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PAR
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IDENTIFICATION ET CARACTÉRISATION DE MUTANTS DE TRIM5α AYANT UNE FORTE ACTIVITÉ DE RESTRICTION CONTRE LE VIH-1

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RÉSUMÉ

Bien que la thérapie antirétrovirale hautement active (HAART) a amélioré l'espérance de vie des patients atteints du VIH-1, un tel traitement est loin d'être idéal avec des effets secondaires notables entraînant une mauvaise observance aux traitements et l'émergence fréquente de mutants viraux résistants aux traitements. Le besoin d'une alternative efficace contre le VIH-1 a donné lieu à de grands développements dans le domaine de la recherche en thérapie génique. Dans cette optique, il serait plus bénéfique de cibler le VIH-1 au début de son cycle de vie, soit au point d'entrée ou avant l'intégration afin de limiter les effets cytotoxiques, de réduire les possibilités d'évolution mutagène pendant la transcription inverse et d'empêcher l'établissement de réservoirs latents.

Les facteurs de restriction sont un ensemble de protéines antivirales qui forment un aspect important du système immunitaire inné. Leur expression constitutive permet une réponse immédiate à une infection rétrovirale. Parmi les facteurs de restriction présentement identifiés, les plus étudiés sont : APOBEC, tétherine/BST-2, SAMHD1 et TRIM5α. Bien que chez l'homme, TRIM5α a peu d'effet inhibiteur sur le VIH-1, il a été démontré que des modifications pouvaient conférer une activité anti-VIH à la protéine TRIM5α humaine (TRIM5α_{hu}) , soit en substituant les séquences critiques PRYSPRY de TRIM5α_{hu} par celui du singe rhésus TRIM5α_{rh} ou par un changement unique d'acide aminé en position 332 de la protéine humaine. De plus, contrairement aux autres facteurs de restriction mentionnés, le VIH n'a pas développé de protéine accessoire qui soit capable de neutraliser les effets restrictifs de TRIM5α, ce qui en fait un candidat idéal pour la thérapie génique contre le VIH-1.

Dans cette étude, nous avons réussi à (1) générer des mutations aléatoires dans la région PRYSPRY de la protéine TRIM5α_{hu}, (2) isoler par un crible fonctionnel d'autres mutants présentant une activité de restriction accrue contre le VIH-1, (3) caractériser l'effet de TRIM5α_{hu} muté sur diverses souches cellulaires et virales. Finalement, nous discuterons ici comment ces protéines pourraient être adaptées et exploitées pour des approches de thérapie génique contre le VIH-1.

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LISTE DES SYMBOLES ET ABRÉVIATIONS

ADN	Acide désoxyribonucléique
AGM	Singe vert africain (A*frican Green Monkey*)
APOBEC	(*Apolipoprotein B mRNA-editing catalytic)*, Facteur de restriction
ARN	Acide ribonucléique
CA	Capside virale
CA-NTD	Domaine N-tenninal de la capside virale
CRF	Formes recombinantes circulante du VIH *(Circulating Recombinant Forms)*
CsA	Cyc1osporine A
CRISPR	(Clustered Regularly Interspaced Short Palindromic Repeat), famille de séquences génétiques répétées
CTL	Lymphocytes T cytotoxiques *(Cytotoxic T Lymphocyte)*
CypA	Cyclophiline A
EIAV	Virus de l'anémie infectieuse équine *(Equine lnfectious Anemia Virus)*
ENV	Enveloppe virale
FACS	Cytométrie en flux (*Fluorescence-activated cell sorting)*
FIV	Virus de l'immunodéficience féline *(Feline lmmunodeficiency Viru)*
FRC	Forme recombinante circulante du VIH
FRU	Forme recombinante unique du VIH
Fv1	(*Friend virus susceptibility* 1), facteur de restriction
gp120	Glycoprotéine 120 du VIH
gp41	Glycoprotéine 41 du VIH
HAART	Traitement antirétroviral hautement actif *(Highly active antiretroviral therapy)*
HF	(*High-Fidelity),* se dit des tampons et polymérases à haute fidélité

HIV	Virus de l'immunodéficience humaine (*Human Immunodeficiency Virus)*
HSC	Cellules souches hématopoïétiques (*Hematopoietic stem cells)*
IFN-I	Interférons de type 1
IN	Intégrase virale
LTR	Séquence terminale longue répétée *(Long Terminal Repeat)*
MA	Matrice virale
MLV	Virus de la leucémie murine (*Murine Leukemia Virus)*
NC	Nucléocapside virale
NF-ĸB	(*Nuclear factor kappa-light-chain-enhancer of activated B cells)* sous-unité régulatrice d'une protein kinase
NMR	Résonance magnétique nucléaire (*Nuclear Magnetic Resonance)*
PIC	Complexe viral de pré-intégration (*Pre-Integration Complex)*
POL	Polymérase virale
PR	Protéase virale
qPCR	PCR quantitative
Rh	Singe rhésus
RT	Transcriptase inverse virale *(Reverse Transcriptase)*
SAMHD1	*(SAM domain and HD domain-containing protein 1*), facteur de restriction
SIDA	Syndrome d'immunodéficience acquise
SIV	Virus de l'immunodéficience simienne (*Simian Immunodeficiency Virus)*
SU	Protéine virale de surface
TM	Protéine virale transmembranaire
TRIM	Motif tripartite (*Tripartite Motif*)
TRIM5α_{hu}	Orthologue humain de TRIM5α
TRIM5α_{rh}	Orthologue du singe rhésus de TRIM5α
VIH	Virus de l'immunodéficience humaine

VSV	Virus de la stomatite vésiculaire (*Vesicular Stomatitis Viru)*
WT	Type sauvage (*wild type)*
ZFN	Nucléases à doigt de zinc (*Zinc Finger Nucleases)*

CHAPITRE I

INTRODUCTION

1.1 Le virus de l'immunodéficience humaine

Le virus de l'immunodéficience humaine (VIH) fait partie de la famille des rétrovirus et du genre des lentivirus. L'infection par le VIH cause le syndrome de l'immunodéficience acquise (SIDA) qui se caractérise par une diminution progressive du taux de lymphocytes T CD4+ circulants (< 200 cellules/µl) résultant en une plus grande susceptibilité face aux infections opportunistes (Girard P.M. *et al.*, 2011).

1.1.1 Épidémiologie et problématique

Même si on observe une légère diminution du nombre de nouvelles infections, qui est attribuable à une diminution des comportements à risque, le SIDA reste un problème important de santé publique. En 2013, le nombre de personnes vivant avec le VIH dans le monde est passé de 34,6 millions en 2012, à 35 millions en 2013 (+1,16 %). On estime qu'entre 2015 et 2030, il y aura 28 millions de gens atteints d'une nouvelle infection par le VIH et 21 millions de morts liées au SIDA (Figure 1.1) (ONUSIDA, 2014).

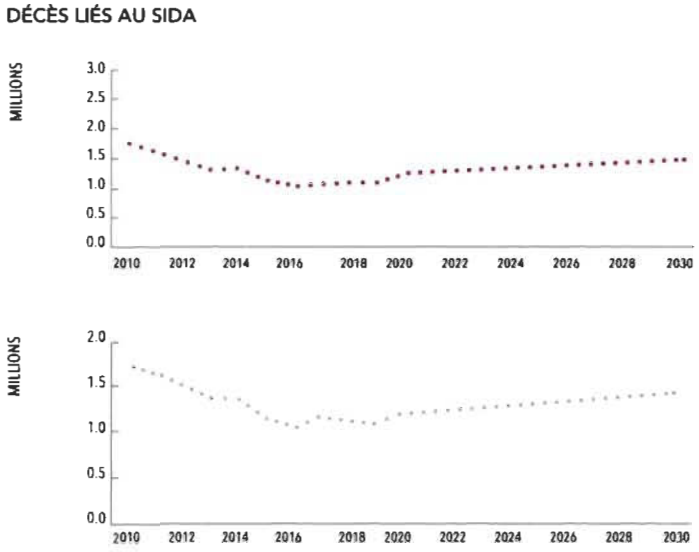

Figure 1.1 Tendance actuelle faisant le point sur l'épidémie mondiale du SIDA (ONUSIDA, 2014).

Aucun traitement n'est capable d'éradiquer ou de prévenir l'infection par le VIH. Les trithérapies actuelles permettent seulement de ralentir l'évolution de la maladie et de diminuer la mortalité liée au SIDA. Toutefois, les traitements présentent de nombreux effets indésirables à moyen et long terme, notamment atteintes rénales, lipodystrophies, risque cardio-vasculaire, anomalies lipidiques, syndrome métabolique, et anomalies osseuses (Jones *et al.*, 2005; Saves *et al.*, 2003; Gerard *et al.*, 2000; Fellay *et al.*, 2001; Friis-Moller *et al.*, 2007; Grund *et al.*, 2009; Deti *et al.*, 2010; Lang *et al.*, 2010). Ces inconvénients ont un impact sur la qualité de vie des patients et par conséquent sur l'adhésion aux traitements (Carrieri *et al.*, 2001; Duran *et al.*, 2001). Ces deux qualités sont pourtant indispensables pour une efficacité optimale et durable des médicaments et pour limiter l'apparition de mutations de résistance sur le virus (Moatti *et al.*, 2004; Paterson *et al.*, 2000).

Parmi les autres problématiques du développement thérapeutique, il y a l'impact de la diversité génétique des lignées virales (polymorphismes naturels) sur l'efficacité des traitements. (Perno *et al.*, 2001; Abecasis *et al.*, 2006; Holguin *et al.*, 2002; Vergen *et al.*, 2006). Les études cliniques d'efficacité et de tolérance des antirétroviraux sont réalisées majoritairement sur le sous-type B du VIH-1 qui est responsable de l'épidémie

aux États-Unis et en Europe (Deschamps *et al.*, 2005). Or, en raison des mouvements migratoires, la proportion des sous-types non-B est croissante dans les pays industrialisés et leur incidence a fortement augmenté ces dernières années (Cazein *et al.*, 2010; Descamps *et al.*, 2010). Il est donc essentiel de trouver de nouvelles stratégies thérapeutiques.

1.1.2 Structure du VIH

Le VIH est un lentivirus faisant partie de la famille des *Retroviridae*. Les rétrovirus sont des particules sphériques enveloppées d'une bicouche phospholipidique recouverte par des protéines de surface (SU) et transmembranaires (TM). L'intérieur de l'enveloppe est tapissé d'une matrice protéique (MA). Vient ensuite une capside virale protéique (CA) à l'intérieur de laquelle se trouve la nucléocapside (NC) qui protège les ARNs génomiques de polarité positive, associés à différentes enzymes virales telles que la transcriptase inverse (RT), l'intrégrase (IN) et la protéase (PR) ainsi que des protéines régulatrices et d'interaction avec l'hôte telles que Vpr ou Vpx (Figure 1.2) (Goujon *et al.*, 2007).

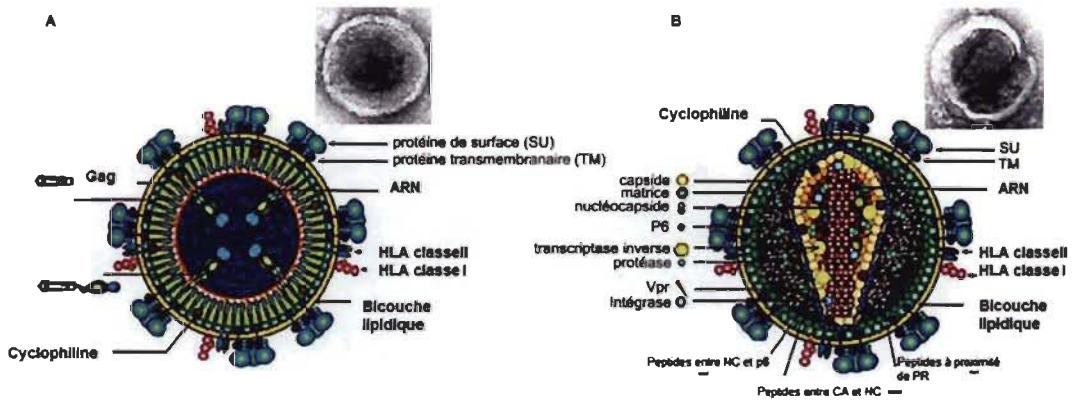

Figure 1.2 Schéma de la structure du VIH-1 immature (A) et mature (B) (adaptée de Sierra *et al.*, 2012).
Les protéines Gag et Gag-Pol sont représentées en différentes couleurs pour indiquer les domaines correspondants aux protéines matures formées à partir de ces précurseurs. Les composantes de surface et transmembranaires de l'enveloppe virale sont présentées au niveau de la membrane lipidique. (b) virion mature caractérisé par la forme en cône de la capside virale.

1.1.3 Génome viral

Le génome rétroviral est diploïde, formé par deux brins d'ARN monocaténaires d'environ 9,7 kpb (Muesing *et al.*, 1985; Ratner *et al.*, 1985). Il est constitué de trois gènes de structures, *gag*, *pol*, *env* et de six gènes codant pour des protéines d'interaction avec l'hôte (vif, vpr, vpu et nef) et régulatrices (tat et rev) (McCune *et al.*, 1991). Après la transcription inverse, on retrouve de part et d'autre du génome viral une région non codante longue et répétée (Figure 1.3) (Marcello *et al.*, 2004).

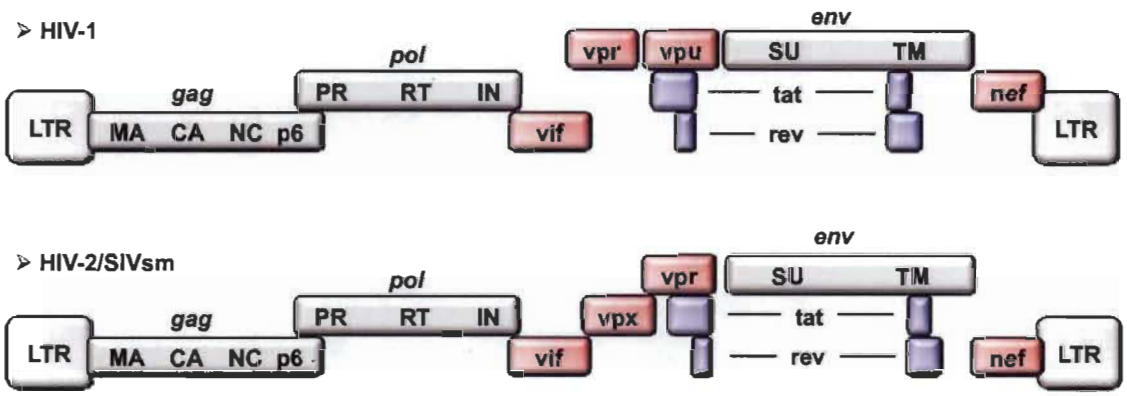

Figure 1.3 La carte génomique du VIH-1 et du VIH-2 (adaptée de Levy *et al.*, 2007).

a) Protéines de structure

Le gène gag code pour une polyprotéine Pr55Gag dont le clivage par la protéase virale permet la production de protéines de structure telles la p17 (protéine matrice), p24 (capside), p7 (nucléocapside) et p6 (Schwartz *et al.*, 1990b). La protéine p6 est essentielle pour le bourgeonnement des particules virales. En effet, grâce à son domaine P(S/T)AP qui interagit avec le domaine ESCRT-I de la protéine Tsg101 ubiquitinée. P6 recrute cette dernière au niveau des sites d'encapsidation, permettant le bourgeonnement de la particule virale grâce au complexe ESCRT. En interagissant avec Vpr ou Vpx, p6 va également permettre leur incorporation dans la particule virale. (Stuchell, Garrus *et al.*, 2004).

Le gène Pro-pol code pour les enzymes impliquées dans la réplication et l'intégration du provirus et dans la maturation des protéines virales : l'ARNase (p66/51), la protéase (p10), la réverse transcriptase et l'intégrase (p32), (Levy *et al.*, 2007).

Le gène env : La protéine d'enveloppe est traduite à partir d'un ARN messager épissé (Arrigo *et al.*, 1989; Hammarskjold *et al.*, 1989; Schwartz *et al.*, 1990b) et est ensuite clivée par la furine ou PC7, une protéase cellulaire, pour former les glycoprotéines de surface gp120 et transmembranaires gp41. Ces protéines sont primordiales dans le processus de reconnaissance et de fusion membranaire entre le virus et la cellule cible (Arrigo *et al.*, 1989; Hammarskjold *et al.*, 1989).

b) Les gènes régulateurs

La protéine Tat est indispensable à la réplication virale. C'est un transactivateur de la transcription (Sodroski *et al.*, 1985a, c). La protéine Tat, en se fixant sur la région TAR du LTR du nouveau brin en cours de transcription va recruter la cycline T1 et la kinase Cdk9 formant un complexe Tat-cycline T-Cdk9 (Wei *et al.*, 1998; Zhou *et al.*, 1998). Ce complexe permet l'activation de l'élongation suite à l'action de la Cdk9 qui va phosphoryler le domaine C-terminal de RNA polymérase II (Herrmann *et al.*, 1995; Wei *et al.*, 1998).

La protéine Tat possède également un fort pouvoir pathogène; en effet, elle peut induire l'apoptose cellulaire (Bartz *et al.*, 1999), activer les cellules quiescentes en les rendant sensibles à l'infection par le VIH (Huang *et al.*, 1998), stimuler l'expression de cytokines pro-inflammatoires comme le TNF-α et l'IL-1β, augmenter l'expression des corécepteurs du VIH (Xiao *et al.*, 2000), induire la production d'IL-10 (Badou *et al.*, 2000), inhiber l'expression de l'IL-12 et d'une enzyme mitochondriale, la manganèse superoxyde dismutase, enzyme dont l'activité est essentielle par la lutte contre les radicaux libres oxygénés.

Rev est impliquée dans l'exportation de transcripts (complets ou épissés) du noyau vers le cytoplasme et en son absence, les transcrits viraux sont épissés en ARNm codant pour des protéines régulatrices (Feinberg *et al.*, 1986).

c) Les gènes codant pour les protéines d'interaction avec l'hôte

Nef : Nef est associée à la face cytoplasmique des membranes cellulaires. Elle interagit avec la queue cytoplasmique de la molécule CD4, permet le recrutement d'AP-2, l'internalisation du CD4 et la dégradation de la molécule par les lysosomes. Ainsi, Nef empêche la réinfection des cellules (Chaudhuri *et al.*, 2007; Garcia and Miller, 1991; Roeth and Collins, 2006). Nef agit également sur les CMH (Complexe Majeur d'Histocompatibilité) de classe I et II en diminuant leur présentation à la surface des cellules (Roeth and Collins, 2006; Schindler *et al.*, 2003; Schwartz *et al.*, 1996). Enfin, Nef régule la présentation du CD3 (récepteur de la cellule T) (Thoulouze *et al.*, 2006). Nef permettrait aussi d'optimiser l'infectivité du virus en facilitant la pénétration du core sur le réseau d'actine durant les premières phases de l'infection (Pizzato *et al.*, 2007). Par ailleurs, SERINC3 et SERINC5 sont des protéines membranaires ayant une activité anti-rétrovirale contre le VIH en bloquant la capacité du virus à infecter de nouvelles cellules (Rosa *et al*, 2015; Usami *et al*, 2015). La protéine Nef rétablit l'infection du VIH en empêchant les proteines SERINCs d'être incorporées dans les particules virales naissantes (Aiken, 2015).

Vpr aide le complexe PIC à intégrer le noyau favorisant ainsi l'infection de cellules qui ne sont pas nécessairement en division (Heinzinger, 1994). Vpr permet également l'arrêt du cycle cellulaire en G2 (He, 1995/Jowett, 1995/Re 1995) maximisant ainsi la production de protéines virales (Hrimech, 2000; Chowdhury, 2003).

Vpu présente uniquement chez VIH-1, tout comme Nef, diminue l'expression des récepteurs CD4 à la surface de la cellule en les séquestrant dans des complexes viraux gp160/CD4 durant la production de nouveaux virions (Willey, 1992a,b) et en favorisant l'ubiquitination ainsi que la dégradation de CD4 par le protéasome (Schubert, 1998).

De plus, Vpu facilite le bourgeonnement des virus en empêchant la tétherine/BST-2 d'agir, responsable de la rétention de particules virales à la membrane cellulaire (Vandamme et Guatelli, 2008; Neil *et al.*, 2008). Enfin, Vpu atténue la réponse immunitaire en diminuant la présentation des CMH de classe I et II (Hussain *et al.*, 2008; Kerkau *et al.*, 1997).

Vif inhibe l'activité anti-rétrovirale d'APOBEC3G, une cytidine déaminase en bloquant son incorporation dans les nouveaux virions (Mangeat *et al.*, 2003; Sheehy *et al.*, 2002). Elle permet également la maturation des virions et diminue la formation de virions défectueux (Hoglund *et al.*, 1994).

d) Extrémités non-codantes

Les régions non codantes sont situées en 5' et en 3'de l'ARN viral. L'extrémité 5' joue le rôle de promoteur de transcription et est composée de six régions : Coiffe, R, U5, PB, L et SD. L'extrémité 3' est le site de polyadénylation et est composée de quatre régions : PP, U3, R et PolyA (Tableau 1.2) (Lenz *et al.*, 1997).

Tableau 1.1

Fonctions et régions des extrémités non-codantes
(adapté de Lenz *et al.*, 1997)

Extrémité 5'	Fonctions
Coiffe	Correspond à la structure m7G5'ppp5'Gm que l'on trouve dans les ARNm des eucaryotes
R (Redondant)	Est important pour la transcription inverse et l'intégration
U5 (séquence unique en 5')	Participe à la dimérisation de l'ARN
PB (Primer Binding Site)	Fixer un ARNt cellulaire par son extrémité 3' complémentaire créant ainsi le site d'initiation de la transcription inverse
L (Leader)	Englobe la séquence d'encapsidation de l'ARN viral et participe à la stabilisation de l'ARNm post transcriptionnel
SD (Site Donneur)	Site donneur pour l'épissage de l'ARNm
Extrémité 3'	**Fonctions**
PP (Poly Purine)	Important lors de l'initiation de la synthèse du second brin d'ADN
U3 (séquence unique en 3')	Englobe les régions « TATA box » et le site de polyadénylation essentielle pour l'initiation de la transcription. Contient également plusieurs sites de fixation de facteurs de transcription dont SPJ et NF-kB
R (Redondant)	Permet la fixation sur l'ARN transcrit, de la protéine de régulation Tat qui est indispensable pour la réplication virale
PolyA	De 100 à 200 Adénines comme pour les ARNm eucaryotes

1.1.4 Variabilité génétique du VIH

Depuis sa découverte, plusieurs variantes génétiques du virus ont été identifiées. Le VIH appartient aux groupes VIH-1 et VIH-2. Le type 1 est largement majoritaire et disséminé sur l'ensemble du globe alors que le type 2, minoritaire, est localisé principalement en Afrique de l'Ouest et plus modérément en Europe de l'Ouest. L'écart de virulence entre ces deux souches virales s'explique en partie par une différence au niveau (1) de la vitesse de propagation du virus dans les cellules, (2) de la charge virale dans le sang et les fluides génitaux, (3) du degré d'activation immunitaire et d'apoptose cellulaire chez les individus infectés (Gilbert *et al.*, 2003, Levy *et al.*, 2007). Il est aujourd'hui admis que le VIH-2 est beaucoup moins pathogène que le VIH-1 (Kanki *et al.*, 1994; Gilbert *et al.*, 2003). L'organisation des deux types de VIH est globalement identique excepté la présence du gène *vpx* pour le VIH-2. On observe également des variations au niveau de certains gènes, notamment *env* qui présente seulement 42 % d'homologie entre les deux types. Les autres gènes sont globalement identiques.

Les variations au sein des gènes *gag*, *pol*, *env* ont permis de classer le VIH-1 en trois groupes, le groupe M (majoritaire), lui-même subdivisé en 10 sous-groupes (notés A-J), le groupe O (outlier) présent en Afrique de l'Ouest et le groupe N (ni M ni O) présent au Cameroun. (Plantier *et al.*, 2009). Les trois premiers groupes (les M, O et N) proviennent de transmissions indépendantes inter-espèces (zoonose) du virus de l'immunodéficience simienne chez le chimpanzé *Pan troglodyte troglodyte* (SIVcpz) à l'homme (Gao *et al.*, 1999) alors que le groupe P est plus proche du SIV infectant le gorille (Plantier *et al.*, 2009). Le VIH-2, quant-à-lui est seulement classé en deux groupes A et B et dérive du SIV du singe sooty mangabey (Reeves *et al.*, 2002). Ces différents groupes ne présentent pas ou peu d'antigénicité croisée entre eux, ce qui peut poser un problème important au niveau du diagnostic et de l'efficacité des traitements (Figure 1.4).

De plus, il peut se produire que deux sous-types différents de VIH infectent une même cellule du corps, le virion nouvellement formé est considéré comme un virus

recombinant. Si la forme recombinante n'a été identifiée que chez un seul individu, on le catégorise alors comme étant une « forme recombinante unique » (FRU). Par contre, si la nouvelle forme recombinante est décrite quand au moins trois personnes sans lien épidémiologique direct entre elles sont infectées par le même virus, le nom attribué est alors « forme recombinante circulante » (FRC). Le terme « cpx » indique les formes de recombinaison de trois sous-types ou plus (Figure 1.4a) (Arien *et al.*, 2007).

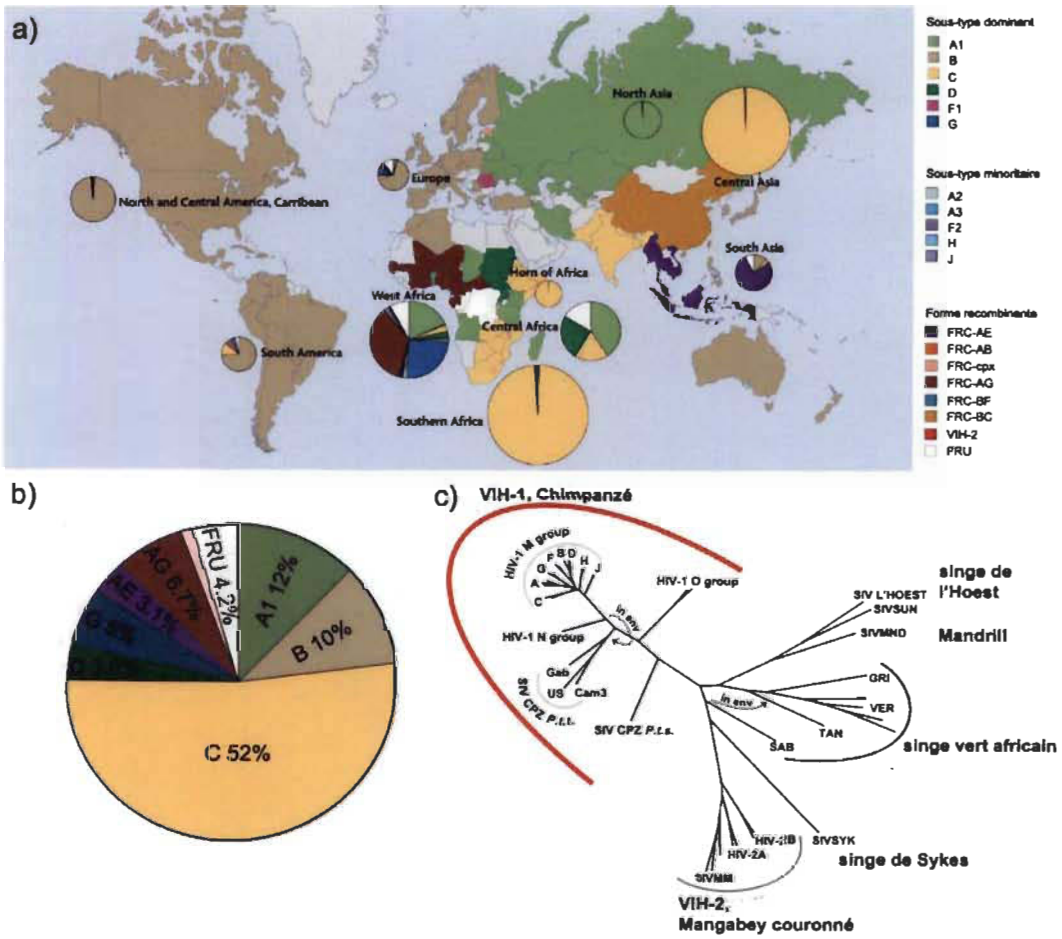

Figure 1.4 Planisphère de la distribution des sous-types du VIH.
a) Les régions sont coloriées en fonction de la dominance du sous-type de VIH-1 groupe M. Les régions en gris représentent des données insuffisantes. Les diagrammes en forme de tarte représentent la proportion de chaque sous-type dans chaque région géographique et la taille est proportionnelle au nombre d'individus infectés dans cette région. **b)** Diagramme résumant la répartition des sous-types de VIH-1 dans le monde. **c)** Associations phylogénétiques existant entre les différentes souches de SIV et VIH (Arien *et al.*, 2007; André *et al.*, 1998).

1.1.5 Le cycle viral

Le cycle de réplication du VIH se compose d'une série d'évènements qui peuvent être divisés en deux phases : (1) la phase précoce allant de la fixation du virus au niveau de la membrane cellulaire jusqu'à l'intégration de l'ADN viral dans le génome de la cellule infectée et (2) la phase tardive allant de l'expression du provirus jusqu'à la libération de nouvelles particules virales (Figure 1.5) (Coiras *et al.*, 2009).

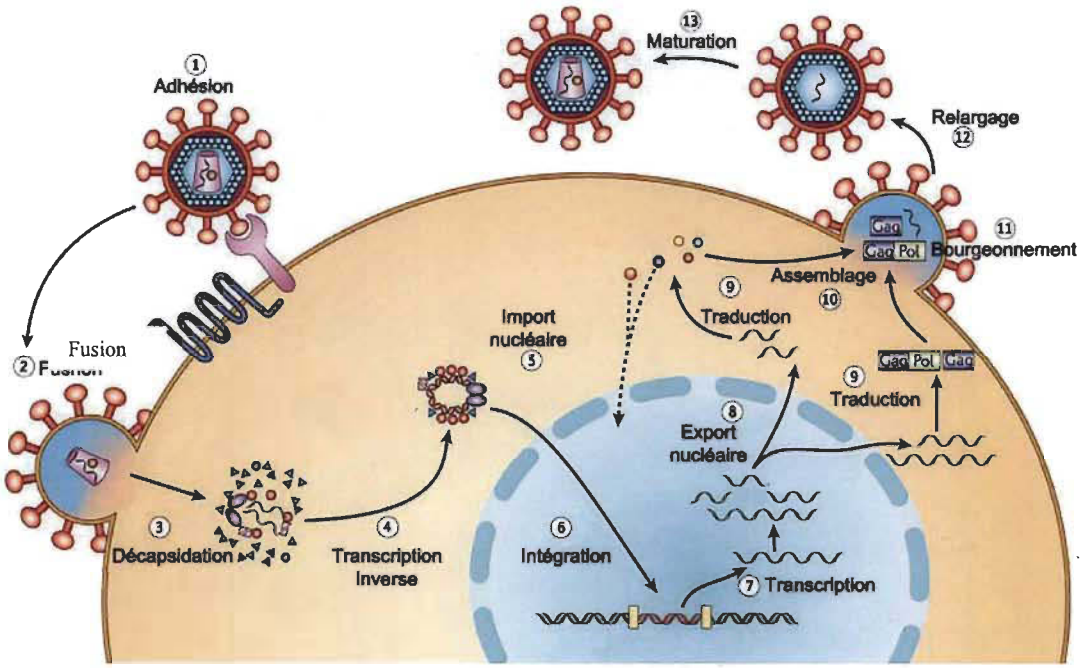

Figure 1.5 Le cycle viral de l'infection par le VIH-1 (adaptée de Coiras *et al.*, 2009).

L'infection commence lorsque les glycoprotéines de l'enveloppe virale engagent le récepteur CD4 et les co-récepteurs transmembranaires (CCR5 ou CXCR4) (étape 1). Cette interaction conduit à la fusion des membranes virales et cellulaires et à l'entrée de la particule virale dans le cytoplasme (étape 2). Décapsidation partielle du cœur viral (étape 3) qui facilite la transcription inverse (étape 4). À la suite de l'import dans le noyau de la cellule (étape 5), l'intégrase associée à PIC orchestre la formation du provirus (étape 6). Transcription provirale (étape 7) médiée par l'ARN polymerase de l'hôte. Exportation de l'ARNm viral (étape 8). L'ARNm sert de matrice pour la production de nouvelles protéines virales (étape 9), et l'ARN génomique et les protéines nouvellement formés sont incorporés dans la particule virale (étape 10). Le bourgeonnement (étape 11) et la libération (étape 12) de la particule virale sont médiés par ESCRT.

a) Phase précoce de l'infection

La phase précoce comporte : (a) l'entrée du virus à l'intérieur de la cellule, (b) la réverse transcription virale et (c) l'intégration de l'ADN viral dans l'ADN génomique de la cellule cible.

Entrée du virus

L'infection commence par la reconnaissance et la fixation des protéines de surface virales aux récepteurs membranaires de la cellule hôte. Ce premier contact va engendrer l'entrée du virus par deux processus possibles : 1) par une fusion directe entre l'enveloppe virale et la membrane cellulaire (Gomez *et al.*, 2005) ou 2) par le processus d'endocytose qui résulte souvent en l'inactivation ou la dégradation par les lysosomes (Maréchal *et al.*, 2001).

Tout d'abord, une interaction se produit entre la protéine virale gp120 et le récepteur CD4 se trouvant sur la cellule cible (Simon *et al.*, 2006). Cette interaction provoque un changement de conformation au niveau du récepteur gp120 résultant en l'exposition d'une région en forme de boucle appelée boucle V_3. Dépendamment de la souche virale du VIH, cette boucle se lie ensuite au corécepteur CCR5 ou CXCR4 situé sur la cellule cible (Markosyan *et al.*, 2003). L'exposition et la liaison des domaines d'interaction V_3 avec les corécepteurs induisent le repliement de la gp41 permettant ainsi la liaison des deux sous-unités (HR1 et HR2) et l'exposition/insertion du peptide de fusion (Melikyan *et al.*, 2000). Ce dernier provoque la fusion de l'enveloppe virale et de la membrane plasmique de la cellule cible entraînant l'introduction de la capside virale dans le cytoplasme de la cellule cible (Simon *et al.*, 2006).

Importance des corécepteurs CCR5 ET CXCR4

Le CD4, bien qu'il soit nécessaire, n'est pas suffisant pour médier l'entrée du virus par fusion. De nombreux travaux ont permis d'identifier les deux principaux corécepteurs de VIH-1 : CCR5 et CXCR4.

La liaison avec le corécepteur CCR5 prédomine dans les phases initiales d'une infection. Plus l'infection progresse et plus il y a apparition de mutations permettant au virus de se lier aux corécepteurs CXCR4 (présents chez 90 % des cellules CD4+ alors que les corécepteurs CCR5 ne sont présents que chez 10 % des cellules CD4+). Le transfert de CCR5 à CXCR4 permet donc d'infecter un plus grand nombre de cellules CD4+ et est associé à la progression accélérée de l'infection (Doms *et al.*, 2004). Le VIH-1 est donc séparé en deux catégories (Asjo, 1986/Gartner, 1986). Les virus utilisant le corécepteur CCR5 sont dits R5 tropique (ou M-Tropique ou NSI-tropique pour non-syncytium inducing). Les virus R5 peuvent infecter les macrophages ainsi que les cellules T primaires. De plus, ils n'induisent pas de syncytium lors de l'infection. Les virus utilisant les corécepteurs CXCR4 sont dits X4-tropique (ou T-Tropique ou SI tropique pour *syncytium inducing*) et sont peu infectieux chez les macrophages et forment des syncitias lors de l'infection (Asjo *et al.*, 1986; Gartner *et al.*,1986; Cocchi *et al.*, 1995; Alkhatib *et al.*, 1996; Berson *et al.*, 1996).

Décapsidation et transcriptase inverse

Suite à l'entrée du virus à l'intérieur de la cellule cible, il y a décapsidation du cœur viral (Simon *et al.*, 2006) et la transcriptase inverse synthétise à partir du brin d'ARN viral positif, un brin d'ADNc simple brin négatif. Un second brin sera synthétisé à partir de la première copie d'ADN (Figure 1.6) (Gotte *et al.*, 1999). De nombreuses mutations dans le génome viral ont lieu au cours de cette étape, car la transcriptase inverse n'a pas d'activité correctrice. Elle incorpore une base non correct pour 2000 à 4000 nucléotides polymérisés. (Mansky *et al.*, 1995). Ces mutations se produisant dès les premiers stades du cycle de réplication, elles sont propagées à tous les virions produits par ce cycle ce qui induit la plupart du temps l'apparition de quasi-espèces chez un même individu infecté. Ces mutations, dans le cas où elles ne sont pas létales pour le virus, lui permettent souvent d'échapper aux tentatives de neutralisation par les produits de la réponse immune (anticorps neutralisants et CTL CD8 spécifiques) ainsi qu'aux différentes drogues (Mansky *et al.*, 1995).

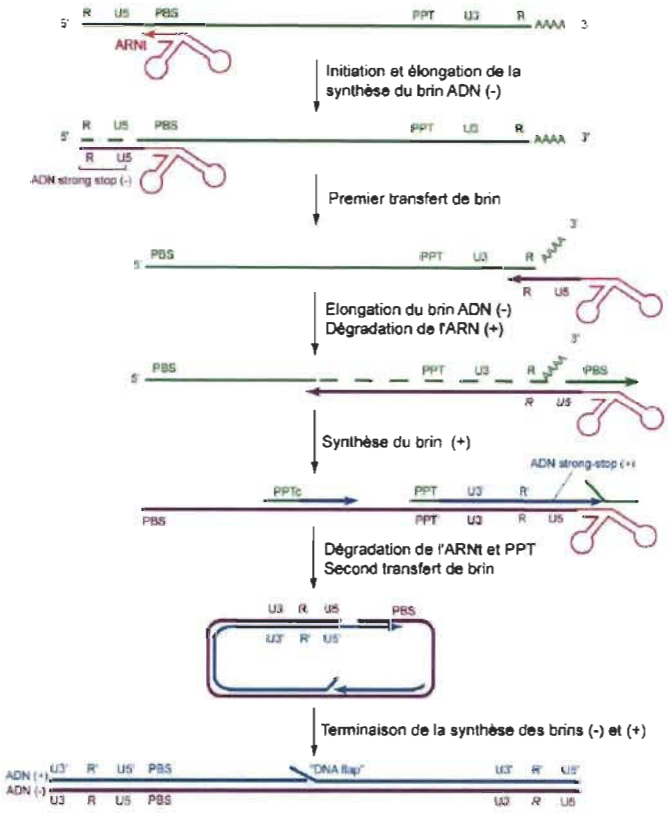

Figure 1.6 Transcription inverse de l'ARN du VIH-1 (adaptée de Gotte *et al.*, 1999).

Transport nucléaire et intégration

L'ADN ainsi formé migre dans le noyau où il est intégré au génome de la cellule hôte grâce à l'action de l'intégrase. L'intégration semble se faire dans des sites peu spécifiques, mais dans des régions qui sont actives pour la transcription. Une fois intégré, le provirus se comportera comme n'importe quel gène cellulaire. Il pourra rester sous forme latente ou s'exprimer dans les cellules activées pour donner de nouvelles particules virales qui, à leur tour, pourront infecter de nouvelles cellules (Gotte *et al.*, 1999, Forshey *et al.*, 2002).

b) Phase tardive de l'infection

La phase tardive de l'infection est tout d'abord amorcée par la synthèse de l'ARN viral. Certaines copies ne sont pas épissées et forment alors le génome viral qui sera

encapsidé avec les précurseurs polyprotéiques Gag et Gag-Pro-Pol dans les virions. D'autres copies subissent un épissage résultant en la synthèse de plusieurs protéines virales telles que : Env, Nef, Vif, Vpr (Vpx), Vpu, Tat, et Rev (Coiras *et al.*, 2009).

Une fois les protéines virales et le génome viral produits, la polyprotéine Env (gp160) traverse le réticulum endoplasmique. Elle est ensuite dirigée vers l'appareil de Golgi où elle est clivée en gp120 et gp41 par les protéases de l'hôte. Ces deux glycoprotéines d'enveloppe se lient ensuite aux corécepteurs CCR5 ou CXCR4 situés sur la cellule cible (Markosyan *et al.*, 2003). Les polyprotéines Gag (p55) et Gag-Pol (p160) ainsi que l'ARN génomique s'associent à leur tour avec la surface interne de la membrane plasmique lorsque les virions commencent à bourgeonner. Au cours de cette étape de bourgeonnement, la maturation du virus a lieu. La protéase du VIH clivent les polyprotéines (Gag, Gag-Pol et Nef) en protéines individuelles et fonctionnelles et les composants de la structure du VIH s'assemblent pour produire un virus mature (Coiras *et al.*, 2009, Levy *et al.*, 2007).

1.2 Les facteurs de restriction

Les facteurs de restriction sont un ensemble de protéines anti-rétrovirales qui forment un aspect important du système immunitaire inné. Leur expression constitutive permet une réponse immédiate à une infection virale. Parmi les facteurs de restriction identifiés au cours de la dernière décennie, les plus étudiés sont TRIM5α, tétherine/BST-2, APOBEC3G et SAMHD1 (Figure 1.7). Ces protéines constitutives sont typiquement exprimées à de faibles niveaux permettant une réponse immunitaire immédiate. Par contre, l'expression de ces facteurs peut être régulée à la hausse par les Interférons (IFN) suite à une infection virale (Chan *et al.*, 2014). Les facteurs de restriction identifiés diffèrent grandement au niveau de leur structure, mais ils ont tous été soumis à des niveaux élevés de sélection naturelle au cours d'un processus d'évolution continue aux côtés des agents pathogènes viraux. L'expression des facteurs de restriction est beaucoup plus répandue qu'on ne le pensait initialement et un large éventail de mammifères expriment ces protéines. Typiquement le VIH-1, le VIH-2 et le

SIV ne sont pas inhibés significativement par les facteurs de restriction de leurs espèces hôtes. Ces rétrovirus ont développé, au cours de leur évolution, des mécanismes pour contourner ces différents mécanismes de restriction. Toutefois, le VIH n'a pas de protéine spécialisée dans la résistance à TRIM5α, ce qui en fait un candidat prometteur pour des approches thérapeutiques (Chan *et al.*, 2014). Certains Facteurs de restrictions seront présentés plus en détails dans les pages qui suivent.

1.2.1 APOBEC3G

Le facteur viral Vif (Viral Infectivity Factor) est essentiel pour l'infection du VIH-1 dans certains types de cellules, incluant les cellules T CD4 + (Gabuzda *et al.*, 1992). En l'absence de Vif, la restriction du VIH-1 est médiée par APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) (Sheehy *et al.*, 2002).

APOBEC3G appartient à la famille des cytidine déaminases. Elle interfère avec la transcription inverse en induisant de nombreuses mutations dans l'ADN du VIH-1. En l'absence de Vif, APOBEC3G est encapsidée dans les virions par son interaction avec la protéine virale Gag. Elle accompagne alors les virions jusqu'à ce qu'ils infectent une nouvelle cellule. C'est à ce moment qu'APOBEC3G va introduire des mutations dans l'ADN viral simple brin négatif en provoquant la déamination des désoxycytidines en désoxyuridines (Goila-Gaur *et al.*, 2008). Les produits de cette déamination suivent alors deux voies : (a) ils sont dégradés par les endonucléases ou (b) l'ADN viral hypermuté est inséré dans le génome viral de l'hôte menant à une production de protéines virales non fonctionnelles (Figure 1.8B) (Goff, 2004).

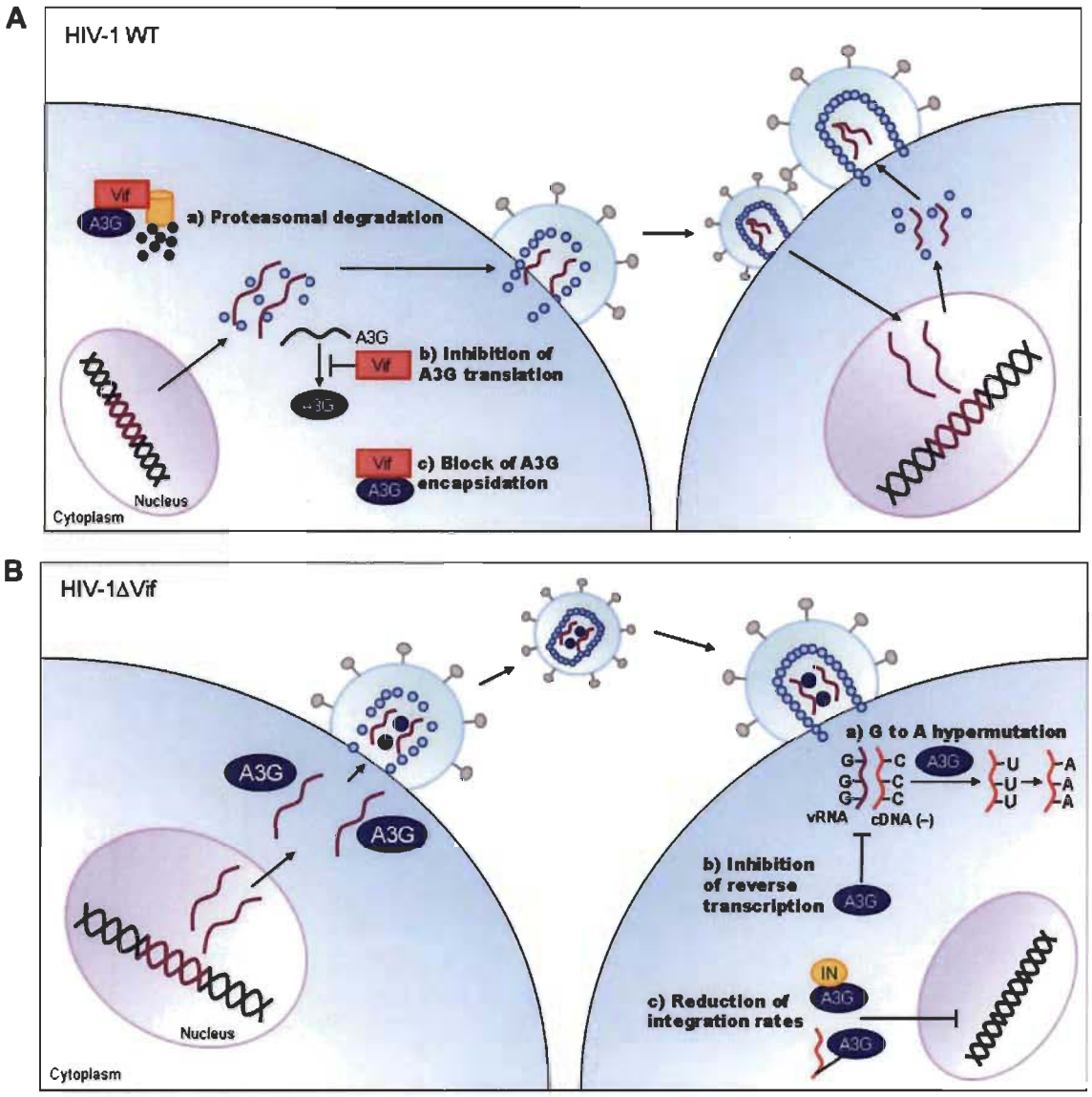

Figure 1.7 **Le mécanisme d'action d'APOBEC3G.**
(A) La protéine virale Vif inhibe l'action d'APOBEC3G (A3G) en limitant son incorporation dans les particules virales. (B) Dans le cytoplasme des cellules productrices de virus, en l'absence de Vif, A3G est efficacement incorporée dans les virions et transportée vers les prochaines cellules cibles, où elle peut agir comme un puissant inhibiteur de la réplication du VIH-1.

Même si cette protéine possède une activité de restriction contre plusieurs rétrovirus, des rétrovirus tels le VIH-1 ont développé des moyens pour contrecarrer le pouvoir d'inhibition d'APOBEC3G. En effet, la protéine virale du VIH-1 appelée Vif inhibe fortement l'activité anti-rétrovirale d'APOBEC3G par une interaction physique qui résulte en l'exclusion de la déaminase des virions. De fait, Vif recrute une ubiquitine

ligase E3 qui provoque la polyubiquitination d'APOBEC3G et dirige cette dernière vers la voie de dégradation par le protéasome (Figure 1.8A) (Goila-Gaur *et al.*, 2008).

Bien que la substitution de l'acide aspartique par une lysine en position 128 d'APOBEC3G humain confère une résistance à Vif du VIH-1 (Schrofelbauer *et al.*, 2004 et Xu *et al.*, 2004), l'intérêt initial pour l'exploitation d'APOBEC3G en vue d'une thérapie génique s'est atténué lorsque d'autres études ont révélé que ses interactions avec le VIH-1 pouvaient être bénéfiques pour le virus. Ainsi, des mutations de résistance aux médicaments se développent dans le VIH-1 en présence d'APOBEC3G *in vitro* (Kim *et al.*, 2010).

1.2.2 Tétherine/BST-2

La tétherine/BST-2 est une protéine exprimée par les cellules stromales de la moelle osseuse, les cellules B matures, certaines lignées cancéreuses, les macrophages dérivés de monocytes ainsi que par les cellules dendritiques plasmacytoïdes (pDCs) (Ishikawa *et al.*, 1995; Evans *et al.*, 2010; Kuhl *et al.*, 2011). En 2008, cette protéine fut caractérisée comme étant un facteur de restriction cellulaire induit par l'interféron α (IFNα) (Neil *et al.*, 2008; Van Damme *et al.*, 2008), et capable de bloquer la libération de virus enveloppés, dont le VIH-1 (Neil *et al.*, 2008; Van Damme *et al.*, 2008; Kawai *et al.*, 2008; Erikson *et al.*, 2011). En absence de Vpu, la tétherine/BST-2 va empêcher les particules virales de quitter la cellule infectée qui les a générées en les retenant à la surface de la cellule hôte, ou en liant les particules virales les unes aux autres (Neil *et al.*, 2008; Van Damme *et al.*, 2008; Fitzpatrick *et al.*, 2010; Hammonds *et al.*, 2010). Une portion de ces virions sera ensuite internalisée par endocytose et va s'accumuler dans les endosomes tardifs $CD63^+$ (Figure 1.9) (Neil *et al.* 2008). Toutefoirs, un certain nombre de virus dont le VIH-1, ont développé une stratégie pour neutraliser ce processus de défense anti-rétrovirale. Ainsi, Vpu neutralise le facteur de restriction Tétherine/BST-2 en se liant à celle-ci et conduit à sa dégradation par le protéasome par la voie Beta-TrCP2-dépendante. Vpu favorise ainsi la libération des particules du VIH-1 et la progression de l'infection (Versteeg *et al.*, 2010; Martin-Serrano *et al.*, 2011).

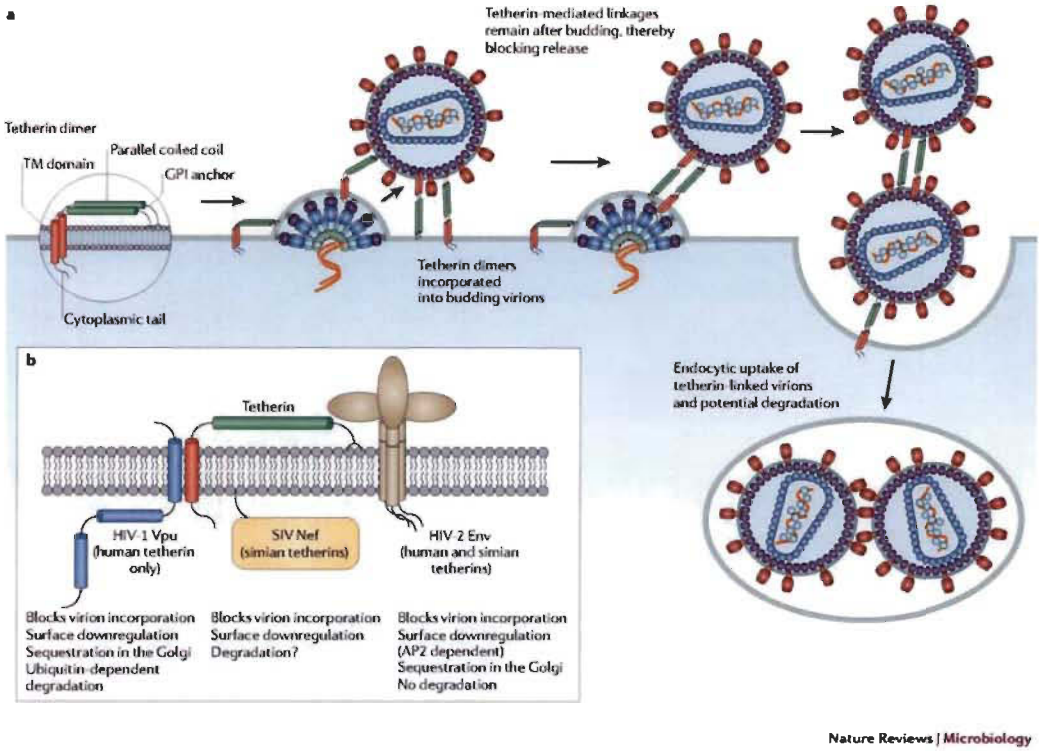

Figure 1.8 **Le mécanisme d'action de la tétherine/BST-2 (Gottlinger *et al.*, 2008).** (a) La tétherine/BST-2 est incorporée dans le virion naissant et forme des réticulations physiques entre la cellule et le virion. Cela conduit à l'accumulation des virions à la surface des cellules et son internalisation dans des endosomes tardifs. (b) Vpu du VIH-1 interagit avec la tétherine/BST-2 et entraîne une inhibition de son activité anti-rétrovirale.

1.2.3 SAMHD1

Récemment, l'isolement des protéines cellulaires interagissant avec Vpx a permis d'identifier SAMHD1 comme un autre facteur de restriction du VIH-1 dans les cellules dendritiques et les macrophages (Hrecka *et al.*, 2011; Laguette *et al.*, 2011). SAMHD1 fonctionne comme un triphosphohydrolase de dNTPs (Goldstone *et al.*, 2011) et réduit donc les niveaux de dNTPs, empêchant ultimement la transcription inverse (Lahouassa *et al.*, 2012). Même si on retrouve également SAMHD1 dans les lymphocytes T CD4+, son activité restrictive est toutefois absente dans les cellules en division. En effet, les différences au niveau de la phosphorylation impliquent que SAMHD1 est seulement active dans les cellules au repos, ce qui limite grandement son utilisation pour des thérapies géniques (Cribler *et al.*, 2013; White *et al.*, 2013). De plus, la protéine virale Vpx est capable d'induire la dégradation de SAMHD1 par le protéasome et permet de

rendre permissives les cellules initialement résistantes à l'infection par le VIH (Hrecka *et al.*, 2011; Laguette *et al.*, 2011).

1.2.4 TRIM5α

TRIM5α fait partie de la famille des TRIMs (*TRIpartite Motif)* constituée d'environ 80 membres qui partagent une structure commune, mais des fonctions différentes. Certaines protéines TRIM sont exprimées de façon ubiquitaire dans l'organisme alors que d'autres sont exprimées dans des organes précis. La localisation des différentes protéines TRIM peut varier en fonction du degré de maturité de l'organisme les exprimant. Elles sont retrouvées au niveau du cytoplasme ou du noyau sous forme de corps cytoplasmiques ou de corps nucléaires. Elles peuvent être également retrouvées sous forme diffuse dans le cytoplasme ou le noyau de la cellule (Reymond *et al.*, 2001).

En 2004, le crible d'une banque d'ADNc de cellules de singe rhésus a permis d'identifier la protéine TRIM5α comme étant le facteur cellulaire responsable de la résistance des singes du Vieux Monde contre le VIH-1 (Stremlau *et al.*, 2004). De plus, il a été démontré que Lv1 et Ref1 étaient en réalité des variantes spécifiques d'espèces d'une seule et même protéine : TRIM5α (Hatziioannou *et al.*, 2004; Keckesova *et al.*, 2004). L'activité de ces facteurs de restriction est spécifique à chaque espèce; l'être humain possède donc un orthologue de cette protéine ($TRIM5\alpha_{hu}$) capable d'inhiber le virus de la leucémie murine N-tropique (MLV-N) mais inefficace contre MLV-B et le VIH-1 (Song *et al.*, 2005; Yap *et al.*, 2005; Schaller *et al.*, 2007).

a) Structure

Le motif tripartite TRIM est constitué d'un domaine RING en position N-terminale suivi des domaines B-Box et Coil-Coiled. Chez de nombreux membres de la famille TRIM, on retrouve en position C-terminale un domaine PRYSPRY, mais pour TRIM5, seul l'isomère α le possède (Figure 1.10) (Towers *et al.*, 2007).

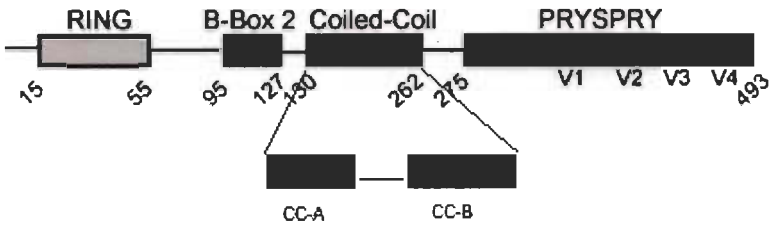

Figure 1.9 La structure de la protéine TRIM5α (adaptée de Maillard *et al.*, 2007).

Le domaine RING (doigt de zinc) lie deux atomes de zinc et possède une activité E3 ubiquitine ligase. La fonction principale des ubiquitines est de marquer d'autres protéines en vue de leur dégradation ou protéolyse (Yamauchi *et al.*, 2008). Le domaine RING contient des cystéines et des histidines critiques à la liaison de zinc et qui t contribuent au repliement approprié de la protéine TRIM5α (Javanbakht *et al.*, 2005; Li *et al.*, 2006a). Pour sa part, le domaine B-box joue un rôle dans l'interaction avec d'autres protéines et coordonnerait l'oligomérisation de TRIM5α (Diaz-Griffero *et al.*, 2006). Le domaine Coiled-Coil permet l'homo et l'hétéro multimérisation de la protéine. Les mutations au niveau du domaine coiled-coil altèrent l'interaction entre TRIM5α et l'hexamère de la capside virale, diminuant ainsi la restriction (Mische *et al.*, 2005). D'après la littérature, TRIM5α formerait des dimères anti-parrallèles, des trimères et des hexamères (Goldstone *et al.*, 2014; Ganser-Pornillos *et al.*, 2011; Nepveu-Traversy *et al.*, 2009; Mische *et al.*, 2005). De plus, RING, B-box et coiled-coil semblent tous trois impliqués dans la formation de corpuscules cytoplasmiques, structures formées d'agrégats de TRIM5α (Reymond *et al.*, 2001). Il semble cependant que ces structures cytoplasmiques participent à l'activité de restriction sans toutefois en être essentielles (Song *et al.*, 2005; Perez-Caballero *et al.*, 2005).

Le domaine PRYSPRY (ou B30.2) est composé d'une combinaison d'un motif PRY fusionné avec un domaine SPRY et il contient quatre régions variables appelées v_1, v_2, v_3 et v_4 (Song *et al.*, 2005b; Yap *et al.*, 2005). Ces régions sont hautement variables en longueur et en séquence d'acides aminés selon les espèces. La région PRYSPRY est en majeure partie responsable de l'interaction physique entre TRIM5α et la protéine de la capside virale et serait donc responsable de la spécificité de la restriction (Stremlau *et al.*, 2006; Ohkura *et al.*, 2006).

b) Spécificité de restriction des différents TRIM

L'activité de restriction de TRIM5α est spécifique à chaque espèce de primate et aucune d'entre elles n'est capable d'offrir une résistance face à tous les types de rétrovirus (Tableau 1.4). Par exemple, TRIM5α_{hu} a une activité de restriction contre MLV-N, mais pas contre VIH-1 et MLV-B. TRIM5α_{rh}, quant à elle, est capable d'inhiber le VIH-1, mais pas MLV-N, ni MLV-B (Song *et al.*, 2005). De plus, certains primates comme le singe-hibou, possèdent une forme singulière de TRIM appelée TRIMCyp. Cette particularité correspond à la substitution du domaine SPRY par la cyclophiline A. Chez les singes hiboux et du vieux monde, l'expression de TRIMCyp confère à ces primates une activité de restriction contre le VIH-1 (Berthoux *et al.*, 2005). Des orthologues de TRIM5 ont également été retrouvés chez les léporidés et les bovins (Song *et al.*, 2005; Yap *et al.*, 2005; Schaller *et al.*, 2007).

Tableau 1.2

Spécificité de restriction par les différents orthologues de TRIM5α
(Yap *et al.*, 2004; Song *et al.*, 2005c; Ohkura *et al.*, 2006, Bouchard, 2008)

Différents orthologues de TRIM5α	Force avec laquelle les différents rétrovirus sont inhibés					
	VIH-1[a]	MLV-N[b]	MLV-B[c]	MoMLV[d]	SIV_{agm}[e]	SIV_{mac}[f]
Humain	-	+++	-	-		-
Chimpanzé	-	+++	-	-		-
Orang-outang	++	+++	-	-		++
Gorille	+	+++				++
Rhésus	+++	++	-	-	+++	-
Agm_{pyg}	++	+++	-	-	-	-
Agm_{tan}	++	+++	-	-	-	+++
Sooty mangabey	++	+++				-
Capucin brun	-	+++				-
Écureuil	-	++	-	-	++	+++
Tamarin cotton-top	++	++	-	-	++	+++
Tamarin empereur	++	++	-	-	++	+++
Goeldi marmoset	-					
Silvery marmoset	-					
Araignée	+++	++	-	-	+++	

[a] Virus de l'immunodéficience humaine de type I
[b] Virus de la leucémie murine de tropisme N
[c] Virus de la leucémie murine de tropisme B
[d] Virus de la leucémie murine de souche Moloney
[e] Virus de l'immunodéficience simienne du singe vert africain
[f] Virus de l'immunodéficience simienne de macaque

Aucune inhibition -
Inhibition faible +
Inhibition modérée ++
Inhibition forte +++

Ces différences de restriction spécifique entre les espèces sont attribuables à la variation de la séquence au niveau de la capside virale (CA) et donc à la capacité de TRIM5α de reconnaître et de se lier aux virus. Cette spécificité de restriction de TRIM5α réside notamment à l'intérieur des quatre régions variables du domaine PRYSPRY, allant d'un changement d'un seul acide aminé à un polymorphisme de longueur important pour toutes les régions variables exceptées v_4 qui ne contient pas de polymorphisme de longueur. De fait, les régions à l'intérieur du domaine B30.2 ont été soumises à des niveaux élevés de sélection positive (Sawyer *et al.*, 2005) suggérant une co-évolution avec des virus. Tout au long de l'évolution, il est possible que TRIM5α, avec sa spécificité variable entre les espèces, ait joué un rôle important dans le contrôle de la transmission inter-espèces des rétrovirus (Johnson *et al.*, 2009).

Pour le gène TRIM5α de primates, la sélection positive s'est effectuée notamment au niveau du domaine B30.2 dans les régions variables v_1, v_2, v_3 et v_4, mais plus particulièrement dans une portion de la région variable v_1 formée par 11 à 13 acides aminés (Figure 1.11). Chez TRIM5α_{hu}, il s'agit des acides aminés 330 à 340 (Sawyer *et al.*, 2005).

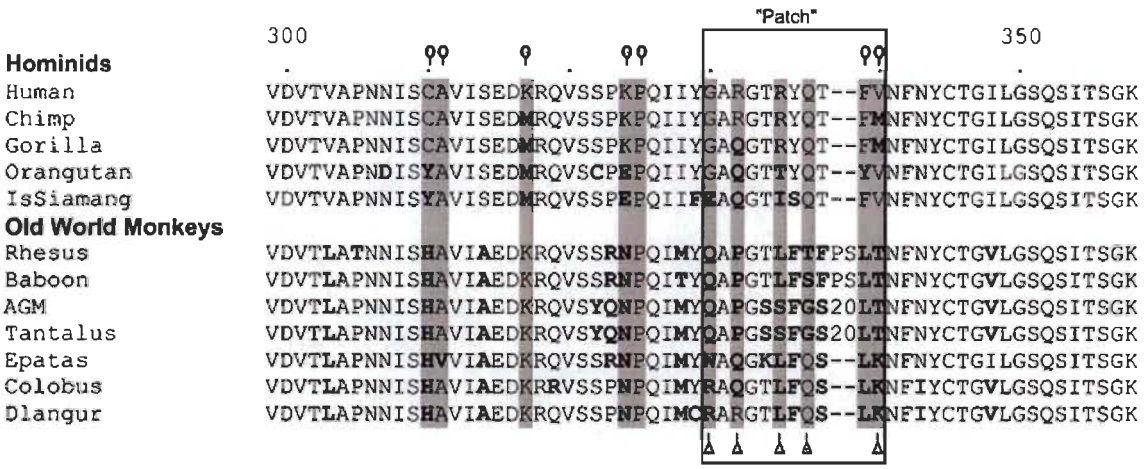

Figure 1.10 La sélection positive de la région variable v_1 de TRIM5α (Sawyer *et al.*, 2005).

Le domaine PRYSPRY est un point chaud «hot spot» pour les insertions/délétions et la sélection positive. La sélection positive des codons de TRIM5α est indiquée sur fond gris. Les changements relatifs à l'humain sont indiqués en gras. La région «Patch» est une région particulièrement abondante en délétion.

c) Mécanisme d'action

L'activité inhibitrice de TRIM5α s'opère très rapidement (15-30 min) après l'entrée de la capside virale dans le cytoplasme et avant la réverse transcription (Stremlau *et al.*, 2006; Perron *et al.*, 2007; Perez-Caballero *et al.*, 2005).

Décapsidation accélérée des cœurs viraux

Lors de l'entrée du virus dans la cellule, TRIM5α reconnaît la capside virale via son domaine B30.2. L'interaction avec TRIM5α induirait la décapsidation accélérée du cœur viral (Stremlau *et al.*, 2006; Perron *et al.*, 2007; Black *et al.*, 2010; Zhao *et al.*, 2011). À cette étape, la présence d'hexamères semble être nécessaire pour une restriction efficace. De fait, elles augmenteraient l'affinité avec la capside virale en permettant à plusieurs domaines de PRYSPRY de couvrir et d'interagir avec le noyau viral entrant. Ainsi, les monomères de TRIM5α présents dans le cytoplasme formeraient, en présence de la capside virale, des dimères qui se transformeraient spontanément en hexamères (Figure 1.12) (Ganser-Pornillos *et al.*, 2011). En l'absence de multimérisation, il y a absence de restriction rétrovirale par TRIM5α (Mische *et al.*, 2005).

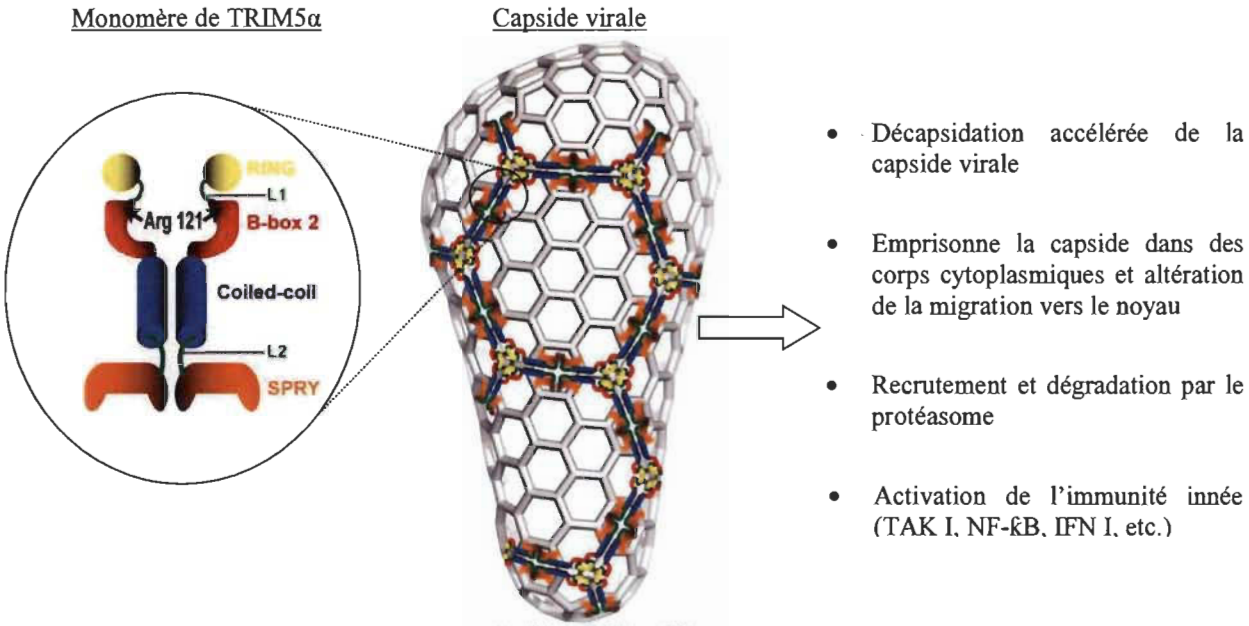

Figure 1.11 Mécanisme d'action de TRIM5α (Ganser-Pornillos *et al.*, 2011).
Modèle schématique de la capside du VIH-1 et de sa restriction par TRIM5α. Notez que seul un petit nombre d'anneaux hexamèriques de TRIM5α est nécessaire pour créer une avidité élevée. La double symétrie, suggère que chaque bord peut être composé de deux dimères de TRIM5α, avec des éléments bispiralés reliant les domaines PRYSPRY et B-BOX.

Favorise la dégradation par le protéasome

Le complexe formé par TRIM5α et la capside virale pourrait être dégradé par le protéasome. Cette hypothèse a été émise suite à différentes observations expérimentales. Tout d'abord, l'inhibition du protéasome entraîne une augmentation des produits de transcription inverse sans toutefois rétablir l'infection (Anderson *et al.*, 2006; Wu *et al.*, 2006). De plus, lorsque le domaine RING (activité d'ubiquitine ligase) est altéré par une mutation, il y a diminution de la restriction (Diaz-Griffero *et al.*, 2006). Finalement, il y a accélération de la dégradation de TRIM5α et de certaines composantes du cœur virale par le protéasome suite à une infection par un rétrovirus soumis à restriction (Wu *et al.*, 2006; Kutluay *et al.*, 2013). Il faut toutefois souligner que la présence seule du virus au niveau de la cellule n'est pas suffisante à la restriction, mais qu'une interaction directe entre TRIM5α et la capside virale est nécessaire (Rold *et al.*, 2008).

Empêche le transport vers le noyau

Une partie de l'ADN viral se referme sur lui-même et forme des cercles à 1 LTR ou 2 LTR. Les cercles à 2 LTR se forment uniquement au niveau du noyau (Wu *et al.*, 2006). Dans un contexte de restriction par TRIM5α et lorsque le protéasome est inhibé, il y a accumulation des produits de la réverse transcription sans toutefois rétablir la formation des cercles à 2 LTR (Rold *et al.*, 2008). Par conséquent, l'inhibition du protéasome permet de rétablir les produits de transcription inverse, mais ne supprime pas l'activité anti-rétrovirale de TRIM5α (Rold *et al.*, 2008; Anderson *et al.*, 2006; Wu *et al.*, 2006). Ceci suggère que TRIM5α serait capable d'empêcher le transport des ADN viraux vers le noyau par un mécanisme autre que celui du protéasome.

L'activité anti-rétrovirale indépendante du protéasome proviendrait de la séquestration du génome viral dans des agrégats préexistants de TRIM5αα, ou par la formation de nouveaux agrégats autour des particules virales. On observe également la présence de TRIM5α quittant des corps cytoplasmiques pour interagir avec le complexe de transcription inverse à proximité. On émet ainsi l'hypothèse que les corps cytoplasmiques de TRIM5α pourraient séquestrer les particules virales et ainsi rendre leur accès au noyau impossible (Campbell *et al.*, 2007 et 2008).

d) Les facteurs agissant sur la restriction et la régulation de TRIM5α

Différents facteurs extérieurs sont capables d'agir sur la restriction et la régulation de TRIM5α tels que les interférons, NF-ĸB et la cyclophiline A.

Régulation de TRIM5α par l'interféron

Les interférons sont des peptides extracellulaires faisant partie de la famille des cytokines. Ils possèdent plusieurs fonctions biologiques telles une activité anti-rétrovirale, une activité antiproliférative et une activité antitumorale. Les IFN se lient à leur récepteur provoquant une transduction du signal jusqu'au noyau où des facteurs de transcription sont activés. Ils sont divisés en trois types d'IFN comportant

des voies de signalisations différentes. Le premier est appelé IFN de type I et il comprend les IFN-α et IFN-β, le deuxième groupe se nomme IFN de type II et comprend les IFN-γ (Goldsby *et al.*, 2003) et le troisième groupe se nomme IFN de type III et comprend les IFN-λ (Bartlett *et al.*, 2005 ; Meager *et al.*, 2005).

TRIM5α est exprimée de façon ubiquitaire dans la plupart des tissus du corps humain, y compris les lymphocytes T. Alors que son niveau d'expression peut être constitutivement faible, il est régulé à la hausse par les IFN de type I (Asaoka *et al.*, 2005). En effet, l'interféron-α augmente le niveau de la transcription du gène TRIM5α_{rh} dans les cellules de singe rhésus FrhK4. De plus, il augmente les activités anti-rétrovirales médiées par TRIM5α_{rh} sur la réplication du VIH-1, en diminuant notamment le nombre de virions produits dans le surnageant. Pour sa part, chez les cellules HeLa et les cellules TE671, l'IFN-α augmente la production d'ARNm de TRIM5α_{hu} et amplifie la restriction du virus MLV-N suite à son entrée dans la cellule (Sakuma *et al.*, 2007). Les IFNs de type III partagent de nombreuses similitudes avec les IFNs de type I (Meager *et al.*, 2005), notamment sur le plan des propriétés anti-rétrovirales (Bartlett *et al.*, 2005 ; Robek *et al.*, 2005).

TRIM5α dans la signalisation cellulaire

Si l'immunité innée conventionnelle peut réguler l'expression de TRIM5α, l'inverse est également vrai. En plus de la reconnaissance de la capside virale, TRIM5α joue un rôle dans la signalisation cellulaire du système immunitaire inné par la voie NF-ƙB. TRIM5α fonctionne en liaison avec UBC13-Uev1A, une enzyme de conjugaison de l'ubiquitine. Ensemble, ils assemblent les chaînes non attachées K63 qui activent le complexe kinase TAK1. TAK1 stimule ensuite les facteurs AP-1 et NF-ƙB impliqués dans la signalisation immunitaire innée (Nepveu-traversy *et al.*, 2014; Pertel *et al.*, 2011). La formation de chaînes d'ubiquitine et l'activation de la signalisation NF-ƙB sont stimulées lors de la reconnaissance de la capside virale. La prévention de la formation de ces chaînes au moyen d'un knockdown de UBC13 ou de Uev1A diminue la restriction rétrovirale médiée par TRIM5α_{hu} (Pertel *et al.*, 2011). Cet effet sur NF-ƙB

par la protéine humaine dépendrait du niveau d'expression de TRIM5α (Jung *et al.*, 2015; Tareen *et al.*, 2011). Grâce à cette fonction, TRIM5α agit comme récepteur de reconnaissance de la CA rétrovirale, améliore l'activation de NF-ƙB et la réponse immunitaire innée.

Cyclophiline A

La cyclophiline A est une peptidyl-propyl isomérase qui catalyse la commutation entre les conformations cis/trans des résidus de proline. CypA interagit avec la capside du VIH-1 au niveau de la liaison peptidique G89-P90 située sur une boucle flexible constituée de neuf acides aminés (P85-P93), et catalyse l'isomérisation de la liaison. Le rôle de la CypA dans la réplication virale est ambigu. En effet, CypA agit en facteur positif pour la réplication du VIH-1 en stabilisant la capside virale après son entrée dans des cellules humaines et en facteur négatif pour la réplication du VIH-1 dans les cellules simiennes en augmentant l'affinité de la liaison avec TRIM5α_{rh} (Keckesova *et al.*, 2006; Towers *et al.*, 2003). Plus récemment, il a été démontré que CypA a un rôle dans la protection du VIH-1 contre la détection par les capteurs immunitaires d'ADN cytoplasmique (cGAS) (Rasaiyaah *et al.*, 2013). Il a été démontré que le mutant CA P90A du VIH-1 déclenchait les cGAS dans les macrophages dérivés de monocytes conduisant à l'activation d'une réponse immunitaire innée. L'infection par le mutant VIH-1 P90A dans ces macrophages conduit à la production du second messager cGAMP, NF-ƙB et IRF3 et à la production d'IFN de type 1, qui supprime complètement la réplication du VIH-1. L'inhibition des IFN par des anticorps spécifiques rétablit l'infection par le VIH-1. Les mécanismes par lesquels CypA empêche l'ADN du VIH-1 de déclencher la réponse immunitaire par la voie cGAS restent encore inconnus (Rasaiyaah *et al.*, 2013; Schaller *et al.*, 2011).

e) TRIMCyp

Le gène TRIM5 est constitué, chez tous les primates, de huit exons et sept introns. Sa transcription permet la synthèse de plusieurs isoformes, générés par épissage

alternatif, désignés TRIM5α, β, etc., la forme α est la plus longue et possède un domaine PRYSPRY complet en C-terminal. Cette règle compte cependant une exception : dans les cellules de singes-hiboux, un pseudogène est présent dans l'intron reliant les exons 7 et 8. Ce pseudogène, inséré vraisemblablement par un événement de rétrotransposition datant de quelques millions d'années, code pour la cyclophiline A (Nisole *et al.*, 2004; Sayah *et al.*, 2004; Berthoux *et al.*, 2005). L'épissage alternatif de l'ARNm codant pour TRIM5 conduit à la synthèse d'une protéine de fusion TRIM5-CypA constituée des trois premiers domaines de TRIM5α et de la CypA en lieu et place du domaine PRYSPRY. Cette protéine de fusion est capable d'inhiber la réplication du VIH-1, mais pas des isolats MLV-N. Dans ce cas, il a été possible d'observer une interaction directe du domaine CypA avec la capside du VIH-1 (Nisole *et al.*, 2004). Cette découverte permet donc d'expliquer l'observation antérieure selon laquelle l'interaction entre la CypA et la capside virale est nécessaire à la restriction du VIH-1 dans les cellules de singe-hibou (Towers *et al.*, 2003). En effet, si l'on bloque l'interaction CypA-CA, la levée de restriction n'est pas due au fait que la CypA cellulaire ne puisse plus reconnaître la protéine de capside du VIH-1, mais est due à l'inhibition de l'interaction de la protéine de fusion TRIM5-CypA avec sa cible virale. L'inhibition peut être surmontée par traitement avec de la cyclosporine (CsA) ou l'utilisation de mutants G89V du VIH-1 (Nisole *et al.*, 2004; Sayah *et al.*, 2004; Berthoux *et al.*, 2005). TRIMCyp restreint également FIV et SIVagm, mais laisse les cellules de ces espèces sensibles à l'infection par SIVmac et EIAV (Diaz-Griffero *et al.*, 2006).

Il y a eu d'autres incidents de rétrotransposition de CypA dans le locus de TRIM5 chez certaines espèces de singe macaque du vieux monde, y compris les macaques rhésus (Macaca mulatta), les macaques à queue de cochon (Macaca nemestrina) et les macaques crabier (Macaca fascicularis). Cette autre orthologue de TRIMCyp est apparu indépendamment de l'événement du singe-hibou en raison de l'emplacement différent de la séquence codante CypA dans le gène TRIM5. De fait, l'ADNc de CypA se trouve en aval de l'exon 8 pour les singes du vieux monde (Brennan *et al.*, 2008; Newman *et al.*, 2008; Langelier *et al.*, 2008; Wilson *et al.*, 2008). La protéine résultante est codée par les exons 2 à 7 avec le remplacement de l'ADNc CypA au niveau de l'exon 8 à la

différence du singe-hibou qui est codé par les exons 2 à 6 de TRIM5, avec le remplacement des l'ADNc CypA au niveau des introns 7 et 8. La spécificité anti-rétrovirale de cette protéine de fusion est également distincte du singe-hibou, avec le TRIMCyp rhésus étant un puissant inhibiteur du VIH-2, le VIH-1 groupe O et FIV, mais pas le VIH-1 du groupe M.

Cette différence de spécificité de restriction est due à des variations dans le domaine de CypA de TRIMCyp-rh par rapport à celle de la CypA génomique, ce qui modifie la conformation de la boucle du site actif (Price *et al.*, 2009). En outre, les allèles TRIMCyp provenant de différentes espèces de macaques ont également différentes spécificités anti-rétrovirales en raison de mutations supplémentaires dans leurs domaines Cyp (Ylinen *et al.*, 2010). Ces incidences de l'évolution convergente à produire des facteurs de restriction de TRIMCyp sur plus d'une occasion et leur subsistance dans le génome suggèrent qu'ils ont connu une forte pression sélective face aux infections rétrovirales au cours de leur évolution (Malfavon-Borja *et al.*, 2013).

La protéine TRIM5α comme candidat pour la thérapie génique

Les progrès récents dans le domaine de la thérapie génique ont renouvelé l'intérêt de développer de nouvelles approches thérapeutiques visant à inhiber l'infection du VIH-1. Plusieurs stratégies sont en cours de développement et un certain nombre a atteint les essais cliniques. L'une de ces stratégies vise à empêcher l'entrée du virus en perturbant l'expression du corécepteur CCR5 soit par l'utilisation d'ARNs interférents ou de ribozymes (Chung *et al.*, 2011; Holt *et al.*, 2010; Thèbes *et al.*, 2014), soit par l'élimination directe du locus de CCR5 par CRISPR (Owens, 2014; Wang *et al.*, 2014; Li *et al.*, 2015; Hou *et al.*, 2015). Puisque cette stratégie inhibe l'expression d'un facteur cellulaire, les risques et les effets secondaires potentiels à long terme ne sont pas connus.

Une autre approche consiste à transduire les cellules avec des inhibiteurs naturels de la réplication du VIH-1. Un tel candidat est le facteur de restriction TRIM5α. En effet, TRIM5α cible le VIH-1 au début de son cycle de vie, soit au point d'entrée et

avant l'intégration afin de limiter les effets cytotoxiques, de réduire les possibilités d'évolution mutagène pendant la transcription inverse et d'empêcher l'établissement de réservoirs latents (Owens *et al.*, 2003; Stremlau *et al.*, 2004). Cependant, la gamme de virus restreints par TRIM5α varie grandement d'une espèce à l'autre. Par exemple, l'orthologue humain de TRIM5α ($TRIM5\alpha_{hu}$) limite modérément le VIH-1 (moins de deux fois), tandis que son homologue du singe Rhésus ($TRIM5\alpha_{rh}$) est très actif contre le VIH-1 (50 à 100 fois) (Stremlau *et al.*, 2004; Yap *et al.*, 2004).

Toutefois, il a été démontré que lorsque le domaine PRYSPRY de $TRIM5\alpha_{hu}$ était substitué par les séquences correspondantes à celles du singe Rhésus et que cette protéine chimérique était exprimée dans les cellules humaines, TRIM5α humaines/rhésus était capable d'inhiber efficacement le VIH-1 (Stremlau *et al.*, 2004; Yap *et al.*, 2004). De plus, l'étude comparative entre la séquence codante de TRIM5α humain à celle du singe rhésus, a permis d'identifier une mutation unique en position 332 de $TRIM5\alpha_{hu}$ pouvant diminuer l'infection du VIH-1 par un facteur de 10. Cependant, cette mutation unique ne suffit pas à restreindre efficacement la réplication du VIH-1 (Stremlau *et al.*, 2004; Yap *et al.*, 2004).

1.3 Hypothypothèses et objectifs

Basé sur la double hypothèse que (i) $TRIM5\alpha_{Rh}$ et d'autres orthologues de TRIM5 provenant des singes du vieux monde pourraient être trop immunogène pour la thérapie génique, (ii) $TRIM5\alpha_{hu}$ pourrait être modifiée génétiquement afin de générer une protéine ayant des effets anti-rétroviraux contre le VIH-1. Notre objectif consiste donc à (i) générer des mutations aléatoires dans la région PRYSPRY de la protéine $TRIM5\alpha_{hu}$, (ii) isoler par un crible fonctionnel d'autres mutants présentant une activité de restriction accrue contre le VIH-1 et (iii) caractériser l'effet de $TRIM5\alpha_{hu}$ mutée sur les diverses souches cellulaires et virales.

CHAPITRE II

GENERATION OF HUMAN TRIM5α MUTANTS WITH HIGH HIV-1 RESTRICTION ACTIVITY

Le contenu de ce chapitre a fait l'objet d'une publication en anglais dans la revue *Gene Therapy*, le 17 juillet 2010.

2.1 Résumé

La protéine TRIM5α est un facteur de restriction présent dans le cytoplasme des cellules hôtes. Elle inhibe les rétrovirus après leur entrée dans la cellule lors de la phase précoce de l'infection. Chez le singe macaque, TRIM5α_{rh} est un puissant inhibiteur de la réplication du VIH-1 alors que son orthologue humain TRIM5α_{hu} a très peu d'effet sur ce virus. Dans cette expérience, nous avons (1) généré des mutations aléatoires par mutagenèse dirigée dans le domaine PRYSPRY de TRIM5α_{hu} et (2) isolé par un criblage fonctionnel les mutants présentant une activité de restriction accrue contre le VIH-1. Ce protocole a conduit à la caractérisation d'une variante de TRIM5α humain contenant une mutation au niveau de l'arginine 335. Le niveau de protection résultant de l'expression de ce mutant a été comparable à celle des mutations décrites précédemment à la position 332. Dans les fibroblastes et dans la lignée de lymphocytes T, lorsque la mutation R335 est exprimée en combinaison avec la mutation Arg332, elle diminue de 20 à 50 fois la susceptibilité à l'infection par le VIH-1 et empêche la propagation du VIH-1 infectieux à un niveau équivalent à TRIM5α du singe macaque. Ces résultats semblent confirmer le potentiel de TRIM5α_{hu} comme étant un bon candidat pour des applications de thérapie génique.

2.2 Premier article scientifique

Generation of human Trim5α mutants with high HIV-1 restriction activity

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Abstract

Rhesus macaque tripartite motif TRIM5α potently inhibits early stages of human immunodeficiency virus (HIV)-1 replication, while the human orthologue has little effect on this virus. We used PCR-based random mutagenesis to construct a large library of human TRIM5α variants containing mutations in the PRYSPRY domain. We then applied a functional screen to isolate human cells made resistant to HIV-1 infection by the expression of a mutated TRIM5α. This protocol led to the characterization of a human TRIM5α variant containing a mutation at arginine 335 as conferring resistance to HIV-1 infection. The level of protection stemming from expression of this mutant was comparable to that of previously described mutations at position 332. R332/R335 double mutants decreased permissiveness to HIV-1 and to other lentiviruses by 20- to 50-fold in TE671 fibroblasts and in the T-cell line SUP-T1, and prevented HIV-1 spreading infection as efficiently as the rhesus macaque TRIM5α orthologue did. The finding that

only two substitutions in human TRIM5α can confer resistance to HIV-1 at levels as high as one of the most potent natural orthologues of TRIM5α removes a roadblock toward the use of this restriction factor in human gene therapy applications.

Keywords: HIV-1; lentivirus; random mutagenesis; TRIM5α; restriction factor; retroviral replication

Introduction

TRIM (tripartite motif) proteins form a family of dozens of members, most of them showing a RING, a B-box and a Coiled-coil domains.[1, 2] TRIM5α was isolated in 2004 as the factor governing the resistance of rhesus macaque monkeys to transduction by human immunodeficiency virus (HIV)-1 vectors.[3] Shortly thereafter, most primates were shown to encode TRIM5α orthologues each having the capacity to restrict a particular range of retroviruses.[4, 5, 6, 7, 8, 9] As would be expected, TRIM5α from a particular species does not inhibit cognate retroviruses, but it can target retroviruses from other species, thereby contributing to the prevention of inter-species transmission. Human TRIM5α inhibits some retroviruses, such as the equine infectious anemia virus (EIAV) and the so-called 'N strains' of the murine leukemia virus (N-MLV)[4, 5, 10, 11] but it only weakly restricts HIV-1, HIV-2 and many simian immunodeficiency viruses (SIVs), including SIVs from macaques (SIV_{mac}), from African green monkeys (SIV_{AGM}) and from chimpanzees (SIV_{cpz}).[5, 12, 13] It is likely that the limited restriction range of $TRIM5\alpha_{hu}$ has made it possible for lentiviruses infecting nonhuman primates to cross-species and thrive in humans, thus causing the current HIV pandemic. It has been suggested that $TRIM5\alpha_{hu}$ derives from an ancestor gene that had evolved to provide resistance against unknown, now-extinct retroviruses, leaving us more vulnerable to infections by modern simian lentiviruses.[14]

Restriction by TRIM5α is initiated by physical recognition of incoming retroviruses by TRIM5α proteins.[15, 16] This interaction occurs within the first hours after virus entry.[17] and involves determinants present in the N-terminal domain of the capsid proteins that

constitute the retroviral outer core structure.[18, 19, 20] After this initial contact, progression of the retroviral life cycle is impeded through several mechanisms. Viral cores seem to undergo accelerated uncoating, as evidenced by the disappearance of capsid in particulate form.[16, 21, 22] The proteasome is also involved and causes a decrease in retroviral complementary DNA accumulation in acutely infected cells.[23] TRIM5α proteins can seemingly self-ubiquitinate[24, 25] and are rapidly degraded by the proteasome on exposure to a restriction-sensitive virus.[26] Finally, TRIM5α interferes with the transport of post-entry retroviral complexes toward the nucleus, and this anti-rétroviral activity is independent from the one involving the proteasome.[27, 28] As retroviral replication is blocked in the cytoplasm and within the first hours of infection, successful restriction by TRIM5α is expected to completely prevent *de novo* transcription of viral genes from integrated or unintegrated viral complementary DNAs in the nucleus.

In addition to the RING, a B-box and a Coiled-coil motif, TRIM5α contains a C-terminal PRYSPRY domain (also called SPRY or B30.2)[29] that is the sole determinant of restriction specificity.[30] PRYSPRY is the most variable domain of TRIM5α, and it contains four highly variable regions (see Figure 1d), labeled v_1–v_4,[31] although only v_1–v_3 are generally considered relevant to restriction specificity. Construction of chimeric genes in which these three variable regions are swapped between TRIM5α orthologues showed that all three of them can potentially contribute to restriction specificity.[32] Particular attention has been given to the v_1 region, which is rich in positively (that is, non-randomly) selected aminoacids.[33, 34] Versions of human TRIM5α in which short stretches of the v_1 region were replaced by those of the macaque orthologue showed restriction activity against HIV-1.[33, 35] In particular, substitution of $TRIM5\alpha_{hu}$ arginine 332 for a proline (the aminoacid present at that position in $TRIM5\alpha_{rh}$) decreased permissiveness to HIV-1 infection.[35, 36, 37] In fact, most substitutions of this residue, excepted to a lysine, resulted in significant HIV-1 restriction, showing that suppression of the positive charge was the mechanism underlying the acquisition of HIV-1 restriction potential.[36] Although the protection provided by mutations at position 332 was strong (10- to 30-fold), these mutants still restricted HIV-1 10-times less efficiently than $TRIM5\alpha_{rh}$ did.[36]

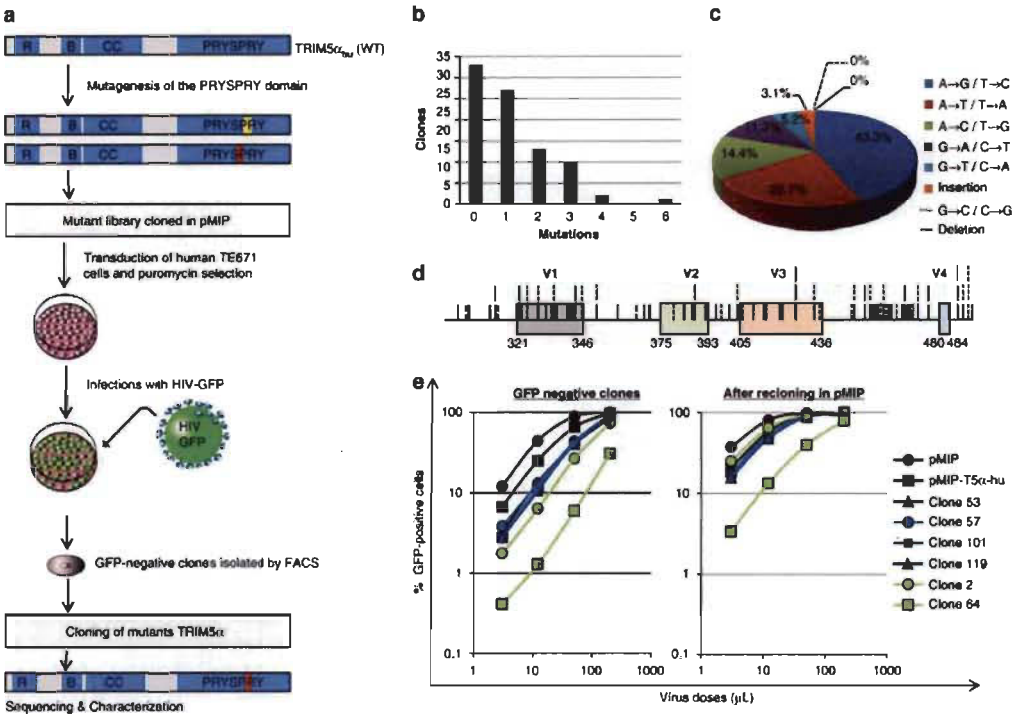

Figure 1. A random mutagenesis screen of TRIM5α_{hu}. (a) Outline of the protocol used. PCR-based mutagenesis was confined to the PRYSPRY domain of TRIM5α_{hu} and followed by a fluorescence-activated cell sorting (FACS)-based functional assay to isolate the cells putatively resistant to HIV-1 infection. (b) Number of mutations per clone before functional screening. In total, 86 clones were sequenced. (c) Analysis of mutations found in the PRYSPRY domain of TRIM5α_{hu}. All 97 mutations found in the 86 analyzed clones were included. (d) Mapping of 94 substitutions along the TRIM5α_{hu} PRYSPRY domain. Non-silent substitutions are indicated with continuous lines while silent mutations are represented by dotted lines. (e) Functional screen and isolation of a TRIM5α_{hu} mutant that restricts HIV-1. Left panel: cell clones that were GFP negative after exposure to a large amount of a GFP-expressing HIV-1 vector were infected with four different doses of HIV-1TRIP_CMV_GFP. Control cells were transduced with wild-type TRIM5α_{hu} or with the parental MIP vector. Shown are some of the clonal populations that showed a twofold or more decrease in permissiveness to HIV-1 replication compared with the cells transduced with wild-type TRIM5α_{hu}. Right panel: TRIM5α_{hu} coding sequences from these cell lines were recloned into pMIP and re-transduced into parental TE671 cells. These cells and control cells were again challenged with the HIV-1 vector expressing GFP. Clone 64 contained the R335G mutation.

Numerous gene therapy clinical trials for the treatment of AIDS have been undertaken to mostly disappointing results, both in terms of viral load and of CD4+T cells counts.[38] In particular, although it is desirable that transgene-expressing cells survive and

proliferate in the recipient patients, the opposite is often observed. For instance, after gene transfer of an anti-HIV ribozyme in hematopoietic stem cells, a fivefold reduction in expression of the ribozyme was observed within 6 months.[39] Rapid elimination of productively HIV-1-infected lymphocytes is well-known[40] and results from the action of cytotoxic T lymphocytes and also from virus-related killing. Therefore, it is likely that to succeed, a gene therapy approach for AIDS would need to disrupt viral replication either at entry or at immediate post-entry steps, before viral antigens are being synthesized. Interfering with the expression of the virus entry co-receptor CCR5, either by RNA interference[41, 42] or by transfer of a zinc-finger nuclease[43] might achieve that goal, but the immunological consequences of completely disrupting CCR5 expression in human cells *in vivo* are unknown. Thus, expression of a TRIM5α-based restriction factor targeting HIV-1 represents an attractive alternative. Whenever an exogenous protein is to be expressed as part of a gene therapy protocol, an additional requirement is for the transgene to be the least immunogenic as possible. Nonhuman primate TRIM5α orthologues such as TRIM5α_{rh} and the African green monkey orthologue (TRIM5α_{AGM}) are potent inhibitors of HIV-1 replication, but their aminoacid sequences diverge from that of the human orthologue (GenBank: ABB90543) by 11.6% (Rhesus; ABG67966) and 16.8% (AGM; AAT81667). To discover new, discrete determinants of HIV-1 restriction by TRIM5α, we have randomly mutagenized TRIM5α_{hu} and then performed a functional screen to isolate variants with high anti-HIV-1 activity.

Results

A screen based on random mutagenesis of TRIM5α

A human TRIM5α mutant library was constructed by error-prone PCR of the PRYSPRY domain (Figure 1a), the only region of this protein known to influence retroviral target specificity. The frequency of mutations was within the desired range of 1–2 per clone (Figure 1b). The mutations introduced were almost exclusively (97%) substitutions, with a strong bias toward mutations at A/T residues (approximately 80%; see Figure 1c), as predicted from this type of mutagenesis. As expected, mutations were scattered all along

the PRYSPRY domain (Figure 1d). Some regions, like the first variable region and the region between v_3 and v_4, appeared to be mutational hot-spots, and this seems to correlate partly with their high A/T content. Among 94 substitutions analyzed, 34 (36%) were silent and 17 (18%) were present more than once (eight were present twice, and one was present three times). Thus, enrichment for particular point mutations, a recurring problem in PCR-based random mutagenesis, was relatively low in our screen by virtue of the PCR conditions used (high amount of template DNA, low number of cycles). No mutations were found in TRIM5α_{hu} regions upstream of the PRYSPRY domain.

The library was retrovirally transduced into human cells rather than in the cell lines of murine or feline origin that are often used to study TRIM5α-mediated restriction. This might seem somewhat counterintuitive at first, considering that human cells express endogenous wild-type TRIM5α, creating the possibility of functional interference between the two proteins.[44] However, considering that the ultimate goal is to inhibit HIV-1 replication in human recipients, we judged that human cells should be used in the screen itself. In addition, published data suggest that overexpressed, exogenous TRIM5α is dominant over endogenous TRIM5α,[44] most probably because of large differences in protein amounts. Untransduced cells were eliminated by puromycin treatment. Functional screening was carried out by infecting the library-expressing cells with large doses (typically leading to 99% infected cells) of an HIV-1 vector expressing green fluorescent protein (GFP) (HIV-$1_{TRIP-CMV-GFP}$) then isolating cells that were GFP negative and thus putatively resistant to HIV-1 infection. This approach is the same one that originally led to the discovery of TRIM5α as an anti-rétroviral protein by the Sodroski laboratory.[3] In a second step of functional selection, 108 GFP-negative cell clones were infected with four doses of the GFP-expressing HIV-1 vector, along with the control cells transduced with wild-type TRIM5α_{hu} or with the empty expression construct (pMIP). In all, 40 of the clonal cell lines were found to show a twofold or more decrease in permissiveness to HIV-1 infection compared with cells transduced with wild-type TRIM5α_{hu} (some examples are shown in Figure 1e). In step three, transgenic TRIM5α_{hu} were PCR amplified from all of these clones and were re-introduced into

naive TE671 cells by retroviral transduction using the MIP vector again. These cells were also challenged with four doses of HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$, and that time we found that only one cell line (clone 64) showed significant resistance to HIV-1 (Figure 1e). Therefore, resistance to HIV-1 infection in the other 39 clonal cell lines from the second step of functional screening was due to factors other than the expression of exogenous TRIM5α. Accordingly, sequencing showed that some of these TRIM5α coding sequences did not even carry mutations (not shown). We did not analyze the 39 false positives any further.

Targeted mutagenesis of Arg332 and Arg335 within PRYSPRY first variable region

TRIM5α complementary DNA in clone 64 contained the A1003G mutation, resulting in an arginine to glycine substitution at position 335 of the protein. It also had a silent mutation (A906C). Arg335 is located in the hypervariable region v_1 of the PRYSPRY domain and has been positively selected during recent evolution.[33] Humans, chimpanzees and gorillas are the only known primates to have a positively charged residue at this position.[31] Partly for that reason, the role of this residue in the inability of $TRIM5\alpha_{hu}$ to restrict HIV-1 had been suspected before,[45] but data published so far has suggested that it was only a minor determinant of HIV-1 restriction. For instance, $TRIM5\alpha_{rh}$ with an arginine at this position instead of a leucine was still fully able to restrict HIV-1.[35] Li *et al.*[36] constructed the reciprocate R335L mutation of $TRIM5\alpha_{hu}$ and found that it weakly inhibited HIV-1; in addition, the double-mutant R332P/R335L restricted HIV-1 less efficiently than R332P alone. Therefore, isolation of an Arg335 mutant in our screen was rather unexpected. We hypothesized that this discrepancy was simply caused by different substitutions (to leucine in Li's paper, to glycine as found in our screen).

A computerized model for the structure of the PRYSPRY domain of $TRIM5\alpha_{hu}$ has been suggested, based on the known crystal structures of similar domains within other proteins.[46] In this model, the variable region v_1 forms a loop that protrudes from the rest of the domain and is essentially an insertion compared with other SPRY domains.

Owing to its flexibility, the exact spatial conformation of this region is more uncertain than that of the more structured globular core. However, the side chains of Arg332 and Arg335 clearly are solvent exposed and would be likely to come to close contact with the viral capsid (Figure 2a). To examine the respective roles of arginines 332 and 335 in the restriction range of TRIM5α_{hu}, we constructed an array of mutations at position 335 and we also constructed double mutations (Figure 2b). First, to insure that R335G was the sole mutation in clone 64 causing the observed phenotypical change, we reintroduced the mutation in the parental plasmid. To analyze the possibility that increased restriction of HIV-1 was caused by suppression of the positive charge at position 335, we made the arginine-to-lysine mutant R335K, which maintains this positive charge. We also constructed the basic-to-acidic mutations R335D and R335E, replacing the positive charge by a negative one. We also substituted Arg335 into two hydrophobic residues, one aromatic (R335F) and one aliphatic (R335L; Li *et al.*[36] mutation), and an additional mutant had Arg335 deleted. Arg332 was mutated to glycine, which was one of the mutations at this position conferring a high HIV-1 restriction phenotype. Finally, we combined R332G with R335G or with R335E. These two double mutations have a –2 and –3 net effect on the protein's charge, respectively. All mutated TRIM5α_{hu} were transduced into TE671 cells and into the lymphocytic T-cell line SUP-T1.

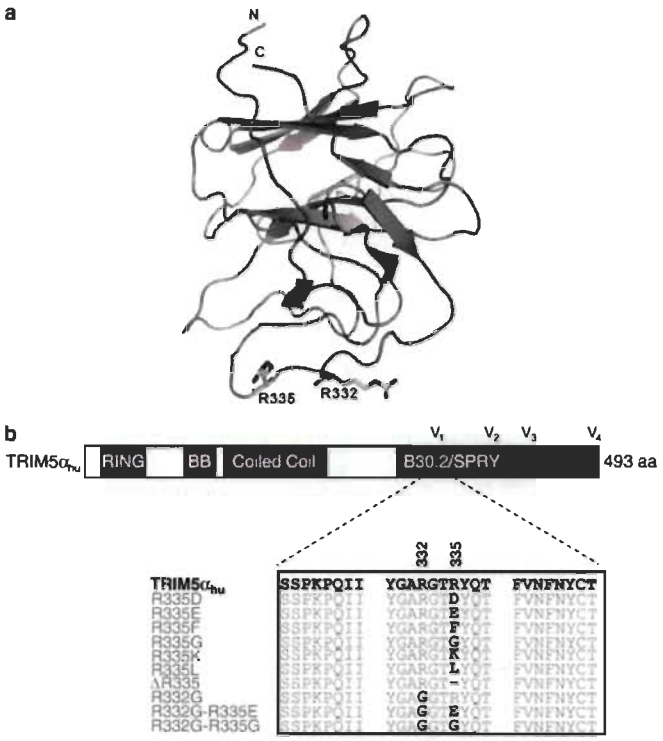

Figure 2. Site-directed mutagenesis of TRIM5α_{hu}. (a) Localization of Arg332 and Arg335 in the structure model for the TRIM5α_{hu} PRYSPRY domain, as suggested by Sebastian *et al.* [46] (b) Mutations introduced in the first variable region (v_1) of TRIM5α_{hu} are in bold letters.

Restriction of HIV-1 by wild-type and mutant TRIM5α_{hu}

All the TE671 cell lines created were challenged with retroviral vectors derived from HIV-1 (Figure 3a and Table 1) and from other retroviruses (Supplementary Figure 1 and Table 1). All retroviral vectors expressed the enhanced GFP, which thus served as a marker to quantitate the percentages of infected cells. As shown in Figure 3a, overexpression of wild-type TRIM5α_{hu} slightly inhibited HIV-1 infection (less than twofold), an effect observed by others before.[13, 35, 47] All mutants caused a significant decrease in permissiveness to HIV-1 (Figure 3a and Table 1). R332G and R335G both restricted HIV-1 by 10- to 20-fold. Clone 64 TRIM5α_{hu} also inhibited HIV-1 to the same extent, confirming that R335G was the only mutation in this clone having a role in the restriction. The least efficient mutant for the restriction of HIV-1 was R335K (sixfold), suggesting that suppression of the positive charge at this position was indeed needed for maximum restriction. R335L and R335F similarly had a weak effect on HIV-1 infection,

suggesting that a hydrophobic residue at this position was detrimental for the restriction of HIV-1. All other mutations decreased HIV-1 infection by up to 35-fold, and the mutant with the strongest effect was the double substitution R332G/R335G, showing that the two mutations had partially additive effects.

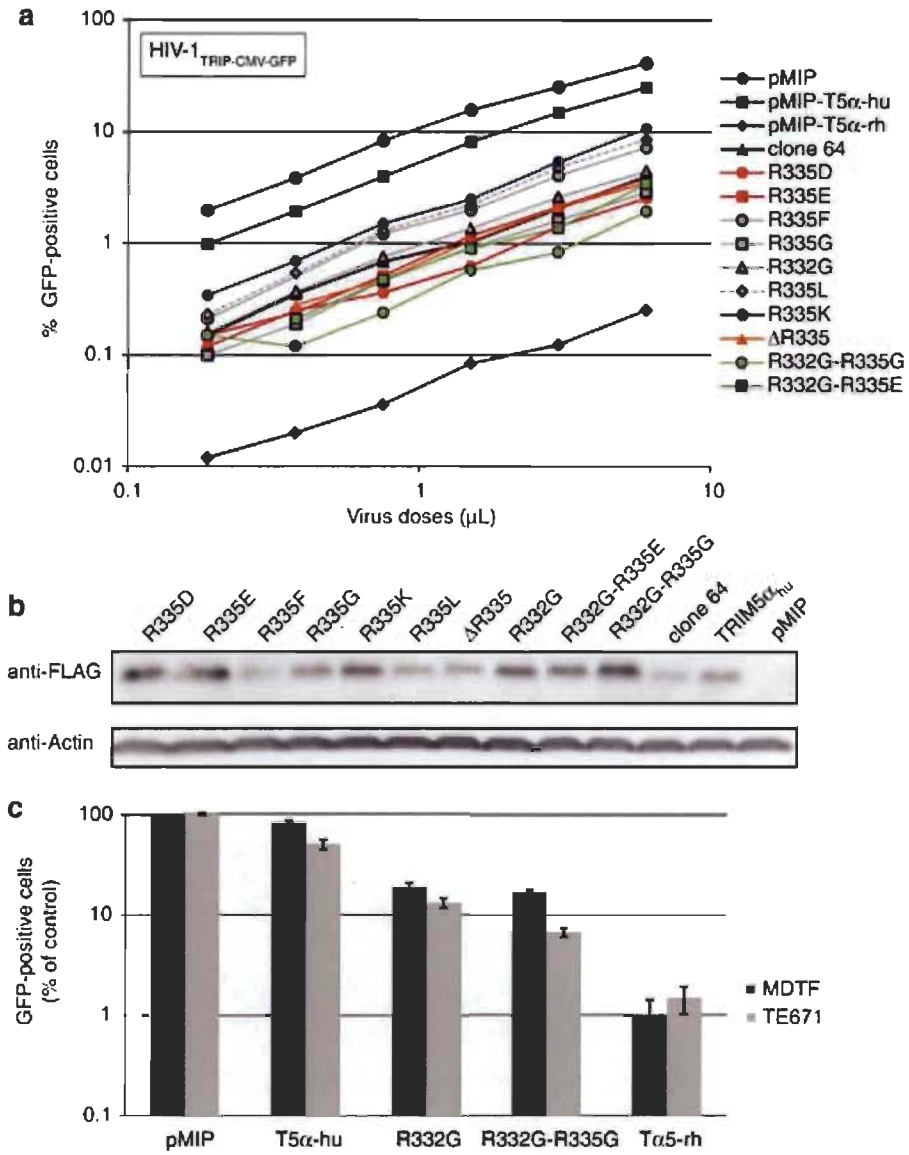

Figure 3. Effect of Arg332 and Arg335 mutations on the anti-retroviral activity of TRIM5α_{hu}. (a) TE671 cells expressing wild-type and mutant TRIM5α_{hu} or transduced with the empty parental vector were challenged with multiple doses of HIV-$1_{TRIP\_CMV\_GFP}$. The percentages of GFPpositive cells were analyzed by fluorescence-activated cell sorting (FACS) two days later. (b) Expression of FLAG-tagged wild-type and mutant TRIM5α_{hu} in transduced TE671 cells was assessed by western blotting using antibodies

directed against the FLAG epitope (top) or actin (bottom). (c) TE671 and MDTF cells expressing the indicated TRIM5α variants were challenged with HIV-$1_{TRIP\_CMV\_GFP}$. The virus dose used was adjusted so that approximately 10% of the control cells transduced with the empty vector MIP were infected. GFP expression was analyzed 2 days later by FACS and data are expressed as % relative to the MIP-transduced controls for each cell line. Bars show the averages of three infections with s.d.

Table 1. Restriction of other retroviruses.

TRIM5α$_{hu}$ variant	***Restriction of:***					
	HIV-1	***HIV-2***	***SIV $_{mac}$***	***EIAV***	***N-MLV***	***B-MLV***
Wild type	+	++	++	++	+++	+
R335D	++++	++++	+++	++	ND	ND
R335E	+++	+++	+	+	++++	–
R335L	++	++	–	+	+++	–
R335F	++	+	–	+	+++	–
R335G	+++	++++	+++	++	+++	+
R332G	+++	++++	++	++	++	–
R335K	++	+++	+++	+++	++++	+++
ΔR335	+++	++++	+++	+++	ND	ND
R332G/R335G	++++	++++	+++	++	++++	–
R332G/R335E	+++	++++	+	+	+++	–

Abbreviations: EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; ND, not determined; N-MLV, N strains of the murine leukemia virus; SIV, simian immunodeficiency virus; TRIM, tripartite motif.

[a] Decrease in permissiveness to retroviral transduction by GFPexpressing vectors derived from the indicated retroviruses, relative to mock-transduced control cells. Cell lines expressing wild-type or mutated TRIM5α$_{hu}$ were infected with the retroviral vectors at a dose leading to about 10% infected cells for the mock-transduced control. The percentages of infected cells were determined 2 days later by flow cytometry. –, less than twofold decrease; +, two- to fivefold decrease; ++, 5- to 10-fold decrease; +++, 10- to 20-fold decrease; ++++, > 20-fold decrease.

Expression of exogenous TRIM5α in these cell lines was assessed by western blotting using an antibody recognizing the C-terminal FLAG tag. Among other similarly FLAG-tagged TRIM5α orthologues, the human version has been found difficult to detect in the past. By optimizing the experimental conditions, we were able to detect all

TRIM5α_{hu} mutants in TE671 cells and all of them were found to have the expected size (Figure 3b). Levels of expression seemed to vary substantially between mutants. Differences in efficiencies of transduction constitute a likely explanation, although we cannot exclude that this region of TRIM5α_{hu} might be important to its stability (see Discussion).

Restriction by various TRIM5α orthologues have often been studied in cell lines of cat or mouse origin, which do not express endogenous TRIM5α. To gain insight into the cell context dependency of HIV-1 restriction by TRIM5α_{hu} mutants, we transduced R332G and R332G/R335G TRIM5α_{hu} in both TE671 and murine *Mus dunni* tail fibroblast (MDTF) cells (Figure 3c). As expected, TRIM5α_{rh} decreased HIV-1 replication by 100-fold in both cell lines, while TRIM5α_{hu} had a small inhibitory effect in both cell contexts (although the restriction was stronger in human cells). R332G TRIM5α_{hu} decreased HIV-1 replication by five- to eightfold in both cell lines, consistently with previous results. Interestingly, in MDTF cells R332G/R335G restricted HIV-1 at levels similar to the single mutant R332G. In TE671 cells, R332G/R335G was again significantly more efficient than R332G alone and decreased HIV-1 replication by approximately 20-fold (Figure 3c). It is not clear whether this observed cell context dependency is due to the presence of low levels of endogenous TRIM5α_{hu} in human cells or to some other cellular factor. However, this observation argues in favor of performing this type of screen directly in human cells, which was the strategy followed here.

Restriction of other retroviruses

As also shown in Supplementary Figure 1 and Table 1, HIV-2 was significantly inhibited (10-fold) by overexpressed TRIM5α_{hu}, an observation previously reported.[13, 48] R332G caused an even greater (60-fold) inhibition of this virus, while R335L and R335F restricted HIV-2 only weakly. All other mutants had an HIV-2 restriction phenotype intermediate between wild-type TRIM5α_{hu} and the R332G mutant. Wild-type TRIM5α_{hu} weakly inhibited SIVmac239 (fivefold), and this restriction was abolished by introducing hydrophobic residues at position 335. A group of mutations that included

R332G, R335G and the deletion of Arg335 decreased SIVmac239 replication by 10- to 20-fold, while R335E and the double-mutation R332G/R335E had a more moderate effect. Thus, arginines 332 and 335 modulate the restriction of not only HIV-1 but also of other primate lentiviruses.

We also examined the effect of our constructs on the replication of EIAV and N-MLV, two viruses that are restricted by the human endogenous TRIM5α. EIAV replication was further decreased five times by overexpression of wild-type TRIM5α_{hu}. Several mutations (R332G, R335K, R335) inhibited EIAV at significantly higher levels (up to 12-fold) while the double-mutation R332G/R335G had a smaller effect (threefold). Thus, arginines 332 and 335 are also determinants in the restriction of nonsimian lentiviruses.

The N-MLV and B-tropic MLV (B-MLV) Gag proteins differ by a few amino acids,[49] and amino acid 110 in the capsid domain determines the sensitivity of MLV to TRIM5α_{hu} and to Fv1, another restriction factor functionally (if not structurally) related to TRIM5α.[50, 51] In our hands, N-MLV replication was decreased by overexpression of wild-type TRIM5α_{hu} (Supplementary Figure 1). This effect was greater at relatively high amounts of viral input (for example, 100 µl of virus) than at lower doses such as 25 µl. This is likely due to the well-known effect of saturation of the restriction at high virus doses:[50, 52] endogenous TRIM5α_{hu} is saturated by large amount of incoming N-MLV capsids but overexpression of TRIM5α_{hu} restores restriction. That both N-MLV and EIAV were significantly inhibited by overexpressed TRIM5α_{hu} suggests that the endogenous protein is expressed at levels too low for optimal restriction, at least in the absence of interferons.[53, 54] Mutations at position 332 and 335 had modest effects on the restriction of N-MLV by TRIM5α_{hu} (Supplementary Figure 1 and Table 1). For instance, in conditions in which wild-type TRIM5α_{hu} inhibited N-MLV infection by 20- to 30-fold, some mutations (R335K, R332G/R335G) increased that inhibition by approximately twofold, while others decreased it by approximately twofold.

Infection with a B-MLV vector was in most cases only marginally affected by mutations at the two arginines, with the exception of R335G and R335K, which inhibited this virus by approximately 5-fold and 20-fold, respectively. All the other mutants behaved like the mock-transduced cells. These data show that Arg335 also influences the sensitivity of N-MLV and B-MLV to the restriction by TRIM5α_{hu}, but in a manner strikingly different from what is observed with lentiviruses: (i) unlike that of lentiviruses, restriction of N-MLV is not significantly affected by the introduction of hydrophobic residues at position 335; (ii) B-MLV is the only virus analyzed here that is specifically inhibited by the R335K mutation. It is noteworthy that another group reported that B-MLV could be restricted by TRIM5α_{hu} bearing a mutation at Tyr336.[55]

Restriction of HIV-1 in a T-cell line

We then analyzed the level of protection conferred by TRIM5α_{hu} mutants in the T-cell line SUP-T1. These cells were transduced with the various TRIM5α_{hu} variants and controls, and untransduced cells were eliminated by puromycin treatment. As shown in Figure 4a, we first challenged these cells with the same HIV-1-derived vector (HIV-$1_{TRIP-CMV-GFP}$) that had been used in TE671 cells. TRIM5α_{rh} caused a decrease in permissiveness to HIV-1 of approximately 25-fold, which is significantly smaller than what is typically found in fibroblasts (100-fold or more). This is consistent with results published by others.[56] R332G and R335G both inhibited HIV-1 by approximately eightfold, while R335K had no restrictive effect at all. Overall, mutants showed phenotypes similar to what had been observed in TE671 cells. The two double mutants restricted HIV-1 to nearly TRIM5α_{rh} levels, confirming that mutations at Arg332 and Arg335 had additive effects.

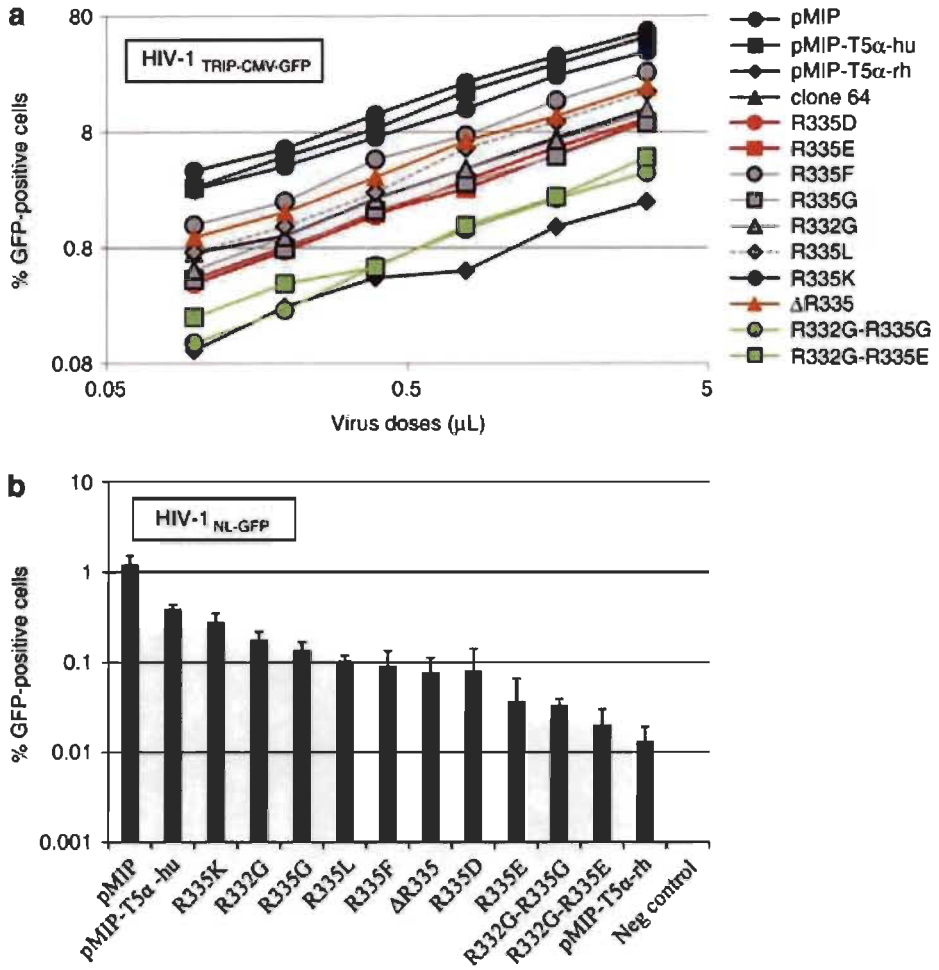

Figure 4. HIV-1 restriction in a T-lymphoid cell line. SUP-T1 cells expressing wild-type or mutant TRIM5α_{hu} were challenged with multiple doses of the VSV G-pseudotyped, multiply attenuated HIV-1TRIP_CMV_GFP vector (a) or with a single dose of HIV-1NL_GFP, a non-pseudotyped NL4-3-derived vector in which only the Nef protein has been deleted and replaced by GFP (b). The percentages of GFP-positive cells were determined by fluorescence-activated cell sorting (FACS) after 48 h (a) or 36 h (b). Error bars in (b) are s.d. from quadruplicate infections.

To analyze the protection conferred by TRIM5α_{hu} mutants in more physiological conditions, we challenged the SUP-T1 cell lines with a replication-competent HIV-1 clone derived from NL4-3 but expressing GFP in place of Nef. This virus enters the cells by virtue of its own envelope proteins instead of being pseudotyped with VSV-G. All but one of the viral proteins is encoded and expression of GFP allows the quantification of cells' permissiveness to HIV-1_{NL-GFP} by fluorescence-activated cell sorting. In this context, a group of mutants that included R335G, R335L, R335F, ΔR335

and R335D all decreased HIV-1 replication by approximately 10-fold, while R332G had a slightly smaller effect. Again, the two double mutants inhibited HIV-1 the most efficiently (approximately 50-fold) and were nearly as restrictive as TRIM5α_{rh}. Collectively, the data in Figures 3 and 4 show that while the inhibition of HIV-1 by particular TRIM5α_{hu} mutants can vary to some extent depending on cell type and virus used, the R332/R335 double mutants consistently show the best prospects as anti-HIV-1 transgenes.

Restriction over multiple replication cycles

We then analyzed the inhibition of HIV-1 propagation by these double mutants on multiple replication cycles. It has been shown recently that in *in vitro* T-cell cultures, and probably in most instances *in vivo*, HIV-1 is transmitted from one cell to the next through virological synapses[57, 58, 59] rather than through cell-free viral particles. This mode of transmission may result in different sensitivities of the incoming viruses to TRIM5α. It may also result in TRIM5α targeting susceptible retroviruses not just at post-entry stages but also at later stages of the retroviral life cycle.[60] We thus aimed at analyzing the potency of the R332/R335 double mutants in the context of cell to cell transmissions. 'Classical' virus spreading assays are initiated by adding cell-free virus on a culture of T cells. In these conditions, the first round of entry events is thus mechanistically different from subsequent rounds. To assay virus spread in more consistent conditions, we infected parental SUP-T1 cells with HIV-1_{NL4-3}. The next day, these cells were washed and then placed in culture at a ratio of 1:10 with the SUP-T1 cells expressing wild-type or mutant TRIM5α_{hu} or expressing TRIM5α_{rh} (Figure 5a). Reverse transcriptase activity was measured in the supernatants of the infected cells for 7 weeks after the initiation of the co-cultures (Figure 5b). Viral replication peaked after 14 days of virus spread in control (mock-transduced) cells. HIV-1 replication in cells expressing exogenous TRIM5α_{hu} was slightly delayed, consistent with the small inhibition effect observed in other experimental settings. Spreading infections in TRIM5α_{rh}-, R332G/R335G and R332G/R335E TRIM5α_{hu}-expressing cells were all strongly delayed as shown by the absence of reverse transcriptase activity until day 28.

In these three cell lines, replication seems to plateau at around day 30, and a second peak is observed at around day 42. The possibility that HIV-1 may have incorporated mutations allowing replication in restrictive conditions is currently being analyzed. The single-mutant R332G was included in this experiment and consistently with the one-cycle assays, we found that it caused a significant replication peak delay (1 week) yet the inhibition was clearly much weaker than with the two double mutants. It is noteworthy that replication of HIV-1 in cells expressing TRIM5α_{rh} and the two double mutants of TRIM5α_{hu} did not induce obvious cytopathic effects, while infection of control cells and of cells transduced with R332G TRIM5α_{hu} resulted in massive loss of cells coinciding with the replication peak.

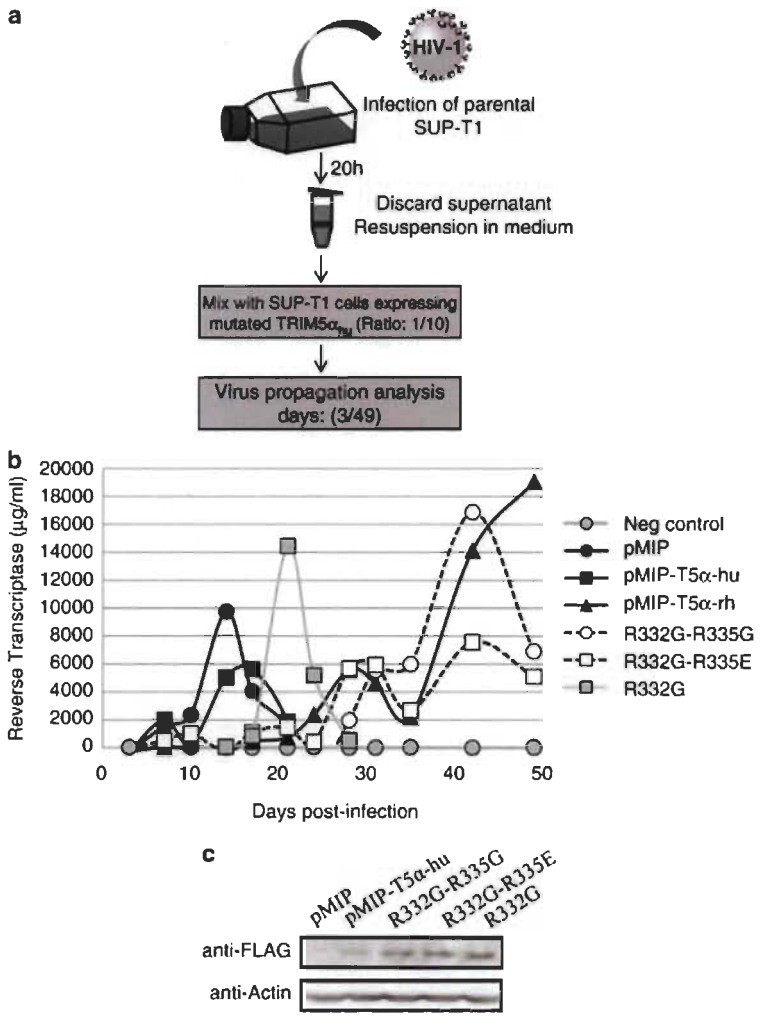

Figure 5. Inhibition of HIV-1 spreading replication. (a) Overview of the protocol used. Parental SUP-T1 cells were infected with HIV-1NL4_3. After 20 h, these

cells were mixed with SUP-T1 cells expressing wild-type or mutated TRIM5α_{hu} at a 1:10 ratio. (b) Cultures were analyzed for reverse transcriptase activity for 49 days. Reverse transcriptase was quantitated with a fluorescence-based kit and using dilutions of pure recombinant reverse transcriptase as a standard. 'Neg control' are uninfected parental cells. (c) Western blot analysis of FLAG-tagged wildtype and mutant TRIM5α_{hu} in transduced SUP-T1 cells.

Expression of transgenic wild-type and mutant TRIM5α_{hu} was assessed by western blotting in SUP-T1 cells. R332G and the two double mutants were expressed at similar levels and at slightly higher levels than the wild-type control. Therefore, and as in the TE671 cells, enhanced HIV-1 restriction by TRIM5α_{hu} containing mutations at Arg332 and Arg335 was not explained by protein levels.

Discussion

In this work, we used targeted *in vitro* molecular evolution to identify and characterize a new mutant of TRIM5α_{hu} (R335G) that shows restriction against HIV-1. Clearly, our results show the usefulness of mutagenic screens in the generation of novel anti-rétroviral gene candidates. Arg332 and Arg335 both seem to prevent targeting of HIV-1 by TRIM5α_{hu} through the presence of a positive charge and removal of the charge at position 335 enhances restriction to the same extent as removal of that of Arg332. One important difference is that all mutations at Arg332 (except R332K) increase HIV-1 restriction to the same extent,[36] while we show that mutations at Arg335 can have very different effects on the restriction of not only HIV-1 but of other retroviruses as well. This particular amino acid of the v_1 region thus seems to represent an important determinant in the interactions between TRIM5α proteins and the viral capsids they target. Removal of the positive charge at Arg332 increases the capacity of TRIM5α_{hu} to bind to HIV-1 capsid,[36] and the mutations at Arg335 described here are expected to have the same effect, although that remains to be shown. It is noteworthy that Arg332 and Arg335 are part of a highly flexible loop (Figure 2a). One could speculate that this loop makes contact with a region of HIV-1 CA that itself is positively charged, hence preventing efficient interactions. In addition, substituting arginines to glycines within

this loop is expected to increase its flexibility even further, perhaps enhancing the capacity of the PRYSPRY domain to adapt to the capsid target protein.

Interpretation of the infectivity assays shown here is potentially complicated by the fact that TRIM5α_{hu} variants were seemingly expressed at different levels. It is noteworthy that R335L, R335F and R335K seemed less stable than the rest of the mutants. Low stability could explain R335L and R335F generally poor restriction potential. However, R335K efficiently restricted B-MLV while the rest of the mutants had little or no activity against this virus. Similarly, R332G was expressed at levels similar to the two double mutants, although it was a less efficient restriction factor against several lentiviruses. Altogether, the correlation between stability and restriction potential appears to be small and probably not relevant at all. Other investigators have found previously that variations in levels of exogenously expressed TRIM5α (for instance by treatment with the stabilizer sodium butyrate) had little effect on restriction activity.[13, 17]

To our knowledge, no study had analyzed the extent of protection provided by mutations at Arg332 in T-cell lines or against a fully replicative strain of HIV-1. Our data show that mutating this arginine alone does not provide adequate resistance against HIV-1 in these settings. In contrast, the R332/R335 double mutations described here seem to confer a level of resistance similar to that of TRIM5α_{rh}, one of the most potent natural HIV-1 restriction factors described so far. Of course, characterization of its protective effect in other T-cell lines, other cell types, and in primary cells will be necessary to fully establish its antiviral potency. Similarly, experiments involving other HIV-1 strains, including non-laboratory-adapted ones, will be needed.

Our mutant TRIM5α_{hu} anti-rétroviral approach begs the comparison with hT5Cyp,[56] the recently constructed TRIMCyp-like restriction factor with very high HIV-1 restriction activity. Both types of gene are likely to inhibit HIV-1 through the same effector mechanisms, although that remains to be assessed. Both are predicted to show low immunogenicity when used *in vivo*. hT5cyp probably has higher potency,[56] certainly not a negligible factor. The single most important determinant for success *in vivo*, however,

is likely to be the emergence (or lack thereof) of HIV-1 variants resistant to the transgenic restriction factor used. In that respect, too, both types of transgene are promising, although for different reasons. One might predict that it would be difficult for HIV-1 to escape being targeted by the cyclophilin A domain of hT5cyp, given that it needs to interact with endogenous cyclophilin A for optimal replication.[61, 62] However, it has been suggested that cyclophilin A-independent strains of HIV-1 are circulating among patients.[63] The advantage of the mutant TRIM5α_{hu} approach, on the other hand, lies in its plasticity: additional TRIM5α_{hu} mutants with anti-HIV-1 activity could be found in different and larger-scale mutagenic screens than the one described here, and the search for variants that do not lead to the appearance of resistant HIV-1 strains could be fully integrated in this discovery process.

Materials and methods

Cells and plasmids DNAs

Human rhabdomyosarcoma TE671 cells, MDTF and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C Human T lymphoid SUP-T1 cells were cultured 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).

pMIP-TRIM5α_{hu}, pMIP-TRIM5α_{rh} and mutants of pMIP-TRIM5α_{hu} all express C-terminal FLAG-tagged versions of complementary DNAs and were gifts from Jeremy Luban.[64] pNL4-3, pNL-GFP, pMD-G, pCIG3N, pCIG3B, pCNCG, pΔR8.9, pTRIP$_{CMV-GFP}$, pCL-Eco, pROD$_{Nef-GFP}$, pSIV$_{mac-GFP}$, pONY3.1 and pONY8.0 have all been extensively described before[44, 65, 66, 67, 68, 69, 70, 71, 72]

Production of retroviral vectors

To produce replication-competent HIV-1, 293T cells plated at 80% confluency in 10-cm Petri dishes were transfected using polyethylenimine (Polysciences, Warringtown, PA, USA) with 10 μg of pNL4-3 or pNL-GFP. All other retroviral vectors were pseudotyped with VSV G. To produce HIV-$1_{TRIP-CMV-GFP}$, 293T cells were co-transfected with 10 μg of $pTRIP_{CMV-GFP}$, 10 μg of pΔR8.9 and 5 μg of pMD-G. To produce MLV vectors expressing GFP, 293T cells were similarly co-transfected with 10 μg of pCNCG, 10 μg of pCIG3 N or B, and 5 μg of pMD-G. HIV-$2_{ROD-GFP}$ and $SIV_{mac-GFP}$ vectors were produced by co-transfection of 10 μg of $pROD_{Nef-GFP}$ or $pSIV_{mac-GFP}$ respectively, and 5 μg of pMD-G. $EIAV_{GFP}$ were produced by co-transfection of 10 μg of pONY3.1, 10 ug of pONY8.0 and 5 μg of pMD-G. Two days post-infection, the supernatants were collected, clarified by low speed centrifugation and stored in 1 ml aliquots at −80 °C.

Library construction

Random mutations were introduced into the PRYSPRY domain of $TRIM5\alpha_{hu}$ using the Diversify PCR Random Mutagenesis Kit (Clontech, Mountain View, CA, USA). Predefined experimental buffer conditions were tested to select the desired rate of one to two mutations per 600 bp. Buffer conditions #2 of the kit were used and consisted in an excess of dGTP combined with 160 μM of $MnSO_4$. Error-prone PCR was performed using 100 ng of pMIP-$TRIM5\alpha_{hu}$ and the following primers: $SPRY_{fwd}$, 5′-ACAGATGTCCGACGCTACTGGGTT-3′and $TRIM5_{rev}$, 5′-TCCTGAATTCTTACTTATCGTCGTCATCCTTGTAATC-3′. DNA was amplified for 20 cycles (94 °C, 15 s; 60 °C, 15 s; 68 °C, 1 min). The rest of $TRIM5\alpha_{hu}$ was independently amplified with 20 units ml^{-1} of high-fidelity Phusion enzyme from New England Biolabs (Pickering, ON, USA) in 1 × high-fidelity buffer. Each PCR reaction contained 1 ng of pMIP-$TRIM5\alpha_{hu}$, 0.2 mM of dNTP and 0.2 μM of each of the oligonucleotides $TRIM5_{fwd}$ (5′-GTTCCTCGAGATGGCTTCTGGAATCCTGGTTAAT-3′) and $SPRY_{rev}$ (5′-AACCCAGTAGCGTCGGACATCTGT-3′). PCR was carried out for 25 cycles (98 °C,

20 s; 56 °C, 20 s; 72 °C, 1 min). PCR products were cleaned of PCR reaction components by using the QIAGEN (Mississauga, ON, USA) QIAQuick PCR purification kit. Aliquots (7 µl out of 50 µl) of each purified product were then mixed together to serve as templates in a second round of PCR, which used the $TRIM5_{fwd}$ and $TRIM5_{rev}$ oligonucleotides. DNA was amplified for 25 cycles (98 °C, 20 s; 56 °C, 20 s; 72 °C, 1 min) using the Phusion enzyme. The resulting PCR fragment was gel-purified (Qiagen QIAQuick kit), digested with Xho1 and EcoR1 and ligated to pMIP cut with the same enzymes. *Escherichia coli* DH5α were transformed with the ligation products, yielding a library of 24 600 clones. A number of colonies were used to start small liquid cultures to prepare DNA for sequencing of individual clones. The remainder were harvested and mixed together in liquid Luria–Bertani (LB) medium, and an aliquot of this resuspension was used to start a 125-ml overnight culture. Plasmid DNA was purified using the Qiagen Midiprep kit and used to produce MLV-derived retroviral vectors. For this, 10 µg of the library DNA were mixed with 10 µg of pCl-Eco and 5 µg of pMD-G and this mixture was transfected using polyethylenimine in 293T cells plated at 80% confluence in a 10-cm plate. Two days later, the supernatant of this plate was collected, aliquoted and frozen at −80 °C. To express the library in human TE671 cells, cells were plated in six-well plates at 250 000 cells per well and infected in duplicates with various amounts of the mutant-library MLV vectors. Two days later, cells were treated with 2 µg ml^{-1} of puromycin. This concentration of puromycin is relatively high, allowing most of the non-transduced cells to die in <24 h. The day after puromycin was added, mortality was estimated visually, and we only maintained in culture the cells infected at a virus dose leading to no more than 10% transduced cells. These cells were pooled and cultured in 10-cm plates. For the functional selection, cells at approximately 10% confluence in 10-cm dishes were challenged three consecutive times with a preparation of $HIV\text{-}1_{TRIP\text{-}CMV\text{-}GFP}$. In all, 5 ml of the undiluted GFP-expressing vector were used per plate and per infection, and infections were allowed to proceed for 16 h each. Approximately a week later, cells were sorted by fluorescence-activated cell sorting and GFP-negative cells (typically 1% of total intact cells) were plated at 1 cell per well in 96-well plates in Dulbecco's modified Eagles's medium-based culture medium supplemented with 10% conditioned medium (0.45 µm-filtered supernatant of

confluent TE671 cells). The surviving clones were grown and challenged with HIV-$1_{TRIP-CMV-GFP}$ in 24-well plates as detailed below.

Site-directed mutagenesis

Mutations were introduced by overlap extension PCR.[73] PCRs were carried out using the high-fidelity Phusion enzyme as detailed above. The following primers were used (the modified codons are underlined):

$R332G_{fwd}$, 5′-ATATGGGGCAGGAGGGACAAGATAC-3′;
$R332G_{rev}$, 5′-CTTGTCCCTCCTGCCCCATATATTA-3′;
$R335D_{fwd}$, 5′-ACGAGGGACAGACTACCAGACATTTGT-3′;
$R335D_{rev}$, 5′-ATGTCTGGTAGTCTGTCCCTCGTGCCC-3′;
$R335E_{fwd}$, 5′-ACGAGGGACAGAATACCAGACATTTG-3′;
$R335E_{rev}$, 5′-TGTCTGGTATTCTGTCCCTCGTGCCC-3′;
$R335F_{fwd}$, 5′-ACGAGGGACATTCTACCAGACATTTGT-3′;
$R335F_{rev}$, 5′-ATGTCTGGTAGAATGTCCCTCGTGCCC-3′;
$R335G_{fwd}$, 5′-ACGAGGGACAGGATACCAGACATTT-3′;
$R335G_{rev}$, 5′-GTCTGGTATCCTGTCCCTCGTGCCC-3′;
$R335K_{fwd}$, 5′-CGAGGGACAAAATACCAGACATTTG-3′;
$R335K_{rev}$, 5′-TGTCTGGTATTTTGTCCCTCGTGCC-3′;
$R335L_{fwd}$, 5′-ACGAGGGACATTATACCAGACATTTGT-3′;
$R335L_{rev}$, 5′-ATGTCTGGTATAATGTCCCTCGTGCCC-3′;
$R332G/R335E_{fwd}$, 5′-ATATGGGGCAGGAGGGACAGAATACCAGACATTTG-3′;
$R332G/R335E_{rev}$, 5′-TGTCTGGTATTCTGTCCCTCCTGCCCCATATATTA-3′;
$R332G/R335G_{fwd}$, 5′-ATATGGGGCAGGAGGGACAGGATACCAGACATTT-3′;
$R332G/R335G_{rev}$, 5′-GTCTGGTATCCTGTCCCTCCTGCCCCATATATTA-3′;
$R335_{fwd}$, 5′-ACGAGGGACATACCAGACATTTGT-3′;
$R335_{rev}$, 5′-ATGTCTGGTATGTCCCTCGTGCCC-3′;
as well as $TRIM5_{fwd}$ and $TRIM5_{rev}$.

Generation of cells stably expressing TRIM5α variants

Retroviral vectors encoding wild-type TRIM5α_{hu}, TRIM5α_{rh} or mutants of TRIM5α_{hu} proteins were created using the pMIP vector. Recombinant viruses were produced by co-transfecting 293T cells in six-well plates with 2 μg of the appropriate pMIP construct, 2 μg of pCL-Eco and 1 μg of pMD-G. Two days later, supernatants were collected, cleared by low-speed centrifugation and used immediately. For TE671 and SUP-T1, 125 000 cells were plated per well in six-well plates and exposed to 2 ml of MIP-TRIM5α retroviral vectors or with the empty parental vector MIP as a control. Two days post-transduction, cells were placed in medium containing 0.5 μg ml^{-1} of puromycin (EMD Biosciences, Gibbstown, NJ, USA). Selection was allowed to proceed for 1 week, although untransduced control cells were killed in 2–3 days. Expression of the transduced TRIM5α was analyzed by western blotting, using antibodies directed against the FLAG epitope (mouse monoclonal; Sigma-Aldrich, St Louis, MI, USA) and actin (rabbit polyclonal; Santa Cruz, Santa Cruz, CA, USA).

Viral challenges

For single-cycle infections of TE671 and MDTF, 25 000 cells were seeded in 400 μl per well of 24-well plates. Cells were infected the next day with multiple doses of HIV-$1_{TRIP-CMV-GFP}$, MLV-N_{GFP}, MLV-B_{GFP}, $SIV_{mac-GFP}$, $EIAV_{GFP}$ or HIV-$2_{ROD-GFP}$. Two days post-infection, adherent cells were trypsinized and fixed in 1% formaldehyde-phosphate-buffered saline while SUP-T1 cells were simply treated with formaldehyde. For HIV-1_{NL-GFP} infections, SUP-T1 cells were plated at 2×10^5 cells in 400 μl per well of 24-well plates and infected with a single dose (150 μl) of virus. To avoid the initiation of a second cycle of replication, cells were fixed at 36 h. All cells were subjected to fluorescence-activated cell sorting analysis on a FC500 MPL instrument (Beckman Coulter, Mississauga, ON, USA) using 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.

For spreading assays, 1.5×10^6 parental SUP-T1 cells were seeded in 2 ml of medium per well of six-well plates. Cells were infected with 15 µl of HIV-1_{NL4-3}. 20 h post-infection, cells were harvested by centrifugation at 1200 revolutions per minute for 5 min, washed once with culture medium, and resuspended in 1.5 ml of fresh medium. ~In total, 1.0×10^5 of these infected cells were mixed with 1.0×10^6 SUP-T1 cells expressing wild-type or mutant TRIM5α_{hu} in 1 ml of medium per well of 12-well plates. Between two-thirds and three-quarters of each culture were harvested at various time points after infection, and cleared by centrifuging at 8000 revolutions per minute for 5 min in a microcentrifuge. Cultures were replenished with fresh medium while aliquots of cleared supernatant were preserved at −80 °C. After 4 weeks, reverse transcriptase activity was quantified with the EnzChek Reverse Transcriptase Assay Kit (Invitrogen, Burlington, ON, USA) according to the manufacturer's instructions and using serial dilutions of recombinant reverse transcriptase (provided by the NIH AIDS Reagents Program) as a standard. Reverse transcriptase assays were performed in black clear bottom microtiter plates (Corning, Corning, NY, USA) which were read in a Synergy HT Multi-Mode microplate reader (Bio-Tek, Winoosk, VT, USA) at an emission wavelength of 520 nm and excitation of 480 nm. Supernatants from uninfected cultures were used as blanks.

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CHAPITRE III

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

Le contenu de ce chapitre a fait l'objet d'une publication en anglais dans la revue *Virus Research,* le 25 janvier 2013.

3.1 Résumé

Les gènes antirétroviraux dérivés de l'humain sont d'une grande 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 (v_1) du facteur de restriction humain TRIM5α. Nous avons utilisé un megaprimer muté afin de créer une librairie de mutants dans la région v_1 de TRIM5α_{hu}. De plus, nous avons isolé un variant présentant une mutation à la position 330 (G330E) et qui inhibe la transduction du 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 dans la région v_1 représente l'élément clé pour la restriction du VIH-l. Le mutant G330E inhibait également la propagation du 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-l. 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 informatisé de la 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 était moins restrictif que le double mutant. Cette étude souligne donc le potentiel des stratégies de mutagénèse aléatoire à isoler des variantes de protéines humaines avec des propriétés antivirales.

3.2 Deuxième article scientifique

A novel aminoacid determinant of HIV-l restriction in the Trim5α variable 1 region isolated in a random mutagenic screen

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Abstract

Human-derived antiretroviral transgenes are of great biomedical interest and are actively pursued. HIV-l is efficiently inhibited at post-entry, pre-integration replication stages by point mutations in the variable region 1 (vI) of the human restriction factor TRIM5α. Here we use a mutated megaprimer approach to create a mutant library of TRIM5αHu vI and to isolate a mutation at Gly330 (G330E) that inhibits transduction of an HIV-l 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 vI charge toward increased acidity was key to inhibiting HIV-l. G330E TRIM5αHu also disrupted replication-competent HIV-l propagation in a human T cellline. 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 TRIM5αHu 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-I inhibitory potential of Gly330 mutants was not predicted by examination of natural TRIM5α orthologs that are known to strongly inhibit HIV-I. 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 (v_1) 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,b; Bérubé et al., 2007; Pham et al., 2010; Sebastian et al., 2006). pNL-GFP, pMD-G, pΔR8.9, pTRIPCMV-GFP and pCL-Eco have all been described elsewhere (Berthoux et al., 2003, 2004, 2005a,b; 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 pTRIPCMV-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′-GTTCCTCGAGATGGCTTCTGGAATCCTGGTTAAT) 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 s; 56°C, 30 s; 72°C, 45 s). 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 s; 56°C, 20 s; 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*Escherichia 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. Two days later, cells were treated with 2 μg/ml of puromycin for 2–3 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× 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):

K324Qfwd, GAGCTCTCCGCAACCACAGATAATA;
K324Qrev, ATCTGTGGTTGCGGAGAGCTCACTT;
I328Sfwd, ACCACAGATATCATATGGGGCACGAG;
I328Srev, TGCCCCATATGATATCTGTGGTTTCG;
Y329Cfwd, CAGATAATATGTGGGGCACGAGGGA;
Y329Crev, CCTCGTGCCCCACATATTATCTGT;

G330Efwd, ATAATATATGAGGCACGAGGGACAA;
G330Erev, CCCTCGTGCCTCATATATTATCTGT;
G333V fwd, GGGGCACGAGTGACAAGATACCAGA;
G333Vrev, GGTATCTTGTCACTCGTGCCCCAT;
Y336Cfwd, GGGACAAGATGCCAGACATTTGTGA;
Y336Crev, AAA TGTCTGGCATCTTGTCCCTCGT;
Q337Pfwd, GGACAAGATACCCGACATTTGTGAA;
Q337Prev, TTCACAAATGTCGGGTATCrTGTCC;
T338Pfwd, CAAGATACCAGCCATTTGTGAA TTTC;
T338P rev, TTCACAAA TGGCTGGTATCTTGTCC;
G330E-R332Gfwd, CAGATAATATATGAGGCAGGAGGGACAAGATACC;
G330E-R332Grev, GGTATCTTGTCCCTCCTGCCTCATATATTA;
G330E-R335Gfwd, ATAATATATGAGGCACGAGGGACAGGATACCAGAC;
G330E-R335Grev, GTCTGGTATCCTGTCCCTCGTGCCTCATATATTA;
G330E-R332G-R335Gfwd,
ATAATATATGAGGCAGGAGGGACAGGATACCAGAC;
G330E-R332G-R335Grev, 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 an 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

10,000–25,000 cells. For the propagation assay, 2×10^6 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 h 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 min at 35,000 × *g*. Nucleic acids were precipitated by adding 0.11 equivalents of 2 M $(NH_4)_2SO_4$ and the same volume of 10% polyethylenimine and then pelleted by centrifugation at 29,500 × *g* for 15 min. Proteins were recovered by adding 0.35 equivalents of saturated $(NH_4)_2SO_4$ and centrifuging at 9820 × *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 8600 × *g* for 5 min. 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 min. Lysates were centrifuged in a refrigerated microcentrifuge (~14,000 × *g*) for 5 min. Supernatants were supplemented with 1/10 volume of 10× 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 h. A portion of this mixture, referred to as "input" was stored. The mixture was spun through a 70% sucrose cushion (70% sucrose, 1× PBS and 0.5 mM DTT) at 100,000 × *g* in an SW55 rotor (Beckman) for 1 h at 4°C. After centrifugation, the supernatant was carefully removed and the pellet was resuspended in 1× 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 v_1-focused mutagenic screen

Previously published data pointed to v_1 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 323–341 of TRIM5α_{Hu} (Fig. 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 (Fig. 1B). Sequencing of the TRIM5α_{Hu} expressed from the retroviral vector showed that 3 of them contained mutations at position 332 (Fig. 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.

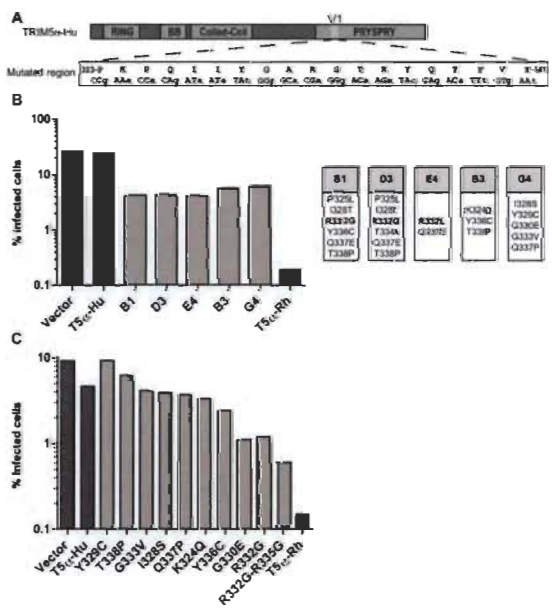

Fig. 1. Isolation of amino acid 330 of TRIM5α_{Hu} as a determinant of HIV-1 restriction. (A) A megaprimer approach to introduce mutations in the v_1 region of TRIM5α_{Hu}. The nucleotide sequence of the region targeted by the primer is shown in the box under the corresponding amino acid sequence. Partly 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 $HIV_{TRIP\text{-}CMV\text{-}GFP}$. 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 $HIV_{TRIP\text{-}CMV\text{-}GFP}$ as above. Previously characterized restrictive TRIM5α_{Hu} mutants R332G and R332G-R335G were included in the assay.

Each of the 7 substitutions found in clones B3 and G4 was individually 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 (Fig. 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 (Fig. 1C).

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 (Fig. 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.

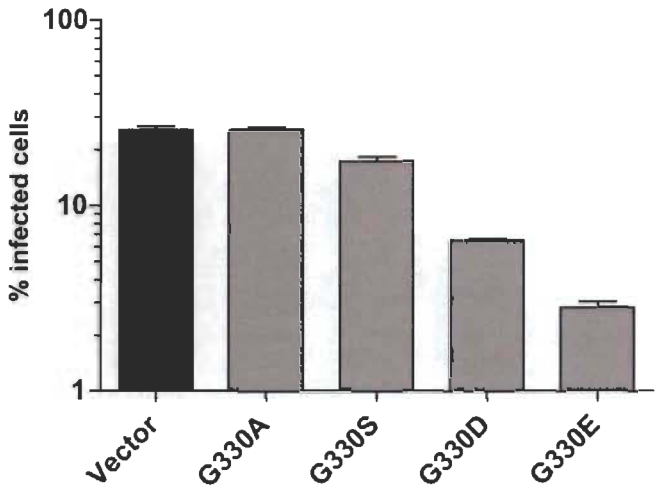

Fig. 2. Restriction of HIV-1 by TRIM5α_{Hu} mutants of Gly330. TE671 cells were transduced to express the indicated TRIM5α and then challenged with a single dose of $HIV_{TRIP\text{-}CMV\text{-}GFP}$ exactly as in Fig. 1. The percentages of infected cells were determined by FACS 2 days later. Shown are 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 Fig. 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 v_1 region.

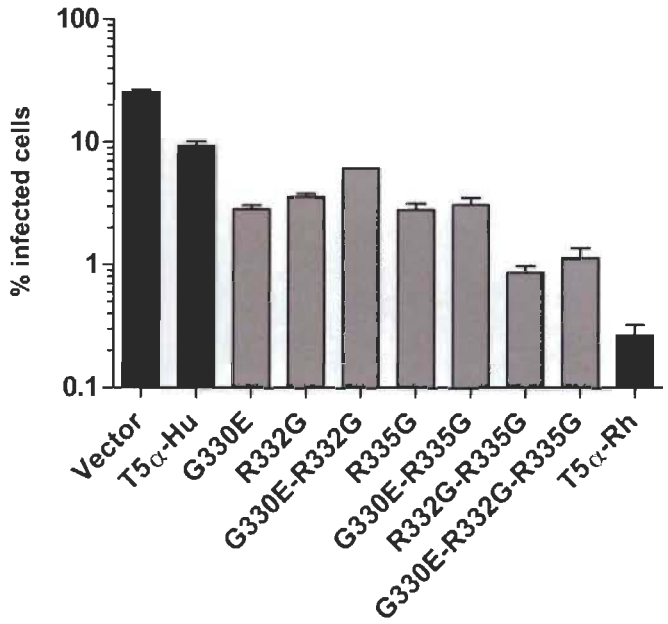

Fig. 3. Antiretroviral effect conferred by G330E when combined with R332G or R335G. The indicated single, double or triple TRIM5α_{Hu} mutants were constructed and retrovirally transduced into TE671 cells. The cell lines created were then challenged with $HIV_{TRIP\text{-}CMV\text{-}GFP}$ as before. 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 (Fig. 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.

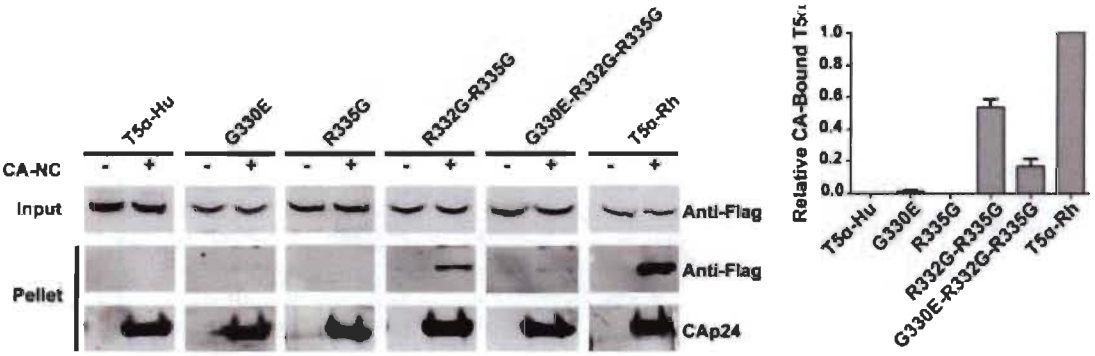

Fig. 4. In vitro TRIM5α binding to HIV-1 CA. CA–NC complexes were assembled in vitro and mixed with lysates from 293T cells transfected with the relevant TRIM5α-FLAG-expressing plasmid constructs. CA–NC complexes were separated from soluble proteins by ultracentrifugation through a sucrose cushion and 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 Fig. 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.

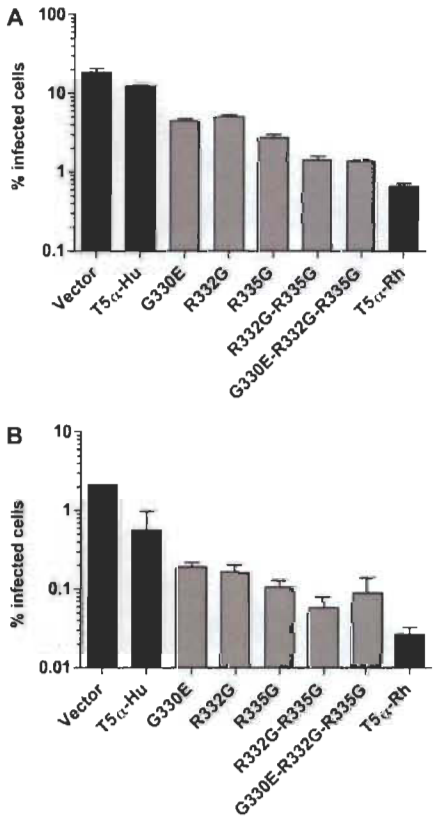

Fig. 5. Restriction of HIV-1 replication in human lymphocytic cells and in nonhuman cells. Human Sup-T1 cells (A) and feline fibroblast CRFK cells (B) were retrovi-rally transduced with the "empty" MIP vector or with MIP expressing the indicated TRIM5α. The cells were then challenged in triplicates with a single dose of HIV-$1_{NL\text{-}GFP}$. Percentages of infected (GFP-positive) cells were determined 2 days later by FACS.

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 (Fig. 5B) closely resembled what had been obtained in human TE671 and Sup-T1 cells (Fig. 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.

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, G330E or R332G–R335G TRIM5α_{Hu} with HIV-$1_{NL4\text{-}3}$ (Fig. 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 (Fig. 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.

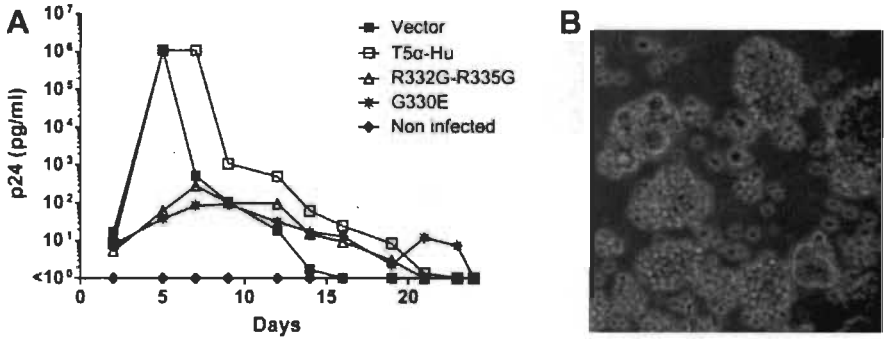

Fig. 6. HIV-1 propagation in human lymphocytic cells expressing G330E TRIM5α_{Hu}. Sup-T1 cells transduced with WT or mutated TRIM5α_{Hu} or untransduced (vector) were then infected with the replication-competent HIV-1 clone NL4-3. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 24 days. (A) CA 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.

Modeling the effect of v_1 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 (Fig. 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 (v_1, v_2, v_3). This was most apparent in the surface representation (Fig. 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.

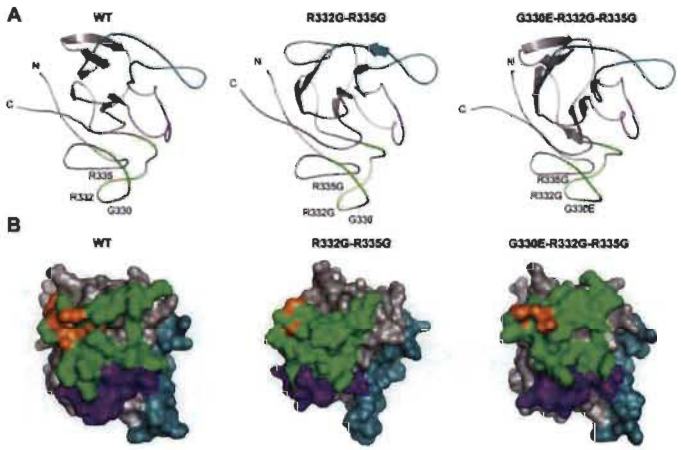

Fig. 7. Model representation of the TRIM5α_{Hu} PRYSPRY domain. The predicted impact of v_1 mutants on the structure of the PRYSPRY domain was modeled in silico based on the published structures of similar domains. (A) Ribbon model and (B) surface representation of WT, R332G–R335G and G330E-R332G–R335G TRIM5α_{Hu}. The v_1, v_2 and v_3 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 v_1 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 v_1 domain of TRIM5α_{Hu}. However, our structure prediction analysis suggests that discrete mutations in v_1 can have long-range effects on the whole CA-interaction surface of the PRYSPRY domain. This explains how single mutations in TRIM5α_{Hu} v_1 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 muta-genesis 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.

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CHAPITRE IV

DISCUSSION ET PERSPECTIVES

Bien que la thérapie antirétrovirale hautement active (HAART) a amélioré l'espérance de vie des patients atteints du VIH-1, un tel traitement est loin d'être idéal avec des effets secondaires notables, des problèmes d'adhésion aux ordonnances médicales et par conséquent l'émergence fréquente de mutants viraux résistants aux traitements. Le besoin d'une alternative efficace contre le VIH-1 a donné lieu à de grands développements dans le domaine de la recherche en thérapie génique. Dans cette optique, TRIM5α semble être un candidat idéal puisqu'il cible le VIH-1 au début de son cycle de vie, soit au point d'entrée et avant l'intégration, ce qui permet (1) de limiter les effets cytotoxiques, (2) de réduire les possibilités d'évolution mutagène pendant la transcription inverse et (3) d'empêcher l'établissement de réservoirs latents. De plus, contrairement aux autres facteurs de restriction (APOBEC, Tétherine/BST-2 et SAMHD1), le VIH n'a pas développé de protéine accessoire qui soit capable de neutraliser les effets restrictifs de TRIM5α. Bien que chez l'homme, TRIM5α a peu d'effet inhibiteur sur le VIH-1 (Speelmon *et al.*, 2006; Van Manen *et al.*, 2008; Sewram *et al.*, 2009), il a été démontré que TRIM5α humain peut être modifié pour fournir une spécificité de restriction contre le VIH-1 par un changement unique d'acide aminé en position 332 de la protéine humaine (Stremlau *et al.*, 2005; Yap *et al.*, 2005; Li *et al.*, 2006). Notre objectif consistait donc à (1) générer des mutations aléatoires dans la région PRYSPRY de la protéine TRIM5α_{hu}, (2) isoler par un crible fonctionnel d'autres mutants présentant une activité de restriction accrue contre le VIH-1, (3) caractériser l'effet de TRIM5α_{hu} muté sur diverses souches cellulaires et virales.

4.1 Le crible génétique et l'isolation de mutants résistants au VIH-1

Dans ce travail, nous avons utilisé deux cribles génétiques (par évolution moléculaire) *in vitro* pour identifier et caractériser de nouveaux mutants de TRIM5α

ayant une activité de restriction accrue contre le VIH-1. Ces expériences nous ont permis d'identifier deux nouvelles mutations ponctuelles (G330E et R335G) dans la région v_1 de TRIM5α humain diminuant l'infection du VIH-1 par un facteur de 10, semblable à ce qui a déjà été réalisé par la mutation ponctuelle Arg332 dans cette région. De plus, bien qu'une mutation ponctuelle au niveau de Gly330, Arg332 ou Arg335 permette d'augmenter l'activité de restriction de $TRIM5\alpha_{hu}$, elle ne suffit pas à restreindre efficacement la réplication du VIH-1. Seule la double mutation en positions 332 et 335 décrite dans cette étude semble conférer une résistance accrue et similaire à son homologue TRIM5α du singe rhésus, l'un des facteurs de restriction le plus efficace contre le VIH-1 décrit à ce jour. Étonnamment, alors que les mutations R332G et R335G ont des effets additifs sur la restriction, l'addition de G330E à R332G et/ou R335G semble diminuer légèrement l'activité de restriction contre le VIH-1 par rapport au double mutant R332G/R335G.

Le double mutant R332G-R335G de $TRIM5\alpha_{hu}$ est significativement plus efficace que le simple mutant R332G à inhiber l'infection par le VIH-1 infectieux et à augmenter la survie des cellules génétiquement modifiées (Pham *et al.*, 2010; Pham *et al.*, 2013; Veillette *et al.*, 2013, Jung *et al.*, 2015). La possibilité d'avoir un transgène encore plus actif nous a poussés à réaliser un crible par mutagénèse aléatoire dans la région variable 1 du domaine de reconnaissance de la capside virale de $TRIM5\alpha_{hu}$. Ceci nous a permis d'identifier une autre mutation, G330E, qui permet de restreindre le VIH-l. 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 (Pham *et al.*, 2013; Veillette *et al.*, 2013). De plus, nos données montrent également que dans les lymphocytes, R332G-R335G restreint le VIH-1 à des niveaux équivalents à $TRIM5\alpha_{rh}$. Les deux mutations étant situées près l'une de l'autre, il devrait être possible d'introduire simultanément les deux mutations dans le génome humain, par l'utilisation de méthodes récentes d'édition génomique telles que celles basées sur la technologie de CRISPR-cas9 (Hsu et autres 2014; Yin et autres 2014).

4.2 Surexpression de TRIM5α

4.2.1 L'effet dominant négatif

À l'état naturel, l'activité de restriction de TRIM5α est spécifique à chaque espèce de primate, ainsi aucun variant de la protéine ne semble conférer une réelle résistance vis-à-vis des rétrovirus isolés chez la même espèce, évoquant l'implication de ces facteurs de restriction comme une barrière potentielle de la transmission inter-espèce. Par exemple, TRIM5α_{hu} endogène a une activité de restriction contre MLV-N mais pas contre le VIH-1 (Johnson *et al.*, 2009). Il serait donc prudent, dans notre effort d'augmenter l'activité de restriction de TRIM5α_{hu} contre le VIH-1, de ne pas affaiblir notre barrière immunitaire naturelle en la rendant susceptible à d'autres infections. Or, il a été démontré que l'expression hétérologue de TRIM5α_{rh} ou de TRYMcyp dans des cellules humaines pouvait interférer avec l'activité anti-MLV-N de TRIM5α_{hu} endogène (Berthoux *et al.*, 2005). Cet effet a également été retrouvé chez notre double mutant R332G/R335E, mais non pas chez notre double mutant R332G/R335G. De fait, alors qu'on observe une légère diminution de la restriction de SIVmac et de EIAV par le mutant R332G/R335E, la diminution de la restriction est totalement absente pour le mutant R332G/R335G. Encore plus étonnant, à l'exception de MLV-B, le mutant R332G/R335G semblerait augmenter de manière significative l'activité de restriction sur l'ensemble des rétrovirus testés soit en diminuant l'infection par un facteur de 5 à 10 pour EIAV, 10 à 20 pour SIVmac et supérieur à 20 pour VIH-1, VIH-2 et MLV-N.

4.2.2 L'activation incontrôlée du système immunitaire

L'activation incontrôlée du système immunitaire par la surexpression de TRIM5α représente potentiellement un autre obstacle majeur au développement de mutants TRIM5α_{hu} comme thérapie anti-VIH-1. De fait, il a été démontré que la transfection de TRIM5α pouvait activer NF-ƙB et AP-1 par l'activation de TAK1 et la génération de chaînes d'ubiquitine liés à K63 (Nepveu-Traversy *et al.*, 2014; Pertel *et al.*, 2011; Tareen *et al.*, 2011a; Uchil *et al.*, 2013). Cependant, des expériences récentes effectuées dans nos laboratoires démontrent que lorsque des mutants de TRIM5α sont surexprimés

par transfection dans des lymphocytes, il y a bien une activation de NF-ĸB. Toutefois, lorsque TRIM5α mutant est exprimée dans les lymphocytes par une transduction stable avec des vecteurs rétroviraux, il n'y a pas d'activation ou de modulation de NF-ĸB ou de TAK1 (Jung *et al.*, 2015).

4.2.3 La résistance virale : le cas de CA-V86M et de CTL

Des mutations de la CA virale au niveau de la Val86 et à d'autres positions confèrent un certain degré de résistance à TRIM5α_{rh} (Pacheco *et al.*, 2010; Soll *et al.*, 2013; Veillette *et al.*, 2013). Ces mutations affectent directement la boucle de liaison CypA-CA (V86A et M96I), qui est connue pour jouer un rôle important dans la restriction médiée par TRIM5α (Berthoux *et al.*, 2004; 2005b; Keckesova *et al.*, 2006; Stremlau *et al.*, 2006b). Ces résultats soulèvent la possibilité que certaines souches de VIH-1 très divergentes du sous-groupe B, grandement utilisées dans la majorité des études précliniques sur le VIH-1, puissent échapper à la restriction par nos mutants de TRIM5α_{hu}. Bien que le mutant CA-V86M n'ait pas été l'objet de mes études, des expériences publiées récemment dans nos laboratoires semblent démontrer que la mutation V86M du VIH-1 peut conférer une résistance partielle contre la restriction rétrovirale par TRIM5α_{rh} et nos mutants de TRIM5α_{hu}. V86M altèrerait les mécanismes d'interaction entre la CypA et la capside virale, résultant en une augmentation du transport nucléaire de l'ADN du VIH dans des conditions restrictives. Cependant, il est important de noter qu'aucune augmentation de la résistance virale n'a été observée pour nos mutants R322G/R335G lorsque testée sur des virus infectieux (plusieurs cycles d'infection) (Veillette *et al.*, 2013).

Pour comprendre la disparité entre l'effet de V86M sur la restriction des souches réplicatives et non-réplicatives, il est important de noter que la région de la boucle de liaison à CypA de la CA semble être importante pour diverses fonctions virales. Il a été récemment démontré que CypA avait en autre un rôle dans la protection du VIH-1 contre la détection par les capteurs d'ADN cGAS du système immunitaire inné de la cellule (Rasaiyaah *et al.*, 2013). Dans cette expérience, alors que la mutation P90A de la

capside virale procure une résistance à TRIM5α, elle entraîne une augmentation de la sensibilité à cGAS qui conduit à la production de seconds messagers et d'IFN de type I, supprimant ultimement la réplication du VIH-1. Ainsi, les mutations dans la boucle d'interaction CypA/CA, permettant au virus d'échapper à la restriction par TRIM5α conduisent souvent à une diminution de la capacité d'infection et de réplication du virus (Rasaiyaah *et al.*, 2013).

Autre fait intéressant, une étude sur les mutations de la capside virale qui permettent au VIH d'échapper aux lymphocytes T cytotoxiques (CTL) a montré que certaines de ces mutations pouvaient augmenter la susceptibilité du virus à TRIM5α_{hu} (Battivelli *et al.*, 2010 et 2011). Des résultats récents démontrent que cet effet peut également s'appliquer à une variante de TRIM5α humaine plus restrictive comme R332G-R335G (Jung *et al.*, 2015). Ces résultats suggèrent qu'un TRIM5α hautement restrictif utilisé dans des applications de thérapie génique pourrait aider à contrôler l'apparition de virus mutant en rendant plus difficile pour le virus d'échapper à la fois aux réponses immunitaires de l'hôte et à l'inhibition médiée par TRIM5α.

Dans l'étude de Jung *et al.*, l'activité de restriction du mutant R332G-R335G avait également été testée contre un vaste éventail de souches dérivées d'isolats cliniques avec des représentants de plusieurs groupes et sous-groupes du VIH, y compris le groupe O. 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 (Jung *et al.*, 2015, en annexe). De plus, l'expression de R332G-R335G semblerait protéger les cellules contre les effets cytopathiques du VIH-1 et conduit à la sélection positive des cellules transduites *in vitro*. En effet, dans l'expérience de *Jung et al* (voir annexe), un mélange de cellules exprimant de manière stable différentes TRIM5α restrictives et non-restrictives a été mixé avec des cellules parentales dans un rapport approximatif de 1:99. Afin de permettre la quantification par cytométrie de flux, les cellules transduites exprimaient également la GFP. Les différentes co-cultures ont ensuite été infectées avec une dose unique de NL4-3 (Adachi et autres 1986). Au départ, la proportion de cellules

GFP-positives était proche de 1 % dans toutes les cultures. Toutefois, plus d'un mois après le début de l'expérience, dans les contrôles non-restrictifs, à savoir les cellules transduites avec TRIM5α_{hu} naïve ou avec le vecteur « vide » pMIG, il était à peine possible de détecter des cellules exprimant la GFP, montrant un manque total d'avantage de survie des cellules transduites. Dans tous les autres cas, la proportion de cellules exprimant la GFP a augmenté de façon significative, allant de ~ 20 % des cellules exprimant TRIM5α_{hu} (R332G) à près de 90 % des cellules exprimant TRIM5α_{Rh}. De plus, le pourcentage de cellules positives pour la GFP était proportionnel au niveau de protection conféré par les variantes de TRIM5α respectives: TRIM5α_{Rh}> R332G-R335G TRIM5α_{hu}> R332G TRIM5α_{hu} (Pham *et al.*, 2010; Pham *et al.*, 2013; Veillette *et al.*, 2013). Cette expérience suggère que le double mutant R332G-R335G de TRIM5α_{hu} peut conférer une protection non seulement contre le VIH-1 infectieux, mais également contre les effets cytopathiques de la propagation du virus actif provenant des cellules non modifiées (TRIM5α_{hu} naïve) (Pham *et al.*, 2010; Pham *et al.*, 2013; Veillette *et al.*, 2013, Jung *et al.*, 2015, en annexe).

4.2.4 Mutation dans la région V_1 de TRIM5α

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 et que l'interaction PRYSPRY-CA est dominée par une région v_1 mobile (Biris *et al.*, 2012). Or, l'Arg332 et Arg335 font partie de cette boucle hyper flexible v_1 et le retrait de la charge positive semble augmenter la capacité de TRIM5α_{hu} de se lier à la capside du VIH-1. Toutefois, l'activité de restriction du double mutant R332G/R335G étant supérieure à R332G/R335D et R332G/R335E, le retrait de la charge positive n'est donc pas le seul déterminant de l'interaction TRIM5α/CA.

4.2.5 Flexibilité conformationnelle de la glycine

En 1997, les structures de 23 enzymes différentes avaient été analysées pour déterminer les propriétés des acides aminés impliqués dans les régions du site actif.

Cette analyse avait démontré que les sites d'interaction enzyme/ligand étaient particulièrement abondants en résidus Gly. La chaîne latérale de la glycine ne contient qu'un atome d'hydrogène et c'est cette caractéristique qui la rend plus flexible que les autres acides aminés. En permettant de modifier légèrement la conformation de la protéine, la glycine permet d'augmenter l'affinité enzyme/ligands tout en conservant sa stabilité structurale (Tsou *et al.*, 1997, Yan *et al.*, 1993). En outre, l'expérience de résonance magnétique nucléaire suggère que l'interaction PRYSPRY-CA est dominée par la boucle v_1 hyper flexible, permettant au domaine PRYSPRY de s'adapter aux diverses conformations du coeur viral (Biris *et al.*, 2012). Hypothétiquement, cette flexibilité conformationnelle de la glycine combinée à la mobilité de la boucle v_1 pourrait également être impliquée dans le potentiel qu'a notre mutant R332G/R335G de (1) restreindre un large spectre de rétrovirus, (2) de s'adapter aux différentes souches du VIH-1 ou même (3) de prévenir l'apparition de virus résistants. L'importance d'une telle flexibilité est d'ailleurs observée dans d'autres composantes du système immunitaire tel que les IgM, leur permettant de réagir à différents antigènes (Biris *et al.*, 2012; Notkins *et al.*, 2004). Toutefois, cette hypothèse reste à être confirmée, car nous avons vu que d'autres mutations qu'une glycine à ces positions causaient également de la restriction (Tableau 2.1). Il faudrait donc tester différentes combinaisons d'acides aminés en position 332/335 et comparer leur activité de restriction avec notre mutant R332G/R335G.

4.3 Perspectives

4.3.1 Poursuivre le crible génétique

La réussite de ces deux cribles montre bien le potentiel qu'ont ces approches d'identifier de nouveaux transgènes humains à caractère antiviral. Bien que les mutations dans les régions Gly330, Arg332 et Arg335 soient des facteurs déterminants dans la spécificité de la restriction, il n'est pas exclu que d'autres mutations dans la région v_1 ou d'autres régions variables du domaine PRYSPRY puissent conduire à la restriction du VIH-l par TRIM5α_{hu}. Parallèlement, il est possible que d'autres mutations qui, une fois

combinées à R332G-R335G, puissent augmenter ou optimiser l'activité de nos transgènes. Un crible par mutation génétique infecté à partir de nos mutants R332G-R335G et infecté avec une dose plus importante de VIH-1 pourrait nous aider à identifier ces candidats potentiels.

4.3.2 L'implication des mutations sur la structure de PRYSPRY

Une meilleure compréhension des transformations structurales induites par la présence de mutations au niveau du domaine PRYSPRY de TRIM5α permettrait d'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.

De plus, il serait intéressant de vérifier si la mutation V86M de la capside virale procure une résistance partielle à TRIM5α en favorisant l'import nucléaire du complexe PIC (Veillette *et al., 2013;* Ambrose *et al.*, 2012; Schaller *et al.*, 2011) ou au contraire, si cette mutation entraîne une augmentation de la sensibilité de la capside virale à cGAS qui conduit ultimement à la suppression de la réplication du VIH-1 par la voie des interférons (Rasaiyaah *et al.*, 2013). Ces expériences permettraient d'expliquer la disparité des résultats entre l'effet de V86M sur la restriction des souches virales réplicatives et non-réplicatives.

4.3.3 La thérapie génique exploitant le mutant R332G/R335G de TRIM5α

La possibilité future d'une thérapie génique contre le VIH chez l'homme repose sur l'existence d'un modèle animal sur lequel des tests cliniques pourront être effectués afin de (1) mieux comprendre les interactions entre l'expression des transgènes de TRIM5α et le système immunitaire de l'hôte et (2) évaluer la faisabilité, la sécurité et l'efficacité de nos mutants de TRIM5α sur le VIH-1 réplicatif dans un contexte *in-vivo*. Or, des expériences effectuées sur des souris humanisées, ont démontré que l'expression de TRYMCyp dans des lymphocytes T CD4+ ou l'expression de chimères de TRIM5α-

humain/Rhésus dans des cellules souches hématopoïétiques CD34+ avaient un effet protecteur contre le VIH-1 et que les cellules greffées avaient un avantage de survie par rapport aux cellules naïves (Neagu *et al.*, 2009; Walker *et al.*, 2012). Il a également été suggéré que les patients soient traités préalablement par une trithérapie (HAART) accompagnée d'une chimiothérapie, ce qui permettrait de diminuer les réservoirs latents et de favoriser la survie des cellules souches exprimant le transgène. Dans ce contexte, notre mutant R332G/R335G de TRIM5α présente certains avantages par rapport à TRYMCyp et à la chimère de TRIM5α-humain/Rhésus par (1) sa capacité à restreindre un large spectre de rétrovirus, (2) par l'absence d'effet dominant négatif et (3) probablement par un effet immunogène moindre. De plus, notre double mutant TRIM5α_{hu} R332G-R335G est aussi puissant que TRIM5α_{rh} dans les lymphocytes (Pham *et al.*, 2010; Pham *et al.*, 2013; Veillette *et al.*, 2013; Jung *et al.*, 2015).

D'autres chercheurs travaillent également sur la construction d'un vecteur rétroviral dérivé du VIH-1 qui pourrait infecter les singes macaques permettant ainsi d'avoir un modèle animal plus proche de l'homme. Récemment, une équipe du Japon aurait réussi à construire un vecteur rétroviral à partir du VIH-1 (mt-VIH-1) résistant à la majorité des facteurs de restriction naturellement retrouvés chez le singe (APOBEC3G, TRIM5α_{rh}, TRIMCyp et tétherine/BST-2) (Nomaguchi *et al.*, 2013). Cette résistance accrue *in vitro* contre TRIM5α_{rh} est due à trois mutations (M94L/R98S/G114Q) dans la région codante de la capside virale du VIH-1 et correspond à celles retrouvées chez son homologue simien SIVmac239. Bien que les vecteurs rétroviraux similaires à celui de l'équipe Nomaguchi aient été créés pour tester des vaccins contre le VIH-1 chez le singe, il n'est pas exclu que l'existence d'un vecteur rétroviral à la fois résistant à APOBEC3G, tétherine/BST-2, TRIM5α_{rh} et TRIMCyp puisse servir à l'étude de nos mutants de TRIM5α_{hu}. En effet, dans nos résultats préliminaires, il semblerait que la double mutation R332G/R335G augmente non seulement la restriction du VIH-1, mais aussi du VIH-2 et de SIVmac. Spéculativement, il est donc possible que notre double mutant ait une activité de restriction accrue contre le vecteur rétroviral mt-VIH-1 par rapport à TRIM5α_{hu} naïve, TRIM5α_{rh} et TRIMCyp. Toutefois, l'intérêt et l'utilité d'une telle étude demeurent spéculative et prématurée puisque l'expérience de Nomugachi

comporte plusieurs lacunes. En effet, même si le clone mt-VIH-1 augmente la résistance du virus aux facteurs de restriction *in vitro*, son niveau d'infection demeure inférieur à celui de SIVmac239 et dans un contexte *in vivo* cet écart peut être suffisant pour que le système immunitaire de l'hôte intervienne et inhibe la réplication rétrovirale. De plus, mt-VIH-1 interagit avec le corécepteur CXCR-4 alors que dans la majorité des cas cliniques, les virus étudiés interagissent avec le corécepteur CCR5. La réponse immunitaire peut également varier selon que l'hôte est d'origine humaine ou simienne. Finalement, la diminution de l'infectivité par notre mutant R332G/R335G de TRIM5α_{hu} sur SIVmac par un facteur entre 10 à 20 n'est probablement pas suffisante pour inhiber un virus SIVmac réplicatif. Toutefois, la possibilité d'avoir un modèle simien pour l'étude *in vivo* de la restriction du VIH-1 par nos transgènes demeure une avenue à prendre en considération.

4.4 Conclusion

Nous avons démontré le potentiel qu'a la mutagénèse aléatoire d'identifier de nouveaux transgènes à activité anti-rétrovirale. TRIM5α agit au début de son cycle de vie, soit au point d'entrée et avant l'intégration permettant ainsi de limiter les effets cytotoxiques, de réduire les possibilités d'évolution mutagène pendant la transcription inverse et d'empêcher l'établissement de réservoirs latents. De plus, contrairement aux autres facteurs de restriction, le VIH n'a pas développé de protéine accessoire qui soit capable de neutraliser les effets restrictifs de TRIM5α. L'effet anti-rétroviral de notre mutant TRIM5α_{hu} semblerait agir par le même mécanisme que TRIM5α du singe rhésus ou de la chimère humaine TRIMcyp. Comme TRIM5α_{rh} et TRIMcyp, TRIM5α_{hu} présente une forte activité de restriction contre le VIH-1. Par contre, la version humaine semble présenter un grand avantage dans le contexte d'une thérapie génique par l'absence d'effet dominant négatif et est potentiellement moins immunogène *in vivo* par rapport à ses homologues simiens. Somme toute, l'ensemble de ces travaux ouvre 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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ANNEXE

PRE-CLINICAL ASSESSMENT OF MUTANT HUMAN TRIM5α AS AN ANTI-HIV-1 TRANSGENE

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Short title: Human TRIM5α mutants as anti-HIV-1 transgenes.

Abstract

Current HIV-1 gene therapy aims at stopping the viral life cycle at its earliest steps, such as entry or immediate post-entry events. Among the most widely adopted strategies are CCR5 down-regulation/knockout or the use of broadly neutralizing antibodies, but long-term efficacy and side effects are still unclear. TRIM5α is an interferon-stimulated restriction factor that can intercept incoming retroviruses within one hour of cytosolic entry and potently inhibits infectivity of restriction-sensitive viruses. The human TRIM5α ($TRIM5\alpha_{hu}$) does not target HIV-1, but point mutations in its capsid-binding domain can confer anti-HIV-1 activity. Although the mechanisms by which $TRIM5\alpha_{hu}$ mutants inhibits HIV-1 are relatively well understood, their properties as potential transgenes for gene therapy have been incompletely characterized. Reports of general immune activation by TRIM5α over-expression have also prevented its broad use as transgene for gene therapy. Here we demonstrate the ability of the R332G-R335G $TRIM5\alpha_{hu}$ mutant to efficiently restrict highly divergent HIV-1 strains, including Group O, as well as clinical isolates bearing cytotoxic T lymphocyte (CTL) escape mutations. R332G-R335G $TRIM5\alpha_{hu}$ efficiently protected human lymphocytes against HIV-1 infection even when expressed at relatively low levels from predominantly single integration events following lentiviral transduction. Most importantly, under these conditions Rhesus macaque TRIM5α ($TRIM5\alpha_{Rh}$) and $TRIM5\alpha_{hu}$ (wild-type or mutated) had no major effect on the NF-κB pathway. Transgenic TRIM5α did not modulate the kinetics of IκBα, JunB and TNFAIP3 expression following TNF-α treatment. Finally, we show that human lymphocytes expressing R332G-R335G $TRIM5\alpha_{hu}$ have a clear survival advantage over unmodified parental cells in presence of pathogenic, replication-competent HIV-1. These results underline the relevance of R332G-R335G and other mutants of $TRIM5\alpha_{hu}$ as candidate effectors for HIV-1 gene therapy.

Introduction

Recent advances in gene therapy technologies have renewed the interest in developing approaches aimed at inhibiting HIV-1 infection. New clinical studies were

started in the past few years, some aiming at impeding viral entry by disrupting expression of the co-receptor CCR5 via RNA interference, ribozymes or by direct knockout of the CCR5 locus (Chung and others 2011; Holt and others 2010; Tebas and others 2014). Instead of down-regulating expression of a necessary cellular co-factor for HIV-1 replication, an alternative approach consists of transducing cells with specialized innate immunity effectors that are natural inhibitors of HIV-1 replication. One such candidate is the retroviral restriction factor TRIM5α, which acts in the immediate post-entry, pre-integration window (Owens and others 2003; Stremlau and others 2004). TRIM5α and the related TRIMCyp (TRIM5-CypA) target the N-terminal domain of viral capsid proteins (CA-NTD) that form the outer surface of the viral core (Cowan and others 2002; Forshey and others 2005; Ikeda and others 2004; Owens and others 2003; Perez-Caballero and others 2005; Shi and Aiken 2006; Stremlau and others 2006a). This interaction leads to several blocks to the progression of the virus life cycle (Anderson and others 2006; Berthoux and others 2004; Black and Aiken 2010; Campbell and others 2008; Diaz-Griffero and others 2006; Perron and others 2007; Rold and Aiken 2008; Stremlau and others 2006a; Zhao and others 2011) while also promoting innate immunity signaling (Pertel and others 2011; Tareen and Emerman 2011b). However, the range of viruses restricted by TRIM5α varies greatly in a species-specific way. For example, the human ortholog of TRIM5α (TRIM5α_{hu}) only moderately restricts HIV-1 (< 2-fold) while its Rhesus monkey counterpart (TRIM5α_{Rh}) is highly active against HIV-1 (50 to 100-fold) (Pham and others 2010; Stremlau and others 2004; Yap and others 2004). Studies have shown that overexpression of TRIM5α_{Rh} in human cells is dominant over the endogenously expressed protein and leads to a potent block to HIV-1 replication (Berthoux and others 2005a; Pham and others 2010; Stremlau and others 2004), a phenotype also seen in hematopoietic progenitor cells (Anderson and Akkina 2005).

Replacing regions within the CA-targeting domain, called PRYSPRY, of TRIM5α_{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 and others 2011). Modeling studies and genetic

screens have also led to the identification of point mutations in the variable region 1 (v1) of the TRIM5α_{hu} PRYSPRY domain that allow it to target HIV-1 for restriction (Li and others 2006; Pham and others 2010; Pham and others 2013; Yap and others 2005). We previously described the R332G-R335G TRIM5α_{hu} mutant as especially efficient at restricting HIV-1 (Pham and others 2010; Pham and others 2013; Veillette and others 2013). Because these mutants differ only slightly from the endogenous form of TRIM5α_{hu}, they are not expected to be immunogenic, thus making them strong candidates for gene therapy applications.

It is well-established that escape mutations often lead to a fitness cost on viral replication (Friedrich and others 2004; Liu and others 2007; Martinez-Picado and others 2006; Troyer and others 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 TRIM5α_{hu} (Battivelli and others 2011). Conversely, CA mutations of Val86 and at other positions that confer some degree of resistance to TRIM5α_{Rh} or R332G-R335G TRIM5α_{hu} have recently been identified (Pacheco and others 2010; Soll and others 2013; Veillette and others 2013). These results raise the possibility that some HIV-1 strains highly divergent from the clade B strains used in the vast majority of HIV-1 pre-clinical studies, or isolates bearing CTL-escape mutations, might be poorly sensitive to restriction by TRIM5α_{hu} mutants. Another common caveat in TRIM5α pre-clinical studies is that it is usually expressed from very strong promoters and following retroviral or lentiviral vector-mediated transduction with little control over the number of integration events per cell. Finally, uncontrolled NFκB/AP-1 activation that can result from TRIM5α over-expression (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011; Tareen and Emerman 2011a) may disqualify TRIM5α as an anti-HIV transgene. Here we demonstrate the antiviral potential of the R332G-R335G mutant of TRIM5α_{hu} against highly divergent viral strains as well as CTL-escape capsid mutants. We show that this mutant efficiently inhibits HIV-1 propagation in human lymphocytes following lentiviral transduction of R332G-R335G TRIM5α_{hu} at a low multiplicity-of-infection and using a relatively weak promoter. Importantly, we show that unlike TRIM5α transfection, stable

oncoretroviral vector or lentiviral vector-mediated transduction of R332G-R335G TRIM5α_{hu} does not activate innate immune pathways. Finally, T lymphocytes stably expressing R332G-R335G TRIM5α_{hu} possess a survival advantage in presence of HIV-1 even when the modified cells are present in relatively low numbers.

Materials and Methods

Plasmid DNAs

pMIP-TRIM5α_{hu} and pMIP-TRIM5α_{Rh} are oncoretroviral vectors that express C-terminal FLAG-tagged versions of the WT or mutant proteins along with puromycin as a selection marker and have been described before (Berthoux and others 2005a; Sebastian and others 2006). The versions of these vectors expressing GFP in place of puromycin were constructed by transferring the TRIM5α sequence into pMIG (Van Parijs and others 1999) using Xho1 and EcoR1. pNL4-3 encodes a fully replication-competent clone of HIV-1 (Adachi and others 1986). pCMV-Δ8.91 -based *gag-pol* expressing vectors were described elsewhere and are a kind gift of Greg Towers and Yasuhiro Ikeda (Ikeda and others 2004). pNL4-3XCS-based 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* are a kind gift of Allan Hance (Battivelli and others 2010). pMD-G and pTRIP$_{CMV-GFP}$ have been described elsewhere (Zennou and others 2000; Zufferey and others 1997).

To produce lentiviral vectors expressing TRIM5α, we first digested pHIV7 (Li and others 2003) with BamHI and DraII and inserted a multiple cloning site generated by PCR using pHIV7 as the template and the following ODNs: HIV_reinsert_fw, 5'-CGCGACTCTAGATCATGGATCCTCCGGACTGCACTCTAGATAGGTCACCACCGTCGACTAGCCGTACCT, and HIV_reinsert_rev, 5'-ACTATAGGGCGAATTGGGTACC. The plasmid generated, called pHIV7-empty, was digested with Kpn2I and Eco91I and a PCR fragment containing the CMV-

intron(premiR30)-GFP-partial polyA cassette from pSM30-GFP (Du and others 2006), generated with primers HIV8miR30_fw (5'-TATTCGCACTGGATACGATCCGGATGATTCTGTGGATAACCGT) and HIV8miR30_rev (5'-AATATCCTCCTTAGTTCCGGTGACCTAGAATGCAGTGAAAAAAATG), was ligated into the backbone to generate pHIV8-C-A. A codon-optimized 31 amino acid long 2A sequence based on Donnelly *et al.* (Donnelly and others 2001) (now referred to as 2A31D) was inserted between the intron and EGFP coding sequences; 2A31D contains an AgeI site, start codon, flag tag and two inverted BsmBI sites to allow flush attachment of an upstream protein to the 2A31D sequence without codon frame change. This was achieved by ligation of overlapping ODNs and subsequent PCR amplification yielding a double stranded fragment of the following sequence: 5'-ATCCACCGGTCGCCACCatgGACTACAAGGACGACGATGAGCAGGAGACGGAAGCCACAGACGTCTCCGTGACCGAGCTGCTGTACCGCATGAAGCGCGCCGAGACCTACTGCCCCCGCCCCCTGCTGGCCATCCACCCCACCGAGGCCCGCCACAAGCAGAAGATCGTGGCCCCCGTGAAGCAGACCCTGAACTTCGACCTGCTGAAGCTGGCCGGCGACGTGGAGAGCAACCCCGGCCCCGTGAGCAAGGGCGAGGAGCTGTTCACCGGG. This fragment was then used in a fusion PCR reaction using the reverse primer ODN 2A-EGFP_rev (5'-AAAGTCCCGGACGTAGCCTTCGGGCATGGCGG). The resulting DNA fragment was then digested with AgeI and PfoI and cloned into pHIV8-C-A to generate pHIV8-C-2A31D-A. Subsequently, the CMV promoter driving transgene expression was replaced with the short human EF1α promoter (EFS promoter) with an added MCS region by fusion PCR. For this, we first generated a long reverse primer by fusion-PCR, using pHIV8-C-A as a template and the primer ODNs EFS_brdg_(fw 5'-GCCGCCAGAACACAGGTGCATGCTTAATTAAATTTAAATTTAATACTAGAAGCTTTATTGCGGTAGTTTATCAC) and Intron_rev (5'-TTGCTCACCATGGTGGC). This fragment was used as the reverse primer in a fusion-PCR reaction using pLV-tTRKRAB (Wiznerowicz and Trono 2003) as a template and EFS_fw (5'-atatatTCCGGAGGCTCCGGTGCCCGT) as the upstream primer. The resulting fragment and pHIV8-C-2A31D-A were digested with Kpn2I and NheI and ligated to

generate pHIV8-ES-2A31D-A. Afterwards, the woodchuck hepatitis regulatory element (WPRE) was introduced into the 3' UTR by PCR, using pHIV7-GFP (Li and others 2003) as a template and primers wpreO_fw (5'-ATATCGGCCGCATCAAGCTGGGCTGCAGG) and wpreO_rev (5'-TATAGGTGACCATCGATGCGGGGAGGC). The PCR product was digested with BsteII and EagI and ligated to pHIV8-ES-2A31D-A cut with the same enzymes to generate pHIV8-ES-2A31D-W.

TRIM5α variants were amplified by PCR from the corresponding pMIP constructs, using the primer ODNs 2A-TRIM5a (5'-ATCCACCGGTCGCCACCATGGCTTCTGGAATCCTGG) and 2A-hT5-fus_rev (5'-gcatatTCGGTCACGGAGACGTCTGTGGCTTCCGTCTCTTCACAGAGCTTGGTGAGCACAGAGT) for human variants and 2A-rT5-fus_rev (5'-gcatatTCGGTCACGGAGACGTCTGTGGCTTCCGTCTCTTCACAAAGCTGGGTGAGCACAGAGT) for the rhesus variant. This PCR step also removed the Start codon-FLAG sequence present upstream of TRIM5α in the pMIP constructs. The PCR products were digested with AgeI and BsmBI and cloned into pHIV8-ES-2A31D-W cut with the same enzymes. The resulting constructs, named pHIV8-ES-T5a(x)-2A31D-W, were used as transgene lentiviral vector plasmids.

Cell lines

Human rhabdomyosarcoma TE671 cells, human embryonic kidney (HEK) 293T cells and Crandell-Rees feline kidney (CRFK) cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Thermo Scientific, Logan, UT, USA) at 37 °C in 5% CO_2. Human T cell lines Sup-T1 and CEM.NKR-CCR5 (Trkola and others 1999) were maintained at a density between 5×10^5 and 2×10^6 cells per ml in RPMI (Corning, Manassas, VA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

Generation of stably transduced cells

TE671 and Sup-T1 cells stably expressing TRIM5α and puromycin N-acetyl-transferase were produced by using pMIP-based retroviral vectors and then maintained in puromycin as described extensively before (Bérubé and others 2007; Veillette and others 2013). To generate CEM.NKR-CCR5 cells stably transduced with lentiviral vectors expressing TRIM5α, we produced vector particles as previously described (Li and others 2003) with the following adjustments. 15 ml of medium containing 20-25million HEK293T cells were seeded in 150 mm plates. 18 hours later, the medium was replaced and cells were then transfected with 35 μg of pCMV-G, 25 μg of pCMV-rev, 25 μg of pCHGP and 35 μg of the pHIV8-ES-T5a(x)-2A31D-W construct using 374.4 μl of $CaCl_2$ in 3 ml total volume. 25 hours post seeding, the medium was replaced and virus particles collected 24 hours later and purified by ultracentrifugation. CEM.NKR-CCR5 cells were transduced at a density of 10^5 cells per ml in presence of 8 μg/ml of polybrene (EMD Millipore, Billerica, MA), using an MOI of 0.5 as determined by titering the concentrated vector on HEK293T cells. GFP-positive cells were sorted by flow cytometry 25 days later, and *gag-pol* PCR confirmed that cells did not carry replication-competent HIV-1.

Production of HIV-1 vectors and viruses

HIV-1 NL4-3 and HIV-1-based vectors were produced through transient transfection of HEK293T cells and collected as previously described (Bérubé and others 2007; Pham and others 2013; Veillette and others 2013). To produce Gag variant 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 generate Gag variant HIV-$1_{NL\text{-}Luc}$, cells were co-transfected with the respective pNL4-3XCS vector and pMD-G. HIV-1_{NL43} was produced by transfection of pNL4-3. HIV-1 IIIB amplified by passage in stimulated peripheral blood mononuclear cells was titered by end-point dilution in MAGI cells, as described before (Kimpton and Emerman 1992).

Single-cycle infection assays

For viral challenges with HIV-$1_{TRIP\text{-}CMV\text{-}GFP}$, cells were plated in 24-wells plates at 50,000 cells per well and infected the next day with the appropriate HIV-1 vectors at an MOI of 0.1, as normalized by titering the viral stocks in feline CRFK cells. Two days post-infection, cells were trypsinized 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-$1_{NL\text{-}Luc}$, CRFK and TE671 cells were plated in 24-wells plates at 50,000 cells per well and infected the next day with 100 µl of the appropriate HIV-1 vectors, a dose that resulted in measurable luciferase activity in all tested conditions. Two days post-infection, cells were washed once with PBS and 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 Assay System (Promega, Madison, WI, USA) according to the manufacturer instructions and read in a Biotek Synergy HT plate-reader.

Replication-competent HIV-1 infection assays and p24 ELISA

10^6 CEM.NKR-CCR5 cells were seeded in 1 ml in a 12 well plate and challenged with HIV-1 virus IIIB (MOI 0.01). After 24 hours, cells were washed three times with PBS. Subsequently, 0.5-1 ml of the cell suspension was removed every 3 or 4 days and cell density readjusted with fresh media to a volume of 1.5-2 ml. On days 8, 15 and 22, the cell suspensions were subjected to low-speed centrifugation (7 min at 250xg) and supernatants were then used for CAp24 ELISA. CAp24 ELISA was performed using the Alliance kit (Perkin Elmer, Santa Clara, CA) according to manufacturer's instructions. For the experiment with HIV-1 NL4-3, Sup-T1 cells transduced with various pMIG-TRIM5α constructs were infected with 15 µl of virus, a dose previously determined to lead to peak infection in about 12 days (Pham and others 2010). Cells were then maintained in culture at a density between $5x10^5$ and $2x10^6$ cells per ml and aliquots were analyzed by FACS for GFP expression at different time-points.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) stimulation assay

TE671 cells stably expressing FLAG-tagged R332G-R335G TRIM5α_{hu}, WT TRIM5α_{Rh} or transduced with the empty vector were plated in 12-well plates at a cell density leading to 80% confluent cells at the time of transfection. TE671 cells stably expressing R332G-R335G TRIM5α_{hu} or WT TRIM5α_{Rh} were transfected with 0.6 μg of the NF-κB luciferase reporter construct (pCEP4-NF-κB-Luc) or the activation-deficient mutant of the reporter construct (pCEP4-ΔNF-κB-Luc). TE671 transduced with the empty vector control were co-transfected with R332G-R335G TRIM5α_{hu} or WT TRIM5α_{Rh} (2 μg) and the NF-κB luciferase reporter construct (0.6 μg). As a control, TE671 transduced with empty vector were co-transfected with R332G-R335G TRIM5α_{hu} (2 μg) and ΔNF-κB-Luc (0.6 μg). Cells were lysed with RIPA buffer 48 hours post-transfection and assessed for luciferase activity using the BrightGlow Luciferase kit (Promega). Luminescence was measured with a Synergy HT multi-detection microplate reader (BioTek, Winooski, VT) and analyzed using the Gen5 software (BioTek). The cellular lysates prepared to quantify luciferase activity were also used to analyze TRIM5α_{Rh} expression. Specifically, the three lysates from each triplicate transfection were pooled and analyzed by western blotting using a rabbit anti-FLAG antibody (Cell signaling, 1: 2000). Actin was used as a loading control and was detected with mouse anti-actin HRP (Sigma, 1:20000).

Tumour necrosis factor (TNF)-α stimulations

For IκBα protein kinetics, CEM.NKR-CCR5 cells were collected unstimulated, after stimulation with TNF-α (10 ng/ml; PeproTech, Rocky Hill, NJ) for 30 minutes and recovery from TNF-α stimulation for 2 hours. For the analysis of NF-κB and activator protein-1 (AP-1) regulated target genes by RT-PCR, cells were collected unstimulated, after stimulation with TNF-α (10 ng/ml) for 4 and 8 hours, and after a recovery phase of 16 hours after TNF-α for 8 hours.

RT-qPCR

Total RNA from 5×10^6 CEM.NKR-CCR5 cells was purified using STAT60 (TEL-TEST, Friendswood, TX) according to manufacturer protocols and 7 µg of RNA were digested with 1 µl of Turbo DNase in 25 µl total volume (AM1907, Ambion). Afterwards, 2.4 µg of RNA were reverse transcribed in 46 µl (3.26 ng/µl random primers, 0.43 mM dNTP, 10 nM DTT, 1.74 U/µl RNAsin, 8.7 U/µl MMLV-RT (#28025-013, Invitrogen) at 25 °C for 10 minutes, 37 °C for 15 minutes and heat inactivated at 70 °C for 15 minutes.)

qPCR was performed in 20 µl with 25 ng cDNA for detection of GAPDH (200 ng for other target genes) with IQ Sybr Green Supermix (170-8882, Biorad, Hercules, CA) and 0.5 µM of primer pairs for human GAPDH (fw 5'CCACTCCTCCACCTTTGAC & rev 5'ACCCTGTTGCTGTAGCCA), human and rhesus TRIM5a (fw 5'AGACATTCTGAAAAGCCTTACGAA & rev 5' ATCAGGAGCTCGAAACACTCTC), EGFP (fw 5' ATCATGGCCGACAAGCAGAAGAAC & rev 5' GTACAGCTCGTCCATGCCGAGAGT (Godbey and others 2008)), human FasL (fw 5'TAAAACCGTTTGCTGGGGC & rev 5' CTCAGCTCCTTTTTTTCAGGGG), JunB (fw 5' GGACGATCTGCACAAGATGA & rev 5' GGGAGTAGCTGCTGAGGTTG) or human TNFAIP3 (fw 5'CCCTCATCGACAGAAACA & rev, 5'GAACGCCCCACATGTACT, all provided by IDT, Coralville, IA) and PCR performed in 96 well plates (HSP9601, Biorad, Hercules, CA) at 95 °C 9', 40 x 95 °C 30", 59 °C 1', 72 °C 30", 72 °C 5".

Results

Targeting of highly divergent HIV-1 CA by R332G-R335G TRIM5α$_{hu}$

In order to assay the sensitivity of diverse HIV-1 isolates to mutated human TRIM5α, we first used a series of HIV-1 vectors carrying *gag* from 12 different strains in an otherwise isogenic context (Ikeda and others 2004). The VSV G-pseudotyped, GFP-expressing HIV-1 vectors were produced by transient transfection as previously

described (Bérubé and others 2007; Pham and others 2010). Strain names, sequence accession numbers, subtypes or clades, and cellular tropism of the HIV-1 isolates used in this study are summarized in Table 1 of Supplementary Information. 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). Sequence alignments of the isolates show multiple variations in the N-terminal domain of capsid that determines sensitivity to TRIM5α (not shown). In particular, some of the strains present mutations at positions known to modulate HIV-1 sensitivity to either Rhesus or human TRIM5α, such as Val86, Iso91 and Gly116 (Pacheco and others 2010; Soll and others 2013; Veillette and others 2013). To analyze the sensitivity of these Gag variants to restriction by either TRIM5α_{Rh} or R332G-R335G TRIM5α_{hu}, we used TE671 cells stably expressing these two variants, while a cell line stably transduced with WT TRIM5α_{hu} was used as non-restrictive control. As shown in Fig. 1A, all viruses tested were susceptible to restriction by both TRIM5α_{Rh} and R332G-R335G TRIM5α_{hu}. Although the efficiency of 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 they interact with HIV-1 CA in a similar fashion. TRIM5α_{Rh}-mediated inhibition was only ~2-times more efficient than with R332G-R335G TRIM5α_{hu} in this assay, showing the potency of the TRIM5α_{hu} mutant approach.

Restriction of HIV-1 bearing CTL escape mutations

Next we tested the sensitivity of NRC1, NRC2 and NRC10 isolates bearing Gag mutations that allow for CTL escape but increase TRIM5α_{hu} sensitivity, to restriction by the same TRIM5α variants. For this, we used a different set of vectors, based on pNL-Luc, a pNL4-3 derivative that contains a deletion in *env* and encodes *Renilla* luciferase in place of *nef*. *Gag* and *Protease* sequences of pNL-Luc were replaced for those from isolates bearing CTL escape mutations, yielding constructs NRC1, NRC2

and NRC10 (Battivelli and others 2011). It was previously established that compared to NL-Luc, the increase in sensitivity to TRIM5α_{hu} was greatest in NRC10, less so in NRC2, and weak in NRC1 (Battivelli and others 2011). NRC10-VMG is a lab-generated mutant of NRC10 in which 3 of the mutations that are known to increase sensitivity to TRIM5α_{hu} (V86A, M96I, G116A) are reverted back to WT. We measured the infectivity of NL4-3-based HIV-1 vectors bearing Gag sequences from those isolates in both feline CRFK cells, which do not express any known TRIM5α ortholog, and in human TE671 cells transduced with the different TRIM5α cDNAs (Fig. 1B). The data gathered in CRFK cells allow us to quantify the TRIM5α-independent decrease in viral fitness stemming from the CTL-escape mutations in Gag and Protease. NRC1 had the same restriction pattern as that of NL4-3, although the former was slightly less sensitive to R332G-R335G TRIM5α_{hu}. As previously observed by Battivelli and colleagues, NRC2 and NRC10 showed a marked increase in sensitivity to TRIM5α_{hu}. Specifically, infectivity of NRC2 and NRC10 in TE671 expressing TRIM5α_{hu} was reduced ~20-fold and ~50-fold, respectively, compared with the respective CRFK controls. Restriction of these two variants by R332G-R335G TRIM5α_{hu} and TRIM5α_{Rh} was also more efficient, although the effect was less pronounced than for WT TRIM5α_{hu}. In particular, NRC-2 and NRC-10 were restricted ~40-fold and ~100-fold by R332G-R335G TRIM5α_{hu}, respectively, while the NL-Luc control was restricted slightly more than 10-fold. As expected, the NRC10-VMG revertant clone showed decreased sensitivity to all three TRIM5α tested. In summary, CTL-escape HIV-1 Gag mutants show a gain of sensitivity to restriction by R332G-R335G TRIM5α_{hu}.

Survival advantage of lymphocytes expressing HIV-1-restrictive TRIM5α variants

The previous experiments use replication-incompetent vectors that can achieve early stages of the infection only. Typical HIV-1 spreading assays performed in our earlier studies (Pham and others 2010; Pham and others 2013; Veillette and others 2013) also do not faithfully represent the situation in *in vivo* gene therapy protocols, in which only a minority of the cells are protected by the presence of the antiviral transgenes. Would these cells be protected against HIV-1-mediated cytopathic effects in a virus

spreading context? We designed experimental conditions allowing a minority of TRIM5α-expressing human lymphocytic Sup-T1 cells to expand due to their resistance to HIV-1-mediated killing. For this, we mixed cells stably expressing various restrictive and non-restrictive TRIM5α with parental cells at an approximate ratio of 1:99 (Table 1). The transduced cells also expressed GFP, allowing for convenient quantification by flow cytometry. We then infected the various co-cultures with a single dose of NL4-3 (Adachi and others 1986) that results in a replication peak at about 2 weeks post infection as measured by reverse transcriptase assay (Pham and others 2010). At the first two time-points for which we performed flow cytometry, the proportion of GFP-positive cells was close to 1% in all cultures, as expected. However, HIV-1 spread very efficiently in all cultures, and there were too few surviving cells for us to perform cytometry between days 11 and 18 post infection. We nonetheless maintained these cells, and more than a month after the beginning of the experiment, recovery was sufficient for us to analyze the proportion of GFP-expressing cells in all cultures. The results were strikingly different depending on whether the initial mixed culture contained cells expressing HIV-1-restrictive TRIM5α or not. In the non-restrictive controls, i.e. cells transduced with WT TRIM5α_{hu} or with the "empty" MIG vector, we could barely detect any cell expressing GFP, showing a total lack of survival advantage of the transduced cells. In all other cases, the proportion of GFP-expressing cells had markedly increased, ranging from ~20% (cells expressing R332G TRIM5α_{hu}) to close to 90% (cells expressing TRIM5α_{Rh}). In addition, the % of GFP-positive cells was approximately proportional to the level of protection conferred by the respective TRIM5α variants: TRIM5α_{Rh} > R332G-R335G TRIM5α_{hu}> R332G TRIM5α_{hu} (Pham and others 2010; Pham and others 2013; Veillette and others 2013). Although the exact proportion of TRIM5α-expressing cells that survived HIV-1 infection in the first 4 weeks of infection is not known, this experiment suggests that R332G-R335G TRIM5α_{hu} can confer protection not only against HIV-1 infection but also against cytopathic effects stemming from the active spread of the virus in the unmodified cells.

Lack of detectable NF-κB activation in TE671 cells stably expressing TRIM5α

TRIM5α expression can activate transcription factors NF-κB and AP-1 (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011; Tareen and Emerman 2011a; 2011b; Uchil and others 2013), and in some cases results in type I interferon production (Pertel and others 2011; Uchil and others 2013). This raises the possibility that stable transduction of TRIM5α following retroviral gene transfer may constitutively activate these pathways, which could result in unregulated inflammation. TRIM5α-mediated activation of NF-κB has been detected following transient transfection of HEK293T cells (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011; Tareen and Emerman 2011a), but it is unknown whether its stable transduction has the same effect. To investigate this, we used cells that were stably transduced with R332G-R335G TRIM5α_{hu} or TRIM5α_{Rh}. Control cells transduced with the empty parental vector were transfected with the vector plasmids encoding R332G-R335G TRIM5α_{hu} or TRIM5α_{Rh}. To assess for NF-κB activation, cells were additionally transfected with a construct expressing luciferase under the control of an NF-κB-inducible promoter. Two days post-transfection, luciferase activity and TRIM5α expression levels were assessed (Fig. 2). A basal NF-κB activity was detectable in control cells transduced with the empty vector and transfected with the NF-κB-luc construct, as compared to cells transfected with the control plasmid bearing a deletion in the NF-κB binding domain (a ~3-fold increase). NF-κB activation levels were statistically indistinguishable in cells stably transduced with R332G-R335G TRIM5α_{hu} or with TRIM5α_{Rh}, compared with the control cells (Fig. 2). However, when cells were transiently transfected with the retroviral vector plasmids expressing these proteins, we observed a large increase (~10-fold) in NF-κB activation levels. This result suggests that expression of TRIM5α by transient transfection, but not following stable transduction, stimulates NF-κB. Interestingly, levels of TRIM5α expression in the transfected cells were similar to that in the stably transduced cells, as judged in a Western blotting analysis (Fig. 2, bottom panels). It is possible, however, that a minor fraction of the transiently transfected cells express very high levels of TRIM5α, and that NF-κB activation occurs mostly in this subset of cells.

Stable expression of TRIM5α from a lentiviral vector with predominantly single integration

We next aimed at testing the anti-HIV-1 and innate immune activation properties of TRIM5α_{hu} mutants, using a vector system in which TRIM5α expression is relatively low and cell-to-cell variation is minimal. For this, we designed a lentiviral vector plasmid, pHIV8-ES-2A31D, to express TRIM5α and GFP under control of the short human EF1α promoter, GFP translation being made possible due to the presence of a 2A domain C-terminal of TRIM5α (see Materials and Methods). We transduced T lymphocytic CEM-based CEM.NKR-CCR5 cells (Trkola and others 1999) with the "empty" lentiviral vector, expressing only GFP, or with the vector also expressing TRIM5α (Fig. S1). In addition to TRIM5α_{Rh}, WT TRIM5α_{hu} and R332G-R335G TRIM5α_{hu}, we also tested the single mutant R332G TRIM5α_{hu} in this novel experimental model. To demonstrate comparable transduction and expression rates, GFP expression was analyzed for intensity (Fig. S1A,B). Results show that transduction levels and GFP expression levels were similar across the various TRIM5α-expressing constructs. Cells transduced with the "empty" vehicle showed higher levels of GFP expression, which could be due to higher transduction levels and/or higher GFP production as a result of the absence of the TRIM5α sequence. In addition, GFP expression profiles following FACS sorting of the GFP-positive cells at day 29 post-infection showed the presence of a single peak when cells were transduced with one of the TRIM5α-expressing vector, suggesting that most cells had only one copy of the integrated vector. In contrast, 2 or more peaks were apparent for cells transduced with the empty vector, suggesting that some of the cells had been transduced with 2 or more copies of the vector (Fig. S1A). In order to determine levels of transgenic TRIM5α expression relative to endogenous TRIM5α_{hu}, we quantified total TRIM5α mRNA by SybrGreen RT-PCR (Fig. S1C,D) Primers used in this experiment can amplify WT or mutant TRIM5α_{hu} sequences, as well as TRIM5α_{Rh} (see Materials and Methods). Using this approach, we determined that transduction with the empty vector had no impact on endogenous TRIM5α mRNA expression levels, while transduction with the various TRIM5α-expressing vectors resulted in a ~6-fold to ~12-fold increase in mRNA levels (Fig. S1C,D). It is unclear why about 2 times less TRIM5α mRNA was detected

in cells transduced with R332G-R335G TRIM5α_{hu} compared with cells transduced with TRIM5α_{hu} or TRIM5α_{Rh} (Fig. S1D), even though GFP expression levels were similar (Fig. S1B). It cannot be excluded that the mutated sites affected reverse transcription or PCR to some degree, or slightly decreased protein stability. Another possibility is that the long half-life of GFP (Corish and Tyler-Smith 1999) masked differences in protein translation levels, which would not be the case for TRIM5α protein which has a notoriously short half-life of about 40 minutes (Diaz-Griffero and others 2007). Thus, transgenic TRIM5α was moderately over-expressed from predominantly single integration events in our experimental system, with the R332G-R335G variant possibly being expressed at slightly lower steady-state levels.

TAK1 is not activated in stably TRIM5α-transduced cells

Loss-of-function experiments have demonstrated that transforming growth factor (TGF)-activated kinase 1 (TAK1) is important for the activation of NF-κB following over-expression of TRIM5α (Pertel and others 2011; Uchil and others 2013), Bst2/Tetherin (Galao and others 2012; Tokarev and others 2013), or treatment with the pro-inflammatory cytokine TNF-α (Hofer-Warbinek and others 2000). The TAK1 kinase complex, which comprises TAK1 as well as TAK1-binding proteins 1 and 2 (TAB1 and TAB2), is activated by K63-linked polyubiquitin chains that are catalyzed by E3 proteins TRAF6 (Wang and others 2001) and TRIM5α (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011). TAK1 phosphorylates IκB kinase (IKK), which in turn phosphorylates IκBα, leading to NF-κB activation (Chen 2012; Wang and others 2001). TAK1 is central to many innate immune pathways, including those downstream of innate sensors of the Toll-like receptor (TLR) and retinoic acid-inducible gene 1 (RIG-I) families (Chen 2012). TAK1 stimulatory functions require phosphorylation at Ser412 (Gu and others 2014; Kobayashi and others 2005). We used Western blotting to analyze the total and Ser412-phosphorylated forms of TAK1 in stably transduced CEM.NKR-CCR5 cells (Fig. 3A). Quantification from 3 independent experiments showed that stable overexpression of the different TRIM5α used did not significantly modulate total levels of TAK1, compared with the untransduced cells (Fig. 3B). Levels of Ser412-

phosphorylated TAK1 were slightly higher in cells transduced with WT and R332G TRIM5α_{hu}, compared with the other cell lines, but this effect did not reach statistical significance (Fig. 3B). In conclusion, results from this experiment do not support abnormal activation of TAK1 in cells stably transduced with TRIM5α.

No effect of transgenic TRIM5α on IκBα kinetics following TNF-α treatment

Although TRIM5α transduction did not appear to significantly modulate TAK1 activation levels, there was still a possibility that it could modulate the kinetics of the NF-κB/AP-1 pathway. To test this, we treated the various CEM.NKR-CCR5 cell lines with TNF-α and then analyzed kinetics of IκBα and of NF-κB-dependent gene expression. IκBα binds to NF-κB in the cytoplasm, preventing its nuclear translocation and transcriptional activity (Scott and others 1993). TNF-α causes the phosphorylation and ultimately the degradation of IκBα (Hoffmann and Baltimore 2006), allowing NF-κB to reach the nucleus where it can potentially activate hundreds of genes (Pahl 1999; Tian and others 2005). Among these are IκBα, hence providing a negative feedback loop that contributes to ending the stimulation in a well-studied mechanism (Hoffmann and others 2002; Werner and others 2008). By controlling the duration of NF-κB activation, the negative feedback loop indirectly controls NF-κB transcriptional activity profiles as well (Saccani and others 2001; Tian and others 2005). Thus, we used an experimental setting that would allow us to study both the activation and recovery phases. We observed that as expected, TNF-α resulted in a significant decrease in IκBα levels after 30 min of treatment (Fig. 4A). Upon repeated experiments, we found that stable transduction with WT or mutant TRIM5α_{hu}, or with TRIM5α_{Rh}, did not alter the capacity of TNF-α to down-modulate IκBα expression (Fig. 4B). Gel densitometry analyses showed that on average, IκBα expression was reduced ~2-fold as a result of the treatment. After 2 hours of post-treatment incubation ("recovery"), IκBα expression returned to normal or slightly elevated levels. One exception was the untransduced cells, in which IκBα expression was about 3-times higher compared to pre-treatment levels. This could be related to the fact that IκBα pre-treatment levels are higher in cells transduced with the lentival vector used (encoding TRIM5α or not) than in untransduced

cells (see Fig. 4A). It is possible that low levels of innate activation are induced by the lentiviral transduction transduction itself, independent of TRIM5α expression (Rossetti and others 2011). In conclusion, the down-modulation/recovery profiles seen in cells transduced with the various TRIM5α variants were similar to that seen in cells transduced with the control vector.

No effect of TNF-α on the kinetics of expression of genes relevant to the NF-κB and AP-1 pathways

Treatment with TNF-α activates the AP-1 transcriptional complex through mechanisms that are both independent and dependent on NF-κB (Fujioka and others 2004). Since TRIM5α over-expression can activate both NF-κB and AP-1 (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011), we investigated whether stably transduced TRIM5α would modulate TNF-α-induced expression of factors relevant to both pathways. We treated the various CEM.NKR-CCR5 cell lines with TNF-α for 4 or 8 hours and then maintained an aliquot of each culture for 16 additional hours in the absence of drug. qRT-PCR was performed to analyze mRNA expression levels for tumor necrosis factor, alpha-induced protein 3 (TNFAIP3*)* (Fig. 4C) and JunB (Fig. 4D). Transcription of TNFAIP3 (also known as A20) is stimulated by NF-κB, and this protein negatively regulates the NF-κB pathway, by a mechanism different from that of IκBα (Heyninck and others 1999). Transcription of JunB, a component found in some AP-1 complexes, is stimulated by both NF-κB and AP-1, and this protein regulates AP-1 activity (Schutte and others 1989; Shaulian and Karin 2001). We observed a ~2- to ~2.5-fold increase in TNFAIP3 mRNA levels after 4 or 8 hours of treatment with TNF-α in most cell lines (Fig. 4C). In cells stably expressing R332G-R335G TRIM5α_{hu}, activation was more efficient than in the cells transduced with the other TRIM5α variants or with the empty vector (Fig. 4C). Expression levels were returned to normal by 16 hours after the end of TNF-α treatment (Fig. 4C). JunB showed delayed activation kinetics compared with TNFAIP3. In most cell lines analyzed, higher JunB mRNA levels were found after 8 hours in presence of TNF-α but not after 4 hours (Fig. 4D). In some cell lines analyzed, JunB levels were still higher than normal 16 hours after drug withdrawal (Fig. 4D). We observed some variation between the various cell lines tested, but these

effects were not statistically significant. Likewise, JunB activation profiles were similar in cells stably expressing TRIM5α and in untransduced or empty vector-transduced cells (Fig. 4D). In summary, stable transduction with WT or mutant TRIM5α_{hu} or with TRIM5α_{Rh} had no major impact on the capacity of TNF-α to down-regulate IκBα or up-regulate NF-κB/AP-1 dependent genes.

R332G-R335G TRIM5α_{hu} strongly restricts HIV-1 IIIB spreading in CEM.NKR-CCR5 cells

Finally, we analyzed how efficiently R332G-R335G TRIM5α_{hu} inhibits HIV-1 over multiple rounds of replication. For this, we infected the various CEM.NKR-CCR5 lines generated with IIIB, a highly pathogenic strain of HIV-1 (Popovic and others 1984). p24 CA in supernatants was quantified by ELISA at days 8, 15 and 22 post infection (Fig. 5). As expected, HIV-1 amounts in control, empty vector-transduced cells increased markedly between day 8 and day 15, and then between day 15 and day 22. Signs of cell death were visible at day 15 and most cells were dead at day 22 (not shown). Compared with these control cells, transduction of WT TRIM5α_{hu} had no effect on HIV-1propagation, as expected. At all 3 time-points, HIV-1 amounts in the supernatant of cells over-expressing WT TRIM5α_{hu} was similar to that in the control cells, or even higher (Fig. 5). Transduction of R332G TRIM5α_{hu} decreased HIV-1 replication levels by approximately 1 Log_{10}, as seen at day 8 and day 15. However, HIV-1 spread efficiently in these cells; by day 22, most cells had died and the amounts of virus detected in the supernatants were similar to that of the control. In contrast, cells stably expressing R332G-R335G TRIM5α_{hu} or TRIM5α_{Rh} were highly resistant to HIV-1 spreading infection. At all time-points analyzed, CAp24 levels were lower by several orders of magnitude in these two cell lines, compared with the empty vector-transduced cells or compared to the cells stably transduced with WT TRIM5α_{hu} (Fig. 5). Interestingly, CAp24 levels were highest at day 15 for R332G-R335G TRIM5α_{hu}, and then apparently decreased as seen at day 22. This opens the possibility that virus replication was sufficiently slowed by R332G-R335G TRIM5α_{hu} that uninfected cells outgrew infected cells. In summary, R332G-R335G TRIM5α_{hu} and TRIM5α_{Rh} but not R332G TRIM5α_{hu} very efficiently restrict HIV-1 spreading infection in CEM cells.

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 and others 2004; Kirmaier and others 2010; Mariani and others 2003). We 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 and others 2006; Maillard and others 2007; Pham and others 2010; Stremlau and others 2005; Yap and others 2005). Because those constructs differ only slightly from their endogenous counterpart, they would probably not be immunogenic, thus making them interesting candidates for therapeutic treatment of HIV-1. However, it was not yet known whether these TRIM5α mutants would target CA belonging to clinical isolates of HIV-1. Our results show that all strains of a diverse panel of HIV-1 variants are potently restricted by R332G-R335G TRIM5α_{hu} and by TRIM5α_{rh}.

Some mutations in CA that were associated with escape from CTLs were previously shown to increase sensitivity to TRIM5α_{hu} (Battivelli and others 2010; Battivelli and others 2011) and our results show that this effect can also be extended to a more restrictive human TRIM5α variant like R332G-R335G. This suggests that a highly restrictive TRIM5α used in gene therapy applications could help control the rate at which CTL escape mutations appear, and make it more difficult for the virus to escape both host immune responses and transgene-mediated inhibition at the same time. CTL escape mutations are often followed by compensatory mutations that reduce the fitness cost of the resistance mutations (Battivelli and others 2011; Brockman and others 2007; Brumme and others 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 and others 2004; 2005b; Keckesova and others 2006; Stremlau and others 2006b). In addition, the residue at position 86 was already shown to play a crucial role in the resistance to both TRIM5α_{Rh} (Pacheco and others 2010; Soll and others 2013) and TRIM5α_{hu} mutants (Veillette and others 2013). Altogether, these data suggest that mutations in or near the

CypA-binding loop of CA play an important role in modulating HIV-1 sensitivity to innate and acquired immune defenses.

Unregulated immune activation by transgenic, over-expressed TRIM5α was, potentially, a major impediment to the development of TRIM5α_{hu} mutants as anti-HIV-1 transgenes. This concern was first raised several years ago, when it was demonstrated that transfecting TRIM5α could activate NF-κB and AP-1 through the activation of TAK1 and the generation of K63-linked ubiquitin chains (Nepveu-Traversy and Berthoux 2014; Pertel and others 2011; Tareen and Emerman 2011a; Uchil and others 2013). Most of these previous experiments were done by transfecting HEK293 cells, however. Here we show that NF-κB can be activated by transfecting TRIM5α into TE671 cells as well. Importantly however, we could not detect any NF-κB activation in these cells when TRIM5α was expressed through stable oncoretroviral vector-mediated transduction rather than through transient transfection. Furthermore, stable lentiviral vector-mediated transduction of TRIM5α in lymphocytes did not lead to any major change in TAK1 activation or in dynamics of the NF-κB pathway following TNF-α treatment. Altogether, our results strongly suggest that stable transduction of TRIM5α does not activate or modulate this pathway. Pertel *et al.* also reported that NF-κB could be activated upon interaction between TRIM5α and an incoming restriction-sensitive virus (Pertel and others 2011). Although we have not attempted to reproduce this observation in this study, it should be pointed out that this effect, if present, would contribute to the specific TRIM5α antiviral activity and thus be desirable.

R332G-R335G TRIM5α_{hu} was significantly more efficient than the single mutant R332G at inhibiting HIV-1spreading infection and at increasing the survival of gene-modified cells. These results confirm our previously published data obtained in single-cycle infection experiments using HIV-1 vectors (Pham and others 2010; Pham and others 2013; Veillette and others 2013) or in spreading infection of a different HIV-1 strain (NL4-3) in a different T cell line (Sup-T1) (Pham and others 2010; Veillette and others 2013). Collectively, our data also show that in lymphocytes, R332G-R335G TRIM5α_{hu} restricts HIV-1 about as much efficiently as TRIM5α_{Rh} does. It also did not

escape our attention that the two mutations being located close to one another, it should be feasible to introduce them both simultaneously into the human genome, through the use of recently developed genome editing methods such as those based on clustered regularly interspersed, short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (CRISPR-Cas9) (Hsu and others 2014; Yin and others 2014).

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Author Disclosure Statement

Lionel Berthoux and Quang Toan Pham are co-owners of a US patent (8,623,815 B2) covering antiviral applications of TRIM5α_{hu} Arg335 mutations.
No competing financial interests exist for the other authors.

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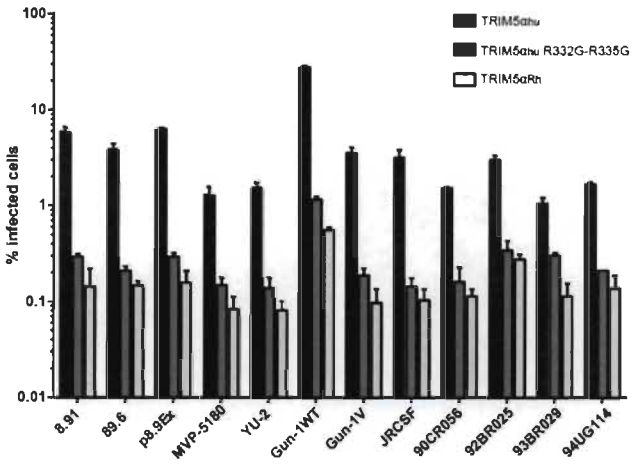
Figure legends

A



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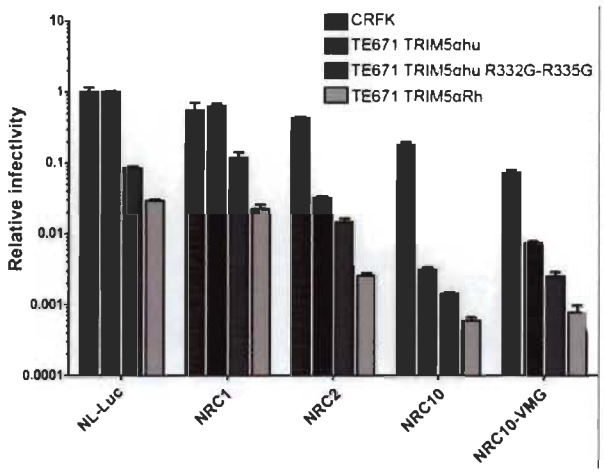

FIG. 1. Restriction of highly divergent HIV-1 capsids and of cytotoxic T lymphocyte escape mutants in human cells expressing R332G-R335G TRIM5α_{hu}. **(A)** TE671 cells were transduced with WT TRIM5α_{hu}, R332G-R335G TRIM5α_{hu} or TRIM5α_{Rh} expressed from the oncoretroviral vector MIP. Cells were then infected at a single multiplicity of infection with 12 different GFP-encoding HIV-1 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. **(B)** TE671 cells expressing the same TRIM5α cDNAs as in (A) were infected with a single dose of the wild-type or CA-mutated HIV-$1_{NL\text{-}Luc}$ vectors. As an additional non-restrictive control, feline CRFK cells that do not express any TRIM5α were infected with the same amounts of virus. Two days post-infection, cells were lysed and the luciferase activity was measured. Relative light unit (RLU) values in TE671 cells are all normalized relative to the value obtained for the NL-Luc control in the cells expressing WT TRIM5α_{hu}. Similarly, RLU values in CRFK cells are normalized to the value obtained for NL-Luc in CRFK cells. Shown are the mean and standard deviation of three independent infections.

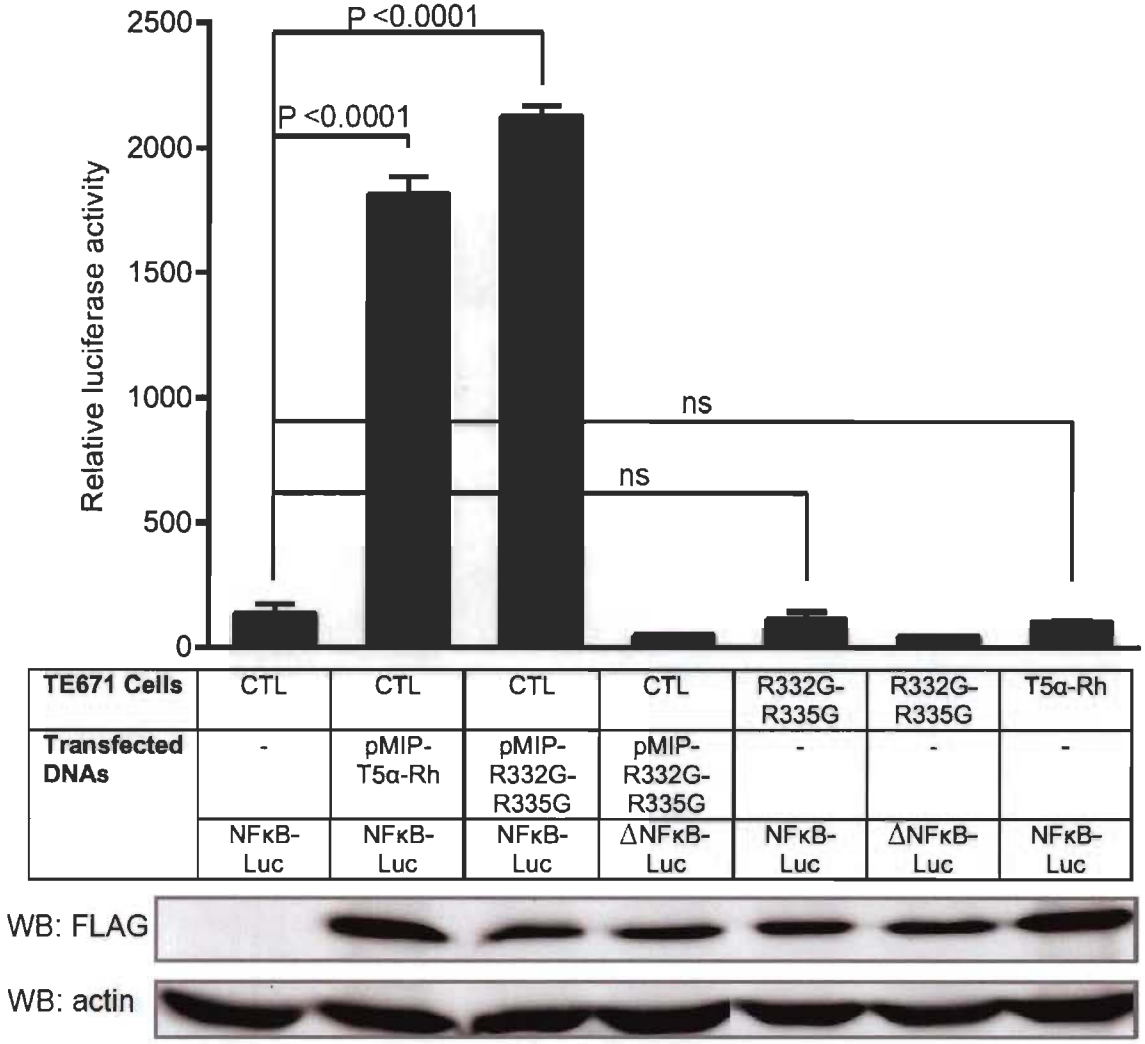

FIG. 2. Activation of the NF-κB pathway by transient transfection of Rhesus or human TRIM5α but not by their stable transduction. TE671 cells stably expressing FLAG-tagged R332G-R335G TRIM5α_{hu}, WT TRIM5α_{Rh} or transduced with the empty vector (CTL) were transfected in triplicates with a plasmid expressing luciferase from an NF-κB-dependent promoter, or with the control plasmid ("ΔNFκB-Luc") bearing a mutation in the NF-κB binding site. Empty vector-transduced cells were also co-transfected with pMIP plasmids expressing FLAG-tagged TRIM5α_{Rh} or R332G-R335G TRIM5α_{hu}. 2 days later, cells were lysed and luciferase activity was quantified (top panel). P-values were determined by the one-way ANOVA test followed by Turkey's multiple comparison test. ns, non-statistically significant. Lysates were also analyzed by Western blotting using an antibody against FLAG to detect exogenous TRIM5α expression and a loading control antibody against actin (bottom panels).

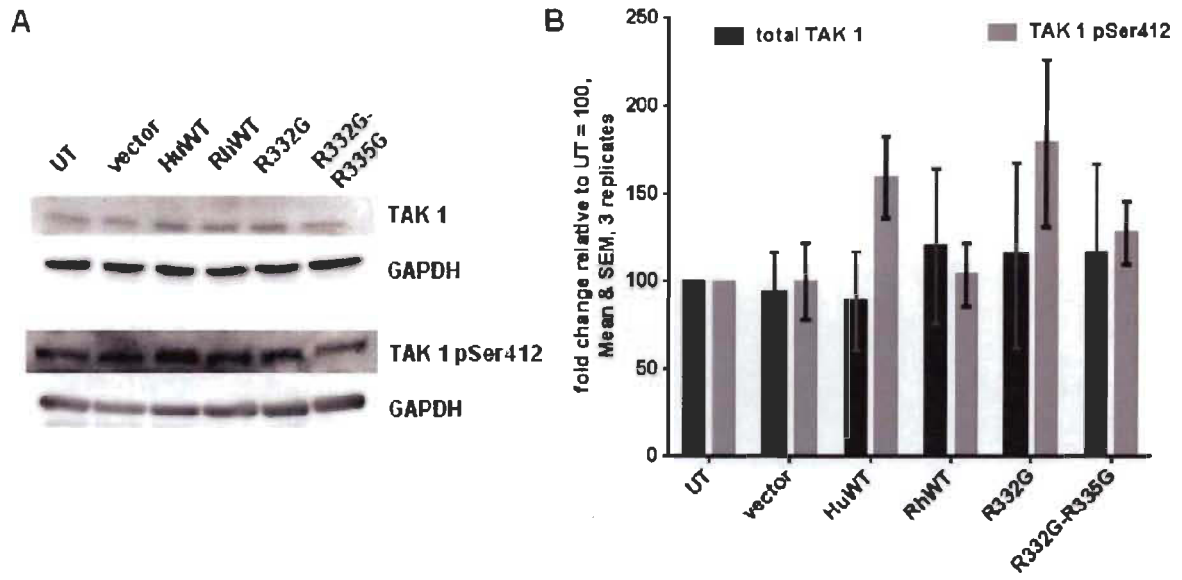

FIG. 3. Stable TRIM5α transduction does not affect TAK1 expression levels nor its Ser412 phosphorylation. **(A)** CEM.NKR-CCR5 cells stably transduced with the indicated TRIM5α variants expressed from the HIV8-ES-2A31D lentiviral vector, or stably transduced with the "empty" vector expressing only GFP ("Vector") were sorted for GFP expression. After sorting, cells were subjected to Western blotting analysis using antibodies against TAK1 and Ser412-phosphorylated TAK1. Blots were re-analyzed using a GAPDH antibody as a loading control. Untransduced, unsorted parental cells ("UT") were used as a control. A representative blot is shown. **(B)** Gel densitometry analysis. Values obtained were normalized to those for GAPDH and then to the value obtained for untransduced cells. Shown are means from 3 independent experiments, with the standard error of the mean (SEM).

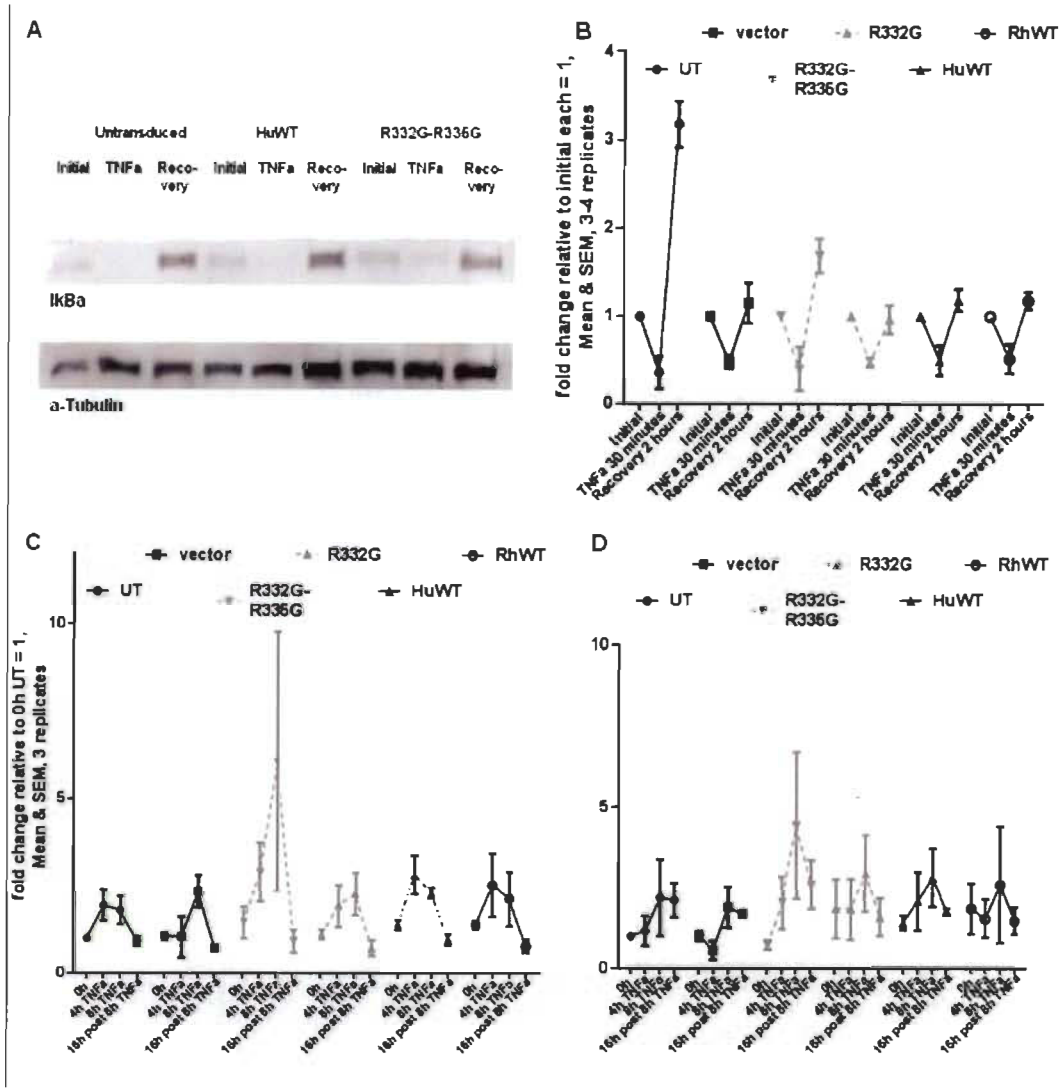

FIG. 4. TRIM5α transduction has no significant impact on the kinetics of IκBα protein levels or on the regulation of the AP-1/NF-kB pathway. Sorted, stably transduced CEM.NKR-CCR5 cells expressing the indicated TRIM5α variants or transduced with the vector expressing GFP only ("vector") were subjected to TNF-α treatment for 30 min. An aliquot of each culture was then lysed for protein analysis, while the remainder was kept in culture for 2 additional hours in the absence of TNF-α. **(A)** Representative Western blot analyses of IκBα expression levels at baseline levels ("Initial"), after TNF-α stimulation ("TNFa") and 2 hours after TNF-α removal ("Recovery"). The protein lysates were also analyzed using an antibody against α-tubulin as a loading control. **(B)** IκBα protein expression levels after normalization to α-tubulin and baseline level. Shown are means from 3-4 independent experiments, with the SEM. UT are untransduced, unsorted control cells. **(C, D)** RT-qPCR analysis of NF-κB regulated genes TNFAIP3 (C) and JunB (D). Total mRNAs were prepared at baseline ("0h"), after 4 or 8 hours of TNF-α treatment, and after 8 hours of TNF-α treatment followed by 16 hours in drug-free medium. Shown are -fold changes relative to untransduced cells at baseline and after normalization to values obtained for GAPDH. Results are means from 3 independent experiments with SEM.

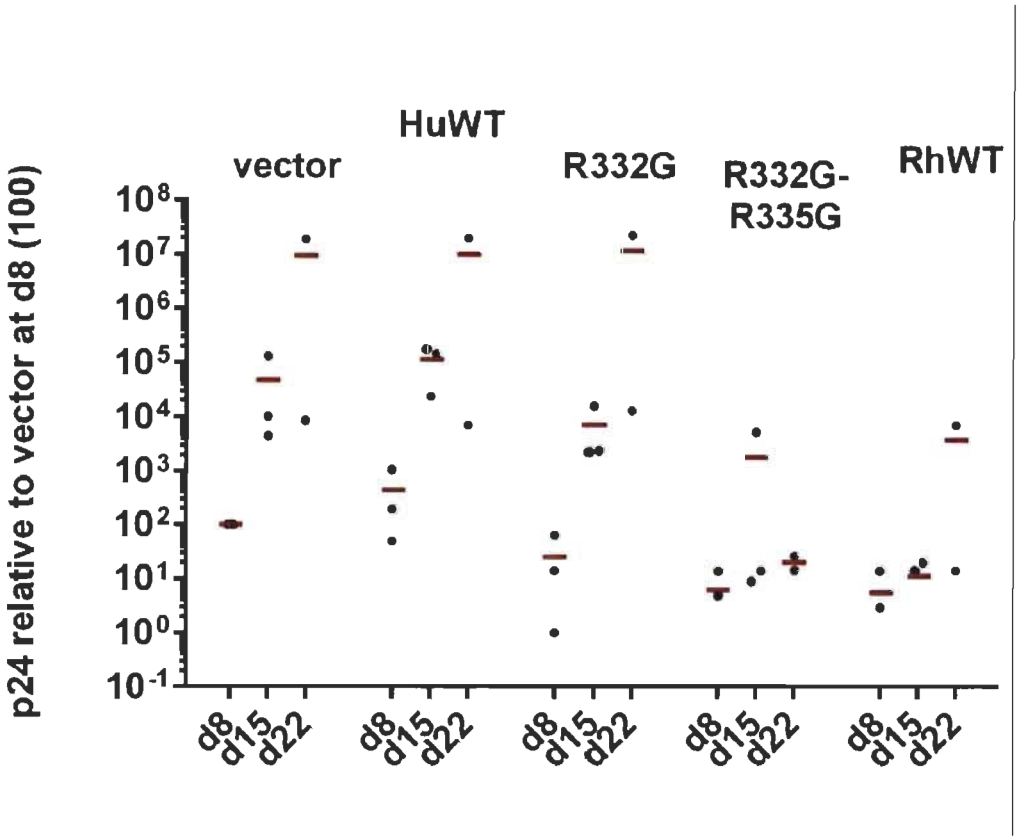

FIG. 5. Different TRIM5α variants show markedly different potency of HIV-1 restriction in propagation assays. CEM.NKR-CCR5 cells stably transduced with the indicated TRIM5α variants, or transduced with the empty parental vector as a control ("Vector"), and sorted for GFP expression, were infected with HIV-1 IIIB (MOI 0.01). CAp24 ELISA was performed on supernatants at days 8, 15 and 22. Shown are relative p24 amounts in 2-3 independent experiments, normalized to the value obtained for the Vector control at day 8 x100.

Table 1. Survival advantage of Sup-T1 cells expressing various TRIM5α following HIV-1 infection[a]

Transgene	Days post infection						
	4	7	11	14	18	33	39
Vector[b]	0.8	1.1				<0.1	0.2
TRIM5α$_{hu}$ WT	0.8	0.8				0.1	<0.1
TRIM5α$_{hu}$ R332G	1.4	1.3	*Very high cell mortality*[c]			18.9	24.1
TRIM5α$_{hu}$ R332G-R335G	0.9	1.3				71.6	64.8
TRIM5α$_{Rh}$	0.5	0.5				89.5	86.6

[a]Mixed cultures containing approximately 1% of cells transduced with MIG-TRIM5α and 99% of unmodified parental Sup-T1 cells were infected with the pathogenic NL4-3 strain of HIV-1. Cells were maintained in culture for 39 days post infection and a fraction of each culture was collected at various times for flow cytometry analysis. Data show the percentage of cells in each sample expressing GFP, which is translated from the same mRNA as TRIM5α. [b]"Vector" refers to the "empty" MIG expressing only GFP. [c]Around the peak of HIV-1 propagation, the mortality rate in the various cultures was too high to collect enough cells for flow cytometry analysis.

Pre-clinical assessment of mutant human TRIM5α as an anti-HIV-1 Transgene

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Supplementary information

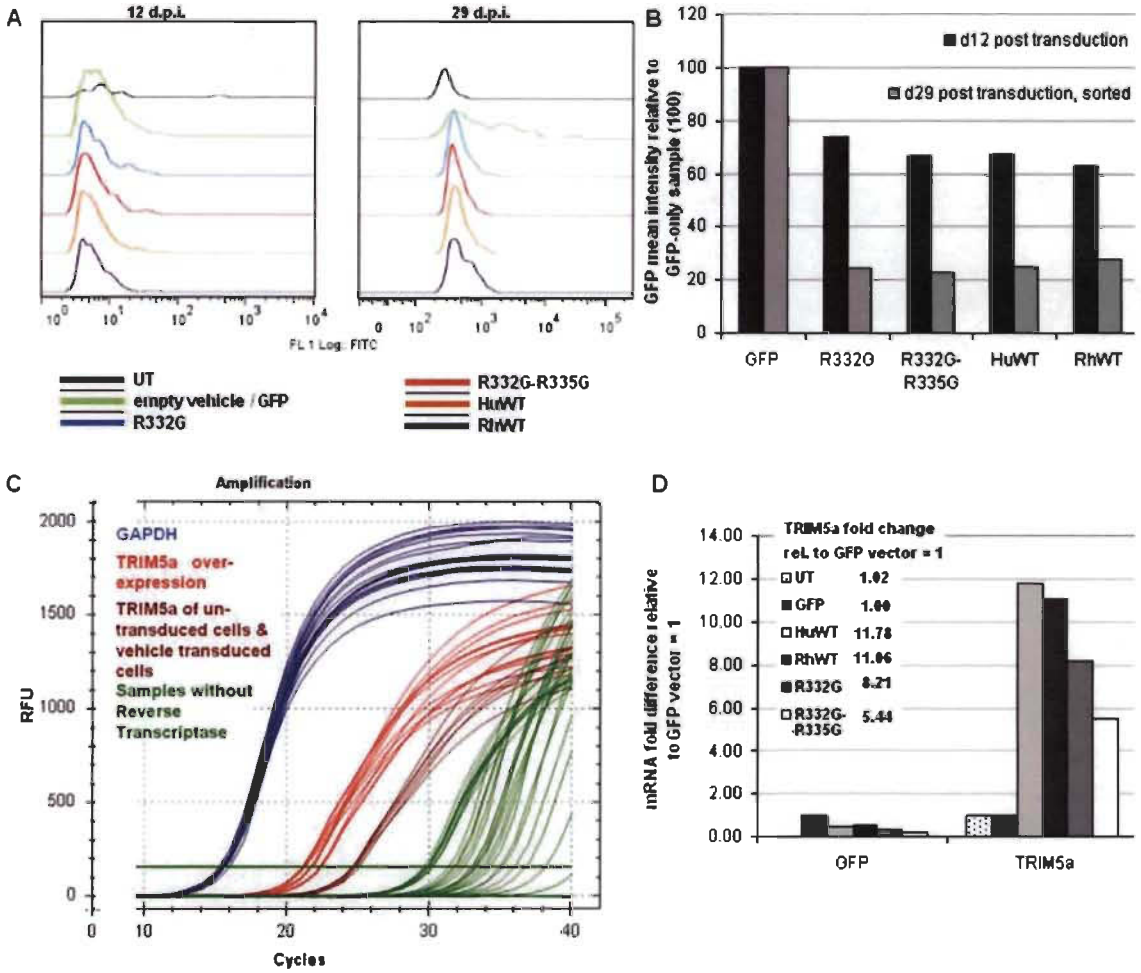

FIG. S1. Characterization of TRIM5α and G 1 FP expression following transduction of CEM-NKR-CCR5 cells with pHIV8-ES-2A31D based vector. **(A)** GFP expression profiles pre- and post-sorting of GFP-positive cells. CEM-NKR-CCR5 cells were left untransduced (UT) or were transduced at a low vector dose with the "empty" HIV8-ES2A31D vector, which expresses GFP but not TRIM5α ("empty vehicle / GFP"), or with HIV8-ES2A31D expressing the following TRIM5α variants along with GFP: R332G TRIM5α_{hu}; R332G-R335G TRIM5α_{hu}; WT TRIM5α_{hu}; WT TRIM5α_{Rh}. GFP expression profiles on unsorted cells were analyzed at 12 days post-infection (d.p.i.). GFP-positive cells were sorted at day 29 and the right panel shows GFP expression in the sorted cells, as compared to the unsorted, untransduced cells. **(B)** Mean GFP

fluorescence intensity in the GFP-positive cells at day 12 and in the sorted cells at day 29, expressed as a percentage of the value obtained in cells transduced with the "empty" vector ("GFP"). **(C)** Real-time RT-PCR profiles of TRIM5α-encoding mRNAs. CEM-NKR-CCR5 cells transduced with pHIV8-ES-2A31D-based vectors and sorted for GFP expression as detailed above as well as untransduced/unsorted cells were submitted to total mRNA extraction followed by RT-PCR using various primer pairs and performed in triplicates. PCR curves are shown as four color-coded groups, as follows. "GAPDH" are RT-PCR reactions performed on all mRNA samples using primers designed to amplify a GAPDH fragment, and serve as a loading control. "TRIM5α over-expression" are RT-PCR performed using TRIM5α-specific primers, on cells transduced with HIV8-ES-2A31D vectors expressing a TRIM5α variant (either WT or mutant). The third group shows RT22PCR performed with the same TRIM5α primers, but on untransduced cells or cells transduced with the "empty" HIV8-ES-2A31D vector; these curves result from the, amplification of endogenous TR 1 IM5α. Finally, the fourth group of curves are control reactions performed in the absence of reverse transcriptase, for all the mRNAs prepared. **(D)** mRNAs extracted from the same cells as in (C) were submitted to RT-PCR using primer pairs specific for GFP and TRIM5α. Results are expressed as mRNA copy numbers relative to the value obtained for the "empty" vector ("GFP").

TABLE S1. Summary information on viral strains used to construct HIV-1 vectors.

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	Unknown	T/M	(Shimizu et al., 1994; Takeuchi et al., 1987)
GUN-1V	D34594	Unknown	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.