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Evaluation of sequence variability in HIV-1 gp41 C-peptide helix-grafted proteins

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Abstract

Many therapeutically-relevant protein-protein interactions (PPIs) have been reported that feature a helix and helix-binding cleft at the interface. Given this, different approaches to disrupting such PPIs have been developed. While short peptides (<15 amino acids) typically do not fold into a stable helix, researchers have reported chemical approaches to constraining helix structure. However, these approaches rely on laborious, and often expensive, chemical synthesis and purification. Our premise is that protein-based solutions that stabilize a therapeutically-relevant helix offer a number of advantages. In contrast to chemically constrained helical peptides, or minimal/miniature proteins, which must be synthesized (at great expense and labor), a protein can be expressed in a cellular system (like all current protein therapeutics). If selected properly, the protein scaffold can stabilize the therapeutically-relevant helix. We recently reported a protein engineering strategy, which we call “*helix-grafted display*”, and applied it to the challenge of suppressing HIV entry. We have reported helix-grafted display proteins that inhibit formation of an intramolecular PPI involving HIV gp41 C-peptide helix, and HIV gp41 N-peptide trimer, which contain C-peptide helix-binding clefts. Here, we used yeast display to screen a library of grafted C-peptide helices for N-peptide trimer recognition. Using ‘hits’ from yeast display library screening, we evaluated the effect helix mutations have on structure, expression, stability, function (target recognition), and suppression of HIV entry. 2009 Elsevier Ltd. All rights reserved.

Graphical Abstract

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Supplementary material

Supplementary material is available free of charge on the Internet

Keywords

gp41; helix; HIV; protein engineering; protein evolution

1. Introduction

The large size, diverse structures, and molecular complexity found in proteins often generates surfaces that potently and selectively bind to therapeutically-relevant targets that challenge—or evade—small-molecules, including protein-protein interactions (PPIs).[1] Additionally, the discovery of protein binders, through laboratory evolution, is often higher-throughput and simpler than the analogous small-molecule centered discovery process.[2] A number of therapeutically significant PPIs feature a cleft that is shape-complementary to an exposed α -helix. Given this, the inhibition of PPIs that feature a helix and helix-binding cleft at the interface, has been the focus of many therapeutic discovery efforts.

One approach to disrupting interfaces that feature a helix and helix-binding cleft is the development of small-molecules[3], helical foldamers[4–7], or conformationally constrained peptides[8–12] that mimic the three-dimensional structure of a folded helix at the interface. In contrast to many proteins, short peptides often do not overexpress in a soluble form in cells (*E. coli*, yeast, mammalian cells), and their chemical synthesis on a therapeutically viable scale remains costly and wasteful. Meanwhile, such ligands are typically unfolded or poorly folded, significantly enhancing their susceptibility to non-specific degradation *in vivo*. Peptides have been fitted with conformational constraints (such as hydrogen bond surrogates[12] and ‘staples’[8]) to increase conformational rigidity (a helix) and increase stability. These approaches require non-trivial chemical synthesis and purification, that limits throughput and elevates costs. Researchers have also described ‘miniature proteins’ as a method for helix stabilization[13–15]. However, these proteins do not express in *E. coli* and must be chemically synthesized.

We recently described a new approach to folded helix display that relies on protein engineering.[16, 17] A stable scaffold protein displaying a helix, which contains at least one solvent-exposed face, serves as a generic canvas upon which to paint, or extend, any desired helical interface. We term this method ‘helix-grafted display’ (Figure. 1a). In developing a general scaffold for helix grafted display, we sought to identify a protein fold that presents a properly folded α -helix in such a way as to permit direct receptor access to one helical face. Ideally, the scaffold would be stable, express well as a soluble protein in *E. coli*, and tolerate multiple mutations and/or extension of the helix. Our initial scaffold search identified Pleckstrin Homology (PH) domains as suitable starting points. From this work, we identified ELMO (Figure 1a), a PH domain amenable to helix-grafting.

We have used helix-grafted display to engineer proteins capable of suppressing HIV-1 entry. [16, 17] This therapeutic activity is the result of inhibiting an intramolecular PPI between HIV-1 gp41 C-peptides (α -helix) and a trimer of HIV-1 gp41 N-peptides, which contain C-peptide α -helix-binding clefts. This PPI generates a 'trimer-of-hairpin' assembly that brings HIV and mammalian cell membranes into close proximity, leading to membrane fusion and HIV-1 entry (Figure 1b).[18] The 'trimer-of-hairpins' assembly generated from mixing three C-peptides and three N-peptides, is shown in Figure 1c[19]. Helix-grafted proteins presenting a C-peptide surrogate bind pre-fusogenic gp41 N-peptide trimer, leading to suppression of gp41 fusogenic assembly.

Cpep-ELMO consists of the ELMO scaffold (with a truncated N-terminal helix) and a genetic fusion between the N-terminal ELMO helix and gp41 C-peptide (Figure 1a). Cpep-ELMO binds tightly to gp41 5-helix ($K_D \sim 90$ nM) and suppresses entry of HIV-1 into Cluster of Differentiation 4 (CD4) positive human cells.[16] In contrast to its peptide counterpart (the FDA approved peptide drug FuzeonTM), Cpep-ELMO is stable in human serum.

During the course of these earlier studies, we questioned whether or not the gp41 C-peptide helix could be evolved for improved function, within the context of helix-grafted display. We reasoned that mutation and optimization of solvent-exposed residues on the grafted C-peptide helix could result in improved N-peptide trimer recognition, improved expression, and/or improved stability of the helix-grafted protein. Additionally, we reasoned that mutating solvent exposed C-peptide residues, and evaluating this protein library for target affinity, would provide valuable information on the relative role each mutated C-peptide residue plays in gp41 N-peptide trimer recognition, and the capacity for variation within this helix. With this in mind, we set out to create a Cpep-ELMO library, with randomized solvent exposed residues in the grafted helix, and use yeast display to evaluate this helix-grafted display protein library for N-peptide trimer recognition.

2. Results and discussion

2.1 Yeast display evolution of a Cpep-ELMO helix-grafted display protein library

As stated above, we first demonstrated that we could graft a portion of gp41 C-peptide onto ELMO, to generate a new protein that reliably replicates C-peptide display and suppresses HIV-1 entry.[16] We next sought to evaluate sequence variability in the grafted helix. Five positions on Cpep-ELMO (Figure 2a) that bury into the C-peptide helix-binding cleft of N-peptide trimer (wild-type sequence WWIYI) were randomized by saturation mutagenesis. In particular, the tryptophan residues (labeled residues 1 and 2 in Figure 2a) we mutated are buried in a deep (and conserved) hydrophobic pocket[19]; their deletion has been reported to significantly reduce affinity[20]. Thus, these mutations, in a sense, serve as a control for amino acid evolution ('hits' should regain those tryptophan residues).

The ~3.2 million member Cpep-ELMO derived protein library was displayed on yeast, and tightest binders were selected by cell sorting. Specifically, the yeast display library was made to express Cpep-ELMO derived library members that contain an C-terminal *cMyc* tag. Following display, yeast was concomitantly incubated with a FITC-labelled anti-*cMyc*

antibody and biotinylated gp41 5-helix. 5-helix is a previously reported protein that covalently tethers five of the six subunits within the gp41 trimer-of-hairpins (three N-peptides and two C-peptides) using simple GlyGlySer connectors.[5, 21] When folded, it features the gp41 coiled coil with two of its binding sides already occupied, and just a single exposed interface (Figure 2b). This reduces the recognition event to a simple 1:1 interaction, simplifying the analysis. Following a brief washing step, streptavidin-phycoerythrin conjugate (SA-PE) was added. Thus, yeast displaying biotin, by virtue of a surface protein/5-helix interaction, will be bound by SA-PE. Yeast were then analyzed by flow cytometry, and sorted for the highest FITC fluorescence intensity (which comments on display efficiency) and PE red pigment intensity (which comments on 5-helix binding efficiency, summarized in Figure 2c). Yeast that display tightest affinity proteins were enriched by flow cytometry over the course of two rounds of screening against 500 nM or 100 nM biotinylated 5-helix, respectively (Figure 2d).

From our flow cytometry experiments, we identified eleven unique sequences that retain affinity for 5-helix in our yeast display screen. The established preference for tryptophan at positions W1 and W2 (Figure 2a) was faithfully replicated, suggesting that the method is sensitive to tightest receptor affinity. Meanwhile, while hydrophobic residues were often selected for, the other positions interrogated in this study are reasonably amenable to mutation, supporting the idea that variation within certain residues on the binding interface is permitted (Figure 2e). We next evaluated the effect helix mutations have on structure, expression, stability, function (5-helix recognition), and suppression of HIV-1 entry in a live virus assay.

2.2 Evolved Cpep-ELMO mutants retain affinity for HIV-1 gp415-helix

Given the findings from our yeast display screening, whether or not solvent exposed residues on the gp41 C-peptide helix are amenable to mutation is no longer in question. What remained a question, however, was what effect these mutations had on the affinity for gp41 5-helix. We assessed binding between Cpep-ELMO mutants and gp41 5-helix by Enzyme-Linked Immunosorbent Assay (ELISA).

Biotinylated gp41 5-helix was first immobilized onto commercially available streptavidin coated plates, which were then washed to remove unbound protein. Enriched mutants (Cpep1-ELMO – Cpep11-ELMO) containing a C-terminal FLAG tag were separately incubated with immobilized 5-helix and binding as then analyzed by measuring luminescence, following incubation with necessary ELISA reagents (see Supporting Information). Of the initial eleven hits, five proteins (Cpep1, 3, 4, 5, 8-ELMO) compared favorably to Cpep-ELMO and warranted further investigation (Figure 3a). Amino acid composition of Cpep-ELMO, and our evolved proteins at mutated positions is shown in Figure 3b.

2.3 Evolved Cpep-ELMO proteins are structured, stable, and in some cases, express more robustly in *E. coli*

Given the findings from our yeast display screening and ELISA experiments, whether or not solvent exposed residues on the gp41 C-peptide helix are amenable to mutation is no longer

in question. What remained in question, however, was what affect these mutations had on the larger protein structure. We began to answer this question by comparing protein expression levels of mutants to our starting protein (Cpep-ELMO). In addition to selecting for improved target affinity, display-based methods often select for proteins with improved protein stability and expression[22]. We expressed five of the tightest binding Cpep-ELMO mutants in *E. coli* as N-terminal His₆ fusions, and purified them by nickel-NTA chromatography. Levels of purified eluted proteins were then measured as mg/L with spectroscopy (nanodrop analysis). As shown in Figure 3c, all but one of our evolved proteins express at higher levels, compared to Cpep-ELMO. While Cpep-ELMO expresses modestly in *E. coli* (~2.5 mg/mL), our best performing mutants, Cpep3-ELMO and Cpep4-ELMO, express more than twice as well.

In addition to expressing better in *E. coli*, evolved mutants retain structural features associated with our starting protein (Figure 3d) as assed by circular dichroism (CD). This makes sense: high efficiency yeast display requires proper protein folding and shuttling to the cell surface. Our yeast display screen concomitantly selects for robust expression, protein stability, and high affinity for gp41 N-peptide.

Having established that mutations selected for in our yeast display experiment do not appreciably alter protein *structure*, we next evaluated the effect yeast display evolution had on protein *stability*. Relative stabilities of Cpep-ELMO, and evolved mutants were measured by thermally denaturing (0 °C – 90 °C) each protein and measuring helicity by CD at 222 nm (Figure 3e). Mutated proteins unfolded within the range 44–57°C, which is not dramatically lower than what is the observed melting temperature for Cpep-ELMO (57 °C). Collectively, these findings show that proteins evolved in our yeast display screen not only retained affinity for gp41 5-helix, but these proteins express—sometimes more robustly—as soluble proteins in *E. coli*, and these proteins retain their structure and stability.

2.4 Evolved Cpep-ELMO mutants suppress HIV infection in a live virus assay

Having demonstrated that some of our evolved C-peptide ELMO helix-grafted display proteins express better than Cpep-ELMO, retain helical structure, and bind to gp41 5-helix *in vitro*, we next measured their ability to suppress HIV infection using a live virus assay we have previously reported[16, 23]. In this method, HIV IIIB is administered to CD4+ mammalian cells stably integrated with a plasmid that encodes the HIV-1 long-terminal repeat (LTR) upstream of green fluorescent protein. Since HIV-1 Tat/TAR-dependent transcription ultimately leads to the expression of GFP, we will be able to see if HIV-1 infects these cells. The number of cells that express GFP correlates to the number of cells infected with the virus and can thus be measured via flow cytometry. As seen in Figure 4, all but one of the evolved helix-grafted display proteins (Cpep8-ELMO) inhibit HIV-1 entry better than the original helix-grafted display protein, Cpep-ELMO. A number of our mutated proteins have dramatically improved Inhibitory Constant 50 (IC₅₀) values for inhibition of HIV-1 entry (IC₅₀: Cpep1-ELMO: 0.43 μM; Cpep3-ELMO: 0.31 μM; Cpep4-ELMO: 0.77 μM; Cpep5-ELMO: 0.19 μM. In contrast, the IC₅₀ of our starting protein, Cpep-ELMO, was not reached over the course of the concentrations used in this experiment.

Conclusion

Many disease-relevant protein-protein interactions (PPIs) utilize interfaces that involve an α -helix and helix-binding groove. In order to disrupt these PPIs, researchers have developed various techniques to stabilize helical display of side chains within a peptide or peptide mimetic. While these techniques, such as peptide ‘stapling’, ‘hydrogen-bond surrogate’ engineering, and helical ‘foldamers’ represent important sectors of biomimetic research, and are important reagents for inhibiting disease-relevant PPI’s that feature a helix-binding groove, their synthesis and purification can be expensive and laborious. Researchers have also described ‘minimal proteins’ with an evolved helix (for tailored recognition); however, these polypeptides do not express as a folded protein in *E. coli*, and must be prepared by solid-phase peptide synthesis, which is also laborious and expensive.

Recently we reported ‘helix-grafted display’, a potentially general solution for displaying a folded therapeutically-relevant helix on a protein scaffold. In recently reported work, we showed that a Pleckstrin Homology (PH) domain can be resourced as a helix-grafted display scaffold, wherein a solvent-exposed helix serves in the wild-type protein serves as a generic canvas upon which to paint, or extend, any desired helical interface. To date, our research has focused on the development of new proteins that display HIV-1 gp41 C-peptide, an established therapeutic reagent. Previously, we showed that Cpep-ELMO, an engineered protein that displays gp41 C-peptide as a fusion to the N-terminal helix of the ELMO PH domain, binds to gp41 5-helix, which mimics the pre-fusogenic state of gp41 and serves as a therapeutically-relevant target. Cpep-ELMO potently binds to gp41 5-helix ($K_D \sim 90$ nM) and suppresses HIV entry into CD4-positive cells, in a concentration-dependent manner.

In this work, we sought to optimize properties of Cpep-ELMO mutants by mutating five C-peptide binding face residues to all possible proteinogenic amino acids, and selecting for efficient display on yeast (which relates to stability) and 5-helix recognition. As a result of this screen, we reasoned that we could not only improve recognition, but also assess the variability of sequences within the solvent-exposed binding face of C-peptide in our helix-grafted protein. After screening a ~3.2 million-member Cpep-ELMO derived library, we found that two neighboring tryptophan residues (labeled W1 and W2 in this work, Figure 2a) are enriched for, supporting previous findings detailing their importance in gp41 5-helix recognition. Thus, their enrichment demonstrated the ability of our yeast display screen to enrich for residues necessary for 5-helix recognition. Other residues, however, (labeled I3, Y4, and I5 in this work, Figure 2a) exhibit sequence variability. While hydrophobic residues are generally selected for at these positions, no single residue emerged as absolutely necessary, or even particularly favored. Using eleven proteins (Cpep1-ELMO–Cpep11-ELMO) enriched in our screen, we measured their expression in *E. coli*, structure, and stability. We also measured the ability of these proteins to recognize gp41 5-helix, and suppress HIV entry using a live virus assay. These characteristics and activities were compared to our starting protein, Cpep-ELMO.

Satisfyingly, 5 of the 11 favor comparably to Cpep-ELMO when it comes to 5-helix recognition; all 5 of these proteins exhibit CD spectra that indicates retention of helical structure; all express as soluble proteins in *E. coli*, and in some cases, expression levels are

improved, in comparison to the starting protein. Finally, 4 of our evolved proteins (Cpep1-ELMO; Cpep3-ELMO; Cpep4-ELMO; Cpep5-ELMO) suppress HIV-1 entry better than our starting helix-grafted protein (Cpep-ELMO). This work shows that solvent exposed residues on C-peptide are amendable to mutation, and that their mutation can generate new proteins with improved properties and therapeutic activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Helix-grafted display technology: a therapeutically-relevant helix (HIV gp41 C-peptide) is grafted onto a solvent exposed helix on ELMO, a Pleckstrin Homology (PH) domain. (b) HIV-1 entry requires structural collapse of gp41 N-peptide and C-peptide trimers, leading to a 'trimer-of-hairpins' assembly, and fusion of HIV-1 and immune cell membranes. (c) Structure of the HIV gp41 'trimer-of-hairpins', generated from assembly of three N-peptides (light brown) and three C-peptides (blue).

Figure 2.

(a) Library design for Cpep-ELMO helix-grafted display library. Residues labeled 1–5, and highlighted as light brown spheres, were mutated to all possible 20 proteinogenic amino acids, generating a ~3.2 million-member protein library. (b) Cartoon depiction of gp41 5-helix, a single polypeptide that contains three N-peptides (brown) and two C-peptides (blue), which presents a single available C-peptide binding site. (c) Flow cytometry enrichment scheme to identify novel Cpep-ELMO helix-grafted proteins that bind to gp41 5-helix. (d) Flow cytometry data from yeast display library screening (round 1: left; round 2: right). Yeast selected in round 1 and round 2, representing 0.4% and 1% of the total population, respectively, are boxed. (e) Sequence logo generated from 11 Cpep-ELMO derived mutants enriched after two rounds of yeast display screening.

Figure 3.

(a) ELISA data showing binding between immobilized gp41 5-helix and Cpep-ELMO, or mutants described in this work. Error bars represent the standard error of the mean from three replicate experiments. (b) Sequence of Cpep-ELMO, and mutants selected for by yeast display in this work (Cpep1-ELMO – Cpep11-ELMO). (c) Expression levels, in *E. coli*, for our starting helix-grafted display protein (Cpep-ELMO) and mutants generated from our yeast display screen (Cpep1-11-ELMO). (d) Circular dichroism spectra for Cpep-ELISA and mutants described in this work. (e) Melting data for Cpep-ELMO and mutants described in this work.

Figure 4. Normalized suppression of HIV-1 entry in CD4-positive cells in a live virus assay by Cpep-ELMO and derivatives. Each data point reports HIV-1 infectivity relative to the peptide hydrating buffer treated control (mean $\pm$ SEM from 3 replicates; error bars not shown are smaller than the data points).