FAT10: a Novel Mediator of Vpr-Induced Apoptosis in Human Immunodeficiency Virus-Associated Nephropathy[▽]

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Human immunodeficiency virus (HIV)-associated nephropathy is a significant cause of morbidity and mortality in HIV-infected persons. Vpr-induced cell cycle dysregulation and apoptosis of renal tubular epithelial cells are important components of the pathogenesis of HIV-associated nephropathy (HIVAN). FAT10 is a ubiquitin-like protein that is upregulated in renal tubular epithelial cells in HIVAN. In these studies, we report that Vpr induces increased expression of FAT10 in tubular cells and that inhibition of FAT10 expression prevents Vpr-induced apoptosis in human and murine tubular cells. Moreover, we found that Vpr interacts with FAT10 and that these proteins colocalize at mitochondria. These studies establish FAT10 as a novel mediator of Vpr-induced cell death.

Antiretroviral therapy has decreased morbidity and mortality due to opportunistic infections in persons with human immunodeficiency virus HIV/AIDS; however, kidney disease is the fourth most common cause of death among Americans with HIV/AIDS (33). HIV-associated nephropathy (HIVAN) is characterized pathologically by glomerulosclerosis and severe tubulointerstitial disease (29, 35). HIV infection of renal tubular epithelial cells (RTEC) has been conclusively demonstrated in HIVAN biopsy specimens (8, 22). The mechanism of viral entry into RTEC is unknown. Infection of RTEC by cell-free virus is inefficient and may involve direct CD4-independent cell-cell transfer (26). Aberrant RTEC proliferation and apoptosis are prominent components of HIVAN pathogenesis (7).

Recent studies have demonstrated a central role for HIV-1 Vpr in HIVAN pathogenesis (12, 14, 15, 40) and that Vpr induces apoptosis and hyperploidy in RTEC (28). However, the mechanisms by which Vpr induces RTEC apoptosis are unknown.

We recently reported that the ubiquitin-like protein FAT10 is expressed in RTEC in HIVAN biopsy specimens, FAT10 is upregulated by HIV infection of RTEC, and blocking *FAT10* expression prevents HIV-induced RTEC apoptosis (30). FAT10 is a ubiquitin-like protein that can form covalent conjugates, thereby targeting proteins for degradation by the 26S proteasome (25, 32). FAT10 has putative roles in regulation of cell cycle (20, 21), innate immunity (9, 24), and apoptosis (25, 30). Since Vpr is known to mediate HIV-induced apoptosis in some cell types, we examined the role of *FAT10* in Vpr-induced RTEC apoptosis.

Given that *FAT10* is one of the most highly upregulated

genes in HIV-infected RTEC (30), we investigated whether Vpr induces *FAT10* expression. Since infection of RTEC by cell-free HIV-1 is inefficient, we transduced human RTEC (HK2; described in reference 31) with lentiviruses pseudotyped with the broadly tropic vesicular stomatitis virus glycoprotein. Lentiviral vectors were used at a multiplicity of infection of 3 according to previously published methods (15). The vectors used in these studies are depicted in Fig. 1. HK2 cells were transduced with lentiviruses expressing Vpr (HR-Vpr), the empty vector control (HR), *gag*- and *pol*-deleted HIV-1 (NL4-3:ΔG/P-EGFP [enhanced green fluorescent protein]), or *gag pol*-deleted HIV-1 with a mutated Vpr start codon (NL4-3ΔVpr:ΔG/P-EGFP). *FAT10* expression was analyzed by quantitative PCR at 5 days postinfection. Quantitative PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen) with the following primers: *cyclophilin* sense (5'-AGGGTGGTGACTTTACACGC-3') and antisense (5'-ATCCAGCCATTCAGTCTTGG-3') and *FAT10* sense (5'-AATGCTTCCTGCCTCTGTGT-3') and antisense (5'-CTGCACAGGAACCTTGGTCT-3'). The following conditions were used: 95°C for 15 min; 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and finally 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Results were analyzed according to previously published methods (30). Transduction of HK2 cells with NL4-3:ΔG/P-EGFP and HR-Vpr increased *FAT10* expression by 5.5-fold, and 7.7-fold, respectively, compared to empty vector ($P < 0.0005$) (Fig. 2A). However, NL4-3ΔVpr:ΔG/P-EGFP did not significantly increase *FAT10* expression. *Vpr* therefore appears to be the HIV-1 gene most important for induction of *FAT10* expression in RTEC. Since our vectors did not contain the *gag* and *pol* genes, we cannot rule out a role for those genes in modulating *FAT10* expression in RTEC infected with full-length HIV. We were not able to definitively determine *FAT10* protein levels after transduction of HK2 with these vectors. Western blotting using anti-FAT10 antibodies revealed bands of variable molecular weights (data not shown), and since *FAT10* becomes covalently attached to as-yet-unidentified tar-

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FIG. 1. Lentiviral vectors used in these studies. LTR, long terminal repeat; IRES, internal ribosome entry site; RRE, Rev-responsive element; cPPT, central polypurine tract; PGK, phosphoglycerine kinase; nEGFP, nuclear EGFP; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

get proteins (25), it was difficult to determine the specificity of these bands.

Previous studies have demonstrated that transduction of HK2 cells with HR-Vpr induces hypodiploidy and that hypodiploidy was reflective of increased apoptosis as detected by annexin V and propidium iodide staining (22). Similarly, transduction of HK2 cells with HR-Vpr induced cleavage of poly(ADP ribose) polymerase 1 (PARP-1; an indicator or caspase-3 activation), whereas no cleaved PARP-1 was detected in cells transduced with empty HR vector (Fig. 2C).

Since *FAT10* influences HIV-induced apoptosis and may have a role in cell cycle regulation (20, 21, 27, 30), we investigated whether *FAT10* expression is necessary for Vpr-induced apoptosis and cell cycle dysregulation. HK2 cells were transduced with a lentiviral short hairpin RNA (shRNA) targeting *FAT10* (VIRHD/E-FAT10.1) or luciferase (VIRHD/E/shLuc) (30) as a control (Fig. 1C). Cells were then transduced with HR-Vpr or empty vector at a multiplicity of infection of 9 and analyzed 5 days later. Cellular DNA content was measured by analyzing propidium-stained cells by flow cytometry (28). Gates defining the hypodiploid, G_1 , S, G_2/M , and $>4N$ cell

FIG. 2. Vpr upregulates *FAT10* expression. (A) Transduction of HK2 cells with Vpr-expressing lentivirus (HR-Vpr) led to a 7.7-fold increase in *FAT10* expression by 5 days postinfection. Transduction of HK2 cells with NL4-3:ΔG/P-EGFP induced upregulation of *FAT10* by 5.5-fold at 5 days postinfection, whereas transduction with NL4-3:ΔVpr:ΔG/P-EGFP did not significantly increase *FAT10* expression at 5 days postinfection. Results represent three separate experiments. *, $P < 0.0005$; **, $P < 0.02$. Error bars represent 1 standard deviation. (B) VIRHD/E-FAT10.1 inhibited Vpr-induced upregulation of *FAT10* expression by 68% compared to VIRHD/E-luc control shRNA. +, $P < 0.005$. *FAT10* expression after transduction with control vector(s) was assigned a value of 1, with each experimental sample depicted as change (fold) relative to control. (C) HR-Vpr transduction induced PARP-1 cleavage (arrow). No cleaved PARP-1 was detected in cells transduced with HR (control) or in cells cotransduced with HR-Vpr and VIRHD/E-FAT10.1. Blots were reprobed with anti-β-actin to confirm equal loading.

FIG. 3. Suppression of *FAT10* expression abrogates Vpr-induced apoptosis. (A) The proportion of hypodiploid cells after transduction with HR-Vpr was lower in cells expressing VIRHD/E-*FAT10.1* (10.6%) than in those expressing control VIRHD/E-luc (22.6%; $P < 0.0001$), whereas the percentage of 4N cells increased modestly from 32.7% to 39.5% ($P < 0.0001$) and that of >4N cells increased from 18.6% to 27.1% ($P < 0.0001$). (B) Five days after transduction of *FAT10*^{-/-} RTEC with HR-Vpr, the hypodiploid fraction did not change significantly compared to control lentivirus, whereas the proportions of 4N and >4N cells increased modestly from 16.4% to 25.3% ($P < 0.0001$) and from 3.62% to 8.94% ($P < 0.0001$), respectively. (C) Restoration of *FAT10* expression by transduction of *FAT10*^{-/-} cells with HR-*FAT10* increased the proportion Vpr-induced apoptosis from 6.8% to 20.2% ($P < 0.0001$) and increased the proportion of >4N cells from 13.0% to 17.6% ($P < 0.0001$).

cycle phases were applied to data from HK2 cells transduced with HR control vector. These gates were subsequently applied to other transduced cell populations to perform comparisons according to previously published methods (22).

The *FAT10* shRNA vector VIRHD/E-*FAT10.1* inhibited Vpr-induced *FAT10* mRNA expression by 68% relative to HR-Vpr-transduced cells treated with the shRNA control, VIRHD/E-luc ($P < 0.0001$) (Fig. 2B). VIRHD/E-*FAT10.1* decreased the percentage of HR-Vpr-transduced cells that became hypodiploid (apoptotic) from 22.6% to 10.6% ($P < 0.0001$). This decrease in Vpr-induced hypodiploidy was associated with concomitant increases in the percentages of G₂/M cells from 32.7% to 39.5% and hyperploid (>4N) cells from 18.6% to 27.1% ($P < 0.0001$) (Fig. 3A). VIRHD/E-*FAT10.1*

also decreased the percentage of hypodiploid cells after transduction with control vector. However, whereas *FAT10* inhibition led to an increase in G₂/M and >4N cells after transduction with Vpr, the decrease in apoptosis that occurred in control-transduced cells after *FAT10* inhibition was accompanied primarily by an increase in the G₀/G₁ population. Therefore, *FAT10* inhibition decreased Vpr-induced apoptosis predominantly in G₂/M and >4N RTEC.

To further explore our hypothesis that *FAT10* is necessary for Vpr-induced apoptosis, we examined the effects of Vpr expression in an RTEC line (referred to as K1) derived from a *FAT10*^{-/-} mouse. *FAT10*^{-/-} mice develop normally, and unstressed mice have no overt phenotype (9). K1 cells express RTEC markers (P. Gong, A. Canaan, J. Leventhal, A. Snyder,

FIG. 4. FAT10 and Vpr coimmunoprecipitate and colocalize to mitochondria. (A) HEK293T cells were cotransfected with pcDNA4-FAT10 and either pFLAG-Vpr or pFLAG-CMV-6c (FLAG control). Cell lysate was immunoprecipitated with anti-FLAG and immunoblotted using anti-FLAG and anti-FAT10. FAT10 was detected in immunoprecipitates from cells expressing FLAG-Vpr but not FLAG alone. Results were confirmed in a reciprocal experiment in which pFLAG-FAT10 or p-FLAG-CMV-6c (FLAG control) was cotransfected with pNL4-3:ΔG/P-EGFP. Immunoprecipitation of cell lysate using anti-FLAG and subsequent immunoblotting using anti-FLAG and anti-Vpr demonstrated that Vpr was present in immunoprecipitate from cells transfected FLAG-FAT10 but not the FLAG control. (B) HEK293T cells were cotransfected with pGFP-Vpr or pHR (GFP control) and pFLAG-FAT10. GFP-Vpr and FLAG-FAT10 colocalized with MitotrackerCMXRos, primarily in large aggregates. (C) Western blotting of mitochondrial and cytoplasmic protein from HEK293T cells after cotransfection with pHA-Vpr and pFLAG-FAT10 demonstrated that FAT10 and Vpr in were present mitochondrial and cytoplasmic fractions. Blots were stripped and reprobed with anti-COX4 and anti-β-actin to confirm the purity of mitochondrial fractions.

B. Wang, M. Kretzler, V. D'Agati, S. Weissman, and M. J. Ross, submitted for publication) and were conditionally immortalized with temperature-sensitive T antigen (30). Transduction of K1 cells with HR-Vpr did not significantly increase apoptosis but did increase the proportion of G₂/M and >4N cells compared to control-transduced cells (Fig. 3B). To determine whether the lack of Vpr-induced apoptosis in K1 cells was correctly attributed to the absence of *FAT10*, we transduced K1 cells with the *FAT10*-expressing lentivirus HR-FAT10 (Fig. 1B) or the HR control 2 days prior to transduction with HR-Vpr. Whereas only 3.7% of K1 cells transduced

with HR-Vpr underwent apoptosis (Fig. 3B), 20.2% of HR-FAT10-transduced K1 cells underwent apoptosis after subsequent transduction with HR-Vpr ($P < 0.0001$) (Fig. 3B). Prior transduction with HR-FAT10 also modestly increased Vpr-induced accumulation of >4N cells ($P < 0.0001$) (Fig. 3C). Having found that FAT10 facilitates Vpr-induced apoptosis and cell cycle dysregulation, we studied whether these proteins interact. To express FLAG-tagged Vpr, we created pFLAG-CMV-6c-Vpr and pFLAG-CMV-6c-FAT10 by subcloning *Vpr* and *FAT10* into pFLAG-CMV-6c (Sigma). pFLAG-CMV-6c-Vpr or pFLAG-CMV-6c (FLAG control)

was cotransfected with pcDNA4-FAT10 (30) into HEK293T cells, and cellular lysate was immunoprecipitated using anti-FLAG. The primary antibodies were to FAT10 (BIOMOL International), FLAG (Sigma), and Vpr (Jeffrey B. Kopp, NIH/NIDDK). Western blotting of immunoprecipitate revealed that FAT10 was detected only in immunoprecipitate from cells expressing FLAG-Vpr. Similarly, immunoprecipitation of lysate from HEK293T cells cotransfected with pNL4-3: Δ G/P-EGFP and either pFLAG-CMV (cytomegalovirus)-FAT10-6c or pFLAG-CMV-6c (FLAG control) using anti-FLAG demonstrated that Vpr coimmunoprecipitated from cells expressing FLAG-FAT10 but not FLAG peptide alone (Fig. 4A).

We then performed immunocytochemistry to examine the subcellular localization of FAT10 and Vpr. Vpr has been shown to localize to the nucleus, nuclear envelope, and/or mitochondria (16). HEK293 cells were cotransfected with pGFP-Vpr plasmid (Lubbertus Mulder, Mount Sinai School of Medicine) or pHR and pFLAG-CMV-6c-FAT10. GFP-Vpr was detected in nuclei and in punctate extranuclear foci. While the distributions of GFP-Vpr and FLAG-FAT10 localization were not identical, the proteins did colocalize in large aggregates that stained with MitotrackerCMXRos, suggesting that Vpr and FAT10 colocalize at clumped mitochondria (Fig. 4B).

To confirm whether Vpr and FAT10 colocalize at mitochondria, HEK293T cells were transfected with pHR-6x-His-HA-Vpr-IRES-GFP (abbreviated pHA-Vpr; gift from Vicente Planelles, University of Utah) alone or cotransfected with pFLAG-CMV-6c-FAT10 (abbreviated pFLAG-FAT10). Mitochondrial and cytoplasmic protein isolation was performed using a Pierce mitochondrial isolation kit. Subsequent Western blotting using anti-FLAG and antihemagglutinin (anti-HA) antibodies demonstrated that FLAG-FAT10 and HA-Vpr were present in mitochondrial and cytoplasmic fractions (Fig. 4C).

Vpr mediates HIV-induced pathology in diverse cell types (2, 19, 23, 40) and induces apoptosis and hyperploidy in RTEC in vivo and in vitro (28). These data implicate the ubiquitin-like protein FAT10 as a critical mediator of Vpr-induced apoptosis in RTEC and define *Vpr* as the HIV-1 gene primarily responsible for *FAT10* upregulation. While the mechanism by which *Vpr* increases FAT10 expression is unknown, it is possible that Vpr upregulates tumor necrosis factor alpha and/or gamma interferon, both of which are known to induce FAT10 expression (24). Alternatively, Vpr has been shown to suppress the activity of p53, a known inhibitor of FAT10 expression (1, 38).

We demonstrated that *FAT10* suppression prevents Vpr-induced apoptosis in human and murine RTEC. This decrease in apoptosis occurred concomitantly with an increase in the proportion of G₂/M and hyperploid (>4N) cells, suggesting that FAT10 facilitates Vpr-induced apoptosis primarily in hyperploid cells. In addition, Vpr-induced apoptosis, as indicated by PARP-1 cleavage, was abrogated by FAT10 inhibition. Our data suggest that in RTEC, apoptosis occurs as a consequence of cell cycle dysregulation. However, the mechanisms by which hyperploidy induces FAT10-dependent apoptosis are unknown and may be cell-type specific (4).

Restoration of *FAT10* expression in RTEC from *FAT10*^{-/-} mice rendered them susceptible to Vpr-induced apoptosis and hyperploidy, suggesting that FAT10 may play a role in both

processes. However, in HK2 cells, FAT10 inhibition prevented apoptosis while increasing the number of hyperploid cells. It is possible that complete absence of FAT10 inhibits Vpr-induced hyperploidy, whereas shRNA-induced suppression of FAT10 was insufficient to prevent Vpr-induced cell cycle effects. Alternatively, there may be differences in Vpr-induced cell cycle dynamics in human versus murine RTEC, as has been reported in other cell types (5, 6, 39).

Our studies demonstrate that a fraction of Vpr and FAT10 proteins colocalize at mitochondria and coimmunoprecipitate, suggesting that they either bind directly or exist in a multiprotein complex. Our results do not suggest that FAT10 can become covalently attached to Vpr, since their coexpression does not lead to an increase in the molecular weight of Vpr. It is unclear why FAT10 and Vpr colocalized primarily at clumped mitochondria. It is possible that since Vpr induces Bax-dependent apoptosis (3) and Bax can induce mitochondrial fusion (18), Vpr and FAT10 localize at mitochondria that have undergone Bax-induced fusion. Alternatively, FAT10 has recently been reported to localize to aggresomes (17), cellular structures that recruit mitochondria and have roles in viral replication (37). Future studies should further define the subcellular localization and trafficking of Vpr and FAT10.

The specific mechanism by which FAT10 mediates Vpr-induced apoptosis remains to be determined. Moreover, it is not known whether FAT10-mediated apoptosis in HIV-infected cells is harmful to the host or if it is an adaptive response that limits spread of viral infection. FAT10 is not expressed in T cells before or after infection with HIV-1 (unpublished observations); therefore, FAT10 is unlikely to play a role in Vpr-induced apoptosis in T cells. Future studies should also determine whether FAT10 interacts with the recently described DCAF1-DDB1-VprBP complex that mediates Vpr-induced G₂/M arrest (10, 11, 13, 34, 36).

HIVAN is a major cause of morbidity and mortality in HIV-1-infected patients, and Vpr has been shown to be an important factor in HIV-induced renal pathogenesis. These studies establish the ubiquitin-like protein FAT10 as a critical mediator of Vpr-induced apoptosis in RTEC and demonstrate for the first time that these proteins interact and colocalize to mitochondria.

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