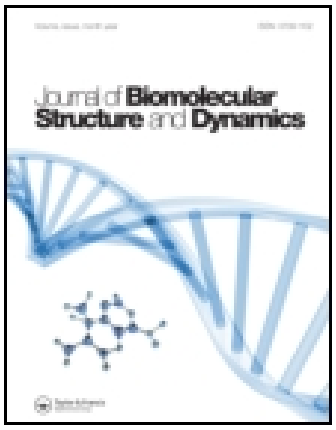

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A Trimeric Structural Subdomain of the HIV-1 Transmembrane Glycoprotein

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A Trimeric Structural Subdomain of the HIV-1 Transmembrane Glycoprotein

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Abstract

HIV-1 infection is initiated by the fusion of viral and cellular membranes with subsequent transfer of viral genetic material into the cell. The HIV-1 transmembrane envelope glycoprotein gp41 plays a major role in this membrane fusion process. Previous studies have shown that a stable, α-helical, trimeric structural domain of gp41 consists of N-terminal 51-residue (N-51) and C-terminal 43-residue (C-43) extraviral segments. This α-helical, trimeric complex has been proposed to form the core of the membrane fusion-active conformation of the HIV-1 envelope. We show here that a stable subdomain can be formed by shorter 36-residue (N-36) and 34-residue (C-34) peptides corresponding to the central regions of N-51 and C-43, respectively. In isolation, N-36 is predominantly aggregated, while C-34 is unfolded. Upon mixing, however, these peptides form a stable, α-helical, discrete trimer of heterodimers (the melting temperature of a 10 μM solution is 64°C at pH 7). Thus, this subdomain displays the salient features of the stable core structure of the isolated gp41 protein. Our results also provide strong support for the notion that short peptides can form unique, cooperatively folded subdomains, in which elements of secondary structure are stabilized by native-like tertiary interactions.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infects $CD4^+$ macrophages and T-helper cells both *in vivo* and in cell culture (for a recent review, see ref. 1). Infection begins with the binding of virus to its primary cell-surface CD4 receptor (1). HIV-1 then gains entry into target cells through fusion of the viral envelope with the plasma membrane (2,3). Both receptor binding and membrane fusion are mediated by the viral envelope glycoprotein (Env). Env is synthesized as a precursor gp160, which is processed proteolytically to yield the soluble surface subunit gp120 and the transmembrane subunit gp41 (4,5). gp120 is non-covalently associated with gp41 that anchors the complex in the viral membrane. The native gp120-gp41 molecule forms oligomers in virions, but the assignment of this oligomerization state is not yet definitive. The gp120 subunit is responsible for the binding of HIV-1 to the cellular CD4 receptor (6,7,8), while the gp41 subunit contains the hydrophobic 'fusion peptide' at its amino-terminus and plays a major role in mediating viral fusion with target cells (9-11). In addition, accessory cell-surface co-receptor molecules, CXCR-4 and CCR-5, have recently been identified and shown to interact specifically with the CD4-gp120 complex (12-18).

The HIV-1 gp41 molecule is a transmembrane glycoprotein of 41 kDa (Figure 1). The ectodomain (that is, the extraviral portion) of gp41 is responsible for association with gp120, and is directly involved in the fusion reaction (19). As in many other viral fusion proteins, the N-terminal fusion peptide of the HIV-1 gp41 is followed by two sequences containing a 4-3 repeat of hydrophobic amino acids predicted to form a coiled coil (20,21). We have identified a stable, α-helical, trimeric structure comprising two peptides, N-51 and C-43, that overlap with the two predicted coiled-coil regions in the gp41 sequence (see Figure 1A) (22). These pep-

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tides associate preferentially and fold autonomously to form a stable, α-helical trimer of heterodimers (22). We have proposed that N-51 forms an interior, parallel, homotrimeric, coiled-coil core, against which three C-43 helices pack in an antiparallel fashion (Figure 1B), and that this α-helical, trimeric complex represents the core of the membrane fusion active conformation of the HIV-1 envelope (22). Wiley and co-workers have also shown that the gp41 extraviral fragment forms an α-helical, rod-like oligomer (23).

Synthetic peptides corresponding to the sequences of the two predicted coiled-coil regions of gp41 have been shown to inhibit HIV-1 syncytium formation and infectivity (24-26). Single proline substitutions in the N-terminal heptad repeat of gp41 abolish Env membrane fusion activity (27-29). Taken together, these results suggest that the two heptad repeat regions in gp41 and hence, formation of the coiled-coil structure, are critical for HIV-1 Env-mediated membrane fusion.

The structures of large proteins are often modular in nature, consisting of multiple structural subdomains (for a review, see ref. 30). Isolated domains or subdomains are generally considered to be the minimal independent structural and functional elements of a protein structure (for reviews, see 31-33). Here we describe the minimization of the trimeric structural domain of the HIV-1 transmembrane envelope glycoprotein gp41 (22). We find that the minimal stable envelope subdomain consists of a 36-residue peptide (N-36) and a 34-residue peptide (C-34), derived from the trimeric N-51/C-43 complex of gp41. In isolation, N-36 is predominantly aggregated, while C-34 is unfolded. However, when these peptides are mixed, they associate preferentially to form a stable, α-helical, discrete trimer of heterodimers,

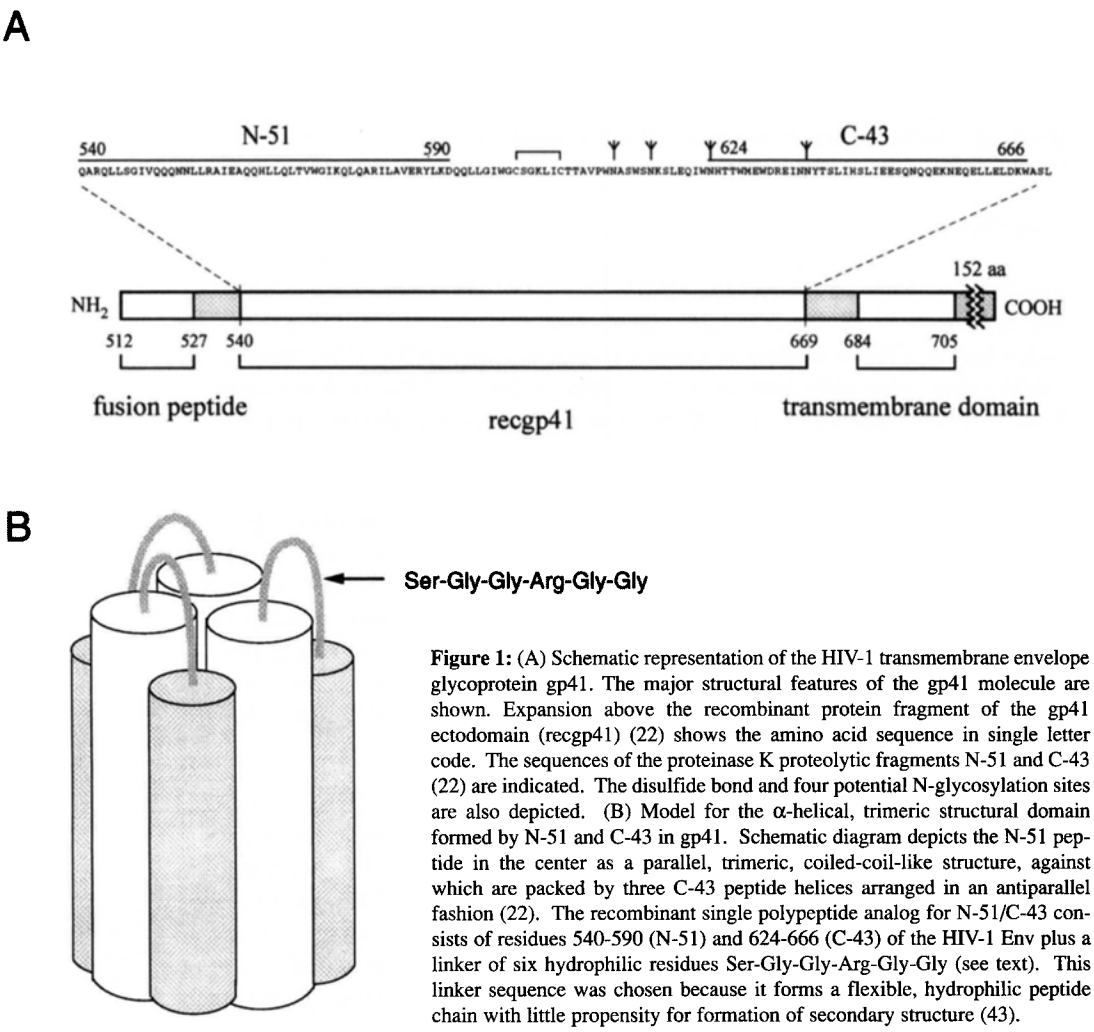

Figure 1: (A) Schematic representation of the HIV-1 transmembrane envelope glycoprotein gp41. The major structural features of the gp41 molecule are shown. Expansion above the recombinant protein fragment of the gp41 ectodomain (recgp41) (22) shows the amino acid sequence in single letter code. The sequences of the proteinase K proteolytic fragments N-51 and C-43 (22) are indicated. The disulfide bond and four potential N-glycosylation sites are also depicted. (B) Model for the α-helical, trimeric structural domain formed by N-51 and C-43 in gp41. Schematic diagram depicts the N-51 peptide in the center as a parallel, trimeric, coiled-coil-like structure, against which are packed by three C-43 peptide helices arranged in an antiparallel fashion (22). The recombinant single polypeptide analog for N-51/C-43 consists of residues 540-590 (N-51) and 624-666 (C-43) of the HIV-1 Env plus a linker of six hydrophilic residues Ser-Gly-Gly-Arg-Gly-Gly (see text). This linker sequence was chosen because it forms a flexible, hydrophilic peptide chain with little propensity for formation of secondary structure (43).

each comprising N-36 and C-34. Thus, the trimeric structural domain of gp41 has been truncated by nearly a quarter. The small size of such a subdomain makes it more amenable to synthetic chemistry and has facilitated its structural determination at atomic resolution (34).

Materials and Methods

Production of HIV-1 gp41 Peptides. Plasmid pN51/C43-L6, encoding the single polypeptide model for the N-51/C-43 complex (see Figure 1B), was constructed by oligonucleotide-directed mutagenesis (35) of p41-130 (22). Plasmid pN41/C34-L6 for the expression of the N41(L6)C34 peptide was derived from pN51/C43-L6. Standard recombinant DNA techniques were used (36). The recombinant proteins N51(L6)C43 and N41(L6)C34 were expressed in *E. coli* BL21(DE3) pLysS using the T7 expression system (37). Cells, freshly transformed with an appropriate plasmid, were grown to late log phase. Protein expression was induced by addition of 1 mM isopropylthio-β-D-galactoside (IPTG). After another 3 hr of growth at 37°C, the bacteria were harvested by centrifugation, and the cells were lysed by glacial acetic acid (38). N51(L6)C43 and N41(L6)C34 peptides were purified from the soluble fraction by gel filtration (Sephadex G-50). Peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography (HPLC), using a Vydac C-18 preparative column and a linear gradient of acetonitrile containing 0.1% trifluoracetic acid. The N-36 and C-34 peptides were synthesized by solid-phase FMOC methods and purified by HPLC as described above. In each peptide, the N-terminus is acetylated and the C-terminus is amidated. The identity of each HPLC-purified peptide was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems).

CD Spectroscopy. Circular dichroism (CD) spectra were recorded on an AVIV Model 62DS CD spectrometer equipped with a thermoelectric temperature controller. The cuvettes used for wavelength spectra and for thermal unfolding studies were 0.1 cm and 1 cm in pathlength, respectively. Peptide concentrations were determined by absorbance at 280 nm in 6 M GuHCl (39). Each CD sample contained 10 μM peptide in phosphate-buffered saline (PBS buffer) at pH 7. The wavelength dependence of molar ellipticity, [θ], was monitored at 0°C by 5 scans in 1 nm increments with a sampling time of 10 seconds. The CD signal at 222 nm was measured as a function of temperature, and thermal melts were performed in 2°C steps, with 2 min of equilibration at each temperature, and an acquisition time of 0.5 min. The midpoint of the thermal unfolding transition (T_m) was estimated from the maximum of the first derivative, with respect to T^{-1} (Kelvin), of the CD signal at 222 nm (40). Helix content was estimated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation by short helices, -33,000 deg cm^2 dmol^{-1} (41).

Analytical Ultracentrifugation. Apparent molecular masses were determined by sedimentation equilibrium with a Beckman XL-A

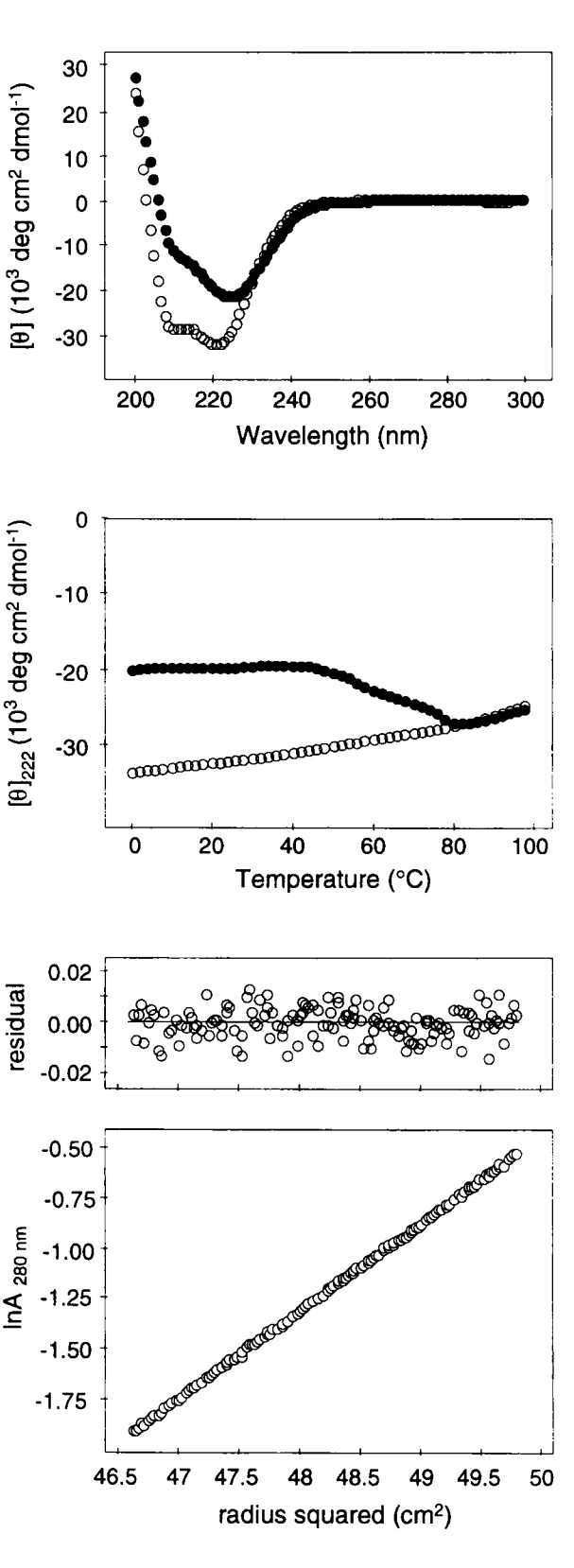

Figure 2: (A) Circular dichroism (CD) spectra of the N51(L6)C43 peptide (10 μM) before heating (closed circles) and after heating (open circles) in PBS buffer (pH 7) at 0°C. (B) Temperature dependence of the CD signal at 222 nm for N51(L6)C43 (10 μM) before heating (closed circles) and after heating (open circles) in PBS buffer (pH 7). (C) Sedimentation equilibrium centrifugation of N51(L6)C43 (after heating) at concentrations of 10 μM in PBS buffer (pH 7) at a rotor speed of 15,000 rpm. The observed molecular mass is 35,038 Da (the calculated mass for a trimer is 35,683 Da). The random distribution of the residuals indicates that the data fit well to an ideal single-species model.

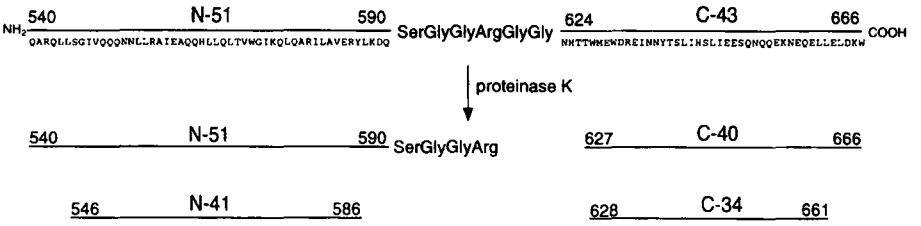

Figure 3: Proteinase K digestion of the aggregated N51(L6)C43 peptide. The proteolytic digestion (see Materials and Methods) produces two major fragments and two minor fragments. The two major fragments with masses of 6427 and 5023 correspond to N-51, spanning residues 540 to 590, plus an N-terminal methionine residue and a C-terminal linker of four residues Ser-Gly-Gly-Arg, and C-40, spanning residues 627 to 666, respectively. The two minor proteolytic fragments with masses of 4746 and 4251 correspond to N-41, spanning residues of 546 to 586, and C-34, spanning residues 628 to 661.

Optima analytical ultracentrifuge at 20°C. Samples were dialyzed against the reference buffer (50 mM sodium phosphate and 150 mM NaCl, pH 7) for at least 12 h. For the N51(L6)C43 peptide (after heating) and the N41(L6)C34 peptide, three samples of total peptide concentration of 10, 25, and 50 μM were analyzed at rotor speeds of 15,000 or 18,000 rpm. For the N-36/C-34 complex, three samples of total peptide concentration of 10, 35, and 100 μM were analyzed at rotor speeds of 20,000 or 23,000 rpm. Data were fit to a single ideal species model plot of ln(absorbance) versus radial distance squared. Nonrandom residuals, indicative of aggregation or derivations from ideality, were observed only for the N41(L6)C34 peptide. Specific volumes and solvent densities were calculated as described by Laue *et al.* (42).

Proteolysis Experiments. For a typical digestion, 2 μg of protein were incubated with 0.1 μg of proteinase K (Boehringer Mannheim) at room temperature for 2 hr in 10 μl of PBS buffer (50 mM sodium phosphate and 150 mM NaCl) at pH 7. Proteolysis was quenched by addition of phenylmethylsulphonyl fluoride (PMSF, Sigma) to a final concentration of 2 mM. The proteolytic fragments were separated and purified by HPLC as described above, and characterized by N-terminal sequencing and mass spectrometry. Five residues at the N-terminus of each proteolytic fragment were sequenced for each identified fragment.

Results and Discussion

Single Polypeptide Model for the Trimeric N-51/C-43 Complex. The ectodomain of the HIV-1 envelope protein gp41 has been shown to fold into a stable, α-helical, trimeric structure comprising the N-51 and C-43 peptides in isolation (22). We designed and constructed a unimolecular (*i.e.*, single polypeptide) analog of N-51/C-43. The single polypeptide version is named N51(L6)C43, in which the C-terminus of the N-51 peptide is connected to the N-terminus of the C-43 peptide, via the six-residue hydrophilic linker Ser-Gly-Gly-Arg-Gly-Gly (see Figure 1B). This recombinant protein was expressed in *E. coli* at high levels (~40 mg of protein per liter of culture) and purified by reverse-phase HPLC (see Materials and Methods).

The recombinant N51(L6)C43 polypeptide forms an insoluble aggregate at concentrations above ~6 μM in PBS buffer. The CD spectrum of this tethered peptide shows a strong minimum at 225 nm, which is not typical of an α-helix (Figure 2A). After heating above 60°C, however, the molecule folds into an α-helical structure, as shown by the minima near 208 and 222 nm (Figure 2). After heating, the peptide is soluble in PBS buffer up to concentrations of 55 μM and is ~ 90% helical with a melting temperature (T_m) over 100°C (Figure 2B). In addition, sedi-

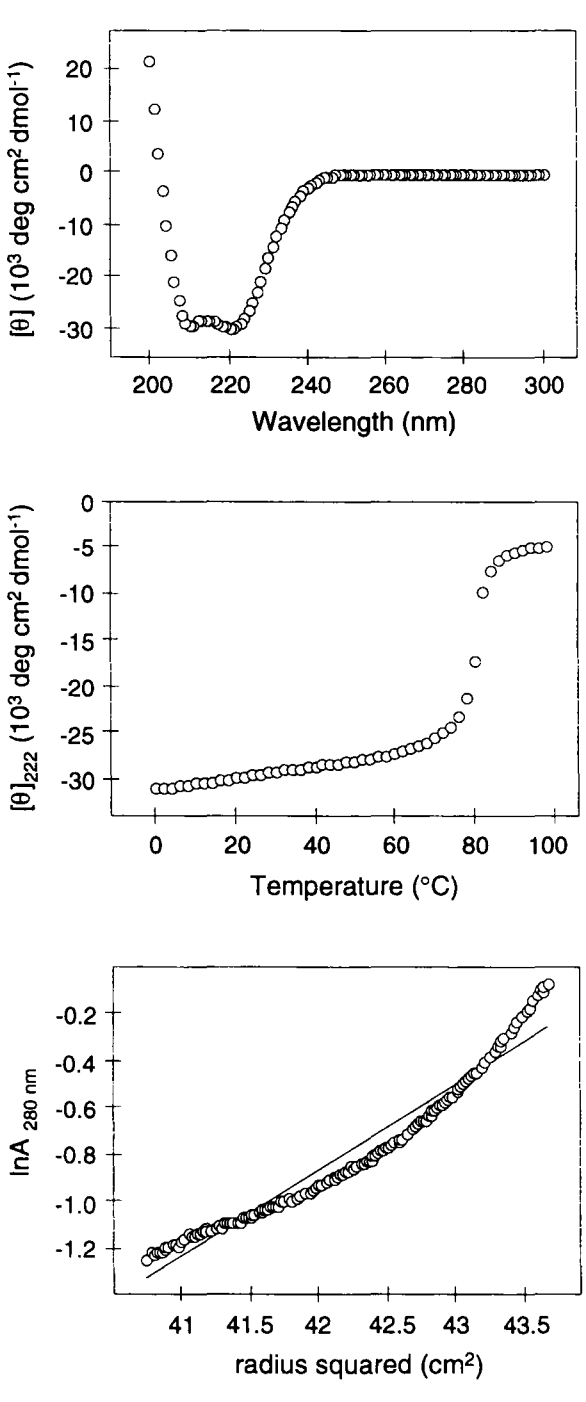

Figure 4: (A) CD spectrum for the N41(L6)C34 peptide (10 μM) in PBS buffer (pH 7) at 0°C. (B) Temperature dependence of the CD signal at 222 nm for N41(L6)C34 (10 μM) in PBS buffer (pH 7). (C) Sedimentation equilibrium centrifugation of N41(L6)C34 at concentrations of 25 μM in PBS buffer (pH 7) at a rotor speed of 15,000 rpm. Nonrandom residuals fit assuming a model for a single ideal species, indicating that higher-order association was occurring.

mentation equilibrium experiments demonstrate that this tethered peptide, after heating, is a trimer in solution over the concentration range measured (10 to 50 μM) (Figure 2C).

Proteolysis Yields N-36 and C-34. We carried out a proteolytic digestion of the N51(L6)C43 aggregate (*i.e.*, before heating) with proteinase K. The peptide fragments were separated and purified by HPLC. Each peptide was identified by N-terminal sequencing (five residues) and mass spectrometry. The digestion yields, in addition to N-51 (residues 540 to 590) with a C-terminal Ser-Gly-Gly-Arg (observed mass: 6428; expected: 6425), and C-40 (residues 627 to 666) (observed mass: 5023; expected 5022), two shorter peptide fragments: N-41, spanning residues 546 to 586 (observed mass: 4746; expected 4743), and C-34, spanning 628 to 661 (observed mass: 4253; expected: 4249) (Figure 3). These shorter fragments correspond to the central regions of N-51 and C-43, respectively.

To further minimize the subdomain structure, we produced a bacterially expressed polypeptide N41(L6)C34. In this model, the C-terminus of the N-41 peptide is connected to the N-terminus of the C-34 peptide by the linker Ser-Gly-Gly-Arg-Gly-Gly. The recombinant protein N41(L6)C34 is soluble in PBS buffer up to concentrations of 350 μM. CD spectra demonstrate that this molecule is ~90% helical and exhibits a cooperative, irreversible thermally-induced unfolding transition with an apparent T_m of 80°C under physiological conditions (Figure 4). In addition, sedimentation equilibrium experiments demonstrate that the apparent molecular weight of N41(L6)C34 changes with concentration, consistent with the formation of higher-order oligomers (Figure 4c and data not shown). Extensive proteolytic digestion of the N41(L6)C34 aggregate with proteinase K generates two peptide fragments with masses of 4124 (expected 4123) and 4719 (expected 4720), corresponding to N-36, spanning residues 546 to 581, and C-34, spanning 628 to 661, with the linker residues Ser-Gly-Gly-Arg-Gly-Gly at the N-terminus, respectively.

A Trimeric N-36/C-34 Subdomain. To investigate these shorter fragments further, synthetic peptides with blocked termini, corresponding to N-36 and C-34, were generated (see Materials and Methods). The CD spectrum of an equimolar mixture of the N-36 and C-34 peptides is typical of an α-helix, displaying the characteristic minima at 208 and 222 nm (Figure 5A). This complex is fully helical (Figure 5A) and very stable, unfolding reversibly with a T_m of 64°C under physiological conditions (Figure 5B). Sedimentation equilibrium centrifugation of the N-36/C-34 complex at 20°C indicates that the complex consists of three molecules each of N-36 and C-34 (Figure 5C). We conclude that the N-36 and C-34 peptides associate preferentially to form a stable, α-helical, discrete trimer of heterodimers, each consisting of N-36 and C-34.

The isolated N-36 peptide does not display a typical α-helix CD spectrum (Figure 5A). Moreover, the CD signal of N-36 is concen-

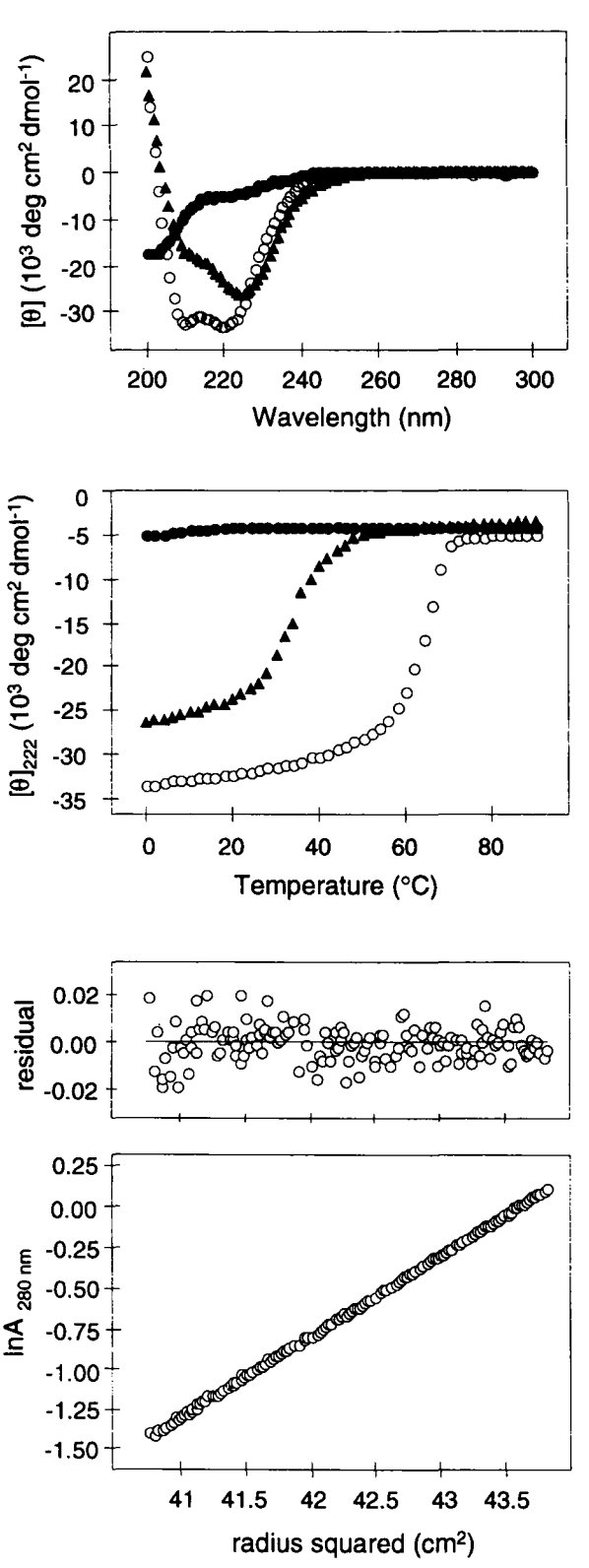

Figure 5: (A) CD spectra for the isolated N-36 (10 μM) (closed triangles) and C-43 (10 μM) (closed circles) peptides, and a mixture of N-36 (10 μM) and C-34 (10 μM) (open circles) in PBS buffer (pH 7) at 0°C. (B) Temperature dependence of the CD signal at 222 nm for the three species described above (10 μM, pH 7). (C) Sedimentation equilibrium centrifugation of the N-36/C-34 complex at concentrations of 35 μM in PBS buffer (pH 7) at a rotor speed of 20,000 rpm. The observed molecular mass is 24,792 Da (the calculated mass for a trimer is 25,360 Da). The random distribution of the residuals indicates that the data fit well to an ideal single-species model.

tration-dependent (data not shown), and the solution becomes turbid upon heat denaturation. These observations indicate that N-36 has a strong tendency to aggregate. The isolated C-34 peptide, on the other hand, is unfolded in solution (Figure 5A) and does not undergo a thermal transition (Figure 5B).

Conclusion

By proteolytic digestion of a single polypeptide analog for the trimeric N-51/C-43 complex, we have identified a stable, α-helical, trimeric structure composed of two shorter peptides, N-36 and C-34, that correspond to the central regions of N-51 and C-43, respectively. The biophysical properties are very similar between the N-36/C-34 and N-51/C-43 complexes (Figure 5 and ref. 22).

It is striking that the information required to specify the fold of the stable core structure of the gp41 ectodomain in isolation is contained within the small N-36/C-34 subdomain. This subdomain therefore can be considered to be an autonomously folding unit, which we propose forms the core of the membrane fusion active conformation of the HIV-1 envelope. This subdomain displays the significant features of the stable core structure of the isolated gp41 ectodomain. Thus, the stable, α-helical, trimeric N-36/C-34 complex identified here provides a simplified and more tractable model for understanding determinants of the structure and function of the transmembrane subunit of the HIV-1 envelope.

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