Inhibition of human immunodeficiency virus type 1 particle formation by alterations of defined amino acids within the C terminus of the capsid protein

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In previous studies, we demonstrated that the substitution of amino acid triplets for alanines in the carboxy-terminal portion (amino acids 341–352: ATL EEM MTA CQC) of the capsid protein domain (p24) of human immunodeficiency virus type 1 (HIV-1) partly led to an inhibitory effect on the capacity to form virus-like particles (VLPs). In these experiments, the uncleaved Pr55<sup>gag</sup> precursor protein was expressed by recombinant vaccinia viruses. We have now investigated the effects of these mutations with respect to a replication-competent HI-provirus system. Substitution of amino acids 344–346 (EEM) for alanines, which was previously shown to lead to an inhibition of VLP formation, completely blocked assembly and release of HIV. A substantial reduction of HIV synthesis was also observed in the proviral system after exchange of amino acids 347–348 [MT(A)] which, in contrast, was formerly shown to result in an increased formation of VLPs. Western blot analysis of lysates of cells transfected with these mutated proviral constructs revealed an abnormal intracellular processing pattern of the Pr55<sup>gag</sup> precursor molecules. Further analyses suggest a structural aberration of these altered polyproteins as the basis for the observed block of virus formation.

Introduction

The Gag proteins of retroviruses harbour the information necessary for virion assembly and budding from the host cell (Delchambre et al., 1989; Haffar et al., 1990; Hansen et al., 1990; Wagner et al., 1992). In human immunodeficiency virus type 1 (HIV-1), they are synthesized as 55 kDa polyproteins (Pr55<sup>gag</sup>) and transported to the inner side of the plasma membrane where they associate with the lipid bilayer by means of an amino-terminally attached myristic acid (Goddard et al., 1989; Göttlinger et al., 1989; Pal et al., 1990). During virus maturation, these Pr55<sup>gag</sup> precursor molecules are cleaved by the viral protease into the matrix protein (p17-MA), the capsid protein (p24-CA), the nucleocapsid protein (p7-NC), the ‘link’ protein (p6-LI) and two small peptides termed p2 and p1, respectively (Overton et al., 1989; Veronese et al., 1988; Wills & Craven, 1991). The carboxy-terminal portion of Pr55<sup>gag</sup>, comprising the sequences for p7 and p6, is dispensable for particle assembly, as deletion still leads to the formation of virus-like particles (VLPs) when the remaining sequence is expressed by recombinant baculoviruses (Royer et al., 1992; Spearman et al., 1994).

Various functions have been assigned to p17-MA, including: the transport of the precursor molecules to the membrane (Gelderblom, 1991; Facke et al., 1993; Yuan et al., 1993; Zhou et al., 1994); interaction of the individual polyproteins for particle formation (Freed et al., 1994; Morikawa et al., 1995); incorporation of the viral glycoprotein complexes via gp41 (Yu et al., 1992a; Wang et al., 1993; Dorfman et al., 1994a); and directing the viral preintegration complex to the nucleus during the early steps of infection (Bukrinsky et al., 1992; Bukrinsky et al., 1993; Yu et al., 1992b). Nevertheless, deletion of about 80% of the p17-MA domain of HIV-1 did not affect particle formation, except for a redirection of the assembly process to the membrane of the endoplasmic reticulum and the abolition of envelope glycoprotein incorporation (Facke et al., 1993).

Although there is still no direct evidence that p24-CA is responsible for the formation of particulate structures, this protein portion is very likely involved in the viral assembly process. Evidence for this comes from its capacity to assemble into oligomeric structures (Ehrlich et al., 1992; Gitti et al., 1996;
Momany et al., 1996) and, in addition, numerous studies showing that several mutations within p24-CA lead to the inhibition of virus formation (Hong & Boulanger, 1993; Wang & Barklis, 1993; Reicin et al., 1995). In particular, mutations located carboxy-terminally to the major homology region (MHR), a highly conserved stretch of amino acids within p24-CA, resulted in decreased virus release (Dorfman et al., 1994b; Reicin et al., 1995; von Poblotzki et al., 1993). We previously substituted defined amino acids within this carboxy-terminal portion of p24-CA (between residues 342 and 352) in triplets for alanines and expressed the altered gag sequences by recombinant vaccinia viruses (von Poblotzki et al., 1993) or recombinant baculoviruses (B. Kattenbeck et al., 1996). In these artificial expression systems, a strong reduction in particle formation and release of particles with an aberrant morphology was observed, at least for some of the mutants. In order to further investigate the effect of these mutations on the synthesis of infectious HIV, we altered these portions of the proviral DNA sequences by exchanging the amino acids 342–352 (TL EEM MTA CQG) in triplets/pairs for alanine residues (see Table 1). The proviral constructs were transfected into COS-7 cells and the ability to release infectious particles was monitored by several approaches. Further analyses of the intracellular appearance of the Pr55^ppg precursors provide possible explanations for the observed effects.

**Methods**

**Cells, transfections and infections.** COS-7 cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% inactivated foetal bovine serum and penicillin (1 U/ml)/streptomycin (1 µg/ml). Cells (1 x 10⁵) in a volume of 0.4 ml were taken for electroporation and transfected with 20 µg of the respective proviral DNA construct using a Bio-Rad gene pulser set at 230 V and 960 µF. Transfected cells were harvested 60–80 h after transfection.

MT4 cells were grown in RPMI medium supplemented with 10% inactivated foetal bovine serum and penicillin (1 U/ml)/streptomycin (1 µg/ml). Cells (1–2 x 10⁷) were infected with aliquots taken from the supernatant of transfected COS-7 cells. In a second approach, infection was performed by co-cultivation of the MT4 cells with the transfected COS-7 cells. A third mode of infection was applied by using purified virus particles which were adjusted for their p24 content and subsequently used to infect 1–2 x 10⁷ MT4 cells. In the case of the mutants p24-EEM (amino acids 344–346) and p24-MT (amino acids 347–348), the whole preparations were used for further infection studies. Cells were maintained and observed for signs of infection for up to 5 weeks.

**Recombinant plasmids.** In this study the HIV wild-type provirus plasmid HX10 (Ratner et al., 1987) was used. The Pr55^ppg open reading frame (830–2429 bp) was excised, except for a short stretch of 42 bp at the 5’-OH terminus, by use of the unique restriction sites ClaI and Bcl1. Inserts containing the respective mutations were excised from vectors plin8p55/1, plin8p55/4, plin8p55/7 and plin8p55/10, generated previously (von Poblotzki et al., 1993) by the use of the same restriction sites and ligated into the proviral plasmid. This resulted in the generation of the proviral constructs p24-TL, p24-EEM, p24-MT and p24-CQG in which the amino acids 342–343 (TL), 344–346 (EEM), 347–348 (MT) and 350–352 (CQG) of the capsid protein were replaced by alanines (Table 1). All constructs were verified by DNA sequencing.

**Analysis of particle release.** Eighty hours after transfection, virus release was monitored by a p24-ELISA (Abbott Laboratories) and also by a non-radioactive reverse transcriptase (RT) assay (Boehringer Mannheim). In order to compensate for variations in the amounts of particles due to varying electroporation efficiencies, cells were transfected in three separate experiments and pooled prior to resuspension. The protein concentration of aliquots of the cell lysates was determined 80 h after transfection (Bio-Rad) and adjusted volumes containing equal amounts of protein were analysed for their p24 concentration. If possible, values of released particles determined by the p24 capture assay or the RT assay were corrected against the values of intracellular p24. In general, p24 values below A₄₉₂ 0.070 were negative and reflected a lack of particle release.

For virus purification, supernatants of transfected cells were cleared from cellular debris and the precleared supernatants were layered on top of 2 ml of 25% sucrose. Virus particles were pelleted by centrifugation at 130,000 g for 4 °C for 2.5 h. The pellets were resuspended in ice-cold PBS containing 1 µg/ml Aprotinin, 10 µg/ml Leupeptin, 1 µg/ml Pepstatin, 1 mg/ml Pefabloc SC and 0.5 mg/ml EDTA (Boehringer Mannheim), and were taken either for infection or further purification. For further purification, pelleted virions were layered on top of a 10–60% sucrose gradient and centrifuged at 120,000 g at 4 °C for 3 h. Twenty fractions were collected, virus particles were precipitated from the individual fractions by acetone and viral proteins were separated by SDS–PAGE. In order to detect the Pr55^ppg precursors and p24-CA in Western blots, monoclonal antibodies (13/5) directed against an epitope located within the amino-terminal part of p24-CA (Wolf et al., 1990) were used. After infection of MT4 cells, the amount of p24-CA in the supernatants was determined immediately after infection and 15 and 29 days thereafter (respectively 4, 19 and 33 days after co-cultivation).

**Analysis of intracellular protein synthesis.** Intracellular viral proteins were analysed 60 h after transfection by p24 capture assays and by Western blotting of the cell lysates. For immunofluorescence microscopy, transfected cells were harvested at 40 g at room temperature, washed twice with PBS, air dried on glass slides and fixed in petroleum/gasoline (1:1) at ~ 20 °C. Fixed cells were incubated with the monoclonal antibodies 13/5. FITC-conjugated anti-mouse antibodies (DAKO) diluted 1:200 were employed as secondary antibodies. For protein sequencing, the proteins in the lystate of cells transfected with mutant p24-MT were separated by SDS–PAGE and electroblotted on a Hybond-

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### Table 1. Exchanged amino acids and corresponding mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Exchanged amino acids</th>
<th>New sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>340-ATL EEM MTA CQG-353</td>
</tr>
<tr>
<td>p24-TL</td>
<td>342–343: TL</td>
<td>340-AAAG EEM MTA CQG-353</td>
</tr>
<tr>
<td>p24-EEM</td>
<td>344–346: EEM</td>
<td>340-ATL AAA MTA CQG-353</td>
</tr>
<tr>
<td>p24-MT</td>
<td>347–348: MT</td>
<td>340-ATL EEM AAA CQG-353</td>
</tr>
<tr>
<td>p24-CQG</td>
<td>350–352: CQG</td>
<td>340-ATL EEM MTA AAA-353</td>
</tr>
</tbody>
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* Alanines replacing the original amino acids are underlined; flanking numbers refer to the position of adjacent amino acids.
PVDF membrane (Amersham). Sequencing of the membrane-bound protein was performed using the Edman degradation procedure (Applied Biosystems) after cleaving the protein with trypsin. The amino acid sequence obtained was matched to the Gag protein sequence derived from the gag nucleotide sequence of the HXB2-clone.

Results

Particle formation and production of infectious HIV

We introduced a series of amino acid exchanges within a highly conserved stretch (von Poblotzki et al., 1993) of the carboxy-terminal portion of the HIV-1 capsid protein (Table 1; Fig. 1). Each mutation comprised the exchange of two or three consecutive amino acids to alanine residues. Determination of the RT activity and of virus-associated p24 in the supernatants of transfected cells showed that the release of virus particles was reduced by up to 40% when the amino acids TL were exchanged and up to 10% when CQG were substituted by alaminates (Fig. 2a). No virions at all were detected in the supernatant of cells transfected with mutant p24-EEM and only marginal amounts of virus were determined in the supernatant of mutant p24-MT.

To further confirm the lack of virus release after substitution of EEM and to monitor the infectivity of the other virus mutants, the ability of released virions to infect MT4 cells was investigated. Three different approaches were pursued to ensure infection of the target cells by released virus particles (see Methods). All three approaches led to comparable results as determined by the p24-ELISA and the RT activity. Fig. 3 shows the p24 values obtained by the co-cultivation experiment. MT4 cells were easily infected by supernatants taken from the HIV wild-type-transfected COS-7 cells as indicated by the strong increase in the amount of p24-CA in the supernatants after 19 days. Mutant p24-CQG infected the CD4-positive cells at a rate similar to the wild-type virus, whereas a significantly lower amount of p24-CA was detected after 19 days of co-cultivation with mutant p24-TL. This corresponds to the observation that this mutation led to a reduced virus release (compare with Fig. 2) and hence to a decreased rate of multiplication within the infected culture. No infection was achieved with the supernatants of COS-7 cells after transfection with mutant p24-EEM or p24-MT. This observation supports the previous finding that substitution of the amino acids EEM and MT to alanine residues prevented virus release. Obviously the low amount of virus released by cells transfected with mutant p24-MT was not able to establish the infection of the MT4 cells.

For immunochemical analysis, virus particles sedimented in a sucrose density gradient were analysed by Western blotting (Fig. 4). HIV wild-type particles accumulated in gradient fractions at a density between 1.15 and 1.20 g/cm³ according to published sedimentation values (Shioda & Shibuta, 1990; Wagner et al., 1992) and displayed the typical processing pattern of Pr55<sup> gag </sup> into p24/25 and the intermediate products p11/12. The same characteristics were observed for the mutants p24-TL and p24-CQG, indicating a rather wild-type-like appearance. As expected, no proteins were detected by the antibodies in any gradient fraction of mutant p24-EEM, and almost no viral proteins were identified by the p24-CA specific antibodies in the respective fractions of mutant p24-MT. Surprisingly, the antibodies here bound to a protein of 41 kDa, although neither the precursor molecule Pr55<sup> gag </sup> nor the processed p24-CA was detected. This may indicate that aberrant processing of the Gag proteins occurred due to the exchange of the amino acids MT.

Taken together the results suggest that the substitution of the amino acids EEM and MT of p24-CA (amino acids 344–348) by alaminates led to serious defects in HIV particle formation.

Expression and intracellular processing of the gag polyprotein precursor Pr55<sup> gag </sup>

Since no virus particles were detected in the supernatants of cells transfected with the construct p24-EEM and only marginal
amounts of particulate structures were determined after transfection of p24-MT, it was essential to prove that the viral gag gene products were expressed within the cells. A preliminary experiment was performed by subjecting transfected cells to indirect immunofluorescence (Fig. 5). In all transfection assays, including those performed with constructs p24-EEM and p24-MT, approximately similar amounts of cells were found to contain the gag gene product or at least the p24 portion of this protein.

In order to compare the intracellular amounts of these proteins with regard to the respective Gag mutant, we applied the p24 capture assay on lysates of the transfected cells, applying equal amounts of total protein for this test (Fig. 2b). Cells transfected with the proviral construct p24-CQG and cells expressing the wild-type provirus contained comparable amounts of p24/Gag, whereas about 15% less p24 was detected within cells expressing p24-TL. Surprisingly, no p24-CA could be detected at all by this test in cells transfected with p24-EEM and only basal amounts were found in the cell lysates after transfection of mutant p24-MT. However, the presence of p24-containing, gag-derived proteins had previously been confirmed for both of these mutants by the immunofluorescence studies. From these results we assume that the mutation led to an interference with epitope recognition of the antibodies provided by the ELISA test.

To investigate further, Western blot analysis of lysates of transfected cells was performed using the antibodies 13/5, which obviously were able to bind to the p24 portion of all of the mutants (Fig. 6). In cells expressing the HIV-wild-type-DNA uncleaved Pr55\textsuperscript{gag}, fully processed p24-CA and intermediates of 41–42 and 25 kDa were present (lane 6). Identical binding patterns were observed for lysates of cells transfected with the mutants p24-TL (lane 2) and p24-CQG (lane 5), although p25 appeared more prominently in the latter case. When the mutated proviral construct p24-EEM was expressed (lane 3), neither the full-length Pr55\textsuperscript{gag} precursor nor p24-CA
Mutations in p24-CA block HIV-1 release

Fig. 5. Immunofluorescence labelling of untransfected cells (negative control) and cells transfected with wild-type proviral DNA or mutants p24-EEM and p24-MT. Cells were fixed 60 h after transfection. Mouse antibodies binding to the amino-terminal portion of p24 were used as first antibodies, followed by FITC-conjugated rabbit anti-mouse antibodies.

Fig. 6. Western blotting of cell lysates of the transfected COS-7 cells: about $1 \times 10^6$ cells were harvested 60 h after transfection and applied on a denaturing 12.5% SDS gel. Protein detection was performed using monoclonal antibodies binding to an epitope within the amino-terminal portion of p24-CA. Lane 1, mock control; lane 2, p24-TL; lane 3, p24-EEM; lane 4, p24-MT; lane 5, p24-CQG; lane 6, wild-type.

was detected. However, intermediates of 30 and 41–42 kDa were identified. The lysate of cells transfected with mutant p24-MT mainly displayed a prominent band of approximately 39 kDa but lacked both unprocessed Pr55$^{gag}$ and p24-CA. Protein sequencing of this 39 kDa protein first revealed that its amino terminus was blocked. After digestion with trypsin further sequencing could be performed. Protein stretches were identified which belonged to the matrix protein and the capsid protein, respectively (p17-MA, K-H-I-V-W-A-S, amino acids 31–37; p24-CA, K-E-T-I-N-E, amino acids 202–207). None of the obtained sequences could be assigned to the nucleocapsid portion of the Gag protein.
Discussion

In the present provirus investigations, several independent methods indicated that, in accordance with previous results (von Poblotzki et al., 1993), no virus particles were released when the amino acid triplet EEM (344–346) was substituted by alanines. Instead of a complete loss of particle formation a drastic reduction in virus release was observed after alteration of the amino acids TL (342–343) to alanines. However, the synthesized particles could be shown to be infectious. In contrast to the results gained by the expression of VLPs using recombinant vaccinia viruses, where an increased level of VLPs was determined after exchange of the amino acids MT (347–348), this mutation displayed a strong negative effect on virus synthesis in the proviral system. Particle formation was almost completely abolished and an aberrant intracellular processing of the precursor protein was observed. Furthermore, no significant effect on virus release and infectivity was determined after alteration of CQG (amino acids 350–352) in the proviral system, indicating that the cysteine residue at position 350, although conserved among retroviruses, is not functionally involved in the formation of infectious virus.

Together with our previously published results, the present data demonstrate that the gag region comprising the amino acids 342–348 has to be intact to allow particle formation. In particular, exchange of EEM (344–346) and MT (347–348) interferes with this process. Nevertheless, it seems likely that in addition to the correct sequence of the Gag protein, additional factors may be important for particle formation, as may be inferred from the difference between results obtained in different expression systems (von Poblotzki et al., 1993). These include cell type specificity and the presence of further viral enzymatic and structural components, and are likely to account for most of the opposite effects observed for the mutants p24-MT and p24-CQG in the two expression systems. The fact that the cleavage site between p24-CA and p15-NC is easily accessible in the mutant proteins p24-EEM and p24-MT suggests that the observed differences in particle formation may be associated with the presence of the viral protease. Furthermore, it should be taken into account that alteration of the nucleotide sequence within gag might influence Gag protein expression at the mRNA level and therefore might lead to a reduced concentration of these proteins within the cells. As this effect would obviously result in a lower level of particle formation, the values representing the amount of particles determined in this work were corrected with respect to the intracellular amount of the Gag precursor proteins. This aspect was not considered when the vaccinia virus system was employed. Nevertheless, the present provirus data are supported by the expression by recombinant baculoviruses of the altered Gag sequences as VLPs (Kattenbeck et al., 1996).

The present studies also provide an explanation for the loss of capacity to synthesize virus particles observed for mutant p24-EEM, and the strong reduction in this capacity seen for mutant p24-MT. From our observations, we suggest that the correct structure of the Gag precursors is affected and this accounts for the inhibition of particle formation. This assumption is supported by several observations. Firstly, we were able to show that p24-containing gag-derived proteins were present within the transfected cells (Fig. 5). Nevertheless, these proteins could not be detected by the p24-ELISA. It is reasonable to conclude that the monoclonal antibodies used here were no longer able to bind to the mutated Gag proteins due to conformational changes of the target protein. Secondly, the Western blot analysis of the cell lysates revealed an incomplete or aberrant processing pattern of the two loss-of-function mutants. It has been shown previously that processing of Pr55$^{gag}$ already occurs intracellularly to a great extent (Kaplan & Swanstrom, 1991). Hence, the appearance of individual Gag-cleavage products indicates that the proteolytic enzymes have access to the respective cleavage sites and, therefore, the wild-type polyprotein displays its native structure. In contrast, neither the full-length precursor molecule Pr55$^{gag}$ nor the cleaved processing product p24-CA was detected intracellularly after expression of p24-EEM and p24-MT. Instead, partially processed forms (p41/42 and p39) were present. This indicates that cleavage of these Pr55$^{gag}$ precursors might be affected by the mutations in different ways. Firstly, the processing site between p17-MA and p24-CA seems to be blocked, leading to the inhibition of cleavage at this site. Secondly, the absence of the uncleaved full-length Pr55$^{gag}$ suggests that other cleavage site(s), in particular between p24-CA and the p7-NC domain, were now readily accessible for the proteolytic enzymes. The 39 kDa fragment observed for mutant p24-MT is probably due to the introduction of a new cleavage site at the carboxy terminus of p24 after alteration of the amino acids methionine and threonine to alanine residues. As none of the mutations directly affected any of the cleavage sites of Pr55$^{gag}$, again an overall structural alteration might be an explanation for these simultaneously occurring effects.

Further evidence for a conformationally based effect is drawn from the observation that deletion of the Gag portion carboxy-terminal to p24-CA was shown to be dispensable for particle formation (Royer et al., 1992). It was also demonstrated that the inhibition of the viral protease results in the release of non-infectious particles, but does not interfere with the processes of virus formation and budding (Schätzl et al., 1991). Therefore, one would expect that expression of p24-EEM and p24-MT, which obviously still led to the intracellular presence of uncleaved Gag fragments containing p17-MA and p24-CA, should result in the formation of non-infectious particles consisting of these fragments. In contrast, an inhibitory effect on virus formation and release in general was observed.

Taken together, the results suggest that substitutions of amino acids to alanines in the region 344–348 of the capsid protein affect virus assembly by conformational changes of the Pr55$^{gag}$ molecule. It must be emphasized that it is still not clear...
from these studies whether this region within the carboxy terminus of p24 is functionally active during the association of the individual Gag molecules in order to yield the particulate structure. Nevertheless, the altered precursor molecules are not able to associate into particles. With respect to these mutants we assume that domains within Gag, which are essential for the interaction between the individual molecules, become covered or altered and thus the intermolecular association is abolished. Interference with these interactions of the Gag molecules was also suggested for synthetic peptides which were derived from the same region of the HIV-1 capsid protein and displayed an inhibitory effect on virion formation (Niedrig et al., 1994). Since the mutations within gag introduced for the present study affected every single Pr55<sup>gag</sup> molecule, a complete block of virus formation may take place if the substituted amino acids are either themselves active during association of the Pr55<sup>gag</sup> precursors or, as we propose, lead to a structural alteration of the whole precursor molecule.

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References


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