厚生労働科学研究費補助金

エイズ対策研究事業

HIV-1 感染のヒトーラット種間バリアーの解明

平成１９年度～２１年度　総合研究報告書

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平成２２年５月

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総　合　研　究　報　告　書

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研究要旨

HIV-1 感染ラットモデルを作成するために、ラットでのHIV増殖の非効率の原因の解明を目的とした。特に、HIV-1の侵入過程と感染性ウイルス粒子の形成に関与している細胞性因子の同定を試みた。また、ヒトCCR5,CXCR4,CD4,CRM1, CycT1遺伝子を発現するTgラットを作製し、ex vivo で primary T細胞とマクロファージにおけるHIV-1複製の解析を行った。

研究目的

エイズの根本的な予防と治療法を開発するために，HIV感染小動物モデルの確立はきわめて有用である。特に、 近交系が確立しているために、詳細な免疫学的な解析が可能であり、遺伝子操作が可能なラットやマウスにウイルスを感染できれば研究開発に大いに役立つ。中でも、ヒトのCD4とケモカイン受容体を発現させてやれば、HIVがわずかに増殖できる事から、ラットは良い感染動物モデルになる可能性を秘めている。

当研究室でヒトCD4/CCR5を発現するラットT細胞株に、HIV RevのコファクターであるヒトCRM1と、Tatのコファクターである CyclinT1を発現させ，HIV-1分子クローンのを導入すると、ヒトT細胞株の約1/3のHIV-1粒子が生産されることが見いだされた。しかし、感染が広がらないことが分かった。このことから、ラットT細胞におけるHIV-１の複製は感染性のある子孫ウイルスの生産、又は、ウイルスの次のターゲット細胞への伝播に種間バリアーが存在すると考えられた。実際にラットT細胞株から生産されたHIV-1 NL4-3株は感染性が低かった。このことは、ラットT細胞にウイルス粒子の感染性に関与する阻害因子があることを予想させる。

他方、ラットT細胞への感染効率は細胞株によって異なるものの、primary T細胞で特に低いことが分かった。さらに、サイクロフィリンA(CypA) の阻害剤サイクロスポリンA(CsA)を作用させると感染効率が上がった。このことはCypAと協力してHIV-1の脱殻と核移行の段階を阻止するサルのTrim5αを想起させる。そこで、ラットTrim5をノックダウンしたところ、感染効率は変わらなかった。Trim6も関与していなかった．これらのことは侵入段階でHIV感染を阻止する新規の因子を有していることを示唆している．そこで、これらの阻害因子を同定し、ノックダウンしてやれば高感受性のラットモデルができると考えられる。本研究の目的はこれらの阻害因子を同定することである。

本研究を進める過程において、ヒトTRIM5αとラットTRIM5がHIV-1の生産を減弱させるとの結果を得た。其の機構の解明を本研究のもう1つの目的として追加した。

研究方法

1. ラットT細胞由来HIV NL4-3株の低感染性の解析

ウイルス粒子の低感染性とEnv蛋白の関与を調べるため、Env 欠損 HIV-1 とVSV-G分子クローンをラットT細胞株に導入し、できたシュードウイルスの感染性を indicator細胞(TZM-bl)で測定した。また、Western blottingによってGagとGタンパク質を検出した。

HIV-1 Env蛋白質の安定性を測定するため、HIV-1ゲノムを導入した細胞を蛋白質合成阻害剤である cycloheximideで処理し、細胞を経時的に回収した。 Cell lysateを Western blotting によって分析した。

2. Tgラット由来primary 細胞における HIV-1複製の解析

ラットprimary細胞へHIV-1を導入するため、hCRM1と CycT1を発現するTgラットからprimary T細胞とマクロファージを調製した。脾臓細胞 からprimaryT細胞をナイロンウールカラムで精製した。そして、抗ラットCD3抗体と CD28 抗体で 活性化し、HIV-1 分子クローンを nucleofectorを用いて導入した。マクロファージを得るため、ラット腹水に含まれるmonocyteを抗ラットCD11b抗体と反応させ、 anti-IgA MicroBeads で分離し，GM-CSF存在下で、接着培養した。そして、VSV-G coated

HIV-1を感染させた。HIV-1の増殖をHIV-1 p24 ELISA 法で測定した。

3. HIVの侵入過程で働く阻害因子の同定

1) マイクロアレイー法による侵入過程で働く阻害因子の同定

HIV-1の侵入効率に関して両極をなし、更にサイクロスポリンA(CsA)の効果が異なるラットT細胞株であるFPM1, C58NTD, NB2のTotal RNAを精製した。Agilent社においてcDNAマイクロアレイを作製し、GeneSpringGX及びGenMAPPのソフトウェアで各細胞の遺伝子発現プロファイルを比較した。

候補cDNA発現コンストラクトを入手し、ヒト293T細胞に発現させ、 VENUSを発現するHIVシュードウイルス(HIV-Venus)を感染させて、FACSでHIV-1感染阻害効果を調べた。

2) Functional Cloning法による侵入過程で働く阻害因子の同定

HIV-1侵入効率の低いFPM1細胞から抽出したmRNAを基にレトロベクターcDNAライブラリーを作成し、ヒトMolt5CCR5細胞にトランスデュースし、ラット遺伝子発現細胞群を構築した。HIV-Venusを感染させて、VENUS$^-$細胞をFACSvantageの自動細胞捕集装置で回収し、細胞をクローニングした。ついで、ベクター部分に設計したプライマーを用いて、VENUS$^-$ 細胞からラットcDNAを回収した。cDNAの発現コンストラクトを作製し、293T細胞にトランスフェクション後、HIV-venusの感染阻害効果を調べた。

4. ヒトTrim5αのHIV-1産生抑制効果の検討

1). ヒト Trim5αの過剰発現よるHIV-1産生の抑制効果

アカゲザル、ヒトTRIM5α、またはSPRYドメインに点変異した変異体をHIV-1 分子クローンと293T細胞に導入し、細胞内または培養上清中にHIV-1 Gagの量をp24 ELISAまたは western blottingで検討した。

2). 内在性ヒトTrim5αのHIV-1 産生の抑制効果

HIV-1分子クローンを導入したヒトHT1080また Jurkat細胞に抗TRIM5α siRNAをlipofectamineまたはnucleofectionにより導入し、培養上清中のHIV-1 Gagの量を測定した。

3). ヒトTrim5αのHIV-1粒子内への取り込み

上記の培養上清を20% sucrose層にのせ、超遠心によってHIV-1粒子を濃縮した。Western blottingによってTrim5αと Gag 蛋白質を検出した。

(倫理面への配慮)

本研究動物実験については、厚生労働省基本指針と北海道大学動物実験機関内規程に沿って行い、動物愛護の精神で動物に与える苦痛の軽減と排除に勤めた。

研究結果

1. ラットT細胞株由来ＨＩＶ－１粒子感染性低下原因の解明。

ラットT細胞株由来NL4-3粒子の感染性が低い原因を解明するため、まず、Envの関与を調べた。Env 欠損 NL4-3分子クローン と VSV-Gをラット T細胞株に導入し、できたシュードウイルスの感染価を調べた。 P24量を基準として同量のウイルスの感染性を測定したところ、ラットT細胞由来のウイルスの感染性は,ヒトT細胞であるMolt4細胞由来のウイルスよりも高い感染性を示し、G蛋白の量と関連していた。この事はVSV-Gでコートされたラット T細胞由来のHIV粒子は十分に感染性を有することを示している。また、ラットT細胞株由来NL4-3ウイルス粒子をWestern blottingで分析したところ、粒子内に取り込まれたEnv蛋白質の量がラット上皮細胞またはヒト細胞由来のHIV-1粒子より少ないことが分かった。さらにcycloheximideを用いてラットT細胞内に発現したEnv蛋白質の安定性を調べた結果、Env蛋白質は不安定で、速やかに分解されることが分かった。それに対し、ラットT細胞で作られたGag蛋白質は他の細胞のGagと同様安定であった。これらの結果から、ラットT細胞で作られるNL4-3 HIV-1粒子の感染性低下はENV蛋白に原因があることが分かった。

しかし、他の種々のHIV株(AD8, JR-CSF, YU-2, 89.6, Lai2等)を用いて、感染性を調べた結果、ラットT細胞株で作られたAD8, JR-CSF, YU-2等のマクロファージ指向性HIV-1は、ヒトT細胞由来のウイルス粒子と同等の感染性を持つことが分かった。一方、ラットT細胞株由来のNL4-3, Lai2などのT細胞指向性HIV-1と両指向性の89.6株は感染性が低いことが確認された。

以上in vitro の研究結果からラットT細胞由来HIV-1粒子感染性低いのは特定のHIV－１株に限られることがわかった。

2. 新規に作成したTgラットにおけるHIV-1複製の解析

当研究室でヒトCD4/CCR5/CXCR4/CyclinT1/CRM1 Tgラットの作製に成功したので、このTgラットにおけるHIV-1の複製を解析した。

まず、本Tgラットから肺胞マクロファージを調製して,EGFPをコードするHIV-1 AD8株を感染し、GFP陽性細胞の割合をFACSで測定する事により、感染効率を調べた。その結果、ラットマクロファージへの感染効率はヒトマクロファージ以上である事が分かった。このことは、ラットマクロファージにおいては侵入過程で阻害因子が存在しないことを示唆している。

また、このTgラットのマクロファージから作られたウイルス産生量を測定したところ,ヒト細胞に准じるものである事（1/6-1/2まで）が分かった。更に、産生されたウイルスはR5 type、X4 typeともに高感染性であることが分かった。これらの事により、ラットマクロファージにおいては侵入過程、粒子形成過程においても阻害因子が存在しないと示唆された

一方、TgラットからT細胞を調製し、Gタンパク質でコートした、GFP発現HIV－１の感染効率を測定したところ、ラットT細胞への感染効率はヒト細胞より低いことを確認した。このことは、ラットT細胞には侵入過程で働く阻害因子があることを示唆している。

しかし、HIVゲノムをTgラットprimary T細胞に導入すると、ヒトPBLの約3分の１のウイルスが生産された。また、primary T細胞からの子孫ウイルス(NL4-3, AD8, JR-CSF)に感染性があることが分かった。このことは、上記ラットT細胞株を用いた結果と異なり、実際にラットにおけるHIV-1感染性粒子を生成する過程で厳密なブロックがないことを示唆している。

3. HIV-1侵入過程で働く阻害因子の同定

ラットT細胞株にはHIV-1の侵入効率の高いものと低いものがある。サイクロフィリンA(CypA)の阻害剤サイクロスポリンA(CsA)を作用させると侵入効率の低いT細胞株への感染効率はヒトT細胞とほぼ同等にまで上がった。侵入効率の高いラットT細胞株では、ヒトT細胞と同様に感染効率がやや下がった。このことは、ラットT細胞は侵入を支持する因子を欠くのではなく、Trim様阻害因子を持つ事を示唆している。

各ラットT細胞株の遺伝子発現プロファイルを入手し、分析した。HIV-1の感染効率が低く、CsA処理により感染効率が上がる細胞で発現が高く、HIV-1感染効率が高く、CsA処理で下がる細胞で発現が低い遺伝子の発現クローンを１８個入手し、293T細胞に発現させ、HIV-1の感染への影響を調べた。その結果一つの分子クローンgene X(特許申請の関係で遺伝子名を伏せさせていただきます。)がHIV-1感染を顕著に抑制した。gene XはDNA, RNAとの結合能を持ち、胚の発達の調節、アポトーシスの制御などに関与すると報告されている。しかし、gene Xノックアウトマウスは生存可能である。

一方、ラットcDNAを導入したHIV-1抵抗性のMolt4CCR5細胞をFACSvantageでスクリーニングした、その中からHIV-1感染効率がサルTRIM5α発現Molt4CCR5と同程度、又はより低い細胞クローンを多数得た。そのなかからいくつかの宿主遺伝子を同定した。しかし、見出した遺伝子をさらに分析したところ、HIV-1 感染耐性とは無関係であることが分かった。現在のところ、残りのクローンのHIV-1感染阻害効果を分析している最中である。

4. ヒトTrim5αのHIV-1産生の抑制効果

本研究過程で過剰発現したヒトTrim5αがHIV-1の生産を抑制することを見出した、そこで、ヒトTrim5αのHIV-1の複製への影響を詳細に調べ、以下のことを明らかにした。過剰発現したヒト野生型のTrim5αは、量依存的にHIV-1 Gagの産生を抑制した。また、437番目アミノ酸がアルギニンからシステインへ変異しているヒトTrim5α変異体（R437C)はGagの産生を抑制しなかった。ヒトHT1080細胞とJurkat細胞ではsiRNA でTrim5αをノックダウンすることにより、Gagの生産量が約２倍から３倍増加した、一方、元々Trim5αの発現が少ない293T細胞では、Gagの生産量に変化はなかった。野生型のヒトTrim5α はわずかにHIV-1粒子に取り込まれた。R437C変異体の粒子への取り込みは更に少なかった。野生型ヒトTrim5α がMLV-Nの感染を抑制するのに対し、R437C変異体は抑制しなかった。

考察

本研究当初ラットT細胞から生産されたHIV粒子は感染性減弱細胞性因子を含むと仮定して実験を進めてきた。そこて、ラットT細胞株由来のNL4-3 粒子の感染性の低い原因が、細胞内でEnv蛋白質が不安定で、粒子内に取り込まれる量が少ない事にある事を明らかにした。しかし、このことは、T細胞指向性または両指向性HIV－１株に限られ、マクロファージ指向性HIV-1 株は、ヒト細胞で作られたHIV-1 株と同等の感染性を持っていた。更にラットprimary T 細胞において、hCRM1とhCycT1を発現するTgラットT細胞から高感染性のHIV-1がヒト細胞に準じる量（1/10-1/3）生産されるとの結果から、ラットでは、HIV-1感染の後期過程において厳密な種間バリアーが存在しないと考えられる。

最近、他の研究グループよりラットCD317(Tetherin)がHIV-1粒子の放出を抑制するとの報告があった。このことから、当研究室で作成したTgラットにCD317をノックダウン／アウトすることより感染性を持つHIV-1粒子のヒト細胞に匹敵する生産が期待できる。

マイクロアレイー法で同定されたラットgene Xは直接HIV-1感染を阻害する可能性を持ち，さらに解析を進める予定である。また、他のレトロやレンチウイルスの感染にも抑制効果を持つ可能性も考えられる。

以上の結果を基にヒトCCR5,CXCR4,CD4,CRM1,CycT1 Tgラットとgene X/CD317ノックダウン／アウトラットを交配することより高感受性HIV-1感染ラットモデルの開発が期待される。

ヒトTRIM5αがヒト細胞でHIV-1産生を阻害することは感染者体内でのHIV-1の生産の抑制にも一定寄与していることを示唆しており、遅い発症の１原因であると考えられる。ラットTRIM5がヒトTRIM5αと共にHIV-1のGag蛋白の生産を抑制することから、ラットTRIM5をノックダウンする事によって、更なるHIV-1感染ラットモデルの改善が期待される。

結論

種々ののHIV-１株とCCR5,CXCR4,CD4,CRM1,CycT1 Tgラット由来のprimary マクロファージとT細胞の解析から、ラットではHIV-1感染の後期過程において厳密な種間バリアーが存在しないことがわかった。

ついで、HIV-1侵入過程で働くT細胞の持つ阻害因子を同定した。同定された阻害因子とTetherinのノックダウン／アウトラットを作製し、当研究室で開発されたCCR5,CXCR4,CD4,CRM1,CycT1 Tgラットと交配することより高感受性HIV-1感染ラットモデルの構築が期待できる。

健康危険情報

特になし

知的所有権の出願・取得状況

新規ラットHIV感染阻害因子について，出願検討中

研究発表

別添リストを参照

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Synergistic effect of human CycT1 and CRM1 on HIV-1 propagation in rat T cells and macrophages

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Published: 12 May 2009

Retrovirology 2009, **6**:43 doi:10.1186/1742-4690-6-43

Received: 11 September 2008
Accepted: 12 May 2009

This article is available from: http://www.retrovirology.com/content/6/1/43

Abstract

Background: *In vivo* studies of HIV-1 pathogenesis and testing of antiviral strategies have been hampered by the lack of an immunocompetent small animal model that is highly susceptible to HIV-1 infection. Although transgenic rats that express the HIV-1 receptor complex hCD4 and hCCR5 are susceptible to infection, HIV-1 replicates very poorly in these animals. To demonstrate the molecular basis for developing a better rat model for HIV-1 infection, we evaluated the effect of human CyclinT1 (hCycT1) and CRM1 (hCRM1) on Gag p24 production in rat T cells and macrophages using both established cell lines and primary cells prepared from hCycT1/hCRM1 transgenic rats.

Results: Expression of hCycT1 augmented Gag production 20–50 fold in rat T cells, but had little effect in macrophages. Expression of hCRM1 enhanced Gag production 10–15 fold in macrophages, but only marginally in T cells. Expression of both factors synergistically enhanced p24 production to levels approximately 10–40% of those detected in human cells. R5 viruses produced in rat T cells and macrophages were fully infectious.

Conclusion: The expression of both hCycT1 and hCRM1 appears to be fundamental to developing a rat model that supports robust propagation of HIV-1.

Background

A small-animal model of HIV-1 infection is needed for development of prophylactic vaccines and more efficient antiviral therapies. Current animal models of HIV infection, including non-human primates [1-4] and severe combined immunodeficiency (SCID) mice transplanted with fetal human cells [5,6], have made significant contributions to our understanding of lentiviral pathogenesis and to the development of vaccines and therapeutic agents. However, these models have shortcomings, such as their limited availability and high cost, their permissivity restricted to related retroviruses of nonhuman primates, as well as the absence or insufficient induction of an immune response against HIV-1. Therefore, a better small-animal model is needed.

Rodents may be useful models if they can be infected with HIV-1. Because they are established experimental animals,

inbred strains are available, and genetic manipulations can be performed. However, a fully permissive model has not been developed yet because of several inherent blocks to HIV-1 replication in rodent cells. One major block to HIV-1 replication is at the level of viral entry into the cell; this may be overcome by introducing human CD4 (hCD4) and CCR5 (hCCR5) [7,8]. Indeed, transgenic (Tg) rats expressing these receptors support some HIV-1 replication, albeit poorly [8], whereas Tg mice expressing hCD4 and hCCR5 do not support any HIV replication [9]. These results suggest that rats may provide a good small-animal model.

Studies on rodent cell-specific defects in the HIV-1 life cycle after viral entry provide the molecular basis for improving the propagation of HIV in rodents. However, several studies using established cells lines [7,10,11] have indicated that there are cell line specific defects in each step of the viral life cycle. Moreover, technical difficulties have hampered detailed analyses of the function of cellular cofactors in rodent T cells and macrophages, particularly primary cells.

A study of the effects of rodent cellular factors on the function of the viral factors Tat and Rev will be of importance because of the essential roles these proteins play in viral propagation. Currently, controversial results have been reported regarding the existence of a profound block affecting Tat function in rodent cells. In early studies, human CyclinT1 (hCycT1), identified as a Tat interacting protein that is crucial for transcription during HIV-1 replication [12], was expressed in mouse NIH 3T3 fibroblasts and transcriptional activity was dramatically enhanced [13,14]. Moreover, hCycT1 Tg mice supported the enhanced expression of an integrated HIV-1 provirus [15]. A single amino acid difference between human and mouse CyclinT1 (mCycT1), which has a tyrosine at residue 261 in place of the cysteine amino acid in hCycT1, causes almost a complete loss of Tat cofactor activity [13,14]. In contrast to mouse cells, rat cells support significant amounts of Tat function, even though rat CyclinT1 (rCycT1) has a tyrosine at residue 261 and shares ~96% sequence homology with mCycT1. Only 2–5 fold enhancement of Tat function by overexpression of hCycT1 in rat cells has been reported. Moreover, since the reported experiments lacked the expression of rCycT1 as a control, uncertainty remains whether it was the quantity or the quality of exogenously-expressed hCycT1 which augmented Tat function [7,16,17]. On the other hand, a substantial increase in Gag protein levels upon hCycT1 expression in a rat myelomonocytic precursor cell line has been reported [18].

Rev function is involved in the expression of the unspliced 9-Kb and partially-spliced 4-Kb RNAs that encode the HIV viral genome and the structural proteins [19]. Rev activity that supports HIV-1 replication in rodent cells has also been debated, although a reduction in the ratio of the unspliced 9-kb transcript to the fully-spliced 2-kb viral transcript in rodent cells has generally been reported [7,10]. Moreover, the role of the rat counterpart of hCRM1, which exports HIV RNAs in cooperation with Rev [20,21], has been incompletely explored. Instead, oversplicing or a reduced stability of unspliced transcripts in rodent cells compared to human cells has been proposed [22], which has been reported to be repaired by the expression of the human p32 protein [23].

In this study, we investigated the effect of human CyclinT1 and CRM1 expressed in rat T cells and macrophages, including primary cells, in order to identify a molecular basis for improving a rat model for HIV-1 infection. Our results show that co-expression of hCycT1 and hCRM1 synergistically promotes Gag p24 production. Interestingly, cell type specific requirements for these two human factors were detected.

Methods

Cells and plasmids

Rat T cell lines, FPM1 [25] and C58(NT)D (ATCC TIB-236), a rat macrophage line, NR8383 (ATCC CRL-2192), and human T cell lines, Jurkat and Molt4R5, were used for propagation of HIV-1. TZM-bl cells were used to measure the infectivity of HIV-1 according to previously described procedures [26]. NR8383hCRM1, FPM1hCRM1, FPM1hCT, and FPM1hCT/hCRM1 expressing hCRM1, hCycT1, or both were constructed as described previously [40].

To construct hemagglutinin (HA)-tagged hCycT1, pβCycT, which harbors the human cyclinT1 cDNA in the pCXN2 vector, was used as a template for PCR with forward (5'-ggtctagagcactatggagggagagaggaag-3') and reverse (5'-gggaattcatgcatagtctggtacatcgtaggggtacttaggaaggggt-ggaagtggtgg-3') primers with the following amplification conditions: 2 min at 94°C, 30 cycles of 30 s at 94°C, 60 s at 64°C, 2.5 min at 72°C, and a final extension for 10 min at 72°C. The amplified DNA was digested and inserted between the *Eco*RI and *Xba*I sites of pCXN2 [41].

Rat Cyclin T1 mRNA was extracted from rat ER-1 neo1 cells using the Absolute RNA extraction Kit (Stratagene) and amplified by RT-PCR using the following primers: 5'-ccgaattcaagcactatggagggagagaggaa-3' and 5'-ccgaattcatg catagtctggtacatcgtaggggtacttaggaagaggtggaagaggtgg-3'. The amplification conditions were: 94°C for 2 min, 30 cycles of 15 s at 94°C, 30s at 60°C, 2.5 min at 68°C, and a final extension for 5 min at 68°C. The amplified DNA was digested and inserted into the *Eco*RI site of pCXN2.

To construct pSRαrCRM1-HA, pSRαrCRM1 was used for PCR with the following primers: 5'-ctggaatcacttggcagct-

gagctctacagagagagtcca-3' and 5'-tatggtaccttaagcataatcaggaacatcgtatgggtagtcacacatttcttct-gggatttc-3'. The amplification conditions were: 2 min at 94°C, 20 cycles of 30 s at 94°C, 1 min at 62°C, 2 min at 68°C, and a final extension for 10 min at 68°C. The amplified DNA was digested and inserted into the SacI and KpnI sites of pSRαrCRM1.

The following plasmids were used in this study: pSRα296 [42]; pCRRE [35]; pΔpol [24]; pMaxGFP (Amaxa) and pCDMβ-gal [43]; pNL4-3 [30]; pYU-2 [28]; p89.6 [32]; pLAI-2 [31]; pYK-JRCSF [27]; and pNLAD8-EGFP [29]. pH1-luc (a gift from Dr. A. Adachi) contains a luciferase coding sequence downstream of the HIV-1 LTR. pSRαhCRM1-HA was a gift from Dr. T. Kimura.

Development of Human Cyclin T1 Transgenic (Tg) Rats

An hCycT1 BAC (RZPD;RZPDB737F032099D) was microinjected into fertilized rat (F344) eggs. To identify Tg rats, total genomic DNA extracted from rat tail snips was examined by PCR using two sets of PCR primers with one primer annealing the BAC backbone vector and the other annealing the 5' or 3' end of hCyclin T1 genomic DNA. Primers CTB3 (gccaacgctcaatccggttctcgc) and CTGB3 (gctattttccagctgttctcgagtg) were used for the 5' end. Primers CTB4 (ttattccctagtccaaggatgac) and CTGB4 (cagacaatagactatcaagacactgtg) were used for the 3' end. PCR was performed using 500 ng of DNA as a template with the following amplification conditions: 94°C for 2 min, 30 cycles of denaturation (94°C for 1 min), annealing (58°C for the 5' end primers and 54°C for the 3' end primers, 30s), extension (72°C, 1 min), and a final extension (72°C, 5 min).

Preparation of rat primary cells and human cells

Rat primary T cells were enriched from splenocytes using a nylon wool column. More than 95% of the cells were $CD3^+$ cells, as evaluated by Flow Cytometry (FACS Calibur; Becton Dickinson). The cells were stimulated for 2 days with an anti-rat CD3 mAb (5 μg/ml) and an anti-rat CD28 mAb (0.5 μg/ml) that had been coated on the culture plates. $CD4^+$T cells were then isolated by negative selection using anti-rat CD8 MicroBeads (Miltenyi Biotec). Isolated $CD4^+CD8^-$T cells were >90% pure, as determined by staining with anti-rat-CD4 (BD Biosciences Pharmingen) and anti-rat-CD8 (BD Biosciences Pharmingen).

Rat peritoneal macrophages were isolated from rats that had been treated with 3% thioglycollate for 3 days. The macrophages were coated with anti-rat CD11b and isolated using goat anti-mouse IgG MicroBeads (Miltenyi Biotec). Isolated $CD11b^+$ peritoneal cells were >90% pure, as determined by staining with mouse anti-rat-ED2 (BD Biosciences). Isolated $CD11b^+$ $ED2^+$ peritoneal cells were cultured for 2 h at 37°C to allow them to adhere to the plates.

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll Paque Plus (Amersham Biotechnology) density centrifugation. The cells were activated with 5 μg/ml phytohemagglutinin-P (PHA-P) (SIGMA) and 20 U/ml IL-2 (PeproTech EC) for 3 days at 37°C. Peripheral blood lymphocytes (hPBLs) were harvested as non-adherent cells.

Human monocytes were isolated from PBMCs using anti-CD14 conjugated to magnetic beads (Miltenyi Biotec), and allowed to adhere on dishes at 37°C for 1 h in RPMI 1640 supplemented with 1% human serum. Human monocyte-derived macrophages (MDMs) were then generated by incubation in RPMI 1640 supplemented with 15% FBS, antibiotics, and GM-CSF (10 U/ml) (R & D) for 5 days.

Electroporation

Cell lines (2×10^6) and primary T cells (1×10^7) were electroporated in 100 μl of Nucleofector Solution (Cell line Solution V, Mouse T cell and human T cell Nucleofector kit, Amaxa Biosystems,) using the conditions (FPM1;T-03, C58(NT)D;T-20, NR8383;T-27, and rat primary T;X-01, Jurkat;X-01, Molt4R5;A-30, hPBL;U-14) and plasmids described in the Figure Legends. After 48 h, p24 in the supernatant and in cells was quantified using a p24 ELISA kit (Zeptometrix). In some cases, the viruses were concentrated by centrifugation at 15,000 rpm for 90 min in a microcentrifuge and p24 was quantitatively recovered from the pellets.

Western Blotting

Cells were lysed in buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.5% NP40, and protease inhibitors or sample buffer without mercaptoethanol and dye, and protein concentrations were determined by BCA assay. Samples containing 50 μg protein were then subjected to Western blotting using anti-CycT1 (Novocastra Laboratories Ltd), anti-CRM1 [42], anti-HA (Behringer), or anti-β-actin (SIGMA).

Infection

Rat peritoneal macrophages and human MDMs were seeded at a density of 5×10^5 cells/well in 24 well plates and cultured for 1 day at 37°C. Macrophages were then inoculated with VSV-G-coated NL43 and NLAD8-EGFP (50 ng), which were prepared by transfection of pNL4-3 or pNLAD8-EGFP along with pVSV-G to 293 T cells with Fugene6, in the absence or presence of 20 μM PMPA [44] overnight at 37°C. Finally, cells were washed gently 5 times and 2 ml of RPMI containing 15% FCS with or without PMPA was added.

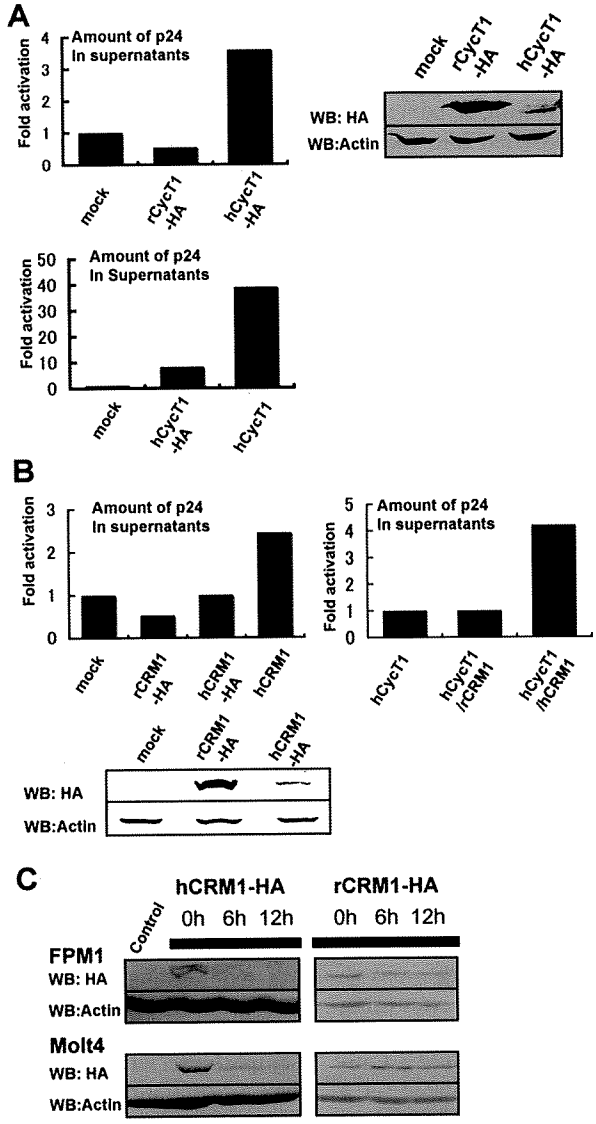

Figure 1

Effect of hCycT1 and hCRM1 expression in rat T cell lines (part 1). (A) FPM1 cells were electroporated with 2 μg pΔpol, 1 μg pMax-GFP, and 1 μg pCXN2, pCXN2hCycT1-HA, pβhCycT1, or pCXN2rCycT1-HA. After 2 days, p24 levels in the medium were measured by ELISA. The percentage of living cells was approximately 18% and approximately 95% of the living cells were GFP+ based on FACS analysis. The ratio of p24 in the CycT1 containing samples relative to mock treated samples was calculated. The total amount of p24 in the hCycT-HA containing sample was 119 pg. Values are means of duplicate samples. rCycT1 and hCycT1 were detected by Western blotting using anti-HA. (B) FPM1 cells were electroporated with 2 μg pΔpol, 1 μg pMax-GFP, and 0.5 μg pSRα296, pSRα hCRM1-HA, pSRαrCRM1-HA, or pSRαhCRM1. The percentage of living cells was approximately 4%, and 60% of the living cells were GFP+. The total amount of p24 in the sample containing hCRM1 was 146 pg. In the right panel, 1 μg pCNXhCycT1 was included. Values are means of duplicate samples. The total amount of p24 in the sample containing hCRM1 was 15.7 ng. (C) pSRα296, pSRαhCRM1-HA, or pSRαrCRM1-HA (0.5 μg) were electroporated into FPM1 and Molt4 cells, and 50 μg/ml cycloheximide was added after 24 h. The cells were then collected at 0, 6, and 12 h after the drug addition, and analyzed by Western blotting. Various amounts of the cell lysates were used for blotting (25 μg of hCRM1-HA containing FPM1, 5 μg rCRM1-HA containing FPM1, and 25 μg of hCRM1-HA or 10 μg of rCRM1-HA containing Molt4, respectively).

Results

Synergistic Effects of hCycT1 and hCRM1 in Rat T cell lines

Since controversial results regarding the activity of Tat in rat cells have been reported, we compared the effect of hCycT1 versus rCycT1expression in rat T cells. To express the HIV-1 genome and CycT1 in rat T cells, we used the electroporation of CycT1 and an HIV-1 genome expressing plasmid, since we experienced very low rates of HIV-1 infection even with VSV-G coated particles. In our hands, electroporation was the only way to introduce enough HIV genome into rat T cells. We co-electroporated pMax-GFP or pCDM-βgal to monitor the efficiency of electroporation. When we electroporated pΔpol, which was constructed by deleting 328 base pairs in the pol gene of the infectious pNL43 genome [24], and HA-tagged hCycT1 or rCycT1 into FPM1 cells, a rat $CD4^+$ T cell line transformed with HTLV-1 [25], Gag p24 production was enhanced several fold in the presence of hCycT1-HA. However, hCycT1 expression was very low. In contrast, rCycT1-HA was efficiently expressed, but did not alter Gag p24 production. Since hCycT1-HA may be unstable, we next used an untagged hCycT1 for co-electroporation. We detected a 40 fold enhancement of Gag production in the presence of hCycT1 (Fig. 1A). The band corresponding to hCycT1 was, however, hardly detected by Western blot analysis (data not shown). The reason why untagged hCycT1 enhanced expression more efficiently than hCycT1-HA is currently unclear, because the intracellular amounts of these hCycT1s cannot be exactly compared due to the different abilities of the anti-HA mAb and anti-hCycT1 antibody.

Next, to assess Rev activity in rat T cells, we compared the effects of hCRM1 and rCRM1 on HIV-1 propagation. When we electroporated HA-tagged CRM1 expression plasmids and pΔpol into FPM1 cells, p24 production was not significantly increased. The level of hCRM1-HA detected by Western blotting was very low. However, we reproducibly observed a 2–4 fold enhancement of p24 production in cells transiently expressing untagged hCRM1, but not rCRM1 (Fig. 1B). These results suggest that endogenous rCRM1 supports p24 production less efficiently than the hCRM1 and that Rev function is not absolutely blocked in rat T cells. To examine the stability of CRM1-HA, we added cycloheximide to inhibit translation in CRM1-transfected T cells and examined CRM1 protein levels over time. In both rat and human T cells, hCRM1-HA was much less stable than rCRM1-HA (Fig. 1C), partly accounting for the lower amounts of hCRM1 (See Fig. 1B).

To examine the effects of both hCycT1 and hCRM1 on HIV-1 propagation in rat T cells, including FPM1 and C58(NT)D cells, we co-electroporated these expression plasmids with pΔpol. Additionally, we co-transfected pH1-Luc, which expresses the luciferase gene driven by the HIV-1 LTR, to examine the effect of hCycT1 and hCRM1 on Tat-directed gene expression. Expression of hCycT1, but not hCRM1, enhanced LTR-derived expression several fold, consistent with the previously reported functions of these proteins. Notably, the enhancement of p24 production by hCycT1 was substantially greater than that of the luciferase activity. Furthermore, levels of extracellular p24 were more enriched than intracellular levels, and hCycT1 synergistically cooperated with hCRM1 to augment the synthesis of p24 by approximately 100 fold (Fig. 2A and 2B). These results suggest that hCycT1 enhanced the transcription of the LTR-driven HIV-1 pre-mRNA. Since the pre-mRNA is the source of mRNAs encoding Gag, Tat and Rev, its increase may trigger positive feedback in the synthesis of HIV-1 pre-mRNA as a result of increased Tat protein levels and in the amounts of unspliced mRNA as a result of increased Rev protein levels. Thus, Gag would be produced much more efficiently than luciferase. Subsequently, the enhanced Gag expression facilitates the more efficient release of viral particles. The level of p24 produced by rat T cells expressing both hCycT1 and hCRM1 was approximately 25–33% of the levels produced by the human T cell line Molt4 (data not shown).

To examine the effect of hCycT1 and hCRM1 on HIV-1 propagation using a full length HIV-1 clone, we electroporated pNL4-3 into FPM1 T cells that continuously expressed hCycT1 and hCRM1, and then quantified the production of p24. Again, hCycT1 greatly augmented p24 production, and hCRM1 had a moderate effect. Notably, the levels of hCycT1 and hCRM1 expression in FPM1 cells were similar to those in Molt4 cells (Fig. 2C). Thus, expression of these human factors should support robust HIV-1 propagation in rat T cells.

Synergistic Effects of hCycT1 and hCRM1 in rat macrophages

We examined the effect of hCycT1 and hCRM1 on p24 production and LTR-driven expression in the rat macrophage cell line NR8383, using the experimental approaches described above. Transient expression of rCRM1-HA in NR8383 cells did not affect p24 production, whereas hCRM1-HA enhanced p24 production 5–10 fold, although the level of hCRM1-HA expression was much less than that of rCRM1-HA (Fig. 3A). Expression of hCycT1 enhanced p24 production by only a few fold. The expression of hCycT1 was readily detected by Western blotting (Fig. 3B), in contrast to the low levels in rat T cells. Neither hCycT1 nor hCRM1 expression significantly affected luciferase expression driven by the HIV LTR (Fig. 3C). We also detected a greater than 10 fold enhancement of extracellular and intracellular p24 production in the presence of untagged hCRM1 (Fig. 3C), but not rCRM1 (data not shown). When hCycT1 and hCRM1 were co-expressed, they synergistically augmented p24 production

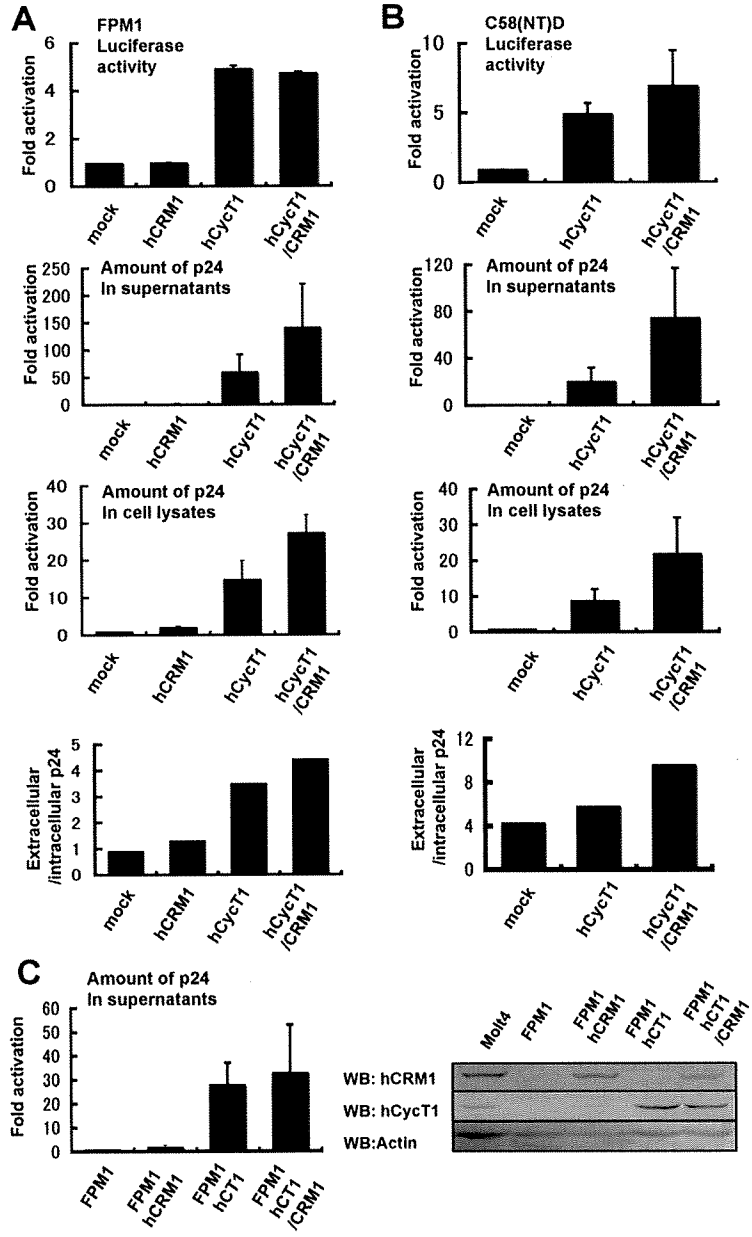

Figure 2

Effect of hCycT1 and hCRM1 expression in rat T cell lines (part 2). (A) FPM1 and (B) C58(NT)D cells were electroporated, as above, with the exception that 0.4 μg pH1-Luc and 0.2 μg pCDMβ-gal were used instead of pMax-GFP. LTR activity and transfection efficiency were measured by luciferase and β-gal assays using cell lysates. The luciferase/β-gal activity or the amount of p24 was calculated, and the value of the mock sample was normalized to 1. Values are means of triplicate samples and the SD was calculated. The amount of p24 in the FPM1 and C58(NT)D samples containing hCycT1/hCRM1 was 3.7 and 2.8 ng, respectively. (C) FPM1 cells continuously expressing hCycT1 and hCRM1 were electroporated with 4 μg pNL4-3 and 1 μg pMaxGFP. The percentage of living cells was approximately 10%, and 50% of the living cells were GFP+. The amount of p24 in the FPM1hCT/hCRM1 sample was 6.0 ng. Approximately 10 μg of each cell lysate were subjected to Western blotting.

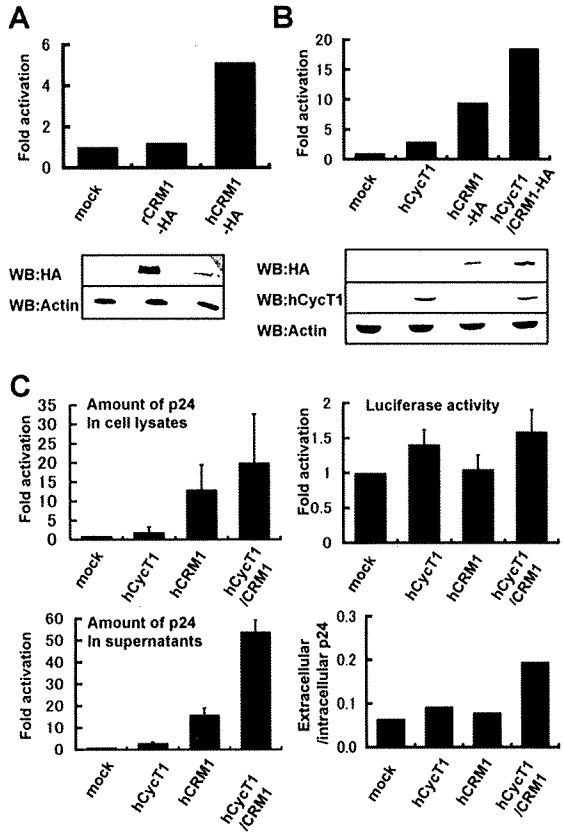

Figure 3
Synergistic effect of hCycT1 and hCRM1 in rat macrophage cell lines. (A) NR8383 cells were electroporated as described in Fig. 1B. The percentage of living cells was approximately 20–40%, and approximately 75% of the living cells were GFP+. The amount of p24 in the sample containing hCRM1-HA was 196 pg. Approximately 50 μg samples of the cell lysates were subjected to Western blotting as described in the Methods. (B) NR8383 cell lines were electroporated as described in Fig. 1A. The percentage of living cells was approximately 15%, and approximately 60% of the living cells were GFP+. The amount of p24 in the sample containing hCRM1-HA/hCycT1 was 56 pg. (C) NR8383 cell lines were electroporated with 2 μg pΔpol, 0.4 μg pH1-Luc and 0.2 μg pCDMβ-gal along with or without 1 μg pβhCycT1 and 0.5 μg pSRαhCRM. pSRα296 was added to adjust the total amount of the plasmids. The amounts of p24 in the cell lysate and medium of the sample containing hCRM1/hCycT1 were 488 and 96 pg, respectively. Values are means of triplicate samples.

by greater than 20–50 fold in NR8383 cells (Fig. 3B and 3C). The amount of extracellular p24 increased more than intracellular p24, as seen in T cells, suggesting that the increase in Gag expression facilitated more efficient release of viral particles. These results clearly indicate that hCRM1 augments p24 production in rat macrophages

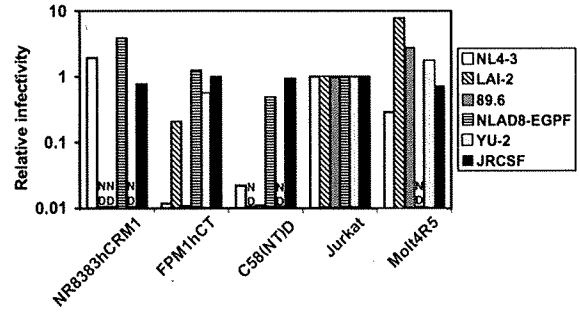

Figure 4
Infectivity of HIV-1 produced in rat and human cells. The medium [containing 50 or 500 pg of p24] from the various cell types electroporated with infectious clones was used to infect TZM-bl cells, and luciferase activity in the TZM-bl cells infected with various progeny viruses was normalized to that in cells infected with HIV-1 released from Jurkat cells. The relative infectivity of HIV-1 from Jurkat cells was normalized to 1. N.D: not determined.

more efficiently than hCycT1, in contrast to the effects of the two proteins in rat T cell lines.

Infectivity of HIV-1 produced by rat cells

To investigate whether HIV-1 produced by rat cells is infectious, we electroporated infectious HIV-1 molecular clones into rat and human cells and evaluated the infectivity of the progeny viruses using the indicator TZM-bl cells, which express luciferase upon HIV infection [26]. Luciferase activity versus inoculated p24 was used as a surrogate marker of infectivity. Interestingly, R5 viruses produced in rat T cells, including the JR-CSF [27], YU-2 [28], and NL-AD8 [29] strains, were equally infectious compared to those produced by human T cells, whereas rat T cell-derived ×4 and dual tropic viruses such as NL4-3 [30], LAI-2 [31], and 89.6 [32] varied in their infectivity. In contrast, both R5 and ×4 viruses produced in the macrophage cell line exhibited infectivities comparable to those from human cells (Fig. 4).

Characterization of hCycT1 and hCRM1 Tg rats

To examine the role of hCycT1 in primary cells, we constructed transgenic (Tg) rats that express hCycT1. Since the regulation of cyclinT1 gene expression is complex [33], a BAC harboring the entire human cyclinT1 gene, which is assumed to contain all the regulatory sequences, was microinjected into fertilized rat eggs. To confirm the expression of hCycT1 in the Tg rats, cells isolated from both thymus and spleen were analyzed by Western blotting using anti-hCycT1. Thymocytes, but not splenocytes, of Tg rats expressed hCycT1 (Fig. 5A). Since hCycT1 is expressed during the activation of human lymphocytes [33], we stimulated the splenocytes with anti-CD3 and anti-CD28. Expression of hCycT1 was detected within 1

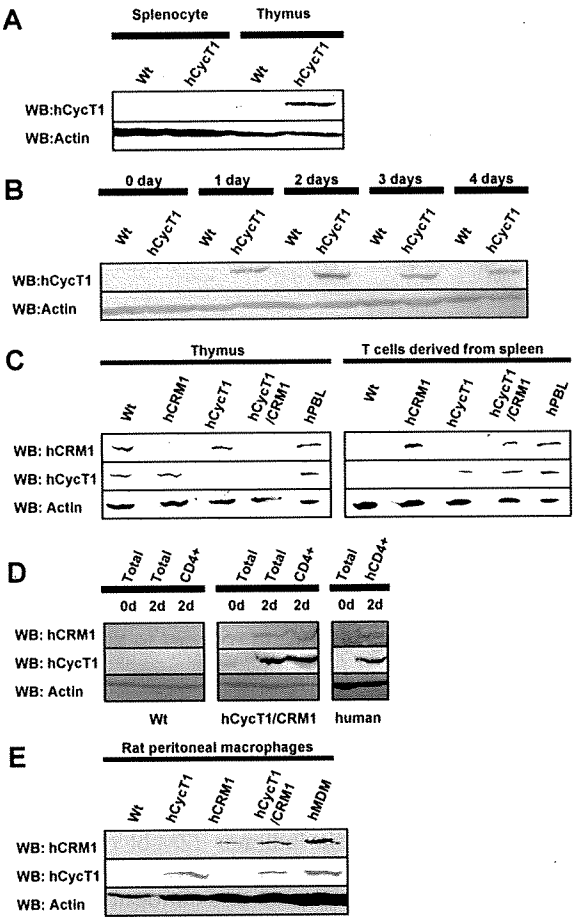

Figure 5
Characterization of hCycT1 and hCRM1 Tg rats. (A) The expression of hCycT1 in spleen- and thymus-derived cells from WT or hCycT1 Tg rats was confirmed by Western blotting using anti-hCycT1. (B) T cells derived from the spleen of WT or hCycT1 Tg rats were stimulated with anti-rat-CD3 and anti-rat-CD28. Cells were collected at the indicated times and subjected to Western blotting using anti-hCycT1. (C) The expression of hCycT1 and hCRM1 in spleen- and thymus-derived cells (C), total T and $CD4^+CD8^-$ T cells (D), and macrophages (E) in WT or Tg rats was confirmed by Western blotting using anti-hCycT1 and anti-hCRM1. T cells derived from the spleen of WT or hCycT1 Tg rats were stimulated with anti-rat-CD3 and anti-rat-CD28.

day and peaked 2 days after stimulation (Fig. 5B). Interestingly, rat splenocytes stimulated with phytohemaglutinin (PHA) and IL-2 did not express hCyCT1 (data not shown).

Expression of hCRM1 in Tg rats was also examined, using a previously established Tg rat [34]. hCRM1 was expressed in both thymocytes and splenocytes activated with anti-CD3/CD28 (Fig. 5C). hCRM1 was not expressed in unstimulated splenocytes (data not shown), consistent with hCRM1 expression in human PBMC [34]. We further characterized total T cells and $CD4^+CD8^-$ T cells prepared from double Tg rats in comparison to rat total T cells and human $CD4^+CD8^-$ T cells 2 days after stimulation. Both hCycT1 and hCRM1 were expressed in activated $CD4^+CD8^-$ T cells prepared from the Tg rat, similar to human $CD4^+CD8^-$ T cells (Fig. 5C and 5D). Both hCycT1 and hCRM1 were expressed in rat peritoneal macrophages at levels equivalent to expression in human monocyte-derived macrophages (MDMs) (Fig. 5E).

Ex vivo p24 production in T cells derived from hCycT1/CRM1 Tg rats

To investigate the effects of hCycT1 and hCRM1 on p24 production in primary T cells, we prepared T cells from splenocytes of wild-type (WT) and Tg rats and stimulated them with anti-CD3/CD28. As a control, isolated human PBLs were activated. In these experiments we used pCRRE [35], which harbors an HIV-1 genome with a deletion in the region from pol to vpr, instead of pΔpol [24], since introducing either pΔpol or the full-sized HIV-1 genome into the primary T cells by any method, including electroporation or VSV-G coated virus, had limited success.

T cells derived from hCycT1 Tg rats produced approximately 10–15 fold more p24 than WT T cells. In T cells derived from hCRM1 Tg rats, p24 production increased approximately 3 fold over WT cells. T cells-derived from hCycT1/CRM1 doubly Tg rats produced p24 at levels 24–40 fold greater than WT, and this level was ~40% of that produced by hPBLs (Fig. 6A). We further examined p24 production by $CD4^+CD8^-$ T cells prepared from double Tg rats in comparison to WT rat and human cells. $CD4^+CD8^-$ T cells prepared from double Tg rats produced p24 in the medium approximately 180 fold more efficiently than WT rat cells; this level was ~11% of the amount of p24 produced by human $CD4^+CD8^-$ T cells (Fig. 6C). These results indicate that the synergistic effects of hCycT1 and hCRM1 promoted the production of p24 in rat primary T cells *ex vivo*.

When intracellular p24 was evaluated by ELISA, increases of approximately 7 and 17 fold were observed in total T and $CD4^+CD8^-$ T cells, respectively (Fig. 6B and 6D), considerably less than the amount of extracellular p24 described above. The ratio of extracellular p24 to intracellular p24 increased gradually as p24 production increased, suggesting a more efficient virus release from the double Tg rat T cells compared to WT rat T cells.

Ex vivo p24 production in peritoneal macrophages derived from hCycT1/CRM1 Tg rats

To investigate HIV-1 propagation in macrophages derived from Tg rats, we prepared $CD11b^+ED2^+$ peritoneal macro-

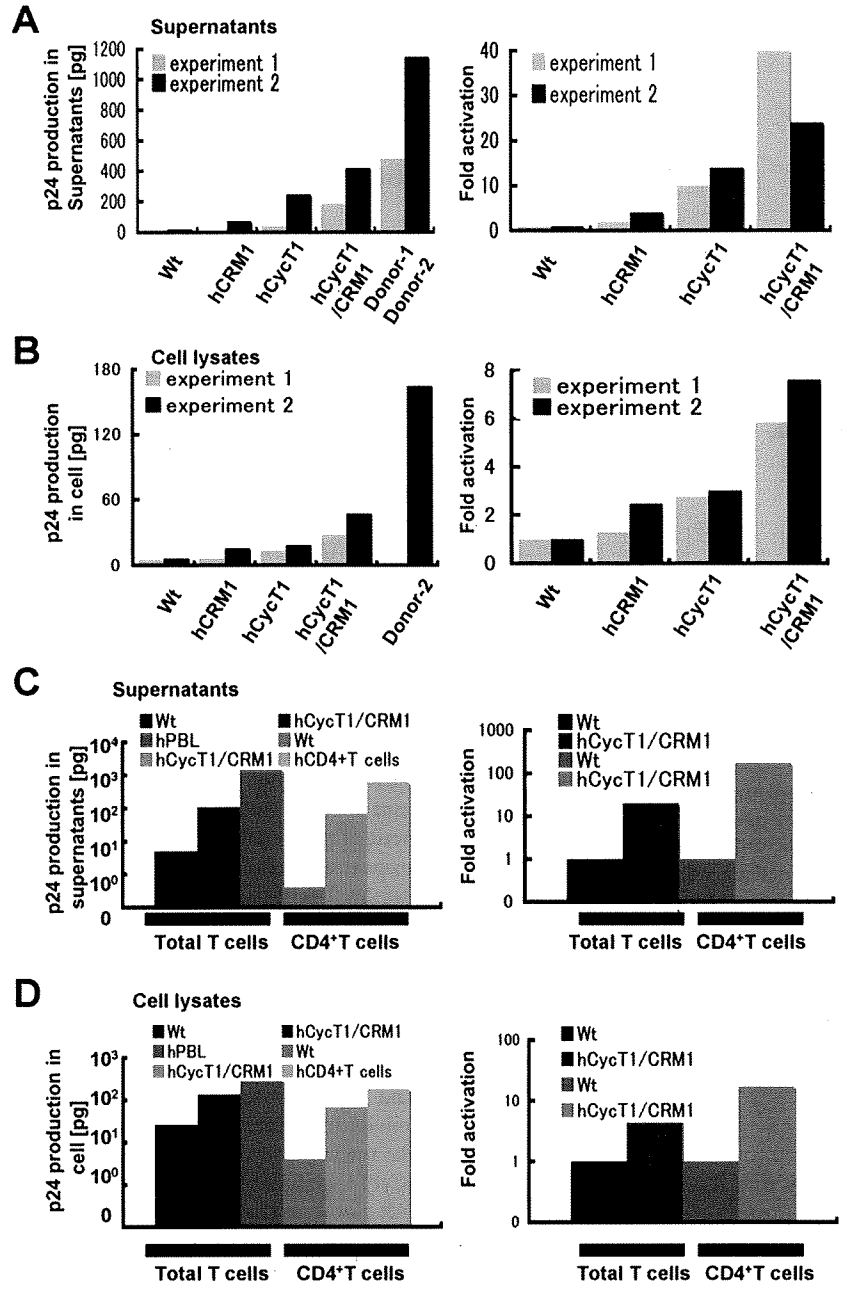

Figure 6

Quantification of p24 production in the total T cell fraction and CD4$^+$CD8$^-$ T cell fraction derived from hCycT1/CRM1 Tg rats. Stimulated spleen-derived T cells from WT or Tg rats and hPBL were electroporated with 4 μg PCRRE and 1 μg pMax-GFP, and p24 production in the supernatants (A) and cell lysates (B) was measured by ELISA (left panel). The percentage of living cells was 30–40%, and 28–40% of the living cells were GFP$^+$. The right panels represent the fold activation of Tg versus WT rats. Stimulated CD4$^+$CD8$^-$ T cells derived from WT, hCycT1/CRM1 Tg rats, and human blood were electroporated, as above, and p24 production in the supernatants (C) and cell lysates (D) was measured. The percentage of living cells was ~10%, and 30–40% of the living cells were GFP$^+$. Values are the means of duplicate samples.

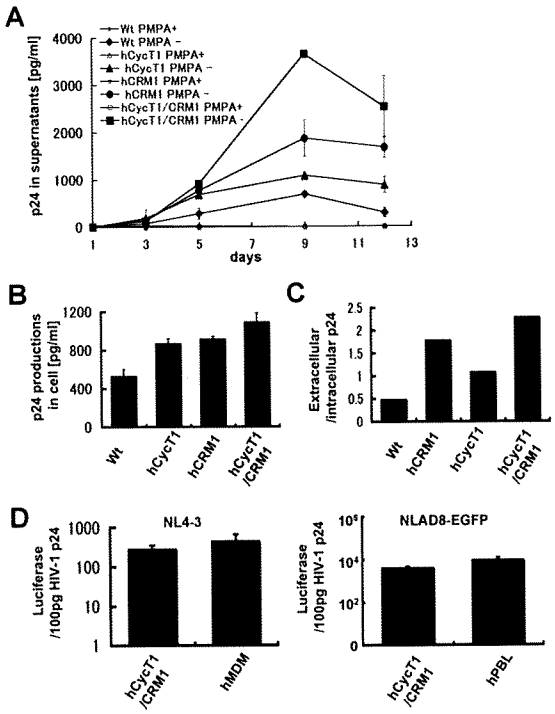

Figure 7
Quantification of p24 production in rat peritoneal macrophages. (A) Rat peritoneal macrophages or human MDMs were infected with VSV-G pseudotyped NL4-3 virus. The amount of p24 in the medium was then measured by ELISA. (B) The infected cells were harvested 12 days after infection and intracellular p24 levels were evaluated. (C) The ratio of the amount of extracellular to intracellular p24 was calculated. (D) Infectivity of viruses present in the medium 5 days after infection was measured using TZM-bl cells. NLAD8-EGFP was used to infect 5×10^5 macrophages from double Tg rats or human PBL, and the medium was recovered 5 days after infection. Values are the means of triplicate samples.

phages and subsequently infected the cells using HIV-1 pseudotyped with VSV G protein. Although WT peritoneal macrophages produced a considerable amount of HIV-1 progeny virus in the absence of hCRM1 and hCycT1 expression, macrophages derived from hCycT1/CRM1 doubly Tg rats produced 6 fold higher levels of p24 at their peak (Fig. 7A). This level corresponds to 20% of the amount of p24 produced by human MDMs (data not shown). Macrophages from hCRM1 Tg rats supported a several fold increase in p24 production, but hCycT1 expression had a smaller effect. Macrophages treated with PMPA, a reverse transcriptase inhibitor, did not produce significant amounts of p24, confirming that the p24 measured represents production of progeny viruses and not inoculum. The amount of intracellular p24 also increased to some extent in the Tg rats, but to a lesser extent than p24 levels in the medium (Fig. 7B). Approximately 67% of the p24 synthesized in the doubly Tg cells was released into the medium and the ratio of extracellular p24 to intracellular p24 increased as viral production increased (Fig. 7C).

The infectivity of the viruses, which were harvested 5 days post infection, was evaluated using TZM-bl cells. Figure 7D shows that both R5 and ×4 viruses produced from rat macrophages retained infectivity levels similar to those from human PBLs and MDMs.

Discussion

In the present study, we demonstrated the effects of hCycT1 and hCRM1 on augmentation of HIV-1 Gag production in both established and primary rat T cells and macrophages. hCycT1 enhanced p24 production profoundly in rat T cells, suggesting that hCycT1 is an essential gene that should be included in the construction of a rat model of HIV-1 infection. Although our results are in contrast to the previous reports of only a 2–5 fold increase in early gene expression in rat primary T cells and epithelial cells expressing hCycT1 [7,10,16,17], the overall effects stemmed from the increased HIV-1 pre-mRNA in response to hCycT1 expression included an increase in Tat/Rev proteins and enhanced efficiency of p24 release from T cells. This may explain the remarkable enhancement of p24 levels in the extracellular milieu. Our results support and extend the effect of hCycT1 expressed in rat primary T cells originally described by Michel et al [17]. In contrast, hCycT1 expression in macrophages had only a minor effect on p24 production. Since the level of LTR-driven luciferase activity in NR8383 cells in the absence of hCycT1 was similar to Molt4 cells (data not shown), the high basal activity of LTR-driven gene expression may explain the diminished effect of hCycT1 expression. These data are consistent with the relatively high HIV-1 LTR activity in primary macrophages [7,16,17]. Since rCycT1, like mCycT1, has a tyrosine at residue 261 in place of the hCycT1 cysteine [7], which is crucial for binding to the TAR element, rCycT1 itself may not be functional in LTR-driven expression. Instead, rat epithelial cells and macrophages may support transcription in a Tat independent manner. Alternatively, other factors in these cells may cooperate with rCycT1 for efficient LTR-driven expression.

The expression of hCRM1 in the rat macrophage line NR8383 profoundly augmented the production of p24, suggesting that Rev function is impaired and that inclusion of the hCRM1 gene in construction of a rat model for HIV-1 infection should be considered. Moreover, the profound effects of hCRM1 expression have been observed in several rat epithelial cell lines (data not shown); rCRM1

may support Rev function less efficiently. However, the effect of hCRM1 was not as great in T cell lines, primary T cells, or macrophages, compared to the macrophage cell line. These observations suggest that CRM1 function may be affected by factors involved in the formation of gag mRNA, such as the cell type-specific efficiency of splicing.

In mouse cells, defects in HIV particle formation and release have been reported [11] due to incorrect transport of gag mRNA from the nucleus to the cytoplasm [36]. The release of viral particles from both primary rat T cells and macrophages was inefficient when p24 production was low. However, when p24 production was enhanced by expression of hCycT1 in T cells or hCRM1/hCycT1 in macrophages, p24 was released more efficiently. These results suggest that the intracellular concentration of Gag protein is critical for efficient virus formation. However, rat tetherin, which is resistant to Vpu-induced degradation, may reduce the release of viral particles, although this effect was demonstrated using tetherin overexpression [37]. Since we observed that the efficiency of viral release was variable under different conditions (compare panels of Fig. 6), the inhibitory effect of rat tetherin may be an important subject for future study.

Both R5 and ×4 viruses produced from rat macrophages are as infectious as those produced by human macrophages, consistent with the report of Keppler et al. [8]. In contrast, ×4 and dual-tropic viruses that were produced in rat T cells had varying infectivities, although several R5 strains produced in rat T cells were as infectious as human T cell-produced viruses. These differences in infectivity may be ascribed to the envelope because the AD8 strain was constructed by substituting M-tropic *env* for the counterpart *env* fragment in pNL4-3 [29]. Investigating the causes of these differences in infectivity will enable us to make a rat model that allows for propagation of various strains of HIV-1.

The efficiency of the early steps of infection, including reverse transcription, nuclear import, and integration in macrophages and T cells of Sprague-Dawley rats is comparable to human cells, in contrast to the low rate of integration in mouse T cells [8,16,38]. We have also efficiently infected rat macrophages using VSV-G-coated viruses. However, the very low rate of infection of primary T cells from the rat F344 strain used in this study has hampered our detailed analysis, and suggested that inhibitory factors affecting viral penetration, similar to monkey Trim5α [39], may be present. Further studies on the mode of HIV infection in each rat strain will be required.

Conclusion

Expression of both hCycT1 and hCRM1 synergistically enhanced p24 production in rat T cells and macrophages to levels approximately 10–40% of those detected in human cells. R5 viruses produced in the rat cells were infectious. Moreover, the efficiency of the early steps of HIV-1 infection in some rat cells has been reported to be comparable to human cells [8,16]. Collectively, these results suggest that rats that express human CD4, CCR5, CycT1, and CRM1 may provide the basis for a good model system that supports multiple cycles of HIV-1 infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HS and TO designed the study. HO conducted the majority of the experiments. XZ performed and analyzed infection experiments. IBF and HS constructed and maintained the transgenic rats. MN constructed HA-tagged CRM1 plasmids. HS and HO wrote the paper. All authors approved the final manuscript.

Acknowledgements

We thank A. Hirano and N. Mizuno for technical assistance. PMPA, pH1-luc, pΔpol, pβCycT, pYK-JRCSF, pNLAD8-EGFP, and pSRαhCRM1-HA were kind gifts from Dr. E. DeClercq (Reg Institute for Medical Research), Dr. A. Adachi (Tokushima University), Dr. Y. Iwakura (Tokyo University), Dr. Y. Koyanagi (Kyoto University), Dr. K. T. Jeang (George Washington University), and Dr. T. Kimura (Ritumeikan University), respectively. Human blood was the kind gift of the Hokkaido Red Cross Blood Center (Sapporo, Japan). The infectious molecular HIV-1 clones and TZM-bl cells were obtained through the AIDS Research and Reference Reagent program. This study was supported by grants from the Ministry of Sports and Culture (Japan), and the Ministry of Health and Welfare (Japan).

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