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SHORT COMMUNICATION

Proteolytic Cleavage of HIV-1 GFP-Vpr Fusions at Novel Sites Within Virions and Living Cells: Concerns for Intracellular Trafficking Studies

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Abstract Fluorescent labelling of the highly conserved HIV-1 accessory protein Vpr (Viral Protein R) with GFP or variants thereof has proved a valuable approach to track Vpr and/or HIV-1 subcellular localisation *in vivo*. Our analysis in transfected mammalian cells expressing GFP-Vpr fusion protein, as well as within virus derived there from, documents site-specific proteolytic cleavage of the GFP-Vpr fusion protein. Western analysis revealed that transfected mammalian cells harbour a C-terminally truncated variant of Vpr in addition to full-length GFP-Vpr. Further, virions derived from these GFP-Vpr expressing cells show protein in which the GFP-tag has been additionally cleaved from the Vpr protein. Endogenous HIV protease (PR) activity was shown to be responsible for the latter, as addition of Saquinavir™, a potent PR inhibitor abolished the cleavage. Since many previous studies have relied on imaging the GFP fluorescence of GFP-Vpr, it would appear that the results may not reflect intact GFP-Vpr.

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Introduction

The HIV-1 accessory protein Vpr (Viral protein R) is the second most highly conserved HIV-1 protein, its major functions during viral replication including the induction of G_2 cell cycle arrest [1], apoptosis [2] and facilitation of pre-integration complex (PIC) nuclear import [3]. Vpr has previously been labelled at both its C- and N-terminus with *Aequorea victoria* green fluorescent protein (GFP) or variants thereof in order to study Vpr and/or HIV-1 subcellular localisation [4–7]. Since virion associated Vpr is specifically incorporated into forming virus particles, fluorescently-labelled virus can be obtained by cotransfecting GFP-Vpr expressing plasmid with an infectious HIV proviral plasmid in mammalian cells [8]. Utilising this method, GFP-Vpr-labelled viruses have been used to track viral binding, fusion and endocytosis of permissive cells [9–12], visualise microtubule trafficking of virions within the cytoplasm of infected cells [8] and observe viral transfer from dendritic to T-cells [13]. Significantly, attempts thus far to use GFP-Vpr-labelled virus to follow nuclear transport of the HIV-1 PIC, of which Vpr is an integral part, have failed [4] which may in part relate to the stability of the GFP-Vpr protein itself (see below) [14]. In this study, we demonstrate for the first time that GFP-Vpr is prone to site-specific proteolysis in both mammalian cells and the virus itself, shedding light on previous failures to track HIV PIC nuclear import *in vivo*.

Materials and methods

Mammalian cell expression vectors encoding pEPI-GFP-Vpr (1–96) or the truncations (1–46) and (46–96), were generated using the Gateway™ cloning technology (Invitrogen) according to manufacturer's guidelines. Briefly, DNA fragments encoding Vpr (1–96), (1–46) or (46–96) flanked by attB recombination sites were generated by standard PCR techniques from the template pUC18-NL4.3 [15]. Fragments were recombined into the pDONR207 vector (Invitrogen) in a BP recombination reaction, and the resultant pDONR207-Vpr vectors recombined into pEPI-GFP-DEST (an in-house vector derived from the episomally-replicating GFP-expression vector pEPI-GFP [16] and modified to be compatible with the Gateway™ system via the Invitrogen Gateway Vector Conversion Kit) [17], resulting in the GFP-Vpr mammalian cell expression constructs pEPI-GFP-Vpr (1–96), (1–46) and (46–96). The integrity of all pEPI-GFP-Vpr constructs was verified by DNA sequencing using an Applied Biosystems 3730S Genetic Analyser. The proviral vector pUC18-NL4.3 was obtained via the Aids Reagents Program (Cat# 114), whilst pUC18-NL4.3FS was kindly donated by Dr. Damien Purcell (Melbourne University, Australia). 293T (SV40 transformed human embryonic kidney 293) cells were transfected in DMEM (Invitrogen) supplemented with 10% Foetal Calf Serum (Thermo-Electron) using Lipofectamine 2000™ (Invitrogen) according to manufacturer's instructions.

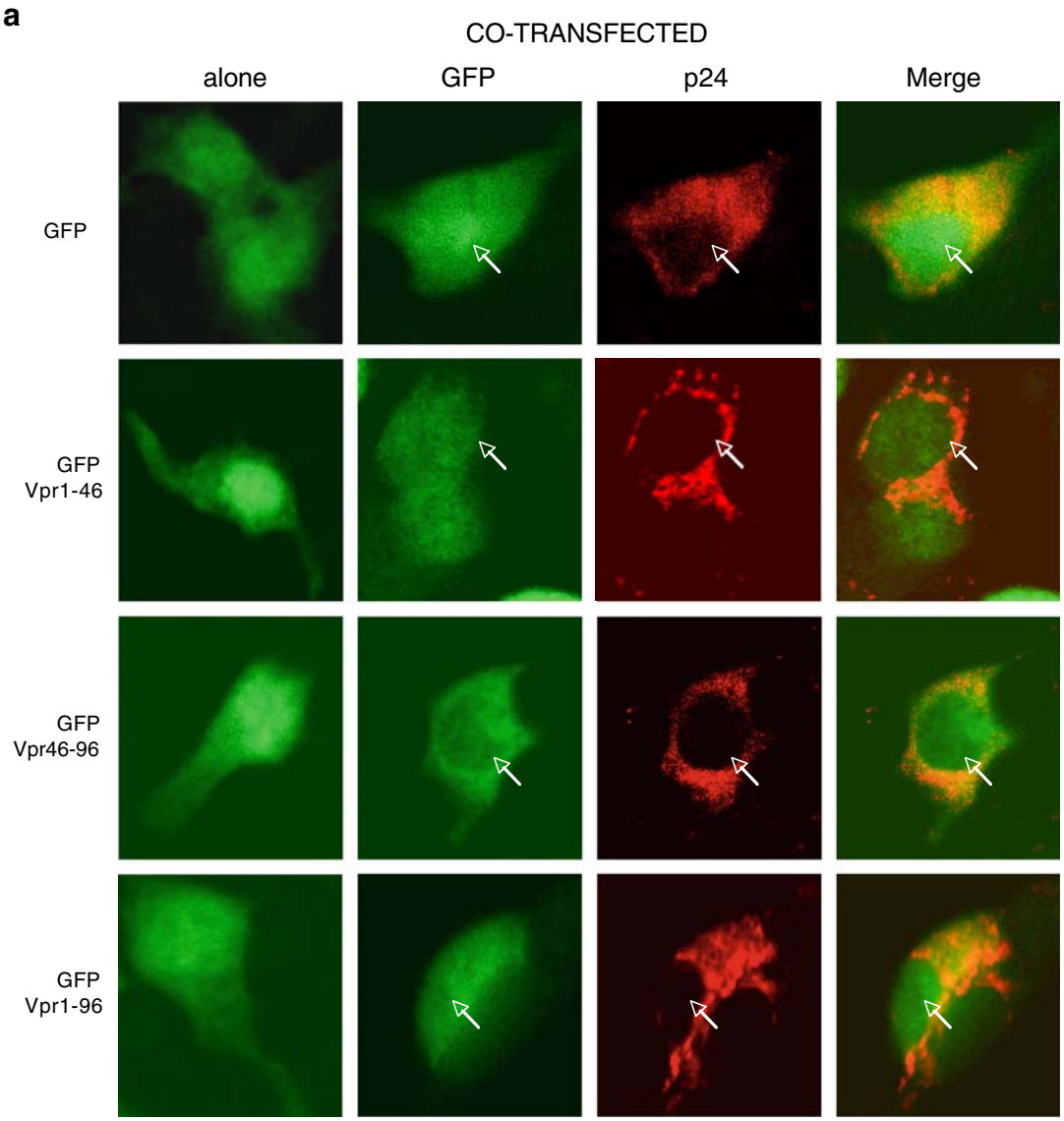
Where indicated, transfected cells were fixed in 4% paraformaldehyde, stained with anti-p24 primary (Cat# 4121 – NIH Aids Reagents Program) and AlexaFluor-568 (Invitrogen) secondary antibodies, and visualised on a BioRad MRC-600 confocal laser scanning microscope (CLSM) using a 60x oil immersion lens. Digital images were analysed using the public domain software ImageJ [18] to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence (Fn/c = [Fn - B] / [Fc - B]; where B = background fluorescence). Virus was obtained from DMEM by first clearing the media using a 0.22 μm filter to remove cellular debris, and pelleting virions via centrifugation of media at 20 000 g for 2 h at 4°C. Virions were lysed in Berman Lysis Buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 in PBS). Western blots were probed with anti-GFP (Zymed, San Francisco, USA), -Vpr (Cat# 3951 - AIDS Research and Reference Reagent Program, NIAID, NIH) or -p24 antibodies, and developed using the Sigma Alkaline Phosphatase based Ex-AP™ and BCIP/NBT-Blue Liquid Substrate System for Membranes™. In some experiments, the HIV-1 protease inhibitor Saquinavir (5 μM) (Cat# 4658 - AIDS Research and Reference Reagent Program, NIAID, NIH) was added to samples 6 h post-transfection.

Results and discussion

HIV virions have previously been fluorescently labelled using GFP-Vpr to track viral particles *in vivo* [19, 20]. Here we derived comparable labelled virions after cotransfecting 293T cells with pEPI-GFP-Vpr or derivatives thereof (including truncated Vpr forms) and pro-viral pUC18-NL4-3 plasmid vectors. Visualisation of CLSM images derived from co-transfected 293T cells (Fig. 1 a), revealed a reduction in GFP-Vpr nuclear localisation in the context of proviral expressing cells compared to expression of GFP-Vpr alone, implying probable cytoplasmic sequestration and viral incorporation of GFP-Vpr. Quantitative analysis of derived images revealed a significant (as indicated by p-values) reduction in Fn/c for all GFP-Vpr fusions (1–96; 1–46; 46–96) tested, but not for GFP alone (Fig. 1b), implying that Vpr sequestration within the context of virally infected cells is mediated by sequences within the N– and C-termini.

To test if the observed reduction in nuclear localisation was due to viral sequestration and not a result of cellular based GFP-Vpr degradation, fusion protein integrity was assessed by Western blot. Analysis of transfected cell lysates revealed not only the presence of intact GFP-Vpr1–96 fusion protein (~41-kDa) but also a lower molecular weight (~35-kDa) C-terminally truncated species detectable with either anti-GFP or anti-Vpr (directed against Vpr amino acids 1–46) antibodies (Fig. 2a). Strikingly, these results are consistent with those from other groups using comparable GFP-Vpr fusions [6, 8], the lower molecular weight species almost certainly corresponding to a c. 30 amino acid C-terminal truncation of the full-length GFP-Vpr fusion protein. Lysates derived from 293T cells cotransfected with pEPI-GFP-Vpr1-96 and the HIV pro-viral plasmids pUC18-NL4.3 [15] and pUC18-NL4.3FS (a Vpr deficient mutant containing a frame-shift (FS) mutation at amino acid 63 of Vpr) revealed that

Fig. 1 Reduced nuclear accumulation of GFP-Vpr in the context of HIV-1 infection. **a** 293T cells cotransfected with pEPI-GFP-Vpr and pUC18NL4.3 were visualised using CLSM, revealing a marked reduction in GFP-Vpr nuclear accumulation (nuclei denoted by white arrows) in the presence of HIV co-expression (observed via p24 staining. **b** Digital images from (A) were analysed using the ImageJ software to determine the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence between GFP-Vpr samples either alone (dark grey) or coexpressed with proviral plasmid pUC18-NL4.3 (light grey); to generate the Fn/c value [(Fn-B)/(Fc-B)] (B denotes background fluorescence). Significant reductions (as determined by p-values) in Fn/c were observed for all GFP-Vpr proteins tested within the context of virus producing cells, indicating that cytoplasmic sequestration of Vpr is mediated by both its N- and C-termini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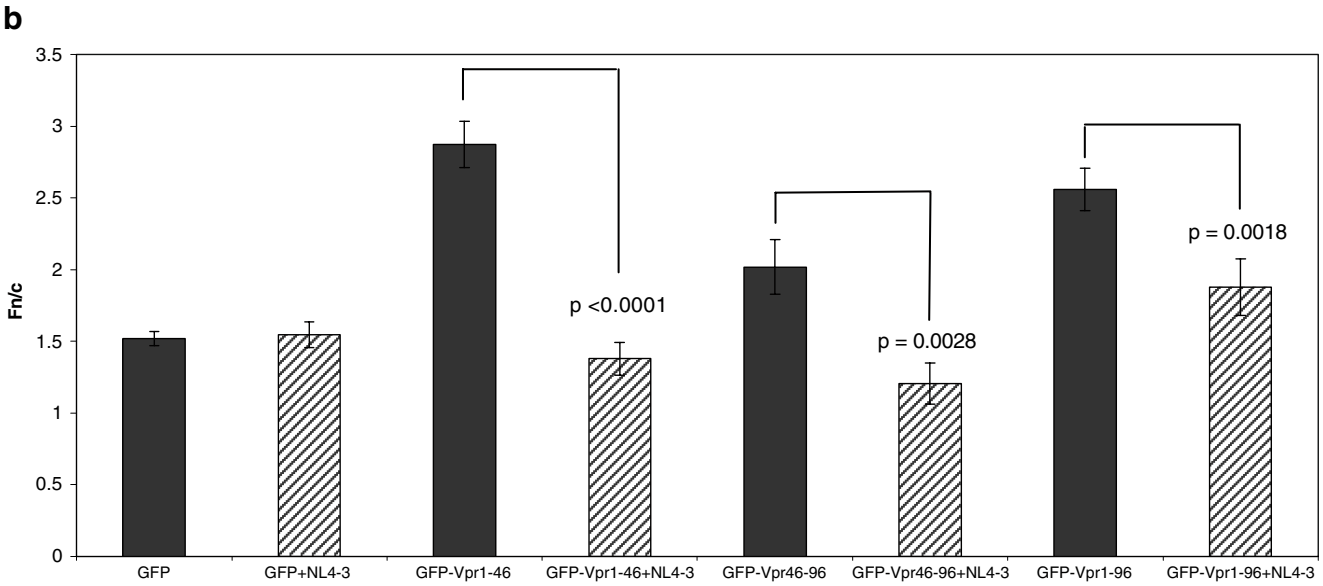
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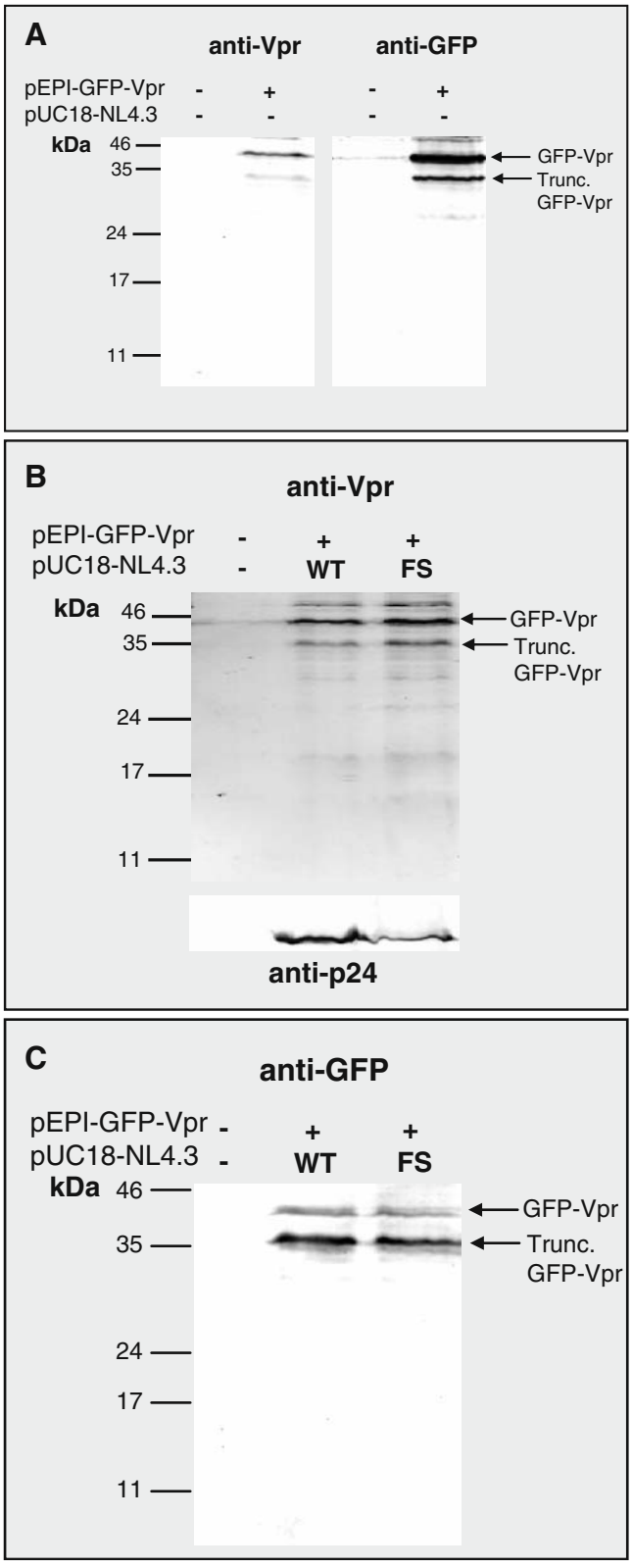

Fig. 2 Western blot analysis of GFP-Vpr expression in 293T cells in the absence or presence of viral expression. Western blot analysis of cellular **a** and viral lysates (**b**, **c**) obtained 48 h post transfection from 293T SV40 transformed human embryonic kidney cells transfected using Lipofectamine 2000™ (Invitrogen). Derived blots were probed with antibodies to GFP, Vpr or p24, and developed using the Sigma Alkaline Phosphatase based Ex-AP™ and BCIP/NBT-Blue Liquid Substrate System for Membranes™. It was observed that the cellular expression of full length GFP-Vpr was not altered in the presence of viral co-expression

expression of full-length or truncated GFP-Vpr1-96 was not altered in the context of viral expression (Fig. 2b,c).

To confirm virion incorporation of expressed GFP-Vpr1-96 protein, viral supernatants were harvested and analysed by Western blotting. Basal levels of viral Vpr expression were determined for both pUC18-NL4.3 and pUC18-NL4.3FS from cells transfected with proviral plasmid alone (Fig. 3a) using Vpr anti-serum; note the absence of virally-derived Vpr in pUC18-NL4.3FS lysates due to instability/degradation of FS-encoded Vpr protein. Western blots of viral samples from cells cotransfected with pEPI-GFP-Vpr1-96 and the pUC18-NL4.3 plasmids indicated that both full-length and truncated GFP-Vpr1-96 were incorporated into virions (Fig. 3b). Interestingly viral lysates were found to contain significantly high amounts of previously unobserved (absent within cellular lysates) GFP and Vpr protein alone. This was especially evident in blots derived from NL4.3FS lysates (Fig. 3b *left panel*), where free Vpr protein can only be derived from co-expressed GFP-Vpr1-96 (compare to Fig. 3a). Detection of free GFP in both cotransfected pUC18-NL4.3(WT/FS) viruses (Fig. 3b *right panel*) at levels comparable to free Vpr, indicates that proteolysis of GFP-Vpr1-96 almost certainly occurs post incorporation and specifically within the virus. The lack of detectable GFP alone and Vpr alone within blots from cells expressing GFP-Vpr1-96 alone (Fig. 2b,c), further supports the virion specificity of this cleavage.

The only active protease within the HIV virion that could be responsible for this observed cleavage is the HIV protease (PR) [21]. To test the role of viral PR in the cleavage of GFP-Vpr1-96, cotransfected 293T cells were treated with 5 μM of the specific PR inhibitor. Saquinavir™ (NIH) [22]. Within treated samples, the p24 (PR derived) cleavage product of pr55 was absent, confirming the ability of Saquinavir™ to inhibit HIV PR activity (Fig. 4c). Virions obtained from Saquinavir-treated cells were found to be devoid of free GFP and Vpr, in stark contrast to control untreated cells (Fig. 4a,b); thus implicating virion PR as the major determinant of GFP-Vpr fusion protein degradation.

Clearly, our results show that although largely ignored in previous studies [6, 8, 19], significant amounts (>50%) of proteolysed forms of GFP-Vpr are present in transfected cells, as well as in virus derived therefrom. Whilst there appears to be several different expression vectors for GFP-Vpr currently being used in HIV studies, all appear to yield C-terminally truncated GFP-Vpr1-96

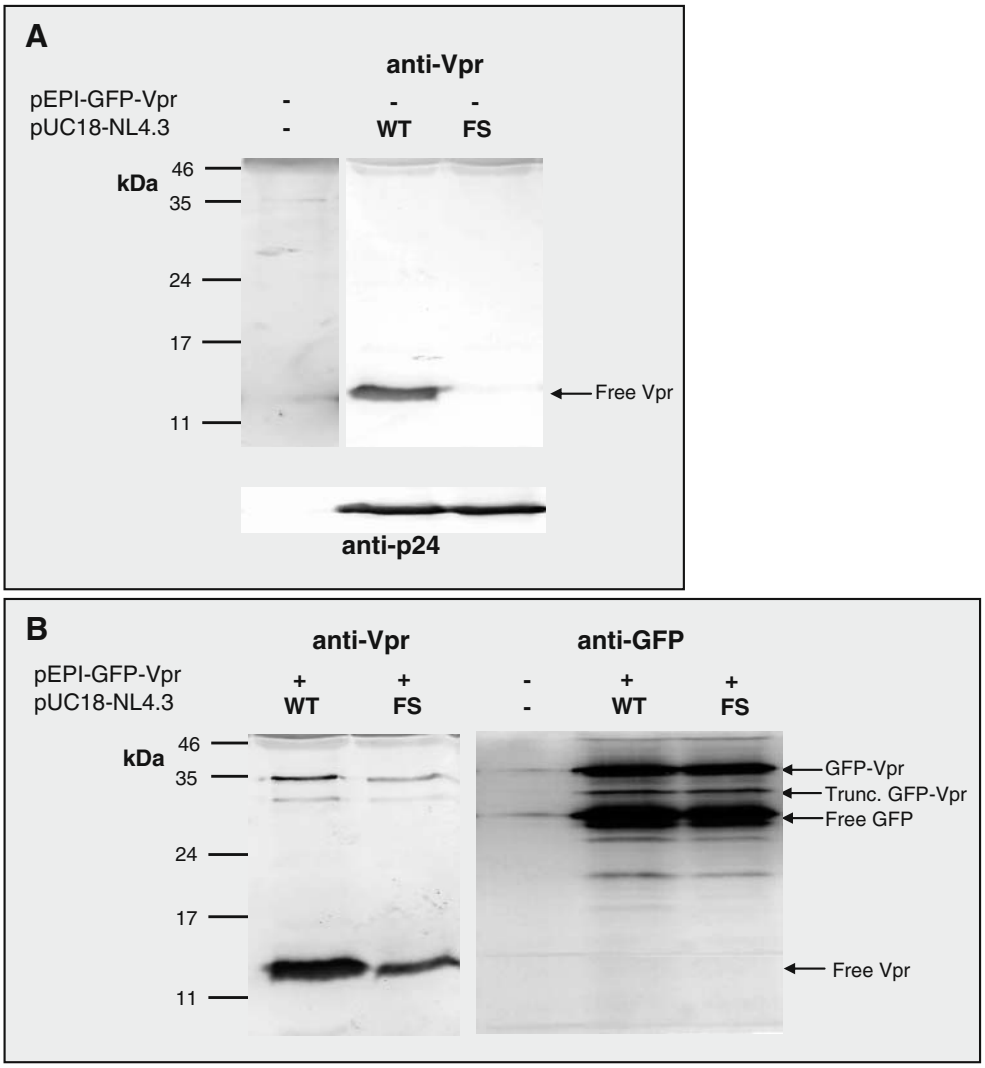

Fig. 3 Western blot analysis of incorporated GFP-Vpr within purified HIV viral lysates.Western blot analysis was performed on viral lysates as per the legend to Fig. 2. **a** Viral lysates from 293T cells solely expressing the proviral constructs pUC-18-NL4.3(WT) or (FS) identified Vpr expression within (WT) virus but not within truncated-Vpr expressing (FS), as expected due to Vpr(FS) protein instability. Loading control for the Capsid protein (p24) indicated equivalent loading of virus in all lanes. **b** Virus derived from 293T cells cotransfected with GFP-Vpr and proviral expression plasmids revealed virion incorporation of full-length GFP-Vpr (~41-kDa) as well as a lower molecular weight species (~35-kDa). Strikingly the presence of free-Vpr within the (FS) virus, which was not present within blots of (FS) virus alone (*compare panel* **A**). The presence of free-GFP to comparable levels of free-Vpr indicated the presence of virion-specific proteolysis towards the incorporated GFP-Vpr fusion protein

[6, 8, 19]. The mechanism by which GFP-Vpr is truncated at the C-terminus is currently unknown, although cellular protease activity or autocatalysis of Vpr are possible candidates. Clearly, this truncation is likely to have very significant effects in terms of using GFP-Vpr labelled viruses to study Vpr functions that rely on its C-terminus, such as PIC nuclear import [23, 24], apoptosis [25, 26], and cell-cycle arrest [27, 28]. The apparent lack of success regarding the labelling and visualisation of PIC nuclear import with GFP-Vpr may in part be due to fluorescent signals wrongly assumed to be intact full-length GFP-Vpr [8], where these species may in fact include functionally deficient C-terminally truncated GFP-Vpr or simply GFP-alone.

Additionally, the fact that our GFP-Vpr fusion appears to be cleaved by the HIV-1 PR implies the existence of a novel protease recognition site located between GFP and Vpr, which is distinct from the classical HIV PR recognition sites [29]. Further examination of this novel site is a focus of further study in this laboratory.

Conclusion

The present study highlights the potentially important, and largely ignored problems regarding the use of GFP-Vpr-labelled HIV for subcellular localisation studies, since it represents a mixture of intact and truncated forms of GFP-Vpr. Detailed analysis of fusion proteins with regards to their expression and stability are clearly necessary to enable conclusions to be drawn with respect to Vpr subcellular localisation based on fluorescent images of cells expressing GFP-Vpr. Similarly, viral incorporation and processing of GFP-Vpr needs to be studied in detail before GFP-Vpr-labelled virus can be used to track virions intracellularly or in functional

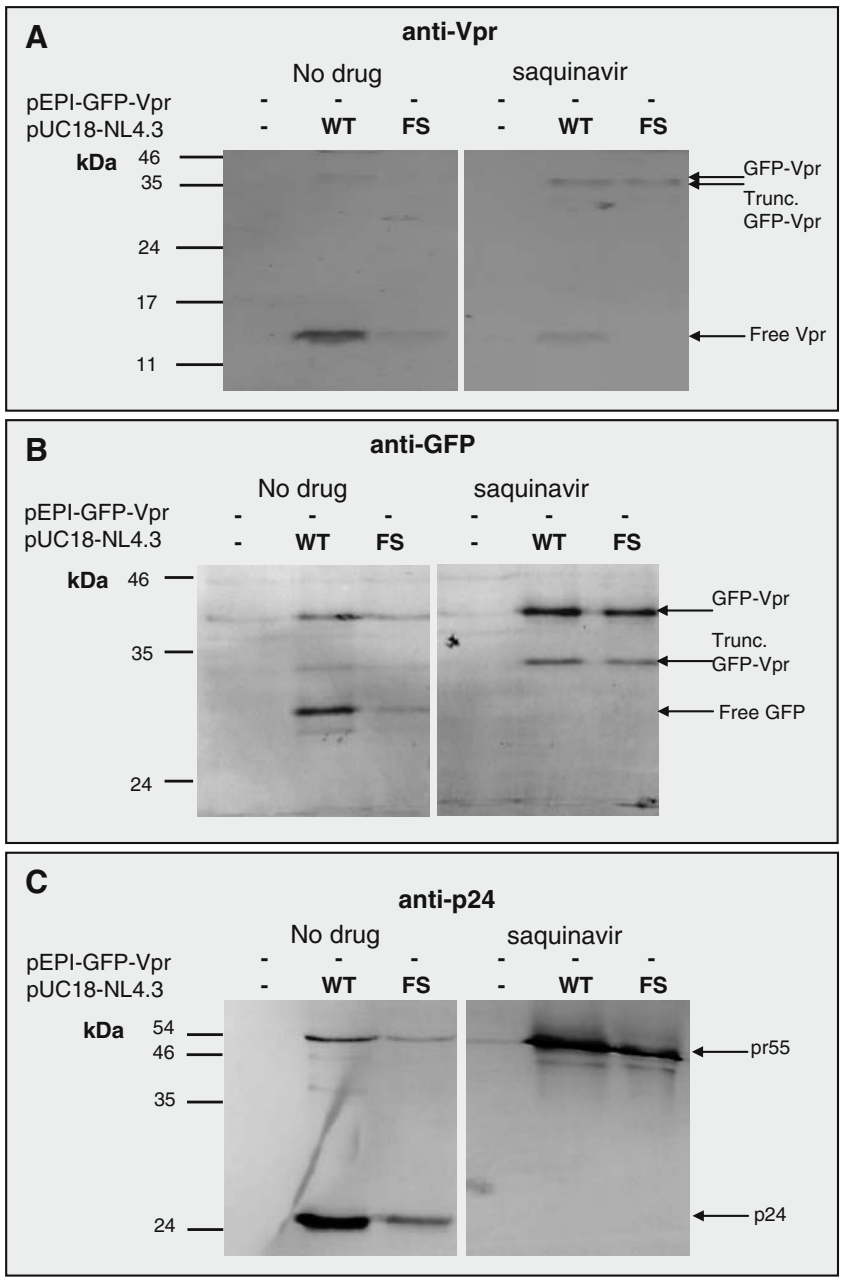

Fig. 4 GFP-Vpr fusion protein cleavage is mediated within the virion by HIV protease. Cotransfected 293T cells (GFP-Vpr + pUC18NL4.3WT/FS) were cultured in the absence or presence of the HIV-1 protease inhibitor Saquinavir (5 μM) (AIDS Research and Reference Reagent Program, NIAID, NIH). Free-Vpr a and free-GFP b proteins were absent from viral lysates obtained from Saquinavir™ treated cells, indicating that HIV-1 PR was responsible for the observed virion-specific proteolysis of GFP-Vpr. **c** The absence of PR mediated proteolysis of pr55 to p24 (a function of HIV-1 PR *in vivo*) confirmed the successful inhibition of PR activity by Saquinavir™

assays [5]. Importantly, the existence of truncated forms of GFP-Vpr, presumably with impaired function, may represent the basis of previous failures to track the HIV-1 PIC inside of cells [5].

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