The Ability of Primate Lentiviruses to Degrade the Monocyte Restriction Factor SAMHD1 Preceded the Birth of the Viral Accessory Protein Vpx

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SUMMARY

The human SAMHD1 protein potently restricts lentiviral infection in dendritic cells and monocyte/macrophages but is antagonized by the primate lentiviral protein Vpx, which targets SAMHD1 for degradation. However, only two of eight primate lentivirus lineages encode Vpx, whereas its paralog, Vpr, is conserved across all extant primate lentiviruses. We find that not only multiple Vpx but also some Vpr proteins are able to degrade SAMHD1, and such antagonism led to dramatic positive selection of SAMHD1 in the primate subfamily Cercopithecinae. Residues that have evolved under positive selection precisely determine sensitivity to Vpx/Vpr degradation and alter binding specificity. By overlaying these functional analyses on a phylogenetic framework of Vpr and Vpx evolution, we can decipher the chronology of acquisition of SAMHD1-degrading abilities in lentiviruses. We conclude that vpr neofunctionalized to degrade SAMHD1 even prior to the birth of a separate vpx gene, thereby initiating an evolutionary arms race with SAMHD1.

INTRODUCTION

HIV-1 and other primate lentiviruses encode accessory genes that serve to enhance virus replication and counteract host immune factors (Malim and Emerman, 2008). Studies of these accessory proteins have led to the identification of important restriction factors encoded by host genomes (Kirchhoff, 2010). The accessory protein Vpx was previously shown to be critical for the ability of primate lentiviruses to efficiently infect monocytes, dendritic cells, and mature macrophages (Ayinde et al., 2010; Sharova et al., 2008). Recently, the target of Vpx has been identified as the restriction factor SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011), where the binding of Vpx to SAMHD1 leads to the proteasomal degradation of SAMHD1.

Humans with missense mutations in SAMHD1 are associated with Aicardi-Goutieres syndrome (AGS), an encephalitis syndrome which mimics a state of viral infection leading to interferon production and an autoimmune syndrome (Rice et al., 2009; Stetson et al., 2008). SAMHD1 has recently been shown to be a deoxynucleotide triphosphohydrolase enzyme which could suppress cellular dNTP pools to inhibit retrovirus reverse transcription (Goldstone et al., 2011; Powell et al., 2011). Thus, by targeting this host protein for degradation, Vpx allows primate lentiviruses to infect key immunomodulatory cells types.

Despite this important function, only two of the eight major lineages of primate lentiviruses (reviewed in Peeters and Courgnaud, 2002) encode Vpx: HIV-2/SIVsm-related viruses and a lineage represented by SIV from red-capped mangabeys (SIVrcm). On the other hand, all extant primate lentivirus lineages encode a paralogous gene called Vpr that causes cell-cycle arrest (Sharp et al., 1996; Tristem et al., 1990, 1998). Both Vpr and Vpx are incorporated into the core of budding viruses (Yu et al., 1988; Yuan et al., 1990), and both bind to the Cul4 complex through interactions with DDB1 and DCAF1 (reviewed in Ayinde et al., 2010). Despite its limited representation in primate lentiviruses, Vpx appears to be more critical than Vpr for replication of SIV in monkeys (Gibbs et al., 1995; Hirsch et al., 1998). The important role played by Vpx has led to a conundrum as to why this protein is missing in lentiviruses like HIV-1. The recent identification of SAMHD1 as the target of Vpx allows us to characterize Vpx function from diverse lentiviruses with SAMHD1 from different hosts. Such an analysis can distinguish between the possibility that SAMHD1 degradation had an ancient origin and was subsequently lost in some lineages due to lack of selective pressure from SAMHD1, or that it was a recent adaptation of some viruses.

The evolutionary analysis of both host and viral proteins combined with functional analysis can reveal the evolutionary dynamics of this arms race, both in terms of its birth and its more recent adaptations. Host defense genes like SAMHD1 that are involved in antagonistic virus-host interactions often display strong signatures of diversifying selection as a result of repeated episodes of selection by viral antagonists (Emerman and Malik, 2010; Meyerson and Sawyer, 2011). This methodology can be used to pinpoint the exact amino acid residues involved in the viral-host interaction (Lim et al., 2010; McNatt...
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RESULTS

Species-Specific Antagonism of SAMHD1 by Diverse Vpx Proteins

A recent study found that SIVrcm Vpx could not degrade human SAMHD1 (Laguet et al., 2011), suggesting that this function might be very limited among primate lentiviruses. We first wished to test if the ability of Vpx to degrade SAMHD1 is conserved, and if there is species specificity to the interaction. Thus, we cloned SAMHD1 from a panel of primate species and assayed for Vpx-mediated SAMHD1 degradation by western blot analysis after transient cotransfection of epitope-tagged SAMHD1 proteins with vpr or vpx from different lentiviruses.

Consistent with previous reports, we found that HIV-2 (Rod9) Vpx degraded human SAMHD1 and that SIVmac Vpx degraded rhesus SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011) (Figures 1A and 1B). We also found that Vpx from a primary strain of HIV-2, 7312a, degraded SAMHD1 (Figure 1A). Surprisingly, we found that while the HIV-2 (Rod9) had a relatively narrow specificity, only degrading SAMHD1 from human and De Brazza’s monkeys among a broader panel of primate SAMHD1 proteins (Table 1), HIV-2 (7312a) Vpx could degrade SAMHD1 from humans and all of the OWMs tested (Table 1). The corresponding Vpr proteins of both viruses were unable to degrade human SAMHD1 (Figure 1A), similar to Vpr proteins from HIV-1 and SIVcpz strains (Figure 1A).

SIVrcm also encodes both Vpx and Vpr. However, SIVrcm Vpx is only 42% identical to HIV-2/SIVsm Vpx at the amino acid level. Nonetheless, we found that SIVrcm Vpx potently degraded SAMHD1 from the host species it naturally infects—the red-capped mangabey (RCM) (Figure 1B). Furthermore, we found that SIVrcm Vpx can degrade SAMHD1 from other OWMs, but not from sooty mangabeys or humans (Table 1). The corresponding Vpr protein of SIVrcm did not have this activity (Figure 1B). Thus, our findings not only suggest that the ability to degrade SAMHD1 is conserved in other clades of Vpx, but also shows species specificity (Table 1).

HIV-1 encodes only vpr, while HIV-2 encodes both vpr and vpx, yet both infect humans. There is an analogous situation in mandrills which are naturally infected by two highly divergent lentiviruses, SIVmnd1, which encodes only vpr, and SIVmnd2, which encodes both vpr and vpx (Souquière et al., 2001; Take mura and Hayami, 2004; Tsujimoto et al., 1988). We found that the Vpx protein from SIVmnd2 was able to degrade mandrill SAMHD1, but neither SIVmnd2 Vpr nor SIVmnd1 Vpr could degrade mandrill SAMHD1 (Figure 1C). Thus, even within a given host, some lentiviruses have a protein with the ability to degrade SAMHD1, while others do not.

Some Vpr Proteins Also Antagonize SAMHD1

Thus far, analyses suggest a clear separation of function between the vpr and vpx genes examined (reviewed in Ayinde et al., 2010), implying that vpx alone evolved the ability to degrade SAMHD1. However, the evolutionary history of these genes is far from clear, in part due to the high diversity of sequences (Sharp et al., 1996; Tristem et al., 1990; Tristem et al., 1998). This raises the possibility that at least some divergent lentiviral vpr genes might share the property of degrading SAMHD1. We used 115 vpr and vpx gene sequences from...
SIVmnd1, SIVmnd2, and SIVolc—which are inactive against their host species (Figure 1 and Figure 2)—were also unable to degrade the panel of SAMHD1 levels. The following Vpr proteins from HIV-1 Lai, HIV-1 Q23-17, HIV-2 Rod9, HIV-2 7312a, SIVcpz 3.1, SIVcpz 2.69, SIVmac239, SIVrcm, cates combinations that resulted in a greater than 90% decrease in SAMHD1 levels. “–” indicates combinations that had no significant changes in SAMHD1. The host species SAMHD1 is listed in the left column, and the Vpx/Vpr proteins tested against SAMHD1 are listed in the top row. “+” indicates combinations that resulted in a greater than 90% decrease in SAMHD1 (data not shown). The Vpr proteins from SIVagmVpr had a broad specificity against primate SAMHD1 (Table 1). Extending these analyses further, we found that Vpr proteins from both SIVagmVpr and SIVmnd1 Vpr. On the other hand, however, we found that Vpr from SIVdeb that infects De Brazza’s monkeys (from the green color group in Figure 2A) not only degraded De Brazza’s monkey SAMHD1 (Figure 2B) but also potently degraded SAMHD1 from all primate species including humans (Table 1). Vpr from SIVmus, in the same group as SIVdeb, also had a broad specificity against primate SAMHD1 proteins (Table 1). Extending these analyses further, we found that Vpr proteins from both SIVagm Grievet (677 strain) and SIVagm Vervet (9648 strain) (yellow group in Figure 2A) also degraded SAMHD1 from their African green monkey (AGM) host (Figure 2B). SIVagmVpr had a narrow specificity only capable of degrading AGM SAMHD1, whereas SIVagmVpr had a broader specificity (Table 1). These data reveal that phylogenetically distinct Vpr proteins functionally degrade SAMHD1, at times with striking species specificity.

We overlaid the functional analysis of Vpr and Vpx proteins that do and do not degrade SAMHD1 on the unrooted phylogenetic tree. Notably, all of the Vpr/Vpx proteins that do degrade SAMHD1 are found on one side of the tree (Figure 2A, blue stars), while all of the Vpr proteins that do not degrade SAMHD1 are found on the other side (Figure 2A, red stars). There is strong bootstrap support for the separation of these two subtrees (bootstrap support [BS] = 90.3, posterior probability [PP] = 1), which argues that there was a single gain/loss event for the function of degrading SAMHD1 in Vpr/Vpx evolution, and this function is not only confined to the previously classified “vpr” genes but is also observed in “vpx” genes from diverse lentiviral lineages.

Table 1. Species-Specific SAMHD1 Degradation by Vpr and Vpx

<table>
<thead>
<tr>
<th>SAMHD1</th>
<th>HIV-2 Rod9 Vpx</th>
<th>HIV-2 7312a Vpx</th>
<th>SIVmac Vpx</th>
<th>SIVdeb Vpr</th>
<th>SIVmus Vpr</th>
<th>SIVagm.Gri Vpr</th>
<th>SIVagm.Ver Vpr</th>
<th>SIVrcm Vpx</th>
<th>SIVmnd2 Vpx</th>
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<td>Red-capped mangabey</td>
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<td>African green monkey</td>
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<td>De Brazza’s</td>
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The table summarizes results of western blot analyses of SAMHD1 degradation phenotype by indicated Vpr and Vpx across a panel of primate species' SAMHD1 (data not shown). The AGM SAMHD1 tested is from the Vervet subspecies matching the SIVagmVer 9648 host strain; SAMHD1 from the Tantalus subspecies was found to be heterozygous for a second allele that was resistant to all HIV/SIV Vpr and Vpx tested (data not shown). The primate lentivirus methods yielded congruent unrooted topologies (Figure 2A, Figure S1A available online). The diverse HIV and SIV isolates to construct phylogenies using both maximum likelihood (ML) and Bayesian (BI) methods; both methods yielded congruent unrooted topologies (Figure 2A, see Figure S1A available online). The primate lentivirus vpr and vpx sequences grouped into seven phylogenetic clusters (cutoff at ML bootstrap >75, BI posterior probability >0.8; shaded by colors in Figure 2A). Phylogenies obtained from an application of the Fast Statistical Alignment (FSA) algorithm that is more conservative in terms of homology assignment (Bradley et al., 2009), or trimmed to the minimal 44 shared amino acid positions from the FSA alignment, yielded the similar seven phylogenetic clades (Figure S1B). In all cases, a subset of the vpr genes clustered closer to the vpx genes than they did to other vpr genes (the yellow and green groups in Figure 2A). Thus, we tested the vpr genes from each of the diverse primate lineages against their own host SAMHD1 as well as other primate SAMHD1 genes.

The Vpr protein from SIVOvlc (from the gray color group in Figure 2A), which infects olive colobus monkeys (OLC) and does not carry Vpx, cannot degrade colobus SAMHD1, similar to HIV-1/ SIVcpz Vpr and SIVmnd1 Vpr. On the other hand, however, we found that Vpr from SIVdeb that infects De Brazza’s monkeys (from the green color group in Figure 2A) not only degraded De Brazza’s monkey SAMHD1 (Figure 2B) but also potently degraded SAMHD1 from all primate species including humans (Table 1). Vpr from SIVmus, in the same group as SIVdeb, also had a broad specificity against primate SAMHD1 proteins (Table 1). Extending these analyses further, we found that Vpr proteins from both SIVagm Grievet (677 strain) and SIVagm Vervet (9648 strain) (yellow group in Figure 2A) also degraded SAMHD1 from their African green monkey (AGM) host (Figure 2B). SIVagmVpr had a narrow specificity only capable of degrading AGM SAMHD1, whereas SIVagmVpr had a broader specificity (Table 1). These data reveal that phylogenetically distinct Vpr proteins functionally degrade SAMHD1, at times with striking species specificity.

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Binding of Diverse Vprs to SAMHD1 Correlates with Degradation

Previous studies have shown that Vpx from SIVsm and HIV-2 are able to bind SAMHD1 directly in order to promote its degradation (Hrecka et al., 2011; Laguette et al., 2011). To determine if the diverse Vpr proteins that degrade SAMHD1 also antagonize through protein–protein interactions, we performed coimmunoprecipitations. As the immunoprecipitation was directed against SAMHD1, the proteasome inhibitor MG132 was added to the cells in an attempt to prevent degradation of SAMHD1. Consistent with our degradation results, we found that SIVdeb Vpr coimmunoprecipitates with SAMHD1 from De Brazza’s monkeys (Figure 2C). Similarly, we found that SIVagm Vpr binds AGM SAMHD1. The AGM-SAMHD1 Vpr complexes, but not the SIVdeb Vpr, also interacted with the Cu4 ubiquitin ligase complex protein, DDB1 (Figure 2C). In contrast, SIVmnd1 Vpr did not bind to Mandrill SAMHD1 (Figure 2C), indicating that only Vprs that degrade SAMHD1 are able to bind SAMHD1. As the degradation of De Brazza’s SAMHD1 was not rescued by MG132, and SIVdeb Vpr was not found to interact with DDB1 by SAMHD1 coimmunoprecipitation, this may suggest an alternate means of degradation by SIVdeb Vpr. However, this is most likely not representative of this clade of vpr, as we found that degradation by the related SIVmus Vpr to be rescued by MG132. Furthermore, we found that SIVmus Vpr interacts with DDB1 in a SAMHD1 complex (data not shown). Thus, the ability of Vpr to cause degradation of SAMHD1 correlates with its ability to bind SAMHD1 and is conserved with the known mechanism of Vpx interaction with SAMHD1.
Figure 2. Some Vpr Proteins Degrade SAMHD1

(A) Unrooted phylogeny of 115 vpr and vpx sequences among diverse primate lentiviruses. Bootstrap values indicate maximum likelihood proportions that are highly supported by Bayesian inference (Figure S1A). Seven phylogenetic clusters are shaded in colors (cutoff at ML bootstrap >75, Bayesian posterior probability >0.87). Vpx sequences form two clades (shaded in light blue and dark blue) that have strong support of monophyly from all other vpr sequences. Functional phenotypes of Vpr and Vpx (Table 1) that degrade SAMHD1 (Blue stars) or do not degrade SAMHD1 (Red stars) are overlaid on the phylogeny. See also Figure S1B.

(B) Western blot analysis of Colobus monkey, De Brazza’s monkey, and African green monkey (AGM) SAMHD1 in the presence of indicated Vpr constructs. The AGM SAMHD1 tested is from the Vervet subspecies matching the SIVagmVer 9648 host strain; the Colobus SAMHD1 tested is from the Colobus guereza subspecies.

(C) Association of SAMHD1 with Vpr and DDB1 by coimmunoprecipitation was detected by western blot analysis of HA-immunoprecipitated SAMHD1 for FLAG-epitope-tagged Vpr and DDB1 association (IP), or input expression (Input). After transfection, cells were treated with 25 μM MG-132 for 12 hr prior to immunoprecipitation. SIVmnd1 Vpr, which fails to degrade mandrill SAMHD1, was assayed as a negative control. Actin and the antibody light chain (Lc) are shown as loading controls.
Vpr/Vpx Antagonism Drove Positive Selection of SAMHD1 in Old World Monkeys

One key to the question of whether antagonism of a host protein by a viral protein is ancient or recent is to determine the selective pressures that have shaped the host protein evolution. Therefore, we investigated whether SAMHD1 is under positive selection by sequencing the coding region of SAMHD1 from 31 primate species representing approximately 40 million years of evolutionary divergence (Figure 3A). The phylogeny constructed from the primate SAMHD1 sequences was congruent with the generally accepted primate species phylogeny (Perelman et al., 2011), confirming that the sequences are orthologous. We found that there was strong evidence of recurrent positive selection on SAMHD1 during primate evolution (Figure 3B, \( p < 0.001 \)), and this conclusion was corroborated with other methods (Figure S2A). This signature of positive selection clearly stemmed from the OWM clade (\( p < 0.001 \)), as neither new world monkey (NWM) (\( p > 0.19 \)) nor hominoid (\( p > 0.35 \)) clades showed significant evidence of positive selection (Figure 3B). The lack of positive selection in NWM or hominoids was not a result of low statistical power from limited evolutionary depth, as the tree length (number of substitutions per codon) of the NWM clade (0.20) and hominoid clade (0.15) was greater than that of the OWM clade (0.13).

In order to investigate the selective pressures across the different primate lineages, we calculated the omega ratio (dN/dS) along each branch by performing a free ratio analysis using PAML, where omega (dN/dS) ratios >1 are indicative of positive selection (Figure 3A and Figure S2B). Aside from OWMs, only the branch leading to orangutans had statistically significant dN/dS >1 (Figure S2C). Strikingly, SAMHD1 has evolved by positive selection in multiple branches of the OWM subfamily Cercopithecinae (Figure 3A, Figure S2D). This suggests that the most dramatic signatures of recurrent positive selection are exhibited by members of the Cercopithecinae primate subfamily (the top clade of OWM in Figure 3A) and occurred after this lineage split from the Colobinae subfamily (the bottom clade of OWM in Figure 3A).

Positive selection analysis identified six amino acid residues (aa 32 and 36 in the N-terminal domain; aa 46, 69, and 107 in the SAM domain; and aa 486 in the C-terminal domain) as having evolved under recurrent positive selection with strong confidence (posterior probability >0.95) (Figure 3C, Figure S3). Furthermore, if we removed all six residue positions from the primate SAMHD1 alignment, the bulk of the gene-wide signature of positive selection was lost (\( p > 0.11 \)), indicating that these greater than 1 (hence under positive selection). Neutral models (M7) were compared to selection models (M8) under the F61 model of codon substitution. Similar results were obtained in a comparison of M1 (neutral) versus M2 (selection) (data not shown). See also Figure S2.

For further analysis, see Figure S3.
amino acids are largely responsible for the signal across the entire gene (Figure 3C).

If amino acid residues under positive selection determine sensitivity to Vpx antagonism, this would strongly argue that a Vpx-like factor was responsible for the signature of positive selection acting on SAMHD1. Alternatively, if the sites under recurrent positive selection did not affect SAMHD1's susceptibility to Vpx, this would strongly suggest that Vpx and Vpx-like factors are too recent to have significantly affected SAMHD1 evolution. Of the six sites identified under strong positive selection, residues 46 and 69 in the SAM domain showed unmistakably strong signals of recurrent positive selection (Figure 4A and Figure S3). These residues also differ in certain primate species' SAMHD1 that show opposite susceptibility to Vpx. In particular, AGM and mandrill SAMHD1 differ at positions 46 and 69, with mandrill encoding the "ancestral state" at both sites, while AGM encodes the "derived" state (Figure 4A).

To determine if the changes at positions 46 and 69 are responsible for the species specificity of SAMHD1 antagonism by Vpx, we investigated SAMHD1 degradation by SIVmnd2 Vpx, which can degrade mandrill but not AGM SAMHD1 (Table 1). We made D46G and Q69R mutations in the AGM-"resistant" SAMHD1 backbone, reverting these two positions to their ancestral state. We found that the introduction of either mutation resulted in increased susceptibility to degradation by SIVmnd2 Vpx (Figure 4B, see AGM D46G, AGM Q69R). This increased sensitivity of SAMHD1 correlated with increased binding to SIVmnd2 Vpx, since SIVmnd2 Vpx strongly coimmunoprecipitated with mandrill SAMHD1, but its interaction with AGM SAMHD1 was much weaker (Figure 4C). However, either single reversion point mutation (AGM D46G, AGM Q69R) resulted in a stronger interaction with SIVmnd2 Vpx (Figure 4C; compare last three lanes). Thus, changes at the positively selected residues 46 and 69 in SAMHD1 determine both binding and susceptibility to Vpx.

We also tested the reciprocal G46D and R69Q mutations in the "sensitive" Mandrill SAMHD1. We found that while neither mutation alone was sufficient to confer resistance to SIVmnd2 Vpx degradation of SAMHD1 Is a Neofunction of Vpr

Figure 4. SAMHD1 Positive Selection Residues Map to Vpx Sensitivity
(A) Alignment of N-terminal and SAM domain regions from indicated primates. Symbols (circle on a stick) represent the positively selected residues marked on the SAMHD1 domains. Sites 46 and 69, which displayed highly significant signals of positive selection, are boxed in the alignment. Stars represent the codons under positive selection with strong support (Figures S3A–S3C). The N-terminal region of SAMHD1 from gray mouse lemur is included to represent amino acid residues encoded by a distantly related prosimian primate, showing that the G is the ancestral state at amino acid 46 and the R is the ancestral state at position 69. (B) Expression of mandrill, AGM, and AGM point mutants (AGM D46G and AGM Q69R) were analyzed by western blot, in the presence or absence of SIVmnd2 Vpx expression. (C) Western blot analysis of HA-immunoprecipitated SAMHD1 for FLAG-epitope-tagged SIVmnd2 Vpx association. Cells were treated with 25 μM MG-132 for 12 hr prior to immunoprecipitation. Heavy chain (Hc) is shown as a loading control. (D) Expression of SAMHD1 from mandrill and mandrill-derived mutations (Mnd G46D, Mnd R69Q, and Mnd G46D, R69Q) in the presence or absence of SIVmnd2 Vpx.
degradation (Figure 4D, Mnd R69Q and Mnd G46D), a combination of both mutations together resulted in the gain of resistance against degradation by SIVmnd2 Vpx (Figure 4D, Mnd R69Q G46D). Thus, these results demonstrate that changes in amino acids evolving under positive selection in SAMHD1 are necessary and sufficient to determine specificity of Vpx antagonism. This strongly suggests that a Vpx-like factor was responsible for the recurrent positive selection on SAMHD1 during primate evolution.

The Ability to Degrade SAMHD1 Preceded the Birth of Vpx in Primate Lentiviruses

We wished to determine whether the ability to degrade SAMHD1 was an ancestral trait common to all Vpr/Vpx proteins, and that function was subsequently lost by some Vpr lineages across evolution; or alternatively, whether the ancestral Vpr/Vpx lacked the ability to degrade SAMHD1, but the trait was gained (neofunctionalized) over the course of primate lentivirus evolution. However, in order to interpret whether there was a gain or a loss of the ability of Vpr/Vpx to degrade SAMHD1, it was necessary to root the vpr/vpx phylogenetic tree from Figure 2A.

Previous studies demonstrated that the endogenous lentivirus in the genomes of lemurs, pSIVgml, is 2–6 million years old and unambiguously forms an outgroup to all extant primate lentiviruses (Gifford et al., 2008; Gilbert et al., 2009; Liegeois et al., 2009). However, pSIVgml does not encode a vpr or vpx gene. Therefore, we performed a phylogenetic analysis of pol sequences and found that SIVolc and SIVwrc, which infect the primate species of the Colobinae subfamily of OWM and contain an existing vpr/vpx gene, are the closest relatives to pSIVgml (Figure S4A). This result is consistent with previous studies (Gifford et al., 2008; Gilbert et al., 2009). Analysis of env sequences (which are 3’ of vpr and pol) showed that the pSIVgml nests with the similar cluster of sequences (Figure S4B). Therefore, we used SIVolc/SIVwrc vpr sequences to root the vpr/vpx tree, reflecting the high likelihood that this clade represented the earliest branching event of extant primate lentiviruses.

Using this rooted tree, we overlaid the SAMHD1 degradation phenotype onto the phylogeny and found that the vpr genes that lacked SAMHD1-degrading ability (Figure 5, red stars) were clearly separable from the SAMHD1 degrading vpr and vpx genes (Figure 5, blue stars). Strikingly, all vpr and vpx genes that shared the ability to degrade SAMHD1 nest within the same monophyletic clade with high confidence (Figure 5, BS, 90.3; Figure S1A, PP, 0.98). Since Vpr from HIV-1, HIV-2, SIVmac, SIVrcm, and SIVmnd2 was unable to degrade SAMHD1 (Figure 5, red stars), the most parsimonious explanation is that the Vpr of their common ancestor (Figure 5, node 3) lacked the SAMHD1 degradation capability. Given that the outgrouping SIVmnd1 Vpr and SIVolc Vpr proteins were incapable of degrading SAMHD1 (Figures 2 and 3), this strongly supports the hypothesis that the ancestral Vpr was “inactive” against SAMHD1 (Figure 5, node 1) and that the ability to degrade SAMHD1 subsequently arose only once during vpr and vpx evolution.

Based on the phylogeny, we can clearly pinpoint that the neofunctionalization of Vpr to degrade SAMHD1 occurred on the
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branch leading up to the split of SIVagm, SIVdeb/mus/mon lineages (Figure 5, node 2). Importantly, based on phylogeny, our results suggest that the birth of the vpx recombination/duplication dated after the neofunctionalization occurred (Figure 5, node 4). Thus, the combined phylogenetic and functional study presented here strongly supports a scenario in which the degradation of SAMHD1 by Vpx was preceded by the neofunctionalization of Vpr in a transitional SIV lineage. Furthermore, this phylogenetic framework argues against a subsequent loss of SAMHD1-degrading ability in any lentiviral Vpr protein; that is, those Vpr proteins that currently lack this ability including HIV-1 Vpr likely never possessed it.

DISCUSSION

Here we show that diverse Vpx proteins as well as some Vpr proteins have the ability to target their host species’ SAMHD1 for degradation. Both Vpx and Vpr antagonists display species-specific degradation of SAMHD1 which, in some cases, is quite specific to the virus’s extant host. Such species specificity is a hallmark of an antagonistic “arms race” between host and virus, in which both sides rapidly evolve to gain an advantage. Indeed, we show that SAMHD1 has been evolving under positive selection in primates. We demonstrate that the residues under positive selection in the SAM domain of SAMHD1 determine the specificity of degradation by Vpx, directly implicating Vpr/Vpx antagonism as the source of the remarkable signature of positive selection detected in SAMHD1, which is most pronounced in the Cercopithecinae subfamily of OWMs. By combining our functional results with phylogenetic analyses, we show that the ability to degrade SAMHD1 is a neofunctionalization of Vpr which preceded the birth of Vpx by recombination/duplication.

Based on our combined phylogenetic and functional analyses, the common ancestor of SIV viruses most likely encoded a single Vpr that was inactive against SAMHD1. The ability to recruit a protein degradation complex is important for Vpr-mediated cell-cycle arrest (reviewed in Dehart and Planelles, 2008) and thus may represent the ancestral function of Vpr/Vpx. Interestingly, although cell-cycle arrest and SAMHD1 degradation functions are segregated into two separate proteins in those viruses that encode Vpr and a Vpx (Aynide et al., 2010), SIVagm Vpr is able to cause both cell-cycle arrest (Planelles et al., 1996; Stivahtis et al., 1997) and SAMHD1 degradation (Figure 2A). This indicates that the two functions are not mutually exclusive. Furthermore, since cell-cycle arrest by Vpr has some species specificity (Stivahtis et al., 1997), it is likely that the substrate used by Vpr to cause cell-cycle arrest will, like SAMHD1, have evolved under positive selection.

While the cellular protein targeted by Vpr to cause cycle-cell arrest is not yet known, the adaptive evolution of SAMHD1 might provide a clue as to why some viruses evolved to encode a separate Vpx and Vpr gene. One scenario we propose is that the neofunctionalization of the ancestral Vpr/x to target SAMHD1 exerted a strong selective pressure on OWMS’ SAMHD1. As a result, variants of SAMHD1 that conferred protection from Vpr/x antagonism were selected for, leading to the signatures of rapid evolution in SAMHD1, especially localized within the SAM domain. This posed a unique challenge to the ancestral Vpr/x that had to recognize both the cell-cycle arrest factor and multiple rapidly evolving variants of SAMHD1. In order to maintain both functional capabilities, a recombination/duplication of Vpr might have given rise to Vpx. This subsequently allowed the subfunctionalization of Vpx to maximize its SAMHD1-targeting capability, while preserving the cell-cycle arrest phenotype in Vpr. This model might explain the complicated evolutionary history of vpr and vpx (Sharp et al., 1996; Tristem et al., 1990, 1998). Thus, we speculate that the “birth” of a new gene in some lineages leading to both vpr and vpx in the same viral genome, a more modern event compared to the neofunctionalization of Vpr, may have been directly driven by the rapid evolution of the SAMHD1 protein.

HIV-1 lacks the capability of degrading SAMHD1, since its Vpr protein is unable to degrade SAMHD1 and it does not encode Vpx. Since SIVcpz Vpr also lacks SAMHD1-degrading ability (Figure 2A), this function was missing in HIV-1 even prior to its cross-species transmission from chimpanzees to humans. Moreover, human SAMHD1 is not special in terms of its resistance to Vpr antagonism, as it is readily degraded by HIV-2 Vpx. This situation is directly analogous to the two lentiviruses that infect mandrills. SIVmnd1 contains only a Vpr gene that has no activity against mandrill SAMHD1 (Figure 1C), whereas SIVmnd2 has both Vpr and Vpx, the latter of which is capable of degrading mandrill SAMHD1 (Figure 1C). Intriguingly, SIVmnd1 appears more pathogenic than SIVmnd2, similar to the higher pathogenicity of HIV-1 relative to HIV-2 (Souquière et al., 2009). One possible explanation is that both HIV-1 and viruses like SIVmnd1 evolved unique antagonistic functions (or more effective countermeasures) that collectively allow HIV-1 to achieve sufficient replicative potential in target cells (including SAMHD1-expressing monocytes) even in the absence of SAMHD1-degrading abilities. On the other hand, Vpx-encoding viruses may have become more dependent on the ability to counteract SAMHD1 to achieve successful replication in target cells and have relaxed selection on alternate measures used by viruses like HIV-1.

Most of the signatures of positive selection in primate SAMHD1 appear to originate from the OWM lineages, specifically the subfamily Cercopithecinae after its split from Colobinae. This highly localized positive selection on the primate phylogeny is unusual. Most previously analyzed host immune genes, such as TRIM5alpha, Tetherin, PKR, and APOBEC3G, display signatures of positive selection throughout many primate lineages including hominoids and NWMs (Eide et al., 2009; Lim et al., 2010; McNatt et al., 2009; Meyerson and Sawyer, 2011; Sawyer et al., 2004, 2005), while others have been restricted to hominoids and OWMS alone (TRIM22). Such a localized signature of positive selection might signal the advent of a highly specialized and unique antagonist. Orangutan SAMHD1 is the only primate species outside of the Cercopithecinae subfamily that also has strong signals of positive selection. However, while there is no evidence of SIVs infecting orangutans to date, there have been reports of simian T-lymphotropic virus (STLV) and simian type D retrovirus (SRV) infecting orangutans (Verschoor et al., 2004; Warren et al., 1998).

Intriguingly, our phylogenetic framework (Figure 5) strongly argues that the Vpr/Vpx proteins’ ability to degrade SAMHD1 arose within the primate lentiviruses, and specifically among
lentiviruses that infect Cercopithecinae and jumped into Hominidae, but not from viruses isolated from Colobinae. Together with our findings that residues in SAMHD1 under positive selection directly determine Vpx sensitivity, this suggests that the birth of the SAMHD1-degrading ability within primate lentiviruses initiated the evolutionary arms race that led to such a highly localized signature of positive selection within Cercopithecinae. Thus, both the positive selection of SAMHD1 and consequently the birth of Vpx may have been driven by the neofunctionalization of Vpr to antagonize SAMHD1.

EXPERIMENTAL PROCEDURES

Sequencing of Primate SAMHD1 Genes

The SAMHD1 genes from the following primates were amplified from RNA isolated from cell lines obtained from Coriell Cell Repositories (Camden, NJ): chimpanzee (Pan troglodytes), bonobo (Pan paniscus), gorilla (Gorilla gorilla), Sumatran orangutan (Pongo pygmaeus), white-cheeked gibbon (Nomascus leucogenys), agile gibbon (Hylobates agilis), Siamese gibbon (Hylobates syndactylus), Rhesus macaque (Macaca mulatta), patas monkey (Erythrocebus patas), talapoin monkey (Miopithecus talapoin), greater white-nosed monkey (Cercopithecus nigroviridis), sooty mangabeys (Cercocebus atys), Francois’ leaf monkey (Lemur catta), proboscis monkey (Nasalis larvatus), tamarin (Saguinus labiatus), pygmy marmoset (Cebuella pygmaea), white-faced saki (Pithecia pithecia), spider monkey (Ateles geoffroyi), owl monkey (Aotus trivirgatus), dusky titi monkey (Calliebus moloch), and woolly monkey (Lagothrix lagotricha). Human, African green monkey (Chlorocebus pygerythrus), and baboon (Papio anubis) SAMHD1 was amplified by reverse transcription-PCR (RT-PCR) from an RNA extract of 293T cells, Vero cell (AGM Vervet subspecies), COS-7 cells (AGM Sabauea subspecies), and B-LCL cells. SAMHD1 was amplified by RT-PCR with SuperScript III One-Step RT-PCR (QIAGEN), and the cDNA derived was directly sequenced. SAMHD1 was amplified with forward primer SAMHD1-Hominoid-F (5′-ATGACGCG GCCGATTCGGAGCAGGACC-3′), SAMHD1-OWM-F (5′-ATGACGCGAAGCCGAC TCCGACGACC-3′) or SAMHD1-NWM-F (5′-ATGACGACGAGCGACTTG AGCAGCC-3′) in combination with reverse primer SAMHD1-Hominoids-r (5′-TACATTGGGTATCTTAAAAAGGTCG-3′), SAMHD1-OWM-r (5′-TACATTGGGTATCTTAAAAAGGTCG-3′) or SAMHD1-NWM-r (5′-TACACCGGG GTACCTTTAAGGTCG-3′).

SAMHD1 Sequence Analysis

SAMHD1 DNA sequences were aligned by ClustalX (42) and were edited by hand based on amino acid sequences or with PhyML (10) by the ML method. The two methods yielded trees with identical topologies. ML analysis was performed with CODEML from the PAML suite of programs (55) as previously described (17). Briefly, SAMHD1 sequences were fitted to NSsites model that disallowed (NSsites model 1 and 7) or permitted (NSsites model 2 and 8) positive selection. Likelihood ratio tests were performed to evaluate whether permitting codons to evolve under positive selection gave a better fit to the data. Data were fitted with an F61 model of codon frequency, and consistent results were obtained when the data were fitted with an F3 x 4 model of codon frequency. These analyses (MB) identified amino acid residues with high posterior probability (p > 0.95) of having evolved under positive selection. Analyses were also validated with PARRIS and REL from the HyPhy package (Pond et al., 2005). Free ratio analysis in PAML was used to calculate the ω0 (dN/dS) ratios of individual branches.

Plasmids

Primate SAMHD1 was cloned from cDNA from the respective species and ligated into pLPCX construct, with a hemagglutinin (HA) epitope tag fused to the C termini. Vpr and Vpx constructs ligated into a pCDNA3.1 expression vector, with a 3xFLAG epitope tag fused to the N termini. The following genes were cloned from provirus plasmids: HIVLai Vpr, SIVagmGrn677 Vpr, SIVagmMlrV648 Vpr, HIV-2 Rod9 Vpr and Vpx, HIV-2 7312a Vpr and Vpx (as previously described [Stavrvitis et al., 1997]); HIV-1 Q23-17 Vpr (a gift from Julie Overbaugh [Pozz and Overbaugh, 1999]); SIVmac239 Vpr and Vpx (provirus plasmid, obtained from NIH AIDS Research and Reference Reagent Program [Regier and Desrosiers, 1990]); and SIVcpzTan2.69 Vpr and SIVcpzTan3.1 Vpr (proviral plasmid, obtained from the NIH AIDS Research and Reference Reagent Program [Takehisa et al., 2007]).

The following genes were codon optimized and synthesized (Genscript): SIVolc 970112 Vpr (FM185200), SIVmnd1 1011 Vpr (M27470), SIVrcm NG411 Vpr and Vpx (AF349868), SIVmmd2 5440 Vpr, and Vpx (AY155922), SIVdeb CM5 Vpr (AY523866), and SIVmus 1 1239 Vpr (EF070330).

Transfection

293T cells were transfected with 100 ng of SAMHD1 (in LPCX expression vector, C-terminal HA-epitope tag) with or without 100 ng of Vpr/Vpx constructs (in pCDNA3.1 expression vector, N-terminal 3xFLAG epitope tag) using TransIT-LT1 (Mirus Bio). The amount of codon-optimized Vpr/Vpx was reduced to normalize for similar levels of protein expression. The total amount of DNA in all transfections was maintained constant with appropriate empty vectors. Forty-eight hours posttransfection, cells were harvested for western blot analysis.

Western Blotting

Western blot analysis was performed as described previously (Lim and Emerman, 2009; Lim et al., 2010) with the following antibodies: HA-specific antibody (Babco), anti-FLAG M2 antibody (Sigma-Aldrich), anti-actin (Sigma-Aldrich), and DDB1 antibody (Cell Signaling). Primary antibodies were detected with a corresponding horseradish peroxidase-conjugated secondary antibody.

Immunoprecipitation

293T cells were transfected by TransIT-LT1 (Mirus Bio) with the appropriate plasmids 36 hr prior to lysis, and were treated with 25 μM MG-132 (Calbiochem) for 12 hr. Cells were washed twice with PBS and lysed with IP lysis buffer (50 mM Tris [pH 7.4], 250 mM NaCl, 0.4% [v/v] NP-40, 1 mM DTT, plus Protease Inhibitor Cocktail [Roche]). Lysates were cleared at 15,500 g for 15 min, and immunoprecipitations were performed for 1 hr at 4°C with EZview Red anti-HA affinity gel (Sigma-Aldrich). Following immunoprecipitation, affinity gel was washed four times with IP lysis buffer; proteins were eluted in 2x Laemmli sample buffer and analyzed by western blotting.

Phylogenetic Analysis

Phylogenetic trees were constructed from amino acid alignments of vpr and vpx sequences obtained from the Los Alamos HIV Sequence Database (Los Alamos HIV Sequence Database, 2011). Alignments were performed by using ClustalX (Thompson et al., 1997) and edited manually or by using FSA (Bradley et al., 2009) for a more conservative alignment that maximizes accuracy. Phylogenies were constructed with PhyML (10) by the ML method, and MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) and BEAST v1.6.2 (Drummond and Rambaut, 2007) using a Bayesian MCMC inference. Support for ML and Bayesian trees was assessed with Tracer v1.5 (Drummond and Andrew, 2009).

Accession Numbers

The sequences of the 31 SAMHD1 genes have been entered into the GenBank database under accession numbers NP_056289 and JQ231123–JQ231152.

Supplemental Information

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/chom.2012.01.004.
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