Department of Laboratory Medicine
Division of Clinical Microbiology
Karolinska Institutet, Stockholm, Sweden

Studies on HIV-1 Core Assembly

Samir Abdurahman

Karolinska Institutet

Stockholm 2007

Cover picture:

Upper panel: Electron micrographs of budding, immature, and mature HIV-1 cultivated by the author, Professor Stefan Höglund took the photos
Middle panel: Graphically manipulated pictures of some of the techniques used in exploring HIV-1 CA assembly and infectivity (from left: part of HPLC chromatogram, Western blot analysis, and RT-ELISA)
Bottom panel: Immunofluorescence analysis of HIV-1 CA in transfected HeLa-tat cells

Published by Karolinska Institutet. Printed by Larseric Digital Print AB
Sundbyberg, Stockholm, Sweden

ISBN: 978-91-7357-363-4

To my family

ABSTRACT

The main objective of this thesis was to define the roles of Gag (p55), in particular, the capsid (CA, p24) protein in human immunodeficiency virus type 1 (HIV-1) particle assembly. More specifically, i) to determine the relative contribution of some specific residues and/or sequences in HIV-1 CA core assembly and virus release, ii) to characterize the importance of two conserved residues with quite opposing intra-molecular contacts with other CA residues in capsid assembly iii) to define the role and significance of a specific amino acid involved in formation of a conserved β-hairpin structure in HIV-1 capsid assembly, and iv) to define the active antiviral metabolite of an antiviral tripeptide amide previously found to affect HIV-1 capsid assembly and infectivity.

The HIV-1 CA plays a crucial role in both assembly and maturation of the virion. Two highly conserved sequences located in the *C*-terminal domain (CTD) of HIV-1 CA were investigated with site directed mutagenesis, a valuable technique used widely to study the structure and function of CA in HIV-1 capsid assembly. We showed that mutations of specific residues within the two conserved sequences in the *C*-terminal domain could affect viral protein expression, virus assembly, release and infectivity. In addition, we showed that these residues are essential for proper proteolytic processing of the Gag/Gag-Pol precursors in a cell-type dependent manner, as well as, for proper morphogenesis of HIV-1 particles.

The importance of two particular amino acid residues, Glu98 and Glu187, located within each of the two CA domains were investigated. In contrast to Glu98 which has no intra-molecular contacts, Glu187 has extensive intra-molecular contacts with eight other CA residues. Furthermore, Glu187 has been shown to be important for a salt-bridge formation in a head-to-tail dimer of HIV-1 CA. We performed detailed analysis to assess the potential effects of mutating these two Glu residues for Ala and Gly, respectively, on Gag processing, virus infectivity, viral cDNA production and virus morphology. In spite of the lack of contact with the other residues of CA as revealed by the structural data, Glu98 was shown herein as a critical element in the action of CA to correctly form mature cores. Our data also showed that the two residues in the study displayed deviated biological properties than the ones being predicted from crystallography and/or analysis of inter-atomic contacts. Thus, Glu187 was found to be dispensable although the residue was predicted to be important for the *N*-*C* CA-dimer formation.

As the CA CTD dimer formation is one of the fundamental interactions driving CA multimerization, which also involves hexamerization of the CA *N*-terminal domains (NTD), we investigated the role and significance of aspartate 51 (D51) which previously has been shown to play a key role in virus assembly and maturation by forming a β-hairpin structure that is highly conserved among retroviruses. In addition to the D51A substitution reported elsewhere, we showed that substitutions of aspartate with glutamate, glutamine, or asparagine, three amino acid residues that are structurally close and have similar properties in protein as aspartate, could not rescue the structural integrity of the protein.

It has previously been shown that addition of the tripeptide GPG-amide, corresponding to a motif found in both conserved sequences described above, could induce non-infectious HIV-1 particles with aberrant core structures. We identified and demonstrated that it is a metabolite of GPG-amide that affects HIV-1 infectivity. This metabolite was purified and its structure determined by NMR to be α-hydroxy-glycineamide (α-HGA). α-HGA binds to the HIV-1 CA and affects its ability to assemble into tubular or core structures *in vitro* and *in vivo*. As an antiviral, α-HGA has an unusually simple structure, a pronounced antiviral specificity and a novel mechanism of antiviral action.

In conclusion, our findings suggest that mutations in CA are lethal when affecting proper CA core assembly and that the semi-stable non-covalent protein interactions in HIV-1 CA can be specifically disrupted by small molecules, such as α-HGA.

Keywords: HIV-1, mutation, capsid, p24, capsid assembly, α-HGA, antiviral

LIST OF ABBREVIATIONS

AIDS	**a**cquired **i**mmuno**d**eficiency **s**yndrome
HIV-1	**h**uman **i**mmunodeficiency **v**irus type-**1**
CA	**ca**psid protein
gag	**g**roup **a**ssociated **g**ene
env	**env**elope gene
pol	**pol**ymerase gene
rev	**re**gulator of **v**irion proteins gene
tat	***t**rans*-**a**ctivator of **t**ranscription gene
vif	**v**irion **i**nfectivity **f**actor gene
vpr	**v**iral **p**rotein **R** gene
vpu	**v**iral **p**rotein **U** gene
nef	**ne**gative regulatory **f**actor gene
gp120	**g**lyco**p**rotein 120
LTR	**L**ong **t**erminal **r**epeat
RNA	**r**ibo**n**ucleic **a**cid
DNA	**d**eoxyribo**n**ucleic **a**cid
SP1	**s**pacer **p**eptide 1 (previously known as p2)
SP2	**s**pacer **p**eptide 2 (previously known as p1)
SDS-PAGE	**s**odium **d**odecyl **s**ulfate -**p**oly**a**crylamide **g**el **e**lectrophoresis
GPG-NH_2	**g**lycine-**p**rolyl-**g**lycineamide
α-HGA	alpha-**h**ydroxy **g**lycine**a**mide
CypA	**Cy**clo**p**hilin **A**
APOBEC3G	**apo**lipoprotein **B** mRNA-editing **e**nzyme, **c**atalytic polypeptide-like **3G** (also known as CEM15)
TRIM5	**Tri**paritate **m**otif protein 5
ESCRT	**E**ndosomal **s**orting **c**omplex **r**equired for **t**ransport
mAb	**m**onoclonal **a**nti**b**ody
AA (aa)	**a**mino **a**cid
WB	**W**estern **b**lot
ELISA	**E**nzyme **l**inked **i**mmuno**s**orbent **a**ssay
PBMC	**P**eripheral **b**lood **m**ononuclear **c**ell

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. Samir Abdurahman, Stefan Höglund, Laura Goobar-Larsson, and Anders Vahlne. Selected amino acid substitutions in the *C*-terminal region of Human immunodeficiency virus type-1 capsid protein affect virus assembly and release. *Journal of General Virology*, 2004, **85**:2903–2913.

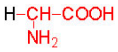
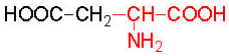
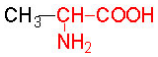
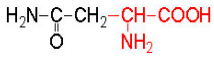
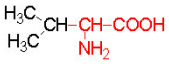
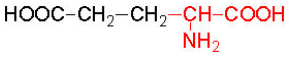
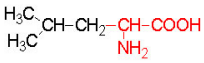
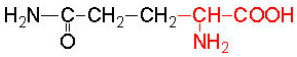
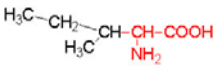
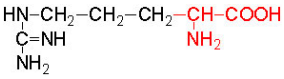
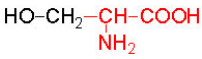
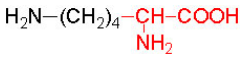
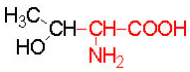
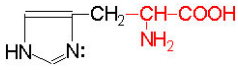
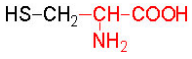
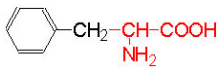
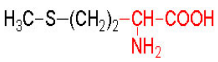
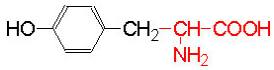
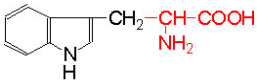
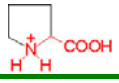
II. Samir Abdurahman, Stefan Höglund, Anders Höglund, and Anders Vahlne. Mutation in the Loop *C*-terminal to the Cyclophilin A Binding Site of HIV-1 Capsid Protein Disrupts Proper Virus Assembly and Infectivity. *Retrovirology*. 2007 Mar 19; **4**:19.

III. Samir Abdurahman, Masoud Youssefi, Stefan Höglund and Anders Vahlne. Characterization of the invariable residue 51 mutations of human immunodeficiency virus type 1 capsid protein on in vitro CA assembly and infectivity. *Retrovirology*. 2007 Sept 28; **4**:69. [Epub ahead of print]

IV. Samir Abdurahman, Ákos Végvári, Masoud Youssefi, Michael Levi, Stefan Höglund, Marita Högberg, Weimin Tong, Ivan Romero, Elin Andersson, Peter Horal, Bo Svennerholm, Jan Balzarini, and Anders Vahlne. Alpha-Hydroxy-Glycineamide is a New Antiretroviral Agent Affecting HIV-1 Core Assembly and Infectivity. *Submitted*.

CONTENTS

AMINO ACIDS

Amino Acid	Symbol	Structure	Amino Acid	Symbol	Structure
Aliphatic R-Groups			**Acidic and their Amides**		
Glycine	Gly - **G**		**Aspartic Acid**	Asp - **D**	
Alanine	Ala - **A**		**Aspara-gine**	Asn - **N**	
Valine	Val - **V**		**Glutamic Acid**	Glu - **E**	
Leucine	Leu - **L**		**Glutamine**	Gln - **Q**	
Iso-leucine	Ile - **I**				
			Basic		
Non-Aromatic with Hydroxyl R-Groups			**Arginine**	Arg - **R**	
Serine	Ser - **S**		**Lysine**	Lys - **K**	
Threo-nine	Thr - **T**		**Histidine**	His - **H**	
Sulfur-Containing R-Groups			**Aromatic**		
Cysteine	Cys - **C**		**Phenyl-alanine**	Phe - **F**	
Methio-nine	Met-**M**		**Tyrosine**	Tyr - **Y**	
			Trypto-phan	Trp-**W**	
Imino					
Proline	Pro - **P**				

1 BACKGROUND

Human immunodeficiency virus (HIV) is the etiological agent for acquired immune deficiency syndrome (AIDS) and its infection has led to the worldwide HIV/AIDS pandemic affecting millions of people, mostly in low and middle income countries. HIV is a relatively small and simple virus, but yet, with a very complex pathogenesis that results in destruction of the immune system of an infected person. The intense research within the field during the past 25 years have resulted in 21 approved HIV-drugs that are in clinical use today [1]. However, despite the significant progress made in understanding the viral replication and pathogenesis, the virus is still spreading and no vaccine is yet available. Current antiretroviral drugs control HIV-1 infection by targeting the viral enzymes protease and reverse transcriptase, a 36 amino acid peptide that inhibit the fusion of the viral envelope with the plasma membrane, and more recently the FDA (August 2007) and EU approved a CCR5 antagonist [2], a new class of HIV drugs. The first integrase inhibitor which also belongs to another class of drugs is soon to be approved by FDA. However, despite the successful treatment with combinations of available drugs, drug toxicity and resistance are still the major reasons for treatment failure. It has also become more common for people who contract HIV to be infected with dual- and triple-class multi-drug resistant HIV strains.

This thesis was focused on understanding the HIV-1 capsid (CA, p24), one of the viral core proteins that could be a potential new target for developing effective drugs and vaccines to fight HIV.

1.1 Discovery and pandemic

In early 1980s, a cluster of unusual diseases such as rare opportunistic infections (*Pneumocyctis carinii pneumonia*) and a rare malignancy (Kaposi's sarcoma) started to appear in previously healthy young homosexual men [3, 4]. Common features among these patients were the profound state of immune suppression and a decline of T cells subsets that harbored the CD4 surface antigen [5, 6]. It was first suggested by Myron Essex and Robert Gallo that a retrovirus might be the cause of the disease. However, although a new retrovirus was first isolated from AIDS patients by Luc Montagaier and co-workers at the Pasteur Institute in Paris in 1983 [7] its link as the cause of acquired immune deficiency syndrome (AIDS) was proved by Gallo and his co-workers at the National Cancer Institute in Bethsada, Maryland [8, 9]. These findings were then confirmed by Levy and his co-workers at the University of California [10]. Although the viruses from all these three groups were characterized by a common morphological appearances, they were named differently: lymphadenopathy-associated virus (LAV), human T cell lymphotropic virus III (HTLV-III), or AIDS-associated retrovirus (ARV), respectively [8, 10, 11]. In 1985, a second novel retrovirus was isolated from West African patients with AIDS [12] and was named LAV-2. These names started to raise concerns and finally in 1986, an international committee on

Taxonomy of Viruses renamed the virus as human immunodeficiency virus (HIV) [13]. Thus, HTLV-III, LAV-1 and ARV are all what we today call HIV-1, whereas LAV-2 is now HIV-2.

HIV is a lentivirus belonging to a larger group of viruses known as retroviruses. Lentivirus isolates from humans are grouped into one of two types, designated HIV-1 and HIV-2. The question of the origin and the initial HIV/AIDS outbreak is still not settled. However, through genetic sequencing, Gao *et al.* have identified the nearest simian immunodeficiency virus (SIV) to HIV-1 in certain chimpanzees (SIVcpz) in Africa, the *Pan trolodytes trolodytes* [14]. All HIV-1 strains known to infect humans, including HIV-1 groups M, N, and O are closely related to just the one found in this particular SIVcpz lineage's, the *Pan trolodytes trolodytes*. Although HIV was first identified in 1983-84, the earliest evidence of the virus comes from the antibodies reactive to HIV-1 discovered from the plasma sample of an African patient in 1959 [15]. It is suggested that the virus likely entered the human population through zoonotic transmission from chimpanzees to humans in Central Africa [14]. In the case of HIV-1, it is currently believed that the virus entered human populations in the early 1930s [16, 17]. The second retrovirus, HIV-2, which is only about 40% homologous to HIV-1, has a closer relationship to the SIV from macaque monkeys, the *sooty mangabey* (SIVsm). Furthermore, in comparision to HIV-1, HIV-2 is more geographically restricted and is primarily found in West Africa. HIV-2 infection of humans has been estimated to date back to 1940 plus minus 16 years [18].

In less than three decades, the HIV/AIDS pandemic has become one of the greatest global threats to human health and development. The disease has now spread to all parts of the world and thus far been responsible for the death of 32 million people worldwide. To date, there are approximately 40 million people infected with the virus and only during last year, 2.9 million people died due to HIV/AIDS. In most developed countries, however, the AIDS death rate is now the lowest since 1996. At the same time, the number of people living with HIV/AIDS in sub-Saharan Africa has steadily increased since the beginning and the AIDS crisis still continues to deepen in developing countries [19, 20].

1.2 Transmission and pathogenesis

HIV is sexually transmitted disease (anal, oral, and vaginal). Concommitent genital infections can also aid the infection through ulceration and inflammation of the mucosal epithelial surfaces. HIV can also be transmitted through parenteral contact (exposure to infected blood or blood products and use of drug injections) and vertically from infected mothers to their offsprings during birth or later through breast-feeding. HIV-1 and HIV-2 cause clinically identical AIDS, however, the latter is less easily transmitted [21], and the period between initial infection and illness appears to be longer [22].

Early after transmission, infected people may or may not develop any symptoms. If the symptoms appear, they usually develop within 3 to 10 weeks

after the initial infection. The symptoms are often characterized as a flu-like illness accompanied by fever, rashes, lymphadenopathy, myalgia, gastrointestinal symptoms, and CNS disorders, which occur at the time of seroconversion [23-26]. The course of the disease is often divided into three phases (**Fig. 1**); an acute/primary, a chronic latent and a final AIDS phase.

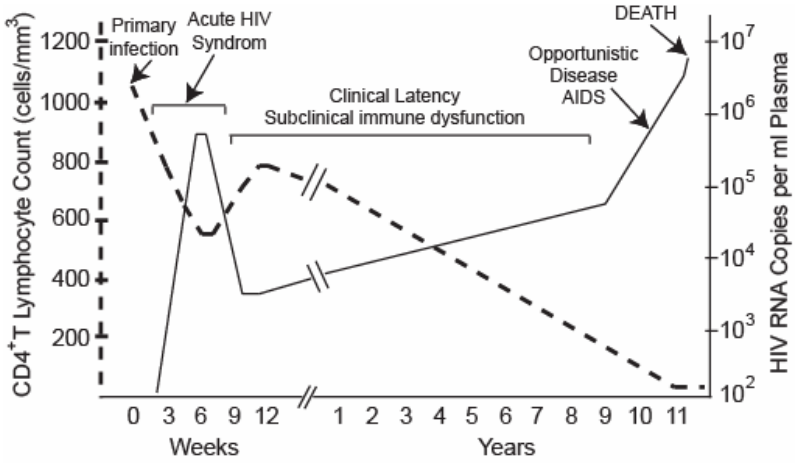


Figure 1. Graph showing HIV-1 copies (solid line) and CD4 cell counts (broken line) over the course of HIV infection

During the acute phase of infection, the viral replication is high reducing the number of circulating CD4+ cells and a peak of viremia occurs weeks after infection [24, 27-30]. At this time, an innate immune reaction develops which is followed by a specific humoral and cellular immune responses to HIV, the result of which is that the level of viral replication is dramatically decreased [31], lowering the viral load and allowing the number of CD4+ T cells to return to near normal levels. After the acute phase, a prolonged period of clinically asymptomatic period follows. During this stage of the disease, the virus replication (viral load) continues slowly to increase. Although there is a variation in the rate of disease progression, it is believed that the intial reaction to the infection during the acute/primary phase of HIV-1 infection determine the disease prognosis [32-34]. Besides declining absolute CD4+ T-cell numbers, a dysregulation in B-cell function has also been observed at this stage of infection [35, 36]. Eventually, in the absence of therapy, the majority of people infected with the virus enters the final phase of AIDS within 8 to 10 years. Some of the clinical manifestations of AIDS that appear during this last phase of infection are various opportunistic infections, Kaposi's sarcoma, lymphoma, extra pulmonary tuberculosis, weight loss and neurological disorders [37, 38].

1.3 VIROLOGY OF HIV-1

1.3.1 Virion structure

The HIV-1 virion has a spherical shape, is about 110 nm in diameter and consists of a host cell derived lipid bi-layer, the envelope that surrounds a cone-

shaped nucleocapsid (**Fig. 2**). The outer membrane or envelope of the virus contains approximately 72 spikes that show triangular symmetry [39]. Each spike is thought to contain homotrimers of the envelope glycoprotein linked together in groups [40]. The homotrimeric protein is composed of a surface (gp120) and a transmembrane subunit (gp41) connected non-covalently [41] . A stable interaction of glycoprotein gp41 with p55(Gag) then ensues in immature HIV-1 [42, 43]. The Gag polyprotein is attached to the inner surface of the virus membrane by a biparitet membrane-binding domain composed of a myristic acid moiety and a highly basic residues in the N terminus of MA [44, 45]. As an immature virus (**Fig. 2A**), the HIV-1 core has a doughnut-like morphology which is transformed into cone-shaped structure upon maturation as a result of Gag processing [46] (**Fig. 2B**). Thus upon maturation, the MA lines the inner surface of lipid membrane, while the CA forms the conical core structure. On the inner side of the viral core structure the HIV-1 genome is contained and consists of two copies of single stranded RNAs tightly bound to the nucleocapsid (NC; p7) protein. Other viral proteins contained here are the reverse transcriptase (RT; p66/p51), protease (PR; p15) and integrase (IN; p31).

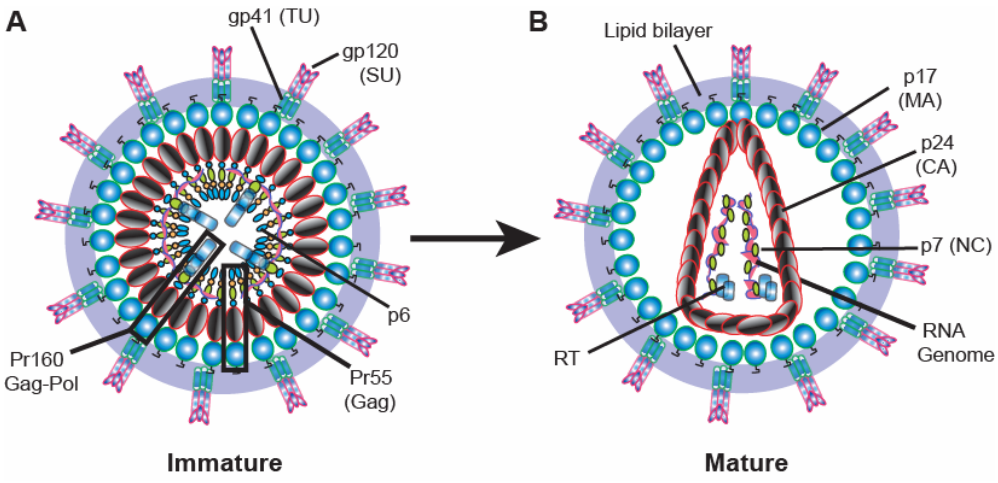


Figure 2. Schematic representation of an immature (A) and mature (B) HIV-1 with their approximate locations of the viral proteins.

1.3.2 Genomic structure

The HIV-1 genome is about 9.2 kb long (**Fig. 3**). It has nine open reading frames (ORF) encoding 15 distinct proteins [47, 48]. Three of these ORFs encode the Gag, Pol, and Env polyproteins, which are subsequently cleaved into individual proteins common to all retroviruses. The Gag and Env polyproteins have structural functions and produce the matrix (MA; p17) proteins, capsid (CA; p24), nucleocapsid (NC; p7), p6 and the surface envelope glycoproteins gp120 and gp41, respectively. The *pol* gene encodes the three indispensable viral enzymes, protease (PR; p31), integrase (IN; p32) and reverse transcriptase (RT; p66/p51). The latter is a heterodimer of p51 and the precursor polyprotein p66 [49-51].

Unlike most retroviruses, HIV-1 encodes six additional genes called the accessory genes [52, 53]. Three of these gene products (Vif, Vpr, and Nef) are incorporated into the budding virus while the others are not [54, 55]. However, Nef has been found only at very low levels in virions, and it is unclear if this is significant. Two other accessory gene products, Tat and Rev, provide essential gene regulatory functions [56-58], while the last gene product, Vpu, indirectly assists in the assembly of the virion [59].

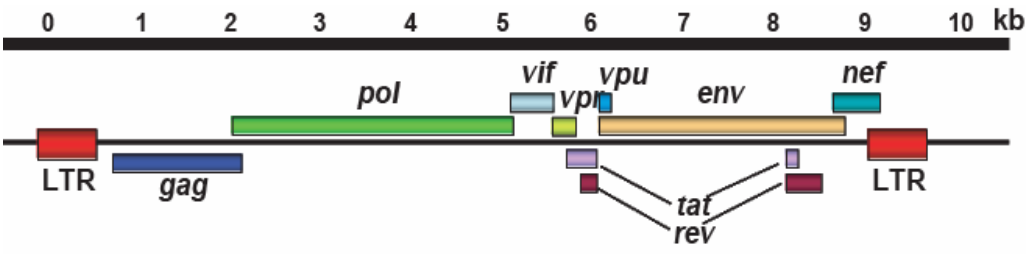


Figure 3. Schematic representation of the HIV-1 genomic structure, showing the locations of open reading frames.

1.3.3 Replication cycle

The first step in the HIV-1 replication (**Fig. 4**) is the virus attachment mediated by the virus glycoprotein gp120 with a cell that express the CD4 molecule [60] and a chemokine receptor belonging to the chemokine receptor family [61, 62].

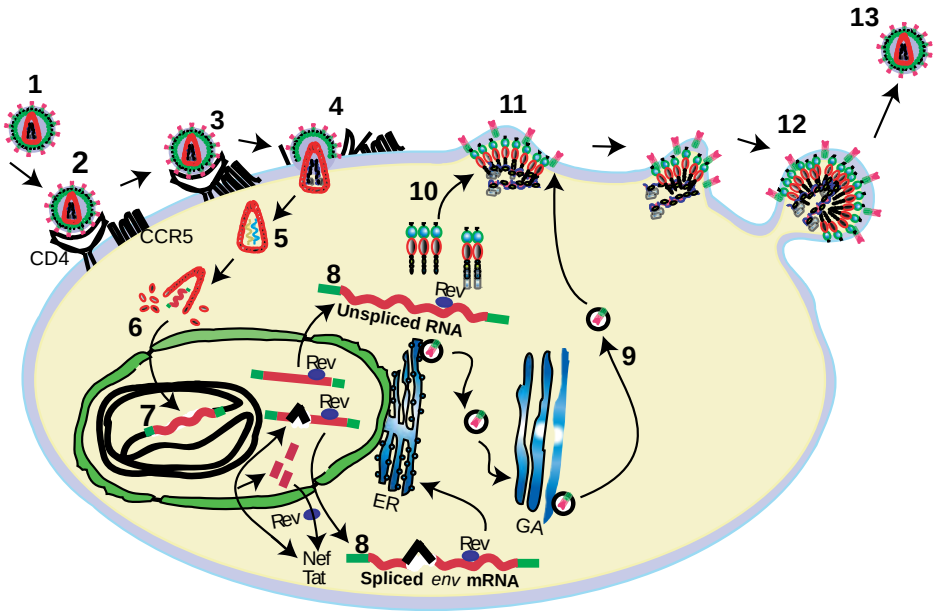


Figure 4. Schematic representation of the HIV-1 replication cycle. 1, infecting virus; 2, virus attachement to CD4 receptor ; 3, fusion of the virus mediated by the co-receptor binding; 4, virus entry and core release; 5, reverse-transcription; 6, nuclear transport of the proviral DNA; 7, integration of the proviral genome into the host genome; 8, rev dependent expression of the structural proteins (unspliced & singly spliced); 9, Env expression through ER-Golgi pathway & membrane transport; 10, expression of Gag & Gag-Pol and transport to the inner surface of the cell membrane; 11, virus assembly; 12, virus release; and 13, mature virion.

The chemokine receptor molecules CCR-5 and CXCR-4 have been identified as the major co-receptors required for HIV-1 entry [63-66] and the differential expression of these receptors on CD4+ cells accounts for virus cell tropism and pathogenesis [61, 62, 67-69].

Binding of gp120 to the CD4 molecule exposes the co-receptor binding domain of gp120, the interaction of which with the chemokine receptor expose the hydrophobic fusion domain of gp41 which then is inserted into the plasma membrane. This in turn triggers a conformational change in gp41, in that a coiled-coil structure of the gp41 triplet is induced drawing the viral envelope into close contact with the plasma membrane, resulting in a fusion between these two membrane structures [70, 71]. As a result, the HIV-1 nucleocapsid core particle is introduced into the cell cytoplasm.

Following entry of the core particle, viral RNA is reversly transcribed into double stranded cDNA by the viral reverse transcriptase (RT) [51] within the intact capsid shell [72]. Only retroviruses use the enzyme RT in order to convert their genomic RNA into double stranded DNA. The HIV-1 RT has three activities in retrovirus replication: It is an RNA dependent DNA polymerase (reverse transcription), DNA dependent DNA polymerase (second strand provial DNA synthesis), and thirdly possess an RNase H activity [51, 73]. Due to the lack of proof reading activity, this is the step where most of the genetic variability appears [74, 75]. Uncoating of the virus core occurs at the nuclear pore upon RT completion [72] and the double stranded viral cDNA is then transported into the nucleus in the form of a nucleoprotein core-DNA complex (or preintegration complex, PIC) consisting of the viral cDNA, matrix protein, integrase and other cellular proteins [76], and the proviral DNA is inserted into the host cell genome preferentially in active transcription genes by the action of the viral integrase. The accessory protein, Vpr, mediates the transfer of the preintegration complex into the cell nucleus [55, 77].

The integrated provirus is flanked by repetitive sequences termed long terminal repeats (LTR) at each 5´ and 3´end of the genome, and contains the promoter sequences which drive viral RNA transcription [78-80]. The HIV-1 transcription is regulated largely by the 5´-LTR of the genome. Once integrated, the transcriptional activity of the provirus is regulated by two virally encoded proteins, Tat and Rev, as well as, other cellular transcription factors (reviewed in [81]) induced during host cell activation. The combined action of the two viral regulatory proteins divides the HIV-1 lifecycle into two phases- early and late. Tat and Rev are encoded by multiply spliced mRNAs [82]. Early on in the viral replication cycle, newly transcribed mRNA is spliced multiple times and the Tat, Rev and Nef proteins are produced [82]. Tat is a sequence-specific trans-activator that binds to a specific RNA stem-loop structure known as *trans*-activation response (TAR) element and activates high level of HIV-1 transcription [57, 83]. Rev acts post-transcriptionally [84, 85] by regulating the transport of HIV-1 transcripts from the nucleus to the cytoplasm. Rev binds also to viral RNA structure called rev response element (RRE) and stabilizes the

production of viral structural proteins encoded by unspliced and singly spliced mRNAs while inhibiting the expression of the regulatory *trans*-activators encoded by multiply spliced mRNAs [86-89]

Once sufficient amounts of these regulatory proteins accumulate, synthesis of the unspliced and singly spliced HIV-1 mRNAs encoding the essential structural and enzymatic proteins can proceed. Translation of the Gag and Gag-Pol are made on free ribosomes in the cytoplasm, whereas Env goes through the ER-Golgi-plasma membrane pathway where it is highly glycosylated post-translationally. The viral envelope precursor proteins are synthesized from singly spliced mRNA as gp160 and subsequently cleaved by the cellular protease into the surface (gp120) and transmembrane (gp41) subunits. The unspliced viral mRNA is translated into Gag and Gag-Pol polyproteins and cleaved by the viral protease into the mature viral structural proteins and enzymes. The Gag and Gag-Pol precursors are transported to the inner surface of the cell membrane where the activity of the viral protease is initiated [44, 90] and productive assembly takes place [91]. Although localization of the Gag at the plasma membrane is evident from numerous studies, the question as to how it arrives there has not been fully settled. However, several recent studies have identified that newly synthesized Gag is transported first to multivesicular bodies (MVB), an endosomal compartments that contain the ESCRT (endosomal sorting complex required for transport) machinery. This suggests that MVB may represent a trafficking intermediate for Gag transport before its appearance at the plasma membrane. Thus, it has been shown that a signal within CA identified as a dileucine-like sorting motif, as well as, specific host-cell lipids and proteins promote MVB localization [92-96]. Finally, as all viral components gather at the cell membrane, new HIV-1 virions are released acquiring the viral Env proteins as they bud from the host cell membrane. During the release or shortly after budding, the Gag and Gag-Pol polyproteins are cleaved by the viral protease [90, 97], transforming the immature virus into a mature infectious virion with a conical core structure. Several proteins of cellular origin are also incorporated into the released virus, including cyclophilin A, tumor susceptability gene 101 (Tsg101), human leukocyte antigen (HLA) class I and II, intracellular adhesion molecule-1 and β_2-microglobulin [98-103]. More recently, similar membrane fission events between HIV-1 budding and cytokinesis has been described [104]. Cytokinesis is a process whereby the cytoplasm of a single animal cell is divided into two daughter cells. Two proteins, Tsg101 and Alix (apoptosis-linked gene 2 interacting protein X), which are involved in HIV-1 budding have also been shown to be recruited to the midbody of a dividing cell during the membrane fission event. Thus, the study concluded that Tsg101 and Alix to be required for efficient completion of cytokinesis, and that the cellular machineries involved in both events share some components and are functionally related [104].

1.4 THERAPY

HIV-1 infection is a chronic infection for which at present there is no cure. Combination therapy, also known as highly active antiretroviral therapy (HAART), is the most effective treatment available for controlling the virus. The treatment is often individualized and may be changed due to side effects or treatment failure. The intense research within the field during the past 25 years have resulted in 21 approved anti-HIV drugs in clinical use today [1]. The antiretroviral drugs used control HIV-1 infection by targeting the viral enzymes protease and reverse transcriptase. In addition, a fusion inhibitor that belongs to another class of drugs has been added to this repertoire. Most recently, the FDA (August 2007) and EU have also approved [2] yet another class of drugs, a CCR5 antagonist that was designed for treatment of adults with CCR5-tropic HIV-1 (R5 virus) and have treatment failure on current HAART therapy.

Usually, the initial therapy consists of two nucleoside reverse transcriptase inhibitors (NRTIs) and one protease inhibitor (PI) or non-nucleoside reverse transcriptase inhibitor (NNRTI) [105]. Although the introduction of HAART has significantly prolonged the life and improved the life quality of most people infected with HIV-1, the ineffectiveness of the therapy in some patients and the emergence of multi-drug resistance HIV-1 strains also in treatment naïve patients [106] underscore a continuing need for other treatment options.

2 THE GAG PRECURSOR IN HIV-1 ASSEMBLY

The inner shell of immature HIV-1 is composed of approximately 5,000 copies of the Gag precursor [107] that provides all of the major internal structural components of the virus and is also sufficient for formation of virus like particles *in vitro* [108, 109]. During or shortly after budding, the Gag and Gag-Pol polyproteins are cleaved to mature protein products by the action of the viral protease (PR; p15). Hence, the specific sequential cleavage of the Gag precursor yields the matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7) and p6 proteins. In addition, there are two small spacer peptides called SP1 and SP2 [110-112] (**Fig. 5**). Proteolytic maturation of Gag also results in that the CA proteins extensively rearrange to form a conical core structure with approximately 1,500 CA molecules assembled into hexameric and pentameric lattices [107, 113, 114].

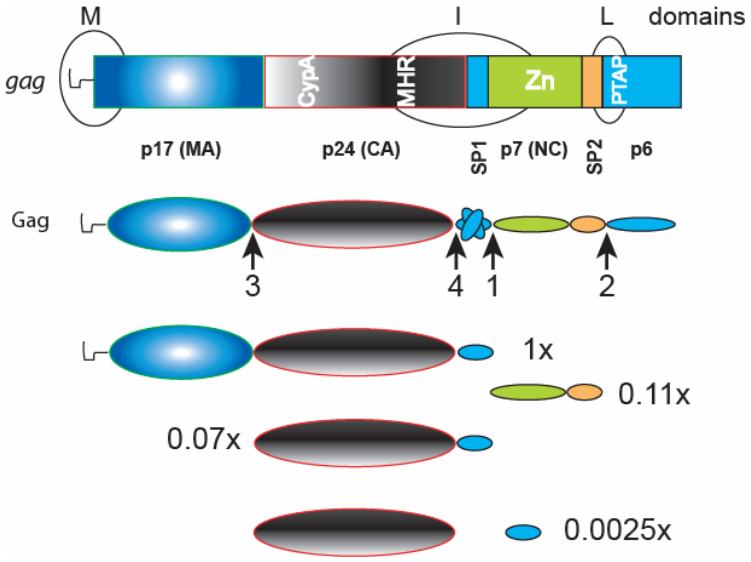


Figure 5. Schematic view of the HIV-1 Gag. Upper, the *gag* gene showing the assembly domains (M, I, and L) and individual domains found in *gag*. The approximate locations of the binding site for cyclophilin A and the major homology region in CA, the zinc finger motifs in NC, and the PTAP motif in p6 are also indicated. The numbers in the lower panel indicate the sequential cleavage sites in the Gag precursor, as well as, the frequency at which the cleavage at each site occurs.

The HIV-1 Gag is a multifunctional polyprotein which is involved both early and late in the viral replication cycle [115]. Three domains contained within the Gag precursor have been shown to be involved in the diverse functions of the virus assembly and maturation process [116]. The membrane binding (M) domain containing an *N*-myristoylation signal in MA, an interacting (I) domain composed of one third of the *C*-terminal CA and the NC region, and the late domain (L) found in p6 are the three determinants most important for virus assembly.

The MA containing the M domain is essential for membrane targeting of the Gag and Gag-Pol precursors and in stabilizing the virus particles during the assembly by binding to the inner surface of the virus membrane [117, 118]. It has been shown that the *N*-terminal myristoylation signal and the basic residues to be important for membrane binding affinity of Gag and particle release [45, 119-123]. Crystallographic studies of the MA has shown that the MA forms a symmetrical trimer [40]. Similar trimerization or assembly to a large-molecular-weight multimeric intermediate of the Gag precursor in solution has also been shown to occur through the action of the MA [124, 125]. It is therefore possible that some Gag self assembly corresponding to a trimer [125, 126] or hexamers [127] may start already in the cell cytoplasm. The MA has been reported also to facilitate nuclear transport of viral DNA due to karyophilic signals that are recognized by the cellular nuclear import machinery [128, 129]. However, their role in nuclear transport of the nucleoprotein core-DNA complex (or preintegration complex, PIC) is disputed [130].

The second Gag-Gag interaction determinant called the I domain comprises one third of the *C*-terminal CA and the *N*-terminal NC region of the Gag precursor [131-133]. The I domain has multiple functions including Gag polymerization, RNA binding and participation in reverse transcription [134, 135]. Most mutations *C*-terminal to the major homology region (aa 153 to 172), which is a highly conserved sequence in most retroviruses, impairs CA/Gag dimerization affecting proper virus assembly and release [136]. Recently, mutagenesis study of two dileucine-like motifs (L189/L190 and I201/L202) located in the vicinity of the CTD dimer interface has been shown to impair virus assembly, CA dimerization, and virion maturation [137]. The CA which is the major building block in virus assembly will be discussed later (see below). There are also two zinc finger motifs termed the Cys-His box in NC which account for the specific interaction of NC with HIV-1 RNA packaging signal, the Psi, [138-142] and act as a scaffold for Gag multimerization. The Psi signal is a ~110 nucleotide segment in the 5' region of the genome that contains four stem-loops (SL1 through SL4), all of which are important for genome packaging [143, 144]. The NC also possesses an additional function that is not related with the assembly of HIV-1, but rather involves an interaction with a cellular protein called APOBEC3G. It is a protein of the RNA editing machinery, a cytidine deaminase that converts cytosines within the minus strand of the viral cDNA to uracils and thereby leads to hypermutations in newly synthesized HIV-1 proviral DNA [145]. Mutagenesis study has shown that specific packaging of APOBEC3G into HIV-1 is mediated by an interaction with the NC domain [146, 147]. The virus has, however, evolved the *vif* gene to counteract the endogenous antiviral activity by targeting APOBEC3G for degradation via the ubiquitin-proteasome pathway [148-150].

The third region called the L domain is found in the *N*-terminal region of p6 and contains a PTAP motif involved in the budding process of the viral replication [151, 152]. Late domains have also been identified in many other viruses, for example PPXY for Rouse sarcoma virus (RSV), Mason-pfizer monkey virus (MPMV) and murine leukemia virus (MLV); YPDL for equine infectious

anemia virus (EIAV). This highly conserved motif is known to mediate protein-protein interactions between cellular proteins and hence several studies have suggested that this domain might function by interacting with host factors [103, 153]. Mutations of this motif are characterized by accumulation of budded but tethered virus particles with an immature morphology [103, 154]. Functional inter-changeability of this motif with other retroviruses' L domain and positional independence [155, 156] may also imply that the L domain is not a structural element in virus assembly, but may rather function as a docking site for host cell factors [157]. Indeed it has been shown that tumor susceptability gene 101 (Tsg101) is the host cell protein that interacts specifically with the PTAP motif [153]. Tsg101 is homologues to VPS28 and both are components of a 350 kDa multi-protein complex termed ESCRT-1 (endosomal sorting complex required for transport) [158-161]. The two proteins together with a third cellular ATPase called VPS4 are involved in the cellular endosomal protein-sorting pathway [162]. The pathway that sorts membrane associated proteins destined for lysosomal degradation by mediating the formation of multivesicular bodies (MVB) from intracellular vesicles budded through the lumen [160]. Mono-ubiquitinylation is a signal for the protein sorting into the MVB [160, 163] and since Gag is mono-ubiquitinylated, the binding of Gag to Tsg101 and VPS28, which normally reside in the cytosol results in their relocation to the cell membrane where virus budding takes place [164]. Since identification and characterization of Tsg101, most of the mammalian ESCRT components have been shown to be required for HIV-1 particle formation [165-168].The p6 also possesses another important function that is not associated with virus release. It mediates the interactions between Gag and Vpr through a Leu repeat motif located near the *C*-terminus of p6, leading to the incorporation of Vpr into the assembling virions [167, 169, 170]

Thus, the key step in the viral replication cycle requires the proper assembly of the virus that allows it to be released, and subsequently infect and colonize new target cells. This in turn is driven by multiple cooperative inter and intra protein contacts involving several Gag domains. Additionally, production of an infectious virus particle relies on several viral and cellular protein-protein interactions, exclusion of an inhibitory cellular factors (for example APOBEC3G and 3F) from the budding virus [149, 171], and the interaction and/or incorporation of cellular proteins (for example CyPA, Tsg101) into the virus in order to complete certain steps in its replication cycle. Summary of all viral gene products and cellular proteins involved in HIV-1 replication are reviewed by Ott et al. [81].

2.1 The HIV-1 capsid protein

The HIV-1 capsid (CA, p24) protein, which forms the building blocks of the viral core [172], is a hydrophobic protein that consists of 231 amino acids (aa) and is separated into two structural domains, the *N*- and *C*-terminal domains (NTD and CTD, respectively). The two individual domains are folded independently of each other and are linked via a 5-residue linker sequence (aa

146 to 150) [173]. The NTD (aa 1-145) which is composed of seven α-helices and one β-hairpin [174], is important for CA assembly early in the replication cycle [175-177] and also binds cyclophilin A, a cellular peptidyl-prolyl *cis-trans* isomerase [100, 178-180]. The CTD (aa 146-231) is composed of four α-helices and a 3_{10} helix [181, 182] and is required for CA dimerization and oligomerization of Gag and Gag-Pol polyproteins [174, 181, 183, 184]. In addition to the CTD of CA, the NC containing cluster of basic residues, SP1 and the basic cluster in MA have also been shown to mediate multimerization of Gag [44, 185, 186].

There are no structural data for the full-length Gag or the mature CA and thus the process of virus maturation that conversts the HIV-1 capsid core from spheres to cones is unknown. The availability of the whole Gag structure would have made it possible to determine the structural rearrangments that take place in mature CA. Nevertheless, available three-dimensional structure of each domains, as well as, studies with image reconstruction from cryo-electron microscopy of purified mature virions and *in vitro* assembled virus like particles [187-192] has allowed proposal of two different dimerization models, a head-to-head (*N*-*N* dimer) [178, 183] and a tail-to-tail (*C*-*C* dimer) [181] (**Fig. 6**). Additionally, mutagenesis combined with assembly and structural studies have provided important insights on the structure-function relationships of HIV-1 CA assembly [181, 193, 194].

A third head-to-tail dimer interface (*N*-*C*) has also been observed in a near complete CA structure [173] (**Fig. 6**). However, the *N*-*C* dimer interface described contradicts the current view of capsid oligomerization and cone formation, both in the immature and mature virion. Currently, the general view is that following protease cleavage of the Gag precursor at the junction between MA and CA creates a salt-bridge between proline 1 and a buried aspartate 51 [113] **(Fig. 7)**. This in turn triggers a profound structural rearrangement in CA thereby allowing core cone formation. The two flexibly linked CA domains allow then self-association of the protein in two different orientations (the *C*-*C* and *N*-*N* dimers). The *N*-terminal domain of CA forms the hexameric rings and each ring is connected to the neighboring rings by the CA *C*-terminal dimerization domain [187]. Abundant mutational mapping and structural studies of the two CA dimer interfaces have also identified several residues/regions that are essential for particle assembly [113, 178, 181, 191, 195]. Further, purified and *in vitro* assembled cones have made it possible to reconstruct images of HIV-1 CA following cryo-electron microscopy, suggesting that CA cones to be formed of hexamers in hexagonal nets and closed by pentamers at both ends [187, 191, 196]. Although it is now evident that certain residues within the two CA domains including the two so called *N*-*N* and *C*-*C* dimer interfaces are important for CA assembly, the exact identities of all amino acids involved in the immature and mature form of CA are unknown.

In most of the studies referred to above, mutagenesis has been one of the key methods used in elucidating the basis of HIV-1 replication, as well as, identifying regions in the CA which are important for core assembly and

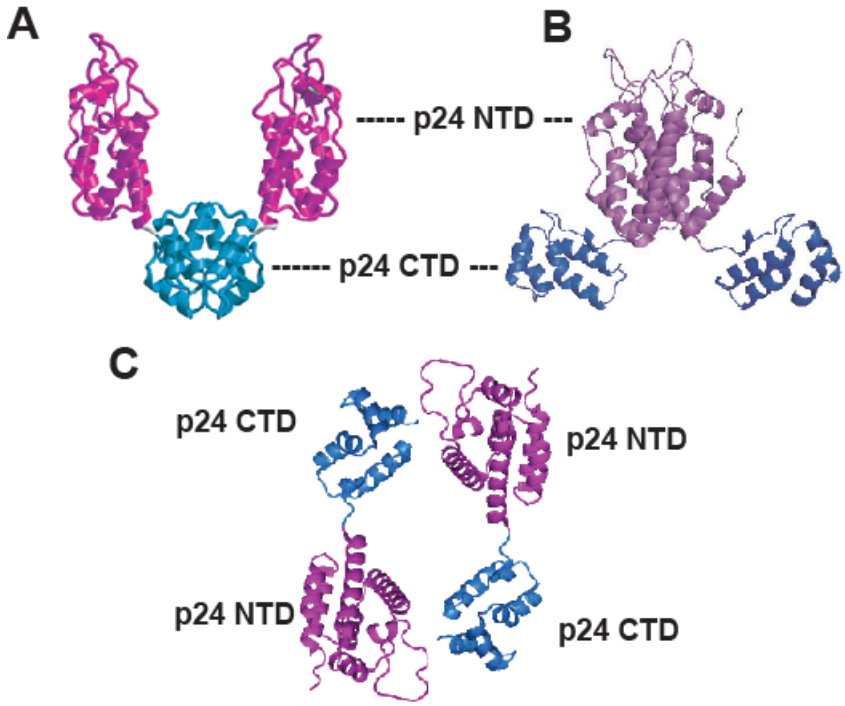


Figure 6. Different dimerization models of HIV-1 CA. (A) *C-C* dimer (head-to-head), (B) *N-N* dimer (tail-to-tail) and (C) *N-C* dimer (head-to-tail).

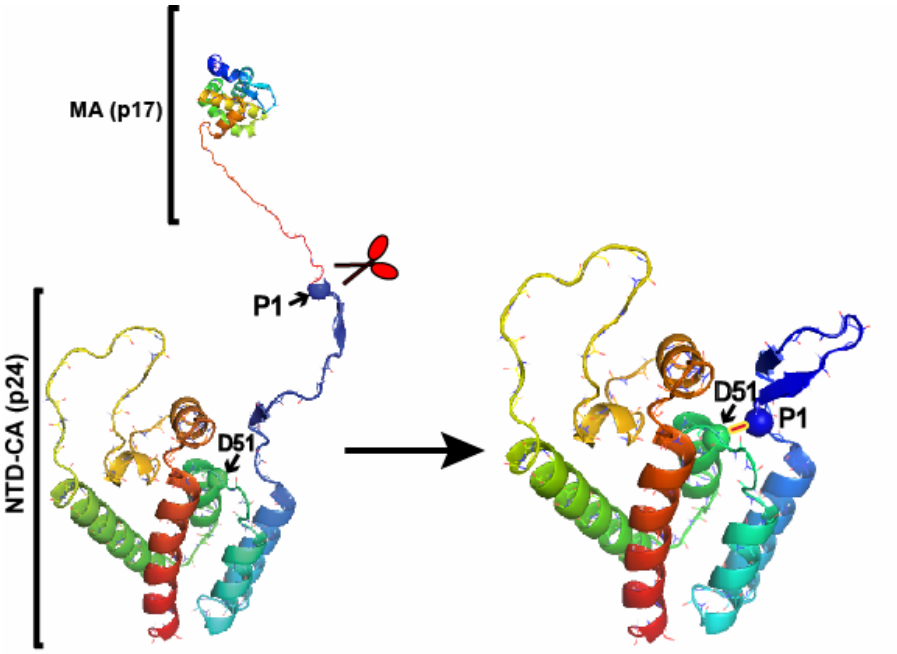


Figure 7. Ribbon diagram of the MA [197] and *N*-terminal domain (NTD) CA [178] depicting the structural rearrangemts that takes place in the NTD of CA upon proteolytic processing at the MA-CA junction (indicated with a sax). The model to the right represents a processed NTD-CA showing the β-hairpin formation which is stabilized by the salt-bridge formation between the imino terminal Pro 1 and Asp 51. For clarity, Pro 1 and Asp 51 are shown as filled circles. The ribbon diagrams were generated with the PyMOL [198] and modified with Adobe Photoshop software.

infectivity. A twenty amino acids long (aa 153 to 172 of CA) conserved sequence, referred to as the major homology region (MHR), located in the *N*-terminus of the CTD has been characterized using this technique. Although this region does not contribute to the dimer interface, it has been shown that mutations in MHR could affect assembly and membrane affinity of HIV-1 Gag [199] and also impair particle formation [200-202]. Similar strategies were also employed in identifying and investigating the binding site for cyclophilin A [100, 174, 178, 203], the function and interaction of cellular proteins in viral assembly and infectivity [204, 205], and for designing and investigating therapeutic strategies targeting viral proteins in virus assembly [206-208].

For a long time, it has been intriguing how certain non-human primates, such as rhesus macaques and owl monkeys, restrict (inactivate) HIV-1 immediately when the virus enters the target cell. This mystery was partly uncovered when Stremlau *et al*. [209] isolated a gene known as TRIM5α (**tri**paritate **m**otif protein 5α), that was responsible for restriction of HIV-1 in rhesus macaques. Although how TRIM5α block or modulate an incoming virus is still unclear (reviwed in [210]), recently it has been demonstrated that TRIM5α binds in a CA-specific manner to retroviral cores [211, 212] and that the hexameric surface lattice is necessary for providing multiple points of contact that involves TRIM5α trimers [213].

Since the discovery of TRIM5α, other orthologues genes from other primates, including humans have been isolated and characterized for restriction activities against various retroviruses, including HIV-1, HIV-2, SIV, N-tropic murine leukemia virus (N-MLV), and equine infectious anemia virus (EIAV) [214-219].

Among all retroviruses, only HIV-1 encodes a CA that binds CypA, a peptidyl-prolyl isomerase that catalyzes the *cis*/*trans* isomerization of peptidy-prolyl bonds [220] that links residues Gly89 and Pro90 of the HIV-1 CA [221]. The importance of CypA as a cellular factor that binds to and regulates HIV-1 infectivity has been well established [100, 180, 222-224]. It has been suggested that the block for HIV-1 in non-human primate cells might take place following virus entry but before or during reverse transcription [225-231]. Elucidating TRIM5α mechanism of action might make it possibile to recreate its effects in future terapeutic treatment.

3 BACKGROUND TO THE THESIS

The HIV-1 capsid (CA, p24) protein which consists of 231 amino acids is a hydrophobic protein with two independently folded domains, the *N*- and *C*-terminal domains, connected via a 5-residue linker sequence (amino acid 146 to 150). Before final maturation, the CA is part of the Gag precursor (p55) and proteolytic cleavage triggers morphological maturation which transform the HIV-1 core from a spherical to a conical structure. While the exact nature of CA-CA contacts in immature particles (as a part of Gag) is not fully known, in mature particles the CA lattice has been modelled based on crystal structures and studies with image reconstruction from cryo-electron microscopy of purified mature virions and *in vitro* assembled virus like particles [114, 187, 196].

Optimal core semi-stability is a prequisite for HIV-1 replication, as acquisition of virion infectivity, reverse transcription, and subsequent dissociation of the capsid core are highly dependent on the just right stability of the capsid cone structure, which is made up of multiple semi-stable non-covalent CA-CA interactions [72]. Mutations *C*-terminus to the major homology region (aa 153 to 172 of CA), a stretch of 20 amino acids which are highly conserved among retroviruses, affect capsid assembly and abolish particle production. We have studied two highly conserved sequences located within the *C*-terminal region of CA (^{204}ALGPGATLEE^{213} and ^{218}CQGVGGPG^{225}). These two sequences are highly conserved among different HIV-1 and HIV-2 isolates, as well as, the simian (SIV) and feline (FIV) immunodeficiency viruses. Previous studies have shown that larger deletions or insertions (three or more amino acids) in these two conserved sequences could affect virus assembly, release and infectivity [232-234]. As the two sequences are located in a dominant assembly region of the HIV-1 CA, our attention focused on the specific residues and therefore we designed eight different HIV-1 mutants and studied virus assembly, release and infectivity. We found that single amino acids in the two conserved sequences to be essential for proteolytic processing of Gag and Gag-Pol precursors and for the proper assembly and release of infectious virus particles.

Based on available structural studies and analysis of intra-atomic contacts, we designed two single amino acid substitution mutations, the E98A and E187G (Paper II). Glu98 has no intra-molecular contacts with other residues, whereas Glu187 has extensive contacts with eight other CA residues. Glu187, which is located within the *C*-terminal dominant assembly region, has previously been shown to be important for formation of a salt-bridge [173]. Currently, the general view is that two different CA dimer interfaces exist, the *N*-*N* (head-to-head) and *C*-*C* (tail-to-tail) dimers. A third *N*-*C* (head-to-tail) dimer interface has also been observed [173], in which an important salt-bridge between Glu187 and Arg18 of the two interacting CA monomers was found in the hydrophobic core of the interface mediating the interactions. Thus, the study in Paper II was designed to investigate the role and significance of the two Glu residues having quite opposing intra-molecular contacts with other CA residues.

Proper structural rearrangement of CA after Gag cleavage is a highly conserved feature in most retroviruses [235]. As a result of this process, a β-hairpin structure formed by a salt-bridge between Pro1 and Asp51 (D51) is important for conformational stability of the *N*-terminal CA structure [113]. Furthermore, proteolytic refolding of CA amino terminal has been suggested to be the key switch for morphological conversion of the virus' internal structure from spheres to cones [113]. As the CTD CA dimer formation is one of the fundamental interactions driving CA multimerizations, which also involves hexamerizations of the NTD CA, in Paper III, we investigated the role and significance of aspartate 51 (D51) by substitutions of the aspartate with glutamate, glutamine, or asparagine. These three amino acids, in contrast to the D51A substitution reported earlier, are structurally close and have similar properties in proteins as D51 (Glu > Asn > Gln).

Site-directed mutagenesis was used to introduce substitution mutations. All HIV-1 CA mutants were then assessed using biochemical, functional and morphological techniques.

Mutations in CA are often lethal, particularly if they inhibit capsid assembly by destabilizing the intra and/or inter molecular CA contacts and thereby affecting proper formation of the conical core structure. These features make CA of interest as a target for developing new antiviral drugs. It has earlier been shown that addition of the amidated tri-peptide glycyl-prolyl-glycine amide (GPG-amide) or glycineamide (G-amide) to the culture medium of infected cells abrogated HIV-1 replication and proper capsid formation [236-240]. In Paper IV of this thesis, we defined the active antiviral metabolite of GPG-amide/G-amide and investigated how it affects HIV-1 capsid assembly using various techniques.

4 AIMS OF THE STUDY

The main objective of this thesis was to define the roles of Gag, in particular, the capsid protein in HIV-1 particle assembly. More specifically,

i) to determine the relative contribution of some specific residues and/or sequences in the HIV-1 capsid core assembly and virus release

ii) to characterize the importance of Glu187 in formation of a salt-bridge between two interacting CA proteins in *N-C* dimer

iii) to investigate the role and significance of Asp51 in formation of a highly conserved β-hairpin structure in HIV-1 capsid assembly

iv) to define the active antiviral small molecule metabolite of GPG-NH_2/G-NH_2 and to investigate its activity and antiviral mechanisms on HIV-1 CA assembly

5 COMMENTS ON METHODOLOGY

5.1 PLASMID DNA CONSTRUCTION (PAPER I, II AND III)

The polymerase chain reaction (PCR) was utilized to develop all *CA* mutants in the study using mutagenic oligonucleotides and the overlap extension technique (paper I) or QuickChange II XL site-directed mutagenesis kit obtained from Stratagene (papers II and III). In paper I, the 560 bp fragment (nucleotides 1451 to 2011 of pNL4-3) generated after the PCR was purified and subcloned into the pCR2.1-TOPO vector (Invitrogen). The 506 bp fragment of the mutated sequences were then cleaved out and cloned directionally into the *Apa*I/*Spe*I sites of the pNL4-3 vector. All plasmid DNAs were then propagated in *Escherichia coli* and the plasmid DNAs were purified using the Maxiprep Purification Kit (Qiagen).

The P207A mutant was back-mutated to the wild type (WT) pNL4-3 (designated BM-P207A). The *Apa*I/*Spe*I fragment of the WT pNL4-3 plasmid was cut out, purified and cloned directionally into the backbone of mutant P207A depleted of its *Apa*I/*Spe*I fragment.

For *in vitro* protein expression, the *gag* gene of three mutants, L205A+P207A, L205A, and P207A, and the WT pNL4-3 were cloned into an *in vitro* expression vector pHM6-CMV (Invitrogen) containing a T7 promoter. The primer pair 5´-CAG-GTACCGGTGCGAGAGCGTCGG and 3´-GAATTC***CTA***TTGTGACGAGGGG-TCG were used for amplification of all *gag* constructs using PCR (*Kpn*I and *EcoR*I sites underlined, the stop codon is shown in boldface italics). The PCR products subcloned into pCR2.1-TOPO vector (Invitrogen), the *gag* genes were then cleaved out using the *Kpn*I and *EcoR*I sites and cloned directionally into the *Kpn*I/*EcoR*I sites of the pHM6-CMV vector.

The *CA* gene was also amplified as above using the primer pair 5´-CAGGTACC-CCTATAGTGCAGAACCTCC and 3´-CGAATTC***CTA***CAAAACTCTTGCTTTA, and cloned directionally into the *Kpn*I/*EcoR*I sites of the pHM6-CMV vector.

In both paper II and III Site-directed mutagenesis kit was used to create all mutants using mutagenic oligonucleotides as recommended by the manufacturer. The HIV-1 CA mutants in paper II were created with the mutagenic oligonucleotide 5'-GGC CAG ATG AGA GCA CCA AGG GGA AG-3' and 5'-TGG ATG ACA GGA ACC TTG TTG-3'. In paper III, mutations in the CA encoding gene that switched the amino acid D51 (aspartate) to E, N, and Q (glutamate, asparagine and glutamine respectively) were done using the mutagenic oligonucleatides 5'-GCC ACC CCA CAA GAG TTA AAT ACC ATG-3', 5'-GCCACC CCA CAA AAT TTA AAT ACC ATG-3' and 5'-GCC ACC CCA CAA CAA TTA AAT ACC ATG-3', respectively. All constructs

were confirmed by sequencing. The mutations in the CA gene representing the dimer interfaces were also mutated using the mutagenic oligonucleotide 5'-CTT CAC AAG AGG TAA AAA ATG CGG CGA CAG AAA CCT TGT TGG TC-3'.

5.2 PCR AND SEQUENCING (II AND IV)

Nested RT-PCR was used to amplify the *RT* and *PR* genes of HIV-1 and drug related resistance mutations were identified and defined according to European guidelines. Sequencing was performed with an ABI PRISM 310 Genetic Analyzer.

In order to detect virus attachment and entry, total RNA was isolated from wild type and mutant E98A infected TZM-bl cells and a 593 bp fragment of the p17 viral RNA was also amplified using nested RT-PCR.

5.3 CELLS AND TRANSFECTION (PAPERS I, II, III, AND IV)

HeLa, HeLa-tat, HeLa-tat III, BHK21, COS7, Vero, HepG2, 293T, and TZM-bl cells were maintained in complete DMEM. The HIV-1 chronically infected ACH-2 and H9 and MT4 cell lines were maintained in RPMI 1640 medium. Periferal blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density gradient centrifugation and stimulated with phytohemagglutinin (PHA) for three days before being infected. Transfection of adherent cells were done in 6-well culture plates using the non-liposomal FuGENE 6 transfection reagent (Roche). The cells were harvested 48 to 72 hrs post-transfection in 1× RIPA buffer [50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, and 0.1% sodium dodecyl sulphate (SDS) supplemented with a complete protease inhibitor cocktail from Roche]. For EM analysis, transfection of adherent cells was performed in a single culture plates as described above.

5.4 RNA ISOLATION AND NORTHERN BLOT ANALYSIS (PAPER I)

Total RNA was isolated from HeLa-tat cells that were harvested using Trizol® LS reagent (Gibco). For Northern blot, 10 µl of total isolated RNA were denatured in RNA loading buffer and subjected to electrophoresis. The gel was transferred overnight to nylon membrane Hybond N (Amersham).

For HIV-1 mRNA hybridisation, a 3.5 kb *Sac*I fragment from pNL4-3 which spans the *vpu-env-nef* was radio-labelled using Amersham's oligonucleotide labelling kit. The hybond-N membrane was then hybridized with the radio labelled probes (2×10^6 cpm/ml) for another 16 hrs and exposed to x-ray films at -80°C.

5.5 *IN VITRO* TRANSCRIPTION AND TRANSLATION (PAPER I)

In a cell free system utilizing rabbit reticulocyte lysate (Promega), the mutants L205A+P207A, L205A, P207A, and WT *gag* constructs were expressed in a coupled transcription and translation system. Plasmid DNAs encoding each mutant was added to a reaction mixture containing rabbit reticulocyte lysate, T7 RNA polymerase, amino acid mixtures, reaction buffer, and RNasin® (Promega), and then incubated at 30°C for 90 min. The translation products were then immunoprecipitated and analyzed by SDS-PAGE.

5.6 IMMUNOPRECIPITATION (PAPER I AND II)

The *in vitro* transcription/translation products were immunoprecipitated with monoclonal antibodies against p24 or hemagglutinin (HA, Roche) at 4°C overnight. Bound antigens were then pelleted using protein A/G-agarose (Santa Cruz), washed and re-suspended in 2× SDS sample buffer before being subjected to SDS-PAGE.

In order to produce virus supernatants free of soluble HIV-1 Tat, HeLa-tat and 293T cells culture supernatants (corresponding 400 ng of p24) were clarified of cell debris, adjusted to 500 µl with DMEM and incubated with or without 20 µl of monoclonal antibody against Tat at room temperature for 2 hrs. Thereafter, 50 µl protein A/G-agarose (Santa Cruz Biotechnology) was added and the suspension was incubated further for 1 h at room temperature. Virus containing culture supernatant free of soluble Tat was collected by centrifugation at 2,000 r.p.m. for 5 min and used to infect TZM-bl cells as described above.

5.7 IMMUNOFLUORESCECE STAINING (PAPER I AND III)

Immunofluorescence staining was performed on mutant and WT pNL4-3 transfected HeLa cells cultured on chambered slides (Nunc). Cells were washed and fixed in acetone-methanol (1:1) followed by incubation with the primary mouse anti-p24 antibody. Cells were also labelled for DNA with 4', 6-diadimino-2-phenylindole dihydrochloride (DAPI; blue). As a secondary antibody, Cy2-conjugated (paper I) and FITC-conjugated (paper III) anti-mouse IgG antibody was used. After final wash, slides were mounted and fluorescent images were acquired using a Nikon Eclipse E600 phase contrast fluorescent microscope.

5.8 RECOMBINANT PROTEIN PRODUCTION (PAPER III AND IV)

Competent *E. coli* Origami (DE3) cells were transformed with mutants or the wild-type pET11a-CA expression plasmid and protein expression was induced by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM. After a 4 hrs incubation period at 37°C, the cells were harvested by centrifugation. Extracted protein solutions were put in four 15 cm long dialysis tubings (Spectrpor, MWCO 6-8000, and 1.7 ml/cm) and dialyzed against 50 mM

Tris pH 8.0 overnight at room temperature. Proteins were then precipitated by addition of saturated $(NH_4)_2SO_4$ and purified on ÄKTA FPLC chromatography system (Amersham Biosecience). In order to increase the purity of the recombinant proteins, samples were loaded onto a gel filtration column, HiLoad 16/60 Superdex 75 prep grade and purified.

5.9 DIALYSIS / INCUBATION (PAPER III AND IV)

In order to produce metabolite-X (Met-X) enzymatically, porcine (PS) or fetal bovine serum (FBS) in dialysis tubing (5 kDa MWCO, SpectraPor) was pre-washed five times in PBS and transferred to beaker containing a 10 mM glycineamide (G-NH_2) solution in PBS. The solution was then incubated at 37°C under constant stirring. At the end of incubation time, the dialysis solutions were filtered before being analyzed with HPLC.

In order to determine the structure of the unknown Met-X, similar procedures were used to produce $^{13}C_2/^{15}N$-labelled Metabolite-X (Met-X) enzymatically from $^{13}C_2/^{15}N$-glycineamide. The dialysis solution was then filtered and lyophilized before being analyzed with NMR.

In paper III, mutant and wild type recombinant CA proteins produced in *E. coli* were also dialyzed against 50 mM Tris pH 8.0 before being subjected to precipitation with saturated $(NH_4)_2SO_4$.

5.10 HPLC ISOLATION AND CHARACTERIZATION OF MET-X (PAPER IV)

The peak fractions containing Met-X or $^{13}C_2/^{15}N$-labelled Met-X produced enzymatically by dialysis against PS or FBS were isolated and analyzed either directly or after lyophylization. All samples were analyzed on Merk-Hitachi LaChrome HPLC system with the use of the D-7000 Chromatography Data Station Software.

The metabolic solutions were injected by an HPLC autoinjector onto a cationic ion-exchange column, Theoquest Hypersil SCX, 5 μm (Thermo), 250 × 10 mm with a mobile phase of 90% 0.1 M KH_2PO_4 pH 4.5 / 10% acetonitrile. The samples were analyzed using an isocratic flow of mobile phase at a flow rate of 5 ml/min and the absorbance was measured at 206 nm by an L-7400 UV-detector.

$^{13}C_2/^{15}N$-labelled Met-X was analyzed as above except that a cationic-exchange column Theoquest Hypersil SCX, 5 μm (Thermo), 250 × 10 mm was used. A flow rate of 5 ml/min and an isocratic flow of 90% 0.1 M KH_2PO_4 pH 4.5 / 10% acetonitrile were used. One ml fractions were collected and the peak fractions containing the compound were pooled and concentrated by lyophylization before being analyzed with NMR.

The metabolic profiles derived from dialysis of G-NH_2 in serum and synthetically produced α-HGA was then compared using retention time as an indicator.

5.11 COMPOUND CHARACTERIZATION BY NMR SPECTROSCOPY (PAPER IV)

Due to the poor results achieved by NMR spectroscopy at the low natural abundance of ^{13}C- and ^{15}N-nuclei in glycine, the commercially available labelled glycine with two 99% ^{13}C- and one 99% ^{15}N-isotopes was purchased from Isotech and used as starting material to produce $^{13}C_2$/ ^{15}N-labelled glycineamide.

The labelled glycine was coupled to a solid support (Rink-Amide MBHA resin), de-protected and cleaved in order to transform it into an intermediate – the labelled glycineamide. This compound was dialyzed against PS or FBS in order to characterize and produce the unknown substance obtained and called for Met-X.

^{1}H, ^{13}C and 2D NMR spectra for compounds $^{13}C_2$/ ^{15}N-labelled glycineamide and Met-X were measured on a Bruker DPX 300 MHz, a Jeol Eclipse^{+}500 MHz and a Bruker DMX 600 MHz spectrometers.

5.12 COMPARISON OF MET-X WITH α-HGA BY CAPILLARY ELECTROPHORESIS (PAPER IV)

Capillary electrophoresis experiments were carried out at 20ºC with a BioFocus 3,000 system (Bio-Rad), which was equipped with a fast scanning UV-Vis detector. Fused silica tubing (50 and 365 μm inner and outer diameter, respectively) was cut to a length of 23 cm (with 18.5 cm effective length). Sodium phosphate buffer (0.05 M) at pH 7.4 was used as a background electrolyte. The polarity was set from positive to negative (the detection point was closer to the cathode). The Met-X solution obtained from the dialysis procedure was diluted two fold in the buffer solution and filtered through a syringe disc Ultrafree-MC filter (5000 NMWL, Millipore) prior injection by pressure (3 psi·s). α-HGA was dissolved in the buffer at 10 mM concentration and injected by pressure (3 psi·s). The applied voltage was 10 kV in all experiments resulting in 50 μA current.

5.13 VIRUS STOCK PREPARATION (PAPER I, II, III, AND IV)

Virus stocks were prepared by collecting culture supernatants from transfected HeLa-tat (papers I, II, and III), COS7 and 293T or H9 cells infected with the laboratory strain SF-2 virus (paper II) and 50% tissue culture infectious dose ($TCID_{50}$) was determined. Titration of the virus stock was carried out by infecting H9 cells with serial dilution of the virus. Culture supernatants from the infected cells were collected on days 4, 7 and 11 and the $TCID_{50}$ was

determined. The p24 antigen content of transfected cell culture supernatants was determined using an in-house p24-ELISA.

5.14 INFECTIVITY ASSAY (PAPER I, II, AND III)

H9 or MT4 cells were infected with the X4 NL4-3 strain of mutant or wild type HIV-1 for one to three hrs at 37°C. Unbound viruses were then removed by centrifuging the cells at 1,200 r.p.m. for 7 min, washed and resuspended in complete RPMI medium. Supernatants were collected at various days post-infection and tested for p24 antigen contents by ELISA.

5.15 ANTIVIRAL ACTIVITY OF MET-X AND α-HGA (PAPER IV)

Initially, the antiviral activity of G-NH_2 was tested in HIV-1 infected H9 cells cultured in medium containing human, porcine or fetal bovine serum (FBS). When FBS or porcine serum (PS) was used, 100 μM G-NH_2 repeatedly abolished HIV infectivity. One mM G-NH_2 was then dialyzed against pre-washed FBS at 37ºC over night. The dialysis solution (DS) obtained was then added to infected H9 cells, cultured in medium void of FBS but containing human serum. Infected cell cultures to which 100 μM G-NH_2 or no drug had been added served as controls. HPLC analysis of DS revealed an unknown peak fraction, called metabolite-X (Met-X). To further investigate the enzyme responsible for converting G-NH_2 to an Met-X, we also studied the antiviral activity of DS by incubating the infected cells in culture medium containing boiled porcine serum. Infected cells with or without G-NH_2 cultured in same conditions served as controls.

Once the structure of the unknown Met-X was determined by NMR, α-HGA was synthetically produced and tested for antiviral activity. Freshly prepared H9 cells (100,000 cells per well in 48-well plates) in complete RPMI medium were infected with the SF-2 at 100 $TCID_{50}$ by incubating for 2 hrs at 37°C. The cells were then pelleted, washed and resuspended in complete RPMI medium containing HS or PS and test compound was added. Cells were cultured for 11 days and growth medium was changed seven days post-infection. The HIV-1 p24 antigen contents were assayed at day 7 and 11 post infection.

Anti-HIV activities of α-HGA against clinical HIV isolates was also accessed. Stocks of clinical HIV-1 isolates were prepared by collecting the supernatants from infected cultures of peripheral blood mononuclear cells (PBMCs) and the 50% tissue culture infective dose (TCID50) was determined by infecting PBMCs with serial, 10-fold dilutions of the virus. PBMC (600,000 cells/ml) were infected with clinical isolates at 100 TCID50 and cultured in medium containing various concentrations (1, 4, 16, 64, and 256 μM) of α-HGA. Infected cultures without test compound served as controls, and each isolate was plated in duplicate. Medium was changed at days 5 to 7 post infection, and the supernatants were monitored for their p24 antigen contents until the termination of incubation at day 14. To determine co-receptor usage, each isolate was

cultured in MT2 cells (500 000 cells/ml). Syncytia formation indicates CXCR4 usage, whereas no cytopathic effect indicates CCR5 usage.

The antiviral activities of other compounds that were structurally related to α-HGA were also tested at 100 μM drug concentrations. These include oxamic acid (also called oxalic acid monoamide), oxamide (also called oxalic acid diamide), α-methoxy-G-NH_2 and Boc-α-methoxy-G-NH_2. The infectivity assay used in this study was essentially as described above; however, culturing medium was changed at days 4 and 7 post-infection and assayed for both p24 contents and RT activity of the culture supernatants.

The antiviral activity of α-HGA was further investigated against several different viruses with different size and genome. The antiviral assays other than anti-HIV assays, were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus, cytomegalovirus, varicella-zoster virus and vesicular stomatitis virus], Vero (parainfluenza-3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus), or HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus) cell cultures. Virus infected cells were cultured in the presence of varying concentrations (5-fold dilutions) of α-HGA. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds.

5.16 ELISA (PAPER I, II, III, AND IV)

The HIV-1 replication in infected cells was assayed by measuring the p24 antigen contents and the RT-activity (Paper I) in the culture supernatants. For p24 assay, in-house p24-ELISA was used and performed essentially as previously described [241]. Briefly, anti-p24-coated microwell plates (MWP) (Nunc) were blocked with 3% BSA in PBS at 37°C for 30 min. Supernatants were added to the plates and incubated at 37°C for 1 h. The MWPs were then washed and biotinylated anti-p24 antibody was added. One hour after incubation, the MWPs were washed and streptavidine-conjugated anti-rabbit antibody (Jackson) was added. The RT activity in the culture supernatant was measured by the Lenti RT-ELISA kit as recommended by the manufacturer (Cavidi Tech).

5.17 TRANSMISSION ELECTRON MICROSCOPY (PAPER I, II, III, AND IV)

For thin section transmission electron microscopy (TEM) studies, transfected HeLa-tat, infected H9 or induced ACH-2 cells were fixed by adding freshly prepared 2.5% glutaraldehyde. The cells were postfixed in 1% OsO_4 upon embedding in epoxy resins, stained with 1% uranyl acetate, dehydrated and polymerized. Importantly, sections were made approximately 60 nm thick to allow accommodation of the volume of the core structure parallel to the section plane. Minimal beam dose technique was employed throughout to reduce beam damage. Numerical evaluation of morphology was done with series of electron

micrographs to depict different categories of virus morphology, specifically focusing on the packing of the virus core structure.

Additionally, isolated CA proteins assembled in vitro (in turbidity assay, see below) were negatively stained with 2% ammonium molybdate at pH 8.0 to study possible polymerization.

5.18 *IN VITRO* AND *IN VIVO* STUDIES ON HIV-1 CA ASSEMBLY (PAPER III AND IV)

An *in vitro* assembly of purified recombinant mature HIV-1 CA protein would result in long tubular or spherical structures depending on the pH and ionic strength of the assembling buffer.

In order to examine the structures formed by HIV-1 CA assembled *in vitro*, the recombinant CA were incubated with and without α-HGA. Polymerization of CA was then induced by addition salt and monitored in a Shimadzu spectrophotometer (Germany, GmbH) as the rate of tube formation increases the sample turbidity in the absence of the assembly inhibitor. Absorbance measurements at 350 nm were recorded at 30 s intervals for up to 60 min. The *in vitro* polymerization of CA cylinders formed in presence and absence of α-HGA were also prepared for TEM analysis. Similar strategies were also used to investigate polymerization of mutant and WT CA proteins in paper III.

For *in vivo* CA assembly study, HeLa-tat cells were seeded in the presence or absence of α-HGA 24 hrs before transfection with the pC37M plasmid expressing the MA-CA fusion protein. Forty-eight hours post transfection, culture supernatants were removed and cells were fixed in 2.5% glutaraldehyde and prepared for TEM analysis.

5.19 IMMUNOBLOTTING (PAPER I, II, III, AND IV)

Denatured whole cell lysates of transfected (HeLa, HeLa-tat, HeLa-tat III, BHK21, COS7, Vero, HepG2, and 293T), infected (H9 and MT4) and induced (ACH-2) cells, viral lysate or immunoprecipitates were resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and immunoblotted with various primary antibodies or a pool of different HIV-1 positive human sera. Bound antibodies were then detected by using appropriate horseradish peroxidase-conjugated secondary antibody. In some cases, the membranes were also re-probed with anti-calnexin or anti-cyclophilin A antibodies.

5.20 VIRUS PURIFICATION (PAPER I, II, III AND IV)

Cultured supernatants from mutant and wild type pNL4-3 transfected cells or induced ACH-2 cells treated with different concentrations of α-HGA (paper IV) were clarified of cell debris by centrifugation, filtered through a syringe filter and mixed (4:1) with Viraffinity (CPG Inc). The mixture was incubated at room

temperature for 5 min, briefly centrifuged and viral pellets were washed three times. Finally, viral pellets were dissolved in 1× RIPA buffer and subjected to SDS-PAGE as described above.

5.21 MITOGENIC ACTIVITY ASSAYS OF α-HGA (PAPER IV)

Human PBMC were exposed to several concentrations of α-HGA for 3 days in the absence or presence of phytohemagglutinin (PHA). After three days, tritium labelled thymidine was added into the culture and further incubated for 20 hrs at 37°C. Finally, the cells were harvested and washed extensively before measuring the thymidine incorporation by liquid scintillation. Stimulation index (SI) was calculated as the mean count of three cultures without α-HGA. The relative SI was calculated as the mean count of three cultures with α-HGA plus PHA divided by the mean count of three cultures with PHA only.

5.22 DETECTION OF PROVIRAL DNAs (PAPER II)

Total DNA was isolated from infected H9 cells sixteen days post-infection. The DNAs were isolated using Qiangen's DNA purification kit as recommended by the manufacturer. An equal amount of DNA from the cells was then subjected to PCR amplification using a set of primers corresponding to the early and late gene replication steps of proviral DNA synthesis. Early HIV-1 gene products were amplified using the forward primer Ra 5'-TCT CTG GTT AGA CCA GAT CTG-3' (459-479) and the reverse primer U5a 5'-GTC TGA GGG ATC TCT AGT TAC-3' (584-604) described previously [242]. Late gene products representing a conserved region of the HIV-1 *gag* was amplified with the forward primer SK-38 5'-ATC CAC CTA TCC CAG TAG GAG AAA T-3' (1090-1117) and the reverse primer SK-39 5'-TTT GGT CCT TGT CTT ATG TCC AGA ATG C-3' (1177-1204) [243] that amplified a 115-bp fragment. To quantify the total cellular DNA present in each sample, human β-globin DNA was amplified using a set of primers PC03 and PC04, which amplified the corresponding sequences of a 110-bp fragment of the first exon of the human β-globin gene [244].

One microgram of each DNA isolated was used as the template for PCR with PCR mastermix (Promega) and were subjected to 29 cycles with denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, extension for 1 min at 72°C, and 10 min extension step at 72°C. Twenty µl of the amplified PCR products was subjected to agarose gel electrophoresis, and the gel was stained with ethidium bromide.

5.23 VIRUS BINDING AND INTERNALIZATION (PAPER II)

Virus binding and internalization was determined on CD4+ TZM-bl cells essentially as described elsewhere [245]. Briefly, cells were seeded one day before and infected with equal amounts of mutant and wild type virus treated

with DNase I. To measure virus attachment, cells were incubated at 4°C for 1 h and infected with equivalent amounts of mutant and wild type virus. For internalization study, same conditions as above were used except that upon removal of unbound virus, cells were transferred to 37°C and further incubated for 2 hrs. At the end of each procedure, cells were harvested in 0.5× RIPA buffer and the amount of cell associated p24 was measured.

5.24 SINGLE REPLICATION CYCLE INFECTIVITY (PAPER II AND III)

TZM-bl cell line is a HeLa cell clone engineered to stably express CD4, CXCR4 and CCR5, and contain an integrated copy of HIV-1 long terminal repeat (LTR) linked to a luciferase and β-galactosidase gene [246]. Expression of the indicator luciferase or β-galactosidase gene is under the control of Tat protein that is activated by Tat protein synthesized from the infecting virus. For relative viral infectivity assay, TZM-bl cells were seeded one day before infection. Following day, medium was removed and target cells were inoculated by adding various amounts of mutant or wild type NL4-3 virus stocks with 20 μg/ml DEAE-dextran. After adsorption period of approximately 2 hrs, input viruses were removed and cells were fed with complete DMEM with or without indinavir and cultured for another 18 to 48 hrs under the same conditions.

At the end of the experiment, the luciferase activity in infected TZM-bl cells was measured with the luciferase assay kit obtained from Promega as recommended by the manufacturer. Briefly, culture supernatants were removed and Glo Lysis Buffer (Promega) was added to each well, and incubated for 5 min at room temperature to allow complete cell lysis. One hundered μl of the cell lysate was transferred to a white 96-well micro well plate (Costar) and a volume of Bright-Glo™ Reagent (Promega) equal to that of the cell lysate was added before measuring the luminescence using the Luminoskan Ascent luminometer (ThermoLabsystem).

6 RESULTS

In this thesis, a total of 13 mutants located in both the *N*- and *C*-termini of HIV-1 capsid (CA, p24) domains were investigated.

In Paper I, the importance of two highly conserved sequences located in the *C*-terminal CA were investigated by constructing eight different mutants (L205A+P207A, L205A, P207A within the ^{204}A***L***G***P***GATLEE^{213} conserved sequence, and 223GPG225AAA, G223A, P224A, G225A and V221G within the ^{218}CQG***V***G***GPG***^{225} sequence) of the infectious clone pNL4-3. For clarity for the reader these mutations will here be referred to as follows. The L205A mutant will be referred to as ^{204}A***L→A***GPGATLEE^{213} the P207A mutant as ALG***P→A***GATLEE, the triple mutant 223GPG225AAA as ^{218}CQGVG***GPG→AAA***^{225} and so on.

In Paper II, the relevance and significance of Glu187 in formation of a head-to-tail dimer (*N*-*C* dimer) between two interacting CA monomers was investigated by mutating Glu187 to Gly (E187G). As a control, Glu98 which has no intra-molecular contacts with other CA residues was also mutated to Ala (E98A).

The role and significance of a highly conserved β-hairpin structure formed between Pro1 and Asp51 of the *N*-terminal CA was also investigated in Paper III, by mutating Asp51 to three other closely related amino acids, Asn, Glu, and Gln.

Lastly, we defined the active antiviral metabolite of GPG-amide/G-amide and investigated how it affects HIV-1 capsid assembly.

We performed detailed analysis to assess the potential effects of all HIV-1 mutants using biochemical, functional and morphological techniques. Various methods were also used in identification and characterization of the active antiviral metabolite of GPG-amide. The results are summerized below.

6.1 EFFECT OF CA MUTANTS ON VIRAL PROTEIN EXPRESSION (PAPER I, II, AND III)

There are two conserved sequences, ALGPGATLEE (aa 204–213) and CQGVGGPG (aa 218–225), located in the *C*-terminal CA region of HIV-1. Previous viral biological studies performed [232-234] were mainly based on large deletions and insertions. Therefore, our attention focused on specific amino acids, and therefore, we constructed several mutants in these two sequences to study their biological effects on viral replication. Eight different mutants of the infectious clone pNL4-3 were constructed and their effects on viral assembly, release and infectivity was studied.

The intracellular level of the Gag precursor in all except three mutants was comparable to that of the WT, whilst the ^{204}A***L→A***G***P→A***GATLEE^{213} double mutant and the ^{204}A***L→A***GPGATLEE^{213} mutant displayed reduced levels of the protein and the ^{218}CQG***V→G***GGPG^{225} mutant displayed no Gag. Processed HIV-1 Gag proteins were detected in all cell lysates, with the exception of the cells transfected with ^{204}ALG***P→A***GATLEE^{213} and ^{218}CQG***V→G***GGPG^{225} mutants that were aberrant in Gag processing. In addition to severely reduced amounts of viral MA and CA, the two mutants showed a band of approximately 30 kDa (^{204}ALG***P→A***GATLEE^{213}) and 46 kDa (^{218}CQG***V→G***GGPG^{225}), respectively. Additionally, the ^{218}CQG***V→G***GGPG^{225} p41^{Gag} levels were almost twice that of the WT.

To further characterize whether the Gag expression and processing pattern of the two mutants ^{204}ALG***P→A***GATLEE^{213} and ^{218}CQG***V→G***GGPG^{225} were cell specific, five different cell lines (BHK21, COS7, Vero, HepG2, and 293T) were transfected with the plasmids. Transfection of HeLa-tat cells with the ^{218}CQG***V→G***GGPG^{225} mutant produced mainly p41, while p25 was additionally detected in BHK21, COS7 and 293T cells. Small amounts of Gag were also observed in COS7 and 293T cells.

Western blot banding patterns of the E187G mutant affecting the salt-bridge formation in *N-C* dimer of p24 and the E98A mutant having no inter-molecular contacts with other CA residues (Paper II) were identical to that of WT pNL4-3 transfected cells, indicating that neither mutation had any substantial effect on viral protein expression and/or Gag processing.

Investigation of the viral protein expression of the three HIV-1 CA mutants carrying the D51N, D51E, and D51Q mutations (Paper III) constructed to study the salt-bridge formation of the β-hairpin structure in the *N*-terminal CA revealed that intracellular levels of the Gag precursor in all mutants were somewhat variable depending on the cell type. The D51N and D51Q mutants displayed reduced levels of the CA as compared to the D51E mutant or wild type pNL4-3 transfected cells. Additionally, in Western blot analysis of the precipitated particulate extracellular material, mature CA was the major product. However, the level of CA in both D51N and D51Q mutants of precipitated material was significantly reduced relative to the wild type and D51E mutant, correlating with the lower intracellular CA levels.

6.2 EFFECT OF CA MUTATIONS ON PARTICLE RELEASE (PAPER I AND II)

The effect on Gag assembly and virus particle release of CA mutations located within the two conserved *C*-terminal CA sequences was analyzed by the p24 level and the RT activity in the culture supernatants. Release of virus particles from ^{204}ALG***P→A***GATLEE^{213} and ^{218}CQG***V→G***GGPG^{225} transfected cells into the culture medium was negligible, whereas a decrease by 60 to 70%, compared to that of the WT-transfected cells, was observed with the

^{204}A***L→A***G***P→A***GATLEE^{213}, ^{204}A***L→A***GPGATLEE^{213}, ^{218}CQGVG***GPG→AAA***^{225}, ^{218}CQGVG***G→A***PG^{225}, ^{218}CQGVGG***P→G***^{225}, and ^{218}CQGVGGP***G→A***^{225} mutants. Additionally, the viral protein contents released into the culture supernatants were precipitated and analyzed. In Western blot, mature CA represented the major product of the precipitated material except for mutant ^{218}CQG***V→G***GGPG^{225}, in which p41 was the major product correlating with the Western blot analysis of the cell lysates of this mutant. To rule out the effects of the ^{204}ALG***P→A***GATLEE^{213} mutation was not owing to accidental mutation at other sites of *gag*, the ^{204}ALG***P→A***GATLEE^{213} mutant was further investigated by analyzing the viral protein expression profiles of a back-mutated plasmid, the BM-P207A. From numerous experiments, it was further possible to deduce that the differences observed between the phenotypes of ^{204}ALG***P→A***GATLEE^{213} and the WT were not due to the amount of DNA used for the transfections or number of cells in the experiment. Furthermore, none of the mutations studied affected the transcription or splicing of HIV-1 RNA as detected by Northern blot analysis of the total RNAs. These results were also confirmed using imunofluorescence assay of transfected cells. In this assay, the staining pattern of viral proteins showed specific signals for CA in all mutants except ^{204}ALG***P→A***GATLEE^{213} and ^{218}CQG***V→G***GGPG^{225} when analyzed with monoclonal antibody against p24.

Release of E187G virons was unaffected and cells transfected with the E98A mutant released approximately 15% higher p24 than cells transfected with the control vector, indicating that the mutations had no substantial effect on particle release. Furthermore, Western blot analysis of viral protein contents released into the culture supernatants showed no effect in both mutants, as judged by the presence of the intermediate and fully processed Gag proteins of the wild type-level.

p24-ELISA was also used to investigate virus release from the three D51 mutants (D51N, D51E, and D51Q). Analysis of p24 antigen contents released into culture supernatants of transfected HeLa-tat III, 293T and COS7 cells revealed that the release of virions from D51N and D51Q transfected cells were negligible in all three cell lines, whereas the virus production of the D51E mutant was reduced by 2- to 6-fold as compared to the wild type.

6.3 IMPACT OF CA MUTATIONS ON VIRAL INFECTIVITY (PAPER I, II AND III)

The efficacy of mutant viral progenies to infect and replicate was also studied in H9 and MT4 cells that were inoculated with the corresponding culture supernatants.

H9 infectivity of all mutants of the two conserved sequences, ^{204}A***L***G***P***GATLEE^{213} and ^{218}CQG***V***G***GPG***^{225}, located in the *C*-terminal CA was either absent or severely reduced, compared to that of the WT virus. We also found that the E98A virions, in contrast to the E187G and wild-type NL4-3

(Paper II), were non-infectious in this CD4 positive H9 cells. Similar results were also obtained when infectivity of the latter two mutants was assessed with infected MT4 cells.

Likewise, the infectivity of all three D51 mutant virions (D51N, D51E, and D51Q) was also completely absent in MT4 cells (Paper III). Similar results were also seen when the infectivity of mutant viruses was tested in H9 cells that were kept in cultures for more than 25 days.

6.4 EFFECT OF CA MUTATIONS ON VIRION MORPHOLOGY (PAPER I, II, AND III)

Transmission electron microscopy (TEM) was used to study viral morphogenesis of all CA mutants in this thesis. Transfection and/or infection of the cells was performed with the respective mutant or wild type NL4-3 virus or the proviral DNA construct pNL4-3.

No virus structures were observed with the ^{204}ALG***P→A***GATLEE^{213} mutant transfected HeLa-tat cells or H9 cells incubated with the ^{204}ALG***P→A***GATLEE^{213} culture supernatant. Four other mutants, ^{218}CQGVG***G→A***PG^{225}, ^{218}CQGVGG***P→G***^{225}, ^{218}CQGVGGP***G→A***^{225}, and ^{218}CQGVG***GPG→AAA***^{225}, showed a limited number of virus particles with aberrant core morphology. Unlike the WT and the ^{204}A***L→A***G***P→A***GATLEE^{213} and ^{204}A***L→A***GPGATLEE^{213} mutants, the ^{218}CQG***V→G***GGPG^{225} formed only intracellular virus-like particles. Numerical evaluation of morphology was done only with two mutants, ^{204}A***L→A***G***P→A***GATLEE^{213} and ^{204}A***L→A***GPGATLEE^{213}, which produced sufficient amounts of virus. In ^{204}A***L→A***G***P→A***GATLEE^{213} and ^{204}A***L→A***GPGATLEE^{213} cultures, 62 and 47% respectively, of all virus particles counted had an aberrant and heterogeneous core appearances as opposed to 2% in WT controls. None of the mutant virus particles had a longitudinal sectioning of the core with normal mature conical structure.

The majority of TEM images of NL4-3 and E187G showed a mixture of both mature particles of normal morphology and immature particles. In contrast, TEM images of E98A showed mostly aberrant and immature particles. Additionally, a substantial increase in percentage of distorted, aberrant and immature-like E98A virus was observed as compared to the WT control.

Among the three D51 mutants, the D51N and D51Q mutant virions showed mostly particles devoid of the typical HIV-1 core structure. Furthermore, the D51N virions showed a large pool of intra-vesicular viruses that were deficient of the electron dense core structure. A limited number of immature-like viruses and occasionally mature-like viruses but with aberrant cores were observed with the D51E mutant. Only the wild type control produced viruses with typical immature- and mature-like HIV-1 virions.

6.5 ANTIVIRAL ACTIVITY OF GLYCINEAMIDE (G-NH_2) AND METABOLITE-X (MET-X) (PAPER IV)

Antiviral activity of G-NH_2 was performed initially in infected cells cultured in human- (HS), procine (PS) or fetal bovine serum (FBS). Virus replication was severely inhibited in infected cells cultured in the presence of PS or FBS but not HS, suggesting that G-NH_2 had to be converted by an enzyme present in PS and FBS but not in HS. This result prompted us to investigate the metabolic profile of G-NH_2 in PS or FBS.

G-NH_2 was dialyzed against PS and the dialysis solution was tested for antiviral activity in infected cells cultured with HS or PS. G-NH_2 showed no antiviral activity in HS, however, infected cells cultured in HS or PS and with dialysis solution (DS) of G-NH_2, virus replication was completely inhibited at a concentration of ~100 μM. Further analysis of DS with HPLC indicated that G-NH_2 had been converted to an at the time unknown molecule which we termed metabolite-X (Met-X). Indeed, the HPLC fraction containing the Met-X was also isolated and tested for antiviral activity. Both dialysis solution derived form G-NH_2 and the isolated HPLC peak fraction could inhibit HIV-1 replication when cultured both in HS and boiled PS.

6.6 PRODUCTION AND ANALYSIS OF MET-X (PAPER IV)

In order to produce Met-X, G-NH_2 was dialyzed against porcine serum at 37ºC. The transformation of G-NH_2 to the unknown Met-X could be monitored and also analyzed with HPLC using the retention time as an indicator. Dialysis of G-NH_2 aginst PS at 4ºC or HS at 37ºC gave no Met-X characteristic peak in the HPLC chromatogram as in PS or FBS at 37ºC. Met-X treatment of ACH-2 and infected H9 cells resulted in significant changes in virion morphology when analyzed with TEM.

6.7 IDENTIFICATION AND CHARACTERIZATION OF α-HGA (PAPER IV)

With the antiviral activity of the unknown Met-X confirmed, it was produced from $^{13}C_2/^{15}N$-labelled glycineamide. The $^{13}C_2/^{15}N$-labelled Met-X was produced in order to increase the sensitivity of NMR for structural determination. Once sufficient amount of $^{13}C_2/^{15}N$-labelled Met-X was produced enzymatically, the dialysis solution was concentrated and lyophilized for the NMR studies. A significant downfield shift in ^{1}H NMR spectrum of Met-X (5.18 ppm in D_2O solution) compared with the original glycineamide (3.86 ppm in D_2O solution) indicated that one proton in the glycine methylene group was replaced by a strong electronegative substituent, such as OH. Furthermore, a relatively large ^{1}H -^{13}C and ^{13}C- ^{13}C couplings also indicated such a substitution. The expected values for NH_3^+ and $CONH_2$ nitrogen resonances obtained by the 2D ^{1}H-^{15}N HSQC experiment and the agreement between ^{1}H and ^{13}C spectra, indicated the existence of a molecule $NH_3^+CH(X)CONH_2$. Thus, based on these results, the possible structure of the unknown compound Met-X was determined as α-hydroxy-glycineamide (α-HGA).

6.8 CHARACTERIZATION OF MET-X AND α-HGA (PAPER IV)

Two different approaches, HPLC and capillary electrophoresis (CE), were employed to compare the biochemical properties of Met-X with α-HGA. The HPLC chromatogram of α-HGA was found to be identical to the enzymatically produced Met-X. Analysis of α-HGA with capillary electrophoresis also produced a symmetrical single peak, which had strikingly similar migration time to Met-X.

6.9 SPECIFICITY AND INHIBITION OF α-HGA ON HIV-1 REPLICATION (PAPER IV)

Strong inhibitory effect of chemically synthesized α-HGA on the replication of HIV-1 in infected H9 cells was obtained. The HPLC chromatogram of synthetically produced α-HGA was also identical to the enzymatically produced Met-X. At 100 μM of α-HGA, the HIV-1 replication was completely inhibited in infected H9 cells. The 50% effective concentration (EC_{50}) of α-HGA was determined in HIV-1 SF-2 infected H9 cells and was in the range of 4 to 6 μM.

The specificity and inhibitory effect of α-HGA on HIV-1 replication was also compared to four other structurally related compounds, oxamic acid, oxamide, α-methoxy glycineamide and Boc-α-methoxy glycineamide. None of the compounds tested showed any significant effect on HIV-1 replication.

6.10 EFFECT OF α-HGA ON HIV-1 MORPHOLOGY AND VIRUS LIKE PARTICLE (VLP) FORMATION (PAPER IV)

α-HGA was chemically synthesized and tested for antiviral activity in different cell lines. Three different approaches were utilized to characterize the antiviral activity of α-HGA. Analysis of HIV-1 progeny produced from infected cells in the presence and absence of α-HGA, inhibition of virus like particle (VLP) formation of *in vitro* assembled recombinant CA proteins, as well as, HeLa-tat cells transfected with the MA-CA expression plasmid, which were all analyzed with TEM.

Electron micrographs of HIV-1 progeny acquired in the presence of α-HGA showed significantly increased number of virus with aberrant core morphology. In α-HGA treated cultures 62% of virus showed aberrant cores as opposed 20% in the WT control.

The effect of α-HGA on *in vitro* CA assembly of purified HIV-1 CA protein was also studied in presence and absence of α-HGA. TEM analysis showed that addition of 100 μM of α-HGA could completely inhibit the *in vitro* capsid tubular formation. Similar results were also seen *in vivo* when HeLa-tat cells transfected with the MA-CA expression plasmids were analyzed in the presence and absence of α-HGA.

Additionally, turbidity assay was used to analyze the assembly and inhibition properties of CA *in vitro*. Absence of α-HGA resulted in an increased turbidity that was monitored spectrophotometrically. In contrast, addition of 100 μM of α-HGA, corresponding to a CA/α-HGA molar ratio of 1/3.3, appeared to completely inhibit tube formation.

6.11 EFFECT OF α-HGA ON CELL PROLIFERATION AND REPLICATION OF OTHER VIRUSES (PAPER IV)

α-HGA had no mitogenic activity against human PBMCs at concentrations up to 2 mM. In contrast, 2 μg/ml PHA markedly stimulated dThd incorporation into PBMC DNA. When the compound was added to PBMCs in the presence of PHA, no effect on PHA-induced stimulation of DNA synthesis (cell proliferation) was observed at concentrations up to 400 μM. At 2 mM, the PHA-induced stimulation was markedly inhibited by the test compounds (presumably due to cellular toxicity).

The antiviral activity of α-HGA against vesicular stomatitis virus, respiratory syncytial virus, parainfluenza, Coxsackie B4, Sindbis virus, herpes simplex virus 1 and 2, vaccinia virus, cytomegalovirus, varicella-zoster virus, reovirus 1 and Punta Toro virus was tested. The EC_{50} values of α-HGA to all these viruses could not be reached at 2 mM (data not shown) and thus, α-HGA was considered not to be active against any of these viruses in cell culture.

7 DISCUSSION

There are two highly conserved sequences within the *C*-terminal CA domain, the ALGPGATLEE (aa 204–213) and CQGVGGPG (aa 218–225). Since previous viral biological studies [232-234] performed on these two sequences were mainly based on large deletions and insertions, our attention focused on the specific amino acids. Our results also showed that the two conserved sequences are essential for the proteolytic processing of Gag and Gag-Pol precursors, as well as, for the proper assembly and release of infectious virus particles.

Mutations in the first conserved sequence ^{204}A***L***G***P***GATLEE^{213}, except for the ^{204}ALG***P→A***GATLEE^{213} mutant in which the imino acid proline was substituted for the more hydrophobic amino acid alanine, seem to have led to increased but defect proteolytic processing of Gag precursor and p41 into mature proteins and hence, no virus structures were released that could be detected with either ELISA or TEM analysis. The *in vitro* transcription and translation, as well as, the steady state viral RNA levels of all mutants were the same as with the WT, suggesting that there were no defects at the transcriptional or translational level of the virus replication. Complementing the ^{204}ALG***P→A***GATLEE^{213} with the WT *gag* or *CA* genes *in trans* could partly restore viral protein expression and virus assembly, but not infectivity. However, co-transfection of ^{204}ALG***P→A***GATLEE^{213} with WT pNL4-3 displayed WT expression profiles and infectivity, suggesting no dominant-negative effect with the ^{204}ALG***P→A***GATLEE^{213} mutant.

All mutations located within the second conserved sequence ^{218}CQG***V***G***GPG***^{225} except for ^{218}CQG***V→G***GGPG^{225}, had no substantial effect on viral protein expression, but yet the release of virus particles was severely reduced. This could be due to a defect linked to p55-p55 or p55-p160 interactions at the cell membrane which is necessary for efficient viral assembly and release. Additionally, although the four mutants ^{218}CQGVG***G→A***PG^{225}, ^{218}CQGVGG***P→G***^{225}, ^{218}CQGVGGP***G→A***^{225}, and ^{218}CQGVG***GPG→AAA***^{225} were assembly-competent, they produced virus particles with aberrant core morphology and no infectivity. The viral protein expression of the ^{218}CQG***V→G***GGPG^{225} mutant produced mainly p41 and also intracellular virus-like particles. This might be linked to an early but incomplete intracellular protease activity before targeting of Gag and Gag-Pol to the cell membrane. It is also possible that this motif might bind to an unknown cellular factor that is necessary for transport of Gag to the cell membrane.

There are no structural data for the full-length Gag or mature CA and thus the process of virus maturation that converts the HIV-1 capsid core from spheres to cones is unknown. The HIV-1 *N*- and *C*-terminal CA dimer interfaces have been pinpointed using both structural and mutagenesis studies [113, 181, 183, 247]. Most of the structural studies that determined the *N-N* (head-to-head) [178, 183] and *C-C* (tail-to-tail) [181] dimers were based on each domain of CA separately

[178, 181, 183]. Nevertheless, available three-dimensional structure of each domains, as well as, studies with image reconstruction from cryo-electron microscopy of purified mature virions and *in vitro* assembled virus like particles [187-192] has allowed modelling of the p24 lattice [114, 187, 196]. Thus, it has been proposed that the *N*-terminal domain of CA forms hexameric rings joined to one another by the CA *C*-terminal dimerization domain.

Proper structural rearrangement of CA after Gag cleavage is a highly conserved feature in most retroviruses [235]. As a result of this process, a β-hairpin structure formed by a salt-bridge between Pro1 and Asp51 (D51) is important for conformational stability of the *N*-terminal CA structure [113]. Thus, upon maturation, the proteolytic refolding of CA amino terminal has been shown to be the key switch for morphological conversion of the virus from spheres to cones [113]. Previous mutagenesis study of D51 in HIV-1 CA, and likewise Asp54 in murine leukemia virus (MLV) or human T-cell leukemia virus-1 (HTLV-1), has been shown to disrupt formation of this β-hairpin structure [113, 248, 249].

We demonstated that D51 substitution of HIV-1 CA with glutamate (D51E), asparagine (D51N), but not glutamine (D51Q) could partly restore *in vitro* p24 assembly but not the infectivity of the virus particles. These three amino acids, in contrast to the D51A substitution reported earlier, are structurally close and have also similar properties in protein as D51 (Glu > Asn > Gln). Analysis of viral protein expression patterns in different cell types indicated that the intracellular concentrations of CA in any of the cells transfected with D51N and D51Q were generally reduced. This could not be explained by the lack of recognition by the antibody used, since detection with various antibodies using different techniques gave similar results. Further, immunofluorescence analysis revealed less intracellular CA staining with the D51 mutants (although somewhat less with the D51E mutant) that may have resulted as a consequence of CA/Gag instability, correlating well with the less amount of CA detected with immunoblotting. However, increased accumulations of dense staining close to or at the plasma membrane (PM) of cells transfected with D51 mutants were observed. This may have been due to improper Gag assembly at the PM, perhaps due to conformational constraints imposed by the mutations, which ultimately blocked virus release that resulted in reduction of virion release observed with all mutants. It is also possible that the virus release may have been blocked as a result of inability to form the stabilizing β-hairpin structure in the *N*-terminal domain of CA upon maturation which is necessary for the virus release. Collectively, our data and previously published observations by others [113, 177, 250] suggest that the invariable D51 residue of HIV-1 is crucial for formation of the β-hairpin structure in matured CA. Additionally, even substitution of D51 with such a similar residue as with glutamate could not restore the integrity of this structure. Furthermore, although our results demonstrated that the D51N and D51E substitutions could restore the *in vitro* CA tubular formation, the infectivity of all D51 mutation were rendered non-infectious indicating that this residue is indispensable.

In addition to the two different CA dimers interfaces described above, a third head-to-tail dimer (*N-C*) of mature CA representing nearly the whole CA has also been described [173]. In light of this study and based on available structural studies and analysis of inter-atomic contacts, we designed two single amino acid substitution mutations, the E98A and E187G. The E187 has been shown to be important for forming a salt bridge between Glu187 and Arg18 of the two interacting CA monomers in the hydrophobic core of the interface mediating the interactions. Additionally, Glu187 has extensive intra-molecular contacts with eight other CA residues. In contrast, Glu98 had no intra-molecular contacts with other CA residues and was expected not to grossly interrupt the CA structure. However, mutation of Glu98 to Ala was found to be lethal for virus assembly and infectivity, despite the fact that available data on crystallography suggested that the residue was not important. Thus, we showed that Glu98 and Glu187 have deviated biological properties than what was predicted with crystallography and/or analysis of inter-atomic contacts [251]. We demonstrated that mutations of some p24 residues that are not involved in such contacts (in this case, the E98A) may also be lethal. We also excluded the possibility that this effect may be due to the lack of cyclophilin A incorporation (since the mutation was near the cyclophilin A binding site). However, this may not necessarily rule out the possibility that other cellular factor may be involved.

Optimal capsid core stability is a critical factor for HIV-1, and acquisition of virion infectivity is critically dependent on proper capsid cone formation. Thus as was demonstrated with most of our mutants mutations in CA are often lethal when they inhibit CA assembly by destabilizing the intra and/or inter molecular CA contacts. These features make CA of interest as a target for developing new antiviral drugs.

Glycyl-prolyl-glycinamide (GPG-NH_2) and glycinamide (G-NH_2) have previously been described to induce non-infectious HIV-1 particles with aberrant core structures when added to the culture medium of infected cells. We here showed that it was a metabolite of GPG-NH_2/G-NH_2 that affected virus replication previously ascribed to these compounds. The metabolite was characterized as a small modified amino acid, alpha-hydroxy-glycineamide (α-HGA) that affected the HIV-1 core assembly and virus maturation, by interfering with CA-CA interactions. Both molecular and biological assays were used to identify and characterize the molecule. Studies on *in vitro* and *in vivo* CA assembly showed that α-HGA could inhibit the formation of tubular structures. Western blot data obtained for α-HGA treated and untreated samples of infected cells were also similar, indicating that it does not affect viral protein synthesis nor interfere with the proteolytic processing of HIV-1 Gag precursor. Furthermore, α-HGA had no antiviral activity against several other viruses with either RNA or DNA genomes, suggesting that the antiviral activity attributed to HIV-1 is rather specific. Additionally, α-HGA has shown a strong inhibitory activity against all HIV-1 subtypes, as well as, several clinical isolates that had acquired resistance against other approved antiretroviral drugs.

In conclusion, this study demonstrated that substitution mutations of some of the determinant residues in the HIV-1 capsid were lethal, affecting proper virus assembly, viral replication and infectivity. The nature of the the HIV-1 capsid core, as being made up of multiple semi-stable protein interactions would make the CA protein a suitable target for antiviral intervention, such as α-HGA, which could be a potential lead compound for a new class of anti-HIV substances.

8 CONCLUDING REMARKS AND FUTURE PERSPETIVES

This thesis has explored some of the determinant residues and/or sequences in HIV-1 capsid core assembly and has demonstrated that:

- Single amino acids of the two conserved sequences located in *C*-terminal region of CA are also important for the proper virus assembly, release and infectivity of HIV-1.
- Mutation of Asp51 of HIV-1 CA to three other structurally related residues could impair virus replication and suggesting this invariable residue to be indispensable.
- Although Asp187 is involved in an *N*-*C* dimer between two HIV-1 CA monomers, substitution of this residue to Gly neither affects CA assembly nor infectivity of progeny viruses.
- Some residues within HIV-1 CA may have deviated biological properties than predicted their functions from crystallography or analysis of inter-atomic contacts.
- The study has further demonstrated that the small compound identified as α-hydroxy glycineamide (α-HGA) could specifically interfere with *in vitro* and *in vivo* CA assembly and inhibit HIV-1 replication.

Assembly and release of infectious HIV-1 particles is highly complex event involving several viral and cellular proteins. In Paper I, II and III, attempts have been made to explore the importance of the two highly conserved sequences in CA, characterize the formation of an important salt-bridge, as well as, investigate the role and importance of a highly conserved motif (β-hairpin structures) on HIV-1 capsid core assembly and virion infectivity. Whereas the D51 mutations in Paper III were found to be lethal and that this residue is indispensable, the E187 (Paper II) was shown to be dispensable despite the fact that it is well conserved and also participates in formation of an important salt-bridge in *N*-*C* CA dimer. However, it is also important to note that HIV-1 assembly and release is a highly complex event, involving several viral protein-protein, protein-RNA, and protein-lipid interactions. The fact that mutation of Glu98 which is not involved in any inter-atomic contacts with other CA residues was found to be lethal, suggests that the E98 might be important for interactions with other cellular or viral factor necessary for HIV-1 CA assembly and/or infectivity. Thus, further experiments will be necessary in order to rule out or proof these possibilities. Furthermore, studies on this viral-cellular protein

interactions and/or functions, might not only elucidate the viral morphogenesis, but may also provide new approaches to inhibit HIV-1 replication.

α-HGA is a small compound with a specific anti-HIV activity against various HIV-1 laboratory strains, as well as, clinical isolates that have aquaired resistance to other antiretroviral drugs. Various biological approaches including *in vitro* and intracellular studies, as well as, MALDI-TOF-TOF analysis suggested that α-HGA could bind to HIV-1 CA and thereby interfere with CA assembly. Although the latter study showed that α-HGA could bind to the hinge region in CA, the exact binding site of α-HGA has yet to be determined. Thus, α-HGA might prove to be a lead compound for a new class of anti-HIV substances.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Det huvudsakliga syftet med denna avhandling var att definiera rollen av Gag-polyproteinet, med fokus på dess delenhet kapsid-proteinet (CA, p24) och dess funktion i HIV-1: s uppbyggnad. Avsikten med studien var att i) att bestämma vilka betydelser vissa specifika aminosyror eller sekvenser i HIV-1 har för kapsidens uppbyggnad och virusets avknoppning, ii) att karakterisera betydelsen av två konserverade aminosyrors vilka har två skilda egenskaper vad gäller kontakten med andra aminosyror i CA vid virusets uppbyggnad, iii) att definiera rollen och betydelsen av en viss aminosyra med stor betydelse vid bildningen av en konserverad β-hairpin-struktur i samband med proteolytisk klyvning av Gag och viruskärnans omstrukturering, samt iv) att definiera den aktiva metaboliten av en antiviral amiderad tripeptid, som visat sig ha effekt på kapsid-bildningen av HIV-1 och virus infektivitet.

HIV-1:s CA har en avgörande betydelse vid virus uppbyggnad och mognad. Vi har studerat två väl konserverade sekvenser som är lokaliserade vid *C*-terminala delen av HIV-1 CA genom en teknik som kallas för riktad mutagenes eller ”site-directed mutagenesis”. Denna teknik har använts flitigt för att studera struktur-funktionsrelationer av HIV-1 CA för viruskapsidens uppbyggnad och virus infektivitet. Vi visade att mutationer av vissa specifika aminosyror i de två konserverade sekvenserna i *C*-terminala delen kunde påverka det virala proteinuttrycket, virus uppbyggnaden, virus utsöndringen samt virus infektivitet. Dessutom visade vi att dessa aminosyror är nödvändiga för den ordnade proteolytiska klyvningen av Gag och Gag-Pol polyproteinerna och att mutationernas egenskaper kan vara celltypsberoende. Mutationerna av dessa aminosyror har också påverkat virus morfogenes och infektiviteten av producerade HIV-1 partiklar.

Vi har också studerat rollen av två specifika aminosyror, glutamat 98 (E98) och 187 (E187) i CA. Till skillnad ifrån E98 som inte har någon intra-molekylär kontakt, har E187 kontakt med åtta andra aminosyror i CA. E187 bildar dessutom en viktig salt-brygga i en potentiell *N-C* (huvud-till-svans) CA-dimer. Vi studerade den potentiella effekten av dessa två mutationer på Gag-polyproteinets klyvning, virus infektivitet, viral cDNA produktion och virus morfogenes. Trots avsaknad av kontakter med andra aminosyror av CA proteinet, E98 har visat sig ha en kritisk betydelse för CA:s veckning och korrekt kapsid bildning. Våra resultat visade också att de två aminosyrorna i denna studie har avvikande biologiska egenskaper än vad som kunde förutsägas genom kristallografi och/eller analys av inter-atomiska kontakter. E187 har alltså visat sig vara utbytbar trots att denna aminosyra tidigare har bedömts ha en viktig roll för en *N-C* CA-dimerbildning, indikerande att *N-C* dimerer inte förekommer i den naturliga infektionen.

Det sker två olika CA-CA kontakter vid kapsidskalets uppbyggnad, en CA-dimer som bildas av *C*-terminala delen och en CA-hexamer som bildas av *N*

terminala delen av CA. CA dimer-bildningen är en förutsättning för hexameriseringen av proteinet när kapsidskalet mognar, d. v. s. när virusets kapsid omvandlas från en sfärisk till en konisk struktur. Aminosyra D51 har i en tidigare studie visat sig spela en viktig roll vid denna omvandling genom att bilda en väl konserverad β-hairpinstruktur. Vi studerade därför betydelsen av denna aminosyra i mer detalj. Till skillnad från D51A-mutationen som beskrivits i en tidigare studie, muterade vi D51 till glutamat (D51E), glutamin (D51Q), och asparagin (D51N), tre aminosyror som strukturellt och biologisk funktionellt har mer eller mindre samma egenskaper som aspartat (D51). Trots detta kunde inte mutationerna till dessa tre aminosyror bibehålla den strukturella integriteten av CA.

Tidigare studier har visat att tillsatts av den antivirala tripeptiden GPG-amid till HIV-1 infekterade cellers medium resulterade i icke-infektiösa partiklar med abnorm kapsidmorfologi. Vi identifierade och visade att det är en metabolit av GPG-amid som orsakade effekten på HIV-1:s infektivitet. Denna metabolit renades och dess struktur bestämdes med NMR till α-hydroxy-glycineamid (α-HGA). α-HGA binder till CA och påverkar dess förmåga att bilda tubulära eller koniska struktur både *in vitro* och *in vivo*. Som en antiviral, har α-HGA en ovanlig enkel struktur, uttalad antiviral specificitet och en ny mekanism av antiviral påverkan.

Sammanfattningsvis visar studierna i detta avhandlingsarbete att mutationer i CA är letala när de påverkar kapsidens uppbyggnad, som består av semi-stabila icke-kovalenta proteininteraktioner och att dessa specifikt kan påverkas av små molekyler som exempelvis α-HGA.

10 ACKNOWLEDGMENTS

This work would not have been possible without help and support of family, friends and co-workers; I would like to thank everyone who directly or indirectly made this work possible, especially to:

My supervisor - Professor Anders Vahlne for his exceptional scientific guidance, critical advice and encouragement, his vast knowledge and expertise and confidence in my work have been crucial to my scientific achievements throughout my PhD work. I am grateful for your generosity, kindness and support. I am also deeply thankful, not only for giving me the opportunity to work in your laboratory but also for the privilege of being part of your team.

Dr Piotr Nowak, thank you for being such a true friend. I am glad I got to know you and your friendship is now indispensable. You made me realize that "science is science; fun is fun and science is fun". I wish also to thank all your friends and your family in Poland for their friendly and open-hearted hospitality.

My special thanks go also to Alenka Jejcic (known as "Banditen") for her presence. Sharing the lab and office with you has made my working environment so stimulating and perfect. Without you, life in the lab would not have been so much fun. Thank you for all your lively, yet constructive and friendly discussions. Your presence, without any doubts, has contributed to my scientific achievements.

My co-authors and collaborators for their active and fruitful collaborations.

My previous co-supervisor Laura Goobar-Larsson for her invaluable advises and open-heartedly scientific discussions at the beginning of my PhD career.

My collaborator and friend, Associate professor Ákos Végvári for your constant help and uplifting humor, as well as, all the scientific and non-scientific discussions. Thank you for your advice, support, and encouragement whenever needed.

Professor Stefan Höglund, for fruitful collaborations, support and kindness. Your collaboration has been an essential contribution to my work. I would like also to thank Anders Höglund for all help and assistance.

All previous and present members of our group, Mojgan, Yiping, Jin, Sung, Yuba, Ulf, Haifa, Masoud, Pia, Maria, my collaborators and others who have previously worked and still do in Tripep AB; Michael, Cattis, Jan, Åsa, Caroline for creating a wonderful and stimulating working environment and your friendly help. Specially, I am deeply thankful to Sung, Yuba, and Pia for all your help, Yiping for help with setting up the infectivity in Paper I and for being such a warm hearted and wonderful person, Mojgan for all fun on various occasions

and pub evenings during various conferences and for sharing your flat with me and the “Bandit” when we were in NY.

I am also thankful to all previous and present researchers and students at our old division (Clinical Virology) for sharing the pleasure of scientific and science-unrelated discussions and for being part of the big team. Specifically, thanks to those students with whom I had the pleasure to enjoy after work parties and other occasions. Thanks also to all new researchers and students who are now part of the big family – Clinical Microbiology.

I wish also to express my deep gratitude to all members of the routine lab at the old division of Clinical Virology, for their friendship and kindness and for generously sharing of equipments and reagents, as well as, nice coffee and lunch brakes. Marit’s excellent help with preparing all my solutions and reagents is also greatly appreciated.

I am also deeply thankful to Tripep AB for all help and the sponsorship.

Kerstin Lindholm, for her cheerful smile and a wonderful secretarial assistance of all kind of paper work.

My friends outside the lab, Joakim (“stora Banditen”) and Samer (“Osmanah”) for sharing so many memories during our time in undergraduate school at KI. I miss our get together party.

I am especially thankful to Menge, Fre and Eleni (Luli) for their constant support and encouragement. I am also thankful to all other friends and families in Ethiopia, in particular, Mekonnen and Eyassu for making my stay there so pleasant and memorable.

All my brothers, sisters and relatives in Sweden, Ethiopia, Canada and the USA , for their constant support and encouraging advice.

Last and the most, my Mom and late Dad who have been always encouraging me to pursue my higher education. Thank you for your Dua’a and support. Nabil and Jody, you are my sunshine and thank you for your unconditional love. I love you too! Jody, thanks for all your support, understanding and putting up with me during a period of hard and late work.

This work was supported by grants from the Swedish Medical Research Council (grant no. HIV-2006-050 and K2000-06X-09501-10B), Swedish International development Cooperation Agency, SIDA (grant no. 2006-0011786), European Commission (HPAW-2002-90005) and Tripep AB.

11 REFERENCES

1. De Clercq, E. (2005). Emerging anti-HIV drugs. Expert opinion on emerging drugs *10*, 241-273.
2. Groeschen, H.M. (2007). Novel HIV treatment approved. Am J Health Syst Pharm *64*, 1886.
3. Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A., and Saxon, A. (1981). Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med *305*, 1425-1431.
4. Hymes, K.B., Cheung, T., Greene, J.B., Prose, N.S., Marcus, A., Ballard, H., William, D.C., and Laubenstein, L.J. (1981). Kaposi's sarcoma in homosexual men-a report of eight cases. Lancet *2*, 598-600.
5. Dalgleish, A.G., Beverley, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature *312*, 763-767.
6. Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J.C., and Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature *312*, 767-768.
7. Montagnier, L., Chermann, JC., Barré-Sinoussi, F., Chamaret, S., Gruset, J., Nugeyre, MT., Rey, F., Dauguet, C., Axler-Blin, C., Brun-Vezinet, F., Rouzioux, C., Saimot, GA., Rozenbaum, W., Gluckman, JC., Klatzman, D., Vilmer, E., Griscelli, C., Foyer-Gazengel, C. and Brunet, JB. (1984). A new human T-lymphotropic retrovirus: Characterization and possible role in lymphadenopathy and acquired immune deficiency syndromes. In "Human T cell leukemia/lymphoma virus" (RC Gallo, ME. Essex and L. Gross, eds). Cold Spring Harbor Laboratory New York, 363-379.
8. Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., et al. (1984). Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science *224*, 500-503.
9. Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science *224*, 497-500.
10. Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M., and Oshiro, L.S. (1984). Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science *225*, 840-842.
11. Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., et al. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science *220*, 868-871.
12. Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.O., Laurent, A.G., Dauguet, C., Katlama, C., Rouzioux, C., et al. (1986). Isolation of a new human retrovirus from West African patients with AIDS. Science *233*, 343-346.
13. Coffin, J., Haase, A., Levy, J.A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., et al. (1986). Human immunodeficiency viruses. Science *232*, 697.
14. Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., et al. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature *397*, 436-441.
15. Nahmias, A.J., Weiss, J., Yao, X., Lee, F., Kodsi, R., Schanfield, M., Matthews, T., Bolognesi, D., Durack, D., Motulsky, A., et al. (1986). Evidence for human infection with an HTLV III/LAV-like virus in Central Africa, 1959. Lancet *1*, 1279-1280.
16. Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B.H., Wolinsky, S., and Bhattacharya, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. Science *288*, 1789-1796.
17. Leitner, T., and Albert, J. (1999). The molecular clock of HIV-1 unveiled through analysis of a known transmission history. Proc Natl Acad Sci U S A *96*, 10752-10757.

18. Lemey, P., Pybus, O.G., Wang, B., Saksena, N.K., Salemi, M., and Vandamme, A.M. (2003). Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci U S A *100*, 6588-6592.
19. UNAIDS (2006). Report on the Global AIDS epidemic.
20. MacNeil, A., Sarr, A.D., Sankale, J.L., Meloni, S.T., Mboup, S., and Kanki, P. (2007). Direct evidence of lower viral replication rates in vivo in human immunodeficiency virus type 2 (HIV-2) infection than in HIV-1 infection. J Virol *81*, 5325-5330.
21. Kanki, P.J., Travers, K.U., S, M.B., Hsieh, C.C., Marlink, R.G., Gueye, N.A., Siby, T., Thior, I., Hernandez-Avila, M., Sankale, J.L., et al. (1994). Slower heterosexual spread of HIV-2 than HIV-1. Lancet *343*, 943-946.
22. Rowland-Jones, S.L., and Whittle, H.C. (2007). Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? Nat Immunol *8*, 329-331.
23. Kinloch-de Loes, S., de Saussure, P., Saurat, J.H., Stalder, H., Hirschel, B., and Perrin, L.H. (1993). Symptomatic primary infection due to human immunodeficiency virus type 1: review of 31 cases. Clin Infect Dis *17*, 59-65.
24. Cooper, D.A., Gold, J., Maclean, P., Donovan, B., Finlayson, R., Barnes, T.G., Michelmore, H.M., Brooke, P., and Penny, R. (1985). Acute AIDS retrovirus infection. Definition of a clinical illness associated with seroconversion. Lancet *1*, 537-540.
25. Tindall, B., and Cooper, D.A. (1991). Primary HIV infection: host responses and intervention strategies. Aids *5*, 1-14.
26. Kahn, J.O., and Walker, B.D. (1998). Acute human immunodeficiency virus type 1 infection. N Engl J Med *339*, 33-39.
27. Little, S.J., McLean, A.R., Spina, C.A., Richman, D.D., and Havlir, D.V. (1999). Viral dynamics of acute HIV-1 infection. J Exp Med *190*, 841-850.
28. Lindback, S., Karlsson, A.C., Mittler, J., Blaxhult, A., Carlsson, M., Briheim, G., Sonnerborg, A., and Gaines, H. (2000). Viral dynamics in primary HIV-1 infection. Karolinska Institutet Primary HIV Infection Study Group. Aids *14*, 2283-2291.
29. Clark, S.J., Saag, M.S., Decker, W.D., Campbell-Hill, S., Roberson, J.L., Veldkamp, P.J., Kappes, J.C., Hahn, B.H., and Shaw, G.M. (1991). High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. N Engl J Med *324*, 954-960.
30. Daar, E.S., Moudgil, T., Meyer, R.D., and Ho, D.D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. N Engl J Med *324*, 961-964.
31. Pantaleo, G., Graziosi, C., and Fauci, A.S. (1993). New concepts in the immunopathogenesis of human immunodeficiency virus infection. N Engl J Med *328*, 327-335.
32. Hel, Z., McGhee, J.R., and Mestecky, J. (2006). HIV infection: first battle decides the war. Trends in immunology *27*, 274-281.
33. Pantaleo, G., Demarest, J.F., Schacker, T., Vaccarezza, M., Cohen, O.J., Daucher, M., Graziosi, C., Schnittman, S.S., Quinn, T.C., Shaw, G.M., et al. (1997). The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. Proc Natl Acad Sci U S A *94*, 254-258.
34. Lemey, P., Kosakovsky Pond, S.L., Drummond, A.J., Pybus, O.G., Shapiro, B., Barroso, H., Taveira, N., and Rambaut, A. (2007). Synonymous substitution rates predict HIV disease progression as a result of underlying replication dynamics. PLoS computational biology *3*, e29.
35. Yarchoan, R., Redfield, R.R., and Broder, S. (1986). Mechanisms of B cell activation in patients with acquired immunodeficiency syndrome and related disorders. Contribution of antibody-producing B cells, of Epstein-Barr virus-infected B cells, and of immunoglobulin production induced by human T cell lymphotropic virus, type III/lymphadenopathy-associated virus. J Clin Invest *78*, 439-447.
36. Vendrell, J.P., Segondy, M., Ducos, J., Reynes, J., Huguet, M.F., Nicolas, J.C., and Serre, A. (1991). Analysis of the spontaneous in vitro anti-HIV-1 antibody secretion by peripheral blood mononuclear cells in HIV-1 infection. Clin Exp Immunol *83*, 197-202.
37. Welch, K., and Morse, A. (2002). The clinical profile of end-stage AIDS in the era of highly active antiretroviral therapy. AIDS Patient Care STDS *16*, 75-81.
38. Bower, M., Palmieri, C., and Dhillon, T. (2006). AIDS-related malignancies: changing epidemiology and the impact of highly active antiretroviral therapy. Curr Opin Infect Dis *19*, 14-19.
39. Ozel, M., Pauli, G., and Gelderblom, H.R. (1988). The organization of the envelope projections on the surface of HIV. Arch Virol *100*, 255-266.

40. Hill, C.P., Worthylake, D., Bancroft, D.P., Christensen, A.M., and Sundquist, W.I. (1996). Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. Proc Natl Acad Sci U S A *93*, 3099-3104.
41. Wyatt, R., and Sodroski, J. (1998). The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science *280*, 1884-1888.
42. Bhattacharya, J., Repik, A., and Clapham, P.R. (2006). Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes. J Virol *80*, 5292-5300.
43. Wyma, D.J., Kotov, A., and Aiken, C. (2000). Evidence for a stable interaction of gp41 with Pr55(Gag) in immature human immunodeficiency virus type 1 particles. J Virol *74*, 9381-9387.
44. Resh, M.D. (2005). Intracellular trafficking of HIV-1 Gag: how Gag interacts with cell membranes and makes viral particles. AIDS Rev *7*, 84-91.
45. Zhou, W., Parent, L.J., Wills, J.W., and Resh, M.D. (1994). Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. J Virol *68*, 2556-2569.
46. Hoglund, S., Ofverstedt, L.G., Nilsson, A., Lundquist, P., Gelderblom, H., Ozel, M., and Skoglund, U. (1992). Spatial visualization of the maturing HIV-1 core and its linkage to the envelope. AIDS Res Hum Retroviruses *8*, 1-7.
47. Frankel, A.D., and Young, J.A. (1998). HIV-1: fifteen proteins and an RNA. Annu Rev Biochem *67*, 1-25.
48. Freed, E.O. (2001). HIV-1 replication. Somat Cell Mol Genet *26*, 13-33.
49. Hill, M., Tachedjian, G., and Mak, J. (2005). The packaging and maturation of the HIV-1 Pol proteins. Curr HIV Res *3*, 73-85.
50. Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., and Steitz, T.A. (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science *256*, 1783-1790.
51. Mulky, A., and Kappes, J.C. (2005). Analysis of human immunodeficiency virus type 1 reverse transcriptase subunit structure/function in the context of infectious virions and human target cells. Antimicrob Agents Chemother *49*, 3762-3769.
52. Seelamgari, A., Maddukuri, A., Berro, R., de la Fuente, C., Kehn, K., Deng, L., Dadgar, S., Bottazzi, M.E., Ghedin, E., Pumfery, A., et al. (2004). Role of viral regulatory and accessory proteins in HIV-1 replication. Front Biosci *9*, 2388-2413.
53. Yu, X.G., Lichterfeld, M., Addo, M.M., and Altfeld, M. (2005). Regulatory and accessory HIV-1 proteins: potential targets for HIV-1 vaccines? Curr Med Chem *12*, 741-747.
54. Liu, H., Wu, X., Newman, M., Shaw, G.M., Hahn, B.H., and Kappes, J.C. (1995). The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. J Virol *69*, 7630-7638.
55. Sherman, M.P., De Noronha, C.M., Williams, S.A., and Greene, W.C. (2002). Insights into the biology of HIV-1 viral protein R. DNA Cell Biol *21*, 679-688.
56. Cullen, B.R. (1998). HIV-1 auxiliary proteins: making connections in a dying cell. Cell *93*, 685-692.
57. Fittipaldi, A., and Giacca, M. (2005). Transcellular protein transduction using the Tat protein of HIV-1. Adv Drug Deliv Rev *57*, 597-608.
58. Nekhai, S., and Jeang, K.T. (2006). Transcriptional and post-transcriptional regulation of HIV-1 gene expression: role of cellular factors for Tat and Rev. Future microbiology *1*, 417-426.
59. Binette, J., and Cohen, E.A. (2004). Recent advances in the understanding of HIV-1 Vpu accessory protein functions. Curr Drug Targets Immune Endocr Metabol Disord *4*, 297-307.
60. Hernandez, L.D., Hoffman, L.R., Wolfsberg, T.G., and White, J.M. (1996). Virus-cell and cell-cell fusion. Annu Rev Cell Dev Biol *12*, 627-661.
61. Broder, C.C., and Collman, R.G. (1997). Chemokine receptors and HIV. J Leukoc Biol *62*, 20-29.
62. Doms, R.W., and Peiper, S.C. (1997). Unwelcomed guests with master keys: how HIV uses chemokine receptors for cellular entry. Virology *235*, 179-190.
63. Coakley, E., Petropoulos, C.J., and Whitcomb, J.M. (2005). Assessing chemokine co-receptor usage in HIV. Curr Opin Infect Dis *18*, 9-15.
64. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., et al. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell *85*, 1135-1148.

65. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., et al. (1996). Identification of a major co-receptor for primary isolates of HIV-1. Nature *381*, 661-666.
66. Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., et al. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature *381*, 667-673.
67. Berger, E.A. (1997). HIV entry and tropism: the chemokine receptor connection. Aids *11 Suppl A*, S3-16.
68. Schwartz, S., Felber, B.K., Fenyo, E.M., and Pavlakis, G.N. (1989). Rapidly and slowly replicating human immunodeficiency virus type 1 isolates can be distinguished according to target-cell tropism in T-cell and monocyte cell lines. Proc Natl Acad Sci U S A *86*, 7200-7203.
69. Moore, J.P. (1997). Coreceptors: implications for HIV pathogenesis and therapy. Science *276*, 51-52.
70. Doms, R.W., and Moore, J.P. (2000). HIV-1 membrane fusion: targets of opportunity. J Cell Biol *151*, F9-14.
71. Shu, W., Ji, H., and Lu, M. (2000). Interactions between HIV-1 gp41 core and detergents and their implications for membrane fusion. J Biol Chem *275*, 1839-1845.
72. Arhel, N.J., Souquere-Besse, S., Munier, S., Souque, P., Guadagnini, S., Rutherford, S., Prevost, M.C., Allen, T.D., and Charneau, P. (2007). HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore. Embo J *26*, 3025-3037.
73. Mulky, A., Sarafianos, S.G., Arnold, E., Wu, X., and Kappes, J.C. (2004). Subunit-specific analysis of the human immunodeficiency virus type 1 reverse transcriptase in vivo. J Virol *78*, 7089-7096.
74. Mansky, L.M., and Temin, H.M. (1995). Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol *69*, 5087-5094.
75. Preston, B.D., Poiesz, B.J., and Loeb, L.A. (1988). Fidelity of HIV-1 reverse transcriptase. Science *242*, 1168-1171.
76. Sherman, M.P., and Greene, W.C. (2002). Slipping through the door: HIV entry into the nucleus. Microbes Infect *4*, 67-73.
77. Sherman, M.P., de Noronha, C.M., Heusch, M.I., Greene, S., and Greene, W.C. (2001). Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. J Virol *75*, 1522-1532.
78. Rohr, O., Marban, C., Aunis, D., and Schaeffer, E. (2003). Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells. J Leukoc Biol *74*, 736-749.
79. Starcich, B., Ratner, L., Josephs, S.F., Okamoto, T., Gallo, R.C., and Wong-Staal, F. (1985). Characterization of long terminal repeat sequences of HTLV-III. Science *227*, 538-540.
80. Jeeninga, R.E., Hoogenkamp, M., Armand-Ugon, M., de Baar, M., Verhoef, K., and Berkhout, B. (2000). Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. J Virol *74*, 3740-3751.
81. Ott, D.E. (2002). Potential roles of cellular proteins in HIV-1. Rev Med Virol *12*, 359-374.
82. Schwartz, S., Felber, B.K., Benko, D.M., Fenyo, E.M., and Pavlakis, G.N. (1990). Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol *64*, 2519-2529.
83. Liang, C., and Wainberg, M.A. (2002). The role of Tat in HIV-1 replication: an activator and/or a suppressor? AIDS Rev *4*, 41-49.
84. Schneider, R., Campbell, M., Nasioulas, G., Felber, B.K., and Pavlakis, G.N. (1997). Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. J Virol *71*, 4892-4903.
85. Schwartz, S., Campbell, M., Nasioulas, G., Harrison, J., Felber, B.K., and Pavlakis, G.N. (1992). Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. J Virol *66*, 7176-7182.
86. Cullen, B.R. (2002). Using retroviruses to study the nuclear export of mRNA. Results Probl. Cell Differ. *35*, 151-168.
87. Cullen, B.R. (2003). Nuclear mRNA export: insights from virology. Trends Biochem Sci *28*, 419-424.
88. Elfgang, C., Rosorius, O., Hofer, L., Jaksche, H., Hauber, J., and Bevec, D. (1999). Evidence for specific nucleocytoplasmic transport pathways used by leucine-rich nuclear export signals. Proc Natl Acad Sci U S A *96*, 6229-6234.

89. Cmarko, D., Boe, S.O., Scassellati, C., Szilvay, A.M., Davanger, S., Fu, X.D., Haukenes, G., Kalland, K.H., and Fakan, S. (2002). Rev inhibition strongly affects intracellular distribution of human immunodeficiency virus type 1 RNAs. J Virol *76*, 10473-10484.
90. Kaplan, A.H., Manchester, M., and Swanstrom, R. (1994). The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. J Virol *68*, 6782-6786.
91. Finzi, A., Orthwein, A., Mercier, J., and Cohen, E.A. (2007). Productive human immunodeficiency virus type 1 assembly takes place at the plasma membrane. J Virol *81*, 7476-7490.
92. Lindwasser, O.W., and Resh, M.D. (2004). Human immunodeficiency virus type 1 Gag contains a dileucine-like motif that regulates association with multivesicular bodies. J Virol *78*, 6013-6023.
93. Goff, A., Ehrlich, L.S., Cohen, S.N., and Carter, C.A. (2003). Tsg101 control of human immunodeficiency virus type 1 Gag trafficking and release. J Virol *77*, 9173-9182.
94. Ono, A., Ablan, S.D., Lockett, S.J., Nagashima, K., and Freed, E.O. (2004). Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proc Natl Acad Sci U S A *101*, 14889-14894.
95. Dong, X., Li, H., Derdowski, A., Ding, L., Burnett, A., Chen, X., Peters, T.R., Dermody, T.S., Woodruff, E., Wang, J.J., et al. (2005). AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. Cell *120*, 663-674.
96. Alroy, I., Tuvia, S., Greener, T., Gordon, D., Barr, H.M., Taglicht, D., Mandil-Levin, R., Ben-Avraham, D., Konforty, D., Nir, A., et al. (2005). The trans-Golgi network-associated human ubiquitin-protein ligase POSH is essential for HIV type 1 production. Proc Natl Acad Sci U S A *102*, 1478-1483.
97. Pettit, S.C., Everitt, L.E., Choudhury, S., Dunn, B.M., and Kaplan, A.H. (2004). Initial cleavage of the human immunodeficiency virus type 1 GagPol precursor by its activated protease occurs by an intramolecular mechanism. J Virol *78*, 8477-8485.
98. Arthur, L.O., Bess, J.W., Jr., Sowder, R.C., 2nd, Benveniste, R.E., Mann, D.L., Chermann, J.C., and Henderson, L.E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science *258*, 1935-1938.
99. Benkirane, M., Blanc-Zouaoui, D., Hirn, M., and Devaux, C. (1994). Involvement of human leukocyte antigen class I molecules in human immunodeficiency virus infection of CD4-positive cells. J Virol *68*, 6332-6339.
100. Franke, E.K., Yuan, H.E., and Luban, J. (1994). Specific incorporation of cyclophilin A into HIV-1 virions. Nature *372*, 359-362.
101. Henderson, L.E., Sowder, R., Copeland, T.D., Oroszlan, S., Arthur, L.O., Robey, W.G., and Fischinger, P.J. (1987). Direct identification of class II histocompatibility DR proteins in preparations of human T-cell lymphotropic virus type III. J Virol *61*, 629-632.
102. Orentas, R.J., and Hildreth, J.E. (1993). Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. AIDS Res Hum Retroviruses *9*, 1157-1165.
103. Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., Cote, M., Rich, R.L., et al. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell *107*, 55-65.
104. Carlton, J.G., and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science *316*, 1908-1912.
105. Josephson, F., Albert, J., Flamholc, L., Gisslen, M., Karlstrom, O., Lindgren, S.R., Naver, L., Sandstrom, E., Svedhem-Johansson, V., Svennerholm, B., et al. (2007). Antiretroviral treatment of HIV infection: Swedish recommendations 2007. Scand. J. Infect. Dis. *39*, 486-507.
106. von Wyl, V., Yerly, S., Boni, J., Burgisser, P., Klimkait, T., Battegay, M., Furrer, H., Telenti, A., Hirschel, B., Vernazza, P.L., et al. (2007). Emergence of HIV-1 drug resistance in previously untreated patients initiating combination antiretroviral treatment: a comparison of different regimen types. Arch. Intern. Med. *167*, 1782-1790.
107. Briggs, J.A., Simon, M.N., Gross, I., Krausslich, H.G., Fuller, S.D., Vogt, V.M., and Johnson, M.C. (2004). The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol *11*, 672-675.
108. Huseby, D., Barklis, R.L., Alfadhli, A., and Barklis, E. (2005). Assembly of human immunodeficiency virus precursor gag proteins. J Biol Chem *280*, 17664-17670.
109. Spearman, P., and Ratner, L. (1996). Human immunodeficiency virus type 1 capsid formation in reticulocyte lysates. J Virol *70*, 8187-8194.

110. Morikawa, Y., Shibuya, M., Goto, T., and Sano, K. (2000). In vitro processing of human immunodeficiency virus type 1 Gag virus-like particles. Virology *272*, 366-374.
111. Wiegers, K., Rutter, G., Kottler, H., Tessmer, U., Hohenberg, H., and Krausslich, H.G. (1998). Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J Virol *72*, 2846-2854.
112. Pettit, S.C., Moody, M.D., Wehbie, R.S., Kaplan, A.H., Nantermet, P.V., Klein, C.A., and Swanstrom, R. (1994). The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J Virol *68*, 8017-8027.
113. von Schwedler, U.K., Stemmler, T.L., Klishko, V.Y., Li, S., Albertine, K.H., Davis, D.R., and Sundquist, W.I. (1998). Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. Embo J *17*, 1555-1568.
114. Briggs, J.A., Wilk, T., Welker, R., Krausslich, H.G., and Fuller, S.D. (2003). Structural organization of authentic, mature HIV-1 virions and cores. Embo J *22*, 1707-1715.
115. Scarlata, S., and Carter, C. (2003). Role of HIV-1 Gag domains in viral assembly. Biochim Biophys Acta *1614*, 62-72.
116. Morikawa, Y. (2003). HIV capsid assembly. Curr HIV Res *1*, 1-14.
117. Hearps, A.C., and Jans, D.A. (2007). Regulating the functions of the HIV-1 matrix protein. AIDS Res Hum Retroviruses *23*, 341-346.
118. Bukrinskaya, A. (2007). HIV-1 matrix protein: A mysterious regulator of the viral life cycle. Virus Res. *124*, 1-11.
119. Provitera, P., El-Maghrabi, R., and Scarlata, S. (2006). The effect of HIV-1 Gag myristoylation on membrane binding. Biophys. Chem. *119*, 23-32.
120. Bryant, M., and Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc Natl Acad Sci U S A *87*, 523-527.
121. Saad, J.S., Loeliger, E., Luncsford, P., Liriano, M., Tai, J., Kim, A., Miller, J., Joshi, A., Freed, E.O., and Summers, M.F. (2007). Point Mutations in the HIV-1 Matrix Protein Turn Off the Myristyl Switch. Journal of Molecular Biology *366*, 574-585.
122. Zhou, W., and Resh, M.D. (1996). Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. J Virol *70*, 8540-8548.
123. Ono, A., Orenstein, J.M., and Freed, E.O. (2000). Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. J Virol *74*, 2855-2866.
124. Morikawa, Y., Hockley, D.J., Nermut, M.V., and Jones, I.M. (2000). Roles of matrix, p2, and N-terminal myristoylation in human immunodeficiency virus type 1 Gag assembly. J Virol *74*, 16-23.
125. Morikawa, Y., Zhang, W.H., Hockley, D.J., Nermut, M.V., and Jones, I.M. (1998). Detection of a trimeric human immunodeficiency virus type 1 Gag intermediate is dependent on sequences in the matrix protein, p17. J Virol *72*, 7659-7663.
126. Datta, S.A.K., Zhao, Z., Clark, P.K., Tarasov, S., Alexandratos, J.N., Campbell, S.J., Kvaratskhelia, M., Lebowitz, J., and Rein, A. (2007). Interactions between HIV-1 Gag Molecules in Solution: An Inositol Phosphate-mediated Switch. Journal of Molecular Biology *365*, 799-811.
127. Alfadhli, A., Huseby, D., Kapit, E., Colman, D., and Barklis, E. (2007). Human immunodeficiency virus type 1 matrix protein assembles on membranes as a hexamer. J Virol *81*, 1472-1478.
128. Gallay, P., Swingler, S., Song, J., Bushman, F., and Trono, D. (1995). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell *83*, 569-576.
129. Haffar, O.K., Popov, S., Dubrovsky, L., Agostini, I., Tang, H., Pushkarsky, T., Nadler, S.G., and Bukrinsky, M. (2000). Two nuclear localization signals in the HIV-1 matrix protein regulate nuclear import of the HIV-1 pre-integration complex. J Mol Biol *299*, 359-368.
130. Depienne, C., Roques, P., Creminon, C., Fritsch, L., Casseron, R., Dormont, D., Dargemont, C., and Benichou, S. (2000). Cellular Distribution and Karyophilic Properties of Matrix, Integrase, and Vpr Proteins from the Human and Simian Immunodeficiency Viruses. Exp. Cell Res. *260*, 387-395.
131. Derdowski, A., Ding, L., and Spearman, P. (2004). A novel fluorescence resonance energy transfer assay demonstrates that the human immunodeficiency virus type 1 Pr55Gag I domain mediates Gag-Gag interactions. J Virol *78*, 1230-1242.

132. Sandefur, S., Smith, R.M., Varthakavi, V., and Spearman, P. (2000). Mapping and characterization of the N-terminal I domain of human immunodeficiency virus type 1 Pr55(Gag). J Virol *74*, 7238-7249.
133. Sandefur, S., Varthakavi, V., and Spearman, P. (1998). The I domain is required for efficient plasma membrane binding of human immunodeficiency virus type 1 Pr55Gag. J Virol *72*, 2723-2732.
134. Liu, H.W., Zeng, Y., Landes, C.F., Kim, Y.J., Zhu, Y., Ma, X., Vo, M.N., Musier-Forsyth, K., and Barbara, P.F. (2007). Insights on the role of nucleic acid/protein interactions in chaperoned nucleic acid rearrangements of HIV-1 reverse transcription. Proc Natl Acad Sci U S A *104*, 5261-5267.
135. Zeng, Y., Liu, H.W., Landes, C.F., Kim, Y.J., Ma, X., Zhu, Y., Musier-Forsyth, K., and Barbara, P.F. (2007). Probing nucleation, reverse annealing, and chaperone function along the reaction path of HIV-1 single-strand transfer. Proc Natl Acad Sci U S A *104*, 12651-12656.
136. Chien, A.I., Liao, W.H., Yang, D.M., and Wang, C.T. (2006). A domain directly C-terminal to the major homology region of human immunodeficiency type 1 capsid protein plays a crucial role in directing both virus assembly and incorporation of Gag-Pol. Virology *348*, 84-95.
137. Joshi, A., Nagashima, K., and Freed, E.O. (2006). Mutation of dileucine-like motifs in the human immunodeficiency virus type 1 capsid disrupts virus assembly, gag-gag interactions, gag-membrane binding, and virion maturation. J Virol *80*, 7939-7951.
138. Harrison, G.P., and Lever, A.M. (1992). The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. J Virol *66*, 4144-4153.
139. Berkowitz, R.D., Luban, J., and Goff, S.P. (1993). Specific binding of human immunodeficiency virus type 1 gag polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. J Virol *67*, 7190-7200.
140. Dannull, J., Surovoy, A., Jung, G., and Moelling, K. (1994). Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. Embo J *13*, 1525-1533.
141. Clever, J.L., Mirandar, D., Jr., and Parslow, T.G. (2002). RNA structure and packaging signals in the 5' leader region of the human immunodeficiency virus type 1 genome. J Virol *76*, 12381-12387.
142. Mark-Danieli, M., Laham, N., Kenan-Eichler, M., Castiel, A., Melamed, D., Landau, M., Bouvier, N.M., Evans, M.J., and Bacharach, E. (2005). Single point mutations in the zinc finger motifs of the human immunodeficiency virus type 1 nucleocapsid alter RNA binding specificities of the gag protein and enhance packaging and infectivity. J Virol *79*, 7756-7767.
143. Amarasinghe, G.K., Zhou, J., Miskimon, M., Chancellor, K.J., McDonald, J.A., Matthews, A.G., Miller, R.R., Rouse, M.D., and Summers, M.F. (2001). Stem-loop SL4 of the HIV-1 psi RNA packaging signal exhibits weak affinity for the nucleocapsid protein. structural studies and implications for genome recognition. J Mol Biol *314*, 961-970.
144. Rulli, S.J., Jr., Hibbert, C.S., Mirro, J., Pederson, T., Biswal, S., and Rein, A. (2007). Selective and nonselective packaging of cellular RNAs in retrovirus particles. J Virol *81*, 6623-6631.
145. Lecossier, D., Bouchonnet, F., Clavel, F., and Hance, A.J. (2003). Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science *300*, 1112.
146. Luo, K., Liu, B., Xiao, Z., Yu, Y., Yu, X., Gorelick, R., and Yu, X.F. (2004). Amino-terminal region of the human immunodeficiency virus type 1 nucleocapsid is required for human APOBEC3G packaging. J Virol *78*, 11841-11852.
147. Schafer, A., Bogerd, H.P., and Cullen, B.R. (2004). Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. Virology *328*, 163-168.
148. Schrofelbauer, B., Yu, Q., and Landau, N.R. (2004). New insights into the role of Vif in HIV-1 replication. AIDS Rev *6*, 34-39.
149. Wiegand, H.L., Doehle, B.P., Bogerd, H.P., and Cullen, B.R. (2004). A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. Embo J *23*, 2451-2458.
150. Santa-Marta, M., da Silva, F.A., Fonseca, A.M., and Goncalves, J. (2005). HIV-1 Vif can directly inhibit apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G-mediated cytidine deamination by using a single amino acid interaction and without protein degradation. J Biol Chem *280*, 8765-8775.

151. Huang, M., Orenstein, J.M., Martin, M.A., and Freed, E.O. (1995). p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J Virol *69*, 6810-6818.
152. Demirov, D.G., and Freed, E.O. (2004). Retrovirus budding. Virus Res. *106*, 87-102.
153. VerPlank, L., Bouamr, F., LaGrassa, T.J., Agresta, B., Kikonyogo, A., Leis, J., and Carter, C.A. (2001). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). Proc Natl Acad Sci U S A *98*, 7724-7729.
154. Demirov, D.G., Ono, A., Orenstein, J.M., and Freed, E.O. (2002). Overexpression of the N-terminal domain of TSG101 inhibits HIV-1 budding by blocking late domain function. Proc Natl Acad Sci U S A *99*, 955-960.
155. Li, F., Chen, C., Puffer, B.A., and Montelaro, R.C. (2002). Functional replacement and positional dependence of homologous and heterologous L domains in equine infectious anemia virus replication. J Virol *76*, 1569-1577.
156. Yuan, B., Campbell, S., Bacharach, E., Rein, A., and Goff, S.P. (2000). Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. J Virol *74*, 7250-7260.
157. Freed, E.O. (2002). Viral late domains. J Virol *76*, 4679-4687.
158. Hurley, J.H., and Emr, S.D. (2006). The ESCRT complexes: structure and mechanism of a membrane-trafficking network. Annu. Rev. Biophys. Biomol. Struct. *35*, 277-298.
159. Bishop, N., and Woodman, P. (2001). TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes. J Biol Chem *276*, 11735-11742.
160. Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145-155.
161. Alam, S.L., and Sundquist, W.I. (2007). Structural biology: ESCRT service. Nature *447*, 921-922.
162. von Schwedler, U.K., Stuchell, M., Muller, B., Ward, D.M., Chung, H.-Y., Morita, E., Wang, H.E., Davis, T., He, G.-P., Cimbora, D.M., et al. (2003). The Protein Network of HIV Budding. Cell *114*, 701-713.
163. Vogt, V.M. (2000). Ubiquitin in retrovirus assembly: actor or bystander? Proc Natl Acad Sci U S A *97*, 12945-12947.
164. Gottwein, E., and Krausslich, H.G. (2005). Analysis of human immunodeficiency virus type 1 Gag ubiquitination. J Virol *79*, 9134-9144.
165. Martin-Serrano, J., Yarovoy, A., Perez-Caballero, D., and Bieniasz, P.D. (2003). Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. Proc Natl Acad Sci U S A *100*, 12414-12419.
166. Eastman, S.W., Martin-Serrano, J., Chung, W., Zang, T., and Bieniasz, P.D. (2005). Identification of human VPS37C, a component of endosomal sorting complex required for transport-I important for viral budding. J Biol Chem *280*, 628-636.
167. von Schwedler, U.K., Stuchell, M., Muller, B., Ward, D.M., Chung, H.Y., Morita, E., Wang, H.E., Davis, T., He, G.P., Cimbora, D.M., et al. (2003). The protein network of HIV budding. Cell *114*, 701-713.
168. Bieniasz, P.D. (2006). Late budding domains and host proteins in enveloped virus release. Virology *344*, 55-63.
169. Checroune, F., Yao, X.J., Gottlinger, H.G., Bergeron, D., and Cohen, E.A. (1995). Incorporation of Vpr into human immunodeficiency virus type 1: role of conserved regions within the P6 domain of Pr55gag. J Acquir Immune Defic Syndr Hum Retrovirol *10*, 1-7.
170. Le Rouzic, E., and Benichou, S. (2005). The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology *2*, 11.
171. Sheehy, A.M., Gaddis, N.C., Choi, J.D., and Malim, M.H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature *418*, 646-650.
172. Briggs, J.A.G., Grunewald, K., Glass, B., Forster, F., Krausslich, H.-G., and Fuller, S.D. (2006). The Mechanism of HIV-1 Core Assembly: Insights from Three-Dimensional Reconstructions of Authentic Virions. Structure *14*, 15-20.
173. Berthet-Colominas, C., Monaco, S., Novelli, A., Sibai, G., Mallet, F., and Cusack, S. (1999). Head-to-tail dimers and interdomain flexibility revealed by the crystal structure of HIV-1 capsid protein (p24) complexed with a monoclonal antibody Fab. Embo J *18*, 1124-1136.
174. Gitti, R.K., Lee, B.M., Walker, J., Summers, M.F., Yoo, S., and Sundquist, W.I. (1996). Structure of the amino-terminal core domain of the HIV-1 capsid protein. Science *273*, 231-235.

175. Douglas, C.C., Thomas, D., Lanman, J., and Prevelige, P.E., Jr. (2004). Investigation of N-terminal domain charged residues on the assembly and stability of HIV-1 CA. Biochemistry *43*, 10435-10441.
176. Fitzon, T., Leschonsky, B., Bieler, K., Paulus, C., Schroder, J., Wolf, H., and Wagner, R. (2000). Proline residues in the HIV-1 NH2-terminal capsid domain: structure determinants for proper core assembly and subsequent steps of early replication. Virology *268*, 294-307.
177. Tang, S., Murakami, T., Agresta, B.E., Campbell, S., Freed, E.O., and Levin, J.G. (2001). Human immunodeficiency virus type 1 N-terminal capsid mutants that exhibit aberrant core morphology and are blocked in initiation of reverse transcription in infected cells. J Virol *75*, 9357-9366.
178. Gamble, T.R., Vajdos, F.F., Yoo, S., Worthylake, D.K., Houseweart, M., Sundquist, W.I., and Hill, C.P. (1996). Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell *87*, 1285-1294.
179. Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., and Goff, S.P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell *73*, 1067-1078.
180. Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C.T., Sodroski, J., and Gottlinger, H.G. (1994). Functional association of cyclophilin A with HIV-1 virions. Nature *372*, 363-365.
181. Gamble, T.R., Yoo, S., Vajdos, F.F., von Schwedler, U.K., Worthylake, D.K., Wang, H., McCutcheon, J.P., Sundquist, W.I., and Hill, C.P. (1997). Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. Science *278*, 849-853.
182. Worthylake, D.K., Wang, H., Yoo, S., Sundquist, W.I., and Hill, C.P. (1999). Structures of the HIV-1 capsid protein dimerization domain at 2.6 A resolution. Acta Crystallogr D Biol Crystallogr *55 (Pt 1)*, 85-92.
183. Momany, C., Kovari, L.C., Prongay, A.J., Keller, W., Gitti, R.K., Lee, B.M., Gorbalenya, A.E., Tong, L., McClure, J., Ehrlich, L.S., et al. (1996). Crystal structure of dimeric HIV-1 capsid protein. Nat Struct Biol *3*, 763-770.
184. Huang, M., and Martin, M.A. (1997). Incorporation of Pr160(gag-pol) into virus particles requires the presence of both the major homology region and adjacent C-terminal capsid sequences within the Gag-Pol polyprotein. J Virol *71*, 4472-4478.
185. Burniston, M.T., Cimarelli, A., Colgan, J., Curtis, S.P., and Luban, J. (1999). Human immunodeficiency virus type 1 Gag polyprotein multimerization requires the nucleocapsid domain and RNA and is promoted by the capsid-dimer interface and the basic region of matrix protein. J Virol *73*, 8527-8540.
186. Ono, A., Demirov, D., and Freed, E.O. (2000). Relationship between human immunodeficiency virus type 1 Gag multimerization and membrane binding. J Virol *74*, 5142-5150.
187. Li, S., Hill, C.P., Sundquist, W.I., and Finch, J.T. (2000). Image reconstructions of helical assemblies of the HIV-1 CA protein. Nature *407*, 409-413.
188. Fuller, S.D., Wilk, T., Gowen, B.E., Krausslich, H.G., and Vogt, V.M. (1997). Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. Curr Biol *7*, 729-738.
189. Wilk, T., Gross, I., Gowen, B.E., Rutten, T., de Haas, F., Welker, R., Krausslich, H.G., Boulanger, P., and Fuller, S.D. (2001). Organization of immature human immunodeficiency virus type 1. J Virol *75*, 759-771.
190. Benjamin, J., Ganser-Pornillos, B.K., Tivol, W.F., Sundquist, W.I., and Jensen, G.J. (2005). Three-dimensional structure of HIV-1 virus-like particles by electron cryotomography. J Mol Biol *346*, 577-588.
191. Ganser-Pornillos, B.K., von Schwedler, U.K., Stray, K.M., Aiken, C., and Sundquist, W.I. (2004). Assembly properties of the human immunodeficiency virus type 1 CA protein. J Virol *78*, 2545-2552.
192. Yeager, M., Wilson-Kubalek, E.M., Weiner, S.G., Brown, P.O., and Rein, A. (1998). Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms. Proc Natl Acad Sci U S A *95*, 7299-7304.
193. Lanman, J., Sexton, J., Sakalian, M., and Prevelige, P.E., Jr. (2002). Kinetic analysis of the role of intersubunit interactions in human immunodeficiency virus type 1 capsid protein assembly in vitro. J Virol *76*, 6900-6908.
194. Lanman, J., Lam, T.T., Barnes, S., Sakalian, M., Emmett, M.R., Marshall, A.G., and Prevelige, P.E., Jr. (2003). Identification of novel interactions in HIV-1 capsid protein assembly by high-resolution mass spectrometry. J Mol Biol *325*, 759-772.
195. von Schwedler, U.K., Stray, K.M., Garrus, J.E., and Sundquist, W.I. (2003). Functional surfaces of the human immunodeficiency virus type 1 capsid protein. J Virol *77*, 5439-5450.

196. Ganser, B.K., Li, S., Klishko, V.Y., Finch, J.T., and Sundquist, W.I. (1999). Assembly and analysis of conical models for the HIV-1 core. Science *283*, 80-83.
197. Massiah, M.A., Starich, M.R., Paschall, C., Summers, M.F., Christensen, A.M., and Sundquist, W.I. (1994). Three-dimensional Structure of the Human Immunodeficiency Virus Type 1 Matrix Protein. Journal of Molecular Biology *244*, 198-223.
198. DeLano, W.L. (2002). The PyMOL Molecular Graphics System.
199. Chang, Y.-F., Wang, S.-M., Huang, K.-J., and Wang, C.-T. (2007). Mutations in Capsid Major Homology Region Affect Assembly and Membrane Affinity of HIV-1 Gag. Journal of Molecular Biology *370*, 585-597.
200. Dorfman, T., Bukovsky, A., Ohagen, A., Hoglund, S., and Gottlinger, H.G. (1994). Functional domains of the capsid protein of human immunodeficiency virus type 1. J Virol *68*, 8180-8187.
201. Mammano, F., Ohagen, A., Hoglund, S., and Gottlinger, H.G. (1994). Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J Virol *68*, 4927-4936.
202. Reicin, A.S., Paik, S., Berkowitz, R.D., Luban, J., Lowy, I., and Goff, S.P. (1995). Linker insertion mutations in the human immunodeficiency virus type 1 gag gene: effects on virion particle assembly, release, and infectivity. J Virol *69*, 642-650.
203. Chiu, H.C., Wang, F.D., Yao, S.Y., and Wang, C.T. (2002). Effects of gag mutations on human immunodeficiency virus type 1 particle assembly, processing, and cyclophilin A incorporation. J Med Virol *68*, 156-163.
204. Hatziioannou, T., Cowan, S., Von Schwedler, U.K., Sundquist, W.I., and Bieniasz, P.D. (2004). Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid. J Virol *78*, 6005-6012.
205. Hatziioannou, T., Perez-Caballero, D., Cowan, S., and Bieniasz, P.D. (2005). Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. J Virol *79*, 176-183.
206. Li, F., Goila-Gaur, R., Salzwedel, K., Kilgore, N.R., Reddick, M., Matallana, C., Castillo, A., Zoumplis, D., Martin, D.E., Orenstein, J.M., et al. (2003). PA-457: a potent HIV inhibitor that disrupts core condensation by targeting a late step in Gag processing. Proc Natl Acad Sci U S A *100*, 13555-13560.
207. Sticht, J., Humbert, M., Findlow, S., Bodem, J., Muller, B., Dietrich, U., Werner, J., and Krausslich, H.G. (2005). A peptide inhibitor of HIV-1 assembly in vitro. Nat Struct Mol Biol *12*, 671-677.
208. Tang, C., Loeliger, E., Kinde, I., Kyere, S., Mayo, K., Barklis, E., Sun, Y., Huang, M., and Summers, M.F. (2003). Antiviral inhibition of the HIV-1 capsid protein. J Mol Biol *327*, 1013-1020.
209. Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature *427*, 848-853.
210. Towers, G.J. (2007). The control of viral infection by tripartite motif proteins and cyclophilin A. Retrovirology *4*, 40.
211. Sebastian, S., and Luban, J. (2005). TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. Retrovirology *2*, 40.
212. Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I., and Sodroski, J. (2006). Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. Proc Natl Acad Sci U S A *103*, 5514-5519.
213. Mische, C.C., Javanbakht, H., Song, B., Diaz-Griffero, F., Stremlau, M., Strack, B., Si, Z., and Sodroski, J. (2005). Retroviral restriction factor TRIM5alpha is a trimer. J Virol *79*, 14446-14450.
214. Keckesova, Z., Ylinen, L.M., and Towers, G.J. (2004). The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. Proc Natl Acad Sci U S A *101*, 10780-10785.
215. Hatziioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., and Bieniasz, P.D. (2004). Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. Proc Natl Acad Sci U S A *101*, 10774-10779.
216. Perron, M.J., Stremlau, M., Song, B., Ulm, W., Mulligan, R.C., and Sodroski, J. (2004). TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc Natl Acad Sci U S A *101*, 11827-11832.

217. Yap, M.W., Nisole, S., Lynch, C., and Stoye, J.P. (2004). Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc Natl Acad Sci U S A *101*, 10786-10791.
218. Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. J Virol *79*, 3930-3937.
219. Ylinen, L.M., Keckesova, Z., Wilson, S.J., Ranasinghe, S., and Towers, G.J. (2005). Differential restriction of human immunodeficiency virus type 2 and simian immunodeficiency virus SIVmac by TRIM5alpha alleles. J Virol *79*, 11580-11587.
220. Fischer, G., Tradler, T., and Zarnt, T. (1998). The mode of action of peptidyl prolyl cis/trans isomerases in vivo: binding vs. catalysis. FEBS Lett. *426*, 17-20.
221. Bosco, D.A., Eisenmesser, E.Z., Pochapsky, S., Sundquist, W.I., and Kern, D. (2002). Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A. Proc Natl Acad Sci U S A *99*, 5247-5252.
222. Braaten, D., Franke, E.K., and Luban, J. (1996). Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. J Virol *70*, 3551-3560.
223. Braaten, D., and Luban, J. (2001). Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. Embo J *20*, 1300-1309.
224. Sokolskaja, E., Sayah, D.M., and Luban, J. (2004). Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. J Virol *78*, 12800-12808.
225. Besnier, C., Takeuchi, Y., and Towers, G. (2002). Restriction of lentivirus in monkeys. Proc Natl Acad Sci U S A *99*, 11920-11925.
226. Hatziioannou, T., Cowan, S., Goff, S.P., Bieniasz, P.D., and Towers, G.J. (2003). Restriction of multiple divergent retroviruses by Lv1 and Ref1. Embo J *22*, 385-394.
227. Himathongkham, S., and Luciw, P.A. (1996). Restriction of HIV-1 (subtype B) replication at the entry step in rhesus macaque cells. Virology *219*, 485-488.
228. Hofmann, W., Schubert, D., LaBonte, J., Munson, L., Gibson, S., Scammell, J., Ferrigno, P., and Sodroski, J. (1999). Species-specific, postentry barriers to primate immunodeficiency virus infection. J Virol *73*, 10020-10028.
229. Cowan, S., Hatziioannou, T., Cunningham, T., Muesing, M.A., Gottlinger, H.G., and Bieniasz, P.D. (2002). Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. Proc Natl Acad Sci U S A *99*, 11914-11919.
230. Shibata, R., Sakai, H., Kawamura, M., Tokunaga, K., and Adachi, A. (1995). Early replication block of human immunodeficiency virus type 1 in monkey cells. J Gen Virol *76 (Pt 11)*, 2723-2730.
231. Towers, G.J., Hatziioannou, T., Cowan, S., Goff, S.P., Luban, J., and Bieniasz, P.D. (2003). Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat Med *9*, 1138-1143.
232. Kattenbeck, B., von Poblotzki, A., Rohrhofer, A., Wolf, H., and Modrow, S. (1997). Inhibition of human immunodeficiency virus type 1 particle formation by alterations of defined amino acids within the C terminus of the capsid protein. J Gen Virol *78 (Pt 10)*, 2489-2496.
233. von Poblotzki, A., Wagner, R., Niedrig, M., Wanner, G., Wolf, H., and Modrow, S. (1993). Identification of a region in the Pr55gag-polyprotein essential for HIV-1 particle formation. Virology *193*, 981-985.
234. Zhang, W.H., Hockley, D.J., Nermut, M.V., Morikawa, Y., and Jones, I.M. (1996). Gag-Gag interactions in the C-terminal domain of human immunodeficiency virus type 1 p24 capsid antigen are essential for Gag particle assembly. J Gen Virol *77 (Pt 4)*, 743-751.
235. Coffin, J.M. (1992). Genetic diversity and evolution of retroviruses. Curr Top Microbiol Immunol *176*, 143-164.
236. Andersson, E., Horal, P., Jejcic, A., Hoglund, S., Balzarini, J., Vahlne, A., and Svennerholm, B. (2005). Glycine-amide is an active metabolite of the antiretroviral tripeptide glycyl-prolyl-glycine-amide. Antimicrob Agents Chemother *49*, 40-44.
237. Andersson, E., Horal, P., Vahlne, A., and Svennerholm, B. (2004). No cross-resistance or selection of HIV-1 resistant mutants in vitro to the antiretroviral tripeptide glycyl-prolyl-glycine-amide. Antiviral Res. *61*, 119-124.
238. Hoglund, S., Su, J., Reneby, S.S., Vegvari, A., Hjerten, S., Sintorn, I.M., Foster, H., Wu, Y.P., Nystrom, I., and Vahlne, A. (2002). Tripeptide interference with human immunodeficiency virus type 1 morphogenesis. Antimicrob Agents Chemother *46*, 3597-3605.

239. Su, J., Andersson, E., Horal, P., Naghavi, M.H., Palm, A., Wu, Y.P., Eriksson, K., Jansson, M., Wigzell, H., Svennerholm, B., et al. (2001). The nontoxic tripeptide glycyl-prolyl-glycine amide inhibits the replication of human immunodeficiency virus type 1. J Hum Virol *4*, 1-7.
240. Su, J., Naghavi, M.H., Jejcic, A., Horal, P., Furuta, Y., Wu, Y.P., Li, S.L., Hall, W.W., Goobar-Larsson, L., Svennerholm, B., et al. (2001). The tripeptide glycyl-prolyl-glycine amide does not affect the early steps of the human immunodeficiency virus type 1 replication. J Hum Virol *4*, 8-15.
241. Horal, P., Hall, W.W., Svennerholm, B., Lycke, J., Jeansson, S., Rymo, L., Kaplan, M.H., and Vahlne, A. (1991). Identification of type-specific linear epitopes in the glycoproteins gp46 and gp21 of human T-cell leukemia viruses type I and type II using synthetic peptides. Proc Natl Acad Sci U S A *88*, 5754-5758.
242. Jacque, J.M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. Nature *418*, 435-438.
243. Ou, C.Y., Kwok, S., Mitchell, S.W., Mack, D.H., Sninsky, J.J., Krebs, J.W., Feorino, P., Warfield, D., and Schochetman, G. (1988). DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science *239*, 295-297.
244. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science *230*, 1350-1354.
245. Guyader, M., Kiyokawa, E., Abrami, L., Turelli, P., and Trono, D. (2002). Role for Human Immunodeficiency Virus Type 1 Membrane Cholesterol in Viral Internalization. J. Virol. *76*, 10356-10364.
246. Wei, X., Decker, J.M., Liu, H., Zhang, Z., Arani, R.B., Kilby, J.M., Saag, M.S., Wu, X., Shaw, G.M., and Kappes, J.C. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother *46*, 1896-1905.
247. Tang, C., Ndassa, Y., and Summers, M.F. (2002). Structure of the N-terminal 283-residue fragment of the immature HIV-1 Gag polyprotein. Nat Struct Biol *9*, 537-543.
248. Bouamr, F., Cornilescu, C.C., Goff, S.P., Tjandra, N., and Carter, C.A. (2005). Structural and dynamics studies of the D54A mutant of human T cell leukemia virus-1 capsid protein. J Biol Chem *280*, 6792-6801.
249. Mortuza, G.B., Haire, L.F., Stevens, A., Smerdon, S.J., Stoye, J.P., and Taylor, I.A. (2004). High-resolution structure of a retroviral capsid hexameric amino-terminal domain. Nature *431*, 481-485.
250. Leschonsky, B., Ludwig, C., Bieler, K., and Wagner, R. (2007). Capsid stability and replication of human immunodeficiency virus type 1 are influenced critically by charge and size of Gag residue 183. J Gen Virol *88*, 207-216.
251. Sobolev, V., Sorokine, A., Prilusky, J., Abola, E.E., and Edelman, M. (1999). Automated analysis of interatomic contacts in proteins. Bioinformatics *15*, 327-332.

12 APPENDIX (ARTICLES I-IV)