Identification of Novel Determinants of HIV-1 Uncoating in the Capsid Protein

By

Ruifeng Yang

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Approved:

Professor Christopher Aiken

Professor Gerald Stubbs

Professor W. Gray Jerome

Professor Mark Denison

Professor Rich D'Aquila

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BBS	BES-buffered saline
BlaM	β-lactamase
BSA	Bovine serum albumin
CA	Capsid protein
CCR5	CC chemokine receptor 5
CD4	Protein marker on surface of some cells
CDC	Centers for Disease Control
CMV	Cytomegalovirus
CypA	Cyclophilin A protein
CTD	Carboxy-terminal domain
CXCR4	CX chemokine receptor 4
DMEM	Dulbecco’s modified Eagle’s Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
Env	Envelope glycoproteins
ER	Endoplasmatic reticulum
FPLC	Fast protein liquid chromatography
Gag	Group specific antigen
GFP	Green fluorescent protein
gp120	Envelope glycoprotein gp120; SU

HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HRP	Horseradish peroxidase
HTLV	Human T-cell leukemia virus
IC50	Median inhibitory concentration
IN	Integrase protein
kb	Kilobases
KD	Knockdown
KDa	KiloDalton
LTR	Long terminal repeat
MA	Matrix protein
MLV	Murine leukemia virus
MOI	Multiplicity of infection
NC	Nucleocapsid protein
Nef	Negative factor
NIH	National Institutes of Health
NLS	Nuclear localization signal
NNRTI	Non-nucleoside reverse transcription inhibitor
NPC	Nuclear pore complex
NRTI	Nucleoside reverse transcription inhibitor
nt	Nucleotide

NTD	Amino-terminal domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Preintegration complex
Pol	Polymerase gene
PR	Viral protease
PVDF	Polyvinylidene difluoridene
REV	Regulator of virion protein expression
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
rpm	Revolutions per minute
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	Reverse transcription complex
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
STE	Sodium/Tris/EDTA buffer
SU	Viral surface envelope protein
TAR	Transctivation-responsive region
Tat	HIV transactivator of transcription
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral infectivity factor
VLP	Virus-like particle

Vpu	Viral protein U
vRNP	viral ribonucleoprotein complex
VSV	Vesicular stomatitis virus
VSV-G	VSV glycoprotein
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

HIV/AIDS

The human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome. During the last 20 years, AIDS has changed from being an unknown disease to a widespread pandemic, with an estimated 33.2 million individuals living with the disease in 2007. The rate of new infections is still increasing. It is estimated that over 2.1 million people died from AIDS in 2007 (UNAIDS, 2007). Over three-quarters of deaths occurred in sub-Saharan Africa. The pandemic is also spreading in Asia and Eastern Europe, and the impact of the disease is felt worldwide.

There is currently no vaccine or cure for AIDS. The major method for prevention is to avoid exposure to HIV virus. Current treatment consists of highly active antiretroviral therapy (HAART). Current optimal HAART options consist of combinations (or "cocktails") of multiple drugs to target multiple viral targets. While treatment is available to control the disease, the cost of the drugs is a barrier to providing medicine to the people most affected. Moreover, HIV mutants can emerge quickly to circumvent the current drug therapy. Therefore, novel drugs that work with different mechanisms, or an effective vaccine, is required in order to contain this disease.

Uncoating is a step of the HIV-1 lifecycle in which the viral capsid dissociates following entry of the viral core into the cell. The uncoating process is poorly understood. My doctoral research was directed toward identification of novel determinants of HIV-1 uncoating in the capsid protein. This study will help to characterize uncoating as a potential pharmacologic target in the HIV-1 life cycle.

Progression of AIDS

HIV is transmitted through contact with HIV-containing bodily fluids. HIV is spread by sexual contact with an infected person, by sharing needles and/or syringes (primarily for drug injection) with someone who is infected, or, less commonly, through transfusions of infected blood or blood clotting factors.

HIV infection leads to a progressive loss of CD4+ T cells and an increased viral load in blood. HIV-1 infection can be divided into three stages, consisting of acute infection, clinical latency, and severe clinical immunodeficiency (UNAIDS, 2007). During acute infection, the initial level of HIV in peripheral blood is commonly high and accompanied by a dramatic loss of CD4+ T cells. Flu-like symptoms are common in this stage, which may include fever, lymphadenopathy, pharyngitis, rash, myalgia, and malaise. Acute infection last about 28 days and is followed by clinical latency. During this phase, the immune system reaches an equilibrium in which CD4+ T cells rebound to around 800 cells per microliter and viral load declines to a steady level. The latency period varies from 2 weeks to 20 years. In the last phase, patients with fewer than 200 CD4+ T cells per microliter

of blood are considered to have AIDS (CDC, 1992). The loss of CD4+ T cells results in the collapse of the immune system and multiple symptoms resulting from opportunistic infections and malignancies. The infections include pneumonia caused by the fungus *Pneumocystis carinii*, brain infection caused by cytomegalovirus (CMV), widespread infection with a bacterium called MAC (mycobacterium avium complex), and widespread diseases with fungi such as histoplasmosis. The malignancies include Kaposi's sarcoma, lymphoma, and others (Afessa et al., 1998; Kaplan et al., 2000; Nelson et al., 1990).

Genomic Structure of HIV-1

The HIV-1 genome consists of two identical copies of a 9200 base pair positive-strand RNA molecule encoding nine open reading frames. Three of these encode the Gag, Pol, and Env polyproteins, which are cleaved by proteases during formation of a mature virion. HIV-1 also encodes four accessory proteins (Vif, Vpr, Vpu and Nef) and two regulatory proteins (Rev and Tat). At both ends of the HIV-1 genome, there are unique sequences 5' (U5) and 3' (U3), with flanking repeats (R) (Fig. 1-1).

Gag is a 55 kiloDalton (KDa) precursor polypeptide, which is cleaved into the structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC),

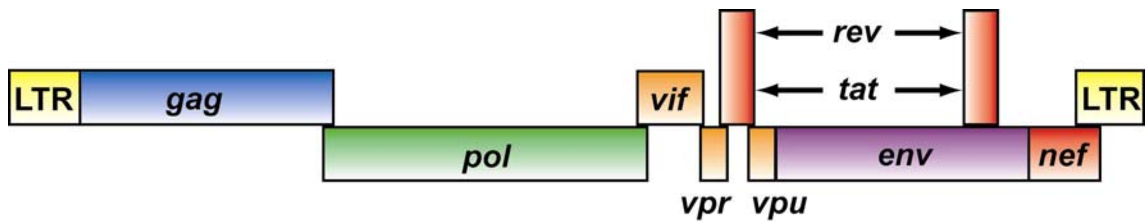

Figure 1-1. Genomic organization of HIV-1. The HIV-1 genome encodes the genes *gag*, *pol*, and *env*, as well as the accessory genes *vif*, *vpr*, *vpu*, *nef*, and regulatory genes *rev* and *tat*. The genome is approximately 9.7 kb long (Drawing provided by David Dismuke, with permission).

as well as the p6 peptide, by the viral protease. The *pol* gene encodes three viral enzymes: these enzymes are necessary for cleavage of viral polyproteins (protease, PR), synthesis of DNA intermediates of viral genome (reverse transcriptase, RT), and integration of the proviral DNA into the host genome (integrase, IN).

The Env proteins are cleaved into gp41 (transmembrane, TM) and gp120 (surface, SU) subunits by cellular proteases. SU binds to the CD4 molecule and co-receptors CCR5 or CXCR4 of the host cell membrane, and TM mediates the subsequent fusion of viral envelope with the cell membrane.

Rev and Tat are regulatory proteins required for viral replication. Rev is responsible for shuttling unspliced and singly-spliced transcribed viral RNA out of the nucleus into the cytoplasm by binding to the Rev-response element, a RNA structure residing within Env-encoding region. Tat acts as a transcriptional antiterminator by binding to the transctivation-responsive region (TAR) present in the 5' of newly transcribed RNA molecule.

Nef, Vpu, Vpr, and Vif are nonessential for replication in culture and are therefore termed accessory proteins. The main functions of Nef include downregulating CD4 and MHC class I levels, and enhancing viral infectivity. Vpu promotes degradation of intracellular CD4 molecules, thus preventing the retention of newly synthesized CD4 in ER as a CD4-Env complex. The role of Vpr in HIV-1 infection is not clear. Vpr is known to induce G2 arrest in cell cycle. Vif is a protein that counteracts the host restriction factor APOBEC3G in infected cells.

HIV-1 Virion

HIV-1 is a retrovirus that belongs to the lentivirus genus. The HIV virion is spherical and approximately 120 nm in diameter. The virion has a lipid envelope derived from the host cell membrane. The envelope also contains the viral glycoproteins gp120 and gp41, which form spike structures that mediate viral entry. HIV-1 MA protein lines the inside of the viral envelope via its covalently attached myristate and a positively charged protein domain. An electron dense conical viral core is present within the mature virion. CA protein forms the capsid shell of the core. Inside the core, a viral RNA ribonucleoprotein (vRNP) complex consisting of genomic RNA associated with NC, IN, RT and Vpr, is present (Fig 1-2).

Overview of the HIV Life Cycle

The life cycle of HIV in host cells, like other retroviruses, can be divided into two distinct phases: early and late (Fig. 1-3). The early events of the HIV-1 life cycle include cell entry via fusion with the cell membrane, uncoating of the viral core, reverse transcription, import into the nucleus, and integration of the proviral DNA into the host genome. The steps following integration are known as late events and include assembly and release of virions. Finally, the virion

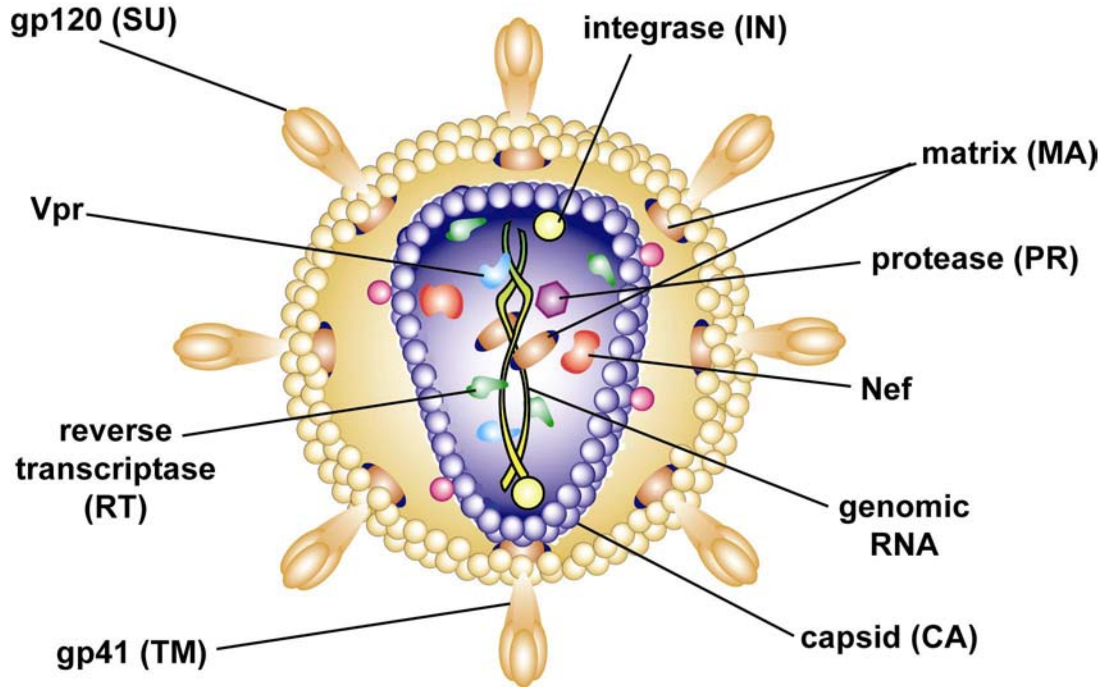

Figure 1-2. The mature HIV-1 virion. The particle is approximately 120 nm in diameter with a lipid bilayer envelope surrounding the viral core. The core is conical and has a capsid comprised of the CA protein. Protruding from the viral membrane are the envelope proteins that mediate attachment and entry. The two copies of the RNA genome are encased within the core along with the core-associated proteins. This figure does not represent all virion-associated proteins or the exact location within the virion of the proteins. The standard two-letter abbreviations for the protein names are shown in parentheses (Drawing provided by David Dismuke, with permission).

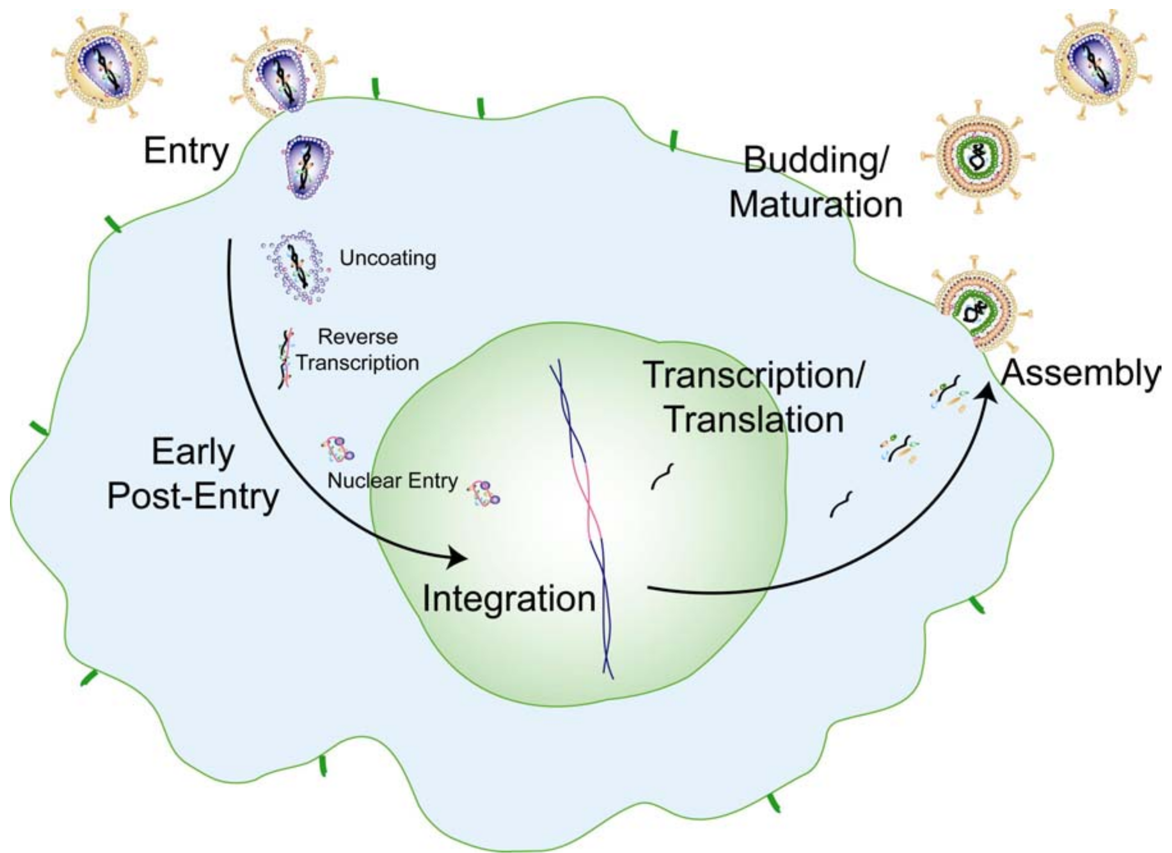

Figure 1-3. Overview of the HIV life cycle. Following attachment and entry into target cells, the virus goes through the early post-entry events of uncoating, reverse transcription, and nuclear entry. Within the nucleus, the viral DNA is integrated into the host genome. Viral transcripts are translated in the cytosol and assembled with viral proteins at the plasma membrane in an immature form. During or shortly after budding, the virus undergoes processing to a mature, infectious virion (Drawing provided by David Dismuke, with permission).

becomes mature upon cleavage of the Gag and Gag-Pol polyproteins during budding from host cells. Maturation leads to a dramatic reorganization of the internal virion structure, resulting in the formation of the conical viral capsid.

During viral entry, the HIV-1 envelope protein gp120 binds to the CD4 molecule of T-lymphocytes or macrophages. Besides CD4, two host co-receptors are also involved in HIV-1 binding. They are designated as the α-chemokine receptor CXCR4 and the β-chemokine receptor CCR5. CXCR4 is expressed in primary T-cells, while CCR5 is expressed in primary T-cells and macrophages. Upon binding, conformational changes in gp41 are induced that allow it to initiate the fusion event (Chan and Kim, 1998).

Upon fusion, viral cores are delivered into the cytoplasm, where they undergo a poorly-understood disassembly process termed uncoating. During uncoating, CA dissociates from the viral core to release a reverse transcription complex (RTC). The RTC then undergoes reverse transcription to become the preintegration complex (PIC) (Bukrinsky et al., 1992; Bukrinsky et al., 1993).

HIV-1 Reverse Transcription

HIV-1 reverse transcription is a step to produce a double-stranded DNA intermediate from the single-stranded RNA. Reverse transcription is catalyzed by the virus-encoded enzyme RT. Reverse transcription is a discontinuous process. The production of DNA begins with short and discrete segments which will be elongated during a process called strand transfer. Strand transfer involves translocation of short DNA product from initial synthesis site to acceptor template

regions at the other end of the genome. This reaction takes place in the RTC. The viral proteins RT, MA, NC, Vpr, IN, and Nef have been reported in this complex (Bukrinsky et al., 1993; Fassati and Goff, 2001). The completion of reverse transcription yields a linear viral DNA product. Cellular and viral proteins associate with the proviral DNA in a complex referred to as the preintegration complex (PIC) (Miller et al., 1997). The proper dissociation of CA from the HIV-1 core is thought to be required for reverse transcription. Through analysis of native viral cores isolated from intact virions, the Aiken lab previously demonstrated that HIV-1 CA mutants with either unstable or hyperstable cores are poorly infectious. For most of the mutants, the defect was attributed to an impaired ability of the virus to undergo reverse transcription in target cells. These data suggested that HIV-1 uncoating is a balanced process, requiring a capsid that disassembles at the proper rate in target cells (Forshey et al., 2002).

Cellular factors are also implicated in HIV-1 reverse transcription. The RTC associates with the cytoskeleton, and reverse transcription occurs almost exclusively in the cytoskeletal compartment (Bukrinskaya et al., 1998). Cell extracts have been shown to stimulate reverse transcription in permeabilized HIV-1 virions *in vitro* (Warrilow et al., 2008). Recently, two cellular proteins, HuR and AKAP149, were identified as RT-binding proteins and were found to be involved in HIV-1 reverse transcription (Lemay et al., 2008a; Lemay et al., 2008b).

HIV-1 Nuclear Entry

For successful infection, the PIC must traverse the nuclear membrane to reach the chromosomal DNA. Some simple retroviruses, such as murine leukemia virus (MLV), require the dissolution of the nuclear membrane during mitosis in order infect the cell. As a lentivirus, HIV-1 is capable of infecting terminally differentiated macrophages and cell cycle-arrested cells (Bukrinsky et al., 1992; Lewis et al., 1992; Schmidtmayerova et al., 1997; Weinberg et al., 1991).

The study of HIV-1 nuclear entry has a long and checkered history. The viral proteins MA, Vpr, and IN, have been implicated in HIV-1 nuclear targeting (Bouyac-Bertoia et al., 2001; Popov et al., 1998; von Schwedler et al., 1994). Most of these results have not withstood the test of time, leaving open the question of what viral function mediates nuclear entry. A later finding implicated a triple-stranded DNA structural element formed during reverse transcription (the "flap") in nuclear import (Zennou et al., 2000). Like the other studies, this claim has been met with skepticism by other investigators (Dvorin et al., 2002). Recently, our lab showed that a CA mutant (Q63A/Q67A) is associated with impaired nuclear import (Dismuke and Aiken, 2006). This mutant exhibited incomplete uncoating in target cells, as indicated by the presence of high levels of CA in the purified PICs.

Other studies have recently confirmed the involvement of the HIV-1 CA protein in infection of nondividing cells. By analyzing chimeras between HIV-1 and MLV, which cannot infect nondividing cells (Lewis and Emerman, 1994), the

Emerman group showed that the MLV CA protein renders HIV-1 unable to infect mitotically-arrested cells (Yamashita and Emerman, 2004). This group then reported that point mutations in CA selective impair the ability of HIV-1 to infect nondividing cells (Yamashita et al., 2007). Surprisingly, the defect was not at the level of nuclear import, suggesting that HIV-1 infection of nondividing cells depends on a function of CA following nuclear penetration. One of the most defective mutants was the Q63A/Q67A, the PICs of which our lab found to exhibit elevated CA and to be impaired for integration *in vitro* (Dismuke and Aiken, 2006). Collectively these studies demonstrate that CA, and perhaps uncoating, plays a crucial role in the ability of HIV-1 to infect nondividing cells. In Chapter III of this dissertation, I present additional evidence for a connection between HIV-1 uncoating and infection of nondividing cells. In Chapter III of this dissertation, I describe experiments in which I exploited the link between CA and HIV-1 infection of nondividing cells to understand the function of a second-site CA suppressor mutant.

Cellular factors have been reported to be involved in HIV-1 nuclear import. By screening IN-interacting proteins or siRNA knockdown, a karyopherin (TNPO3) have been identified as host proteins required for HIV-1 nuclear entry (Brass et al., 2008; Christ et al., 2008; Emiliani et al., 2005; Turlure et al., 2004). However, the role of host factor/virus interactions in nuclear import is not well understood.

HIV-1 Uncoating

Following HIV-1 fusion with cell membrane, it is thought that the viral core undergoes an uncoating process in which the core disassembles and releases the viral genome to continue the life cycle. Uncoating of the HIV-1 core has been defined as the dissociation of CA from the viral core resulting in a RNP complex (Fig. 1-4) (Aiken, 2006). By contrast to many other steps of HIV-1 infection, uncoating represents a relatively poorly understood process. One of the major reasons for this is the lack of a useful *in vivo* assay. Our laboratory has pioneered the use of biochemical assays to study HIV-1 uncoating *in vitro* (Aiken, 2009; Forshey and Aiken, 2003; Forshey et al., 2002; Kotov et al., 1999). HIV-1 cores can be purified by detergent treatment of virions and sucrose-gradient ultracentrifugation. Wild-type HIV-1 cores purified from wild-type HIV-1 particles were found to contain 15-20% of the CA present in the starting suspension of HIV-1 particles. This percentage of CA is defined as “yield of cores”. The CA of these purified cores can dissociate from vRNP spontaneously at 37°C. HIV-1 mutants with aberrant yield of cores after sucrose-gradient ultracentrifugation purification also exhibited defects in infectivity. In addition, the mutant cores also exhibited aberrant uncoating kinetics in the *in vitro* disassembly assay. These observations suggest a requirement for balanced intrinsic capsid stability for HIV-1 infection. The replication block for most of those mutants is prior to or at reverse transcription, indicating a dependence of reverse transcription on proper uncoating.

Many questions related to HIV-1 uncoating remain to be answered. For example, what viral and cellular factors regulate HIV-1 uncoating? What are the

domains in CA required for proper uncoating? What is the dependence of reverse transcription and nuclear import on HIV-1 uncoating?

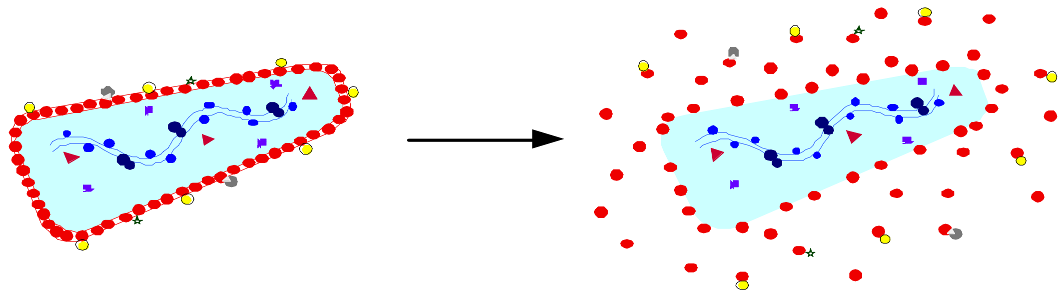

Fig. 1-4. Scheme of HIV-1 core uncoating. Disassembly of the capsid and the release of viral ribonucleoprotein complex (RNP) (Aiken, 2006).

Two unstable CA mutants, P38A and Q63A/Q67A, were competent for reverse transcription yet impaired for infectivity. Q63A/Q67A was found to be impaired for both nuclear import and integration (Dismuke and Aiken, 2006). Furthermore, PICs purified from cells inoculated with the Q63A/Q67A mutant contained elevated levels of CA, suggestive of an uncoating impairment *in vivo*. The viral DNA flap has also been implicated in uncoating (Arhel et al., 2007). Mutations preventing formation of the flap resulted in accumulation of viral cores at the nuclear membrane and a failure to enter the nucleus. These studies suggested that uncoating is a continuous process that occurs before and after reverse transcription which may also be involved in multiple post-entry steps of the viral life cycle. The viral MA protein may also play a role in uncoating. Mutations in MA have been associated with early post-entry defects in HIV-1 infection (Davis et al., 2006; Yu et al., 1992). Phosphorylation of multiple serine residues in MA was linked to an early, postentry step of infection (Kaushik and Ratner, 2004).

Host factors have also been implicated in HIV-1 uncoating. One such protein is cyclophilin A (CypA). CypA is a cellular peptidylprolyl isomerase that binds to the HIV-1 CA NTD via interaction with an exposed loop between helices 4 and 5 (Gamble et al., 1996; Luban et al., 1993). Disruption of the CypA-CA interaction, via mutations in CA (G89V or P90A) or addition of cyclosporin A (CsA), inhibits HIV-1 replication (Franke et al., 1994; Towers et al., 2003). An initial model held that incorporation of CypA into HIV-1 particles is necessary for

proper uncoating in target cells (Luban, 1996). . However, biochemical studies failed to reveal a difference in intrinsic stability between G89V and wild-type (Wiegers et al., 1999). More recent studies have demonstrated that a requirement for the CypA-CA interaction is manifested following entry of the core into target cells, and incorporation of CypA into virions appears to be biologically irrelevant (Hatziioannou et al., 2005; Sokolskaja et al., 2004). Despite many years of study, the precise role of CypA in promoting HIV-1 infection remains obscure.

Other factors influence uncoating likely exist. A recent study found that uncoating of purified HIV-1 cores is stimulated by lysate from activated CD4+ lymphocytes, while quiescent CD4+ lymphocyte lysate lack this activity (Auewarakul et al., 2005). Cell lysates also stimulate HIV-1 reverse transcription in permeabilized virions (Warrilow et al., 2008). These two biochemical systems may prove useful for identifying novel roles for host factors in early steps of infection, including uncoating.

Two proteins from simian cells, TRIM5α and TRIM-Cyp, inhibit HIV-1 infection by targeting the viral capsid (Nisole et al., 2004; Sayah et al., 2004; Stremlau et al., 2004). TRIM5α and TRIM-Cyp recognize CA and block replication prior to reverse transcription but after viral entry (Hatziioannou et al., 2004; Owens et al., 2004; Owens et al., 2003). TRIM5α and TRIM-Cyp are thought to promote premature uncoating, which is detrimental for the virus (Stremlau et al., 2006a).The restrictive action of TRIM5α and TRIM-Cyp can be

saturated with high doses of decoy virus particles (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002). The ability of decoy viruses to function in abrogation assays is dependent upon their having a stable core (Forshey et al., 2005; Shi and Aiken, 2006). This abrogation-of-restriction assay provides a tool to evaluate capsid stability in target cells, which I exploited in studies described in Chapter III.

HIV-1 CA

A HIV-1 core contains approximately 1500 molecules of the CA protein which encapsidate the vRNP (Briggs et al., 2004). CA protein is composed two distinct domains, N-terminal domain (NTD) and C-terminal domain (CTD), connected by a flexible linker (Gamble et al., 1996; Gamble et al., 1997; Ganser-Pornillos et al., 2007; Gitti et al., 1996) (Fig. 1-5). CA-CA interactions are involved in stabilizing HIV-1 cores. CryoEM studies have shown that CA is arranged as a hexameric lattice in mature HIV-1 cores (Li et al., 2000). Initial modeling studies suggested that NTD-NTD interactions are mediated through helices 1 and 2, which localized near the center of a hexamer (Li et al., 2000). Consistent with cryoEM studies, genetic and biochemical assays also provide evidence for the NTD-NTD interaction. Alanine substitutions of surface-exposed residues in helices 1 and 2 disrupt HIV-1 CA assembly *in*

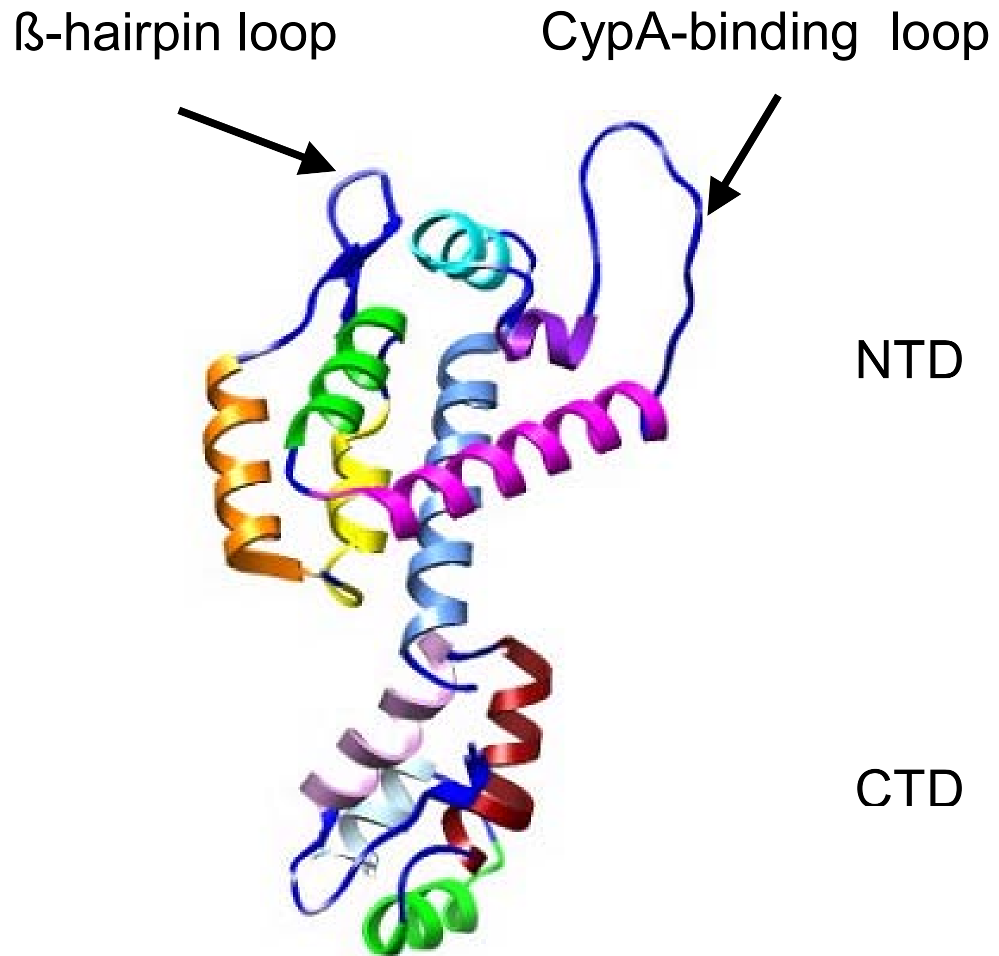

Fig. 1-5. Ribbon diagram of the mature HIV-1 CA protein. The structure is based on data from Gitti, et al., 1996, Gamble, et al., 1997 and Ganser-Pornillos, et al., 2007.

(Ganser-Pornillos et al., 2004; von Schwedler et al., 2003). Recently, a high-resolution crystal structure of a hexamer of MLV CA NTD revealed a novel interaction between helices 2 and 3 of adjacent subunits (Mortuza et al., 2004). Since MLV and HIV-1 CA proteins exhibit highly similar tertiary structures, I hypothesized that CA-CA interaction between helices 2 and 3 also exists in HIV-1 CA since HIV-1 CA also forms a hexamer lattice. Consistent with this notion, amide protons in helices 1, 2, and 3 are protected from deuterium exchange upon assembly (Lanman et al., 2004). Experiments described in Chapter II were performed to test this hypothesis.

HIV-1 CA molecules can also form dimeric contacts between adjacent CTDs. Purified HIV-1 CA forms a dimer in solution. The dimeric interface was mapped to helix 9 of the CTD (Gamble et al., 1997). Alanine substitutions at W184 and M185 in helix 9 of CA abolished dimer formation *in vitro* and impaired viral infectivity *in vivo* (Gamble et al., 1997; von Schwedler et al., 1998; von Schwedler et al., 2003). Thus, CTD-CTD interactions may be involved in regulating HIV-1 capsid stability.

The tertiary structure of CA is conserved among major retroviruses (Kingston et al., 2000).The existence of NTD-CTD interactions was initially inferred from studies of Rous sarcoma virus CA in which a lethal mutation in helix 9 of CTD was rescued by a second-site suppressor in helix 4 at NTD (Bowzard et al., 2001). An NTD-CTD intermolecular interaction in HIV-1 CA was detected between helices 4 and 9 by chemical cross-linking (Lanman et al., 2003). Deuterium protection assay also revealed the existence of an NTD-CTD

interaction *in vitro* and *in vivo* (Lanman et al., 2004). Recently, a medium-resolution cryoEM study revealed novel NTD-CTD interdomain interactions between neighboring CA molecules (Ganser-Pornillos et al., 2007). Potential contacts between helix 4 and regions of helices 8, 9, 10 and 11 were revealed. Portions of helices 8 and 11 were also found in proximity to helix 7 and the loop between helices 3 and 4. These observations identify new potential intersubunit contacts that may affect HIV-1 capsid stability, and thus, uncoating.

Research Objectives

Following fusion at the plasma membrane, HIV-1 particles undergo an uncoating process that is poorly understood. It is thought that viral and cellular factors are involved in the uncoating process. A properly balanced capsid stability appears to be critical for infection. A better understanding of viral uncoating process will likely provide new targets for AIDS therapy.

The main objective of my dissertation research was to identify novel CA determinants of HIV-1 capsid stability in order to better understand the regulation of HIV-1 uncoating during infection. I took two approaches to accomplish this goal. First, I performed alanine-scanning mutagenesis to further analyze a panel of CA mutants in helix 3. I identified a CA mutant, T54A, which exhibited reduced core-associated CA and was defective for infectivity. My studies, described in Chapter II of this dissertation, showed that the infectivity of T54A is CsA dependent. I also determined that T54A has a post-entry defect. Thus, T54 is a CA determinant outside the CypA-binding loop that can modulate the

dependence of HIV-1 infection on CypA. In studies described in Chapter III, I selected for second-site suppressors of HIV-1 uncoating mutants P38A and E45A by forward genetics. I identified second-site mutations T216I for P38A and R132T for E45A which rescued viral replication. This study identified novel determinants in CA that modulate HIV-1 uncoating. It also provided genetic evidence for functional interactions between the NTD and CTD of adjacent CA molecules in the HIV-1 capsid.

CHAPTER II

A MUTATION IN ALPHA HELIX 3 OF CA RENDERS HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 CYCLOSPORIN A RESISTANT AND DEPENDENT: RESCUE BY A SECOND-SITE SUBSTITUTION IN A DISTAL REGION OF CA

Introduction

Previous studies have shown that substitutions in the CypA-binding loop, A92E and G94D, confer HIV-1 resistance to CsA (Aberham et al., 1996; Braaten et al., 1996). Interestingly, infection by these mutants is actually enhanced by CsA in some cell lines, such as HeLa and H9, but not others, such as HOS and Jurkat (Aberham et al., 1996; Hatziioannou et al., 2005). Positions 92 and 94 reside in the CypA-binding loop in CA, but these mutations do not affect CypA-CA binding (Braaten et al., 1996), which suggests that CsA resistance is independent of this interaction. The CsA dependence implies that CypA binding to CA has a detrimental effect on infection by these mutants in some cell types.

To identify novel determinants of HIV-1 uncoating, I analyzed the phenotype of a poorly infectious HIV-1 mutant, T54A, encoding a Thr-to-Ala substitution in helix 3 of the CA NTD. The mutant particles contained cores of lower capsid stability than the wild type virus. I also found that the effects of the T54A substitution resemble those of previously characterized CypA-binding loop mutations A92E and G94D in that infection was enhanced by disruption of the

CypA-CA interaction.

Results

Phenotypic analysis of HIV-1 CA helix 3 mutants

To determine whether α-helix 3 plays a role in HIV-1 capsid stability, I performed alanine-scanning mutagenesis to analyze CA mutants in this helix. Gag processing and particle release were monitored by measurement of the yield of HIV-1 CA in the transfected cell supernatants and western blotting of sucrose cushion-pelleted virions from culture supernatants. Fully processed CA proteins were detectable in Q50A, T54A, and T58A virions, while L52A, N53A, M55A, L56A, and N57A contained increased levels of incompletely processed Gag intermediates. Decreased levels of particles were detected in supernatants of cells transfected with the L52A, N53A, M55A, L56A, and N57A proviral constructs. Single-cycle infectivity assays of the viruses showed that Q50A and T58A were fully infectious, while L52A, N53A, M55A, L56A, N57A were less than 2% as infectious as WT. T54A was about 10% as infectious as WT. Thus, T54A was competent for viral assembly and release but was moderately reduced in infectivity (Table 2-1). I therefore selected this mutant for further analysis of a potential HIV-1 uncoating defect.

HIV-1 containing the T54A substitution in CA is poorly infectious and is impaired for replication

To further characterize the infectivity impairment associated with the T54A

Mutation	Particle release	Gag processing	Infectivity
Q50A	**+**	**+**	**~100%**
L52A	**-**	**-**	**<2%**
N53A	**-**	**-**	**<2%**
T54A	**+**	**+**	**<10%**
M55A	**-**	**-**	**<2%**
L56A	**-**	**-**	**<2%**
N57A	**-**	**-**	**<2%**
T58A	**+**	**+**	**~100%**

Table 2-1. Gag processing, particle release, infectivity of HIV-1 CA helix 3 mutants.

mutant, I generated particles from wild-type and the mutant proviruses by transfection of 293T cells and assayed infectivity by titration on HeLa-P4 indicator cells. The specific infectivity of the T54A mutant, determined as the number of infected cells per ng of p24 in the inoculum, was reduced 10-fold relative to the wild type (Fig. 2-1A). To evaluate the effects of the T54A mutation on the efficiency of HIV-1 replication, I determined the kinetics of viral spread in the CEM human T-cell line. By contrast to the wild type virus, the T54A mutant particles failed to replicate within the 3-week culture period (Fig. 2-1B).

The T54A mutant is impaired at an early postentry step ininfection

Previous studies have demonstrated that HIV-1 particles containing substitutions in CA are frequently impaired in early steps in infection (Fitzon et al., 2000; Forshey et al., 2002; Tang et al., 2001). To further define the defect associated with the T54A mutation, I first analyzed entry of the mutant particles using the β-lactamase-based reporter fusion assay (Cavrois et al., 2002). Wild-type and T54A mutant particles were generated by cotransfection of 293T cells with proviral DNA and a BlaM-Vpr expression construct. The BlaM-carrying HIV-1 particles were then incubated with HeLa-P4 cells, and fusion was detected by quantifying the conversion of a cell permeable fluorescent BlaM substrate. The wild-type and T54A mutant viruses exhibited nearly equivalent activities in this assay (Fig. 2-1C), indicating that the T54A mutation does not impair the efficiency of HIV-1 fusion.

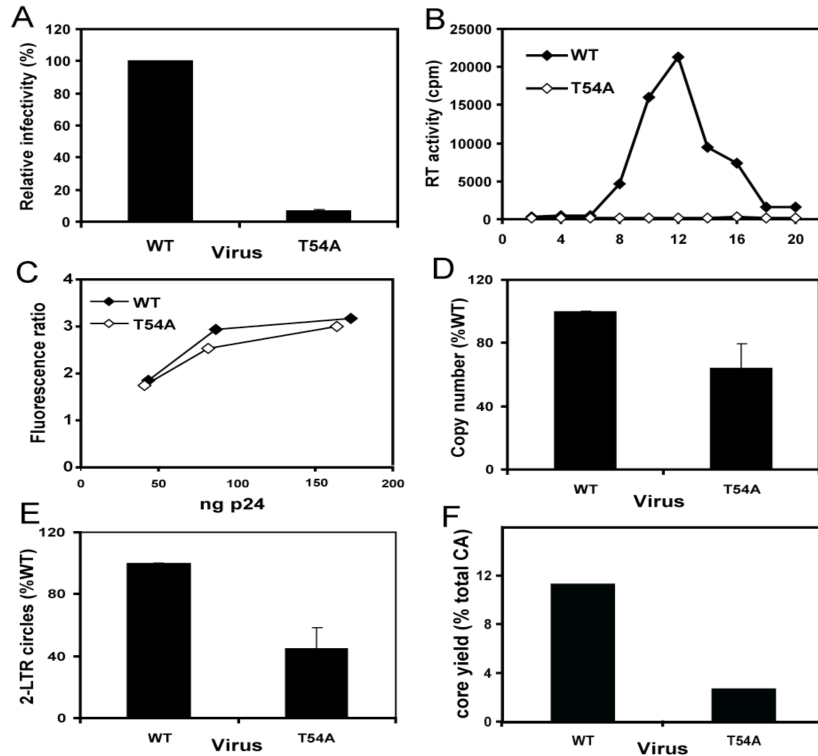

FIG. 2-1. The HIV-1 T54A mutant is impaired at an early postentry step of infection. (A) Single-cycle infectivity was assayed in HeLa-P4 target cells. Infectivity was determined as the number of infected cells per ng of p24 in the inoculum, and values are expressed as percentage of wild-type (WT) HIV-1 infectivity. The results shown are the mean values of three independent experiments, with error bars representing 1 standard deviation. (B) Replication kinetics of T54A and WT viruses in CEM cells. Cultures were inoculated with equal quantities of WT and T54A mutant viruses. Supernatants were sampled on the days indicated and assayed for RT activity. Shown is a representative growth curve for each virus from duplicate cultures. (C) BlaM reporter assays of HIV-1 fusion. The results are expressed as the extent of conversion of the cell-permeable BlaM substrate CCF2-AM and are representative of two independent experiments. (D and E) Real-time PCR quantitation of HIV-1 DNA synthesis in target cells. Cells were inoculated with DNAse I-treated virus stocks, and the total DNA was harvested at the indicated time postinoculation and analyzed for synthesis of late products of reverse transcription (D) and for two-LTR circular forms (E). The results shown are the mean values of three independent experiments, with error bars representing 1 standard deviation. (F) T54A mutant virions contain unstable capsids. Wild type (WT) and T54A mutant particles were concentrated and subjected to equilibrium ultracentrifugation through a layer of 1% Triton X-100 into a sucrose density gradient. Fractions were collected, and the p24 content was determined by ELISA. The yield of CA present in the fractions corresponding to cores was calculated as a percentage of the total CA quantity in each gradient. The values shown are the means of three independent experiments, with error bars representing 1 standard deviation.

I next asked whether the T54A mutant is competent for reverse transcription in target cells. To quantify the rate and extent of reverse transcription, wild-type and T54A mutant particles were added to cultures of HeLa-P4 cells, and the cells were cultured for various times and harvested. DNA was isolated from cell pellets, and HIV-1 DNA was subsequently quantified by real-time PCR using primers and probes specific for late products of DNA synthesis and the two-LTR circular form, a marker for nuclear import. Relative to wild type HIV-1, the synthesis of late reverse transcripts was only moderately reduced for the T54A mutant (Fig. 2-1D). Furthermore, the accumulation of two-LTR circles was reduced to a similar extent for the mutant (Fig. 2-1E). These results indicate that the T54A mutant particles are impaired, albeit only moderately, for reverse transcription in target cells and that the reverse transcribed products efficiently enter the nucleus.

T54A mutant particles contain unstable capsids

Previous studies in our laboratory have shown that mutations that alter the stability of the HIV-1 capsid are associated with impaired infection, likely due to aberrant uncoating in target cells (Fitzon et al., 2000; Forshey et al., 2002; Tang et al., 2001). To determine whether the T54A mutation alters the stability of the viral capsid, I quantified the yield of CA protein associated with cores released from wild-type and T54A mutant virions upon treatment with nonionic detergent and equilibrium density gradient sedimentation. For the wild-type virus, approximately 12% of the CA protein was detected in fractions

containing HIV-1 cores (Fig. 2-1F). For the T54A mutant, this value was reduced to approximately 3% of virion-associated CA. The decreased recovery of CA in these fractions suggests that the mutant virions contain cores with capsids that are less stable than those of the wild type.

A second-site suppressor mutation, A105T, restores replication and single-cycle infectivity to the T54A mutant

To further understand the defect associated with the T54A mutant, I selected a pseudorevertant by serial passage of the T54A mutant virus in CEM cells. Virus supernatants were harvested immediately following the peak of growth of the T54A mutant virions and were inoculated into fresh cells, and the cultures were maintained until reemergence of HIV-1 in the cultures. To determine whether phenotypic reversion was a result of acquisition of compensatory HIV-1 mutations, DNA was purified from CEM cells harvested near the peak of growth, and a DNA product fragment spanning the matrix protein and most of the N terminus of the CA coding region was generated by PCR, digested with *Bss*HII and *Spe*I, and inserted into the corresponding region of the wild-type R9 plasmid. Sequencing of several clones identified a mutation at codon 105 resulting in a substitution of Thr for Ala, in addition to the original T54A mutation. To determine whether the A105T substitution was responsible for the accelerated growth kinetics observed upon passage of T54A-derived virus, I assayed replication of the double mutant in CEM cells. The T54A/A105T double

mutant exhibited accelerated growth relative to the original T54A mutant yet was significantly delayed relative to the wild type (Fig. 2-2A). I also tested whether the A105T mutation could relieve the T54A infectivity impairment in the single-cycle infection assay. The results showed that A105T enhanced the infectivity of T54A to nearly that of the wild-type virus (Fig. 2-2B).

Infection by T54A, but not the double mutant T54A/A105T, is enhanced by CsA

Previous studies identified two mutations, A92E and G94D, which render HIV-1 capable of replicating in the presence of CsA (Aberham et al., 1996). Interestingly, the replication of these mutants is dependent on the presence of. CsA in some cell lines, such as H9 (Hatziioannou et al., 2005; Sokolskaja et al., 2004). An early study reported that adaptation of the A92E mutant for growth in the absence of CsA resulted in the acquisition of additional substitutions, including V86A and A105T (Aberham et al., 1996). Because the A105T mutation restored the ability of the T54A mutant to replicate in T cells, I hypothesized that T54A might also exhibit a CsA-resistant/dependent phenotype. To test this, I first analyzed the effects of addition of CsA on infection by the T54A mutant virus in HeLa-P4 cells. The results showed that infection by the T54A mutant increased by approximately threefold in the presence of CsA. The stimulating effect of CsA on infection was lost upon addition of the second-site compensatory mutation A105T, which rendered the virus CsA sensitive (Fig. 2-3A). As a control, I also constructed the A105T single mutant and tested its infectivity with or without CsA,

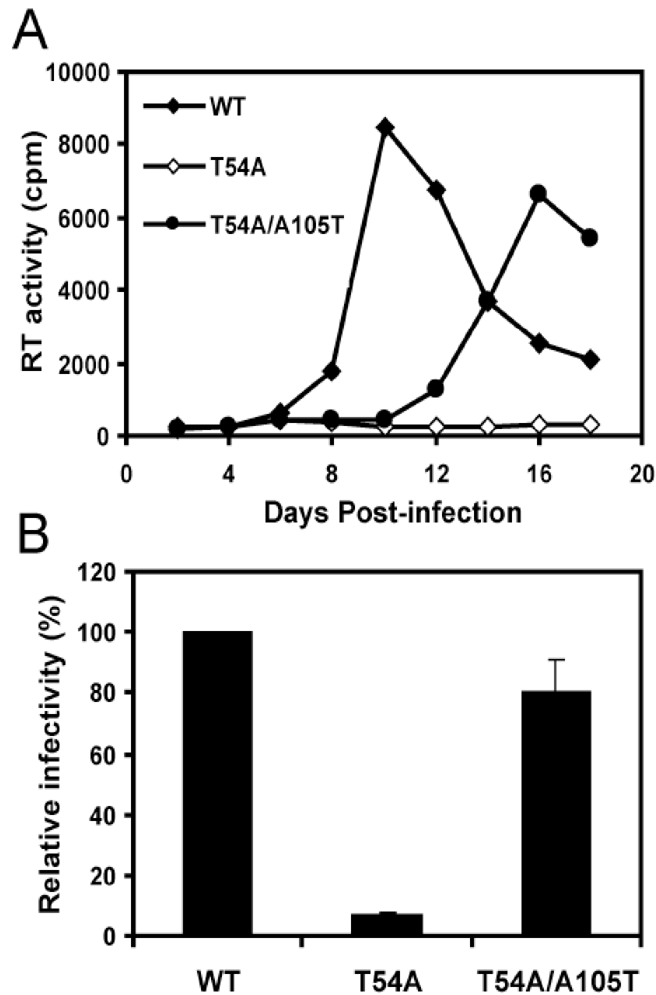

FIG. 2-2. Rescue of T54A replication by a second-site mutation. (A) Replication kinetics of wild-type and mutant HIV-1 in CEM cells. Samples were collected on the days indicated and analyzed for exogenous RT activity. Shown are the average RT values obtained from duplicate parallel cultures. (B) Single-cycle infectivity was determined by titration of viruses on HeLa-P4 reporter target cells. The results shown are the mean values of three independent experiments, with error bars representing 1 standard deviation.

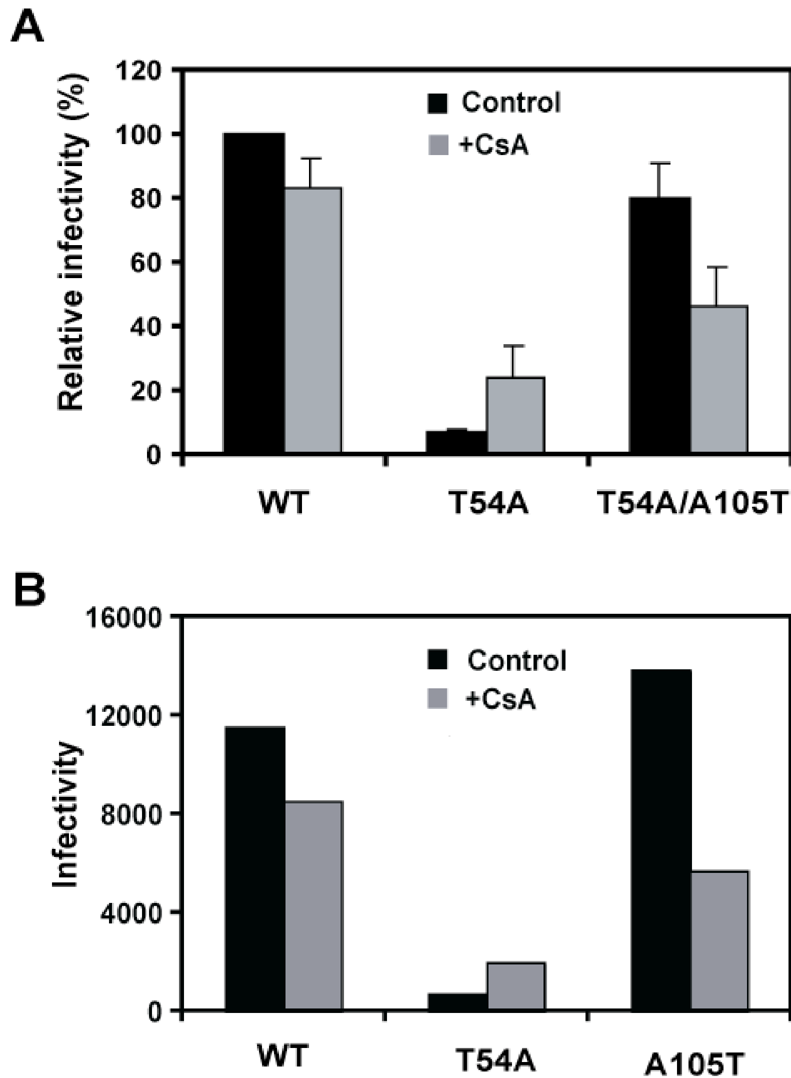

FIG. 2-3. Infection by T54A, but not T54A/A105T and A105T, is enhanced by CsA. An infectivity assay was performed in HeLa-P4 cells in the presence or absence of CsA (5 μM) during the infection. (A) WT, T54A and T54A/A105T. The results shown are the mean values from three independent experiments and are expressed as a percentage of the wild-type (WT) infectivity value from cultures lacking CsA. (B) WT, T54A and A105T. The results shown are from one of two independent experiments and are expressed as the number of X-gal-positive cells per nanogram of p24 in the inoculum.

which was similar to that of WT (Fig. 2-3B). Since the previously characterized CsA-resistant mutant A92E exhibits CsA dependence in HeLa or H9 cells (Aberham et al., 1996; Yin et al., 1998), I next assessed the effect of CsA on the replication of T54A and T54A/A105T in H9 cells. In H9 cells, the wild-type virus replicated efficiently, with a peak in p24 production at day 8 of postinoculation (Fig. 2-4A). CsA-containing cultures exhibited a moderate reduction in wild-type virus yield in the cultures. By contrast, T54A exhibited only low levels of replication, but its growth was markedly stimulated by CsA, resulting in a peak at day 12 (Fig. 2-4B). By contrast, the T54A/A105T double mutant replicated moderately efficiently in the absence of CsA, and its replication was partially inhibited by CsA (Fig. 2-4C). The kinetics of the double mutant resembled that of the wild type, but the virus yield was approximately one-fourth
that of the wild type. Collectively, these results indicate that infection and replication of the T54A mutant are stimulated by CsA.

A105T complements the CsA-resistant/dependent mutant A92E

My results demonstrated that CsA enhances infection of viruses containing mutations in two distinct domains of CA: T54 in helix 3 and A92E in the CypA-binding loop. My results also indicate that addition of the A105T mutation rescues the impaired infectivity of the T54A mutant. The A105T substitution has also been observed in a virus resulting from adaptation of the CsA (Aberham et al., 1996). An additional mutation, V86A, was also observed in that study; however it was not determined whether one or both mutations were

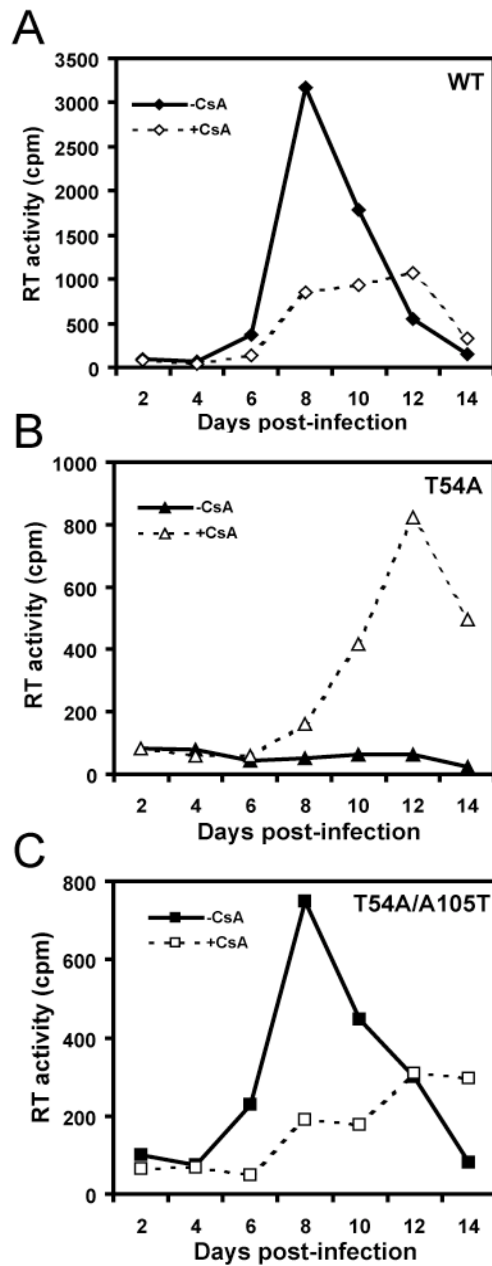

FIG. 2-4. CsA enhances replication of T54A in H9 cells. Replication of wild type (WT) (A), T54A (B), and T54A/A105T (C) HIV-1 in H9 cultures. CsA (2.5 μM) was present in the media of the indicated cultures for the duration of the experiment. resistant/dependent A92E mutant for growth in HeLa-CD4 cells in the absence of Samples were collected on the days indicated and analyzed for RT activity. Shown are the average RT values obtained from duplicate parallel cultures.

required to rescue the replication of the A92E mutant virus. To test whether the A105T mutation is sufficient to complement the A92E infectivity defect, I constructed the double mutant A92E/A105T and tested the virus for single-cycle infectivity in HeLa-P4 cells. The double mutant was nearly as infectious as wild-type HIV-1 in the absence of CsA (data not shown). The A105T mutation also rescued the ability of the A92E mutant to replicate in H9 cells in the absence of CsA, and replication of the A92E/A105T virus was not dependent on CsA (Fig. 2-5). Thus, the A105T mutation enhanced the replication of both T54A and A92E HIV-1 mutants.

The enhancing effect of CsA on infection by T54A mutant HIV-1 is dependent on the target cell

Previous studies have demonstrated that the ability of CsA to stimulate infection by the A92E mutant depends on the identity of the target cells (Hatziioannou et al., 2005; Sokolskaja et al., 2004). My findings that T54A replication is enhanced by CsA, and that the A105T mutation restores replication to T54A and A92E mutant viruses, suggest that these mutants have similar phenotypes. To further test this hypothesis, I assayed infection of different adherent human cells, HeLa-P4 and HOS cells, with VSV-G-pseudotyped reporter viruses. In HeLa-P4 cells, CsA stimulated infection by the T54A mutant virus; by contrast, in HOS cells, a slight inhibition of the mutant was observed in the presence of CsA (Fig. 2-6). As previously reported (Sokolskaja et al., 2004), infection of HeLa-P4 cells by wild-type HIV-1 was not affected by CsA, whereas

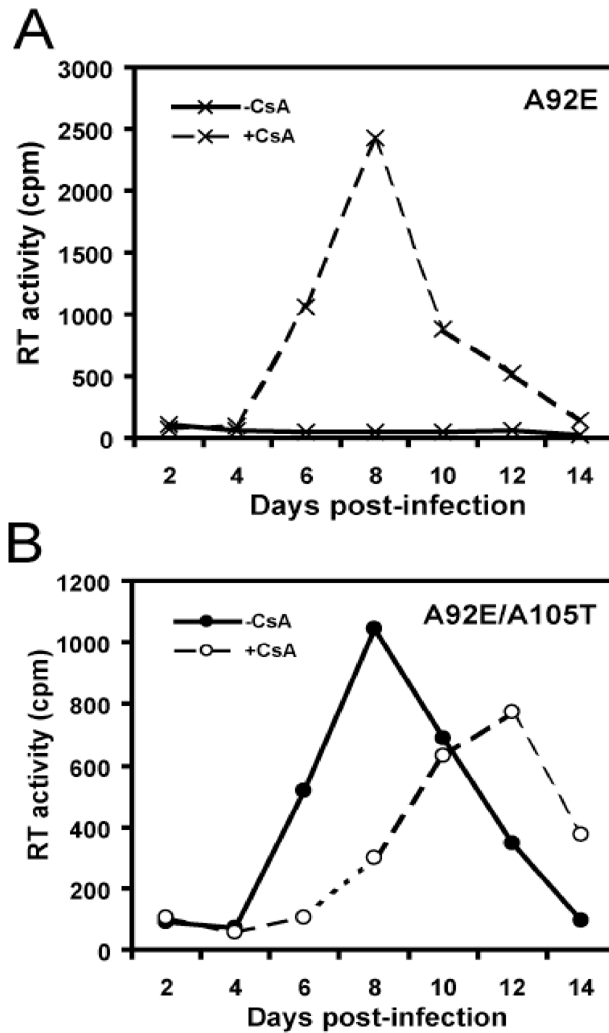

FIG. 2-5. A105T rescues A92E replication in H9 cells. H9 cultures were inoculated with (A) A92E and (B) the double mutant A92E/A105T. Cultures were maintained with and without CsA at 2.5 μM. Samples were collected on the days indicated and analyzed for RT activity. Shown are the average RT values obtained from duplicate parallel cultures.

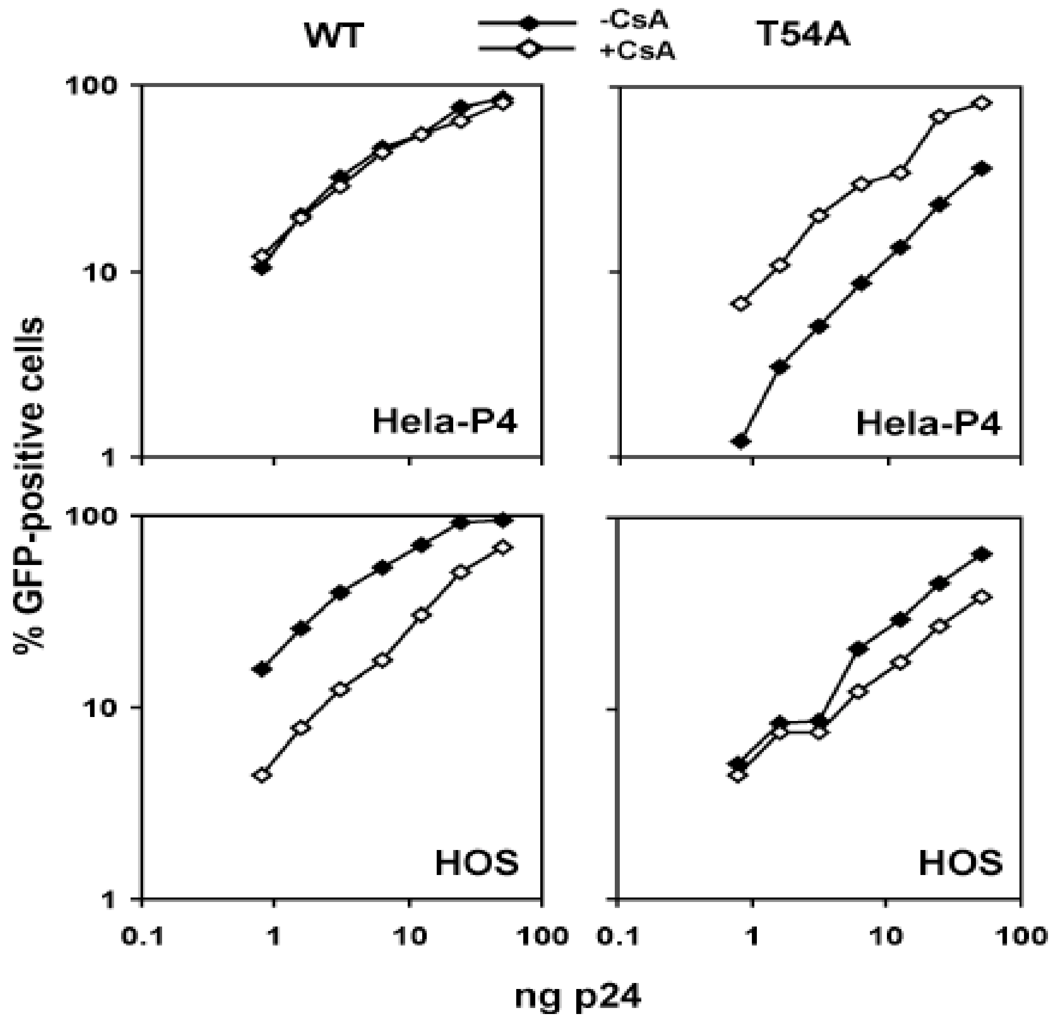

FIG. 2-6. CsA enhances infection by T54A in a target cell-dependent manner. Titration of VSV-G-pseudotyped HIV-GFP in HeLa-P4 or HOS target cells in the presence and absence of CsA (5 μM). Two days later, the percentage of infected cells was determined by flow cytometric analysis of GFP expression. The percentage of infected GFP-positive cells is plotted as a function of the input virus dose.

infection of HOS cells was inhibited by three- to fourfold (Fig. 2-6). In additional studies, I confirmed that the infectivity of the A92E mutant virus was enhanced by CsA in HeLa-P4 but not in HOS cells, as previously reported (Song and Aiken, 2007).These results indicate that the infectivity of the T54A mutant, like that of A92E, is enhanced by CsA in a target cell-specific manner.

To determine whether the enhancing effect of CsA on infection by the T54A HIV-1 mutant is mediated through CypA, I assayed infection of HeLa cells in which CypA expression was inhibited by a specific short-hairpin RNA (HeLa-CypA KD). These cells were previously shown to exhibit enhanced permissiveness to CsA-resistant/dependent mutants (Hatziioannou et al., 2005; Sokolskaja et al., 2004). HeLa-CypA KD cells were approximately 10-fold more permissive to infection by the T54A mutant than control HeLa cells (Fig. 2-7). Addition of CsA did not further enhance infection in the CypA KD cells. By contrast, the wild-type virus was equally infectious on HeLa and HeLa-CypA KD cells, and CsA had little to no effect on this virus in both cell types. Collectively, these results indicate that HeLa cells restrict infection by the T54A mutant virus by a mechanism that depends on CypA.

Structural analysis of binding of CypA to CA-NTD

To determine whether CypA binding induces structural changes in T54A and A92E mutants, ^{1}H-^{15}N HSQC NMR experiments were performed to assess the effects of CypA binding on the structure of CA. Uniformly ^{15}N-labeled CA-NTD recombinant proteins of WT, T54A, A105T, T54A/A105T, A92E, and

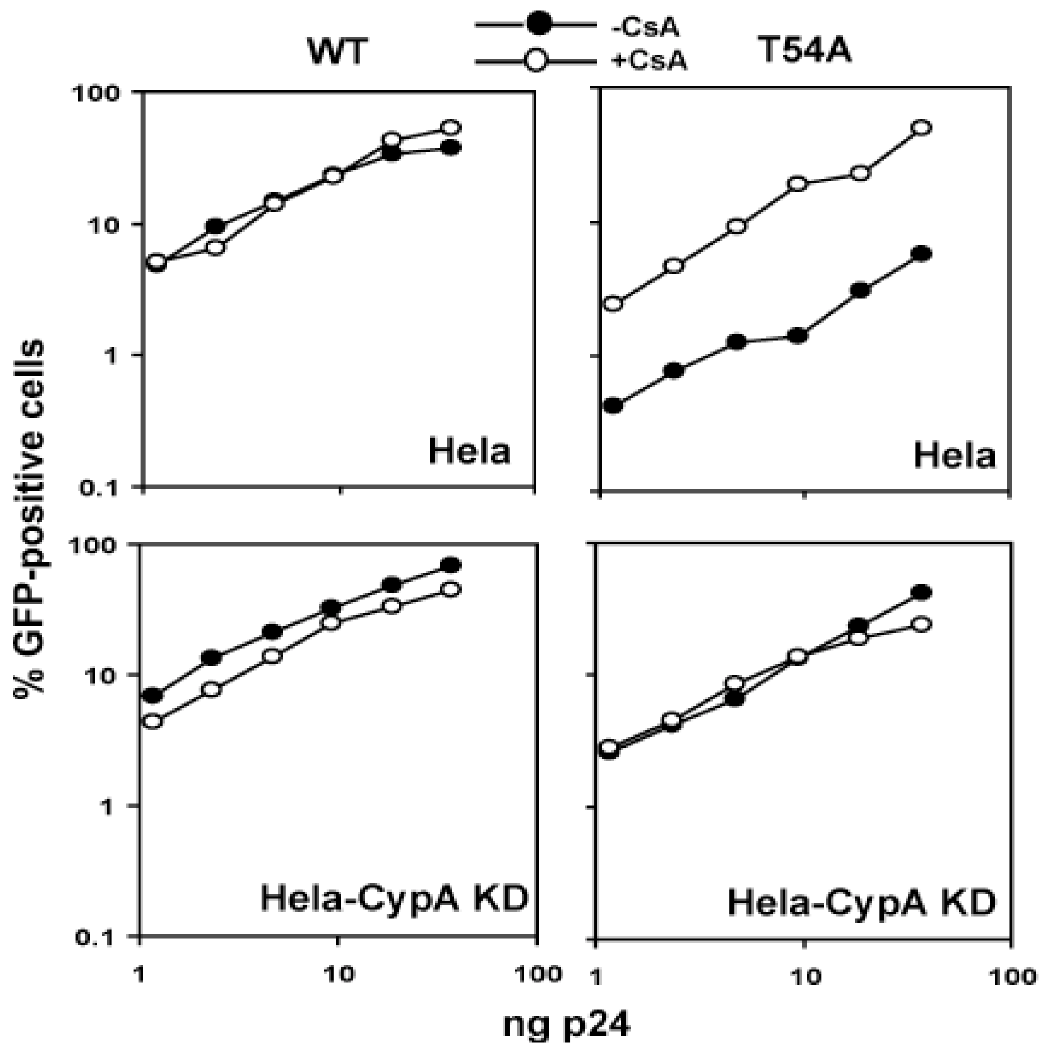

FIG. 2-7. Reduction of CypA expression enhances cellular permissiveness to infection by T54A mutant virions. VSV-G-pseudotyped wild-type and T54A and A92E mutant viruses encoding GFP were titrated onto control HeLa cells (top) and HeLa-CypA KD cells (bottom) in the presence and absence of 5 μM CsA. Infected cells were quantified by flow cytometry for GFP expression.

A92E/A105T were expressed in E. coli and purified by Jinwoo Ahn of the University of Pittsburgh. This study was performed in cooperation with Drs. In-Ja Byeon, Jinwoo Ahn, and Angela Gronenborn at the University of Pittsburgh. NMR HSQC spectra were acquired. The results shows a superposition of the ^{1}H-^{15}N HSQC spectra of CA-NTD of CypA-bound and unbound forms (Fig. 2-8). The results indicated that those mutations did not significantly alter the CA-NTD structure. Furthermore, CypA did not induce significant structural changes upon binding to the CA-NTD structures.

Discussion

In this study, I discovered that a HIV-1 mutant with a substitution of Ala for Thr54 in α-helix 3 of CA is poorly infectious due to an early postentry defect in the virus life cycle. Assays of viral DNA synthesis in target cells revealed a twofold reduction of reverse transcription of the mutant relative to the wild type, with no apparent defect in nuclear entry. The magnitude of the reverse transcription impairment does not seem to account for the 10-fold reduction in infectivity associated with the mutant, suggesting the possibility of an additional integration defect, as recently reported for another CA mutant (Dismuke and Aiken, 2006).

Addition of CsA enhanced infection by the T54A mutant particles in a target cell-dependent manner. This result was unexpected, since the previously identified CsA-resistant/dependent mutants both mapped to the CypA-binding

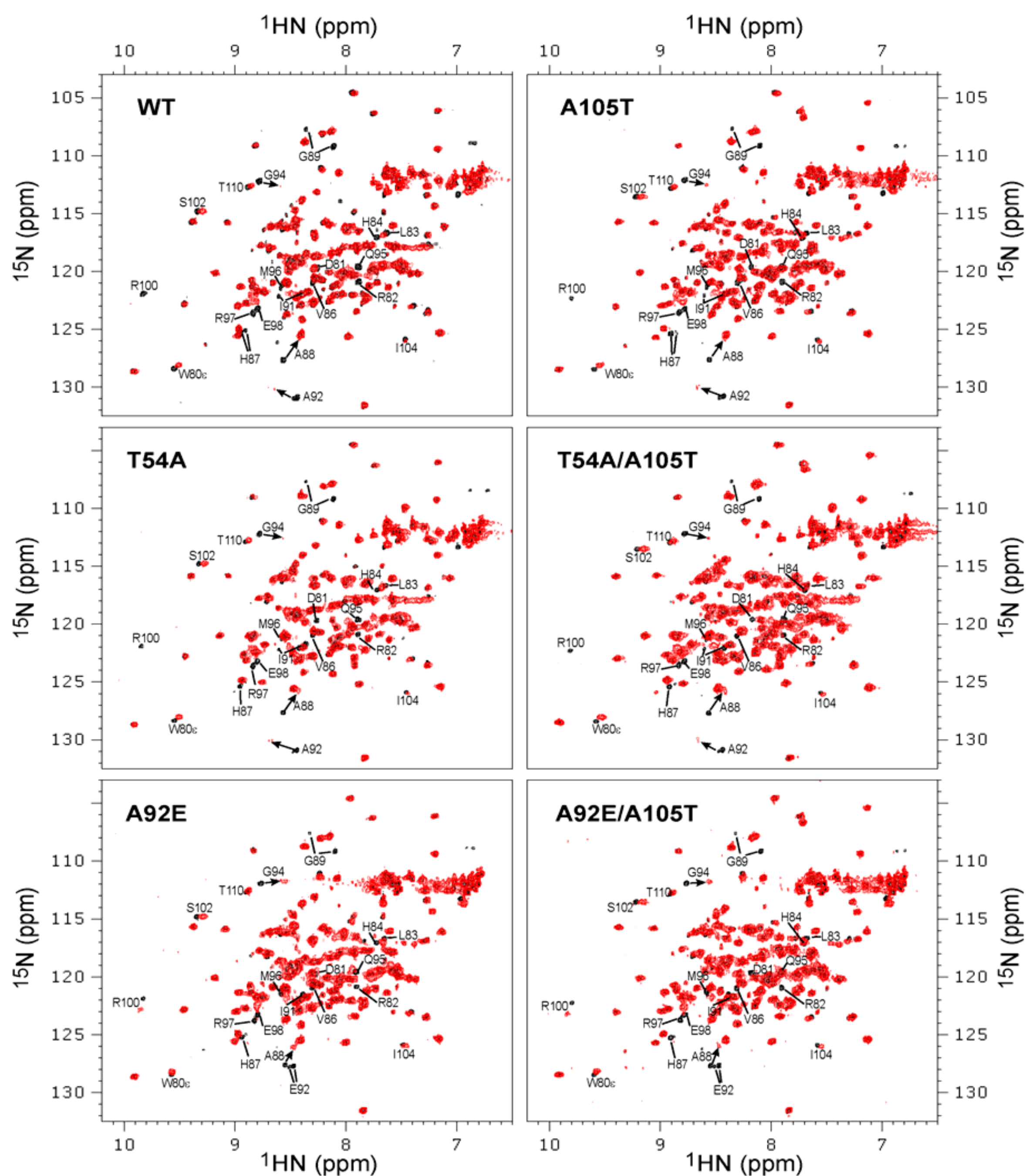

Fig. 2-8. ^{1}H-^{15}N HSQC NMR monitoring structural perturbations of CA-NTD caused by mutations. Superimposed ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled in the absence (red) and presence (black) of unlabelled CypA.

domain of CA, and T54A resides on the outer face of helix 3. I also identified the second-site mutation A105T that complements the T54A infectivity impairment, as well as that of the previously characterized CsA-resistant/dependent mutant A92E. Like the two previously identified CsA dependent/resistant mutants, the enhancing effect of CsA on infection by T54A was observed in HeLa, but not HOS, target cells. CypA-deficient HeLa cells were permissive for infection by the T54A mutant virus, but the mutant virus was still slightly impaired relative to wild-type HIV-1. Collectively, my results indicate that infection by the T54A mutant is restricted at a postentry stage of infection by a CypA-dependent mechanism. Because the addition of CsA did not restore the replication of the mutant to wild-type levels in H9 cells, I cannot exclude the possibility of additional impairments to replication resulting from the mutation, such as a potential uncoating defect resulting from the reduced stability of the mutant viral capsid.

Inhibition of the CA-CypA interaction by CsA is detrimental to the infectivity of most wild-type HIV-1 isolates, yet CsA promotes infection by the CA mutants A92E, G94D, and T54A in some cells. These observations have suggested the possibility of a host cell factor that inhibits the infection of the mutants in a CypA-dependent manner in target cells (Hatziioannou et al., 2005; Sokolskaja et al., 2004). The identity of the host factor is unknown, but the CsA-dependent phenotype appears to be independent of TRIM5α-mediated restriction (Hatziioannou et al., 2005; Sokolskaja et al., 2006). Somewhat paradoxically, the current view holds that CypA protects the wild-type HIV-1 capsid from an

unknown restriction factor in human cells (Towers et al., 2003). Interestingly, a subclone of the human TE671 cell line exhibited loss of TRIM5α-dependent restriction of N-tropic murine leukemia virus (i.e., Ref1 activity), as well as CypA-dependent infection by wild-type HIV-1 (Sayah and Luban, 2004). The observation that the cell subclone was unaltered in expression of TRIM5α suggests that an unidentified common host factor may be necessary for both apparent restrictions (Sayah and Luban, 2004; Sokolskaja et al., 2006).

The molecular basis for the opposing effects of CA-CypA binding on virus replication in wild-type and CsA-resistant/dependent HIV-1 mutants is currently unknown. The A92E and G94D mutations do not inhibit incorporation of CypA into HIV-1 particles and thus do not alter the affinity of CypA for the CA NTD (Jinwoo Ahn, personal communication). It has been proposed that A92E and G94D mutations perturb the formation of a type II tight turn in the CypA-binding loop, potentially resulting in different effects of the CA-CypA interaction on HIV-1 growth (Bukovsky et al., 1997). Consistent with this hypothesis, NMR structural studies revealed alterations in the type II turn in the G94D mutant CA protein (Campos-Olivas and Summers, 1999). The A105T suppressor identified in my study may compensate for these structural changes in CA proteins or may directly prevent binding of a host cell factor that restricts infection by the CsA-resistant/dependent mutants. Interaction of CA with CypA may also result in conformational changes outside the CypA-binding loop (Bosco et al., 2002). In support of this view, transfer of a small region of the CypA-binding loop of HIV-1 CA to the corresponding region of simian immunodeficiency virus renders this

virus CsA dependent (Bukovsky et al., 1997). My identification of the T54A mutation, which resides in a CA domain distinct from the CypA-binding loop and renders HIV-1 resistant and dependent on CsA, is consistent with the notion that CypA binding results in allosteric changes in the structure of CA, thereby modulating HIV-1 susceptibility to host cell restriction. However, our NMR data indicated that T54A mutation did not alter CA-NTD secondary and tertiary structures. Furthermore, NMR analysis was not able to reveal significant structural changes upon CypA binding to CA-NTD of T54A *in vitro*. Mutations in α-helix 3 have also been reported to reduce the susceptibility of HIV-1 to postentry restriction in simian cells (Owens et al., 2004). Restriction is dependent on TRIM5α and is modulated by CypA binding (Berthoux et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006b). Thus, the negative effect of CypA binding on infection by the T54A mutant is reminiscent of TRIM5α-dependent restriction in simian cells, yet restriction of the phenotypically similar A92E mutant is independent of TRIM5α in human cells. A detailed understanding of the mechanism of CsA-dependent infection by HIV-1 mutants in human cells will likely await identification of the putative restriction factor.

CHAPTER III

SECOND-SITE SUPRESSORS OF HIV-1 UNCOATING MUTANTS: GENETIC EVIDENCE FOR INTERDOMAIN INTERACTIONS IN THE VIRAL CAPSID

Introduction

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In this study, I employed forward genetics in order to identify novel mutations modulating HIV-1 uncoating. Here I identified single substitutions in CA which rescue the ability of virus containing unstable (P38A) and hyperstable (E45A) capsids to replicate in T cells. This study provides evidence of interdomain interactions in the viral CA.

Results

Second-site suppressor mutations restore replication to the P38Aand E45A mutants

To probe the possible role of domain interactions in maintaining optimal HIV-1 capsid stability, second-site suppressor mutations were isolated by serial passage of P38A and E45A mutant viruses in CEM cells. Virus supernatants were harvested immediately following the peak of growth of the mutant virions and were inoculated into fresh cells and the cultures were maintained until reemergence of HIV-1 in the cultures. To determine whether phenotypic

reversion was associated with acquisition of compensatory HIV-1 mutations, DNA was purified from infected CEM cells harvested near the peak of viral growth. Sequence analysis of PCR product from P38A culture revealed the presence of a mutation at codon 216 resulting in a substitution of Thr to Ile (T216I) in addition to the original P38A mutation. Sequence analysis of the PCR product from the E45A culture revealed the presence of a mutation at codon 132 resulting in a substitution of Arg to Thr (R132T) in addition to the original E45A mutation. No additional mutations were observed in the PCR-amplified viral sequences. To determine whether these second-site substitutions were responsible for the accelerated growth kinetics of the adapted viruses, a 500-bp PCR product spanning part of CA and NC coding region containing the additional mutation, was digested with *Spe*I and *Apa*I, and used to replace the corresponding region of the wild-type R9 plasmid. A replication assay was performed with the double mutant viruses in CEM cells. By contrast to the original P38A and E45A mutants, which failed to replicate in CEM cells within 26 days, the P38A/T216I and E45A/R132T double mutants both replicated yet were delayed relative to wild-type (Fig. 3-1A, B). These results demonstrate that the second-site mutations, T216I and R132T, permit the replication of P38A and E45A mutant viruses, respectively.

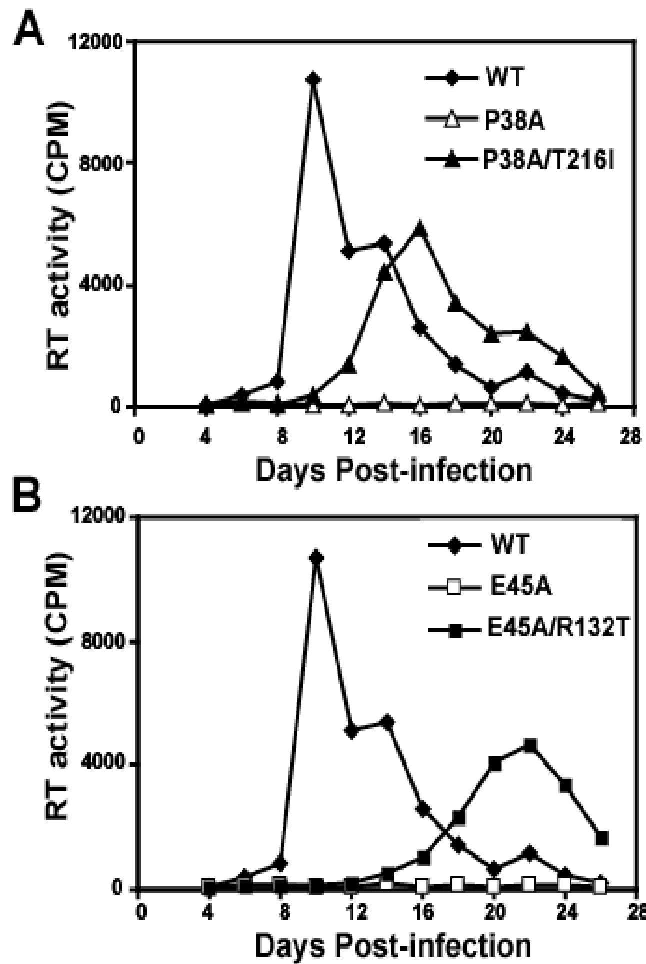

Fig. 3-1. Rescue of P38A (A) and E45A (B) mutations by second-site mutations T216I and R132T, respectively. Replication kinetics of the mutant and wild-type viruses in CEM cells. Samples were collected on the days indicated and analyzed for exogenous RT activity. Shown are the average RT values obtained from duplicate parallel cultures.

Second-site suppressor mutations enhance the infectivity of the P38A and E45A mutants in a single-cycle assay

Our group previously showed that P38A and E45A mutant viruses are impaired at an early post-entry stage of infection (Forshey et al., 2002). Therefore, I sought to determine whether the second-site mutations rescue P38A and E45A infection in a single-round reporter assay. The single-cycle assay requires completion of steps in the virus life cycle up to and including Tat expression and is therefore useful for probing defects in the early phase of infection. Quantitation of virus infectivity using the Hela-P4 cell line (HeLa-CD4/LTR-*lac*Z) revealed that second-site mutations T216I and R132T markedly enhance the infectivity of P38A and E45A, respectively (Fig. 3-2A). I also constructed and measured the infectivity of the single mutants T216I and R132T. Both single mutants showed similar infectivity to the wild-type (Fig. 3-2B), indicating that the suppression is specific for the defects associated with the original mutants and is not due to a general enhancement of HIV-1 infectivity. I conclude that the second-site mutations function by enhancing a step prior to integration.

Second-site suppressor mutations do not restore normal capsid stability *in vitro*

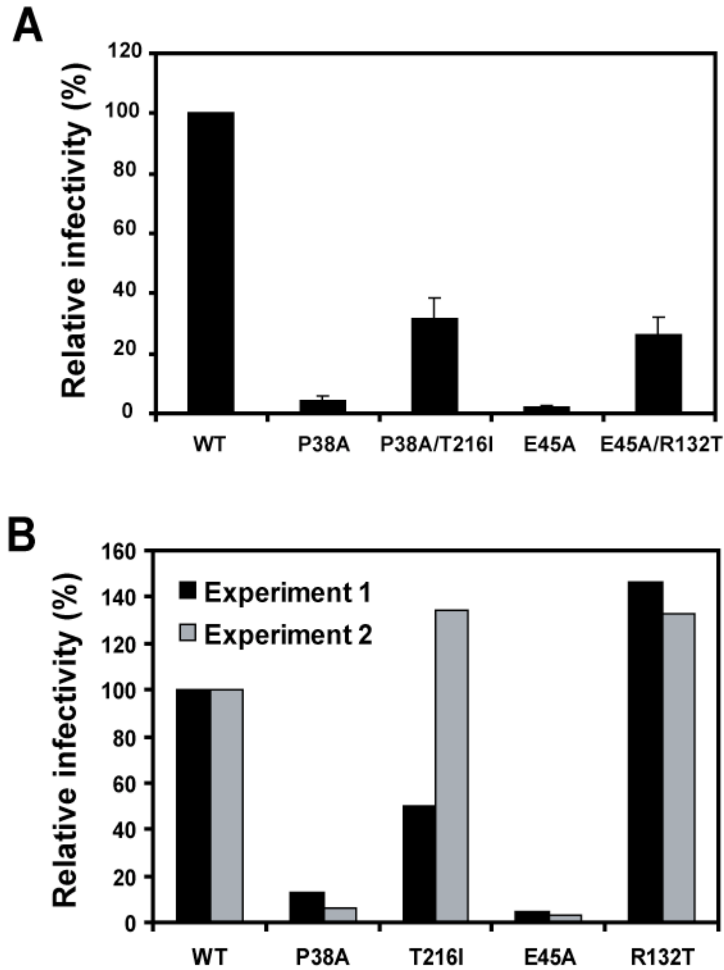

Fig. 3-2. Rescue of P38A and E45A infectivity defects by second-site mutations T216I and R132T, respectively. Single-cycle infectivity was assayed in Hela-P4 target cells. Infectivity was determined as the number of infected cells per ng of p24 in the inoculum, and values are expressed as percentage of wild-type HIV-1 infectivity. (A) Infectivity of WT, P38A, P38A/T216I, E45A, and E45A/R132T. Results shown are the mean values of three independent experiments, with error bars representing one standard deviation. (B) Infectivity of WT, P38A, T216I, E45A, and R132T. Results shown are the values from two independent experiments.

To determine whether the compensatory mutations restore normal capsid stability, I performed studies of cores purified from concentrated virions. I isolated cores from each of the mutant viruses. The level of core-associated CA was recovered in the fractions corresponding to the viral cores. This value was approximately 10% for wild-type HIV-1. By contrast, the P38A-derived cores exhibited a decreased level of CA (about 2%), while the CA content of E45A-derived cores was elevated to about 20% due to its elevated capsid stability (Fig. 3-3). Surprisingly, cores from double mutants P38A/T216I and E45A/R132T also exhibited CA levels that were similar to the single mutants (Fig. 3-3A, B). To further evaluate the relative stability of E45A mutant HIV-1 cores, I studied the kinetics of uncoating of purified cores *in vitro* in collaboration with Jiong Shi in the Aiken lab. The poor recovery of core-associated CA in the P38A mutant precluded its analysis. Cores isolated from E45A and E45A/R132T both exhibited slower uncoating *in vitro* compared to wild-type cores (Fig. 3-3C). Collectively, these results indicate that the suppressor mutations do not rescue the intrinsic capsid stability defects associated with the P38A and E45A mutants.

A second-site mutation T216I restores the activity of P38A for saturation of restriction in OMK cells

Previous studies have shown that HIV-1 mutants with unstable capsids, including P38A, are less effective at abrogating host restriction in simian cells. This defect is not due to a direct effect on CA recognition by restriction factors, as it was rescued by mutations which prevent cleavage of Gag between CA and NC

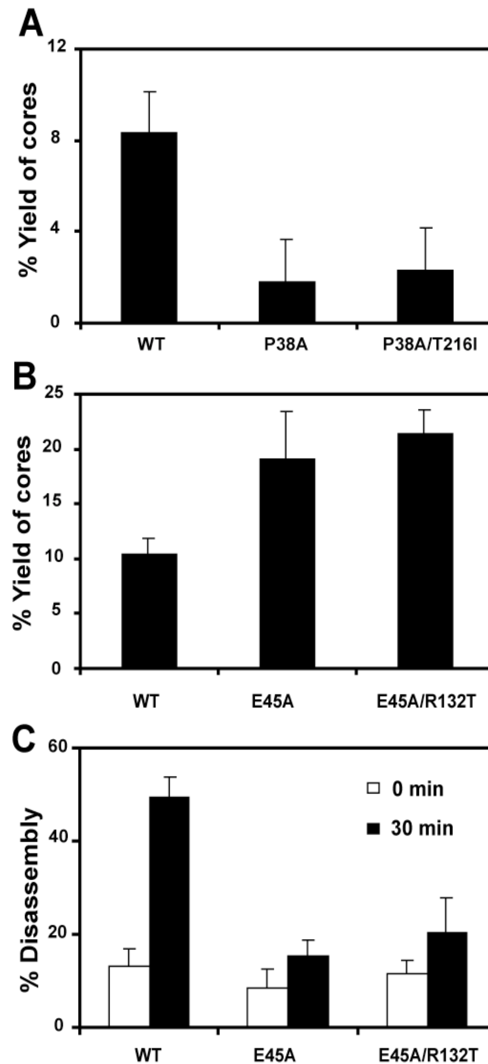

Fig 3-3. Second-site suppressor mutations do not restore capsid stability *in vitro*. (A and B) Concentrated virions were subjected to ultracentrifugation through a detergent layer into a sucrose density gradient. Yield of cores were calculated as percentage of total CA detected in the peak fractions of cores. Results shown are the mean values of three independent experiments, with error bars representing one standard deviation. (C) Kinetic of HIV-1 uncoating *in vitro*. Purified HIV-1 cores were incubated at 37°C for the indicated times, followed by separation of free and core-associated CA by ultracentrifugation. Supernatants and pellets were analyzed by p24 ELISA. The extent of disassembly was determined as the percentage of the total CA protein in the reaction detected in the supernatant. Results shown are the mean values of two independent experiments with duplicates in each experiment. Error bars represent one standard deviation.

by viral protease during particle maturation (Forshey et al., 2005; Shi and Aiken, 2006). I therefore asked whether the T216I mutation restores the ability of P38A virus to abrogate restriction in OMK cells, which restrict HIV-1 via TRIMCyp. Cells were inoculated with a fixed quantity of HIV-GFP reporter virions together with increasing amounts of wild-type or mutant non-GFP-encoding HIV-1 particles. This assay was performed by Jiong Shi in the Aiken lab. As previously reported, titration of wild-type particles led to a marked enhancement of infection by the reporter virus. By contrast, P38A mutant particles were approximately 50% less effective in enhancing infection by HIV-GFP (Fig. 3-4). Addition of the T216I mutation restored the abrogation activity of P38A mutant particles to nearly that of the wild type (Fig. 3-4). These results suggest that T216I restores the stability of unstable mutant P38A in target cells, despite the apparent lack of capsid stabilization *in vitro*.

***In vitro* capsid assembly analysis**

Thermodynamically, the stability of a polymeric complex is determined by the cumulative interactions in the complex. Purified HIV-1 CA protein can assemble into tubelike structures in a high salt buffer system *in vitro* (Campbell and Vogt, 1995; Ehrlich et al., 1992; Gross et al., 1997; von Schwedler et al., 1998). The E45A mutation was reported to accelerate HIV-1 CA assembly *in vitro* (Douglas et al., 2004), suggesting that this mutation enhances protein-protein interactions between CA subunits. Therefore, I asked whether the intrinsic

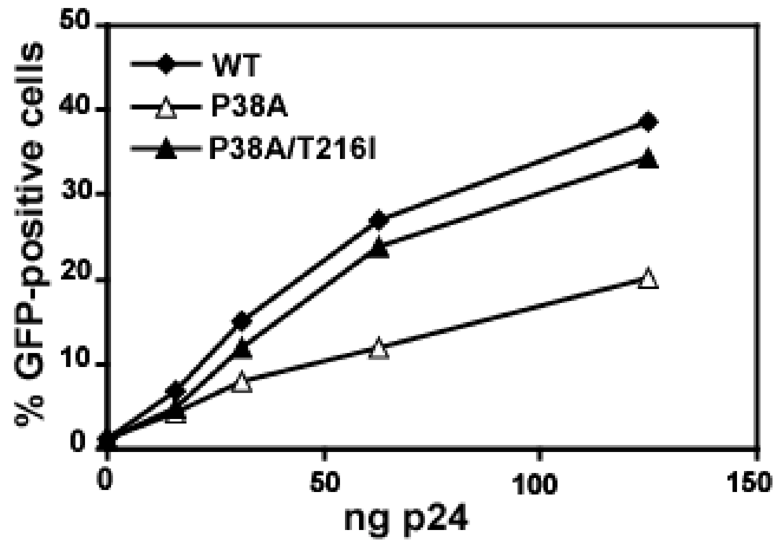

Fig. 3-4. The second-site mutation T216I restores the activity of P38A for saturation of restriction in OMK cells. OMK cells were infected with the indicated numbers of mutant particles and HIV-GFP particles. Results shown are the averages of duplicate determinations and are representative of two independent experiments.

stability of HIV-1 cores is related to the kinetics of CA assembly *in vitro* and whether the suppressor mutations would alter the rates of polymerization. Purified recombinant CA proteins encoding the E45A and E45A/R132T mutations were diluted into a high salt buffer, and the turbidity of the solution was monitored for 15 minutes following initiation of the reactions. The wild-type CA proteins assembled with moderate kinetics, with the reaction nearly complete within 10 minutes. By contrast, the E45A mutant reaction was complete within 2 minutes. However, the accelerated rate of assembly of E45A was not compensated by the suppressor mutation R132T as shown in mutant E45A/R132T. The unstable P38A mutant also exhibited a significant accelerated rate assembly, reacting completely within 3-4 minutes. The double mutant P38A/T216I also showed a similar accelerated rate, which is similar to P38A (Fig. 3-5A, B). This result demonstrated that the capsid stability mutants E45A and P38A accelerated the kinetics of assembly *in vitro*, yet the assembly kinetics of these mutant proteins were not affected by their corresponding suppressor mutations. Thus, the rate of CA assembly *in vitro* was not correlated with the impaired infectivity of P38A and E45A mutant viruses.

NMR characterization of CA^{151} (P38A, E45A, E45A/R132T and R132T) and comparison with CA^{151}

To determine whether P38A and E45A mutations cause altered secondary or tertiary structure changes, ^{1}H-^{15}N HSQC NMR experiments were performed to assess the effects of the E45A, P38A, E45A/R132T, and R132T mutations on the

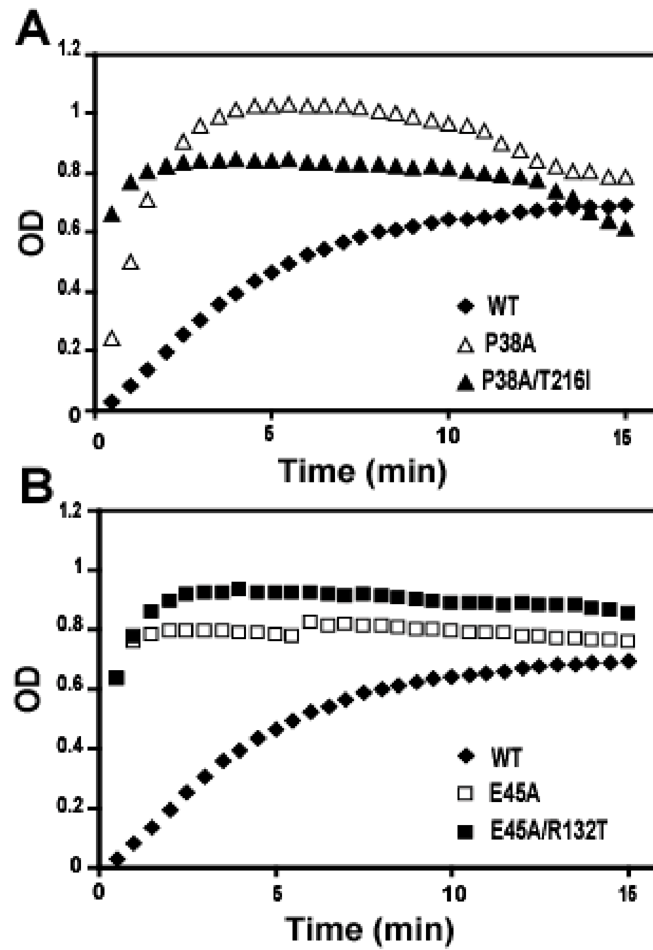

Fig. 3-5. *In vitro* capsid assembly analysis at 50 μM. Rate of CA protein assembled at a final concentration of 2.25 M NaCl. The results were averages of two trials and are representative of 3 experiments.

CA-NTD structure. This study was performed in cooperation with Drs. In-Ja Byeon, Jinwoo Ahn, and Angela Gronenborn at University of Pittsburgh. The results shows a superposition of the ^{1}H-^{15}N HSQC spectra of wild-type and E45A (Fig. 3-6A), P38A (Fig. 3-6B), E45A/R132T (Fig. 3-6C) and R132T (Fig. 3-6D). The spectrum of each of these mutants is very similar to that of wild type, clearly showing conservation of the overall global fold. However, these mutants are expected to show local structural changes. The E45A, E45A/R132T and R132T mutants show a spectral perturbation only in the region around the mutation site. Interestingly, P38A exhibits significant spectral changes on not only the region around mutation site (such as I37 and M39 on helix 2) but also the preceding loop (A31-S33), helix 1 (W23-V24), the loop connecting helices 3 and 4, and the vicinity (L56 and G60) and the C-terminal end region (V142 on helix 7). This rather substantial structural perturbation created by the P38A mutation may be responsible for the reduced stability of its capsid.

Second-site suppressor mutation R132T relieves the cell-cycle dependence of the E45A mutant

In a previous study, E45A was one of several specific CA mutants found to be selectively impaired for infection of growth-arrested cells (Yamashita et al., 2007). I therefore asked whether introduction of the R132T mutation in CA would relieve the cell-cycle-dependence of infection. My results showed that the E45A

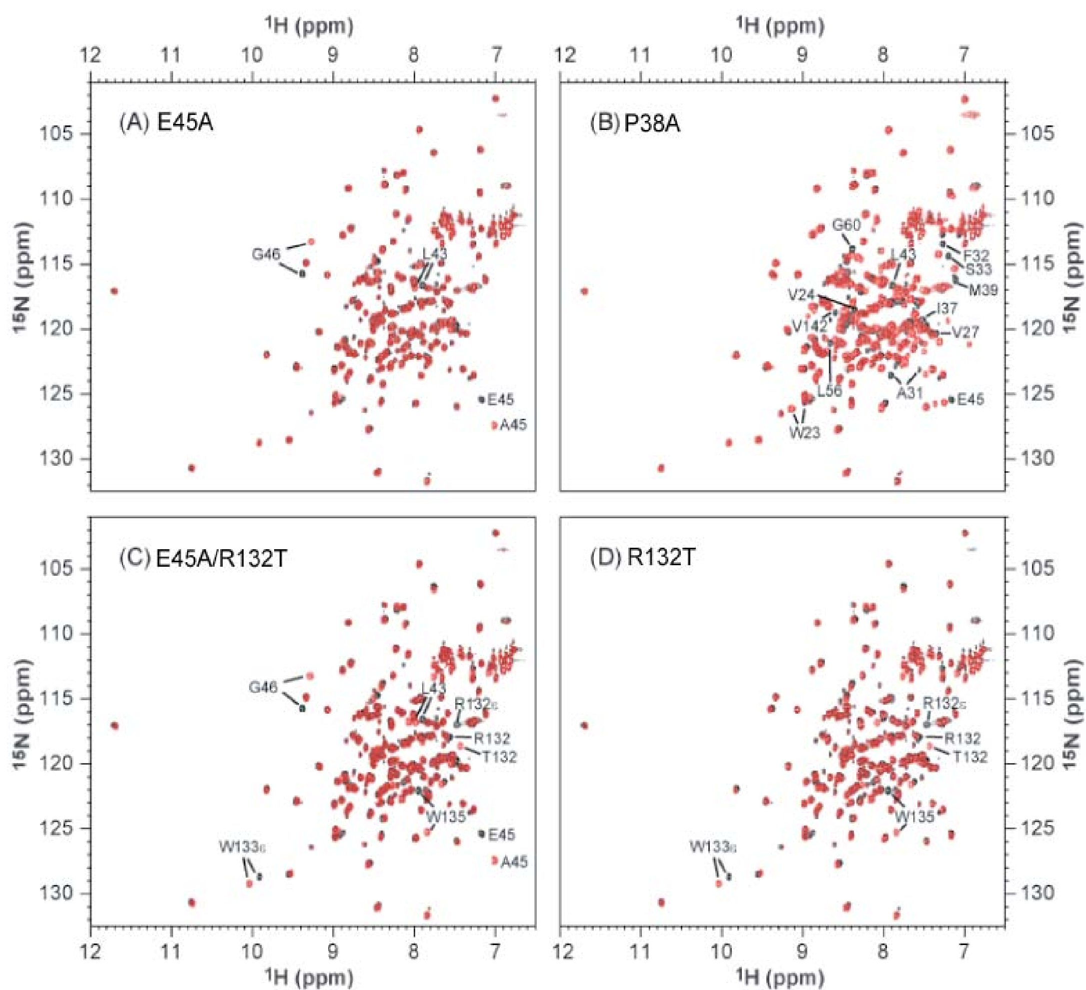

Fig. 3-6. Monitoring structural perturbations of CA-NTD caused by mutations with ^{1}H-^{15}N HSQC NMR. Superimposed ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled wild-type (black) and mutants (red), (A) E45A, (B) P38A, (C) E45A/R132T and (D) R132T. The peaks showing substantial perturbation due to the mutation are labeled with assignments.

exhibited ~8 fold decrease for infectivity in aphidicolin-arrested Hela-P4 cells relative to non-treated cells, while the wild-type and double mutant E45A/R132T viruses each exhibited only a slight decrease (less than 2 fold) in infection in arrested cells. Thus, the second site mutation, R132T, relieves the cell cycle-dependence of E45A. By contrast, the unstable mutant, P38A, and the double mutant, P38A/T216I, each exhibited similar behavior to the wild-type virus (Fig. 3-7).

Second-site suppressor mutation R132T reverses the resistance of the E45A mutant to an antiviral compound targeting CA

An experimental compound (PF-03450074) has been shown to bind HIV-1 CA and inhibit HIV-1 infection (Pfizer, unpublished data). Treatment of HIV-1 particles with this compound destabilized the capsid, suggesting that PF-03450074 inhibits HIV-1 infection by inducing premature uncoating in target cells (J. Shi and C. Aiken, unpublished data). I therefore reasoned that the infectivity of an HIV-1 mutant with hyperstable cores would be less sensitive to the compound, while the mutant with unstable cores might be more easily inhibited. To test this, I analyzed the effects of addition of PF-03450074 on infection by the E45A and P38A mutant viruses and the double mutants containing second-site suppressors in HeLa-P4 cells. Infection by the WT virus exhibited an IC50 about 0.3 μM (Fig. 3-8). In contrast, the hyperstable E45A mutant was unaffected by the compound until the concentrations of up to 5 μM. Addition of the second-site mutation R132T reversed the effect (Fig. 3-8A), redering the virus sensitive to the

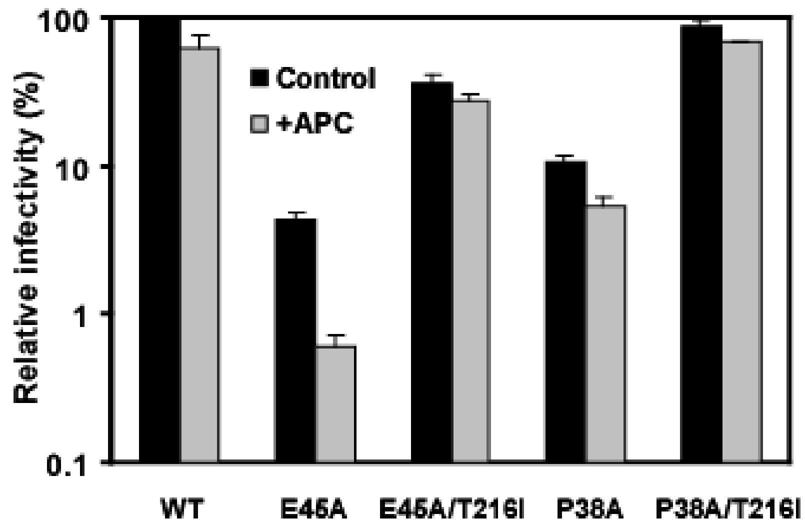

Fig. 3-7. Second-site suppressor mutation R132T relieves the cell-cycle dependence of the E45A mutant in a single-cycle infectivity assay. As controls, the unstable mutant, P38A and double mutant, P38A/T216I, were tested. Hela-P4 cells were inoculated in the presence and absence of aphidicolin (2 µg/ml). Infectivity was determined as the number of infected cells per ng of p24 in the inoculum, and values are expressed as percentage of wild-type HIV-1 infectivity. Shown are the mean values of triplicate infections, with error bars representing one standard deviation. The results shown are from one representative of three independent experiments.

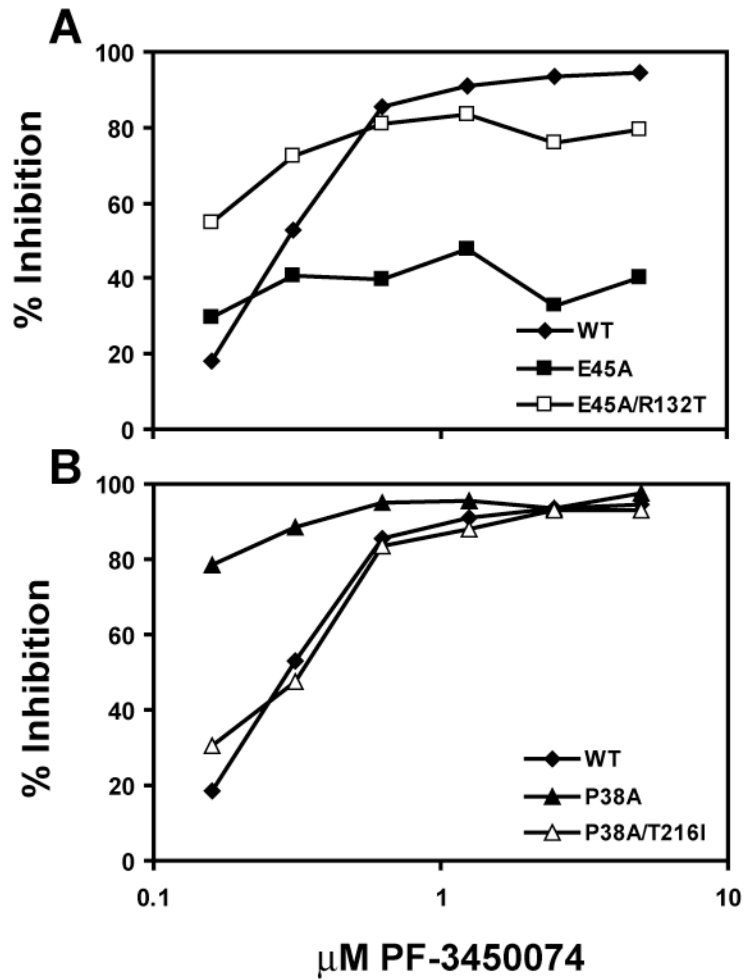

Fig 3-8. Drug sensitivity experiment. An infectivity assay was performed in HeLa-P4 cells in the absence or presence of PF-3440074 with various concentrations (0.32, 0.63,1.25, 2.5, 5 μM) during the infection. The results shown are from one representative of three independent experiments.

drug. Compared to the WT, the P38A mutant was more sensitive to the compound, with 80% inhibition at a concentration of 0.15 μM, while the double mutant P38A/T216I exhibited drug sensitivity similar to WT (Fig. 3-8B). These results suggest that the second-site mutations restore the infectivity of E45A and P38A by reversing the capsid stability defects in target cells.

Discussion

In this study, I isolated second-site suppressors of HIV-1 CA mutants P38A and E45A, which were previously shown to destabilize and hyperstabilize the viral capsid. These and other CA mutations that altered capsid stability also reduced viral infectivity, leading to the conclusion that the stability of the capsid must be properly balanced for retroviruses to complete the early postentry steps in infection (Craven et al., 1995; Dismuke and Aiken, 2006; Forshey et al., 2005; Tang et al., 2003b; Wacharapornin et al., 2007). The biological relevance of the biochemical stability studies was further established by subsequent studies establishing that capsid-destabilizing mutations impair the ability of HIV-1 to saturate restriction by TRIMCyp and TRIM5α (Forshey et al., 2005; Shi and Aiken, 2006). Thus, mutations that destabilize the viral capsid *in vitro* also lead to premature capsid disassembly in target cells.

Remarkably, the second site suppressors which I identified did not correct the intrinsic stability defects associated with the original mutations, yet the respective double mutants (P38A/T216I and E45A/R132T) were capable of

replicating in T cells and were markedly enhanced in infectivity relative to the corresponding single mutants in single-cycle reporter assays. Viruses encoding only the suppressor mutations T216I and R132T were similar in infectivity to wild type HIV-1, indicating that the rescue was specific for the original mutants. This observation suggested that the suppressors might act by altering interactions in the capsid that are not detected in our biochemical assays. Similar observations were reported for an RSV mutant (Bowzard et al., 2001). Mutation L171V in CA, which rendered the viral core hypersensitive to detergent, was rescued by A38V, but the suppressor only partially restored the capsid stability. I was also unable to detect an effect of the suppressor mutations on the assembly of purified mutant CA proteins *in vitro*. While both E45A and P38A CA proteins assembled more rapidly than wild type CA, the double mutant proteins E45A/R132T and P38A/T216I exhibited assembly kinetics that resembled that of the corresponding single mutant proteins. Thus, it appears that neither biochemical assay system recapitulates all of the requirements for proper HIV-1 uncoating in target cells. Nonetheless, for the P38A mutant, it seems likely that the stability defect in target cells is corrected by the T216I mutation. The P38A/T216I double mutant was fully competent for abrogation of host restriction, suggesting that the capsid does not undergo rapid disassembly in the cytoplasm. For this reason, I suspect that the T216I mutation alters the virus interaction with host factors, resulting in effective stabilization of the capsid in target cells despite its instability *in vitro*.

Other evidence that suggests the second-site suppressors restore the capsid stability in target cells is based on the drug sensitivity experiment with

compound PF-03450074. The binding of the drug to these mutants was not altered relative to WT HIV-1 (unpublished data, J. Zhou and C. Aiken). This observation suggest that the second–site suppressors restore the infectivity by stabilizing capsid stability in target cells, not by changing the binding of the compound.

HIV-1 CA consists of two distinct domains (NTD and CTD) connected by a flexible linker. Structural studies of assembled HIV-1 CA suggest that several intersubunit interactions may control capsid stability. Helices 1, 2 and 3 in the NTD appear to form one intersubunit interface. Helix 9 in the CTD also forms a dimeric interface. Interestingly, the P38A mutation in the NTD was rescued by T216I in the CTD. NMR analysis of purified NTD proteins demonstrated that the P38A mutation does not grossly alter the secondary and tertiary structure. An analogous interdomain suppressor mutant was previously reported for RSV, providing the first genetic evidence for a functional NTD-CTD interaction in a retroviral capsid. Two suppressor mutations in the NTD (P69Q, helix 4; A38V, helix 2) rescued two CTD mutations (R170Q and L171V in helix 9, respectively) (Bowzard et al., 2001). Structural studies have identified intermolecular NTD-CTD contacts in CA dimer crystals, yet these structures are difficult to reconcile with models of the retroviral capsid that are supported by low resolution image reconstructions (Li et al., 2000). Crosslinking studies have identified an interaction of helix 4 in NTD and helix 9 in the CTD of HIV-1 CA (Lanman et al., 2003). The same group also reported an NTD-CTD interaction within HIV-1 particles by hydrogen-deuterium exchange assay. Consistent with the

crosslinking experiment, the NTD-CTD interface was also mapped to helices 3 and 9 (Lanman et al., 2004). Docking the high-resolution structures of the two HIV-1 CA domains into a higher resolution cryoEM density map enabled construction of a full-length CA hexamer model (Ganser-Pornillos et al., 2007). Besides the previously detected helix 4-helix 9 interaction, the model also revealed other potential NTD-CTD interactions involving helices 3, 7, 8, and 11. In the context of the structural model, my results suggest that T216I in the CTD (helix 11) rescues P38A in NTD (helix 2) by altering intersubunit contacts to modulate capsid stability (Fig. 3-9A). The perturbations in helices 1, 2, and 7 induced by P38A suggest that the substitution alters the CA-CA interactions, and the second-site mutation T216I may compensate for the disrupted CA-CA interactions by promoting hydrophobic interactions between helices 11 and 7. These hypotheses can be tested by further mutational analysis of amino acids mediating these putative contacts. A structure of the assembled CA protein at higher resolution may also help clarify these issues.

Suppression of the E45A mutation by R132T is also likely due to altered virus-host cell interactions. Our lab has speculated that a loss of electrostatic repulsion in the E45A capsid renders it hyperstable (Forshey et al., 2002). This notion was supported by an *in vitro* assay in which the purified CA protein of E45A mutant exhibited accelerated assembly compared to the wild-type protein. The R132T mutation failed to correct the accelerated assembly rate observed for the E45A CA protein *in vitro*. E45A and E45A/R132T mutants were both fully active in saturating restriction, consistent with my observation that both mutant

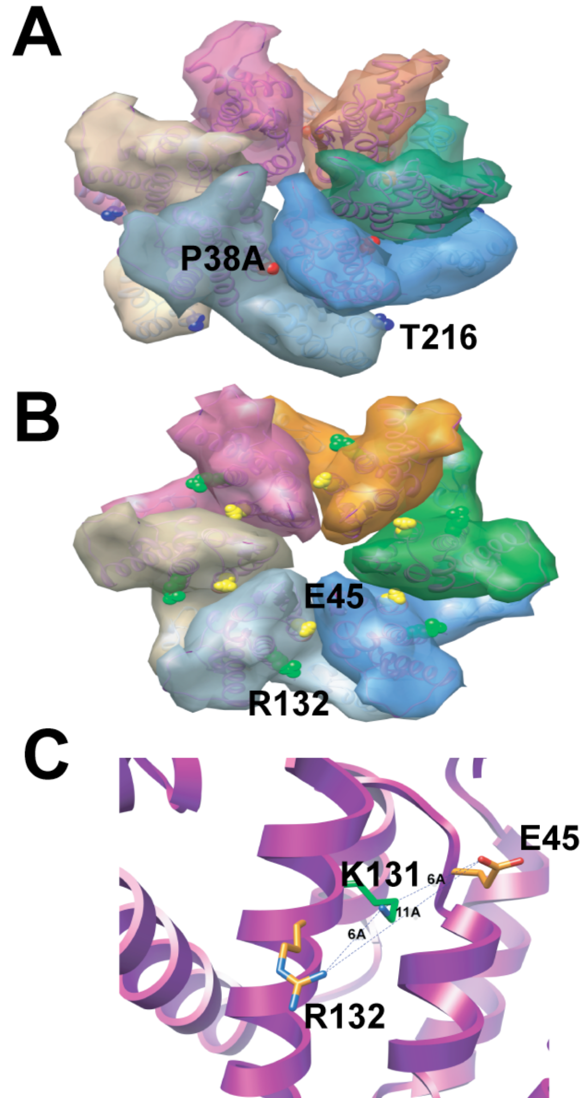

Fig. 3-9. Structural models of CA. Model of a novel NTD-CTD interdomation interaction in a CA hexamer (Ganser-Pornillos et al., 2007). (A) P38A (red) in helix 2 is shown located on the CA NTD-NTD interface and is close to helix 3 in the adjacent CA. T216I (blue) in helix 11 is shown located on the CA NTD-CTD interface and is close to helix 7 in the adjacent CA. (B) E45A (yellow) and R132T (green) in a CA hexamer. ((C) Detailed view of E45A and R132 are shown on a CA NMR structure (PDB ID: 1GWP).

viruses exhibit hyperstable capsids *in vitro*. I reasoned that a loss of positive charge in R132T mutation would offset an increase in capsid stability induced by a potential loss of electrostatic repulsion resulting from E45A. Nonetheless, inspection of the recently reported model of the hexameric CA lattice (Ganser-Pornillos et al., 2007) failed to identify a negatively charged residue in close proximity to E45 that could result in a direct intersubunit electrostatic repulsion (Fig. 3-9B). However, these residues are on the same face of the NTD, suggesting that they may directly affect intersubunit contacts. Furthermore, E45A and R132 are bridged by another positively charged residue (K131) in a CA crystal structure; thus R132T could rescue E45A by altering the CA-CA interaction via this E45-K131-R132 interface (Fig. 3-9C). Nonetheless, NMR analysis revealed that the mutations did not significantly alter the NTD protein structure in solution, suggesting that the biological effects are likely the result of subtle changes in protein-protein interactions.

Despite the lack of an effect of R132T on stability of the E45A capsid *in vitro*, I found that the R132T CA mutation rescued the ability of E45A to infect mitotically-arrested cells. Yamashita *et al.* (2007) recently identified several CA mutants, including E45A, that exhibit a selective impairment for infection of nondividing cells. It was inferred that inability of these mutants to infect nondividing cells might be due to increased level of CA in the RTC/PIC resulting from defective uncoating (Yamashita et al., 2007). We previously established that purified E45A cores retain more CA and uncoat slowly *in vitro* than the wild

type (Forshey et al., 2002). We also reported that the Q63A/Q67A mutant cores, while unstable *in vitro*, undergo incomplete uncoating in target cells as revealed by the stable association of CA with isolated preintegration complexes (Dismuke and Aiken, 2006). Collectively, these observations support the hypothesis that completion of uncoating is a prerequisite for efficient infection of nondividing cells. I speculate that the ability of E45A/R132T to infect nondividing cells results from reversal of the uncoating defect in target cells. The restored sensitivity of the E45A/R132T mutant to the CA-targeting antiviral compound adds further support to this conclusion, as the compound appears to act by destabilizing the viral capsid. It should be noted that because R132T partially rescued the E45A infectivity impairment (Fig. 3-2) yet fully reversed the cell-cycle dependence (Fig. 3-7), it is possible that the impaired infectivity of E45A mutant particles in cycling cells results from an additional defect that is not corrected by R132T.

The viral capsid plays a key role in HIV-1 infection, as evidenced by the existence of potent inhibitory cellular factors that target it. Uncoating of the viral core following penetration of the target cell is a critical step in HIV-1 infection that is likely to depend on positive-acting host factors. For these reasons, HIV-1 uncoating represents an attractive area for therapeutic discovery and development.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Introduction

HIV/AIDS is now a pandemic that threats human health around the world. There is no vaccine or cure for AIDS. A few antiviral drugs have developed that markedly delay the onset of HIV/AIDS. Since drug-resistant HIV-1 mutants can emerge during antiviral therapy, it is critical to use multiple drugs that blocks multiple viral life cycles. Some drugs are currently widely used, such as PR and RT inhibitors. Further understanding of different steps of the HIV-1 life cycle will help to identify novel targets for drug design. Recently, the capsid has emerged as an attractive target for therapy. A peptide assembly inhibitor, CAI, that targets CA, has been identified (Braun et al., 2008; Sticht et al., 2005). In addition, a small molecule (CAP-1) has been identified as a CA-specific inhibitor (Tang et al., 2003a). Neither of these compounds has yet been developed as a potent HIV-1 antiviral, and the specificity of inhibition has not be established. Therefore, more research is required to identify promising inhibitors which target the viral uncoating step.

HIV-1 uncoating is a poorly understood step in HIV-1 infection. While previously developed biochemical assays have linked capsid stability *in vitro* to viral infectivity *in vivo*, our understanding of uncoating within an infected cell

remains limited. Previous studies from this laboratory demonstrated that productive HIV-1 infection requires a viral capsid of optimal stability. The main goal of my doctoral research has been to identify novel determinants of HIV-1 uncoating in CA. To achieve this, I employed the approach of selecting for second-site mutations by virus adaptation. The motive for this work has been that a better understanding of HIV-1 uncoating could lead to rational drug design aimed at targeting capsid functions. The identification of suppressor mutations in my studies described in Chapter II and Chapter III will help to inform the selection of drug targets and design inhibitors to block HIV-1 infection. In addition, my work also suggests that uncoating is dependent on cellular factors. Future work should lead to identify the cellular factors for HIV-1 uncoating.

Characterization of the T54A mutant

Our lab has developed a spin-through method in which the virus particles are spun through a sucrose density gradients containing a small layer of Triton X-100 at the top of the gradient. Upon centrifugation, the viral cores pass through the detergent, separate from the lipid envelope, and then sediment to a density of 1.24-1.26 g/mL (Kotov et al., 1999). About 15% of total CA is recovered with the cores in this method (Forshey et al., 2002). Mutant viruses that give rise to either an increased or decreased quantity of core-associated CA also are impaired for infectivity, suggesting that a metastable core stability is crucial for viral infection. Those mutants with aberrant core-associated CA are defined as uncoating mutants. Consistent with this notion, detailed studies indicated that those mutant

viruses are blocked at a post-entry step of the viral life cycle. Most of these noninfectious viruses were blocked for reverse transcription in target cells (Forshey et al., 2002). However, the Q63/67A mutant was competent for reverse transcription but impaired for nuclear entry and integration (Dismuke and Aiken, 2006).

HIV-1 capsid stability is thought to be maintained by CA intersubunit interactions. Recently, a high-resolution crystal structure of a hexamer of MLV CA NTD revealed a novel interaction between helices 2 and 3 of adjacent subunits (Mortuza et al., 2004). Since MLV and HIV-1 CA proteins exhibit highly similar tertiary structures, I hypothesized that CA-CA interaction between helices 2 and 3 also exists in HIV-1 CA since HIV-1 CA also forms a hexamer lattice. To determine whether mutations in helix 3 result in altered capsid stability, I performed alanine-scanning mutagenesis of helix 3 and measured the viral infectivity and level of core-associated CA. I identified one mutant, T54A, that was impaired for infectivity and exhibited a decrease in core-associated CA.

To further understand the basis for the T54A infectivity impairment, I selected a second-site suppressor mutation of this mutant virus by culturing in a T-cell line (CEM). A pesudo-revertant emerged eventually and was identified as T54A/A105T by sequencing a PCR-amplified segment of the integrated provirus from CEM cells. I also determined that A105T is sufficient for rescue of T54A by contructing the T54A/A105T double-mutant in a wild-type provirus background. Previously, a revertant V84H/A92E/A105T has been identified in a CsA dependent/resistant CA mutant, A92E. A92E is localized within the CypA-

binding loop of CA. CsA blocks the binding of cellular protein CypA to CA (Aberham et al., 1996). The binding of CypA is responsible for the CsA-dependent/resistant phenotype which was confirmed in CypA KD cells in previous studies. Depending on the target cell type, A92A infection is dependent on or resistance to CsA (Hatziioannou et al., 2005; Sokolskaja et al., 2004). I hypothesized that T54A is also CsA-dependent for infectivity. I performed infectivity assays in the presence and absence of CsA. My results showed that T54A infection is enhanced by CsA. This result was surprising because T54A is localized at distal end of the cyclophilin-binding site. I also showed that, like A92E, the T54A phenotype is also cell type-dependent. The block to T54A was post-entry, and was associated with a modest impairment for reverse transcription.

The current view holds that cellular factors determine the CsA-dependent/resistant phenotype (Hatziioannou et al., 2005; Sokolskaja et al., 2004). It has been hypothesized that CypA recruits some cellular factors to the A92E HIV-1 CA and restricts viral infection during a post-entry step. My results suggest that the T54A and A92E mutants have a similar phenotype. Recent studies further suggest that the cell-dependent restriction of A92E is genetically dominant. A92E was shown to be CsA-dependent in HeLa cells while CsA-resistant in HOS cells. Heterokaryons generated by fusion of HeLa and HOS cells retained the CsA-dependent phenotype (Song and Aiken, 2007). This result suggests that an inhibitor of A92E infection is present in HeLa but not in HOS cells.

Future directions should focus on identification of the putative restriction factor which confers the CsA-dependent phenotype. The identification of T54A and its suppressor will provide useful tools for such efforts. To this end, I propose to identify host proteins from HeLa cells that bind selectively to cores of CsA-dependent HIV-1 mutants. I will isolate HIV-1 cores from WT, T54A, A92E, T54A/A105T, A92E/A105T virions and incubate those cores with lysates of HeLa or HOS cells. After pelleting to remove unbound proteins, I will employ mass spectrometry to identify proteins bound to each sample of cores and search for proteins that are present in complexes with T54A and A92E cores but not the others. Such proteins will then be tested for their role in CsA-dependent infection using RNA interference in HeLa cells.

Recently, it was reported that the CypA level dictates infection efficiency of A92E and G94D mutants (Ylinen et al., 2008). The TE671 cell line expresses less CypA than HeLa cells and does not restrict infection by CsA-dependent mutants. Overexpression of CypA in TE671 cells resulted in CsA-dependent infection by the mutants. This study appears to argue against the hypothesis that a cell-specific factor determines the CsA-dependent phenotype. Previous study showed that overexpression of CypA in 293T cell line did not result in restriction of CsA-dependent mutants {Song, 2007 #147}. Since TE671 cells express higher level of TRIM5α than 293T cells, I reason that the discrepancy could be resulted from different levels of endogenous human TRIM5α. To test this, I can performed infectivity assay with CypA-overexpressed TE671 cells with TRIM5α

KD to determine whether there will be a lack for restriction for CsA-dependent mutants.

Isolation and characterization of suppressor mutants of P38A and E45A mutants

Previous studies in our lab have shown that formation of an HIV-1 core of optimal capsid stability is crucial for viral replication. Cores isolated from HIV-1 CA mutants with faster or slower capsid disassembly rates are less infectious than the wild type (Forshey et al., 2002). To probe the possible role of domain interactions in maintaining optimal HIV-1 capsid stability, HIV-1 uncoating mutants, with unstable or hyperstable cores (P38A or E45A, respectively), were serially passaged to isolate mutants adapted for efficient replication. The CA-encoding regions of adapted mutant viruses were sequenced and identified as P38A/T216I and E45A/R132T. To test whether the second-site substitutions were responsible for the restoration of replication, I cloned the DNA fragment containing the second-site mutations and the original mutations to HIV-1 wild-type backbone. These second-site mutations partially rescued viral infectivity in single-cycle infectivity assay and restored viral replication of viruses with the original mutations.

P38A and E45A mutant viruses exhibit unstable and hyperstable cores, respectively. To test whether the second-site mutations restore normal core stability, a biochemical assay was performed to measure the recovery of CA from a sucrose gradient ultracentrifugation fraction containing HIV-1 cores. However,

P38A/T216I and E45A/R132T exhibited similar levels of core-associated CA as the single mutants. Thus, my data showed that the second-site mutations T216I and R132T rescued viral replication but not the intrinsic defects in capsid stability in our biochemical assay. This result was surprising, since our previous work strongly suggested that HIV-1 infectivity is dependent on optimal core stability. One possible explanation for this apparent paradox is that the double mutant cores may be stable in target cells without necessarily being stable *in vitro*. To test this, I employed a previously developed assay to study the mutant cores in target cells. This assay is based on the requirement for a stable viral core for efficient abrogation of host restriction in simian cells (Forshey et al., 2005; Shi and Aiken, 2006). Mutant virions were titrated onto target cells with a fixed quantity of the HIV-GFP reporter virus. As previously reported, the unstable mutant P38A was reduced in the ability to abrogate restriction relative to wild-type HIV-1. This result was observed in both owl monkey (OMK) and rhesus monkey (FRhK-4) cells. In contrast, the double mutant P38A/T216I showed an increased ability to abrogate restriction. This experiment suggests that T216I partially rescues the impaired capsid stability of P38A mutant in target cells.

To determine whether the elevated capsid stability of the E45A mutant is reduced by R132T, I studied the intrinsic stability of the mutant viral cores purified from virions. I determined the yield of core-associated CA protein. The results showed that addition of R132T did not reduce the elevated yield of E45A mutant cores and did not accelerate the delayed uncoating of mutant cores *in vitro.*

As an alternative approach to study HIV-1 capsid stability, I studied the assembly of purified recombinant CA protein assembly *in vitro*. This approach has been used to study HIV-1 capsid stability by measuring the rate of assembly of the soluble form of CA (Douglas et al., 2004). Those authors found that the E45A mutation led to accelerated CA assembly in vitro. Based on this report, I hypothesize that the isolated second-site mutations will restore the rate of assembly of E45A and P38A mutant CA proteins to that of the wild type. For this study, I cloned the full-length CA mutant to an expression vector and purified the 7 proteins by FPLC. In agreement with the previous report, E45A showed a markedly accelerated kinetics of assembly *in vitro*. However, the accelerated rate of assembly of E45A was not reduced by addition of the suppressor mutation R132T. Surprisingly, the unstable P38A mutant also demonstrated a significant accelerated rate of assembly. The double mutant P38A/T216I also showed an accelerated rate, which was similar to P38A. These results demonstrated that the E45A and P38A mutations accelerated the kinetics of assembly *in vitro,* yet the assembly kinetics of these mutant proteins were not affected by their respective suppressor mutations. Thus, I was unable to account for the rescue of the viral mutants using this biochemical approach.

A high-resolution cryoEM docking study has revealed novel CA NTD-CTD interdomain interactions (Ganser-Pornillos et al., 2007). Besides the previously revealed interactionas between helices 4 and 9 by chemical cross-linking (Lanman et al., 2003), the model also revealed that other NTD-CTD interactions extending to helices 3, 7, 8, and 11 and P38A and T216I could potentially

influence CA-CA interactions. Consistent with this model, my results suggest that T216I (in CTD, helix 11) rescues the phenotype of P38A (in NTD, helix 2) by modulating direct intersubunit contacts to restore HIV-1 capsid stability in target cells.

It was reported that the E45A mutant is selectively dependent on mitosis for HIV-1 infection (Yamashita et al., 2007). In this study, I found that the CA mutation R132T reverses the selective impairment of E45A for infection in aphidicolin-arrested cells. Yamashita *et al.* (2007) also showed that the Q63A/Q67A CA mutant is also dependent on cell cycle for infection. Work in our laboratory found that this mutant undergoes slower uncoating in target cells (Dismuke and Aiken, 2006). Another cell cycle-dependent mutant (T54A/T57A), exhibited faster uncoating kinetics in vivo, but was sensitive to CsA for longer than the wild type. Thus, it was speculated that the gain of cell-cycle-dependence might be due to increased level of CA in the RTC/PIC during an aberrant uncoating process.

In this study, I identified suppressor mutations that rescue the replication of two uncoating mutants P38A and E45A. My data suggest that proper uncoating can be restored by contacts between the NTD and CTD on adjacent molecules in the P38A mutant capsid. My data also suggest that HIV-1 uncoating plays a role on the infection in nondividing cells.

My results will be informative for design of drug screening approaches. For example, we can make two small peptides derived from the HIV-1 CA region containing P38 and T216. We will then screen for the different compounds that

bind to these two peptides and inhibit HIV-1 infection. This approach could lead to identification of potent uncoating inhibitors. The use of a combination of compounds targets P38 and T216 region could help to eliminate the chance of the emergence of drug-resistant mutants.

Our current understanding of HIV-1 uncoating comes mainly from biochemical and structural studies. Suppressor mutations of P38A and E45A identified in this study did not restore the normal capsid stability *in vitro*. It is possible that cellular factors are involved in the uncoating process that are not reflected by the biochemical assays *in vitro*. To test this possibility, we can infect multiple human cell lines to determine whether the infection of HIV-1 uncoating mutants is cell type-dependent. It would be informative if we can find unstable and hyperstable mutants have different tropisms in different cell lines. As an alternative approach, I can select cell clones that are permissive for the uncoating mutants. Identification of a cell clone that is permissive to an uncoating mutant would represent novel evidence for the involvement of cellular factors in HIV-1 uncoating. The cells could then be deployed for studies of other uncoating mutants. I could also identify cellular factors that regulate HIV-1 uncoating by comparing microarray analysis data on mutant and WT cells. Based on my studies described in this dissertation, I could make recombinant proteins of WT and single and double CA mutants containing suppressor mutations, then use these recombinant CA proteins to pull down host proteins from cell lysates and perform proteomic analysis. By comparing the presence or absence of proteins

pulled down from these CA proteins, I might identify cellular factors regulating HIV-1 infection.

Future studies also should be focused on developing and robust assays for HIV-1 uncoating *in vivo* that could be adapted to further understand the mechanism of HIV-1 uncoating. Previous studies have shown that DNA flap mutant exhibited accumulated perinuclear CA, suggestive of an uncoating defect (Charneau et al., 1992). A similar experiment can be done to examine the HIV-1 single mutants and the suppressor mutants in my studies. This experiment might reveal differences in perinuclear CA level among those mutants. If it does, it can be developed as an *in vivo* assay to better understand HIV-1 uncoating. Co-localization of CA with potential cellular factors could be examined by fluorescence confocal microscopy to determine whether the host factor associates with the viral core.

CHAPTER V

MATERIALS AND METHODS

Cells and viruses

293T, HeLa-CD4/LTR-lacZ (HeLa-P4), and HeLa-CypA KD cells (Sokolskaja et al., 2004) were cultured in Dulbecco's modified Eagle medium (Cellgro) supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 µg/ml) at 37°C with 5% CO2. CEM and H9 cells were cultured in RPMI 1640 medium with the same supplements. The wild-type HIV-1 proviral DNA construct R9, encoding full-length open reading frames for all HIV-1 structural and accessory genes, was used for these studies. Q50A, L52A, N53A, M55A, L56A, N57A, and T58A point mutations were created by PCR site-directed mutagenesis. The point mutation T54A in the CA region of R9 was previously reported (von Schwedler et al., 2003) and was the generous gift of Wes Sundquist. In some experiments, HIV-GFP, an envelope-defective HIV-1 reporter virus clone encoding green fluorescent protein (GFP) in place of Nef (He et al., 1997), was employed. Viruses were produced by calcium phosphate transfection of 293T cells (20 µg of plasmid DNA per 2 X10^6 cells) as previously described (Chen and Okayama, 1987). Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped reporter virus particles were produced by cotransfection of HIV-GFP plasmid DNA with the VSV-G expression plasmid pHCMV-G (Yee et al., 1994). One day after transfection, the culture supernatants were harvested and

clarified by being passed through 0.45-μm-pore-size filters, and aliquots were frozen at -80°C. The CA contents of the virus stocks were quantified by p24 enzyme-linked immunosorbent assay (ELISA), as previously described (Wehrly and Chesebro, 1997). The HeLa-P4 cell line, a HeLa cell clone engineered to express CD4 and an integrated long terminal repeat (LTR)-*lacZ* reporter cassette (Charneau et al., 1992), was used to quantify HIV-1 infectivity, as previously described (Charneau et al., 1992).

HIV-1 infection assays

HIV-1 stocks were serially diluted in culture medium, and samples (0.125 ml) were used to inoculate HeLa-P4 target cells seeded the day before (20,000 cells per well in 48-well plates). Two hours after inoculation, the cultures were supplemented with additional medium (0.5 ml) and cultured for another 48 h prior to being stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to detect infected cells. To determine the number of infected cells per well, individual wells were visualized using a Scion grayscale digital camera (model no. CF 1312M) equipped with a Navitar Macro Zoom 18- to 108-mm lens, and images were captured with a Power Macintosh 8500 computer. Blue cells were counted using NIH Image software in the particle-counting mode. Infections were performed in triplicate, and only values within the linear range of the infection assay (up to 1,000 blue cells per well) were used to calculate infectivity. Infections with HIV-GFP reporter viruses were analyzed by flow cytometric analysis of GFP expression as previously described (Forshey et al., 2005).

Viral replication assay

Viral growth was determined by inoculation of cultures of CEM cells (2 x 10^5 cells in 0.2 ml) or H9 cells with quantities of virus corresponding to 2 ng of p24 antigen. Prior to addition to tissue culture medium, CsA was dissolved in dimethyl sulfoxide to produce a stock concentration of 1 mM. The stock was added to the medium to achieve a final concentration of 2.5 μM or 5 μM when needed. Mock-treated controls in each experiment received the same final concentrations of dimethyl sulfoxide as those in the treated cultures. Every 2 days, samples of culture supernatants (100 μl) were withdrawn and replaced with an equal volume of fresh medium. Reverse transcriptase activity in culture supernatants was quantified as previously described (Aiken and Trono, 1995).

Virus-cell fusion assay

The β-lactamase (BlaM)-Vpr HIV-1 fusion assay was performed essentially as previously described (Wyma et al., 2004). Quantities of wild-type and mutant reporter viruses were normalized by p24 content and used to inoculate P4 target cells for 2 h at 37°C. The cells were then loaded with the CCF2-AM fluorogenic substrate overnight at room temperature. The supernatant was removed and replaced with phosphate-buffered saline (PBS). Cellular fluorescence was determined in a microplate fluorometer. The background levels of blue (no virus) and green (no cells or virus) fluorescence were determined at 410 nm and 520 nm, respectively, and were subtracted from the experimental samples. Fluorescence ratios were calculated for each well. For each virus

dilution, triplicate determinations were performed, and values typically agreed to within 10%.

Quantitative analysis of HIV-1 reverse transcription in target cells

HIV-1 reverse transcription in target cells was quantified essentially as described previously (Dismuke and Aiken, 2006). One day prior to infection, 100,000 HeLa-P4 cells per well were seeded in 12-well plates. Virus stocks were treated with 20 µg/ml of DNase I and 10 mM MgCl2 at 37°C for 1 h to remove contaminating plasmid DNA. Inocula were normalized by p24 content to 100 ng per well for HIV-1 in medium containing DEAE-dextran (10 µg/ml). At 8 h postinfection, the cells werewashed with 1 ml of PBS and then detached with trypsin. The trypsin was deactivated by the addition of 0.5 ml of complete medium, and the cells were pelleted and washed once with 500 µl of PBS. The cell pellets were resuspended in 200 µl of PBS, and DNA was isolated using a DNeasy kit (QIAGEN) according to the manufacturer's instructions. Viral DNA was quantified by real-time PCR using an MX-3000p thermocycler (Stratagene) utilizing TaqMan chemistry. Reverse transcription products (U5-Gag) were detected using the forward primer MH531 (5'-TGTGTGCCCGTCTGTTGTGT-3') and the reverse primer MH532 (5'-GAGTCCTGCGTCGAGAGAGC-3'), with the probe LRT-P (5'-6 carboxyfluorescein [FAM]-CAGTGGCGCCCGAACAGGGA-6-carboxytetra

methylrhodamine [TAMRA]-3') as previously described (Butler et al., 2001). Thermal-cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by

40 cycles of 95°C for 15 s and 60°C for 60 s.

Assays of HIV-1 nuclear targeting

Assay of HIV-1 nuclear targeting was performed essentially as previously described (Dismuke and Aiken, 2006). DNA from acutely infected cells was isolated at 24 h postinfection by using the DNeasy kit (QIAGEN) and analyzed for the presence of two-LTR circles. Two-LTR circle DNA was detected by quantitative real-time PCR utilizing primers and a TaqMan probe specific for the LTR-LTR junction. The forward primer MH535 (5'-ACTAGGGAACCCACTGCTTAAG-3'), the reverse primer MH536 (5'-TCCACAGATCAAGGATATCTTGTC-3'), and the two-LTR probe MH603 (5'-[FAM]-ACACTACTTGAAGCACTCAAGGCAAGCTTT-[TAMRA]-3') were used. Thermal-cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 90 s.

Isolation of HIV-1 cores

Cores were isolated from concentrated virions as previously described (Kotov et al., 1999). Briefly, supernatants from transfected 293T cells were filtered to remove cellular debris, and HIV-1 particles were concentrated by ultracentrifugation (100,000 x *g* for 3 h at 4°C) through a cushion of 20% (wt/vol) sucrose in STE buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA). The viral pellets were resuspended in STE buffer (0.4 ml), and the concentrated virions were subjected to ultracentrifugation (130,000 x *g* for 16 h at 4°C) through a layer of 1% Triton X-100 into a linear sucrose density gradient (10 ml of STE

buffer containing 30 to 70% sucrose). Fractions (1 ml) were collected from the top of the gradient and analyzed for CA content by p24 ELISA. The yield of cores was determined as the CA content (as determined by p24 ELISA) in the peak fractions of cores as a percentage of the total CA content in the gradient.

Disassembly of purified HIV-1 cores *in vitro*

The kinetics of disassembly of HIV-1 cores were measured as described previously (Forshey et al., 2002). Purified cores were diluted in STE buffer and incubated at 37°C. Following incubation, the particles were subjected to ultracentrifugation to separate free CA from intact cores. Both supernatants and pellets were analyzed for CA content by ELISA to determine the percentage of total CA released from the cores during incubation.

HIV-1 adaptation studies

Virus stocks were normalized for reverse transcriptase (RT) activity and used to infect CEM cells. Virus supernatant was harvested at the peak of RT activity and normalized. Two subsequent passages of infection in fresh CEM cells were performed until accelerated growth was observed. Cells were harvested immediately following the peak of RT activity from the third passage. Proviral DNA was purified with the DNeasy kit (QIAGEN), a 900-bp fragment spanning the BssHII and SpeI sites (encoding the matrix protein and most of the NTD of CA) was amplified by PCR, and a BssHII-SpeI restriction fragment was transferred into R9. Mutants A105T and A92E/A105T were created by PCR-

based mutagenesis in the R9 background. The A105T single mutant was created by using the sense primer 5'-CAAGATTTAAATACCATGCTAA ACACAGT-3' and the antisense primer 5'-TAGCATGGTATTTAAATCTTG TGGGGTGG-3' with the R9-T54A/A105T plasmid as a template. A92E/A105T was created by using the sense primer 5'-GGGCCTATTGAACCAGGCCAGATGAGAGA-3' and the antisense primer 5'-CTGGCCTGGTTCAATAGGCCCTGCATGCA-3' with the R9-A105T plasmid as a template. The envelope-defective HIV-GFP/T54A reporter virus construct was created by transferring the BssHII-SpeI restriction fragment from R9.T54A into HIV-GFP. The sequences of the replaced regions of all viral constructs were experimentally verified.

Similar procedure was performed to isolated revertants of the P38A and E45A mutants. Virus stocks were normalized for RT activity and used to infect CEM cells. Virus supernatant was harvested at peak of RT activity, and normalized quantities of viruses were re-inoculated in to new CEM cultures. Two subsequent passages of infection in fresh CEM cells were performed until accelerated growth was observed. Cells were harvested immediately following the peak of RT activity from the third passage. Proviral DNA was purified with the DNeasy kit (Qiagen), and regions of Gag were PCR amplified using *pfu* DNA polymerase (Stratagene) to a 1000-bp *BssH*II-*Spe*I fragment spanning MA and part of CA coding region and a 500-bp *Spe*I-*Apa*I fragment spanning part of CA and NC coding region. The *BssH*II-*Spe*I fragment was amplified using sense primer 5'-GGAGATCTCTCGACGCAG-3' and anti-sense primer 5'-TTTAATCCCAGGATTATCCAT-3'. The *Spe*I-*Apa*I fragment was amplified using

sense primer 5’-GCATGCAGGGCCTATTGC -3’ and anti-sense primer 5’-CCTGTCTCTCAGTACAATC-3’. Following identification of additional mutations, the 500-bp Spe*I*-Apa*I* fragments were transferred into R9.E45A or R9.P38A. The individual single R132T and T216I mutants were constructed by transferring the mutant *Spe*I-*Apa*I segments into the wild-type R9 plasmid. DNA sequences of the replaced regions of all viral constructs were verified by sequencing of the proviral clones.

Analysis of HIV-1 infection by flow cytometry

The wild-type HIV-GFP and mutant HIV-GFP.T54A (2) pseudotyped by VSV-G were used to assay infection of HeLa-P4 or HOS cells. Cultures (20,000 cells per well in 12-well plates) were inoculated with various concentrations of reporter viruses in the presence of polybrene (8 μg/ml) in a total volume of 300 μl. One day later, complete medium (1 ml) was added. Two days after infection, the cells were detached using trypsin and fixed by the addition of an equal volume of PBS containing 4% paraformaldehyde.GFP expression was quantified by flow cytometry by using a FACSCalibur instrument (Becton Dickinson), and the percentage of GFP-expressing cells was quantified with Cellquest software. A minimum of 5,000 cells were analyzed for each sample.

Protein expression and purification

pET21a-based plasmids were constructed for full-length CA protein expression in *E. coli* BL21-DE3. Plasmids for expression of the full-length HIV-CA proteins were obtained by PCR mutagenesis. WT and mutant plasmids were

used as template for PCR. An NdeI/XhoI fragment was amplified using sense primer 5'- GGAATCCCATATGCC TATAGTGCAGAACCTCCAGGGG-3' and anti-sense primer 5'- CCC CTCGAG TCACAAA ACTCTTGCTTTATGGCCGGG-3'. The PCR amplified DNA was cloned into the *Nde*I/*Xho*I site of the pET21a and verified by DNA sequencing. CA proteins were expressed and purified as previously described (Ganser et al., 1999), except where noted. The proteins were eluted from the UnosphereQ column (Bio-Rad) at NaCl concentrations ranging from 70 to 90 mM. Most of the E45A protein was found in the flow-through step. The proteins were then dialyzed into 50 mM Na_2PO_4 buffer, pH 8.0. The dialyzed proteins were concentrated by Centriprep centrifugal filter (Millipore) to a concentration of ~300-500 µM. Concentrated protein solutions were stored frozen at -80°C until needed. The proteins were >99% pure by SDS-PAGE.

pET11a-based plasmids were constructed for CA-NTD protein expression in *E. coli* BL21-DE3 for NMR studies. Plasmids for expression of the CA-NTD proteins were obtained by PCR mutagenesis. WT and mutant plasmids were used as template for PCR. An *Nde*I/*Bam*HI fragment was amplified using sense primer 5'-GGAATCCCATATGCCTATAG TGCAGAACCTCC AGGGG-3' and anti-sense primer 5'-CCCGGATCCTCACAGAATGCT GGTAGGGCTAT ACAT-3'. The PCR amplified DNA was cloned into the *Nde*I/*Bam*HI site of the pET11a and verified by DNA sequencing. For isotopic labeling, proteins were expressed in M9 minimal medium using $^{15}NH_4Cl$ as a sole nitrogen source in *E. coli* Rosetta 2 (DE3) at 23 ºC for 16 h with 0.4 mM IPTG. Soluble CA-NTD proteins were

obtained by sonication in lysis buffer containing 25 mM sodium phosphate, pH 7.0, 1 mM DTT, and 0.02% sodium azide followed by ultracentrifugation at 127,000Xg. Proteins were purified by using 10 mL Hi-Trap QP (GE Healthcare, Piscataway, NJ) at pH 7.0 and pH 8.5 with 1M NaCl gradient. The fractions with CA-NTD were further purified using Hi-Load Superdex75 26/60 (GE Healthcare, Piscataway, NJ) equilibrated with 25 mM sodium phosphate, pH 6.5, 100 mM NaCl, 1 mM DTT, and 0.02% sodium azide. The molecular weight of each CA-NTD protein was confirmed by LC-ESI-TOF mass spectrometry (Bruker Daltonics, Billerica, MA) and isotope labeling efficiency was estimated to grater than 99%.

***In vitro* capsid assembly**

Purified wild-type and mutant capsid protein were assembled by rapid dilution into concentrated NaCl solutions at 23°C to yield a final concentration of NaCl to 2.25 M and protein of 50 μM, and the turbidity of the solution was monitored. For kinetic analysis, the reaction was rapidly mixed and placed into a 1-mm quartz cuvette. Approximately 20 s elapsed between the time of the addition of salt and the first time point measured. The increase in optical density was monitored at 350 nm for 30 min as previously described (Douglas et al., 2004).

NMR analysis

^{1}H-^{15}N HSQC NMR experiments were conducted at 25°C on ^{15}N-labeled CA-NTD samples in the NMR buffer (25 mM sodium phosphate buffer, pH 6.5, 100 mM NaCl, 2 mM DTT, and 0.02 % sodium azide) using Bruker Avance 700 and 600 MHz spectrometers, equipped with 5 mm, triple resonance and z-axis gradient cryoprobes.

Cell cycle arrest

To generate cell cycle-arrested target cells for infectivity assay, HeLa-P4 cells (4×104 cells/well) were plated in 48-well plates. Aphidicolin was added to a final concentration of 2 μg/ml upon seeding (Qi et al., 2008; Yamashita et al., 2007). Cultures were exposed to viruses overnight; aphidicolin was also present during this period. Cells were fed with 0.5 ml fresh media for another 24 hours prior to staining.

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