The PI3K pathway acting on alternative HIV-1 pre-mRNA splicing

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HIV-1 mediates pro-survival signals and prevents apoptosis via the phosphatidylinositol-3-kinase (PI3K) pathway. This pathway, however, also affects phosphorylation of serine-arginine (SR) proteins, a family of splicing regulatory factors balancing splice site selection. We now show that pharmacologic inhibition of PI3K signalling alters the HIV-1 splicing pattern of both minigene- and provirus-derived mRNAs. This indicates that HIV-1 might also promote PI3K signalling to balance processing of its transcripts by regulating phosphorylation of splicing regulatory proteins.

HIV-1 infection of host cells is closely linked to a redirection of cellular signalling pathways to establish an environment efficiently supporting viral replication (Chugh et al., 2008; Jäger et al., 2012; Kim et al., 2011; Schrager et al., 2002; Witte et al., 2008). A well-described target of HIV-1 (and also other viruses) is the phosphatidylinositol-3-kinase (PI3K) signalling pathway, which relays receptor-mediated activation and survival signals, for instance, by cytokines (Diehl & Schaal, 2013; Dunn & Connor, 2012). Active PI3K heterodimers are then redistributed to the plasma membrane to catalyse the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3). These provide docking sites for the pleckstrin homology (PH) domain containing proteins such as the Akt kinase, which controls the activity of downstream effectors (Engelman et al., 2006). The importance of this pathway in HIV-1 replication is highlighted by the fact that several HIV proteins mediate its activation. This is achieved by gp120/CD4 interactions during attachment (Briand et al., 1997; François & Klotman, 2003), by trans-activator of transcription (Tat), which binds to receptors (Borgatti et al., 1997) or downregulates phosphatase and tensin homologue (PTEN) (Chugh et al., 2008), or by negative regulatory factor (Nef), which directly binds and activates PI3K (Linnemann et al., 2002; Schibeci et al., 2000; Wolf et al., 2001).

Upon epidermal growth factor (EGF)-induced Akt activation, the PI3K pathway also affects the splicing pattern of more than 200 genes (Zhou et al., 2012), and also activates protein kinases phosphorylating serine-arginine (SR) rich splice accessory proteins (Blaustein et al., 2004, 2005; Jang et al., 2009; Patel et al., 2001, 2005; Zhou et al., 2012).

HIV-1-mediated PI3K signalling has yet not been linked to splice site selection, although this seems very likely given its high dependence on splicing regulatory proteins. Due to the presence of a single promoter driving production of a single HIV-1 primary transcript, extensive processing is critical to generate all protein-coding mRNAs serving as templates for the viral proteome. By the use of at least four different 5′ splice sites (5′ss) and eight different 3′ss, alternative splicing of HIV-1 pre-mRNA leads to more than 40 different mRNA isoforms, which can be separated by their intron content into three major classes (unspliced 9 kb, intron-containing 4 kb and intronless 2 kb species) (Purcell & Martin, 1993; Stoltzfus, 2009; Widera et al., 2013). Moreover, like cellular splice sites, viral splice sites are controlled by the activities of splicing regulatory elements (SREs), commonly found in close proximity to the splice sites (Erkelenz et al., 2013a, b; Stoltzfus, 2009; Widera et al., 2013). These SREs are bound by SR and heterogeneous nuclear ribonucleoproteins (hnRNPs) and affect splicing by either enhancing or repressing the viral splice sites (Stoltzfus, 2009).

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To investigate an involvement of PI3K signalling in HIV-1 splice site selection, we first reaffirmed the inhibitory impact of LY294002 on PI3K activity by analysing the phosphorylation status of PI3K downstream effectors, the Akt kinase and SR proteins, in HEK 293T cells by Western blotting using phospho-specific antibodies. LY294002 treatment (12 h, 50 μM) did not affect cell viability as revealed by Alamar blue (data not shown), yet strongly reduced phosphorylation levels of the Akt kinase and also to some extent SR proteins including SRSF4 and SRSF3 (Fig. 1a, cf. lanes 1, 2). This was not observed with DMSO used as the solvent for LY294002 (Fig. 1a, cf. lanes 1, 3).

To further determine functional consequences of PI3K inhibition for HIV-1 splice site selection, we analysed HIV-1 leader exon 2 recognition because (i) amongst others, it is regulated by SRSF4 (Exline et al., 2008; Kammler et al., 2006) and (ii) within the minigene SV40 ds ex2 (Fig. 1b), exon 2 recognition is weak allowing analysis of both increase and decrease in splice site usage. HEK 293T cells were transiently transfected with SV40 ds ex2, and LY294002 (or DMSO) was added 2 h after transfection for 12 h. Following total RNA extraction (Chomczynski & Sacchi, 1987) and cDNA synthesis (Roche; Invitrogen), exon 2 recognition was determined by reverse transcription (RT)-PCR analyses. As expected, untreated and DMSO-treated cells showed considerable activation of 3′ss A1, but only minimal exon 2 recognition due to inefficient recognition of 5′ss D2. PI3K inhibition, however, resulted in significantly impaired recognition of the 3′ss A1 and/or the upstream 5′ss, demonstrated by the shift towards mRNAs with retained upstream intron (Fig. 1c, lane 2, Fig. 1d). Constitutive splicing of co-expressed GH1, containing

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**Fig. 1.** The PI3K pathway affects SR protein phosphorylation and reduces exon 2 recognition. (a) Western blot analysis of lysates of HEK 293T cells untreated or exposed to LY294002 (50 μM) or DMSO for 12 h using anti-Akt1 (Akt; c-20, Santa Cruz Biotechnology), anti-phospho-Akt (Ser473) (pAkt; 193H12, Cell Signaling Technology) or phosphorylated SR proteins (pSR; 1H4, Invitrogen). The respective SR proteins are indicated. Detection of ERK2 (c14, Santa Cruz Biotechnology) was used as the loading control. (b) Schematic drawing of SV40 ds ex2 containing HIV-1 exon 2. The LTR promoter of LTR ds ex2 (Kammler et al., 2006) was swapped following digestion with BssHII/PvuI and insertion of the SV40 early promoter. D, HIV-1 splice donor; A, HIV-1 splice acceptor; small open boxes, enhancer elements. (c) HEK 293T cells were transfected with SV40 ds ex2 and pXGH5 (encoding GH1; loading control). After treatment with LY294002 or DMSO, total RNAs were isolated and used for RT-PCR analyses using either primer pairs #1544/2588 (5′-CTTTACGATGCCATTGGGA) or #1544/2123 (5′-CTTTACGATGCCATTGGGA). PCR amplicons were separated on a non-denaturing polyacrylamide gel (10%) and stained with ethidium bromide. (d) Relative amounts of spliced mRNAs and mRNAs with retained first intron from three independent experiments.
constitutive spliced introns appeared to be unchanged, indicating that PI3K-dependent phosphorylation of SR proteins specifically affects splice site selection rather than acting generally repressively on pre-mRNA splicing.

To confirm that PI3K signalling specifically affects HIV-1 splice site choice, we extended our analysis to the processing of the entire genomic HIV-1 pre-mRNA. To do so, total RNA isolated from pNL4-3 transfected HEK 293T cells treated 2 h post-transfection with LY294002 (or DMSO) for 12 h was analysed by RT-PCR. Inhibition of PI3K signalling resulted in a specific shift towards an increase in 5’ss D2b-containing tat- and vpr-mRNA isoforms: tat2b, tat4b and vpr4b (Fig. 2b, lane 3) (Purcell & Martin, 1993; Widera et al., 2013). Unlike in the minigene (Fig. 1c), the leader exon 2 splice site D2 was moderately enhanced upon PI3K inhibition (e.g. tat2, tat4), still enabling upstream 3’ss A1 use via exon-definition (Widera et al., 2013). An increase in splice site activation was also observed for splice sites A2 and D3, flanking the downstream leader exon 3 (e.g. tat4, tat4b), which lacks the minigene. Usually, inclusion of exon 2 or 3 into viral mRNAs seems to be mutually exclusive; however, PI3K inhibition enabled combined inclusion of the non-coding leader exons. To exclude the possibility that insufficient amounts of viral Tat or Rev protein might be responsible for the LY294002-induced HIV-1 splicing phenotype, we co-expressed either Tat or Rev from cDNA expression vectors and still observed PI3K-inhibition-related changes in the splicing pattern (Fig. 2b, cf. lanes 1, 2, 5, 6), confirming that the altered splice site choice is caused by interference with the PI3K signalling rather than insufficient amounts of Tat or Rev.

It should be noted that splicing of 5’ss D2b and the leader exon 3 splice sites A2 and D3 are both negatively regulated by hnRNP proteins targeting both, the G-run G12-1 within intron 2 (Widera et al., 2013) and the exonic splicing silencer V (ESSV) within exon 3 (Madsen & Stoltzfus, 2005). Since LY294002 exposure of HEK 293T cells partially mimicked the viral splicing pattern seen following inactivation of the hnRNP F/H-dependent G12-1 silencer element (data not shown), these results indicated interference with the balance of viral splicing enhancer and silencer activities. However, the question remains of how PI3K signalling affects hnRNP protein-dependent viral silencer activities.

Since inhibition of PI3K specifically exerted changes in the HIV-1 pre-mRNA splicing pattern, we next wished to determine possible consequences for viral replication. HEK 293T cells were transiently transfected with proviral DNA and incubated with LY294002 or DMSO as described above or for 6 h, 12 h or 24 h. Total RNA and protein samples were isolated and used for Northern and Western blotting analyses to detect levels of viral mRNA and p24-CA capsid protein (Aalto Bio Reagents). Furthermore, supernatants of pNL4-3-transfected cells were collected to quantify viral release by Western blotting or capture ELISA. Levels of total viral mRNA expressed within pNL4-3 transfected cells were found somewhat reduced upon PI3K inhibition, while those of rRNAs used as a control remained unaffected (Fig. 2c, cf. lanes 2, 3) indicating that PI3K inhibition affects not only splice site choice. Strikingly, however, accumulation of the intronless 2 kb class was reduced upon PI3K inhibition, while that of the intron-containing 4 and 9 kb classes was slightly increased (Fig. 2c, cf. lanes 1, 2). Calculating the ratios of the amounts of viral mRNA classes with or without LY294002 treatment, we observed a significant reduction in the 2 kb mRNA class compared with the 9 kb and 4 kb classes (Fig. 2d), supporting our results that PI3K inhibition selectively impacts on viral splice site usage.

In line with the overall slightly reduced amounts of total viral RNA following LY294002 treatment, the levels of intra- and extracellularly detected p24-CA protein were more than twofold decreased (Fig. 2e, cf. NL4-3 and +LY294002), indicating that PI3K activity is required for efficient viral replication. Neither viral RNA expression nor p24-CA protein production was rescued when we co-transfected pcRev or pcTat, showing that the reduced p24-CA level could not simply be explained by insufficient amounts of Tat and Rev.

Although balanced viral splice site activation is critical for efficient HIV-1 replication (Erkelenz et al., 2013b; Exline et al., 2008; Madsen & Stoltzfus, 2005; Mandal et al., 2010; Widera et al., 2013; Zahler et al., 2004), it has to be taken into account that the PI3K downstream effector Akt is also involved in cellular translational regulation. The phosphorylation of TSC2 by Akt results in the activation of the GTPase Rheb and consequently in the activation of the mammalian target of rapamycin Complex 1 (mTORC1) (Huang & Manning, 2009). Therefore, inhibition of the PI3K pathway may affect, even to a larger extent, the cellular translation and thus the translation of viral proteins, e.g. p24-CA protein (Fig. 2d), which makes it hard to determine to what extent the PI3K inhibition-dependent changes in the viral splicing pattern are involved in viral replication interference.

To further analyse whether PI3K inhibition also affects Akt and SR protein phosphorylation as well as the HIV-1 splicing pattern in T cells, we isolated peripheral blood mononuclear cells (PBMCs) from two healthy donors. At 6 h post-infection (m.o.i. 0.01), PBMCs were treated with 50 µM LY294002 or DMSO as control for 16 h. Detection of Akt and SR protein phosphorylation was performed as described above. As seen for HEK 293T cells, a reduced Akt and SR protein phosphorylation could be observed after LY294002 treatment in both primary cell samples (Fig. 3a, cf. lanes 1, 2, 5, 6). When evaluating the splicing pattern by RT-PCR, samples of both donors also displayed specific changes within the HIV-1 splicing pattern upon PI3K inhibition (Fig. 3b). As seen before in HEK 293T cells, we detected increased amounts of the vpr4b transcript, albeit at a lower level and with a decrease in vpr3 mRNA. However, a striking increase in levels of tat4b or tat2b mRNAs could not be observed (Fig. 3b, cf. lanes 1, 2, 5, 6). The differences in the HIV-1 splicing
**Fig. 2.** Interference with PI3K signalling dysregulates HIV-1 alternative splicing and affects viral replication. (a) Organization of the HIV-1 genome with ORFs as open boxes and schematic drawing of the analysed region by respective RT-PCR primers #1544/#3632 (5’-TGGATGCTTCCAGGGCTC) (small open boxes, enhancer element; small filled boxes, silencer element). (b) HEK 293T cells were transfected either with pNL4-3 alone or together with pcTat or pcRev cDNA expression plasmids. For each sample, human GH1 reporter mRNA was co-expressed as control (#1224, 5’-TCTTCCAGCTCCCATCAGGCT). After treatment with LY294002 total RNA was used for RT-PCR analysis. HIV-1 amplicons were sequenced after gel elution (*) or assigned as previously described (Purcell & Martin, 1993; F. Hillebrand and others 1992; Journal of General Virology 95).
pattern between HEK 293T cells and PBMCs after LY294002 treatment may have originated from donor-to-donor variability in the expression of splicing regulatory proteins (Dowling et al., 2008), which is even greater between primary and stable cell lines. This is obvious as evidenced by differences in the cellular composition of splicing regulatory proteins within these cells, as demonstrated by the slightly different splicing patterns in the absence of any compound (Fig. 2b, lane 2, and Fig. 3b, lanes 1, 5). This, and a possible difference in the response to the PI3K inhibitor by the different cell types, may have contributed to the modestly changed HIV-1 splicing pattern in the PBMCs analysed here compared with HEK 293T cells. Nevertheless, these results demonstrate that blocking of

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**Fig. 3.** Inhibition of PI3K activity alters the HIV-1 splicing pattern in Peripheral blood mononuclear cells (PBMCs). PBMCs from two healthy donors were isolated and activated as described in Widera et al. (2013). Activated PBMCs were infected with HIV-1 NL4-3 (m.o.i., 0.01) and after 6 h incubated with 50 μM of LY294002 (or DMSO) for 16 h followed by isolation of total RNA and proteins. (a) Western blot analysis using specific antibodies against Akt (Akt), phosphorylated Akt (pAkt) or phosphorylated SR proteins (pSR) (see Fig. 1). (b) RT-PCR analysis of the HIV-1 2 kb mRNA class (primers #1544/#3632). Detection of ERK2 protein and mRNA was used as loading control for both Western blot and RT-PCR analysis (primers #4646, 5’-CAACGTGTGGCCACATATTCT/#4647, 5’-CTGCTTATGATAATGTCAACAAAGTTCG).
PI3K signalling also influences HIV-1 splice site selection in infected T cells.

To date, it is unclear whether HIV-1 alters PI3K signalling in order to coordinate alternative splicing of its own pre-mRNA. However, we showed that changes in splice site activation are linked to changes in PI3K signalling. Since viral Nef and Tat were shown to upregulate PI3K signals and these in turn impact the HIV-1 splicing pattern, it is conceivable that PI3K can specifically be targeted to modulate viral splice site activation.

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References


