HIV-1 Vpr: a novel role in regulating RNA splicing

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ABSTRUCT

Pre-mRNA splicing is a critical step in gene expression for metazoans. Several viral proteins regulate the splicing of pre-mRNAs through its complex interactions between the virus and the host cell RNA splicing machinery. Here, we focus on a novel function of HIV-1 Vpr, that selectively inhibit cellular and viral pre-mRNA splicing, via interactions with components of functional spliceosomal complexes. This review discusses our current knowledge of how RNA splicing regulation is accomplished by Vpr-host factors interactions.

INTRODUCTION

The relatively small genome of HIV-1 encodes structural and enzymatic proteins, such as Gag, Pol and Env, several regulatory proteins, such as Tat, Rev and accessory proteins such as Vif, Vpr, Vpu and Nef, through alternative splicing and alternate codon usage. In recent years, considerable attention has been paid to these accessory genes and great deals of evidences had been found to discover the great contributions of these proteins to HIV-1 replication. Vif, for example, was found recently to overcome the sabotage effect of APOBEC3G against HIV reverse transcription (Sheehy et al, 2003). Vpu enhances HIV particle assembly or release in a variety of human cells by antagonize a host factor, tetherin's antiviral activity (Neil et al 2008). Nef manipulate the expression of host cell surface molecules to benefit the viral propagation.

Vpr, a virion-associated viral protein 96 amino-acids in length, has multiple biological functions including its nuclear localization activity (Ijima *et al*, 2004; Jenkins *et al*, 1998; Kamata and Aida, 2000; Kamata et al, 2005; Popov *et al*, 1998), arrest at the G2/M phase of the cell cycle (Sherman *et al*, 2000; Poon *et al*, 1998), increasing the activity of the HIV-1 long terminal repeat (LTR) (Goh *et al*, 1998), positive and negative regulation of apoptosis (Ayyavoo *et al*, 1997; Azuma *et al*, 2006; Jacotot *et al*, 2000; Nishizawa *et al*, 1999, 2000a, 2000b; Stewart *et al*, 2000) and induction of cellular nuclear envelope herniation (de Noronha *et al*, 2001). Recently, we discovered a novel role for Vpr in the selective inhibition of cellular pre-mRNA splicing both *in vivo* and *in vitro* (Kuramitsu *et al*, 2005). The role, if any, of Vpr in splicing of the HIV-1 genome is unknown. In this review, we will describe our

current understanding of the novel role of Vpr in regulating RNA splicing via Vpr-host factors interactions.

WHAT WE KNOW ABOUT SPLICING REACTION

Protein-coding sequences (exons) in primary transcripts are interrupted by intervening sequences (introns) that are eliminated from the pre-mRNA by splicing in the nucleus (Krämer 1996). The splicing reaction is carried out by the spliceosome, which comprises the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/U6 and U5, and over 150 proteins of non-snRNP proteins termed splicing factors. During splicing, successive recognition of splice sites occurs through the stepwise assembly of spliceosomal complexes E, A, B, and C on pre-mRNA (Hastings and Krainer, 2001). In the commitment complex E, U1 snRNP is bound to the 5' splice site and the U2 snRNP auxiliary factor (U2AF) and splicing factor-1 (SF1) are attached to the polypyrimidine tract upstream of the 3' splice site and the branchpoint sequence (BPS), respectively. As U2 snRNP binds to the branch site and complex E is converted to presplicing complex A, a short helix forms between a single-stranded region in U2 snRNA and the intron branch site. The next step is binding of U4/U5/U6 tri-snRNP at the 5' splice site to form the B complex. Just prior to splicing, conformational changes within complex B destabilize the association between U4 and U2/U5/U6 and transform complex B into complex C. The U2 snRNP, one of the key factors in the spliceosome assembly pathway, is classified to two complexes, SF3a and SF3b, SF3a consists of three subunits, SAP60, 62 and 114, and SF3b consists of at least four subunits, SAP49, 130, 145 and 155 an essential component of the SF3b subunit, can bind to pre-mRNA (Staknis *et al*., 1994; Hastings and Krainer, 2001; Will *et al*., 2001) and is implicated in the tethering of U2 snRNA to the branch point

site (Champion-Arnaud and Reed, 1994). The large catalytic spliceosome performs two transesterification reactions on the splice sites and subsequently result in the ligation of the exons and the excision of the intron which formed as lariat RNA with its 5' phosphate joined to the 2' ydroxyl at the branch point.

COMPLICATE SPLICING PATTERN OF HIV-1

A complex pattern of splicing generates more than 30 mRNA species from the single primary mRNA transcript of the HIV genome (Purcell *et al*, 1993, Schwartz *et al*, 1990; Tang *et al*, 1999). This complex pattern of splicing is required to generate mRNAs for viral structural, regulatory and accessory proteins, and undoubtedly plays a critical role in the regulation of HIV-1 gene expression. Viral mRNA species are classified into three main groups. The 2kb-class of mRNAs, which are fully spliced and constitutively exported to the cytosol early in the HIV-1 replication cycle, encode the regulatory proteins Tat, Rev and Nef. The singly spliced 4kb-class of mRNAs are bicistronic, and encode Env, Vpr, Vif and Vpu. The unspliced 9kb-class of mRNA encodes Gag and Gag-Pol polyproteins, and also serves as the source of HIV genomic RNA for packaging into virions. Nuclear export and translation of the 4kb- and 9kb-class of mRNAs is dependent on Rev, which binds to the Rev responsive element (RRE) present in these mRNAs. Correct splicing to produce HIV-1 viral mRNAs is dependent on alternative utilization of four 5'-donor sites, termed D1 to D4, and eight 3'-accept sites, A1, A2, A3, A4a, A4b, A4c, A5 and A7 (Purcell *et al*, 1993). An additional 3'-splice site, A6, has been identified in only one HIV-1 strain to date (Benko *et al*, 1990). Donor sites D1, D2 and D3 can couple to any of the A1 to A5 acceptor sites, whereas donor site D4 is exclusively coupled to site A7, and to site A6 when this site is present.

Well-regulated expression of all viral genes is critical for efficient viral replication and successful infection. The complex process of alternative splicing of HIV-1 mRNA is regulated primarily by the efficiencies at which the alternative splice sites of HIV-1 are utilized. Several features of suboptimal HIV-1 acceptor sites have been described, such as short and interrupted polypyrimidine tracts (O'Reilly *et al*, 1995; Si *et al*, 1997; Staffa *et al*, 1994), and irregular branch point sequences (Damier *et al*, 1997; Dyhr-Mikkelsen and Kjems, 1995). In addition, there is abundant evidence for the existence of several positive and negative *cis*-acting elements within the viral genome, which have been termed exonic splicing silencer (ESS), intronic splicing silencer (ISS), and exonic splicing enhancer (ESE) elements (Amendt *et al*, 1995; Caputi *et al*, 1999; Pongsoki *et al*, 2002; Tange *et al*, 2001). Members of the heterogeneous nuclear ribonucleo-protein (hnRNP) family such as hnRNP A/B or H selectively bind ESS and ISS elements and suppress the removal of adjacent introns (Bilodeau *et al*, 2001; Jacquenet *et al*, 2001a, 2001b). Members of the SR family of splicesome factors have been shown to selectively bind to ESE elements and recruit additional cellular factors, and ultimately suppress nearby splicing silencers (Faustino and Cooper, 2003; Manley and Tacke, 1996). However, there have also been some recent reports that SR proteins are involved in negative regulation of viral pre-mRNA splicing, while the hnRNPs exert a positive effect on splicing (Graveley *et al*, 1999; Krecic and Swanson, 1999). Recently, it was shown that sequential removal of intronic sequences from the 5' end of the HIV-1 genome was critical for efficient viral gene expression (Bohne *et al*, 2005). However, the mechanism for this remains to be elucidated

VPR BINDS NUMEROUS PROTEIN PARTENERS IN HOST CELLS

Being a multi-functional protein, Vpr, is well known to modulate the host intracellular environment: A large body of research had revealed the ability of Vpr to delay or arrest cells in the G2 phase and induce the apoptosis of host cells. Numerous Vpr binding partners have subsequently been found in host cells. The glucocorticoid receptor (GR) (Mahalingam et al, 1998) and uracil DNA glycosylase (UNG) (Gragerov eta al 1998, Withers-ward et al, 1997) had been reported to associate with Vpr and induce the G2 arrest. Another protein, Hsp70, has been proposed to inhibit Vpr induced G2 arrest, in contrast to other Vpr-binding partners (Iordanskiy, 2004). A plausible protein family, named DDB1- and Cullin 4A-associated factor (DCAF)-1 has been discovered. According to several laboratories reports, Vpr associate with a cul4A-containing E3 ligase complex via its interaction with DCAF-1 or DDB1. (Dehart et 2007, Le Rouzic et al, 2007, Schrofelbauer et al, 2007, Belzile et al, 2007). Moreover, it was suggested that Vpr induces checkpoint activation and G2 arrest by binding to the SAP145, a subunit of SF3b (Terada and Yasuda, 2006).

VPR IS REGULATOR OF CELLULAR pre-mRNA SPLICING

As mentioned above, SAP145 is an essential component of SF3b, a key factor in the spliceosome assembly. Even, Terada and Yasuda (2006) revealed that inhibition of SAP145 had no effect on the splicing of Cyclin B1, Ddk1 or cdc25, that are essential for cell cycle progression. Aida and colleagues do found that SAP145 is required for Vpr-induced inhibition of splicing of pre-mRNA. In their recent report by Hashizume they found that Vpr play a novel role as a regulator of pre-mRNA splicing both *in vivo* and *in vitro* (Kuramitsu *et al.,* 2005; Hshizume *et al.,* 2007) as follows. Given SAP145, an essential component of the SF3b factor in U2 snRNP, can bind to pre-mRNA and tethers U2 snRNP to the branch point site required for

complex A assembly (Champion-Arnaud and Reed, 1994), it has therefore been predicted that in addition physical interaction Vpr and SAP145 a splicing functional relationship exists. Likewise, it has been demonstrated that Vpr binds to ribonucleic acid via a process that requires the carboxy-terminal basic domain of the protein (de Rocquigny, 2002), which suggests the possibility of a functional association with pre-mRNA. Does Vpr control a splicing? As expected, our subsequent analyses has shown that Vpr inhibits the splicing of certain pre-mRNAs, such as *immunoglobulin M* pre-mRNA and α, *β-globin* pre-mRNA and caused the accumulation of incompletely spliced forms. In our view, because these experiments are performed during the time when Vpr is not able to induce significant G2 arrest, these novel effects of Vpr should be considered to occur via a pathway that is distinct from arrest of the cell cycle at G2. As is already our evidence, Vpr expressed from an HIV-1 provirus, in fact, was sufficient to inhibit splicing of *α-globin 2* pre-mRNA in infected cells.

VPR INHIBITS SELECTIVELY SPLICING OF THE HIV-1 GENOME

Interestingly, in Kuramitsu's research, it was proposed that the inhibition effect of Vpr on splicing is intron specific: the splicing of intron 1 of *α-globin 2* pre-mRNA was modestly inhibited by Vpr but the splicing of intron 2 was unaffected. Similarly, it was found Vpr regulated Env expression by interfering with *env* pre-mRNA splicing. Vpr selectively inhibit the removal of 3' intron of HIV-1 but not 5' intron, and subsequently increase the accumulation of env mRNA, which belongs to the singly spliced 4-kb class of mRNA. As a result the expression of Env protein is enhanced providing more antigenic Env to incorporated into virion and subsequently maintaining the high infectivity of the virus. (Zhang et al submitted).

PROPOSED MECHANISM FOR RNA PROCESSING REGUlATED BY VPR

An extending investigation by Hashizume revealed that Vpr interacts with SAP145, resulting in the inhibition of binding between SAP145 and the splicing factor SAP49 which has two RNA recognition motifs that located on the surface of SF3b and interacts directly and highly specifically with both SAP145 and pre-mRNA within the essential splicing factor SF3b. This finding is in consistence with Terada and Yasuda's report. However, the interfering of Vpr to association between SAP145 and SAP49 may have more contribution to RNA processing rather than leading to G2 arrest, since, a Vpr mutant, named R80A, could bind to SAP145 and inhibit pre-mRNA processing but failed to induce G2 arrest (Hashizume, 2007).

It is conceivable Vpr may exert its splicing inhibition effect by interfering with the assembly of the spliceosome. Actually, in our recent research (Zhang et al., submitted), Vpr bound to U1-70K (a component of the U1 snRNP), U2AF65 (associated with the polypyrimidine tract), U2B'' (a component of the U2 snRNP), U1 and U2 snRNAs, as well as SAP145, indicating that Vpr associates with functional spliceosomes to regulate splicing. Here we proposed a possible model for Vpr mediated splicing regulation (Fig 1). SAP49 tethers U2 snRNP to the branch site. The U1 and U2 snRNPs are critical for spliceosome assembly, in which U1 snRNP is tightly associated with the 5' splice site, and U2AF65 directly contacts the 3' acceptor site of the pre-mRNA during Complex E assembly. Therefore, the interaction of Vpr with a component of the snRNP may impair the tethering of the U2 snRNP to the branch point site upstream of the acceptor site (Fig1). The Herpes Simplex Virus type 1 (HSV-1) ICP27 protein, an essential HSV-1 regulatory protein, has also been

reported to inhibit splicing prior to the first catalytic step through its association with SAP145 (Smith *et al*., 2005, Bryant *et al*., 2001), while it is likely that ICP27 was also inhibits pre-mRNA splicing from its interaction with SRPK1, a member of a family of conserved kinases that are highly specific for arginine/serine dipeptides, resulting in hypophosphorylation of Serine-arginine rich (SR) proteins, which impairs their role in spliceosome assembly (Smith *et al*.,2005). NS1, an influenza virus non-structural protein, inhibits the splicing of the major class of mammalian pre-mRNAs (GU-AU introns) by binding to a specific stem-bulge in U6 snRNA, thereby blocking the formation of U4/U6 and U2/U6 complexes (Lu *et al*.,1994; Qiu *et al*., 1995). Therefore, these observations raise the possibility that Vpr may block spliceosome assembly by interfering with the function of the SAP145 and SAP49 complex in host cells.

Another question should be solved is that how the inhibition of splicing by Vpr is intron specific. Splicing of cellular *α-globin 2 pre-mRNA* removes 2 introns. Interestingly, only removal of intron 1 was modestly inhibited by Vpr. Also in our recent data, Vpr selectively increased the level of *env* mRNA by inhibiting HIV-1 *env* pre-mRNA splicing, that is, removal the 3' intron of HIV-1 primary transcripts. A splicing site can be regulated by consensus sequence up or downstream of the splice site, and splicing factors. HIV-1 A7 site is regulated by ESS3a, ESS3b, ISS and ESE3 (20), and many cellular splicing factors, such as the hnRNP family and SR family proteins, bind to these sites (21). It is still not clear whether or not the specificity of inhibition of splicing by Vpr is related to these elements or host factors. Vpr also displays high affinity for nucleic acids but no specific DNA sequence targeted by Vpr has been yet identified. Vpr might bind specific DNA sequence once associated with cellular partners to subsequently drive expression of both host cell and viral genes.

Further investigation to define whether there exist synergistic interactions between Vpr and consensus sequence(s) and splicing factors will help to better define the mechanism of pre-mRNA splicing inhibition by Vpr.

STRUCTURE OF VPR AND ITS ESSENTIAL DOMAINS FOR SPLICING REGULATION

An NMR structural analysis showed that full-length Vpr forms three amphipathic alpha helices folded around a hydrophobic core (Morellet et al, 2003). The N terminal domain is flexible and negatively charged, flanking to the helices. The C-terminal domain is also flexible, with positive charge and rich in arginine residues (Morellet, 2003). Two domains of the arginine-rich C-terminal domain from aa 77 to aa 96 and the third α-helical domain αH3, extending from aa 56 to aa 77 our of these domains, have been identified to be critical for inhibition of pre-mRNA splicing by Vpr (Hashizume, 2007). For exmaple, Leu residue at position 67 in αH3 of Vpr, might be critical for Vpr to interact with SAP145 and exert it effect on splicing regulation, since replacement of Leu residue by Pro at position 67, which weekly retained its ability to induce G2 arrest and apoptosis (Nishizawa *et al*., 2000) but lost its ability to target to the perinuclear region (Kamata and Aida., 2000; Kamata *et al*., 2005), remarkably impaired the splicing inhibition, the interaction with SAP145 and the inhibition of SAP49 and SAP145 complex formation (Hashizume 2007). By contrast, although the arginine-rich C-terminal domain appeared to be involved in splicing inhibition, two Vpr mutants with a single mutation in this domain, R80A and R88A, are defective for G2 arrest but could bind SAP145 and inhibit mRNA splicing, indicating that Arg residues at positions 80 and 88 are dispensable for the splicing inhibition via an interaction between Vpr and SAP145. Thus, the association of Vpr to

SAP145 to inhibit pre-mRNA processing seemed to be independent of Vpr's G2 arrest function.

On the other hand, The CUS1 domain on SAP145 has been identified to be required to associate with Vpr. (Terada and Yasuda, 2006). On the other hand, the CUS1 domain on SAP145 has been identified to be required to associate with Vpr. (Terada and Yasuda, 2006). This CUS1 domain is highly conserved (Nurse P, 1990). Further investigation on the structure of binding between SAP145 and Vpr may provide us great information to understand the mechanism how Vpr regulate pre-mRNA processing.

A PROSPECTIVE ROLE IN FUTURE ANTIVIRAL THERAPY

Additional work is needed for the complete understanding of the novel role of Vpr. For instance, how the inhibitory effect of Vpr on RNA splicing can be intron specific; whether regulation of cellular mRNA processing benefit the virus replication and whether it closely related with G2 arrest or apoptosis of host cells. It is now well agreed that Vpr might be critical for pathogenesis of HV-1 infection in vivo.

Vpr could potentially be an attractive target for the development of novel therapeutic strategies. Multiple host factors have been reported to interact with Vpr. The structural information of binding of Vpr with its partners may greatly facilitate the design of antiviral therapy. Development of compound that are potentially block the function of Vpr to induce G2 arrest or nuclear import are in process in several laboratories. The information of Vpr are involved in RNA splicing provide us a novel choice to screen antiviral drugs. Actually, a certain small-molecule chemical has been found to have anti-viral effect via inhibition of HIV pre-mRNA splicing. (Bakkour N

et al, 2007). The understanding of the novel role of Vpr may make great contribution to design a novel antiviral therapy.

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Figure legend:

Vpr inhibit RNA splicing via interaction with SAP145. Normally when initiate the assembly of the spliceosome at the early step of RNA splicing, the 5’ splice site of an intron is recoginized by the U1 small nuclear ribonucleoprotein (snRNP), followed by the binding of U2 auxiliary factor (U2AF) to 3’ splice site and its upstream ploypyrimidine tract and form a complex E, then U2AF recruit the u2snRNP, base pair the U2RNA with branch point to complete the formation of complex A, among recruited U2RNP, SAP145 bind SAP49 to form the subunit of SF3b. Subequent, binding of the U4-U5-U6 snRNP and form a large splicosome, which perform a reaction resulting in the ligation of the exons and excision of the intron. (left), however, in the presence of Vpr, the binding of Vpr with SAP145 block the recruitment of SAP49 and result in failure of catalytic spliceosome formation. Subsequently inhibit the splicing reaction (right).