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Supporting Information

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Investigation of the Structure and Dynamics of the Capsid–Spacer Peptide 1–Nucleocapsid Fragment of the HIV-1 Gag Polyprotein by Solution NMR Spectroscopy**

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Supporting Information

MATERIALS AND METHODS

Materials. Single-stranded DNA, the DNA (-) Primer binding site, 5'-dGTCCCTGTTCGGGC, and the alternating DNA TG motif, 5'-d$(TG)_{15}$, were purchased from Integrated DNA Technologies, Inc. Oligonucleotides were dissolved in deionized water, and dialyzed overnight (Spectra/Pro® DispoDialyzer®, 500 Da cut-off) in a buffer containing 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 0.1 mM $ZnCl_2$, and 1 mM dithiothreitol (DTT). *E. Coli tRNA* was purchased from Sigma-Aldrich (catalog #R1753). The HIV-1 protease inhibitor Darunavir was obtained from the NIH AIDS Reagent Program.

Protein expression and purification. HIV-1 CA-SP1-NC (plasmid pNL4-3), nucleocapsid (plasmid HXB2) and protease (plasmid HXB2 with point mutations Q7K, L33I, L63I, C67A and C95A, to prevent auto-proteolysis and thiol oxidation)[1] were sub-cloned in a pET-11a vector and expressed in BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technologies). Uniformly $^2H/^{15}N/^{13}C$-labeled proteins were expressed at 37°C using our earlier protocol.[2] Briefly, cells were grown in 1 L minimal M9 medium containing 0.3 g/L $^2H/^{15}N/^{13}C$ Isogro (Sigma-Aldrich), ^2H_2O, 1g/L $^{15}NH_4Cl$ and 3g/L $^2H_7, ^{13}C_6$-D-glucose for $^2H/^{15}N/^{13}C$ labeling; 0.3 g/L $^2H/^{15}N$ Isogro (Sigma-Aldrich), ^2H_2O, 1g/L $^{15}NH_4Cl$ and 3g/L $^2H_7, ^{12}C_6$-D-glucose for $^2H/^{15}N$ labeling; and 1g/L $^{15}NH_4Cl$ for ^{15}N labeling. Cells, induced with 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) at an optical density of A_{600} ~0.6, were harvested 8 hours later.

For both CA-SP1-NC and nucleocapsid constructs, cells were re-suspended in a lysis buffer containing 20 mM Tris, pH 8.0, 0.1 mM $ZnCl_2$, 5 mM β-mercaptoethanol (BME), and 1 cOmplete Protease Inhibitor Cocktail tablet (Roche Applied Science). Nucleic acids were precipitated by adding 4% (w/v) polyethyleneimine, pH 8.0 (Sigma-Aldrich) to a final concentration of 0.4% (w/v). Proteins were purified by combination of ion exchange and size exclusion chromatography. The cell lysates were loaded onto a HiPrep 16/10 Q FF column (GE Healthcare) with a 0 to 1 M NaCl gradient in buffer containing 20 mM Tris, pH 8.0, 0.1 mM $ZnCl_2$ and 5 mM BME. Relevant flow-through fractions were loaded onto a HiPrep 16/10 SP FF column (GE Healthcare) with a 0 to 1 M NaCl gradient in buffer containing 20 mM Tris, pH 8.0, 0.1 mM $ZnCl_2$. In the case of CA-SP1-NC, the eluted protein was concentrated (Amicon ultra-15, 10 kDa cut-off) and loaded onto a HiLoad 26/60 Superdex 200 column (GE Healthcare) pre-equilibrated with 20 mM Tris, pH 8, 0.1 mM $ZnCl_2$, 5 mM BME and 50 mM NaCl. For the isolated nucleocapsid construct, the eluted protein from the HiPrep 16/10 SP FF column was dialyzed in 20 mM Tris, pH 8, 0.1 mM $ZnCl_2$, and 5 mM BME. Both proteins were further purified using a Mono S™ 10/100 GL column (GE Healthcare) with a 0 to 1 M NaCl gradient in buffer containing 20 mM Tris, pH 8, 0.1 mM $ZnCl_2$. This purification scheme results in ≥ 99% pure CA-SP1-NC protein with a yield of ≥ 25 mg/Liter ($^2H/^{15}N$ labeled protein); extreme care, however, is required while handling the protein owing to its susceptibility to proteolysis.

For HIV-1 protease, a modification of the protocol from Gulnik et al[3] was employed. Briefly, cells were resuspended in buffer containing 50 mM Tris, pH 8.0 (buffer A), sonicated and centrifuged. Inclusion bodies were first washed with buffer A, then with buffer A containing 0.1% Triton X-100, 1 M NaCl, and 1 M urea respectively, and finally with buffer A alone. Purified inclusion bodies were then resuspended in 8 M urea and purified using combination of anion/cation exclusion chromatography under

denaturing conditions. Eluted protein from a HiPrep 16/10 SP FF column (GE Healthcare) was refolded in buffer containing 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 5 mM BME, and 10% glycerol.

All constructs were verified by DNA sequencing and mass spectrometry (using an Agilent 1100 LC/MS system equipped with an Agilent Zorbax 300SB-C3 column coupled to a quadrupole mass analyzer).

Analytical ultracentrifugation. Solutions of CA-SP1-NC in 50 mM NaCl, 20 mM sodium phosphate (pH 6.5), 0.1 mM $ZnCl_2$ and 1 mM DTT at concentrations of 104, 42, 24, 12 and 4 μM were prepared by dilution of a 0.25 mM stock solution. Concentrated samples were loaded into 3 mm 2-channel epon centerpiece cells (100 μL), whereas low concentration samples were loaded into 12 mm 2-channel epon centerpiece cells (400 μL). Sedimentation velocity experiments were conducted at 50 krpm at temperatures of 20 and 35°C on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge using both the absorbance (280 nm) and Rayleigh interference (655 nm) optical systems. Time-corrected data[4] were initially analyzed in SEDFIT 14.3e[5] in terms a continuous c(*s*) distribution covering an *s* range of 0.0 – 6.0 S with a resolution of 120 and a confidence level (F-ratio) of 0.68. Excellent fits were obtained and *c(s)* profiles were consistent with a reversible CA-SP1-NC monomer-dimer self-association. Data were subsequently exported into SEDPHAT 10.58f[6] for further analysis. The solution density (ρ) and viscosity (η) were measured experimentally at 20ºC on an Anton Paar DMA 5000 density meter and an Anton Paar AMVn rolling ball viscometer, respectively, and corrected for temperature. The partial specific volumes (*v*) for CA-SP1-NC at the various temperatures studied were calculated based on the amino acid composition using SEDNTERP 1.09[7] and corrected for uniform ^{15}N labeling. Sedimentation coefficients were corrected to standard conditions $s_{20,w}$. Global absorbance and Rayleigh interference data were analyzed separately in terms of a reversible monomer-dimer self-association by direct Lamm equation modeling[8] in SEDPHAT 10.58f to obtain the equilibrium constant. Excellent fits were obtained with r.m.s.d. values ranging from 0.004 to 0.015 fringes (Figure S4) or 0.002 to 0.007 absorbance units. The protein extinction coefficient at 280 nm and interference signal increment[9] used for the calculations were determined based on the amino acid composition in SEDNTERP 1.09 and SEDFIT 14.3e, respectively.

To investigate the effects of nucleotides, samples of CA-SP1-NC at natural isotopic abundance were treated with two equivalents of ΔP(-)PBS or 5'd-$(TG)_{15}$ DNA. Mixtures with ΔP(-)PBS DNA were studied at loading concentrations of 90, 45, 22 and 10 μM, whereas mixtures with 5'-d$(TG)_{15}$ DNA were studied at loading concentrations of 68, 22 and 10 μM. Under these conditions, the DNA binding sites on the protein were saturated (1:1 stoichiometry, see below). Interference data were collected and analyzed globally as above, except that the self-associating species is now a 1:1 DNA: protein complex (Figure S4). Excess free DNA was also accounted for in the model, which assumed a partial specific volume of 0.55 cm^3g^{-1} and a signal increment *dn/dc* of 0.185 cm^3g^{-1}. Excellent fits were obtained with r.m.s.d. values ranging from 0.006 to 0.022 fringes.

To confirm the CA-SP1-NC-DNA stoichiometry, sedimentation studies were also carried out using isolated nucleocapsid construct and ΔP(-)PBS or 5'd-$(TG)_{15}$ DNA (Figure S5). Samples were loaded into 12 mm 2-channel epon centerpiece cells (400 μL) and sedimentation velocity experiments were conducted at 55 krpm and 20°C on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge using both the absorbance (280 or 286 nm) and Rayleigh interference (655 nm) optical systems. Time-corrected data[4] were analyzed in SEDFIT 14.3e[5] in terms a continuous c(*s*) distribution covering an *s*

range of 0.0 – 4.0 S with a resolution of 100 and a confidence level (F-ratio) of 0.68. The partial specific volume (v) for nuleocapsid was calculated based on the amino acid composition using SEDNTERP 1.09[7]. Excellent fits were obtained with r.m.s.d. values ranging from 0.0033 to 0.0057 absorbance units and 0.0022 to 0.0073 fringes. All experiments were carried out in 50 mM NaCl, 20 mM sodium phosphate, pH 6.5, 0.1 mM $ZnCl_2$ and 1 mM DTT.

NMR Sample Preparation. All heteronuclear NMR experiments were performed on uniformly $^{15}N/^{13}C/^{2}H$ labeled samples (unless stated otherwise) prepared in buffer containing 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 0.1 mM $ZnCl_2$, 93% H_2O/7% D_2O and 1 mM DTT. Aligned samples were prepared using DMPC/06:0 Diether PC bicelles (q=3) (Avanti polar lipids) doped with 0.1% PEG-2000-PE (Avanti polar lipids) to improve bicelle stability.[10] Backbone amide ($^{1}D_{NH}$) RDC data were measured on samples containing 0.2, 0.1 and 0.5 mM (in subunits) CA-SP1-NC and 0.1 mM nucleocapsid. For samples with DNA, a protein:DNA molar ratio of 1:2 was used. For heteronuclear ^{15}N-$\{^{1}H\}$ NOE measurements, a protein concentration of ~ 0.3-0.35 mM in subunits was employed.

NMR Spectroscopy. All heteronuclear NMR experiments were carried out at 35°C on Bruker 500 and 800 MHz spectrometers equipped with z-gradient triple resonance cryoprobes. Spectra were processed using NMRPipe[11] and analyzed using the CCPN software suite.[12] Sequential ^{1}H, ^{15}N, and ^{13}C backbone resonance assignments were performed using conventional TROSY-based[13] through-bond three-dimensional triple resonance experiments.[14] Backbone amide ($^{1}D_{NH}$) RDCs were measured on $^{2}H/^{15}N$-labeled proteins using the TROSY-based ARTSY technique[15] and analyzed using Xplor-NIH.[16] Heteronuclear ^{15}N-$\{^{1}H\}$ NOE measurements were carried out on uniformly $^{2}H/^{15}N$-labeled CA-SP1-NC protein at ^{1}H frequency of 800 MHz. The ^{15}N-$\{^{1}H\}$ NOE and reference spectra were recorded with a 10 second saturation time for the NOE measurement and equivalent recovery time for the reference measurement in an interleaved manner, each preceded by an additional 1 sec recovery time.

Table S1. Monomer-dimer equilibrium for HIV-1 CA-SP1-NC, full-length capsid (CA_{FL}) and the C-terminal domain ($CA_{276-363}$ respectively) obtained by analytical ultracentrifugation.

Construct	K_{dimer} (μM)	
	20°C	35°C
CA-SP1-NC		
no DNA	10±3	46±9[c]
+ ΔP(-)PBS DNA	25±3	68±18[c]
+ 5'd-$(TG)_{15}$	17±2	44±14
CA_{FL}[b]	20±3	82±9
$CA_{276-231}$[b]	7±3	16±3

[a]The confidence intervals in the K_{dimer} are reported as ±1 standard deviation.
[b]From ref. S2.
[c]The fraction of subunits that are dimeric in the NMR RDC experiments, conducted at 35°C and a subunit concentration of 0.2 mM, is 0.71 and 0.66 in the absence and presence of ΔP(-)PBS DNA, respectively. See Fig. S4 for additional details.

Table S2. Comparison of backbone amide ($^1D_{NH}$) RDCs and ^{15}N-{^1H} Heteronuclear NOE values for the C-terminal tail of capsid (residues 353-363) and SP1 (residues 364-377) measured on CA-SP1-NC in the absence and presence of ΔP(-)PBS DNA[a,b,c].

	no DNA		with ΔP(-)PBS DNA[d]	
Residues	$^1D_{NH}$ (Hz)[d]	^{15}N-{^1H} NOE	$^1D_{NH}$ (Hz)[e]	^{15}N-{^1H} NOE
V353	-6.8±0.31	0.53±0.05	-6.6±0.33	0.52±0.06
G354	0.9±1.21	0.45±0.12	1.1±0.68	0.37±0.11
G355	-1.9±0.16	0.34±0.03	-3.4±0.12	0.37±0.03
G357	-3.1±2.90	0.23±0.15	-0.9±1.03	0.27±0.13
A360	-2.3±0.29	0.24±0.04	-2.2±0.19	0.29±0.04
R361			-1.5±0.13	0.38±0.03
V362			-1.7±0.08	0.38±0.02
L363	-3.7±0.28	0.23±0.05	-3.3±0.15	0.27±0.04
A364	-2.0±0.08	0.22±0.01	-2.0±0.05	0.22±0.01
E365	-3.2±0.23	0.19±0.04	-2.6±0.12	0.16±0.02
M367	-0.5±0.10	0.22±0.02	-1.5±0.08	0.20±0.02
A374	-1.6±0.09	0.28±0.02	-0.9±0.05	0.29±0.01
T375	-0.3±0.12	0.25±0.02	-0.8±0.08	0.25±0.02
I376	-4.4±0.08	0.23±0.02	-4.1±0.06	0.25±0.02
M377	-5.0±0.13	0.20±0.02	-4.1±0.08	0.23±0.02

[a]All RDCs (measured in lipid bicelles) and ^{15}N-{^1H} NOE data were acquired at protein concentrations of 0.2 and ~0.3-0.35 mM, respectively, in sub-units.
[b]Cross-peaks for residues 358, 359, 366 and 368-372 were not observed due to line broadening. Residues 356 and 373 are prolines.
[c]Residues in bold were predicted to form a helix[17] but judging from the low (<0.4) ^{15}N-{^1H} NOE values, values close to zero for the backbone amide ($^1D_{NH}$) RDCs, and minimal deviations from random coil backbone chemical shifts (see Figure 4, main text), it can be concluded that this region is intrinsically disordered in solution even in presence of nucleic acids.
[d]Protein:DNA molar ratio of 1:2.

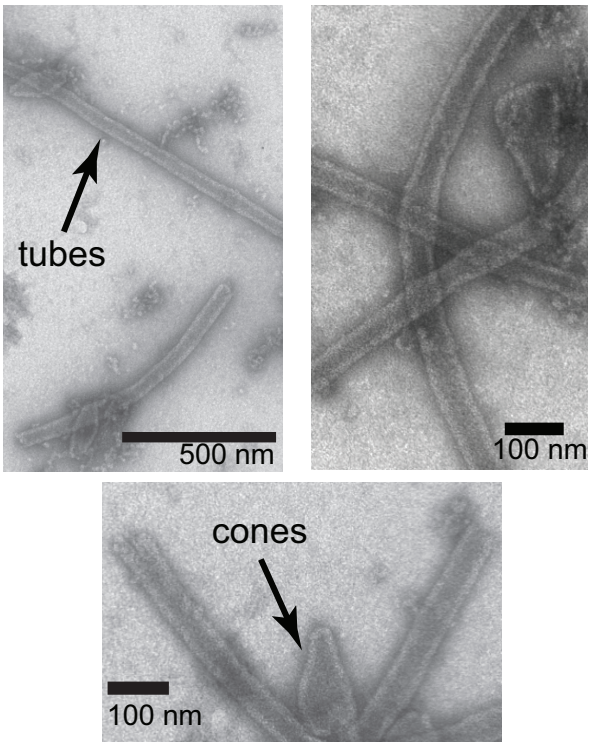

Figure S1. Negatively stained electron microscopy images illustrating tube and cone assemblies observed for the CA-SPI-NC fragment of HIV-1 Gag. Assembly was initiated by the addition of ~0.3 mM *E. coli* tRNA and 0.5 M NaCl to ~0.15 mM (in subunits) CA-SP1-NC in 50 mM Tris, pH 8.0. Samples were incubated at 37°C for 15-30 min after addition of tRNA and salt, and negatively stained with 2% uranyl acetate. Images were acquired as described in ref. 18.

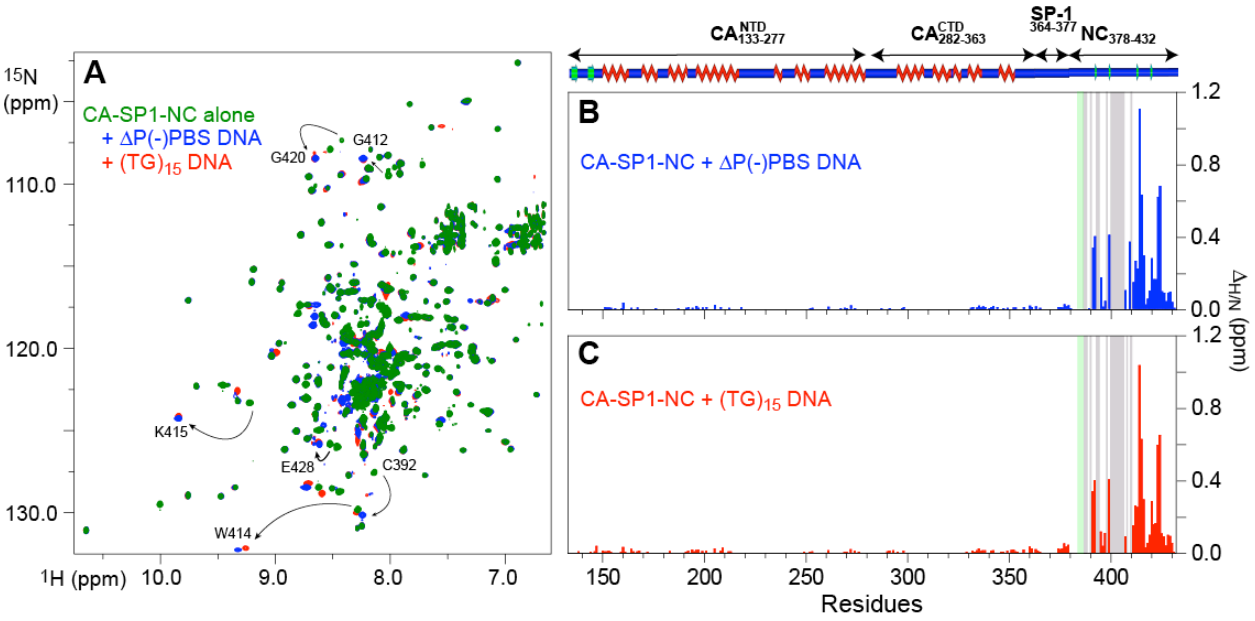

Figure S2. NMR analysis of HIV-1 CA-SP1-NC. (*A*) Overlay of the ^{1}H-^{15}N TROSY spectra of $^{2}H/^{15}N$-labeled CA-SP1-NC in the absence (green) and presence of ΔP(-)PBS (blue) and 5'd-$(TG)_{15}$ (red) DNA. Excellent spectral quality is obtained through careful protein purification, uniform $^{2}H/^{15}N$-labeling and maintaining the concentration below 0.35 mM (in subunits) to avoid aggregation. Experimental conditions were as follows: protein concentration ~0.3-0.35 mM in subunits, protein-DNA molar ratio 1:2, 35°C, in buffer containing 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 0.1 mM $ZnCl_2$, 7% D2O / 93% H2O and 1 mM DTT. A few of the cross-peaks from the nucleocapsid domain exhibiting large chemical shift perturbations upon DNA binding are labeled. (*B*) $^{1}H_N/^{15}N$ chemical shift perturbation profile upon addition of ΔP(-)PBS (blue) and 5'd-$(TG)_{15}$ (red) DNA. $\Delta_{H/N}$ is calculated using equation $[(\Delta\delta_H)^2 + (0.154 \times \Delta\delta_N)^2]^{1/2}$. Semi-transparent grey bars indicate nucleocapsid residues (387-388, 390, 393, 394, 398, 400-405, and 410) that exhibit line broadening in the presence of DNA, green bars indicate nucleocapsid residues (383-386) that could not be assigned unambiguously in the absence of DNA.

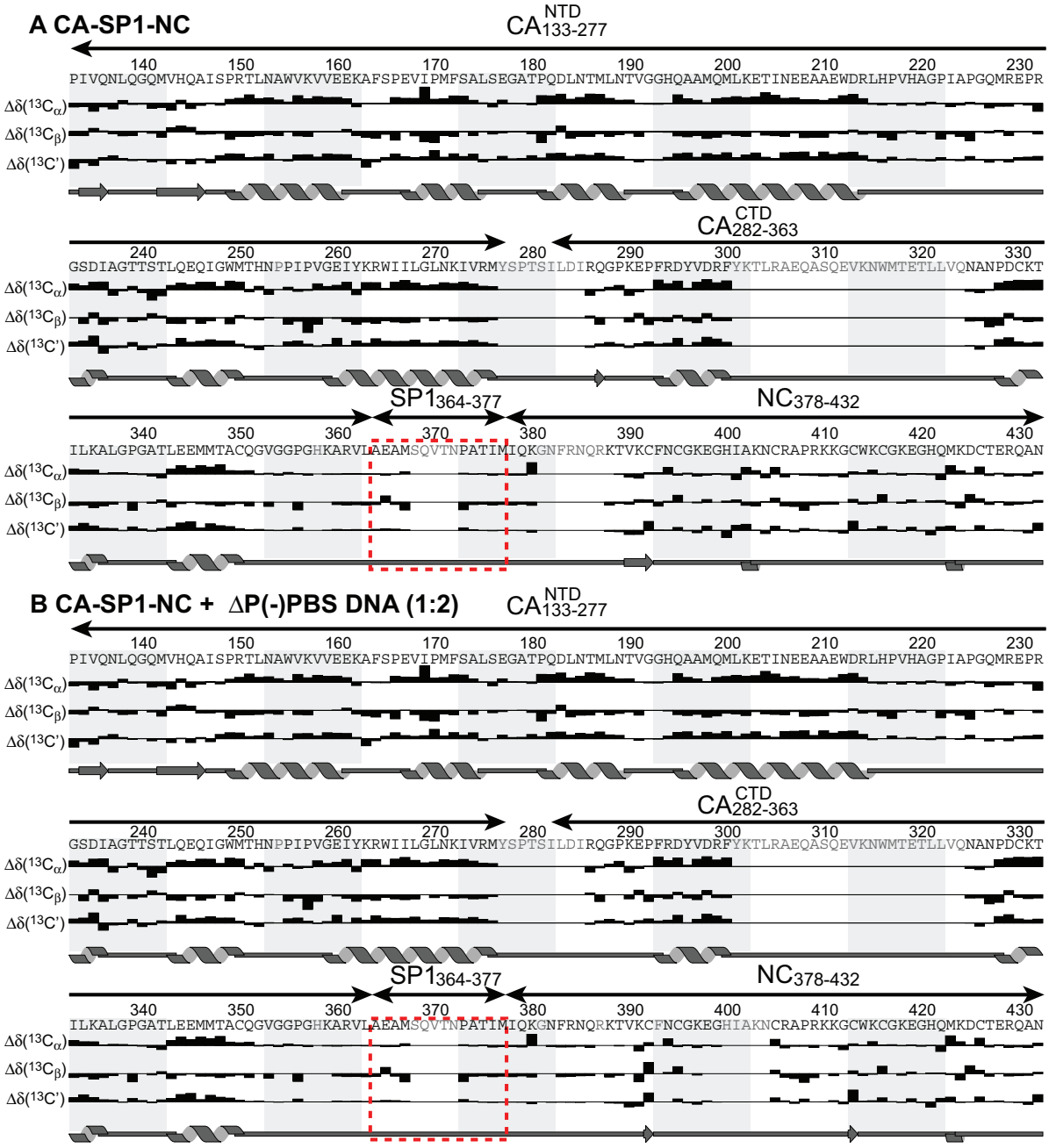

Figure S3. Summary of NMR-derived secondary structure indicators for HIV-1 CA-SP1-NC (*A*) in the absence and (*B*) presence of ΔP(-)PBS DNA. Missing/unassigned residues are shown in grey. Near random chemical shift values of SP1 (highlighted in red, residues 364-377) with and without DNA are indicative of an intrinsically disordered state. The location of secondary structure elements (helix and sheet) is derived from the backbone chemical shifts using Talos+.[19] Only predictions with a confidence level ≥ 0.3 are shown.

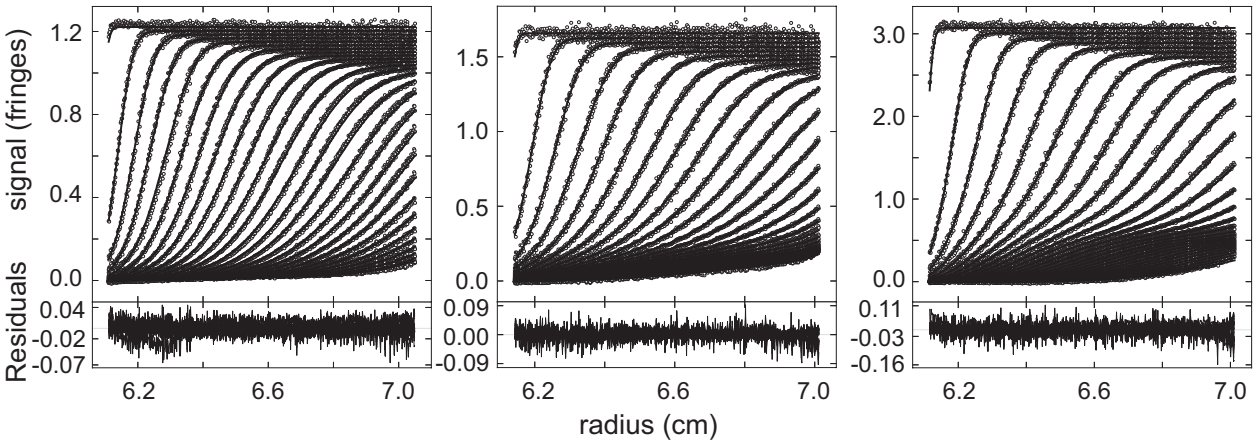

Figure S4. Representative sedimentation velocity data for CA-SP1-NC collected at 35°C, analyzed in terms of a reversible monomer-dimer self-association using Lamm equation modeling. Interference data for CA-SP1-NC at a loading concentration of 42 μM (left panel); interference data for CA-SP1-NC at a loading concentration of 45 μM in the presence of two molar equivalents of ΔP(-)PBS DNA (center panel); interference data for CA-SP1-NC at a loading concentration of 68 μM in the presence of two molar equivalents of 5'd-$(TG)_{15}$ DNA (right panel). In each case, every other data point and scan is shown together with the global best-fit (solid line) and corresponding residuals (bottom panel). Each data set shown is part of a global analysis (e.g. CA-SP1-NC alone includes absorbance and interference data at four other loading concentrations). Data were plotted using GUSSI (obtained from http://biophysics.swmed.edu/MBR/software.html).

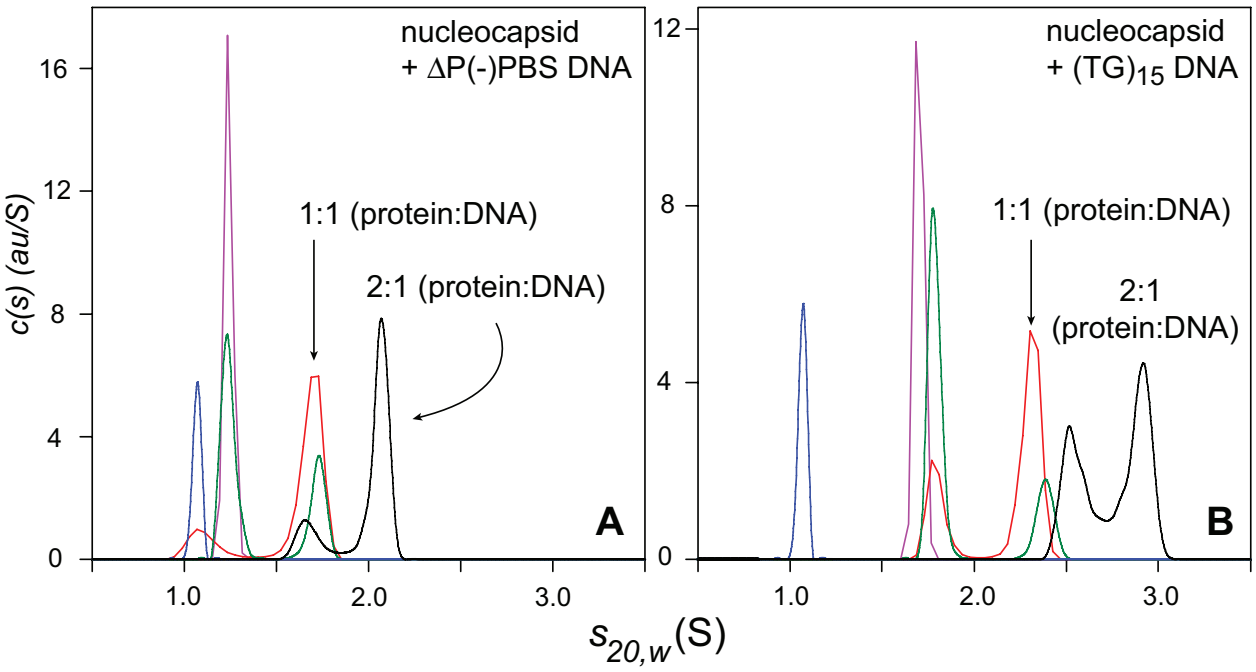

Figure S5. Stoichiometry of the nucleocapsid-DNA interaction. *c(s)* absorbance profiles for (*A*) nucleocapsid (blue, ~52 µM), ΔP(-)PBS DNA (magenta, ~10 µM) and mixtures containing ~10 µM ΔP(-)PBS DNA with nucleocapsid (black, 25 µM, red, ~10 µM, and green, ~5 µM respectively); (*B*) nucleocapsid (blue, ~52 µM), 5'd-$(TG)_{15}$ DNA (magenta, ~10 µM) and mixtures containing ~10 µM 5'd-$(TG)_{15}$ DNA with nucleocapsid (black, 25 µM, red, ~7 µM and green, ~3.5 µM respectively). Profiles for nucleocapsid exhibit a single species at 1.06 S with an estimated molar mass of ~7.0 kDa (M_{calc} = 6.288 kDa). Both ΔP(-)PBS and 5'd-$(TG)_{15}$ DNA also show single species at 1.25 S and 1.70 S with a molar masses of 4.5 and 9.7 kDa respectively (M_{calc} = 4.247 kDa and 9.439 kDa, respectively). Addition of ~1 equivalent of nucleocapsid to ΔP(-)PBS DNA results in a faster sedimenting complex at 1.69 S having a molar mass of ~9.5 kDa consistent with a 1:1 nucleocapsid:DNA complex (M_{calc} = 10.535 kDa). With a two-fold excess of ΔP(-)PBS DNA, the *c(s)* shows the presence of free ΔP(-)PBS DNA at 1.24 S along with a 1:1 complex at 1.73 S. No evidence for larger complexes is observed indicating that nucleocapsid forms a 1:1 complex with excess of ΔP(-)PBS DNA. Addition of at least two equivalents of nucleocapsid, however, results in a complex at 2.05 S with a molar mass of 15.2 kDa indicative of a 2:1 nucleocapsid: ΔP(-)PBS DNA complex (M_{calc} = 16.823 kDa). Similary in case of 5'd-$(TG)_{15}$ DNA, addition of ~0.7 equivalents of nucleocapsid results in a faster sedimenting complex at 2.31 S with a molar mass of ~15.8 kDa consistent with a 1:1 nucleocapsid:$(TG)_{15}$ complex (M_{calc} = 15.727 kDa). With ~0.35 equivalents of nucleocapsid, the *c(s)* shows the presence of free DNA at 1.79 S along with 1:1 complex at 2.39 S. No evidence is observed for larger complexes demonstrating that nucleocapsid forms a 1:1 complex with excess 5'd-$(TG)_{15}$ DNA. In the presence of excess nucleocapsid, however, a complex at 2.89 S is formed with a best-fit molar mass of 22.7 kDa indicative of a 2:1 nucleocapsid:$(TG)_{15}$ complex (M_{calc} = 22.015 kDa). All experiments were carried out in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1 mM DTT, and 0.1 mM $ZnCl_2$.

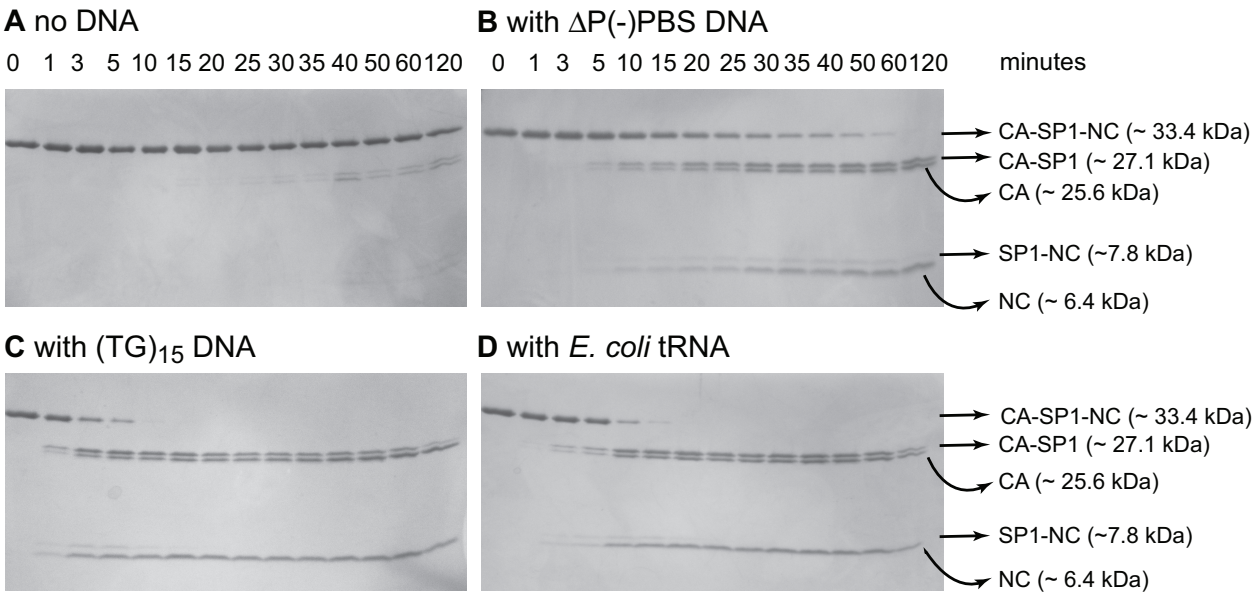

Figure S6. Cleavage of CA-SP1-NC by HIV-1 protease in the (*A*) absence and (*B*, *C*, *D*) presence of nucleic acids. Protein was incubated with HIV-1 protease (molar ratio 100:1) with and without nucleic acids (protein:nucleic acid molar ratio of 1:2) for 2 hours at room temperature. Aliquots were taken at regular time intervals and visualized by Coomassie Blue staining (18% tris-glycine gel, Invitrogen). The cleavage products of the reaction, CA-SP1, CA, SP1-NC and NC, were also verified by LC-MS (the reaction was terminated by addition of the protease inhibitor darunavir prior to loading onto an HPLC column). The buffer conditions were 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 0.1 mM $ZnCl_2$, and 1 mM dithiothreitol.

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