Structural and Dynamics Studies of the D54A Mutant of Human T Cell Leukemia Virus-1 Capsid Protein*

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The human T cell leukemia virus and the human immunodeficiency virus share a highly conserved, predominantly helical two-domain mature capsid (CA) protein structure with an N-terminal β-hairpin. Despite overall structural similarity, differences exist in the backbone dynamic properties of the CA N-terminal domain. Since studies with other retroviruses suggest that the β-hairpin is critical for formation of a CA-CA interface, we investigated the functional role of the human T cell leukemia virus β-hairpin by disrupting the salt bridge between Pro1 and Asp54 that stabilizes the β-hairpin. NMR 15N relaxation data were used to characterize the backbone dynamics of the D54A mutant in the context of the N-terminal domains, compared with the wild-type counterpart. Moreover, the effect of the mutation on proteolytic processing and release of virus-like particles (VLPs) from human cells in culture was determined. Conformational and dynamic changes resulting from the mutation were detected by NMR spectroscopy. The mutation also altered the conformation of mature CA in cells and VLPs, as reflected by differential antibody recognition of the wild-type and mutated CA proteins. In contrast, the mutation did not detectably affect antibody recognition of the CA protein precursor or release of VLPs assembled by the precursor, consistent with the fact that the hairpin cannot form in the precursor molecule. The particle morphology and size were not detectably affected. The results indicate that the β-hairpin contributes to the overall structure of the mature CA protein and suggest that differences in the backbone dynamics of the β-hairpin contribute to mature CA structure, possibly introducing flexibility into interface formation during proteolytic maturation.

All retroviruses encode a precursor polyprotein designated the group-specific antigen (Gag)1 that is sufficient to direct the formation and release of immature viral particles (1). In the presence of the virus-encoded protease, the Gag precursor is cleaved as the immature virion buds, generating three products: matrix (MA), capsid (CA), and nucleocapsid (NC), which refold and organize spatially to form an infectious mature virion. The CA protein condenses around the NC-RNA complex at the center of the virion to form a core structure within a shell composed of MA proteins. Whereas the noninfectious immature particles of all retroviruses are generally spherical, the mature infectious particles are morphologically distinct and are characterized by the shape of the CA core structure. The mature virion is conical for human immunodeficiency virus type 1 (HIV-1) and other lentiviruses and spherical or irregularly polyhedral for human T cell leukemia virus type 1 (HTLV-1) and other members of the retrovirus group, including Rous sarcoma virus and Moloney murine leukemia virus. The morphology of the core and viral infectivity are closely correlated; mutations that result in loss of the characteristic core structure also result in loss of infectivity (2, 3).

The CA protein that forms the core consists of two independently folded domains joined by a flexible linker peptide (4). The structure is highly conserved among retroviral CA proteins that have been examined (5–10); mutations in the C-terminal domain of HIV-1 impair CA protein dimerization and particle assembly (3, 11–13). Mutations in the N-terminal domain (NTD) of HIV-1 block CA protein assembly to a significantly lesser extent, but the assembled particles are noninfectious (3, 11, 13, 14). In contrast, mutations in the C-terminal domain of the HTLV-1 CA protein had little effect on viral particle assembly and release, whereas mutations throughout the NTD of the protein had much greater impact (15). NTD insertion mutations completely blocked particle release of assembled particles; NTD substitution mutations reduced release of mature particles by 5–50%. In particular, a substitution in helix 3 (Asp54-Leu55 to Arg-Ser) produced a 10-fold greater defect than substitutions elsewhere in the NTD and blocked particle assembly almost completely (15). These observations suggest that the NTD and C-terminal domain in the HIV-1 and HTLV-1 CA proteins do not function in the same manner.

The N-terminal residues of the HTLV-1 (8), HIV-1 (4), and Rous sarcoma virus (6) CA proteins form a β-hairpin structure. It has been proposed that upon proteolysis from the Gag pre-

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The abbreviations used are: Gag, group-specific antigen; MA, matrix; CA, capsid; NC, nucleocapsid; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T cell leukemia virus type 1; NTD, N-terminal domain; VLP, virus-like particle.
The refolded amino terminus is hypothesized to create a new CA-CA interface that is essential for building the condensed conical core that will initiate replication in the next round of infection (2). The fact that the spumavirus subgroup lacks the conserved Pro and Asp residues and does not undergo proteolytic maturation supports the hypothesis that the sequence conservation is linked to the refolding function and core assembly. Interestingly, a 2-amino acid insertion next to the conserved Asp51-Leu52 residues in helix 3 of the HTLV-1 CA protein significantly reduced formation of viral particles (15). In contrast, an insertion next to the conserved Asp51-Leu52 residues in the NTD of the HIV-1 CA protein had no detectable effect on viral particle formation (13).

These several observations suggest that structurally equivalent regions can form functionally distinct assembly interfaces during capsid assembly, despite conservation of the secondary and tertiary structures. However, it seems unlikely that the insertion perturbed the β-hairpin structure, since a point mutation in the HIV-1 CA helix 3 contact residue, Asp54, to Ala, reduced viral particle assembly to ~20−100% of WT efficiency, reduced viral infectivity correspondingly, and blocked formation of the typical cone-shaped core structure (20). The fact that each of the Gag precursor incorporated into the viral particles was processed aberrantly to truncated CA protein suggests that the mutation exposed the N terminus to inappropriate proteolysis and possibly accounts for the reduced assembly efficiency.

In this study, we adopted an approach that combines structural, biochemical, and functional techniques to study the effect of disrupting the β-hairpin loop on the structure and dynamics of HTLV-1 CA in solution and on the ability to assemble virus-like particles (VLPs) in mammalian cells. We examined the effect of disrupting the HTLV-1 CA Pro3-Asp54 salt bridge by mutating Asp54 to Ala. Consistent with the notion that the HTLV-1 NTD is a less flexible structure (16), we found that the D54A mutation completely destabilized the β-hairpin in the HTLV-1 NTD and caused localized conformational and dynamical changes in the region near the mutation. We observed significantly smaller changes (~0.2-ppm amide proton shifts) in helices 1, 2, 3, and 6, which are proximal to the β-hairpin in the three-dimensional structure. These findings contrast with the greater perturbation of the equivalent regions that result from the D51A mutation in the HIV-1 CA NTD (2). The conformational alterations detected by NMR in CA134 were maintained in the Gag protein expressed in 293T cells, as indicated by differential monoclonal antibody recognition of the WT and D54A mutated CA proteins following proteolytic maturation. The mutated CA protein was assembled and released into VLPs formed by the Gag and Gag-Pro proteins. Moreover, processing of D54A Gag polyprotein, in contrast to HIV-1 D51A Gag, was not affected. Thus, our data indicate that differences in the backbone dynamics of the β-hairpin of retroviral capsids correlate with differences in the course of mature CA assembly, processing, and release.

MATERIALS AND METHODS

Protein Expression, Purification, and Preparation of NMR Samples—The DNA fragment encoding the D54A mutant was cloned into the NdeI/SalI sites of the pT7B1 plasmid vector (New England Biolabs), as described previously (8, 16). The recombinant plasmid was transformed into Escherichia coli ER2566 strain for protein expression, and the purified D54A mutant was obtained by the method described previously (8, 16). The uniformly 15N- and 15N/13C-labeled NMR samples (0.8−10 m mol protein concentration) were prepared in 250 μl of 10 mm Tris acetate, 0.1 mM NaCl, pH 6.0, containing 92.5% H2O and 7.5% D2O.

NM R Spectroscopy—NMR experiments were carried out either on a Bruker AVANCE 600, operating at the 1H frequency of 600 MHz, or on a Bruker AVANCE 800, operating at the 1H frequency of 800 MHz, both equipped with a 5-mm shielded triple gradient triple resonance probe head. The NMR experiments were performed at 27 °C. The spectra were acquired using the NMRPipe package (18) and analyzed with FIPPI/STAPP (19). Chemical shifts were referenced to the internal water signal (4.735822 ppm at 27 °C).

Chemical Shift Assignments—The sequence backbone NMR resonance assignments were derived from the three-dimensional HNCA (20), CBCA(CO)NH (21), and HNCCAB spectra. The side chain 13C and 1H assignments were obtained from analysis of the three-dimensional HBHACONH (23), CBCA(CO)NH, and HNCAAB spectra.

 Backbone 15N Relaxation Measurements—Longitudinal (T1) and transversal (T1w) relaxation times for the backbone 15N nuclei were measured using conventional pulse sequences (24), adapted to include the Watergate scheme (25), pulsed field gradient (26), and a semiconstant time evolution period in T1 (23). The T1w experiments used continuous 15N spin-lock field strength of 2.5 kHz (27). 15N NMR data were acquired as 768*(t1) × 128*(t2) data sets with 16, 32, and 64 scans per t1 point, respectively. Eight spectra were recorded for the T1w experiments using relaxation delays of 12, 84, 324, 524, 724, 884, 1084, and 1284 ms. For T1w values, eight spectra were recorded in an interleaved manner (to minimize any effects from spectrometer field drifting and sample heating) using the following 15N delays: 4.69, 14.49, 26.89, 43.29, 59.29, 74.49, 93.69, and 117.29 ms. 15N-{1H} NOE values were measured using a relaxation delay of 3.76 s with the water flip-back NOE pulse sequence described by (28), yielding data sets of 768*(t1) × 128*(t2) after accumulation of 64 scans per t1 point.

600-MHz Data—All experiments were acquired as 512*(t1) × 128*(t2) data sets with 32, 64, and 128 scans per t1 point for T1, T2, and NOE experiments. The following 15N delays were used for the T1 experiments: 11, 83, 123, 323, 403, 563, 803, and 1003 ms. The T1w relaxation delay was sampled at eight different time points: 4.84, 14.44, 26.44, 38.44, 60.04, 88.84, 112.84, and 139.24 ms. 15N-{1H} NOE values were measured using a relaxation delay of 3.76 s with the water flip-back NOE pulse sequence.

Relaxation times were calculated by nonlinear fittings of the delay-dependent peak intensities to an exponential decay function with a peak intensity base line of zero. 15N-{1H} NOE values were calculated as the intensity ratios of the 15N-{1H} correlation peaks from pairs of spectra acquired with and without 1H presaturation during the recycle time. The saturated and unsaturated experiments were interleaved; the absence of saturation was obtained by shifting the proton frequency off-resonance by ~3 MHz during the recycle time. 15N-{1H} NOE values were corrected as described previously to compensate for the effects of incomplete 1H magnetization recovery (28).

Construction of Mutated HTLV-1 gag and gag-pro—HTLV-1 Gag and Gag-Pro mammalian cell expression plasmids were constructed as described previously (15). Briefly, the fragment containing the entire HTLV-1 gag or gag and pro genes was cut from p5X13 plasmid (a gift from Therese Astier-Gin, University of Bordeaux, France) using AvrII and ApaI sites, and the cDNA fragment was inserted into the plasmid pSV between XbaI and SmaI sites. The plasmids obtained were named pSVgag and pSVgag-pro. ptax, prx, and pX plasmids that express the HTLV-1 X region accessory proteins Tax and Rex were gifts from Dr. David Derse (NCI-Frederick). Mutants in which alamine was substituted for the aspartic acid residue in position 54 (D54A) and alamine or isoleucine was substituted for the
glycine residue in position 7 (G7A and G7I, respectively) were constructed using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s recommendations.

Cell Culture and Transfection—Human 293T cells were maintained at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfections were performed in 10-cm dishes containing ~2 × 10⁶ cells by using either calcium phosphate or Fugene 6 transfection reagent (Roche Applied Science) following the manufacturer’s recommendations.

Preparation of Cytoplasmic Extracts, Isolation of VLPs, and Protein Analysis—Cells were lysed on ice in 300–500 μl of radioimmune precipitation buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS), sonicated, and centrifuged for 20 min at 4 °C at 13,000 rpm. HTLV-1 Gag proteins were immunoprecipitated with rabbit anti-HTLV-1 capsid antibody (Advanced Biosciences Laboratories) and collected on protein A-Sepharose beads. Proteins were analyzed on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore Corp.). Gag was subsequently detected by Western blot analysis using mouse anti-CA or goat anti-CA antibodies. To detect HTLV-1 Gag proteins released from transfected cells, the culture medium was collected 48 h post-transfection, filtered through a 0.45-μm filter, and spun through a 20% sucrose cushion for 1 h at 25,000 rpm. The pellets were resuspended in phosphate-buffered saline and further analyzed by SDS-PAGE as described above, negative staining and electron microscopy, or rate zonal centrifugation. HTLV-1 Gag proteins were then visualized by Western blot analysis using mouse anti-CA, goat anti-CA, or mouse anti-MA antibodies.

RESULTS

Experimental Approach—Initial studies were conducted to determine whether the substitution to A of the Asp⁵⁴ residue in the NTD of the HTLV-1 CA 134 fragment changed the structure of the protein. The [¹H-¹⁵N] HSQC spectrum, a “fingerprint” of the protein that correlates backbone amide ¹H and ¹⁵N resonances, is a very good indicator of whether the protein undergoes a substantial or minute change(s). This is due to the sensitivity of the NMR resonances to the local chemical environment. A substantial change in the HSQC spectrum can mean global or local structural changes close to the mutation site. This can further be evaluated by looking at the full backbone (amide H and N as well as Cα, Cβ, and Cγ) resonances and comparing them to the wild type structure. The areas in the structure associated with the largest chemical shift changes are the ones experiencing the largest perturbation due to the mutation. Furthermore, since the backbone chemical shifts are indicative of the secondary structure, they will also provide a measure of possible changes in the secondary structure caused by the mutation. However, backbone resonance data do not differentiate a local geometric change (such as reorientation of a secondary structure element) from local unfolding. Thus, we also obtained NMR backbone relaxation data to complement the above data sets and to address whether the observed changes were associated with folding or unfolding of parts of the protein structure.

Backbone Resonance Assignments for the CA¹³⁴ D54A Mutant—The effect of mutating HTLV-1 CA¹³⁴ residue Asp⁵⁴ to Ala on the β-hairpin structure was determined. The [¹H-¹⁵N] HSQC spectrum of the D54A mutant was obtained (Fig. 1B) and is compared with that of wild type (WT) CA¹³⁴ in Fig. 1A. 116 of 121 possible ¹H and ¹⁵N resonances (134 residues minus 13 prolines) were assigned (97.5%). Sequential connectivities for the ¹H, ¹⁵N, ¹³Cα, and ¹³Cγ nuclei were derived from HNCA, CBCA(CO)NH, and ¹³Cα/¹³Cγ analyses. The assignments of ¹H, ¹⁵N, and ¹³Cα resonances were confirmed using two-dimensional ¹H-¹H NOESY spectra. The ¹³Cα frequencies were identified from three-dimensional HNCO analysis. The assignments of ¹H and ¹⁵N resonances were obtained from a combination of HNCO and HNCA-CNHA analysis. The assignments of ¹³Cα/¹³Cγ and ¹H/¹⁵N were checked using the ¹H-¹³C-¹⁵N HSQC analysis. In total, 94% of the backbone assignments as well as 90% of the ¹³Cα and ¹⁵N resonances were obtained. Interestingly, two residues downstream of Asp⁵⁴ and ¹⁵N resonances Gln⁵⁶ and Asp⁵⁷, could not be assigned due to exchange. This is
in contrast to the WT protein, in which these residues do not exhibit exchange (8). Leu<sup>55</sup> did not exhibit exchange in either protein. The fact that a comparable percentage of backbone assignments could be made for the WT and mutated proteins indicates that the substitution of Ala for Asp<sup>54</sup> did not cause gross aggregation or unfolding.

**Secondary Structure for the CA<sub>134</sub> D54A Mutant**—The predicted φ and ψ torsion angles using TALOS (29) together with the analysis of the <sup>15</sup>N-edited NOE spectroscopy (that showed no long range NOEs for residues 1–15 that form the β-hairpin in the wild type HTLV-1 CA and the deviation of the C<sup>α</sup> and C<sup>β</sup> chemical shifts from the random coil values suggest the presence of six α-helices (residues 17–27, 35–48, 52–62, 65–83, 98–101, and 105–121) and seven loops (Fig. 1D), whereas the wild type adopts a βαααααα fold (Fig. 1C). Therefore, the secondary structure of the D54A mutant was exclusively helical. This finding is consistent with the conclusion that the D54A mutation disrupted the β-hairpin.

**Chemical Shift Changes**—Backbone <sup>1</sup>H<sup>α</sup>, <sup>15</sup>N, <sup>13</sup>C<sup>α</sup>, and <sup>13</sup>C<sup>β</sup> chemical shift differences between the WT and the mutant are presented in Fig. 2. A total of 44 of 121 residues exhibited displacements larger than 0.2 ppm in <sup>1</sup>H<sup>α</sup> and 0.3 ppm in <sup>15</sup>N chemical shifts. The changes in chemical shifts of backbone <sup>13</sup>C<sup>α</sup>, <sup>13</sup>C<sup>β</sup>, <sup>15</sup>N, and <sup>13</sup>C<sup>β</sup> reflect the difference in local chemical environment due to the D54A mutation. As might be expected, the largest changes were observed for Asp<sup>54</sup>. In addition, substantial changes also were detected at the N-terminal residues (Pro<sup>1</sup>–Met<sup>20</sup>) that form the β-hairpin in the WT protein. Smaller differences occurred throughout the α-1 region (residues Asp<sup>19</sup>–Ser<sup>28</sup>), at the carboxyl terminus of α-2 (residues Ile<sup>41</sup>–Asp<sup>49</sup>), at α-5 (residues Gln<sup>100</sup>–Asn<sup>102</sup>) and at the beginning of α-6 (residues Gln<sup>107</sup>–Tyr<sup>113</sup>). A worm representation of the WT protein structure (colored red) with the locations of residues exhibiting significant <sup>1</sup>H and <sup>15</sup>N chemical shift changes (colored green and blue) is shown in Fig. 3. Changes due to D54A mutation were mainly localized to the immediate environment of the β-hairpin.

**Backbone <sup>15</sup>N Relaxation Measurements of CA<sub>134</sub> and CA<sub>134</sub> D54A**—To assess the effect of D54A mutation on backbone dynamic properties, the longitudinal relaxation time, <i>T</i><sub>1p</sub>, the transverse relaxation time, <i>T</i><sub>r</sub>, and the steady-state <sup>15</sup>N-[<sup>1</sup>H] NOE were obtained for 107 and 112 of the 121 protonated backbone <sup>15</sup>N nuclei at 600 and 800 MHz, respectively. These quantitative measurements were needed to establish whether the changes observed in the chemical shifts were associated with a simple reorientation or an unfolding of a structural element. Data for residues His<sup>14</sup>, Leu<sup>55</sup>, Lys<sup>12</sup>, and Asp<sup>130</sup> could not be fitted satisfactorily because of either significant signal overlapping or broadening due to chemical exchange. The experimental values of <i>T</i><sub>1p</sub> at both fields are plotted versus the residue number in Fig. 4. As a reference, the WT <i>T</i><sub>1p</sub> data (8) are plotted as well (as open diamonds). The value of <i>T</i><sub>1p</sub> for the rigid part of the molecule (residues 15–125) did not change dramatically with the sequence position and exhibited an average value of 753.89 and 986.53 ms at 600 and 800 MHz, respectively. The averages of <i>T</i><sub>1p</sub> values for residues 15–125, which are well defined in the solution structure, were 84.97 and 73.61 ms at 600 and 800 MHz, respectively. Statistically significant larger <i>T</i><sub>1p</sub> values were observed for residues 1–14 and 126–134 in the highly disordered N- and C-terminal regions, respectively, indicating that these regions were more flexible than the rest of the protein on the picosecond/nanosecond time scale (Fig. 4). Residue Thr<sup>98</sup> had a <i>T</i><sub>1p</sub> value smaller than the average, whereas the <i>T</i><sub>1p</sub> value for Leu<sup>55</sup> was greater, in both fields. The average backbone <sup>15</sup>N <i>T</i><sub>1p</sub> value of 85 and 74 ms at 600 and 800 MHz, respectively, is consistent with the size of a monomeric protein. The NOE values were −0.65–0.85 and 0.70–0.90 at 600 and 800 MHz, respectively, for the ordered residues 15–125 and −2.6 at 600 MHz for the highly disordered N-terminal region (Fig. 4). The decrease in the NOE mirrors the pattern obtained for the <i>T</i><sub>1p</sub>, indicating increased flexibility for this region on the picosecond/nanosecond time scale. These data showed that the mutation did not cause the protein to aggregate, and its comparison with the WT data confirmed the unfolding of the N-terminal β-hairpin in the D54A mutant.

**Internal Motion Analysis**—Assessment of relevant diffusion parameters such as an order parameter that measures the amplitude of the motion and its time scale permitted us to determine whether there were any correlated changes in the dynamics of the protein far away from the mutation site. Long range changes resulting from the mutation should also be reflected in global diffusion parameters such as overall correlation time and anisotropy. In the case of D54A, the unfolding of the N-terminal β-hairpin should result in an effectively smaller protein with less anisotropy compared with the WT protein. The relaxation times and NOEs are very sensitive to fluctuations of the backbone NH vector on the time scale 10<sup>−3</sup> to 10<sup>−12</sup> s (30). Therefore, they are influenced by the internal dynamics of the individual NH bond vector as well as the overall rotational diffusion of the molecule. NOE values exceeding the maximum theoretical value reflect highly restricted....
Fig. 4. Experimental $T_1$, $T_2$, and NOE values at 600 MHz (filled circles), 800 MHz (open circles) for the D54A mutant, and 600 MHz (asterisks) for the wild type HTLV-1 are plotted against residue number.

Fig. 5. $T_1/T_2$ ratios measured at 600 MHz (filled circles) and 800 MHz (open circles) versus residue number.

internal motion, whereas NOE values smaller than 0.65 indicate significant internal motion. The averages of the NOE values at the N and C termini (residues 1–16 and 126–134, respectively) of the D54A mutant were $-0.36$ and $0.23$ at 600 MHz and 0.07 and 0.35 at 800 MHz, indicating rapid internal motion for the N and C termini. At 600 MHz, the average of the NOE values for the well-defined secondary structure region was 0.77, whereas at 800 MHz, the average was 0.82. In both fields, the loop that connects $\alpha_1$ to $\alpha_9$ (residues 29–34) and the homologue of the CyP A binding loop (residues 84–96) exhibited intermediate values. The distribution of NOE values for the mutant protein is quite similar to the WT with the exception of residues 1–16. These residues that make up the $\beta$-hairpin in the WT exhibited NOE values similar to the helical region of the protein.

The rotational motion of a nonspherical protein like CA$_{134}$ is described through two independent rotation axes. One slow and one fast diffusion axis lie along the short part and long part of the protein, respectively. Since the relaxation of a $^{15}$N nucleus on the backbone of a protein is dominated by the dipolar interaction along the NH bond, an NH bond oriented parallel to the long axis of the protein will not experience the fast rotation around that axis but will be very sensitive to the slow rotation around the short axis. The reverse will be true for NH bonds oriented along the short axis of the protein. This differentiation will govern how the relaxation rates (i.e., the $T_1$, $T_2$, or their ratio) will be distributed throughout the protein.

The $^{15}$N $T_1/T_2$ ratio depends on the orientation of the NH bond vector relative to the rotational diffusion tensor; this has been called the molecular frame (31). The $^{15}$N $T_1/T_2$ ratio is, to a first approximation, independent of the rapid internal motions and magnitude of the chemical shift anisotropy. Since the presence of very rapid internal motions causes almost the same fractional increase in $T_1$ and $T_2$, $T_1/T_2$ ratios can be used to derive the rotational diffusion tensor. Residues with a significant contribution from internal motion to the observed $T_1/T_2$ ratios must be eliminated. Residues with NOE $< 0.65$ and residues that exhibit conformational exchange on a microsecond/millisecond time scale must be excluded. We found 17 residues with NOE values lower than 0.65 (Met$^8$, His$^9$, His$^{24}$, Gly$^7$, Ala$^8$, Asn$^{11}$, Arg$^{13}$, Gln$^{29}$, Gln$^{46}$, Leu$^{55}$, Ile$^{67}$, Gly$^{126}$, Ala$^{128}$, Lys$^{129}$, Ser$^{132}$, Trp$^{133}$, and Ala$^{134}$). The same criteria were used to identify the WT residues with slower internal motions (16) were used in evaluation of the mutant.

The residues that do not fulfill these criteria exhibit additional line broadening, commonly described by the exchange term $R_{ex}$ (32). The $R_{ex}$ contribution increases proportionally with the magnetic field. Thus, for the 800-MHz data, the following residues were excluded: Ile$^{41}$, Arg$^{42}$, Thr$^{98}$, Asn$^{94}$, Arg$^{99}$, Ala$^{101}$, Arg$^{110}$, and Gln$^{116}$. The remaining 82 (or 87; based on 112 residues at 800 MHz) residues at 600 MHz (800 MHz) were used to obtain the rotational diffusion tensor.

Since the orientation of the NH bond defines the relative relaxation rates with respect to the average for the whole protein, one can choose a helix in which typically all of the NH bond vectors are nearly parallel to the helix axis (thus, their $T_1/T_2$ ratios are uniform) to provide a qualitative measure of how this helix should be oriented with respect to the long axes of the molecule. Different helices exhibit different average $T_1/T_2$ values corresponding to the orientation relative to the rotational diffusion tensor or the frame of the molecule; the helix with highest average $T_1/T_2$ value is nearly collinear with the principal component of the diffusion tensor (D), whereas the helix with the lowest average $T_1/T_2$ value is nearly perpendicular to it (Fig. 5). The observed average $T_1/T_2$ values for the different helices in the mutant are as follows: $\alpha_1 = 9.29$, $\alpha_2 = 9.47$, $\alpha_3 = 8.57$, $\alpha_4 = 8.88$, $\alpha_5 = 9.12$, and $\alpha_6 = 9.29$ at 600 MHz and $\alpha_1 = 13.69$, $\alpha_2 = 14.33$, $\alpha_3 = 13.02$, $\alpha_4 = 13.49$, $\alpha_5 = 14.29$, and $\alpha_6 = 14.19$ at 800 MHz. The average values for these helices do not vary as much as they do for the WT protein. This qualitatively suggests that the anisotropy ($D/D_\parallel$) value for the mutant will be smaller than the WT protein (i.e., the mutant has a more compact structure).

**Diffusion Tensor**—To check for consistency, we determined the diffusion tensor. A small reduction in the effective size of the molecule (thus, its diffusion tensor) is predicted if the $\beta$-hairpin is unfolded. Since the NMR relaxation parameter is quite sensitive to changes in the effective size of a protein in solution, the measurements for D54A should be different from that of the WT protein. The rotational diffusion tensor was obtained from the $T_1/T_2$ ratios using a similar procedure as for the native protein (16). First, the diffusion tensor for the mutant was constrained to all three models (isotropic, axial, and fully asymmetric) separately for each field (see Table I for 600 MHz and Table II for 800 MHz data). Then the combined 600- and 800-MHz data were applied to the model that showed a statistically significant fit to our data.

We first assumed the isotropic model. For the 600-MHz data, the one-dimensional Powell minimization generated a $\tau = 9.06$ ns and a normalized error function $\chi^2 = 7.32$; for the 800-MHz data, $\tau = 8.54$ ns and a normalized error function $\chi^2 = 8.27$. Second, the data were fitted into the axially symmetric model, resulting in a significant reduction of the normalized error
function relative to the isotropic model. Use of the fully asymmetric model, six-parameter fit, resulted in a further reduction of $\chi^2$. We used the F-test (33) to evaluate whether the decrease in the error function obtained with an increase in the number of parameters was statistically significant. When the diffusion tensor was constrained to the axially symmetric model, the improvement in the error function was clearly significant for both fields: $F = 7.32, P_F = P(F, 3, 76) = 0.0028$ at 600 MHz, and $F = 8.30, P_F = P(F, 3, 81) = 0.0051$ at 800 MHz. However, adding two more degrees of freedom, for the fully asymmetric model, the improvement was not statistically significant. In this case, the use of the F-test resulted in $F = 1.22, P_F = P(F, 2, 76) = 0.31$ at 600 MHz and $F = 0.80, P_F = P(F, 2, 81) = 0.53$ at 800 MHz. Fitting the 600- and 800-MHz data simultaneously into the axially symmetric model, the overall correlation time is $\tau_e = 8.63$ ns. We can now identify the principal components of the axially symmetric diffusion tensor: $D_{xx} = 2.14 \times 10^7$ s$^{-1}$ and $D_{yy} = 1.82 \times 10^7$ s$^{-1}$. This is a significant reduction relative to those values of the WT. A $D_{xy}/D_{zz}$ of 1.18 for the mutant is 6% smaller than the value of the WT. This is consistent with what one would expect if the N-terminal $\beta$-hairpin adopts an unfolded conformation.

The model free internal diffusion parameters are shown in Fig. 6. The square of the generalized order parameter ($S^2$) had average values of 0.87 for the whole molecule, neglecting the high flexibility regions (i.e. the N and C termini). Within the cone model of Brainard and Szabo (34), this average value would correspond to motion within a semiangle of 18°. The N-terminal 13 residues and C-terminal 7 residues of D54A had significantly lower $S^2$ values. In contrast, only the C-terminal region of the WT protein possessed low $S^2$ values. Two residues in the homologue CyP A binding loop (Gly$^{96}$ and Gly$^{97}$) as well as Leu$^{55}$ showed $S^2$ values that are slightly less than average for the well-structured region of the mutant protein, whereas in the WT only Gly$^{96}$ exhibited lower than average $S^2$ value.

Effective correlation time ($\tau_e$) values for internal motions are below 100 ps and range as far as 360 and 450 ps for residues Gln$^{29}$ and Leu$^{55}$, respectively. The sites with higher $\tau_e$ values also have lower $S^2$ values, with the exception of residue Gln$^{29}$, which exhibits high $\tau_e, 360$ ps, and $S^2$ values within the average, 0.88. The values of order parameters are completely independent of secondary structure. There are no obvious changes in the order parameters of the mutant compared with the WT values that can be correlated to any secondary structure. These results indicate that, outside of the N-terminal 13 residues, there were no other parts of the D54A structure that underwent conformational unfolding due to the mutation.

**Differential Antibody Recognition of WT and Mutated CA$^{134}$**—The chemical shift analysis indicated that disruption of the salt bridge by mutation of Asp$^{54}$ affected regions of $\alpha_2, \alpha_3, \alpha_4$, $\alpha_5$, and $\alpha_6$, regions that are distal to the mutation. The mutation apparently affected an antigenic site in the NTD, since the mutated CA$^{134}$ Protein (Fig. 7A, lane 2) was no longer recognized in Western analysis by a monoclonal antibody that detected the WT protein (Fig. 7A, lane 1). In contrast, a polyclonal antibody detected both the WT (Fig. 7B, lane 1) and the mutated (Fig. 7B, lane 2) proteins, as revealed by stripping the blot and reprobing with a rabbit polyclonal anti-CA antibody. The results suggested that disruption of the salt bridge resulted in global changes in the protein that, directly or indirectly, affected the overall protein surface.

**D54A Mutation in HTLV-1 CA Did Not Affect VLP Assembly and Release**—To determine whether the conformational alterations detected in the D54A mutant by NMR affected its function, the Asp$^{54}$ mutation was engineered into HTLV-1 DNA constructs that express the Gag polyprotein precursor alone or the Gag and Gag-Pro polyprotein precursors. The latter construct contains the active virally encoded protease and allows for proteolytic processing of the precursors. The DNAs were transfected into 293T cells, and cytoplasmic extracts were prepared and examined for proteolytic maturation of the Gag precursors. In addition, viral release was analyzed by harvesting the media in which the cells were grown and examining for VLPs. Proteins were detected by Western analysis.

Gag and Gag-Pro precursor polyproteins of both the WT and the D54A mutant were blotted and then probed with either a mouse monoclonal antibody (Fig. 8A) or a goat polyclonal antibody (Fig. 8B). As expected, no specific signals were detected

### Table I

Calculated diffusion parameters for the 600-MHz data (76 residues)

| Model                  | $\tau_e$ | $2D_{zz}/D_{zz} + D_{yy}$ | $D_{xx}/D_{zz}$ | $\theta$ | $\phi$ | $\psi$ | $\chi^2$ | $F$ | $P_F$ |
|-----------------------|----------|---------------------------|-----------------|---------|-------|-------|----------|-----|-------|
| Isotropic             | 9.06     | 1                         | 1               | 63      | 179   |       | 7.32     | 9.6 | 0.0028 |
| Axially symmetric     | 8.86     | 1.18                      | 1               | 63      | 178   | -3    | 5.23     | 1.7 | 0.31  |
| Fully asymmetric      | 8.86     | 1.17                      | 1.08            | 63      | 178   | -3    | 5.05     | 1.7 | 0.31  |

### Table II

Calculated diffusion parameters for the 800-MHz data (81 residues)

| Model                  | $\tau_e$ | $2D_{zz}/D_{zz} + D_{yy}$ | $D_{xx}/D_{zz}$ | $\theta$ | $\phi$ | $\psi$ | $\chi^2$ | $F$ | $P_F$ |
|-----------------------|----------|---------------------------|-----------------|---------|-------|-------|----------|-----|-------|
| Isotropic             | 8.54     | 1                         | 1               | 65      | 179   |       | 8.27     |     |       |
| Axially symmetric     | 8.33     | 1.19                      | 1               | 67      | 178   | -44   | 6.12     | 0.80| 0.53  |
| Fully asymmetric      | 8.37     | 1.17                      | 1.09            | 67      | 178   | -44   | 6.12     | 0.80| 0.53  |
in mock-transfected cells (lanes 1 and 4). In contrast, cells transfected with DNA encoding the WT (lanes 2 and 5) or the mutant (lanes 3 and 6) expressed the HTLV-1 Gag precursor protein (p53Gag). The selectivity of the monoclonal antibody for WT over mutated CA was maintained in the natural Gag and Gag-Pro context, as indicated by the differential recognition of the WT CA protein (lanes 2 and 5) but not the mutated D54A CA protein (lanes 3 and 6). This defect was directly related to disruption of the salt bridge between Pro1 and Asp54, because whereas the monoclonal antibody recognized the WT mature D54A CA protein (i.e., the unprocessed pre-cursor) almost comparably (Fig. 8A, lanes 2 and 5), the antibody recognized the mature mutant D54A CA protein in the cell lysate and in VLPs poorly (compare lane 2 with lane 3 and lane 5 with lane 6, respectively). In contrast, the polyclonal antibody recognized the WT and the mutant D54A p53 Gag precursor protein as well as the WT and the mutant D54A mature CA proteins to comparable extents (Fig. 8B, compare lane 2 with lane 3 and lane 5 with lane 6), as was the case for CA134. Moreover, although the mutated mature D54A CA protein was not efficiently recognized by the monoclonal antibody (Fig. 8A, lanes 5 and 6), comparable amounts of the WT and mutated CA proteins were detected by the polyclonal antibody (Fig. 8B, lanes 5 and 6). These results indicate that destabilization of the β hairpin did not prevent release of VLPs at WT levels.

To further explore the differential recognition of the Gag precursor and the mature CA protein due to disruption of the salt bridge, the effects of two additional mutations were analyzed (Fig. 9). Gag and Gag-Pro precursor proteins containing mutations in the CA Gly7 residue (i.e., within the residue in CA that makes the turn of the β hairpin) to Ile or Ala were included in this analysis. Substitution of Ile for Gly is predicted to significantly change the dihedral angle and prevent the turn, whereas substitution of Ala should have little effect (35). As shown in Fig. 8A, the WT and both mutant p53 Gag proteins were all recognized by the monoclonal antibody (A and B) or a monoclonal anti-MA antibody (C).
Consistent with the results above, the WT mature CA protein (lane 6) was also recognized, but not the CA protein bearing the Asp\(^{54}\) mutation (lane 7). Interestingly, the CA proteins bearing the Gly\(^{7}\) mutations (lanes 8 and 9) were recognized as well as the WT CA protein, both in cytoplasmic extracts (Fig. 8A) and in VLPs (Fig. 8, B and C). Analysis of the media by probing with an antibody against the HTLV-1 MA protein indicated that, although the CA protein in VLPs assembled by the D54A mutant was not detected by the anti-CA monoclonal antibody (Fig. 8B, lane 7), all of the mutants assembled particles at WT levels (Fig. 8, B (lanes 2–5 and 6, 8, and 9) and C (lanes 1–4)).

D54A Mutation in HTLV-1 CA Did Not Affect VLP Size or Density—We next determined whether the conformational change in the mature CA protein resulting from the D54A mutation affected the size of the assembled particles. VLPs released from cells following expression of the WT and D54A Gag-Pro constructs were isolated from the media by pelleting through a sucrose cushion, stained with uranyl acetate and then examined by electron microscopy (Fig. 10). No significant differences in VLP size or overall morphology were apparent. The WT (A) and the mutant (B) particles both contained roughly spherical electron-dense cores that were 60–70 nm in diameter. Physical properties reflected in particle density also were not affected, as indicated by examination of the particles by rate zonal centrifugation in 10–55% sucrose gradients (Fig. 11). This analysis indicated that the WT (Fig. 11A) and the D54A (Fig. 11B) VLPs both sedimented as a broad band that spanned a density range of 1.11–1.16 (fractions 14–17). Control experiments done in parallel with HTLV-1 proviral DNA encoding all of the viral genes except \textit{env} revealed particles of similar size and density (data not shown). The infectivity of cell-free HTLV-1 WT particles is intrinsically very low (15), making it difficult to assess the effect on infectivity of the D54A mutation with any degree of confidence. Nevertheless, the results suggest that whereas disruption of the salt bridge in the D54A mutant resulted in conformational alterations that affected recognition of the mature CA protein, the mutation had no effect on VLP release, size, or density.

DISCUSSION

Previous structural studies of retroviral capsid proteins have demonstrated that despite the low sequence conservation, especially in the NTD, the structure is highly conserved through the family. In particular, all retroviral CA proteins conserve an N-terminal \(\beta\) hairpin structure. This structural element was at first suggested to function as a morphological switch that allowed the formation of a CA-CA interface in the assembly of the conical cores that are characteristic of the lentiviral subgroup. However, as the oncovirus HTLV-1 forms a spherical core, the salt bridge is apparently not conserved for this function. Moreover, our previous studies showed that although the same D residue defines the orientation of the hairpin in the HTLV-1 and HIV-1 CA proteins, the HTLV-1 hairpin is oriented away, rather than toward, the helical core of the protein. In this study, we determined the structural and functional effects of disrupting the salt bridge that stabilizes the \(\beta\) hairpin. We found evidence for structural alterations throughout the NTD
resulting from the mutation but, in contrast to the defective assembly resulting from the same mutation in HIV-1 CA (D51A) (2), the alterations to the HTLV-1 CA protein did not prevent correct proteolytic processing or efficient release from the cell of VLP that exhibit WT size and density. These observations, together with our previous studies, suggest that the role of the salt bridge in determining the orientation of the β-hairpin may be critical during the late stage of viral assembly when morphogenetic rearrangements related to particle maturation occur.

In the current study, the amino-terminal residues (Pro1–Arg13) of the D54A mutant exhibited relaxation times and NOE values that are characteristic of highly disordered regions in contrast to the WT protein, where that region is well defined. The Cα and Cβ chemical shifts for these residues are close to their random coil values. In addition, long range NOEs that are characteristics for β-hairpin are absent in the D54A mutant. Therefore, all of these data indicate that the amino-terminal region of the mutated CA protein does not form a β-hairpin, and, in the context of the Gag precursor, it did not refold into a β-hairpin following proteolytic processing. Although the D54A mutation is located near the amino terminus of α-3, we observed chemical shift changes (i.e. structural modifications) throughout the β-hairpin and its neighboring helices. For the β-hairpin region, the chemical shifts were displaced in general toward random coil values, indicating that the disruption of the salt bridge favored the unfolding of the amino terminus of the CA protein and completely destabilized the formation of the β-hairpin. Changes in the neighboring helices α2, α3, and α4 reflect differences in packing within them due to the unfolding of the β-hairpin. In addition, we also observed secondary effects in α3 and α4 due to changes in the first three helices.

Several observations can be made by comparing the 600-MHz relaxation data (T1, T2, and NOE) obtained for the mutant and the native proteins. The average T1 value for the well structured region (residues Lys18 to Ala121) was higher in the mutant than in the WT protein (754 and 708 ms for the mutant and wild type, respectively); the average T2 for the mutant was lower than the WT value (85 versus 92 ms, respectively); the pattern of relaxation data for the CyP A binding loop homologue (residues Glu39–Pro96) was identical; and the region near the mutation (residues Lys53 and Ala54) exhibited higher mobility than its counterpart in the WT. These observations suggest that disruption of the salt bridge does not affect the central loop. Curiously, the differences in average T1 and T2 values of the two proteins suggest that a small amount of the mutant protein might be aggregated. Considering the average T1 and T2 values at the high protein concentration at which the experiments were carried out as well as the 2–3% experimental errors, the estimated maximum dimer population is 5% (36). A determination of the concentration dependence of protein oligomerization not be helpful in this case, because the experimental error, which is already close to the relative variation of the T1 and T2 averages (ΔT1/T1 ≈ 6.5%; ΔT2/T2 ≈ 7.6%), will be higher at lower concentrations. Alternatively, the differences in average T1 and T2 values might reflect significant differences in the hydrodynamic properties of the two proteins. On the other hand, it is important to point out that the principal axis of diffusion is the same for both WT and mutant proteins (within 5°). Nevertheless, the anisotropy for the mutant is significantly smaller. This can be attributed to the lack (i.e. disruption) of structured β-hairpin at the N-terminal end of the mutated protein. Furthermore, the residual in the diffusion tensor fitting to the measured relaxation rates for the mutant is not as good as the WT, as measured by the χ2. This suggests that there is some local structural difference between the two proteins. This is also supported by long τc for Glu29 and Leu55, suggesting the presence of slow motion that was absent in the WT protein as well as changes in chemical shifts of residues away from the β-hairpin and the mutation site. Thus, although the axis of diffusion is the same for the two proteins, the overall shape or topology and local structural as well as dynamic details are not. This result is consistent with the differential antibody recognition between the WT and the D54A mutant proteins observed using Western analysis (Figs. 7–9). As noted above, there may be sufficient renaturation of the protein during the Western analysis procedure to permit these differences to be distinguished by the antibody probes.

Our study shows that destabilization of the β-hairpin by the Asp54 to Ala mutation alone did not affect Gag processing, caused no detectable reduction in VLP assembly or release, and did not detectably alter particle size or morphology. Asp54 and other residues within α-3 may be involved in formation of the maturation interface without participation of the β-hairpin. Interestingly, Rayne et al. (15) found that mutation of Asp54-Leu55 to RS significantly impaired VLP morphogenesis and release from 293T cells. As already noted, mutations in the homologous site in HIV-1, Asp51-Leu54-Asn55, had much smaller effects compared with effects observed by Rayne et al. (13). The observed differences in the flexibility of the NTD structures may explain the more drastic effect of the mutation on HIV-1 particle production. Conformational alterations in helices 3 and 6 have been associated with the morphological switch from tubes to spheres that occurs upon proteolytic maturation of the HIV-1 Gag precursor. The observation that these regions were only minimally perturbed by disruption of the salt bridge in HTLV-1 is consistent with the fact that both the immature and mature capsids of this virus are spherical (i.e. no apparent morphological transition occurs). Thus, we hypothesize that the function of the β-hairpin may be to control formation of interfaces during the proteolytic maturation process to ensure proper core formation. It seems likely that understanding the role of the highly conserved β-hairpin and the neighboring helices could provide additional insights into how the retroviral CA proteins form structurally and functionally distinct assemblages.

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