Second-Site Compensatory Mutations of HIV-1 Capsid Mutations

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The human immunodeficiency virus (HIV) capsid (CA) protein assembles into a hexameric lattice that forms the mature virus core. Contacts between the CA N-terminal domain (NTD) of one monomer and the C-terminal domain (CTD) of the adjacent monomer are important for the assembly of this core. In this study, we have examined the effects of mutations in the NTD region associated with this interaction. We have found that such mutations yielded modest reductions of virus release but major effects on viral infectivity. Cell culture and in vitro assays indicate that the infectivity defects relate to abnormalities in the viral cores. We have selected second-site compensatory mutations that partially restored HIV infectivity. These mutations map to the CA CTD and to spacer peptide 1 (SP1), the portion of the precursor Gag protein immediately C terminal to the CTD. The compensatory mutations do not locate to the molecularly modeled intermolecular NTD-CTD interface. Rather, the compensatory mutations appear to act indirectly, possibly by realignment of the C-terminal helix of the CA CTD, which participates in the NTD-CTD interface and has been shown to serve an important role in the assembly of infectious virus.

Retroviruses are composed of a glycoprotein-studded lipid membrane enveloping a ribonucleoprotein core. The major structural protein of retroviruses is the PrGag polypeptide, which is composed of several subdomains. At the N terminus, the matrix (MA) domain interacts with the membrane via a myristoyl moiety and N-terminal basic amino acids (3, 6, 18, 19, 22, 25, 50, 56, 57, 61, 64, 73, 74). The capsid (CA) domain, which includes an N-terminal domain (NTD) and a C-terminal domain (CTD) connected by a flexible linker, is the major component responsible for Gag-Gag protein interactions (24, 29, 31, 32, 34, 39, 67, 70). Following capsid is spacer peptide 1 (SP1), which joins to nucleocapsid (NC). Nucleocapsid binds the viral RNA; through this interaction, NC both initiates PrGag oligomerization and assembly and ensures that the viral genome is packaged in budding virions (4, 12, 14, 20, 23). Nucleocapsid is connected to spacer peptide two (SP2), which is followed by the p6, or late, domain, at the C terminus of the Gag polypeptide. The p6 domain is involved in protein interactions with cellular components that ensure PrGag budding at the plasma membrane, the site of productive assembly (28, 33, 35, 44, 53, 61, 65, 71).

PrGag undergoes proteolytic cleavage during the maturation of the virus, which is critical for infectivity. This process may occur during assembly at the plasma membrane or shortly after budding from the producer cell. Prior to cleavage by the HIV protease, immature virions are spherical in nature, with as many as 5,000 densely packed PrGag proteins assembled into a hexameric lattice (10, 11, 54). After cleavage, the spherical shell of PrGag reassembles to form a cone or tube; this viral core is composed of ~1,500 CA proteins (32). The CA proteins in the core are also assembled as hexamers, but with 12 pentamers at the ends to enable closure of the cone (11, 32, 43, 52). In electron micrographs of HIV produced from cells, mature HIV cores can be seen in both tube and cone forms, with the cone form predominating (16, 17, 42, 70). Within viral cores, NC proteins are bound to two copies of viral genomic RNA, which associate with viral reverse transcriptase (RT) and integrase (IN) proteins as well as other viral and cellular factors that contribute to infectivity.

The structural organization of CA accommodates both the immature and the mature forms of hexameric assembly. The seven alpha helices of the NTD (CA helices 1 to 7) are connected via a linker to the four alpha helices (CA helices 8 to 11) of the CTD (Fig. 1A). At the N-terminal end of the NTD, 13 residues fold after proteolytic cleavage to form a β-hairpin, which is required for infectivity (1, 37, 41, 49, 51, 67). The first three helices of the NTD interact with the first three helices of other CA NTDs, forming an 18-helix bundle in a hexameric lattice (30, 52, 58). The CA CTD dimerizes to connect the hexameric subunits to each other (13, 30, 58). Intermolecular interactions between NTD helices 3 and 4 and helix 8 of the adjacent CTD stabilize the hexamer (30, 58). Further stabilization is provided from contacts between helix 7 of the NTD and helix 11 of the CTD (30, 58). A further interaction is between the NTD-CTD interface and helix 9 of the CTD: this connection is proposed to have a role in cooperativity of assembly (30). Evidence of the importance of the intermolecular NTD-CTD interface in vivo comes from a study of the Rous sarcoma virus capsid: mutations in the CTD major homology region (MHR) elicited second-site revertants in the NTD (7). Further support for the importance of NTD-CTD interactions derives from studies of the assembly inhibitor CAP-1. CAP-1 binds to a hydrophobic groove formed by the N-terminal end of helix 4 and the C-terminal end of helix 7; this same groove is normally occupied by the CTD. Disruption of this interaction in cell culture by incu-

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bation with CAP-1 leads to inhibition of HIV-1 infectivity and abnormal core morphologies (46, 58, 66).

To further understand how the NTD-CTD interface contributes to HIV-1 assembly, we mutated a key residue, histidine 62 (H62), within the loop between NTD helices 3 and 4 (Fig. 1A). This residue is of particular interest for several reasons. It is highly conserved (27, 62), and it has been shown to move upon CAP-1 binding. Furthermore, histidine is unique in that the pKa of its imidazole side chain is 6.04, within physiologic levels. Protonation of histidine, occurring at a pH that is thought to trigger the “mature” form of CA in vitro, could be responsible for rearrangements of the capsid hexamer lattice involved in maturation (27). Indeed, structural evidence suggests that the imidazole group of H62 forms ring-stacking interactions with F32 and Y145 of the NTD, which is buttressed by the guanidium group of the CTD at R162 (58). In our study, mutations of H62 to alanine, cysteine, tryptophan, lysine, tyrosine, or phenylalanine had modest effects on assembly and release but pronounced effects on infectivity. Remarkably, we discovered three independent second-site compensatory mutations of H62 mutants. These second-site mutations bracketed helix 11 of the CTD: one was in SP1, and remarkably, two mapped to the same G208 residue in the CTD. The compensatory mutants showed enhanced viral replication and assembly in vitro over the respective H62 mutants but were not better than wild-type (WT) HIV-1. The locations of the compensatory mutations, in SP1 and in the loop between helices 10 and 11, are surprising, as they are not spatially located near the H62 residue or directly involved in any CA NTD-CTD interactions. Our data suggest that compensatory changes for H62 mutations act indirectly by repositioning CTD elements to offset NTD structural alterations.

MATERIALS AND METHODS

Recombinant DNA constructs. A vesicular stomatitis virus (VSV) glycoprotein (G) expression construct, pVSV-G, was a generous gift from Randy Taplitz. The parental HIVLuc construct (pNL-LucE-R+H9252) was kindly provided by Nathaniel Landau. The parental NL4-3 construct (2) is available from the AIDS reagent database. The bacterial expression vector used in the in vitro assembly assay, pWISP-WTCAH6, has been described previously (5).

To create the H62 mutants in the HIVLuc and pNL4-3 backgrounds, the BssHII-SpeI fragments then were subcloned into HIVLuc and pNL4-3. The nucleotides covering CA residues 60 to 65 (NL4-3 nt 1363 to 1377) are as follows, with mutations underlined for each variant: for H62A, 5'GGGGGAGCTCAAGCA3'; H62C, 5'GGGGGATTTCAAGCA3'; H62K, 5'GGGGGATGGCAAGCA3'; H62F, 5'GGGGGATATCAGCA3'; H62W, 5'GGGGGATTCGCAAGCA3'; and H62Y, 5'GGGGGATACGCAAGCA3'.

Compensatory mutants cloned from longitudinal passages were isolated by extraction of DNA from infected cells. Cells were lysed by 250 μl IPB (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). An equal volume of water was added, and cells were then digested with 50 μg of proteinase K for 2 h. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The sequence between nt 671 and nt 2096 was amplified using overlap PCR with primers mutagenic at the nt 1369-to-1371 (H62) site. All nucleotide numbers refer to NL4-3 as a reference sequence. Mutant fragments were cloned into the pGEMTeasy vector (Promega), and mutations were confirmed by sequencing. The BssHII-SpeI fragments then were subcloned into HIVLuc and pNL4-3. The nucleotides covering CA residues 60 to 65 (NL4-3 nt 1363 to 1377) are as follows, with mutations underlined for each variant: for H62A, 5'GGGGGAAGCTCAGCA3'; H62C, 5'GGGGGAATTCAGCA3'; H62K, 5'GGGGGAAATTCAGCA3'; H62F, 5'GGGGGAATTTCAGCA3'; H62W, 5'GGGGGAATTCAGCA3'; and H62Y, 5'GGGGGAATTCAGCA3'.

FIG. 1. Effects of mutations on virus particle assembly and release. (A) Shown is a pair of CA proteins (Protein Data Bank [PDB] accession no. 3GV2) in HIV-1 CA hexamers viewed roughly from the center of the hexamer outward. NTDs (blue) and CTDs (green) are marked, as are the β-hairpin (β) and cyclophilin A binding (CypA) loops and helices 1 to 11 on one of the capsid proteins. H62 residues are indicated in red. (B) HEK 293T cells were transfected with the indicated HIVLuc constructs. At 72 h posttransfection, virus samples were collected, and Gag proteins were detected after gel electrophoresis by immunoblotting using a primary anti-CA antibody. Full-length PrGag and CA bands are as indicated. (C) For quantification of viral particle release at 72 h posttransfection, virus and cell samples were collected and subjected to electrophoresis and immunoblotting using an anti-CA antibody. Cell and virus Gag levels were quantitated densitometrically; raw virus release levels (Virus Gag/Cell PrGag) were calculated and are shown, normalized to WT HIV release levels. Results derive from two (H62K and H62W), three (H62C and H62F), four (H62Y), five (H62A), or eight (WT) separate experiments. Standard deviations are as shown.
Cell culture and transfections. HEK 293T (26) and HJ (45) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES (pH 7.4), penicillin, and streptomycin at 37°C/5% CO2. CEM-SS and MT-4 T cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum, 10 mM HEPES (pH 7.4), penicillin, and streptomycin at 37°C/5% CO2.

Quantification of viral particle assembly and release was performed using calcium phosphate transfections of HEK 293T cells by 24 μg of HIVLuc plasmid constructs as described previously (68, 69). Virus samples were collected at 72 h by filtering them through a 0.45-μm filter to remove cell debris and then centrifuged at 13,000 rpm to pellet the insoluble membrane material, and then stored at −80°C. Virus and cell samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (62, 63) and subsequently immunoblotted using a primary anti-CA antibody (183-H12-5C; available from the AIDS Reagent and Reference program) (15), followed by secondary alkaline phosphatase-conjugated anti-mouse antibodies (Promega). 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, and virus release levels were calculated as virus Gag/cell Gag ratios and normalized to the level of WT samples run in parallel.

For the longitudinal infections, 24 μg of pNL4-3 WT or H62 variants was transfected via calcium phosphate into HEK 293T cells. At 3 days posttransfection, viral supernatants were collected and filtered to remove cell debris. Infections were tracked by anti-CA immunoblotting of infected cell lysates. Potential compensatory mutations in the NTD of H62 mutants were monitored by densitometric comparison of stained SDS-PAGE protein bands versus known standards.

RESULTS

Analysis of capsid mutants. There are five histidines in HIV-1 capsid, three of which are conserved (H12, H62, and H84) (27). Of these three, our previous work delineated a role in assembly for H84, located on an external surface of the NTD hexamer (62). The residue H62 is also in the NTD (Fig. 1A) and locates to the assembly inhibitor CAP-1 binding region. Additionally, H62 forms ring-stacking interactions with F32 and Y145, which are thought to be critical for the stability of the NTD-CTD intermolecular interface (46, 58). We interro-gated the role of H62 in this interaction by substituting alanine, cysteine, lysine, tyrosine, phenylalanine, and tryptophan for histidine and assessed these mutants in vitro and in cell culture. To measure the effects of mutations on the release of viral-like particles (VLP), we transfected 293T cells with each mutant construct in the context of an HIV Luc background. Levels of virus release were monitored by anti-CA immunoblotting of SDS-PAGE-fractionated VLP samples taken from the supernatants of transfected cells. As shown in Fig. 1B, the VLP levels of the H62 mutants were slightly reduced compared to those of the WT, and there appeared to be minor variations in CA-to-ProGag ratios. To quantify the viral release, parallel cell and VLP samples were electrophoresed and immunoblotted for ProGag and CA detection. Gag band intensities were determined via densitometry, and virus-to-cell Gag ratios were calculated, after which the mutant construct ratios were normalized to those of the WT (Fig. 1C). Whereas H62 mutants exhibited slight decreases in virus release, the defects appeared moderate in that all of the mutant viruses were released at least half as efficiently as the WT. Thus, none of the H62 mutants appear to be significantly impaired for VLP release. As a second step of analysis, we assessed the impact of H62 mutations on virus infectivity. To do so, HIVLuc luciferase reporter (21) versions of WT and mutant constructs were cotransfected into 293T cells with a VSV envelope glycopep-
tein expression construct to generate pseudotyped virus. Viruses were used to infect CD4\(^+\) HiJ cells (45), and after 72 h, infected and transfected cells were assayed for luminescence. While these assays did not correct for the minor release effects observed in Fig. 1C, Fig. 2A demonstrates that several H62 mutants exhibited substantial defects in their abilities to support infection. Consistent with the notion that H62 forms important aromatic interactions with F32 and Y145, aromatic substitutions for H62 (H62F, H62W, and H62Y) yielded viruses that were at least 5% as infectious as the WT. In contrast, substitutions lacking an aromatic side chain (H62A, H62C, and H62K) were at most 1% as infectious as the WT.

**Examination of core structures.** As mentioned previously, H62 locates to a region involved in intermolecular NTD-CTD interfaces, which are crucial for the stabilization of the hexameric CA lattices (7, 30, 31, 47, 48). Therefore, we examined the core structures of released VLP for possible anomalies. Consequently, VLP from cells transfected with HIVLuc variants were collected, filtered through 0.45-\(\mu\)m sterile filters, and used to infect HiJ cells. At 3 days postinfection, HiJ cells were collected and processed for luciferase assays. Results are depicted as normalized luciferase activity signals in infected cells, relative to signals from cells infected with WT HIVLuc virus in parallel: they are not corrected for variations observed in virus particle release. Results are derived from 46 (WT), 16 (H62A and H62F), 2 (H62C and H62F/G208A), 3 (H62K), 4 (H62W and H62Y), 5 (G208A), 8 (G208R and H62F/S1P10L), and nine (H62A/G208R) separate experiments. Standard deviations are as shown.

![FIG. 2. Analysis of virus infectivity. Luciferase gene-transducing HIV viruses were produced by cotransfection of HEK 293T cells with a VSV-G expression vector plus WT and H62 mutant HIVLuc constructs (A) or the indicated HIV variants that derived from selection of H62A or H62F second-site revertants (B). At 3 days posttransfection, virus-containing medium supernatants were collected, filtered through 0.45-\(\mu\)m sterile filters, and used to infect HiJ cells. At 3 days postinfection, HiJ cells were collected and processed for luciferase assays. Results are depicted as normalized luciferase activity signals in infected cells, relative to signals from cells infected with WT HIVLuc virus in parallel: they are not corrected for variations observed in virus particle release. Results are derived from 46 (WT), 16 (H62A and H62F), 2 (H62C and H62F/G208A), 3 (H62K), 4 (H62W and H62Y), 5 (G208A), 8 (G208R and H62F/S1P10L), and nine (H62A/G208R) separate experiments. Standard deviations are as shown.](http://jvi.asm.org/content/85/11/4733)
of virions, we utilized an in vitro assembly assay with purified CA proteins. We chose to compare the WT CA versus the H62A mutant, as H62A exhibited a large defect in infectivity (Fig. 2A), but the protein was as readily purified from bacteria as the WT protein. For analysis, purified proteins (100 μM) were incubated in assembly reactions. Following assembly incubations, samples were processed and imaged by EM at low magnification (A, B, D, E, G, H, J, and K; size bar in panel J) or high magnification (C, F, I, and L; size bar in panel L). For each capsid variant, assembly efficiency was assessed by the appearance of capsid tubes, and tube assembly was quantified by tube coverage in EM images as described in Materials and Methods. Average tube coverage percentages, normalized to the WT level, were calculated from 12 pictures each and are as follows: WT, 100%; H62A, 0%; H62A/G208R, 11%; G208R, 68%.

Characterization of compensatory mutations. Although our luciferase-based infectivity assays (Fig. 2A) were informative, they reflect only a single round of infection. To examine the role of H62 in the context of a more physiologically relevant infection model, we cloned H62 mutations into the HIV-1 NL4-3 provirus. We then transfected the proviral mutants into 293T cells, collected viral supernatants, and normalized the Gag levels. The H62W and H62F viruses both peaked around 11 days, reflecting the greater defects observed in single-round infectivity assays (Fig. 2A). Finally, the H62A mutation again failed to replicate. A small amount of CA was detected after 27 days of virus passage, but this appeared to represent a compensatory mutation, as discussed in further detail below.

The H62A, H62F, and H62W mutants were chosen for extended serial passages to examine the capability of the virus to evolve compensatory mutations. We chose these mutants because their rates of replication in MT4 cells were distinguishable from those for the WT and because the replication delays that we observed (Fig. 5B) could have resulted from the acquisition of second-site mutations. For these studies, MT4 cells were infected with equalized stocks of virus and split every 3 to 4 days for several weeks. Late-passage infections were pelleted to remove cell debris, and the supernatants were added to fresh MT4 cells. This was repeated twice, for a total of three sequential series of infections. With the H62W virus, serial passage resulted in the derivation of a virus with enhanced replication relative to that of the mutant. To identify mutations that might account for this phenotypic change, the gag gene of the virus was cloned and sequenced. Our analysis indicated that the virus had reverted to the original H62 sequence and contained no other mutations (data not shown).

During our analysis of H62F replication, two of the H62F replicates were much more infectious than the H62F mutant. The first replicate was sequenced as before, and we confirmed the maintenance of the H62F mutation. Interestingly, we discovered a P10L mutation in the SP1 spacer peptide; this mutation also has arisen in response to an HIV mutant lacking stem-loop 1 of viral genomic RNA, a region involved in dimerization and packaging into virions (59). The second replicate also maintained the H62F mutation but encoded a G208A mutation in the C-terminal domain of capsid and an E21K mutation in NC. It is noteworthy that residue 208 is also an alanine in other HIV strains. Further sequencing confirmed that this was not a viral contaminant but indeed a compensatory mutation (see below). Remarkably, another compensatory mutation arose at the same codon from the H62A mutant during the third round of serial passages. After cloning and sequencing, the stock was found to have a G208R mutation in addition to the original H62A mutation.

To determine the roles of the above-described mutations in counteracting the H62F and H62A defects, we first cloned them into the HIVLuc background. In doing so, we separated the NC(E21K) and CA(G208A) mutations in the H62F background, as it was possible that only one was responsible for correcting the H62F defect. Indeed, the H62F/E21K mutant was still defective in single-round and longitudinal infectivity assays (data not shown). In contrast, the H62F/G208A, H62F/
SP1P10L, and H62A/G208R double mutations improved single-round infectivities more than 6-fold over the respective H62F and H62A viruses (Fig. 2B). Because the G208R and G208A variants identified the same residue as being important for overcoming the effects of H62 mutations, we focused on these in the studies described below. To ensure that the G208A and G208R mutations were compensating for the H62 defects and not independently enhancing HIV-1 infectivity, we assessed the single-round infectivities of G208R and G208A in the context of the WT H62 sequence. As seen in Fig. 2B, the G208A and G208R single mutations were not superinfectious; if anything, the mutants exhibited slight defects relative to WT infections (Fig. 2B).

To compare compensatory mutants with the original mutants in T cell infections, we cloned the single and double mutants into the HIV-1 strain NL4-3 background for passages in MT4 cells. The results for these investigations are shown in Fig. 6. With the H62F set of viruses (Fig. 5C), the original H62F virus showed delayed kinetics, as expected, but the double H62F/G208A virus replicated nearly as fast as the WT (day 7 versus day 4 peaks), and the single G208A mutant virus was similar to the WT. These results indicate that G208A compensates for the H62F defect. A similar pattern was observed for the H62A set of viruses (Fig. 5D). Specifically, the original H62A virus was noninfectious, the G208R virus mimicked the WT, and the H62A/G208R double mutant showed intermediate replication kinetics. The compensatory mutations thus converted replication-defective viruses into replication-competent viruses.

To examine how at least one of our compensatory mutants might affect CA protein interactions, we examined the cores of H62A/G208R mutant viruses by EM. Relative to the H62A mutant, which showed predominantly immature-type cores, the double mutant yielded an increased number of cone-like or tube-like cores (47% versus 19%) (Table 1 and Fig. 3). To further examine the ability of proteins with the G208R mutation to compensate for the assembly defect of H62A CA proteins, we utilized our in vitro assembly assay. Importantly, while H62 CA proteins assembled no tubes in vitro (Fig. 4D to F), the H62A/G208R double mutant proteins assembled tubes (Fig. 4G to I), albeit at 10.8% of WT levels. Because the single G208R mutant CA proteins did not assemble any more efficiently than the WT (Fig. 4J to L) (68% of WT levels), our data indicate that mutation at CA CTD residue 208 specifically rescues the assembly defect of mutations at CA NTD residue H62.
are as labeled. blue N-terminal domains and light and dark green C-terminal domains perpendicular to the capsid surface from the outside. Light and dark surface up and the inner surface down. Panel B is viewed roughly parallel to the plane of capsid coats, with the predicted outer capsid (PDB accession no. 3GV2) are depicted. Panel A is viewed roughly orthogonal ring-stacking interactions at the NTD-CTD inter-

not surprising, as the aromatic rings of H62 are implicated in chains (phenylalanine, tryptophan, and tyrosine) were less af-

core morphologies visualized by electron microscopy (Fig. 3 and CTD helices 8 and 11. Although H62 is highly conserved H62 locates to this interface, which involves NTD helices 3 and 4 and CTD helices 8 and 11. Although H62 is highly conserved and has the potential to act as a pH switch, mutation of histidine did not have major effects on release of virus-like particles from transfected cells (Fig. 1A and B). However, replacement of H62 in single-round and longitudinal infectivity studies pro-

and Table 1). In each assay, substitutions with aromatic side pro-

of H62 in single-round and longitudinal infectivity studies pro-

duced a more dramatic phenotype, with significant defects in survival of HIV: the virus will evolve mutations to compensate for structural defects caused by losing this interaction. However, atomic models indicate that CTD residue 208 is not in a position to interact directly with NTD residue 62 (Fig. 6B). How then do these second-site mutations compensate for the structural defects caused by a change of H62? One possibility is that they alter a newly described 3-fold axis of symmetry defined by CTD-CTD dimers (13). Alternately, residue 208 mutations may reposition helix 11 so that it accommodates H62 NTD mutation. In effect, this compensatory mutant may act in the same manner as the one we have proposed above for the P10L mutation and may help explain the pronounced effects that helix 11 mutations have been shown to have on virus infectivity (24, 55, 60). In summary, our work here supports the idea that NTD-CTD intermolecular interactions of the CA core are crucial to the survival of HIV: the virus will evolve mutations to compensate for structural defects caused by losing this interaction.

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