Central to our understanding of human immunodeficiency virus-induced fusion is the high resolution structure of fragments of the gp41 fusion protein folded in a low energy core conformation. However, regions fundamental to fusion, like the fusion peptide (FP), have yet to be characterized in the context of the cognate protein regardless of its conformation. Based on conformation-specific monoclonal antibody recognition, we identified the polar region consecutive to the N36 fragment as a stabilizer of trimeric coiled-coil assembly, thereby enhancing inhibitory potency. This tertiary organization is retained in the context of the hydrophobic FP (N70 fragment). Our data indicate that the N70 fragment recapitulates the expected organization of this region in the viral fusion intermediate (N-terminal half of the pre-hairpin intermediate (N-PHI)), which happens to be the prime target for fusion inhibitors. Regarding the low energy conformation, we show for the first time core formation in the context of the FP (N70 core). The α-helical and coiled-coil stabilizing polar region confers substantial thermal stability to the core, whereas the hydrophobic FP does not add further stability. For the two key fusion conformations, N-PHI and N70 core, we find that the FP adopts a nonhelical structure and directs higher order assembly (assembly of coiled coils in N-PHI and assembly of bundles in the N70 core). This supra-molecular organization of coiled coils or folded cores is seen only in the context of the FP. This study is the first to characterize the FP region in the context of the folded core and provides a basic understanding of the role of the elusive FP for key gp41 fusion conformations.

The crystal structure of the HIV gp41 core, presented nearly a decade ago (1–3), served as the basis for the follow-

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1Recipient of the Harold S. and Harriet B. Brady Professorial Chair in Cancer Research. To whom correspondence should be addressed. Tel.: 972-8-934-2711; Fax: 972-8-934-4112; E-mail: yechiel.shai@weizmann.ac.il.

2The abbreviations used are: HIV, human immunodeficiency virus; SHB, six-helix bundle; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; FP, fusion peptide; PHI, pre-hairpin intermediate; DTT, 1,4-dithiothreitol; PBS, phosphate-buffered saline; mAb, monoclonal antibody; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; Fmoc, N-(9-fluorenyl)methoxycarbonyl; MESNA, 2-mercaptoethanesulfonic acid; HPLC, high pressure liquid chromatography; Boc, t-butoxycarbonyl; MBHA, methylbenzhydrylamine.
to hemi- and full fusion states with the apposed viral membrane. The tendency of the FP to self-assemble in membranes is the basis for the ability of exogenous FP-containing fragments to inhibit in vivo fusion by oligomerizing with viral FP, thereby inhibiting proper assembly of viral gp41 required for fusion (17). Based on these findings, it is believed that the FP self-assemblies in membranes, directing higher order oligomerization of gp41 trimers at the fusion locus. The molecular mechanism of FP self-assembly in membranes is based on in-register parallel β-sheet organization of the hydrophobic N-terminal stretch (~16 residues) (27). The robust oligomerization interface created by the extended parallel β-sheet of the hydrophobic FP in membranes is believed to provide the seed for supramolecular gp41 organization or alternatively for interference in the presence of exogenous FP-containing fragments.

Adjacent to the N-terminal FP is the NHR region, which is an auto-oligomerization domain (28) directing parallel trimeric coiled-coil organization in gp41 (1). In model systems, the NHR significantly boosts the fusogenic function of the FP, for consecutive FP/NHR fragments (24), in part by pre-organizing the FP region (29). The β-structure adopted by the FP in membranes, when stabilized by the NHR, is likely that which is required during viral fusion (30). FP/NHR constructs model the N-terminal half of the PHI conformation (target of commercially available fusion inhibitor, Fuzeon), provide information on functional synergy between distinctly different regions of gp41 (24), and may be useful for screening fusion inhibitors. A basic gap in our understanding of HIV fusion centers on the role of the viral fusion peptide, for distinct gp41 conformations, during fusion.

This study characterizes the FP region in the context of the folded core and the N-terminal half of the PHI in solution. Using CD, we analyzed core formation between free peptides corresponding to the CHR (C34) and different N-peptides. N-peptides include the NHR (N36) and extensions of N36 to include the following: (i) the adjacent polar region (17-70) or (ii) complete N-terminal extension to include polar and hydrophobic FP regions (N70) (see Fig. 1). Core stability was analyzed by following thermal denaturation. Native gels were used to analyze the oligomerization character of the various folded cores and the free peptides that constitute them. Propensity of the N-peptides to spontaneously form trimeric coiled coils was monitored using a conformation-specific monoclonal antibody. NHR peptides soluble at physiologic pH were compared for their ability to inhibit gp41-induced cell-cell fusion. We discuss sequence- and structure-specific determinants toward both coiled-coil and core stability. This study is the first to analyze the fusion peptide in the context of the folded core.

EXPERIMENTAL PROCEDURES

Materials—Boc and Fmoc amino acids, Boc MBHA resin, and Fmoc rink amide MBHA resin were purchased from Nova- biochem. S-Trityl-β-mercapto propionic acid was purchased from Peptides International (Louisville, KY). Other reagents for peptide synthesis, TCEP, the sodium salt of 2-mercaptoethane- sulfonic acid (MESNA), proteinase K, phosphate-buffered saline (PBS), and 1,4-dithiothreitol (DTT) were purchased from Sigma. 5-(and -6)-Carboxytetramethylrhodamine, succinimidy ester, was purchased from Molecular Probes. All other reagents were of analytic grade. Buffers were prepared using double glass-distilled water.

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Cell Lines and Reagents—Cell culture reagents and media were from Biological Industries, Israel (Beit-Haemek Ltd.). Steady-Glow Luciferase detection kit was from Promega (Madison, WI). HL2/3 cells (31) (a HeLa cell line) expressing cleaved HIV-1 molecular clone HXB2/3gpt (containing HIV-1 Gag, Env, Tat, Rev, and Nef proteins) were obtained through the AIDS Research and Reference Reagent Program from Dr. Barbara Felber and Dr. George Pavlakis. The TZM-bl indicator HeLa cell line (32), which expresses high levels of CD4 and CCR5 along with endogenously expressed CXCR4, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme, Inc. The cells were cultured every 3–4 days by trypsinization and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C with 5% CO2 in a humidified incubator.

Peptide Synthesis and Fluorescent Labeling—All peptides used in this study, including those chemically ligated to produce longer constructs, were synthesized by solid phase methodology. The “linker peptides” (34-residue N-terminal fragment as well as fragment spanning residues 17–34) were synthesized by a manual solid phase method on MBHA resin using Boc chemistry as described previously (33, 34), with modifications outlined by Hackeng et al. (35), required for inclusion of the linker moiety (S-trityl-β-mercapto propionic acid). The peptides N36, C34, and 17-34 were acetylated at the N terminus. In addition, some 17-34 was labeled at the N terminus with rhodamine (17-34Rho) as described previously (36) to be used in subsequent ligation. All peptides except linker peptides were amidedated at the C terminus. Boc peptides were cleaved from the resin by HF. All other peptides were synthesized by an Fmoc solid phase method on rink amide MBHA resin, using an ABI 433A automatic peptide synthesizer, and then cleaved from the resin using a mixture made of trifluoroacetic acid:double distilled water:TES (18.5:1:0.5 (v/v)). All peptides were purified by reverse phase-HPLC on a C4 Bio-Rad semi-preparative column to >98% homogeneity, and the mass of each peptide was confirmed by platform LCA electrospray mass spectrometry.

Native Chemical Ligation—N70 was prepared by ligating 1-34 “linker” with N36(S35C); 17-70 was prepared by ligating 17-34 linker with N36(S35C), and N-terminal rhodamine-labeled 17-70 (17-70Rho) was prepared by ligating 17-34Rho with N36(S35C). The ligation of unprotected fragments was carried out as described (35) with the following alterations. 1) We used MESNA as the catalyst (37) (2). Prior to addition of lysophilized peptides, the pH of the reaction mixture, made of 6.0 M guanidine HCl, 0.1 M Na2HPO4, and 1% MESNA (w/v), was adjusted to ~7.2 using 1 M NaOH (3). Ligations were carried out at peptide concentrations ranging from 0.2 to 0.8 mM (4). The various ligations were incubated over a range of temperatures, from 4 to 37 °C, and for a period of 4–5 days. For each ligation, the product resolved as a single isolated peak and
was purified to >98% homogeneity using reverse phase-HPLC. Product identity was confirmed by mass spectrometry.

**CD**—Free peptides at 10 μM, or mixtures of N- and C-peptides at 10 μM each, were prepared by dialysis overnight against reference buffer (50 mM sodium formate with 50 μM TCEP, pH 3.0) prior to measurements. CD spectra were recorded at 25 °C in a quartz cuvette on an Aviv 202 spectropolarimeter at 1 nm increments, 10 s averaging time, and in triplicate. The signal of buffer alone was subtracted from the averaged triplicate signals of sample in buffer. Buffer corrected spectra for between 2 and 5 different sets of samples were averaged for each peptide alone and mixture. Following spectral acquisition, thermal melts were recorded at 222 nm wavelength and were based on the following parameters: temperature range (25–105 °C), temperature step of 2 °C, 20-s averaging time, 2-min incubation prior to each measurement, and 1-min ramp for each 2 °C temperature increase. α-Helical content was estimated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation (33,000 degrees cm² dmol⁻¹) (38).

**Native Gel and Western Blot**—10% polyacrylamide continuous native gels at pH 3.4 were prepared. Gels were cast using 30 mM β-alanine and 42 mM formic acid and then pre-run in the same 30 mM β-alanine, 42 mM formic acid running buffer containing 100 μM TCEP as a reducing agent. The free peptides and pre-formed cores were prepared under identical conditions as those used for CD measurements, except 10% glycerol was added prior to loading. Identical gels were run in parallel (differing only in the amounts loaded), and complementary gels were either stained with Coomassie Blue or transferred by Western blot to polyvinylidene difluoride membranes (Millipore). Membranes were probed with NC-1 monoclonal mouse primary antibody (kind gift of Dr. Shibo Jiang) and then with goat anti-mouse secondary antibody conjugated to horseradish peroxidase. Bound NC-1 was monitored using the ECL-based “Phototope-HRP Western Blot Detection System” from Cell Signaling Technology. The amounts of peptides or pre-formed cores loaded (from 0.35 to 10 μg) were calibrated based on both Coomassie Blue stain and reactivity with NC-1 mAb (see Fig. 3 legend).

**Fusion Assay**—Fusion was monitored by using a reporter gene assay based on activation of HIV long terminal repeat-driven luciferase cassette in TZM-bl (Target) cells by HIV-1 tat from the HL2/3 (Effector) cells. Fusion assays were performed in a serum-free medium to minimize nonspecific association of peptides with serum proteins. N36 was solubilized in Me₂SO, whereas 17-70 was solubilized in Me₂SO with 10× molar DTT at all dilutions. The TZM-bl cells, plated in 96-well clusters (2.5 × 10⁴ per well) overnight at 37 °C, were placed in 50 μl of serum-free medium per well prior to fusion assays. The target cells were co-cultured with 50 μl of HL2/3 cells (2.5 × 10⁴ per well in serum-free medium) for 6 h at 37 °C in the presence or absence of various concentrations of peptides (1 μl of peptide per well). Efficiency of fusion was determined by measuring luciferase activity in the wells following the manufacturer’s instructions using the steady-glow luciferase determination kit (Promega, Madison, WI). The light intensity was measured using VICTOR² 1420 multilabel counter (PerkinElmer Life Sciences). Background luminescence in TZM-bl cells was determined without addition of HL2/3 cells.

**Fluorescence Assay**—The N-terminally rhodamine-labeled 17-70 (17-70Rho) was initially dissolved in a small volume of MeOH and DTT at 20× molar excess and then diluted with PBS containing 1 μM DTT at physiologic pH. 17-70Rho dilutions ranged from 2 μM to 16 nM and contained a maximum of 2% MeOH at the highest concentration that was serially diluted in the lower concentrations. Samples were equilibrated overnight in the dark, and fluorescence was measured on a SLM-AMINCO Bowman series 2 luminescence spectrometer with excitation set at 530 nm and emission set at 580 nm. Measurements were carried out in duplicate, and then proteinase K was added to a final concentration of 62 ng/μl, and samples were allowed to incubate for 7 h in the dark with subsequent re-measurement of fluorescence.
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RESULTS AND DISCUSSION

This study describes the contribution of the FP-containing region (N-terminal 34 residues of gp41) toward stability of the well characterized HIV SHB (N36/C34 core). We further demarcate the N-terminal 34-residue region into the canonical FP, which constitutes the highly hydrophobic N-terminal 16 residues (hydrophobic or FP region), and the adjoining polar stretch of residues 17–34 (polar region). Fig. 1 outlines the gp41 fragments used in this study. Because of its large hydrophobic contribution, especially in a trimeric organization, the FP region significantly affects the solubility of both the N70 free peptide and its corresponding N70/C34 core complex in solution. Therefore, free peptides and folded cores were prepared at pH 3.0 in 50 mM formate buffer (solubility of N70 and N70/C34 core at least 30 μM). It has been shown previously that the structure and oligomerization state of gp41 are not affected by low pH (39).

Core Formation in Context of the Fusion Peptide—To analyze the effect of the polar and hydrophobic FP regions on core formation, we employed CD to follow the ability of free N- and C-peptides to form cores. C34 was the constitutive C-peptide for all mixes of N- and C-peptides. Core formation is qualitatively determined by induction of α-helical signal (primarily in unstructured C34) upon mixing of N- and C-peptides (17, 40–42). Fig. 2A compares the additive CD spectra of free peptides versus the spectra of mixed peptides (Fig. 2, A1–A3). We show the N36/C34 core (Fig. 2A1) as a positive control to indicate that at low pH this core forms at physiologic pH, as we have shown previously (42). This is supported by the fact that the analogous simian immunodeficiency virus gp41 folds into an SHB core in either pH 7.5 (43) or 3.0 (44) solutions. As we extend the N-peptide N-terminally, we see inducible core formation. The 17–70 core represents an extension of N36 to include the “polar” intervening region (Fig. 2A2). The N70 core represents an extension of N36 to the N terminus of gp41, which includes both polar and hydrophobic FP regions (Fig. 2A3). Stable cores form with either the additional polar region alone (17–70 core) or both polar and hydrophobic FP regions (N70 core). This is the first evidence showing HIV core formation with the fusion peptide included.

Role of the Polar and Hydrophobic FP Regions toward Stabilizing the Core—We analyzed core thermal stability by monitoring the melting profile at 222 nm using CD. Fig. 2B compares the thermal denaturation profiles of the inducible cores. For the discrete N36/C34 core, we see the same induction and relative T_m at pH 3 as we have shown at physiologic pH in PBS (42) (see Table 1). This indicates that the hydrophobic forces governing the six-helix bundle formation are independent of the ionization state of the solvent-exposed surface (dictated primarily by C34). Extending the N36 substrate N-terminally to include the polar but not hydrophobic FP region (17–70) results in significant core stabilization (T_m of 83 °C). The 17–70 core is also soluble at pH 7, and we find a comparable T_m of 89 °C in PBS at 10 μM with 50 μM DTT (data not shown). The increase in core thermal stability upon extending N36 N-terminally may be due to a more stable N-helix coiled coil or to enhanced C34 binding to a more robust N-helix substrate, or both. Interestingly, the 17–70 and N70 cores have comparable thermal stabilities, although for the N70 core, the N-peptide is extended to include the N-terminal hydrophobic FP region as well. The hydrophobic FP region (16 N-terminal residues) is not part of the heptad repeat, and our data show that it does not contribute to further core stability.

Sequence- and Structure-specific Determinants of Core Stabilization by FP Region—The ability of the N-peptides to present a suitable trimeric coiled coil is correlated to their ability to form cores (8). We therefore analyzed the α-helical nature of each N-peptide and its corresponding core to probe for a structural component to core formation and stabilization. The CD spectra of each free N-peptide as well as C34 C-peptide are shown in Fig. 2C. The estimated α-helical content of each core and corresponding N-peptide is given in Table 1. At physiologic pH, the N36/C34 core is ~95% α-helical (42). As a control, we show here the same approximate α-helical content at pH 3 that correlates with the same thermal stability at either pH 3 (Table

FIGURE 2. A core formation by CD. C34(10 μM) was mixed with N-peptide (10 μM) in 50 mM formate buffer at pH 3.0, and CD spectra were recorded at 25 °C (solid line). The CD spectra of individual C34(10 μM) and corresponding N-peptide(10 μM) were summed and corrected to normalize for path length (broken line). A1, N36 + C34; A2, 17-70 + C34; A3, N70 + C34. B, thermal stability by CD. Temperature dependence of the molar ellipticity at 222 nm for mixtures of C34 and N peptides(10 μM each, 50 mM formate buffer, pH 3.0). N36 + C34 (filled squares), 17-70 + C34 (filled circles), N70 + C34 (filled triangles). C, CD spectra of individual peptides at 10 μM in 50 mM formate buffer, pH 3.0, and 25 °C. C34 (solid line), N36 (filled squares), 17-70 (filled circles), N70 (filled triangles).
TABLE 1
Relative helicity of free N-peptides and their corresponding cores with C34 and thermal stability of formed cores

| Peptide | Helical fraction of free peptide\(^a\) | Helical fraction of core with C34\(^b\) | Melting temperature of core with C34\(^b\) |
|---------|--------------------------------------|--------------------------------------|--------------------------------------|
| N36     | 33                                   | 93                                   | 58                                   |
| 17-70   | 92                                   | 96                                   | 82                                   |
| N70     | 67                                   | 78                                   | 81                                   |

\(^a\) Data are based on molar ellipticity at 222 nm wavelength for free peptide.
\(^b\) Data are based on molar ellipticity at 222 nm wavelength for an equimolar mixture of the indicated peptide with C34.

1) or pH 7 (42). Although the free N36 peptide exhibits partial \(\alpha\)-helical character and aggregates at physiologic pH (40, 45), it retains the ability to organize into a fully \(\alpha\)-helical discrete parallel trimeric coiled coil thereby directing core formation when co-incubated with C34 (1, 40). We show that at pH 3, N36 is mainly unstructured with low \(\alpha\)-helical character (Table 1) but still folds into a core indistinguishable from that at pH 7.

Extending the relatively unstructured N36 to include the adjoining polar region stabilizes the free N-peptide (17-70) to 92% helicity indicating that the polar region is likely involved in coiled-coil organization, which would specifically identify it as a key component of the NHR and not the FP. The core formed with 17-70 is likewise 96% helical indicating that the polar appendage continuous with N36 is helical in the folded core. Taken together, the polar region contributes to core stability by first stabilizing \(\alpha\)-helical structure in the NHR, thereby providing an optimal binding substrate for the CHR to pack against.

Complete N-terminal extension of N36 to include both polar and hydrophobic FP regions results in a core that is \(\sim\)78% \(\alpha\)-helical for the N70 core (leaving \(\sim\)23 residues non-helical of 104 for the core). The reduction from pure helicity (seen for both N36 and 17-70 cores) indicates that the hydrophobic FP is not \(\alpha\)-helical in the context of the folded core. This pattern is reflected in the free peptides themselves. Although 17-70 is fully \(\alpha\)-helical, N70 is \(\sim\)67% \(\alpha\)-helical (\(\sim\)23 residues nonhelical out of 70 for N70 alone) indicating that extension to include the 16-residue hydrophobic FP does not contribute to the \(\alpha\)-helical structure. If the structure of N70 is similar alone to that in its core with C34, then the data indicate that the N-terminal \(\sim\)23 residues are non-helical in both conformations (six helix bundle and N-terminal half of the pre-hairpin intermediate).

Regions that do not contribute directly to either N-substrate trimerization or \(\alpha\)-helicity do not contribute to six-helix bundle stabilization. Comparable thermal stability between 17-70 and N70 cores is a function of the preserved \(\alpha\)-helical N-helix binding substrate in N70 dictated by the 17-70 stretch of residues. The switch from a fully \(\alpha\)-helical 17-70/C34 core to a less \(\alpha\)-helical N70/C34 core may be explained by the hydrophobic 16 N-terminal residues of N70 adopting \(\beta\)-sheet structure with a short unstructured stretch linking the \(\beta\)-sheet hydrophobic FP to the \(\alpha\)-helical coiled coil. For the N70 construct alone, we have shown previously that the hydrophobic N-terminal 16 residues adopt parallel in-register \(\beta\)-sheet conformation in membranes, whereas the NHR region remains \(\alpha\)-helical (27). It is highly likely that the hydrophobic FP of the N70 core, which represents \(\sim\)15% of residues, is adopting parallel \(\beta\)-sheet structure in solution as well.

Oligomerization Character of N-peptides and Their Conjugate Cores by Native Gel—Native gels were used to analyze the oligomerization character of the various folded cores and the free peptides that constitute them. Samples were prepared under identical low pH conditions used for CD and were run on continuous native polyacrylamide gels at pH 3.4. Samples were loaded in duplicate, electrophoresed in parallel, and then gels were either stained with Coomassie Blue or transferred by Western blot to membranes followed by probing with NC-1 monoclonal antibody at physiologic pH. The Coomassie Blue-stained gels were either stained with Coomassie Blue or transferred by Western blot, amounts loaded were as follows: 1.5 \(\mu\)g of N70, 70, and 17-70/C34 core; 2.8 \(\mu\)g of N70/C34 core; 3.2 \(\mu\)g of C34; and 4 \(\mu\)g of N36 and N36/C34 core. For the gel transferred by Western blot, amounts loaded were as follows: 0.35 \(\mu\)g of N70/C34 core, 0.7 \(\mu\)g of N70, and 17-70/C34 core; 1.5 \(\mu\)g of 17-70, 3.5 \(\mu\)g of C34, 5.0 \(\mu\)g of N36, and 10 \(\mu\)g of N36/C34 core.
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is localized near the well, but it remains in equilibrium with the discretely running species. These results are consistent with recent data showing the ability of the FP to direct higher order oligomerization (above the trimeric) in the context of N70, even in the presence of dissociating SDS (30). Our present findings, based on Coomassie Blue staining of free peptides and folded cores in native gels, suggest that the hydrophobic FP region, adopting non-α-helical structure, directs a degree of assembly of folded cores.

Transfer of gel slabs to polymer membranes and probing with NC-1 mAb was used to monitor the oligomeric nature of the free peptides and cores. Fig. 3, A2 and B2, shows the results of probing with NC-1 mAb at physiologic pH. We see that NC-1 binds specifically to bands corresponding to each core, to both free N-peptides 17-70 and N70 but not to free N36 or C34. Our data showing NC-1 interactions with the N36/C34 core, but not with its constituent free peptides, are in direct agreement with both initial characterization of NC-1 (46) as well as recent findings (47). Blumenthal and co-workers (47) delineated the binding substrate of NC-1, showing that it binds the N-helix coiled coil when restricted to trimeric assembly but does not bind the dimeric or monomeric forms. Because NC-1 also binds the six-helix bundle, its target motif must be the outer surface-exposed region of the coiled-coil N-helix trimer (47). Free N36 is a negative control because it either does not form discretely trimeric assemblies (runs as a smear; see Fig. 3B1) or is unstructured in the NC-1 binding region and therefore does not bind NC-1 (Fig. 3B2) as has been shown previously (46). Extending N36 to include the adjacent polar region (17–70) stabilizes the coiled coil of the free peptide to a trimeric organization, based on α-helical contributions shown using CD (Fig. 2C). This is indicated by the ability of NC-1 to interact with free 17-70 (Fig. 4A2). These findings identify the polar region consecutive and N-terminal to N36 as belonging specifically to the NHR and stabilizing trimeric coiled-coil assembly (illustrated in Fig. 5).

Complete extension to include the hydrophobic FP (non-α-helical contributor) does not compromise coiled-coil trimerization of the free N70 peptide, indicated by strong NC-1 binding (Fig. 3A2). This finding indicates that the organization of N70 in solution recapitulates that which is expected to occur for this region in the PHI, which happens to be the key target for fusion inhibitors. Interestingly, the FP adds a sticky component that directs a degree of higher order assembly of trimers in solution that remain in equilibrium with the discrete trimeric (directed by the 17-70 region). Specifically, we interpret the two Coomassie Blue bands of N70 (Fig. 3A1) to represent the discrete oligomeric trimer (fast band), and a dimer of trimers (slow band). In addition, higher order associations of trimers are localized near the well. This interpretation is based on the ability of NC-1, which binds specifically to trimeric NHR coiled coils, to interact strongly with all N70 Coomassie Blue-stained bands (both discrete bands as well as heaviest component localized near the well; compare Fig. 3, A1 with A2).

The bands representing mixed N- and C-peptides are indicative of folded cores in each case based on the following: (i) signature core stability shown using CD under the same conditions; (ii) NC-1 binds in each case indicating that at least a trimeric NHR coiled coil is present; (iii) bands for mixed N- and C-peptides run different from individual peptides alone. The FP drives higher order association of the folded core, as it does the coiled-coil NHR. We see the same fast (single core) and slow (dimer of cores) running bands for the N70 core as seen with N70 alone, except the equilibrium is shifted to the fast, single core band. NC-1 binds strongly to both bands as well as the higher order component localized near the well, despite 8-fold less N70 core loaded in the gel transferred by Western blot. Because of very strong binding of NC-1 to N70 and its core, the film was exposed briefly (Fig. 3A2). Extended exposure clearly outlines NC-1 binding to the slower N70 core band (not shown). This supra-molecular organization of cores is seen only in the context of the FP.

17-70 Is More Efficacious than N36 in Inhibiting gp41-driven Cell-Cell Fusion—Stabilizing the trimeric organization of an N36-derived peptide through covalent interactions significantly boosts its ability to inhibit cell-cell fusion, presumably by enhancing binding to its CHR counterpart exposed during initial stages of fusion (48). The assay is carried out under physiologic conditions, necessitating the solubility of exogenously added test compounds at physiologic pH. Extension of N36 to include the adjacent polar region does not compromise solubility at pH 7. However, it does stabilize both α-helicity and trimeric coiled-coil organization, thereby priming it to bind exposed CHR regions. Therefore, we analyzed the inhibitory efficacy of 17-70 in a cell-cell fusion assay. In Fig. 4, we see that 17-70 inhibits fusion with an IC50 of 391 ± 33 nM, an approximate 2-fold enhancement relative to N36 (866 ± 81 nM). We would expect 17-70 (forms discrete trimers) to be significantly more inhibitory relative to N36 (oligomerizes nonspecifically (40)) given the very low IC50 values for constructs that present a stable coiled-coil trimer either through engineered disulfide bridges (N35–CCG–N13 (48) and NCCG-gp41 (49)) or by the presence of two C-helices in a contiguous construct (5-helix (10)). These differences may be due in part to variation between
target the exposed gp41 CHR region, and perhaps to a lesser degree on monomer interaction with the exposed gp41 NHR region (8). Regardless of the specific target(s), the ability of 17-70 to effectively inhibit gp41-driven cell-cell fusion adds further support to our findings that 17-70 folds into a biologically relevant conformation.

Concluding Remarks—Two general findings are reported in the present study (summarized in Fig. 5). First, we find that the polar region consecutive to N36 adopts α-helical structure and stabilizes coiled-coil organization of the NHR, which serves to form a more stable core with C34 and to more effectively inhibit cell-cell fusion.

Second, and of fundamental importance, this report is the first to identify SHB folding in the context of the FP and to characterize effects of the FP on key gp41 fusion conformations in solution as follows: the SHB (N70/C34 core) and the N-terminal half of the PHI (N70). We find that the FP, adopting nonhelical structure, directs higher order assembly in both conformations, whereas coiled-coil organization is preserved in N70, and trimer of dimer organization is preserved in the N70/C34 core (see Fig. 5). As expected, the nonhelical FP does not further stabilize the fully helical SHB. Clearly, the function of the FP in the context of N70 mirrors its effect on the SHB. These findings are in agreement with recent characterization of N70 in membranes, which directly identified parallel β-sheet organization for the FP, which serves to direct self-organization, whereas the NHR remained α-helical (27).

Although in this study we do not specifically assign β-structure to the FP in solution, we identify its structural contribution as nonhelical, which drives supra-molecular organization, findings that weigh heavily in favor of FP β-structure in the context of both N70 and its cognate core in solution. This study pushes our understanding of gp41-induced viral fusion one step closer to the actual in vivo events by characterizing the elusive FP in the context of key gp41 fusion conformations.

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