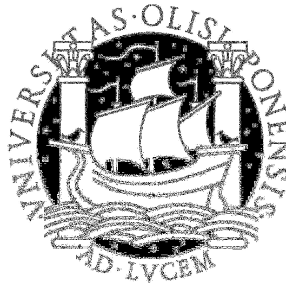
# UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

DEPARTAMENTO DE MICROBIOLOGIA


# The structure-function relationship of HIV-1 Vif protein and its regulation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Tese orientada pelo Prof. Doutor João Gonçalves


DOUTORAMENTO EM FARMÁCIA

MICROBIOLOGIA

2008

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Carla Iris Cadima Couto was financially supported by a Ph.D scholarship SFRH/BD/18805/2004 from Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal.

## Preface

The research work described in this dissertation was performed from October 2004 until September 2008 under the supervision of Prof. João Gonçalves at the CPM/URIA of the Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal.

During the period of my research I spent three months (from September 2007 to December 2007) in Prof. Moshe Kotler laboratory at the Hadassah Medical School of the University of Jerusalém, Israel, and the work developed during this period is included in chapter 2 of this thesis and in a manuscript already submited.

The results described in this thesis were included in manuscripts already submited for publication:

**Iris Cadima-Couto,** Acilino Freitas-Vieira, Roni Nowarski, Elena Britan-Rosich, Moshe Kotler, and João Gonçalves. (2008). Modelling APOBEC3G intracellular steady-state shows a differential inhibition of HIV-1ΔVif. *Journal of Biological Chemistry. (Submited)*.

**Iris Cadima-Couto,** Soraia Oliveira, Saraiva N, João Gonçalves. (2008). Cooperation of amino and carboxyl-terminal domains of Vif is essential for its role in A3G degradation. *Journal of Biological Chemistry. (Submited)*.

**Iris Cadima-Couto,** Saraiva N, Gonçalves J. (2008). Assessment of HIV-1 Vif-APOBEC Interactions Based on a β-Lactamase Protein Fragment Complementation Assay. *Retrovirology. (Submited)*

According to the “Decreto-Lei 388/70”, article 8 point nº 2, the data presented in this dissertation is the result of the authors work and it is clearly acknowledged in the text whenever data or reagents produced by others were used. The author participated in the planning and execution of the experimental procedures such as in the results interpretation and in manuscripts preparation. The opinions expressed in this publication are from the exclusive responsibility of the author and have not been previously submitted for any degree at this or other university.

## Acknowledgments/Agradecimentos

Ao meu orientador, Professor João Gonçalves, pela sua orientação durante o desenvolvimento deste trabalho. Obrigada por todas as recomendações e por me ter dado liberdade na autogestão do meu tempo de trabalho no laboratório, que foi fundamental para que este trabalho tivesse sido concluído dentro dos prazos limites.

Ao Professor José Moniz Pereira por permitir a realização desta tese na Faculdade de Farmácia da Universidade de Lisboa.

À Fundação para a Ciência e Tecnologia pelo apoio financeiro no âmbito da bolsa de doutoramento, SFRH/BD/18805/2004.

Ao Acilino por ter sido um excelente colega de trabalho, pelo seu companheirismo e amizade e por ter estado sempre presente durante os bons e maus momentos ao longo destes 4 anos. Obrigada por tudo!

Aos meus restantes colegas de laboratório que me acompanharam ao longo deste trabalho: Ana Fonseca, Andreia, Cláudia, Filipa, Frederico, Maria João Leite, Maria João, Mariana, Paula, Rita, Sara, Sofia Coelho, Sofia CR, Soraia, Sylvie. A todos, obrigada! Obrigada a todos os meus restantes companheiros da unidade de microbiologia, com os quais partilhei as muitas horas no P3.

À Madalena e ao João Vital pela sua simpatia e boa disposição.

À Professora Aida Esteves, obrigada por ter disponibilizado o seu P3 durante um período do meu doutoramento. Obrigado também à Sandra, ao Rui, ao João e ao Ricardo, do grupo da Prof. Aida, por me terem sempre recebido tão bem.

I am deeply grateful to Professor Moshe Kotler, from the University of Jerusalem, for receiving me so well in his lab, for his support and friendship and for making me feel at home away from home. I would also like to thank to all the members of Prof. Moshe lab: Roni and Elena, thank you so much for being so kind in your guidance during my work in the lab. Maia and Shira, thank for your friendship!

À minha família, por me terem sempre apoiado em todas as decisões da minha vida. Obrigada aos meus amigos que acompanharam de perto todos os momentos do meu doutoramento….obrigada pela conversas, por todos os momentos de lazer que passámos e que foram essências durante este período.

Finalmente, um especial agradecimento ao Rafael. Obrigada pelo teu constante apoio, paciência, e por nunca teres duvidado de que eu iria conseguir ultrapassar esta etapa da minha vida. Obrigada por me teres dado ânimo nos momentos mais difíceis deste doutoramento e por teres partilhado comigo todos os bons momentos.

## Estrutura-Função da proteína Vif do HIV-1 e sua regulação


### SUMÁRIO

O Vírus da Imunodeficiência Humana tipo 1 (HIV-1) é um retrovírus complexo que codifica uma proteína acessória, Vif (Viral Infectivity Factor), necessária para uma produtiva infecção. A presença de Vif na célula produtora de vírus é um dos factores essenciais para o aumento da infecciosidade viral, podendo esta ser aumentada até cerca de 1000 vezes.

Há mais de uma década que é sabido que a proteína Vif é necessária para a replicação do HIV-1 em algumas células, chamadas não permissivas. No entanto em outras, chamadas permissivas, a replicação viral ocorre mesmo que na ausência de Vif. A razão para este facto permaneceu desconhecida até á descoberta, em 2002, da desaminase de citidina humana, *human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G* (APOBEC3G) como sendo a proteína responsável por restringir a replicação de HIV-1 que não expressa Vif. Este factor de restrição celular encontra-se presente maioritariamente no baço, gónadas, monócitos e linfócitos periféricos.

Na ausência de Vif, A3G, é encapsidado no virião em formação por um sistema dependente de Gag. Desta forma, A3G é transportado para a próxima célula alvo onde vai actuar no momento da transcrição reversa, desaminando a cadeia simples, negativa do cDNA viral. Este processo resulta na inactivação do processo replicativo por hipermutação do genoma ou mesmo pela degradação do cDNA viral por um mecanismo dependente da enzima DNA uracil glicosilase.

Após a identificação de A3G e mais tarde de APOBEC3F (A3F), ficou claro que o mecanismo pelo qual o HIV-1 escapava á acção destes factores de restrição era através da proteína Vif. Vif actua inibindo a entrada de A3G e A3F nos viriões através da diminuição dos níveis celulares destes factores de restrição. Um dos mecanismos que a Vif utiliza implica a degradação proteossomal destas enzimas. Para isso, a Vif recruta uma série de proteínas celulares para formar um complexo Culina5- os desta forma para degradação proteossomal.

Desta forma, torna-se essencial compreender de que forma a proteína A3G, documentada como sendo a que exerce maior actividade contra o HIV-1, pode ser estimulada na célula de maneira a poder escapar aos efeitos da Vif e consequentemente inibir a replicação viral. Assim, decidimos modelar o tempo de meia-vida do A3G como forma de monitorizar a sua acção na célula desde o momento em que é traduzido até á sua encapsidação no virião. Este mecanismo de modelação do tempo de meia-vida de uma proteína é conhecido pelo nome de "N-end Rule" e consiste na introdução de diferentes amino ácidos no inicio da região N-terminal de uma proteína resultando em diferentes tempos de meia-vida da mesma, de acordo com a identidade do resíduo introduzido. Ao interferir com a estabilidade desta proteína, fomos capazes de a direccionar para degradação no proteossoma duma forma independente da Vif. Os resultados apresentados neste trabalho confirmaram a importância do tempo de meia-vida do A3G para a manutenção da sua actividade antiviral. Ao diminuir os tempos de vida do A3G na célula, fomos capazes de determinar que o tempo de meia-vida mínimo do A3G para que este seja incorporado nos viriões é de aproximadamente 13 minutos. Neste caso, concluímos que a Vif terá que actuar nos estágios iniciais da vida do A3G de forma a impedir que este seja encapsidado nos viriões. A análise da actividade catalítica dos nossos diferentes A3Gs revelou que a sua actividade enzimática no citoplasma da célula não parece ser importante para a sua actividade antiviral e que a actividade catalítica destes variantes não aumenta de acordo com o aumento do tempo de meia-vida destes. Esta estratégia, permitiu-nos compreender de que forma o tempo de meia-vida do A3G pode influenciar a sua actividade antiviral, quer ao nível da sua encapsidação nos viriões como ao nível da sua actividade catalítica na célula.

A identificação de padrões de interacção entre a Vif e o A3G é um dos maiores desafios para o desenvolvimento de novas terapias antivirais. Tem sido demonstrado que o impedimento da interacção Vif-A3G resulta num aumento da actividade antiviral deste último. Assim, decidimos estudar a interacção entre estas duas proteínas através de duas estratégias que, embora já tenham sido utilizadas em estudos semelhantes com outras proteínas, foram aqui usadas pela primeira vez no contexto da interacção Vif-A3G.

A primeira estratégia consistiu na fusão de fragmentos da Vif e do A3G a domínios de interacção do factor de transcrição de levedura GCN4 (Zip), que forma homodimeros. A presença de Zip em fusão com estas proteínas obrigará estas a se encontrarem espacialmente permitindo-nos assim mutagenizar partes de ambas as proteínas e concluir acerca da importância de certos domínios na interacção. Esta estratégia permitiu-nos demonstrar a importância da região C-terminal da Vif na interacção com o A3G. Os resultados obtidos indicam que esta região da Vif, por si só, não é suficiente para induzir a degradação do A3G e sugerem que a interacção Vif-A3G deverá envolver um co-factor celular adicional que se deverá ligar á região N-terminal da Vif.

Estudámos também a orientação espacial da interacção Vif-A3G através da fusão destes, a domínios interactivos de Zips anti-paralelos. Os resultados obtidos mostraram a importância da orientação espacial das duas proteínas, demonstrando que esta deverá ocorrer em orientação paralela. Demonstrámos também que, para uma eficaz interacção entre Vif e A3G, estas deverão se encontrar espacialmente próximas uma da outra.

De forma a mostrar a especificidade da interacção Vif-A3G, decidimos estudar a interacção da Vif com uma proteína com a qual Vif não interage. Desta forma, utilizámos a proteína EGFP em fusão com Zip. Desta interacção não resultou degradação, levando-nos a concluir que a interacção Vif-A3G é específica reforçando uma vez mais a nossa hipótese de existência de um factor celular essencial para uma eficaz interacção e degradação do A3G.

A segunda estratégia usada no estudo da interacção Vif-A3G consiste num sistema de complementação proteica baseado na enzima TEM-1 β-lactamase. Desta forma, os domínios inactivos da enzima são fundidos a cada uma das proteínas em causa, através de uma ponte de aminoácidos que fornece plasticidade à fusão. Se ocorrer interacção proteína-proteína os dois domínios inactivos da enzima tornam-se funcionais e capazes de hidrolizar o anel β-lactâmico do substrato comercial CENTA. Desta forma, introduzimos mutações em resíduos previamente descritos como tendo um papel fundamental na actividade de ambas as proteínas.

Os resultados obtidos mostram que alguns resíduos no A3G, descritos como sendo importantes para a interacção com a Gag do HIV-1, estão também envolvidos na interacção com a Vif.

Para além da interacção Vif-A3G, o método explorado neste trabalho permitiu testar interacções da Vif com outros membros da família APOBEC, nomeadamente A3F, A3C e A2. Destes, apenas o A3G e o A3F estão descritos como possuindo actividade antiretroviral. Por outro lado a relevância da interacção da Vif com A3C ou A2 permanece por esclarecer. Através do nosso ensaio, não fomos capazes de detectar interacção entre a Vif e o A3C. No entanto, conseguimos recuperar esta interacção ao substituir a região DRMR da Vif por alaninas. Tendo em conta os nossos resultados, colocámos a hipótese de esta região da Vif estar envolvida na selecção do APOBEC a inactivar. Surpreendentemente, a Vif foi capaz de interagir com A2, apesar de esta proteína não ter qualquer efeito sobre a replicação do HIV-1. Isto faz-nos colocar a hipótese que a interacção da Vif com os vários APOBECs parece ser promíscua. Ou seja, a Vif pode possivelmente ligar-se com diferentes afinidades, a muitos dos APOBECs conhecidos. Então, é possível que o facto de a Vif interagir com certas proteínas da família APOBEC não ter de ser necessariamente devido ao facto de estas exercerem actividade antiviral sobre o HIV-1. Neste caso particular do A2, é possível que, apesar de interagir com Vif, esta enzima não tenha qualquer actividade antiviral devido á ausência de um co-factor que poderá ser fundamental na actividade desta enzima.

Palavras Chave: HIV-1; proteossoma; N-end Rule; Factor de transcrição de levedura GCN4; Ensaio de Complementação Proteica.

# The Structure-function relationship of HIV-1 Vif protein and its regulation


## SUMMARY

One of the critical determinants of HIV-1 infectivity is the capacity of the virus to infect new cells. The viral Vif protein is one of the critical factors involved in the increase infectivity of the virus. Vif protein is required for HIV to replicate in some human cell types (termed 'non-permissive' cells), but not others ('permissive' cells). The reason for this is the expression, in non-permissive cells, of a potent antiretroviral enzyme: APOBEC3G (A3G) and its paralog APOBEC3F (A3F). Each of these enzymes contains two RNA-binding motifs and incorporates into assembling HIV-1 capsids where they cause lethal dC-to-dU hypermutations in the single-stranded viral DNA that transiently forms during reverse transcription. One of the mechanisms that Vif uses to overcome this viral block is by reducing A3G/A3F intracellular expression levels recruiting it for polyubiquitination and proteasomal degradation in the 26S proteasome.

In order to understand how A3G half-life can influence its antiviral activity, both at the level of viral encapsidation and catalytic activity in the cell, we decided to model the intracellular half-life of A3G by targeting it to the N-end rule pathway. This system allowed us to show that A3G variants with different stabilities are capable to be targeted for proteasomal degradation in a Vif-independent manner. Our results confirmed the importance of A3G steady-state expression for the maintenance of its antiretroviral activity indicating that in order to counteract A3G activity, Vif has to act at the very early stages of A3G life-time, probably within the first 13 minutes after its synthesis, in order to impede its incorporation into virions. In addition, we show that active deamination in the cytoplasm does not seem to be important for antiviral activity of A3G and deamination activity does not increase with the half-life of A3G.

Identification of protein biding partners between Vif and A3G proteins is one of the major challenges for the development of new antiviral drugs, as disruption of A3G-Vif interaction is predicted to stimulate natural antiviral infectivity.

In this work, we used two different approaches to study the interaction of Vif with A3G. One strategy was based on an oligomerization-assisted interaction whereby fragments of Vif and A3G were fused to independently folding and interacting domains of homodimerizing GCN4 leucine zipper-forming sequences. This allowed us to show that the C-terminal region of Vif alone is not sufficient to induce A3G degradation and that binding of Vif to A3G must involve an additional co-factor that is probably linked to the N-terminus of Vif and may be essential for the interaction and consequent degradation of A3G.

The other strategy involved a Protein Complementation Assay (PCA). We produced several mutants of both Vif and A3G bearing mutations on key residues that are thought to be crucial for the interaction between these two proteins. By using this strategy we were able to show that some mutations in A3G protein that had been described as being involved in the interaction with HIV-1 Gag, were also important for the interaction with Vif. As a result, we were able to achieve a quantitative assessment of HIV-1 Vif-A3G interactions, which allowed us to do a fine dissection of the regions involved in this interaction.

Finally, we found that a region in Vif (DRMR) may be important for the selection of the APOBEC protein to inactivate. By studying the interaction of Vif with A2 protein we reached the conclusion that an additional co-factor may be required in order to A2 to be able to exert antiviral action. Therefore, we provided supporting information for a new direction of the study of Vif-A3 problematic regarding the involvement of an additional co-factor.

Keywords: HIV-1; Vif; APOBEC3G; proteosome; N-end rule; GCN4 leucine zipper;
Protein Complementation Assay

# Abbreviations

## Reagents

| | |
|---|---|
| **DMEM** | Dulbeco`s Modified Eagle Medium |
| **DMSO** | Dimethyl Sulfoxide |
| **HRP** | Horseradish Peroxidase |
| **PAGE** | PolyAcrylamida Gel Electrophoresis |
| **PBS** | Phosphate Buffer Saline |
| **SDS-PAGE** | Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis |

| | |
|---|---|
| **AGM** | African Green Monkey |
| **AID** | Activation-Induced Cytidine Deaminase |
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **APOBEC1 (A1)** | Apolipoprotein (apo) B Editing Cytidine Deaminase |
| **APOBEC2 (A2)** | Apolipoprotein B mRNA-Editing Catalytic polypeptide-like 2 |
| **APOBEC3B (A3B)** | Apolipoprotein B mRNA-Editing Catalytic polypeptide-like 3B |
| **APOBEC3C (A3C)** | Apolipoprotein B mRNA-Editing Catalytic polypeptide-like 3C |
| **APOBEC3F (A3F)** | Apolipoprotein B mRNA-Editing Catalytic polypeptide-like 3F |
| **CA** | Capsid |
| **CD** | Catalytic Domain |
| **CD4** | Cluster Designation 4 |
| **cDNA** | Complementary DNA |
| **CPRG** | Chlorophenolred-β-D-galactopyranoside |
| **DNA** | Deoxyribonucleic  Acid |
| **DIS** | Dimerization Initiation Signal |
| **Cul5** | Cullin 5 |
| **DNA** | Deoxyribonucleic Acid |
| ***E. coli*** | *Escherichia coli* |
| **EGFP** | Green Fluorescent Protein |
| **EIAV** | Equine Infectious Anemia Virus |

| | |
|---|---|
| **EloC** | **Elonguin C** |
| **Env** | Envelope polyprotein |
| **Gag** | Group specific antigen polyprotein |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GST** | Glutathione *S*-Transferase |
| **HA** | Hemagglutinin |
| **HIV-1** | Human Immunodeficiency Virus type-1 |
| **HIV-2** | Human Immunodeficiency Virus type-2 |
| **HMM** | High Molecular Mass |
| **IN** | Integrase |
| **LMM** | Low Molecular Mass |
| **LTR** | Long Terminal Repeats |
| **MA** | Matrix |
| **mRNA** | Messenger Ribonucleic Acid |
| **NC** | Nucleocapsid |
| **Nef** | Negative regulator factor |
| **NLS** | Nuclear localization signal |
| **OD** | Optical Density |
| **ORF** | Open Reading Frame |
| **PABPC1** | Polyadenylate-binding protein 1 |
| **P-bodies** | mRNA processing bodies |
| **PBS** | Primer Binding Site |
| **PCD** | PseudoCatalytic Domain |
| **PCR** | Polymerase Chain Reaction |
| **PIC** | Pre-Integration Complex |
| **cPPT** | Central Polypurine Tract |
| **PPT** | Polypurine Tract |
| **Pol** | Polymerase |
| **PR** | Protease |
| **RBX2** | RING-box-2 |
| **Rev** | Regulator of virion |
| **Rh** | Rhesus |
| **RNA** | Ribonucleic Acid |
| **RNP** | Ribonucleoprotein |
| **RRE** | Rev Response Element |
| **RT** | Reverse Transcriptase |

| | |
|---|---|
| **SIV** | **Simian Immunodeficiency Virus** |
| **ssDNA** | Single-stranded DNA |
| **TAR** | Trans-Activating Response region |
| **TM** | Transmembrane protein |
| **tRNA** | Transfer RNA |
| **Tat** | Transcriptional transactivator protein |
| **Ub** | Ubiquitin |
| **Vif** | Viral Infectivity Factor |
| **Vpr** | Viral protein R |
| **Vpu** | Viral protein U |
| **Vpx** | Viral protein X |
| **VSV-G** | Vesicular Stomatitis Virus Glycoprotein G |
| **Zip** | GCN4 Leucine Zipper |

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# Chapter 1

## GENERAL INTRODUCTION

# 1. The Human Immunodeficiency type-1 virus

## 1.1. HIV-1

The Human Immunodeficiency virus type-1 (HIV-1) belongs to the family *Retroviridae*, generus *Lentivirus*. Lentiviruses are small, enveloped, positive-strand RNA viruses that differentiate from other retroviruses by the presence of a large number of non-structural proteins. These viruses code for the reverse transcriptase enzyme (RT), which enables them to replicate their RNA genome through a DNA intermediate.

HIV-1 was first discovered in 1983 and was described as the causative agent of the Human Acquired Immunodeficiency Syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983). The course of HIV-1 infection is characterized by a progressive loss of CD4-positive cells leading to immune dysfunction and opportunistic infections (Teeuwsen *et al.*, 1990; Clerici *et al.*, 1991). HIV-1 is the prevalent virus and is the responsible for an actual global pandemic. A small percentage of HIV cases are attributed to a less virulent HIV virus, the HIV virus type 2 (HIV-2). HIV-2 was first identified in 1986 from samples isolated from west african patients (Clavel *et al.*, 1986). HIV-2 infections are most prevalent in some countries such as Guinea-Bissau and Portugal.

Substantial progress has been made in the past 25 years since the discovery of HIV-1. Studies toward understanding the biological function of each protein, and the three-dimensional structures of many components, such as portions of the RNA genome, have been determined. Despite this significant progress, new challenges continue to arise and more efforts have to be done to continue with these studies.

### 1.2. HIV-1 life cycle

HIV-1 begins its life cycle by binding to a CD4 receptor and one of two co-receptors, CCR4 or CXCR5, on the surface of a CD4$^{+}$ T - lymphocyte. The virus enters the cells by fusion with the host cell and releases its genetic material, two copies of RNA, into the cytoplasm of the cell. The HIV-1 enzyme called reverse transcriptase (RT) will then convert the single stranded HIV RNA to double-stranded HIV DNA. The newly formed HIV DNA will then enter the host cell´s nucleus where it will be integrated within the host cell's DNA by the action of another HIV enzyme called, integrase (IN). Once integrated into the host's genome the HIV DNA may remain inactive for several years, producing a few or no copies of HIV. The integrated HIV DNA is called provirus. Activation of HIV provirus requires a host enzyme called RNA polymerase II. When the host cell receives a signal to become activated, the provirus will use the cell's RNA polymerase to create copies of its genetic material as well as viral transcripts that will be expressed from the promoter located in the 5' long terminal repeat (LTR), with Tat greatly enhancing the rate of transcription. A set of spliced and genomic-length RNAs are then transported from the nucleus to the cytoplasm, where they can be translated into long chains of HIV proteins, or polyprecursors. This step is regulated by Rev. After translation of viral mRNAs, the Gag and Gag-Pol polyprecursors become localized to the cell membrane. The core viral particle is then assembled from the

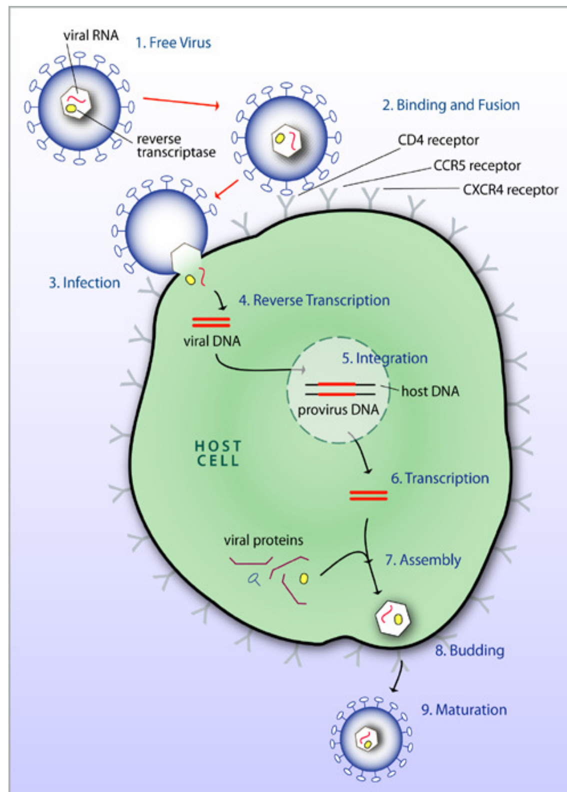



**Fig 1.** Schematic representation of HIV-1 life-cycle. *(From: Allison O'Brien)*

Gag and Gag-Pol polyprecursors (later processed to MA, CA, NC, p6, PR, RT, and IN), Vif, Vpr, Nef, and the genomic RNA, and an immature virion begins to bud from the cell surface. The newly assembled virion is released from the host cells through a process called budding. During budding, the new virion “steals” part of the cell’s outer envelope. This envelope, which acts as a covering, is studded with protein/sugar combinations called HIV glycoproteins. These glycoproteins are essential for the virus to infect a new target cell as they are necessary to bind CD4 and co-receptors. A new cycle of infection is now ready to begin.

### 1.3. The HIV-1 structure and genome

The viral genome contains two strands of RNA of positive polarity and the mature virus consists of a bar-shaped electron dense core which contains the viral genome along with the enzymes RT, IN, PR, and ribonuclease. The viral core is surrounded by a lipid envelope containing the antigen gp120 that is involved in the binding of the virus to the target cell by interactions between CD4 and CC or CXC chemokine co-receptors (Fig. 2).

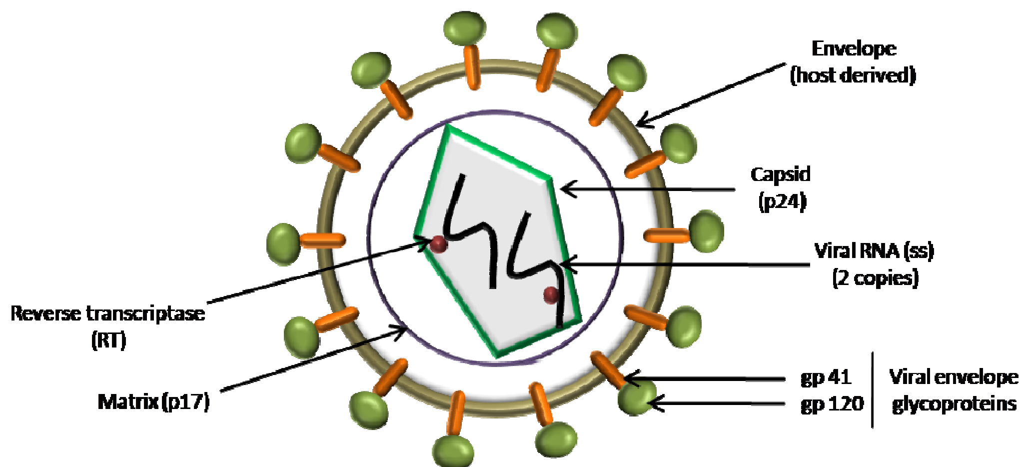
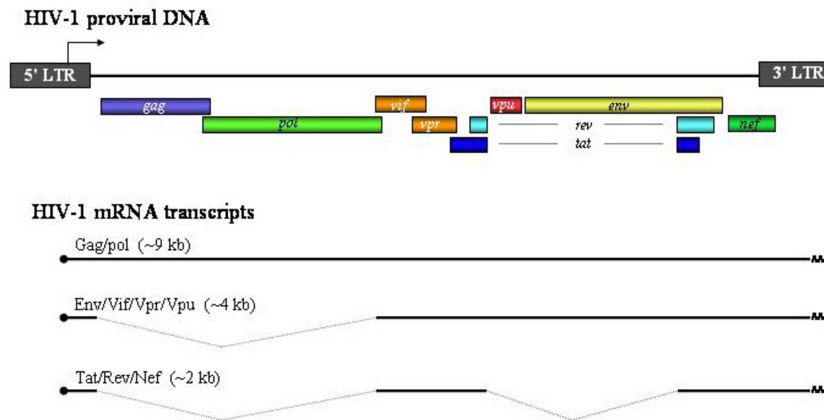



**Fig.2. Structure of HIV-1 virion**

HIV-1 genome, like other retrovirus, has three major structural genes: Gag, Pol and Env. The major components coded by the Gag gene include the core proteins p55, p40, p24 (capsid, CA), p17 (matrix, MA), and p7 (nucleocapsid, NC). The Pol gene codes for the enzyme proteins p66 and p51 (RT), p11 (PR), and p32 (IN). Finally, the Env gene codes for the envelope glycoproteins, which include the outer envelope glycoprotein gp120 and transmembrane glycoprotein gp41 derived from the glycoprotein precursor gp160. The genome also contains several non-structural genes such as, Tat (trans-activator), Rev (regulator of expression of virion protein), Nef (negative factor), Vif (virion infectivity), Vpr and Vpu (viral proteins R and U, respectively). In addition to the structural and non-structural proteins, the viral genome contains a number of *cis*-acting sequences, such as the Tat-acting region (TAR), the primer binding site (PBS), the dimer initiation site (DIS), the packaging sequence (psi), the polypurine tract (PPT located at the 3' end of the genome and a second cPPT present at the center of the genome) and the Rev-responsive element (RRE). These sequences play important roles during the viral life-cycle such as transcription (TAR), translation and nuclear export (RRE), packaging (psi), dimerization of viral genomic RNA (DIS) and reverse transcription (PBS, PPT and cPPT).




*(Figure from: Journal of Virology 2006* ***3****:60)*

**Fig.3. Schematic representation of the HIV-1 genome and viral mRNA transcripts.** Polyprotein precursors of HIV-1, Gag/Pol, Env/Vif/Vpr/Vpu, and Tat/Rev/Nef. Viral mRNAs are first produced and most are doubly spliced to produce the Tat, Rev and Nef proteins. The U3 region of the 5' LTR is responsible for the initiation of viral transcripts and basal transcription activity is modulated by the availability of specific host cell factors acting on the LTR. The first viral transcripts to appear in the cytoplasm of the infected cell are the so called 2kb mRNAs that will originate the non-structural proteins Tat, Rev and Nef.

### 1.4. Non-structural proteins

The first viral transcripts originated from the viral LTR are multiply spliced mRNAs that will originate the non-structural proteins Tat, Rev, and Nef. Upon translation in the cytoplasm, Tat reaches the nucleus where it is responsible for the trans-activation of viral RNA transcription and enhancing the processivity of transcribing

RNA polymerases. Under some conditions, Tat increases the production of viral mRNAs up to 100-fold being essential for viral replication.

The Rev protein is important for the regulation of viral gene expression. Unspliced viral genomic RNA and single spliced mRNAs are exported from the nucleus into the cytoplasm where translation of the structural genes and remaining non-structural genes will take place. Rev is important in this switch because it overcomes the default pathway in which mRNAs are spliced prior to nuclear export and functions by binding to the RRE site located in the Env coding region. This will promote the export of viral-containing mRNAs from the nucleus. There is evidence that entry into the splicing pathway may also be important for Rev function because mutating 5' splice sites on RRE-containing mRNAs eliminates Rev activity. Furthermore, Rev can directly inhibit splicing by preventing entry of additional snRNPs during the later stages of spliceosome assembly (Kjems J, Sharp PA. 1993).

The HIV Nef, like Tat and Rev, is expressed early in the HIV life-cycle. It's an N-terminally myristoylated protein that is involved in reducing the levels of cellular CD4 by facilitating the routing of CD4 from the cell surface and Golgi apparatus to lysossomes. This process results in receptor degradation and prevents inappropriate interactions with Env. By down-regulating CD4, Nef facilitates Env incorporation into the virions, promotes particle release and possibly affects $CD4^+$ T-cell signalling pathways (Mangasarian A, Trono D. 1997). Nef contains a consensus SH3 domain binding sequence (PXXP) that mediates binding to several Src-family proteins such as, Src, Lyn, Hck, LcK, and Fyn, indicating an involvement in the regulation of tyrosine kinase activities. These interactions appear to be important for enhancing viral infectivity but not for downregulating CD4 (Greenway A and McPhee D. 1997; Moarefi I. *et al.*, 1997; Benichou S. *et al.*, 1997).

It was also shown that Nef is involved in the down-regulation of MHC class I molecules ([103] Le Gall S, Heard JM, Schwartz O. 1997), which may help protect infected cells from killing by cytotoxic T cells (Le Gall S. *et al.*, 1997). Nef mutant viruses also exhibit decreased levels of viral DNA synthesis following infection (Guatelli JC. 1997). This defect can be overcome if Nef is supplied in *trans* in virus-producing cells but not in target cells, suggesting possible roles in virus assembly, maturation, or entry. Such roles are consistent with the observation that approximately 70 Nef molecules are incorporated *per* virion; these virion-associated proteins are cleaved by PR at residue 57 to generate a soluble C-terminal fragment (Guatelli JC. 1997). The mechanism of Nef incorporation has not been defined but is probably relatively nonspecific, because Nef can also be incorporated into Mo-MLV particles (Bukovsky A. *et al.*, 1997).

### 1.5. Structural proteins

The structural proteins Gag, Pol, and Env are translated in the form of polyprotein precursors that, with exception of Env, are processed by the viral-encoded protease. Gag is initially expressed into a polyprotein precursor of 55 kDa that upon cleavage by viral protease yields the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6. The Env polyprecursor is cleaved by viral protease and originates the surface protein or gp120 (SU), and the transmembrane or gp41 protein (TM). These proteins are structural components that make up the core of the virion and outer membrane envelope. The three Pol proteins, PR, RT, and IN, provide essential enzymatic functions and are also encapsidated within the particle.

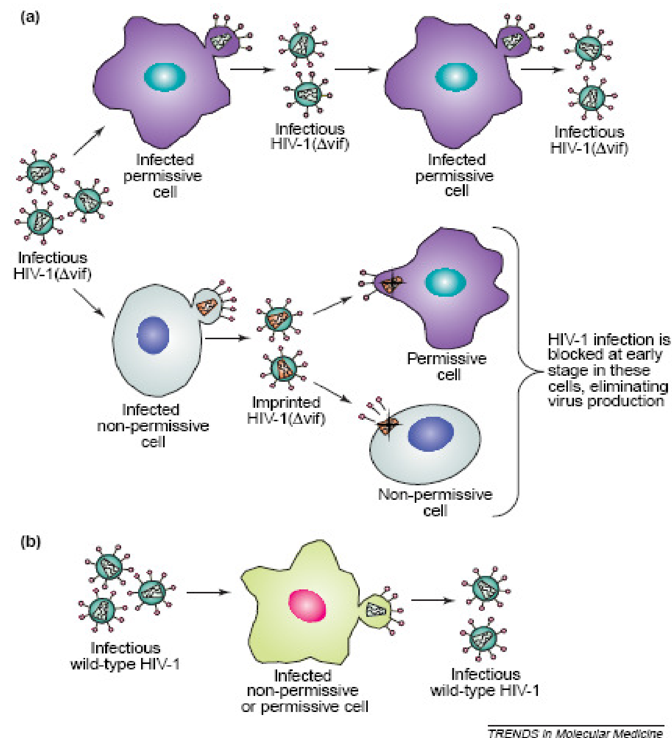
## 2. The HIV-1 Viral Infectivity Factor (Vif) protein

### 2.1. The viral Vif protein

"Vif" stands for Viral Infectivity Factor and it is a 23-kDa, primarily cytoplasmic protein that is expressed from a partially spliced mRNA in a Rev-dependent manner in the late phase of HIV-1 replication (Trono D. *et al.*, 1995 1995; Emerman M. *et al.*, 1998). Vif was originally discovered in the mid 1980s and was initially called *sor* (Fisher A. G. *et al.*, 1987; Strebel K. *et al.*, 1987). It is found among all the lentiviruses with exception of equine infectious anemia virus. Vif was named after the first functional studies demonstrated that virions generated in the absence of this protein were 1,000 times less efficient in establishing an infection (Fisher, A. G. *et al*, 1987; Strebel, K. *et al.*, 1987). Cell-to cell transmission was also reported to require Vif activity (Fisher, A. G. *et al*, 1987) and several studies demonstrated that the requirement for Vif was cell type dependent (Fan L., and K. Peden. 1992; Gabuzda D. *et al.*, 1992; Sakai H. *et al.*, 1993; Sova P., and D. J. Volsky, 1993; Von Schwedler *et al.*, 1993). HIV-1ΔVif viruses are able to replicate in most $CD4^+$ cell lines (permissive cells) although in other cell such as, macrophages, H9, and peripheral blood mononuclear cells (PBMCs) (nonpermissive) these mutants are unable to replicate. Many studies showed that when ΔVif and wild-type viruses were produced in semipermissive cells, no quantitative difference was observed during the first

replication cycle up to virus production, inclusive. However, ΔVif virus progeny was strongly restricted for the pre-retrotranscription steps, indicating a severe impairment in the construction of the viral particle in the absence of Vif (Sova, P. and D. J. Volsky, 1993; Von Schwedler *et al.*, 1993; Marianne C. *et al.*, 1995; Borman, A. M. *et al.*, 1995). Also, Vif defective virions, when produced from nonpermissive cells, have an abnormal protein profile (Sakai H. *et al.*, 1993; Borman A. M. *et al.*, 1995) and an altered ultrastructural morphology (Borman A. M. *et al.*, 1995; Hoglund S. *et al.*, 1994). The cellular specificity of Vif function could theoretically have two possible explanations: (I) permissive cells might express a protein that functions similarly to Vif to enhance HIV-1 infectivity 50-100 fold (Madani N. *et al.*, 1998; Gabuzda D. *et al.*, 1992; Simon J. H. *et al.*, 1998; Sheehy A. M. *et al.*, 2002), such that the viral Vif is unnecessary, or (II) nonpermissive cells might contain a potent inhibitor of HIV-1 that is neutralized by Vif (Rose M. K. *et al.*, 2004).

A major clue to the solution of this enigma came in 1998 by two independent groups, Madani and Kabat and Simon *et al.*, which discovered that in somatic cell fusion experiments, heterokaryons formed by the fusion of permissive and non-permissive cells were found to be non-permissive (Madani N. *et al.*, 1998; Simon JH *et al.*, 1998). These findings suggested the presence of an endogenous cellular factor that would confer a non-permissive phenotype to the cell that was overcome by the viral Vif protein. The cellular specificity of HIV-1 Vif protein is represented in the figure below (Fig. 4 a, b).




*(Figure from: Kristine M. Rose et al., 2004)*

**Fig. 4. Schematic representation of the cell-specific replication behaviour of ΔVif and Wt HIV-1 Virions.** **(a)** In the absence of Vif (HIV-1 ΔVif), infectious viral particles produced in permissive cells are able to infect permissive and non-permissive cells. Although, progeny virions from infected non-permissive cell that appear to have a normal protein and genomic RNA content, are unable to successfully complete proviral DNA synthesis in the next cycle of infection, regardless of whether the target cells are permissive or non-permissive. **(b)** In the presence of Vif (HIV wt) infectious viral particles are able to infect permissive and non-permissive cells and produce infectious particles that are able to complete the next round of infection, regardless of cell type.

### 2.2. Structural motifs important for Vif function

The HIV-1 Vif protein contain two conserved motifs $S^{144}LQXLA^{149}$ and $S^{23}L(I/V)X4YX9Y^{40}$, which are common among all lentivirus (Oberst and Gonda, 1992), and two basic motifs $W^{89}RKKR^{93}$ and $P^{156}KKIKP^{161}$ (Huvent I. *et al.*, 1998). In addition, there are two well conserved antigenic sequences: $I^{87}EWRKKRY^{94}$ and $D^{172}RWNKPQ^{178}$ (Wieland U. *et al.*, 1991). The $W^{89}RKKR^{93}$ motif is homologous to the nuclear localization sequence (NLS) of SV40 T antigen (Huvent I. *et al.*, 1998). The Vif molecule also contains two cysteines, at positions 114 and 133, which are extremely well conserved among HIV-1, HIV-2, and in several SIV isolates (Ma X. *et al.*, 1994). Some authors have demonstrated that both residues are required for Vif function (Ma X. *et al.*, 1994), whereas others showed that $Cys^{133}$ is not essential to HIV-1 infectivity in non-permissive cells (Sakai K. *et al.*, 1999).

Vif is phosphorylated *in vivo* and *in vitro* at residues $Ser^{144}$, $Thr^{155}$, and $Thr^{188}$ by serine/threonine protein kinases (Yang X. *et al.*, 1996) and at residues $Thr^{96}$ and $Ser^{165}$ by p44/42 mitogen-activated protein kinase (MAPK) (Yang X. and Gabuzda D., 1998). Mutations of the $Ser^{144}$ and $Thr^{96}$ residues result in impairment of Vif activity and inhibition of viral replication (Yang X. and Gabuzda D., 1998; Yang X. and Gabuzda D., 1999; Jacqué JM. *et al.*, 1998). However, it remains unclear whether these mutations interfere with the primary structure of the protein or instead, lead to loss of phosphorylation.

Studies using Vif mutants showed that Vif does not tolerate more than five or six amino acid deletions (with exception to the carboxyl-terminal truncations) without loss of its function. Also, Vif substitutions of amino acids distributed throughout the protein lead to disruption of its function.

Finally, the proline-rich C-terminal region of Vif (amino acids 151-164) is essential for multimerization (Yang *et al.*, 2001). Deletions in this region of Vif lead to viruses that are replicative- defective, indicating an important role of this region for HIV-1 replication (Yang *et al.*, 2001).

### 2.3. Cellular localization

Vif is predominantly a cytoplasmic protein, which can exist associated with the cellular membrane. However, Vif is not an integral part of the membrane but it's binding might involve membrane-associated proteins (Michaels FH. *Et al*., 1993; Goncalves J. *et al*., 1994). Association of Vif with the cytoplasmic site of cellular membranes requires basic residues that are located at the C-terminal region of the protein (amino acids 171-192) (Goncalves J. *et al*., 1995). Thus, this region appears to be essential for a stable association with the cellular membranes and for Vif function during viral replication (Goncalves J. *et al*., 1995). Vif can also exist as a soluble cytosolic protein. A small portion of Vif protein can also be found in the nucleus of the cell. Although, the specific role of Vif inside the nucleus remains to be elucidated. Vif can also be found associated with microvesicles present into the extracellular culture medium (M K Karczewski and K Strebel., 1996).

### 2.4. Vif incorporation into HIV-1 viral particles

The issue of Vif incorporation into viral particles has always been controversial. Initial reports suggested that the amount of Vif protein packaged into virions was estimated to be on the order of 1 molecule of Vif for every 20 to 30 molecules of p24, or between 60 and 100 molecules of Vif *per* viral particle (Liu, H. *et al*., 1995). Some authors reported that Vif was involved in the late stages of viral assembly and maturation (Gabuzda, D. *et al*., 1992; Hoglund, S. *et al*., 1994; Sakai, H. *et al*., 1993; Simm, M. *et al*., 1995; von Schwedler, U. *et al*., 1993). In addition, it was also demonstrated that Vif colocalizes or interacts with Gag (Bouyac, M. *et al*., 1997; Huvent, I. *et al*., 1998; Simon, J. *et al*., 1997). In contrast, some authors referred that virions released from infected cells contained a small amount or no Vif (Camaur, D., and D. Trono., **1996;** Dettenhofer, M., and X. F. Yu., **1999;** Simon, J. *et al*., 1998).

This apparent contradiction may have been resolved by Kao *et al*. who reported that Vif was efficiently packaged into virions from acutely infected cells (60 to 100 copies per virion), while packaging into virions from chronically infected H9 cells was near the limit of detection (4 to 6 copies per virion) (Kao *et al*., 2003). In addition, Kao *et al*. demonstrated that *de novo* synthesis of cytoplasmic Vif was rapidly translocated to a

detergent-resistant cellular compartment, where it was accumulated in a packaging-resistant form (Kao *et al.*, 2003).

Sucrose density gradients have showed that Vif cosediments with capsid proteins even after detergent treatment of virus preparations, suggesting that Vif associates with the inner core of HIV particles (M K Karczewski and K Strebel., 1996; **Liu, H. *et al.*, 1995**). These results indicate that Vif represents an integral component of HIV and SIV particles and raise the possibility that it plays a direct role in early replication events. Further, Khan *et al*. demonstrated that packaging of Vif was dependent on the packaging of viral genomic RNA in both permissive and restrictive HIV-1 target cells (Khan A. *et al.*, 2001). In addition, mutations in the nucleocapsid zinc finger domains abrogated packaging of viral genomic RNA and abolished packaging of Vif (Khan A. *et al.*, 2001). Thus, Vif incorporation into virions seems to be dependent on the zinc-finger domain of NC protein, which is responsible for viral RNA packaging and it is also dependent on the RNA packaging signal of the viral genomic RNA.

In contrast, Sova *et al*. demonstrated that deletion of the nucleocapsid (NC) domain does not affect Vif incorporation, and that Vif are also incorporated into the virus-like particles (VLPs) **(Sova P. *et al.*, 2001)**.

Despite controversy surrounding the Vif incorporation issue, it is now widely accepted that Vif is specifically packaged into viral particles through interactions with the viral genomic RNA, co-packaged cellular RNAs and the nucleocapsid (NC) domain of Gag (Svarovskaia ES. *Et al.*, 2004; Khan A. *et al.*, 2001; Dettenhofer M. *et al.*, 2000; Zhang H. *et al.*, 2000; Bouyac M. *et al.*, 1997; Simon JH. *Et al.*, 1999; Henriet S. *et al.*, 2005). Vif defective viruses produced from non-permissive cells display defects not only on viral assembly events but also during post-entry steps of infection, resulting in a failure to complete reverse transcription and integration (Chowdhury IH. *Et al.*, 1996; Courcoul M. *et al.*, 1995; Gonçalves J. *et al.*, 1996; Simon JH. And Malim M., 1996; Von Schwedler *et al.*, 1993; Carr JM *et al.*, 2006). Moreover, viral particles produced in the absence of Vif show structural defects such as aberrant core morphology and reduced stability (Simon JH, Malim MH. *Et al.*, 1996; Ohagen A, and Gabuzda D., 2000; Hoglund, S., *et al.*, 1994).

### 2.5. Interaction of Vif with Gag precursor

Vif interacts with the cytoplasmic side of plasma membrane, an association mediated by intrinsic membrane components and the C-terminal domain of Vif (Goncalves *et al.*, 1994, 1995; Simon *et al.*, 1997). Vif was shown to directly interact with the HIV-1 pr55Gag precursor using *in vitro* GST systems, *in vitro* translated proteins, and *in vivo* in the context of infected cells, as well as in transfected cells co-expressing Gag and Vif (Bouyac M. *et al.*, 1997). In contrast, co-immunoprecipitation assays from HIV-1 infected cells did not find a direct association between Vif and Gag, suggesting that Vif and Gag may associate through a protein mediator (Henzler T. *et al.*, 2001). Indeed, Zimmerman *et al*. demonstrated that the host cell protein, HP68, specifically interacts with Gag and Gag-Pol precursors after translation in HIV-1 infected cells promoting their assembly into immature capsids (Zimmerman C. *et al.*, 2002). In addition, HP68-Gag and HP68-Gag-Pol complexes were also found to contain Vif molecules indicating its pivotal role during virus assembly (Zimmerman C. *et al.*, 2002).

The affinity of Vif for RNA was shown to decrease in the presence of Gag precursors, suggesting a displacement and exchange of RNA-bound proteins during genome packaging (Zhang *et al.*, 2000). Strebel and co-workers showed that virus-associated Vif had the ability to interact with Gag precursor molecules and to modulate Gag maturation (Akari H. *et al.*, 2004).

Despite some controversy, it is now usually accepted that Vif and pr55Gag co-localize in the cytoplasm (Simon JH. *et al.*, 1999) and mutations in the NC domain of pr55Gag significantly reduce Vif packaging. In addition, Vif also associates with the HIV-1 genomic RNA *in vitro* and *in vivo* (Dettenhofer, M. *et al.*, 2000; Khan, M. *et al.*, 2001). Vif binds the viral RNA in the cytoplasm of infected cells, forming a 40S mRNP complex that most likely mediates viral RNA interactions with HIV-1 $pr55^{Gag}$ (Zhang H. *et al.*, 2000). Finally, RNA binding properties of Vif and its association with the nucleoprotein complex during virus assembly seems to be crucial for efficient virions assembly and subsequent optimization of viral particles morphology (Hoglund H. *et al.*, 1994).

### 2.6. Interaction of Vif with viral Envelope Glycoproteins

Initial studies proposed that Vif could target envelope glycoproteins, mediating the processing of the C-terminus of gp41 protein (Guy B. *et al.*, 1991) or the incorporation of gp120 glycoprotein (Sakai H. *et al.*, 1993; Borman AM. *et al.*, 1995).

Despite these initial studies, it was demonstrated that Vif does not influence virus binding/entry and virion incorporation of Env. It is generally accepted that Vif enhances HIV-1 infectivity at the post-entry step(s) independently of the Env function (Akari H. *et al.*, 1999; **Camaur, D., and D. Trono,** 1996).

### 2.7. Interaction of Vif with viral Protease (PR)

Protease-dependent proteolytic processing of Vif was demonstrated both *in vitro* and *in vivo* (Khan M. *et al.*, 2002). *In vivo* processing of Vif is cell type independent and is restricted to cell-free virus preparations (Khan M. *et al.*, 2002). Vif is incorporated into the virion where it will be proteolytic processed by the viral PR originating a 17-KDa and a 7-KDa processing sub-products (Khan M. *et al.*, 2002; Baraz L. *et al.*, 2002). The processing site in Vif is mapped to $Ala_{150}$, which is located in a highly conserved domain among HIV-1, HIV-1, and SIV Vif isolates (Khan M. *et al.*, 2002). Mutations in Vif that have an effect on proteolytic processing also impair Vif function (Khan M. *et al.*, 2002). This seems to indicate a pivotal rule of the intravirion processing of Vif protein for the production of infectious viruses (Khan M. *et al.*, 2002). Vif inhibits the autoprocessing of truncated HIV-1 Gag-Pol polyproteins expressed in bacterial cells (Kotler M. *et al.*, 1997), and interacts with the N-terminal domain of HIV-1 PR through its central region. This was demonstrated by Baraz L. *et al.*, which showed that peptides derived from two regions of N-Vif encompassing residues $Tyr^{30}Val^{65}$ and $Asp^{78}Val^{98}$, inhibited PR activity both *in vitro* and *in vivo* (Baraz L *et al.*, 1998). Later, in 2004, Kotler and co-workers showed that peptides derived from the N-terminal region of PR abrogate Vif function in non-permissive cells (Hutoran M. *et al.*, 2004).

## 2.8. The role of Vif in Reverse Transcription

Reverse transcription in HIV infected cells occurs in a nucleoprotein complex termed the reverse transcription complex (RTC). RTCs containing RT and integrase activity are heterogeneous in size and density on sucrose velocity and equilibrium gradients (Carr JM. *et al*, 2006). RTCs from ΔVif virus produced in non-permissive cells display a reduction in the major RTC form and more of the reverse transcription products in rapidly sedimenting structures (Carr JM. *et al*, 2006). ΔVif virions can assemble in permissive cells and infect non-permissive cells and initiate the process of reverse transcription, but this process fails to complete (Frankel, A. D. and Young, J. A., 1998). This Vif-dependent infectivity is manifested only in primary lymphocytes and macrophages and in a limited number of T-cell lines (Gabuzda, D. H. *et al*., 1994; Inubushi, R. and Adachi, A., 1999).

Analysis of HIV-1 ΔVif reverse transcription process in non-permissive cells showed a specific block in the synthesis of minus- and plus-strand viral DNA products, being uncapable to produce full-length viral DNA genomes (Trono D., 1992; Goncalves J. *et al*., 1996; Nascimbeni M. *et al*., 1998). The Vif protein was found to modulate the RNA- and DNA-dependent DNA synthesis activity of the viral reverse transcriptase in two ways: (i) by stimulating the binding of the $tRNA^{lys}$ primer on the viral template, affecting the initiation of reverse transcription, (ii) by increasing the polymerization rate of HIV-1 RT (Reynel C. *et al*., 2004). Vif has been shown to bind HIV-1 RNA (Dettenhofer, M. *et al*., 2000) and to form multimers *in vivo* through a self-association domain located at its C-terminus (Yang, S. *et al*., 2001). This domain was also found to be essential for the Vif-dependent stimulation of HIV-1 infectivity. Reynel *et al*. showed that a Vif mutant lacking the C-terminal 56 amino acids failed to stimulate reverse transcription. Conversely, another Vif mutant lacking the N-terminal 43 amino acids, which are involved in RNA binding and interaction with the viral protease, was able to stimulate RT activity. In addition, Vif was found to promote the bypass of an abasic site by HIV-1 RT (Reynel C. *et al*., 2004). Despite all these findings, it remains unclear whether Vif plays a direct role in reverse transcription. Recently, Henriet *et al*. have showed that Vif does play a role during the early phase of reverse transcription. This activity is performed in coordination with other components of the viral core such as Gag and its maturation products and during the dimerization of viral genomic RNA (Henriet S. *et al*., 2007). In addition, Henriet *et al*. raised the possibility that Vif might be a temporal regulator during viral assembly, suggesting a model in which Vif interacts with genomic RNA and NC-derived products preventing RNA

dimerization/packaging and premature initiation of reverse transcription. Vif, together with Gag precursors, may also promote the placement of $tRNA^{Lys3}$ on to the PBS, stabilizing NC intermediates to increase the efficiency of the early steps of reverse transcription (Henriet S. *et al.*, 2007).

## 3. Vif and APOBEC3G (A3G)

### 3.1. The discovery of A3G as an anti-retroviral factor

Since identification of HIV-1 in the early 1980`s (Barre-Sinoussi, F. *et al.*, 1983), scientists have only been able to grow the virus in a sub-set of immortalized human T-cell lines, termed “permissive”. The reason why some cell-lines were permissive for HIV-1 replication whereas others did not support a productive viral growth (nonpermissive) remained unclear for many years until the function of the HIV-1 Vif protein was unveiled. HIV-1 Vif protein has a major role in HIV-1 replication in all of its *in vivo* cellular targets (nonpermissive cells), and Vif defective viruses (HIV-1ΔVif) are unable to replicate in some cell lines but grow unhindered in others. These findings were made over 15 years ago but the molecular mechanism underlying this cell-specific difference was maintained a mystery until 1998, when heterokaryon experiments suggested that Vif acted to overcome an inhibitory host cell factor (Madani N. *et al.*, 1998; Simon JH *et al.*, 1998). Later, in 2002, Malim and co-workers (Sheehy A.M. *et al.*, 2002) reported the identification of the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) as the HIV-1 replication inhibitor. APOBEC3G (A3G), initially named CEM15, is a member of the cytidine deaminase family of nucleic acid-editing enzymes, with an unknown physiological role in normal cells. A3G protein is present mainly in lymphocytes and monocytes (Jarmuz A. *et al.*, 2002). However, A3G mRNA can also be found in the thymus, suggesting that it may be induced late in the maturation of T cells (Mariani R. *et al.*, 2003).

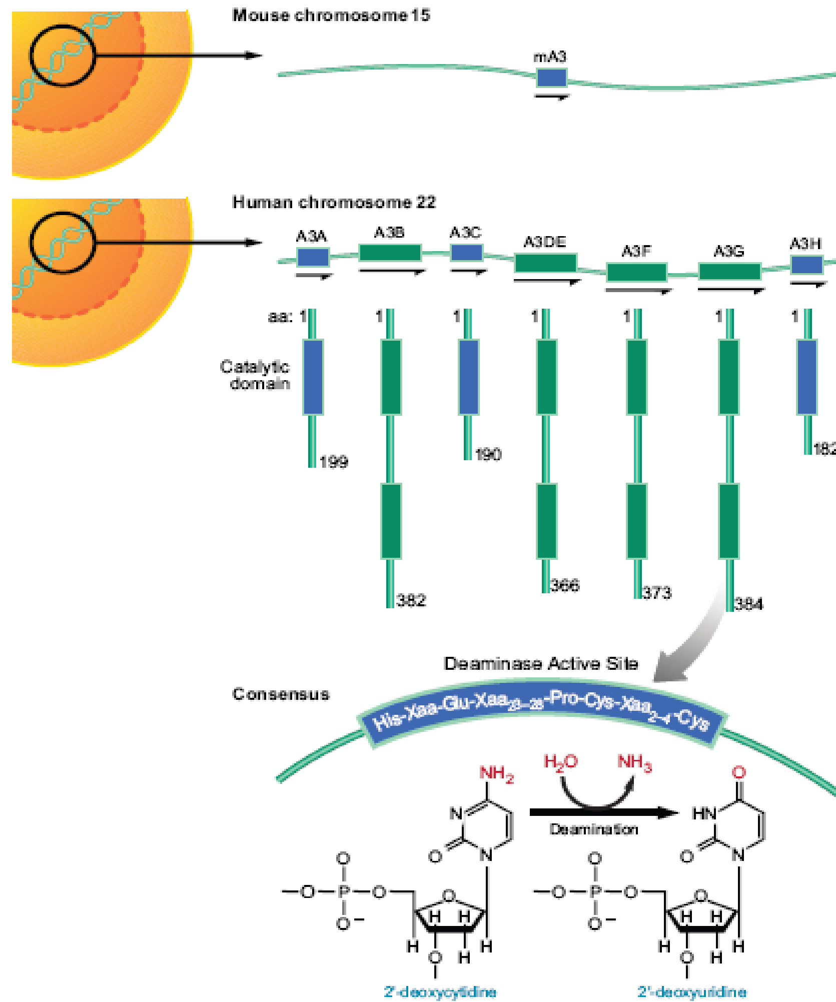
### 3.1.1. APOBEC3G family and evolution

A3G belongs to a family of polynucleotide cytidine deaminases (CDAs), whose members include APOBEC1 (A1), APOBEC2 (A2), APOBEC4 (A4), which edit apolipoprotein B mRNA and generate a premature stop codon; activation induced deaminase (AID), which is expressed in B cells and is needed for somatic hypermutation and class switch recombination during antibody gene diversification, and seven other related genes (Conticello *et al.*, 2005; Harris and Liddament, 2004; Jarmuz *et al.*, 2002; Rogozin *et al.*, 2005). A3G and other APOBEC3 (A3) family members are encoded by genes clustered in chromosome 22, which have evolved from a single gene in mice to eight genes (A3A-H) in primates during mammalian evolution (Jarmuz A. *et al.*, 2002; Conticello SG. *et al. et al.*, 2005(a)). Surprisingly, expansion of the APOBEC3 gene cluster (Jarmuz A. *et al.*, 2002; Conticello SG *et al.*, 2005 (b)) contrasts with the decline in retro-transposon activity in primates (Maksakova IA *et al.*, 2006) raising the possibility that these genes may have evolved to prevent genomic instability caused by endogenous retro-elements (Sawyer SL *et al.*, 2004).

Endogenous retro-elements in mammals are mobile through retro-transposition, an intracellular process that involves reverse transcription. They include autonomous long interspersed nucleotide elements (LINEs), non-autonomous short interspersed nucleotide elements, and elements with long terminal repeats, such as endogenous retrovirus. It is likely that they have played an important role in genome evolution as they occur in high copy number in ancestral genomes (Kazazian HHJ, 2004). However, it is still a mystery whether A3G and other APOBEC3 proteins can target or edit other cellular sequences that are unrelated to viral elements and how these activities are regulated in the cell. In this regard, it is of great importance to identify potential cellular factors that may be involved in A3G regulation or contribute to its cellular and antiviral functions.

CDAs catalyze the deamination of cytosine to uracil in the DNA and/or RNA strands, affecting multiple physiological functions. All members of the APOBEC protein family contain one or two copies of a His/Cys-X-Glu-$X_{23\text{-}28}$-Pro-Cys-$X_{2\text{-}4}$-Cys coordination motif that is characteristic of CDAs. These proteins also contain a key glutamate required for proton shuttling during catalysis and two key aromatic residues involved in RNA substrate binding (Bennett *et al.*, 2006; Huthoff and Malim, 2005; Lehmann *et al.*, 2007; Smith *et al.*, 2004; Teng *et al.*, 1993; Wedekind *et al.*, 2003, 2006). A1 was the first CDA to be identified and is involved in lipid

metabolism (Teng *et al.*, 1993). A1 is expressed in human gastrointestinal tissues and specifically edits C6666 in apolipoprotein B (APOB) mRNA to generate a truncated functional form of APOB (Lee *et al.*, 2004; Teng *et al.*, 1993). AID is expressed in B cells and is essential for both somatic hypermutation and class switch recombination during antibody gene diversification through C-to-U editing of DNA at the immunoglobulin loci (Di Noia and Neuberger, 2007; Di Noia *et al.*, 2007; Eto *et al.*, 2003; Harris *et al.*, 2002; Lee *et al.*, 2004; Rada *et al.*, 2004). These processes are an integral part of the DNA-level modifications that drive maturation of the vertebrate antibody response to pathogens. A2 is expressed most prominently in cardiac and skeletal muscle and is regulated by NF-*k* -B in human hepatocytes (Liao *et al.*, 1999; Matsumoto *et al.*, 2006; Prochnow *et al.*, 2007). A4, which is a new member of APOBEC family, is expressed primarily in the testis (Rogozin *et al.*, 2005). However, physiological functions of A2 and A4 are still unknown. A3s are capable not only of retroviral defenses but are also active against mobile genetic elements (Bogerd *et al.*, 2006a,b; Esnault *et al.*, 2006; Kinomoto *et al.*, 2007; Muckenfuss *et al.*, 2006; Niewiadomska *et al.*, 2007; Stenglein and Harris, 2006). A3G, A3F and A3C are expressed in many tissues, including spleen, peripheral blood lymphocytes, ovary and testis (Hill *et al.*, 2006; Peng *et al.*, 2006; Pido-Lopez *et al.*, 2007; Sheehy *et al.*, 2002; Stopak *et al.*, 2007; Ying *et al.*, 2007). However, little or no A3B mRNA is detectable in these tissues and it is more prevalent in various cancer cells. A3A is highly expressed in immature monocytes (Peng *et al.*, 2007).




*(Figure from: Ya-Lin Chiu and Warner Greene, Annu. Rev. Immunol. 2008)*

**Fig. 5. Domain organization of A3 proteins and their relative locations on the human chromosome 22.** A3 proteins have evolved from one gene in mice to eight genes (A3A-H) in primates. A3B, A3D, A3E, A3F, and A3G exhibit additional duplications of the cytidine deaminase domain (CDA), the intervening linker and pseudoactive domains. CDA motifs harbour one histidine and two cysteine residues, and the glutamic acid residue that are involved in the hydrolytic deamination of cytosine. A3G, followed by A3F, displays the most potent effects against HIV. Other A3 family members can inhibit weakly the wild-type HIV (A3B and A3D/E). The presence of two cytidine deaminase domains seems to be important for the antiretroviral activity of APOBEC enzymes, as none of the deaminase members bearing only one catalytic domain have shown inhibitory effects on HIV-1.

### 3.1.2. APOBEC3G cellular localization

A3G is located in the cytoplasm of the cell and has been shown to be an exclusive DNA mutator (Suspene R, *et al.*, 2004). A3G exists has either an enzimatically active low-molecular-mass (LMM) form consistent in size with monomers or dimmers of the enzyme, or as an enzimatically inactive high-molecular-mass (HMM) ribonucleoprotein complex larger than 2 MDa (Chiu YL. *Et al.*, 2005). LMM A3G is encountered in resting CD4 T cells and monocytes where it acts as a powerful antiviral restriction factor for HIV-1 (Chiu YL. *Et al.*, 2005). Conversely, resting $CD4^{+}$ T cells in lymphoid tissues and macrophages are permissive to HIV-1 infection as A3G is expressed predominantly in HMM complexes due to the lymphoid microenvironment (Kreisberg JF *et al.*, 2006). In lymphoid tissues, cytokines such as IL-2 and IL-5 are responsible for the stimulation of HMM complexes, which in turn will confer the permissive phenotype for HIV-1 infection (Kreisberg JF *et al.*, 2006). These findings may explain the reason why A3G does not attack reverse transcripts from incoming viruses in activated CD4+ T cells and macrophages.

A3G has been shown to localize throughout the cytoplasm and to concentrate within punctuate cytoplasmic bodies (Wichroski,M.J. *et al.*, 2005), which were found to be mRNA processing (P) bodies (Wichroski M.J. *et al.*, 2006). P-bodies are specialized compartments found in the cytoplasm of yeast and mammalian cells where nontranslating mRNAs accumulate and are subject to degradation or storage (Cougot N. *et al.*, 2004; Sheth U. *et al.*, 2003; Teixeira D. *et al.*, 2005). In addition to P-bodies, biochemical studies carried out by Wichroski *et al*. (Wichroski M.J. *et al.*, 2006) showed evidence that APOBEC3G localizes to a ribonucleoprotein (RNP) complex with other P-body proteins which are involved in *cap*-dependent translation (Andrei MA. *Et al.*, 2005), translation suppression (Minshal N. *et al.*, 2001; Coller J. and Parker R., 2005), RNA interference-mediated post-transcription gene silencing (Lian A. S. *et al.*, 2005; Liu J. *et al.*, 2005 (a); Liu J *et al.*, 2005 (b); Meister G. *et al.*, 2005; Pillai R. S. *et al.*, 2005; Rehwinkel J. *et al.*, 2005; Sen G. L. *et al.*, 2005) and mRNA decapping (Cougot N *et al.*, 2004; Sheth U. *et al.*, 2003; Coller J. Parker R, 2004).

A3G recruitment to HMM RNP complexes leads to inhibition of its enzymatic activity. However, *in vitro* treatment with RNase (Chelico, L. *et al.*, 2006; Chiu, Y. L. *et al.*, 2005) has been shown to lead to complex disassembly suggesting that the recruitment of A3G to large RNP complexes may be a mechanism of enzymatic activity regulation.

In order to identify potential cellular regulatory mechanisms for controlling A3G function, Malim and co-workers have used tandem-affinity purification (TAP) to

identify proteins that interact with A3G, and confocal microscopy to examine sub-cellular localization and relative proximities of these proteins in intact cells (Gallois-Montbrun S. *et al.*, 2006). Interestingly, a number of RNA binding proteins were found to interact with A3G suggesting an indirect interaction through common RNA molecules (e.g. PABPC1, HuR, YB-1, and the hmRNP proteins) as treatment with RNase resulted in the loss of many of these interactions (Gallois-Montbrun S. *et al.*, 2006). Some of these proteins are involved in mRNA biogenesis and metabolism, whereas other proteins such as Ro60 and La are known to interact with some RNA Polymerase III transcripts (Wolin, S.L., and Cedervall T., 2002; Wolin, S. L., and Steitz J. A., 1983). RNA binding proteins such as, PABPC1, RNA helicase A and hmRNP U that were identified to interact with A3G have previously been implicated in various steps in the HIV-1 life cycle (Afonina, E. *et al.*, 1997; Hartman, T. R. *et al.*, 2006; Valente, S. T. and Goff S. P., 2006), although it still remains to be addressed whether these proteins can influence the antiviral activities of A3G.

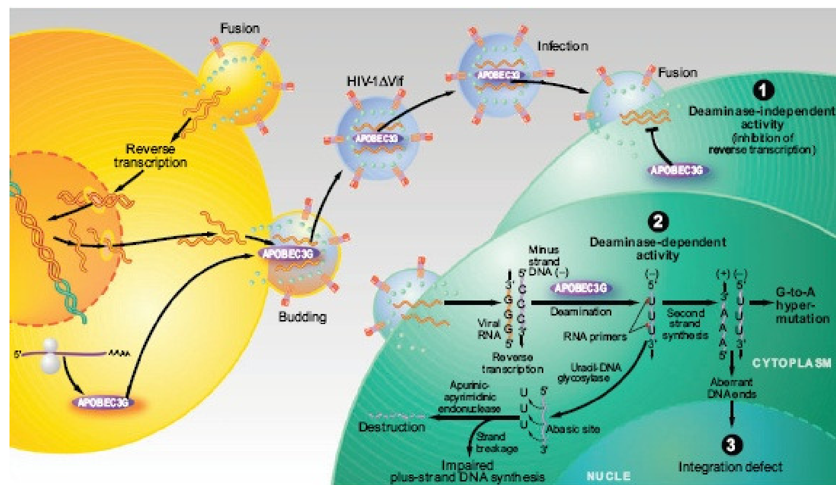
Localization studies (Gallois-Montbrun S. *et al.*, 2006) revealed that A3G is not only dispersed throughout the cytosol but is also concentrated in cytoplasmic P-bodies interacting with other constituent proteins, which is in agreement with the work developed by Wichroski *et al.* (Wichroski M.J. *et al.*, 2005; Wichroski M. J. *et al.*, 2006). Although, in contrast to the work developed by Wichroski *et al.*, not all the interactions with A3G were RNase sensitive reflecting the importance of direct protein-protein contact in complex formation (Gallois-Montbrun S., *et al.*, 2006). Malim and co-workers also reported that heat-shock induces redistribution of A3G and certain P-body proteins towards other RNP-rich cytoplasmic microdomains termed stress granules (SGs) (Gallois-Montbrun S., *et al.*, 2006).

### 3.2. HIV-1 restriction by A3G and A3F proteins

The antiviral activity of A3G is suppressed in the presence of HIV-1 Vif protein, which prevents its packaging into assembly virus particles through protein degradation in the proteasome (Marin M. *et al.*, 2003; Sheehy AM. *Et al.*, 2003; Stopak *et al.*, 2003). In the absence of Vif (HIV-1ΔVif), A3G is recruited into budding virions through its interaction with the nucleocapsid (NC) component of the Gag Polyprotein and/or HIV genomic RNA (Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. L. and Greene W. C., 2006).

In non-permissive cells, A3G is incorporated into the progeny ΔVif viruses and will block infection in the next target cell by deamination dependent and deamination independent mechanisms (Fig. 6). HIV-1 WT viruses encoding an active Vif protein successfully replicate in non-permissive cells in the presence of A3G because Vif mediates proteasome degradation of A3G and it is able to replicate in both permissive and non-permissive cells. However, Vif may also inhibit A3G activity through mechanisms independent of proteasomal degradation (Kao S. *et al.*, 2003; Kao S. *et al.*, 2004; Mehle A. *et al.*, 2004b; Santa-Marta M. *et al.*, 2005; Stopack K. *et al.*, 2003), as it will be discussed later in this section.

More recently, two other members of the APOBEC family, APOBEC3F (A3F) and to a lesser degree, APOBEC3B (A3B), were reported to have similar antiviral activities (Bishop *et al.*, 2004; Liddament *et al.*, 2004; Zheng *et al.*, 2004).




*(Figure from: Ya-Lin Chiu and Warner Greene, Annu. Rev. Immunol. 2008)*

**Fig. 6. Schematic representation of the multifaceted antiviral actions of virion incorporated A3G.** In the absence of Vif, A3G present in the cytoplasm of HIV-1 producer cells is incorporated into budding virions. A3G incorporated into viral particles will then be carried to the next target cell where it will be released and act as a potent of HIV-1 replication. Two independent mechanisms of HIV-1 inhibition are represented here: the deamination-independent mechanism (1), and the deamination-dependent mechanism of HIV-1 restriction (2).

### 3.2.1. A3G incorporation into HIV-1 Virus particles

During the HIV-1ΔVif deficient replication, A3G associates with the nucleocapsid (NC) component of the Gag Polyprotein and/or HIV genomic RNA during viral assembly and is packaged into progeny virions RNA (Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. L. and Greene W. C., 2006). Nevertheless, some authors have reported that viral genomic RNA is not necessary for A3G incorporation into virions and that binding of A3G to RNA is nonspecific (Cen S. *et al.*, 2004; Navarro F. *et al.*, 2005). The interaction between A3G and the NC domain of HIV Gag is essential for this process and was demonstrated by *in vitro* GST pull-down experiments (Alce T. M. *et al.*, 2004; Douaisi M. *et al.*, 2004), coimmunoprecipitation assays (Cen S. *et al.*, 2004; Luo K. *et al.*, 2004; Luo K *et al.*, 2004; Zennou V *et al.*, 2004), and *in vitro* translated binding assay (Schafer A, *et al.*, 2004). A3G interacts with the first 11 amino acids of the N-terminal domain of NC on the membrane of the producer cell and incorporates into budding virions (Cen S. *et al.*, 2004; Luo K. *et al.*, 2004; Luo K *et al.*, 2004; Zennou V *et al.*, 2004).

#### 3.2.1.1. Intravirion A3G complexes

In activated, virus producing T cells, A3G resides in HMM RNP complexes of 5 to 15 MDa. A different situation occurs in resting CD4 T cells and monocytes, where it acts as a powerful antiviral restriction factor for HIV-1 (Chiu Y.L. *et al.*, 2005).

It is widely agreed that to be able to exert its antiviral activity, A3G needs to be incorporated into the budding viral particles. However, the molecular form of A3G that is actually incorporated into virions remained unclear for some time. Recently, the work developed by Soros *et al*. demonstrated that newly-synthesized A3G (LMM) is incorporated into the viral particles, and not the pre-existing HMM A3G (Soros V. B. *et al.*, 2007). Pulse-chase radiolabeling and biochemical fractionation studies showed that A3G is initially LMM and is recruited within 30 min into stable cellular HMM RNA-protein complexes. In less than 90 minutes, newly synthesized A3G is preferentially incorporated into virions, whereas “mature” A3G, already assembled into HMM complexes, is apparently less available for virion incorporation (Soros V. B. *et al.*, 2007). In addition, Fast Protein Liquid Chromatography (FPLC) analysis of virion lysates containing A3G revealed the presence of large RNA-protein complexes, distinct from

the cellular HMM complexes, which contained A3G (Soros V. B. *et al.*, 2007). This finding was surprising since it was thought that A3G present into viral particles would remain in an enzymatically active LMM form as it deaminates the viral minus-strand DNA in the target cell. Instead, A3G incorporated into virions assembles into large RNA-protein complexes distinct from the cellular HMM complexes. In this large intravirion A3G complex (IVAC) the enzymatic activity of A3G is negatively regulated by RNA binding, indicating that A3G is incorporated into virions as a large enzymatic-latent RNP complex (Soros V. B. *et al.*, 2007). The enzymatic activity of A3G was shown to be restored by addition of RNase H, indicating that HIV-1 RNase H may play a central role in triggering the activity of virion associated A3G (Soros V. B. *et al.*, 2007). Finally, as a consequence of this work, two mechanisms of A3G activity were proposed: (I) enzymatically inactive A3G bound to HIV RNA may impair the generation of minus-strand DNA by blocking movement of RT on its viral RNA template; and (II) minus-strand DNA is eventually generated setting the stage for enzyme-dependent antiviral action of A3G. RNase H degrades viral RNA allowing the A3G to deaminate the minus-strand DNA (Soros V. B. *et al.*, 2007)

Several mechanisms of action were also proposed for Vif protein, which include: (I) Vif targeting of newly LMM A3G (unbound to RNA); (II) Vif targeting ribosome-associated A3G, and degrading newly LMM A3G; or (III) Vif targeting the RNA-associated A3G (Soros V. B. *et al.*, 2007). Indeed, such scenario is consistent with the observation that Vif partially inhibits the synthesis of A3G (Stopak K. *et al.*, 2003; Kao S. *et al.*, 2003).

#### 3.2.1.2. Deamination-dependent mechanism of HIV-1 restriction

Once packaged, A3G acts as a potent inhibitor of HIV-1 infectivity by a mechanism that results in genome degradation, incomplete cDNA synthesis, and a detrimentally high mutation rate within the HIV-1 genome, which has been largely attributed to deamination of the viral cDNA [Fig. 5 (2)] (Mariani R. *et al.*, 2003; Goff SP 2003; Gu Y. and Sundquist WI. 2003; Harris R. S. *et al.*, 2002; Klarman G. J. *et al.*, 2003; Mangeat B. *et al.*, 2003). Viral A3G targets ssDNA for degradation by deamination of deoxycytidines (dC) converting them into deoxyuridines (dU) primarily at dCdC sites (Cullen BR. 2006; Mangeat B. and Trono D. 2005; Chiu YL. and Greene WC. 2006). The presence of dU residues may initiate non-template DNA repair by uracil DNA

glycosylase (Yu B. Q. *et al.*, 2005) in order to eliminate the unwanted uracil residues. Glycosylase action will then produce abasic sites, which are targeted by host cell endonucleases for strand cleavage leading ultimately to degradation of minus ssDNA abrogating the HIV life cycle. However, this process is apparently imperfect as some deaminated minus-strand ssDNA can escape degradation and serve as a template for plus-strand synthesis. Due to the presence of deoxyuridines instead of deoxycytidines, massive dG→dA hypermutations are introduced into the plus strand viral DNA, compromising HIV production.

#### 3.2.1.3. Deamination-independent mechanism of HIV-1 restriction

Recently, two independent studies have indicated that A3G antiviral function can be dissociated from its enzymatic activity, suggesting a distinct mechanism for HIV-1 restriction (Chiu Y. L. *et al.*, 2005; Newman E. N. *et al.*, 2005). The study carried by Newman and colleagues showed that certain amino acid substitutions in the C-terminal cytidine deaminase “core” domain of A3G (A3G has two such domains) originate mutant proteins that are unable to mutate DNA, yet maintained the antiviral activity (Newman E. N. *et al.*, 2005). On the other hand, when unstimulated CD4 T cells (where A3G is expressed as an active LMM form), are treated with A3G-specific small interfering RNAs (siRNA) the early replication block encountered by HIV-1 was greatly relieved (Chiu Y. *et al.*, 2005). When HIV-1 reverse transcripts formed in resting primary $CD4^+$ T cells from blood were examined for evidence of A3G-induced dG→dA hypermutations only 8% of the transcripts contained such mutations suggesting an antiretroviral activity independent of deoxycytidines deaminase activity (Chiu Y. L. *et al.*, 2005). Studies carried out by Iwatani and colleagues indicated that initiation of HIV reverse transcription and/or processivity of reverse transcriptase (RT) could be inhibited by A3G (Iwatani *et al.*, 2007). Furthermore, other groups showed that the CDA-deficient A3 proteins reduce the accumulation of viral reverse transcription products in target cells, and this effect is most obvious for A3F proteins (Bishop *et al.*, 2006; Holmes *et al.*, 2007). However, CDA independent effects cannot be observed when the mutant proteins are expressed at physiological levels (Miyagi, E. *et al.*, 2007), making these non-enzymatic A3 effects on RT controversial.

Recently, Judith Levi and co-workers demonstrated that A3G inhibits all RT-catalyzed DNA elongation reactions, with no effect on RNase H activity or NC's ability to promote tRNA annealing (Iwatani *et al.*, 2007). They reported that A3G inhibits (−) ssDNA and (+) ssDNA synthesis, minus and plus-strand cDNA transfer, and the elongation of minus and plus strand DNAs (Fig. 7). Also, Guo *et al*. showed that the reduction of early reverse transcripts in A3G-expressing cells infected with HIV-1 Vif is correlated with decreased tRNA priming *in vitro* (Guo *et al.*, 2006). These studies indicate that the presence of A3G results in the formation of aberrant viral 3'-long terminal repeat (LTR) DNA ends, and suggest that A3G interferes with cleavage and removal of the tRNA primer.

Sequence analyses of 2-LTR circle junctions from unintegrated DNA synthesized in the presence of A3G showed that DNA at the U5 end sometimes had an additional six RNA bases derived from the 3'-terminus of tRNALys3 (Mbisa *et al.*, 2007). This result suggested that A3G causes a defect during tRNA removal that limits plus-strand transfer, resulting in viral DNA ends that are will not be able to efficiently integrate in the host cell DNA (Mbisa *et al.*, 2007). All this process of successive inhibition of reverse transcription has a cumulative effect and could explain the reduction of integrated viral DNA. However, the decrease in plus-strand DNA transfer does not account for all observed decreases in viral cDNA synthesis by A3G (Aguiar R. S. and Peterlin B. M., 2008). In parallel, A3G could be co-immunoprecipitated with NC and integrase (IN) in HIV-1 Vif-positive viruses (Luo *et al.*, 2007). Indeed, A3F co-immunoprecipitation with virion-associated integrase (IN) was observed. GST-pull down assays do not show binding between A3G and RT, suggesting that interactions between A3G and viral proteins may inhibit the process of reverse transcription (Iwatani *et al.*, 2007).

In summary, these studies strongly support additional mechanisms that could account for the observed CDA-independent A3G-mediated antiviral activity. Despite these studies, recent work using cell lines that express stably comparable amounts of both proteins suggests that the antiviral activity of CDA defective A3G is significantly lower than its wild-type counterpart (Miyagi *et al.*, 2007). Therefore, Miyagi *et al*. concluded that despite some A3G deaminase-independent effect on HIV-1 reverse transcription, efficient inhibition of Vif-defective HIV-1 requires catalytically active A3G (Miyagi *et al.*, 2007).

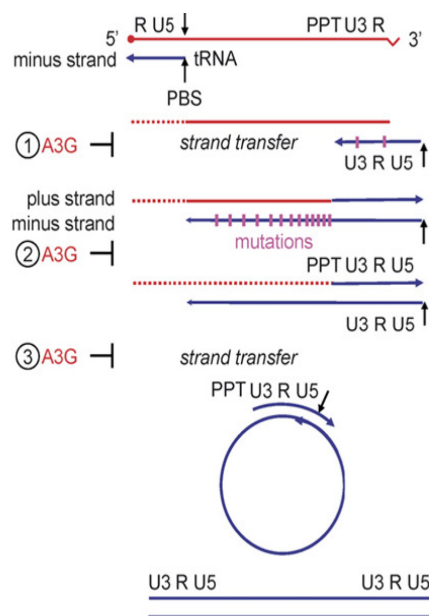



**Fig.7. Schematic representation of HIV-1 restriction mechanism independent of A3G deamination.** Negative strand DNA synthesis begins with tRNALys3 priming in primer-binding site (PBS). After the strong stop, the DNA is transferred to the 3-LTR and RNaseH degrades the viral RNA. RT copies the entire viral genome to the PBS. Some positive strand DNA synthesis occurs from the PPT. After additional RNase H digestion, the transcribed DNA anneals with the positive strand DNA, which completes the synthesis of double strand DNA. Pink dashes in the minus strand DNA denote areas of hypermutation (G-to-A) induced by the CDA activity of A3 proteins. However, A3G can interfere at different steps of reverse transcription reactions in a CDA independent manner. Thus, A3G inhibits (−) ssDNA and (+) ssDNA synthesis (represented as numbers (1) and (2)), minus- and the plus strand cDNA transfer (numbers (1) and (3)), as well as the elongation of minus and plus-strand DNAs. Also, A3G interferes with cleavage and removal of the tRNA primer (step (3)). These findings suggested that A3G causes a defect during tRNA removal that limits plus-strand transfer, resulting in viral DNA ends that are inefficient substrates for integration. (Aguiar R. S. and Peterlin B. M.. 2008).

### 3.3. How does Vif counteract A3G activity?

A3G needs to be incorporated into the HIV-1 budding virions in order to have access to the target cell and exert its antiviral action. A3G protein has an antiviral activity by introducing deleterious mutations in the viral minus strand DNA during reverse transcription and /or by impairing accumulation of its products.

The first indication of Vif function was the finding that in its presence, HIV-1 wild-type virions contained 100-fold less A3G than Vif defective virions and were >100-fold infectious (Mariani R. *et al.*, 2003; Marin M. *et al.*, 2003; Stopack *et al.*, 2003; Sheehy AM. *Et al.*, 2003; Mehle A. *et al.*, 2004b; Kao S. *et al.*, 2003). It soon became clear that Vif acts by depleting A3G from cells. Therefore, Vif counteracts A3G antiviral action both by inhibiting its synthesis (Stopak K. *et al.*, 2003) and stimulating its degradation by the 26S proteasome (Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. and Greene W., 2006). Consequently, A3G is no longer available for recruitment into the virions. The mechanism by which Vif stimulates A3G degradation in the 26S

proteasome has long been deciphered (Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. and Greene W., 2006). Vif binds A3G and induces its degradation by forming an E3 ubiquitin ligase complex with cullin 5 (Cul5), elongin B (EloB), and elongin C (EloC) that targets these proteins for degradation by the ubiquitin-proteasome pathway (Bogerd H. *et al*., 2004; Lui B. ert al., 2005; Marin M. *et al*., 2003; Mehle A. *et al*., 2004a, b; Sheehy *et al*., 2003; Stopack K., *et al*., 2003; Yu X. *et al*., 2003; Yu Y. *et al*., 2004).

### 3.3.1. Vif domains involved in the interaction with A3G.

The Vif-A3G interaction is of particularly interest in a way that it provides a compelling target for novel therapeutic strategies for treating HIV-1 infections. Disruption of A3G-Vif interaction is predicted to rescue A3G expression and virion packaging, therefore stimulating natural antiviral infectivity. Similarly, pharmacologic studies to suppress A3G proteasome-mediated degradation have been shown to enhance A3G expression and consequently inhibit HIV-1 infection (Yu, X. *et al*., 2003). In order to facilitate the rational design of inhibitors for A3G-Vif interaction, molecular biology experiments have been employed to define features of A3G that are involved in the interaction with Vif.

In order to protect against A3G, Vif uses multiple regions for protein interaction such as the N-terminal region, the SLQ(Y/F)LA motif and a highly conserved H-X5-C-$X^{17-18}$C- $X_{3-5}$-H motif upstream of the BC box. The N-terminal region of HIV-1 Vif is important for binding and neutralization of A3G and A3F and also contributes to species-specific recognition (Marin M, *et al*., 2003; Schrofelbauer, B. *et al*, 2006; Simon, V., 2005; Tian, C. *et al*, 2006; Indrani P. *et al*, 2006).

The highly conserved cysteine residues at positions 114 and 133 and the SLQ(Y/F)LA motif at residues 144 – 149 are required for Vif function and HIV-1 replication (Goncalves, J. *et al*., 1994; Ma, X. *et al*., 1994). In contrast, a deletion in the Vif region 142 to 154 that removes the SLQ(Y/F)LA motif shows a remarkably effect in reducing A3G levels and support virus replication. This suggests a relationship between the ability of Vif to reduce A3G levels and to support virus replication.

Vif associates with the Cul5-EloB-EloC complex by binding directly to EloC via a BC box motif at positions 144 to 153 and to Cul5 via hydrophobic residues at positions 120, 123, and 124 within a zinc-binding region (residues 100 to 142) formed by a conserved H-X5-C-X17–18C-X3–5-H (HCCH) motif (Mehle A. *et al*., 2004a; Mehle A. *et al*., 2006). The $S^{144}$ $LQXLA^{150}$

motif is essential for targeting A3G for proteasomal degradation. Substitution of the SLQ portion of the SLQXLA motif has been reported to be sufficient to prevent A3G degradation (Kobayashi, M. *et al.*, 2005; Sheehy AM. *et al.*, 2003; Yu, X. *et al.*, 2003). The zinc binding-motif HCCH has also been reported to be involved in A3G degradation and necessary for the specificity of Vif-Cullin 5 interaction (Luo, K. *et al.*, 2005; Xiau Z. *et al.*, 2006).

The species-specific recognition by Vif is determined by a single amino acid at position 128 in A3G, which is aspartic acid in humans and lysine in African green monkey (AGM) (Bogerd H. *et al.*, 2004; Mangeat B. *et al.*, 2004; Schrofelbauer B. *et al.*, 2004; Xu H. *et al.*, 2004). Substitution of D128 in human A3G for K128, found in African Green Monkey (AGM) A3G, results in a mutant (D128K-A3G) deaminase that is resistant to the effect of Vif. This may be either because the mutant protein is no longer able to interact with Vif or due to subsequent downstream steps that may be inhibited (Schrofelbauer, B. *et al.*, 2006; Bogerd, H. *et al.*, 2004; Mangeat, B. *et al.*, 2004; Xu, H *et al.*, 2004).

Mutations in the Vif conserved region $D^{14}RMR^{17}$ similarly alter its species-specific effect. Alteration of the Vif conserved sequence DRMR to SERQ or SEMQ, which is the sequence in $SIV_{AGM}$ Vif, causes Vif to interact with AGM A3G, rhesus (Rh) A3G, as well as D128K-A3G (Schrofelbauer B. *et al.*, 2006). This loss of species restriction is probably caused by an overall increase in the negative charge of amino acids 14-17 in HIV-1 Vif that promotes effective interaction with the positive charge of lysine present at residue 128 in AGM A3G and Rh A3G. The DRMR region, localized at the N-terminal domain of Vif is important for interaction with A3G (Schrofelbauer B. *et al.*, 2006). Although, additional binding sites may be required for stabilization of this interaction (Schrofelbauer B. *et al.*, 2006). Schrofelbauer *et al.*, have also reported the importance of the $D^{14}RMR^{17}$ in A3F inhibition and in the D128K-A3G Vif resistant mutant (Schrofelbauer B. *et al.*, 2006; Bogerd, H. *et al.*, 2004; Mangeat, B. *et al.*, 2004; Xu, H., *et al.*, 2004).

More recently, Pathak and co-workers have shown that the HIV-1 Vif region $Y^{40}RHHY^{44}$ is also important for binding to A3G and that a mutation in this region increases Vif ability to suppress A3F. Although, the region reported as important for A3F-Vif binding is $D^{14}RMR^{17}$**.** Pathak and co-workers showed that Vif binding to D128K-A3G region is dependent on the $Y^{40}RHHY^{44}$ but not the $D^{14}RMR^{17}$ region. Consistent with previous observations, they showed that substitution of Vif`s region $D^{14}RMR^{17}$ with SEMQ in Vif, similar to SERQ amino acids in $SIV_{AGM}$ Vif, is required for subsequent neutralization of

D128K-A3G. Therefore, $Y^{40}RHHY^{44}$ domain is necessary for binding to A3G and the $D^{14}RMR^{17}$ domain may be involved in a secondary step that is necessary for A3G degradation (Russell A. and Pathak V., 2007).

### 3.3.2. Mechanisms of Vif action not involving degradation of A3G.

Vif-mediated A3G degradation plays an important role in overcoming the antiviral action of A3G. However, Vif may also act by sequestering A3G from sites of virion assembly, impairing A3G incorporation into viral particles (Kao S. *et al.*, 2007, Mehle A. *et al.*, 2004b; Stopak K. *et al.*, 2003). Moreover, Vif has been reported to be associated with the plasma membrane, the site of virion assembly, localizing Vif in proximity to A3G (Simon JH *et al.*, 1997).

One study indicates that Vif can enhance virion infectivity under conditions where it only moderately reduces the steady-state levels of A3G (Kao S. *et al.*, 2004). In addition, the non-phosphorylated Vif S144A mutant leads to progeny virions with poor infectivity despite being able to effectively deplete A3G (Mehle A. *et al.*, 2004b). More recently, the work developed in Klaus Strebel group showed that Vif was able to inhibit virion encapsidation and the antiviral activity of an A3G degradation resistant mutant (C97A) (Opi S. *et al.*, 2007).

Two major mechanisms have been suggested to explain this alternative mechanism of Vif action: (I) physical exclusion of A3G from sites of virion assembly and budding; and/or (II) inhibition of A3G encapsidation by competing for binding to NC or viral genomic RNA (Mariani R. *et al.*, 2003; Opi S. *et al.*, 2007; Kao S. *et al.*, 2004).

Additionally, two studies have showed a possible link between Vif and Vpr accessory proteins and HIV-1 cytopathicity and cell cycle arrest. These findings were supported by Terry Finkel and co-workers (Wang J. *et al.*, 2007), which demonstrated that Vif contributes to the arrest of HIV-1 infected cells in the $G_2$ phase of the cell cycle. However, the mechanism by which Vif induces $G_2$ arrest or why HIV-1 uses two accessory proteins (Vif and Vpr) for the same purpose still remains to be explained. One possible explanation is the need for producing as much virus as possible in the short period that the infected cell is alive in order to ensure viral propagation. Cells arrested in the $G_2$

phase have shown to produce more virus (Goh, W.C. *et al.*, 1998), ultimately leading to cell death (Stewart, S. A. *et al.*, 1997; Yuan, H. *et al.*, 2003).

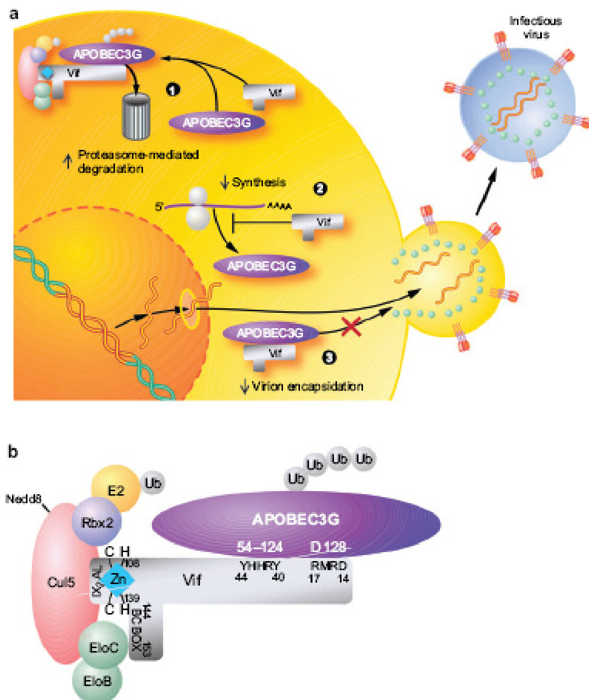



**Fig. 8. Schematic representation of Vif mediated inhibition of A3G. (a) (1), (b).** Vif binds A3G and promotes the coordinated activation of three classes of enzymes: E1, E2, and E3. E1 is the ubiquitin activating enzyme and is responsible for transferring ubiquitin to the E2 ubiquitin-conjugating enzyme. E3 is a multiprotein complex that specifically recognises the substrate and mediates its ubiquitin-dependent degradation. E3 is the ubiquitin ligase, which transfers ubiquitin to the substrate. Part of the E3 complex is composed by Vif, elongin B, elongin C, cullin-5 (CUL5) and RING-box-1 (RBX1). Vif binds A3G and targets the RING-type E3 complex to A3G promoting its ubiquitination by bringing the activated E2 into close proximity to the substrate. **(2)** Vif may also impair translation of A3G mRNA. **(3)** Vif action of sequestering A3G from sites of virion assembly impairs A3G incorporation into viral particles. *(Figure from: Ya-Lin Chiu and Warner Greene, Annu. Rev. Immunol. 2008)*

### 3.3.3. Additional Vif domains involved in the interaction with A3G.

The Thr[96], Ser[144], and Thr[188], are highly conserved phosphorylation sites in Vif. A mutation of Thr[96] or Ser[144], impairs but is not sufficient to abolish viral replication (Yang X. *et al*., 1996; Yang X. and Gabuzda D., 1998). The T96A, S144A, and T188A mutants retain the ability to decrease A3G levels (Mehle A. *et al*., 2004b), indicating that some mutations that reduce Vif function during HIV-1 replication still retain the ability to decrease A3G levels.

The central hydrophilic domain, E[88]WRKKR[93], and the proline-rich P[161]PLP[164] domain, have been shown to be important for enhancing the steady-state levels of Vif and for binding to tyrosine kinases, respectively (Douaisi M., *et al*., 2005; Fujita M., *et al*., 2003). One may speculate on the E[88]WRKKR[93] motif function for maintaining sufficient levels of Vif for A3G inhibition. Mutations in the Vif PPLP motif were shown to reduce the infectivity of virions produced in T-cells and eliminate Vif-Vif interaction *in vitro* (Yang *et al*., 2001). It has been suggested that multimerization of Vif may be necessary for A3G binding and that the PPLP region may be essential for this behaviour (Miller J. H. *et al*., 2007). In addition, this region has also been identified as part of a novel "SOCS-box" motif implicated in binding to EloC (Mehle A. *et al*., 2004 a; Yu Y. *et al*., 2004) and to be involved in the Vif interaction with the cellular Hck tyrosine kinase (Hassaine G. *et al*., 2001; Yang B. *et al*., 2003). Recently, Donahue J. *et al*. demonstrated that mutation of the Vif PPLP motif impaired the ability to bind to A3G, and did not affect EloC and Cullin5 binding (Donahue J. *et al*., 2008).

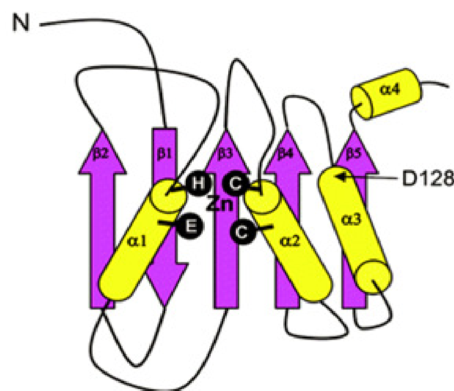
Other studies have identified other regions in the Vif protein that are responsible for A3G and A3F inhibition but are not located in the SLQXLA and HCCH motifs. As an example, Simon *et al*., reported that any single amino acid substitutions in Vif sequences isolated from HIV-1 infected patients were sufficient to prevent AG3 neutralization (Simon V., *et al*., 2005). Also, a subset of mutants has been reported to be functional against A3G but not A3F and vice versa (Russel R. and Pathak V., 2007).

### 3.4 Structural domains involved in A3G functionality

All members of the APOBEC protein family have one or two copies of His/Cys-X-Glu-X $_{23\text{-}28}$-Pro-Cys-X$_{2\text{-}4}$-Cys coordination motif that is characteristic of CDAs. As mentioned before, A3B, A3D, A3E, A3F, and A3G contain duplications of the CDA domain.

Although, the two CDA domains are not equivalent, as only the C-terminal CDA motif displays deaminase activity (Holmes, R.K. *et al.*, 2007; Newman E.N. *et al.*, 2005).

Cristal structures of CDAs from *Escherichia coli* (Betts *et al.*, 1994), *Bacillus subtilis* (Johansson *et al.*, 2002), *Saccharomyces cerevisiae* (Xie *et al.*, 2004) and most recently humans (Chung *et al.*, 2005) are available. These structures offer an opportunity to perform homology modelling and help to determine common trends and differences among the organization of APOBEC family members. Analysis of these structures revealed a core β1β2α1β3α2β4β5 arrangement in which the five β-strands form a mixed sheet that supports the parallel positioning of two α-helices that contain the His, Cys and Glu residues that are required for zinc-coordination, proton transfer and catalysis (Wedekind *et al.*, 2003). Based on these structures the model for APOBEC proteins was predicted (Huthoff H. and Malim M., 2005) and is shown in Fig.9.



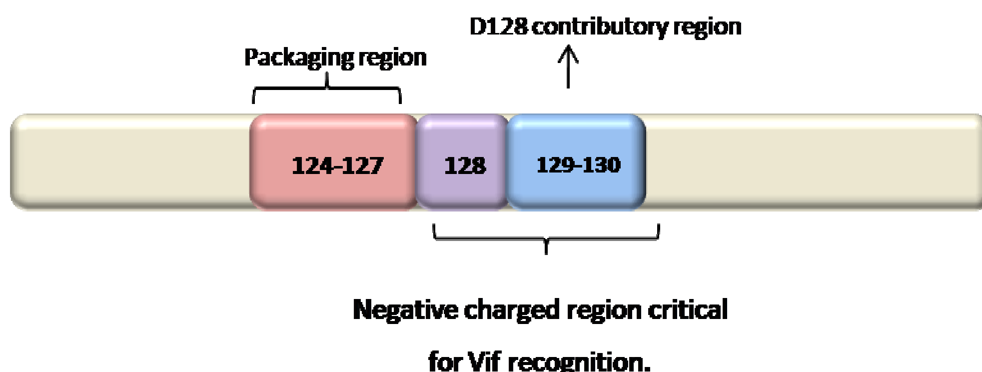

*(Figure from: Huthoff H. and Malim M., J. Virology, 2007)*

**Fig. 9. Model of the N-terminal CDA domain of A3G.** The predicted positions of α-strands (yellow) and β-strands (purple) are indicated. The amino acid D128, responsible for the species-specific recognition of A3G by Vif is indicated. The Zinc-coordinating motifs are indicated by dark circles.

Substitution of the aspartic residue (D128), which naturally occurs in human A3G with the lysine residue found in AGM A3G abrogates the interaction with HIV-1 Vif and confers sensitivity to the $SIV_{AGM}$ Vif protein. Experiments, by alanine-scaning and multiple substitutions at the A3G residues P129 and D130 confirmed the central role

played by the aspartic acid at position 128 and showed the crucial role of proline-129 and aspartic acid-130, as important contributory residues (Huthoff H. and Malim MH. 2007) (Fig. 10).

In addition, a four amino-acid sequence comprising residues 124 to 127 was shown to be responsible for an efficient A3G incorporation into HIV-1 virions (Huthoff H. and Malim MH. 2007) (Fig. 10).




*(Adapted from Hendrik Huthoff and Michael Malim, J.V, 2007)*

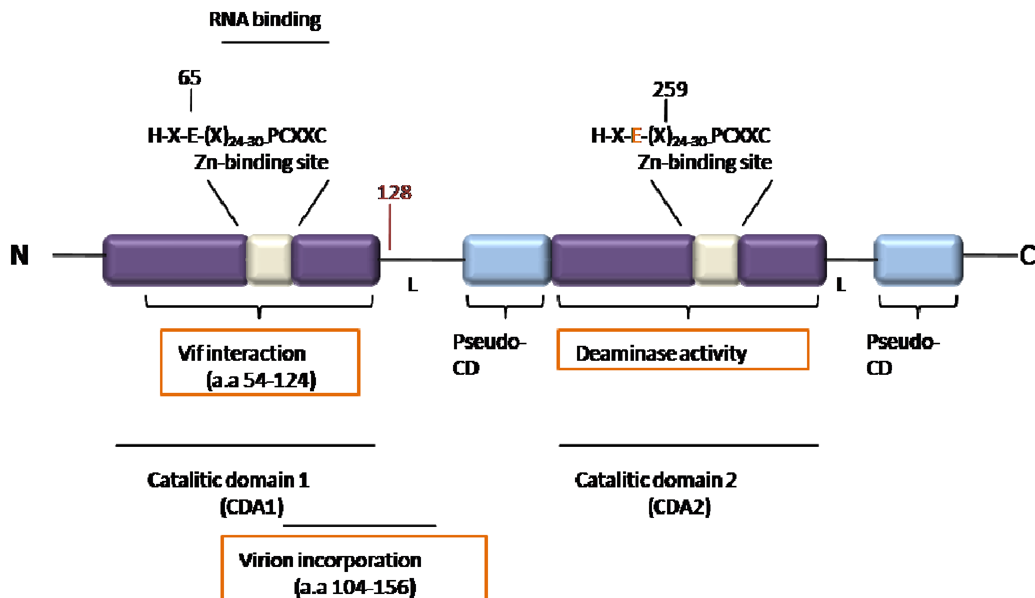
**Fig. 10. Schematic representation of amino acids involved in Vif recognition of A3G and in virion incorporation.** The amino acid region 124 to 127 is strongly conserved among A3G proteins from different species and is involved in A3G packaging into HIV-1 virions. Alanine substitutions in this region generated proteins with reduced level of antiviral activity against HIV-1ΔVif. The presence of tryptophan in position 127 was shown to be particularly important for the maintenance of A3G antiviral activity (Huthoff H. and Malim M, 2007). The negative charge of D128 and D130 region appears to be crucial for an efficient Vif recognition.

Several groups have shown that Vif-induced degradation of A3G requires their physical interaction of the two proteins and that a single amino acid change in A3G at residue 128 was sufficient to abolish this interaction (Bogerd, H. P. *et al.*,2004; Mangeat, B. *et al.*, 2004; Schrofelbauer, B. *et al.*, 2004). Amino acid substitutions in the region 128 to 130 by amino acids carrying

side chains with different properties indicated that the interaction between Vif and A3G is largely determined by electrostatic interactions involving residues 128 and 130 (Huthoff H. and MalimM., 2007). Substitution of proline in residue 129 to alanine or glycine displayed a strong Vif-resistant phenotype indicating that a specific structural interaction is also required for an efficient inhibition of A3G by Vif (Huthoff H. and MalimM., 2007). , In addition to electrostatic determinants, one study reported that A3G residues 54 to 124 were sufficient to coimmunoprecipitate with Vif, which suggests the presence of additional interaction domains between Vif and A3G (Conticello SG. *Et al*.,2003). Interestingly, two research groups have shown that HIV-2 and $SIV_{MAC}$ Vif proteins were able to mediate degradation of both wild-type A3G and the D128K mutant (Gaddis N.C. *et al*., 2004; Xu, H. *et al*., 2004). This observation indicates that Vif proteins have different requirements for functional interactions with A3G.

Vif neutralization of A3F requires different amino acids regions in Vif, indicating that the interaction between Vif and A3F is different from the interaction with A3G (Simon V. *et al*., 2005; Tian C. *et al*., 2006). In fact, A3F contains an ERD motif instead of the DPD motif found in A3G. The DPD motif, found at residues 128 to 130 in A3G, is involved in Vif-response and was shown to be unique of this protein (Huthoff H. and Malim M., 2007).

A3G is recruited into budding virions through its interaction with the NC component of the Gag polyprotein and nonspecific RNA binding (Cen S. *et al*., 2004; Navarro F. *et al*., 2005; Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. L. and Greene W. C., 2006). A3G proteins, with mutations at positions 124, 125 and 126 were shown to interact with HIV-1 Gag in a RNA-dependent manner (Doehle B. P. *et al*.,2005; Zennou V. *et al*., 2004). However, the mutant W127A did not show this property (Huthoff H. and Malim M., 2007). This observation by Huthoff H. and Malim M. suggests that Gag-A3G-RNA interaction cannot be the sole responsible for A3G packaging. It is possible that a spatial proximity between A3G and the membrane budding sites also may be a contributing factor for A3G packaging. Therefore, it is conceivable that this motif may be involved in determining the correct subcellular localization of A3G (Huthoff H. and Malim M., 2007). In addition, it is possible that the motif encompassing amino acids 124 to 127 of A3G may bind to an as-yet-unidentified molecular partner that may specify virus incorporation (Huthoff H. and Malim M., 2007). A schematic representation of A3G structure involved in recognition of Vif is indicated in figure 11.



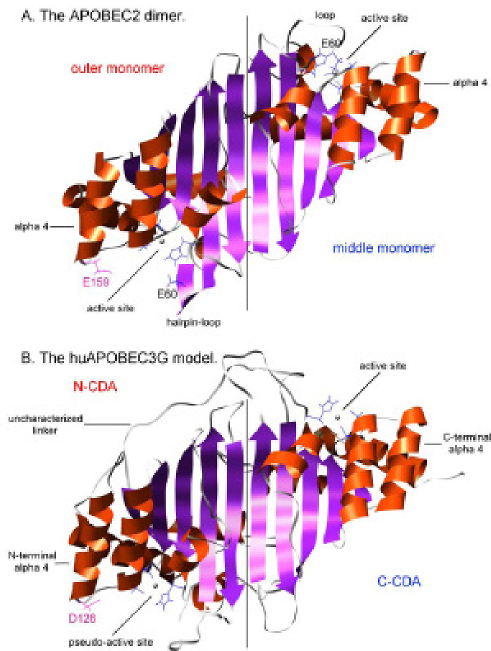

*(Adapted from: Melanie Kremer and Barbara S. Schnierle, Curr. HIV. Res. 2005)*

**Fig. 11. Structural domains of A3G involved in the interaction with Vif and in virion incorporation.** A3G has two CDA domains, the catalytically inactive amino-terminal domain (CDA 1) is responsible for the interaction with Vif and viral incorporation; the C-terminal domain (CDA 2) is responsible for the catalytic activity of the protein.

### 3.4.1 Model structure of A3G based on the crystal structure of APOBEC2 (A2).

Based on the crystal structure of A2 (Prochnow C. *et al.*, 2006), the N-terminal domain of A3G was predicted by theoretical methods (Zhang K. L. *et al.*, 2007). While Huthoff H. and Malim M. provided a genetic analysis of the A3G N-terminal residues 119 to 146 (as described above) (Huthoff H. and Malim M., 2007), Zhang K. L. *et al.* analysed 48 of the 194 residues of the N-CDA domain (Zhang K. L. *et al.*, 2007). The model developed by Zhang K. L. *et al.*, was based on a sequence identity of 27%, which should be satisfactory in its global fold as supported by the good ANOLEA energy score profile (Zhang K. L. *et al.*, 2007). This latest analysis confirmed the results obtained by the first authors and extended it. They

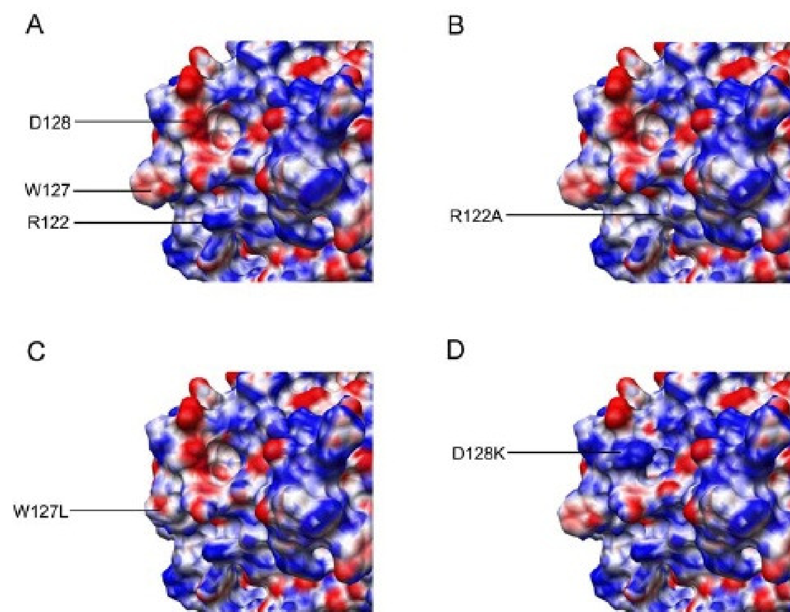
defined residues 124 to 127 of A3G as having a role in its viral packaging, and extended it to include residue R122. Residues 128 to 130 were confirmed as crucial for the interaction with Vif. However, in this analysis, D130 mutants did not display a Vif-resistant phenotype.




*(Figure from Zhang K. L. et al, PLoS ONE.2(4):e378, 2007)*

**Fig. 12. Structure of A2 dimer and the predicted model of A3G. (A)** A2 protein is composed of two outer monomers and two middle monomers. Modelling of the A3G N-CDA domain was based on the outer monomer of A2 in order to resolve the structure between residues 22 and 33 of A3G. (B) The resulting A3G model displays a very similar pattern to the A2 dimer. The active and pseudo active sites represented here were based on the corresponding homologous active site of A2.

As previously shown, amino acid substitutions in residue P129 displayed a strong Vif-resistant phenotype and this region was indicated as being important for a structural interaction with Vif (Huthoff H. and MalimM., 2007). In the study developed by Zhang K. L. *et al.*, this region was not tested but its biological relevance was highlighted by its inclusion in a patch of residues under positive selective pressure in primates, together with D128 (Zhang K. L. *et al.*, 2007).




*(Figure from Zhang K. L. et al, PLoS ONE.2(4):e378, 2007)*

Fig. 13. Representation of surface charge changes and structure alterations on A3G model. Mutational analysis of 48 out of 194 residues of A3G N-CDA showed that residues R122, W127 and D128 form a cluster at the surface of the protein and contribute to changes in surface charge or structure. (A) Model of A3G wild-type N-CDA region. (B-D) Effect of mutations R122A, W127L, and D128K on surface charge and structure. The red colour represents negative potential, the blue colour represents positive potential, and the white colour expresses zero potential (Zhang K. L. *et al.*, 2007).

As it can be seen in the figure above, mutations in amino acids R122, W127 and D128 modified the shape and the charge of the protein indicating that those differences in this region are the molecular basis for disruption of A3G interaction with Vif and packaging into HIV-1 virions.

Detailed models of the A3G structure are important for a rational drug design. Pharmacological inhibition of the Vif-A3G interaction appears to one of the best therapeutic approaches, as disruption of this interaction would enable A3G to be free to exert its antiviral action in the cell.

## Aims of the Study

The aim of this dissertation is to study the structure-function relationship of HIV-1 Vif protein and to determine how its function is regulated in the cell. In order to achieve this, we focused mainly in understanding the cellular interplay between Vif and one of its targets, the endogenous antiretroviral factor: A3G.

To achieve this goal we conducted three research projects that are described in detail in chapters 2, 3, and 4 of this thesis.

The main objectives are summarized below:

**Chapter 2:** the objective is to understand how A3G half-life can influence its antiviral activity, both at the level of viral encapsidation and catalytic activity in the cell.

**Chapter 3:** the objective is to study the *in vivo* interaction of Vif-A3G using a novel approach based on oligomerization assisted by the interaction between two GCN4 leucine zipper domains. We want to approach the degree of importance of Vif C-terminal region for an efficient degradation of A3G, and to ascertain on the topology of Vif-A3G interaction.

**Chapter 4:** the objective is to use a Protein Complementation Assay (PCA) to study specific domains that may have a key role in Vif-A3G interaction. Furthermore, we also want to study Vif interaction with others members of APOBEC family, namely: APOBEC3F, APOBEC3C, and APOBEC2.

# CHAPTER 2

## Modelling APOBEC3G intracellular steady-state shows a differential inhibition of HIV-1ΔVif.

**Iris Cadima-Couto**[1], Acilino Freitas-Vieira[1], Roni Nowarski[2], Elena Britan-Rosich[2], Moshe Kotler[2], and João Gonçalves[1]. (2008). Modelling APOBEC3G intracellular steady-state shows a differential inhibition of HIV-1ΔVif. *Journal of Biological Chemistry. (Submited)*.

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## ABSTRACT

The human APOBEC3G (A3G) is a potent inhibitor of HIV-1 replication and its antiviral activity is suppressed in the presence of HIV-1 Vif protein. One of the mechanisms by which Vif overcomes A3G, is by reducing A3G intracellular expression levels recruiting it for polyubiquitination and proteasomal degradation in the 26S proteasome.

In this study, we used a system that allowed us to insert different A3G N-terminal residues resulting in different steady-states according to the identity of the amino acid used. By modelling the intracellular half-life of A3G, we showed that A3G can also be targeted by the ubiquitin proteolytic system in a Vif-independent manner. Furthermore, our results revealed that A3G variants with a half-life of approximately 13 minutes were still able to incorporate into viral particles, whereas less than 5 minutes old A3G variants were not. Interestingly, in an *in vitro* deamination assay, all A3G variants displayed similar catalytic activity, despite the differences of their intracellular steady-state. This indicates that newly synthesized A3G, with less than five minutes of life, is already active in the cell. These findings showed us the importance of the A3G steady-state levels for the maintenance of its antiretroviral activity and point out that modelling A3G steady-state can predict how protein half-life can influence the antiviral activity of the deaminase, both at the level of its catalytic activity in the cell and viral encapsidation.

## INTRODUCTION

The human apolipoprotein B mRNA-editing enzyme, APOBEC3G (A3G), is a potent antiretroviral enzyme that, when incorporated into HIV-1 virions, is associated with the hypermutation of viral DNA through cytidine deamination of newly synthesized reverse transcripts (Mariani R. et al, 2003; Stopak K. et al, 2003; Soros V., et al, 2007; Xu H et al, 2007; Yu Q. et al, 2004; Suspene R et al, 2004).. HIV-1 virus encodes a 23-KDa protein, Vif, which has been shown to counteract the antiviral action of A3G preventing this mutational onslaught. One of the mechanisms by which Vif counteracts the antiviral action of A3G is by stimulating its degradation by the 26S proteasome (Stopak K. *et al*., 2003; Marin M. *et al*., 2003; Sheehy AM. *et al*., 2003; Conticello SG. *et al*., 2003; Mehle A. *et al*., 2004; Yu X. *et al*., 2003). Consequently, A3G is no longer available for recruitment into the virions. Vif binds A3G through the activation of a coordinated action of three classes of enzymes: E1, E2, and E3. E1 is the ubiquitin (Ub) activating enzyme and is responsible for transferring ubiquitin to the E2 ubiquitin-conjugating enzyme. E3 is a multiprotein complex that acts as an ubiquitin ligase, which specifically recognises the substrate and mediates its ubiquitin-dependent degradation. The E3 complex is composed of Elongin B, Elongin C, Cullin-5 (CUL5), RING-box-2 (RBX2), and SOCS-box proteins (Vif), which binds to A3G and promotes its polyubiquitination on lysine residues (Conticello S. G. *et* al., 2003; Bogerd H. *et al*., 2004; Lui B. ert al., 2005; Marin M. *et al*., 2003; Mehle A. *et al*., 2004a, b; Sheehy *et al*., 2003; Stopack K., *et al*., 2003; Yu X. *et al*., 2003; Yu Y. *et al*., 2004).

It was recently proposed that Vif could function as a vehicle to transport A3G for proteasome degradation, but that required polyubiquitination of Vif instead of A3G (Dang Y. *et al*., 2008). Indeed, Vif is polyubiquitinated and subject to proteasome degradation (Akari H. *et al*., 2004; Mehle A. *et al*., 2004), and this reaction is catalyzed by the same Cul5-E3 ligase (Mehle A. *et al*., 2004). This raises the debate about the mechanism that Vif uses to destabilize A3G and take it for degradation in the proteasome.

Despite some controversy, the mechanism of action of A3G in the cell has always been studied in the context of the Vif protein and the later has always been addressed as the sole responsible for the degradation of A3G in the 26S proteasome.

In this study, we decided to model the intracellular half-life of A3G in order to better understand its mechanism of action. We used a novel approach that allowed us to introduce any desired residue at the amino-terminal domain of A3G originating a protein with a different half-life according to the identity of its N-terminal residue. This

system consists in linear Ub-protein fusions that are rapidly cleaved by Ub-specific proteases at the Ub-protein junction, making possible the production of otherwise identical proteins bearing different N-terminal residues.

This mechanism was first described by Varshavsky and co-workers and relates the intracellular half-life of a protein to the identity of its N-terminal residue (Bachmair A. *et al.*, 1986). The set of individual amino acids can be ordered according to the half-life that they confer to the protein when present at its amino-terminus and that is called the "N-end rule". Short-lived proteins usually contain destabilizing N-terminal residues that function as essential determinants of an N-terminal degradation signal, called N-degron (Bachmair A. *et al.*, 1986; Bachmair, A. and Varshavsky, A. 1989; Gonda D. *et al.*, 1989; Kwon Y. T *et al.*, 1998; Tasaki T. *et al.*, 2005; Varshavsky A., 1996). N-degrons can be created from a pre-N-degron through specific N-terminal modifications that will destabilize otherwise stable polypeptides. The selectivity of ubiquitylation is determined mainly by the E3 Ub ligase that recognises a degradation signal of the target protein leading to the conjugation of Ub, in the form of a multi-Ub chain, to one or more of the internal Lys residues. The resulting Ub-containing protein is then processively degraded by the 26S proteasome.

This pathway was first discovered in 1986, and was based on the observation that when a chimeric gene encoding an ubiquitin-beta-galactosidase fusion protein was expressed in the yeast *Saccharomyces cerevisiae*, ubiquitin was cleaved off the nascent fusion protein, yielding a deubiquitinated beta-galactosidase (β- gal) (Bachmair A. *et al.*, 1986). This cleavage was shown to take place at the Ub-β-gal junction, making it possible to expose different residues at the amino-termini of the otherwise identical β-gal proteins. The β-gal proteins produced displayed different half-lives *in vivo*, from more than 20 hours to less than 3 minutes, depending on the nature of the amino acid at the amino-terminus of β-gal (Bachmair A. *et al.*, 1986).

The aim of this study was to understand how A3G half-life can influence the antiviral activity, both at the level of viral encapsidation and catalytic activity in the cell. To achieve this, we used a panel of seven different N-terminal residues classified as primary (Arg, Phe and Leu), secondary (Asp), tertiary (Asn) and stabilizing (Met and Gly) residues, primary residues being the least stable. By using this strategy we were able to mimic the effect of Vif on A3G steady-state allowing us to monitor A3G intracellular activity in a Vif-free environment.

We showed evidence that newly synthesized A3G, with a half-life of approximately 13 minutes, is still able to incorporate into HIV viral particles. As previously showed by Soros V *et al*., newly synthesized A3G molecules are selectively incorporated into virions within 1.5 hours after synthesis (Soros V. *et al.*, 2007). Ours results showed that incorporation of A3G molecules occurs in a very short time after synthesis and that regardless of its half-life, A3G molecules are catalytically active in the very early stages after its synthesis. In addition, this shows evidence that Vif has to act at the very early stages of A3G life-time in order to avoid its incorporation into virions.

# EXPERIMENTAL PROCEDURES

### Plasmids

The full-length molecular clone of HIV-1 (pNL4.3) was obtained from the NIH AIDS Research and Reference Reagent Program (catalog number 114), and was used for the production of infectious virus. Vif defective molecular clone pNL4.3ΔVif (Karczewski, M. K., and K. Strebel. 1996) was used for the production of Vif-defective HIV-1 virus. pcDNA3.1-APOBEC3G-HA (A3G) (Stopak K *et al*, 2003) was used for the expression of C-terminally epitope-tagged human A3G proteins and was a kindly supplied by Dr. Strebel K.. pcDNA-HVif was obtained from the NIH AIDS Research and Reference Reagent Program (Nguyen, K. L., 2004 ). pcDNA3.1 Zeo(+) was obtained from the NIH (Catalog: 10077). pEGFP*IRES-Ub-X (where X stands for the different A3G N-terminal residues) cloning vector was used as the cloning vector for the subsequent cloning steps and was kindly supplied by Dr. Mark A. Muesing (Lubbertus C. F. *et al*, 2000).

### DNA constructs

For the insertion of A3G downstream of the pEGFP*IRES-Ub-X sequence, A3G was amplified by PCR using oligos: 5’-GAGGAGGAGGCGGGGCCCAGGCGGCCATGAAGCCTCACTTCAGAACACAGTG-3’ and 5’-GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCGTTTTCCTGATTCTGGAGAATGGC-3’, containing the restriction site for SfiI and ligated to the cloning vector linearized by restriction digestion with SfiI . This originated a bicistronic A3G expression vector, where transcription of the mRNA is initiated at the cytomegalovirus promoter/enhancer upstream of the EGFP coding sequence, along with an internal ribosome entry site (IRES) upstream of the Ub-X-A3G gene segment. Seven different amino acid sequences coding for, Met, Gly, Asn, Asp, Arg, Phe, and Leu, were inserted downstream of the Ub sequence and were introduced by site-directed mutagenesis. All constructs were subjected to sequencing analysis for the detection of undesirable mutations.

**Chemicals**

Proteasome inhibitor MG-132 (10mM in DMSO) was purchased from Calbiochem (cat. 474790). Cycloheximide was purchased from Aldrich (cat. C1988-1G) and stock solution was prepared in DMSO. Cell Proliferation Reagent WST-1 was purchased from ROCHE (cat.11644807001).

**Cell culture and transfections**

Human embryonic kidney (HEK) cell line 293T, and P4 cells (HeLa-CD4 LTR-LacZ) were obtained from the NIH AIDS Research and Reference Reagent Program Catalog and were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1X penicillin-streptomycin-fungizone mixture and L-Glutamine (Bio Whittaker). For transfections, 293T cells were grown in 6-well or 60-mm plates to about 70% confluency, as appropriate. Cells were transfected using the calcium phosphate precipitation method. A total of 1 to 10 µg of plasmid DNA was used for transfections in 6-well or 60-mm plates, unless otherwise stated. To generate VSV-G-pseudotyped virions, 293T cells were cotransfected with a plasmid expressing the VSV-G envelope. Total amounts of transfected DNA were kept constant in all transfections by adding empty-vector DNA (pcDNA 3.1Zeo+) (Invitrogen), when appropriate. Cells were harvested typically 40-44 h post-transfection.

**Virus production and infectivity assays**

Virus stocks were prepared by transfection of 293T cells with pNL4.3 or pNL4.3ΔVif in the presence of wild-type A3G-HA or pEGFP-IRES-Ub-X-APOBEC3GHA, as appropriate. Virus-containing supernatants were harvested 40 to 44 h after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 X *g*), and unconcentrated clarified supernatants were used for infectivity assays in P4 cells. For immunoblot analysis of viral proteins, virus-containing supernatants (2 ml) were concentrated by ultracentrifugation through 1 ml of 20% sucrose in phosphate-buffered saline (PBS) as described previously (26). To determine viral infectivity, viral stocks were collected and the viral titer determined and normalized by the amount of the p24 protein detected

using the AIDS Vaccine Program HIV-1 $p24^{CA}$ Antigen Capture Assay Kit. Hela P4 cells were infected with 100µl of unconcentrated virus supernatant. Briefly, 1.5 X $10^4$ cells were plated in a 96-well plate the day before infection. Quantification of the β-galactosidase activity was performed in cell lysates forty-eight hours after infection, using a colorimetric assay based on the cleavage of chlorophenolred-β-D-galactopyranoside (CPRG) by β-galactosidase (Roche) (27). Briefly, P4 cells were placed in 100 µl of lysis buffer ($MgCl_2$ 5mM, NP40 0.1% in phosphate buffered saline) and after 30 min on ice cell lysates were incubated with 100µl of reaction buffer (CPRG 6mM in lysis buffer) between 5 min and 2 h at 37ºC. Optical densities in the reaction wells were read at 550nm with a reference filter set at 620nm.

**Antisera**

For detection of APOBEC3G HA-epitope-tagged, an Anti-HA HRP monoclonal antibody (Roche) was used. Anti-HA High Affinity Matrix (ROCHE) was used for immunoprecipitation of HA-epitope-tagger proteins. Detection of GFP protein was carried by using anti-GFP mouse monoclonal antibody (Roche). The HIV-1 capsid p24 protein was detected using the anti-p24 anti-mouse supplied by the NIH AIDS Research and Reference Reagent Program (cat. 530). GAPDH was used as a loading control and the antibody used was purchased from Santa-Cruz Biotechnology (GAPDH 6C5).

**Immunoblotting.**

For the detection of intracellular proteins, whole-cell-lysates were prepared as follows. Transfected 293T cells were washed once with warm phosphate-buffered saline (PBS) and were suspended in RIPA supplemented with 0.1% of Triton X-100 and protease inhibitor (ROCHE) (500µl/$10^6$ cells). Cell lysates were mixed with equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125mM Tris-HCL [ph= 6,8], 10% 2-mercaptoethanol, 10% glycerol, and 0,02% bromphenol blue). Proteins were solubilized by boiling for 10 min at 95ºC. Proteins were separated by 12% SDS-PAGE (National Diagnostics) and were transferred to nylon membranes (Hybond, Amersham) and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies

(Amersham Biosciences) and visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences).

**Total RNA extraction and qRT-PCR.**

Total RNA was extracted from transfected 293T cells using Trizol reagent (Invitrogen) following manufacturer's instructions. cDNA products were synthesized from RNA isolated from 2 x $10^6$ cells, using random hexamers and Superscript II RNA polymerase (Invitrogen). A3G mRNA levels were quantified using SYBR Green (Invitrogen) and according to the manufacturer´s instructions. The primers used for the amplification of the different A3G constructs were as follows: A3G forward primer: 5´-GGCTCCACATAAACACGGTTTC -3´ (nucleotides 735 to 756); A3G reverse primer: 5´-AAGGGAATCACGTCCAGGAA – 3´ (nucleotides 803 to 784). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizing control with the following primers: 5´- GGTGGTCTCCTCTGACTTCAACA-3´, and 5´-GTTGCTGTAGCCAAATTCGTTGT-3´. Amplification of Vif was carried using the following primers: 5´- TGGCATTTGGGTCAGGGAGTC -3´, and 5´- GTT GGTCTGCTAGGTCAGGGTC -3´. Each SYBR Green reaction contained, 12.5µl 2x SYBR Green PCR mix (Invitrogen), 0.25 of each 10µM primer, and 1µl of 1:4-diluted cDNA products. Absolute mRNA copy numbers were calculated by generating standard curves generated by serial dilutions of a plasmid containing the APOBEC3G gene and the cDNA product (GAPDH). Each sample was run in triplicate in an ABI 7000 (Applied Biosystems) with one cycle at 50ºC (2min) followed by 95ºC (10 min) and 40 at 95ºC (15s) followed by 60ºC (1 min) (Nicholas O. Davidson, 2006).

**Degradation Rate Quantitation**

For the quantification of the degradation rate of each of the Ub-X-A3GHA mutants, 293T cells were transfected in a 60 mm plate with 10µg of plasmid DNA encoding the different A3G mutants. 16-20 h after transfection, cells were split in 6-well plates, and the next day they were treated with 100µg/ml of cycloheximide for 0, 1, 2, 3, 4 hours for the A3G WT, Ub-Met-A3G, and Ub-Gly-A3G transfections; 0, 1, 2, 3 hours for the

Ub-Asn-A3G and Ub-Asp-A3G transfections; 0, 20, 40, 60 mins for the Ub-Arg-A3G transfection, and 0, 5, 10, 20 mins for the Ub-Leu-A3G transfection.

For each sample, cellular lysates were obtained and protein content analyzed by WB using the appropriate antibodies (as described above in material and methods). Quantification of band density was performed using Quantity One 4.6.3 software (Bio-Rad Laboratories, USA).

**Determination of A3G deaminase activity**

For measuring A3G deaminase activity we used an *in vitro* sensitive and quantitative assay developed in Prof. Moshe Kotler lab (Norwarski R. *et al.* 2008). The assay was performed in a cell-free system and is based on PCR amplification of a synthetic ss-deoxyoligonucleotide harboring the 5’ CCCA 3’ target sequence for A3G deamination referred to as substrate. For protein expression of A3G WT and Ub-X-A3G mutants, transfection of 293T cells was carried out as described in the section cell culture and transfections. Briefly, 10 µg of each plasmid DNA was used to transfect 293T cells in 60 mm plates. Whole-cell-lysates were prepared as described above. Immunoprecipitation and purification of HA-tagged A3G proteins was performed by incubation of A3G protein from crude lysates with Anti-HA High Affinity Matrix (ROCHE), according to the manufacture instructions. Purified A3G proteins were treated with DNAse I (Invitrogen, Cat. No. 18047-019) according to the manufacturer’s instructions prior to incubation with a deamination substrate. Briefly, purified protein (40 Fmol/µl) was incubated with a ss-deoxyoligonucleotide substrate (10 Fmol/µl)) in the presence of RNAse H (40 µM), Tris buffer (25mM), BSA (0.1 mg/ml), for 30 mins at 37ºC. After incubation, A3G reaction products were amplified by PCR and the PCR product was digested with Eco147I. Briefly, one round of PCR was performed at 94ºC (3 min) followed by fifteen rounds at 94ºC (30 sec) and 61ºC (30 sec). The restriction reaction products were separated by non-denaturing (TBE) SDS-PAGE.

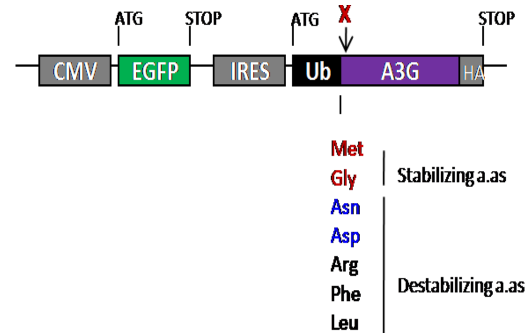
# RESULTS

**APOBEC3G expression can be manipulated by the N-end rule pathway.**

We sought to manipulate the expression of A3G to assess whether the amount of protein correlates with the inhibition of viral infectivity, viral incorporation and deaminase activity. We conditionally destabilized A3G to ensure that its total quantity in the cell is similar. To accomplish this goal, we employed a technique previously designed to express proteins that have an N-terminal amino acid different from methionine (Bachmair, A., *et al*, 1986). This strategy will express A3G proteins with a dissimilar intracellular half-life. Mechanistically, it takes advantage of deubiquitylating enzymes that potently and rapidly cleave the junction between the C terminus of ubiquitin and the N terminus of a fused protein domain, in a process called deubiquitination. Therefore, in our work the coding sequence for human ubiquitin was fused with A3G to generate Ub-X-A3G, where X represents any class of destabilization amino acids. According to the identity of the amino acid used at N-terminus, this arrangement allowed us to obtain A3G variants with different steady-states. In this study, we used a panel of seven different amino acids that have been described to be involved in the N-end rule pathway (Bachmair, A., *et al*, 1986; Gonda D.. *et al*, 1989; Varshavsky A., 1996). The schematic representation of the Ub-X-A3G fusion construct and the amino acids used at the N-terminus of A3G is represented in figure 1.

We used an expression vector constructed by Mark Muesing's (Lubbertus, C. F and Muesing M, 2000) laboratory containing the coding sequence of EGFP under the control of the cytomegalovirus promoter, followed by an internal ribosome entry site (IRES) upstream of the Ub-X-A3G fragment. This bicistronic expression vector is central to check DNA transfection efficiency and provides the means to control expression of Ub-X-A3G gene.

**Fig.1. Schematic representation of the A3G bicistronic expression vector, pEGFP*IRES-Ub-X-A3G-Ha.** Transcription of the mRNA is initiated at the cytomegalovirus promoter/enhancer upstream of the EGFP coding sequence. The translation start and stop codon for EGFP and Ub-X-A3G is indicated. The site for the post-translational cleavage between Ub and X-A3G is indicated with a downward arrow. The amino acid residues used in this study are indicated, which in eukaryotes are classified as stabilizing and destabilizing according to the half-life that they confer to the protein (Varshavsky A., 1996).




Vif has been proposed to target A3G for accelerated degradation in the 26S proteasome via the ubiquitin-proteasome pathway (Conticello S. *et al*., 2003; Liu B. *et al*., 2005; Marin M. *et al*., 2003; Mehle A. *et al*., 2004; Sheehy *et al*., 2003; Stopack K. *et al*., 2003; Yu X. *et al*., 2003; Yu Y. *et al*., 2004). A similar situation occurs with the N-end substrates that are bound by a targeting complex, followed by formation of a substrate-linked multi-Ub chain. The ubiquitylated substrate is processively degraded by the 26S proteasome.

To determine whether Ub-X-A3G follows the 26S proteasome degradation pathway in the absence of HIV-1 Vif protein, we substituted the first amino acid in A3G sequence by six different residues. We have also maintained the amino acid methione at the N-terminus of the A3G sequence as a control for expression of wild-type deaminase in this system. The corresponding recombinant plasmids EGFP-IRES-Ub-X-A3G are isogenic except for the codon sequence of the amino acid present between Ubiquitin and A3G. The A3G N-terminal residues introduced synthetically cover the four classes of amino acids described by Varshavsky and co-workers in the N-end rule: the least stable or primary (Leu, Phe and Arg), followed by secondary (Asp and Glu), tertiary (Gln and Asn), and stabilizing (Met, Gly and Val) (Bachmair, A., *et al*, 1986; Gonda D. *et al*, 1989; Varshavsky A., 1996).

In order to determine the steady-state expression of A3G fusions to ubiquitin, according to the amino acid at the Ub-A3G junction, we transfected equal amounts of different Ub-X-A3G plasmids in 293T cells. The expression profiles obtained for the different A3G variants showed that the proteins containing stabilizing residues (Met and Gly), tertiary residue (Asn) and secondary residue (Asp) have higher expression

levels compared to proteins that have the least stable residues (Arg, Phe and Leu) (Fig. 2 A).

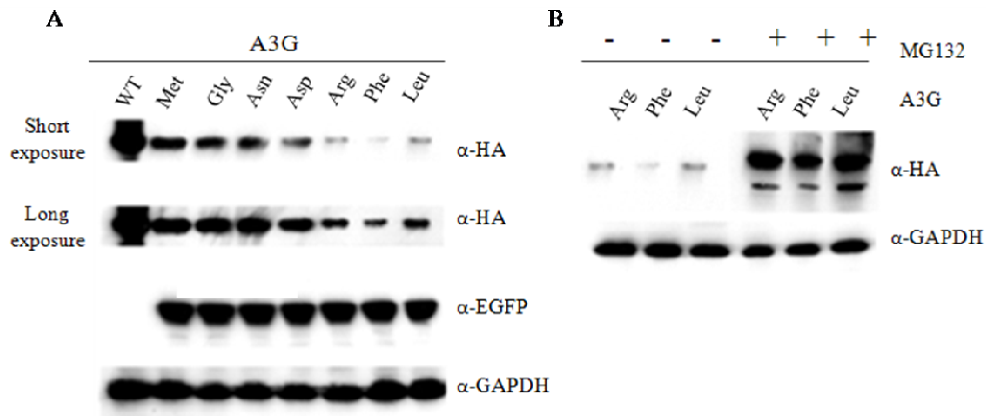



**Fig.2. Expression of A3G mutants. (A)** Wild-type A3G and N-terminal A3G mutants are indicated above as wt, Met, Gly, Asn, Asp, Arg, Phe, Leu. EGFP expression is shown as a control for the transfection. GAPDH is shown as a general control to the amount of protein loading in Western blotting. Briefly, 2µg of each plasmid was used to transfect 293T cells in 6-well plates. 40-44 hrs after transfection cells were washed twice with room-temperature PBS and lysed in cold RIPA lysis buffer. Lysates were subjected to further WB analysis. **(B) The stability of A3G variants is regulated by the proteasome.** Inhibition of proteasome function using MG-132 (20 µM) greatly increased the steady-state of the least-stable A3G variants: Arg-A3G, Phe-A3G, and Leu-A3G. α-GAPDH antibody was used as a loading control.

Similar levels of EGFP expression show a similar transcription level for the different constructs confirming that differences in X-A3G expression were not due to the efficiency of the transfection or other experimental artefact (Fig. 2A). The molecular weight of different X-A3G proteins was similar to wild-type deaminase showing that Ubiquitin was cleaved from A3G. In addition, GAPDH control indicates that differences regarding Ub-X-A3G expression were not due to protein loading. To discard a possible toxicity effect due to the chimeric A3G expression, a cell viability and proliferation

assay using the tetrazolium salt WST-1 (Roche) showed that the differences observed were not due to abnormal cell physiology.

Figure 2B shows that the steady-state levels of least stable proteins Arg-A3G, Phe-A3G and Ub-Leu-A3G were detectable only when transfected cell cultures were pre-treated with proteasome inhibitor. Briefly, 293T cells were transfected with equal amounts of Ub-Arg-A3G, Ub-Phe-A3G and Ub-Leu-A3G isogenic plasmids. 36 hrs after transfection, cells were treated with proteasome inhibitor MG-132 (20 µM) for 4 h and analyzed by SDS-PAGE after lysis. Albeit more detectable than Phe-A3G, proteins Leu-A3G and Arg-A3G were barely evident in the absence of inhibitor, whereas in the presence of MG132 levels of similar proteins were, as expected, correspondingly higher. Taken together, these results indicate that X-A3G proteins are physiological substrates of ubiquitin-proteasome degradation pathway dependent on the N-end rule. Therefore, the low level expression detected with Arg-A3G, Phe-A3G and Ub-Leu-A3G was indeed a consequence of the amino-terminal destabilizing residues and not dependent on the lower protein expression in a given moment.

**Stability of X-A3G variants targeted to N-end rule pathway.**

The observation that A3G is incorporated into the virion core suggested several possible cellular sources of the deaminase. Most likely, the mechanism of A3G virion incorporation depends on the amount of free deaminase in the cytoplasm capable to be targeted to the virion. One important variable for this mechanism is the time necessary for this recruitment into the virion core. It is conceivable that when A3G is bound to the cellular high molecular mass (HMM) complex cannot be readily rescued for virion incorporation. Therefore, fine-tuning different half-lives of A3G proteins can help to understand the time-space relationship of A3G targeting to the viral particle. The N-end rule of X-A3G should provide the total amount of deaminase in the cell with similar half-life. In order to assess whether X-A3G mutants can be used with this intention the kinetics of X-A3G degradation was evaluated. To monitor the *in vivo* decay of A3G proteins we performed a cycloheximide chase analysis, as described in experimental procedures. We used this approach, instead of pull-chase analysis, because we aimed to monitor the turnover of existing proteins and to examine newly synthesized molecules. Due to the expected rapid degradation of X-A3G with unstable

amino acids, some of our constructs could only be detected by this method if the pulse-labeling time was less than 5 minutes and the chase times very short. This rational, together with the fact that the steady-state amount of unstable X-A3G would be very low, leaded us to use this methodology in order to avoid misinterpretations.

In Figure 3, cycloheximide assay showed that the *in vivo* half-life of the different A3G proteins ranged from more than 4 hours to less than 5 minutes, depending on the nature of the amino acid residue present at the amino-terminus of A3G. Specifically, deubiquitinated X-A3G proteins with either Met or Gly at the amino-terminus displayed a relatively long half-life of more than 4 hours, similar to the half-life of control wt A3G. In contrast, X-A3G mutants bearing N-terminal destabilizing residues classified as primary amino acids exhibited very short half-lives. Cycloheximide chase analysis showed that Arg-A3G half-life was approximately 28 minutes, 13 minutes for Phe-A3G and less than 5 minutes for Leu-A3G. In addition, the half-life of X-A3G proteins bearing amino-terminal residues Asn and Asp was approximately 85 minutes and 65 minutes, respectively. Despite some controversy, previous authors showed that intracellular half-life of wt A3G in the presence of Vif was reduced from the original 8 h to 1-2 minutes (Marin M. *et al.*, 2003). Interestingly, the half-lives showed in the assay by the different A3G variants reflected an effect similar to the one observed in the presence of Vif. It is notorious the sharp reduction detected in the half-life of the least stable proteins (Arg-A3G, Phe-A3G, and Leu-A3G). Thus, it is possible to mimic the effect of Vif on A3G intracellular steady-state by changing the first amino acid residue in A3G.

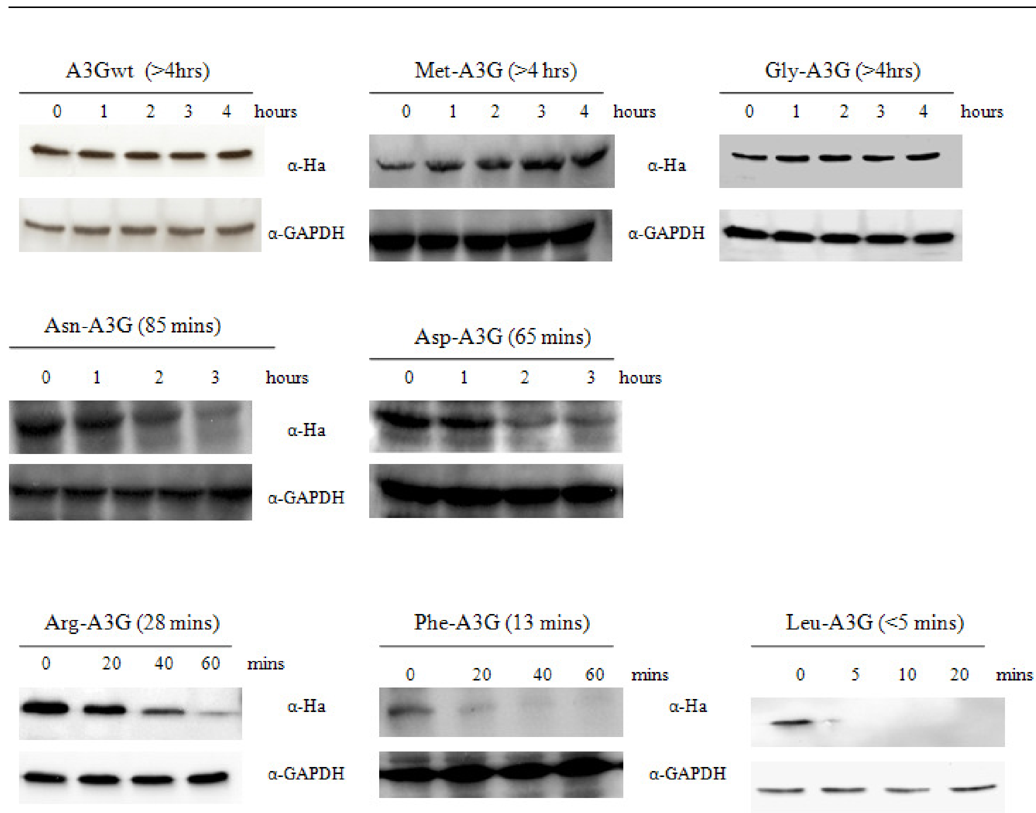



**Fig. 3. Cycloheximide chase analysis.**

A3G wt and N-terminal A3G variants were expressed in 293T cells (60-mm plates) and, the second day after transfection the cells were split in 6-well-plates and the translational inhibitor cycloheximide (100 µg/ml) was added to each of the cell cultures. 293T cells expressing the A3G proteins were incubated with cycloheximide for 1, 2, 3, and 4 hours for A3G wt, Met-A3G, and Gly-A3G; 1, 2, and 3 hours for Asn-A3G and Asp-A3G; 20, 40, and 60 minutes for Arg-A3G and Phe-A3G, and 5, 10 , and 20 minutes for Leu-A3G. The zero time-point received drug-free diluent DMSO. Cellular lysates were obtained at each time point and the protein content was analyzed by WB using the anti-Ha HRP monoclonal antibody. α-GAPDH antibody was used as a loading control. The approximate half-life value of each protein is indicated between brackets. Each value represents the mean relative value of three independent experiments obtained by band densitometry.

**Amino-terminal A3G variants are targeted to degradation by HIV-1 Vif protein.**

Due to the nature of the N-end rule, it is conceivable the X-A3G variants could somehow escape the action of Vif by a different pathway of degradation. It could result that the alteration of the first N-terminal residue in X-A3G may affect its subcellular distribution allowing the protein to escape the action of Vif. In this way, and according to the fact that wild-type A3G stability is impaired in the presence of Vif protein (Stopack K. *et al.*, 2003), we sought to determine if X-A3G constructs were capable to be targeted for degradation by Vif. Based on the expression profiles obtained for the different X-A3G constructs, those that exhibited higher steady-state (Met-A3G, Gly-A3G, Asn-A3G, and Asp-A3G) were expressed in the presence of Vif. Results in Figure 4 show that Vif accelerates degradation of all X-A3G variations similarly, even though they were under the N-end rule. Even with a low steady-state, the amount of the three least stable constructs (Arg-A3G, Phe-A3G, and Leu-A3G) was also reduced by Vif (data not shown). These results seem to indicate that Ub-X-A3G fusions and its cleavage products do not have deleterious effects on deaminase folding, being capable of targeted degradation by Vif. Moreover, Vif shows a capacity to recognize ubiquitinated X-A3G and accelerate its proteasomal elimination regardless of the altered pathway of degradation presented by the N-end rule.

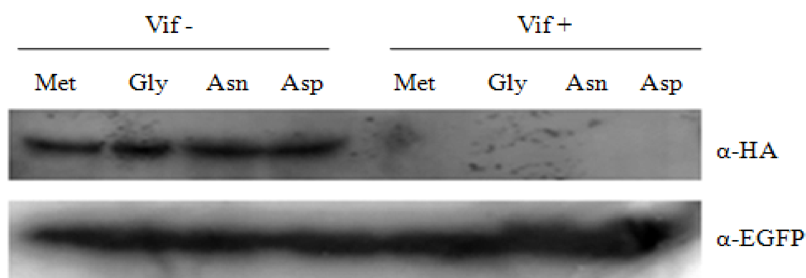



**Fig. 4. Detection of Ub-X-A3G proteins in the presence of Vif.** 293T cells were cotransfected in a 6-well plate with 1 µg of Ub-Met-A3G, Ub-Gly-A3G, Ub-Asn-A3G, Ub-Asp-A3G, and 3 µg of pcDNA-HVif. 40-44 h after transfection cells were lysed and protein content was analysed by SDS-PAGE.

**N-terminal A3G variants incorporation into viral particles**

According to Soros V. *et al.*, newly synthesized low molecular mass (LMM) A3G is preferentially recruited into HIV virions within 1.5 h after synthesis (Soros V. *et al.*, 2007). Moreover, it was elegantly shown that the amount of A3G decreased substantially after the 1 hour peak, suggesting that once it is assembled into the cellular high molecular mass (HMM) complexes, it is no longer available for virion encapsidation. Therefore, we sought to investigate whether the least stable X-A3G proteins were still able to incorporate into the HIV-1 viral particles.

Equal amounts of N-terminal X-A3G variants in the presence of HIV-1 ΔVif plasmid DNA were transfected into 293T cells. Two days after transfection, virions were harvested and after p24 normalization were analyzed for the presence of incorporated X-A3G. Results obtained in this experiment showed that X-A3G with a half-life of 13 min is incorporated into viral particles, but the least stable Leu-A3G mutant (half-life < 5min) was absent from all the samples analyzed (Fig. 5C).

In addition to results of Soros V. *et al*., we presented data that implies a time-window for A3G incorporation into virions that is relatively short. Therefore, Vif must act during this time period to avoid A3G incorporation. However, it is not clear whether Vif acts before, during or after the assembly of A3G into HMM complexes. Based on previous work (Soros V. *et al.*, 2007), we assumed that the least stable X-A3G proteins were associated into LMM and able to exert antiviral activity (Fig. 5 B).

As shown in Figure 5, Vif-mediated degradation of A3G has to occur between 0 and 13 minutes after A3G synthesis before its assembly into HMM complexes and virion incorporation, given those 13 minutes is the minimum half-life of X-A3G to be incorporated into virions. It is interesting to note that the amount of X-A3G incorporation into virions is increasing from Phe-A3G to Asn-A3G, possibly reflecting a controlled inclusion of APOBEC into HIV-1 particles. Because, 85 minutes is the determined half-life of Asn-A3G, that is consistent with previous results that show preferentially recruitment of A3G into HIV virions within 1.5 h after synthesis (Soros V. *et al.*, 2007). Hence, artificial fine-tuning of protein steady-state expression is a useful tool to monitor A3G its activity over-time inside the cell the time by providing evidence of the shortest time-frame necessary for its incorporation into HIV-1ΔVif virions.

**Phenotypic analysis of N-terminal A3G mutants**

We have shown that the alteration of the first amino acid in A3G is capable of modelling its half-life and consequently manipulate its time-dependent activity in the cell. While all X-A3G, with the exception of Leu-A3G, were capable to be incorporated into HIV-1ΔVif virions, we hypothesized that viral infectivity could be reduced similarly. This proposal stems from the assumption that the presence of A3G into virions is sufficient and necessary to influence its antiviral activity.Therefore, to examine if X-A3G variants were active against HIV-1, we tested all X-A3G isogenic constructs for their ability to inhibit viral infectivity in the absence and presence of Vif protein. Viral stocks were produced by transfection of pNL4.3ΔVif and pNL4.3 into 293T cells with A3G wt and N-terminal A3G variants. Infectivity values were obtained after infection of HeLa P4 cells with the viral stocks produced (Fig. 5 A, B). In the presence of Vif, it is shown that all X-A3G constructs were unable to inhibit infection when compared to A3G wt and variations observed among the different constructs were not significant ($p<0.001$) (Fig. 5 A). In contrast, the relative viral infectivity obtained in the absence of Vif and presence of different X-A3G showed differential effects. As illustrated in Figure 5 B, X-A3G proteins bearing stabilizing amino acids, Met and Gly, were able to inhibit HIV-1ΔVif infection comparably to A3G wt. Interestingly, X-A3G proteins bearing tertiary (Asn) and secondary (Asp) amino terminal residues also exhibited antiviral activities similar to A3G wt, Met-A3G, and Gly-A3G. We were expecting a reduction in the antiviral activity of these A3G variants, since Asn and Asp are considered destabilizing residues with lower half-life and lower steady state expression. Nevertheless, the results consistently showed an activity of these A3G variants comparable to A3G wt. Thus, the total amount of Asp-A3G and Asn-A3G in the cell at 65 min and 85 min of half life, respectively, was enough to be incorporated into virions and have antiviral effect similar to wild-type. In contrast, X-A3G variants bearing the least stable residues, Arg and Phe, only vaguely inhibited HIV-1ΔVif infection (33% of A3G wt activity), and the Leu-A3G protein did not display any antiviral effect in the absence of Vif (Fig. 5 B). Hence, X-A3G show an ability to be incorporated shortly into virion particles but that does not correlate directly with its antiviral effect.

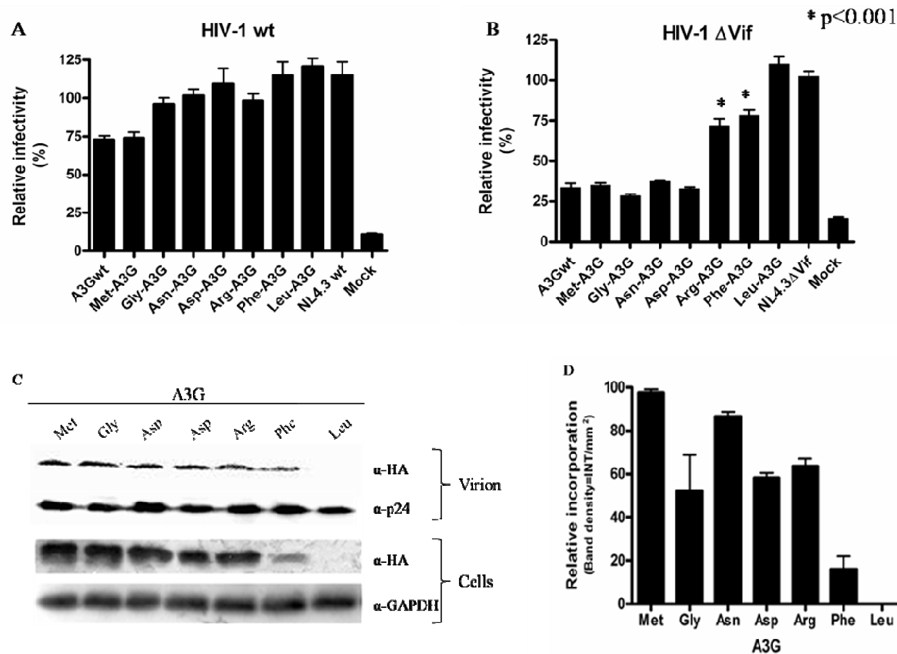



**Fig. 5. Infectivity assay and A3G virion incorporation.** HIV-1 virions were produced by co-transfecting 293T cells in 6-well plates, with equal amounts (1 µg) of A3G wt and Ub-X-A3G plasmids in the presence of 3 µg of pNL4.3 **(A)** or pNL4.3ΔVif **(B)**. VSV-G plasmid (0.1 µg) was used in all transfections to generate VSV-G-pseudotyped virions. DNA amounts were adjusted to 5 µg using empty pcDNA3.1 vector DNA. 40-44 hours after transfection, VSV-G pseudo-typed virions were collected (100 µl), normalised for the presence of p24 protein, and used to infect Hela P4 cells ($1.5X10^4$ cells *per* well). 40 h after infection of P4 cells, the single-cycle titter of viruses produced was determined by quantification of the β-galactosidase activity in P4 lysates, using CPRG assay. β-gal activity was determined by measuring the absorbance at 550/620nm. A3G wt was used as a control for infection inhibition. Relative infectivity values presented here are the result of the four independent experiments. **(C)** The remaining virus stocks (1.9 ml) were used for detection of intravirion A3G protein. After collecting virus-containing supernatants cellular debris was removed by centrifugation (3 min, 3,000 X *g*), and clarified supernatants (1.9 ml) were concentrated by ultracentrifugation through 1 ml of 20% sucrose in phosphate-buffered saline (PBS) as described previously (Kao, S., *et al.*, J.Virol. 2003). After centrifugation, viral pellet was subjected to immunoblot detection of intravirion A3G content using anti-Ha HRP monoclonal antibody. We performed this assay four times and the results obtained were always similar to the one represented here. The data are averages of the four replicates with the indicated standard deviations. **(D)** Relative virion incorporation was determined by calculating the mean relative values of the three independent experiments measured by band densitometry of incorporated X-A3G proteins.

It was possible that differences in X-A3G expression could somehow be related to differences in transcription. Although, EGFP was measured and normalized, to overcome this hypothesis. We performed real-time PCR in order to confirm the presence of equal amounts of mRNA transcripts in all the samples used for the production of viral stocks. Total RNA was obtained from the transfected 293T cells and was analysed for the presence of equal amounts of A3G wt and X-A3G variants. Equal X-A3G and GAPDH mRNA transcripts (Fig. 6 A, B) were detected in all the samples analysed showing that the variations observed in figure 5 B were not due to variations in the amount of cells transfected. We also confirmed the presence of equal Vif mRNA transcripts in cells transfected with HIV-1 wt (Fig. 6 C). Quantification of mRNA levels from all the samples analysed showed that variations observed were not significant ($p<0.001$). These results confirmed that the observed differences in infectivity showed in Figure 5 B were indeed due to the steady-state of each X-A3G variant that was used.

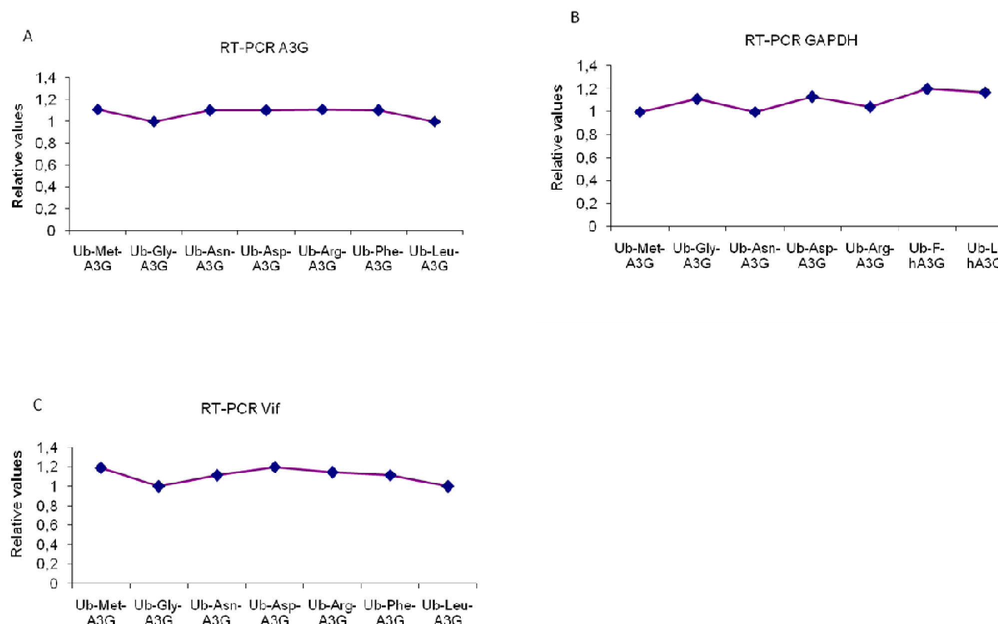



**Fig. 6. qRT-PCR.** A3G, GAPDH, and Vif mRNA levels were quantified using SYBR Green (Invitrogen) and according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizing control. Absolute mRNA copy numbers were calculated by generating standard curves generated by serial dilutions of plasmids containing the A3G, and Vif sequences and the cDNA product (GAPDH). Each sample was run in triplicate in an ABI 7000 with one cycle at 50ºC (2min) followed by 95ºC (10 min) and 40 at 95ºC (15 s) followed by 60ºC (1 min). (Nicholas O. Davidson, 2006). SD A3G= 0.928; SD GAPDH= 1.656; SD Vif= 2.08.

### Deaminase activity of A3G amino-terminal variants

There is a clear link between A3G deaminase activity and its antiviral activity. Moreover, it has been shown by Soros *et al* that newly synthesized A3G and not pre-existing A3G already assembled into inactive cellular high molecular mass complexes, is encapsidated into budding virions (Soros V. *et al.*, 2007). Therefore, we sought to determine if all X-A3G proteins displayed enzymatic activity in an *in vitro* deamination assay. This was a qualitative assessment to evaluate if shorter and longer half-life variants of the deaminase might show different catalytic activities.

To investigate the enzymatic activity of X-A3G variants, we performed an *in vitro* deamination assay developed in Kotler's lab (Norwarski R. *et al.* 2008). In this procedure a synthetic ss-deoxyoligonucleotide substrate harboring a 5' CCCA 3' target sequence for A3G deamination was incubated with immunoprecipitated X-A3G constructs. The objective of this assay was to determine if newly synthesized, or more mature, A3G proteins displayed intracellular enzymatic activity. Surprisingly, the results obtained showed that the enzymatic activity of individual X-A3G proteins was similar, despite different half-lives exhibited. As demonstrated in Figure 7, Leu-A3G with a half-life of less than 5 minutes was able to deaminate 5' CCCA 3' substrate. Moreover, when different X-A3G that exhibit increasing half-lives was used, the amount of resultant enzymatic products was maintained.

These results indicate that enzymatic activity of A3G in the cell is not dependent on its half-life. Since the amount of resultant small oligonucleotide is maintained steady from 5 minutes to 85 minutes of A3G half-life, it is possible that this quantity of deamination results from enzyme that is active in LMM.

Therefore, the increasing amount of A3G in steady state expression does not correlate with the enzymatic activity of A3G indicating that between 5 minutes and 13 minutes A3G can be assembled into inactive cellular HMM complexes.

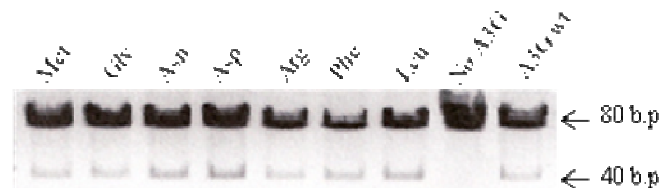



**Fig. 7. A3G catalytic activity is not dependent on the protein steady-state. (A)** A synthetic ss-deoxyoligonucleotide (10 Fmol/µl) containing a central 5' CCCA 3' target sequence for A3G deamination was incubated with immunoprecipitated A3G protein. Deamination of $C_3$ in the 5' $C_1$ $C_2$ $C_3$ A 3' sequence, which introduces an Eco147I restriction site in the deaminated substrate following PCR amplification, is shown by the formation of a 40b.p cleavage product.

**DISCUSSION**

In this work we sought to investigate the relationship between A3G half-life and its antiviral activity. It has been well demonstrated that Vif protein is capable of recruiting A3G to degradation via ubiquitination/proteasome. Therefore, reducing A3G steady state is a major strategy to eliminate antiviral activity by impeding its virion incorporation. The time A3G needs to be incorporated into virus particles is important to assess how promptly Vif can act to induce its degradation. In addition, these results provide evidence for how long A3G should exist in the cytoplasm in order to be a component of a viral particle.

It was elegantly demonstrated by Warner Greene's laboratory that newly synthesized A3G is preferentially recruited into HIV virions within 1.5 h after synthesis (Soros V. *et al.*, 2007). By pulse-chase radiolabeling it was possible to show that assembly of A3G in cytoplasmic high molecular mass (HMM) complexes occurred after 30 minutes of synthesis, restricting the enzyme to be included in the viral particle. Therefore, we wanted to ask whether viable A3G proteins with a half-life shorter than 30 minutes could be incorporated into virions and have an antiviral activity.

In order to understand the mechanism of short-lived A3G and to monitor over-time its activity inside the cell, we decided to fine-tune the intracellular half-life of A3G by targeting it to the N-end rule pathway. Moreover, to evaluate the time required for A3G incorporation into virion particles, fine-tuning the half-life of A3G alone has the advantage to test its activity independently from Gag synthesis.

The method used in this study showed that A3G variants with different stabilities are capable to be targeted for proteasomal degradation in a Vif-independent manner. These results provide evidence that N-end rule can be a useful tool to study A3G protein and its antiviral effects in a Vif-free environment.

Ubiquitylation by the N-end rule pathway has been shown to be mediated by multiple N-recognins that contain a zinc-finger-like domain termed the UBR box (Tasaki T *et al.*, 2005; Kwon YT. *et al.*, 2003). The mammalian genome encodes at least seven UBR box-containing proteins, named UBR1 through to UBR7 (Tasaki T *et al.*, 2005). UBR box proteins generally contain specific signatures unique to E3 Ub ligases or a substrate recognition subunit of the E3 complex (Tasaki T *et al.*, 2005). HIV-1 Vif protein is post-translationally modified by

ubiquitin and has been shown to interact with proteins belonging to the HECT E3 ubiquitin ligase family (Nedd4 and AIP4), and with a ring finger E3 ubiquitin complex containing Elongin B and C, Cullin 5, and Rbx 2 (Conticello S. G. *et* al., 2003; Bogerd H. *et al*., 2004; Lui B. ert al., 2005; Marin M. *et al*., 2003; Mehle A. *et al*., 2004a, b; Sheehy *et al*., 2003; Stopack K., *et al*., 2003; Yu X. *et al*., 2003; Yu Y. *et al*., 2004). Since all these E3 ubiquitin ligases are expressed in T lymphocytes, and are involved in Vif-targeted degradation of A3G, we raise the question as to whether A3G is targeted by the same, or other, E3 ligases, in the absence of Vif. In this context, it is interesting to note that even if the degradation pathway is altered it does not alter the capacity of A3G to have an antiviral activity of be degraded by Vif. Therefore, A3G degradation might follow a common pathway whether it is caused by the N-end rule or Vif-induced.

Results obtained in infectivity assays confirmed the importance of A3G steady-state expression for the maintenance of its antiretroviral activity. Ours results with homogenous populations of A3G having different half-lives show that its incorporation into virions occurs in a very short time after synthesis. We showed evidence that A3G with a half-life of approximately 13 minutes, is able to be incorporate into HIV viral particles. These results indicate that to counteract A3G activity and impede its incorporation into virions, Vif has to act at the very early stages of A3G life-time.

Our results are somewhat different from Soros *et al* that previously showed by pulse-chase that A3G is selectively incorporated into virions within 1.5 hours after synthesis (Soros V. *et al*. 2007). However, it is technically difficult to detect specific low amount of A3G incorporation into virions when pulse-chase is performed, which does not occur when all A3G population in a cell has the same intracellular half-life. Moreover, we are observing A3G incorporation into viral particles independently of Gag synthesis. We observe in our assays short-lived A3G proteins with t1/2=13 min already incorporated into virions indicating that it can be recruited by Gag during virus assembly between 5 to 13 minutes after A3G synthesis. Thus, it seems likely that A3G can be associated with Gag during virus assembly and not necessarily simultaneous with Gag expression. These results also bring the possible conclusion that Vif must act during 13 minutes after A3G synthesis to promote its degradation and reduce its half-life to less than 5 minutes, which seems to be the minimum time from A3G incorporation into virions.

The recruitment of A3G into virions is ultimately disadvantageous to the virus. Therefore we assessed whether A3G half-life affected its incorporation into virions and

consequently viral infectivity. Our results show that the amount of A3G into virion is not directly correlated with its half-life as A3G variants with t1/2=30 minutes to t1/2=85 minutes have similar virion incorporation. Moreover, it is interesting to observe the discrepancy between intracellular expression of X-A3G variants and its viral incorporation. In contrast to other X-A3G variants, the amount of Phe-A3G present in virions is very similar to that expressed intracellularly. The fact that the majority of Phe-A3G with t1/2=13 min is incorporated into virions suggests that it is present in intracellular LMM and available in totality for virus inclusion. This observation confirms previous the results of Warner Green's laboratory showing that A3G is present in LMM complexes during the first 30 minutes after synthesis (Soros V. *et al.* 2007). In contrast to Phe-A3G, all other X-A3G variants are highly expressed in the cell and the amount of X-A3G incorporated into virions is steadily similar. The result showing that the amount of A3G into virions increases from t1/2=13 min to t1/2=28 min and then remains constant is compatible with the assumption that A3G can only be incorporated into virions between 13 to 28 minutes after synthesis. Since it was showed that A3G is present on HMM after 30 minutes, these results are attuned with the hypothesis proposed by Soros *et al* that the presence of X-A3G into HMM complexes may impede its virion incorporation (Soros V. *et al.* 2007).

The higher amount of A3G into virions appears to be reached with a half-life superior to 28 minutes. When the antiviral properties of these A3G variants are assayed, we observe that a minimum infectivity is obtained with t1/2=65 min. Therefore, a discrepancy is present between the amount of A3G into virions and its antiviral activity from 13 minutes to 65 minutes of half-life. The incorporation of Arg-A3G (t1/2=28 min) into virions is higher than that of Phe-A3G (t1/2=13 min) but an increase in the antiviral effect is not observed. Furthermore, when similar comparison is made between Arg-A3G (t1/2=28 min) and Asp-A3G (t1/2=65 min), the amount of protein into virions is alike but the antiviral activity is higher with Asp-A3G. This disparity among virion-incorporated A3G and viral infectivity may indicate that a threshold of A3G capable of antiviral activity can only be overcome when the protein half-life is around 65 minutes. Conversely, it can also be hypothesized that cellular co-factors required for A3G antiviral activity are not readily incorporated into virions, but are rescued by the deaminase to the particle at a later stage of viral assembly between 28 to 65 minutes after A3G synthesis.

It was reported that virion-associated A3G is derived from intracellular LMM complexes (Soros V. *et al.*2007). Our results show that enzymatic activity of intracellular A3G is constant from t1/2=13 min until t1/2=4 h. Therefore, it is possible that the amount of A3G in LMM active form is available for virion incorporation during this half-life interval, confirming previous results of A3G virion incorporation.

It has been suggested that cytokines, such as IL-2 and IL-5, produced in lymphoid tissues during cell-cell interactions are responsible for the formation of HMM complexes (Kreisberg JF et al., 2006). Therefore, A3G could be inactivated in HMM RNA/protein complexes which in turn might confer the permissive phenotype for HIV-1 infection (Kreisberg JF et al., 2006). Thus, it would be interesting to test whether the activity of specific E3 ligases could explain why in certain cells A3G is present in LMM complexes and in others it exists as HMM. Therefore, the reduction of A3G half-life to escape recruitment to the HMM and regulate its activity in the cell is a conceivable hypothesis. How the cell can accomplish this goal is a matter of discussion. We have shown in this work that X-A3G can follow the proteasome degradation pathway in a Vif-independent manner. This indicates that A3G can be targeted to degradation by cellular specific E3 ligases, other than Vif. Although we have artificially modelled A3G half-life by introducing destabilizing residues at the amino-terminus, post-translational modifications can occur in proteins targeted by the N-end rule to proteasome degradation. Thus, in the absence of Vif we cannot exclude the possibility that A3G suffers a post-translational modification that could remove, or introduce, an amino-acid residue different for methionine. Thus, post-translational modification could expose amino acid residues and consequently trigger a cascade of events that ultimately would lead A3G to proteasome degradation.

Although the above hypothesis might seem acceptable, our results show that that the activity of A3G is very similar for every half-life variants. We showed that the antiviral activity of A3G does not seem to increase with the diminishing of protein half-life. We should stress that these experiments were performed at the late stages of viral replication during viral incorporation. Hence, there is a lack of information about the role of cellular A3G half-life during initial stages of viral infection, mainly during reverse transcription. If deamination is a major antiviral strategy for viral infectivity, it is conceivable that an increase amount of cellular A3G might abrogate initial steps of HIV-1 infectivity. Therefore, based on the results presented in this work we speculate that

to increase the putative antiviral activity of cellular A3G during reverse transcription, a strategy should be implemented to disassemble HMM and consequently release active A3G. In this case the total amount of A3G is increased with consequent improvement of its antiviral activity.

# CHAPTER 3

## Cooperation of amino and carboxyl-terminal domains of Vif is essential for its role in A3G degradation

**Iris Cadima-Couto,** Soraia Oliveira, Saraiva N, João Gonçalves. (2008). Cooperation of amino and carboxyl-terminal domains of Vif is essential for its role in A3G degradation. *Journal of Biological Chemistry. (Submited)*.

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## ABSTRACT

Identification of protein biding partners in Vif and A3G proteins is one of the major challenges for the development of new antiviral drugs. Here, we present a novel approach to study the *in vivo* interaction of Vif and A3G. The strategy used in this study is based on an oligomerization-assisted interaction whereby fragments of Vif and A3G are fused to independently folding and interacting domains of homodimerizing GCN4 leucine zipper-forming sequences. Reassembly of the two interacting domains was assessed *in vivo* and proved to be a useful tool for a rapid screening of Vif-A3G interacting domains. We show that the C'terminal region of Vif alone is not sufficient to induce A3G degradation and that binding of Vif to A3G must involve an additional co-factor that is probably linked to the N-terminus of Vif and may be essential for the interaction and consequent degradation of A3G. We also show the importance of the topological orientation of Vif-A3G for an efficient interaction indicating that it must occur in a parallel orientation when both proteins are in close proximity. Our results show that Vif-targeted degradation of A3G is specific and supports the previous hypothesis of an involvement of a specific co-factor in this interaction.

## INTRODUCTION

The Human Immunodeficiency Virus type-1 (HIV-1) Viral Infectivity Factor protein (Vif) is a 23KDa protein that plays an important role in HIV-1 replication. Vif is expressed late in the retrovirus life cycle and is required to overcome the antiviral activity of the host cell's APOBEC3G (A3G) and APOBEC3F (A3F). In the absence of Vif, A3G and A3F are incorporated into HIV-1 virions and carried to the target cell where they will deaminate cytidines in viral minus-strand DNA during reverse transcription (RT), resulting in G-to-A hypermutation, which will lead to the premature degradation of the newly synthesized viral DNA (Harris RS *et al.*, 2003; Mangeat B *et al.*, 2003; Mariani R *et al.*, 2003; Zhang H *et al.*, 2003). Vif neutralizes the antiviral activity of A3G and A3F by forming a RING-finger E3 ubiquitin complex containing Elongin B and C, Cullin 5, and Rbx2 to form an E3 ubiquitin ligase complex, and uses all these key domains to recruit these proteins for polyubiquitination and proteasomal degradation (Conticello SG *et al.*, 2003; Liu B *et al.*, 2005; Marin M *et al.*, 2003; Mehle A *et al.*, 2004; Sheehy AM *et al.*, 2003; Stopak *et al.*, 2003; Yu X *et al.*, 2003; Yu X *et al.*, 2004). Vif may also neutralize A3G antiviral through other independent mechanisms that do not involve proteasomal degradation (Kao S. *et al*, 2003; Kao S. *et al*, 2004; Mehle A. *et al*, 2004; Santa-Marta M. *et al*, 2005; Stopak K. *et al*, 2003).

The SLQ$^{144}$LQXLA$^{150}$ Vif motif is the most conserved sequence in Vif and it is required for degradation of A3G and A3F by the Vif-Cul5-E3 ligase complex but unnecessary for binding (Mariana Marin *et al*, 2003). Conversely, binding of Vif to A3G and A3F is necessary for their degradation by the Vif-Cul5-E3 ligase (Mariana Marin *et al*, 2003). It was previously suggested that one region of Vif was responsible for binding to A3G, whereas another region comprising the conserved SLQLQXLA motif was responsible for mediating A3G degradation in the proteasome (Mariana Marin *et al*, 2003).

The N-terminal region of Vif has been implicated in its binding to an N-terminal region of A3G (amino acids 54-124) and also contributes to species-specific recognition (Marin M, *et al.*, 2003; Schrofelbauer, B. *et al*, 2006; Simon, V., 2005; Tian, C. *et al*, 2006; Indrani P. *et al*, 2006). Although, the regions of Vif involved in binding to A3G remain poorly defined. The DRMR region of Vif, localized at the N-terminal region of Vif (a.as 14-17), was showed to be critical for the strength of binding with A3G (Schrofelbauer, B. *et al*, 2006). However, coimmunoprecipitation assays have demonstrated that additional binding sites were required for a stabilization of this interaction, and that the region 128 of A3G could interact with Vif

at amino acid 15 or 17 (Schrofelbauer B. *et al*, 2006). More recently, the interaction of Vif with A3G has been mapped to amino acids 40 to 44 (YRHHY) of HIV-1 (Mehle A., *et al*, 2007; Russel R. and Pathak V., 2007). However, other amino acids in Vif may also contribute to A3G binding.

A striking feature of HIV-1 Vif is the high concentration of conserved tryptophans in its N-terminal region (Lee T. H., *et al*, 1986), which may play a role in a differential recognition of A3G and A3F, by Vif (Tian, C. *et al*, 2006). Furthermore, Mehle *et al*. demonstrated that amino acids 40 to 71 in the N-terminus of Vif contain a nonlinear binding site for A3G and that His 42/43 are important for Vif-A3G binding and Vif-mediated degradation of A3G *in vivo* (Mehle A., *et al*, 2007). Recently, a new Vif motif comprising amino acids 52 to 72 was identified, and was shown to be important for the interaction and for the Vif-mediated degradation and virion exclusion of A3G (He Z. *et al.* 2008).

In this work we aimed to study the *in vivo* interaction between Vif and A3G through oligomerization assisted by the interaction between two GCN4 leucine zipper domains.

Homodimerizing GCN4 parallel coiled-coil leucine zipper-forming sequences (ZIP) are known to interact *in vivo* and this type of coiled coil interaction has been intensively used as a rapid and efficient *in vivo* screening strategy for identification of interacting pairs of heterodimerizing polypeptides (Pelletier JN, e tal., 1999; Newman JR, 2003; Alber T. 1992; Arndt KM,*et al.*, 2000). In addition, leucine zippers have also been found to associate in an anti-parallel orientation. In this study, we decided to use both parallel and antiparallel leucine zipper-sequence domains to study the importance of interacting domains between Vif and A3G, as well as to ascertain on the spatial orientation of this interaction.

# EXPERIMENTAL PROCEDURES

### Plasmids

pcDNA3.1-A3G-HA (1) was used for the expression of C-terminally epitope- tagged wild type human A3G protein and was a kindly supplied by Strebel K.. pcDNA-HVif was obtained from the NIH AIDS Research and Reference Reagent Program (Nguyen, K. L., 2004 ). pcDNA3.1 Zeo(+) was obtained from the NIH (Catalog number: 10077). Homodimerizing GCN4 parallel coiled-coil leucine zipper (Zip) in fusion with β-lactamase fragments was kindly supplied by W. Michnick and has been previously described (Galarneau *et al*, 2002). The plasmid pEGFP-C1 was obtained from Clontech. Anti-parallel GCN4 leucine zipper domains were kindly supplied by Regan L. (Thomas J., *et al.*, 2004). The Vif-defective molecular clone pNL4.3ΔVif has been previously described (Karczewski, M. K., and K. Strebel. 1996) and was used for the production of virus stocks.

### DNA constructs

GCN4 leucine zipper (Zip) was obtained from the original plasmid pcDNA3.1-Zip-15a.a-BLF[1] (Galarneau *et al*, 2002) and was used as the cloning vector for the subsequent steps. For the insertion of A3G, Vif, Vif SLQ, Vif C-terminal region (amino acids 98 to 192), and EGFP downstream of the Zip sequence and in fusion with a 15 a.a flexible polypeptide linker $(GGGGS)_3$, each sequence was amplified by PCR using specific oligonucleotides (A3G: Zip-Apo-F- XhoI and Zip-Apo-R-ClaI; Vif and Vif SLQ: Zip-Vif-Full-XhoI and Zip-Vif-R-ClaI; Vif 92-192: Zip-VifC-XhoI and Zip-Vif-R-ClaI) containing the restriction site XhoI/ClaI and ligated to the Zip vector linearized with XhoI/ClaI to remove the BLF[1] sequence. This originated the plasmids Zip-A3G, Zip-Vif, Zip-VifSLQ, Zip-Vif 98-192, and Zip-EGFP. Mutation of SLQ region in pcDNA-HVif was accomplished by PCR-based mutagenesis of pcDNA-HVif originating the Vif SLQ>AAA mutant. Vif SLQ>AAA mutant was generated using oligos: SLQ-Vif-NIH-F and SLQ-Vif-NIH-R.

For the insertion of A3G and Vif upstream of the Zip sequence, both sequences were PCR amplified with specific oligonucleotides (A3G: APO-F-RI and APO-GGG-R-NotI; Vif: Vif-R1 and Vif-GGG-R-NotI) containing restriction site EcorI/NotI and coding in frame

for a 15 a.a flexible polypeptide linker $(GGGGS)_3$. PCR product was digested and ligated into the EcorI/NotI site of the linear vector, originating the plasmids A3G-Zip and Vif-Zip.

The anti-parallel GCN4 leucine zipper (CZ) was covalently linked to the Vif sequence by linearization of the plasmid (Zip-Vif) with the restriction enzymes NotI/AscI to remove the original Zip sequence and replace it by CZ, which had been PCR amplified using specific primers (Zip-AP-NotI-F and Zip-AP-AscI-R) containing the restriction site NotI/AscI. This led to the creation of the plasmid CZ-Vif.

A PCR-generated anti-parallel GCN4 leucine zipper (NZ) was inserted downstream of the A3G sequence by one round of PCR using specific oligos (A3G-HA-Not-F and A3G-GGG-AP-R), which introduced an A3G epitope tagged N-terminal HA and 15 a.a flexible polypeptide linker $(GGGGS)_3$ at the C-terminal domain of A3G originating the plasmid A3G-NZ. PCR product was gel purified and was subjected to a second PCR (A3G-HA-amp-F and A3G-AP-R), which inserted the antiparallel leucine zipper (NZ) downstream of the A3G and linker sequences. The final PCR product was purified and digested with the restriction enzymes NotI/XhoI and inserted into the linear vector pcDNA3.1 Zeo+. All constructs were subjected to sequencing analysis for the detection of undesirable mutations. The sequence of primers used is listed in table 1 at the end of this section.

**Antisera**

For detection of APOBEC3G HA-epitope-tagged, an anti-HA HRP monoclonal antibody (Roche) was used. Vif protein was detected using the anti-Vif anti-rabbit antibody (cat# 2221) supplied by the NIH AIDS Research and Reference Reagent Program Catalog. Detection of GFP protein was carried by using anti-GFP mouse monoclonal antibody (Roche). GAPDH was used as a loading control and the antibody used was purchased from Santa-Cruz Biotechnology (GAPDH 6C5: sc-32233).

**Cell culture and transfections**

Human embryonic kidney (HEK) cell line 293T, and P4 cells (HeLa-CD4 LTR-LacZ) were obtained from the NIH AIDS Research and Reference Reagent Program Catalog and were cultivated in Dulbeco`s modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1X penicillin-streptomycin-fungizone mixture and L-Glutamine (Bio Whittaker). For transfections, 293T cells were grown in 6-well plates to about 70% confluency. Cells were transfected using the calcium phosphate precipitation method. A total of 2 to 3 µg of plasmid DNA per well was used, unless otherwise stated. Total amounts of transfected DNA were kept constant in all samples of any given experiment by adding empty-vector DNA (pcDNA3.1Zeo$^+$) as appropriate. Cells were harvested typically 40-44hrs post-transfection.

**Immunoprecipitation analysis**

For coimmunoprecipitation analysis of A3G and Vif, cell lysates were prepared as follows. Cells were washed once with PBS and lysed in 300 µl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100). The cell extracts were clarified at 13,000 _ *g* for 3 min, and the supernatant was incubated on a rotating wheel for 1 h at 4°C with Anti-HA High Affinity Matrix (ROCHE). Immune complexes were washed three times with 50 mM Tris, 300 mM NaCl, and 0.1% Triton X-100, pH 7.4. Bound proteins were were eluted from beads by heating them in sample buffer for 5 min at 95°C and analyzed by immunoblotting.

**Immunoblotting**

For the detection of intracellular proteins, whole-cell-lysates were prepared as follows. Transfected 293T cells were washed once with room-temperature phosphate-buffered saline (PBS) and were suspended in RIPA supplemented with 0, 1% of Triton X and anti-protease (500µl/$10^6$ cells). Cell lysates were mixed with equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125mM Tris-HCL [ph= 6,8], 10% 2-mercaptoethanol, 10% glycerol, and 0,02% bromphenol blue). Proteins were solubilized by boiling for 10 min at 95ºC. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis

and were transferred to polyvinylidene difluoride membranes and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences, Piscataway,NJ) and visualized by enhanced chemiluminescence (Amersham Biosciences).

**Preparation of virus stocks**

Virus stocks were prepared by transfection of 293T cells with appropriate plasmid DNAs. Virus-containing supernatants were harvested 40 to 44 hours after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 X *g*), and unconcentrated clarified supernatants were used for infectivity assays. For determination of viral infectivity, unconcentrated clarified supernatants were used for the infection of HeLa P4 cells.

**Infectivity assay**

To determine viral infectivity, viral stocks were collected and the viral titer determined and normalized by the amount of the protein p24 detected by immunoassay. Hela P4 cells were infected with 100µl of unconcentrated virus supernatant. Briefly, $1{,}5X10^4$ cells were plated in a 96-well plate the day before infection. Forty-eight hours after infection of P4 cells, the single-cycle titer of viruses produced was determined by quantification of the β-galactosidase activity in P4 lysates, using a colorimetric assay based on the cleavage of chlorophenolred-β-D-galactopyranoside (CPRG) by β-galactosidase (Eustice D., *et al.* 1991). Briefly, following elimination of the supernatant, the P4 cells were lysed in 100µl of lysis buffer ($MgCl_2$ 5mM, NP40 0,1% in phosphate buffered saline) and 100µl of reaction buffer (CPRG 6mM in lysis buffer) was added to the cell lysates and incubated from between 5 min and 2 h at 37ºC. Optical densities in the reaction wells were read at 550nm with a reference filter set at 620nm.

**Table 1.**

| *Oligonucleotide* | *Sequence (5' – 3')* |
|---|---|
| Zip-Apo-F- XhoI | ccg ctc gag aag cct cac ttc aga aac aca gtg g |
| Zip-Apo-R-ClaI | cca tcg atg gtt aag cat aat cag gaa cat cgt aag gat atc cac cgt ttt cct gat tct gga gaa tgg |
| SLQ-Vif-NIH-F | ctg cta  gtg cca agt atg ctg ctg ctc cta cct tgt tat gtc ctg ctt |
| SLQ-Vif-NIH-R | gca gca tac ttg gca cta gca gca tta ata aaa |
| Zip- Vif-Full- XhoI | ccg ctc gag gaa aac aga tgg cag gtg atc att g |
| Zip-Vif-R- ClaI | cca tcg atg gct aca gat ctt ctt cag aga tga gtt tct gct cgt gtc cat tcg ttg tat ggc tc |
| Zip-VifC-F-XhoI | ccg ctc gag gac cta gca gac caa cta att cat ctg |
| Zip-Vif-R-ClaI | cca tcg atg gct agt gtc cat tcg ttg tat ggc |
| GFP-XhoI-F | ccg ctc gag gtg agc aag ggc gag gag c |
| GFP-ClaI-R | cca tcg atg gtc act tgt aca gct cgt cca tgc cga g |
| Vif-R1 | cgg aat tcc gat gga gaa ccg gtg gca gg |
| Vif-GGG-R-NotI | ttt tcc ttt tgc ggc cgc ggt gtc cat tca ttg tat ggc tcc |
| APO-F-RI | cgg aat tcc gat gaa gcc tca ctt cag aaa cac ag |
| APO-GGG-R-NotI | ttt tcc ttt tgc ggc cgc gtg aac ctc cac ctc cgg acc cac cac ctc cag agc cac cgc cac cgt ttt cct gat tct gga gaa tgg c |
| Zip-AP-NotI-F | aag gaa aaa acg cgg ccg cac cat ggc aag cga gca gct gg |
| Zip-AP-AscI-R | agg cgc gcc cct gcg cga gtt ttt ttt cca atg |
| A3G-HA-Not-F | aag gaa aaa agc ggc cgc taa cct ata tgt atc ctt acg atg ttc ctg att atg ctt cta agc ctc |
| A3G-GGG-AP-R | gcc tgc aat tct ttt ttg agg gct gaa cct cca cct cct ccg gac cca cca cct cca gag cca ccg cca ccg ttt tcc tga ttc tgg aga atg gc |
| A3G-AP-R | ccg ctc gag tta ctg cgc cag ttc ctt ttt cag agc ttg taa ctc cca ctt cag ctg cgc aag ttc ttt ttt gtt tgc ctg caa ttc ttt ttt gag gg |

# RESULTS

**Functional analysis of leucine zipper fusion proteins.**

In order to study the *in vivo* interaction between Vif and A3G we used a strategy that allows for the spatially directed association of Vif and A3G proteins by oligomerization-assistance of two dimerizing GCN4 leucine zipper (Zip) domains.

This way, we fused both proteins to the N-terminal and C-terminal domains of the Zip sequence. Both N- and C-terminal fusions were expressed *in vivo* and tested for their ability to functionally interact with each other.

First, we examined the ability of Vif fusion proteins to degrade A3G WT and Z-A3G in 293T cells (Fig. 1). Western blot analysis showed that both Vif fusion proteins were able to degrade A3G WT indicating that the presence of Zip in fusion with Vif did not have any deleterious effect on its activity (Fig. 1 C). The same was observed for Z-A3G fusion protein, which was equally degraded by Vif WT and Z-Vif (Fig. 1 D). The Z-VifSLQ mutant, in which the SLQ domain in Vif was replaced by alanines, was used as a control for the absence of A3G degradation confirming the integrity of our constructs (Fig. 1 D).

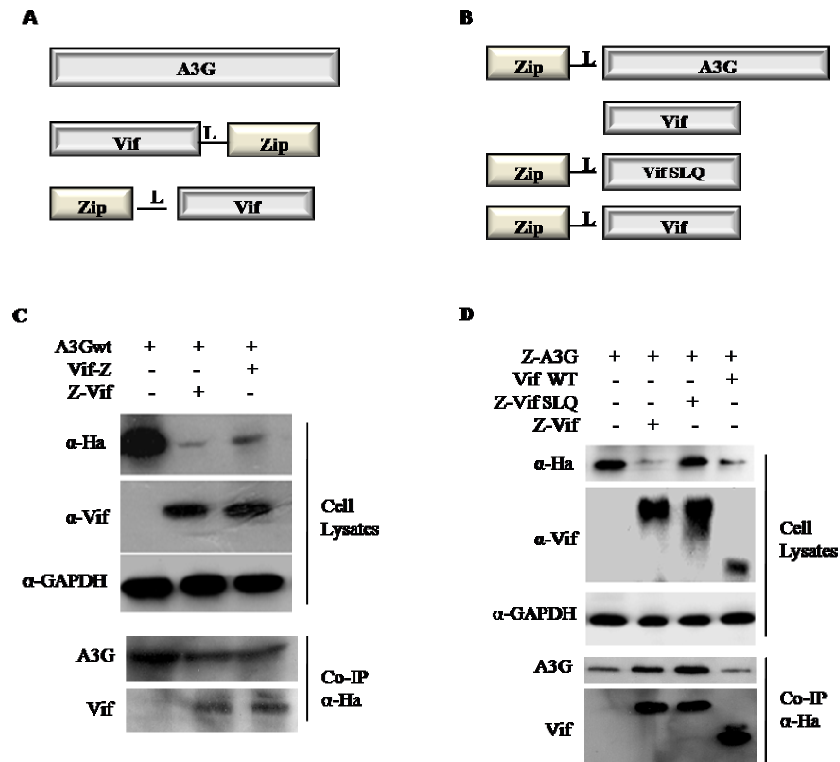


**Fig.1. Analysis of Vif fusion proteins functionality.** (A), (B) Schematic representation of A3G and Vif leucine zipper fusion proteins. 293T cells were cotransfected in 6-well-plates with the plasmids (C) A3G WT (1 µg) in the presence of Vif-Z (2 µg) and Z-Vif (2 µg) or (D) Z-A3G (1 µg) in the presence of Vif WT (2 µg), Z-VifSLQ (2 µg), and Z-Vif (2 µg). 40-44 h later cells were lysed and subjected to western blot analysis.

## C-terminal domain of Vif is necessary but not sufficient to suppress A3G

Previous work demonstrated that the N-terminal region of Vif was involved in binding to A3G (Marin M, *et al.*, 2003; Schrofelbauer, B. *et al*, 2006; Simon, V., 2005; Tian, C. *et al*, 2006; Indrani P. *et al*, 2006). Regarding this, we constructed a Vif fusion protein where the N-terminal region had been replaced by the Zip sequence. In this way, the presence of leucine zipper sequence at the N-terminal region of Vif would allow the protein to functionally

interact with Z-A3G. Therefore, we used this system to evaluate whether the C-terminal domain of Vif alone was sufficient to suppress the activity of A3G. To achieve this, the C-terminal portion of Vif was fused to Zip (Z-Vif 98-192) and the ability of the fusion protein to carry Z-A3G for degradation and inhibit its antiviral activity was tested (Fig. 2). Interestingly, Z-Vif 98-192, which contains the HCCH and the SLQ motifs, had no detectable effect on Z-A3G steady-state (Fig 2 B, lane 2) and was not able to suppress its antiviral activity (Fig. 2 D).

In contrast, the same was not observed when the Z-Vif was used instead of Z-Vif 98-192. In fact, degradation of Z-A3G protein was higher than the one observed in the presence of Vif WT, as seen in figure 2 B (lanes 3 and 4). This seems to indicate that the fusion of A3G and Vif to Zip allowed a stabilization of both proteins in a way that favoured this interaction. Therefore, we conclude that despite the interaction of Vif and A3G through the N-terminal Zip domain, the C-terminal portion of Vif was not sufficient to carry Z-A3G for degradation. This result indicates that the C-terminal domain of Vif is necessary but not sufficient to suppress A3G, suggesting that the interaction between Vif and A3G must require specific domains localized at the N-terminal region of Vif protein that were absence in this construct.

**Vif-A3G interaction through oligomerization assisted by antiparallel leucine zippers**

Parallel and antiparallel coiled coils usually associate because of the interaction of hydrophobic residues that are buried at the peptide-peptide interface and also due to charge-charge interactions between "edge" positions (Aber T. 1992; Adamson J. G. *et al.*, 1993; Lupas A. 1996).

In order to study the topology of the Vif-A3G interaction we fused strongly interacting antiparallel leucine zippers to the N- and C-terminal domains of Vif and A3G, respectively. By changing the placement of charge-charge interactions, that could result in charge-charge clashes in the parallel orientation, we aimed to test whether antiparallelism would favour the interaction between Vif and A3G in order to better understand the topology of this interaction. The results obtained showed that the antiparallel orientation of Vif-A3G did not favour the interaction between the two fusion proteins resulting in less than 25 % degradation of A3G. (Fig. 2 C, lane 2).

Interestingly, antiparallel interaction resulted in an efficient inhibition of A3G antiviral activity (Fig. 2 D). This was the only situation where A3G activity was inhibited in the absence of degradation. As seen in Fig 2 D, A3G activity was greatly inhibited when both proteins interacted in a parallel orientation. The only exception occurred when the N-terminal domain of Vif was absent (Z-Vif 98-192).

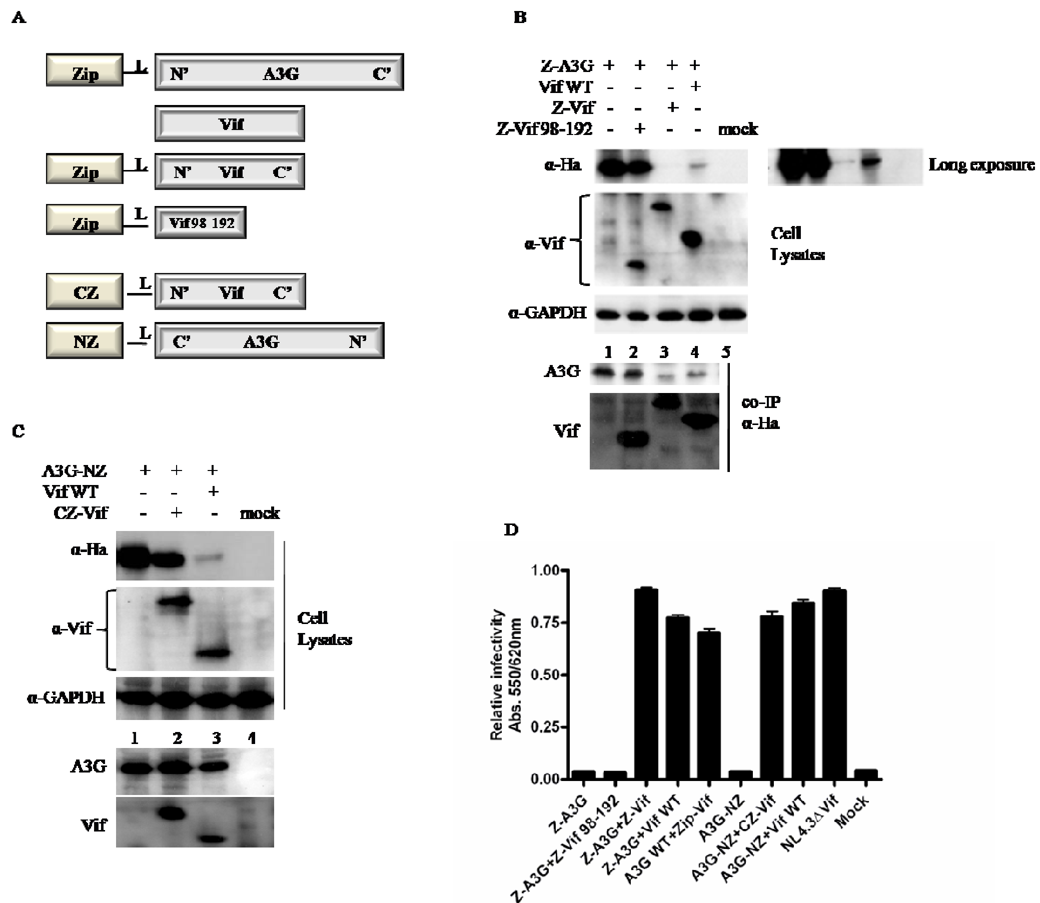


**Fig. 2. Analysis of A3G fusion protein degradation by parallel and antiparallel leucine zipper orientations.** (A) Schematic representation of the A3G and Vif leucine zipper fusion proteins used. 293T cells were cotransfected in 6-well-plates with the plasmids: (B) Z-A3G (1 µg) in the presence of Vif WT (2 µg), Z-Vif (2 µg), or Zip-Vif 98-192 (2 µg) plasmids; (C) A3G-NZ (1 µg) in the presence of CZ-Vif (2 µg), or Vif WT (2 µg) plasmids. 40-44 h later cells were lysed and subjected to western blot analysis.(D) Phenotypic analysis of A3G Zip fusions antiviral activity: HIV-1ΔVif virions were produced by cotransfecting 293T with pNL4.3ΔVif (3 µg) in the presence of WT or Zip fusion proteins in the same conditions as described above. 48 h after transfection viruses were collected and used to infect HeLa P4 cells ($1.5X10^4$). Relative infectivity values were detected by measuring the absorbance of β-gal activity at 550/620nm, in a CPRG assay.

One of the advantages of this strategy is that it allowed us to fuse both Vif and A3G to the C- and N- terminal domains of the Zip sequence. First, we showed that Z-Vif was able to degrade Z-A3G protein and inhibit the antiviral activity of the latest (Fig. 2 B, D).

In a different attempt to assess the topology of the *in vivo* interaction of both proteins, we decided to express both proteins upstream and in fusion with the leucine zipper sequence. The schematic representation of the Zip fusions is represented in figure 3 A.

Initially, we expected to observe the same degradation profile in both cases, as all the fusion proteins were shown to be active in the context of their ability to inhibit HIV-1 infection (Z-A3G and A3G-Z) and A3G WT antiviral activity (Z-Vif and Vif-Z) (Fig. 3 E). However, when we cotransfected A3G-Z in the presence of Vif-Z the same was not observed. In fact, Vif-Z only slightly reduced the expression of A3G-Z in 293T cells, when compared to Vif WT (Fig. 3 C, lane 2).

The explanation for this discrepancy must rely on the inability of Vif-Z protein to interact with the Cullin5-EloB/C complex and carry A3G-Z protein for degradation. Homodimerizing leucine zipper interactions are very strong interactions and we hypothesized that when Vif-Z is coexpressed with A3G-Z, the C-terminal domain of both fusion proteins is in such a close proximity that does not allow the Vif-Cul5-BC complex to be formed and consequently, ubiquitination of A3G-Z protein does not occur. In the first situation, when Z-Vif is coexpressed *in vivo* with Z-A3G, the interaction occurs through the N-terminal domain of both proteins, allowing the C-terminal domain of Z-Vif protein to be free to recruit the Cullin5-EloB/C complex and suppress the activity of Z-A3G.

These results show, once more, the importance of the C-terminal domain of Vif to carry an efficient degradation of A3G protein and are in agreement with previously work that showed that the interaction between Vif and A3G alone is not sufficient to suppress the activity of A3G (Yu, X., *et al.*, 2003; Mariana Marin *et al*, 2003).

Furthermore, following the same approach used for Z-A3G, we coexpressed A3G-Z in the presence of Vif WT, Z-Vif, and Z-Vif 98-192 in 293T and looked for A3G-Z degradation. The schematic representation of the expected spatial orientation of the fusion proteins is represented in figure 3 B. Interestingly, when compared with the results showed in figure 2 B, neither Z-Vif or Z-Vif 98-192 were able to take A3G-Z for

degradation (Fig. 3 D) and inhibit its antiviral activity (Fig. 3 E). This seems to indicate that both proteins need to be in close proximity in order for Vif to be able to inhibit A3G.

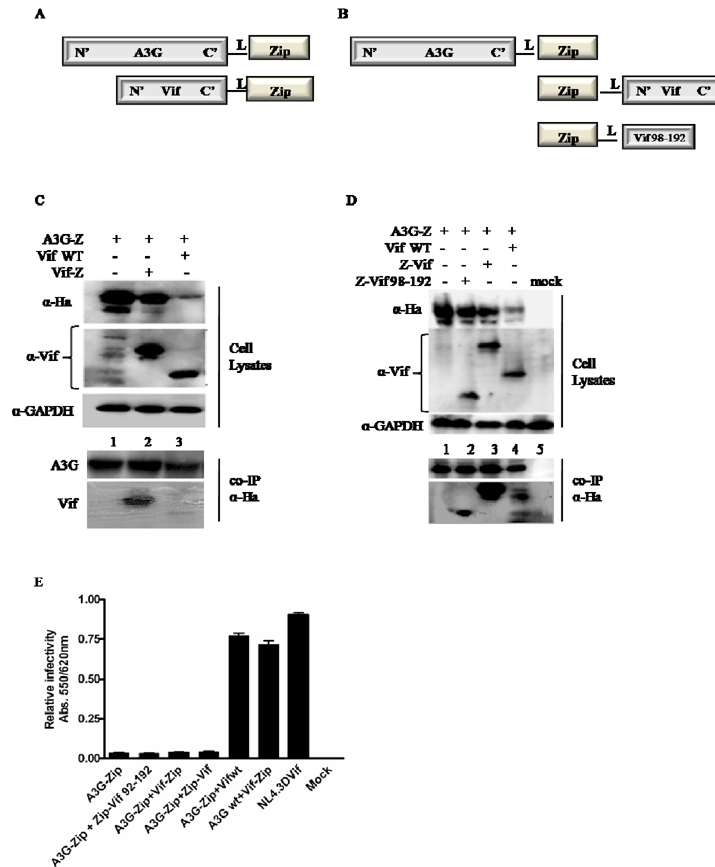


**Fig. 3. Analysis of degradation profiles of A3G-Z fusion protein.** (A), (B) Schematic representation of A3G and Vif leucine zipper fusion proteins used. 293T cells were cotransfected in 6-well-plates with the plasmids A3G-Z (1 µg) in the presence of Vif WT (2 µg), Vif-Z (2 µg) (C) or with Z-Vif (2 µg) and Z-Vif$_{98-192}$ (2 µg) plasmids (D). 48 h later cells were lysed and subjected to western blot analysis. (E) Phenotypic analysis of A3G-Z antiviral activity in the presence HIV-1ΔVif virus. Viral stocks were produced by transfection of 293T in the same conditions as described above. 48 h after transfection viruses were collected and used to infect HeLa P4 cells (1.5X10$^4$). Relative infectivity values were detected by measuring the absorbance of β-gal activity at 550/620nm, in a CPRG assay.

### Specific *in vivo* interaction between Vif and A3G

To determine whether the interaction between Vif and A3G was specific for these two proteins we constructed a plasmid encoding the protein EGFP downstream and in fusion with the Zip sequence (Z-EGFP). Next, we coexpressed Z-EGFP and Z-Vif in 293T cells and determined if the EGFP fusion protein was degraded by Vif.

The result obtained showed that the steady-state of the Z-EGFP protein was not affected by the presence of Vif, indicating that the Vif protein was unable to take EGFP protein for degradation despite the interaction through their N-terminal Zip domain.

This led us to conclude that the interaction between Vif and A3G is specific for these two proteins requiring the involvement of specific domains in the N-terminal region of Vif.

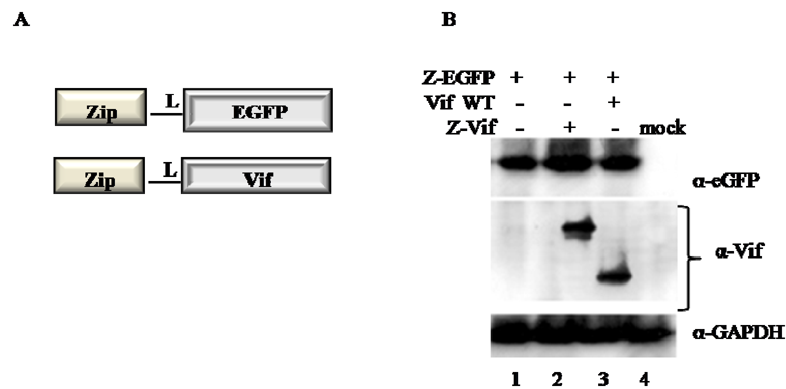


**Fig. 4. Determination of EGFP degradation by Vif.** (A) Schematic representation of EGFP and Vif Zip fusions. (B) 293T cells were cotransfected in 6-well-plates with the plasmids Z-EGFP (1 µg) in the presence of Vif WT (2 µg) or Z-Vif (2 µg). 48 h later cells were lysed and subjected to western blot analysis.

**DISCUSSION**

*In vitro* binding assays and cell based assays have been commonly used to identify binding regions between Vif and A3G. However, these approaches offered some disadvantages as the *in vitro* processing steps make it difficult to assess the *in vivo* significance of the results. In addition, cell based assays are often complicated due to the relative instability or mislocation of Vif deletion mutants, probably due to improper folding.

In this study, we used a novel approach for studying the Vif-A3G interaction *in vivo*. The strategy used ensures that both proteins will be brought together, regardless of their structure, in a directed and productive manner and provided a valuable toll in understanding the role of Vif's N- and C-terminal domains in the *in vivo* interaction with A3G.

Our results showed that both Vif and A3G were able to interact through their leucine zipper N-terminal domain. However, Z-Vif 98-192, which contains only the C-terminal domain of Vif, was not able to induce the degradation of A3G despite having all the domains required for the recruitment of Cul5, EloB and EloC E3 ubiquitin ligase complex. Therefore, we concluded that the reduced ability of Vif to mediate A3G degradation could not be attributed to a reduced interaction with the E3 ligase complex. It is possible that the absence of one or more specific domains in the N-terminus of Vif may have interfered with the binding to A3G. In fact, a previous work reported the existence of a Vif mutant, VifL72S, that had its ability to suppress A3G reduced in 75% despite being able to efficiently bind to A3G and assemble with the ubiquitin ligase complex (He Z. *et al.* 2008).

In addition, it was recently found that in one particular human T cell line, CEM-T4, A3G and A3F were unable to block HIV-1 replication, even in the absence of Vif (Han Y. *et al.*, 2008). Further analysis indicated that this cell line lacked a cellular factor that was very critical for A3G/A3F antiviral activity. They suggested that in order to A3G/A3F inhibit HIV-1 replication it required the presence of a cell factor that was present in some human cell lines but absent in others.

Thus, it is possible that the binding of Vif to A3G must involve an additional co-factor that is probably linked to the N-terminus of Vif and may be responsible for the interaction and consequent degradation of A3G.

When we tested the topology of Vif-A3G interaction, antiparallel orientation resulted in only approximately 25% degradation of A3G indicating that this orientation did not favour the interaction of Vif with A3G. However, despite not being degraded, A3G antiviral activity was inhibited. Thus, Vif may use an alternative mechanism to inactivate A3G without accelerating its destruction in the 26S proteasome.

From an evolutionary perspective, it is possible that the topology of Vif-A3G interaction may have evolved from an antiparallel to a parallel interaction favouring inactivation of A3G through proteasomal degradation.

In general, these results underline the importance of the topological orientation of Vif and A3G for an efficient degradation of the latest. We suggest, based on our results, that Vif-A3G interaction is indirect and can be abrogated by changing the spatial orientation of the two proteins. In addition, the presence of a co-factor favouring the interaction between both proteins is an appealing hypothesis.

Interaction of Vif with EGFP through the Zip domain did not result in EGFP degradation. This result shows that the Vif-targeted degradation of A3G is specific and supports the previous hypothesis of an involvement of a specific additional co-factor in the interaction between Vif and A3G.

Specific residues in both Vif and A3G have been previously described as being involved in the binding between the two proteins. However, in the present study, binding between both proteins through specific regions was overcome by the presence of Zip domain. Then, if the C-terminal domain of Vif is not sufficient to target A3G for degradation in the proteasome, it is possible that the N-terminal domain of Vif may be involved in the binding to A3G through a specific co-factor that it's linked to it.

In conjunction, our results support a previous report that raises the hypothesis of the existence of an endogenous co-factor that may be required for an efficient interaction and subsequent inactivation of A3G in the cell.

This strategy also proved to be efficient in providing a general approach for protein dissection and interaction domains studies, and can be used in the future as a novel approach for a rapid *in vivo* screening of Vif-A3G interacting domains. It also offers the advantage of allowing it to be done in an *in vivo* environment without any deleterious effect on the structure and activity of the proteins.

# CHAPTER 4

## Assessment of HIV-1 Vif-APOBEC Interactions Based on a β-Lactamase Protein Fragment Complementation Assay.

**Iris Cadima-Couto,** Saraiva N, Gonçalves J. (2008). Assessment of HIV-1 Vif-APOBEC Interactions Based on a β-Lactamase Protein Fragment Complementation Assay. *Retrovirology. (Submited)*

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## ABSTRACT

The Vif-APOBEC3G interaction is of particularly interest in a way that it provides a compelling target for novel therapeutic strategies for treating HIV-1 infections. Disruption of A3G-Vif interaction is predicted to rescue A3G expression and virion packaging, therefore stimulating natural antiviral infectivity. In order to facilitate the rational design of inhibitors of the A3G-Vif interaction molecular genetics experiments using alanine-scanning mutations and multiple substitutions at key residues have been employed to define features of APOBEC3G that are involved in the interaction with Vif. To study this interaction, we used a β-lactamase complementation assay in which the inactive domains of the enzyme are fused to each of the proteins in question. The interaction of our complementary proteins will result in a functional β-lactamase capable of hydrolysing a substrate measurable by spectrophotometry. In this study, we produced several mutants of both Vif and A3G bearing mutations on key residues that are thought to be crucial for the interaction between these two proteins. As a result, we were able to achieve a quantitative assessment of HIV-1 Vif-A3G interactions, which allowed us to do a fine dissection of the regions involved in this interaction. We also used this strategy to study the interaction of Vif with other members of the APOBEC family. The results obtained indicate that the DRMR region in Vif may be involved in the selection of the APOBEC to inactivate. In addition, interaction of Vif with APOBEC2 (A2) indicates that the antiviral activity of the enzyme may not be necessarily dependent on the interaction with Vif. We suggest that despite the ability of A2 to interact with Vif, an additional co-factor may be required in order to A2 to be able to exert antiviral action.

## INTRODUCTION

APOBEC3G (A3G) is a single-stranded DNA cytidine deaminase that targets retroviral minus-strand DNA and has potent antiviral activity against diverse retroviruses. In order to successfully replicate in their hosts, lentiviruses such as HIV-1 and simian immunodeficiency virus (SIV) encode the Vif protein, which induces polyubiquitination and degradation of multiple APOBEC3 (A3) molecules (Conticello SG. *et al*, 2003; Liu B. *et al.* 2005; Liu B. *et al.* 2004; Marin M. *et al.* 2003; Mehle A. *et al.* 2004; Sheehy A. *et al.* 2003; Stopak A. *et al.* 2003; Yu X *et al.* 2003). All members of the A3 family have one (A3A, A3C, and A3H) or two (A3B, A3DE, A3F, and A3G) cytidine deaminase motifs containing a conserved consensus sequence that coordinates $Zn^{2+}$, a glutamic acid residue involved in proton shuttling, and critical aromatic residues involved in RNA binding (Jarmuz A *et al.* 2002). Followed by A3G, APOBEC3F (A3F) displays the most potent effects against HIV (Bishop KN *et al* 2004). Other A3 family members, A3A, A3B, and A3C have weaker anti-HIV-1 activities and are only partially degraded by Vif (Marin M. *et al.* 2008). APOBEC2 (A2) is a member of the family of polynucleotide cytidine deaminases (CDAs). However, like other editing enzymes of the cytidine deaminase superfamily, A2 has low, but definite, intrinsic cytidine deaminase activity (Liao W. *et al.* 1999).

Disruption of A3G-Vif interaction is predicted to rescue A3G expression and virion packaging, therefore stimulating natural antiviral infectivity. Identification of protein biding partners between Vif and A3G proteins is one of the major challenges for the development of new antiviral drugs.

*In vitro* binding assays and cell based assays have been commonly used to identify binding regions between Vif and A3G. However, these approaches offer some disadvantages as the *in vitro* processing steps make it difficult to assess the *in vivo* significance of the results, and cell based assays are often complicated due to the relative instability or mislocation of Vif deletion mutants, probably due to improper folding (Marin M *et al*, 2003). The yeast two-hybrid analysis method it's been commonly used in the study of protein-protein interactions (Fields S and Song O. 1989), although this method has significant limitations, in particular because it must be made in yeast, it requires nuclear importation and function, it can be confounded by proteins that activate transcription in the absence of a binding partner, and originates abundant false positives (Magliery T. J., *et al*, 2004). In order to circumvent some of these problems a number of

assays have been developed, which include bacterial two-hybrid systems and functional interaction traps based on fusion of dissected fragments (Hays, L. *et al*. 2000; Pelletier *et al*., 1999; 9; Zhu, H. *et al*. 2001).

In this work we used a new strategy based on interaction-assisted folding of rationally designed fragments of enzymes for studying Vif-A3G interactions. Protein Fragment Complementation Assays (PCA) have been used for many purposes, including the study of protein folding (Ladurner, A. *et al*, 1997; de Prat Gay *et al*, 1994), gene structure (Ullmann A. *et al*, 1967), the role of primary sequence in determining tertiary structure of proteins (Taniuchi, H. *et al*, 1971), the role of tertiary structure elements in enzyme catalysis (Shiba, K. *et al*, 1992), to probe macromolecular assembly (Tasayco, M. L. *et al*, 1992), and to test theories on protein evolution (Bertolaet, B. L. *et al*, 1995). More recently, Pelletier and others (5, 6, 7, 18, 20) have used the protein fragment complementation approach for the *in vivo* detection of protein-protein interactions. PCA relies on engineering reporter protein fragments that exhibit no functional activity by themselves and do not spontaneously fold. When these fragments are fused to interacting proteins, the interaction of the hybrid proteins will bring the two reporter fragments into proximity. The fragments will then fold into the active 3D structure of the complete reporter protein. Several proteins have been described as reporter proteins in PCAs such as, β-lactamase, dihydrofolate reductase, *Renilla*, luciferases, and GFP and yellow fluorescent protein (YFP) (Galarneau *et al*, 2002; Spotts, J. M., *et al*, 2002; Remy, I. *et al*., 2001; Paulmurugan, R., *et al*, 2002; Luker, K. E., *et al*, 2004; Ghosh, I., *et al*, 2000; Hu, C. D., *et al*, 2002; Remy, I., *et al*, 2004). GFP and YFP PCAs have particularly been simple for detection and library screening of cytosolic, membrane, and nuclear protein-protein and protein-RNA interaction (Remy, I., *et al*, 2004; Remy, I., *et al* 2004; de Virgilio, M., 2004; Rackham, O. *et al*, 2004).

We used the PCA technique based on the enzyme TEM-1 β-Lactamase to study the interaction between HIV-1 Vif and the human A3G protein. This strategy is well described elsewhere (Michnick SW *et al.* 2000; Pelletier JN *et al*, 1998) and offers the advantage of allowing us to perform the assays in an *in vivo* system, without the requirement for co-immunoprecipitation assays. In parallel, we have also used co-immunoprecipitation in order to do a comparative analysis and validate the results obtained.

# EXPERIMENTAL PROCEDURES

### Plasmids

pcDNA3.1-A3G-HA (1) was used for the expression of C-terminally epitope- tagged wild type human A3G protein and was a kindly supplied by Strebel K.. pcDNA-HVif was obtained from the NIH AIDS Research and Reference Reagent Program (Nguyen, K. L., 2004 ). pcDNA3.1 Zeo(+) was obtained from the NIH (catalogue: 10077). The *Vif*-defective molecular clone pNL4.3ΔVif has been previously described (Karczewski, M. K., and K. Strebel. 1996) and was used for the production of virus stocks. Homodimerizing GCN4 parallel coiled-coil leucine zipper (Zip) in fusion with β-lactamase fragments was kindly supplied by W. Michnick and has been previously described (Galarneau *et al*, 2002). HVif mutants YRHHY>A5, DRMR>A4 and DRMR>SEMQ without the SLQ>A3 mutation used in the degradation assays were a kind gift from Pathak V.

### DNA constructs

Fragments of β-lactamase were obtained from the original plasmid pcDNA3.1-Zip-15a.a-BLF[1] ($ZIP_{-B1}$ and $ZIP_{-B2}$) and pcDNA3.1-Zip-15a.a-BLF[2] (Galarneau *et al*, 2002) and was used as the cloning vector for the subsequent steps. Each cloning vector was linearized with restriction enzymes NotI/AscI to remove Zip sequence. For the insertion of HVif, A3G-HA, A3F-HA, A3C-HA, A2-HA in fusion with β-lactamase fragment , each sequence was amplified by PCR using specific oligonucleotides (HVif: Not-HVif-F and Asc-HVif-R; A3G-HA: apo3g-amp-F and 4BL-B1-Asc-R; A3F-HA: F-not and R-Asc; A3C-HA: Apo3C-F-Not and Apo3C-R-Asc; A2-HA: a2F-Not and a2R-Asc). PCR products were digested with NotI/AscI restriction enzymes and ligated to linear vector. This originated the plasmids: $Vif_{-B2}$, $A3G_{-B1}$, $A3G_{-B2}$, $A3F_{-B1}$, $A3C_{-B1}$, and $A2_{-B1}$.

Mutation of SLQ region in pcDNA-HVif was accomplished by PCR overlap method of pcDNA-HVif originating the Vif SLQ>AAA mutant. Vif SLQ>AAA mutant was generated using oligos: SLQ-Vif-NIH-F and SLQ-Vif-NIH-R. Substitution of Vif YRHHY and DRMR regions by alanines was accomplished by PCR using specific oligonucleotides (Vif YRHHY: Vif-YRHHY-F and Vif-YRHHY-R; Vif DRMR: Vif-DRMR-F and Vif-DRMR-R). Vif

DRMR region was replaced by SEMQ sequence by PCR using oligonucleotides: Vif-SEMQ-F and Vif-SEMQ-R, originating Vif SEMQ plasmid DNA. Vif YRHHY, DRMR, and SEMQ mutations were introduced as described in the previous originated Vif SLQ>AAA sequence.

A3G mutants R122A, W127L, and D128K were generated by PCR using specific oligonucleotides (A3G R122A: A3g-R122A-F and A3g-R122A-R; A3G W127L: A3g-127L-F and A3g-127L-R; A3G D128K: A3g-D128K-F and A3g-D128K-R). All Vif and A3G PCR generated mutants were digested with restriction enzymes NotI/AscI and ligated to the linear vector in fusion with β-lactamase fragment. This originated the plasmids: $Vif_{SLQ>A3-B2}$, $Vif_{SLQ>A3 + YRHHY>A5-B2}$, $Vif_{SLQ>A3 + DRMR>A4-B2}$, $Vif_{SLQ>A3 + SEMQ>A4-B2}$, $A3G_{R122A-B1}$, $A3G_{W127L-B1}$, $A3G_{D128K -B1}$. All constructs were subjected to sequencing analysis for the detection of undesirable mutations. The sequence of primers used is listed in table 2 at the end of this section.

**Antisera**

For detection of A3G HA-epitope-tagged, an anti-HA HRP monoclonal antibody (Roche) was used. Anti-HA High Affinity Matrix (ROCHE) was used for co-immunoprecipitation assays. Vif protein was detected using the anti-Vif anti-rabbit antibody (catalogue number: 2221) supplied by the NIH AIDS Research and Reference Reagent Program Catalogue. Detection of GFP protein was carried by using anti-GFP mouse monoclonal antibody (Roche). GAPDH was used as a loading control and the antibody used was purchased from Santa-Cruz Biotechnology (GAPDH 6C5: sc-32233).

**Cell culture and transfections**

Human embryonic kidney (HEK) cell line 293T, and P4 cells (HeLa-CD4 LTR-LacZ) were obtained from the NIH AIDS Research and Reference Reagent Program Catalogue and were cultivated in Dulbeco`s modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1X penicillin-streptomycin-fungizone mixture and L-Glutamine (Bio Whittaker). For transfections, 293T cells were grown in 60-mm plates to about 70% confluency. Cells were transfected using the calcium phosphate precipitation method.

A total of 30 µg of plasmid DNA was used, unless otherwise stated. Total amounts of transfected DNA were kept constant in all samples of any given experiment by adding empty-vector DNA pcDNA3.1 empty plasmid DNA as appropriate. Cells were harvested typically 40-44hrs post-transfection.

**Immunoprecipitation analysis**

For coimmunoprecipitation analysis of A3G and Vif, cell lysates were prepared as follows. Cells were washed once with PBS and lysed in 500 µl of lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM NaCl, and 1% Triton X-100) containing complete protease inhibitor cocktail (Roche). The cell extracts were clarified at 13,000 _ *g* for 3 min, and the supernatant was incubated on a rotating wheel overnight at 4°C with Anti-HA High Affinity Matrix (ROCHE). Immune complexes were washed three times with 50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM NaCl, and 1% Triton X-100 pH 7.4. Bound proteins were eluted from beads by heating them in sample buffer for 5 min at 95°C and analyzed by immunoblotting.

**Preparation of virus stocks**

Virus stocks were prepared by transfection of 293T cells with appropriate plasmid DNAs. Virus-containing supernatants were harvested 40 to 44 hours after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 X *g*), and unconcentrated clarified supernatants were used for infectivity assays. For determination of viral infectivity, unconcentrated clarified supernatants were used for the infection of HeLa P4 cells.

**Infectivity assay**

To determine viral infectivity, viral stocks were collected and the viral titer determined and normalized by the amount of the protein p24 detected by immunoassay. Hela P4 cells were infected with 100µl of unconcentrated virus supernatant. Briefly, $1{,}5X10^4$ cells were plated in a 96-well plate the day before infection. Forty-eight hours after infection of P4 cells, the single-cycle titer of viruses produced was determined by quantification of the β-galactosidase activity in P4 lysates, using a colorimetric assay based on the cleavage of chlorophenolred-β-D-galactopyranoside (CPRG) by β-galactosidase (Eustice D., *et al.* 1991). Briefly, following elimination of the supernatant, the P4 cells were lysed in 100µl of lysis buffer ($MgCl_2$ 5mM, NP40 0,1% in phosphate buffered saline) and 100µl of reaction buffer (CPRG 6mM in lysis buffer) was added to the cell lysates and incubated from between 5 min and 2 h at 37ºC. Optical densities in the reaction wells were read at 550nm with a reference filter set at 620nm.

**β-Lactamase PCA colorimetric assay**

HEK 293T cells were split 24h before transfection at $1x10^5$ in 6-well plates in DMEM containing 10% fetal calf serum, 1X penicillin-streptomycin-fungizone mixture and L-Glutamine (Bio Wittaker). Cells were transfected with 1µg of each plasmid DNA using the calcium phosphate precipitation method. 48 hrs after transfection $5x10^6$ cells were washed twice with PBS and resuspended in 100µl of 100mM phosphate buffer, ph 7.0, then lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation at 16,000g for 2 min at 4ºC. To test B-Lactamase activity, 100µl of phosphate buffer (100mM, ph 7.0) was added to each well to a final concentration of 60 mM, containing 2µl of 10 mM nitrocefin (final concentration 100µM) and 20 µl of cell lysates, and diluted with deionised water.

**Table 2.**

| *Oligonucleotide* | *Sequence (5' – 3')* |
|---|---|
| apo3g-amp-F | ata aga atg cgg ccg cta aac tat atg aag cct cac ttc aga aac aca gtc |
| 4BL-B1-Asc-R | acc gcc acc ggc gcg cca aga agc gta gtc cgg aac gtc |
| F-not | ata aga atg cgg ccg cta aac tat atg tac ccg tac gac gtt ccg gac tac gct tct gcc cag aag gaa gag gct gc |
| R-asc | acc gcc acc ggc gcg cca ctc gag aat ctc ctg cag ctt g |
| Apo3C-F-Not | ata aga atg cgg ccg cta aac tat atg tac ccg tac gac gtt ccg gac tac gct tct aat cca cag atc aga aac ccg atg |
| Apo3C-R-Asc | acc gcc acc ggc gcg cca ctg gag act ctc ccg tag cct tc |
| a2F-Not | ata aga atg cgg ccg cta aac tat atg tac ccg tac gac gtt ccg gac tac gct tct gcc cag aag gaa gag gct gc |
| a2R-Asc | acc gcc acc ggc gcg cca ctt cag gat gtc tgc caa ctt ctc |
| Not-HVif-F | ata aga atg cgg ccg cta aaa tgg aga acc ggt ggc agg |
| Asc-HVif-R | acc gcc acc ggc gcg ccc gtg tcc att cat tga atg gct ccc |
| A3G-R122A-F | cgt tgc cgc act cta cta ctt ctg |
| A3G-R122A-R | cag aag tag tag agt gcg gca acg |
| A3G-127L-F | cct cta cta ctt ctt gga ccc aga tta c |
| A3G-127L-R | gta atc tgg gtc caa gaa gta gat gag g |
| A3G-D128K-F | cta ctt ctg gaa gcc aga tta cca g |
| A3G-D128K-R | ctg gta atc tgg ctt cca gaa gta g |
| SLQ-Vif-NIH-R | ctg cta gtg cca agt atg ctg ctg ctc ct acct tgt tat gtc ctg ctt |
| SLQ-Vif-NIH-F | gca gca tac ttg gca cta agc agc att taa taa aa |

| | |
|---|---|
| Vif-SEMQ-F | agc gaa atg cag att aac acc tgg aag cgc ctg |
| Vif-SEMQ-R | cca ggt gtt aat ctg cat ttc gct cac ctg cca cac aat cat cac c |
| Vif-YRHHY-F | gca gct gca gct gct gag agc acc aac ccc aag att ag |
| Vif-YRHHY-R | cg caa agc taa gga ctg gtt cgc agc gct gca gca gct gct gag agc |
| Vif-DRMR-F | gca gct gca gct att aac acc tgg aag cgc ctg |
| Vif-DRMR-R | cca ggt gtt aat agc tgc agc tgc cac ctg cca cac aat cat cac c |

# RESULTS

### The Protein Fragment Complementation (PCA) strategy.

In order to study the interaction of Vif with A3G and other APOBEC proteins, a new strategy based on protein-assisted folding of rationally designed fragments of enzymes was used based on the enzyme TEM-1 β–lactamase (EC :3.5.2.6). This involves a simple colorimetric *in vitro* assay using the CENTA substrate. TEM-1 β–lactamase is a relatively small monomeric protein that has been well characterized functionally and structurally (Matagne A. *et al.*, 1998; Philipon A. *et al.*, 1998). It has been shown to be easily expressed and it is not toxic to prokaryotic and eukaryotic cells (Matagne A. *et al.*, 1998; Philipon A. *et al.*, 1998). Furthermore, PCA based on β–lactamase can be used in eukaryotes and many prokaryotes without any detectable background activity (Galarneau A. *et al.*, 2002). A schematic representation of the PCA principle is represented in figure 1.

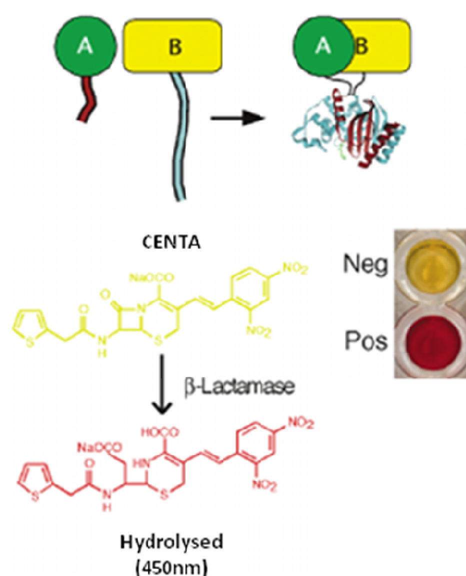



**Fig.1. β-lactamase protein fragment complementation.** Interaction between two proteins (A and B) fused to fragments of β-lactamase brings fragments into proximity allowing correct folding and reconstitution of enzyme activity. The *in vivo* assay uses a substrate (CENTA) that is hydrolysed by the of β-lactamase enzyme. Hydrolysis rates are determined by measuring absorbance at 450 nm. *Figure adapted from: Galarneau, A. et al., 2002. Nat. Biotechnol.* ***20:*** *619-622.*

**Detection of HIV-1 Vif and human A3G interactions using the PCA principle.**

The interaction between Vif and A3G has been extensively studied and characterization of specific domains involved in the binding between the two proteins is crucial for the design of new therapeutic strategies. In order to study this interaction, we replaced the GCN4 leucine zipper (ZIP) sequence from the original plasmid by Vif and A3G proteins. Interaction between Vif and A3G proteins will bring the two fragments of the β–lactamase, termed here as B1 and B2, together allowing the reconstitution of the reporter enzymatic activity. Therefore, by using the PCA principle we aim to characterize several specific domains that have been previously shown to be involved in Vif-A3G interaction. First, we wanted to validate PCA technique by studying the *in vivo* interaction between A3G and Vif wild-type proteins. This way, we fused both proteins to each of the fragments of the β-lactamase enzyme and expressed them *in vivo* in 293 T cells. Two days after transfection cell lysates were analyzed for the presence β-lactamase enzymatic activity, which would be indicative of an interaction between Vif and A3G. We can see from figure 2 B, that β-lactamase hydrolysis rate was highest in the presence of Vif $_{SLQ>AAA\text{-}B2}$ and A3G$_{\text{-}B1}$. *In vivo* detection of Vif-A3G interaction by PCA it's possible when we replace the SLQ region in Vif by alanines, as Vif is no longer able to carry A3G for proteasomal degradation. This will allow us to increase the detection of both proteins in the cell lysates and consequently, if interaction occurs, the two fragments of β-lactamase will come into proximity and enzymatic activity is restored. As shown in the lower panel of figure 2 B, when Vif wild-type is used, interaction with A3G will lead to the degradation of both proteins in the proteasome and consequently no enzymatic activity is detected. Therefore, we decided to continue our assays using the SLQ mutated version of Vif instead of wild-type Vif.

In addition, when A3G$_{\text{-}B1}$ and A3G$_{\text{-}B2}$ were expressed together in 293T we detected a high hydrolysis rate of CENTA substrate, similar to the one obtained for Vif $_{SLQ>AAA\text{-}B2}$ and A3G$_{\text{-}B1}$ (Fig. 2 B upper panel). Thus, *in vivo* dimerization of A3G can be used in order to test the general applicability of PCA assay.

In addition, we also tested the generalizability of PCA by testing the *in vivo* interaction of two known interacting pairs of GCN4 homodimerizing leucine zipper (ZIP) proteins

(Fig. 5 C). Non-interaction proteins, $A3G_{-B1}$ and $Zip_{-B2}$ were also used as a negative control of interaction (Fig. 5 C).

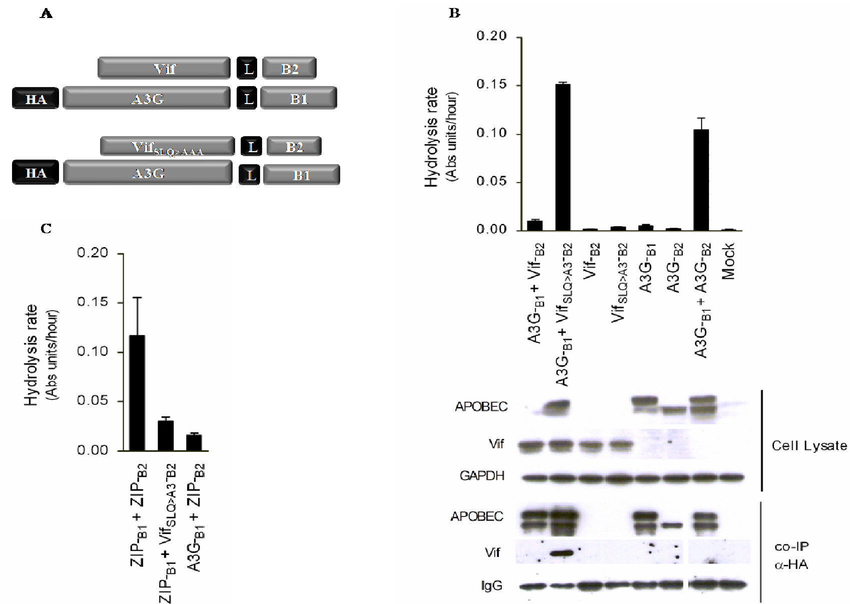



**Fig 2. Validation of PCA colorimetric assay. A.** Schematic representation of Vif, $Vif_{SLQ>A3}$ and A3G fusions to the β-lactamase fragments B2 and B1, respectively. **B**. Upper panel: *In vivo* hydrolysis rate (abs. unit measured at 450 nm) of CENTA for Vif, $Vif_{SLQ>A3}$ and A3G interacting pairs of proteins. Hydrolysis rate of CENTA for fusion proteins alone is also represented as negative controls. Mean hydrolysis rate was determined for three independent experiments and normalized for cell number and protein content**.** Briefly, 20 µg of each plasmid, $Vif_{-B2}$, Vif $_{SLQ>A3-B2}$, $A3G_{-B1}$, $ZIP_{-B1}$, and $ZIP_{-B2}$, and 10 µg of each $A3G_{-B1}$ and $A3G_{-B2}$ was used for transfections in 60-mm plates. DNA amounts were adjusted to 30 µg using empty pcDNA3.1 vector DNA. 48 hrs after transfections, media was removed and cells were washed twice in PBS and were suspended in 100 µl of 100 mM phosphate buffer ($K_2HPO_4$ 50mM, pH=7) for the PCA assay. Lower panel: Western blotting analysis of cell lysates and co-immunoprecipitation. 1/3 of the HEK 293T cells transfected for the PCA assay were collected for co-immunoprecipitation of Vif and A3G proteins using Anti-HA-High-Affinity-Matrix (ROCHE). **C.** Homodimerizing GCN4 leucine zipper are known to interact *in vivo* and were tested to assess the generalizability of the assay. The data are averages of triplicates with the indicated standard deviations.

**PCA efficiency is dependent on protein concentration.**

Detection of Vif-A3G interaction using PCA is dependent on the hydrolysis rate of CENTA substrate by active β-lactamase enzyme. Therefore, intracellular concentration of proteins is crucial for a reliable quantification of protein-protein interactions. In order to determine the best concentration of intracellular Vif and APOBEC proteins we tested different concentrations of intracellular Vif and A3G fusion proteins for the intensity of CENTA hydrolysis rate. We transfected different rations of $Vif_{-B2}$ and $A3G_{-B1}$ in 293T cells and looked for the intensity of the signal obtained for CENTA hydrolysis. The results obtained indicate that the higher the concentration of both proteins the higher the signal obtained (Fig. 3). Thus, we decided to use ratio A3G (10 µg):Vif (20 µg) in the subsequent experiments as this was the ratio that allowed us to better detect A3G-Vif interaction.

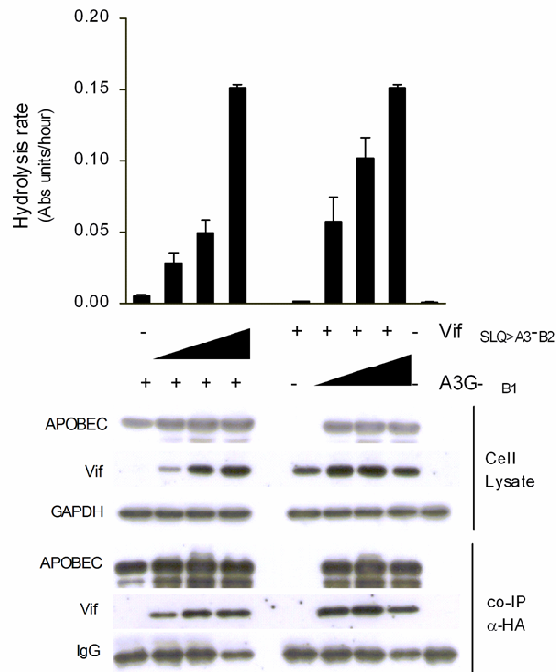



**Fig.3. Intensity of the signal obtained by the hydrolysis rate of CENTA substrate it's directly related to the amount of both proteins expressed in the cells.** 293 T were transfected with increasing amounts of either Vif $_{SLQ>A3-B2}$ or A3G$_{-B1}$ protein: A3G plasmid DNA quantity was kept constant (10 µg) and increasing amounts of Vif was used (0, 5, 10, 20 µg). In parallel, we kept Vif plasmid DNA quantity constant (20 µg) and added increasing amounts of A3G (0, 3, 5, 10 µg). DNA quantities were adjusted to a total of 30 µg by adding empty pcDNA3.1 vector DNA, when appropriate. 48 hours after transfection media was removed and cells were washed twice in PBS and were suspended in 100 µl of 100 mM phosphate buffer ($K_2HPO_4$ 50mM, pH=7) for the PCA assay. Upper panel: *in vivo* hydrolysis rate (abs. unit measured at 450 nm) of CENTA for the combination of protein concentration used. Lower panel: The data are averages of triplicates with the indicated standard deviations. Lower panel: Western blotting analysis of cell lysates and co-immunoprecipitation. 1/3 of the HEK 293T cells transfected for the PCA assay were collected for co-immunoprecipitation of Vif and A3G proteins using Anti-HA-High-Affinity-Matrix (ROCHE). The data are averages of triplicates with the indicated standard deviations.

**Analysis of A3G and Vif mutations using PCA**

Different approaches have been employed to define regions in Vif and A3G that are required for their *in vivo* interaction. One of the key residues that have been extensively studied is the residue 128 in A3G. This region is responsible for the species-specific recognition of A3G by Vif, which is aspartic acid in humans and lysine in African green monkey (AGM) (Bogerd H. *et al.*, 2004; Mangeat B. *et al.*, 2004; Schrofelbauer B. *et al.*, 2004; Xu H. *et al.*, 2004). In addition, Huthoff and Malim demonstrated that amino acids 124-127 are involved in A3G encapsidation into virions, whereas amino acids 129-130 are involved in Vif response (Huthoff H. and Malim M., 2007). It has been suggested that amino acid 122 may also be involved in virion encapsidation (Huthoff H. and Malim M., 2007) but this remains to be confirmed.

Therefore, we used the mutants A3G R122A and A3G W271L to study the interaction with Vif by PCA. This way, each protein sequence was fused to one of the fragments of β-lactamase enzyme.

As shown in figure 4 A, there was a sharp reduction in the ability of the mutants, $A3G_{R122A\ -B1}$ and $A3G_{W271L\ -B2}$, to interact with $Vif_{-B2}$, $Vif_{YRHHY>A5\text{-}B2}$, $Vif_{DRMR>A4\text{-}B2}$, and $Vif_{DRMR>SEMQ\text{-}B2}$. This seems to indicate that A3G residues 122 and 127 may also play a role in the interaction with Vif. The effect of A3G R122A, W127L mutations was also tested in an infectivity assay in which both A3G mutants were co-expressed with Vif wt and HIV-1ΔVif plasmids. The ability of A3G mutants to inhibit HIV-1 infection was absent (Fig. 4 B). This must be due to the inability of both mutants to be encapsidated into viral particles as it was previously suggested by Huthoff and Malim (Huthoff H. and Malim M., 2007).

According to our results (fig. 4 A) alanine-substitutions in Vif YRHHY and DRMR regions resulted in a weak interaction with A3G wt and mutants indicating that the two distinct regions in Vif may be equally important for the interaction with A3G. This was also confirmed in an infectivity assay, which showed the equal inability of both Vif YRHHY and DRMR mutants to overcome A3G wt protein (Fig. 4 B). This is interesting in a way that both Vif YRHHY and Vif DRMR regions have been previously described as being involved in the differential inhibition of A3G and A3F, respectively (Russel R. and Pathak V., 2007). The ability of those Vif mutants to overcome A3G R122A, W127L, and D128K mutants was also assessed in an infectivity assay (Fig. 4 B). As seen in figure 4 B, HIV-1 infectivity was slightly reduced by A3G R122A and W127L in the presence of both YRHHY and

DRMR Vif mutants (Fig. 4 B). However, this reduction was minimal when compared to A3G wt and D128K mutant proteins.

Finally, it is interesting to note that despite the weak interaction between Vif mutants and A3G wt, interaction with A3G R122A, W127L mutants is always weaker when compared to A3G wt (Fig. 4 A). This again, may reinforce our suggestion that A3G residues 122 and 127 may also play a role in the interaction with Vif.

A3G D128K mutant protein was unable to interact with $Vif_{-B2}$ and that is in accordance with previous work (Schrofelbauer at al., 2004). In addition, Vif SEMQ mutant, which has been shown to interact with A3G wt and D128K mutant (Schrofelbauer B. *et al.* 2006), displayed a weak interaction with A3G D128K protein when compared to A3G wt (Fig. 4 A). However, Vif SEMQ was able to counteract both A3G wt and D128K mutant in an infectivity assay rescuing infectivity nearly at the same level detected for W127L A3G mutant (Fig. 4 B). Therefore, residue 128 may be important for the species-specific recognition of A3G by Vif, but may not necessarily be involved in the interaction with Vif.

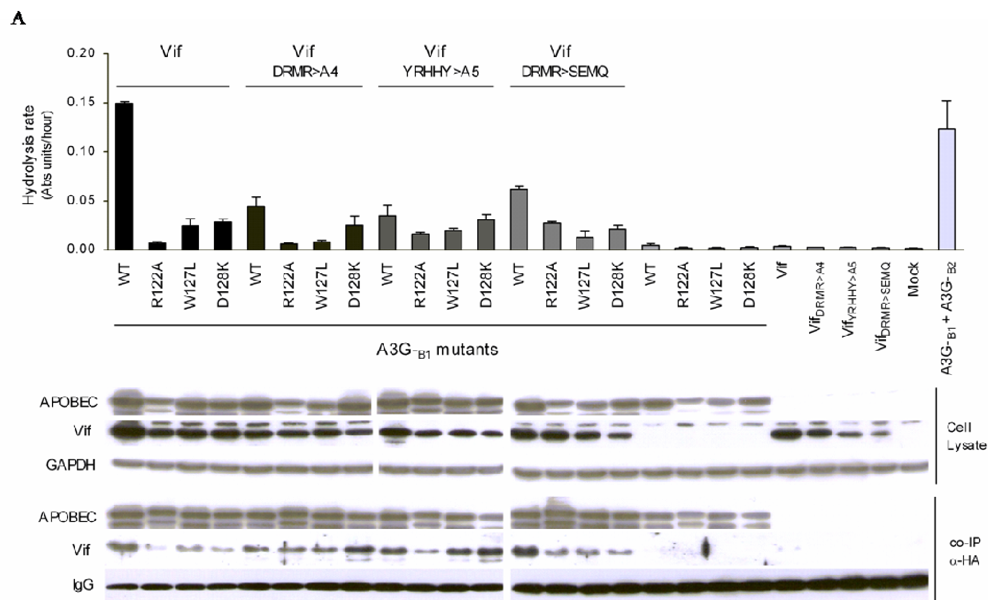



**Fig. 4. A. Effect of Vif and A3G mutations in the interaction.** Top panel: *In vivo* hydrolysis rate (abs. unit measured at 450 nm) of CENTA for the interaction of Vif, $Vif_{DRMR}$, Vif $_{YRHHY}$, and $Vif_{SEMQ}$ with and A3G wt, R122A, W127L, and D128K mutants. Hydrolysis rate of CENTA for fusion proteins alone is also represented as negative controls. Mean hydrolysis rate was determined for three independent experiments and normalized for cell number and protein content**.** Briefly, 10 µg of each A3G plasmid, and 20 µg of each Vif plasmid DNA were used for transfections in 60-mm plates. DNA amounts were adjusted to 30 µg using empty pcDNA3.1 vector DNA when appropriate. 48 hrs after transfections, media was removed and cells were washed twice in PBS and were suspended in 100 µl of 100 mM phosphate buffer ($K_2HPO_4$ 50mM, pH=7) for the PCA assay. The data are averages of triplicates with the indicated standard deviations. Lower panel: Western blotting analysis of cell lysates and co-immunoprecipitation. 1/3 of the HEK 293T cells transfected for the PCA assay were collected for co-immunoprecipitation of Vif and A3G proteins using Anti-HA-High-Affinity-Matrix (ROCHE).

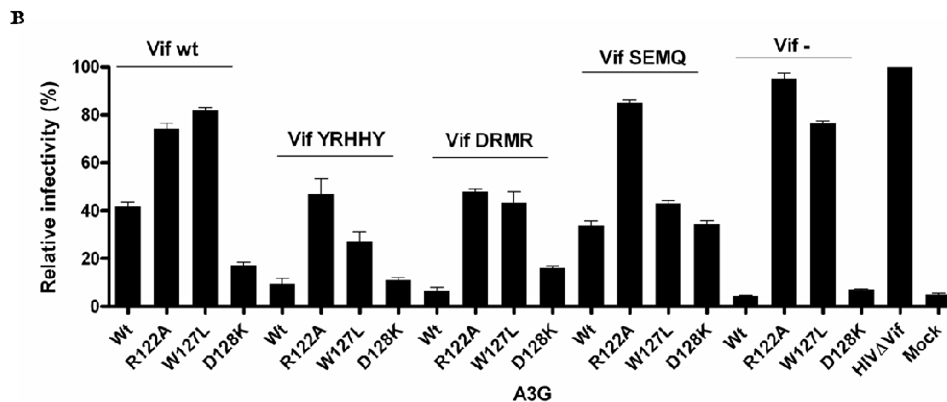



**Fig. 4 B. Infectivity assay.** HIV-1ΔVif virions were produced by cotransfecting 293T with pNL4.3ΔVif (15 µg) in the presence of 5 µg of each A3G plasmid, and 10 µg of each Vif plasmid. DNA amounts were adjusted to 30 µg using empty pcDNA3.1 vector DNA when appropriate. 48 h after transfection viruses were collected and used to infect HeLa P4 cells ($1.5X10^4$). Relative infectivity values were detected by measuring the absorbance of β-gal activity at 550/620nm, in a CPRG assay. The data are averages of triplicates with the indicated standard deviations.

**PCA analysis of Vif interaction with A3F, A3C, and A2.**

The involvement of some deaminases, other than A3G, in HIV-1 infection, as well as the role of Vif in overcoming their action is still poorly understood. Therefore, we decided to use PCA assay to study the interaction of human and SIV Vif with A3F, A3C, and A2, and compare it with the results obtained for A3G.

We have showed that any of the Vif DRMR and YRHHY mutations were able to abolish the interaction with A3G wt. This way, we decided to use the same strategy to study the importance of those regions for the interaction with A3F, A3C, and A2. As seen in figure 5 A, the strongest interaction is detected between Vif and A3G wt proteins. Interaction of Vif wt with A3F was detected, although with a lower signal (Fig. 5 A), which is in accordance with previous reports showing that the anti-HIV-1 effect of A3F is significantly lower than that of A3G (Holmes R. *et al.* 2007; Zennou V. *et al.* 2006). As observed for A3G, Vif DRMR and YRHHY mutants were also unable to interact with

A3F (Fig. 5 A) indicating an equal involvement of those regions in the interaction with both A3G and A3F.

Interaction of Vif wt with A3C was shown to be very weak when compared to A3G, A3F, and A2 (Fig 5 A) and no inhibition of HIV-1ΔVif infection was detected (Fig. 5 B). This is in accordance with previous reports which showed that A3C is relatively resistant to Vif action (Langlois MA *et al.* 2005; Yu Q. *et al.* 2004; Doehle BP. *et al.* 2005). However, when we replaced Vif DRMR region by alanines it was notable the increase in the interaction with A3C (Fig. 5 A). The increase in the signal detected may be a result of the substitution of DRMR region by alanines that may have altered the charge of the protein allowing its interaction with A3C. If this is the reason why Vif DRMR>A4 was able to interact with A3C, then it is possible that this region of Vif may be important for the selection of the deaminase to which it will interact. In addition, A3C was not able to interact with Vif SEMQ (Fig. 5 A). However, it has been reported has having a potent antiviral activity against SIV (Yu Q. *et al.* 2004). In addition, Vif SEMQ was not able to rescue HIV-1Δvif infectivity in the presence of A3C (Fig. 5 B). In fact, only Vif DRMR>A4 mutant was able to rescue infectivity to a level near to what was obtained in the presence of A2.

Substitution of Vif YRHHY to alanines resulted in a reduction in the interaction with all the APOBEC proteins analyzed (Fig. 5 A). We had seen before that Vif YRHHY>A5 mutant was unable to interact with both A3G and A3F. Interestingly, this mutation also abolished interaction with A2 by PCA (Fig. 5 A). In fact, interaction of A2 with Vif wt, Vif DRMR>A4 and Vif SEMQ was also detected, with exception to Vif YRHHY>A5 mutant. It appears that, with exception for Vif YRHHY>A5 mutant, A2 follows a similar interaction pattern to A3G. However, A2 was not able to inhibit HIV-1 ΔVif infection in an infectivity assay (Fig. 5 B), which is not surprising as there is no evidence of an effect of A2 on HIV-1 replication. This is an indication that APOBEC antiviral activity may not be necessarily dependent on the interaction with Vif.

When, we replaced the DRMR region in Vif by SEMQ it was detected a reduction in its ability to interact with A3F and A3C (Fig. 5 A). Also, Vif SEMQ protein was not able to rescue infectivity in the presence of A3F indicating that this region is not involved in the interaction with A3F (Fig. 5 B).

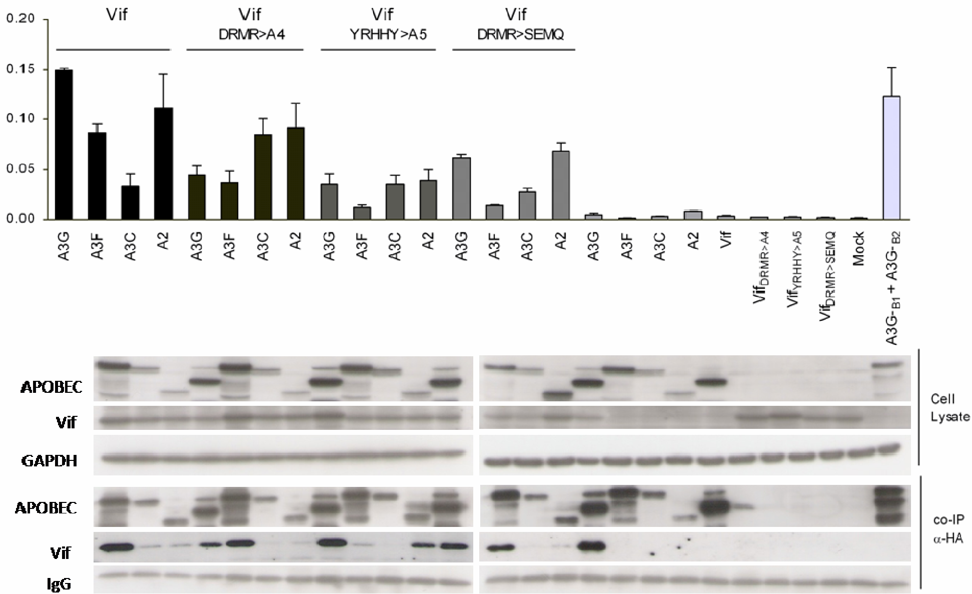



**Fig. 5. A. Interaction of Vif wt and Vif mutants with A3F, A3C, and A2 deaminases.** Top panel: *In vivo* hydrolysis rate (abs. unit measured at 450 nm) of CENTA for the interaction of Vif, $Vif_{DRMR}$, Vif $_{YRHHY}$, and $Vif_{SEMQ}$ with and A3G, A3F, A3C, and A2 deaminases. Hydrolysis rate of CENTA for fusion proteins alone is also represented as negative controls. Mean hydrolysis rate was determined for three independent experiments and normalized for cell number and protein content**.** Briefly, 20 µg of each Vif plasmid and 10 µg of each A3G plasmid DNA was used for transfections in 60-mm plates. DNA amounts were adjusted to 30 µg using empty pcDNA3.1 vector DNA when appropriate. 48 hrs after transfections, media was removed and cells were washed twice in PBS and were suspended in 100 µl of 100 mM phosphate buffer ($K_2HPO_4$ 50mM, pH=7) for the PCA assay. Mean hydrolysis rate was determined for three independent experiments and normalized for cell number and protein content**.** The data are averages of triplicates with the indicated standard deviations. Lower panel: Western blotting analysis of cell lysates and co-immunoprecipitation. Co-immunoprecipitation of Vif and A3G proteins was performed using Anti-HA-High-Affinity-Matrix (ROCHE).

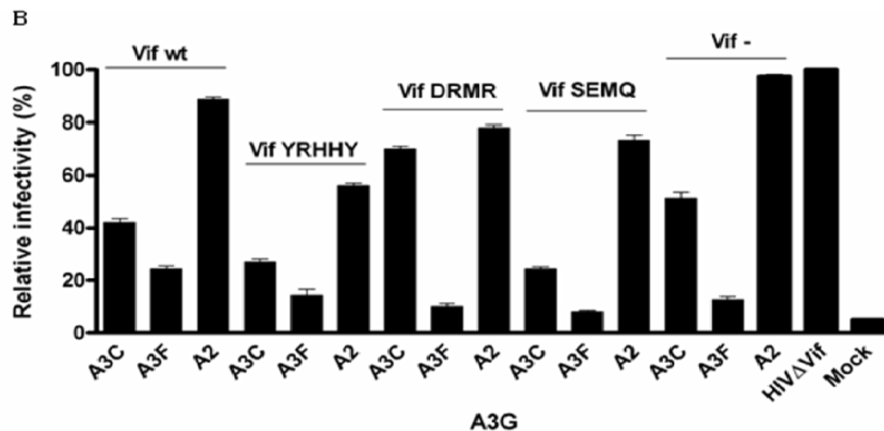



**Fig. 5 B. Infectivity assay.** HIV-1ΔVif virions were produced by cotransfecting 293T with pNL4.3ΔVif (15 µg) in the presence of 5 µg of each APOBEC plasmid, and 10 µg of each Vif plasmid. DNA amounts were adjusted to 30 µg using empty pcDNA3.1 vector DNA when appropriate. 48 h after transfection viruses were collected and used to infect HeLa P4 cells ($1.5X10^4$). Relative infectivity values were detected by measuring the absorbance of β-gal activity at 550/620nm, in a CPRG assay. The data are averages of triplicates with the indicated standard deviations.

**DISCUSSION**

The interaction of Vif with A3G has been extensively studied. However, the role of other deaminases such as A3F, A3C and A2, in HIV-1 replication is still poorly understood. Of these, only A3G and A3F are described as having antiretroviral activity. A3C is a good candidate to study as it is expressed in cells targeted by HIV-1 (Jarmuz A *et al.*2002) and A3C-induced mutations occur at levels that allow replication to persist and may therefore play a role in driving viral diversity (Bourara K. *et al.* 2007). Furthermore, it has been shown that A3C exerts potent antiviral activity against SIV (Yu Q. *et al.* 2004) but it is relatively resistant to human Vif (Langlois MA *et al*. 2005; Yu Q. *et al.* 2004; Doehle BP. *et al.* 2005).

In order to clarify the mechanism involved in the interaction of Vif with A3G, we used both proteins in fusion to fragments of β-lactamase, carrying mutations in regions that

had been previously described as being crucial for this interaction. Particularly, we studied the importance of Vif D$^{14}$RMR and Y$^{40}$RHHY regions for the *in vivo*

interaction with A3G wild-type and mutant proteins. We showed by PCA that both Vif DRMR and YRHHY regions were equally involved in the interaction with A3G and A3F, as substitution to alanines resulted in inability to interact with those proteins. Our results contradict a previous report by Russel R. and Pathak V.., which identified Vif DRMR and YRHHY regions as being critical for independent interactions with A3F and A3G, respectively (Russel R. and Pathak V., 2007). It was also reported by the same authors that Vif binding to A3G D128K mutant was dependent on the YRHHY region of Vif (Russel R. and Pathak V., 2007). However, we did not detect interaction between Vif mutants and A3G D128K mutant protein, despite Vif SEMQ being able to counteract both A3G wt and D128K mutant in an infectivity assay. An explanation for that may be that residue 128 may be important for the species-specific recognition of A3G by Vif, but may not necessarily be involved in the interaction with Vif. Therefore, we suggest that further clarification must be carried in the future in order to clarify the involvement of those two regions in Vif interaction with A3G, A3F, and A3G D128K.

We have also studied the involvement of A3G regions 122, 127 and 128 in the interaction with Vif wt and mutants. Alteration of 128 residue in A3G from aspartic acid to lysine resulted in inhibition of the interaction with Vif wt, which is in agreement with previous work (Bogerd H. *et al*., 2004; Mangeat B. *et al*., 2004; Schrofelbauer B. *et al*., 2004; Xu H. *et al*., 2004). In addition, mutations in both regions of A3G protein, R122A and W127L, resulted in a sharp reduction in the ability to interact with Vif. This led us to conclude that those two regions in A3G may not only be important for the interaction with Gag, as previously demonstrated by Huthoff and Malim (Huthoff H. and Malim M., 2007) but also for the interaction with Vif.

Interaction of Vif wt, Vif YRHHY, and Vif SEMQ with A3C was shown to be very weak when compared to A3G, A3F, and A2 and no inhibition of HIV-1ΔVif infection was detected. However, it is interesting to note that when we replaced Vif DRMR region by alanines interaction with A3C was recovered. This led us to suggest that the DRMR region in Vif may be involved in the selection of the APOBEC to inactivate. This was also clear in our infectivity assay, as Vif DRMR was able to rescue HIV-1 infectivity in the presence of A3C.

The function of A2 is still unknown, although it was proposed to be an RNA-editing enzyme in analogy with APOBEC1 (Anant S. *et al.*2001; Liao W. *et al.* 1999). In this case, A2 would be the only proposed RNA-editing enzyme apart from adenosine deaminases (Schaub M. and W. Keller, 2002) to be widely expressed among vertebrates. This way, it could be interesting to study the effects of A2 on HIV-1 replication and its relation to Vif. Therefore, we have used PCA to assess the interaction of A2 with Vif. In our PCA assay, A2 displayed ability to interact with all Vif proteins used, with exception to Vif YRHHY mutant. This is interesting in a way that A2 is not reported as having activity against HIV-1 and that was confirmed by us in an infectivity assay showing that A2 was not able to inhibit HIV-1 infectivity in any of the assays performed. In this way, it is possible that the antiviral activity of a deaminase may not be a pre-requisite for Vif to interact with it. Alternatively, it is possible that despite the ability of A2 to interact with Vif, an additional co-factor may be required in order to A2 to be able to exert antiviral action. Our hypothesis is also supported by a recent report suggesting that in order to A3G/A3F inhibit HIV-1 replication it required the presence of a cell factor that was present in some human cell lines but absent in others (Han Y. *et al.*, 2008). If this is the case, there is an urgent need for clarification of which co-factor that is present in certain cell lines and absent in others, is playing a role in APOBEC HIV-1 restriction.

Overall, PCA strategy was proven to be a simple and sensitive assessment of the *in vivo* interaction of Vif with A3G and other members of the APOBEC family. This technique was shown to be highly reproducible with a low probability of false-negatives and we believe that it could be used in future to clarify about specific domains that may be involved in the interaction between Vif and APOBEC proteins.

# CHAPTER 5

## GENERAL DISCUSSION

# 1. General Discussion

## 1.1. Background

HIV-1 is a complex retrovirus that encodes an accessory protein, Vif, which is required for production of infectious virus in a cell-type specific manner (Fisher A. G. *et al.*, 1987; Strebel K. *et al.*, 1987). In the past few years, remarkably advances in elucidating Vif function were made based on the finding that it inactivates a cellular antiviral factor named APOBEC3G (A3G). Tremendous progress has been made since the identification of A3G as a novel innate defense mechanism against HIV-1 infection. It is now widely known that viruses lacking functional Vif genes have their ability to replicate in non-permissive cell types (cells without A3G) severely impaired. Conversely, other cell lines (e.g. SupT1, Jurkat, CEM-SS) are permissive to HIV-1 infection and allow replication of Vif-defective viruses.

One of the mechanisms that Vif uses to counteract A3G is by stimulating its degradation in the 26S proteasome (Cullen BR, 2006; Mangeat B and Trono D., 2005; Chiu Y. and Greene W., 2006). If A3G escapes Vif action, it will be free to be incorporated into budding HIV-1 virions and exert its antiviral activity in the next infected cell. Therefore, understanding how A3G functions in the cell and how Vif reacts to counteract its activity *in vivo* is crucial for the design of novel antiretroviral therapies.

In this dissertation we aimed at understanding the mechanism of action of A3G, the structure-function relationship between Vif and A3G, as well as the *in vivo* interaction of both proteins.

The major conclusions of this work are discussed in detail at the end of each chapter and will be stated next as general conclusions.

## 2. General Conclusions

**In chapter 2**, we modelled A3G half-life in order to better understand the initial steps of its mechanism of action. This was an artificial fine-tuning of A3G steady-state that proved to be useful as a model to study its mechanism of action and to monitor its activity inside the cell over the time as well as its incorporation into the HIV-1 virions.

- We have showed that A3G follows the proteasome degradation pathway in a Vif-independent manner, indicating that A3G may be targeted by specific cellular E3 ligases, which do not include Vif.

- We showed that newly synthesized A3G, with less than five minutes of life, is already active in the cell and that A3G, with a half-life of approximately 13 minutes, is still able to incorporate into HIV viral particles. These results indicate that, in order to counteract A3G activity, Vif has to act at the very early stages of A3G life-time in order to avoid its incorporation into virions.

These findings showed us the importance of the A3G steady-state levels for the maintenance of its antiretroviral activity and point out that modelling A3G steady-state can predict how protein half-life can influence the antiviral activity of the deaminase, both at the level of its catalytic activity in the cell and viral encapsidation.

**In chapter 3**, we used a strategy to study the *in vivo* interaction of Vif-A3G based on an oligomerization-assisted interaction whereby fragments of Vif and A3G were fused to independently folding and interacting domains of homodimerizing GCN4 leucine zipper-forming sequences.

- We showed that the C-terminal region of Vif alone is not sufficient to induce A3G degradation and that binding of Vif to A3G must involve an additional co-factor that is probably linked to the N-terminus of Vif and may be essential for the interaction and consequent degradation of A3G.

- We showed the importance of the topological orientation of Vif-A3G for an efficient interaction indicating that it must occur in a parallel orientation and both proteins must be in close proximity.

- We showed that Vif-targeted degradation of A3G is specific for these two proteins supporting the hypothesis of an involvement of a specific co-factor in this interaction.

This was a novel approach that proved to be efficient in studying Vif-A3G interaction domains *in vivo*.

**In chapter 4** we also assessed the Vif-A3G interaction using a Protein Complementation Assay (PCA). To study this interaction, we used a β-lactamase complementation assay in which the inactive domains of the enzyme were fused to each of the proteins in question. The interaction of our complementary proteins will result in a functional β-lactamase capable of hydrolysing a substrate measurable by spectrophotometry.

- We were able to achieve a quantitative assessment of HIV-1 Vif-A3G interactions, which allowed us to do a fine dissection of the regions involved in this interaction.

- We showed that some mutations in A3G protein that had been described as being involved in the interaction with HIV-1 Gag, were also important for the interaction with Vif.

- We also studied the interaction of Vif with other members of the APOBEC family. By using PCA strategy we found a new region in Vif that may be important for the selection of the APOBEC protein to inactivate.

- By studying the interaction of Vif with A2 protein we reached the conclusion that an additional co-factor may be required in order to A2 to be able to exert antiviral action. This hypothesis had also been suggested by us in chapter 3 and it is also supported by other authors. Therefore, we provided supporting information for a new

direction of the study of Vif-A3 problematic regarding the involvement of an additional co-factor.

We will next discuss the excitement new opportunities that stand for the development of novel anti-HIV-1 therapies that exploit the interplay between Vif and A3G. We will also discuss it in light of the results presented in this dissertation.

# 3. Integrative Discussion

## 3.1. Therapeutic strategies based on Vif and A3G.

To date, the most successful HIV antiviral drugs in the market are those that target the HIV enzymes reverse transcriptase (RT) and protease (PR). More recently, drugs that target HIV integrase (IN) (β- diketo compounds [Hazuda DJ. *et al.*, 2000] or pyranodipyrimidines [Pannecouque C. *et al.*, 2002; Witvrouw M. *et al.*, 2004]) and HIV entry, including T20, a synthetic peptide that disrupts virus-to-cell fusion (Wild CT. *et al.*, 1994) were also developed.

Recent advances in the study of the biological role of HIV-1 Vif and A3G, together with progress in deciphering how Vif counteracts A3G opened new opportunities to develop anti-HIV drugs. The most obvious target is the interaction between Vif and A3G. However, understanding the mode of action of Vif and A3G alone can also provide a number of attractive targets.

In chapter 2 of this dissertation, we assessed the mechanism of action of A3G. By monitoring A3G activity inside the cell over the time, we were able to do a fine-tuning of the temporal distribution of A3G since the time it is translated until its incorporation into the virion. This was an innovative approach that extended the actual knowledge on the mechanism of action of A3G allowing the future development of new drugs. By showing that A3G follows the proteasome degradation pathway in a Vif-independent manner, we suggest that A3G may be targeted by cellular specific E3 ligases, which do not include Vif. Therefore, blocking the interaction of A3G with its specific E3 ligase could be a therapeutic approach to increase the amount of the antiviral protein in the

cell. However, a lot of work would have to be done to find which E3 ligase is indeed interacting with A3G.

Vif forms an E3 ubiquitin ligase complex containing Elongin B and C, Cullin 5, and Rbx2, and uses all these key domains to recruit A3G for polyubiquitination and proteasomal degradation (Conticello SG *et al.*, 2003; Liu B *et al.*, 2005; Marin M *et al.*, 2003; Mehle A *et al.*, 2004; Sheehy AM *et al.*, 2003; Stopak *et al.*, 2003; Yu X *et al.*, 2003; Yu X *et al.*, 2004). Therefore, the interaction of Vif with the E3 ligase complex it's another potential target, which could prevent A3G ubiquitination and degradation.

In addition, based on our results, it would be interesting to determine if A3G protein, in the absence of Vif, would suffer a post-translational modification that could expose amino acid residues that could trigger a cascade of events that would ultimately lead to proteasome degradation. If that is proved to happen, a therapeutic approach using dipeptide inhibitors of the N-end rule pathway could be used to inhibit Ub conjugation and consequently increase the intracellular half-life of the protein providing a better anti-retroviral response.

Enhancing A3G, and A3F (also an HIV-1 inhibitor), activities and inhibiting Vif function are attractive therapeutic strategies. However, care must be taken when attempting to upregulate A3G expression as A3G mRNA is highly expressed in some tumor cells (Jarmuz A. *et al.*, 2002) and this could potentially induce tumor formation.

#### 3.1.1. High molecular mass A3G as a therapeutic target.

A3G exists has either an enzimatically active low-molecular-mass (LMM) form or as an enzimatically inactive high-molecular-mass (HMM) ribonucleoprotein complex larger than 2 MDa (Chiu YL. *et al.*, 2005). It's the LMM A3G form that is responsible for the post-entry block to HIV-1 infection in resting CD4 T cells. In the HMM complexes A3G is inactive in its ability to inhibit HIV-1 replication.

It was recently found that A3G molecules are synthesized as a LMM form and within 1.5 hours after synthesis they are assembled into HMM complexes where they become inactive (Soros V. *et al.*, 2007). Apparently, it is during this period, before assembling into HMM complexes, that A3G molecules are selectively incorporated into virions (Soros V. *et al.*, 2007).

Our results extended this knowledge by giving more detailed information on the time-window between A3G synthesis and incorporation into virions. We showed that A3G molecules with approximately 13 minutes of life are already incorporated, whereas less than 5 minutes old A3G are not. Based on previous authors (Soros V. *et al*., 2007) we assumed that those were LMM newly synthesized molecules that were able to exert antiviral activity in the cell and that was confirmed by us through an infectivity assay. Therefore, our results indicate that in order to prevent encapsidation of A3G Vif has to act within a 5 to 13 minute time-window after its synthesis.

The fact the LMM A3G is a post-entry restriction factor suggests a potential therapeutic strategy. Therapeutic strategies that could induce the formation of LMM A3G complexes, or instead, disrupt HMM A3G complexes could enable A3G to exert its antiviral activity, rendering a cell resistant to HIV-1 infection.

However, the conversion of LMM into HMM complexes after T cell activation requires careful assessment in terms of the signals and molecular pathways that induce this response.

### 3.1.2. *In vivo* Vif-A3G interaction as a potential therapeutic target.

Blocking the *in vivo* binding of Vif to A3G is certainly one of the most obvious therapeutic strategies. Several authors reported that Vif may function at several levels to prevent incorporation of A3G into viral particles, either by promoting degradation of A3G in the proteasome or by sequestering A3G from sites of virion assembly impairing A3G incorporation into viral particles. (Kao S. *et al*., 2007; Santa-Marta M. *et al*., 2005; Mehle A. *et al*., 2004b; Stopak K. *et al*., 2003). Preventing the binding of Vif to A3G may result in two outcomes: 1) inhibition of A3G proteasomal degradation and, 2) increase the amount of A3G at the membrane site of viral assembly and budding resulting in a higher level of A3G incorporation into virions. However, it is important to note that Vif is also taken for degradation in the proteasome since it binds directly to the SCF complex and other E3-Ub-ligases independently of A3G. Therefore, disruption of Vif-A3G interaction in a way that would prevent the proteolytic degradation of A3G could also prevent Vif degradation and the effect of increasing levels of Vif in the cell is still unclear.

Features of Vif and A3G domains involved in the interaction between the two proteins have been extensively studied in the last few years. A detailed knowledge of the interacting domains involved in this interaction is extremely important for the rational design of new antiviral drugs. To date, several regions in Vif and A3G proteins have been mapped and the effect on the interaction has been studied.

The first region being described as essential for the *in vivo* interaction of Vif and A3G was the amino acid 128 in A3G. This region is responsible for the species-specific recognition of A3G by Vif, which is aspartic acid in humans and lysine in African green monkey (AGM) (Bogerd H. *et al.*, 2004; Mangeat B. *et al.*, 2004; Schrofelbauer B. *et al.*, 2004; Xu H. *et al.*, 2004). Substitution of D128 in human A3G for K128, found in African Green Monkey (AGM) A3G, results in a mutant (D128K-A3G) deaminase that is resistant to the effect of Vif. It is the charge of this amino acid at position 128 in A3G that markedly influences the interaction with Vif indicating that the Vif-A3G interaction is dependent on electrostatic forces (Shcrofelbauer B. *et al.*, 2006). Thus, therapeutic compounds that could target this region of A3G could be effective in preventing Vif-A3G interaction. Importantly, molecules targeting amino acid 128 are not predicted to interfere with A3G enzymatic activity, which is conferred by other domains of the protein (Navarro F. et al., 2005; Li J. et al., 2004).

In addition to this region of A3G, other regions in Vif and A3G have been identified that proved to be potential targets for drugs.

The N-terminal region of Vif has been implicated in binding to A3G, whereas the SLQ$^{144}$LQXLA$^{150}$ motif in the C-terminal domain is responsible for degradation of A3G and A3F by the Vif-Cul5-E3 ligase complex (Marin M, *et al.*, 2003; Schrofelbauer, B. *et al*, 2006; Simon, V., 2005; Tian, C. *et al*, 2006; Indrani P. *et al*, 2006). Several regions have been described as being involved in the Vif-A3G interaction and are described in more detail in chapters 3 and 4.

In chapter 3, we confirmed the previous observation that the N-terminal region of Vif is involved in binding to A3G, and extended it by showing that binding of Vif to A3G must involve an additional cofactor that is probably linked to the N-terminus of Vif. It was recently found that in one particular human T cell line, CEM-T4, A3G and A3F were unable to block HIV-1 replication, even in the absence of Vif (Han Y. *et al.*, 2008). Further analysis indicated that this cell line lacked a cellular factor that was very critical for A3G/A3F antiviral activity. These results indicated that in order to A3G/A3F inhibit HIV-1 replication it required the presence of a cell factor that was present in some human

cell lines but absent in others. Our results support this previous report, indicating that the binding of Vif to A3G must involve an additional cofactor, and extended it by showing that it is probably linked to the N-terminus of Vif. The involvement of one (or more) cofactor may then be responsible for the interaction and consequent degradation of A3G. This observation leads to a new research direction that could result in the discovery of new drug targets.

Despite the extensive work that has been developed by many authors to define regions that are crucial for the interaction between the two proteins, there are still controversial data. Therefore, clarification of conflicting results is required prior to the rational design of molecules to efficiently block this interaction.

The work presented in this dissertation (chapters 3 and 4) represent novel approaches that proved to be efficient in providing a general approach for protein dissection and interaction domains studies, and can be used in the future as a novel approach for a rapid *in vivo* screening of Vif-A3G interacting domains. It also offers the advantage of allowing it to be done in an *in vivo* environment without any deleterious effect on the structure and activity of the proteins.

### 3.2. Potential consequences of Vif-based drugs.

HIV-1 is a highly recombinant virus with a huge evolutionary potential. One of the key factors responsible for this, is the high mutational rates due to the poor fidelity of reverse transcriptase (Mansky LM. And Temin HM., 1995). Recombination is also another factor responsible for the evolutionary potential of HIV-1 and some authors suggest that it's even more powerful in shaping HIV-1 evolutionary patterns that mutation (Levy DN. Et al., 2004). The high rates of HIV-1 replication ($10^{10}$ viral particles are produced each day within an infected individual), together with the high rates of recombination and mutation, can result in a substantial cost to the virus.

Previous reports suggested that the HIV-1 population in infected individuals is mainly composed of non-infectious, mutated viruses (Bourinbaiar AS., 1994; Li Y., 1992; Sanchez G. et al., 1997). It has been suggested that even a small increase in the mutational rate of HIV-1 could

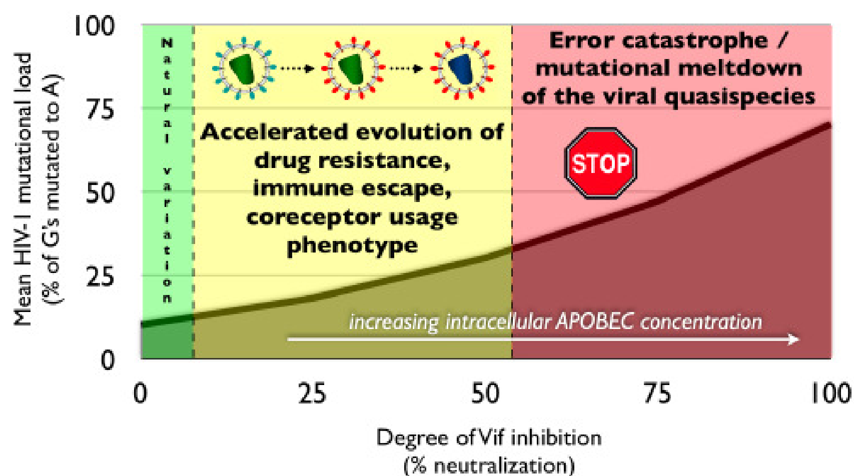
lead to a genetic meltdown of the viral quasispecies in a phenomenon called "error catastrophe" (Eigen M., 1971; Eigen M., 2002).

Therefore, antiviral therapies that could enhance the mutational rate of HIV-1 are attractive. One possible strategy would be to inhibit Vif action and consequently allow A3 proteins to exert their mutational onslaught during HIV-1 reverse transcription. A3G and, to a lesser extend A3F, antiviral mechanisms result in genome degradation, incomplete cDNA synthesis, and a detrimentally high mutation rate within the HIV-1 genome, which has been largely attributed to deamination of the viral cDNA (Mariani R. *et al*., 2003; Goff SP 2003; Gu Y. and Sundquist WI. 2003; Harris R. S. *et al*., 2002; Klarman G. J. *et al*., 2003; Mangeat B. *et al*., 2003).

Antiviral drugs that could inhibit Vif and enhance A3G/A3F activity are emerging as attractive targets (Mezei M. and Minarovits J., 2006; Carr JM et al., 2006; Stopak K. and Greene WC., 2005; Fessel J., 2005). However, care must be taken when designing new antiviral drugs as incomplete Vif inhibition could result in an increase in A3G intracellular concentration just enough to exert an intermediate level of mutational pressure on the HIV-1 genome resulting in a "sub-error catastrophe". This could accelerate viral evolution instead of inducing a population collapse (Fig.1) resulting in antiretroviral resistance (Berkhout B. and de Ronde A., 2004) and in immune escape.

Therefore, the potential outcome of a Vif-based intervention must be examined rigorously both *in vitro* and *in vivo* prior to clinical deployment. In addition, A3G and A3F may also function through other mechanisms that do not necessarily require deamination (Chiu YL. Et al., 2005; Bishop KN. Et al., 2006; Soros VB. Et al., 2007).

Despite some controversy remaining on that subject, a therapeutic strategy that could amplify this non-mutagenic phenotype without enhancing cytidine deamination may be an alternative to suppress viral replication.




*(Figure from: Pillai SK. et al., Retrovirology.2008)*

**Fig. 1. Possible effects of Vif inhibition on HIV-1 population.** The extent of HIV-1 hypermutation is directly proportional to the concentration of A3 in the cell (Suspene R. et al., 2004). A highly effective Vif inhibitor could result in a significant increase in A3 intracellular levels leading to viral error catastrophe (red zone). However, if a drug is not efficient enough in inhibiting Vif, that could fail to induce a mutational meltdown in HIV-1 and instead it will accelerate viral evolution and drug resistance (yellow zone).

In summary, A3G and A3F proteins have appeared in the war against HIV-1 as part of our innate immune system. The antiretroviral action of these proteins is overtaken by the action of HIV-1 Vif protein. In the past 6 years, since the discovery of A3G as an antiviral factor, many advances have been made in trying to understand its mechanism of action in the cell and how Vif acts in order to counteract its activity.

However, despite Vif-A3G interplay being an attractive target for future therapeutic strategies, there are still questions that remain to be answered and controversial points that need to be clarified. Determination of A3G and Vif high-resolution structure will certainly give a huge impulse in the clarification of many unanswered questions and will be crucial for the development of novel therapeutic strategies.

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