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Regulation of APOBEC3G by host and viral factors

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Regulation of APOBEC3G by Host and Viral Factors

by

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DISSERTATION

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Acknowledgments

I have heard that catastrophic events can cause your life to pass before your eyes—clear and distinct moments appear and fade, as if an impatient child were flipping through flash cards. Writing one's thesis can provoke a similar response—a parade of faces and moments that contributed to your development as a scientist and as a human being appear and fade, yet somehow remain with you. When I think of the people who have helped me, guided me, or inspired me these past years, the amount of time and energy provided by countless individuals is overwhelming. I have been privileged to have met each one of these kind, generous, brilliant, and altruistic people. And what amazes me is that they all behave this way on a daily basis. Helping others is an integral part of their daily lives and they expect little, if anything, in return. I have found that this is true of so many people at the Gladstone, including the scientists, the research associates, the graduate students, the laboratory aides, the administrative staff, and the janitors. There is good will in the air, in the water, and perhaps even embedded in the stones and mortar that make up the foundation of the building.

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needed to carry the project to completion. But what I have most received from Warner was simply being able to know him, to learn about his values; to hear his thoughts on everything from science to career development to the benefits of drinking water; and to witness first-hand how a great scientist and leader thinks and behaves in the modern world.

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Chapter 2 contains a reprint of a manuscript that appeared in Molecular Cell. I was co-first author on this manuscript along with Dr. Carlos de Noronha. Dr. Wes Yonemoto was also an author on this paper. Chapter 3 contains a reprint of a review I co-authored with Warner Greene that has previously appeared in the journal Current Opinion of Investigational Drugs and also contains experiments performed by myself, Louise Ogle, and Dr. Wes Yonemoto. Chapter 4 contains a reprint of a manuscript that appeared in The Journal of Biological Chemistry. I was first author of this paper. The co-authors were Dr. Ya-Lin Chiu, Jerry Kropp and Robert Grant. Dr. Warner C. Greene directed and supervised all of the work that appears in every chapter of this manuscript. The individual contributions of each co-author will be described in the Introduction to each chapter.

Abstract

As the acquired immunodeficiency syndrome (AIDS) pandemic continues to grow, physicians and patients must contend with the emergence of drug-resistant viral strains and with adverse side-effects associated with current drugs. The identification of new targets for drug development is thus critical at this juncture. Human CD4+ T cells harbor an enzyme, APOBEC3G ("A3G"), which has the potential to completely block the spread of HIV. A3G is a largely cytoplasmic protein that can be incorporated into budding virions, where it later exerts its antiviral activity. The HIV-1 Vif protein counteracts the activity of A3G, enabling wild-type HIV-1 virus to replicate in such targets as primary CD4+ T cells.

We observed that HIV-1 Vif caused the elimination of endogenous A3G from infected T cells. We further determined that Vif binds A3G and causes its disposal by 26S proteasomes, and that it inhibits the synthesis of A3G protein. These findings enabled the design of a high-throughput screen (HTS) for inhibitors of Vif action. The screen involves co-expressing Vif and A3G-luciferase in cells suitable for HTS. In the third set of studies, we switched our attention from how the virus regulates cellular A3G expression, to how the cell does so. We tested a panel of cytokines, chemicals naturally produced by human cells, for their effect on the expression of A3G. IL-2, -7, and –15 all significantly enhanced the expression of A3G protein and mRNA through a Jak/MAPK signaling pathway. Interestingly, these agents also render resting CD4+ T cells permissive to HIV infection. When we studied the activity of A3G, we found that the cytokines caused A3G to shift into an inactive complex that removed the restriction to HIV infection.

The finding that Vif causes the removal of most of the A3G from T cells provides a starting point for a highthroughput screen to identify inhibitors of Vif action. The work examining host-factor regulation of A3G identified several factors that enhance the expression of A3G and showed that some (such as IL-2 and –15) also inactivate A3G, enabling HIV infection. Together, our work has provided some insights that might be valuable for the future development of therapeutics.

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Chapter 1

Introduction

I. Summary

Despite significant advances in our understanding of human immunodeficiency virus (HIV), the causative agent of AIDS, and despite advances in drug development and their wider dissemination, HIV infections continue to be on the rise in every region of the world. Nearly 40 million people world-wide are currently HIV-infected, according to the World Health Organization and UNAIDS; and nearly 25 million people have died of AIDS since the disease was first reported in 1981. The majority of the new cases appear in Sub-Saharan Africa, but HIV infection rates have experienced sharp increases in other regions including Central Asia/Eastern Europe, India, and China with rates increasing as much as 70% since 2004 in some areas.

In the US, the news is more encouraging. Although infections are still on the rise, the availability of highly active antiretroviral therapy (HAART), involving the administration of drug combinations, has dramatically increased the life expectancy of HIV-infected individuals (115). Unfortunately, this gain is accompanied by significant financial costs. An HIV-1-infected person receiving HAART can expect to incur a total health care bill exceeding $600,000 over the course of his or her lifetime.

The costliness of HAART makes effective world-wide distribution difficult, and a very small percentage of the people in need actually receive treatment. For those who receive treatment, the effectiveness of HAART can be undermined by the toxicity of the drugs, which can impede patient adherence (20), and by emerging single-drug-resistant and cross-resistant strains of HIV (42, 62).

Although there are now over 20 HIV drugs available, most target only two viral enzymes, either Reverse Transcriptase or Protease. New drugs targeting different parts of the viral lifecycle are desperately needed.

Our work began with a simple research question, but we ended with a fresh perspective into potential strategies for combating the HIV virus. At the beginning of this project, it was known that the HIV Vif protein was critical to the lifecycle of the HIV virus; but its mechanism of action was poorly understood. Our lab and others identified the exact mechanism of Vif's action, namely to eliminate an antiviral factor (APOBEC3G (A3G)) from immune cells. This work provided the basis for a high-throughput screen for Vif inhibitors, an approach that we hope will lead to the identification of a new class of drugs in the future. One of the exciting aspects of this work is that such a therapeutic would not only target a viral protein, but would enable the A3G already present in human immune cells to do a job it is quite able at performing: defeating the HIV virus. After determining how the viral Vif protein regulated A3G, we next turned to how host factors regulate A3G, and learned that certain cytokines enhance the expression of A3G in some cell types, but that they also inactivated it, rendering cells permissive to HIV infection. We also identified another group of host factors (the interferons) that enhance A3G expression without impairing its antiviral activity. Our hope is that the combined understanding of how Vif and host cellular factors regulate A3G will assist in the search for new therapeutics that could improve the lives of the many people suffering from HIV/AIDS.

II. Introduction to Lentiviruses

The past century of research into the biology of lentiviruses began with a sick horse in the French countryside. In 1904, a "filterable agent" was isolated from a horse that had fallen ill with equine infectious anemia, a disease characterized by cycles of fever, thrombocytemia, and severe wasting (19, 147). Since the identification of Equine Infectious Anemia Virus (EIAV), related viruses have been isolated from sheep (visna-maedi virus), goats (Caprine arthritis/encephalitis virus, CAEV), cows (bovine immunodeficiency virus), cats (feline immunodeficiency virus, FIV), nonhuman primates (simian immunodeficiency virus, SIV), and, of course, humans (HIV) (59-61, 147).

Lentiviruses are complex retroviruses that are associated with certain chronic disorders of the immune and nervous systems. Simple retroviruses contain at least three genes, *gag, pol,* and *env*, which encode the core and surface structural proteins as well as enzymes, reverse transcriptase enzyme (RT), Integrase (IN), and protease (PR), necessary for viral replication. A common feature of all retroviruses is the use of RT to convert their RNA genomes into a DNA intermediate that is subsequently integrated into the host chromosomal DNA via the action of IN. The integrated provirus generally uses host cell machinery for replication, expression and protein production. Lentiviruses differ from simple retroviruses, however, in that they encode additional genes that improve viral replication and persistence. HIV-1 encodes six such additional genes (*tat, rev, vif, vpr, vpu,* and *nef*).

III. A Brief Summary of the HIV Lifecycle

The HIV-1 life-cycle begins when an infectious virion attaches and enters a target cell; in the next phase the viral genomic RNA is reverse-transcribed into double-stranded DNA which is subsequently integrated into the host DNA; following integration, proviral DNA can be transcribed into viral proteins and genomic RNA that assemble into a budding virion that exits the cell (Fig. 1).

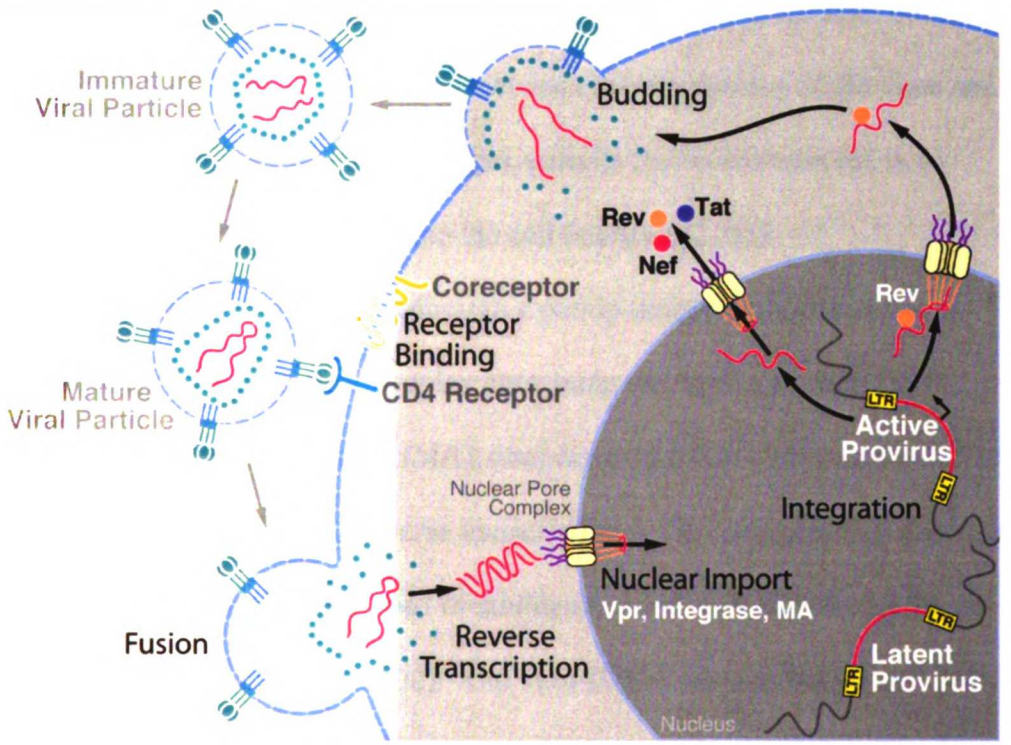

Fig. 1 The HIV-1 Lifecycle

HIV-1 entry into target cells requires attachment, interaction with specific cell-surface receptors, and fusion. The initial attachment process is mediated both by cell-surface adhesion molecules that are incorporated into HIV-1 virions (16-18, 44, 45, 116,

162) and by the viral Env gp120 protein. Env gp120 interacts with CD4 glycoprotein expressed on the surface of T cells, monocytes, dendritic cells and brain microglia (31, 86, 162). Interaction with CD4 triggers a conformational change in gp120 that exposes the binding site to chemokine co-receptors such as CXCR4 or CCR5(128, 150), although some laboratory isolates of HIV-1 and –2 can bind chemokine receptors independent of an interaction with CD4 (27, 40, 121). Chemokine receptor engagement triggers an additional conformational change in the HIV-1 gp41 ectodomain, resulting in the insertion of the trimeric coiled-coil (preexisting or newly-formed) into the lipid bilayer of the target cell (21, 48, 49, 88, 156). This bridge between the membranes of the virus and cell, along with additional conformational changes, permits fusion between the two entities and the extrusion of the viral core into the cell interior (22, 63).

Uncoating of the virus occurs next through a poorly understood process, generating a viral reverse transcription complex containing the double-stranded RNA genome, tRNAlys primer, RT, IN, matrix (MA), nucleocapsid (NC), viral protein R (Vpr) and various host proteins(79, 80, 92). Reverse transcription is then completed giving rise to a preintegration complex (PIC) composed of double-stranded viral cDNA and IN, as well as other viral and host proteins(14, 102). The viral cDNA encodes the entire viral genome and is flanked at either end by a 5' and 3' long-terminal repeat (LTR) (Fig. 2).

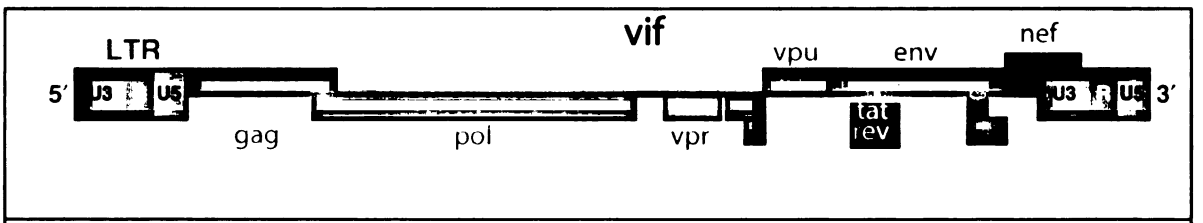

Fig. 2 The HIV-1 Genome.

The PIC is then imported into the nucleus through nuclear pores. IN binds the ragged ends of viral cDNA generated by RT and catalyses a cleavage reaction that results in a

two-base-pair recess at each end (14). Then, with the cooperation of cellular DNA repair enzymes, IN catalyzes joining reactions necessary for the integration of the double-stranded viral cDNA into the host chromosome (14). Alternatively, the viral DNA may form one of three types of circular structures (single LTR, 2LTR, or an autointegratrated circular structure) that fail to lead to the production of infectious virus. Some of these circular forms could be transcriptionally active and could generate the transcriptionally transactivator Tat and Nef (161).

Following integration, the provirus can either be actively transcribed or, on rare occasions, remain latent. The exact causes of latency and the factors responsible for activating transcription of latent proviruses *in vivo* are unclear. Some latency could be due to integration into repressive, heterochromatin-rich regions of the genome (76); but there are other causes, such as an absence of some aspect of transcriptional machinery, that could also be at play.

An integrated provirus is generally similar to other multi-exon cellular genes and relies on host-cell machinery for transcription and translation. The promoter elements within the 5' LTR, including several NF-kB responsive sites, activate the start of transcription, which proceeds through to the 3' LTR. Unlike ordinary cellular mRNA, the splicing and nuclear export process of HIV viral RNA is not tightly coupled. At early points, most viral mRNAs are doubly-spliced and encode "early" products such has Tat (a transcriptional activating protein), Rev, and Nef (a major modulator of expression of cell-surface proteins, including CD4, and a regulator of cell-signaling) (30). At later points, Rev binds to a Rev responsive element (RRE) within the *env* coding region and enhances the export of unspliced mRNAs by shunting the mRNA into a CRM1-

dependent nuclear export pathway. Incomplete splicing of these transcripts could be due to suboptimal splice-donor and acceptor sites within the viral transcripts. Rev could also inhibit splicing by interacting with alternative splicing factor/splicing factor 2 (ASF/SF2) (119) and p32 protein (94). As a result, unspliced, intact viral genomic RNA can be exported into the cytoplasm for later packaging into the budding virion. Several other viral transcripts also remain incompletely spliced including those encoding proteins Gag structural proteins and accessory proteins such as Vif, Vpu and Vpr.

The dependence of Vif on Rev has made transient expression of Vif difficult in the absence of other viral proteins. However, as is described in Chapter 3, our lab and others have devised a strategy to promote Vif expression in transiently-transfected cells.

Viral assembly occurs after Gag and Gag-Pol precursor polyproteins are targeted to the plasma membrane, where budding occurs. The Gag and Gag-Pol polyproteins assemble into a core virion containing two strands of viral genomic RNA (+) as well as Vpr and Nef accessory proteins (47). Budding begins as a lipid coat derived from the plasma membrane and containing Env gp120/gp41 trimeric complexes forms around the core virion. These protrusions are then pinched off with the help of tumor suppressor gene 101 (TSG101) (54, 154), a cellular protein that assists in vacuolar-sorting pathways by forming the ESCRT-1 complex, to form immature, noninfectious particles that become mature when the viral Protease enzyme cleaves Gag and Gag-Pol polyproteins.

The HIV-1 lifecycle is also highly dependent on the ability of the virus to evade the immune system, a process mediated by several different viral proteins. HIV-1 Nef down-modulates cell-surface molecules include in CD4 and MHC I, causing infected cells to bypass ordinary immune surveillance. The downmodulation of CD4 may also

protect T cells from superinfection, which could be a way of enhancing the reservoir of viral-producing cells (160). HIV-1 Env is heavily glycoslyated, obstructing antibody recognition. Finally, HIV-1 RT is highly error-prone, contributing mutations that make HIV a moving target for immune recognition.

IV. HIV-1 Reverse Transcription

Reverse transcription is the transition of an RNA genome into a double-stranded DNA molecule. Retroviruses are typically RNA viruses that use reverse transcription to replicate their genomes, a process first characterized in RNA tumor viruses by Baltimore (4, 148) and Temin and Mizutani (4, 148).

HIV-1 viruses contain two plus-stranded genomic RNA molecules (~9kb) flanked at either end by two short, redundant (R) sequences that are adjacent to unique sequences at the 5' (U5) and 3' (U3) termini (75). During reverse transcription, duplication of the U5 and U3 sequences results in proviral double-stranded DNA with U3-R-U5 LTR at both termini. U3 contains *cis* elements necessary to initiate transcription of the provirus and needs to be upstream of the 5' R, the starting point for transcription. The duplication event occurring during reverse transcription is therefore essential because it permits U3 to be in the proper position to direct the later transcription of the HIV provirus.

Initiation of HIV reverse transcription occurs when a cellular $tRNA^{Lys3}$ anneals to the primer binding site (PBS) in the 5' untranslated region of the genomic RNA (5) (Fig. 3). The genomic RNA from the PBS to the 5' cap provides a template for RT to synthesize minus-strand strong-stop DNA (-sssDNA). Minus-strand synthesis continues with a strand-transfer reaction, in which the R region of the –sssDNA pairs with a 3' R. a process that can occur either intramolecularly with the 3' R from the original RNA strand or intermolecularly with the 3'R within the second RNA genome in the virion (152). Synthesis of minus strand DNA then proceeds from the 3' to the 5' end of the RNA genome. During this process the RNaseH activity of RT degrades most of the RNA in the RNA:DNA hybrids, except for two polypurine tracts (PPT) that are RNaseH resistant (50). The PPT in the

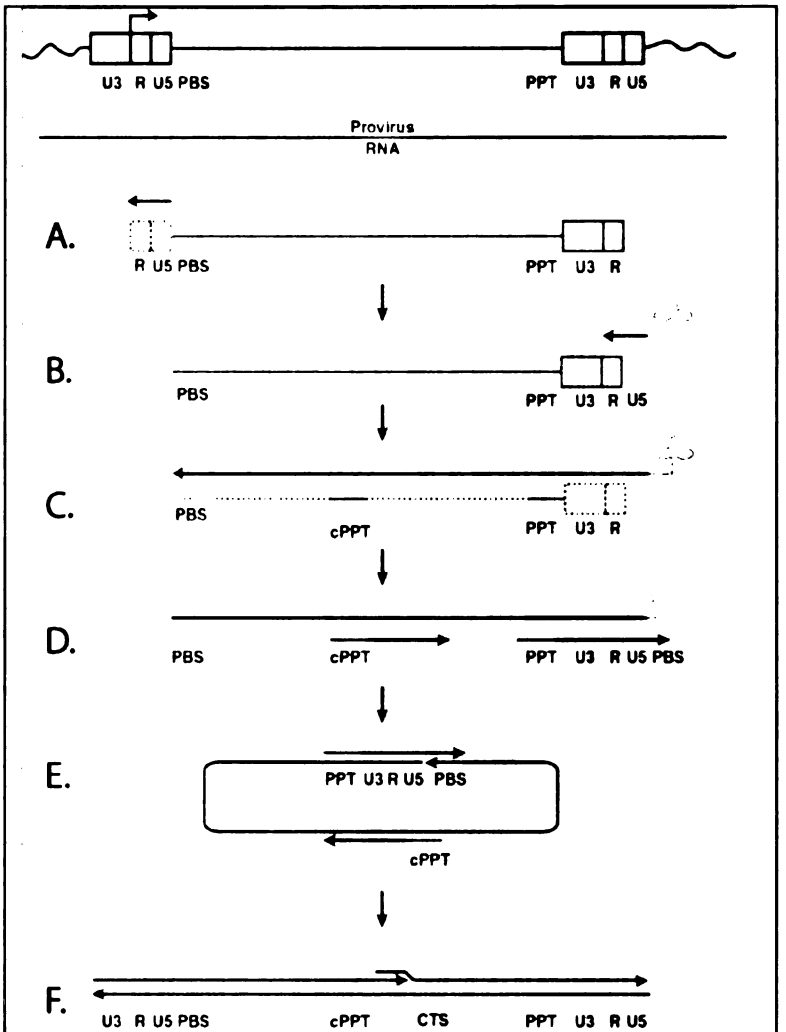

Fig. 3 Overview of Reverse Transcription. A. Primer binding and minus-strand strong-stop synthesis; B. Minus-strand transfer reaction; C. Completion of minus-strand synthesis; D. Positive-strand strong-stop synthesis; E. Second-strand transfer reaction; F. Completion of synthesis of double-stranded DNA

U3 domain of the 3' LTR and the c PPT at the center of the genome then serve as primers for the next phase of reverse transcription, + strand synthesis. Synthesis originating at the 3' PPT generates (+) strong-stop DNA, which undergoes a second strand transfer after base pairing with the PBS in the minus-strand DNA. RT then continues to elongate the (+) strand to the central termination signal (CTS), displacing 100 nucleotides of the + strand DNA that had been initiated at the c PPT. These two discrete segments are then joined together, presumably by cellular enzymes, resulting in the generation of double-stranded proviral DNA, flanked by an LTR at each end.

V. Introduction to HIV-1 Vif

HIV-1 encodes an accessory protein Vif, 23 kDa in size, that plays a major, but until recently, enigmatic role in HIV pathogenesis. *Vif* is a viral late gene (along with *vpr*, *gag-pol-env*) that is singly-spliced and is expressed in a Rev-dependent manner (2, 53, 130). Vif-specific antibodies have been detected in the sera of HIV-1 infected patients at all stages of infection, indicating that *vif* is expressed during natural infections (3, 109, 159). All lentiviruses, except EIAV, encode Vif proteins; and Vif proteins of closely-related lentiviruses are highly conserved (110). Vif also contains several short sequence motifs (including a SOCS-box BC box SLQXLA . . . .PPLP)) that are highly conserved across even distantly-related lentiviruses (67, 99, 110, 166) and a highly-conserved upstream zinc-coordinating motif (100, 166).

Vif is interesting in that in some settings it is absolutely necessary for the lifecycle of the virus. Without a functioning Vif protein, HIV is unable to replicate in cultured primary CD4+ T cells and macrophages, which are natural targets of HIV-1 infection as well as certain immortalized T cell lines (43, 51, 52, 143, 145, 155). ΔVif virions from

these cell-types are unable to complete reverse transcription in the target cell (36, 39, 58, 138). The importance of Vif for pathogenesis has also been demonstrated in simian models (35). When the relative importance of Vif for viral infectivity has been compared *in vitro* to other viral accessory proteins such as Vpu, Vpr, and Nef, the effect of deleting *vif* was dramatically more detrimental to HIV replication than of deleting any one of the other genes (56).

VI. Correlation between Vif and Progression to AIDS in Patients

The *in vitro* and animal-model data of Vif is highly predictive of an important role for Vif in the development of AIDS in patients. Studies of viral isolates from human patients have also been informative, but inconsistent. Some studies involved examining inter- and intra-individual sequence variations and conserved motives in the Vif genes of HIV-1 infected individuals. Although the results have varied, most studies have found at least some significant proviral sequence conservation in the vif gene, especially in the 5' end region, among infected patients and a direct correlation between certain vif sequences and the course of disease (67, 114, 158). Another study reported frequent, sporadic inactivation of Vif in infected patients and an association with viral diversification (141).

Other studies have involved comparing viral isolates from normal HIV-1 patients with those from HIV-1-infected long-term nonprogressors (LTNPs), which represent 5-10% of all HIV-1-infected individuals. LTNPs are patients who have been infected for 10 or more years, but are clinically healthy without antiretroviral treatment. The ability of LTNPs to control HIV-1 infection is likely due to a number of host or viral factors. For example, 25-30% of LTNPs have T cells expressing mutant forms of CCR5 or

CCR2, which are co-receptors for the HIV-1 virus (33, 69). In other studies, LTNP has been associated with superior anti-HIV immune responses such as robust helper T-cell and cytotoxic T-lymphocyte responses (65) and high levels of interferon producer cells or plasmacytoid dendritic cells (90, 91, 142). In addition, LTNP-status has been associated with HIV-1 strains containing defective genes, such as a defective *nef* gene, suggesting that these individuals were exposed to attenuated viruses unable to establish productive infections (85, 163).

There have been several reports correlating some instances of long-term nonprogression with defects in the Vif gene (1, 41, 67, 126). One such study involving the comparison of *vif* gene sequences from 42 LTNPs with 8 late progressors revealed a correlation between viral load within LTNPs and the amino acid at position 132 of the Vif protein (67). Some other studies have failed to find a correlation between Vif and LTNP status, but it should be noted that these studies tended to involve analysis of isolates from only a few individuals, whose LTNP status could be explained by other variables (141, 169).

VII. Evidence of an Endogenous Antiviral Factor Counteracted by Vif

Early studies revealed that the necessity of Vif is cell-type specific and that the "imprinting" of virions is determined by some unknown entity present in certain cell types, but not others. Primary CD4+ T cells and macrophages and T-cell lines such as H9, CEM and HUT78 are considered "nonpermissive" because Vif is required for the production of fully infectious virus from these cell-types (43, 51, 52, 143, 145, 155). However, several "permissive" cell lines, including SupT1, CEM-SS, and Jurkat T cells and HeLa, COS and HEK-293T cells, are able to sustain normal HIV replication even in

the absence of Vif. A similar permissive/nonpermissive cell dichotomy exists when SIVmac virus is used to infect the same cell types (171).

The existence of nonpermissive and permissive cell-types raised two possibilities for the Vif phenotype. Either permissive cells harbored a positive factor necessary for HIV replication that was lacking in nonpermissive cells and that Vif was capable of mimicking, or nonpermissive cells contained an inhibitory factor that Vif could counteract.

Another line of investigation also pointed to the existence of a Vif-sensitive cellular factor controlling the infectivity of HIV-1 virions. Viral complementation studies using HIV and simian immunodeficiency virus (SIV) Vif proteins produced in human or African green monkey (AGM) cells demonstrated that the interaction between Vif and a species-specific cellular factor--as opposed to a species-specific viral factor—governed the infectivity of these viruses (140). As expected, SIV_{AGM} Vif modulated the infectivity of SIV_{AGM} when both are produced in African Green Monkey Cells. However, SIV_{AGM} Vif had no effect on the same virus produced by human PBMCs (140). This result suggested that SIV_{AGM} Vif was interacting with a cellular factor. But again, whether the factor was positive or negative remained unclear. The AGM cells possibly contained a positive factor that cooperated with SIV_{AGM} Vif, or the human PBMCs contained a negative factor counteracted by SIV_{AGM} Vif.

The positive/negative dilemma was resolved in 1998, when two groups published the results of transient heterokaryon studies indicating the existence of a strong negative factor that inhibited the infectivity of HIV-1ΔVif virus but that was overcome by HIV-1 Vif. The elegant approach of Simon *et al* was to first express wt or Δ*vif* viral "cores"

(HIV-1Δ*env*) in CEM-SS (permissive) or HUT78 (nonpermissive) T cells(137). The core-producing T cells were mixed with Env/Vif or Env/ΔVif-expressing 293T cells; and heterokaryon formation was then encouraged by the interaction between CD4/CXCR4 on the T cells with the Env on the surface of the 293T cells. This strategy ensured that infectious particles could be produced only by the heterokaryons and not by either of the parental cell lines. They found that virions produced from the HUT78/293T heterokaryons in the absence of Vif were 10-fold less infectious than all of the other combinations tested, which included virions produced by CEM-SS/293T cells as well as virions produced in the presence of Vif either in the core-producing T cells or Env-expressing 293T cells. These results strongly indicated that a negative factor was present in the HUT78 cells and was acting to block the infectivity of HIV-1 ΔVif viruses. However, since it was also possible that a positive factor was being extinguished by the heterokaryon formation process, time-course experiments and experiments involving varying the ratios of parental cells were also performed. These additional experiments ruled out the possibility of a positive factor, and strengthened the case for the presence of a mystery endogenous HIV inhibitor that was counteracted by Vif. Madani and Kabat used a different strategy to make the heterokaryons, but came to a similar conclusion (95).

VIII. Identification of APOBEC3G

In a 2002 paper that launched a new direction in HIV biology, Sheehy *et al* reported that the identity of the mystery negative factor was APOBEC3G, a 47 kDa protein that had significant homology to the cytidine deaminase family of editing enzymes(133). They used a two-pronged strategy to identify the gene. First, they

isolated total mRNA from two genetically-related T cell lines, the parental CEM (nonpermissive) line and the CEM-SS line, a subclonal isolate that was permissive Vif. After a reverse transcription step to obtain cDNA, they identified cDNAs present in CEM cells but not in CEM-SS cells using subtractive PCR hybridization. In the next prong of the strategy, northern blots using each of the candidate cDNAs to probe total RNA from a panel of permissive and nonpermissive cell types revealed that one cDNA consistently hybridized with nonpermissive but not permissive cell types. This cDNA corresponded to the APOBEC3G gene. Subsequent testing of APOBEC3G revealed that it was ably incorporated into HIV-1Δ Vif virions and rendered them noninfectious. However, it had a minimal effect on HIV-1 wild-type virus, indicating that its action was counteracted by the HIV Vif protein.

IX. The Cytidine Deaminase Superfamily

A3G is a member of a larger family of cytidine deaminases (CyDa) that includes APOBEC1, 2, 3A-H, and activation-induced cytidine deaminase (AID). The CyDa class of proteins includes enzymes known to edit RNA or DNA. RNA editing through C-U and other means has also been observed in the mitochondrial RNA of trypanosomes (7), marsupial $tRNA^{gly}$(73), and in pre-mRNAs for several ion channels(101), neurotransmitter receptors (13), hepatitis-delta virus (55), and tRNA (82, 118).

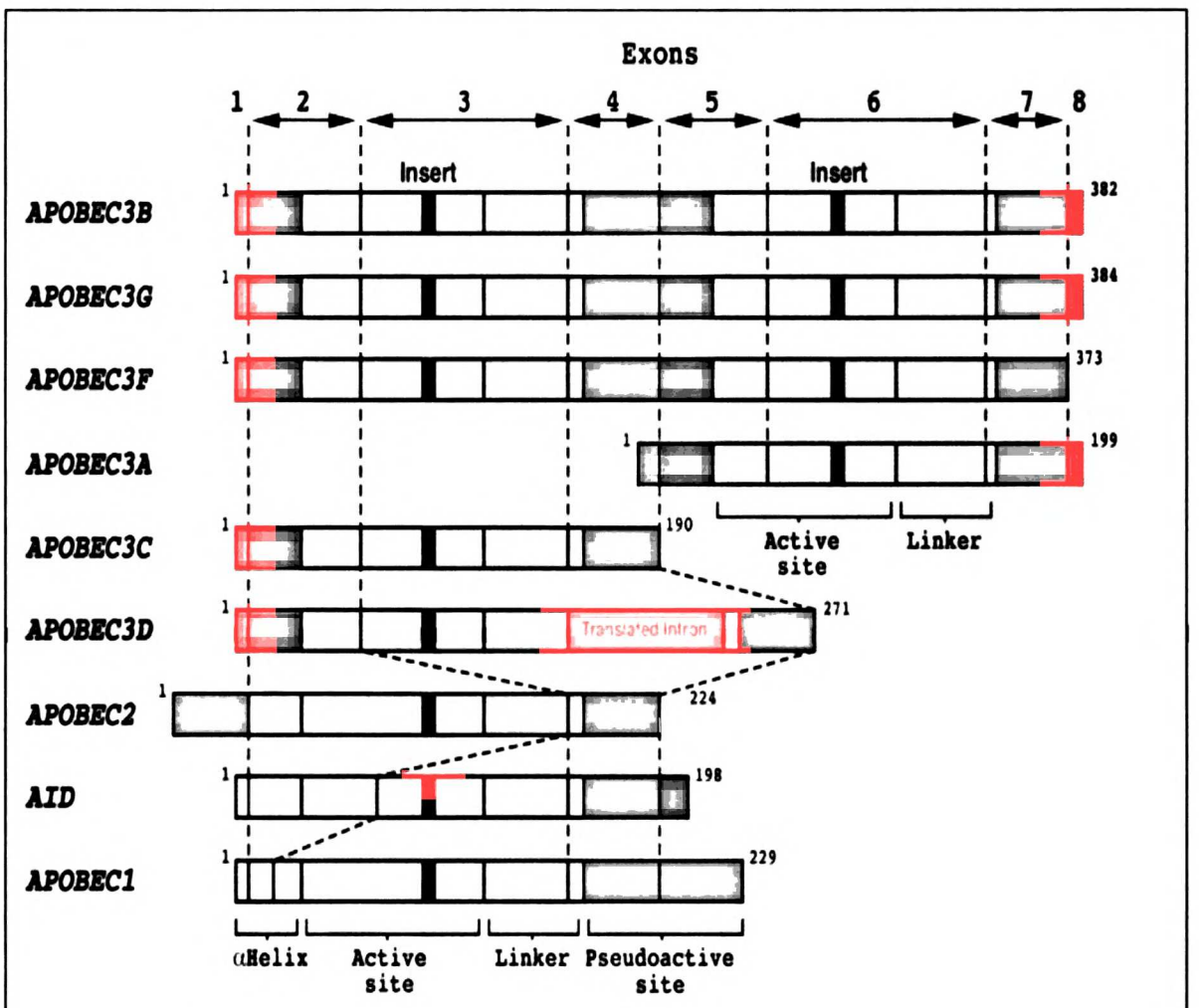

Fig. 4 Domain Organization of APOBEC3-family proteins.

APOBEC proteins contain similar domain organization consisting of active sites, pseudo-active sites and linker domains (74) (Fig. 4). The active sites contain a cytidine deaminase consensus sequence, His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys, containing zinc ligands and a glutamate residue thought to be involved in proton shuttling during the catalysis of the editing reaction (74).

In humans, the most highly-characterized C-U editing mechanism is mediated by APOBEC1 (apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1, A1), which promotes more efficient lipid metabolism by modulating the expression of apolipoprotein (apo) B in a tissue-specific manner (24, 120, 149). A1 inserts a premature stop codon in apoB mRNA by making a C-to-U edit at Cytidine^{6666}, resulting in a shortened form of apoB lipoprotein to be exclusively located in the small intestine, the site of A1 expression. The truncated apoB48 protein promotes lipid transport but avoids formation of low-density-lipoproteins (LDLs), which correlates with coronary heart disease.

Unlike A1, which edits RNA, AID edits DNA and catalyzes dC-to-dU deamination of immunoglobulin genes to induce somatic hypermutation and class-switch recombination in B cells (74, 104, 117).

The APOBEC3A-H family is expressed in a tandem array on chromosome 22, most likely as a result of expansion and tandem duplication with unequal crossover (74). Certain of the APOBEC3 proteins (A3B, A3G, and A3F) have a complete duplication of the active site, linker region, and part of the pseudoactive site, while others (A3A, A3C, A3D, and A3E) have only one of these regions and have been described as "half" genes. An addition hybrid double domain A3DE protein has also been identified.

The A3 proteins have been reported to perform a variety of functions. A3G, A3F, and to a lesser extent A3DE and A3B all exhibit anti-HIV activity (9, 32, 38, 68, 122, 157, 170). A3B and A3C, as well as A3G, inhibit SIV replication (164). A3A and A3B are reportedly powerful inhibitors of LTR-retrotransposition in human cells (10), while A3G is a powerful inhibitor of Alu retrotransposition (26). As such, these A3 proteins can be considered to be defenders of the genome against instability perpetrated by rogue retroelements.

X. Biological Functions of A3G

a. A3G's antiviral activity

Of all the A3 proteins, A3G has demonstrated the most potent anti-HIV activity; A3F, A3B, and A3DE also inhibit HIV replication but to a lesser extent (9, 32, 38, 68, 122, 157, 170). A3G disables a broad range of retroviruses, including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), murine leukemia virus (MLV), foamy virus, and HTLV-I, as well as hepatitis B virus, a pararetrovirus (34, 66, 87, 97, 98, 106, 123, 125, 127, 131, 133, 146, 168). Several of the other A3 proteins are also able to inhibit replication of other viruses. For example, A3B and A3C can inhibit SIV replication (164).

A3G is incorporated into newly-formed HIV-1-ΔVif virions, making it available to act in the subsequent target cell. This mechanism is Vif-sensitive, and wt HIV is able to replicate even in the presence of A3G in certain cell types. In addition to this producer-cell dependent mechanism, A3G can also act as a post-entry restriction factor. In resting CD4+ T cells, A3G is present in a low-molecular mass (LMM), active form

that is able to disable the reverse transcription of incoming virus (25). In activated T cells, A3G shifts into an inactive, high-molecular mass (HMM) complex, incapable of protecting cells from infection. Surprisingly, the post-entry restricting activity of A3G is successful even against wild-type virus. The lack of significant quantities of Vif in the virion, may prevent wild-type virus from counteracting the post-entry restricting action of A3G.

A3G has very strong cytidine deaminase activity. In target cells, APOBEC3G induces extensive dC to dU mutations in the viral minus (first cDNA) strand DNA during reverse transcription, resulting in massive dG to dA substitutions in the viral plus (genomic) strand (89, 97, 98, 168) (Fig. 5). A3G binds and edits single-stranded DNA, but not double-stranded DNA or DNA:RNA hybrids (165). The dC-to-dU changes appear with graded frequency in the 5'-3' direction, possibly due to differences in exposure time of ssDNA (165).

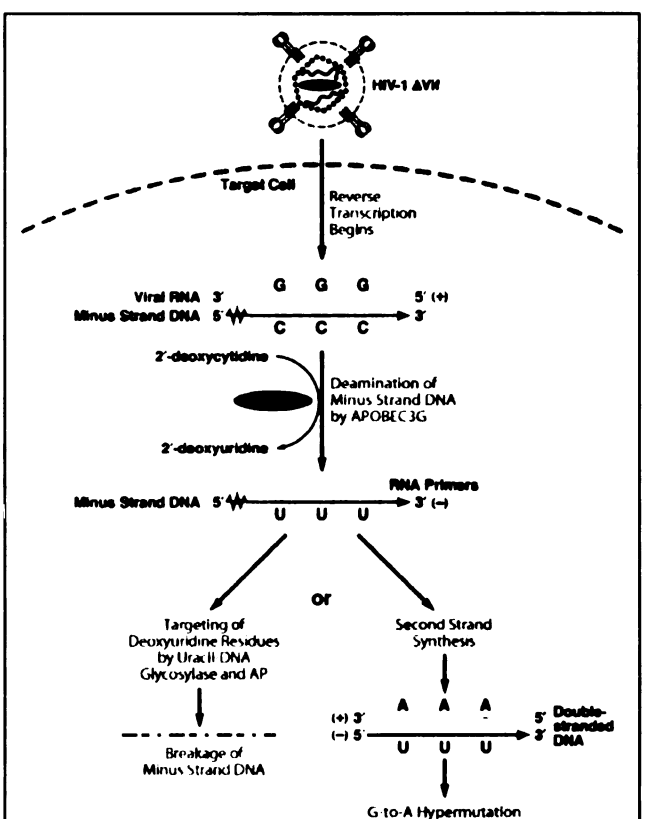

Fig. 5 Mechanism of Hypermutation by A3G.

b. A3G's anti-retroelement activity

The original biological function of A3G was likely not to combat modern primate lentiviruses, which are thought to have originated no more than a million years ago (132). Instead, there is evidence of a genetic conflict exerting positive selection pressure on the A3G gene (as well as A3E and A3DE) throughout the history of primate evolution, which dates back tens of millions of years (129).

Long interspersed nucleotide elements 1 (LINE1), also called "jumping genes", are the only autonomously active human mobile genetic elements (113). LINE1 elements can contribute to genetic diversity, but excessive insertional activity could also lead to genomic instability. Interestingly, the expansion of the A3 gene cluster (28, 74) correlated with a decline in retrotransposon activity in primates (96), suggesting a role for A3 proteins in protecting against genomic insults mediated by endogenous retroelements. Diverse A3 proteins are now known to inhibit the retrotransposition of LTR and non-LTR retroelements (10, 23, 26, 70, 103). Research performed in our laboratory indicates that activation of CD4+ T cells results in enhanced expression of nonautonomous retroelement RNAs (Alu and hY), and that HMM A3G protects against them by sequestering them from the LINE1 machinery required for retrotrasnposition(26).

c. A3G's possible contribution to viral diversity

While it is clear that A3G has powerful antiviral effects *in vitro* and *in vivo*, sub-optimal levels of A3G editing could actually be helpful to viral survival. HIV-1 sequences from infected patients often display dG-to-dA mutations at consensus target sequences for A3G and A3F, suggesting that both proteins contribute to viral diversification in patients (72, 84, 93, 153). Such dG-to-dA mutations in patients has also been correlated with sporadic but consistent inactivation of the viral *vif* gene (141),

indicating that suboptimal activity of Vif could lead to enhanced viral diversity. Total inactivation of Vif would likely not contribute to viral diversity, however, since the consequence would be completely defective virions, incapable of replicating. The influence of A3F and A3G is also suggested by the highly AT-rich nature of the HIV-1 genome, an indication that the two proteins have historically exerted evolutionary pressure on HIV and have shaped its overall sequence composition (165).

d. A3G's non-enzymatic activity

The massive hypermutation induced by A3G may not be the only cause of its antiviral activity. The introduction of stop-codons and other changes caused by hypermutation would likely prevent the production of viral proteins. However, it may not sufficiently explain the reduced levels of viral RT products, a hallmark of A3G action. A prevailing theory had been that the dC-to-dU changes in the viral minus-strand DNA rendered it vulnerable to cellular uracil DNA glycosidases such as UNG2, resulting in degradation of RT products. However, recent data demonstrating that UNG2 does not contribute to the A3G antiviral effect (77) casts doubt on that hypothesis.

While it is possible that hypermutation reduces viral RT products by some other mechanism, such as by interfering with base-pairing necessary for strand-transfer reactions, there is now growing support for the idea that a second, non-enzymatic activity contributes to the antiviral actions of A3F and A3G. The A3G present in resting CD4+ T cells can block reverse transcription in these cell types without causing substantial increases in dG-to-dA changes in viral cDNAs (25). In several cases, A3F and A3G appear to retain antiviral activity in the absence of any evidence of editing activity. A3F and A3G have duplicated cytidine deaminase-like domains; but the first, more N'

terminal domain does not catalyze editing reactions (8, 68, 107). However, both proteins retain antiviral activity when the editing activity of the C' terminal domain is abrogated (8, 107). Interestingly, A3G is more detrimentally affected than A3F by such changes, indicating that a non-enzymatic activity plays a greater role in the antiviral activity of A3F (68).

Furthermore, the activity of A3 proteins against viral species other than HIV does not always seem to require hypermutation. A3G and A3F can reportedly inhibit the replication of hepatitis B virus (HBV) with little-to-no evidence of dG-to-dA hypermutation although this varies depending on the cell-type tested (108, 123, 124, 146, 151). Similarly, LTR and non-LTR retroelements are reportedly inhibited by several APOBEC3 proteins by non-editing mechanisms (10, 23, 26, 70, 103).

Together, this data indicates that A3G can block reverse transcription by a mechanism unrelated to its capacity to edit genomic DNA. There is evidence that A3G-mediated inhibition of early viral cDNA synthesis is due to its inhibition of $tRNA_3^{Lys}$ priming, possibly due to decreased primer annealing to viral RNA (64). The reduction in late viral cDNA synthesis could likewise be due to reduced pirmer annealing during strand-transfer reactions or, perhaps A3G physically obstructs the processivity of RT.

XI. A3G and Vif

The identification of A3G in 2002 provided answers to many of the lingering questions that plagued a decade's worth of biochemical and genetic studies of the Vif protein. Work investigating the phenotype of Vif, its subcellular localization, virion

incorporation, and conserved sequences could all now be explained, at least in part, by A3G and by the mechanism used by Vif to defeat it.

Vif impairs the infectivity, but not the quantity, of virions released by virion-producer cells (29). Without Vif, the first round of viral production from nonpermissive cells is normal, but the viruses themselves are inherently defective and unable to establish proviral DNA in target cells (36, 46, 58, 105, 138, 143, 155). The defect is due to a flaw in the reverse transcription process, since both early and late RT products are diminished in target cells infected by Vif-defective virions and in ΔVif virions analyzed by exogenous RT assays (36, 39, 58, 138). RT assays using ΔVif virions supplemented with nucleotides and an exogenous template indicated that Vif has no effect on the activity of RT itself (36, 39, 58). Viral RNA incorporation and dimerization are similarly unaffected by the presence or absence of Vif (57, 155).

This information, along with the producer-cell dependence of the Vif phenotype, led many investigators to search for impairments earlier in the viral lifecycle; and a growing body of work began to appear in support of the idea that Vif promoted the proper assembly of the budding virion, ultimately affecting the stability of the viral core, with drastic—but unexplained—effects on reverse transcription. One line of evidence to support this were confocal microscopy and biochemical studies indicating that Vif colocalizes with Gag in the cytoplasm (6, 11, 135, 144), although conflicting reports make it unclear whether Vif and Gag directly bind to each other (71, 135). These studies indicated that Vif colocalized with P^{55} Gag precursor proteins and therefore may play a role in early events of viral assembly (6, 11, 135, 144). Vif, which is highly basic

(approx. pi 10.7), is also reportedly an RNA binding protein and thus might form a large ribonucleoprotein (RNP) complex with HIV genomic RNA (36, 83, 167).

Other studies indicated that Vif impairs the integrity of virion cores (12, 112, 138). Studies of detergent-permeabilized wt and ΔVif HIV virions indicated enhanced stability of wt virion cores compared to ΔVif (112). ΔVif virion cores were more easily disrupted by detergents, high salt, basic pH, S100 cytosol, dNTPs and RNase. Simlarly, electron micrographs indicated that ΔVif cores have an aberrant morphology compared to that of wt virus (12).

Taken together, this work appeared to be building a case for Vif interfering with the maturation of Gag with ultimate effects on the stability of the virion core and the integrity of the preintegration complex. Unfortunately, the validity of this hypothesis remains unclear, but unlikely, given the studies indicating that Vif has no effect on the integrity or modification of virion components including Gag. Extensive comparisons between wild-type and Vif-deficient viruses revealed that Vif has little effect on packaging, modification, processing or function of viral-associated proteins including Gag, Pol and Env (12, 46, 111, 134, 155). The hypothesis also falters to the extent it relies on Vif incorporation in the virion. There have been some reports of low to moderate amounts of Vif in HIV-1 virions and even an association of Vif with virion cores (78, 81, 83). However, other studies suggest that the quantity of Vif in the virion is negligible, at best, and not important for its function, especially since, unlike other virion-packaged viral proteins, Vif proteins are not specifically incorporated into virions (15) (139). Similarly, when highly-purified virions are obtained using iadixonal gradients, Vif

is virtually undetectable in virion preparations, suggesting that the virion preparations in previous studies were contaminated with Vif-containing microvesicles (37).

Of course, the biggest blow to the hypothesis that Vif's primary role is to promote Gag assembly came with the identification of A3G. The lack of appreciable quantities of Vif in the virion, combined with its cytoplasmic localization (57, 136, 167) and the producer-cell dependence of the Vif phenotype, all suggest the Vif acts primarily in the cytoplasm of producer cells. But its role in the cytoplasm is more likely to combat A3G, another cytoplasmic protein, rather than to play a role in Gag assembly. Why Vif might associate with Gag and RNA remains unclear but one explanation for the colocalization of Vif with Gag precursors and viral RNA is that viral RNA/Gag complexes provide a scaffold from which Vif could attack residual A3G in close proximity to the assembling virion.

The identification of A3G also provides an explanation for the minimal levels of Vif found in virions. As will be shown in this thesis, our work and that of others has shown that Vif targets A3G for degradation by the 26S proteasome, and does so in an almost absolute manner, thereby preventing A3G incorporation into virions. Given the efficiency with which Vif carries out this role in the cytoplasm, there may be no functional reason for Vif to be incorporated into virions.

The work presented in this thesis began in 2002, at the inception of the A3G field. Since then, a tidal wave of publications has dramatically transformed our understanding of A3G and the other A3 proteins and their relationship to retroviruses and other exogenous and endogenous species. Our work began with the small question of how the

HIV-1 Vif counteracts the activity of A3G (Chapter 2). We then shifted to designing a high-throughput screen for Vif inhibitors (Chapter 3). And we ended by studying the means by which A3G's expression and activity are regulated by cellular factors (Chapter 4). Together, this information has hopefully added to our understanding of A3G and Vif and may pave the way to future inquiries, especially those geared to the development of anti-HIV/AIDS therapies.

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Chapter 2

HIV-1 Vif causes the degradation of APOBEC3G via the 26S proteasome

Introduction

The HIV-1 Vif protein plays a major role in the HIV-1 lifecycle. Without Vif, the replication of HIV-1 and other primate lentiviruses is severely compromised in certain cell culture and animal model systems (1-3, 5-7). Although the importance of Vif for viral replication has been known for over a decade, the exact mechanism employed by Vif had long been a mystery. At the beginning of this work, it was known that Vif functions by counteracting the antiviral activity of A3G (4). However, the mechanism employed by Vif was not known.

The purpose of the work presented in this chapter was to study the mechanism employed by Vif to counteract A3G. To answer this question we examined (1) whether Vif interacts with A3G; (2) whether Vif prevents A3G from being incorporated into HIV-1 virions; (3) whether Vif affects the intracellular stability of A3G; and (4) if so, whether other viral proteins were also necessary for this process.

We used standard biochemical and viral-infection techniques to answer these questions. The work presented in the accompanying paper was performed by myself and Drs. Carlos de Noronha and Wes Yonemoto. As first author of this work, I designed and performed the experiment showing the interaction between HIV-1 Vif and A3G, the infection study demonstrating that HIV-1 Vif causes the degradation of endogenous A3G in infected T cells, the proteasome-inhibitor study and the virion incorporation study. Co-first author Dr. Carlos de Noronha preformed the *in vitro* translation study, the nuclear/cytoplasmic localization study, and the Northern analysis. Dr. Wes Yonemoto performed the pulse-chase study and several of the remaining western blot experiments. Dr. Warner Greene directed and supervised the entire project.

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HIV-1 Vif Blocks the Antiviral Activity of APOBEC3G by Impairing Both Its Translation and Intracellular Stability

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Summary

The human immunodeficiency virus type 1 (HIV-1) relies on Vif (viral infectivity factor) to overcome the potent antiviral function of APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G, also known as CEM15). Using an APOBEC3G-specific antiserum, we now show that Vif prevents virion incorporation of endogenous APOBEC3G by effectively depleting the intracellular levels of this enzyme in HIV-1-infected T cells. Vif achieves this depletion by both impairing the translation of APOBEC3G mRNA and accelerating the posttranslational degradation of the APOBEC3G protein by the 26S proteasome. Vif physically interacts with APOBEC3G, and expression of Vif alone in the absence of other HIV-1 proteins is sufficient to cause depletion of APOBEC3G. These findings highlight how the bimodal translational and posttranslational inhibitory effects of Vif on APOBEC3G combine to markedly suppress the expression of this potent antiviral enzyme in virally infected cells, thereby effectively curtailing the incorporation of APOBEC3G into newly formed HIV-1 virions.

Introduction

The function of HIV-1 Vif is to suppress a powerful antiviral host defense mechanism mediated by the DNA editing enzyme APOBEC3G. In the absence of Vif, the replicative capacity of HIV-1 and other primate lentiviruses is severely impaired in most cell culture (Fisher et al., 1987; Strebel et al., 1987; von Schwedler et al., 1993) and in vivo systems (DesRosiers et al., 1998).

Early studies demonstrated that HIV-1 requires Vif for effective viral spread in primary $CD4^+$ T cells, which are natural targets of HIV-1 infection. Similarly, some cell lines such as the H9 T cell line are also "nonpermissive" because Vif is required for the production of fully infectious virus from these cells. However, HIV-1 does not require Vif for viral spread in "permissive" T cell lines like SupT1 or Jurkat T cells (Gabuzda et al., 1992; Sova and Volsky, 1993).

Infectivity studies of virions produced by heterokaryons formed between permissive and nonpermissive T cell lines revealed that nonpermissive cells produce an inhibitory factor(s) that impairs the infectivity of HIV-1 virions (Madani and Kabat, 1998; Simon et al., 1998). These studies laid the groundwork for the seminal identification of this antiviral factor as APOBEC3G (CEM15) (Sheehy et al., 2002). APOBEC3G is homologous to the APOBEC1 RNA editing enzyme (Sheehy et al., 2002), and both APOBEC3G and APOBEC1 are able to mutate DNA (Harris et al., 2002). When APOBEC3G expression vector DNA is transfected into permissive cells, these cells are converted to the nonpermissive phenotype (Sheehy et al., 2002).

APOBEC3G is incorporated into newly formed virions making it available to act in the subsequent target cell (Sheehy et al., 2002). In these targets, APOBEC3G induces extensive dC to dU mutations in the viral minus (first cDNA) strand DNA during reverse transcription, resulting in G to A substitutions in the viral plus (genomic) strand (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003).

The mutations induced by APOBEC3G are so extensive that they effectively terminate the viral life cycle during or at some point after reverse transcription (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). Antiviral effects of APOBEC3G are not limited solely to HIV-1 but also extend to other retroviruses including murine leukemia virus (MLV), simian immunodeficiency virus, and equine infectious anemia virus (EIAV) (Harris et al., 2003; Mangeat et al., 2003). Of note, MLV and EIAV do not encode Vif or Vif-like proteins and thus may be particularly susceptible to the antiviral effects of APOBEC3G.

Although the DNA-hypermutating activity of APOBEC3G appears to explain its potent antiviral effects, little is known about how Vif overcomes the effects of APOBEC3G. Our studies have focused on this key question and demonstrate that Vif reduces the level of APOBEC3G protein in virally infected cells through inhibitory effects on both APOBEC3G translation and intracellular survival.

Results

Native APOBEC3G Is Selectively Expressed in Nonpermissive T Cells and Is Principally Localized in the Cytoplasm

In order to analyze the endogenous APOBEC3G protein, we developed a rabbit polyclonal antibody that specifically reacts with the C-terminal 16 amino acids of this ~46 kDa enzyme. Although APOBEC3G mRNA is expressed in nonpermissive but not in permissive cells (Sheehy et al., 2002), it is not known whether the corresponding APOBEC3G protein is stably expressed in nonpermissive cells. We assessed the expression pattern of APOBEC3G protein in nonpermissive and permissive cell lines by immunoblotting whole-cell lysates with the anti-APOBEC3G antibody (Figure 1A). In nonpermissive T cells including PHA-stimulated primary peripheral blood lymphocytes (PBLs) and H9 cells, the antibody reacted with a ~46 kDa protein (Figure 1A, lanes 2 and 3) that was not recognized by preimmune serum isolated from the same rabbit prior to immunization with C-ter-

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Figure 1. Expression and Localization of Endogenous APOBEC3G Protein

(A) Immunoblot analysis of APOBEC3G expression in nonpermissive and permissive cells. Cells were lysed and analyzed by SDS-PAGE and immunoblotting with anti-APOBEC3G antiserum, anti-tubulin, and anti-$p56^{lck}$. To confirm specificity of the antiserum, 293T cells were transfected with a HA-APOBEC3G expression vector. PBL, peripheral blood lymphocytes; +, PHA-stimulated cells; −, unstimulated cells.

(B) Subcellular localization of APOBEC3G. Nuclear and cytoplasmic fractions were prepared from H9 (nonpermissive) or SupT-1 (permissive) cells and analyzed by immunoblotting with antiserum reacting with the native APOBEC3G protein. Note that APOBEC3G is predominantly localized in the cytoplasm although small amounts of this protein are also present in the nucleus.

minal APOBEC3G peptide (data not shown). The ~46 kDa protein band was absent or barely detectable in lysates prepared from the permissive cell lines SupT-1, Jurkat, or 293T cells (Figure 1A, lanes 4–6). α-tubulin was expressed at similar levels in both the nonpermissive and permissive cell lysates confirming equal loading of cellular proteins. Interestingly, PHA stimulation of PBLs increased the expression of endogenous APOBEC3G (compare lanes 1 and 2) as well as of α-tubulin. Since α-tubulin was upregulated by PHA stimulation, we used $p56^{lck}$ as a loading control. Although $p56^{lck}$ was expressed at nearly equivalent levels in stimulated and unstimulated PBLs, APOBEC3G expression appeared to be moderately induced following mitogen activation.

To further confirm the specificity of the anti-APOBEC3G antibody, cellular lysates from 293T cells transfected with either control vector DNA (mock) or HA-APOBEC3G expression vector were immunoblotted with the anti-APOBEC3G antiserum. An ~50 kDa protein was detected in the lysate from the HA-APOBEC3G-transfected cells but was not detected in the lysate from mock-transfected 293T cells (Figure 1A, lanes 6 and 7). The decreased mobility of this protein band was consistent with the inclusion of a triple-HA epitope tag in the APOBEC3G cDNA. Together, these findings demonstrate that the rabbit anti-APOBEC3G antibody specifically reacts with the endogenous human APOBEC3G enzyme, confirm that the APOBEC3G protein is stably expressed in nonpermissive cells, and reveal that APOBEC3G enzyme levels are moderately upregulated after PHA stimulation of primary PBLs.

Next, we analyzed the subcellular localization of APOEC3G by immunoblotting nuclear and cytoplasmic fractions isolated from nonpermissive H9 cells and permissive SupT1 cells (Figure 1B). Tubulin and histone H3 were employed as cytoplasmic and nuclear markers, respectively, to monitor the purity of the subcellular fractions. Although endogenous APOBEC3G was not evident in permissive SupT1 cells, it was readily detected in the cytoplasm of nonpermissive H9 cells. In addition, small amounts of APOBEC3G were present in the nuclear lysate. Since expression of tubulin was exclusively restricted to the cytoplasmic fraction and histone H3 to the nuclear fraction, the small amount of nuclear APOBEC3G detected does not appear to reflect cross-contamination of the nuclear lysate with cytoplasmic proteins during the purification. Therefore, APOBEC3G is principally expressed in the cytoplasm although small amounts of APOBEC3G are also present in the nucleus.

Vif Prevents Incorporation of Endogenous APOBEC3G into HIV-1 Virions

Two reports have appeared that disagree as to whether Vif prevents APOBEC3G from being incorporated into virions (Mariani et al., 2003; Sheehy et al., 2002). Both groups produced virus from transiently transfected 293T cells to evaluate whether APOBEC3G is incorporated into virions. However, one group found that Vif had no effect on virion incorporation of APOBEC3G (Sheehy et al., 2002), while the second group reported that Vif excludes APOBEC3G from virions (Mariani et al., 2003). Because of the unnaturally high levels of APOBEC3G produced in transfected 293T cells, this system may not faithfully recapitulate events occurring with the endogenous APOBEC3G enzyme. Therefore, we examined the levels of native APOBEC3G in virions produced from

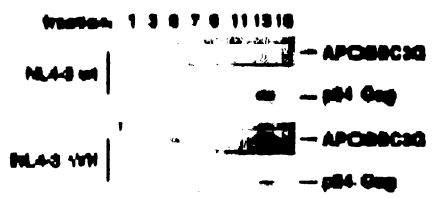

Figure 2. Vif Prevents Incorporation of Endogenous APOBEC3G into Virions

HIV-1 and HIV-1 ΔVif virions were prepared by ultracentrifugation fractionation of H9 cells with VSV-G pseudotyped HIV-1 or HIV-1 ΔVif viruses. After 48 hr, virus-containing supernatants were collected, and virions were purified on discontinuous step iodixanol gradients. Gradient fractions were immunoblotted with anti-p24 Gag antibody to identify virions (fraction 13 of both gradients). The gradients were also probed with anti-APOBEC3G. Note that APOBEC3G was present in HIV-1 ΔVif virions but was undetectable in HIV-1 wt virions.

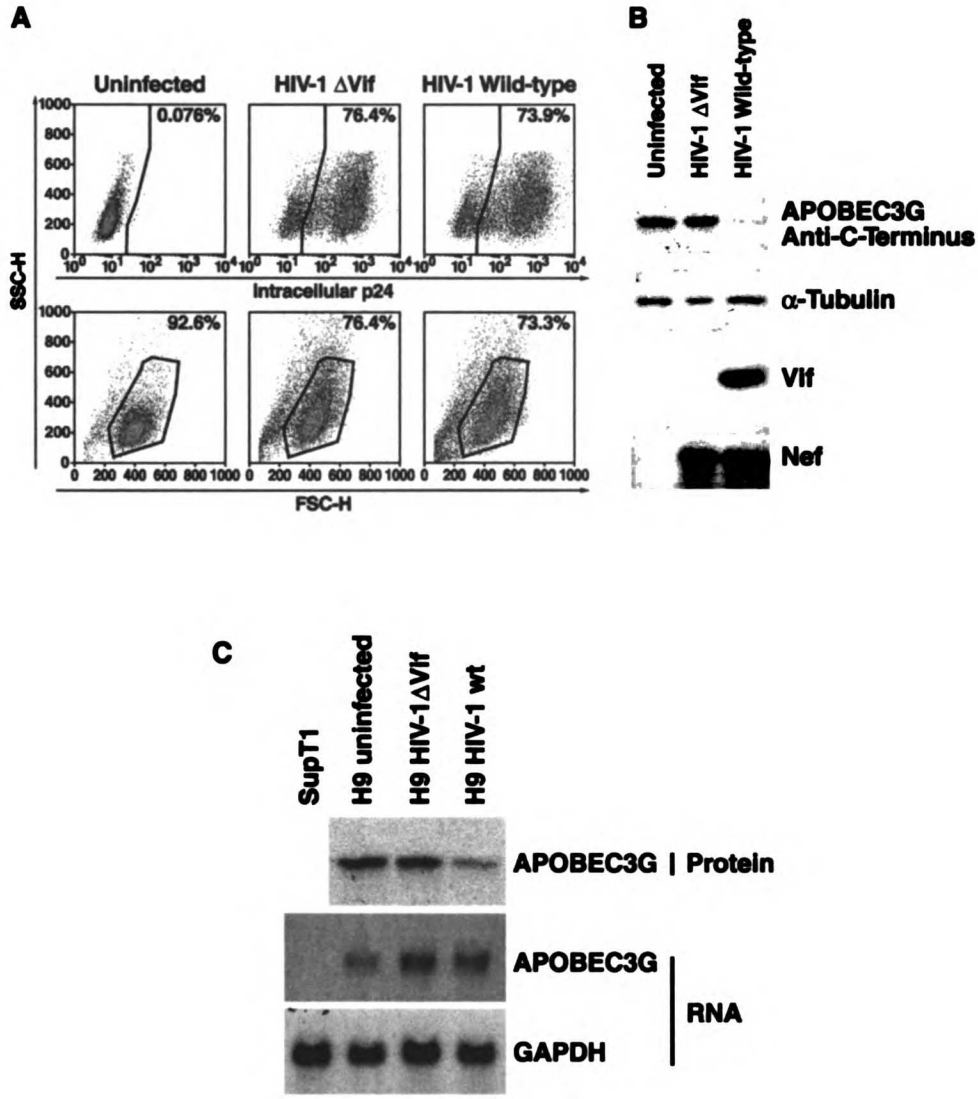

Figure 3. Endogenous APOBEC3G Is Nearly Eliminated from H9 Cells Infected with HIV-1-wt but Not from Cells Infected with HIV-1 ΔVif

(A) H9 cells were infected with VSV-G pseudotyped HIV-1-wt or HIV-1 ΔVif virus by spinoculation and analyzed by flow cytometry using intracellular Gag expression to monitor the extent of viral infection and using forward and side scattering of light (FSC/SSC) to identify live cells. H9 cells infected with HIV-1 wt and HIV-1 ΔVif were comparably infected and displayed equivalent numbers of cells in the live cell gate.

(B) Analysis of endogenous APOBEC3G expression in uninfected H9 cells or H9 cells infected with HIV-1 wt and HIV-1 ΔVif viruses. Cellular lysates from the cultures in (A) were immunoblotted with anti-C-terminal APOBEC3G antiserum and with anti-α-tubulin, anti-Vif, or anti-Nef antibodies. The bands were quantified using Scion Image software (version 1.62), which showed a sharp decline in the intensity of the HIV-1 wt-infected sample (0.24) when compared to that of the HIV-1 ΔVif (1.0).

(C) Vif does not alter APOBEC3G mRNA expression or integrity. Total RNA was extracted from lysates of H9 cells that were either uninfected or infected with VSV-G pseudotyped HIV-1 or HIV-1 ΔVif viruses and then subjected to Northern blot analysis using a ^{32}P-labeled APOBEC3G cDNA probe. Note that APOBEC3G protein expression is decreased in H9 cells infected with HIV-1 wt, but the level of APOBEC3G mRNA in these same cells is equivalent to that in cells infected with HIV-1 ΔVif. Radiolabeled GAPDH probes were used as a control for mRNA loading and integrity.

infected H9 T cells. These cells were infected with vesicular stomatitis protein G (VSV-G) pseudotyped HIV-1_{NL4-3} wt or VSV-G-HIV-1_{NL4-3}-ΔVif viruses. To isolate highly purified virions, supernatants from the infected H9 cells were pelleted through a 5% iodixanol cushion and then applied to discontinuous iodixanol gradients. Sequential fractions from the gradients were analyzed for p24-Gag content as a marker of HIV-1 wt and Δvif virions (Figure 2, fraction 13). Immunoblotting of these fractions with the APOBEC3G-specific antiserum revealed that endogenous APOBEC3G is incorporated in HIV-1 ΔVif virions (Figure 2, fraction 13, third panel). Conversely, no full-length APOBEC3G protein was detected in the HIV-1 wt virions (Figure 2, fraction 13, top panel). Small quantities of APOBEC3G were detected in fractions that did not contain p24-Gag (fractions 3 through 7), likely corresponding to APOBEC3G residing in microvesicles. However, these contaminants were well separated from the virion fractions. These findings demonstrate that the presence of Vif in infected T cells prevents the native

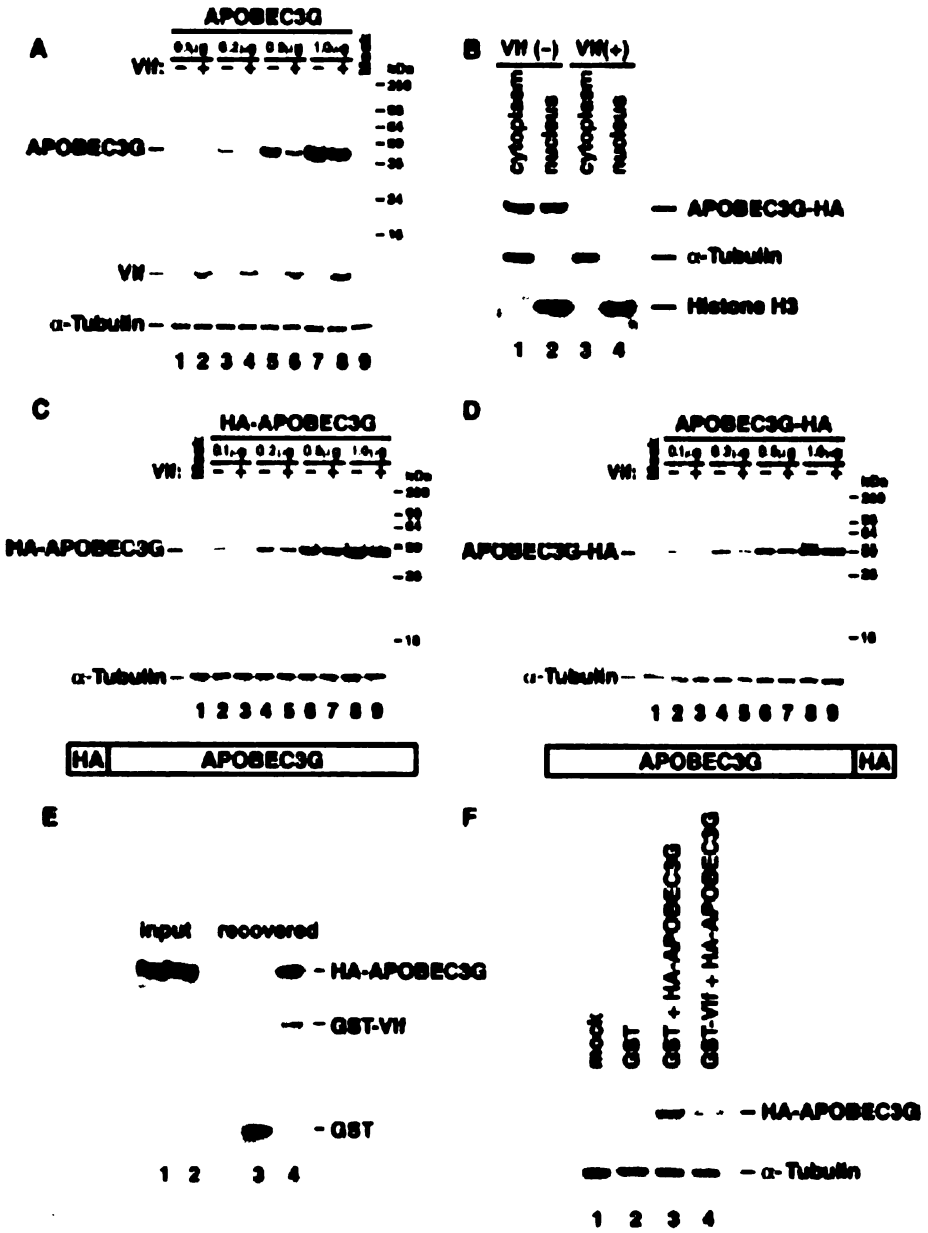

Figure 4. Vif Does Not Require the Participation of Other Viral Proteins to Eliminate Intracellular APOBEC3G, the Disappearance of APOBEC3G Occurs at Both Ends of the Protein and Is Not Due to Its Translocation into the Nucleus, and Vif Physically Interacts with APOBEC3G

(A) Vif alone is sufficient to deplete APOBEC3G from transfected 293T cells. 293T cells were transfected with graded amounts of APOBEC3G expression vector DNA (0.1–1 μg) in the presence or absence of a constant amount (2 μg) of Vif expression vector. Cellular lysates from these cultures were immunoblotted with anti-APOBEC3G antiserum. Vif alone induced a decline in APOBEC3G expression over the range of APOBEC3G inputs, although the effect is most pronounced at the lower levels of APOBEC3G expression.

(B) Nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic proteins were purified from transfected 293T cells and probed with anti-HA (to detect APOBEC3G-HA), anti-tubulin, or anti-histone H3 antibodies. Note that Vif induced APOBEC3G depletion in both the nuclear and cytoplasmic fractions.

(C and D) Vif promotes degradation or extensive proteolysis of APOBEC3G as reflected by the loss of both N- and C-terminal immunoreactive epitopes. The experiment in (A) was repeated using N- or C-terminally HA-tagged APOBEC3G. Cellular lysates were analyzed by immunoblotting with anti-HA antibodies. Vif expression induced a loss of anti-HA immunoreactivity regardless of whether the HA epitope was positioned at the N or C terminus of APOBEC3G.

(E) APOBEC3G interacts with GST-Vif. Lysates from 293T cells transfected with HA-APOBEC3G expression vector and either GST or GST-Vif

APOBEC3G enzyme from being encapsidated into newly formed virions.

Infection of Nonpermissive T Cells with HIV-1-Expressing Vif Results in the Apparently Complete Depletion of Intracellular APOBEC3G

To further elucidate the mechanism by which Vif prevents the incorporation of APOBEC3G into HIV-1 virions, we analyzed the intracellular expression of the endogenous APOBEC3G enzyme in H9 T cells 2 days after infection with either HIV-1 wt or HIV-1 ΔVif. For these studies, we employed H9 cell cultures displaying comparable levels of viral infection (76.4% for HIV-1 ΔVif versus 73.9% for HIV-1 wt assessed by intracellular anti-p24-Gag immunostaining) (Figure 3A, top panel) and equivalent viability as assessed by forward and side light scattering (Figure 3A, bottom panel). Equivalent amounts of endogenous APOBEC3G were detected in uninfected H9 cells and H9 cells infected with HIV-1 ΔVif (Figure 3B, top panel). However, APOBEC3G expression was markedly reduced in H9 cells infected with HIV-1 wt. The intensity of the APOBEC3G band from the HIV-1 wt infected sample was 24% of that from the uninfected sample. Considering that approximately 26% of the cells present in the HIV-1 wt infected sample were uninfected (p24-Gag-negative), the small amount of APOBEC3G present in the HIV-1 wt sample likely derives from the uninfected cells. Therefore, a likely interpretation of this data is that the endogenous APOBEC3G is nearly completely depleted in the virally infected subset of cells. This conclusion is further supported by the absence of detectable APOBEC3G encapsidation into virions produced by H9 T cells infected with HIV-1 wt viruses (Figure 2).

α-tubulin levels were comparable in each of the cell lysates, indicating equal loading of lysate proteins (Figure 3B). Moreover, Vif expression was observed in the H9 cells infected with HIV-1 wt but not in the cells infected with HIV-1 ΔVif, and equivalent levels of Nef were detected in both of the virally infected samples, further demonstrating comparable infection of these cultures (Figure 3B). Accordingly, infection of nonpermissive cells with HIV-1-expressing Vif leads to the apparently complete depletion of native APOBEC3G protein from the infected subset of cells.

APOBEC3G mRNA Levels Are Not Altered following Infection of Nonpermissive Cells with HIV-1-Expressing Vif

To further dissect the mechanism underlying the intracellular depletion of APOBEC3G in HIV-1 wt-infected H9 cells, we considered the possibility that Vif alters the expression or integrity of APOBEC3G mRNA. RNA and protein were simultaneously prepared from uninfected and infected nonpermissive H9 T cells, and the levels of APOBEC3G mRNA and protein were assessed (Figure 3C). As expected, APOBEC3G protein expression was decreased in the H9 cells infected with HIV-1 wt in comparison to the HIV-1 ΔVif-infected samples. Quantification of these immunoblots revealed that the band corresponding to APOBEC3G in the HIV-1 wt sample was 48% of the intensity of the APOBEC3G bands in the uninfected and HIV-1 ΔVif-infected samples (data not shown). Since intracellular p24-Gag immunostaining revealed that 47% of the H9 cells were infected with HIV-1 wt in this experiment (data not shown), we again conclude that Vif induced complete or almost complete disappearance of APOBEC3G within the infected subset of cells. Despite these changes in APOBEC3G protein expression, there was no apparent difference in the level of expression of APOBEC3G mRNA among the three samples (Figure 3C). Equivalent RNA loading was confirmed by hybridization with a radiolabeled DNA probe specific for GAPDH, and RNA extracted from the permissive cell line SupT1 was used as a negative control (Figure 3C). These findings indicate that Vif does not impair APOBEC3G protein expression by interfering with the production or stability of APOBEC3G mRNA.

Vif Alone Is Sufficient to Deplete Intracellular APOBEC3G

We next examined whether Vif requires the participation of other viral components to cause the depletion of intracellular APOBEC3G. 293T cells were transiently transfected with increasing doses of untagged APOBEC3G expression plasmid DNA (0.1–1.0 μg) in the presence of a fixed amount of Vif expression vector DNA (2 μg) (Figure 4A). The expression of Vif alone reduced the intracellular levels of the APOBEC3G protein (Figure 4A, lanes 5 and 6), although this effect was less apparent as APOBEC3G input levels increased. Vif expression was comparable in each of the cellular lysates transfected with the Vif expression plasmid. Since these studies were performed with cytoplasmic extracts, we considered the possibility that the Vif-induced depletion of APOBEC3G was caused by translocation of the APOBEC3G enzyme into the nucleus. However, analysis of subcellular fractions revealed that Vif virtually eliminated APOBEC3G from both the cytoplasm and the nucleus (Figure 4B). In this experiment, we observed more APOBEC3G in the nucleus than we did in the previous experiment (Figure 1B). The increase in nuclear APOBEC3G is likely due to protein overexpression as a consequence of transient transfection. These results indicate that Vif does not require participation of other viral components to impair APOBEC3G protein expression. In addition, the observed depletion is not due to translocation of cytoplasmic APOBEC3G into the nuclear compartment.

Vif-Induced Depletion of APOBEC3G Involves Disappearance of the Entire Protein

Next, we investigated whether the apparent loss of APOBEC3G in Vif-expressing cells could be explained by

expression vectors were incubated with glutathione-Sepharose beads. Immunoblotting with anti-HA antiserum revealed that HA-APOBEC3G was precipitated by GST-Vif but not GST.

(F) GST-Vif is biologically active. Lysates from 293T cells cotransfected with HA-APOBEC3G expression plasmid and either GST or GST-Vif expression plasmid were analyzed by immunoblotting. Expression of GST-Vif but not GST induced a decline in intracellular HA-APOBEC3G levels.

proteolytic clipping of the C terminus, which would result in a loss of the epitope used for immunodetection. When 293T cells were cotransfected with graded doses of HA-APOBEC3G or APOBEC3G-HA expression vector DNA in the presence or absence of Vif expression plasmid DNA, both the N- and C-terminally tagged versions of the APOBEC3G enzyme were diminished in the presence of Vif (Figures 4C and 4D). These findings indicate that Vif targets the entire APOBEC3G protein for elimination rather than inducing proteolysis only at the C terminus.

Vif Physically Assembles with APOBEC3G

To evaluate whether HIV-1 Vif physically interacts with APOBEC3G, 293T cells were cotransfected with HA-APOBEC3G and GST-Vif or control GST expression plasmid DNA (Figure 4E). Cellular lysates were prepared and incubated with glutathione–Sepharose beads. Immunoblotting of proteins bound to these beads demonstrated recovery of HA-APOBEC3G by GST-Vif but not by GST (Figure 4E). To assess whether the GST-Vif protein retained biological activity, we monitored HA-APOBEC3G levels in the presence and absence of GST-Vif and observed lower levels of this enzyme in the presence of GST-Vif (Figure 4F). These findings indicate that Vif is able to associate with the APOBEC3G protein in living cells and further that the chimeric GST-Vif protein retains the ability to impair intracellular APOBEC3G expression.

Vif Shortens the In Vivo Half-Life of APOBEC3G

We hypothesized that Vif might deplete the intracellular levels of APOBEC3G by accelerating its degradation. To investigate this possibility, [^{35}S]methionine/cysteine pulse-chase radiolabeling studies were performed in HEK293 cells cotransfected with APOBEC3G-HA and pNL-A1 (which expresses wild-type Vif) or pNL-A1-ΔVif. After incubation in starvation medium followed by a 30 min pulse, the cultures were chased for up to 7 hr in media containing excess unlabeled methionine and cysteine. APOBEC3G-HA was then immunoprecipitated from lysates of cells harvested at different intervals (Figure 5A). In the absence of Vif, the radiolabeled APOBEC3G protein was relatively stable, and about 80% of the protein was present even after 7 hr of chase. However, in the presence of Vif, the radiolabeled APOBEC3G disappeared more rapidly, displaying a $T_{1/2}$ that consistently was less than 2 hr (Figure 5A). These findings demonstrate that Vif shortens the half-life of APOBEC3G.

Proteasome Inhibitors Partially Block Vif-Mediated Depletion of APOBEC3G

We hypothesized that the 26S proteasome might mediate the Vif-induced degradation of APOBEC3G. To explore this possibility, H9 cells were infected with HIV-1 wt or were mock infected. Forty-four hours after infection, the cells were treated for 16 hr with either DMSO (0.1%) or 0.25 μM epoxomicin, a specific inhibitor of three proteolytic activities in the proteasome. Whole-cell lysates were subsequently prepared by boiling the cells in Laemmli lysis buffer, followed by protein separation by SDS-PAGE and immunoblotting. As expected, APOBEC3G levels were much lower in the infected sample when compared to the uninfected sample (Figure 5B, lanes 1 and 3). However, APOBEC3G levels were elevated in infected cells treated with epoxomicin when compared to DMSO-treated cells (Figure 5B, lanes 1 and 2), while epoxomicin did not appear to cause a similar increase in the level of APOBEC3G in the uninfected cultures (Figure 5B, lanes 3 and 4). Interestingly, epoxomicin also caused an increase in Vif levels. We then performed a similar experiment using 293T cells transiently transfected with an APOBEC3G-HA expression vector and either a Vif expression vector or a control vector. The cells were then treated with a panel of different proteasome inhibitors for 16 hr. We observed that the proteasome inhibitors MG132 (12.5 μM) and ALLN (25 μM) as well as the more specific proteasome inhibitor clasto-lactacystin β-lactone (10 μM, 20 μM) enhanced the levels of APOBEC3G-HA in the presence of Vif when compared to the diluent control sample (0.1% DMSO) (Figure 5C, lanes 7–12). In contrast, calpain inhibitor III (50 μM), which inhibits a separate proteolytic pathway (Figure 5C, lane 12), did not cause an increase in APOBEC3G levels. We also observed that proteasome inhibitors enhanced APOBEC3G levels (including a lower molecular weight APOBEC3G band) even in the absence of Vif (Figure 5C, lanes 2–6). Taken together, our results indicate that the Vif-mediated depletion of APOBEC3G is at least partially mediated by the proteasome, or, alternatively, that Vif accelerates the natural turnover of APOBEC3G by the proteasome.

Vif Also Impairs Translation of APOBEC3G mRNA

In the course of the in vivo pulse-chase radiolabeling experiments, we observed that 15%–40% less radiolabeled APOBEC3G was recovered after the pulse-labeling phase in cells expressing Vif as compared to cells not expressing Vif. (This finding was not presented in the graph in Figure 5A since the time = 0 samples were normalized for better comparison of the relative degradation rates.) These results raised the possibility that Vif might inhibit APOBEC3G expression by impairing the translation of APOBEC3G mRNA. To further investigate this possibility, we performed shorter pulse-labeling experiments (15 min) in the presence of increasing amounts of Vif (0–8 μg) (Figure 6A, left panel). These studies revealed that Vif impaired [^{35}S]methionine/cysteine radiolabeling of APOBEC3G-HA in vivo in a dose-related manner, consistently causing a 30%–60% decline in APOBEC3G translation during the pulse-labeling period. Immunoblotting analysis of the same samples revealed an 80% decline in the steady-state levels of APOBEC3G-HA (Figure 6A, right panel). Since a 30%–50% decline in translation does not fully account for the 80% decline in the steady-state levels of APOBEC3G-HA, Vif must function both by partially blocking the synthesis of the APOBEC3G protein and by inducing APOBEC3G degradation after translation.

To further examine the effects of Vif on APOBEC3G mRNA translation, we employed an in vitro–coupled transcription/translation system. Rabbit reticulocyte lysates were programmed with APOBEC3G DNA in the presence of pretranslated Vif or GST control protein (Figure 6B, left panel). In this in vitro system, Vif impaired APOBEC3G translation by approximately 70%–75%. To confirm that the effect was due to a translational impair-

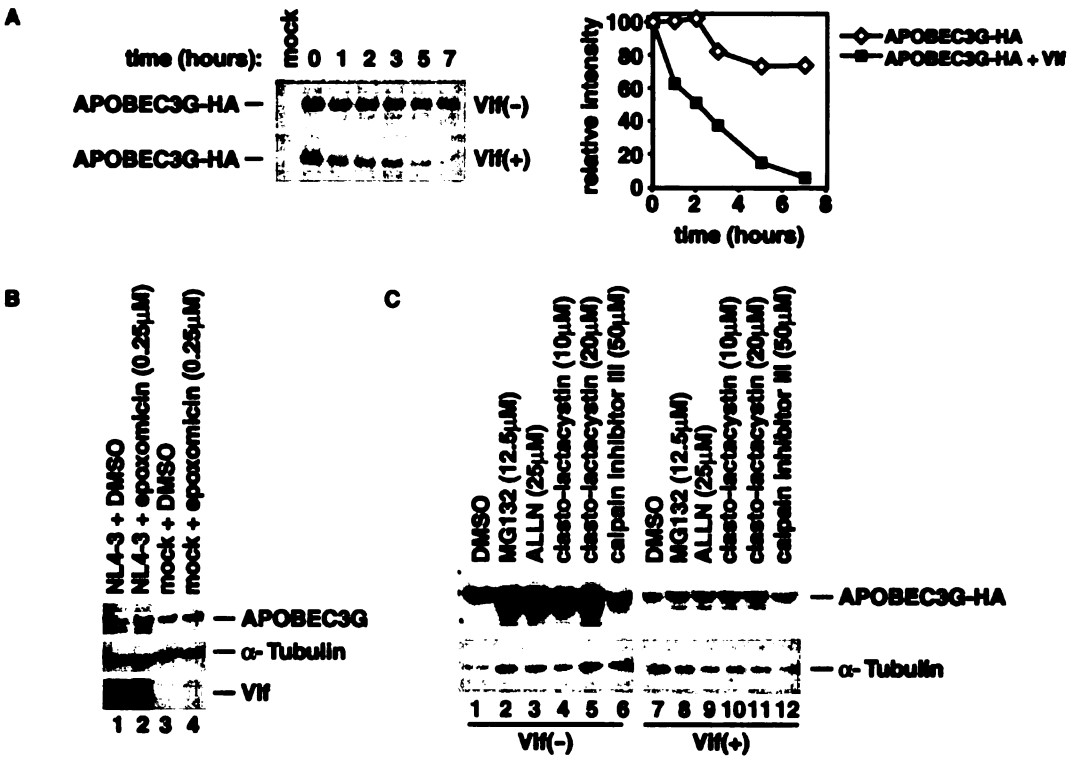

Figure 5. Vif Shortens the Half-Life of APOBEC3G, and Proteasome Inhibitors Partially Block the Intracellular Depletion of APOBEC3G-HA

(A) Pulse-chase radiolabeling. HEK293 cells transiently transfected with APOBEC3G-HA and pNL-A1 or pNL-A1ΔVif DNA were pulse-labeled and then incubated with chase media for the indicated time points (left panel). After immunoprecipitation with anti-HA antibody, the proteins were separated by SDS-PAGE, and the gels were subjected to autoradiography. The relative intensity of the bands was quantified, and the Vif (+) and Vif (−) samples were normalized to 100 and graphed (Figure 5, right panel).

(B) Proteasome inhibitor studies using infected H9 cells. Mock or HIV-1 wt-infected H9 cells were treated with epoxomicin (0.25 μM) or DMSO (0.1%) 44 hr after infection. Whole-cell lysates were prepared by boiling the cells in Laemmli lysis buffer. Note that epoxomicin caused an increase in endogenous APOBEC3G levels in the infected sample (compare lane 2 with lane 1).

(C) Proteasome inhibitor studies using transiently transfected 293T cells. After transfection of 293T cells with 0.1 μg APOBEC3G and 2 μg Vif or control vector DNA, the cells were treated with the indicated concentrations of inhibitor for 15 hr. APOBEC3G-HA levels (including a minor molecular weight band derived from APOBEC3G) were elevated in the presence of the proteasome inhibitors MG132, ALLN, and clasto-lactacystin β-lactone (lanes 8–11), but not in the presence of calpain inhibitor III (lane 12).

ment, we performed a similar experiment in the presence or absence of 5 μM epoxomicin, a potent proteasome inhibitor (Figure 6B, right panel). Although epoxomicin did slightly increase APOBEC3G levels in both samples, the Vif-mediated translation inhibition was not diminished.

Together, these results demonstrate that Vif depletes intracellular APOBEC3G in a bimodal manner by both inhibiting translation of APOBEC3G mRNA and by accelerating proteasome-mediated degradation of the APOBEC3G enzyme. In combination, these two mechanisms cause the essentially complete elimination of the APOBEC3G enzyme from infected T cells.

Discussion

APOBEC3G mediates extensive hypermutation of viral DNA during reverse transcription but has no apparent effect on the full-length genomic RNA packaged into the virion (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). It follows that APOBEC3G must be incorporated into the virion in order to mutate DNA in the subsequent target cell during viral DNA synthesis. Our results demonstrate that HIV-1 Vif effectively prevents native full-length APOBEC3G from being incorporated into HIV-1 virions. These results agree with the recently published work of Mariani and colleagues (Mariani et al., 2003) who made similar observations in transiently transfected cultures.

Our studies are also most consistent with the conclusion that Vif prevents virion incorporation of endogenous APOBEC3G by causing the near-complete depletion of the intracellular levels of this enzyme in HIV-1-infected T cells. We suggest that the dramatic reduction in the native APOBEC3G levels that occurs in HIV-1-infected T cells lies at the heart of Vif action and explains why APOBEC3G is not encapsidated in progeny HIV-1 virions. This conclusion is further supported by our finding that the intracellular levels of APOBEC3G are not altered in cells comparably infected with HIV-1 ΔVif viruses and that the virions derived from these cells contain readily detectable APOBEC3G.

Mariani et al. observed only a modest decrease in cellular APOBEC3G levels in the presence of Vif. We also found that in transiently transfected cells, moderate expression of APOBEC3G can diminish the Vif effect.

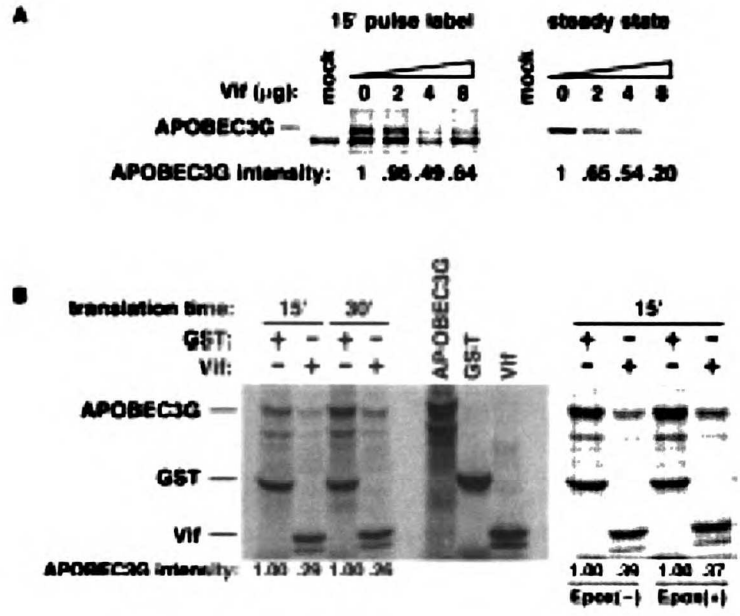

Figure 6. Vif Impairs the Translation of APOBEC3G-HA mRNA

(A) In vivo pulse label studies. HEK293 cells transiently transfected with 1 μg of APOBEC3G-HA expression vector DNA and 0–8 μg Vif expression vector DNA were pulsed with radiolabeled methionine and cysteine for 15 min and then immediately harvested. After immunoprecipitation with anti-HA antibody, the samples were either (I) subjected to SDS-PAGE and analyzed by autoradiography (left panel) or (II) subjected to SDS-PAGE and analyzed by immunoblotting (right panel). Higher concentrations of Vif caused a significant (20%–60%) impairment in the in vivo translation of APOBEC3G-HA (left panel). Vif caused an even greater (80%) reduction in the steady-state levels of APOBEC3G-HA (right panel).

(B) In vitro transcription/translation studies. Vif or control GST protein was initially transcribed and translated for 90 min using the Promega TNT T7-Coupled Reticulocyte Lysate System according to the manufacturer's instructions. The preysynthesized Vif or GST was then added to a new transcription/translation mix programmed with APOBEC3G DNA on ice in the presence or absence of 5 μM epoxomicin. The reactions were allowed to proceed for 15 or 30 min. The proteins were then separated by SDS-PAGE, and the gel was analyzed by autoradiography. The relative intensity of the bands was quantified, and all of the Vif(−) samples were normalized to 1. Note that Vif produced an ~70%–75% impairment in the in vitro synthesis of APOBEC3G (left panel) even in the presence of epoxomicin (right panel).

However, at lower doses of APOBEC3G, Vif caused a marked reduction in the intracellular levels of APOBEC3G even in this system. Mariani and colleagues have suggested that Vif assembly with APOBEC3G somehow prevents the incorporation of this enzyme into the virion (Mariani et al., 2003). We similarly find that Vif physically assembles with APOBEC3G. However, our studies reveal that the biological consequences of the Vif-APOBEC3G interaction are not simply to block virion incorporation. Rather, we find that Vif targets the APOBEC3G enzyme for essentially complete elimination in HIV-1-infected T cells. We also show that Vif does not require the participation of other viral components to reduce the levels of APOBEC3G.

Intriguingly, our studies indicate that Vif-mediated depletion of intracellular APOBEC3G involves bimodal inhibitory effects with Vif both triggering the accelerated degradation of the APOBEC3G protein and impairing the translation of APOBEC3G mRNA (summarized in Figure 7). Pulse-chase radiolabeling studies revealed that Vif markedly shortens the half-life of APOBEC3G, which is relatively stable in the absence of Vif. Our pulse-chase results differ from those of Mariani et al. One potential explanation is that we employed different transfection conditions than Mariani et al., who used a viral plasmid-to-APOBEC3G plasmid ratio of 2:1. Since we had previously observed that the levels of APOBEC3G and Vif achieved under these conditions resulted in negligible Vif-induced depletion of APOBEC3G and that the same 2:1 ratio of plasmids in the studies of Mariani et al. similarly did not rescue full viral infectivity, we performed additional experiments in which the Vif-to-APOBEC3G plasmid ratio was increased. Under these conditions, we consistently observed that Vif markedly shortened the intracellular half-life of APOBEC3G.

Our studies further support the notion that Vif-induced degradation of APOBEC3G involves the proteasome since addition of epoxomicin, a drug that specifically inhibits each of the three proteolytic activities present in the 26S proteasome, partially blocks the ability of Vif to deplete APOBEC3G in the cell. Since we also observed some effect of proteasome inhibitors on APOBEC3G levels in the absence of Vif, it is also possible that Vif accelerates the normal turnover of APOBEC3G by the 26S proteasome. Such targeted degradation of a host factor by a viral protein is not without precedent. For example, both the STAT1 transcription factor and the Rb tumor suppressor are targeted for proteasome degradation by the simian virus 5 V protein and human papilloma virus (HPV) E7 proteins respectively (Boyer et al., 1996; Didcock et al., 1999).

However, Vif-induced degradation of APOBEC3G by the proteasome does not appear to be the sole mechanism by which Vif depletes APOBEC3G in the infected host cell. Pulse-labeling studies and in vitro translation experiments support an additional inhibitory effect of Vif occurring at the level of APOBEC3G mRNA translation. In the in vitro translation system, Vif produced up to a 70%–75% reduction in APOBEC3G mRNA translation while in the pulse-labeling experiment, a 30%–40% in-

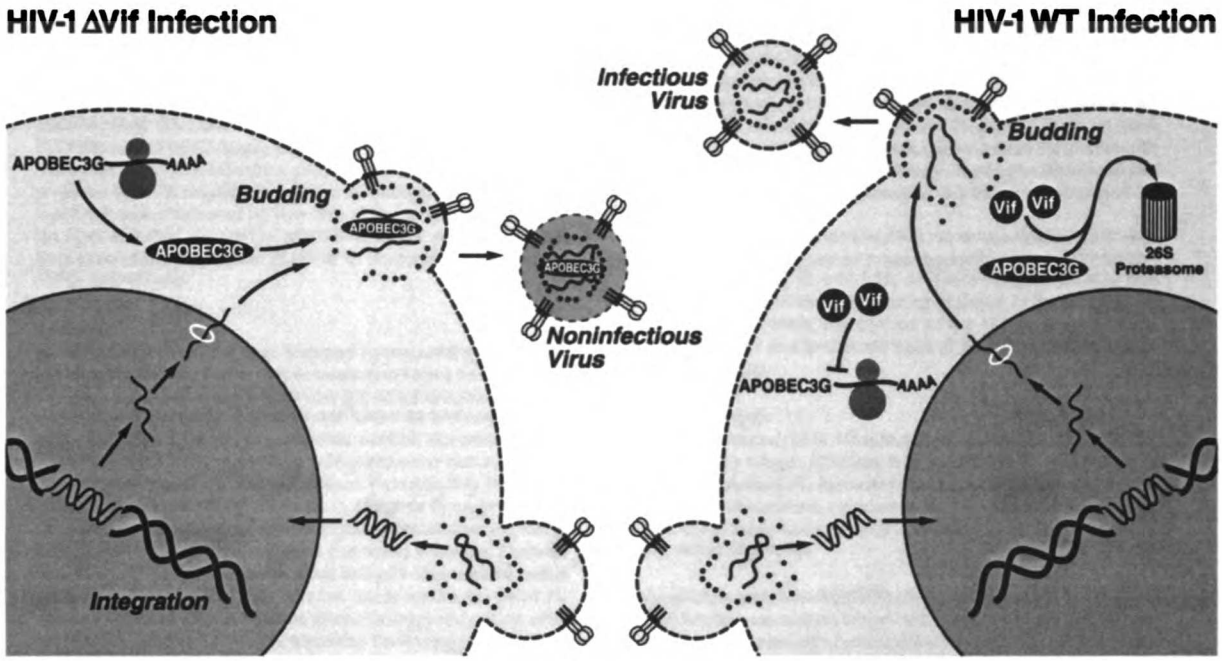

Figure 7. Schematic Summary Depicting How Vif Both Impairs the Translation of APOBEC3G mRNA and Targets the Remaining APOBEC3G Protein for Degradation by the Proteasome

The combined effect of these two activities of Vif effectively eliminates APOBEC3G from the cell leading to a failure of APOBEC3G encapsidation in virions. Through these combined effects, Vif prevents APOBEC3G from inducing devastating mutations in the viral DNA synthesized during reverse transcription in the subsequent target cell.

pairment in translation was observed. Interestingly, an even greater decline (80%) in the steady-state level of APOBEC3G was observed in samples from the in vivo pulse-labeling experiment. These findings further support the bimodal nature of Vif action since the impairment of translation is not sufficient to account for the even greater decline in steady-state levels of APOBEC3G. Thus, Vif targets two different steps in the APOBEC3G cycle, one producing inhibition of APOBEC3G mRNA translation and the second increasing turnover of the synthesized APOBEC3G protein. Whether the accelerated turnover of the APOBEC3G protein could be the result of Vif-mediated inhibition of translation of a second cellular factor required for stability of the APOBEC3G protein remains a possibility that is under further study. Nevertheless, such a two-pronged strategy allows HIV-1 to maintain genetic economy while ensuring the essentially complete elimination of the potent antiviral activity of APOBEC3G within the HIV-1-infected host cell.

Other viruses also attack key intracellular proteins in a bimodal manner. For example, the HPV type 16 E6 oncoprotein inhibits the p53 tumor suppressor by two distinct mechanisms (Zimmermann et al., 1999). Specifically, the E6 protein abrogates p53 function both by binding to CBP/p300, which represses the transcriptional activity of p53, and by directly targeting the p53 protein for degradation by the 26S proteasome (Zimmermann et al., 1999).

It remains unknown whether the impairment of translation produced by Vif is mediated through binding to APOBEC3G mRNA or to the APOBEC3G primary translation product. An impairment before translation is unlikely in view of our finding that Vif does not alter the levels or stability of APOBEC3G mRNA. However, it is possible that Vif targets a more general process such as the initiation of translation. In this regard, poliovirus (PV) induces marked inhibition of host cell cap-dependent protein synthesis by cleaving several components necessary for efficient translation (Etchison et al., 1982; Kuyumcu-Martinez et al., 2002). Interestingly, PV-induced cleavage of an essential translation initiation factor, eIF4GI, results in only a 50% decrease in the translation of cellular proteins (Bonneau and Sonenberg, 1987; Bovee et al., 1998). Whether a similar mechanism could underlie the partial impairment of APOBEC3G mRNA translation induced by Vif is currently under study.

Taken together, our findings shed new light on the mechanism by which HIV-1 Vif defeats the function of APOBEC3G, a powerful innate antiretroviral factor. It might be possible in the future to exploit these mechanistic insights for the development of a new class of antiviral agents that interfere with the ability of Vif to reduce APOBEC3G levels in the cell. Such small molecules would lead to increased virion encapsidation of APOBEC3G and allow this cytidine deaminase to induce debilitating mutations during viral DNA synthesis in the subsequent target cell. By suppressing the activity of Vif, this class of antiviral drugs would restore an essential feature of the innate immune system and enable host cells to utilize endogenous APOBEC3G to inhibit HIV replication.

Experimental Procedures

Plasmids

The pNL4-3 and pNL4-3 ΔVif (also known as p-NL-ND) expression vectors have been described (Adachi et al., 1991; Sakai et al., 1993).

The pNL-A1 and pNL-A1-ΔVif plasmids were a generous gift from Dr. K. Strebel (Khan et al., 2001). The APOBEC3G-HA expression vector was produced by subcloning an APOBEC3G-HA cassette, isolated from the NG/C15 retroviral vector (a generous gift from Dr. M. Malim) (Sheehy et al., 2002) into pcDNA3.1 (Invitrogen). A stop codon was introduced at the end of the APOBEC3G cDNA to produce the untagged pcDNA3.1 APOBEC3G vector. To prepare the pCMV4-HA-APOBEC3G vector, APOBEC3G cDNA was amplified by PCR from an H9 cDNA library and then inserted into the HindIII and XbaI sites of the pCMV4 vector. pCMV4-Vif and pEG-GST-Vif were prepared by PCR amplification of the Vif coding region present in a pNL4-3 vector followed by insertion of the amplicon into either the KpnI and XbaI sites of the pCMV4-his vector or the EcoRI and NheI sites of the pEG vector (a gift of G. Kadinger) (Chatton et al., 1995), respectively.

Antibodies

Anti-APOBEC3G antibody was prepared by immunizing and boosting rabbits with a synthetic peptide corresponding to the C-terminal 15 amino acids of human APOBEC3G ([C]-GDLSGRLRAILQNQEN). Serum was obtained after 42 days and tested for immunoreactivity with H9 cellular lysates. As a negative control, the same lysates were tested for immunoreactivity with preimmune serum isolated from the same rabbits (Antibody Solutions, Palo Alto, CA). Polyclonal anti-Vif antiserum was a gift from Dr. D. Gabuzda through the AIDS Research and Reference Reagent Program (Goncalves et al., 1994). Rabbit anti-Nef antiserum has been previously described (Greenberg et al., 1998). Mouse monoclonal anti-p24 Gag ascites was a generous gift from Beckman Coulter. Other antibodies used included rabbit anti-HA (Santa Cruz Biotechnology) and mouse anti-HA (12CA5, Roche) (3F10, Cell Signaling Technology). FITC-KC57 anti-p24 antibody (Coulter) was used in the intracellular anti-p24 Gag immunostaining studies.

Cell Lines, Spinoculation, and Preparation of Highly Purified Virions

H9, Supt-1, Jurkat, and 293T cells were maintained using standard tissue culture techniques. Fresh human PBLs were maintained in complete RPMI medium (10% FBS) supplemented with IL-2 (5 µg/l) (Roche Diagnostics). A portion of PBLs was activated with phytohemagglutinin (PHA) (5 µg/ml) for 24 hr before use. HIV-1 wt and HIV-1 ΔVif viruses were pseudotyped with the VSV-G envelope by calcium phosphate-mediated cotransfection of 293T cells with expression vector DNAs encoding VSV-G and pNL4-3 or pNL4-3 ΔVif. After 48 hr of culture, virion-containing supernatants were harvested, clarified by centrifugation at 500 × g for 5 min, and filtered through 0.2 µm filters. The virus-containing supernatant was then incubated with 0.4 × 10⁶ H9 cells/well in 48-well plates and centrifuged at low speed for 90 min at 24°C. The cells were then washed, incubated in complete medium for 40 hr at 37°C, pelleted by centrifugation at 500 × g for 5 min, washed, and lysed for immunoblotting or Northern blotting studies. Highly purified virions were obtained from the virus-containing supernatant using the discontinuous iodixanol step gradient purification method previously described (Dettenhofer and Yu, 1999).

Protein Expression Assays

293T cells were transfected with the indicated amounts of expression vector DNA using the calcium phosphate method. Lysis buffers used for immunoblotting were (1) 100–400 mM NaCl, 50 mM HEPES, 0.5% NP40, 0–5 mM EDTA, 0.1 mM PMSF, plus complete protease inhibitor cocktail (Roche) (1 tablet/50 ml buffer) or (2) 50 mM HEPES (pH 7.5), 125 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 10% glycerol, 1 mM EDTA, 1× protease inhibitor cocktail (Calbiochem). Samples were analyzed using standard SDS-PAGE techniques.

For the proteasome inhibitor experiments in transiently transfected cells, 293T cells were transiently transfected with 0.1 µg of APOBEC3G-HA DNA, 2 µg of control vector DNA, and 2 µg of either pcDNA3.1 Vif DNA vector or an additional 2 µg of control vector DNA. Twenty-four hours after transfection, the cells were treated with the indicated inhibitors and harvested 16 hr later. For the proteasome inhibitor experiments using infected H9 cells, H9 cells were infected by spinoculation as described above. Forty-four hours after infection, the cells were treated for 18 hr with either DMSO (0.1%) or 0.25 µM epoxomicin. All of the inhibitors (ALLN, MG132, epoxomicin, calpain inhibitor II, and clasto-lactacystin β-lactone) were purchased from Calbiochem and were dissolved in DMSO.

Intracellular anti-p24 Gag immunostaining of H9 cells was performed as described (Eckstein et al., 2001). In brief, H9 cells were harvested, washed, and permeabilized by incubation at room temperature for 30 min in PermeaFix (Ortho Diagnostic Systems, Raritan, NJ). After two additional washes, the cells were incubated with FITC-conjugated anti-p24-Gag antibody (Coulter) for 30 min at room temperature. After additional washes, the cells were analyzed by flow cytometry.

To analyze subcellular distribution, H9 or transfected 293T cells were washed and suspended in swelling buffer (10 mM HEPES [pH 7.5], 1.5 mM MgCl₂, 10 mM KCl, protease inhibitor cocktail and PMSF) for 10 min on ice before being vortexed in the presence of 0.4% NP-40. The lysate was layered on top of 1 ml of 1% sucrose in swelling buffer and then centrifuged at 3,500 RPM in a Beckman GS-6R centrifuge.

Northern Analysis

RNA was extracted from 10⁷ infected or uninfected H9 or Supt-1 cells using the Qiagen RNAeasy RNA extraction kit. The RNA was then separated on a 1% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and probed with a ^{32}P-labeled APOBEC3G cDNA. Hybridizing bands on the membrane were then visualized using autoradiography.

Pulse-Chase and Pulse Radiolabeling Experiments

HEK293 cells were cotransfected with 1 µg of APOBEC3G-HA expression vector and 8 µg of either pNL-A1 or pNL-A1ΔVif. The transfected HEK293 cells were preincubated for 1 hr in labeling media (DMEM without methionine and cysteine [Gibco] plus 10% dialyzed FBS). Each sample was subsequently pulse-labeled for 30 min with 350 µCi EasyTag EXPRESS ^{35}S Protein Labeling Mix (PerkinElmer). The initial pulse-labeled (t = 0) samples were harvested. The remaining radiolabeled samples were incubated with chase media (DMEM plus 10% FBS with 4.02 mM methionine [20×] and 3 mM cysteine [15×]) and harvested at various time points. For the pulse-label experiment, each sample was pulse-labeled for 15 min and then harvested immediately.

The harvested cell pellets were lysed in buffer A (50 mM HEPES, 125 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA [pH 7.5] plus protease inhibitor cocktail set I [Calbiochem]) for 15 min on ice and clarified at 14,000 × g, at 4°C for 10 min. Immunoprecipitations (IPs) were set up at equal protein concentration/volume in the presence of anti-HA monoclonal antibodies (BAbCO) and incubated on ice. The IPs were washed four times with buffer A and eluted. The samples were analyzed by SDS-PAGE, and the gels were fixed before treatment with Amplify fluorographic reagent (Amersham Biosciences). The dried gels were subjected to autoradiography and the scanned images were quantified using Scion Image software (version 1.62).

In Vitro Translation

Vif or control GST protein was initially transcribed and translated for 90 min using pcDNA-Amp Vif and pEG vectors and the Promega TNT T7-coupled reticulocyte lysate system according to the manufacturer's instructions. The presynthesized Vif or GST was then added to a new transcription/translation mix programmed with APOBEC3G DNA on ice in the presence or absence of 5 µM epoxomicin. The reactions were then transferred to a 30°C water bath and allowed to proceed for 15 or 30 min. The proteins were separated by SDS-PAGE, and the gel was analyzed by autoradiography.

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Chapter 3

Design of a high-throughput screen for inhibitors of Vif-mediated degradation of APOBEC3G

Introduction

In the past decade, the availability of highly active antiretroviral therapy (HAART), has improved the mortality and morbidity rates of HIV-infected individuals (14). Current HAART involves the administration of a combination of drugs targeting the Protease (Pr) or Reverse Transcriptase (RT) proteins of the HIV virus or targeting the process of virion fusion to cells. RT inhibitors include nucleoside RT inhibitors (NRTI) that block elongation of the growing polynucleotide chain by mimicking natural nucleotides and non-nucleoside RT inhibitors (NNRTI) that act by allosterically binding RT just outside the catalytic domain. The entry inhibitor Fuzeon (T-20, enfuvirtide), a peptide that blocks virion fusion to cells by impairing gp41/Env hairpin formation, has been recently approved by the FDA and is included as part of salvage therapy (10, 20). Despite the benefits of HAART, and the availability of over 20 anti-HIV drugs, obstacles such as cost, drug toxicity(3), and emerging single-drug-resistant and cross-resistant strains of HIV (4, 6) underscore the necessity of new drug targets.

Drugs targeting other aspects of the virus are currently under development, but are not available for widespread use. One such drug, BMS-378806, which is an attachment inhibitor that interferes with gp120 and CD4, has demonstrated a reasonable safety profile in early animal toxicology studies (7, 10); a related inhibitor, BMS-499043, has improved pharmacokinetic properties and is currently in Phase 2a clinical trials (7, 10). Other promising drugs currently under development target the viral integrase (IN) and RNAseH proteins and the interaction between gp120 and the CCR5 chemokine receptor (6, 10).

Several groups have also studied the potential of the HIV-1 Vif protein to be a possible anti-HIV drug target. A Vif-specific single-chain antibody has been developed that showed anti-HIV potential when expressed intracellularly (5). Similarly, siRNA designed to block the intracellular expression of Vif has shown significant anti-HIV effects (1). Although of questionable practicality given current drug technologies, both of these studies at least highlight the suitability of the HIV-1 Vif protein for drug targeting.

The identification of A3G (16) and the discovery that Vif causes the virtual elimination of A3G from infected cells (18) provides a new vantage point for the development of anti-HIV drugs.

Potential Development of a Drug Targeting Vif-mediated Degradation of A3G

HIV-1 Vif prevents virion incorporation of the endogenous A3G enzyme by causing the intracellular depletion of A3G from infected T cells (18). Vif reduces intracellular levels of A3G both by accelerating the degradation of A3G by the 26S proteasome and by partially impairing the synthesis of the A3G protein (18). By employing this bimodal mechanism, Vif succeeds in removing A3G from the cell before it has the chance to be incorporated into the virion. Vif also interacts with A3G (18), and functions as an adaptor molecule, bringing A3G in contact with a E3 ubiquitin ligase complex, which promotes the polyubiquitylation of A3G (9, 17, 22). This ubiquitylation reaction targets A3G for rapid degradation by the 26S proteasome.

One advantage of our highthroughput screen is that it will be able to identify molecules that block any one of a number of steps in the Vif-mediated depletion of A3G. Small molecule antagonists could be identified that act by 1) blocking the assembly of

Vif with A3G, 2) blocking the interaction of Vif with the E3 ligase machinery, 3) binding A3G in a manner that prevents its polyubiquitylation, or 4) preventing Vif-mediated inhibition of the synthesis of A3G.

The first two scenarios, and possibly the fourth, would involve identifying an inhibitor that blocks protein-protein interactions. Several recent studies support that it is possible to disrupt protein-protein interactions with small molecules. Small molecular antagonists of the interaction between the E3 ligase MDM2 and the p53 tumor suppression protein have recently been identified. (19). And, as mentioned earlier, there are several small molecule inhibitors of HIV already in development that target protein-protein interactions. Small-molecule inhibitors that block viral attachment by interfering with the interaction between gp120 and CD4 are currently in clinical development (7, 10). Small molecule inhibitors targeting CCR5-gp120 interactions are also showing clinical promise (10).

The Vif:A3G interaction might be especially conducive to inhibition by a small molecule. Several laboratories have reported that mutation of a single amino acid (Asp 128) in the A3G sequence suffices to make it resistant to Vif, allowing it to function as an antiviral agent against wild-type human HIV-1 (2, 8, 15, 21). While it remains unclear whether this single amino acid forms a key component of the Vif binding site or instead affects the overall conformation of A3G, it is certainly possible that small molecules might either block this site or induce similar conformational changes. It is also promising that there is a potential target within this single site rather than in an extended interface between the two proteins.

Recent work clarifying the exact domains in Vif that participate in the degradation of A3G provide additional places a candidate drug from our screen could target. Vif binds to A3G and targets it for ubiquitination through a cullin-dependent ubiquitin ligase complex containing Cullin 5 (Cul5), Elongins B and C (EloBC) and Rbx (11, 22, 23). Ubiquitination is a post-translational modification that requires the coordinated activity of three enzymes. E1 ubiquitin activating enzymes transfer ubiquitin to E2 conjugating enzymes. E2 conjugating enzymes transfer ubquitin to the target protein with the help of E3 ubiquitin ligases. Cul5, EloBC are core subunits of a Skp1-Cullin-F-box (SCF)-like E3 complex. HIV-1 Vif and other lentiviral Vif proteins contain a highly-conserved BC Box motif, SLQXL, that is necessary for viral replication and allows Vif to directly interact with EloC (11, 22, 23) Fig. 1A. A longer sequence, ending with a C terminal P/L-rich region makes up a novel SOCS (suppressor of cytokine signaling)-box, but the functional significance of the P/L-rich sequence to Vif is unclear (11, 23) Fig. 1A. ΔSLQYLAL-mutant Vif proteins are still able to bind A3G, but do not cause degradation. Interestingly, several single mutations in this region prevent binding to the Cul5 eloBC complex including L145A, A149G, L150A, A149L (11, 23). Although the BC box is sufficient to bind EloC, an additional HCCH zinc-binding region upstream of the BC-box mediates Vif binding to Cul5 (12, 23) Fig.1A(lowerpanel). Mutations of His/Cys residues in this region impair zinc coordination, Cul5 binding and A3G degradation.

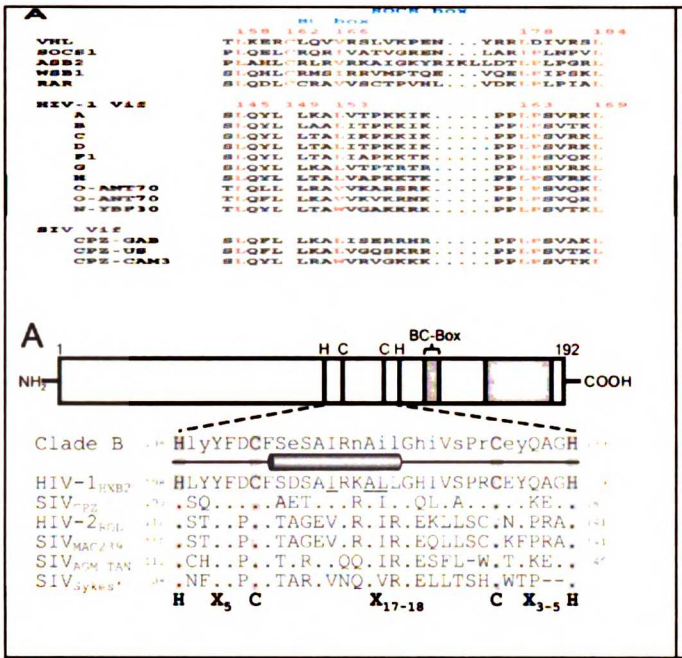

Fig.1A Sequence of conserved SOCS box motifs across lentiviral Vif proteins including BC Box and C-term P/L-rich region (*upper panel*). Sequence of Zinc-coordinating motif in different lentiviral Vif proteins (*lower panel*).

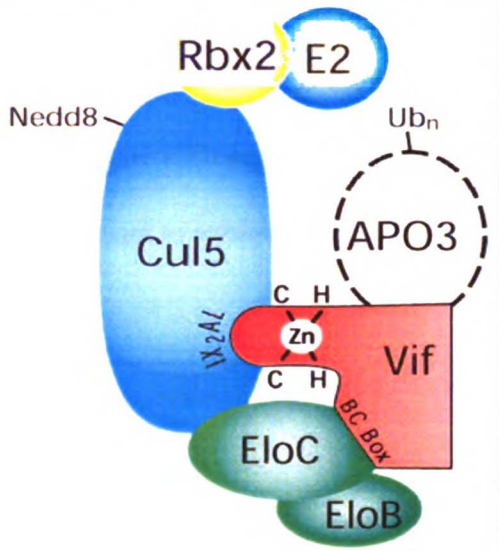

Fig.1B Model of Vif interaction with A3G and recruitment of E3 ligase complex containing Cul5, Elongins B and C, RBX2 and E2.

Design of The High-throughput Screen

The high-throughput screen for Vif inhibitors will rely on the construction of an adherent cell line stably transfected with a Vif expression vector as well as a vector expressing A3G fused to the renilla luciferase enzyme ("A3G-Luc"), a sensitive reporter protein (Figure 2). In the absence of an inhibitor, Vif will trigger the degradation of the A3G-Luc in these cells; and the cells will lack a significant positive signal for luciferase action. Conversely, an effective small-molecule inhibitor will block the action of Vif, resulting in the accumulation of A3G-Luc within the cells. Preserved expression of A3G-Luc will be detected by an increase in luciferase activity. Provided that a cell line is constructed that passes rigorous quality assurance and quality control standards, these cells will be used in automated, single well screening assays involving approximately

200,000 compounds displaying high pharmacore diversity. One advantage of this approach is that since it is cell-based, only compounds disrupting the activity of Vif in its native conformation will be selected. In addition, since only cells with enhanced signal will score positive in our screen, it contains a built-in mechanism to exclude small molecules that are toxic to cells.

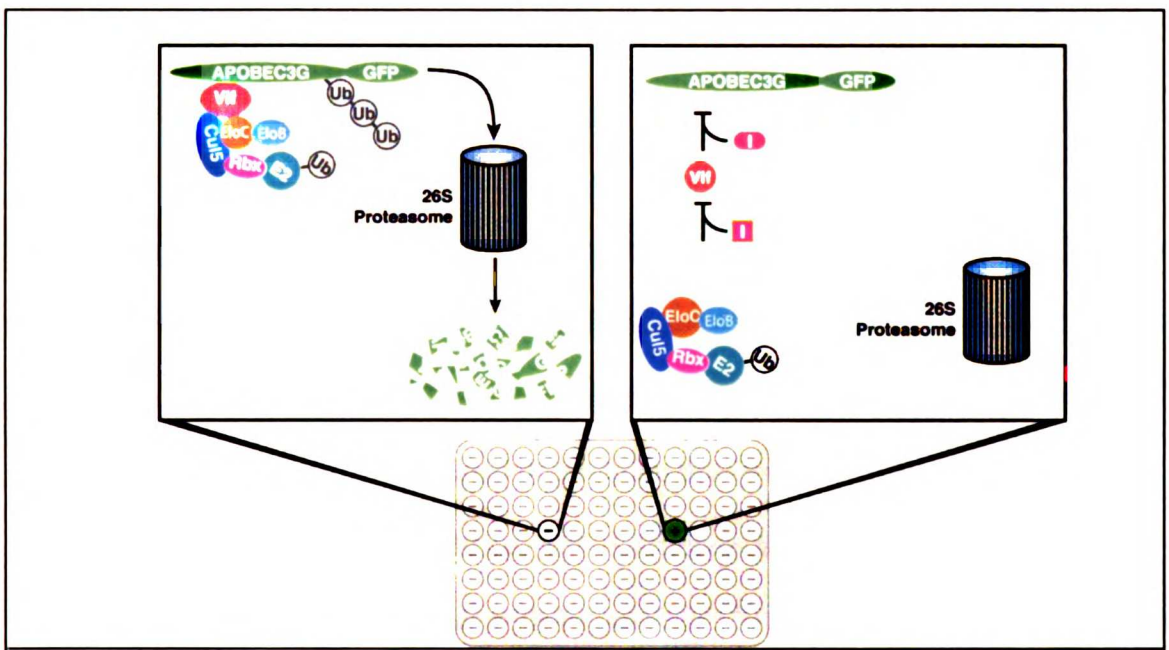

Fig. 2. Model of High-throughput Screen. The basis of the screen will be a cell line stably expressing both Vif and a version of A3G tagged with luciferase. Under basal conditions, Vif will induce the depletion of A3G-Luc by targeting this enzyme for polyubiquitylation and degradation by the 26S proteasome (left panel). If a small molecule succeeds in blocking Vif-mediated depletion of A3G-Luc, then the levels of A3G-Luc will accumulate to detectable levels in the cell (right panel). The screen is designed to identify molecules that have blocked the physical assembly between Vif and A3G, the assembly between Vif and the E3 ubiquitin ligase machinery, or any other event in the Vif-mediated cascade leading to A3G depletion.

The data presented in this chapter was performed by myself, Dr. Wes Yonemoto and Louise Ogle. The original idea for the screen was my own, with the help of Dr. Carlos de Noronha, and stemmed from my identification of the mechanism employed by Vif to counteract the activity of A3G. The data relating to the original monoclonal cell line, including construction of the A3G-Luc and Luc-A3G plasmids was performed by

myself and Louise Ogle. The engineering of the human-codon-optimized version of Vif used for the next versions of the cell line was done by myself with the help of Dr. Wes Yonemoto. Figure 5 was done by Wes Yonemoto using my reagents. He subsequently assumed responsibility for this project and has, on his own, produced a cell line expressing the human-codon-optimized Vif under an inducible promoter along with A3G-Luc, which is now being developed for screening purposes. Dr. Warner Greene directed and supervised this project.

Results

In order to construct our cell line, we cloned a series of vectors expressing Vif and APOBEC3G ("A3G"). The vectors encoded A3G tagged with the reporter protein Renilla Luciferase at either the C' or N' terminal. Given that A3G-Luciferase (C' terminal tag) was more susceptible to Vif-mediated degradation than was Luciferase-A3G (Fig. 5(b) and data not shown), we decided to use A3G-Luciferase in our cell line. Our initial strategy was to transfect HeLa cells with Vif and A3G-Luciferase expression vectors at a 5:1 ratio. Since the A3G-Luciferase contained a neomycin resistance gene, we subjected the transfected cells to G418 (neomycin) drug selection (1.0 mg/ml). Once we obtained a parental line stably expressing both proteins, we obtained subclones by limiting dilution. We also attempted to select the clones using the histidinol selection marker present on the Vif plasmid, but this method did not yield viable cells. However, since we had transfected the Vif and A3G-Luciferase vectors at a high ratio, we reasoned that any cells containing A3G-Luciferase should also have Vif.

Western analysis of one of our clonal lines (Clone "78") revealed the presence of both Vif and A3G-Luciferase (Fig. 3A). In addition, we observed significant

upregulation of the A3G-Luciferase protein in the presence of MG132, an inhibitor that blocks the 26S proteasome, thereby also blocking the activity of Vif (Fig. 3A, lane 3 versus 4). Luciferase assays also revealed significant induction of A3G-Luciferase after treatment of Clone 78 with MG132 (Fig. 3B).

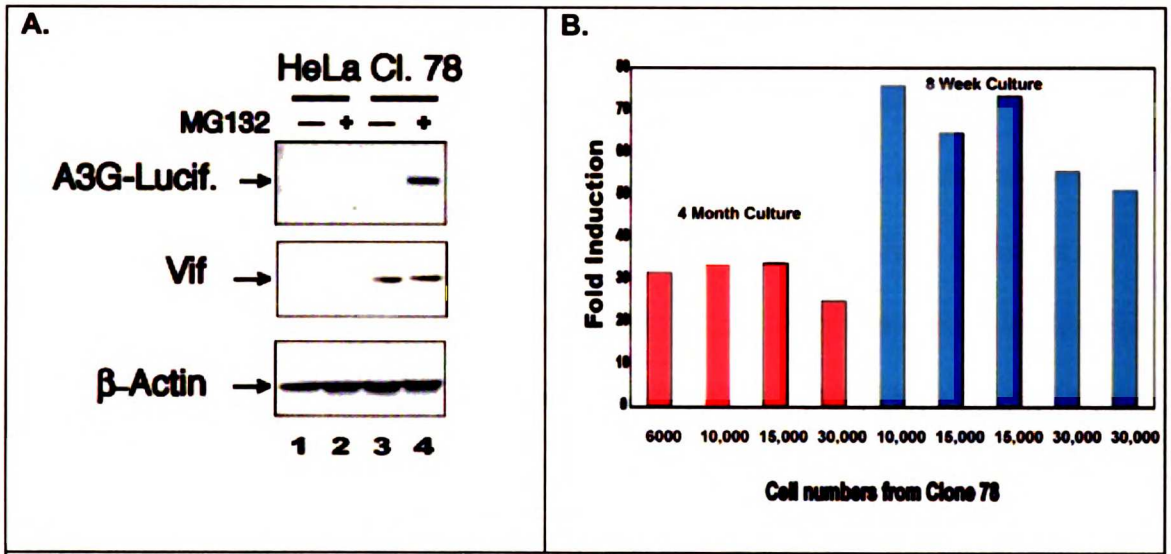

Fig. 3. Initial Testing of Monclonal Cell Line. **(A)** Western Blot of Monoclonal Cell Line ("Clone 78") stably expressing both A3G-Luc and Vif. Clone 78 and untransfected HeLa cells were treated with either MG132 or DMSO. Cellular lysates were prepared and subjected to SDS-PAGE and western blotting with anti-Vif, anti-A3G, or anti-β-Actin antibodies. Comparison of lanes 3 and 4 reveals MG132-induced A3G-Luciferase expression in Clone 78. β-Actin was used as a loading control. **(B)** Luciferase Assays of Clone 78. Clone 78 was cultured for either 8 weeks (blue bars) or 4 months (red bars) and then treated with MG132 or DMSO. The fold-induction of A3G-Luc activity of the MG132-treated cells over DMSO-treated is indicated on the y' axis. The x' axis notes the number of cells used in each assay.

For these experiments, the G418 selection medium was removed from cells that had been in culture for either 8 weeks or 4 months. The cells were then treated for 16 hours with MG132 (12.5 μM) before analysis by luciferase assay. In cells that had been cultured for 8 weeks, MG132 enhanced A3G-Luciferase expression by as much as 75-fold. In cells that had been in culture for 4 months, MG132 caused a 30-fold increase in

A3G-Luciferase expression (Fig. 3B). Although the level of induction had fallen over time, it remained robust, indicating that the cell line was relatively stable.

Our next step was to perform Z' series tests on our cell line to assess its suitability for high-throughput screening. The Z' value is an industry standard that measures the quality of a high-throughput assay. The Z' value reflects the assay's signal dynamic range as well as the data variation associated with the signal measurements (Fig. 4A). In our assay, large deviations in luciferase activity from sample to sample would reduce the Z' value, indicating that the assay is unsuitable for HTS. Similarly, a high induction of A3G-Luciferase by MG132 would improve the Z' value. To perform the Z' series test, we plated Clone 78 in a 96-well plate with the indicated number of cells per well (Fig. 4B). We then treated the first and fourth quadrants of the 96-well plate with MG132. These quadrants served as our experimental "positive" wells. Our "negative" control wells were in the second and third quadrants, which were treated with DMSO. The average MG132-induction of A3G-Luciferase in our positive wells was up to 70-fold (Fig. 4B). The Z' values for Clone 78 ranged from 0.8 to 0.9, indicating that our line was highly suitable for HTS (Fig. 4B).

Before sending Clone 78 to our collaborators at the Southern Research Institute for HTS, we decided to perform one last test given that the MG132 experiments were not definitive proof that our phenotype had been caused by Vif. It was possible that the observed induction of A3G-Luciferase upon MG132 treatment had been due to rapid turnover of the protein by a Vif-independent mechanism. To assure that the phenotype was truly Vif-dependent, we developed small-interfering RNA that would knock-down Vif levels in our cell line. When these experiments were performed, the results were

inconclusive. The hope had been that reducing the intracellular level of Vif would cause the A3G-Luciferase levels to increase. Given the stakes involved, we decided to abandon Clone 78 and pursue a better strategy to develop a cell line.

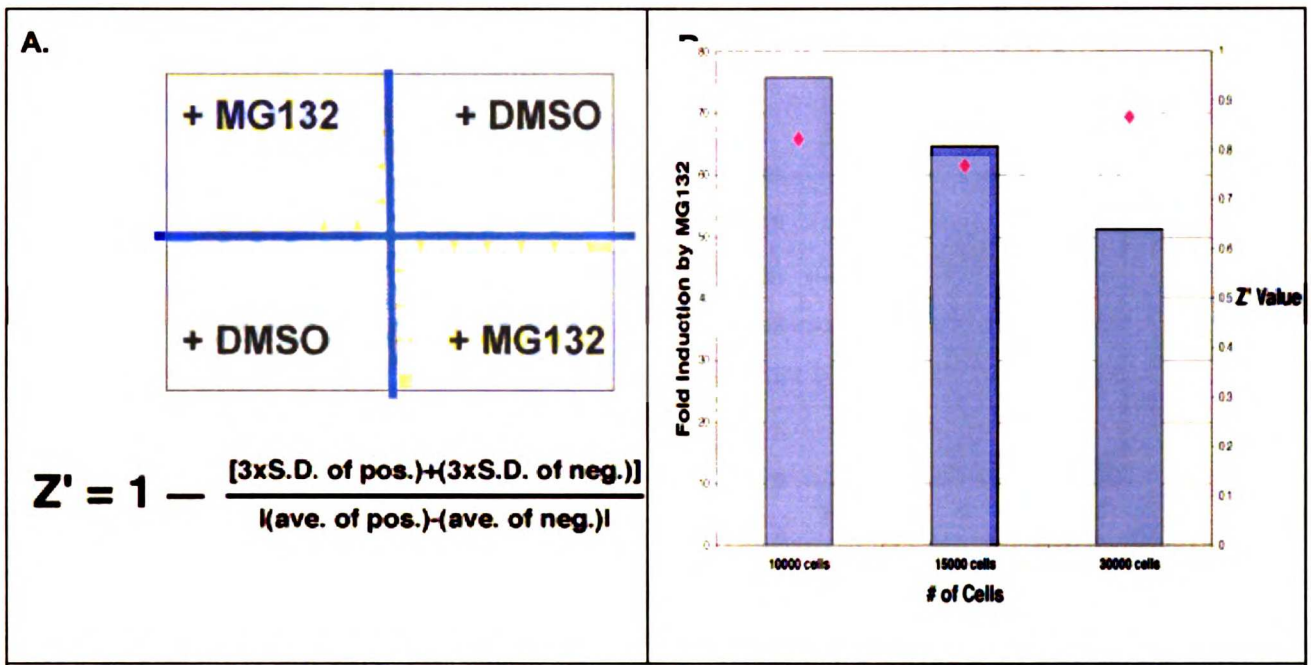

Fig. 4. Z' Series Analysis of Clone 78. (A) Diagram of 96-well plate format used to calculate Z' value (top panel) and Equation for Calculation of Z' factor (lower panel). Optimal Z' values are as close to 1 as possible. ***(B)*** Z' series analysis of Clone 78. Clone 78 was seeded in a 96-well plate. The first and fourth quadrants were treated with MG132, and the second and third quadrants were treated with DMSO (as shown in (A)). The average fold induction by MG132 (blue bars) and the individual Z' values (pink diamonds) were then calculated and represented by the y' axis. Cell numbers used for the assays are indicated on the x' axis.

For phase two of the project, we improved the expression of Vif by engineering a human codon-optimized version of Vif ("cVif"). The expression of cVif was superior to hVif, a partially-human-codon-optimized protein that had been created by another laboratory (Fig. 5A, compare lane 8 with lane 10). Western blot analysis revealed that cVif not only expressed well but was equally active against A3G-Luc as it was against HA-A3G (Fig. 5A, lanes 2-8). The effectiveness of cVif at depleting A3G-Luc was

confirmed by Luciferase assay (Fig. 5B, lanes 4,5). The cVif protein was not effective at depleting Luciferase-A3G (Fig. 5B, lanes 6,7).

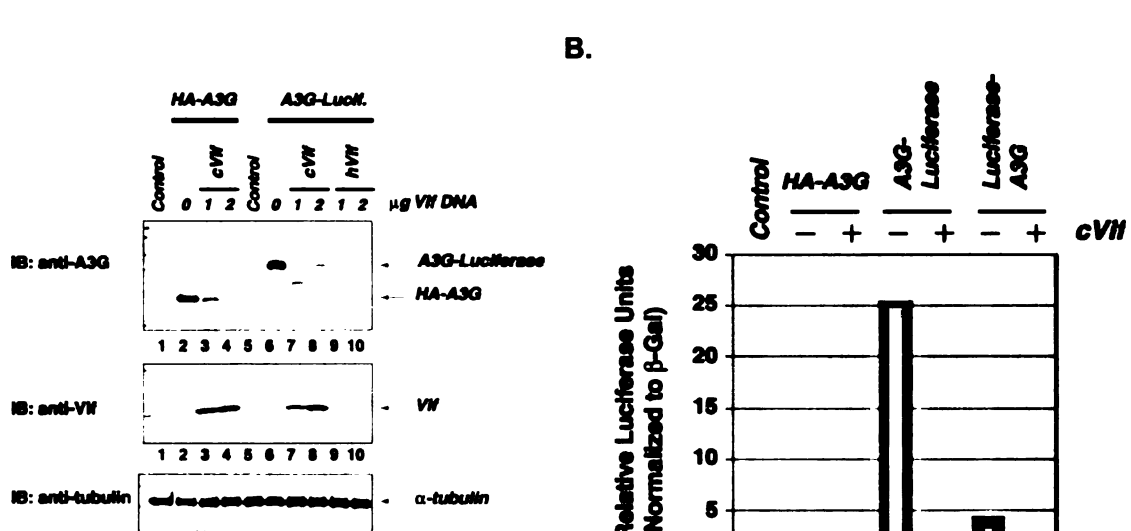

Fig. 5. cVif Expression and Activity against A3G-Luciferase. ***(A)*** Western blot analysis of cVif expression and activity against A3G-Luciferase. 293T cells were transfected with expression vectors encoding A3G-Luciferase or HA-A3G either alone or in combination with the indicated amount of cVif or hVif expression vectors. The cells were lysed and analyzed by SDS-PAGE, followed by immunoblotting to determine the intracellular level of A3G-Luciferase or HA-A3G. The activity of cVif against HA-A3G (lanes 2-4) and A3G-Luciferase (lanes 6-8) is presented next to the activity of a lower-expressing version of Vif (hVif) (lanes 9,10). αTubulin was used as a loading control. ***(B)*** Luciferase assay demonstrating the activity of cVif against A3G-Luciferase or Luciferase-A3G. 293T cells transfected with the indicated A3G expression vector plus-or-minus cVif were harvested. The level of the A3G fusion protein was then determined by Renilla Luciferase assay. The cells were also transfected with β-galactosidase, which was used to normalize the data.

Discussion

The development of the cell line has had two distinct phases. In the first phase, we followed a single-selection procedure in which we selected cells co-transfected with Vif and APOBEC3G-luciferase vectors at once and then proceeded to clone out monoclonal cell populations by limiting dilution. Using this procedure, we isolated a clonal population ("clone 78") that met all initial requirements for the cell line. Western analyses revealed that both APOBEC3G-luciferase ("A3G-luc") and HIV-1 Vif proteins

were expressed in clone 78. In addition, both western analyses and luciferase assays revealed high induction of A3G-Luciferase expression following treatment with MG132 to block the activity of the 26S proteasome. Since Vif mediates degradation of A3G by the 26S proteasome, this assay suggested that Vif was causing significant degradation of A3G-Luc in the cell line and that this process was being blocked by MG132. Furthermore, this clone passed Z' series tests, an industry standard to measure the quality of a high-throughput assay. The Z' value reflects the assay's signal dynamic range as well as the data variation associated with the signal measurements. Unfortunately, the clone did not pass the very last test we performed, which was an siRNA study. In this study, we used siRNA to knock down the Vif protein in our cell line. If our assay had been working correctly, the loss of Vif should have resulted in increased expression of A3G-Luciferase. However, the siRNA studies were not conclusive, suggesting that the induction observed with MG132 treatment may have been an artifact of the system. It was possible that the induction of A3G-Luciferase we had previously observed may have been due to rapid turnover of the protein in the cell that had been caused by some other, Vif-independent mechanism.

We decided to abandon this cell line and initiate a second stage for developing the cell line. We concluded that there were two areas that could have been improved in order to assure that the phenotype was truly Vif-dependent. One was that instead of using a one-step selection system, it would be preferable to first derive a clonal population expressing A3G-Luciferase and then, in a second step employing a second drug-resistance marker, introduce Vif. In this way, we could directly compare the A3G-Luc clone with the A3G-Luciferase plus Vif clone to ensure that any differences in A3G-

Luciferase expression were truly due to the presence of Vif. We also decided to explore ways to improve expression of the Vif protein in our line. We had previously observed only weak expression of Vif in our clone. Since Vif is a notoriously difficult protein to express in the absence of a viral protein to facilitate the export of Vif mRNA from the nucleus, the poor expression of Vif was not surprising. We have since engineered a "cVif" expression plasmid containing a fully-human codon-optimized version of the *vif* gene. The expression of cVif is vastly superior to the expression of Vif from the plasmid we had previously employed. During the first phase of our project, another laboratory developed a plasmid containing a partially human codon-optimized *vif* gene ("hVif")(13). For proprietary reasons, the hVif expression plasmid was unavailable for use in our HTS assay. However, our cVif vector expresses even better than the hVif, making it a great candidate for use in our cell line. Using both western analyses and Luciferase assays, we have also confirmed that cVif ably mediates the degradation of A3G-Luc.

We are currently in the next phase of the project, which has been taken over by Dr. Wes Yonemoto, who also contributed to the early studies. Dr. Yonemoto has developed a new monoclonal cell line expressing human-codon-optimized Vif protein under an inducible promoter along with A3G-Luc. This cell line is currently being screened for candidate small molecule inhibitors by our collaborators at The Southern Research Institute.

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Protecting APOBEC3G: A potential new target for HIV drug discovery

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Introduction

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Vif versus APOBEC3G

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Advantage of Vif

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Conclusions

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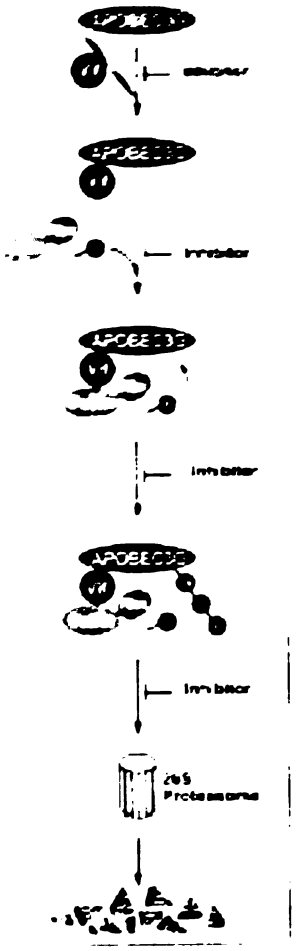
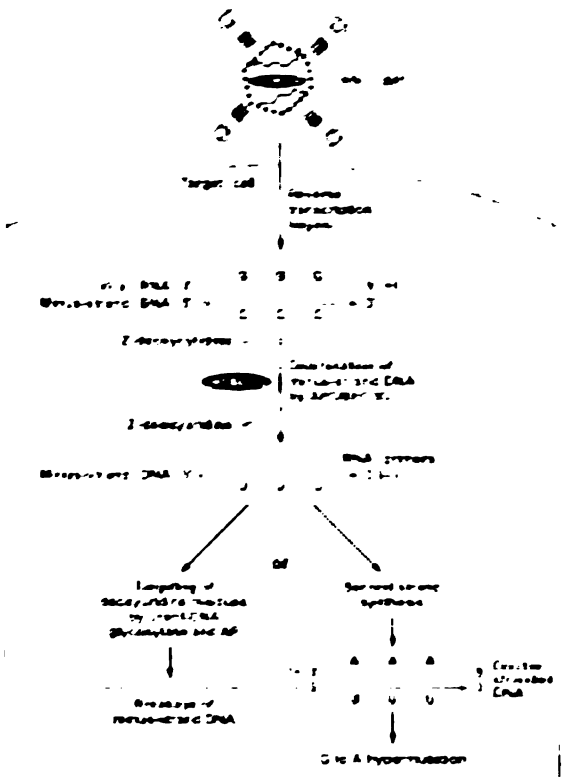


Vif-APOBEC3G as a potential drug target



Chapter 4

Regulation of APOBEC3G expression and activity by cytokines

Introduction

APOBEC3G (A3G) is a component of the innate immune system that provides a defense against both exogenous retroviruses and endogenous retroelements. The exogenous viruses inhibited by A3G include retroviruses such as HIV, SIV, EIAV, MLV, foamy virus and HTLV-1 as well as hepatitis B virus, a pararetrovirus (3, 4, 6, 8-10, 12, 14-17, 20, 22). A3G is also a potent inhibitor of Alu retrotransposition in human cells (2). Although natural levels of A3G would suffice to block some retroviral replication, suboptimal levels of A3G may actually promote viral replication by contributing to viral diversification (18). Given the diverse roles played by A3G, an understanding of how A3G is regulated at the cellular level may be useful.

A3G is primarily expressed in cells of lymphoid and myeloid lineages, although some also appears in ovary and testes (5). The A3G gene and other related A3 proteins are also inducible. Both A3A and A3B are modulated by phorbol esters and consequently were originally termed phorbolin-1 and –2, respectively (5). Another CyDA family member, AID, is induced by IL-4 (23) as well as by cell division (13).

We have previously reported that treating resting peripheral blood lymphocytes (PBLs) with phytohemmagglutinin (PHA) and IL-2 results in enhanced expression of the A3G protein (19). Another group has published that treatment of the H9 T-cell line with phorbol myristate acetate (PMA) induces expression of A3G mRNA and protein (11). PMA activation of A3G gene expression in H9 cells relies on the MAPK signaling pathway (11).

In order to determine whether A3G regularly induced in physiologic settings, we applied a panel of cytokines to primary resting PBLs and examined the effects on A3G

mRNA and protein expression. IL-2 and IL-15 both significantly enhanced expression ofA3G mRNA and protein. IL7 also had an effect, but at somewhat slower kinetics. Since the MAPK pathway is one of the pathways activated by ligation of the IL-2 and –15 receptors, we also interrogated the contribution of MAPK signaling members (7).

A curious aspect of the finding that IL-2 and –15 upregulate the expression of A3G, a known antiviral factor, is that it was already known that both of these agents also enhance the permissivity of CD4+ T cells to infection by HIV-1 (21). In previous work, our lab reported that mitogenic stimulation of CD4+ T cells causes A3G to become inactivated (1). More precisely, A3G exists in an active low-molecular mass (LMM) form in resting CD4+ T cells, which enables it to act as a post-entry restriction factor to HIV-1 (1). Mitogenic activation of T cells enhances A3G expression, but also causes it to shift into an inactive, highmolecular mass (HMM) complex that is no longer able to protect T cells from infection by HIV-1 (1). In order to determine whether a similar process was at work in cytokine- treated cells, we stimulated CD4+ T cells with IL-2, -7, or –15 and analyzed the complex status of A3G by fast performance liquid chromatography (FPLC). Since these experiments involved cells with mixed population of activated and less-activated cells, we also sought to determine the A3G complex status of the infected population. To do this, we infected activated CD4+ T cells with GFP lentiviral vectors, sorted the infected from the uninfected cells and analyzed the A3G complex status in each population.

In a different line of investigation, we also analyzed expression of A3G in interferon-treated PBLs and macrophages and in polyI;C/TNFα-matured dendritic cells.

I was first author of this paper and designed and performed all of the experiments except that Dr. Ya-Lin Chiu provided the FPLC analysis of the dendritic cells and Jerry Kropp performed quantitative RT PCR analyses of samples I prepared. The manuscript that follows was reprinted from *The Journal of Biochemistry* (Stopak, KS, Chiu, YL, Kropp, J, Grant RM and WC Greene, Distinct Patterns of Cytokine Regulation of APOBEC3G Expression and Activity in Primary Lymphocytes, Macrophages, and Dendritic Cells, *J Biol. Chem.*, 10.1074/jbc.M610138200 (November 16, 2006)), with permission from The American Society for Biochemistry and Molecular Biology.

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DISTINCT PATTERNS OF CYTOKINE REGULATION OF APOBEC3G EXPRESSION AND ACTIVITY IN PRIMARY LYMPHOCYTES, MACROPHAGES, AND DENDRITIC CELLS

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Running Title: Cytokine-Mediated Regulation of APOBEC3G

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Human APOBEC3G (A3G), a deoxycytidine deaminase, is a broadly-acting antiretroviral factor expressed in a variety of cells. Mitogen activation of CD4 T cells enhances A3G expression and leads to recruitment of low-molecular-mass (LMM) A3G, which functions as a post-entry HIV restriction factor, into enzymatically inactive, high-molecular-mass (HMM) RNA-protein complexes that include Staufen RNA transporting granules. We now report that IL-2 and IL-15, and to a lesser extent IL-7, enhance the expression of A3G in peripheral blood lymphocytes and that this effect is blocked by inhibitors of the JAK and MAPK signaling pathways. In mixed cultures of CD4+ T cells containing either HMM or LMM A3G, we find that HIV preferentially infects cells containing HMM A3G. A3G shifts into a HMM complex when IL-2, -7, or -15 are added to resting T cells, likely explaining how cytokine treatment renders resting CD4+ T cells permissive to HIV infection. Similarly, poly (I:C)/TNFα-induced maturation of dendritic cells (DCs) is associated with a sharp increase in A3G expression; however, this induction leads to accumulation of the LMM form of A3G. Together, these results highlight the distinct inductive effects of select cytokines on A3G gene expression and A3G complex assembly that occur in natural cellular targets of HIV infection.

Human APOBEC3G (A3G), a powerful antiretroviral factor, is a deoxycytidine deaminase that induces dC-dU mutations in minus-stranded DNA formed during reverse transcription. A3G employs this enzymatic activity, along with a possible non-enzymatic activity, to disable a broad range of retroviruses, including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), murine leukemia virus (MLV), foamy virus, and HTLV-I, as well as hepatitis B virus, a pararetrovirus. (1-13).

A3G can exist in two different forms in cells: a low molecular mass (LMM) form that restricts HIV spread and an enzymatically-inactive, high molecular mass (HMM) complex containing one or more inhibitory RNAs that lacks HIV restricting activity (14). HMM A3G complexes are found in mitogen-activated CD4 T-cells and in macrophages while LMM A3G is present in blood-derived resting CD4+ T cells and monocytes, which are poorly permissive for HIV replication (14). In activated CD4+ T cells infected with HIV lacking Vif, A3G is incorporated into budding virions and exerts its potent antiviral activity in the subsequent target cell either by causing lethal hypermutation in nascent viral DNA transcripts formed during reverse transcription (1,7,9,15), or by a non-editing mechanism (16,17,18). A major function of HIV Vif is to deplete A3G from the cytoplasm of virus-producing cells, thereby preventing A3G from being incorporated into virions (19-23). Vif is thus absolutely required for the production of infectious virus emanating from A3G-expressing cells (24-29). Vif binds to A3G and promotes its polyubiquitylation and

subsequent degradation by the 26S proteasome (19-23).

A3G is a member of larger family of cytidine deaminases (CyDA) that includes APOBEC1, 2, 3A-H, and activation-induced cytidine deaminase (AID). The APOBEC3A-H family is expressed in a tandem array on chromosome 22 (30). Several of the genes encoding these enzymes are expressed in an inducible manner. For example, both A3A and A3B are upregulated by phorbol esters and consequently were originally termed phorbolin-1 and –2, respectively (30). Another CyDA family member, AID, which is present on chromosome 12 and plays a critical role in class-switch recombination and somatic hypermutation in B cells, is induced by IL-4 (31) as well as by cell division (32).

The A3G gene is also inducible. Treatment of resting peripheral blood lymphocytes (PBLs) with phytohemagglutinin (PHA) and IL-2 increases expression of the A3G protein (19). Similarly, treatment of the H9 T cell line with phorbol myristate acetate (PMA) induces expression of A3G mRNA and protein (33), a result that correlates with the effects of phorbol esters on A3A and A3B. Further, PMA activation of A3G gene expression is dependent on activation of the MAPK signaling pathway in H9 leukemic T cells (33).

Mitogenic activation relieves the A3G-mediated post-entry restriction block in CD4+ T cells and causes these cells to become permissive to HIV infection (14). The mitogen effect is caused by shifting A3G from its active LMM form into inactive HMM complexes that include Staufen RNA transporting granules and Ro ribonucleoprotein complexes (34). Certain cytokines including IL-2, IL-7, and IL-15 have been reported to render resting CD4+ T cells permissive for HIV infection (35-38). We have explored the possibility that these cytokines activate A3G gene expression and recruit the LMM A3G present in primary resting CD4 T-cells into HMM A3G complexes. In addition, we have examined A3G expression in interferon-treated monocyte-derived macrophages and in dendritic cells (DCs).

Experimental Procedures

Isolation and Culture of Primary Cells - Buffy coats from individual healthy donors were processed on Ficoll-Hypaque density gradients to isolate peripheral blood mononuclear cells. CD14- peripheral blood lymphocytes (PBLs) were prepared by negative selection with anti-CD14+ microbeads (Miltenyi) according to the manufacturer's instructions using Automacs technology (Miltenyi). Residual CD14+ cells were further depleted by adherence to plastic for 3 hours. To enrich CD4+ cells, PBLs pre-incubated with anti-CD4+ microbeads were positively selected with the Automacs. The purity of these populations was >95% when analyzed by flow cytometry. Cells were routinely cultured ($2x10^6$ cells/ml) in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 ug/streptomycin.

Immature DCs were derived by culturing CD14+ monocytes for 6 days in complete medium supplemented with 25 ng/ml IL4 (R&D Systems) and 50 ng/ml GM-CSF (Biosource)(39). DC maturation was induced by incubating the immature DCs for 24 h with 25 μ g/ml poly(I:C) (Amersham) and 5 ng/ml TNF-α (Biosource) (40). The phenotype of these DCs was verified by immunostaining with antibodies specific for immature and mature DCs. Macrophages were derived from CD14+ blood monocytes by culture of these cells for 7-12 days in medium supplemented with 5% human AB serum (Omega).

Cytokine Stimulation - To study potential changes in A3G expression over time, freshly isolated CD14- PBLs were preincubated overnight at an approximate density of $2x10^6$ cells/ml in medium and then stimulated with IL-2 (50 ng/ml), IL-6 (50 ng/ml), IL-7 (62.5 ng/ml), IL-9 (50 ng/ml), IL-15 (12.5 ng/ml) or phytohemagglutinin (PHA) (5 µg/ml). All cytokines were purchased from R&D Systems. Samples were collected 0, 6, 12, 18, 24, 48, 120, and 168 hours following treatment and analyzed by either quantitative real-time PCR

to assess changes in A3G mRNA levels or by immunoblotting using a rabbit anti-human A3G specific antibody to detect changes in protein expression (14). Dose response experiments with these cytokines as well as IL-4, IL-12, and IL-13 (R&D Systems) were similarly performed with collection of samples at 48 hours for real-time PCR analysis and at 120 hours for immunoblotting studies. Potential effects of interferon on A3G expression were studied in macrophages treated with interferonα (1000 U/ml)(Schering, intron a); interferon-β (1000 U/ml) (R&D), or interferon-γ (1000 U/ml)(R&D). For the kinase inhibitor experiments, CD14- PBLs were pre-treated for 1 hour with 12.5, 50, or 100 μM AG490 (CalBiochem) or 2.5, 10, or 20 μM U0126 (Cell Signaling) before being stimulated with the indicated cytokine or PMA. To analyze A3G complexes in cytokine-treated cells, freshly isolated CD4+ T cells were treated with the indicated cytokine or mitogen for 5 days, washed with ice-cold 1X PBS, and lysed prior to analysis by fast protein liquid chromatography as described below.

Immunoblotting - Harvested cells were washed in ice-cold PBS and then lysed in lysis buffer (LB) containing 50 mM Hepes, pH 7.4, 125 mM NaCl, 0.2% NP-40, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1x EDTA-free protease inhibitor cocktail (CalBiochem) or, for the phosphorylation studies, RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% Sodium deoxycholate, 150 mM NaCl, 2 mM $NaVO_3$, 25 mM NaF, 0.1 mM PMSF, and 1x EDTA-free protease inhibitor cocktail (CalBiochem). After clarification of the lysates, protein concentrations were determined using the BCA Protein Assay Kit (Pierce); an equal amount of lysate protein from different samples was loaded in individual lanes of 12.5% SDS-polyacrylamide gels followed by electrophoretic separation of proteins. Proteins were then transferred to Immobilon-P PVDF (Millipore) membranes followed by immunoblotting with anti-A3G antibodies (19), anti-ERK1/2 (Santa Cruz), anti–phospho-ERK1/2 (Cell Signaling), or anti-PKR (Santa Cruz). The specificity of the anti-A3G antibody has been previously described (41). Membranes were subsequently stripped and re-probed with anti-β-actin antibodies (Sigma) to assess comparability of protein loading.

Quantitative Real-time PCR - A3G mRNA transcripts were assayed using quantitative kinetic reverse transcription PCR methodology (Applied Biosystems) and Taqman chemistry (Roche). Total RNA was extracted from pelleted cells using RNeasy (Qiagen) and subjected to on-column DNasing prior to elution of the purified RNA. A portion of the RNA was reverse transcribed and subjected to real-time PCR using an ABI Prism 7700 Sequence Detector (Applied Biosystems). A quantification standard prepared by run-off *in vitro* transcription of a molecular clone containing the A3G open reading frame was assayed along with the cellular RNAs and used to construct a standard curve. Total RNA concentrations in the extracts were measured on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and A3G mRNA was reported as copies per nanogram of total RNA.

Infection and Sorting of CD4+ T Cells - Reporter ppt-GFP viruses were produced by calcium phosphate transfection of HEK 293T cells with VSV-G envelope expression plasmid (provided by Dr. J. Burns (University of California, San Diego, La Jolla, CA)), the packaging construct CMVR8.2 (provided by Dr. D. Trono, University of Geneva, Geneva, Switzerland and described in (42)), and the lentiviral vector pRRL.cPPT.hPGK.GFP.Wpre (provided by L. Naldini, Turin, Italy). Freshly purified CD4+ T cells were stimulated with PHA for 24 hours, followed by stimulation with IL-2 for an additional 72 hours and then infected with *vsv-g* ppt-GFP virus. Cells were harvested 24-48 hours later and sorted according to GFP-positivity using a FACS DiVa (Becton Dickinson (BD)) instrument. The GFP positive, GFP negative, or unsorted fractions were then either (1) washed, lysed in LB, and analysed by fast protein liquid chromatography or (2) stained for CD3, CD4, CD25, or CD69 positivity with fluorescent-conjugated antibodies (BD) and analysed by

flow cytometry on a FACSCaliber (BD) instrument. The data were acquired using CellQuest software and analyzed with FlowJo software (TreeStar).

Analysis of A3G HMM and LMM Complexes by Fast Protein Liquid Chromatography - FPLC analysis was performed as previously described (14). Briefly, CD4+ T cells or DCs were washed in PBS (1x) and lysed in LB. Lysates were applied to a calibrated Superose 6 HR 10/30 gel filtration column attached to an FPLC apparatus (AKTA, Amersham Biosciences). The FPLC running buffer consisted of 50 mM Hepes, pH 7.4, 125 mM NaCl, 0.1% NP-40, 1 mM dithiotreitol, and 10% glycerol. One column volume (24 ml) was collected in 1 ml fractions.

RESULTS

A3G is induced by IL-2, -7, and -15. A range of individual cytokines were first tested to assess whether any altered the expression of A3G in primary CD14- PBLs. Cells were cultured for up to 120 hours with four escalating doses of each cytokine. Lysates were then prepared and subjected to SDS-PAGE and immunoblotting with anti-human A3G antibodies. IL-2 (5, 12.5, 25, 50 ng/ml) and IL-15 (1, 5, 12.5, 25 ng/ml) consistently induced moderate upregulation of A3G protein expression (Fig. 1*A*). Treatment with IL-7 (1, 12.5, 25, 62.5 ng/ml) also produced an increase in A3G protein expression at this time point (Fig. 1*A*). Immunoblotting with anti-β-actin antibodies confirmed comparable protein loading (Fig. 1*A*). Stimulation of the cells with IL-4 (5, 12.5, 25, 50 ng/ml), IL-6 (5, 12.5, 25, 50 ng/ml), IL-9 (5, 12.5, 25, 50 ng/ml), IL-12 (0.4, 1, 2, 4 ng/ml), or IL-13 (2, 5, 10, 20 ng/ml) did not significantly alter levels of A3G expression (Fig. 1*A*). All of the cytokines were biologically active based on their ability to stimulate STAT tyrosine phosphorylation (data not shown). Three independent experiments were performed with three different donors and the protein bands were quantitated using Scion Image 1.63c software. A3G protein was enhanced in PBLs treated with IL-2 (~3.5 fold) or –15 (~4 fold), while a lesser increase (~2-fold) was observed in PBLs treated with IL-7 (Fig. 1*B*).

When changes in A3G protein expression were monitored over time, IL-2 and IL-15 enhanced expression as early as 12-18 hours (Fig. 1*C*). The response to these cytokines peaked after 48 hours and persisted for 120-168 hours (Fig. 1*C*). The time course following PHA stimulation was similar (Fig. 1*C*). In contrast, IL-7 required more than 48 hours of stimulation to enhance A3G expression and this response did not peak until 7 days after initial stimulation (Fig. 1*C*). A3G protein levels in the mock- and IL-6-treated samples remained constant over the entire time course (Fig. 1*C*). Together, these findings demonstrate that IL-2 and IL-15 enhance A3G expression in primary PBLs as early as 12 hours after stimulation, with a peak response occurring approximately 48 hours after stimulation. IL-7 also increases A3G expression, but induction by this cytokine occurs with much slower kinetics and could be the result of an indirect pathway.

Interferon-treatment of monocyte-derived macrophages, but not PBLs, results in enhanced A3G expression. We next assessed the regulation of A3G protein levels in monocyte-derived macrophages. A3G is recruited into a HMM complex when monocytes differentiate into macrophages, likely contributing to the enhanced permissivity of these cells to HIV infection (14). Macrophages contain significantly less A3G protein than do monocytes relative to total levels of protein and β-actin (Fig. 1*D*). However, when macrophages were treated with interferon-α, -β, or -γ, A3G protein levels markedly increased, almost achieving the level found in monocytes (Fig. 1*D*). These results suggest that at least in macrophages, A3G may form part of the interferon response to viral infection. Similar induction of A3G expression by interferon-α and -γ has recently been described by Peng et al. (43). When PBLs were treated with interferon -α, -β, -γ, or both -α and -γ, no effect on A3G protein expression was observed, despite an increase in PKR expression (Fig. 1*E*). These results

highlight differences in the inducible A3G response occurring in macrophages and PBLs.

Enhanced A3G protein expression in PBLs correlates with higher levels of A3G mRNA and is not the result of enhanced mRNA stability. A3G mRNA levels in the same PBLs treated with the escalating doses of cytokine shown in Fig. 1*A* were assessed by quantitative real time PCR. IL-2 induced an ~8-fold increase in A3G mRNA at the highest dose (50 ng/ml), while the highest dose of IL-15 (25 ng/ml) stimulated a ~4-fold increase in A3G mRNA (Fig. 2*A*). IL-7 caused an increase in A3G mRNA, but displayed greater donor-to-donor variability at this timepoint, likely because IL-7 effects require more than 48 hours to become manifest in most donors (Fig. 2*A*). When A3G mRNA levels were analyzed over time, IL-2 and IL-15 induced upregulation of A3G mRNA as early as 6 hours following treatment, while the effects of IL-7 were slower and less-pronounced (Figs. 2*B-D*). A3G mRNA levels in the mock- , IL-6 and IL-9-treated samples remained relatively unchanged over the entire time course (Fig. 2*E*). In order to assess whether the observed increase in A3G mRNA was due to increased synthesis of mRNA or increased stability of mRNA transcripts, PBLs were stimulated with either IL-2 or IL-15 to enhance A3G mRNA levels and then tracked over time for the persistence of these transcripts after inhibiting new mRNA synthesis with Actinomycin D. A3G mRNA transcripts in the IL-2- and IL-15-treated samples decayed at a similar rate as the mock-treated sample, indicating that increased mRNA synthesis, and not enhanced mRNA stability, likely underlies the observed increase in A3G expression (Figs. 2*F,G*). Rose et al. similarly found that PMA stimulation of H9 leukemic T cells stimulated increased A3G mRNA synthesis with no effect on mRNA stability (33). These findings highlight the ability of IL-2 and IL-15 to activate *de novo* A3G gene expression in primary PBLs.

Regulation of A3G expression is sensitive to inhibitors of the JAK and MAPK signaling pathways. Although IL-2 and IL-15 play distinct roles in immunologic responses, they also share some overlapping biological properties and have nearly identical effects on T cell proliferation (44-46). The IL-2 and IL-15 receptors share common β and γ_c chains but contain different alpha chains (IL-2Rα and IL-15Rα) (47). Interaction of IL-2 with its intermediate- (IL2R$\beta\gamma_c$) or high (IL2R$\alpha\beta\gamma_c$)-affinity receptor or of IL-15 with its equivalent intermediate or high affinity receptor leads to the initiation of a variety of signaling cascades, beginning with a rapid activation of Janus kinase-1 (JAK-1) and JAK-3 bound respectively to the β and γc receptor chains (47). Activated JAK-1 and JAK-3 phosphorylate select tyrosines on both the IL-2Rβ and γ_c cytoplasmic tails thus forming docking sites for various signaling intermediates including Stat5A/B. JAK-mediated phosphorylation of tyrosine residues on the cytokine receptor also initiates other signaling cascades including activation of the mitogen-activated protein kinase (MAPK) cascade. Specifically, JAK-induced phosphorylation of the cytokine receptor recruits the adaptor protein Shc, which in turn associates with Grb2, another adaptor protein, and mSos, a guanine nucleotide exchange factor (47). These serial binding events ultimately culminate in activation of the Ras-Raf-MEK-MAP kinase pathway.

To assess whether IL-2 or IL-15 upregulation of A3G gene expression involves signaling through the JAK/MAPK signaling pathway, PBLs were pretreated with either AG490, a pan-JAK inhibitor, or U0126, a MEK inhibitor that blocks downstream activation of MAPK. Addition of either of these small molecule inhibitors effectively inhibited IL-2- and IL-15-mediated upregulation of A3G protein (Fig. 3*A*) and mRNA expression (Fig. 3*C*). IL-7 had only a slight effect on A3G protein and mRNA levels at this time point, so the action of the inhibitors could not be evaluated (Fig. 3*A* and *C*). PMA activates the MAPK pathway but not through the JAK pathway. As such, U0126, but not AG490, blocked PMA-mediated upregulation of A3G protein expression (Fig. 3*A*). When U0126 and AG490 were added in increasing amounts, a dose-related inhibition of IL-2 and IL-15-mediated upregulation of

A3G protein expression was detected (Fig. 3*B*). Similarly, both U0126 and AG490 blocked IL-2 and –15-mediated upregulation of A3G mRNA but only U0126 blocked the effects of PMA (Fig. 3*C*). The quantitation and standard error bars reflect the results of three independent experiments performed with cells from three different donors. We confirmed that IL-2 and IL-15 activate the MAPK pathway by analyzing the expression of phospho-ERK1/2, a known intermediate in the pathway. Enhanced phospho-ERK1/2 was observed in PBLs treated with IL-2 or IL-15 (Fig. 3*D*). Phospho-ERK1/2 was also elevated in PBLs treated with PMA, which was employed as a positive control (Fig. 3*D*). In addition, both AG490 and U0126 inhibited the induction of phospho-ERK1/2 in the cytokine treated cells (Fig. 3*D*). However, since PMA does not act through a JAK kinase pathway, only the MEK inhibitor U0126, and not the JAK inhibitor AG490, impaired ERK1/2 phoshorylation (Fig. 3*D*). Taken together, these results indicate that the JAK/MAPK kinase pathway may play a role in cytokine-mediated upregulation of A3G gene expression. Interestingly, IL-7-mediated upregulation of A3G occurs with significantly slower kinetics than that observed with IL-2 and IL-15, suggesting that either IL-7 less-potently triggers the JAK/MAPK kinase pathway (36) or that A3G upregulation by IL-7 involves a different, or possibly indirect, pathway.

A3G is recruited into HMM complexes following cytokine stimulation of CD4+ T cells. Since treatment of resting CD4+ T cells with cytokines or mitogens relieves a post-entry block and renders these cells permissive to HIV infection, we hypothesized that, like mitogens, cytokines may inactivate LMM A3G by triggering its recruitment into HMM RNA-protein complexes. We used gel filtration to assess the size of A3G complexes in resting CD4+ T cells that were either mock treated or treated with IL-2, IL-7, or IL-15. Samples were harvested 5 to 7 days later depending on the time required for at least 50% of the cells to express the activation marker CD25 (data not shown). We observed that all three cytokines (IL-2, IL-7 or IL-15) promoted HMM A3G formation reflected by a shift of a portion of A3G from fractions 14-17 (LMM) to fractions 7-8 (HMM) (Fig. 4). The fact that a significant fraction of A3G remained in the LMM form is consistent with the prior finding that cytokine treatment of resting T cells results in only 5 to 22% of cells acquiring permissiveness to HIV infection (37).

HIV-1 preferentially infects cells containing HMM A3G. PHA/IL-2-activated CD4+ T cells contain inactive, HMM A3G and are therefore more susceptible to infection by HIV-1 than resting CD4+ T cells, which contain an active, LMM form of the enzyme that restricts HIV infection. Since the cytokine-treated cells in our system contained a mixture of activated and less-activated cells, we sought to determine whether HIV-1 preferentially infects the cells containing HMM A3G over those with LMM A3G in such mixed population. To emulate the cytokine-treated conditions, CD4+ T cells were treated with PHA (24 hours) and IL2 (72 hours). Cells were then infected with vsv-g ppt-GFP lentiviral vectors. Two days later, the cells were harvested. 57% of the cells were CD25+, indicating that the culture contained a mixed population of activated and less-activated cells (Fig. 4*B*). The cells were then sorted based on GFP expression and assessed for purity. The GFP negative cells were nearly 100% pure, while the GFP positive cells were 90% pure, indicating a 10% contamination with uninfected cells (Fig. 4*B*). The samples were then lysed in LB, and A3G complex formation was assessed using FPLC. In the GFP positive, or infected, cells, A3G was primarily localized in a HMM complex (Fig. 4*C*). Although a small percentage of the A3G appeared to be LMM, this could stem from the 10% contamination of this population by non-GFP expressing cells or other experimental irregularities. Conversely, the vast majority of the A3G in the GFP negative populations was LMM (Fig. 4*C*). The experiment presented is representative of four independent experiments performed with cells from four different donors.

A3G expression increases during the maturation of DCs; and LMM A3G appears in

mature DCs. Potential changes in A3G expression during DC maturation were also evaluated. A3G protein levels were much higher in mature DCs compared to immature DCs (Fig. 5*A*). The presence of LMM versus HMM complexes was next evaluated in these two populations of DCs. In immature DCs, A3G was primarily localized to HMM complexes (Fig. 5*B*). This finding is consistent with the known permissivity of immature DCs to R5-tropic HIV infection, which reflects high levels of expression of the CCR5 chemokine receptor on these cells. In contrast, as reported earlier, A3G in monocytes was distinctly LMM, eluting in fractions 14-17 likely corresponding to a monomeric or dimeric form of A3G (Fig. 5*B*) (14). Interestingly, in mature DCs, which are less permissive to HIV infection than immature DCs, a higher proportion of A3G appeared in a LMM form. Further, intermediate-sized complexes present in fractions 13-15, roughly 155 kDa in size, were also more present. These findings reveal that DC maturation is associated with an upregulation of A3G expression and the appearance of LMM A3G, perhaps reflecting saturation of a limiting host factor(s) required for HMM A3G complex formation in mature DCs.

DISCUSSION

Our studies indicate that IL-2, IL-7, and IL-15 induce upregulation of A3G protein expression in PBLs. In contrast, other cytokines including IL-4, -6, -9, -12, 13 and IFN-α, β, γ and TNF-α did not alter A3G expression in PBLs. Since the IL-2 and IL-15 receptors share the same β-chain and γ_c subunits, it is perhaps not surprising that IL-2 and IL-15 exert similar inducing effects on A3G protein expression. These cytokines increased A3G expression even when they were administered in the absence of prior mitogen stimulation. Quantitative real-time PCR studies indicate that the increase in A3G protein expression is due to increased synthesis of A3G mRNA. IL-2 and -15 did not appear to alter the overall stability of A3G mRNA transcripts. In addition, since inhibiting both the activation of JAK and MAPK impaired IL-2 and IL-15 upregulation of A3G mRNA and protein in PBLs, the observed upregulation is likely the result of cytokine triggering of the JAK/MAPK signaling pathway. We have found that IL-7-stimulated upregulation of A3G occurs with much slower kinetics than the response triggered by IL-2 or IL-15, perhaps implicating a different signaling pathway or an indirect pathway. A3G expression was also enhanced during DC maturation and in macrophages treated with interferon α, β, or γ, indicating that A3G expression can be influenced by Toll-like receptor signaling and is a component of the interferon response in certain cell types.

A curious feature of our T-cell findings is that while IL-2, -7, and -15 increase the expression of A3G, which is a potent antiviral factor, these same agents render T cells permissive to HIV infection (37,38). Like cytokines, mitogens also enhance A3G protein expression in T cells (19,33); and such mitogen-treated T cells also become permissive to HIV infection. The mechanism by which mitogens act is now known: they inactivate LMM A3G by promoting its recruitment into enzymatically inactive HMM complexes that are unable to interfere with reverse transcription (14). Similarly, we find that treatment of T cells with IL-2, -7, or –15 leads to the formation of HMM A3G complexes, likely explaining how these cytokines act to render resting T cells permissive to infection by HIV-1 in the absence of full-fledged cellular activation. Thus, although these cytokines induce higher levels of A3G expression, the post-entry restricting activity of A3G is forfeited due to cytokine-induced recruitment of A3G into enzymatically inactive HMM A3G complexes. In addition, we provide additional evidence indicating that HIV-1 preferentially infects activated cells containing HMM A3G complexes in mixed populations of activated and less-activated cells.

Our recent studies demonstrate that Staufen-containing RNA transporting granules form one important component of the HMM A3G complex (34). Of note, endogenous Alu and hY retroelement RNAs are recruited to

these granules in an A3G specific manner. This recruitment interdicts Alu retrotransposition apparently by sequestering the retroelement RNA away from the nuclear LINE enzymes required for the retrotransposition of non-autonomous Alu retroelements. These findings suggest that HMM A3G complexes form to protect activated T cells from the threat posed by select mobile genetic elements. Unfortunately, this response removes the post-entry restriction block provided by LMM A3G and thus renders these cells permissive for HIV infection.

In related studies, we have found that the local action of much lower concentrations of IL-2, IL-15 and one or more additional cytokines present in lymphatic tissues underlies the permissive state of naïve CD4+ T cells to HIV infection, which contrasts sharply with the nonpermissiveness of these same cells circulating in the blood stream (48).

In the setting of A3G induction during DC maturation, low- and intermediate-sized forms of A3G appear. Whether A3G plays a role in the replicative block observed in mature DCs seems likely but is unproven. Certainly, other explanations for the differences in permissivity between immature and mature DCs are also possible, including variations in receptor expression. Nevertheless, our findings suggest that the high levels of A3G protein occurring as a result of DC maturation may saturate one or more of the cellular factors required for HMM A3G complex formation. Alternatively, the differentiation signals underlying DC maturation may actively promote HMM A3G complex disassembly and give rise to LMM and intermediate-sized forms of A3G.

Together, our findings highlight the action of distinct cytokines as inducers of A3G gene expression in different cellular targets of HIV infection. IL-2 and IL-15 play an inductive role in T-cells; the interferons in macrophages; and polyI:C/TNF-α in DCs. Our findings also reveal distinct differences in the regulation of A3G activity in these different cell populations. In cytokine-stimulated CD4 T cells, induction of A3G gene expression correlates with HMM (inactive) A3G complex formation, while in DCs the increase in A3G levels associated with maturation appears to result in the increased appearance of intermediate and LMM (active) forms of A3G. Cytokine-induced recruitment of A3G into HMM complexes correlates with the acquisition of permissiveness to HIV infection in CD4 T cells, while the appearance of LMM A3G in mature DCs correlates with the decreased permissivity of these cells to HIV infection. Whether it will be possible to manipulate the form of A3G expressed in HIV target cells for therapeutic benefit remains unclear, however exploration of this possibility merits investigation.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Inducibility of A3G Protein Expression by IL-2, IL-7 and IL-15. (*A*) Survey of Effects of Cytokines on A3G Protein Expression. Resting, CD14- PBLs were treated with increasing doses of the indicated cytokine for 5 days before harvest and immunoblotting with anti-A3G or anti-β-actin antibodies. Cytokine concentrations tested were as follows: IL-2 (5, 12.5, 25, 50 ng/ml), IL-4 (5, 12.5, 25, 50 ng/ml), IL-6 (5, 12.5, 25, 50 ng/ml), IL-7 (1, 12.5, 25, 62.5 ng/ml), IL-9 (5, 12.5, 25, 50 ng/ml), IL-12 (0.4, 1, 2, 4 ng/ml), IL-13 (2, 5, 10, 20 ng/ml), and IL-15 (1, 5, 12.5, 25 ng/ml). (*B*) Quantitative Assessment of Effects of Cytokines on Expression of A3G protein. Studies were performed as in Fig. 1(*A*) using cells from three independent donors. The A3G bands were quantitated and normalized to the β-actin loading control bands and graphed as shown. Error bars representing standard error of the mean for the three donors were also included. (*C*) Effect of Cytokines on Expression of A3G Protein Over Time. Resting, CD14- PBLs were treated with the indicated cytokine (IL-2 (50 ng/ml), IL7 (62.5 ng/ml) or IL15 (12.5 ng/ml) and then harvested 0, 6, 12, 24, 48, 120, or 168 hours after treatment. As negative controls, cells were either mock-treated or treated with IL-6 (50 ng/ml) and harvested in parallel. PHA (5 μg/ml) was employed as a positive control. The results presented were obtained with lymphocytes from a single donor and are representative of results obtained from three independent donors. (*D*) Effects of interferon on monocyte-derived macrophages. CD14+ macrophages were treated with interferon -α, -β, or -γ for 24 hours and analyzed by immunoblotting for changes in A3G protein expression. β-actin was employed as an internal protein loading control. (*E*) Effects of interferon on PBLs. CD14- PBLs were treated with interferon -α, -β, -γ, or both α and γ for 24 hours and then analyzed by immunoblotting for changes in A3G expression. PKR was used as a control to confirm that the interferons tested were biologically active.

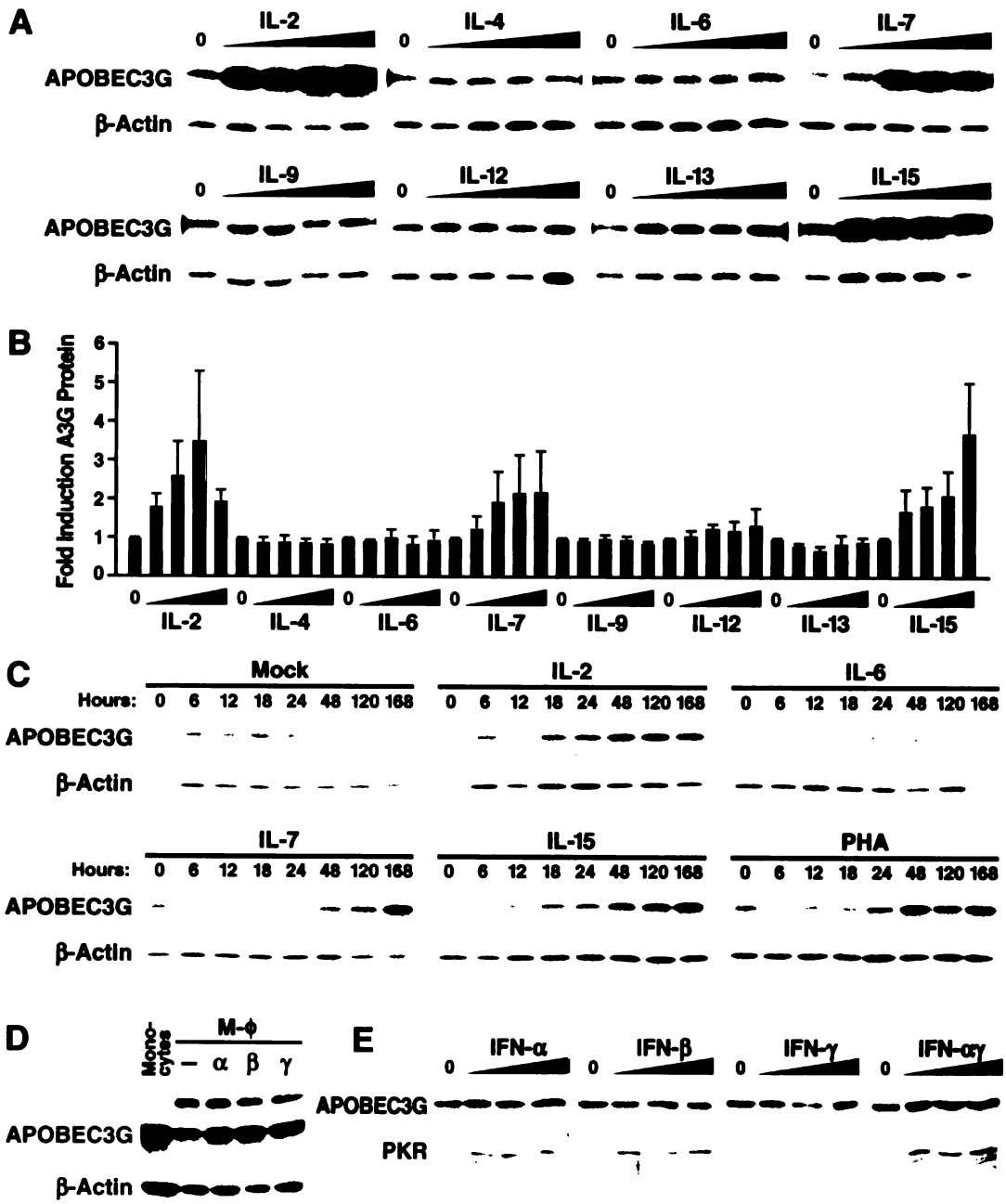
Figure 2. Induction of A3G mRNA Expression by IL-2, IL-7, and IL-15. (*A*) Effects of Cytokines on Expression of A3G mRNA. Samples were treated identically to those in Figure 1(*A*), but were collected 48 hours after treatment for analysis of A3G mRNA by quantitative real-time PCR. The data reflects the results from three independent experiments using three different donors. Error bars representing standard deviation (SD) data from three donors were included. (*B-E*) Effects of Cytokines on Expression of A3G mRNA Over Time. Samples were treated identically to those in Figure 1(*C*), and then harvested and subjected to analysis of A3G mRNA levels by quantitative real-time PCR. The results presented reflect samples taken from a single donor and are representative of results obtained from three independent donors. (*F,G*) CD14- PBLs were incubated with IL-2 (50 ng/ml) or IL-15 (12.5 ng/ml) for 24 hours before being treated with Actinomycin D (5 g/ml) at T=0. Samples were collected at the indicated time and analyzed by real-time PCR for A3G mRNA expression. The results are representative of two independent experiments.

Figure 3. JAK/MAPK Activation and Induction of A3G Gene Expression. (*A*) Effect of Inhibiting JAK or MAPK Signaling on Induction of A3G Protein Expression. CD14- PBLs were pre-treated with either AG490 (JAK inhibitor) or U0126 (MEK inhibitor) for 1 hour prior to stimulation with the indicated cytokine (IL-2 (50 ng/ml), IL-7 (62.5 ng/ml) or IL-15 (12.5 ng/ml)). Samples were harvested 48 hours later and analyzed by immunoblotting for expression of A3G protein. β-actin was employed as an internal protein loading control. The results presented are representative of two independent experiments. (*B*) Dose-dependent Effects of Kinase Inhibitors on Induction of A3G Protein Expression. CD14- PBLs were pre-treated with escalating amounts of AG490 (12.5, 50, or 100 μM) or U0126 (2.5, 10, or 20 μM) and then were analyzed as in Figure 3(*A*). (*C*) Effect of Inhibiting JAK or MAPK Activation on Induction of A3G mRNA. Samples were

treated the same as in (*A*), except cells were harvested after 24 hours and analyzed by quantitative real time PCR for induction of A3G mRNA. The results presented reflect three independent experiments conducted using three different donors. Error bars represent SD for the three donors. (*D*) Effect of AG490 and U0126 on the MAPK Kinase Pathway. CD14- PBLs were pre-treated for 1 hour with the indicated inhibitor and then incubated with the indicated cytokine or mitogen for 45 minutes. Samples were washed and lysed in RIPA buffer and then analyzed by immunoblotting for phospho-ERK1/2. Immunoblotting of ERK1/2 was performed to assess comparability of protein loading between the different samples.

Figure 4. Cytokine-Induced Formation of A3G HMM Complexes in Primary Human T Cells. (*A*) Purified CD4+ T cells were cultured 5 to 7 days with the indicated cytokine or with PHA/IL2 as a positive control. Cell lysates were loaded on a gel-exclusion column for FPLC analysis. Twenty-four one-ml fractions were collected and analyzed by immunoblotting for expression of the A3G protein. Two different donors are shown. (*B*) Separation of Infected CD4+ T Cells. Purified CD4+ T cells were infected with vsv-g pseudotyped ppt-GFP lentiviral vectors. Infected (GFP+) cells were separated from uninfected (GFP-) cells using a FACS DiVa instrument. Cells were stained for the CD25 or CD69 activation markers, or for CD3/CD4 and analyzed by flow cytometry. (*C*) FPLC Analysis of A3G Complexes in Infected versus Uninfected Cells. The sorted cells from Fig. 4(*B*) were lysed, and the samples were then subjected to FPLC analysis and analyzed by anti-A3G immunoblotting.

Figure 5. A3G Expression and Complex Formation in DCs. (*A*) Immature or mature monocyte derived DCs were analyzed for changes in A3G protein expression. β-actin was employed as an internal control for protein loading. The two donors shown are representative of results obtained in three independent experiments. (*B*) FPLC analysis of A3G Complexes in DCs. Lysates from monocytes or immature or mature DCs were analyzed by FPLC to assess the size of A3G-containing complexes. The results presented were obtained with cells from a single donor and are representative of results obtained with three independent donors.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IL-6
IL-7
APOBEC3G
β-Actin
IL-9
IL-12
IL-13
IL-15
B
Fold Induction A3G Protein
C
Mock
IL-2
IL-6
Hours: 0 6 12 18 24 48 120 168
IL-7
IL-15
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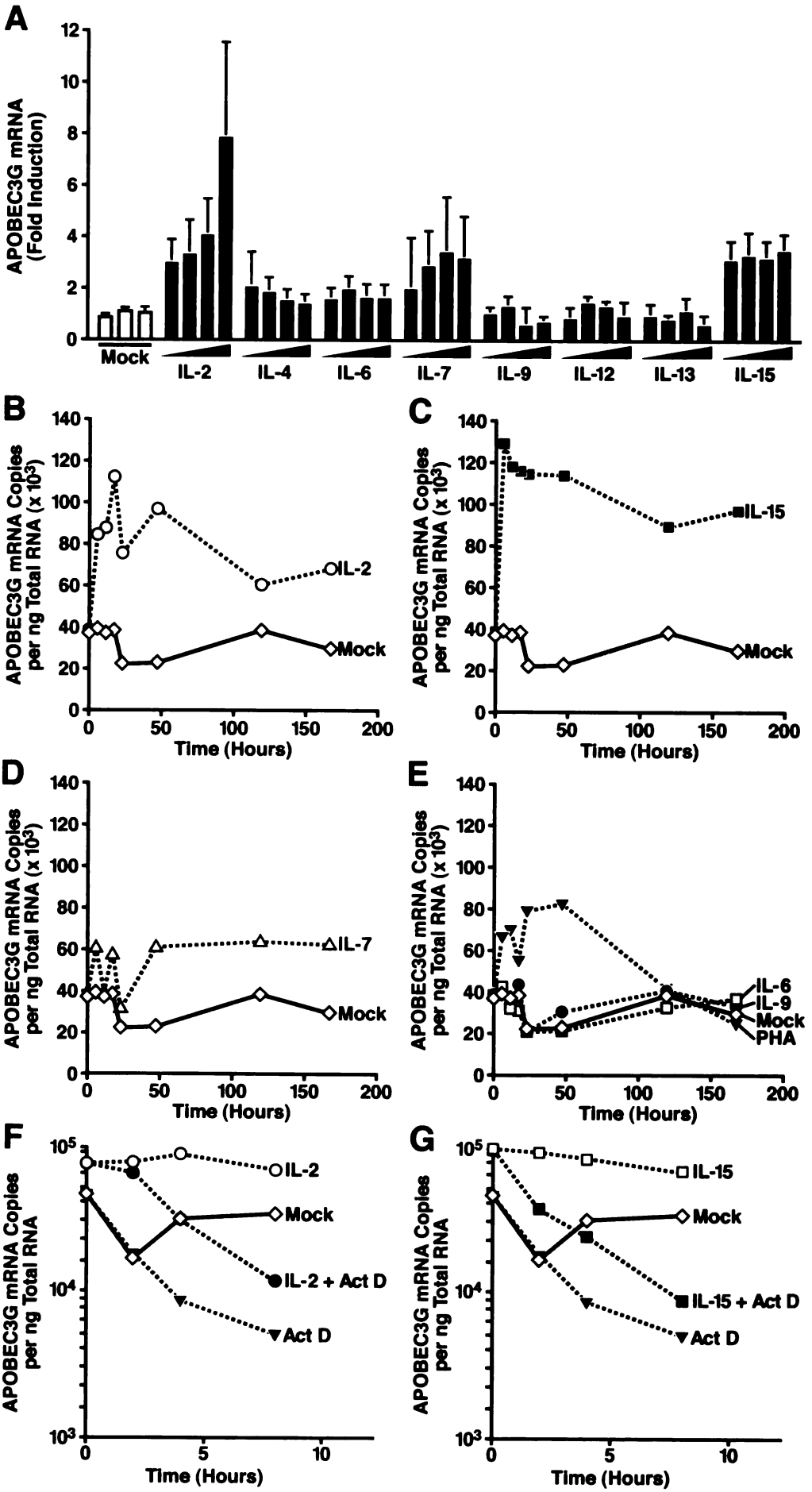
IFN-α
IFN-β
IFN-γ
IFN-αγ
PKR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IL-4
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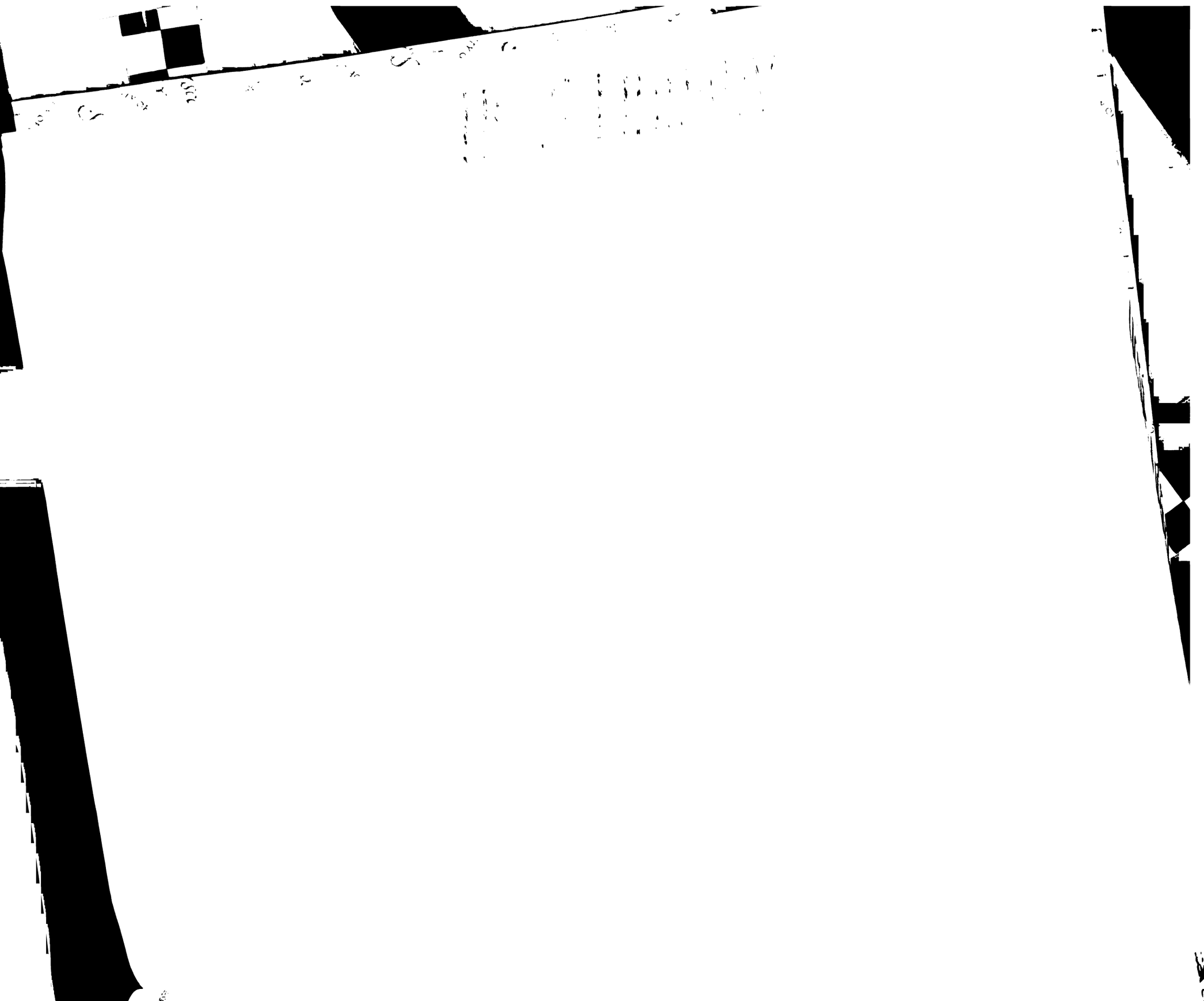
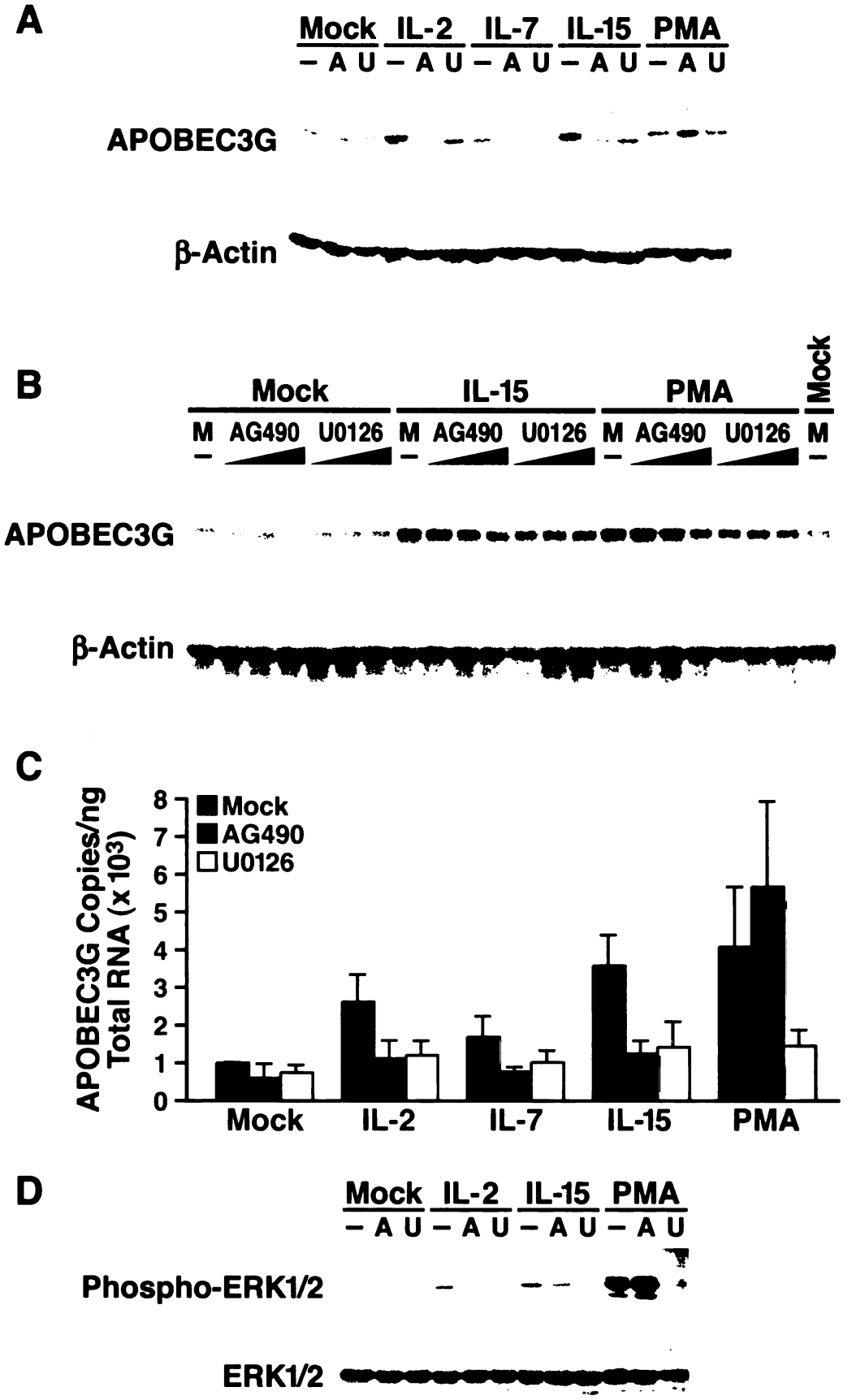
IL-9
IL-12
IL-13
IL-15
B
APOBEC3G mRNA Copies per ng Total RNA (x 10³)
IL-2
Mock
Time (Hours)
C
IL-15
Mock
D
IL-7
Mock
E
IL-6
IL-9
Mock
PHA
F
APOBEC3G mRNA Copies per ng Total RNA
IL-2
Mock
IL-2 + Act D
Act D
G
IL-15
Mock
IL-15 + Act D
Act D




A
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IL-2
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APOBEC3G
β-Actin
C
APOBEC3G Copies/ng Total RNA (x 10³)
Mock
AG490
U0126
0
1
2
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4
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8
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D
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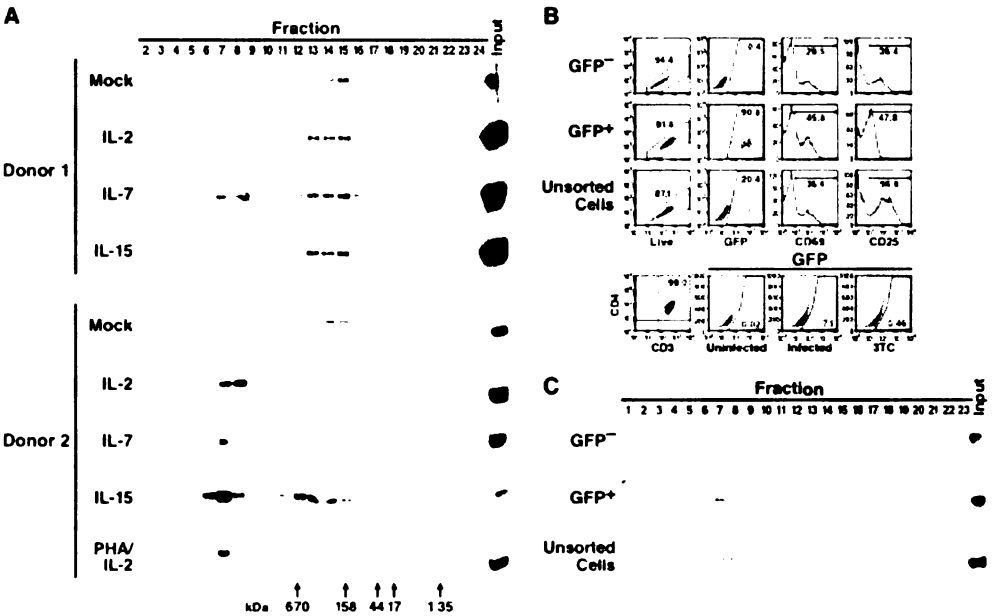
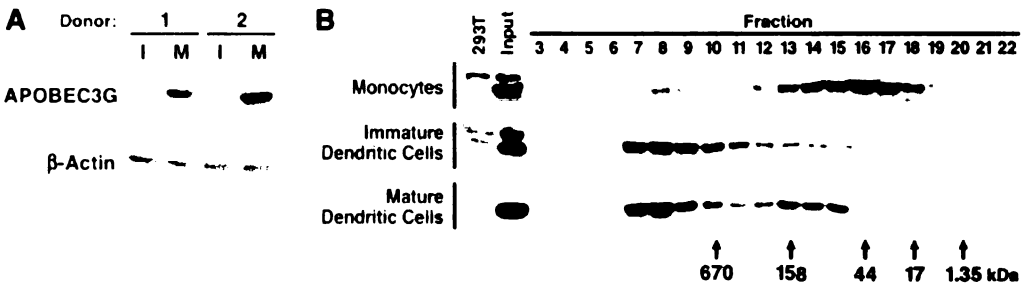
Phospho-ERK1/2
ERK1/2

Stopak et al. Figure 4



Stopak et al. Figure 5



Chapter 5

Conclusions

When we began the work presented in this dissertation, it was known that Vif played a very important role in the HIV-1 lifecycle and that it counteracted the antiviral activity of A3G. It was also known that in activated T cells, A3G needs to be incorporated into virions before exerting its antiviral activity in the subsequent target cell. We set out to determine the mechanism employed by Vif to inhibit A3G. We learned that Vif binds A3G and prevents A3G from being incorporated into T cells (12). When we compared HIV-1-infected T cells to ΔVif-HIV-1-infected cells, we observed a marked decrease in A3G expression in the presence of Vif. Measuring the decrease revealed a near 70% reduction in A3G protein in cells. Given that only 70% of the cells were infected in these studies, we concluded that Vif causes the virtual elimination of A3G from infected T cells and the residual A3G reflected protein that was present in the uninfected cells. Further investigation revealed that A3G mRNA was intact in HIV-1 infected cells, indicating that the elimination occurs at the protein level. Studies using proteasome inhibitors revealed that Vif caused marked degradation of A3G through the 26S proteasome; and pulse-chase studies revealed that Vif caused rapid turnover of A3G. We also observed that Vif partially blocked the synthesis of new A3G proteins. Together these findings revealed a coordinated effort by Vif to deplete T cells of A3G, thereby preventing virion incorporation of the A3G protein.

We next turned from the academic question of the mechanism of Vif action to the more practical question of whether these findings could help in the identification of new anti-HIV-1 drugs. We reasoned that a fairly simply method of high-throughput screening

could be developed based on the observation that Vif causes the degradation of A3G. The screen would involve a cell line co-expressing HIV-1 Vif as well as the A3G protein tagged with luciferase (A3G-Luc), which would act as a reporter in the screen. Under basal conditions, the action of Vif would prevent the expression of A3G-Luc. However, in the event that a candidate small molecule inhibited the action of Vif, the cells would score positive for A3G-Luc. Work published subsequently to our original findings lended further support to the possibility of finding a small molecule that would inhibit either the protein-protein interactions between Vif and A3G or between Vif and other elements of the E3 ligase machinery, particularly Cul5 and EloC, both of which bind directly to Vif (6, 7, 15). Single amino acid substitutions in either Vif or A3G can abrogate these interactions, indicating a short interface between Vif and A3G (1, 5, 11, 13) or Vif and the E3 ligase machinery (6, 7, 14). Our first attempts at the screen were unsuccessful for a variety of reasons. One important hurdle we solved in our next attempt was the engineering of human codon-optimized Vif, which was expressed very well, even in the absence of viral proteins such as Rev. In addition, Dr. Wes Yonemoto has engineered a new cell line using the human-codon optimized Vif under an inducible promoter. This strategy avoided the toxicity that might occur with constitutively-expressed Vif, and also provided an elegant means of ensuring that any decrease in A3G-Luc expression was actually due to the presence of Vif.

In the third set of studies, we switched our attention from how the virus regulates cellular A3G expression, to how the cell does so. We tested a panel of cytokines, chemicals naturally produced by human cells, for their effect on the expression of A3G in cells. We found that IL-2, -7, and –15 all significantly enhanced the expression of A3G

protein and mRNA through a Jak/MAPK signaling pathway. A curious aspect of this finding is that these agents also render resting CD4+ T cells permissive to HIV infection, which is counterintuitive given the enhanced presence of A3G, a powerful antiviral factor. When we studied the activity of A3G, we found that the cytokines caused A3G to shift into an inactive complex that removed the restriction to HIV infection, which correlated with the effects of mitogens on CD4+ T cells (2). When we looked at mixed populations of activated and less-activated CD4+ T cells, the more-activated cells containing a higher proportion of inactive HMM A3G complexes were more easily infected by a lentiviral vector.

In another line of investigation, we learned that interferon-treatment of macrophages, but not PBLs, leads to a marked enhancement of A3G expression. These findings were also reported by another group, who found that A3G forms a part of an anti-HIV-1 interferon response in macrophages (8).

Finally, when we compared A3G expression in immature monocyte-derived dendritic cells (DCs) with DCs that had been matured by treatment with polyI:C/TNFα, we observed a marked enhanced of A3G protein expression in the mature DCs. Interestingly, FPLC studies revealed that while immature DCs, which are permissive to HIV-1 infection, contained HMM A3G, a higher abundance of LMM and intermediate molecular mass A3G was present in mature DCs, which are more resistant to infection by HIV.

The PBL and DC data was very distinct. In the case of PBLs, enhanced A3G expression correlated with the appearance of inactive, HMM A3G complexes and enhanced permissivity to HIV-1 infection. In contrast, enhanced A3G expression in DCs

led to the appearance of active LMM and intermediate-sized A3G and correlated with enhanced resistance to HIV-1 infection. The DC data is intriguing in that it indicates that increased expression of A3G may saturate a nucleating factor in the HMM complex, permitting the appearance of overflow A3G in more active forms. Alternatively, maturation may trigger a signaling pathway in DCs leading to dissolution of HMM A3G complexes.

In summary, our studies began with the narrow question of the method employed by Vif to block the antiviral activity of A3G. Our finding that Vif mediates the degradation of A3G led to the development of a cell-based high-throughput screen for inhibitors of Vif activity. In our last set of studies, we switched focus from how the virus regulates A3G expression to how cellular factors regulate A3G expression. We found that cytokines regulate both the expression and activity of A3G in PBLs, macrophages, and DCs, and that the cellular milieu determined whether enhanced expression of A3G would lead to enhancement or repression of A3G activity.

Although an enormous amount of progress has been made in just the last four years in the Vif-A3G field, there are still many interesting questions that remain unexplored. In addition, as the years move forward, our previous understanding of Vif and A3G has also undergone some transformations. It was originally supposed that since A3G induced such massive hypermutation in viral minus-strand DNA during reverse transcription, editing was the primary means used by A3G to block viral replication. However, increasing evidence suggests that A3G may act through a non-editing mechanism as well. Deciphering that mechanism will likely be the subject of much research over the next few years. In addition, given that Vif virtually eliminates A3G

from cells, it was also originally supposed that this was the primary mechanism employed by Vif to block virion incorporation of A3G. However, increasing evidence suggests that Vif might also block A3G function through non-degradative means as well (9).

The future may also involve further investigation of the mechanism employed by A3G in interferon-treated macrophages to counteract the activity of HIV-1. Although it is clear that interferon treatment upregulates A3G expression and antiviral activity in macrophages, it is not clear whether A3G acts as a post-entry restriction factor in these cell-types or by some other mechanism. It is possible that the enhanced presence of the A3G protein in these cell-types overwhelms the Vif effect and enables some A3G to be incorporated into virions. Alternatively, interferon treatment could lead to the appearance of LMM, active forms of A3G. In preliminary studies, we have observed a slightly enhanced presence of LMM forms, but more work in this area still needs to be done.

The post-entry restricting function of the A3 proteins will likely also be the subject of further research in the future. Our lab has demonstrated that removing A3G from resting CD4+ T cells removes a post-entry block to HIV-1. However, it is also possible that other post-entry blocks mediated by other A3 proteins are also at play in these cell-types and others. Notably, investigators studying the anti-HIV potential of the A3A-H proteins performed experiments investigating virion incorporation and producer-cell-dependent antiviral activity. Whether the other A3 proteins can exert post-entry restricting behavior in T cells and other natural targets of HIV-1 infection, remains to be determined.

More research will also likely focus on the natural role played by A3G and the other A3 proteins, The presence of A3G and the other A3 proteins in primates predates modern lentiviruses (10), indicating that these proteins play a role outside of their antiviral function. Work in our lab and others has demonstrated that several of these proteins, including A3G, inhibit the retrotransposition of LTR and non-LTR retroelements (3) and that they thus play a role in defending the germ-line from retrotransposon-mediated insults. Additional functions of the A3 proteins may still be uncovered. For example, it has been previously reported that A3G is present in ovary and testis(4), yet the role played by A3G in these tissues is not fully understood.

Finally and most importantly, we hope that the culmination of our research and that of others' will result in the development of a therapeutic of benefit to AIDS patients. Perhaps the drug will be a Vif inhibitor that prevents interaction of Vif with A3G or of Vif with other members of the E3 ligase machinery. Perhaps the drug will encourage the appearance of LMM A3G, so that even activated T cells will harbor a post-entry restriction factor against HIV. Perhaps the drug will promote expression of A3G, enabling the sheer amount of A3G to overwhelm the activity of Vif. Or perhaps the drug will work in different way beyond our current imagination. Whatever the mechanism, adding Vif to the current menu of viral factors targeted by HAART will hopefully aid the effort to defeat the deadly games played by HIV.

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