EXPRESSION AND ANTIVIRAL ACTIVITIES OF THE APOBEC3 CYTIDINE DEAMINASES ARE REGULATED BY HIV-1 VIF AND CELLULAR FACTORS

by

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CERTIFICATE OF APPROVAL

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Table of Contents

Acknowledgements i

Abstract ii

Chapter 1: Introduction

I. Human Immunodeficiency Virus Type I

A. HIV-1 pathogenesis 1

B. Tools for studying HIV-1 3

C. HIV-1 life cycle 4

D. HIV-1 immune evasion strategies 6

E. Biological relevance of HIV-1 encoded proteins 8

F. Restriction of HIV-1 and other retroviruses by cellular factors 11

II. HIV-1 viral infectivity factor (Vif)

A. Cell-type specificity of Vif 13

B. Nonpermissive cell derived HIV-1(Δ*vif*) virions 16

C. Role of Vif in completion of reverse transcription 16

D. Evolution and species-specificity of Vif function 17

E. Posttranslational modifications of Vif 18

F. Nonpermissive cells contain a potent inhibitor of HIV-1 21

III. APOBECs

A. A potent cellular inhibitor of HIV-1 infectivity 22

B. APOBEC family of cytidine deaminases 23

C. APOBEC3G has a broad range of antiviral activity 29

D. Ancient Evolution of APOBEC3s 31

IV. Regulation of APOBECs and antiviral factors

A. Regulation of APOBEC-1 and AID cytidine deaminase activity 32

B. Antiviral proteins regulated by the innate immune response 34

C. Regulation by additional extracellular mitogens 35

V. Summary 40

Chapter 2: Manuscript #1 42

Chapter 3: Manuscript #2 78

Chapter 4: Manuscript #3 102

Chapter 5: Discussion 131

Appendix I 174

Appendix II 181

Appendix III 187

Appendix IV 193

Appendix V 196

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Abstract

Acquired immunodeficiency syndrome (AIDS) research has been substantially advanced by the discovery of APOBEC3G (A3G) as a key component of a potent antiretroviral pathway that occurs in the natural cellular targets of human immunodeficiency virus type 1 (HIV-1). A3G is a cytidine deaminase that causes lethal dC-to-dU deamination in the HIV-1 negative single strand DNA. Expression of A3G inactivates HIV-1(Δ*vif*) [these lack a *vif* gene that encodes the viral infectivity factor] released from cells by a factor of 50-100 fold and can be neutralized by the viral infectivity factor (Vif) encoded by HIV-1.

A key issue we addressed was the mechanism by which HIV-1 Vif was able to effectively counteract the antiviral activity of A3G. Vif binds directly to A3G and induces its degradation by a proteasome-dependent pathway. As a result, A3G is packaged specifically into HIV-1(Δ*vif*) virions and efficiently excluded from HIV-1(wt) virions. Moreover, Vif contains at least two functionally distinct domains, an amino terminal region important for binding to A3G and a carboxyl terminal region with a highly conserved SLQ(Y/F)LA motif that is essential for A3G degradation.

A series of gene duplications and domain shuffling gave rise to A3G cytidine deaminase paralogs (A3A, A3B, A3C, and A3F). We confirm that A3F has antiviral activity similar to A3G, and is counteracted by Vif. In addition, A3A and A3C weakly inactivate HIV-1(Δ*vif*) virions. A3B has antiviral activity against both HIV-1_{HXB2}(wt) and HIV-1_{HXB2}(Δ*vif*), although A3B is a more potent inhibitor of HIV-1_{HXB2}(Δ*vif*) . Interestingly, investigation of a panel of cell lines reveals that some cells highly permissive for HIV-1(Δ*vif*) contain A3G mRNA and protein at levels similar to the

nonpermissive cell line H9. Furthermore, cellular mRNA expression levels of antiviral A3B and A3F did not correlate with their ability to inactivate HIV-1 (Δ*vif*) virions. Endogenous A3G in highly permissive cells is degraded by Vif, and is specifically packaged in low but significant amounts into HIV-1(Δ*vif*) virions, suggesting that A3G is not in a sequestered site. Notably, A3G and A3B heterooligomerize with each other, and also heterooligomerize promiscuously with A3A, A3C, and A3F. However co-expression of A3A, A3B, A3C, or A3F did not interfere with the antiviral potency of A3G. These results validate other evidence that the anti-HIV-1 activities of A3G, A3F, and A3B can be strongly influenced by cellular and/or viral factors other than Vif.

Since activities of related cytidine deaminases are determined by their intracellular expression levels, one fundamental goal was to uncover factors that influence A3G expression. Interestingly, we found that levels of A3G mRNA and protein were unaffected by treatment of proliferating H9 cells with interferons or tumor necrosis factor-α, but were enhanced up to 20-fold by phorbol myristate acetate. This induction was mediated at the transcriptional level by a pathway that required activation of the protein kinase C α/βI (PKCα/βI) isozymes, mitogen-activated protein kinase kinase 1 and 2 (MEK), and extracellular signal-regulated kinase (ERK). Taken together, our data shows that expression and antiviral activity of A3G is dynamically controlled by cellular factors in human T lymphocytes.

Chapter 1

Introduction

I. Human Immunodeficiency Virus

I. A. HIV-1 pathogenesis

The World Health Organization estimates that 4.9 million individuals worldwide were infected with human immunodeficiency virus (HIV) in the year 2004, while there are an astonishing 39.4 million people living with HIV. The number of new infections in sub-Saharan Africa and Asia has increased by nearly 50% in 2004. The acquired immunodeficiency syndrome (AIDS) epidemic took the lives of 3.1 million people last year (http://www.who.int/topics/hiv_infections/en/). Although effective drug therapies are dramatically increasing the live spans of people living with HIV, these therapies are not available in underdeveloped countries. In addition, the persistence of the viral reservoir permitting the emergence of drug resistance continues to be a major obstacle to virus eradication. Attempts to develop safe and effective vaccines to circumvent HIV infection, and additional drug therapies to suppress viral replication are still crucial to the ongoing fight against the HIV-1 epidemic.

Since HIV type 1 (HIV-1) was discovered as the cause of AIDS in 1984 (7, 90), there have been thousands of publications covering every aspect of viral pathogenesis. Yet we have only begun to realize the complexity of HIV-1 infection and the difficulty of finding a true weakness in the virus that can be exploited as a target for antiretroviral therapies. The primary human response against invasion by HIV-1 is a combination of innate and adaptive immunity. The innate immune system is the first line of defense that reacts rapidly (minutes to days) to destroy viruses or control infection, although the

innate immune system has received only minor attention in HIV-1 infection until recently (25, 153). The innate immune response to HIV-1 includes induction of soluble factors (e.g. interferons) and activation of specific immune cells, mainly macrophages and dendritic cells. In addition to the conventional innate responses to retroviruses, collections of novel retroviral restriction factors have been recently identified. These restriction factors are an array of constitutively expressed genes that suppress or prevent retroviral infections (96). Adaptive immunity is a delayed immune response (days to weeks) that recognizes cells in which infection has been established and HIV-1 proteins are being readily expressed. The adaptive immune response includes antibodies directed against HIV-1 proteins, and cytotoxic T lymphocyte (CTL) responses (76). CTLs are a type of T-cell capable of recognizing and eliminating infected cells (178). However, the immune system itself is the primary target of HIV-1 (CD4+ T-lymphocytes and macrophages), which leads to the depletion of CD4+ T-lymphocytes. A decline in CD4+ T-lymphocytes significantly increases susceptibility to opportunistic infections which results in progression to AIDS (72). The success of HIV-1 is derived from its ability to evade immune recognition using several strategies, which include mutation of HIV-1 epitopes recognized by CTLs and downregulation of immune surveillance molecules CD4 and major histocompatibility complexes (MHC) from the cell surface(140, 207). Findings in this dissertation reveal a mechanism HIV-1 has evolved to avoid attack by a novel antiretroviral factor intrinsic to T-lymphocytes and macrophages.

I. B. Tools for studying HIV-1

A large number of immunological, biochemical, genetic techniques, and model systems have been developed to advance our understanding of this complex and devastating retroviral infection. Transfection of cell cultures (including 293T, COS7, and HeLa) with HIV-1 proviral DNA or expression vectors for individual viral proteins has been important in (i) identifying association of viral proteins with other viral or host proteins, (ii) dissecting the stages of envelope-mediated fusion and entry, (iii) monitoring cellular localization and trafficking of viral proteins, (iv) unraveling viral immune evasion strategies, and (v) measuring the assembly and release of viral particles. A better understanding of the HIV-1 life cycle will unveil weaknesses in the virus that can be exploited during treatment of the disease, and define novel cellular proteins and systems interacting with the virus.

Since HIV-1 establishes infection only in humans and chimpanzees and can only induce disease in humans, an animal model system to verify hypotheses and answer vital questions about disease progression has been challenging (104, 190). Even though chimpanzees are the closest genetic model to humans, their immune responses to HIV-1 tend to differ from those observed in infected humans. In addition, their use as primate model is not feasible because of the high cost of maintaining the animals and the risk of reducing an already endangered species (190). Infection of rhesus macaques with a related simian immunodeficiency virus (SIV_{MAC}), however, closely resembles HIV-1 infection and pathogenesis. The rhesus macaque model has been essential for evaluating the progression of the infection that leads to destruction of the immune system, the

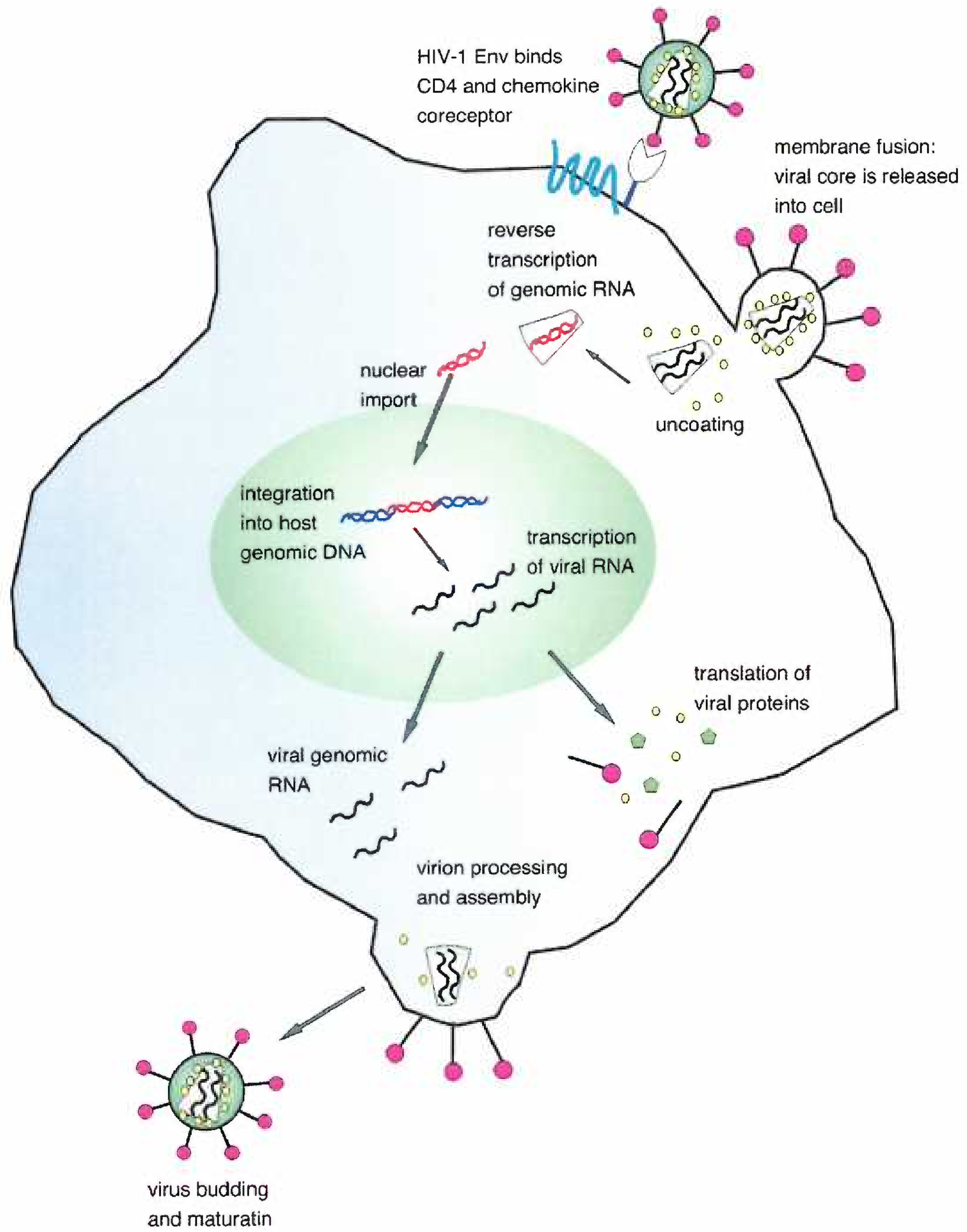
biological roles of many HIV-1 proteins, and the evaluation of various HIV-1 vaccine and therapeutic approaches (104, 190).

I. C. HIV-1 life cycle

HIV-1 infection is initiated by adsorption of virions onto CD4 expressed on the surface of cells, macrophages and T-lymphocytes being the primary viral target *in vivo*. Although the interaction of CD4 and the HIV-1 gp120/gp41 envelope glycoproteins is necessary for viral entry, the envelope must make a second contact with a chemokine receptor (usually CCR5 or CXCR4) for fusion with the cellular membrane and entry (63). Following entry the viral particle is partially uncoated in the cytoplasm and reverse transcription of the RNA genome is initiated (1). The double-stranded reverse transcription product becomes part of a nucleoprotein complex that is actively transported into the nucleus of a non-dividing cell. Full-length proviral cDNA is incorporated into the host chromosome and serves as a template for cellular RNA polymerase II (1, 37, 250). The coordinated activities of HIV-1 encoded Tat protein, cellular transcriptional activators (e.g. NFκB and Sp1), and RNA polymerase II produce high levels of HIV viral RNAs (224). HIV encodes a unique protein, Rev, to ensure that sufficient levels of both partially-spliced and unspliced viral RNAs are transported out of the nucleus (92, 93). Before adequate Rev levels accumulate in the infected cells, only fully spliced HIV-1 mRNAs encoding Tat, Rev, and Nef enter the cytosol. Unspliced RNAs are either packaged into virions as genomic viral RNA or serve as the mRNA template for Gag and Pol proteins. Partially spliced RNAs encode Vif, Vpr, Env, and Vpu proteins that are produced later in the viral infection (85) (Figure 1).

Figure 1

HIV-1 life cycle



Mature HIV-1 envelope (env) consists of two subunits, a surface glycoprotein (SU) responsible for binding to receptors and a transmembrane (TM) glycoprotein critical for fusion. The HIV-1 Env precursor, gp160, is translated into the endoplasmic reticulum (ER) and is heavily post-translationally modified while traveling through the ER and Golgi network. HIV-1 Env acquires its CD4-binding conformation prior to polyprotein cleavage (into gp120 and gp41) and transport to the plasma membrane (63). Gag and Gag-Pol precursors join with a dimer of the viral RNA genome and other viral proteins to condense at the plasma membrane and induce budding of an immature, spherical particle. During or immediately after release of the particle, proteolytic processing of Gag and Gag-Pol polyproteins by the virion incorporated HIV-1 protease (PR) generates the individual structural proteins that form the cone-shaped core characteristic of a mature HIV particle (133) (Figure 1 and 2).

I. D. HIV-1 immune evasion strategies

Through extensive efforts, we have learned a great deal about viral entry, replication, integration, as well as, budding and release of HIV-1 (Figure 1). Several factors inherent to HIV-1 pathogenesis contribute to the difficulty in treating this disease. HIV-1 infects various cell types and tissues (macrophages, T-lymphocytes, dendritic cells; gut, brain, thymus, and cervical tissues) and shortly thereafter infected cells invade the lymph nodes and begin a gradual destruction of the lymphoid tissue (55). Infected cells are normally detected by surface presentation of viral peptides by MHC class I and by CTLs of the immune system. This process is only temporarily successful in HIV-1

infection because HIV-1 destroys the T lymphocyte pool thereby preventing proper function of both CTLs and B cells producing anti-HIV-1 antibodies (99, 229). HIV-1 employs numerous mechanisms to evade detection by the immune system, including downregulation of MHC class I from the cell surface, mutations in epitopes recognized by neutralizing antibodies or CTLs, or hiding in niches that are inaccessible to CTLs (glial cells and resting T-lymphocytes) (140, 207).

A further complexity results from the inherent ability of the virus to accumulate and tolerate a large degree of mutations due to the inefficiency of reverse transcriptase and genetic recombination (46, 127, 172). An almost complete turnover of the circulating viral pool occurs each day (120) and the accelerated mutation rate of 1.6×10^{-2} nucleotide substitutions per site per year (80, 172, 298) leads to the development of viral quasi-species within an individual that can escape immune surveillance and confer drug resistance (44, 123, 140, 207).

The ever-changing face of HIV-1 has made development of therapeutic strategies difficult. Using drug combinations that target distinct and essential stages of the viral life cycle has been successful at reducing viral loads in patients, although escape variants resistant to all inhibitors eventually arise in treated patients (44, 123). Furthermore the genetic diversity within and among HIV-1 and HIV-2 clades that are spread around the world make it difficult to design drugs that will abrogate a broad spectrum of viral strains. Conserved sequence motifs that occur in widely diverse lentiviruses provide hope for sustainable and generally applicable therapeutic strategies (146, 195, 223).

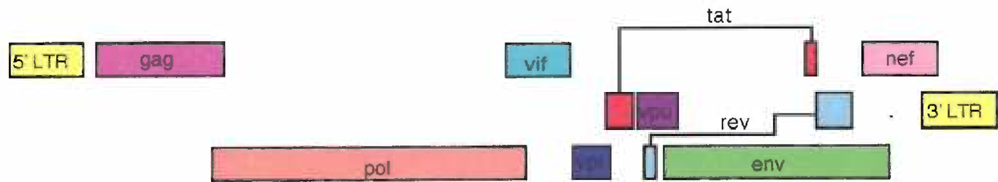
I. E. Biological relevance of HIV-1 encoded proteins

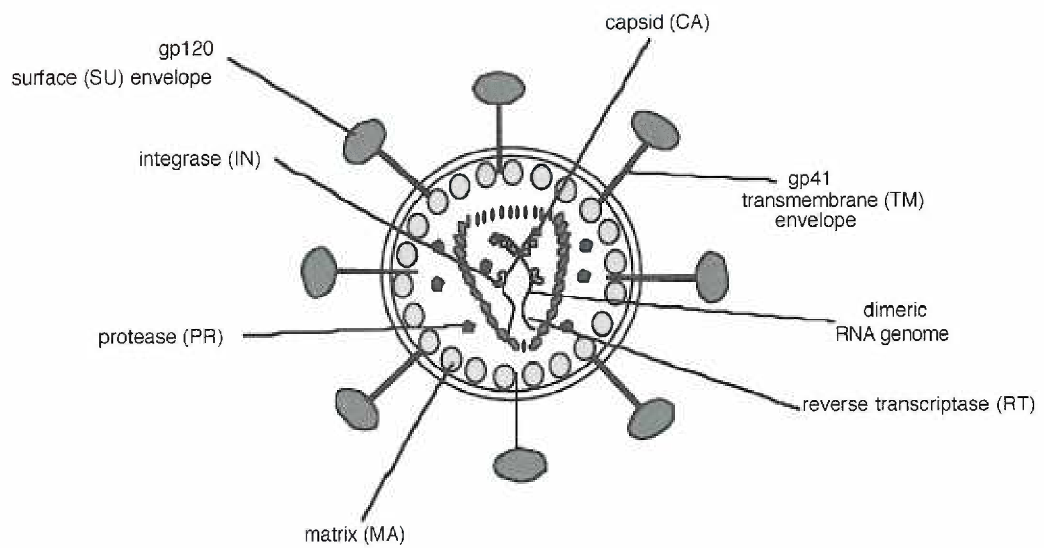
HIV-1 encodes an amazing level of complexity in its approximately 9.7 kbp genome by the use of 3 open reading frames, and singly and multiply spliced mRNAs (85) (Figure 2). The major genetic components of all retroviruses are shared by HIV-1 including *gag*, *pol*, and *env* (288). The virion structural components p24 capsid (CA), p17 matrix (MA), and p7 nucleocapsid (NC) are cleavage products of the $p55^{Gag}$ precursor protein, while *env* encodes the gp120/gp41 surface and transmembrane domains of the HIV-1 Env protein. Pol encodes four essential enzymatic products, reverse transcriptase (RT), integrase (IN), protease (PR), and RNase H (Figure 2) (133). Their unique expression in retroviruses and essential roles in early stages of HIV-1 infection make them valuable targets for drug inhibition without interfering with normal cellular function. Consequently, focused efforts have enabled successful combinatorial drug therapies that block both RT and PR functions and decrease viral loads in HIV-1 infected patients (123). Unfortunately, accumulation of 1-2 mutations within the HIV-1 genome upon each replication cycle increases the probability of generating a virus with greater fitness (80, 120, 172, 298), thereby, leading to the rapid evolution of drug resistant viral strains and the necessity for development of further therapeutic strategies (14).

Lentiviruses such as HIV-1 possess a greater genetic complexity than other groups of retroviruses, expressing six additional genes that all have essential roles in infectivity *in vivo*. *Tat* and *rev* are two regulatory genes that are essential for transcriptional regulation and transport of late mRNAs from the nucleus, respectively

Figure 2

HIV-1 genome and virion structure





(92, 93, 224). The remaining four genes were initially categorized as "accessory genes" because they were thought to be dispensable for infection *in vitro*. Subsequent comprehensive studies utilizing the natural targets of HIV-1, macrophages and T-lymphocytes, revealed that *vif* makes a vital contribution to HIV-1 replication and pathogenesis (75, 136, 151, 260, 268). Conversely, *vpr*, does not appear to play a significant role in HIV-1 growth in cycling T-cells or peripheral blood lymphocytes; however, is important in tissue macrophages (250). Despite the functional diversity of these viral proteins that could potentially be exploited for antiviral therapy, drugs are not currently available to counteract these critical viral proteins. Briefly, Vpr arrests infected cells in the G_2 stage of the cell cycle, and is involved in targeting the viral preintegration complexes to the nucleus of non-dividing cells, although Vpr's role in this process is highly contentious (8, 37, 225, 250). Vpu degrades the HIV-1 receptor CD4 through an ER degradation pathway and downregulates major histocompatibility complex (MHC) class I from the cell surface (43, 173) thereby facilitating viral release from infected cells and protection of cells from CTLs (32, 59, 242). The most studied, Nef, is essential *in vivo*, influences signaling processes, reduces cell surface expression of CD4 and MHC (43, 162, 169, 271), and enhances cell-free virus infectivity (2, 290). Despite three decades of dedicated research, however, the functional significance of HIV-1 viral infectivity factor (Vif) remained elusive. Finally, seminal experiments done independently by two groups established that nonpermissive cells contained an antiviral factor or system that HIV-1 Vif was able to efficiently counteract (166, 254), although it would take three years to discern the source of the intrinsic antiviral activity (248). This key contribution made by Sheehy et al has led to the rapid elucidation of Vif function and

revealed the absolute necessity for Vif in HIV-1 infectivity (248). Modulation of Vif function presents probably the most promising target for antiretroviral therapies to date. This dissertation describes Vif function as it relates to counteraction of a novel system of innate immune factors.

I. F. Restriction of HIV-1 and other retroviruses by cellular factors

Similar to all viruses, HIV-1 relies heavily on interaction with host cell factors in nearly all stages of the viral life cycle (1, 198, 230, 267). HIV-1 has become highly adapted to exploit cellular machinery and major advances have been made in identifying cellular factors that promote infectivity at various stages post-entry. However, not all virus-host interactions are beneficial to the virus. The constant onslaught of endogenous and exogenous infectious agents has forced an adaptive evolution in the host of innate immune strategies to combat infectious agents. Generally, innate antiviral components of the immune system have been difficult to detect, in part because the viruses that we study have by necessity become resistant to their effects. Nevertheless, tools have been developed to reveal these innate defenses and to exploit them in pharmacological and vaccine strategies. Consequently, innate immunity has become a major frontier in virus research.

A new category of innate or intrinsic antiviral factors was recently described based on species-specific restriction of retroviral infections (25, 96). Although the mechanism by which these factors operate to abolish retrovirus infection remains unclear, they all appear to interact with the CA of incoming viruses and to inhibit virus replication at a stage that precedes integration (25, 199). This retroviral restriction system originally

described in mice was called Fv-1 (Friend virus susceptibility-1) (27, 110, 208), although similar systems have since been characterized in humans (Ref-1, restriction factor-1), and Old and New World monkeys (Lv-1, Lentivirus susceptibility factor-1) (26, 52, 113, 186, 269, 279). Fv-1 is derived from a retrovirus *gag* gene encoded by an endogenous retrovirus found in multiple copies in the mouse genome (23) [for further discussion of endogenous retroviruses see section IIIC]. Two major allelic variants of Fv-1 (called Fv-1^a and Fv-1^b), were shown to permit or restrict infection by specific tropic strains of murine leukemia virus (MLV) (110, 208). Similarly, a splice variant of the cellular tripartite interaction motif 5, TRIM5α (previously called Lv-1), expressed in rhesus monkeys and African green monkeys restricts infections by lentiviruses including HIV-1(22, 113, 121, 138, 269, 302). Human (previously called Ref-1) and nonhuman primate orthologs of TRIM5α can neutralize other lentiviruses and MLV (113, 138, 203, 302).

Finally, the previously uncharacterized rat protein called zinc-finger antiviral protein (ZAP), was shown to inhibit accumulation of MLV mRNA posttranscriptionally in the cytoplasm thereby blocking the late stage of the viral life cycle (91). Although the mechanism of action is unclear, ZAP binds specifically to viral RNAs and possibly directs their degradation in exosomes (102). Additionally, ZAP conveys a broad resistance to alphaviruses (including Sindbis, Semiliki Forest, Ross River, and Venezuelan equine encephalitis virus) by blocking translation of incoming viral RNAs (24). Discovery of these intrinsic restriction factors has uncovered the existence of critical cellular defense systems against retroviruses, with additional examples perhaps still to be discovered.

II. HIV-1 Viral infectivity Factor (Vif)

II. A. Cell-type specificity of Vif

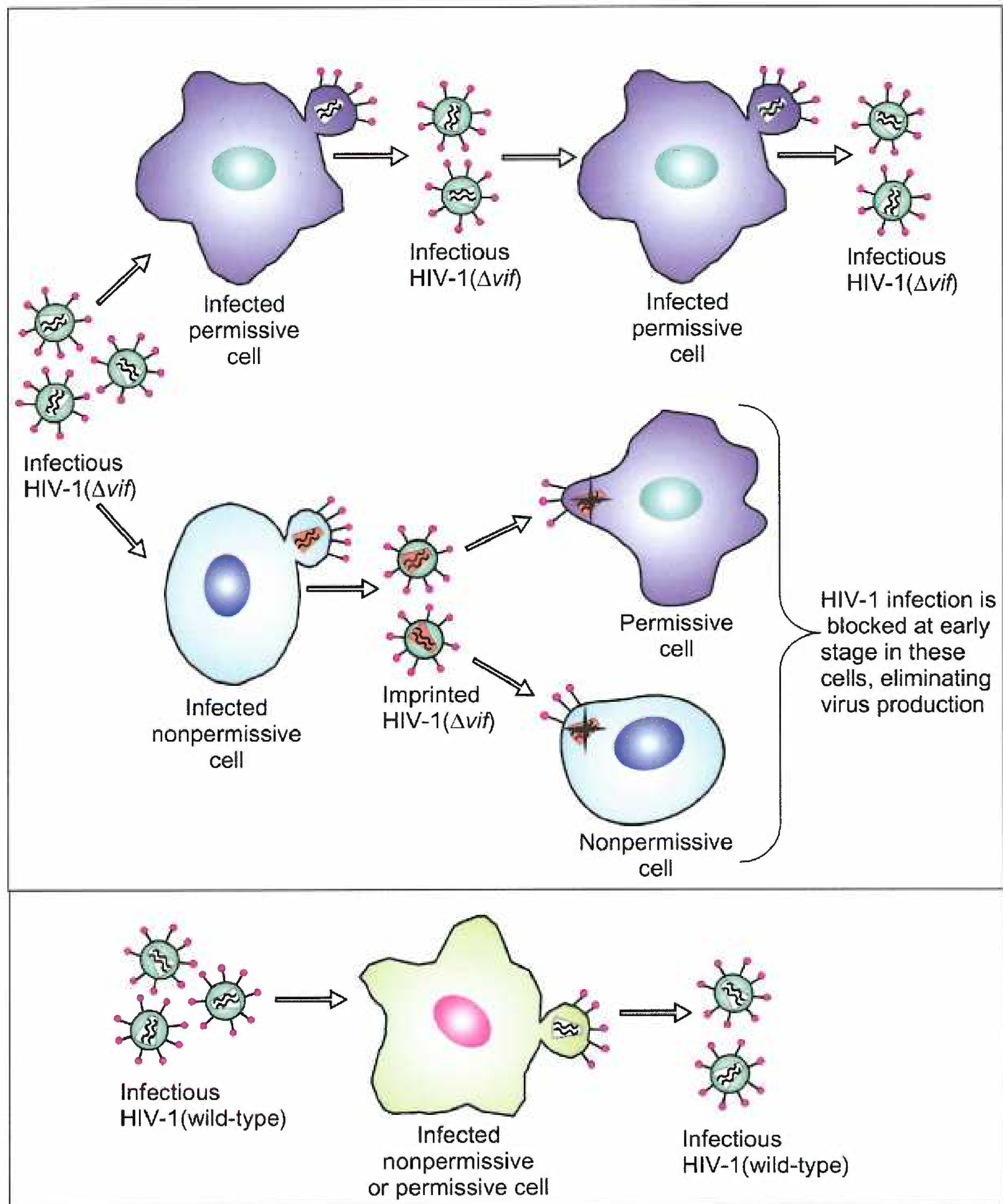
Vif is a basic M_r~23,000 phosphoprotein that is expressed in a Rev-dependent manner late in the virus life cycle (93, 130, 243). Indirect immunofluoresence showed Vif was associated with cell membranes, intermediate filaments, and that it was predominantly localized to the cytoplasm but also in the nuclei to a lesser extent (97, 135, 184, 253). Recent investigations visualizing fluorescently tagged Vif in live non-hematopoetic cells (293T and HeLa) and the T-cell line HUT78 suggest that a more substantial portion of Vif localizes to the nucleus than previously described (221, 291). These results are consistent with evidence that Vif interacts with nuclear proteins Sp140 (167) and zinc-finger protein inhibiting NFκB (ZIN) (74). The proline-rich C-terminal region of Vif is essential for membrane association and multimerization; however, it is not clear whether either of these biological properties are important for Vif function (97, 296, 297). Thorough association with HIV-1 genomic RNA and the $p55^{Gag}$ polyprotein, Vif is packaged in trace amounts (10-100 molecules/virion) into HIV-1(wild-type) virion cores (15, 31, 33, 39, 61, 78, 135, 139, 158), however, Vif's incorporation into virions has no known functional relevance.

Vif is required for HIV-1 replication in cells called nonpermissive, which include lymphocytes and macrophages and some leukemic T cell lines (86, 87, 119, 165, 256). In contrast, Vif is irrelevant for viral replication in cells termed permissive, which include other leukemic T cell lines and commonly used non-hematopoietic cell lines such as HeLa-CD4, 293T, and COS7 (31, 33, 78, 82, 86, 87, 94, 184, 219, 231, 256, 287).

However, agreement as to the permissivity of some cells lines has been complicated due to a lack of consistency in infectivity protocols and clonal differences between cell lines (226). An oddity of the cellular specificity of Vif function is that the infectivity of HIV-1(Δ*vif*) virions [these have a deletion or inactivating mutation in their *vif* gene] is determined or imprinted by the cell in which the virus is produced, rather than the cell that is infected. HIV-1(Δ*vif*) virions replicate as efficiently as wild-type HIV-1 in permissive cells (86, 287). The HIV-1(Δ*vif*) virions produced in permissive cells can infect nonpermissive cells, resulting in high fidelity proviral DNA synthesis and in production of progeny virions that have a substantially normal protein and RNA composition (78, 88, 196). In contrast, HIV-1(Δ*vif*) virions made in nonpermissive cells are negatively imprinted in a manner that severely impairs completion of reverse transcription in the subsequent cycle of infection (51, 62, 78, 88, 98, 255). These nonpermissive-derived HIV-1(Δ*vif*) virions are irreversibly inactivated, regardless of whether they are used to infect permissive or nonpermissive cells or whether these target cells contain Vif (51, 86, 287). In contrast, HIV-1(wild-type) virions replicate efficiently in nonpermissive cells without being imprinted. This imprinting phenomenon, which is diagrammed in Figure 3, has made elucidation of Vif function difficult because it is imposed in the cells producing virions while its outcome only becomes evident in subsequently infected target cells.

Notably, variants in *vif* have been linked to long term non-progression (LTNP) in HIV-1 infected patients. A single amino acid polymorphism at position 132 in Vif was correlated with LTNP in a cohort of HIV-1 infected patients (111), and inhibits HIV-1 replication in nonpermissive cell culture (83). In addition, a two amino acid insertion into

Figure 3



Vif was associated with a mother and child who were LTNPs, and the same amino acid insertion also dramatically reduced HIV-1 infection in cultured peripheral blood mononuclear cells (PBMCs) (6).

II. B. Nonpermissive cell derived HIV-1(Δ*vif*) virions

Studies to elucidate Vif function have required comparisons of wild-type and Δ*vif* virions made in nonpermissive cells. The levels of HIV-1(Δ*vif*) virions that were produced in nonpermissive cells such as H9, HUT78 (a derivative of H9), and PBMCs were comparable to the level of wild-type virions produced by the same cells (31, 51, 86, 88). Furthermore, indistinguishable levels of viral and cellular proteins and genomic RNA were detected in purified virions from both HIV-1(Δ*vif*) and HIV-1(wild-type) virions (4, 33, 34, 51, 61, 78, 79, 88, 139, 165, 196, 287). In addition, endogenous and exogenous reverse transcription assays confirmed HIV-1(wild-type) and (Δ*vif*) virion incorporated reverse transcriptase were fully functional (31, 51, 64, 88, 135, 189, 287). Finally, pseudotyping HIV-1 with the efficient VSV-G envelope (4) or MLV envelope (287) did not eliminate the Vif-dependent defect in HIV-1 infectivity. These observations confirm earlier reports that virions are not deficient in envelope-mediated entry, and implied that the block to infection is at an early step of viral replication that follows entry.

II. C. Role of Vif in completion of reverse transcription

After extensive studies, the most prominent defect in nonpermissive cell derived HIV-1(Δ*vif*) virions appeared to be at a step that closely followed viral entry into cells.

Early observations led to the hypothesis that nucleoprotein (preintegration) complexes were improperly formed resulting in an abortive reverse transcription process (31, 51, 255). More specifically, these imprinted virions were unable to fully synthesize proviral DNA (31, 189, 255, 287). Correspondingly, the viral DNA synthesis defect increased progressively as the products were representative of longer reverse transcription DNA species (88, 98, 189). In contrast to a wild-type infection, HIV-1(Δ*vif*) reverse transcription products in nonpermissive cells are not maintained for prolonged periods which led to initial suggestions that viral nucleic acids are cleared by a host degradation mechanism (31, 258, 189).

II. D. Evolution and species-specificity of Vif function

Vif (also called *sor* in HIV-1 or Q in other lentiviruses) has been evolutionarily conserved in all lentiviruses including the closely related HIV-2, simian (SIV), feline (FIV), and bovine immunodeficiency viruses (BIV), caprine arthritis encephalitis virus (CAEV), and Maedi-visna virus (visna virus), and is absent only in the equine infectious anemia virus (EIAV) (105, 106, 144, 160, 217, 246, 278). HIV-2 and SIV_{MAC} Vif share 30% sequence identity to HIV-1 Vif while BIV and visna virus Vifs share only 8% identity (Figure 4). Highly conserved motifs; however, were located in these widely diverse Vifs. 34 of 38 lentivirus Vif sequences analyzed contained the SLQXLA motif (X being F or Y in primate viruses and R in nonprimate viruses) located in the C-terminus (195). Consequently, deletion of part or all of this domain abolishes HIV-1 infection in nonpermissive cells (82, 257). In addition HIV-1, HIV-2, most SIVs, and BIV share another conserved domain (SL(I/V)X_4YX_9Y) near the Vif amino terminus

(195). Finally, two conserved cysteine residues at position 114 and 133 are critical for Vif function (82, 83, 142, 163, 232, 257). (Figure 4) Analysis of HIV-1 sequences isolated from patients shows Vif has evolved rapidly by positive selection, which is consistent with selective pressure from an antagonistic interaction (298). The conservation of the Vif reading frame throughout evolution substantiates its essential role in the viral life cycle, while the variable regions could be just as important for determining species-specificity, for example, interaction of Vif with host cellular proteins.

HIV-2 and SIV_{MAC} Vif are able to compensate for the HIV-1(Δ*vif*) replication defect when expressed *in trans* in nonpermissive human cells producing virus (89, 258). Similarly, a chimeric SIV_{MAC} encoding HIV-1 Vif, replicates with efficiency comparable to wild-type SIV_{MAC} in rhesus monkey PBMCs (134). In contrast, SIV_{AGM} (African green monkey) Vif, SIV_{SYK} (Sykes' monkey) Vif or nonprimate Vif proteins from BIV, FIV, and visna virus could not stimulate the replication of *vif*-deleted HIV-1 or HIV-2 in nonpermissive human cells (89, 256, 258). However, HIV-1 Vif expressed in nonpermissive human cells was able to rescue *vif*-deleted SIV_{AGM} replication (256). Taken together, this represents compelling evidence for a species-specific interaction between Vif and producer cell component(s) that may influence functionality of Vif

II. E. Posttranslational modifications of Vif

Vif is phosphorylated *in vivo* at four sites in the C-terminus Ser^{144}, Thr^{155}, Thr^{188}, and Ser^{165},and one site in the N-terminus Thr^{96} (299, 300). Extracellular signal-regulated kinases p44/42 (ERK-1/2) phosphorylates Ser^{165} and Thr^{96}. Mutations of Thr^{96} or Ser^{144}

Figure 4. Alignment of lentiviral Vif sequences

Shaded regions indicate conserved sequences among lentiviral Vifs identified by Oberste et al (195). The grey shaded region is the N-terminal conserved sequence (SL(I/V)X_4YX_9Y). Pink shaded residues represent a conserved SLQ(Y/F)LA motif essential for Vif function. Yellow shaded cysteines (C) are conserved and also important for Vif function.

Figure 4

```
HIV-1 HXB2     1 M-EN--R-WQV-MIVWQV-DR--MRIRTWKS-LVKHHM--YVSG------  33
HIV-2 ROD      1 MEEDK-R-WIV-VPTWRV-PG--R-MEKWHS-LVKYLK--YKTK------  34
SIV mac239     1 MEEEK-R-WIA-VPTWRI-PE--R-LERWHS-LIKYLK--YKTK------  34
SIV AGM        1 MNPNK-E-WVM-RVTWKV-PG--DLITKWQG-IVRYWM--RQ-R------  34
BIV            1 MERTL-Q--SV-VGRRRG-SS--NRGRGKNS-LISTPS--YALH------  34
FIV            1 MSEE--D-WQVSKGLFAVLQGGVHSAMLYISELPEMEKEQYKKEFKKRLL  47
CAEV           1 M-QNLFRHRRN-RRDRRIGPE--LPLSLW-T-YTAYSI--NK--------  34
                 *

HIV-1 HXB2    34 KAR-GWFYRHHYE--SPHPRIS-SEV--HIPL---GD--AR-LVITTY--  69
HIV-2 ROD     35 DLE-KVCYVPHHK--VGWAWWTCSRV--IFPL---KGN-SH-LEIQAY--  72
SIV mac239    35 DLQ-KVCYVPHFK--VGWAWWTCSRV--IFPL---QEG-SH-LEVQGY--  72
SIV AGM       35 NL--KWNYYMHYQ--ITWAWYTMSRY--VIPI---GKH-GE-ICVDLY--  71
BIV           35 PP--PRFRYPRWEF-VRQTEYS-MTA--CVRK---GK------LVLTYQ-  68
FIV           48 DKETGFIRRLRKAEGIKWSFHTRDYYMGYVKELVAGSSTPDSLRLYIYIS  97
CAEV          35 DP--AWYTTLRLQQ-MMWHRRGNKLT--YVRE---NAQYEE-WEMTSYE-  74
                           .                                  .  *

HIV-1 HXB2    70 ---WGLH-----TG--ERDWHLGQGVSIE--WRK---KRY-STQVDP-EL 102
HIV-2 ROD     73 ---WNL------TP--EKGWLSSYSVRIT--WYT---EKF-WTDVTP-DC 104
SIV mac239    73 ---WHL------TP--EKGWLSTYAVRIT--WYS---KNF-WTDVTP-NY 104
SIV AGM       72 ---WHL------TP--EQGWLSTYAVGIQ--YYSNLESKY-RTELDP-AT 106
BIV           69 ---YAI-------W--KRVWTIETGFTDP-SLFM---TPA-GTHTTE-EI 100
FIV           98 NPLWHGK----YRPG-LKNFNKEWPFVNM--WIK---TGFMWDDIEKQKI 137
CAEV          75 ---WRIRMRRDKTKSHPRGHTSPWQYRRQDGWKD---VGT-WF-LQPGDY 116
                                   .

HIV-1 HXB2   103 --ADQLIHLYY-----------FDC---FSDS----AIRKALLG-HI--- 128
HIV-2 ROD    105 --ADVLIHSTY-----------FPC---FTAG----EVRRAIRG-EK--- 130
SIV mac239   105 --ADILLHSTY-----------FPC---FTAG----EVRRAIRG-EQ--- 130
SIV AGM      107 --ADSIIHGHY-----------FNC---FKER----AIQQALRG-HR--- 132
BIV          101 GHLD-LFWLRY-----------CSC---PHEMP---PWLDFLRGTLN--L 130
FIV          138 C-VGGEISPGWGPGMVGIAIKAFSCGERKIEATPVMIIREEIDP-KK--W 183
CAEV         117 RKADQQFWFAWRI---------VSCSC-KKEGF---NIREFMLGTHRWDL 153
                                          *                   .

HIV-1 HXB2   129 VSPRC-EYQAGHNK---VGSLQYLALAALI--TPKKIK-PPLPSVTK-LT 170
HIV-2 ROD    131 LLSCC-NYPRAHRA--QVPSLQFLALVVVQQNDRPQRD-STTRKQRR-RD 175
SIV mac239   131 LLSCC-RFPRAHKY--QVPSLQYLALKVVS-DVRSQGE-NPTWKQWR-RD 174
SIV AGM      133 FVFC--QFPEGHKSTGQVPSLQYLALLAHQNGLRERSKRGKTRRSRN-LG 179
BIV          131 RISCRRALQASVLTSTPRHSLQRLAALQLCTNACLCWY--PLGRIND--T 176
FIV          184 CGDCW-NLMCLRNS--PPGTLQRLAMLACG-RKAKCWR--GCCNQRF--V 225
CAEV         154 CKSCC-QGEVVKRT--QPYTLQRLTWLKLTEDHVFQVM--PLWRARKGIT 198
                                    .** *.

HIV-1 HXB2   171 ED-RW----NKPQ-KTKG--HRGSH---------T-MN---G-------- 191
HIV-2 ROD    176 YR-RG--LRLAKQ-DSRSHKQRSSE------SPTPRTY-FPG-------- 206
SIV mac239   175 NR-RG--LRMAKQ-NSRGDKQRGGK------PPTKGAN-FPG-------- 205
SIV AGM      180 SK-QGAVGQMAKRYVTRS--QPGGEAAFWERTPVPSMELLSGGRRKTWYS 226
BIV          177 TP-LW---------LNFS---SGKE---------PTIQQLSG-------- 196
FIV          226 SP-YR-----TPA-DLEV--IQYKP--------GWNLL-WLG-------- 249
CAEV         199 IDFPW------CR-DTKGFLEPWTT------QECWQIE-YPL-------- 226

HIV-1 HXB2   192 ----H       192
HIV-2 ROD    207 -VAEVLEILA  215
SIV mac239   206 -LAKVLGILA  214
SIV AGM      227 HDGKGLQIL   235
BIV          197 ----HP      198
FIV          250 ----EL      251
CAEV         227 ----EDE     229
```

(contained within the SLQXLA motif), which are highly conserved among HIV-1, HIV-2, and SIV, resulted in significant loss of Vif activity and HIV-1 replication. Phosphorylation of Ser^{144},Thr^{155}, Thr^{188} are regulated by unknown cellular serine/threonine kinases (299, 300).

Recent evidence revealed that Vif is monoubiquitinated (67, 180) through its association with two HECT E3 ubiquitin ligases, Nedd4-1 and AIP4, that are both expressed in T-lymphocytes (67). Several recent publications suggest that steady-state Vif levels increase upon proteasome inhibition, implying that monoubiquitinated Vif could be a target for degradation. Data supporting this mechanism, however, are controversial (3, 67, 81, 180, 291). Instead, ubiquitin modification may alter Vif's subcellular localization or function (67). The topic of Vif ubiquitination and its influence on infectivity is reviewed further in the discussion section.

II. F. Nonpermissive cells contain a potent inhibitor of HIV-1

The cellular specificity of Vif function diagrammed in Figure 3 could theoretically have two explanations. Permissive cells might have a cellular protein that functions like viral Vif to enhance HIV-1 infectivity by a factor of 50-100 fold (31, 33, 78, 82, 86, 87, 94, 119, 166, 219, 231, 248, 254, 287), so that the viral Vif is dispensable. Alternatively, nonpermissive cells might contain a potent inhibitor of HIV-1 that is neutralized by Vif. These possibilities were distinguished by fusing permissive and nonpermissive cells. The nonpermissive phenotype was dominant in the heterokaryons, implying that nonpermissive cells contain an antiviral factor that is absent in permissive cells (166, 254). This suggested that lymphocytes and macrophages, the major natural

cellular targets of HIV-1, contain a powerful means to destroy HIV-1 and that this innate protective system is neutralized by Vif.

The search for the elusive innate antiviral factor that was counteracted by Vif, led to discovery of multiple Vif interacting proteins. Several include intermediate filament vimentin (135), ATP-binding protein HP68 (315), tyrosine kinase Hck (112), nuclear body component Sp140 (167), and zinc-finger protein inhibiting NF-κB (ZIN) (74). The implication of these proteins in terms of Vif-dependent HIV-1 infectivity remains unclear, although their significance may soon be uncovered with rapid advances in understanding Vif's role in infectivity.

III. APOBECs

III. A. A potent cellular inhibitor of HIV-1 infectivity is blocked by HIV-1 Vif

A major contribution to this field was the discovery by Sheehy et al of a nonpermissive cellular protein called CEM15, also termed APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) (248). This was accomplished by subtractive cDNA hybridization of cDNAs of the nonpermissive cell line CEM with the closely related permissive cell line CEM-SS. Consistent with Figure 3, APOBEC3G (A3G) does not inhibit production of HIV-1 virions, although it drastically and specifically reduces the infectivity of HIV-1(Δ*vif*) virions. More importantly, they showed that A3G overexpression in permissive cell lines converted them to the nonpermissive phenotype. In addition, they reported that A3G was expressed in nonpermissive cell lines in addition to CEM and that it was absent or undetectable in the permissive cell lines tested. These results suggested that A3G is necessary and sufficient

to confer the nonpermissive phenotype on cells. Because only lymphocytes and macrophages are believed to be nonpermissive, the idea that A3G is necessary and sufficient for the nonpermissive phenotype is difficult to reconcile with evidence of A3G expression in various tissues (testes, ovary, spleen) and cell lines (Appendix I) (226) and numerous cancer cells (109, 126). This broad range of expression suggested (i) A3G could be active against a broader range of viruses (ii) A3G may have other cellular functions (iii) A3G could be inactive or ineffective in some cells. For example, A3G is apparently present in low concentrations in resting T cells and is strongly induced when they are activated with phytohemagglutinin (Appendix I) (266). Points (ii) and (iii) were investigated further in this dissertation.

Initially, it was reported that A3G is incorporated equally into HIV-1(Δ*vif*) and HIV-1(wild-type) virions derived from nonpermissive cells (248). Recent studies, however, have unambiguously established that A3G is incorporated into HIV-1(Δ*vif*) virions by interaction with HIV-1 NC and possibly viral genomic RNA or cellular RNA (5, 41, 65, 238, 273, 310) and is specifically and efficiently excluded by Vif from HIV-1(wild-type) virions (132, 157, 174, 175, 180, 249, 266, 309). Additionally, A3G phosphorylated by cellular tyrosine kinases Fyn and Hck is preferentially incorporated into HIV-1 virions (68), suggesting these kinases play an important role in A3G antiviral activity.

III.B. APOBEC family of cytidine deaminases

A3G is closely related to APOBEC1, a cytidine deaminase that causes a specific cytosine-to-uracil (C-to-U) editing change in the apolipoprotein B mRNA (212). Other

members of this protein family include APOBEC2, APOBEC3A, -3B, -3C, -3D, -3E, and 3F (A3A-A3F), and the AID enzyme that causes class switch recombination and hypermutation of immunoglobulin genes (Figure 3) (109, 126, 154, 185, 220). A3G is encoded on chromosome 22 in a region that also contains the genes for APOBEC3A to 3F (126). A3D is believed to be unexpressed and the A3E locus encodes a pseudogene (109, 126). Phylogenetic analysis identified AID and APOBEC2 as the likely ancestral root of all the cytidine deaminases, with APOBEC3 members being mammalian-specific derivatives that arose through gene duplications and complex domain shuffling (Figure 5) (50, 126). APOBEC family members share a consensus catalytic domain (H/C)-(A/V)-E-$(X)_{24-30}$-P-C-$(X)_2$-C containing two zinc-coordinating residues, and a glutamate residue that acts as a proton donor for the deamination reaction (109, 126). Interestingly, A3G, A3F, and A3B all contain a duplication of the catalytic domain in their C-terminus (Figure 5) (126). Cytidine deaminases contain conserved characteristics including the ability to bind Zn^{2+} and RNA or DNA, and form dimers or multimers (126). Recent reports suggest the functionality of the N-terminal and C-terminal catalytic domains of A3G are separable, although conclusions as to the critical roles of each domain for the antiviral activity of A3G were conflicting (191, 192, 251). These results will be reviewed further in the Discussion section. In a yeast two-hybrid system A3G interacted with itself and A3B but not APOBEC1(126), and A3B homodimerizes *in vitro* (305). Similar to APOBEC1, purified A3G and A3B expressed in baculovirus efficiently bind Zn^{2+} and apolipoprotein B mRNA and AU-rich RNA substrates but not an HIV-1 RNA substrate (126). A3A, A3B and A3G lack RNA cytidine deaminase activity *in vitro* (126), although an *E. Coli* based assay system revealed that APOBEC1, A3G, A3B, and A3C

Figure 5 **(A)** Alignment of human APOBEC3 paralogs. Grey shaded residues indicate the catalytic domains of APOBEC3s. A3B, A3G, and A3F contain a second copy of the catalytic domain in the N-terminus. The chart values represent the percent amino acid sequence identity between APOBEC3s. **(B)** Alignment of A3B, A3F, and A3G.

Figure 5 (A)

```
A3A     1                                                      0
A3B     1 MNPQIRNPMERMYRDTFYDNFENEPILYGRSYTWLCYEVKIKRGRSNLLW  50
A3C     1                                                      0
A3F     1 MKPHFRNTVERMYRDTFSYNFYNRPILSRRNTVWLCYEVKTK-GPSRPRL  49
A3G     1 MKPHFRNTVERMYRDTFSYNFYNRPILSRRNTVWLCYEVKTK-GPSRPPL  49

A3A     1                                                      0
A3B    51 DTGVFRGQVYFKPQYHAEMCFLSWFC-GNQLPAYKCFQITWFVSWTPCPD  99
A3C     1                                                      0
A3F    50 DAKIFRGQVYSQPEHHAEMCFLSWFC-GNQLPAYKCFQITWFVSWTPCPD  98
A3G    50 DAKIFRGQVYSELKYHPEMRFFHWFSKWRKLHRDQEYEVTWYISWSPCTK  99

A3A     1         MEASP-------AS----------------------------   7
A3B   100 CVAKLAEFLSEHPNVTLTISAARLYYYWERDYRRALCRLSQA--G--ARV 145
A3C     1                                                      0
A3F    99 CVAKLAEFLAEHPNVTLTISAARLYYYWERDYRRALCRLSQAG----ARV 144
A3G   100 CTRDMATFLAEDPKVTLTIFVARLYYFWDPDYQEALRSLCQKRDGPRATM 149

A3A     8 -------------------G----P----------------------RHL  12
A3B   146 TIMDYEEFAYCWENFVYNEGQQFMPWYKFDENYAFLHRTLKEIL---RYL 192
A3C     1   MNPQ--------------------------------------IRNPMKA  11
A3F   145 KIMDDEEFAYCWENFVYSEGQPFMPWYKFDDNYAFLHRTLKEILRNPMEA 194
A3G   150 KIMNYDEFQHCWSKFVYSQRELFEPWNNLPKYYILLHIMLGEIL---RHS 196

A3A    13 MDPHIFTSNFNNG---IGRHKTYLCYEVERLDNGTSVKMDQHRGFLHNQA  59
A3B   193 MDPDTFTFNFNNDPLVLRRRQTYLCYEVERLDNGTWVLMDQHMGFLCNEA 242
A3C    12 MYPGTFYFQFKNLWEANDRNETWLCFTVEGIKRRSVVSWK--TGVFRNQV  59
A3F   195 MYPHIFYFHFKNLRKAYGRNESWLCFTMEVVKHHSPVSWK--RGVFRNQV 242
A3G   197 MDPPTFTFNFNNEPWVRGRHETYLCYEVERMHNDTWVLLNQRRGFLCNQA 246
          * *  *   * *     *  . **. .* .   . *      *   *.

A3A    60 KNLLCGFY-GRHAELRFLDLVPSLQLDPAQIYRVTWFISWSPCFSWGCAG 108
A3B   243 KNLLCGFY-GRHAELRFLDLVPSLQLDPAQIYRVTWFISWSPCFSWGCAG 291
A3C    60 DSET-----HCHAERCFLSWFCDDILSPNTKYQVTWYTSWSPCPD--CAG 102
A3F   243 DPET-----HCHAERCFLSWFCDDILSPNTNYEVTWYTSWSPCPE--CAG 285
A3G   247 PHKH-GFLEGRHAELCFLDVIPFWKLDLDQDYRVTCFTSWSPCFS--CAQ 293
                      ***  **       *     * ** . *****    **

A3A   109 EVRAFLQENTHVRLRIFAARIYDY-DPLYKEALQMLRDAGAQVSIMTYDE 157
A3B   292 EVRAFLQENTHVRLRIFAARIYDY-DPLYKEALQMLRDAGAQVSIMTYDE 340
A3C   103 EVAEFLARHSNVNLTIFTARLYYFQYPCYQEGLRSLSQEGVAVEIMDYED 152
A3F   286 EVAEFLARHSNVNLTIFTARLYYFWDTDYQEGLRSLSQEGASVEIMGYKD 335
A3G   294 EMAKFISKNKHVSLCIFTARIYDD-QGRCQEGLRTLAEAGAKISIMTYSE 342
          *.  *.  . .* * **.**.*        .* *. *   *  . ** * .

A3A   158 FKHCWDTFVDHQGCPFQPWDGLDEHSQALSGRLRAILQNQGN* 200
A3B   341 FEYCWDTFVYRQGCPFQPWDGLEEHSQALSGRLRAILQNQGN* 383
A3C   153 FKYCWENFVYNDNEPFKPWKGLKTNFRLLKRRLRESLQ*     191
A3F   336 FKYCWENFVYNDDEPFKPWKGLKYNFLFLDSKLQEILE*     374
A3G   343 FKHCWDTFVDHQGCPFQPWDGLDEHSQDLSGRLRAILQNQEN* 385
          * **.**  **.** **  .  * * *.
```

APOBEC3 paralogs (percent identity)

	A3B	A3C	A3F	A3G
A3A	45	36	20	34
A3B		19	58	57
A3C			39	20
A3F				49

Figure 5 (B)

```
A3B    1 MNPQIRNPMERMYRDTFYDNFENEPILYGRSYTWLCYEVKIKRGRSNLLW  50
A3F    1 MKPHFRNTVERMYRDTFSYNFYNRPILSRRNTVWLCYEVKTK-GPSRPRL  49
A3G    1 MKPHFRNTVERMYRDTFSYNFYNRPILSRRNTVWLCYEVKTK-GPSRPPL  49
         * *. ** .********  ** * ***  *   ******* * * *

A3B   51 DTGVFRGQVYFKPQYHAEMCFLSWFCG-NQLPAYKCFQITWFVSWTPCPD  99
A3F   50 DAKIFRGQVYSQPEHHAEMCFLSWFCG-NQLPAYKCFQITWFVSWTPCPD  98
A3G   50 DAKIFRGQVYSELKYHPEMRFFHWFSKWRKLHRDQEYEVTWYISWSPCTK  99
         *. .******     * ** *  **.   .*   . ...**..**.**

A3B  100 CVAKLAEFLSEHPNVTLTISAARLYYYWERDYRRALCRLSQA--G--ARV 145
A3F   99 CVAKLAEFLAEHPNVTLTISAARLYYYWERDYRRALCRLSQA--G--ARV 144
A3G  100 CTRDMATFLAEDPKVTLTIFVARLYYFWDPDYQEALRSLCQKRDGPRATM 149
         *   .* **.* * *****  *****.*. **. **  *.*   *  * .

A3B  146 TIMDYEEFAYCWENFVYNEGQQFMPWYKFDENYAFLHRTLKEILR---YL 192
A3F  145 KIMDDEEFAYCWENFVYSEGQPFMPWYKFDDNYAFLHRTLKEILRNPMEA 194
A3G  150 KIMNYDEFQHCWSKFVYSQRELFEPWNNLPKYYILLHIMLGEILR---HS 196
          **  .**  **  *** . . * **      *  **  * ****

A3B  193 MDPDTFTFNFNNDPLVLRRRQTYLCYEVERLDNGTWVLMDQHMGFLCNEA 242
A3F  195 MYPHIFYFHFKNLRKAYGRNESWLCFTMEVVKHHSPVSWKR--GVFRNQV 242
A3G  197 MDPPTFTFNFNNEPWVRGRHETYLCYEVERMHNDTWVLLNQRRGFLCNQA 246
         * *  * *.* *      * .. **. .* . . . *   .  *   *.

A3B  243 K-NLLCGFYGRHAELRFLDLVPSLQLDPAQIYRVTWFISWSPCFSWGCAG 291
A3F  243 DPETHC-----HAERCFLSWFCDDILSPNTNYEVTWYTSWSPCP--ECAG 285
A3G  247 P-HKHGFLEGRHAELCFLDVIPFWKLDLDQDYRVTCFTSWSPCF--SCAQ 293
                    ***  **       *     * ** . *****    **

A3B  292 EVRAFLQENTHVRLRIFAARIYD-YDPLYKEALQMLRDAGAQVSIMTYDE 340
A3F  286 EVAEFLARHSNVNLTIFTARLYYFWDTDYQEGLRSLSQEGASVEIMGYKD 335
A3G  294 EMAKFISKNKHVSLCIFTARIYD-DQGRCQEGLRTLAEAGAKISIMTYSE 342
         *.  *.  . .* * **.**.*       .* *. *   ** . ** * .

A3B  341 FEYCWDTFVYRQGCPFQPWDGLEEHSQALSGRLRAILQNQGN* 383
A3F  336 FKYCWENFVYNDDEPFKPWKGLKYNFLFLDSKLQEILE*     374
A3G  343 FKHCWDTFVDHQGCPFQPWDGLDEHSQDLSGRLRAILQNQEN* 385
```

could deaminate DNA (109, 126). Taken together the evidence strongly suggested that A3G is also a cytidine deaminase, and implied that it might mutagenize HIV (248). Similarly, interferon-inducible adenosine deaminases can inactivate several RNA viruses (234), and phage DNAs are degraded by restriction endonucleases (194). Thus, host defenses that involve enzymatic attack on viral nucleic acids are widespread.

This hypothesis was rapidly confirmed in several laboratories, who found that A3G (as well as A3F and A3B) causes extensive deoxyC-to-deoxyU mutations in the HIV-1 negative-strand DNA that is made during reverse transcription (148, 174, 251, 307, 311). Deamination requires packaging of A3G into HIV-1 virions (157, 309), an intact C-terminal catalytic domain (103, 191, 192, 251), and was prevented by Vif (28, 108, 174, 251, 311). DNA repair processes involving HIV-1 virion incorporated cellular uracil DNA glycosylase-2 (UNG-2) and apurinic/apyrimidinic endonucleases can eliminate dU erroneously incorporated by RT or generated by cytidine deamination (145, 214). UNG-2 works in concert with HIV-1 RT and RNaseH to eliminate and repair dU misincorporations into RNA/DNA or DNA/DNA duplexes (214). In contrast, a recent report suggests that dU resulting from deamination of negative ssDNA can be excised by UNG-2, but the resulting abasic site cannot be repaired because of the lack of a complimentary DNA template (214). RT preferentially inserts dA into some abasic sites (38) generating a G-to-A mutation in the positive DNA strand. Alternatively, uracilated DNA may be cleaved by cellular DNA repair enzymes (145), thus terminating synthesis of the provirus (53, 100). Most early reverse transcription products are thought to be degraded at this point, and heavily hypermutated proviruses that become incorporated into the host genome likely encode nonfunctional or truncated proteins (125, 174).

Analysis of hypermutated proviruses abundant in HIV-1 infected patient PBMCs reveals two heavily favored dinucleotide targets on the minus strand, CpC and TpC (underlined residue is mutated) (125, 284). Since CpC is the preferred target of A3G and TpC is favored by A3B and A3F, the latter APOBECs are suspected to make dominant contributions to cytidine deamination *in vivo* (19, 28, 103, 155, 272).

III. C. APOBEC3G has a broad range of antiviral activity

Cytidine deaminase activity of a bacterially expressed A3G revealed promiscuous editing of A3G on a wide-range of DNA targets (109). Moreover, overexpression of human A3G in human cells can mutagenize other retroviruses including MLV and EIAV (107, 170), which lack Vif genes. This mutagenesis; however, may not occur efficiently in all circumstances or in T cells of the natural hosts (165, 174). Gammaretroviruses including MLV and FLV replicate with substantial fidelity in T cells of mice or cats where they often induce thymic lymphomas (48, 304). Similarly, mouse mammary tumor viruses efficiently replicate in mouse B cells that also express an A3G ortholog (54), and EIAV replicates efficiently in equine macrophages (244). These viruses may have alternative means of evading the A3G orthologs that occur in their natural hosts. Mouse APOBEC3 is excluded from MLV virions by an unknown mechanism (142). Notably, human A3G also has antiviral activity against the ancient endogenous retroviruses and acts by inhibition their retrotransposition in murine cells and yeast (69, 70) (see section III.D.).

Several lines of evidence suggest that A3G's DNA editing activity is dispensable for its antiviral activity. Point mutations in the C-terminal catalytic domain that disable

A3G's ability to deaminate DNA retain 50-97% of antiviral activity against HIV-1 (103, 192, 306). Antiviral activity depends on at least one intact active site with no apparent discrimination for either site (306), while only the C-terminal domain is essential for deaminase activity (192, 251, 306). Thus, the two activities are separable whereas cytidine deamination is not essential for antiviral activity. Furthermore, production of the hepadnavirus, hepatitis B, was dramatically reduced by expression of A3G (245, 282). However, no nucleotide changes were detected in the newly synthesized viral DNA, providing additional support for an alternative antiviral function for A3G that is distinct from cytidine deamination. The implications of these results for natural hepatitis B virus infections is uncertain, in part because A3G is believed to be absent in normal hepatocytes (126).

Presumably, other APOBEC family members might also have antiviral functions. Several other primate and non-primate species encode APOBEC orthologs, but artiodactyls (such as cows) and rodents have only one APOBEC3 gene (108). In fact, recent investigations into the anti-retroviral potentials of APOBECs from rodents as well as humans revealed that human A3F has anti-HIV-1 activity similar to A3G (28, 226, 292, 313). A3F is bound by HIV-1 Vif, and targeted for proteasome-mediated degradation (313). Moreover, human A3A and A3C have only a weak anti-HIV-1(Δ*vif*) affect (28, 226, 305), and AID and APOBEC1, have no significant anti-HIV-1 properties (28, 226, 305). Interestingly, rat APOBEC1, mouse APOBEC3, African green monkey A3G and human A3B have strong antiviral activity against both HIV-1(wild-type) and HIV-1(Δ*vif*), with the murine orthologs exhibiting a more potent antiviral effect (28, 174, 226, 305). Interestingly, murine orthologs have no antiviral activity against MLV (28,

174). These results suggest a resistance of these APOBEC family members to HIV-1 Vif that is thought to result from an inability to interact with Vif. A single amino acid in A3G at position 128 (aspartic acid in human and lysine in african green monkey) restricts interaction of Vif with the A3G expressed in a particular species (30, 171, 240, 293). Meanwhile, substitution of human A3G D128 with Ala, Glu, His, or Arg does not disrupt interactions with Vif, suggesting that a negative or neutral charge at position 128 is necessary to sustain species-specific interaction with human A3G (30, 240). In agreement with this finding, A3F and A3B encode a glutamic acid at position 128 (Figure 5) but both maintain interaction with Vif and have anti-HIV-1 activity (Appendix II) (226, 292, 313). Interestingly, A3F is subject to neutralization by Vif-mediated proteasome degradation (313), while A3B seems to be resistant to certain strains of HIV-1 Vif and can significantly inhibit both HIV-1(wild-type) and HIV-1(Δ*vif*) (176).

III. D. Ancient Evolution of APOBEC3s

Analysis of primate A3G sequences reveals a positive selection acting on A3G (also A3C and A3F) that predates modern retroviruses (50, 237, 312). Nearly half of the human genome is composed of transposable elements, remnants of retroviral infections of germ line cells million of years ago (57). Endogenous retroviruses are retrotransposons that contain a long-terminal repeat (LTR) structurally similar to infectious retroviruses and comprise 8% of the human genome, while non-LTR retrotransposons known as LINE-1 (long interdispersed nucleotide element-1) occupy 20% of the human genome. Other retroelements include pseudogenes and SINE (short interdispersed nucleotide elements) (236). Notably, A3G inhibits retrotransposition of murine and yeast

endogenous retroviruses by cytidine deamination (69, 70). In contrast, A3G has no effect on LINE-1 transposition, probably because reverse transcription of these elements occurs in the nucleus, whereas A3G is predominantly expressed in the cytoplasm (283). Consequently, many suspect that a decline of retrotransposition in primates resulted from the expansion of the APOBEC3 family (69, 70). Due to the antagonistic interaction of A3G with Vif, one suspects that Vif could be the driving force for A3G selection, although no differences in selective pressure were seen between organisms susceptible or resistant to HIV/SIV (312). Furthermore, the excess of nonsynonymous amino acid substitutions spread throughout the gene and not localized to a single domain suggests A3G may be targeted for elimination by more than one virus therefore it requires multiple substitutions to evade recognition by all these viruses (50, 237, 312). Although most human endogenous retroviruses (HERVs) have undergone extensive mutations and deletions, they retain the ability to express some transcripts and proteins (137). Based on this precedence, we can speculate that APOBEC3s may have evolved to restrict endogenous retroviruses and ancient retroelements.

IV. Regulation of APOBECs and antiviral factors

IV. A. Regulation of APOBEC-1 and AID cytidine deaminase activity

Unrestricted cytidine deamination in cells could introduce numerous non-functional gene products; therefore restriction of enzymatic activity to a specific time and place is critical. In fact, aberrant and elevated levels of APOBEC expression are detected in cells derived from numerous cancerous tissues, psoriasis, and in patients with schizophrenia (126, 168, 281). Specific factors have been shown to regulate the

expression and to limit the enzymatic activity of APOBEC1 and AID (21, 40, 56, 152, 181, 205). Cell-specific and time-dependent expression, *cis*- and *trans*-acting factors, and intracellular localization all contribute to the specificity and regulation of cytidine deamination (40, 205). In most species including humans, APOBEC1 is expressed exclusively in endothelial cells of the small intestine and is responsible for deamination of a single nucleotide in apolipoprotein B mRNA (277). The substrate specificity of the well-studied APOBEC1 enzyme is controlled by APOBEC-1-complementation factors (ACFs) (56, 152, 181), which recruit the enzyme to a specific site in the apolipoprotein B mRNA based on RNA secondary structure and an 11-nucleotide mooring sequence (13, 247). It is only when APOBEC1 is aberrantly expressed in additional cell types or in excess of the ACFs that it deaminates other mRNAs (263, 295). Interestingly, overexpression of APOBEC1, AID, and other cytidine deaminases can cause rampant mutagenesis, cellular toxicity, and lead to tumorogenisis (197, 295, 303). Additional positive and negative regulatory factors for APOBEC-1 editing activity have been identified (for review see (9, 289)).

AID enzymatic activity is thought to be dependent on its translocation from the nucleus (21, 204). In addition, normal expression of AID is limited to activated B-cells in germinal centers and is induced by IL-4 and engagement of CD40L and repressed by antibodies against CD45 (58, 185, 314). Similar to the enzymatic activity of A3G, AID cytidine deamination occurs at multiple sites along ssDNA of immunoglobulin and has a bias for WR<u>C</u> (W is A or T; R is a purine) with no additional sequence requirements (205). Recruitment of AID to actively transcribed regions where ssDNA is exposed, may result from the interaction of AID with RNA polymerase II (204). Based on this

precedence, we hypothesize that cellular factors and expression levels of APOBEC3 cytidine deaminases can regulate their functions.

mRNA expression of more closely related APOBEC family members, A3A and A3B (also called phorbolin-1 and -2 respectively), are upregulated in psoriasis, and through stimulation of normal keratinocytes with the phorbol ester phorbol-12-myristate-13-acetate (PMA) (168). In addition, A3A is upregulated in peripheral blood lymphocytes by interferon-α treatment (276). Therefore, we analyzed the distribution of APOBEC3 family members mRNA in cell lines and primary blood cell fractions (Appendix I) (226), as well as how expression of APOBEC3s mRNAs could be regulated by HIV-1 infection (175, 180, 266) (Appendix I) and cellular factors (227). Controlled expression of antiviral APOBEC3s may enable cells to combat invading retroviruses by saturating inhibitory factors such as Vif, to tip the arms race in favor of the cell.

IV. B. Antiviral proteins regulated by the innate immune response

A thorough investigation of the processes that regulate expression and cytidine deaminase activity of the innate antiviral defense system of APOBEC3 enzymes will facilitate efforts to define effective targets for therapeutic intervention. In light of the obvious necessity to tightly control editing specificity, one would anticipate similar cellular constraints to regulate A3G activity. We have developed a system to look at A3G interacting proteins by purifying a tandem-affinity tagged A3G in the presence and absence of HIV-1(wild-type) followed by 2-demensional gel analysis of associated factors (Appendix III).

Interferons (INFs) were discovered as antiviral agents nearly 50 years ago (234). The Type I IFN response (INF-α,-β, and in some cell types INF-γ) induced by virus infection or dsRNA represents an early innate host defense prior to the onset of adaptive immunity (234). In addition to a significant role in the ensuing immune response, TNF-α emulates the actions of INF-γ against invading viruses (101, 234). Several INF-induced proteins implicated in antiviral action include, protein kinase R, the 2',5'-oligoadenylate synthetase (OAS) and RNaseL, the Mx protein GTPases, and most interestingly, the RNA-specific adenosine deaminase (ADAR1) (234).

The TAR RNA sequence in the 5'-terminal region of HIV-1 is able to activate both OAS and PKR (20, 164). Nevertheless, HIV-1 has two mechanisms to counteract this TAR-mediated activation of PKR. First, PKR function is down-regulated by the cellular TAR-binding protein (TRBP) which forms an inactive heterodimer with PKR (20). Second, HIV-1 encodes the Tat protein that counteracts the activation of PKR (35). Many other RNA and DNA viruses encode proteins to antagonize these INF-induced proteins which illustrates the fundamental importance of the interferon system in defense against viral infection.

IV. C. Regulation by additional extracellular mitogens

Initial evidence that A3A and A3B expression can be influenced by the extracellular mitogens such as phorbol esters, suggests that protein kinase C (PKC) activation may be involved in APOBEC3 gene expression (168). PKC isozymes can be classified into three groups. The most well studied are the Ca^{2+}-dependent or conventional PKCs (cPKCs), which include PKC-α, -βI, -βII, and -γ (PKCβ is

alternatively spliced to produce 2 gene products). In contrast, the novel PKC (nPKC) isozymes, PKC-δ, -ε, -η, -μ, lack a Ca^{2+} binding domain. The third group, atypical PKCs (aPKCs) (PKC-λ,-ι, and -ζ) are also Ca^{2+}-independent and thought to have some constitutive activity. nPKCs and cPKCs respond to both DAG and phorbol esters, however aPKCs are insensitive to both and are instead activated by phosphatidylinoditides, phosphatidylserine, and unsaturated fatty acids (159).

Tumor promoting phorbol esters mimic the action of 1,2-diacglycerol (DAG) by activating the cPKC and nPKC isozyme groups as well at PKC-independent pathways (36, 159, 182). At least five alternative receptors bind PMA and/or DAG with high affinity: chimerins, protein kinase D, RasGRPs, Munc13s and DAG kinase γ (36). Since PKC and all five alternative receptors contain the high affinity DAG/PMA interaction domain (C_1), the ability to functionally separate PKC-dependent and -independent pathways is dependent on the use of specific pharmacological reagents. PKC ATP-binding site inhibitors and indolocarbazoles (e.g. Gö6976) are considerably PKC specific and some of these inhibitors have a preference for certain isozymes (17, 131).

Activation of PKC leads to regulation of cell growth, differentiation, vesicle trafficking and gene expression. One of the most important and well-characterized events induced by extracellular mitogens or stress signals is activation of mitogen-activated protein kinases (MAPKs) (Figure 6). In mammals there are at least six independent functional MAPK signaling pathways. The properties of three of these pathways, the p44/p42 extracellular signal-regulated kinases (ERK-1 and -2), the c-Jun stress-activated protein kinases (JNK), and the p38 MAPK have been biochemically characterized (47, 122, 280). ERKs can be activated by extracellular mitogens such as

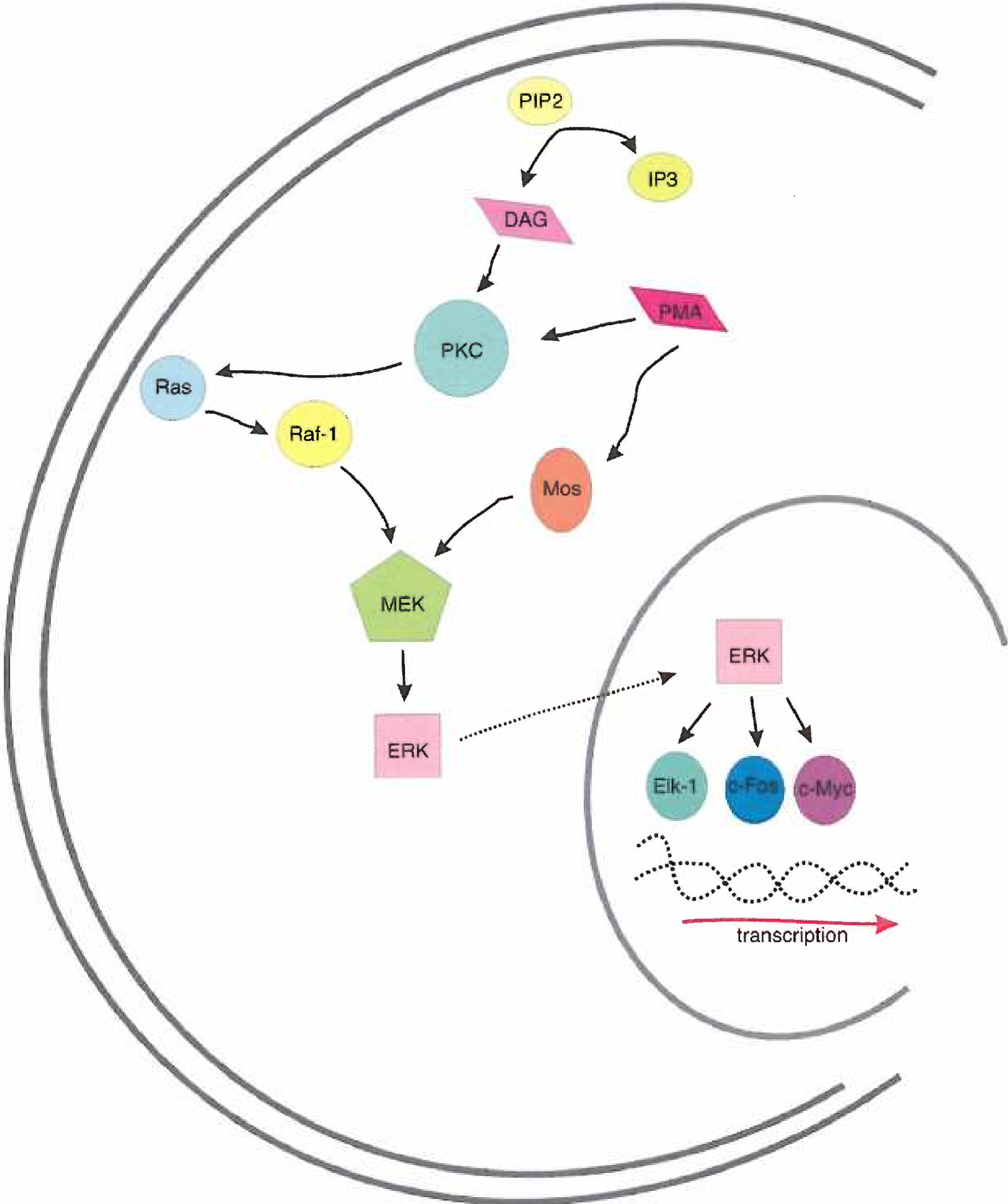
growth factors or phorbol esters. Unlike ERKs, JNK and p38 MAPKs are preferentially activated by stress responses such as UV radiation, heat shock, osmostic shock, and inflammatory cytokines (122). The MAPK signaling cascade is a hierarchy of protein kinases that consists of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). MAPKKK phosphorylates and activates the MAPKK, which in turn phosphorylates and activates MAPK. Activated MAPK then influences gene expression and cellular activities by phosphorylating and thereby regulating transcription factors and downstream kinases (47, 280).

PKC is used by diverse receptors to regulate the MAPK (ERK) cascade. The effects of phorbol esters can be propagated through the GTP-bound G-protein coupled receptor Ras, which binds the protein kinases Raf-1 and B-Raf. Although PKC is a potent activator of Ras (143) the protooncogene product Mos can also be activated by phorbol esters (84). Ras, Mos, and MEKK1 can all phosphorylate MEK-1 and -2 (hereafter referred to as MEK) on serine residues 218 and 222, activating the dual specificity protein kinases to phosphorylate ERK. Despite the similar arrangement of activating phosphorylation sites, other closely related kinases, such as p38 and JNK are poor substrates for MEK (156). A maximally activated ERK (phosphorylated on T183 and Y185) functions as a serine/threonine kinase that can directly or indirectly influence protein stability, mRNA stability, DNA binding, regulation of transcriptional repression, and transcriptional activation (280). Due to the diverse group of transcription factors that are activated by ERK (c-Fos, CREB, AP-1, NFκB, c-Myc, Elk-1, and many others), the Raf/MEK/ERK signaling cascade has become a target for intervention in leukemia and other cancers (42, 280). In agreement with this observation, APOBEC mRNAs are

upregulated in many cancer tissues and have recently been correlated as disease markers in psoriasis and schizophrenia (126, 168, 276).

Figure 6

PKC cell signaling pathway



V. Summary

This dissertation uncovers the mechanism by which HIV-1 counteracts a novel antiviral system of APOBEC3 cytidine deaminases, with particular emphasis on the regulation of expression and antiviral activities of APOBEC3 cytidine deaminases by HIV-1 Vif and other viral/cellular factors. We have defined a mechanism by which Vif binds A3G and causes its polyubiquitination and rapid degradation by a proteasome-dependent pathway. This complete loss of A3G from infected cells expressing Vif makes A3G unavailable for incorporation into virions, thereby preventing lethal cytidine deamination (hypermutation) of the nascent HIV-1 DNA in the subsequent host cell. Previous reports suggest expression of A3G is necessary and sufficient for the nonpermissive phenotype and that expression of A3G transcripts were restricted to nonpermissive cells (248). In contrast, analysis of a large panel of cell lines reveals A3G is broadly expressed in cells displaying varying degrees of anti-HIV-1 activities that do not correlate with A3B, A3C, A3F, or A3G mRNA expression levels. We have confirmed reports of A3F anti-HIV-1 activity and found that A3B is resistant to certain strains of HIV-1 Vif, therefore inhibiting both HIV-1(wild-type) and HIV-1(Δ*vif*). Taken together these data suggest compelling evidence for additional factors that regulate APOBEC3 antiviral activities. Finally, in both nonpermissive and permissive cells regulation of A3G and A3B, but not A3F mRNA translation is controlled by activation of the PKCα/βI/MEK/ERK pathway. In fact, we believe involvement of this pathway restricts basal A3G expression to the early G_1 through G_1/S transition of the cell cycle where MEK is active, whereas resting cells in G_0 express low or undetectable levels of

A3G. This strongly suggests that culture conditions and growth state of the cells at time of infection could contribute to the antiviral activity of A3G and possibly A3B. Overall, data presented in this dissertation exposes a potent intrinsic antiretroviral system of cytidine deaminases that can be regulated at the level of expression and enzymatic and/or antiviral activity. Discovery of this family of proteins exposes a promising new target for retroviral therapy with far-reaching applications in other diseases such as cancer.

Chapter 2

The Vif Protein of HIV-1 Binds the Editing Enzyme APOBEC3G and Induces its Degradation

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Abstract

The viral infectivity factor (Vif) encoded by human immunodeficiency virus (HIV-1) neutralizes a potent antiviral pathway that occurs in human T lymphocytes and in several leukemic T cell lines that are termed nonpermissive, but is not present in other cells. In the absence of Vif, this antiviral pathway efficiently destroys HIV-1. Recently, it was reported that APOBEC3G (also known as CEM-15), a cytidine deaminase nucleic acid editing enzyme, confers this antiviral phenotype on permissive cells. Here, we describe evidence that Vif binds to APOBEC3G (A3G) and induces its rapid degradation, thus eliminating it from cells and preventing its incorporation into HIV-1 virions. Studies of Vif mutants imply that it contains two domains, one that binds A3G and another with a conserved SLQ(Y/F)LA motif that mediates A3G degradation by a proteasome-dependent pathway. These results provide promising approaches for drug discovery.

Introduction

The viral infectivity factor (Vif) encoded by HIV-1 is a small basic Mr 23,000 protein that is necessary for HIV-1 replication *in vivo* and in nonpermissive cells, which include T lymphocytes and macrophages and several leukemic T cell lines, but it is irrelevant in many other cells termed permissive (86, 166, 248, 254). Consequently, HIV-1(Δ*vif*) that has a deletion or mutation in its *vif* gene can efficiently replicate in permissive cell lines. Furthermore, the resulting HIV-1(Δ*vif*) virions can also infect nonpermissive cells, resulting in proviral DNA integration and in production of virus-encoded proteins that are packaged with viral RNA into progeny virions that appear to have a normal composition (89, 196). However, these HIV-1(Δ*vif*) virions that are derived from nonpermissive cells have been imprinted in a manner that severely inhibits reverse transcription during the subsequent cycle of infection (51, 62, 98, 255, 287). Because these virions are inactive in all target cells including permissive or nonpermissive cells that contain Vif, the imprinting may be irreversible (51, 86, 287).

The nonpermissive phenotype is dominant in permissive x nonpermissive heterokaryons (166, 254). This suggested that nonpermissive cells have a potent antiviral defense system that would efficiently inactivate HIV-1 were it not neutralized by Vif. Recently, Sheehy et al. (248) identified APOBEC3G (previously termed CEM-15), a member of the cytidine deaminase family of nucleic acid editing enzymes (109, 277), as the specific antiviral factor in nonpermissive cells. Most significantly, they found that expression of APOBEC3G (A3G) in permissive cell lines converted them to nonpermissive (248). Additionally, they reported that A3G is incorporated into HIV-1 virions regardless of whether Vif is present or absent in the producer cells. Since Vif is

incorporated in small amounts into HIV-1 virions (88, 139, 158), and since it binds to RNA (61, 139), they suggested that Vif might bind to the HIV-1 genomic RNA and shield it from inactivation by A3G in the producer cells and/or in the released virions (89, 248), thus acting on the target of A3G rather than directly on the antiviral protein. Recently, it was shown that A3G causes cytidine deamination of HIV-1 negative strand DNA during the process of reverse transcription (107, 148, 170, 311).

Here, we describe studies of the mechanism by which Vif neutralizes A3G. We confirm that A3G efficiently inactivates HIV-1(Δ*vif*) and that its antiviral activity is neutralized by Vif. We report that Vif binds to A3G and induces its rapid degradation. The most conserved sequence in Vif, an SLQ(Y/F)LAΦΦΦΦ motif (Φ is a hydrophobic amino acid) (195), is unnecessary for binding to A3G but is required for its degradation. Accordingly, HIV-1 virions made in the presence of Vif specifically lack A3G.

Methods

Expression vectors

pHIV-*gpt*(wt) and its derivative pHIV-*gpt*(Δ*vif*) were previously described (165, 166, 200). pcDNA3.1-Vif was donated by D. Gabuzda (Dana-Farber Cancer Institute, Harvard, Boston, MA). Rev-dependent plasmids for expression of the Vif mutants Δ2, Δ5, Δ6, Δ7, Δ9, Δ10, Δ12 and Δ13 (257) were provided by M. Malim (Medical Research Council, Cambridge, UK) and were coexpressed with pHIV-*gpt*(Δ*vif*). A3G cDNA was cloned from H9 cells (nonpermissive) by reverse transcriptase-PCR (16, 233) using primers complementary to the 5' and 3' ends of A3G coding region (248) (upstream primer, 5'-GGGCTCGAGAGGATGAAGCCTCACTTCAGAAAC-3' containing an *Xho*I restriction site [underlined]; downstream primer, 5'-GGGTTCGAAGTTTTCCTGATTCTGGAGAATGGC-3' containing a *Sfu*I restriction site [underlined]). The cDNA was cloned between *Xho*I and *Sfu*I into the pcDNA3.1/Myc-His C mammalian expression vector (Invitrogen) to obtain the pcDNA3.1-A3G-Myc vector. The pSVIIIEnv vector was used to pseudotype HIV-*gpt* virions (116). Transfections employed PolyFect reagent (Qiagen, Inc.) according to the manufacturer's instructions, with equimolar ratios of all plasmids and with harvests after 36h unless otherwise mentioned.

Viruses

HIV-*gpt* virions were produced and titered as previously described (200, 201, 210). For viral purifications, 27 ml of the virus-containing media from 293T cells were pelleted through 2 ml of a 20% sucrose at 100,000g for 1.5 h. Virions were resuspended

in TSE (0.1M NaCl, 1mM EDTA, 0.01M Tris-Cl, pH 7.4), and centrifuged to equilibrium in 5 ml 20 – 60 % sucrose gradients in TSE at 200,000g for 2.5 h. Virus-containing fractions were detected by immunoblotting using HIV Immunoglobulin serum (AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by NABI and NHLBI). Peak fractions were pooled, diluted with TSE, and pelleted through 20% sucrose at 100,000g for 2h.

Analysis of proteins and RNAs

Extracts of cotransfected 293T and COS7 cultures were prepared using either TX (1% Triton X-100, 150mM NaCl, 10mM Tris-Cl, pH 7.5, 1mM MgCl) or RIPA (50mM Tris-Cl pH7.4, 1% Nonidet P40, 0.1% sodium deoxycholate, 150 mM NaCl) buffers with complete protease inhibitors (Roche) followed by centrifugation at 1,500g for 5 min to sediment nuclei. Extracts were adjusted to equivalent protein concentrations using the Bradford reagent (BioRad Laboratories), and equal aliquots were then used for Western immunoblotting (177) or for immunoprecipitations (141) using the antibodies HIV-1 HXB2 Vif rabbit antiserum #2221, (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by Dr. D. Gabuzda), *Myc*-specific monoclonal antibody clone 9E10 (Sigma) (177), or the ubiquitin-specific mouse monoclonal antibody (Zymed). This procedure for ensuring equal loading of proteins into the lanes of our gels was verified by immunoblotting with an antiserum specific for α-tubulin (Sigma). Where indicated, cultures were preincubated with 50 μM concentrations of the proteasome inhibitors ALLN, MG-132, or Proteasome Inhibitor-I (Calbiochem). For immunoprecipitations, cell lysates were precleared by adsorption onto

protein A-coated Sepharose 4B (Sigma) followed by incubations with Myc-specific antibody 9E10 and subsequent addition of protein A-coated Sepharose 4B. For Apobec-3G-Myc purifications based on its 6xHis tag, culture extracts adjusted to equal volumes and protein concentrations were incubated with 100 ul/ml of Ni-NTA-agarose beads (50% slurry), followed by thorough washing (100mM NaH_2PO_4, 10mM Tris-Cl, 8M urea, pH 6.3) and elution (100mM NaH_2PO_4, 10mM Tris-Cl, 8M urea, pH 4.5) in denaturing conditions known to remove contaminants (Qiagen, Inc.). For immunofluorescence, COS7 cells were used because they are more adherent than 293T cells. Cells cultured in Permanox chamber slides (Nalge Nunc International) were fixed in 5% formaldehyde and 2% sucrose in PBS (Invitrogen) at room temperature for 20 min, and permeabilized with 1% Triton X-100 in PBS with 10% sucrose for 30 min. Primary antibodies were Vif antiserum #2221 or the *Myc*-specific monoclonal antibody 9E10. Secondary fluorescent antibodies were Alexa Fluor 488 goat anti-rabbit IgG(H+L) and Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes Inc,), which reacted only with their species-specific primary antibodies. Slides were mounted in FluoroGuard (Bio-Rad Laboratories), and observed with a Zeiss Axiovert 200M deconvolution microscope. The percentage of double-positive cells in **Fig. 2c** is defined as the percentage of the Vif-positive cells and that contained A3G-Myc. RNA extraction and Northern blot analyses were previously described (274). A cDNA probe for the S2 ribosomal protein was used as a loading control.

Pulse-chase experiments

Tran^{35}S-label (ICN Biochemicals, Inc.) was diluted with unlabeled L-cysteine and L-methionine to a final specific radioactivity of 20 Ci/mmole. Cultures were washed and incubated with DMEM lacking cysteine and methionine for 60 min at 37°C. They were pulse-labeled in the same medium with 80 uCi/ml of the Tran^{35}S-label solution for 4 min and rapidly washed and chased in complete growth medium supplemented with 10X unlabeled methionine and cysteine plus cycloheximide (50 ug/ml) (Sigma). Cytosolic RIPA buffer extracts were immmunoprecipitated with Myc antibody 9E10. Low exposure autoradiograms were scanned by densitometry.

Results

Expression of APOBEC3G-Myc converts human and monkey permissive cells to nonpermissive

To analyze its antiviral activity, we transiently cotransfected human 293T and African green monkey COS7 cells with pcDNA3.1-A3G-Myc in the presence of vectors for expression of HIV-*gpt*(wt) or HIV-*gpt*(Δ*vif*) [these are derivatives of wild-type (wt) or *vif* deleted (Δ*vif*) HIV-1 with the bacterial *gpt* gene replacing the viral *env* gene]; and we subsequently harvested virions from the culture media and analyzed their infectivities in HeLa-CD4 (clone HI-J) cells (165, 166, 200). A3G-Myc reproducibly decreased the titers of HIV-*gpt*(Δ*vif*) virions by approximately 25-100-fold but had no effect on titers of HIV-*gpt*(wt), strongly suggesting that it converts human and African green monkey permissive cells to nonopermissive (see **Supplementary Fig. 1**). Consistent with this conclusion, A3G-Myc had no significant or reproducible effect on synthesis of HIV-1 encoded proteins in the producer cells or on their packaging into virions. Similar results were obtained with owl monkey kidney cells (results not shown). Thus, HIV-1 Vif neutralizes human A3G-Myc in both human and monkey cells.

Vif eliminates the APOBEC3G protein from cells

We used this transient cotransfection system to analyze the mechanism by which Vif neutralizes the antiviral activity of A3G. Extracts of cultures that had been cotransfected with these vectors were adjusted to equal protein concentrations prior to Western blot analysis. Cultures that had been cotransfected with pHIV-*gpt*(wt) reproducibly contained approximately 4-10-fold less A3G-Myc than cultures that had

been cotransfected with pHIV-*gpt*(Δ*vif*) (**Fig. 1**). Vif-induced downmodulation of A3G-Myc was largest when the pcDNA3.1-A3G-Myc and pHIV-*gpt* plasmids were cotransfected in equimolar amounts, but was still substantial when the pHIV-*gpt* plasmids were reduced 27-fold (**Fig. 1b**). A3G-Myc downmodulation also occurred when the cells were cotransfected with a pcDNA3.1-Vif vector, suggesting that it did not require HIV-1-encoded proteins in addition to Vif (**Fig. 1c**). Vif had no effect on expression of LacZ-Myc expressed from the same pcDNA3.1 vector or on the quantities of A3G or LacZ mRNAs in the cell cultures (**Fig. 1c**) or in HIV-1-infected H9 leukemic T cells (nonpermissive) (results not shown). A3G-Myc had no effect on expression of Vif (**Fig. 1a, b**). The accuracy of our protein concentration adjustments was substantiated by immunoblotting of the control protein α-tubulin (**Fig. 1a**).

Immunofluorescence microscopy indicated almost complete segregation of A3G-Myc from wild-type Vif within the transiently cotransfected cultures, with some cells containing A3G-Myc but no Vif, and with others containing Vif but no A3G-Myc (e.g., see **Fig. 2a**). Moreover, the percentage of cells with A3G-Myc was approximately 4 times lower in the cultures that had been cotransfected with pHIV-*gpt*(wt) than in cultures that had been cotransfected with a negative control vector or with pHIV-*gpt*(Δ*vif*) (**Fig. 2b**). This Vif-dependent reduction in the proportion of cells with A3G-Myc corresponds within experimental error to the degree of A3G-Myc downmodulation seen by immunoblotting. This implies that A3G-Myc expression is eliminated in cells that contain wild-type Vif, and that the residual A3G-Myc that remains in the cultures is in a small population of cells that lack Vif. Consistent with this interpretation, mutations in Vif that eliminate its activity all blocked its ability to downmodulate A3G-Myc (see

below), and these mutant Vif proteins were extensively coexpressed with A3G-Myc within single cells (**Fig. 2a**). For example, approximately 95% of the cells that expressed the previously described (257) inactive Δ12Vif mutant contained A3G-Myc, whereas only approximately 10% of the cells with wild-type Vif had A3G-Myc (**Fig. 2c**). The latter double-positive cells may have been overestimated because they had only trace amounts of wild-type Vif that were difficult to distinguish from the background staining with this antiserum. We presume that these cells might have only recently begun to synthesize wild-type Vif.

Vif and the HIV-1 core and envelope proteins accumulate coordinately in a Rev-dependent manner late in the infection cycle (93). Therefore, production of progeny virions would be expected to occur almost exclusively in cells that contain large amounts of Vif and that consequently lack A3G (see **Fig. 2a**). In agreement with this prediction, we reproducibly detected A3G-Myc in the HIV-*gpt*(Δ*vif*) virions but not in the HIV-*gpt*(wt) virions (**Fig. 3**). A control using cells that express A3G-Myc alone excluded a contribution of contaminating cell-derived microvesicles (95) to our results.

Vif binds to APOBEC3G-Myc

Vif coimmunoprecipitates with A3G-Myc from RIPA buffer extracts of cell cultures that contain these proteins. This is a specific association that requires both proteins as well as the monoclonal antibody to Myc (**Fig. 4a**). Vif did not coimmunoprecipitate with LacZ-Myc using the same monoclonal antibody. Initially, this was confusing because our immunofluorescence results indicated that Vif and A3G-Myc are segregated into different cells within the cultures (**Fig. 2**). This was resolved by

finding that the association of Vif with A3G-Myc occurs in cell extracts and is not indicative of pre-existing intracellular complexes. Thus, complexes were coimmunoprecipitated from mixtures of two cell extracts that individually contained the discrete proteins (see **Supplementary Fig. 2**). We emphasize that these results do not establish whether the binding of Vif to A3G is direct or is mediated by other factors. Our attempts to immunoprecipitate these complexes with our Vif antiserum were unsuccessful because the Myc epitope bound nonspecifically to the serum as well as to preimmune rabbit serum.

We analyzed a series of small deletion mutations at different positions in Vif, which all severely inhibit its activity by at least 93% (257). Coimmunoprecipitation assays indicated that most of these Vif mutations prevented association with A3G-Myc (**Fig. 4a**). All mutant Vif proteins were detected in the cell extracts, except the Δ6 mutant which may be rapidly degraded or unreactive with our antiserum (**Fig. 4b**). However, the Δ12 and Δ31 deletion mutants clearly bound to A3G-Myc. Interestingly, these two deletions overlap and they both remove the SLQ(Y/F)LAΦΦΦΦ motif that is the most conserved site in Vif proteins of HIV-1 and other lentiviruses (195) (**Fig. 4c**). All mutations that prevent Vif function blocked Vif-induced downmodulation of A3G-Myc (**Fig. 4d**).

Vif induces rapid degradation of APOBEC3G

Based on the above evidence, we postulated that association with wild-type Vif might induce rapid A3G-Myc degradation. However, because our transiently cotransfected cultures contain a small proportion of cells that lack wild-type Vif and

therefore accumulate A3G-Myc (**Fig. 2**), we anticipated that this residual A3G-Myc would have a relatively long lifespan. To analyze this background, we treated the cotransfected 293T cultures with cycloheximide to block protein synthesis, and we subsequently analyzed the quantities of A3G-Myc by Western immunoblotting. These preliminary studies established that the residual A3G-Myc in the cultures cotransfected with pHIV-*gpt*(wt) and the A3G-Myc in the cultures cotransfected with pHIV-*gpt*(Δ*vif*) both turn over slowly with half-lives of approximately 8h.

To initially determine whether Vif induces rapid A3G-Myc degradation, we incubated A3G-Myc-positive cultures that contained or lacked Vif in the continuous presence of L-[^{35}S]methionine plus L-[^{35}S]cysteine, and we measured the labeling of A3G-Myc as a function of time. When the labeling periods were 5 min or less, the amounts of [^{35}S] A3G-Myc were usually very similar in these cultures, but by 60 min the cultures without Vif contained 4-5 times more [^{35}S]A3G-Myc (See **Supplementary Fig. 3**). These studies suggested that a large proportion of the A3G-Myc synthesized in the cultures that contained Vif had a very short half-life.

Pulse-chase labeling experiments confirmed these conclusions, and indicated that the 293T and COS7 cultures with Vif contain a substantial pool of A3G-Myc that is degraded within minutes of its synthesis (**Fig. 5**). In contrast, newly synthesized A3G-Myc is stable in the cultures that lack Vif. Indeed, the Vif-dependent component of A3G-Myc degradation was so rapid that it was difficult to detect unless the pulse-labeling time was 5 min or less and the chase times were also very short. Furthermore, optimal detection of this degradation required that the chase medium function quickly to terminate incorporation and that delays associated with rinsing the culture plates be

minimized. If these conditions are not all met, as in a recent report (174), only the slow degradation that occurs in the cells lacking Vif can be detected. Our conclusion based on many pulse-chase analyses is that the Vif-dependent component of A3G-Myc degradation is extremely rapid ($t_{1/2} \sim$ 1-2 min).

Consistent with these interpretations, treatments with the proteasome inhibitors ALLN, MG-132, or Proteasome Inhibitor-I for 6-10 h reproducibly increased the quantities of A3G-Myc in the cultures that contained Vif but not in the cultures that lacked Vif, and had no effect on the control protein α-tubulin (**Fig. 6a**). These inhibitors were active in both cultures as indicated by their enhancements in the amounts of total polyubiquitinated proteins (see **Supplementary Fig. 3**). Immunofluorescence microscopy confirmed that ALLN also caused a dramatic increase in the percentage of cells that coexpressed wild-type Vif and A3G-Myc (see **Fig. 2a–c**). Because our A3G-Myc protein contains a 6xHis tag adjacent to the Myc epitope, we were able to purify it away from Vif and other associated factors in highly denaturing conditions containing 8M urea. Interestingly, these preparations contained large polyubiquitinated proteins in amounts that were increased by Vif and by proteasome inhibitors (**Fig. 6b**). These results and sequence evidence described below suggest that Vif-induced A3G degradation occurs by a proteasome-dependent pathway that may involve ubiquitination.

Figure 1. Expression of Vif downmodulates A3G-Myc. 293T cells that had been cotransfected with plasmids for expression of A3G-Myc and for HIV-*gpt*(wt) or HIV-*gpt*(Δ*vif*) were analyzed for A3G-Myc and for Vif by Western immunoblotting. (**a**) shows that A3G-Myc is strongly downmodulated by HIV-*gpt*(wt) compared to HIV-*gpt*(Δ*vif*). The accuracy of our protein loading methods was verified by immunoblotting for α-tubulin. The HIV-*gpt* and A3G-Myc vectors were transfected in a 1:1 molar ratio. (**b**) shows the effects of altering quantities of pHIV-*gpt* plasmids relative to the A3G-Myc plasmid. Downmodulation of A3G-Myc is most extensive when the plasmid molar ratio is 1:1, but it remains substantial even when pHIV-*gpt* plasmids are reduced by 27-fold. (**c**) (left portion) shows that A3G-Myc downmodulation also occurs with a pcDNA3.1-Vif vector (Vif only) in the absence of other HIV-1-encoded proteins, and that Vif has no effect on expression of LacZ-Myc encoded by the same vector. (**c**) (right portion) is a Northern blot analysis of cellular RNAs. S2 is a loading control.

Figure 1

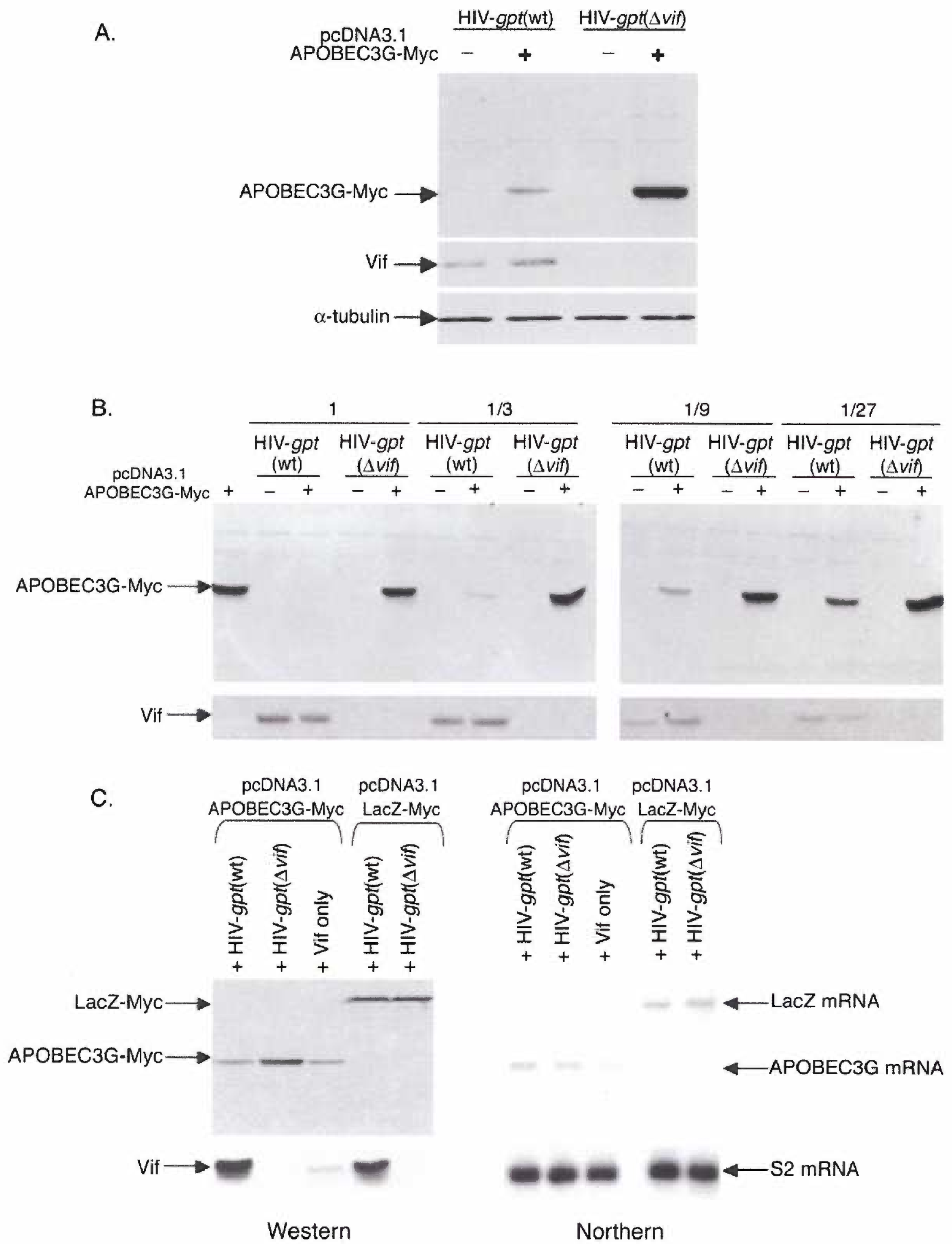


Figure 2. Effects of Vif on A3G-Myc analyzed by immunofluorescence microscopy. (**a**) shows representative images of COS7 cultures that had been cotransfected with vectors for expression of A3G-Myc and either wild-type Vif or inactive mutant Δ12Vif. With wild-type Vif, there is a segregation within the culture, with some cells containing A3G-Myc but no Vif and with others containing Vif but no A3G-Myc. In contrast, almost all of the cells with Δ12Vif coexpress A3G-Myc. Additionally, cultures cotransfected with vectors for A3G-Myc and wild-type Vif and treated with the proteasome inhibitor ALLN showed a dramatic increase in coexpression of both proteins within single cells. (**b**) shows results of an experiment in which we quantitatively analyzed effects of wild-type Vif on A3G-Myc expression at the cellular level. Whereas approximately 20% of the cells contained A3G-Myc in the cultures cotransfected with the negative control vector or with pHIV-*gpt*(Δ*vif*), a much smaller percentage of cells contained A3G-Myc in cultures that contained wild-type Vif. (**c**) We quantitatively analyzed effects of wild-type Vif and mutant Δ12Vif on coexpression of A3G-Myc. In addition, we examined effects of the proteasome inhibitor ALLN dissolved in dimethylsulfoxide (DMSO) and of DMSO alone. Approximately 25% of cells were Vif-positive in all of the cultures. Vif-positive cells were examined for the percentages that coexpressed A3G-Myc (double-positive). Wild-type Vif caused a large decrease in A3G-Myc coexpression independently of DMSO, and this decrease was alleviated by ALLN. For **b** and **c**, five fields of cells with at least 100 cells/field were examined for each culture; error bars are ± s.e.m.

Figure 2

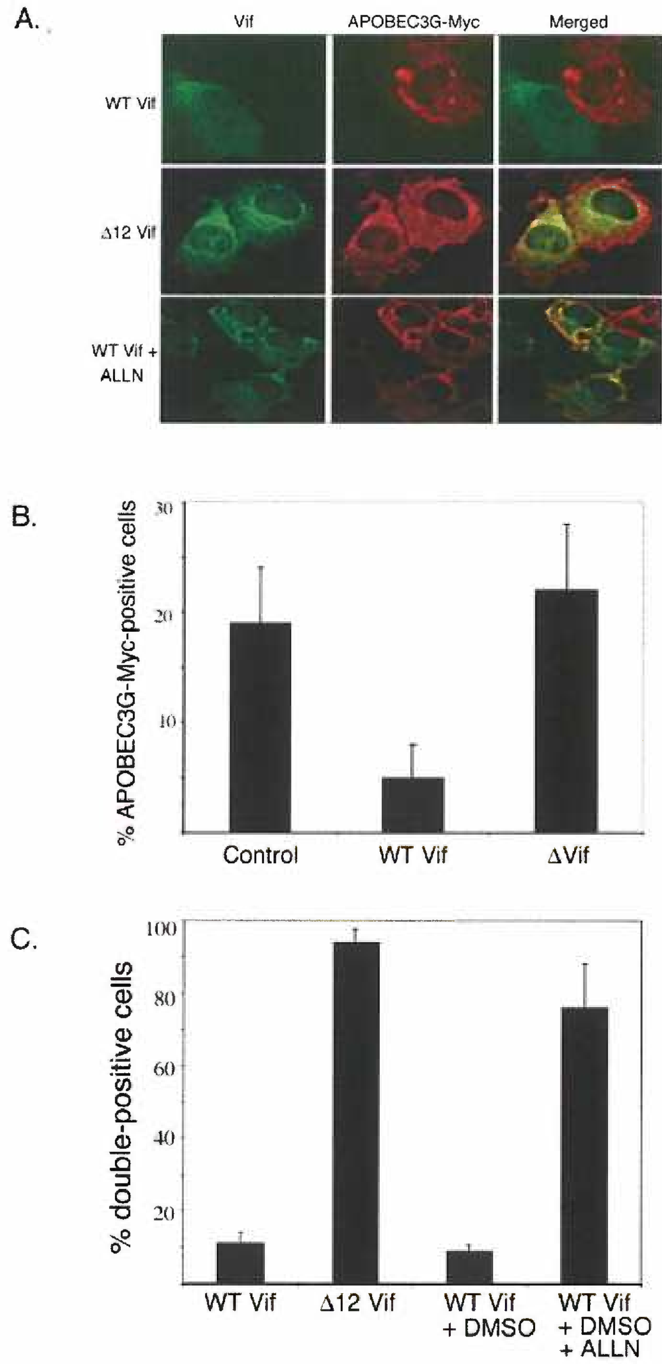


Figure 3. Vif blocks incorporation of A3G into purified HIV-1 virions. Cultures of 293T cells were cotransfected with pcDNA3.1-A3G-Myc in the presence of vectors for expression of HIV-*gpt*(wt) or HIV-*gpt*(Δ*vif*). Virions from the culture media were purified by equilibrium density centrifugation. The HIV-*gpt*(wt) virions lacked A3G-Myc, whereas the HIV-*gpt*(Δ*vif*) virions contained A3G-Myc. The virion samples contained similar amounts of HIV-1 Gag protein p24. A control preparation from the culture medium of cells expressing A3G-Myc alone was negative, indicating the absence of A3G-Myc in cell-derived microvesicles that heavily contaminate HIV-1 preparations (95).

Figure 3

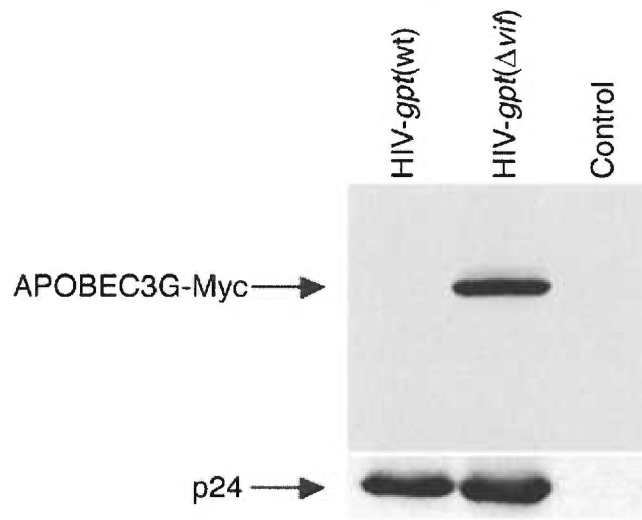


Figure 4. Vif binds to A3G-Myc. A3G-Myc was immunoprecipitated with a Myc-specific monoclonal antibody from extracts of cultures that contained or lacked Vif, and the immunoprecipitates were subsequently analyzed by Western immunoblotting using a Vif-specific antiserum. (**a**) Wild-type Vif coimmunoprecipitated with A3G-Myc but not with LacZ-Myc. A series of small in-frame Vif deletion mutants, which all eliminate Vif activity by at least 93% (257), were also analyzed. Only the Δ12 and Δ31 mutants bind to A3G. (**b**) shows immunoblot analysis of Vif in alliquots of the cell extracts that were not immunoprecipitated. (**c**) is a linear map of Vif with positions of the deletion mutations. (**d**) shows the effects of wild-type and mutant Vif proteins on downmodulation of A3G. Only wild-type Vif downmodulated A3G.

Figure 4

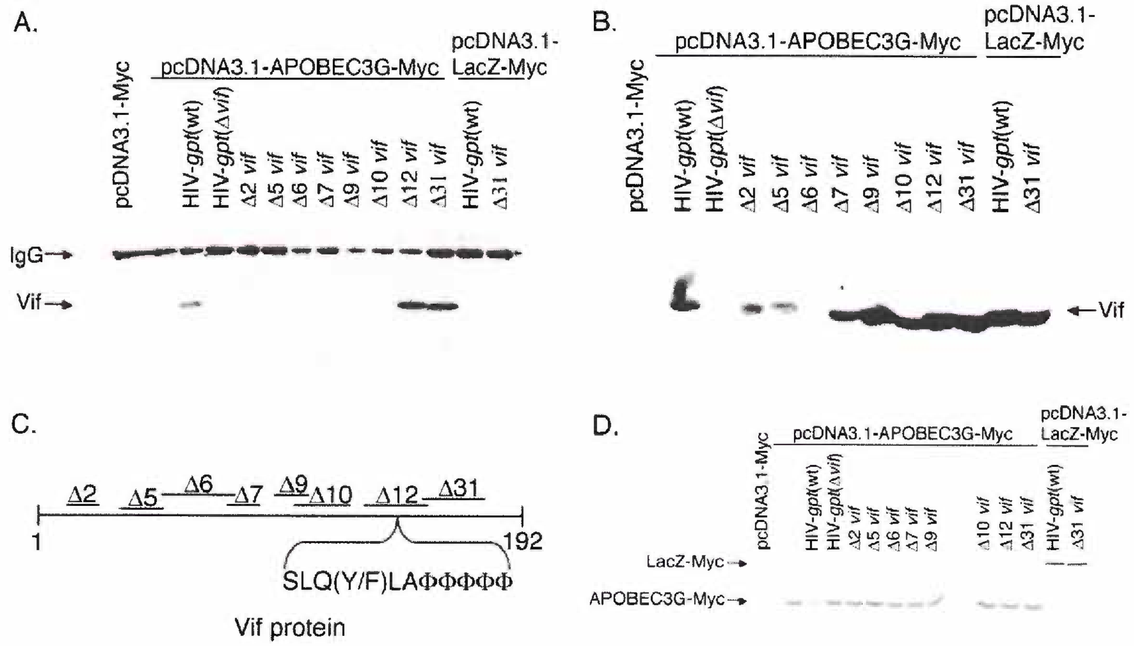
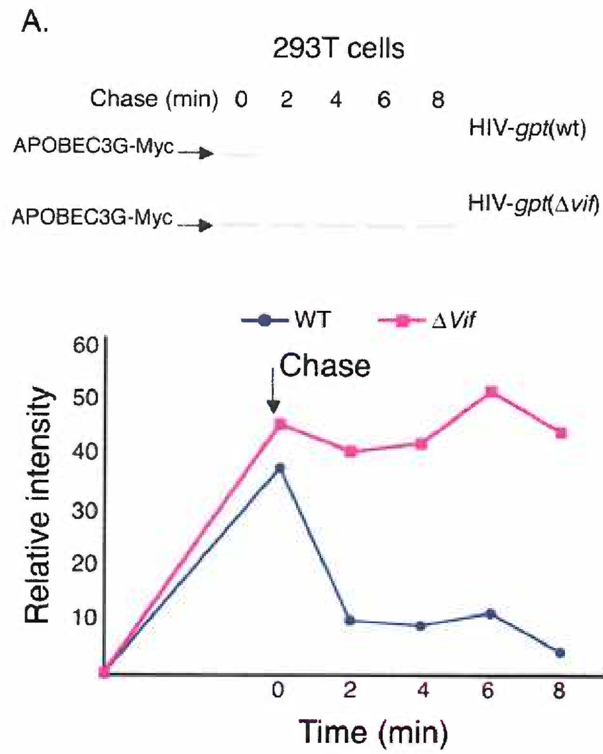


Figure 5. Vif causes rapid degradation of A3G-Myc. 293T (**a**) and COS7 (**b**) cell cultures were cotransfected with vectors for expression of A3G-Myc and HIV-*gpt*(wt) or HIV-*gpt*(Δ*vif*). After 36 h, the cells were pulsed-labeled by incorporation of L-[^{35}S]amino acids for 4 min and chased in the presence of cycloheximide for the times indicated. A3G-Myc was immunoprecipitated from the cell extracts and the immunoprecipitated proteins were analyzed by electrophoresis followed by autoradiographic detection. The relative intensities of the A3G-Myc bands were determined by densitometry.

Figure 5



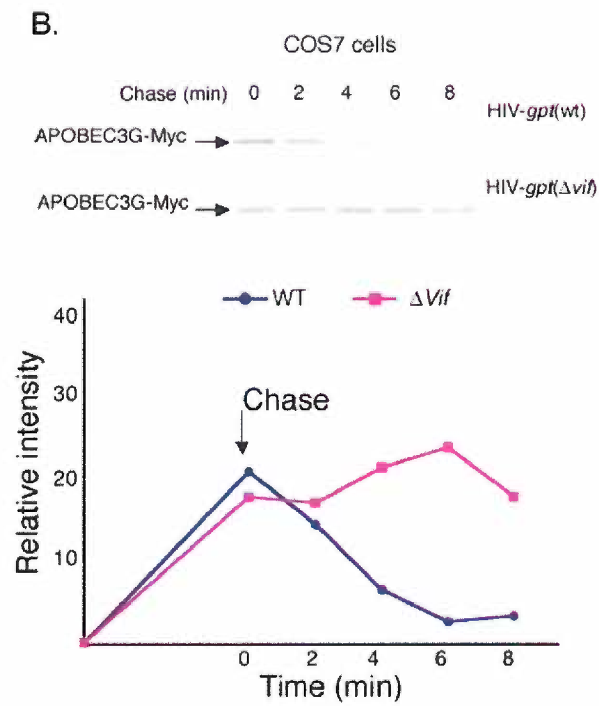
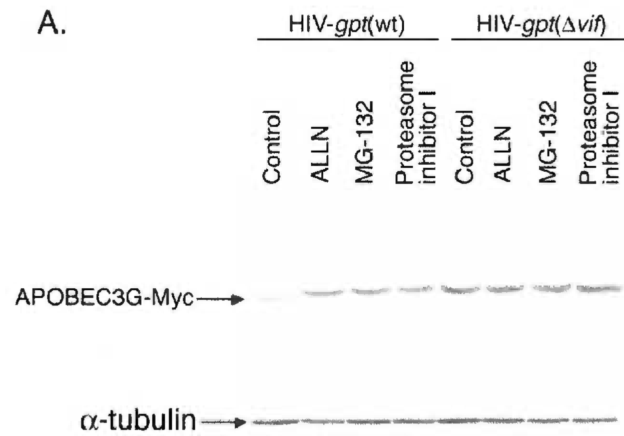
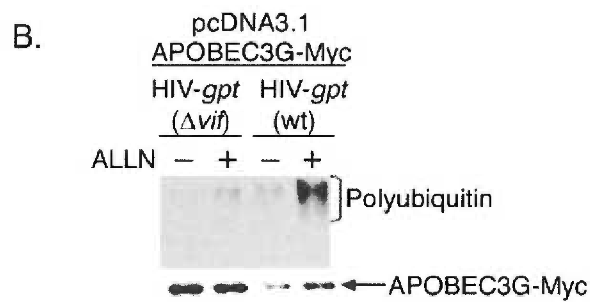


Figure 6. A3G-Myc degradation by Vif involves a proteasome-dependent pathway. 293T cell cultures were cotransfected with vectors for expression of A3G-Myc and HIV-*gpt*(wt) or HIV-*gpt*(Δ*vif*). (**a**) Cultures were incubated for 10 h with the proteasome inhibitors ALLN, MG-132, or Proteasome Inhibitor-I prior to analysis of lysates by immunoblotting of A3G-Myc or the α-tubulin loading control. (**b**) Cultures were incubated for 10h with the proteasome inhibitor ALLN prior to purification of A3G-Myc from cell extracts using Ni-NTA agarose and washing and elution buffers that contained 8M urea. Aliquots of the eluted protein were then analyzed by Western immunoblotting using Myc-specific or ubiquitin-specific antibodies.

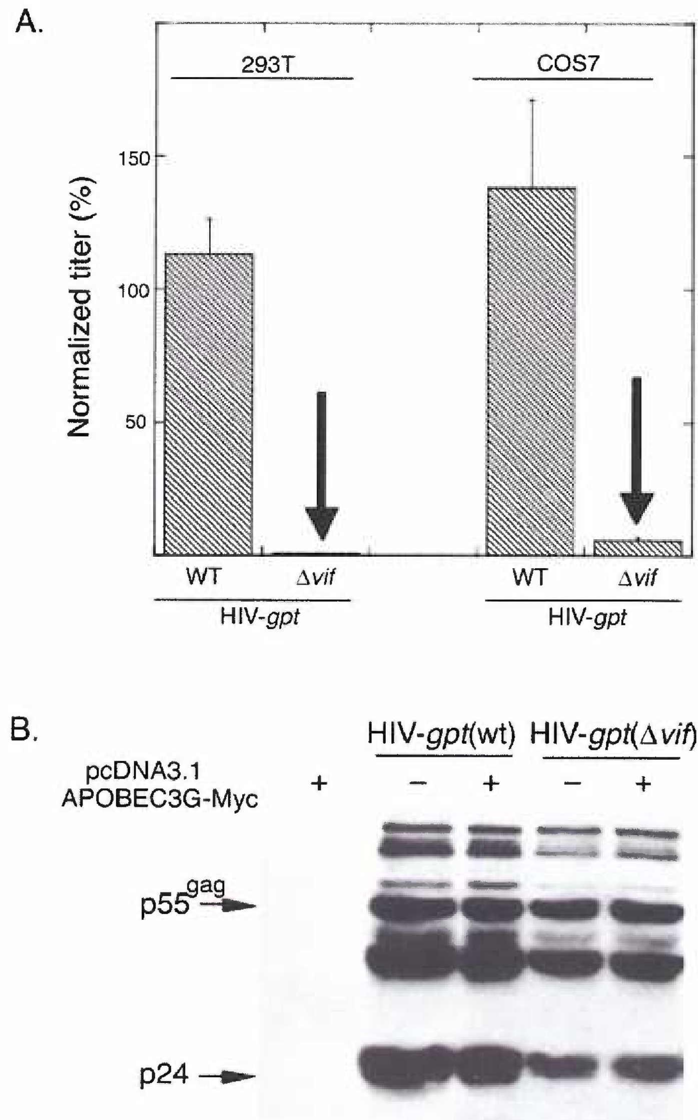
Figure 6





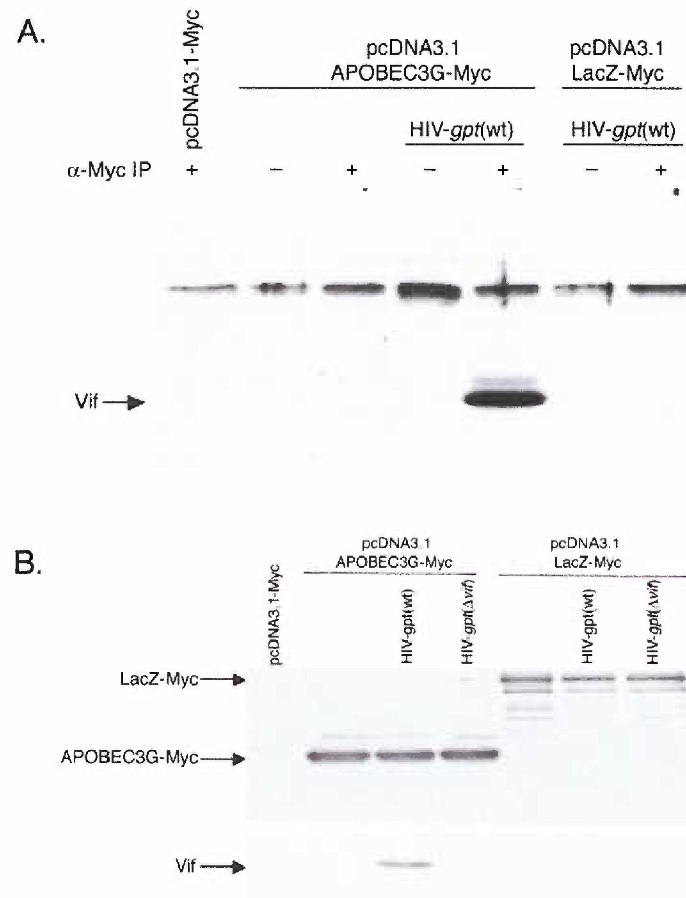
Supplementary Fig. 1.. Expression of human A3G-Myc in human 293T or African green monkey COS7 cells converts them to the nonpermissive phenotype._ The cells were cotransfected with a plasmid for expression of A3G-Myc or pcDNA3.1-Myc in the presence of plasmids for expression of HIV-*gpt*(wild-type) or HIV-*gpt*(Δ*vif*) viruses. Virions were subsequently harvested from the media. The titer of each virus made in the presence of A3G-Myc was normalized relative to the titer of the same virus made in the absence of A3G-Myc (n=6 for 293T cells and n=5 for COS7 cells; error bars are ± s.e.m.). As shown in (**a**), A3G had no significant effect on titers of HIV-*gpt*(wild-type) but strongly reduced titers of HIV-*gpt*(Δ*vif*). (**b**) extracts of cotransfected 293T cells were analyzed for HIV-1-encoded Gag proteins by immunoblotting. A3G-Myc had no effect on synthesis of Gag proteins, consistent with the classical NP phenotype.

Supplemental Figure 1



Supplementary Fig. 2. Specificity controls for the Vif – A3G-Myc coimmunoprecipitation assay. 293T cells were cotransfected with pcDNA3.1-Apobecc3G-Myc, pcDNA3.1-LacZ-Myc, or a negative control pcDNA3.1-Myc vector, in the presence where indicated of pHIV-*gpt*(wild-type) or pHIV-*gpt*(Δ*vif*). (**a**) The cell extracts were precleared by adsorption onto protein A-Sepharose 4B beads. The precleared extracts were then divided into two portions, one that received the 9E10 anti-Myc antibody (labeled +) and the other without (labeled -) before adsorption onto additional protein A-Sepharose 4B beads. The eluted proteins were analyzed by immunoblots that were developed using the Vif-specific rabbit antiserum. Vif coimmunoprecipitates with A3G-Myc in a highly specific manner. (**b**) 293T cell cultures were separately transfected with the individual plasmids, and cell extracts were then prepared from these cells and from the negative control cells. The extracts were then mixed for 30 min at 0^0 C prior to preclearing and immunoprecipitation using the Myc-specific monoclonal antibody, and the immunoprecipitated proteins were then analyzed for Vif by Western immunoblotting. The results show that Vif and A3G made in separate cell cultures formed a specific complex when the cell extracts were mixed prior to immunoprecipitation. Vif had no effect on the quantity of A3G-Myc in these mixed extracts incubated at 0^0 C.

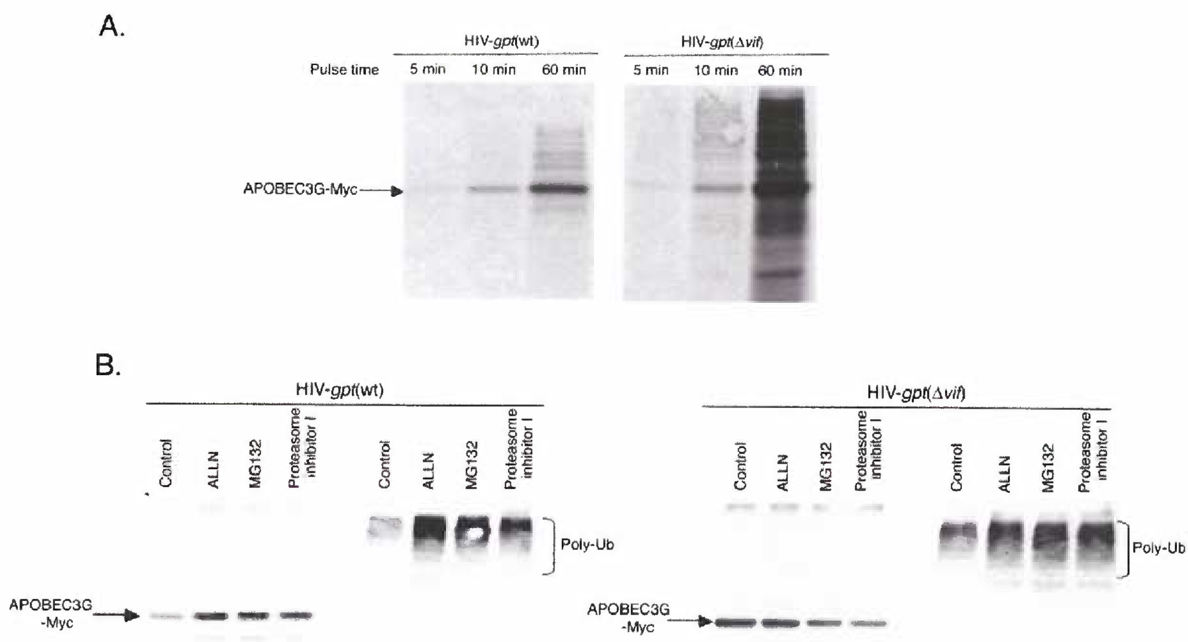
Supplemental Figure 2



Supplementary Fig. 3. Control studies concerning Vif-induced A3G-Myc degradation. 293T cell cultures were cotransfected with vectors for expression of A3G-Myc and with HIV-*gpt*(wild-type) or HIV-*gpt*(Δ*vif*). (**a**) After 36 h, the cultures were washed 2X with DMEM lacking cysteine and methionine and incubated with the same media for 60 min at 37°C. The cells were then labeled in the continuous presence of L-[^{35}S]amino acids (final specific radioactivity of 10 Ci/mmole) for 5, 10, or 60 min. A3G-Myc was immunoprecipitated from the cell extracts and the immunoprecipitated proteins were analyzed by electrophoresis followed by autoradiographic detection. The amounts of [^{35}S]A3G-Myc in the two cultures were similar after 5 min of labeling. However, by 60 min of continuous incorporation the culture lacking Vif had accumulated approximately 4 – 5 times more [^{35}S]A3G-Myc than the culture containing Vif. The results suggest that a large proportion of the [^{35}S]A3G-Myc synthesized in cultures that contain Vif is rapidly degraded. The remainder accumulates in cells that lack Vif (see **Fig. 2**). (**b**) is an analysis of proteasome inhibitor activities in 293T cultures that express A3G-Myc in the presence or absence of Vif. The cultures were preincubated for 6h with the proteasome inhibitors ALLN, MG-132, or Proteasome Inhibitor-I prior to lysis and Western blot analyses of the total cell culture extracts. In the left panels of each pair of blots, we used the antibody specific for Myc. The proteasome inhibitors increased the quantities of A3G-Myc in the cultures that contained Vif but not in the cultures that lacked Vif. This supports the conclusion that Vif induces relatively rapid degradation of A3G-Myc by a proteasome-dependent pathway. The right panels of each pair of blots are analyses of proteins in the total cell culture extracts that bind to the ubiquitin-specific antibody. The proteasome inhibitors were active in both cultures, as indicated by their augmentations of the

quantities of polyubiquitinated proteins. The polyubiquitinated proteins in these extracts consist of numerous cellular proteins and are not specifically related to A3G-Myc.

Supplemental Figure 3



Discussion

These results substantially clarify the mechanism by which Vif neutralizes an innate antiviral pathway in human T lymphocytes. Specifically, Vif binds to the A3G cytidine deaminase and targets it for rapid degradation by a proteasome-dependent pathway (**Figs. 1–6**). The rate of this Vif-induced degradation ($t_{1/2}$~ 1-2 min) was similar to that of ornithine decarboxylase, which is the most rapidly degraded protein previously known (286). Thereby A3G is eliminated from cells that contain wild-type Vif (**Fig. 2**) and it is consequently absent from HIV-1 progeny virions that are produced by these cells (**Fig. 3**). Although additional investigations of the mechanism of Vif-induced degradation of A3G are necessary, our results suggest that this pathway may involve ubiquitination. Specifically, we have detected large polyubiquitinated derivatives of A3G-Myc in amounts that are substantially increased by Vif and by the proteasome inhibitor ALLN (**Fig. 6b**). Since there is much less A3G-Myc in the samples purified from the Vif-positive cultures (**Fig. 6b**) and since steady-state levels of A3G-Myc are very low in the cells that contain Vif (**Fig. 2**), the polyubiquitination that we have detected must be extremely accelerated and efficient in the cells that are rapidly degrading A3G-Myc. Nevertheless, a relatively slow and inefficient process of A3G-Myc polyubiquitination also occurs in the absence of Vif.

Accordingly, we have noticed that the SLQ(Y/F)LAΦΦΦΦ motif in Vif and the downstream proline-rich region is very similar to the BC-box sequence SLQYLC---Φ in human SOCS6 (suppresser of cytokine signalling) that also occurs in other proteins including the von Hippel-Lindau tumor suppressor (129). BC-box proteins associate with Elongins B and C and a cullin to target other proteins for ubiquitination and degradation

(124). This correspondence strongly supports our results and leads us to propose that Vif may function in association with these or related proteins as an E3 ubiquitin-protein isopeptide ligase. Interestingly, the HIV-1 encoded Vpu protein targets CD4 for degradation by a different ubiquitin-dependent pathway (173, 241).

Additional considerations support these interpretations. Two Vif mutants Δ12 and Δ31 that have overlapping deletions of the conserved SLQ(Y/F)LAΦΦΦΦ core sequence retain ability to bind A3G but have lost the capability for inducing its degradation (**Fig. 4**). This indicates that Vif binding to A3G is insufficient for neutralizing the antiviral phenotype and that the conserved core region of Vif then mediates the targeted degradation, as proposed above. A catalytic mechanism is also suggested by the observation that small amounts of Vif expression vectors suffice for efficient A3G elimination and that a pcDNA3.1-Vif vector that causes relatively little Vif expression also strongly downmodulates A3G (**Fig. 1**). The concentration of Vif is unaffected by coexpression of A3G (**Fig. 1a, b**), which also implies that Vif functions repetitively.

Our conclusion that Vif eliminates A3G from cells is consistent with our studies of HIV-1 virions (see **Fig. 3**). Thus, in controlled studies we reproducibly find that A3G is absent from HIV-1 virions made in cells that contain Vif, whereas it occurs in HIV-1 virions purified from cells that lack Vif. Although our data do not establish whether the A3G-mediated attack on HIV-1(Δ*vif*) occurs in nonpermissive producer cells or later in virion particles or in subsequently infected target cells, recent evidence supports the latter alternative (107, 148, 170, 311). Specifically, these reports indicate that A3G causes cytidine deamination of the negative DNA strand during reverse transcription in the target cells. Therefore, we believe that the Vif-induced elimination of A3G from virus-

producing cells and thereby from progeny virions is the primary mechanism for control of viral infectivity in nonpermissive cells.

These results are encouraging from a drug development perspective, because they imply that Vif has a conserved core with an SLQ(Y/F)LAΦΦΦΦ motif that mediates A3G degradation. The conservation of this core during lentiviral evolution (195) suggests that it has been difficult to mutate without loss of viral fitness, implying that drug escape mutants might have low replicative efficiencies. Moreover, A3G is clearly a grave threat to HIV-1 as indicated by its need for the *vif* gene (60, 86, 287). Consequently, drug selection strategies focused on the binding and degradative processes described here might be useful.

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Chapter 3

Transcriptional Regulation of APOBEC3G, a Cytidine Deaminase that Hypermutates Human Immunodeficiency Virus

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Rose KM, Marin M, Kozak, SL, Kabat D, 2004, *Journal of Biological Chemistry*, 279: 41744-9.

Abstract

APOBEC3G (A3G) is an antiretroviral deoxycytidine deaminase that lethally hypermutates human immunodeficiency virus type 1 (HIV-1), but is itself neutralized by the HIV-1 encoded viral infectivity factor (Vif). Accordingly, A3G occurs specifically in human T lymphocytic cell lines including H9 that contain this antiviral defense. Since the substrate specificities of related cytidine deaminases are strongly influenced by their intracellular quantities, we analyzed the factors that control A3G expression. The levels of A3G mRNA and protein were unaffected by treatment of proliferating H9 cells with interferons or tumor necrosis factor-α, but were enhanced up to 20-fold by phorbol myristate acetate. This induction was mediated at the transcriptional level by a pathway that required activation of the protein kinase Cα/βI isozyme (PKC), mitogen-activated protein kinase kinase 1 and 2 (MEK), and extracellular signal-regulated kinase (ERK). Correspondingly, induction of A3G was blocked by multiple inhibitors that act at diverse steps of this pathway. The PKCα/βI / MEK / ERK pathway also controlled basal levels of A3G mRNA and protein, which consequently declined when cells were treated with these inhibitors or arrested in the G_0 state of the cell cycle by serum starvation. We conclude that expression of the antiviral A3G editing enzyme is dynamically controlled by the PKCα/βI / MEK / ERK protein kinase cascade in human T lymphocytes.

Introduction

APOBEC3G (A3G) is a potent antiretroviral deoxycytidine deaminase that occurs in human T lymphocytes and macrophages that lethally hypermutates the viral negative DNA strand shortly after the DNA is synthesized by reverse transcriptase (148, 170, 174, 251, 306, 311). Furthermore, A3G is specifically incorporated into the cores of progeny virions that are produced by these cells, a location that is critical for its antiviral activity (132, 157, 174, 175, 180, 249, 266, 308). Human immunodeficiency virus (HIV-1) and nearly all other members of the lentiviral genus of retroviruses encode a viral infectivity factor (Vif). HIV-1 Vif binds specifically to human A3G and induces its polyubiquitination and rapid degradation by proteasomes (157, 175, 180, 249, 266, 308), thereby ridding the virus-producing cells of A3G and saving the viral progeny (49, 132, 157, 174, 175, 180, 249, 266, 308). This effect is species-restricted, since HIV-1 Vif cannot bind to A3G from African green monkeys or mice (30, 171, 174, 240, 293).

Although A3G occurs in T lymphocytes and in several leukemic T cell lines including HUT78 and its derivative H9 that contain this antiviral defense system, it is absent in many other cell lines that have been examined (249, 266). Consequently, these other cell lines are permissive for replication of HIV-1(Δ*vif*) that contains an inactivating mutation or deletion of the *vif* gene (86, 166, 254). Expression of A3G in these permissive cell lines converts them to the nonpermissive phenotype, as determined by their ability to efficiently inactivate HIV-1(Δ*vif*) but not wild-type HIV-1 (49, 132, 157, 174, 175, 180, 249, 266, 308). Recently, another member of the cytidine deaminase family, APOBEC3F, was also shown to have antiretroviral activity (292, 313). Similarly,

the RNA-specific adenosine deaminases (ADARs), which are induced by interferons in response to double-stranded RNA, inactivate several RNA viruses (234).

The factors that control expression of A3G have not previously been investigated. Furthermore, it is unknown whether the A3G that occurs naturally in T lymphocytes is constitutively active or whether its anitiviral function is induced by infection or by signal transduction processes such as the classical innate antiviral responses involving type I interferons (INF) and tumor necrosis factor-α (TNF-α) (117, 234). A3G occurs in a family of cytidine deaminases that includes APOBEC1, 2, 3A, 3B, 3C, 3E, 3F, ARCD-1, and AID. It is encoded on chromosome 22 in a region that also contains the genes or pseudogenes for APOBEC3A to 3F (11, 126). Previously, it was shown that phorbol esters cause increases in the amounts of APOBEC3A and APOBEC3B mRNAs by an unknown mechanism. Consequently, these enzymes were initially termed phorbolin-1 and phorbolin-2, respectively (168, 218). Phorbol myristate acetate (PMA) activates several protein kinase C (PKC)-dependent and -independent intracellular signaling pathways (36, 159, 182). The most extensively studied PKC-dependent pathway involves activation of Ras and the downstream kinases Raf, mitogen-activated protein kinase kinase 1 and 2 (collectively termed MEK) and the extracellular signal-regulated kinase (ERK) (42, 150, 239). Activated ERK stimulates specific transcription factors that have been shown to upregulate expression of a diverse array of genes in different tissues (42, 280). ERK is a member of the mitogen-activated protein kinase (MAPK) family, which also includes JNK and p38. The latter are not activated by PMA but respond to stress stimuli and inflammatory cytokines (36, 280).

Specific factors have been shown to regulate the expression and to control the functions of APOBEC1 and AID (21, 56, 152, 181, 187). The substrate specificity of the well-studied APOBEC1 enzyme is controlled by APOBEC-1-complementation factors (ACFs), which recruit it to a specific site in the apolipoprotein B mRNA (56, 181). It is only when APOBEC1 is expressed in excess of the ACFs that it deaminates other mRNAs (262, 294). Interestingly, overexpression of A3G and other cytidine deaminases can cause mutagenesis and cellular toxicity (109, 294, 303). Thus, there is precedence for the hypothesis that cellular factors and expression levels of cytidine deaminases can regulate their functions.

Here we describe our initial characterization of the factors that control A3G expression levels in T cells. Interestingly, A3G mRNA synthesis is dynamically controlled by a protein kinase cascade that is induced by extracellular signals and that is inhibited when cells become arrested in the G_0 stage of the cell cycle.

Methods

Cell culture and Reagents. H9 cells were maintained in RPMI with 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (Invitrogen). Phorbol-12-myristate-13-acetate (PMA) (100nm-1μM), an inactive phorbol ester, 4α-phorbol-12,13-didecanoate (4αPMA) (100nm) (Calbiochem), TNF-α (50ng/ml) (R&D Biosystems), INF-α (1μg/ml), INF-γ (1μg/ml) (Peprotech, Inc.), INF-β (1μg/ml) (PBL Biomedical Laboratories), were added to the culture media of H9 cells for 2-24hrs. H9 cells were treated with PKC inhibitors Calphostin C (100nM), PKC inhibitor 20-28 (also known as myristolated pseudosubstrate 20-28) (20μM), Ro-32-0432 (200nM), or Gö6976 (50nm), or specific MEK inhibitors PD98059 (50μM) and U0126 (20μM) (Calbiochem) for 1hr prior to and for the duration of PMA treatment. Cyclohexamide (10μg/ml) or actinomycin D (5μg/ml) (Sigma) were added to the media of H9 cells in culture 1hr prior to mock treatment or treatment with PMA for up to 16hrs.

Northern blot analysis and qRT-PCR. RNA extraction and northern blot analysis were previously described (274). [^{32}P]-labeled cDNA probes were used to detect A3G, protein kinase R (PKR), or the S2 ribosomal protein mRNAs (used as a loading control). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed according to standard protocols (114). Briefly, 1μg of total RNA was reverse transcribed using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). The cDNAs of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and A3G were amplified using 2X SYBR Green Master Mix (2X PCR Buffer, 4mM $MgCl_2$, 0.4mM dNTPs, 0.005% SYBR Green, 2X Rox reference dye, 16% DMSO, 0.04U/μl Platinum

Taq polymerase) and an ABI Prism 7700 sequence detection system (Applied Biosystems). The sensitivity of the PCR was tested by amplification of the target from serially diluted cDNAs generated from reverse transcription of human reference RNA (Stratagene). qRT-PCR amplification within samples was normalized using GAPDH amplification levels as an endogenous control. Each sample was assayed in triplicate using the primer pair 5'-TCAGAGGACGGCATGAGACTTA-3', 5'-AGCAGGACCCAGGTGTCATT-3'specific for A3G and the primer pair 5'-GAAGGTGAAGGTCGGAGT-3', 5'-GAAGATGGTGATGGGATTTC-3' for GAPDH. Data analysis and calculations were done following the $2^{-\Delta\Delta CT}$ comparative method outlined in the ABI Prism 7700 user bulletin #2.

Western blot analysis. Western blot analysis was previously described (177). Briefly cells were lysed with RIPA buffer containing phosphatase inhibitors (50mM Tris-Cl (pH 7.4), 1% Nonident P40, 0.1% sodium deoxycholate, 150mM NaCl, 2mM $NaVO_3$, and 25mM NaF) and complete protease inhibitors (Roche). Cell lysates were adjusted to equal protein concentrations by using Bradford reagent (Biorad) and equal amounts were used for Western immunoblotting. A3G was detected using a rabbit peptide antibody (a gift from Warner Greene, UCSF). MAPK were detected using antibodies for MEK-1, ERK-1, and phospho-specific antibodies for detecting the phosphorylated forms of both MEK and ERK (Santa Cruz Biotechnology). Activation of p38 was monitored using phospho-specific antibodies for p38 MAPK (a gift from Bruce Magun, OHSU). Equivalent loading of proteins was shown using a mouse antibody specific for α-tubulin (Sigma).

Results

Control of APOBEC3G Expression by Specific Protein Kinase C Isozymes

Northern blot analyses in stringent conditions and real-time quantitative reverse transcription-PCR (qRT-PCR) were both used to evaluate A3G mRNA levels in cells. Furthermore, because sequence similarities exist among APOBEC family members (126), we used oligonucleotide primers that were specific for A3G, and we cloned and sequenced multiple PCR products from independent reactions and confirmed that they all corresponded to A3G. As shown in Fig. 1A and B, A3G mRNA levels in H9 cells were unaffected by treatments with interferons (INF)-α, -β, or-γ, which were active as indicated by their inductive effects on protein kinase R (PKR) mRNA levels (Fig. 1B). Similarly, tumor necrosis factor-α (TNF-α) had no effect on the A3G mRNA level, although it was also active in these cells as indicated by its ability to phosphorylate and activate p38 MAPK (data not shown). In contrast, phorbol myristate acetate (PMA) induced strong increases in A3G mRNA levels within 2hrs that continued until at least 10hrs post-treatment. This effect was specific for PMA because an inactive phorbol ester, 4α-phorbol-12,13-didecanoate (4αPMA) had no effect on A3G mRNA levels (Fig. 2B). Prolonged exposure to PMA causes elimination of protein kinase C (PKC) (159)which may explain the diminished effect of PMA on A3G expression levels at 24hrs post-treatment (Fig. 1).

PMA was previously shown to increase mRNA levels of APOBEC3A and APOBEC3B (168, 218). PMA mimics the second messenger diacylglycerol that binds to and activates PKC. There are eleven known PKC isozymes, nine of which are PMA sensitive (α,βI,βII,θ,γ,δ,ε,η,μ) (159). To determine whether enhancement of A3G

mRNA levels by PMA treatment is mediated by PKC, general PKC inhibitors (Calphostin C, PKC inhibitor 20-28) as well as inhibitors specific for PKCα/βI (Gö6976, Ro-32-0432) were incubated with H9 cells 1 hour prior to and for the duration of PMA or a mock treatment. All PKC inhibitors abolished the induction of A3G mRNA by PMA (Fig. 2A). Inhibitors that are highly selective for PKCα /βI isozymes were as effective as general PKC inhibitors at preventing PMA enhancement of A3G mRNA, suggesting that PKCα/βI isozymes are solely responsible for this effect.

MEK regulates APOBEC3G mRNA levels

Activation of PKCα/βI is critical for the regulation of mRNA levels for many genes in numerous cell lines (42, 150, 280). The major downstream target of PKCα/βI is MEK (47, 239). To evaluate the possible role of MEK as a downstream target of PMA treatment, H9 cells were treated for 1 hour with specific MEK inhibitors U0126 or PD98059 (66, 73) prior to and during PMA treatment. These MEK inhibitors efficiently blocked the PMA-induced increase in A3G mRNA (Fig. 2B and C). Indeed, A3G mRNA levels in H9 cells treated with U0126 or PD98059 declined below the basal level within 4h post-treatment and continued to decline over a 24hr period (Fig. 2B and C), suggesting that MEK-dependent signal transduction is necessary both to maintain the basal level of A3G and to enhance the A3G mRNA level in response to PMA.

We also did Western immunoblot analyses of H9 cell extracts using a previously characterized antiserum made to a peptide corresponding to the carboxyl terminal 16 amino acids of A3G. This antiserum is highly specific for A3G (results not shown) (266) and detected a single protein in the cell extracts in relative amounts that correlated closely with the cellular A3G mRNA levels (Fig. 3). Serum-starvation arrests

cultured cells at the G_0/G_1 transition of the cell cycle and causes dephosphorylation and consequent inactivation of MEK, whereas progression to G_1 coincides with MEK activation (147, 183, 202). Accordingly, serum starvation of H9 cells caused a reproducible decline in the A3G protein content and PMA treatment of serum-starved H9 cells caused an induction of A3G protein that was blocked by the MEK inhibitors PD98059 and U0126 (Fig. 3, left panel). Although these treatments had no effect on the total cellular quantity of MEK, PMA induced the phosphorylation of MEK and this was also inhibited by PD98059 and U0126 (Fig. 3, right panel). This result is consistent with previous evidence that these compounds can inhibit the phosphorylation of MEK as well as the activity of the phosphorylated MEK (73). The only known downstream substrates of phosphorylated MEK are ERK1/2 (156). As shown in Fig. 3 (right panel), serum starvation caused almost complete elimination of phosphorylated ERK, whereas PMA induced its phosphorylation in these serum-starved cells. This induction was prevented by the MEK inhibitors PD98059 and U0126 (Fig. 3, right panel). In contrast, total cellular ERK levels were unaffected by these treatments.

PMA induces increased APOBEC3G mRNA synthesis

Activation of ERK has been reported to increase levels of mRNAs by either transcriptional enhancements or by stabilization of mRNAs (150, 280). To distinguish between these possibilities, H9 cells were mock treated or treated for 8 hours with PMA before actinomycin D was added to block further transcription. Aliquots of cells were taken at subsequent times to monitor A3G mRNA stability. At the time of actinomycin D addition, the level of A3G mRNA was approximately four times higher in the PMA-

treated cells than in the untreated control cells. Consequently, the blot exposures were normalized to show equal intensities of the A3G mRNAs at the time of actinomycin D addition. As shown in Fig. 4A, no significant differences in A3G mRNA stability were observed over 16 hours in the presence or absence of PMA. Therefore, we conclude that PMA does not function by stabilizing A3G mRNA. To evaluate the synthesis of A3G mRNA in the presence of PMA, H9 cells were cultured in the absence of serum for 24 hours, followed by treatments with the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide, in combination with specific MEK inhibitors. After 1 hour, cells were stimulated with PMA. PMA caused an increase in A3G mRNA that was blocked by all of the inhibitors (Fig. 4B). These results suggest that PMA induces an increase in A3G mRNA synthesis by a pathway that requires continued protein synthesis and activated MEK.

Similar inductive effects of PMA on A3G levels were seen in several other proliferating T cell lines that contained basal amounts of A3G in the absence of PMA. However, PMA did not induce significant amounts of A3G expression in cells that had very low or negligible basal amounts of this protein, implying that PMA alone may be unable to activate fully repressed A3G genes (results not shown). In agreement with this observation, we also found in a preliminary study that PMA did not convert permissive cells to the nonpermissive phenotype. Conversely, we found that H9 cells did not become permissive when they were starved for serum or treated with MEK inhibitors for 24h in conditions that cause reductions in intracellular levels of A3G. We consider the latter studies preliminary because these treatments caused only modest reductions in the

intracellular amounts of A3G (see Fig. 3), presumably because A3G mRNA and protein turn over relatively slowly (see Figs. 2 and 4) (175, 266).

Figure 1. APOBEC3G mRNA levels are enhanced by PMA treatment. Total RNA was isolated from H9 cells at the indicated times following exposures of the cultures to PMA (1μM), INF-α (1μg/ml), INF-γ (1μg/ml), INF-β (1μg/ml), or TNF-α (50ng/ml) and was analyzed by Northern blotting (A), and by quantitative real-time PCR (B) to determine the quantities of A3G mRNA. Northern blots were subsequently probed with [^{32}P]-labeled DNA probes for A3G, protein kinase R (PKR), and S2 ribosomal RNA (loading control). PKR is a positive control for INF signal transduction.

Figure 1

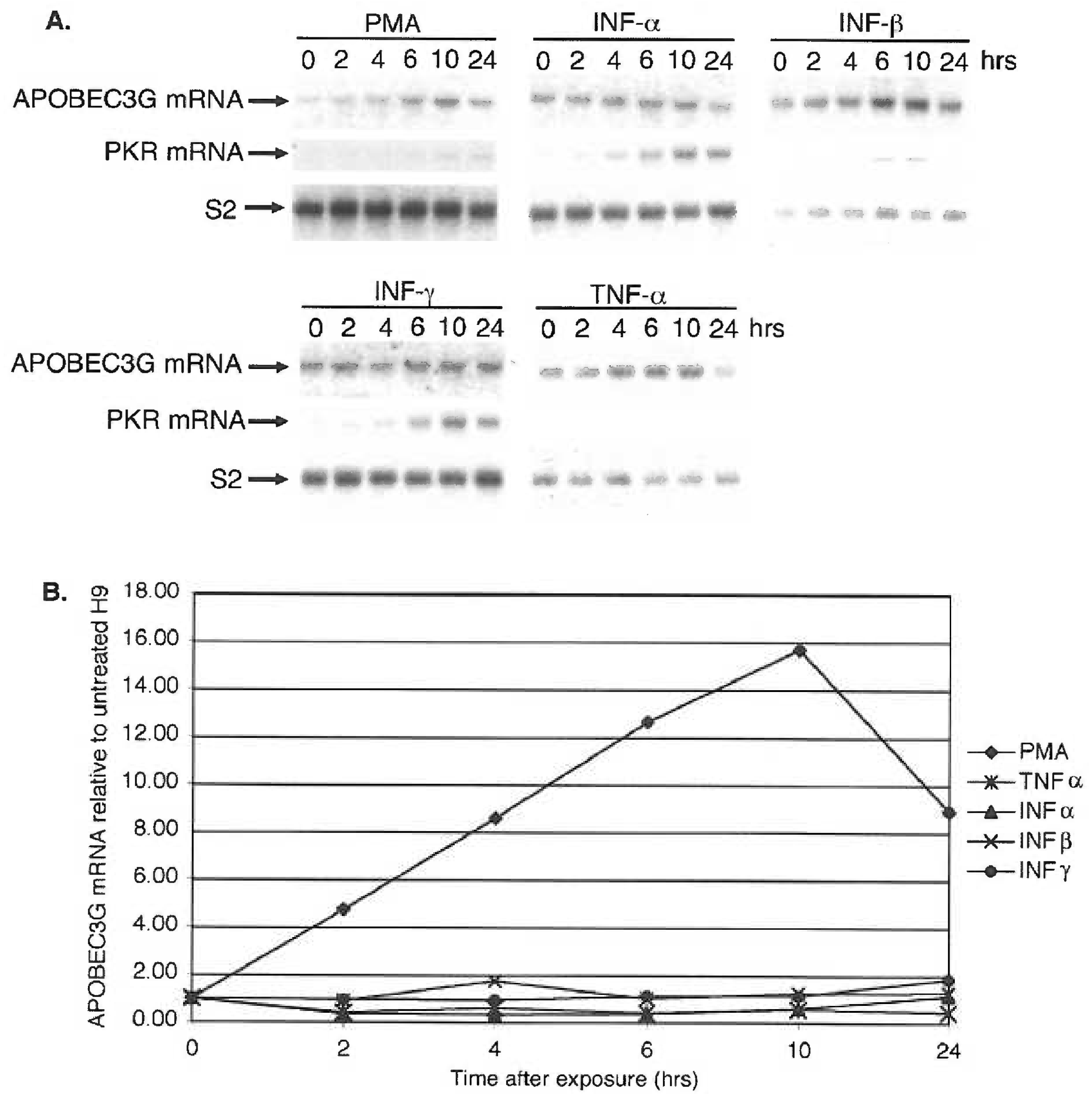


Figure 2. APOBEC3G mRNA levels are regulated by PKC and MEK1/2. (A) H9 cells were treated with the general protein kinase C (PKC) inhibitors, PKC inhibitor 20-28 (100nm), or Calphostin C (50nM), or with the PKCα/βI specific inhibitors, Gö6976 (200nM), and Ro-32-0432 (100nM) for 1hr prior to addition of PMA (100nM). (B) H9 cells were treated with the specific MEK inhibitors U0126 (20μM) or PD98059 (50μM) or mock treated with DMSO alone for 1hr prior to addition of PMA (100nM) or 4αPMA (100nM), an inactive phorbol ester. The DMSO concentrations were equal in all of the cultures. RNA was isolated from cells at the indicated times and was analyzed by quantitative real-time PCR for A3G mRNA levels. (C) Northern blot analysis of H9 cells treated with U0126 (20μM) or mock treated for 1hr prior to addition of PMA (100nM) using a [^{32}P]-labeled DNA probe specific for A3G. The northern blot was subsequently probed with a [^{32}P]-labeled DNA probe for the S2 ribosomal RNA loading control.

Figure 2

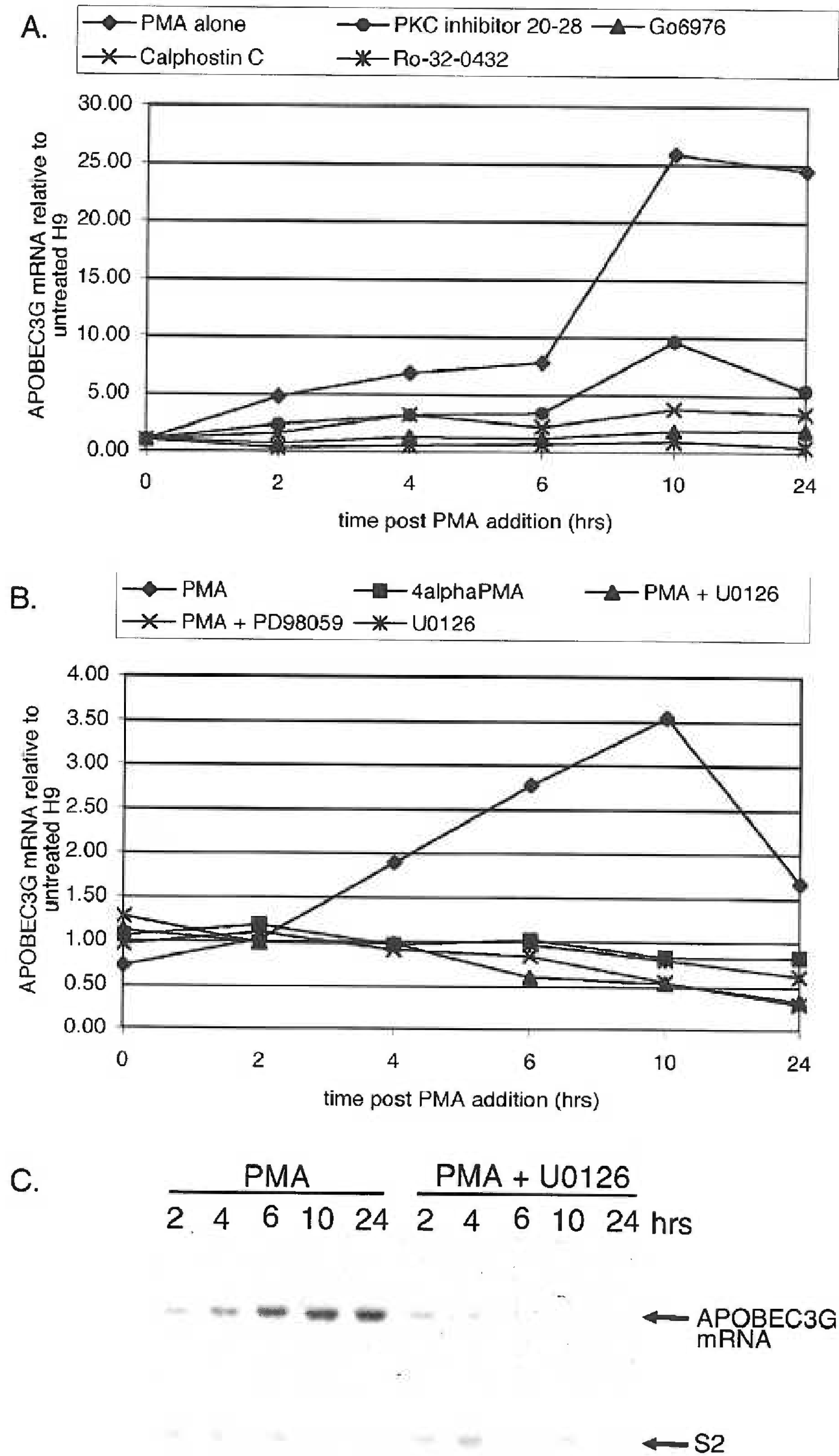


Figure 3. PMA enhances APOBEC3G protein levels in a PKC and MEK-dependent manner. H9 cells were cultured in the absence of serum for 24hrs followed by addition of PD98059 (50μM) or U0126 (20μM) for 1hr prior to treatment with PMA (100nM) for 12hrs. Cell lysates were analyzed by Western blotting using antibodies specific for A3G, MEK-1, phosphorylated MEK1/2 (p-MEK1/2), extracellular signal-regulated kinase-1 (ERK-1), phosphorylated ERK-1(pERK-1), and α-tubulin.

Figure 3

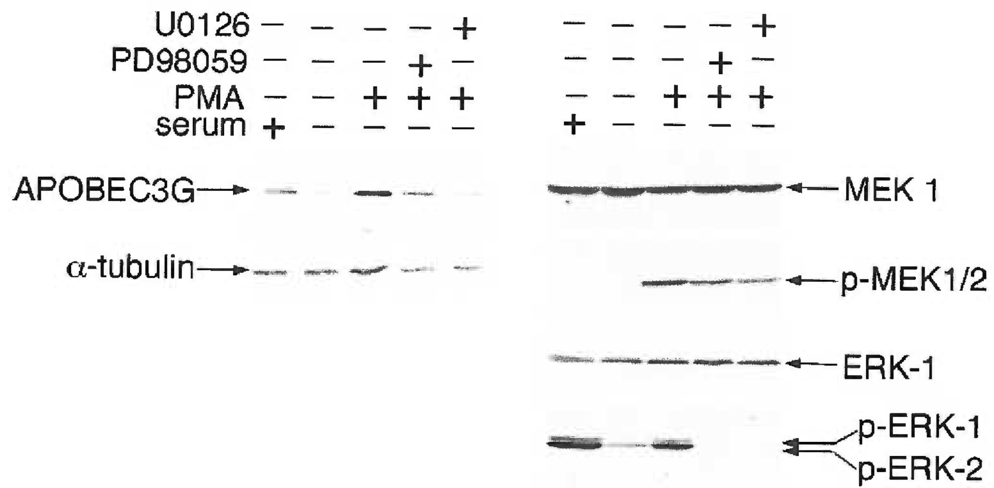
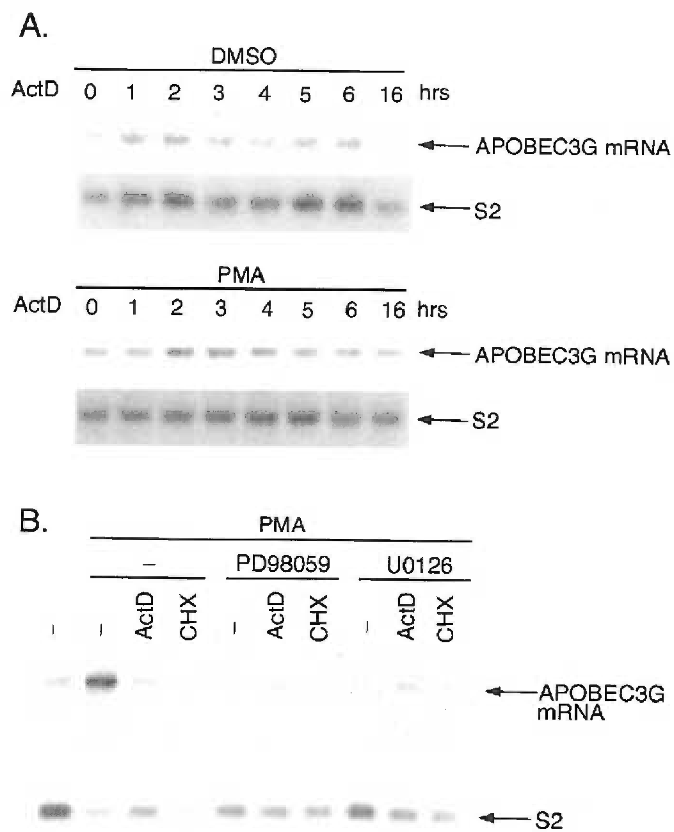


Figure 4. PMA enhances APOBEC3G mRNA synthesis. (A) H9 cells were mock treated with DMSO alone or were treated with PMA (100nM) for 8hrs prior to addition of 5μg/ml actinomycin D. RNA was isolated at indicated times and analyzed by Northern blot analysis with a [^{32}P]-labeled DNA probe specific for A3G. The northern blot was re-probed to detect the S2 ribosomal RNA loading control. (B) H9 cells were mock treated (-), or treated with 5μg/ml actinomycin D (ActD), or 10μg/ml cyclohexamide (CHX) in the presence or absence of 50μM PD98059 or 20μM U0126 for 1hr prior to addition of 100nM PMA. RNA was isolated from cells after a 4hr incubation with PMA and analyzed by Northern blot analysis with [^{32}P]-labeled DNA probes specific for A3G mRNA and for the S2 ribosomal RNA loading control.

Figure 4



Discussion and Conclusions

These studies provide initial evidence concerning the dynamic regulation of A3G mRNA and protein levels in the human T lymphocytic cell line H9. Unlike many other antiviral factors (e.g., ADARs, ribonuclease L, Mx GTPase, PKR) (234), the syntheses of A3G mRNA and protein were unaffected by type 1 or type 2 interferons (Fig. 1). These interferons were active in H9 cells as indicated by their abilities to induce the synthesis of PKR. In addition, A3G was unaffected by TNF-α, which was also active in these cells (Fig. 1). In addition, A3G mRNA levels do not change in response to HIV-1 infection (180, 266). In striking contrast, the quantity of A3G mRNA was enhanced approximately 20-fold by treatment of the cells with PMA (Fig. 1 and 2). This induction was mediated by the PKCα/βI isozymes and by the downstream protein kinase cascade involving MEK and ERK and it was consequently inhibited by the PKCα/βI inhibitors Gö6976 and Ro-32-0432 and by the MEK inhibitors U0126 and PD98059 (Figs. 2-4). These inductive and inhibitory effects were seen using Northern blot analyses in stringent hybridization conditions as well as qRT-PCR assays using primers that were specific for A3G (Figs. 1, 2C, and 4), and were confirmed by protein immunoblot analyses using an anti-peptide antiserum made to the specific carboxyl terminal region of A3G (Fig. 3) (266).

Interestingly, the basal expression of A3G also appeared to depend on this same protein kinase cascade. Thus, the basal level of A3G mRNA was reduced in the presence of these inhibitors (Fig. 2B and C). In accordance with these conclusions, it is known that MEK becomes dephosphorylated when cells enter the G_0 state of the cell cycle (147, 183, 202), and we verified this using serum starved cells as shown in Fig. 3.

This treatment caused accumulation of the cells in the G_0 state and resulted in dephosphorylation of the downstream target ERK (Fig. 3). PMA treatment of these growth arrested cells induced the phosphorylation of MEK and of ERK and the accumulation of A3G mRNA and protein in a manner that was inhibited by the MEK inhibitors U0126 and PD98059 (Figs. 3 and 4B). Considered together, these data strongly indicate that the expression levels of A3G mRNA and protein are regulated by a protein kinase cascade involving PKCα/βI, MEK1/2, and ERK1/2 and by the interactions of this cascade with the normal cell cycle. These results may potentially explain the increase in A3G previously reported to occur when resting T lymphocytes are activated with interleukin-2 and phytohemmagluttinin (266).

Our data further suggest that the effects of PKCα/βI and MEK are mediated by changes in synthesis of A3G mRNA rather than by alteration of the mRNA stability (Fig. 4A and B). Thus, the rate of A3G mRNA degradation was not significantly altered despite the substantial increase in A3G mRNA caused by the PMA-dependent activation of PKCα/βI. Previous evidence has shown that the MAPK pathway implicated by our results can activate numerous transcription factors including CREB, NFκB, AP-1 (c-Jun, c-Fos), c-Myc, Ets-1 and Elk-1 (149). A domain search of the 5'-untranslated region of A3G (accession # NM_0218252) using web-based search engines, TFSEARCH (115) and TESS (18), revealed several binding sites for Ets-1, c-Myc, and Elk-1. Further investigations will be needed to determine whether these putative binding sites are bound by transcription factors and whether any of these ERK regulated transcription factors are responsible for the PMA-induced increase in A3G transcription implied by our data.

It was previously shown that APOBEC3A and APOBEC3B are induced by phorbol esters (168, 218). Our results extend this to A3G, which is encoded in the same gene cluster on chromosome 22 (126). Although we presume that the mechanisms of the APOBEC3A and APOBEC3B inductions may be similar to that described here for A3G, the PMA induction pathways for these other cytidine deaminases have not been investigated. In any case, the close linkage of these genes that are activated by PMA raises the possibility that they might be coordinately regulated by a PKCα/βI / MEK / ERK-dependent locus control region as well as by promotor-specific transcription factors that influence their tissue- and cell-specific patterns of expression. This differential pattern of expression clearly is important in the case of A3G, which is present only in some lymphoid and myeloid cell lines(126).

It is unknown whether A3G has a normal cellular function in addition to its role in inhibiting the retroviruses and retroelements that continually invade the genomes of mammals. However, in view of its powerful antiretroviral activity, it is intriguing that A3G is so strongly and dynamically regulated by a protein kinase cascade that is known to be activated by extracellular signaling molecules (42, 149). The functions and substrate specificities of other cytidine deaminases can be controlled by their expression levels and interactions with accessory factors (13, 152, 181, 197, 247, 294). Further studies will be needed to learn how these or other regulatory pathways might control the antiviral functions of A3G.

Acknowledgements

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Chapter 4

Regulated Production and Anti-HIV-1 Activities of Cytidine Deaminases APOBEC3B, 3F and 3G

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Abstract

APOBEC3G and 3F (A3G and A3F) cytidine deaminases incorporate into retroviral cores where they lethally hypermutate nascent DNA reverse transcripts. As substantiated here, the viral infectivity factor (Vif) encoded by human immunodeficiency virus type-1 (HIV-1) binds A3G and A3F and induces their degradation, thereby precluding their incorporation into viral progeny. Previous evidence suggested that A3G is expressed in H9 and other nonpermissive cells that contain this antiviral defense but not in several permissive cells, and that overexpression of A3G or A3F makes permissive cells nonpermissive. Using a broader panel of cell lines, we confirmed a correlation between A3G and cellular abilities to inactivate HIV-1(Δ*vif*). However, there was a quantitative discrepancy because several cells with weak antiviral activities had similar amounts of wild-type A3G mRNA and protein compared to H9 cells. Antiviral activity of H9 cells was also attenuated in some conditions. These quantitative discrepancies could not be explained by the presence of A3F or other A3G paralogs in some of the cell lines. Thus, A3A, A3B, and A3C had weak but significant anti-HIV-1 activities and did not dominantly interfere with A3G or A3F antiviral functions. Control of A3G synthesis by the protein kinase C/ mitogen-activated protein kinase kinase / extracellular signal-regulated kinase pathway was also similar in permissive and nonpermissive cells. A3G in highly permissive cells is degraded by Vif, suggesting that it is not in a sequestered site, and is specifically incorporated in low amounts into HIV-1(Δ*vif*). Although A3G and/or A3F inactivate HIV-1(Δ*vif*) and are neutralized by Vif, the antiviral properties of cell lines are also influenced by other cellular and viral factors.

Introduction

The viral infectivity factor (Vif) encoded by human immunodeficiency virus type 1 (HIV-1) neutralizes a potent antiretroviral defense that occurs only in lymphocytes and macrophages and some leukemic T cell lines (157, 174, 175, 180, 227, 248, 249, 266, 308). These cells are nonpermissive for replication of HIV-1(Δ*vif*) that has a deletion or inactivating mutation in its *vif* gene, whereas other cells termed permissive lack this defense (71, 86, 231, 287). Recently, APOBEC3G (A3G) was identified as a component of this antiretroviral system (248). A3G is related to APOBEC1, a cytidine deaminase that causes a single C-to-U change in apolipoprotein B mRNA (247). Additional members of this family include APOBEC2, APOBEC3A-3F (A3A to A3F), and the activation-induced cytidine deaminase (AID) that hypermutates immunoglobulin genes (11, 126). A3G incorporation into virions requires RNA and the HIV-1 nucleocapsid protein (5, 41, 65). When these A3G-containing virions infect cells, lethal dC-to-dU hypermutations occur in HIV-1 DNA reverse transcripts (5, 41, 65, 170, 174, 251, 306, 311). A3F has similar anti-HIV-1(Δ*vif*) activity and is also neutralized by Vif, (28, 155, 292, 313) whereas A3A, A3C, and A3B have been reported to lack or have only weak anti-HIV-1 activities (28, 292, 313). In contrast, A3B and A3C strongly inhibit simian immunodeficiency viruses (305).

Vif accumulates late in the HIV-1 infection cycle coordinately with Gag, Pol, and Env virion components (93). The amino terminal domain of Vif binds A3G, whereas its carboxyl terminal domain recruits a multisubunit ubiquitin-protein isopeptide ligase that causes A3G polyubiquitination and degradation (157, 175, 180, 266, 308). Consistent with roles in infectious diseases, *A3G, A3F,* and *A3B* genes have evolved rapidly with

high frequencies of nonsynonymous substitutions (237, 312). Accordingly, lentiviral Vif proteins only bind and neutralize A3G orthologs of their natural hosts and closely related species (30, 171, 174, 240, 293). Identification of A3G as a determinant of the nonpermissive phenotype relied on subtraction of cDNAs from the nonpermissive cell line CEM with cDNAs from related permissive CEM-SS cells (248). A3G mRNA was also expressed in the nonpermissive cell line H9 and peripheral blood lymphocytes, but not in several analyzed permissive cells (248). Additionally, overexpression of A3G in permissive cell lines made them nonpermissive (49, 157, 174, 175, 180, 248, 249, 251, 266, 308). Although these results implied that A3G may be necessary and sufficient to confer the nonpermissive phenotype, this idea is difficult to reconcile with its presence in numerous nonhematopoietic cells (109, 126) and with recent evidence that A3F rather than A3G might be most important *in vivo* (155). While overexpression of human A3G can hypermutate retroviruses that lack *vif* genes, including murine leukemia viruses and equine infectious anemia virus, (107, 170) the latter replicate with fidelity in T cells and macrophages of their natural hosts (228). Murine A3G is excluded from murine leukemia viruses by an unknown viral mechanism (142). Furthermore, HIV-2 Vif functions interchangeably with HIV-1 Vif, implying a common activity (219, 222, 258) . However, some cells nonpermissive for HIV-1(Δ*vif*) are permissive for HIV-2(Δ*vif*) (219). These results imply that at least some retroviruses have Vif-independent means to overcome normal cellular amounts of A3G and/or A3F. Here we confirm that Vif binds to A3G and A3F and downmodulates their expression and antiviral activities. In addition, we describe evidence that A3G, A3F, and A3B occur in substantial amounts in some cells that have only weak antiviral activities. The antiviral activity of H9 cells also becomes attenuated

in some culture conditions.

Materials and Methods

Cells and reagents. Cells were maintained according to supplier's specifications. Human T-cell lines were H9, HSB-2, A3.01, HUT78, MT-2, SP, SupT1, and CEM-A [AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, NIH (ARRRP)]. A3.01 is a derivative of CEM, whereas CEM-A was derived by fusion of CEM with peripheral blood lymphocytes. Other cells were umbilical cord blood lymphocytes C8166-45, peripheral blood cells Molt4/8, human promonocytes U937, astroglioma U87MG, B-cell AA-2 (ARRRP), HeLa-CD4 (211), 293T (ATCC), and H9LVifSN (H9.Vif) (Didier Trono, CMU, Geneva) (287). Human peripheral blood lymphocytes were prepared according to standard procedures and stimulated with phytohemagglutinin-P and hIL-2. Phorbol-12-myristate-13-acetate (PMA) (100nM) (Calbiochem) was added to cultures for 12-24 h. MEK inhibitors PD98059 (50μM) and U0126 (20μM) (Calbiochem) were added 1h prior and during PMA treatment.

Northern blot analysis and qRT-PCR. RNA extraction and Northern blot analysis were described (274) using [^{32}P]-labeled cDNA probes for A3G or S2 ribosomal protein loading control. Quantitative real-time reverse transcription PCR (qRT-PCR) was described (227). Samples were assayed in triplicate using the specific A3G primer pair 5'-TCAGAGGACGGCATGAGACTTA-3', 5'-AGCAGGACCCAGGTGTCATT-3'; the specific A3B primer pair 5'-CTACGACAACTTTGAAAACGAACCC-3', 5'-ACTGAGGCTTGAAATACACCTGGC-3'; the specific A3F primer pair 5'-CCTACGCAAAGCCTATGGTCGG-3', 5'-CCAGGAGACAGGTGAGTGGTGC-3'; and the glyceraldehyde-3-phosphate dehydrogenase primer pair 5'-GAAGGTGAAGGTCGGAGT-3', 5'-GAAGATGGTGATGGGATTTC-3'.

Viruses and plasmids. Strain NL4-3 HIV-1(wild-type) and HIV-1 (Δ*vif*) were prepared using p83-2, p83-10, and p197-1 (ARRRP) (165). pHIV-*gpt*(wt) and pHIV-*gpt*(Δ*vif*) were pseudotyped using pSVIII Env (165). A3G-Myc and LacZ-Myc vectors were described (175). A3G-Luciferase (A3G-Luc) was made by subcloning A3G in frame in the N-terminus of the firefly luciferase gene in pSP-luc+NF (Promega) followed by cloning into pcDNA3 (Invitrogen) at *Kpn*I and *Xho*I restriction sites. A3B cDNA was amplified from A3.01 cells with primers from the 5' and 3' ends of A3B (database entry NM_004900) and was cloned at *Hind*III and *Xho*I restriction sites into pcDNA3.1/Myc-His (Invitrogen). A3A-HA, A3C-HA, A3F-HA (292) (a gift from Bryan Cullen, Duke University Medical Center), and codon optimized Vif (HVif) expression vectors (193) (a gift from Stephan Bour, National Institute of Allergy and Infectious Diseases) have been previously described. Transfections used PolyFect (Qiagen) with equimolar ratios of plasmids and media collection 36 h post-transfection unless otherwise noted.

Protein analyses. Western blots and immunoprecipitations were described [Marin, 2003 Nat Med #320]. Briefly, cells were lysed with RIPA (50mM Tris-Cl (pH 7.4), 1% Nonident P40, 0.1% sodium deoxycholate and 150mM NaCl) with complete protease inhibitors (Roche) and were adjusted to equal protein concentrations. Phosphatase inhibitors $NaVO_3$ (2mM), and NaF (25mM) were added for phosphospecific Western blots. A3G was detected using a rabbit peptide antibody (gift of Warner Greene, Gladstone Institute, UCSF) (266). Protein loading was assessed with mouse antibody for α-tubulin (Sigma). Additional antibodies were anti-Myc 9E10 (Sigma), anti-pERK-1,

anti-ERK-1 (Santa Cruz Biotechnology), anti-luciferase, anti-HA clone HA-7 (Sigma), HIV-IgG 3957, and Vif antiserum 2221 (ARRRP).

Infection assays and virion preparations. HIV-1(wild-type) and HIV-1(Δ*vif*) were grown in HeLa-CD4 cells and stocks were stored at -80^{0}C and titered by a focal infectivity assay (165). Equal concentrations of these viral stocks were used to infect each test cell line (1x10^6 cells/well in 6-well plate) as well as the control standard permissive cell line HeLa-CD4 by spinoculation at 500xg, 20^{0}C, for 60min, followed by incubation for 12h at 37^{0}C. Cells were washed and cultured at 37^{0}C for 24h, and the media were titered. The titers of the released HIV-1(wild-type) and HIV-1(Δ*vif*) in each test cell line were normalized relative to the titers of these same viruses released from the control HeLa-CD4 cells that were analyzed in the same experiment. Virions were purified by buoyant density centrifugation (175).

Results

A3G, A3F, and A3B occur in permissive and nonpermissive cells

To analyze antiviral activities, we compared infectivities of HIV-1(wild-type) and HIV-1(Δ*vif*) virions made by a broad panel of cell lines during the first cycle of infection after they were incubated with equal titers of these viruses (Fig. 1A). As we and others have previously shown, the nonpermissive phenotype specifically reduces infectivity of released HIV-1(Δ*vif*) virions but not the number of virions made by the cells (86, 88, 165, 228). These data confirm that our H9, SP, and MT-2 cells inactivate HIV-1(Δ*vif*) much more efficiently than our Jurkat, A3.01, U937, and CEM-A cells. Surprisingly, however, there was a large variability in efficiency of HIV-1(Δ*vif*) inactivation by H9 cells in different experiments (e.g., Fig. 1A). Furthermore, early virus harvests following infection were more completely inactivated than later harvests. For example, in a representative experiment the ratio of HIV-1(wild-type)/HIV-1(Δ*vif*) infectivity was 57±16 for a 36 h harvest and only 7.8 ± 2.2 for a 48 h harvest from the same H9 culture.

Although the extents of HIV-1(Δ*vif*) inactivation were relatively very low in A.301, CEM-A, U937, and Jurkat cells (e.g., Fig. 1A), this slight inactivation appeared to be reproducible in comparison to parallel control assays using fully permissive HeLa-CD4 cells. For example, in a series of assays in A3.01 cells that were normalized using HeLa-CD4 cells (see Materials and Methods), the ratio of HIV-1(wild-type)/HIV-1(Δ*vif*) infectivities was 2.8 ± 0.6 (N =7), which was significantly different from the HeLa-CD4 data (P > 0.95). Such small amounts of HIV-1(Δ*vif*) inactivation in a single cycle of infection could become more significant in assays that require viral replication for many

days or in chronic infections *in vivo* (88). Based on these considerations, data described below, and previous compatible evidence concerning these cells,(78, 82, 86, 88, 119, 184, 219, 258, 287) we conclude that A3.01, CEM-A, Jurkat, and U937 cell lines are highly permissive rather than fully permissive in assays that use a single cycle of infection. Clearly, these cells inactivate HIV-1(Δ*vif*) only very weakly compared to nonpermissive cells in a single cycle of infection.

Fig. 1B shows Northern blots of A3G mRNAs from a broad panel of cell lines. Interestingly, A3G mRNA occurs in all nonpermissive cells, including H9, HUT78, AA-2, SP, MT-2, and peripheral blood lymphocytes, and also in highly permissive CEM-A, Jurkat, A3.01, and U937 cells. Surprisingly, as confirmed by qRT-PCR, A3G mRNA levels in standard nonpermissive cell lines H9, AA-2, SP, and MT-2 were similar on normalized bases to amounts in CEM-A and A3.01 (Fig. 1B and C). A3F and A3B mRNAs were also present in both permissive and nonpermissive cells in amounts that did not correlate with cellular antiviral properties (Fig. 1C). Multiple full-length wild-type A3G cDNAs were cloned from Jurkat, HSB-2, U937, CEM-A, and A3.01 cells, whereas mutant A3Gs were not detected.

A previously characterized antiserum made to a 16 amino acid peptide corresponding to the carboxyl terminus of A3G (266) detected a single protein with the size of A3G in amounts that closely correlated with A3G mRNA levels (Fig. 2A). Thus, both the protein and mRNA were present in H9, MT-2, SP, AA-2, CEM-A, Jurkat, A3.01, and U937 but not in SupT1, HeLa, or 293T cells. Although this peptide sequence is specific to A3G, the carboxyl terminus of A3B differs by two nonconservative changes. However, A3B occurs in substantial amounts in HeLa cells (Fig. 1C) (126),

which lack the protein we detected (Fig. 2A). A3B reacts weakly with the anti-peptide antiserum and electrophoreses faster than A3G (Fig. 2B). The results in Figs 1 and 2 are consistent with the suggestion of Sheehy et al (248) that A3G plays a major role in the inactivation of HIV-1(Δ*vif*) in cell cultures. However, because the magnitudes of HIV-1(Δ*vif*) inactivation do not closely correspond with A3G or A3F expression levels, we analyzed this disparity in more detail.

Antiviral properties of A3A, A3B, A3C, A3G, and A3F alone and in combinations

APOBEC1 heterodimerizes with APOBEC2 [also called ARCD-1] and is thereby strongly inhibited (11). Consequently, it is intriguing that A3B and A3F heterodimerize with A3G (126, 292) and are coexpressed with A3G in cells that have only weak anti-HIV-1(Δ*vif*) activities (Fig. 1). Therefore, we tested whether these or other cytidine deaminases can dominantly interfere with A3G function. In cotransfected 293T cultures, A3B-Myc formed a complex with A3G-Luc that immunoprecipitated with antibodies against either Myc or Luc (Fig. 3A). Using a similar assay, we confirmed that A3G and A3F formed a complex (results not shown). As shown in Fig. 3B, we also found that both A3G and A3F were strongly and specifically downmodulated by coexpression with HIV-1 Vif. A similar downmodulation of A3F was previously reported by Zheng et al, who found that it was efficiently prevented by proteasomal inhibitors (313), in agreement with similar evidence for A3G (49, 157, 175, 249, 266, 308). In addition, Vif bound specifically to A3G-Myc and A3F-HA as seen by its coimmunoprecipitation from cell extracts using antibodies to the Myc and HA epitope tags, respectively (Fig. 3C). We then tested A3A, A3B, A3C, A3F, and A3G for their antiviral activities alone and in

several combinations. Control studies demonstrated that all of these epitope-tagged cytidine deaminases were highly expressed in the transfected 293T cultures (results not shown). As shown in Fig. 3D, we found that both A3G and A3F specifically inhibited HIV-1(Δ*vif*) infectivity. In agreement with a recent report (28), we also found that A3B significantly inactivates both HIV-1(wild-type) and HIV-1(Δ*vif*). In addition, A3A and A3C have relatively low but significant anti-HIV-1 activities. Importantly, coexpression studies indicated that A3A, A3B, and A3C do not dominantly interfere with the anti-HIV-1(Δ*vif*) activity of A3G (Fig. 3D). On the contrary, these cytidine deaminases cooperate with A3G and A3F to reduce infectivities of both HIV-1(wild-type) and HIV-1(Δ*vif*).

Control of cytidine deaminase syntheses

Conceivably, the antiviral activities of cytidine deaminases could be influenced not only by their basal levels, but also by factors that modulate their syntheses and degradations. However, we did not observe significant differences in regulation of A3G synthesis in cells with widely different antiviral properties. Thus, as we previously reported for H9 cells (227), phorbol myristate acetate (PMA) also induced A3G mRNA and protein in A3.01, Jurkat, and CEM-A cells coordinately with enhanced phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Fig. 4A). In contrast, PMA had no effect on total cellular levels of ERK1/2 (Fig. 4A). Equivalent loading of proteins was confirmed by detection of the control protein α-tubulin. Both basal and PMA-induced levels of A3G mRNA and protein synthesis require activated protein kinase C (PKC) α/βI / MAP kinase kinase (MEK)/ ERK as shown by diverse inhibitors

that block this pathway (227). Accordingly, the PMA-induced increase in A3G in CEM-A and A3.01 cells was prevented by the MEK inhibitors PD98059 (Fig. 4B) and U0126 (results not shown). PMA did not induce A3G in SupT1 or HeLa cells, which lack basal expression of this enzyme (Fig. 4A). Thus, phosphorylated ERK cannot override the transcriptional repression in the latter cells. A3B mRNA was also induced by PMA, whereas A3F mRNA was not (Fig. 4C) (218).

Vif downmodulates A3G in both H9 and A3.01 cells.

We analyzed A3G in A3.01 and H9 cells after infection with HIV-1(wild-type) and HIV-1(Δ*vif*). Approximately 60-75% of the cells were infected in the optimal conditions of this experiment. A3G levels in the A3.01 and H9 cell extracts were downmodulated to approximately this extent by HIV-1(wild-type) compared to HIV-1(Δ*vif*) and mock infected cells (Fig. 5A). This is consistent with evidence that Vif efficiently eliminates A3G from cells that express both proteins, and that the residual A3G in infected or transfected cultures is principally in cells lacking Vif [Marin, 2003 Nat Med #320]. In agreement with this interpretation, A3G was almost completely eliminated from H9.Vif cells that constitutively express Vif in all cells (Fig. 5B). These results suggest that A3G is not substantially sequestered from Vif within infected highly permissive A3.01 cells.

A3G is incorporated into HIV-1(Δvif) virions in highly permissive cells

We analyzed A3G contents of HIV-1(wild-type) and HIV-1(Δ*vif*) virions that were made in A3.01 and H9 cells (Fig. 5C). Consistent with previous evidence in

nonpermissive cells (157, 175, 180, 249, 266, 308), A3G was present in HIV-1(Δ*vif*) but not HIV-1(wild-type) virions. Reproducibly, after infection in the same conditions, virion production as indicated by Gag p24 was approximately 10 times greater in A3.01 than in H9 cells (Fig. 5C, lower panel). Although these cultures released similar total amounts of HIV-1(Δ*vif*)-associated A3G, the A3G levels/virion, as determined by normalizing A3G relative to virion p24, were much greater for the virions from H9 cells than for those from A3.01 cells (Fig. 5C). This was clearly evident when the virions from A3.01 cells were diluted to approximately the same p24 concentration as those derived from H9 cells. Thus, the HIV-1(Δ*vif*) virions made in the permissive A3.01 cells contain relatively low amounts of A3G, consistent with their increased infectivity.

Figure 1. A3G, A3B, and A3F mRNAs are expressed in permissive and nonpermissive cells. (**A**) Titers of viruses released from cells infected with HIV-1(wild-type) (gray) or HIV-1(Δ*vif*) (black). Shown for each cell line is the ratio ± standard deviation of HIV-1(wild-type)/HIV-1(Δ*vif*) infectivity. Error bars are standard deviations based on counting statistics. wt, wild-type (**B**) Northern blots of A3G mRNA in permissive (**P**) and nonpermissive (NP) cells. Antiviral phenotypes of HSB-2 and C8166-45 cells were ambiguous because of resistance to infection and apparent clonal diversity, respectively. As described in Results, A3.01, CEM-A, U937, and Jurkat are highly permissive although they have very weak anti-HIV-1(Δ*vif*) activities. S2 ribosomal protein mRNA is a loading control. (**C**) qRT-PCR analyses of A3G, A3B, A3F mRNAs in cell lines relative to H9 cells. Values represent the average ± SEM (n=2).

Figure 1

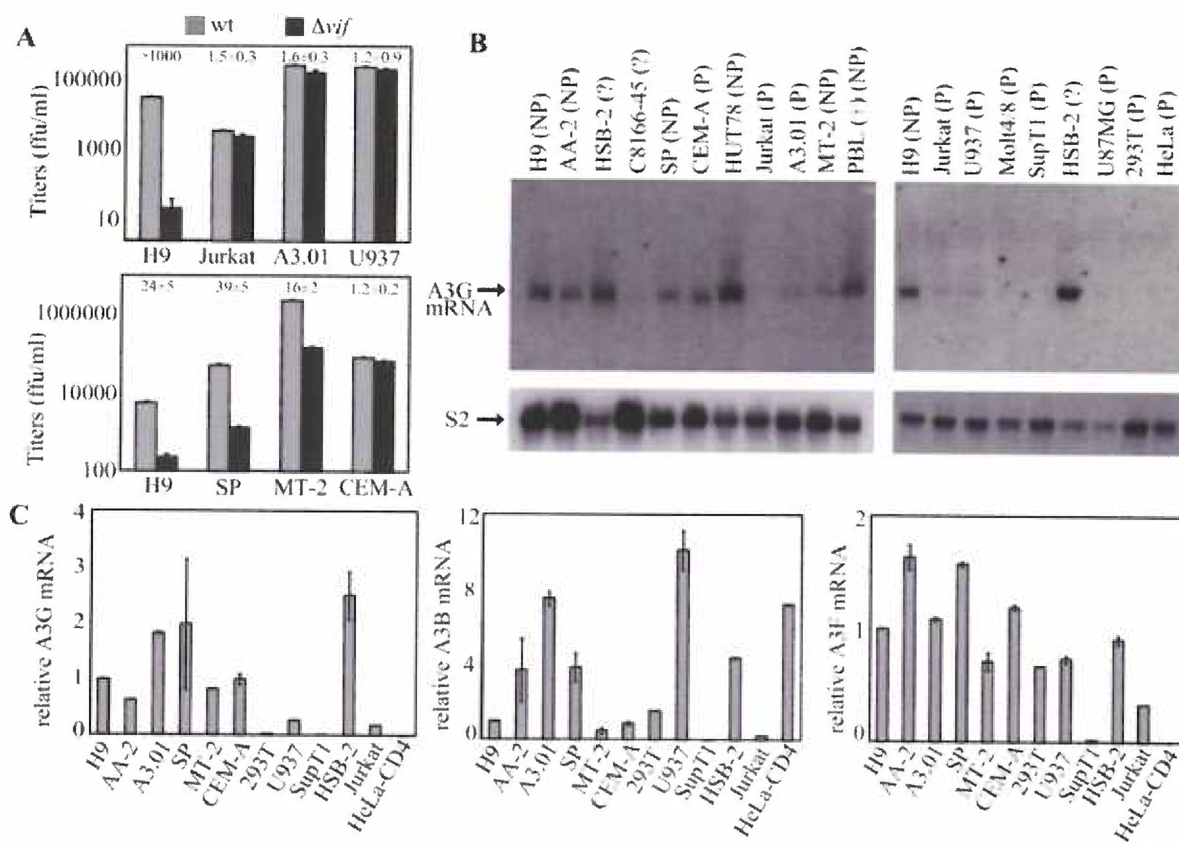


Figure. 2. A3G protein in permissive and nonpermissive cell lines. (**A**) Western blot analysis using anti-A3G peptide antibody. (**B**) Specificity of anti-A3G peptide antibody. 293T cells were transfected with A3G-Myc, A3B-Myc, or negative control pcDNA3.1-Myc (mock) vectors. Cell lysates were analyzed by immunoblotting with anti-Myc (upper panel) and anti-A3G peptide antibody (middle panel), which binds weakly to A3B. The α-tubulin blot is a loading control.

Figure 2

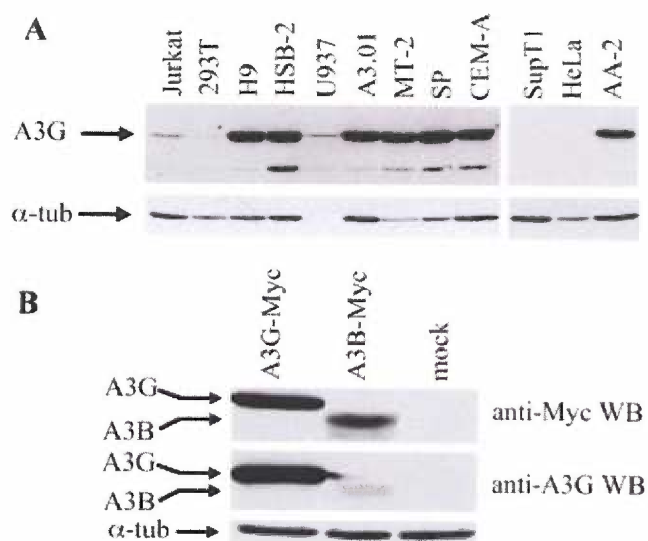


Figure. 3. Interactions and antiviral activities of cytidine deaminases A3A-A3F. **(A)** A3B and A3G heterodimerize. 293T cells were cotransfected with A3G-Luc and A3B-Myc vectors. Immunoprecipitates using either anti-Luc or anti-Myc were analyzed by Western blot with anti-Myc (upper panel) or anti-Luc (lower panel). **(B)** A3G and A3F are downmodulated by HIV-1 Vif. 293T cultures were tranfected with A3G-Myc (left panel) or A3F-HA (right panel) in the presence of identical quantities of a pcDNA3.1 control vector, a pcDNA3.1-HVif vector for codon optimized HIV-1 Vif, or with pHIV-*gpt*(wild-type) or pHIV-*gpt*(Δ*vif*), respectively. Cell lysates were analyzed by blotting for A3G-Myc or A3F-HA (upper blots), Vif (middle blots), or α-tubulin loading control (lower blots). **(C)** Vif binds to A3G-Myc and A3F-HA. 293T cells were transfected with A3G-Myc or A3F-HA or with a negative control vector in the presence of a HIV-*gpt*(wild-type) or HVif vector. Anti-epitope tag immunoprecipitates of cell lysates were analyzed by blotting with antibodies to the epitope tags (upper panels) or with anti-Vif. IgG, immunoglobulin heavy chain. **(D)** Infectivities of HIV-*gpt*(wild-type) and HIV-*gpt*(Δ*vif*) viruses produced in the presence of equal total quantities of plasmids for different cytidine deaminases alone and in the indicated combinations. The titer of each virus normalized relative to the titer of the same control virus that was made in the absence of cytidine deaminase vectors (n=3; error bars are ± SEM). wt, wild-type

Figure 3

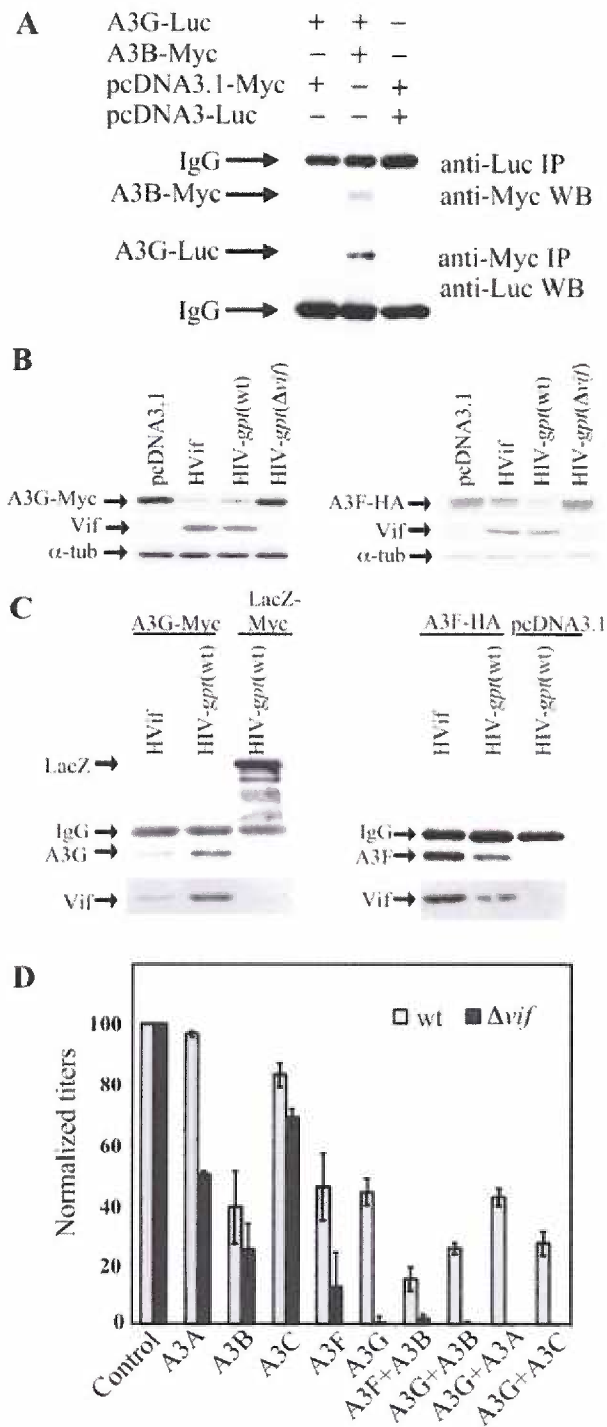


Figure 4. PMA induces A3G protein in a MEK and ERK-dependent manner. **(A)** H9, HeLa, A3.01, Jurkat, SupT1, and CEM-A cells were induced with 100nM PMA. Cell lysates were collected at indicated times and analyzed by Western blotting using antibodies specific for A3G, ERK-1, phosphorylated ERK-1 (pERK-1), and α-tubulin. ERK levels were constant, whereas pERK-1 was increased by PMA in all cells. **(B)** MEK inhibitor blocks PMA-induced A3G synthesis. As previously described for H9 cells (227), A3.01, and CEM-A cells were cultured with the specific MEK inhibitor PD98059 (50μM) for 1h prior to treatment with PMA (100nM) for 24h. Cell lysates were analyzed by immunoblotting as above. **(C)** qRT-PCR analyses of A3G, A3B, and A3F mRNAs in H9 cells incubated for different times with 100nM PMA.

Figure 4

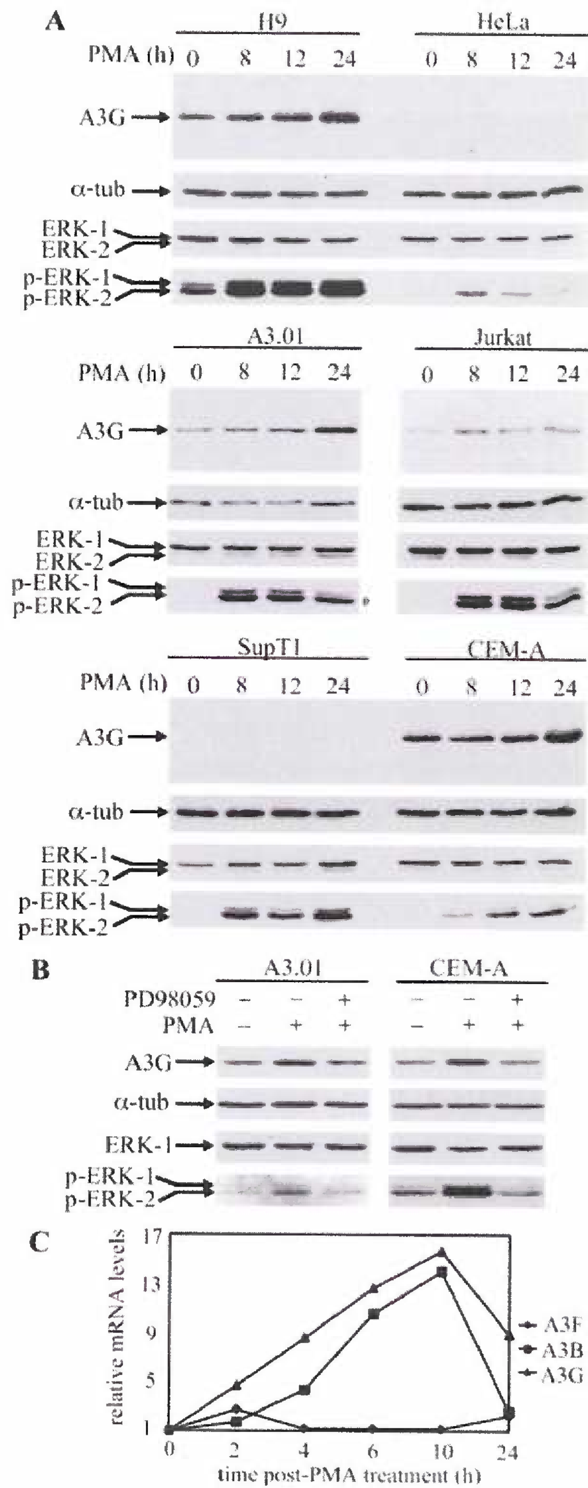
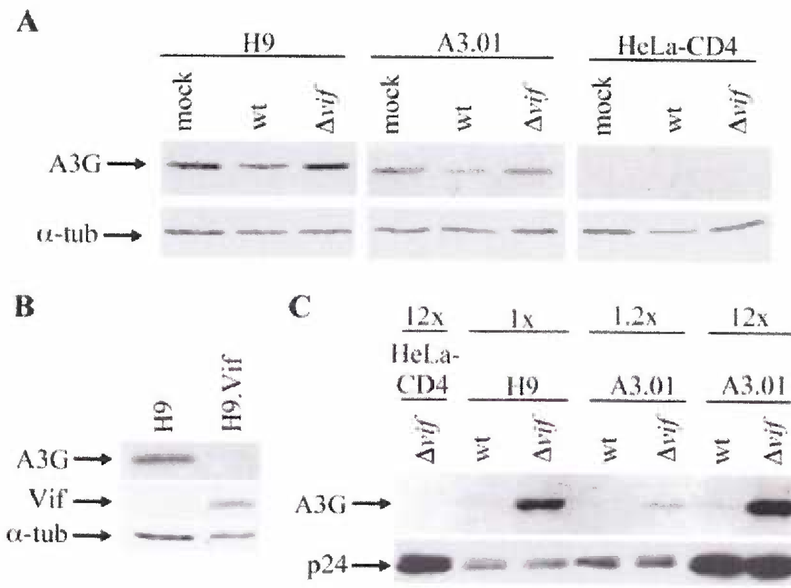


Figure 5. Vif downmodulates expression and inhibits viral encapsidation of A3G in nonpermissive and permissive cells. (**A**) Lysates of HIV-1 infected cells analyzed by immunoblotting show that A3G is strongly downmodulated by HIV-1(wild-type) compared to HIV-1(Δ*vif*) in H9 and A3.01 cells. HeLa-CD4 cells lack A3G. (**B**) Western blot analyses of H9 and H9.Vif cell lysates using anti-A3G, anti-Vif, and anti-α-tubulin. (**C**) Purified virions from HIV-1 infected cells analyzed by immunoblotting. Densitometric analyses of p24 were used to measure relative amounts of virions in the samples. wt, wild-type

Figure 5



Discussion

Although A3G makes a major contribution to the anti-HIV-1 activities of human cell lines, our results reveal significant disparities between the cellular levels of A3G and the efficiencies of HIV-1*(Δvif)* inactivation in the released viral particles. For example, A3.01 and CEM-A cells have weak anti-*HIV-1(Δvif)* activities in our single cycle infectivity assays, but often contain similar amounts of A3G mRNA and protein compared to nonpermissive H9, AA-2, MT-2, and SP cells (Figs 1,2, and 4). Similarly, A3F and A3B, which have significant anti-HIV-1 activities (Fig 3), are expressed in relatively large amounts in several cell lines that are highly permissive (Fig. 1C). Moreover, the efficiency of *HIV-1(Δvif)* inactivation in H9 cells becomes highly attenuated in some culture conditions. These results support the idea that A3G and/or A3F and other cytidine deaminases comprise a potential antiviral defense system with multiple components that may not be constitutively active and that can be influenced by other factors. Other recent evidence also supports this conclusion (89). Because these issues could be important in control of HIV-1 infections, we analyzed these cellular controls in greater detail.

The substrate specificities and activities of APOBEC1 and AID are restricted by associated cellular factors (10, 40, 181, 259). For example, APOBEC1 heterodimerizes with APOBEC2 and is thereby inhibited (11). Thus, when APOBEC1 and AID occur in normal cells, their activities and substrate specificities are strictly limited. However, when they are overexpressed in cell cultures or *in vivo*, they can cause abnormal mRNA editing, mutations, and oncogenesis (118, 197, 262, 301, 303). Based on this precedence, we tested the effects of the A3G paralogs A3A, A3B, A3C, and A3F on the antiviral

potency of A3G (Fig. 3). Our data confirmed that A3G heterodimerizes with A3B (Fig 3) and A3F, that Vif binds to both A3G and A3F and downmodulates their expressions in 293T cell cultures, and that both of these cytidine deaminases preferentially inactivate HIV-1*(Δvif)* (28, 155, 175, 180, 249, 266, 292, 308, 313). The Vif-dependent downmodulations of both A3G and A3F are reversed by proteasome inhibitors (28, 157, 175, 180, 249, 266, 292, 308, 313). A3A, A3B, and A3C all have relatively weak anti-HIV-1*(Δvif)* activities when expressed alone, (28, 292, 313) and do not dominantly interfere with A3G or A3F activities (Fig. 3). On the contrary, these cytidine deaminases function in a collaborative or additive manner to inhibit HIV-1 infectivity (Fig 3).

One mechanism for cellular control derives from the dependency of A3G mRNA synthesis on activation of the PKCα/βI /MEK /ERK protein kinase cascade (Fig. 4), which is activated by extracellular factors (42, 227). Because MEK1/2 and ERK become dephosphorylated when cells enter a stationary or G_0 state of proliferative arrest (183, 202), basal levels of A3G decline when sera are depleted and cells cease proliferating (227). Thus, A3G levels depend on recent culture conditions. Unidentified signaling or stress pathways might also control quantities or activities of these cytidine deaminases. However, A3G synthesis is unaffected by interferons or tumor necrosis factor α, which induce other antiviral proteins (227). The control of A3G expression by this signaling cascade occurs in all T cell lines that we have examined, including A3.01, CEM-A, Jurkat, and H9 (Fig. 4).

A3Gs in cells that are permissive or nonpermissive have identical sequences and are similar in other tested properties. A3G in both cell types is downmodulated by Vif (Fig. 5A and B) and incorporated selectively into HIV-1(*Δvif*) but not HIV-1(wild-

type) virions (Fig. 5C). However, in the conditions of our experiments the HIV-1(Δ*vif*) made in A3.01 cells contained approximately 10 times less A3G/virion than those made in H9 cells. We believe that this low quantity of A3G/virion may explain the increased infectivity of the HIV-1(Δ*vif*) virions from A3.01 cells compared to those derived from H9 cells. Therefore, the incorporation of A3G into HIV-1(Δ*vif*) virions appears to be restricted in permissive compared to nonpermissive cells.

Although we have not identified mechanism(s) responsible for modulating antiviral activities of A3G and A3F or other cytidine deaminases, our results are compatible with recent evidence of Alce et al that high intracellular Gag concentrations can reduce soluble A3G levels due to adsorption onto the nucleocapsid protein and possible export of these complexes from cells (5). Accordingly, our results suggest that A3.01 cells produce and accumulate much larger amounts of Gag proteins than H9 cells at similar multiplicities of infection, which might competitively reduce A3G levels per virion (e.g., see Fig. 5C). In addition, it was recently shown that incorporation of the uracil glycosylase UNG-2 into HIV-1 virions functions in conjunction with an intrinsic apurinic/apyrimidinic endonuclease activity of reverse transcriptase to efficiently repair low frequency dC-to-dU mutations in viral DNA (44, 215). In contrast, these same repair processes can cause DNA fragmentation if the DNA has been extensively hypermutated by a large amount of A3G (107). Conceivably, cells might produce virions with different amounts of UNG-2 and with correspondingly different tolerances for A3G.

The control of A3G by signaling pathways that are coupled to cell proliferation (227) (Fig. 4) implies that antiviral properties of cell lines might be variable, and that cell lines classified as permissive might be capable in certain conditions of using A3G or A3F

to inactivate HIV-1(Δ*vif*). This has been difficult to evaluate because the factors that alter A3G levels including serum starvation, inhibitors of the PKCα/βI /MEK /ERK pathway, and PMA require prolonged treatments and have nonspecific effects on cell viabilities and infectious virus yields (227). However, in agreement with this expectation of variability, the efficiencies of HIV-1(Δ*vif*) inactivation in H9 cells differ substantially in independent virus preparations (e.g., Fig. 1A), and within the same infected culture depending upon the time of viral harvest. In this context, we emphasize that our standard permissivity assay measures infectivities of virions released early during a single cycle of infection. In contrast, several groups have inoculated cultures with small amounts of HIV-1(wild-type) and HIV-1(Δ*vif*), and have compared the accumulations of virions in the media after many cycles of viral replication (71, 86, 89, 231, 248, 249, 287). Such methods exponentially amplify minor differences in viral growth rates, including secondary effects of Δ*vif* mutations (261) and small differences in titers of initial inocula, and are also perturbed by extraneous factors such as cell growth rates, media changes, and differences between cell lines in levels of virion production (45, 89). Consequently, we believe that our single cycle assays provide a relatively accurate and direct measurement of the abilities of cell lines to inactivate HIV-1(Δ*vif*). An important implication of our data is that the efficiencies of HIV-1(Δ*vif*) inactivation are surprisingly low for some cells that have substantial amounts of A3G and is often weak even for nonpermissive H9 cells in some conditions. Nevertheless, a small inhibition in a single cycle assay could have a major influence on a chronic infection that requires continuous viral replication. These considerations suggest that treatments that even slightly enhance A3G or A3F activities or that inhibit Vif might have major clinical benefits in control of

chronic HIV-1 infections. For these same reasons, small inhibitory effects of these cytidine deaminases and of A3B and A3C on wild-type HIV-1 (Fig. 3), could also have important effects on disease progression. Therefore, the latter cytidine deaminases should also be considered in the quest to suppress replication of HIV-1.

Acknowledgements

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APOBEC3B accession # AY743217

Chapter 5

Discussion

Discovery of APOBEC3s as a potent intrinsic defense system against HIV has transformed the field of HIV research, by uncovering a novel innate antiviral system that is extremely effective at inhibiting not only HIV but other diverse viruses (28, 69, 70, 107-109, 175, 226, 266, 282). APOBEC3s belong to a family of cytidine deaminases with several members (A3G, A3F) capable of severely hypermutating HIV-1 negative strand DNA during reverse transcription (28, 107, 148, 155, 174, 251, 311). When A3G and A3F are overexpressed in P cells they efficiently reduce HIV-1(Δ*vif*) viral replication by 50-100 fold. However, expression of HIV-1 Vif in virus producing cells effectively neutralizes A3G activity and A3F activity to a lesser extent (28, 155, 157, 175, 180, 249, 266, 308, 313).

Cytidine deaminases A3G and A3F drastically increase levels of G-to-A hypermutations in HIV-1 positive strand DNA, resulting in abortive reverse transcription followed by fragmentation of the DNA or integration of severely mutated proviruses (28, 107, 148, 155, 174, 251, 311). These highly mutated proviruses are frequently isolated from HIV-1 patients (125, 284), and they likely encode defective proteins that could possibly act in a dominant negative manner to inhibit superinfections. Retroviruses including MLV and EIAV that lack *vif* genes are subject to cytidine deamination by human A3G and must employ alternative mechanisms to inhibit or exclude from virions A3G in their natural hosts (170). Murine APOBEC3 is excluded from murine viruses by an unknown mechanism (142). Conversely, HTLV-1 incorporates significant levels of human A3G into virions but its infectivity is not affected (191). Significantly, A3G can

inhibit retrotransposition of endogenous retroviruses (69, 70), implying that A3G could have evolved millions of years ago to control related endogenous retroviruses (50, 312). Antiviral activity of A3G requires its incorporation into virions (170,107,28,155), an intact C-terminal catalytic domain (191), and possibly additional cellular regulatory factors (89, 226). However, several reports provide compelling evidence for a mechanism of A3G antiviral activity that is distinct from its ability to deaminate DNA (191, 192, 251, 282).

Neutralization of A3G by HIV-1 Vif

Disabling Vif's essential role of blocking A3G activity, would release this robust antiviral protein to attack the virus. Therefore, defining the mechanism by which Vif counteracts A3G antiviral activity could lead to important insights for anti-HIV-1 therapies. We present substantial evidence describing a mechanism by which Vif eliminates A3G from cells. Without the aid of other HIV-1 encoded proteins, Vif binds directly to A3G and induces its rapid degradation in human and African green monkey cells (157, 175, 180, 249, 266, 308). In fact, the half-life of A3G in the presence of HIV-1 Vif is dramatically reduced from 8 hours to approximately 2 minutes, making A3G one of the most short-lived proteins (175, 286). A similar reduction in A3G protein levels occurs in HIV-1 infected T-cell lines (107, 180, 226, 249, 266, 308). Additionally, the fact that small amounts of Vif can degrade large quantities of A3G implies that this mechanism is catalytic (132) (107, 180, 226, 249, 266, 308). Addition of proteasome inhibitors restores A3G expression levels in cells expressing Vif, suggesting that A3G is targeted for proteasome-dependent degradation (49, 180, 226, 249, 266, 308). Consistent

with this evidence, large amounts of polyubiquitinated A3G-Myc accumulate after addition of the proteasome inhibitor ALLN or co-expression of HA-tagged ubiquitin only when Vif is present (49, 175, 180, 249).

However, alternative mechanisms of Vif-mediated A3G downmodulation have been proposed and may exist in certain experimental systems (132, 235, 266). For example, Stopak et al show translation of A3G is hampered by expression of Vif in a cell-free *in vitro* translation assay (266). However, using the same reticulocyte lysate system we show that Vif also inhibits translation of other unrelated proteins (Appendix VI). In addition, A3G mRNA levels in 293T or T-cell lines are unaffected by HIV-1 infection or presence of Vif (Appendix I) (132, 175, 180, 266). More importantly, expression of Vif in cells has no effect on the rate of A3G synthesis (175), implying that A3G downmodulation occurs at a post-translational level. Therefore, we suggest that Vif-mediated degradation of A3G is the dominant mechanism by which HIV-1 neutralizes this potent antiviral factor.

Substantial insight into the Vif-mediated degradation of A3G was gained by evaluation of A3G and Vif expression in individual cells by indirect immunofluorescence. Most significantly, A3G is expressed in the cytoplasm and is completely eliminated from cells expressing wild-type Vif with an efficiency of over 100-fold (175, 291). As a result, A3G is specifically incorporated into HIV-1 virions in cells that lack Vif (157, 175, 180, 249, 266). This observation is initially difficult to reconcile with evidence that Vif and A3G co-immunoprecipitate in co-transfected cell lysates because these results imply complete segregation of wild-type Vif and A3G in these cell cultures. However, variations in Vif and A3G expression are observed in the

transfected cell culture, thus some cells that express A3G lack Vif and vice versa. Thus, when the culture is lysed, mixing of Vif and the residual A3G occurs. Furthermore, the association of Vif and A3G occurs rapidly in cell lysates mixed at $0^{0}C$ (Appendix II) (175).

Furthermore, treatment of cells with proteasome inhibitor ALLN or co-transfection of a nonfunctional Vif mutant Δ12 restores A3G and Vif coexpression within the same cells and encapsidation of A3G into released virions (157, 175, 291). Mutations in HIV-1 NC (5, 41, 65, 161, 238, 273, 310) or the N-terminal catalytic domain of A3G (191), severely disrupt A3G packaging into virions and eliminates A3G antiviral activity. Together these results emphasize that incorporation of A3G is necessary for its antiviral activity. As outlined in Figure 3 of the introduction, A3G expressed in the cytoplasm of a target cell is unable to attack invading HIV-1 possibly because reverse transcription occurs within the protection of the viral core where A3G is unable to penetrate (175). Therefore, A3G encapsidation into HIV-1(Δ*vif*) virions allows it to be delivered to the precise location in the host where it is poised to attack the virus during reverse transcription.

Using a series of nonfunctional Vif mutants we identified two functionally distinct domains in Vif necessary for A3G degradation: an amino terminal domain is essential for binding A3G (49), and a carboxyl terminal domain containing the highly conserved SLQ(Y/F)LA motif that mediates Vif-dependent A3G downmodulation (157, 175, 180, 308). Specifically, Vif mutants Δ12 and Δ31 that retained the ability to bind A3G but lacked the SLQ(Y/F)LA motif were unable to degrade A3G and therefore had no effect on HIV-1 infectivity(175). We noted that the SLQ(Y/F)LA motif shares

significant homology to the BC-box domain (also called SOCS-box) contained in the SOCS family proteins and in many other proteins including the von Hippel Lindau tumor suppressor (VHL) (124). The BC-box functions as an assembly platform to link proteins targeted for degradation to a multisubunit E3 ubiquitin isopeptide ligase that contains elongins B and C, cullins, and Rbx-1. An E1 activating enzyme transfers ubiquitin to an E2 ubiquitin-conjugating enzyme and by association with the catalytic core (cullin and Rbx-1) covalently links ubiquitin to Lys side chains of the substrate protein. Transfer of multiple ubiquitin moieties results in a polyubiquitinated protein that is transferred to proteasomes for degradation (206). We hypothesized that Vif might function in a manner similar to SOCS, by binding and recruiting A3G into an E3 ligase complex for subsequent ubiquitination and degradation by the proteasome.

Our hypothesis was rapidly confirmed by experiments showing the SLQ(Y/F)LA motif of Vif functioned as a BC-box and interacted directly with elongin C in a complex containing elongins B and C, cullin 5, and Rbx-1 (142, 179, 308, 309). Significantly, a SLQ-to-AAA mutation in the Vif BC-box consensus site caused dissociation of elongins B and C and cullin 5 from the complex (179, 308, 309). Poly-ubiquitination of A3G could be reconstituted *in vitro* with purified Vif-elonginB/C-cullin 5 complexes (142). Moreover, phosphorylation of Ser^{144} in the Vif BC-box abolishes its interaction with elongin C, thereby negatively regulating the E3 ligase complex assembly (179). Also, dominant-negative mutants of cullin 5 that were either incapable of being modified by the Ub-like small modifier Nedd8 or of association with Rbx-1 prevented the degradation of A3G and restored its incorporation into progeny virions and antiviral activity (179, 308, 309). Finally, both non-functional Vif mutants C114S and C133S retain the ability to

bind A3G and assemble into an elongin B/C-cullin 5 complex, but do not induce ubiquitination of A3G. Kobayahsi et al suggest that C114S and C133S mutations perturb Vif conformation thereby altering the positioning of A3G in the E3 ligase complex, resulting in loss of E3 activity (142). Together these data present striking evidence that this degradation pathway is necessary and sufficient for the Vif-mediated neutralization of A3G.

Interestingly, Vif is itself monoubiquitinated by HECT E3 ligases. Evidence that intracellular Vif levels are elevated by proteasome inhibition implies Vif is a substrate for proteasome-mediated degradation (81, 175, 180, 291). In fact, Mehle et al suggest a portion of the intracellular Vif is simultaneously degraded with A3G (180). However, Dussart et al conclude Vif is not a target for the proteasome and suggest instead that ubiquitination of Vif may alter its subcellular localization, structure, or activity(67). Therefore, the fundamental significance of Vif ubiquitination still remains unclear.

Broad antiviral activities of APOBEC3 cytidine deaminases

A3G belongs to a family of cytidine deaminases (A3A-A3F) that are tandemly arrayed on human chromosome 22 (108, 126). Due to the apparent antiretroviral potency of A3G, investigations were rapidly initiated to test the potential antiviral activities of other APOBEC family members from humans, other primates, and rodents (28, 29, 155, 226, 305). We have confirmed evidence that human A3F has anti-HIV-1(Δ*vif*) activity similar to A3G (28, 29, 155, 226, 292, 313) and undergoes Vif-mediated proteasome degradation and inhibition of antiviral activity (313). Similarly, human A3G and A3F are potent inhibitors of SIV_{AGM} and SIV_{MAC}, and are only counteracted by SIV_{MAC} Vif (305).

In contrast, A3A and A3C have relatively weak anti-HIV-1 activities (305) (28, 226), and human APOBEC1, APOBEC2, and AID have no affect on HIV-1, SIV_{MAC}, or MLV infectivity (28, 305). However, A3B and A3C strongly inhibit SIV_{MAC} and SIV_{AGM}, although only A3C can be neutralized by SIV Vif (305). Surprisingly, expression of human A3B (28, 176, 226), rat APOBEC1, or mouse APOBEC3 (28, 29, 142) significantly inhibits both HIV-1(wild-type) and HIV-1(Δ*vif*) viruses, suggesting that these APOBECs are resistant to Vif encoded by $HIV\text{-}1_{IIIB}$. Similarly, HIV-2(Δ*vif*) isolates are only minimally restricted by human A3G (222), and some cells nonpermissive for replication of HIV-1(Δ*vif*) are permissive for HIV-2(Δ*vif*) replication (184, 219, 222). These results suggest that lentiviruses are differentially susceptible to APOBEC3 paralogs. In fact, we recently noticed that different laboratory-adapted and patient-derived isolates of HIV-1 have distinct vulnerabilities to antiviral activities of A3G and other APOBEC3s (176).

The APOBEC3 genes of primates have evolved rapidly, with extremely high frequencies of nonsynonymous mutations including large duplications and deletions, consistent with a hypothesis that they have diversified in response to coevolutionary adaptations (50, 237, 312). In fact, a single amino acid at position 128 of A3G determines a species-specific interaction of Vif with its natural host and closely related species, that is independent of additional species-specific cellular factors (30, 171, 240, 293). Moreover, analysis of HIV-1 sequences indicates that Vif has undergone positive selection consistent with adaptation to an antagonistic interaction (298). These results suggest that Vif binds directly to APOBEC3G, and that coevolutionary changes in these proteins contribute to the species barriers that limit lentivirus zoonoses.

APOBEC3s represent a collection of cytidine deaminases that exist in primates and several non-primates species. While expression and antiviral activities of some APOBEC3s make a significant contribution to anti-HIV-1 activities, promiscuous editing of cellular DNAs by these cytidine deaminases could lead to production of nonfunctional proteins, and disruption of essential cellular functions. Similarly, overexpression of APOBEC1 or AID leads to unrestricted editing of cellular RNA and DNA, respectively, and development of carcinomas (197, 262, 294, 301, 303). Deaminase activity of both APOBEC1 and AID are tightly controlled by cell-specific and time-dependent expression, as well as, complex regulatory mechanisms to restrict enzymatic activity to favorable sites on RNA or DNA (21, 204, 259). Therefore, we postulated that similar regulatory mechanisms must be in place to restrict A3G deaminase activity and mRNA and protein expression.

Regulation of A3G expression in the H9 T-lymphocytic cell line

Other antiviral proteins (such as PKR, ADAR-1, RNase L, and Mx GTPases) are induced by viral infection or the presence of dsRNA by the classical innate immune response involving Type I interferons (INF-α, INF-β, INF-γ) and to an extent TNF-α (234). In contrast, treatment of H9 cells with TNF-α or INFs had no affect on A3G mRNA levels. However, H9 cells were sensitive to both stimuli as shown by induction of PKR mRNA in response to INF and phosphorylation of MAPK p38 as a result of TNF-α treatment (Appendix V) (227). Similarly, expression of HIV-1 in P or NP cells or HIV-1 infection of T-cell lines has no affect on A3G mRNA levels (Appendix I) (175, 180, 266). In stark contrast, PMA enhanced A3G mRNA levels up to 20-fold with a

corresponding increase in the A3G protein levels. Enhancement of APOBEC3G mRNA levels required activated PKCα/βI and the dual-specificity protein kinase MEK, which results in phosphorylation and activation of the downstream protein kinase ERK. Consequently, diverse inhibitors acting at specific steps of the PKCα/βI/MEK/ERK pathway abolish A3G induction (227).

Importantly, the pathway implicated above appears to link A3G synthesis to the cell cycle, thereby influencing basal A3G expression levels in H9 cells. Activation of ERK is dynamically regulated during the cell cycle and thought to be necessary for cell cycle progression (183, 202). For instance, ERK is inactive during the G_0 quiescent phase of the cell cycle and transitions into its active phosphorylated state in early G_1 through late G_1 (183). Thus, H9 cells arrested in G_0 by serum starvation have severely reduced or negligible levels of A3G mRNA and protein that correspond with a decrease in phosphorylation of MEK and ERK. Stimulation of quiescent cells with PMA results in phosphorylation of ERK and a parallel increase in A3G mRNA and protein levels. The control of A3G expression by a signal transduction pathway that is linked to the cell cycle suggests that intracellular levels of A3G could be variable. In agreement with these observations, the extent to which H9 cells can inhibit HIV-1(Δ*vif*) varies in different culture conditions or at various time points after infection (226, 227). Furthermore, these results may explain a previous report that A3G levels increase when resting T lymphocytes are activated with interleukin-2 and phytohemmagluttinin (266). Also, preliminary data suggests A3G mRNA may be enhanced specifically in differentiated macrophages exposed to HIV-1 for 7 days (Appendix I), although further investigation is be needed to confirm these results. We suggest that cell culture conditions contribute to

A3G expression levels which dictates the intracellular levels of A3G available for packaging into virions and therefore the potency of antiviral activity.

In light of this, it is important to consider other aspects of HIV-1 pathogenesis that alter cell cycle control. Most importantly, HIV-1 encoded Vpr arrests infected cells in G_2 of the cell cycle (128) (183, 202) where ERK is inactive, thus creating an environment where A3G synthesis is not favored. Although this theory requires thorough investigation, in this situation Vpr may assist in reduction of intracellular A3G levels.

Despite a significant increase in A3G mRNA levels upon PMA treatment, the overall stability of A3G mRNA is unaffected, but transcription of A3G is dramatically increased in a manner that requires *de novo* protein synthesis. Additionally, enhanced A3G mRNA transcription is dependent on activation of the PKCα/βI/MEK/ERK cascade (227). Activated ERK can stimulate a number of transcription factors including Elk-1, Ets-1, NFκB, CREB, and c-Myc (159). In fact, domain searches of the 5' untranslated region of A3G reveal binding sites for Ets-1, Elk-1, and c-Myc. Further investigation is needed to verify a role for these transcription factors in this activation pathway. We present compelling evidence of the dynamic regulation of A3G translation in a human T-lymphocytic cell line by a specific protein kinase cascade. Further investigations into A3G expression patterns and regulation of antiviral capabilities may elucidate alternative activities of A3G and possible identify a cellular function similar to APOBEC1.

Regulated expression and antiviral activities of APOBEC3 cytidine deaminases

In a limited study, A3G mRNA was shown to be present in macrophages, PBMCs, H9 and HUT78 cell lines that exhibited anti-HIV-1(Δ*vif*) activity and absent in cells such as SupT1, HeLa, and 293T that lacked the antiviral phenotype (248). However, our investigation of a larger panel of cells revealed significant inconsistencies between the cellular levels of A3G and the efficiencies of HIV-1(Δ*vif*) inactivation in the released virions. This evidence suggests that although A3G may make important contributions to the anti-HIV-1 phenotype of the cell and that it may not be constitutively active in all cells (226). For example, A3.01 and CEM cells weakly inactivate HIV-1(Δ*vif*) but express A3G mRNA and protein at levels comparable to cells such as H9, SP, and MT-2 that strongly inhibit HIV-1(Δ*vif*). In addition, A3B and A3F which have strong anti-HIV-1 activities (28, 155, 226, 292, 313) are expressed at significant levels in many cells that are highly permissive (Appendix I) (28, 226, 275, 292, 313). Together this evidence suggests that in certain cell lines this network of antiretroviral enzymes is dependent on additional factors for its activity. Due to the importance of these observations in control of HIV-1 infectivity, we examined more closely the behavior of A3G in the highly permissive A3.01 cells.

The substrate specificities and deaminase activities of the related APOBEC1 deaminase is restricted by recognition of specific RNA sequences and associated cellular factors (9, 40, 181, 259). However, when overexpressed in cell cultures or *in vivo*, it can cause abnormal mRNA editing, mutations, and oncogenesis (197, 262, 301, 303). Furthermore, Although homodimerization of APOBEC1 generates the functional form of the enzyme, heterodimerization with APOBEC2 dominantly inhibits APOBEC1

enzymatic activity (11). Based on this observation and evidence for co-expression of APOBEC3s within cell lines (226) (Appendix I), we determined the effects of expressing A3A, A3B, A3C, or A3F on the antiviral potency of A3G. Our results show that A3G and A3F heterodimerize with each other and A3B (226). Furthermore, A3G, A3F, and A3B heterodimerize with the weakly antiviral A3A and A3C (Appendix II) (176). Importantly, co-expression of APOBEC3s does not dominantly interfere with anti-HIV-1 activity of A3G, A3F, or A3B (226). Actually, these cytidine deaminases seem to collaborate or function additively to increase the overall effectiveness of inhibition of viral replication (155, 226).

Multiple A3G cDNAs cloned from H9, Jurkat, HSB-2, U937, CEM-A, and A3.01 cells all have identical sequences and are similar in other tested properties. As originally observed in NP H9 cells, regulation of A3G mRNA synthesis and protein levels in Jurkat, CEM-A and A3.01 could be enhanced by PMA and was dependent on activation of the PCKα/βI/MEK/ERK protein kinase cascade. Similarly, we confirmed A3B mRNA expression could be induced by PMA (276) but A3F mRNA levels were unaffected (226). This observation reinforces the idea that diverse and yet undefined regulatory mechanisms control these closely related cytidine deaminases. Furthermore, A3G in both H9 and A3.01 cells undergoes Vif-mediated proteasome dependent degradation and A3G is incorporated specifically into HIV-1(Δ*vif*) virions and efficiently excluded from HIV-1(wildtype) virions. However, under these conditions A3.01 virions contained 10 times less A3G/virion than those virions derived from H9 cells. We believe that the low level of A3G encapsidated into A3.01 virions could explain the relatively weak inactivation of HIV-1(Δ*vif*) observed in these cells (226). Minimal reductions in HIV-1(Δ*vif*)

infectivities in a single cycle assay; however, could translate into substantial reductions in infectivity in assays requiring multiple rounds of infection or viral replication *in vivo* (89, 226, 176).

Although the above evidence does not determine the mechanism that attenuates A3B, A3F, or A3G activity in certain cells, our data are consistent with an observation made by Alce et al (5). Specifically, they report high intracellular Gag expression can reduce intracellular A3G concentrations by adsorption onto the NC protein and transport of these complexes from the cell in virus-like particles (5). At similar multiplicities of infection, A3.01 cells produce and accumulate much higher levels of Gag than H9 cells which could consequently reduce intracellular A3G levels through this competing mechanism.

Together these observations suggest that some cells expressing A3B, A3G, or A3F could be incapable of inhibiting HIV-1 under specific circumstances. In agreement with this, Gaddis et al observed SIVtan and SIVmnd1 (isolated from African green monkey species, tantalus monkeys and mandrils) were resistant to A3G and A3F endogenously expressed in HUT78 cells irrespective of Vif function (89). Therefore, it seems plausible that certain cell types must either require additional cellular factors for their antiviral functions or must overcome a negative regulatory mechanism. Moreover, the dynamic interplay between APOBEC3 homologs expressed in different species and the viral pathogen may be more complex than originally envisioned. This apparent strict regulation of APOBEC3 antiviral activity led us to develop assays that would uncover A3G associated factors.

Strategies for purifying A3G associated factors

Attempts to obtain highly purify A3G-Myc protein complexes by immunoprecipitation with a monoclonal anti-myc conjugated agarose resulted in preparations highly contaminated with proteins non-specifically bound to the conjugated agarose. Following numerous attempts to clarify the A3G complexes through modification of the binding stringency and elution methods, it became apparent that an additional strategy was required. We have constructed a pCMV-TAP (tandem affinity purification) vector that expresses A3G with an N-terminal tandem affinity tag consisting of a calmodulin binding domain and two IgG binding domains separated by a TEV protease cleavage site (77, 216). This vector was expressed in 293T cells and then A3G-TAP was purified from cell lysates using both tags by IgG affinity chromatography followed by TEV protease cleavage and then calmodulin affinity chromatography. TCA precipitated proteins were analyzed by 2-demensional isoelectric focusing/SDS gel electrophoresis. The proteins in the gels were stained with fluorescent dye SYPRO Orange (Molecular Probes) which interacts with the SDS coat surrounding proteins in the gel (264, 265), and gels were analyzed with a fluoroimager (Appendix III). In these, preliminary studies, we could clearly detect several protein spots that were specifically copurified with A3G that were absent from negative control, and proteins that copurified with the complexes that contained Vif_{HXB2} (data not shown). We propose to pick these protein spots with an automatic spotcutter, and to identify the relevant proteins and tryptic peptides by electrospray mass spectrometry. Our preliminary results suggest the likelihood of identifying potentially important A3G-interacting proteins with the tandem affinity purification technique.

Potential implications for drug development

Knowledge of the mechanism by which Vif counteracts A3G has revealed promising new targets and assays for drug development. However, growing evidence suggests that HIV-1 encounters a host of APOBEC3 enzymes that may function individually or in concert to partially disable HIV-1 replication *in vivo* (28, 155, 226). Recent findings reveal a complex interplay between the rapidly evolving Vif variants and their disparate abilities to counteract the individual APOBEC3 enzymes in this defense system (176). A greater understanding of the regulation of this effective antiviral network and the specific tissue niches occupied by individual members will have a significant impact on development of successful antiviral therapies.

HIV-1 and other lentiviruses have a high inherent rate of G-to-A mutation, which enables escape from the immune system, adaptation to diverse tissue niches, and expansion of the coreceptor repertoire (14). This variability is clearly advantageous for HIV-1, which is believed to operate near the threshold for error catastrophe beyond which its survival would become impossible (125). Consequently, a drug that interferes even slightly with the Vif-mediated degradation of A3G, A3F or A3B might be very useful for increasing the mutation frequency and reducing viral fitness.

In evaluating potential drug targets it is important to consider the critical role of proteasomes in removing damaged and misfolded proteins and in regulating the cell cycle and apoptosis. Disruption of normal proteasome-mediated degradation pathways leads to numerous diseases including neurodegenerative disorders and cancer. Nevertheless, general proteasome inhibitors have provided effective therapies for cancer, without the

pleiotropic effects that might be expected (188). Therefore, inhibition of proteasomes might be beneficial. An additional benefit of these studies is that any drugs for these pathways could have potential uses in investigation or treatment of other diseases in which proteasome functions are implicated.

In principle, the most efficient strategy would employ a small molecule inhibitor that could selectively disrupt association of Vif and A3G or A3F. Although interfering with protein-protein interactions is often difficult to achieve, the CCR5 inhibitors TAK779 and SHC351125 work by this mechanism to block binding of HIV-1 gp120 (12, 270). Compounds that prevent MDM2-mediated polyubiquitination and degradation of p53 by blocking their association have recently been developed(285). However, we observe that Vifs of patient HIV-1 isolates bind promiscuously to the structurally diverse APOBEC3 paralogs A3A, A3B, A3C, A3F, and A3G, using nonidentical sites for these interactions (176). Based on this precedence, an inhibitor with wide-ranging activity to block association of Vif variants and the distinct APOBEC3s will be difficult to achieve.

Other levels of intervention in the Ub-dependent degradation pathway also offer a varied degree of specificity for APOBEC3G degradation. Targets upstream of the proteasome, which include E1 and E2 Ub-conjugating enzymes, have been well studied and the wealth of information on their structure and enzymatic function could direct the design of effective inhibitory agents. In particular, there are numerous E2 enzymes (~50 identified to date) that interact with specific E3 ligases to contribute to target specificity, but factors determining this specificity are poorly understood (188, 213). (179, 188, 213. Further investigations are needed to identify the crucial E2 enzyme(s) involved in APOBEC3G degradation. Additionally, the E3 ligase components Elongins B and C,

Cul5, and Rbx-1 that associate with Vif and are essential for APOBEC3G degradation (142, 308, 309) present multiple points of intervention (142, 176, 179, 308, 309). Cul5 belongs to a family of proteins that are covalently modified by the Ub-like protein Nedd8. Neddylation, which is critical for cullin recruitment of the E2-Ub conjugating enzyme to the E3 ligase, occurs by an enzymatic cascade similar to that of ubiquitination, thus providing additional potential targets for inhibition (188). A compound that eliminated interaction of the BC-box of Vif with Elongins B and C would also restore APOBEC3G antiviral activity. However, because BC-box sequences occur in many important proteins, a drug specifically targeting the BC-box of Vif might be difficult to obtain. Although recent evidence suggests dephosphorylation of Ser^{144} in the Vif BC-box positively regulates its association with elongin C (179). Moreover, a strict confirmation of the individual components of the E3 ligase complex, particularly accessibility of A3G to the E2 Ub-conjugating enzyme, may be critical for polyubiquitination of A3G (142), implying that disruption of Vif interaction with E3 ligase components may not be entirely necessary to rescue A3G from the degradation pathway.

The obvious importance of the PKCα/βI/MEK/ERK pathway for regulating A3G and possibly A3B synthesis indicates this protein kinase cascade could be manipulated to enhance expression of these potent antiviral proteins thereby augmenting their activity. Although, due to the broad spectrum of critical cellular activities regulated by activation of ERK, interference with this pathway must be highly specific. Additional information concerning the specific transcriptional regulators or repressors that affect A3G synthesis is essential to define an appropriate target. Furthermore, overexpression of A3G and other cytidine deaminases could lead to promiscuous editing of cellular DNAs and/or

RNAs, which could effectively damage genes essential to normal cellular functions. For instance, overexpression of APOBEC1 and AID lead to rampant deaminase activity and often tumorogenesis (197, 262). In this regard, aberrant expression of APOBEC3s is found in numerous human cancer cells including colorectal adenocarcinoma, chronic myelogenous leukemia, and lung carcinoma (126). Therefore, it will also be important to identify factors that regulate APOBEC3 enzymatic and antiviral activity such as barriers that limit its diffusion into HIV-1 cores and factors that severely diminish its activity in certain cells.

Highly mutated proviruses isolated from PBMCs of HIV-1 infected patients contain G-to-A mutations that correspond to the preferred deamination context of A3G, A3F, and A3B, with the latter two making the major contribution. Although APOBEC3 enzymes contribute to sequence variation *in vivo*, it is not to a level that would eliminate or reduce HIV-1 to a chronic benign infection.

Summary and Conclusions

APOBEC3G (A3G) is a fundamental component of a potent antiretroviral pathway that occurs in the natural cellular targets of HIV-1, macrophages and lymphocytes. A3G functions as a cytidine deaminase and induces extensive lethal dC-to-dU deamination in the HIV-1 negative ssDNA made during reverse transcription. HIV-1 encoded Vif binds directly to A3G and induces its degradation by a proteasome-dependent pathway, effectively eliminating A3G from cells by a factor of 100-fold. As a result, A3G is packaged specifically into HIV-1(Δ*vif*) virions and efficiently excluded from HIV-1(wild-type) virions. Mutagenesis of Vif revealed at least two functionally distinct domains, an amino terminal region important for binding to A3G and a carboxyl terminal region with a highly conserved SLQ(Y/F)LA motif is essential for A3G degradation. Mutations in either region can prevent A3G degradation. Vif binds A3G and recruits E3 ligase components, elongins B/C, cullin 5, and Rbx-1 through interaction with its BC-box (SLQ(Y/F)LA motif) resulting in subsequent polyubiquitination and degradation. Vif effectively neutralizes potent antiretroviral activity by eliminating its expression from cells and consequently from progeny HIV-1 virons.

Since the substrate specificities of related cytidine deaminases are strongly influenced by their intracellular quantities, we analyzed factors that control A3G expression. APOBEC3G mRNA and protein levels were unaffected by treatment of proliferating H9 cells with INFs (-α,-β, and -γ) or TNF-α, but were enhanced up to 20-fold by PMA. This induction was mediated at the transcriptional level by a pathway that required activation of the protein kinase Cα/βI isozyme (PKC), mitogen-activated protein kinase kinase 1 and 2 (MEK), and extracellular signal-regulated kinase (ERK). In

addition, basal levels of APOBEC3G mRNA and protein declined when cells were treated with specific inhibitors of this protein kinase cascade or when cells were arrested in the G_0 state of the cell cycle by serum starvation. Expression of the antiviral APOBEC3G editing enzyme is dynamically controlled by the PKCα/βI / MEK / ERK protein kinase cascade in human T lymphocytes.

A3F has antiviral activity similar to A3G, can be bound by Vif, and is degraded by a proteasome-dependent mechanism. Surprisingly, A3B has antiviral activity that is unaffected by the Vif_{HXB2} strain used in these experiments. In addition, A3A and A3C weakly inactivate HIV-1(Δ*vif*) virions. Analysis of a panel of cell lines revealed that cells highly permissive for HIV-1(Δ*vif*) contained A3G mRNA and protein at levels similar to the nonpermissive cell line H9. In fact, cellular mRNA expression levels of antiviral A3B and A3F did not correlate with their ability to inactivate HIV-1 (Δ*vif*) virions released from these cells. A3G in highly permissive cells is degraded by Vif, suggesting that it is not in a sequestered site, and is specifically packaged in low but significant amounts into HIV-1(Δ*vif*) virions. In all cells tested, PMA induced A3G mRNA and protein expression requires activation of the PKCα/βI/MEK/ERK protein kinase cascade. A3B mRNA is also enhanced by PMA in T cells, while A3F mRNA is not. We suggest the dependency of A3G on the activation PKCα/βI/MEK/ERK signaling cascade could explain the varied and attenuated antiviral activity of H9 cells in some culture conditions. We confirmed that A3G heterodimerizes with A3F and A3B. In addition, A3B and A3G also heterodimerize with the weakly antiviral A3A and A3C. However co-expression of A3A, A3B, A3C, or A3F did not interfere with the antiviral potency of A3G. These results support other evidence that the anti-HIV-1 activities of A3G, A3F, and A3B can

be strongly influenced by cellular and/or viral factors other than Vif. The presence of these APOBEC3s, even in substantial amounts is insufficient to confer the nonpermissive phenotype in some human T cell lines.

Most importantly, discovery of this intrinsic and powerful response against HIV-1 reveals many new targets for therapeutic intervention. We have identified at least 3 distinct stages at which A3G activity could be modulated. First, our group and others have verified that Vif contains a conserved BC-box that links A3G to E3 ligase components and the proteasomal degradation pathway. Preventing ubiquitination and/or degradation of A3G would free this cytidine deaminase and allow its incorporation into virions. Second, transcription of A3G depends on activation of the PKCα/βI/MEK/ERK protein kinase cascade that is coordinately regulated with the cell cycle. Although not characterized, ERK activated transcription factors likely influence A3G synthesis. Thus, A3G expression levels could be enhanced to overcome inhibiting factors such as Vif. Finally, the significant levels of APOBEC3s expressed in some highly permissive cells do not correlate with their ability to inactivate HIV-1(Δ*vif*) virions released from cells, suggesting yet unidentified cellular and/or viral factors interact with APOBEC3s to restrict their activities. Further characterization of these factors may uncover important regulatory mechanisms that could control APOBEC3 activities *in vivo*.

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APPENDIX I

mRNA expression profile of APOBEC3 family members

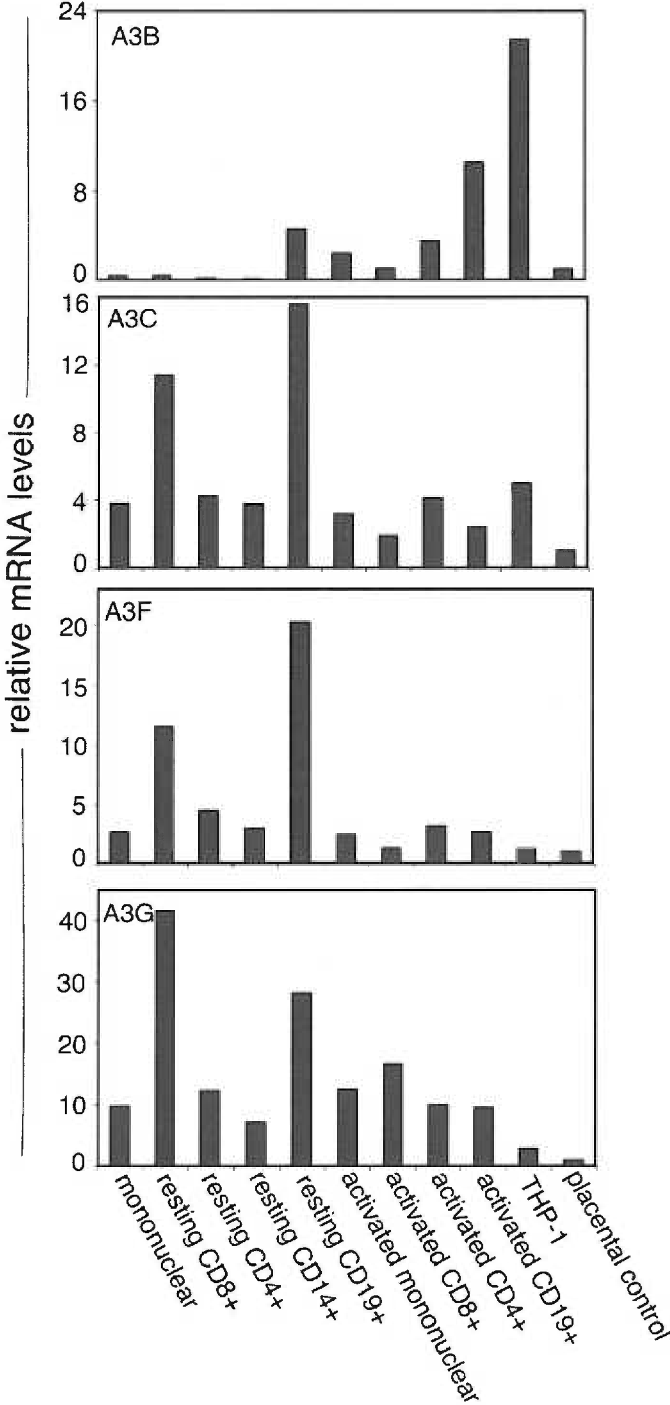
Appendix I

(A) Expression of APOBEC3s in resting and activated blood cell fractions.

A panel of cDNAs representing resting or activated blood cell fractions (BD Biosciences), and cDNA from the THP-1 monocytic cell line (a kind gift provided by Ashlee Moses, ATCC) were used for qRT-PCR analysis of A3C, A3B, A3F, A3G mRNA levels (226, 227). Cells in the "activated" groups were treated for 3-4 days as follows: $CD4^+$ T-lymphocytes with 5μg/ml concanavalin A, $CD8^+$ T-lymphocytes with 5μg/ml phytohemmagglutinin, mononuclear cells (B- and T-lymphocytes, and monocytes) with 2μl/ml pokeweed mitogen (GIBCO) and 5μg/ml concanavalin A (ICN), and $CD19^+$ (B-lymphocytes) with 2μl/ml pokeweed mitogen. CD14+ (monocytes) were not activated. Values represent mRNA levels relative to levels in a placental control cDNA sample (Clonetech).

Appendix I

(A) qRT-PCR analysis of APOBEC3s in blood cell fractions



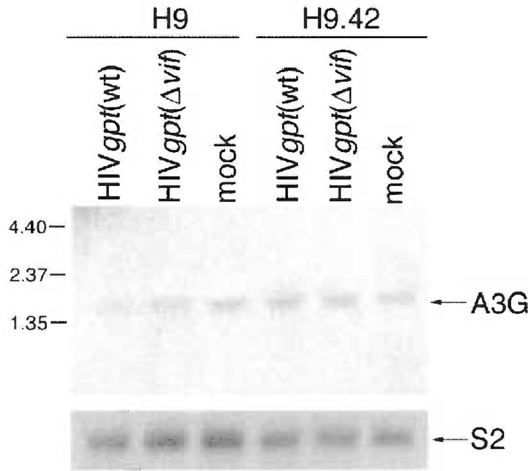
Appendix I

(B) HIV-1_{NL4-3} infection does not affect endogenous A3G mRNA levels in the nonpermissive H9 T-cell line.

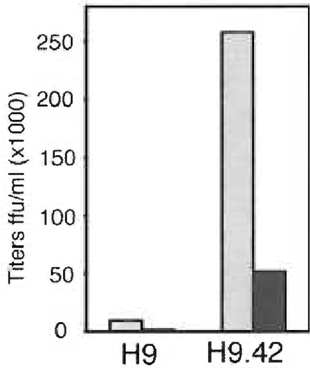
H9 or H9.42 (209) T-cell lines (H9.42 cells ten times more CD4 receptor) were treated for 20 min with 8μg/ml DEAE-Dextran then exposed to equal titers of NL4-3(wt) or NL4-3(Δ*vif*) and incubated for 48h at 37^0C with 5% CO_2. Virus containing supernatants were collected and titered on HeLaCD4+ cells using a focal infectivity assay (165, 166). Titers are represented as focus-forming units per milliliter (ffu/ml) of virus-containing supernatant. Titers of NL4-3(wt) released from H9.42 were 27.3-fold higher than H9 titers. Although, H9 and H9.42 cells inactivated NL4-3(Δ*vif*) to similar extents (14% and 20% respectively). Cells were pelleted and divided into two portions for RNA isolation using Absolutely RNA RT-PCR mini-prep kit (Stratagene) and cell lysates using RIPA buffer (175). Total RNA (10μg) was analyzed by Northern blot analysis using an A3G specific probe to detect endogenous A3G levels. The Northern blot was stripped and re-probed with the S2 ribosomal RNA to determine equal loading. Endogenous A3G levels in H9 and H9.42 were unaffected by HIV-1_{NL4-3} infection in the presence or absence of Vif. RIPA cell lysates were analyzed by western blot analysis using a polyclonal anti-p24 antibody #4121 (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Jonathan Allan) (252) to determine level of HIV-1 $p24^{CA}$ expressed in H9 versus H9.42. Although, a larger proportion of H9.42 cells are expressing HIV-1 proteins, steady-state A3G mRNA levels are not changed.

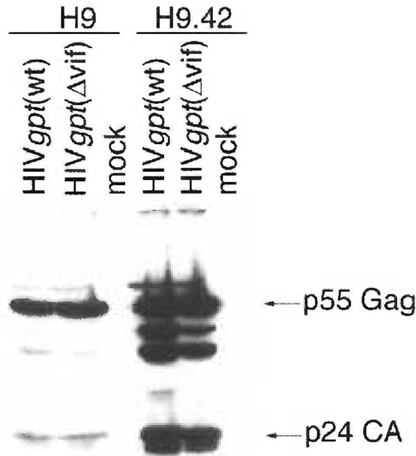
Appendix I

(B) Steady-state A3G mRNA levels are unaffected by HIV-1 infection of H9 or H9.42



Northern blot





anti-p24 western blot

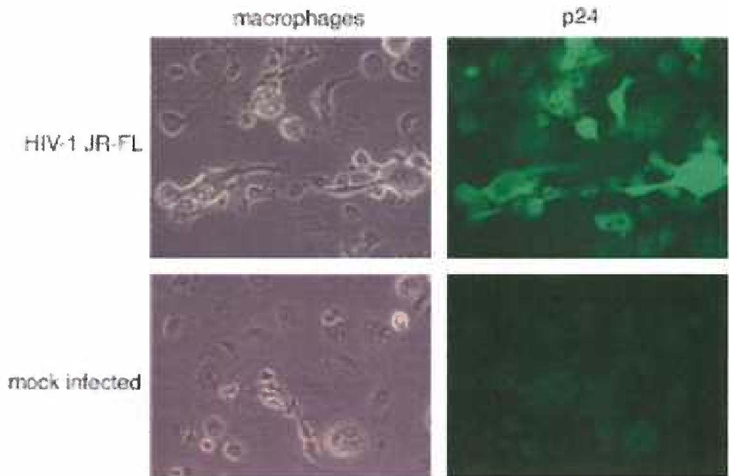
Appendix I

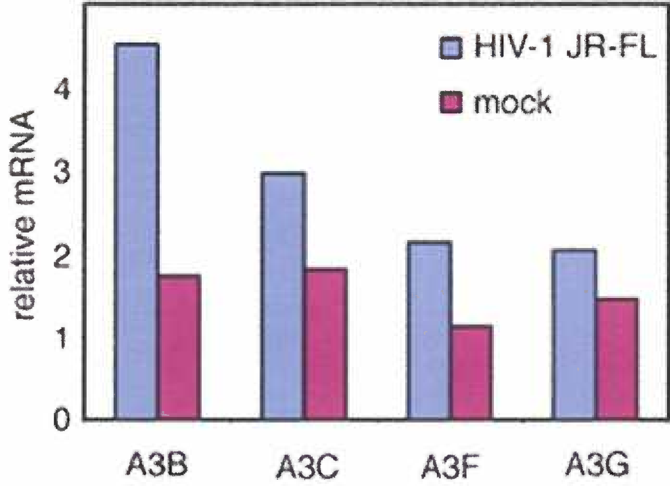
(C) A3G mRNA levels are enhanced in differentiated macrophages infected with $HIV\text{-}1_{JR\text{-}FL}$.

Cells were enriched for monocytes by adherence onto tissue culture plates and then grown for several days to differentiate into a more macrophage-like state. Cells were infected with $HIV\text{-}1_{JR\text{-}FL}$ at m.o.i. of approximately 1. Infected cells were stained with anti-p24 antibody to detect infected cells, and visualized by immunofluorescence microscopy. Cells were lysed and RNA was isolated. qRT-PCR analysis determined that A3G mRNA levels were induced in these macrophages after a 7 day exposure to $HIV\text{-}1_{JR\text{-}FL}$, while A3G mRNA levels in mock infected cells were unchanged. qRT-PCR analysis procedures have been previously described (226, 227).

Appendix I (C)

A3G mRNA levels are enhanced in HIV-1 infected macrophages





APPENDIX II

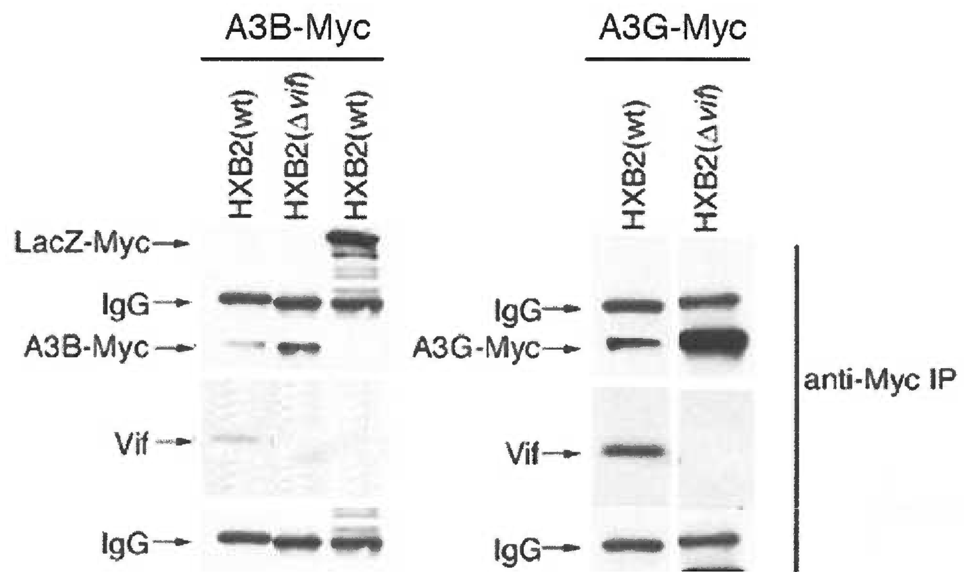
A3B and A3G bind HIV-1_{HXB2} Vif and heterooligomerizes with APOBEC3 family members

Appendix II

(A) A3B and A3G bind HIV-1HXB-2 Vif. 293T cells are co-trasfected using polyfect reagent (QIAGEN) with equimolar ratios of HIV*gpt*(wt) or HIV*gpt*(Δvif) and pcDNA3.1-A3B-Myc or pcDNA3.1-A3G-Myc. 36h post-transfection, cells are lysed in RIPA buffer and lysates are coimmunoprecipited with a monoclonal anti-Myc antibody (9E10) (Sigma) (175). Immunoprecipitated proteins were separated on a 10% SDS PAGE gel and analyzed by western blot using anti-Myc antibody and Vif antiserum #2221 (AIDS Research and Reference Reagent Program, NIH, NIAID, Dr. Dana Gabzda). A3G and A3B both bind to Vif_{HXB2}. **(B)** A3G binds wild-type Vif and Δ12 Vif in cell lysates at $0^{0}C$. Ni-NTA-agarose beads were incubated with A3G-Myc-6xHis containing cell lysates at $4^{0}C$ for 2h. Ni-NTA-agarose that contain adsorbed A3G-Myc-6xHis were thoroughly washed and incubated for the indicated times with 293T cell lysates expressing wild-type Vif or the BC-box mutant Vif (Δ12). The samples were analyzed by Western blot for Vif, A3G-Myc, and Δ12 Vif. A3G binds to both Vif and Δ12 Vif rapidly in cell lysates at $0^{0}C$. In addition, the BC-box region of Vif is necessary for binding of elongins B/C, but has not effect on binding to A3G.

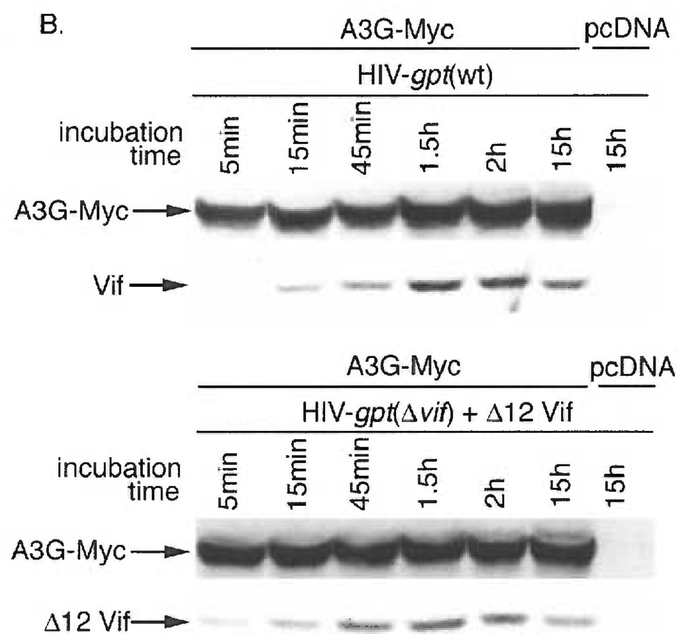
Appendix II

(A) A3G and A3B bind HIV-1_{HXB2} Vif



Appendix II

(B) A3G binds wild-type Vif and Δ12Vif mutant rapidly at 0^0C

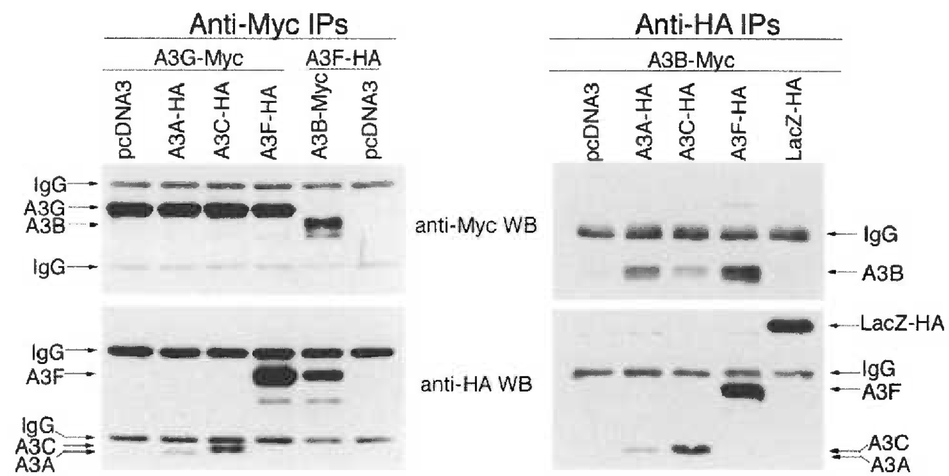


Appendix II

(C) A3G and A3B promiscuously heterooligomerize with APOBEC3 paralogs. 293T cotransfected cultures were lysed, immunoprecipitated either with anti-Myc (left panel) or anti-HA (right panel) and analyzed by Western blot with anti-Myc (upper panels) or anti-HA (bottom panels).

Appendix II (C)

A3G and A3B heterooligomerize with APOBEC3 paralogs



APPENDIX III

Tandem affinity purification of A3G protein complexes

Appendix III Tandem affinity purification of A3G protein complexes followed by 2-demensional gel electrophoresis.

(A) A3G was cloned into the XhoI and EcoRI restriction sites in pCMV-TAP to generate a recombinant A3G with an N-terminal tandem affinity tag (calmodulin and two IgG-binding domains separated by a TEV cleavage site) (77). **(B)** 293T cells transiently co-transfected (polyfect reagent, QIAGEN) with pCMV-TAP or pCMV-TAP-A3G are lysed in IPP150 buffer (10mM Tris-Cl pH8.0, 150mM NaCl, 0.1% NP40, 1X complete protease inhibitors) and applied to an IgG Fastflow 6 sepharose column (Amersham Biosciences). The column is rotated for 2h at 4^0C, drained by gravity flow, and washed with 30ml IPP150 (without protease inhibitors) followed a wash with 20ml TEV clevage buffer (10mM Tris-Cl pH8.0, 150mM NaCl, 0.1%NP40, 0.5mM EDTA, 1mM DTT). IgG-bound TAP-A3G was cleaved in 2ml TEV cleavage buffer, 200U AcTEV protease (Invitrogen) rotating at 4^0C for 3-12h. TAP-A3G was recovered by gravity flow from the IgG sepharose. Three volumes of IPP150 calmodulin binding buffer (10mM β-mercaptoethanol, 10mM Tris-Cl pH 8.0, 150mM NaCl, 1mM Mg acetate, 1mM imidazole, 2mM $CaCl_2$, 0.1% NP40) and 3mM $CaCl_2$ are added to recovered TAP-A3G before applying partially purified samples to the calmodulin affinity column (Amersham Biosciences). The calmodulin affinity column is rotated at 4^0C for 1h, drained by gravity flow, and washed with 30ml IPP150 calmodulin binding buffer. TAP-A3G is eluted in 6 column volumes calmodulin elution buffer (10mM β-mercaptoethanol, 10mM Tris-Cl pH 8.0, 150mM NaCl, 1mM Mg acetate, 1mM imidazole, 2mM EDTA, 0.1% NP40), TCA precipitated, and resuspended 2D gel rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 2% IPG buffer pH3-10, 0.3% DTT). **(C)** Purified A3G is isoelectrically focused

on Immobiline drystrips pH3-10 (Amersham Biosciences), followed by separation on a 10% SDS-PAGE gel. Proteins are stained with SYPRO orange (Molecular Probes) and imaged on a fluroimager. Arrows indicate proteins that have been cut out of the gel and processed for anlysis by eletrospray mass spectrometry.

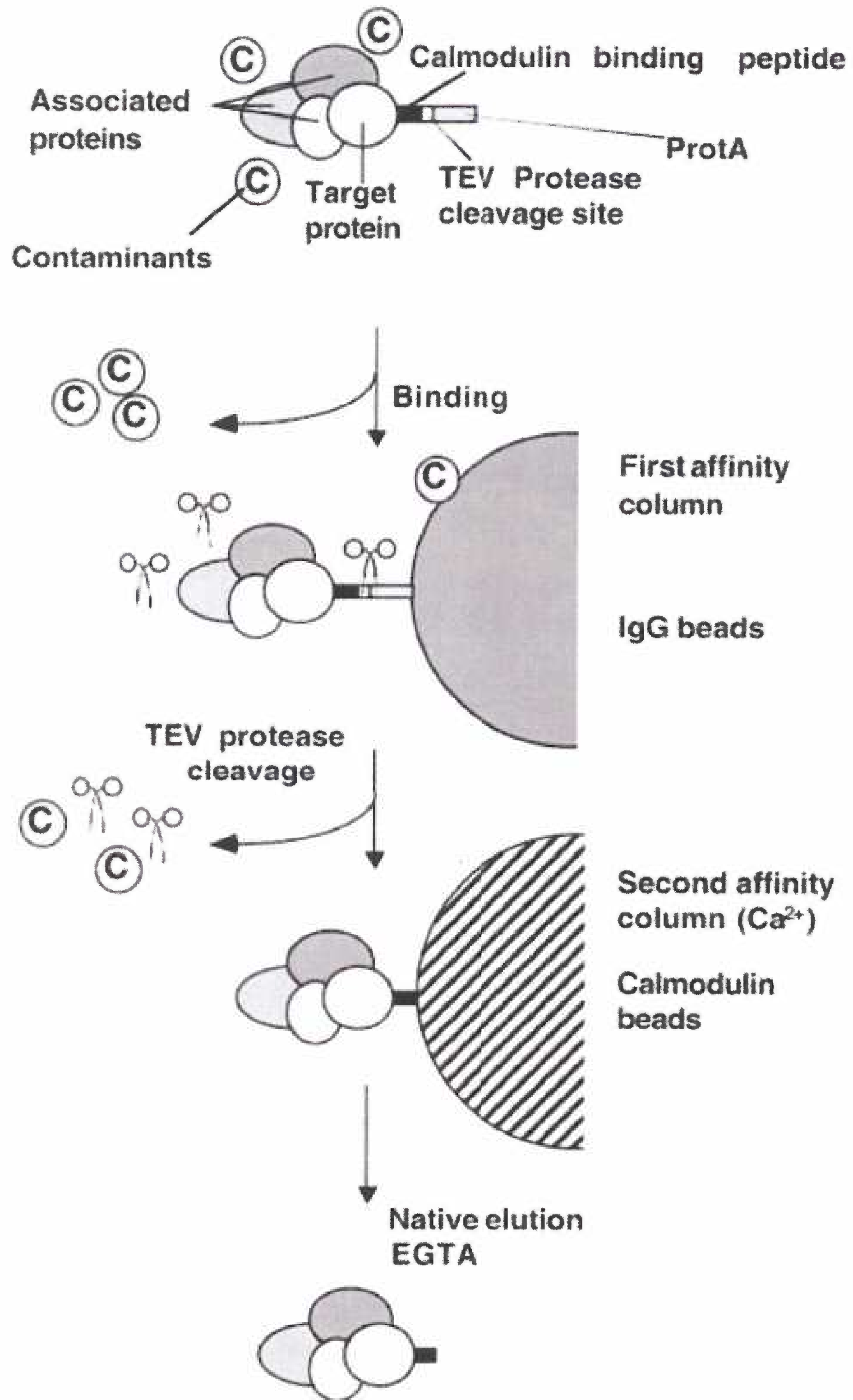
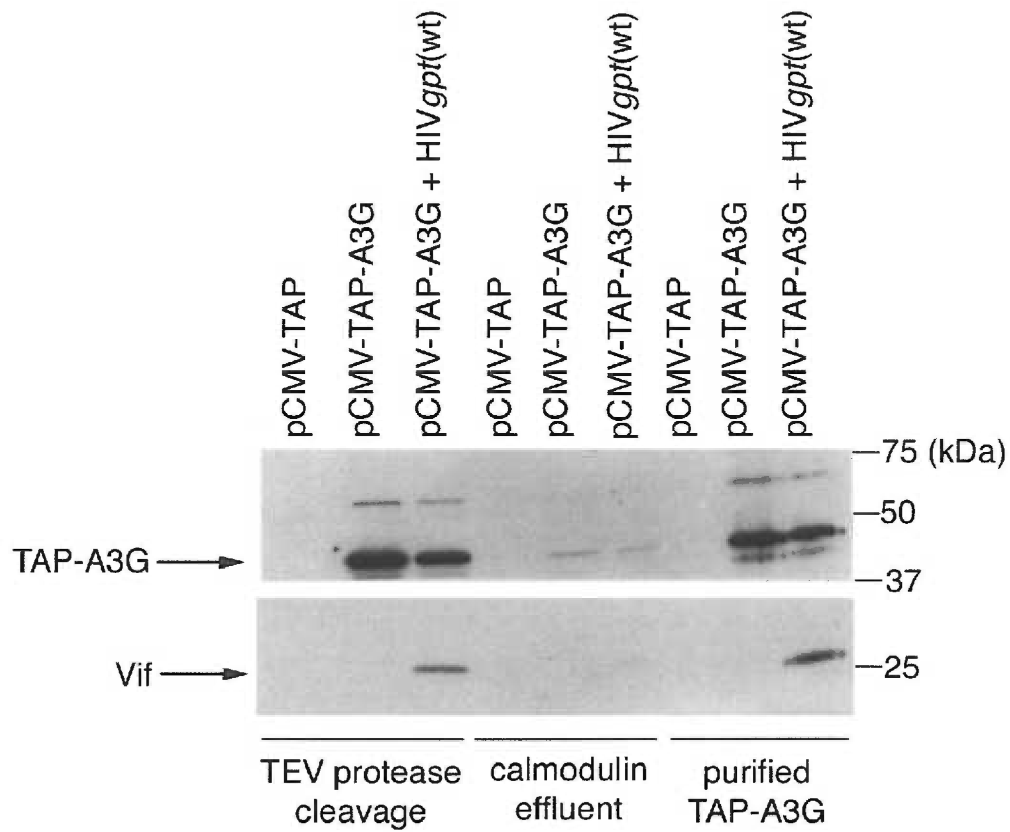


Figure taken from reference 216

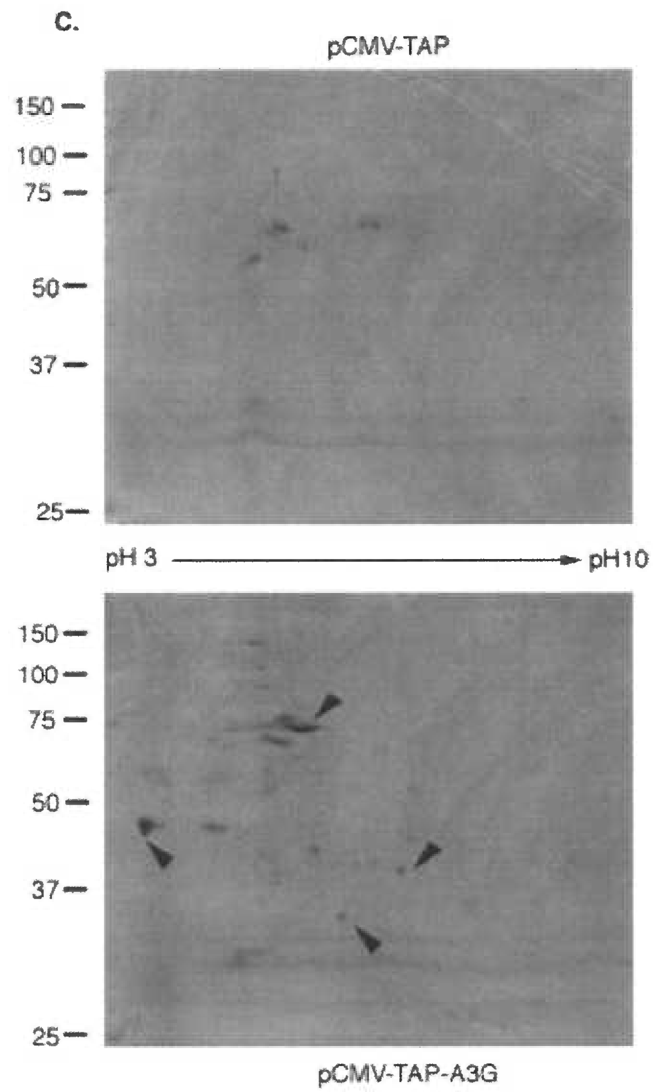
Appendix III

(B) TAP purification of A3G



Appendix III

(C) 2D gels of TAP purified A3G



APPENDIX IV

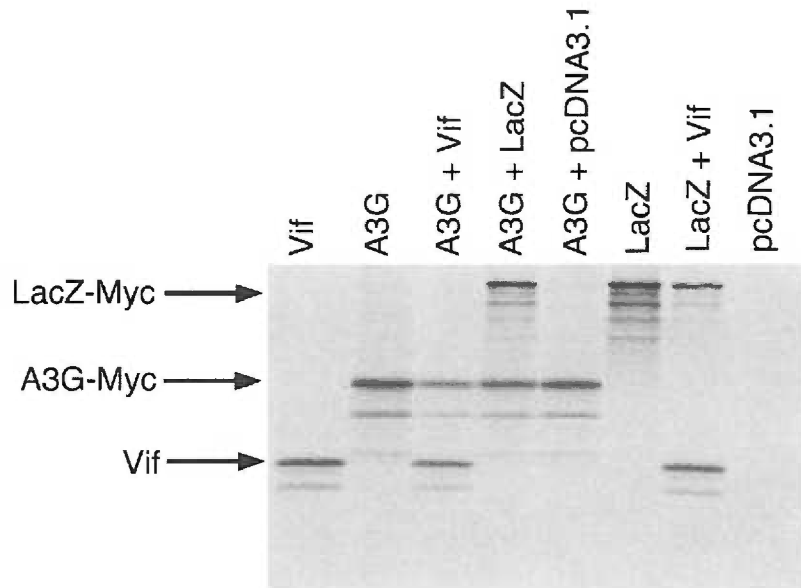
Vif inhibits translation of proteins non-specifically *in vitro*

Appendix IV Vif inhibits translation of proteins non-specifically in a reticulocyte lysate system *in vitro.*

pcDNA3.1-A3G-Myc, pcDNA3.1-LacZ-Myc, pcDNA3.1 A1 Vif, pcDNA3.1 vectors were translated using the TNT rabbit reticulocyte lysate system (Promega) and 1μg of each vector. Translations were done independently or in combinations indicated. Co-translation of Vif inhibited A3G-Myc, as well as, LacZ-Myc translation in vitro; therefore, co-translation of Vif in this system hampers translation of non-specific genes inserted into the pcDNA3.1-Myc expression vector.

Appendix IV

Vif inhibits translation of non-specific proteins *in vitro*



APPENDIX V

TNF-α activated pathways are functional in H9 cells

Appendix V TNF-α activated pathways are functional in H9 cells.

Cultured H9 cells were treated with TNF-α (50ng/ml) (R&D Biosystems) for 20 min. Cells were collected before treatment and 20 min after TNF-α treatment, lysed in RIPA buffer (175), and cell lysates were analyzed by Western blot using a phospho-specific p38 MAPK antibody (a kind gift from the Bruce Magun lab). Detection of phosphorylated p38 in H9 cells after TNF-α treatment confirms that H9 cells are sensitive to TNF-α treatment.

Appendix V

TNF-α stimulates phosphorylation of p38 in H9 cells