Residues in the HIV-1 Capsid Assembly Inhibitor Binding Site Are Essential for Maintaining the Assembly-competent Quaternary Structure of the Capsid Protein*

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Morphogenesis of infectious HIV-1 involves budding of immature virions followed by proteolytic disassembly of the Gag protein shell and subsequent assembly of processed capsid proteins (CA) into the mature HIV-1 core. The dimeric interface between C-terminal domains of CA (C-CA) has been shown to be important for both immature and mature assemblies. We previously reported a CA-binding peptide (CAI) that blocks both assembly steps in vitro. The three-dimensional structure of the C-CA/CAI complex revealed an allosteric effect of CAI that alters the C-CA dimer interface. Based on this structure, we now investigated the phenotypes of mutations in the binding pocket. CA variants carrying mutations Y169A, L211A, or L211S had a reduced affinity for CAI and were unable to form mature-like particles in vitro. These mutations also blocked morphological conversion to mature virions in tissue culture and abolished infectivity. X-ray crystallographic analyses of the variant C-CA domains revealed that these alterations induced the same allosteric change at the dimer interface observed in the C-CA/CAI complex. These results point to a role of key interactions between conserved amino acids in the CAI binding pocket of C-CA in maintaining the correct conformation necessary for mature core assembly.

Human immunodeficiency virus (HIV)³ assembles into immature, non-infectious virions at the plasma membrane of the infected cell. These particles contain several thousand Gag polyproteins (1–3) organized radially beneath the viral membrane (4). Concomitant with particle budding, Gag proteolysis is initiated by the viral protease leading to separation of the individual domains matrix (MA), capsid (CA), nucleocapsid (NC), p6 and two spacer peptides (SP1 and SP2) (5). This maturation process results in a morphological conversion to the infectious virion, where the MA shell remains attached to the viral membrane (6–8), whereas the ribonucleoprotein core with the NC protein is condensed (9) and encased by a cone-shaped protein shell made of 1000–1500 CA molecules (2). Formation of the cone-shaped mature capsid represents a second, independent assembly step; CA cleavage from Gag leads to disassembly of the immature Gag layer and subsequent formation of the mature CA lattice, which requires only part of the available CA molecules.

Our understanding of HIV assembly and structure has been strongly advanced by the development of in vitro assembly systems (10–12) that faithfully mimic formation of the immature and mature protein lattice of HIV-1, respectively. Several recent studies using cryoelectron microscopy (1–3) and image reconstruction of enveloped virions and in vitro assembled particles together with the determination of high resolution structures (13–16) of individual Gag subdomains revealed a general picture of the architecture of immature and mature HIV-1. The Gag lattice of the immature virion is hexagonally arranged with a spacing of 8 nm and appears to cover only part of the inner virion membrane (2, 17). The lattice of the mature, cone-shaped capsid exhibits a fullerene geometry with a 9.6-nm spacing (18, 19). Assembly of the immature and mature protein shell is mainly driven by weak protein-protein interactions. The CA segment within Gag plays a key role in both processes. Its modular structure with separately folded N- and C-terminal domains (N-CA; C-CA) connected by a flexible hinge allows for an essential conformational plasticity. Structural details of the protein interfaces of the immature shell are currently not

nelscope; MALACANCS-2, polyprotein composed of the matrix protein with deletion of amino acid residues 16–99 followed by capsid, spacer peptide 1, nucleocapsid, spacer peptide 2; NC, nucleocapsid protein; N-CA, N-terminal domain of capsid protein; PEG, polyethylene glycol; TCID₅₀, tissue infectious dose 50; MES, 4-morpholineethanesulfonic acid.
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**Experimental Procedures**

**Proteins and Peptides**—All expression vectors were derived from pET11c (Novagen) (11, 12) and carried coding sequences of Gag-derived proteins based on HIV-1 NL4-3 (28) and for ΔMACANCSP2 on HIV-1 BH10 (29); the plasmids were transformed for expression into *Escherichia coli* BL21(DE3) CodonPlus-RIL (Stratagene). All mutations were introduced by PCR mutagenesis and confirmed by sequence analysis; protein variants of CA, C-CA, CANC, and ΔMACANCSP2 proteins were purified as described (11, 12, 25). Peptides were obtained as lyophilized trifluoroacetic acid salts (PSL GmbH, Heidelberg, Germany). Protein and peptide concentrations were estimated spectrophotometrically using calculated extinction coefficients.

**Determination of Dissociation Constants by Fluorescence Polarization**—CAI was C-terminal-extended by a cysteine residue and labeled with 5-iodoacetamido fluorescein. The labeled peptide showed no significant difference in assembly inhibition compared with CAI. Labeled CAI was dissolved in DMSO at 10 mM concentration. CA proteins were stored in 30 mM MES, pH 6.0, 1 mM EDTA, 2 mM dithiothreitol at concentrations 250–500 μM. The final reaction buffer consisted of phosphate-buffered saline containing 0.05% Tween 20 and 2 mM dithiothreitol with 10–20% protein storage buffer. HIV-1 CA variants (0–60 μM) were incubated with 30 nm fluorescein-labeled CAI for 2 h at room temperature in triplicate in black 384-well ProxiPlates (PerkinElmer Life Sciences) in a final volume of 15 μl. The fluorescence polarization signal was detected on an EnVision™ Multilabel Plate Reader (PerkinElmer Life Sciences) at excitation wavelength 495 nm and emission wavelength 520 nm. Median values were fitted to a one-site binding model from which the dissociation constant was directly calculated (GraphPad Prism).

**Cell Culture and Detection Methods**—All mutations were introduced into the infectious proviral plasmid pNL4-3 (28) by introducing the Splh/Apal fragment from the respective ΔMACANCSP2 constructs. 293T cells in 6 wells (5 × 10⁵ cells/well) were transfected with 2 μg of plasmid using standard calcium phosphate precipitation. Released particles were harvested 48 h after transfection and purified from filtrated supernatant through a 20% sucrose cushion. Cell and particle lysates were resolved on 17.5% low cross-linking polyacrylamide gels and probed with anti-CA (1:5,000) followed by horseradish peroxidase-conjugated secondary antibody (1:10,000) to produce a chemiluminescent signal (SuperSignal™ CL-HRP System, Pierce). For quantitative Western blots, donkey anti-rabbit antibody (IRDye 800CW; 1:20,000) was used as a secondary antibody, and the infrared signal was detected using the Odyssey Imaging System (LI-COR™). For TCID₅₀ infectivity assays, 3 × 10⁶ C8166 cells/well were plated into 96-well plates 1 day before infection. Transfection supernatants were cleared by low speed centrifugation and titrated in 10-fold serial dilutions (10⁻¹–10⁻¹⁰) in quadruplicate on C8166 cells. Cells were microscopically examined for cytopathic effects and formation of syncytia for 6–7 days. Virus titer was calculated using the Karber formula (30).
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**A**

![Diagram of the structure of the C-CA/CAI complex](image)

**B**

![Sequence alignment of C-CA consensus sequences of HIV-1, HIV-2 and SIV](image)

**FIGURE 1.** Mutations in the CAI binding site. **A**, ribbon diagram of the structure of the C-CA/CAI complex (PDB code 2BUO (27)) in two orientations with the 2-fold axis of the dimer drawn as black line. CA residues selected for mutagenesis are highlighted. The α-helices are color-coded as follows: red, helix 1; pink, helix 2; blue, helix 3; green, helix 4; yellow, CAI peptide. **B**, sequence alignment of C-CA consensus sequences of HIV-1, HIV-2 and simian immunodeficiency virus (SIV; Los Alamos sequence database (sequences from 2006/2007); www.hiv.lanl.gov) prepared using the program ClustalX2 (37). Strictly conserved residues are shown in black, conserved residues of the “strong” amino acid groups are in green (37), conserved residues of the “weaker” amino acid groups are in blue (37), and variable residues are in black. The relative contribution of individual residues to CAI binding is depicted by yellow bars. Residues selected for mutagenesis are indicated by an asterisk. Secondary structure elements are shown underneath the sequences.

In Vitro Assembly and EM Analysis—In vitro assembly of ΔMACANCSP2, CANC, and CA was induced by dialysis as described (11, 12); assembled material was pelleted by centrifugation for 5 min at 17,600 × g and resuspended in dialysis buffer in a quarter of the original volume. Assembled particles were adsorbed onto glow-discharged, Formvar, and carbon-coated copper grids and stained with 2% uranyl acetate for 5 min. Samples were examined using a Zeiss EM 10 transmission electron microscope. To quantify the in vitro assembly efficiencies of wild-type and L211S CA proteins and their mixtures (at molar ratios from 2:1 to 1:10; final CA concentration 3 mg/ml), the number and length of tubular particles was determined for at least 25 view fields at a magnification of 20,000, and the average per view field was calculated.

For EM analysis of HIV-1 producing cells, HeLaP4 cells were transfected using FuGENE 6 as specified by the manufacturer (Roche Applied Science). Cells were briefly washed with phosphate-buffered saline and fixed with cold 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, 36 h after transfection. Samples were stained in situ with 2% OsO₄ in 0.1 M sodium phosphate, pH 7.2, followed by dehydration with ethanol, post-staining with 6% uranyl acetate, and incubation with propylene oxide. Subsequently, cells were scraped from the plates, and the cell pellet was embedded in EPON and processed by ultrathin sectioning. Micrographs were taken using a Zeiss EM 10 transmission electron microscope. Viral particles (200–250 for each variant) were grouped as mature, immature, and budding particles, and mature particles were subsequently classified according to core shape (conical, diffuse, eccentric, or empty core).

Crystallization—Protein concentrations used for crystallization were 2 mM for unliganded proteins and 1 mM for the C-CA/CAI complexes. The latter were formed at a 1:2 molar ratio by incubating for 1 h at room temperature. All crystals were obtained at room temperature by the hanging-drop vapor diffusion method, mixing 1 μl of protein or complex and 1 μl of the reservoir solution. Crystallization conditions of the N183A and E187A variants were similar to those that led to crystals of the wild-type C-CA/CAI complex. The conditions for variants N183A and E187A were 30%/32% PEG 4000, 100 mM ammonium acetate, pH 4.6/5.0, and 10 mM MgCl₂, respectively. The Y169A and L211S crystallization conditions were 20% PEG 3350, 200 mM sodium iodide and 30% PEG 4000, 100 mM sodium HEPES, pH 7.5, 200 mM CaCl₂, respectively. The complexes C-CA N183A/CAI and C-CA E187A/CAI were crystallized in 32%/30% PEG 4000, 100 mM ammonium acetate, pH 4.2, 10 mM MgCl₂, respectively. Crystallization conditions for the C-CA Y169A/CAI and C-CA L211S/CAI complexes were 30% PEG 4000, 200 mM ammonium acetate, 100 mM sodium acetate, pH 4.6, and 30% PEG 4000, 200 mM ammonium sulfate, respectively.

X-ray Diffraction Data Collection—Crystals were transferred into a cryoprotectant solution containing the reservoir solution plus 15% (w/v) PEG 400 before flash-cooling in liquid nitrogen. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility beamlines (ID-14-1, ID14-3, ID23, ID29, and BM30) and the Swiss Light source PX beamline X06SA.

Structure Determination and Refinement—The AMoRe (31) and PHASER (32) programs were used to determine the structures by molecular replacement using two different models; the high affinity C-terminal domain dimer of the HIV-1 capsid protein CA₁⁻²³₁ (PDB code 1A43) and the C-terminal domain dimer in complex with the CAI peptide (PDB code 2BUO). The atomic models were built with the program COOT (33) and refined with program Refmac (34) from the CCP4 suite (35) except for the unliganded variant E187A, for which the resolution limits of the diffraction pattern did not allow refinement. Crystallographic data, Protein Data Bank accession codes, and refinement statistics are indicated in Table 2. All illustrations were prepared with the PyMOL Molecular Graphics System.
RESULTS

In Vitro Assembly Properties of CAI Binding Mutants—Based on the structure of C-CA in complex with CAI (27), CA residues Tyr-169, Asn-183, Glu-187, and Leu-211 (Fig. 1A) were selected for mutagenesis experiments. All four residues are conserved among the lentiviruses (with conservative Y169F and E187Q exchanges; Fig. 1B), and Leu-211 is also conserved in most other retroviruses except for Rous sarcoma virus. The changes were predicted to affect CAI binding for the following reasons; the aromatic ring of Tyr-169 forms a stacking interaction with its counterpart from CAI residue Tyr-10. Asn-183 provides an N-terminal cap to the CAI/H9251-helix, thus helping stabilization of the α-helical conformation of the peptide. Glu-187 is located at the kink of helix 2, which forms the C-CA dimer interface, a helix that becomes straightened upon CAI binding. Furthermore, the charged side chain of Glu-187 interacts with the electrostatic dipole of the CAI α-helix. Finally, Leu-211 is an essential part of the hydrophobic binding pocket, interacting closely with CAI residues Ile-1 and Leu-6. Alanine was chosen as substitute because it is most likely neutral for the secondary structure of the protein; in the case of Leu-211, an additional change to serine was introduced to analyze the effect of a polar amino acid on the nonpolar groove.

The individual mutations were introduced into a CA expression plasmid, and mutant CA proteins were purified from E. coli. The dissociation constants of wild-type and mutant complexes were determined using fluorescence polarization. Increasing concentrations of the respective CA variant were incubated with fluorescently labeled CAI, and the increase in polarization of the emitted signal dependent on complex formation was determined. The data were fitted to a one-site binding model, and the K_d values were calculated (Table 1). The complex of wild-type CA and CAI exhibited a K_d value of 1.6 ± 0.1 μM. This number is about 10-fold lower than the K_d determined by NMR analysis for the complex of CAI with a dimerization defective C-CA variant (C-CA_W184A/M185A) (25). Using fluorescence polarization, we confirmed our previous result for the latter complex (9.2 ± 1.5 μM), indicating a tighter interaction of CAI with full-length wild-type CA. All CA variants with changes in the binding pocket displayed a lower affinity for CAI except for E187A. The most severe effect was observed for the mutation L211S with a 40-fold increase in K_d. Intermediate values were obtained for variants Y169A, N183A, and L211A, which showed a 4–9-fold reduction in CAI binding (Table 1).

The effect of the mutations on particle assembly was analyzed using two established in vitro assays (11, 12). For assembly of immature-like spherical particles, all mutations were cloned into a CA expression plasmid, and mutant CA proteins were purified from E. coli. The dissociation constants of C-CA variant proteins binding fluorescently labeled CAI and measured by fluorescence polarization

| Variant      | K_d (μM) |
|--------------|----------|
| CA wild type | 1.6 ± 0.1|
| CA Y169A    | 6.0 ± 0.4|
| CA N183A    | 14.9 ± 0.9|
| CA E187A    | 2.0 ± 0.1|
| CA L211A    | 6.3 ± 0.4|
| CA L211S    | 64.5 ± 21.8|

FIGURE 2. In vitro assembly of immature- and mature-like capsids. A, electron micrographs of negatively stained particulate structures after in vitro assembly of ΔMACANCSP2 variants (immature-like assembly of spherical particles; scale bar, 200 nm). wt, wild type. B, electron micrographs of negatively stained particulate structures after in vitro assembly of CAI variants (mature-like assembly of tubular particles; scale bar, 200 nm). C, electron micrographs of in vitro assembly products from wild-type CANC and variants N183A and Y169A. Scale bar, 200 nm. D, quantitative analysis of the relative assembly efficiency of mixtures of wild-type and L211S variant CA proteins at different molar ratios. Assembly reactions were performed and analyzed as in panel B. The graph depicts the relative amounts of assembled particles per view field at a magnification of 20,000. The assembly efficiency of wild-type CA was set to 100%, and the efficiencies of the different mixtures were calculated as percentage of wild-type assembly.
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FIGURE 3. Effects of CAI binding site mutations on HIV-1 morphogenesis and infectivity in tissue culture. A, Western blot analysis of lysates of transfected 293T cells (left panel) and released viral particles (right panel) after transfection with wild-type pNL4-3 or the indicated variants. The CA domain of the Gag polyprotein was detected with a polyclonal antiserum. Molecular mass standards (in kDa) are shown on the left. The reactive bands represent the Pr55Gag polyprotein, a MACA intermediate of 41 kDa, and the processed CA and CASP1 proteins (p24/p25). An additional cleavage product of 20 kDa in the case of Y169A, L211A, and L211S is indicated by an asterisk. B, the relative infectivity of the released particles was determined in quadruplicate for wild-type HIV-1 (NL4-3) and the respective variants were determined by endpoint titration on C8166 cells. TCID₅₀ values per ml of cell culture supernatant harvested 48 h after transfection are shown. In the case of variants Y169A, L211A, and L211S no infectious virus could be obtained.

FIGURE 4. Ultrastructure of budding sites and released particles. A, representative section of a plasma membrane region of HeLaP4 cells transfected with wild-type pNL4-3 showing budding sites and immature virions. These structures appeared similar for wild-type HIV-1 and all mutant constructs. B, representative sections depicting normal mature virions released from wild-type pNL4-3 transfected cells (left panel) and aberrant HIV-1 structures released from variant L211S transfected cells (right panel). Similar phenotypes were observed for the other assembly-competent (N183A, E187A) and assembly-incompetent (Y169A, L211A) variants, respectively. C, enlarged examples of HIV-1 particles displaying regular cone-shaped (a and b) or aberrant cores (c–h). Quantification of the relative numbers of different structures observed for all variants is shown in supplemental Fig. S2. Bar size, 200 nm.

into an expression vector for ΔMACANCSP2 (12), and the respective proteins were purified and used in the in vitro assay. No difference was observed for wild-type and mutant proteins in this assay, and all variants assembled spherical particles of similar size and morphology as the wild-type protein and at similar efficiency (Fig. 2A). The situation was different for mature-like assembly using wild-type and variant CA proteins. Only wild-type CA and E187A were capable of assembling hollow tubes, whereas the other CAI binding pocket mutations abolished assembly of regular tubes, yielding exclusively aggregates and aberrant structures (Fig. 2B). The result was generally similar when variant CANC proteins were used instead of CA and in vitro assembly was induced by nucleic acid addition at a lower protein concentration and lower ionic strength. The only exception was mutation N183A, which assembled into regular hollow tubes at these conditions (Fig. 2C). In contrast, no ordered structures were observed for variants Y169A, L211A, and L211S in the CANC assembly assay (Fig. 2C and data not shown). These results indicate that positions 169 and 211 of CA appear critical for mature-like particle assembly, whereas Asn-183 is less important, and Glu-187 does not seem to be relevant.

To determine whether the defective variants had a dominant negative effect on mature-like assembly in vitro we performed mixing experiments of CA L211S with wild-type CA at different ratios (Fig. 2D). Assembly was virtually abolished at an equimolar ratio and ≥20-fold reduced at a 2-fold molar excess of wild-type CA over the L211S protein. Increasing the molar excess of wild-type CA gradually restored assembly, but assembly efficiency was still ~50% reduced at a 10-fold molar excess of the wild-type protein.

Effect of the Mutations on HIV-1 Morphogenesis and Infectivity—To investigate their effect on HIV-1 assembly, virion morphology, and infectivity in cell culture, the described mutations were introduced into the infectious proviral plasmid pNL4-3. Virus particles were produced by transfection of 293T or HeLa cells and analyzed for release efficiency, protein composition and processing, infectious titer, and virion morphology. Immunoblots of cell and particle extracts from transfected cells demonstrated that similar amounts of CA-reactive proteins were produced in all cases (Fig. 3A). Furthermore, particle release also appeared to be similar in all cases, and this result was confirmed by enzyme-linked immunosorbent assay-based detection of released antigen (data not shown). The pattern and efficiency of polyprotein processing appeared to be generally similar with the exception of an additional CA-reactive band at
TABLE 2
Crystallization, x-ray diffraction data collection, and refinement statistics

The PDB codes of each of the structures are indicated.

|                   | N183A | N183A + CAI | E187A | E187A + CAI | L211S | L211S + CAI | Y169A | Y169A + CAI |
|-------------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|
| Data collection   |       |             |       |             |       |             |       |             |
| Space group       | P1    | P4,2,2      | P1    | P4,2,2      | P1    | P4,2,2      | P1    | P4,2,2      |
| Unit cell parameters |       |             |       |             |       |             |       |             |
| α (Å)             | 51.36 | 42.84       | 51.6  | 42.89       | 29.84 | 31.51       | 29.67 | 112.8       |
| β (Å)             | 51.32 | 42.84       | 51.67 | 42.89       | 74.9  | 69.27       | 75.66 | 112.8       |
| γ (Å)             | 109.2 | 90          | 109.94| 90          | 90    | 90          | 90    | 90          |
| α (°)             | 109.63| 90          | 108.48| 90          | 99.97 | 97.7        | 99.92 | 90          |
| β (°)             | 109.54| 90          | 110.2 | 90          | 90    | 90          | 90    | 120         |
| γ (°)             | 2     | 0.5a        | 2     | 0.5a        | 1     | 1           | 1     | 1           |
| Number of C-CA dimers in asymmetric units |       |             |       |             |       |             |       |             |
| % Solvent         | 56    | 32          | 56.9  | 28.6        | 36.2  | 47.9        | 35.6  | 60.6        |
| Resolution (Å)    | 42.2  | 38.7-1.6    | 30.2-4.0| 38.8-1.6    | 37.4  | 2-1.2       | 37.8  | 12.2-2.85   |
| Last resolution bin (Å)b | (2.49-2.4) | (1.66-1.6) | (4.14-4.0) | (1.66-1.6) | (2.07-2.0) | (1.19-1.12) | (1.26-1.2) | (2.85-2.7) |
| Total observations | 146,750| 161,806     | 35,981| 296,318     | 33,130| 240,309     | 131,790| 44,564      |
| Unique reflections | 13,571| 11,638      | 2,160 | 11,802      | 9,396 | 63,254      | 38,077| 6,718       |
| Completeness (%)b | 86.3  | (100)       | 62.2  | (65.5)      | 99.7  | (98.6)      | 98.9  | (91.8)      |
| Redundancyb       | 3.1   | (2.2)       | 13.9  | (10.9)      | 2.4   | (2.5)       | 25.1  | (19.7)      |
| (I/s)              | 36.4  | (5)         | 25.6  | (7.2)       | 5.3   | (5.3)       | 73.8  | (9.7)       |
| Rmerge (%)h,i      | 2.5   | (13.9)      | 7.9   | (36.2)      | 9.2   | (15.8)      | 4.3   | (36.8)      |
| B Wilson (Å2)      | 61.8  | 22.9        | 68.3  | 22.5        | 16.4  | 10.8        | 10.3  | 71.3        |
| Refinement        |       |             |       |             |       |             |       |             |
| PDB accession code| 3DS5  | 3DS0        | 3DTJ  | 3DS1        | 3DPH  | 3DS4        | 3DS2  | 3DS3        |
| Resolution (Å)    | 42.2  | 38.7-1.6    | Not refined | 31.2-1.6 | 37.4-1.2 | 35.4-1.12 | 27.3-1.2 | 36.9-2.7   |
| Last resolution bin (Å)b | (2.46-2.4) | (1.64-1.6) | Not refined | (1.64-1.6) | (2.06-2.0) | (1.15-1.12) | (1.23-1.2) | (2.77-2.7) |
| Number of reflections | 12,892| 11,086     |       | 11,189      | 8,931 | 62,969      | 36,840| 6,570       |
| B refinement      | Isotropic | Isotropic | Anisotropic | Isotropic | Isotropic | Anisotropic | Isotropic | Isotropic |
| Rmerge (%)h,i      | 22.0  | (29.4)      | 20.6  | (19.4)      | 21.2  | (25.2)      | 21.0  | (31.4)      |
| Rfree (%)h,i       | 26.9  | (33.9)      | 24.5  | (31.5)      | 27.2  | (35.2)      | 24.6  | (32.7)      |
| Number of protein atoms | 2,293| 734        |       | 731         | 1,244 | 1,303       | 1,312 | 1,345       |
| Number of waters   | 21    | 78          |       | 86          | 84    | 321         | 208   | 80          |
| Mean B-value (Å2)  | 60.25 | 24.3        |       | 24.75        | 16.9  | 17.0        | 14.5  | 65.5        |
| Root mean square deviations |       |             |       |             |       |             |       |             |
| Bond lengths (Å)   | 0.014 | 0.009       |       | 0.011        | 0.023 | 0.007       | 0.027 | 0.005       |
| Bond angles (°)    | 1.617 | 1.305       |       | 1.357        | 1.941 | 1.072       | 2.201 | 0.782       |

a In these crystals, the dimer axis is crystallographic.
b Values in parentheses refer to the outer resolution shell.
Rmerge (%) = Σ|I(hkli) − <I(hkli)>|/ΣI(hkli), where I(hkli) are the measurements contributing to the mean reflection intensity, <I(hkli)>.

Thin section EM analysis of transfected HeLaP4 cells was performed to determine the morphology of viral budding sites and released immature and mature virions for the various mutations. The morphology of individual budding sites and immature particles was similar in all cases (Fig. 4A), whereas more budding sites and immature particles were detected in the case of all variants compared with wild type. Major differences were seen for mature particles, on the other hand (Fig. 4B). They were classified according to their core morphology as particles with either conical, eccentric, diffuse, or empty cores. Examples of the various morphologies are shown in Fig. 4C, and quantification of the different structures is displayed in supplemental Fig. S2. Regular cone-shaped cores were observed in ~70% of all mature virions released from cells transfected with wild-type pNL4-3 with the remaining cores being aberrant. The proportion of cone-shaped cores was similar but slightly reduced (to ~50%) in the case of variants N183A and E187A, whereas virtually no cone-shaped cores were detected in case of variants Y169A, L211A, and L211S (supplemental Fig. S2). Aberrant core structures were mostly diffuse or eccentric in these cases, and no regular core morphologies were detected.

X-ray Crystallography of C-CA Variants—To relate the observed phenotypes to the structure of the variant proteins and to verify that the changes did not introduce gross folding.

~20 kDa (CAp20) in the case of the Y169A, L211A, and L211S variants. This protein is smaller than fully processed CA (CAp24) and must be due to aberrant cleavage within CA. A similar product was obtained upon in vitro cleavage of the respective purified, mutant CA proteins with HIV-1 protease (data not shown). Mass spectrometry analysis mapped the cleavage site to residues Leu-189/Leu-190 of CA. This position is distant from the sites of both mutations, and the result, therefore, indicates conformational changes making it accessible to HIV-1 protease-mediated cleavage. The relative amount of the aberrantly cleaved product was quantified by determining the ratio of the 20-kDa product to all CA-reactive species in the respective lane using quantitative Western blot analysis (supplementary Fig. S1). Virion extracts of variants Y169A, L211A, and L211S contained the aberrant processing product at 2.3, 10.6, and 22.8% of all CA-reactive species, respectively.

The relative infectivity of the different viruses was determined by titration on the T-cell line C8166. Wild-type HIV-1 exhibited an infectious titer of 2.5 × 10^6 infectious units/ml. This number was similar for variant E187A and slightly reduced (~2.5-fold) for N183A (Fig. 3B). Infectivity was completely lost, on the other hand, for variants Y169A, L211A, and L211S (Fig. 3B), and no infectious virus could be recovered even upon prolonged incubation.
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defects in the proteins, we carried out crystallization experiments of the variant C-CA domains (spanning CA residues 146–231) both alone and in complex with the CAI peptide. All variants and their complexes with CAI yielded crystals, most of which diffracted X-rays to a resolution sufficient to determine the three-dimensional structure, except for L211A. The best diffraction observed was close to 1.1 Å for crystals of variants L211S and Y169A in complex with CAI, whereas the E187A crystals were the worst, diffracting only to 4 Å resolution. The diffraction data were collected at synchrotron sources and led to the determination by molecular replacement of eight new crystal structures to various resolutions, corresponding to each of the four variants in the presence and absence of bound CAI. Table 2 provides the parameters of each crystal and the statistics concerning diffraction data collection and refinement of the atomic model. In the case of variant E187A, for which the cell parameters are the same as N183A, it was clear from the molecular replacement solution that its conformation was native-like, as indicated in Fig. 5.

The overall structures of the variant C-CA proteins were not altered, presenting all of the previously identified secondary structure elements (Fig. 5). The described phenotypes are, therefore, not due to protein misfolding but, rather, due to more subtle changes in the tertiary and quaternary protein structure, with the possible exception of L211A for which we did not obtain diffraction quality crystals. As shown in Fig. 5, wild-type C-CA and all variant structures in complex with CAI displayed the same features with a characteristically altered dimer interface and a straightened helix 2 with respect to the unliganded wild-type structure. There was a striking difference between the structures of the assembly-competent (wild-type, E187A, and N183A) and assembly-incompetent variants (Y169A and L211S) in the absence of CAI, however; the two assembly-incompetent variants displayed the CAI-induced dimeric conformation even in the absence of CAI, in contrast to the assembly-competent variants (Fig. 5). This result suggests that the perturbation introduced by changing the side chains at these positions caused the same type of alteration as did the binding of CAI.

The structures of the C-CA variants in complex with CAI resembled very closely the wild-type C-CA/CAI complex (Fig. 5). The location of CAI was the same for all variants, except in the complex with Y169A which exhibited a slight shift of the C-terminal end of CAI toward the binding pocket (Fig. 5). This shift is very likely due to the loss of the aromatic packing between CAI residue Tyr-10 and the Tyr-169 side chain of C-CA.

With the exception of the crystals of the N183A and E187A variants in complex with CAI, in which the C-CA dimer axis is coincident with a strict crystallographic 2-fold axis, in all other cases there was a dimer in the asymmetric unit of the crystal (supplemental Table S1). These dimers were more or less symmetric, with a root mean square deviation after superposing the subunits varying by about an order of magnitude, from 0.1 Å (which is within the positional error) to 1.5 Å, which reflects real differences in conformation (see supplemental Table S1). The Y169A dimer was found to be fairly symmetric, and the two subunits superimpose relatively well on each other both in the presence and absence of CAI (supplemental Fig. S3). In contrast, the structure of L211S in complex with CAI is special because only one of the two CAI binding sites was found occupied (see Fig. 5, lower right panel). The L211S variant also showed the lowest affinity for CAI (Table 1). This observation suggests that binding of CAI to one site induced an allosteric conformational change of the dimer, altering the 2-fold symmetry and consequently reducing the affinity for the second site. In the crystal, the packing is such that the presence of the second peptide would collide with CAI bound to the neighboring dimer in the crystal lattice. This also suggests that the crystallization process may have selected dimers having a single CAI molecule bound. The root mean square differences after superimposing individual subunits in the different variant dimers with wild type and with themselves are provided in supplemental Table 1, and various superpositions of the L211S
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subunits with wild type and between individual subunits of the variant (unliganded and CAI bound) are displayed in Fig. 6. These comparisons show that the C-CA subunit in the unbound L211S dimer is in an intermediate conformation between unbound and CAI-bound wild type. Interestingly, the asymmetry observed for the CAI-bound L211S dimer is such that the unliganded subunit has become closer to unbound wild type, and the other one has become closer to CAI-bound wild type. Despite this asymmetry in the individual conformation of the subunits, the dimer interface of unliganded and CAI-bound L211S, as is the case for the Y169A variant, remains close to that of the CAI-bound wild type, as seen in Fig. 5. This suggests that the latter is a conformation that the polypeptide chain will adopt by default in the absence of specific interactions between side chains in the CAI binding pocket that maintain it in the wild-type conformation. Overall, our study indicates that relatively subtle alterations of the individual subunits in the variant proteins give rise to the same allosteric change of the quaternary structure as induced by CAI binding. The most likely explanation is that these perturbations disrupt specific interactions necessary to maintain the assembly-competent conformation.

DISCUSSION

In this study we carried out a biochemical, structural, and functional characterization of the conserved, reactive groove of HIV-1 CA that is the binding site of CAI, an inhibitor of immature- and mature-like particle assembly (25, 27). We used a structure-based, site-directed mutagenesis approach to alter residues that are contacted by CAI and examined the functionality of the resulting proteins using in vitro assembly and virion infectivity assays. Furthermore, we performed an extensive structural characterization of each of the variants in the presence and absence of bound CAI. Most of the engineered proteins indeed showed reduced affinity for CAI (Table 1). The assembly studies showed that all variants were competent for immature-like assembly in vitro and budding of immature particles in tissue culture. However, mutations at two positions, namely Tyr-169 and Leu-211, abolished mature-like assembly and gave rise to noninfectious virions lacking regular mature cores.

We observed that Gag processing of variants Y169A, L211A, and L211S by HIV-1 protease led to additional proteolysis within CA, resulting in a minor fraction of a protein of 20-kDa (CAp20) instead of the normal CAp24. Although this observation could suggest a possible dominant negative effect of CAp20 on mature assembly, our result that the Y169A, L211A, and L211S variants did not assemble in the mature-like assembly studies showed that all variants were competent for immature-like assembly in vitro and virion infectivity assays. Furthermore, we performed an extensive structural characterization of each of the variants in the presence and absence of bound CAI. Most of the engineered proteins indeed showed reduced affinity for CAI (Table 1). The assembly studies showed that all variants were competent for immature-like assembly in vitro and budding of immature particles in tissue culture. However, mutations at two positions, namely Tyr-169 and Leu-211, abolished mature-like assembly and gave rise to noninfectious virions lacking regular mature cores.

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such an effect is not required for the observed phenotype. Taken together, our assembly data suggest that mutations at these two positions render Cap24 incompetent for mature assembly because they alter the structure in a way that is incompatible with this process. This alteration also affects the region at the new cleavage site (Leu-189/Leu-190), distant from the introduced mutations, making it accessible to HIV-1 protease.

The fact that the mutations affected neither immature assembly in vitro nor morphogenesis of immature virions suggests an indirect effect of CAI binding on assembly of the immature lattice, which is not mimicked by mutations in the binding pocket. The recent study with a cell-penetrating CAI derivative (26, 36) indicates that this CAI effect is not limited to the in vitro system. Determining the structural correlate of the CAI effect on immature assembly requires a better understanding of the assembly contacts in the immature virion, however. In contrast, all results concerning mature assembly in vitro and in tissue culture indicate that the CAI effect in this case is due to direct binding to the conserved pocket in C-CA. The phenotype in vitro and in tissue culture generally showed a good correlation. Variant N183A exhibited an intermediate phenotype and lacked assembly competence in vitro, when CA was used for the assembly reaction, but was assembly-competent when CANC was used. Accordingly, mature cores were formed in tissue culture but with a slight reduction in infectious titer. The fact that all mutations exhibiting a significant loss of CAI binding also affected infectivity suggests that resistance against a CAI-derived antiviral compound may not be easily achieved by single point mutations in the binding pocket without compromising viral fitness. The loss of infectivity was not very pronounced for N183A, however, and this mutation may, thus, confer some resistance to a CAI-derived inhibitor.

The recent electron crystallographic analysis of two-dimensional lattices formed by an intact CA variant (R18L) (24) revealed that the CAI binding pocket of CA is involved in C-CA/N-CA cross-domain contacts. These involve two neighboring CA subunits within hexameric rings of CA, which are in turn connected by 2-fold interactions via C-CA dimers, the subunits of which belong to neighboring hexamers. The resulting model of the mature CA lattice nicely explains that binding of CAI can inhibit mature-like assembly simply by steric hindrance. In this model it is not necessary to invoke any alteration of the C-CA dimer interface to explain assembly inhibition. Our new results suggest, however, that both effects may play a role and that the CAI-induced conformation of the C-CA dimer is not compatible with mature assembly even if CAI did not interfere directly with the binding to N-CA. Although residues Tyr-169 or Leu-211 may play an important role in directly contacting N-CA, it appears more likely that it is the altered conformation of C-CA in these variants that is responsible for the inhibition. Both Tyr/Phe-169 and Leu-211 are conserved in HIV-1 and HIV-2/simian immunodeficiency virus, and Leu-211 is also strictly conserved in most other retroviruses. Their presence appears responsible for maintaining the assembly-competent conformation of the C-CA dimer seen in Fig. 5, left column, top three rows. Binding of CAI alters the interaction between these amino acids, resulting in the adoption of a nonfunctional conformation of the polypeptide chain. Mutating one of these two residues had the same effect on the C-CA dimer interface, making the protein assembly incompetent. The observation that the mutations caused a more subtle change in the C-CA tertiary structure than CAI binding (as seen in Fig. 6 and supplementary Fig. 3) suggests that the C-CA dimer conformation is very sensitive to the details of the contacts within the CAI pocket. Altering these interactions by different means led to the same overall quaternary structure of C-CA, which could, therefore, be viewed as an inert “default” conformation. This altered conformation apparently has a dominant negative effect as the in vitro assembly block is evident even when it is present in minority.

Overall, our data suggest that CAI binds to a reactive groove in the C-terminal domain of HIV-1 CA that controls the protein conformation necessary for formation of infectious virions. On the one hand, CAI directly interferes with a physical contact that is needed for assembly of the mature core by competing with the natural binding region in the N-CA domain. On the other hand, it interferes indirectly by modifying the C-CA dimer interface that is important in propagating the assembly. This second effect is also present in the Y169A and L211S mutants, explaining why they are not competent for mature assembly.

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