Structural Basis of Clade-specific Engagement of SAMHD1 (Sterile α Motif and Histidin/Aspartate-containing Protein 1) Restriction Factors by Lentiviral Viral Protein X (Vpx) Virulence Factors*

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**Background:** Lentiviral Vpx binding to primate SAMHD1 is under positive selection.

**Results:** Different Vpx protein variants interact with the N-terminal domain or the C-terminal tail of SAMHD1 in ubiquitin-ligase-substrate receptor complexes in a unique fashion.

**Conclusion:** Vpx antagonizes SAMHD1 by recruiting it via two separate regions for proteasomal degradation.

**Significance:** Our findings shed light on how lentivirus virulence factors intersect with host innate immunity.

Sterile α motif (SAM) and histidine/aspartate (HD)-containing protein 1 (SAMHD1) restricts human/simian immunodeficiency virus infection in certain cell types and is counteracted by the virulence factor Vpx. Current evidence indicates that Vpx recruits SAMHD1 to the Cullin4-Ring Finger E3 ubiquitin ligase (CRL4) by facilitating an interaction between SAMHD1 and the substrate receptor DDB1- and Cullin4-associated factor 1 (DCAF1), thereby targeting SAMHD1 for proteasome-dependent down-regulation. Host-pathogen co-evolution and positive selection at the interfaces of host-pathogen complexes are associated with sequence divergence and varying functional consequences. Two alternative interaction interfaces are used by SAMHD1 and Vpx: the SAMHD1 N-terminal tail and the adjacent SAM domain or the C-terminal tail proceeding the HD domain are targeted by different Vpx variants in a unique fashion. In contrast, the C-terminal WD40 domain of DCAF1 interfaces similarly with the two above complexes. Comprehensive biochemical and structural biology approaches permitted us to delineate details of clade-specific recognition of SAMHD1 by lentiviral Vpx proteins. We show that not only the SAM domain but also the N-terminal tail engages in the DCAF1-Vpx interaction. Furthermore, we show that changing the single Ser-52 in human SAMHD1 to Phe, the residue found in SAMHD1 of Red-capped monkey and Mandrill, allows it to be recognized by Vpx proteins of simian viruses infecting those primate species, which normally does not target wild type human SAMHD1 for degradation.

Critical components in the intricate interplay between viruses and the host machinery are restriction factors and innate immune effectors as well as virulence and permissivity factors (1–4). In SIV/HIV infection, five restriction factors have been identified to date: Tripartite motif-containing protein 5 α (Trim5α) (5), apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) (6, 7), bone stromal tumor protein 2 (BST2)/Tetherin (8, 9), sterile alpha motif and histidine-aspartic acid (HD)-containing protein 1 (SAMHD1) (10–12), and myxovirus resistance 2 (MX2) proteins (13–15). To counteract their anti-viral activities, HIV and SIV encode virulence factors that target many of these for proteasome-dependent degradation: viral infectivity factor (Vif) targets APOBEC3G, viral protein u (Vpu) targets BST2/ Tetherin, and a subset of viral protein t (Vpr) or viral protein X (Vpx) targets SAMHD1 using the same general mechanism (16–19). In particular, the substrate specificity of E3 ubiquitin ligases is altered and exploited either by interaction with the substrate adaptors or substrate receptor components of the ligases. For example, Vpx binds DDB1- and Cullin4-associated factor 1 (DCAF1), a substrate receptor of Cullin4 RING E3 ubiquitin ligase (CRL4) and loads SAMHD1 for polyubiquitination, the first step toward degradation (10, 11, 20).

Host-pathogen co-evolution leads to positive selection, the degree of which can be identified through interspecies sequence alignments (21, 22). Positive selection at the interfaces of host-pathogen complexes is present if the ratio of nonsynonymous changes to the number of synonymous changes...
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per site is greater than 1 (23). Thus, interfaces that are used in the interaction between host and pathogen proteins accumulate more frequent amino acid changes than would occur at random. Evolutionary signatures resulting from positive selection permit differentiation of broad acting anti-viral proteins from narrowly effective virus-specific antagonists. In particular, strong positive selection signals at several sites in a single host gene suggest that the gene product is targeted by different viruses at multiple regions. This is noted for SAMHD1, in which two separate regions, the SAM domain and the C terminus, interact with Vpx from distinct HIV/SIV strains. For example, Vpx proteins from the clade that includes HIV-2 and SIVsm (isolated from the sooty mangabey monkey) target a sequence element in the C terminus of SAMHD1, whereas those from the clade that includes SIVrcm (isolated from the Red-capped monkey (RCM)) and SIVmnd (isolated from the Mandrill monkey) interact with the SAM domain, with key residues mapping to different sites of the protein (20, 24–29).

Here, we investigated the positioning and distribution of selection signals using biochemical and structural approaches to elucidate the molecular basis of Vpx/SAMHD1 co-evolution. Our data show that the large N-terminal region of MND SAMHD1, including the SAM domain, interacts with VpxSIVrcm and VpxSIVmnd in the context of DCAF1. Changing several amino acids residues in the N-terminal region of human (Hu) SAMHD1 to those found at equivalent positions in the simian proteins revealed that substitution of only a single residue, Ser-52 to Phe, is sufficient to allow recognition by SIVrcm and SIVmndVpx of and recruitment to the CRL4-DCAF1 E3 ubiquitin ligase, whereas most other changes were invariant for recruitment. Our combined structural and biochemical results provide important molecular insights into how Vpx proteins from different HIV/SIV clades engage different elements in the cellular SAMHD1 protein to overcome the cell’s antiviral defense.

Experimental Procedures

Cloning and Plasmid Construction—The N-terminal regions (NTD, residues 1–115) of Hu, RCM, and MND SAMHD1 were cloned into pET41 (EMD Biosciences) with GST and His6 tags at their N termini and C termini, respectively. The NTDs were also cloned into pET21 (EMD Biosciences) with His6 tags at their C termini. The C terminus of RCM SAMHD1 (residues 595–626, CTD) was cloned into a modified pET32 vector containing a tobaccoetch virus protease site at the C terminus of His6-tagged thioredoxin fusion. Full-length VpxSIVrcm (residues 1–108) or residues 1–90 (Vpx(ΔC)SIVrcm) from red-capped monkey were cloned into pET34 vectors with a C-terminal His6 tag modified to include a tobacco etch virus protease site between the N-terminal NusA fusion and Vpx proteins, as described previously (20). Full-length VpxSIVmnd (residues 1–99) and residues 1–89 (Vpx(ΔC)SIVmnd) were also cloned into pET34 vector as described above. DCAF1, human and monkey SAMHD1, VpxSIVmnd, and VpxSIVrcm were also cloned, separately, into pCDNA with a HA tag at the N terminus. The cDNAs for monkey SAMHD1, VpxSIVmnd, and VpxSIVrcm were provided by M. Emerman (Fred Hutchison Cancer Center, Seattle, WA) (25, 27). Site-specific mutants of Hu and RCM SAMHD1 were prepared using the QuikChange mutagenesis kit (Agilent Technologies). All other clones were described previously (30).

Protein Expression and Purification—The various SAMHD1 proteins, including the GST fusions and thioredoxin fusion, and NusA-Vpx fusion proteins were expressed in Escherichia coli Rosetta 2 (DE3) grown in Luria-Bertani medium with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C for 16 h. Alternatively, the same Rosetta 2 (DE3) cells were grown, and proteins were expressed in autoinduction medium at 18 °C for 24 h. All proteins were purified as described previously (20, 30). Protein complexes, including DDB1-DCAF1CA (DCAF1 residues 1045–1396), DDB1-DCAF1CA-VpxSIVrcm, and DDB1-DCAF1CA-Vpx(ΔC)SIVrcm were prepared as described previously (20, 27, 30). Mixtures of DCAF1CA, NusA-Vpx(ΔC)SIVmnd, and MND SAMHD1-NTD at molar ratio of 1:2:5:5 were incubated and digested with tobacco etch virus protease. The complex was purified over an 8-ml Mono Q column (GE Healthcare) at pH 7.5 using a 0–1 M NaCl gradient with a buffer containing 25 mM Tris-HCl, 1 mM Tris(2-carboxyethyl)phosphine, 5% glycerol, and 0.02% sodium azide. For isotopic labeling of SAMHD1 NTDs, proteins were expressed in modified minimal media using [U-13C6,2H7]glucose or [U-13C6]glucose as the sole nitrogen and/or carbon sources, respectively. Protein concentrations were determined using theoretical molecular extinction coefficients based on amino acid sequences using the ExPASy Proteomics Server.

Isothermal Titration Calorimetry—Typically, protein complexes, DDB1-DCAF1CA-VpxSIVrcm, were placed in the sample cell of the calorimeter (MicroCal Inc., Northampton, MA), and aliquots of SAMHD1 NTD or CTD were added at 25 °C. Titrations were carried out in 10 mM HEPES buffer, pH 8.0, 1 mM Tris(2-carboxyethyl)phosphine, 100 mM NaCl, and 0.02% sodium azide. All samples were dialyzed against this buffer and concentrated using Amicon concentrators (Millipore) before titrations. Titration of SAMHD1 NTD or CTD into the buffer was used for base-line correction. Data analyses were performed using Origin 7 software (OriginLab Corp).

NMR Spectroscopy—NMR data were collected on Bruker 600-, 700-, 800-, and 900-MHz AVANCE spectrometers. All spectrometers were equipped with a z axis gradient, triple resonance cryoprobes. Experiments were performed at 298 K. Heteronuclear two-dimensional 1H, 15N HSQC and TROSY-HSQC and three-dimensional HNCACB, TROSY-HNCA, TROSY-HN(CO)CA, and HNCA experiments were performed on a 15C,2H,15N-labeled MND SAMHD1 NTD sample to obtain the backbone assignments. For mapping the DCAF1CA-VpxSIVmnd and DCAF1CA-VpxSIVrcm binding sites on SAMHD1 NTD, two-dimensional 1H,15N TROSY-HSQC spectra were recorded for 13C,15N-labeled MND SAMHD1 NTD in the presence of unlabeled DCAF1CA-VpxSIVmnd or DCAF1CA-VpxSIVrcm at 1:0.5, 1:1, and 1:1.5 molar ratios. All data were processed with NMRPipe and analyzed with Collaborative Computing Project for NMR.

X-ray Crystallography—Crystallization of the ternary complex consisting of DCAF1CA, Vpx(ΔC)SIVmnd, and MND SAMHD1 NTD was carried out at room temperature by the sitting drop vapor diffusion method using drops comprising 2
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Table 1: Data collection, refinement, and Ramachandran statistics for DCAF1-VpxSIVmnd-MND SAMHD1 NTD

| Complex | Data collection | Refinement | Ramachandran |
|---------|----------------|------------|--------------|
|         | Space group | Cell dimensions | Resolution (Å) | No. unique reflections | Rwork/Rfree | No. atoms | Protein | Ligand/ion | Water | Average B-factors (Å²) | Protein | Ligand/ion | Water | Root mean square deviations | Bond lengths (Å) | Bond angles (°) | MolProbity statistics | Cβ deviation | PDB accession code |
|         | P3₁          | a, b, c (Å) | 36.72-2.60 (2.72-2.60) | 33,447 | 1.005 | 2 | 7,712 | 114 | 49.19 | 62.57 | 38.76 | 1,007 | 1.428 | 23.54 | 2.96 | 0 | 4Z8L |

Note: Data were obtained from the best diffracting crystal according to crystallization condition (see “Experimental Procedures” for details) and collected at ~180°C. The crystallographic phase problem was solved by molecular replacement using PHASER (see “Experimental Procedures” for details). Refinement was carried out using PHENIX.

Results

VpxSIVmnd Facilitates the Interaction between SAMHD1 and DDB1-DCAF1 via the N-terminal Domain of SAMHD1—We and others previously reported that VpxSIVmnd and VpxHIV-2 preferentially interact with the CTD of SAMHD1, whereas VpxSIVrcm and VpxSIVmnd interact with the NTD of SAMHD1 (Fig. 1A) (20, 25–28). The interactions between these particular Vpx and the respective SAMHD1 proteins require the substrate transfection with 7 µg of a mixture of pCDNA plasmids encoding SAMHD1, DCAF1CA (residues 1040–1400), and Vpx using LipofectAMINES 2000 (Life Technologies). After 48 h cells were lysed with a buffer containing 25 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA, 0.3% Nonidet P-40, 1% Tween, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science). For immunoprecipitation, agarose beads conjugated with anti-FLAG antibodies (Sigma) were added to the cell lysates. Proteins were analyzed as described for GST pulldown assays, with appropriate antibodies, including anti-HA (Covance) and anti-actin (Sigma) antibodies.
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adaptor and receptor component (DDB1-DCAF1) of CRL4, with the E3 ligase mediating proteasome-dependent down-regulation of SAMHD1. Because RCM and MND SAMHD1, but not Hu SAMHD1, were effectively down-regulated by Vpx \textsuperscript{SIVrcm} and Vpx \textsuperscript{SIVmnd} (25), we speculated that the NTD of RCM and MND SAMHD1 share a sequence motif that is targeted by Vpx \textsuperscript{SIVrcm} and Vpx \textsuperscript{SIVmnd}. To identify this motif, we aligned the amino acid sequences of the NTD of Hu, RCM, and MND SAMHD1 proteins (Fig. 1A). Despite being highly homologous, several residues, including amino acids at positions 3, 7, 15, 24, 32, 52, 60, 63, 72, 85, 107, and 108 are different between the monkey and human sequences. The two monkey

FIGURE 1. Vpx from \textsuperscript{SIVrcm} (Vpx\textsuperscript{SIVrcm}) binds to the N terminus of RCM and MND SAMHD1 and recruits SAMHD1 to DDB1-DCAF1. A, schematic representation of the SAMHD1 structure and amino acid sequence alignment of the NTD (residues 1–115) and the CTD (residues 595–626) of MND, RCM, and Hu SAMHD1. Amino acid residues that vary between monkey and human SAMHD1 are colored in red, and the single residue that differs between MND and RCM SAMHD1 is colored in orange. B, isothermal titration calorimetry traces for RCM SAMHD1 NTD, MND SAMHD1 NTD, and RCM SAMHD1 CTD binding to DDB1-DCAF1\textsubscript{CA}-Vpx\textsuperscript{SIVrcm}. Aliquots of RCM SAMHD1 NTD (30 \mu M) were added to the DDB1-DCAF1\textsubscript{CA}-Vpx\textsuperscript{SIVrcm} complex (4.6 \mu M) in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.02% sodium azide at 12 °C (left panel). Aliquots of MND SAMHD1 NTD (30 \mu M) and RCM SAMHD1 CTD (residues 595–626 at 30 \mu M) were added to the DDB1-DCAF1\textsubscript{CA}-Vpx\textsuperscript{SIVrcm} complex (2 \mu M) in the same buffer, respectively (middle panel and right panel). The heat of dilution at each concentration and buffer condition was determined and subtracted. The data in the left panel and middle panel were best fitted to a single-site binding isotherm yielding a \textit{K}_d of 170 ± 22 nM and 22 ± 2 nM, respectively. C, amino sequence alignment of Vpx\textsuperscript{SIVmnd}, Vpx\textsuperscript{SIVrcm}, Vpx\textsuperscript{HIV-2}, and Vpx\textsuperscript{SIVsm}. The sequence motif essential for SAMHD1 C-terminal binding is colored in red (29, 30).
sequences, on the other hand, contain only one conservative change at position 36 (Leu to Val). Thus, the NTD of RCM SAMHD1 and MND SAMHD1 are essentially identical. We, therefore, hypothesized that DDB1-DCAF1CA-VpxSIVrcm could bind to the NTD of both RCM and MND SAMHD1. To quantitatively determine binding affinities between SAMHD1 and DDB1-DCAF1-VpxSIVrcm, we performed isothermal titration calorimetry (Fig. 1B). As expected, both the NTDs of RCM and MND SAMHD1 bound tightly to DDB1-DCAF1-VpxSIVrcm, with \( K_d \) values of 170 ± 22 nM and 22 ± 2 nM, respectively. In contrast, the CTD of RCM SAMHD1, which is homologous, and the sequence motif, essential for recruitment, is those that are different between monkey and human proteins. Indeed, the amino acid sequences of VpxSIVmnd and VpxSIVrcm are highly homologous, and the sequence motif, essential for recruitment, of the C terminus of SAMHD1 to DCAF1, is not present in either of the two Vpx proteins (Fig. 1C).

Structural Mapping of DCAF1-VpxSIVmnd and DCAF1-VpxSIVrcm Binding to the SAM Domain of SAMDH1—To identify the region of the SAM domain involved in the binding by DCAF1-VpxSIVmnd or DCAF1-VpxSIVrcm, we utilized NMR spectroscopy. Fig. 2 displays a superposition of the \(^1\text{H},^{15}\text{N}\) TROSY-HSQC spectra of the MND SAMHD1 NTD in the absence (blue spectrum) and presence (red spectrum) of DCAF1-VpxSIVmnd at a 1:1 molar ratio. More than half of the \(^1\text{H},^{15}\text{N}\) resonances from the SAM domain (55 out of 105 residues) exhibited noticeable chemical shift perturbations (>0.070 ppm), indicating that binding affects a large portion of the SAM domain. Furthermore, when DCAF1-VpxSIVmnd was added at a submolar amount (~0.5) to the NTD, the \(^1\text{H},^{15}\text{N}\) resonances from the free and bound states are in slow exchange on the NMR chemical time scale (data not shown). Thus, NMR (Fig. 2) and isothermal titration calorimetry (Fig. 1B) analyses yielded consistent results. Residues whose resonances experience chemical shift changes upon binding (e.g., Gly-3, Ser-24, Val-36, Phe-52, Gly-60, and Gly-108 resonances, marked with solid ovals in Fig. 2) are indicated on the SAM domain structure (amino acids 23–118) of Hu SAMHD1 (PDB code 2E8O), with changes colored in red (>0.15 ppm) and orange (between 0.07 and 0.15 ppm). As can be easily appreciated, binding involves one face of the structure, forming a large extended binding site for DCAF1-VpxSIVmnd (Fig. 2). Several of the affected residues are those that are different between monkey and human SAM proteins. Indeed, some of which reside on \( \alpha \) helices \( \alpha_1 \) and \( \alpha_2 \). However, not all divergent residues are affected by the DCAF1-VpxSIVmnd binding, in particular those residing on \( \alpha \) helices \( \alpha_3 \) and \( \alpha_4 \). For example, His-85 in the monkey sequences is replaced by Arg-85 in human (Fig. 1A), but its amide resonance exhibited negligible (<0.035 ppm) chemical shift changes upon complex formation (dashed oval in Fig. 2), demonstrating that His-85 and the surrounding region are not part of the binding interface.

In our NMR analysis, we also obtained information on the 30-residue N-terminal tail of the SAM domain that is flexible in the solution structure. The associated amide resonances appear
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as sharp and intense cross-peaks in the central, poorly dispersed, random coil region of the $^{1}H,^{15}N$ TROSY-HSQC spectrum of the free protein (blue resonances, enclosed by the dashed rectangle in Fig. 2). Interestingly, many of these N-terminal tail resonances exhibited large chemical shift perturbations upon binding DCAF1-Vpx$^{SIVmnd}$, indicating that the N-terminal region is involved in complex formation and changes conformation in the complex.

A similar NMR study, mapping the binding of DCAF1-Vpx$^{SIVrcm}$ to MND SAMHD1 NTD, shows that essentially the same regions of the SAM domain are involved in both complexes (data not shown). Note that the MND SAMHD1 and RCM SAM HD1 exhibit identical amino acid sequences for the SAM domain, except for the L36V change.

Crystal Structure of the DCAF1-Vpx$^{SIVmnd}$-SAMHD1 NTD Complex—To further delineate the details of SAMHD1 recruitment via its NTD for degradation by Vpx$^{SIVmnd}$, we determined the crystal structure of the DCAF1-Vpx$^{SIVmnd}$-SAMHD1 NTD complex at 2.6 Å resolution. Two ternary complexes are found in the asymmetric unit (data not shown), and given their near identity, only one is described below. Several important features are noted: two critical anchoring points are used by the SAM HD1 NTD to interact with DCAF1 and Vpx, involving the N-terminal tail (residues Gln-2–Arg-20) and the core SAM domain, in particular residues residing in helices $\alpha$1 and $\alpha$2. The N-terminal tail up to residue Arg-20 contacts both DCAF1 and Vpx residues and is intertwined between these two proteins (Fig. 3, A and B), whereas residues Gly-35–His-39 and the first two helices ($\alpha$1, residues Gly-46–Gly-57, and $\alpha$2, residues Gly-60–Glu-70) of the SAM HD1 NTD interact solely with Vpx (Fig. 3A). This mode of interaction is different from what was observed in the structure of the DCAF1-Vpx$^{SIVsm}$-SAMHD1 CTD (Fig. 3C) (29). On the other hand, despite sequence differences between Vpx$^{SIVmnd}$ and Vpx$^{SIVsm}$ (Fig. 1C), both Vpx proteins interact with DCAF1 in a very similar way (Fig. 3D) for recruitment of SAM HD1 to DCAF1 via the SAM HD1 NTD and CTD, respectively.

The above structural observations were validated in co-immunoprecipitation experiments. In particular, Vpx$^{SIVmnd}$ co-immunoprecipitated with MND SAM HD1 and WT DCAF1 as well as the F1330A/F1355A mutant DCAF1, whereas Vpx$^{HIV-2}$ failed to immunoprecipitate Hu SAM HD1 with the F1330A/F1355A DCAF1 mutant (Fig. 3E). On the other hand, the D1092A/E1093A DCAF1 mutation abolished both Vpx$^{SIVmnd}$, SAM HD1 and Vpx$^{HIV-2}$, SAM HD1 interactions (Fig. 3E). These co-immunoprecipitation results can be explained based on the DCAF1-Vpx$^{SIVmnd}$ interface in the crystal structure. In the structure, the side chain of DCAF1 Asp-1092 is hydrogen-bonded to the backbone amide of Phe-15 and the Ne of the Arg-14 side chain of MND SAM HD1 NTD as well as to the hydroxyl group of the Tyr-62 side chain of Vpx$^{SIVmnd}$, whereas the side chain of DCAF1 Glu-1093 hydrogen bonds with the NH$_2$ group of Arg-66 and the Nζ group of Lys-73 of MND SAM HD1 NTD (Fig. 3F). Similar hydrogen-bonding patterns are present in the DCAF1-Vpx$^{SIVsm}$ structure (29). Therefore, changing Asp-1092 and Glu-1093 to Ala eliminates these H-bonds (Fig. 3F). Similarly, residues Phe-1330 and Phe-1335 of DCAF1 engage in hydrophobic contacts with Tyr-76 in Vpx$^{SIVmnd}$ (Fig. 3F). The same hydrophobic triad can be formed with Phe-80 in Vpx$^{HIV-2}$ (29), and removal of the two aromatic side chains on DCAF1 will abrogate the interaction with Vpx$^{HIV-2}$. This loss may possibly be compensated for in Vpx$^{SIVmnd}$, where the hydroxyl group of the equivalent tyrosine is close to the O$_2$ hydroxyl group of Thr-1097 in DCAF1 (Fig. 3G). All together, the structural data clearly visualize how the distinct regions of SAM HD1 are targeted by clade-specific Vpx proteins.

A Single Residue Change in the Human SAM HD1 NTD Mediates Proteasome-dependent Down-regulation by Vpx$^{SIVrcm}$—To identify the human SAM HD1 residue(s) that is responsible for the lack of recognition by Vpx$^{SIVrcm}$ and Vpx$^{SIVmnd}$, N-terminal SAM HD1 residues that varied between human and monkey were exchanged, and GST pulldown assays with mixtures of GST-Hu SAM HD1 NTD and DDB1-DCAF1-Vpx($\Delta$)SIVrcm or DDB1-DCAF1-Vpx($\Delta$)SIVmnd were performed (Fig. 4, A and B). Specifically, Hu SAM HD1 NTD amino acids were individually changed to those at the corresponding position in RCM and MND SAM HD1, i.e., S52F, E60G, V63A, E72K, and R85H (Fig. 1A). The S52F change substantially enhanced the binding of GST-Hu SAM HD1 NTD with DDB1-DCAF1-Vpx($\Delta$)SIVrcm as well as with DDB1-DCAF1-Vpx($\Delta$)SIVmnd (Fig. 4, A and B), whereas none of the other changes produced a similar effect. We verified that the mutation at residue 52 does not affect the structural integrity of the SAM domain by NMR and ascertainment that no significant changes in the $^{1}H,^{15}N$ TROSY-HSQC spectrum of the S52F mutant protein, compared with that of WT human SAM HD1 NTD, occurred (data not shown). Similarly, the $^{1}H,^{15}N$ TROSY-HSQC spectra of WT and F52S MND were also essentially identical (data not shown).

Once recruited to DDB1-DCAF1, a substrate adaptor-receptor component of CRL4 E3 ubiquitin ligase, by Vpr or Vpx, SAM HD1 is polyubiquitinated and degraded via the ubiquitin-proteasome system (UPS). HIV-2 Vpx significantly decreases

**FIGURE 3.** Complex formation between DCAF1-Vpx$^{SIVmnd}$ and MND SAM HD1. A, crystal structures of the DCAF1-Vpx$^{SIVmnd}$-MND SAM HD1 NTD complex. All proteins are displayed in ribbon representation. DCAF1, Vpx$^{SIVmnd}$, and MND SAM HD1 NTD are colored in light gray, yellow, and green, respectively. B, electron density for residues Lys-11–Glu-30 of MND SAM HD1 NTD and residues Glu-7–Leu-17 of Vpx$^{SIVrcm}$, contoured at 1 $\sigma$. C, crystal structures of the DCAF1-Vpx$^{SIVsm}$-Hu SAM HD1 CTD complexes (29). DCAF1, Vpx$^{SIVsm}$, and Hu SAM HD1 CTD are colored in light gray, yellow, and green, respectively. D, superposition of the structures of DCAF1-Vpx$^{SIVmnd}$-MND SAM HD1 NTD and DCAF1-Vpx$^{SIVmnd}$-Hu SAM HD1 CTD. In the DCAF1-Vpx$^{SIVmnd}$-MND SAM HD1 NTD complex, the individual components DCAF1, Vpx$^{SIVmnd}$, and MND SAM HD1 NTD are colored in light gray, yellow, and green, respectively. In the DCAF1-Vpx$^{SIVsm}$-Hu SAM HD1 CTD complex, the individual components DCAF1, Vpx$^{SIVsm}$, and Hu SAM HD1 CTD are colored in blue, magenta, and orange, respectively. E, immunoprecipitation (IP) of complexes. FLAG-tagged WT or mutant DCAF1 proteins (residues 1040–1400) were transiently expressed in the presence of MND SAM HD1 and Vpx$^{SIVmnd}$ or Hu SAM HD1 and Vpx$^{SIVmnd}$ as indicated. Cells were treated with MG132 for 6 h before cell lysates were subjected to immunoprecipitation with anti-FLAG antibody and analyzed by immunoblotting (IB). F and G, hydrogen bonds formed by DCAF1 residues Asp-1092/Glu-1093 (F) and hydrophobic interactions involving DCAF1 residues Phe-1330/Phe-1355 (G) in the DCAF1-Vpx$^{SIVmnd}$-MND SAM HD1 NTD complex. DCAF1, Vpx$^{SIVmnd}$, and Hu SAM HD1 NTD are colored in light gray, yellow, and green, respectively.
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A

[Image of gel electrophoresis with bands labeled "GST-Hu SAMHD1 NTD", "DCAF1CA", and "Vpx(SIVmnd)"

B

[Image of gel electrophoresis with bands labeled "Hu", "GST-Hu SAMHD1 NTD", and "DCAF1CA"

C

[Graph showing response (RU) over time (Sec) for dissociation at various concentrations of ligand: RCM SAMHD1 NTD-GST

D

[Graph showing response (RU) over time (Sec) for dissociation at various concentrations of ligand: Hu SAMHD1 NTD-GST

E

[Graph showing response (RU) over time (Sec) for dissociation at various concentrations of ligand: Hu SAMHD1 NTD(S52F)-GST

F

[Graph showing response (RU) over time (Sec) for dissociation at various concentrations of analyte (nM)

G

[Image of gel electrophoresis with bands labeled "WT", "S52F", "CRL4-DCAF1ca-Vpx(SIVmnd)"

H

[Image of gel electrophoresis with bands labeled "DCAF1ca", "Vpx(SIVmnd)", "Hu SAMHD1", "SAMHD1-Ubn" and "actin"

I

[Image of protein structure with residues W45, F42, F52, V36, L38 and distance 2.91]
the dissociation rate of SAMHD1 from DDB1-DCAF1, as seen by SPR (30). The increased residence time in the complex may contribute to a more efficient polyubiquitination by the CRL4 E3 ubiquitin ligase. We hypothesized that the interaction between DDB1-DCAF1-Vpx and RCAM1 may also influence the dissociation rate, and we, therefore, performed similar SPR analyses using the NTD of RCAM1 and its cognate DDB1-DCAF1-Vpx(SIVrcm) complex (Fig. 4C). Vpx(SIVrcm) substantially decreased the dissociation of the complex from the RCAM1 NTD, whereas the same virus-host protein complex dissociated from the human SAMHD1 NTD much faster (Fig. 4D). After introduction of the S52F amino acid change into human SAMHD1 NTD, a reduced dissociation rate from the DDB1-DCAF1-Vpx(SIVrcm) complex was observed (Fig. 4E). Analysis of the overall dissociation curves also suggests that the S52F amino acid change in human SAMHD1 increased the binding affinity to the virus-host protein complexes (Fig. 4F). Because the S52F mutant of human SAMHD1 was able to bind to the DDB1-DCAF1-Vpx(SIVrcm) complex, we also tested whether in vitro and in vivo polyubiquitination by the CRL4-DCAF1-Vpx E3 ubiquitin ligase occurs. As illustrated in Fig. 4, G and H, this is clearly the case. The importance of a Phe residue at position 52 is supported by the structure of the DCAF1-Vpx(SIVmnd)-SAMHD1 NTD complex. As illustrated in Fig. 4I, Phe-52 of MND SAMHD1-NTD engages in hydrophobic contacts with Phe-42 (as well as Trp-45) of Vpx(SIVmnd) (distances <4 Å), and identical contacts should be made with Vpx(SIVrcm), as no differences in sequence in this region of Vpx(SIVmnd) and Vpx(SIVrcm) exist (Fig. 1B). Therefore, replacement by a Ser, the amino acid present at this position in human SAMHD1, will interfere with this interaction.

**Discussion**

One of the hallmarks revealing the presence of the evolutionary interplay between virulence factors and host restriction factors is the presence of positive selection at protein-protein interaction interfaces. This is seen by an adaptive mutation(s) in the viral proteins and localized sequence variation of the restriction factors in different host species (for reviews, see Refs. 23, 39, and 40). In particular, the co-evolution of primate SAMHD1 and Vpx of various SIVs resulted in the use of two distinctive protein-protein interfaces: NTD/Vpx and the CTD/Vpx (27). Specifically, Vpx from SIVrcm and SIVsm preferentially interact with the SAMHD1 NTD, whereas Vpx from SIVsm and HIV-2 requires the SAMHD1 CTD to establish antagonism, and a stable interaction only occurs when a third component, DCAF1, is present (30). The crystal structure of the DCAF1-Vpx(SIVmnd)-SAMHD1 CTD elegantly illustrated how Vpx binding to DCAF1 creates a new molecular interface for the SAMHD1 CTD and corroborated previous biochemical findings (29). In our current work, NMR studies reveal that a large portion of the SAMHD1 NTD is affected by binding to the DCAF1-Vpx complex (Fig. 2). The crystal structure of DCAF1-Vpx(SIVmnd)-SAMHD1 NTD complex confirmed the NMR data. The distinct differences in the interaction of Vpx from SIVmnd and SIVsm with SAMHD1 are in stark contrast to the very similar mode observed for binding of these Vpx proteins to DCAF1 (Fig. 3, A, C, and D). Therefore, the distinct modes of SAMHD1 recruitment most likely are the result of sequence variations in Vpx, which have led to creation of unique interfaces for different regions of SAMHD1 upon DCAF1 binding. In addition to structural causes for the distinct engagement of SAMHD1 by the Vpx variants from the two species, dynamics may also play a differentiating role; real-time kinetic analyses by SPR suggest that Vpx substantially decreases the dissociation rate of SAMHD1 from the CRL4-DCAF1 E3 ubiquitin ligase, depending on whether SAMHD1 is recruited via its SAM domain (Fig. 4) or the C terminus (30).

![FIGURE 4. A single residue in the SAM domain of human SAMHD1 is critical for binding to DDB1-DCAF1-Vpx(SIVmnd) and DDB1-DCAF1-Vpx(SIVrcm), A and B, GST-pulldown assay of GST-Hu SAMHD1 NTD interacting with DDB1-DCAF1(Vpx(ΔC))SIVmnd (A) and DDB1-DCAF1(Vpx(ΔC))SIVrcm (B), Hu SAM domain residues that differ from the simian protein were individually changed to those present in MND SAMHD1 and RCAM1 (see Fig. 1A). Pulldown assays of DDB1-DCAF1CA-Vpx(ΔC)SIVmnd or DDB1-DCAF1CA-Vpx(ΔC)SIVrcm protein complexes with GST-Hu SAMHD1 NTD were immunoblotted with the appropriate antibodies. C–E, real-time kinetic analysis DDB1-DCAF1-Vpx(SIVmnd) binding to NTD. SPR sensorgrams of DDB1-DCAF1CA-Vpx(ΔC)SIVmnd binding to RCAM1 SAMHD1 NTD-GST (solid circles, Kd = 322 ± 25 nM), to Hu SAMHD1 NTD-GST (solid squares, Kd = 2260 ± 370 nM), and to Hu SAMHD1 NTD52(S52F)-GST (solid triangles, Kd = 256 ± 42 nM) were generated by plotting the response levels with different analytic concentrations at 120 s (marked as dissociation in C, D, and E). RU, response units. Dissociation constants were determined from two independent series of experiments. G, in vitro ubiquitination (Ubn) of full-length Hu SAMHD1 WT or S52F with CRL4-DCAF1CA-Vpx(ΔC)SIVmnd E3 ubiquitin ligase. T7-epitope-tagged SAMHD1 proteins were incubated with E1, E2, ubiquitin, and the appropriate E3 ligases in ubiquitination buffer, as described under “Experimental Procedures.” H, HEK293 cells were transiently co-transfected with DCAF1CA (residues 1040–1400), Hu WT or S52F SAMHD1, and Vpx(SIVmnd) as indicated. The levels of expressed proteins were determined by immunoblotting with appropriate antibodies. I, residue Phe-52 of MND SAMHD1 NTD (green, displayed in stick representation) is involved in hydrophobic contacts with Phe-42 and Trp-45 of Vpx(SIVmnd) (yellow, displayed in stick representation).]
residue 52, along with residue 15, was under positive selective pressure (28).

In conclusion, our studies presented here, in conjunction with several previous reports (20, 25–28), provide mechanistic and structural insight at the molecular level into how Vpx proteins from different SIV lineages have adapted to sequence variations in their cellular target.

While this manuscript was in preparation a similar structure of DCAF1-VpxSIVmand-SAMHD1 NTD complex was reported (41).

Author Contributions—J. A. and A. M. G. conceived and coordinated the study. Y. W. designed, performed, and analyzed the experiments shown in Figs. 1 and 4. C. H. B. and I. L. B. designed, performed, and analyzed the experiments shown in Fig. 2. L. M. I. K., J. M. M., and G. C. designed research and performed and analyzed the experiments shown in Fig. 3. Y. W., L. M. I. K., I. L. B., J. A., J. M., M. D., and G. C. wrote the paper, and all authors approved its final version.

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Note Added in Proof—Many of the values in Table 1 in the version of the article that was published as a Paper in Press on June 4, 2015 have been changed because PDB entry 4Z8L was re-refined with better data processing and refinement statistics. The updated Table 1 is now available. These changes do not affect the interpretation of the results or the conclusions.

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