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GLUE That Sticks to HIV: A Helix-Grafted GLUE Protein That Selectively Binds HIV gp41 N-Terminal Helical Region

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Abstract

Methods for the stabilization of well-defined helical peptide drugs and basic research tools have received considerable attention in the last decade. Here, we report the stable and functional display of an HIV gp41 C-peptide helix mimic on a GRAM-Like Ubiquitin-binding in EAP45 (GLUE) protein. C-peptide helix-grafted GLUE selectively binds the N-terminal helical region of gp41, a well-established HIV drug target, in a complex cellular environment. Additionally, the helix-grafted GLUE is folded in solution, stable in human serum, and soluble in aqueous solutions, and thus overcomes challenges faced by a multitude of peptide drugs, including those derived from HIV gp41 C-peptide.

Keywords

protein engineering; protein-protein interaction; HIV/AIDS; gp41; helix-grafting

The ongoing discovery of new drug therapies is of vital importance to human health. The traditional pharmaceutical paradigm for this centers on small molecules binding to well-defined protein pockets, typified by enzyme active sites. However, there remain countless important targets largely beyond the reach of this strategy, principally due to extended contact surfaces. Such interactions are often collected under the heading of ‘protein-protein interfaces’ or PPIs.^[1]

A medically significant subset of these PPIs feature binding of one protein to an exposed helix on another, which has sparked considerable interest in the synthetic replication of helical epitopes as a route to novel therapeutics. Various strategies have been employed, including oligomeric organic scaffolds that project side chains along appropriate vectors^[2], covalently constrained (or ‘stapled’) peptides^[3], and helical ‘foldamers’^[4] employing

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natural or unnatural backbone architectures. Each has produced effective agents, but requires non-trivial synthetic effort and expense.

A more accessible suite of ligands might be had by exploiting the flexibility and feasibility of protein expression. If one could identify a scaffold protein bearing a helix with at least one solvent exposed face, and if that protein was simple and stable enough to facilitate easy expression and tolerate varying the exposed helical residues and helix length, it could serve as a generic canvas upon which to paint any desired helical interface. The result would be a 'protein graft' in which residues critical to recognition of a particular epitope are grafted onto the host protein in appropriate positions. The general concept of grafting is an established method for mimicking protein surfaces.^[5]

Here we report successful application of our specific 'helix-grafting' technique to a PPI crucial for HIV infection. The HIV fusion protein gp41 operates in part by binding a C-terminal helix (C-peptide) onto a trimeric N-terminal coiled coil. We show that grafting of gp41 C-peptide residues onto the exposed helical face of a suitable host affords a new ligand that expresses well in *E. coli*, exhibits excellent serum stability and is capable of replicating the native interaction.

Following initial attachment to a target cell, HIV entry is effected by fusion of the respective cell membranes, mediated by gp41.^[6] In the prefusogenic state, gp41 is trimerized via an N-terminal heptad repeat (NHR), and an N-terminal fusion peptide inserts into the target membrane. The protein then undergoes a conformational rearrangement in which the C-peptide packs as an antiparallel helix against the surface of the NHR trimer.^[7] Anchoring of the respective protein termini into viral and cell membranes ensures that this rearrangement requires membrane juxtaposition, and it thus provides a mechanism for promoting fusion (Figure 1).^[8] Peptides derived from this C-peptide helix (some as short as 12–16 residues) have been shown to bind the coiled coil and inhibit membrane fusion by HIV in human cells.^[8–9] The best known of these, Enfuvirtide (FuzeonTM), consists of 36-residues, and is an FDA-approved treatment.^[10] However, like other short peptide drugs, its chemical synthesis is extraordinarily expensive and it exhibits poor serum stability ($t_{1/2}$ ~3.8 hours).^[11] We reasoned that a helix-grafted alternative might retain similar specificity but have improved stability, solubility, and availability.

In designing our first-generation helix-grafted protein, we focused on a Pleckstrin homology (PH) domain called GLUE (GRAM-Like Ubiquitin-binding in EAP45, Figure 2A, gray), which is derived from a subunit of the endosomal sorting complex.^[12] Like other members of the PH family, the GLUE domain contains a C-terminal amphipathic helix resting in a cleft formed by two opposed beta sheets, with one face presented to solvent. This relatively rare arrangement is well suited to serve as a helix grafting scaffold. Although the native GLUE helix is only 16 residues, known structures of other PH domains with helices up to 29 residues suggested that it could be extended to a length comparable to the C-peptide without structural compromise. In addition to its well positioned helix platform, GLUE is a relatively small (~15 kDa) and stable protein. Finally, native GLUE function relies on an affinity for phospholipids that can be abolished by a single Arg107Ala mutation, making it suitable for future intracellular targets without fear of disrupting lipid trafficking.^[12]

We began by aligning the native helix on GLUE (Figure 2A, grey) with a single C-peptide helix from gp41 (Figure 2A, purple). Backbone atoms from the GLUE helix (PDB code 2CAY) were aligned with the corresponding number from the N-terminal segment of the gp41 C-peptide (PDB 1AIK), using PyMol's pair_fit algorithm. The overlay was very good (RMSD of 0.44 Å over 60 atoms), and it allowed us to confidently select six positions on the GLUE helix at which we could install side chains from the gp41 sequence in such a way as to replicate their native three-dimensional positions. We then extended the helix by attaching a pure gp41 sequence to the C-terminus of GLUE (Figure 2B) such that the total length of the new helix was appropriate for binding to the trimeric N-terminal coiled coil (Figure 2C). The final sequence expressed as a soluble protein in *E. coli*.

We characterized both wild-type GLUE (wtGLUE) and the helix-grafted variant (referred to as GLUE-Cpep herein) by circular dichroism (CD), to probe for macroscopic structural changes (Figure 3A). Both proteins display a similar overall signal, suggesting that the grafting process does not compromise the GLUE domain fold. Since one element of our design was the expectation that a well folded protein domain would exhibit improved serum stability compared to an isolated short peptide, we next conducted a serum stability test using a standard assay^[13]. FLAG-tagged GLUE-Cpep incubated with human serum for up to 12 hours showed no appreciable degradation by Western blot analysis (Figure 3B). This supports a significant serum stability enhancement for the grafted protein compared to isolated peptides such as Enfuvirtide.

Direct analysis of the binding interaction between GLUE-Cpep and the NHR receptor by simple mixing of the two components is complicated by several factors: proper self-assembly of the N-terminal peptide, the potential for one, two, or three GLUE-derived ligands per complex, and the known susceptibility of unbound N-peptide trimers to aggregation/precipitation. Fortunately these challenges have long been recognized, and several solutions exist. We chose to use a construct called 5-helix, based on initial work by Kim and coworkers.^[14] It solves the problem of multiple equilibria and binding sites by covalently tethering five of the six subunits with short Gly/Ser loops. Thus a single polypeptide contains three copies of the NHR domain and two C-peptides, such that when folded it features the coiled coil with two of its binding sides already occupied, and just a single exposed interface (Figure 4A). Throughout, we use 5-helix as a receptor to assess complex formation with GLUE-Cpep. Initial CD characterization of the GLUE-Cpep/5-helix complex demonstrates binding-induced gains in helicity and thermal stability. The wavelength spectrum (Figure 4B) exhibits a notably deeper signal for the 1:1 mixture than for either component alone, and the corresponding melt data (Figure 4C) reveal a dramatic increase in thermal stability, as evidenced by a significant shift of the overall melting curve, though the change in T_m is more modest (observed T_m values of ~77 °C, ~79 °C, ~83 °C for 5-helix, GLUE-Cpep, and the complex, respectively). The melting transition for the 1:1 sample is also highly cooperative, further supporting a well-defined assembly.

Having validated the GLUE-Cpep/5-helix interaction, we moved on to probe its viability in more complex environments. Binding in living cells (*E. coli*) was first assessed by split-superpositive Green Fluorescent Protein (split-spGFP) reassembly, a technique we recently reported.^[15] *E. coli* were co-transformed with plasmids encoding 5-helix fused to the N-

terminal half of spGFP (N-spGFP-5-helix) and one of two C-spGFP fusions: GLUE-Cpep or the gp41 C-peptide by itself. Interaction-dependent reassembly of GFP fragments (to generate a fluorescent signal) was measured by flow cytometry. Cells expressing either ligand construct are highly fluorescent, in contrast to a control with nothing fused to C-spGFP (Figure 4D). We further characterized this interaction using an Enzyme-Linked Immunosorbant Assay (ELISA). The grafted GLUE binds 5-helix with slightly better affinity than the native C-peptide (Figure 4E, columns 3 and 4, respectively), while the wild type GLUE exhibits no appreciable affinity (Figure 4E, column 2), confirming the need for the grafted domain. This ELISA signal is observed even for a GLUE-Cpep sample that was pre-incubated with human serum (Supporting Information), confirming that the degradation-resistant form of the protein remains functional. Taken together, these experiments show that the helix-grafted GLUE binds 5-helix in the context of a complex cellular milieu, in a manner comparable to the native ligand, and with improved serum longevity.

Binding selectivity was assessed by measuring the amount of protein that is co-purified from *E. coli* expressing an untagged GLUE-Cpep (~17.1 kDa) and His_{6x}-tagged 5-helix (~25.4 kDa). As seen in Figure 4F, the tagged 5-helix co-purifies with a single protein, which was identified as GLUE-Cpep by mass spectrometry (Supporting Information, Figure S2). The similar amounts of each co-purified protein (as determined by densitometry measurements of each protein band) further indicates that the complex involves a 1:1 ratio of proteins. The relatively miniscule levels of other co-purified cellular proteins indicates excellent selectivity for this interaction, even in a complex cellular environment, suggesting a reasonably strong mutual affinity.

In conclusion, we have demonstrated that the solvent exposed C-terminal alpha helix of the GLUE protein scaffold can be dramatically modified and extended, so as to mimic the function of the gp41 C-peptide. ELISA and co-purification data indicate that GLUE-Cpep selectively binds 5-helix, a protein that mimics the native C-peptide receptor. Unlike the isolated C-helix of Enfuvirtide, GLUE-Cpep is soluble and well-folded in aqueous solution at room temperature (~25 °C), and is resistant to degradation in human serum at physiological temperature (~37 °C). Thus, this protein drug lead overcomes challenges faced by traditional peptide reagents and may represent a new reagent for inhibition of HIV entry. Additionally, helix-grafting onto PH and PH-like domains, such as GLUE, may be a general approach to the development of new reagents of interest to a diverse set of diseases that rely on helix-driven assembly. Finally, GLUE-Cpep serves as a starting point for the generation of higher affinity and more selective mutants through the application of high-throughput screening or selection methods. Such experiments are currently underway, and will be reported in due course.

Experimental Section

Detailed experimental methods, sequences of all proteins used in this work, and PAGE gels of all purified proteins are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) HIV viral fusion via formation of a trimer-of-hairpins assembly involving the N-terminal Helical Region (NHR, orange) and C-terminal Helical Region (CHR, purple) regions of gp41. (b) crystal structure of the NHR/CHR complex (PDB code 1AIK).

Figure 2.

Helix-grafting HIV gp41 C-peptide helix onto a stable PH-like domain protein. (a) Wild-type GLUE (grey) and gp41 C-peptide (purple). Sequences of the GLUE helix and the corresponding region of the gp41 helix are shown in gray and purple, respectively. Spheres indicate the C-alpha positions for each. (b) Helix grafted GLUE-Cpep, produced by backbone alignment of the independent structures. Spheres indicate C-alpha positions of GLUE residues mutated to those from gp41 (also color coded in the sequence).

Figure 3.

(a) Circular dichroism (CD) data for wtGLUE (red) and GLUE-Cpep (blue). (b) Western blot of FLAG-tagged helix-grafted GLUE that isn't incubated with human serum (lane 1), or after 0.5 to 12 hours of incubation (lanes 2–7).

Figure 4.

(a) Depiction of 5-helix, a single protein consisting of three copies of gp41 N-peptide (orange) and two copies of gp41 C-peptide (purple). When folded, this protein presents a single binding site for a C-peptide (or mimic thereof), which is depicted as a gray column with dashed border. (b) Circular dichroism spectra of GLUE-Cpep (blue circles), 5-helix (red circles) and a pre-mixed 1:1 ratio of 5-helix and GLUE-Cpep (open circles) (c) Circular dichroism data (222 nm) showing temperature-dependent melting of the solutions in part (b). (d) Flow cytometry data of *E. coli* following split-spGFP reassembly experiments. Negative = NscGFP-5-helix/CscGFP-; Cpep = NscGFP-5-helix/CscGFP-C-pep; GLUE-Cpep = NscGFP-5-helix/CscGFP-GLUE-Cpep. (e) ELISA data from *E. coli* cell lysate that contain an empty pET DUET plasmid, or pET DUET that encodes His_{6x}-tagged 5-helix along with wtGLUE, grafted GLUE, or C-peptide. (f) Co-purification of His_{6x}-tagged 5-helix and untagged GLUE-Cpep from *E. coli* cell lysate.