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Cytoplasmic localization of HIV-1 Vif is necessary for Apobec3G neutralization and viral replication

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DEDICATION

This thesis is dedicated to

My parents

Bruce and Jeanne Farrow

And my beloved friend

Heidi Lippold

Your unwavering support and unconditional love

have meant the world to me

Thank you, I love you

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ABSTRACT

The binding of HIV-1 Vif to the cellular cytidine deaminase Apobec3G and subsequent prevention of Apobec3G virion incorporation have recently been identified as critical steps for the successful completion of the HIV-1 viral life cycle. This interaction occurs in the cytoplasm where Vif complexes with Apobec3G and directs its degradation via the proteasome pathway or sequesters it away from the assembling virion, thereby preventing viral packaging of Apobec3G.

While many recent studies have focused on several aspects of Vif interaction with Apobec3G, the subcellular localization of Vif and Apobec3G during the viral life cycle have not been fully considered. Inhibition of Apobec3G requires direct interaction of Vif with Apobec3G, which can only be achieved when both proteins are present in the same subcellular compartment.

In this thesis, a unique approach was utilized to study the impact of Vif subcellular localization on Vif function. The question of whether localization could influence function was brought about during the course of studying a severely attenuated viral isolate from a long-term non-progressor who displayed a remarkable disease course. Initial observations indicated that this highly attenuated virus contained a mutant Vif protein that inhibited growth and replication. Upon further investigation, it was found that the Vif defect was atypical in that the mutant was fully functional in in vitro assays,

but that it was aberrantly localized to the nucleus in the cell. This provided the basis for the study of Vif localization and its contribution to Vif function.

In addition to the unique Vif mutant that was employed, while determining the localization and replication phenotypes of the differentially localized Vif proteins, a novel pathway for Vif function was defined. Copious publications have recently defined the mechanism for Vif inhibition of Apobec3G. Vif is able to recruit Apobec3G into a complex that is targeted for degradation by the proteasome. However, this directed degradation model did not fully explain the complete neutralization of Apobec3G observed in cell culture. Other recent works have proposed the existence of a second, complementary pathway for Vif function. This pathway is defined here as formation of an aggresome that prevents Apobec3G packaging by binding and sequestering Apobec3G in a perinuclear aggregate. This second mechanism is believed to work in parallel with the already defined directed degradation pathway to promote complete exclusion of Apobec3G from the virion.

The data presented here provide insight into two areas of HIV research. First, the work on the naturally occurring Vif mutant isolated from a long-term non-progressor confirms the importance of Vif in in vivo pathogenesis and points to Vif as a potentially useful gene for manipulation in vaccine or therapy design due to its critical contributions to in vivo virus replication. Additionally, the work done to address the subcellular

localization of Vif led to the proposal of a second pathway for Vif function. This could have implications in the field of basic Vif research in terms of completely understanding and defining the functions of Vif. Again, a more complete knowledge about Vif can help in the development of novel therapies aimed at disrupting Vif function and abrogating HIV-1 replication.

TABLE OF CONTENTS

Copyright Notice.....ii

Approval Page.....iii

Dedication.....iv

Acknowledgements.....v

Abstract.....vii

Table of Contents.....x

List of Figures.....xi-xii

Abbreviations.....xiii

CHAPTER I. Introduction.....1

CHAPTER II. Materials and Methods.....52

CHAPTER III. Nuclear localization of HIV-1 Vif isolated from a long-term asymptomatic individual and potential role in virus attenuation...67

CHAPTER IV. Cytoplasmic localization of HIV-1 Vif is required for Apobec3G degradation and viral replication.....95

CHAPTER V. HIV-1 Vif sequesters Apobec3G into aggresomes to prevent virion incorporation of Apobec3G.....121

CHAPTER VI. Discussion.....144

References.....155

LIST OF FIGURES

Figure 1.1 The three phases of HIV-1 infection....4

Figure 1.2 Comparison of CD4+ T cell counts in a hemophilia cohort....6

Figure 1.3 HIV-1 genome....15

Figure 1.4 HIV-1 viral life cycle....16

Figure 1.5 HIV-1 reverse transcription process....21

Figure 1.6 HIV-1 virion structure....33

Figure 1.7 Apobec3G packaged in the virion deaminates nascent viral transcripts resulting in G→A hypermutation or degradation....41

Figure 1.8 Vif mediated degradation of Apobec3G....45

Figure 3.1 Viral loads and CD4+ T cell counts for LTNP4....71

Figure 3.2 LTNP4 exhibits a Vif enhanced growth phenotype and unique mutations in Vif....74-75

Figure 3.3 Subcellular localization of KKRK and WT Vif fusion proteins....79

Figure 3.4 Infectivity of NL4.3/WT versus NL4.3/KKRK viruses....84-85

Figure 3.5 Comparison of NL4.3/WT and NL4.3/IF infectivity....89

Figure 4.1 Localization of NL4.3/WT or NL4.3/KKRK Vif in infected cells....98-99

Figure 4.2 Degradation of GFP-Apobec3G by NL4.3/KKRK and NL4.3/WT.103-105

Figure 4.3 Binding of Apobec3G by rKKRK-Vif and rWT-Vif....108

Figure 4.4 Colocalization of Apobec3G and NL4.3/WT or NL4.3/KKRK Vif.....113

Figure 4.5 Site of GFP-Apobec3G Vif colocalization.....115

Figure 4.6 Packaging of Apobec3G into the virion.....119

Figure 5.1 Apobec3G perinuclear aggregates recruit ubiquitin.....121

Figure 5.2 Vif-Apobec3G aggregates are aggresomes.....127

Figure 5.3 Vif-Apobec3G aggresomes are regulated by the microtubule network..131

Figure 5.4 Vif mediates aggresome assembly.....134

Figure 5.5 Disrupting aggresome formation reduces infectivity.....138

ABBREVIATIONS

AIDS- Acquired Immnuodeficiency Syndrome

Apobec3G- apolipoprotein B mRNA editing catalytic polypeptide 3G

CA- capsid

CTL- cytotoxic T lymphocyte

DAPI- 4',6-Diamidino-2-phenylindole

DsDNA- double stranded deoxyribonucleic acid

DMEM- Dulbecco's Modified Eagle Media

ECL- enhanced chemiluminescence

ELISA- enzyme linked immunosorbent assay

FACS- fluorescence-activated cell sorter

FBS- fetal bovine serum

FITC- fluorescein isothiocyanate

GFP- green fluorescent protein

HDAC6- histone deacetylase 6

HIV- Human Immunodeficiency Disease

HLA- human leukocyte antigen

HRP- horseradish peroxidase

IN- integrase

LANL- Los Alamos National Laboratory

LTNP- long-term non-progressor

LTS- long-term survivor

MA- matrix

MHC- Major Histocompatibility Complex

NC- nucleocapsid

NLS- nuclear localization sequence

PBMC- peripheral blood mononuclear cell

PBS- phosphate buffered saline

PBS- primer binding site

PCR- polymerase chain reaction

PE- phycoerythrin

PHA- phytohemaglutinnin

PR- protease

RRE- Rev response element

RT- reverse transcriptase

RT-PCR- reverse transcription polymerase chain reaction

SBBC- Sydney Blood Bank Cohort

SDF-1- stromal cell-derived factor 1

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIV- simian immunodeficiency virus

TRITC- tetramethylrhodamine isothiocyanate

UNG2- uracil DNA glycosylase 2

WT- wild type

CHAPTER I

INTRODUCTION

A. *Retroviridae*

Retroviruses belong to the family *Retroviridae*. These viruses are defined by their virion encapsidated, genomic, positive strand RNA that is reverse transcribed by the enzyme Reverse Transcriptase into double-stranded DNA (dsDNA) upon entry into the host cell. This reverse transcribed dsDNA is transported to the nucleus where it becomes integrated into the host genome via the virally encoded Integrase enzyme. The integrated form of viral DNA is known as the provirus. This provirus drives production of viral RNA and viral proteins. The integrated provirus allows for production of new viral particles, persistence of the virus in the host cell, and vertical transmission to progeny cells.

Retroviral virion particles are spherical and consist of an outer lipid membrane bilayer studded with Envelope glycoproteins. These Envelope glycoproteins determine host cell tropism. Beneath this membrane layer, there is a spherical shell composed of the Matrix protein. The viral core is made up of Capsid encompassing the genomic RNA, which is coated with Nucleocapsid. Also contained in the viral core are the Reverse Transcriptase, Protease, and Integrase enzymes.

Retroviruses can be divided into two main classifications based on their genome composition. The retroviral genome consists of a dimer of single stranded, positive sense

RNA. All Retroviruses contain a 5' cap and 3' poly-A tail similar to mRNA, a R region at the 5' and 3' ends, 5' and 3' unique regions containing the *att* integration sites, a primer binding site for minus-strand DNA synthesis, a polypurine tract for plus-strand DNA initiation, a Psi element that governs viral genome packaging, and the gag, pol, and env genes that code for the structural proteins, viral enzymes, and envelope glycoproteins, respectively. Viruses that contain only these basic elements, such as alpha-, beta-, or gammaretroviruses, are known as simple retroviruses. Those that encode additional auxiliary genes are complex retroviruses. Members of the genus epsilonretrovirus, spumavirus, or lentivirus are complex retroviruses.

B. Human Immunodeficiency Virus type 1 Infection

1. Background

The genus *Lentivirus* belongs to the Retrovirus family. Lentiviral characteristics include extended latent periods and eventual development of diseases associated with the immune, nervous, or hematopoietic systems [1]. The Human Immunodeficiency Virus type 1 (HIV-1) is a lentivirus that infects the immune system of humans. A complex retrovirus, the genome of HIV-1 is 9.2kb and encodes the gag, pol, and env genes as well as the six accessory genes, vif, vpr, vpu, tat, rev, and nef. HIV-1 targets the $CD4^+$ T lymphocytes and macrophage cells of the immune system.

HIV-1 was initially isolated and identified as a retrovirus in 1983 [2]. The following year, three independent groups reported the discovery of a cytopathic retrovirus that infected $CD4^+$ T lymphocytes that potentially caused the acquired immunodeficiency syndrome (AIDS). These three prototype viruses, lymphadenopathy-associated virus

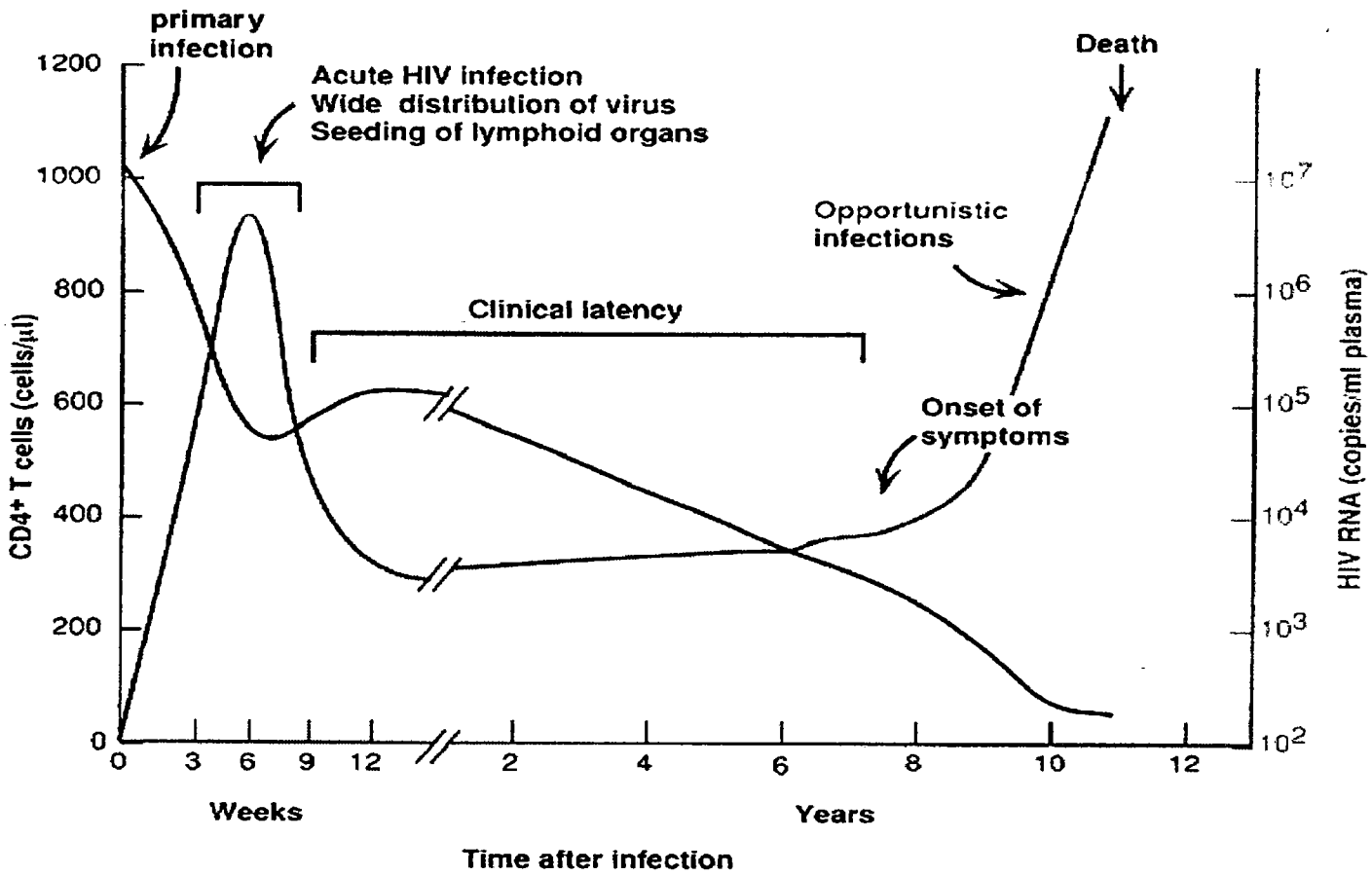
(LAV) [3], human T-cell leukemia virus III (HTLV-III) [4-7], and AIDS related virus (ARV) [8], were closely related and identified as a lentivirus associated with AIDS [1]. This AIDS virus was later given the name Human Immunodeficiency Virus (HIV) in 1986.

HIV-1 is the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). Currently, it is estimated that 39.4 million people are infected with HIV, resulting in 3.1 million AIDS related deaths in 2004 with an estimated total of 20 million deaths due to AIDS since 1981[9].

HIV-1 infection can be segregated into three distinct phases (Figure 1.1). Upon contraction of the virus, an acute, primary infection develops. Primary infection often correlates with a high viral burden and rapid loss of $CD4^+$ T cells. This stage typically lasts for several weeks as the virus-host interaction evolves. Initially, there is a burst of viremia in the absence of an adaptive immune response. This rapid production of a high titer of virus results in widespread cytopathicity as well as the establishment of a viral reservoir. Within a few weeks of acute infection, the adaptive immune system mounts a response and begins to control viral replication and spread. Once the immune system and virus have found an equilibrium state, the viral setpoint is established and the disease enters a latent phase [10-13].

The hallmark of lentivirus infection is a latent phase that generally lasts five to ten years with patients remaining asymptomatic during this period. The latent phase is actually a phase of dynamic interaction between the virus and immune system. During this time, the virus continues to replicate and induce cytopathicity. However, the adaptive immune system is able to respond and prevent rampant spread of the virus. As

Figure 1.1



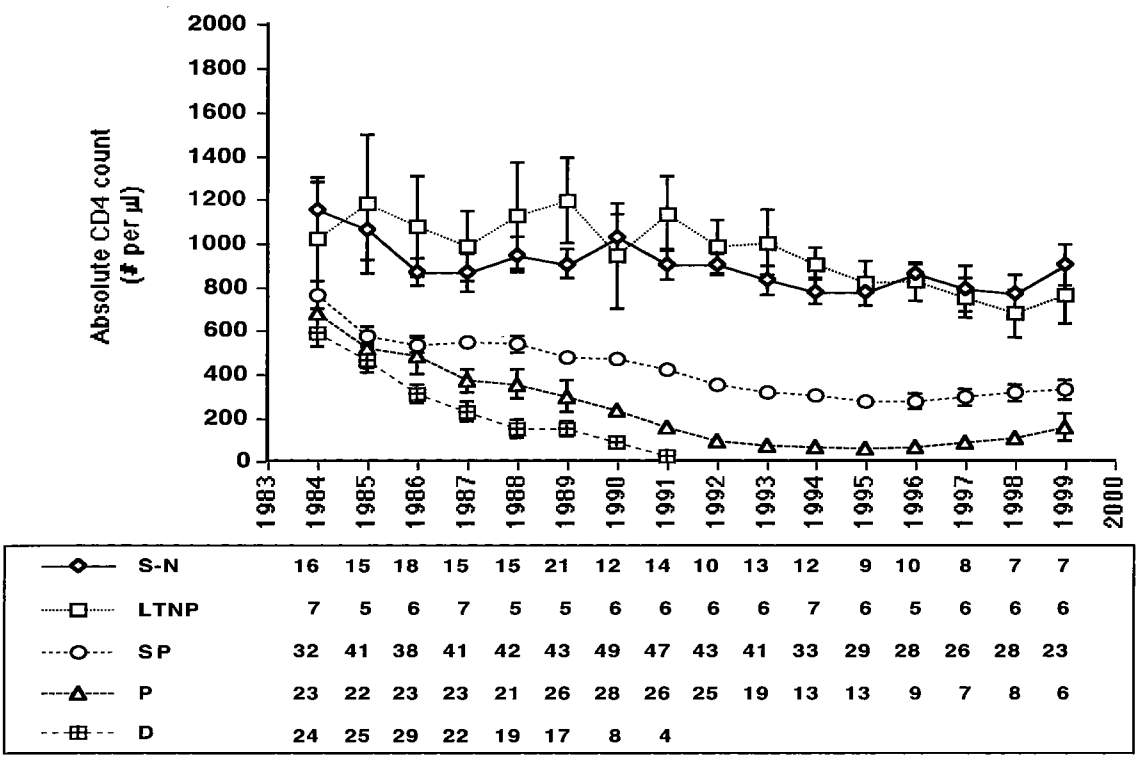
The three phases of HIV-1 infection. (from G. Pantaleo et. al. New England Journal of Medicine 1993 328 (5): 327-335)

the virus continues to replicate, it acquires mutations throughout the genome. The HIV-1 RT enzyme is error prone and generally introduces one error per replication cycle. As these mutations accumulate throughout the genome, naturally occurring escape mutants are generated. These escape mutants are able to evade the host adaptive immune response mediated by cytotoxic T lymphocytes (CTL) and begin to replicate rapidly [14, 15]. This rapid spread of virus results in a precipitous decline in $CD4^+$ T cells. A recent study has mathematically determined that a typical host can withstand 1,300 viral years before experiencing a complete depletion of $CD4^+$ T cells [16], demonstrating a direct connection between viral load and $CD4^+$ T cell number. The loss of $CD4^+$ T cells results in the onset of the clinical manifestation known as AIDS [17-21]. Once a patient has entered the AIDS state, they will typically succumb to opportunistic infections resulting in AIDS related death.

2. Long-Term Non-Progressors (LTNP)

Approximately 5-10% of the HIV-1 infected population exhibit an aberrant disease course [22]. These individuals are asymptomatic for greater than ten years, maintain stable $CD4^+$ T cell counts above 500cells/μl, and demonstrate strong immune responses in the absence of antiretroviral therapy [23-28]. When the $CD4^+$ T cell counts of LTNP from a hemophilia cohort are compared to slow progressors (SP), normal progressors (P), rapid progressors (D), or seronegative individuals (SN), the profile closely resembles that of HIV-1 seronegative donors while all other HIV-1 infected groups show decline in $CD4^+$ T cell counts over time (Figure 1.2). Typically, these long-term non-progressors (LTNP) have low to undetectable viral loads and virus isolation from these patients is often complex or unattainable [25, 29, 30]. This low level of

Figure 1.2



Comparison of CD4+ T cell counts in a hemophilia cohort. (courtesy of T. C. Greenough, University of Massachusetts Medical School)

plasma virus can be attributed to a number of factors including resistance factors in the host or infection mediated by an attenuated strain of HIV-1. In most cases, it is assumed that a combination of an effective immune response and infection with a quasi species containing attenuated virus is responsible for the remarkable disease course [30, 31].

One host resistance factor is the CCR5Δ32 mutation [32, 33]. The CCR5Δ32 mutation is characterized by a 32-bp deletion that results in the expression of a truncated CCR5 protein. Individuals who are homozygous for CCR5Δ32 do not properly express the CCR5 chemokine co-receptor on the cell surface [33]. Since the predominant HIV-1 strains that are transmitted between individuals establish initial infection use CCR5 as a co-receptor to enter the cell, the cell is protected from HIV infection by its lack of CCR5 on the cell surface. Homozygotes for CCR5Δ32 are rarely infected with HIV-1 [34-37]. However, since they remain susceptible to infection with strains of HIV-1 that use the alternate CXCR4 chemokine co-receptor to enter the cell, some rare instances of infection have been detected [32, 33, 38-40]. Additionally, heterozygotes for CCR5Δ32 are no less susceptible to HIV infection, but they do have delayed progression of disease with a recent study showing that CCR5Δ32 heterozygotes have a 30% lower occurrence of AIDS [38, 41, 42]. This protective effect could be attributed to detention of wild-type CCR5 in the endoplasmic reticulum where it is complexed with the CCR5Δ32 protein, leading to decreased CCR5 density on the cell surface [43]. Genetic polymorphisms in the CCR5 promoter region may also deter HIV disease progression [44].

A second chemokine co-receptor mutation, the CCR2-V64I mutation, has also been implicated in retarded progression to AIDS [41, 45]. How this mutation contributes to delayed progression is not understood.

There also exists a mutation in the stromal cell-derived factor-1 (SDF-1) protein that impacts binding of HIV-1 to the CXCR4 co-receptor. SDF-1 functions as the primary ligand for CXCR4. A variant of this gene, SDF-1 3'α, has a mutation in the untranslated region that may allow upregulation of SDF-1 expression. This creates an environment where SDF-1 is in abundance and can effectively compete with HIV-1 for binding to the CXCR4 co-receptor [44]. SDF-1 3'α homozygotes demonstrate a delayed progression to AIDS, but again there is no protective effect during initial infection where CCR5 is the more important co-receptor [46, 47].

Patient Human Leukocyte Antigen (HLA) types may also help define the rate of disease progression. In general, HLA class I homozygosity at one or more of the HLA-A, HLA-B, or HLA-C loci is associated with rapid progression to AIDS whereas heterozygosity at at least one loci provides some protection and a delayed development of disease [48-50]. This could be explained by the diversity of antigenic peptides that can be presented by heterozgotes versus the more limited repertoire presented by homozygotes, which may curtail the appearance of CTL escape mutants [51]. Additionally, individual HLA alleles and haplotypes have discordant influence on disease progression. The alleles B35 and Cω04 appear to expedite the development of disease [48]. Conversely, HLA B27 and B57 have most commonly been found in association with long-term non-progression [52-56]. When a cohort of LTNP were studied and compared to progressors, it was found that 85% of LTNP, compared to only 9.5% of progressors, had the HLA B*5701 allele, suggesting a protective effect for this allele [57]. A comprehensive study of HLA class I and II alleles identified HLA B14 and C8 as strong correlates of long-term non-progression [58].

In addition to host factors that may manipulate the disease course, viral factors can also play a role in sluggish disease development. If infection is mediated by an attenuated strain of the virus, it may not be able to establish as robust an initial infection or spread as effectively. It has been shown that the level of HIV-1 in recent seroconverters can be as low or lower than that in LTNPs. However, only LTNP are capable of maintaining low levels of HIV-1 replication, which impacts their development of a long-term asymptomatic condition [59]. Additionally, another study demonstrated a direct correlation between in vitro replicative capacity of biological clones isolated from long-term survivors (LTS) and in vivo viral load [60]. A more recent study confirmed this observation that the replicative capacity of LTNP virus is significantly lower than that of wild-type controls [61].

One of the first reports pertaining to the passage of a defective strain of HIV-1 in a long-term non-progressing cohort was that of the Sydney Blood Bank Cohort (SBBC). Deacon et al. reported the isolation of a HIV quasi species with deletions in nef and the overlapping U3 region of the LTR that had been passed from a donor to multiple recipients via blood transfusion [62]. Both the donor and the recipients remained asymptomatic for a period of at least ten years or more. However, the donor and some of the recipients have shown progression to disease, perhaps due to the emergence of quasi species with different nef genotypes [63]. The SBBC report was in agreement with an earlier report in which rhesus macaques were exposed to nef deleted Simian Immunodeficiency Virus (SIV). The monkeys maintained stable $CD4^+$ T cell counts and low viral loads, indicating that nef was important for disease progression [64].

A second early report on long-term non-progressors also implicated the nef gene as a determinant of disease progression. An initial study identified a long-term non-progressor who harbored a virus with a large nef deletion. Follow-up studies in the same cohort revealed that an additional patient had only nef alleles with unusual polymorphisms [65, 66]. Observations relating to nef defective viruses and attenuation continue, a second cohort in Australia consisting of three LTNP have been shown to harbor virus carrying nef deletions [67].

The observation that nef defective virus was less infectious raised the question of the contribution of other viral accessory genes to in vivo disease progression. While there does not appear to be a common origin for attenuated HIV-1 viruses [68], many of those recovered from LTNP do harbor mutations in the accessory gene region that range from large deletions and premature stop codons to subtle, single amino acid changes.

The idea that accessory gene mutations play a critical role in viral replication can be seen when viral isolates from LTNP are sequenced and analyzed. A study that directly examined transmission of mutant genomes found that mother-to-child vertical transmission did not occur when the mother had virus that carried vif and vpr mutations. On the other hand, viruses from transmitting mothers were found to maintain a high conservation of intact accessory genes [69]. When the accessory gene region of vif, vpr, vpu, tat1, and rev1 were assessed for mutations in a single LTNP, 64% of the clones carried missense or nonsense mutations [31, 70]. A cohort of Japanese hemophiliacs revealed elevated levels of mutations in nef, vpu, vpr, vif, and rev-2 when compared to progressors. Interestingly, vif had the highest rate of mutation with 36.7% in the LTNP group and only 0.4% in progressors [71]. In the recent Australian cohort study, one of

the LTNP had viral sequences that revealed nef deletions in conjunction with mutations resulting in expression of truncated Vif protein [67].

In agreement with the results above, several in vitro studies have provided data implicating accessory gene defects in viral attenuation. In the monkey model, attenuated SIV constructs of multiple or single gene mutants were used to establish a ranking of accessory gene mutations based on their contribution to replication from most attenuating to least attenuating- nef, vpx, vpr, US,vif>nef, vpx, vpr, US; vif> nef, vpx, US> nef, vpr, US>vpr, vpx; nef>vpx>vpr [72]. Also, the effect of mutations in nef, vpr, vpu, and vif was assessed using mutated HIV-1 in tissue culture assays. Vpu mutants showed an impaired growth while vif mutants showed no detectable growth in both peripheral blood mononuclear cells and macrophages [73].

While attenuating mutations in the accessory gene region diminish the replicative capacity of the virus, they do allow the virus to establish an infection that is persistent. It has been shown that mutants defective in vif, vpr, vpu, or a combination triple mutant defective in all three of the central region accessory genes could establish a transient infection with increased cytopathicity followed by a persistent infection with no cytopathicity. These persistent viruses had acquired additional accessory gene mutations that allowed for establishment of a long-lived, persistent infection as opposed to a short-lived, highly cytopathic infection as seen in nef mutant or wild-type viruses [74]. These reports clearly establish that multigenic defects in the accessory genes, particularly those of the central region (vif, vpr, vpu), can lead to a persistent, low-level infection that does not lead to cytopathicity in the host.

While it is clear that multiple mutations working in concert can lead to highly attenuated strains of HIV-1, some single gene mutations can also be equally effective in attenuating the virus.

The vpr gene has been implicated as a possible source of attenuation in LTNP. Specifically, the R77Q substitution has been linked to long-term non-progression as 75% of the identified LTNP in the Los Alamos National Laboratory (LANL) HIV database have the R77Q substitution. It is proposed to play a role in reduced cytopathicity since it resides in the $(H(F/S)RIG)_2$ domain that has been linked to apoptosis, therefore leading to less $CD4^+$ depletion in vivo [75]. Another report showed a correlation between non-progression in spite of high viral loads and Vpr mutations. In this study, a LTNP with stable $CD4^+$ cells and high viral loads was found to have virus that contained vpr mutations including early termination codons and the unusual polymorphism, Q3R. It was found that this Q3R mutation rendered the virus less cytopathic without disrupting its replicative capacity [76]. Again, it was proposed that the Vpr mutations were responsible for decreased cytopathicity in the host, thus allowing for maintenance of stable $CD4^+$ levels. A non-progressing mother-child pair also revealed only mutated vpr sequences at amino acids 83-89, as opposed to only wild-type sequences at 83-89 in people who had progressed to AIDS, that potentially affect the ability of Vpr to induce G2 cell cycle arrest, secondary structure of Vpr, and the overlapping tat gene [77, 78]. These studies indicate a potential role for Vpr in maintaining high levels of circulating $CD4^+$ cells, even when viral replication is not controlled, by disrupting the ability of Vpr to induce apoptosis of infected $CD4^+$ cells.

The vif gene was actively investigated as it is known to influence HIV-1 replication in vivo [73, 79-81]. In one study, a single amino acid difference was found to distinguish LTNP from progressors [68, 82]. Non-progressors associate predominantly with an Isoleucine at position 123 while a Threonine at the same position was found less frequently in LTNP and in about half of the progressors studied. A second single amino acid determinant was found to affect viral loads within the LTNP group and between the LTNP cohort and the progressors. A Serine at position 132 was found in LTNP with low viral load while an Arginine at 132 was associated with high viral load LTNPs or progressors [82]. A mother-child pair who both displayed LTNP characteristics were studied for viral determinants of non-progression. In both the mother and the child, a two amino acid insertion in Vif was identified. This insertion mutant was studied for possible attenuation of viral replication and it was found that viruses containing the mutant vif gene were less infectious in vitro [83]. A LTNP in a Japanese hemophilia cohort was found to carry virus that had inactive vif in all clones sequenced [84].

While it is clear that accessory gene mutations contribute to viral attenuation, which in turn leads to delayed disease progression, one of the biggest questions surrounding LTNPs is how the virus maintains those mutations in light of the high error rate of the RT enzyme. Viral evolution is directly related to the amount of viral replication occurring in LTNP. In LTNP with extremely low levels of replication, average of 275 copies HIV-1 RNA/ml plasma, there is little detectable evolution of the viral sequences. However, LTNP with more moderate levels of replication, average of 3420 copies HIV-1 RNA/ml plasma, show an evolution from the ancestral sequences to modern sequences. It is proposed that the LTNP who demonstrate this viral diversity and

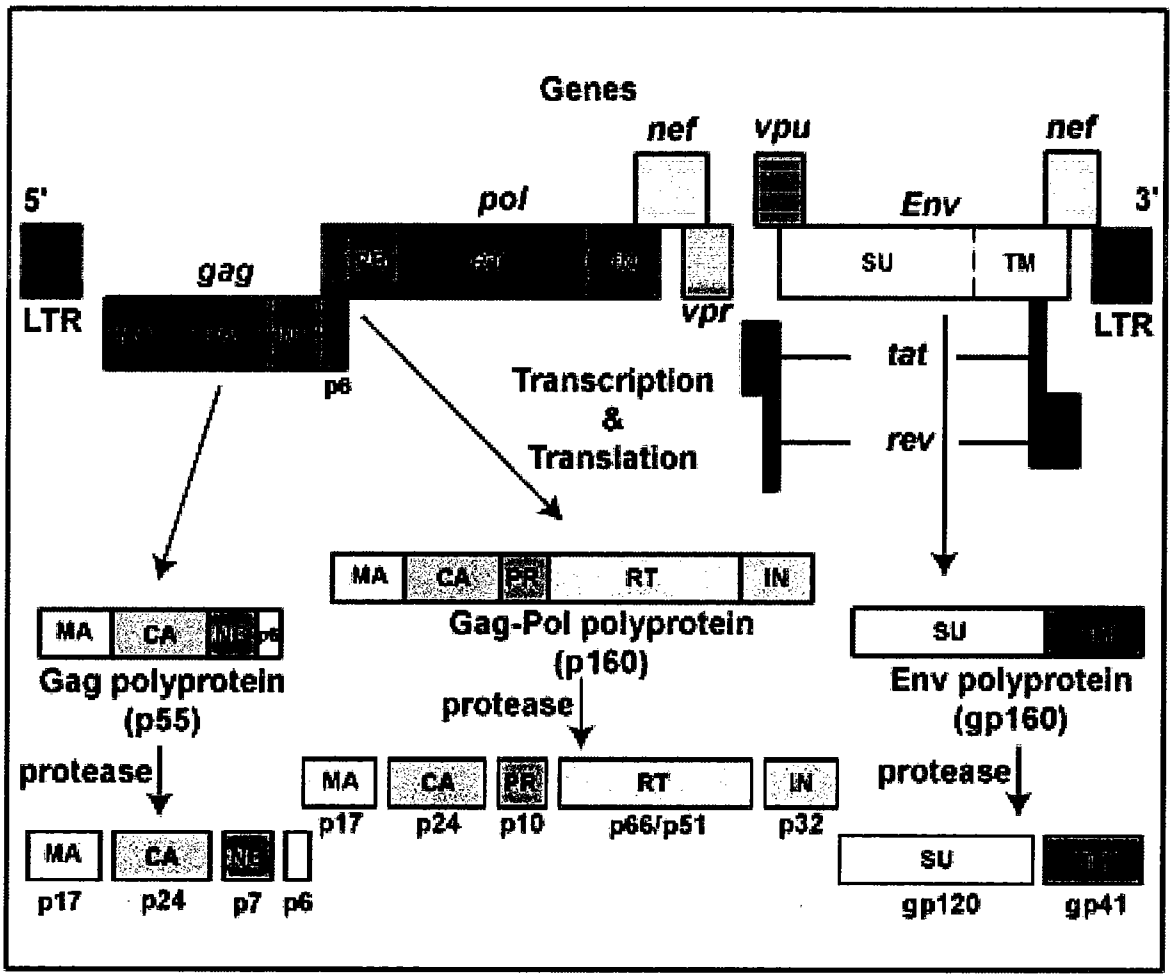
evolution are actually slow progressors, while those that maintain ancestral sequences are true long-term non-progressors [22].

C. Viral Life Cycle

A complex retrovirus, HIV-1 encodes the gag, pol, and env genes as well as the accessory genes vif, vpr, tat, rev, vpu, and nef (Figure 1.3). The objective of HIV-1 is to enter the target cell, utilize the host cell machinery to generate viral transcripts, proteins, and genomic RNA, and ultimately assemble and release progeny virions. Although simplistic in its objective, the viral life cycle is extremely complex with extensive interplay amongst viral proteins and to a greater degree, between viral proteins and host cell factors (Figure 1.4). These interactions with host factors involve both the exploitation of cellular proteins to perform functions necessary for completion of the viral life cycle and the neutralization of inhibitory antiviral factors present in the target cell and the host immune system.

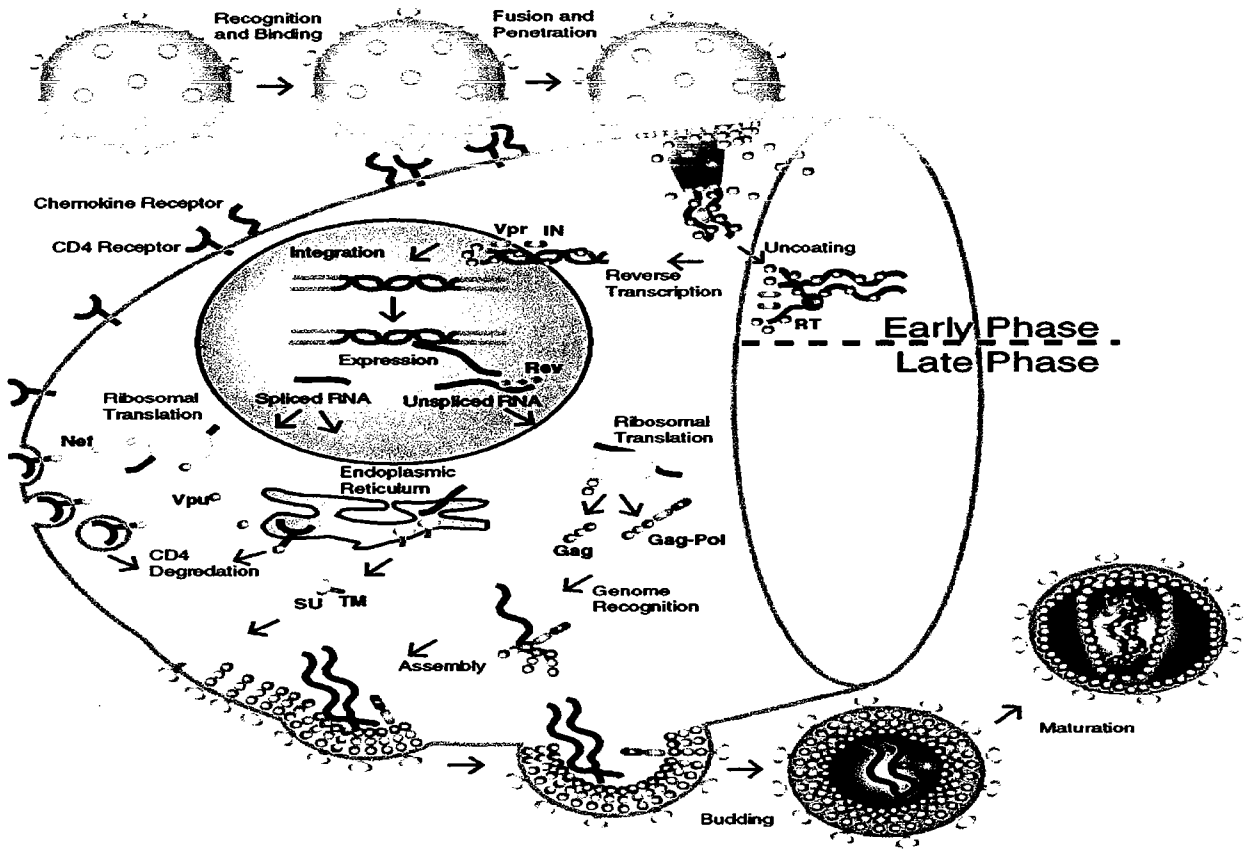
The viral life cycle begins when the virion attaches to the target cell surface. Initial binding is mediated by interaction of the viral surface glycoprotein gp120 to the CD4 protein on the cell surface [85-87]. Specifically, the fourth conserved region (C4) of gp120 binds to the CD4 molecule [88, 89]. Conformation of gp120 at this binding stage is critical as additional noncontiguous regions are also involved in contacting the CDR2 domain of CD4 [90], providing further support to the gp120-CD4 anchor. The importance of the gp120-CD4 interaction has been confirmed by competitive binding tests using soluble CD4 which leads to a loss of HIV-1 infectivity [91-94].

Figure 1.3



HIV-1 genome. (from G. E. Kaiser Microbiology website http://www.cat.cc.md.us/courses/bio141/lecguide/unit2/viruses/hivgenes.html)

Figure 1.4



HIV-1 viral life cycle. (from B. G. Turner and M. F. Summers Journal of Molecular Biology 1999 285: 1-32)

Following attachment of the virion to the cell surface via the gp120-CD4 binding event, the gp120 co-receptor binding site becomes exposed as the env V1/V2 loops become repositioned [95]. Once the binding site is exposed, the V3 loop of env can contact the chemokine co-receptor on the cell surface.

There are a number of chemokine co-receptors for HIV-1 entry. The two major chemokine co-receptors are the CXCR4 co-receptor expressed on the surface of CD4 lymphocytes [96, 97] and the CCR5 co-receptor expressed on the surface of macrophages [98-101]. The env V3 loop sequence determines viral tropism. HIV-1 strains that bind CXCR4 are classified as T-tropic, syncytium inducing (SI) strains. Those that bind CCR5 are M-tropic, non-syncytium inducing strains (NSI). The V3 loop of X4-tropic viruses is usually highly, positively charged. This is because the high positive charges on the gp120 interact with the negatively charged E2 extracellular loop of CXCR4. This electrostatic interaction helps to stabilize the gp120:CXCR4 interaction [95]. For the R5-tropic viruses, there is a lower net positive charge on the V3 loop.

Viral tropism plays a major role in disease progression as the initial infection and asymptomatic phase are generally mediated by NSI strains [102-104]. The AIDS phase generally develops following a switch in co-receptor use. This switch in tropism leads to predominant use of the CXCR4 co-receptor, therefore leading to an increase in the number of infected $CD4^+$ cells. This ability to broadly infect $CD4^+$ cells leads to depletion of the host $CD4^+$ cell number and development of the clinical AIDS stage [105-107]. The importance of the R5 to X4 switch can be seen in the LTNP cohort as most LTNP have R5-tropic, NSI viruses [108]. In two studies, it was shown that non-

progessors with nef-deleted virus who eventually progressed to AIDS underwent an R5 to X4 tropic switch that correlated with disease progression [63, 109].

The next step in the virus-cell fusion pathway is the exposure of the fusion peptide in gp41. Since the gp120 surface glycoprotein and gp41 transmembrane glycoprotein are noncovalently linked, perturbing the gp120 subunit results in a conformational change that exposes the fusion peptide in gp41. The gp41 ectodomain contains the fusion peptide as well as two regions, one N-terminal and the other C-terminal, with heptad repeats. The fusion peptide inserts into the host cell membrane, resulting in the pre-hairpin intermediate complex [110]. The gp41 heptad repeats now assume the fusion-competent arrangement of a six helix bundle (SHB) with the N-terminal peptides assembling into three central helices surrounded by three helices formed by the C-terminal peptides [111, 112]. The SHB hairpin structure can now draw the viral and cellular membranes close together to allow for membrane fusion and entry of the viral core. The formation of the six helix bundle is critical to virus entry. When the SHB intermediate state is exposed to the peptide inhibitor T-20 that binds to the N-terminal peptide region, it occludes the interacting sites for the C-terminal peptide. This disruption prevents formation of the SHB and the virus cannot fuse to or enter the target cell [63].

Once the virus fuses to the cell, the virion core is deposited into the host cell cytoplasm. An ill-defined uncoating process, which may involve phosphorylation of MA to release the virion core from the plasma membrane [113], then occurs resulting in a complex that is now competent for reverse transcription, the reverse transcription complex (RTC). This RTC contains the necessary components to engage in the critical

step of reverse transcribing the encapsidated viral RNA genome into a double-stranded DNA species that can be integrated into the host genome. The reverse transcription process is a complex, highly regulated event that involves the use of the genomic RNA, the virally encapsidated $tRNA^{Lys3}$ primer, and the Reverse Transcriptase (RT) enzyme.

The RT enzyme catalyzes the entire process of reverse transcription [114, 115]. The holoenzyme is composed of two subunits, a heterodimer of the p51 and p66 subunits. The p51 subunit is derived from the p66 subunit by proteolytic processing, it does not have RNaseH activity or a polymerizing cleft. The structure of the p66 subunit is often described as a right hand with the palm containing the polymerase active site and the template binding cleft formed by the palm, fingers, and thumb. The RNaseH domain is tethered to the polymerase domain in the palm. The polymerase active site has three Asp residues and two coordinated Mg^{2+} ions that are absolutely critical for polymerizing activity. The template and dNTPs are trapped in the binding cleft by the fingers. Following addition of the dNTP to the elongating chain, the fingers "relax" and open up to allow for the next dNTP to enter for elongation to continue [116].

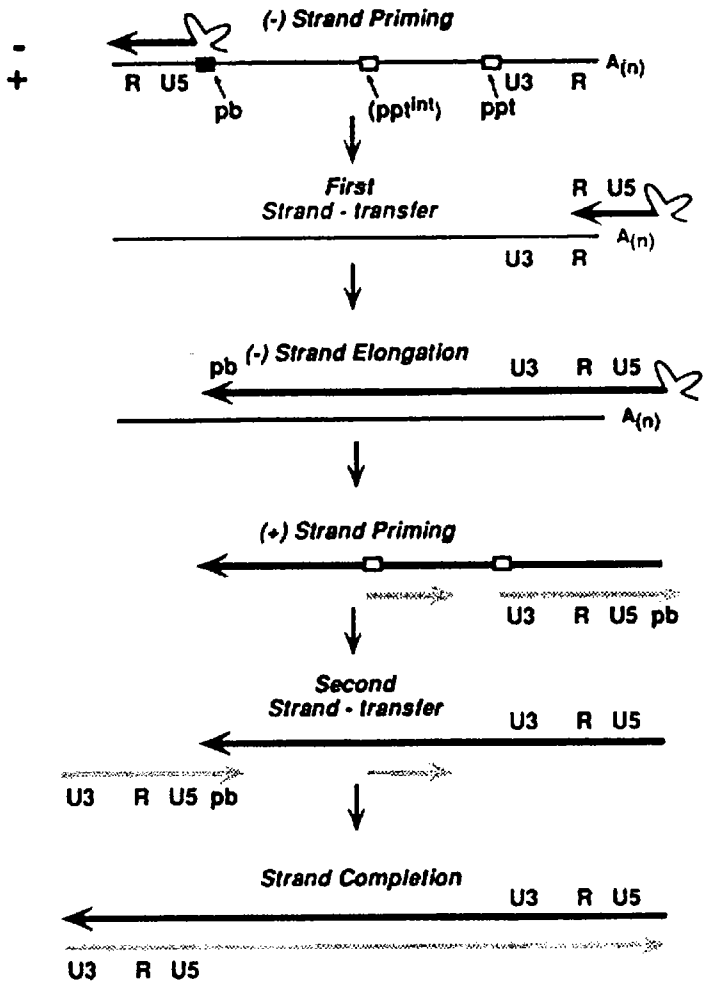
Since RT does not have exonuclease proofreading activity, it is prone to errors [117, 118]. The calculated mutation rate, including substitutions, frameshifts, deletions, and deletions with insertions, is $3x10^{-5}$ mutations/cycle of replication [119]. In addition to the error prone RT enzyme, intermolecular jumps and intramolecular switches during reverse transcription drive recombination. Also, the activity of the Apobec3G enzyme can result in production of hypermutated genomes [120]. Taken together, these changes introduced during the reverse transcription process lead to rapid evolution of the viral genome that may be important in generating immune escape variants [121, 122].

The first step of reverse transcription is the annealing of the $tRNA^{Lys3}$ primer to the primer binding site (PBS) in the 5' termini of the viral RNA, a process directed by NC and RT (Figure 1.5). Minus-strand DNA synthesis then proceeds from the 3'-OH of the $tRNA^{Lys3}$ primer towards the 5' end of the viral genome. This results in a RNA:DNA duplex. RNaseH then degrades the RNA component of the duplex, thereby liberating a short fragment of ssDNA known as the minus-strand strong-stop DNA. This ssDNA encodes the R region that is homologous to the 3' end of the viral genome, which allows hybridization of the minus-strand strong-stop DNA to the viral RNA template. This hybridization is known as the first strand transfer.

Minus-strand synthesis occurs from the hybridization site at the 3' end towards the PBS region at the 5' end. RNaseH continues to degrade the RNA from the resulting RNA:DNA duplexes. However, short purine-rich sequences of RNA are resistant to RNaseH degradation. These polypurine tracts (ppt) oligonucleotide binds the minus-strand strong-stop DNA and serves as the primer for plus-strand DNA synthesis. Here, the minus-strand DNA now serves as the template with the reaction proceeding toward the 5' end of the minus-strand strong-stop DNA. This results in the production of a DNA fragment known as the plus-strand strong-stop DNA consisting of the U3, R, and U5 sequences of the terminal region plus a segment of the PBS with elongation terminating at position 19 of the $tRNA^{Lys3}$ primer [123].

The $tRNA^{Lys3}$ primer is degraded by RNaseH, exposing the PBS of the plus-strand DNA. The plus-strand DNA can now engage in the second strand transfer reaction where the plus-strand DNA hybridizes to its homologous region at the 3' end of the minus-strand DNA. Plus-strand synthesis proceeds to the end of the minus-strand DNA

Figure 1.5



HIV-1 reverse transcription process. (from E. Acheampong et. al. Frontiers in Bioscience 2003 8: s156-174)

where it encounters the central termination signal downstream of the central ppt region [124]. The displacement of the plus-strand DNA at the central ppt results in a DNA triplex, 99 nucleotides long, known as the flap that influences nuclear import of the viral dsDNA [125]. The integrity of the dsDNA is maintained by coating of the viral DNA with the NC protein, which is important for efficient integration into the host genome [126-129]. Once the reverse transcription process has been completed, the viral dsDNA must be transported to the nucleus where it can be imported for integration in the host genome.

The reverse transcription process is complex and error prone due to the infidelity of the RT enzyme. In addition to this, the RT reaction is also open to modifications by cellular proteins. The function of Vif, detailed later in this chapter, is to indirectly protect the RT process from attack by the protein Apobec3G. Apobec3G, also detailed later in this chapter, is a cellular cytidine deaminase that can package in the virion if Vif is absent or non-functional in the producer cell. Apobec3G deaminates cytosine to uracil in single-stranded DNA templates. Since there are a number of ssDNA species created during reverse transcription, the nascent viral transcripts are vulnerable to deamination by Apobec3G. This deamination results in two potential outcomes. The first of which is the creation of G→A hypermutations throughout the genome. This leads to the integration of a defective provirus. Alternatively, the presence of uracil in DNA could activate cellular DNA repair pathways that eventually lead to degradation of the DNA, effectively halting the reverse transcription process and ultimately the viral life cycle.

Following reverse transcription, the large nucleoprotein complex consisting of the linear dsDNA, the viral proteins MA, RT, IN, NC, and Vpr, and the cellular protein

HMG I(Y) is now termed the Pre-integration Complex (PIC). This PIC must be directed to the nucleus where it is actively imported to allow for integration of the viral dsDNA into the host genome [130-132]. The lentiviruses are unique amongst retroviruses in that they are able to infect non-dividing cells by active transport through the nuclear pore complex unlike other retroviruses, which must wait for nuclear envelope breakdown during mitosis to gain access to the host chromatin.

The PIC undergoes size changes as it transits to the nucleus, thereby suggesting that other viral and/or cellular proteins are associated with it, the MA, IN, and Vpr proteins are considered to be critical for nuclear import. However, many studies have shown conflicting results pertaining to nuclear localization of MA, IN, and Vpr as fusion proteins. This has compounded efforts to elucidate the exact mechanisms involved in nuclear import of the PIC. Whether one, all, or none of these proteins is involved in nuclear import remains unclear, but it is clear that the HIV dsDNA genome does enter the nucleus where it can undergo two events.

Following entry into the nucleus, the linear dsDNA can serve as a substrate for two reactions. The first reaction results in a dead-end product and termination of the viral life cycle. This reaction involves the formation of a circular form of the dsDNA driven by ligation of the two ends to form a 2-LTR circle, formation of a 1-LTR circle by homologous recombination, or self-integration resulting in rearranged circles [133-135]. The second reaction that can occur is integration of the linear dsDNA genome into the host chromatin. This reaction leads to production of viral transcripts and continuation of the viral life cycle.

The Integrase enzyme (IN) catalyzes the integration of the HIV-1 provirus into the host cell genome to allow for transcription of viral genes for production of virions and propagation of infection to daughter cells. IN is composed of three domains, an N-terminal domain containing the Zinc finger, a core domain, and a non-conserved C-terminal domain. IN multimerizes, possibly mediated by the Zinc finger domain [136]. The core domain contains the D,D-35-E catalytic triad motif that is responsible for IN catalytic activity [137, 138]. The C-terminal domain is capable of binding DNA [139], it is necessary for 3' end processing of the provirus and is proposed to mediate IN association with the provirus[140].

Integration proceeds as a regulated, three step process [140, 141]. First, 2-3nt are removed form the 3' termini of the linear, proviral DNA. This step occurs while the pre-integration complex is still in the cytoplasm. Once the provirus is in the nucleus, IN cleaves the host chromosomal DNA resulting in processed 5' ends. The 3' recessed ends of the proviral DNA can now be ligated to the cleaved 5' ends of the host DNA and the provirus is integrated into the chromosomal DNA. The LTR regions have *att* sequences that mediate insertion into the chromosomal DNA [142]. Finally, cellular DNA repair mechanisms become activated to fill in the gaps resulting from the integration reaction [140, 141, 143]. A number of cellular cofactors have been identified that help to catalyze the integration reaction including the non-chromosomal histone protein HMG 1-a [144], the SWI/SNF chromatin remodeling complex member Ini-1 [145], the MA interacting protein EED [146], barrier to autointegration factor BAF [147], and most recently LEDGF/p75 [148, 149].

Once the provirus has been successfully integrated, the viral DNA can be transcribed using the host cell transcription machinery. The long-terminal repeat regions created during reverse transcription contain cis elements that regulate viral RNA synthesis. The 5' LTR contains the transcriptional promoter and regulatory regions while the 3' LTR encodes the poly-A signal and addition site.

Transcription of the viral RNA is controlled much like transcription of cellular RNA due to the fact that they use the same cellular machinery. The first step in viral transcription is the binding of TFIID to the TATA box located -29 to -24nt upstream of the transcription start site. TFIIB then binds the TFIID and acts to recruit the DNA dependent RNA polymerase, Pol II [150]. The TATA box plus three Sp1 sites constitute the core promoter region. Mutating all three Sp1 sites can result in inhibition or delay of virus production in some cell lines, confirming the importance of these sites in transcriptional activation [151, 152]. In addition to the core promoter, there is an enhancer region composed of two NF-KB sites upstream of the Sp1 sites. The NF-KB and Sp1 sites cooperate to promote binding of the transcriptional activators to the LTR [153]. If both NF-KB and all three Sp1 sites are eliminated, no virus is produced [154, 155]. Additional regions downstream of the transcription start site including Ap-3-like, DBF-1 and Sp1 sites are responsible for maintaining an open chromatin region to facilitate transcription [156, 157].

Low-level, basal transcriptional activity can be driven by the binding of the factors mentioned above to produce short, abrogated transcripts. However, the processivity of Pol II is not optimal in this setting. Transcriptional activity is controlled by the phosphorylation state of the Pol II complex. Active transcription occurs when Pol

II is hyperphosphorylated. Initially, hypophosphorylated Pol II is recruited to the transcription start site. It is this hypophosphorylated Pol II that drives the low-level, basal transcription. The Tat gene product is one of the short transcripts produced during the basal activation period. Tat is translated in the cytoplasm and then imported back into the nucleus via its nuclear localization signal. The HIV-1 Tat protein recruits a CTD kinase complex that drives phosphorylation at the C-terminal domain (CTD) of Pol II.

Tat is recruited to the site of transcription by binding to the HIV-1 TAR RNA element. The TAR element is unique as a transcriptional regulator as it is located downstream of the transcription start site [158]. TAR is derived from the first 59nt that fold into a stable stem-loop structure. The minimal TAR element is comprised of a stem region of base-paired nucleotides, a UCU bulge region, and a G-rich loop at the apex of the loop structure [159]. Tat requires the UCU bulge as well as the G-rich loop for function.

Tat acts as a scaffold to recruit cellular factors that will hyperphosphorylate Pol II to enhance its transcriptional activity, this complex is known as the Tat associated kinase (TAK) complex. Specifically, the cellular protein cyclin T1 binds the Tat activation domain [160]. Cyclin T1 serves two functions following Tat binding. First, it greatly enhances the affinity of Tat for the TAR element as cyclin T1 binds to the TAR loop region while Tat binds the UCU bulge [160-162]. Additionally, cyclin T1 serves to recruit the cyclin-dependent kinase Cdk9 [160]. It is the kinase activity of Cdk9 that leads to hyperphosphorylation of the CTD of Pol II [163, 164]. This is the critical step in Tat transactivation as hyperphosphorylated CTD allows Pol II to release from the promoter and engage in processive transcription and active elongation of the transcript.

Following LTR driven transcription of the viral genes, the viral transcripts must be selectively exported out of the nucleus for translation. Typically, cellular transcripts are subjected to post-transcriptional modification where introns are spliced out of the transcript prior to nuclear export. However, both the HIV genomic RNA and some of the viral transcripts must be exported unspliced to allow for proper production of the viral particle and viral proteins. The completely unspliced RNA serves as the genomic RNA for packaging and also is the template for production of the Gag and Pol proteins. Partially spliced transcripts encode the Vif, Vpr, Vpu, and Env proteins. The initial, fully spliced transcription products that produce Tat also produce the Rev and Nef viral proteins. Rev is analogous to Tat in that it is produced early during transcription and is imported back into the nucleus to facilitate downstream processes in the transcription pathway.

Rev is a nuclear-cytoplasmic shuttling protein that possesses an Arginine rich nuclear localization sequence that drives nuclear import as well as a Leucine rich nuclear export sequence that mediates export out of the nucleus. Rev functions by binding to the Rev response element (RRE) present in all unspliced and partially spliced viral transcripts. The RRE is a stem-loop structure found in the env gene. Initially, a single Rev protein binds a purine rich bubble in stem loop II of the RRE. Then, more Rev molecules are recruited leading to multimerization and nucleation of the RNA [165-168].

The Rev-RRE complex is then exported out of the nucleus by interaction with the CRM-1 nuclear export factor. Once Rev binds the importin-β family member CRM-1, CRM-1 binds RanGTP and interacts with the nuclear pore complex to achieve nuclear export of the unspliced RNA [169-173]. Nuclear translocation of the complex results in

the unspliced RNA being exported into the cytoplasm where RanGTP is hydrolyzed and the complex dissociates, releasing the RNA for translation and production of viral proteins that will assemble to form new viral particles at the cell surface [174, 175].

Gag, Pol, and Env are all initially produced as polyprotein precursors. Gag and Gag-Pol are translated by cytoplasmic ribosomes from the unspliced RNA while Env is on a partially spliced transcript that is translated by ribosomes residing on the rough endoplasmic reticulum. This leads to co-translational insertion into the lumen of the ER and eventually Env transits through the Golgi. All three precursors must be present at the cell surface for assembly and budding of infectious virions. Also, all three precursors must undergo proteolytic modifications to produce mature forms that are necessary for viral infectivity.

Gag is synthesized as a $Pr55^{Gag}$ polyprotein precursor or as a $Pr160^{Gag\text{-}Pol}$ precursor as a result of a -1 ribosomal frameshift [176]. This frameshift occurs at a low frequency due to a cis acting slippery sequence in the genomic RNA leading to a Gag:Gag-Pol ratio of about 20:1. The -1 frameshift allows the ribosome to read through the Gag stop codon and produce the Gag-Pol precursor. Maintenance of this ratio is critical for infectivity to ensure that the Pol enzymes are present at appropriate levels in the virion [177]. Both precursors oligomerize and traffic to the inner side of the plasma membrane. The trafficking of Gag and Gag-Pol is mediated by myristoylation of the N-terminal Glycine residue in the MA region at the N-terminus of the polyproteins. Once the polyproteins reach the surface, a cluster of basic residues participate in membrane binding by interacting with phospholipids in the lipid bilayer to stabilize the interactions [178-180].

Gag is composed of four domains, matrix (MA), capsid (CA), nucleocapsid (NC), and p6. While MA directs trafficking to the cell surface, CA also plays a critical role in forming the virion as the C-terminal dimerization domain is important for virion assembly [181]. In addition, the CA N-terminal core domain is responsible for virion incorporation of the necessary cellular protein cyclophilin A [182, 183], recently shown to provide protection against the host restriction factor Ref-1 [184]. The NC domain contains two CCHC zinc fingers that are critical for genome encapsidation and subsequent infectivity [185, 186]. The zinc fingers bind stem loop 3 of the Psi packaging site located in the genomic RNA between the 5' LTR and gag genes [187]. It is proposed that NC acts as an adaptor molecule that binds the genomic RNA and the cytoskeletal protein F-actin to promote trafficking to the cell surface [188-190]. Additionally, NC actually nucleates the genomic RNA to promote dimerization of the two packaged RNA strands [191, 192]. NC is important during the initiation of reverse transcription for enhancing the annealing of the tRNA primer to the PBS [193, 194]. And, NC stimulates strand transfers [195, 196] and RT processivity during reverse transcription [197]. The p6 protein also serves multiple functions. A PTAP motif in p6 has been shown to be necessary for virion budding and release [198, 199]. Additionally, p6 recruits Vpr to be packaged in the virion [200].

The Gag and Gag-Pol precursor polyproteins traffic to the cell surface by usurping the cellular pathway normally involved in the creating of multivesicular body (MVB) vesicles in the late endosomal compartment. The p6 domain contains a PTAP motif that recruits the cellular protein Tsg101 [201-203]. Tsg101 normally functions in the cell to sort proteins into the MVB. The sorting of proteins into the MVB typically

involves the use of ubiquitin tags. The Gag proteins are monoubiquitinated at several sites and ubiquitin is incorporated into the viral particle, providing indirect evidence that ubiquitylation is important for HIV budding [204, 205]. Additionally, ubiquitin mutations and application of proteasome inhibitors that deplete ubiquitin pools in the cytoplasm can inhibit virus budding [206-209]. Although the exact mechanisms of HIV budding remain to be elucidated, it is clear that the interaction between the p6 PTAP motif and Tsg101 and the involvement of ubiquitin are critical for proper trafficking of the Gag polyproteins to the surface for assembly and budding.

The Env glycoproteins are initially produced as a gp160 precursor. The gp160 precursor is glycosylated in the ER. Also while in the ER, gp160 associates with BiP/GRP78, forms disulfide bonds, and oligomerizes, usually as a trimer [210, 211]. Once it enters the Golgi network, gp160 is cleaved by a furin-like protease to yield the gp120 SU and gp41 TM proteins [212]. The gp120 and gp41 proteins are non-covalently associated and are directed to the plasma membrane by the Golgi vesicles.

Env is recruited into rafts at the plasma membrane by two palmitoylated Cysteine residues in gp41 [213]. This recruitment increases the local concentration of Env at the site of viral budding, leading to interaction with the Gag precursor and incorporation of Env in the budding virion. The interaction of Env and Gag at the cell surface prevents internalization of Env via the Y-X-X-L internalization motif by masking the motif [214-216]. MA directly binds the gp41 cytoplasmic tail to promote virion incorporation [217-220].

Budding occurs at specific sites of the plasma membrane. In some cases, this site is known as the virological synapse as the release site is where cell-to-cell contact is

occurring. It is proposed that this directed release at the virological synapse provides the virus with a direct route for passage to uninfected T cells. The site of virus release typically occurs at lipid raft sites in the plasma membrane [213, 221, 222]. Lipid rafts are proposed to serve as platforms for recruiting the necessary viral and cellular proteins involved in budding. This allows the budding machinery to concentrate at a specific site to facilitate viral release.

The Gag-Pol precursor also contains the viral enzymes RT, IN, and PR. The IN domain of the Gag-Pol precursor has been shown to recruit the cellular uracil DNA glycosylase (UNG2) enzyme into the budding virion [223]. The packaging of UNG2 into the virion is important for counteracting dUTP misincorporation into the genome during reverse transcription. UNG2 can excise uracil bases from DNA and set off a DNA repair pathway [224]. Once the virion has assembled and budded from the host cell, it undergoes a maturation process directed by the PR enzyme. This maturation leads to the evolution of a morphologically distinct particle that is fully infectious. PR is similar to the cellular aspartic proteases with two Asp residues at the active site [225]. HIV-1 PR functions as a dimer with the two monomers coming together to form flaps that surround the binding site to help stabilize the substrate [226-229]. The Asp residues lie in the center of the dimer at the catalytic site. PR expression levels and function are tightly regulated to insure that proper timing of PR activation occurs [230]. PR must first liberate itself from the Gag-Pol precursor by autocatalysis [231, 232]. Next, it engages in an ordered series of proteolysis to free the components of the Gag and Gag-Pol polyprotein precursors. Cleavage occurs to yield the p17 MA, p24 CA, p2 spacer, p7

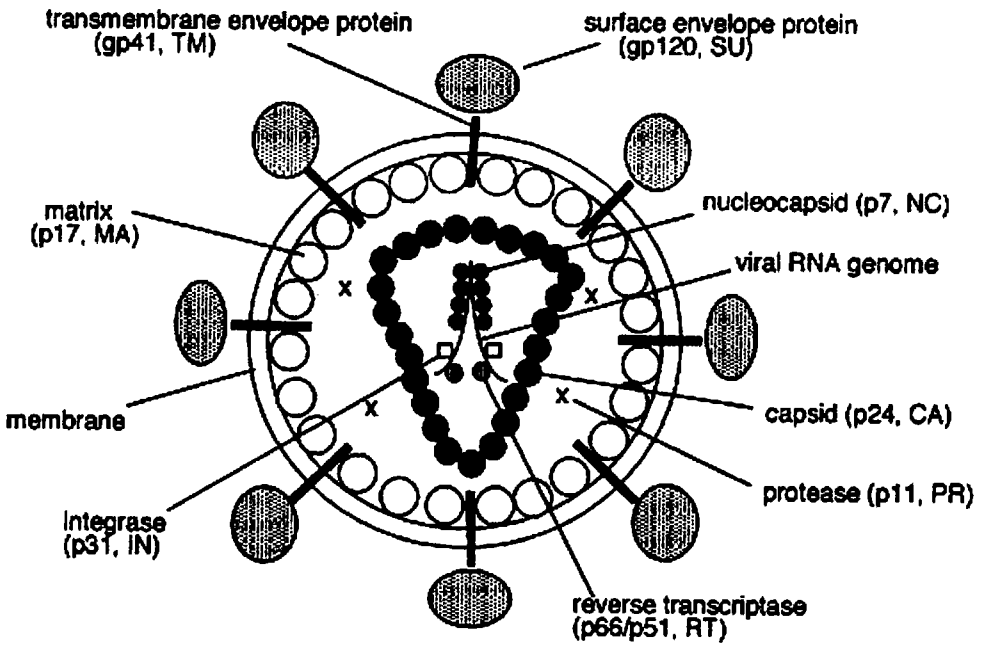
NC, p1 spacer, and p6 from Gag and the p11 PR, p66/p51 RT/RNaseH, and p31 IN enzymes from the Pol region of Gag-Pol [233].

While the object of the immature polyprotein precursors was to oligomerize, recruit additional viral or cellular proteins, and target to the cell surface for virion assembly and budding, the liberated, mature forms of the proteins must now reorganize to form a morphologically mature viral particle that is infectious (Figure 1.6) [234, 235]. MA must interact to form the outer shell that underlies the lipid bilayer membrane. The critical step in virion maturation is condensation of the core. The core is surrounded by CA proteins and contains the two strands of positive sense RNA nucleated with the NC protein as well as the RT/RNaseH, IN, and PR enzymes. Once condensation of the core is complete, the virion is now competent for infection of the next target cell. Any mutations that disrupt the catalytic activity of PR and therefore perturb virion maturation significantly reduce infectivity [236].

In addition to the Gag, Gag-Pol, and Env precursors, the Tat, Rev, Vif, Vpr, Vpu, and Nef proteins are also translated and participate in early and late events in the viral life cycle. Tat, Rev, and Nef are produced early in infection from the short, fully spliced transcripts. Tat and Rev are important for transcription and translation of the viral proteins as described above. Nef participates in many phases of the life cycle. Vif, Vpr, and Vpu are made from Rev-dependent transcripts later in the infection cycle.

Nef is a multi-functional protein that plays a role in many processes during the viral life cycle and interacts with multiple cellular pathways. It is a small, myristoylated protein that binds membranes and is necessary for pathogenesis in vivo [64, 237, 238]. Nef, an early gene, is active in all stages of the viral life cycle including possible roles in

Figure 1.6



HIV-1 virion structure. (from E. Acheampong et. al. Frontiers in Bioscience 2003 8: s156-174)

transcriptional regulation [222, 239], increased replication rates [240-242], increased particle infectivity [243, 244], immune evasion [245, 246], and completion of reverse transcription [247, 248].

Nef mediates the redistribution of both CD4 and Major Histocompatibility Complex I molecules in infected cells. Nef redirects CD4 from the cell surface and Golgi network to lysosomes where it is degraded [244, 249]. Nef interacts with the adaptor protein (AP) complexes of the endosomal sorting machinery. This interaction leads to transport of CD4 and Nef to lysosomes where degradation occurs [250]. Downregulating CD4 molecules increases the level of free Env available for virion incorporation on the cell surface by preventing CD4-Env complexes from forming intracellularly, thereby increasing the infectivity of released virions. Nef can also participate in immune evasion by endocytosing or intercepting MHC I molecules in the Golgi, thereby decreasing the expression of MHC I on the cell surface, which reduces susceptibility of virus infected cells to recognition and killing by cytotoxic T cells [245, 246]. Interestingly, although these processes of CD4 and MHC I downregulation both involve the use of AP complexes and endosomal sorting pathways, they are mediated by distinct motifs in Nef, the dileucine C-terminal motif for CD4 downregulation and an acidic cluster for MHC I downregulation [245, 250-253].

Nef is also important in infected macrophages. Here, Nef stimulates the release of CC- chemokines MIP-1α/β that act as chemotactic agents to recruit T cells to the infected macrophage. The recruitment of uninfected T cells to a site of high virus production allows for efficient infection of the substrate T cells [254]. Not only does Nef mediate recruitment of substrate T cells, but it can also induce macrophages to produce

soluble factors that allow resting, quiescent T cells that are ordinarily refractory to HIV infection to become permissive for infection. Nef does so by intercepting the CD40 signaling pathway and activating NF-κB to induce the secretion of soluble CD23 and ICAM. Both sCD23 and sICAM stimulate B cells to upregulate co-stimulatory molecules that in turn drive resting T lymphocytes out of G(0) of the cell cycle and allow them to become permissive to infection by HIV-1 [255].

Another interesting feature of Nef is its presence in the virion [256, 257]. Virion associated Nef is important for infectivity as Nef defective virions show decreased levels of viral cDNA synthesis [247, 248]. One study has shown that Nef is associated with the mature core following CA dissociation [258]. Other studies have suggested possible roles for Nef in proper disassembly of the core prior to initiation of reverse transcription [259] or enhancement of RT affinity for the RNA substrate [260].

The viral protein U (Vpu), encoded only by HIV-1 [261, 262], also participates in CD4 downregulation [263] and enhancement of viral particle release from the cell surface [264, 265]. Vpu is a small, oligomeric, integral membrane phosphoprotein. It participates in CD4 degradation in the endoplasmic reticulum to liberate Env from CD4-Env complexes. These CD4-Env complexes retain Env in the ER until Vpu degrades the CD4, which allows Env to proceed to the cell surface. Vpu binds the CD4 and the cellular protein h-βTrCP simulataneously. The complex is directed to the proteasome via Skp1 and the F box motif on h-βTrCP [266]. A recent study has shown that Vpu acts to promote viral release by interacting with the K^+ channel TASK-1. Here, it was shown that Vpu has regions of homology to TASK-1. This homology allows Vpu to bind TASK-1 as it multimerizes to form a K^+ channel. Intercalation of Vpu into TASK-1

channels disrupts the current of the channel and leads to changes in the membrane that allow for enhanced release of HIV-1 virions from the cell surface [267].

The Vpr protein of HIV-1 is a small, basic protein that is critical in the pathogenesis of AIDS [268]. Much like the Nef protein, Vpr is a multitasker that functions throughout the viral life cycle and is packaged in the virion. The incorporation of Vpr is controlled by its interaction with the p6 protein in the Gag precursor [200]. Once the virion has undergone maturation, Vpr associates with the genomic RNA in the virion core [256, 269, 270]. Following entry and uncoating, Vpr remains associated with the RTC and the PIC post-reverse transcription [271, 272]. It has also been shown that Vpr indirectly affects the fidelity of the reverse transcription process by recruiting the cellular DNA repair enzyme uracil DNA glycosylase (UNG2) to be packaged in the virion [119, 273, 274]. Within the RTC, UNG2 functions to suppress the mutation rate of the error-prone RT by excising uracil from DNA. Vpr directed UNG2 incorporation remains open to debate as other groups have shown that it is the IN domain of the Gag-Pol precursor that directs UNG2 packaging [223, 224].

PIC associated Vpr could be important in mediating nuclear import of the viral DNA [275, 276]. Vpr may play a role in the docking of the PIC with the nuclear envelope, rather than the actual translocation process [277, 278]. The virion packaged Vpr is also able to induce G2 cell cycle arrest by inactivating p34/cdc2-cyclinB kinase [279-282]. It is believed that this provides a favorable environment for viral transcription since the HIV-1 LTR is more active in G2 [283]. Vpr has also been shown to transactivate the viral LTR through the NF-κB, Sp1, C/EBP, and GRE enhancer sequences in the promoter region [284-286]. In addition to LTR activation, Vpr directly

interacts with the transcription factor TFIIB, which is part of the basal transcription machinery [287].

In addition to the virion incorporated Vpr, the newly synthesized Vpr also plays a role in the infected host cell. Vpr is a pro-apoptotic protein and is believed to induce death, which may contribute to $CD4^+$ depletion in vivo [76, 288]. Two divergent viewpoints on how Vpr induces cytotoxicity exist. One suggests that apoptosis is a byproduct of the G2 cell cycle arrest by aberrantly activating cdc2 [289] or the ATR pathway [290]. The alternate pathway involves Vpr disrupting the mitochondrial membrane leading to release of cytochrome c, which then activates the caspase pathway, and pro-apoptotic factors into the cytoplasm [291, 292]. Vpr crosses the outer mitochondrial membrane and then interacts with the adenine nucleotide translocator, a transmembrane protein on the inner membrane, to induce membrane permeabilization and release of apoptotic factors [292, 293].

D. HIV-1 Vif and Apobec3G

1. Background

The viral infectivity factor (Vif) protein of HIV-1 is critical for infectivity *in vivo* as well as in certain non-permissive T cell lines such as H9, HUT78, and CEM. However, Vif is dispensable in the permissive T cell lines SupT1, Jurkat, and CEM-SS [73, 79-81, 294-296]. Vif is a 27kDa protein that is present in the cytoplasm. It has been shown to associate with the cytoskeletal protein vimentin, the Pr55Gag precursor, and the viral RNA genome.

Early experiments aimed at elucidating the function of Vif utilized heterokaryons to determine if non-permissive cell lines encoded a restrictive factor, or if permissive lines provided a homologous protein that could substitute for Vif. The result of the heterokaryon experiment suggested that non-permissive cell lines express a restrictive factor that Vif must counteract [297].

Sheehy et al. performed genetic screens on the non-permissive cell line CEM and compared it to the related, permissive cell line CEM-SS to identify the candidate inhibitory protein, which they termed CEM15. They went on to show that CEM15 is differentially expressed in non-permissive cell lines, but not permissive cell lines. Exogenous expression of CEM15 in permissive cell lines was sufficient to render them non-permissive [298]. It was later revealed that CEM15 is actually the apolipoprotein B mRNA editing catalytic polypeptide 3G (Apobec3G) [299]. Apobec3G is a cytidine deaminase that deaminates cytosine to uracil in single-stranded DNA [300, 301]. Subsequent experiments revealed that in addition to Apobec3G, the related family members Apobec3B and Apobec3F also demonstrate antiviral activity against HIV-1 [302-305].

2. Apobec3G

The Apobec superfamily consists of Apobec1, Apobec2, Apobec3A-G, and activation induced deaminase (AID) in humans. Each of these family members deaminates cytosine to uracil in either an RNA or DNA template. All members have at least one catalytic site with a zinc-dependent cytidine deaminase domain of $HXEX_{23-28}PCXXC$, while Apobec3B/F/G have undergone gene duplication to produce larger proteins with two catalytic sites [299].

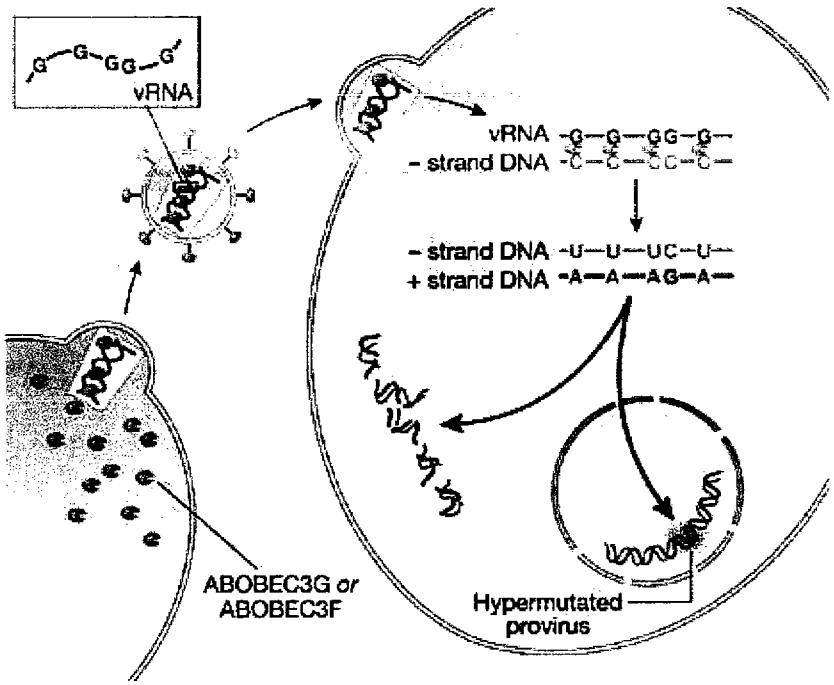
Apobec3G is expressed in lymphoid and myeloid cell lines. It is not constitutively expressed, but rather its transcription is regulated by an inducible PKCα/βI/MEK/ERK pathway that can activate downstream transcription factors. This protein kinase cascade can be induced by treatment with the phorbol ester PMA. T cell activation, but not HIV-1 infection, upregulates Apobec3G expression [306]. Apobec3G is expressed exclusively in the cytoplasm of cells, possibly to avoid editing of endogenous DNA [307]. Evolutionary studies of the Apobec3G gene in non-human primates and hominids suggests that a positive selective force was exerted on the gene prior to the emergence of modern lentiviruses. It is proposed that Apobec3G initially evolved to target endogenous retroviruses in humans (HERV), and only later became an antiviral factor directed against HIV as well as hepatitis B virus [307].

Early studies revealed that Apobec3G is incorporated into virions in the absence of Vif [308, 309] via an interaction with the NC domain of the $Pr55^{Gag}$ precursor [310-313]. Gag alone, in the absence of viral proteins and genomic RNA, is sufficient for packaging Apobec3G into virus like particles (VLP) [311, 313, 314]. Although it was shown that binding is mediated by the first eleven residues in the N-terminus of NC and the first linker region of Apobec3G [311, 312], the ability of divergent Gag molecules from MLV, SIV, and different HIV subtypes to efficiently package Apobec3G suggested that there was not a direct interaction between the two [312]. Subsequent work revealed that there appears to be an RNA bridge that facilitates the interaction of NC and Apobec3G in the cell [313, 315]. One recent study showed that Apobec3G is mono-ubiquitinated by Nedd4-1 and that this modification may address Apobec3G to a compartment where $Pr55^{Gag}$ precursor is also present [310, 316]. Apobec3G has been

found at the cell surface as an aggregate with Gag as well as in late endosomal compartments where Gag is present [297]. These studies have led to a model where $Pr55^{Gag}$ precursor interacts with Apobec3G via cellular RNA in specific compartments of infected cells. This interaction allows the NC domain of $Pr55^{Gag}$ precursor to direct the packaging of Apobec3G into the budding virions.

The objective of Apobec3G is to associate with the viral core so that it can enter the target cell and remain associated with the RTC. Exploiting an interaction with NC guarantees passage into the virion core. Once in the RTC in the target cell, Apobec3G can utilize the minus strand DNA produced during reverse transcription as a template for cytosine deamination (Figure 1.7). Apobec3G will deaminate cytidine to uracil on the minus strand cDNA [120, 122, 301, 308, 317]. The presence of uracil in DNA typically sets off a pathway that results in degradation of the uracil containing DNA. This would explain the observed defect in ΔVif virions where reverse transcription is either incomplete or the transcripts are unstable [318, 319]. Alternatively, the presence of uracil in DNA could trigger a pathway that recruits the cellular repair enzyme uracil DNA glycosylase to excise the uracil, leaving an abasic site in its place. Since the RNA is degraded rapidly during reverse transcription by RNaseH, there is no template available to repair the abasic site. So, HIV-1 RT will preferentially insert an adenine across from an abasic site. The end result of cytidine to uracil deamination is a G-to-A mutation in the coding, plus strand of the viral DNA. Apobec3G has a preference for GG dinucleotides, usually in the consensus sequence T/A<u>G</u>GG where the <u>G</u> is mutated to an A [320]. While this explained most of the G-to-A hypermutation that was detected in the HIV-1 genome following reverse transcription, it was the identification of Apobec3F as

Figure 1.7



Apobec3G packaged in the virion deaminates nascent viral transcripts resulting in G→A hypermutation or degradation. (from D. Trono EMBO 2004 5(7): 679-680)

an antiviral factor that helped to explain the additional G-to-A mutations that were frequently detected in sequences that were not Apobec3G targets. Apobec3F functions in a manner analogous to Apobec3G, but its preference for target nucleotides is the cytidine in the context of TC dinucleotides [302]. Hypermutation of the genome often leads to the generation of premature stop codons such that provirus that is integrated is attenuated or completely defective [301].

Apobec3G contains two cytidine deamination (CD) sites. The N-terminal CD1 site is involved in RNA binding, virion incorporation, and antiviral activity while the C-terminal CD2 site is responsible for cytidine deaminase activity and the editing mediated antiviral effect [321]. Either of these sites is capable of exerting an antiviral effect, suggesting that while editing of the genome will inhibit the viral life cycle, Apobec3G is capable of impacting the reverse transcription process in other manners [322]. Interestingly, it has been shown that Apobec3G inhibition of hepatitis B virus is independent of the deamination activity of Apobec3G, supporting the idea of a second antiviral effect that supplements the hypermutation induced by cytidine deamination [323]. It was recently demonstrated that the enzymatic activity of Apobec3G is critical for its antiviral activity, but it is not the only determinant. Deletion mutants that were impaired for DNA editing activity maintained an ability to inhibit HIV infection [324]. It is possible that this secondary antiviral effect is the result of Apobec3G interaction with NC. The hallmarks of ΔVif virions are defects in intravirion reverse transcription and endogenous reverse transcription [318, 325]. One study also showed that ΔVif virions undergoing reverse transcription had compromised capacity for proper tRNA placement [326], although a second study shows limited defects in the endogenous RT assay for

ΔVif virions [327]. NC is necessary for the production of full-length reverse transcripts and is involved in placement of the tRNA primer on the PBS. Therefore, it is possible that the interaction of Apobec3G with NC, in the absence of Vif, results in packaging of Apobec3G and downstream disruption of NC function during subsequent reverse transcription.

3. HIV-1 Vif

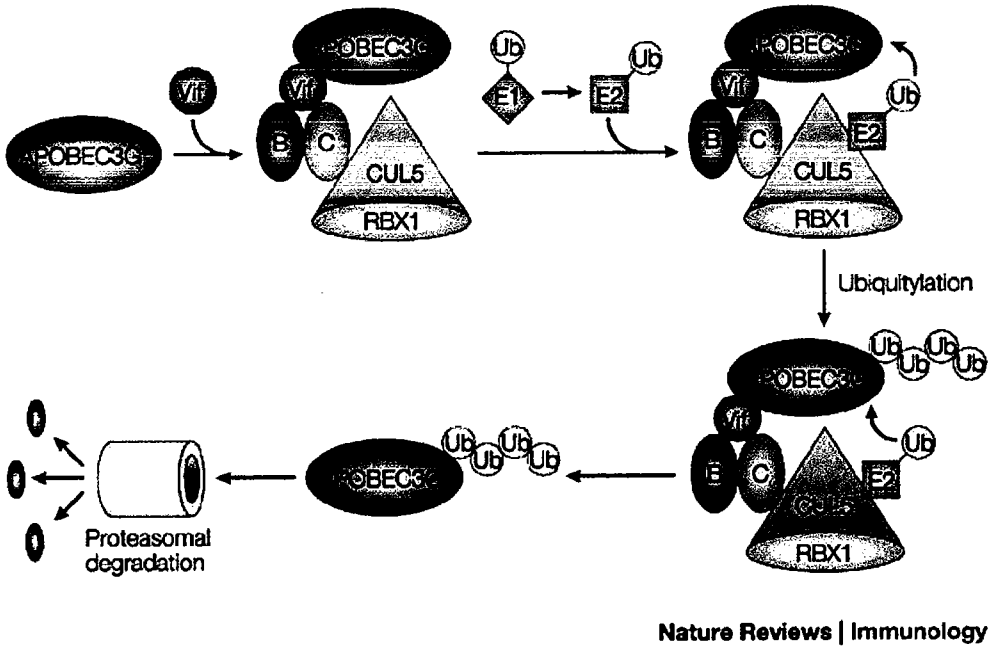
Clearly, packaging of Apobec3G into the virion is suicide for the virus. Therefore, the virus utilizes its Vif protein to neutralize Apobec3G inhibition by preventing its incorporation into the virion [309, 328, 329] . The ability of Vif to prevent virion incorporation of Apobec3G packaging is critical as ΔVif viruses are defective in post-entry events in the life cycle due to the presence of Apobec3G in the virion. Once Apobec3G has been encapsidated into the virion, it can inhibit the reverse transcription steps, even if Vif is also present in the virion [330]. Initially, it was shown that Vif mediates Apobec3G degradation via the proteasome pathway to reduce available Apobec3G for packaging [329, 331-333]. Additional studies have also pointed to the ability of Vif to block Apobec3G packaging by direct interaction and sequestration of the protein away from NC and the virus assembly site [334, 335].

Initial studies looking at Vif neutralization of Apobec3G were focused on the active degradation of Apobec3G promoted by interaction with Vif. When intracellular expression levels of Apobec3G were compared in cells infected with a WT or ΔVif virus, the levels were consistently much lower in the presence of WT Vif. Also, when these cells were treated with proteasome inhibitors, this Apobec3G downregulation could be partially rescued [329, 331-333]. Additionally, it was found that Apobec3G is subject to

modification by polyubiquitination in the presence of Vif. This led to the conclusion that Vif directs Apobec3G degradation via the cellular proteasome pathway [331-333].

While the above studies suggested that Vif and Apobec3G might directly interact to drive Apobec3G degradation, the mechanism by which Vif actively promotes proteasomal degradation was defined in a series of papers examining Vif interaction with the Cul5-ElonginB-ElonginC E3 ubiquitin ligase complex (Figure 1.8). The first observations indicated that Vif intereacts with Apobec3G and the Cul5 pathway to promote degradation of Apobec3G. The nonfunctional SLQ Vif mutant was still competent for Apobec3G binding, but not Cul5 interaction, suggesting that Apobec3G binding alone was not sufficient for proteasomal degradation of Apobec3G [336]. Building on this data plus a previous observation that the highly conserved and critical SLQ motif in Vif resembles a SOCS-box motif [332], the Yu group went on to show that Vif assembles with Cul5 via a novel SOCS box motif. Here, it was shown that Vif utilizes its atypical SLQ SOCS box motif to interact with Elongin C. Elongin C then serves as a scaffold for recruitment of Elongin B and Cul5 along with Rbx1 and an E3 ubiquitin ligase. In addition to the SOCS box motif, upstream Cysteine residues at positions 114 and 133 in Vif are critical for stabilizing the Vif-Cul5 complex [337]. The assembly of the Vif-Cul5 complex is subject to regulation by phosphorylation. Also, when Vif assembles with Cul5, both Vif and Apobec3G are ubiquitinated by the E3 ubiquitin ligase [338]. Finally, an in vitro assay was developed to confirm that Apboec3G could serve as a substrate for the Vif-Cul5-E3 ubiquitin ligase complex [339]. This suggests a model where Vif interacts with the Cul5 complex and recruits Apobec3G

Figure 1.8



Vif mediated degradation of Apobec3G. (from R. S. Harris and M. T. Liddament Nature Reviews Immunology 2004 4(11): 868-877)

to the complex for polyubiquitination. This would then target both Vif and Apobec3G to the proteasome for degradation.

While it is clear that Vif can mediate direct degradation of Apobec3G via recruitment of an EloBC-Cul5-E3 ubiquitin ligase complex, several studies noted that blocking this pathway did not restore Apobec3G levels completely. Therefore, it was suggested that Vif has a second function that does not involve the proteasome pathway for Apobec3G inhibition. Mehle et al. proposed that Vif regulation by phosphorylation could indicate the existence of an alternative, phosphorylated form of Vif that could still bind Apobec3G, but might sequester Apobec3G in a distinct subcellular compartment rather than direct its degradation [338]. To further explore this idea of an alternative pathway for Apobec3G neutralization by direct binding in the absence of degradation, a study was performed in E. coli cells that lack proteasomal machinery. In this study, Vif and Apobec3G were both expressed in E. coli and Apobec3G cytidine deaminase activity was monitored by following cytidine deamination in an Apobec3G substrate template. It was found that in this system, Vif tightly binds Apobec3G and this is sufficient to decrease Apobec3G deaminase activity. Previous work had established that a D128K mutation in Apobec3G does not disrupt its catalytic activity, but renders it resistant to HIV-1 Vif [340-343]. The Vif induced inhibition of Apobec3G catalytic activity could be alleviated by using D128K Apobec3G, confirming that Vif-Apobec3G interaction is sufficient to inhibit Apobec3G activity [335]. Also in support of a supplemental antiviral effect by interaction in the absence of degradation, Kao et al. has shown that Vif and Apobec3G can be efficiently co-expressed in cells and that virus produced from these cells is infectious [334]. Taken together, this data indicates the existence of two distinct

pathways that Vif can exploit for Apobec3G inhibition. This is not unique as the MDM2-p53 interaction in cells can be disrupted by two separate, independent mechanisms under different conditions [344].

It is clear that Vif functions to prevent virion encapsidation of Apobec3G. This can be achieved by two mechanisms, either directed degradation of Apobec3G by a Vif induced EloBC-Cul5-E3 ubiquitin ligase complex or by the direct engagement of Apobec3G by Vif. Since both of these approaches are necessary, but not sufficient for prevention of Apobec3G into virions, Vif probably utilizes both of these approaches simultaneously in the cell to ensure that Apobec3G is not available for interaction with NC and packaging into the virion. Once in the virion, Apobec3G can exert its antiviral effects by either directly modulating NC or inducing hypermutation of the nascent viral reverse transcripts leading to abortive reverse transcription or the generation of hypermutated, defective genomes.

In addition to the recently derived knowledge of Vif function, many studies have been performed to address the subcellular localization of Vif in an effort to understand how this might contribute to Vif function. Early work in the field of Vif subcellular localization revealed that Vif is a predominantly cytoplasmic protein that shows diffuse staining throughout the cytoplasm as both a soluble and membrane associated protein [345-347]. Other studies suggested cytoplasmic colocalization of Vif with the HIV Gag protein, perhaps mediated by the NC domain of Gag [348-350]. In addition to the noted cytoplasmic localization of Vif, it has also been reported to form vimentin caged, perinuclear aggregates in some cells [348, 351, 352]. Functionally, it was found to be critical for Vif to localize to both an insoluble and soluble cytoplasmic fraction. Viruses

harboring mutant Vif proteins that localized only to the soluble fraction were rendered significantly less infectious [353] Most of these studies were performed in permissive Hela or 293T cells prior to the identification of Apobec3G as the cellular target for Vif function. As such, they were unable to provide a correlation between subcellular localization of Vif and Apobec3G neutralization by Vif. Two recent studies have shown that cytoplasmic localization of Vif is absolutely critical for Vif function [352, 354]. The need for Vif in the cytoplasm is obvious when its function is considered. Vif interacts with Apobec3G, a cytoplasmic protein, and sequesters it in a cytoplasmic compartment or drives its degradation via the cytoplasmically located proteasome pathway. Also, the observations that Vif and Gag colocalize and that this colocalization may be mediated by NC can now be better understood when Apobec3G is considered in the equation. Vif must prevent Apobec3G from binding NC and packaging in the virion. Therefore, Vif-Gag colocalization may be important for preventing NC interaction with Apobec3G by direct occlusion of the Apobec3G binding site on NC or by competitive binding of Apobec3G by Vif.

In summary, much work has been done in the Vif field recently. Several studies have provided insight into the cytoplasmic localization of Vif. Many recent works have dissected the function of Vif in counteracting the cellular cytidine deaminase Apobec3G. Taken together, we can now consider a model for Vif where it interacts with Apobec3G in the cytoplasm to neutralize Apobec3G. This interaction may occur by two distinct pathways involving active degradation or sequestration that may function synergistically, or under different physiological conditions. Regardless of the pathway, the ultimate objective of Vif is to prevent Apobec3G from interacting with the NC domain of the Gag

precursor and packaging into the virion. Once Apobec3G has packaged in the virion, it is capable of exerting an antiviral effect by inducing cytidine deamination of the nascent retroviral transcripts, or perhaps by influencing other components of the reverse transcription machinery leading to abortive reverse transcription or the production of hypermutated, dead-end viral genomes.

E. Thesis Aims

The objective of this thesis was to determine the impact of Vif subcellular localization on function. The recent insight into the Vif-Apobec3G interaction and its contribution to the replication status of virions has demonstrated the critical nature of the Vif protein. While many studies have aimed at elucidating the mechanism by which Vif degrades Apobec3G, here the goal was to determine where Vif encounters Apobec3G and how altering the subcellular localization of Vif might disrupt its function by preventing a Vif-Apobec3G interaction.

In Chapter III, the initial studies pertaining to Vif subcellular localization are presented. Here, the importance of Vif localization was derived by investigating a naturally occurring Vif mutant present in the virus isolated from a unique long-term non-progressor. The Vif protein from this LTNP was non-functional and contributed to the low viral growth phenotype observed in tissue culture assays. The amino acid sequence of this Vif mutant revealed a unique set of mutations at the central region of ^{90}KKRK^{93}. This KKRK motif was found to be a functional nuclear localization sequence that mislocalized Vif from the cytoplasm to the nucleus. Isogenic clones of NL4.3 that differed only at the central region revealed that NL4.3/KKRK virus was significantly less

infectious than NL4.3/WT virus when produced from non-permissive cells. These findings suggested that the presence of a functional Vif in the cytoplasm is necessary for production of infectious virions.

Based on the above observations, the NL4.3/KKRK mutant was further characterized to determine its capacity for Apobec3G neutralization. In Chapter IV, it is shown that NL4.3/KKRK Vif is less capable of interacting with Apobec3G in the cell, although in vitro binding assays reveal that it is fully competent for Apobec3G binding. Additionally, data are presented that demonstrate a diminished capacity for NL4.3/KKRK Vif to downregulate Apobec3G by degradation or sequestration. Also, it was found that Apobec3G was efficiently packaged into the NL4.3/KKRK virion, but not the NL4.3/WT virion. This study is the first to show an aberrant interaction between a naturally occurring Vif mutant and Apobec3G leading to Apobec3G virion encapsidation and decreased virus infectivity. Additionally, it highlights the importance of Vif cytoplasmic localization in inhibiting Apobec3G virion packaging.

Chapter V builds upon the above findings to further investigate the role of NL4.3/WT Vif in the cytoplasm of infected cells. Initial studies suggested that Vif functions by directing the degradation of Apobec3G by exploiting the EloBC-Cul5-E3 ubiquitin ligase to ubiquitylate Apobec3G for degradation by the proteasome. However, we and others have observed conditions where Vif is still capable of inhibiting Apobec3G in the absence of complete degradation. This suggested a second pathway for Vif neutralization of Apobec3G. Work presented here shows that Vif can also inhibit Apobec3G by sequestering it into a perinuclear aggregate. To accomplish this, we prove that Vif can exploit the cellular aggresome pathway to recruit Apobec3G into an

aggregate. This would effectively prevent virion packaging of Apobec3G by sequestering it at a perinuclear site distant from the virus assembly and budding site. Disruption of aggresome assembly leads to decreased infectivity of virions produced in from cells expressing Apobec3G. This alternative pathway would serve as a parallel mechanism for Apobec3G inhibition in conjunction with the previously demonstrated proteasome pathway.

The data presented here confirm that cytoplasmic localization of Vif during the viral life cycle is critical. Additionally, evidence of a second pathway for Vif mediated Apobec3G neutralization is presented. These findings could influence both vaccine and therapy design as abrogating Vif function leads to inhibition of viral replication as well as having an impact on the Vif biology field by defining alternate pathways for Vif mediated Apobec3G neutralization.

CHAPTER II

MATERIALS AND METHODS

A. Subject

Long-Term Non-Progressor 4 (LTNP4) is an HIV-1 seropositive hemophiliac. LTNP4 has been followed as part of the New England Hemophilia Center cohort since 1983. LTNP4 is also hepatitis B and C seropositive. The patient was infected with HIV-1 via infusion with contaminated factor VIII concentrates prior to 1984. LTNP4 is antiretroviral therapy naïve.

B. Immunological Assays

$CD4^+$ and $CD8^+$ T cells were enumerated by labeling whole blood with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse monoclonal antibodies (Becton Dickinson, Mountain View, CA) and analyzing by fluorescence-activated cell sorting (FACS). $CD4^+$ and $CD8^+$ T cells counts are presented as cells/μl. $CD4^+$ T cell counts were monitored semi-anually from 1983 to the present. $CD8^+$ T cell counts were derived annually as were $CD4^+/CD8^+$ ratios.

C. Quantification of Plasma Viral Load

Plasma viral load was determined by quantitative PCR using the Amplicor HIV-1 Ultrasensitive Monitor Test, according to manufacturer's instructions (Roche

Diagnostics, Branchburg, NJ). Plasma samples were processed and viral RNA was amplified using the reverse transcriptase polymerase chain reaction (RT-PCR). Amplified products were quantified by comparison to a standard panel of known HIV-1 RNA copy numbers. Results presented in Chapter III are HIV-1 RNA copies/ml. The limit of detection for the assay was improved from 400 copies/ml to 50 copies/ml in 1997.

D. Cell culture and co-culture assays

All parental T cell lines were cultured in RPMI media supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. Vif-complementing T cell lines (kindly provided by Dr. Dana Gabuzda, Dana Farber Cancer Institute) were maintained as above with the addition of puromycin at 1μg/ml. The permissive SupT1 cell line, as well as the non-permissive H9 and HUT78 cell lines, and their Vif-complementing derivatives were used in infection and co-culture studies presented in Chapter 3. The paired cells lines CEM, the non-permissive, parental line, and CEM-SS, a CEM derived, permissive line, were used for infection studies presented in Chapter V.

The adherent Hela cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. The Hela derivative MAGI was cultured in DMEM/10% FBS plus 200μg/ml G418, and 100μg/ml Hygromycin B. The GHOST-X4 reporter cell line, an adherent cell line that are stably transfected to allow constitutive expression of CD4 and CXCR4 and that harbor the GFP protein under the control of the HIV-1 Tat promoter, was cultured in DMEM supplemented with 10% FBS plus 1μg/ml puromycin, 500μg/ml G418, and 50μg/ml hygromycin B. Hela-Apo lines are Hela cells

stably transfected with an Apobec3G expression vector. Lines are maintainted in DMEM supplemented with 10% FBS under selection with 400μg/ml G418.

Blood from both HIV seronegative and seropositive individuals were collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation. PBMC were stimulated with phytohemagglutinin (PHA) at 5μg/ml for 3 days followed by $CD8^+$ T cell depletion by magnetic beads coated with an antibody specific for CD8 (Dynal, Inc, Great Neck, NY). PBMC were cultured at $2x10^6$ cells/ml in RPMI supplemented with 10% FBS, 10U/ml rIL-2, penicillin G (100U/ml), and streptomycin (100μg/ml) (all cell culture reagents from Sigma-Aldrich, St. Louis, MO except for rIL-2 from Roche Applied Diagnostics, Indianapolis, IN). Co-cultures were performed according to the AIDS Clinical Trial Group (ACTG) virology lab manual protocol for isolating LTNP virus by co-culturing LTNP PBMC with normal, seronegative donor cells.

For growth of LTNP4 virus presented in Chapter III, a modified co-culture technique was developed for co-culture of patient cells with T cell lines. $CD4^+$ T cell enhanced LTNP4 PBMC were re-stimulated overnight with PHA (5μg/ml). $2x10^6$ patient cells were co-cultured with $2x10^6$ cells of the parental or Vif-complementing T cell lines and maintained in RPMI/10% FBS supplemented with 10U/ml recombinant interleukin 2 in a 12-well culture plate. One week post-co-culture, cells were switched to RPMI/10% FBS for the parental T cell lines and RPMI/10% FBS supplemented with 1μg/ml puromycin for the Vif-complementing T cell lines. For co-culture of transfected cells with T cell lines, $2x10^6$ parental or Vif complementing HUT78 cells were washed once in RPMI/10% FBS and then added to $2x10^6$ transfected HUT78 cells in a 24 well

plate. All cell cultures were incubated at 37°C in a 5% CO_2 humidified incubator. Culture supernatants were assayed for virus production by RNA PCR and p24 antigen detection assays every 3 to 4 days post-co-culture.

E. Amplification and sequencing of LTNP4 viral sequences

RNA from cultured virus as well as patient plasma virus was isolated by lysing virions with lysis buffer (Tris-HCl/68%guanidine thiocyanate/3%DTT) for 10 minutes at room temperature. The RNA was precipitated with isopropanol and resuspended in a Tris buffer. Reverse transcription was carried out using the ThermoScript RT-PCR System from Gibco-BRL (Grand Island, NY) according to manufacturers instructions with random hexamers.

2μl of the RT reaction containing the PCR-amplified cDNA was then used in a PCR reaction using Taq polymerase (Gibco) with two HIV-1 vif-specific primers, IS5'GTACAAATGGCAGTATTCATCCAC3' and IA5'TTATTATGGCTTCCACTC CTGCCC3', in a 100μl reaction. Amplification was achieved using 25 cycles each consisting of 94° for 1 minute for denaturation, 50° for 1.5 minutes for annealing, 72° for 2 minutes for extension, and a final extension step of 72° for 10 minutes in a Perkin Elmer 9700 Thermal Cycler. A nested PCR reaction was then performed under similar PCR parameters using 5μl PCR product from the previous reaction with the vif specific primers Vif-F $^{5'}$GGTGAAGGGGCAGTAGTAATACAA$^{3'}$ and Vif-R$^{5'}$GTGTCCATTCATTGTATGGCTCCC$^{3'}$.

To determine the sequence of proviral DNA in patient cells, whole cell lysates of patient $CD4^+$ T cells were prepared using Tris-HCl (pH7.3), 1%Triton X-100, 7.5mM

$MgCl_2$, 0.01% Proteinase K, and 0.05% sodium azide for 30 minutes at 60°C followed by 30 minutes at 100°C. 5µl of whole cell lysate was used as the template in a nested PCR reaction using the same Vif-IS/IA and F/R primers, polymerase, and cycling protocol described above.

The final PCR product was purified using the QIAquick PCR Purification kit from Qiagen (Valencia, CA) following the manufacturers protocol. Sequencing was performed using the following primers, Vif-IIF $^{5'}$GTTCAGAAGTACACATCCC AC$^{3'}$and Vif-IIR$^{5'}$GTGGGATGTGTACTTCTGAAC$^{3'}$, in a Big Dye Terminator sequencing reaction. All sequencing was performed on an ABI 377 XL Prism at the UMass Center for AIDS Research Molecular Biology Core Facility. Sequences were analyzed using the Sequencher software package.

Sequences presented in Chapter III were aligned against the consensus clade B vif sequence from the Los Alamos National Laboratory (LANL) database. The frequency of mutations in the population was also derived from the sequences deposited in the LANL database.

F. Plasmid construction and mutagenesis

To generate isogenic clones of NL4.3 that differed only at the KKRK motif in Vif, site directed PCR mutagenesis was used. Site directed PCR mutagenesis was achieved using the full length HIV-1 plasmid NL4.3 as a template in the Stratagene QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The NL4.3 plasmid template was propagated in Stbl II competent cells from Invitrogen (Carlsbad, CA) to ensure methylation of the template. Methylated template NL4.3 was used with

the following gel purified primers, VifK1[5']TCCATAGAATGGAAGAAAAGAAAAT ATAGCACACAA[3'] and Vif-K2[5']TTGTGTGCTATATTTTCTTTTCTTCCATTCTAT GGA[3'] to generate substitution mutations of arginine to lysine, lysine to arginine, and arginine to lysine as indicated by the underlined nucleotides. 20 amplification cycles of 95° for 1 minute, 45° for 1 minute, and 68° for 35 minutes followed by a final extension of 68° for 7 minutes were performed. Following amplification, the PCR product was subjected to 3 hours of digestion by DpnI at 37°C to degrade the methylated input template. 50 to 200 ng of the mutagenized plasmid was then used to transform INVαF' competent cells from Invitrogen (Carlsbad, CA). Clones were selected and the sequence confirmed by full length sequencing of the plasmid.

The same site directed mutagenesis approach was used to generate the NL4.3/IF plasmid. Here, the primers used were VIF-HF [5']CATATCTATTCATCAAGGAAAGC TAAGGACTGGATATATAGACATC[3'] and VIF-HR [5']GATGTCTATATATCCA GTCCTTAGCTTTCCTTGATGAATAGATATG[3'].

For the initial subcellular localization studies in Chapter III, the pDsRed1-N1 fusion vector from Clontech (Palo Alto, CA) was used to generate in-frame, N-terminal DsRed-Vif fusion proteins. Wild type and mutant vif sequences were amplified from the NL4.3 templates using Vif-Nhe [5']GGCTAGCTTATGGAAAACAGATGG[3'] and Vif-Eco [5']CGGAATT CGCTGTCCATTCAT[3'] in a 100µl PCR reaction using 25 cycles of 94° for 1 minute, 48° for 1 minute, 72° for 3 minutes, and a final extension of 72° for 10 minutes. PCR products were digested with EcoRI and NheI and ligated into the EcoRI/NheI digested DsRed vector using T4 DNA ligase from New England Biolabs

(Beverly, MA). The pCMV/myc/nuc/GFP plasmid from Invitrogen (Carlsbad, CA) was used as a control for nuclear localization.

The HL8 Vif shuttling construct used in Chapter V was constructed by amplifying and cloning the DsRed-WT vif fusion gene into a vector that contained a linker region. The stop codon of DsRed was removed and it was inserted N-terminal to a linker region with a stop codon in its C-terminus. By inserting a linker region, the molecular weight of the DsRed-WT Vif construct was elevated above the cutoff for passive nuclear diffusion. A GFP-tagged Rev protein (kindly provided by Dr. Maria Zapp, UMass Medical School) was included as a nucleocytoplasmic shuttling protein control.

The GFP-tagged Apobec3G fusion construct used in Chapters IV and V was generated by amplifying the pcDNA-Apobec3G plasmid (kindly provided by Dr. Michael Malim, King's College) with the primers GFP-HindIII 5'CCC AAG CTT GGG ATG AAG CCT CAC TTC3' and GFP-EcoRI 5'GCG GAA TTC CTG TCA GTT TTC CTG 3' and cloned into the eGFP-C3 vector (Clontech, Palo Alto, CA). An empty vector (kindly provided by Dr. Maria Zapp, UMass Medical School) was also included in those studies to insure that equal amounts of total DNA were transfected in each co-transfection experiment.

For rVif constructs, vif was amplified from either the NL4.3/KKRK or NL4.3/WT plasmid using the primers pHAT-EcoRI 5'GGA ATT CCC TAC TGT CCA TTC ATT 3' and pHAT-BamHI 5'GGG ATC CTT ATG GAA AAC AGA TGG 3' and cloned into the pHAT11 vector (Clontech, Palo Alto, CA). For the rApo construct, apobec3G was amplified from the pcDNA-Apobec3G plasmid with the primers pHAT-KpnI 5'CGG

GGT ACC CCG ATG AAG CCT CAC TTC 3' and pHAT-EcoRI 5'GCG GAA TTC CTG TCA GTT TTC CTG 3'.

All constructs were confirmed by amplification and sequencing as above and Western blot or immunofluorescence microscopy for expression.

G. Production of viral stocks

Virus stocks for NL4.3/WT, NL4.3/KKRK, or NL4.3/IF were generated by transfection of permissive or non-permissive cell lines. Non-permissive HUT78, H9, or CEM or permissive CEM-SS cells were seeded 24 hours prior to transfection and the SuperFect Transfection Reagent kit from QIAgen (Valencia, CA) was used. Hela cells were seeded 24 hours prior to transfection with the PolyFect Transfection Reagent kit from QIAgen. Culture supernatant was harvested 72 hours post-transfection and assayed for production of virus by a p24 ELISA detection assay. For the aggresome studies presented in Chapter V, CEM or CEM-SS cells were infected with 100ng p24 for two days prior to being washed three times and split into four fractions. One group for each cell line received 10μM lactacystin, 10μM nocodazole, or 10μM Scriptaid for 18 hours.

H. Infectivity assays

For the spreading infection assay presented in Chapter III, H9 or CEM-SS cells ($2x10^6$) were infected with 5ng of p24 with 8μg/ml DEAE-dextran for 3 hours followed by five washes in media. Replication was followed for two weeks by culture supernatant sampling and p24 ELISA detection assay. The viral rescue assay presented in Chapter III

utilized a co-culture of transfected HUT78 cells with HUT78 or HUT78xVif cell lines. $2x10^6$ parental or Vif complementing HUT78 cells were washed once in RPMI/10% FBS and then added to $2x10^6$ transfected HUT78 cells in a 24 well plate. All cell cultures were incubated at 37°C in a 5% CO_2 humidified incubator. Culture supernatants were assayed for virus production p24 ELISA detection assays every 3 to 4 days post-co-culture. For the single cycle infectivity assays presented in Chapter III and V, GHOST-X4 cells were infected overnight with culture supernatant containing virus normalized to 100ng of p24, or for 3 hours in the presence of 8μg/ml DEAE-dextran followed by three media washes. Cultures were washed and assayed by FACS analysis for %GFP+ cells three days post-infection.

I. Subcellular localization of Vif and Apobec3G

The localization studies shown in Chapter III utilized DsRed tagged WT or KKRK Vif constructs. For these experiments, MAGI cells were seeded to subconfluency on coverslips in a six well plate and allowed to adhere at 37°C for 12 hours prior to transfection. 20ug of the plasmid construct was ethanol precipitated and resuspended in 450μl sterile water and 50μl 2M $CaCl_2$. The DNA mixture was then added dropwise to 500μl 2xHBS pH 7.08 while vortexing and incubated for 20 minutes on ice. The mixture was then added dropwise to the cells and incubated at 37°C for 16 hours. The cells were washed three times with PBS and twice with media. 5ml of DMEM/10% FBS supplemented with 200μg/ml G418, and 100μg/ml Hygromycin B was added back.

Forty-eight hours post-transfection, the coverslips were fixed in 1% formaldehyde for 15 minutes at room temperature and washed 6x 30s in PBS/1%BSA/0.1% Tween.

Ice-cold methanol was added for 15 minutes followed by a 6x 30s wash as above. DAPI stain was applied for 1 minute and then the coverslip was washed 3x in sterile water. The coverslip was then mounted on a slide using the Pro-long Antifade Kit from Molecular Probes (Eugene, OR). Image acquisition was performed at the UMass Digital Imaging Core Facility on an Olympus IX 70 inverted microscope equipped with a Physik LVPZT piezo-electric focus drive. A series of planes were acquired at 0.2μm intervals using a PlanoApo 100x oil immersion lens with a DAPI, FITC, or TRITC filter. All images were stacked and deconvolved using the Universal Imaging MetaMorph software package. Colocalization values were determined using the colocalization feature in MetaMorph.

For the colocalization of NL4.3/WT and NL4.3/KKRK Vif with GFP-Apobec3G shown in Chapter IV, Hela cells were grown on coverslips and transfected at a 1:1 ratio of NL4.3/KKRK or NL4.3/WT and GFP-Apobec3G. 48-60h post-transfection, coverslips were washed and fixed in -20°C methanol. Following a 30min block in PBSAT (PBS/1%BSA/0.1%Tween-20) at 37°C, coverslips were stained with the 319 mAb against Vif, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Vif Monoclonal (#319) from Dr. Michael H. Malim, and a Cy-3 conjugated anti-mouse Ig whole molecule (Sigma, St. Louis, MO). DAPI stain was applied and the coverslips were washed and mounted using Pro-Long Antifade (Molecular Probes, Eugene, OR).

For the subcellular localization studies of NL4.3/WT and NL4.3/KKRK presented in Chapter IV, GHOST-X4 cells were grown on coverslips and infected with 100ng p24 NL4.3/KKRK or NL4.3/WT plus 8ug/ml DEAE-dextran for 48-60h. Following infection, coverslips were fixed and stained for Vif as above.

HUT78 cells were infected with 50ng p24 NL4.3/KKRK or NL4.3/WT plus 8ug/ml DEAE-dextran for three hours. Coverslips were washed three times in media and incubated in RPMI/10%FBS. Three days post-infection, HUT78 cells were spotted onto coverslips and spun down at 2400xg for 15min. Coverslips were washed three times with PBS, fixed in methanol, and washed three times in PBS, all steps were performed in a six-well plate on ice. Coverslips were then blocked with PBSAT at 37°C, two times ten minutes, and stained for Vif and DAPI as above.

Differential lysis of Hela-Apo cells transfected with NL4.3/WT or NL4.3/KKRK was achieved by first treating the cells with permeabilization buffer (29mM Hepes, pH 7.4, 110mM KOAc, 2mM MgOAc, 1mM EGTA, 200mM NaCl, 0.01% digitonin) for seven minutes on ice. This detergent system will allow lysis of the plasma membrane while leaving the nuclear membrane intact. The lysates were centrifuged at 3000xg for five minutes at 4°C to pellet the nuclear fraction. The post-nuclear supernatant was removed and the pelleted fraction was washed twice with permeabilization buffer. After the second wash, the nuclear fraction was lysed with a 1%NP-40 buffer for 30 minutes on ice. Both the post-nuclear and nuclear fractions were immunoprecipitated with α-Vif (kindly provided by Dr. Klaus Strebel) and resolved on a 12% SDS-PAGE gel and transferred to PVDF membrane. The membranes were probed with α-Vif (kindly provided by Dr. Dana Gabuzda) or α-tubulin mAb (Sigma, St. Louis, MO) followed by a HRP-conjugated anti-mouse secondary. The Enhanced Chemiluminescence (ECL) Kit (Pierce, Rockford, IL) was used to detect signal.

The aggresome studies presented in Chapter V were performed by growing Hela cells on coverslips and co-transfecting with NL4.3/WT and GFP-Apobec3G at a 1:1 ratio

or with GFP-Apobec3G or DsRed-Vif alone. Or, Hela-Apo cells were transfected with NL4.3/WT. Coverslips were processed as above, except for HDAC6, which was incubated overnight at 4°C in primary antibody. Staining for ubiquitin was done with a α-ubiquitin polyclonal Ab, Vif was detected using a polyclonal antibody generously supplied by Klaus Strebel or the 319mAb, HDAC6 detection was achieved with a α-HDAC6 mAb, and tubulin was detected with a α-tubulin mAb (unless noted, all antibodies are from Sigma, St. Louis, MO). Secondary antibodies used were Cy-3 conjugated α-mouse IgG, Cy-3 conjugated α-rabbit IgG, or FITC conjugated α-mouse IgG (Sigma, St. Louis, MO). Leptomycin treatment was done with 5ng/ml leptomycin for 2.5 hours. Nocodazole treatment utilized 10μM nocodazole for 3 hours.
Image acquisition was performed at the UMass Digital Imaging Core Facility on an Olympus IX 70 inverted microscope using a PlanoApo 100x oil immersion lens with a DAPI, FITC, or TRITC filter for fluorescence images and a phase filter for phase contrast images. All images were processed using the Universal Imaging MetaMorph software package. Colocalization values were determined using the colocalization feature in MetaMorph.

J. FACS analysis

The GHOST infection assay, shown in Chapter III was done by using FACS to determine the number of GFP+ cells in each culture. Infected GHOST-X4 cells were collected, washed twice with PBS, resuspended in 250 to 300ul PBS, and analyzed by one-color flow cytometry using FACSort and Cell Quest software (BD Biosciences, San Diego, CA). Uninfected GHOST-X4 cells were included as a control for setting the gate

to determine GFP+ cells and for background fluorescence levels. Relative infectivities were established by subtraction of background fluorescence followed by setting the NL4.3/WT value at 1.

The GFP degradation assay presented in Chapter IV utilized FACS to follow the GFP signal in cultures over a period of six days. GFP vector or GFP-Apobec3G were co-transfected at a 1:1 ratio with NL4.3/KKRK, NL4.3/WT, or empty vector in Hela cells. Cells were collected and washed twice with PBS, resuspended in 250 to 300ul PBS, and analyzed by one-color flow cytometry using FACSort and Cell Quest software (BD Biosciences, San Diego, CA). Untransfected Hela cells were included as a control for setting the gate to determine GFP+ cells and for background fluorescence levels. Cells were analyzed on days 2, 3, 4, and 6 post-transfection.

K. Virion incorporation of Apobec3G

Virion incorporation of Apobec3G was assayed by collecting virions by ultracentrifugation and lysing the virion pellet in 1%NP-40 lysis buffer overnight at 4°C. Proteins were concentrated with a Centricon YM-10 centrifugal filter unit. Equal amounts of p24 were added to a 12% SDS-PAGE gel. Blots were probed with a monoclonal antibody against Apobec3G (ImmunoDiagnostics, Inc, Woburn, MA) and a monoclonal antibody against p24 obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Hybridoma 183 (Clone H12-5C) from Dr. Bruce Chesebro. Enhanced Chemiluminescence was used to detect signal (Pierce, Rockford, IL). Packaging ratios were determined by using the Kodak 1D 3.0 software.

L. Expression of rVif and rApo

BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA) were transformed with pHAT-KKRK, pHAT-WT, or pHAT-Apobec3G expression vectors. Transformed cultures were selected and a 20ml overnight culture was setup in LB broth plus ampicillin in a 37°C shaking incubator. This culture was used to seed 1L of LB broth supplemented with glucose (10g/L) for 2-3h. IPTG (Sigma, St. Louis, MO) was added to the 1L culture to a final concentration of 1mM. Following a 5 hour incubation at 37°C, cells were harvested by centrifugation at 2000-3000g at 4°C for 15-20 minutes at 4°C. Cells were resuspended in extraction buffer (50mM Na2HPO4, 300mM NaCl pH7.0), treated with 0.75mg/ml lysozyme (Sigma, St. Louis, MO) for 25min at room temperature, and then lysed by sonication. The cell supernatant was clarified by centrifugation at 12,000g for 20 minutes at 4°C before being applied to a TALON resin (Clontech, Palo Alto, CA)

M. Immobilized pull-down for Vif-Apobec3G interaction and aggresome markers

Interaction of rVif or rApo with GFP-Apobec3G, Hsp70, HDAC6, or vimentin was determined by incubating cell lysates from unifected HUT78, infected HUT78, or GFP-Apobec3G transfected Hela with immobilized rVif or rApo. For GFP-Apobec3G binding, purification, and recovery, eluates were concentrated with the Centricon YM-10 centrifugal filer unit (Millipore, Bedford, MA) and a Bradford assay was done to determine protein concentration. For all other pull-down assays, a Bradford assay was performed on all eluates. Following the Bradford assay, equal amounts of protein from the eluates were resolved on a 10% or 12% SDS-PAGE gel followed by transfer to a PVDF membrane. The membranes were probed with the mAb 319 against HIV-1 Vif

and a monoclonal antibody against Apobec3G (ImmunoDiagnostics, Inc, Woburn, MA). Blots were also probed with antibodies against Apobec3G, Hsp70, HDAC6, or vimentin (Sigma, St. Louis, MO).

CHAPTER III

NUCLEAR LOCALIZATION OF HIV-1 VIF ISOLATED FROM A LONG-TERM ASYMPTOMATIC INDIVIDUAL AND POTENTIAL ROLE IN VIRUS ATTENUATION

A. Introduction

As one of the six accessory genes of HIV-1, Vif performs a critical function necessary for production of infectious virions in primary blood lymphocytes and macrophages as well as certain T cell lines, termed non-permissive [73, 80, 81, 294-296, 355]. Permissive cell lines such as SupT1 do not require Vif for production of infectious virions. HIV-1 carrying nonfunctional Vif is defective for completion of reverse transcription in non-permissive cells [319, 356-359]. Heterokaryon experiments revealed that Vif is necessary to neutralize an inhibitory cellular factor recently identified as CEM15 or Apobec3G, a cellular cytidine deaminase expressed only in non-permissive cells [297, 298, 360].

Vif prevents the incorporation of Apobec3G into virions by inducing the degradation of Apobec3G via the proteasome pathway [329, 332, 333, 361]. In the absence of Vif, Apobec3G is packaged in the virion, and upon infection the encapsulated Apobec3G edits the single-stranded DNA during reverse transcription by inducing deoxycytidine to deoxyuridine deamination. These G to A hypermutations in the viral genome lead to activation of DNA repair pathways that result in premature degradation of the newly synthesized reverse transcripts [120, 308]. Recent reports implicate an

additional member of the Apobec3 family as an antiretroviral factor. It has been shown that Apobec3F is also expressed in non-permissive cells where it is able to heterodimerize with Apobec3G, package into the virion, and induce degradation of the nascent viral reverse transcripts by deamination. As with Apobec3G, Vif suppresses the antiviral effect of Apobec3F and prevents its virion incorporation [302, 304].

Since Vif functions by interacting with Apobec3G in the cytoplasm, it's localization there is critical. Vif is localized in a diffuse pattern throughout the cytoplasm [345], with a fraction that may be associated with cytoplasmic side of membranes [346, 349, 350], co-localized with Gag [348-350, 362-364], or in association with the intermediate filament vimentin [351, 365].

Since Vif is necessary for production of infectious virions in vivo, it has often been studied in the context of patient isolates that exhibit diminished replicative capacity. Such viruses are typically isolated from a group of infected, but asymptomatic individuals known as long-term non-progressors (LTNP); individuals who maintain low viral loads and stable $CD4^+$ T cell counts in the absence of antiretroviral therapy. Some viruses isolated from LTNP have been found to harbor mutations in vif that may compromise the replicative and/or cytopathic abilities of HIV-1 [82, 83, 366, 367]. These Vif mutant viruses are useful tools in investigating the functions of Vif. In this study, a viral isolate from a long-term non-progressor containing vif mutations was used to study Vif trafficking and how this affects Vif function.

B. LTNP4 isolate shows Vif-enhanced growth phenotype.

One individual (LTNP4) in the New England Area Hemophilia Cohort has displayed a particularly remarkable course of infection for more than twenty years. The clinical, virological and immunological characteristics of LTNP4 were first described in detail in 1994 [368], emphasizing the inability to isolate virus and potent virus-specific cellular immune responses. Ten years later, this individual shows no evidence of HIV-related disease. On the majority of study visits, LTNP4 has had plasma viral RNA levels that were undetectable at a threshold of 50 copies/ml. On occasion, viremia was detected at levels < 400c/ml, and had a single peak value of 1,404c/ml (Figure 3.1A). LTNP4 has maintained $CD4^+$ T cell counts that range between 500 and 1500 cells/μl with an average of 1229 cells/μl (Figure 3.1B). While receiving interferon therapy for hepatitis C virus in late 2003, absolute $CD4^+$ T cell count fell to 432 cells/μl, but the percentage of $CD4^+$ T cells remained stable at 50% with a $CD4^+/CD8^+$ ratio > 2, and the plasma viral RNA level was < 50c/ml. Virus-specific immune responses remain vigorous with cytolytic $CD8^+$ T cell responses to Gag, Pol, and Env, and significant $CD4^+$ and $CD8^+$ proliferation to viral antigens.

Exhaustive attempts to culture the LTNP4 virus using a variety of co-culture techniques proved unsuccessful until enhanced techniques were developed that involved selection of donor cells with high levels of CD4 and CCR5 expression after PHA activation [369]. Early culture attempts employed several types of donor cells as well as Tat and Rev-complementing T-cell lines [368-370]. Virus replication in T-cell lines was only observed when Vif-complementing cell lines were co-cultured with LTNP4 PBMC,

and RNA PCR was used to detect virus in culture supernatant (Figure 3.2A). While the virus did not replicate to detectable levels in parental T-cell lines, WT Vif provided in trans was sufficient to rescue the virus. Thus, virus from LTNP4 exhibited a Vif-mutant replication phenotype.

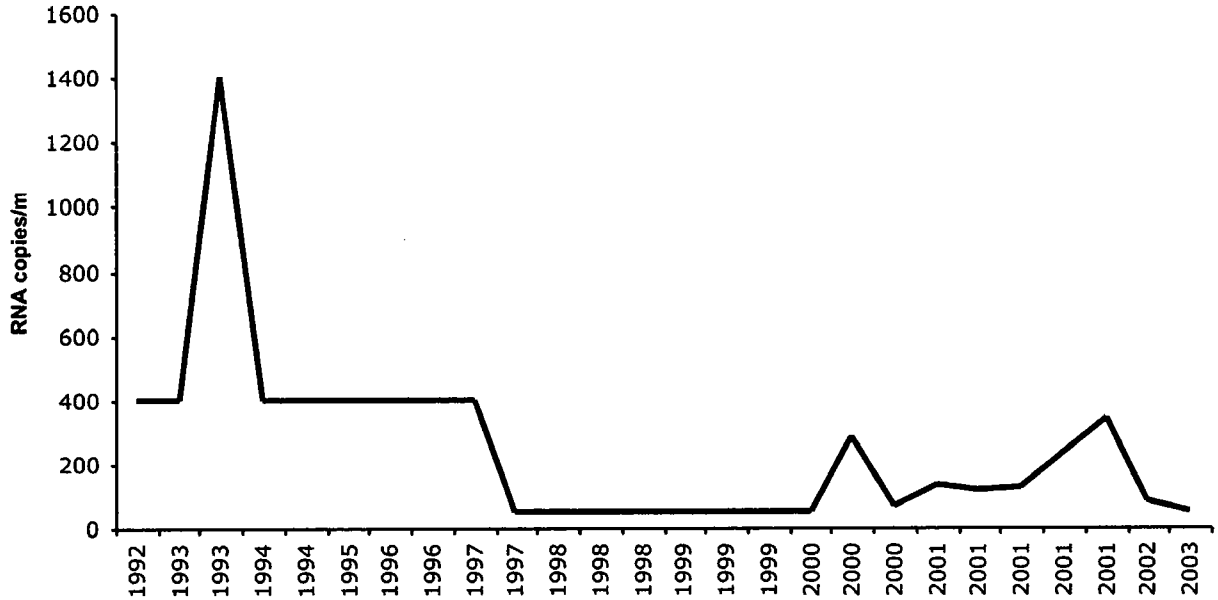
To exclude the possibility that the ability to grow the LTNP4 virus was due to a non-specific cellular effect as a result of Vif over-expression, another slow-growing viral isolate from a different long-term non-progressor, LTNP6, that has been shown to have unique, difficult to revert polymorphisms, in regions other than Vif, was tested for the ability to grow in the parental and Vif-complementing cell lines [367]. Growth of this virus was observed in both cell lines at low levels, providing evidence that growth of the LTNP4 isolate was enhanced by Vif expression. In these experiments, the low level growth of the LTNP viruses was attributed to the fact that they are known to use CCR5 as a co-receptor, and the T cell lines primarily express the CXCR4 co-receptor. This growth phenotype of LTNP4 virus was consistently observed at multiple time points. Based on this observation, the vif gene of the LTNP4 isolate was further characterized genotypically and phenotypically to explore the role of Vif in viral replication.

C. LTNP4 isolate contains a unique motif in the vif gene.

Sequence analysis of the vif gene of the LTNP4 isolate revealed a complete open reading frame with no frameshift mutations or early termination codons. A number of point mutations resulting in amino acid substitutions were observed in comparison to the consensus clade B Vif sequence (Figure 3.2B). Substitution mutations are classified by their frequency of occurrence in the HIV database with mutations of the lowest frequency

Figure 3.1

A.


RNA copies/m
1600
1400
1200
1000
800
600
400
200
0
1992
1993
1993
1994
1994
1995
1996
1996
1997
1997
1998
1998
1998
1999
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2001
2001
2002
2003

B.

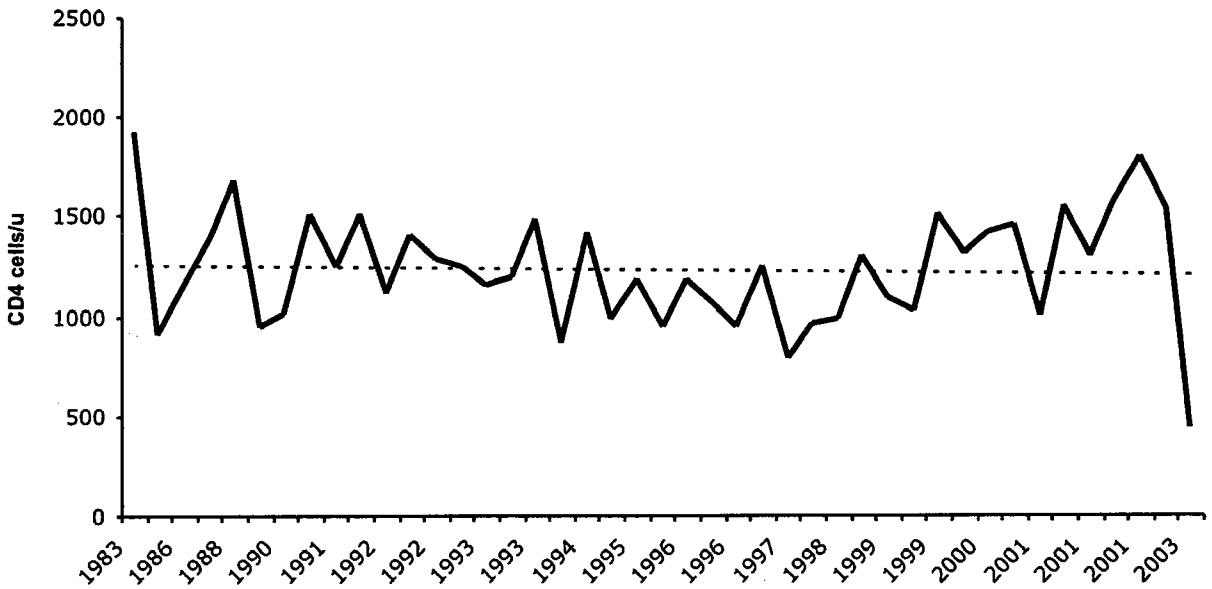

CD4 cells/u
2500
2000
1500
1000
500
0
1983
1986
1988
1990
1991
1992
1992
1993
1993
1994
1995
1996
1996
1997
1998
1999
1999
2000
2001
2001
2001
2003

Figure 3.1: Viral loads and $CD4^+$ T cell counts for LTNP4. (A) LTNP4 plasma viral loads were determined by PCR using the Roche Amplicor HIV-1 detection kit. Prior to 1997, the limit of detection for the assay was <400copies/ml. The sensitivity of the assay was then improved to <50 copies/ml. (B) LTNP4 CD4 cell counts were determined by flow cytometry (FACScan, Becton Dickinson). The average cell count gives a value of 1229 cells/μl as represented by the trendline.

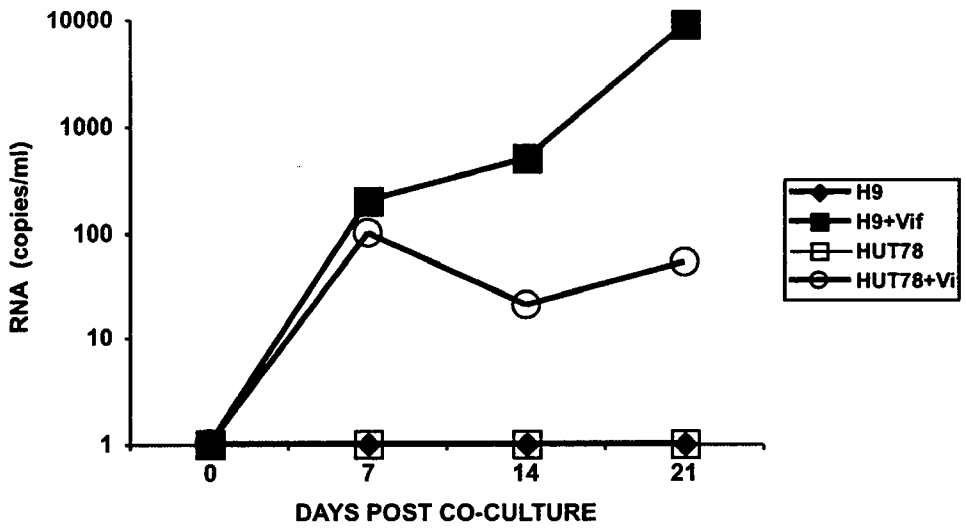
representing the most interesting to explore in terms of structure or function while common mutations are seen as potential byproducts of high error rates of the reverse transcriptase enzyme or as escape mutants that confer a fitness advantage to the virus. A motif not previously reported in Vif was observed at amino acid positions 90 to 93 where the clade B sequence of RKRR was replaced with KKRK, a motif that commonly functions as a nuclear localization signal (NLS) in many viral and cellular systems [371]. This unique substitution was observed in the majority of viral sequences obtained from LTNP4 plasma, PBMC DNA, and cultured virus at multiple timepoints in 1991, 1997, 2000, 2001, and 2003 (Figure 3.2B).

D. Nuclear localization of KKRK Vif.

Based on the observation that KKRK is a nuclear localization sequence, we hypothesized that Vif would be localized aberrantly to the nucleus, thereby rendering Vif non-functional. WT Vif is found primarily in the cytoplasm where it plays a critical role in the production of infectious virions [81, 295, 348, 355, 359, 372]. To determine the subcellular localization of the KKRK Vif, N-terminal DsRed fusion proteins were made using the WT and KKRK Vif sequences. As a control for nuclear localization, a GFP-tagged protein harboring the myc NLS of DPKKRKV was included. These constructs were used to transfect MAGI cells and localization of the fluorescent fusion proteins was determined by fluorescence microscopy. A Z-series of images was acquired at 0.2μm intervals for the DAPI stain and the DsRed or GFP fluorescence. Following image processing on MetaMorph software, the images were overlaid to determine subcellular localization. The subcellular localization patterns of the DsRed protein alone, the KKRK

Figure 3.2

A.


10000
1000
100
10
1
RNA (copies/ml)
0
7
14
21
DAYS POST CO-CULTURE
H9
H9+Vif
HUT78
HUT78+Vi

B.

```
Clade B              MENRWQVMIVWQVDRMRIRTWKSLVKHHMYISGKAKGWFYRHHYESTHP
1991 PBMC            ----------------------------I----------------S----------I----------
1997 CD4             -----------------------------------S----------S----------I----------
1999 Culture sup                                                              ----------
2000 Culture sup     ------------------------------------------------S----------I----------
2000 PBMC            ------------------------------------------------S----------I----------
2001 Culture sup     ------------------------------------------------S----------I----------
2001 CD4             ----------I----------C-I-----------------------S----------I----------
2001 PBMC
2002 PBMC            ------------------------------------------------S----------I----------
2003 Plasma          ------------------------------------------------S----------I----------

Clade B              RISSEVHIPLGDARLVITTYWGLHTGERDWHLGQGVSIEWRKRRYSTQVDPDLA
1991 PBMC            ------------------------------------------------------SK----K----------G----
1997 CD4             ------------------------------------------------------K----K----------G----
1999 Culture sup     ------------------------------------------------------K----K----------G----
2000 Culture sup     ------------------------------------------------------K----K----------G----
2000 PBMC            ------------------------------------------------------K----K----------G----
2001 Culture sup     ------------------------------------------------------K----K----------G----
2001 CD4             ------------------------------------------------------K--KK----------G----
2001 PBMC            ----------------------------------------------R---------SK----K------P-----G----
2002 PBMC            ------------------------------------------------------K----K----------G----
2003 Plasma          ------------------------------------------------------K----K----------G----

Clade B              DQLIHLYYFDCFSESAIRNAILGHIVSPRCEYQAGHNKVGSLQYLALAALITPKKI
1991 PBMC            ----------------------------------------S-------------------------------------
1997 CD4             ----------------------------------------S-------------------------------------
1999 Culture sup     ----------------------------------------K---S-----------------------------------
2000 Culture sup     ----------------------------------------R---S-------------------------------N---
2000 PBMC            ----------------------------------------R---S-------------------------------N---
2001 Culture sup     ----------------------------------------R---S-------------------------------N---
2001 CD4             ----------------------------K----------S--K----------K-----------------------
2001 PBMC            ----------------------------------------S-------------------------------N---
2002 PBMC            ----------------------------------------S-------------------------------N---
2003 Plasma          ----------------------------------------S-------------------------------N---

Clade B              KPPLPSVTKLTEDRWNKPQKTKGHRGSHTMNGH
1991 PBMC            ----------------------T-----------------------
1997 CD4             ----------R----------N----------P-------Q
1999 Culture sup
2000 Culture sup     ------------------------------------------------Q
2000 PBMC            ------------------------------------------------Q
2001 Culture sup     ------------------------------------------------Q
2001 CD4             ----------NK------------------------------------Q
2001 PBMC            ------------------------------------------------Q
2002 PBMC            ----------------------------------------K-----Q
2003 Plasma          ------------------------------------------------Q
```

Figure 3.2: LTNP4 exhibits a Vif enhanced growth phenotype and unique mutations in Vif. (A) $2x10^6$ IL-2 stimulated, CD8-depleted PBMC of LTNP4 were co-cultured with $2x10^6$ parental or Vif-complementing H9 or HUT78 cells for 21 days. Culture supernatant was sampled for virus production by viral RNA quantification at days 7, 14, and 21. (B) LTNP4 Vif sequence was determined by RT-PCR of culture supernatant or plasma virus for replicating viral species or by direct PCR of cell lysates for integrated virus. The consensus clade B strain was used for alignment. Sequences that occur at low frequencies in the Los Alamos National Laboratory HIV-1 database are highlighted and underlined, sequences unique to the database are highlighted and italicized. Positions 90 to 93 represent a nuclear localization sequence, the underlined Ser^{95} and Thr^{96} residues designate potential cAmp-dependent protein kinase A or casein kinase II phosphorylation sites, respectively.

and WT Vif fusion proteins, and GFP nuclear protein are shown (Figure 3A-L). DAPI staining was used to specifically identify the nucleus (Figure 3.3A-D). The vector construct revealed DsRed fluorescence throughout the cytoplasm (Figure 3.3E). Overlaying the DAPI and DsRed fluorescence images showed dispersed DsRed fluorescence throughout the cytoplasm (Figure 3.3I). The DsRed-WT Vif construct exhibited a similar pattern of dispersed fluorescence throughout the cytoplasm as well as the perinuclear compartment (Figure 3.3B, F, and J). However, the DsRed-KKRK Vif construct demonstrated a dramatically different pattern. DsRed images revealed a punctate DsRed fluorescence apparently in the nuclear compartment (Figure 3.3G). Overlaying the DsRed image with the DAPI image confirms that the localization of DsRed-KKRK Vif is predominantly nuclear (Figure 3.3K). This fluorescence pattern was observed only in DsRed-KKRK Vif transfected cells. The GFP-tagged nuclear control (Figure 3.3D, H, and L) exhibits an exclusively nuclear stain.

To confirm the observation that DsRed-KKRK Vif appeared to localize predominantly to the nucleus, the localization for each construct was quantitatively determined using the colocalization feature of the MetaMorph Software package. For this analysis, the amount of colocalization between the fluorescent signal and nuclear DAPI stain was calculated for each cell completely in the field. The DsRed vector gave the expected pattern with exclusive cytoplasmic localization. The GFP-myc nuclear control protein showed a distinct nuclear distribution with 96% of the signal being detected in the nucleus. DsRed-WT Vif also exhibited the expected distribution pattern of predominantly cytoplasmic. The distribution ratio was 66% cytoplasmic to 34% nuclear. However, this ratio was inverted for the DsRed-KKRK construct which

exhibited a 27% cytoplasmic to 73% nuclear distribution. The ratios presented here are consistent with a series of images analyzed for each construct. While the localization of DsRed-KKRK Vif is not exclusively nuclear, it does demonstrate an aberrant localization when compared to the DsRed-WT Vif. This finding supports the hypothesis that Vif is localized to the nucleus in the presence of the KKRK substitutions. Since the KKRK mutation is unique and could have functional consequences due to aberrant localization of Vif, experiments were undertaken to investigate possible effects on viral replication.

E. Presence of KKRK motif reduces virus infectivity.

Following the genotypic analysis of the LTNP4 viral species and subsequent analysis of the impact of the KKRK mutation on Vif localization, the effect of the KKRK mutation on viral infectivity was investigated. Two experimental approaches were employed to assess the contribution of KKRK to viral infectivity. The first approach involved using a co-culture system similar to the one used in the initial isolation of the LTNP4 virus. Although not a direct assay of Vif function, this experiment replicated the conditions under which the LTNP4 KKRK Vif mutation was first detected and was performed to confirm the original viral growth data shown in Figure 3.2A. The second set of experiments involves the traditional spreading infection assay and a single-cycle infectivity assay using the GHOST-X4 indicator system to directly assess the replication phenotype of a KKRK Vif virus.

Mutagenesis was performed using the X4 tropic, cytopathic HIV-1 clone NL4.3 to determine the contribution of the KKRK mutation on the replicative capacity of the virus. Isogenic NL4.3 clones were generated that differed only with respect to the Vif

Figure 3.3

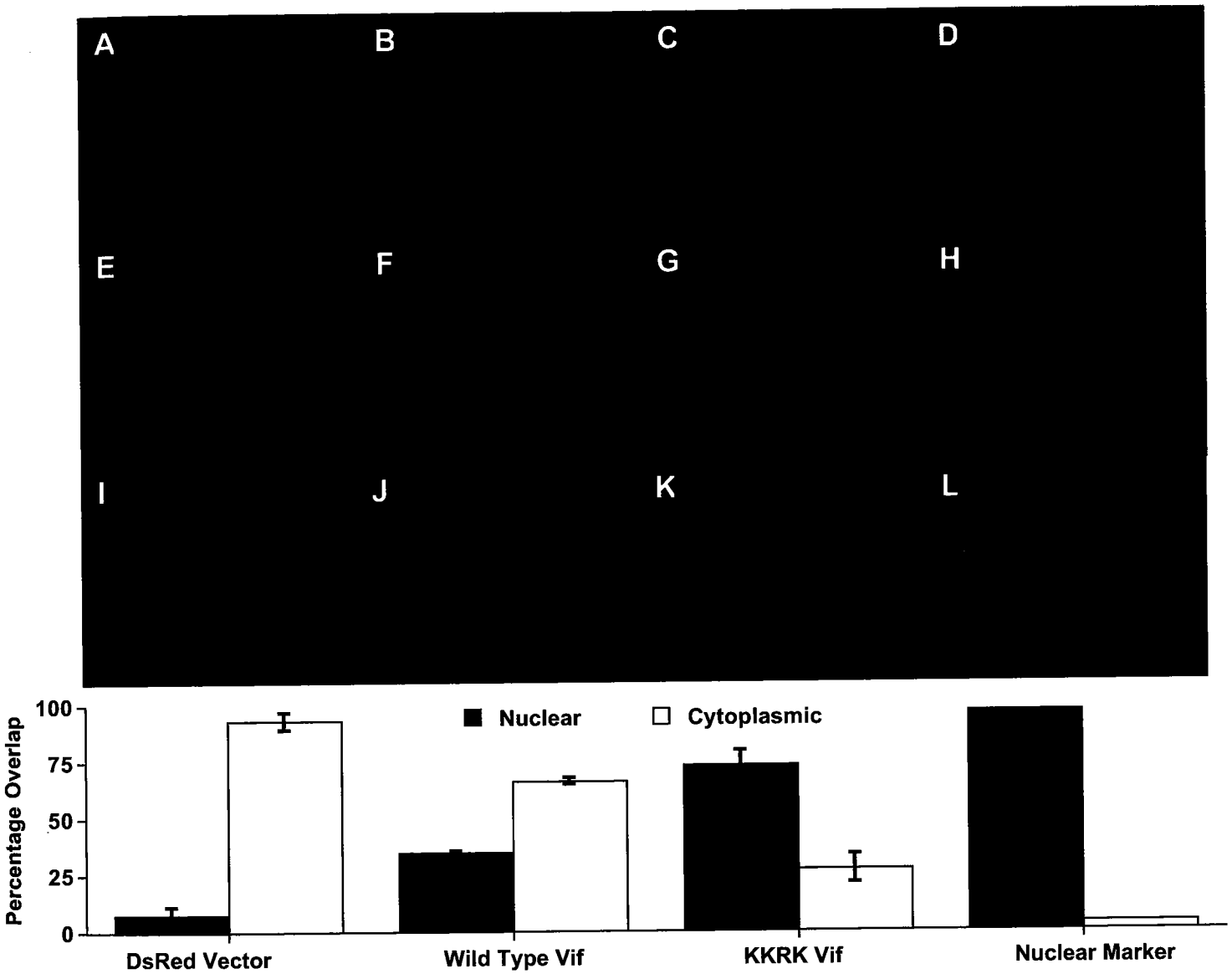

A
B
C
D
E
F
G
H
I
J
K
L
Percentage Overlap
100
75
50
25
0
Nuclear
Cytoplasmic
DsRed Vector
Wild Type Vif
KKRK Vif
Nuclear Marker

Figure 3.3: Subcellular localization of KKRK and WT Vif fusion proteins. DsRed fusion proteins were made with NL4.3/WT Vif or with NL4.3/KKRK Vif and used to transfect MAGI cells to determine localization of WT versus KKRK Vif. DsRed vector and a GFP-nuclear protein were included as controls A Z series of images was acquired at 0.2um intervals and images were processed using the MetaMorph software. Fluorescence signals for DsRed vector (E), DsRed-WT Vif (F), DsRed-KKRK Vif (G), and GFP-nuclear (H) are shown. DAPI staining of the nucleus is shown in Panels A-D. To qualitatively assess subcellular localization, overlays of DAPI and the fluorescence signals were done for DsRed vector (I), DsRed-WT Vif (J), DsRed-KKRK Vif (K), and GFP-nuclear (L). Subcellular localization was quantified by determining the percent overlap of the fluorescence signal with the DAPI nuclear stain and presented as nuclear or cytoplasmic.

KKRK motif. Analysis of culture supernatants from transfected non-permissive HUT78 cells and permissive HeLa cells revealed that the NL4.3/KKRK clone produced virus upon transfection to equivalent levels as wild-type NL4.3 transfections 48 hour post-transfection.

The basis of the co-culture experiment is that non-permissive cells harboring a ΔVif virus can effectively transmit virus via the cell-cell transmission route. Although the producer cell is non-permissive, i.e. expresses Apobec3G, and cell-free virus produced from these cells is non-infectious [372], it has been previously shown that ΔVif virus can be efficiently transmitted to a permissive target cell by co-culture [347, 373]. Once the virus enters the permissive cell and is successfully integrated into the host genome, all virions produced will be infectious since there is no Apobec3G present for packaging into the virion. In our system, the exogenously supplied wild-type Vif in the HUT78 Vif-complementing cell line renders that cell permissive. Therefore, a Vif rescued virus produced by the HUT78 Vif-complementing cells can initiate a spreading infection driven by production of infectious, cell-free virions.

If NL4.3/KKRK is a Vif-mutant virus, co-culture of transfected HUT78 cells with parental HUT78 cells should not enhance viral replication. However, transfected HUT78 cells co-cultured with HUT78xVif cells should show enhanced replication levels due to the permissive environment in the target HUT78xVif cell. HUT78 cells that were transfected with NL4.3/KKRK and co-cultured with parental HUT78 cells gave peak p24 levels that were consistently lower than NL4.3/WT transfected HUT78 cells co-cultured with parental HUT78 cells. However, when NL4.3/KKRK transfected HUT78 cells were co-cultured with the HUT78xVif cells, the amount of p24 in the supernatant was elevated

to a level comparable to NL4.3/WT. When peak p24 values observed in the Vif-complementing HUT78 co-cultures were normalized based on peak p24 levels in the parental HUT78 co-cultures, the NL4.3/WT virus gained no replication advantage from the presence of exogenous Vif. However, NL4.3/KKRK exhibited an enhanced replication phenotype similar to that of the primary isolate from LTNP4 during co-culture with T cell lines expressing Vif (Figure 3.4A).

To determine if the NL4.3/KKRK virus exhibits a Vif-mutant phenotype (i.e. less infectious when produced from non-permissive cells, but infectious when produced from permissive cells), viral stocks were produced from both non-permissive H9 or HUT78 and permissive HeLa cells and used in spreading infection and single-cycle infectivity assays.

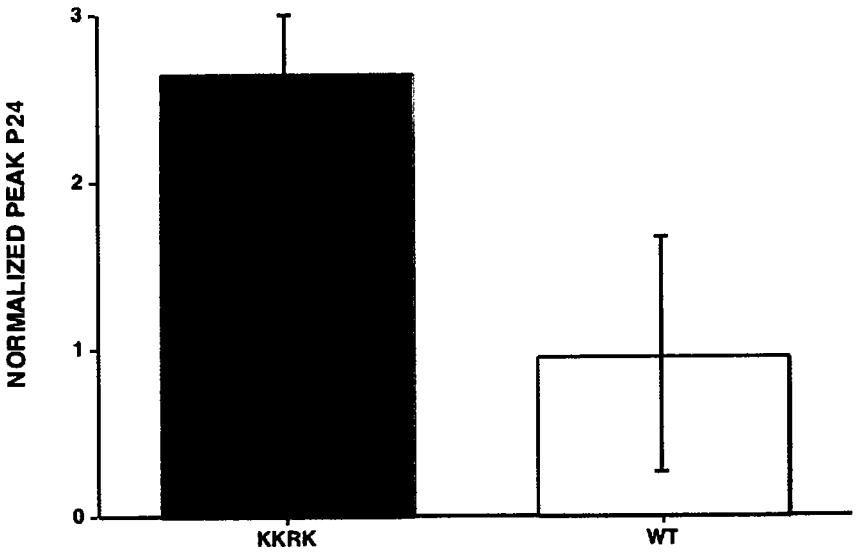
Initial experiments indicated that the NL4.3/KKRK virus was less infectious when derived from or passaged on non-permissive cells. To confirm this, a spreading infection assay was performed using virus from a non-permissive or permissive source to infect either the non-permissive H9 or permissive CEM-SS cell line. Replication was followed for two weeks by the p24 ELISA culture supernatant assay. When NL4.3/WT virus is derived from either a non-permissive or permissive source, it is infectious when passaged on non-permissive and permissive target cells (Figure 3.4B and 3.4C). NL4.3/KKRK from permissive cells is infectious when passaged on permissive target cells (Figure 3.4C). On the other hand, the NL4.3/KKRK virus produced from non-permissive cells exhibits a delayed and significantly lower replicative capacity when used 4321q to infect a non-permissive target. Here, spreading infection is delayed for one week and detected at a tenfold lower level compared to NL4.3/WT (Figure 3.4B).

The single-cycle GHOST assay was also used to investigate differences in infectivity between NL4.3/WT and NL4.3/KKRK derived from non-permissive as well as permissive sources. Virus was normalized to 100ng of p24 and used to infect GHOST-X4 cells for 24 hours. GHOST-X4 cells express both the CD4 receptor and the CXCR4 co-receptor that allow HIV-1 infection. Additionally, these cells harbor a reporter plasmid that expresses the GFP protein under control of the HIV-1 LTR. Upon successful infection of these cells by HIV-1, expression of HIV-1 Tat protein leads to activation of the LTR and expression of GFP. When viruses derived from permissive HeLa cells were used for infection, the NL4.3/KKRK virus was equally as infectious as the WT virus, as expected (Figure 3.4D). However, when viral stocks produced by the non-permissive HUT78 cells were used, the relative infectivity was significantly impaired compared to the NL4.3/WT (unpaired t-test, $p < 0.05$). To confirm the observations that both the LTNP and NL4.3/KKRK constructs are capable of infection by cell-cell spread, transfected HUT78 cells were co-cultured with GHOST-X4 cells and assayed for relative infectivity as above. Again, the NL4.3/KKRK virus was equally as infectious as the NL4.3/WT virus when cell-cell transmission, rather than cell-free virus, was utilized.

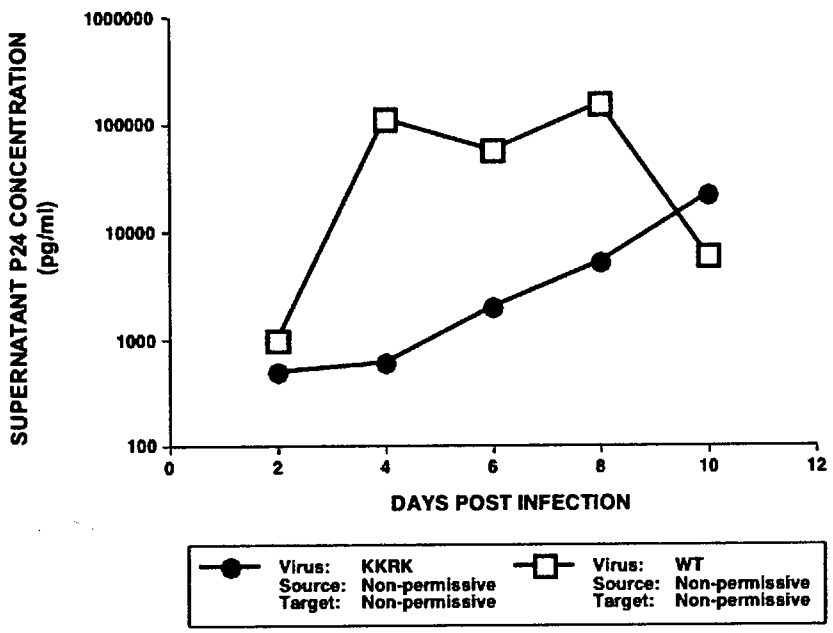
These experiments demonstrate a Vif-dependent effect where the "permissivity" of a cell line determines the infectivity of progeny virus. Taken together, these results demonstrate that the NL4.3/KKRK virus exhibits a Vif-mutant phenotype and is significantly less infectious than the NL4.3/WT virus.

Figure 3.4

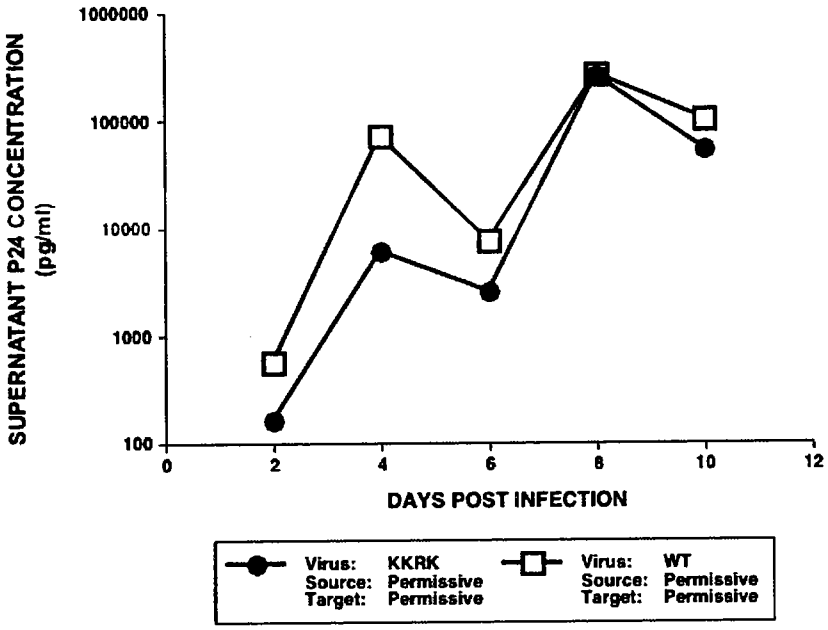
A.


NORMALIZED PEAK P24
3
2
1
0
KKRK
WT

B.


SUPERNATANT P24 CONCENTRATION (pg/ml)
1000000
100000
10000
1000
100
0
2
4
6
8
10
12
DAYS POST INFECTION
Virus: KKRK
Source: Non-permissive
Target: Non-permissive
Virus: WT
Source: Non-permissive
Target: Non-permissive

C.


SUPERNATANT P24 CONCENTRATION (pg/ml)
1000000
100000
10000
1000
100
0
2
4
6
8
10
12
DAYS POST INFECTION
Virus: KKRK
Source: Permissive
Target: Permissive
Virus: WT
Source: Permissive
Target: Permissive

D.

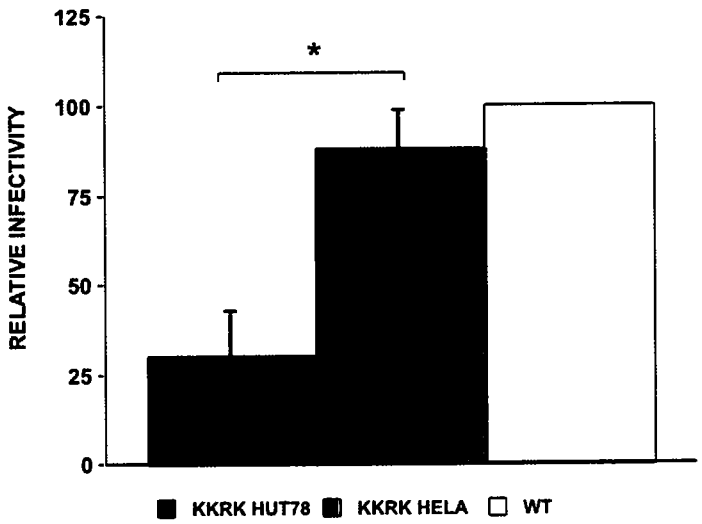

RELATIVE INFECTIVITY
125
100
75
50
25
0
*
KKRK HUT78
KKRK HELA
WT

Figure 3.4. Infectivity of NL4.3/WT versus NL4.3/KKRK viruses. (A) HUT78 cells were transfected with NL4.3/WT or NL4.3/KKRK and co-cultured with HUT78 parental or Vif-complementing cells. Virus replication was followed by p24 production. Peak p24 data for the HUT78-Vif cell line cultures were normalized to that of the HUT78 parental culture. Enhanced replication of NL4.3/KKRK construct is indicated by a normalized value greater than one. Data are from two experiments with standard error shown. (B) Non-permissive H9 (source) cells were transfected with NL4.3/WT or NL4.3/KKRK. Cell-free virus normalized to 5ng of p24 was used to infect H9 (target) cells. The accumulation of p24 in culture supernatant was measured using a quantitative ELISA assay. (C) Permissive HeLa (source) cells were transfected with NL4.3/WT or NL4.3/KKRK. Infections of permissive CEM-SS (target) cells were performed and assayed as in B. (D) NL4.3/WT or NL4.3/KKRK viruses were produced by transfection of the non-permissive HUT78 cell line, or the permissive HeLa cell line. Viruses were normalized to 100ng p24 and used to infect GHOST-X4 cells that harbor the GFP protein under the control of the HIV-1 LTR. Three days post-infection, GFP positive cells were enumerated by FACS. Data were normalized to the NL4.3/WT values to derive a relative level of infectivity. Data for the NL4.3/KKRK construct are shown as a percentage of that observed for the NL4.3/WT construct (* indicates $p<.05$).

F. Additional Vif mutations identified in LTNP4 virus contribute to attenuation

While the KKRK mutation found in LTNP4 Vif proved to be the most interesting to explore due to its unique absence in the database, other rare mutations were also found in the LTNP4 vif gene. Two rare mutations were found in the N-terminus of Vif, an Isoleucine to Serine substitution at position 31 and a Phenylalanine to Isoleucine substitution at position 39. Both of these mutations are present at a low frequency in the LANL database, neither segregates with LTNPs.

Although these mutations are not unique, they are still interesting due to their low frequency in the database and that they are present in an attenuated virus that shows Vif enhanced growth. The importance of these additional mutations was determined by infectivity assays analogous to those used with NL4.3/KKRK. The NL4.3 template was mutagenized to generate the plasmid NL4.3/IF containing the Ile→Ser and Phe→Ile substitutions at positions 31 and 39 (Figure 3.2B). NL4.3/IF was used to produce viral stocks from permissive Hela cells and non-permissive HUT78 cells. Equal amounts of p24 were used to infect GHOST-X4 reporter cells and infectivity was measured three days post-infection as %GFP+ cells. Values were normalized to set NL4.3/WT at a value of one. When NL4.3/IF virus was produced from permissive Hela cells, it was equally as infectious as NL4.3/WT. However, when NL4.3/IF from non-permissive HUT78 cells was used, infectivity was reduced to 49% that of NL4.3/WT (Figure 3.5). While this reduction in infectivity is not as significant as it was with the NL4.3/KKRK construct, it is still indicative of an attenuated virus.

When viewed separately, both the KKRK and IF mutations contribute to attenuated viral phenotypes. In the LTNP4 isolate, the attenuation is severe, with limited

viral replication. This would suggest that both sets of mutations work synergistically to reduce Vif function in the LTNP4 virus. Future work using a NL4.3 plasmid containing both the KKRK and IF mutations would be interesting to study to determine if the affect of both mutations is additive and does render the virus completely non-infectious.

F. Discussion

The studies presented here establish that the presence of a functional NLS in HIV-1 Vif is sufficient to render the virus significantly less infectious. Previous work has shown that WT Vif is distributed throughout the cytoplasm. It has also been well established that Vif is necessary for production of infectious virions. Our working hypothesis is that the presence of a functional NLS renders Vif non-functional by retaining it in the nucleus during the viral life cycle, therefore not allowing it to perform its critical function in the cytoplasm of inducing Apobec3G degradation. Alternatively, the KKRK mutations may interfere with the direct interaction of Vif with Apobec3G. This model would account for the observation that the patient isolate, which harbors a KKRK motif, displays a Vif-mutant replication phenotype.

Figure 3.5

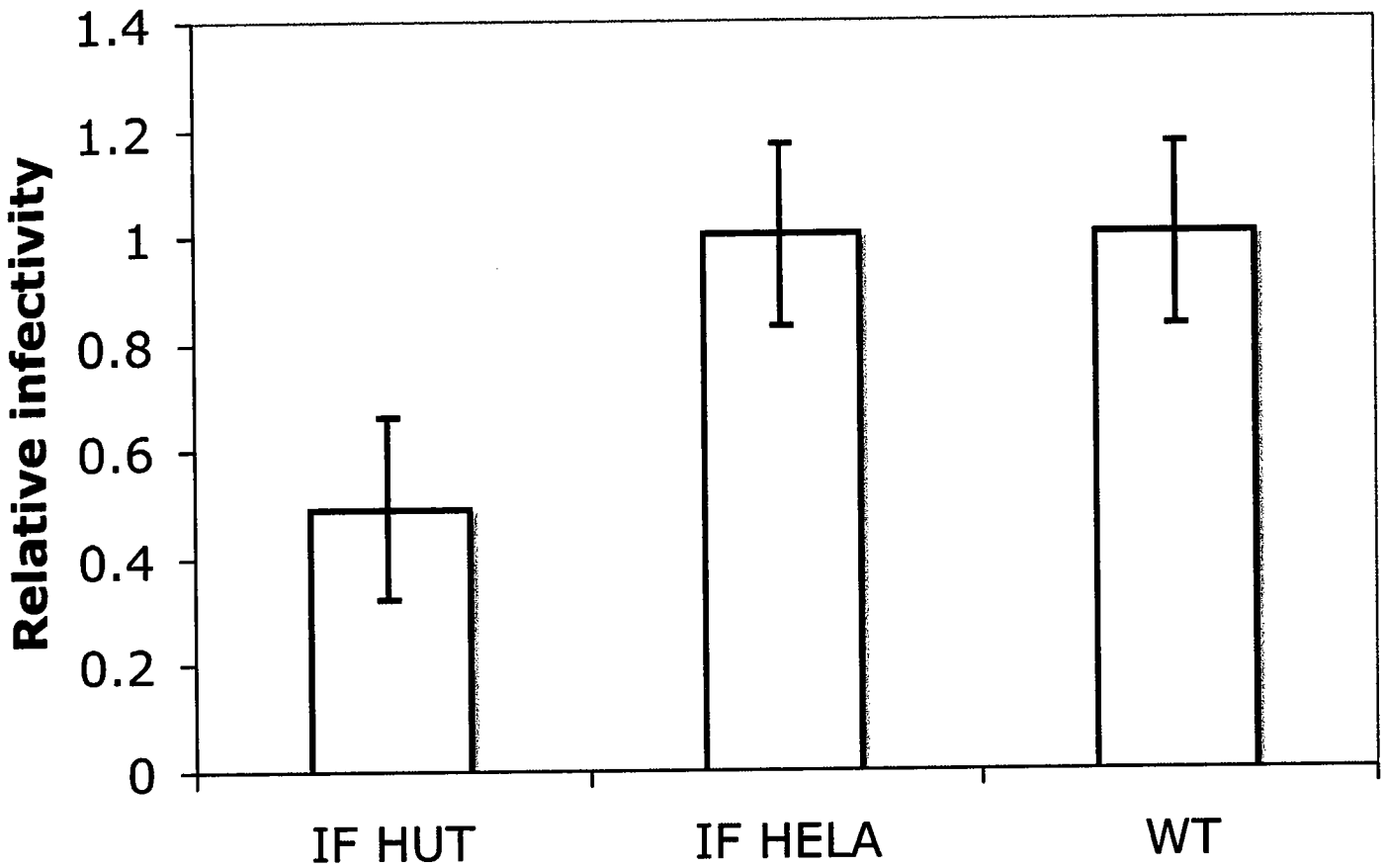


Figure 3.5: Comparison of NL4.3/WT and NL4.3/IF infectivity. NL4.3/WT or NL4.3/IF viruses were produced by transfection of the non-permissive HUT78 cell line, or the permissive HeLa cell line. Viruses were normalized to 100ng p24 and used to infect GHOST-X4 cells. Three days post-infection, the number of GFP positive cells was determined by FACS. Data were normalized to the NL4.3/WT values to derive a relative level of infectivity. Data for the NL4.3/IF construct are shown as a percentage of that observed for the NL4.3/WT construct.

The KKRK motif serves as a common NLS for viral and cellular proteins [371]. The KKRK motif reported here is most similar to that of the cellular transcription factor LEF-1 and the splice variant of Bcl-2 family member Bfl-1, Bfl-1S. Both contain a NLS composed of only a KKRK motif with no flanking proline residue [371, 374]. The prototypical viral KKRK NLS is that of SV40 T Ag. The SV40 large T Ag NLS is dependent on phosphorylation sites flanking the KKRK motif [375-379]. A sequence homologous to one of these phosphorylation sites, a consensus site for phosphorylation by casein kinase 2 (CK2), is present immediately downstream of the KKRK at positions 91-98 (KRKYSTQVD) in Vif. The Thr^{96} residue of Vif is phosphorylated by p44/42 mitogen-activated protein kinase (MAPK) in stimulated cell lines [380]. Mutation of this conserved residue significantly reduces the activity of Vif. A sequence homologous to a second phosphorylation site, a consensus site for phosphorylation by cAmp-dependent protein kinase A (PKA), critical for the *Drosophila* morphogen Dorsal NLS [378], is also found downstream in the Vif sequence at positions 92 to 99 (RKYSTQVDP) (PROSITE database, http://www.expasy.ch/prosite/). It remains to be determined whether phosphorylation sites flanking the KKRK motif in this unusual Vif protein play a role in the nuclear localization function of this motif.

The SV40 T Ag utilizes the Importin α/β pathway for nuclear import [381, 382]. Upon activation of T cells (e.g. viral infection), the expression of Importins is upregulated [383]. The presence of critical phosphorylation sites juxtaposing the KKRK motif in the mutant Vif is consistent with the hypothesis that this sequence functions as a NLS and relocates the protein to the nucleus, thereby removing it from its site of action. This may be relevant in view of the observation that T cell activation results in

upregulation of the components of the Importin α/β pathway for nuclear import, the receptors that recognize this particular motif.

In contrast to the KKRK motif, the NL4.3 wild-type sequence at these positions, RKKR, is unable to mediate nuclear translocation. The presence of lysines at the first and fourth positions, seen in the mutant but not the WT, are the critical energy determinants for NLS binding to nuclear Importins [384]. Preliminary molecular modeling based on the crystal structure of a KKRK peptide bound to Importin α suggested that when RKKR was substituted [385], the lysine at position 3 might not make favorable contacts with the receptor (C. Schiffer, University of Massachusetts Medical School, personal communication). Previous reports have examined the potential of RKKR to function as a nuclear transport inhibitory signal due to the highly basic nature of the motif. The initial report found that the RKKR motif could function as a nuclear transport inhibitory signal (NTIS) by associating with Importin α/β and that Vif could then function by occluding the nuclear import pathway of the cell [386]. However, Stephens et al reported that it is unlikely that the RKKR acts as a NTIS based on the genetic diversity of this region when different clades and groups are analyzed [387]. The experimental conditions utilized and the IC50 determined with the RKKR peptide make it unlikely that the RKKR motif can successfully bind Importins under physiologically relevant conditions. This is supported by the genetic diversity of the region, which makes it highly unlikely that it plays a functional role by inhibiting nuclear traffic. Experimental data presented here reveals that the presence of KKRK in place of RKKR is sufficient for the aberrant localization of Vif from the cytoplasm to the nucleus and the

reduction of replication due to the production of significantly less infectious virions from non-permissive cells.

The presence of Vif in the cytoplasm of infected cells and its necessary function in producing infectious virions has been clearly established. Vif interacts with the cellular protein Apobec3G and induces its degradation via the proteasome pathway [329, 332, 333, 361]. By altering the localization of Vif during the viral life cycle, virus that is released is less able to spread in culture via cell-free transmission. The ability to manipulate viral infectivity by altering Vif localization will be useful for future studies aimed at elucidating biological functions of Vif, as well as understanding its cellular trafficking during the viral life cycle. Studies are ongoing to determine the contribution of the additional Vif substitution mutations along with the KKRK mutation to the localization and function of Vif and their effects on interactions with Apobec3G.

The remarkable disease course of LTNP4 is probably due to multigenic defects in the virus including the KKRK mutation in Vif, mutations observed in the env and nef genes, and perhaps also the I to S and F to I mutations also present in Vif. While all of these mutations probably contribute to the severe attenuation of the LTNP4 virus, it is clear that the KKRK mutation is at least partly responsible for the attenuated growth phenotype.

Studying unique, naturally occurring mutations, that are present in poorly replicating primary virus isolates, provides insights into the *in vivo* function of HIV proteins. Our analysis of the vif gene of this slow-growing primary isolate confirms the critical role of Vif localization to the cytoplasm for production of infectious virions. The consistency and high frequency with which this mutation in Vif was found in the majority

of vif genes in this long-term non-progressor with asymptomatic HIV-1 infection argues that Vif has an important function in *in vivo* disease progression. Further characterization of this Vif mutant may help to elucidate determinants of HIV infectivity and mechanistic aspects of Vif function.

CHAPTER IV

CYTOPLASMIC LOCALIZATION OF HIV-1 VIF IS REQUIRED FOR APOBEC3G DEGRADATION AND VIRAL REPLICATION

A. Introduction

The function of Vif is to neutralize Apobec3G, either by direct interaction [334, 335] or by directing the degradation of Apobec3G via the proteasome pathway [329-333, 352, 361]. While many recent studies have focused on the interaction of Vif and Apobec3G, the proteasomal degradation of Apobec3G, species specificity of Vif, and specific Vif mutants, the localization of Vif and Apobec3G during these events has only begun to be explored. Previous work has established a predominantly cytoplasmic localization for both proteins [345, 346, 351-353]. Additionally, it has been suggested that Vif drives the ubiquitylation of Apobec3G via the Cul-SCF5 complex and that this results in proteasomal degradation of Apobec3G [337, 338]. This pathway functions in the cytoplasm of cells with the ubiquitylation machinery being in the cytosol and the proteasome residing in a perinuclear region at or near the centrosome.

This study looks at the impact of Vif localization on Apobec3G degradation and virion packaging. In Chapter III, the viral isolate from LTNP4 was shown to contain a ^{90}KKRK^{93} motif in the central region. Phenotypic studies showed that this motif, a common nuclear localization signal, was capable of mediating nuclear localization of the Vif protein. Viral constructs demonstrated a reduced infectivity when this KKRK motif

was present. These observations led to the hypothesis that KKRK Vif located in the nucleus would not be able to mediate degradation of the cytoplasmically located Apobec3G. This would leave a free pool of Apobec3G in the cytoplasm where it could interact with NC and be packaged into the virion where it would disrupt reverse transcription in the target cell, rendering the virus less infectious.

B. Subcellular localization patterns of Vif in infected cells

A previous study indicated that NL4.3/KKRK Vif was nuclear and exhibited reduced infectivity compared to that of NL4.3/WT. However, tagged Vif proteins were transfected into cells to assess localization. To confirm that the previously reported Vif localization patterns are the same during an infection, the subcellular distribution of NL4.3/WT and NL4.3/KKRK Vif in infected GHOST-X4 cells were determined (Figure 4.1A). The NL4.3/KKRK Vif staining pattern demonstrates an area of intense staining surrounded by a less intense, dispersed region of staining (panel I). The fluorescent intensity profile (panel II) gives a topographical rendering of the most intense areas of stain. This again shows that there exists a region of intense staining surrounded by a much less intense, more dispersed stain. The intensity profile coupled with the overlay of Vif and DAPI (panel III) show that the most intense area of Vif staining occurs in the nucleus. The NL4.3/WT Vif staining shows a dispersed stain throughout with a concentrated area of high intensity in the perinuclear space (panel IV). Based on this and the overlay of Vif with DAPI (panel VI), it is apparent that the intense area of Vif stain is in the cytoplasm, with an intense area in the perinuclear space and less intense areas of staining extending toward the cell membrane. The NL4.3/WT Vif showed the expected

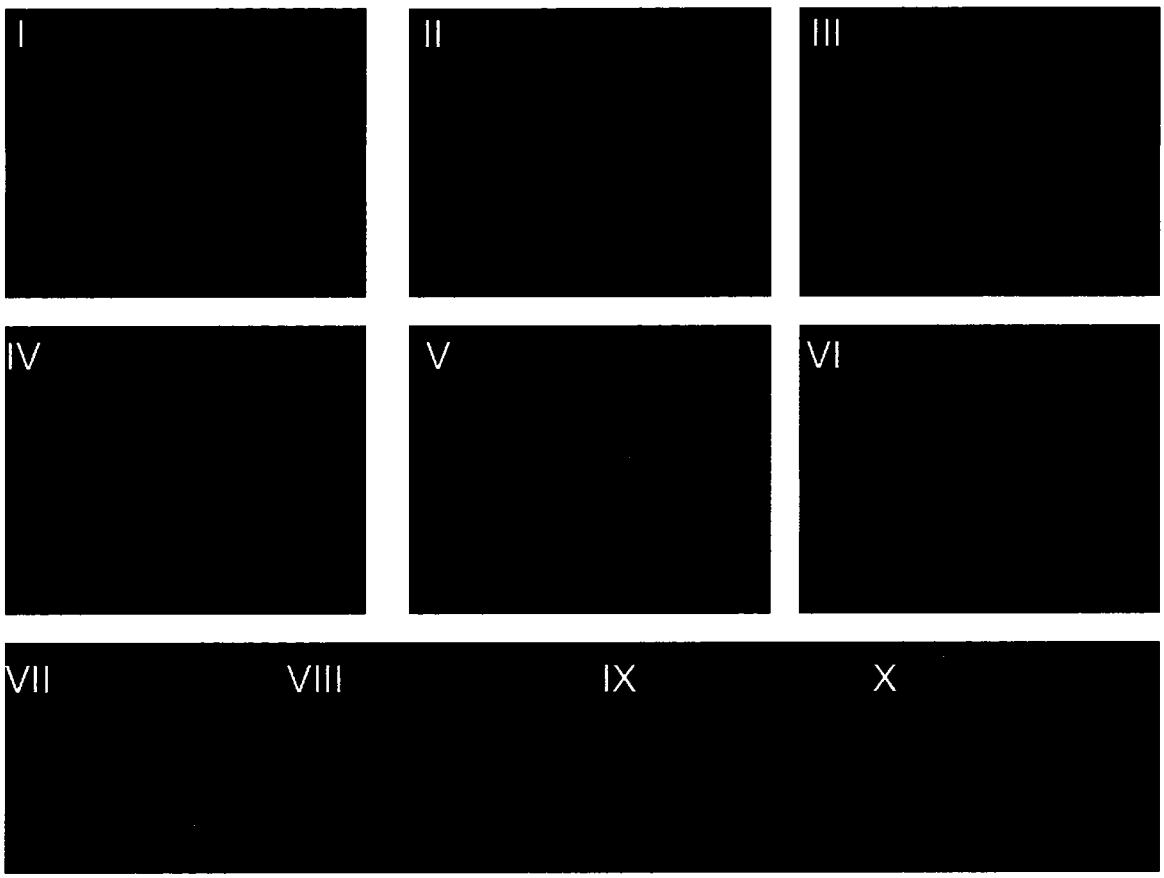
predominant cytoplasmic partitioning, while the NL4.3/KKRK Vif demonstrated an aberrant localization phenotype with the nuclear compartment being enriched in Vif versus the cytoplasm.

The infection of GHOST-X4 cells provides a clearer picture of the localization of Vif in a setting where all viral proteins are present. However, GHOST-X4 cells are not natural target cells for HIV-1 infection. Therefore, the staining pattern of the NL4.3/WT and NL4.3/KKRK Vif proteins in infected T cells was examined. The T cell line HUT78 was infected with NL4.3/WT or NL4.3/KKRK virus derived from permissive Hela cells. T cells have limited cytoplasmic space with large nuclei, therefore cytoplasmic staining is not as clear as it was in the GHOST-X4 cells. However, there is an absence of Vif stain throughout most of the nucleus in the NL4.3/WT cells (panel IX) with cytoplasmic staining apparent when the Vif and nucleus images are overlaid (panel X). The NL4.3/KKRK infected cells demonstrate an opposite staining pattern. Here, it is clear that the nucleus is intensely stained throughout for Vif with some staining in the cytoplasm (panel VII and VIII).

NL4.3/KKRK Vif and NL4.3/WT Vif transfected Hela-Apo cells were also differentially lysed for subcellular fractionation analysis. Both the nuclear and post-nuclear fractions were separated on a SDS-PAGE gel and probed for Vif signal. Again, the NL4.3/WT Vif shows a strong localization in the post-nuclear, cytoplasmic compartment. However, the NL4.3/KKRK Vif preferentially partitions to the nuclear fraction (Figure 4.1B). Fractionation and loading controls confirm that the observed localization patterns are not due to cross-contamination of the nuclear compartment

Figure 4.1

A.



B.

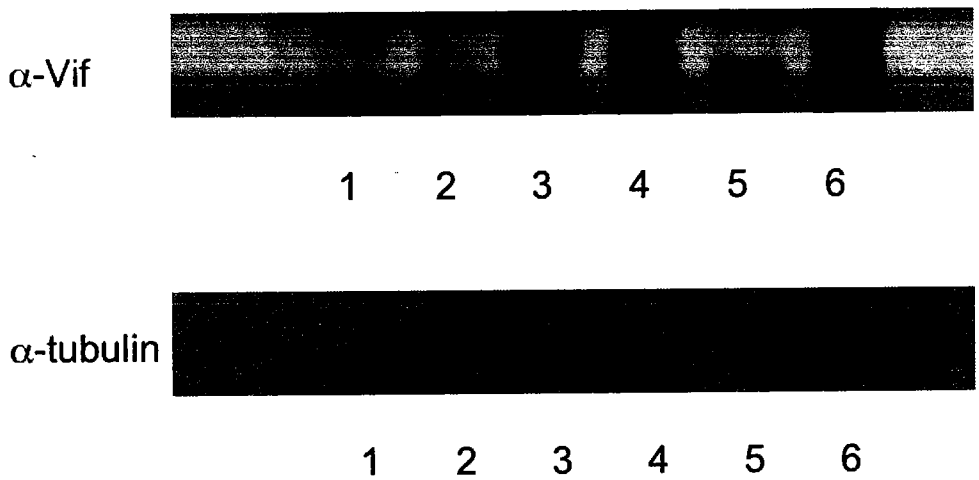


Figure 4.1: (A). Localization of NL4.3/WT or NL4.3/KKRK Vif in infected cells. GHOST-X4 cells were infected with NL4.3/WT or NL4.3/KKRK and stained for Vif three days post-infection. Vif staining patterns show that both the NL4.3/KKRK (panel I) and NL4.3/WT (panel IV) signals have distinct areas of intense stain surrounded by more diffuse areas. Panels II and V are intensity topography maps that render the most intense regions of stain in three dimensions. The overlay of Vif stain with DAPI revealed a more concentrated nuclear stain for NL4.3/KKRK (panel III) compared to the intense perinuclear stain apparent for NL4.3/WT (panel VI). HUT78 cells were infected with NL4.3/WT or NL4.3/KKRK and stained as above (panels VII-X). NL4.3/KKRK Vif shows staining throughout the cell (panel VII). The NL4.3/WT Vif stain shows an area of exclusion surrounded by an area of staining (panel IX). The overlays of Vif stain with DAPI show that NL4.3/KKRK Vif is present in the nucleus (panel VIII) while NL4.3/WT appears to be excluded from the nucleus (panel X) in infected HUT78 T cells. (B). Hela-Apo cells were transfected with NL4.3/WT or NL4.3/KKRK and lysed into a nuclear and post-nuclear fraction, immunoprecipitated, and resolved on a SDS-PAGE gel. Blots were probed for Vif (upper panel) or tubulin (lower panel). For the Vif panel, lanes 1 and 2 are the nuclear and cytoplasmic fractions of uninfected Hela-Apo, lanes 3 and 4 are the NL4.3/KKRK and NL4.3/WT nuclear fractions, lanes 5 and 6 are the NL4.3/KKRK and NL4.3/WT cytoplasmic fractions. The blots were also probed for tubulin as control for lysis and sample loading with lanes 1-3 being the uninfected, NL4.3/KKRK, and NL4.3/WT nuclear fraction and lanes 4-6 being the uninfected, NL4.3/KKRK, and NL4.3/WT post-nuclear fraction.

during lysis or unequal sample loading. Together with the staining information, this confirms that NL4.3/WT Vif is cytoplasmic while NL4.3/KKRK Vif is nuclear.

C. Vif mediated degradation of Apobec3G

Since the NL4.3/KKRK Vif demonstrates an aberrant localization pattern and is less infectious, its ability to mediate degradation of Apobec3G was examined. If cytoplasmic localization of Vif is important for Apobec3G degradation, which in turn influences infectivity, then NL4.3/KKRK should be impaired in its ability to degrade Apobec3G. A lack of Apobec3G degradation indicates the presence of either a non-functional Vif that cannot bind Apobec3G or a functional Vif that is binding competent, but not accessible to the Apobec3G protein at a high enough concentration to mediate degradation.

For the Apobec3G degradation assay, Hela cells, which contain no endogenous Apobec3G, were co-transfected with the NL4.3/WT or NL4.3/KKRK construct and either GFP-vector or GFP-Apobec3G plasmid. Two days post-transfection, %GFP+ cells were enumerated by FACS. This value was taken as the baseline reading for GFP-vector or GFP-Apobec3G expression. Using GFP fluorescence as a surrogate marker for Apobec3G degradation, the loss of GFP signal was followed over the next four days. Figure 4.2A shows the GFP signal over time for each of the cultures. Both the control GFP-vector and GFP-Apobec3G showed no significant fluctuation in %GFP+ cells over time in the absence of any Vif. Some loss of signal was detected for the GFP-vector and GFP-Apobec3G plus NL4.3/KKRK cultures. However, the fold reduction in signal for the GFP-Apobec3G was not significantly different from that of the GFP-vector ($p>.05$),

indicating that there was no active degradation of GFP-Apobec3G. For the NL4.3/WT cultures, loss of GFP signal was elevated in both the GFP-vector and GFP-Apobec3G co-transfections to levels significantly above those in the control transfections. Importantly, the fold reduction in %GFP+ cells was almost twice as high for the GFP-Apobec3G sample as it was for the GFP-vector sample. This difference is clearly shown in Figure 4.2B where the GFP-Apobec3G values have been normalized against GFP-vector. The normalized fold reduction for GFP-Apobec3G in the NL4.3/KKRK co-transfection remains at or around one over the course of sampling. The normalized fold reduction for the GFP-Apobec3G in the presence of NL4.3/WT is slightly elevated at day 2, and is significantly elevated at day 4.

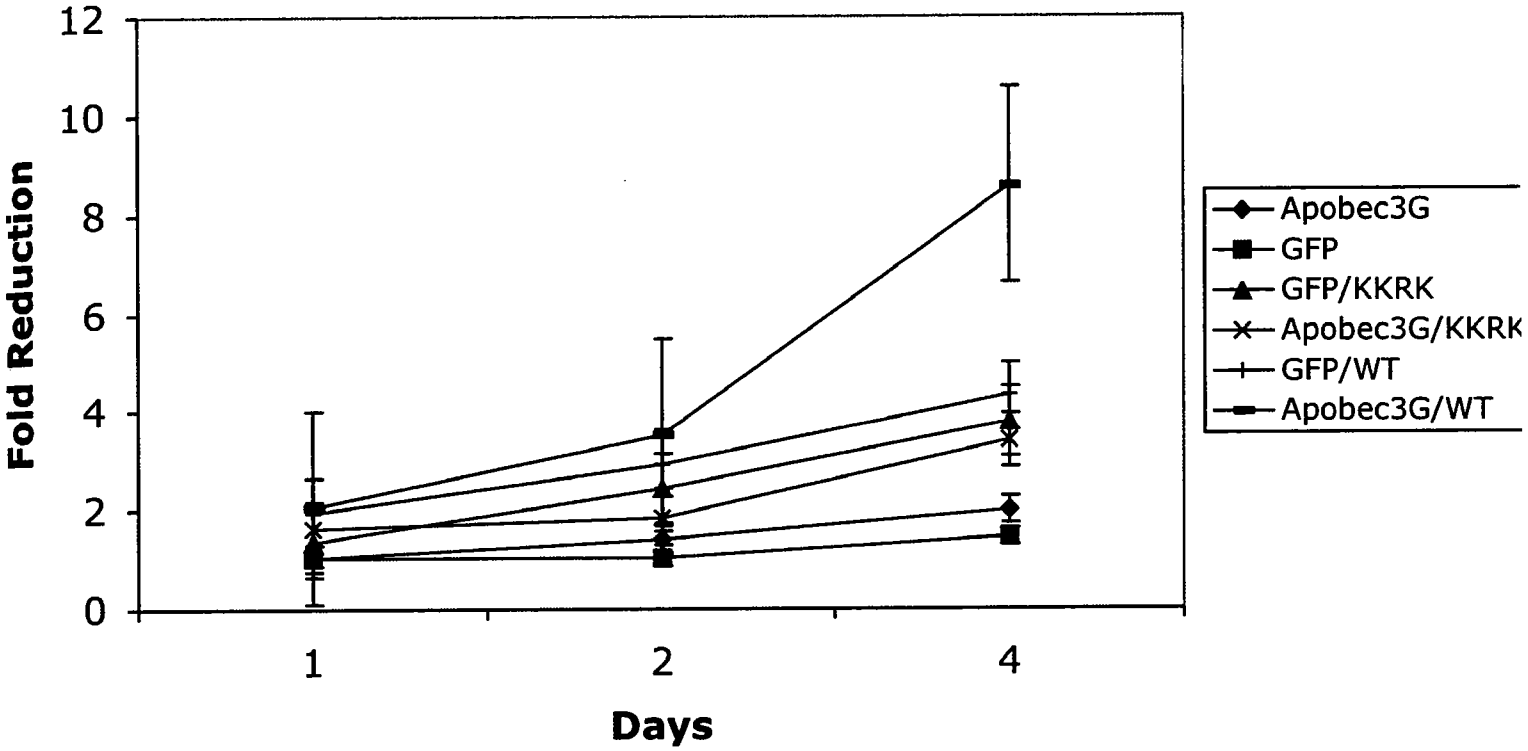
It is clear that NL4.3/WT Vif is capable of degrading GFP-Apobec3G at a high level on day four. To conclusively show this, the fold reduction on day four for GFP-Apobec3G alone or with either construct was compared. As can be seen in Figure 4.2C, the difference between loss of signal in GFP-Apobec3G and GFP-Apobec3G plus NL4.3/KKRK is minimal. There is a clear and statistically significant difference ($p<.05$) between the fold reduction in GFP signal when the GFP-Apobec3G alone is compared to the GFP-Apobec3G plus NL4.3/WT. The inability to degrade Apobec3G demonstrated by the NL4.3/KKRK Vif could lead to the packaging of Apobec3G in NL4.3/KKRK virions resulting in non-infectious virions.

D. Binding of NL4.3/WT and NL4.3/KKRK Vif to Apobec3G

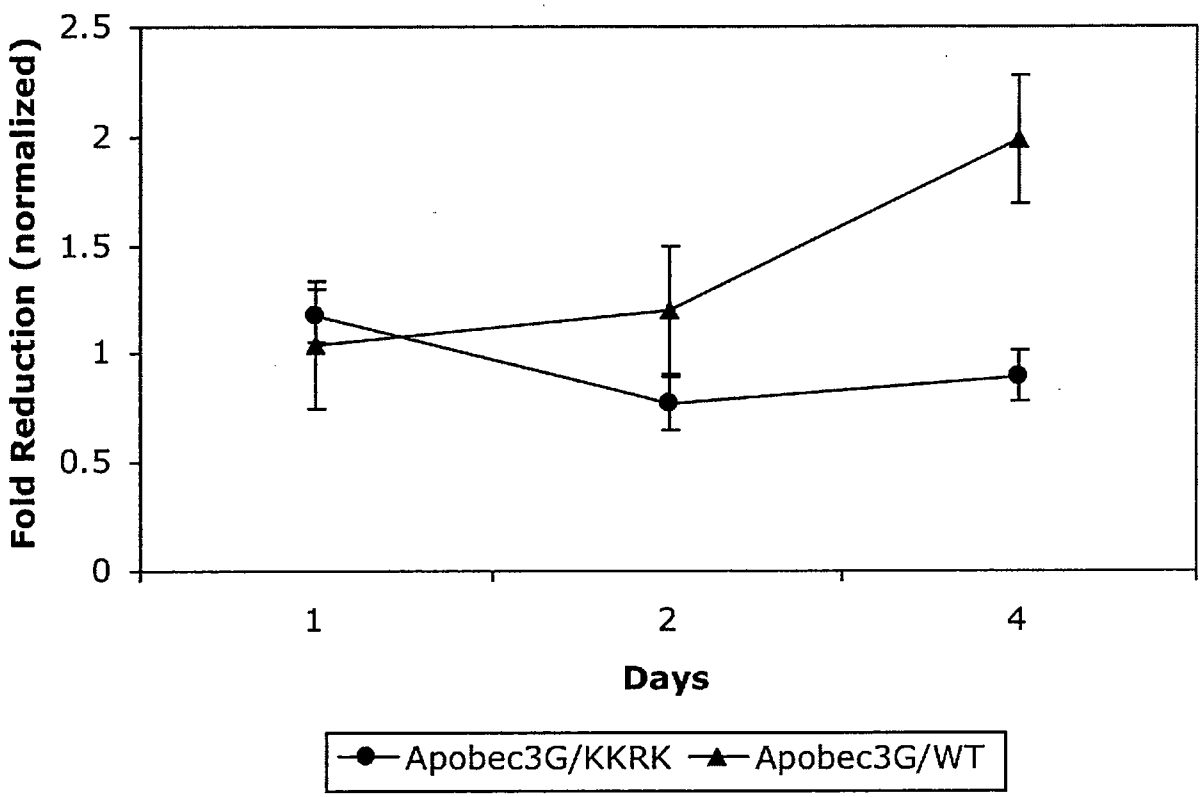
Based on the above data, NL4.3/KKRK Vif does not mediate degradation of Apobec3G. This can be attributed to either the localization phenotype of NL4.3/KKRK

Figure 4.2

A.


12
10
8
6
4
2
0
Fold Reduction
1
2
4
Days
Apobec3G
GFP
GFP/KKRK
Apobec3G/KKRK
GFP/WT
Apobec3G/WT

B.


Fold Reduction (normalized)
2.5
2
1.5
1
0.5
0
1
2
4
Days
Apobec3G/KKRK
Apobec3G/WT

C.

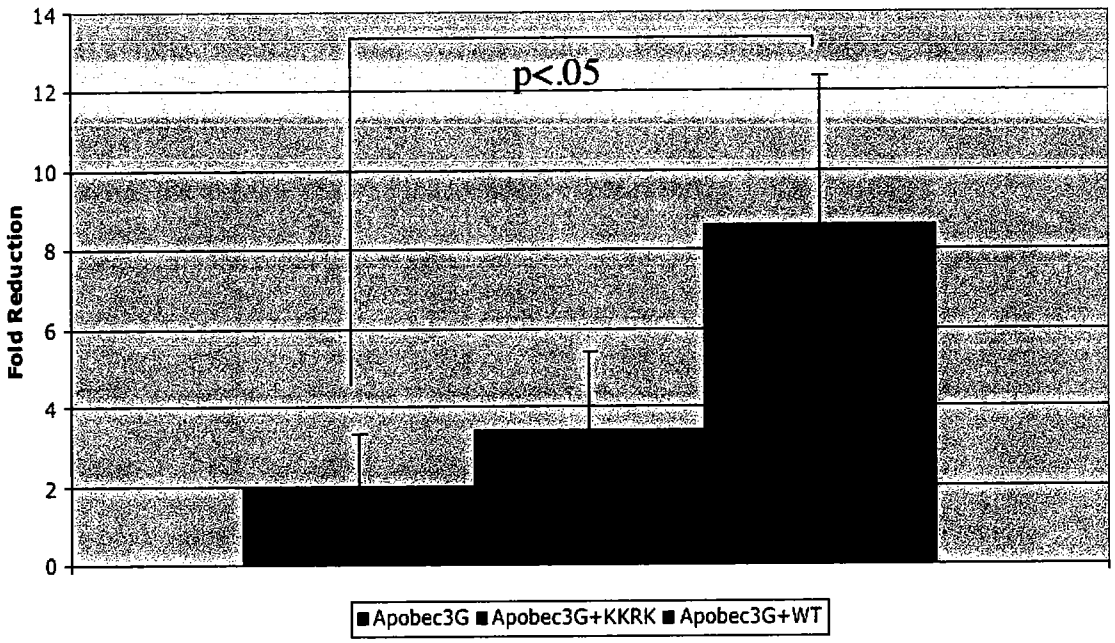

14
12
10
8
6
4
2
0
Fold Reduction
p<.05
Apobec3G
Apobec3G+KKRK
Apobec3G+WT

Figure 4.2: Degradation of GFP-Apobec3G by NL4.3/KKRK and NL4.3/WT. (A). Hela cells were co-transfected with GFP vector or GFP-Apobec3G plus an empty vector, NL4.3/KKRK, or NL4.3/WT. The number of GFP+ cells was enumerated by FACS for a 7 day period following transfection. All transfections were normalized to day two post-transfection and the fold reduction of GFP over the following five days was determined. Data shown is the average of three independent experiments.

(B). To determine the reduction of GFP-Apobec3G independent of normal loss of GFP fluorescence over time, fold reduction of GFP-Apobec3G cotransfectants was normalized against GFP vector cotransfectants. Data shown is the average of three independent experiments.

(C). The peak value for fold reduction of GFP-Apobec3G for GFP-Apobec3G+vector, GFP-Apobec3G+NL4.3/KKRK, and GFP-Apobec3G+NL4.3/WT on day four is shown. The fold reduction was statistically significantly different between GFP-Apobec3G+vector and GFP-Apobec3G+NL4.3/WT ($p<.05$), but not between GFP-Apobec3G+vector and GFP-Apobec3G+NL4.3/KKRK ($p>.05$).

Vif or NL4.3/KKRK Vif may not be capable of directly interacting with Apobec3G. To address the possibility that NL4.3/KKRK Vif is a binding mutant, an in vitro pull-down assay using His-tagged rKKRK-Vif or rWT-Vif proteins with lysates from GFP-Apobec3G transfected Hela cells was performed. Relative amounts of Vif and Apobec3G were determined by Western blot and densitometry analysis (Figure 4.3). The results showed that rKKRK-Vif is capable of binding Apobec3G more efficiently than rWT-Vif in an in vitro setting. Therefore, NL4.3/KKRK Vif is not a binding mutant for Apobec3G.

E. Colocalization of Apobec3G with NL4.3/WT or NL4.3/KKRK Vif

In light of the above finding that NL4.3/KKRK Vif is competent for binding to Apobec3G, but can not mediate the degradation of Apobec3G, the localization patterns of both NL4.3/KKRK and NL4.3/WT Vif proteins with Apobec3G were investigated to determine if localization influences the ability of Vif to counteract Apobec3G.

Hela cells were co-transfected with NL4.3/KKRK or NL4.3/WT plasmid along with GFP-Apobec3G plasmid, or with GFP-Apobec3G plasmid alone. Two days post-transfection, cells were fixed and stained for Vif and the nucleus (Figure 4.4). In the absence of Vif, the GFP-Apobec3G protein showed a punctate pattern that was dispersed throughout the cytoplasm (panels I and II). However, when the GFP-Apobec3G protein was co-transfected with NL4.3/WT, fewer cells expressing GFP-Apobec3G were detected. In cells where signal was detected, the GFP-Apobec3G signal now occurred as a compact aggregate in the perinuclear space (panel VI). The NL4.3/WT Vif protein also

Figure 4.3

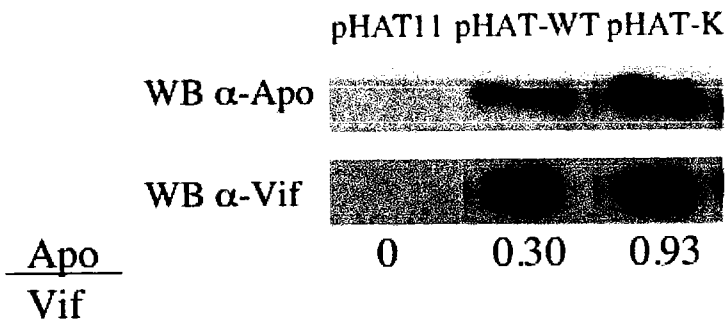


Figure 4.3: Binding of Apobec3G by rKKRK-Vif and rWT-Vif. Lysates of Hela cells transfected with GFP-Apobec3G were applied to columns of immobilized rKKRK-Vif or rWT-Vif. The columns were eluted and purified rVif plus any interacting proteins were run on a SDS-PAGE gel. The gel was subjected to Western blot using a Vif or Apobec3G specific antibody to detect the GFP-Apobec3G fusion protein. The ratio of Vif to Apobec3G for each rVif was determined using the Kodak imaging software. rKKRK-Vif was found to have a Apobec3G/Vif ratio of 0.93 indicating strong interaction. The rWT-Vif exhibited an Apobec3G/Vif ratio of 0.30 indicating a slightly weaker interaction. No interaction was observed between Apobec3G and the empty pHAT11 vector.

exhibited a strong perinuclear, aggregated localization in these co-transfected cells (panel VII). When the two signals are overlaid, it is clear that the GFP-Apobec3G and NL4.3/WT Vif are colocalized as a perinuclear aggregate (panel VIII). In a majority of cells containing perinculear aggregates, the nucleus was invaginated in the area of the aggregate, a hallmark of the aggresome. This formation of an aggresome may be functionally important as a storage site for bound Apobec3G or, for the recruitment of proteasomal machinery to degrade both proteins. When the GFP-Apobec3G signal was determined in NL4.3/KKRK co-transfected cells, it demonstrated a significantly different localization pattern. Here, the GFP-Apobec3G protein appears to be dispersed throughout the cytoplasm with some areas of intense signal in the nucleus (panel III). The cytoplasmic signal is dispersed in the region outside of the nucleus as was seen in the cells where Vif was not expressed. The Vif immunostaining revealed that NL4.3/KKRK Vif was predominantly nuclear (panel IV) and that colocalization of GFP-Apobec3G with NL4.3/KKRK Vif was less than that with NL4.3/WT Vif. Additionally, the limited area of GFP-Apobec3G colocalization with NL4.3/KKRK Vif does not appear as a perinuclear aggregate (panel V).

The images presented are representative of a series of images analyzed. In order to address the question of colocalization in a more quantitative manner, the percent colocalization for each set of transfections was calculated. Figure 4.3B shows that while the NL4.3/WT Vif efficiently colocalizes with the GFP-Apobec3G with 79% overlap, the NL4.3/KKRK exhibits only a 51% overlap. This difference in overlap is significant ($p<.005$) and could be a reason for the observed inhibition of NL4.3/KKRK mediated Apobec3G degradation.

While the differences in the colocalization were clear, there was still colocalization of GFP-Apobec3G with NL4.3/KKRK Vif. If the colocalization was occurring in the nucleus, this would leave a pool of free Apobec3G in the cytoplasm. To determine where the colocalization was occurring, a region of overlap was created by overlaying the Vif and Apobec3G images, and then subtracting out all light not corresponding to the colocalizing areas. This region of overlap was then overlaid with the phase image to determine if the colocalization was nuclear or post-nuclear (Figure 4.5A). The overlap of NL4.3/WT Vif and Apobec3G is extensive (Figure 4.4B) and occurs exclusively at a perinuclear site (panel IV). This is in agreement with previous reports that the degradation of Apobec3G occurs via the proteasome pathway, which is known to be concentrated in the perinuclear region [388, 389]. The region of overlap image for the NL4.3/KKRK Vif and GFP-Apobec3G was distinct from that of the NL4.3/WT Vif. Instead of an aggregated, perinuclear signal, the signal is a collection of smaller foci (panel I). When this image is overlaid with the phase image (panel II), it is clear that these small foci are concentrated in the nucleus with a very small, light area dispersed around the nuclear envelope.

A quantitative analysis for this breakdown of colocalization was performed as above. The region of overlap and DAPI nuclear stain were used to quantify where the Vif and Apobec3G colocalization was occurring for each Vif construct. Figure 4.5B shows that the colocalization that occurs between NL4.3/WT Vif and GFP-Apobec3G is almost exclusively cytoplasmic (88.2%) with minimal colocalization being detected in the nucleus. This ratio is inverted for the NL4.3/KKRK Vif with 88.6% of the colocalization now occurring in the nucleus.

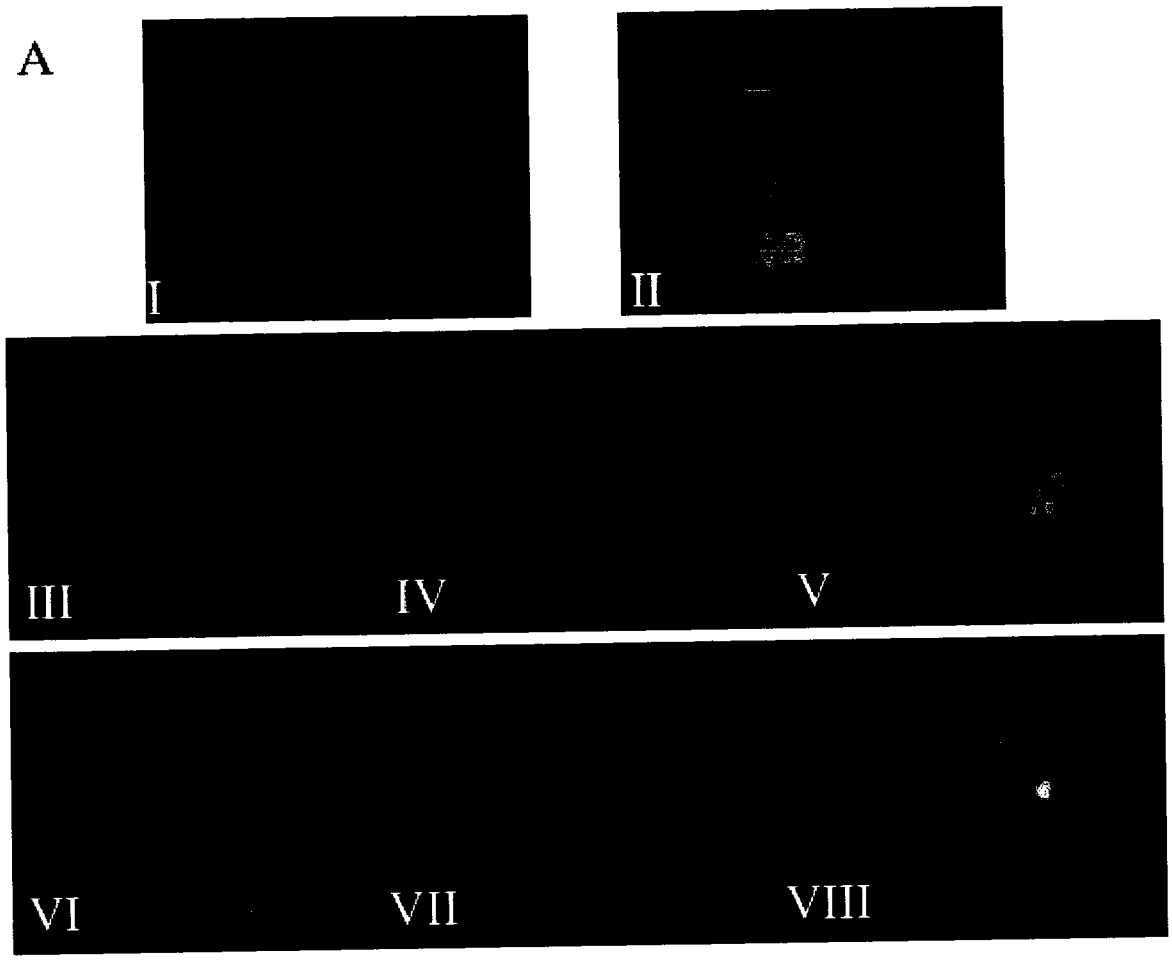
This disparity in colocalization could impact the viral packaging of Apobec3G. In the NL4.3/KKRK, cells some of the Apobec3G is sequestered in the nucleus. But the remaining Apobec3G that does not colocalize with NL4.3/KKRK Vif exists as a cytoplasmic pool of free Apobec3G that is available for packaging in the virion. In the NL4.3/WT cells there is no free Apobec3G in the cytoplasm for virion packaging as it is completely sequestered in a perinuclear aggregate.

F. Packaging of Apobec3G in NL4.3/WT or NL4.3/KKRK virions

Since NL4.3/KKRK Vif does not mediate degradation of Apobec3G, a free pool of Apobec3G exists in NL4.3/KKRK transfected cells, and NL4.3/KKRK virions are less infectious than NL4.3/WT, it was important to determine if Apobec3G was being packaged into NL4.3/KKRK virions.

Virions were harvested from the supernatant of Hela cells co-transfected with GFP-Apobec3G plus NL4.3/WT or NL4.3/KKRK and assayed for the amount of GFP-Apobec3G packaged. Following concentration by ultracentrifugation and a p24 detection assay, the virions were lysed and gels were loaded with equal amounts of p24 for each sample. Levels of GFP-Apobec3G and p24 were determined by Western blot and densitometry. The amount of GFP-Apobec3G packaged was established by normalizing the GFP-Apobec3G signal to the p24 signal for each sample. As shown in Figure 4.6, NL4.3/KKRK virions package Apobec3G at a 25-times higher level than NL4.3/WT virions. This could be explained by the availability of Apobec3G in the cytoplasm of NL4.3/KKRK transfected cells. The packaging of Apobec3G into NL4.3/KKRK virions

Figure4.4


A
I
II
III
IV
V
VI
VII
VIII

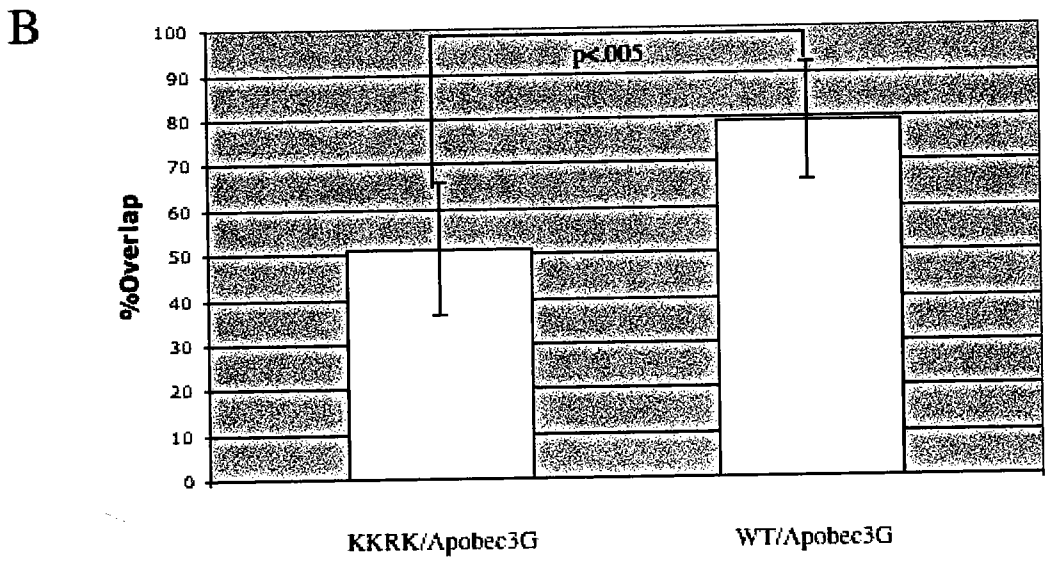
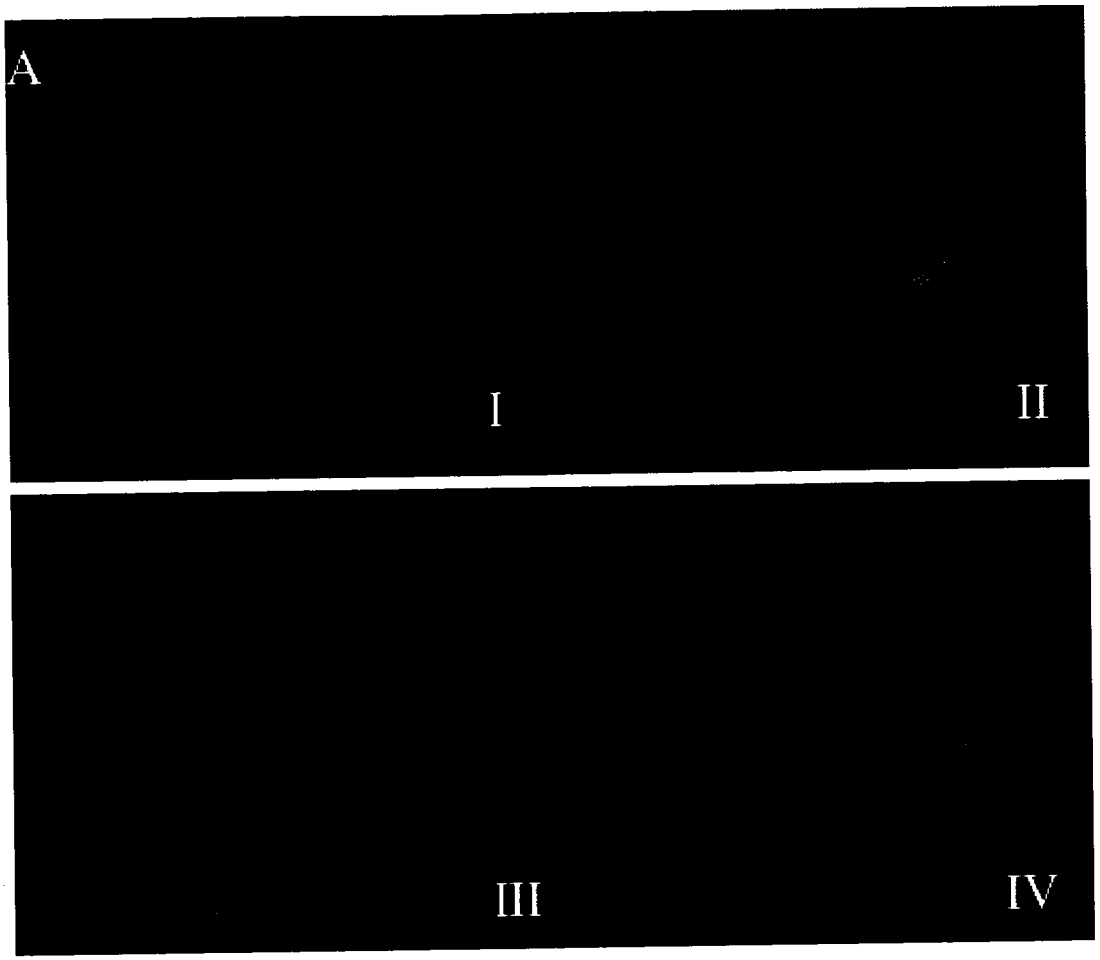

B
p<.005
%Overlap
100
90
80
70
60
50
40
30
20
10
0
KKRK/Apobec3G
WT/Apobec3G

Figure 4.4: Colocalization of Apobec3G and NL4.3/WT or NL4.3/KKRK Vif. (A). GFP-Apobec3G was transfected alone (panels I-II), or co-transfected with NL4.3/KKRK (panels III-V) or NL4.3/WT (panels VI-VIII) into Hela cells. Two days post-transfection, cells were stained for Vif and evaluated for colocalization of Apobec3G and Vif. Panels I, III, and VI are the GFP-Apobec3G signal, panels IV and VII are Vif signal. Panel II shows the overlay of GFP-Apobec3G and DAPI, demonstrating that Apobec3G is distributed in the cytoplasm in the absence of Vif. Panel V is an overlay of NL4.3/KKRK Vif and Apobec3G signals showing that there are limited areas of colocalization. The overlay of NL4.3/WT Vif and Apobec3G signals (panel VIII) reveals an extensive colocalization of the signals in a perinuclear aggregate.

(B). Quantification of colocalization. The percent overlap for the GFP-Apobec3G and NL4.3/KKRK Vif and GFP-Apobec3G and NL4.3/WT Vif was determined using the measure colocalization feature of MetaMorph. GFP-Apobec3G shows a 51% overlap with the NL4.3/KKRK Vif and an 79% overlap with the NL4.3/WT Vif ($p<.005$). The error bars represent standard deviation (n=5).

Figure 4.5


A
I
II
III
IV

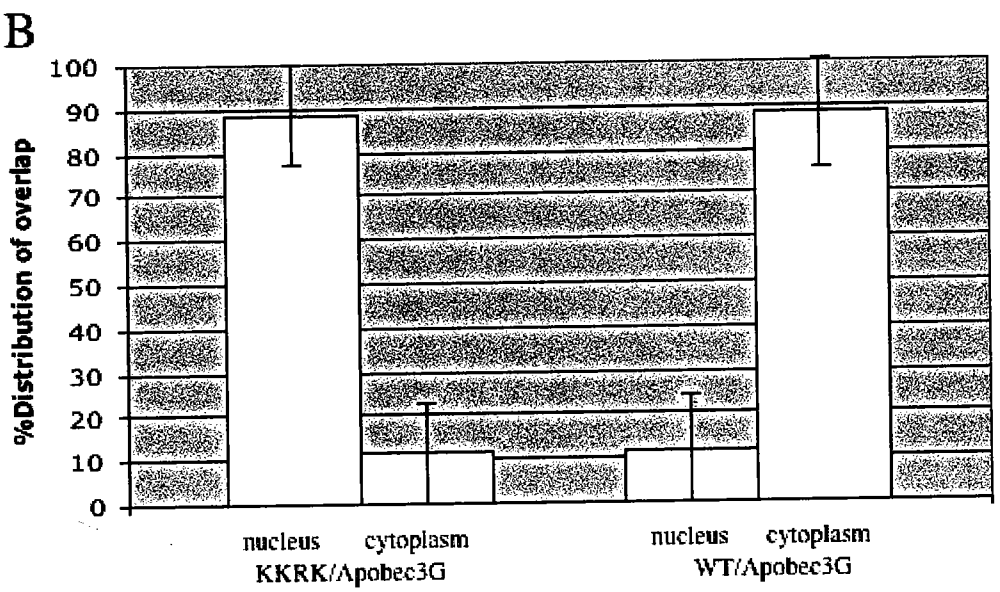

B
%Distribution of overlap
100
90
80
70
60
50
40
30
20
10
0
nucleus
cytoplasm
KKRK/Apobec3G
nucleus
cytoplasm
WT/Apobec3G

Figure 4.5: Site of GFP-Apobec3G Vif colocalization. (A). The subcellular site of colocalization for was determined by creating a region of interest image that showed only the area where overlap occurred for NL4.3/KKRK Vif and GFP-Apobec3G (panel I) and NL4.3/WT Vif and GFP-Apobec3G (panel III). This region of interest site was then overlaid with the phase contrast image to determine the subcellular site of colocalization. The overlay of the NL4.3/KKRK Vif region of interest with phase (panel II) shows a predominantly nuclear localization. The NL4.3/WT Vif region of interest overlay (panel IV) shows an exclusively perinuclear aggregate.

(B). Quantification of subcellular site of colocalization. The percent distribution of overlap for each construct was determined by comparing the region of interest to the nuclear DAPI stain. For NL4.3/KKRK Vif colocalization with GFP-Apobec3G, 88.6% of the overlap occurred in the nucleus. The NL4.3/WT Vif colocalization with GFP-Apobec3G demonstrated a 11.8% overlap in the nucleus with 88.2% of the overlap occurring outside of the nucleus. The difference in localization is statistically significant, $p<.0005$. The error bars represent standard deviation (n=5).

would result in reduced infectivity of NL4.3/KKRK compared to NL4.3/WT, as has been previously reported.

G. Discussion

The work presented here points to the critical nature of HIV-1 Vif localization during the viral life cycle. Previous work has detailed the importance of Vif interactions with Apobec3G, here it is shown that Vif must be available in the cytoplasm to bind and counteract the inhibitory effects of Apobec3G by preventing its packaging into the virion. Although NL4.3/KKRK Vif is expressed at a normal level and is fully capable of binding Apobec3G in an in vitro setting, its aberrant localization during the viral life cycle renders it less effective against Apobec3G, thus demonstrating that mislocalization of HIV-1 Vif is sufficient to confer a ΔVif phenotype.

Previous work on determinants of viral fitness in long-term non-progressors, presented in Chapter III, showed that the KKRK Vif mutation was capable of mislocalizing Vif to the nucleus and rendering virions produced from non-permissive cells significantly less infectious. The work presented here extends that study to include analysis of the impact of mislocalization of Vif on its ability to neutralize the cellular cytidine deaminase Apobec3G. NL4.3/KKRK Vif is mislocalized to the nucleus in infected cells. This quarantining of Vif in the nucleus prevents it from interacting with a significant amount of Apobec3G in the cytoplasm. Lack of interaction leads to diminished Apobec3G degradation, which allows virion packaging of Apobec3G. When Apobec3G is packaged into the virion, it can disrupt the reverse transcription process in the target cell leading to a non-productive infection.

It is also important to note that while the KKRK Vif mutation is being studied in the context of Vif localization and its influence on function, this is the first report that characterizes the impact of a naturally occurring Vif mutation on its ability to counteract Apobec3G. While other studies have shown that naturally occurring Vif mutations can diminish viral infectivity [31, 82-84], this is the first study to directly assess the ability of a naturally occurring Vif mutant to interact with and degrade Apobec3G. Interestingly, NL4.3/KKRK virus does not have a complete ΔVif phenotype when spreading replication assays are followed for a period in excess of one week. This could be due to the fact that the NL4.3/KKRK Vif retains the capacity to interact with Apobec3G and sequester some of it into the nucleus. But, its altered subcellular localization does not allow NL4.3/KKRK Vif to access all of the Apobec3G, as seen in the NL4.3/WT Vif model, and a free pool of Apobec3G is still available in the cytoplasm. The eventual emergence of replication competent NL4.3/KKRK virus after several days in tissue culture could indicate that in some cells, enough interaction is occurring with Apobec3G and infectious virus is produced. This highlights the critical balance between Vif and Apobec3G that must be maintained during virus assembly.

Figure 4.6

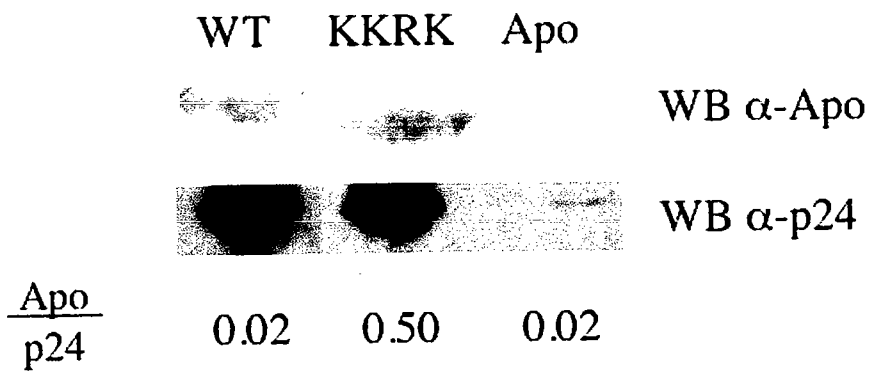


Figure 4.6: Packaging of Apobec3G into the virion. Virus produced by co-transfection of GFP-Apobec3G and NL4.3/KKRK or NL4.3/WT was harvested, concentrated, and lysed. Virion associated proteins were resolved on an SDS-PAGE gel. Levels of virion associated Apobec3G and p24 protein were determined by Western blot. The ratio of p24 to Apobec3G was calculated using the Kodak imaging software. The Apobec3G packaged into the NL4.3/KKRK virion at a ratio of 0.50, the NL4.3/WT virion packaged Apbec3G at a ratio of 0.02 which is not above the negative control lane using culture supernatant from Hela transfected with GFP-Apobec3G vector only.

CHAPTER V

HIV-1 VIF SEQUESTERS APOBEC3G INTO AGGRESOMES TO PREVENT VIRION INCORPORATION OF APOBEC3G

A. Introduction

In light of the recent advances in determining Vif function, understanding the mechanisms and pathways involved in Vif-Apobec3G interaction has become critical. It has been shown that Vif is necessary for the proteasomal degradation of Apobec3G [329-333, 352, 361] . Additionally, there is evidence that a direct interaction between Vif and Apobec3G could inhibit Apobec3G function [334, 335]. Both of these processes appear to occur in the cytoplasm.

While the directed proteasomal degradation of Apobec3G has been the focus of several recent studies, the ability of Vif to neutralize Apobec3G without degradation has been noted, but remains poorly defined. The experiments presented in Chapters III and IV demonstrate the importance of cytoplasmic localization of Vif during the viral life cycle in preventing virion incorporation of the cellular inhibitory factor Apobec3G. Interestingly, while conducting these experiments, it was found that Vif and Apobec3G form perinuclear aggregates that resemble aggresomes. Recently, Rana's lab published a similar observation of Vif-Apobec3G colocalization reminiscent of aggresomes [352]. In addition to that study, an earlier paper reported that Vif localized in a perinuclear space, surrounded by a vimentin cage [351], a typical aggresome structure [390]. Taken

together with previous observations, it is proposed that the observed Vif-Apobec3G perinuclear aggregates are aggresomes and that the formation of aggresomes could be mechanistically important for Vif function. This second inhibitory effect on Apobec3G would be achieved by sequestering it into an aggresome in the perinuclear space to prevent Apobec3G interaction with NC and subsequent virion packaging.

B. Vif-Apobec3G aggregates contain ubiquitin

If the Vif-Apobec3G perinuclear aggregates are aggresomes, then aggresome markers such as ubiquitin should also be present in the aggregates [390, 391]. To determine if ubiquitin was present in the perinuclear aggregates, Hela cells were co-transfected with Apobec3G and NL4.3/WT and analyzed for Apobec3G and ubiquitin colocalization in perinuclear aggregates (Figure 5.1). Imaging revealed that when GFP-Apobec3G is transfected into Hela cells, it is distributed throughout the cytoplasm (panels I and II), as previously observed. However, when GFP-Apobec3G is co-transfected with NL4.3/WT, cells exhibiting perinuclear aggregates of Apobec3G are detected (panel IV). Ubiquitin staining reveals that the perinuclear aggregates contain ubiquitylated protein (panels V and VI). This observation is in agreement with several recent studies that have shown Apobec3G ubiquitination when Vif is present [336, 337, 339] .

In addition to detection of the aggresome marker ubiquitin, analysis of the phase image shows a dense body in the perinuclear region (panel VIII) that overlaps with the Apobec3G (panel VIII) and ubiquitin signals (panel IX). Such bodies are not present in

GFP-Apobec3G transfected cells in the absence of Vif (panel III). Again, dense perinuclear bodies are indicators of aggresome formation [392].

Figure 5.1

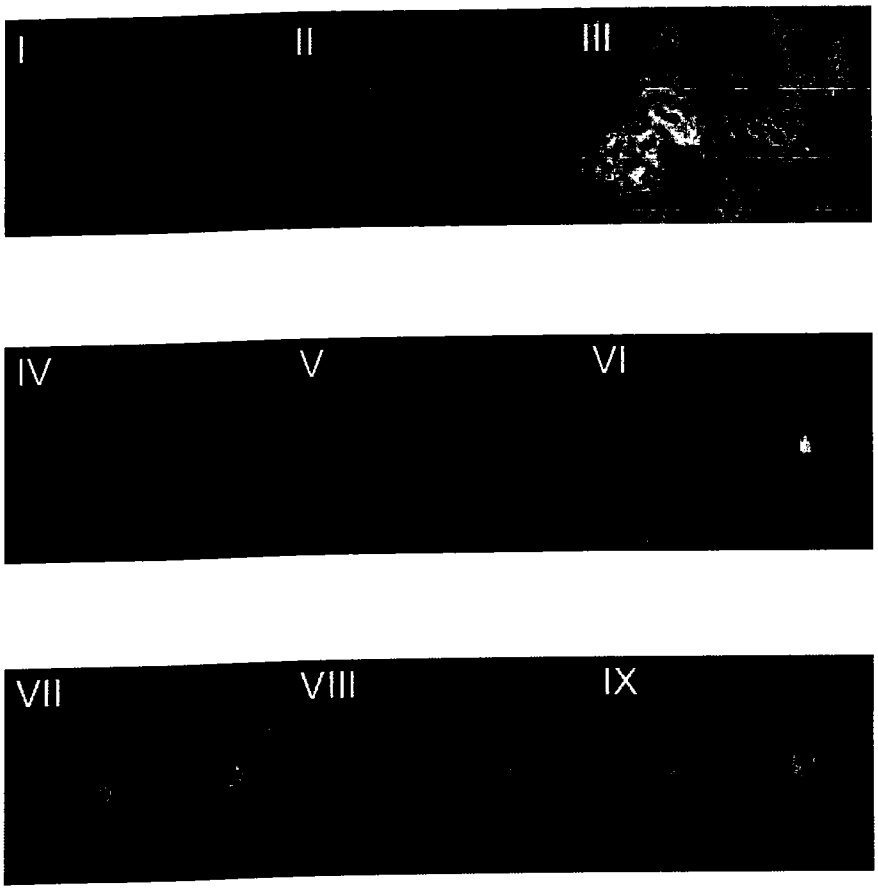


Figure 5.1: Apobec3G perinuclear aggregates recruit ubiquitin. Hela cells were transfected with GFP-Apobec3G (panels I-III) or cotransfected with NL4.3/WT and GFP-Apobec3G (panels IV-IX). The GFP-Apobec3G signal is cytoplasmically distributed in the absence of Vif (panel I). Overlaying the Apobec3G signal with DAPI nuclear stain (panel II) or with DAPI and phase (panel III) shows that there is no Apobec3G in the nucleus. Cotransfected cells were stained for ubiquitin (panel V). The GFP-Apobec3G signal demonstrates a perinuclear aggregate (panel IV). When the Apobec3G and ubiquitin signals are overlapped, it is clear that ubiquitin is also present in the perinuclear aggregate (panel VI). A dense structure is apparent in this cell under phase (panel VII) that colocalizes with the aggregated Apobec3G (panel VIII) and ubiquitin (panel IX) signals in this cell.

C. Vif and Apobec3G form aggresomes

While the presence of ubiquitin in the aggregates does suggest aggresome formation, its presence alone is not sufficient to confirm this. It is possible that these aggregates are just colocalizing Vif and ubiquitylated Apobec3G, not specifically formed aggresomes. To address this possibility, the presence the aggresome assembling protein HDAC6 [392, 393], was determined. The Hela-Apo cell line that is stably transfected with Apobec3G to allow for constitutive expression of Apobec3G was used. Hela-Apo cells were transfected with NL4.3/WT and stained for Vif plus HDAC6.

Transfection of Hela-Apo with NL4.3/WT again demonstrated the presence of perinuclear aggregates containing Vif (Figure 5.2, panels I and V). When cells were stained for the ubiquitously expressed HDAC6, it was found distributed throughout the cytoplasm for most of the cells (panel II). However, in cells containing Vif aggregates, HDAC6 also demonstrated a perinuclear, aggregated staining (panel II). When the Vif and HDAC6 signals were overlaid, it was clear that Vif and HDAC6 were present in the same perinuclear aggregate (panel III). The aggregate of Vif and HDAC6 is distinguishable as a dense structure in the phase images (panel IV). The presence of the specific aggresome marker HDAC6, in addition to ubiquitin, demonstrate that the Vif-Apobec3G aggregates are aggresomes.

D. Vif-Apobec3G aggresomes are regulated by the microtubule network

Aggresomes form in the cytoplasm and are then transported to the perinuclear region via the microtubule network [390, 393]. Inhibition of microtubule trafficking by

Figure 5.2

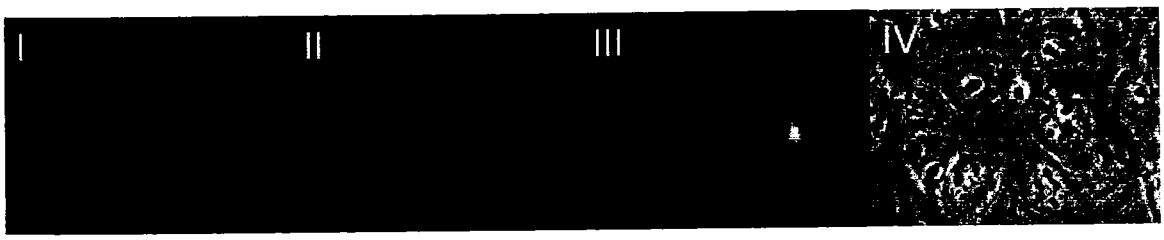


Figure 5.2: Vif-Apobec3G aggregates are aggresomes. Hela-Apo cell lines were transfected with NL4.3/WT and stained for HDAC6 (panel I) or Vif (panel II). Both signals demonstrated an aggregated signal that colocalized when the images were overlapped (panel III). A dense structure is also present under phase (panel IV).

nocodazole treatment, which induces microtubule network collapse, causes dispersion of the aggresome into smaller vesicles that are excluded from the perinuclear space and redistributed throughout the cytoplasm [390, 393]. While it was evident that Vif and Apobec3G were forming aggresomes, it was unclear whether these Vif induced aggresomes behaved in a manner analogous to aggresomes formed by cellular proteins. The role of microtubule trafficking in Vif-Apobec3G aggresome formation was investigated by exposing Hela cells transfected with NL4.3/WT to nocodazole (Figure 5.3). First, the impact of aggresome formation on microtubules was assessed by staining transfected cells for Vif and tubulin. In all cells, including those that exhibited Vif aggresomes, the tubulin network was not perturbed (panels I and II). However, when the cells were treated with nocodazole prior to fixation, the microtubule network collapsed and tubulin staining was now present as an intense signal in an area consistent with the microtubule organizing center (MTOC) (panel VI). While this result is expected for nocodazole treated cells, a more interesting observation is the dispersal of Vif stain in these cells (panel V). The Vif aggregate is not completely disrupted, but it is no longer located in the perinuclear space and it has begun to dissipate with the emergence of smaller, punctate foci rather than the tight, aggregated signal previously seen (panel V).

To determine if this dispersal of Vif signaling is an artifact of treatment with nocodazole, Hela cells were transfected with a DsRed-Vif protein and treated with leptomycin. Leptomycin inhibits nuclear shuttling by preventing nuclear export and trapping shuttling proteins in the nucleus. Some groups have proposed that Vif is capable of shuttling into and out of the nucleus. The use of leptomycin treatment provides a control for the nocodazole experiment and allows for investigation of the proposed

nuclear shuttling capacity for Vif. In cells transfected with the DsRed-Vif construct that were untreated, Vif aggregates in the perinuclear region were detected (panel X). In the presence of leptomycin treatment, Vif aggregates were still detected in a perinuclear region (panel XII). These results show that aggresome formation and disruption are not experimental artifacts and are controlled by the microtubule network. Additionally, it argues against a nuclear shuttling function for Vif.

E. Vif mediates aggresome formation

While the experiments in Hela cells demonstrate that Vif and Apobec3G form aggresomes, the question of which component was mediating aggresome formation still remained. To determine whether Vif or Apobec3G was driving aggresome formation, pull-down assays using rVif or rA3G were performed with lysates from H9 that endogenously express Apobec3G or H9 infected cells. rVif or rA3G were immobilized on columns and allowed to incubate with cell lysates. The rVif or rA3G were eluted from the column, along with any bound protein, and equal amounts of protein for each eluted sample were resolved on a SDS-PAGE gel. A total cell lysate and vector only plus lysate were included as controls. Blots were probed with α-Hsp70, α-vimentin, or α-HDAC6. Additionally, the blots were also probed with α-Apobec3G or α-Vif 319mAb to confirm expression.

Hsp70 is a cellular chaperone that is recruited to the aggresome along with ubiquitin and the proteasome [389, 393]. The Hsp70 blot (Figure 5.4, middle panel) showed that while Apobec3G is capable of interacting with Hsp70 (lane 2), this interaction is enhanced when the cell is infected and Vif is present (lane 3). When Vif is

Figure 5.3

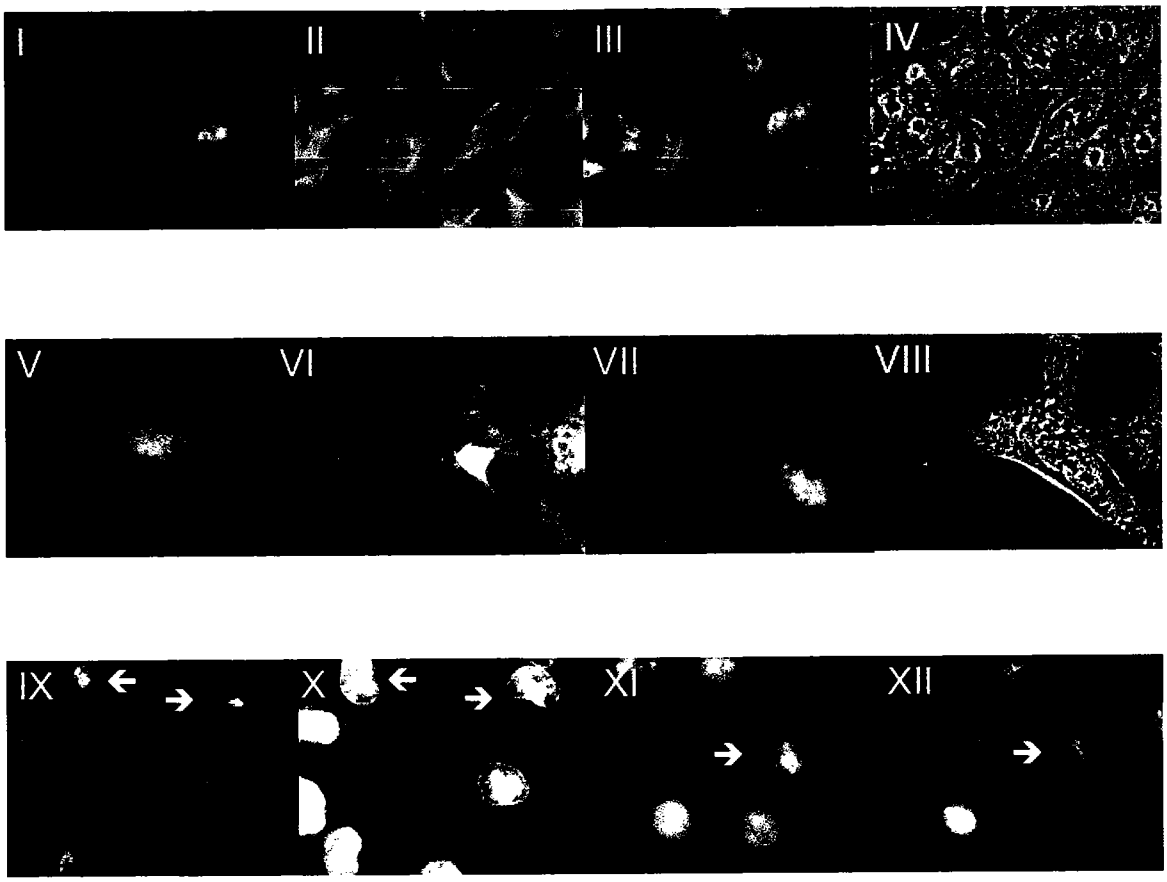


Figure 5.3: Vif-Apobec3G aggresomes are regulated by the microtubule network. Hela cells were transfected with NL4.3/WT and stained for Vif (panels I and V) or tubulin (panels II andVI). The cells in panels V-VIII were treated with 10μM nocodazole for 3 hours. When the microtubule network is intact (panel II), aggregated Vif stain can be detected (panel I) with the characteristic nuclear invagination (panel III) and dense phase structure (panel IV). When the cells are treated with microtubule depolymerizing drug, the microtubule network collapses (panel VI) and the Vif signal is displaced from the perinuclear space to a cytoplasmic location and the signal begins to disperse (panel V). The nucleus is no longer invaginated (panel VII) and there are now two dense phase structures corresponding to the collapsed tubulin signal and displaced Vif signal (panel VIII). In panels IX-XII, Hela cells were transfected with a DsRed-Vif construct and treated with 5ng/ml leptomycin for 2.5 hours (panels XI and XII). Vif is capable of forming perinuclear aggregates in the absence (panel IX, arrows) or presence (panel XI, arrow) of leptomycin. In both cases, invagination of the nucleus is also detectable (panels X and XII, arrows).

used as the bait protein, an equal amount of Hsp70 is detected from pulldowns of infected cell lysates (lane 5). The strongest signal is obtained when rVif is incubated with uninfected H9 cells (lane 6). This indicates that both proteins bind Hsp70, but that the presence of Vif enhances interaction with Hsp70.

The HDAC6 blot (Figure 5.4, upper panel) provided further insight into this question of whether Apobec3G or Vif were mediating the aggresome formation. HDAC6 regulates aggresome formation, so binding to HDAC6 would be the initial step in forming the aggresome. Neither of the rA3G lanes were capable of interacting with HDAC6 (lanes 2 and 3). However, the rVif proteins were competent for HDAC6 binding. Interestingly, we again detect a stronger interaction when uninfected H9 lysates were used (lane 4). These observations were unexpected in terms of uninfected cell lysates giving stronger signals. However, the lysis that was performed does not involve the extraction of detergent resistant fractions, which is where aggresomes are located. Therefore, we propose that infected cells having preformed aggresomes located in detergent resistant compartments have less free HDAC6 or Hsp70 available in the lysates. Taken with the observations above, we propose that Vif interacts with HDAC6 to drive aggresome formation.

Pull-down assays were also performed to determine if the previously reported observation of Vif interaction with vimentin held true in our system [351]. Permissive SupT1 or non-permissive HUT78 T cells, the stably transduced cell lines SupT1xVif or HUT78xVif, or infected SupT1 or HUT78 T cells were lysed and incubated with immobilized rVif. The eluted proteins were gel resolved and the blots were probed with α-vimentin (Figure 5.4, lower panel). Vif was able to pull down vimentin from each of

Figure 5.4

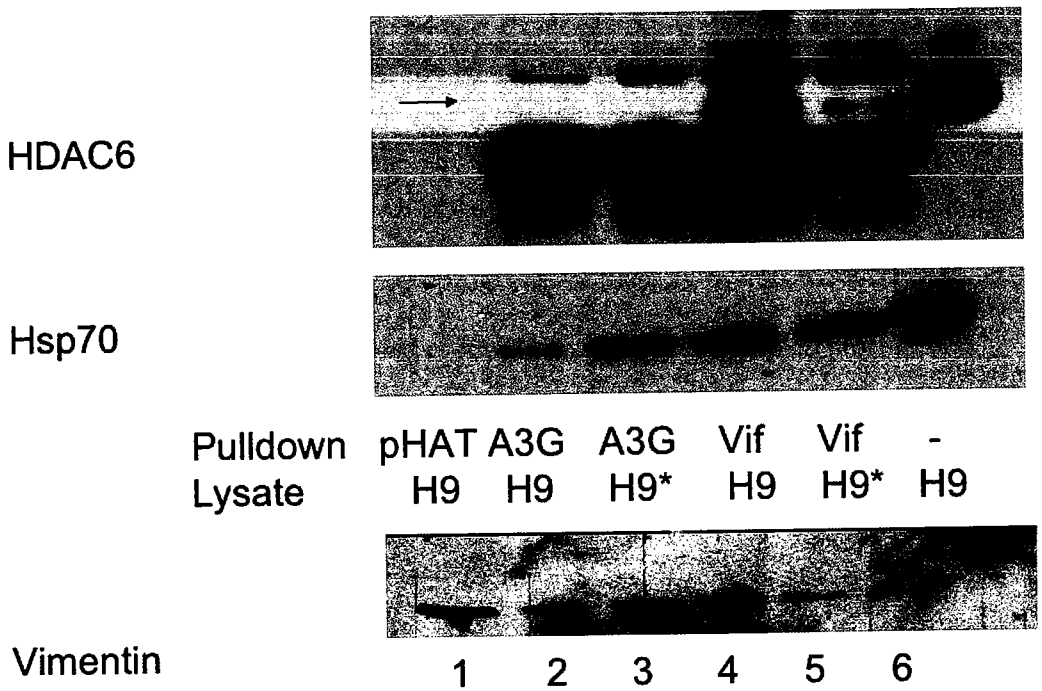

HDAC6
Hsp70
Pulldown pHAT A3G A3G Vif Vif -
Lysate H9 H9 H9* H9 H9* H9
Vimentin
1 2 3 4 5 6

Figure 5.4: Vif mediates aggresome assembly. rApobec3G or rVif were expressed and immobilized on a TALON column. For the Hsp70 and HDAC6 blots, lysates of H9 infected (*) or uninfected cells were added to the column, eluates were recovered, and equal amounts of protein were resolved on a SDS-PAGE gel. A cell lysate that was applied to a vector only column (lane 1) and a cell lysate that was not run over a column (lane 6) were included as controls. For the vimentin blot, uninfected HUT78 (lane 1) or SupT1 (lane 4), HUT78xVif (lane 2) or SupT1xVif (lane 5), or infected HUT78 (lane 3) or SupT1 (lane 6) lysates were added to immobilized Vif columns and processed as above. Blots were probed for HDAC6 (upper panel), Hsp70 (middle panel), or vimentin (lower panel).

the samples, regardless of the presence of Apobec3G or other viral proteins. However, the strongest signal was from HUT78 cells, suggesting that the presence of Apobec3G increases the affinity of Vif for vimentin. Again the signal is stronger in uninfected cells, as was seen with Hsp70, perhaps indicating that vimentin is more accessible from in cells where Vif is not being expressed and aggresomes are not being formed. This shows that Vif and vimentin can interact in vitro in addition to the previous reports demonstrating an interaction in the cell.

F. Functional relevance of aggresome formation

The experiments detailing colocalization of Vif, Apobec3G, and aggresome markers suggest that Vif forms an aggresome and can recruit Apobec3G into that aggresome. The question remained as to whether formation of this aggresome had any functional relevance for Vif.

To address this, the non-permissive CEM cell line that expresses Apobec3G and its permissive derivative CEM-SS that does not express Apobec3G were infected, treated with aggresome disrupting drugs, and assayed for viral infectivity. Two days post-infection, cells were washed extensively and treated with the proteasome inhibitor lactacystin, the microtubule depolymerizing drug nocodazole, or Scriptaid, an inhibitor of HDAC6. The cells were allowed to produce virus in the presence of the drugs for eighteen hours. Culture supernatants were harvested and used in the GHOST-X4 infectivity assay. When cultures were normalized against infection mediated by untreated cell supernatants, it was clear that each of the drugs was inhibitory in the CEM cells with varying efficacy (Figure 5.5, panel A).

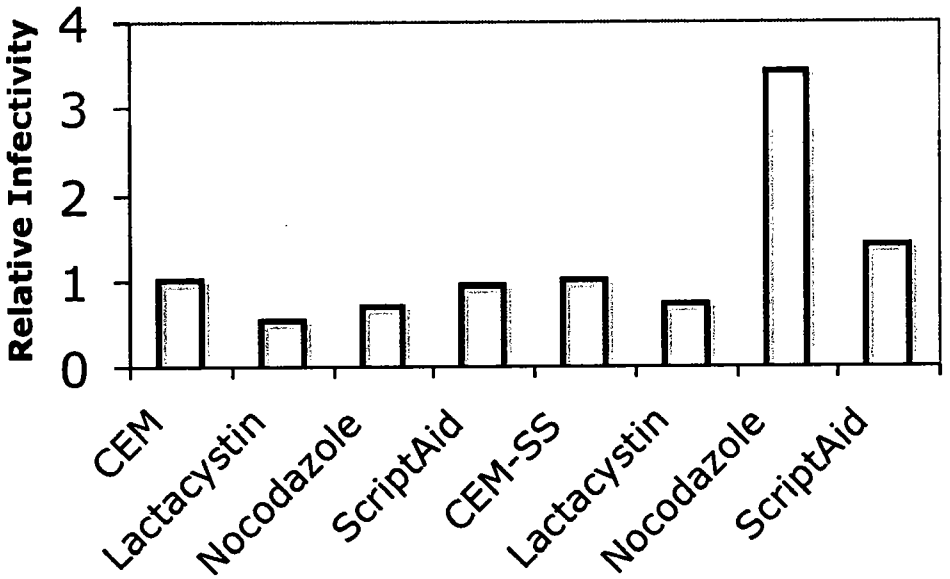
However, the full extent of this inhibition was revealed when the normalized inhibitions for CEM cells were compared to normalized CEM-SS infections (Figure 5.5, panel B). This shows that while lactacystin does have inhibitory effects on virus produced from CEM cells, it also has modest inhibitory effects on virus released from the permissive line CEM-SS. Nocodazole and Scriptaid also show diminished infectivity for virus released from CEM cells, but both treatments actually enhance infectivity for CEM-SS cells. By comparing the effect of the drug treatments on both cell types, the nocodazole and Scriptaid treatments appear to have more of an impact on virus infectivity than lactacystin treatment. While this could be due to a suboptimal dose of lactacystin or be attributed to the high levels of Apobec3G used in this study , it is still evident that nocodazole and Scriptaid treatment dramatically reduce viral infectivity when Apobec3G is present.

G. Discussion

While previous studies suggested that Vif and Apobec3G may form aggresomes, here it is shown that Vif can bind Apobec3G and form an aggregated structure with characteristics of a cellular aggresome including association with ubiquitin, vimentin, Hsp70, and HDAC6. Apobec3G present in the aggresome is sequestered to a perinuclear region away from the virus assembly site. The potential functional relevance of Vif mediated aggresome formation is demonstrated when producer cells are treated with drugs to disrupt the proteasome or aggresome formation. In this study, disrupting aggresome formation had more of an impact on infectivity than inhibiting the proteasome.

Figure 5.5

A.


Relative Infectivity
0
1
2
3
4
CEM
Lactacystin
Nocodazole
ScriptAid
CEM-SS
Lactacystin
Nocodazole
ScriptAid

B.

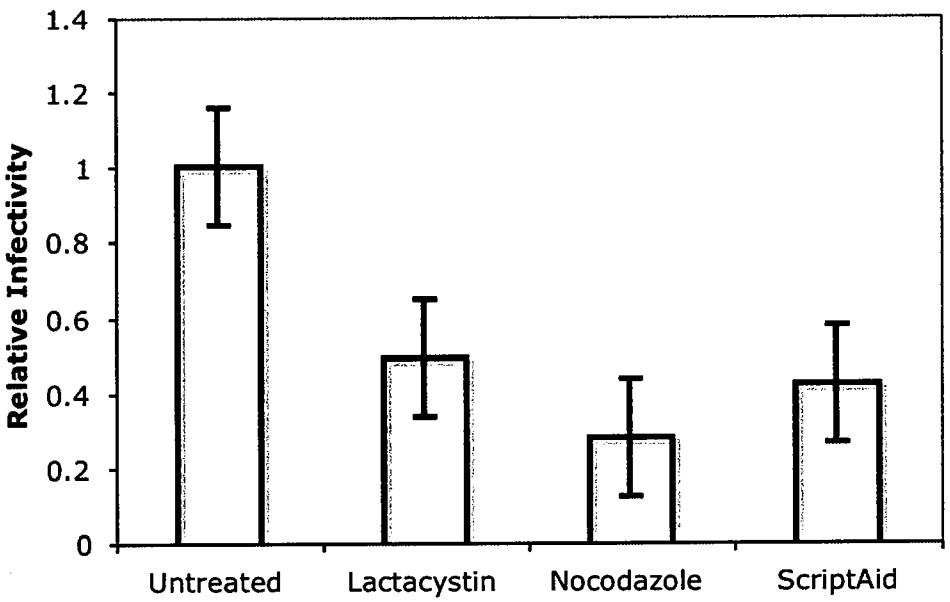

Relative Infectivity
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Untreated
Lactacystin
Nocodazole
ScriptAid

Figure 5.5: Disrupting aggresome formation reduces infectivity. (A). CEM or CEM-SS cells were infected with NL4.3/WT for two days before being washed and treated with lactacystin, nocodazole, or Scriptaid for 18h. Equal amounts of p24 were used to infect GHOST-X4 reporter cells. Three days post-infection, the number of GFP+ cells was determined by FACS analysis. Infectivities were normalized against virus from untreated cells for each cell type. (B). CEM infectivity was then normalized against CEM-SS infectivity for each treatment tested (n=2).

Aggresome formation has been the focus of several recent studies as aggresomes are believed to participate in pathogenesis of diseases such as Alzheimer's [394], Huntington's [395], and Parkinson's [396]. It has been shown that the aggresome is formed in response to proteasome inhibition or when the proteasome becomes saturated and misfolded protein continues to accumulate in the cell [390, 391]. It is believed that aggresomes serve as long-term storage sites for misfolded protein to protect the cell from the adverse effects of misfolded proteins in the cytoplasm. The aggresome localizes to a perinuclear site where it can eventually access the proteasome machinery [389, 397].

In addition to these studies, other work has shown that some viral systems are involved in protein sequestration by aggresome-like cytoplasmic body formation. Adenovirus E1B inhibits p53-mediated apoptosis by binding and sequestering p53 into a cytoplasmic body that resembles an aggresome, although it does not have all of the attributes of a cellular aggresome formed in response to misfolded protein stress [398]. Also, a C-terminal fragment of the cellular protein TSG101 is able to block retrovirus budding for PTAP- and PPPY-dependent retroviruses by sequestering viral Gag proteins into an aggresome like cytoplasmic aggregates [399]. In both cases, it is believed that one protein is prone to aggregation and actively recruits the substrate protein into an aggresome structure to inhibit its function in the cell. This model is similar to the one we are proposing for Vif induced aggregation of Apobec3G in order to sequester Apobec3G away from the virus assembly site.

Vif aggregation can be seen both in the presence and absence of Apobec3G, while Apobec3G aggregation occurs only when Vif is present. This observation is supported by the finding that Vif interacts with the aggresome assembling protein HDAC6. Several

lines of evidence have suggested that Vif is inherently unstable and could be prone to aggregation based on this instability. First, the intracellular half-life of soluble Vif is very short as it is rapidly degraded by the proteasome or shuttled into more stable complexes that associate with the cytoskeleton [400]. Additionally, Vif is a highly basic protein with a predicted pI of around 9.9 (http://prospector.ucsf.edu/ucsfbin4.0/msdigest.cgi). Our experiences in attempting to overexpress rVif in vitro revealed that Vif is prone to forming insoluble aggregates at neutral pH, which may indicate a tendency to aggregate in the cell where the cytoplasmic environment is also around neutral pH (personal communication, Jared Auclair, University of Massachusetts Medical School). All of these observations suggest that Vif is unstable in the cell and is prone to rapid degradation due to unfolding or misfolding.

We propose a model whereby Vif is inherently unstable and prone to aggregation. When Apobec3G is present, Vif interacts with Apobec3G and recruits it into the aggresome structure. This sequestration would effectively deplete the pool of free Apobec3G available in the cytoplasm for interaction with nucleocapsid and subsequent virion packaging. Having an aggresome structure available to sequester Apobec3G and supplement the direct proteasomal degradation of Apobec3G would confer an advantage to the Vif protein as it would have a means of storing Apobec3G while it also actively directed its degradation. If the proteasome became saturated during the viral life cycle, the aggresome sequestration pathway would still limit Apobec3G virion packaging and perhaps eventually lead to proteasomal degradation. Wichroski et al. has recently demonstrated the ability of Vif to sequester Apobec3G into vimentin caged, perinuclear aggregates when proteasome function is impaired or when the Vif C114S mutant that is

unable to interact with the EloBC-Cul5-E3 ubiquitin liagse complex to mediate proteasomal degradation of Apobec3G are used.

Alternatively, the existence of two distinct Vif pathways for Apobec3G neutralization may be due to separate pools of Apobec3G in the cell that are accessible by different means. This is not unique as the MDM2-p53 interaction in cells can be disrupted by two separate, independent mechanisms under different conditions [344]. Vif may utilize the different pathways, either the Cul5 complex assembly or HDAC6 mediated aggresome pathway, to access and neutralize the different stores of Apobec3G. The existence of different forms of Apobec3G has been suggested in the Wichrowski study where they detected Apobec3G as both a dispersed stain throughout the cytoplasm as well as in specific cytoplasmic bodies. Also, it has recently been proposed that Apobec3G can exist in both high and low molecular weight complexes in the cell (Retro conference).

This bimodal action of Vif may be regulated by its phosphorylation state. Recently, Mehle et al. found that unphosphorylated, but not phosphorylated Vif was capable of interacting with the EloBC-Cul5-E3 ubiquitin ligase complex that is responsible for directing the degradation of Apobec3G via the proteasome. The authors of that study proposed that Vif regulation by phosphorylation could indicate the existence of an alternative, phosphorylated form of Vif that could still bind Apobec3G, but might sequester Apobec3G in a distinct subcellular compartment rather than direct its degradation [338]. The phosphorylation state of Vif may influence its ability to bind either the EloBC complex or HDAC6 and allow Vif to exploit distinct pathways for Apobec3G neutralization simultaneously in a synergistic fashion.

The work presented in Chapter V shows that Vif is capable of neutralizing Apobec3G via a distinct pathway involving direct binding and sequestration of Apobec3G into an aggresome structure. The aggresome structure is formed and stored in the cytoplasm, again highlighting the critical importance of Vif localization in the cytoplasm during infection. Having a second, complementary pathway for Apobec3G neutralization may be important for Vif function depending on the phosphorylation state of Vif, the existence of discrete pools of Apobec3G, and the activation state of the cell. This alternate pathway may allow Vif to access secondary sources of Apobec3G or to provide long-term storage of Apobec3G when the proteasome is saturated or when Apobec3G levels are in excess.

CHAPTER VI

DISCUSSION

A. Thesis Overview

Recent work in the HIV-1 Vif field has focused on the interaction of Vif with the cellular protein Apobec3G. If Apobec3G is packaged in the HIV-1 virion, it inhibits the reverse transcription process in the target cell and effectively disrupts the viral life cycle. Vif counteracts Apobec3G by preventing its virion incorporation. This can be achieved by two pathways, recruiting Apobec3G to a Vif-Cul5 ubiquitin ligase complex for directed proteasomal degradation of Vif and Apobec3G [331-333] or the direct binding of Vif to Apobec3G and sequestration of Apobec3G away from the virus assembly site [334, 335, 338]. In either case, the objective of Vif is to limit the amount of free Apobec3G in the cytoplasm that is available to bind the HIV-1 nucleocapsid and be packaged into the virion. The work presented here has focused on the subcellular localization of Vif during the viral life cycle and how this impacts the ability of Vif to neutralize Apobec3G by either pathway.

As shown in Chapter III, the localization of HIV-1 Vif in the cytoplasm is extremely important for the production of infectious virus. Here, a naturally occurring Vif mutant was identified and it was demonstrated that aberrant localization of Vif in the nucleus resulted in the production of significantly less infectious virions. These initial

observations suggested that Vif must be present in the cytoplasm to allow for production of infectious virions.

The initial work presented in Chapter III highlighting the importance of Vif localization during the viral life cycle was extended in Chapter IV to determine if Vif localization influenced its ability to neutralize Apobec3G and prevent its incorporation into the virion, thus explaining the observed defect in virus infectivity. Here, the localization mutant identified in Chapter III was compared to a wild-type Vif protein in a series of assays that examined colocalization of Vif and Apobec3G, degradation of Apobec3G, and virion packaging of Apobec3G. The result of this study indicated that the aberrantly located mutant protein was impaired in its ability to colocalize with Apobec3G, direct Apobec3G degradation, and impair virion packaging of Apobec3G. This confirmed the importance of cytoplasmic localization of Vif during the viral life cycle to perform its function of Apobec3G neutralization.

During the course of the studies conducted in Chapters III and IV, perinuclear aggregates of wild-type Vif and Apobec3G were observed. These observations, in addition to the recent literature, suggested that Vif could also impair Apobec3G virion incorporation independent of degradation, but rather by a direct binding. The result of the experiments shown in Chapter V indicate that Vif can sequester Apobec3G into an aggresome structure. This aggresome is located in the perinuclear space where it serves as a long-term storage compartment during periods of proteasome malfunction or saturation. While aggresome formation does not deplete Apobec3G from the cytoplasm, it does sequester it away from the virus assembly site which could also prevent virion incorporation.

B. Rationale for studying HIV-1 Vif subcellular localization

Several studies have demonstrated that Vif is cytoplasmically localized during the viral life cycle [345-347]. Additionally, it is known that Vif is a critical determinant of viral replication and spread [73, 79-81, 294-296]. Many previous studies have utilized Vif mutants that were truncated or had large regions deleted or substituted. Specific amino acids and motifs were found to be important for Vif function. However, no study had fully considered the impact of Vif subcellular localization on function. In Chapter III, the localization of HIV-1 Vif was initially explored using an isolate from a highly unique long-term non-progressor. This LTNP was shown to display a remarkable disease course over a twenty year period of infection with stable $CD4^+$ T cell counts, low to undetectable viral loads, and an extremely difficult to culture virus. Based on these characteristics, viral defects that contributed to the attenuated phenotype were investigated. It was found that providing Vif in trans was sufficient to rescue this virus in co-culture assays. This growth phenotype indicated that the vif gene may be defective. Sequencing of the viral genome revealed that the virus harbored a unique set of mutations resulting in a KKRK nuclear localization sequence being present in the central region of the Vif protein. This NLS was found to be functional and sufficient to render the majority of the Vif protein nuclear. Additionally, it was shown that the presence of this unique KKRK motif rendered the virus significantly less infectious in a number of infectivity assays. Based on the observed localization phenotype and infectivity defect of the KKRK mutant, the importance of Vif localization in terms of function was determined.

C. Cytoplasmic localization of HIV-1 Vif is necessary for Apobec3G neutralization

Importantly, the defect in NL4.3/KKRK virus was only found when the virus was produced from non-permissive cells. This indicated that subcellular localization of Vif may be important for Vif-Apobec3G interactions since the replication defect was only observed in the presence of Apobec3G. Since Apobec3G is cytoplasmically located [307], and the NL4.3/KKRK Vif mutant is predominantly nuclear, it was proposed that NL4.3/KKRK Vif sequestered in the nucleus would not be available to interact with Apobec3G.

The experiments conducted in Chapter IV were aimed at elucidating the importance of the aberrant localization of NL4.3/KKRK Vif on Vif function. By comparing the mislocalized NL4.3/KKRK Vif to a normal NL4.3/WT Vif, a better understanding of the importance of Vif localization during the viral life cycle could be determined. First, the nuclear localization of NL4.3/KKRK Vif was confirmed in the presence of other viral proteins to insure that the KKRK NLS was not occluded by Vif interaction with other viral proteins. Also, the ability of NL4.3/KKRK Vif to bind Apobec3G was determined. Since it is competent for interacting with Apobec3G in vitro, but is unable to colocalize with Apobec3G in the cell, it could be concluded that any defect in NL4.3/KKRK function is a localization defect.

At this point, it was clear from the work shown in Chapter III that NL4.3/KKRK viruses were significantly less infectious than NL4.3/WT viruses. Extensive work present in the literature indicated that the difference between infectious and less infectious virions, pertaining to Vif, is governed by the packaging of Apobec3G in the virion. Viruses with WT Vif proteins are able to inhibit Apobec3G packaging, whereas

non-functional Vif proteins allow virion incorporation of Apobec3G. When the NL4.3/KKRK and NL4.3/WT Vif proteins were compared in terms of their ability to mediate Apobec3G degradation, it was clear that NL4.3/KKRK was not capable of directing Apobec3G degradation. Since Apobec3G was not being degraded and there was free Apobec3G available in the cytoplasm, virion packaging levels of Apobec3G were compared. This provided the final piece of evidence that the NL4.3/KKRK virus is a Vif deficient virus since Apobec3G was packaged more efficiently into NL4.3/KKRK virions than NL4.3/WT virions. The packaging of Apobec3G into NL4.3/KKRK virions explains the diminished infectivity of these virions. This finding can be extrapolated to explain one possible defect in the LTNP4 virus. However, this one mutation alone cannot fully explain the extreme attenuation of the LTNP4 virus as other mutations in Vif and across the viral genome have been found, but remain to be investigated. Also, such a remarkable disease course as the one exhibited by LNTP4 could only be attributed to multigenic defects.

The data presented are in good agreement with the previously reported cytoplasmic location of Vif. However, most of those observations were made prior to the identification of Apobec3G as the cellular partner for Vif and the understanding of Vif function. This study is unique in that it directly correlates subcellular localization and Vif function by comparing distinctly localized Vif proteins and their ability to mediate Apobec3G degradation and virion packaging. It conclusively shows that in addition to understanding the structure-function relationship of Vif, one must also consider the subcellular localization of the protein when defining function.

This is also the first study to not only identify a naturally occurring Vif mutant, but to mechanistically define that mutant. Previous studies were unable to provide direct evidence that their observed Vif mutants were functionally defective. Instead, those studies relied on production of less infectious virus as a surrogate marker for Vif function. Here, it has been shown that NL4.3/KKRK Vif is aberrantly localized to the nucleus and that this mislocalization is sufficient to render Vif non-functional and allow packaging of Apobec3G leading to less infectious virus being produced.

Interestingly, this mutation does not render the virus completely replication deficient. Rather, the virus appears to overcome the Vif defect after several rounds of replication. The delay in kinetics raises some interesting points to consider. First, the NL4.3/KKRK Vif is capable of sequestering some Apobec3G in the nucleus. This observation was made within the first few days of infection. Perhaps as the infection continues, Vif continues to be produced at a high level while Apobec3G production remains steady or declines. A recent study by Sheehy et al. did demonstrate that Vif expression levels peaked approximately ten days post-infection [333]. Having Vif available in excess could lead to a situation where Vif is saturating and able to interact with increasing amounts of Apobec3G. If that were the case, the NL4.3/KKRK Vif would then eventually overcome its inability to drive degradation of Apobec3G by exploiting its ability to sequester Apobec3G in the nucleus. This would be equally as effective since Apobec3G would no longer be in the cytoplasm for virion packaging. It is known that the NL4.3/KKRK virus that eventually grows out does not contain reversions at the KKRK motif or compensatory mutations in Vif. Therefore, it is clear that the

kinetics of Apobec3G and Vif expression must also be considered when investigating Vif function.

D. Vif can utilize a second pathway for Apobec3G neutralization

One recent study suggested that Vif may be able to form aggresome like structures under certain physiological conditions [352]. However, the authors of this study felt that this was a non-specific event. At the same time, additional publications indicated the potential for a second pathway for Vif function [334, 335, 338]. Based on the observations made while examining subcellular localization of Vif in Chapter IV, it was proposed that aggresome formation was specific and could serve as a potential second pathway for Vif function.

The data presented in Chapter V shows that Vif forms aggresomes, recruits Apobec3G into aggresomes, and that aggresome formation is functionally important for production of infectious virions. Some of these observations are in agreement with those presented by Wichroski et al. and a previous study by Karczewski et al. The Karczewski study was the first to show perinuclear aggregates of Vif surrounded by vimentin [351]. However, this work was published prior to the identification of Apobec3G and the authors were unable to assign any relevance to these structures. The Wichroski publication confirmed the presence of Vif perinuclear aggregates and suggested that they were indeed aggresomes [352]. While this study did show that Apobec3G also colocalized to these aggresome structures, they provided no conclusive evidence that the structures were aggresomes and suggested that they were non-specifically formed. Here, it is shown that the observed Vif-Apobec3G perinuclear aggregates contain the

aggresome marker ubiquitin and that Vif can associate with the additional aggresome markers Hsp70 and vimentin. Importantly, the Vif aggregates are shown to contain the aggresome regulating protein HDAC6 and in vitro assays demonstrate that Vif and HDAC6 bind. Additionally, formation of these aggresomes is dependent on an intact microtubule network, also a hallmark of cellular aggresomes. This conclusively shows that the perinuclear aggregates of Vif and Apobec3G are aggresomes.

While the presence of aggresome markers confirms that the perinculear aggregates are cellular aggresomes, the question of functional relevance of these structures remained. It is clear that a second pathway for Apobec3G neutralization must exist since even when Vif is present and capable of degrading Apobec3G via the proteasome, a significant amount, up to 26%, of Apobec3G is still detected in the cell [329, 330]. The studies presented in Chapter IV highlight the importance of complete neutralization of Apobec3G to allow for production of infectious virions, leaving a pool of free, cytoplasmic Apobec3G disrupts viral replication. Also, when the degradation assays were performed in Chapter IV, NL4.3/WT did drive degradation of Apobec3G at a significantly higher level than NL4.3/KKRK, but the observed Apobec3G degradation was not complete. This again indicates that a second pathway must function in conjunction with the proteasome degradation of Apobec3G. Therefore, a second pathway, independent of proteasomal degradation, must be available for Vif inhibition of Apobec3G. The proposal of a second pathway for Apobec3G neutralization by direct interaction with Vif provided support for the functional relevance of aggresome formation [334, 335, 338]. Vif interaction with Apobec3G in the cytoplasm may not be sufficient to disrupt Apobec3G binding to NC if the two binding sites are separate, as

may be the case since Vif-Apobec3G binding appears to be direct [335] while NC-Apobec3G binding may be mediated by an RNA bridge [313, 315]. Therefore, Vif would need to not only bind Apobec3G, but also to sequester it at a site distant from the virus assembly and budding area. Since the aggresomes are located in a perinuclear region and are trapped within a vimentin cage, this would allow Vif to trap Apobec3G in an area of the cell where it could not access NC. Disrupting aggresome formation by inducing collapse of the microtubule network or by inhbiting the HDAC6 protein resulted in production of less infectious virions from cells where Apobec3G was expressed. These treatments did not inhibit virus produced from cells where Apobec3G was not expressed.

One interesting observation here is that proteasome inhibition resulted in decreased infectivity for viruses produced from both non-permissive and permissive cell lines. This could be attributed to a suboptimal dose of proteasome inhibitor, or it could be due to the levels of Apobec3G present. Most previous experiments done with proteasome inhibitors used cells that were cotransfected with Vif and Apobec3G or used cells that were stably transfected with Apobec3G and typically Vif was in excess in those studies [328-332, 361]. Here, cell lines that endogenously express Apobec3G were used, allowing for a more relevant evaluation of the system. The results of these experiments indicate that aggresome formation could be the second pathway for Apobec3G inhibition. This would serve as a parallel pathway for preventing Apobec3G incorporation into the virion.

The ability of Vif to inhibit Apobec3G by aggresome formation is not unique as it has previously been reported that the adenovirus E1B protein inhibits cellular p53 by

sequestering p53 into an aggresome like cytoplasmic structure [398]. Vif may have evolved two distinct mechanisms for accessing separate pools of Apobec3G present in the cell. The ability to use two pathways may be modulated by phosphorylation of Vif as proposed by Mehle et al [338]. The need for proteasomal degradation of Apobec3G has been clearly established. Here, a second pathway for Apobec3G inhibition by sequestration into an aggresome structure has been demonstrated. This leads to the proposal of a model whereby Vif can exploit two pathways for Apobec3G neutralization, either directed degradation via assembly with a EloBC-Cul5-E3 ubiquitin ligase complex or via interaction with HDAC6 and formation of an aggresome as a storage unit.

E. Conclusions

In conclusion, the subcellular localization of HIV-1 Vif is critical for Vif function. By mislocalizing Vif to the nucleus during the viral life cycle, it is unable to prevent virion incorporation of Apobec3G. Viruses that package Apobec3G are significantly less infectious due to the ability of the Apobec3G cytidine deaminase to disrupt the reverse transcription process in target cells. This conclusively shows that the cytoplasmic localization of Vif is imperative for proper Vif function.

Also, a second pathway for Vif function has been defined. This pathway involves the interaction of Vif with HDAC6 to form an aggresome. Vif can recruite Apobec3G into the aggresome and neutralize Apobec3G by sequestration. The aggresome assembles in the periphery and is transported along the microtubule network until it reaches the centrosome. It is then surrounded by a vimentin cage and serves as a long-term storage compartment until the proteasome can degrade the proteins.

These studies were initiated based on a viral isolate from a unique LTNP. Studying isolates from LTNP provides insight into mutations that occur during the natural course of infection. Utilizing the knowledge gained from studying these viruses, the development of more effective therapies and vaccines can be achieved.

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