The central region of human immunodeficiency virus type 1 p6 protein (Gag residues S14–I31) is dispensable for the virus in vitro

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The human immunodeficiency virus type 1 p6 region encodes p6Gag and the transframe p6Pol protein. The Gag frame encodes an N-terminal late assembly L domain and a C-terminal Vpr binding domain. In the Pol frame, substitution at a C-terminal motif decreases protease autocleavage. The role of the highly polymorphic central region of p6, comprising amino acids S14–I31 (p6Gag) and R20–D39 (p6Pol), is unclear. Analysis of this central region demonstrated that 35 % of p6Gag appears to be dispensable for virus propagation in vitro and smaller deletion and insertion polymorphisms can be tolerated in vivo. Extensive Pol deletion (∆R20–D39, 42 % of p6Pol) did not alter protease autocleavage.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) p6 region encodes two proteins, p6Gag (52 amino acids) and the transframe p6Pol (48 amino acids). Two functional domains have been mapped to the Gag frame: an N-terminal PT(S)AP motif, which represents the late (L) virus assembly domain (Göttlinger et al., 1991; Yu et al., 1995, 1998) and a C-terminal LXXLF sequence, which participates in the incorporation of Vpr into particles (Lu et al., 1999; Kondo & Göttlinger, 1996). PTAP binds Tsg101, a protein involved in vacuolar cell sorting (Vogt, 2000; Garrus et al., 2001; VerPlank et al., 2001). In addition, a fairly conserved KELY motif is present at the boundary between the hydrophilic and the hydrophobic domains of p6Gag. Substitution of Y36 of KELY in association with a substitution at downstream L41 results in reduced infectivity and failure to incorporate the Env protein into virus particles (Ott et al., 1999). In the Pol frame, the transframe protein encoded by the p6pol region acts as a regulator of protease activation (Partin et al., 1991; Louis et al., 1999; Paulus et al., 1999). The C-terminal SNSF motif has been identified as critical for protease autocleavage (Partin et al., 1991). The role of the central region of p6, comprising amino acids S14–I31 of p6Gag or R20–D39 of p6Pol, is unclear. Except when specifically referring to p6Pol experiments, p6 substitutions will be described using p6Gag amino acid numbering, although Gag substitutions may be accompanied by frame shift p6Pol changes.

The identification of extensive deletion and insertion polymorphisms in the central protein region, including deletions of up to six amino acids and duplication of the conserved motif K27Q28E29 (KQE) (Peters et al., 2001), led us to analyse the role of this region in the virus life cycle. For this purpose, we constructed HIV-1 molecular clones carrying: (i) p6 deletions observed in circulating virions in HIV-1-infected individuals; (ii) experimental deletions that spanned up to 20 amino acids; and (iii) the KQE duplication.

METHODS

Analysis of p6 sequences. RNA from plasma virions from HIV-1-infected patients (n = 296) was isolated, reverse transcribed, amplified via nested PCR and sequenced as previously described (Bleiber et al., 2001).

Virus culture conditions. Peripheral blood lymphocytes (PBLs) were cultured in RPMI 1640 supplemented with Glutamax (2 mM) (Gibco-BRL Life Technologies), gentamicin (50 μg ml−1), foetal calf serum (FCS; 20 %, v/v) and IL-2 (10 U ml−1). 293 T cells were maintained in RPMI 1640, Glutamax and gentamicin, with the addition of 10 % FCS. HeLa, COS-7 and GHOST cells (stably transduced with chemokine receptor CXCR4 and with the green fluorescence protein (GFP) linked to the HIV-1 long terminal repeat; obtained through the NIH AIDS Research and Reference Reagent Program from Drs D. Littman and V. K. Ramani) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with Glutamax (2 mM), gentamicin (50 μg ml−1) and 10 % FCS. For GHOST cells, selective medium also contained puromycin (1 μg ml−1; Sigma), hygromycin (100 μg ml−1; Gibco-BRL Life Technologies) and G-418 (500 μg ml−1; Gibco-BRL Life Technologies).

Recombinant clones. Deletions of two to six amino acids observed in vivo, the duplication K27Q28E29 and a number of experimental deletions of up to 20 residues in p6 were introduced into pNLA-3 by site-directed mutagenesis (Stratagene). The small deletions ΔS14–E19, ΔG18–T21, ΔS14–R16, ΔF17–G18, ΔT21–T23, ΔP30–I31, ΔE12–E13 and ΔK27–E29 were introduced by a one-step single mutagenesis reaction. The larger deletion ΔS14–T23 used
of GFP-positive cells. Infectious titre was determined by FACS analysis as the proportion
with complete protease inhibitor cocktail (Roche Diagnostics). Cells
EDTA, 150 mM NaCl, 50 mM Tris/HCl, pH 8
(90 min, 10,000 g) were concentrated by ultracentrifugation
EXPRESS; NEN Life Science Products) for 12–17 h. Virions in the
culture supernatant were collected to monitor virus replication using an
HIV-1 p24 antigen ELISA (HV AG-1 Monoclonal; Abbott).

**Virion infectivity.** GHOST/CXCR4 cells in 48-well plates (3 x 10⁴
cells per well) were infected in triplicate with recombinant virus
(3000 pg p24 antigen) as described earlier (Bleiber et al., 2001). The
infectious titre was determined by FACS analysis as the proportion of
GFP-positive cells.

**Replication kinetics.** PBLs (3 x 10⁶ cells) were infected with virus
(1500 pg p24 antigen) in 1 ml of supplemented RPMI 1640 for 2 h.
Residual inoculum was removed by washing. Aliquots of culture supernatant were collected to monitor virus replication using an
HIV-1 p24 antigen ELISA (HV AG-1 Monoclonal; Abbott).

**Protease autoprocessing.** Auto cleavage efficiency of viral protease
was assessed by expression of a nucleocapsid-transframe-p6-pol-pr
(NT-TF-p6pol-PR) polyprotein in a transcription and translation TNT
T7 rabbit reticulocyte lysate (Promega), following a published protocol
(Peters et al., 2001). Deletions and duplication in p6 were introduced
into pET3/NC-TF-p6pol-PR by site-directed mutagenesis. Constructs
were confirmed by sequencing. Samples of the TNT reaction were
resolved by 15 % SDS-PAGE and processing efficiency evaluated by
radioimmunoprecipitation assay buffer and
radioimmunoprecipitation assay buffer and
radioimmunoprecipitation assay buffer and
cellular debris was removed by centrifugation. Particle- and cell-
associated viral proteins were immunoprecipitated from lysates
using anti-HIV human immunoglobulin (NIH AIDS Research and
Reference Reagent Program) and protein A-Sepharose CL-4B beads
(Amersham Pharmacia Biotech) and separated by SDS-PAGE (5–
15 % gradient gel). Radioactivity content was quantified using
Instant Imager.

**RESULTS**

**Recombinant p6 clones**

Among 296 patients, we identified 17 (5.7 %) with viruses carrying
dele tions polymorphisms of two to six amino acids in the region from S14 to I31 of p6⁶⁶⁸. Six representative
natural deletions were introduced by site-directed muta-
genesis in pNL4-3. The collection of deletion clones was completed by introducing five extensive experimental
deletions in NL4-3. In addition, we investigated insertion
polymorphisms observed at or around the KQE conserved motif. In 14 (4.7 %) patients, the majority of circulating
viruses presented the following insertions, generally after
treatment (no.) Current Peak†

<table>
<thead>
<tr>
<th>Deletion/insertion</th>
<th>Number of observations</th>
<th>Virus subtype (no.)</th>
<th>Viraemia (log RNA copies ml⁻¹)*</th>
<th>CD4 cell count (cells ml⁻¹)*</th>
<th>Anti-retroviral treatment (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔS14-R16</td>
<td>1</td>
<td>A (1)</td>
<td>Current 2·8</td>
<td>4·8</td>
<td>85</td>
</tr>
<tr>
<td>ΔS14-E19</td>
<td>1</td>
<td>F (1)</td>
<td>Current 4·6</td>
<td>5·7</td>
<td>147</td>
</tr>
<tr>
<td>ΔF17-G18</td>
<td>7</td>
<td>B (7)</td>
<td>Current 4·7 (2·6–5·1)</td>
<td>5·3 (3·6–5·9)</td>
<td>274 (158–649)</td>
</tr>
<tr>
<td>ΔG18–T21</td>
<td>1</td>
<td>A (1)</td>
<td>Current 2·8</td>
<td>4·3</td>
<td>250</td>
</tr>
<tr>
<td>ΔT21–T23</td>
<td>1</td>
<td>AG (1)</td>
<td>Current 5·1</td>
<td>5·2</td>
<td>262</td>
</tr>
<tr>
<td>ΔP30–I31</td>
<td>6</td>
<td>A (2)</td>
<td>Current 4·9 (4·3–5·6)</td>
<td>5·4 (4·9–5·8)</td>
<td>158 (92–387)</td>
</tr>
<tr>
<td>Ins at KQE</td>
<td>14</td>
<td>A (3)</td>
<td>Current 4·6 (2·8–5·9)</td>
<td>5·7 (4·3–6·4)</td>
<td>194 (26–415)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>J (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA (3)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Median (range).
†Peak and nadir correspond to the highest level of viraemia and the lowest counts of CD4 cells registered for the patient.
NA, Not available.

**Early virus cycle and replication phenotype of p6 clones**

There was no profound defect in infectivity for clones with natural or experimental polymorphisms in p6 (Fig. 1A).

Table 1. Clinical characteristics of patients carrying viruses with deletion/duplication polymorphisms in p6
With respect to wild-type (100%), ΔS14–E19 exhibited the highest (mean ± SEM, 161 ± 13%, P=0.02), and ΔE12–E13 the lowest (74 ± 14%, P=0.02) infectivity. The coefficient of variation of the single-cycle infectivity assay used was 15% (intra-assay) and 13% (inter-assay), which could account for some of the observed differences. An apparently normal infectivity does not exclude a small defect in the late virus cycle that would be amplified during replication kinetics.

In replication kinetics, the various clones representing natural variants displayed a mean ± SEM relative replication rate of 98 ± 4% compared with wild-type (100%) (Fig. 1B). Among clones representing experimental deletions, only constructs lacking amino acid residues E12 and E13 (ΔE12–E13 and ΔE12–I31) exhibited a statistically significant diminished replication pattern as demonstrated by a reduction in peak p24 production to 54 ± 5 and 25 ± 2%, respectively, with respect to wild-type (P<0.001 for both). Deletion or insertion at KQE did not result in a measurable deficit. The coefficient of variation of the replication kinetics assay was 17% (intra-assay) and 7% (inter-assay), which could explain some of the observed differences.

**Late virus cycle phenotype of p6 clones**

p6Pol has been implicated in the regulation of viral protease autoactivation (Louis et al., 1999; Paulus et al., 1999). As changes introduced in p6Gag may also modify p6Pol, we assessed the rate of protease autocleavage for the various constructs using an *in vitro* transcription and translation assay (Fig. 2). A range of autocleavage activity was observed with respect to the wild-type (100%), with ΔF17–G18 exhibiting the highest (mean ± SEM, 150 ± 1%, P=0.02) and ΔE12–E13 the lowest (73 ± 5%, P=0.02) activity. Particularly relevant was the observation that the clone with the most profound replication impairment and the largest deletion (ΔR20–D39, 42% of p6Pol) did not present a deficit in autoprocessing (Fig. 1C). The inter-assay coefficient of variation was 7.5%. Overall, only clone ΔE12–E13 presented deficits in infectivity, replication and protease autocleavage (Fig. 2).

Analysis of protein maturation in infected cells and virions was carried out for the clones with the most profound impairment, ΔE12–E13 and ΔE12–I31, and for ΔS14–I31. SDS-PAGE analysis did not reveal major defects or delays in Gag–Pol precursor processing, as assessed by gel separation of labelled cell-associated and supernatant viral proteins (Fig. 3). Electron microscopic analysis of wild-type and mutant clones demonstrated comparable budding activity and particle morphology (data not shown).

We assessed the phosphorylation status of p6Gag by virus-associated kinases for clone ΔS14–E19 (deletion of one of 12 putative phosphorylation sites) (Muller et al., 2002), ΔT21–T23 (deletion of three of 12 sites), ΔS14–T23 (deletion of four of 12 sites) and ΔS14–I31 (deletion of five of 12 sites). Phosphorylation was observed for all clones with the possible exception of the large deletion ΔS14–I31 (results not shown). However, the low degree of phosphorylation obtained *in vitro* did not allow a formal quantitative analysis.

**DISCUSSION**

The conserved p6Gag motifs PTAP (the viral L domain), LXXLF (Vpr packaging) and in the Pol frame, SNSF, with a role in protease activation have been extensively evaluated. In addition, mutational analysis of p6 function has shown that truncation of p6 at various positions or specific amino acid changes alter virus production and propagation significantly (Gottlinger et al., 1991; Huang et al., 1995; Yu et al., 1995, 1998; Garnier et al., 1998; Dettenhofer & Yu, 1999; Ott et al., 1999; Peters et al., 2001). The present work contributes data on the central region of the p6 proteins, including the conserved motif KQE.

In *vivo*, viruses with deletions of up to six amino acids are not associated with unusual patterns of disease progression in patients infected with those strains. As T helper and CTL recognition epitopes have been described in p6 (HIV molecular immunology database, http://hiv-web-lanl.gov), one possibility is that deletion in this region contributes to escape from immune surveillance. *In vitro*, no deleterious phenotype of the corresponding molecular clones was observed. It is conceivable that there is a fitness cost associated with such deletions and that it could have been better estimated by competitive replication kinetics. However, testing of sequence variation outside its original genetic context makes interpretation of small differences in replication problematic. Surprisingly, more extensive deletions in the region between S14 and I31 or of the conserved KQE motif – polymorphisms that are never observed *in vivo* – had little effect on virus propagation *in vitro*. The central region of p6 could be relevant in determining the level of phosphorylation of p6Gag (Muller et al., 2002). However, analysis of the phosphorylation status of various clones did not yield reliable quantitative data to assess the relevance of this phenomenon in the retention of this region in nature. The significance of the infectivity deficit of natural clone ΔP30–I31 in the absence of a replication deficit is unclear. We did not test for a potential role of central p6Gag region on Vpr incorporation, as no such impairment has been identified in previous analyses (Lu et al., 1995; Kondo & Gottlinger, 1996).

We assessed the effect of KQE domain deletion or duplication on virus replication. The lysine residue is the substrate for ubiquitin modification. Ubiquitin is attached by an isopeptide linkage between the C-terminal carboxyl group and the ε-amino acid group of lysine. An estimated 2% of the mature p6Gag of HIV-1 is monoubiquitinated (Ott et al., 1998) and there is a relationship between the level of free ubiquitin in the infected cell and the efficiency of virus release (Schubert et al., 2000). We have observed duplication of this motif in 4-7% of clinical strains, a
variation not associated with a specific in vitro or in vivo phenotype. KQE duplication could lead to changes in the interaction with the ubiquitination machinery and therefore the ubiquitination pattern of Gag. In contrast, KQE is never deleted in nature, although its absence in molecular clones was not deleterious in vitro. This is
consistent with previous work indicating that substitution of the two lysine residues in p6\textsuperscript{Gag} that are substrates for ubiquitin modification (K27 and K33) had no apparent effect on virus assembly or release (Ott \textit{et al.}, 2000). It has been proposed that when the preferred sites of ubiquitination are not available, other lysine residues may be used (Hou \textit{et al.}, 1994; Vogt, 2000; VerPlank \textit{et al.}, 2001).

Only extension of the deletion to include amino acid residues E12 and E13 led to markedly diminished replication. The role of the acidic motif (E)E is not well established. This motif is present as AE, ME, VE or LE following the L domain PTAP among various lentiviruses: simian immunodeficiency virus, feline immunodeficiency virus and caprine arthritis encephalitis virus. Rarely, EE, ME or AD can follow a PPPY or a double PPPY/PTAP late domain characteristic of various non-lentiviral retroviruses or some enveloped RNA viruses: vesicular stomatitis virus, Ebola and Marburg virus. The human T cell leukaemia virus 1 has a VE motif within a complex PPPYVEPTAP L domain.

Work by Garrus \textit{et al.} (2001) indicates that substitution of E13 for alanine resulted in a modest reduction in binding affinity of Tsg101. Work by Martin-Serrano \textit{et al.} (2001) described a GG for EE substitution leading to a minor decrease in Tsg101–Gag interaction and virion production and a moderately diminished viral infectivity. In our work, deletion of EE diminished viral infectivity and replication without evidence of major changes in protein maturation. Electron microscopy did not reveal changes in budding reminiscent of those reported for mutants lacking L-domain function – a failure of particles to ‘pinch off’. Demirov \textit{et al.} (2002) indicated that premature termination at E12 markedly disrupts particle production, whereas truncations immediately downstream have no effect on

\[ \text{Fig. 1.} \] Infectivity, replication and rate of protease autocleavage of p6 clones with natural and experimental polymorphisms. (A) One-cycle infectivity of p6 clones was determined by FACS analysis of GHOST/CXCR4 cell fluorescence and expressed as a percentage of the wild-type NL4-3 value (black bar). Error bars indicate the range of values (± SEM) obtained from triplicate data points in one of two experiments, in which comparable results were obtained. (B) Replication kinetics were performed in PBLS. Cells were infected with p24-normalized amounts of virus particles. The data are from one of three experiments, in which comparable results were obtained. (C) Protease autocleavage in selected clones ΔR20–R21, ΔE22–D39 and ΔR20–D39 (p6\textsuperscript{Pol}) amino acid numbering) was estimated by using an NC-TF-p6\textsuperscript{Pol}-PR expression vector in a reticulocyte lysate transcription and translation (TnT) system. Shown are the processing patterns after 30 min of transcription/translation, including the unprocessed product of a D25E construct, generating an inactive viral protease (PR\textsuperscript{<}). pETWT carries the NL4-3 wild-type sequence.

\[ \text{Fig. 2.} \] Summary of data on infectivity, replication and autocleavage analyses of natural (black bars) and experimental (grey bars) constructs. Results are given as percentages relative to the wild-type NL4-3 (100%). *, Statistically significant differences (P < 0.05) compared with wild-type NL4-3.
virus production yet impaired virus replication, a phenotype reminiscent of \( \Delta E12–E13 \). Similarly, mutations in p9 of equine infectious anaemia virus have been reported that have no apparent effect on virus production yet impair virus replication (Chen et al., 2001).

Thus, this paper highlights the fact that regions of the p6 gag open reading frame and the overlapping region in pol can be deleted without major consequences for virus replication in vitro or in vivo. The tolerance for polymorphism in this region may reflect a role of the central region of p6 as a linker structure that accommodates the constraints of the overlapping gag/pol open reading frames. Finally, this study underlines the need to investigate the conserved motif KQE using new approaches, given the questions that remain about its precise role in virus physiology.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation. We thank A. Ciuffi and M. Muñoz for help and encouragement. We are grateful to the NIH AIDS Research and Reference Reagent Program for providing various reagents for this study.

REFERENCES


