Amino acid substitutions in human immunodeficiency virus type 1 (HIV-1) Gag cleavage sites have been identified in HIV-1 isolated from patients with AIDS failing chemotherapy containing protease inhibitors (PIs). However, a number of highly PI-resistant HIV-1 variants lack cleavage site amino acid substitutions. In this study we identified multiple novel amino acid substitutions including L75R, H219Q, V390D/V390A, R409K, and E468K in the Gag protein at non-cleavage sites in common among HIV-1 variants selected against the following four PIs: amprenavir, JE-2147, KN-272, and UIC-94003. Analyses of replication profiles of various mutant clones including competitive HIV-1 replication assays demonstrated that these mutations were indispensable for HIV-1 replication in the presence of PIs. When some of these mutations were reverted to wild type amino acids, such HIV-1 clones failed to replicate. However, virtually the same Gag cleavage pattern was seen, indicating that the mutations affected Gag protein functions but not their cleavage sensitivity to protease. These data strongly suggest that non-cleavage site amino acid substitutions in the Gag protein recover the reduced replicative fitness of HIV-1 caused by mutations in the viral protease and may open a new avenue for designing PIs that resist the emergence of PI-resistant HIV-1.

Combination antiretroviral therapy using reverse transcriptase inhibitors and protease inhibitors (PIs) produces substantial suppression of viral replication in HIV-1-infected patients. However, the emergence of drug-resistant HIV-1 variants in such patients has limited the efficacy of combination chemotherapy. HIV-1 variants resistant to any of the currently available antiretroviral therapeutics have emerged both in vitro and in vivo (1). In particular, HIV-1 resistant to one PI is often cross-resistant to another PI, presenting formidable challenges in the therapy of HIV-1 infection. Indeed, HIV-1 protease has been shown to tolerate extensive sequence variations, remaining functional even with as many as 15 amino acid substitutions accumulated in a molecule composed of 99 amino acids (2, 3).

Amino acid substitutions in HIV-1 Gag precursor p7-p1 and p1-p6 cleavage sites have been identified in HIV-1 isolated from patients with AIDS failing chemotherapy including PIs (4, 5). Those substitutions are believed to compensate for the enzymatic impairment of protease per se resulting from the acquisition of PI resistance-conferring amino acid substitutions within the protease-encoding region of the HIV-1 genome. However, a number of highly PI-resistant HIV-1 variants lack such cleavage site amino acid substitutions. Furthermore, recombinant HIV-1 clones to which PI resistance-conferring substitutions in the protease-encoding region were introduced are known to often fail to propagate in vitro (6). Therefore, we thought that as yet unidentified amino acid substitutions in the Gag-Pol polyprotein, the substrate for the enzyme, compensate for the altered enzymatic function caused by the acquisition of amino acid substitutions in the viral protease. To this end, we generated PI-resistant HIV-1 variants by exposing HIV-1 to four different PIs including amprenavir (APV) (7), JE-2147 (2), KN-272 (8), and UIC-94003 (7), identified up to 23 amino acid substitutions in Gag and protease, generated a variety of infectious HIV-1 clones containing such amino acid substitutions, and characterized their replication profiles. We conclude that Gag amino acid substitutions such as H219Q and R409K, located outside the cleavage sites, contribute to the development of HIV-1 resistance to PIs and are essential for the replication of HIV-1 variants in the presence of PIs.
EXPERIMENTAL PROCEDURES

**Cells and Antiviral Agents**—MT-2 and H9 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (HyClone, Logan, UT), 50 units/ml penicillin, and 50 μg/ml streptomycin. Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated by phytohemagglutinin (PHA) in RPMI 1640-based medium containing interleukin-2 (5 ng/ml) (R & D Systems, Minneapolis, MN) for 2 days before HIV-1 exposure. APV was a kind gift from Glaxo Wellcome. JE-2147 and KNI-272 were synthesized as previously described (2, 7, 11, 12). Generation of HIV-1 Resistant to PIs—The wild type cloned HIV-1, HIV-1NL-43, obtained from COS-7 cells transfected with pHIV-1NL4-3, was propagated in human CD4 + MT-2 cells in the presence of increasing concentrations of PIs as described previously (2, 7). Briefly, MT-2 cells (5 × 10^6) were exposed to HIV-1NL4-3 (500 50% tissue culture infective dose (TCID_{50})) and cultured in the presence of APV, KN-272, or UIC-94003 at initial concentrations of 0.0005–0.003 μM. Viral replication was monitored by observation of the cytopathic effect (CPE) in MT-2 cells. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased. This selection was carried out for a total of 27–62 passages. For the generation of JE-2147-resistant virus, HIV-1NL4-3/I84V (2) was employed instead of HIV-1NL4-3. Proviral DNAs from the lysates of infected cells from several passages were sequenced as indicated.

**Determination of Nucleotide Sequences**—Molecular cloning and determination of nucleotide sequences of HIV-1 passaged in the presence or absence of antiretroviral agents was performed as described previously (2). In brief, high molecular weight DNA was extracted from HIV-1-infected MT-2 cells using High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals) and the entire Gag and protease-encoding regions of the HIV-1 genome was amplified with primers 5'-ATG TAA AAG ACA CCA AGA AAG C-3' and 5'-TAT TT TTT TTC TTT TGT CAT AAG GCC-3' (Fig. 1). The forward primer had a βsHsi site, whereas the SmaI site was introduced in the reverse primer with synonymous substitutions at nucleotides 2590 and 2593. The PCR products were purified with PCR Select III columns (5 Prime →3 Prime, Inc., Boulder, CO) and subjected to molecular cloning using Original TA Cloning Kit (Invitrogen), followed by sequence determination using an Applied Biosystems model 373 automated DNA sequencer.

**Generation of Recombinant HIV-1 Clones**—The PCR products obtained as described above were digested with two of three enzymes BssHII, ApaI, and SmaI, and the obtained fragments were introduced into pHIV-1NL4-3, designed to have a SmaI site by changing two nucleotides at the 5' end of pHIV-1NL4-3 to generate HIV-1 cloning vectors containing the mutations, site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was performed, and the mutation containing genomic fragments were introduced to pHIV-1NL4-3. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each recombinant plasmid was transfected into COS-7 cells with GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA), and thus obtained infectious viruses were harvested 48 h after transfection and stored at −80 °C until use.

**MAGI Assay**—MAGI assay was employed to determine the infectivity of the stock HIV-1 preparations as described previously (11, 12). HeLa-Cd4-LTR-β-gal cells (10/500,000) were plated in 96-well flat-bottomed microtitre culture plates. On the following day, the medium was aspirated, and the cells were exposed to HIV-1 in a total volume of 50 μl. After incubation at 37 °C for 2 h, fresh complete Dulbecco’s modified Eagle’s medium (50 μl) was added to each well. Forty eight hours after viral exposure, the total number of blue cells in each well was determined. All assays were performed in triplicate.

**Replication Kinetics Assay**—MT-2 cells (10^5) or PHA-stimulated PBMCs (5 × 10^6) were exposed to each infectious virus preparation (500 and 2000 blue cell-forming units defined in the MAGI assay for MT-2 cells and PBMCs, respectively) for 12 h, washed twice with PBS, and cultured in 5 ml of complete medium in the presence or absence of PI. Culture supernatants (200 μl) were harvested every other day, and p24 Gag amounts were determined using a commercially available radioimmunoassay kit (Du Pont/NEN Research Products, Boston, MA). An enzyme-linked immunosorbent assay kit (Beckman Coulter, Inc., Fullerton, CA) was also used for the determination of p24 Gag amounts as needed. **MTT Assay**—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine the CPE of each stock virus preparation. MT-2 cells (4 × 10^4 in 200 μl complete medium) were plated on 96-well flat-bottomed microtitre culture plates. After 16 h, the medium was aspirated, and each virus preparation (20 blue cell-forming units/well) was inoculated. In 10 days of culture, medium (100 μl) was removed, and MTT solution (10 μl, 7.5 mg/ml) was added to each well. The cells were incubated at 37 °C for 2 h and exposed to 100 μl of acidified isopropyl alcohol-containing 4% (v/v) Triton X-100 to dissolve the formazan crystals (absorbance ω wavelength, 570 nm) was then measured in a microplate reader. All assays were performed in triplicate.

**Competitive HIV-1 Replication Assay (CHRA)**—Freshly prepared H9 cells (3 × 10^5) were exposed to infectious clones to be examined for their replicative ability and cultured in the presence or absence of APV as described previously (12). To ensure that the infectivity of paired infectious clones was equal or comparable, a fixed amount (200 TCID_{50}) of one infectious clone was combined with three different amounts (100, 200, and 300 TCID_{50}) of the second infectious clone. On day 1 in culture, one-third of infected H9 cells were harvested and washed twice with PBS, and cellular DNA was extracted and subjected to nested PCR for amplification of the p7-1 p6-encoding gag region plus the protease-encoding gene, and direct sequencing was performed. The first primer pair used was CM5 (5'-AGC GTG CAA ACA CAT CCT TGA GCT ACG ACC-3') and CM4 (5'-TAT TT TTT TTC TTT TGT CAT AAG GCC-3'), which included the M13 forward and reverse sequences, respectively (2). The HIV-1 culture, which best approximated a 50:50 mixture on day 1, was further propagated, and the remaining cultures were discarded. Every 7 or 10 days, the supernatants of the virus co-culture were transmitted to fresh uninfected H9 cells. The cells harvested at each stage were subjected to direct DNA sequencing, and viral population changes were determined. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay.

**Western Blot Analysis**—To analyze whether HIV-1 polyproteins in various HIV-1 clones were cleaved by protease, Western blot analysis was conducted (13). It is of note that when the virus was propagated in the presence of high concentrations of PIs, the amounts of Prs55Gag, p41, and p24 varied vastly, and no normalization was possible. Therefore, by assuming that the transfection efficiency was comparable, the 48-h supernatants of the culture of COS-7 cells transfected with the same amount of each plasmid using the same conditions were directly subjected to Western blot analysis as described previously (13). Briefly, 48 h after transfection with plasmid preparations, the culture supernatant was centrifuged at 13,800 × g for 10 min and passed through a 0.45-μm pore-size filter to remove cellular debris. The filtrate was centrifuged at 20,380 × g for 4 h to pellet virions. The pelleted virions were lysed in lysis buffer (10 mM Tris (pH 7.4), 50 mM NaCl, 100 mM KCl, 1% NP40, 1 mM dithiothreitol, 1% aprotinin, and 1 μg/mL benzamidine). Transfected COS-7 cells were washed with PBS and lysed in lysis buffer at 4 °C for 30 min, followed by centrifugation at 13,800 × g to remove cell debris (13). Protein concentrations of the cell lysates were determined with the bicinchoninic acid protein assay kit (Pierce). Cell lysates (30 μg of protein) were subjected to electrophoresis on SDS–12% polyacrylamide gradient gels (Bio-Rad) under reducing conditions, followed by electroblotting onto nitrocellulose membranes. The HIV-1 Gag proteins were visualized by SuperSignal WestPico (Pierce) using anti-p24Gag antisera (Advanced Biotechnologies, Inc., Columbia, MD) or anti-p6_{core} antisera (a kind gift from Louis E. Henderson). When needed, anti-cyclophilin A (CypA) antisera was also used (Bioulom Research Laboratories, Plymouth, PA).

**Northern Blot Analysis**—To determine whether HIV-1 genomic RNA was properly packaged in virions, Northern blot analysis was performed as described previously (14). The above-mentioned pelleted virus preparations were lysed in Tris buffer (pH 7.4) containing 10 mM EDTA, 1% (v/v) SDS, 100 mM NaCl, 50 μg/ml trypsin, 100 μg/ml proteinase K for 4 h at 37 °C. Lysates were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then ethanol-precipitated overnight at −20 °C. The amount of RNA used for each lane was adjusted based on the p24 amount in the lysates for normalization. RNA precipitates were collected by centrifugation and fractionated on a denaturing formaldehyde/agarose gel. The fractionated RNAs were transferred to nitrocellulose membranes, and the membrane was hybridized with ^32P-labeled 5088bp AseI fragments of pHIV-1NL4-3 prepared using the DECAPrime Kit (Ambion, Austin, TX).
Gag Mutations Outside Cleavage Sites of HIV-1

Amino Acid Mutations Identified in Gag in PI-resistant HIV-1 Variants—In an attempt to determine whether unidentified amino acid substitutions were involved in the development of HIV-1 resistance to various PIs, we generated HIV-1 variants highly resistant to APV and examined whether the resultant HIV-1 had amino acid substitutions in the Gag protein. The wild type HIV-1NL4-3 was propagated in MT-2 cells in the presence of increasing concentrations of APV. By passage 10 in culture where HIV-1 was propagating in the presence of 0.14 μM APV (HIV-1P10/APV), four amino acid substitutions (L10F, V32I, M46I, and I84V) in the protease and three mutations (V35I, L75R, and H219Q) in the Gag protein emerged (Table I). Interestingly, 5 of 13 clones examined for HIV-1P10/APV had L75R plus H219Q in Gag but no substitutions in its protease, showing that these two Gag substitutions emerged earlier than the substitutions in protease did in these five clones. By passage 20 (with 2.5 μM APV), the overall frequency of the 4 mutations in protease increased, and so did that of the two Gag mutations except V35I. In addition, two amino acid substitutions (R409K and E468K), which have not been previously reported, emerged in Gag together with the p1-p6 cleavage site mutation (L449D) reported by Doyon et al. (4). At passage 31 (with 10 μM APV), two additional mutations (I54M and A71V) in protease and two mutations (E12K and V390D) in Gag were seen in HIV-1 clones (Table I).

The five mutations in Gag (L75R, H219Q, R409K, L449F, and A71V) that were detected in the resultant HIV-1 had amino acid substitutions in the Gag protein except V35I. These substitutions played a significant role in the development of HIV-1 resistance against APV. We next asked whether the mutations seen in APV-resistant HIV-1 (HIV-1R) described above also emerged in HIV-1 propagated in the presence of other PIs, JE-2147 (2), KNI-272 (8), and UIC-94003 (7, 10). HIV-1 variants resistant to each of the three PIs were generated under conditions similar to those when HIV-1R was generated. In these HIV-1 variants, different protease mutation profiles were seen compared with that of HIV-1R (Table I) (2, 7, 17). In HIV-1 propagated in the presence of JE-2147, one mutation (M46I) in protease and two mutations (H219Q and V390D) in Gag were detected at passage 6 (0.04 μM JE-2147). By passage 33 (2.2 μM JE-2147), the frequencies of these mutations increased and three additional mutations (V32I, I47V, and V82I) in protease and two additional mutations (R409K and L449F) in Gag emerged (Table I).

In HIV-1 propagated in the presence of KNI-272 or UIC-94003, two mutations (H219Q and R409K) in Gag, which were seen in both HIV-1R and HIV-1R (JE-2147-resistant HIV-1), were identified at high frequencies (60–100%). These data suggest that although Gag mutation profiles may vary depending upon PI in use, certain Gag mutations such as H219Q and R409K develop in common and may facilitate the development of PI resistance.
The primer pair used for amplification of the gag gene was also shown as a pair of arrows at the top. The forward primer (LF3) contains a BsuBI site and the reverse primer (XM1) an SmaI site.

Resistance. The locations where the amino acid substitutions occurred are illustrated in Fig. 1.

L75R and H219Q Mutations in Gag Confer Replication Advantages on HIV-1—To analyze the effects of the above described three Gag mutations (L75R, H219Q, and V390D) in HIV-1, we generated five infectious HIV-1 clones, which had all or a subset of the three mutations in addition to six protease mutations (L10F, V32I, M46I, I54M, A71V, and I84V) plus three other Gag mutations (R409K, L449F, and E468K) (see Table I and Fig. 1). As shown in Fig. 2A, when propagated in the absence of PIs, among the five, two clonal HIV-1s containing both L75R and H219Q substitutions (HIV-1NL4-3; HIV-1NL75/H219Q/V390) replicated even at a greater level compared with the wild type HIV-1NL4-3, whereas two clonal HIV-1s containing L75R or H219Q substitutions (HIV-1NL75/H219Q/V390) replicated comparably to HIV-1NL4-3. An HIV-1 clone containing both H219Q and L75R (HIV-1NL75/H219Q/V390) replicated comparably to HIV-1NL75/H219Q/V390. Another HIV-1 clone containing L75R (HIV-1NL75R/H219Q/V390) had moderately greater replicative fitness compared with HIV-1NL4-3. In the presence of APV, however, all these HIV-1NL clones failed to propagate (Fig. 2E). These data indicate that both L75R and H219Q mutations confer replication advantages on HIV-1NL4-3 in the absence of not in the presence of PIs.

Role of L75R and H219Q Mutations in HIV-1 Replication in PBMCs—To evaluate the possible biological relevance of the replication kinetics data shown above using immortalized and long term cultured MT-2 cells, we conducted similar experiments using PHA-PBMCs freshly prepared from two healthy donors. As shown in Fig. 2F, HIV-1NL75R/H219Q/V390, which replicated only poorly in MT-2 cells (Fig. 2A), moderately replicated compared with the wild type HIV-1NL4-3 in the absence of PI. In contrast, HIV-1NL75/H219Q/V390 quickly replicated compared with HIV-1NL75R/H219Q/V390. These data are in agreement with the data with MT-2 cells showing that the two amino acid substitutions, L75R and H219Q, recover the compromised replicative ability of HIV-1NL75/H219Q/V390. It was noted, however, that HIV-1NL75R/H219Q/V390 did not outgrow HIV-1NL4-3, a difference from the replication profiles shown in Fig. 2A. The reason for this difference is as yet unknown, but cell-specific difference seems to be involved, and this issue is a subject of future research.

Cytopathic Effect of Various HIV-1 Clones—To define further the significance of the Gag mutations seen in this study, the MTT assay was performed using clonal HIV-1 preparations. MT-2 cells (4 x 10^6/well) were exposed to virions, and CPE of each HIV-1 clone was determined using the MTT assay (Fig. 3). Two clonal HIV-1NL4-3 containing both L75R and H219Q in Gag (HIV-1NL75R/H219Q/V390 and HIV-1NL75/H219Q/V390) exerted significant CPE in the presence and absence of APV, whereas two HIV-1 clones containing either L75R or H219Q (HIV-1NL75R/H219Q/V390 and HIV-1NL75/H219Q/V390) showed CPE comparable with that of wild type HIV-1NL4-3 (HIV-1NL4-3). The HIV-1NL4-3 clone containing none of the three Gag mutations (HIV-1NL75/H219Q/V390) failed to exert CPE in the presence and absence of drug. In contrast, the HIV-1NL4-3-containing HIV-1NL4-3 (HIV-1NL75R/H219Q/V390), HIV-1NL4-3, and HIV-1NL75/H219Q/V390 showed significant CPE in the presence and absence of APV, whereas the HIV-1NL4-3 containing HIV-1NL4-3 (HIV-1NL75R/H219Q/V390), HIV-1NL4-3, and HIV-1NL75/H219Q/V390 showed only moderate CPE in the absence of APV. Two HIV-1NL4-3 clones carrying wild type protease and containing H219Q (HIV-1NL75R/H219Q/V390 and HIV-1NL75/H219Q/V390) also showed potent significant CPE, whereas another clone containing only L75R (HIV-1NL75R/H219Q/V390) showed moderate CPE in the absence of APV. In the presence of APV, how-
ever, all HIV-1NL clones failed to infect cells, and no CPE were seen. These data corroborate the data of replication kinetics illustrated in Fig. 2, A–E.

HIV-1 Mutants That Recover Replicative Fitness with Gag Mutations Show No Recovery in Gag Processing—To define the mechanism by which L75R and H219Q Gag mutations recover replication competence of HIV-1AR and HIV-1JR in the presence of APV and JE-2147, respectively, we examined Gag cleavage patterns using the supernatants and cell lysates of COS-7 cells transfected with each plasmid employing Western blot analysis. In this assay, assuming that the transfection efficiency was comparable, the 48-h supernatants of the culture of COS-7 cells transfected with each plasmid using the same conditions were directly subjected to Western blot analysis as described previously (13). As expected, there was no significant difference in p24 amounts (Fig. 4A, lanes 1, 3, and 5–8). The pattern of HIV-1NL4–3 showed distinct cleavage as described previously (13) (Fig. 4A and B, lane 1), and 2.5 mM APV completely blocked the processing of p24 Gag (Fig. 4A, A and B, lane 2). HIV-1NL4–3 and HIV-1NL L75R/H219Q/V390, which
replicated more efficiently than HIV-1 NL4-3 (Fig. 2E), gave comparable cleavage patterns in the presence and absence of 2.5 μM APV (Fig. 4, A and B, lanes 3 and 4). The replication-incompetent HIV-1 R175/H219/V390 gave a pattern with more immature p41 Gag protein (Fig. 4, A and B, lane 5), differing from the pattern of HIV-1 NL4-3. Unexpectedly, HIV-1 R175/H219/V390, which recovered its replicative fitness with L75R and H219Q substitutions (Fig. 2A, lane 6), even in the presence of APV (2.5 and 10 μM), there was no significant difference in cleavage pattern between HIV-1 R175/H219/V390 and HIV-1 R175/H219/V390, although the amounts of p24 Gag and p41 immature proteins were decreased, and the amount of Pr55Gag polyprotein was increased in both viruses (Fig. 4C, lanes 3–4). HIV-1 R175/H219/V390, which replicated more efficiently both in the presence and absence of JE-2147 than HIV-1 R175/H219/V390, also produced profiles comparable with those of HIV-1 R175/H219/V390 (Fig. 4, A and B, lanes 7 and 8, and C, lanes 5–8).

Northern Blot Analysis of HIV-1 Viral Genome Packaging—Because two Gag mutations L75R and H219Q did not significantly alter the Gag cleavage profiles as shown above (Fig. 4), we next asked whether viral RNA genomes were properly recognized and packaged into virion particles produced in transiently transfected COS-7 cells.

The wild type HIV-1 NL4-3 proved to contain a substantial amount of genomic RNA and showed a strong signal at 9.2 kb, which corresponds to the size of full genomic RNA (HIV-1 NL4-3 undiluted in Fig. 5). When the sample of HIV-1 NL4-3 was serially diluted (5-, 25-, and 125-fold), the signal decreased in a dose-response manner. Under the same conditions, virtually the same level of signal was seen at 9.2 kb among four viral preparations examined (HIV-1 R175/H219/V390, HIV-1 R175/H219/Q/V390, HIV-1 R175/H219/V390, and HIV-1 R175/H219/Q/V390) regardless of the presence of L75R and/or H219Q mutations (Fig. 5). These data strongly suggest that neither mutations in protease nor those in Gag affected the recognition and packaging of HIV-1 RNA genome into virions.

Fig. 3. Cytotoxic effects of 11 HIV-1 clones. MT-2 cells were exposed to each clone and cultured for 10 days in the absence of PI (open bars) and presence of 2.5 μM APV (closed bars) or 0.5 μM JE-2147 (hatched bars). Cell survival was assessed using the MTT assay. The values shown are means (±1 S.D.) of three independent experiments.

Fig. 4. Western blot analysis of Gag protein cleavage profiles. Western blot analysis was performed using anti-p24Gag antiserum with virus pellets (A) and lysates of COS-7 cells transfected (B and C). A and B, COS-7 cells were transfected with pHIV-1 R175/H219/V390 (lanes 1 and 2), pHIV-1 R175/H219/Q/V390 (lanes 3 and 4), pHIV-1 R175/H219/V390 (lanes 5), pHIV-1 R175/H219/Q/V390 (lanes 6), pHIV-1 R175/H219/Q/V390 (lanes 7), or pHIV-1 R175/H219/Q/V390 (lanes 8). After 48 h of culture in the presence (lanes 2 and 4) or absence (other lanes) of 2.5 μM APV, the virions in the culture supernatant (A) and the lysates of COS-7 cells (B) were harvested. Note that no cleavage of Pr55Gag polyprotein was detected, and none or the least amount of p24 was seen in lanes 2 and 4, whereas p24 and p41 (p17 + p24) were seen in lanes 1 and 3 and 5–8. C, COS-7 cells were transfected with pHIV-1 R175/H219/V390 (lanes 1 and 2), pHIV-1 R175/H219/Q/V390 (lanes 3 and 4), pHIV-1 R175/H219/Q/V390 (lanes 5 and 6), or pHIV-1 R175/H219/Q/V390 (lanes 7 and 8). After 48 h of culture in the presence of 2.5 (lanes 1 and 3) or 10 μM (lanes 2 and 4) APV, 0.5 (lanes 5 and 7) or 2.5 μM (lanes 6 and 8) JE-2147, COS-7 cells were harvested and lysed, and the cell lysates were subjected to Western blot analysis.

No Difference in RNA Copy Numbers or p24 Contents in Virions among HIV-1 Clones—To corroborate the data of Northern blot analysis (Fig. 5), we propagated five infectious clones in H9 cells (HIV-1 R175/H219/V390, HIV-1 R175/H219/Q/V390, HIV-1 R175/H219/V390, HIV-1 R175/H219/Q/V390, and HIV-1 NL4-3), determined viral particle numbers using electron microscopy (negative staining), counted viral RNA copies using RT-PCR, and measured p24 amounts in each culture (Table III). The culture supernatant of HIV-1 R175/H219/V390 contained the greatest virion number, followed by that of HIV-1 R175/H219/Q/V390, in agreement with the replication kinetics data shown in Fig. 2. The remaining three cultures contained less but varying virion numbers. It should be noted that the variability in virion numbers determined with electron microscopy is up to 3-fold.

K. Nagashima, unpublished data.
significant morphological differences were noted among the five virus preparations examined under electron microscopy. The culture supernatant of HIV-1_RL75R/H219Q/V390 contained the greatest p24 amount, but there was no significant difference by 2–3-fold in RNA copies and p24 amounts as determined in supernatants of cultures prepared in multiple replicates (Fig. 2).3 When RNA copy number per virion was calculated, a difference by up to 8.3-fold (2.11 for HIV-1_RL75R/H219Q/V390 versus 17.5 for HIV-1_RL75R/H219Q/V390) was identified among the cultures, but considering the estimated variability in the determination methods for both virion numbers and RNA copies, it appears that there was no significant difference in RNA copy numbers per virion. We found that the same was true for p24 amounts per virion and RNA copy numbers per p24. Thus, we concluded that there was no difference in the incorporation of RNA genomes in virions in association with the mutations in the gag-pol gene we examined in this study.

Decreased Cyclophilin A Incorporation into HIV-1 with Gag Mutations—Although L75R and H219Q mutations exerted positive effects on the replication of HIV-1NL4-3 and its PI-resistant variants, no significant changes in Gag cleavage or genomic RNA packaging profiles were detected. Considering that His219 is located in the cyclophilin A (CypA) binding loop (Fig. 1) and that the incorporation of CypA into HIV-1 particles is thought to be indispensable for efficient viral replication (20, 21), we asked whether the Gag mutations altered the incorporation of CypA into HIV-1 particles. HIV-1_RL75R/H219Q/V390, which has mutant protease and wild type Gag protein, and HIV-1_RL75R/H219Q/V390, which has mutant protease plus L75R and H219Q mutations, were lysed, diluted, and subjected to Western blot analysis. With comparable amounts of Pr55Gag, p41, and p24 detected (see lanes 3 and 6 in Fig. 6), a substantially less amount of CypA was found incorporated in HIV-1_RL75R/H219Q/V390 than in HIV-1_RL75R/H219Q/V390. These results suggest that the H219Q mutation might change the conformation of CypA binding loop and decrease CypA incorporation into virion particles.

Effects of Three Gag Mutations (R409K, L449F, E468K) on the Replication Kinetics of HIV-1_RL75R/H219Q/V390—We also examined the effect of R409K, L449F, and E468K on HIV-1NL4-3 in MT-2 cells. To this end, we generated various HIV-1_RL75R/H219Q/V390 clones containing all or a subset of three mutations in addition to three Gag mutations (L75R, H219Q, and V390D) and six protease mutations (L10F, V32I, M46I, I54M, A71V, and I84V) (see Table I and Fig. 1). When propagated in the absence of APV, a clone lacking the cleavage site amino acid substitution L449F (HIV-1_RL409K/L449F/E468K) failed to propagate, whereas the clonal HIV-1 with L449F (HIV-1_RL409K/L449F/E468K) showed a markedly improved fitness (Fig. 7A), indicating that this cleavage site mutation is essential for the fitness of HIV-1_RL75R/H219Q/V390. Two clones that lacked either R409K or E468K (HIV-1_RL409K/L449F/E468K and HIV-1_RL409K/L449F/E468K) also had a markedly improved replication fitness, although the latter appeared to be less fit in the absence of APV (Fig. 7A).

When propagated in the presence of 2.5 μM APV, HIV-1_RL409K/L449F/E468K, as expected, predominated all other clones examined (Fig. 7B). We found that the clone lacking R409K (HIV-1_RL409K/L449F/E468K), which exhibited the greatest level of replication in the absence of drug (Fig. 7A), had a moderate level of fitness in the presence of APV, whereas the replication pattern of HIV-1_RL409K/L449F/E468K was apparently comparable with that of HIV-1_RL409K/L449F/E468K (Fig. 7B). It was noted that in the presence of APV, the R409K mutation provided HIV-1_RL75R/H219Q/V390 with a replication advantage compared with E468K.

It has been reported that when the cleavage site mutation L449F was introduced to HIV-1 carrying a wild type protease, its replication capability was significantly reduced (4). We therefore asked whether the replication profile of HIV-1NL4-3, containing the wild type protease was altered when R409K and E468K were introduced. In our assays, the replication of HIV-1NL4-3_L175R/H219Q/V390D exhibited a high level of replication regardless of the presence of these two mutations (Fig. 7C).

Furthermore, to evaluate the possible biological relevance of the replication kinetics data using MT-2 cells shown above, we also conducted experiments using PHA-PBMCs freshly prepared from two healthy donors. As shown in Fig. 7D, the replication profiles of four infectious clones, HIV-1_RL409K/L449F/E468K, HIV-1_RL409K/L449F/E468K, HIV-1_RL409K/L449F/E468K, and HIV-1_RL409K/L449F/E468K, were virtually the same as those shown in Fig. 7A.

Competitive HIV-1 Replication Assays for HIV-1_RL75R Clones—To define further the significance of R409K and E468K, the viral fitness was compared among the HIV-1 clones described above in the presence and absence of 2.5 μM APV using CHRA (12). In the absence of APV, HIV-1_RL409K/L449F/E468K propagated comparably to HIV-1_RL409K/L449F/E468K (Fig. 8A) but readily outgrew HIV-1_RL409K/L449F/E468K (Fig. 8B). In the presence of APV, HIV-1_RL409K/L449F/E468K predominated HIV-1_RL409K/L449F/E468K and HIV-1_RL409K/L449F/E468K, corroborating that the presence of R409K and E468K in addition to the cleavage site mutation confers on HIV-1_RL75R a significant replication advantage in the presence of APV (Fig. 8, C and D).

We also asked whether the cleavage of the Gag precursor polyprotein was impaired when the HIV-1_RL75R lacked either R409K or E468K. However, Western blot analyses using anti-p6 antisera detected no difference in cleavage patterns among these three viruses (data not shown).

To delineate how these two mutations affect the replication kinetics of wild type protease-containing viruses, we also compared the fitness of two HIV-1 clones, HIV-1NL4-3_RL409K/L449F/E468K, which was slowly outgrown by HIV-1NL4-3_RL409K/L449F/E468K (Fig. 8E), suggesting that R409K/E468K mutations compromise the fitness of HIV-1NL4-3_RL409K/L449F/E468K.
acquired in HIV-1 clones during the assays, and such mutations might have affected the replication profiles of HIV-1 clones examined. However, when we determined the nucleotide sequence of the entire gag and protease-encoding region, no additional mutations were identified in any of HIV-1 clones at the conclusion of CHRA.

DISCUSSION

The role and impact of amino acid substitutions in the gag gene of HIV-1 genome which emerge during therapy with protease inhibitors in patients with AIDS have been poorly understood. This is mainly due to the findings that most of the amino acid substitutions detected in the gag gene are not seen in common among clinical HIV-1 strains isolated from patients and even among HIV-1 clones generated from HIV-1 of a single patient. More problematic is that the functions and tertiary structures of HIV-1 Gag proteins remain largely to be determined. In the present study, we used a single HIV-1 clone obtained from newly transfected COS-7 cells, HIV-1NL4-3, as a starting HIV-1 strain and propagated it in the presence of increasing concentrations of four different PIs, APV, JE-2147, KNI-272, and UIC-94003 over 27–62 passages; and we studied 14 mutations identified in Gag (Table I), among which 9 mutations were seen in more than two PI-exposed HIV-1 preparations. We focused on six major Gag amino acid substitutions, L75R, H219Q, V390D/V390A, R409K, L449F, and E468K in the present study (Table I).

When HIV-1 was passaged under APV pressure, L75R and H219Q mutations in the Gag protein emerged prior to the emergence of protease mutations, and we found that such Gag mutations were indispensable for the efficient replication of APV resistant HIV-1 (HIV-1AR). We thought that Gag polyprotein containing L75R or H219Q would be more sensitive to the cleavage by mutant protease because such a protease has impaired enzymatic activity due to the accumulation of amino acid substitutions within itself. Indeed, HIV-1AR whose mutant gag gene was substituted with a wild type gag gene (but with the mutant protease being intact) (HIV-1ARL75/H219/V390) failed to replicate (Fig. 2A, C, and D) or presence of 2.5 μM APV (B). Each HIV-1 clone contained three mutations, L75R, H219Q, and V390D in Gag. The results shown are representative of three independent experiments (A–C). Data shown in D represent geometric means (±1 S.D.) of two independent experiments.

There are several lines of evidence suggesting that trimerized matrix proteins (MAs) serve as a fundamental building block for the formation of the MA shell within the mature HIV-1 (19). The x-ray structural analysis of HIV-1 MA suggests that residues Pro-66 and Gly-71, which are highly conserved,

FIG. 6. Western blot analysis of virion-incorporated CypA. Two HIV-1 clones were lysed, diluted (HIV-1RL75R/H219Q/V390: neat, 75, 50, and 25% in lanes 1–4, respectively, and HIV-1RL75R/H219Q/V390: neat, 80, 60, and 40% in lanes 5–8, respectively), and subjected to Western blot analysis using anti-p24 Gag and anti-CypA antisera. Note that with comparable amounts of Pr55Gag, p41, and p24 detected (see lanes 3 and 4), substantially less CypA was found in HIV-1ARL75R/H219Q/V390 than in HIV-1ARL75/H219/V390.

FIG. 7. Replication kinetics of HIV-1AR clones. MT-2 cells (A–C) or PBMCs (D) were exposed to various HIV-1AR clones and cultured in the absence (A, C, and D) or presence of 2.5 μM APV (B). Each HIV-1 clone contained three mutations, L75R, H219Q, and V390D in Gag. The results shown are representative of three independent experiments (A–C). Data shown in D represent geometric means (±1 S.D.) of two independent experiments.

### Table III

| Virion numbers, RNA copies, and p24 amounts in culture supernatants of various HIV-1 clones |
|---------------------------------------------|
| HIV-1RL75R/H219Q/V390 | HIV-1RL75R/H219Q/V390 | HIV-1RL75R/H219Q/V390 | HIV-1RL75R/H219Q/V390 |
| Numbers of virions in supernatants of chronically HIV-1-infected H9 cell culture were determined with negative stain electron microscopy. | RNA copies (×10⁹/ml)² | 50.3 | 150 | 11.8 | 62.0 | 31.4 |
| RNA copies per 10³ virus particles | 0.198 | 0.317 | 0.207 | 0.294 | 0.261 |
| p24 (ng/ml) | 88.03 | 113.51 | 34.03 | 69.53 | 96.31 |
| p24 (pg) per 10³ virus particles | 3.94 | 2.11 | 17.5 | 4.74 | 8.31 |
| RNA copies/p24 (pg p24) | 1.75 | 0.757 | 2.88 | 1.12 | 3.07 |

* Numbers of virions in supernatants of chronically HIV-1-infected H9 cell culture were determined with negative stain electron microscopy.
* RNA copy numbers were determined using RT-PCR.
function as "hinges" that allow a structural reorientation of MA at the trimer interface and that Ser-72 and Leu-75 have a tight interaction, and when the distance between these amino acids changes, the conformational interconversion of Gag occurs (22). Yu et al. (23) constructed a mutant HIV-1 with a wild type protease lacking 10 Gag amino acids (codons 68–77), which showed an impaired viral production, suggesting that that stretch of amino acids is essential for the replicative fitness of HIV-1. In the present study, the L75R substitution in Gag conferred replication advantages on HIV-1, particularly when combined with H219Q substitution. Taken together, one can speculate that the L75R substitution may alter the trimerization of MA and/or the interaction of MA with a lipid membrane of HIV-1 resulting in its "membrane anchoring" through altered electrostatic interactions. Such changes, with HIV-1_R, may ultimately confer a replication advantage on the virus.

The H219Q substitution in p24 was identified in all clones derived from four PI-resistant HIV-1 variants. In this regard, the amino acids 217–225 reportedly made a single exposed loop in the capsid protein that binds to the enzymatic active site of cyclophilin A (24) whose incorporation into HIV-1 particles seems to be indispensable for the infectivity of HIV-1. In fact, H219Q substitution is often seen particularly in clades F and G isolates (27). In the present study, we found that His-219 in HIV-1NL4-3 obtained diminished CypA binding. It should be noted that H219Q represents a polymorphic amino acid substitution and is strongly suggested that the H219Q substitution brings about a conformational change in the loop formed by the stretch of the amino acids positioned 217–225 (corresponding to CypA binding loop).

Based on these data, we postulated that the exposed loop of p24 might exert a negative effect(s) on the replication of HIV-1 either at the stages of assembly or disassembly and that the binding of CypA to the loop might induce conformational changes so that the negative effect(s) are canceled, thus restoring replicative fitness. Therefore, we compared the amounts of viral particle-associated CypA in H219Q-carrying and H219Q-lacking HIV-1 (HIV-1ARL75/H219Q/V390 and HIV-1ARL75/H219/Q/V390, respectively) using the Western blot assay, and we found that HIV-1ARL75/H219Q/V390 contained less CypA (Fig. 6) but replicated faster than HIV-1ARL75/H219Q/V390 (Fig. 2, A and F). These data suggest that H219Q substitution changed the loop conformation so that the rate of replication became greater and presumably relatively independent of the binding of CypA. Indeed, in our preliminary structural characterization of HIV-1 matrix-capsid antigens, the N terminus of capsid antigen appears to take multiple conformations. It is possible that the cleavage of matrix and capsid antigens shifts the equilibrium toward a certain conformation (28), and CypA binding is required for the stabilization of that conformation. In this respect, the H219Q substitution per se might stabilize that conformation, and the requirement of CypA binding is reduced. It is worth noting that Western blot analysis has a limited utility in quantification and that enzyme-linked immunosorbent assay or its related method should enable us to quantitate more precisely the levels of virion-associated cyclophilin A than Western blot analysis. However, to date, no cyclophilin-specific monoclonal antibody has been generated or reported, and the establishment of a more quantitative method for cyclophilin A is awaited for further detailed investigation into the role of cyclophilin A in the replication of HIV-1.

Following the emergence of protease gene mutations in HIV-1NL4-3, it was outgrown by HIV-1ARL75/H219Q/V390 (Fig. 2, A and F). These data suggest that H219Q substitution changed the loop conformation so that the rate of replication became greater and presumably relatively independent of the binding of CypA. Indeed, in our preliminary structural characterization of HIV-1 matrix-capsid antigens, the N terminus of capsid antigen appears to take multiple conformations. It is possible that the cleavage of matrix and capsid antigens shifts the equilibrium toward a certain conformation (28), and CypA binding is required for the stabilization of that conformation. In this respect, the H219Q substitution per se might stabilize that conformation, and the requirement of CypA binding is reduced. It is worth noting that Western blot analysis has a limited utility in quantification and that enzyme-linked immunosorbent assay or its related method should enable us to quantitate more precisely the levels of virion-associated cyclophilin A than Western blot analysis. However, to date, no cyclophilin-specific monoclonal antibody has been generated or reported, and the establishment of a more quantitative method for cyclophilin A is awaited for further detailed investigation into the role of cyclophilin A in the replication of HIV-1.

Following the emergence of protease gene mutations in HIV-1NL4-3, it was outgrown by HIV-1ARL75/H219Q/V390 (Fig. 2, A and F). These data suggest that H219Q substitution changed the loop conformation so that the rate of replication became greater and presumably relatively independent of the binding of CypA. Indeed, in our preliminary structural characterization of HIV-1 matrix-capsid antigens, the N terminus of capsid antigen appears to take multiple conformations. It is possible that the cleavage of matrix and capsid antigens shifts the equilibrium toward a certain conformation (28), and CypA binding is required for the stabilization of that conformation. In this respect, the H219Q substitution per se might stabilize that conformation, and the requirement of CypA binding is reduced. It is worth noting that Western blot analysis has a limited utility in quantification and that enzyme-linked immunosorbent assay or its related method should enable us to quantitate more precisely the levels of virion-associated cyclophilin A than Western blot analysis. However, to date, no cyclophilin-specific monoclonal antibody has been generated or reported, and the establishment of a more quantitative method for cyclophilin A is awaited for further detailed investigation into the role of cyclophilin A in the replication of HIV-1.

The H219Q substitution in p24 was identified in all clones derived from four PI-resistant HIV-1 variants. In this regard, the amino acids 217–225 reportedly made a single exposed loop in the capsid protein that binds to the enzymatic active site of cyclophilin A (24) whose incorporation into HIV-1 particles seems to be indispensable for the infectivity of HIV-1. In fact, H219Q substitution is often seen particularly in clades F and G isolates (27). In the present study, we found that His-219 in HIV-1NL4-3 obtained diminished CypA binding. It should be noted that H219Q represents a polymorphic amino acid substitution and is strongly suggested that the H219Q substitution brings about a conformational change in the loop formed by the stretch of the amino acids positioned 217–225 (corresponding to CypA binding loop).

Based on these data, we postulated that the exposed loop of p24 might exert a negative effect(s) on the replication of HIV-1 either at the stages of assembly or disassembly and that the binding of CypA to the loop might induce conformational changes so that the negative effect(s) are canceled, thus restoring replicative fitness. Therefore, we compared the amounts of viral particle-associated CypA in H219Q-carrying and H219Q-lacking HIV-1 (HIV-1ARL75/H219Q/V390 and HIV-1ARL75/H219Q/V390, respectively) using the Western blot assay, and we found that HIV-1ARL75/H219Q/V390 contained less CypA (Fig. 6) but replicated faster than HIV-1ARL75/H219Q/V390 (Fig. 2, A and F). These data suggest that H219Q substitution changed the loop conformation so that the rate of replication became greater and presumably relatively independent of the binding of CypA. Indeed, in our preliminary structural characterization of HIV-1 matrix-capsid antigens, the N terminus of capsid antigen appears to take multiple conformations. It is possible that the cleavage of matrix and capsid antigens shifts the equilibrium toward a certain conformation (28), and CypA binding is required for the stabilization of that conformation. In this respect, the H219Q substitution per se might stabilize that conformation, and the requirement of CypA binding is reduced. It is worth noting that Western blot analysis has a limited utility in quantification and that enzyme-linked immunosorbent assay or its related method should enable us to quantitate more precisely the levels of virion-associated cyclophilin A than Western blot analysis. However, to date, no cyclophilin-specific monoclonal antibody has been generated or reported, and the establishment of a more quantitative method for cyclophilin A is awaited for further detailed investigation into the role of cyclophilin A in the replication of HIV-1.

Following the emergence of protease gene mutations in HIV-1NL4-3, it was outgrown by HIV-1ARL75/H219Q/V390 (Fig. 2, A and F). These data suggest that H219Q substitution changed the loop conformation so that the rate of replication became greater and presumably relatively independent of the binding of CypA. Indeed, in our preliminary structural characterization of HIV-1 matrix-capsid antigens, the N terminus of capsid antigen appears to take multiple conformations. It is possible that the cleavage of matrix and capsid antigens shifts the equilibrium toward a certain conformation (28), and CypA binding is required for the stabilization of that conformation. In this respect, the H219Q substitution per se might stabilize that conformation, and the requirement of CypA binding is reduced. It is worth noting that Western blot analysis has a limited utility in quantification and that enzyme-linked immunosorbent assay or its related method should enable us to quantitate more precisely the levels of virion-associated cyclophilin A than Western blot analysis. However, to date, no cyclophilin-specific monoclonal antibody has been generated or reported, and the establishment of a more quantitative method for cyclophilin A is awaited for further detailed investigation into the role of cyclophilin A in the replication of HIV-1.
Gag precursor p7-p1-p6 and made the cleavage site more accessible to the mutated protease, the rate-limiting step for virus maturation (29–31). However, Western blot analysis failed to reveal the difference in Gag cleavage profiles. There is a possibility that the functions of Gag proteins are altered by L75R, H219Q, R409K, or E468K substitutions, resulting in enhanced viral replication. One of known Gag substitutions at non-cleavage sites. Such non-cleavage site Gag proteins may contribute to the HIV-1 acquisition of resistance to PIs, but it appears that HIV-1 resistance to PIs is acquired with multiple mechanisms.

Acknowledgment—We thank Louis E. Henderson for helpful discussions.

REFERENCES

1. Mitsuya, H., and Erickson, J. (1999) in Textbook of AIDS Medicine (Merigan, T. C., Bartlett, J. G., and Bolognesi, D., eds) pp. 751–780, Williams & Wilkins, Baltimore

2. Yoshimura, K., Kato, R., Yusa, K., Kavik, M. F., Maroun, N., Nguyen, A., Mimoto, T., Ueno, T., Shintani, M., Falloon, J., Maier, H., Hayashi, H., Erickson, J., and Mitsuya, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8675–8680

3. Erickson, J. W., Gulnik, S. V., and Markowitz, M. (1999) AIDS 13, 189–204

4. Doyon, L., Croteau, G., Thibeault, D., Polein, F., Pilote, L., and Lamarre, D. (1990) J. Virol. 70, 3763–3769

5. Zhang, Y. M., Imami, H., Imami, T., Lane, H. C., Falloon, J., Vasudevachari, M. B., and Salzman, N. P. (1997) J. Virol. 71, 6662–6670

6. Rose, R. E., Geng, Y. F., Greystak, J. A., Beckold, C. M., Terry, B. J., Robinson, B. S., Alam, M., Coleman, R. J., and Lin, P. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 93, 1648–1653

7. Yoshimura, K., Kato, R., Kavik, M. F., Nguyen, A., Maroun, V., Maeda, K., Hussain, K. A., Ghosh, A. K., Erickson, J., and Mitsuya, H. (2002) J. Virol. 76, 1349–1358 in press

8. Kageyama, S., Mimoto, T., Murakaya, Y., Nomizu, M., Ford, H., Jr., Shirasaka, T., Gulnik, S., Erickson, J., Takada, K., Hayashi, H., Broder, S. K., and Mitsuya, H. (1993) Antimicrob. Agents Chemother. 37, 810–817

9. Mimoto, T., Imai, J., Kisanuki, S., Enomoto, H., Hattori, N., Akaji, K., and Kiso, Y. (1992) J. Virol. 66, 2232–2239

10. Koo, Y., Cho, H., Walters, D. E., Krishnan, K., Hussain, K. A., Koo, Y., Cho, H., Rudall, C., Holland, L., and Bathud, J. (1998) Bioorg. Med. Chem. Lett. 8, 687–690

11. Kimpton, J., and Emerman, M. (1999) J. Virol. 70, 2223–2239

12. Kosalaraksak, P., Kavik, M. F., Maroun, V., Le, R., and Mitsuya, H. (1999) J. Virol. 73, 5356–5363

13. Sei, S., Yang, Q. E., O’Neill, D., Yoshimura, K., Nagashima, K., and Mitsuya, H. (2000) J. Virol. 74, 4621–4633

14. Gorelick, R. J., Gagliardi, T. D., Bosche, W. J., Wiltrout, T. A., Coren, L. V., Chabot, D. J., Lifson, J. D., Henderson, L. E., and Arthur, L. G. (1999) Virology 256, 92–104

15. Palmer, E. L., and Martin, M. L. (1988) Electron Microscopy in Viral Diagnos.

16. Hayat, M. A. (1989) Principles and Techniques of Electron Microscopy. Biological Application, 3rd Ed., pp. 352–376, CRC Press, Inc., Boca Raton, FL

17. Gulnik, S. V., Suvorov, L. I., Liu, B., Yu, B., Anderson, B., Mitsuya, H., and Erickson, J. W. (1990) J. Virol. 64, 77–80

18. Barrie, K. A., Perez, E. E., Lamers, S. L., Farmeire, W. G., Dunn, B. M., Sleasman, J. W., and Goodenow, M. M. (1996) Virology 219, 407–416

19. Turner, B. G., and Summers, M. F. (1999) J. Mol. Biol. 285, 1–32

20. Braaten, D., Abercrom, C., Franke, E. K., Yin, L., Phares, W., and Luban, J. (1999) J. Virol. 70, 5170–5176

21. Massiah, M. A., Worthylake, D., Christensen, A. M., Sundquist, W. I., Hill, C. P., and Summers, M. F. (1996) Protein Sci. 5, 2391–2398

22. Yu, X., Yuan, M., Matsuda, Z., Lee, T. H., and Essex, M. (1992) J. Virol. 66, 4690–4691

23. Gamble, T. R., Vajdos, F. F., You, S., Worthylake, D. K., Housewearth, M., Sundquist, W. I., and Hill, C. P. (1996) Cell 87, 1285–1294

24. Bukovsky, A. A., Weimann, A., Acela, M. A., and Gottlinger, H. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1043–1048

25. You, S., Myszka, D. G., Yeh, C., McMurray, M., Hill, C. P., and Sundquist, W. I. (1997) J. Mol. Biol. 269, 780–795

26. Kuiken, C., Foley, B., Hahn, B. H., McCutchan, F., Mellors, J., Mallins, J., Wolinsky, S., and Korber, B. (1999) in Human Retroviruses and AIDS (Bradac, J., ed) pp. 201–289, Les Alamos National Laboratory, Los Alamos, NM

27. Dietrich, L., Ehrlich, L. S., LaGrassa, T. J., Ebbets-Reed, D., and Carter, C. (2001) J. Virol. 75, 4721–4733

28. Darke, P. L., Nutt, R. F., Brady, S. F., Garsky, V. M., Ciccarone, T. M., Leu, C. T., Lumma, P. K., Freidinger, R. M., Veber, D. F., and Sigal, I. S. (1998) Biochem. Biophys. Res. Commun. 156, 297–303

29. Tozser, J., Blaha, I., Copeland, T. D., Wondrak, E. M., and Oroszlan, S. (1991) FEBS Lett. 281, 77–80

30. Wondrak, E. M., Louis, J. M., de Rocquigny, H., Chermann, J. C., and Roques, B. P. (1993) FEBS Lett. 333, 21–24
Amino Acid Substitutions in Gag Protein at Non-cleavage Sites Are Indispensable for the Development of a High Multitude of HIV-1 Resistance against Protease Inhibitors

Hiroyuki Gatanaga, Yasuhiro Suzuki, Hsinyi Tsang, Kazuhisa Yoshimura, Mark F. Kavlick, Kunio Nagashima, Robert J. Gorelick, Sek Mardy, Chun Tang, Michael F. Summers and Hiroaki Mitsuya

J. Biol. Chem. 2002, 277:5952-5961.
doi: 10.1074/jbc.M108005200 originally published online December 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108005200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 14 of which can be accessed free at http://www.jbc.org/content/277/8/5952.full.html#ref-list-1