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Biochemical and Biophysical Characterization of the Interaction between Human Immunodeficiency Virus Type 1 Integrase and Capsid

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Biochemical and Biophysical Characterization of the Interaction between Human Immunodeficiency Virus Type 1 Integrase and Capsid

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Bioengineering

By

Xiaowen Xu

2017
ABSTRACT OF DISSERTATION

Biochemical and Biophysical Characterization of the Interaction between Human Immunodeficiency Virus Type 1 Integrase and Capsid

by

Xiaowen Xu

Doctor of Philosophy in Bioengineering

University of California, Los Angeles, 2017

Professor Samson A. Chow, Chair

Upon infection of host cells by human immunodeficiency virus type 1 (HIV-1), the viral membrane fuses with the cell plasma membrane and the viral core is released into the cytoplasm, where uncoating of viral capsid (CA) core takes place. Numerous studies have shown that optimal core stability is a key determinant in the uncoating. However, the underlying factors and mechanisms governing uncoating are poorly understood. We have previously shown that HIV-1 integrase (IN) is involved in uncoating of the viral core and required for optimal core stability. In this study, we have demonstrated that IN interacts with in vitro assembled CA tubes and preferentially binds to CA hexamers. Our biochemical and biophysical analyses have further determined that the reaching dimer of IN is required for interacting with CA hexamer. Moreover, we show that both NTD and CTD of IN are involved in interacting with CA assemblies, while IN NTD contributes to the preferential recognition towards CA hexamers. This project provides
useful information to understand crucial but yet poorly characterized processes during HIV-1 lifecycle. By characterizing the IN-CA interaction, we may provide a mechanical basis for the requirement of IN during HIV-1 uncoating. The IN-CA interaction also indicates that IN may play a role during late stage of HIV-1 replication, as recent studies in the field suggested that IN may be involved in virion maturation. Finally, this finding highlights the potential for exploiting the CA and IN interaction as a new therapeutic target.
The dissertation of Xiaowen Xu is approved

Thomas G. Graeber
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James Akira Wohlschlegel
Samson A. Chow, Committee Chair

University of California, Los Angeles
2017
To my parents, who have given me the courage, strength and invaluable gift of education, to become the strong woman who I am today.

To my husband, who have always been my No.1 fan.

To my son, James, who wakes me up early every morning.
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Chapter 1: Introduction: Capsid Uncoating and the Role of Integrase in Uncoating
Life cycle of HIV-1

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus which belongs to the retrovirus family. It is the virus responsible for acquired immune deficiency syndrome (AIDS). A mature HIV-1 viral particle is made up of a glycoprotein decorated envelope and a capsid (CA) core, which encloses two positive single strand viral RNA, reverse transcriptase (RT), integrase (IN), protease (PR) and other viral proteins. In this dissertation, the term “CA” will used to describe the protein that makes up the core and “capsid core” to describe the assembled form of viral core. The virus life cycle is initiated with envelope glycoproteins recognition and binding of host cell receptors and co-receptors. Upon fusion of the viral envelop with host membrane, the capsid core is released into the host cell cytoplasm, followed by uncoating and reverse transcription. Uncoating is the disassembly of the capsid core. The exact mechanism of uncoating is not completely clear yet, but it has been established that optimal uncoating is required for completion of reverse transcription and viral infectivity. Reverse transcription of the viral RNA genome is carried out by the viral protein RT in the reverse transcription complex (RTC). The pre-integration complex (PIC), which involves viral cDNA, IN, RT and other viral proteins, is then formed and transported to the nuclear envelope. Although the complete process of nuclear import of PIC is unclear, some studies have shown additional viral and cellular players include CA, matrix protein, viral protein R, nuclear pore complex 153, lens epithelium-derived growth factor (LEDGF)/p75 are also involved in this process\(^1-6\). IN then catalyzes the integration of viral cDNA into the genome of the host cell, forming the provirus. The proviruses further exploit the host transcriptional and translational machinery to produce viral genomic RNA and viral proteins, which then assembled at the cellular membrane to form the immature viral particles. Upon release of the immature virions, the viral enzyme PR is then activated and
cleaves Gag polyprotiens to form mature viruses, a process known as proteolytic processing and maturation. (Fig 1.1)

Uncoating is a critical step during HIV-1 life cycle

Capsid core structure

During maturation, CA spontaneously assembles into hexameric lattice which further forms a conical shaped cone. The core, which consists about 200-300 CA hexamers and 12 pentamers, encapsulates viral RNA genome and three important viral enzymes, IN, RT and PR, along with other accessory proteins\textsuperscript{7-9} (Fig 1.2). The CA protein is a 25 kDa protein consisting of two domains, a N-terminal domain (amino acid 1-148) and a C-terminal domain (amino acid 149-231). CA in solution spontaneously forms dimer through CTD-CTD interaction\textsuperscript{10,11}. During viral assembly, CA forms hexameric lattice and conical core; while, in solution, CA hexameric lattice mostly assembles into tubular assemblies\textsuperscript{12-14}. A recent study also showed that protease cleavage of Gag polyprotein leads to formation of trimer of CA dimers, which is suggested to be the basic building block of mature CA hexameric lattice\textsuperscript{15,16}. In the context of viral particles, the CA hexameric lattice is further sealed with 12 CA pentamers, resulting in a conical shaped core.

Uncoating

Upon viral membrane fusion, the HIV-1 capsid core is released into the cytoplasm, followed by uncoating and reverse transcription. Although the exact timing of uncoating is still unclear, it is general consensus that uncoating is a crucial step during early HIV-1 life cycle and is intimately linked with reverse transcription\textsuperscript{17-19}. Studies have shown that either hyper-stable or unstable core leads to loss of early reverse transcription products and results in loss of viral infectivity\textsuperscript{20,21}. Evidences early on have supported arguments from both sides that capsid core
uncoats immediately upon fusion\textsuperscript{22,23}, or uncoating happens later on as CA is found in PIC and is possibly associated with nuclear transportation\textsuperscript{24}. Some biochemical evidence and imaging studies suggested that capsid core undergoes “biphasic uncoating”, where the core partially disassembles to allow reverse transcription to complete, while somewhat remains contained to the RTC and PIC\textsuperscript{3,25}.

**Role of IN in viral life cycle**

**IN structure**

IN is an essential enzyme for viral integration and replication. It is a 32kDa protein containing three distinct domains, N-terminus (NTD) consisting of amino acid 1-49, catalytic core domain (CCD) consisting of amino acid 50-212, C-terminal domain (CTD) consisting of amino acid 212-288. The structures of isolated NTD, CCD and CTD have been determined either by NMR or X-ray crystallography\textsuperscript{26–29}. A recent discovery of atomic model of HIV-1 intasome structure has been made possible through a cryo-electron microscopy (EM) study\textsuperscript{30}. Unfortunately, the full structure of IN is still unavailable due to its poor biophysical property. A structure study combining small and wide-angle X-ray scattering suggesting that IN forms two different types of dimers: a reaching and a core-core dimer\textsuperscript{31}. The tetramer consists two stacked reaching dimers, stabilized by core-core interactions. The enzymatic activities of IN are dependent on its active form of multimers, as in vitro complete 3’-end processing and strand transfer requires a tetramer minimally\textsuperscript{32–34}.

**Integration**

Integration of viral DNA into host genome has been well studied and characterized. The reaction is initiated with a cleavage of two nucleotide at each 3’-end by IN, a process termed 3’-
prime processing. Following that, IN cuts at the target DNA 5 bases apart, and joins the processed viral 3’-ends to the 5’ overhang of the cleaved host DNA. This reaction, termed strand transfer, results in a gapped product, which is repaired by host enzymes, completing the final steps of integration. Both 3’-end processing and strand transfer can be recapitulated in vitro using recombinant HIV-1 IN\(^{35,36}\).

**Pleotropic effect of IN mutation during viral replication**

Besides integration, studies also showed that mutations of integrase affect other steps during viral replication, including early steps like uncoating and reverse transcription, as well as late steps such as assembly and maturation\(^{18,19,37-41}\).

Our lab has previously reported a new role of IN during HIV-1 uncoating\(^{40}\). Briones *et al* reported that HIV-1 viral mutant carries IN C130S point mutation or viruses lack IN are replication defective, with a loss of early reverse transcription products. Capsid cores isolated from those two viral mutants were functional in carrying out reverse transcription under in vitro conditions. However, those isolated cores displayed defects in uncoating with poor yield and decreased stability, as well as decreased CypA incorporation. These results suggested that IN is required for optimal stability for viral core.
Figure 1.1 HIV-1 life cycle.

https://www.intechopen.com/source/html/16788/media/image3.jpeg
Figure 1.2 The conical capsid core containing hexameric and pentameric CA subunits.

Chapter 2: CA-IN interaction requires higher-order assembly of CA
Abstract

During human immunodeficiency virus type-1 (HIV-1) infection, the viral core disassembles after entering the cytoplasm, a process known as uncoating. Previous studies demonstrated that the viral enzyme integrase (IN) is required during uncoating for optimal core stability. We have obtained preliminary data suggesting that IN-capsid (CA) interaction can be detected within isolated viral cores but not with purified recombinant IN and CA. We report here that we have identified a direct interaction between HIV-1 IN and assembled CA. We show that IN preferentially binds to CA hexamers over pentamers and WT CA. Furthermore, our data suggests that the interaction between IN and CA hexamer may involve complex oligomerization of both proteins. Our study supports previous findings that IN plays an important role in viral core uncoating and provides additional insights in understanding the uncoating process.
HIV-1 is the causative agent of the acquired human immune deficiency syndrome (AIDS). A mature HIV-1 viral particle is composed of a spherical viral envelope and a conical shaped capsid (CA) core, enclosing two copies of plus strand viral RNA and viral enzymes including integrase (IN), reverse transcriptase (RT), protease (PR), and other viral proteins.

After cell entry, the HIV-1 virion undergoes two major events, uncoating and reverse transcription. Although the precise timing and location of uncoating is still under debate, it has been shown to be a dynamic and highly regulated process that affects viral infectivity. CA mutants that display either unstable or hyperstable phenotypes are shown to have an altered core yield and impaired replication as a result of reverse transcription deficiency. These results suggest that core stability is crucial in the uncoating process and plays an important role in HIV-1 replication. Like many other steps in the viral replication cycle, uncoating has been shown to be affected by various cellular factors. Two cellular proteins, cyclophilin A (CypA) and tripartite motif-containing protein alpha (TRIM5\(\alpha\)), have been shown to interact with cores directly and regulate uncoating both positively and negatively.

The CA protein is a 25 kDa protein consisting of two domains, an N-terminal domain (NTD) and a C-terminal domain (CTD). CA in solution spontaneously forms dimers through CTD-CTD interactions and under high salt conditions, CA forms hexameric lattice and tubular assemblies, recapitulating the viral core. During viral maturation, CA forms a hexameric lattice consisting of 200-300 CA hexamers and the lattice is further sealed with total of 12 pentamers on two ends, forming the conical shaped core.
Our lab has previously reported that the viral protein IN also plays an important role in modulating capsid core disassembly and is required for optimal core stability\textsuperscript{40}. We have demonstrated that two non-infectious IN mutant viruses, C130S and ΔIN, showed decreased core stability and lower CypA incorporation. In addition, our preliminary data suggests that IN interacts with CA in purified viral cores (unpublished, Briones M.). This study is aimed to better characterize the CA-IN interaction, both biochemically and structurally, in order to better understand the biological significance of the CA-IN interaction during the HIV-1 life cycle. The information provided by this study will shed some light on the mechanism governing the uncoating, which may lead to the discovery of new drug targets for anti-HIV therapy.
Results

**Purified IN binds to in vitro assembled CA and CA-NC tubes**

As described above, IN and CA interact in viral cores, but the interaction does not exist between purified IN and CA proteins. Since CA forms higher order assemblies in the context of the viral particles, we hypothesized that the IN-CA interaction *in vivo* requires such assembly and multimerization.

Native CA exhibits a monomer-dimer equilibrium with a dissociation constant of 18 μM\(^{11}\). The homo-dimerization of CA forms via its C-terminal domain, which has an apparent dissociation constant of 10 μM, similar to the full-length protein\(^{11}\). Moreover, CA-nucleocapsid (CA-NC) fusion protein forms tubular assemblies under high salt condition in the presence of oligos, which recapitulates authentic mature cores\(^ {12,13}\) (Fig 2.1 A). Therefore, we first examined the IN-CA interaction through a co-immunoprecipitation (Co-IP) assay, where the purified recombinant fusion protein CA-NC was present either in the form of tubular assemblies or at the same concentration but in the form of dimers. A negative control was included using either agarose beads without CA-NC or recombinant CA-NC at a lower concentration, which predominately forms monomers. Electrophoresis and Western blot analysis of the eluted protein complex showed that both IN and CA were present only when CA-NC was in the form of assemblies (Fig 2.1 B). Un-assembled recombinant CA-NC at the same concentration, which predominantly forms dimers, had only background level of binding to IN, similar to the negative control when beads were used without pre-incubation with CA-NC. The result suggests that higher order assemblies of CA is required for the IN-CA interaction *in vitro*.

To further support the conclusion, we assessed the IN-CA interaction using a binding assay with tubular assemblies that are formed by either CA or CA-NC *in vitro*. In the binding assay, IN
was added to the core-like assemblies and followed by centrifugation. CA and CA-NC assemblies were spun to the bottom of a sucrose cushion while any non-interacting protein remains in the supernatant. SDS-PAGE and western blot analysis showed that IN appeared in the pellet along with CA or CA-NC assemblies (Fig 2.1 C and D). This is consistent with the observation that assembled CA and CA-NC is important for the IN-CA interaction \textit{in vitro}.

**HIV-1 IN preferentially interacts with CA hexamers**

The conical shaped viral core consists of CA hexameric lattice that is decorated with CA pentamers on both ends. To further determine the differential ability of IN to recognize multimeric vs. monomeric CA, we employed a pull-down assay that takes advantage of two known CA mutants that can selectively form hexamers or pentamers under non-reducing conditions. The two CA mutants each bearing four substitutions A14C/E45C/W184A/M185A and N21C/A22C/W184A/M185A. The two substitutions (W184A and M185A) at the CTD-CTD interface, weakened the lattice growth but leave the hexamer structure unchanged. The double cysteine substitutions for each mutant aids the formation of disulfide bond under oxidizing condition, stabilizing assembled CA hexamers and pentamers. Both of the CA mutants were purified and sequentially dialyzed to remove salt and reducing agent to promote hexamer and pentamer formation respectively. Assembled hexamers and pentamers were further confirmed using non-reducing SDS-PAGE (Fig 2.2 A).

IN were then conjugated to a solid support of magnetic beads via amine coupling chemistry and subjected to incubation with assembled CA hexamers, CA pentamers and WT CA. Blank beads without protein conjugation was used as a control. As shown in Fig2.2 C, IN pulled down
more CA hexamers compared to pentamer or WT CA, indicating IN preferentially interacts with CA hexamers.

To further confirm the observation, IN and CA multimers were exchanged in the pull-down assay. Similarly, CA multimers were conjugated to magnetic beads and subjected to incubation with purified WT IN. Beads conjugated with WT CA were also included for comparison, and beads without protein were served as negative control. As shown in Fig2.2 B, beads conjugated with CA hexamers had the most binding to IN, while pentamers and WT showed significantly weaker binding. In addition, MLV IN were included here, further confirming the interaction is specific for HIV IN and CA hexamers. Taken together, we conclude that IN preferentially interacts with CA hexamers over pentamers or monomeric CA.

**Surface Plasmon Resonance (SPR) analysis of binding kinetics**

To further investigate the kinetics of the IN-CA interaction, we examined IN binding to WT CA, CA pentamers, as well as CA hexamers. Due to the poor biophysical properties of WT IN, a viable mutant IN (F185H/C280S) with greater solubility was used in the SPR analysis. The IN mutant is both viable when introduced into the viruses and is catalytically active in vitro. Three CA variations were immobilized on the SPR chip and IN mutant F185H/C280S was injected at different concentrations to assess the binding. A blank channel was included as a control. The signal generated from the control channel was subtracted from IN binding to WT CA, CA pentamers and CA hexamers. The resultant sensorgrams suggest that all three types of CA bind to IN to a certain level (Fig 2.3). However, the data could not be fitted into a simple 1:1 binding model, suggesting a more complicated mode of interaction.
Material and methods

Protein expression and purification

The expression plasmids that contain CA and CA-NC coding sequence are kind gifts from Dr. Wesley Sunquist (University of Utah, UT). The expression plasmids that contain CA mutation A14C/E45C/W184A/M185A and N21C/A22C/W184A/M185A are kind gift from Dr. Owen Pornillos (University of Virginia, VA). Expression plasmids were transformed into BL21 Competent E. coli cells and grown in 1 L LB medium at 37 °C. Expression was induced in log-phase (OD600 ~0.8) for 4 hr by adding Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1mM concentration. For CA purification, cells were harvested and resuspended in 40 mL lysis buffer containing 20 mM Tris, pH 7.5, 10 mM β-mercaptoethanol (β-ME) and EDTA-free protease inhibitor tablets (Roche; 1 tablet/10 mL lysis buffer) followed by sonication. The lyate was clarified by centrifugation at 35,000 g for 1 hr. CA protein was then precipitated by addition of 0.35 equivalent (v/v) of saturated ammonium sulfate and re-dissolved in a buffer containing 20 mM KMops (pH 6.9) and 10 mM βME. The protein was further purified through cation-exchange chromatography on a HiTrap SP HP column (GE Healthcare Life Sciences).

For CA-NC purification, cells were harvested by centrifugation, and then lysed in 30 mL of 0.5 M NaCl in buffer A (20 mM Tris-HCl, pH 7.5), 1 mM ZnCl2, 10 mM βME, EDTA-free protease inhibitor). Insoluble cellular debris was removed by centrifugation at 35,000 g for 1 hr. Nucleic acids were precipitated from the soluble protein by the addition of 0.11 equivalents (v/v) of 2 M (NH4)2SO4, followed by addition of the same volume of 10% polyethylenimine. The nucleic acid was then removed by centrifugation at 29,500 g for 15 min. The CA-NC was recovered by the addition of 0.35 equivalents saturated (NH4)2SO4 solution to soluble protein and collected by centrifugation. The protein was then dialyzed overnight and further purified through cation-
exchange chromatography. Purified CA and CA-NC protein was dialyzed against storage buffer with 20 mM Tris-HCl, pH 7.5 and concentrated through Amicon Ultra centrifugal filter units (EMD Millipore). For CA mutants A14C/E45C/W184A/M185A and N21C/A22C/W184A/M185A, higher βME concentration (200 mM) was used in all buffers.

WT IN and IN F185H/C280S were prepared as mentioned previously. CodonPlus E. coli cells (Agilent) were transformed with expression constructs encoding either full-length IN or its mutant derivatives containing N-terminal His tags for facilitating protein purification. Both WT IN and IN F185H/C280S were purified under non-denaturing conditions as previously described, with few modifications. Briefly, transformed cells were grown in LB medium at 32 °C until the optical density at 600 nm was between 0.8 and 1.0. Protein expression was then induced by adding 0.4 mM IPTG and left to grow overnight at 18 °C. Pelleted cells were suspended in a lysis buffer containing 20 mM HEPES-Na, pH 7.5, 5 mM βME, 1 M NaCl, 0.2 mM EDTA, 10% glycerol, 0.5% IGEPAL® CA 630 (Sigma-Aldrich), and EDTA-free protease inhibitor tablets (Roche; 1 tablet/10 mL lysis buffer), and further disrupted by sonication. Lysates were then clarified by centrifugation at 100,000 x g for 1 hr at 4 °C. Purified IN or its mutant derivatives were then obtained from clarified lysates using Ni²⁺ nitrilotriacetic acid (NTA) immobilized metal affinity chromatography (IMAC) and cation exchange chromatography. Purified proteins were quantitated using Bradford assay, and purity determined by SDS-PAGE and Coomassie staining.

**Preparation of in vitro tubular assembly**

CA was diluted to 3 mg/ml with assembly buffer containing 50 mM Tris, pH 7.5, 1 M NaCl. For CA-NC assemblies, CA-NC was diluted to 300 µM with a buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl and 60 µM (TG)₅₀ DNA oligo. The mixture was then incubated on a roller
overnight at 4 °C to promote assembly formation. The CA and CA-NC tubular assemblies were analyzed using EM.

**Preparation of CA pentamers and hexamers**

Pentamers and hexamers of CA were assembled through sequential dialysis. 10 mg/ml CA mutant (A14C/E45C/W184A/M185A and or N21C/A22C/W184A/M185A) was first dialyzed against assembly buffer (50 mM Tris, pH 8, 1 M NaCl) containing 200 mM β-ME overnight. Then it was dialyzed against assembly buffer to remove β-ME and finally with 20 mM Tris, pH 8. All the dialysis steps were performed at 4 °C and for at least 8 hr. The assembled pentamers and hexamers were analyzed by non-reducing SDS-PAGE and quantified through Bradford assay. Assembled proteins were stored at 4 °C until used.

**CA binding assay**

Briefly, 40 pmol WT IN or IN mutants was added to 2 nmol assembled CA tubes in a total volume of 100 µl binding buffer. The mixture was incubated at 4 °C on a roller for 1 hr. The protein mixture was then pelleted though a 40% (weight/volume) sucrose cushion by at 20,000 g at 4 °C for 30 min. The supernatant was discarded and the pellet was collected and analyzed by SDS-PAGE gel and Coomassie staining or immunoblotting against anti-IN antibody.

**Surface plasmon resonance (SPR)**

Binding studies were performed on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Proteins were immobilized on CM5 sensor chips by amine coupling. The solution phase analytes were dissolved in HBS-EP buffer, which contained 150 mM NaCl, 20 mM HEPES, pH 7.5, and 0.5% TritonX-100. The solutions traversed the sensors at a flow rate of 50 µl/min. Costar low-retention polypropylene tubes (Corning, 3207) were used throughout.
Binding results were expressed in resonance units. Kinetics and dissociation constants were analyzed and calculated with BIAevaluation Software Version 4.1.

**Pull down assay**

WT CA, CA hexamers, and CA pentamers were covalently conjugated to Dynabeads M-270 Epoxy using a coupling kit (Life Technologies, catalog # 14311D) at a ratio of 40 pmol CA (monomer) to 1 mg magnetic beads following the manufacturer instructions. CA-conjugated beads were stored at a concentration of 1 mg/100 µL in SB buffer (supplied with the Kit) at 4 °C until use. For the pull-down assay, 100 µL of the CA-conjugated beads (1 mg) were initially washed with 900 µL binding buffer (20 mM HEPES-Na, pH 7.5, 150 mM NaCl, 0.5% Triton X-100), and then incubated with 20 pmol of IN in 500 µL binding buffer at 4 °C for 1 h. Following incubation, the supernatant fraction containing unbound IN ("Unbound" fraction) was collected. Any bound INs were then eluted from the CA-conjugated beads by the addition of 46 µL elution buffer (100 mM glycine, pH 2.5) and incubating at room temperature for 5 min. The eluate ("Bound" fraction) was neutralized by adding 4 µL 1 M Tris-HCl, pH 9.5 and resuspended in 10 µL 6x loading buffer. All samples were heated in loading buffer at 95 °C for 3 min before analysis by SDS-PAGE and immunoblotting using anti-HIV human serum (Scripps Laboratories, # P0224) for IN 220-270 and IN 50-212, and polyclonal anti-IN antibody (AIDS Reagent Program, # 757) for full-length IN and other derivative mutants. Pull down of CA variations with IN-conjugated beads was done in a similar fashion.

**Discussion**

Previous studies have demonstrated that IN plays an important role in viral core uncoating, but how exactly IN associates with CA was unclear. Here, we report that CA interacts with IN
directly and the interaction requires higher order assemblies of CA. We have demonstrated with a binding assay that both CA and CA-NC tubular assemblies can interact with IN. To further investigate the basic binding unit for IN-CA interaction, we took advantage of two CA mutants that form stable hexamers and pentamers in vitro. A pull-down assay revealed that CA hexamers preferentially bind to IN. Attempted kinetic analysis suggests that WT CA, CA hexamer, CA pentamer may bind to IN at different rates. However, the interaction cannot be fitted into a simple 1:1 binding model, most likely due to the complicated multimers associations of both CA and IN. We will discuss more about the multimeric form of IN and study the stoichiometry of the IN-CA interaction in Chapter 3.

Upon entering the cell, the viral core is released into the cytoplasm where uncoating and reverse transcription take place. Some early biochemical studies suggested that the core disassembly begins immediately upon entry and completes soon after fusion\textsuperscript{22,23}. Later studies showed that uncoating is a regulated process and optimal uncoating is required for viral infectivity. Additional evidence now supports a biphasic uncoating model where the core disassembles to some degree in the cytoplasm but a measurable amount of the CA remains associated with the reverse transcription complex (RTC)\textsuperscript{22,25,52}. Our data provides evidence for this second theory, such that IN is able to interact with CA hexamer and CA dimer/monomer with different affinity. The IN-CA interaction may also contribute to the CA-RTC association and mediate interaction with critical host factors and nuclear import. Although the knowledge of the mechanism governing HIV-1 core uncoating is still incomplete, the discovery and characterization of the CA-IN interaction provides structural information in understanding of early and late events in the HIV-1 life cycle, and may present new opportunities for the development of novel targeted inhibitors.
Figure 2.1. Capsid binding assay to assess the interaction of IN and CA interaction *in vitro*.

A. High resolution EM images of CA-NC (a, b) and CA (c, d) assemblies. Both CA and CA-NC form tubular assemblies that are ~50 nm in diameter with various length that resemble the CA hexameric lattice of HIV-1 cores. B. Purified IN and CA-NC or its assemblies were first incubated and then subjected to Co-IP using anti-CA conjugated agarose beads. Eluted proteins were analyzed by SDS-PAGE. CA-NC was detected with Coomassie blue staining and IN was assessed by blotting against a polyclonal anti-IN antibody. C and D. Assemblies made with CA (C) or CA/NC (D) were incubated with IN for binding. The input and pellet were analyzed by Western blotting with anti-p24 (CA) and anti-p32 (IN). A significant amount of IN was pelleted when core assemblies were present, indicating a direct interaction between IN and core assemblies *in vitro*. 
Figure 2.1.

A.

B.

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>Dimer</th>
<th>Tube</th>
<th>ctrl</th>
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<tbody>
<tr>
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α-IN

CB
Figure 2.2. Interaction of WT IN with CA hexamer, CA pentamer, and WT CA.

A. CA mutants can form stable hexamers or pentamers under non-reducing conditions. The CA quadruple mutants A14C/E45C/W184A/M185A and N21C/A22C/W184A/M185A were subjected to sequential dialysis to remove salt and reducing agent. WT CA was included as a control. After dialysis, the assembled multimers were confirmed by non-reducing SDS-PAGE and blotting with anti-CA antibody.

B. Pull down of IN with CA multimers. CA hexamer and pentamer along with WT CA were covalently conjugated to magnetic beads and then incubated with HIV-1 IN. MLV IN was included as a negative control.

C. Pull down of CA multimers with IN. WT IN were covalently conjugated to magnetic beads and then incubated with CA multimers. Blank beads without conjugation were included as a negative control.
Figure 2.2.

A.

B.

C.
Figure 2.3. SPR analysis of IN interaction with WT CA and CA hexamers.

A.

B.

C.
Chapter 3: Tetrameric IN is important for interacting with CA hexamer
Abstract

Integrase (IN) is an essential enzyme that catalyzes the integration of viral cDNA into host genome during HIV-1 replication cycle. Besides, numerous studies have identified the pleotropic effect of IN mutations on viral replication, including reverse transcription, protein composition, virion morphology. In addition, we previously identified an important role of IN during viral core uncoating. Recent studies on allosteric integrase inhibitors reveals IN’s role in viral core morphogenesis and maturation. Here, we characterize the interaction between in vitro assembled capsid (CA) and IN to better understand the mechanism underlying viral uncoating and. First, we identify the active multimeric IN responsible for binding to CA hexamer is a tetramer. Further analysis show that CA hexamer selectively interacts with IN reaching dimer but not with IN core-core dimer. A set of IN single amino acid substitutions on the IN CTD dimer interface have also been examined for their ability to interact with core assemblies and CA multimers, further supporting our observation that IN reaching dimer contains the structural domain required for CA hexamer binding. To map the binding domain on IN, we introduced five IN truncation mutants 1-50, 1-234, 50-288, 50-212 and 220-270. Our data suggest that both CTD and NTD domain of IN bind to CA hexamers. However, only NTD domain retains the specificity towards CA hexamers. These findings provide additional insights on the structural basis for CA-IN interaction during HIV-1 life cycle.
Introduction

Integration of viral c-DNA into host genome is catalyzed by the viral enzyme integrase (IN) and is a crucial step during HIV-1 life cycle. The mechanism of HIV-1 integration has been extensively studied and well characterized. This reaction is carried out in two steps. First step, termed 3’-end processing, is initiated with a cleavage of two nucleotides at each 3’-end by IN. Following that, IN cuts at the target DNA 5 bases apart, and joins the processed viral 3’-ends to the 5’ overhang of the cleaved host DNA. This reaction, termed strand transfer, results in a gapped product, which is repaired by host enzymes, completing the final steps of integration.

Due to the low solubility of full length IN, there are limited amount of structural information available on the full length IN. However, the structure of each individual domains of IN has been solved. The N-terminal domain, amino acid 1-49, contains a zinc binding domain. The catalytic core domain, amino acid 50-212, contains the catalytic site of integration. The C-terminal domain, amino acid 220-288, binds DNA non-specifically. In the absence of DNA substrate, full length IN in solution exists as dimers and tetramers. Dimer of IN is responsible for 3’-end processing\textsuperscript{34,53,54}, while a tetramer of IN is required to catalyze the strand transfer reaction and complete integration\textsuperscript{33,51}. A structural study employing small angle x-ray scattering suggests that IN can assemble into two alternate dimer forms: a core-core dimer characterized by interaction between two CCD domains, and a reaching dimer stabilized by interaction of NTD and CTD from two monomers\textsuperscript{31}. The resulted IN tetramer is comprised of two stacked reaching dimers, stabilized by core-core interactions. During HIV-1 integration, IN interacts with the reverse-transcribed DNA and forms the stable synaptic complex (SSC) or intasome. The correct oligomerization of intasome is essential for the catalytic activity of IN\textsuperscript{54,55}. A very recent study
reveals a high resolution cryo-EM structure of the tetrameric strand transfer complex intasome, resolving previous conflicting models of intasome assembly\textsuperscript{30}.

IN has been shown to have pleiotropic effect on viral replication besides integration. Mutational analyses of HIV-1 IN showed that IN mutations can affect virion morphology, levels of particle-associated integrase, reverse transcription, and viral DNA synthesis\textsuperscript{19,37–41,56}. Our previous study has demonstrated that IN plays an important role in modulating capsid core disassembly and is required for optimal core stability\textsuperscript{40}. Moreover, a few recent studies show that some IN mutant viruses as well as allosteric IN inhibitor treated viruses share similar aberrant core morphology, indicating participation of IN during maturation stage of HIV-1 life cycle\textsuperscript{57,58}.

Results

**The active multimeric form of IN required for interacting with CA hexamer is a tetramer**

The enzymatic activities of IN are dependent on its active form of multimers, as \textit{in vitro} 3’- end processing requires IN dimers minimally and strand transfer requires a tetramer. Since both IN and CA forms high level multimers in solution, it is difficult to assess different species in such a complex situation. Here, to determine the preferred oligomeric states of IN and CA involved in the binding event, we employed size exclusion chromatography in combination with multi-angle light scattering (SEC-MALS). Unlike SEC alone, MALS determines molecular weight through scattered light without the requirement for calibration standards. Since the biophysical properties of WT IN is notoriously poor, a viable mutant IN F185H/C280S with greater solubility is used in the biophysical characterization\textsuperscript{51}. This particular mutant is both catalytically active \textit{in vitro} and viable when introduced into viruses. CA hexamer and IN F185H/C280S were mixed and incubated on ice, and loaded on to a size exclusion column.
followed by light scattering measurement. 0.5 ml fractions were collected throughout the entire run and selected fractions were analyzed with SDS-PAGE and Western blotting. CA hexamer and IN F185H/C280S were also loaded on SEC-MALS separately as controls.

IN F185H/C280S alone showed two overlapping peaks, corresponding to molecular weight 46.8 kDa and 71.0 kDa (Fig 3.1 A). Due to the limitation of resolution and sample concentration, the low molecular weight species cannot be determined with good accuracy. The choice of column range was made based on the assumption that multimers of IN or CA forms complexes that fall into higher molecular weight range. As shown in figure 3.1 B, CA hexamer alone presented two separate peaks at molecular weight 153 kDa and 284 kDa. The first peak corresponds well with CA hexamer whose theoretical molecular weight is 154 kDa. Further analysis of the results has confirmed that the second peak at 284 kDa is a result of dimer association of CA hexamers, and is present even at very low concentration. The WT CA and IN mixture presented a single peak at 37.3 kDa, likely a mixture of CA monomer and IN monomer/dimer (Fig 3.1 C). The CA hexamer and IN mixture eluted three peaks. First peak is at 55.1 kDa, likely a mixture of small molecular weight species. Second peak is at 151 kDa, corresponding to CA hexamer. The third peak, although eluted at the similar position as dimer of CA hexamers, has a significantly lower molecular weight at 261 kDa. This is most likely due to the emergence of a new species which has a smaller molecular weight compared to CA hexamer dimer. Further gel analysis confirmed that the co-existence of IN and CA at the peak 3 (Fig 3.1 D). Theoretical molecular weight of IN tetramer combined with CA hexamer is 290 kDa, which is also about 20 kDa lower than theoretical molecular weight of CA hexamer dimer. Moreover, the mass ratio of peak 2:3 is 65.8% to 16% (1:0.24) for IN-CA mixture compared to 82.6% to 17.4% (1:0.21)
when CA was analyzed alone (Table 3.1). The increase in mass content of the 3rd peak is a result of IN tetramer binding to CA hexamers.

**Reaching dimer of IN is responsible for CA hexamer binding**

Previous investigation revealed two different forms of IN dimers in the absence of DNA substrates. First, a core-core dimer is observed when E11K substitution was introduced into the NTD. The other dimer, termed reaching dimer, involves interaction of the NTD of one monomer with the CCD and CTD of the second monomer, as well as CTD-CTD interactions. Amino acid substitution of F181T interrupts the interaction at core-core interface, resulting in homogeneous reaching dimers. In this study, we purified both IN E11K and IN F181T and examined their interaction with CA hexamer and WT CA with a pull-down assay. Mutant INs and WT IN were incubated with magnetic beads covalently-conjugated with either CA hexamers or WT CA. Bounded proteins were eluted with an acidic wash buffer and analyzed on gel. Analysis showed that WT and IN F181T bind similarly to CA hexamers, while E11K, which forms monodisperse core-core dimers, had much weaker interaction with CA hexamers. In addition, IN F181T retained the preference binding to CA hexamers, similar to that of WT IN, while IN E11K did not.

A previous study which solved the structure of IN CTD domain, has mapped a hydrophobic CTD dimer interface composed of a group of hydrophobic residues. In addition, a study on combined IN CCD and CTD domain (52-288) also revealed a CTD dimer interface mediated by Phe-243 stacking. Based on those observations, we prepared four IN single amino acid substitution mutants (W243E, G247E, A248E, V250E). All four residues are located at the hydrophobic region of proposed IN CTD dimer interface and viral mutants that harbor these substitutions were tested replication deficient with loss of reverse transcription products.
mutant variations and WT IN were subject to pull down assay and CA binding assay. Although all four residues were found at the dimer interface, only IN V250E and W243E showed significant reduction in interaction with CA hexamers (Fig 3.2 A and B). Then, we examined the multimer composition of the four IN mutants using a crosslinking assay. The reagent used in the experiment, BS3, is crosslinker that covalently links the amine groups in the side chains and the N-terminus of each polypeptide. The crosslinking reactions were performed at four BS3 concentrations (0, 1nM, 2.5 nM, and 5 nM). The gel analysis of crosslinked products showed that WT IN forms both dimer and tetramers under the experimental conditions. Compared to WT IN, IN V250E has increased dimer species but decreased tetramer species at 1 nM and 2.5 nM crosslinker concentration. It also lost some high order multimers that were observed with WT IN at all three concentrations. At high crosslinker concentration (5 nM), V250E showed reduction in all species including monomer and dimer. This reduction is likely due to formation of large aggregations that cannot enter the gel. IN W243E, which also failed to interact with CA hexamers, has significantly lowered both dimer and tetramer formation throughout all concentrations of crosslinker (Fig 3.2 C). Moreover, monomer species of W243E remains similar to that of WT, indicating a different mechanism from V250E mutant.

N-terminal domain of IN preferentially recognizes CA hexamers

To investigate which domain on IN is responsible for the preferential recognition of CA hexamers, we utilized five IN truncation mutants, IN 1-49, IN 1-234, IN 50-212, IN 50-288 and IN 220-270. CA hexamer, pentamer and WT CA conjugated magnetic beads were subjected to pull down assay with each IN truncation mutant. Results showed that IN mutants contain NTD domain (1-49 and 1-234) displayed differential binding affinity similar to that of WT IN, while IN CTD (50-212) showed abolished binding among all three CA variations (Fig 3.3). On the
other hand, IN mutants that contain the CTD domain (220-270 and 50-288) showed similar affinity to all three CA variations above background level. Therefore, we conclude that NTD of IN contributes to preferential recognition of CA hexamers.

Material and Methods

Protein purification

WT IN and IN mutants were prepared as mentioned previously. The expression plasmids encoding IN F185H/C280S and IN truncation mutants 50-212, 1-234 and 220-280 are kindly provided by Dr. Robert Craigie (NIH, MD) and plasmid encoding IN 1-49 is a kind gift from Dr. Alan Engelman (Harvard University, MA). Plasmids encoding IN mutants F181T and E11K is a kind gift from Dr. Mark Andrake (Fox Chase Cancer Center, PA). IN single amino acid substation W243E, G247E, A248E, V250E were prepared in house based on the QuickChange site-directed mutagenesis protocol. CodonPlus E. coli cells (Agilent, CA) were transformed by expression constructs encoding either full-length IN or its mutant derivatives sequences having an N-terminal His tag for facilitating protein purification. All full-length IN and IN truncation constructs were purified under non-denaturing conditions as previously described, with few modifications. Briefly, transformed cells were grown in LB medium at 32 °C until the optical density at 600 nm was between 0.8 and 1.0. Protein expression was then induced by adding 0.4 mM IPTG and left to grow overnight at 18 °C. Pelleted cells were suspended in a lysis buffer containing 20 mM HEPES-Na, pH 7.5, 5 mM βME, 1 M NaCl, 0.2 mM EDTA, 10% glycerol, 0.5% IGEPAL® CA 630 (Sigma-Aldrich), and EDTA-free protease inhibitor tablets (Roche; 1 tablet/10 mL lysis buffer), and further disrupted by sonication. Lysates were then clarified by centrifugation at 100,000 x g for 1 hr at 4 °C. Purified IN and its mutant derivatives were then
obtained from clarified lysates using Ni\textsuperscript{2+} nitrilotriacetic acid (NTA) immobilized metal affinity chromatography (IMAC) and cation exchange chromatography. Purified proteins were quantitated using Bradford assay, and purity determined by SDS-PAGE and Coomassie blue staining.

The expression plasmids that contain CA coding sequence is a kind gift from Dr. Wesley Sunquist (University of Utah, UT). The expression plasmids that contain CA mutation A14C/E45C/W184A/M185A and N21C/A22C/W184A/M185A are kind gift from Dr. Owen Pornillos (University of Virginia, VA). Expression plasmids were transformed into BL21 Competent E. coli cells and grew in 1 L LB medium at 37 °C. Expression was induced in log-phase (OD600 ~0.8) for 4 hr by adding Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1mM concentration. For CA purification, cells were harvested and resuspended in 40 ml lysis buffer containing 20 mM Tris, pH 7.5, 10 mM β-mercaptoethanol (βME) and EDTA-free protease inhibitor tablets (Roche; 1 tablet/10 mL lysis buffer) and followed by sonication. The lyate was clarified by centrifugation at 35,000 g for 1 hr. CA protein were then precipitated by addition of 0.35 equivalent (v/v) of saturated ammonium sulfate and re-dissolved in a buffer containing 20 mM KMops (pH 6.9) and 10 mM βME. The protein was further purified through cation-exchange chromatography on a HiTrap SP HP column (GE Healthcare Life Sciences).

**Preparation of CA pentamers and hexamers**

Pentamers and hexamers of CA were assembled through sequential dialysis. 10 mg/ml CA mutant (A14C/E45C/W184A/M185A and or N21C/A22C/W184A/M185A) were first dialyzed against assembly buffer (50 mM Tris, pH 8, 1 M NaCl) containing 200 mM βME overnight. Then it was dialyzed against assembly buffer to remove β ME and finally with 20 mM Tris, pH 8. All the dialysis steps were performed at 4 °C and for at least 8 hr. The assembled pentamers
and hexamers were analyzed by non-reducing SDS-PAGE and quantified through Bradford assay. Assembled proteins were stored at 4 °C until used.

**SEC-MALS**

SEC experiments were performed on a Shodex Protein KW-803 column (Showa Denko K.K., Japan) at 0.5 ml/min at RT in a buffer containing 20 mM HEPES pH 7.5, 450 mM NaCl, 0.1 mM EDTA, and 10 µm ZnCl₂, 5mM Chaps. The detergent concentration has been confirmed to be at submicellar concentrations at this ionic strength. All buffer used in this experiment was filtered through 0.2 µm syringe filter. The scattered light intensity of the eluant was recorded with a DAWN-HELEOS multi-angle light scattering detector (Wyatt Technology Corp.), which was calibrated with a bovine serum albumin standard (ThermoFisher Scientific). Concentration of the eluant was determined using an in-line Optilab DSP interferometric refractometer (Wyatt Technology Corp.). The weight-averaged molecular weight of species of each chromatographic peak was calculated using the ASTRA software version 5.2 (Wyatt Technology Corp.).

**Pull down assay**

WT CA, CA hexamers and pentamers were covalently conjugated to Dynabeads M-270 Epoxy using a coupling kit (Life Technologies, catalog # 14311D) at a ratio of 40 pmol CA (monomer) to 1 mg magnetic beads following the manufacturer instructions. CA-conjugated beads were stored at a concentration of 1 mg/100 µL in SB buffer (supplied with the Kit) at 4 °C until use. For the pull-down assay, 100 µL of the CA-conjugated beads (1 mg) were initially washed with 900 µL binding buffer (20 mM HEPES-Na, pH 7.5, 150 mM NaCl, 0.5% Triton X-100), and then incubated with 20 pmol of IN in 500 µL binding buffer at 4 °C for 1 h. Following incubation, the supernatant fraction containing unbound IN (“Unbound” fraction) was collected. Any bound INs were then eluted from the CA-conjugated beads by adding 46 µL elution buffer
(100 mM glycine, pH 2.5) and incubating at room temperature for 5 min. The eluate ("Bound" fraction) was neutralized by adding 4 µL 1 M Tris-HCl, pH 9.5 and resuspended in 10 µL 6x loading buffer. All samples were heated in loading buffer at 95 °C for 3 min before analysis by SDS-PAGE and immunoblotting using anti-HIV human serum (Scripps Laboratories, # P0224) for IN 220-270, IN 50-212 and IN 50-288, and polyclonal anti-IN antibody (AIDS Reagent Program, # 757) for full-length IN and other derivative mutants.

Discussion

In this study, we determined that the active multimeric form of IN required for interacting with CA hexamer is a tetramer. In the SEC-MALS experiment, due to the limitation in resolution of size exclusion chromatography, proteins fall under ~MW 100 kDa, including monomer CA, monomer and dimer, are likely to appear as one single peak, thus cannot be determined with accuracy. Biophysical characterization techniques, such as MALS, usually require high sample concentration. In our study, we utilized an active IN double amino acid substitution mutant F185H/C280S with greatly improved solubility to minimize large aggregations. Under our experimental conditions, IN alone most likely exists as monomer and dimer in solution and CA hexamers alone also exhibit detectable level of dimer association (Fig 3.1 A and B). The combination of IN and CA hexamers resulted a shift of a peak in the high MW range (Fig 3.1 D). Although this peak (261 kDa) has similar elution profile as dimer of CA hexamers (284 kDa), it has a ~20 kDa drop in molecular weight and the western blot analysis confirmed IN presence at that peak (Fig 3.1 E). This higher molecular weight species of IN is absent when IN was
analyzed alone or in the presence of WT CA. Together, the SEC-MALS analysis indicated tetramer of IN is required to interact with CA hexamers.

We introduced four single amino acid substitutions to full length IN (W243E, G247E, A248E, V250E), which have been previously reported in a hydrophobic region of CTD dimer interface. The crosslinking study showed that IN V250E has increased dimer species but decreased tetramer species, and W243E has lowered both dimer and tetramer formation. The gel analysis of crosslinked products further indicated that IN V250E forms aggregations that may prevent CA hexamer interaction. However, IN W234E affected oligomerization of IN in a different fashion, such that IN W243E fail to form IN dimer or other higher order multimers. Both V250E and W243 lead to abolished interaction with CA tubular assemblies and CA hexamers. This observation further supported that tetramer of IN is important for IN-CA interaction.

CTD of IN has been shown to be important for IN active dimer formation. Mutants harbors point mutations on CTD were found with strongly reduced 3’ processing and strand-transfer activity. A structural study on IN 50-288 crystallization indicated that conserved residues W243 together with L242 was found in a hydrophobic surface in CTD domain interactions mediating IN dimer-dimer interaction. Based on previous findings, our crosslinking study further confirmed that residue W243 is important in dimer formation of IN. A structural study has suggested two models of IN dimers, a core-core dimer characterized by interaction between two CCDs, and a reaching dimer stabilized by interaction of NTD of one monomer and CTD from the other monomer. The resulted IN tetramer is comprised of two stacked reaching dimers, stabilized by core-core interactions. Based on the reaching dimer model, we speculate that residues W243 is involved in IN reaching dimer formation, so that
W243E substitution likely brought structure change to CTD domain of integrase that affects both dimer and tetramer formation.

The domain mapping study took advantage of five truncated IN mutants IN 1-49, IN 1-234, IN 50-212, IN 50-288 and IN 220-270. IN NTD domain showed preferential binding to hexameric CA, while CTD exhibited non-specific binding to CA multimer assemblies and WT CA. All three domains of IN form dimers on their own, and the dimer structure of a single domain deviates from what has been reported for multi-domain. Although the precise structure model is not available for full length IN dimer or tetramer, the IN reaching dimer model suggests that NTD and CTD of two IN dimers are closely located in the center while two CCDs are separated on each side on the IN dimer. This structure model could potentially be used in further mapping the CA hexamer binding domain on IN.

Taken together, our study has demonstrated that the reaching dimer of IN tetramer is important for interacting with CA hexamer and the domain responsible for preferential binding to CA hexamer is NTD of IN. These finds will contribute to the continuing efforts to elucidate the conformation and multimerization of IN, as well as to understand IN’s role in multiple steps during HIV-1 life cycle, including uncoating and maturation.
Figure 3.1. SEC-MALS analysis of CA and IN interaction.

SEC profiles of A. IN F185H/C280S, B. CA hexamer, C. WT CA + IN F185H/C280S, D. CA hex + IN F185H/C280S. Molecular weight of each species was determined through multi-angle light detection device coupled to a Shodex Protein KW-803 column. E. Each 0.5 ml fraction was collected for the run of CA hex + IN F185H/C280S. Selected fractions were analyzed on SDS-PAGE and blotted against anti-IN (top) and anti-CA antibodies. IN was detected in fraction 15 (7.5 ml elution volume) along with CA.
Figure 3.1

A.

B.

C.
D.

E.

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Elution volume

- \(\alpha\)-IN
- \(\alpha\)-CA
Table 3.1. SEC-MALS profile of CA hexamer alone and CA hexamer and IN F185H/C280S mixture. Comparison of molecular weight and mass ratio of each peak.

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Figure 3.2. Single amino acid substitutions on IN that affect correct multimer formation also diminish IN-CA interaction.

A. Two IN mutants (E11K and F181T) which form the core-core dimer and the reaching dimer respectively were subject to pull down with \textit{in vitro} assembled CA hexamer, pentamer and WT CA. WT IN was included as a control. 10% input of IN was used as a loading control. Eluted IN proteins were analyzed with SDS-PAGE and blotted against anti-IN. B and C. Individual amino acid substitutions are introduced to full length IN and IN mutants are subjected to CA binding assay (B) and pull-down assay (C). D. Crosslinking profiles of WT IN and IN mutants. 640 nM WT IN and full length IN mutants were titrated with increasing amount of BS3 crosslinker (0, 1 nM, 2.5 nM, 5 nM). The reaction was allowed to go for 20 min at RT. Proteins were analyzed by denaturing SDS-PAGE and blotted against anti-IN.
Figure 3.2

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α-IN

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α-IN

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C.

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α-IN
### Table D.

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Figure 3.3. WT IN and truncated IN mutants interact with CA hexamers.

A. Pull down of IN mutants (1-49, 1-234, 50-212, 220-270, 50-288) and magnetic beads conjugated with *in vitro* assembled CA hexamer, pentamer and WT CA. WT IN was included as a control. 10% input of IN was used as a loading control. Eluted IN proteins were analyzed with SDS-PAGE and blotted against anti-IN or HIV-1 anti-serum. Quantitative analysis of Western blot image is shown in B.
Figure 3.3

A.

B.
Chapter 4: Summary and Conclusions
Although progresses have been made in novel anti-HIV prevention and treatment approach, our knowledge regarding some crucial steps during HIV-1 life cycle is still lagging. The goal of this dissertation is to characterize the interaction between two essential viral proteins, CA and IN, and to provide additional clues to understand the biological significance of the interaction. Our lab has previously reported that IN plays an important role in modulating viral core uncoating and is required for optimal core stability. In addition, an increasing number of studies have suggested that IN is involved in viral core morphogenesis during maturation.

In chapter two, we determined that CA interact with IN directly and the interaction requires higher order assembly of CA. We also demonstrated with a binding assay that both CA and CA-NC tubular assemblies interact with IN. To further understand the basic binding unit for IN-CA interaction, we took advantage of two CA mutants that assemble into hexamers and pentamers \textit{in vitro} respectively. A pull-down assay analysis revealed that IN preferentially binds to CA hexamers over CA pentamers or WT CA. Our data provides evidence for the biphasic model of viral core uncoating, in which core partially disassemble but remains associated with reverse transcription complex (RTC), modulating uncoating at an optimal rate. IN-CA interaction may contribute to the CA-RTC association as well as initial uncoating of the core.

In chapter three, we determined that the active multimeric form of IN required for interacting with CA hexamer is a tetramer and the reaching dimer of IN is responsible for the IN-CA interaction. The molecular weight of the complex formed by CA hexamer and IN tetramer was determined through SEC-MALS. Further analysis showed that CA hexamers interact with IN mutant that forms reaching dimers rather than core-core dimers. In addition, we introduced four single amino acid substitutions to full length IN (W243E, G247E, A248E, V250E) which have been previously reported to be buried in a hydrophobic region of CTD dimer interface.
The crosslinking study showed that both V250E and W243 lead to diminished interaction with CA tubular assemblies and CA hexamers, likely through different mechanisms. IN V250E led to aggregation of IN while IN W243E attenuated all forms of multimer formation. This observation further supported that reaching dimer of IN is important for IN-CA interaction. These findings support other biochemical and biophysical studies on IN which show that correct multimerization is important for viral core maturation. The domain mapping study took advantage of five truncated IN mutants IN 1-49, IN 1-234, IN 50-212, IN 50-288 and IN 220-270. IN NTD domain showed preferential binding to hexameric CA, while CTD exhibit nonspecific binding to CA multimer assemblies and WT CA.

The work presented in this dissertation is part of an effort to better understand the steps that are involved with CA-IN interaction during HIV-1 life cycle, namely uncoating and maturation. In addition, information gained in this study provides structural basis and highlight the significance for exploiting CA and IN interaction as a new therapeutic target.
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