., 2001; Kotler et al., 1997; Mougel et al., 2009). This could be achieved either by direct transient interactions with PR and $Pr55^{Gag}$ or by modulating $Pr55^{Gag}$–RNA interactions. Thus, a competition mechanism preventing the majority of Vif from being recruited into viral particles can be envisioned where Vif acts in a transient manner.

3. Structural organization of Vif

3.1. Biochemical properties of Vif

The HIV-1 Vif protein is a small basic protein (pI = 10.7) composed of 192 amino acids (23 kDa) where the N-terminus is enriched in tryptophan residues (Fig. 2C). The presence of a high number of conserved hydrophobic amino acids (38.5% of Vif, including 8 tryptophan and 16 leucine residues) might well explain why Vif has a strong tendency to form aggregates in solution (Fig. 2C). Interestingly, each of these tryptophan residues has been shown to be important for the binding of either A3G or A3F (see below and Tian et al., 2006). Several amino acids (T_{96}, S_{144}, T_{155}, S_{165}, T_{188}) are potential targets for cellular kinases (such as MAPK) and their phosphorylation seems to play important roles in HIV replication (Yang and Gabuzda, 1998; Yang et al., 1996). Indeed, mutating T_{96} or S_{144} leads to a marked decrease in virus infectivity. Finally, the L_{150} residue has been shown to be a processing site for HIV-1 PR (Khan et al., 2002) since mutating this residue affected viral infectivity, suggesting that Vif processing is important for function.

Alignment of HIV-1 Vif sequences highlights several conserved domains that correlate with the predicted secondary structure of Vif (Barraud et al., 2008). At the N-terminus, amino acids ^{63}RLVITTYW^{70} and ^{86}SIEW^{89} are important to form β-sheet structures that are involved in the regulation of Vif expression and in viral infectivity (Fujita et al., 2003). The last two amino acids, E_{88} and W_{89} of this β-sheet structure are also part of a hydrophilic region ^{88}EWRKKR^{93} and are critical for the replication of HIV-1 in target cells by enhancing the steady-state expression of Vif (Fig. 2B) (Fujita et al., 2003). In the central region, residues 108–139 constitute a non-consensus HCCH zinc finger motif that binds zinc and Cullin 5 (Mehle et al., 2006; Xiao et al., 2006). Next to this HCCH motif is the so-called SOCS box (^{144}SLQYLA^{149}) (Fig. 2C) due to high similarities with boxes present in SOCS (suppressor of cytokine signaling) proteins. The last highly conserved domain is the ^{161}PPLP^{164} motif also known as the Vif multimerization domain (Fig. 2C and D) (Yang et al., 2001, 2003). Interestingly, this motif belongs to the C-terminal region of Vif, predicted to be intrinsically disordered (Reingewertz et al., 2009), while being involved in many important interactions (see below).

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3.2. Structured and disordered domains of Vif

Due to its intrinsic properties, characterization of the 3D-structure of Vif is still a challenging problem and a matter of intense research. In fact Vif has a high tendency to aggregate in solution, which hampers the production of large amounts of soluble Vif required for crystallization assays and NMR studies. The 3D-structure of Vif has been predicted on the basis of model proteins that share similar secondary structure elements (Fig. 2E) (Balaji et al., 2006; Lv et al., 2007). High secondary structure similarities were found between both the N- and C-terminal domains of Vif and NarI and the SOCS Box from the VHL protein, respectively (Iwai et al., 1999). These proteins can also bind to Elongin B and C. Moreover, this model correctly fits with the secondary structure prediction shown in the alignment (Fig. 2B), contrary to the model of Balaji and collaborators which predicts only one helix (Fig. 2E). If such models are helpful to understand Vif protein organization, they unfortunately lack important Vif sequence information such as the HCCH zinc finger motif, as well as the long C-terminal region predicted to be disordered (Reingewertz et al., 2010), which constitutes another limiting factor for the crystallization of the full length Vif protein.

Yet only a very short fragment of Vif corresponding to the SOCS Box (residues 139–179) has been crystallized in a complex with Elongin B and C (Stanley et al., 2008). The crystal structure of a shorter fragment (residues 140–156) has been solved at 2.4 Å resolution (PDB: 3DCG), showing a loop-helix structure that forms a highly hydrophobic interface allowing binding to Elongin C (Fig. 2E). Interestingly, the structure of this peptide could fit in the predicted model of Lv and collaborators (in place of another helix) and could also be included in place of an unstructured sequence in the model of Balaji and collaborators (Fig. 2E – SOCS box in orange). Despite the lack of full-length Vif 3D-structure, the function of Vif has been extensively investigated by mutational and functional domain analyses. As for other HIV-1 accessory proteins, Vif appears to be a multifunctional protein with several domains involved in specific and/or overlapping functions, notably the disordered C-terminus (Tompa and Kovacs, 2010).

3.3. Functional domains of Vif

3.3.1. The N-terminal tryptophan rich domain: A3G, genomic RNA and CBF-β binding

As mentioned above, the N-terminal region of Vif is enriched in hydrophobic amino acids, notably in tryptophan residues that are conserved. Mutagenesis studies have shown that these residues are important for binding to either A3F or A3G. Binding to A3F requires residues W_{11}, W_{79}, Q_{12} and the ^{14}DRMR^{17} motif (Fig. 2C) (Russell and Pathak, 2007; Schrofelbauer et al., 2006; Tian et al., 2006). Interestingly, binding to A3G involves other residues, namely W_5, W_{21}, W_{38}, W_{89}, I_9, K_{22}, E_{45}, N_{48} and the ^{40}YRHHY^{44} and ^{85}VSIEW^{89} motifs that are the key residues for A3G binding (Fig. 2C) (Mehle et al., 2007; Russell and Pathak, 2007; Santa-Marta et al., 2005; Simon et al., 2005; Wichroski et al., 2005). Other domains have been described to be important for A3G binding, especially the PPLP motif in the C-terminal domain (Fig. 2C). Such discontinuous interacting sites suggest that the correct folding of Vif is crucial to generate the binding platform allowing interaction with APOBEC3 proteins. More recently, Vif residues important for interaction with A3H, A3C and A3DE have also been identified in the N-terminal region (Binka et al., 2012; Pery et al., 2009; Zhang et al., 2008; Zhen et al., 2010).

The N-terminal region of Vif and more precisely the first 64 amino acids are also involved in genomic RNA binding both *in vitro* (Bernacchi et al., 2007; Henriet et al., 2005) and in infected cells (Dettenhofer et al., 2000; Zhang et al., 2000). Deletion analysis underlined the importance of amino acids 75–114 for the binding of Vif to RNA in infected cells (Khan et al., 2001). CBF-β (core binding factor β), a partner of Vif involved in the E3 ubiquitin ligase recruitment, has recently been identified and the interaction domain mapped to the N-terminal domain of Vif, with an important role for W_{21} and W_{38} (Hultquist et al., 2011a; Jager et al., 2011; Zhang et al., 2011). Finally, Vif binds to the viral PR through residues 78–98 (Baraz et al., 2002) and to the cellular MDM2 E3-ligase involved in its degradation by the proteasome (Izumi et al., 2009).

3.3.2. The HCCH central zinc finger domain

One of the major functions of Vif is to recruit an E3 ubiquitin ligase in order to target A3G for proteasomal degradation. The E3 ligase recruited by Vif is composed of Elongin B, Elongin C, CBF-β, Cullin 5, and Rbx2 proteins (Yu et al., 2003). The Cullin 5 binding domain (Yu et al., 2004b) involves residues C_{114} and C_{133} of the conserved HCCH zinc finger motif (Xiao et al., 2007) of the form H^{108}-$(X)_5$-C^{114}-$(X)_{18}$-C^{133}-$(X)_5$-H^{139}, that is different from a conventional zinc finger. In fact its primary structure is not found in other classes of zinc binding proteins and thus can be considered to be Vif specific. This motif represents a *bona fide* zinc finger (Mehle et al., 2006; Xiao et al., 2007), where each zinc-coordinating residue has been shown to be important for the binding of Cullin 5. Changing sequences and spacing between HCCH residues affected the binding to Cullin 5, viral infectivity, and A3G degradation (Mehle et al., 2006). Interestingly, the conformation of both the HCCH motif and the whole Vif protein is altered upon zinc binding (Paul et al., 2006). Moreover, the aggregation of Vif is favored by zinc, but in a reversible manner (Paul et al., 2006).

3.3.3. The SOCS box: Elongin C binding domain

This highly conserved sequence is composed of residues ^{144}SLQ-(Y/F)-LA^{149} and alanine substitution in the SLQ motif leads to important defects in virus infectivity (Schmitt et al., 2009). This region promotes the interaction with the heterodimer Elongin C/Elongin B (Yu et al., 2004b) and is therefore important for the recruitment of the E3 ubiquitin ligase that targets A3G for degradation. This interaction occurs *via* a hydrophobic platform on both proteins (Wolfe et al., 2010), requiring the SLQ residues and the last A_{149} of the motif (Yu et al., 2004b) together with α-helix 4 of Elongin C (Stanley et al., 2008). Very recently, it has been shown that interactions between Vif and Elongin C/B also involve downstream elements such as the Cullin 5 box and the ^{161}PPLP^{164} motif (see below and (Bergeron et al., 2010; Wolfe et al., 2010))

3.3.4. The intrinsically disordered C-terminal domain

The C-terminal region of Vif is intrinsically disordered (Fig. 2A) and starts with a putative Cullin 5 box (residues 159–173), that is able to recruit Cullin 5 (Stanley et al., 2008) but with a lower efficiency compared to the HCCH zinc finger domain (Wolfe et al., 2010). This region encompasses the ^{161}PPLP^{164} motif involved in Vif multimerization (Fig. 2D) (Yang et al., 2001, 2003). This PPLP motif is also involved in the interaction with various partners such as A3G (Donahue et al., 2008; Miller et al., 2007), Elongin B/Cullin 5 (Bergeron et al., 2010; Wolfe et al., 2010), HIV-1 RT (Kataropoulou et al., 2009) and the cellular kinase Hck (Douaisi et al., 2005; Hassaine et al., 2001). Finally, the last 25 residues are important for Vif interaction with $Pr55^{Gag}$ (Bouyac et al., 1997; Simon et al., 1999; Syed and McCrae, 2009), cytoplasmic membranes (Goncalves et al., 1995) and RT (Kataropoulou et al., 2009).

The disordered nature of the C-terminal region (see Fig. 2A) is in line with secondary structure prediction models (Reingewertz et al., 2010) and the 3D-structure analysis of Vif where only the first 15 amino acids of the analyzed peptide (40 residues) have been observed. No electron density was detected for the rest of the

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protein, reflecting the intrinsically disordered nature of the C-terminus (Stanley et al., 2008). Deuterium incorporation assays showed that this small part of Vif folds upon binding to Elongin C, which is also refolded upon Vif binding (Marcsisin and Engen, 2010). Such Vif-induced folding has also been observed in our laboratory upon Vif binding to specific RNA sites such as the Trans-acting Responsive element (TAR) (see below and Bernacchi et al., 2011).

4. Vif multimerization

Vif multimerization was initially detected *in vitro* by GST pull-down, co-immunoprecipitation and two hybrid assays (Yang et al., 2001). A deletion mutagenesis approach allowed to characterize residues 151–164, encompassing the conserved proline-rich region ^{161}PPLP^{164}, as the domain governing Vif multimerization. The importance of this ^{161}PPLP^{164} motif has been later highlighted using competitor peptides and deletion/substitution mutants (Bernacchi et al., 2011; Yang et al., 2003). Recently, this motif has been shown to be required for the assembly of an active E3 ubiquitin ligase complex, as a second binding motif for the Elongin C/B and Cullin5 (Bergeron et al., 2010; Wolfe et al., 2010).

4.1. Importance of the Vif PPLP motif in HIV-1 replication

This motif was found to be important for Vif function and virus infectivity since mutating this motif resulted in a 2.5-fold reduction of infectivity in non-permissive cells (Donahue et al., 2008) and antagonist peptides containing the ^{161}PPLP^{164} motif drastically diminished virus replication (Miller et al., 2007; Yang et al., 2003). The reduced virus infectivity could be explained in both cases by the lost of Vif A3G-degradation function. Indeed, Miller and coworkers (Miller et al., 2007) showed that the antagonist peptides were associated with an augmented A3G packaging into viral particles. Donahue and collaborators showed that substituting AALP for PPLP impaired Vif-induced degradation of A3G, which was mainly due to the reduced binding of A3G, without affecting the interaction with Elongin C and Cullin 5 (Donahue et al., 2008). Similarly, this substitution favored A3G incorporation into virions but did not abrogate the translational regulation of A3G mRNA by Vif (Mercenne et al., 2010). However, the PPLP motif does not seem to be the only one to be involved in Vif multimerization as a recent study pointed out the involvement of domains such as the HCCH motif, the BC box and downstream residues (S165 and V166) in this property of Vif (Techtmann et al., 2012). It is also interesting to notice that while the PPLP motif is conserved in all HIV-1 isolates, this motif is absent in HIV-2 and SIV Vif (Barraud et al., 2008) and the C-terminal regions are the most divergent, beside the fact that both HIV-2/SIV Vif proteins are 25 amino acids longer than the HIV-1 Vif (Yamamoto et al., 1997). Whereas the HIV-1 Vif C-terminal region is highly hydrophilic (and seems important for the association with membranes), the same region of HIV-2 Vif is hydrophobic. Finally, while both Vif proteins have the same functions and are interchangeable in some cellular conditions (Reddy et al., 1995), differences in some structural domains (oligomerization domain for instance) may suggest that the mechanism of action of HIV-1 and HIV-2/SIV Vif proteins may not be completely identical. Up to now, oligomerization properties of HIV-2/SIV Vif proteins are unknown.

4.2. RNA binding and Vif folding

As mentioned above, the PPLP motif is located in a strategic binding platform for Vif partners. With respect to the involvement of the PPLP motif in Vif oligomerization, it is conceivable that the functions of those partners could be linked to the multimerization state of Vif. We recently report that alanine substitution of the PPLP motif (Vif-AALA) did not significantly affect the overall secondary structure of Vif, but affects its oligomerization state (Bernacchi et al., 2011). Nevertheless, Dynamic Light Scattering experiments showed that the AALA Vif mutant was still able to form protein dimers, suggesting that other domains are involved in Vif-Vif interactions, as mentioned in another study using deuterium exchange assays (Marcsisin and Engen, 2010). Mutating the PPLP motif also led to a decrease of Vif binding affinity and specificity for nucleic acids (Bernacchi et al., 2011).

Interestingly, the high affinity binding of wild-type Vif to the TAR element led to the formation of high molecular mass complexes and was associated with Vif folding since the proportion of disorder, defined by circular dichroism experiments, decreased from 40% in absence of RNA to less than 20% in presence of TAR, and β-sheet structures increased from 40% to 70%. This folding process was not observed when AALA was substituted for the PPLP motif (Bernacchi et al., 2011). Altogether, these results showed that both the PPLP motif and RNA binding are important to induce, at least in part, Vif folding. As mentioned above, the Vif-Elongin C interacting domain has also been shown to undergo structural rearrangements upon binding (Marcsisin and Engen, 2010).

These observations strongly suggest that Vif (or at least its C-terminal domain) is disordered in its free form and can fold upon interactions, thus ensuring multiple functions (He et al., 2009). These properties are characteristic of proteins with RNA and/or protein chaperone activities (Tompa and Kovacs, 2010): as a general mechanism, such protein–protein and protein–RNA interactions induce the folding of both the chaperone and the target molecules upon binding, forming a functional complex (Semrad, 2011; Tompa and Csermely, 2004). This has already been described for several HIV-1 proteins such as the NC protein (Muriaux and Darlix, 2010; Rein, 2010) and more recently proposed for the Tat protein (Xue et al., 2011). This new potential role of Vif and its impact on HIV-1 replication are described below.

5. RNA chaperone activity of Vif

The three-dimensional structure of cellular and viral RNA molecules is crucial for function, but RNA molecules tend to adopt non-functional structures due to kinetic and thermodynamic folding traps (Herschlag, 1995). In order to prevent and resolve these non-functional conformations, proteins with RNA chaperone activity are essential during the RNA folding process (Cristofari and Darlix, 2002). An RNA chaperone is defined as a partially disordered protein that binds transiently and non-specifically to RNA molecules, preventing their misfolding in an ATP independent-manner. Once the RNA has been correctly folded, the protein is no longer needed and the RNA conformation remains stable (Cristofari and Darlix, 2002).

Proteins with RNA chaperone activity are widespread and are implicated in essential cellular functions (Rajkowitsch et al., 2007). The list of RNA chaperones is constantly increasing and a database has been constructed (http://www.projects.mfpl.ac.at/rnachaperones/index.html) in which RNA chaperones are classified into families according to their primary function (Rajkowitsch et al., 2007). Here, we will focus on retrovirus-encoded RNA chaperones, specifically those encoded by HIV-1.

Nucleocapsid proteins (NCp) from Rous sarcoma virus (RSV) and murine leukemia virus (MuLV) were the first retrovirus-encoded RNA chaperones to be described (Prats et al., 1988). Later, the RNA chaperone activity of HIV-1 NC was documented using different approaches. *In vitro*, HIV-1 NC increases hammerhead-ribozyme

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cleavage of an RNA substrate (Tsuchihashi et al., 1993), facilitates annealing of complementary DNA oligonucleotides and promotes strand exchange between complementary double-stranded and single-stranded DNA oligonucleotides (Tsuchihashi and Brown, 1994). In cell culture, NCp efficiently increases splicing of a thymidylate synthase mRNA mutant in a HIV-1 folding trap assay (Clodi et al., 1999). It has also been reported that in addition to NCp7, Vif and the transcription activator (Tat) possess RNA chaperone activity (Henriet et al., 2007; Kuciak et al., 2008).

Next, we will briefly summarize the RNA chaperoning activity of Vif in parallel with that of NCp7 and Tat, and discuss how this could influence virus assembly and Gag processing, thus regulating the final stage of virion formation including reverse transcription by means of a molecular crowding phenomenon where Vif in excess has a negative impact on NC functions, which is relieved once virions are released from the cells and matured (Darlix et al., 2011).

5.1. Dimerization of the genomic RNA

During virus assembly, the full-length viral genomic RNA is packaged in the form of a dimeric RNA (Chen et al., 2009; Housset et al., 1993) where the two copies are physically linked through a site called the dimer linkage structure (DLS) located close to their 5′ ends. Dimerization and subsequent packaging of the HIV-1 RNA dimer are mediated by interactions between the NC domain of $Pr55^{Gag}$ and the 5′-end of the viral genome (reviewed in Lu et al., 2011; Paillart et al., 1996, 2004; Russell et al., 2004). The HIV-1 genomic RNA can form two different kinds of dimers *in vitro*, termed loose and tight dimers, which are defined by their low or high thermostability, respectively (Paillart et al., 2004). This dimeric structure is important for viral infectivity (Shen et al., 2000). It has been shown that NCp promotes the formation of HIV-1 RNA loose and tight dimers and stabilizes the resulting dimeric structure (Muriaux et al., 1996). *In vitro*, Vif alone was also capable of stimulating the formation of the loose HIV-1 RNA dimer, but not formation of the tight dimer, at least when using long RNA fragments (600-nt long RNA). Interestingly, Vif also significantly inhibited NCp-induced tight RNA dimer formation. This inhibition might temporally prevent premature tight dimer formation in the cytoplasm of infected cells and thus assembly (Henriet et al., 2007; Cimarelli and Darlix, 2002).

5.2. Initiation of reverse transcription

Reverse transcription is primed by the annealing of the cellular $tRNA^{Lys,3}$ onto the primer binding site (PBS) in the 5′ region of the viral RNA genome. The $tRNA^{Lys,3}$ is selectively packaged into virions and used as a primer for reverse transcriptase (RT) to initiate minus-strand strong stop DNA synthesis ((−) ssDNA) (reviewed in Isel et al., 2010). HIV-1 NCp chaperones the annealing of the $tRNA^{Lys,3}$ primer onto the PBS by facilitating structural changes in the $tRNA^{Lys,3}$ as well as in the viral RNA (Barraud et al., 2007; Brule et al., 2002; Hargittai et al., 2004; Isel et al., 1995; Tisné, 2005; Tisné et al., 2004).

Henriet and collaborators showed that Vif can also promote the annealing of $tRNA^{Lys,3}$ to the PBS, which generates a functional $tRNA^{Lys,3}$/RNA complex, even though Vif was less efficient than the NC proteins used in this study (NCp15, NCp9 and NCp7) (Henriet et al., 2007). Interestingly, Vif also significantly inhibited NC-induced $tRNA^{Lys,3}$ annealing. This inhibition was observed using a Vif/NC ratio of 1/3, which is similar to the 1/2 Vif/$Pr55^{Gag}$ ratio in the assembly complexes in producing cells (Fouchier et al., 1996; Simon et al., 1999). However, in HIV-1 virions the Vif/NC ratio drops down to 1/20 to 1/40 and the inhibitory effect is then relieved. It has thus been proposed that Vif might be a temporal regulator, preventing premature initiation of reverse transcription (Henriet et al., 2007). During virus assembly, the high concentration of Vif would inhibit NC-mediated $tRNA^{Lys,3}$ annealing through an interaction with the genomic RNA and the NC domain of $Pr55^{Gag}$ (Henriet et al., 2007). This hypothesis could explain that mutations in NC cause premature reverse transcription (Didierlaurent et al., 2008; Houzet et al., 2008) since such mutations could destabilize the interaction between Vif, the genomic RNA and the NC domain of $Pr55^{Gag}$.

In a manner similar to NCp7 and Vif (Beltz et al., 2003), Tat can anneal primer $tRNA^{Lys,3}$ onto the PBS of the HIV-1 RNA genome (Kameoka et al., 2002). Similarly to Vif, Tat might well regulate premature initiation of reverse transcription by inhibiting the RT activity (Kameoka et al., 2001). This inhibition is observed at high concentrations of Tat and during the late stages of viral replication (Kameoka et al., 2001). However, at a low concentration possibly found in viral particles (Chertova et al., 2006), Tat might be able to promote $tRNA^{Lys,3}$ annealing onto the PBS (Kameoka et al., 2002) and to stimulate reverse transcription through an interaction with RT (Apolloni et al., 2007).

5.3. Minus-strand DNA transfer

Once the (−) ssDNA has been synthesized by RT, it must be transferred onto the 3′-end of the viral genome to resume elongation of the (−)-strand DNA. This transfer is achieved by the annealing of the repeat regions (R) (containing the TAR element) at the 3′ ends of the (−) ssDNA and the (+) sense viral RNA (Basu et al., 2008). NCp7 chaperones minus-strand DNA transfer by promoting the annealing of the complementary (+) and (−) TAR sequences and preventing non-specific self-priming reactions (Driscoll and Hughes, 2000; Guo et al., 1997; Li et al., 1996). Vif is also able to stimulate (−) ssDNA transfer, although with a reduced efficiency compared to NC proteins (NCp15, NCp9 and NCp7) (Henriet et al., 2007). It was later demonstrated that Tat can also anneal complementary TAR DNA sequences and to promote DNA strand displacement (Kuciak et al., 2008). A recent fluorescence study shed light on the mechanism of action (Boudier et al., 2010). In the presence of Tat, the complementary sequences anneal through their stem termini, forming an intermediate with 12 intermolecular base pairs that is finally converted to an extended duplex. This mechanism is similar to that of NCp-induced annealing, except that Tat only marginally destabilizes the TAR structure and acts at much lower oligonucleotide fractional saturation (Boudier et al., 2010). In the same study Tat was also shown to cooperate with NCp in annealing the complementary TAR sequences. In addition, it was reported that the TAR DNA annealing activity of NCp7 was efficiently inhibited by small 2′O-methylated oligoribonucleotides (mODN). These mODNs are potent inhibitors of the HIV-1 reverse transcription complex (Grigorov et al., 2011).

5.4. Minus-strand DNA elongation

During (−) strand DNA elongation, RT has to resolve secondary structures in the RNA template causing pausing and eventually premature termination of DNA synthesis. The nucleic acid chaperone activity of NCp reduces RT pausing by transiently destabilizing these RNA secondary structures, thus promoting minus-strand DNA elongation (Ji et al., 1996; Wu et al., 1996). Vif is also capable of reducing RT pausing and enhancing minus-strand DNA elongation (Henriet et al., 2007). To support this observation, it was shown that Vif and NCp7 bind to several secondary structure motifs in the 5′-UTR of the genomic RNA (Bernacchi et al., 2007; Henriet et al., 2005). These results indicate that both NCp and Vif reduce RT pausing and promote RT processivity (Bampi et al., 2006).

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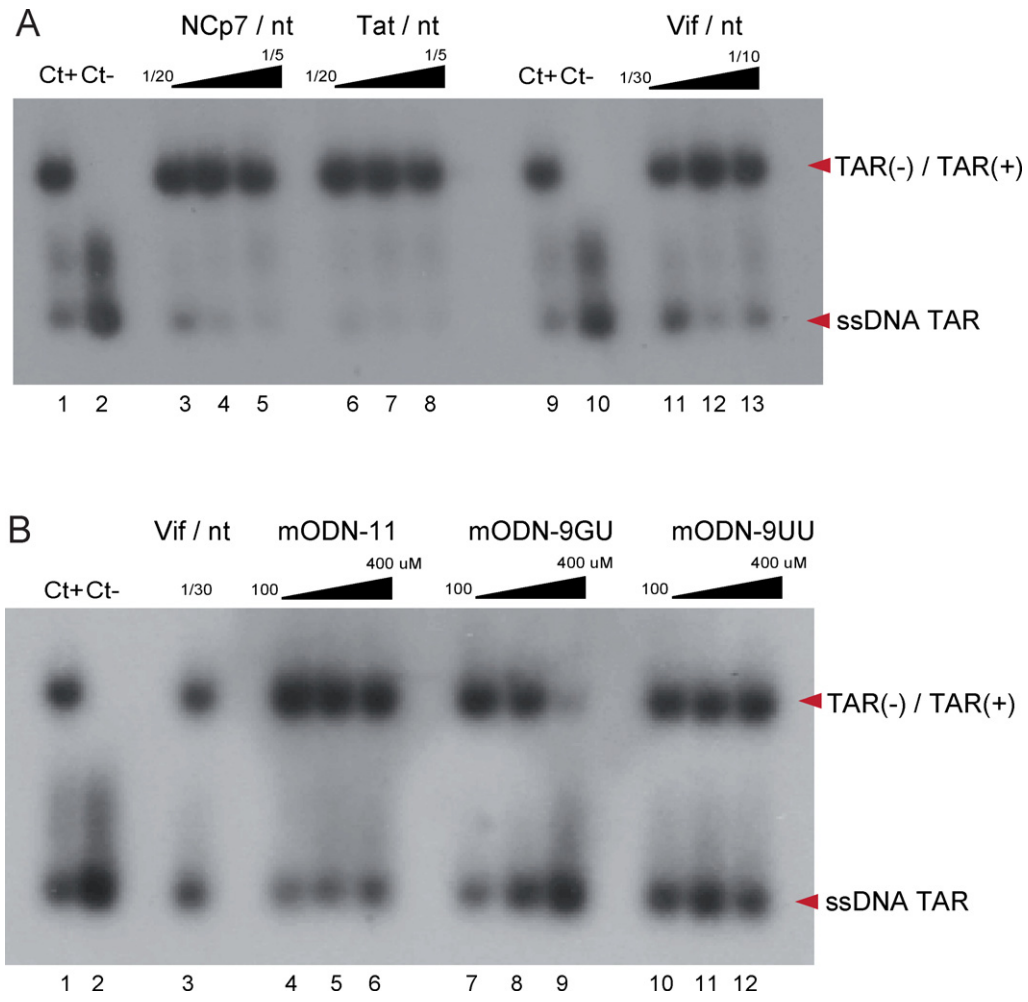

Fig. 3. Stimulation of HIV-1 TAR(+)/TAR(−) DNA annealing by Vif. (A) TAR(+) and ^{32}P-labeled TAR(−) DNA oligonucleotides were co-incubated in the presence of increasing concentrations of NCp7, Tat and Vif. In the absence of protein, double-stranded TAR DNA was formed by annealing upon incubation at 65 °C for 30 min (Ct+, lanes 1 and 9) but not at 37 °C (Ct−, lanes 2 and 10). Lanes 3–5: TAR(+)/TAR(−) annealing at 37 °C for 5 min with HIV-1 NCp7 at protein/nt molar ratios of 1/20, 1/10 and 1/5. Lanes 6–8: TAR(+)/TAR(−) annealing at 37 °C for 5 min with HIV-1 Tat at protein/nt molar ratios of 1/20, 1/10 and 1/5. Lanes 11–13: TAR(+)/TAR(−) annealing at 37 °C for 5 min with HIV-1 Vif at protein/nt molar ratios of 1/30, 1/15 and 1/10. (B) Inhibition of Vif-mediated TAR DNA annealing by methylated ODNs (mODN). TAR(+) and ^{32}P-labeled TAR(−) DNA were co-incubated in the presence of HIV-1 Vif and increasing concentrations of either one of the mODNs. Upon annealing in the absence of protein at 65 °C for 30 min a double-stranded TAR DNA was formed (Ct+, lane 1) but not at 37 °C (Ct−, lane 2). Lane 3–12: TAR(+)/TAR(−) annealing at 37 °C for 5 min with HIV-1 Vif at protein/nt molar ratio of 1/30 in the absence of mODN (lane 3) or with increasing concentrations (100–400 μM) of mODN-11 (lanes 4–6), mODN-9GU (lanes 7–9), and mODN-9UU (lanes 10–12), respectively.

5.5. Plus-strand DNA transfer

As (−) strand DNA synthesis proceeds, RT initiates (+) strand DNA synthesis using a polypurine tract (PPT) as a primer. The resulting short DNA product is known as the positive strong-stop DNA ((+) ssDNA). In order to complete (+) strand DNA synthesis, (+) ssDNA must be transferred to the 3′ end of the (−) strand DNA. During this process, the PBS sequence at the 3′ end of (+) ssDNA anneals to the (−) PBS sequence at the 3′ end of the (−) strand DNA to form a circular intermediate (reviewed in Basu et al., 2008). These complementary PBS sequences cannot be annealed unless the primer tRNA is cleaved off from the 5′-end of the (−) strand DNA. Acting as a nucleic acid chaperone, NCp favors tRNA primer removal and chaperones PBS annealing (Isel et al., 2010).

In the next section, we present experimental evidences that extend our knowledge on the RNA chaperone properties of Vif by analyzing the annealing of DNA oligonucleotides TAR(−)/TAR(+), the dimerization of HIV-1 RNA fragments and the enhancement of ribozyme-directed RNA substrate cleavage by Vif, in parallel with HIV-1 NCp7.

6. The RNA chaperone activity of HIV-1 Vif: experimental evidence

6.1. Materials and methods

6.1.1. DNA and RNA substrates

Oligodeoxynucleotides (ODNs) used for the TAR(−)/TAR(+) DNA annealing assay corresponded to nucleotides 1–56 of the HIV-1 RNA sequence (MAL isolate) in the sense and antisense orientations, as previously described in Kuciak et al. (2008). RNA fragments used in the RNA dimerization assay corresponded to nts 1–311 of the HIV-1 MAL isolate. The R3 ribozyme and the S14 substrate RNAs were used for the hammerhead ribozyme cleavage assay. Methylated oligoribonucleotides mODN-11, mODN-9GU, mODN-9UU and non-methylated ODN-12 are described in Grigorov et al. (2011).

6.1.2. In vitro transcribed RNAs

RNAs used for the ribozyme assay were synthesized by *in vitro* transcription with T7 RNA polymerase as previously published (Kuciak et al., 2008). For dimerization assay, RNA 1–311 was obtained from run-off transcription of plasmid pJCB that was

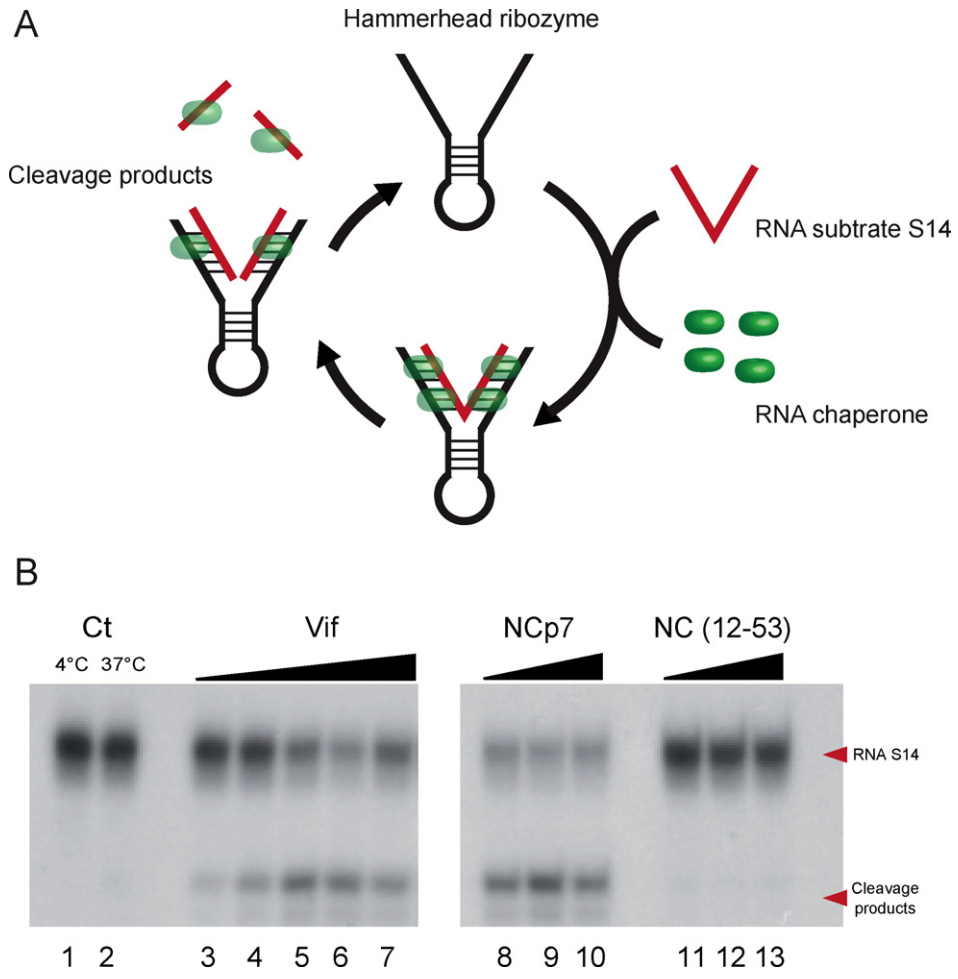

Fig. 4. Enhancement of hammerhead ribozyme-induced cleavage by HIV-1 Vif protein. (A) Schematic representation of hammerhead ribozyme cleavage of RNA. (B) R3 ribozyme and an excess of the S14 substrate RNA were co-incubated with increasing concentrations of Vif, NCp7 and NCp(12–53). RNA S14 is 61 nts in length, while the cleavage product is 49 nts long. Lanes 1 and 2: control cleavage of the RNA S14 substrate by the hammerhead ribozyme for 30 min at 4 °C or 37 °C. Lanes 3–13: incubation for 10 min with Vif at protein/nt molar ratios of 1/50, 1/40, 1/30, 1/20 and 1/15 (lanes 3–7), with NCp7 at protein/nt molar ratios of 1/20, 1/10, 1/5 (lanes 8–10) and with NCp(12–53) at protein/nt molar ratios of 1/10, 1/5, 1/2 (lanes 11–13).

linearized using Rsa I restriction endonuclease (Henriet et al., 2005). Reverse transcription of a viral RNA fragment corresponding to the very 3′ 620 nts of the HIV-1 genomic RNA was as described in Kuciak et al. (2008).

6.1.3. Proteins

Wild-type HIV-1 Vif protein was expressed in *Escherichia coli* with an N-terminal 6-His tag and purified as previously described (Bernacchi et al., 2011). HIV-1 NCp7 and NC(12–53) were synthesized and purified as described in Ivanyi-Nagy et al. (2008). HIV-1 Tat protein was synthesized as reported in Ivanyi-Nagy et al. (2008) and Kuciak et al. (2008).

6.1.4. TAR(−)/TAR(+) DNA annealing assay

The DNA annealing assay was performed as described in Grigorov et al. (2011). Briefly, 0.03 pmol of the TAR(+) and ^{32}P-TAR(−) ODNs were incubated in 10 μl of buffer A (25 mM Tris-HCl, 0.2 mM $MgCl_2$ and 30 mM NaCl) with increasing concentrations of Vif, NCp7 or Tat proteins, as indicated in the figure legends (Fig. 3). In an attempt to inhibit the nucleic acid chaperoning activity of Vif, we used 2′O-methylated oligoribonucleotides (mODN) at increasing concentrations (100–300 nM) since such mODNs were found to strongly inhibit NCp7 *in vitro* and HIV-1 replication in T cells and macrophages (Grigorov et al., 2011). First viral proteins of interest were pre-incubated with the mODNs and subsequently used in the annealing assay (see below). Annealing reactions were performed at 37 °C for 5 min except for the positive control that was incubated at 65 °C for 30 min and put at 4 °C. To stop the reaction and remove the protein from the ^{32}P-ODN, 5 μl of a solution containing 20% glycerol, 20 mM EDTA pH 8.0, 2% SDS, 0.25% bromophenol blue and 0.4 mg/ml calf liver tRNA were added. Samples were resolved by 8% native PAGE in 50 mM Tris–borate pH 8.3, 1 mM EDTA at 4 °C. Subsequently, gels were autoradiographed and the amounts of labeled single-stranded and double-stranded DNA were assessed by phosphorimaging.

6.1.5. Hammerhead ribozyme cleavage assay

Hammerhead ribozyme cleavage assay was carried out as previously described (Kuciak et al., 2008). Briefly, ribozyme and substrate RNAs were independently heated for 1 min at 90 °C in water. The reaction buffer was added to final concentrations of 5 mM $MgCl_2$, 100 mM NaCl, 20 mM Tris–HCl, pH 7.5. After slow cooling to 37 °C, RNAs were incubated for 5 min at 20 °C. Ribozyme (0.02 pmol) and RNA substrate (0.08 pmol) were then combined in a final volume of 10 μl. NCp7, NC(12–53), Vif or Tat were added at the final protein to nucleotide molar ratios indicated in the figure legends. Incubation was for 20–30 min at 37 °C. Reactions were stopped by adding 20 μl of the stop solution (0.3% SDS, 15 mM EDTA), and extracted with 30 μl of phenol and 15 μl of chloroform. The aqueous phase was precipitated with ethanol and the pellet

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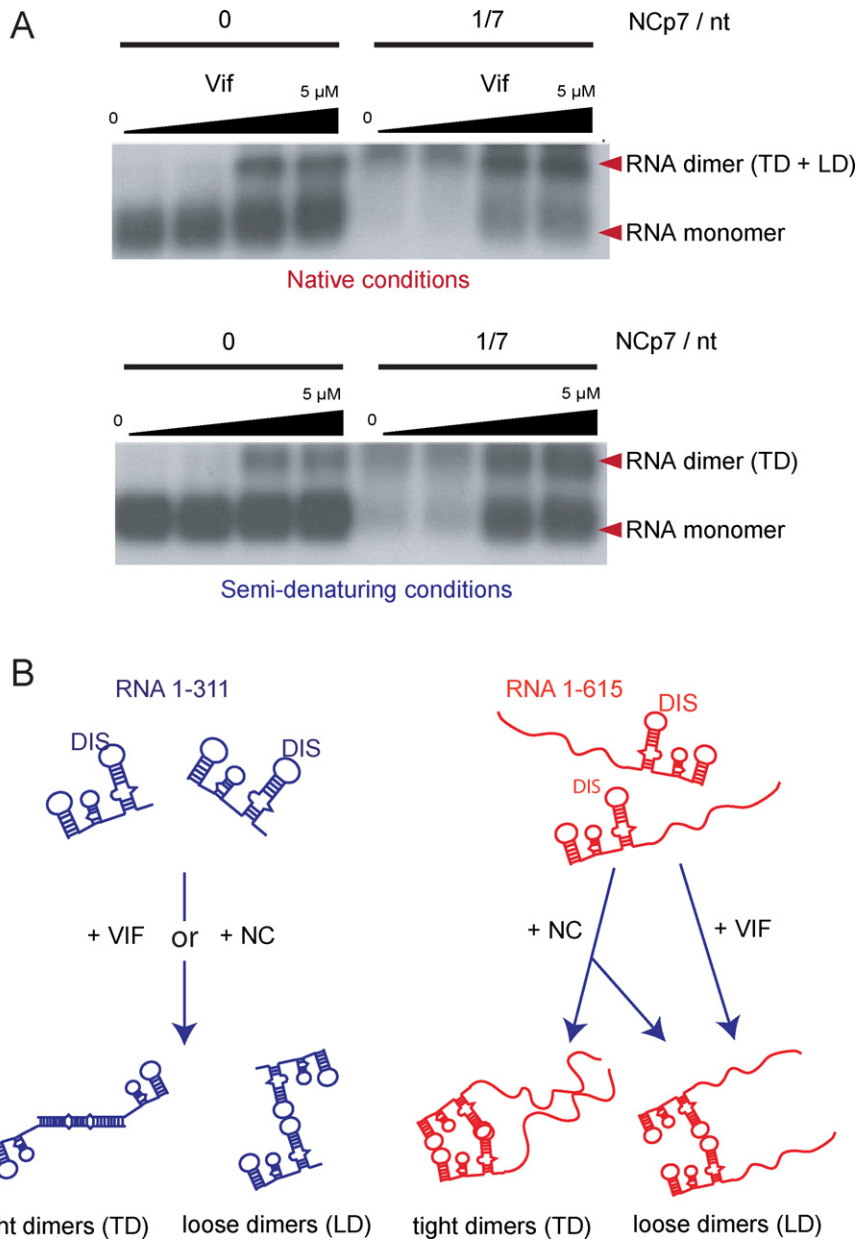

Fig. 5. RNA dimerization induced by Vif and NCp7. (A) HIV RNA 1–311 was allowed to dimerize in the presence of increasing concentration of Vif (0, 0.2, 2.0 and 5.0 µM), in the absence or in presence of NCp7 at a protein/nt molar ratio of 1/7. After protein removal, RNA was visualized under native and semi-denaturing electrophoresis conditions. (B) Schematic representation of Vif and NCp-induced RNA dimerization under native and semi-denaturing conditions.

re-suspended in 45% formamide, 0.5× TBE and 0.1% dyes. ^{32}P-labeled RNAs were analyzed on 8% PAGE in 7 M urea and 0.5× TBE. After electrophoresis, gels were autoradiographed and the efficiency of RNA cleavage was analyzed by phosphorimaging.

6.1.6. RNA dimerization assay

RNA dimerization was performed as described previously (Henriet et al., 2007). Briefly, 100 nM of unlabeled HIV-1 1–311 RNA fragment were diluted in 10 µl of water together with the corresponding labeled RNA (5000 cpm, 3–5 nM). Samples were denatured for 2 min at 90 °C, and snap-cooled on ice for 2 min. Dimerization was initiated by addition of Vif and/or NCp7 under conditions disfavoring salt-induced RNA dimerization (50 mM sodium cacodylate pH 7.5, 50 mM NaCl, 0.1 mM $MgCl_2$). When both proteins were present in the same reaction, the order of addition of the two proteins did not influence the final result. RNA samples were incubated 30 min at 37 °C and deproteinized as above, then resuspended in glycerol-containing loading buffer, split in two equal volumes and analyzed on a 0.8% agarose gel in native (Tris–Borate 0.5×, $MgCl_2$ 0.1 mM, run at 4 °C) or semi-denaturating (TBE 1×, and run at 20 °C) electrophoresis conditions. These two electrophoresis conditions have been tested in order to discriminate loose/unstable RNA dimers (only visible on native conditions) from tight/stable RNA dimers (visible under both electrophoresis conditions). Gels were fixed in 10% trichloroacetic acid for 10 min and dried for 1 h under vacuum at room temperature. Radioactive bands corresponding to monomeric and dimeric species were visualized and quantified using a FLA 5000 (Fuji).

6.2. Results and discussion

6.2.1. HIV-1 Vif activates TAR(+)/TAR(−) annealing

The annealing of the complementary TAR sequences is essential to achieve (−) strand DNA transfer. To better understand

Vif-induced (−) strand DNA transfer, we compared the ability of Vif to promote the annealing of two complementary TAR sequences with that of NCp7 and Tat. In the absence of protein, TAR(+)/TAR(−) annealing occurred at 65 °C in high salt conditions in the positive control (Fig. 3A, Ct+). No TAR(+)/TAR(−) annealing was observed at 37 °C in the negative control (Fig. 3A, Ct−). Incubation with NCp7 or Tat at 37 °C for 5 min favored TAR(+)/TAR(−) annealing at protein to nucleotide molar ratios of 1/20, 1/10 and 1/5 (Fig. 3A, lanes 3–8). In the presence of Vif, TAR(+)/TAR(−) annealing was also observed, but at higher protein to nucleotide molar ratios of 1/30, 1/15 and 1/10 (Fig. 3A, lanes 11–13), indicating that Vif annealing activity was slightly less efficient than that observed for NCp7 or Tat.

6.2.2. Inhibition of Vif-mediated TAR(+)/TAR(−) annealing by 2′O-methylated ODNs

In an attempt to characterize molecules capable of inhibiting the chaperoning activity of Vif, we used methylated oligonucleotides (mODN) found to bind to NCp7 and extensively impair its chaperoning activity *in vitro* and virus replication in T-cells and macrophages (Grigorov et al., 2011). To this end, we monitored the effect of mODNs on Vif-mediated *TAR(+)/TAR(−) annealing* as previously reported for NCp7 (Fig. 3B) (Grigorov et al., 2011). In the positive control of Vif-mediated TAR(+)/TAR(−) annealing, Vif was used at a protein to nucleotide molar ratio of 1/30 (Fig. 3B, line 3). We observed no effect on the TAR(+)/TAR(−) annealing by Vif after addition of increasing concentrations (100–400 μM) of mODN-11 and mODN-9UU (Fig. 3B, lanes 4–6 and 10–12, respectively). However, addition of mODN-9 GU drastically reduced TAR(+)/TAR(−) annealing by Vif (Fig. 3B, lanes 7–9). This result suggests that mODNs could be used to inhibit not only the nucleic RNA chaperone activity of NCp7 (Grigorov et al., 2011) but also that of Vif.

6.2.3. Vif activates hammerhead ribozyme-directed RNA cleavage

The hammerhead ribozyme cleavage assay was used to monitor two RNA chaperone properties: the annealing of the RNA substrate to the ribozyme, and the subsequent dissociation of the RNA products (Fig. 4A) (Cristofari and Darlix, 2002). We observed enhanced ribozyme-directed cleavage of RNA S14 after addition of increasing concentrations of Vif at protein to nucleotide molar ratios between 1/50 and 1/3 (Fig. 4B, lanes 3–7). As expected, we observed the same effect with NCp7 (Fig. 4B, lanes 8–10). No hammerhead ribozyme-directed RNA cleavage was detected using NC(12–53) which has only little nucleic acid chaperone activity (Fig. 4B, lanes 11–13) (Cristofari and Darlix, 2002; Darlix et al., 2011; Kuciak et al., 2008).

6.2.4. Vif induces the formation of HIV-1 RNA loose and tight dimers

Previously, we reported that Vif is able to induce the formation of loose RNA dimers in a RNA dimerization assay using RNA fragments containing the first 615 nts of the HIV-1 genomic RNA (Henriet et al., 2007). Moreover, in the presence of NCp7, Vif was able to inhibit NC-induced tight RNA dimer formation (Henriet et al., 2007). We now report that Vif is able to form both loose and tight RNA dimers when dimerization assays were carried out with an RNA corresponding to the first 311 nts of the HIV-1 genome (Fig. 5A). The dimerization experiment was carried out with increasing concentrations of Vif (0–5 μM) and in the absence or presence of NCp7 (NCp7/nucleotide ratio of 1/7). We analyzed RNA dimerization using two different electrophoresis conditions: (1) native conditions in which both loose and tight dimers are stable (Fig. 5A, upper panel) and (2) semi-denaturing conditions in which only tight dimers are preserved (Fig. 5A, lower panel). In the absence of NCp7, Vif stimulated both loose and tight RNA dimers formation, as observed by the presence of RNA dimers under both electrophoresis conditions, with a dimerization yield of ∼40% at 5 μM Vif (Fig. 5A). In the absence of Vif (Fig. 5A, lane 5), NCp7 induced >90% RNA dimer under both electrophoresis conditions. When increasing concentrations of Vif were added, we observed a reduction of the NCp7-induced tight RNA dimer formation (Fig. 5A, lanes 5–8), indicating a competition between Vif and NCp7, as previously observed (Henriet et al., 2007). These observations suggest that Vif favors the formation of tight RNA 1–311 dimers (Fig. 5B), contrary to what was observed for RNA 1–615 (Henriet et al., 2007). Indeed, in the RNA 1–311 context, the dimerization initiation site (DIS), responsible for RNA dimerization, is located directly at the 3′-end of the molecule, which probably induced a reduction of topological constraints and could thus allow the formation of an extended duplex (Fig. 5B). Conversely, the DIS of RNA 1–615 is located in the middle of the sequence, and in this context, Vif and/or NCp7 may stabilize the RNA dimer *via* different loop-loop interactions. It is thus likely that the tight dimers formed by RNA 1–311 differ from those formed by RNA 1–615, and presumably by full length genomic RNA.

7. Conclusion and future directions

Results presented here (Figs. 3–5) indicate that Vif is an authentic nucleic acid chaperone that may be required at various stages of the HIV-1 replication cycle (Fig. 1). As a matter of fact, Vif may balance the potent RNA chaperoning activities of NC in order to regulate genomic RNA dimerization and maturation (Fig. 5). Through interactions with Gag (125), Vif would at the same time regulate assembly and Gag processing, in turn preventing premature initiation of reverse transcription by RT (90) during virus formation. Work is presently in progress in our laboratories to characterize domains, notably the disordered C-terminal region, involved in the RNA chaperone activity of Vif and evaluate their implications in virus replication.

HIV-1 Vif and the cellular APOBEC3 proteins have been extensively studied in the past 10 years and the understanding on mechanisms restricting viral replication largely benefited to the scientific community. We are just starting to decipher the multiple functions of Vif associated with its biochemical and biophysical properties and the next step will be the resolution of its tridimensional structure, either alone, or most probably in complex with A3G and the E3 ubiquitine ligase (Jager et al., 2012). The discovery and the development of new drug candidates that would inhibit Vif functions and restore the innate cellular restriction will then become an attractive possibility.

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