



Fig. 1. Alignment of Vif HCCH protein sequences from HIV and SIV subtypes and secondary structural prediction for HIV-1_{HXB2} Vif. Conserved potential metal-binding residues are in bold type. A horizontal line marks the conserved BC box motif. The HCCH sequence is underlined. Secondary structural prediction for the HCCH motif was generated by using the Psi-Pred algorithm (29).

peptide reversibly binds zinc and find that zinc binding generates a conformation capable of forming high-order protein assemblies.

Results and Discussion

The 192-aa HIV-1_{HXB2} Vif sequence is homologous to other lentiviral Vif proteins but has little similarity to other proteins in the National Center for Biotechnology Information nonredundant database. Alignment of Vif sequences from HIV-1, HIV-2, SIV, and subtypes thereof highlights several highly conserved residues at the C terminus (Fig. 1). In particular, a conserved HCCH motif is evident. Mutation of many residues within this motif has been shown to affect Vif function (16). This motif has recently been implicated in the interaction of Vif with the Cul5-based E3 ligase that targets Apo3G for proteasomal destruction (12). Others (17, 18) have suggested that the Cys residues in the HCCH motif are involved in Vif function.

Cys Reactivity in Full-Length Vif. HIV-1_{HXB2} Vif was produced as an N-terminal His-tag fusion protein in *Escherichia coli* and purified under denaturing conditions as described (24), except that Mes buffer (50 mM Mes/200 mM NaCl) was used throughout the purification and dialysis steps (*Materials and Methods*). Purified Vif (27.5 kDa) was boiled in SDS buffer with 200 mM DTT ($E^{\circ} = -330$ mV vs. NHE) and analyzed by gel electrophoresis (Fig. 2A). In addition to the monomeric Vif protein band ($\approx 90\%$ of total band intensity), we noticed the presence of a faint band with an apparent molecular weight of 55 kDa, consistent with dimeric Vif. When Vif was boiled in SDS buffer supplemented with thionin ($E^{\circ} = +60$ mV vs. NHE) and subsequently analyzed by gel electrophoresis, protein bands corresponding to dimer (55 kDa), trimer (82.5 kDa), and tetramer (110 kDa) became more apparent (Fig. 2A). The data suggest that C114 and C133 in Vif are capable of forming inter-

molecular disulfide bonds (there are no other Cys residues in the protein). We also observed a band with increased electrophoretic mobility ($M_r < 16.5$ kDa), but the intensity of this band did not change within the range of redox potential tested (-330 to $+60$ mV vs. NHE), suggesting that it is either a minor contaminant ($\approx 6\%$ of the total protein detected) or a proteolytic N-terminal fragment of Vif that lacks one or both of the Cys residues in the full-length sequence. Because the higher-molecular-weight bands appear to be oxidized Vif species, we estimate that the protein is $\approx 94\%$ pure. Approximately 5% of total Vif remained in a dimeric form under reducing electrophoretic conditions, suggesting that the participating Cys residues are quite reactive.

Most studies to date support the notion that the conserved Cys residues in Vif (C114 and C133) are required for biological function (10, 16–18, 25). However, their exact role in the structure and function of Vif is unclear. We and others (17, 18) have noted the ability of C114 and C133 to form disulfide bonds. Considering the reducing environment of the cytosol (26), and that the oxidized forms of Vif are not detected in virus-infected cells or in virions (17), it seems unlikely that the homooligomeric forms of Vif are biologically relevant. It remains possible that Vif forms cystine-linked heterooligomeric complexes with other viral or host proteins (e.g., Apo3G, nucleocapsid).

Vif Metal-Binding Properties. The conserved His residues of the HCCH motif (H108 and H139) appear to be critical for function, because their mutation to Ala inhibits the Vif–Cul5 interaction (12). The alignment of Vif HCCH motifs (Fig. 1) reveals that the spacing between the conserved His and Cys residues is highly conserved as is the position of several hydrophobic residues. Taken together, the conservation of His/Cys and hydrophobic residues and the spacing between them is reminiscent of the well characterized zinc-finger motif. Zinc fingers are zinc-binding domains that bind metal and fold by an energetically coupled mechanism (27). We postulated that the conserved His and Cys residues in the HCCH motif could be involved in the coordination of a metal ion (e.g., cobalt and zinc) and that metal binding might affect protein structure.

Many zinc-binding proteins bind cobalt to yield complexes that undergo d-d and ligand-to-metal charge transfer transitions in the UV/visible region of the spectrum (28). We initially attempted to detect metal binding to Vif using cobalt as a spectroscopic probe. However, using this approach, we found no evidence of cobalt binding. The cobalt-binding spectroscopic assay is most sensitive when protein ligation alters the geometry of cobalt from octahedral to tetrahedral or square pyramidal. Thus, we cannot rule out the possibility that cobalt binds to Vif without significant perturbation in the ligand geometry.

When purified Vif was mixed with 10 molar equivalents (eq) of zinc, protein precipitation was visually evident after 5 min at room temperature. Light scattering was used to further examine this phenomenon. Zinc-mediated Vif aggregation was rapid and biphasic (Fig. 3A, open circles). An exponential function was fit to the zinc data revealing a fast phase ($k_{\text{obs}} = 0.90 \pm 0.06 \text{ min}^{-1}$) and slow

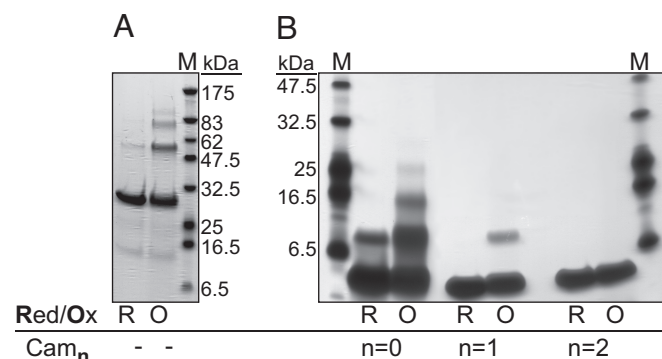


Fig. 2. SDS/PAGE analysis of full-length Vif and HCCHp under reducing and oxidizing conditions. Proteins were purified as described, diluted to a final concentration of $8 \mu\text{M}$ in SDS sample buffer containing 200 mM DTT (Red) or 2 mM thionin (Ox), and boiled for 10 min. Protein was electrophoresed on a 12% polyacrylamide Bis-Tris gel (Invitrogen) for 90 min at 130 V. (A) Vif protein bands were stained for 3 h with colloidal blue (Invitrogen). (B) HCCHp protein bands were silver-stained (46).

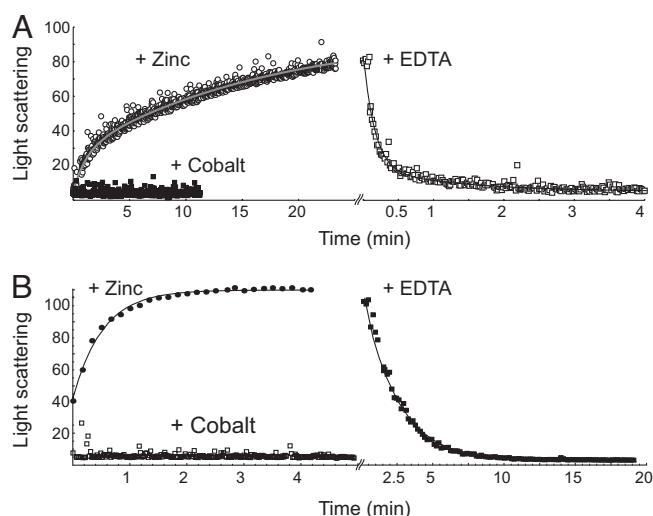


Fig. 3. Zinc- and cobalt-binding kinetics of full-length Vif and HCCHp. The y axis is normalized light scattering intensity. (A, open circles) Full-length Vif (8 μ M) was reacted with 10 eq $\text{Zn}(\text{NO}_3)_2$. Solid line is double-exponential fit to the data ($k_{\text{obs}} = 0.90 \pm 0.06 \text{ min}^{-1}$, $0.060 \pm 0.003 \text{ min}^{-1}$). (Open squares) EDTA (20 eq) was added to the zinc aggregate. Solid line is a single-exponential fit to the data ($k_{\text{obs}} = 4.5 \pm 0.2 \text{ min}^{-1}$). (Closed squares) Vif (8 μ M) was reacted with 10 eq $\text{Co}(\text{NO}_3)_2$. (B, closed circles) HCCHp (20 μ M) was reacted with 1 eq $\text{Zn}(\text{NO}_3)_2$. Solid line is a single-exponential fit to the data ($k_{\text{obs}} = 1.9 \pm 0.1 \text{ min}^{-1}$). (Closed squares) EDTA (2 eq) was added to the zinc aggregate. Solid line is a single-exponential fit to the data ($k_{\text{obs}} = 0.41 \pm 0.01 \text{ min}^{-1}$). (Open squares) HCCHp (20 μ M) was reacted with 10 eq $\text{Co}(\text{NO}_3)_2$.

phase ($k_{\text{obs}} = 0.060 \pm 0.003 \text{ min}^{-1}$) of the aggregation reaction. The origin of the slow phase of aggregation is not clear. It may be the result of protein oligomerization or a conformational change that precedes more rapid aggregation steps. Remarkably, EDTA was found to efficiently reverse aggregation (Fig. 3A, open squares). Analysis of the EDTA kinetic data highlights a single exponential phase ($k_{\text{obs}} = 4.5 \pm 0.2 \text{ min}^{-1}$) of the reaction.

Addition of 10 eq of cobalt did not induce Vif aggregation (Fig. 3A, filled squares) and did not alter the kinetics of zinc-mediated aggregation (not shown). The simplest interpretation of the data is that under our experimental conditions, cobalt and zinc do not compete for the same binding site in Vif. A kinetic barrier or unfavorable thermodynamics may prevent cobalt from binding to Vif. Zinc binding induces the formation of a protein aggregate that is in rapid equilibrium with soluble protein, presumably apoprotein.

Design of a Minimalist HCCH Peptide. Our finding that Vif interacts with zinc is consistent with two recent studies (14, 15) in which zinc and Vif were found to interact. We were concerned, however, that the observed zinc binding by full-length Vif could be due to one or more of the 17 His residues not contained in the HCCH motif (in addition to the 6x His tag, HIV-1_{HXB2} Vif contains 11 His residues outside the HCCH motif). In such a scenario, the HCCH motif could play an indirect structural role in metal binding. To reduce the number of potential metal-binding ligands and to address these issues, a peptide fragment encompassing the HCCH motif of HIV-1_{HXB2} Vif was constructed. The peptide sequence boundaries were chosen based on a secondary structural prediction (29) of Vif (Fig. 1). Based on this prediction, the HCCH motif is located within an α -helix forming region of the Vif sequence. A peptide fragment [hereafter referred to as HCCHp (synthetic peptide acid corresponding to amino acids 101–142 of HIV-1_{HXB2} Vif)] was chosen to include the entire α -helix-forming sequence and the conserved Cys and His residues (Fig. 1, underlined sequence). The 42-aa

HCCHp peptide was synthesized as the free acid by Fmoc-based solid-phase peptide synthesis, obtained in crude-powder form, and purified as described in *Materials and Methods*.

Cysteine Reactivity in HCCHp. HCCHp was $\approx 95\%$ pure as judged by analytical C18 HPLC. The peptide sequence was verified by MALDI-TOF/MS. However, analysis by SDS/PAGE under reducing conditions revealed two bands (Fig. 2B, $n = 0$). Under oxidizing conditions the number of bands increased to at least four. As further support of their direct role in the oligomerization process, modification of both HCCHp cysteines with the carboxyamidomethyl moiety (Cam) group inhibited oligomerization under reducing and oxidizing conditions (Fig. 2B, $n = 2$). Using mild alkylation conditions HCCHp-Cam was prepared and chromatographically separated from HCCHp modified at C114 and C133 with Cam [HCCHp-(Cam)₂] and unreacted HCCHp (*Materials and Methods*). HCCHp-Cam was capable of forming dimers (Fig. 2B, $n = 1$) but did not form the higher-order (trimeric or tetrameric) species formed by unmodified HCCHp. Thus, under oxidizing conditions, HCCHp molecules become covalently crosslinked by disulfide bonds between Cys residues in neighboring polypeptides. Both Cys residues in HCCHp (and full-length Vif) are capable of forming intermolecular disulfide bonds.

We observed a buildup of HCCHp-Cam under mild alkylation conditions that may be due to the relative accessibility or reactivity of the Cys residues in HCCHp. According to the secondary structural prediction in Fig. 1, C133 is located in a random coil region whereas C114 is part of an α helix. The site of Cys modification in HCCHp-Cam was determined by digesting the protein with trypsin and analyzing the peptides by MALDI/TOF-MS (*Materials and Methods*). When HCCHp-Cam was trypsin digested, two of the resulting peptides were found to increase in mass by 57 atomic mass units, corresponding to the Cam moiety: H_3N^+ -ALLGHIVSPRC₁₃₃(Cam)EYQAGHNKV-CO₂[−] (2,249.0 Da observed, 2,249.1 Da expected), and H_3N^+ -C₁₃₃(Cam)EYQAGHNKV-CO₂[−] (1,205.7 Da observed, 1,205.6 Da expected). Unmodified C114-containing peptides H_3N^+ -ELADQLIHLYYFDC₁₁₄FSDSAIRK-CO₂[−] (2,648.1 Da observed, 2,647.3 Da expected) and H_3N^+ -ELADQLIHLYYFDC₁₁₄FSDSAIRK-CO₂[−] (2,520.0 Da observed, 2,519.2 Da expected) were also detected in the HCCHp-Cam sample. Based on these results, we conclude that C133 is the site of Cam modification in HCCHp-Cam. C114 may be less reactive toward iodoacetamide due to its engagement in hydrogen-bonding or hydrophobic interactions within the predicted α helix or with other structural elements in the protein.

HCCHp Metal-Binding Properties. Because of the simplicity of HCCHp compared with full-length Vif, we expected that metal binding would occur in a stoichiometric fashion. Unexpectedly, upon addition of 1 eq of zinc to HCCHp, insoluble protein aggregates appeared as evidenced by light-scattering measurements (Fig. 3B, closed circles). Zinc induced the formation of HCCHp aggregates, at a rate ($k_{\text{obs}} = 1.9 \pm 0.1 \text{ min}^{-1}$) comparable to that for the fast phase of zinc-mediated full-length Vif aggregation ($k_{\text{obs}} = 0.90 \pm 0.06 \text{ min}^{-1}$). HCCHp-Zn aggregates were efficiently solubilized with EDTA (Fig. 3B, closed squares) at a rate ($k_{\text{obs}} = 0.41 \pm 0.01 \text{ min}^{-1}$) slower than that for full-length Vif ($k_{\text{obs}} = 4.5 \pm 0.2 \text{ min}^{-1}$). This difference may be due to the large number of potential metal-binding sites and possible cooperative effects in full-length Vif. This is supported by the fact that HCCHp aggregation was induced with stoichiometric zinc, whereas full-length Vif aggregation required excess (10 eq) zinc. That HCCHp and full-length Vif aggregate in the presence of zinc strongly supports the conclusion that a Zn-HCCH complex is a building block for the construction of larger metalloprotein aggregates.

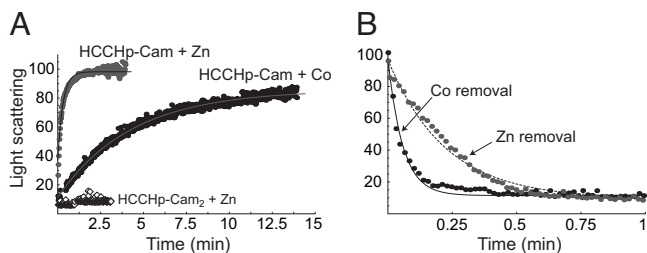


Fig. 4. Metal-binding and EDTA chelation kinetics of HCCp-Cam and HCCHp-(Cam)₂. The y axis is normalized light-scattering intensity. (A) Protein (20 μM) was mixed with 1 eq Zn(NO₃)₂ or Co(NO₃)₂, and light scattering was measured. Solid lines are single-exponential fits to the data. Best-fit aggregation rates: HCCHp-Cam + Zn ($k_{\text{obs}} = 3.3 \pm 0.1 \text{ min}^{-1}$); HCCHp-Cam + Co ($k_{\text{obs}} = 0.23 \pm 0.01 \text{ min}^{-1}$). (B) Metal aggregates were reacted with 2 eq EDTA. Solid lines are single-exponential fits to the data. Best-fit EDTA chelation rates: HCCHp-Cam-Zn + EDTA ($k_{\text{obs}} = 4.5 \pm 0.1 \text{ min}^{-1}$); HCCHp-Cam-Co + EDTA ($k_{\text{obs}} = 18 \pm 1 \text{ min}^{-1}$).

HCChp did not aggregate in the presence of up to 10 eq of cobalt (Fig. 3B, open squares). However, when the length of time that light scattering was measured was increased from 20 min to 5.5 h, we did observe very slow aggregation induced by cobalt (Fig. 6, which is published as supporting information on the PNAS web site). The rate of cobalt induced HCChp aggregation ($k_{\text{obs}} = 0.013 \text{ min}^{-1}$) was 2 orders of magnitude slower than that of zinc induced HCChp aggregation ($k_{\text{obs}} = 1.9 \text{ min}^{-1}$). EDTA resolubilized cobalt-aggregates at a rate ($k_{\text{obs}} = 0.23 \text{ min}^{-1}$) close to that for zinc-aggregates ($k_{\text{obs}} = 0.41 \text{ min}^{-1}$).

Because the rate of cobalt-induced HCChp aggregation was much slower than that for zinc-induced aggregation, we were able to analyze the cobalt-bound form in solution by UV/vis optical spectroscopy (Fig. 7, which is published as supporting information on the PNAS web site). The spectrum exhibits a ligand-to-metal charge transfer (LMCT) absorbance at 293 nm, consistent with thiol coordination. The visible region of the Co^{2+} spectrum exhibits a maximal absorbance at 627 nm, indicative of S_2N_2 ligand coordination (30). The extinction coefficient of this transition ($240 \text{ M}^{-1}\text{cm}^{-1}$) is suggestive of tetrahedral coordination (31). Because of the complex nature of the system (involving binding and subsequent aggregation) further experiments are required to accurately determine the extinction coefficients for the Co^{2+} d-d and LMCT transitions. Our results support the conclusion that the geometry of the cobalt binding site in HCChp is tetrahedral and contains Cys (S) ligands.

Role of C114 and C133 in Metal Binding. To test whether the conserved Cys residues of HCCHp are required for metal binding, the metal-binding activities of HCCHp-Cam and HCCHp-(Cam)₂ were examined by using the light-scattering assay. HCCHp-(Cam)₂ did not aggregate in the presence of 1 eq of zinc (Fig. 4A). However, HCCHp-Cam rapidly bound zinc and formed protein aggregates (Fig. 4A) that were resolubilized with EDTA (Fig. 4B). HCCHp-Cam bound zinc 1.7 times faster than HCCHp and released zinc to EDTA 10 times faster than HCCHp. Assuming a simplistic model for zinc-induced HCCHp aggregation [$\text{HCCHp} + \text{Zn}^{2+} \rightleftharpoons (\text{HCCHp-Zn})_{\text{aggregate}}$], the apparent affinity of zinc for HCCHp-Cam is reduced by ≈ 6 -fold, corresponding to a modest destabilization of the HCCHp-Cam-Zn complex by +1 kcal/mol relative to the HCCHp-Zn complex.

HCCHp-Cam must bind metal by a mechanism distinct from that of HCCHp. One possibility involves zinc coordination by the nonconserved His residue at position 127 (Glu in the homologs shown in Fig. 1). The existence of alternate zinc-binding modes in a zinc-finger motif of neural zinc-finger factor-1 (NZF-1) has been demonstrated (32). Although structural data (33) suggest

NZF-1 coordinates zinc with three Cys residues and the second His residue of its conserved CCHHC metal-binding domain, point mutation of either His residue does not significantly effect metal-binding affinity (32). The altered zinc-binding and release kinetics of HCCHp-Cam may also reflect a mode of metal binding in which the fourth metal ligand is supplied by hydroxide/water. Metal ion coordination by exogenous ligands has been observed for other zinc-finger peptides (34, 35). If zinc is coordinated by three protein ligands and one solvent-derived hydroxide, higher solvent accessibility of the HCCHp-Cam metal-binding pocket could explain the 10-fold increase in the rate of zinc chelation.

We further tested HCCHp-Cam for its ability to bind cobalt. The rates of cobalt and zinc-induced HCCHp-Cam aggregation differed by 14-fold, with the rate for cobalt being slower (Fig. 4A). Upon addition of EDTA, soluble HCCHp-Cam was rapidly formed ($k_{\text{obs}} = 18 \pm 1 \text{ min}^{-1}$; Fig. 4B). It is estimated that cobalt binding to HCCHp-Cam is ≈ 60 times weaker ($+2.4 \text{ kcal/mol}$) compared with zinc binding. The fact that alkylation of C133 lowers metal binding specificity and affinity is likely the result of changes in coordination number, geometry, and possibly protein conformation.

HCCHp Secondary Structural Properties. The zinc-binding domains of zinc finger proteins undergo thermodynamically coupled metal-binding and protein-folding reactions in the presence of zinc or cobalt (36). Metal ion dehydration and favorable covalent and noncovalent interactions accompany metal binding and are thought to drive the entropically unfavorable process of achieving the zinc-finger fold. Far-UV CD spectropolarimetry was used to determine whether metal binding to HCCHp induces changes in protein secondary structure.

The CD spectrum of HCCHp (Fig. 5A, dark circles) resembles that for an α helical protein, having ellipticity minima at ≈ 208 and 222 nm (37) and is consistent with the secondary structural prediction of HCCHp shown in Fig. 1. Upon addition of 1 eq of zinc to HCCHp, a different CD signal appeared (Fig. 5A, light circles), with a minimum ellipticity centered at 219 nm. The observed CD signal is consistent with the presence of β sheet structure. These results indicate that zinc binding induces the formation of an alternate conformation of HCCHp with β sheet character. Remarkably, addition of 2 eq of EDTA regenerated the α helical signature of apo-HCCHp (Fig. 5A, squares). This provides evidence that the metal-bound conformation of HCCHp is in equilibrium with apo-HCCHp.

Protein nucleation is an energetically unfavorable process. However, calculations based on nucleation theory (38, 39) suggest that destabilization of a protein fold by as little as 1–2 kcal/mol can increase the probability of aggregate nucleation by as much as 10^5 fold. We propose that zinc binding destabilizes the HCCHp structure and generates a β sheet conformation that is the nucleating structure for molecular aggregation.

Role of C114 and C133 in HCCHp Structure. The average secondary structure of HCCHp resembles that of an α -helical protein. Zinc binding to HCCHp leads to β -sheet formation (Fig. 5A) and protein aggregation (Fig. 3B). HCCHp-Cam and HCCHp-(Cam)₂ were analyzed by CD spectroscopy to determine whether the Cys residues play a part in stabilizing the α -helical structure observed for HCCHp. HCCHp-Cam was found to have an α -helical CD signature similar to that of HCCHp (Fig. 5B) suggesting that C133 does not significantly contribute to the stabilization of the α -helical conformation of HCCHp. In contrast, the CD spectrum of HCCHp-(Cam)₂ was dramatically altered (Fig. 5B) indicating that C114 plays a critical role in the stabilization of the HCCHp protein fold. As indicated by our light-scattering assays, Vif, HCCHp, and HCCHp-Cam bind metal to form metal-protein aggregates. The conformation of HCCHp-(Cam)₂ was insensitive to added zinc (not

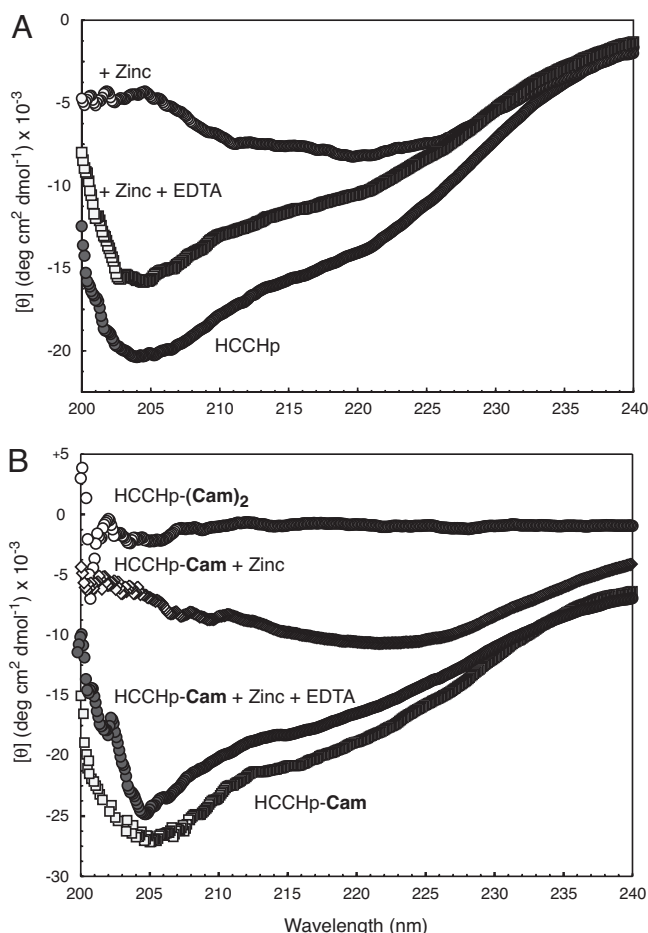


Fig. 5. Far-UV CD spectra for HCCHp, HCCHp-Cam, and HCCHp-(Cam)₂. (A) Spectra are shown for HCCHp in the absence of metal (filled circles), after addition of 1 eq of ZnCl₂ (open circles), and after EDTA treatment (open squares). (B) Spectra are shown for HCCHp-Cam in the absence of metal (open squares), after addition of 1 eq of ZnCl₂ (open diamonds), and after EDTA treatment (filled circles). A value of 5 mdeg cm²·dmol^{−1} was subtracted from latter two data sets for clarity. The CD spectrum of HCCHp-(Cam)₂ is represented by open circles.

shown), but addition of zinc to HCCHp-Cam generated a β -sheet conformation similar to that observed for HCCHp (Fig. 5B). Coordination of zinc by C114 would lead to thiol deprotonation and could destabilize the α -helical structure of HCCHp and HCCHp-Cam by disrupting a hydrogen-bond network. Treatment of the HCCHp-Cam-Zn aggregates with EDTA regenerated the apo-HCCHp-Cam α -helical signature (Fig. 5B). The data are consistent with a model in which zinc binding requires C114 and induces the formation of a β -sheet protein conformation that appears to be a precursor to larger metalloprotein aggregates.

Models of Vif/HCCHp Oligomerization. We find that the Cys residues (C114 and C133) of recombinant Vif (Fig. 2A) and HCCHp (Fig. 2B) are capable of forming intermolecular disulfide bonds. The $n > 2$ oligomeric species result from protein stacks that are covalently crosslinked by cystine bonds. Two Cys residues per polypeptide are required to form these high-order species. Consistent with this model, HCCHp-Cam (which is selectively modified at C133) cannot form the high-order species but is capable of dimerizing, and HCCHp-(Cam)₂ is monomeric under reducing and oxidizing conditions (Fig. 2B).

Zinc binding leads to protein oligomerization and aggregation. If the mechanism of aggregation involves seeding by a single

HCCHp-Zn complex, we would expect that the metal-protein template for aggregation would not be solvent accessible due to crowding by neighboring polypeptides. However, because EDTA-metal chelation is rapid and efficiently reverses aggregation, we favor a model in which aggregates are formed by metal-bound polypeptide monomers. Based on this model, aggregate breakdown at the solid-solution interface would occur readily in the presence of a metal chelator such as EDTA. The proposed metal-mediated polymerization of Vif may be due to bridging of monomers in a fashion similar to that envisioned for cystine-mediated oligomerization. In this model, each metal ion is coordinated by residue side chains from two (or more) polypeptides. Alternatively, the aggregate may be formed from noncovalent forces between stoichiometric HCCHp-Zn complexes. Each metal ion would be tetrahedrally coordinated by four ligands (e.g., H108, C114, C133, and H139). Further studies to elucidate the mode(s) of zinc interaction with Vif are underway.

Cullins act as molecular scaffolds to assemble a functionally diverse array of CRL complexes (40). Viral “hijacking” of CRLs represents a common mechanism of immune evasion by which specific cellular proteins are targeted for destruction. Although there are many examples of viral proteins that redirect CRL activity (13), very few involve the Cul5-based CRL. The only other documented case of Cul5 hijacking involves the human papilloma virus oncoprotein E6 (E4orf6; refs. 41 and 42). E4orf6 acts in concert with the protein E6-AP to target and degrade p53. E4orf6 contains two copies of the unusual Cys-rich motifs (C-X₂-C-X₂₉-C-X₂-C) in its N and C termini. The recent solution structure of the C-terminal domain of E4orf6 (43) reveals that this motif binds zinc to stabilize a unique α/β fold that exposes several clusters of conserved residues to the solvent.

Although further experiments are required to understand the effect of metal binding on Vif function, we propose that zinc binding alters the native protein conformation to expose a protein-protein interaction domain. The resulting conformation displays surface side-chain residues that confer binding specificity for target proteins (e.g., Cul5). Because of the importance of CRL involvement in virus-host interactions, identification and biochemical dissection of viral modulators of CRL activity is expected to yield insights into cellular processes that are controlled by ubiquitination.

Materials and Methods

Protein Purification. Unless stated otherwise, all chemicals were obtained in high purity from Sigma/Aldrich, St. Louis, MO. Synthetic HCCHp (Fig. 1, underlined sequence) was obtained from SynPep (Dublin, CA) in crude powder form, and resolubilized for 10 min at 50°C in 20% acetonitrile/0.1% TFA/1 mM Tris-carboxyethyl phosphine (TCEP). A nonlinear gradient (Waters gradient curve 5) from 32–42% acetonitrile (supplemented with 0.1% TFA) eluted the peptide from a C18 reversed-phase column. HCCHp was immediately transferred into an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and concentrated under vacuum (Thermo Savant). The peptide was found to be >95% pure by analytical C18-HPLC and gel electrophoresis. Peptide identity was confirmed by MALDI/TOF MS on an Applied Biosystems (Foster City, CA) Voyager-DE STR Biospectrometry Workstation. Peptides were mixed 1:1 (vol/vol) with a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA, applied to a stainless steel sample plate (Applied Biosystems) and allowed to dry. Analysis in linear mode gave m/z value of 4,821 Da (expected 4,822 Da). The doubly charged ion ($m/z = 2,412$ Da) was also observed. HCCHp was titrated to pH 7.4 in 10 mM 4-morpholinepropanesulfonic acid (Mops) and stored at -70°C in aliquots. Peptide concentrations were determined in 30 mM Mops, pH 7.0/6 M guanidine hydrochloride, as described (44).

The 576-bp *vif* gene was amplified from pNL-A1 (provided by Klaus Strebel, National Institute of Allergy and Infectious Diseases,

National Institutes of Health) and cloned into a pET28a+ derivative (Novagen, San Diego, CA). *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA) were transformed and grown in LB media (Qbiogene, Irvine, CA) with 1% glucose/50 μ g/ml kanamycin at 37°C. When the OD₆₀₀ reached 0.6, expression was induced with 1 mM isopropyl β -D-thiogalactoside for 5 h. Subsequent steps of Vif purification were performed as described (24), except that 50 mM Mes/200 mM NaCl was used during all purification and dialysis steps, and the final pH of protein solutions was adjusted to 6.2. Protein samples were stored at -70°C in aliquots. Protein concentration was determined by the methods of Bradford (45) and Edelhoch (44) and gave similar results.

Carboxyamidomethyl Modification of Cys residues in HCCHp. Modification of both Cys residues was achieved by incubating purified HCCHp with 15 eq of TCEP and 150 eq of iodoacetamide in 200 mM Mops/80 mM NaCl, pH 7.8 at 30°C for 16 h in an anaerobic chamber. Under mild conditions, HCCHp was reacted with 1.5 eq iodoacetamide for 1 h at room temperature in 200 mM Mops/80 mM NaCl, pH 7.4. The reaction products, HCCHp-Cam and HCCHp-(Cam)₂, were purified by HPLC and verified by MALDI-TOF MS.

Tryptic Digestion of HCCHp-Cam. HCCHp-Cam (1 μ g) was digested with trypsin (40 ng) (Promega, Madison, WI) at 30°C in 20 μ l 50 mM Tris-HCl, pH 8.0/50 mM DTT/5 mM EDTA/10 mM CaCl₂ for 24 h. The pH of the reaction was reduced to 4 with HCl, and 0.1% TFA was added. The tryptic digest was then drawn through a C18 ZipTip (Millipore, Billerica, MA) and washed extensively with 0.1% TFA. Peptides were eluted with 75% acetonitrile 0.1% TFA, spotted onto a stainless steel sample plate, and analyzed by MALDI/TOF MS.

Metal-Binding and Light-Scattering Assays. HCCHp and Vif sample preparation was carried out inside an anaerobic glove box (Coy Laboratory Products) operated under an atmosphere of 95% N₂ and 5% H₂. Standard zinc and cobalt solutions were purchased from Sigma. Metal binding to Vif was carried out in 50 mM Mes,

pH 6.2/200 mM NaCl/1.8 mM TCEP. HCCHp samples were prepared in 200 mM Mops, pH 7.4/80 mM NaCl/1.8 mM TCEP. The assay components were mixed in a 2.5-ml quartz cuvette with a 1-cm pathlength and a flea stir bar. A 1- to 2- μ l bead of the zinc or cobalt metal ion solution was placed on the bottom of the Teflon cap, which was inverted and seated into the top of the cuvette. Light scattering was measured by using an ISS PC1 single-photon counting fluorometer (ISS, Champaign, IL) with $\lambda_{\text{Ex}} = 400$ nm, $\lambda_{\text{Em}} = 398$ nm, and excitation/emission band pass = 4 nm/4 nm. The sample was stirred in the cell holder and scanned for 1–2 min before mixing with the metal ion solution. After protein aggregation was complete (judged by a plateau in the light-scattering signal), the sample was transferred back into the glove box where EDTA was carefully added to the cuvette cap. The cuvette was placed back into the fluorometer where light scattering was measured for 1–2 min before EDTA mixing.

Fitting Details. Aggregation and resolubilization kinetics were analyzed by fitting exponential functions to the raw data (intensity vs. time) by using Mathematica software (Wolfram Research, Champaign, IL).

CD. HCCHp, HCCHp-Cam, and HCCHp-(Cam)₂ samples were prepared anaerobically in 10 mM Mops, pH 7.4/20 mM NaCl/40 μ M TCEP at a final concentration of 15 μ M. CD spectra were measured in a Jasco J-810 spectropolarimeter (Jasco, Easton, MD). Samples were placed in rectangular quartz cuvettes of 2-mm path length and maintained at 20°C by using a NESLAB circulating water bath (Thermo Electron, Waltham, MA). For each measurement, three spectra were acquired at 100 nm/min with a time constant of 2 s.

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