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A Metallopeptide Assembly of the HIV-1 gp41 Coiled Coil Is an Ideal Receptor in Fluorescence Detection of Ligand Binding**

Miriam Gochin,* Rodney Kiplin Guy, and Martin A. Case

The transmembrane subunit, gp41, of the envelope glycoprotein of HIV-1 plays a crucial role in the fusion of the virus to the target cell during infection.[1–3] During the conformational changes that accompany virus–cell binding, the coiled-coil domain of three HR1 helices of trimeric gp41 becomes exposed and vulnerable to attack by antiviral drugs that could prevent fusion and infection of cells.[4] To date, the only successful drug against fusion is the peptide T-20 (DP178), derived from the HR2 region of gp41.[5–8] T-20 binds tightly to the HR1 coiled coil, thus interfering with formation of the fusion-competent six-helix bundle structure of gp41, comprising three HR1 and three HR2 helices. There is considerable interest in the discovery and development of nonproteolytic alternatives to T-20, which is very expensive to produce and not orally bioavailable.

A key challenge to screening compound libraries for drugs that target the three stranded coiled coil of gp41 arises from the fact that it is unstable when excised from full-length gp41 and buried in intact gp41. Peptides from gp41 HR1 are hydrophobic and tend to aggregate in solution. Previous approaches for accessing the coiled coil domain have involved stabilizing it as part of a larger soluble protein construct. Prior work has included the addition of GCN4 trimeric helices which extend the coiled coil by several heptad repeats (IQN17),[9] the preparation of 5-helix, a gp41 protein lacking one HR2 domain,[10] and the use of mutant 6-helix protein in which the HR1–HR2 interaction is destabilized, thus allowing for transient exposure of the HR1 coiled coil.[11] We report herein on the design of a stable fragment of the HR1 coiled-coil domain that does not involve extra protein domains, and that can be used in a fluorescence assay for rapid screening of potential fusion inhibitors.

The basis of the design is depicted in Figure 1. A metal-ion-binding bidentate ligand, 2,2′-bipyridyl (bpy) is attached to the N-terminus of a 31 residue peptide that contains residues 565–584 of gp41. The region selected contains a prominent cavity within the grooves of the coiled coil.[12] Addition of a metal ion such as Fe^{II} or Ni^{II} should result in formation of a tris-bipyridyl metal complex, which stabilizes the coiled-coil structure, as has been shown previously for several designed peptides.[13–18]
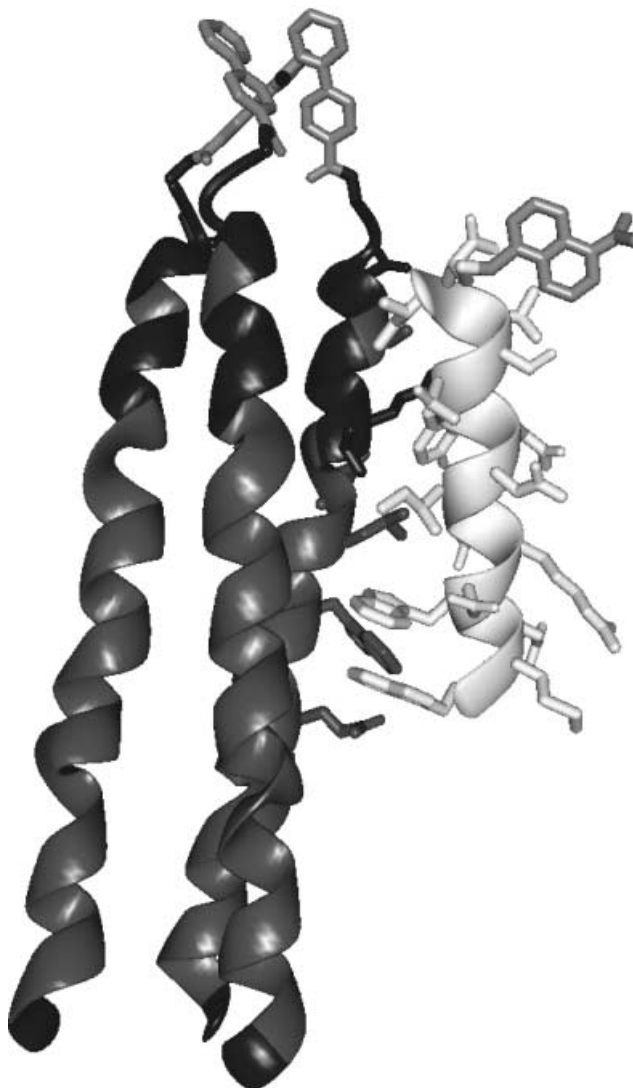

Figure 1. Model of the expected Fe-env2.1—dansylated C-peptide interaction, derived by homology modeling from pdb2ez0. Residues of Fe—env2.1 corresponding to the native sequence of the gp41 coiled coil are shown in gray, and all other coiled coil residues in black. Iron(II) is shown in black, coordinated to the bipyridine groups in light gray. The C-peptide residues are off-white, with the dansyl group shown in light gray attached to the cysteine side chain in an arbitrary conformation. For clarity, side chains of coiled coil residues not in direct contact with the C-peptide are omitted. The distance between iron(II) and the dansyl group is estimated from the model to be about 15 Å.

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The Fe^{II}–bipyridyl complex is magenta, which corresponding to an absorbance maximum at 545 nm. This absorbance agrees well with the emission maximum of a dansyl fluorophore, which absorbs at 340 nm and emits at 542 nm (for the free probe). Thus, quenching of the dansyl fluorescence by fluorescent resonance energy transfer (FRET)[19] should occur if it is brought within 30 Å of the Fe^{II}–bipyridyl center. This is accomplished by using a C-peptide from the HR2 region of gp41, labeled with a dansyl fluorophore at a C-terminal cysteine (Figure 1). The C-peptide is expected to bind to the grooves of a properly folded coiled coil, thus inducing fluorescence quenching.

UV, Circular Dichroism, NMR and fluorescence spectroscopy have been used to characterize this system and demonstrate the effectiveness of the design. The peptide shown in Figure 1 has the sequence bpy–GQA *V*QA*L*EKL *L*QL*T*VWG *I*KQ*L*QAR *I*LA*V*EK-amide. It was prepared according to previously published procedures.[20] Residues native to gp41 are underlined, and positions a and d of the heptad repeat are highlighted in bold. In the first heptad repeat, the native gp41 sequence was substituted with a sequence designed to stabilize three-helix bundle formation.[21] The additional residues bpy–GQA at the N-terminal end were designed to fulfill structural requirements for metal–bpy ligation (unpublished results). This peptide is referred to as env2.1. Metal ion binding was readily observed in the UV spectrum with a red shift of the 291 nm π–π* band and a metal–ligand charge transfer band at 314 nm for Ni^{II} and 545 nm for Fe^{II} centers. The secondary structure was elucidated by measuring the mean residue ellipticity in the CD spectrum at 222 nm. A relatively high intrinsic helical content of 76% was obtained for the apo-peptide and it increased to 87% and 90% upon addition of Fe^{II} and Ni^{II} ions, respectively.

The formation of discrete trimeric structure was assessed by NMR spectroscopy. Shown in Figure 2 are aromatic regions of the NMR spectra of env2.1 with and without Ni^{II} ions. Line broadening of the Trp and bipyridyl resonances in the apo-peptide is indicative of nonspecific hydrophobic aggregation. In the presence of paramagnetic Ni^{II} ions, which shift the aromatic bpy resonances far downfield,[18] the five Trp resonances sharpen significantly, thus demonstrating the alleviation of aggregation. NMR diffusion experiments conducted on Ni^{II} ion-bound env2.1 confirmed the trimeric structure. The molecular weight estimated from diffusion lies within 12% of the expected molecular weight of 11.08 kDa for the trimer. Thus the metal-bound form of this peptide should be able to present the binding surfaces of the hydrophobic coiled coil required for binding HR2 peptides or alternative drug candidates. This is a critical test for success of the design in creating a properly folded coiled coil.

The ability of the designed coiled coil to bind to known target peptides was examined by using CD and NMR spectroscopy. Target peptides included a 16-residue flanking peptide from the C-terminal heptad repeat of gp41 and a previously designed D-peptide, known as D10-p1–2K,[22] which binds to the hydrophobic pocket. The C-peptide has the sequence Ac-WMEWDRKIEEYTKKIC. It is altered from the native sequence Ac-WMEWDREINNYTSLIH according to a
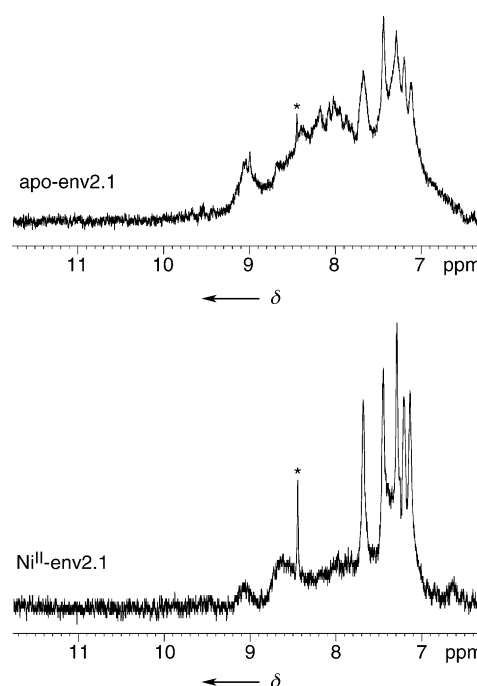

Figure 2. Aromatic region of the ^{1}H NMR spectra of 200 μM env2.1, free (top) and in the presence of 67 μM NiCl$_2$ (bottom). Spectra were taken at 500 MHz and 25 °C in 25 mM deuterated Tris/acetate buffer at pH 6. A spurious signal is indicated with an asterisk.

design suggested by Otaka et al.,[23] in which amino acids nonessential to the HR1–HR2 binding interaction are mutated to permit $i, i + 3$ Lys-Glu salt bridges. These mutations are intended to stabilize the helical conformation and improve the solubility of the hydrophobic C-peptide. The C-terminal cysteine was introduced for dansyl-aziridine labeling.[25] The important binding interactions are hydrophobic contacts involving Trp 1, Trp 4 and Ile 8 in the hydrophobic pocket, as well as a salt bridge between Asp 5 and Lys 574 of the coiled coil.

Figure 3 shows the results of CD experiments to test for binding between the 16 residue C-peptide and env2.1. The short C-peptide has no apparent helicity, despite the presence of three putative salt bridges. In the absence of a metal ion, the spectrum of an equimolar mixture of C-peptide and env2.1 is indistinguishable from the sum of the spectra of the two individual components, thus indicating that no interaction can be detected. This correlates with the result from NMR spectroscopy, in which aggregation of apo-env2.1 was observed. Aggregation is expected to interfere with formation of the correct helical fold or obscure the binding site. In the presence of Fe^{II} ions, however, and in accordance with the NMR result, clear evidence of binding is obtained. The spectrum of the mixture of C-peptide and Fe-bound env2.1 differs from the sum of component spectra. There is a concomitant increase in the overall helicity of the system, presumably due to the increased helicity of the C-peptide upon binding.

The binding of the D-peptide D10-p1-2K to env2.1 was followed by NMR and compared to the results obtained with IQN17.[22] D10-p1-2K has the sequence
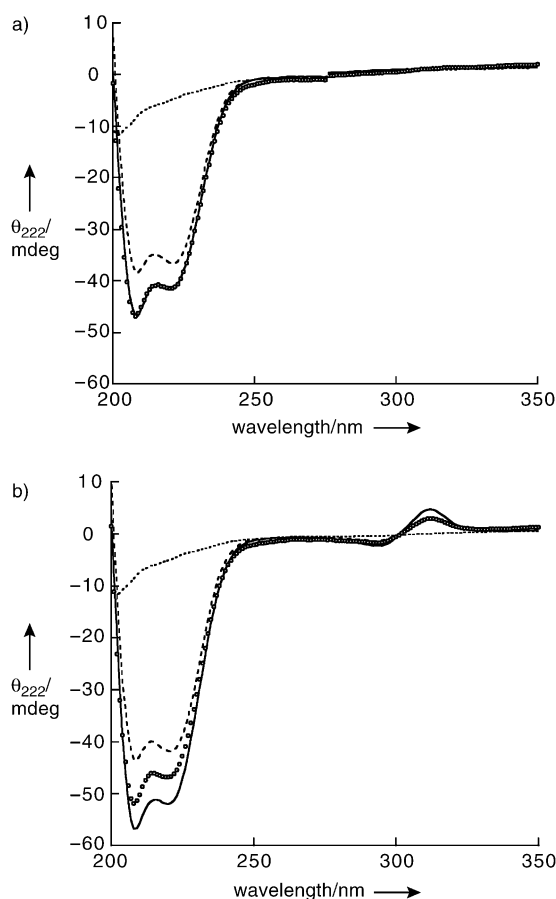
Figure 3. CD spectra of env2.1 (----) and C-peptide (•••••) alone and combined in an equimolar ratio (——). Also shown is the sum of the env2.1 and C-peptide spectra (○).a) Results for apo-env2.1; b) results for Fe^{II}—bound env2.1.

Figure 4. 500 MHz aromatic ^1H NMR spectra of: (top) 0.8 mM Ni^{II}-bound env2.1; (middle) 0.5 mM D10-p1–2 K; (bottom) a mixture of 0.8 mM Ni^{II}-bound env2.1 and 0.4 mM D10-p1–2 K. A spurious peak is indicated with an asterisk.

groups interacting in the hydrophobic pocket and not along the first heptad repeat of the coiled coil (Figure 1).

Figure 5b demonstrates a competitive inhibition assay with this system. Titration of unlabeled C-peptide was followed by measuring the fluorescence intensity in solutions containing 100 μM dansylated C-peptide and 5 μM Fe^{II}-env2.1. Recovery of the dansyl fluorescence at high C-peptide concentrations was observed, with an IC_{50} value of 273 ± 35 μM. Most of the difference between IC_{50} and K_d can be accounted for by the effect of substrate concentration on IC_{50}

Ac-KKGACEARHREWAWLCAA-NH_2 with all D-amino acids. Binding between D10-p1-2K and env2.1 is characterized by line broadening and upfield shifting of Trp resonances (Figure 4), as was described previously with IQN17. The shifts were attributed to ring-current effects of the D-peptide on the resonances of Trp571 in the hydrophobic pocket. There is additional upfield shifting of a methyl group, possibly of Ile 573 by the same mechanism (data not shown).

The ability of metal-bound env2.1 to recognize and bind the C- and D-peptides confirms the integrity of the construct. We then evaluated FRET as a means of detection of peptide binding. Figure 5a shows the observed fluorescence intensity of dansylated C-peptide as a function of Fe^{II}-env2.1 concentration. A 3.5-fold reduction in fluorescence intensity was observed as a result of peptide–coiled-coil binding. The curve was fit to a standard binding isotherm with a dissociation constant, K_d, of 0.40 ± 0.09 μM. It should be noted here that an alternative coiled-coil construct of the same length as env2.1 but with the native gp41 sequence in the first heptad repeat, binds the C-peptide with a similar K_d (0.65 ± 0.20 μM). This observation implies that the bulk of the binding affinity resides with the
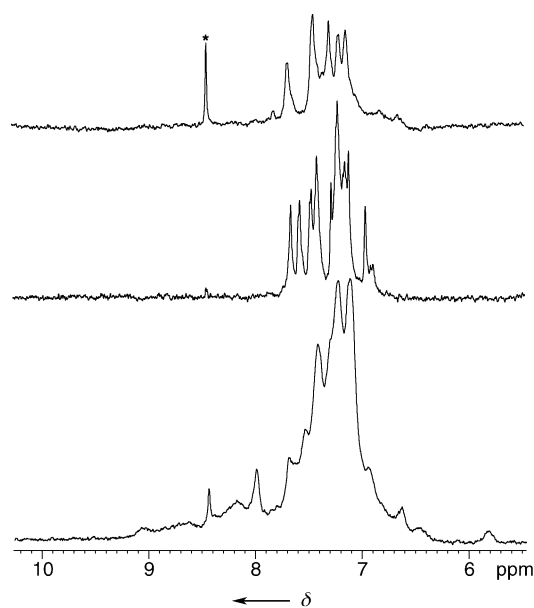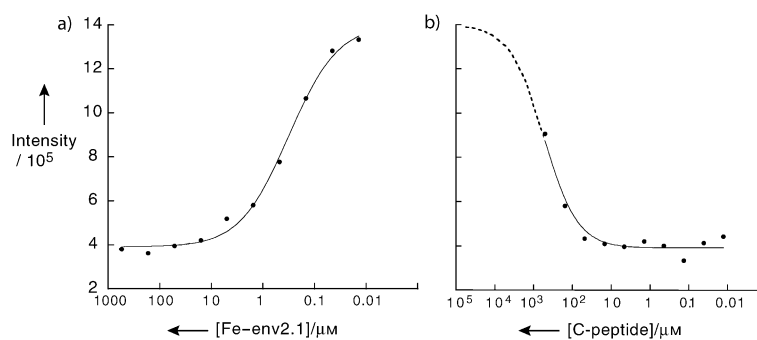

Figure 5. a) Titration of Fe^{II}-env2.1 into wells of a 396-well plate containing a final concentration of 100 μM dansylated C-peptide in 25 mM Tris/acetate buffer, pH 6.6. Fe^{II}-env2.1 concentration is shown along the x-axis and fluorescence intensity above 400 nm following excitation at 340 nm is shown along the y-axis. The binding curve shown was fit to the binding isotherm $\Delta F = c_1 (P_t - c_2 \Delta F)/(K_d + P_t - c_2 \Delta F)$ where ΔF is the reduction in fluorescence intensity compared to maximum, $P_t = [Fe^-env2.1]$ and c_1, c_2 are constants. b) A competition experiment in which unlabeled C-peptide was titrated into wells of a 396-well plate containing a final concentration of 100 μM dansylated C-peptide and 5 μM Fe^-env2.1. The data was fit to a competitive inhibition curve $\Delta F = \Delta F_0/(1 + L_t/IC_{50})$. ΔF_0 is the maximum fluorescence change and $L_t = [C$-peptide]. Part of the curve was inaccessible owing to limited solubility of the C-peptide.

in competitive inhibition kinetics.[24] In this experiment, the dansylated peptide concentration is 250 times the value of K_d. The additional factor of $\approx 2–3$ in the observed ratio of IC_{50} to K_d may arise from a slightly different binding affinity of the dansylated peptide. IC_{50} values are typically used to obtain the relative ranking of a series of ligands in a high-throughput assay. Translating IC_{50} into K_d must be approached cautiously, with accurate knowledge of substrate K_d and component concentrations.

In summary, we have demonstrated that a peptide from the gp41 coiled coil region can be excised from the intact protein and stabilized by using metal ion–bipyridyl coordination at the peptide N-terminus. The resulting protein exhibits characteristics of the intact gp41 coiled coil, that is, it forms a trimeric unit in which the grooves along the coiled coil are accessible to binding by a small flanking peptide from the gp41 C-heptad repeat, as well as by a designed D-peptide known to bind to the coiled coil. Labeling the C-peptide with a fluorescent probe then enables direct determination of binding by using the phenomenon of FRET. Compounds which are able to bind to the coiled coil and displace the C-peptide can be measured with a competitive inhibition assay by following the increase in dansyl fluorescence intensity or lifetime with compound concentration. Naturally fluorescing organic compounds are not expected to interfere at the observed dansyl–peptide fluorescence maximum of 570 nm. Any residual fluorescence can be taken into account by using a negative control.

The molecules described here represent relatively short segments of the HR1 and HR2 regions of HIV-1 gp41. The design can be adapted to longer segments if desired, as well as to different regions of the gp41 coiled coil, and coiled coil domains in fusion proteins of other class 1 enveloped viruses. These include retroviruses, pneumoviruseses, orthomyxoviruses, paramyxoviruses and filoviruses, to which the particularly virulent Influenza, Ebola and Respiratory Syncytial Viruses belong, to name a few. In each case, the full-length fusion protein is unsuitable for biophysical binding studies, as the region of interest for drug targeting is masked by outer domains. This study suggests that the metallo-peptide design demonstrated here is a promising strategy for isolating the coiled coil domain and providing a built-in probe for high-throughput fluorescence screening of potential fusion inhibitors.

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