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The N-terminal Zinc Finger and Flanking Basic Domains Represent the Minimal Region of HIV-1 Nucleocapsid Protein for Targeting Chaperone Function

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Abstract

The human immunodeficiency virus type-1 (HIV-1) nucleocapsid (NC) protein is a chaperone that facilitates nucleic acid conformational changes to form the most thermodynamically stable arrangement. The critical role of NC in many steps of the viral life cycle makes it an attractive therapeutic target. The chaperone activity of NC depends on its nucleic acid aggregating ability, duplex destabilizing activity and rapid on/off binding kinetics. During the minus-strand transfer step of reverse transcription, NC chaperones the annealing of highly structured transactivation response region (TAR) RNA to the complementary TAR DNA. In this work, the role of different functional domains of NC in facilitating 59-nucleotide TAR RNA/DNA annealing was probed by using chemically-synthesized peptides derived from full-length (55 amino acids) HIV-1 NC: NC(1-14), NC(15-35), NC(1-28), NC(1-35), NC(29-55), NC(36-55) and NC(11-55). Most of these peptides displayed significantly reduced annealing kinetics, even when present at much higher concentrations than wild-type (WT) NC. In addition, these truncated NC constructs generally bind more weakly to single-stranded DNA and are less effective nucleic acid aggregating agents than full-length NC, consistent with the loss of both electrostatic and hydrophobic contacts. However, NC(1-35) displayed annealing kinetics, nucleic acid binding, and aggregation activity that were very similar to that of WT NC. Thus, we conclude that the N-terminal zinc finger, flanked by the N-terminus and linker domains, represents the minimal sequence that is necessary and sufficient for chaperone function *in vitro*. In addition, covalent continuity of the N-terminal 35 amino acids of NC is critical for full activity. Thus, although the hydrophobic pocket formed by residues proximal to the C-terminal zinc finger has been a major focus of recent anti-NC therapeutic strategies, NC(1-35) represents an alternative target for therapeutics aimed at disrupting NC's chaperone function.

HIV-1 nucleocapsid protein (NC) is a 55-amino acid highly basic protein generated as a result of proteolytic cleavage of the gag precursor protein during viral maturation¹⁻⁶. HIV-1 NC has been shown to play roles in different steps of the viral life cycle including specific

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viral RNA packaging^{7, 8}, virus assembly⁹, reverse transcription of viral RNA^{10–12}, and proviral DNA integration^{13, 14}. NC consists of two well-conserved CCHC-type zinc finger motifs that are separated by a short basic linker. The aromatic residues present in the zinc finger motifs stack with nucleobases present in single-stranded (ss) regions of nucleic acids^{15–18}. NC also possesses a 14-amino acid flexible N-terminal domain, which has a high local density of basic residues (5 out of 14). This N-terminal domain forms a 3_{10} -helix when NC binds to stemloops 2 and 3 of the viral packaging signal^{15, 17}. NC's numerous roles in the viral life cycle depend, in part, on its nucleic acid chaperone activity, which facilitates nucleic acid rearrangements to form the thermodynamically most stable conformation^{10–12, 19}. Due to the critical role of NC in HIV-1 infection, targeting NC's chaperone function is a viable therapeutic strategy^{20–23}.

The nucleic acid chaperone function of NC depends on two main activities: nucleic acid duplex destabilization and aggregation, which are associated with the zinc fingers and basic N-terminal domain, respectively^{11, 12}. In addition, rapid nucleic acid binding kinetics has also been shown to be a critical component of NC's chaperone activity^{24, 25}. Previous studies have suggested that NC's duplex destabilization activity is more important for reactions where the rate-limiting step involves significant melting of nucleic acid structure¹². For example, the zinc finger motifs were shown to be important for annealing of the highly structured transactivation response region (TAR) RNA and TAR DNA stemloops²⁶ and also for RNA removal reactions during reverse transcription^{26–28}, but are not needed for human tRNA^{Lys3} annealing to the primer binding site (PBS) in the viral RNA genome^{29, 30}. The role of zinc fingers was tested by employing a SSHS NC mutant wherein all the Cys residues of the two zinc fingers are mutated to Ser, thus abolishing the capability to bind zinc²⁶. The relative contributions of the N-terminal and C-terminal zinc finger motifs in the chaperone function of NC have also been studied, and the N-terminal zinc finger has been found to play a major role in destabilizing structured nucleic acids^{31–33}.

In addition to the destabilization activity of NC's zinc fingers, the capability of NC to aggregate nucleic acids facilitates the nucleation step of the annealing reaction^{34–38}. This nonspecific nucleic acid aggregation activity of NC is a major component of its chaperone function and contributes to annealing of both structured and non-structured nucleic acids¹¹. While the zinc fingers of NC have also been shown to play a role in aggregating nucleic acids by contributing to NC's binding strength^{36, 37}, the N-terminal domain of NC is most critical for this activity^{11, 34, 35}.

In addition to the documented roles of the N-terminal domain and zinc fingers in the chaperone activity of NC, the function of synthetic peptides derived from different domains of NC has been investigated in a variety of assays^{39–51}. For example, Surovoy et al. performed *in vitro* binding studies using chemically synthesized peptides NC(1-19), NC(36-55) and NC(20-55) and an RNA construct representing the 5' end of the HIV-1 genome⁴⁴. Both NC(1-19) and NC(20-55) bound to RNA, albeit with 50- to 200-fold lower affinity than WT, while NC(36-55) displayed almost no RNA binding. In a related study, the importance of the N-terminal zinc finger of NC and the flanking basic residues (i.e., NC(1-35)) in promoting specific binding to the ψ -packaging sequence was elucidated⁴⁵.

Peptides representing the proximal (residues 13 to 30) and distal zinc finger (residues 34 to 51) motifs were found to be inactive in *in vitro* assays of HIV-1 RNA dimerization, as well as in annealing of primer tRNA^{Lys3} to the PBS³⁹. Rocquigny et al. demonstrated the importance of the basic residues flanking the proximal zinc finger of NC in tRNA^{Lys3}/PBS annealing and RNA binding⁴¹. Complete deletion of the zinc fingers did not have any effect on the tRNA annealing activity of NC and it was concluded that inhibition of the basic residues flanking the first zinc finger of NC could be a model for the design of antiviral

agents. This study is consistent with a later study showing that zinc binding was not required for tRNA annealing^{29,30}. However, the role of different domains of NC in annealing structured nucleic acids (e.g. TAR RNA/DNA annealing), where zinc fingers play a vital role²⁶, was not investigated. In addition, the removal of zinc fingers involved complete deletion of all the amino acids forming the fingers instead of simply removing zinc, thereby maintaining the primary sequence of NC.

In the present work, NC fragments representing different domains of NC (Figure 1) were chemically synthesized and their capability to chaperone the annealing of 59-nt TAR RNA to complementary 59-nt TAR DNA was tested. As mentioned above, this reaction is known to require the destabilization activity of zinc fingers of NC²⁶. Nucleic acid binding and aggregation activity were also evaluated separately to independently gauge their contributions toward the chaperone activity. The quantitative results reported here indicate that the N-terminal zinc finger flanked with basic N-terminal and linker domains is sufficient to drive the annealing kinetics to a similar extent as WT NC, and suggest that the C-terminal zinc finger is not important for annealing of structured nucleic acids under the *in vitro* conditions investigated here. Thus, targeting the N-terminal 35 residues of NC is a viable therapeutic strategy aimed at abolishing both specific Gag- ψ RNA binding⁴⁵ and NC's chaperone function.

Experimental Procedures

Synthesis of Full-Length and Truncated NC Constructs

The following NC constructs were synthesized using 9-fluorenyl methoxy carbonyl (Fmoc) chemistry: NC(1-55), NC(1-14), NC(1-28), NC(15-35), NC(36-55), and NC(11-55). Solid-phase synthesis was performed on a 433A peptide synthesizer (Applied Biosystems, Foster City, CA) following the general procedure described previously⁵¹. The crude peptides were purified by reversed-phase HPLC and analyzed using mass spectrometry. NC(1-35) and NC(29-55) were purchased from Genscript Corp., Piscataway NJ. All lyophilized peptides were reconstituted in 40 mM HEPES pH 7.5, 5 mM dithiothreitol (DTT) and 0.1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The reducing agents DTT and TCEP were included in the reconstitution buffer to prevent the formation of disulfide bonds involving the Cys-containing peptides. The concentrations of peptides with a tryptophan residue in the sequence were determined by measuring the absorbance at 280 nm using an extinction coefficient of 6050 M⁻¹cm⁻¹. The concentrations of NC(1-35), NC(1-28) and NC(15-35) were determined by treatment with Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)], which reacts with sulfhydryl group of accessible Cys residues in a 1:1 ratio and generates a product that can be quantified by measuring the absorbance (extinction coefficient of 14150 M⁻¹cm⁻¹ in 0.1 N NaOH) at 412 nm⁵². The concentration of NC(1-14), which is devoid of Trp or Cys residues, was determined using quantitative amino acid analysis (Scientific Research Consortium, Inc, Roseville, MN). Stock solutions of peptides were aliquoted and stored at -80 °C. Poly-L-lysine was purchased from Sigma Aldrich (St. Louis, MO).

Nucleic Acid Preparation

The sequences and predicted structures (using mfold⁵³) of 59-nt TAR RNA and TAR DNA used in this study are shown in Figure 2. The TAR RNA hairpin was generated by *in vitro* transcription of a DNA template encoding the RNA sequence under the control of a T7 RNA polymerase promoter. This DNA template was prepared by PCR amplification of chemically-synthesized oligonucleotides purchased from Integrated DNA Technologies (Coralville, IA). Following transcription, TAR RNA was purified on a 12% denaturing polyacrylamide gel, and its concentration determined using an extinction coefficient of 533,700 M⁻¹cm⁻¹ at 260 nm. Internally ³²P-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. TAR DNA was obtained from Integrated DNA Technologies and purified on a 12% polyacrylamide gel. The concentration of TAR DNA was determined using an extinction coefficient of $564,800 \text{ M}^{-1}\text{cm}^{-1}$ at 260 nm. The 20-mer sequence 5'-CTTCTTTGGGAGTGAATTAG-3' (5'-FAM-DNA20) labeled at the 5' end with the succinimidyl ester of carboxyfluorescein (FAM) was obtained from Trilink BioTechnologies (San Diego, CA).

Gel-Shift Annealing Assay

Prior to each experiment, TAR RNA (1.5 μM) (spiked with ^{32}P -labeled TAR RNA) and TAR DNA (6 μM 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 100 mM MgCl_2 (to 10 mM final) and placement on ice. For annealing assays, ^{32}P -labeled TAR RNA (15 nM) and TAR DNA (45 nM) were combined in a solution containing 20 mM HEPES, pH 7.5, 20 mM NaCl, and 5 mM DTT. The final concentration of MgCl_2 (from the initial folding step) was 0.2 mM. The solution was incubated at 37 °C for 5 min before addition of NC to a final concentration of 0.88 μM , corresponding to a 4:1 nt:NC ratio. Annealing reactions were performed under these standard conditions of nucleic acids, NC and buffer composition, unless otherwise indicated. At desired time points, an aliquot (27 μl) of the reaction mixture was quenched by addition of 3 μl of 10% SDS (final concentration of 1%), 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 a 12% polyacrylamide gel (19% acrylamide: bisacrylamide). The gels were visualized using a Typhoon Trio phosphorimager (GE Healthcare) and quantified with Imagequant TL (v2005) Software. Rate constants were calculated by fitting the data to a single exponential equation.

Sedimentation Assay

Both ^{32}P -labeled TAR RNA and TAR DNA were folded as described for annealing assays. The sedimentation assays to measure aggregation were performed as described previously ³⁸, by incubating ^{32}P -labeled TAR RNA and TAR DNA to a final concentration of 15 nM and 45 nM, respectively, in a buffer containing 20 mM HEPES pH 7.5 and 20 mM NaCl in the absence or presence of different concentrations of NC. The final MgCl_2 concentration was 0.2 mM, unless otherwise indicated. The samples (30 μl) were incubated at 37 °C for 30 min and then centrifuged in a Micromax RF rotor at a speed of 10,000 rpm for 10 min. An aliquot (4 μl) of supernatant was taken and the amount of radioactivity measured using scintillation counting.

Fluorescence Anisotropy (FA)-Based Binding Assay

Protein binding to 5'-FAM-DNA20 was measured by incubating 20 nM DNA in the presence of varying concentrations of peptide in 20 mM HEPES pH 7.5, 50 mM NaCl, 10 μM TCEP, 5 mM β -mercaptoethanol and 1 μM zinc acetate for 30 min at 25 °C. FA measurements were 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 was calculated using SoftMax® Pro (Molecular Devices) software. The resulting plot of anisotropy vs. NC concentration was fit using Kaleidagraph (Synergy Software, Reading, PA) according to the following one-site binding model ⁵⁴ to obtain apparent dissociation constant (K_d):

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

where T, N, K_d , A_{bound} , A_{free} , and R represent the concentrations of oligonucleotide and protein, equilibrium dissociation constant, anisotropy of completely bound and unbound oligonucleotide, and the ratio of fluorescence intensity of completely bound oligonucleotide relative to unbound nucleotide, respectively. For binding assays performed using zinc-less NC constructs and NC(1-14), zinc acetate was omitted from the reaction buffer.

Results

NC preferentially binds to ss nucleic acids and this binding preference is crucial for its chaperone function, which involves nucleic acid aggregation and duplex destabilization activities. Binding capability general correlates closely with aggregation activity, which is facilitated mainly by NC's basic residues. In contrast, the zinc finger structures are primarily responsible for nucleic acid destabilization capability, which is essential for annealing of stable stem-loop substrates such as TAR RNA/DNA investigated here. Chemically synthesized full-length HIV-1 NC (55 amino acids) and NC fragments (Figure 1) were used to explore in more detail the specific roles of different domains of NC in mediating nucleic acid binding, aggregation and complementary TAR RNA/DNA annealing. The theoretical isoelectric point (pI) of all the NC constructs was calculated using Protparam web-based program⁵⁵ and these values are given in Table 1. Unless otherwise noted, all of the NC constructs containing CCHC motif(s) were reconstituted with three equivalents of zinc prior to the assays.

Nucleic Acid Binding

The results of fluorescence anisotropy (FA) binding studies carried out with the NC fragments and 5'-FAM-DNA20 are summarized in Table 1. A non-specific ss DNA was chosen for these studies to avoid any complications due to RNA folding or due to NC's duplex destabilization activity. WT NC binds to this DNA with a dissociation constant (K_d) of 57 nM and NC(1-35) demonstrated a similar binding affinity, with a K_d of 86 nM. NC(15-35) bound with at least ~116-fold lower affinity than NC(1-35), confirming an important role in nucleic acid binding for the electrostatic interactions provided by the highly basic N-terminal domain. Removal of the linker domain from NC(1-35) to generate NC(1-28) led to ~8.5-fold reduction in binding affinity. Thus, at 50 mM NaCl, the N-terminal and linker domains flanking the proximal zinc finger provide the major contribution to binding. The results for NC(15-35) and NC(1-28) also reveal the greater contribution of the N-terminal domain to binding relative to the linker region. Surprisingly, no binding could be detected by the highly basic domain NC(1-14) alone.

NC(29-55) has ~15-fold lower ss DNA binding affinity than WT NC, with a K_d of 867 nM. Although, NC(15-35) and NC(29-55) both include the basic linker domain and have similar pI values, the binding affinity of NC(15-35) was measured to be at least ~12-fold weaker than NC(29-55). The hydrophobic pockets formed by the residues of the zinc fingers of NC have been shown to interact specifically with unpaired guanosine residues of single-stranded sequences^{8, 12, 18}, and the aromatic residues Phe16 and Trp37 stack with the guanosine bases. The results reported here are consistent with the previously observed stronger stacking interactions of Trp 37 (present in NC(29-55)) relative to Phe16 (present in NC(15-35))^{56, 57}.

The effect of zinc-coordination to the CCHC motif(s) present in WT NC, NC(1-35), NC(1-28) and NC(29-55) was probed by performing binding studies in the absence of zinc (i.e., the synthetic peptides were not reconstituted with zinc, and the reaction buffer did not contain Zn^{2+}). In the case of WT NC, the zinc-bound protein has a slightly lower affinity (~4-fold) compared to the zinc-less form (Table 1), suggesting that the zinc finger structure is not critical for nucleic acid binding under the low salt conditions employed here, where non-specific electrostatic interactions likely dominate. Interestingly, zinc coordination has the opposite effect on binding by NC(1-35), NC(1-28) and NC(29-55) sequences; the zinc-bound forms have slightly higher affinities (~2- to 3-fold) for 5'-FAM-DNA20, relative to the zinc-less variants (Table 1). This is likely due to the enhanced role of the folded zinc fingers for these peptides, which lack the additional electrostatic interactions conferred by the basic residues in the deleted regions.

Nucleic Acid Aggregation

NC has been shown to form ordered aggregates with nucleic acids^{37, 38, 58–61} and this activity facilitates nucleation of the product duplex during annealing reactions. Saturated NC binding is required for maximum aggregation activity and the NC-concentration dependence of fractional nucleic acid aggregation reflects the protein's binding strength³⁸. To monitor the contribution of different domains of NC in nucleic acid aggregation, sedimentation assays were performed using varying concentrations of WT NC and NC fragments under conditions similar to those used for TAR RNA/DNA annealing assays (see Experimental Procedures and results described below). All of the NC constructs containing CCHC motif(s) were reconstituted with 3 equivalents of zinc, unless noted otherwise. Results of sedimentation assays are depicted in Figure 3. WT NC displayed very high levels (~97%) of nucleic acid aggregation, even at 0.88 μM concentration. As expected, aggregation levels did not change appreciably upon increasing the concentration of WT NC to 3 μM and 10 μM . Similar to WT NC, NC(1-35) aggregated ~96% of the nucleic acids, even at 0.88 μM peptide (Figure 3A). This result suggests that the distal zinc finger is not important for nucleic acid aggregation. Under these relatively low salt conditions, close to 100% aggregation was observed for WT NC and NC(1-35) at the lowest concentration tested (0.88 μM), precluding comparison of their relative activities. Therefore, we also studied aggregation activities of these proteins at much lower protein concentrations and in the presence of 2 mM MgCl_2 . Mg^{2+} competes effectively with low concentrations of both WT NC and NC(1-35), making it possible to observe any differences in binding and aggregation capability, which appear to be minor (Figure 3C).

NC(1-14) and NC(15-35) demonstrated concentration-dependent aggregation behavior over the 10 μM to 50 μM peptide concentration range with lower levels observed for NC(1-14) (Figure 3A). Previous studies showed that deletion of part of the N-terminal domain from the WT sequence resulted in almost total abrogation of the nucleic acid aggregating capability of NC^{34, 35}. However, the aggregation behavior of the isolated N-terminal domain was not addressed previously. Results presented here for NC(1-14) suggest that the N-terminal domain by itself is not sufficient for efficient nucleic acid aggregation. When present at high concentration (50 μM), NC(15-35) showed almost WT levels (~95%) of aggregation, whereas NC(1-14) only achieved ~80%. When NC(1-14) and NC(15-35) are present in *trans*, their capability to induce nucleic acid aggregation does not exceed that of NC(15-35) (Figure 3A). Thus, covalent continuity of NC(1-14) and NC(15-35) is required for efficient aggregation activity.

NC(1-28) showed ~2.2-fold lower aggregation levels than NC(1-35) at 0.88 μM , suggesting a role for the basic linker domain in the aggregation activity (Figure 3A and B). The aggregation activity of NC(1-28) approached that of NC(1-35) at concentrations of 20 μM

and above, in good agreement with the annealing activity results (see below). NC(36-55) and NC(29-55) displayed extremely poor aggregation capability, and showed no appreciable change in aggregation levels when their concentrations were increased from 10 μ M to 50 μ M (Figure 3B); the levels of aggregation at 50 μ M remained at ~0% and ~8% for NC(36-55) and NC(29-55), respectively. The slightly higher level of aggregation for NC(29-55) is likely conferred by the additional basic linker domain, which may enhance non-specific electrostatic interactions with nucleic acids.

Previous studies have shown that the aggregation capability of WT NC does not strongly depend on its folded zinc fingers^{29, 30, 34}. To assess if this holds true for NC fragments as well, we performed sedimentation assays using NC(1-28), NC(1-35), and NC(29-55) in the absence of zinc, under the same concentrations employed for the zinc-bound forms. In the case of NC(1-35), no appreciable differences in aggregation levels were observed in the presence or absence of zinc, and essentially complete aggregation was observed in both cases (data not shown). In the case of NC(29-55), zinc does appear to modulate nucleic acid aggregation behavior, since the levels of aggregation observed for the zinc-less form are higher than the zinc-bound form by ~2- to 4-fold at all concentrations tested (Figure 3B). In addition, and in contrast to the zinc-bound form, the zinc-less form of NC(29-55) showed concentration-dependent aggregation behavior, with aggregation levels increasing from 40% to 72% upon raising the peptide concentration from 10 μ M to 50 μ M, respectively. NC(1-28) showed ~1.7-fold lower aggregation activity than its zinc-less form at the lowest concentration tested (0.88 μ M), but both forms showed similar activities at high concentrations. Taken together, these results suggest that the absence of zinc may enhance the aggregation behavior of some NC-derived peptides by transforming their rigid zinc-bound structures to more mobile peptides. In addition, the C-terminal zinc finger is modulated more strongly by zinc binding than the N-terminal zinc finger, in agreement with the more hydrophobic and less cationic character of the C-terminal domain nucleic acid binding interaction.

TAR RNA/DNA Annealing

The overall chaperone function of WT NC and truncated NC fragments was determined by performing time-course annealing assays to measure the capability of the peptides to anneal complementary TAR RNA and TAR DNA hairpins (Figure 2) to form a 59-bp duplex. Figure 4 shows the percent of TAR RNA annealed over time and the annealing rate constants are presented in Table 2. Under saturating binding conditions (0.88 μ M; 4:1 nt:NC ratio¹¹), WT NC displayed very fast annealing kinetics, with 80% of the RNA annealed within 30 sec and 86% annealed product formed at 30 min (Figure 4A). Due to the fast reaction rate observed for WT, we could only measure the lower limit for the annealing rate constant under these conditions (Table 2).

NC(1-14), which has five basic residues in its sequence, showed very poor capability to anneal TAR RNA/DNA even at 20 μ M concentration (~20-fold higher than WT), with only 3% RNA annealed after 30 min (Figure 4A). The level of annealing after 30 min did not change appreciably even when the concentration of NC(1-14) was raised to 50 μ M (data not shown), in agreement with the extremely poor binding observed for this peptide (Table 1). NC(1-14) represents the major aggregation domain of WT NC^{11, 12} and behaves as a flexible short polycation. In order to evaluate if longer polycations could better facilitate the annealing reaction, we performed annealing assays using zinc-less WT NC and poly-lysine (Figure 4B). The latter has been shown to be a highly efficient nucleic acid aggregating agent⁶². The results showed that these longer unstructured polycations have 2- to 3-fold reduced annealing activity compared to WT NC (Figure 4B and Table 2). These results support a role for the zinc fingers in destabilizing the TAR stem-loop structures during the annealing reaction¹².

The annealing activity of NC(36-55), even at 50 μ M, is also very poor, with final levels of annealing after 30 min reaching only ~1.5% (Figure 4C), consistent with this variant's low net positive charge density ($pI = 7.9$) due to four basic and three acidic residues. Addition of the highly basic linker domain to NC(36-55), thus generating NC(29-55), did not significantly improve the annealing activity. The final levels of RNA annealed after 30 min for NC(29-55) approached 6% (Figure 4C). Results obtained for NC(36-55) and NC(29-55) suggest that the distal zinc finger may not be important for annealing of TAR RNA/DNA under the low salt conditions used here.

In contrast, at 20 μ M concentration, NC(15-35) displayed significant annealing activity, despite the fact that it lacked the C-terminal zinc finger; ~38% RNA/DNA duplex was formed after 30 min (Figure 4A). Previous studies suggested that the N-terminal zinc finger is more important for destabilizing structured nucleic acids than the C-terminal zinc finger^{31, 33}, in accord with the results obtained here.

Even in the presence of 20 μ M NC(15-35), the rate of annealing of this variant is significantly reduced (> 600-fold) relative to WT NC (Table 2), which suggests that the presence of the proximal zinc finger connected to the basic linker contributes to the annealing, but is not sufficient for optimal chaperone activity. In contrast, NC(1-35), which consists of the proximal zinc finger flanked by the basic N-terminal and linker domains, displays annealing kinetics at or close to the lower limit established for WT NC at the same concentration (0.88 μ M), with ~75% of RNA annealed within 30 sec (Figure 4A and Table 2). To better differentiate the annealing activities of NC(1-35) and WT NC, annealing assays were carried out at higher Mg^{2+} concentration (2 mM $MgCl_2$), conditions where the kinetics of annealing are known to be reduced^{60, 63}. While TAR binding by WT NC and NC(1-35) is likely saturating under these conditions, Mg^{2+} is known to stabilize the TAR RNA and DNA hairpins, thereby slowing the annealing reaction. Even under these conditions, NC(1-35) showed similar annealing activity as WT NC (Figure 4D). The final levels of annealing were ~63% and ~55% for WT and NC(1-35), respectively, and the annealing rate constant for NC(1-35) was only ~1.3-fold lower than WT (Table 2). Thus, as for the binding and aggregation assays, NC(1-35) represents the minimal region, containing the necessary sequence and structural elements required for efficient TAR RNA/DNA annealing.

To determine the effect of adding NC(1-14) and NC(15-35) together, both peptides were used at equal concentrations of 20 μ M in the annealing reaction (Figure 4A). Activity of the two domains *in trans* was much lower than the activity of NC(1-35) at 0.88 μ M. The final level of annealing and the k_{obs} were very similar to that of NC(15-35) (Table 2). This suggests that as for aggregation activity, covalent continuity of NC(1-35) is essential for optimal chaperone function. Consistent with this observation, NC lacking the N-terminal 10 amino acid residues, NC(11-55), is highly deficient in annealing TAR RNA/DNA, even at five-fold higher protein concentration compared to WT NC (Figure 4B). These observations indicate that the N-terminal region of NC plays an important role in NC's annealing activity presumably by promoting nucleic acid aggregation. Comparison of the two peptides containing the first or the second zinc finger in combination with the cationic linker, i.e. NC(15-35) and NC(29-55) in their TAR RNA/DNA annealing (Fig. 4A and C) highlights much stronger activity of the first zinc finger, in agreement with its stronger TAR RNA/DNA binding under the same solution conditions (Fig. 3A and B).

To probe the role of the linker domain in the activity of NC(1-35), annealing assays were performed using NC(1-28), which lacks the basic linker region. This variant demonstrated very poor annealing activity at 0.88 μ M, suggesting an important role for the linker domain in the chaperone activity of NC(1-35) (Figure 4C). However, 20 μ M NC(1-28) showed activity almost equal to 0.88 μ M NC(1-35) (Figure 4A and C). Under these conditions, the

annealing extents at the shortest time point tested (30 sec) were ~63% and ~75% for NC(1-28) and NC(1-35), respectively. This result suggests that the basic residues of the linker domain (R29, R32, K33 and K34) contribute to nucleic acid binding and not to destabilization activity by increasing the overall positive charge of NC(1-35).

Discussion

The present study using NC fragments was performed to delineate the roles played by different domains of NC in its nucleic acid chaperone function. Targeting this critical function of NC is a promising therapeutic strategy. Studies of TAR RNA/DNA annealing, nucleic acid aggregation, and ss DNA binding suggest that the C-terminal domain of NC (i.e., residues 36–55) is not required for NC's chaperone function. The presence of the N-terminal zinc finger flanked by the highly basic N-terminus and linker domains is sufficient to achieve binding, aggregation, and annealing activities similar to that of WT NC. Previous studies have shown that folded zinc fingers are important for TAR RNA/DNA annealing²⁶ and that the N-terminal zinc finger is especially crucial for annealing structured substrates^{31, 33}. The similar nucleic acid chaperone abilities of WT NC and NC(1-35) suggest that the C-terminal zinc finger is dispensable for annealing structured nucleic acids such as TAR RNA/DNA.

Since complete nucleic acid aggregation was observed by WT NC or by NC(1-35), with and without bound zinc, it appears that zinc coordination is not required for aggregation activity. Indeed, polycations with little, if any, apparent structure have been shown to be effective nucleic acid aggregating agents^{11, 64, 65}, which correlates with the present results. Furthermore, the zinc-less variants of NC(29-55) and NC(1-28) are more efficient in aggregating nucleic acids than the zinc-bound forms, which may be due to the presence of greater flexibility in the peptide backbone of the zinc-less forms. Our conclusion that the folded zinc finger structures of NC are not required for effective nucleic acid aggregation is not in agreement with previous electron microscopy studies³⁷. The previous work employed higher Mg^{2+} concentrations, as well as much longer nucleic acid sequences, which may result in a different mechanism of NC-induced nucleic acid aggregation.

Another interesting finding from our work was that the isolated NC(1-14) peptide is less effective in promoting nucleic acid aggregation, binding, or annealing than WT NC or NC(1-35), despite the fact that this is the main "aggregation domain" in the context of full-length NC^{11, 12}. Indeed, annealing studies with NC(11-55) support the key role played by the N-terminal residues in chaperone function. Adding NC(1-14) to NC(15-35) *in trans* also failed to improve activities of the isolated domains. Thus, the N-terminal domain of NC cannot function as an independent chaperone protein but can strongly enhance the chaperone function of NC when covalently connected to the zinc fingers.

The nucleic acid binding affinity of NC is closely coupled to its nucleic acid aggregating activity¹¹. Results of binding measurements using a ss DNA oligonucleotide indicate that, with the exception of NC(1-35), which has a K_d within 2-fold of WT NC, truncated constructs have significantly lower affinity (>10-fold) compared to the full-length sequence. Although an unstructured ss DNA oligonucleotide was used in the present work, the binding affinities of different NC constructs could still be compared, given that nucleic acid chaperones generally do not recognize a specific sequence. Previous studies examining binding to the psi RNA packaging signal also concluded that specific binding by HIV-1 NC requires NC(1-35)⁴⁵.

Whereas WT NC displays somewhat stronger binding in the absence of zinc, enhanced binding is observed for zinc-bound forms of the truncated constructs tested. These binding

results for NC fragments [except NC(1-35)] are in contrast to their aggregation capabilities, where zinc-less forms are generally better in aggregating nucleic acids compared to zinc-bound forms. With fewer basic residues in NC fragments, the presence of at least one folded zinc finger domain enhances binding to ss DNA, whereas unstructured fragments are more capable of aggregating nucleic acids than their structured counterparts. NC(15-35) has appreciable TAR RNA/DNA annealing and aggregation activities, even though it binds to ss DNA very poorly. This is in agreement with the conclusion that the N-terminal zinc finger is important for chaperone activity.

NC has been proposed to be an effective therapeutic target due to its important role in HIV-1 replication^{12, 20–23}. NC possesses two invariant CCHC zinc fingers and several studies have focused on development of small molecule inhibitors that function by ejecting zinc and thus inhibiting NC's activity^{23, 66, 67}. The challenge here is to generate compounds that specifically interact with NC, without inadvertently affecting the zinc fingers associated with cellular proteins such as transcription factors. Inhibitors that function by binding to the NC protein without chelating zinc may therefore represent a more promising strategy. Recently, efforts to target different aspects of NC function such as nucleic acid binding, annealing, and duplex destabilization activities have been reported^{68–79}. Several of these inhibitors function via binding to the hydrophobic pocket of NC formed by the zinc finger motifs^{72, 74, 76, 77, 79}.

The present study has identified the first 35 residues of NC as sufficient for its chaperone function. Since this is also the minimal domain for conferring specific psi RNA binding⁴⁵, small molecule inhibitors targeting this minimal domain could potentially block two important NC activities, i.e., genomic RNA packaging and nucleic acid chaperone function. Alternatively, this minimal construct, with its specific cationic and hydrophobic features, could be used as a starting point to synthesize peptidomimetic compounds with enhanced binding affinities to NC substrates. Although our results show that the C-terminal zinc finger is not essential for NC's chaperone activity *in vitro*, the numerous functions of NC *in vivo* likely involve important contributions of both zinc fingers. Nevertheless, the identification of the minimal domain required for chaperone function might provide a platform for the development of a new class of NC-targeted therapeutics.

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Abbreviations

NC	nucleocapsid
HIV-1	human immunodeficiency virus type-1
TAR	transactivation response region
nt	nucleotide(s)
PBS	primer-binding site
Fmoc	9-fluorenyl methoxy carbonyl
DTT	dithiothreitol

TCEP	tris(2-carboxyethyl)phosphine hydrochloride
FAM	carboxyfluorescein
ss	single-stranded
K_d	dissociation constant

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Figure 1.

Sequences of chemically-synthesized HIV-1 NC (NL4-3 isolate) constructs used in this work: WT NC(1-55), NC(1-14), NC(15-35), NC(1-35), NC(1-28), NC(29-55), NC(36-55) and NC(11-55). The basic residues are indicated by arrows. The aromatic residues in the zinc fingers are boxed. The zinc binding residues are shown in gray. Peptides NC(1-35) and NC(29-55) were purchased commercially from Genscript Corp. (Piscataway NJ), whereas all the other peptides were synthesized in house.

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Figure 3.

RNA aggregation assays for WT NC and truncated NC peptides. (A) Assays performed with 0.88–20 μM WT NC and NC(1-35), and 10–50 μM NC(1-14) and NC(15-35) alone or added together *in trans*. (B) Assays with 0.88–50 μM NC(1-28) in the presence and absence of zinc, 10–50 μM NC(29-55) in the presence and absence of zinc, and 10–50 μM NC(36-55). (C) Assays performed with 23–600 nM WT NC and NC(1-35) in the presence of 2 mM MgCl_2 .

Figure 4.

TAR RNA/DNA annealing time courses for WT NC and truncated NC peptides. (A) Reactions with WT NC and NC(1-35) were performed with $0.88 \mu\text{M}$ peptide. NC(1-14), NC(15-35) and NC(1-14) + NC(15-35) were all present at $20 \mu\text{M}$. (B) Reactions using $4 \mu\text{M}$ of WT NC in the presence and absence of zinc (Zinc-less), $3 \mu\text{M}$ Poly-Lysine, and $20 \mu\text{M}$ NC(11-55) performed in the presence of 90 nM TAR DNA and 2 mM MgCl_2 . (C) Reactions with WT NC and NC(1-28) were with $0.88 \mu\text{M}$ peptide, NC(1-28)* was present at $20 \mu\text{M}$, and NC(29-55) and NC(36-55) were at $50 \mu\text{M}$. (D) Reactions using $0.88 \mu\text{M}$ WT NC and NC(1-35) performed in the presence of 2 mM MgCl_2 . Results are the average of three trials with standard deviations indicated

Table 1

Theoretical Isoelectric Points (pI) of WT NC and NC Fragments and their Apparent Binding Dissociation Constants (K_d) to 5'-FAM-DNA20.

NC Variant	pI ^a	K_d ^b (nM)
WT	9.93	57 ± 22
WT (zinc-less)	9.93	16 ± 5
NC(1-14)	12.02	1 × 10 ⁴
NC(15-35)	9.75	1 × 10 ⁴
NC(1-35)	10.71	86 ± 19
NC(1-35) (zinc-less)	10.71	296 ± 170
NC(1-28)	9.93	731 ± 150
NC(1-28) (zinc-less)	9.93	1600 ± 309
NC(29-55)	9.65	867 ± 3
NC(29-55) (zinc-less)	9.65	1835 ± 370
NC(36-55)	7.95	ND
NC(11-55)	9.54	ND

^a pI values were computed using the ProtParam program⁵⁵.

^b FA Binding experiments were performed in 20 mM HEPES pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol, 10 μM TCEP and 1 μM zinc acetate at 25 °C. For binding studies using the zinc-less NC constructs or NC(1-14), no zinc acetate was present. Values reported represent the average of three trials with the standard deviation indicated. ND is not determined.

Table 2Annealing Rate Constants (k_{obs}) of WT NC and NC Variants

NC Variant, conc.	Final % Annealing	k_{obs} (min^{-1}) ^a	Fold decrease ^b
WT, 0.88 μM	86	> 4	-
NC(1-14), 20 μM	3	ND	
NC(1-35), 0.88 μM	84	> 4	-
NC(15-35), 20 μM	37	0.15 ± 0.03	600
NC(1-14) + NC(15-35), 20 μM each	46	0.15 ± 0.01	600
NC(1-28), 20 μM	88	2.7 ± 1.5	33
NC(36-55), 50 μM	1.5	ND	
NC(29-55), 50 μM	6	ND	
WT, 0.88 μM ^c ,	63	0.22 ± 0.04	-
NC(1-35), 0.88 μM ^c	54	0.17 ± 0.07	1.3
WT, 4 μM ^d	72	0.12 ± 0.01	-
WT (zinc-less), 4 μM ^d	22	0.07 ± 0.02	2
Poly-lysine, 3 μM ^d	28	0.03 ± 0.01	3
NC(11-55), 20 μM ^d	5	0.009 ± 0.002	~70

^aValues reported represent the average of three trials with the standard deviation indicated. ND is not determined.^bValues are normalized for difference in protein concentration used. The fold decrease was calculated relative to the rate constant for WT NC obtained under the same conditions.^cExperiments were performed at 2 mM MgCl₂.^dExperiments were performed using 90 nM TAR DNA and 2 mM MgCl₂.