Selected amino acid substitutions in the C-terminal region of human immunodeficiency virus type 1 capsid protein affect virus assembly and release

Samir Abdurahman,1 Stefan Höglund,2 Laura Goobar-Larsson3 and Anders Vahlne1

1,3Divisions of Clinical Virology1 and Clinical Chemistry3, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden
2Department of Biochemistry, Biomedical Center, Uppsala University, Uppsala, Sweden

Correspondence
Anders Vahlne
Anders.Vahlne@labmed.ki.se

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The capsid protein (CA or p24) of human immunodeficiency virus type 1 (HIV-1) plays a major role both early and late in the virus replication cycle. Many studies have suggested that the C-terminal domain of this protein is involved in dimerization and proper assembly of the viral core. Point mutations were introduced in two conserved sites of this region and their effects on viral protein expression, particle assembly and infectivity were studied. Eight different mutants (L205A + P207A, L205A, P207A, 223GPG225AAA, G223A, P224A, G225A and V221G) of the infectious clone pNL4-3 were constructed. Most substitutions had no substantial effect on HIV-1 protein synthesis, yet they impaired viral infectivity and particle production. The two mutants P207A and V221G also had a profound effect on Gag–Pol protein processing in HeLa–tat cells. However, these results were cell line-specific and Gag–Pol processing of P207A was not affected in 293T cells. In HeLa–tat cells, no virus particles were detected with the P207A mutation, whereas the other mutant virus particles were heterogeneous in size and morphology. None of the mutants showed normal, mature, conical core structures in HeLa–tat cells. These results indicate that the two conserved sequences in the C-terminal CA domain are essential for proper morphogenesis and infectivity of HIV-1 particles.

INTRODUCTION

The inner protein shell of immature human immunodeficiency virus type 1 (HIV-1) is composed of approximately 1500 copies of the Gag precursor protein, p55gag. On maturation, p55gag undergoes specific cleavage by the HIV-1 protease, yielding the matrix (MA or p17gag), capsid (CA or p24gag), nucleocapsid (NC or p7gag) and p6gag proteins and two small spacer peptides, SP1 and SP2 (Freed, 1998). Subsequently, this process gives rise to the formation of condensed, conical cores (Gelderblom et al., 1987; Höglund et al., 1992).

The CA protein, which forms the building blocks of the viral core, is largely a hydrophobic protein that consists of 231 aa that may be separated into two functionally and structurally distinct domains. The N-terminal domain (aa 1–150) contributes to viral core formation and binds cyclophilin A, a cellular peptidyl-prolyl cis–trans isomerase (Gamble et al., 1996; Yoo et al., 1997). The C-terminal domain (aa 151–231) mediates the oligomerization of Gag and Gag–Pol precursors that is necessary for virion budding (Srinivasakumar et al., 1995; Chen et al., 1997; Huang & Martin, 1997; Borsetti et al., 1998; Chiu et al., 2002).

There is a 20 aa conserved sequence, referred to as the major homology region (MHR), in the C-terminal CA domain. It has been shown that most mutations that are N-terminal to the MHR prevent virus replication. Such mutant viruses, however, are assembled and released as efficiently as wild-type (WT) virus (Dorfman et al., 1994). By contrast, mutations or deletions in the MHR or C-terminal to this conserved region block virus assembly and release and, thereby, reduce particle yields significantly (Chen et al., 1997; Furuta et al., 1997; Chiu et al., 2002). Mutations or deletions in the region that lies C-terminal to the MHR may also affect Gag–Pol incorporation and expression of reverse transcriptase (RT) (Huang & Martin, 1997; Chiu et al., 2002).

The tertiary structures of both the N- and C-terminal domains of the p24gag molecule have been determined (Gitti et al., 1996; Gamble et al., 1997) and, more recently, the crystal structure of a complex of p24gag with a monoclonal Fab fragment has been resolved (Berthet-Colominas et al., 1999). Based on mutagenesis and X-ray crystallographic data of p24gag, some defined amino acids in the C-terminal CA region and conserved residues located in the MHR were found to be important during virus assembly (Gamble et al.,...
C-terminal to the MHR of CA, the two sequences ALPGATL and CQQGGGP were shown to be highly conserved among different HIV-1 and HIV-2 isolates, as well as the simian (SIV) and feline (FIV) immunodeficiency viruses (Talbott et al., 1989; Myers et al., 1991; von Poblotzki et al., 1993; Zhang et al., 1996). The effects of overlapping tripeptides of the p24<sup>ΔN</sup>-C-terminal domain on HIV-1 replication have been tested. The two peptides ALG and GPG, which correspond to the two conserved regions, were shown to inhibit HIV-1 replication by >90 % at a concentration of 100 μM (Su et al., 2000) and disturb proper virion core formation (Höglund et al., 2002). In this study, we investigated whether a series of HIV-1 mutations located within the two conserved sequences in the C-terminal region of CA may be important for viral protein expression, virion assembly and release and virus infectivity.

**METHODS**

**Plasmid DNA construction.** PCR was utilized to develop all CA mutants in the study by using mutagenic oligonucleotides and the overlap extension technique, as described by Ho et al. (1989). The same 5’ and 3’ outer primers, which contain the Apal and SpeI sites, respectively, were used for all mutants. The 560 bp fragment (nt 1451–2011 of pNL4-3) that was generated by the PCR was then purified and subcloned into the vector pCR2.1-TOPO (Invitrogen). The 506 bp fragment of the mutated sequences was then cleaved out and cloned directionally into the SpeI/Apal sites of the pNL4-3 vector. All plasmid DNAs were propagated either in *Escherichia coli* DH5α, TOP10 or HB101 and purified by using a Maxiprep Purification kit (Qiagen) as recommended by the manufacturer. All mutants were confirmed by sequencing (Cybergene).

The P207A mutant was also back-mutated to the WT pNL4-3 (designated BM-P207A) as follows. The Apal/SpeI fragment of the WT pNL4-3 plasmid was cut out, purified and cloned directionally into the backbone of mutant P207A, deleted of its Apal/SpeI fragment.

For *in vitro* protein expression, the gag gene of three mutants, L205A + P207A, L205A and P207A, and the WT pNL4-3 were cloned into the *in vitro* expression vector pHM6-CMV (Invitrogen), which contains a T7 promoter. The primer pair 5’-CAGGTACCCCATATGTTGAGAACCTCC and 3’-GAATTCCTCTATTGTTGAGGCTCG were used for amplification of all gag constructs by using PCR (KpnI and EcoRI sites are underlined; stop codon is shown in bold italic type). PCR products were purified by using a Qiagen gel extraction kit and subcloned into pCR2.1-TOPO (Invitrogen). The gag genes were then cleaved out by using the KpnI and EcoRI sites and cloned directionally into the KpnI/EcoRI sites of the pHM6-CMV vector. The CA sequence was also amplified as above by using the primer pair 5’-CAGGTACCCCATATGTTGAGAACCTCC and 3’-GAATTCCTCTATTGTTGAGGCTCG and cloned directionally into the KpnI/EcoRI sites of the pHM6-CMV vector. All constructs were confirmed by sequencing (Cybergene).

**Cell transfection and protein expression.** HeLa–tat, BHK21, COS7, Vero, HepG2 and 293T cells were transfected with the plasmids indicated in six-well culture plates by using the non-liposomal transfection reagent FuGENE 6 (Roche), following the manufacturer’s instructions. Cells were harvested 72 h post-transfection in 1× RIPA buffer [50 mM Tris/ HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS, supplemented with a complete protease inhibitor cocktail from Roche].

**Western blotting.** Denatured whole-cell lysates (normalized for co-transfected β-galactosidase activity), viral lysates (normalized for p24 content) or immunoprecipitates were resolved by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane and immunoblotted for enhanced chemiluminescence with the following primary antibodies: an anti-p24<sup>ΔN</sup> mouse mAb (anti-p24<sup>ΔN</sup> mAb) or rabbit polyclonal antiserum, anti-gp120/gp160 mouse mAb (kindly provided by Jorma Hinkula; Hinkula et al., 1990), HIV-1 Vif (Transgene) and RT (NIH, catalogue no. 7373) mAbs or a pool of three different HIV-1-positive human sera. Bound antibodies were then detected by using an appropriate horseradish peroxidase-conjugated secondary antibody, raised against mouse (DAKO; 1:4000), human (Pierce; 1:20000) or rabbit (Sigma; 1:4000) IgG. Band densities of total gag protein levels in the cells from immunoblots were quantified by using GeneTools analysis software (SynGene).

**RNA isolation and Northern blot analysis.** Two days post-transfection, HeLa–tat cells were harvested by using Triazol LS reagent (Gibco) and total RNA was isolated as recommended by the manufacturer. For Northern blotting, 10 μl total isolated RNA was denatured in RNA loading buffer (which contained 50 % formamide, 20 mM MOPS and 9 % formaldehyde) by incubating at 70 °C for 5 min. Samples were then subjected to electrophoresis (3 V cm<sup>−1</sup>) in a denaturing gel that was made with 1:25 % agarose, 1× MOPS buffer, 6-2 % formaldehyde and 0.1 μg ethidium bromide ml<sup>−1</sup>. The gel was transferred overnight to nylon membrane Hybond-N (Amersham Biosciences) with 20 × SSC and hybridized.

For HIV-1 mRNA hybridization, a 3.5-kbp SacI fragment from pNL4-3 was radio labelled by using an oligonucleotide labelling kit (Amersham Biosciences). The SacI fragment, which spans vpu–env–nef, hybridizes to all HIV-1 transcripts (Hadzopoulou-Cladaras et al., 1989; Schwartz et al., 1991). The Hybond-N membrane was prehybridized in 10 ml hybridization solution in a Hybrid oven at 45 °C for 3 h and followed by hybridization in fresh hybridization solution, supplemented with radiolabelled probes (2 × 10<sup>6</sup> c.p.m. ml<sup>−1</sup>) that were denatured by boiling for 5 min. Hybridization was continued for another 16 h and terminated by washing the membrane twice in 250 ml 2 × SSC at room temperature for 30 min each, and then twice in 250 ml 0.2 × SSC containing 0.2 % SDS at 55 °C for 30 min. The membrane was then sealed in a plastic bag and exposed to X-ray film at −80 °C.

**In vitro transcription and translation.** In a cell-free system that utilized rabbit reticulocyte lysate (Promega), mutants L205A + P207A, L205A, P207A and WT gag constructs were expressed in a coupled transcription/translation system. Briefly, 1 μg DNA was added to a reaction mixture of 50 μl, which contained rabbit reticulocyte lysate, T7 RNA polymerase, amino acid mixtures, reaction buffer and RNasin (Promega), and then incubated at 30 °C for 90 min. Translation products were then immunoprecipitated as described below.

**Immunoprecipitation.** In *in vitro* transcription/translation products were diluted with 0.8 ml immunoprecipitation solution (IP solution;
150 mM NaCl, 10 mM EDTA, 1% Nonidet P40 and a complete protease inhibitor cocktail obtained from Roche) and incubated with mAbs against p24<sup>gag</sup> or haemagglutinin (HA; Roche) at 4°C overnight. The following day, 50 μl Protein A/G-agarose (Santa Cruz Biotechnology) was added and the suspension was incubated further for 3 h at 4°C. The immunocomplex was collected by centrifugation at 4000 r.p.m. for 5 min at 4°C, washed three times in 1 ml IP solution, resuspended in 2× SDS sample buffer and boiled for 5 min before being subjected to SDS-PAGE.

**Virus purification.** Culture supernatants (normalized for p24<sup>gag</sup> content except for P207A and V221G, where no such protein was detected) were first clarified from cell debris by centrifugation at 1200 r.p.m. for 10 min, filtered through 0.45 μm filters and mixed (4:1) with Viraffinity (CPG). The mixture was incubated at room temperature for 5 min, centrifuged at 1000 g for 10 min and viral pellets were washed three times in a buffer that contained 60 mM HEPES and 150 mM NaCl (pH 6.5). Finally, the viral pellets were dissolved in 1× RIPA buffer and subjected to SDS-PAGE.

**Viral infectivity assay.** Infectivity of HIV-1 particles carrying a WT or mutated CA gene that had been released into the supernatants from transfected HeLa–tat cells was assayed. After 72 h, virus-containing supernatants were harvested, clarified by centrifugation, filtered through 0.45 μm filters and used to infect H9 cells after normalization for p24<sup>gag</sup> content. Cells were pelleted 3 h post-infection, washed three times in PBS and incubated further. Infections were performed in triplicate in 48-well plates and supernatants were collected on days 1, 5, 7, 9, 13 and 20 and tested for p24<sup>gag</sup> content by ELISA.

**ELISA.** For p24<sup>gag</sup> ELISA, rabbit anti-p24<sup>gag</sup>-coated microwell plates (MWP) (Nunc) were blocked with 2% BSA in PBS at 37°C for 30 min. Supernatants from transfected or infected cells were added to the plates, followed by incubation at 37°C for 1 h. The MWP were then washed and biotinylated anti-p24<sup>gag</sup> antibody (1:16 000) was added, before being incubated further at 37°C for 1 h. Finally, the MWP were washed and streptavidine-conjugated anti-rabbit antibody (Jackson; 1:2000) was added, before being incubated further. The plates were read in a Labsystems multispan MS spectrophotometer. For RT-ELISA, the manufacturer’s procedure was followed (Cavidii Tech).

**Immunofluorescent staining.** HeLa cells cultured on chambered slides (Nunc) were transfected with mutant and WT pNL4-3 pro-viral DNA constructs. Forty-eight hours post-transfection, cells were fixed in acetone/methanol (1:1) for 10 min, washed three times in PBS and permeabilized in PBS that contained 0.1% Triton X-100 for 5 min. Slides were then incubated with primary antibody that was diluted in blocking solution for 15 min, washed three times in PBS and further incubated with secondary antibody for 30 min. All incubations were carried out at room temperature. Anti-p24<sup>gag</sup>mAb was used as the primary antibody and cells were also labelled for DNA with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Cy2-conjugated anti-mouse IgG antibody (Jackson) was used as secondary antibody. After the final wash, slides were mounted and fluorescent images were acquired by using a Nikon Eclipse E600 phase-contrast fluorescent microscope and analysed by using Spot Advanced software (Diagnostic Instruments). Images from six to ten different sites within each well were collected.

**Transmission electron microscopy (TEM) analysis.** Transfected cells were prepared for electron microscopy essentially as described previously (Höglund et al., 2002). Evaluation of morphology was done with series of electron micrographs to depict different categories of virus morphology, specifically focusing on the packing of the virus core structure. Three-dimensional visualization of the internal HIV structure was elicited from several TEM projections, taken at evenly spaced tilt angles and followed by computer processing, essentially as described previously (Höglund et al., 2002).

**RESULTS**

**Effect of CA mutants on viral protein expression**

To investigate the expression profiles of mutant and WT pNL4-3-transfected HeLa–tat cells, proteins were immunoblotted and assayed by using pools of HIV-1-positive human sera from three different individuals. A representative experiment performed with the different mutants is shown in Fig. 1c, as well as their position in Gag (Fig. 1a). Similar results were observed when cell lysates were analysed with a rabbit anti-p24<sup>gag</sup> polyclonal antiserum (data not shown). Processed HIV-1 Gag proteins were detected in all cell lysates 3 days post-transfection, with the exception of cells that were transfected with the P207A and V221G mutants, where HIV-1 Gag proteins were partially processed (Fig. 1c). With the two latter mutants, additional protein bands with apparent molecular masses of approximately 30 (P207A) and 46 (V221G) kDa were seen (Fig. 1c). By using polyclonal antibodies, an additional band with a molecular mass slightly lower than that of p24<sup>gag</sup> was also observed in cell lines that were transfected with the L205A + P207A and L205A mutants (indicated with an arrow in Fig. 1c). However, this band was not detected by using the anti-p24<sup>gag</sup> mAb (data not shown).

Levels of the p17<sup>gag</sup> and p24<sup>gag</sup> proteins that were detected with mutants P207A and V221G in transfected HeLa–tat cell lines were severely reduced (Fig. 1c, lanes 4 and 11). A reduction in expression of p17<sup>gag</sup> was also observed with the other mutants, except for G225A (Fig. 1c, lane 10).

To investigate the relative expression of each protein, the densities of each Western blot band, expressed as the ratio of the sum of all Gag protein bands obtained with the WT, are depicted in Fig. 1d. The relative concentrations of WT Gag proteins were 30% of p55<sup>gag</sup>, 24% of p41<sup>gag</sup>, 30% of p24<sup>gag</sup> and 16% of p17<sup>gag</sup>. The intracellular percentage of p55<sup>gag</sup> in all mutants was comparable to that of the WT, whilst the L205A + P207A and L205A mutants displayed reduced levels of the protein and the V221G mutant displayed no p55<sup>gag</sup>. The relative concentration of p41<sup>gag</sup> was also reduced for L205A + P207A and L205A. In contrast, V221G p41<sup>gag</sup> levels were almost twice that of the WT; additionally, a protein slightly larger than p41<sup>gag</sup> was observed (Fig. 1c, lane 11). A faint band was detected for p24<sup>gag</sup> content in the P207A and V221G mutants. However, the intracellular levels of p24<sup>gag</sup> for the other mutants were comparable to that of the WT. A reduction in the p17<sup>gag</sup> level was observed in mutants P207A and V221G, whereas the 223GPG225AAA mutation demonstrated a 50% reduction in p17<sup>gag</sup> content. A V3 loop-specific mAb could detect HIV glycoproteins in all mutants with the exception of V221G, where gp160 was not detected (Fig. 1b, lane 11).
Fig. 1. Positions of mutated amino acids and Western blot analysis of transfected HeLa–tat cell lysates and precipitated viruses. (a) Schematic drawing of the Gag precursor protein and its cleavage products. Initial cleavage of the Gag precursor takes place at the junction of SP1 and NC (thick arrowhead), yielding the N-terminal p41\(^{\text{gag}}\) and the C-terminal p15\(^{\text{gag}}\). The sequence of the two conserved regions in the C-terminal CA domain containing all the mutated amino acids (underlined) are also shown. (b, c) Western blot analysis of HeLa–tat cells transfected with mock control (lane 1), L205A\(^{+}\)P207A (lane 2), L205A (lane 3), P207A (lane 4), WT (lane 5), BM-P207A (lane 6), 223GPG225AAA (lane 7), G223A (lane 8), P224A (lane 9), G225A (lane 10) and V221G (lane 11), detected with a mouse anti-V3 mAb (b) and a pool of HIV-1-positive human sera (c). This experiment was repeated more than ten times using both mAbs and polyclonal antibodies, which gave similar results. (d) Band densities of Gag proteins in cells from immunoblots were quantified by using GeneTools analysis software; percentages of Gag proteins were obtained by dividing the individual value by the total Gag protein value of the WT and multiplying by 100. All values were derived from four independent experiments. (e, f) Western blot analysis of precipitated particulate material (normalized for p24\(^{\text{gag}}\)) from supernatants of HeLa–tat cells that were transfected with plasmids as in (b) and detected with the same antibodies. This experiment was repeated more than six times using both mAbs and polyclonal antibodies, which gave similar results. (g) Western blot analysis of \(^{35}\text{S}\)-labelled proteins from \textit{in vitro} transcription/translation products of mutant \textit{gag} constructs of L205A\(^{+}\)P207A (lane 1), L205A (lane 2), P207A (lane 3), WT (lane 4) and a mock control (lane 5). (h) Denatured cell lysates were run on a 7.5\% SDS-PAGE gel, transferred onto a nitrocellulose membrane and detected with primary murine antibodies against RT and Vif. The positions of specific viral proteins are indicated to the right in (b), (c), (e), (f), (g) and (h). Numbers to the left depict positions of molecular mass markers (in kDa).

<table>
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<tr>
<th>Lane</th>
<th>Constructs</th>
<th>p55(^{\text{gag}}) (%)</th>
<th>p41(^{\text{gag}}) (%)</th>
<th>p24(^{\text{gag}}) (%)</th>
<th>p17(^{\text{gag}}) (%)</th>
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<tr>
<td>5</td>
<td>WT</td>
<td>30 ± 4</td>
<td>24 ± 2</td>
<td>30 ± 3</td>
<td>16 ± 3</td>
<td>100</td>
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<tr>
<td>6</td>
<td>BM-P207A</td>
<td>28 ± 3</td>
<td>21 ± 4</td>
<td>29 ± 2</td>
<td>16 ± 3</td>
<td>95</td>
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<tr>
<td>2</td>
<td>L205A(^{+})P207A</td>
<td>21 ± 4</td>
<td>11 ± 4</td>
<td>28 ± 2</td>
<td>13 ± 4</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>L205A</td>
<td>22 ± 3</td>
<td>13 ± 5</td>
<td>29 ± 2</td>
<td>13 ± 4</td>
<td>76</td>
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<tr>
<td>4</td>
<td>P207A</td>
<td>31 ± 0</td>
<td>24 ± 4</td>
<td>5 ± 4</td>
<td>2 ± 4</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>223GPG225AAA</td>
<td>29 ± 2</td>
<td>22 ± 3</td>
<td>25 ± 2</td>
<td>8 ± 6</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>G223A</td>
<td>28 ± 3</td>
<td>22 ± 2</td>
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<td>87</td>
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<td>10</td>
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To further characterize the Gag expression and processing pattern of the two mutants P207A and V221G, five different cell lines (BHK21, COS7, Vero, HepG2 and 293T) were transfected with plasmids encoding the mutants. Fully processed, major viral proteins were detected in transfected COS7 and 293T cells (Fig. 2), yet the level of p24gag was reduced slightly in transfected COS7 cells. Transfection of HeLa–tat cells with the V221G mutant produced mainly p41gag, whereas p25gag was additionally detected in BHK21, COS7 and 293T cells. Small amounts of p55gag were also observed in COS7 and 293T cells that were transfected with this mutant. Although the levels of viral proteins that were detected in transfected Vero and HepG2 cells were reduced, the expression pattern observed was similar to that detected in HeLa–tat cells.

**Effect of CA mutations on particle release and p55\textsuperscript{gag} processing**

To evaluate the effects of HIV-1 CA mutations on Gag assembly and virus particle release, levels of p24\textsuperscript{gag} and RT from transfected HeLa–tat cells were assayed by ELISA.

Release of virus particles from P207A- and V221G-transfected cells into the culture medium was negligible, whereas a decrease by 60–70 %, compared to that of WT-transfected cells, was observed with the other mutants (Fig. 3).

Mature p24\textsuperscript{gag} represented the major product of the precipitated material from the cell-culture supernatants (normalized for p24\textsuperscript{gag} content) except for mutant V221G (in which p41\textsuperscript{gag} was the major product), as was also seen in cell lysates with this mutant (Fig. 1c and f, lane 11). In addition, no p17\textsuperscript{gag} was detected in V221G precipitated material. Almost no proteins were detected in mutant P207A precipitated material (Fig. 1f, lane 4). For the other mutants, p41\textsuperscript{gag}, p24\textsuperscript{gag} and p17\textsuperscript{gag} were present in similar amounts to the WT. Precipitates of the two CA mutants L205A + P207A and L205A also displayed the extra band just below p24\textsuperscript{gag} (indicated with an arrow in Fig. 1f, lanes 2 and 3) that was also observed in their corresponding cell lysates.

To verify that the observed effects of the P207A mutant were not due to an additional mutation, we investigated the viral protein expression profiles of a back-mutated plasmid (designated BM-P207A). By using the latter construct, all HIV-1 proteins were expressed and virus particles were released as efficiently as with the WT construct (Fig. 1c and f, lane 6). From numerous experiments, it was deduced that the differences observed between the phenotypes of P207A and WT were not due to the amount of DNA that was used for the transfections or the number of cells used in the experiment. Furthermore, none of the mutations studied affected transcription or splicing of HIV-1 RNA, as detected by Northern blot analysis of total RNA (Fig. 4).

The potential dominant-negative effect of the P207A mutant was also investigated. Co-transfection of P207A with WT pNL4-3 had no effect on HIV-1 viral protein expression (Fig. 5a) or infectivity (data not shown). Transfection in trans with WT gag or CA partially rescued viral protein expression and virus assembly (Fig. 5), but not infectivity (data not shown). Complementation of P207A with WT gag in trans resulted in the appearance of globular and condensed core structures (data not shown). In
contrast, empty cone-shaped cores were seen when WT CA was used for in trans complementation (Fig. 5b and c).

In vitro expression of gag mutants by using rabbit reticulocyte lysate

As a protein slightly smaller than p24gs was detected in Western blots with the two mutants L205A+P207A and L205A (Fig. 1c and f), the expression profiles of the corresponding gag constructs were examined in a cell-free system, in order to exclude the possibility of premature termination. The P207A mutant, which expresses almost no intracellular p24gag, was also included in this experiment. The three gag mutants, including P207A, were expressed and detected as efficiently as the WT gag construct (Fig. 1g). In this experiment, the protein slightly smaller than p24gs was no longer detected. These results were further confirmed by using proteins that were tagged with an immunogenic sequence from influenza HA and detected with an anti-HA antibody (data not shown).

Viral infectivity assay

The effect of the mutations on virus infectivity was also tested with culture supernatants (normalized for p24gs content except for P207A and V221G, where all supernatant material was used) from transfected HeLa–tat cells. Such culture supernatants were used to infect H9 cells, which were then screened for virus production up to several weeks post-infection. Infectivity of all mutants was absent or severely reduced, compared to that of the WT virus. The progeny of infectious virions from BM-P207A-transfected cells was of the same order as was obtained with the WT construct. The results are summarized in Fig. 6.

Fig. 4. Northern blot analysis of HIV-1-specific RNA. Total RNA was isolated 2 days post-transfection of HeLa–tat cells with the proviral DNA constructs indicated. (a) Detection of HIV-1-specific RNA by using [32P]dCTP-labelled oligonucleotides from the env region (a 3-5 kbp SacI fragment cleaved with BamHI into two parts). This fragment hybridizes with full genomic RNA (approx. 9 kbp), single-spliced RNA (4–5 kbp) and multiply spliced RNA (approx. 2 kbp) (Hadzopoulou-Cladaras et al., 1989; Schwartz et al., 1991). (b) Ethidium bromide staining of the gel, where integration of rRNAs is confirmed by 28S rRNA bands. NT, Non-transfected control.

Fig. 5. Analysis of in trans complementation of P207A with WT pNL4-3, gag- and CA-encoding genes. (a) Western blot analysis of HeLa cells that were transfected with both the P207A mutant and WT, gag or CA constructs and detected by using a pool of HIV-1-positive human sera. (b) A typical particle with an empty cone-shaped core, obtained after transfection with P207A in trans with CA. (c) Three-dimensional reconstruction of (b).

Fig. 6. Infectivity of mutant and WT virus particles. H9 cells ($5 \times 10^5$) were infected with equal amounts of cleared and filtered virus stocks that were collected from transfected HeLa–tat cells on day 3 (normalized for p24gs content), as described in Methods. Supernatants were then collected on the days indicated and assayed by p24gs ELISA. The experiment was repeated three times, with similar results. NT, Non-transfected control. Only WT and BM-P207A showed any replication.
Fig. 7. CA immunofluorescence of HeLa cells 48 h post-transfection with mutant or WT proviral clones, as indicated. A mouse anti-p24<sup> gag </sup>mAb and a Cy2-conjugated (green) donkey anti-mouse IgG were used as primary and secondary antibodies, respectively. DAPI (4',6-diamidino-2-phenylindole dihydrochloride; blue) was used to stain cell nuclei.

Fig. 8. TEM of WT and mutant virus particles. (a) With the WT, a rim of dense material was shown inside the envelope of immature virus (left) and a dense and cone-shaped core structure of mature virus (right). Mutants L205A + P207A and L205A showed a heterogeneous population of particles, ranging from 120 to 200 nm in size. No virion structures could be seen with mutant P207A. (b) Enumeration of particles (%) with respective morphology of WT and the mutants above. (c) Mutants G223A, 223GPG225AAA, G225A, P224A and V221G showed a limited number of heterogeneous particles with aberrant core structures. Bars, 100 nm.
**Immunofluorescence assay**

The staining pattern of viral proteins in transfected cells was examined by using mAbs against p24\(^{Gag}\). All mutants except P207A and V221G showed specific signals in the cytoplasm when stained with an anti-p24\(^{Gag}\) antibody. Representative staining patterns are shown in Fig. 7.

**Effect of HIV-1 CA mutations on virion morphology**

Morphogenesis of all mutant viruses was examined by TEM. Neither P207A-transfected HeLa–tat cells nor H9 cells that were incubated with mutant P207A culture supernatant showed any virus structures. A limited number of the other mutant virus particles were obtained, with a heterogeneous population of particles between 120 and 200 nm in size. Most strikingly, no mature virus particles with conical core structures were detected with any of the mutants. Instead, various forms of aberrant core structures were observed (Fig. 8a and c). Only the L205A + P207A and L205A mutant viruses produced a sufficient number of particles to perform a numerical analysis of particles with respective morphology (Fig. 8b).

**DISCUSSION**

In this study, we have shown that single amino acids in the two C-terminal conserved sequences ALGPGATLEE (aa 204–213) and CQGVGGPG (aa 218–225) of the HIV-1 CA protein are essential for the proteolytic processing of Gag and Gag–Pol precursors and for the proper assembly and release of infectious virus particles.

Northern blot analyses showed that steady-state viral RNA levels of HeLa–tat cells that were transfected with any of the mutants in this study were the same as those obtained with the WT, suggesting that the defects observed were not at the transcriptional level. In the *in vitro* system studied, transcription and translation of mRNAs of these mutants were also functional. Translation in this system is not cell-dependent and therefore cannot be used alone to assess *in vivo* phenotypes; however, the results indicated that there was probably no defect at this stage of the virus replication cycle.

In the first conserved sequence, substitution of leucine for alanine alone, as in L205A, or together with proline (L205A + P207A) seems to have led to increased proteolytic processing of Gag precursors, which may be evidenced by a decreased amount of p55\(^{Gag}\) and p41\(^{Gag}\). In addition to the expression of normal Gag proteins, lysates and precipitated viruses from cells that were transfected with these two mutants demonstrated a protein that migrated with an apparent molecular mass of approximately 20 kDa. This band probably does not represent a C-terminally truncated form of p24\(^{Gag}\), as it was not recognized by the anti-p24\(^{Gag}\) mAb, which maps to a linear epitope in the MHR (Hinkula *et al.*, 1990), i.e. approximately one-third from the C-terminus of p24\(^{Gag}\). Western blot analysis of HeLa–tat cells that were transfected with the two mutants exhibited also a substantial increase in RT content (p66/p55), as was demonstrated with the Pol-specific antibody.

The P207A mutant, in which the imino acid proline was substituted for the more hydrophobic amino acid alanine, showed defects in proteolytic processing of p55\(^{Gag}\) and p41\(^{Gag}\) into mature viral proteins. This could be a consequence of a block in intracellular transport of the Gag precursor to the cell membrane (Göttlinger *et al.*, 1989; Karacostas *et al.*, 1993; Yuan *et al.*, 1993), where activation of the viral protease occurs simultaneously with viral particle release (Kaplan *et al.*, 1994). The effects observed might also be the result of other defects in the intracellular processing of Gag proteins (Lee *et al.*, 1998). The P207A mutant showed no immunofluorescent staining by using the anti-p24\(^{Gag}\) mAb, indicating that this antibody only recognizes p41\(^{Gag}\) in its reduced and denatured condition, as in the Western blots. Complementing the P207A mutant with the WT gag or CA *in trans* led to partially restored viral protein expression and assembly, despite the fact that these two constructs lacked the Rev response element, which is needed for mRNA stabilization by Rev. In our hands, no proteins were expressed when transfecting HeLa cells with either the WT gag or CA constructs. Even though the expression of CA or gag alone does not result in cone formation (Schneider *et al.*, 1997; Kotsopoulos et al., 2000), the mutated pNL4-3 construct appeared to restore WT CA formation to a certain degree. This, in turn, allowed the chimeric CA/p41/p55\(^{Gag}\) interaction to occur in the present study. However, co-transfection of P207A with WT pNL4-3 displayed WT expression profiles and infectivity, suggesting no dominant-negative effect with P207A, as has been reported for other Gag mutants (Furuta *et al.*, 1997).

Surprisingly, the second amino acid substitution in the L205A + P207A double mutant restored some of the defects of P207A. We have no explanation for this, but perhaps the additional replacement of L205 with alanine somehow allowed the restoration of a tertiary conformation that is necessary for proteolytic cleavage of p41\(^{Gag}\)/p55\(^{Gag}\).

No virus structures were observed with the P207A mutant. The other two mutants, L205A + P207A and L205A, showed only particles with aberrant core structures. Infectivity of the virus particles was also severely reduced, suggesting that the first conserved sequence in the C-terminal CA is important for proper core assembly and hence infectivity of released virus particles.

In a recent study that was published after the present work was done, Forshey *et al.* (2002) have also described a P207A single amino acid substitution in an infectious clone of HIV-1. In contrast to our results, they found no effect on Gag–Pol protein expression, virus assembly or infectivity. Forshey *et al.* (2002) used the R9 WT proviral DNA construct for their mutagenesis studies and tested for virus production by transfecting 293T cells. We cannot explain...
the discrepancy in the phenotype of the P207A mutants that were obtained by these authors and the results obtained in the present study. However, at least three previous reports have shown cell line-specific defects in intracellular Gag processing and infectivity (Sakuragi et al., 1995; Lee et al., 1998; Parker & Hunter, 2000). In order to investigate this, we transfected five different cell lines and found that the P207A mutant could be expressed and processed fully in two of the cell lines, COS7 and 293T. Also, for the V221G mutant, small amounts of p55\textsuperscript{gag} were observed in BHK21, COS7 and 293T cells. It is therefore possible that some mutations may behave differently in different cell lines, due to differences in the intracellular milieu or other cellular and/or viral factor(s) that may be necessary for transport or membrane targeting. Although many cellular proteins that are involved in HIV replication have been identified, many of the cell systems that they are involved in are still poorly understood [reviewed by Ott (2002)]. Further work is under way to elucidate these cell-dependent differences in gag expression patterns with the mutants described here.

The five mutants G223A, P224A, G225A, V221G and 223GPG225AAA were located within the second conserved sequence, CQVGPGG (aa 218–225). Although the mutations in four of the mutants, G223A, P224A, G225A and 223GPG225AAA, had minimal effects on viral protein expression and immunofluorescence-staining patterns, the release of virus particles was reduced. This could suggest a defect that is linked to p55\textsuperscript{gag}, p55\textsuperscript{gag} or p55\textsuperscript{gag}–Pr160\textsuperscript{gag}–pol interactions at the cell membrane or to destabilization of the p55\textsuperscript{gag} and Pr160\textsuperscript{gag}–pol associations with the cell membrane, thereby affecting the budding process (Kaplan & Swanstrom, 1991; Karacostas et al., 1993; Luban et al., 1993; Yuan et al., 1993; Vogt, 1996). Although these four mutants were assembly-competent, they produced virus particles with aberrant core morphology. The virus particles were able to incorporate HIV-1 glycoprotein, but infectivity of the virus particles was severely reduced, suggesting that the infectivity of the mutant viruses was impaired at a post-entry step of the virus replication cycle.

The V221G mutant displayed no p55\textsuperscript{gag} or p17\textsuperscript{gag} and only low levels of p24\textsuperscript{gag}, but an increase in p41\textsuperscript{gag} and a protein slightly larger than p41\textsuperscript{gag} were detected, indicating defects in proteolytic processing of the Gag and Gag–Pol precursors, as evidenced by Western blot analysis with a Pol-specific anti-RT antibody. Unlike the WT and the other mutants, this mutant formed intracellular virus-like particles that probably consisted of assembled p41\textsuperscript{gag} protein. This may be due to early (Kaplan & Swanstrom, 1991; Karacostas et al., 1993; Yuan et al., 1993) but incomplete intracellular protease activity before targeting of Gag and Gag–Pol precursors to the cell membrane. It is also noteworthy that the extracellular precipitated, particulate material contained almost only the p41\textsuperscript{gag} intermediate.

Previous studies have shown that larger deletions or insertions (three or more amino acids) in the two conserved sequences of the C-terminal CA domain affect virus particle assembly, release and infectivity (von Poblotzki et al., 1993; Zhang et al., 1996; Kattenbeck et al., 1997). The results presented here demonstrate that single amino acids of the two conserved sequences at the C-terminus are also important for proper assembly, release and maturation of virus particles from transfected HeLa cells and may be important targets for antiviral interventions and vaccine development.

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