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Cell surface assembly of HIV gp41 six helix bundles for facile, quantitative measurements of hetero-oligomeric interactions

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Abstract

Helix-helix interactions are fundamental to many biological signals and systems, found in homo- or hetero-multimerization of signaling molecules as well as in the process of virus entry into the host. In HIV, virus-host membrane fusion during infection is mediated by the formation of six helix bundle (6HB) from homo-trimers of gp41, from which a number of synthetic peptides have been derived as antagonists of virus entry. Using a yeast surface two-hybrid (YS2H) system, a platform designed to detect protein-protein interactions occurring through a secretory pathway, we reconstituted 6HB complex on the yeast surface, quantitatively measured equilibrium and kinetic constants of soluble 6HB and delineated the residues influencing homo-oligomeric and hetero-oligomeric coiled-coil interactions. Hence, we present YS2H as a platform for facile characterization and design of antagonistic peptides for inhibition of HIV and many other enveloped viruses relying on membrane fusion for infection, as well as cellular signaling events triggered by hetero-oligomeric coiled coils.

Many proteins naturally fold into varying degrees of helix bundles, which are indispensable to normal physiology and to the onset of diseased states¹. Of the well-known examples are viral membrane fusion proteins like influenza virus hemagglutinin (HA) and HIV-1 envelope glycoprotein gp41. HIV infection critically depends on the attachment and fusion of virus to host cells through the gp120/gp41 complex. The extracellular domain of gp41 consists of a fusion peptide, an N-terminal heptad repeat (NHR), a loop region, a C-terminal heptad repeat (CHR), and a membrane-proximal external region (MPER) (Figure 1a). Binding of gp120 to CD4 and subsequent interaction with co-receptors (CCR5 or CXCR4) lead to the dissociation of gp120 from gp41 and insertion of the viral gp41 fusion peptide into the target cell membrane, which then forms a prehairpin intermediate. Fusion of viral and cellular membrane is provided by the formation of a gp41 six helix bundle (6HB), a conformation described by three CHR packing in an antiparallel manner to a central three-stranded NHR coiled coil^{2–3} (Figure 1b, c). gp41 prehairpin intermediate is transiently accessible to viral fusion inhibitors derived from NHR or CHR, named as N or C peptide accordingly^{2,4–8} (Figure 1c, d). T20 is one of the early C peptides developed for HIV fusion inhibition^{9–10}. CP32M, a rationally designed C peptide, extends to the upstream region of CHR compared to T20, and contains mutations to enhance affinity to NHR. These resulted in higher thermostability and greater inhibition of viral infection against diverse HIV strains¹¹. Compared with the inhibitors derived from the C peptides, there are fewer based

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ASSOCIATED CONTENT

Supporting Information. Experimental details and analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

on the N peptides due to their low solubility. 5-Helix (5H) peptide generated by linking 6HB peptides in tandem without the last C peptides is used to mimic N peptide, and is shown to be highly stable and potent in viral inhibition¹². Other N-peptide derivatives have been based on fusion to synthetic trimerization sequences¹³. While much interest has been focused on developing synthetic inhibition peptides, natural peptides circulating in the blood have been reported to possess a capacity to inhibit virus fusion¹⁴.

Developing biochemical assays to examine the potency of inhibitors of 6HB formation have been hampered by low solubility of N- and C-peptides and by their tendency to aggregate¹⁵. As a facile and quantitative platform to study 6HB formation and to aid in the design of antagonistic peptides, we used yeast surface two hybrid (YS2H) system and expressed gp41 N- and C-peptides on yeast cell surface. In YS2H, a system designed to express a pair of proteins for measuring protein-protein interactions¹⁶⁻¹⁷, one protein is fused to yeast agglutinin and is thereby displayed on yeast surface as the 'bait', while the other protein is secreted in soluble form and serves as the 'prey' (Figure 2a, b). In the presence of interaction, two proteins associate with each other within the secretory pathway, and the prey is captured on the cell surface by the bait. The affinity of the interaction between the prey and the bait can be quantitatively estimated from the relative abundance of short tags fused to bait and prey, typically measured by flow cytometry¹⁷. This system has previously been used to study heterodimeric interactions; however, its applicability to study higher order, complex oligomerizations has not been demonstrated.

In order to reconstitute gp41 6HB complex on the yeast surface, we expressed N36 and C34¹⁸, peptides derived from regions in NHR and CHR, respectively (Figure 1d, Figure 2). N36 and C34 are known to exclusively form a 6-helix bundle complex devoid of any aggregates^{3,18}. Surface expression of N36, fused to Aga2 (a subunit of agglutinin) as the 'bait', was confirmed by antibody binding to Flag tag appended to the C-terminus of N36. With C34 co-expressed as the 'prey', antibody binding to Myc tag indicated that C34 was captured by N36 (denoted as N36/C34 to refer to a pair of Aga2-fused bait and soluble prey; Figure 2b-c). N36/C34 association was further recognized by conformation-specific monoclonal antibodies (mAb) of gp41: NC-1¹⁹ and D50²⁰ (Figure 2c). Although the presumed epitope for D50 is at the C-terminus of CHR (Ile642-Lys665)²⁰, yeast cells expressing C34 alone (C34/-) were not recognized by D50, indicating that C34 without N36 did not adopt a conformation existing in the context of 6HB. With N36 and C34 swapped for the role of bait and prey (*i.e.*, C34/N36), similar levels of binding of tag-specific antibodies and mAbs NC-1 and D50 were achieved (Figure 2c). We have chosen to anchor N peptide as a fusion to Aga2 and secrete C peptide as itself, a configuration more suitable for evaluating the potency of C peptide-based antagonists.

To validate that in our YS2H system N- and C-peptides were assembled into the conformation of 6HB previously seen in solution and crystals^{3,18,21}, we performed Western blot on N36/C34 peptides cleaved by TEV protease from the surface of yeast (Figure 3). The staining pattern of the N36/C34 by antibodies against Flag and Myc tags closely matched that of the synthetic peptides (N36+C34) of identical amino acid sequence: one of the major bands from N36/C34 was identical to ~40 kDa band from the synthetic peptides, which have been shown to reconstitute 6HB in solution¹⁸. The identity of the 40 kDa band to be 6HB was also corroborated by the staining of mAb NC-1. Compared to the synthetic peptides that formed one major band of 6HB, the N36/C34 assembled in yeast did exhibit higher molecular weight bands stained by both anti-tag antibodies. It can be speculated that during the secretory pathway, not all N36 and C34 peptides would associate with each other to assemble into 6HB but some of N36 may also form a higher order oligomers, which were still able to associate with C34 and retain NC-1 binding. In contrast to N36/C34 cleaved

from yeast, N36 alone (N36/-) or N36 with 6HB disrupting mutation I559P (I559P/C34; see below for details) did not produce a presumed 6HB complex band.

In order to examine if YS2H would provide a quantitative readout to a change in hetero-oligomeric interactions, we have introduced point mutations at various positions and measured the binding of antibodies against reporter tags and different oligomerization states of N36/C34 complexes (Figure 4). Physical forces contributing to 6HB or coiled-coil conformation in general are from the combination of van der Waals (vdW) or hydrophobic contacts at the helix-helix interface and electrostatic attraction among the residues positioned outside of the interface. In a homotrimeric coil of NHR, amino acids in the position of 'a' and 'd' in a helical wheel or heptad repeat form a helix-helix interface at the core (Figure 1b). The residues at 'e' and 'g' of NHR would normally be those that promote electrostatic interaction, but instead they are mainly hydrophobic so that CHR packs onto NHR to form 6HB. The mutations that disrupt hydrophobic or electrostatic forces would completely or partially disrupt coiled-coil interactions. Mutations at the interface between NHR and CHR (e.g., L556A (at position 'e'), A558G ('g'), and V570D ('e') of NHR and S649D ('a') of CHR) led to a modest (A558G) to almost complete (L556A, V570D, S649D) loss of C34 association with N36 (Figure 4). 5HB-specific mAb D5²², which largely recognizes the hydrophobic pocket present at the C-terminal end of NHR, displayed binding to 6HB mutants different from anti-Myc and D50. To cells expressing only N36 (N36/-), D5 showed strong binding (Figure 4), revealing that without an assembly with C34, N36 adopts a conformation seen in the context of gp41. This is an important result that underscores the utility of yeast display, which circumvents the difficulty of studying isolated N-peptide derivatives in solution because of their hydrophobic, aggregation prone nature. The mutations that disrupted a homotrimeric N peptide conformation (e.g., I559P and I573D at position of 'a' of NHR), therefore, led to a complete loss of both anti-Myc and D5 antibodies (Figure 4). Such disruption to 6HB formation by I559P was also confirmed by Western blot (Figure 3). L568A, a mutation introduced at position 'c' of NHR, least compromised 6HB formation. In contrast to various mutations that led to partial to complete perturbation of 6HB formation, N656L led to an increase in the level of Myc expression (Figure 4), attributed to the introduction of a hydrophobic residue that would form vdW contacts with Ile548 and Val549 (Figure 1d).

After validating the assembly and perturbation of gp41 6HB, we examined the pairing of N36 or full-length NHR (Met530 – Gln590) with the variants of C34, such as T20 and CP32M as antagonistic C peptides, which have been developed for inhibiting HIV entry (Table 1). N36/T20 helix-helix interface is formed with three heptad repeats of α -helix (Figure 1d), too short to produce a strong interaction, and this resulted in marginal binding of anti-Myc antibody and D50. In contrast, NHR/T20, spanning longer than four heptad repeats of α -helix at the interface, resulted in the level of Myc expression comparable to or less than the levels seen in NHR/C34 and N36/C34. The observation that NHR/T20 led to maximum binding with mAb D50 is consistent with the fact that D50 epitope is at the C-terminal end of CHR (Ile642-Lys665), fully included in T20 but only partially present in C34²⁰. When paired with either N36 or NHR, CP32M, a peptide spanning Gln621 to Gln652 of CHR and containing mutations to enhance electrostatic and hydrophobic interactions with NHR, exhibited elevated levels of Myc expression. The lack of mAb D50 binding to N36/CP32M and NHR/CP32M is due to the epitope for D50 being almost absent in CP32M. In YS2H, the levels of reporter tags (Myc & Flag) is directly related by Langmuir equation to equilibrium dissociation constant (K_D) for the interaction between bait and prey, given as $\text{Flag/Myc} = \alpha(1 + K_D[\text{prey}])$, where α is the ratio of Flag/Myc for $K_D \sim 0$. With the estimated values of $\alpha = 4$ and $[\text{prey}] = 10 \text{ nM}$ in YS2H¹⁷, the values of K_D were obtained for NHR or N34 binding to C34, T20, or CP32M (Table 1). Although direct measurements of solution affinity for these pairs are not available, the difference in affinity

is consistent with reported potency of C-peptide based inhibitors in the order of CP32M > C34 > T20¹⁷. The ability to quantitatively measure equilibrium binding constants highlights YS2H as a facile platform for the prediction of the potency of antagonistic peptides.

In addition, YS2H can be adapted as a platform for measuring dissociation kinetics for hetero-oligomeric interactions. To minimize rebinding of dissociated C peptides to N peptides, yeast cells were washed and resuspended in a much larger volume of binding buffer (333-fold dilution of yeast culture) and antibody binding to reporter tags was measured at different time points (Figure 5). Notably, the decrease in antibody binding to Myc followed a biphasic behavior, characterized by a rapid reduction of Myc within the first 10 min, followed by a slower decrease. An initial rapid dissociation of C peptide may be due to a loss of small percentage (~15%) of C peptides that were associated with the N peptides forming non-ideal 6HB complexes, some of which may correspond to higher molecular weight bands detected in Western blot (Figure 3). When a two phase dissociation model ($Y = A_1 \times \exp(-t/T_1) + A_2 \times \exp(-t/T_2)$) was used to fit the data, in agreement with the overall trend of dissociation, the slower kinetics were dominant ($A_1 \approx 15$ and $A_2 \approx 85$) for all interaction pairs measured (Table 1). Following a similar trend with equilibrium affinities, a dissociation rate of C34 from NHR (NHR/C34; $16.2 \times 10^{-6} \text{s}^{-1}$) was 3.5-fold slower than that from N36 (N36/C34; $56.7 \times 10^{-6} \text{s}^{-1}$), and a dissociation rate of NHR/C34 was 3.9-fold slower than that of NHR/T20 ($62.7 \times 10^{-6} \text{s}^{-1}$). Notably, although CP32M exhibited equilibrium affinity to N36 higher than C34 or T20, a dissociation rate of CP32M from N36 ($242.7 \times 10^{-6} \text{s}^{-1}$) was 4.3-fold faster than that of C34 ($56.7 \times 10^{-6} \text{s}^{-1}$) (Table 1), indicating that CP32M binding to N36 is comparatively dominated by its on-rate.

In summary, we have demonstrated a construction of a pair of α helices in the YS2H system, and the assembly of viral 6HB structure on the yeast cell surface. The equilibrium binding strength between coiled coils within the bundle as well as the kinetics of soluble peptides could be directly and quantitatively characterized. Subtle alterations that resulted from single point mutations perturbing homo-trimeric as well as hetero-oligomeric coiled coils could also be detected in YS2H, which demonstrates its utility as a platform for the design, optimization, and evaluation of antagonistic peptides as drug candidates. Besides HIV, many enveloped viruses including influenza, respiratory syncytial virus, and Ebola virus require helix bundles for membrane fusion during virus entry into the host²³. In the past, a lot of effort has been directed towards the *de novo* synthesis of various types of helix bundles^{24–28}. The assembly of helix bundles or hetero-oligomeric peptides on the yeast surface would allow rapid screening and design of candidate inhibitors, without impediments from often unreliable *in vitro* protein refolding and costly chemical synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

HIV-1 gp41 fusion complex driven by the formation of trimer-of-hairpins comprised of NHR (blue) and CHR (red). (a) Schematic view of gp41 elements. Residue numbers at the boundaries of each element are shown. (b) Ribbon drawings of side and top views of 6HB are shown (PDB ID: 1AIK³). N- and C-termini of helices are labeled. A helical wheel diagram is shown on the right, viewed from the C-terminal end of NHR. (c) Depicted are the events of HIV-1 membrane fusion and the mechanism of fusion inhibition by soluble N- or C-peptides. (d) Segments of NHR and CHR of gp41 are shown. 'a'-'g' denote the positions of heptad repeats in a helical wheel. Interacting pairs are delineated with dashed lines. N and C peptides (orange) as well as single point mutations (green) used in this study are indicated. CP32M contains rationally designed mutations, shown in pink, some of which were made to introduce intramolecular salt bridges, shown in pink square brackets. FP=fusion peptide; NHR=N-terminal heptad repeat; CHR=C-terminal heptad repeat; MPER=membrane-proximal external region; TM= transmembrane domain; CP=cytoplasmic domain.

Figure 2.

Assembly of 6HB on yeast surface. (a) A schematic view of 6HB displayed on the yeast surface by YS2H is depicted. (b) In YS2H, the bidirectional promoter Gal1/Gal10 drives the expression of surface-anchored 'bait' as a fusion to Aga2 and of secretory 'prey', the level of which is measured by antibody binding to the short epitope tags, Flag and Myc, which are fused to bait and prey, respectively. SS=signal sequence. (c) Immunofluorescence flow cytometry histograms of antibody binding to Flag and Myc tags and gp41-specific antibodies (NC-1, D50). White and grey filled curves are histograms of uninduced and induced yeast cells, respectively. '-' denotes no peptide.

Figure 3.

Confirmation of 6HB assembly in YS2H by Western blot. A TEV cleavage site was introduced immediately after Flag tag following N36 and prior to Aga2 to permit enzymatic release of N36 from yeast cell surface. Synthetic peptides containing identical amino acid sequence as cell surface-cleaved N36/C34 were used as a control. N36/C34 before the induction of protein expression (control), N36 alone (N36/-), and N36 with a 6HB disrupting mutation I559P were also included for comparison. Peptides were run on 12% native polyacrylamide gel. Molecular weight standards were marked based on their different colors (Kaleidoscope Standards).

Figure 4.

Immunofluorescence flow cytometry histograms of antibody against Flag and Myc tags and antibodies (D50, D5) reactive to oligomeric forms of gp41. White and grey filled curves are histograms of uninduced and induced yeast cells, respectively. '*' denotes Aga2-fused bait; '-' denotes no peptide; wt: wild type.

Figure 5. Dissociation kinetics of C peptide inhibitors measured in YS2H. The loss of antibody binding to Myc (MFI_{Myc}) is plotted as a percentage after normalization to the level of antibody binding to Flag (MFI_{Flag}) (n = 5). A two phase kinetic model was used to fit the data, shown in solid or dotted lines.

Table 1

Quantification of the binding kinetics of C peptides to N peptides

N peptide	C peptide	Flag (a.u.)	Myc (a.u.)	D50 (a.u.)	K _D (nM)	A ₁	k _{off1} (10 ⁻⁶ s ⁻¹)	A ₂	k _{off2} (10 ⁻⁶ s ⁻¹)	k _{on} (10 ³ s ⁻¹ M ⁻¹)*
N36	T20	100±3	1±0	2±0	433.3±81.2	NA	NA	NA	NA	NA
N36	C34	111±15	14±3	111±1	10.8±2.8	13	6×10 ⁴	87	56.7±5.0	5.8±2.0
N36	CP32M	141±13	22±1	0±0	6.1±0.6	21	9×10 ⁴	79	242.7±19	40.5±7.1
NHR	T20	125±2	13±1	150±5	16.6±1.9	22	8×10 ⁴	78	62.7±9.2	3.9±1.0
NHR	C34	150±3	22±2	134±4	7.2±1.1	12	6×10 ⁴	88	16.2±2.2	2.4±0.7
NHR	CP32M	141±19	28±4	0±0	2.8±2	16	1×10 ⁴	84	20.7±3.2	16.8±13.1

Numbers in a.u. are mean fluorescence intensity ± standard error of anti-Flag, anti-Myc, and D50 binding (n = 3). N peptide is fused to Aga2 as a bait. a.u.: arbitrary unit; NA: not analyzed;

* Computed from k_{off2}/K_D