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Original article

A rapid recombination assay of HIV-1 using murine CD52 as a novel biomarker

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Abstract

Biomarkers are commonly used for verification of infection in conjunction with the development of viral vectors or experiments involving virus infection. Leukocyte surface antigens (CDs) are a prime option for biomarkers since they can be easily visualized and analyzed by flow cytometry after indirect fluorescent staining. For analyses of human cells, murine CD24 (Heat Stable Antigen: HSA) and CD90.2 (Thy-1.2) are currently being used. In the study reported here, we attempted to develop a rapid system for measuring retroviral genome recombination efficiency. For this purpose, we looked for an alternative CD molecule which could be used as a marker on a viral vector concurrently with other markers. We found that murine CD52 is suitable for this purpose because of its small gene size, low inhibitory effect on virus production, and measurable level of surface expression. With this novel biomarker, we succeeded in developing a rapid viral recombination measuring system using a flow cytometer.

Keywords: Retrovector; Biomarker; CD52; Recombination; Flow cytometry

1. Introduction

The use of retroviral vectors for gene delivery has become very common in recent years. To determine the efficiency of gene induction and/or to monitor the fate of induced cells, a variety of selectable markers have been incorporated into retroviral vectors. The genes of products that confer resistance to toxic compounds are widely used from the start of vector development, since their stable expression enables positive selection of cells induced by prolonged treatment with antibiotics [1]. Fluorescent proteins such as eGFP and RFP are widely used as markers because of their high stability, minimal toxicity, and non-invasive detection [2].

Cell surface molecules, such as leukocyte surface antigens (CDs), constitute another type of commonly used selectable marker antigens. Staining cells with fluorescent dye-conjugated antibodies and analyzing them with a flow cytometer enables the rapid and quantitative detection of transferred gene expression in the desired target cells while they are still alive. However, most of the genes used as selectable markers are relatively large, leaving limited space in the retroviral vector for other genes of interest. The human hematopoietic cell surface antigen CD24 and its murine homologue, the heat stable antigen (HSA), are two of the few exceptions because of their relatively small gene size (about 0.24 kb) and potential for cell surface expression. Because of these properties, CD24 and HSA are widely used as biomarkers [3,4]. Another surface antigen, CD90.2 (Thy-1.2), is also in common use as a biomarker although its gene size (488 bp) is larger than that of HSA [5].

To monitor retroviral infection and viral genome recombination in cells, several good vector assay systems have been developed [6,7]. Rhodes et al. described an attractive method using two similar vectors with mutated eGFPs and surface markers to measure their infectivity and recombination rate [8]. These systems are based on the principle that only a single

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DNA is formed in an infectious retroviral particle [9]. Although this system promises reasonable and stable results, it requires multiple cell sorting and expansion, so that it takes considerable time and effort to perform. In addition, the vectors carry IRES sequences for marker gene expression, sequences which do not exist in the native retroviral genome. We therefore wanted to modify this recombination assay system since we intended to evaluate HIV-1 genome recombination in a situation more similar to viral physiological conditions and to simplify the experiments. We attempted to generate retrovectors which could express two or more biomarkers at the same time for the development of the recombination assay system. Since it was therefore necessary to find a new biomarker, we explored the database and found that murine CD52 (mCD52), a protein of the CD24/HSA family, might have the characteristics required for our purpose.

2. Materials and methods

2.1. DNA constructs

The replication competent HIV-1 proviral clone pNL4-3 [10] and pMSMBA [11], a derivative of pNL4-3, were used as progenitors for the mutant constructs described below. The HSA gene was amplified from the plasmid pNLrHSAS [12], also a derivative of pNL4-3, the vpr gene of which was replaced with the HSA gene, with a pair of primers (XbaHSAonF: 5′-TCTAGAGCCGCCATGGGCAGAGCG-3′, and EcoRIHSAstpR: 5′-GAATTCTCTAACAGTAGAGATG-3′). pNLrHSAS was digested with NheI, blunt-ended with a Blunting-High kit (Toyobo, Osaka, Japan), and ligated to generate pNLrHSASNh. The amplified fragment was replaced with the XbaI-EcoRI region of pNLrHSASNh to add a Kozak sequence upstream of the ATG codon of the HSA gene to enhance its expression, and the resultant plasmid was named pNLrH. pNLrH was then digested with HpaI and XhoI, and the HpaI-XhoI fragment of pGEMHnGX [13] including the EGFP gene was inserted in the corresponding position to construct pNLrHnG.

The murine CD52 (mCD52) gene coding fragment was generated by synthesizing three oligonucleotide probes (CD52atg-95: 5′-ATGAAGAGCTTCCTCCTCTTCCTCAC TATCATTCTTCTGGTTGTGATTCAGATACAAACAGGAT CCTTGGGACAAGCCACTACGGCCGCTTCTGG-3′, CD52 cmpl170-116: 5′-GGCACCCGCATCGATGATGGATGAGG CCCCACTCTTTAAGGGGGTTTTTTTGGTGGAGGTGCTG TTTTTGTTAGTACCAGAAGCGGCCGTAGTGG-3′, and CD52fwd151-stp: 5′-CCATCATCGATGCGGGTGCCTGCAGTTT CCTCTTCTTTGCCAATACCTTAATGTGCCTCTTCTACC TCAGCTGA-3′) following sequential PCR amplification with two pairs of primers (XbaKozCD52F: 5′-TCTAGAGCCGC CATGAAGAGCTTCCTCCTCTTCC-3′, CD52RevCla: 5′-GG CACCCGCATCGATGATGGATG-3′, CD52FwdCla: 5′-CCAT CATCGATGCGGGTGCCTGC-3′, and CD52RevStp: 5′-TCA GCTGAGGTAGAAGAGGCAC-3′). The 0.23 kb amplified fragment was purified, ligated to the pGEMTeasy vector (Promega, Madison, WI) to construct pGEMmCD52, and verified for its sequence authenticity. pNLrHnG was digested with XbaI and EcoRI, and the XbaI and EcoRI fragment of pGEMmCD52, including the mCD52 gene, was inserted at the corresponding position to construct pNLrCnG. A base substitution mutation was then introduced into the start codon of the eGFP gene of pNLrHnG to eliminate eGFP expression (ATG to TAA) in order to allow for the construction of pNLrHnGΔN. A frame shift mutation of the eGFP gene at the 0.6 kb position from the start codon was introduced as reported elsewhere [8] (mutant pON-H6: one-base substitution and one-base insertion to introduce a stop codon and a frame shift) into pNLrCnG for the construction of pNLrCnGΔC. Furthermore, two-base substitution mutation at the hairpin loop of SL1 (GCGCGC to GTGCAC) was introduced into pNLrCnGΔC to construct SL1MrCnGΔC.

2.2. DNA transfection

293T cells [14] (approximately 3×10^6) were seeded on dishes (diameter 100 mm) the day before transfection with plasmid DNA (total 5 μg) using the calcium phosphate precipitation method [15]. The day after transfection, the supernatant was replaced with fresh medium.

2.3. Virus infection

At 48–72 h post-transfection, the media was centrifuged and the supernatant was used for infection into T-cell lines (MT-4 and M8166).

2.4. RT-PCR assay

Two days after transfection, 293T cells were harvested and total cellular RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). RNAs were treated with RQ1-DNaseI (Promega) for removal of contaminated DNAs. Reverse transcription (RT) reaction using 5 μg of total RNA with Superscript III (Invitrogen) was performed according to the manufacturer's instructions, and one-twentieth of the RT products was used for the PCR template. Two sets of primer pairs were prepared to detect the mCD52 gene (Forward: 5′-TCTAGAGCCGC CATGAAGAGCTTCCTCCTCTTCC-3′; reverse: 5′-TCAGC TGAGGTAGAAGAGGCAC-3′) and the GAPDH gene (forward: 5′-CCACATCGCTCAGACACCAT-3′; reverse: 5′-GGC AACAATATCCACTTTACCAGAGT-3′). Amplified products were subjected to agarose gel electrophoresis and visualized by means of ethidium bromide staining.

2.5. Flow cytometric analysis

Mock-infected cells, an empty vector, and HSA/eGFP/mCD52/Thy-1.2-infected cell populations in growth medium were first centrifuged and washed twice in PBS(−) supplemented with 10% Blocking One solution (Nacalai Tesque Inc., Kyoto, Japan). Aliquots of the cells were then stained with an anti-mCD52 rat monoclonal antibody (MBL Co. Ltd., Nagano, Japan) for 30 min, washed twice, incubated with Allophycocyanin (APC)-labeled anti-rat Ig polyclonal

antibody (BD Biosciences, San Jose, CA) for an additional 30 min, and washed twice. The cells were then stained with directly conjugated anti-murine HSA-Phycoerythrin (PE) antibody and anti-murine Thy-1.2-biotin antibody (both from BD Biosciences) for an additional 30 min, washed twice, and stained with PerCP-Cy5.5-conjugated Streptavidin (BD Biosciences). After antibody labeling, two further washes in PBS(−) were performed, the last together with 1% formaldehyde to fix the cells. Finally, the cells were analyzed on a FACSCalibur (BD Biosciences).

3. Results

3.1. Generation of mCD52 expressing HIV-1 vectors

To construct multi-marker carrying retrovectors, we initially tried to use the existing biomarkers. Within the HIV-1 genome, the vpr, env, and nef coding regions were replaced with HSA, Thy-1.2, and eGFP genes in various combinations. While the HSA gene was well expressed under any conditions, the Thy-1.2 and eGFP genes performed well only when located within the nef coding region (data not shown). Any vector carrying the Thy-1.2 or eGFP gene in the vpr or env region produced little or no viral particles. Hence, we needed to find a novel marker with approximately the same potential as CD24/HSA and that could be used concurrently with them.

Human and murine CD52s (h/mCD52) belong to a group of very small GPI-anchored sialoglycoproteins which include CD24/HSA and with size and protein properties resembling those of CD24. Human CD52 is abundantly expressed on lymphocytes and monocytes, and is also expressed in non-lymphoid tissue in epithelial cells of the distal epididymal and deferent ducts from which it is transferred to the surface of sperm [16]. The gene size of mCD52 is only 222 bp and encodes 74 peptides (Fig. 1A). A computer search found no significant homology of mCD52 to any known molecules except hCD52 at either the DNA or amino acid sequence level [17]. Although it retains a certain homology to hCD52 within the N- and C-terminal signal region, the amino acid sequence of the mature peptide region of mCD52 is significantly different from that of its human homologue [16]. In addition, it has been suggested that the monoclonal antibody BTG-2G, which is the only commercially available anti-mCD52 monoclonal antibody (mAb) [17], can recognize peptides containing KKTPL [18]. This sequence is unique to mCD52 (Fig. 1A) and thus no cross-reaction of the antibody with hCD52 can be expected. These characteristics suggested to us that mCD52 could be a candidate for a novel selectable marker of gene transfer in human cells. We cloned the mCD52 gene by synthesizing and amplifying its DNA primers and inserting it in place of the vpr gene of HIV-1 to construct various retrovectors. Fig. 1B shows representative schematics of the vectors we constructed for the experiments described below. We confirmed expression of the mCD52 gene by means of an RT-PCR assay (Fig. 1C). A transcription of the mCD52 gene was clearly detected in cells transfected with the vectors carrying the mCD52 gene. The production of the viral antigen from cells transfected with vectors carrying the mCD52 insertion was only moderately reduced compared to that from cells transfected with the wild-type or the vectors carrying HSA (data not shown). This indicated that the effect of mCD52 insertion into the viral genome on virus production was nearly negligible.

3.2. Detection of cell surface expression of mCD52 by mAb

We first used a flow cytometer to verify surface expression of the mCD52 protein and its detection with an mAb. 293T cells were then transfected with pNLrCnG, which carries both the mCD52 and eGFP genes as biomarkers. Two plasmids, pNLNh and pNLrHnG, were used for transfection as parallel controls. Unlike pNLrCnG, pNLrHnG carries an HSA gene instead of an mCD52 gene. Forty-eight hours after transfection, the cells were stained with anti-mCD52 mAb, APC-anti-RatIg antibody, and phycoerythrin (PE)-conjugated anti-HSA mAb. The cells were fixed with 1% formaldehyde-containing PBS(−), and analyzed with a flow cytometer. The expressions of eGFP, HSA, and mCD52 were detected through channels FL1, FL2, and FL4, respectively. The results showed good expression and separation of the three marker genes (Fig. 1D), suggesting that these markers could be utilized concurrently within a cell for discrimination of their expression. Double marker positive cells accounted for about 11% (NLrCnG) and 14% (NLrHnG) of total cells.

3.3. Four-color analysis of transduced cells using three surface markers and eGFP

As expression of mCD52 was clearly distinguishable from the expressions of HSA and eGFP, we attempted to identify gene transductions mediated by multiple retrovectors by means of four biomarkers using all fluorescence channels (FL1–4) of a flow cytometer. For this purpose, the vectors pNLrCnGΔC (NLC) and pNLrHnGΔN (NLH) were prepared. NLC carries the mCD52 gene and NLH the HSA gene, and both carry inactivated eGFP genes. In addition, another surface marker gene, murine Thy-1.2, was employed for analysis. The vector pNLΔBgThy [5] (NLT) carries the Thy-1.2 gene in place of the nef gene, features deletion of the Env gene of pNL4-3 and does not carry the eGFP gene. Biotin-conjugated anti-Thy-1.2 mAb and Avidin-PerCP-Cy5.5 were used for staining and labeling Thy-1.2. The order of cell staining is shown in Fig. 2A. 293T cells were transfected with pCG-VSVG and various combinations of retrovectors. Two days post-transfection, the cells and supernatants were harvested, and the cells were stained with the mAbs and analyzed with a flow cytometer (data not shown). As expected, four biomarkers were detected independently and no cross-reaction was observed. The harvested supernatants were then used for the infection assay with MT-4 cells (Fig. 2B). Similar to the finding for 293T, infection of retrovectors resulted in a satisfactory expression and detection of marker genes with a flow cytometer. The bottom row of the panel in Fig. 2B shows

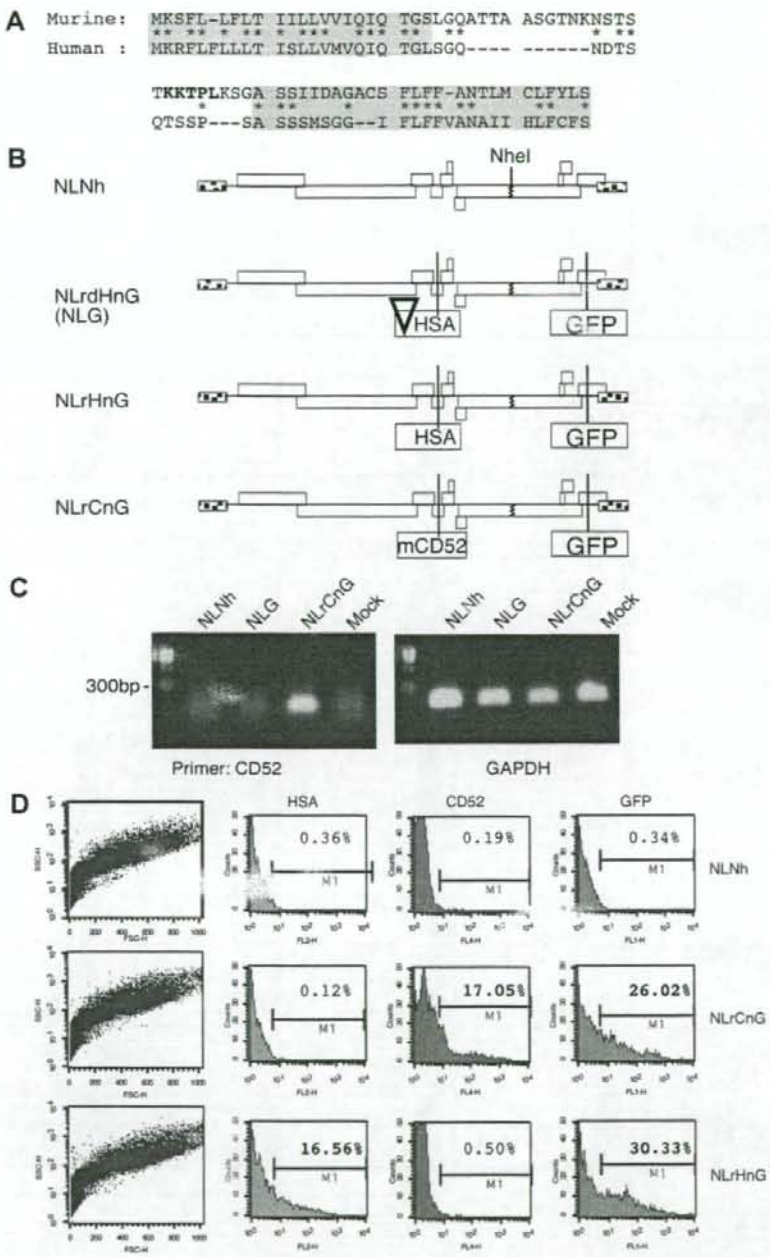

Fig. 1. Construction of murine CD52 expressing HIV-1 vector. A) Comparison of murine and human CD52 protein sequences. N-terminus signal sequences and C-terminus GPI-addition sequences [22] are shaded. The BTG-2G monoclonal antibody recognition sequence [18] are shown in bold. B) Schematic of constructed HIV-1 vectors. Plain boxes represent ORFs of HIV-1 genes and stippled boxes represent LTRs. The env gene in each vector was inactivated by frameshift mutation introduced at the NheI site. The boxes labeled HSA, GFP, and mCD52 represent marker genes inserted in the vectors. The triangle represents the location of mutations introduced to inactivate the gene. C) RT-PCR assays to confirm mCD52 expression. 293T cells were transfected with the plasmids shown in Fig. 1B. Cells were harvested 2 days post transfection and total cellular RNA was extracted. RT products of 5 μg of total cellular RNA were used for PCR amplification. Detection of the expression of the GAPDH gene was used for control. Faint signals for mCD52 in control lanes were non-specific since they also appeared in RT negative controls (data not shown). D) Surface expression of mCD52 molecules caused by transfection. 293T cells were transfected, harvested, and stained with anti-mCD52 Rat monoclonal antibody, APC-conjugated anti-Rat IgG antibody, and PE-conjugated anti-HSA monoclonal antibody. Four charts in one row represent one set of analyses of one sample. Plasmids used for transfection and molecules detected by cytometric analysis are indicated to the left of the rows and above the columns respectively. Percentages of positive cells are shown in each histogram.

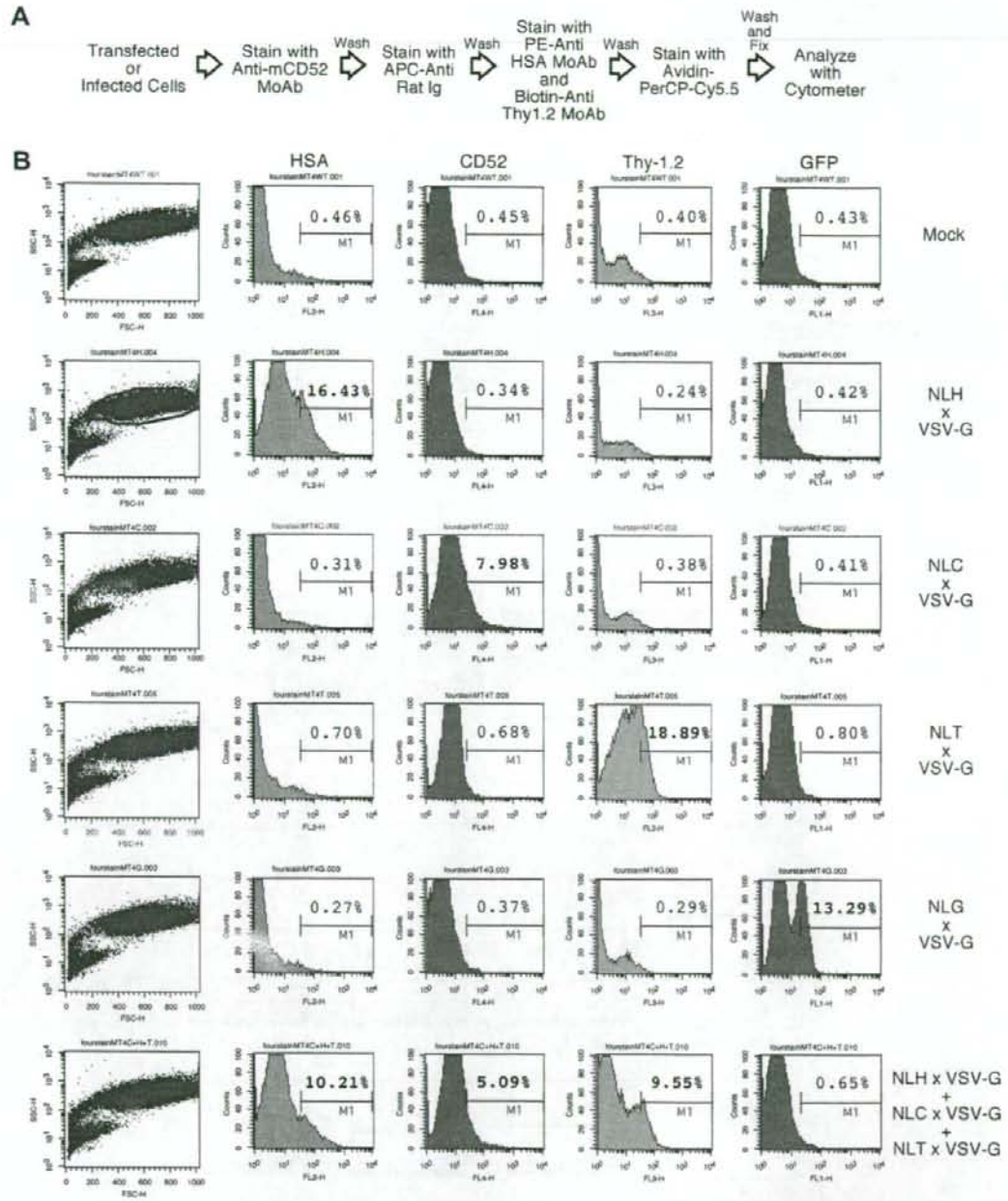

Fig. 2. Simultaneous detection of four biomarkers transduced by retrovectors. A) Flowchart of cell staining of the assay. B) MT-4 cell infection experiment. The supernatants of 293T cells transfected with pCG-VSVG and various retrovectors were collected and used for infection into MT-4. Six charts in one row represent one set of analyses of one sample. The notations are the same as those for Fig. 1D. The "x" between two or three plasmids indicates co-transfection of the plasmids.

that co-infection of the mixture of three vectors resulted in a lack of eGFP expression, thus indicating that no recombination between GFPΔN and GFPΔC from different virus particles had occurred. We therefore proved that these three surface markers could be conveniently leveraged to distinguish the gene induction of different vectors simultaneously, and that their utilization could be expected to have a wide range of applications.

3.4. Construction of HIV-1 genome recombination assay

We then attempted to develop a simple system to monitor retroviral infection and viral genome recombination in cells. The system we designed is shown in Fig. 3. Two similar vectors with the same or different dimerization signals (DLS) were constructed (Fig. 3A) and co-transfected together with or without the VSV-G expression vector (pCG-VSVG) (Fig. 3B). Vector A carries a surface biomarker (Mark-A) and an inactivated eGFP gene with amino-terminal mutation (GFPΔN), while vector B carries another biomarker (Mark-B) and an inactivated eGFP gene with carboxyl-terminal mutation (GFPΔC). After transfection, the released virions can be

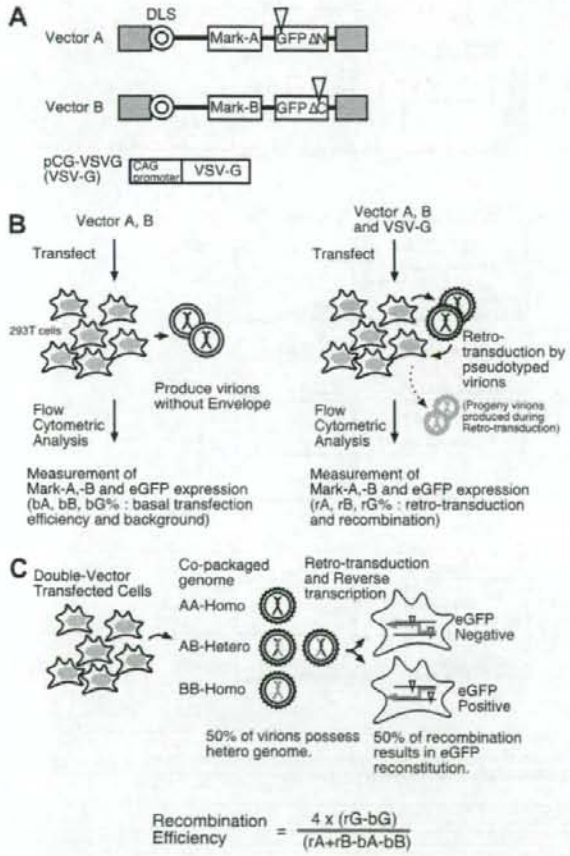

Fig. 3. The system for estimating HIV-1 recombination efficiency using retro-transduction realized by means of pseudotyping. A) Schematics of the vectors planned for the system. Symbols are the same as those for Fig. 1B. Concentric circles represent encapsidation/dimerization signals (E/DLS). B) Experimental design of the system. Without VSV-G, co-transfection of the vectors results in production of non-infectious virions and expression of marker genes (left). With VSV-G pseudotyping, the generated virions infect the cells within the transfected cell culture (retro-transduction), and marker gene expressions are enhanced. C) Estimation of recombination efficiency. Co-transfected cells produce 25% A-A and 25% B-B homo-dimerized genomes, as well as 50% A-B hetero-dimerized genomes containing virions. Fifty % of the opportunity for genome recombination results in reconstitution of the eGFP gene.

expected to co-package the homo- or hetero-dimerized vector genome, while the ratio of homo- to hetero-dimerization should be one-to-one if the genome expression efficiency of the two vectors is similar (Fig. 3C). Without VSV-G, the expression of Mark-A and -B indicates transfection efficiency of the vectors, and no eGFP expression should be observed. With VSV-G expression, pseudotyped virions have been observed to cause retro-transduction to the producer cells [13] and the number of marker genes expressing cells in the transfectant increases with an increase in the occurrence of retro-transduction. Recombination of the two vectors can be assumed to occur only in retro-transduced cells, and is monitored in terms of further restoration and expression of the eGFP gene (Fig. 3C). Transfection and retro-transduction efficiency are measured by expression of Mark-A and -B, while the transduction efficiency is estimated by subtracting marker gene expression ratios of the VSV-G negative sample (bA, bB%) from those of the positive sample (rA, rB%). Finally, the recombination efficiency is estimated by calculating the ratio of eGFP expressing cells (rG−bG%) in vector-transduced cells. If we assume that one of the recombination events always occurs between the ΔN and ΔC mutation of eGFPs during reverse transcription, 50% of the recombination events should result in reconstitution of the eGFP gene. In addition, 50% of the virions from doubly transfected cells possess a hetero-dimerized genome, and thus have the potential to reconstitute eGFP. This means that a ratio of eGFP positive cells of 25% in Mark-A or -B positive cells should be the maximum value for recombination. We therefore adopted this maximum ratio for easy indexing by quadrupling the numeric results (Fig. 3C).

We assessed the efficacy of this system by constructing and testing several vectors derived from HIV-1. We found that the combination of vpr substitution with HSA or mCD52 as surface markers and nef substitution with mutated eGFP genes yielded satisfactory results in terms of virion production, infectivity, and marker expression. We also used this system to verify the recombination between the wild-type retrovectors and those derived from a dimerization initiation site (DIS) mutant of HIV-1 (Fig. 4). DIS is located in DLS and it has been suggested that it performs core functions in viral genome recombination [19]. The vectors NLC and NLH carry the same DIS, whereas pSL1MrCnGΔC (SL1MC) carries a two-base substitution on DIS (Fig. 4A), so that heterogenome dimer formation between NL- and SL1M-vectors can be assumed to be reduced. In this experiment (Fig. 4B), 3–5% of all cells were surface marker positive cells in non-pseudotyped samples (NLH × NLC, NLH × SL1MC), which constitutes evidence of the efficiency of transfection and marker expression. eGFP fluorescence was not detected in these samples, confirming that ΔN or ΔC mutation inactivated the functional eGFP expression. In contrast, HSA/mCD52 expression was dramatically enhanced by about 80% in pCG-VSVG co-transfected samples due to retro-transduction. Efficient reconstitution of eGFP was observed in the homo-DIS sample (20% of total cells) whereas only limited eGFP expression was detected in the hetero-DIS sample (4%), thus indicating diminished occurrence of recombination as also reported elsewhere [19]. Recombination efficiency was

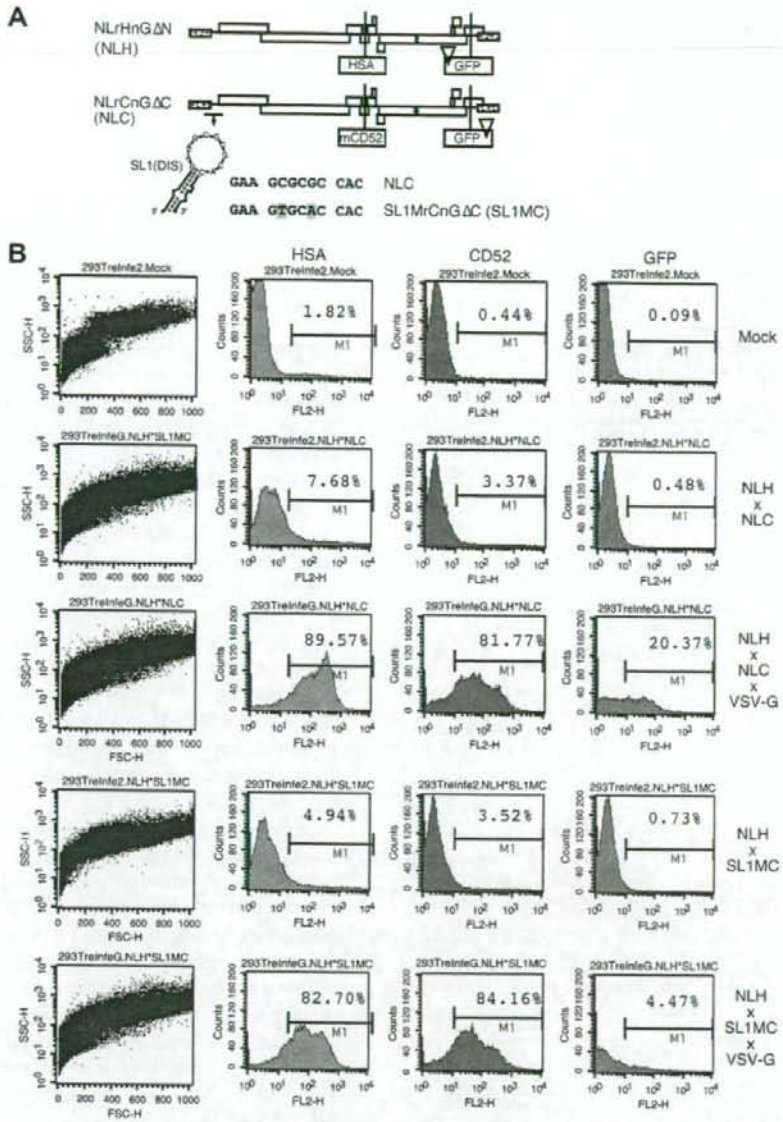

Fig. 4. Detection of HIV-1 recombination. A) Schematic of the vectors actually constructed for the recombination estimation system. Two nucleic acids on DIS of pNLrCnGΔC were substituted for each other (5′-GCGCGC-3′ for 5′-GTGCAC-3′) to construct pSL1MrCnGΔC. B) Genome recombination assay using 293T cells. The notations are the same as those for Fig. 2B.

estimated at 0.5 for NL-NL [4(20.37 − 0.48)/(89.57 + 81.77 − 7.68 − 3.37) = 0.496] and 0.09 for NL-SL1M [4(4.47 − 0.73)/(82.70 + 84.16 − 4.94 − 3.52) = 0.094]. The efficiency of heterogenome dimerization between NL and SL1M mutant estimated with the system devised by us [20] was reduced to about 30% of that of homo-dimerization (data not shown), which suggests there is a parallel interrelation between genome dimerization and recombination. These results clearly showed that our system utilizing retro-transduction is effective and quite practical for evaluation of recombination.

4. Discussion

In this report, we described the practical use of a new reporter gene, mCD52, and the development of a rapid recombination assay system for HIV-1. We focused on the mCD52, which is a protein of the HSA family, since its gene is as small as that of HSA and was assumed to possess similar properties. The results of our study demonstrated that mCD52 can be a useful biomarker since its insertion into the vpr region resulted in its expression from the HIV-1 genome similar to that of HSA.

The recombination assay system we developed is relatively simple, easy and fast. Infection to the transfected cells (=retro-transduction) is the key component of the system, and without it, this system cannot be operated. Retro-transduction is inevitable as far as using the VSV-G protein for pseudotyping, since the VSV-G pseudotyped vector can infect virtually any kind of cell [13]. On the other hand, it is not easy to attain high enough infectivity of the vectors to calculate the recombination rate without VSV-G. We therefore constructed this system with VSV-G by taking advantage of retro-transduction for a quick estimation. For reliability of the calculation, however, the efficiency of retro-transduction is a matter of concern. If the titer of the vectors is too high, multiple infections would occur in a single cell. As the estimate of the recombination rate is based on the assumption of a "single-hit" infection per cell, too high an efficiency of vector production might bias the estimate. In several reports, the recombination frequency of HIV-1 in cultured cells is estimated at about once per 0.9–1 kb of viral genome [6,8]. Our result for the normal recombination rate was 0.5 times per 0.6 kb, so that the estimated frequency is once per 1.2 kb. Since this value represents a lower efficiency than previously reported, it may be the result of multiple infections reflecting a high infection rate (most of the cells in the culture were marker positive as seen in Fig. 4). To prevent biased estimates, a reduction in vector infectivity is needed for this system. In fact, when we reduced the titer of the vector appropriately to minimize "multiple-hit" infection, the calculated recombination ratio was as one event per 0.8 kb, which was very close to the previously published data (data not shown).

There is a possibility that viral genome-derived cDNA and transfected plasmid DNA recombine in retro-transduced cells. We performed an additional experiment to verify this possibility. The 293T cells were cotransfected with the plasmid NLH, pCG-VSVG, and the plasmid carrying only GFPΔC gene. After three-day incubation, we observed no appearance of GFP positive cells in retrotransduced cell culture, whereas a certain number of HSA positive cells appeared (data not shown). This clearly showed that the recombination between the GFPΔN gene derived from viral cDNA and the GFPΔC gene derived from the transfected plasmid was undetectable during retro-transduction. Thus, we believe that plasmid-cDNA recombination is negligible in this system. Like in other established systems [6,8], the recombination rate estimated by our system only reflects that in the nef coding region. By changing the structure of the vectors, it may be possible to further study the recombination rate in regions other than the nef region.

We did not encounter any practical problems such as alterations of cell viability caused by the expression and staining of mCD52. Specific mAb (Campath-1H) treatment of human CD52 has been shown to lead to extensive eradication of CD52 positive cells by complement activation and is thus utilized for bone marrow transplantation therapy [21]. Although our system includes anti-mCD52 mAb treatment, the mAb we used was different from Campath-1H, and the treatment of cells in our study lasts only for a short time prior to cell fixation. In addition, complement components in cell culture media were inactivated by heat inactivation of serum. Although mCD52 seemed to cause no serious defects in the experiment, mCD52 expression may have some deleterious effect under certain conditions such as in *in vivo* experiments. The potential problems associated with wider application of our system thus need to be investigated.

In conclusion, mCD52 constitutes a novel option for a reporter gene which can be leveraged concurrently with other biomarkers. We could demonstrate that mCD52 is a useful marker for transduction by retrovectors, and the utility of this system may be extended to various viral and non-viral gene transfer systems. With this new marker, we developed an easy-to-use HIV-1 recombination assay system, which is expected to be useful for studying and determining the recombination ratios of many viral strains and/or mutants at one and the same time.

Acknowledgements

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TRIM5α-independent anti-human immunodeficiency virus type 1 activity mediated by cyclophilin A in Old World monkey cells

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ABSTRACT

Cyclophilin A (CypA) is a peptidyl-prolyl isomerase that binds to the capsid protein of human immunodeficiency virus type 1 (HIV-1). TRIM5α is an antiretroviral factor influencing species-specific retroviral replication in Old World monkey (OWM) cells. In the study reported here, we investigated the role of CypA in anti-HIV-1 activity of OWM cells. Exogenous expression of CypA inhibited HIV-1 infection in OWM cells but not in human cells when the function of TRIM5α was suppressed by overexpression of dominant negative form of TRIM5α as well as by using RNA interference. This inhibitory action depended upon the interaction of the CypA moiety with HIV-1 capsid and disruption of CypA and capsid interaction by cyclosporine A enhanced the HIV-1 susceptibility of OWM cells even in the absence of functional TRIM5α. These results point to the presence of novel TRIM5α-independent anti-HIV-1 activity mediated by CypA in OWM cells.

Introduction

The hydrophobic pocket of cyclophilin A (CypA) makes direct contact with the proline residue at the 90th position and adjacent residues on the surface-exposed loop between α-helices 4 and 5 (the h4/5 loop) of human immunodeficiency virus type 1 (HIV-1) capsid (CA) (Franke et al., 1994; Gamble et al., 1996; Luban et al., 1993). This interaction can be disrupted experimentally by mutations that alter CA proline 90 or adjacent residues (Franke et al., 1994), by competitive inhibitors of the interaction including cyclosporine A (CsA) (Braaten et al., 1996a; Franke and Luban, 1996; Thali et al., 1994), by CypA knockout by means of gene targeting (Braaten and Luban, 2001), or by CypA knock-down with RNA interference (RNAi) (Sokolskaja et al., 2006; Sokolskaja et al., 2004). Each of these interventions reduces HIV-1 susceptibility in human cells (Braaten et al., 1996a; Braaten and Luban, 2001; Franke and Luban, 1996; Franke et al., 1994; Rosenwirth et al., 1994; Sokolskaja et al., 2006, 2004), with the block occurring early, at the time of reverse transcription (Braaten et al., 1996b). Recent data have led to the hypothesis that, by binding to CA, CypA protects HIV-1 against antiviral restriction activity in human cells (Luban, 2007; Towers, 2007).

In contrast with the situation in human cells, HIV-1 replication in cells from several Old World monkeys (OWM) encounters a block before completion of reverse transcription due to their tripartite motif (TRIM) 5α (Stremlau et al., 2004). TRIM5α is a member of the tripartite motif protein family, which comprises RING, B-box and a coiled-coil domains (Reymond et al., 2001). Rhesus (Rh) and cynomolgus monkey (CM) TRIM5αs inhibit HIV-1 but not simian immunodeficiency virus from macaque (SIVmac), whereas African green monkey (AGM) TRIM5α prevents replication of both HIV-1 and SIVmac (Hatziioannou et al., 2004; Keckesova et al., 2004; Nakayama et al., 2005; Stremlau et al., 2004). Human (Hu) TRIM5α shows very weak antiviral activity against those viruses (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2004, 2005; Yap et al., 2005) but confers strong resistance against N-tropic murine leukemia virus (N-MLV) (Hatziioannou et al., 2004; Perron et al., 2004; Yap et al., 2004). Among several splicing variants of TRIM5, an α isoform carries the SPRY or B30.2 domain that determines virus specificity of this intracellular factor (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2004, 2005; Yap et al., 2005).

CsA treatment has been reported to increase HIV-1 replication in OWM cell lines (Berthoux et al., 2004; Kootstra et al., 2003; Towers et al., 2003). Recently, two groups investigated the role of CypA in OWM TRIM5α antiviral activity and suggested that CypA acts in trans to promote TRIM5α-mediated restriction of HIV-1 (Berthoux et al., 2005; Keckesova et al., 2006). Berthoux et al. reported that HIV-1 susceptibility increased in response to CypA knock-down to the same extent as observed in response to TRIM5 knock-down by RNAi in Rh FRhK4 cells. However, simultaneous knock-down of both CypA and TRIM5 caused minimal additional increase, suggesting that CypA inhibits HIV-1 replication in Rh cells in a TRIM5α-dependent manner. These findings suggest that CypA is required for efficient CA recognition by TRIM5α (Berthoux et al., 2005). Keckesova et al. (2006) also failed to observe additional increase in HIV-1 susceptibility in TRIM5α-knocked-down AGM and Rh cells in response to CsA treatment. Neither of these reports, however, used the overexpression system of CypA protein.

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In contrast to OWM cells, owl monkeys of the New World monkey possess CypA as a fusion protein with TRIM5 (TRIMCyp) (Nisole et al., 2004; Sayah et al., 2004). In a mutagenesis study of TRIMCyp, deletion of the RING domain of TRIMCyp only partially reduced the ability of the TRIMCyp protein to restrict HIV-1, suggesting that overexpression of CypA-like proteins can results in a diminished susceptibility of cells to HIV-1 (Diaz-Griffero et al., 2006). These findings prompted us to test the effect of exogenous expression of CypA in OWM cells, especially in the absence of functional TRIM5α and we were able to show that CypA suppresses HIV-1 infection in OWM cells even in the absence of functional TRIM5α.

Results

Exogenous expression of CypA reduces HIV-1 susceptibility in OWM cells in TRIM5α-independent manner

To examine the effect of exogenously expressed CypA on HIV-1 infectivity in OWM cells, Sendai virus (SeV) vector system was used to express CypA. HIV-1 susceptibility was then tested by using vesicular stomatitis virus G (VSV-G) protein-pseudotyped HIV-1 vector encoding green fluorescence protein (GFP). AGM-TRIM5-specific siRNA transfection was used to reduce TRIM5 levels. Real time PCR analysis revealed that TRIM5 mRNA levels in siRNA transfected cells were reduced to 23.6% of those of control siRNA transfected cells. As shown in Fig. 1, the TRIM5-specific siRNA transfection greatly enhanced HIV-1 susceptibility in AGM CV1 cells, suggesting the critical role of TRIM5α to restrict HIV-1 in CV1 cells. Exogenous expression of CypA apparently reduced HIV-1 infectivity in the absence of TRIM5α expression, suggesting that CypA may restrict HIV-1 in OWM cells in a TRIM5α-independent manner. On the other hand, CypA expression showed no additive effect on HIV-1 restriction in the presence of TRIM5α. This indicates that the amount of endogenous CypA in the presence of TRIM5α in CV1 cells is sufficient to restrict HIV-1.

Since there is one concern that the residual TRIM5α after siRNA knock-down might interact with exogenous CypA, we used SeV expressing CM TRIM5α lacking the SPRY domain (CM-SPRY(-)-SeV) to eliminate function of endogenous TRIM5α. It was reported previously that TRIM5 splicing variant TRIM5γ, which lacks SPRY domain, dominantly interfered with the antiviral activity of TRIM5α (Stremlau et al., 2004). CM-SPRY(-)-SeV had been shown to efficiently interfere with HIV-1 suppression mediated by over-expressed CM, AGM, Rh and human TRIM5αs (Maegawa et al. submitted). In addition, CM-SPRY(-)-SeV was able to introduce dominant negative TRIM5 mutant to virtually all the cells in a dish, and was also able to suppress anti-HIV-1 activity of TRIM5a much more efficiently than siRNA (Maegawa et al. unpublished result). Effects of CM-SPRY(-)-SeV on HIV-1 infection was not observed in human cells (Fig. 2), confirming that SPRY(-) mutant TRIM5 specifically interfere with TRIM5α function. SeV expressing AGM TRIM5α lacking the coiled-coil region (AGM-CC(-)-SeV), was used as a non-interfering control. AGM cell lines Vero and CV1, Rh cell line LLC-MK2 and human cell lines 293T and MT4, were tested for their HIV-1 susceptibility in the presence of exogenous CypA. We also constructed SeV expressing CypA, which carries an HA tag (YPYDVPDYAA) at its C-termini (CypA-HA). Cells were first infected with AGM-CC(-)-SeV or CM-SPRY(-)-SeV, mixed with a SeV expressing CypA or CypA-HA, or with an empty SeV vector parental Z strain, incubated at 37 °C for 16 h, and then challenged with VSV-G-pseudotyped HIV-1 vector encoding GFP. Consistent with our previous finding, CM-SPRY(-)-SeV infection enhanced HIV-1 susceptibility in OWM cells but not in human cells (Fig. 2). As shown in Figs. 2B, D, and F, exogenous expression of CypA in OWM cells dramatically reduced HIV-1 susceptibility when endogenous TRIM5α were interfered with overexpression of the dominant negative form of TRIM5α. In contrast, the exogenous expression of CypA showed only a slight additional enhancement of HIV-1 susceptibility in the presence of endogenous TRIM5α (Figs. 2A, C, E). The exogenous expression of the HA-tagged version of CypA had a similar suppressive effect on HIV-1 infection, even though the effect was slightly weaker than that of the non-tagged version of CypA (Figs. 2B, D, and F). These results are consistent with the findings from the siRNA experiment shown in Fig. 1. In contrast to OWM cell lines, human cell lines were not affected by the exogenous expression of CypA or CypA-HA in HIV-1 susceptibility either in the presence or absence of functional endogenous TRIM5α (Figs. 2G to J). Similar results were observed when we used the empty SeV vector in stead of AGM-CC(-)-SeV (data not shown). We clearly detected expression of exogenous CypA-HA as well as those of endogenous CypA in human and OWM cell lines, although the levels of expression of exogenous CypA were slightly low in human cells than in OWM cells (Fig. 2K). Taken together, these results indicate that the exogenous expression of CypA in OWM cells has a potent antiviral activity in TRIM5α-independent manner.

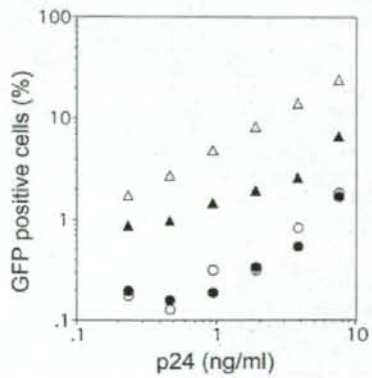

Fig. 1. CV1 cells were transfected with siRNA against AGM TRIM5 (triangles) or SiCONTROL Non-targeting siRNA#2 as a negative control (circles). Two days after transfection, cells were infected with SeV expressing CypA (black triangles or black circles) or empty SeV vector parental Z strain (white triangles and white circles) at a multiplicity of infection (MOI) of 10 plaque forming units (PFUs) per cell and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were counted with a flowcytometer.

Endogenous CypA also restricts HIV-1 infection in OWM cells in TRIM5α independent manner

CsA treatment disrupts the interaction between CypA and viral capsid and has been reported to increase HIV-1 replication in OWM cells and reduce that in human cells (Berthoux et al., 2004, 2005; Keckesova et al., 2006; Kootstra et al., 2003; Stremlau et al., 2006; Towers et al., 2003). We treated AGM-CC(-)-SeV or CM-SPRY(-)-SeV infected Vero, CV1, LLC-MK2, 293T or MT4 cells with 5 μM of CsA for 1 h and then inoculated them with the VSV-G-pseudotyped HIV-1 vector encoding GFP. As shown in Fig. 3, CsA treatment affected HIV-1 susceptibility in opposite directions in human and OWM cells. CsA treatment enhanced HIV-1 infection in OWM cells (Figs. 3A, C, and E) but reduced HIV-1 infection in human cells in the presence of functional TRIM5α (Figs. 3G and I). These results were consistent with previous findings (Berthoux et al., 2004, 2005; Keckesova et al., 2006; Kootstra et al., 2003; Stremlau et al., 2006; Towers et al., 2003). In the absence of functional TRIM5α, CsA treatment also enhanced the susceptibility of the OWM cells to HIV-1 infection (Figs. 3B, D, and F) and diminished that of the human cells (Figs. 3H and J). These results suggest that endogenous CypA, like exogenous CypA, can also restrict HIV-1 in OWM cells but not in human cells.

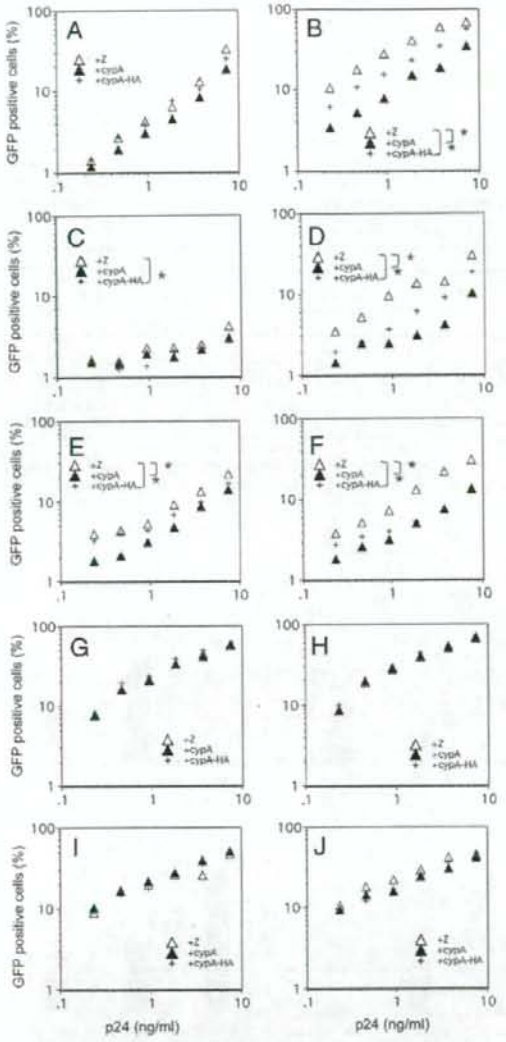

Fig. 2. Vero (A, B), CV1 (C, D), LLC-MK2 (E, F) 293T (G, H) and MT4 (I, J) cells were infected with SeV expressing AGM TRIM5α lacking the coiled-coil domain (A, C, E, G, I) or CM TRIM5α lacking the SPRY domain (B, D, F, H, J) mixed with SeV expressing CypA (black triangles), CypA-HA (crosses) or the empty SeV vector parental Z strain (white triangles) at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were counted with a flowcytometer. Asterisks indicate statistically significant differences ($p<0.05$, paired t-test). Representative data of at least three independent experiments is shown. (K) Lysates of CV1 (lane 1), Vero (lane 2), LLC-MK2 (lane 3), 293T (lane 4) and MT4 (lane 5) cells infected with SeV expressing CypA-HA were visualized by western blotting with an antibody against CypA. The lower and upper bands represent endogenous CypA and exogenous CypA-HA, respectively.

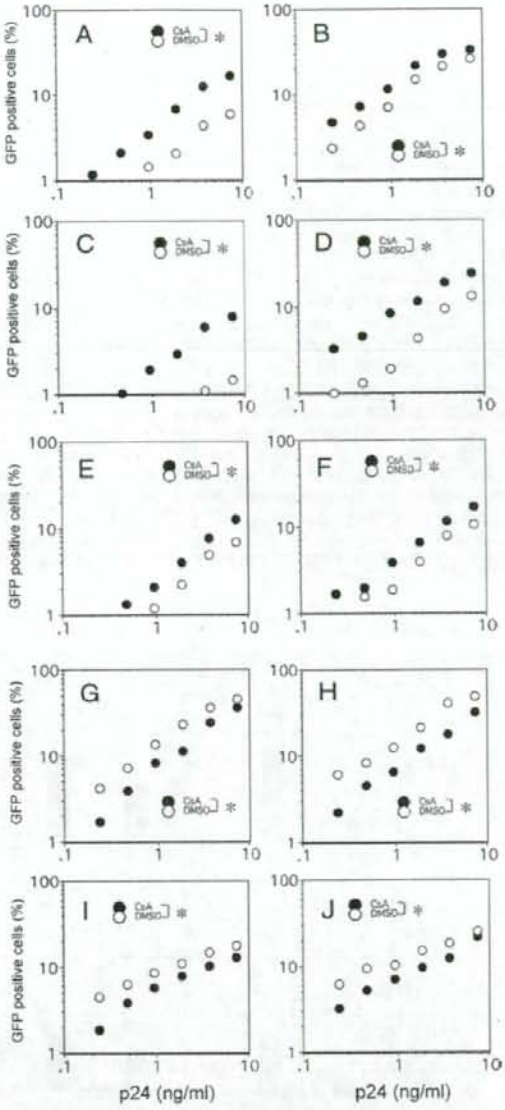

Fig. 3. Vero (A, B), CV1 (C, D), LLC-MK2 (E, F), 293T (G, H) and MT4 (I, J) cells were infected with SeV expressing AGM TRIM5α lacking the coiled-coil domain (A, C, E, G, I) or CM TRIM5α lacking the SPRY domain (B, D, F, H, J) at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Cells were treated with 5 μM of Cyclosporin A (black circles) or vehicle control, 0.5% of dimethyl surfoxide (DMSO, white circles), for 1 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP in 5 μM of Cyclosporin A (black circles) or 0.5% of DMSO (white circles) were inoculated for 2 h. After washing out of the inoculum, cells were cultivated for 40 h and GFP positive cells were counted with a flowcytometer. Asterisks indicate statistically significant differences ($p<0.05$, paired t-test). Representative data of at least three independent experiments is shown.

CypA binding to CA is critical for restriction by CypA in OWM cells

The hydrophobic pocket of CypA makes direct contact with HIV-1 CA proline 90 and adjacent residues on the surface-exposed h4/5 loop. Replacement of the h4/5 loop with SIVmac reportedly eliminates CypA

binding to CA (Bukovsky et al., 1997). In order to further elucidate the role of CypA and CA interactions in the antiviral activity of CypA, we investigated whether HIV-1 mutant (NL-ScaVR) or HIV-2 (GH123), whose CAs are unable to bind CypA, are restricted to human and OWM cells. NL-SVR, which can bind CypA and is an HIV-1 NL43 variant carrying SIVmac vif, was used as a control virus. NL-ScaVR is a NL43 variant carrying both SIVmac vif and the SIVmac gag h4/5 loop and has been shown to be able to grow in CM cells (Kamada et al., 2006). Consistent with our previous finding, expression of TRIM5α lacking the SPRY domain enhanced HIV-1(NL-SVR, NL-ScaVR) and HIV-2 infectivity in OWM cells (Figs. 4Ad, Bd, and Cd) compared with non-interfering control (Figs. 4Ac, Bc, and Cc). Exogenous expression of CypA suppressed the NL-SVR virus, which is able to bind CypA, in OWM cells (Fig. 4Ad) but not in human cells (Fig. 4Ab). In contrast, both NL-ScaVR (Fig. 4B) and GH123 (Fig. 4C), those cannot bind CypA, were not suppressed in either human or OWM cells by exogenously expressed CypA. Instead, those viruses tended to show slightly higher titers in cells expressing exogenous CypA.

As for the CypA protein, it is known that H54, R55, and H126 are crucial for the incorporation of CypA into HIV-1 virion (Dorfman et al., 1997). When we introduced R55A substitution into an HA-tagged version of CypA, the suppressive effect of exogeneous CypA was eliminated in CV1 and LLC-MK2 cells (Fig. 5A), although the level of expression in those cells was comparable to that of wild type CypA (Fig. 5B). Expression levels of TRIM5α lacking the SPRY domain in mutant CypA and the wild type CypA expressing cells were also comparable (Fig. 5B). These results indicate that CypA and CA interaction is essential for HIV-1 restriction by CypA in OWM cells.

CypA multimerization is not required for the HIV-1 restriction in OWM cells

In a mutagenesis study of TRIMCyp, overexpression of CypA-like protein reportedly reduced susceptibility of cells to HIV-1 (Diaz-Griffero et al., 2006). Javanbakht et al. (2007) reported that multimerization of CypA increased the anti-HIV-1 activity of CypA. Thus, it is possible that multimerization of CypA occurs only in OWM cells but not in human cells. To determine the extent of multimerization of CypA in human and OWM cells, we infected 293T and LLC-MK2 cells with SeV expressing CypA-HA and fixed the cell lysates with glutaraldehyde. CypA-HA proteins were then immunoprecipitated by using the anti-HA antibody. As shown in Fig. 6, most of the CypA-HA protein was monomeric in both 293T and LLC-MK2 cells, suggesting that the multimerization of exogenous CypA is not required for HIV-1 restriction in OWM cells.

Discussion

In the study reported here, we investigated the role of CypA in HIV-1 restriction of OWM cells. We demonstrated that exogenous

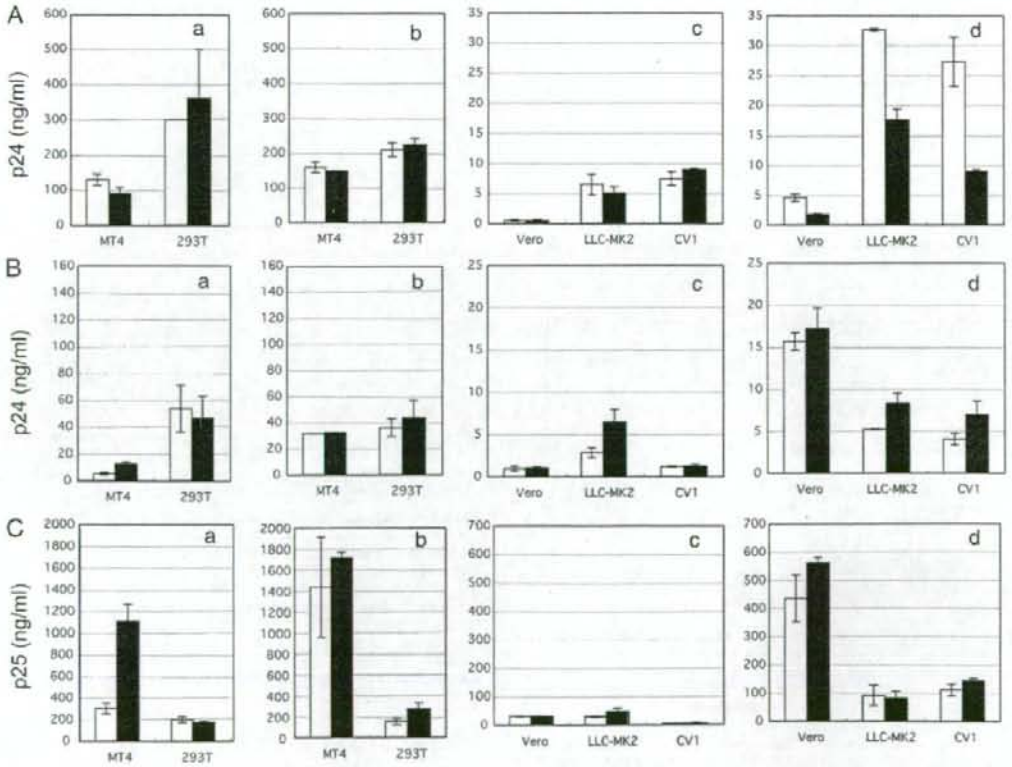

Fig. 4. MT4, 293T, Vero, LLC-MK2 and CV1 cells were infected with SeV expressing AGM TRIM5α lacking the coiled-coil domain (a and c) or CM TRIM5α lacking the SPRY domain (b and d) mixed with the empty SeV vector parental Z strain (white bars) or SeV expressing CypA (black bars), at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Cells were then superinfected with 20 ng of p24 of VSV-pseudotyped HIV-1-NL-SVR (A), HIV-1-NL-ScaVR (B) or p25 of HIV-2 GH123 (C). The culture supernatants were collected 3 days after infection, and the level of p24 or p25 was measured wth a RETROtek antigen ELISA kit. Data points show means of triplicate samples with SD.

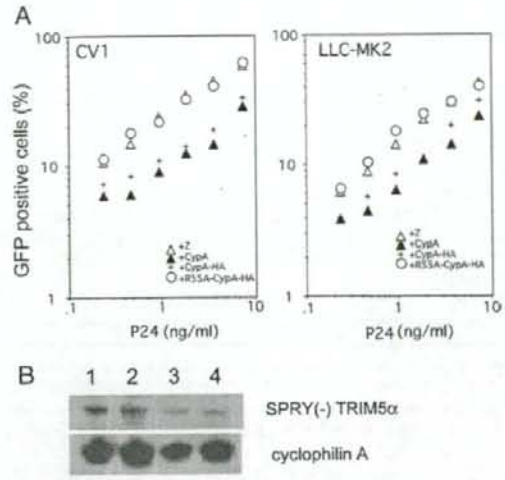

Fig. 5. (A) CV1 and LLC-MK2 cells were infected with SeV expressing CM TRIM5α lacking the SPRY(-) domain mixed with SeV expressing CypA (black triangles), CypA-HA (crosses), R55A-CypA-HA (white circles) or the empty SeV vector parental Z strain (white triangles) at a MOI of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were counted with a flowcytometer. Representative data of at least three independent experiments is shown. (B) Lysates of CV1 (lanes 1 and 2) and LLC-MK2 (lanes 3 and 4) cells infected with SeV expressing CM TRIM5α lacking the SPRY(-) domain mixed with SeV expressing CypA-HA (lanes 1 and 3) or R55A-CypA-HA (lanes 2 and 4) were visualized by western blotting with an antibody against HA. Representative data of two independent experiments is shown.

expression of CypA suppresses HIV-1 infection in OWM cells in the absence of functional TRIM5α and that the inhibitory activity of CypA depends upon the interaction of CypA moiety with HIV-1 capsid. In addition, disruption of CypA and capsid interaction by CsA treatment enhanced the HIV-1 susceptibility of OWM cells even in the absence of functional TRIM5α. These results suggest the presence in OWM cells of TRIM5α-independent anti-HIV-1 activity mediated by CypA.

Knock-down of TRIM5 alone or dominant negative suppression of functional TRIM5α caused enhancement of HIV-1 infection and exogenous expression of CypA partially compensating for the absence of TRIM5α in HIV-1 infection, even though the expression levels of exogenous CypA were overwhelmingly higher than those of TRIM5α. This result suggests that TRIM5α may play a pivotal role in HIV-1 restriction in the OWM cells used in our study. We speculate that the mechanisms of anti-HIV-1 activity mediated by TRIM5α-independent CypA is static, for example, by trapping the virion core in cytoplasm and interfering with the nuclear transport of reverse transcription complex. Although CypA itself is not strong enough to eliminate HIV-1 infection, endogenous TRIM5α can attack HIV-1 more effectively if CypA traps virion in cytoplasm and reinforces restriction process.

Recently, two groups reported that the simultaneous knock-down of both CypA and TRIM5 in OWM cells caused minimal additional increase of HIV-1 infection compared with knock-down of TRIM5 alone, suggesting that CypA inhibits HIV-1 replication in OWM cells in a TRIM5-dependent manner. They suggested that CypA is required for CA recognition by TRIM5α. We agree that both CypA and TRIM5α are able to suppress HIV-1 infection in OWM cells, since the most potent suppression of HIV-1 infection in our study was observed in the presence of both CypA and TRIM5α. However, our findings also indicate the presence in OWM cells of TRIM5α-independent anti-HIV-1 activity mediated by CypA. At present, the reason for the discrepancy between their results and ours is not clear. It is possible that the dominant negative protein we used interfered more effectively with TRIM5α function than did the RNAi used in the other studies. In this respect, it is noteworthy that one of the two groups did observe slightly higher HIV-1 infection after simultaneous knock-down of CypA and TRIM5 than after knock-down of TRIM5 alone (Berthoux et al., 2005).

The effect of CypA on HIV-1 infection in human cells is opposite to the effect that in cells from OWM. It has been proposed that interaction of CypA with CA protects HIV-1 against restriction by human TRIM5α or other unknown antiviral factors (Luban, 2007; Towers, 2007). Our findings did not support this hypothesis that CypA protects HIV-1 from human TRIM5α, since we found that CsA treatment of human cells suppressed the HIV-1 infection even in the absence of functional human TRIM5α. In case of a virus unable to bind CypA, overexpression of CypA led to slightly higher virus replication in both human and OWM cells, although the enhancement varied among cells. It has also been reported that CypA augmented cell proliferation and gene expression in cancer cells (Li et al., 2005; Yang et al., 2005). It is possible that CypA itself affects the cell signaling or innate immune pathway to support high virus replication in both human and OWM cells. In the case of HIV-1, which is able to bind CypA, however, overexpression of CypA showed suppression of HIV-1 replication only in OWM cells. This suggests that the HIV-1 restricting activity of exogenously expressed CypA surpasses the HIV-1 enhancing effect of CypA in OWM cells. The effect of CsA treatment was more prominent in the presence of TRIM5α than in the absence of TRIM5α. It is therefore likely that CsA treatment in the presence of TRIM5α disrupted both the TRIM5α-dependent and -independent effects of CypA, whereas CsA treatment in the absence of TRIM5α disrupted only the TRIM5α-independent effect.

The amino acids sequence of human CypA is identical to the sequences of OWM CypAs. The cell type specific difference in the effect of exogenous CypA expression must thus be caused by the difference in cellular factor(s) interacting with CypA. Diaz-Griffero et al. (2007) showed exogenous expression of CypA suppressed HIV-1 in feline CRFK cells. On the other hand, Javanbakht et al. (2007) reported that multimerized CypA but not monomeric CypA suppressed HIV-1 in canine Cf2Th cells. These findings also suggest that the effect of exogenously expressed monomeric CypA on HIV-1 infection varies widely among cells or species. It is further possible that a certain OWM-specific restriction factor binds to CypA in OWM cells and thus

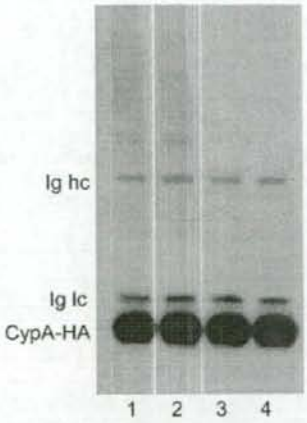

Fig. 6. Lysates of CV1 (lanes 1 and 3) and 293T (lanes 2 and 4) cells infected with SeV expressing CypA-HA were first fixed with 2.5 mM glutaraldehyde and then subjected to immunoprecipitation with an anti-HA High Affinity rat monoclonal antibody. CypA-HA proteins were visualized by western blotting with an antibody against HA. Ig hc and Ig lc indicate the IgG heavy chain and light chain used for immunoprecipitation, respectively.

inhibits HIV-1 infection. The identification of factor(s) reacting with CypA in OWM cells is therefore important to gain an understanding of the precise mechanisms of OWM resistance against HIV-1 as well as the physiological function of CypA.

Materials and methods

Cloning and expression of CypA

CypA cDNA was amplified by RT-PCR from the human T cell line MT4 by using 5′-GCGGCCGCAGCCATGGTCAACCCC-3′ as the forward primer and 5′-ACGGCGGTCTTTTCATTCGAGTTGTCC-3′ as the reverse primer. The amplified product was then cloned into pCR-2.1TOPO (Invitrogen, Carlsbad, CA) and verified for nucleotide sequence authenticity. The resultant cDNA clone served as a template for mutagenesis to generate a hemagglutinin (HA;YPYDVPDYAA) tagged version and an active site mutant which replaced the 55th arginine residue with alanine. Those cDNA fragments were then cloned into the pSeV18+b(+) vector, and recombinant SeV carrying CypA, CypA-HA tag, and R55A-CypA-HA were recovered with a slightly modified previously described method (Nakatsu et al., 2006; Nakayama et al., 2004; Takeda et al., 2005). Generation of SeV expressing CM-TRIM5α lacking the SPRY domain (CM-SPRY(-)-SeV) (Song et al., 2007) and SeV expressing AGM-TRIM5α lacking the coiled-coil domain (AGM-CC(-)-SeV) were described previously (Maegawa et al submitted).

RNA interference

Si-TRIM5 was a mixture of five siRNA targeting TRIM5: si-TRIM5-1 (5′-CUGAGAAUAUACAGCCUAAdTdT-3′), si-TRIM5-2 (5′-CGGCAGAUUUUGAGCAACUdTdT-3′), si-TRIM5-3 (5′-GCAAUGAGCUGCAGAACCUdTdT-3′), si-TRIM5-4 (5′-GGUUAUAGGGUUACAGGAAdTdT-3′) and si-TRIM5-5 (5′-GUUACAGGAAGGAGAUAAAdTdT-3′). All siRNAs were directed against AGM TRIM5 coding sequences. The 5′ ends of siTRIM5-1, -2, -3, -4, and -5 were located at positions 194, 551, 596, 1266, and 1275, respectively, of the TRIM5 open reading frame. For negative control, "SiCONTROL Non-targeting siRNA#2 (Si-Cont.)" (5′-UAAGGCUAUGAAGAGAUACUU-3′) was used (Dharmacon, Lafayette, CO). 0×10^5 CV1 cells in 6-well plates were transfected with 60 pmol of siRNA using credia-TF (Credia Japan, Kyoto, Japan) according to the manufacturer's instructions.

Real time PCR

Total RNA extracted from siRNA transfected CV1 cells were examined for the expression of TRIM5α using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect TRIM5α were as follows. forward primer: 5′-AACCTGGAGAAGGAGGAAGAAGA-3′, reverse primer: 5′-CTGGGTCTGCTGCACCAT-3′ and probe: 5′-FAM-TCCGTTTCAGACTTCG-TAMRA-3′. These primers amplify the coiled-coil region of TRIM5 gene.

Viruses and HIV-1 lentivirus vector

VSV-G-pseudo typed HIV-1-NL-SVR, HIV-1-NL-ScaVR, or HIV-2-GH123 was prepared by transfection of 293T cells with a combination of pMD.G (Miyoshi et al., 1998, 1997) and pNL-SVR (Kamada et al., 2006), pNL-ScaVR (Kamada et al., 2006) or pGH123 (Shibata et al., 1990), respectively. pNL-SVR and pNL-ScaVR were generated by PCR-based mutagenesis by using pNL432 (Adachi et al., 1986) as a template as reported by Kamada et al. (2006). HIV-1 vector expressing GFP was prepared as described previously (Miyoshi et al., 1998, 1997). Two days after transfection, culture supernatants of 293T cells were collected and assayed for their p24 or p25 level by using a RETROtek antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY).

Viral infection

Assays for the HIV-1 vector expressing GFP were performed in 24-well plates containing 4×10^4 Vero, CV1, LLC-MK2, MT4 or 293T cells. Target cells were infected with AGM-CC(-)-SeV or CM-SPRY(-)-SeV mixed with SeV expressing CypA, CypA-HA or the empty SeV vector parental Z strain at a multiplicity of infection of five plaque forming units per cell for each virus and incubated at 37 °C for 16 h. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were then inoculated, and infected cells were counted with a flowcytometer (FACScan, Becton Dickinson Biosciences, San Jose, CA) 40 h after infection.

4×10^4 Vero, CV1, LLC-MK2, MT4 or 293T was infected with AGM-CC(-)-SeV or CM-SPRY(-)-SeV mixed with SeV expressing CypA or the empty SeV vector parental Z strain at a multiplicity of infection of five PFUs per cell for each virus and incubated at 37 °C for 16 h. Cells were then superinfected with 20 ng of p24 of HIV-1-NL-SVR, HIV-1-NL-ScaVR or p25 of HIV-2 GH123. The culture supernatants were collected 3 days after infection, and the level of p24 or p25 was measured with the aid of a RETROtek antigen ELISA kit.

Immunoprecipitation and western blot analysis

We analyzed the expression levels of CypA in Vero, CV1, LLC-MK2, 293T or MT4 cells infected with recombinant SeV expressing CypA-HA by western blot analysis by using anti-CypA polyclonal antibody (Affinity BioReagents, Golden, CO). For analysis of the oligomeric CypA, cell lysates were first fixed with 2.5 mM glutaraldehyde and then precipitated with anti-HA High Affinity rat monoclonal antibody (Roche Diagnostics, Indianapolis, IN) as described previously (Mische et al., 2005). Materials were subjected to SDS-PAGE on a 4-12% NuPAGE Bis-Tris gel (Invitrogen) and proteins in the gel were then electronically transferred to a PVDF membrane (Immobilon; Millipore, Billerica, MA). Blots were blocked and probed with anti-HA antibody overnight at 4 °C, and then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA) and developed using the Chemilumi-one L HRP chemiluminescent kit (Nacali, Kyoto, Japan). Visualized images were recorded by with LAS1000 (Fujifilm, Tokyo, Japan).

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Silencing of tripartite motif protein (TRIM) 5α mediated anti-HIV-1 activity by truncated mutant of TRIM5α

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ABSTRACT

Tripartite motif protein (TRIM) 5α is a restriction factor of human immunodeficiency virus type 1 in Old World monkey cell. It was found that both naturally occurring and artificial TRIM5α variants lacking the SPRY domain could silence TRIM5α activity. Specifically, the artificial TRIM5α mutant could suppress TRIM5α activity of various primate species with even higher efficiency than could small interfering RNAs. The findings indicate that TRIM5α variants lacking the SPRY domain are useful for silencing TRIM5α activity.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) encounters post-entry block in Old World monkey (OWM) cells (Himathongkham and Luciw, 1996; Hofmann et al., 1999; Shibata et al., 1995), which is mediated by tripartite motif protein (TRIM) 5α (Stremlau et al., 2004). TRIM5 consists of RING, B-box 2, and coiled-coil domains, but only the α isoform possesses the SPRY (B30.2) domain at its C-terminus (Reymond et al., 2001). Several recombinant studies between human and rhesus monkey (Rh) TRIM5α revealed that the determinant of the species specificity lies in the SPRY domain of TRIM5α (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). It was also demonstrated that a specific region in the SPRY domain of AGM TRIM5α determined species-specific restriction of SIVmac (Nakayama et al., 2005). Previous biochemical studies showed that TRIM5α binds viral capsid in a SPRY domain dependent manner (Sebastian and Luban, 2005; Stremlau et al., 2006). Since the SPRY domain is thought to recognize viral capsid, TRIM5α is the only isoform with anti-retroviral activity. Several isoforms of TRIM5 lack the SPRY domain, and a γ isoform of TRIM5 has been shown to exert a dominant-negative effect on anti-HIV-1 activity of rhesus monkey TRIM5α (Stremlau et al., 2004).

RNA interference is a potent and highly specific gene-silencing tool that is triggered by double-stranded RNAs of 21–23-nt. These small interfering (si)RNAs trigger gene silencing by binding to their target-mRNA sequences and cleaving the target, so that the use of siRNA for silencing specific genes in mammalian cells has become a standard laboratory procedure. However, the knockdown effect of siRNA greatly depends on the RNA sequence and transfection efficiency. In addition, the extent of the knockdown effect by siRNA may seriously affect conclusions drawn from study results. In this study, we applied the dominant-negative effect of TRIM5α variants lacking the SPRY domain on TRIM5α to silencing of TRIM5α mediated anti-HIV-1 activity.

2. Materials and methods

2.1. Cloning and expression of TRIM5s

African green monkey (AGM) TRIM5γ cDNA was amplified by reverse transcription-PCR from AGM CV1 cells by using 5′-GCGGC-CGCTACTATGGCTTCTGG-3′ and 5′-AGACTTGAGAGAAAACTGG-3′. The amplified products were cloned into pCR-2.1 TOPO (Invitrogen, Carlsbad, CA). A PCR-based mutagenesis was used to generate AGM TRIM5γ cDNA with a hemagulutinin (HA) tag (YPYDVPDYAA) at its N-terminus, a C-terminally HA-tagged AGM-TRIM5α lacking the coiled-coil domain (132nd to 233rd position) (AGM-TRIM5α-CC(−)-HA), and human TRIM5α with R332P

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mutation. AGM-TRIM5α carrying the myc tag at its C-terminus was described previously (Nakayama et al., 2006). The entire coding sequences of those TRIM5-tags were then transferred to the NotI site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) expressing various TRIM5-tags were obtained using a method described previously (Shioda et al., 2001). Recombinant SeVs expressing AGM-TRIM5α-HA, cynomolgus monkey (CM)-TRIM5α-HA, CM-TRIM5α-SPRY(−)-HA, and Rh-TRIM5α-HA were previously described (Kono et al., 2008; Nakayama et al., 2005; Song et al., 2007).

2.2. Immunoprecipitation and Western blot analysis

For protein expression analysis, human T-cell line MT-4 was infected with SeV at a multiplicity of infection (MOI) of 10 plaque forming unit (PFU) per cell, and incubated at 37 °C for 16 h. Cells were then lysed and lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were then transferred electronically to a membrane (Immobilon; Millipore, Billerica, MA). Blots were blocked and probed with the anti-HA High Affinity rat monoclonal antibody (Roche, Indianapolis, IN) overnight at 4 °C. Blots were then incubated with peroxidase-conjugated anti-rat IgG (American Qualex, San Clemente, CA). Bound antibodies were visualized with the chemilumi-one L chemiluminescent kit (Nacalai Tesque, Kyoto, Japan).

For co-immunoprecipitation analysis, CV1 cells were infected with recombinant SeV expressing various TRIM5s at a MOI of 5 PFU per cell, followed by incubation at 37 °C for 22 h. Cells were then lysed and TRIM5 proteins in the lysates were precipitated with the anti-myc mouse monoclonal antibody (9B11; Cell Signaling Technology, Danvers, AM) using a Protein G-immunoprecipitation kit (Roche, Indianapolis, IN). Precipitated proteins were detected with the same procedure as above except that the anti-myc mouse monoclonal antibody and peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used for visualizing myc-tagged TRIM5α protein.

The anti-TRIM5 antibody was prepared by immunization of rabbit with a peptide (CRISYQPENIQPNRH) corresponding to the amino acid sequence from the 59th to 72nd position between the RING and the B-box 2 domains of AGM TRIM5α. The antigen was immunized with Freund's complete adjuvant. Sera from immunized rabbits were used as the anti-TRIM5 antibody in this study.

2.3. Virus and HIV-1 lentiviral vector

HIV-1-NL43 was prepared by transfection of 293T cells with pNL432 (Adachi et al., 1986). The HIV-1 vector expressing green fluorescence protein (GFP) (HIV-1-GFP) was prepared as described previously (Miyoshi et al., 1997, 1998). The viral titer was determined by measuring p24 with a RETROtek antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

2.4. Viral infection

Serially diluted HIV-1-GFPs were inoculated into 5×10^4 CV1 cells in 24-well plates, and 40 h after infection, the infected cells were counted with a flow cytometer (FACScaliber; Becton Dickinson, Franklin Lakes, NJ).

For the HIV-1 infection assay, 2.0×10^5 MT-4 cells were infected with SeV expressing various TRIM5s. Nine hours after SeV infection, the cells were superinfected with 20 ng of p24 of HIV-1 NL43. The culture supernatants were collected 3 days after infection, and the p24 levels were measured.

2.5. RNA interference

si-TRIM5 was a mixture of five siRNA targeting TRIM5: si-TRIM5-1 (5′-CUGAGAAUAUACAGCCUAAdTdT-3′), si-TRIM5-2 (5′-CGGCAGAUUUUGAGCAACUdTdT-3′), si-TRIM5-3 (5′-GCAAUGAG-CUGCAGAACCUdTdT-3′), si-TRIM5-4 (5′-GGUUAUAGGGUUACAGG-AAdTdT-3′), and si-TRIM5-5 (5′-GUUACAGGAAGGAGAUAAAdTdT-3′). All siRNAs were directed against AGM TRIM5 coding sequences. The 5′ ends of si-TRIM5-1, si-TRIM5-2, si-TRIM5-3, si-TRIM5-4, si-TRIM5-5 were located at positions 194, 551, 596, 1266, 1275, respectively, of the TRIM5 open reading frame. For negative control, "SiCONTROL Non-targeting siRNA#2 (Si-Cont)" (5′-UAAGGCUAUGAAGAGAUACUU-3′) was used (Dharmacon, Lafayette, CO).

1.0×10^5 CV1 cells in six-well plates were transfected with 60 pmol of siRNA using credia-TF (Credia Japan, Kyoto, Japan) according to the manufacturer's recommendations. After 48 h, gene knockdown was confirmed with a previously described real-time PCR method that amplifies the coiled-coil region of TRIM5 (Nakayama et al., 2005).

To compare knockdown effect by siRNA and AGM TRIM5γ or CM-TRIM5α-SPRY(−), we prepared (1) CV1 cells transfected with Si-Cont or si-TRIM5, (2) CV1 cells infected with SeV expressing AGM TRIM5γ, CM-TRIM5α-SPRY(−) or AGM-TRIM5α-CC(−), or (3) CV1 cells transfected with si-TRIM5 and subsequently infected with SeV expressing AGM TRIM5γ, CM-TRIM5α-SPRY(−).

For the preparation of (1) cells, 1.0×10^5 CV1 cells were seeded in six-well plates. After 24 h, the cells were transfected with Si-Cont or si-TRIM5. Forty-nine hours after transfection, the cells were trypsinized, reseeded in 24-well plates, and incubated for 8 h.

For the preparation of (2) cells, 1.0×10^5 CV1 cells were seeded in six-well plates. After 72 h, the cells were infected with SeV expressing AGM TRIM5γ, CM-TRIM5α-SPRY(−) or AGM-TRIM5α-CC(−). One hour after infection, the cells were trypsinized, reseeded in 24-well plates, and incubated for 8 h.

For the preparation of (3) cells, 1.0×10^5 CV1 cells were seeded in six-well plates. After 24 h, the cells were transfected with si-TRIM5. Forty-eight hours after transfection, the cells were infected with SeV expressing AGM TRIM5γ or CM-TRIM5α-SPRY(−). One hour after infection, the cells were trypsinized, reseeded in 24-well plates, and incubated for 8 h.

These (1)–(3) cells were then infected with HIV-1-GFP. Forty hours after infection, the infected cells were counted with a flow cytometer.

2.6. Gene reporter fusion assay

A recombinant vaccinia virus (Vac)-based gene activation assay using β-galactoside gene as a reporter was performed as described previously (Nussbaum et al., 1994; Nakayama et al., 2004). Briefly, mouse fibroblast L cells were transfected with β-galactosidase reporter plasmid pGINT7β-gal and infected with a recombinant Vac expressing gp160 of an X4 HIV-1 strain NL43 or parental WR strain. At the same time, CV1 cells were infected with SeVs expressing various TRIM5s and human CXCR4, and with Vacs expressing T7 RNA polymerase and human CD4, and cultured at 31 °C overnight. Then, L and CV1 cells were mixed, incubated at 37 °C for 3 h, and β-galactosidase activities in the cell lysate were measured by using chlorophenol red-β-D-galactopyranoside as substrate.

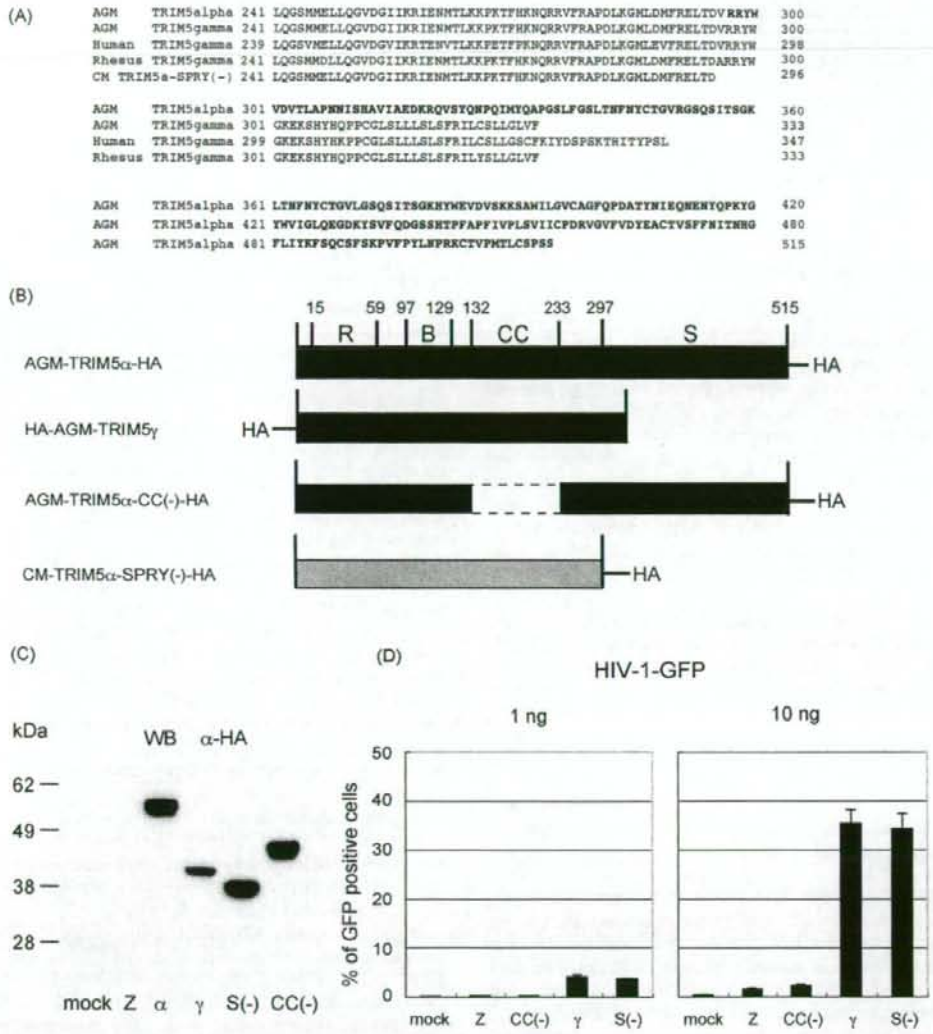

Fig. 1. (A) Alignment of amino acid sequence of the C-terminal portion of African green monkey TRIM5α (AGM TRIM5alpha), TRIM5γ (AGM TRIM5gamma), human TRIM5γ (Human TRIM5gamma), and cynomolgus monkey CM-TRIM5α lacking SPRY domain (CM-TRIM5a-S(−)) predicted from the cDNA sequences. The rhesus monkey TRIM5γ (Rhesus TRIM5gamma) sequence (Stremlau et al., 2004) is also shown. Bold face letters indicate amino acid residues in SPRY domain of AGM TRIM5α. (B) Schematic representation of TRIM5 constructs. Black and gray bars denote AGM and cynomolgus monkey (CM) sequences, respectively. *Abbreviations for domains*: R, RING; B, B-box 2; CC, coiled-coil; S, SPRY. The numbers of the amino acid residues at the boundaries of the TRIM5α domains (Song et al., 2005) are shown. Dotted boxes denote deletion of corresponding amino acid. CM-TRIM5α-SPRY(−) is composed of 296 amino acid residues. (C) Expression of various TRIM5s. TRIM5 proteins in MT-4 cells mock infected (mock) or infected with parental Z strain of SeV (Z), or with SeV expressing AGM TRIM5α (α), AGM TRIM5γ (γ), CM-TRIM5α-SPRY(−) (S(−)), and AGM-TRIM5α-coiled-coil(−) (CC(−)), at MOI of 10 PFU per cell were visualized by Western blotting with antibody to HA. (D) Dominant-negative effect of TRIM5γ and truncated mutant of TRIM5α. CV1 cells mock infected (mock) or infected with parental Z strain of SeV (Z), or with SeV expressing AGM TRIM5α (α), AGM TRIM5γ (γ), CM-TRIM5α-SPRY(−) (S(−)), or AGM-TRIM5α-CC(−) (CC(−)) at MOI of 10 PFU per cell were exposed to 1 ng (left) or 10 ng (right) of p24 of the HIV-1-GFP. GFP-positive cells were counted with a flowcytometer. Error bars show the S.D. of triplicate values for representative results of three independent experiments.

2.7. Immunofluorecence confocal microscopy

CV1 cells infected with SeV expressing several TRIM5s at MOI of 10 PFU per cell were fixed 24 h after infection in 3% paraformaldehyde in PBS, permeabilized with 0.05% saponin and 0.2% bovine serum albumin in PBS, and incubated with the anti-HA rat monoclonal antibody. Bound antibodies were then detected with the FITC-conjugated goat antibody directed against rat IgG (American Qualex Antibodies, San Clemente, CA). Indirect immunofluorescence was visualized with a Radiance 2000 laser confocal microscope system (Bio-Rad Laboratories, Hercules, CA).

For colocalization analysis, CV1 cells were infected with SeV expressing several TRIM5s at MOI of 5 PFU per cell. Colocalization of TRIM5s was visualized with the same procedure as the one described above except for the use of anti-myc mouse monoclonal antibody and Cy5-conjugated mouse antibody directed against mouse IgG (Amersham-Pharmacia Biotech, Freiburg, Germany) for visualizing myc-tagged TRIM5α protein as well as anti-HA antibody and FITC-conjugated goat antibody.

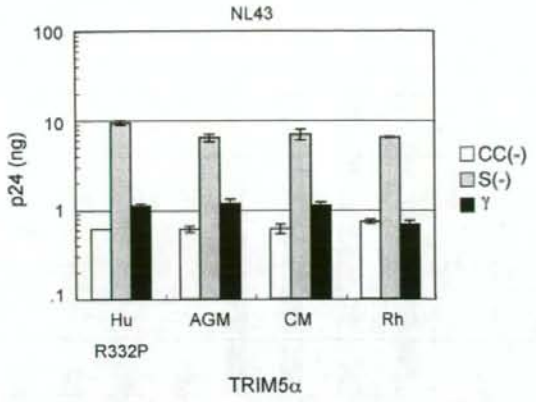

Fig. 2. Dominant-negative effect of truncated mutant of TRIM5α on TRIM5α of different primate species. MT-4 cells infected with SeV expressing indicated TRIM5α and AGM-TRIM5α-CC(−) (white bar), CM-TRIM5α-SPRY(−) (gray bar), or AGM TRIM5γ (black bar) at MOI of 5 PFU per cell were infected with NL43. Three days after infection, culture supernatants were assayed for p24 levels. The representative results of two independent experiments with similar results are shown. Error bars denote S.D. of duplicate samples.

3. Results

3.1. Dominant-negative effect of truncated mutant of TRIM5α and of TRIM5γ

Rh TRIM5γ had been shown to suppress the restriction of HIV-1 in Rh cells in a dominant-negative manner (Stremlau et al., 2004). To examine whether AGM TRIM5γ also shows a dominant-negative effect on TRIM5α-mediated HIV-1 restriction in AGM cells, TRIM5γ cDNA was cloned from AGM cell line CV1. The predicted amino acid sequence from the 241st position of TRIM5γ is shown in Fig. 1A. AGM TRIM5γ is composed of 333 amino acid residues and shares the N-terminal 300 amino acid residues with TRIM5α, while it was 16 amino acids shorter than Hu TRIM5γ. In the γ-specific region, AGM TRIM5γ showed the same amino acid sequence as that of Rh TRIM5γ except for the 326th position. A recombinant SeV expressing AGM TRIM5γ (Fig. 1B) was then constructed by first constructing AGM TRIM5γ fused with the HA tag at the C-terminus. Although AGM TRIM5γ could be detected by the anti-TRIM5 antibody in immunoblotting, it could not be detected by the anti-HA antibody (data not shown). This failure to detect the C-terminal HA tag may be due to steric hindrance of the HA tag by hydrophobic amino acid residues specific to TRIM5γ. An N-terminally HA tagged TRIM5γ was constructed and it was found that the N-terminally HA tagged TRIM5γ could be clearly detected by the anti-HA antibody at a reduced level compared to that of TRIM5α in human T-cell line MT-4 (Fig. 1C).

To examine whether AGM TRIM5γ and CM TRIM5α lacking the SPRY domain [CM-TRIM5α-SPRY(−)-HA] (Song et al., 2007), whose composition resembled that of TRIM5γ, shows a dominant-negative effect on TRIM5α-mediated HIV-1 restriction in AGM cells, CV1 cells were infected with SeV expressing HA-AGM-TRIM5γ or CM-TRIM5α-SPRY(−)-HA. Nine hours after infection, the cells were superinfected with HIV-1-GFP and infected cells were quantified by counting the GFP-positive cells. Fig. 1D shows the effect of various TRIM5 proteins on AGM TRIM5α mediated restriction. HIV-1-GFP infection was restricted in cells with both viral inoculation doses (1 ng and 10 ng of p24), while infection with the parental

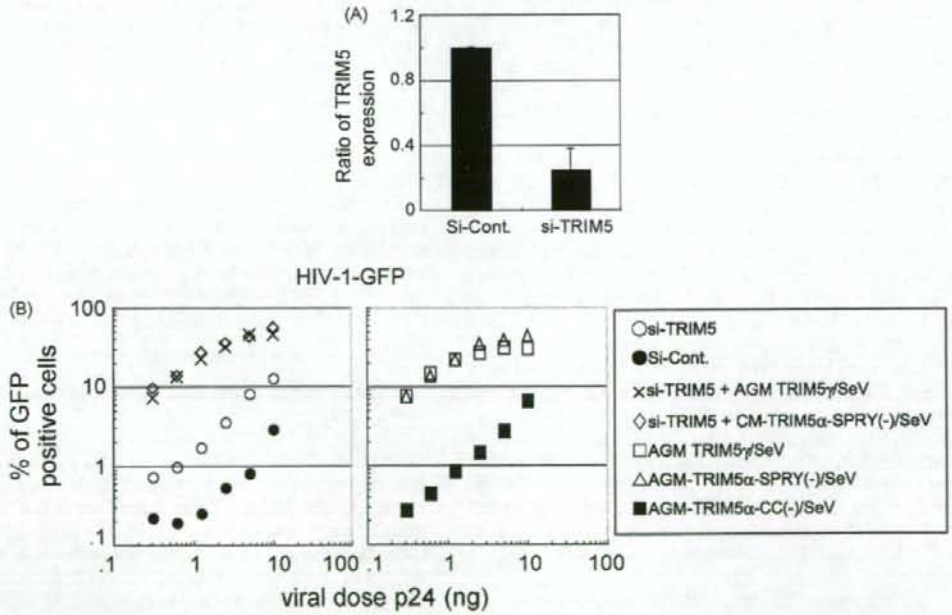

Fig. 3. (A) Reduction of TRIM5 expression by siRNA. CV1 cells were transfected with the si-TRIM5 or Si-Cont. Forty-eight hours after transfection, TRIM5 expression levels were measured by real-time PCR as described in Section 2. Data show the means of three independent experiments. Error bars denote S.D. of three independent experiments. (B) Comparison of knockdown effect by siRNA and CM-TRIM5α-SPRY(−) or AGM TRIM5γ. CV1 cells were transfected with si-TRIM5 (white circles), Si-Cont (black circles), si-TRIM5, and subsequently infected with SeV expressing AGM TRIM5γ (×) or CM TRIM5α-SPRY(−) (white diamonds) (left graph), or infected with SeV expressing AGM TRIM5γ (white squares), CM TRIM5α-SPRY(−) (white triangles), or AGM-TRIM5α-CC(−) (black squares) (right graph). These cells were then infected with HIV-1-GFP and GFP-positive cells were counted with a flowcytometer. The representative results of two independent experiments with similar results are shown.