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A ZINC-BINDING REGION IN VIF BINDS CUL5 AND DETERMINES CULLIN SELECTION

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SUMMARY

HIV-1 Vif overcomes the antiviral activity of APOBEC3G by targeting it for ubiquitination via a Cullin 5-ElonginB-ElonginC (Cul5-EloBC) E3 ligase. Vif associates with Cul5-EloBC through a BC-box motif that binds EloC, but the mechanism by which Vif selectively recruits Cul5 is poorly understood. Here we report that a region of Vif (residues 100-142) upstream of the BC-box binds selectively to Cul5 in the absence of EloC. This region contains a zinc-coordination site H-x_5-C-x_{17-18}C-x_{3-5}-H (HCCH), with His/Cys residues at positions 108, 114, 133, and 139 coordinating one zinc ion. The HCCH zinc-coordination site, which is conserved among primate lentivirus Vif proteins, does not correspond to any known class of zinc-binding motif. Mutations of His/Cys residues in the HCCH motif impair zinc coordination, Cul5 binding, and APOBEC3G degradation. Mutations of conserved hydrophobic residues (I120, A123, and L124) located between the two Cys residues in the HCCH motif disrupt binding of the zinc-coordinating region to Cul5 and inhibit APOBEC3G degradation. The Vif binding site maps to the first cullin repeat in the N-terminus of Cul5. These data suggest that the zinc-binding region in Vif is a novel cullin-interaction domain that mediates selective binding to Cul5. We propose that the HCCH zinc-binding motif facilitates Vif-Cul5 binding by playing a structural role in positioning hydrophobic residues for direct contact with Cul5.

APOBEC3G is a cellular cytidine deaminase with potent antiviral activity (1). APOBEC3G and several related APOBEC3 family members are packaged into HIV-1 virions and deaminate cytidine to uracil in newly synthesized viral DNA, inducing massive G-to-A hypermutation in the viral genome and triggering DNA repair pathways that lead to degradation of viral cDNA (2-10). APOBEC3 proteins may also exert anti-viral activity via mechanisms independent of their cytidine deaminase activity (11,12). The Vif protein encoded by HIV-1 and other lentiviruses counteracts APOBEC3F and 3G by inducing their degradation via the ubiquitin-proteasome pathway (8,10,13-18). Vif binds directly to APOBEC3G and targets it for ubiquitination by a cullin-dependent ubiquitin ligase containing Vif, Cullin 5 (Cul5), Elongins B and C (EloBC), and Rbx (17,19,20). Mechanisms independent of proteasomal degradation may also contribute to the anti-APOBEC3G effects of Vif (15,21-23).

Ubiquitination requires the coordinated activity of an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and a substrate-specific E3 ubiquitin ligase (24). Cullin-dependent ubiquitin ligases contain a cullin scaffold, the RING protein Rbx, adaptor molecules, and specificity subunits. In the SCF (Skp1–Cul1–F-box) complex, a prototypical cullin-dependent ubiquitin ligase, the adaptor molecule Skp1 links Cul1 to specificity-determining F-box proteins by binding the N-terminus of Cul1 and an ~40-amino acid F-box motif (25). Analysis of the SCF^{Skp2} crystal structure reveals that the F-box protein Skp2 also makes important contacts with the N-terminus of Cul1 (25,26). Additional binding domains in the F-box protein (e.g. WD-40 domains, Leu-rich repeats) recruit specific targets for ubiquitination. EloBC–Cul2/5–BC-box ubiquitin ligase complexes are formed in a similar manner. The EloBC dimer serves as the adaptor molecule that bridges BC-box proteins

to the cullin scaffold by binding the N-terminus of the cullin and the BC-box, an ~10-amino acid degenerate sequence motif [(A/P/S/T)L-x_3-C/A-x_3-(A/I/L/V)]. EloBC interacts with both Cul2 and Cul5, but under physiological conditions most BC-box proteins selectively form complexes with only Cul2 or Cul5. Analogous to F-box proteins, BC-box proteins contain regions that bind both EloBC and the N-terminus of the cullin (26). Sequence elements downstream of the BC-box designated the Cul2- or Cul5-box confer specificity on cullin binding. Most BC-box proteins can thus be divided into those that contain a VHL-box (BC-box and Cul2-box) and preferentially utilize Cul2 or those with a SOCS-box (BC-box and Cul5-box) that interact with Cul5.

HIV-1 Vif contains a BC-box motif that is critical for association with Cul5-EloBC and APOBEC3G ubiquitination (19,20). The BC-box motif is conserved in all Vif proteins. The downstream proline/leucine-rich sequence identified as a potential Cul5-box is conserved in HIV-1 and SIV_{CPZ} Vif proteins. However, HIV-2 and other SIV Vif proteins that lack the proline/leucine rich sequence preferentially interact with Cul5 (27). The mechanism by which Vif selectively recruits Cul5 is poorly understood. We show here that a zinc-binding region upstream of the BC-box in Vif binds directly to Cul5 and mediates cullin selection. The data suggest a model in which a novel H-x_5-C-x_{17-18}C-x_{3-5}-H (HCCH) zinc-coordination motif positions conserved hydrophobic residues for direct binding to the first cullin repeat in the N-terminus of Cul5. These results provide insights into the regulated assembly of cullin E3 ubiquitin ligases and also identify new targets for development of antiviral therapies.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids– Antibodies used were rabbit anti-Vif (28), 3F10 anti-HA (Roche), anti-T7 (Novagen), 9B11 anti-myc (Cell Signal), M2 anti-FLAG (Sigma), rabbit anti-Cul5 (a kind gift from M. Fay) and 1D4 anti-C9. Proviral plasmids and expression vectors for Vif, APOBEC3G, Cul2, Cul5, Cul7, EloB, and EloC have been described previously (14,15,19,29). The replication-defective proviral plasmid pNLA1.Vif-FLAG was constructed by deleting the *gag* and *pol* genes from the replication- competent HIV-1 molecular clone pNL4-3 and inserting a FLAG epitope sequence at the 3' end of *vif* (a gift from K. Strebel). Vif and Cul5 truncations and Vif mutants were created by PCR-based mutagenesis and confirmed by sequencing.

Co-precipitation and Western Blot Analysis– 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. At 36-48 h after calcium phosphate transfection, lysates were prepared in lysis buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 0.5% NP-40, and 1% protease inhibitor cocktail). Identical amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and detected by standard techniques. For co-immunoprecipitation experiments, identical amounts of lysate were subjected to immunoprecipitation or GST- pull down followed by Western blotting.

Zinc Blots and Immobilized Metal Affinity Chromatography (IMAC)–Zinc blots were performed as described (30). Briefly, GST-Vif fusions were purified by affinity chromatography (19), separated by SDS-PAGE, and transferred to nitrocellulose. Equivalent amounts of SOD (Sigma), CA (Sigma), HIV-1 NC (NIH AIDS Research and Reference Reagent Program), and HIV-1 IN (a kind gift from A. Engelman) were included as controls. Membrane-bound proteins were refolded in three 1 h changes of renaturation buffer (100 mM Tris pH 7, 50 mM NaCl, 10 mM DTT). The membranes were probed with 35 μM $^{65}Zn^{2+}$ in 100 mM Tris pH 7, 50 mM NaCl for 3 h. After binding, membranes were washed with renaturation buffer. The pH of binding and wash buffers was altered where indicated. Following exposure to film, membranes were stained with amido black to control for protein loading. Chelating Sepharose FF was used for IMAC experiments following the manufacturer's instructions (Amersham

Pharmacia). Similar amounts of purified protein were incubated for 3 h with matrix in lysis buffer. Binding experiments with GST-Vif 100-142 proteins were performed in lysis buffer supplemented to 500 mM NaCl and 10 mM imidazole. Following extensive washing, bound proteins were detected by western blot or coomassie staining. Where indicated, proteins were first eluted with 500 mM imidazole. All buffers were pre-treated with Chelex 100 Resin (BioRad) to remove free metals.

In Vitro Binding Assay– 293T cell lysates or in vitro translation products (TnT Quick System, Promega) were incubated with similar amounts of purified GST fusion proteins in lysis buffer for 2 h. Complexes were captured with glutathione Sepharose 4 Fast Flow, washed with lysis buffer, separated by SDS-PAGE, and detected by western blot, autoradiography, or phosphorimaging.

Atomic absorption spectroscopy (AAS)– GST-Vif 100-142 was purified by affinity chromatography in 50 mM Hepes pH 7.4, 50 mM NaCl, 10% glycerol, 1 mM DTT, 50 μM $ZnCl_2$, washed, dialyzed 4 times against an excess of metal-free 50 mM Hepes pH 7.4, 50 mM NaCl, 10% glycerol, and quantitated by comparison against protein standards of known concentration after SDS-PAGE and coomassie staining. Zinc content was determined by AAS. Zinc was measured with a graphite furnace atomic absorption spectrometer (Perkin Elmer AAnalyst 600) and analyzed with AA Winlab software, as described (31). Zinc concentrations were determined using a standard curve prepared from a zinc standard solution (Sigma). Measurements were taken using two independent protein preparations.

Viruses and Infections– Viruses were produced by co-transfecting 293/APOBEC3G cells (17) with Vif-deficient proviral plasmid pNLXΔvif and WT or mutant pCDNA3.Vif. Viruses were quantitated by reverse transcriptase assays, and normalized amounts were used to infect the reporter cell line Cf2-luc (15). Infectivity was measured 48 h after infection by performing luciferase assays (Promega).

RESULTS

Vif is a zinc-binding protein

The BC-box motif of HIV-1 Vif binds directly to EloC, but regions of Vif that selectively recruit Cul5 have not been defined. The conserved cysteines at positions 114 and 133 upstream of the BC-box (Fig. 1*A*) are required for Cul5 association (19,20,27), APOBEC3G degradation, and viral replication (15,16,32). These cysteines are part of a highly conserved H-x_5-C-x-$_{17\text{-}18}$C-$x_{3\text{-}5}$-H (HCCH) motif present in all primate lentivirus Vif proteins (Fig. 1*A* and Supplemental Fig. 1). Recently, this conserved HCCH motif was shown to be required for assembly of the Vif-Cul5 E3 ligase that ubiquitinates APOBEC3G (19,20,27). The HCCH motif contains two conserved His/Cys pairs flanking a predicted α-helix that contains a cluster of conserved hydrophobic residues. His and Cys are classical zinc-coordinating residues, and the presence of conserved His and Cys residues in proximity is commonly associated with structural tetrahedral zinc-binding sites in which four His/Cys residues function in concert to bind zinc (33). Structural zinc-binding sites accelerate and stabilize protein folding. To our knowledge, however, the HCCH motif does not correspond to any known class of zinc-binding site. We therefore performed experiments to determine if Vif is a zinc-binding protein.

Zinc blot assays that detect the ability of proteins to coordinate zinc were performed using recombinant GST-Vif (30). Proteins transferred to nitrocellulose were re-folded and probed with radioactive ^{65}Zinc (^{65}Zn). GST-Vif was detected by blotting membranes with ^{65}Zn (Fig. 1*B*). The positive control zinc-binding proteins superoxide dismutase (SOD), HIV-1 nucleocapsid (NC), carbonic anhydrase (CA), and HIV-1 integrase (IN) bound zinc in this assay, whereas the negative controls GST, ovalbumin, and bovine serum albumin did not (Fig. 1*B* and data not shown). The positive control proteins utilize diverse binding sites to coordinate zinc, including a nucleic acid-binding zinc finger in NC (CCHC), a catalytic zinc-

binding site in CA (HHH-H_2O), and a structural zinc-binding site in SOD (HHHD), demonstrating the ability of the zinc blot assay to detect a wide range of zinc coordinating motifs. Zinc binding can be inhibited by protonation of the coordinating residues at low pH. We therefore tested zinc binding by Vif at pH values ranging from 7.0 to 5.5. Zinc binding was pH-dependent (Fig. 1*C*), with highest levels of binding occurring at pH 7, suggesting the involvement of specific coordinating residues (30). To further investigate the zinc-binding potential of Vif, immobilized metal affinity chromatography (IMAC) was performed using zinc as an affinity ligand. GST-Vif bound to zinc-charged matrix, but bound to uncharged matrix only at background levels similar to that of the GST control (Fig. 1*D*). GST alone failed to bind to zinc-charged matrix (data not shown). Bound GST-Vif was eluted with imidazole, suggesting that binding was specific and not due to protein aggregation or precipitation onto the matrix. Together, these results suggest that Vif contains a novel zinc-binding motif.

The HCCH motif in Vif is conserved in HIV-1, HIV-2, and SIV Vif (Fig. 1*A*). To determine if other primate lentiviral Vif proteins bind zinc, we performed a zinc blot assay using Vif derived from HIV-1, HIV-2, and SIV_{AGM}. Zinc blots of GST fusion proteins demonstrated that HIV-1, HIV-2, and SIV_{AGM} Vif all bound zinc (Fig. 1*E*). The protein doublet observed in HIV-2 Vif samples resulted from co-purification of truncated Vif proteins. HIV-1 Vif appeared to bind higher levels of zinc compared to HIV-2 and SIV_{AGM} Vif, despite the presence of additional potential zinc-coordinating cysteines in these proteins (Fig. 1*A* and Supplemental Fig. 1*B*). Different metalloproteins can exhibit different affinities for ^{65}Zn in zinc blot assays, but zinc blots do not provide accurate quantitation of zinc-binding capacity (30). Thus, determination of the relative zinc-binding affinities of these Vif proteins awaits further experimentation. These data indicate that diverse primate lentiviral Vif proteins are zinc-binding proteins.

Histidine and cysteine residues in the HCCH motif form a novel zinc coordination site

Our previous studies demonstrated that GST-Vif binds directly to recombinant APOBEC3G and recombinant EloC (15,19), but the biological activity of this fusion protein is unknown. Therefore, to confirm the biological relevance of the preceding results and identify amino acids important for zinc binding, we mutated potential zinc-coordinating residues that are conserved between HIV-1, HIV-2, and SIV Vif proteins (E76, H80, D104, H108, C114, C133, and H139 in HIV-1 Vif) in pCDNA3.Vif and tested the ability of these mutants to rescue viral infectivity in the presence of APOBEC3G. Vif mutants E76N, H80N, and D104N retained near-WT ability to enhance viral infectivity (Fig. 2*A*). In contrast, mutations in the HCCH motif (H108N, C114S, C133S, and H139N) severely impaired Vif function, with viral infectivity indistinguishable from that of a Vif-deleted virus. These data demonstrate that the conserved His/Cys residues of the HCCH motif are essential for viral infectivity.

We next performed experiments to identify a minimal zinc-binding region in Vif and further investigate the role of the HCCH motif. The truncation mutant GST-Vif 100-142, which spans residues 100-142 of HIV-1 Vif and contains the HCCH motif but not the downstream BC-box sequence, was purified and used for IMAC. GST-Vif 100-142, but not GST alone, bound zinc-charged matrix, while GST-Vif 100-142 failed to bind uncharged matrix (Fig. 2*B*). These findings suggest that GST-Vif 100-142 represents a minimal zinc-binding region. Additional IMAC experiments were performed using WT and zinc-coordination site mutants. Mutation of conserved H108 or H139 significantly impaired zinc binding, reducing levels of binding to background levels, implicating these residues as potential zinc ligands (Fig. 2*C*). These results were confirmed by measuring the zinc content of WT and mutant GST-Vif 100-142 in solution. Atomic absorption spectroscopy showed that purified GST-Vif 100-142 contains approximately 0.89 molar equivalents of zinc, suggesting that Vif binds zinc with a 1:1 molar stoichiometry. Inductive coupled plasma mass spectrometry

also detected zinc in full-length WT GST-Vif (data not shown). Mutation of the conserved His/Cys residues in the HCCH motif significantly reduced zinc content, approaching zinc levels detected for GST alone (Fig. 2*D*). These results identify the HCCH motif as a zinc-coordination site, and suggest that H108, C114, C133, and H139 are the zinc-coordinating ligands.

The HCCH zinc-binding motif is required for assembly of the Vif-Cul5 ubiquitin ligase

The Cys residues in the HCCH motif are required for association with Cul5 (19,20,27), raising the possibility that this motif is important for formation of the Vif-Cul5-EloBC complex. We therefore investigated the effects of His/Cys mutations on Vif association with Cul5 and EloBC. Mutation of the four zinc-coordinating residues significantly impaired APOBEC3G degradation when compared to WT Vif (Fig. 3*A*), but did not affect the ability of Vif to bind APOBEC3G ((19) and data not shown). His/Cys mutations significantly reduced co-precipitation of Vif by Cul5 when compared to WT (Fig. 3*B*) suggesting that defects in APOBEC3G degradation resulted from impaired Cul5 association. Paired Cys and His residues frequently form the zinc-binding motifs in zinc fingers, small independently folded domains that recognize nucleic acids (33). Vif binds RNA (34-36) and has been identified as part of an RNase-sensitive high-molecular weight complex containing APOBEC3G and the Cul5 complex (37). Treatment of lysates with the non-specific RNA and DNA endonuclease benzonase did not affect Cul5 co-precipitation (data not shown), suggesting that RNA-binding is not required for interaction with Cul5. To confirm the biological relevance of the preceding results, we performed experiments to detect binding to endogenous Cul5 when Vif is expressed by a replication-defective HIV-1 clone. WT Vif, but not H108/139N and C114/133S mutant Vifs, expressed by a replication-defective HIV-1 molecular clone co-precipitated endogenous Cul5 (Fig. 3*C*), providing evidence that the HCCH motif is important for Cul5 binding in infected cells. Equivalent levels of Vif expression were confirmed by western blot of cell lysates (Fig. *3*C) and α-FLAG immunoprecipitates (data not shown). In contrast, the H108/139N and C114/133S Vif mutants bound EloB and EloC similar to WT Vif (Fig. 3*D*). This finding is consistent with our previous work demonstrating that the BC-box of Vif (aa 144-158) binds directly to EloC (19). EloC binds directly to Cul5, but EloBC binding was not sufficient to bridge Vif to Cul5. These findings suggest that both Vif and EloC must bind Cul5 to form a stable complex. Thus, the HCCH motif is required for association with Cul5, but not EloBC binding. These results are in agreement with a recent study which demonstrated that the HCCH motif of SIV Vif is important for selective association with Cul5 (27). As in cellular BC-box proteins (26), distinct regions of primate lentivirus Vif proteins are required for EloC binding and Cul5 association.

The zinc-binding region in Vif binds selectively to Cul5

The BC-box motif of Vif binds directly to EloC and is predicted to form a helix that interacts with a hydrophobic binding pocket on EloC (19,20). In addition to the BC-box, cellular SOCS-box proteins contain a downstream proline/leucine rich element termed the Cul5-box that has been shown to mediate Cul5 selection and binding. Vif proteins, however, lack a clear Cul5-box sequence. HIV-1 and SIV_{CPZ} Vif proteins contain a conserved PPLP motif (HIV-1 aa161-164), but lack other conserved regions of the Cul5-box. Therefore, it is unclear if these sequences function similarly to cellular Cul5-boxes. The preceding experiments demonstrate that the zinc-binding HCCH motif is important for recruiting Cul5 to the Vif-EloBC complex, suggesting that Vif may have evolved an alternative Cul5-binding domain. We performed experiments to determine if the zinc-binding region interacts with Cul5 in the absence of EloBC. Purified GST-Vif 100-142, which encompasses the HCCH motif but not the BC-box sequence, precipitated Cul5 expressed in 293T cells, whereas GST alone did not (Fig. 4*A*). GST-Vif 100-142 did not interact significantly with Cul2 or Cul7. Similar results were obtained using in

vitro translated cullin proteins. Cul5, but not Cul2, was specifically co-precipitated by GST-Vif 100-142 (Fig. 4*B*). These experiments suggest that the zinc-coordinating GST-Vif 100-142 binds Cul5 independent of EloC and might therefore determine cullin selection.

We further investigated regions in Cul5 and Vif required for their interaction. The N-terminal domain of cullin proteins forms a long stalk-like structure composed of three cullin repeats, which are novel 5-helix structural motifs. The crystal structure of the Cul1 complex shows that helices 2 and 5 of cullin repeat 1 form a binding site for the adaptor molecule Skp1 (25). This region is predicted to be an adaptor binding site in other cullins. This prediction is supported by mutational analysis of Cul3 and Cul5 demonstrating that these regions are required for binding BTB proteins and EloC, respectively (20,38,39). Cul2- and Cul5-box motifs are thought to interact with sequences in the first cullin repeat (26). Structure-based alignment was used to identify the cullin repeats in Cul5. In vitro translated Cul5 cullin repeats were used in binding assays to determine if the HCCH motif also interacts with this region of Cul5. GST-Vif 100-142 specifically bound truncation mutants containing all three cullin repeats (aa 1-400), cullin repeats 1-2 (aa 1-274), and the first cullin repeat alone (aa 1-158) (Fig. 4*B*). In contrast, Vif did not bind Cul5ΔCR1, which lacks the first cullin repeat (Fig. 4*B*). These data together with Cul5 structural predictions (26) suggest that the zinc-binding region in Vif binds directly to the first cullin repeat in the N-terminus of Cul5, possibly adjacent to the predicted EloC binding site.

To further investigate the interaction between Vif and Cul5, we performed experiments to identify Cul5-binding residues in the zinc-binding region. The conserved His and Cys residues coordinate zinc (Fig. 2), suggesting that these residues do not directly bind Cul5 but may instead play a structural role. The two His/Cys pairs within the HCCH motif flank a central region containing several conserved hydrophobic and basic residues (Fig. 1*A*). We assessed the role of these conserved residues in Cul5 binding assays. Mutation of conserved hydrophobic residues I120, A123, and L124 in GST-Vif 100-142 significantly impaired binding to Cul5 expressed in 293T, whereas mutation of the basic residues R121 and K122 induced only minor defects in binding (Fig. 4*C*). Similar results were obtained using in vitro translated Cul5 (Fig. 4*C*), suggesting that conserved hydrophobic residues in the zinc-binding region interact directly with Cul5. Cul5 association is essential for APOBEC3G degradation (17,19,20). We therefore tested the ability of these mutants to induce the degradation of APOBEC3G. In the context of full length Vif, mutating conserved hydrophobic residues (aa 120/123/124), but not basic residues (aa 121/122), in the zinc-binding motif abrogated Vif-dependent APOBEC3G degradation (Fig. 4*D*). Both mutants retained WT ability to bind APOBEC3G (Fig. 4*E*), suggesting that Vif aa 120-124 are not required for APOBEC3G binding and that Vif associates independently with APOBEC3G and Cul5. Thus, the 121/122 mutant would be expected to rescue viral replication while the 120/123/124 mutant would be expected to have lost this ability. Indeed, a recent study by Xiao et al. showed that Vif mutants I120S and A123S/L124S fail to rescue infectivity of a ΔVif virus in the presence of APOBEC3G, whereas the mutant R121A/K122A functions similarly to WT Vif (40). These results suggest that conserved hydrophobic residues in the zinc-binding motif are critical for Cul5 binding and APOBEC3G degradation, but not APOBEC3G binding, although we cannot formally exclude the possibility that the HCCH motif also contributes directly to Cul5 binding. These hydrophobic residues are part of a Φ_2-X-Gly motif (where Φ represents any hydrophobic amino acid) present in nearly all Vif proteins, including those from FIV, BIV, and Maedi-Visna virus (Supplemental Fig. 1*C*), suggesting that Vif proteins from distantly-related viruses interact with Cul5 from different hosts in a similar manner. Like cellular specificity subunits that form Cul5 E3s, Vif contains unique sequences that bind EloC (BC-box) and Cul5 (zinc-binding region) (Fig. 4*F*). Accordingly, we term the combined zinc-binding region and BC-box the cullin-selection zinc-binding (CZ)-box domain.

The CZ-box differs from the VHL-box and SOCS-box domains in that the cullin-binding zinc-coordinating domain is N-terminal of the BC-box and contains the HCCH motif. Together, these data suggest that the CZ-box is a novel zinc-binding domain that facilitates assembly of a Vif-Cul5-EloBC E3 ubiquitin ligase.

DISCUSSION

Vif functions as a substrate receptor in the Vif-Cul5-EloBC ubiquitin ligase complex, specifically targeting APOBEC3 family members for ubiquitination and degradation (8-10,17,19,20). The BC-box motif in Vif binds EloC, but additional regions of Vif are required for selective association with Cul5. Here we report that a zinc-binding region of Vif (residues 100-142) upstream of the BC-box binds selectively to Cul5 in the absence of EloC. This region contains a novel HCCH zinc-binding motif, with His/Cys residues at positions 108, 114, 133, and 139 coordinating one zinc ion. Mutations of His/Cys residues in the HCCH motif impair zinc coordination, Cul5 binding, and APOBEC3G degradation. Mutations of conserved hydrophobic residues (I120, A123, and L124), but not basic residues (R121 and K122) located between the two Cys residues in the HCCH motif disrupt binding to Cul5 and inhibit APOBEC3G degradation. These results suggest that the zinc-binding region in Vif functions similarly to the Cul2- and Cul5-box found in cellular BC-box proteins, mediating selective cullin recruitment (26). The zinc-binding motif in combination with a downstream BC-box sequence forms the CZ-box, a new cullin-interaction domain that selectively binds and recruits Cul5.

The HCCH motif is a unique zinc-binding motif, both in the order and spacing of the zinc ligands. The four conserved His and Cys residues coordinate a single zinc ion per Vif monomer, suggesting tetrahedral zinc coordination geometry. Tetrahedral zinc coordination typically fulfills a structural role, as opposed to the catalytic role of zinc-binding sites in metallo-enzymes (33,41). Structural zinc-binding domains primarily use His and Cys residues as metal ligands in a diverse array of coordination spheres. Zinc binding in these motifs accelerates and stabilizes protein folding. Zinc chelation introduces cross-links within the unfolded polypeptide chain, limiting the number of accessible conformations and facilitating the formation of compact, independent domains (42). Structural zinc-binding domains frequently mediate homo- and heterotypic protein-protein interactions. For example, the CCHH zinc fingers in the lymphoid transcription factors Ikaros and Aiolos mediate multimerization in *cis* and in *trans* (43,44). More complex coordination sites also mediate protein binding. The RING domain of Rbx proteins and some E3s utilizes eight chelating residues and two zinc ions in a "cross-brace" topology to assume the conformation required for E2 binding (24). Our data suggest that like other tetrahedral zinc-binding sites, zinc-binding by the HCCH motif fulfills structural requirements for protein:protein interactions.

Zinc binding is emerging as a common characteristic of diverse viral proteins that interact with cullin-dependent E3 ubiquitin ligases. The adenovirus protein E4orf6 is a BC-box protein that targets p53 for ubiquitination by Cul5-EloBC (45,46). E4orf6 is an unusual BC-box protein in that it contains two BC-box motifs, both of which appear to be required for function, and no obvious Cul5-box (47). E4orf6 is also a zinc-binding protein that contains conserved Cys and His residues implicated in zinc coordination, although the specific zinc-coordination motif has not yet been identified (48). These conserved residues are required to bind EloC and degrade p53 via a E4orf6-Cul5 complex (47,48), suggesting that zinc binding might fulfill a similar role for E4orf6 by mediating Cul5 binding. A cysteine-rich zinc-coordinating domain in the paramyxovirus V protein is also important for assembly of a V-Cul4a-DDB1 E3 that ubiquitinates STAT proteins (49-52). In addition, polyomavirus T Ag, which interacts with Cul7 (29), is a zinc-binding protein (53). However, zinc-coordinating domains in the V protein and T Ag appear to mediate homo-multimerization important for cullin association, and not necessarily direct cullin binding (53,54). In each

case, zinc binding is thought to fulfill structural requirements for protein:protein interactions. Thus, zinc coordination appears to be an important characteristic of certain viral proteins that interact with the cellular ubiquitin machinery by forming interfaces necessary for assembly into E3 ubiquitin ligases complexes.

HIV-1 and SIV_{CPZ} Vif proteins, but not those from HIV-2 or other SIVs, contain a downstream PPLP sequence that shares similarity to the Cul5-box present in SOC-box proteins. The Cul5-box mediates specific binding to a Cul5-Rbx2 module (26), but the PPLP sequence of Vif may not be directly involved in cullin binding (Fig. 4 and (19)) and is not required for Cul5-binding by Vif derived from SIV_{AGM}, SIV_{MAC}, or $SIV_{Syke's}$ (27). Alternatively, the PPLP region has been implicated in Vif multimerization (55,56). Vif proteins have a strong tendency to multimerize, forming dimers in cell lysates and tetramers or higher-order multimers in *in vitro* binding studies (55). Mutations in the PPLP sequence disrupt Vif multimerization and significantly impair HIV-1 replication in APOBEC3G-expressing cells, suggesting that multimerization is important for Vif function (55,56). Several cellular and viral specificity subunits require the formation of homo- and hetero-oligomeric assemblies, generating combinatorial diversity and enhanced affinity for substrate recruitment to a cullin-dependent E3 (53,54,57). It is possible that Vif is incorporated into the Cul5-EloBC complex as a multimer. In this case, the PPLP region would be important for Vif self-assembly, but might also enhance APOBEC3G recruitment due to the presence of multiple APOBEC3G binding sites.

In conclusion, we have demonstrated that a zinc-binding region in Vif binds directly to Cul5 and mediates cullin selection. Vif contains distinct sequences that bind EloBC (BC-box) and Cul5 (zinc-binding region). The BC-box is required for association with both Cul5 and EloBC (19,20), whereas the HCCH motif that forms the zinc-binding site is required only for Cul5 binding (Fig. 3). These results suggest an ordered assembly in which the BC-box first mediates formation of a Vif-EloBC complex, followed by Cul5 recruitment via the zinc-binding region. Structural predictions, preliminary circular dichroism analysis (data not shown), and distance constraints imposed by zinc binding suggest a model in which the zinc-binding region forms a stalk-like structure with the two cysteines in the HCCH motif flanking an α-helix that contains the Cul5-binding site. In this model, the zinc-coordinating His/Cys residues located at the base of the stalk play a structural role in positioning conserved hydrophobic residues for direct binding to Cul5. The hydrophobic Cul5-binding residues are located at the apex of the predicted α-helix in the Φ_2-X-Gly motif. Gly, one of the most abundant C-cap residues, likely terminates the helix (58). Based on average distances from the zinc ions to His nitrogens or Cys thiols, the domain folds to position C133 and H139 at the base of the stalk, ~2.3 Å and ~2.09 Å from the zinc ion, respectively (41). This model suggests that the zinc-binding region forms a compact structure that facilitates Vif-Cul5 binding. In summary, the zinc-binding region in Vif is a cullin-interaction domain that binds selectively to Cul5 to form the Vif-Cul5-EloBC complex that ubiquitinates APOBEC3G. The requirement of this domain for Vif function, its predicted surface exposure, and the presence of a novel zinc-binding motif make it an attractive target for the development of antiviral compounds.

FOOTNOTES

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Fig. 1 Vif is a zinc-binding protein. *A,* Schematic of HIV-1 Vif and sequence alignment of the conserved HCCH motif. Based on 414 HIV-1 clade B Vif sequences from the LANL database, a consensus sequence was determined with conserved residues shown in lowercase (>50%) and uppercase (>95%). A predicted α-helix is depicted below. The conserved His and Cys residues are shaded and a central hydrophobic sequence is underlined. *B,* Zinc blot performed using purified GST, GST-Vif_{HXB2}, and positive control proteins SOD and NC. Bound ^{65}Zn was detected by autoradiography. Membrane bound proteins were stained with amido black. *C,* Vif exhibits pH-dependent zinc binding. Zinc blot using GST-Vif was performed with binding buffer of decreasing pH. Binding was quantitated by phosphorimaging and expressed as a percent of binding at pH 7. Autoradiograms of ^{65}Zn-labeled Vif are shown. *D,* Zinc IMAC captures Vif. Purified GST-Vif was incubated with zinc-charged or uncharged media, and washed. Protein bound to the media (Bound) or a parallel sample where protein was eluted with imidazole (Eluate) were analyzed by western blotting. GST-Vif was detected by western blotting. Results were quantitated and expressed as a percentage of the input. *E,* Zinc blot analysis was performed as in *A* with GST-Vif fusions derived from HIV-1, HIV-2_{ROD}, and SIV_{AGM}.

Fig. 2 The HCCH motif is a novel zinc-binding site. *A,* Virus was produced from 293/APOBEC3G cells following transfection with a *vif*-deleted proviral plasmid and WT or mutant pCDNA3.Vif. Virus was normalized and infectivity was measured in Cf2-luc cells. Equivalent expression of WT and mutant Vif proteins was determined by western blot. *B,* Vif 100-142 is a minimal zinc-binding domain. Purified GST and GST-Vif 100-142 were used in IMAC experiments with zinc-charged (+) or uncharged media. Proteins were detected by Coomassie staining. *C,* and *D,* Conserved His and Cys residues form a zinc-binding site. Zinc binding by WT and mutant GST-Vif 100-142 was determined by IMAC in *C,* and atomic absorption spectroscopy in *D,* Zinc content was corrected for minor variations in protein concentration and expressed as a percentage of WT Vif zinc content. Results are representative of measurements from two independent protein preparations.

Fig. 3 The HCCH zinc-binding motif is required for APOBEC3G degradation and Cul5 association, but not EloBC binding. *A,* APOBEC3G-HA was expressed in 293T cells with WT and mutant Vif proteins. Protein levels were determined by western blot. *B,* The HCCH motif is required for co-precipitation by Cul5. 293T cells were co-transfected with Vif expression vectors and pCDNA3.HA-Cul5. Lysates were immunoprecipitated with antibodies recognizing epitope tags on the indicated proteins and probed by western blotting. Equivalent levels of Vif expression were confirmed by western blot of cell lysates. *C*, The HCCH motif is required for binding to endogenous Cul5. Vif-FLAG was expressed in 293T cells from a replication-defective HIV-1 molecular clone and immunoprecipitated with an antibody recognizing the FLAG epitope. Co-precipitation of endogenous Cul5 was detected by western blotting with rabbit anti-Cul5. Equivalent levels of Vif and Cul5 expression were confirmed by western blot of cell lysates. *D*, EloBC binding does not required the HCCH motif in Vif. 293T cells were co-transfected with Vif expression vectors, pCDNA3.HA-EloB and pCDNA3.T7-EloC. Co-precipitation was detected as in *B*.

Fig. 4 Conserved hydrophobic residues in the zinc-binding region are required for selective recruitment of Cul5. *A,* Lysates were prepared from 293T cells expressing HA-Cul2, Cul5-C9, or HA-Cul7 and incubated with recombinant GST or GST-Vif 100-142. GST proteins were recovered with glutathione-Sepharose. Co-precipitating proteins were detected by western blot, and GST proteins were detected by staining with Coomassie Blue. *B,* GST and GST-Vif 100-142 were used in pull-down assays with in vitro translated HA-Cul2, HA-Cul5, or HA-Cul5 cullin repeat (CR) truncations CR1-3 (aa 1-400), CR1-2 (aa 1-274), CR1 (aa 1-158), and Cul5ΔCR1 (deletion of aa 1-158). In vitro translation products were detected by autoradiography and GST proteins were visualized by Coomassie staining. *C,* GST, GST-Vif 100-142, and GST-Vif 100-142 mutants (I120S/A123S/L124S or R121S/K122S) were incubated with

Cul5 expressed in 293T cells (top) or in vitro translation reactions (bottom) and detected by western blot and autoradiography, respectively. *D,* 293T cells were co-transfected with pAPOBEC3G-HA and WT or mutant pCDNA3.A1Vif. Protein levels were determined by western blot. E. WT and mutant Vif-FLAG were expressed with APOBEC3G-HA in 293T cells, subjected to anti-FLAG immunoprecipitation, and detected by western blotting. *F,* Model of the Vif-Cul5-EloBC complex. The CZ-box, composed of the zinc-binding region and BC-box, selectively recruits Cul5 to form an E3 ubiquitin ligase that targets APOBEC3G.

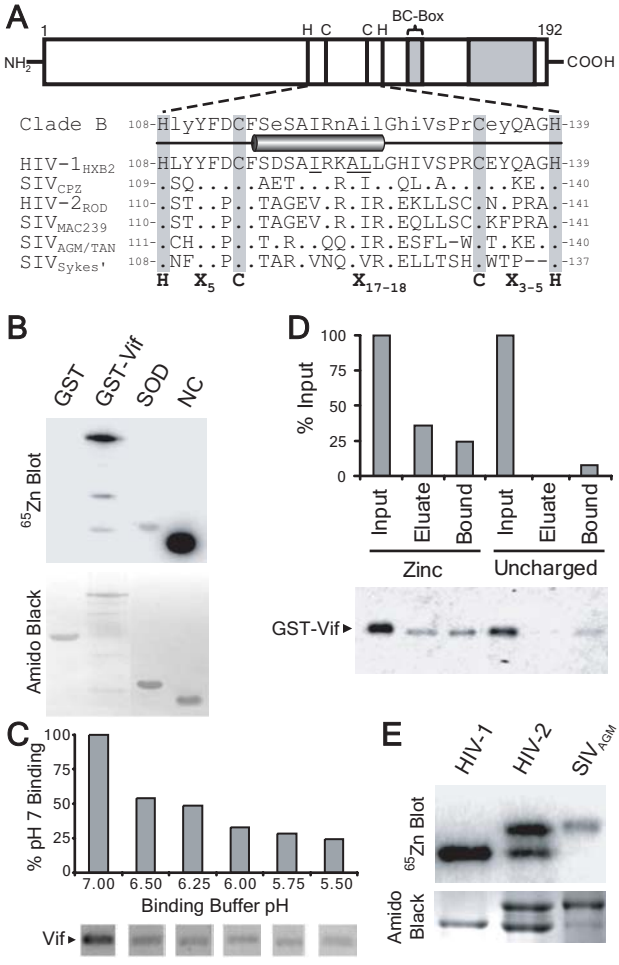

Figure 1

Mehle

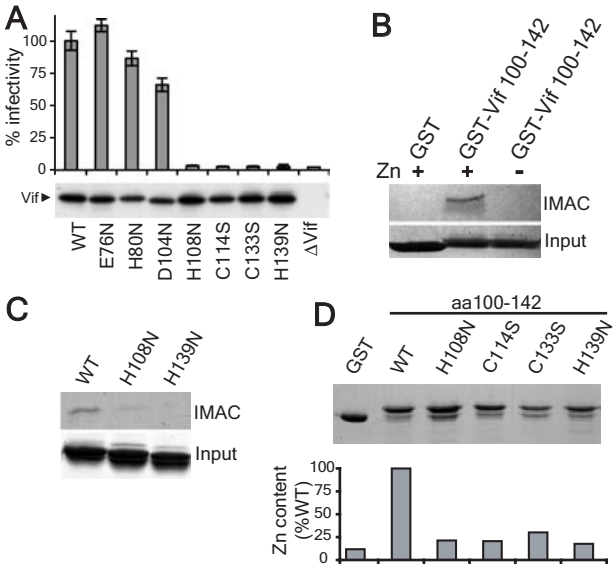

Figure 2

Mehle

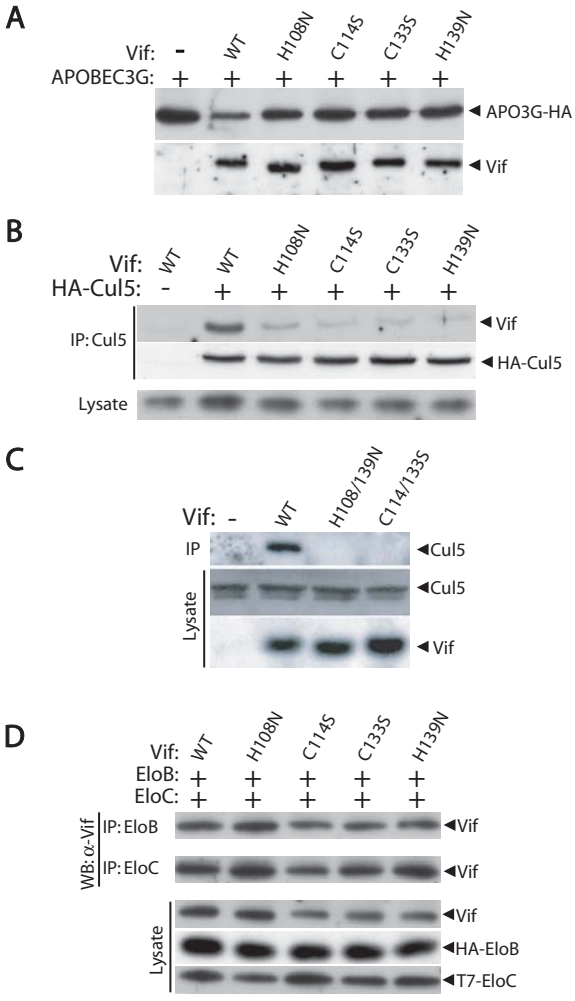

Figure 3

Mehle

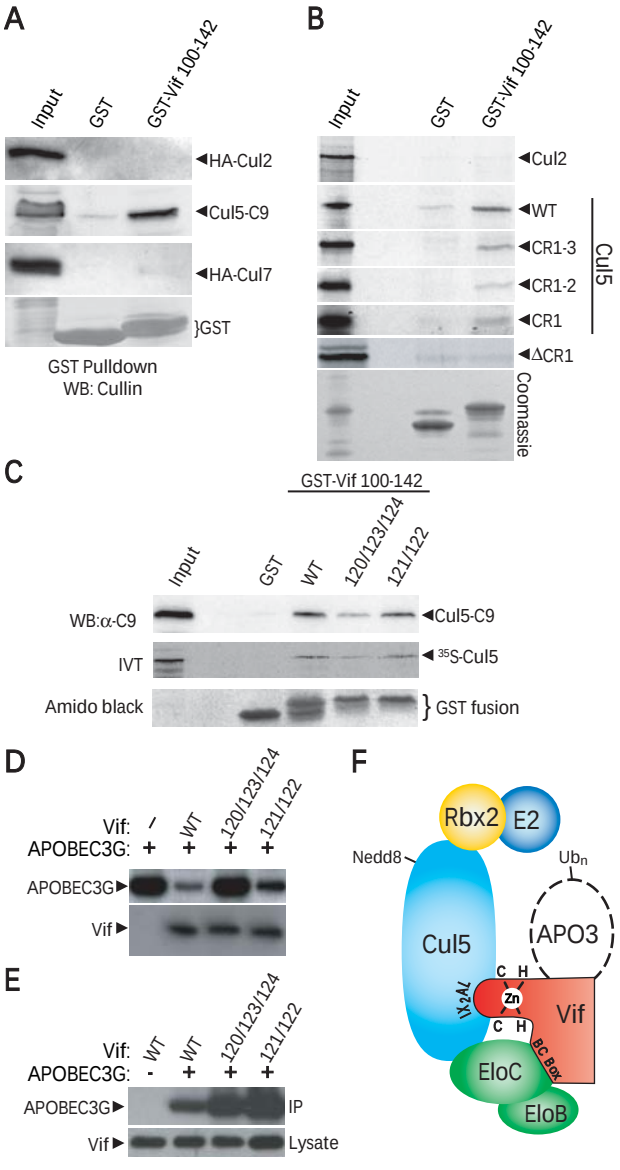

Figure 4

Mehle

A zinc-binding region in Vif binds CUL5 and determines cullin selection

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