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Aromatic residue mutations reveal direct correlation between HIV-1 nucleocapsid protein's nucleic acid chaperone activity and retroviral replication

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Abstract

The human immunodeficiency virus type 1 (HIV-1) nucleocapsid (NC) protein plays an essential role in several stages of HIV-1 replication. One important function of HIV-1 NC is to act as a nucleic acid chaperone, in which the protein facilitates nucleic acid rearrangements important for reverse transcription and recombination. NC contains only 55 amino acids, with 15 basic residues and two zinc fingers, each having a single aromatic residue (Phe16 and Trp37). Despite its simple structure, HIV-1 NC appears to have optimal chaperone activity, including the ability to strongly aggregate nucleic acids, destabilize nucleic acid secondary structure, and facilitate rapid nucleic acid annealing. Here we combine single molecule DNA stretching experiments with ensemble solution studies of protein-nucleic acid binding affinity, oligonucleotide annealing, and nucleic acid aggregation to measure the characteristics of wild-type (WT) and aromatic residue mutants of HIV-1 NC that are important for nucleic acid chaperone activity. These *in vitro* results are compared to *in vivo* HIV-1 replication for viruses containing the same mutations. This work allows us to directly relate HIV-1 NC structure with its function as a nucleic acid chaperone *in vitro* and *in vivo*. We show that replacement of either aromatic residue with another aromatic residue results in a protein that strongly resembles WT NC. In contrast, single amino acid substitutions of either Phe16Ala or Trp37Ala significantly slow down NC's DNA interaction kinetics, while retaining some helix-destabilization capability. A double Phe16Ala/Trp37Ala substitution further reduces the latter activity. Surprisingly, the ensemble nucleic acid binding, annealing, and aggregation properties are not significantly altered for any mutant except the double aromatic substitution with Ala. Thus, elimination of a single aromatic residue from either zinc finger strongly reduces NC's chaperone activity as determined by single molecule DNA stretching experiments without significantly altering its ensemble-averaged biochemical properties. Importantly, the substitution of aromatic residues with Ala progressively decreases NC's nucleic acid chaperone activity while also progressively inhibiting viral replication. Taken

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2012.07.008>.

together, these data support the critical role of HIV-1 NC's aromatic residues, and establish a direct and statistically significant correlation between nucleic acid chaperone activity and viral replication.

Keywords

HIV-1; Infectivity; Nucleic acid chaperone; Nucleocapsid; Single-molecule DNA stretching; Zinc finger

1. Introduction

The nucleocapsid protein (NC) of HIV-1 performs functions in many of the steps of viral replication (Thomas and Gorelick, 2008). As a domain of Pr55^{Gag} it binds to and directs packaging of genomic RNA (D'Souza and Summers, 2005), as well as placement of the primer tRNA^{Lys,3} (Hargittai et al., 2004) during viral assembly. When the viral protease (PR) cleaves Gag during maturation, the evolving precursor and mature forms of the NC protein (NCp15, NCp9, and NCp7) facilitate formation of a stable dimer of the genomic RNA (Feng et al., 1996; Fu et al., 1994; Fu and Rein, 1993), which is essential for reverse transcription and subsequent infection processes (Kafaie et al., 2008).

The NC protein of HIV-1 is a small, highly basic nucleic acid binding protein that contains 15 basic residues out of a total of 55 amino acids and two strictly conserved zinc-binding motifs of the form –Cys–X₂–Cys–X₄–His–X₄–Cys–. Its amino-terminal tail contains a region of high charge density, which is known to contribute significantly to its ability to aggregate nucleic acids (Darlix et al., 1995; Lapadat-Tapolsky et al., 1993, 1995; Le Cam et al., 1998; Stoylov et al., 1997). Each zinc finger contains a CCHC motif, each of which coordinates one zinc ion. Additionally, the other amino acid residues in these motifs are almost completely conserved as well (Leitner et al., 2003; Thomas and Gorelick, 2008). This sequence conservation indicates important roles in viral replication. Of particular interest for the current report is the aromatic residues present in the first loop of each motif, a Phe at position 16 in the amino-terminal zinc binding motif and a Trp at position 37 in the carboxy-terminal Zn²⁺-finger (Fig. 1A). It is important to note that in virtually every other orthoretrovirus, the second amino acid of the amino-terminal zinc-binding motif is aromatic, although the aromatic residue is less well conserved in the carboxy-terminal motif. Thus, due to their high conservation, it is presumed that these aromatic residues play a critical role in NC function, which we investigate in this work.

Numerous NMR studies have been performed on HIV-1 NC, both with the free- and nucleic acid bound-protein forms (Amarasinghe et al., 2000, 2001; Bazzi et al., 2011; de Guzman et al., 1998; Lee et al., 1998; Morellet et al., 1994, 1998, 1992; South and Summers, 1993; Summers et al., 1990; Tisne et al., 2001). Based on these studies, three key observations have been made: first, the protein becomes more structured when bound to nucleic acids; second, the Trp residue in the carboxy-terminal zinc finger and the Phe residue in the amino-terminal zinc finger form hydrophobic platforms and interact with nucleobases through both stacking and hydrogen bonding interactions; third, the two zinc fingers are in close spatial proximity. Intercalation of the aromatic residue at position 37 with nucleobases is also supported by Trp-fluorescence quenching studies (Mely et al., 1994). Based on additional biophysical characterizations, the aromatic residues play a non-electrostatic role in the binding of NC to nucleic acids (Avilov et al., 2008; Bourbigot et al., 2008; Fisher et al., 2006; Godet and Mely, 2010; Lam et al., 1994; Mely et al., 1993), so that eliminating these residues virtually eliminates this component of binding (Fisher et al., 2006; Remy et al., 1998; Urbaneja et al., 1999). Intriguingly, these experiments have demonstrated that

elimination of these aromatic residues can be just as detrimental to interactions between NC and nucleic acids as elimination of the zinc-binding residues.

HIV-1 NC functions as a nucleic acid chaperone, facilitating the rearrangement of nucleic acids into the structure of lowest energy, which should contain the maximum number of base pairs (Levin et al., 2005; Rein et al., 1998; Tsuchihashi and Brown, 1994). These nucleic acid rearrangements are essential for many viral replication processes, including reverse transcription and recombination (Anderson et al., 1998; Bampi et al., 2004; Mark-Danieli et al., 2005; Negroni and Buc, 1999, 2001). They are especially important given the recently characterized complex structures formed by HIV-1 genomic RNA in vivo (Watts et al., 2009). There are a number of steps that require significant rearrangement of nucleic acid secondary structure during retroviral reverse transcription: tRNA primer annealing, genome dimer formation, minus-strand transfer, plus-strand transfer, and whenever recombination occurs during reverse transcription. For example, HIV-1 NC can accelerate tRNA annealing (Hargittai et al., 2004) and minus-strand transfer by over three orders of magnitude in vitro (You and McHenry, 1994).

We have shown that HIV-1 NC's nucleic acid chaperone activity is composed of three major components: nucleic acid aggregation, nucleic acid destabilization, and rapid protein-nucleic acid interaction kinetics (Cruceanu et al., 2006a,b; Levin et al., 2005; Vo et al., 2006; Williams et al., 2001, 2002a,b). We have also shown that NC proteins from other retroviruses exhibit only some of these activities, suggesting that HIV-1 NC has optimal chaperone activity relative to that of other NC proteins (Stewart-Maynard et al., 2008). The current study addresses the role of HIV-1 NC's aromatic residues in its function as an efficient nucleic acid chaperone, as well as the role of these elements in overall retroviral replication. The few published cell-culture based mutagenesis studies investigating the aromatic residues revealed that they are essential for virus replication—but the mechanism of this replication block was not investigated. Viral genomic RNA packaging was reduced to about 15% of WT, which was similar to other mutations that eliminated replication (Dorfman et al., 1993). However, when one considers 10^8 viral particles, an 85% reduction in genomic RNA still leaves 1.5×10^7 particles with genomic RNA. Why therefore are the remaining particles replication defective? By combining in vitro studies of NC's nucleic acid chaperone activity with in vivo replication studies, we establish the importance of nucleic acid chaperone activity for retroviral replication, while dissecting the specific role of aromatic residues in HIV-1 NC's nucleic acid chaperone function.

2. Materials and methods

2.1. Plasmids, mutagenesis, and recombinant protein production

Site-directed mutagenesis was performed using pNL4-3 (Gen-Bank accession no. AF324493, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Malcolm Martin) (Adachi et al., 1986) for analysis of mutant HIV-1 in cell culture. For mutant recombinant NC protein production, the NC expression plasmids, pDB1403 or pDB295 [pET32a-based expression plasmids (Novagen, EMD Biosciences, San Diego, CA) with either the tobacco etch virus protease or enterokinase cleavage sites, respectively] used previously for the production of WT HIV-1 NCp7 (NL4-3) were altered. Mutagenesis was performed using the Agilent Technologies QuickChange Site-Directed Mutagenesis kit (Santa Clara, CA) according to the manufacturer's instructions and standard cloning procedures. The resulting mutations were confirmed by nucleic acid sequencing. Numbering throughout refers to nucleotide positions in the pNL4-3 sequence, GenBank accession no.: AF324493. The F16A mutation was introduced by changing nucleotides 1965–1968 (5'-tttc-3') to 5'-cgca-3', which altered the codon at position 16 from Phe to Ala in NC and also introduced an *Fsp*I restriction site at

position 1963 that was used for tracking the mutation. F16W was made by changing nucleotides 1967–1968 (5′-tc-3′) to 5′-gg-3′. The W37A mutation was incorporated by changing nucleotides 2029–2030 (5′-tg-3′) to 5′-gc-3′ and the W37F mutation changed nucleotides 2030–2031 (5′-gg-3′) to 5′-tt-3′. The F16W/W37F and F16A/W37A mutations were generated by consecutive mutagenesis of the requisite plasmids. Recombinant proteins were expressed and purified as described previously (Carteau et al., 1999; Guo et al., 2000; Stewart-Maynard et al., 2008). The mutant NC amino acid sequences examined are presented in Fig. 1 and the plasmids used in this study are summarized in supplementary information, Table S1.

NC proteins were stored at -80°C in 20 mM HEPES pH 7.5, 5 mM β -ME, 0.1 mM tris (2-carboxyethyl) phosphine (TCEP) and 2 mol of zinc per mole of NC. The concentrations of all proteins, except for the W37A and F16A/W37A aromatic mutants, were determined using an extinction coefficient of $6050\text{ M}^{-1}\text{ cm}^{-1}$ at 280 nm. The concentrations of W37A and F16A/W37A, which lack tryptophan residues, were determined by amino acid analysis.

2.2. DNA stretching experiments

We used dual beam optical tweezers to stretch bacteriophage λ DNA (McCauley and Williams, 2007; Williams et al., 2001, 2002a), which was labeled with biotin on both 5′ ends. Because of strong non-covalent bonds between biotin and streptavidin, we can stretch λ DNA up to 300 pN by manipulating streptavidin-coated polystyrene beads (Bangs Laboratories, Inc., Fishers, IN) with optical tweezers after catching a DNA between two beads. Solutions of specific protein concentrations were exchanged with buffer surrounding a single DNA molecule to investigate protein effects on DNA stretching curves (Fig. 2A). Double-stranded DNA stretching and release curves are shown in the presence and absence of 30 nM WT HIV-1 NCp7 in Fig. 2B, while a single and multiply stretched single-stranded DNA are shown in the presence of 10 nM WT HIV-1 NCp7 in Fig. 2C. All stretching experiments were performed at a pulling rate of $\sim 100\text{ nm/s}$ in 10 mM HEPES, 50 mM Na^+ , pH 7.5 buffer.

2.3. Nucleic acid substrates

The TAR RNA hairpin (Fig. 3) was generated by in vitro transcription of a DNA template encoding the RNA sequence in front of a T7 RNA polymerase promoter. This DNA template was prepared by PCR amplification of chemically synthesized oligonucleotides purchased from Integrated DNA Technologies (IDT, Coralville, IA). Following transcription, TAR RNA was purified using 12% denaturing polyacrylamide gel electrophoresis (PAGE), and its concentration determined using an extinction coefficient of $533,700\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm. Internally- ^{32}P -labeled TAR RNA was prepared using the same procedure as described above, except that α - ^{32}P GTP was employed in the in vitro transcription reaction. The 59-nt complementary TAR (cTAR) DNA (Fig. 3) was obtained from IDT and further purified using 12% PAGE. The concentration of cTAR DNA was determined using an extinction coefficient of $564,800\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm. The microTAR (18-mer RNA) (see Fig. 3) conjugated with fluorescein at the 3′ end, was purchased from Dharmacon Inc. (Thermo Fisher Scientific, Lafayette, CO) and its concentration was determined using an extinction coefficient of $189,300\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm. The 8-mer (TG)₄ sequence labeled at the 5′ end using carboxyfluorescein (FAM), was purchased from IDT and its concentration was determined by absorbance using an extinction coefficient of $96,360\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm.

2.4. Fluorescence anisotropy assays

Fluorescein-labeled microTAR RNA (20 nM) or 5′-FAM-(TG)₄ (10 nM) was incubated in the absence or presence of varying concentrations of WT or mutant NC proteins at RT (20

°C) in 20 mM HEPES pH 7.5, 50 mM NaCl, 10 μ M TCEP, 5 mM β -mercaptoethanol and 1 μ M zinc acetate for 30 min. Fluorescence anisotropy (FA) measurements were carried out in duplicate for each protein concentration using 20 μ l of solution per measurement performed in Corning® 384-well low volume polystyrene NBS™ microplates (Corning, NY) using a SpectraMax® M5 multimode microplate reader (Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were set at 485 nm (9 nm bandwidth) and 525 nm (15 nm bandwidth), respectively. Anisotropy (A) was calculated using SoftMax® Pro (Molecular Devices) software. The resulting plot of A vs. NC concentration (N) was fit using Kaleidagraph according to the following one-site binding model (Cruceanu et al., 2006b):

$$A = \frac{A_{\text{free}} + ([(T+N+K_d) - \{ (T+N+K_d)^2 - (4TN) \}^{1/2}] / 2T) \cdot (RA_{\text{bound}} - A_{\text{free}})}{(R-1) \cdot [(T+N+K_d) - \{ (T+N+K_d)^2 - (4TN) \}^{1/2}] / 2T + 1} \quad (1)$$

where T , A_{bound} , A_{free} , K_d and R represent the concentration of oligonucleotide, anisotropy of completely bound oligonucleotide, anisotropy of unbound oligonucleotide, dissociation constant and the ratio of fluorescence intensity of completely bound oligonucleotide relative to unbound nucleotide, respectively.

2.5. Sedimentation assays

Sedimentation assays to monitor nucleic acid aggregation activity were performed as previously described (Vo et al., 2006). Briefly, cTAR DNA (unlabeled) was combined with 32 P-labeled TAR RNA to a final concentration of 1.5 μ M. Both TAR RNA and cTAR DNA (6 μ M) were folded as described for annealing assays. The aggregation assays were performed by incubating TAR RNA and cTAR DNA to a final concentration of 15 nM and 45 nM, respectively, in a buffer containing 20 mM HEPES pH 7.5, 20 mM NaCl and 0.2 mM MgCl_2 , in the absence or presence of 0.88 μ M NC unless otherwise indicated. The samples (30 μ l) were incubated at 37 °C for 30 min and then spun in a Micromax RF (Thermo IEC) centrifuge rotor at a speed of 12,000 rpm for 10 min at 4 °C. An aliquot (4 μ l) of supernatant was taken and the amount of radioactivity measured using scintillation counting. The percent RNA remaining in the supernatant was determined by the ratio of the amounts of radioactivity remaining in the supernatant with and without NC.

2.6. Electrophoretic mobility gel-shift annealing assays

Immediately prior to each experiment, TAR RNA and cTAR DNA (6 μ M each) solutions were refolded in 25 mM HEPES, pH 7.5, and 20 mM NaCl by heating at 80 °C for 2 min, then cooling to 60 °C for 2 min, followed by addition of MgCl_2 (to 10 mM final) and placement on ice. For annealing assays, 32 P-labeled TAR RNA (15 nM) and cTAR DNA (45 nM) were combined in a solution containing 20 mM HEPES, pH 7.5, 20 mM NaCl, 0.2 mM MgCl_2 and 5 mM DTT. The solution was incubated at 37°C for 5 min before addition of NC to a final concentration of 0.88 μ M, corresponding to a 4:1 nt:NC ratio, unless otherwise indicated. At desired time points, an aliquot of the reaction mixture was quenched by addition of 1% SDS, followed by incubation at room temperature (RT) for 5 min and placement on ice. The aliquots were phenol–chloroform extracted twice (to remove any NC bound to the nucleic acids) followed by addition of glycerol (5%) and separation on 12% PAGE (19:1 acrylamide:bisacrylamide). The gels were visualized using a Bio-Rad (Hercules, CA) Molecular Imager FX and quantified with Bio-Rad Quantity One Software. The percent TAR RNA annealed for a certain time was determined by the band intensities for the duplex product and free RNA and fit to a single exponential function.

2.7. Cell lines, transfections, virus isolations, and infectivity assays

The 293T (DuBridge et al., 1987; Pear et al., 1993), H9 (Popovic et al., 1984), TZM-bl [obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme, Inc. (Derdeyn et al., 2000; Platt et al., 1998; Takeuchi et al., 2008; Wei et al., 2002)], and HeLa CD4⁺ Clone 1022 [obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Bruce Chesebro; (Chesebro et al., 1990; Chesebro and Wehrly, 1988; Chesebro et al., 1991)] lines were cultured as recommended. Transfections of 293T cells by calcium phosphate coprecipitation and virus isolations were performed as described by Gorelick et al. (1999) or Thomas et al. (2008).

Single-round limiting dilution TZM-bl virus infectivity assays were performed as described (Morcock et al., 2008). To monitor multiple-round infections (viral replication), limiting dilution infection assays (10-fold serial dilutions) of H9 cells were performed in 24-well plates by incubating 1 mL of diluted and undiluted virus containing supernatants with 1×10^6 H9 cells in the presence of 2 µg/mL hexadimethrine bromide (polybrene, Sigma–Aldrich, St. Louis MO). The next day, 1 mL of media (RPMI media with 5% (v/v) heat inactivated fetal bovine serum, 2 mM L-glutamine, antibiotics, and 2 µg/ml polybrene) was added. Cultures were split 1:2 twice weekly, feeding with the same media as above, samples were taken weekly for monitoring of viral spread, and virus in undiluted samples was detected using the TZM-bl assay mentioned above. Infection of HeLa CD4⁺ Clone 1022 cells was performed as described (Thomas et al., 2006b).

2.8. Detection of viral proteins

Exogenous template assays for the detection of viral reverse transcriptase activity were performed as described either by Gorelick et al. (1990) using [³H]TTP, or using 3000 Ci/mmol [α -³²P]TTP (Perkin Elmer Life Sciences, Waltham, MA), spotting the resulting products onto DEAE filtermats, and counting the filters using a Wallac 1450 Microbeta scintillation counter (Thomas et al., 2011).

Immunoblot analysis was performed essentially as described (Gorelick et al., 1990) using goat antisera against NCp7 (ACVP #77, lot R196099) at a 1:5,000 dilution, the blot was stripped and reincubated with goat antisera against CAp24 (ACVP #81, lot 000805) at a 1:10,000 dilution, then stripped and incubated for a final time with a mouse monoclonal antibody against SUGp120 (ACVP 3F5-05-F8) at a dilution of 1:1000. Detection of immunoreactive bands was performed using the requisite rabbit anti-goat (BioChain, Hayward, CA) or goat anti-mouse (BioRad Laboratories, Hercules, CA) horseradish peroxidase conjugated secondary antibody and visualized by chemiluminescence using Immuno-Star HRP detection reagent (BioRad Laboratories) and X-ray film.

2.9. Detection of viral nucleic acids

Viral genome quantities, intravirion DNA levels, and assessment of reverse transcription intermediate quantities in either virions or virus-infected cells were determined by quantitative PCR as described previously (Buckman et al., 2003; Thomas et al., 2008, 2006a). For examining intracellular reverse transcription processes in this study, HeLa CD4⁺ clone 1022 cells were used instead of HOS cells (Thomas et al., 2006a), therefore viruses were not pseudotyped with VSV-G.

3. Results

3.1. Single molecule studies of WT and mutant NC-DNA interactions

3.1.1. WTNC interactions—In order to characterize the extent to which WT and mutant NC proteins act as nucleic acid chaperones, we first performed single molecule DNA stretching using optical tweezers to determine the extent to which these proteins altered the stability of double-stranded DNA (dsDNA), as well as the kinetics of DNA annealing after stretching to high forces. Fig. 2B shows a stretching and relaxation cycle for bacteriophage λ DNA in the absence and in the presence of a saturating concentration of WT HIV-1 NC. In the absence of protein, at extensions much less than the B-form contour length of dsDNA (0.34 nm/bp), very little force is required to stretch the DNA. As the contour length is approached, the force increases dramatically, reflecting the elasticity of the double helix. The plateau from 0.34 nm/bp to 0.6 nm/bp at a constant force of about 60 pN represents a transition from dsDNA to single-stranded DNA (ssDNA), or a force-induced melting transition (McCauley and Williams, 2007; Shokri et al., 2008; Williams and Rouzina, 2002; Williams et al., 2002b). In the absence of protein, the transition is cooperative, and as the DNA is relaxed back to lower extensions, the force-extension curve is almost completely reversible, showing little hysteresis.

In the presence of WT HIV-1 NC, the DNA stretching curves are altered dramatically, as previously reported (Cruceanu et al., 2006a,b; Williams et al., 2001, 2002a,b) and also shown in Fig. 2B. At extensions below the B-form DNA contour length, the dsDNA stretching curve is only slightly altered by HIV-1 NC binding. However, at higher forces the DNA molecule stretches as a polymer with a contour length longer than that of B-form DNA, but shorter than that of ssDNA. In addition, the hysteresis of the DNA stretch/release cycle, as determined by the difference between the force-extension curve during stretching and release, is small. The relatively small hysteresis, along with the lower force required to stretch DNA beyond its B-form contour length (>0.34 nm/bp) illustrates that HIV-1 NC destabilizes the DNA duplex, and these results were previously correlated with the optimum chaperone activity of this protein (Cruceanu et al., 2006b; Stewart-Maynard et al., 2008). The small hysteresis of the DNA stretch/release cycle with HIV-1 NC further diminishes after several subsequent stretching cycles, indicating saturated HIV-1 NC protein binding to both DNA strands, which we will refer to as the HIV-1 NC/2x ssDNA complex. A typical HIV NC/2x ssDNA stretch/release curve (only the stretch curve is shown as they are identical within error) is presented in Fig. 2C (solid black line). These curves were generated by repeating stretch/release cycles until there was no observable hysteresis. This curve differs significantly from the saturated HIV-1 NC/ssDNA stretching curve presented for comparison on the same panel (red line). The latter curve was obtained by first dissociating one of the dsDNA strands under high tension in high pH to obtain one ssDNA strand, then reducing the pH to 7.5. HIV-1 NC was then added and the ssDNA was stretched with NC bound. Interestingly, this saturated HIV NC/ssDNA curve is very similar to the ssDNA stretching curve obtained in the absence of protein (data not shown). The difference between the black and red curves on Fig. 2C suggests that saturating HIV-1 NC binding is capable of keeping the two DNA strands together at forces up to ~ 80 pN, which in the absence of protein would induce strand separation, while also strongly elongating and deforming the duplex, as indicated by the increase in extension beyond the B-form contour length. The combined capabilities of HIV-1 NC to destabilize the B-form DNA duplex by deforming it, while also keeping the two strands together may make it an optimum NA chaperone by allowing it to decrease nucleic acid re-folding transition barriers, while also facilitating annealing of the new structure.

3.1.2. Mutant NC interactions: aromatic-to-aromatic alterations—We hypothesize that the capability of HIV-1 NC to destabilize duplex DNA while keeping both of its strands together is due to the presence of NC's specific aromatic residues, which are known to form a hydrophobic platform for stacking with the unpaired nucleotides of ssDNA (de Guzman et al., 1998; Fisher et al., 1998; Shubsda et al., 2002; Vuilleumier et al., 1999; Yuan et al., 2003). To test this hypothesis, we constructed several mutants in which one or both aromatic residues are replaced by either Ala or by another aromatic residue: F16W, F16W/W37F, F16A, W37A, and F16A/W37A, as shown in Fig. 1B – G.

Fig. 4 shows representative DNA stretching and relaxation cycles in the presence of saturating amounts of F16W (panel A) or F16W/W37F (panel B) NC. The saturated concentration value was obtained by measuring the change in slope of the force–extension curve with concentration. At saturated concentration and above, the measured slope did not change significantly. The reduced DNA binding affinity of the mutant NC proteins compared to WT HIV-1 NC is demonstrated by the fact that while the effects of WT HIV-1 NC on the DNA stretching curves saturate at 10 nM concentration (Cruceanu et al., 2006b), it takes 30 nM of HIV-1 F16W and F16W/W37F to reach saturation (data not shown). At saturation, these aromatic to aromatic NC mutants lead to DNA stretching curves with very similar stretch and release curves compared to WT HIV-1 NC, with only small changes in the amount of hysteresis, as will be quantified below.

3.1.3. Mutant NC interactions: aromatic to Ala alterations—The DNA stretching and release curves in the presence of mutants with aromatic residues replaced with Ala are shown in Fig. 5. Both of the single mutants (panels A and B) required 20–30 nM protein to observe a saturated effect on DNA stretching. Interestingly, the DNA stretching curves look very much like the protein-free curves, showing a relatively flat force-induced DNA melting plateau that is just a bit more sloped than that observed without protein. The binding of these variants is apparent from the DNA release curves measured within the same stretching cycle, which are clearly different from the protein-free case and display greatly increased hysteresis. This result likely reflects the relatively slow kinetics with which these mutant HIV-1 NCs dissociate from ssDNA, which interferes with strand re-annealing. Thus, the mutant NC dissociation rate is slower than the ~1 s time step of DNA release.

The small change in slope of the DNA stretch/release curves in the presence of saturating amounts of the single Ala NC mutants also demonstrates the inability of these proteins to keep the two stretched DNA strands together, in marked contrast with WT HIV-1 NC and the aromatic-to-aromatic substitution mutants. This very small slope of the force-extension curve at high forces is even more pronounced in the stretching curves of the double aromatic to Ala substitution mutant, F16A/W37A, presented in Fig. 5C. In this case, the protein has almost no effect on the DNA force-induced melting transition even up to 30 nM protein. Thus, it binds only weakly to dsDNA and is unable to deform and alter its stretching characteristics at any force. The inability of this mutant to hold the two DNA strands together is additionally reflected by the shape of the DNA relaxation curve and the large hysteresis (Fig. 5C), reflecting slow re-annealing kinetics, significant DNA strand separation, and slow dissociation of F16A/W37A from ssDNA.

3.2. Quantitative analysis of DNA melting and annealing in the presence of HIV-1 NC mutants

To further characterize the effects of WT and mutant HIV-1 NC on DNA interaction kinetics, we quantified the concentration-dependent kinetics of protein dissociation by measuring the amount of hysteresis observed. To account for differences in the extent of stretching observed for different molecules, we calculated the relative hysteresis, which is

the amount of hysteresis observed relative to the total amount of hysteresis possible in a given stretch/release cycle. The experimentally observed DNA extension at a given force can be expressed as a linear combination of the extension of pure ssDNA, given by the freely jointed chain model (FJC) for ssDNA elasticity, and the extension of pure dsDNA given by the wormlike chain model (WLC) for dsDNA elasticity (Chaurasiya et al., 2010). The fraction of ssDNA from fits of the DNA release curve was used to determine the amount of hysteresis observed relative to the total amount of hysteresis that would be observed if the release curve were pure ssDNA. The fraction of ssDNA at the end of the stretching cycle was determined by plotting a linear combination of the FJC and WLC models, as previously described (McCauley et al., 2008). As shown in Fig. 6, this curve was then used to calculate the maximum hysteresis that could be observed for a given DNA elongation cycle, represented by the area between the hybrid FJC and WLC curve and the DNA stretching curve, A_2 . The actual hysteresis observed, A_1 , is the area between the stretching (red, solid line) and relaxation (red, dotted line) curves of Fig. 2B, Fig. 4 and Fig. 5. The hysteresis area ratio, or relative hysteresis, is $R = A_1/A_2$, and this value is plotted in Fig. 7 as a function of concentration for all of the proteins studied. This method assesses the overall protein–DNA interaction kinetics, including both the on and off rates. The larger the hysteresis, the larger the value of R , so R increases as the protein–DNA interaction kinetics become slower. For aromatic-to-aromatic mutants, the hysteresis is only slightly greater than that observed for WT HIV-1 NC (Fig. 7A). In contrast, for all three mutants in which the aromatics are replaced with Ala, the hysteresis is significantly increased relative to that observed in the presence of WT HIV-1 NC, and this effect saturates near 30 nM protein concentration (Fig. 7B). Thus, the capability of HIV-1 NC to deform and destabilize duplex DNA and keep its two DNA strands together is significant and is affected by replacement of aromatic residues with Ala. Therefore, the aromatic residues on both zinc fingers contribute to efficient annealing of DNA that is destabilized by force.

3.3. Ensemble solution studies of nucleic acid binding affinity, annealing, and aggregation in the presence of wild type and mutant HIV-1 NC

To determine the effect of HIV-1 NC's aromatic residues on its chaperone activity, we also examined the binding, annealing, and aggregation of short nucleic acid constructs in the presence of the proteins that showed significant changes in DNA stretching compared to WT NC. First, we used FA to determine the equilibrium binding affinity of WT and NC variants for specific nucleic acid constructs. As shown in Table 1, the microTAR RNA binding affinity of the W37A and F16A/W37A aromatic replacement mutants is within about a factor of two of that observed for WT NC, while the affinity of the F16A mutant is about 7-fold greater than that of WT NC. In contrast, the affinity for the single-stranded (TG)₄ sequence, which WT NC binds to with high affinity (Fisher et al., 2006, 1998), is very significantly reduced by aromatic residue substitutions with Ala. F16A, W37A, and F16A/37A NC display 2-, 32-, and ~1000-fold reduced affinity for (TG)₄, respectively. This is consistent with previous results showing that NC's zinc fingers are responsible for its high affinity for (TG)₄ (Vuilleumier et al., 1999). Note that F16A has a higher affinity than WT NC for microTAR, but binds similarly to TG₄ as WT (Table 1). In contrast, W37A NC displays reduced binding to both nucleic acids, with a larger reduction in TG₄ binding. While both aromatic residues have been shown to be important for stabilizing NC-nucleic acid interactions, in the six complexes investigated by NMR to date (Bazzi et al., 2011), W37 always displays strong stacking with guanine residues, whereas F16 mediates interactions with either a G or a T/U and generally contributes less to binding affinity (Avilov et al., 2008). Thus, although the higher binding affinity of F16A NC to microTAR is unexpected, the variable and nucleic acid sequence-dependent contributions of F16 and W37 to binding observed here are consistent with previous results. One can also speculate that binding may be tighter to microTAR for F16A compared to WT NC, as the mutant protein

may be able to adopt additional conformations that (i) are sterically hindered due to the larger side chain of Phe compared to Ala and (ii) may also be constrained due to the aromatic ring interactions with nucleobases. The study by Avilov et al. (2008) showed that while WT NC could restrict the local mobility of a flexible oligodeoxynucleotide, the F16A NC mutant could not.

We also measured the ability of the aromatic-to-Ala mutants and WT NC to aggregate RNA using a sedimentation assay with results presented in Fig. 8A and also in Table 1. As shown in Table 1, at low concentrations the mutants are defective in aggregating RNA. However, at 5 μ M and above, all of the mutants aggregate RNA to an extent similar to WT NC (Fig. 8A). Finally, the annealing of TAR RNA to cTAR DNA was measured, as shown in Fig. 8B. All the aromatic mutants show a decrease in the overall annealing rate (see Table 1), with the double aromatic mutant displaying the largest (>30-fold) decrease in rate relative to WT NC. However, when a higher concentration of F16A/W37A NC was used to compensate for reduced binding affinity, the rate of annealing approached that of WT NC. Thus, within the virion, where the local NC concentration is estimated to be 100 mM, the decreased annealing activity of F16A/W37A NC is not likely to be important. The average biochemical properties of the NC aromatic mutants do not differ significantly from WT HIV-1 NC. Therefore, the slow DNA interaction kinetics observed in the single molecule experiments are the primary feature of the aromatic substitution mutants that are observed in vitro.

3.4. NC-mutant and WT HIV-1 virions

3.4.1. Viral protein characterization—Transfection of the mutant and WT proviral plasmids into 293T cells resulted in the production of virus and Table 2 shows the levels of exogenous template RT activities from a typical experiment. The levels are similar to WT HIV-1 for all mutants except for W37A and F16A/W37A, which are 2- to 5-fold lower, respectively.

Western blotting of pelleted viruses, adjusted for equivalent levels of reverse transcriptase activities (Table 2) for the most part showed that the levels of viral proteins probed (NCp7, CAp24, and SUgp120) were similar in amounts and sizes, compared to WT (Fig. 9). There are some uncleaved (Pr55^{Gag}) and partially cleaved Gag precursors detected for the F16A-, W37A- and F16A/W37A-mutant viruses. Additionally, the NCp7 band for the F16A/W37A mutant appeared less intense than the others, which may be due to less efficient processing of the Gag precursor (Pr55^{Gag}) and/or possible alteration of epitopes recognized by the goat antiserum that was used.

3.4.2. Viral infectivity and replication—Viral expression, protein levels, and processing appear only slightly altered in the mutants with aromatic residues changed to Ala. Thus, to assess whether these factors influenced other properties of the mutant viruses, their infectivity was examined and these results are also presented in Table 2. In the single-round TZM-bl assay, the W37F mutant was as infectious as WT HIV-1, and the F16W and F16W/W37F mutants had 2- and 5-fold reduced titers, respectively. The F16A mutant was ~90% reduced in titer and the other two mutants W37A and F16A/W37A had titer reductions of >99% compared to WT. This shows that removing the aromatic character of the residues at positions 16 and/or 37 in NC results in large drops in single-round infectivity titers. Replication of the mutants in H9 cells was next assessed and these results are in agreement with the observed TZM-bl titers. Aromatic-to-aromatic mutants had titers between 10^5 and 10^6 , similar to that for WT HIV-1 (10^6 TCID₅₀/mL). The F16A mutant has a titer that is 10^4 -fold lower than wild type, and no replication was detected in the W37A and F16A/W37A mutants, even in the undiluted samples.

3.4.3. Viral nucleic acid analysis—Viral RNA genome quantities were next assessed to determine the effects of mutations on genome packaging. RNA was quantified by qRT-PCR (Thomas et al., 2006a) with levels being normalized to equivalent RT activities. Table 2 shows that duplicating the same aromatic residue at positions 16 and 37 results in a ~40% decrease in genome packaging whereas switching the positions of the aromatic residues reduced packaging by ~75%. Mutation of the amino-terminal Zn²⁺-finger aromatic residue to Ala reduced packaging by ~65%, while mutating the carboxy-terminal residue or both residues 16 and 37 to Ala resulted in a reductions in genome packaging by ~80% and ~90%, respectively. The decreased RNA packaging caused by these mutations does not completely account for the reductions in either single- or multiple-round infectivity assays. Therefore additional properties of these mutant virions were assessed.

Fig. 10 shows the viral nucleic acid contents in mutant and WT HIV-1 particles as well as their ability to carry out endogenous reverse transcription (ERT). Extra-virion nucleic acids were removed and the levels of genomes and vDNA within virions were assessed as described (Thomas et al., 2008). Fig. 10A shows the levels of vDNA on a per genome basis, relative to WT, examining R-U5 (minus-strand strong-stop) and R-5' UTR (plus-strand transfer) vDNA targets (Buckman et al., 2003). The levels of intravirion DNA are similar to WT for the F16W and the W37F mutants and slightly elevated (~12-fold for R-U5 and 5-fold for R-5'UTR) for the F16W/W37F mutant. In contrast, the mutants where Ala residues substitute for the aromatic amino acids, the levels of R-U5 are increased 150- to 430-fold and R-5'UTR levels are increased 30-to 90-fold. This indicates that premature reverse transcription has taken place, prior to release of the mutant virions from the producer cells, similar to that observed previously with several other HIV-1 NC-mutant viruses (Didierlaurent et al., 2008; Houzet et al., 2008; Thomas et al., 2008), although not quite to the same extent. Panel B of Fig. 10 shows ERT levels after addition of dNTPs to isolated viruses over a 24h period, again examining the R-U5 and R-5'UTR vDNA targets. As was observed with other HIV-1 NC mutants, virions that contain high levels of intravirion DNA typically have reduced ERT activity, presumably due to consumption of genomes during the premature reverse transcription. This same phenomenon is observed with the mutants in this study with near WT levels of R-U5 and R-5'UTR ERT with the F16W and W37F mutants and a slight reduction (~5- to 9-fold) in ERT activity with the F16W/W37F mutant. The mutants where the aromatic amino acids were replaced by Ala had a 180- to 1600-fold reduction in ERT activity for R-U5 and a 26- to 150-fold reduction in R-5'UTR copies compared to WT HIV-1.

3.4.4. Reverse transcription of NC-mutant and WT viruses in infected cells—

We next assessed the levels of reverse transcription intermediates generated 24h after infecting the CD4+ HeLa clone 1022 cell line. Total DNA was isolated from the infected cells, and R-U5, U3-U5 (minus-strand transfer), Gag (late minus-strand synthesis), R-5' UTR, and total 2-LTR circle (TOT) intermediates were assessed using the qPCR analyses described previously (Buckman et al., 2003; Thomas et al., 2006a). The F16W, W37F, and F16W/W37F mutants were very similar to the WT sample in these analyses (Fig. 11). In contrast, the aromatic mutants substituted with Ala showed a number of interesting properties. Fig. 11A shows that the progression of reverse transcription from R-U5 to later products (reported as the percentage of R-U5) is only reduced slightly with all of the mutant infections compared to WT with the exception of F16A/W37A, which has a more severe effect. The double mutant shows a ~10-fold reduction in the production of the plus-strand intermediate R-5'UTR, and 2-LTR circles were undetectable; the limit of detection for the assay was 10 copies, which amounts to a minimum detection limit of 0.005% in the analysis in Fig. 11 A. With all of the mutants except F16A/W37A (Fig. 11A), overall the levels of 2-LTR circles (TOT) are quite similar to WT virus on a percent R-U5 basis, suggesting that completion of full-length vDNA leading to circles is not significantly affected; the lack of

circles for the F16A/W37A mutant is inconclusive with regard to end-degradation or LTR completion as the quantities of circles were consistently below the level of detection of our qPCR assay. The same data were analyzed by comparing reverse transcription intermediates to the same intermediate in WT virus (Fig. 11B). Since there were differing levels of genomes among the mutants and WT viruses (Table 2) reverse transcription intermediate levels were normalized based upon the numbers of virions assessed using exogenous template RT assay results. The mutants where the aromatic character of residues 16 and 37 are maintained show levels of reverse transcription intermediates that are very similar to WT with a minor reduction for the F16W/W37F mutant. On a per particle basis, the F16A mutant is reduced slightly as well, and the W37A mutant is even lower. The F16A/W37A variant has the greatest reduction of all, with a 625-fold reduced production of the R-5' UTR plus-strand transfer intermediate. Once again, 2-LTR circles were undetectable.

4. Discussion

Recent studies of HIV-1 NC have shown that aggregation, duplex destabilization and rapid DNA annealing kinetics are three major components of nucleic acid chaperone activity (Cristofari and Darlix, 2002; Cruceanu et al., 2006a,b; Qualley et al., 2010; Stewart-Maynard et al., 2008). Single molecule DNA stretching experiments with WT HIV-1 NC demonstrate these properties, based on the observed lowering of the force required to elongate the DNA and the hysteresis between the stretching and relaxation curves. To investigate the hypothesis that the aromatic residues of HIV-1 NC play an important role in these capabilities, aromatic residue mutants were investigated.

The shape of the DNA stretching/relaxation curves in the presence of F16W and F16W/W37F HIV-1 NC are similar to that observed in the presence of WT NC, (compare Fig. 2B to 4A and B). These mutants also retained NC's duplex destabilizing ability. Therefore, our data suggest that F16W and F16W/W37F NC are still excellent nucleic acid chaperone proteins. In line with these observations, single- and multiple-round replication of viruses with these same mutations are similar to virus containing WT NC when the decrease in genome packaging is taken into account (Table 2).

Contrasting results were obtained when we replaced each of the aromatics by Ala, the mutations denoted F16A and W37A. While the stretching and release curves in the presence of these aromatic substitution mutants continued to exhibit weak DNA duplex destabilization, significantly more hysteresis was observed, particularly at higher protein concentrations. Therefore, aromatic residues on both zinc fingers are required for rapid kinetics. Finally, a mutant lacking both aromatic residues, F16A/W37A, induced significant hysteresis between DNA stretching and relaxation curves. Importantly, this mutant did not significantly alter the shape of the DNA melting transition, suggesting a strong reduction in helix-destabilization capability. As the concentration is increased, the hysteresis increases, showing that additional binding does not restore the ability to keep the strands together, which is responsible for rapid kinetics. Ensemble solution studies of RNA binding affinity, RNA aggregation, and TAR RNA/DNA annealing showed relatively small effects upon single Ala substitution but more pronounced effects upon double substitution.

Taken together, the results show that both of the aromatics play a critical role in the NA chaperone activity of NC. While a single aromatic residue in one of the zinc fingers is sufficient for the protein to aggregate and weakly destabilize DNA, both zinc fingers are required for the protein to exhibit rapid kinetics. This result suggests that the two zinc fingers work together to facilitate rapid nucleic acid annealing, which may explain why HIV-1 NC exhibits optimal chaperone activity compared to other retroviral NC proteins. For example, some retroviral NC proteins only have one finger (e.g., murine leukemia virus).

These results also correlate well with in vivo replication assays, suggesting that NC's chaperone activity, mediated in part by its aromatic zinc finger residues, is critical for HIV-1 replication.

To confirm the correlation between NC chaperone function and infectivity, the correlation coefficient ρ is defined as:

$$\rho = \frac{\sum_{i=1}^n (X_i - \bar{X})(Y_i - \bar{Y})/n}{\sigma_X \sigma_Y} \quad (2)$$

where $\bar{X}$ and $\bar{Y}$ are the average values of X and Y , σ_X and σ_Y are the standard deviations of X and Y (Bevington and Robinson, 1992).

This variable determines the extent to which two variables are correlated. If $\rho = 0$, the two variables are completely uncorrelated. If $\rho = \pm 1$, there is a perfect positive or negative correlation. We find that, according to Eq. (2), the coefficient between the measured hysteresis at saturation (30 nM) for all of the aromatic mutants and the TZM-bl titer (single round infectivity) measurement is -0.74 . The observed negative correlation coefficient is within the 95% confidence interval for six measurements assuming a one-tail distribution (Bevington and Robinson, 1992). Therefore the single round infectivity is negatively correlated with hysteresis. Because hysteresis correlates negatively with nucleic acid chaperone activity (Cruceanu et al., 2006a), this result shows that nucleic acid chaperone activity as measured by DNA stretching correlates directly with in vivo infectivity. As a side note, the other measurement that strongly anti-correlates with hysteresis is genomes (% of wild type, Table 2), which has a correlation coefficient of -0.77 .

Previously it was demonstrated that eliminating the aromatic residues could give rise to a replication defective virus, but the reason for this was not well understood (Dorfman et al., 1993). Based on NMR data and Trp fluorescence-quenching studies it was likely that this type of mutation would have a significant effect on the ability of NC to interact with nucleic acids (Amarasinghe et al., 2000, 2001; Avilov et al., 2008; Bourbigot et al., 2008; Demene et al., 1994; Fisher et al., 2006; Mori et al., 2010). Such changes would be expected to reduce the non-electrostatic aspect of NC's binding to nucleic acids while preserving the electrostatic component. It is important to point out the ΔG of the electrostatic binding component is much greater than that of the non-electrostatic binding component in physiological salt concentration (Mely et al., 1993; Privalov et al., 2011; Urbaneja et al., 1999). The non-electrostatic binding component is generally more important for specific binding to nucleic acids as it forms particular contacts with nucleobases (Beltz et al., 2005; Godet et al., 2011; Remy et al., 1998; Tisne, 2005). Thus, it is very likely that the aromatic residues are important in conveying a certain degree of sequence specificity on NC binding.

Once NC is bound to these preferred sites it can form specific contacts with the nucleic acid and enhance the ability of other NC molecules to interact with the nucleic acid as well—perhaps by forcing the DNA to adapt a slightly less stable structure. These initial sites of binding would act as nucleation sites for more preferential NC binding, thereby enhancing the global destabilization of the dsDNA structure. This model is in line with investigations showing that WT NC favors binding to internal loops or bulges in nucleic acid structure (Kanevsky et al., 2011).

These results indicate that the non-electrostatic component of nucleic acid binding is necessary for NC to be able to possess more transient interactions with nucleic acids in order to function as an effective nucleic acid chaperone during reverse transcription. The zinc-binding residues have previously been shown to be important for helix destabilization and

the DNA stretching curves shown in Fig. 5 are similar to those measured with H23C HIV-1 NC (Williams et al., 2002a). This indicates that even subtle changes to the zinc finger structures can impact the hydrophobic platform structure (Bourbigot et al., 2008) that contributes to optimal nucleic acid chaperone activity. NC's capability to restrict local oligonucleotide dynamics, which may be the first step in NC's duplex opening/closing activity, has also been associated with the hydrophobic platforms (Avilov et al., 2008).

Biologically, the aromatic to Ala substitution mutants result in several specific defects. Most notably, they cause a premature reverse transcription phenotype similar to that observed with mutations to zinc-binding residues (Didierlaurent et al., 2008; Thomas et al., 2008). In addition, we see that these mutants display less ERT activity than WT, but this is likely because the majority of genomic RNA was consumed during the premature reverse transcription process. The activity of these NC mutants to participate in ERT is less likely to be impaired due to the high effective concentration of NC within the virion complex where ERT is occurring. Thus, it is not surprising that the ensemble measurements show relatively small effects of these mutations on properties important for reverse transcription.

However, examination of reverse transcripts after infection of cells showed that the viral DNA that is made by Ala substitution mutants, especially W37A and F16A, W37A, could be a great deal more unstable. In the cell, the RTC is shedding NC as reverse transcription progresses (Mirambeau et al., 2007), resulting in, at some point, non-saturating levels of NC. In this case, slow DNA interaction kinetics that were demonstrated by the stretching studies (Section 3.1) with the Ala-substituted NC mutants may come into play, resulting in easier access to the nascent viral DNA by cellular proteins, such as DNases, that can target the reverse transcription complexes. Alternatively, the synthesis of the vDNA by virions that have aromatic-to-Ala mutations could be reduced and this could again be due to slow DNA interaction kinetics. In either case, a reduction in apparent vDNA synthesis is observed, but a direct correlation of the in vitro results with reverse transcription processes upon infection are confounded by the premature reverse transcription that is occurring with the aromatic-to-Ala mutants. Nevertheless, it is clear that there is a specific requirement for NC's aromatic residues for infection and replication processes.

In addition to the alteration of NC's interactions with nucleic acids upon mutation of the aromatic residues there may also be other interactions that are modified, which could be contributing to the replication defects observed. These include direct or nucleic acid mediated interactions with RT (Druillennec et al., 1999; Lener et al., 1998), IN (Carteau et al., 1997, 1999; Gao et al., 2003), Staufien (Chatel-Chaix et al., 2008, 2004; Moulard et al., 2000), ABCE1 [HP68; (Lingappa et al., 2006; Lingappa and Thielen, 2009; Zimmerman et al., 2002)], Vif (Henriet et al., 2007; Levin et al., 2010) and APOBEC3G (Cullen, 2006; Levin et al., 2010), and are certainly of interest for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5'UTR	5' untranslated region between the LTR and <i>gag</i>
A	Ala
BCFU	blue cell focus units
CA	capsid
cTAR	complementary trans-activation response DNA element
dsDNA	double-stranded DNA
ERT	endogenous reverse transcription
F16	Phel6
FA	fluorescence anisotropy
FAM	carboxyfluorescein
FJC	freely jointed chain model for ssDNA elasticity
Gag	group specific antigen
HIV-1	human immunodeficiency virus type 1
LTR	long terminal repeat
NC	nucleocapsid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PR	protease
qRT-PCR	quantitative reverse transcriptase PCR
R	repeat region
RT	reverse transcriptase
ssDNA	single stranded DNA
SU	surface glycoprotein
TAR	trans-activation response RNA element
TCIU	tissue culture infectious units
U3	3' untranslated region of LTR
U5	5' untranslated region of LTR
W37	Trp37
WLC	wormlike chain model for dsDNA elasticity
WT	wild type

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Fig. 1. Sequences of wild type (A), F16W (B) W37F (C), F16W/W37F (D), F16A (E), W37A (F), and F16A/W37A (G) HIV-1 NC proteins. Substitutions are denoted by orange text and highlighted with an arrow, while basic residues are presented in blue and zinc coordinating residues in red.

Fig. 2.

(A) Schematic diagram of single-molecule DNA stretching experiment. First, a single DNA molecule is caught between the two beads and held by its ends as shown. Then a solution of clean buffer is injected into the flow cell, removing all other DNA molecules except the one held between the beads. Finally, HIV-1 NC proteins (orange figures) at a specific concentration are introduced into the cell. The flow is then stopped and the proteins in solution interact with the DNA as it is stretched. (B) Force-extension (solid) and relaxation (dashed) curves of DNA in the absence (black) and in the presence of 30 nM WT HTV-1 NCp7 (red). (C) DNA stretching curve obtained at 10 nM NC by repeated stretching and relaxation several times until there was no hysteresis, generating two strands of ssDNA

saturated with NC (black). The red line shows ssDNA only stretched in the presence of 10 nM NC.

Fig. 3. Structures of 59-nt TAR RNA hairpin (left), complementary 59-nt TAR (cTAR) DNA hairpin (center) and 18-nt microTAR RNA (right), as predicted by *mfold* (Zuker, 2003) at 37 °C that are used in this study. Sequences are derived from pNL4-3 (Gen-Bank accession no.: AF324493).

Fig. 4. DNA stretching (solid line) and relaxation (dotted line) in the presence (red) and absence (black) of HIV-1 NC mutants with aromatics substituted for other aromatics: (A) 30 nM F16W NC and (B) 30 nM F16W/W37F NC.

Fig. 5. DNA stretching (solid line) and relaxation (dotted line) in the absence (black) and presence (red) of HIV-1 NC variants with Ala substitutions: (A) 30 nM W37A, (B) 30 nM F16A and (C) 30 nM F16A/W37A.

Fig. 6.

Method for quantifying the DNA annealing hysteresis for a given extension and relaxation curve. The red solid and dotted curves are the force extension and relaxation curves of DNA in the presence of 30 nM F16A/W37A NC, respectively. The black line is the standard WLC model for dsDNA elasticity, while the green line is the standard FJC model for ssDNA elasticity, at a normal contour length 0.55 nm/bp. The purple line is a linear combination of WLC and FJC models representing the fraction of DNA melted in the initial stretch. The relative hysteresis is determined by calculating the ratio of the area between the extension and relaxation curves (red solid and dotted lines, respectively) and the area between the extension curve and the linear combination of FJC and WLC models (solid red line and solid purple line, respectively).

Fig. 7. Protein concentration dependence hysteresis area ratio in the presence of WT and aromatic mutant HIV-1 NC proteins. (A) WT NC compared to aromatic to aromatic substitution mutants and (B) WT NC compared to Ala substitution mutants.

Fig. 8.

(A) Results of sedimentation assays using WT and variant HIV-1 NC. Plot of percent RNA aggregated vs. NC concentration for WT, F16A, W37A, and F16A/W37A NC. All experiments were performed at 37 °C. Results are the average of three trials with standard deviations indicated. (B) Results of TAR RNA/DNA gel-shift annealing assays using WT and variant HIV-1 NC. Plot of percent TAR RNA annealed vs. time for WT, F16A, W37A, and F16A/W37A NC under standard conditions with 0.88 μM NC. The data for F16A/W37A* were obtained at 15.3 μM NC concentration. All reactions were performed at 37 °C.

Fig. 9.

Western blotting of mutant and WT HIV-1. Viruses were harvested at 48 and 72 h after transfection of 293T cells, pooled, and collected by ultracentrifugation in a Beckman SW32 Ti rotor (BeckmanCoulter, Brea, CA) through a 5 mL, 20% (wt/vol) sucrose cushion (in phosphate buffered saline) at $140,000 \times g$ for 1 h at 4°C. The equivalent of 6.2 mL of WT sample was utilized. The mutants were loaded at an equivalent RT activity to that loaded for WT. The antisera used for detection of specific proteins are indicated at the right, molecular weight marker positions are indicated at the left and the sample identifiers at the top. The positions of partially cleaved Gag (denoted by an asterisk) and Pr55^{Gag} are indicated at the right of the NC and CA blots.

Fig. 10.

Intravirion DNA and ERT analysis of mutant and WT HIV-1. Virus containing supernatants were treated with DNase I and subtilisin and then nucleic acids were isolated as described previously (Thomas et al., 2008). The levels of R-U5 and R-5'UTR vDNA (indicated within the graph area of panel A) and genomic RNA were assessed by qPCR and qRT-PCR methods described previously (Thomas et al., 2006a) for the mutant and WT viruses indicated at the bottom of the figure. (A) Intravirion DNA levels, on a per genome basis are reported relative to WT virus. The levels of intravirion DNA for WT, on a per genome basis were 3.97×10^{-4} and 2.47×10^{-4} for R-U5 and U3-U5, respectively. (B) The same virus samples were subjected to ERT by the addition of dNTPs as described previously (Thomas

et al., 2008). The average ratios of ERT to preERT are reported. Results in both panels are averages with error bars representing the standard deviation of two independent experiments.

Fig. 11.

Reverse transcription of mutant and WT HFV-1 over a 24h infection period. Virus containing supernatants were DNase I treated then applied to HeLa clone 1022 cells, and incubated for 24h. Total DNA was harvested from the infected cells and the levels of the various reverse transcription intermediates (indicated at the right of the figure) were assessed by qPCR (Buckman et al., 2003; Thomas et al., 2006a). The mutant and WT viruses used for the infections are indicated at the bottom of the figure. (A) Viral DNA levels, reported as a percentage of R-U5 for a particular infection sample. (B) Viral DNA intermediate levels reported as a percentage of the same intermediate for WT virus. Intermediate quantities were adjusted for equivalent exogenous template RT activities and DNA recoveries (based on CCR5 copies (Thomas et al., 2006a)). Results in both panels are averages with error bars representing the standard deviation of two independent experiments with the exception of F16A/W37A where only a single experiment was performed.

Table 1

Ensemble measurements of nucleic acid interaction properties for WT NC and NC variants in which aromatic residues are replaced with Ala.

HIV-1 NC	K_d (microTAR, nM) ^a	K_d ((TG) ₄ , nM) ^a	% RNA aggregated (at 30 min) ^b	TAR RNA/DNA annealing rate (min ⁻¹) ^c
WT	315 ± 48	5 ± 1	94	>6
F16A	44 ± 20	10 ± 3	78	2.6 ± 0.3
W37A	561 ± 88	163 ± 110	53	1.4 ± 0.9
F16A/W37A	623 ± 130	5446 ± 1970	40	0.2 ± 0.1

^aData obtained from FA binding assays in 50 mM NaCl using a fluorescein-labeled microTAR RNA hairpin (18-mer) or single-stranded FAM-labeled (TG)₄.

^bData obtained from standard sedimentation assays using ³²P-TAR RNA and unlabeled cTAR DNA as described (Vo et al., 2006). The % RNA aggregated following a 30-min incubation with 0.88 μM NC is indicated.

^cData obtained from gel-shift annealing assays using TAR RNA and DNA hairpins. Assays were performed at 37 °C in 20 mM NaCl using 0.88 μM protein (4:1, nt:NC). Only a lower limit for the rate constant could be obtained for WT NC under these conditions.

Table 2

Properties of mutant and WT viruses.

NC mutant	RT activity ^a	TZM-bl titer (% of wild type) ^b	H9 titer (TCIU/mL) ^c	Genomes (% of wild type) ^d
(-)-Control ^e	0.00×10^0	0.0	<1	0
F16W	1.60×10^7	50.0 ± 10.7	10^6	63 ± 6
W37F	1.69×10^7	98.6 ± 11.2	10^5	62 ± 5
F16W/W37F	1.68×10^7	22.7 ± 1.8	10^6	25 ± 2
F16A	1.25×10^7	14.8 ± 0.1	10^2	35 ± 3
W37A	4.06×10^6	0.70 ± 0.07	<1	18 ± 2
F16A/W37A	1.97×10^6	0.01 ± 0.01	<1	7.6 ± 1.0
WT	1.01×10^7	100.0	10^6	100

^a Exogenous template RT activity cpm [³H]TMP incorporated per mL of viral supernatant.

^b Single-round TZM-bl titer in blue cell focus units (BCFU) per mL of supernatant from two representative assays performed as described (Morcock et al., 2008). Titers have been normalized for equivalent RT activities. Errors represent the standard deviation.

^c Replication of 10-fold serial dilutions of virions in H9 cells, monitored over a 9-week period. Titers are expressed as tissue culture infectious units (TCIU) per mL and are from a representative experiment. The RT activity of the undiluted inoculum for each sample is reported in the "RT activity" column of this table.

^d Genome quantities were determined by normalizing real-time RT PCR quantities (Thomas et al., 2006a) for equivalent RT activities. Errors reported are the standard error of the mean, for 6 or 7 independent virus preparations.

^e Cell cultures transfected with sheared salmon sperm DNA.