Second site reversion of a mutation near the amino terminus of the HIV-1 capsid protein

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During HIV-1 morphogenesis, the precursor Gag protein is processed to release capsid (CA) proteins that form the mature virus core. In this process, the CA proteins assemble a lattice in which N-terminal domain (NTD) helices 1–3 are critical for multimer formation. Mature core assembly requires refolding of the N-terminus of CA into a β-hairpin, but the precise contribution of the hairpin core morphogenesis is unclear. We found that mutations at isoleucine 15 (I15), between the β-hairpin and NTD helix 1 are incompatible with proper mature core assembly. However, a compensatory mutation of histidine 12 in the β-hairpin to a tyrosine was selected by long term passage of an I15 mutant virus in T cells. The tyrosine does not interact directly with residue 15, but with NTD helix 3, supporting a model in which β-hairpin folding serves to align helix 3 for mature NTD multimerization.

Introduction

Expression of the human immunodeficiency virus type 1 (HIV-1) gag gene is sufficient to generate immature viral particles that are released from infected cells (Wills and Craven, 1991; Spearman et al., 1994; Recin et al., 1996; Hermida-Matsumoto and Resh, 1999; Freed, 1998; Mervis et al., 1988; Wang et al., 1998; Ono et al., 2000; Huseby et al., 2005). Immature particles contain a lipid bilayer envelope surrounding a shell formed primarily by precursor Gag (PrGag) proteins (Puller et al., 1997; Wilk et al., 2001; Wright et al., 2007; Briggs et al., 2009). Shortly after budding, the viruses undergo a maturation step, in which the HIV-1 protease (PR) cleaves PrGag into its mature Gag protein components: matrix (MA), capsid (CA), spacer peptide 1 (SP1), nucleocapsid (NC), spacer peptide 2 (SP2), and p6 (Wills and Craven, 1991; Mervis et al., 1988; Freed, 1998). Electron microscopy (EM) studies of mature virus particles have demonstrated that maturation results in the formation of electron dense conical or cylindrical cores that are composed of about 1000–1500 capsid proteins surrounding a ribonucleoprotein complex derived from viral RNAs and tRNA primers, and enzymatic functions (Wills and Craven, 1991; Freed, 1998; Li et al., 2000; Benjamin et al., 2005; Briggs et al., 2003, 2006, 2009; Zhao et al., 2013).

HIV-1 CA is a 24 KDa protein that possesses two independently folded domains connected by a flexible linker. The N-terminal domain (NTD) is important for formation of CA hexamers and pentamers, while the C-terminal domain (CTD) contributes to hexamer formation and also fosters the interconnection of hexamers, in part via a CTD dimer interface (Gamble et al., 1996, 1997; Gitti et al., 1996; Momany et al., 1996; Berthet-Colomina et al., 1999; Worthylake et al., 1999; Ganser-Pornillos et al., 2007; Pornillos et al., 2009, 2010, 2011; see Fig. 1). The CA NTD is composed of seven alpha helices (Fig. 1A), and includes a cyclophilin (CypA) binding loop between helices 4 and 5, and an N-terminal β-hairpin loop composed of the first thirteen residues of the mature CA protein. The interface formed by NTD helices 1–3 makes NTD–NTD contacts that have proven to be essential for mature core assembly (von Schwedler, 1998; Lanman et al., 2002; Ganser-Pornillos et al., 2007; Abdurahman et al., 2007; Pornillos et al., 2009, 2010, 2011; López et al., 2011). These helices form an 18-helix bundle in which residues near the N-terminal end of helix 1 are located towards hexamer centers, and have been shown to be essential for the hexamer formation (Fig. 1B; Ganser-Pornillos et al., 2004, 2007; Pornillos et al., 2009, 2010, 2011; Zhao et al., 2013). The CA CTD is smaller than the NTD, and is composed of a short 3α helix followed by an extended strand and four α helices (Momany et al., 1996; Gamble et al., 1997, Berthet-Colomina et al., 1999; Worthylake et al., 1999; Alcaraz et al., 2007; Wong et al., 2008; Pornillos et al., 2009; Byeon et al., 2009). The CTD comprises the dimerization interface, which relies on amino acid residues W184 and M185 (Gamble et al., 1997, Alcaraz et al., 2008; Byeon et al., 2009; Yu et al., 2009). Interestingly, Bharat et al. (Bharat et al., 2012) recently reported that significant rotations and translations of the two CA domains occur during the maturation process of the Mason–Pfizer monkey retrovirus.
One critical event that occurs during HIV-1 maturation is the proteolytic processing of the N-terminus of the CA protein, which results in the refolding of the first 13 CA residues from an extended conformation into a \( \beta \)-hairpin loop. Biochemical and mutational studies have shown that this \( \beta \)-hairpin loop is stabilized by a salt bridge formed between a proline located at position 1 and an aspartic acid at position 51, on the NTD helix 3 (Gamble et al., 1996; Gitti et al., 1996, Gross et al., 1998; von Schwedler et al., 1998; Tang et al., 2002; Abdurahman et al., 2007; Monroe et al., 2010; Wong et al., 2012). Interestingly, the crystal structure of CA proteins shows no evidence of CA intersubunit interactions involving the hairpin loop (Pornillos et al., 2009). However, several laboratories including ours have shown that mutations, deletions and/or extensions that affect the \( \beta \)-hairpin loop formation are detrimental for in vitro CA assembly reactions and also for maturation and infectivity (Gross et al., 1998; von Schwedler et al., 1998; Abdurahman et al., 2007; Cortines et al., 2011; López et al., 2011). Therefore, it has been postulated that the presence of the \( \beta \)-hairpin loop correlates with mature core assembly although the molecular basis for this requirement remains unclear (Gross et al., 1998; von Schwedler et al., 1998; Cortines et al., 2011; López et al., 2011). One possibility proposed for the Equine Infectious Anemia Virus (EIAV) CA protein is that refolding of the \( \beta \)-hairpin loop extends helix 1 to enhance the oligomerization of the CA NTD, but other models for the role of the \( \beta \)-hairpin exist (Chen et al., 2013).

We recently showed that mutation of I15 near the N-terminal end of helix 1 (Fig. 1) skewed the assembly pathway such that spheres and short tubes were assembled rather than the typical long tubes assembled by wild type (WT) proteins (Campbell and Vogt, 1995; Gross et al., 1998; Li et al., 2000; Barklis et al., 2009; López et al., 2011). Moreover, I15 mutant proteins also had the capacity to dominantly interfere with the assembly of WT proteins (López et al., 2011). These observations suggested that I15 side chain interactions are essential to proper core assembly, and to further analyze the structure and function of the helix 1 N-terminus, I15 mutants were evaluated in cell culture.

To analyze the effects of I15 mutations on virus infectivity, HEK 293T cells were transfected with WT and mutant HIV-1 strain NL4-3 constructs, and CA protein-normalized amounts of virus produced were used to infect MT4 T cells. Virus spread was then monitored by measuring CA levels via anti-CA immunoblotting from samples of cells that were passaged at 3–4 d intervals. As shown in Fig. 2A, WT HIV-1 rapidly infected MT4 T cells with virus levels peaking at 2–4 d post-infection, prior to cell death. In contrast, the CA I15A mutant virus levels did not peak until 21–31 d post-infection (Fig. 2A), while an I15D variant (López et al., 2011) was never detected after serial passages in MT4 T cells at any virus:cell input ratio (data not shown).

**Results**

**Analysis of HIV-1 capsid I15 variants**

In a recent study (López et al., 2011), we examined the effects of CA NTD helix 1 mutations on the in vitro assembly of HIV-1 CA proteins. In particular, we found that mutations at NTD isoleucine 15 (I15) near the N-terminal end of helix 1 (Fig. 1) skewed the assembly pathway such that spheres and short tubes were assembled rather than the typical long tubes assembled by wild type (WT) proteins (Campbell and Vogt, 1995; Gross et al., 1998; Li et al., 2000; Barklis et al., 2009; López et al., 2011). Moreover, I15 mutant proteins also had the capacity to dominantly interfere with the assembly of WT proteins (López et al., 2011). These observations suggested that I15 side chain interactions are essential to proper core assembly, and to further analyze the structure and function of the helix 1 N-terminus, I15 mutants were evaluated in cell culture.
In the case of the I15A mutant, the delayed kinetics of virus replication observed in Fig. 2A could have been due either to a stochastic lag in I15A replication or to the generation of revertant viruses. To distinguish between these alternatives, virus supernatants of the I15A variant from days 21–24 post-infection were recovered and used to re-infect new MT4 T cells. Significantly, virus obtained from the late time points of the initial I15A infections replicated with much faster kinetics than the original I15A virus stock, suggestive of a reversion and/or compensatory mutations. These putative revertants were characterized as described below.

Characterization of compensatory mutations

To examine the capacity of the I15A viruses to evolve compensatory mutations, we characterized I15A-derived viruses from two independent cultures that replicated with faster kinetics than the parental I15A stock. To do so, proviral DNAs from MT4 T cells infected with the rapidly spreading I15A variants were PCR-amplified, and CA sequences were analyzed. Our analysis indicated the presence of two different revertants. In the first one (I15V/S16N), the alanine codon at position 15 had mutated to a valine codon, and the neighboring codon (serine 16) had mutated to an asparagine codon. In the second revertant (H12Y/I15A/M68I), the I15A mutation was conserved, but this revertant possessed two other mutations: one was a H12Y mutation in the β-hairpin loop, and the second was a M68I mutation in NTD helix 4. To verify that the capsid mutations that were sequenced actually compensated for the I15 variations, the I15V/S16N and H12Y/I15A/M68I mutations were cloned into otherwise WT clones of the NL4-3 parental HIV-1 strain, and tested for their replication efficiencies. As shown in Fig. 2B, the molecularly cloned I15V/S16N variant replicated with the same kinetics as the WT NL4-3 strain, and the H12Y/I15A/M68I variant was only slightly delayed. In contrast, the control I15A virus again showed a replication defect, with virus spread not occurring until 21–31 d post-infection.

The above results demonstrate that the I15V/S16N HIV-1 variant is capable of efficient replication in MT4 T cells, and imply that either H12Y, M68I or both mutations compensate for the original I15A defect. To test the individual contributions of the selected mutants, molecular clones of H12Y/I15A and I15A/M68A were constructed and tested in a new experiment. Consistent with the aforementioned results, the H12Y/I15A/M68I virus replicated with WT kinetics, and the I15A virus replication was delayed (Fig. 2C). Significantly, the I15A/M68I variant replicated with somewhat delayed kinetics, with a peak at 9–16 d post-infection, whereas the H12Y/I15A variant replicated similarly to WT HIV-1 (Fig. 2C), suggesting that the H12Y β-hairpin loop mutation was predominantly responsible for compensation for the original I15A mutant. These data could be interpreted to indicate that H12Y and to a certain extent M68I specifically correct the I15A defect, or that the H12Y and M68I viruses are just globally better replicating viruses in MT4 cells. To distinguish between these alternatives, replication profiles of H12Y and M68I single mutation viruses were compared with the WT strain. Fig. 2D demonstrates that replication of the M68I variant was comparable to WT, while H12Y virus replication was slightly delayed. These results support the notion that H12Y, and to a lesser extent M68I, specifically compensate for the I15A defect.

Single step infection assays

As a complementary analysis of the effects of I15 variants, single round replication assays were performed. To do so, HIV-1
envelope-deleted HIV-Luc luciferase reporter constructs were cotransfected into human embryonic kidney (HEK) 293T cells with a VSV envelope glycoprotein expression construct to generate pseudotyped viruses (Scholz et al., 2005, 2008; Noviello et al., 2011) that were used to infect HeLa cell-derived CD4+ H1J cells (Kabat et al., 1994). Not surprisingly, WT HIV-1 viruses were more than fifty times as infectious as their I15A counterparts (Fig. 3). In general, results with the other capsid variant mimicked MT4 infection results (Fig. 2). Notably, the I15V/S16N, H12Y/I15A/M68I and H12Y/I15A variants showed single round infectivity levels of 50–70% WT levels, while the I15A/M68I variant was only slightly more infectious than the I15A parent. These results support the observations with replication competent NL4-3 viruses (Fig. 2), and demonstrate that the I15A defect is not specific to T cells, and can not be salvaged by the alternative entry route of VSV G-pseudotyped virus (Brun et al., 2008).

Analysis of virus particle and release

To measure potential effects of the CA mutations on the release of virus-like particles (VLPs), 293T cells were transfected with each CA variant construct (WT, I15A, I15V/S16N, H12Y/I15A/M68I, I15A/H12Y, I15A/M68I, H12Y and M68I) in the context of the replication defective HIV-Luc background. At 72 h post-transfection, virus and cells samples were collected and processed. Levels of virus release were monitored by immunoblotting of SDS-PAGE-fractionated VLP and cell samples using an anti-CA antibody. As shown for cellular samples (Fig. 4A–K), we observed some minor variations in WT PrGag, p41 and CA protein levels, depending on the transfection date. (Compare lanes A, D, L) Nevertheless, none of the CA variants showed major differences in either cellular Gag expression or processing levels (Fig. 4A–K). Similarly, we noted no consistent reductions in VLP release or VLP Gag processing when VLP samples (Fig. 4, L–V) were surveyed. However, it is worth mentioning that I15A VLP samples often showed a species slightly smaller than the size of WT CA (see especially Fig. 4, lane M), which may represent a CA degradation product (Scholz et al., 2005), potentially indicative of a virus core defect as described below.

Effects of I115 CA mutations on virus core morphologies and in vitro assembly

As shown above, I115A HIV-1 viruses were defective for infection in both NL4-3 and HIV-Luc backgrounds, while the H12Y mutation compensated for this defect (Figs. 2 and 3). Moreover, I115A VLP capsid protein bands showed evidence of abnormalities on SDS-PAGE gels (Fig. 4), suggestive of a possible virus core defect. To extend these observations, we examined the core structures of WT, I15A and H12Y/I15A viruses for possible abnormalities. To do so, VLP from cells transfected with HIV-Luc variants were isolated and subjected to EM analysis as we have described previously (Scholz et al., 2005, 2008; Noviello et al., 2011). As we have seen in the past (Scholz et al., 2005, 2008; Noviello et al., 2011), WT HIV-1 conical cores were observed both associated with virus particles (Fig. 5, top left), and as free cores (Fig. 5, top right). From a total of 169 WT VLPs observed, 63.9% exhibited conical or cylindrical cores, consistent with previous observations. For I115A VLPs, considerably fewer particles showed conical or cylindrical cores (46.6% of 161 particles). Frequently, particles with no obvious cores were observed (Fig. 5, middle left panel), and even when cores were evident, they often appeared anomalous (middle right panel). However, when I115A was expressed in the presence of the compensatory HIV12Y mutation (H12Y/I115A), particle-associated cores and free cores appeared normal (Fig. 5, bottom panels). Additionally, a higher percentage of H12Y/I115A virus (61.7% of 167 particles) showed conical or cylindrical cores, suggesting that the H12Y change in the ␤-hairpin loop corrected the I115A defect at the N-terminus of NTD helix 1.

We further examined the role of the H12Y compensatory mutation by exploiting in vitro assembly assays using purified CA proteins. For this approach, WT, I115A and H12Y/I115A CA proteins were expressed in bacteria, purified, and employed in in vitro assembly reactions (Barklis et al., 2009; López et al., 2011). To do so, purified proteins at 60 µM were induced to assemble at 4 °C for 48 h and then processed for visualization by EM. As shown in Fig. 6 (top panel) WT CA proteins assembled long tubes, which are representative of a mature assembly phenotype (Gross et al., 1998; Li et al., 2000; Ganser-Pornillos et al., 2004; Barklis et al., 2009; López et al., 2011). In contrast, I115A CA proteins (Fig. 6) assembled short tubes and spheres, indicative of a
mature core assembly defect. However, the double H12Y/I15A mutant yielded tube numbers and morphologies similar to WT, demonstrating that H12Y counteracts the I15A defect. To quantify in vitro assembly results, tube lengths and areas covered by tubes were determined and normalized to WT tube values (Fig. 6, bottom panels). As illustrated, the I15A CA mutant gave lower numbers of CA tubes than WT or H12Y/I15A, as measured by tube coverage. Additionally, the average tube length for the few I15A tubes obtained was 50% shorter relative to the significantly higher numbers of WT and H12Y/I15A tubes. Thus, in vitro assembly results (Fig. 6) correlate with our virus core morphology analysis (Fig. 5), and support a model in which the H12Y mutation rescues the I15A replication defect by restoring normal virus core morphology. Details of the H12–I15 interaction are discussed below.

Discussion

Structural analysis has indicated that the first three helices of the HIV-1 CA NTD coordinate CA hexamer and pentamer formation in mature virus cores (Ganser-Pornillos et al., 2004, 2007; Pornillos et al., 2009, 2010, 2011). Interestingly, evidence based on the Mason-Pfizer monkey virus (MPMV) suggests that helices 1–3 do not serve such a role in immature virus particle lattices (Bharat et al., 2012). This is consistent with the fact that virus maturation is accompanied by the formation of the NTD β-hairpin loop and the establishment of a salt bridge between CA proline 1 (P1) and aspartate 51 (D51) in helix 3. In EIAV, the formation of the β-hairpin loop serves to extend NTD helix 1 several residues in the N-terminal direction (Chen et al., 2013), potentially stabilizing mature capsid hexamers, but the precise structural contributions of retroviral β-hairpin and nearby residues with regard to viral morphogenesis are still unclear.

HIV-1 CA NTD residue I15 occupies a position near the N-terminus of helix 1, and adjacent to the P1-D51 salt bridge. Previously, we had shown that purified I15D and I15A proteins assembled immature-like spheres and small tubes rather than mature form long tubes in in vitro assembly reactions (López et al., 2011). Here, we have demonstrated that while I15 mutant Gag proteins are capable of assembling and releasing virus particles from cells (Fig. 4), the virions are replication defective and their mature cores are aberrant (Figs. 2, 3). Selection for revertants of the I15A mutation yielded I15V/S16N and H12Y/I15A/M68I variants (Fig. 2). The selection of the I15V/S16N variant was not unexpected, since a variety of hydrophobic amino acids at CA residue 15 have been found in HIV-1 strains (Table 1). The H12Y/I15A/M68I variant was more interesting because it indicated that H12Y and/or M68I compensated for the I15A defect. Perhaps not surprisingly, separation of the two secondary mutations showed that M68I, located away from helix 1 (Fig. 7A), yielded only a small compensatory effect (Figs. 2 and 3), whereas H12Y was predominantly responsible for rescue of infectivity and virus core structure (Figs. 2, 3, 5 and 6).

How does the H12Y mutation in the β-hairpin loop rescue the I15A mutant? Examination of the I15 side group (Fig. 7B), suggests that it is positioned to make important intramolecular contacts rather than critical intermolecular CA–CA contacts. Specifically, it is positioned to help align the N-terminal portion of NTD helix 1, potentially through interactions with T19 and L20, and to align helix 3 through interactions with T54. Of these two roles, the H12Y change appears more likely to affect the helix 3 alignment. In particular, a tyrosine at CA residue 12 is capable of hydrogen bonding to helix 3 residue Q50 (Fig. 7C), next to the P1-D51 salt bridge. We thus envision that while P1–D51 and I15–T54 interactions ordinarily help align the CA helix 3 N-terminus in mature virions, the I15–T54 interaction is replaced by the H12Y–Q50 hydrogen bonding in the H12Y/I15A double mutant. One consequence of having neither of these interactions is that a fraction of the CA I15A proteins in virions appears to be clipped (Fig. 4, Lane M), potentially near the β-hairpin-helix 1 juncture. In contrast, the consequences of having both I15 and H12Y do not prohibit HIV-1 replication, but may impart a very slight replication disadvantage (Figs. 2 and 3). This may be related to the fact that of the 2% of HIV-1 strains that carry H12Y (Table 1), all also have either a leucine or valine rather than an isoleucine at residue 15.

In terms of how alterations of the NTD helix 3 alignment might perturb HIV-1 morphogenesis, the percentage of conical, albeit anomalous cores formed with the I15A mutant suggest that I15A does not trap CA in an immature conformation. Instead, we speculate that the small variance in the I15A alignment of helices 1 and 3 yields CA proteins that either increase the frequency of pentamers versus hexamers (Ganser-Pornillos et al., 2004, 2007; Pornillos et al., 2009, 2010, 2011), or increase the probability that growth of mature core lattices will be terminated prematurely (Barklis et al., 2009; López et al., 2011). Either way the sensitivity of this region of CA to mutation suggests that the cavity occupied by I15 would be a worthwhile target for antivirals.
Materials and methods

Recombinant DNA constructs

The Vesicular Stomatitis virus (VSV) glycoprotein (G) expression construct, pVSV-G, used for envelope pseudotyping was described previously (Scholz et al., 2005, Noviello et al., 2011) and was a generous gift from Randy Taplitz. The parental HIV-Luc construct (pNL-LucE-Rþ; Connor et al., 1995) was kindly provided by Nathaniel Landau. The parental HIV-1 NL4-3 molecular clone was obtained from the NIH AIDS reagent program. The parental construct used for HIV-1 CA expression in bacteria is pWISP-WTCAH6 (Barklis et al., 2009) which expresses C-terminally histidine-tagged (his-tagged) CA proteins. It derives from pWISP-98-85 (Li et al., 2000; Ganser-Pornillos et al., 2004), which was kindly provided by Wes Sundquist.

The I15A point mutation was created by polymerase chain reaction (PCR) approaches as described previously (López et al., 2011) and was verified by sequencing. Its sequence is given below, where bold nucleotides correspond to the mutated residues, and underlined nucleotides represent silent mutations that create or destroy restriction enzyme cleavage sites: CAT CAG GCC GCT AGC CCT.

The I15A point mutation was cloned from longitudinal passages of MT4 T cells infected with the NL4-3 I15A virus. To do so, total DNA samples were isolated from infected MT4-T cells. For this, cells were lysed in 250 μl cold IPB buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.02% sodium azide, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) for 5 min on ice after which 250 μl of water was added. Cellular extracts were then treated with 2.5 μl of proteinase K (20 mg/ml) for 2 h at 50°C. DNA was isolated by two 500 μl phenol–chloroform extractions followed by a 500 μl chloroform extraction. The aqueous phase was then

Table 1

Amino acid frequency at CA positions 12 and 15. HIV-1 genome sequences obtained from the HIV sequence compendium HIV-1/SIVcpz (http://www.hiv.lanl.gov/content/index) were analyzed for frequency of amino acids in capsid positions 12 and 15.

<table>
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<th>Position</th>
<th>Val</th>
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AGC and CAA AT

Cell culture, transfections and infections

into pWISP-6H using NdeI genes, subcloned into pGEMTeasy, sequenced, and then subcloned HEPES (pH 7.4), penicillin, and streptomycin at 37°C. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), penicillin, and streptomycin at 37°C and 5% CO₂. The MT4 T cell line used for longitudinal passages was maintained treated with 1/10 volume (40 μl) of plasmid-free 3 M sodium acetate pH 7.6 and 2.5 vol (1 ml) of cold plasmid-free 100% ethanol. The DNA was precipitated for 1 h at −80°C, pelleted by centrifugation for 5 min at 16000g, and the ethanol excess was dried using a speed-vac. The DNA samples were resuspended in 150 μl of plasmid-free 10 mM Tris pH7.4, 0.1 mM EDTA, aliquoted and stored at −20°C. Purified DNAs (1–5 μl) then were used as templates for PCR amplification reactions. The sequences between nt 671 and 2121 (pNL4-3 nt sequence) were amplified using Thermopol Polymerase (New England Biolabs) using standard PCR protocols and subcloned into pGEMTeasy vector (Promega) for sequence analysis. The obtained capsid revertants were then subcloned back into both HIV-Luc and pNL4-3 constructs using BssHII–ApaI sites (nt 711 to 2006), and sequenced again. The sequences obtained for the compensatory mutants are as follows: H12Y/I15A: TTA AAA for M68I where bold nucleotides correspond to the mutated residue and underlined nucleotides represent silent mutations that create or destroy restriction enzyme cleavage sites.

To create the double H12Y/I15A and I15A/M68I HIV-Luc and pNL4-3 constructs BssHII–ApaI sites (nt 711 to 2006) and sequenced again. The templates for PCR amplification were created using BssHII (nt 1248–2006) sites into WT parental constructs. For the latter construct HIV-Luc and pNL4-3-115A variants were used as parents. The single M68I variant HIV-Luc and pNL4–3 constructs were created by using NsiI–ApaI (nt 1248–2006) sites into WT parental constructs. For the single H12Y construct, the sequences between nt 671 and 1248 (pNL4-3 nt sequence) were amplified using Thermopol Polymerase using standard PCR protocols and subcloned into pGEMTeasy vector for sequence analysis. To create the H12Y HIV-Luc and pNL4-3 constructs, BssHII–NsiI (nt 711–1248) sites were used. For bacterial CA protein expression, the compensatory mutations were amplified from their respective HIV-Luc constructs by PCR to create Ndel sites at the 5’ ends of the capsid genes, subcloned into pGEMTeasy, sequenced, and then subcloned into pWISP-6H using Ndel–SpeI (nt 1187–1511) sites.

Cell culture, transfections and infections

HEK 293T (DuBridge et al., 1987) and H12 (Kabat et al., 1994) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), penicillin, and streptomycin at 37°C and 5% CO₂. The MT4 T cell line used for longitudinal passages was maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 10 mM HEPES (pH 7.4), penicillin, and streptomycin at 37°C and 5% CO₂.

For analysis of virus from cells, HEK 293T cells were transfected with 24 μg HIV-Luc plasmids using calcium phosphate as described previously (Scholz et al., 2005, 2008; Noviello et al., 2011). At 72 h post-transfection, virus samples were collected by filtering supernatants through 0.45 μm filters, pelleted at 100,000g through 20% sucrose cushions at 4°C and then resuspended in 100 μl of cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 2 mM KH₂PO₄, pH 7.4), stored at −80°C and mixed 1:1 with loading buffer (40 mM DTT, 0.25% bromophenol blue, 4% SDS, 20% glycerol, 125 mM Tris pH 6.8, 10% β-mercaptoethanol) prior to electrophoresis. Cell samples from 10 cm plates were washed in 2 ml of cold PBS, collected in 1 ml of cold PBS, and 200 μl aliquots of the suspended cells were centrifuged and lysed by suspension for 5 min in 50 μl of cold IPβ buffer on ice. Insoluble cellular debris was removed via centrifugation for 15 min at 16,000g at room temperature. Cell lysates were mixed with equal amounts of loading buffer and then stored at −80°C. Both virus and cell samples were fractionated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently immunoblotted as described previously (Scholz et al., 2005, 2008; Noviello et al., 2011). For immunoblotting, the monoclonal mouse anti-CA antibody Hy183 (kindly provided by Bruce Chesebro) was used at a 1:10 dilution (from hybridoma supernatants) as the primary antibody. Secondary antibodies were alkaline phosphatase-conjugated anti-mouse antibodies (Promega) used at a 1:10,000 dilution. BCIP (5-bromo-4-chloro-3-indolyl-phosphate) was used in conjunction with NBT (nitro blue tetrazolium) for the colorimetric detection of alkaline phosphatase activity. Cell and virus Gag levels were quantified densitometrically using NIH ImageJ. Mean gray values for each CA band were measured for the same-sized areas on blots.

To perform the luciferase-based infectivity assays, HEK 293T cells were transfected as described above using 18 μg of HIV-Luc and 6 μg of pVSV-G plasmids. At 72 h post-transfection, supernatants containing viral particles were filtered through 0.45 μm syringe filter, aliquoted and stored at −80°C. Aliquots of the transfected cells were collected in cold luciferase assay buffer (LAB) (100 mM sodium phosphate [pH 8.0], 4 mM adenosine triphosphate, 1 mM sodium pyrophosphate, 6 mM magnesium chloride, 0.2% Triton X-100) and frozen at −80°C to be used as transfection controls. For the infections, confluent HEK cells were transferred from a 10 cm dish to a 6 well plate at a 1:4 dilution the day before the infections. One day later, HEK cells were incubated with 1 ml of virus, 1 ml of culture media, and 10 μl of 1.6 mg/ml polybrene in duplicate and then incubated at 37°C, 5% CO₂.
for 72 h. Three days post-infection cells were washed with cold PBS, lysed in 250 μL LAB buffer and frozen at −80°C before assays. In all cases samples from transfected and infected cells were diluted at 1:10 or 1:20 in LAB buffer lacking Triton X-100 and run on a Centra XS3 LB 960 Microplate Luminometer (Berthold Technologies). The 96-well plates used were Microfluor 1 white (Nunc # 7705). The luciferin volume injected was set for 100 μL per well of a freshly prepared 1 mM luciferin solution (BD Monolight) with counting times of 15 s per well. The obtained relative light units were calculated as (average of infection duplicate counts)/(average of transfection counts) and subsequently normalized to WT samples.

For the longitudinal infections of virus in MT4 T cells, 24 μg of pNL4-3 wild type DNA or H15 variants were transfected via calcium phosphate into HEK 293T cells. At 3 d post-transfection, viral supernatants were filtered through 0.45 μm filters and aliquots of each stock were subjected to SDS-PAGE and immunoblotting for virus quantitation. For viral inoculum normalization, CA bands were quantitated as described above. Pelleted virus samples were gently resuspended in 20% sucrose cushions as described above. Virus particle analysis

HIV-Luc wild type (WT) and H15 variant virus-like particles were produced by calcium phosphate transfection of 24 μg of plasmid DNA of HEK 293T cells. Supernatants were filtered through 0.45 μm filters, and subjected to ultracentrifugation through 20% sucrose cushions as described above. Virus particle analysis

pWISP-6H plasmids containing WT or mutant CA sequences were transfected into the E. coli strain BL21(DE3)/pLysS (Novagen) for protein expression and purification, which followed previous procedures (Barklis et al., 2009; Noviello et al., 2011; López et al., 2011). Briefly, bacteria were grown in Luria-Bertani (LB) broth supplemented with 0.2% maltose and 10 mM MgSO4 to a 600 nm optical density (OD) of about 0.4, and then induced at 25°C for 3 h via the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Roche #114114 46001). Induced bacteria were collected by centrifugation (Sorvall GSA 5000 rpm, 3000g, 4°C) and frozen at −80°C. Bacterial pellets were suspended in 10 ml of ice cold 50 mM NaH2PO4 pH 7.8, 300 mM NaCl containing 0.5 mg of DNaseI (Roche #10104159001) plus protease inhibitors (15 μg/ml leupeptin, 36 μg/ml egg-white trypsin inhibitor, 36 μg/ml soybean trypsin inhibitor, 30 μg/ml aprotinin, and 1.5 mM phenylmethylsulfonyl fluoride [PMSF; Sigma]). After a 10 min incubation on ice, bacterial suspensions were French-pressed twice, cleared by centrifugation at 23,000g (Sorvall SS34, 14,000 rpm) for 15 min at 4°C, and purified by one to two rounds of nickel chelate chromatography (Qiagen Ni-NTA resin, #30210). Non-specific protein contaminants were washed by increasing the imidazole concentration (10 mM, 25 mM and 50 mM) in wash buffer (50 mM NaH2PO4 pH 6, 300 mM NaCl, 10% glycerol), and CA proteins subsequently were eluted in the same buffer containing 250 mM imidazole. Purified fractions were subjected to three rounds of buffer exchange by dialysis in 20 mM Tris pH 8.0, 5 mM β-mercaptoethanol for 6 h at 8°C and protein aliquots were flash frozen on dry ice and stored at −80°C. Protein identities were ascertained by immunoblotting, and protein purities of >90% were verified by Coomassie blue staining of samples fractionated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined via 280 nm absorbance readings assuming a molar extinction coefficient of 33,580, and by densitometric comparison of stained SDS-PAGE protein bands versus known standards.

Analysis of in vitro CA assembled products

In vitro assembly capsid incubations were performed as described previously (Barklis et al., 2009; Noviello et al., 2011; López et al., 2011). Typically, 10 μl reactions included 1.5 mg/ml protein (final concentration) and 1× assembly buffer (50 mM Tris pH 7.0, 1 M NaCl and 5 mM β-Me), and were incubated 48 h at 4°C. After this incubation step, the samples were lifted 3 min onto UV-irradiated 400-mesh carbon-formvar grids (Ted Pella 01822-F), washed with 1 mM water, wicked on filter paper, stained 45 s in filtered 1.33% uranyl acetate, washed, and air dried. Samples were viewed at 100 kV on a Philips CM120 Biotwin and images were collected as described above.

Tube lengths for each CA variant were manually measured using at least ten 4800× gray scale TIF images corresponding to areas of 4.136×4.136 μm2. Tube area coverage was determined as described previously (López et al., 2011). Briefly, image features were highlighted using gray scale TIF images and the feature J/F Laplacian command with a smoothing step of 10, and the zero crossings parameter unselected. Laplacian images were thresholded to low values of 0.10 and maximum high values. For tube coverage tabulation, the Analyze/Analyze Particles command was used with the Analyze/Set Measurements area and Feret’s diameter options highlighted. The Analyze Particles command was called with size and circularity arguments, and results (including particle ID numbers, particle areas, and Feret diameters) were saved in table format. Tube candidates then were excluded if they did not meet the criteria of having a Feret’s diameter of at least 50, and a (Feret2)/area value of at least 4. After performing these qualification steps, total areas covered by tubes were summed and divided by total areas to obtain area fractions covered by tubes. Results are depicted as areas covered by tubes normalized to the results with wild type incubations.

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