HIV-1 Vpr-Induced Apoptosis Is Cell Cycle Dependent and Requires Bax but Not ANT

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The HIV-1 accessory protein viral protein R (Vpr) causes G2 arrest and apoptosis in infected cells. We previously identified the DNA damage–signaling protein ATR as the cellular factor that mediates Vpr-induced G2 arrest and apoptosis. Here, we examine the mechanism of induction of apoptosis by Vpr and how it relates to induction of G2 arrest. We find that entry into G2 is a requirement for Vpr to induce apoptosis. We investigated the role of the mitochondrial permeability transition pore by knockdown of its essential component, the adenine nucleotide translocator. We found that Vpr-induced apoptosis was unaffected by knockdown of ANT. Instead, apoptosis is triggered through a different mitochondrial pore protein, Bax. In support of the idea that checkpoint activation and apoptosis induction are functionally linked, we show that Bax activation by Vpr was ablated when ATR or GADD45α was knocked down. Certain mutants of Vpr, such as R77Q and I74A, identified in long-term nonprogressors, have been proposed to inefficiently induce apoptosis while activating the G2 checkpoint in a normal manner. We tested the in vitro phenotypes of these mutants and found that their abilities to induce apoptosis and G2 arrest are indistinguishable from those of HIV-1NL4-3 vpr, providing additional support to the idea that G2 arrest and apoptosis induction are mechanistically linked.

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Introduction

Loss of CD4⁺ lymphocytes is a hallmark of progression to acquired immune deficiency syndrome (AIDS). The mechanisms proposed to explain the loss and dysfunction of CD4⁺ T cells are multiple and include indirect effects of viral infection, such as generalized activation of the immune system, CD8⁺ cytotoxic T lymphocyte–mediated killing of infected cells, as well as direct effects of infection such as virus budding, and expression of certain viral genes (reviewed in [1–3]). Analysis of virus dynamics in vivo has revealed that a significant portion of CD4⁺ T-cell death is due to virus-induced cytotoxicity in the infected cells [4–6], and the estimated half-life of infected lymphocytes is on the order of 2 d (reviewed in [7]). Therefore, studies that seek to understand the molecular and cellular basis of HIV-1-induced death in T cells are critical toward explaining how immune deterioration results from HIV-1 infection.

The HIV-1 viral protein R (Vpr) has emerged as a major proapoptotic gene product (reviewed in [8,9]). In an effort to characterize the apoptotic signaling cascade induced by Vpr downstream of the mitochondria, Muthumani et al. [10,11] demonstrated that Vpr induces apoptosis via the intrinsic pathway. This pathway is characterized by cytochrome c release and caspase 9 activation and is triggered in the absence of death receptor ligation.

Vpr induces a second type of cytopathic effect by blocking the cell cycle in the G2 phase. We, and others, have previously shown that Vpr causes activation of the G2 checkpoint protein, ATR (ataxia and telangiectasia mutated and Rad3 related), a serine/threonine kinase responsive to DNA damage and replication stress [12–14]. Furthermore, activation of ATR by Vpr is required for Vpr-induced G2 arrest and involves the ATR-associated molecules Rad17 and the Rad9-Rad1-Hus1 trimer [14–16].

A cause–effect relationship between the two deleterious actions of Vpr (G2 arrest and apoptosis) has not been established. Indirect evidence supports the notion that induction of G2 arrest and apoptosis are linked. For example, treatment of cells with either caffeine, an inhibitor of ATR/ATM checkpoint function, or small interfering RNA (siRNA) specific to ATR, relieves both Vpr-induced G2 arrest and apoptosis [17,18]. In addition, Yuan et al. [19,20] demonstrated that siRNA knockdown of Wee1, a Cdk1 inhibitor that is activated by DNA damage, abrogated both Vpr-induced G2 arrest and apoptosis. Taken together, these data suggest a model in which checkpoint activation and apoptosis signaling...
by Vpr are functionally associated and that such an association stems from the ability of Vpr to activate ATR [12,14,17].

On the other hand, evidence from mutagenesis studies suggests that checkpoint activation and apoptosis may be separable effects of Vpr. Thus, mutants of Vpr have been described that induce normal levels of G2 arrest but are partially impaired for induction of apoptosis [21,22]. One model to explain the proapoptotic activity of Vpr was proposed by Jacotot et al. [23] and Vieira et al. [24], who observed that recombinant Vpr associates with the adenine nucleotide transporter (ANT) on purified mitochondria, to directly promote release of mitochondrial-associated cytochrome c and apoptosis. This finding suggested that Vpr initiated the commitment to apoptosis at the mitochondrial membrane by binding to ANT, rather than by activating upstream stress signaling pathways derived from checkpoint activation.

In the present study, we examine the relationship between induction of G2 arrest and onset of apoptosis by Vpr. We find that entry into G2 is a requirement for Vpr to induce apoptosis. Since the requirement for entry into G2 seemed inconsistent with the reported ability of Vpr to bind to ANT and promote apoptosis in a cell cycle–independent manner, we then examined the requirement for ANT. We find that Vpr-induced apoptosis is unaffected by knockdown of ANT. Instead, Vpr-induced apoptosis is dependent on the presence of Bax and is concomitant with Bax activation. Furthermore, Bax activation is a result of proapoptotic signals transduced by Vpr through the upstream stress proteins ATR and GADD45α (growth arrest and DNA damage–responsive protein α) because knockdown of ATR or GADD45α prevented activation of Bax. We also demonstrate that, despite the striking similarities between the signaling events induced by Vpr and those induced by genotoxic stress, important differences can be found. Specifically, Vpr-induced apoptosis is abrogated by checkpoint inhibition, while apoptosis induced by genotoxic agents is exacerbated by checkpoint inhibition. Taken together, these results demonstrate that Vpr activates the ATR-initiated DNA damage–signaling pathway to link checkpoint activation and commitment to apoptosis.

**Results**

**G2 Arrest Precedes the Release of Mitochondrial Smac and Caspase Activation**

Treatment with caffeine, an inhibitor of ATR and ATM, or siRNA targeted to ATR, abrogates Vpr-induced G2 arrest and apoptosis [14,17,18]. One possible explanation for these observations would be that Vpr induces apoptosis as a downstream consequence of sustained G2 checkpoint activation. This idea, however, is inconsistent with a previously published model in which Vpr was proposed to directly bind to the mitochondrial ANT to cause release of proapoptotic factors in a cell cycle–independent manner [23,24].

To begin to differentiate between these models, we studied the kinetics of induction of G2 arrest and apoptosis. If apoptosis were a consequence of G2 checkpoint signaling, we would predict that G2 arrest should precede the onset of apoptosis. However, if Vpr were binding ANT to induce apoptosis, we would predict that commitment to apoptosis should be independent of cell cycle status and would occur early following expression of Vpr. We transduced a human CD4+ T-lymphocyte cell line, SupT1, with lentiviral vectors (pHR-VPR-G or pHR-VPR-R) expressing Vpr and either green fluorescent protein (GFP) or monomeric red fluorescent protein (mRFP), respectively, with an intervening internal ribosome entry site (Figure 1A). Having essentially identical vectors with different fluorescent tags allowed us to stain infected cells with a variety of fluorescent indicators of cell cycle profile and apoptosis. As a control, we used isogenic vectors expressing a mutant form of Vpr, pHR-VPR(R80A), wherein Vpr(R80A) is defective in induction of G2 arrest and apoptosis [17,25–27]. These vectors were previously described in detail [13,17,26]. In parallel experiments, we transduced SupT1 cells with VSV-G pseudotyped, env-defective vector derived from HIV-1NL4-3 (DHIv3; Figure 1A) or a mutant form of the previous vector with Vpr deleted (DHIv3-AVPR). At various time points, cultures were harvested and partitioned for further analyses. Analyses included cell cycle profile, induction of apoptosis as measured by caspase activation, and Western blot to detect release of Smac (second mitochondria-derived activator of caspase) into the cytoplasm.

Accumulation of Vpr-expressing cells at G2 began at 24 h post-transduction, peaked at 48 h, and persisted throughout the remainder of the experiment (Figure 1B). In contrast, caspase activation in Vpr-expressing cells was first detected at 36 h and continued to increase throughout the remainder of the experiment (Figure 1C). Release of Smac (Figure 1D) also occurred late, between 72 and 96 h post-transduction. Samples of flow cytometry charts from experiments shown in Figure 1C at the 72-h time point are presented in Figure 1E.

Therefore, induction of G2 arrest is an early Vpr-mediated event, whereas release of Smac and caspase activation lag in
Figure 1. Vpr-Induced Caspase Activation and Smac Release from the Mitochondria Are Temporally Delayed in Relation to G2 Arrest

(A) pHr-VPR-G and pHr-VPR-R are bicistronic lentiviral vectors that encode HIV-1NL4–3 vpr, an internal ribosome entry site, and either the gene for GFP or that for mRFP, respectively; pHr-VPR(R80A) was derived from pHr-VPR (both the mRFP and the GFP versions) by site-directed mutagenesis; DHIV3 is an envelope-truncated (see gray box) version of HIV-1NL4–3; DHIV3-ΔVPR was derived from DHIV-3 by introducing a frameshift mutation in vpr.

(B) SupT1 T lymphocytes were transduced by spin-infection in the presence of 10 µg/ml Polybrene with indicated vectors. Mock-infected cells were subjected to spin-infection in the presence of 10 µg/ml Polybrene without virus. Cells were collected at specified time points post-transduction, stained with propidium iodide, and analyzed for DNA content by flow cytometry to determine cell cycle profiles. The percentage of cells transduced with pHr vectors and DHIV3 viruses ranged between 70% to 80% and between 65% to 70%, respectively, as determined by mRFP or GFP expression (with pHr vectors) or intracellular p24 staining (with DHIV3 vectors).

(C) Caspase activation was measured as an indication of apoptosis. SupT1 cells were infected with indicated vectors, harvested, and incubated with FITC-VAD-FMK. The percentage of caspase-active cells at each time point was measured by flow cytometry.

(D) Infected SupT1 cells were lysed, and lysates were fractionated into mitochondrial (m) and cytoplasmic (c) fractions and then assayed by Western blot. Western blots were probed with antibodies specific to Smac to measure release from mitochondria and with anti-VDAC antibodies to measure mitochondrial contamination in the cytoplasmic fractions. As a positive control for apoptosis, SupT1 cells were treated with 0.8 µg/ml doxorubicin (dox) for 48 h.

(E) Examples of flow cytometric analysis of caspase activation, corresponding to the 72-h time points from (C).

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Figure 2. The Apoptotic Effect of Vpr Is Lost in Cells Synchronized in G1/S

(A) HeLa cells were transduced with pHR-VPR-G or mock-transduced and then incubated with 2 mM thymidine. After 24 h of incubation, cells were harvested, stained with PI, and analyzed for DNA content by flow cytometry to determine the percentage of cells in G1/S and G2/M. Transduction efficiency of pHR-VPR in both thymidine-treated and cycling cells was 70% to 75% as determined by analysis of GFP expression by flow cytometry (unpublished data).

(B) Cells from experiments shown in (A) were stained for DAPI at 72 h postinfection, in order to evaluate apoptosis via chromatin morphology.

(C) Quantitation of apoptosis in DAPI-stained samples shown in (B); incubation with 25 μM etoposide for 48 h or 1 μM staurosporine for 8 h was included in both cycling and thymidine-treated cells, for comparative purposes.

(D) Cycling or thymidine-synchronized HeLa cells were mock-transduced or transduced with either pHR-VPR-R or pHR-VPR(R80A) for apoptosis analysis, using FITC-VAD-FMK. As positive controls for apoptosis, cycling and synchronized cells were treated with etoposide or staurosporine as shown in (C).

(E) Cycling or thymidine-synchronized HeLa cells expressing Vpr or treated with 25 μM etoposide for 48 h were lysed and analyzed by Western blot for PARP cleavage as a marker of apoptosis/caspase activity. The caspase-cleaved PARP band is observed at 89 kDa.

(F) Cell lysates from pHR-VPR–transduced cells, with or without thymidine block, were harvested at 12 and 24 h post-transduction and analyzed by Western blot for Vpr expression with antibodies specific to the amino-terminal hemagglutinin tag.

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G1/S Block Abolishes the Induction of Apoptosis by Vpr

We designed an experiment to prevent Vpr-expressing cells from entering G2 and then asked whether these cells would still be susceptible to Vpr-induced apoptosis. We chose to perform the experiment in HeLa cells since these cells can be synchronized by incubation with 2 mM thymidine with minimal toxicity [28]. HeLa cells represent an appropriate cell type to study Vpr-induced apoptosis, because ATR activation, breast cancer-associated protein 1 (BRCA1) phosphorylation, and GADD45α upregulation are conserved from HeLa cells to primary human CD4+ lymphocytes [14,17,26,29]. HeLa cells were transduced with pHR-VPR-G and, 4 h later, treated with 2 mM thymidine, which arrests cells at the G1/S boundary [28]. DNA content analysis showed that thymidine treatment effectively synchronized uninfected cells in G1/S (Figure 2A). Thymidine synchronization of pHR-VPR-G-infected cells resulted in 82.9% cells in G1/S and 17.0% cells in G2/M. The comparatively high number of cells in G2/M in the synchronized, pHR-VPR–infected culture (17%) reflects cells that reached G2/M prior to synchronization and arrested due to Vpr expression. To evaluate the frequency of apoptotic cells in these cultures, cells were stained with the nuclear dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). DAPI binds to chromatin and reveals the presence of a conspicuous pyknotic nuclear morphology that is associated with late stages of apoptosis. We have previously described the use of DAPI for analysis of Vpr-induced apoptosis [17,30]. Figure 2B shows fluorescence microscopy photographs from this analysis, and quantitations are shown in Figure 2C. DAPI staining revealed that at 48 h posttransduction, 23% of cells transduced with Vpr were apoptotic if allowed to cycle, whereas only 7% were apoptotic if treated with thymidine (Figure 2C). The background level of apoptosis in uninfected cells treated with thymidine was 4%.

To address whether the decreased sensitivity of G1/S-arrested cells to Vpr-induced apoptosis could be due to thymidine treatment itself, cells were treated with a pharmacologic apoptosis inducer (etoposide or staurosporine) and then allowed to cycle, or not (thymidine incubation). Staurosporine is a kinase inhibitor and potent inducer of apoptosis with no known cell cycle specificity. Etoposide is a topoisoererase II inhibitor and an inducer of double-strand breaks (DSBs) and, like Vpr, causes G2 arrest [31,32]. Treatment with staurosporine or etoposide effectively induced apoptosis in both G1/S-arrested and cycling cells (Figure 2C).

To confirm the previous apoptosis results, we performed parallel experiments using pHR-VPR-R and staining for caspase activity with FITC-VD-AM-FMK (Figure 2D) and by measuring poly-ADP-ribose polymerase (PARP) cleavage (Figure 2E). Analysis of Vpr expression by Western blot revealed that thymidine treatment did not affect expression of Vpr (Figure 2F). These results are in agreement with those obtained by DAPI staining and confirm that synchronization of cells in G1/S alleviates Vpr-induced apoptosis.

Release of Vpr-Expressing Cells from G1/S Block Leads to G2 Arrest-Dependent Apoptosis

The previous results indicate that the proapoptotic effect of Vpr is lost when cells are artificially maintained in G1/S. To determine whether induction of apoptosis by Vpr specifically requires transition into G2, cells were synchronized as in the previous experiment and then were released from thymidine block and allowed to reenter the cell cycle. Released cells were harvested at specified intervals to examine cell cycle progression (Figure 3A) and apoptosis (Figure 3B). Both mock-treated and Vpr-expressing cells entered G2 at 12 h postrelease. Mock-infected cells completed the first division cycle at 18 h postrelease and continued to cycle (Figure 3A, upper panels), while cells expressing Vpr persisted in G2 for the remainder of the experiment (Figure 3A, lower panels). A small amount of cells (17.4%) in the Vpr-transduced culture reached G1 at 18 h postrelease. The reason for this is that the efficiency of transduction with pHR-VPR was about 80% and therefore about 20% are untransduced.

We then asked when, after release from the thymidine block, Vpr-expressing cells would enter apoptosis. Cells which were synchronized, maintained in G1 for 48 h, and then released (Figure 3B, lanes 2–6) began to display detectable PARP cleavage by 12 h postrelease (Figure 3B, lane 5). Therefore, the onset of apoptosis in Vpr-expressing cells is concomitant with entry into the G2 phase. In the absence of synchronization in G1 (Figure 3B, lanes 7–11), Vpr-expressing cells begin to display PARP cleavage by 24 h posttransduction. Synchronized cells, in the absence of Vpr expression, did not display PARP cleavage after release (unpublished data).

Parallel samples from the previous synchronization experiment were also analyzed by DAPI staining (Figure 3C). These experiments revealed chromatin fragmentation/condensation in Vpr-expressing cells at 24 and 48 h postrelease (Figure 3C).

Taken together, these data indicate that Vpr-induced apoptosis requires entry into the G2 phase and artificially maintaining cells in G1 effectively prevents the onset of apoptosis.

Inhibition of ATR Prevents Vpr-Induced Apoptosis but Exacerbates Genotoxic-Induced Apoptosis

Due to the phenotypic similarities between Vpr and genotoxic stress, many studies on Vpr have been modeled based on the current understanding of DNA damage signaling [12,14,33]. Several reports have shown that suppression of G2 checkpoint-activating kinases after exposure to genotoxic agents overrides cell cycle arrest but exacerbates apoptosis [34–37]. The reason for this increase in cell death is thought to be the inability of cells to successfully duplicate DNA or to align and segregate damaged chromosomes during mitosis (reviewed in [35]). Thus, we set out to compare in parallel the effect of ATR suppression on Vpr- versus genotoxic-induced apoptosis. We reasoned that if Vpr causes irreparable DNA damage, then the effect of checkpoint overriding on Vpr-induced apoptosis should mirror that of cells treated with genotoxic agents. To test this, we treated cells with siRNAs specific to ATR, ATM, or nonspecific siRNA. At 48 h following siRNA transfection, we treated cells with 25 μM N-methyl-N’-nitro-N-nitosoguanidine (MNNG), an SN1-type methylating agent and inducer of G2 arrest, or 25 μM etoposide. At 24- and 48-h time points following the addition
**Figure 3.** Release of Vpr-Expressing Cells from Thymidine Block Leads to Reentry into the Cell Cycle and Vpr-Induced, G2-Dependent Apoptosis

(A) Thymidine-synchronized HeLa cells transduced with pHR-VPR-G or mock-transduced were released from thymidine block and harvested at specified time points postrelease. Cells from each time point were stained with PI and analyzed for DNA content by flow cytometry.

(B) pHR-VPR-transduced HeLa cells from the same experiment were monitored by Western blot for PARP cleavage at specified time points. Time points labeled under “Thym + pHR-VPR” indicate hours following release from thymidine block; time points under “pHR-VPR” indicate hours following transduction, as these cells were not synchronized.

(C) Cells treated as those in (A) and (B) were harvested at 24 and 48 h and analyzed for apoptosis by DAPI staining, and the results are quantified.

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of etoposide or MNNG, we measured caspase activation (Figure 4A and 4B; for simplicity, only one time point is shown in 4A). Knockdown of ATR dramatically exacerbated etoposide- and MNNG-induced apoptosis (14.5% to 60.7% and 6.2% to 35.4%, respectively, at 48 h). Knockdown of ATM led to a modest increase in genotoxin-induced apoptosis (14.5% to 21.3% for etoposide and 6.2% to 10% for MNNG, at 48 h). In contrast, Vpr-induced caspase activation was dramatically inhibited by ATR knockdown (from 41.2% to 14.2%) but was unaffected by ATM knockdown (41.2% to 39.4%). DAPI staining showed a similar ATR-dependent decrease in Vpr-induced apoptosis (Figure 4C). The level of knockdown achieved by each siRNA was determined by Western blot with antibodies specific for the endogenous proteins (Figure 4D).

In conclusion, we demonstrated the requirement for ATR checkpoint activation through two different methodologies: (a) manipulation by G1/S synchronization and (b) ATR knockdown. We also conclude that this aspect of Vpr function represents a departure from the manner in which checkpoint activation relates to apoptosis in the context of genotoxic agents.

Vpr-Induced Apoptosis Does Not Require ANT but Is Mediated via Bax, Downstream of ATR Activation

Early reports suggested that the permeability transition pore complex (PTPC), which consists of VDAC, ANT, and cyclophilin D, was involved in release of apoptotic factors from the mitochondria in response to various apoptotic stimuli (reviewed in [38]) and, in particular, for Vpr [23]. However, later reports have put in question the role of the PTPC in DNA damage–induced apoptosis [39–42]. For example, mouse cells deficient in ANT are fully capable of undergoing apoptosis in response to DNA damage and, instead, show increased resistance to necrosis in response to high intracellular Ca\(^{2+}\) [39]. Mouse cells deficient in cyclophilin D, which is required for VDAC function, are fully capable of undergoing apoptosis in response to genotoxins [40,42]. Large oligomeric complexes containing Bax and possessing apoptotic pore function do not contain ANT or VDAC [43]. Bax is required for activation of the mitochondrial pore-forming complex that responds to DNA damage (reviewed in [44,45]) and the pore-forming function of Bax is independent of ANT or VDAC [43,46–48].

Thus, we asked whether Vpr-induced apoptosis is mediated through ANT or, alternatively, through Bax. We addressed this question using siRNAs that would specifically down-regulate Bax or ANT, in the context of pHr-VPR-R transduction. Bax knockdown led to a dramatic decrease in Vpr-induced apoptosis (from 41.2% in the presence of nonspecific siRNA to 12.6% in the presence of Bax siRNA; Figure 4A), whereas ANT knockdown had no appreciable effect on apoptosis (41.2% versus 39.5%; Figure 4A). These results were also confirmed by analysis of DAPI-stained nuclei (Figure 4C). The levels of knockdown achieved by each siRNA are shown in Figure 4D.

Activation of Bax is associated with a conformational change that exposes an N-terminal epitope detected by monoclonal antibody Bax6A7 [49]. Thus, reactivity with Bax6A7 provides a direct measurement of Bax activation. To probe for Bax activation in the presence of Vpr, peripheral blood CD4\(^+\) lymphocytes were infected with either DHIV3 or DHIV3-ΔVPR, cells were lysed, and immunoreactivity with Bax6A7 antibody was tested by immunoprecipitation followed by Western blot (Figure 4E). Reactivity with Bax6A7 was increased following infection with DHIV3, when compared with that of mock- or DHIV3-ΔVPR–infected cells.

Our previous studies established that Vpr-induced ATR activation leads to BRCA1 phosphorylation and GADD45α upregulation and that both ATR and GADD45α are required for Vpr-induced apoptosis [14,17]. Based on previous findings and data reported here, we propose that ATR activation is an upstream signaling event in the pathway leading to Vpr-induced cell cycle arrest and apoptosis. If this were correct, then it would follow that Bax activation by Vpr is also ATR dependent. In order to examine the requirement of ATR in Vpr-induced Bax activation, we treated cells with siRNAs specific to either ATR or GADD45α prior to transduction with pHR-VPR–R. We observed that the increase in Bax6A7 reactivity was reduced to basal levels in the presence of either ATR or GADD45α knockdown but not when using a nonspecific siRNA (Figure 4F). Therefore, these results provide additional support for a model in which Bax activation is a principal effector of Vpr-induced apoptosis downstream of G2 checkpoint activation by the ATR kinase.

Effect of Vpr on Mitotic Entry

It is unclear whether cells arrested in G2 by Vpr transition into mitosis. Early measurements of the mitotic index suggested that Vpr induced G2 arrest and concomitantly inhibited entry into mitosis [50–53]. Later studies, however, showed that cells expressing Vpr develop mitotic abnormalities such as multipolar spindles, mislocalization of certain spindle pole body proteins, and defects in cytokinesis [54,55], collectively indicating mitotic entry. To reexamine the issue of mitotic entry in the context of Vpr, we measured phosphorylation of histone 3 (H3) at serine-10 [56,57] in the presence of Vpr (Figure 5). As positive and negative controls, we incubated cells with nocodazole (lanes 2 and 6) and doxorubicin (lanes 3 and 7), respectively. Doxorubicin is a genotoxic agent that intercalates into DNA, inhibits topoisomerase 2 and induces G2 arrest via Cdc2 Tyr15 phosphorylation, without allowing mitotic entry. Nocodazole arrests cells in mitosis and exerts its effect by depolymerizing microtubules. HeLa cells transduced with pHR-VPR displayed a high level of H3 phosphorylation (lane 4), whereas transduced SupT1 cells displayed no detectable H3 phosphorylation (lane 8). Therefore, Vpr expression leads to mitotic entry in HeLa cells following G2 arrest, whereas Vpr causes sustained G2 arrest in SupT1 cells without subsequent mitotic entry.

Analysis of G2 Arrest and Apoptosis Induction by Clinically Relevant Vpr Alleles and by a Vpr-GFP Fusion Chimera

Several natural or laboratory-constructed mutants of HIV-1 Vpr have been reported that selectively ablate either G2 arrest or apoptosis. These mutants can be categorized into two groups. The first group includes vpr alleles with amino acid substitutions that have been observed in long-term nonprogressors, with a reduced ability to cause apoptosis but with normal induction of G2 arrest. These substitutions include Q3R [22], R77Q [21], and I47A [58]. The second group includes Vpr-GFP and GFP-Vpr fusion proteins. In particular, it was reported that while a GFP-Vpr (whereby Vpr is
Figure 4. siRNA-Mediated Knockdown of ATR or Bax, but Not of ANT, Suppresses Vpr-Induced Apoptosis

(A) HeLa cells were transfected with nonspecific (NS) siRNA or siRNA targeted to ATR, or ATM as indicated. At 48 h post-transfection, cells were either treated with 25 μM etoposide, treated with 25 μM MNNG, mock-transduced, or transduced with pHR-VPR-R. Additionally, cells transfected with siRNAs targeted to Bax or ANT were transduced with pHR-VPR-R or mock-transduced (lower left dot plots). Cells from each treatment were assayed for caspase activity as in Figure 2B.

(B) Cells treated as in (A) were harvested at specified time points post-transduction and assayed for caspase activity.

(C) Cells treated as in (A) were stained with DAPI, and the results were quantified by microscopy.

(D) Cells treated with the indicated siRNAs were lysed and analyzed by Western blot to verify knockdown efficiency.

(E) Primary human CD4+ lymphocytes were infected with DHIV3, infected with DHIV3ΔVPR, or mock-infected. At 48 h postinfection, cells in each treatment were lysed and assayed for protein concentration. Equal amounts of protein from each treatment were incubated with Bax6A7 monoclonal antibody. Antibody–protein complexes were precipitated with agarose beads and boiled and then subjected to Western blot analysis with a polyclonal antibody to Bax.

(F) HeLa cells treated with the indicated siRNAs and transduced with pHR-VPR or mock-transduced were lysed; reactivity to Bax6A7 antibody was assayed as described in (E).

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ylation BRCA1 at residue Ser1423 by the ATR kinase and that induction, when compared to wild-type Vpr.

mutants tested here resulted in lower levels of apoptosis Vpr(I74A). Therefore, we conclude that neither of the Vpr 0.83 for wild-type Vpr, 0.91 for Vpr(R77Q), and 0.92 for.

constructed in the background of pHR-VPR-R (which represents a gain-of-function phenotype and is not repre-

sentative of the biology of wild-type Vpr. Collectively, the data obtained with both groups of mutants would appear to support that induction of G2 arrest and apoptosis are independent, separable functions of Vpr. We wished to examine the phenotype of these mutants and chimeras using the experimental paradigms we described in Figure 1.

The Vpr mutants, R77Q and I74A, were separately constructed in the background of pHR-VPR-R (which encodes HIV-1 vpr). Lentiviral vectors were then produced and used to infect target cells. Transduced cells were then evaluated for cell cycle stage at 48 h and for apoptosis at 72 h (Figure 6A). The levels of apoptosis, as judged by staining with FITC-VAD-FMK, were very similar between wild-type Vpr, Vpr(R77Q), and Vpr(I74A) and the levels of G2 arrest were also similar. To more directly assess whether the frequencies of G2 arrest and apoptosis correlated with each other, we calculated the ratio of cells in apoptosis over cells in G2 for each mutant Vpr (% apoptotic cells/ % cells in G2/M). If a Vpr mutant induces normal levels of G2 arrest, but reduced levels of apoptosis, then the Apo/G2M ratio should decrease. The Apo/G2M ratios were 0.83 for wild-type Vpr, 0.91 for Vpr(R77Q), and 0.92 for Vpr(I74A). Therefore, we conclude that neither of the Vpr mutants tested here resulted in lower levels of apoptosis induction, when compared to wild-type Vpr.

We previously demonstrated that Vpr induced phosphorylation BRCA1 at residue Ser1423 by the ATR kinase and that this phosphorylation correlated with induction of apoptosis [17]. To examine whether the Vpr mutants had a normal ability to induce BRCA1 phosphorylation, we performed Western blot on lysates from cells infected with the above lentiviral vectors. Wild-type Vpr and mutants were able to induce BRCA1 phosphorylation, and this phosphorylation was, in all three cases, ablated by treatment with caffeine (Figure 6B). Therefore, we were unable to find any detectable differences in the functions of Vpr(R77Q) or Vpr(I74A) when compared with Vpr(R77).

We then examined the phenotype of a Vpr-GFP fusion protein. It is well known that the addition of recombinant amino acid sequences as small as the influenza hemagglutinin tag at the carboxyl terminus of Vpr results in failure of Vpr to activate the G2 checkpoint [25]. Carboxyl-terminal addition of larger fusion partners, such as luciferase [25] or GFP [59], also results in ablation of the G2 arrest. Waldhuber et al. [59] found that carboxyl-terminal addition of GFP resulted in a Vpr chimera that was fully able to induce apoptosis. We constructed a Vpr-GFP chimera and observed that it was, as expected, unable to induce G2 arrest (unpublished data). We then examined the induction of apoptosis by the fusion protein. To generate an appropriate control, we introduced the R80A mutation in the fusion construct, to generate Vpr(R80A)-GFP, and tested its ability to induce apoptosis (Figure 6C). Both Vpr-GFP and Vpr(R80A)-GFP were able to induce apoptosis. Since Vpr(R80A) that is not fused to GFP is unable to induce apoptosis (Figures 1 and 2), we expected that the introduction of the R80A substitution in the Vpr-GFP fusion protein [Vpr(R80A)-GFP] would also result in a protein devoid of apoptosis induction. Surprisingly, Vpr(R80A)-GFP is also capable of inducing apoptosis. Because the fusion with carboxyl-terminal GFP turns Vpr(R80A), a nonapoptotic protein, into an apoptotic one, it appears that the induction of apoptosis by Vpr-GFP represents a gain-of-function phenotype and is not representative of the biology of wild-type Vpr.

In conclusion, our results suggest that induction of G2 arrest and apoptosis by HIV-1 Vpr are functionally interrelated and not genetically separable.

Discussion

The Functional Relationship between G2 Arrest and Apoptosis

One gap in our current understanding of checkpoint signaling and apoptosis lies between the activation of nuclear checkpoint sentinels, such as ATR and ATM, and the involvement of Bcl-2 family of apoptosis regulators at the mitochondria. Previous reports have suggested that nuclear proteins, such as Rad9 or Histone H1, may translocate from the nucleus to the cytoplasm in response to DNA damage, to interact with mitochondrion-associated Bcl-2 family proteins and promote cytochrome c release and apoptosis [60,61]. In other instances of DNA damage, activated p53 was shown to directly promote Bax activation [62,63]. However, loss of p53 does not abrogate induction of apoptosis by Vpr [30,64], and we have been unable to observe Rad9 or histone H1 translocation to the mitochondria in response to Vpr (J. L. Andersen and V. Planelles, unpublished data). These observations, taken together with the results presented here, indicate that Vpr links checkpoint activation and apoptosis.

Figure 5. Vpr Induces Histone 3 Phosphorylation in HeLa but Not in SupT1 Cells

HeLa or SupT1 cells were transduced with pHR-VPR-G and at indicated time points, lysed, and analyzed by Western blot with a phospho-specific antibody that recognizes phosphorylation of H3 at serine-10. Nocodazole (250 ng/ml) and doxorubicin (1 μM) were used as positive and negative controls, respectively.

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carboxyl-terminal to GFP) fusion protein was inactive in both G2 arrest and apoptosis induction, the reciprocal chimera, Vpr-GFP, was able to induce apoptosis in the absence of G2 arrest [59]. Collectively, the data obtained with both groups of mutants would appear to support that induction of G2 arrest and apoptosis are independent, separable functions of Vpr. We wished to examine the phenotype of these mutants and chimeras using the experimental paradigms we described in Figure 1.

The Vpr mutants, R77Q and I74A, were separately constructed in the background of pHR-VPR-R (which encodes HIV-1 vpr). Lentiviral vectors were then produced and used to infect target cells. Transduced cells were then evaluated for cell cycle stage at 48 h and for apoptosis at 72 h (Figure 6A). The levels of apoptosis, as judged by staining with FITC-VAD-FMK, were very similar between wild-type Vpr, Vpr(R77Q), and Vpr(I74A) and the levels of G2 arrest were also similar. To more directly assess whether the frequencies of G2 arrest and apoptosis correlated with each other, we calculated the ratio of cells in apoptosis over cells in G2 for each mutant Vpr (% apoptotic cells/ % cells in G2/M). If a Vpr mutant induces normal levels of G2 arrest, but reduced levels of apoptosis, then the Apo/G2M ratio should decrease. The Apo/G2M ratios were 0.83 for wild-type Vpr, 0.91 for Vpr(R77Q), and 0.92 for Vpr(I74A). Therefore, we conclude that neither of the Vpr mutants tested here resulted in lower levels of apoptosis induction, when compared to wild-type Vpr.
in a novel manner and that a key element is the requirement for entry into G₂ (Figure 7A).

In support of a cause–effect relationship between Vpr-induced G₂ arrest and apoptosis, several reports demonstrate that the apoptotic effect of Vpr can be overridden by suppressing G₂-specific cell cycle–regulating kinases [17–20]. Yuan et al. [19,20] found that Wee1, a kinase which negatively regulates Cdk1 activity and thus regulates the G₂-to-M transition, is activated by Vpr. Furthermore, Yuan et al. [19,20] showed that suppression of Wee1 in the presence of Vpr abrogated both Vpr-induced G₂ arrest and apoptosis.

The Role of ATR in Vpr-Induced Apoptosis Differs from Its Role in Genotoxic Stress-Induced Apoptosis

We also demonstrate that ATR knockdown exacerbates genotoxin-induced apoptosis but, surprisingly, relieves Vpr-induced apoptosis [17]. The different effects of ATR knockdown on Vpr- versus genotoxin-induced apoptosis suggest that Vpr activity fundamentally differs from genotoxic stress. More specifically, it is tempting to speculate that such a difference resides in the fact that Vpr does not cause physical DNA damage (such as DSBs) whereas genotoxic agents do. In fact, three lines of evidence indicate that Vpr activates ATR in a manner that does not involve generation of DSBs: (1) Pulse-field gel electrophoresis revealed no DSBs in vpr-expressing cells [12]. (2) Vpr fails to induce phosphorylation of ATM at serine-1981, a residue linked to DSB signaling and potently phosphorylated in response to ionizing radiation [12]. (3) And, as we have shown here, in contrast with etoposide-induced apoptosis, Vpr-induced apoptosis is relieved under conditions of checkpoint suppression.

Models for Vpr activity that would be in agreement with
the lack of DSB formation by Vpr have been proposed in the past. For example, Vpr may perturb nuclear envelope integrity, as suggested by de Noronha et al. [65]. Perturbation of nuclear envelope integrity may, in turn, lead to ATR activation. In addition, Lai et al. proposed that Vpr binds directly to chromatin in a manner that results in ATR activation [12].

Role of the Mitochondria in Vpr-Induced Apoptosis

Previous reports have suggested that Vpr induces apoptosis via a direct interaction with ANT at the inner mitochondrial membrane, which results in release of cytochrome c from fractionated mitochondria [23]. These observations would indicate that Vpr induces mitochondrial depolarization directly, rather than activating upstream stress signals, and suggest that Vpr may induce apoptosis rapidly after being expressed (Figure 7B). We reasoned that if Vpr induced apoptosis by interacting directly with the mitochondria, it would efficiently induce apoptosis regardless of cell cycle status. In contrast with the previous expectation, we observed that cell cycle transition into G2 is required for Vpr to induce apoptosis. Furthermore, blockade of the cell cycle in G1 for 48 h effectively prevented apoptosis, and subsequent release of the block allowed apoptosis induction, coinciding with entry into G2.

Jacotot et al. [23] proposed that the mitochondrial channel activated by Vpr is the PTPC. In sharp contrast, we find that efficient removal of ANT (an essential domain of PTPC) did not affect Vpr apoptosis to any degree. Instead, removal of Bax, an independent mitochondrial pore-forming protein, led to nearly complete suppression of apoptosis. Furthermore, Bax activation is dependent on the presence of ATR, as is induction of apoptosis. Taken together, our data strongly support a model in which the apical step toward Vpr-induced apoptosis is activation of ATR, followed by the activation of downstream apoptotic mediators such as Bax, Smac, and caspases (Figure 7).

Role of Vpr in HIV-1 Pathogenesis

Depletion of CD4 lymphocytes due to HIV-1 infection leads to immune suppression in AIDS patients (reviewed in [66]). The cause of T-cell depletion in AIDS patients is a question under intense research but remains poorly understood. The discovery of mutations in Vpr that may be associated with long-term nonprogressors and impair the apoptosis-inducing ability of Vpr suggests a critical role for Vpr in AIDS pathology [21,22,58]. However, for the Vpr mutation, R77Q, a recent report has questioned its association with long-term nonprogressors [67]. Fisher et al. [67] proposed that R77Q is associated with certain viral subtypes, such as subtype A, and not with disease progression. A more recent analysis of the GenBank database revealed that R77 predominates in subtype B, whereas Q77 predominates in clades A, C, D, G, and H and groups O and N viruses, and subtype F and K strains frequently encode H77 [68]. Rajan et al. [68] also reported, using isogenic, full-length viruses, that HIV-1 carrying Q77 displayed reduced cytopathicity if the virus had R5 tropism but not if it had X4 tropism. The previous experiments did not uncouple the cytopathic potential of the virus strains from their replication kinetics. Therefore, in our studies, we wished to examine the proapoptotic potential of mutant Vpr proteins in the context of a nonreplicating lentiviral vector and in the absence of other viral genes. Our results conclusively show that in our experimental paradigm, the proapoptotic potentials of Vpr(R77Q) and Vpr(I74A) are indistinguishable from that of Vpr(R77). Thus, the results by Rajan et al. [68] would seem to indicate that the R77Q substitution has a moderate, positive effect on cell viability, and this effect may be related to viral replication efficiency rather than to proapoptotic signaling.

Materials and Methods

Western blotting procedures. For PARP and HA-Vpr detection, cells were detached with trypsin, washed once with media containing 10% fetal bovine serum, and then washed twice in PBS. Detached cells were pelleted by centrifugation and then resuspended in ice-cold lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA) in the presence of Complete Protease Inhibitors (Roche, http://www.roche.com). Cell lysates were passed through a 25-gauge needle ten times and then resuspended in Criterion loading buffer (Bio-Rad, http://www.bio-rad.com). Next, 20 μg of cell lysate per sample was loaded on a Criterion XT 10% polyacrylamide gel (Bio-Rad). Following electrophoresis (at 50 amps/gel), proteins were transferred to Immobilon FL PVDF membranes (Millipore, http://www.millipore.com) and then immunostained with anti-PARP (Cell Signaling Technologies, http://www.cellsignal.com) and anti-rabbit 680 (Invitrogen, http://www.invitrogen.com) antibodies. The primary anti-PARP antibody was diluted 1:1,000 in TBPS (PBS, 0.1% Triton-X 100) plus 5% nonfat dry milk (NFDM). The secondary anti-rabbit 680 antibody was diluted 1:10,000 in TBPS plus 5% NFDM and 0.1% sodium dodecyl sulfate (SDS). Blots were incubated with secondary antibody for 1 h at 4°C and then washed 5 times with TBPS. Protein bands were visualized by the Odyssey infrared system (LI-COR Biosciences, http://www.licor.com). Protein concentrations of all samples were determined by BCA protein assay (Pierce Biotechnology, http://www.piercenet.com) prior to loading.

For all other Western blots, the above protocol was followed with...
the exception that secondary HRP-conjugated antibodies were diluted in PBBS plus 5% NFDM and bands were visualized by ECL detection, using ECL plus (Amersham Biosciences, http://www.amershambiosciences.com). For Bax 6A7 immunoprecipitation, cells were lysed in CHAPS buffer (10 mM HEPES, 150 mM NaCl, 1% CHAPS, protease inhibitors) for 10 min, followed by ten passes through a 25-gauge needle. Then 2 μg of Bax 6A7 (Abcam, http://www.abcam.com) was added to 1 mg of cellular protein and incubated 8 h at 4 °C under constant mixing followed by incubation with protein A/G beads (Santa Cruz Biotechnology, http://www.scbt.com) according to the manufacturer’s protocol. Protein-bead complexes were then washed with lysis buffer 3 times, boiled for 3 min, and subject to Western blot with anti-Bax antibody (Cell Signaling Technologies) and ECL detection according to methods described above. Antibodies used were anti-ATR (obtained from Dr. Paul Nghiem, Harvard University, Boston, Massachusetts, United States), anti-GADD45 (Santa Cruz Biotechnology), anti-cytochrome c (Santa Cruz Biotechnology), anti-Smac/Diablo (Imgenex Corporation, http://www.imgenex.com), and Bax 6A7 (Abcam).

Cell cycle analysis. Cells were harvested and counted; 1 × 10^6 cells were aliquoted into tubes and washed once with cold PBS. Washed cells were resuspended in 0.5 ml of cold PBS, following which 2 ml of ice-cold ethanol was slowly added with gently vortexing. Ethanol-fixed cells were left overnight at −20 °C. The following day, cells were centrifuged at 1000 g for 10 min, and ethanol was washed from the cells. Cells were then resuspended in propidium iodide staining buffer (10 μg/ml propidium iodide, 2% FBS, 11.25 U RNase A/ml, 0.02% sodium azide) and allowed to incubate for 10 min on ice prior to analysis of DNA content by flow cytometry. Cell cycle profiles were further analyzed using Modfit software (Verity Software, http://www.verity.com) to derive percentiles of cells in different phases of cell cycle.

Viral vectors and virus. DHIV3 and pHRLentiviral vectors were produced by transient transfection of HEK293T cells. For production of pHRL vectors, pHRL-VPR or pHRL-VPR (R8.2) was cotransfected with pCMVARS:2AVPR [69], and pCMV-Luciferase [70] by calcium phosphate–mediated transfection [18]. Virus-containing supernatants were harvested at 24, 48, and 72 h post-transfection. Supernatants were cleared of cellular debris by centrifugation at 828 × g for 10 min, and virus in cleared supernatants was concentrated by centrifugation at 115,889 × g for 2 h at 4 °C. Virus was titrated by infection of HeLa cells and subsequent flow cytometric analysis of the reporter molecule GFP. Vector titers were calculated with the equation \((T × C) / V × D\), where \(T\) is the frequency of GFP-positive cells found by flow cytometry, \(C\) is the total number of target cells at the time of infection, \(V\) is the volume of inoculum, and \(D\) is the virus dilution factor. The virus dilution factor used for titrations was 10. The total number of target cells at the time of infection for titer was 0.5 × 10^9. Infections were performed at a multiplicity of infection (MOI) of 2.5 with 10 μg of Polybrene. Infections with siRNA-treated cells were performed 48 h after siRNA transfection. T cells and HeLa cells were transduced with virus diluted in cell culture media with 8 μg/ml and 10 μg/ml Polybrene, respectively. For HeLa cell transduction, virus-containing media was washed after 8 h and replaced with fresh culture media. HeLa cells were transduced by spinoculation as previously described [71]. The HIV-1 molecular clone HIV-1NL4-3 was transfigured into 2 × 10^5 HIV-1NL4-3 cells by calcium phosphate transfection. At 48 h after transfection, HEK293FT cells were co-cultured with 10^5 MT-2 cells for 5 h. MT-2 cells were then cultured alone until approximately 75% of cell clumps sludge–syncytia. Virus-containing supernatants were then cleared of cells and debris by centrifugation at 2,000 rpm for 10 min. Viral stocks were then frozen at −80 °C. Spin infections were performed as described above.

Apoptosis assays. For analysis of apoptotic nuclear morphology, cells were fixed in the well with 2% paraformaldehyde (in PBS) for 15 min at room temperature (RT). Fixed cells were then permeabilized in 0.1% Triton X-100 (in PBS) for 15 min at RT, gently washed 2 times in PBS, and then incubated in 0.5 μg/ml DAPI (Invitrogen) for 45 min at 37 °C. Apoptotic nuclei were identified by fluorescence microscopy and quantitated and divided by total cells to determine percent apoptosis. For cell counting, fields were chosen at random, and a minimum of 1,000 cells were counted per treatment in each experiment. Standard deviations were derived from three separate experiments. PARP cleavage was assayed by Western blot as described above. For analysis of caspase activity, cells were stained with FITC-VAD-FMK (Promega, http://www.promega.com) or RED-VAD-FMK (Calbiochem/EMD Biosciences, http://www.emdbiosciences.com) according to manufacturer’s protocols. Caspase-stained cells were analyzed by flow cytometry.

Phosphotyrosine exposure at the cell surface was analyzed as previously described [72] by flow cytometry using phycocyanin-conjugated annexin V (Annexin V-PE, Bender MedSystems, http://www.bendermedsystems.com).

siRNA treatments. All siRNA treatments were performed with Dharmacore anti-manus smart pool siRNA duplexes (Dharmaco, http://www.dharmaco.com). Smart pool siRNAs were transfected at a final concentration of 100 nM into exponentially growing HeLa cells with Oligofectamine (Invitrogen) according to the manufacturer’s protocol. Cells were split 1:3 at 24 h post-transfection. At 48 h post-transfection, cells were harvested to verify knockdown by Western blot or subject to experimental treatments.

Cell culture. For experiments in which cell synchronization or the use of siRNAs was to be employed, we used the human cervical cancer cell line HeLa, which was maintained in Dulbecco’s modified Eagle’s medium (Cambrex BioScience (formerly BioWhittaker), http://www.cambrex.com), supplemented with 10% FCS and 2 mM l-glutamine. For experiments in which viral transduction and measurement of cell cycle/apoptosis were the aims, we used the human T-cell line SupT1, which was maintained in RPMI 1640 (Cambrex BioScience), supplemented with 10% FCS and l-glutamine.

Peripheral blood mononuclear cells were obtained from leukopaks from unidentified, healthy donors (American Red Cross, http://www.redcross.org), and CD4+ lymphocytes were purified using anti-CD4 magnetic beads (Dynal/Invitrogen) according to the manufacturer’s instructions. CD4+ T cells were activated by culture in RPMI plus 10% FCS plus 1 mM l-glutamine with 3 anti-CD3anti-CD28 beads per cell (Invitrogen) for 2 d, changing medium daily. After 2 d, recombinant interleukin 2 was added to the culture medium at a concentration of 100 units/ml.

Drug treatments. Thymidine was diluted to a concentration of 2 mM in culture media and then added to exponentially growing HeLa cells. For experiments in which thymidine treatment was combined with viral gene expression, thymidine-containing media was added immediately following transduction. For experiments in which thymidine treatment was combined with genotoxic drugs, cells were incubated in thymidine-containing media for 4 h prior to the addition of genotoxins. Etoposide (Sigma Aldrich, http://www.sigmaaldrich.com) and MNNG (Sigma Aldrich) were diluted in culture media to a concentration of 25 μM and incubated with cells for 3 h. Following incubation, genotoxin-containing media was removed and replaced with fresh media without or with thymidine.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the proteins discussed in this paper are ANT (P12235 [GenPept]); ATM (NM_000051.1), ATR (NM_001184), BRCA1 (NP_000923.5), GADD45 (NM_001294), HIV-1_1.5-3 VPR (AAB6574 [GenPept]), and VDAC (P21796 [GenPept]).

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Author contributions. JLA, GJ, SB, and VP conceived and designed the experiments. JLA, JLD, GJ, and SB performed the experiments. JLA, JLD, GJ, and SB performed the experiments. JLA, JLD, SB, and VP performed the data analysis. JLA, JLD, ESZ, OA, BK, and VP contributed reagents/materials/analysis tools. JLA and VP wrote the paper.

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