Interaction of human immunodeficiency virus type 1 Vif with APOBEC3G is not dependent on serine/threonine phosphorylation status

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The human immunodeficiency virus type 1 accessory protein Vif is important for viral infectivity because it counteracts the antiviral protein APOBEC3G (A3G). 32P metabolic labelling of stimulated cells revealed in vivo phosphorylation of the control protein, whereas no serine/threonine phosphorylation was detected for Vif or the A3G protein. These data were confirmed by in vitro kinase assays using active recombinant kinase. Mitogen-activated protein kinase/ extracellular signal-regulated kinase 2 efficiently phosphorylated its target ELK, but failed to phosphorylate Vif. Putative serine/threonine phosphorylation point mutations in Vif (T96, S144, S165, T188) using single-round infection assays demonstrated that these mutations did not alter Vif activity, with the exception of Vif.T96E. Interestingly, T96E and not T96A was functionally impaired, indicating that this residue is critical for Vif–A3G physical interaction and activity. Our data suggest that Vif and A3G are not serine/threonine phosphorylated in human cells and phosphorylation is not linked to their functional activities.

Vif is a protein of human immunodeficiency virus type 1 (HIV-1) that is required for replication in non-permissive cells and primary peripheral mononuclear cells that express antiviral APOBEC3G (A3G) (Courcoul et al., 1995; Fisher et al., 1987; Gabuzda et al., 1992; Strebel et al., 1987; von Schwedler et al., 1993). In addition to HIV-1, most other lentiviruses also possess vif genes. HIV-1 Vif targets A3G for polyubiquitination and subsequent degradation through a proteasome-dependent pathway. Vif binds A3G and acts as an A3G substrate receptor molecule by mimicking the SOCS-box component of a cellular E3 ubiquitin ligase in the Vif–Cul5–EloB/C ubiquitin ligase complex (Marin et al., 2003; Mele et al., 2004; Sheehy et al., 2003; Yu et al., 2003, 2004). The SOCS-box in Vif (residues 144–173, Fig. 1), which consists of the BC-box and the Cullin-box, is required for multimerization. The BC-box recruits EloC and the Cullin-box interacts with Cul5 (Donahue et al., 2008; Mele et al., 2004; Stanley et al., 2008; Wolfe et al., 2010; Yang et al., 2001; Yu et al., 2004). The binding of Cul5 to Vif is also mediated by the zinc-binding HCCH-box (residues 108–139) and the T(Q/D/E)x2ADx2(I/L) motif (residues 96–107) (Dang et al., 2010b; Luo et al., 2005; Mele et al., 2006; Wolfe et al., 2010; Xiao et al., 2006, 2007; Yu et al., 2004). The interaction of Vif with A3G is organized by the G-box (residues 40–44), together with the WxSLVK motif (residues 21–26), the FG-box (residues 55–72), the LGxGxxIxW motif (residues 81–89) and the T(Q/D/E)x2ADx2(I/L) motif (Fig. 1) (Dang et al., 2009, 2010a, b; He et al., 2008; Pery et al., 2009; Russell & Pathak, 2007). Vif mutants that have a defect in binding to either A3G, Cul5 or EloC are unable to counteract the antiviral activity of A3G. However, other reports indicate that the physical assembly of Vif to A3G without subsequent degradation is sufficient to inhibit cytidine deamination, particle encapsidation or translation of A3G. Several putative phosphorylation sites in Vif have been described (Yang et al., 1996; Yang & Gabuzda, 1998), suggesting that serine/threonine phosphorylation of Vif at Ser114, Thr155, Thr188, Thr96 and Ser165 may occur in vivo in cultures of human cells (Fig. 1). In addition, two studies suggested that A3G can be phosphorylated by protein kinase A (PKA) at Thr32 (Shirakawa et al., 2008) or at Thr218 by PKA and calcium calmodulin-dependent kinase II (CaMKII) (Demorest et al., 2011).

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To determine whether Vif is phosphorylated in vivo, we radiolabelled Vif [derived from HIV-1 strain NL4-3, not codon-optimized, containing a C-terminal V5 tag (Zielonka et al., 2010)] via metabolic labelling with...
Yang & Gabuzda (1998) previously showed that recombinant Vif expressed in *Escherichia coli* can be phosphorylated *in vitro* by ERK2. To investigate the role of ERK2 in the phosphorylation of Vif, we performed *in vitro* kinase assays using recombinant active ERK2. The Vif protein, derived from transfected HEK-293T cells, was immunoprecipitated and eluted [using V5-peptides (1 μg ml⁻¹)] at 20 °C for 30 min, and was incubated with 2.5 ng active ERK2 (p42 MAPK, Upstate) for 20 min at 30 °C in kinase reaction mixture [10 μl Vif protein and 10 μCi [γ-³²P]ATP (370 kBq) in kinase buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT), PhosSTOP (Roche) and a protease inhibitor mixture (Roche)]. ELK1, which is a standard phosphorylation substrate of ERK2, served as a positive kinase reaction control. A signal derived from protein phosphorylation was only detected for ELK1 (Fig. 2b) and not for Vif, suggesting that Vif is not a substrate for phosphorylation by ERK2.

We also analysed the phosphorylation status of A3G by performing kinase assays *in vivo*. A3G [with a C-terminal HA tag (Mariani et al., 2003)] was expressed by transfection in HEK-293T cells and labelled with ³²P. Cell lysis was performed using RIPA. After immunoprecipitation of A3G and separation by SDS-PAGE, phosphorylation was examined by autoradiography. There was no detectable phosphorylation of A3G upon stimulation by dB-cAMP or TPA (Fig. 2c). Identical results were obtained in experiments without dB-cAMP or TPA stimulation (data not shown).

Two studies suggest that Vif is phosphorylated at multiple serine/threonine residues (Yang et al., 1996; Yang & Gabuzda, 1998). These reports motivated us to investigate the role of the putative phosphorylated amino acids in a quantitative single-round infection assay using the HIV-1 luciferase reporter virus HIVΔvifluc [pNL4-3-luc R⁻Δvif (Mariani et al., 2003)]. Expression plasmids of Vif containing mutations in single threonine or serine residues (T96A, T96E, S144A, S144E, S165A, S165E, T188A and T188E) were generated by fusion PCR. Alanine mutations in those sites prevent a potential phosphorylation and the negatively charged glutamate residues may mimic a constitutive phosphorylation. To functionally test the Vif serine/threonine point mutants, HEK-293T cells were cotransfected with HIVΔvifluc together with A3G and Vif plasmids, along with a VSV-G expression plasmid (pMD.G) as described by Zielonka et al. (2010). Two days post-transfection, the virus-containing supernatants were collected, human osteosarcoma (HOS) cells were transduced with equal amounts of each virus [normalized for reverse transcription activity (Zielonka et al., 2010)] and 3 days later the infectivity of these viruses was determined by quantification of intracellular luciferase activity, as described by Zielonka et al. (2010). Substitution of the putative phosphorylated amino acids for alanine did not modulate the ability of Vif to counteract A3G (Fig. 3a), which is in agreement with data reported by Mehle et al. (2004) for T96A, S144A and T188A. However, the ability of Vif to counteract A3G was impaired by the glutamate substitution of T96 (Vif.T96E), as was reported by Yang & Gabuzda (1998), resulting in reporter virus transductions.
with significantly reduced infectivity. Supporting the importance of this residue, Dang et al. (2010b) recently reported that changing T96 to either D (negatively charged) or R (positively charged) prevented the interaction of Vif with A3G. In contrast, glutamate exchanges at S144, S165 and T188 did not alter the capacity of Vif to inhibit A3G (Fig. 3a). Immunoblots of transfected cells and viral particles demonstrated that Vif.T96E reduced the amount of cellular A3G less efficiently than did wild type (wt) Vif (see cell lysates in Fig. 3b, c). A3G was barely detectable in virions produced in the presence of wt and mutant Vifs (see virus lysates in Fig. 3b). HIV particles made with Vif.T96E contained slightly more A3G than particles derived from wt Vif transfections (Fig. 3b).

To determine whether residue T96 modulates the interaction of Vif with A3G, A3G was pulled down by HA beads (Roche), and bead-associated proteins were identified by immunoblotting (Fig. 3c). We found that wt, T96A and T96E mutants interacted with A3G, but Vif.T96E bound more weakly (~25% less) than wt Vif to A3G (data from two independent experiments, shown in Fig. 3c, d). Vif.T96E itself, expressed without A3G, did not reduce the infectivity of HIV-1 (Fig. 3e). The hampered neutralization of A3G by Vif.T96E supports the theory that the charge is very important in the T(Q/D/E)x2ADx2(I/L) motif (Fig. 1) and that the Vif interaction with A3G depends on electrostatic interaction (Dang et al., 2010b). The T96E Vif mutant did not completely lose the ability to bind and induce degradation of A3G. These observations are similar to the unexplained activity of YFP–Vif (yellow fluorescent protein fused to Vif) that effectively degraded A3G but was severely impaired in its ability to direct the production of infectious HIV-1 particles from A3G-expressing cells (Kao et al., 2007).

In contrast with previous studies of Vif and A3G that reported phosphorylation of both proteins on serine/threonine residues (Demorest et al., 2011; Shirakawa et al., 2008; Yang et al., 1996; Yang & Gabuzda, 1998), our results do not support the theory that serine/threonine residues of Vif or A3G undergo phosphorylation. It is unclear why our results differ from those of previous studies. Possible explanations include the use of different expression and detection systems. Yang et al. (1996) and Yang & Gabuzda (1998) analysed histidine-tagged Vif strongly overexpressed from a vaccinia virus expression system and used E. coli recombinant Vif for in vitro kinase assays. In contrast, our in vivo phosphorylation assay was based on transient expression of Vif by a plasmid carrying the cytomegalovirus promoter, which expresses amounts of Vif similar to full-length HIV-1 (data not shown). To reduce the Rev-dependency of the vif RNA, we included a post-transcriptional regulatory element of the woodchuck hepatitis virus (Donello et al., 1996) in the 3′ untranslated region of the vif mRNA (Zielonka et al., 2010). In addition, our in vitro kinase assays used Vif protein immunoprecipitated from human cells and active recombinant ERK2. If Vif is subject to phosphorylation by ERK2 as reported by Yang & Gabuzda (1998), our in vitro kinase assay should have detected it. Thus, based on results of in vivo phosphorylation and single point mutations in the putative phosphorylated serine/threonine residues in Vif, we conclude that Vif is probably not a phosphoprotein.

Shirakawa et al. (2008) detected putative A3G phosphorylation with antibodies against phospho-PKA substrates, but they did not perform mass spectrometry detection of phosphorylation or metabolic labelling in cell culture. Demorest et al. (2011) showed that an A3G-derived peptide (12 aa) containing the predicted Thr218 can be
phosphorylated in vitro with recombinant PKA and CaMKII. However, prior to our study, no one had tested for the phosphorylation of A3G with radiolabelling. Our approach to radiolabel A3G expressed in HEK-293T is in principle a non-ambiguous and sensitive assay. We cannot rule out that very small and undetectable subpopulations of A3G or Vif proteins may be phosphorylated, even at different residues than those studied herein. However, based on our findings, the majority of Vif and A3G proteins are not subject to serine/threonine phosphorylation in HEK-293T cells. Our results indicate that the serine/threonine phosphorylation of Vif and A3G is not required for the interaction of Vif with A3G for Vif-dependent degradation of A3G and the antiviral activity of A3G. The potential specific functional consequences of any Vif or A3G phosphorylation are therefore unclear. Whether phosphorylation of Vif or A3G in human cells occurs under more physiological conditions (e.g. in primary cells) remains an open question.

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