

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

**ALTERING HIV-1 ENVELOPE
GLYCOPROTEIN MATURATION
AND
ITS EFFECTS ON VIRAL
INFECTIVITY**

Alenka Jejcic



**Karolinska
Institutet**

Stockholm 2011

Cover picture: Electron micrograph of an HIV infected cell and released viral particles.
Professor Stefan Höglund took the photo.

All previously published papers were reproduced with permission from the publisher

Published by Karolinska Institutet. Printed by [Larserics Digital Print AB]

© Alenka Jecic, 2011
ISBN 978-91-7457-516-3

To honesty, integrity, love and tolerance



To all the victims of HIV

ABSTRACT

HIV-1 is dependent on its envelope glycoprotein (Env) to initiate infection. Env binds to cellular receptors and mediate the following fusion of the viral envelope with the cell plasma membrane. In an attempt to inhibit these events the tri-peptide glycyl-prolyl-glycine amide (GPG-NH₂) was designed to block the interaction of Env with its secondary co-receptor. Although the GPG-NH₂ was shown to have antiviral properties, its mode of action was found to be other than the intended. It was observed that GPG-NH₂ acted late in the viral replication cycle and that it affected the cellular expression of Env, but its antiviral mechanism remained unclear. Therefore, the main objectives of this thesis were:

1) To elucidate the effect of GPG-NH₂ on Env and determine if this affected virus infectivity. **2)** To examine if the antiviral mechanism and the specific effect on Env was owing to GPG-NH₂ or its metabolites G-NH₂ or α HGA. **3)** To examine the regulatory importance of the native Env signal sequence for cellular Env expression, viral particle incorporation of Env and viral replication.

In this thesis it is shown that treatment of HIV-1 infected cells with GPG-NH₂ results in production of viral particles with dramatically reduced infectivity. This is in part a consequence of reduced viral incorporation of Env, which disables the viral entry into cells. The mechanism was uncovered by examining Env expression in GPG-NH₂ treated cells, which revealed a significant reduction in Env steady-state levels and its processing to gp120/gp41 but also a decrease in its molecular mass as a result of glycan removal. Taken together the results show that GPG-NH₂ impairs Env maturation, which targets it for endoplasmic reticulum-associated protein degradation (ERAD), where Env is deglycosylated en route to its destruction. This effect of GPG-NH₂ was further shown to be a result of its metabolizing via the intermediate G-NH₂ into the active metabolite α HGA, by enzymes in the fetal bovine serum (FBS) added to the cell culture medium. It was further shown that in the presence of human serum or in the absence of any serum only the final metabolite α HGA was capable of directing Env for destruction. These observed effects were all found to be dependent on the native Env signal sequence and the proteasome.

The 30 residue long Env signal sequence of the precursor Env, gp160, targets it for co-translational translocation into the endoplasmic reticulum (ER). We found that the ER targeting function of the signal sequence was remarkably tolerant to large N-terminal truncations. Its first 8 N-terminal residues were entirely dispensable for adequate gp160 expression levels. However, they provide the signal sequence with regulatory functions detected first when examining the viral particles. The wild type virus incorporated ~80 % more of the precursor gp160 and 20 % less of its processed form, gp120/gp41, compared to the 8 residue truncated signal sequence virus. By promoting viral incorporation of the inactive precursor gp160 over the fusogenic gp120/gp41 the wt signal sequence down regulate the viral particle infectivity by ~40 %. This indicates that the signal sequence may have post ER targeting functions that permit significant amounts of gp160 trafficking through Golgi without being processed and become incorporated into the viral particles. Interestingly, the intra cellular capsid protein levels were initially lower and the viral particle release was initiated later in the presence of the native Env signal sequence than in its absence or in the presence of truncated Env signal sequences.

In conclusion these data illustrate that changes in the viral particle Env content and composition has a profound effect on the HIV-1 infectivity, which can be achieved by targeting selective steps in its biosynthesis and that small molecules may be utilized therapeutically to target unwanted pathogenic proteins for degradation by the existing cellular machinery.

Keywords: HIV-1, Env, gp160, gp120, gp41, signal sequence, ERAD, GPG-NH₂, G-NH₂, α HGA

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

- I. Alenka Jejcic, Robert Daniels, Laura Goobar-Larsson, Daniel N. Hebert, Anders Vahlne. **Small Molecule Targets Env for Endoplasmic Reticulum-Associated Protein Degradation and Inhibits Human Immunodeficiency Virus Type 1 propagation.** *Journal of Virology*, 2009 Oct; 83(19):10075-84. Epub 2009 Jul 29.
- II. Alenka Jejcic, Stefan Höglund, Anders Vahlne. **GPG-NH₂ acts via the metabolite α HGA to target HIV-1 Env to the ER- associated protein degradation pathway.** *Retrovirology*. 2010 Mar 15;7:20.
- III. Alenka Jejcic and Anders Vahlne. **Unexpected Effects of gp160 Signal Sequence truncations on HIV-1 replication.** *Manuscript*.

CONTENTS

| | |
|--|-----------|
| INTRODUCTION | 1 |
| THE HIV PREVALENCE IN THE WORLD..... | 1 |
| THE DISCOVERY OF HIV | 2 |
| THE ORIGIN OF HIV | 2 |
| TRANSMISSION AND PATHOGENESIS..... | 3 |
| THE HIV-1 VIRUS | 4 |
| CLASSIFICATION..... | 4 |
| THE HIV GROUPS AND SUBTYPES | 4 |
| HIV-1 GENOME AND PROTEINS..... | 5 |
| HIV-1 PARTICLE STRUCTURE..... | 6 |
| HIV-1 REPLICATION CYCLE | 7 |
| THE HIV-1 ENVELOPE GLYCOPROTEIN..... | 11 |
| BIOSYNTHESIS | 11 |
| VIRAL ENV INCORPORATION | 11 |
| ENDOPLASMIC RETICULUM | 13 |
| ER QUALITY CONTROL | 13 |
| ER-ASSOCIATED DEGRADATION (ERAD)..... | 14 |
| GPG-NH ₂ , GPG-NH ₂ AND α HGA | 15 |
| AIMS OF THIS THESIS..... | 16 |
| MATERIAL AND METHODS | 17 |
| CELL LINES | 17 |
| PLASMIDS AND CLONING | 17 |
| TRANSFECTION AND DRUG TREATMENTS..... | 19 |
| VIRUS PRODUCTION AND VIRUS PRECIPITATION..... | 19 |
| INFECTIVITY ASSAY | 19 |
| SYNCYTIA FORMATION ASSAY | 20 |
| ENZYME-LINKED IMMUNOSORBENT ASSAY | 20 |
| UPR AND ENZYMATIC DEGLYCOSYLATION | 20 |
| WESTERN BLOT AND GLYCOPROTEIN BLOT..... | 20 |
| SUBCELLULAR FRACTIONATION AND ALKALINE EXTRACTION | 21 |
| RESULTS | 22 |
| PAPER I..... | 22 |

| | |
|--|-----------|
| PAPER II | 28 |
| PAPER III | 31 |
| DISSCUSSION | 37 |
| CONCLUDING REMARKS AND FUTURE PERSPECTIVES..... | 42 |
| POPULÄRVETENSKAPLIG SAMMANFATTNING..... | 44 |
| ACKNOWLEDGEMENTS..... | 47 |
| REFERENCES | 49 |

LIST OF ABBREVIATIONS

| | |
|---------------------|--|
| aa | Amino acids |
| α HGA | alfa hydroxy-glycine amide |
| AIDS | Acquired Immunodeficiency Syndrome |
| bp | Base pares |
| CA | Capsid protein, p24 |
| CAT | Chloramphenicol acetyltransferase |
| DNA | Deoxyribonucleic acid |
| ds | Double stranded |
| EM | Electron microscopy |
| <i>env</i> | HIV-1 envelope glycoprotein gene |
| Env | HIV-1 envelope glycoprotein |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum associated protein degradation |
| FBS | Fetal bovine serum |
| G-NH ₂ | Glycine amide |
| <i>gag</i> | Group associated gene |
| GPG-OH | Glycyl-prolyl-glycine |
| G-NH ₂ | Glycine amide |
| GPG-NH ₂ | Glycyl-prolyl-glycine amide |
| gp160 | Glycoprotein 160 (precursor protein) |
| gp120 | Glycoprotein 120 (surface protein) |
| gp41 | Glycoprotein 41 (transmembrane protein) |
| HIV-1 | Human immunodeficiency virus type 1 |
| HIV-2 | Human immunodeficiency virus type 2 |
| HS | Human serum |
| IN | Integrase |
| kDa | kilo Dalton |
| LAMP-1 | Lysosom associated membrane protein type 1 |
| LTR | Long terminal repeat |
| MA | Matrix protein |
| <i>nef</i> | Negative regulatory factor gene |

| | |
|------------|---|
| Nef | Negative regulatory factor protein |
| PCR | Polymerase chain reaction |
| <i>pol</i> | Polymerase gene |
| PR | Protease protein |
| p24 | Protein 24, core capsid protein |
| p55Gag | Protein 55 group specific antigen (precursor protein) |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase |
| <i>rev</i> | Regulator of virion proteins gene |
| Rev | Regulator of virion proteins |
| ss | Single stranded |
| <i>tat</i> | Viral transcriptional transactivator gene |
| Tat | Viral transcriptional transactivator protein |
| UPR | Unfolded protein response |
| UNAIDS | Joint united nations program in HIV/AIDS |
| <i>vpr</i> | Viral protein R gene |
| Vpr | Viral protein R |
| <i>vpu</i> | Viral protein U gene |
| Vpu | Viral protein U |
| WHO | World health organization |
| wt | Wild type |
| XBP-1 | X-box binding protein homolog |

INTRODUCTION

Like all viruses, the human immunodeficiency virus (HIV) must infect cells to multiply. HIV mainly infects CD4 expressing T-cells, which are the cells within the human immune system that regulate the triggering of immune reactions towards bacteria, virus, fungi and tumor cells etc. As HIV infection progressively leads to killing of these cells the immune system becomes nonfunctional and the infection has thereby developed into the life threatening disease condition called acquired immunodeficiency syndrome (AIDS).

THE HIV PREVALENCE IN THE WORLD



WHO/UNAIDS estimates that in 2009 2.6 million adults and children worldwide to have become newly infected with HIV. In total 33.3 million people were living with HIV and 1.8 million died of AIDS. Although this virus has spread all over the world its prevalence is unevenly distributed (Fig. 1). In the worst affected area, the sub-Saharan Africa, 22.5 million people are infected while in North America 1.5 million people and in western and central Europe 820,000 people carry HIV [1]. In comparison, about 5,000 are living with HIV in Sweden today, where in total 8,935 HIV cases have been reported until 2009. Approximately half of these were infected prior to coming to Sweden [2].

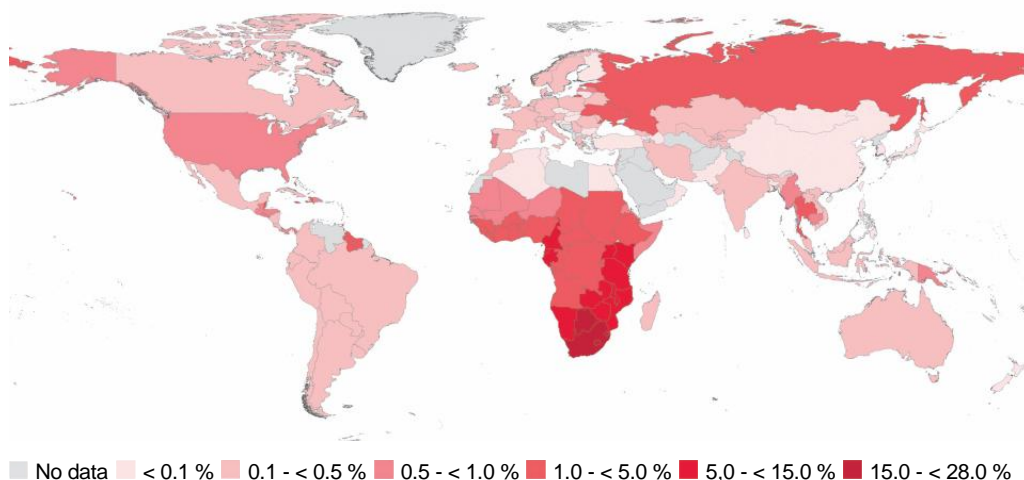


Figure 1. HIV-1 prevalence (%) among adults aged 15-49 years old in the world in 2009.

Source: UNAIDS report 2010

THE DISCOVERY OF HIV

The two oldest documented cases with HIV infections are from 1959. One is from what today is the Democratic Republic of Congo and the other from Manchester, UK [3, 4]. Further retrospective studies have also shown that in 1969 a teenager died in AIDS in St. Louis, USA, and a year later an entire family in Norway, although at the time the disease and its cause were not known [5, 6].

The ongoing of an epidemic was brought to attention first in 1981 when an increased incidence of rare opportunistic infections (*Pneumocystis carinii pneumonia*, mucosal candidiasis) and malignancy (*Kaposi's sarcoma*) in young homosexual men was identified in the USA [7, 8]. In 1982, the U.S. Centers for Disease control and prevention introduced the term AIDS to describe the newly recognized disease. The year after, in 1983, French scientists led by Luc Montagnier were the first to isolate HIV [9], which soon after was shown to be the cause of AIDS by Robert Gallo and his team in the USA [10]. Several different names were used for the virus at the time, (lymphadenopathy-associated virus (LAV), human T cell lymphotropic virus III (HTLV-III), AIDS-associated retrovirus (ARV)), until the International Committee on Taxonomy of viruses in 1986 proposed the virus to be called human immunodeficiency virus [11].

In 1986, another immunodeficiency virus was discovered [12]. It was found to be very similar yet distinct from HIV. To distinguish the two viruses the first discovered was therefore termed HIV-1 and the second HIV-2. However, while HIV-1 has spread over the entire world the HIV-2 has remained relatively uncommon and is usually only found in West Africa.

THE ORIGIN OF HIV

HIV-1 and HIV-2 are believed to have been introduced to humans by transmission of simian immunodeficiency viruses (SIVs) from African non-human primates.

Phylogenetic studies indicate that such zoonotic transmissions have taken place several times and that the specific transmissions that have resulted in the present pandemic took place about 100 years ago [13-15]. HIV-1 has been found to most likely originate from SIV found in chimpanzees (*Pan troglodytes troglodytes*) [16], while HIV-2 from SIV found in Sooty mangabey monkeys (*Cercocebus atays*) [17].

TRANSMISSION AND PATHOGENESIS

HIV is a blood borne virus and, therefore, can be transmitted between individuals by direct blood to blood contact or via blood transfusion, blood products and contaminated syringes, etc. The virus can further be transferred from mother to child during birth and also by breast feeding. However, the main viral transmission route, by which HIV has spread worldwide, is heterosexual unprotected intercourse (anal, oral and vaginal) as the virus is also prevalent in vaginal secretion and semen.

HIV-1 targets mainly CD4⁺ T-lymphocytes, but infects also monocytes, macrophages, and microglial cells in the brain [18-21]. The rate of the disease progression is highly variable among HIV infected, but its course can usually be divided into the three phases: the primary infection phase, the clinically latent phase and the final stage, the AIDS phase [22]. During the first phase (primary infection), which usually takes place 2-10 weeks after the infection, there is a high level of viral replication [23, 24]. The symptoms are similar to many other acute viral infections such as flu-like illness, fever, rash, lymph node enlargement and headache etc [25, 26]. The viremia subsides after a few weeks as do the symptoms of illness [25]. The second phase (clinically latent) may last even over 10 years, during which time the infected individual may remain healthy [27]. During this phase the viral load drops to a lower but stable level and a slow but continuous loss of CD4⁺ cells takes place (Fig. 2). The third phase (AIDS) is characterized by an accelerated loss of CD4⁺ cells and increased viral replication. As the immune system gradually becomes weaker, the infected individual becomes increasingly susceptible to opportunistic infections, malignancies and neurological disorders [28, 29]. Without treatment the patient will most likely die within a few years.

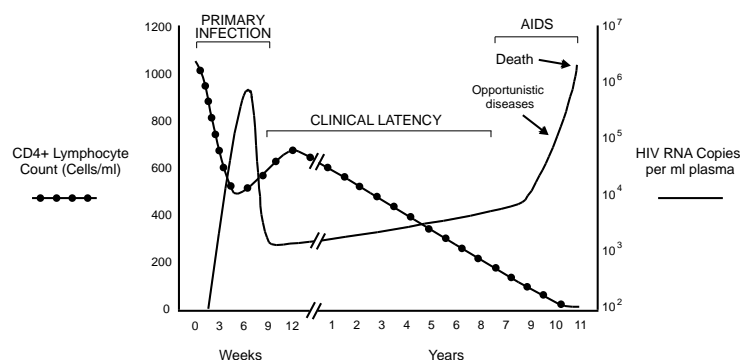


Figure 2. Graf showing the average clinical course of untreated HIV-1 infection depicting CD4 cell count (—●—●—●—) and viral load (———).

THE HIV-1 VIRUS

CLASSIFICATION

HIV-1 belongs to the family of retroviruses (Retroviridae) and is further grouped into the subfamily of Orthoretrovirinae and finally the genus of Lentiviruses (www.ictvonline.org) (Table 1). Lentivirus means slow virus, which refers to the slow onset of the adverse symptoms from the infection.

| FAMILY | SUBFAMILY | GENUS | SPECIES (examples) |
|---------------------|--------------------------|-------------------|---------------------------------------|
| Retroviridae | Spumaretrovirinae | Spumavirus | Simian foamy virus |
| | | | |
| | Orthoretrovirinae | Alpharetrovirus | Rous sarkoma virus |
| | | Betaretrovirus | Jaagsiekte sheep retrovirus |
| | | Epsilonretrovirus | Bovine leukemia virus |
| | | Gammaretrovirus | Walleye dermal sarcoma virus |
| | | Deltaretrovirus | Murine leukemia virus |
| | | | |
| | | Lentivirus | Human immunodeficiency virus 1 |
| | | | Human immunodeficiency virus 2 |
| | | | Simian immunodeficiency virus |

Table 1. Classification according to International Committee on Taxonomy of Viruses (ICTV).

THE HIV GROUPS AND SUBTYPES

Upon comparing the genetic sequences HIV-1 has been divided into three main groups; M (major), O (outlier) and N (non-M non-O). Recently a new group has been identified, group P [30, 31]. Group M is spread all over the world and is further divided into the subtypes A, B, C, D, F, G, H, J and K, as well as recombinants between the subtypes, so called circulating recombinant forms (CRFs) [32, 33]. The O and N groups have not been divided into subgroups as they still contain very few strains. HIV-2 is divided into seven subtypes (A-G).

HIV-1 GENOME AND PROTEINS

The genome of HIV-1 particles constitutes of two single stranded and positive sense RNA molecules of approximately 9.2 kb each. The provirus, which is the viral RNA reversely transcribed into double stranded DNA, is flanked on both sides by identical Long Terminal Repeats (LTR) and is therefore slightly larger than the original RNA [34]. The 5'-LTR serves as promoter/enhancer and directs HIV-1 transcription while the 3'-LTR acts in transcription termination and polyadenylation of the viral mRNAs [35]. The HIV-1 genome encodes the genes *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env* and *nef* (Fig. 3). These nine genes, organized in nine open reading frames which overlap each other to various degrees, result in 15 proteins upon post translational processing [36].

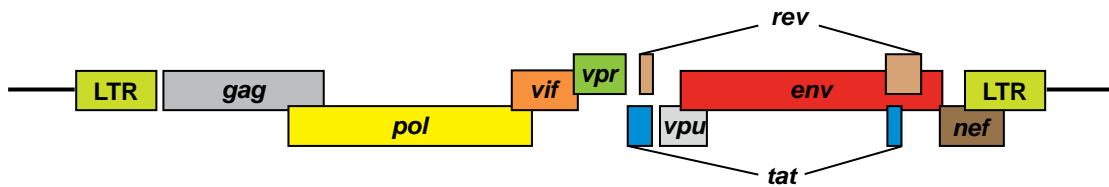


Figure 3. The organization of the HIV-1 provirus.

The HIV-1 proteins are divided into the 3 groups:

- 1) The regulatory proteins, which are **Tat** (trans-activator of transcription) and **Rev** (regulator of virion protein expression).
- 2) The accessory proteins, which are the four proteins **Vpu** (Viron protein U), **Vpr** (Viron protein R), **Vif** (Virion infectivity factor) and **Nef** (negative regulatory factor).
- 3) Major structural proteins, which all are expressed as the precursor proteins **p55Gag** (group specific antigen), **Gag-Pol** (polymerase) and **Env** (envelope glycoprotein).

The p55Gag is further processed to Matrix protein (**MA**, p17), Capsid protein (**CA**, p24), Nucleo capsid protein (**NC**, p7) and p6. Processing of Gag-Pol yields Reverse

transcriptase (**RT**, p55/p66), Integrase (**IN**, p32) and Protease (**PR**, p10). The processing of Env results in gp120 (surface protein) and gp41 (transmembrane protein). The processing of the major structural proteins is further described under “HIV-1 replication cycle” on page 7.

HIV-1 PARTICLE STRUCTURE

The HIV-1 particle has a spherical shape and is approximately 110 nm in diameter. The particle may be in an immature or a mature state. The immature particle has a doughnut-like morphology (Fig. 4A), which upon processing of its internal polyproteins matures into a cone-shaped structure surrounded by an envelope (Fig. 4B). The maturation process is necessary for the virus to become infectious. Moreover, its mature morphology distinguishes lentiviruses from other retroviruses.



Figure 4. Electrone micrographs of A) immature and B) mature HIV-1 viral particles.

The mature and infectious particle has two ssRNA molecules that each is encapsulated by the NC. These, together with the essential viral enzymes RT, IN and PR, are surrounded by the CA protein that forms the conical shaped capsid. The cone is in turn surrounded by a spherical capsid consisting of the MA protein that is enveloped by a lipid bilayer acquired from the host cell. Embedded in the envelope are the virally encoded envelope glycoproteins (Env). These protrude out of the viral surface as trimeric heterodimers consisting of the transmembrane protein, gp41, to which the heavily glycosylated surface protein, gp120, is non-covalently attached.

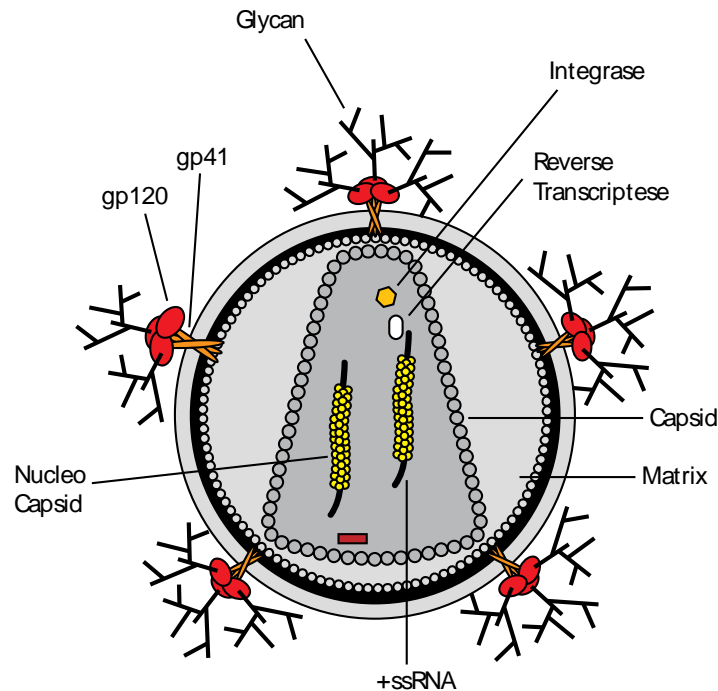


Figure 5. Morphological structure of mature HIV-1 particle.

HIV-1 REPLICATION CYCLE

The HIV-1 particle initiates its replication cycle (Fig. 6, 7) by binding with its gp120 to the cellular receptor CD4 [20, 37]. This will induce a conformational change in the gp120 that leads to its binding to either of the two main co-receptors CCR5 or CXCR4. This in turn will expose gp41 and allow its insertion into the cellular plasma membrane [38-41]. A following conformational change of gp41 into a six helix bundle will pull the virus into close contact with the cell. This will lead to fusion of the viral envelope and the cellular plasma membrane, which subsequently releases the viral content into the cell [40, 41]. In the cytosol, but within the intact capsid shell, the viral RNA is reverse transcribed into dsDNA by the viral reverse transcriptase (RT) [42, 43]. The RT has three separate activities: a RNA dependent DNA polymerase activity, a DNA dependent DNA polymerase activity and a RNase H activity [43, 44]. These activities are all required for its ability to synthesize dsDNA from ssRNA. However, the RT lacks proof reading, which consequently introduces mutations in the provirus and results in a high genetic variability of HIV-1 [45, 46]. This may be is beneficial for the virus as it can quickly adapt to the environment in which it replicates and thereby escape immune, as well as, drug pressure. The capsid core subsequently disintegrates

and the dsDNA forms a preintegration complex (PIC) with MA, IN and cellular proteins which is transported into the nucleus [47, 48]. The proviral DNA will preferentially be inserted into a transcriptional active region of the host genome by the viral IN. However, upon insertion the provirus may stay silent for years.

Once the transcription is activated the whole proviral genome will be transcribed into one mRNA, which at first will become multiply spliced prior to export from the nucleus [49]. This will allow the expression of the two regulatory proteins Tat and Rev. Tat will strongly increase the transcription efficiency of the provirus by binding during transcription to the trans-activation response element (TAR) in the viral mRNA [50, 51]. The main function of Rev is to bind to the Rev-responsive element (RRE) within the *env* region of single and unspliced mRNAs to facilitate their nuclear export [52]. This rescues the mRNAs from multiple splicing and allows expression of the major structural and accessory proteins.

The major structural proteins Gag and Gag-Pol are translated in the cytoplasm into the precursor protein p55Gag and the enzyme precursor protein p160 respectively. Both p55Gag and p160 are then transported to the inner face of the plasma membrane where the viral assembly takes place [53]. The third structural protein Env, is co-translationally translocated into the endoplasmic reticulum (ER) as the transmembrane precursor protein gp160. In the ER it undergoes an extensive glycosylation and folding process and also forms trimers prior to its export to Golgi [54, 55]. In the Golgi gp160 becomes processed into the surface protein gp120 and the transmembrane protein, gp41, which remain non-covalently associated to each other [56]. The gp120/gp41 trimers are then transported to the cell surface, where they become incorporated into the assembling viral particles along with p55Gag and Gag-Pol. During or shortly after the particle has budded off from the cell surface, the processing of p55Gag and Gag-Pol by the viral protease within Gag-Pol is induced [57, 58]. The processing of the p55Gag results in the Matrix protein (MA, p17), the Capsid protein (CA, p24), the Nucleo Capsid protein (NC, p7) and the p6 protein [59, 60]. The proteolytical processing of p55Gag results in an extensive structural rearrangement within the viral particles, where the CA proteins form a conical core structure [61, 62].

The accessory protein Vpu is mainly found in the endoplasmic reticulum (ER) membrane to which it is anchored with its N-terminal end, while the remaining and the

majority of the protein protrudes out in the cytosol [63]. Vpu interacts in the ER membrane with CD4, the primary HIV-1 receptor, which results in the targeting of CD4 for destruction via the ubiquitine-mediated proteasomal degradation pathway [64-66]. This prevents the viral Env glycoprotein from being trapped in the ER as a result of premature binding to CD4. Vpu has also been found to counteract the host cell restriction factor Tetherin/CD317/BST-2 by down-regulating it from the cell surface [67, 68]. Tetherin is expressed as a heterodimer that is anchored N-terminally in the plasma membrane by a one-pass transmembrane domain and C-terminally by the fatty acid glycosylphosphatidylinositol (GPI) [69]. It exerts its antiviral effect by being anchored both in the viral membrane and in the plasma membrane of the host cell, which prevents release of budded viral particles and their spreading to yet uninfected cells. Like Vpu, Vif also acts as an antagonist of a cellular anti-viral factor. It prevents viral incorporation of APOBEC3G. This protein catalyses cytidine deamination of the negative DNA strand during the reverse transcription, which results in hypermutation of the proviral DNA and subsequently production of non-infectious virus [70-72]. The function of Vpr is still unclear and controversial. It has frequently been found dispensable for the viral replication in various cell types, including monocyte-derived macrophages and primary T lymphocytes. However, it has been described to be involved in nuclear import of the viral DNA, transactivation of LTR and cell cycle arrest during viral replication [73, 74]. Nef is a membrane associated protein due to its N-terminal myristoylation. It is expressed abundantly and early along with the regulatory proteins. It has been reported to have several functions among which are down-regulation of CD4 and major histocompatibility complex I and II from the plasma membrane, as well as enhancement of virion infectivity and stimulation of viral replication [75-77].

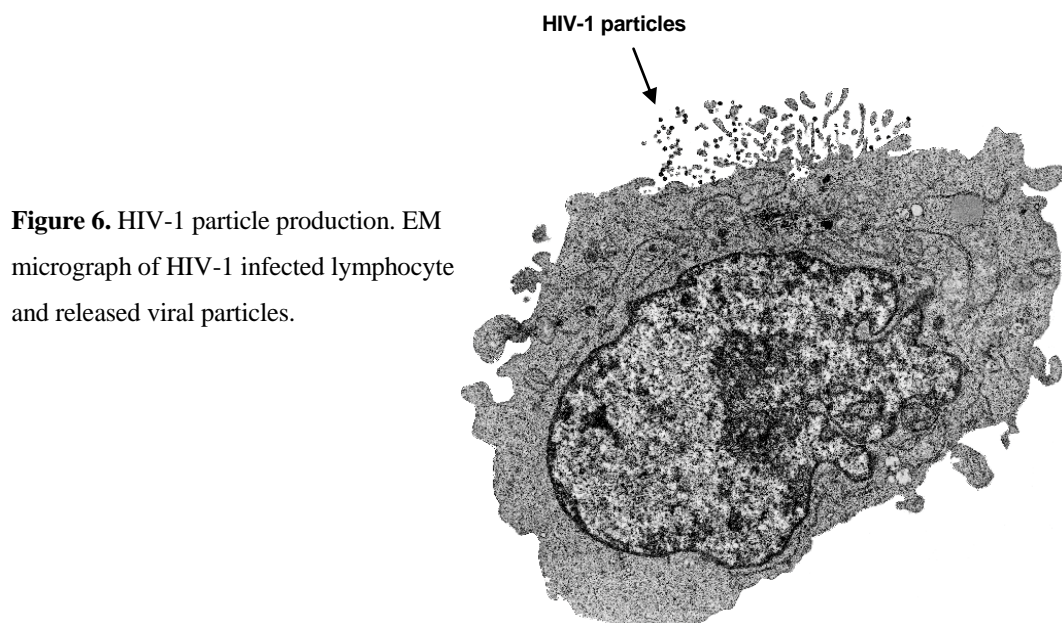


Figure 6. HIV-1 particle production. EM micrograph of HIV-1 infected lymphocyte and released viral particles.

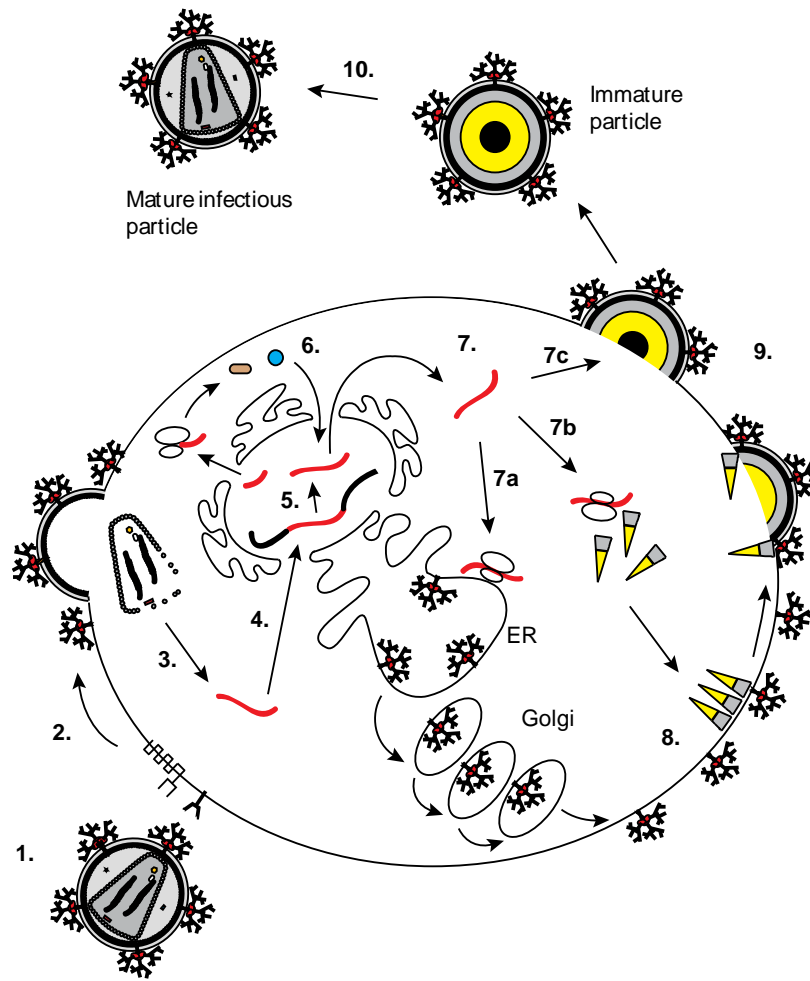


Figure 7. Schematic representation the HIV-1 replication cycle. **1)** HIV-1 binds to the cellular CD4 receptor and subsequently to the co-receptor CCR5 or CXCR4. **2)** The viral envelope membrane fuses with the host cell membrane, which releases the viral content into the cell. **3)** The viral RNA is reverse transcribed into ds DNA that is **4)** transported into the nucleus where it is integrated into the host cell genome. **5)** The provirus is transcribed and its mRNA is multiply spliced, which lead to expression of Tat and Rev. **6)** Tat increases the transcription efficiency and Rev allows unspliced and singly spliced mRNA to be exported out of the nucleus. **7a)** The unspliced RNA is incorporated into the viral particles or **7b)** used for translation of Gag/Gag-Pol in the cytosol and **7c)** the single spliced RNA allows translation of Env into the ER. **8)** Viral assembly at the cell surface. **9)** Virus particle buds off from the cell surface. **10)** Maturation of viral particle.

THE HIV-1 ENVELOPE GLYCOPROTEIN

BIOSYNTHESIS

The HIV-1 Env glycoprotein is a type 1 membrane protein that is translated from a singly spliced bicistronic *vpu/env* mRNA into the ~860 aa long precursor protein, gp160 [78-80]. Gp160 is targeted for co-translational translocation into the ER lumen by its ~30 aa long N-terminal signal sequence [81]. During its translocation into the ER ~30 N-linked glycans will be coupled to its polypeptide backbone and constitute nearly half of the gp160 molecular mass. The N-linked glycans play an important role in the folding process of gp160 by masking hydrophobic patches and recruiting the lectin chaperones calnexin and calreticulin, which along with Bip assist gp160 as it undergoes an extensive and exceptionally slow maturation process [54, 82-84]. Unlike most signal sequences the gp160 signal sequence is not cleaved off during, but after completed translation and certain folding of gp160 [54, 85]. Once gp160 has reached its native state with 10 disulphide bonds it assembles predominantly into trimers, although dimers and tetramers have been observed [55, 86-88]. The gp160 complex is then exported from the ER to Golgi, where its high mannose N-linked glycans acquire complex modifications. In the Golgi the gp160 complex is additionally cleaved by furin or furin-like proteases at its highly conserved motif K/R-X-K/R-R into the surface protein gp120 and the transmembrane protein gp41 [56, 89, 90]. Upon the processing gp120 and gp41 remain associated by noncovalent interactions. The proteolytical processing of gp160 is absolutely necessary for the fusogenic activity of Env and is therefore essential for the viral infectivity[56]. The gp120/gp41 complexes are thereafter transported further to the plasma membrane where they are incorporated into the viral particles.

VIRAL ENV INCORPORATION

The Env content in the viral particles is considered to be controversial as it has only been studied by two research groups, which reported very dissimilar findings. The first study from late 1980's reports that HIV-1 particles incorporate 72 Env spikes, assumed to be trimers of gp120/gp41, that are evenly distributed over the particle surface [91]. However, a recent study found that HIV-1 particles on the average only incorporate approximately 10 spikes per viral particle and that these are not evenly distributed, but clustered on the viral surface [92-94]. The trimers of gp120/gp41 are considered to be

the functional oligomeric form of Env that mediate viral entry, although, the viral particles have been found to also incorporate various non-functional forms of Env such as gp120/gp41 monomers, uncleaved gp160 and gp41 stumps as a result of gp120 shedding [95, 96].

However, the mechanism by which Env is incorporated into the viral particles is unclear, but four general models have been suggested [97]: 1) The passive incorporation model. According to this model Env becomes incorporated into the viral particles only as a result of being present at the site of viral assembly. This is supported by the observation that host cell membrane proteins are readily incorporated into HIV-1, as well as other non-HIV-1 viral glycoproteins [98-100]. In addition removal of the cytoplasmic tail of gp41 has been found to only have a minor effect on the viral Env incorporation, which may indicate that no active interaction is necessary. [101].

2) The direct Gag-Env interaction model. In this model it is proposed that the Env incorporation is facilitated by direct interaction of the cytoplasmic tail of gp41 with the MA domain of Gag. Support for this model derives from studies demonstrating that deletions or mutations in MA prevent Env incorporation. However, this effect has been found to be reversed if the cytoplasmic tail of gp41 is deleted. This may indicate that the wt gp41 require interaction with MA for viral incorporation, but with its long cytoplasmic tail deleted gp41 is incorporated into the viral particle by other mechanisms [102-105]. Further indications of interaction between Env and Gag come from studies in polarized cells where Env directs the viral budding to the basolateral plasma membrane, while in the absence of Env the viral particles are released in a nonpolarized manner [106]. Although, there is plenty of indicative data in support for this model there is limited biochemical evidence.

3) The Gag-Env co-targeting model. In this model Gag and Env are independently of each other targeted to lipid rafts or other plasma membrane domains where the viral assembly takes place. This is supported by microscopical analyses that have shown that Env and Gag co-localize with cellular raft components. In addition, treatment of HIV-1 producing cells with agents depleting or binding cholesterol impairs HIV-1 production [107-109].

4) The indirect Gag-Env interaction model proposes that Env is incorporated into the viral particles by interacting with Gag via host cell proteins that function as linkers. Several cellular proteins have been reported to interact with MA and / or the cytoplasmic tail of gp41, which potentially could function as links that connect the two proteins and permit the viral incorporation of Env [110-112]. It is possible that each

model contributes to Env incorporation into the viral particles to varying degrees and that the mechanism dominating is depending on the cell type.

ENDOPLASMIC RETICULUM

ER QUALITY CONTROL

About 20 % of the human genes are predicted to encode secretory proteins [113]. These proteins are generally targeted for co-translational translocation into the endoplasmic reticulum by a N-terminal signal sequence. The ER targeting is initiated when the hydrophobic region of the signal sequence, which protrudes out from the translating ribosome, is recognized by the signal recognition particle (SRP). The SRP-ribosome complex is then targeted to the ER membrane via the SRP receptor. The signal sequence is subsequently transferred to the translocon, which upon this interaction creates a channel for the growing peptide chain to traverse the ER membrane and emerge into the lumen [114, 115]. Once the growing peptide chain enters the ER lumen it starts to fold and may undergo several co- and post-translational modifications such as N-linked glycosylation, signal sequence cleavage and disulphide bond formation. The ER contains a number of molecular chaperones and folding factors including Bip, GRP 94, PDI, ERp57 and calnexin/calreticulin that aid in the maturation of proteins. This maturation process is strictly monitored by the ER quality control system, which functions to prevent immature or misfolded proteins from being exported out of the ER to cellular compartments where their presence could be toxic to the cell [116-118]. In the ER quality control process the N-linked glycans serve as tags, which signal the folding status of the glycoproteins [119].

Most secretory proteins are modified during their co-translational translocation into the ER by the addition of N-linked glycans. These consist of glucose₃-mannose₉-N-acetylglucosamine₂ and are transferred *en bloc* to asparagine residues on Asn-X-Ser/Thr motifs of the translocating polypeptide by the Oligosaccharyl transferase (OST). Immediately after being coupled to the peptide backbone, the two outer glucoses of the N-linked glycan are removed by glucosidase I and glucosidase II. This results in monoglycosylated N-linked glycans that are capable of recruiting the lectin

chaperones calnexin and calreticulin [120-123]. Due to its localization to the membrane, calnexin associates with glycans on the maturing protein that are in close proximity to the ER membrane, whereas the soluble calreticulin associates with those glycans that are further away. Together, these two homologous chaperones aid in the folding of peptide chains. In addition they recruit other folding factors such as the thiol-disulphide oxidoreductase ERP57, which catalyses transient disulphide bonds with the glycoproteins associated to calnexin/calreticulin [124-126]. Upon release from the chaperones the final glucose is removed by glucosidase II. If the protein has acquired its native conformation, the ER mannosidase I and II will further modify the N-linked glycans and enable the protein to exit the ER for further transport to Golgi. However, if the glycoprotein has not acquired its native conformation, it will be recognized and re-glucosylated by the UDP-glucose:glycoprotein glucosyltransferase (UGGT or GT) [127, 128]. This will lead to re-association of the immature or misfolded glycoprotein with calnexin/calreticulin and allow further folding or refolding of the protein [129]. This way the ER quality control ensures that only properly matured proteins are exported to Golgi.

ER-ASSOCIATED DEGRADATION (ERAD)

Despite the fact that the ER contains a number of molecular chaperones and folding factors to help glycoproteins to fold and mature properly, the protein maturation is an error-prone process. To alleviate the burden of misfolded proteins in the ER lumen, these chaperone systems have developed a mechanism to target terminally misfolded proteins for destruction via a pathway termed the ER-associated degradation (ERAD) [130]. The ERAD pathway can be divided into the following steps in sequential order: substrate recognition and targeting of substrate for ERAD, retrotranslocation of substrate across the ER membrane to the cytosol, where it is ubiquitinated by an E3 ligase and deglycosylated by an N-glycanase prior its degradation by the proteasome [130].

Currently, not all of the components of the ERAD machinery and their function have been elucidated, but it is generally believed that misfolded glycoproteins are recognized by lesions that are created by the exposure of hydrophobic patches, unpaired cysteines, or by the presence of immature glycans. The immature glycans are thought to be one of the more common signals for misfolding as they result from the persistence of the

misfolded substrates within the quality control system. In this way, the glycan, which has undergone additional processing due to its prolonged existence within the ER, is recognized by one of the ERAD lectins OS9 or XTP3-B. These lectins then target the misfolded substrate to the ER membrane where it is retrotranslocated through a channel (*e.g.* Derlin-1,-2 or -3 or Hrd1) to the cytosol [131, 132]. Once protruding out from the retrotranslocon, the proteosomal 19S cap or the AAA-ATPase p93 binds to the misfolded protein and extracts it from the channel into the cytoplasm [133, 134]. In parallel with the extraction process the HRD1 complex ubiquitylates the misfolded protein and the N-glycanase deglycosylates it prior to degradation by the 26S proteasome [119]. By removing the terminally misfolded proteins, ERAD as a process, helps maintain cellular homeostasis by providing a link between the maturation environment within the ER lumen and the degradation machinery present in the cytoplasm.

GPG-NH₂, GPG-NH₂ AND α HGA

The tri-peptide GPG-NH₂ was originally designed to mimic the amino acid sequence of the tip of the V3-loop in gp120 in order to disturb its interaction with its secondary co-receptor and thereby block HIV-1 entry into cells. Although the peptide was found to inhibit HIV-1 propagation in cell cultures involving multiple viral replication cycles, its mode of action was surprisingly found to be other than it had been designed for [135, 136]. As a result of the method employed for the synthesis of GPG, its C-terminal end ended in -CONH₂ instead of the natural form -COOH. This modification was found to be crucial for the antiviral activity as the peptide with a normal C-terminal end showed no inhibition of HIV-1 replication in cell cultures [135]. Eventually it was further found that GPG-NH₂ is not the active anti HIV-1 compound, but rather functions as a pro-drug. When GPG-NH₂ is added to cell culture media it will be processed into G-NH₂ by the CD26 (peptidyl peptidase V) and subsequently hydroxylated by an unidentified enzyme into the active compound alpha hydroxy glycine amide (α HGA) [137-140]. The enzymes required for the metabolism of GPG-NH₂ are provided to the cell culture medium by the supplementation of 10 % fetal bovine serum, which is according to standard cell culturing procedures.

AIMS OF THIS THESIS

The main objective of this thesis has been to elucidate the antiviral mode of action of GPG-NH₂ and its metabolites G-NH₂ and α HGA against HIV-1. More specifically:

- To determine the mechanism by which GPG-NH₂ decreases the Env molecular weight, steady-state levels and processing to gp120/gp41 in HIV-1 infected cells.
- To elucidate the effects of GPG-NH₂ on viral particle production, viral infectivity and viral Env incorporation.
- To examine if the specific effect on Env was owing to GPG-NH₂, its intermediate metabolite G-NH₂ or its final metabolite α HGA.
- To study the native Env signal sequence and its importance for the Env expression levels, processing, viral Env incorporation and the viral replication cycle.

MATERIAL AND METHODS

Presented below is a summarized description of the methods used in this thesis, however, the details of the specific experiments performed can be found in the respective paper.

CELL LINES (Paper I, II, III)

The HeLa and HT1080 cell lines that derive from human cervical epithelial carcinoma and human fibrosarcoma (negative for CD317/BST-2/Tetherin), respectively, were purchased from European Collection of Cell Cultures (ECACC/Sigma). The 293T derives from HEK-293, a human embryonic kidney cell line transformed with adenovirus 5 DNA, which constitutively expresses the simian virus 40 (SV40) large T antigen. The following cell lines were obtained through NIH AIDS and Reference Reagent Program: HeLa-tat III derives from HeLa and constitutively expresses the regulatory HIV-1 protein Tat. The TZM-bl derives from HeLa and stably expresses the receptors CD4 and CCR5. The TZM-bl additionally expresses luciferase and Beta-galactosidase under the control of the HIV-1 promoter LTR. The ACH-2 is a human CD4 negative T-cell line derived from acute lymphoblastic leukemia and is chronically infected with the HIV-1 subtype B strain, LAV. SupT1 is a Non-Hodgkin's T-cell lymphoma, which expresses high levels of CD4. The T-cell lines were maintained in RPMI (Gipco) and the others in DMEM (Gipco). Both media were supplemented with 100 U penicillin, 100 µg/ml streptomycin and 10 % fetal bovine serum (FBS).

PLASMIDS AND CLONING (Paper I, II, III)

The plasmid, pNL4-3 [141], expresses infectious HIV-1 subtype B and was obtained from NIH AIDS and Reference Reagent Program. pNL1.5EU [78], pBrev and pCMVTat, which express Env from the HIV-1 strain NL43, Rev and Tat respectively, were kindly provided by S. Schwartz (Uppsala University, Uppsala, Sweden). Δ nSS-gp160 was created from pNL1.5EU by mutating the start codon ATG to ATA [95]. PCR^R3.1/CAT expresses chloramphenicol acetyltransferase (CAT) (Invitogen). For construction of Vpu expression deficient proviral clones with truncations in the gp160 signal sequence, site directed mutagenesis was performed to change various aa into

methionines or existing methionines into isoleucines using Quick Change II XL (Stratagene). The EcoRI-BamHI fragment from pNL4-3 was cloned into pUC18 in which the Vpu start codon was mutated from ATG to ATA. The XmaI restriction site was additionally introduced immediately upstream from the Vpu start codon creating the vector pUCEnvXΔU. The same mutations were introduced in the vector ΔnSS-gp160 from which the fragment XmaI-NheI was directionally transferred into pUCEnvXΔU creating the pUCEnvXΔUss11. All the following mutations in the gp160 signal sequence region were performed in pUCEnvXΔUss11 from which the fragment EcoRI-NheI was subcloned into p83-10. The p83-10 encodes tat, rev, vpu, env and nef from pNL4-3 and was acquired from NIH AIDS and Reference Reagent Program. Finally the mutation containing fragments were cut out with Sal I and Nco I and cloned directionally into pNL4-3 creating pNLHIV, pNLHIVxΔu, pNLHIVxΔuss22, pNLHIVxΔuss19, pNLHIVxΔuss15, pNLHIVxΔuss11, pNLHIVxΔuss5, pNLHIVxΔuΔss. See the respective primers designed for the site directed mutagenesis below.

XΔu (sense) 5'-GCAGTAAGTAGTACCCGGGATACAACCTATAATAGTAGCAATAG-3'
 (α-sense) 5'-CTATTGCTACTATTATAGGTTGTATCCCGGGTACTACTTACTGC-3'

ss22 (sense) 5'-GAAGGAGAAGTATCAGATGTTGTGGAGATGGGGGTGGAAATG-3'
 (α-sense) 5'-CATTTCACCCCCATCTCCACAACATCTGATACTTCTCCTTC-3'

ss19 (sense) 5'-GAGAAGTATCAGCACTTGTGGATTGTGGGGGTGGAAATGG-3'
 (α-sense) 5'-CCATTTCACCCCCACATCCACAAGTGCTGATACTTCTC-3'

ss15 (sense) 5'-GAGATGGGGGTGGATGTGGGGCACCATGCTCCTTG-3'
 (α-sense) 5'-CAAGGAGCATGGTGCCCCACATCCACCCCCATCTC-3'

ss5 (sense) 5'-GGAAATGGGGCACCATACTCCTTGGGATATTG-3'
 (α-sense) 5'-CAATATCCCAAGGAGTATGGTGCCCCATTTC-3'

Δss (sense) 5'-GGATATTGATAATCTGTAGTGCTATGGAAAAATTGTGGGTC-3'
 (α-sense) 5'-GACCCACAATTTTCCATAGCACTACAGATTATCAATATCC-3'

TRANSFECTION AND DRUG TREATMENTS (Paper I, II, III)

Cells were seeded in dishes upon which various concentrations of GPG-NH₂, GPG-OH or α HGA were immediately added. Alternatively, α HGA was added 2-5 h post transfection. The cells were transfected with 0,5-1,5 μ g plasmid totally either immediately after cell adhesion (~5 h post seeding) or the next day using transfection reagent FuGENE6 or FuGENE HD (Roche). The cells were harvested at the earliest 18 h after transfection using the lysis buffer radio immune precipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7,4), 1 % Triton X-100, 1 % deoxycholate, 150 mM NaCl, 1 mM EDTA, 0,1 % sodium dodecyl sulphate (SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche). The lysates were disrupted by aspiration through syringe needles followed by centrifugation at 16,000 g for 20 min at 4 °C. The lysates were stored at -20 or -80 °C until further analysis.

VIRUS PRODUCTION AND VIRUS PRECIPITATION (Paper I, II, III)

Virus was produced by inducing the chronically infected ACH-2 cells with 100 nM 12-phorbol-13-myristate acetate (PMA) for 2-3 days. Alternatively, HeLa-tat III cells were transfected with either pNL4-3, pNLHIVx Δ u or the gp160 signal sequence derivatives (see above) and allowed to produce virus for 24-72 h. The cell culture supernatants were collected, centrifuged for 10 min at 300 x g and passed through 0.45- μ m-pore-size filters. The viral particles were left to precipitate at 4 °C for 24-48 h in 1:5 (vol/vol) of 40 % polyethylene glycol 6000 containing 0.667 M NaCl. The precipitated viral particles were thereafter centrifuged at 16,000 x g for 20 min at 4 °C. The virus pellets were dissolved in RIPA buffer and stored at -80 °C until further analysis.

INFECTIVITY ASSAY (Paper I, II, III)

TZM-bl cells were seeded and allowed to adhere over night. Virus culture supernatants were added to the cells either unstandardized or standardized to equal p24 levels and incubated at 4 or 37 °C for 2-5 h and thereafter replaced with fresh culture media followed by incubation at 37 °C for 1-2 days. Alternatively, the virus culture supernatants were left to incubate with the cells for 1-2 days. The virus culture supernatants added to the cells alone or supplemented with 5 μ M indinavir and 15 μ g/ml DEAE. The cells were subsequently analyzed for intra cellular luciferase activity using the One-Glo Luciferase assay system (Promega).

SYNCYTIA FORMATION ASSAY (Paper I)

ACH-2 cells were cultured in 10 μ M Indinavir (HIV-1 protease inhibitor) and in the absence or presence of 1000 μ M GPG-NH₂ for 24 h. Thereafter 20,000 ACH-2 cells were collected and co-cultured with 250,000 SupT1 cells at 37 °C. Syncytium formations were monitored after 24-40 by light microscopy.

ENZYME-LINKED IMMUNOSORBENT ASSAY (Paper I, II)

CAT concentrations in cell lysates were measured by using the CAT-ELISA Kit (Roch) according to manufacturers manual. The p24 concentrations in cell lysates or cell culture supernatants were analyzed by either a p24-ELISA [142] or by the automated system Architect® (Abbott)

UPR AND ENZYMATIC DEGLYCOSYLATION (Paper I)

HeLa-tat III cells were either treated with various concentrations of GPG-NH₂ for 48 h or dithiothreitol (DTT) for 3 h. The total cellular RNA was isolated using the RNeasy isolation kit (Qiagen) followed by reverse transcriptase PCR (RT-PCR) using the primer mXBP1 804AS and thereafter PCR amplification of spliced and unspliced XBP-1 using the primers 804AS XBP1 and 383S XBP as described previously [143]. For deglycosylation cells were lysed in RIPA buffer as described above. The lysates were supplemented with 0.5 % SDS and 1 % β -mercaptoethanol prior to incubation for 10 min at 100 °C. Lysates to be deglycosylated with PNGase F were adjusted to 1 % NP-40 and with EndoH to 50 mM sodium citrate (pH 5.5) and followed by incubation at 37 °C for 1 h with 16 U per μ l lysate of either PNGase F or EndoH (New England Biolabs).

WESTERN BLOT (Paper I, II, III) AND GLYCOPROTEIN BLOT (Paper I, II)

Cells or precipitated virus lysed in RIPA buffer were incubated for 3 min at 100 °C under reducing conditions and usually standardized to CAT or p24 levels prior to protein separation by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. For immunodetections the following primary antibodies were used: mAb to gp160/gp41 (Chessie 8) [144] or p24 (EF7) [145] obtained through NIH AIDS and Reference Reagent Program. The mAb against gp160/gp120 (F58/H3)

[146] has been described elsewhere. The pAb against LAMP-1 and Calnexin were purchased from BD Biosciences, Santa Cruz Biotechnology. Bound antibodies were then detected with appropriate horseradish peroxidase-conjugated secondary antibody purchased from Dako. The membranes were exposed to film for various times and band intensities were quantified using Gene Tool analysis software.

SUBCELLULAR FRACTIONATION AND ALKALINE EXTRACTION (Paper I)

HeLa-tat III cells were resuspended in ice-cold 10 mM HEPES (pH7,4), 1 mM EDTA, 0.25 M sucrose, 125 μ M phenylmethylsulfonyl fluoride, 2.5 μ g/ml of aprotinin and leupeptin and homogenized with a Dounce homogenizer. To pellet and discard nondisrupted cells and nuclei the homogenate was centrifuged at 1,500 x g for 10 min at 4 °C. The supernatant was then centrifuged at 180,000 x g for 1 h at 4 °C, which separate membrane vesicles from cytosolic components. The cytosolic components in the supernatant were then precipitated with 15 % trichloroacetic acid, rinsed in acetone and subsequently dissolved in reducing sample buffer. The pelleted membrane vesicles were resuspended in 0.5 mM sucrose containing 50 mM TEA (pH 7.5) and 1 mM DTT. To further separate integral membrane proteins from soluble proteins the membrane vesicles were diluted 20 times in 0.1 NaCO₃ (pH 11,5), incubated on ice for 30 min and centrifugated through 0.5 M sucrose cushion for 1 h at 180,000 x g and 4 °C. The pellet was resuspended in reducing sample buffer and the supernatants precipitated with TCA as described above

RESULTS

The most significant results of this thesis are described below. However, further details of the findings and controls used in this thesis can be found in the respective papers.

PAPER I: SMALL MOLECULE TARGETS ENV FOR ENDOPLASMIC RETICULUM-ASSOCIATED PROTEIN DEGRADATION AND INHIBITS HUMAN IMMUNODEFFICIENCY VIRUS TYPE 1 PROPAGATION

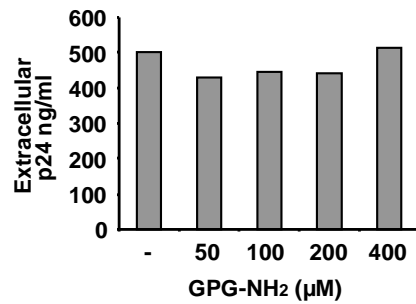
STUDY BACKGROUND

The GPG-NH₂ was originally designed to block viral entry into the target cells by inhibiting gp120 interaction with the secondary receptor. Although GPG-NH₂ owned antiviral properties its mechanism of action was other than the intended. It was observed that GPG-NH₂ acted late in the viral replication cycle and that it affected the cellular expression of Env. In this study the effect of GPG-NH₂ on Env and its mechanism was elucidated.

GPG-NH₂ DECREASES THE HIV-1 PARTICLE INFECTIVITY

The GPG-NH₂ was shown to not interfere with the viral attachment to or fusion with the cells when added simultaneously with the virus to the cell cultures in concentrations up to 1 mM. It was also found that the amount of viral particles produced from the infected cell was not diminished in the presence of GPG-NH₂ (Fig. 7A). However, the particles produced by cells treated with GPG-NH₂ had a reduced infectivity and this effect was found to be dose-dependent, where 50 µM GPG-NH₂ nearly decreased the viral particle infectivity by 50 % (Fig. 7B).

A



B

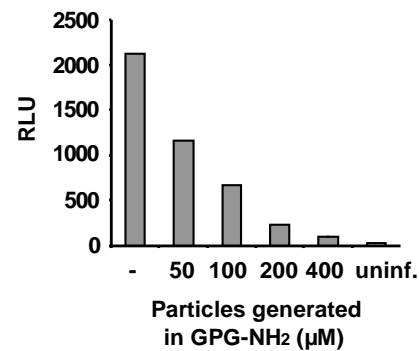


Figure 7. Infectivity of HIV-1 particles produced in the presence of GPG-NH₂ is reduced. **(A)** HIV-1 production by the chronically infected ACH-2 cells in the indicated concentrations of GPG-NH₂ was determined by measuring the extracellular p24. **(B)** Equal amounts of respective HIV-1 particles, generated in the indicated GPG-NH₂ concentrations, were tested for their infectivity by infecting TZM-bl cells. The infectivity was determined by measuring the HIV-1 induced luciferase production in the TZM-bl cells.

GPG-NH₂ REDUCES THE INCORPORATION OF ENV INTO THE HIV-1 PARTICLES, WHICH DISRUPTS THEIR ABILITY TO FUSE WITH CELLS

HIV-1 particles, produced by several cell lines treated with GPG-NH₂, were found to contain less of their viral envelope glycoprotein Env compared to particles produced in the absence of GPG-NH₂ or in the presence of the inactive analog GPG-OH (Fig. 8). GPG-NH₂ was found to decrease the viral incorporation of both the Env precursor protein gp160 and its processed form gp120/gp41 (Fig. 8).

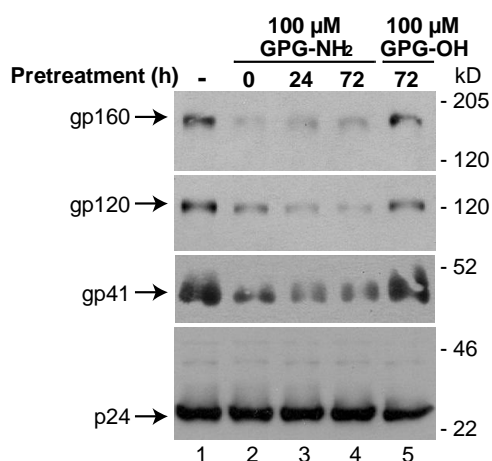


Figure 8. Env glycoprotein analysis by Western blot from equal amounts of HIV-1 particles. The virus was produced by the chronically infected ACH-2 cells in the absence or presence of GPG-NH₂ or GPG-OH. The drugs were added for the indicated hours prior to stimulation of the ACH-2 cells to produce virus.

By using a cell fusion model it was indirectly shown that a reduced content of the gp120/gp41 on the viral surface disables the viral ability to fuse with cells. For the cell fusion model, the ACH-2 cells were employed as they express gp120/gp41 on their cell surface but lack CD4 expression and can therefore not fuse with themselves. If the ACH-2 cells are co-cultivated with the CD4 expressing SupT1 cells they will start fusing and forming many large multinuclear cells with the SupT1, so called syncytia (Fig. 9). However, if the ACH-2 cells were treated with 1 mM GPG-NH₂ prior to co-cultivation with the SupT1 cells, they were incapable of forming any syncytia with the SupT1 (Fig. 9).

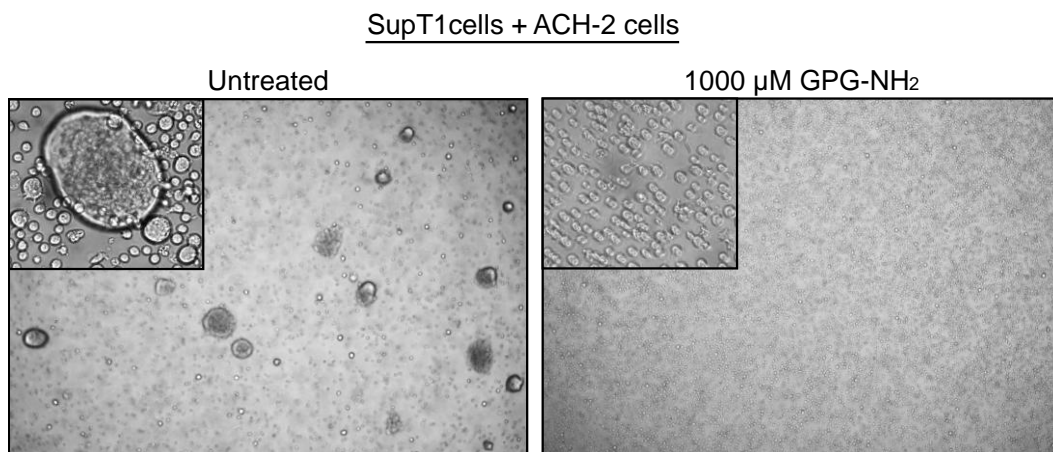


Figure 9. Light microscopy photos of syncytium formation between ACH-2 and supT1 cells. The ACH-2 cells were either untreated or pretreated with 1000 μ M GPG-NH₂ prior to co-cultivation with SupT1 cells.

GPG-NH₂ AFFECTS THE CELLULAR EXPRESSION OF THE HIV-1 ENV PRECURSOR PROTEIN GP160

When examining the cellular expression of Env in gp160 transfected HeLa-tat III cells, treated with 20-1000 μ M GPG-NH₂, it was detected that the molecular mass of gp160 decreased in a dose-dependent way, as well as its steady-state levels and processing to gp41 (Fig. 10A). The decreased molecular mass appeared as a “smear” consisting of products with various and gradually decreasing molecular mass. This variation in mass was shown to be due to an altered glycan status as the peptide backbones were found to have equal molecular mass when deglycosylated with PNGaseF or Endo H (Fig. 10B).

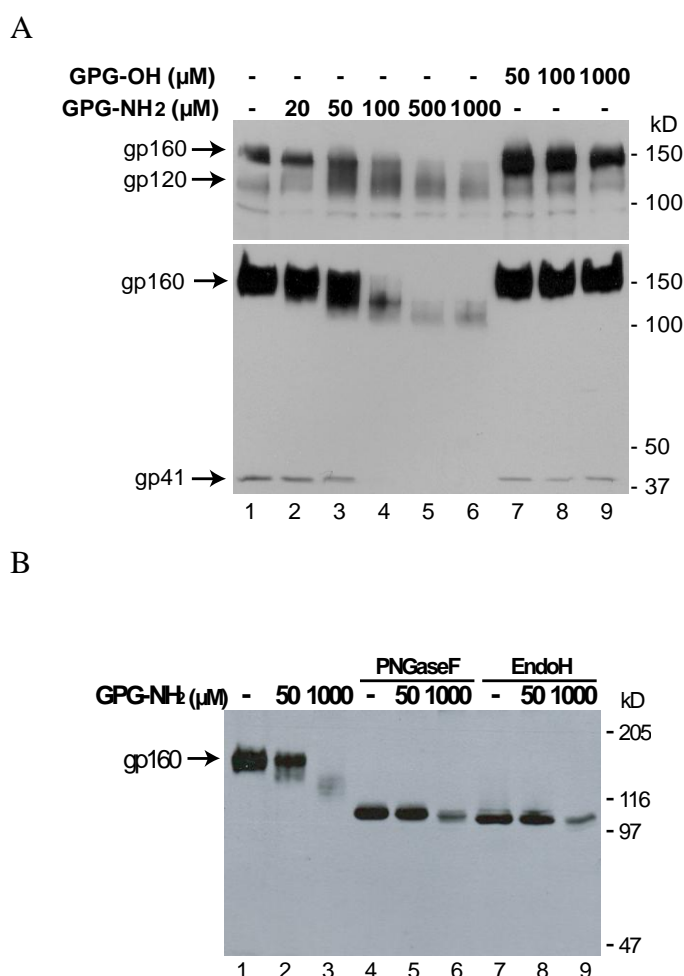
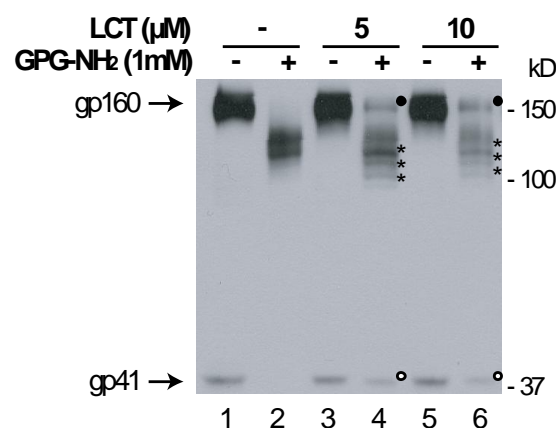


Figure 10. Analysis of intracellular Env expression by Western blot. **(A)** HeLa-tat III cells transfected to express gp160 in the presence of the indicated concentrations of GPG-NH₂ or GPG-OH. The top panel shows gp160 and gp120 and the bottom panel gp160 and gp41. **(B)** Cell lysates from cells treated as in (A) were deglycosylated by PNGaseF or EndoH as indicated and detected for gp160.

GPG-NH₂ TARGETS GP160 FOR DESTRUCTION

Partial inhibition of proteasomes with lactacystein in GPG-NH₂ treated HeLa-tat III cells, stabilized the full molecular mass of gp160, as well as enabled its processing to gp41. The inhibition of the proteasomal activity also allowed visualization of smaller gp160 degradation intermediates (Fig. 11A). Gp160 was further rescued from both deglycosylation and degradation by the proteasome inhibitor epoxomicin (Fig. 11B). This showed that the GPG-NH₂ does not cause inefficient glycosylation of gp160, but that it targets gp160 for ER-associated protein degradation, where the N-linked glycans are removed prior to the protein destruction by the proteasomes.

A



B

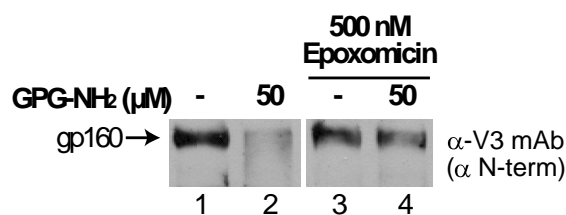


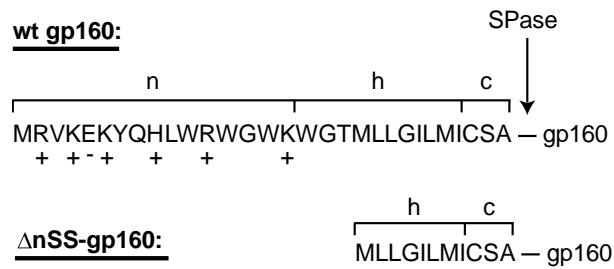
Figure 11. Analysis of intracellular Env expression by Western blot.

(A) HeLa-tat III cells transfected to express gp160 in the absence or presence of GPG-NH₂ and the proteasome inhibitor lactacystein (LCT) or (B) epoxomicin.

GP160 WITH PARTIALLY TRUNCATED SIGNAL SEQUENCE RESISTS THE GPG-NH₂ INDUCED DESTRUCTION

Truncation of 2/3 of the gp160 signal sequence did not destroy the targeting of gp160 to the ER nor its processing in the Golgi to gp41 (Fig. 12A, B). However, the truncated signal sequence left gp160 completely unaffected by the GPG-NH₂ treatment, even at as high concentration as 1 mM (see Paper I) (Fig. 12B).

A



B

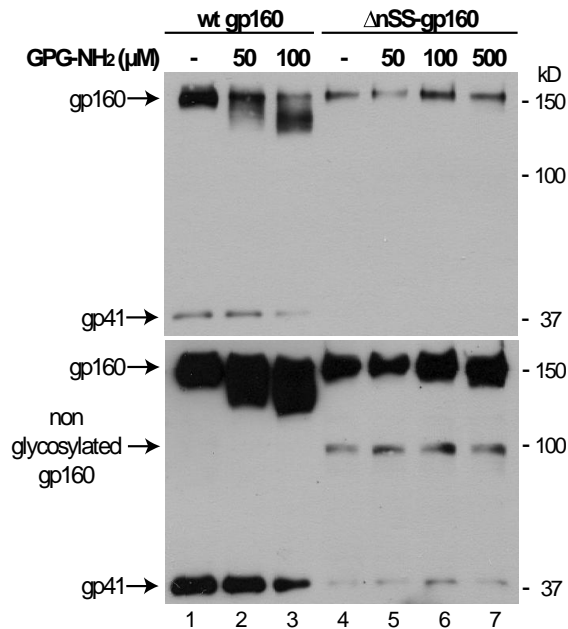


Figure 12. (A) The amino acid sequence of the gp160 native signal sequence (wt gp160) and the truncated signal sequence (ΔnSS-gp160). (B) Analysis of intracellular Env expression by Western blot. HeLa-tat III cells were transfected to express wt gp160 or ΔnSS -gp160 and treated with indicated GPG-NH₂ concentrations.

**PAPER II: GPG-NH₂ ACTS VIA THE METABOLITE αHGA TO TARGET HIV-1 ENV
TO THE ER-ASSOCIATED PROTEIN DEGRADATION PATHWAY**

STUDY BACKGROUND

GPG-NH₂ had in previous studies been shown to be metabolized via the intermediate metabolite G-NH₂ into the final metabolite αHGA due to enzymes in cell culture medium supplemented with 10 % fetal calf serum (FBS) (Fig. 13). As both metabolites retained the ability to inhibit HIV-1 propagation in the presence of FBS and the antiviral mechanism uncovered in Paper I was only shown for GPG-NH₂, in Paper II we examined whether the ability to target gp160 to ERAD is owing to GPG-NH₂ or its metabolites.

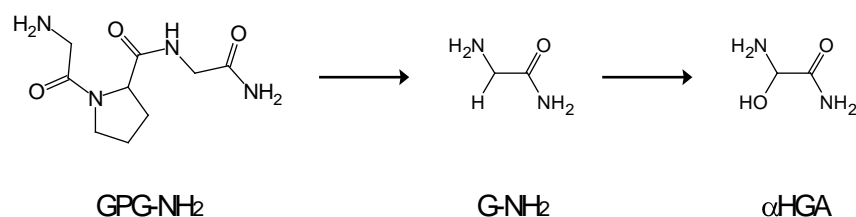


Figure 13. Scheme of GPG-NH₂ being metabolized in cell culture medium supplemented with 10% FBS.

**THE GPG-NH₂ AND ITS METABOLITES G-NH₂ AND αHGA ALL DECREASE THE
STEADY-STATE LEVELS, MOLECULAR MASS AND PROCESSING OF GP160**

In this study it was shown that both G-NH₂ and αHGA retained the ability of GPG-NH₂ to decrease the gp160 molecular mass, steady-state levels and processing to gp41. However, in comparison to GPG-NH₂, both G-NH₂ and αHGA showed a more potent activity (Fig. 14).

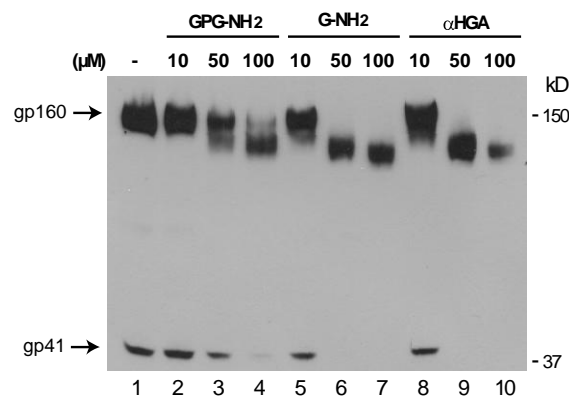


Figure 14. Analysis of intracellular Env expression by Western blot. HeLa-tat III cells were transfected to express gp160 and treated with GPG-NH₂, G-NH₂ or αHGA.

αHGA DOES NOT REQUIRE FBS TO AFFECT GP160

Both G-NH₂ and αHGA have, in a separate study, been shown to be active in serum from several other species than cow, however, only αHGA retained the anti HIV-1 activity in human serum (HS). It was here shown that the anti HIV-1 activity of αHGA in HS correlates with a preserved ability to target gp160 to ER-associated protein degradation, while both GPG-NH₂ and G-NH₂ had, as expected, no effect on gp160 in HS (Fig. 15, left panel). The effect of αHGA was further shown to be completely independent of HS or any sera at all as its effect on gp160 was observed in the absence of serum in the culture medium (Fig. 15, right panel).

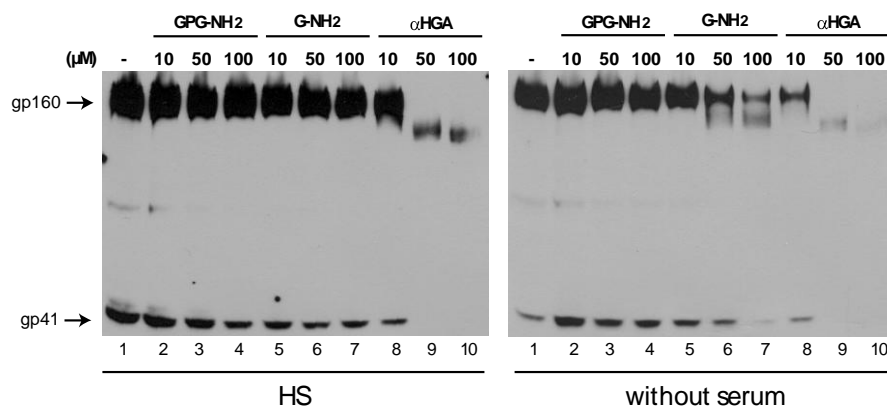


Figure 15. Analysis of intracellular Env expression by Western blot. HeLa-tat III cells were transfected to express gp160, cultured in cell culture medium containing 10 % HS (left panel) or no serum (right panel) and treated with GPG-NH₂, G-NH₂ or αHGA

αHGA TARGETS GP160 FOR DESTRUCTION FASTER THAN GPG-NH₂

The required time for cellular exposure to GPG-NH₂ and αHGA for a detectable effect on gp160 was shown to be significantly shorter for αHGA than GPG-NH₂. While the effect on gp160 benefited from addition of GPG-NH₂ as early as 18 h pre-transfection (Fig. 16A) the effect of αHGA was the strongest when added to the cell cultures 4 or 8 h post-transfection (Fig. 16B).

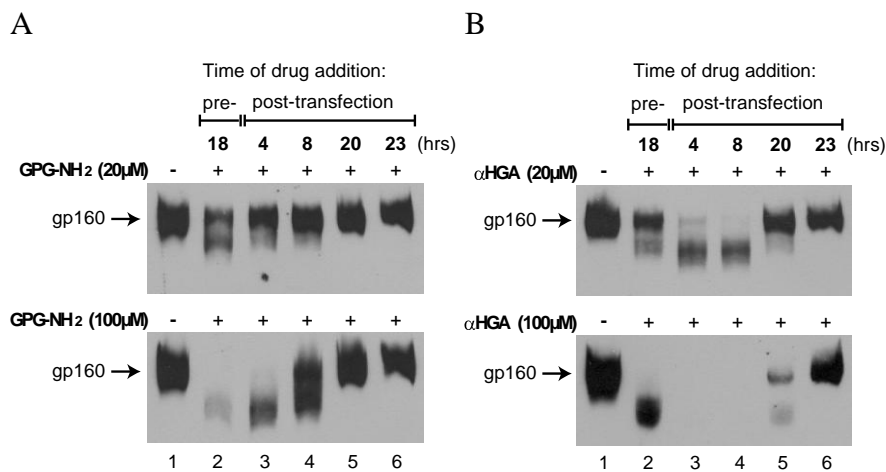


Figure 16. Analysis of intracellular gp160 expression by Western blot. HeLa-tat III cells were transfected to express gp160. GPG-NH₂ (**A**) or αHGA (**B**) was added at indicated concentrations and hours pre- or post-transfection.

αHGA DECREASES THE ENV CONTENT IN HIV-1 PARTICLES

Producing HIV-1 particles from the ACH-2 cell line in the presence of 20 μM αHGA showed that the viral particle content of gp41 was only about 15 % of the particles produced by untreated cells (Fig. 17). This was in Paper I shown to be the consequence of gp160 being targeted to for destruction via the ERAD pathway.

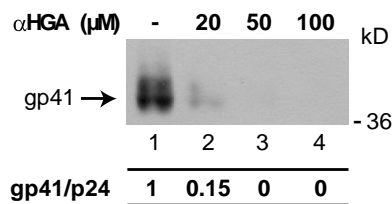


Figure 17. Env glycoprotein analysis by Western blot from equal amounts of HIV-1 particles produced by the chronically infected ACH-2 cells at the indicated αHGA concentrations.

PAPER III: UNEXPECTED EFFECTS OF GP160 SIGNAL SEQUENCE TRUNCATIONS ON HIV-1 REPLICATION

STUDY BACKGROUND

It was found in Paper I that 2/3 of the 30 amino acid long gp160 signal sequence (Fig. 18) could be truncated without destroying the ability of gp160 to co-translationally translocate into the ER. This raised the question of whether the missing 2/3 of the signal sequence could, apart from targeting gp160 to the ER, have additional functions important for the viral replication and the viral particle infectivity. This study explored this by creating viral mutants with various N-terminally truncated gp160 signal sequences.

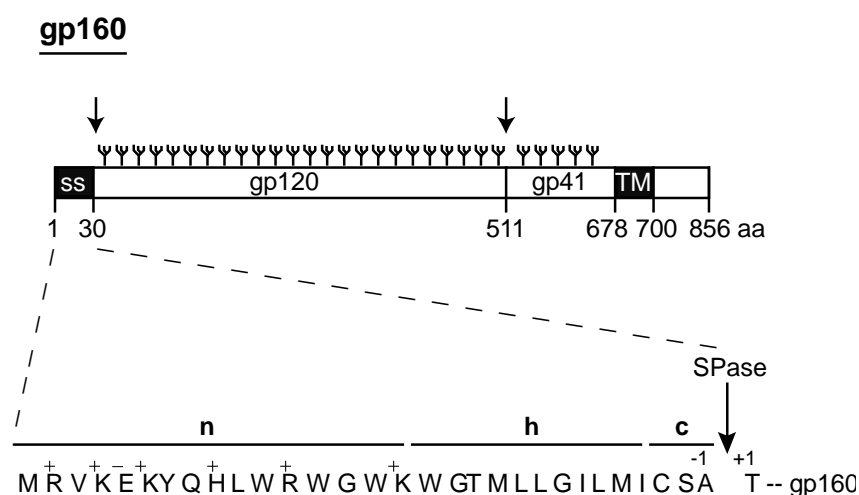


Figure 18. Linear structure of the Env precursor protein gp160 with depicted amino acid sequence of the signal sequence, the proteolytic cleavage sites (arrows) and the N-linked glycans (Ψ).

LOW PARTICLE RELEASE OF VPU DEFICIENT HIV-1 CAN BE OVERCOME IN CELLS CONSTITUTIVELY EXPRESSING TAT

As the gp160 signal sequence region overlaps parts of the Vpu gene, the Vpu start codon was mutated to prevent its expression (Fig. 19).

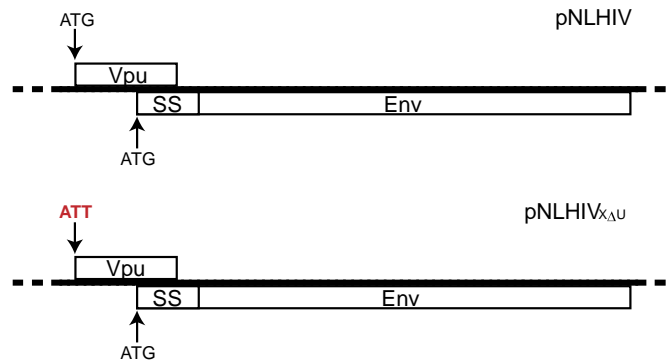


Figure 19. Schematic representation of the overlapping region of Vpu and Env open reading frames in the wild type proviral vector pNLHIV derived from pNL4-3. Depicted is the introduced mutation of the Vpu start codon in pNLHIVx Δ u.

However, Vpu down regulates the cellular protein Tetherin, which otherwise tethers budded HIV-1 particles to the cell surface and may result in poor viral particle release into the cell culture supernatant. It was here found that the Vpu deficient HIV-1 viral particle release was poor both from the Tetherin deficient cell line HT1080 and the Tetherin expressing HeLa cell. However, the Tetherin expressing HeLa-tat III cell line, which constitutively expresses the HIV-1 Tat protein, had at 24 h a poor release of Vpu deficient viral particles (pNLHIVx Δ u) but after 48 h the particle release had nearly reached 2/3 of the Vpu expressing HIV-1 virus (pNLHIV) (Fig. 20).

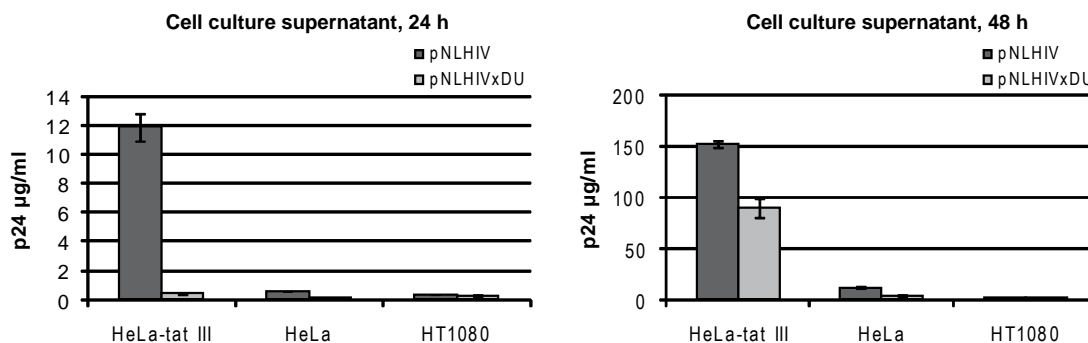


Figure 20. Cell lines tested for optimal Vpu deficient HIV-1 particle production. HeLa-tat III, HeLa and HT1080 were transfected to express wild type HIV-1 (pNLHIV) or Vpu deficient HIV-1 (pNLHIVx Δ u). The particle production was monitored by measuring the p24 levels after 24 h (left chart) and 48 h (right chart).

THE FULL LENGTH WILD TYPE SIGNAL SEQUENCE IS NOT REQUIRED TO TARGET GP160 TO THE ER

The gp160 signal sequence was gradually truncated from its N-terminal end, which created the viral mutants ss22, ss19, ss15, ss11, ss5 and Δ ss (the numbers indicate the number of residues remaining in the signal sequence) (Fig. 21).

| | | |
|--------------------------------------|---|------------|
| <u>pNLHIVΔss30</u> | M R V K E K Y Q H L W R W G W K W G T M L L G I L M I C S A | T -- gp160 |
| <u>pNLHIVΔss22</u> | ML W R W G W K W G T M L L G I L M I C S A | T -- gp160 |
| <u>pNLHIVΔss19</u> | M W G W K W G T M L L G I L M I C S A | T -- gp160 |
| <u>pNLHIVΔss15</u> | M W G T M L L G I L M I C S A | T -- gp160 |
| <u>pNLHIVΔss11</u> | ML L G I L M I C S A | T -- gp160 |
| <u>pNLHIVΔss5</u> | M I C S A | T -- gp160 |
| <u>pNLHIVΔss</u> | | M -- gp160 |

Figure 21. The amino acid sequence of the full length wild type gp160 signal sequence (pNLHIV Δ ss30) and the various truncated signal sequences created in the indicated proviral vectors.

Surprisingly, all the signal sequence truncations allowed intra cellular expression of gp160 and its targeting to the ER. However, the gp160 steady-state levels expressed from all the signal sequence mutants decreased, except for ss22 (Fig.22). In comparison to the wild type signal sequence (ss30 wt) the gp160 expression from ss22 showed that the first 8 aa are not necessary for adequate intra cellular gp160 expression. The 22 aa long signal sequence even increased the gp160 steady-state levels by about 12 % and its processing to gp41 by about 45 %, at 24 h post transfection. The deletion of another three aa (ss19), however, significantly decreased the expression of gp160 to only 26 % of the ss30 wt. Further truncations of the signal sequence drastically reduced the gp160 levels as seen for ss15, ss11, ss5 and Δ ss. However, of all the truncations it was only the complete removal of the signal sequence in Δ ss that inhibited gp160 processing to

gp41, which indicates altered features of the protein, as well as the distinct appearance of two other protein bands of lower molecular weight.

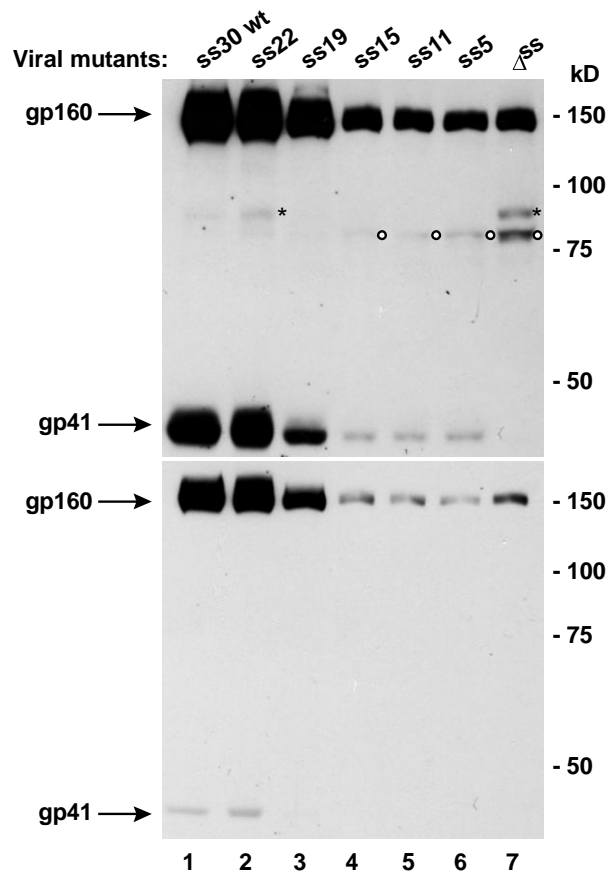


Figure 22. Analysis of intracellular Env expression by Western blot monitoring gp160 and gp41. HeLa-tat III cells were transfected to express gp160 from the indicated viral signal sequence mutants for 24 h. The top panel represents a longer and the bottom a shorter exposure time of the same immunoblot.

TRUNCATION OF THE FIRST EIGHT RESIDUES IN THE GP160 SIGNAL SEQUENCE INCREASES VIRAL PARTICLE INFECTIVITY

The gp160 signal sequence was found to strongly influence both the gp160 and the gp120/gp41 incorporation into the viral particles (Fig. 23A). Truncation of the first 8 N-terminal residues of signal sequence (ss22) nearly decreased the total Env content by 60 %. This loss was due to a reduction in the incorporation of gp160, which decreased by

approximately 80 %. The gp120/gp41 content on the other hand increased by about 20%. This altered composition of gp160 and gp120/gp41 content increased the ss22 particle infectivity by ~40 % compared to the ss30 wt particles (Fig. 23C). Further truncations strongly decreased the gp160 and gp120/gp41 incorporation into the ss19, ss15, ss11 and ss5 viral particles, as well as, their infectivity (Fig. 23B, C). No Env was detected in the Δ ss particles and as expected they were completely non-infectious.

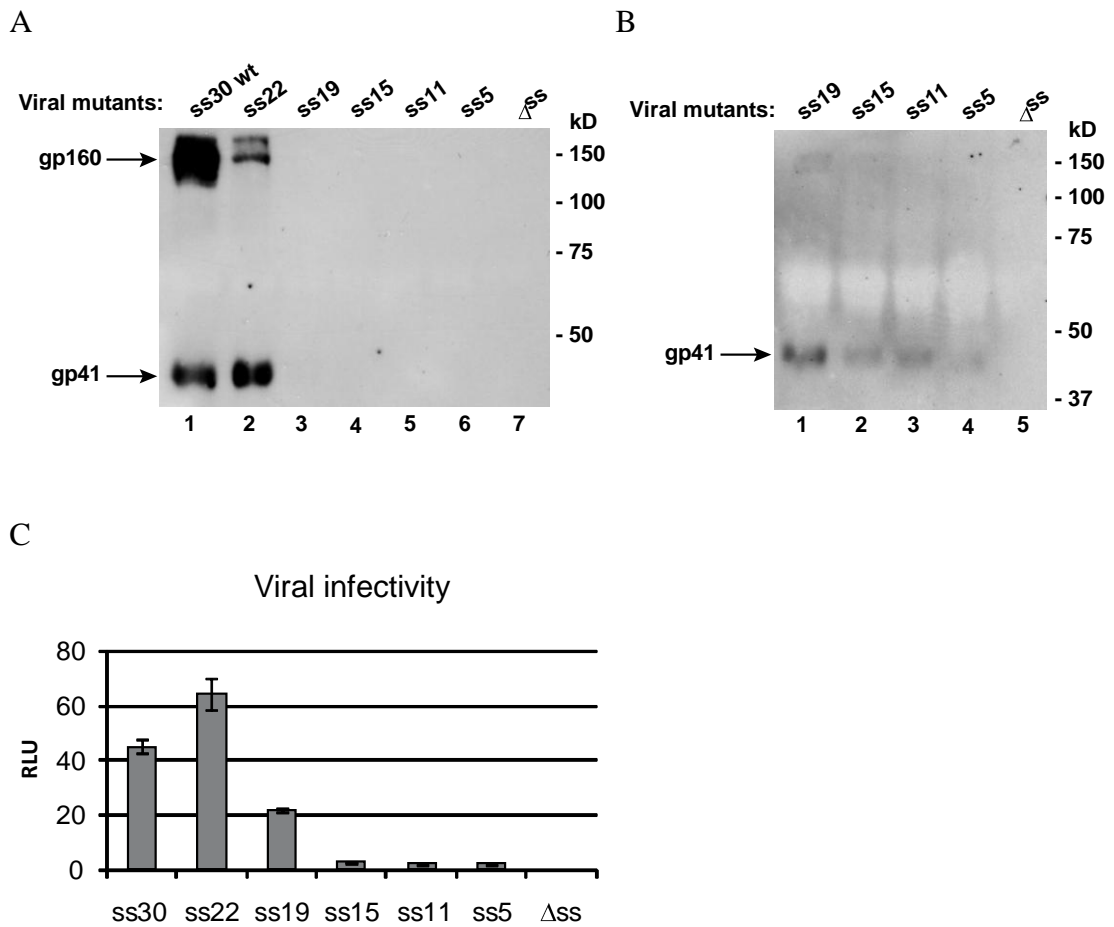
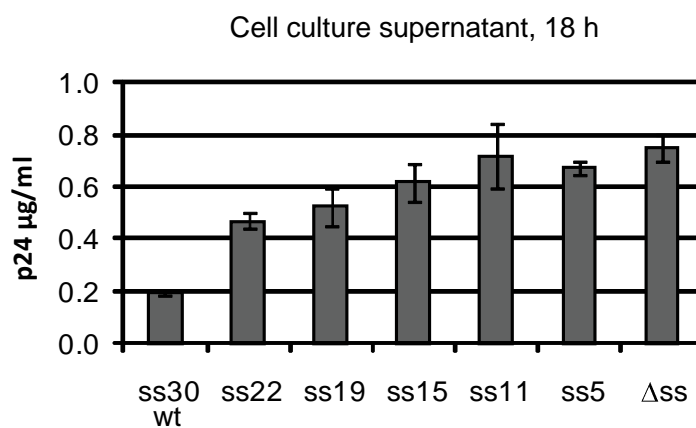


Figure 23. (A) Analysis by western blot of equal amount of viral particles for their content of gp160 and gp41. (B) Indicated viral mutants were re-analyzed as in (A) but with increased amount of virus. (C) Equal amounts of viral particles were used to infect TZM-bl cells. The relative viral particle infectivity was analyzed after 48 h by measuring the HIV-1 Tat driven luciferase expression in the TZM-bl cells.

THE WILD TYPE GP160 SIGNAL SEQUENCE AFFECTS THE VIRAL CAPSID PROTEIN (P24) EXPRESSION

The ss30 wild type virus was found to express lower levels of extra cellular capsid protein p24 compared to the truncated signal sequence mutants (Fig. 24 A). The lower extra cellular p24 levels corresponded to lower intra cellular p24 levels (Fig. 24 B). However, this difference between the ss30 wt and the truncated signal sequence mutants became less pronounced over time (at 48 h). This initial low p24 expression by the ss30 wt was shown not to be caused by cell death as the cellular release of lactate dehydrogenase was not elevated in these cells compared to the cells expressing the truncated signal sequence mutants.

A



B

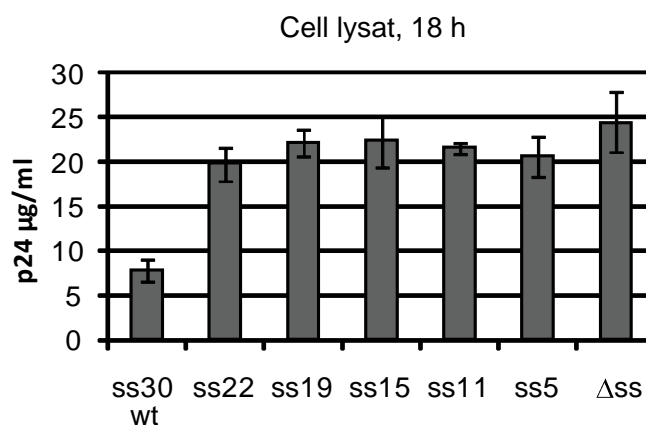


Figure 24. Analysis of p24 expression. Hela-tat III cells were transfected to produce the ss30 wt virus and the indicated truncated signal sequence mutants. (A) The extra cellular and (B) intra cellular p24 levels were analyzed 18 h post transfection.

DISSCUSSION

HIV-1 infections can today successfully be controlled for a long period of time by multidrug antiretroviral therapy. However, the inherent ability of HIV-1 to mutate and adapt to the environment in which it replicates will in some patients eventually give rise to multidrug resistant virus. This results in therapy failure and progression to AIDS. Therefore, development of new antiviral drugs with other mechanisms of action from those available is constantly needed.

The tri-peptide GPG-NH₂, which was originally designed to inhibit Env to interact with its secondary co-receptor to prevent viral fusion with the host cell, was found to indeed have antiviral properties. However, it did not block the viral entry as anticipated. In this thesis the antiviral action of GPG-NH₂ was further examined, unraveling a previously unexplored mechanism for suppressing HIV-1 propagation.

Although the GPG-NH₂ itself did not block viral fusion with cells, the viral particles produced by cells treated with the drug had a drastically reduced ability to fuse with cells. This was shown to be the consequence of reduced Env incorporation into the viral particles, which in turn was caused by the specific ability of GPG-NH₂ to direct maturing Env for destruction via the ERAD pathway. These findings are summarized in Figure 25, and illustrate the subsequent proteasome-mediated retrotranslocation of gp160 from the ER lumen into the cytoplasm, where gp160 becomes deglycosylated prior to its degradation by the proteasome. This corresponds with the shown requirement of proteasomal activity for the gp160 molecular mass, processing and steady-state levels to decrease upon GPG-NH₂ treatment.

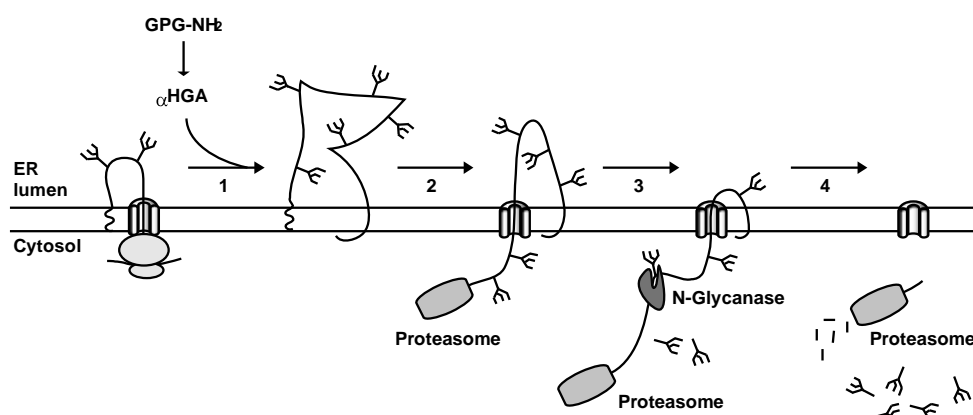


Figure 25. A proposed model for how the GPG-NH₂ metabolite αHGA targets gp160 for ERAD. Initially, gp160 is co-translationally translocated into the ER, where its growing peptide backbone becomes glycosylated and starts to fold. (1) In the presence of αHGA gp160 folds incorrectly, which targets it to ERAD. (2) Subsequently, gp160 is retro-translocated to the cytoplasm in a proteasome-mediated fashion, (3) where it becomes deglycosylated by the cytosolic N-glycanase prior to (4) degradation of its peptide backbone by the proteasome.

The GPG-NH₂ was in separate studies found to gain its ability to suppress HIV-1 propagation first upon being metabolized via the intermediate G-NH₂ to αHGA, by enzymes in FBS present in the cell culture media [137, 139, 140]. It was therefore tested and found that both G-NH₂ and αHGA retained the ability of GPG-NH₂ to target gp160 to the ERAD pathway, but at a higher efficiency. However, of the three compounds, only αHGA was capable of directing gp160 for destruction in human serum (HS). This was in line with separate findings showing that only αHGA inhibited viral propagation in the presence of HS [137](unpublished data). Like GPG-NH₂, αHGA did not suppress viral particle production from a single replication cycle, but did drastically reduce the Env incorporation into the viral particles. In addition, the ability of αHGA to target gp160 for destruction was not only found to be more potent than of GPG-NH₂, but also significantly faster, which further supports that the antiviral activity require GPG-NH₂ to convert to αHGA.

The effect of GPG-NH₂ was observed to be selective for gp160 as the tested cytosolic and non-glycosylated ER proteins remained unaffected and the cellular glycoprotein

LAMP-1 and an unidentified glycoprotein (~150 kD) were only slightly affected at concentrations as high as 0.5 to 1 mM. As for GPG-NH₂, αHGA showed no general effect on proteins as again only an unidentified glycoprotein of ~150 kD slightly increased its mobility. The significantly higher sensitivity of gp160 to the GPG-NH₂/αHGA treatment compared to the endogenous glycoproteins implies that gp160 has divergent features in its maturation process. One such known feature is that gp160 undergoes a rare posttranslational signal sequence cleavage in the ER that takes place first after substantial maturation and disulphide bond formations [54]. This unusually late signal sequences removal is directed in part by several positively charged residues in its n-region, which contribute to the prolonged existence of gp160 within the ER [81, 85]. This extends the exposure time of gp160 to GPG-NH₂ /αHGA and may thereby enhance its sensitivity to the drug. To examine the importance of these positively charged residues the entire n-region was truncated, which was interestingly found to completely abolish the effect of the drug. This may indicate that the prolonged exposure of gp160 to αHGA in the ER is the key factor for the effect. Alternatively, αHGA may disrupt the signal sequence insertion into the ER membrane by interacting with its n-region and that way prevent proper maturation of gp160.

In addition, in separate studies the GPG-NH₂ was observed to negatively influence on proper viral capsid formation [147]. This effect was again found not to be induced by GPG-NH₂, but by its metabolite αHGA by its binding to the hinge region of the capsid protein p24 [138]. Thus, the viral particles produced in the presence of αHGA apparently are rendered non-infectious by two separate modes of actions, i.e. their inability to bind to and fuse with cells due to reduced Env content, as well as, their distorted capsids. However, we have not noted any sequence similarities between the p24 and the Env signal sequence that could explain this dual effect of the αHGA.

The signal sequence, the main task of which is to target the protein for co-translational translocation into the ER, has for some viral glycoproteins been shown to have post ER targeting functions in the viral replication cycle [148, 149]. The observation that the entire n-region, which is 2/3 of the gp160 signal sequence, did not abolish the ER targeting function nor the processing of gp160 to gp120/gp41 in the Golgi, indicated a possibility of additional, yet unknown, functions of the signal sequence. Therefore, to investigate the importance of the entire native gp160 signal sequence for the viral

replication, it was gradually truncated from the N-terminal end in an HIV-1 proviral vector coding for infectious virus. The initial truncation of 8 aa showed that the remaining 22 aa of the signal sequence were more than sufficient for adequate targeting of gp160 (ss22) to the ER as the gp160 expression levels even somewhat increased compared to the gp160 wild type signal sequence (ss30 wt). However, the major and surprising effects of this truncation were observed within the viral particles. The ratio of incorporated gp120/gp41 increased by ~20 %, while the gp160 content decreased by ~80 %, compared to the signal sequence wild type particles. Hence, the total viral glycoprotein content decreased by nearly 60 % in the ss22 compared to the ss30 wt particles. This altered gp160 and gp120/gp41 composition in the ss22 particles increased their infectivity by 40 %. Presumably, this is a consequence of their increased gp120/gp41 content as only gp120/gp41 and not the precursor gp160 can mediate viral entry into the cells. Since signal sequences are cleaved off from proteins in the ER and the gp160 processing to gp120/gp41 takes place in the Golgi, this suggests that the native gp160 signal sequence possesses post ER targeting function in the Golgi that decreases the efficiency by which gp160 is processed by the cellular endoproteases. Alternatively, the signal sequence regulates the gp160 or gp120/gp41 transport to the site of viral assembly or the viral Env incorporation during viral assembly. Although, this regulatory effect of the wt signal sequence was not beneficial for the viral particle infectivity, it may help the virus in evading host immune responses or simply restrict its infectivity to prolong the viral coexistence with its host. Further truncations of the signal sequence, however, strongly decreased the gp160 expression levels, which consequently decreased the Env content in the particles and thereby also their infectivity.

The native gp160 signal sequence was observed to have an additional effect on the viral replication cycle unrelated to gp160 expression and trafficking. In the presence of the native gp160 signal sequence the intracellular p24 levels were initially lower and the viral particle release initiated later than in its absence or presence of the truncated gp160 signal sequences. However, further studies are required to elucidate the mechanism of this effect, as well as, to understand its beneficial function for the viral replication.

In conclusion these data illustrate that changes in the viral particle Env content and composition has a profound effect on the HIV-1 infectivity, which can be achieved by

targeting selective steps in its biosynthesis and that small molecules may be utilized therapeutically to target unwanted pathogenic proteins for degradation by the existing cellular machinery.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In summary, the main findings of this thesis are:

- GPG-NH₂ treatment of HIV-1 infected cells results in Env being targeted for degradation via the ERAD pathway, which reduces Env incorporation into the HIV-1 particles and results in decreased viral particle infectivity.
- The observed effect on Env and viral particle infectivity is not owing to GPG-NH₂, but to its active metabolite α HGA.
- The wild type signal sequence of Env_{NL4-3} has a regulatory function on the viral particle content and ratio of incorporated gp160 vs gp120/gp41, which regulates HIV-1 particle infectivity.
- The wild type Env_{NL4-3} signal sequence suppresses early intra and extracellular expression of the capsid protein p24.

Development of new antiviral compounds with different modes of action from those currently available drugs is necessary to combat HIV-1 infection as multidrug resistance poses a significant problem. This thesis illustrates that small molecules may be utilized therapeutically to specifically target unwanted pathogenic proteins for degradation by the existing cellular machinery. The ability of the GPG-NH₂/ α HGA to target Env to the ERAD pathway, which ultimately reduces the HIV-1 particle infectivity, was shown to require the entire native Env signal sequence. N-terminal truncation of the signal sequence by 2/3 rendered Env insensitive to the drug. Although, this finding provides a lead, further studies are required to localize the exact site of the α HGA interaction to fully understand the mechanism by which it targets Env for destruction.

In this thesis it was further shown that the Env signal sequence regulates the gp160 and gp120/gp41 incorporation into the viral particles by promoting incorporation of gp160

and reducing incorporation of gp120/gp41. Truncation of the first 8 N-terminal aa decreased the gp160 content by ~ 80% and increased the gp120/gp41 content by ~20% in the particles. However, the mechanism behind this regulatory function is unknown and requires further studies to be unraveled.

The wild type signal sequence was additionally found to suppress early expression of both extra and intra cellular p24. This effect was abolished when the signal sequence was truncated. Further studies are needed to understand both the mechanism and the viral benefit of this effect.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Humant immunbrist virus (HIV) måste liksom alla virus infektera celler för att föröka sig. Detta för att de själva saknar både förmåga att alstra energi ur näringsämnen och maskineri som krävs för att tillverka sina egna beståndsdelar, så som tex proteiner. HIV infekterar främst en typ av celler som ingår i immunförsvaret och kallas T-celler. När flertalet av dessa celler på grund av HIV infektionen försvinner utvecklas ett sjukdomstillsånd som kallas för förvärvad immunbristsjukdom (AIDS). Det är ett tillstånd då immunförsvaret tappat sin skyddande förmåga mot virus-, bakterie-, parasitinfektioner och maligna sjukdomar. En HIV infektion går idag inte att bota, men dess utvecklingen till AIDS går att fördröja m h a läkemedel som därför kallas bromsmediciner.

HIV har en hög mutationsfrekvens, vilket gör att viruset snabbt anpassar sig efter och smiter undan den infekterade individens immunförsvär. Av samma anledning blir HIV lätt motståndskraftigt mot läkemedel. Det finns idag ett flertal läkemedel att tillgå som hämmar virusets livscykel i olika skeden. För att fördröja uppkomst av läkemedelsresistent virus ges vid behandling en kombination av dessa olika läkemedel. Det är nämligen svårare för virus att utveckla resistens mot flera läkemedel sammtidigt. Dock uppkommer multiresistent HIV till slut och då går det inte längre att hindra att infektionen leder till AIDS. Multiresistensutvecklingen är ett problem vid behandlingen av HIV och behovet av nya läkemedel med nya verkningsmekanismer är därför också stort.

Det sfäriskt formade HIV viruset har ett yttre hölje, som är uppbyggt på samma sätt som cellens plasmamembran. Från virus höljet sticker proteinkomplex ut. Dessa består av det höljeförankrade proteinet gp41 till vilket ytproteinet gp120 är bundet. Med hjälp av gp120-gp41 komplexet binder viruset till T-cellen och smälter samman sitt hölje med cellens. På så vis töms virusets innehåll in i cellen och infektionen är därmed påbörjad.

Den infekterade cellen kommer för virusets räkning att producera alla de protein och byggstenar virusets arvsmassa kodar för. HIV virusets ytproteinkomplex gp120-gp41 syntetiseras initialt till proteinet, gp160, som utgörs av ca 860 aminosyror ihopsatta till

en lång kedja. De första 30 aminosyrorerna fungerar som en signalsekvens, som skickar proteinet till den cellulära avdelningen endoplasmatiska retiklet (ER). Där kommer signalsekvensen, när aminosyrakedjan syntetiserats klart, att klyvas av. I ER kommer aminosyrakedjan vidare att veckas på ett bestämt sätt tills proteinet har fått rätt form. När gp160 är färdigt transporteras det vidare till den cellulära avdelningen Golgi. I Golgi finns cellulära enzymer som klyver gp160 till gp120 och gp41. Gp120-gp41 komplexet transporteras därefter till cellytan och fogas in i viruspartiklarna, som knoppas av från cellhöljet och lösgör sig därmed från cellen. De nya HIV partiklarna är därmed redo att binda till och smälta ihop med nya ännu oinfekterade celler.

Det har i tidigare påvisats att den syntetiska molekylerna glycyL-prolyl-glycinamid (GPG-NH₂) hämmar tillväxten av HIV i cellkulturer. I första studien av denna avhandling visas att den enskilda infekterade cellen trots allt producerar HIV-1 partiklar i oförminskad takt, i närvaro av GPG-NH₂. Dock avstannar virusspridningen, då dessa HIV partiklar är oförmögna att ta sig in i nya celler. Denna oförmåga visade sig bero på att viruspartiklarna saknar just sin viktigaste komponent för detta ändamål, nämligen ytproteinkomplexet gp120-gp41. För att förstå mekanismen bakom detta behandlades infekterade celler med GPG-NH₂, var efter deras produktion av gp160 undersöktes. Vi fann att GPG-NH₂ stör veckningen av gp160 i ER, som därmed antar en felaktig form. Dessa felformade gp160 protein känns igen av cellen, som skickar dem till nedbrytning. Följdaktligen uppstår brist på gp120-gp41, vilket i sin tur resulterar i produktion av icke infektiösa viruspartiklar.

Avhandlingens andra studie påvisar att det inte är själva GPG-NH₂ som orsakar nedbrytningen av gp160, utan molekylerna alfa hydroxyglycinamid (α HGA). Denna uppstår då GPG-NH₂ metaboliseras av enzymer i cellkulturen.

I avhandlingens tredje studie undersöks betydelsen av gp160 proteinets signalsekvens för syntesen av gp160 i cellen, mängden infogat gp160 och gp120-gp41 i viruspartiklarna och till följd även deras förmåga att ta sig in i celler. En stor andel gp160 infogas HIV partiklar, trots att det endast är dess klyvda form gp120-gp41 som kan mediera virusets inträde i cellen. Studien visade de första 8 av signalsekvensens 30 aminosyror minskar viruspartiklarnas infektivitet med ca 40 % genom att öka andelen gp160 och minska andelen gp120-gp41 i viruspartiklarna. Detta visar att gp160

proteinets signalsekvens har en reglerande funktion på viruspartiklarnas sammansättning av gp160 och gp120-gp41 och därmed även på virusets infektivitet.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone, who directly or indirectly has contributed to this work, especially to:

My supervisor Professor Anders Vahlne, thank you for recruiting me to your group and giving me the chance of becoming a scientist and to study HIV. I am deeply grateful for the scientific freedom you have given me, everything you have taught me, the financial security and your generosity.

Robert Daniels, I most of all want to thank you for your friendship and endless encouragements. You helped me to believe in myself. I am grateful for all that you have taught me and particular for putting so much effort in helping me writing my first paper. THANK YOU!

Samir Abdurahman (known as Kepsen), I can't imagine what I would have done without you all these years. Your wonderful sense of humor kept me sane! Thank you for all the laughter, scientific discussions, company in the lab and in the office, jokes, help, frustration sharing and cookies!

Prof. Dan Hebert, thank you for our collaboration and for your guidance and kind hospitality while I was working in your lab. You have been very supportive and encouraging. It was a great time and truly a great experience for me.

My previous co-supervisor Laura Goobar-Larsson, thank you for your support, belief in and enthusiasm for my research results! I am grateful for our scientific discussions, your support and guidance.

Prof. Stefan Höglund, thank you for a fruitful collaboration, great EM work, for your friendly support, honesty and kindness.

Annika Lindkvist, most of all for your friendship, but also for all our interesting and entertaining debates on political, environmental and broken heart issues! ☺ Going for lunch has not been the same since you graduated.

Maria Perdomo, it has been a real pleasure to be in the same group and share the daily events in the lab and office all these years with you. Thank you!

Selina Poon, thank you for your friendly, kind and fun company. How would I have held out in the p3 plasmid lab without you?! ☺

All the past and present members of our group and staff at Tripep AB: Yi-Ping, Mojgan, Jin, Ulf, Haifa, Massoud, Ivana, Valteri, Pia, Mikael, Yuba, Cattis, Jan, Åsa, Sung-Oun and Gabi.

All the past and present students and co-workers at the division of Clinical Virology/
Microbiology for creating a great atmosphere!

Also Jim Cormier, Bradley Pearse and Ning Wang for your friendly help in the lab and
making my time at UMASS so nice!

Gudrun Rafi and Kerstin Lindholm for all your help with formalities and paper work!

The former and present personnel at the Clinical Microbiology/Virology, F68, with
special thanks to: Charles and the staff at HIV/Hepatitis, Eileen, Claes, Anne Q,
Hamsa, Pascallis and Marit.

Mina käraste vänner Jessica och Ann-Charlotte som har funnits med i alla år...ja, redan
sen tidiga barnsben! Tack för att ni aldrig glömde bort mig fastän jag jämt och ständigt
var upptagen med att "leka" med mina provrör.

Anna och lilla Gråbert, tack för att ni så många gånger fyllt på med positiv energi, när
jag varit sliten av doktorandlivet.

Sung-Oun, tack för att du alltid ger mig positiv energi så fort vi ses eller hörs!

Mest av alla vill jag tacka mina underbara föräldrar, Veri och Joze, och min bror
Davorin. Allt jag är och allt jag någonsin har uppnått är tack vare er, även denna
examen!

REFERENCES

1. UNAIDS. [cited; Available from: <http://www.unaids.org/>.
2. Smittskyddsinstitutet. [cited; Available from: www.smittskyddsinstitutet.se.
3. Corbitt, G., A.S. Bailey, and G. Williams, *HIV infection in Manchester, 1959*. Lancet, 1990. **336**(8706): p. 51.
4. Zhu, T., et al., *An African HIV-1 sequence from 1959 and implications for the origin of the epidemic*. Nature, 1998. **391**(6667): p. 594-7.
5. Froland, S.S., et al., *HIV-1 infection in Norwegian family before 1970*. Lancet, 1988. **1**(8598): p. 1344-5.
6. Garry, R.F., et al., *Documentation of an AIDS virus infection in the United States in 1968*. Jama, 1988. **260**(14): p. 2085-7.
7. Gottlieb, M.S., et al., *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency*. N Engl J Med, 1981. **305**(24): p. 1425-31.
8. Hymes, K.B., et al., *Kaposi's sarcoma in homosexual men-a report of eight cases*. Lancet, 1981. **2**(8247): p. 598-600.
9. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)*. Science, 1983. **220**(4599): p. 868-71.
10. Vahlne, A., *A historical reflection on the discovery of human retroviruses*. Retrovirology, 2009. **6**: p. 40.
11. Coffin, J., et al., *Human immunodeficiency viruses*. Science, 1986. **232**(4751): p. 697.
12. Clavel, F., et al., *Isolation of a new human retrovirus from West African patients with AIDS*. Science, 1986. **233**(4761): p. 343-6.
13. Korber, B., et al., *Timing the ancestor of the HIV-1 pandemic strains*. Science, 2000. **288**(5472): p. 1789-96.
14. Wertheim, J.O. and M. Worobey, *Dating the age of the SIV lineages that gave rise to HIV-1 and HIV-2*. PLoS Comput Biol, 2009. **5**(5): p. e1000377.
15. Worobey, M., et al., *Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960*. Nature, 2008. **455**(7213): p. 661-4.
16. Hahn, B.H., et al., *AIDS as a zoonosis: scientific and public health implications*. Science, 2000. **287**(5453): p. 607-14.
17. Hirsch, V.M., et al., *An African primate lentivirus (SIVsm) closely related to HIV-2*. Nature, 1989. **339**(6223): p. 389-92.
18. Ho, D.D., T.R. Rota, and M.S. Hirsch, *Infection of monocyte/macrophages by human T lymphotropic virus type III*. J Clin Invest, 1986. **77**(5): p. 1712-5.
19. Klatzmann, D., et al., *Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes*. Science, 1984. **225**(4657): p. 59-63.
20. Klatzmann, D., et al., *T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV*. Nature, 1984. **312**(5996): p. 767-8.
21. Patterson, S. and S.C. Knight, *Susceptibility of human peripheral blood dendritic cells to infection by human immunodeficiency virus*. J Gen Virol, 1987. **68** (Pt 4): p. 1177-81.
22. Pantaleo, G. and A.S. Fauci, *Immunopathogenesis of HIV infection*. Annu Rev Microbiol, 1996. **50**: p. 825-54.
23. Clark, S.J., et al., *High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection*. N Engl J Med, 1991. **324**(14): p. 954-60.

24. Lindback, S., et al., *Viral dynamics in primary HIV-1 infection*. Karolinska Institutet Primary HIV Infection Study Group. *Aids*, 2000. **14**(15): p. 2283-91.
25. Gaines, H., et al., *Clinical picture of primary HIV infection presenting as a glandular-fever-like illness*. *Bmj*, 1988. **297**(6660): p. 1363-8.
26. Kinloch-de Loes, S., et al., *Symptomatic primary infection due to human immunodeficiency virus type 1: review of 31 cases*. *Clin Infect Dis*, 1993. **17**(1): p. 59-65.
27. Brostrom, C., A. Sonnerberg, and M. Sallberg, *Human immunodeficiency virus type 1-infected patients with no disease progression display high-avidity antibody production to autologous V3 sequences*. *J Infect Dis*, 1995. **171**(2): p. 509-11.
28. Bower, M., C. Palmieri, and T. Dhillon, *AIDS-related malignancies: changing epidemiology and the impact of highly active antiretroviral therapy*. *Curr Opin Infect Dis*, 2006. **19**(1): p. 14-9.
29. Welch, K. and A. Morse, *The clinical profile of end-stage AIDS in the era of highly active antiretroviral therapy*. *AIDS Patient Care STDS*, 2002. **16**(2): p. 75-81.
30. Plantier, J.C., et al., *A new human immunodeficiency virus derived from gorillas*. *Nat Med*, 2009. **15**(8): p. 871-2.
31. Vallari, A., et al., *Confirmation of putative HIV-1 group P in Cameroon*. *J Virol*. **85**(3): p. 1403-7.
32. Stebbing, J. and G. Moyle, *The clades of HIV: their origins and clinical significance*. *AIDS Rev*, 2003. **5**(4): p. 205-13.
33. Requejo, H.I., *Worldwide molecular epidemiology of HIV*. *Rev Saude Publica*, 2006. **40**(2): p. 331-45.
34. Muesing, M.A., et al., *Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus*. *Nature*, 1985. **313**(6002): p. 450-8.
35. Klaver, B. and B. Berkhout, *Comparison of 5' and 3' long terminal repeat promoter function in human immunodeficiency virus*. *J Virol*, 1994. **68**(6): p. 3830-40.
36. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA*. *Annu Rev Biochem*, 1998. **67**: p. 1-25.
37. Dalgleish, A.G., et al., *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus*. *Nature*, 1984. **312**(5996): p. 763-7.
38. Broder, C.C. and R.G. Collman, *Chemokine receptors and HIV*. *J Leukoc Biol*, 1997. **62**(1): p. 20-9.
39. Kozak, S.L., et al., *CD4, CXCR-4, and CCR-5 dependencies for infections by primary patient and laboratory-adapted isolates of human immunodeficiency virus type 1*. *J Virol*, 1997. **71**(2): p. 873-82.
40. Chan, D.C. and P.S. Kim, *HIV entry and its inhibition*. *Cell*, 1998. **93**(5): p. 681-4.
41. Shu, W., H. Ji, and M. Lu, *Interactions between HIV-1 gp41 core and detergents and their implications for membrane fusion*. *J Biol Chem*, 2000. **275**(3): p. 1839-45.
42. Arhel, N.J., et al., *HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore*. *Embo J*, 2007. **26**(12): p. 3025-37.
43. Mulky, A. and J.C. Kappes, *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*, 2005. **49**(9): p. 3762-9.
44. Mulky, A., et al., *Subunit-specific analysis of the human immunodeficiency virus type 1 reverse transcriptase in vivo*. *J Virol*, 2004. **78**(13): p. 7089-96.

45. Mansky, L.M. and H.M. Temin, *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase*. J Virol, 1995. **69**(8): p. 5087-94.
46. Preston, B.D., B.J. Poiesz, and L.A. Loeb, *Fidelity of HIV-1 reverse transcriptase*. Science, 1988. **242**(4882): p. 1168-71.
47. Sherman, M.P. and W.C. Greene, *Slipping through the door: HIV entry into the nucleus*. Microbes Infect, 2002. **4**(1): p. 67-73.
48. Miller, M.D., C.M. Farnet, and F.D. Bushman, *Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition*. J Virol, 1997. **71**(7): p. 5382-90.
49. Schwartz, S., et al., *Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1*. J Virol, 1990. **64**(6): p. 2519-29.
50. Garcia, J.A., et al., *Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat*. Embo J, 1988. **7**(10): p. 3143-7.
51. Rosen, C.A., *Regulation of HIV gene expression by RNA-protein interactions*. Trends Genet, 1991. **7**(1): p. 9-14.
52. Suhasini, M. and T.R. Reddy, *Cellular proteins and HIV-1 Rev function*. Curr HIV Res, 2009. **7**(1): p. 91-100.
53. Finzi, A., et al., *Productive human immunodeficiency virus type 1 assembly takes place at the plasma membrane*. J Virol, 2007. **81**(14): p. 7476-90.
54. Land, A., D. Zonneveld, and I. Braakman, *Folding of HIV-1 envelope glycoprotein involves extensive isomerization of disulfide bonds and conformation-dependent leader peptide cleavage*. Faseb J, 2003. **17**(9): p. 1058-67.
55. Lu, M., S.C. Blacklow, and P.S. Kim, *A trimeric structural domain of the HIV-1 transmembrane glycoprotein*. Nat Struct Biol, 1995. **2**(12): p. 1075-82.
56. McCune, J.M., et al., *Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus*. Cell, 1988. **53**(1): p. 55-67.
57. Kaplan, A.H., M. Manchester, and R. Swanstrom, *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, 1994. **68**(10): p. 6782-6.
58. Pettit, S.C., et al., *Initial cleavage of the human immunodeficiency virus type 1 GagPol precursor by its activated protease occurs by an intramolecular mechanism*. J Virol, 2004. **78**(16): p. 8477-85.
59. Morikawa, Y., et al., *In vitro processing of human immunodeficiency virus type 1 Gag virus-like particles*. Virology, 2000. **272**(2): p. 366-74.
60. Wiegers, K., et al., *Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites*. J Virol, 1998. **72**(4): p. 2846-54.
61. Briggs, J.A., et al., *Structural organization of authentic, mature HIV-1 virions and cores*. Embo J, 2003. **22**(7): p. 1707-15.
62. von Schwedler, U.K., et al., *Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly*. Embo J, 1998. **17**(6): p. 1555-68.
63. Maldarelli, F., et al., *Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein*. J Virol, 1993. **67**(8): p. 5056-61.
64. Fujita, K., S. Omura, and J. Silver, *Rapid degradation of CD4 in cells expressing human immunodeficiency virus type 1 Env and Vpu is blocked by proteasome inhibitors*. J Gen Virol, 1997. **78** (Pt 3): p. 619-25.

65. Margottin, F., et al., *A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif*. Mol Cell, 1998. **1**(4): p. 565-74.
66. Schubert, U., et al., *CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway*. J Virol, 1998. **72**(3): p. 2280-8.
67. Mangeat, B., et al., *HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation*. PLoS Pathog, 2009. **5**(9): p. e1000574.
68. Lv, M., et al., *Polarity changes in the transmembrane domain core of HIV-1 Vpu inhibits its anti-tetherin activity*. PLoS One. **6**(6): p. e20890.
69. Kupzig, S., et al., *Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology*. Traffic, 2003. **4**(10): p. 694-709.
70. Gaddis, N.C., et al., *Comprehensive investigation of the molecular defect in vif-deficient human immunodeficiency virus type 1 virions*. J Virol, 2003. **77**(10): p. 5810-20.
71. Harris, R.S., et al., *DNA deamination mediates innate immunity to retroviral infection*. Cell, 2003. **113**(6): p. 803-9.
72. Lecossier, D., et al., *Hypermutation of HIV-1 DNA in the absence of the Vif protein*. Science, 2003. **300**(5622): p. 1112.
73. Fujita, M., et al., *Multifaceted activity of HIV Vpr/Vpx proteins: the current view of their virological functions*. Rev Med Virol. **20**(2): p. 68-76.
74. Le Rouzic, E. and S. Benichou, *The Vpr protein from HIV-1: distinct roles along the viral life cycle*. Retrovirology, 2005. **2**: p. 11.
75. Garcia, J.V. and A.D. Miller, *Serine phosphorylation-independent downregulation of cell-surface CD4 by nef*. Nature, 1991. **350**(6318): p. 508-11.
76. Kirchhoff, F., et al., *Role of Nef in primate lentiviral immunopathogenesis*. Cell Mol Life Sci, 2008. **65**(17): p. 2621-36.
77. Leonard, J.A., et al., *HIV-1 Nef disrupts intracellular trafficking of major histocompatibility complex class I, CD4, CD8, and CD28 by distinct pathways that share common elements*. J Virol. **85**(14): p. 6867-81.
78. Schwartz, S., et al., *Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs*. J Virol, 1990. **64**(11): p. 5448-56.
79. Berman, P.W., W.M. Nunes, and O.K. Haffar, *Expression of membrane-associated and secreted variants of gp160 of human immunodeficiency virus type 1 in vitro and in continuous cell lines*. J Virol, 1988. **62**(9): p. 3135-42.
80. Haffar, O.K., D.J. Dowbenko, and P.W. Berman, *Topogenic analysis of the human immunodeficiency virus type 1 envelope glycoprotein, gp160, in microsomal membranes*. J Cell Biol, 1988. **107**(5): p. 1677-87.
81. Li, Y., et al., *Control of expression, glycosylation, and secretion of HIV-1 gp120 by homologous and heterologous signal sequences*. Virology, 1994. **204**(1): p. 266-78.
82. Earl, P.L., B. Moss, and R.W. Doms, *Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein*. J Virol, 1991. **65**(4): p. 2047-55.
83. Li, H., et al., *Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes*. J Immunol, 2008. **180**(6): p. 4011-21.
84. Otteken, A. and B. Moss, *Calreticulin interacts with newly synthesized human immunodeficiency virus type 1 envelope glycoprotein, suggesting a chaperone function similar to that of calnexin*. J Biol Chem, 1996. **271**(1): p. 97-103.

85. Li, Y., et al., *The HIV-1 Env protein signal sequence retards its cleavage and down-regulates the glycoprotein folding*. Virology, 2000. **272**(2): p. 417-28.
86. Earl, P.L., R.W. Doms, and B. Moss, *Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein*. Proc Natl Acad Sci U S A, 1990. **87**(2): p. 648-52.
87. Pinter, A., et al., *Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1*. J Virol, 1989. **63**(6): p. 2674-9.
88. Schawaller, M., et al., *Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV*. Virology, 1989. **172**(1): p. 367-9.
89. Freed, E.O., D.J. Myers, and R. Risser, *Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160*. J Virol, 1989. **63**(11): p. 4670-5.
90. Hallenberger, S., et al., *Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160*. Nature, 1992. **360**(6402): p. 358-61.
91. Ozel, M., G. Pauli, and H.R. Gelderblom, *The organization of the envelope projections on the surface of HIV*. Arch Virol, 1988. **100**(3-4): p. 255-66.
92. Chertova, E., et al., *Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus*. J Virol, 2002. **76**(11): p. 5315-25.
93. Zhu, P., et al., *Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions*. Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15812-7.
94. Zhu, P., et al., *Distribution and three-dimensional structure of AIDS virus envelope spikes*. Nature, 2006. **441**(7095): p. 847-52.
95. Jelicic, A., et al., *Small molecule targets Env for ER-associated protein degradation and inhibits HIV-1 propagation*. J Virol, 2009.
96. Moore, P.L., et al., *Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1*. J Virol, 2006. **80**(5): p. 2515-28.
97. Checkley, M.A., B.G. Luttge, and E.O. Freed, *HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation*. J Mol Biol. **410**(4): p. 582-608.
98. Cronin, J., X.Y. Zhang, and J. Reiser, *Altering the tropism of lentiviral vectors through pseudotyping*. Curr Gene Ther, 2005. **5**(4): p. 387-98.
99. Ott, D.E., *Cellular proteins detected in HIV-1*. Rev Med Virol, 2008. **18**(3): p. 159-75.
100. Page, K.A., N.R. Landau, and D.R. Littman, *Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity*. J Virol, 1990. **64**(11): p. 5270-6.
101. Wilk, T., T. Pfeiffer, and V. Bosch, *Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product*. Virology, 1992. **189**(1): p. 167-77.
102. Freed, E.O. and M.A. Martin, *Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix*. J Virol, 1995. **69**(3): p. 1984-9.
103. Freed, E.O. and M.A. Martin, *Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions*. J Virol, 1996. **70**(1): p. 341-51.
104. Mammano, F., et al., *Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains*. J Virol, 1995. **69**(6): p. 3824-30.

105. Reil, H., et al., *Efficient HIV-1 replication can occur in the absence of the viral matrix protein*. *Embo J*, 1998. **17**(9): p. 2699-708.
106. Owens, R.J., et al., *Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells*. *Proc Natl Acad Sci U S A*, 1991. **88**(9): p. 3987-91.
107. Holm, K., et al., *Human immunodeficiency virus type 1 assembly and lipid rafts: Pr55(gag) associates with membrane domains that are largely resistant to Brij98 but sensitive to Triton X-100*. *J Virol*, 2003. **77**(8): p. 4805-17.
108. Ono, A. and E.O. Freed, *Role of lipid rafts in virus replication*. *Adv Virus Res*, 2005. **64**: p. 311-58.
109. Pickl, W.F., F.X. Pimentel-Muinos, and B. Seed, *Lipid rafts and pseudotyping*. *J Virol*, 2001. **75**(15): p. 7175-83.
110. Camus, G., et al., *The clathrin adaptor complex AP-1 binds HIV-1 and MLV Gag and facilitates their budding*. *Mol Biol Cell*, 2007. **18**(8): p. 3193-203.
111. Lopez-Verges, S., et al., *Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions*. *Proc Natl Acad Sci U S A*, 2006. **103**(40): p. 14947-52.
112. Wyss, S., et al., *The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]*. *J Virol*, 2001. **75**(6): p. 2982-92.
113. Lander, E.S., et al., *Initial sequencing and analysis of the human genome*. *Nature*, 2001. **409**(6822): p. 860-921.
114. Brodsky, J.L., *Translocation of proteins across the endoplasmic reticulum membrane*. *Int Rev Cytol*, 1998. **178**: p. 277-328.
115. Schwartz, T.U., *Origins and evolution of cotranslational transport to the ER*. *Adv Exp Med Biol*, 2007. **607**: p. 52-60.
116. Bichet, D., et al., *The I-II loop of the Ca²⁺ channel $\alpha 1$ subunit contains an endoplasmic reticulum retention signal antagonized by the beta subunit*. *Neuron*, 2000. **25**(1): p. 177-90.
117. Zerangue, N., et al., *A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels*. *Neuron*, 1999. **22**(3): p. 537-48.
118. Ellgaard, L. and A. Helenius, *Quality control in the endoplasmic reticulum*. *Nat Rev Mol Cell Biol*, 2003. **4**(3): p. 181-91.
119. Daniels, R., S. Svedine, and D.N. Hebert, *N-linked carbohydrates act as lumenal maturation and quality control protein tags*. *Cell Biochem Biophys*, 2004. **41**(1): p. 113-38.
120. Hammond, C., I. Braakman, and A. Helenius, *Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control*. *Proc Natl Acad Sci U S A*, 1994. **91**(3): p. 913-7.
121. Hebert, D.N., B. Foellmer, and A. Helenius, *Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum*. *Cell*, 1995. **81**(3): p. 425-33.
122. Spiro, R.G., et al., *Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi*. *J Biol Chem*, 1996. **271**(19): p. 11588-94.
123. Ware, F.E., et al., *The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins*. *J Biol Chem*, 1995. **270**(9): p. 4697-704.

124. Molinari, M. and A. Helenius, *Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells*. Nature, 1999. **402**(6757): p. 90-3.
125. Oliver, J.D., et al., *ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin*. Mol Biol Cell, 1999. **10**(8): p. 2573-82.
126. Oliver, J.D., et al., *Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins*. Science, 1997. **275**(5296): p. 86-8.
127. Caramelo, J.J., et al., *UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates*. Proc Natl Acad Sci U S A, 2003. **100**(1): p. 86-91.
128. Taylor, S.C., et al., *Glycopeptide specificity of the secretory protein folding sensor UDP-glucose glycoprotein:glucosyltransferase*. EMBO Rep, 2003. **4**(4): p. 405-11.
129. Caramelo, J.J. and A.J. Parodi, *Getting in and out from calnexin/calreticulin cycles*. J Biol Chem, 2008. **283**(16): p. 10221-5.
130. McCracken, A.A. and J.L. Brodsky, *Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP*. J Cell Biol, 1996. **132**(3): p. 291-8.
131. Lilley, B.N., D. Tortorella, and H.L. Ploegh, *Dislocation of a type I membrane protein requires interactions between membrane-spanning segments within the lipid bilayer*. Mol Biol Cell, 2003. **14**(9): p. 3690-8.
132. Ye, Y., et al., *A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol*. Nature, 2004. **429**(6994): p. 841-7.
133. Lee, R.J., et al., *Uncoupling retro-translocation and degradation in the ER-associated degradation of a soluble protein*. Embo J, 2004. **23**(11): p. 2206-15.
134. Ye, Y., H.H. Meyer, and T.A. Rapoport, *The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol*. Nature, 2001. **414**(6864): p. 652-6.
135. Su, J., et al., *The nontoxic tripeptide glycyl-prolyl-glycine amide inhibits the replication of human immunodeficiency virus type 1*. J Hum Virol, 2001. **4**(1): p. 1-7.
136. Su, J., et al., *The tripeptide glycyl-prolyl-glycine amide does not affect the early steps of the human immunodeficiency virus type 1 replication*. J Hum Virol, 2001. **4**(1): p. 8-15.
137. Abdurahman, S., et al., *Isolation and characterization of a small antiretroviral molecule affecting HIV-1 capsid morphology*. Retrovirology, 2009. **6**: p. 34.
138. Abdurahman, S., et al., *Activity of the small modified amino acid alpha-hydroxy glycineamide on in vitro and in vivo human immunodeficiency virus type 1 capsid assembly and infectivity*. Antimicrob Agents Chemother, 2008. **52**(10): p. 3737-44.
139. Andersson, E., et al., *Glycine-amide is an active metabolite of the antiretroviral tripeptide glycyl-prolyl-glycine-amide*. Antimicrob Agents Chemother, 2005. **49**(1): p. 40-4.
140. Balzarini, J., et al., *Obligatory involvement of CD26/dipeptidyl peptidase IV in the activation of the antiretroviral tripeptide glycylprolylglycinamide (GPG-NH(2))*. Int J Biochem Cell Biol, 2004. **36**(9): p. 1848-59.
141. Adachi, A., et al., *Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone*. J Virol, 1986. **59**(2): p. 284-91.
142. Horal, P., et al., *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, 1991. **88**(13): p. 5754-8.

143. Lee, K., et al., *IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response*. Genes Dev, 2002. **16**(4): p. 452-66.
144. Abacioglu, Y.H., et al., *Epitope mapping and topology of baculovirus-expressed HIV-1 gp160 determined with a panel of murine monoclonal antibodies*. AIDS Res Hum Retroviruses, 1994. **10**(4): p. 371-81.
145. Devito, C., et al., *Mapping of B-cell epitopes in rabbits immunised with various gag antigens for the production of HIV-1 gag capture ELISA reagents*. J Immunol Methods, 2000. **238**(1-2): p. 69-80.
146. Broliden, P.A., et al., *A monoclonal antibody to human immunodeficiency virus type 1 which mediates cellular cytotoxicity and neutralization*. J Virol, 1990. **64**(2): p. 936-40.
147. Hoglund, S., et al., *Tripeptide interference with human immunodeficiency virus type 1 morphogenesis*. Antimicrob Agents Chemother, 2002. **46**(11): p. 3597-605.
148. Caporale, M., et al., *The signal peptide of a simple retrovirus envelope functions as a posttranscriptional regulator of viral gene expression*. J Virol, 2009. **83**(9): p. 4591-604.
149. Ulbrecht, M., et al., *Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis*. J Immunol, 2000. **164**(10): p. 5019-22.