The effect of capsid mutations on HIV-1 uncoating

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Abstract

Efficient uncoating requires not only an optimal cellular environment, but also some intrinsic properties of the viral capsid protein itself. Using an in vitro uncoating model, we demonstrated that substitution of each serine residue with alanine at the three major phosphorylation sites of HIV-1 capsid protein, i.e. Ser-109, Ser-149 and Ser-178, could significantly reduce uncoating activity of purified core particles. We also showed that the core stability of mutant viruses was lower than that of the wild-type virus so that the lack of efficient uncoating of each mutant could not be due to an increase in capsid physical stability. However, serine-to-aspartic acid mutation to mimic the negative charge of phosphor-serine could not restore either uncoating activity or infectivity, and treatment of purified core particles with a phosphatase did not alter the uncoating activity. Our data indicated that mutations at phosphoacceptor sites of capsid disturbed the uncoating mechanism, but the defect may not be directly caused by the lack of phosphate on the core particles undergoing uncoating.

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Introduction

Capsid (CA) protein is the major structural component of human immunodeficiency virus type 1 (HIV-1), forming a cone-shaped core structure that surrounds the RNA genome at the center of the virion. The HIV-1 CA must be able to assemble properly and stable enough to protect the fragile genome while being transmitted between cells or hosts, but must disassemble or alter in the conformation sufficiently to allow genome release after entry into target cells. The process that HIV-1 conical capsid dissociates in order to release the viral genome is known as uncoating. We previously reported the requirement of cell cycle-dependent specific factors for HIV-1 uncoating. This suggests that the uncoating process is not a spontaneous process; on the other hand, this process is tightly regulated and requires some specific triggers (Auewarakul et al., 2005). Beside the requirement of the cellular environment that supports HIV-1 uncoating, the capsid protein itself seems to play an important role in the uncoating, too. Several HIV-1 capsid functions have been analyzed by mutagenesis. In addition to the mutations that perturb normal particle assembly, maturation and structure, several mutants have been reported to support normal capsid assembly but were nevertheless noninfectious by implicating at the early steps of infection (Forshey et al., 2002). These data indicate that the functions of capsid protein do not only limit to the structural properties, but also important for the regulation of viral life cycle such as uncoating and subsequent reverse transcription.

Phosphorylation of viral protein often plays an important role in regulation of viral replication. Several investigators reported the requirement of virion structural protein phosphorylation, e.g. herpes simplex virus type 1 (HSV-1) tegument protein VP22 (Morrison et al., 1998), vesicular stomatitis virus (VSV) matrix (M) protein (Beckes et al., 1989; Kaptur et al., 1992) and moloney murine leukemia virus (MMLV) p12 protein (Yueh and Goff, 2003), for early post-entry event of the replication cycle. One of the possible roles of the phosphorylation is modifying these structural proteins leading to uncoating (Hui, 2002). For HIV-1, a number of viral proteins have been shown to be phosphorylated, including structural proteins, matrix (MA) (Camaur et al., 1997; Kaushik and Ratner, 2004; Mervis et al., 1988) and CA (Cartier et al., 1999; Henderson et al., 1992; Mervis et al., 1988; Overton et al., 1989; Veronese et al., 1988). The presence of cellular serine/threonine protein kinases incorporated in HIV-1 particles was also demonstrated (Cartier...
et al., 1997, 2003; Devroe et al., 2005). The virion-associated catalytic subunit of cAMP-dependent protein kinase (C-PKA) was found to interact with and phosphorylate the CA protein. C-PKA activity affected infectivity but was not required for maturation and assembly (Cartier et al., 2003). In addition, it has been demonstrated that the target of phosphorylation is on serine residues (Cartier et al., 1999; Mervis et al., 1988). Mutagenesis performed on highly conserved serine residues could uncover the three major phosphorylation sites within CA protein, i.e. Ser-109, Ser-149 and Ser-178. Substitution of each serine residue with alanine did not affect viral budding nor viral structure, by contrast, it affected viral infectivity by preventing the reverse transcription process to be completely achieved (Cartier et al., 1999). These data led to a hypothesis that HIV-1 CA phosphorylation may be required for viral uncoating process.

We sought to identify residues on capsid protein that are essential for the uncoating by testing specific capsid mutants that were previously shown to support normal core assembly but lack infectivity, including the phosphoacceptor site mutants.

Results

CA phosphorylation level and viral infectivity of the wild-type and mutant viruses

To authenticate that CA point mutations at serine residues 109, 149 and 178 affected phosphorylation status of CA protein, wild-type and the CA mutated viruses were immunoprecipitated by anti-HIV p24 and detected for phosphorylation using Pro-Q Diamond phosphoprotein gel stain. As demonstrated in Fig. 1a, phosphorylation status of the mutant CA proteins was significantly less than that of wild-type CA protein (upper panel). Similar amounts of CA protein from each virus were added and were identified by silver stain (middle panel). Band densitometry was performed and the phosphorylation of each mutant CA protein relative to wild-type CA protein was calculated from band densitometry of the silver stain and Pro-Q phosphorylation staining gels (lower panel). The results, therefore, substantiated that mutation at serine 109, 149 and 178 reduced the phosphorylation level of CA protein.

These mutants were then determined for the viral infectivity. H9 cells were incubated with the wild-type and mutant viruses that had been harvested from supernatant of 293T cells. Cultured medium was tested for p24 released in supernatant by ELISA. As shown in Fig. 1b, no p24 released into supernatant of H9 cell cultures which inoculated with S109A, S149A and S178A mutants. On the other hand, wild-type virus could replicate efficiently in cultured H9 cells. Thus, these three mutants were not infectious in H9 cell and suggested that phosphorylation of CA protein was required for HIV-1 infectivity.

Mutation at CA phosphor-acceptor sites affects uncoating activity

To characterize whether the substitution of these serine residues blocks HIV-1 infection at the uncoating step, the uncoating activity of core particles derived from each virus were measured using the in vitro uncoating assay described previously in our laboratory (Auwarakul et al., 2005). Because the uncoating process is defined as the disassembly of viral core in an intracellular environment, an approach to study this process is to detect the disassembly of core particles in the presence of cellular constituent. Core particles purified from each mutant virus were incubated with H9 cell lysate. The extent of uncoating was determined by measuring the level of p24 in the supernatant after ultracentrifugation. We have previously verified that the assay indeed measured a structural disassembly of core particles. The disassembly was not due to any non-specific physical conditions and required a specific cellular component from permissive cells. The assay should therefore accurately reflect the viral uncoating process. Using
this assay, we could show that mutation at these three serine residues led to a decrease in uncoating activity. As shown in Fig. 2, uncoating activity of S109A, S149A and S178A mutant viruses was 6, 3 and 1.6 times lower than that of wild-type virus respectively. This indicated that CA phosphorylation played an important role in HIV-1 uncoating.

Effect of CA phosphor-acceptor site mutations on core stability

To exclude the possibility that the decrease in uncoating activity of these mutants was due to increase in stability of virion core, we characterized the core stability of these wild-type and mutant viruses by comparing yield of core particles of each virus after purification by the spin-through technique. The yields of cores were calculated by determining the amount of CA pellets as a percentage of total input CA (Forshey et al., 2002). As shown in Fig. 3, the core yield from wild-type virus was approximately 11% of the input virus. In contrast, the yields of core particles from these mutant viruses were around 4% to 6% of the input virus. This result indicated that the core stability of mutant viruses was lower than that of the wild-type virus and the lack of efficient uncoating of each mutant could not be due to an increase in capsid physical stability.

Substitution of serine with aspartic acid cannot restore infectivity and uncoating activity

Phosphorylation of a protein causes an increase in negative charge. Therefore, it is possible that the effects of phosphorylation could be mimicked by the addition of negative charge at the phosphorylation site. To determine this hypothesis, we replaced the Ser-149 with aspartic acid residue. We used this construct to studied viral infectivity as well as uncoating activity and stability of purified core particle. S149D mutant could not restore the viral infectivity (Fig. 1b). Substitution of serine residue with aspartic acid could only slightly restore the uncoating activity. As shown in Fig. 2, the uncoating activity of S149D was a little higher than S149A, but still significantly less than that of wild-type virus: approximately half of wild-type virus. The stability of this mutant core particle was also less than wild-type core and was comparable to that of S149A core particle (Fig. 3). This suggested that a negative charge residue could not mimic the roles of CA phosphorylation in viral infectivity and uncoating.

Phosphatase treatment did not affect the uncoating

In order to test whether the uncoating defect was directly due to the lack of phosphate group at the phosphoacceptor sites or indirectly due to other changes caused by the mutations, we treated purified core particles from the wild-type virus and tested for the uncoating activity. The enzymatic treatment successfully got rid of phosphate from the core as shown by a marked reduction in band intensity in a Pro-Q phosphorylation staining gel (Fig. 4). This also served as a control that the staining could semiquantitatively detect phosphorylation status of proteins. However, the uncoating of the treated and untreated core was not significantly different. This indicated that dephosphorylated cores could still undergo normal uncoating process, and the presence of phosphate on the CA protein was not required for the uncoating.

Other mutations with decreased physical stability did not show uncoating defect

The three phosphoacceptor site mutations showed decreased physical stability while resisting to uncoating by cell lysate. In
order to provide additional data supporting that the observed defect was specific to the uncoating mechanism and not simply due to non-specific structural changes, we tested other two CA mutations (K170A and K203A) that were previously shown to be defective in an early post-entry event and have decreased physical stability (Forshey et al., 2002). These two mutants were uncoated at least as efficiently as the wild-type virus (Fig. 5). This suggested that altered physical stability could not cause uncoating defect that was observed in the phosphoacceptor site mutants.

Discussion

The uncoating process of many viruses was shown to require specific cellular factors as well as intrinsic viral properties (Greber et al., 1994; Sansom et al., 1998; Singh and Helenius, 1992; Smyth and Martin, 2002; Wharton et al., 1994). For HIV-1, we previously showed that the uncoating required the specific cellular factors (Auewarakul et al., 2005). In the present study, we identified specific residues on the CA protein required for the uncoating.

Cartier et al. has previously demonstrated that alanine substitution at the phosphorylation sites of serine residue 109, 149 and 178 of the CA protein could suppress the viral ability to replicate in T cell lines at an early post-entry step. The assembly and maturation process of these mutants were demonstrated to be intact (Cartier et al., 1999). We demonstrate here that the step in viral life cycle that is likely to be affected by phosphorylation site mutations was the uncoating process.

Efficient uncoating requires these three serine residues simultaneously. The mature capsid comprises hexameric rings of CA protein held together by a dimeric interaction of CA between adjacent rings (Li et al., 2000). The C-domain to C-domain dimeric interaction is stable while the N-domain to N-domain hexameric interaction is not and could only exist with intact C-domain to C-domain and N-domain to C-domain intermolecular interactions (Lanman et al., 2003). According to the high-resolution structure, Ser-149 is located in the unstructured inter-domain linker, which is disordered in the monomer but forms structured interactions upon assembly (Berthet-Colominas et al., 1999; Lanman et al., 2003). Ser-178 is situated between helix 8 and 9 in the C-domain near the C-domain dimerization interface (residues 179–192) (Gamble et al., 1997; Momany et al., 1996). Therefore, structural modification at these two serine residues may facilitate the disassembly of core particle, possibly by alteration the C-domain to C-domain interaction as we have proposed in the previous report (Auewarakul et al., 2005). The uncoating might not be due to the repulsion force from the negatively charged phosphate group as we demonstrated that substitution of Ser-149 with aspartic acid could not restore infectivity and uncoating activity. On the other hand, the substitution might not only mimic the positive charge but also alter some essential conformation, which led to the loss of uncoating and infectivity. The Ser-109 might be involved in the binding site of the putative uncoating factor since the position of Ser-109 is between helix 5 and 6 in the amino-terminal which is likely to
be exposed to the surface of the capsid and is not situated at the protein–protein interface.

The lack of effect of phosphatase treatment on the uncoating suggests that incoming core does not need to be phosphorylated in order to be uncoated. However, it is intriguing that all the mutations at the phosphoacceptor sites showed uncoating defect, while mutations at other positions showing similarly decreased core physical stability were not defective in uncoating. This suggests that the phosphorylation may be important for the uncoating. It is also possible that the CA protein is phosphorylated while undergoing uncoating in the cell lystate or the target cell. Recently, Amella et al. (2005) demonstrated that inhibition of the kinase C-PKA in target cells significantly inhibited synthesis of HIV-1 DNA without affecting viral entry. More studies are needed to elucidate this obscure process in the HIV replication.

Materials and methods

DNA constructs and site-directed mutagenesis

The plasmid pNL4-3 was used as the parental viral DNA clone. The pNL4-3 gag fragment containing nucleotide number 113 to 2010 was inserted into pGEM-T vector and propagated in *Escherichia coli* JM109. To generate the two oligonucleotides used to prime DNA synthesis by *Pfu* DNA polymerase on a denatured DNA template. The oligonucleotide primers that were used to generate each mutant are shown in Table 1. The thermal cycle was 16 cycles of 95 °C for 30 s, 55 °C for 60 s and 68 °C for 10 min. Because of the background of wild-type plasmid DNA, the mutant molecules were enriched by using the restriction enzyme DpnI, which specifically cleaved fully methylated G\(^{Me6}\)ATC sequences. DpnI digested the bacterially generated DNA used as template for amplification, but it did not digest DNA synthesized during the course of the reaction in *vitro*. The reaction was transformed into *E. coli* JM109. Individual clones were picked up and the mutants were then verified by DNA sequencing. The correct mutants were cloned back into the parental infectious clone, pNL4-3.

### Table 1

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>S109A</td>
<td>Sense</td>
<td>5′-AACTACTGCTACCCTTCAGGAAACAATA-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TGAAGGATGCGTAGGATCTGCATATG-3′</td>
</tr>
<tr>
<td>S149A</td>
<td>Sense</td>
<td>5′-AGCTTACCTGCCATTCTGAGCTAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TCCAGAATGGCGTAGGCTATACCT-3′</td>
</tr>
<tr>
<td>S178A</td>
<td>Sense</td>
<td>5′-AGCAAGCTGAGCACAGAATTTG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TACCTTGCTGACGCTGCTCCTG-3′</td>
</tr>
<tr>
<td>S149D</td>
<td>Sense</td>
<td>5′-AGCTTACCTGCCATTCTGAGCTAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TCCAGAATGGCGTAGGCTATACCT-3′</td>
</tr>
<tr>
<td>K170A</td>
<td>Sense</td>
<td>5′-CAGCTATGCTGACCACTTGAAGGCG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TCATGCTGACCACTTGAAGGCG-3′</td>
</tr>
<tr>
<td>K203A</td>
<td>Sense</td>
<td>5′-ACTATTTACACAGTGTCAGGGCAGGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5′-TCCCAATGCTGCTAAATAGGTTCAACATC-3′</td>
</tr>
</tbody>
</table>

The underlined codons were the mutated codons.

Cell culture and virus production

To generate the viruses, the DNA constructs were transfected into 293T cells using SuperFect® Transfection Reagent (QIAGEN). Five micrograms of each plasmid DNA was diluted with DMEM without serum, proteins or antibiotics to a total volume of 150 μl and then mixed with 30 μl of SuperFect® Transfection Reagent. The samples were incubated for 5–10 min at room temperature to allow transfection complex formation, and 1 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) was added to the reaction tube containing the transfection complex. After 2 h of incubation of 293T cells (40% confluence) with the transfection complexes at 37 °C, DMEM containing the remaining complexes was removed from the cells and replaced by 5 ml of DMEM supplemented with 10% FBS and antibiotics. The supernatants were harvested on day 3 and assayed for p24 level using an antigen-captured ELISA (Vironostika®, Organon Teknika).

Phosphorylation assay

CA proteins of wild-type and mutant virions were immunoprecipitated by anti-HIV p24 (MoAb2 ARP454) and detected for phosphorylation using Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, Oregon). Virions were lysed in 1% NP-40 at room temperature for 1 h. The lysates were incubated with NET buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA pH 8.0) containing anti-HIV p24 antibody for 1 h at 4 °C on a rocking platform. Fifty microliters of protein A-Sepharose was added to the antigen/antibody mixture, and the mixture was incubated for 1 h at 4 °C on a rocking platform. The complex then were collected by centrifugation at 12,000 rpm for 20 s at 4 °C in a microfuge, and washed twice with 1 ml NET buffer then once with 10 mM Tris–Cl pH 7.5, 0.1% NP-40 for 20 min per wash. The pellet was mixed with Western blot loading buffer, heated in boiling water bath for 5 min, centrifuged and the amount of p24 in the supernatant was measured by an antigen-captured ELISA (Vironostika®, Organon Teknika). Supernatant with equal amount of p24 was loaded onto a 12% SDS-polyacrylamide gel.

Fluorescent staining of SDS-polyacrylamide gels using Pro-Q Diamond phosphoprotein gel stain was performed by fixing the gels in 45% methanol, 5% acetic acid overnight, washing with three changes of deionized water for 10 to 20 min per wash, followed by incubation in Pro-Q Diamond phosphoprotein gel stain for 180 min and destaining with three successive washes of 4% acetonitrile in 50 mM sodium acetate pH 4.0 (Schulenberg et al., 2003). The state of phosphorylation was visualized under a UV transiluminator.

The phosphorylation of the CA protein in virus produced from each mutant relative to wild-type virus was calculated from band densitometry data as the band intensity of the Pro-Q/Silver ratio from each mutant relative to wild-type virus was calculated.
Virus infectivity assay

Equal amount of wild-type and mutant viruses normalized by amount of p24 was incubated with 1 × 10^6 H9 cells at 37 °C for 3 h. The input viruses were removed by washing with four changes of 1 ml PBS and plated in 2 ml of RPMI1640 with 10% FBS in a 6-well plate. Virus infectivity was determined by measurement of p24 released in supernatant every 4 days using the antigen-captured ELISA.

Purification of HIV-1 core particles

Viral core particles were purified by a spin-through technique with some modification as previously described (Auewarakul et al., 2005). Three milliliters of cell-free supernatant containing wild-type and mutant virions was overlaid on a discontinuous sucrose density gradient. The gradient was composed of 1 ml of 50% sucrose at the bottom and 1 ml of 10% sucrose containing 0.1% Igepal CA-630 on the top. The overlaid gradient was then centrifuged at 100,000×g in a SW50.1 rotor (Beckman™) for 2 h at 4 °C. The pellet containing viral core particles was then resuspended in 10 μl of PBS, pooled and stored in aliquots at −80 °C. The core particles were quantitated by measuring the capsid p24 antigen with ELISA.

Cell lysate preparation

H9 cell lysate (25 × 10^6 cells/ml) was prepared by resuspending washed cell pellets in hypotonic lysis buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl2, 10 mM KCl and 0.5 mM DTT). The cell suspension was frozen and thawed, incubated on ice for 10 min and centrifuged at 12,000 rpm in a refrigerated microcentrifuge for 5 min. The supernatant was then stored at −80 °C until used in uncoating assay.

Uncoating assay

Wild-type and mutant core particles (1 ng in 2 μl of PBS) were incubated with 2 μl of H9 cell lysate at 37 °C for 30 min, then diluted in 5 ml of PBS and centrifuged at 100,000×g in a SW50.1 rotor (Beckman™) for 2 h at 4 °C. Incubations with hypotonic lysis buffer and p24 disruption buffer (Vironostika®, Organon Teknika) were done in parallel as negative and positive controls. The level of core disintegration was determined by measuring the level of p24 in the supernatant after centrifugation. The complete disruption of core by p24 disruption buffer was considered 100%, and percent disruption by lysates was calculated accordingly.

Phosphatase treatment

Core particle containing 5 ng of p24 antigen was treated with 50 units of Antarctic phosphatase (New England Biolab) in the supplied buffer at 37 °C for 60 min in a 20 μl reaction. A control reaction without the enzyme was performed in parallel. One microliter of the reactions was diluted in 2.5 μl of PBS, and 1 μl of these diluted cores was then used in the uncoating assay. The rest of the treated cores were immunoprecipitated and were detected for their phosphorylation status by Pro-Q Diamond phosphoprotein gel stain.

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References


