TABLE DES MATIÈRES

LISTE DES FIGURES ET TABLEAUX

Tableau **Page**

LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

AML	Leucémie aiguë myéloblastique (A*cute Myeloid Leukemia*)
CA	Capside virale
CA-NTD	Domaine N-terminal de la capside virale
CCR5-Δ32	Délétion de 32pb dans le gène CCR5
CRF	Formes recombinantes circulante du VIH (*Circulating Recombinant Forms*)
CsA	Cyclosporine A
CTL	Lymphocytes T cytotoxiques (*Cytotoxic T Lymphocytes*)
CypA	Cyclophiline A
EIAV	Virus de l'anémie infectieuse équine (*Equine Infectious Anemia Virus*)
FACS	*Fluorescence-activated cell sorting*
FIV	Virus de l'immunodéficience féline (*Feline Immunodeficiency Virus*)
Fv1	*Friend Virus Susceptibility* 1, facteur de restriction
gp120	glycoprotéine 120 du VIH
gp41	glycoprotéine 41 du VIH
HF	*High-Fidelity*, se dit des tampons et polymérase à haute fidélité
HIV	*Human Immunodeficiency Virus*
HSC	Cellules souches hématopoïétiques (*Hematopoietic stem cells*)
IFN-I	Interférons de type 1
IL2-RG	Récepteur gamma de l'interleukine 2
IN	Intégrase virale
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LTR	*Long Terminal Repeat*

MA	Matrice virale
MLV	Virus de la leucémie murine (*Murine Leukemia Virus*)
NC	Nucléocapside virale
NCBI	National Center for Biotechnology Information
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
NMR	Résonance magnétique nucléaire (*Nuclear Magnetic Resonance*)
OMS	Organisation mondiale de la santé
PIC	Complexe viral de pré-intégration (*Pre-Integration Complex*)
PR	Protéase virale
qPCR	PCR quantitative
RT	Transcriptase inverse virale (*Reverse Transcriptase*)
SCID-X	*Severe Combined Immunodeficiency* liée au chromosome X
SIDA	Syndrome d'immunodéficience acquise
SIV	Virus de l'immunodéficience simienne (*Simian Immunodeficiency Virus*)
$TRIM5\alpha_{Hu}$	Orthologue humain de TRIM5α
$TRIM5\alpha_{Rh}$	Orthologue du singe rhésus de TRIM5α
vDNA	ADN virale
VIH	Virus de l'immunodéficience humaine
VSV	Virus de la stomatite vésiculaire (*Vesicular Stomatitis Virus*)
WT	Type sauvage (*wild type*)
ZFN	Nucléases à doigt de zinc (*Zinc Finger Nucleases*)

CHAPITRE I

INTRODUCTION

1.1 Problématique

1.1.1 L'épidémie du VIH/SIDA

Voilà déjà plus de 25 ans, l'Agence de la santé publique du Canada rapportait les premiers cas d'infection au virus de l'immunodéficience humaine (VIH) au Canada. Malgré tous les efforts déployés par les diverses agences gouvernementales, des milliers de Canadiens sont nouvellement infectés à chaque année. À l'échelle mondiale, les données de l'Organisation mondiale de la santé (OMS) montrent que malgré une tendance à la baisse au cours de la dernière décennie, il y avait en 2009 plus de 2,5 millions de personnes nouvellement infectées par le VIH/SIDA (Figure 1.1).

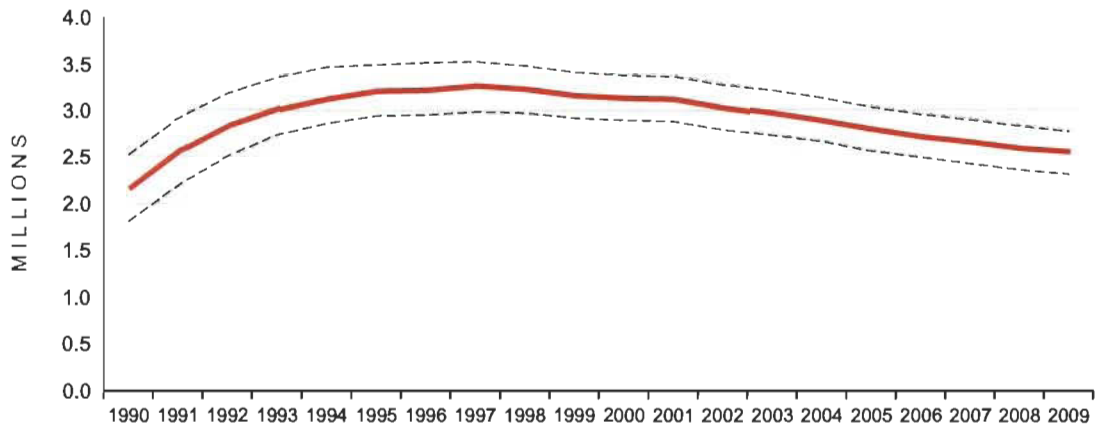

Figure 1.1 Tendance de l'incidence mondiale du VIH-1 [1].

1.1.2 Un traitement alternatif au VIH/SIDA : une réelle nécessité

Les données récentes sur l'incidence du VIH/SIDA démontrent bien que malgré une efficacité grandissante des agents antirétroviraux et de leurs combinaisons, un

nombre important de nouvelles infections est toujours présent au niveau mondial. De plus, bien que le nombre de décès reliés au VIH/SIDA présente une tendance à la baisse dans certaines régions du monde (en Amérique du Nord, entre autres), une importante hausse de la mortalité est observée dans d'autres régions, incluant l'Europe de l'Est et l'Asie (Figure 1.2).

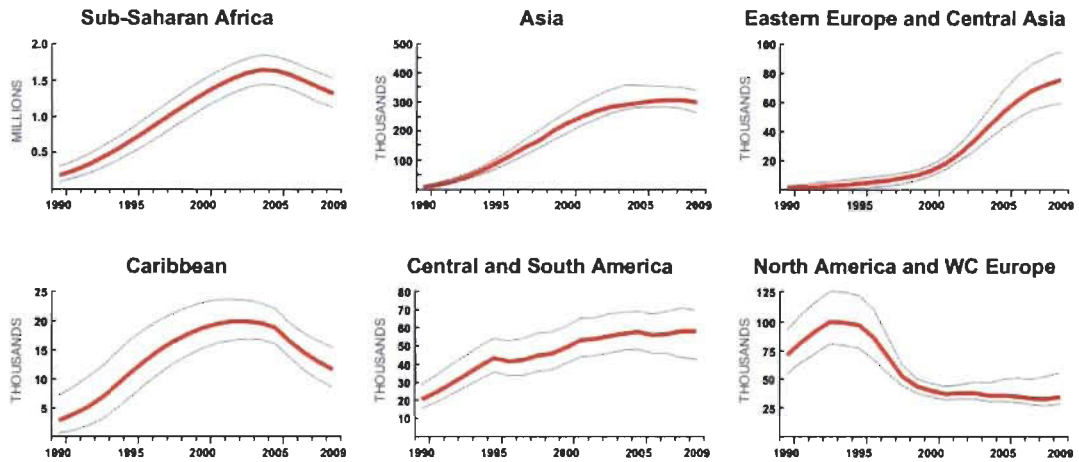

Figure 1.2 Nombre de décès associés au VIH/SIDA selon les régions du monde [1].

De surcroît, aucun traitement actuellement répandu contre le VIH/SIDA ne permet son éradication chez les personnes atteintes. Elles doivent donc continuellement adhérer au traitement, ce qui entraîne une lourde charge économique et augmente la fréquence des interruptions de traitement, ce qui favorise l'émergence de virus résistants aux traitements, en plus de faciliter une éventuelle transmission du virus par l'augmentation de la charge virale [2-4]. La fréquence d'émergence de tels virus est difficile à estimer, compte tenu de la grande quantité de facteurs l'influençant (géographie, contexte socioéconomique, génétiques de l'individu et des différentes souches virales). Néanmoins, des études ont signalé la présence de mutations conférant une résistance à un agent antiviral chez 20 à 50 % des participants à leurs cohortes [5-7]. De plus, la prévalence de la transmission de souches virales portant une mutation de résistance à un agent antiviral augmente au fil du temps, et a récemment été évaluée entre 4 et 12 % selon la localisation géographique [8]. L'élaboration et la mise en place de nouvelles stratégies thérapeutiques sont donc d'une importance capitale dans la lutte contre le VIH/SIDA.

1.1.3 Le cas du « patient de Berlin »

Le cas du « patient de Berlin » était le seul cas jusqu'à plus tôt cette année où un patient a officiellement été guéri du VIH/SIDA. Il s'agit en fait d'un citoyen américain vivant en Allemagne et qui était infecté depuis plus de 10 ans par le VIH-1. Or, ce même patient était atteint d'une leucémie aigüe myéloblastique (AML). L'un des traitements utilisés pour traiter une AML consiste en l'éradication des cellules lymphatiques cancéreuses par radiothérapie suivi d'une repopulation des cellules lymphatiques par greffe de moelle osséuse d'un donneur sain. Or, le docteur traitant ce patient a décidé de trouver un donneur présentant une certaine particularité génétique : le donneur était homozygote pour une délétion de 32 pb dans le gène CCR5 (CCR5-Δ32). CCR5 est un corécepteur essentiel à l'infection de la plupart des souches transmises du VIH-1, sa délétion rendant ainsi résistant à l'infection par le VIH-1 [9-11]. La repopulation des cellules lymphatiques n'exprimant pas CCR5 chez le patient infecté par le VIH-1 a entraîné sa rémission complète et jusqu'à ce jour aucune trace du virus n'a été détectée dans les divers tissus analysés [12]. Suite à cette réussite médicale, plusieurs équipes de recherche consacrent maintenant beaucoup de temps et d'énergie à développer des approches thérapeutiques qui visent à réintroduire des cellules résistantes au VIH-1 chez les patients infectés.

1.1.4 La thérapie génique

En général, la thérapie génique est une stratégie visant à introduire des modifications ou voire même de nouveaux gènes dans des cellules humaines qui demeureront chez le patient et qui pourront apporter un bénéfice thérapeutique. Cette approche se veut complexe alors que les chercheurs doivent composer avec les risques inhérents et l'efficacité parfois faible des stratégies [13].

Le premier cas de thérapie génique « réussie » est sans aucun doute le traitement des « enfants bulles », jeunes enfants atteints du SCID-X (*Severe Combined Immunodeficiency* liée au chromosome X). Une mutation dans le gène codant pour le récepteur gamma de l'interleukine 2 (IL2-RG) induit une perte de fonction de cette

protéine, rendant ainsi impossible la maturation correcte des lymphocytes T [14]. Sans ces lymphocytes T, les autres cellules du système immunitaire ne peuvent jouer leur rôle, induisant ainsi un état de susceptibilité aux infections opportunistes : l'immunodéficience sévère. Vers la fin des années 1990, le Dr Alain Fischer utilisa un vecteur rétroviral basé sur le virus de la leucémie murine (MLV) qu'il modifia pour y insérer une version fonctionnelle du gène IL2-RG. L'introduction de ce vecteur dans les cellules souches hématopoïétiques des patients a permis de rétablir la maturation et la fonction normale des lymphocytes T chez huit des neuf jeunes enfants traités [15]. Or, les vecteurs rétroviraux s'insèrent d'une manière (quasi) aléatoire dans le génome de cellules infectées, laissant ainsi un risque de dérégler l'équilibre pro-oncogène/suppresseur de tumeurs qui différencie les cellules saines de cellules cancéreuses. C'est ce qui se produisit chez quatre des enfants traités, ces derniers ont développé une leucémie des suites de leur traitement [16]. Malheureusement, l'un d'entre eux en est décédé. Suite à des traitements de chimiothérapie, les trois autres se sont rétablis. Des huit enfants ayant survécu au traitement, sept présentent maintenant un système immunitaire quasi-normal, leur permettant ainsi de se développer normalement dans le monde extérieur. Malgré les graves effets secondaires observés, cette thérapie est néanmoins vue comme un succès pour la thérapie génique.

Plusieurs années se sont écoulées depuis ce premier succès et de nouveaux vecteurs viraux plus sécuritaires sont maintenant disponibles [17, 18]. De plus, de récentes avancées dans le domaine de la modification du génome permettent aujourd'hui de modifier les gènes d'une cellule sans avoir à y introduire de manière permanente des vecteurs viraux exogènes. Ces nouveaux outils, jumelés avec le besoin criant de développer de nouvelles approches thérapeutiques contre le VIH/SIDA, réaffirment la nécessité d'identifier des transgènes antiviraux qui pourront être utilisés dans ce type d'application.

1.2 Les rétrovirus

1.2.1 Définition

Les rétrovirus sont une famille de virus infectant les vertébrés. Ils se distinguent des autres virus entre autres par leur mode de réplication particulier. Ce sont des virus à ARN qui possèdent deux copies de leur génome. Ils codent pour une ADN polymérase ARN dépendante (la transcriptase inverse, RT) qui rétrotranscrit les brins d'ARN en ADN viral qui pourra ainsi être intégré de façon définitive dans le génome de la cellule cible [19]. La famille des rétrovirus se divise en deux sous-familles, soit les orthoretrovirus et les spumaretrovirus. Le virus de l'immunodéficience humaine appartient au genre des *lentivirus* de la sous-famille des orthoretrovirus. Il existe deux types de VIH, soit le VIH-1 et le VIH-2. Le VIH-1 est responsable de la pandémie mondiale alors que le VIH-2, qui est moins facilement transmissible, se limite plutôt à l'Afrique de l'Ouest.

1.2.2 Structure

Ce sont des virus enveloppés d'une bicouche phospholipidique recouverte de protéines de surface (la glycoprotéine 120, gp120) et transmembranaire (glycoprotéine 41, gp41). Cette enveloppe est acquise de la cellule productrice lors du bourgeonnement du virus et sa surface interne est recouverte d'une protéine structurale : la matrice (MA). Au sein de cette enveloppe se trouve le cœur viral, formé de la multimérisation de la protéine de la capside (CA) virale. Cet assemblage hexamèrique de la CA en cœurs viraux contient diverses enzymes associées : la RT, l'intégrase (IN) et la protéase (PR), ainsi que le génome viral entouré de protéines de nucléocapside (NC, Figure 1.3).

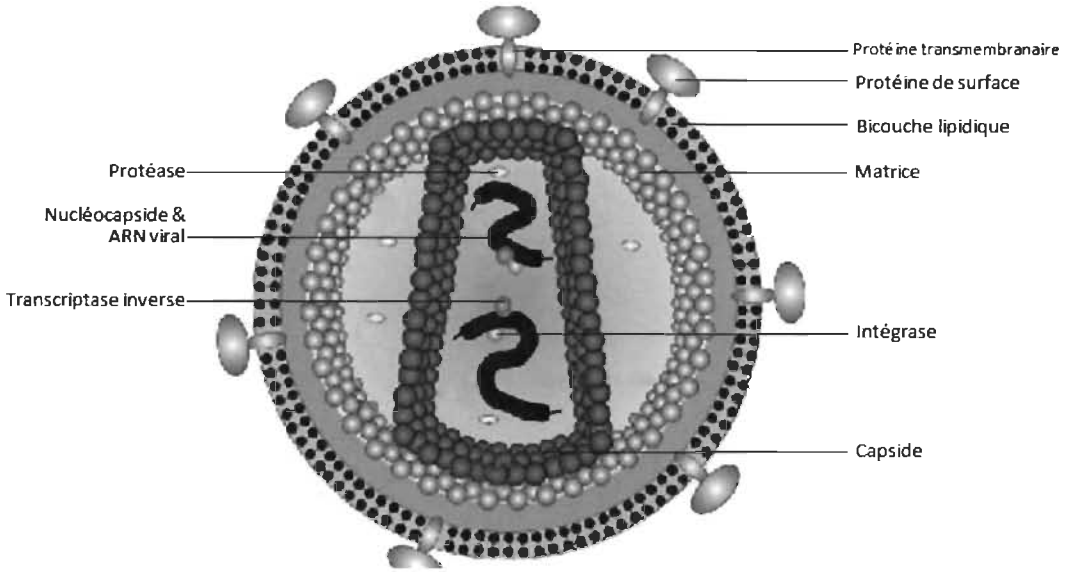

Figure 1.3 Schéma de la structure du VIH-1 [20].

1.2.3 Génome

Le génome des rétrovirus est encodé en deux copies identiques d'ARN simple brin de polarité positive. On y distingue trois gènes principaux qui codent pour des polyprotéines essentielles à la réplication des rétrovirus (Figure 1.4). Ce sont les gènes *gag* (*group-specific antigen*), *pro-pol* (polymérase) et *env* (glycoprotéines d'enveloppe). Ces trois gènes codent pour des précurseurs dont le clivage enzymatique permettra d'obtenir les protéines virales actives (voir Figure 1.4). Le génome complet est encastré entre deux régions nommées LTR (*Long Terminal Repeat*) qui renferment le promoteur (en 5') et le site de polyadénylation (en 3'). Le génome des lentivirus code pour des protéines supplémentaires dites accessoires (pour le VIH-1 : Vif, Vpr, Vpu, Tet, Rev, Tat et Nef) puisqu'elles permettent la régulation de diverses étapes de la réplication virale et de l'interaction avec les protéines de l'hôte.

Figure 1.4 Schéma du génome commun des rétrovirus [20].

1.2.4 Le cycle viral du VIH-1

Bien que les rétrovirus se répliquent selon le même cycle viral, certaines différences existent entre eux. Cette section est donc écrite en fonction du VIH-1 afin d'explorer les particularités de ce virus, facilitant ainsi la compréhension du reste de l'ouvrage. On distingue habituellement deux phases dans le cycle de réplication viral :

- La phase *précoce* allant de la fusion de la particule virale avec la membrane cellulaire jusqu'à l'intégration de l'ADN viral dans le génome cellulaire;
- La phase *tardive* allant de l'expression des protéines du provirus jusqu'au bourgeonnement des nouveaux virions.

Au début de la phase précoce, l'interaction entre la protéine virale de surface gp120 et le récepteur cellulaire CD4 induit un changement de conformation de gp120, permettant ainsi le recrutement du corécepteur cellulaire CCR5 (ou CXCR4 pour certaines souches virales). Le recrutement du corécepteur cellulaire entraîne d'autres changements conformationels importants qui mènent à l'attachement de la glycoprotéine transmembranaire de l'enveloppe, gp41, à la membrane cellulaire. Grâce à ses deux régions homologues, la gp41 agit comme un ressort et son repliement sur elle-même mène à la fusion des deux membranes lipidiques, permettant ainsi le relargage du cœur viral dans le cytoplasme.

S'en suivent les étapes de la décapsidation du cœur viral et de la transcription inverse de l'ARN viral. Bien que relativement peu connue, la régulation précise de ces étapes est essentielle à l'infectivité maximale du virus [21]. La transcription inverse mène à la formation d'un double-brin d'ADN viral (vDNA) qui fera partie du complexe de pré-intégration (PIC). Les protéines MA et IN ainsi que la protéine accessoire Vpr sont présentes dans le PIC. Plusieurs composantes de ce complexe présentent un signal de localisation nucléaire, lui permettant ainsi de migrer vers le noyau. Une fois arrivé à destination, ce complexe permet l'intégration du vDNA à un site quasi-aléatoire du génome cellulaire.

L'ADN viral ainsi intégré tire profit de la machinerie de transcription et de synthèse protéique de la cellule infectée. Grâce au LTR 5' jouant le rôle de promoteur et du signal de polyadénylation provenant du LTR 3', l'ADN viral est transcrit en ARN. Les transcrits sont alors épissés ou non, puis exportés vers le cytoplasme où ils seront traduits en protéines structurales ou enzymatiques. Ces nouvelles protéines virales seront assemblées à deux brins d'ARN viral près de la membrane plasmique pour ainsi former de nouveaux virions et procéder au bourgeonnement de ces particules jusque-là non-infectieuses. Après le bourgeonnement hors de la cellule, la PR clive les précurseurs protéiques (Gag et Gag-Pol) : le virus est maintenant mature et infectieux. La Figure 1.5 présente une schématisation du cycle de réplication viral.

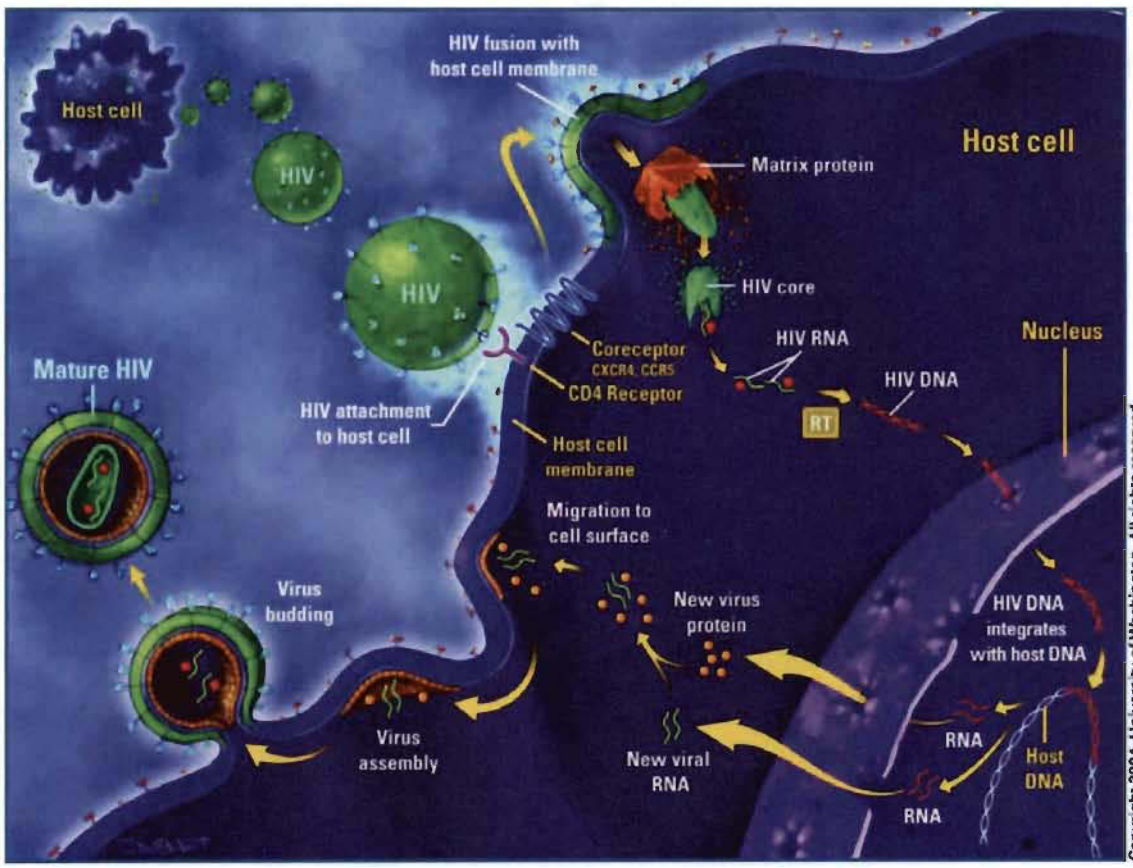

Figure 1.5 Cycle de réplication du VIH-1 [20].

1.2.5 La pandémie de VIH : pandémie divisée en groupes et sous-groupes

Le haut taux de mutations qu'apporte la rétrotranscription de l'ARN en ADN résulte en un très grand nombre de souches virales différentes. Celles-ci sont regroupées en groupes qui partagent des caractéristiques communes de séquences et propriétés phénotypiques. Le groupe M est responsable de la majorité de la pandémie mondiale et on peut y discerner 9 sous-groupes en plus des formes recombinantes circulantes (CRF), qui sont issues de la recombinaison de deux souches de différents sous-groupes au sein d'un même individu. La Figure 1.6 présente un aperçu des différents niveaux de classification utilisés.

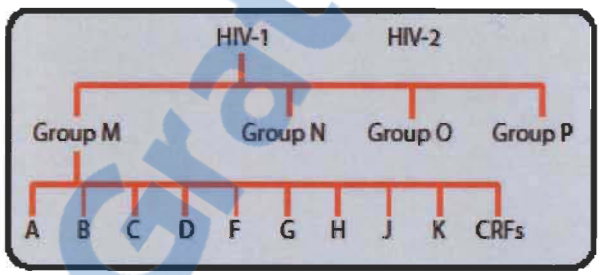

Figure 1.6 Les différents niveaux de classification du VIH.

1.3 Le facteur de restriction TRIM5α

Des millions d'années d'infection par les rétrovirus ont permis d'exercer une sélection positive sur les mammifères afin qu'ils développent des mécanismes de résistance à ces infections. Ces stratégies sont divisées en deux grandes catégories : l'immunité innée et acquise. Importants acteurs de cette première catégorie, les facteurs de restriction permettent d'empêcher l'infection ou du moins réduire le potentiel infectieux de certains virus. L'un de ces facteurs est un membre de la famille des protéines à motif tripartite, TRIM5α.

1.3.1 Historique

Vers la fin des années 1960, le croisement de souris Balb/c à des souris NIH-3T3 a permis l'observation d'une activité de restriction présentant un caractère héréditaire. En effet, alors que les souris NIH-3T3 présentaient une résistance face au virus MLV dit B-tropique (B-MLV) et que les Balb/c étaient plutôt résistantes au virus N-tropique (N-MLV), la première génération fille de ce croisement était complètement résistante aux deux souches de virus. Des expériences supplémentaires ont permis de mettre en évidence que le déterminant viral pour cette restriction était la protéine de capside, plus précisément deux de ses acides aminés (positions 109 et 110) [22]. Toutefois, le gène responsable pour cette restriction n'était toujours pas identifié.

C'est en 1996 qu'il a finalement été cloné; il a été nommé Fv1 pour *Friend Virus Susceptibility*. Les similitudes des caractéristiques de la restriction par Fv1 avec ce qui était observé chez les cellules de primates ont amené les chercheurs à introduire deux autres termes désignant un phénotype de restriction semblable à Fv1 chez l'humain (Ref1) et d'autres primates (Lv1) [23, 24]. Ce n'est cependant pas avant 2004 que TRIM5 isoforme α a été identifié dans des publications démontrant sa capacité de bloquer la réplication du VIH-1 aux étapes de pré-intégration du virus dans les cellules du singe rhésus, identifiant ainsi les phénotypes de Lv1 et Ref1 comme étant bel et bien causés par TRIM5α [25, 26].

1.3.2 Structure

La famille des protéines à motifs tripartite (*tripartite motif*, TRIM) est caractérisée par la présence des domaines RING-finger, B-Box et Coiled-Coil [27, 28] (voir Figure 1.7). L'épissage du gène TRIM5 donne lieu à l'expression de plusieurs isoformes, dont seul TRIM5α possède un domaine supplémentaire en C-terminal, nommé PRYSPRY. Cet isoforme est également le seul à avoir une activité de restriction contre le VIH-1 [29].

Figure 1.7 Schéma général de la protéine TRIM5α.

Le domaine RING confère une activité E3-ubiquitine ligase à la protéine, activité qui est requise pour la restriction des virus [30-32]. Pour sa part, le domaine B-Box peut s'auto-assembler en dimères qui sont essentiels dans l'assemblage des complexes multimériques de TRIM5α sans quoi la protéine ne peut exercer une restriction maximale sur le virus [33-35].

Le domaine PRYSPRY (aussi appelé B30.2) est responsable de la reconnaissance de la capside virale [36, 37]. Il est donc le seul élément duquel découle la spécificité d'action de TRIM5α (voir 1.3.3 ci-dessous) [38]. Le domaine Coiled-Coil permet quant à lui la dimérisation de la protéine, ce qui est essentiel pour que le PRYSPRY puisse convenablement reconnaître la capside virale [39-41].

1.3.3 Spécificité d'action

Au cours de l'histoire et alors que les différents mammifères étaient infectés par des rétrovirus différents d'une espèce à l'autre, la pression sélective exercée sur les mécanismes de défense a entre autres engendré la divergence de la spécificité d'action des orthologues de TRIM5α [42]. Bien souvent, les virus restreints ne sont pas des virus qui infectent normalement l'hôte ce qui suggère que TRIM5α puisse jouer un rôle dans la prévention de la transmission inter-espèces des rétrovirus [43]. Alors que leur structure et leur mécanisme d'action semblent identiques, l'alignement de la séquence des domaines PRYSPRY révèle l'existence de régions variables (Figure 1.8) [42, 44, 45]. C'est au sein de ces régions que l'on retrouve les acides aminés responsables de la spécificité d'action de TRIM5α, la pression sélective exercée sur ces gènes ayant ainsi favorisé la formation de ces régions variables [46].

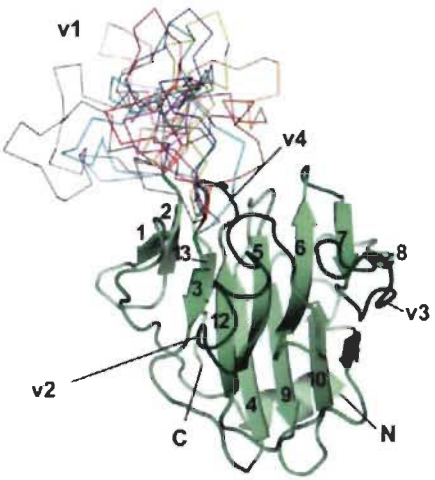

Figure 1.8 Structure du domaine PRYSPRY de la protéine TRIM5α$_{Rh}$ indiquant la position des quatre régions variables (v1-v4).
Les dix conformations de la boucle v1 avec la plus faible énergie sont présentées en différentes couleurs [36].

Tel que l'illustre le Tableau 1.1, le VIH-1 est fortement inhibé par TRIM5α du singe rhésus (TRIM5α$_{Rh}$) alors que la version humaine (TRIM5α$_{Hu}$) ne parvient pas à exercer une activité antivirale efficace contre ce virus. Les efforts visant à identifier des mutations de TRIM5α$_{Hu}$ lui permettant de bloquer efficacement le VIH-1 font l'objet de la section 1.3.7.

Tableau 1.1
Résumé des virus restreints par divers orthologues de TRIM5α

Orthologue	Espèce	Virus restreints
TRIM5α$_{Hu}$	Humain	N-MLV, EIAV, FIV
TRIM5α$_{Rh}$	Singe rhésus	VIH-1, FIV
TRIM5α$_{AGM}$	Singe vert africain	N-MLV, HIV-1, HIV-2, SIV, EIAV

1.3.4 Mécanisme d'action

La restriction d'un rétrovirus par TRIM5α s'initie après reconnaissance de la capside virale par le domaine PRYSPRY. Il a été démontré que la protéine TRIM5α n'interagit que faiblement avec les monomères de CA [29]. Au contraire, l'assemblage

in vitro de la CA en structures multimériques (tubes ou sphères) qui rappellent la structure des cœurs viraux permet une très forte interaction TRIM5α-CA [40, 47, 48]. Cette observation suggère que TRIM5α reconnaît un épitope formé lors de l'assemblage multimérique de CA, ou alors l'avidité de la reconnaissance nécessite plusieurs épitopes. TRIM5α serait donc un récepteur de reconnaissance de motifs moléculaires de pathogènes. Le fait que la multimérisation de TRIM5α soit essentielle pour la restriction vient plutôt confirmer ce deuxième modèle. De plus, des études biochimiques et de microscopie électronique ont permis de mettre en évidence que les TRIM5α restrictifs s'assemblent en structures hexagonales qui sont complémentaires en symétrie et en dimensions avec la CA multimérique (voir Figure 1.9) [35].

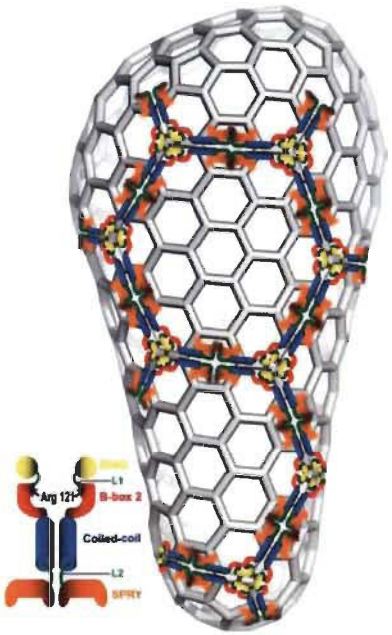

Figure 1.9 Schéma de l'assemblage hexamèrique de TRIM5α sur un cœur viral de CA [35].

L'interaction TRIM5α-CA entraîne un désassemblage accéléré de la particule virale à ses interfaces inter-hexamèriques [40, 48-50], ce qui a été démontré comme empêchant l'accumulation des transcrits viraux et donc nuit à l'infectivité [21, 51, 52]. L'interaction du facteur de restriction avec le PIC induit l'activité d'auto-ubiquitination de TRIM5α, dirigeant ainsi le PIC vers la dégradation par le protéasome [53, 54]. De plus, cette interaction nuit au transport du PIC vers le noyau cellulaire, empêchant ainsi

l'intégration de l'ADN viral dans le génome cellulaire [55] (voir Figure 1.10). Une facette indirecte de l'activité de TRIM5α a également été récemment identifiée. Jouant le rôle de système de reconnaissance de motifs étrangers (*pattern-recognition*), il activerait la signalisation de l'immunité innée amenant via la protéine NF-κB, facteur de régulation de la réponse immunitaire [56].

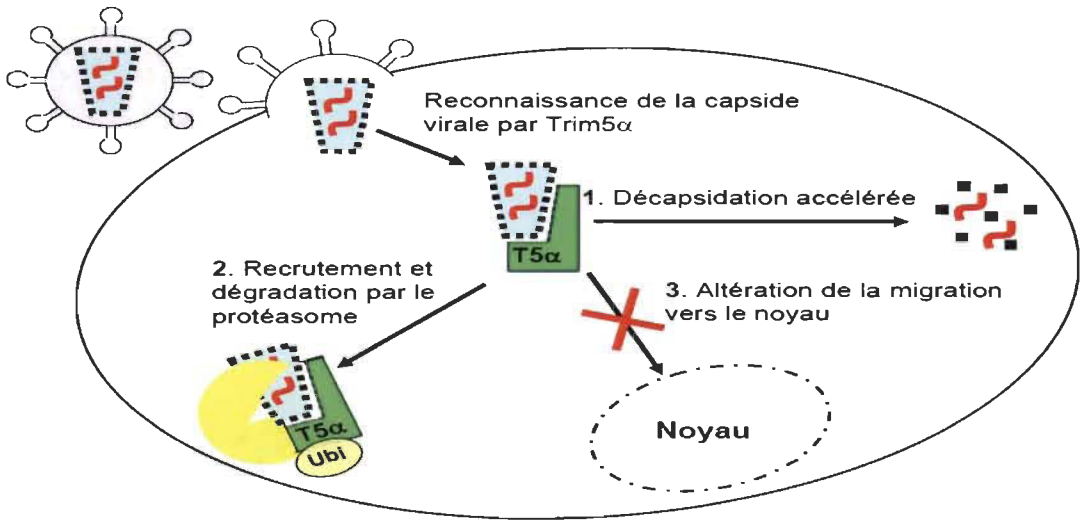

Figure 1.10 Schéma présentant les différents effets de TRIM5α sur le virus [20].

1.3.5 Régulation de l'expression de TRIM5α et autres facteurs de restriction

Les interférons de type 1 (IFN-I) comprennent une vaste famille de protéines impliquées dans la signalisation de l'immunité innée. Ils jouent divers rôles dans la réponse antivirale, incluant l'activation des cellules NK et des macrophages [57]. Une autre facette de leur implication dans l'effort antiviral est la régulation qu'ils exercent sur l'expression de différents facteurs de restriction. En effet, le niveau d'expression de TRIM5α est augmenté en réponse aux IFN-I, ce qui se traduit chez l'humain par une augmentation de la restriction des virus sensibles, notamment N-MLV et quelques isolats du VIH-1 [58-60]. Plusieurs autres facteurs de restriction voient également leur niveau d'expression augmenté en réponse aux IFN-I, notamment APOBEC3G, lequel sera le sujet de la section 5.1.2 [61].

1.3.6 La cyclophiline A

La cyclophiline A (CypA) est une isomérase cis/trans peptidyl-propyl de l'hôte qui est exprimée d'une manière ubiquitaire dans les tissus. Elle peut lier une boucle riche en prolines sur la CA et catalyser l'isomérisation cis/trans du lien peptidique G89-P90 [62, 63]. Le traitement avec l'inhibiteur pharmacologique de CypA, la cyclosporine A (CsA), diminue la restriction par TRIM5α_{Rh} alors que le même traitement diminue l'infectivité du VIH-1 dans les cellules humaines. CypA est donc, dans un contexte cellulaire-dépendant, nécessaire à la restriction par TRIM5α ou essentiel à l'infectivité maximale du virus [64-66]. En ce sens, de récentes études suggèrent que CypA pourrait jouer un rôle dans la régulation de la décapsidation du cœur viral [67, 68].

1.3.7 Des mutants de la protéine TRIM5α humaine actifs contre le VIH-1

Considérant l'ampleur de la problématique qu'implique le VIH/SIDA, il n'est pas étonnant que des chercheurs se soient penchés sur la question d'identifier des déterminants de la restriction du VIH-1 par TRIM5α. Une équipe a d'abord réalisé des chimères TRIM5α_{Hu} – TRIM5α_{Rh} afin de cartographier les différences et déterminants. Cette approche a permis l'identification d'une mutation en position 332 (R332G) de la protéine humaine qui lui confère une activité contre le VIH-1 d'environ 5 fois supérieure à celle de la protéine sauvage [69].

La position 332 fait partie de la région variable 1 (v1) du domaine PRYSPRY. Ceci implique que d'autres positions au sein de v1 ou autres régions variables pourraient jouer un rôle dans la détermination du potentiel restrictif de TRIM5α_{Hu} sur le VIH-1, bien qu'elles puissent ne pas avoir été identifiées dans l'approche comparative avec TRIM5α_{Rh}. Notre laboratoire a donc réalisé un crible par gain de fonction basé sur une mutagénèse aléatoire du domaine PRYSPRY de TRIM5α_{Hu}. Une nouvelle mutation a été identifiée, R335G [70]. Lorsque combinée avec la mutation en position 332 (mutant R332G-R335G, voir Figure 1.11), son efficacité s'est trouvée augmentée (20 fois comparativement à la protéine sauvage). Fait notable, dans les deux cas le changement d'acide aminé venant supprimer une charge positive dans la région de v1 était important

pour l'augmentation de l'activité de restriction. Le chapitre de cet ouvrage présente une deuxième stratégie de notre laboratoire qui a mené à l'identification d'une troisième mutation dans la région v1.

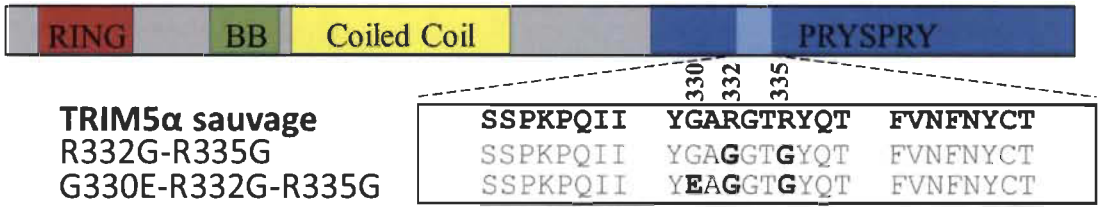

Figure 1.11 Schéma général de TRIM5α_{Hu} identifiant les mutations conférant une activité contre le VIH-1.

1.3.8 TRIM5α : transgène potentiel pour thérapie génique?

Alors que les technologies de thérapie génique s'améliorent et que l'identification de nouveaux transgènes antiviraux continue, il est important de comprendre et d'étudier les prérequis pour un traitement efficace et sécuritaire.

Du point de vue sécurité et considérant que la stratégie d'administration du transgène serait idéalement intrinsèquement sécuritaire, le transgène en lui-même ne devrait pas avoir d'effets négatifs sur l'hôte. De ce côté, l'introduction d'une chimère TRIM5α_{Hu} – TRIM5α_{Rh} dans des cellules souches hématopoïétiques (HSC) humaines s'est révélée comme ne nuisant pas à la différentiation et l'activation de ces cellules [71]. Cependant, une récente étude a démontré que la surexpression constitutive de TRIM5α provoque l'activation de la signalisation liée à l'immunité innée, résultant ainsi en une activation constitutive de NF-κB [56]. La dérégulation de l'activation de NF-κB peut avoir de lourdes conséquences, allant du cancer aux maladies auto-immunes [72, 73]. L'utilisation de vecteurs rétroviraux pour introduire les transgènes de TRIM5α chez l'hôte, d'où ils seraient généralement exprimés grâce à de puissants promoteurs tels que CMV-IE, est donc risquée en soi. Une connaissance plus approfondie des mécanismes par lesquels la surexpression de TRIM5α induit l'activation de NF- κB pourrait permettre d'identifier un moyen de contrôler cet effet indésirable dans une thérapie génique.

Du point de vue de l'efficacité, plusieurs critères sont à prendre en considération. Tout d'abord, étant donné que les rétrovirus sont de par leur cycle de réplication des virus qui mutent et s'adaptent rapidement à la pression qui est exercée sur eux, l'émergence de virus résistant est un risque réel qui doit être limité. Pour ce faire, une meilleure connaissance des mécanismes permettant au VIH-1 d'échapper à la restriction médiée par TRIM5α est essentielle. Le chapitre III de cet ouvrage présente notre étude de ces mécanismes de résistance d'un virus résistant aux mutants de $TRIM5\alpha_{Hu}$. Plus spécifiquement, nous y avons étudié la mutation virale V86M qui se situe au sein de la boucle de liaison à CypA de CA (voir Figure 1.12).

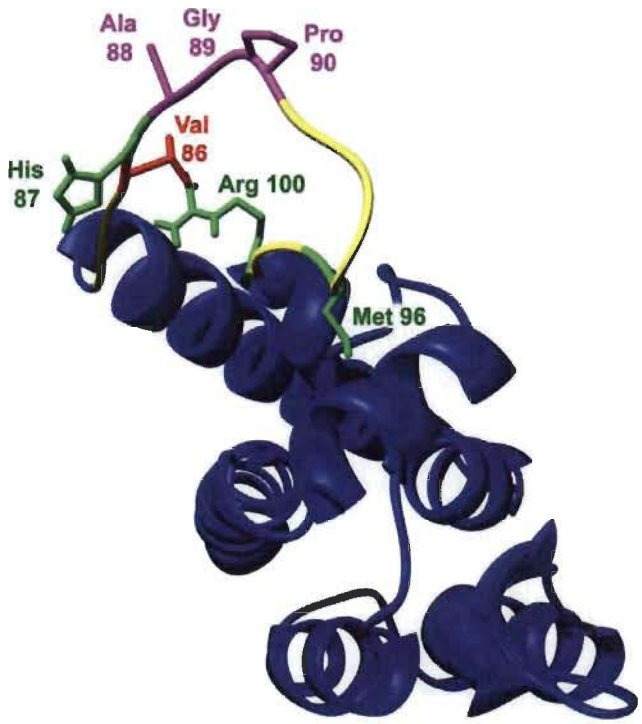

Figure 1.12 Position de l'acide aminé V86 dans la CA virale [74].

Une autre facette de l'efficacité repose sur la variabilité entre les différentes souches de VIH-1 qui infectent les individus à l'échelle mondiale. Les transgènes candidats devraient donc idéalement être capables de bloquer l'infection par plusieurs voire toutes ces différentes variations. Nous avons également étudié cet aspect important de la démarche vers une thérapie génique, étude qui est présentée au chapitre IV.

CHAPITRE II

A NOVEL AMINOACID DETERMINANT OF HIV-1 RESTRICTION IN THE TRIM5α VARIABLE 1 REGION ISOLATED IN A RANDOM MUTAGENIC SCREEN

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Résumé

Les gènes antirétroviraux dérivés de l'humain sont d'une haute importance biomédicale et sont activement recherchés. Le VIH-1 est efficacement inhibé aux étapes précoces du cycle de réplication virale par des mutants de la région variable 1 (v1) du facteur de restriction humain TRIM5α. Dans cette étude, nous avons utilisé un megaprimer muté afin de créer une librairie de mutants de la région v1 de TRIM5α_{Hu}. Nous avons isolé un variant présentant une mutation à la position 330 (G330E) qui inhibe la transduction d'un vecteur VIH-1 aussi efficacement que les mutations déjà décrites aux positions 332 et 335 (R332G et R335G). À l'instar de ces dernières, l'ajout d'une charge acidique locale dans la région v1 était la clef pour la restriction du VIH-1. TRIM5α_{Hu} G330E inhibait également la propagation d'un virus VIH-1 réplicatif dans une lignée de lymphocytes T humains. Fait intéressant, la combinaison de G330E avec les mutations aux positions 332 et 335 n'a pas augmenté la restriction du VIH-1. Par ailleurs, le triple mutant G330E-R332G-R335G liait les tubes de capside recombinante purifiée moins efficacement que le double mutant R332G-R335G. Dans un modèle de la

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structure du domaine PRYSPRY de TRIM5α_{Hu}, l'ajout de la mutation G330E au double mutant R332G-R335G a causé d'importants changements à la surface de liaison à la capside, ce qui pourrait expliquer pourquoi le triple mutant n'était pas plus restrictif que le double mutant. Par ailleurs, le potentiel des mutants de Gly330 d'inhiber le VIH-1 n'a pas été prédit par l'étude d'orthologues de TRIM5α qui sont connus pour inhiber fortement le VIH-1. Cette étude souligne donc le potentiel des stratégies de mutagénènese aléatoire à isoler des variants de protéines humaines avec des propriétés antivirales.

Abstract

Human-derived antiretroviral transgenes are of great biomedical interest and are actively pursued. HIV-1 is efficiently inhibited at post-entry, pre-integration replication stages by point mutations in the variable region 1 (v1) of the human restriction factor TRIM5α. Here we use a mutated megaprimer approach to create a mutant library of TRIM5α_{Hu} v1 and to isolate a mutation at Gly330 (G330E) that inhibits transduction of an HIV-1 vector as efficiently as the previously described mutants at positions Arg332 and Arg335. As was the case for these other mutations, modification of the local v1 charge toward increased acidity was key to inhibiting HIV-1. G330E TRIM5α_{Hu} also disrupted replication-competent HIV-1 propagation in a human T cell line. Interestingly, G330E did not enhance restriction of HIV-1 when combined with mutations at Arg332 or Arg335. Accordingly, the triple mutant G330E-R332G-R335G bound purified recombinant HIV-1 capsid tubes less efficiently than the double mutant R332G-R335G did. In a structural model of the TRIM5α_{Hu} PRYSPRY domain, the addition of G330E to the double mutant R332G-R335G caused extensive changes to the capsid-binding surface, which may explain why the triple mutant was no more restrictive than the double mutant. The HIV-1 inhibitory potential of Gly330 mutants was not predicted by examination of natural TRIM5α orthologs that are known to strongly inhibit HIV-1. This work underlines the potential of random mutagenesis to isolate novel variants of human proteins with antiviral properties.

Introduction

It has long been recognized that treatment with type I IFN (IFN-α especially) efficiently inhibits HIV-1 propagation *in vitro*, suggesting an important role for innate immunity in the control of this infection (Chakrabarti and Simon, 2010). Several mediators of the anti-HIV-1 activity of type I IFNs in human cells have been discovered in the last 15 years. These proteins, sometimes called "restriction factors", can inhibit the replication of retroviruses at various stages. All the restriction factors isolated so far are dominant and some are antagonized by HIV-1 gene products (Goffinet et al., 2009; Mangeat et al., 2009; Marin et al., 2003; Mehle et al., 2004). Some restriction factors show a high level of specificity and typically target non-cognate retroviruses more efficiently, implying an important role in the prevention of inter-species transmission (Cullen, 2006; Evans et al., 2010; Mariani et al., 2003).

TRIM5α was isolated as a retroviral restriction factor in 2004 (Stremlau et al., 2004) and acts within the post-entry, pre-integration window (Owens et al., 2003; Perez-Caballero et al., 2005b). The viral molecular target of TRIM5α is the correctly matured N-terminal domain of capsid (CA) proteins forming the outer surface of the retroviral core (Cowan et al., 2002; Forshey et al., 2005; Ikeda et al., 2004; Owens et al., 2003; Shi and Aiken, 2006; Stremlau et al., 2006). A direct interaction between the two proteins, each present as high molecular weight multimers, occurs shortly after entry and is required for downstream inhibition of viral replication (Kar et al., 2008; Langelier et al., 2008; Perez-Caballero et al., 2005a; Sebastian and Luban, 2005; Stremlau et al., 2006). The mechanism of TRIM5α-mediated restriction can be broken down to discrete events, some of them inter-dependent: (i) virus entrapment into TRIM5α cytoplasmic bodies (Campbell et al., 2008), (ii) decreased stability of the virus core (Black and Aiken, 2010; Perron et al., 2007; Stremlau et al., 2006; Zhao et al., 2011) (iii) targeting to a proteasome-dependent degradation pathway (Anderson et al., 2006; Diaz-Griffero et al., 2006; Lienlaf et al., 2011; Rold and Aiken, 2008); , and (iv) inhibition of nuclear import (Anderson et al., 2006; Berthoux et al., 2004; Wu et al., 2006). The linear structure of TRIM5α reveals a partition between effector domains at the N-terminus and

a target recognition domain called PRYSPRY (also known as SPRY or B30.2) found at the C-terminus. The PRYSPRY domain contains evolutionarily variable regions that determine binding to CA and restriction potency (Diaz-Griffero et al., 2008; Ohkura et al., 2006; Sawyer et al., 2005; Song et al., 2005).

The human ortholog of TRIM5α moderately restricts HIV-1 (~2-fold), even when over-expressed (Pham et al., 2010; Stremlau et al., 2004; Uchil et al., 2008), but many natural isolates of HIV-1 are more sensitive to TRIM5α_{Hu} (Battivelli et al., 2011). Thus, stable transduction of HIV-1-targeting TRIM5α has been proposed as a way to decrease the permissiveness of human cells to HIV-1 infection in medical applications (Rossi et al., 2007). Rhesus macaque TRIM5α (TRIM5α_{Rh}) over-expressed exogenously is dominant over its human endogenous counterpart and efficiently restricts HIV-1, including upon transduction into hematopoietic progenitor cells (Anderson and Akkina, 2005). Human/rhesus chimeric TRIM5α, in which regions of the PRYSPRY domain originate from the rhesus ortholog, have also been used (Kambal et al., 2011). Other human/rhesus mapping experiments led to the discovery that mutations at Arg332 in the variable 1 (v1) region of PRYSPRY conferred HIV-1 restriction activity (Li et al., 2006; Yap et al., 2005), although not to the level of TRIM5α_{Rh} (Li et al., 2006; Pham et al., 2010). Based on the dual hypothesis that (i) TRIM5α_{Rh} and other Old world primate TRIM5α might be too immunogenic for human gene transfer, (ii) novel discrete mutations in TRIM5α could increase its affinity for HIV-1 CA, we previously introduced a random mutagenic screen to isolate point mutants of TRIM5α_{Hu} targeting HIV-1 (Pham et al., 2010). Using this approach, we found that several mutations abrogating the positive charge at position 335 of the PRYSPRY domain restricted HIV-1 in human cell lines. Here we describe a second, different mutagenic screen that led to the identification of additional HIV-1-restrictive mutants.

Material and Methods

Plasmid DNAs

pMIP-TRIM5α_{Hu} and pMIP-TRIM5α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before (Berthoux et al., 2005a; Berthoux et al., 2005b; Bérubé et al., 2007; Pham et al., 2010; Sebastian et al., 2006). pNL-GFP, pMD-G, pΔR8.9, pTRIP$_{CMV-GFP}$ and pCL-Eco have all been described elsewhere (Berthoux et al., 2005a; Berthoux et al., 2004; Berthoux et al., 2005b; Berthoux et al., 2003; Naviaux et al., 1996; Zufferey et al., 1997). The proviral clone pNL4-3 (Adachi et al., 1986) was used for propagation experiments.

Cells and retroviral vectors production

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C. Human T lymphoblast Sup-T1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 37 °C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA). VSV G-pseudotyped HIV-1 $_{NL4-3}$ HIV-1 and MLV-based vectors were produced through transient transfection of 293T cells and collected as previously described (Bérubé et al., 2007; Pham et al., 2010). To produce HIV-1$_{TRIP-CMV-GFP}$, cells were co-transfected with pTRIP$_{CMV-GFP}$, pΔR8.9 and pMD-G. To produce MIP or MIP-TRIM5α vectors, cells were co-transfected with the relevant pMIP vector, pCl-Eco and pMD-G.

Library construction and screening

The following oligodeoxynucleotide (ODN) named "38-doping" was ordered from Sigma-Genosys: 5'-aga caa gtg agc tct CCg AAa CCa CAg ATa ATa TAt GGg GCa CGa GGg ACa AGa TAc CAg ACa TTt GTg AAt ttc aat tat tgt act. This ODN was designed to introduce random mutations in the coding region for TRIM5α_{Hu} (NCBI

accession # NM_033034) residues Pro323 to Asn341. The manufacturer was instructed to introduce random mutations at nucleotides shown in capital letters in the sequence above. At each of these positions, 95% of the ODNs were requested to have the correct residue, while 5% would have any of the 3 other ones. Mutations were confined to the first and second residues of each codon in order to minimize the occurrence of silent mutations. Because 38 residues were targeted for mutagenesis, and assuming a 5% chance of being mutated for each, the expected ODNs synthesized should bear 1.9 mutations on average. However, we found that TRIM5α_{Hu} generated by this procedure had 3.2 mutations on average.

Sewing PCR was used to transfer the mutant library to a retroviral vector expressing TRIM5α_{Hu}. For this, TRIM5α_{Hu} upstream of the mutated region was amplified with primers TRIM5fwd (5'-GTTCCTCGAGATGGCTTCTG-GAATCCTGGTTAAT) and TRIM5$_{963-940}$ (GCTCACTTGTCTCTTATCTTCAGA). The rest of TRIM5α_{Hu} was amplified using the 38-doping ODN and TRIM5rev (5'-TCCTGAATTCTTACTTATCGTCGTCATCCTTGTAATC). Each PCR reaction was done in 1× High Fidelity (HF) buffer, contained 170 ng of matrix DNA, 20 units/ml of high fidelity Phusion enzyme from New England Biolabs (Pickering, ON), 0.2 mM of dNTP, 0.2 μM of each primer and proceeded for 25 cycles (98 °C, 10 sec; 56 °C, 30 sec; 72 °C, 45 sec). PCR products were agarose gel-purified using the QIAquick gel extraction kit. About 1/10 of the purified product from each PCR were combined and used to perform a second PCR reaction with ODNs TRIM5fwd and TRIM5rev. DNA was amplified for 25 cycles (98 °C, 20 sec; 56 °C, 20 sec; 72 °C, 1 min) using the Phusion enzyme. The PCR product was purified using the QIAGEN QIAquick PCR purification kit, then digested with PshA1 and EcoR1. Digested DNA was again gel-purified and then ligated overnight to pMIP-TRIM5α_{Hu} cut with the same enzymes. The ligation product was divided in 6 aliquots and then entirely transformed in *E. coli* DH5α bacteria by electroporation, yielding a 21,000-clone library.

MLV-based vectors carrying the mutant library were produced by transfection of 293T cells in a 10-cm plate with 10 μg of mutated pMIP-TRIM5α_{Hu}, 10 μg of pCL-Eco

and 5 μg of pMD-G. 100,000 TE671 cells in 6-well plates were infected with various amounts of the MLV-TRIM5α_{Hu}, in triplicata. 2 days later, cells were treated with 2 μg per ml of puromycin for 2 to 3 days, at which point we visually examined the cultures and discarded the ones for which transduction efficiency was higher than 15%. The remaining cells were pooled. Puromycin-resistant cells were subsequently challenged with a preparation of the GFP-expressing HIV-1 vector HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ at a high multiplicity of infection, exactly as before (Pham et al., 2010). GFP-negative cells were then sorted out by FACS, and 0.18% of intact cells were recovered. These cells were individually distributed into 2 x 96-wells plates and the surviving clones were challenged again with HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$. Cell clones that expressed mutated versions of TRIM5α_{Hu} and that were confirmed as being more than 2-times resistant to transduction by HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ were analyzed further.

Site-directed mutagenesis

The R332G, R335G and R332G-R335G mutations were described before (Pham et al., 2010). Other mutations were introduced in MIP-TRIM5α_{Hu} by using the following primers through the same "sewing PCR" procedure as before (the modified codons are underlined):

K324Q_{fwd}, GAGCTCTCCG<u>CAA</u>CCACAGATAATA;
K324Q_{rev}, ATCTGTGG<u>TTG</u>CGGAGAGCTCACTT;
I328S_{fwd}, ACCACAGATA<u>TCA</u>TATGGGGCACGAG;
I328S_{rev}, TGCCCCATA<u>TGA</u>TATCTGTGGTTTCG;
Y329C_{fwd}, CAGATAATA<u>TGT</u>GGGGCACGAGGGA;
Y329C_{rev}, CCTCGTGCCCC<u>ACA</u>TATTATCTGT;
G330E_{fwd}, ATAATATAT<u>GAG</u>GCACGAGGGACAA;
G330E_{rev}, CCCTCGTGC<u>CTC</u>ATATATTATCTGT;
G333V_{fwd}, GGGGCACGA<u>GTG</u>ACAAGATACCAGA;
G333V_{rev}, GGTATCTTGT<u>CAC</u>TCGTGCCCCAT;
Y336C_{fwd}, GGGACAAGA<u>TGC</u>CAGACATTTGTGA;
Y336C_{rev}, AAATGTCTG<u>GCA</u>TCTTGTCCCTCGT;

Q337P$_{fwd}$, GGACAAGATACCCGACATTTGTGAA;

Q337P$_{rev}$, TTCACAAATGTCGGGTATCTTGTCC;

T338P$_{fwd}$, CAAGATACCAGCCATTTGTGAATTTC;

T338P$_{rev}$, TTCACAAATGGCTGGTATCTTGTCC;

G330E-R332G$_{fwd}$, CAGATAATATATGAGGCAGGAGGGACAAGATACC;

G330E-R332G$_{rev}$, GGTATCTTGTCCCTCCTGCCTCATATATTA;

G330E-R335G$_{fwd}$, ATAATATATGAGGCACGAGGGACAGGATACCAGAC;

G330E-R335G$_{rev}$, GTCTGGTATCCTGTCCCTCGTGCCTCATATATTA;

G330E-R332G-R335G$_{fwd}$,

ATAATATATGAGGCAGGAGGGACAGGATACCAGAC;

G330E-R332G-R335G$_{rev}$,

GTCTGGTATCCTGTCCCTCCTGCCTCATATATTA.

TE671, CRFK and Sup-T1 cells stably expressing these TRIM5α_{Hu} variants were generated by transduction with the MLV-based vector MIP-TRIM5α_{Hu} followed by puromycin selection, exactly as described previously (Pham et al., 2010).

Viral challenges

Cells expressing various TRIM5α orthologs or mutants were challenged with single doses of HIV-1$_{TRIP\text{-}CMV\text{-}GFP}$ or HIV-1$_{NL\text{-}GFP}$ as described previously (Pham et al., 2010). The viral dose used was typically set so that approximately 10% of control permissive cells are infected, thus allowing reliable quantification of infection when the magnitude of restriction is up to 100-fold. Two days post-infection, adherent cells were trypsinized and fixed in 2% formaldehyde-phosphate-buffered saline while Sup-T1 cells were PBS-washed and then treated with 2% formaldehyde. All cells were subjected to fluorescence-activated cell sorting analysis on a FC500 MPL instrument (Beckman Coulter, Mississauga, ON, USA) using the MXP/CXP software. On the basis of light scatter profiles, only intact cells were included in the analysis. GFP-positive cells were gated and counted as a percentage of total intact cells following analysis of 10,000 to 25,000 cells. For the propagation assay, 2 x 10^6 Sup-T1 cells were infected with 50 µl of

supernatant from 293T cells transfected with pNL4-3, containing approximately 10 ng of CAp24 as measured by ELISA. The next day, cells were washed to remove input virus and then kept in culture for 4 weeks. Cell-free supernatants were collected every 2 or 3 days for analysis. p24 (CA) concentrations in the supernatants were determined by a home-made ELISA assay using the 183 CA monoclonal antibody (Bounou et al., 2002).

HIV-1 CA-NC expression and purification

The HIV-1 capsid-nucleocapsid (CA-NC) protein was expressed, purified and assembled as previously described (Ganser-Pornillos et al., 2004; Ganser et al., 1999). Briefly, a pET11a-based vector (Novagen) expressing the CA-NC protein of HIV-1 was used to transform BL-21(DE3) *E. coli*. CA-NC expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture reached an optical density of 0.6 at 600 nm. After 4 hours of induction, cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.5), 1 μM $ZnCl_2$, 10 mM 2-mercaptoethanol and protease inhibitors (Roche). Lysis was performed by sonication, and debris were pelleted for 30 minutes at 35,000 x *g*. Nucleic acids were precipitated by adding 0.11 equivalents of 2M $(NH_4)_2SO_4$ and the same volume of 10% polyethylenimine and then pelleted by centrifugation at 29,500 x *g* for 15 minutes. Proteins were recovered by adding 0.35 equivalents of saturated $(NH_4)_2SO_4$ and centrifuging at 9,820 x *g* for 15 min. Pellets were resuspended in 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol. Finally the CA-NC protein was dialyzed against 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol, and stored at -80 °C.

In vitro assembly of HIV-1 CA-NC complexes and binding to TRIM5α variants

HIV-1 CA-NC particles were assembled *in vitro* by diluting the CA-NC protein to a concentration of 0.3 mM in 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl and 2 mg/ml DNA oligo-(TG)50. The mixture was incubated at 4°C overnight and centrifuged at 8,600 x *g* for 5 minutes. The pellet was resuspended in assembly buffer (50 mM Tris-HCl

(pH 8.0), 0.5 M NaCl) at a final protein concentration of 0.15 mM and stored at 4 °C until needed. 293T cells were transfected with plasmids expressing wild-type or mutant TRIM5α proteins. 48 h after transfection, cell lysates were prepared as follows. Cells were PBS-washed and resuspended in capsid-binding buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT). Cells were disrupted by freeze-thawing, and incubated on ice for 10 minutes. Lysates were centrifuged in a refrigerated microcentrifuge (~ 14,000 x *g*) for 5 minutes. Supernatants were supplemented with 1/10 volume of 10X PBS and then used in the binding assay. In some cases, samples containing the TRIM5α variants were diluted with extracts prepared in parallel from untransfected cells. To assay binding, 5 μl of CA-NC particles assembled *in vitro* were incubated with 200 μl of cell lysate at room temperature for 1 hour. A portion of this mixture, referred to as "input" was stored. The mixture was spun through a 70% sucrose cushion (70% sucrose, 1X PBS and 0.5 mM DTT) at 100,000 x *g* in an SW55 rotor (Beckman) for 1 hour at 4 °C. After centrifugation, the supernatant was carefully removed and the pellet was resuspended in 1X SDS-PAGE loading buffer. Levels of TRIM5α proteins were determined by western blotting with an anti-FLAG antibody as described above. Levels of HIV-1 CA-NC protein in the pellet were assessed by western blotting with an anti-p24 capsid antibody.

Structural models of the PRYSPRY domain

Prediction structures were generated using the web-based I-TASSER system (Roy et al., 2010; Zhang, 2008) with default parameters. Molecular graphics and analyses were performed with the UCSF Chimera 1.6.2 package (Pettersen et al., 2004).

Results

A v1-focused mutagenic screen

Previously published data pointed to v1 as the major region determining retroviral restriction specificity (Ohkura et al., 2006; Sawyer et al., 2005; Stremlau et al., 2005).

We thus developed a novel mutagenic screen specifically targeting this domain. A megaprimer was used to introduce mutations at residues 323 to 341 of TRIM5α_{Hu} (Figure 2.1A). A library of more than 20,000 clones was generated, each carrying about 3 mutations on average. The library was retrovirally transduced in human TE671 cells and we applied the same functional screen that was used previously by us (Pham et al., 2010) and others (Stremlau et al., 2004) to isolate cells with decreased permissiveness to transduction by the GFP-encoding, VSV G-pseudotyped HIV-1 vector TRIP-CMV-GFP. Following two rounds of functional selection to retain only the clones that restricted HIV-1 by 2-fold or more compared to the control transduced with the "empty" vector, we recovered 5 cell lines in which GFP transduction by HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ was inhibited about 5-fold (Figure 2.1B). Sequencing of the TRIM5α_{Hu} expressed from the retroviral vector showed that 3 of them contained mutations at position 332 (Figure 2.1B). Two of those were the R332G mutation that has been well-characterized by others (Li et al., 2006). These three clones were not analyzed any further as their restriction potential was entirely explained by Arg332 mutations.

Each of the 7 substitutions found in clones B3 and G4 was introduced in the parental retroviral vector expressing TRIM5α_{Hu} by site-directed mutagenesis and then transduced into TE671 cells. Permissiveness to GFP transduction by HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ was then analyzed for each of the mutants. Only the mutation G330E of G4 conferred a strong level of HIV-1 resistance, close to 10-fold (Figure 2.1C). The fact that G330E was more restrictive than the parental clone G4 was probably due to the presence of mitigating mutations in clone G4, such as Y329C. This mutation alone abolishes the slight level of restriction (2-fold) conferred by over-expression of the wild-type (WT) TRIM5α_{Hu}, and thus it is likely that it would also decrease restriction when combined with G330E, although we did not test this. Thus, HIV-1 restriction in TE671-B3 cells was due to a combination effect involving 2 or 3 of the substitutions found in this clone, while restriction in TE671-G4 cells was explained by mutation G330E. In this preliminary analysis, G330E was found to be as efficient at inhibiting HIV-1 as previously described mutation R332G. However, it was approximately 2-times less restrictive than the double mutation R332G-R335G and 10-times less restrictive than the Rhesus macaque ortholog (Figure 2.1C).

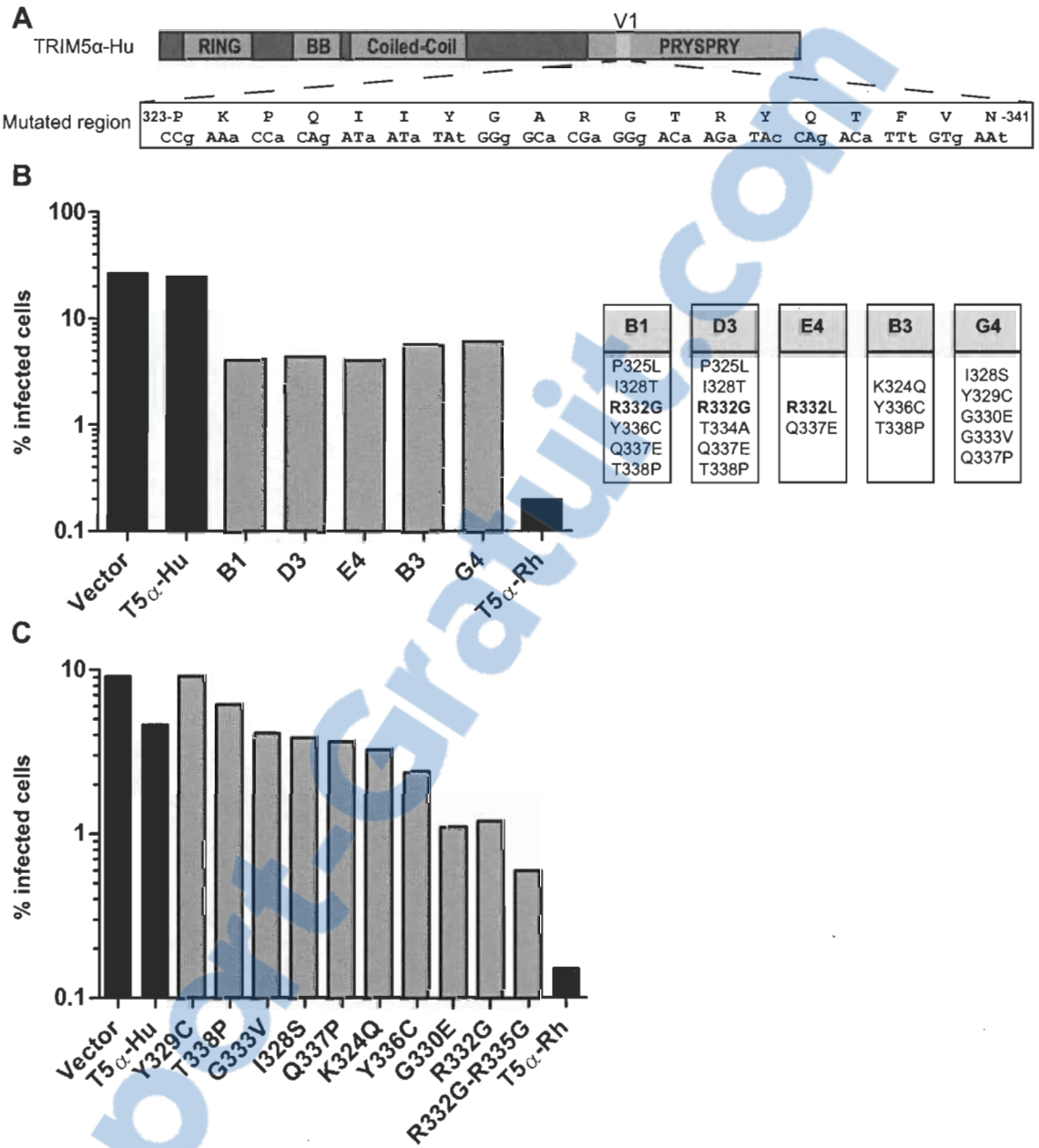

Figure 2.1 Isolation of amino acid 330 of TRIM5α_{Hu} as a determinant of HIV-1 restriction.

(A) Megaprimer approach to introduce mutations in the v1 region of TRIM5α_{Hu}. The nucleotide sequence of the region targeted by the primer is shown in the box under the corresponding amino acid sequence. Partly degenerated primer synthesis was used to introduce random mutations at the 38 nucleotide positions shown in capital letters, and the mix of mutated megaprimers were cloned into the retroviral vector construct pMIP-TRIM5α_{Hu} by sewing PCR, and then transduced into TE671 cells. (B) HIV-1 restriction potential of 5 TE671 clones isolated in a functional screen. Presumed HIV-1-resistant cell clones were grown and tested for HIV-1 permissiveness. The left panel shows percentages of infected

(GFP-positive) cells two days after challenging the 5 clones with a single dose of HIVTRIP-CMV-GFP. Control cells transduced with the empty MIP vector or cells transduced with the weakly restrictive wild-type TRIM5α_{Hu} or with restrictive TRIM5α_{Rh} were included. The right panel lists mutations found in the TRIM5α_{Hu} variants expressed by each of the TE671 cell clones isolated. (C) HIV-1 restriction potential of individual point mutations found in cell clones B3 and G4. Mutations were introduced into pMIP-TRIM5α_{Hu} and TE671 cells transduced with the constructed vectors were challenged with HIVTRIP-CMV-GFP as above. Previously characterized restrictive TRIM5α_{Hu} mutants R332G and R332G-R335G were included in the assay.

Effect of alternative mutations at Gly330

Previous mutations modulating the capacity of TRIM5α_{Hu} to inhibit restriction of HIV-1 affected the charge of the protein. Removal of the positive charge conferred by Arg332 or Arg335 was, indeed, key to HIV-1 restriction (Li et al., 2006; Pham et al., 2010). The negatively charged glutamic acid brought by the G330E mutation likewise affects the protein charge. In order to test whether restriction was primarily due to the removal of the glycine or to the introduction of negatively charged residue, we tested the restriction potential of other substitutions at position Gly330. We found that introduction of an aspartic acid significantly (3- to 4-fold) inhibited HIV-1 replication, while G330A or G330S had little or no effect (Figure 2.2). These data suggest that introducing a negative charge at that position was important to inhibit HIV-1. However, G330D was not as restrictive as G330E, and only the latter mutant was retained for further investigation.

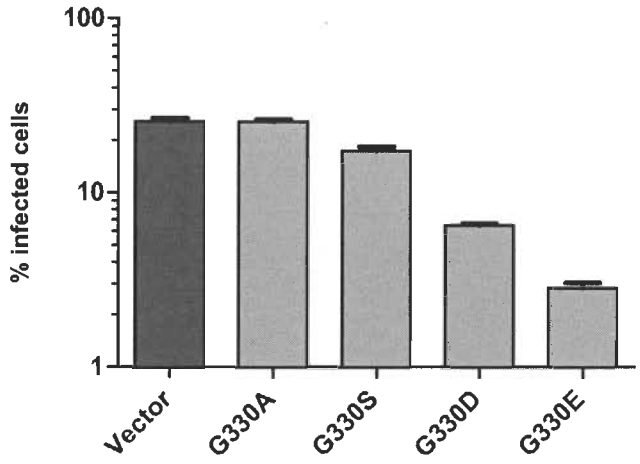

Figure 2.2 **Restriction of HIV-1 by TRIM5α$_{Hu}$ mutants of Gly330.**
TE671 cells were transduced to express the indicated TRIM5α and then challenged with a single dose of HIV$_{TRIP\text{-}CMV\text{-}GFP}$ exactly as in Figure 2.1. The percentages of infected cells were determined by FACS 2 days later. Shown are average values from triplicate infections with standard deviations.

Combining G330E with mutations at Arg332 or Arg335

Previously, we found that R332G and R335G had additive effects on HIV-1 replication (Pham et al., 2010). Thus, we aimed at analyzing whether the inhibition of HIV-1 conferred by G330E was, similarly, additive to that of R332G, R335G, or the double mutant R332G-R335G. As shown in Figure 2.3, however, any combination including G330E was no more restrictive, and even slightly less restrictive, than the relevant controls lacking it. Therefore, G330E has a mitigating effect on HIV-1 restriction stemming from removing positive charges in the v1 region.

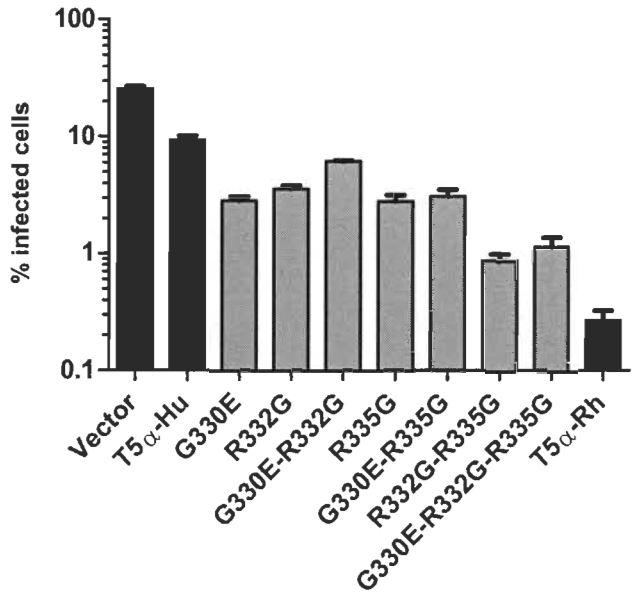

Figure 2.3 Antiretroviral effect conferred by G330E when combined with R332G or R335G.
The indicated single, double or triple TRIM5α_{Hu} mutants were constructed and retrovirally transduced into TE671 cells. The cell lines created were then challenged with $HIV_{TRIP\text{-}CMV\text{-}GFP}$ as before. Bars show averages of percent infected cells after single-dose infections in triplicates, with standard deviations.

In vitro binding analysis

Mutations at Arg332 of TRIM5α_{Hu} have been demonstrated to promote HIV-1 restriction through increased binding of TRIM5α_{Hu} to its molecular target, the CA protein of HIV-1 (Li et al., 2006). Presumably, mutations at positions 330 and 335 would also increase CA recognition, but this had not been demonstrated for Arg335 mutations. We analyzed the potential of TRIM5α_{Hu} mutants to bind *in vitro* assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes, as described before (Diaz-Griffero et al., 2009). TRIM5α_{Rh} and both R332G-R335G and G330E-R332G-R335G mutants of TRIM5α_{Hu} clearly bound *in vitro* assembled CA-NC complexes, and the strength of the interaction roughly correlated with the magnitude of restriction (Figure 2.4). G330E-R332G-R335G was less efficient than R332G-R335G at binding *in vitro* assembled HIV-1 CA-NC complexes, supporting the finding that the G330E mutation does not increase restriction when it is combined with Arg332 and Arg335 mutations.

Single mutants G330E and R335G were not observed to significantly bind *in vitro* assembled HIV-1 CA-NC complexes in these experimental conditions, but this may be simply due to a lack of sensitivity of our assay.

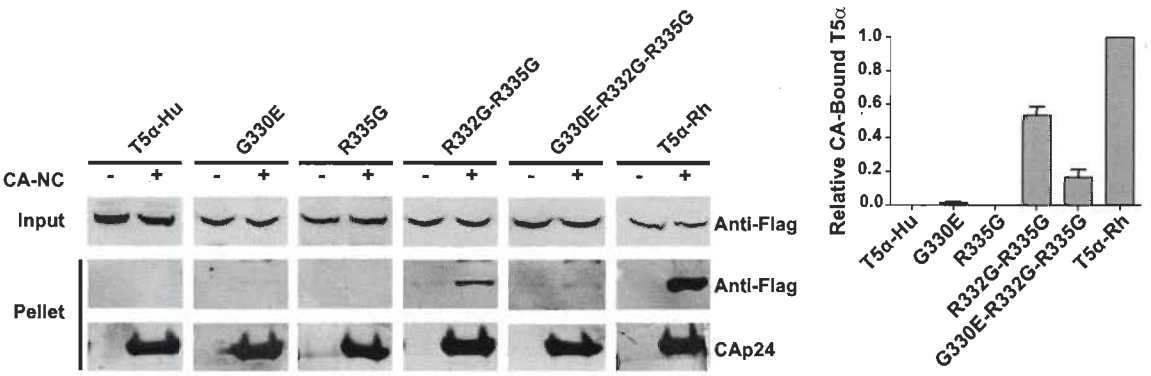

Figure 2.4 ***In vitro* TRIM5α binding to HIV-1 CA.**
CA-NC complexes were assembled *in vitro* and mixed with lysates from 293T cells transfected with the relevant TRIM5α-FLAG-expressing plasmid constructs. CA-NC complexes were separated from soluble proteins by ultracentrifugation through a sucrose cushion and analyzed by Western blotting using CA and FLAG antibodies (pellet). A fraction of the pre-centrifugation mix was analyzed by Western blot for CA content (input). The graph on the right shows relative CA-bound TRIM5α as determined by the ratio between pellet-associated and total (input) TRIM5α, relative to that obtained for TRIM5α_{Rh}. Bars represent the average of 3 experiments with standard deviations.

Restriction in other viral and cellular environments

T lymphocytes are the main host cells in natural HIV-1 infection. To analyze whether G330E and other mutants would inhibit HIV-1 when stably expressed in lymphocytes, Sup-T1 cells were transduced with the MIP-TRIM5α vectors. Like before, untransduced cells were eliminated by antibiotic treatment and cells were then challenged with a single dose of HIV-$1_{NL\text{-}GFP}$. This HIV-1 vector is similar to HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ in that it is derived from a subtype B HIV-1 strain and it does not encode a viral envelope. However, HIV-$1_{NL\text{-}GFP}$ resembles wild-type HIV-1 since all viral proteins except for Nef and Env are expressed following infection. As shown in Figure 2.5A, the infectivity data obtained closely mirrored those generated with HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ in TE671 cells. Although the overall level of restriction was smaller

than in TE671 cells, as observed before (Pham et al., 2010), it is worth noting that expression of either R332G-R335G or G330E-R332G-R335G in Sup-T1 cells inhibited HIV-1 almost as efficiently as TRIM5α_{Rh}. Like before, G330E inhibited HIV-1 as efficiently as mutations in Arg332 or Arg335, but G330E-R332G-R335G was not more efficient than R332G-R335G.

Human cells express low levels of endogenous TRIM5α that do not significantly affect HIV-1 replication, and over-expressed transgenic TRIM5α is known to be dominant over its endogenous counterpart (Berthoux et al., 2005a). However, it was still possible that some of the mutants constructed might behave differently in the absence of endogenous TRIM5α. To test this directly, we transduced feline cells, which do not express any TRIM5, with the same TRIM5α orthologs and mutants that had been tested in human cells. These cells were then challenged with HIV-$1_{NL\text{-}GFP}$ and the percentages of infected cells were determined. We found that the restriction pattern in feline cells (Figure 2.5B) closely resembled what had been obtained in human TE671 and Sup-T1 cells (Figure 2.5A). Specifically, G330E restricts HIV-1 in these cells about as efficiently as R332G and R335G do (about 10-fold compared with the empty vector control), and G330E does not potentiate HIV-1 restriction by the R332G-R335G double mutant.

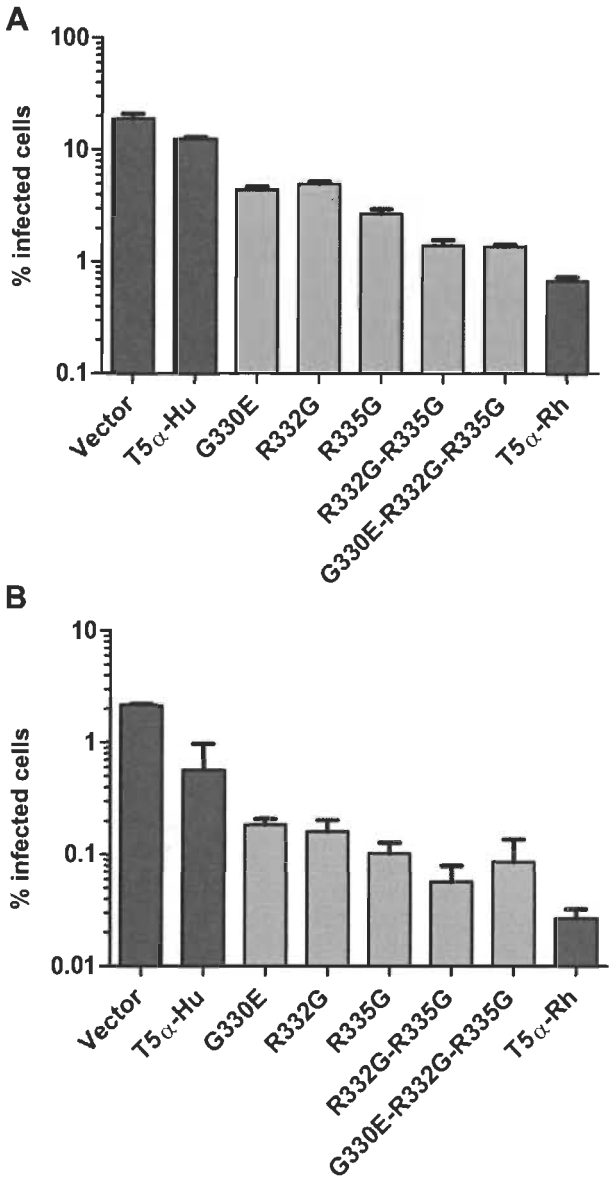

Figure 2.5 **Restriction of HIV-1 replication in human lymphocytic cells and in nonhuman cells.**

Human Sup-T1 cells (A) and feline fibroblast CRFK cells (B) were retrovirally transduced with the "empty" MIP vector or with MIP expressing the indicated TRIM5α. The cells were then challenged in triplicates with a single dose of HIV-$1_{NL\text{-}GFP}$. Percentages of infected (GFP-positive) cells were determined 2 days later by FACS, as described in Materials and Methods.

Restriction of a propagating infection

Experiments using VSV G-pseudotyped HIV-1 vectors may not always faithfully recapitulate TRIM5α-mediated restriction of WT viruses. Indeed, TRIM5α may also interfere with late (assembly) steps of the retroviral cycle (Sakuma et al., 2007) although this conclusion has been rebutted by others (Zhang et al., 2008). In addition, HIV-1 propagation occurs mostly through so-called viral synapses forming between cells, rather than by transmission of cell-free viruses (Groot et al., 2008; Haller and Fackler, 2008; Rudnicka et al., 2009). It is unclear whether TRIM5α restricts HIV-1 originating from viral synapses the same way it would restrict infection by cell-free viruses, but there are some indications that it does not (Richardson et al., 2008). In order to test whether G330E TRIM5α_{Hu} would efficiently disrupt the spread of HIV-1 in susceptible cells, we infected Sup-T1 cells expressing WT, G330 or R332G-R335G TRIM5α_{Hu} with HIV-1_{NL4-3} (Figure 2.6A). Over-expression of WT TRIM5α_{Hu} slightly delayed the peak of replication from ~5 to ~7 days post infection, an effect seen before (Pham et al., 2010). Spreading of HIV-1 in cells expressing G330E or R332G-R335G TRIM5α_{Hu} was apparent but no clear peak was visible during the course of the experiment. Indeed, while the p24 concentration in the supernatants of these two cultures was between 10 and 100 pg/ml from day 5 to day 16 of the propagation assay, these concentrations were more than 10,000-times smaller than those measured at the peak of replication in control cells and in cells over-expressing WT TRIM5α_{Hu}. Yet, viral replication did occur in these cells, as evidenced by the presence of syncytia (Figure 2.6B) followed by widespread cell death (not shown). Therefore, transduction of G330E or R332G-R335G TRIM5α_{Hu} is able to efficiently attenuate the spread of HIV-1 in susceptible cells, without completely disrupting it. That HIV-1 was roughly equally inhibited in G330E and R332G-R335G cells, despite the fact that the latter is more efficient at inhibiting HIV-1 single-cycle vector transduction, indicates that restriction of vector transduction may not always be a faithful model for the restriction of HIV-1 propagation.

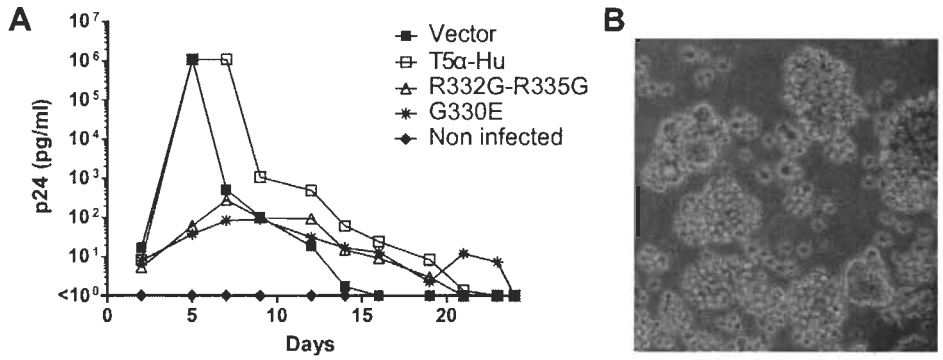

Figure 2.6 HIV-1 propagation in human lymphocytic cells expressing G330E TRIM5α_{Hu}.
Sup-T1 cells transduced with WT or mutated TRIM5α_{Hu} or untransduced (vector) were then infected with the replication-competent HIV-1 clone NL4-3. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 24 days. (A) CA (p24) content in the supernatant of infected cells and of uninfected cells as a control were determined periodically by ELISA. (B) Micrograph of R332G-R335G TRIM5α_{Hu}-expressing cells 14 days post-infection, showing the presence of syncytia.

Modelling the effect of v1 mutations on the TRIM5α_{Hu} PRYSPRY domain

The 4 hyper-variable regions of TRIM5α are present at the surface of the protein and constitute the binding interface for interactions with retroviral capsids, as has been confirmed recently (Biris et al., 2012). We used an *in silico* assay to predict the impact of mutations at positions 330, 332 and 335 on this CA-binding interface (Figure 2.7). The I-TASSER program integrated more than 10 published structures of domains similar to the WT TRIM5α_{Hu} PRYSPRY domain. Among those was the recently published structure of the Rhesus macaque TRIM5α PRYSPRY (Biris et al., 2012). According to this prediction tool, introduction of the R332G and R335G mutations caused significant changes in all 3 main variable regions (v1, v2, v3). This was most apparent in the surface representation (Figure 2.7B). Interestingly, the triple mutant (G330E-R332G-R335G) had a surface structure that was very different from that of the R332G-R335G mutant and, in fact, more closely resembled that of the WT protein. These observations help explain why G330E-R332G-R335G was slightly less restrictive than the 332-335 double mutant.

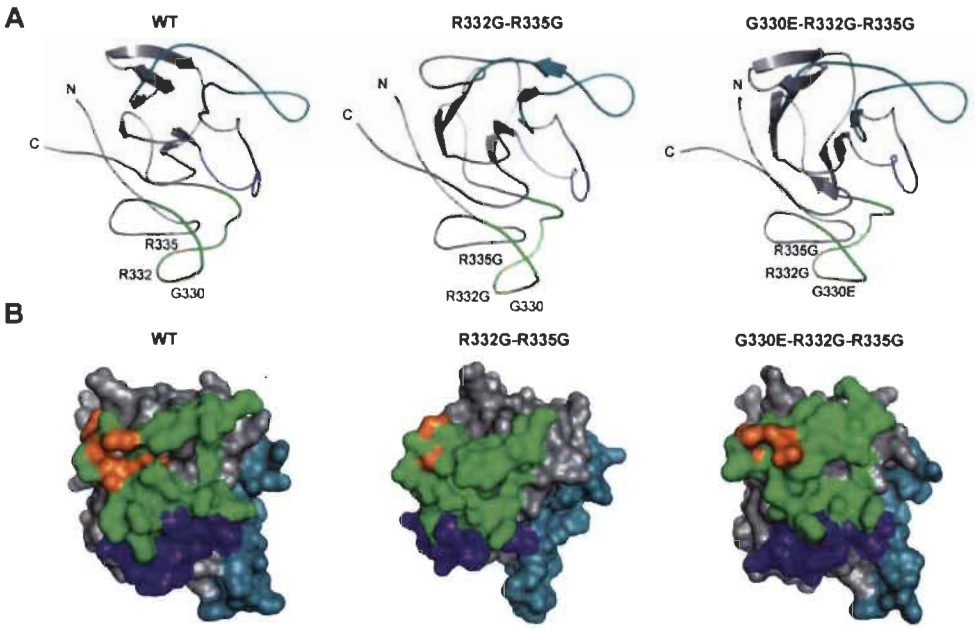

Figure 2.7 **Model representation of the TRIM5α$_{Hu}$ PRYSPRY domain.**
The predicted impact of v1 mutants on the structure of the PRYSPRY domain was modeled *in silico* based on the published structures of similar domains. (A) Ribbon model and (B) surface representation of WT, R332G-R335G and G330E-R332G-R335G TRIM5α$_{Hu}$. The v1, v2 and v3 regions are shown in green, blue and cyan, respectively. The aminoacids at positions 330, 332 and 335 are shown in orange.

Discussion

Our data reveal that a novel mutation in the v1 region of the human TRIM5α results in a 10-fold decrease in permissiveness to HIV-1 vector transduction, similar to what was previously achieved by other point mutations in this region (Li et al., 2006; Pham et al., 2010; Yap et al., 2005). This mutation, G330E, was isolated using a degenerated oligonucleotide, a technique which to our knowledge had never been applied to generate anti-HIV-1 restriction factors. The change in charge associated with the substitution is crucial to G330E inhibitory activity, as was previously found to be the case for other mutants in this region (Li et al., 2006; Pham et al., 2010). Interestingly, the inhibitory effect of G330E is not additive to that of R332G or R335G, while R332G and R335G do have additive effects. In fact, G330E slightly decreased restriction stemming from Arg332 and/or Arg335 mutations and seemed to decrease binding of

R332G-R335G to *in vitro* assembled HIV-1 CA-NC complexes. The molecular interactions between TRIM5α and its molecular target, the N-terminal region of CA, have not been elucidated at the structural level despite some recent advances (Biris et al., 2012; Ganser-Pornillos et al., 2011). Future progress in that field is a prerequisite to the full understanding of phenotypes associated with mutations in the v1 domain of TRIM5α_{Hu}. However, our structure prediction analysis suggests that discrete mutations in v1 can have long-range effects on the whole CA-interaction surface of the PRYSPRY domain. This explains how single mutations in TRIM5α_{Hu} v1 can modulate the affinity of TRIM5 binding to CA even though this binding depends on multiple weak interactions involving all the variable regions (Biris et al., 2012; Ohkura et al., 2006).

Neither E330 nor D330 are found in TRIM5α of primates that do or do not restrict HIV-1 (Song et al., 2005); therefore, the mutation found in our screen could not have been predicted from the available literature, an indication of the usefulness of random mutagenesis approaches. However, TRIM5α from Rhesus macaques and from African green monkeys, two orthologs that strongly restrict HIV-1, bear the following residues at the corresponding positions: Q330, P332, L335. Because this particular combination is different from what we have been testing, it would be valuable to test all the possible combinations (Q330-G332-G335, E330-P332-L335, etc.) in order to isolate (i) the most potent anti-HIV-1 TRIM5α_{Hu} variants and (ii) the least likely to allow HIV-1 escape. In addition, our spreading infection experiment suggests that assays with fully replicative viruses cannot be circumvented in the search for the most efficient HIV-1 restriction factors. HIV-1 vector transduction assays are convenient and informative but do not provide definitive predictions of antiviral activity.

Conclusion

An innovative random mutagenesis screen has led to the isolation of a novel determinant of HIV-1 restriction by TRIM5α_{Hu} with potential applications in gene therapy and genome editing.

Acknowledgements

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CHAPITRE III

THE V86M MUTATION IN HIV-1 CAPSID CONFERS RESISTANCE TO TRIM5α BY ABROGATION OF CYCLOPHILIN A-DEPENDENT RESTRICTION AND ENHANCEMENT OF VIRAL NUCLEAR IMPORT

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Résumé

Le VIH-1 est inhibé rapidement après l'entrée du virus dans les cellules qui expriment certains orthologues simiens de TRIM5α. Des mutants de l'orthologue humain (TRIM5α_{Hu}) peuvent également induire une protection contre le VIH-1. Une protéine de l'hôte, la cyclophiline A (CypA), se lie aux protéines de la capside (CA) virale et favorise les étapes précoces de la réplication du VIH-1 par des mécanismes encore inconnus. D'un autre côté, l'interaction CA-CypA augmente la susceptibilité du VIH-1 à la restriction par TRIM5α. Préalablement, la mutation V86M dans la boucle de liaison à CypA de la CA du VIH-1 a été sélectionnée après des passages en série du VIH-1 dans des cellules exprimant TRIM5α du singe rhésus (TRIM5α_{Rh}). Les objectifs de cette étude étaient de (i) analyser si V86M permet au VIH-1 d'échapper aux mutants de TRIM5α_{Hu}, et (ii) de caractériser le rôle de CypA dans la résistance à TRIM5α par V86M.

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Nos résultats montrent que dans des expériences de transduction de vecteurs VIH-1 non-réplicatifs, V86M confère une résistance partielle contre TRIM5α_{Hu} R332G-R335G et d'autres mutants de la région variable 1 qui ont été isolés précédemment. Par contre, V86M ne semble pas être résistant à TRIM5α_{Hu} R332G-R335G dans un contexte de propagation virale. De manière surprenante, la restriction des vecteurs VIH-1 V86M par les mutants de TRIM5α_{Hu} est surtout insensible à la présence de CypA dans les cellules infectées. Des expériences de RMN montrent que V86M modifie l'interaction CypA-CA ainsi que l'isomérisation de CA par CypA. D'un autre côté, V86M n'affecte pas l'augmentation de la réplication du VIH-1 médiée par CypA dans les cellules humaines. Finalement, des expériences de qPCR montrent que V86M augmente le transport de l'ADN viral au noyau des cellules exprimant un TRIM5α restrictif.

Somme toute, notre étude montre que V86M sépare les deux fonctions normalement associées avec l'interaction CA-CypA. V86M favorise les étapes précoces de la réplication du VIH-1 dans des cellules restrictives en améliorant le transport nucléaire. Nos résultats suggèrent donc que V86M échappe à la restriction médiée par TRIM5α en empêchant sélectivement l'augmentation CypA-dépendante du blocage de l'importation nucléaire médié par TRIM5α.

Abstract

Background: HIV-1 is inhibited early after entry into cells expressing some simian orthologues of the tripartite motif protein family member TRIM5α. Mutants of the human orthologue (TRIM5α_{hu}) can also provide protection against HIV-1. The host protein cyclophilin A (CypA) binds incoming HIV-1 capsid (CA) proteins and enhances early stages of HIV-1 replication by unknown mechanisms. On the other hand, the CA-CypA interaction is known to increase HIV-1 susceptibility to restriction by TRIM5α. Previously, the mutation V86M in the CypA-binding loop of HIV-1 CA was found to be selected upon serial passaging of HIV-1 in cells expressing Rhesus macaque TRIM5α (TRIM5α_{rh}). The objectives of this study were (i) to analyze whether

V86M CA allows HIV-1 to escape mutants of TRIM5α_{hu}, and (ii) to characterize the role of CypA in the resistance to TRIM5α conferred by V86M.

Results: We find that in single-cycle HIV-1 vector transduction experiments, V86M confers partial resistance against R332G-R335G TRIM5α_{hu} and other TRIM5α_{hu} variable 1 region mutants previously isolated in mutagenic screens. However, V86M HIV-1 does not seem to be resistant to R332G-R335G TRIM5α_{hu} in a spreading infection context. Strikingly, restriction of V86M HIV-1 vectors by TRIM5α_{hu} mutants is mostly insensitive to the presence of CypA in infected cells. NMR experiments reveal that V86M alters CypA interactions with, and isomerisation of CA. On the other hand, V86M does not affect the CypA-mediated enhancement of HIV-1 replication in permissive human cells. Finally, qPCR experiments show that V86M increases HIV-1 transport to the nucleus of cells expressing restrictive TRIM5α.

Conclusions: Our study shows that V86M de-couples the two functions associated with CA-CypA binding, i.e. the enhancement of restriction by TRIM5α and the enhancement of HIV-1 replication in permissive human cells. V86M enhances the early stages of HIV-1 replication in restrictive cells by improving nuclear import. In summary, our data suggest that HIV-1 escapes restriction by TRIM5α through the selective disruption of CypA-dependent, TRIM5α-mediated inhibition of nuclear import. However, V86M does not seem to relieve restriction of a spreading HIV-1 infection by TRIM5α_{hu} mutants, underscoring context-specific restriction mechanisms.

Background

TRIM5α was isolated as a retroviral restriction factor in 2004 [1] and acts within the post-entry, pre-integration window [2, 3]. The viral molecular target of TRIM5α is the correctly matured N-terminal domain of capsid (CA) proteins forming the outer surface of the retroviral core [2, 4-8]. A direct interaction between the two proteins, each present as high molecular weight multimers, occurs shortly after entry and is required

for downstream inhibition of viral replication [8-12]. The mechanism of TRIM5α-mediated restriction can be broken down to discrete events, some of them inter-dependent: (i) virus entrapment into TRIM5α cytoplasmic bodies [13], (ii) decreased stability of the virus core [8, 14-16], (iii) targeting to a proteasome-dependent degradation pathway [17-20], and (iv) inhibition of nuclear transport [17, 21, 22].

CypA, a host peptidyl-prolyl *cis/trans* isomerase that is ubiquitously expressed in tissues, is known to play roles in both HIV-1 infection of human cells and in HIV-1 restriction by TRIM5α in monkey cells. In dividing permissive human cells, CypA enhances HIV-1 infectivity by regulating the disassembly of its core [23-25] independently of TRIM5α [26, 27]. On the other hand, restriction of HIV-1 by simian TRIM5α orthologues is enhanced by CypA, and inhibition of CypA expression or of its activity partially rescues infectivity in restrictive conditions [21, 26, 28-30]. CypA binds to an exposed proline-rich loop on the viral CA [31, 32] and catalyzes the isomerisation of the peptide bond G89-P90 [33, 34]. The mutation V86M in the CypA-binding loop of HIV-1 CA has been identified as conferring partial resistance to TRIM5α_{Rh} [35]. The mechanism of HIV-1 resistance to TRIM5α_{Rh} conferred by V86M CA was not addressed in this study. However, it was established that this mutation in the CypA-binding loop did not disrupt CA-CypA interactions *in vitro* or in cell cultures [35].

We and others have proposed that point mutations in the variable region 1 (v1) of TRIM5α_{Hu} could confer HIV-1 restriction capability [36-41]. These mutations were discovered by mapping of HIV-1 restriction determinants in non-human TRIM5α orthologues [36, 39-41] or through the use of random mutagenesis-based screens [38, 42]. Such antiviral genes are promising candidates for gene therapy applications, owing to a few key characteristics: (i) They block replication early after virus entry and before integration can occur; (ii) They are human-like and thus probably nonimmunogenic; (iii) They inhibit HIV-1 by a well-established, natural mechanism with little side effect expected. However, it is currently unknown whether HIV-1 can acquire resistance to TRIM5α_{Hu} mutants. Here we investigate the extent and mechanism of resistance of the HIV-1 CA mutant V86M to R332G-R335G TRIM5α_{Hu} and other mutants of the

v1 domain. Our data show that this mutation affects physical and functional interactions with CypA in order to decrease HIV-1 sensitivity to TRIM5α while retaining replication-enhancement functions also conferred by CypA binding.

Results

CA-V86M HIV-1 is partially resistant to restriction by TRIM5α$_{Hu}$ mutant R332G-R335G

We analyzed whether V86M could protect HIV-1 against restriction by TRIM5α$_{Hu}$ mutant R332G-R335G in human TE671, human Sup-T1 and feline CRFK cells. The choice of human cells as an experimental model was justified by the need to gather data in cells representative of the HIV-1 natural host, while the analysis in cat cells was prompted by the absence of an endogenous TRIM5α protein in this species, thus allowing us to analyse restriction in the absence of potentially interfering effects from the endogenous gene. All the cell lines created were challenged by wild-type (WT) or V86M HIV-1 NL43-based vectors (Figure 3.1). The two vectors had almost identical titers in non-restrictive human or cat cells. In addition, when those vector preparations were normalized according to reverse transcriptase activity or titer in CRFK cells, they were found to have similar titers in activated human lymphocytes or in human M0, M1 and M2 macrophages (Figure 3.9S). However, V86M HIV-1$_{NL\text{-}GFP}$ was about 4-times more infectious than its WT counterpart in cells expressing R332G-R335G TRIM5α$_{Hu}$, and this was true in all the three cell lines tested (Figure 3.1). In other words, restriction of V86M HIV-1$_{NL\text{-}GFP}$ was 4-times less efficient than restriction of WT HIV-1$_{NL\text{-}GFP}$.

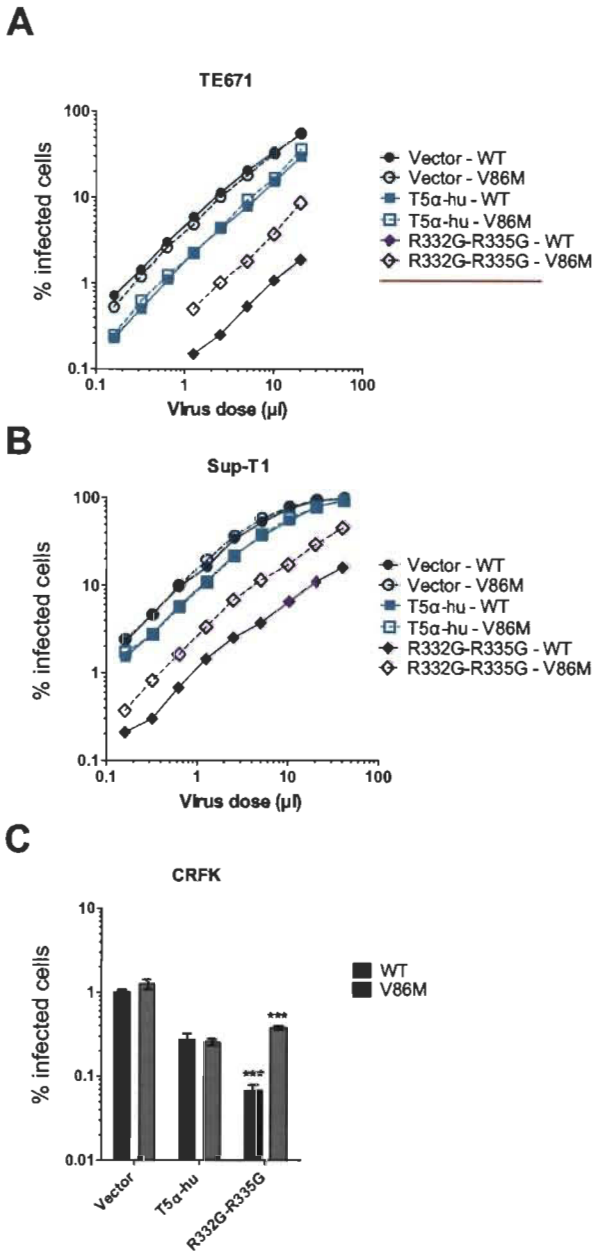

Figure 3.1 **V86M mutation in HIV-1 capsid confers partial resistance against R332G-R335G TRIM5α_{Hu}.**

TE671 (A), Sup-T1 (B) and CRFK (C) cells were transduced with either WT TRIM5α_{Hu}, R332G-R335G TRIM5α_{Hu}, or with the "empty" vector as a control. Following antibiotic selection, cells were infected with multiple doses (A, B) or with a single dose (C) of WT or V86M HIV-1NL-GFP. The two viral preparations had similar virion content as determined by RT assay. The percentage of infected (GFP-positive) cells was determined two days later by flow cytometry. In (C), infections were done at a multiplicity of infection of ~0.1 as determined for the WT virus in parental cells. Bars show the average data from 3 infections with standard deviations (***, P-value < 0.0001).

Restriction of V86M CA HIV-1 by R332G-R335G TRIM5α$_{Hu}$ is independent of cyclophilin A

Although V86 is located in the CypA-binding loop of HIV-1 CA, this mutant was previously shown to retain CA binding to CypA [35]. Accordingly, transduction of lymphocytes and macrophages by V86M HIV-1 was significantly affected by treatment by 5 μM of cyclosporine A (CsA), a drug that competes with CA for binding to CypA (Figure 3.9S). We nonetheless hypothesized that the mutation might affect functional interactions between CypA, TRIM5α and CA. To directly analyze the role of CypA in V86M-mediated resistance, we knocked down its expression in TE671 and in Sup-T1 cells that had been transduced with WT or R332G-R335G TRIM5α$_{Hu}$ or with the empty vector. The knockdown was efficient for all the TRIM5 variants and in both cell contexts (Figure 3.10S). Non-transduced cells were eliminated by antibiotic treatment, and cells were then challenged with either WT or V86M NL4-3 vectors in the presence or absence of 2 μM CsA (Figure 3.2). In control permissive cells, replication of both WT and V86M HIV-1$_{NL-GFP}$ was decreased (25 to 35%) either by expression of the CypA shRNA or by CsA treatment [25, 43] (Figure 3.2, left panels). As expected, combining CsA treatment and CypA knockdown had no additional inhibitory effect on HIV-1 replication in the two cell lines, confirming that CsA inhibits incoming HIV-1 in human cells by interfering with its interactions with CypA [25, 27, 43]. In TE671 and Sup-T1 cells expressing R332G-R335G TRIM5α$_{Hu}$, replication of WT HIV-1 was strongly reduced compared with the control "vector" cells (~50-fold and 20-fold, respectively) and like before, V86M partly rescued infection of HIV-1 in these cells (5-fold in TE671 cells, 2.5-fold in Sup-T1 cells). CypA knockdown enhanced infectivity of WT HIV-1$_{NL-GFP}$ by 5-fold in TE671 cells and by 3-fold in Sup-T1 cells, but had no significant effect on the replication of the V86M mutant. Likewise, CsA treatment strongly increased replication of WT HIV-1$_{NL-GFP}$ in R332G-R335G TRIM5α$_{Hu}$ cells (about 10-times in TE671, 5-times in Sup-T1 cells) but had a much smaller effect on V86M HIV-1$_{NL-GFP}$ (1.5- to 2-fold in both cell lines). Therefore, replication of V86M CA HIV-1 in cells expressing R332G-R335G TRIM5α$_{Hu}$ was not efficiently rescued by depletion or inhibition of CypA.

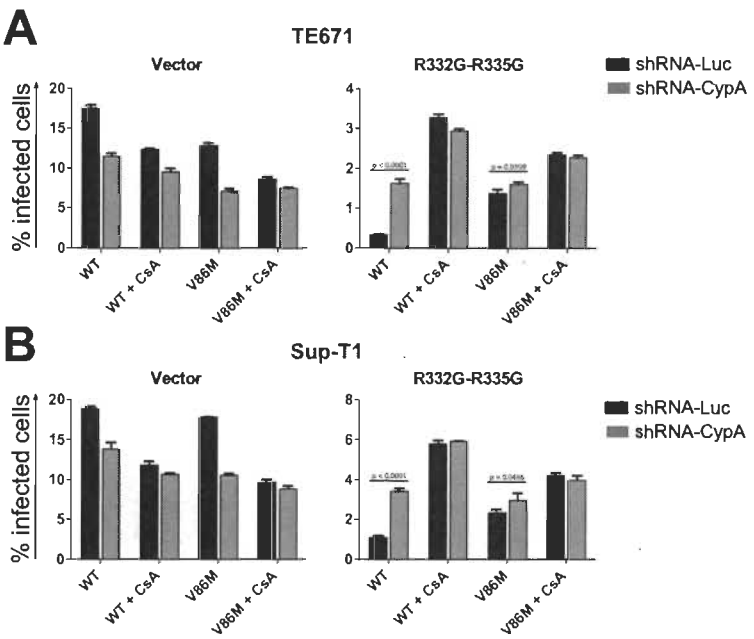

Figure 3.2 **Restriction of CA-V86M HIV-1 by R332G-R335G TRIM5α_{Hu} is independent of cyclophilin A.**
TE671 cells (A) and Sup-T1 cells (B) transduced with either WT TRIM5α_{Hu}, R332G-R335G TRIM5α_{Hu} or with the "empty" vector were also transduced with shRNAs targeting CypA or the non-relevant luciferase mRNAs. Following antibiotic selection, cells were challenged with a single dose of WT or V86M HIV-$1_{NL\text{-}GFP}$, in the presence or absence of 2 μM CsA. A multiplicity of infection of ~0.2 was used, as determined for the WT virus in parental cells and in the absence of drug. WT and V86M virus preparations were adjusted by RT assay. Cells were analyzed by FACS two days after infection, and the bars show the average % infected cells from 3 infections with standard deviations. Note the difference in scale between the y-axis of different graphs.

CsA concentration-dependent assays

In this experimental setting, instead of using a single volume of HIV-$1_{NL\text{-}GFP}$ for all infections as in Figure 3.2, we adjusted the amount of virus used for each virus/cell combination so that approximately 1% of cells were infected in the absence of drug, and we then repeated the infection in presence of increasing CsA concentrations (Figure 3.3). Like before, CsA decreased replication of both WT and V86M HIV-$1_{NL\text{-}GFP}$ in the permissive control "vector" cells by 30% to 50% in both TE671 cells (Figure 3.3A) and Sup-T1 cells (Figure 3.3B). CsA rescued replication of WT HIV-$1_{NL\text{-}GFP}$ in cells expressing R332G-R335G TRIM5α_{Hu}, by up to 6-fold in TE671 cells and

8-fold in Sup-T1 cells. In contrast, the impact of the drug on the restriction of the V86M mutant by this variant of TRIM5α_{Hu} was much smaller: up to 1.5-fold and 2-fold in TE671 and Sup-T1 cells, respectively.

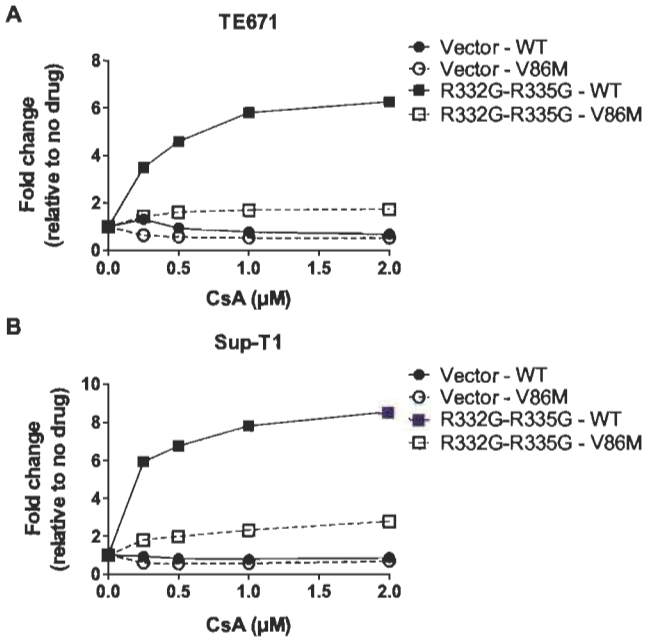

Figure 3.3 CsA dose-dependent analysis.
TE671 cells (A) and Sup-T1 cells (B) expressing R332G-R335G TRIM5α_{Hu} or transduced with the "empty" vector were challenged with WT or V86M HIV-$1_{NL\text{-}GFP}$ in the presence of increasing concentrations of CsA. Virus doses were adjusted so that approximately 1% of cells were infected in the absence of CsA. The percentages of infected cells were determined two days later as before and results are shown as –fold increases relative to the relevant no-drug controls.

Altogether, data in Figure 3.2 and Figure 3.3 show that restriction of V86M HIV-1 replication by R332G-R335G TRIM5α_{Hu} is poorly sensitive to the presence of CypA compared to restriction of the WT control. However, replication of V86M HIV-1 in permissive human cells is as much sensitive as WT HIV-1 to the presence of CypA.

The sensitivity of HIV-1 restriction by TRIM5α_{Hu} mutants to the V86M mutation correlates with its sensitivity to CsA treatment

Data shown in Figure 3.1 and Figure 3.2 revealed that restriction of HIV-1 by R332G-R335G TRIM5α_{Hu} was counteracted either by the V86M mutation or by CsA treatment, and suggested that the two interventions had redundant effects. In order to better characterize the role of CypA in the TRIM5α resistance conferred by V86M, we analyzed the effect of this mutation on other mutants of TRIM5α_{Hu}. We also analyzed the effect of CsA treatment on restriction by these same mutants. G330E, R332G, R335G, and G330E-R332G-R335G TRIM5α_{Hu} all restricted HIV-1 when expressed in TE671 cells, albeit with various efficacies (Figure 3.11SA), and as shown before [38, 42]. Replication of HIV-$1_{NL\text{-}GFP}$ in these cells was enhanced either by introducing the V86M mutation (Figure 3.11SA) or by CsA treatment (Figure 3.11SB). When we plotted the increase in HIV-$1_{NL\text{-}GFP}$ infectivity resulting from V86M against the increase in infectivity resulting from CsA treatment in TE671 expressing these various TRIM5α_{Hu}, we observed a highly significant correlation (Figure 3.4). Also, the level of rescue of HIV-$1_{NL\text{-}GFP}$ infectivity by either V86M mutation or CsA treatment was grossly proportional to the level of restriction. This analysis supports our findings that V86M and CsA treatment rescue HIV-1 replication in restrictive conditions by similar mechanisms. However, the slope of the linear curve (1.89) also shows that CsA is more efficient than V86M at rescuing HIV-1 replication.

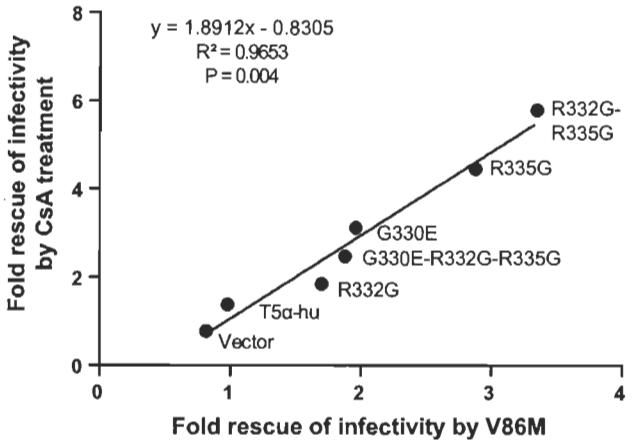

Figure 3.4 Direct correlation between the effect of mutation V86M and the effect of CsA treatment.
The increase in HIV-1 infectivity in TE671 cells expressing the indicated TRIM5α_{Hu} in presence of 2 μM CsA was plotted against the increase in infectivity resulting from the V86M mutation. All values are –fold increases relative to the untreated WT control virus. Data were gathered at a multiplicity of infection yielding close to 1% infected cells for the untreated WT HIV-1 in the cell lines expressing each the various TRIM5α. The correlation is significant, as shown by the R^2 obtained and the Spearman's P-value. The linear regression equation is shown.

Restriction of V86M CA HIV-1 by R332G-R335G TRIM5α_{Hu} in cat cells is insensitive to CsA

In human cells expressing an HIV-1-restrictive TRIM5α, CsA treatment might cause dual, opposite effects on HIV-1: enhancement by counteracting TRIM5α, and inhibition by a yet unclear, CypA-dependent mechanism. In addition, although endogenous TRIM5α_{Hu} is thought to be recessive against the over-expressed TRIM5α in our experimental system, there was still a slight possibility of interference effects. To address these issues, we analyzed the rescue of HIV-1 replication by V86M mutation and/or by CsA treatment under restriction by R332G-R335G TRIM5α_{Hu} in CRFK cat fibroblasts. These cells do not express any TRIM5α, and CsA treatment does not inhibit WT HIV-1 infection in cat cells [26, 44]. We found that R332G-R335G TRIM5α_{Hu} strongly restricted HIV-$1_{NL\text{-}GFP}$, and the mutation V86M in CA decreased restriction by about 3-fold (Figure 3.5). WT HIV-$1_{NL\text{-}GFP}$ replication was enhanced 10-times by CsA treatment while the effect of the drug on the replication of V86M HIV-$1_{NL\text{-}GFP}$ was much smaller (1.5-fold), thus mirroring the data obtained in human cells.

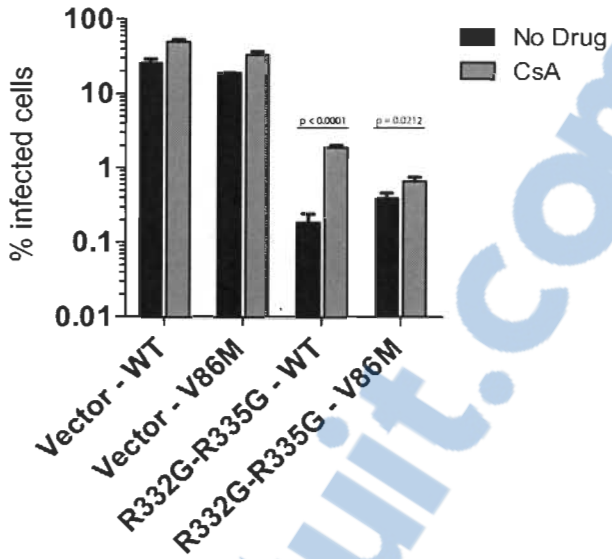

Figure 3.5 V86M HIV-1 restriction by R332G-R335G TRIM5α$_{Hu}$ in cat cells is not counteracted by CsA treatment.
CRFK cells were transduced with R332G-R335G TRIM5α$_{Hu}$ or with the empty vector as control and subsequently challenged with a single dose of WT or V86M HIV-1$_{NL\text{-}GFP}$ in presence or absence of 2 μM CsA. Infections were done at a multiplicity of infection of ~0.2 as determined in parental cells infected with the WT virus, and the amounts of viruses used were normalized by RT assay. The percentages of infected cells were determined two days later by FACS.

V86M CA interactions with CypA studied by NMR

Data in Figures 3.1-3.5 showed that V86M made HIV-1 resistant to CypA-dependent, TRIM5α-mediated restriction mechanisms. Thus, we hypothesized that V86M could modify the interactions between HIV-1 CA and CypA, or the isomerisation of the former by the latter [33, 45]. The interaction of CA with CypA was studied by 2D $^{1}H,^{15}N$ ZZ-exchange NMR spectroscopy (Figure 3.6). In the absence of CypA, we observed two G89 N-H correlation peaks in the V86M capsid spectra, indicating that, as in WT, the GP bond exists in both *cis* and *trans* states. However, the peak positions for both states in V86M were chemically shifted compared to WT, suggesting that the mutation influences the conformations adopted by the cyclophilin-binding loop (Figure 3.6A). Furthermore, there is evidence of a second minor G89$_{cis}$ peak in the V86M spectra that is indicative of discrete CypA-binding loop isomers.

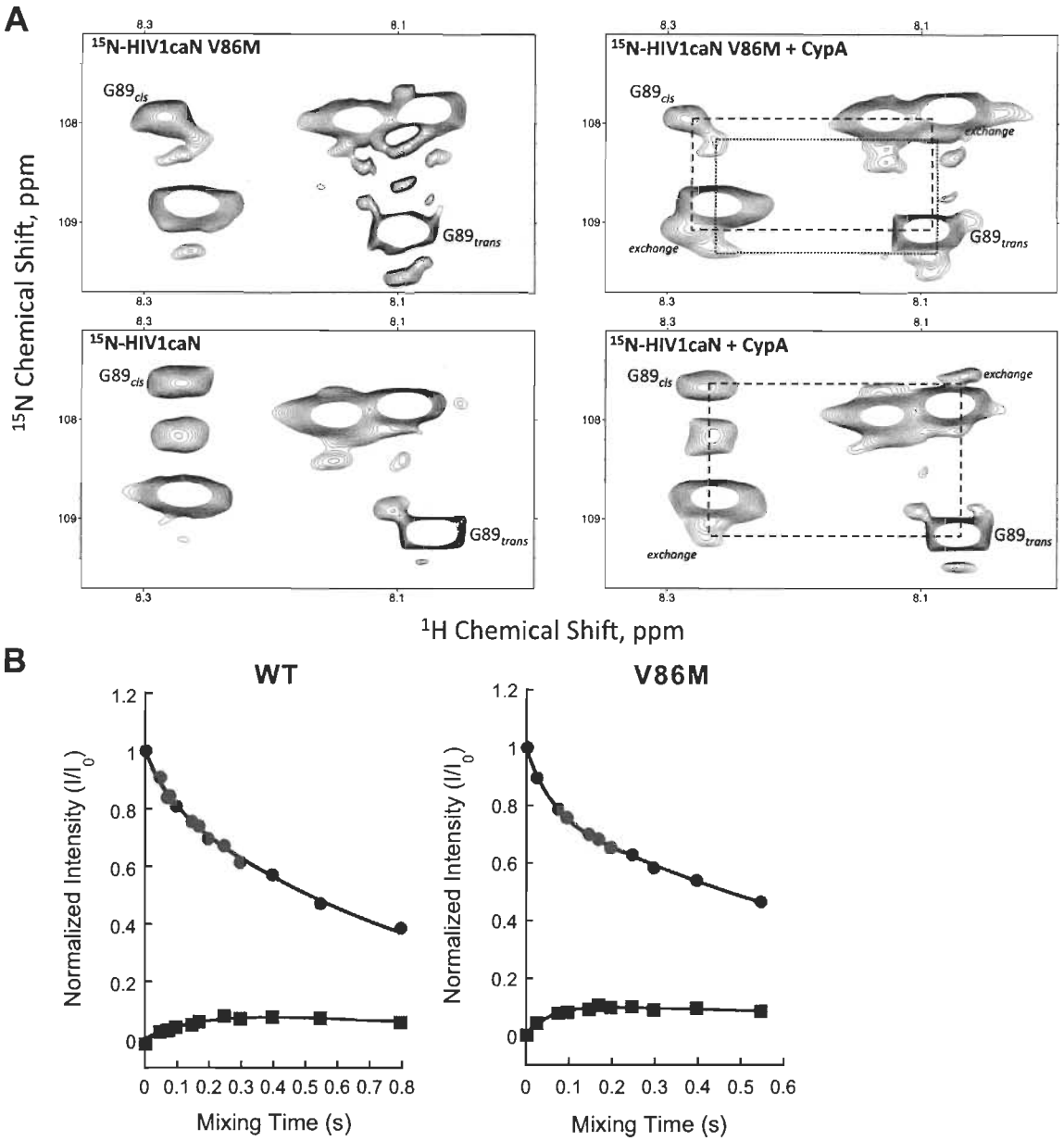

Figure 3.6 **V86M CA interactions with CypA studied by NMR.**
(A) Expansions of 2D ^{1}H-^{15}N ZZ-exchange spectra illustrating CypA catalysed *cis-trans* proline isomerisation of P90 in HIV1caN. The four panels represent backbone amide 1H,15N correlations for the preceding G89 residue in HIV1caN and in HIV1caN V86M in the presence and absence of CypA. In the absence of CypA, the isomerisation reaction is slow and only distinct G89 *cis*- and *trans*- correlation peaks are observed. The presence of CypA leads to an increase in the isomerisation rate and the accumulation of *cis* and *trans* exchange peaks. The dotted box denotes the location of the exchange peaks and indicates their symmetrical distribution with regard to $G89_{cis}$ and $G89_{trans}$. Note that in the case of HIV1caN V86M a broad G89cis peak and an additional cis-exchange peak indicates a second conformer. All spectra were acquired at the same

mixing time (69 ms) and processed with the same contour levels. (B) Evaluation of exchange rates for CypA catalyzed isomerisation of G89-P90 in HIV1caN and HIV1caN V86M. Normalised peak intensities of *trans* auto peaks and the corresponding exchange peaks for HIV1caN/CypA and HIV1caN V86M/CypA were plotted as a function of mixing time (s). Exchange rates (k_{ex}) in the order of 5 s^{-1} for HIV1caN/CypA and 20 s^{-1} for HIV1caN V86M/CypA were extracted by fitting auto peaks and exchange peaks as described by Bosco et al (2002).

Upon addition of CypA, symmetrical exchange peaks were observed for WT and V86M indicating that both capsids are a substrate for CypA and that increased *cis-trans* exchange occurs during the ZZ-exchange experiment. However, in the V86M mutant, at least two *cis* exchange peaks were observed compared to a single peak in the WT. Furthermore, the intensities and line shapes of these additional peaks were directly affected by addition of CypA and dependent upon the mixing time of the experiment (Figure 3.6B). This supports the hypothesis that the V86M mutation results in distinct populations of conformers for the G89-P90 bond.

Next, we determined the catalysed isomerisation rate of the G89-P90 bond in both WT and V86M capsids in the presence of CypA (Figure 3.6B). For WT capsid we observed a similar rate as previously published, of ~5 s^{-1}. For the V86M capsid, the exchange rate for the major G89 peak was ~20 s^{-1}. This suggests that the V86M mutation has both altered the conformations adopted by the CypA-binding loop and increased the rate at which it is isomerised. The presence of an additional $G89_{cis}$ peak in V86M in the absence of CypA suggests that the mutant capsid may also undergo faster uncatalysed isomerisation, however we were not able to quantify this under the conditions tested.

Effects of V86M on early stages of viral replication

qPCR assays were performed on DNA extracted from TE671 cells expressing either WT TRIM5α_{Hu}, R332G-R335G TRIM5α_{Hu} or, as a positive control, TRIM5α_{Rh}, and then infected with WT or V86M HIV-$1_{NL\text{-}GFP}$ (Figure 3.7). We quantified GFP DNA as a marker for late reverse transcription products and 2-LTR circles as a marker for nuclear viral DNA. As expected, accumulation of HIV-1 DNA was reduced in cells

expressing TRIM5α_{Rh} and R332G-R335G TRIM5α_{Hu}, and levels of nuclear HIV-1 DNA were even more strongly decreased. This is consistent with HIV-1 replication being impeded by TRIM5α at both the reverse transcription and nuclear transport stages [17, 22]. The amounts of viral DNAs were decreased further in TRIM5α_{Rh} cells than in R332G-R335G TRIM5α_{Hu} cells, consistent with the greater magnitude of restriction conferred by TRIM5α_{Rh}. V86M had no effect on total DNA levels but significantly increased relative amounts of 2-LTR nuclear DNA species. However, V86M 2-LTR DNA levels in restrictive cells were still reduced 5- to 10-times compared with levels of WT 2-LTR DNA in non-restrictive conditions, as expected from the observation that V86M only partially rescues HIV-1 in restrictive conditions. In summary, escape from restriction by this mutant is associated with increased HIV-1 transport to the nucleus.

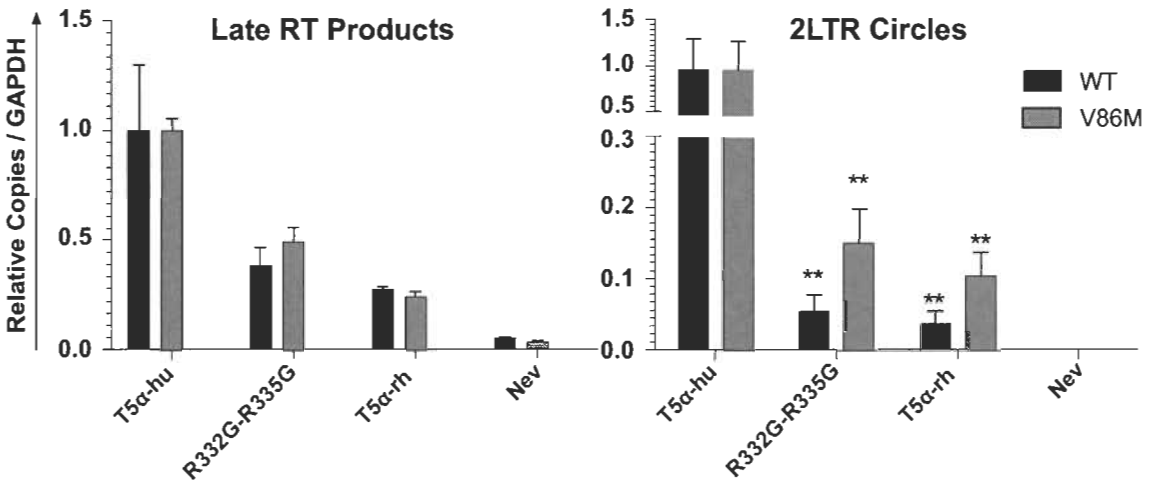

Figure 3.7 V86M enhances nuclear transport of HIV-1 DNA in restrictive conditions.

TE671 cells expressing the indicated TRIM5α were challenged with WT or V86M HIV-$1_{NL\text{-}GFP}$. An RT inhibitor (nevirapine) control was included to show the absence of contaminating DNA. The levels of HIV-1 late reverse transcription products and 2-LTR circles were determined by real-time quantitative PCR using primers to detect GFP or 2-LTR junctions, respectively. DNA amounts were calculated using serially diluted, linearized DNAs as standards. Data are presented as mean ratio of late RT or 2-LTR DNA copies over the number of GAPDH DNA copies, normalized to the permissive control TRIM5α_{Hu}. Shown are values with standard deviations of 3 or 5 independent infections (late RT products and 2-LTR, respectively). Student's t-test was used to test for significance of data, specifically WT vs V86M viruses in cells expressing R332G-R335G or TRIM5α ($p = 0.0020$ and $p = 0.0015$, respectively).

Effect of CypA and V86M on a spreading HIV-1 infection

Single-cycle experiments using HIV-1 vectors are useful to investigate restriction events taking place between entry and integration. However, restriction of fully replicating HIV-1 may be more complex than what is reflected in these assays. Indeed, TRIM5α may also interfere with late stages of the retroviral cycle [46] although these conclusions have been rebutted [47]. Perhaps even more significantly, HIV-1 propagation occurs mainly through so-called virological synapses forming between cells [48-50]. It is still unclear whether TRIM5α restricts HIV-1 originating from these viral synapses in the same way that it targets incoming capsids from cell-free viruses, but there are some indications that it does not [51]. In order to investigate the role of CypA and the effect of V86M on HIV-1 restriction in a spreading infection context, we infected Sup-T1 cells with the NL4-3 strain of HIV-1 (Figure 3.8). The cell lines included in the experiment expressed R332G or R332G-R335G TRIM5α_{Hu} and were co-transduced with the shRNA against CypA or the control shRNA targeting luciferase. The V86M mutation in CA prevented the use of a sensitive CAp24 assay to monitor replication. Instead, we performed a reverse transcriptase (RT) assay [38]. Replication of WT NL4-3 in the control (vector) shRNA-Luc cells peaked at about 18 days post-infection, while replication of the V86M mutant in the same cells peaked at about 15 days. Thus, V86M did not affect the fitness of HIV-1 in Sup-T1 cells. Replication of either WT or V86M NL4-3 was not detectable in the shRNA-Luc cells also expressing R332G-R335G TRIM5α_{Hu} (Figure 3.8, top panel). Therefore, if the V86M mutation conferred some level of resistance to R332G-R335G TRIM5α_{Hu} in a spreading infection context, it did not do so at levels high enough to allow detection of replication. In cells expressing R332G TRIM5α_{Hu}, active replication was detected but only at a single time-point (day 35 post-infection). Replication of V86M NL4-3 was not detected in the same cells, which suggests that V86M does not provide protection against this particular mutant of TRIM5α_{Hu} in a spreading infection assay. In cells transduced with the shRNA targeting CypA, viral replication was delayed compared to the control shRNA-Luc cells, as expected. This was more obvious for the V86M virus which peaked at about 18 days, confirming that CA-V86M HIV-1 depends on CypA for optimal replication in human cells. Interestingly, replication of WT and V86M NL4-3 in shRNA-CypA/R332G

TRIM5α_{Hu}-expressing cells was highly increased compared with replication in the shRNA-Luc cells. Specifically, replication of WT NL4-3 peaked at 28 days post-infection, compared to 35 days post-infection in the control cells, and the amount of viruses in the supernatants was also much higher. V86M NL4-3 peaked at about 35 days post-infection in the shRNA-CypA/R332G TRIM5α_{Hu}-expressing cells while its replication was not detected in the shRNA-Luc/R332G cells. This suggests that CypA knockdown can rescue replication of these two viruses in TRIM5-restrictive conditions and also that V86M NL4-3 is more sensitive to restriction by R332G TRIM5α_{Hu}, in contradiction with the data obtained in vector transduction experiments. In shRNA-CypA/R332G-R335G TRIM5α_{Hu}-expressing cells, replication of both WT and V86M NL4-3 was detected, although at low levels. Replication of V86M NL4-3 was no more efficient than that of its WT counterpart in these cells. Altogether, this experiment shows that restriction of a spreading HIV-1 infection by mutant TRIM5α_{Hu} in Sup-T1 cells is enhanced by CypA, and suggests that the V86M mutation does not rescue HIV-1 replication in this context.

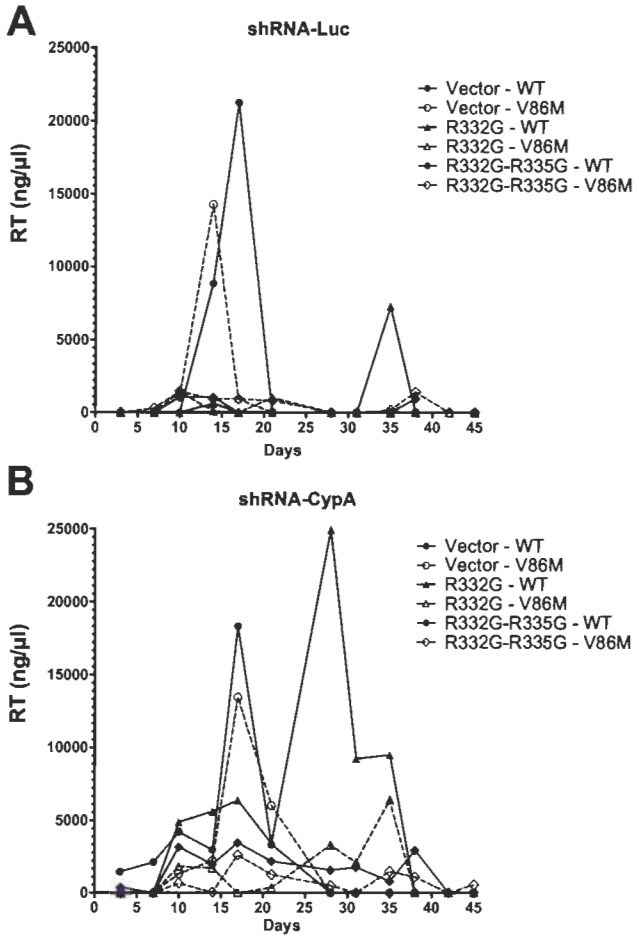

Figure 3.8 Restriction of a spreading CA-V86M HIV-1 infection.
Sup-T1 cells transduced with a control shRNA against luciferase (A) or with an shRNA targeting CypA (B) and expressing the indicated TRIM5α proteins were infected with an identical dose of WT or V86M HIV-1_{NL4-3} normalized by RT activity. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 45 days. Reverse transcriptase activity in the supernatant of infected cells and of uninfected cells as a control were determined periodically. Background noise as determined from the uninfected cells was subtracted from the other samples at each time-point.

Discussion

HIV-1 resistance to treatment is a hallmark of pharmacological interventions against this virus. Invariably, mutations appear in the coding sequences of the proteins targeted by antiretroviral drugs, including protease, reverse transcriptase and integrase [52]. It is expectable that genetic interventions will similarly lead to the occurrence of

viral resistance. Indeed, restriction of HIV-1 by CPSF6-358, a truncated form of the RNA processing factor, cleavage and polyadenylation specific factor 6 (CPSF6), is counteracted by the mutation N74D in CA [53]. This mutation, isolated by serial passages of HIV-1 in cells expressing CPSF6-358, abrogates direct binding of CA to CPSF6-358 [54]. Several groups, including ours, have produced mutants of TRIM5α_{Hu} to be used as antiviral transgenes [36, 38-41]. In order to predict potential HIV-1 escape from inhibition mediated by these TRIM5α_{Hu} variants, it is important to isolate those escape mutants *in vitro*, and to understand by which mechanism they decrease sensitivity to restriction. Our initial efforts had not led us to isolate R332G-R335G TRIM5α_{Hu}-resistant HIV-1 by serial passaging. We then decided to test whether V86M, an HIV-1 CA mutant isolated from cells expressing TRIM5α_{Rh} by the Sodroski group and shown to confer some level of protection against this orthologue, would also make HIV-1 resistant to TRIM5α_{Hu} mutants.

Our data show that V86M indeed confers some level of protection against various mutants of TRIM5α_{Hu}, at least in the context of single-cycle infections with HIV-1 vectors. We saw no protection in the context of HIV-1 spreading infections, although we tested only one HIV-1 strain (NL4-3) in one cell line (Sup-T1). Sodroski and collaborators [35] have isolated the V86M mutant in HeLa-CD4 cells, which we have not tested here. They observed modest levels (2-fold) of protection against TRIM5α_{Rh} in these HeLa-derived cells when infectivity was measured in single-cycle assays, and they saw an even more modest effect in canine Cf2Th cells [35]. Altogether, V86M can confer HIV-1 protection against restriction by various TRIM5α proteins in specific replication settings. However, even in situations in which protection takes place, restriction still occurs, albeit weakened. On the basis of our data, we do not expect V86M to be highly significant in an *in vivo* context, although this is of course rather hazardous to predict.

In order to investigate the mechanism of CA-V86M HIV-1 resistance to R332G-R335G TRIM5α_{Hu}, we analyzed the role of CypA in the restriction. Altogether, our results show that restriction of V86M CA HIV-1 is largely insensitive to the

presence of functional CypA, while the same virus is still inhibited in human cells devoid of CypA. In other words, HIV-1 is able to subtly alter its interactions with CypA in order to downregulate a mechanism of restriction while preserving other benefits associated with this interaction. Accordingly, our NMR data showed that the molecular interactions between CA and CypA were altered by the V86M mutation, as was the isomerisation reaction catalysed by CypA.

Finally, our qPCR data correlate modifications in CypA-CA interactions with effects on nuclear transport in restrictive conditions. Interestingly, experiments pre-dating the discovery of TRIM5α restriction had demonstrated that CsA treatment of Old World monkey cells increased nuclear transport of HIV-1 in these cells [21]. More recently, Lin and Emerman similarly observed that CsA treatment of the sMAGI Rhesus macaque cell line increases HIV-1 nuclear transport more than it does enhance reverse transcription [55]. Other recently published data also link nuclear transport and CA-CypA interactions in permissive human cells. Specifically, CypA knockdown and CsA treatment reduce HIV-1 dependency toward nucleopore components Nup153 and Nup358 for its nuclear transport [56, 57]. It should be informative to analyze whether V86M modifies the interactions between HIV-1 CA and these nucleoporins.

Acknowledgements

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Methods

Plasmid DNAs

pMIP-TRIM5α_{Hu} and pMIP-TRIM5α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before [28, 38, 44, 58, 59]. pMIP-TRIM5α_{Hu} with the mutation R332G-R335G has been described [38], and additional mutants have been described as well [42]. pSRBl-CypA expresses a short hairpin RNA (shRNA) targeting the human CypA mRNA. Shortly, it is the previously described pSRP-CypA plasmid [27] in which the puromycin resistance cassette has been changed to one conferring resistance to blasticidin. The control plasmid, pSRBl-Luc, encodes a shRNA targeting luciferase [60]. pNL-GFP, pMD-G and pCL-Eco have all been described elsewhere [21, 28, 58, 61-63]. The fully replication-competent HIV-1 clone pNL4-3 [64] was used for propagation experiments.

Cells

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C. Human T lymphoblast Sup-T1 cells and monocytic cells THP-1 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 37°C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA). Human PBMCs were isolated from diluted whole blood of a healthy donor by Ficoll density gradient centrifugation as previously described [65]. $CD4^+$ T cells were then isolated using a negative isolation kit (Dynabeads Untouched Human CD4 T Cells, Invitrogen) according to manufacturer specifications. The isolated cells were cultivated in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. Activation and proliferation of $CD4^+$ T cells were performed by supplementing the culture medium with 60 U/ml IL-2 (Peprotech, Canada) and incubating the cells with anti-CD3/CD28 coated beads (Dynabeads Human T-Activator CD3/CD28, Invitrogen) according to the manufacturer instructions. Infections of these cells were performed in the absence of

IL-2 and activating beads. THP-1 monocytic cells (7.5×10^5) were differentiated into M0 macrophages by incubation in serum-free medium supplemented with 50 ng/ml PMA and 10 ng/ml GM-CSF for 48 h. Polarization into pro-inflammatory M1 macrophages was induced by addition of 100 ng/ml LPS and 25 U/ml IFN-γ. Anti-inflammatory M2 macrophages were induced by addition of 20 ng/ml IL-4 and 20 ng/ml IL-10 (all reagents were from Peprotech). In all cases, non-adherent cells were washed away before infection.

Retroviral vectors production

HIV-1 and MLV-based vectors were produced through transient transfection of 293T cells and collected as previously described [38, 44]. To produce $HIV\text{-}1_{NL\text{-}GFP}$, cells were co-transfected with pNL-GFP and pMD-G. To produce the MLV-based vectors SRBl and MIP, cells were transfected with the relevant pMIP or pSRBl plasmid and co-transfected with pCL-Eco and pMD-G. To produce HIV-1 VLPs, cells were co-transfected with pΔR8.9 and pMD-G. When necessary, amounts of viruses were normalized using the nonradioactive EnzChek RT Assay Kit (Invitrogen, Burlington, ON), or based on their titer in permissive human cells.

RNA interference

pSRBl-based retroviral vectors encoding an shRNA targeting either CypA or Luc mRNAs were produced in 293T cells as described [28, 38]. TE671 and Sup-T1 cells were plated at 250,000 cells per well in 12-well plates and exposed to 1 mL of either SRBL-CypA or -Luc vector preparation. Two days post-transduction, cells were placed in medium containing 10 μg/ml of blasticidin (Sigma-Aldrich). Selection was allowed to proceed for 1 week. Efficiency of the knockdown was verified by assaying CypA expression levels in the transduced cells by western blotting, using antibodies directed against CypA (rabit polyclonal IgG; Sigma-Aldrich, StLouis, MI) and actin (mouse monoclonal; Chemicon International).

Viral challenges

Cells were plated in 24-well plates at 50,000 cells /well (TE671 and CRFK), 100,000 cells/well (Sup-T1), 200,000 cells/well (primary CD4 T cells) or 750,000 cells/well (differentiated THP-1) and infected with HIV-1 vectors (either HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$ or HIV-$1_{NL\text{-}GFP}$) with or without CsA treatment (2 or 5 μM). Drugs were added 15 min prior to infections. In CsA dose-dependent experiments, virus doses were adjusted for each virus-cell combination so that approximately 1% of the cells would be infected in the absence of CsA. Two days post-infection, cells were trypsinised when necessary, fixed in 1 to 2% formaldehyde in a PBS solution. The % of GFP-positive cells were then determined by analyzing 10,000 to 20,000 cells with a FC500 MPL cytometer (Beckman Coulter) using the CXP software (Beckman Coulter). For spreading infections, quantification of RT activity in the supernatants of infected cells was done exactly as before [38].

Quantitative real-time PCR of HIV-1 DNA

Cells were plated in 12-well plates at $3x10^5$ cells/well and infected with HIV-$1_{NL\text{-}GFP}$ WT or V86M vectors. Viruses were passed through 0.45 μm filters and pretreated for 1 hour at 37°C with 20U/ml DNAse I (New England Biolabs) to prevent contamination by carry-over plasmid DNA. In addition, control infections were performed in presence of 80μM nevirapine to ascertain the absence of such contaminating plasmid DNA. Total cellular DNA was prepared after 6 hours of infection (late RT products) or 6 hours of infection followed by 18 hours of incubation in virus-free medium (2LTR-circles) and DNA preparation was done using the DNeasy Blood and Tissue Kit (Qiagen, California).

The primer sets to detect each DNA species were as follows: GFP forward, GACGACGGCAACTACAAGAC; GFP reverse, TCGTCCATGCCGAGAGTGAT; 2LTR-circles forward (MH535), AACTAGGGAACCCACTGCTTAAG [66]; 2LTR-circles reverse (MH536), TCCACAGATCAAGGATATCTTGTC; GAPDH forward, GTCAGTGGTGGACCTGACCT; GAPDH reverse, TGAGCTTGACAAAGTGGTCG.

In each experiment, a standard curve of the amplicon being measured was run in duplicate ranging from 30 to 3 × 10^5 copies plus a no-template control. Reactions contained 1× SensiFAST SYBR Lo-Rox kit (Bioline, UK), 300nM forward and reverse primers, and 5µl template DNA (100-300ng) in 20µl-sized reactions. After initial incubation step of 3 min at 95°C, 40 cycles of amplification were carried out at 10s at 95°C, 10s at 62°C (GFP) or 65°C (2LTR, GAPDH) and 10s (GFP) or 15s (2LTR, GAPDH) at 72°C on a MX3000P qPCR system (Agilent Technologies, California). Results were analyzed with the MxPro software (Agilent Technologies).

Statistical analyses

Statistical data were calculated using GraphPad Prism version 5.03. Student's unpaired t-tests were used for tests of difference between means.

2D ^{1}H-^{15}N Heteronuclear (ZZ) Exchange Spectroscopy

Uniformly ^{15}N-labeled CA^N domain of HIV1 (^{15}N HIV1caN) was expressed in DE3 bacteria in K-MOPS buffer supplemented with 20 mM $^{15}NH_4Cl$ as a sole source of nitrogen, 4 mM pH 8.0 KPOi, 0.1 ‰ sodium ampicillin salt, vitamins and 0.4% glucose. Bacterial culture was grown until 0.500-0.700 OD_{600} at 37°C, induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein was expressed at 20°C overnight followed by purification as previously described [67] except that the final step of purification, i.e. gel filtration, was run in 50 mM potassium phosphate, pH 6.5, 100 mM NaCl and 1 mM DTT. Uniformly ^{15}N-labeled CA^N domain of HIV1 V86M (^{15}N HIV1caN V86M) was expressed and purified as per ^{15}N HIV1caN. Non-isotopically labeled N-terminally his_6-tagged human CypA was expressed in DE3 bacteria in 2xTY medium supplemented with 0.6% glucose and 0.1 ‰ sodium ampicillin salt, induced at 0.6-0.8 OD_{600} at 37°C with 1 mM IPTG, expressed at 20°C and purified using Ni-NTA beads (Qiagen) and gel-filtration chromatography in 75 mM Tris pH8.0, 50 mM NaCl and 1 mM DTT buffer. For all NMR experiments, proteins were dialysed against the same 50 mM KPOi, pH 6.5 buffer and supplemented with

1 mM DTT. The capsids were used at 12-fold excess concentration over CypA as previously described [33, 68]: 430 μM CA^N/35 μM Cyp A or 430 μM CA^N only. All NMR samples contained 5% D_2O.

2D ^1H-^{15}N heteronuclear (ZZ) exchange experiment is described in detail in [69] and was previously applied for a similar model [33, 68]. The experiments were performed on a Bruker 800 MHz spectrometer at 298 K using in-house written pulse program with the following mixing times in a randomised order: 3 ms, 25 ms, 47 ms, 69 ms, 75 ms, 97 ms, 147 ms, 169 ms, 197 ms, 247 ms, 297 ms, 397 ms, 547 ms and 796 ms. The first time-point was acquired twice to assess the error. G89 assignment for ^{15}N HIV1caN was assumed from ppm chemical shifts referred to in [33, 68]. It was also assumed that chemical shifts for G89 in ^{15}N HIV1caN V86M did not change dramatically. The data was processed in TopSpin 2.0 (Bruker, Karlsruhe) and after analysis in Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

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Supplementary data

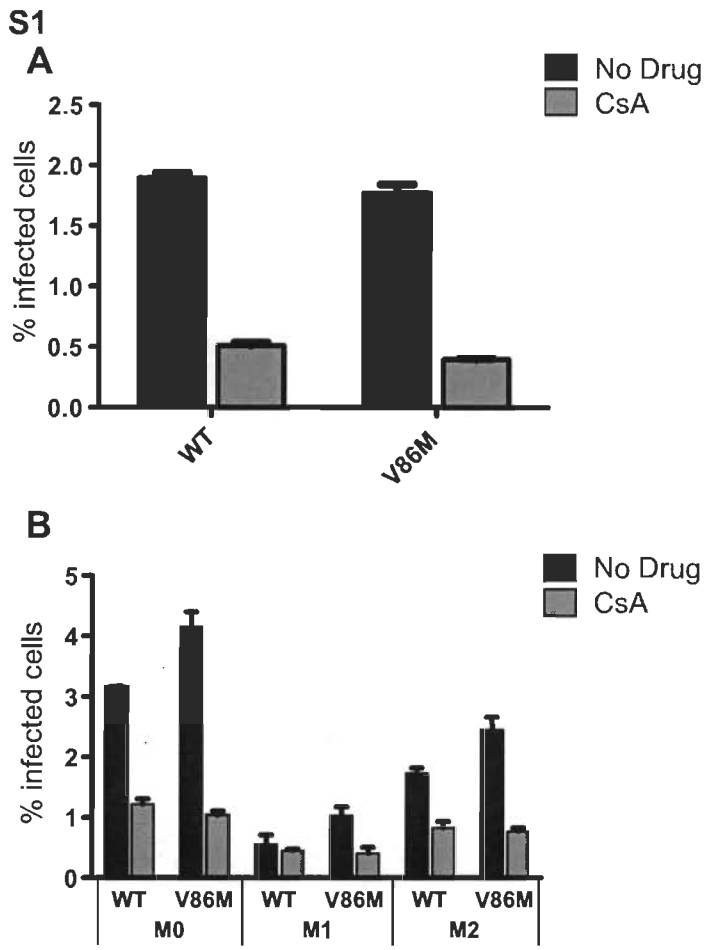

Figure 3.9S CA-V86M HIV-1 is inhibited by CsA in human lymphocytes and macrophages.

(A) Activated human $CD4^+$ lymphocytes were challenged with WT or V86M HIV-1NL-GFP at a multiplicity of infection of ~0.1 in the absence or presence of 2 μM CsA. WT and V86M viral preparations were adjusted by reverse transcriptase assay. The percentage of infected (GFP-positive) cells was determined two days later by flow cytometry. Shown are average values from three independent infections with standard deviations. (B) THP-1 cells differentiated and polarized into either macrophages (M0), pro-inflammatory macrophages (M1) or anti-inflammatory macrophages (M2) were challenged with WT or V86M HIV-1NL-GFP exactly like above but at an M.O.I of ~0.02.

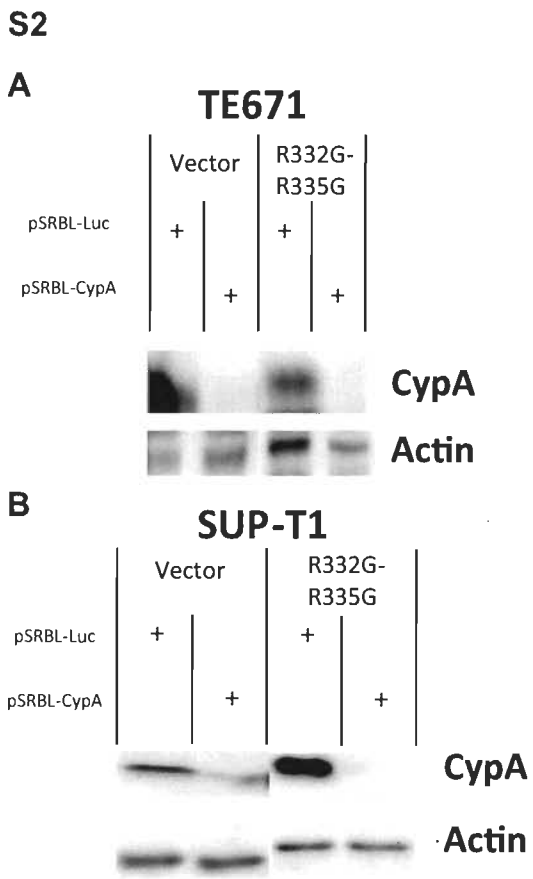

Figure 3.10S Western blotting analysis of CypA knockdown.
TE671 cells (A) and SupT1 cells (B) expressing the indicated TRIM5α_{Hu} mutants and controls were stably transduced with retroviral vectors expressing shRNAs targeting either CypA or the non-relevant control Luciferase. Untransduced cells were eliminated; then, whole cell lysates were prepared from a similar numbers of cells and processed for western blotting using antibodies against CypA or X-actin as a loading control.

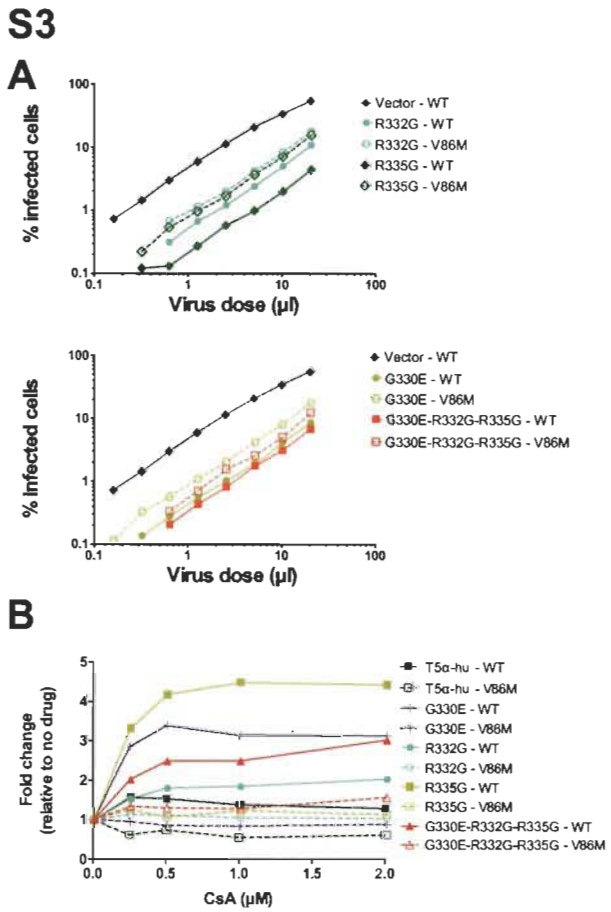

Figure 3.11S Restriction of WT and CA-V86M HIV-1 by a panel of TRIM5α$_{Hu}$ mutants.

(A) TE671 cells transduced with the indicated TRIM5α cDNAs were challenged with multiple doses of WT or CA-V86M HIV-1NL-GFP as described in Figure 3.2. Cells were analyzed by FACS 2 days later. Control permissive cells transduced with the "empty" vector and infected with WT HIV-1NL-GFP are included. (B) TE671 cells expressing the indicated TRIM5α$_{Hu}$ mutants and controls were challenged with WT or V86M HIV-1NL-GFP in the presence of increasing concentrations of CsA. Virus doses were adjusted for each virus-cell combination so that approximately 1% of the cells were infected in the absence of CsA. The percentage of GFP-positive cells was determined by FACS 2 days later and results are shown as –fold increases relative to the no-drug controls.

CHAPITRE IV

RESTRICTION OF A DIVERSE PANEL OF HIV-1 CLINICAL ISOLATES BY A MUTATED HUMAN TRIM5α

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Manuscrit en préparation pour soumission dans *Human Gene Therapy*.

Résumé

De récentes avancées dans le domaine de la thérapie génique amènent de nouvelles possibilités d'utiliser des transgènes humains contre le VIH-1. À cette fin, l'identification et la caractérisation des transgènes potentiels sont primordiales. Des mutations ponctuelles dans le domaine de reconnaissance de la capside virale conférant une activité anti-VIH au facteur de restriction humain TRIM5α ont été décrites. Puisque ces mutants ont préalablement été testés exclusivement contre des souches virales adaptées à la recherche en laboratoire, nous avons étudié le potentiel du mutant R332G-R335G de TRIM5α humain à restreindre des souches du VIH-1 hautement divergentes, incluant les membres du groupe O. Nos résultats montrent que toutes les souches testées étaient efficacement restreintes par le transgène. De plus, des isolats cliniques présentant des mutations permettant d'échapper au lymphocyte T cytotoxique (CTL) et qui ont été montrés comme étant plus susceptible à TRIM5α_{Hu} sauvage étaient également plus sensible à R332G-R335G, suggérant ainsi qu'une thérapie génique basée sur TRIM5α pourrait réduire la probabilité que le virus acquière des mutations permettant une résistance au CTL *in vivo*.

Abstract

Recent advances in the gene therapy field open up novel possibilities to use human-based transgenes against HIV-1. In order to achieve this, the identification and characterization of potent transgenes is essential. Point mutations in the capsid-recognizing domain of the human restriction factor TRIM5α have been described as conferring anti-HIV-1 activity. Because those mutants were previously exclusively tested against laboratory-adapted HIV-1 strains, we investigated the ability of the human R332G-R335G TRIM5α mutant to restrict highly divergent HIV-1 strains, including Group O. Our results show that all of the strains tested were potently inhibited by the transgene. In addition, clinical isolates bearing cytotoxic T lymphocyte (CTL) escape mutations that were previously shown to be more susceptible to the wild-type human TRIM5α were also found to be more susceptible to restriction by R332G-R335G and rhesus TRIM5α, suggesting that TRIM5α-based therapy might decrease the probability of acquiring CTL escape mutations *in vivo*.

Introduction

Recent advances in gene therapy technologies have renewed the interest in developing approaches aimed at inhibiting HIV-1 infection. Of note, several new clinical studies were started in the past years, with the vast majority of those aiming to impede viral entry by disrupting the expression of the coreceptor CCR5 via RNA interference or by direct knockout of the CCR5 locus (Didigu and Doms, 2012; Kiem et al., 2012). Another approach that has been pursued by several groups consists of using human-based transgenes in order to efficiently impede HIV-1 replication. One such candidate is the retroviral restriction factor TRIM5α, which acts in the immediate post-entry, pre-integration window (Owens et al., 2003; Perez-Caballero et al., 2005; Stremlau et al., 2004). It targets the N-terminal domain of viral capsid proteins (CA-NTD) that form the outer surface of the viral core (Cowan et al., 2002; Forshey et al., 2005; Ikeda et al., 2004; Owens et al., 2003; Shi and Aiken, 2006; Stremlau et al., 2006a). The interaction between the two proteins leads to several blocks to the viral cycle (Anderson et al.,

2006; Berthoux et al., 2004; Black and Aiken, 2010; Campbell et al., 2008; Diaz-Griffero et al., 2006; Perron et al., 2007; Rold and Aiken, 2008; Stremlau et al., 2006a; Zhao et al., 2011) while also promoting innate immunity signaling (Pertel et al., 2011). However, the range of viruses restricted by TRIM5α varies greatly in a species-specific way. For example, the human ortholog of TRIM5α ($TRIM5\alpha_{Hu}$) only moderately restricts HIV-1 (~2-fold) while its rhesus monkey counterpart ($TRIM5\alpha_{Rh}$) is highly active against HIV-1 (50 to 100-fold) (Pham et al., 2010; Stremlau et al., 2004; Yap et al., 2004). Studies have shown that overexpression of $TRIM5\alpha_{Rh}$ in human cells is dominant over the endogenously expressed protein and leads to a potent block to HIV-1 replication (Berthoux et al., 2005a; Pham et al., 2010; Stremlau et al., 2004), a phenotype also seen in hematopoietic progenitor cells (Anderson and Akkina, 2005). Replacing regions within the CA-targeting domain, called PRYSPRY, of $TRIM5\alpha_{Hu}$ by the corresponding sequences from its rhesus ortholog has resulted in human/rhesus chimeric TRIM5α proteins that can efficiently restrict HIV-1 when transduced in human cells (Kambal et al., 2011). Modeling studies and genetic screens have also led to the identification of point mutations in the variable region 1 of the $TRIM5\alpha_{Hu}$ PRYSPRY domain that allow it to target HIV-1 for restriction (Pham et al., 2010; Pham et al., submitted; Yap et al., 2005). We previously described the R332G-R335G $TRIM5\alpha_{Hu}$ mutant as especially efficient at restricting HIV-1 (Pham et al., 2010). Because these mutants differ only slightly from the endogenous form of $TRIM5\alpha_{Hu}$, they would probably not be immunogenic, thus making them ideal candidates for gene therapy applications.

It is well-established that escape mutations often lead to a fitness cost on viral replication (Friedrich et al., 2004; Liu et al., 2007; Martinez-Picado et al., 2006; Troyer et al., 2009). Interestingly, a study of CA mutations that allow viral escape from cytotoxic T lymphocyte (CTL) has shown that some of these mutations could increase the virus susceptibility to $TRIM5\alpha_{Hu}$ (Battivelli et al., 2011). Conversely, a single CA mutation at position 86 (V86M) that confers resistance to both $TRIM5\alpha_{Rh}$ and R332G-R335G $TRIM5\alpha_{Hu}$ has recently been identified (Pacheco et al., 2010; Veillette et al., submitted). These data raise the possibility that some HIV-1 clinical isolates or

clones highly divergent from clade B laboratory-adapted strains might be poorly sensitive to restriction by TRIM5α_{Hu} mutants. Potential applications for TRIM5α_{Hu} mutants necessitate that they show potency on a wide range of HIV-1 strains. Here we investigate the susceptibility of a diverse panel of HIV-1 strains to restriction by R332G-R335G TRIM5α_{Hu} and the impact of CTL escape mutations on restriction by this mutant.

Results

In order to assay the sensitivity of diverse HIV-1 isolates to mutated human TRIM5α, we obtained *gag* or *gag-pol* expressing vectors from 14 independently derived isolates. Those vectors were previously constructed in two different backbones. The first set of vectors encodes the full-length *gag* gene from 11 isolates in an otherwise isogenic context (pCMV-Δ8.91) (Ikeda et al., 2004). The other set is based on a NL4-3 vector that contains a deletion in *env* and expresses *Renilla* luciferase in place of *nef*, and in which the *Gag-PR* sequence is derived from three isolates bearing CTL escape mutations (Battivelli et al., 2011). The two sets were used to produce either GFP- or Renilla-encoding, VSV-G pseudotyped HIV-1 vectors by transient transfection as previously described (Berube et al., 2007; Pham et al., 2010). Strains name, sequence accession numbers, subtypes or clades, and cellular tropism are summarized in Tableau 4.1.

Tableau 4.1
Summary information on viral strains used.

Name of strain	GenBank accession no.	Subtype/clade	Tropism[a]	Reference(s)
p8.9Ex (HXB2)	K03455	B	T	
89.6	U39362	B	U	(Kim et al., 1995)
JRCSF	M38429	B	T	(Chesebro et al., 1991; Gao et al., 1998)
YU-2	M93258	B	M	(Gao et al., 1998; Li et al., 1991)
GUN-1WT	D34592	U	T/M	(Shimizu et al., 1994; Takeuchi et al., 1987)
GUN-1V	D34594	U	T/M	(Fu et al., 1999; Shimizu et al., 1994; Takeuchi et al., 1987)
MVP-5180	L20571	Group O	T/M	(Gurtler et al., 1994)
90CR056	AF005496	H	U	(Gao et al., 1998)
92BR025	U52953	C	U	(Gao et al., 1998)
93BR029	AF005495	B/F	U	(Gao et al., 1998)
94UG114	U88824	D	U	(Gao et al., 1998)
NRC1	JN408075	B	U	(Matsuoka et al., 2009)
NRC2	JN408076	B	U	(Matsuoka et al., 2009)
NRC10	JN408077	B	U	(Matsuoka et al., 2009)

[a] T, T-Tropic; M, M-Tropic; U, Unknown.

The genetic diversity of the panel of HIV-1 isolates used in this study was first determined by phylogenetic sequence analysis. For all strains for which information was available, the full-length *gag* nucleotide sequence was retrieved and aligned to randomly chosen reference strains. The alignment was manually checked, and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). As shown in Figure 4.1A, the panel of HIV-1 isolates used in this study includes representatives of different group M subtypes (B, C, D and H) and one evolutionary distant strain from group O. Also, some are known to be macrophage-tropic (CCR5-tropic) while others are T cell-tropic (CXCR4-tropic) (Table 4.1). In order to analyze specific differences in the CA sequence of the strains used, the first 150 amino acids of CA (CA-NTD) were aligned (Figure 4.1B). The isolates in the panel present mutations in all of the seven helical regions except one (helical region 5). Interestingly, three of the strains used

present mutations at the same position as the TRIM5α-resistant V86M (MVP-5180, V86P; NRC1 and NRC10, V86A). In addition, the mutations at position 86 and two others (96 and 116) are reverted back to wild-type (NL4-3) in the NRC10-VMG strain. Previous studies did not investigate whether other mutations at position 86 could also impact infectivity of HIV-1 under TRIM5α-mediated restriction.

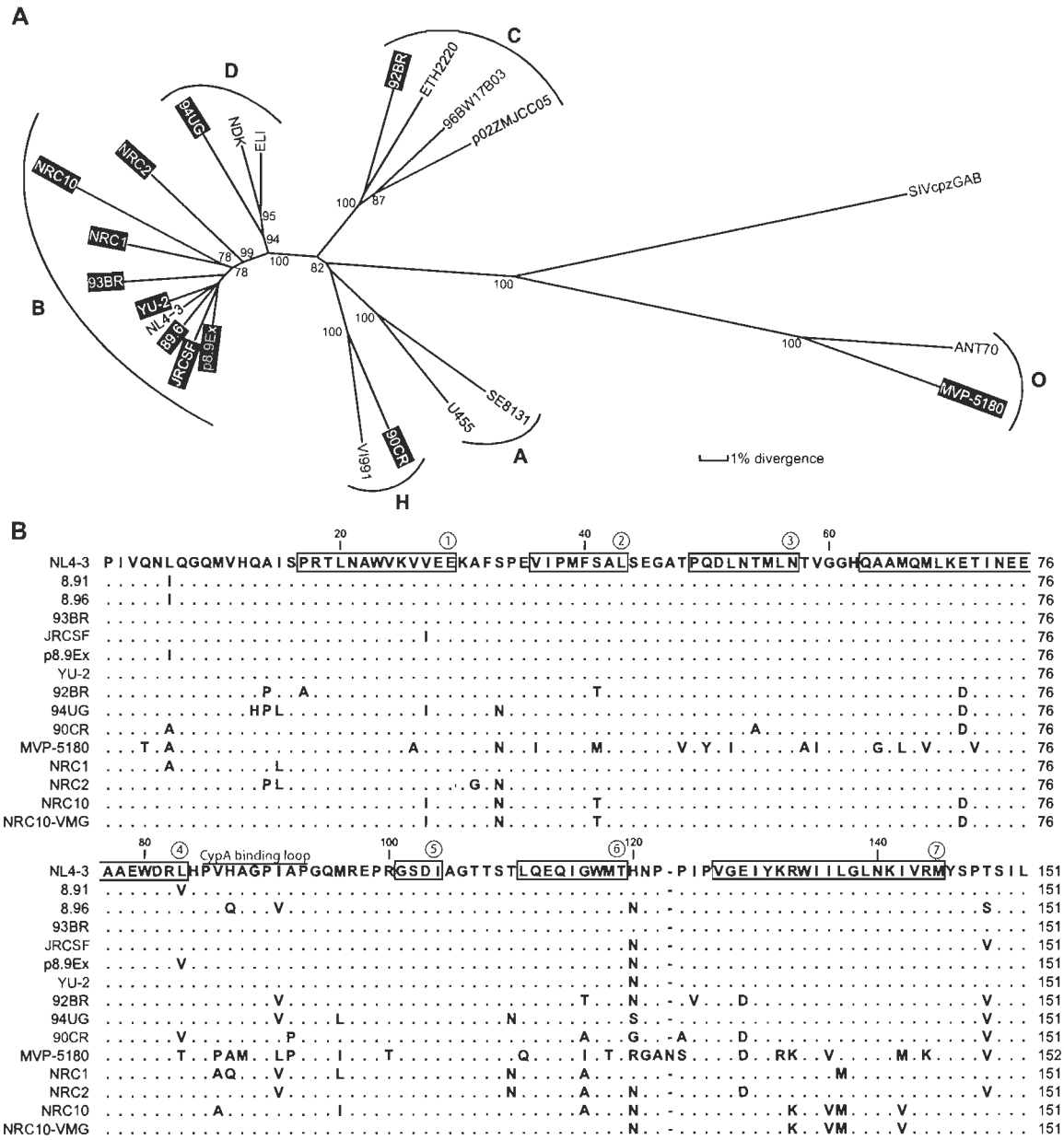

Figure 4.1 Phylogenetic relationships and sequence alignment of the virus strains used in this study.

(A) Phylogenetic relationships of the virus strains used in this study to randomly-chosen reference strains. Trees were constructed from

full-length *gag* nucleotide sequences by using the neighbor-joining method (see Materials & Methods for details). Branch lengths are drawn to scale, with the bar representing 0.01 nucleotide substitution per site. Percent bootstraps values in which the adjacent branching was supported are indicated at the nodes (values of 75% and higher are shown). Strains with a highlighted name were used in this study. Brackets indicate either HIV-1 group O or subtypes of group M. (B) Annotated sequence alignment of the first 150 CA amino acids of strains used in this study and for which sequences were available. The NL4-3 sequence is shown as the reference sequence and the seven helical regions of N-terminal CA are boxed and numbered (numbers in circles). Amino acids are numbered according to the beginning of CA.

Using the GFP-encoding HIV-1 virions produced from the first set of vectors, we infected human TE671 cells expressing either wild-type TRIM5α_{Hu}, R332G-R335G TRIM5α_{Hu} or TRIM5α_{Rh} and quantified GFP-positive cells two days post-infection by flow cytometry. As shown in Figure 4.2, all viruses tested were susceptible to restriction by both TRIM5α_{Rh} and R332G-R335G TRIM5α_{Hu}. Although the extent of the restriction varied slightly from strain to strain, R332G-R335G TRIM5α_{Hu} inhibited all HIV-1 strains by more than 10-fold, with the exception of 93BR. In addition, the pattern of restriction by TRIM5α_{Rh} was similar to that of R332G-R335G TRIM5α_{Hu}, supporting the idea that these two orthologs interact with HIV-1 CA in a similar fashion.

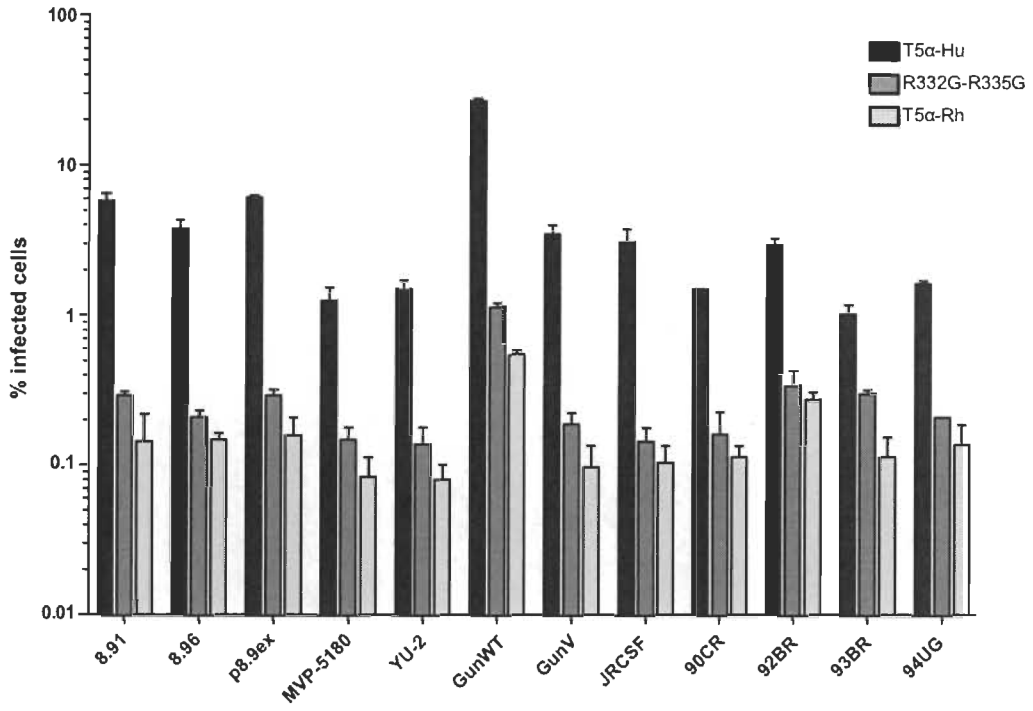

Figure 4.2 Infectivity of different HIV-1 strains in cells expressing a TRIM5α$_{Hu}$ mutant.
TE671 cells were transduced with the indicated TRIM5α cDNAs. Cells were then infected at a single MOI with the different GFP-encoding viral vectors. The percentage of infected cells was determined two days post-infection by flow cytometry. Shown are the mean and standard deviation of three independent infections.

Assaying the sensitivity of the strains that used *Renilla* luciferase as a reporter gene required a different approach. We measured the infectivity of those strains in both feline CRFK cells, which do not express any known TRIM5α ortholog, and in human TE671 cells transduced with the different TRIM5α cDNAs. Results were normalized to 1 for the NL4-3 virus in each cell type (CRFK and TE671 expressing TRIM5α$_{Hu}$, see Figure 4.3). The NRC1 clone had the same restriction pattern as that of NL4-3, although the former was a bit less sensitive to R332G-R335G TRIM5α$_{Hu}$ (11.7- vs. 4.8-fold). Our results were consistent with what was previously described (Battivelli et al., 2011), as NRC2 and NRC10 showed increased sensitivity to TRIM5α$_{Hu}$ (13.5- and 56-fold, respectively). Moreover, increased susceptibility to restriction by TRIM5α$_{Hu}$ was linked with higher restriction by R332G-R335G TRIM5α$_{Hu}$ and TRIM5α$_{Rh}$. The NRC10-VMG clone, in which three mutations (V86A, M96I and G116A) of NRC10 are reverted back

to NL4-3 (Figure 4.1B) showed decreased sensitivity to all three TRIM5α, supporting the idea that human (mutated or WT) and rhesus TRIM5α recognize NRC10 CA through, at least in part, the same interaction domains. In addition, the increase in restriction in cells expressing R332G-R335G TRIM5α_{Hu} or TRIM5α_{Rh}, compared with cells expressing WT TRIM5α_{Hu}, was less important for the two strains (NRC2, NRC10) that were already more sensitive to WT TRIM5α_{Hu}. This again suggests that CA from these two HIV-1 strains bind WT TRIM5α_{Hu} in a fashion similar to their interaction with R332G-R335G TRIM5α_{Hu} or TRIM5α_{Rh}.

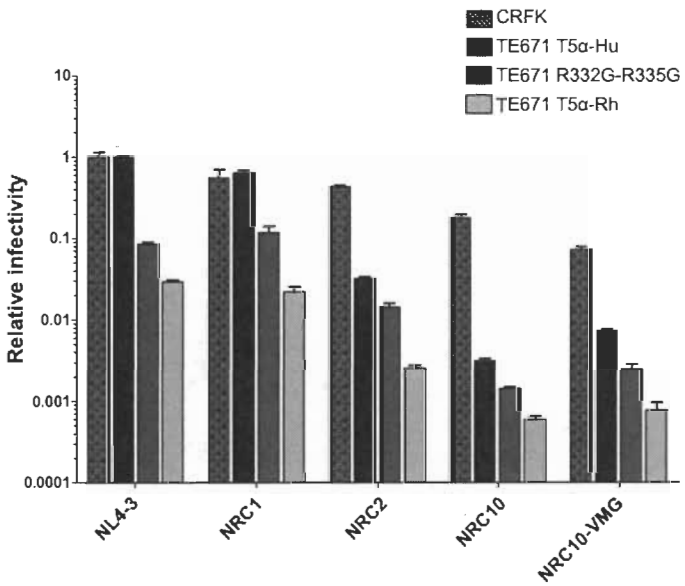

Figure 4.3 **Sensitivity of CTL escape mutants to mutated TRIM5α_{Hu}.**
In order to assay the sensitivity of the HIV-$1_{NL\text{-}Luc}$ strains to restriction by WT or R332G-R335G TRIM5α_{Hu}, feline CRFK cells and TE671 cells expressing the indicated TRIM5α cDNAs were infected with a single dose of the different HIV-$1_{NL\text{-}Luc}$ strains. Two days post-infection, cells were lysed and luciferase activity was measured. The relative light units (RLU) values obtained were normalized to NL4-3 in non-restrictive conditions (CRFK and TE671 expressing TRIM5α_{Hu} WT). Shown are the mean and standard deviation of three independent infections.

Discussion

Restriction factors such as TRIM5α are thought to play an important role in protecting against non-host retroviruses and limiting cross-species transmission (Cullen,

2006; Hatziioannou et al., 2004; Kirmaier et al., 2010; Mariani et al., 2003). Our lab and others have previously shown that point mutations in the PRYSPRY domain of the human TRIM5α protein can increase restriction of lab-adapted HIV-1 viral strains (Li et al., 2006; Maillard et al., 2007; Pham et al., 2010; Stremlau et al., 2005; Yap et al., 2005). Because those constructs differ only slightly from their endogenous counterpart, they would probably not be immunogenic, thus making them ideal candidates for therapeutic treatment of HIV-1. However, it was not yet known whether these TRIM5α variants would restrict clinical isolates of HIV-1. Our results show that all strains of a diverse panel of HIV-1 variants are potently restricted by a mutated TRIM5α_{Hu} (R332G-R335G) and by TRIM5α_{Rh}. In addition, the conserved restriction pattern across all strains tested suggests that these two HIV-1 restrictive variants of TRIM5α share the same CA determinants. However, the HIV-1 strains used here presented variations across almost all of the helical regions of CA-NTD (except helix 5) and we found no correlation between individual mutations and restriction levels.

In a clinical context, predicting how a treatment will interfere or synergize with other natural processes is of high importance. Some mutations in CA that were associated with escape from CTLs were previously shown to increase sensitivity to TRIM5α_{Hu} (Battivelli et al., 2010; Battivelli et al., 2011) and our results show that this increased sensitivity can also be extended to other, more restrictive, TRIM5α variants. This suggests that a highly restrictive TRIM5α used in gene therapy applications could help mitigate the rate at which CTL escape mutations appears, thus probably making it significantly harder for the virus to escape both the acquired immunity and gene therapy-based protections. CTL escape mutations are often followed by compensatory mutations that reduce the fitness cost of the resistance mutations (Battivelli et al., 2011; Brockman et al., 2007; Brumme et al., 2009). Interestingly, such compensatory mutations in the NRC10 strain directly affect the CypA-binding loop of CA (V86A and M96I), which is known to play an important role in TRIM5α-mediated restriction (Berthoux et al., 2004, 2005b; Keckesova et al., 2006; Stremlau et al., 2006b). In addition, the residue at position 86 was already shown to play a crucial role in the resistance to both TRIM5α_{Rh} (Pacheco et al., 2010) and mutated TRIM5α_{Hu} (Veillette et al., submitted). Although the

NRC1 strain shares some mutations that apparently render NRC10 more susceptible to TRIM5α restriction (V86A and G116A), the former presents the same restriction pattern as NL4-3. Without excluding the possibility of context-specific effects of other mutations, this phenotype could also be explained by the presence in NRC1of the H87Q mutation, which was already described as conferring resistance to TRIM5α (Chatterji et al., 2005; Ikeda et al., 2004). Taken together, this suggests that mutations in or near the CypA-binding loop of CA play an important role in modulating HIV-1 interactions with both innate and acquired immune defenses.

Materials and Methods

Plasmids DNAs

pMIP-TRIM5α_{Hu} and pMIP-TRIM5α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before (Berthoux et al., 2005a; Berthoux et al., 2005b; Berube et al., 2007; Sebastian et al., 2006). For all HIV-1 strains used in this study except NRC1, NRC2 and NRC10, pCMV-Δ8.91 -based *gag-pol* expressing vectors were described elsewhere and are a kind gift of Greg Towers and Yasuhiro Ikeda (Ikeda et al., 2004). pNL4-3XCS-derived proviral vectors containing the *Renilla* luciferase gene in place of *nef*, that present a 580-bp deletion in *env* and code for either NL4-3, NRC1, NRC2 or NRC10 *gag-pol* were previously described and are a kind gift of Allan Hance (Battivelli et al., 2010). pMD-G and pTRIP$_{CMV\text{-}GFP}$ have been described elsewhere (Berthoux et al., 2005a; Berthoux et al., 2004, 2005b; Berthoux et al., 2003; Naviaux et al., 1996; Zufferey et al., 1997).

Cell lines

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

Retroviral vectors production

HIV-1-based vectors were produced through transient transfection of 293T cells and collected as previously described (Berube et al., 2007). To produce strain-specific HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$, cells were co-transfected with $pTRIP_{CMV\text{-}GFP}$, the respective pCMV-Δ8.91 vector and pMD-G. To produce strain-specific HIV-$1_{NL\text{-}Luc}$, cells were co-transfected with the respective pNL4-3XCS vector and pMD-G.

Viral challenges and measurement of sensitivity to TRIM5α

For viral challenges with HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$, cells were plated in 24-wells plates at 50,000 cells/well and infected the next day with the appropriate HIV-1 vectors at an MOI of 0.1. Virus doses were normalized by titering the viral stocks in feline CRFK cells (data not shown). Two days post-infection, cells were trypsinised and fixed in 1 to 2% formaldehyde in a PBS solution. The % of GFP-positive cells were then determined by analyzing 10,000 to 20,000 cells with a FC500 MPL cytometer (Beckman Coulter) using the CXP software (Beckman Coulter).

For viral challenges with HIV-$1_{NL\text{-}Luc}$, cells were plated in 24-wells plates at 50,000 cells/well and infected the next day with 100µl of the appropriate HIV-1 vectors, a dose that resulted in measurable luciferase activity in all tested conditions. Two days post-infection, the cells were washed once with PBS then lysed in 100µl of Glo Lysis Buffer (Promega, Madison, WI, USA) following manufacturer recommendations. Relative light units (RLU) were measured using the Renilla-Glo Luciferase Asssay System (Promega, Madison, WI, USA) according to the manufacturer instructions and read in a Biotek Synergy HT plate-reader.

Phylogenetic tree analysis

Phylogenetic relationships of the strains used in this study were estimated by sequence comparison with randomly-chosen reference strains from the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov). *Gag* sequences of all the strains were

aligned using ClustalX2 (2.0) and a phylogenetic tree was constructed by the neighbor-joining method. The reliability of the topology was estimated by performing bootstrap analysis with 1,000 replicates. iTOL v2.2 was used to draw the tree for illustration purposes(Letunic and Bork, 2007, 2011).

Protein sequence alignment

The first 150 amino acids of the CA protein of each strain were aligned using the CLC Mainworkbench software (v6.5) using default parameters.

Conclusions

The screening of a panel of HIV-1 vectors expressing *gag* sequences derived from different isolates allowed us to determine that a broad range of strains can be restricted by TRIM5α_{Hu} mutants, re-affirming their interest as gene therapy candidates. Increased sensitivity of CTL escape mutants to TRIM5α_{Hu} was also shown to correlate with increased sensitivity to both mutated TRIM5α_{Hu} and TRIM5α_{Rh}, hinting that clinical applications using TRIM5α-based transgenes might also help prevent or at least delay acquisition of CTL escape mutations by HIV-1.

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CHAPITRE V

DISCUSSION ET PERSPECTIVES

S'inscrivant dans l'effort concerté de lutte contre la pandémie de VIH-1, l'identification et la caractérisation de transgènes humains efficaces contre le VIH-1 est un enjeu de taille. L'un d'entre eux, le facteur de restriction TRIM5α, a déjà fait l'objet d'efforts qui ont permis d'identifier des mutations lui conférant une activité anti-VIH. Entre autres, notre laboratoire a identifié le mutant R332G-R335G qui permet une diminution d'environ 20 fois de l'infectivité du VIH-1. La possibilité d'avoir un transgène encore plus actif nous a poussés à réaliser un crible par mutagénèse aléatoire de la région variable 1 du domaine de reconnaissance de la capside virale de TRIM5α. Ceci nous a permis d'identifier une autre mutation, G330E, qui permet de restreindre le VIH-1. Cependant, la combinaison de cette nouvelle mutation avec R332G-R335G n'est pas parvenue à augmenter le potentiel de ce transgène. Au contraire, une réduction de l'efficacité a été observée sous certaines conditions. Le meilleur candidat, R332G-R335G, a donc été retenu pour nos études subséquentes.

Le VIH-1 étant un virus hautement mutagénique, il est normal et prévisible d'identifier des virions ayant acquis des mutations leur permettant d'échapper à une pression sélective. Pour TRIM5α, la littérature a présenté une mutation de la capside virale, V86M, qui permet une résistance à ce facteur de restriction chez le singe rhésus [74]. Considérant que ce TRIM5α a le même mécanisme d'action que notre transgène, nous avons étudié si cette mutation pouvait également conférer une résistance aux mutants de TRIM5α_{Hu}, dont le mutant R332G-R335G. Nos résultats ont confirmé notre hypothèse et nous avons ainsi voulu déterminer le mécanisme de cette résistance. L'étude de l'effet de V86M sur le transport nucléaire du PIC et de l'implication de la CypA a permis de suggérer que V86M résiste à TRIM5α par l'abrogation des mécanismes de restriction CypA-dépendant, rétablissant ainsi le transport nucléaire du PIC.

Alors que les études sur l'activité de restriction des transgènes de TRIM5α_{Hu} n'avaient été effectuées qu'avec des souches du VIH-1 adaptées au laboratoire, aucune donnée n'était disponible sur l'efficacité de ces transgènes contre des souches cliniques. Nous avons donc testé cette activité contre un vaste éventail de souches dérivées d'isolats cliniques avec des représentants de plusieurs groupes et sous-groupes du VIH. Ces expériences ont montré que R332G-R335G permet la restriction efficace de toutes les souches testées, suggérant ainsi que la restriction par ce mutant n'est pas limitée aux souches de laboratoire ou quelques sous-groupes du VIH.

5.1 L'identification de transgènes aux propriétés antivirales

Depuis l'essor des technologies de biologie moléculaire, de plus en plus de techniques différentes sont disponibles pour identifier, modifier et améliorer des gènes d'intérêts et les protéines qui en découlent. Ainsi, les scientifiques ont pris avantage de ces technologies pour améliorer divers aspects de la condition humaine, ce qui inclut autant des applications médicales qu'industrielles. D'un point de vue médical, les maladies infectieuses demeurent à ce jour un problème d'envergure et la biologie médicale offre des approches novatrices afin d'identifier de nouvelles stratégies thérapeutiques. De plus, elle permet de mieux caractériser ces nouvelles stratégies, accélérant ainsi le processus vers une application clinique.

5.1.1 Transgènes de TRIM5α

Alors que la comparaison de la séquence d'un orthologue restrictif de TRIM5α avec celle de la protéine humaine a permis l'identification d'une mutation, deux approches différentes par mutagénèse aléatoire ont résulté en l'identification de deux autres de ces mutations. Malgré le fait qu'elles proviennent de trois stratégies différentes, ces mutations sont situées au sein d'une même portion de la région variable 1 de TRIM5α (acides aminés 330 à 335). Récemment, des expériences de résonance magnétique nucléaire (NMR) ont permis d'affirmer que le domaine PRYSPRY de TRIM5α interagit par ses quatre régions variables avec la CA virale [36].

Or, de simples mutations ponctuelles dans la région v1 permettent une restriction du VIH-1. Ce qui semble à priori contradictoire est en partie expliqué par le modèle structural présenté en Figure 2.7 qui suggère que ces mutations ponctuelles peuvent avoir un effet répandu sur les trois autres régions variables sans toutefois affecter le repliement de la protéine. Il est possible que le mécanisme par lequel TRIM5α reconnaît la capside virale, via un assemblage multimérique et de faibles interactions, puisse permettre à l'activité du facteur de restriction de résister à des mutations induisant des changements conformationels marqués. De plus, les mêmes travaux de NMR suggèrent que l'interaction PRYSPRY-CA est dominée par une région v1 mobile, donnant ainsi une flexibilité au domaine PRYSPRY ce qui lui permet de s'adapter aux diverses conformations du cœur viral. Il n'est donc pas surprenant que des mutations ponctuelles dans cette région qui semble si importante induisent de tels changements sur le potentiel de restriction.

Cette flexibilité pourrait également être impliquée dans le potentiel qu'a TRIM5α de restreindre différents virus ou encore de s'adapter aux différences présentes chez un même virus (différents groupes et sous-groupes du VIH, par exemple). Une telle flexibilité est d'ailleurs observée dans d'autres composantes du système immunitaire tels que les IgM [36, 75]. C'est en effet cette flexibilité qui leur permet de réagir à différents antigènes.

Bien que la région v1 semble être un facteur déterminant dans la spécificité de la restriction, il n'est pas exclu que d'autres mutations dans les autres régions variables du domaine PRYSPRY puissent conduire à la restriction du VIH-1 par TRIM5α_{Hu}. D'ailleurs, il est important de rappeler que le crible présenté dans cet ouvrage était limité à une mutagénèse de la région v1. Cependant, le crible par Pham *et al.* [70] a plutôt utilisé une mutagénèse du domaine PRYSPRY. Bien qu'il ait identifié qu'une mutation dans la région v1, il est possible que la méthodologie utilisée dans le crible ait favorisé une identification des mutations dans les régions les plus déterminantes seulement. Néanmoins, la réussite de ces deux cribles montre bien le potentiel qu'ont ces approches d'identifier de nouveaux transgènes humains à caractère antiviral.

5.1.2 Transgènes d'autres facteurs de restriction

Alors que notre étude se limite à TRIM5α, d'autres facteurs de restriction font néanmoins l'objet de travaux dans d'autres laboratoires. L'un d'entre eux, APOBEC3G, est une cytidine déaminase qui induit une hypermutation de l'ADN virale en interférant avec le processus de la transcription inverse. Avec l'évolution, le VIH-1 a cependant développé un mécanisme de résistance à ce facteur de restriction : Vif. En effet, la protéine virale Vif est capable d'interagir et de lier APOBEC3G et favorise sa dégradation par le protéasome. La forme humaine d'APOBEC3G n'est donc pas efficace contre le VIH-1.

À l'instar de TRIM5α, on retrouve chez le singe rhésus un orthologue d'APOBEC3G qui est actif contre le VIH-1. Un groupe de recherche a donc introduit des mutations chez la protéine humaine par comparaison de séquence avec la version simienne. Cette approche leur a permis d'identifier une mutation (D128K) qui rend APOBEC3G résistant à l'action de Vif, rétablissant ainsi son activité antivirale [76]. Bien que cette approche ait porté fruit, il est probable qu'un crible par mutagénèse aléatoire, tel que nous l'avons réalisé pour TRIM5α, serait un outil précieux pour l'identification de mutations supplémentaires conférant une résistance à Vif.

5.1.3 D'autres candidats se cachent dans le génome

Bien qu'il est utile d'utiliser des cribles par mutagénèse aléatoire pour modifier des facteurs de restriction connus, d'importants travaux ont été réalisés afin d'identifier de nouveaux facteurs de restriction ou protéines cellulaires essentielles à la réplication virale. En ce sens, un crible ciblant plus de 19 000 gènes humains a permis d'identifier 114 facteurs qui présentaient un impact significatif sur l'infection du VIH-1 [77]. De ce nombre, les auteurs ont choisi de cibler leurs expériences supplémentaires sur un seul d'entre eux, laissant donc un vaste nombre de possibilités inexplorées. Considérant qu'il faut énormément d'efforts et de temps pour caractériser de nouveaux facteurs de restriction, il est probable que de mieux comprendre les cofacteurs cellulaires essentiels à l'infection du VIH, tels la protéine LEDGF qui est essentielle à l'intégration optimale

de l'ADN virale dans le génome cellulaire, puisse ouvrir de nouvelles voies vers des stratégies thérapeutiques, qu'elles soient pharmacologiques ou via thérapie génique [78].

5.2 La résistance virale et le cas de CA-V86M

5.2.1 Transduction, virus réplicatif et TRIM5α : ce que nous apprend V86M

D'un point de vue pratique, il existe principalement deux stratégies différentes pour analyser la restriction de facteurs tels que TRIM5α. Afin de permettre une manipulation plus aisée et sécuritaire, et puisque TRIM5α agit aux étapes précoces du cycle de réplication, la plupart des expériences sont réalisées avec des souches non-réplicatives du VIH. Le génome de ces souches ne code pas pour l'enveloppe virale, laquelle est introduite sur un autre vecteur utilisé lors de la production des virions. L'enveloppe naturelle du VIH, qui limite l'infection aux cellules exprimant CD4 et un corécepteur spécifique, est bien souvent remplacée par l'enveloppe du virus de la stomatite vésiculaire (VSV) qui elle permet l'entrée du virus dans plusieurs types cellulaires. Utilisant un tel virus modifié, l'analyse de la restriction par TRIM5α se fait alors en observant la diminution du nombre de cellules infectées dans l'échantillon. La deuxième stratégie, qui reflète un peu plus la réalité du virus, utilise un génome viral complet. Ces virus seront donc réplicatifs et l'analyse de la restriction par TRIM5α se fera indirectement en mesurant la quantité de virus produits par ces cellules.

Bien que l'on puisse observer l'efficacité des mutants de TRIM5α dans ces deux contextes expérimentaux, d'importantes différences restent à prendre en considération et sont soulevées par les expériences réalisées avec CA-V86M. En effet, une étude antérieure démontrant que la mutation V86M confère une résistance à $TRIM5\alpha_{Rh}$ présente l'observation de cette résistance sous les deux contextes expérimentaux, non- et réplicatif [74]. Or, nous n'avons pas observé la résistance de V86M aux mutants de $TRIM5\alpha_{Hu}$ lors d'expériences de réplication. Il y a donc disparité entre i) les résultats des deux contextes et ii) nos résultats et ceux déjà publiés. L'explication du premier point est plus aisée, puisque malgré que TRIM5α agisse aux

étapes précoces du cycle de réplication virale, il n'est pas exclu qu'il puisse exercer une activité à une autre étape du cycle viral. En ce sens, certaines études ont suggéré que TRIM5α puisse jouer un rôle inhibiteur dans l'assemblage des nouvelles particules virales [79, 80].

Pour ce qui est de la disparité observée entre nos résultats et ceux du groupe ayant identifié V86M, il est important de noter que nous n'avons pas utilisé le même type cellulaire qu'eux. En effet, leur système était basé sur l'infection de cellules HeLa-CD4 exprimant TRIM5α_{Rh}. Pour notre part, nous avons privilégié un modèle basé sur l'infection d'une lignée cellulaire de lymphocytes T (Sup-T1). Un des facteurs ayant pu résulter en la disparité observée est la transmission virale par ce qui est appelé les synapses virales. Ce type de transmission est différent de la transmission classique en ce sens qu'il se fait de cellule à cellule, sans que les virus aient besoin d'être relâchés dans le milieu extracellulaire. À ce jour, peu est connu sur les conséquences qu'a la transmission synaptique sur le virus. Considérant que le temps de maturation du virus et que la méthode d'entrée cellulaire sont différents, il est possible que ce type de transmission vienne modifier le déroulement des étapes post-entrée, pouvant donc ainsi altérer la susceptibilité à la restriction par TRIM5α.

5.2.2 La résistance virale dans un contexte clinique

Il est également important de reconnaître et d'évaluer la signification d'une mutation de résistance isolée *in vitro* sur l'efficacité potentielle des traitements thérapeutiques qui découleraient d'une utilisation de TRIMα. Premièrement, nous n'avons pas observé de réplication accrue du virus V86M dans un contexte réplicatif en présence des transgènes de TRIM5α, ce qui tend à diminuer l'impact clinique qu'aurait cette mutation. De plus, le contexte clinique est de plusieurs ordres de grandeur plus complexe, faisant intervenir les fonctions immunes du patient et les drogues antirétrovirales qui seraient toujours utilisées. La probabilité de voir resurgir une telle mutation résistante à TRIM5α en est donc d'autant réduite. Néanmoins, il ne faut pas

sous-estimer le potentiel mutagénique du virus, alors que l'une des principales causes de l'échec des traitements actuels est la résistance virale.

De plus, à la lumière des analyses d'isolats cliniques effectués au chapitre IV (voir Figure 4.1), la région de la boucle de liaison à CypA de la CA virale semble être importante pour diverses fonctions virales. En effet, alors que la mutation V86M procure une résistance à TRIM5α, la mutation V86A permet d'échapper aux CTL, mais induit une sensibilité plus grande à TRIM5α [81]. Les effets opposés observés par des mutations à cette position pourraient rendre la résistance virale à l'un ou l'autre des facteurs plus difficile.

5.3 Thérapie génique : implications des résultats obtenus et perspectives

5.3.1 Transgènes de TRIM5α : leur potentiel clinique

Sans expériences concrètes, l'étude des transgènes de TRIM5α dans un contexte clinique n'est que spéculative. Cependant, puisque TRIM5α agit aux étapes précoces de la réplication virale il réduit donc dramatiquement les niveaux de transcrits viraux produits. La synthèse des nouvelles protéines virales en est donc réduite et le risque de voir surgir une mutation de résistance est faible. De plus, la thérapie génique vise à repeupler le patient de cellules immunitaires résistantes au virus, rétablissant ainsi les fonctions immunes. S'il demeure actif, le système immunitaire est capable de bâtir une forte réponse contre le virus. En effet, plusieurs études ont présenté des données suggérant que les anticorps neutralisants et la réponse cytotoxique dépendante des anticorps exercent une forte pression sélective sur le virus [82, 83]. Combinée à la restriction par un transgène de TRIM5α, il est probable que le potentiel qu'aurait le virus d'échapper à toutes ces pressions simultanées soit dramatiquement réduit.

Un autre déterminant essentiel au succès d'une thérapie génique impliquant TRIM5α est la survie et la prolifération des cellules modifiées. Les déterminants de ce point sont répartis en deux facettes. Premièrement, afin que les cellules modifiées

puissent être acceptées et non rejetées par l'hôte, leur présentation antigénique ne devrait pas être modifiée d'une manière à présenter des antigènes du non-soi. Spéculativement, l'utilisation de transgènes modifiés par quelques mutations devrait réduire les probabilités que les cellules modifiées ne soient rejetées par l'hôte. Deuxièmement, peu est connu sur le devenir des antigènes du VIH après restriction par TRIM5α. Théoriquement, il est possible que des antigènes du virus soient incorporés et présentés par le complexe majeur d'histocompatibilité. Cette présentation d'antigène pourrait résulter en une activation de cellules cytotoxiques contre la cellule ayant interrompu l'infection par le VIH, diminuant ainsi l'avantage sélectif des cellules modifiées.

Une équipe de recherche a par ailleurs utilisé une nouvelle approche de modification génique afin de réaliser l'introduction de plusieurs gènes anti-VIH tout en empêchant l'expression du corécepteur CCR5 [84]. À cette fin, ils ont utilisé des nucléases à doigt de zinc (ZFN) afin de cibler spécifiquement le gène CCR5 et y induire un bris du double brin d'ADN. Tirant profit de la machinerie de réparation de l'ADN par homologie, ils ont introduit un vecteur exogène où étaient placés des bras d'homologie pour CCR5 entourant une cassette d'expression pour une chimère humaine – rhésus de TRIM5α, APOBEC3G D128K et Rev M10. Ce dernier est une version dominante négative de la protéine virale Rev, et lorsqu'exprimée empêche l'activité de la protéine Rev virale, ce qui inhibe la réplication en altérant l'export nucléaire des transcrits viraux non épissés. Cette approche a résulté en l'insertion permanente de ces transgènes dans le gène CCR5 et a rendu les cellules modifiées à première vue totalement résistantes à l'infection par le VIH-1. Néanmoins, cette stratégie demeure loin d'être complètement viable cliniquement puisqu'elle emploie une surexpression des facteurs de restrictions alors que TRIM5α peut engendrer de graves dérèglements de la signalisation de l'immunité innée dans ce contexte [56].

5.4 Perspectives générales

Bien que les travaux présentés dans cet ouvrage pavent la voie vers de potentielles approches cliniques utilisant des transgènes de TRIM5α, plusieurs étapes et analyses

restent à faire. Tout d'abord, mieux comprendre les modifications à la structure de TRIM5α qu'apportent les mutations dans le domaine PRYSPRY : ceci pourrait aider à identifier avec précision les déterminants de l'interaction TRIM5α – CA. Pour ce faire, l'utilisation de techniques de biologie structurale telles que la cristallographie protéique serait un atout.

Concernant la résistance de V86M à TRIM5α, nous avons pu cibler le mécanisme de cette résistance à l'interaction CypA – CA modifiée. Cependant, le rôle exact de CypA dans la réplication virale n'est pas connu. Les récentes études et la nôtre tendent à suggérer que CypA pourrait favoriser l'import nucléaire du PIC [68, 85]. Puisque CypA joue également un rôle important dans la restriction par TRIM5α, mieux comprendre le rôle de CypA dans la réplication virale pourrait aider à mieux cerner les mécanismes de restriction et de résistance à TRIM5α. Également, puisque la mutation de résistance étudiée ici a été identifiée après passage en série du virus dans des cellules exprimant $TRIM5\alpha_{Rh}$, il serait pertinent d'essayer d'identifier de nouvelles mutations conférant une résistance à $TRIM5\alpha_{Hu}$ R332G-R335G. Ceci pourrait permettre de mieux prédire des mutations de résistance qui pourraient se développer *in vivo*.

La dernière facette de cet ouvrage concernait la restriction de différents isolats cliniques du VIH-1 par nos transgènes de TRIM5α. Bien que nous ayons analysé la restriction de souches représentant les divers groupes et sous-groupes du VIH, effectuer cette expérience sur un vaste nombre d'échantillons isolés d'une même cohorte de patients infectés permettrait de mieux cerner les variations de restriction auxquelles on pourrait s'attendre dans un contexte clinique.

CONCLUSION

La recherche de nouvelles approches thérapeutiques contre le VIH/SIDA est un enjeu de taille. Les travaux présentés dans cet ouvrage permettent d'avancer les connaissances sur le facteur de restriction TRIM5α, notamment sur sa spécificité d'action et les mécanismes de résistance virale. Nous avons également démontré le potentiel qu'a la mutagénèse aléatoire d'identifier de nouveaux transgènes à activité antivirale. Somme toute, l'ensemble de ces travaux continue de paver la voie vers une thérapie génique utilisant des transgènes de TRIM5α, laquelle semble être un objectif de plus en plus réaliste.

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