

Table 2
Alignment of HEAT repeats

Repeat [*]	Species ^{**}	AA position		Fragment [†]	Score	E-value
		From	To			
HEAT_AAA	Hs - Htt	124	162	QKLLGIAMELFLLLCSDDAESDVRMVADECLNKVICALMD QKLLGIAMELFLLLCSDDAESDVRMVADECLNKVICALMD	1510	1.26E-06
	Cj - Htt	112	150		1590	5.48E-07
HEAT_AAA	Hs - Htt	205	243	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	1.03E-04
	Cj - Htt	193	231		1990	2.37E-08
HEAT_AAA	Hs - Htt	247	285	DNEIKVLLKAFIANLKSSSPTIRRTAAGSAVSIQCQHSRR DNEIKVLLKAFIANLKSSSPTIRRTAAGSAVSIQCQHSRR	1590	5.48E-07
	Cj - Htt	235	273		1590	1.97E-06
HEAT_AAA	Hs - Htt	317	355	LLTLRYLVPLLLQKQVQKDTSLKGSFGVTRKEMEVSPSAEQ LLTLRYLVPLLLQKQVQKDTSLKGSFGVTRKEMEVSPSAEQ	1620	1.11E-06
	Cj - Htt	305	343		1570	1.46E-07
HEAT_ADB	Hs - Htt Cj - Htt	734	771	YPPEQYVSDILNYIDHGDQPVRGATAILCGTLVCSILS	1450	3.29E-07
HEAT_AAA	Hs - Htt	803	841	TFSLADCIPLLRRTLKDESSVTCKLACTAVRNCVMSLCS TFSLADCVPLLRRTLKDESSVTCKLACTAVRHCVMSLCS	1500	5.78E-07
	Cj - Htt	791	829		1449	2.90E-06
HEAT_AAA	Hs - Htt	904	942	KLQERVLNNVVIHLLGDEDPVRVHVAASLIRLVPKLPFY TLQERVLNSVVIHLLGDEDPVRVHVAASLIRLVPKLPFY	1930	6.69E-08
	Cj - Htt	892	930		2150	2.51E-05
HEAT_AAA	Hs - Htt	984	1025	RIYRGYNLLPSITDVTMENNLSRVIAAVSHELITSTTRALTF RIYRGYNLLPSIIDVTMENNLSRVIAAVSHELITSTTRALTF	1370	9.05E-06
	Cj - Htt	972	1013		1410	2.71E-06
HEAT_AAA	Hs - Htt	1425	1463	RLFEPVLVIALKQYTTTTCVQLQKQVLDLLAQLVQLRVN RLFEPVLVIALKQYTTTTCVQLQKQVLDLLAQLVQLRVN	1370	5.62E-06
	Cj - Htt	1413	1451		1580	3.20E-07
HEAT_AAA	Hs - Htt	2798	2836	DDTAKQLIPVISDYLLSNLKGIAHCVNIHSQQHVLVMCA DDTAKQLIPVISDYLLSNLKGIAHCVNIHSQQHVLVMCA	1430	3.51E-06
	Cj - Htt	2785	2823		1430	3.29E-06

* HEAT_AAA and HEAT_ADB indicate subsets of HEAT repeats representing PP2A and adaptin families, respectively.

** Hs-Htt and Cj-Htt indicate the human and common marmoset Htt proteins, respectively.

† Amino acids which are different from the sequence of Hs-Htt are indicated in red.

cDNA have significant sequence homology to that of other species' *Htt* genes. In addition, it should be noted that *Htt* sequences between the human and common marmoset (colored in yellow in Table 1) appear to be particularly conserved as compared with sequence conservation within non-primate *Htt* genes, suggesting that the *Htt* gene is highly conserved in primates.

Fig. 2 shows the alignment of amino acid sequences encoded by *Htt* exon 1 and its corresponding region in various species. From the alignment, *Cj-Htt* appears to possess a short polyglutamine tract of nine glutamines compared with that of the human and chimpanzee *Htt* genes; but other than the polyglutamine tract, the exon 1 corresponding region in *Cj-Htt* exhibits high sequence homology to the human *Htt* exon 1. It is also interesting that polyproline region adjacent to the polyglutamine tract has differences between primates and non-

primates: amino acid substitutions and deletions are observed, and the lack of the polyproline region in the *Callus gallus Htt* exon 1 is particularly remarkable. These differences may influence folding and aggregation of the Htt protein, and might represent adaptive evolution of *Htt* to each species. The difference in the exon 1 among various species may provide us with a hint for understanding the expansion of the polyglutamine tract in Huntington's disease in human.

Other than the exon 1 region, we also investigated the HEAT repeats possessing tandem arrayed bihelical structure, which appear to wrap around target substrates (Andrade and Bork, 1995; Neuwald and Hirano, 2000), and found that the HEAT repeats are also conserved in the *Cj-Htt* protein (Table 2). In addition, it may be interesting that HEAT_ADB, a subset of HEAT repeats representing adaptin family, is present in *Cj-Htt*, but not in Hs-Htt.

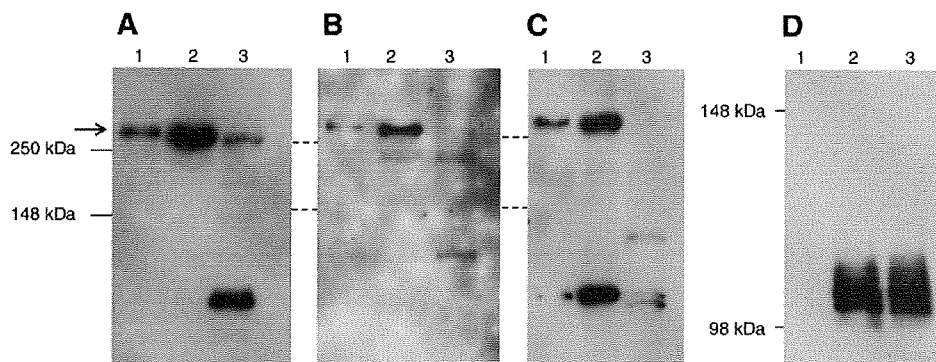


Fig. 3. Assessment of anti-human Htt antibodies against the common marmoset Htt polypeptide. Cell lysate derived from the common marmoset cell line (HSCj-110) (lane 1), brain tissue (lane 2), and mouse brain tissue as a control (lane 3) was examined by Western blotting with anti-human Htt antibodies. Tested antibodies were as follows: (A) MAB2166 (Chemicon), (B) MAB2170 (Chemicon), and (C) ab7666 (Abcam). Arrow indicates the signals of Htt proteins. The same results as those of HSCj-110 were also obtained when HSCj-002 and-009 were used (data not shown). After detection of signals, blotted membranes were subjected to antibody removal and then incubated with anti-APP antibody [MAB348 (Chemicon)] (D) followed by the same procedure as in the anti-human Htt antibodies described above.

3.2. Detection of *Cj-Htt* gene products

It is important to be able to properly detect the *Cj-Htt* gene and its gene products. Since the nucleotide and predicted amino acid sequences of the *Cj-Htt* cDNA showed significantly high sequence homologies to those of the human *Htt* gene, we tested whether

commercially-available TaqMan probe and antibodies against human *Htt* gene products (mRNA and protein) were also able to detect *Cj-Htt* gene products. As a result, the TaqMan probe (Fig. 5A) appears to be able to detect *Cj-Htt* mRNA. In addition, newly designed PCR primers, which are perfectly matched to *Cj-Htt*, also appear to be able to detect *Cj-Htt* mRNA (Fig. 5B).

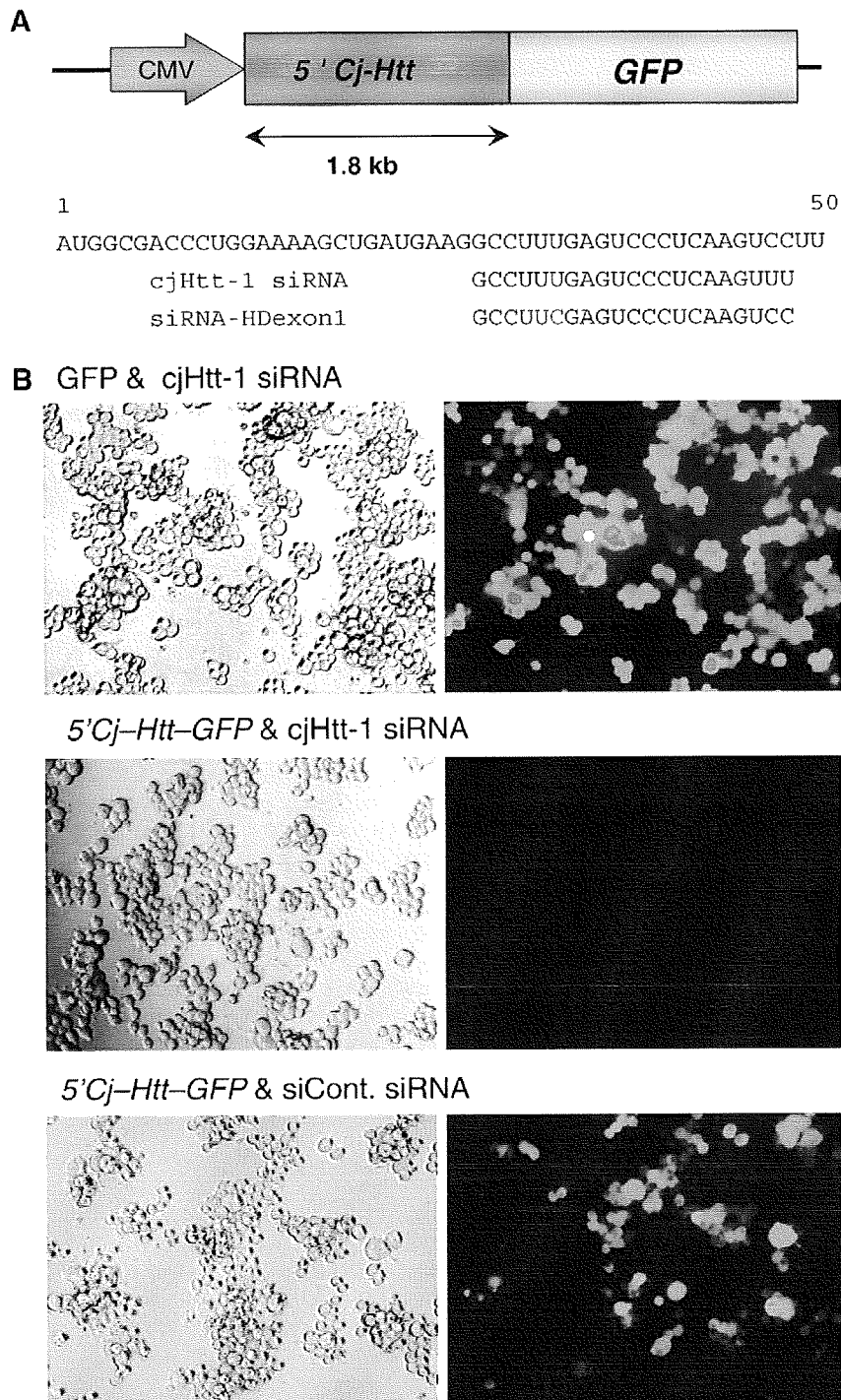


Fig. 4. Gene silencing against the 5'*Cj-Htt*-GFP fusion gene. (A) Schematic drawing of the 5'*Cj-Htt*-GFP fusion gene and designed cjHtt-1 siRNA. The fusion gene is composed of the 5' terminal region of the *Cj-Htt* ORF and GFP, and driven by the Human cytomegalovirus promoter. The *Cj-Htt* sequence from the first ATG to position 50 is shown together with sequences of cjHtt-1 siRNA and siRNA-HDexon1 targeting human *Htt*. A variant nucleotide between the siRNAs is indicated in red. (B) Effect of cjHtt-1 siRNA on gene silencing. Reporter genes [5'*Cj-Htt*-GFP or GFP (empty reporter)] and siRNAs [cjHtt-1 or siCont. (non-silencing siRNA)] were introduced into mouse neuroblastoma Neuro2a (N2a) cells. Two days after transfection, the cells were examined by a fluorescent microscope. Left (differential interference contrast) and right (fluorescence image) panels are identical in the visual field.

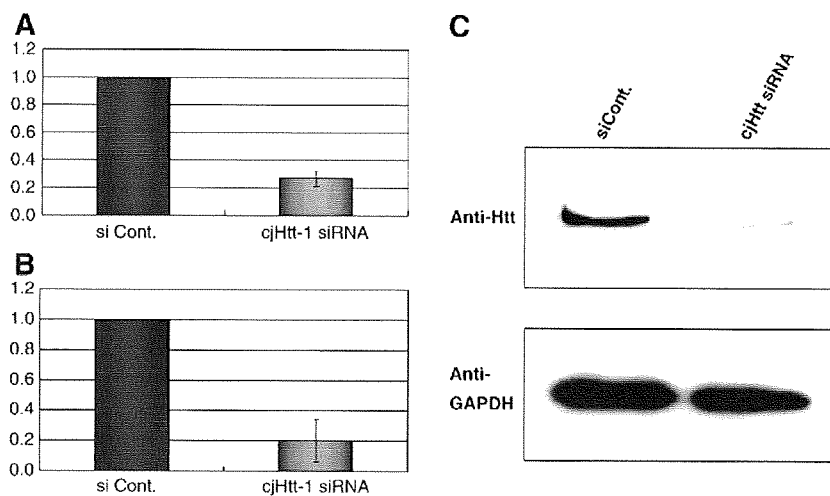


Fig. 5. Inhibition of expression of endogenous *Cj-Htt* by RNAi. The cjHtt-1 siRNA was introduced into HSCj-009 cells by means of electroporation. Two days after transfection, total RNA and cell lysate were prepared and examined by RT-real time PCR and Western blotting, respectively. Total RNA was subjected to cDNA synthesis as in Fig. 1. The resultant cDNA was examined by real time PCR with a TaqMan probe for the human *Htt* gene (A) and newly designed PCR primers [cjHtt(1139)-F and cjHtt(1254)-R] (B). The expression of *Gapdh* as a control was also examined using Perfect Real Time Primers for the human *GAPDH* gene (TAKARA BIO). The expression level of *Cj-Htt* was normalized against that of *Gapdh*, and the ratios of *Cj-Htt* expression level in the presence of cjHtt-1 siRNA were normalized against the ratio obtained in the presence of the siControl siRNA (siCont.). Data are means of at least three independent determinations. Error bars represent standard deviations. (C) Western blot. Cell lysate was examined by Western blotting with anti-human Htt antibody (MAB2166; Chemicon) as in Fig. 3. After detection of signals, the expression of GAPDH as a control was also examined by anti-GAPDH antibody (AM4300; Ambion).

Western blot analyses indicate that a polypeptide of approximately 350 kDa, which is almost the same as the molecular weight estimated from the amino acid sequence (346 kDa) in the *Cj-Htt* cDNA, can be detected in the common marmoset specimens by the antibodies tested, suggesting that the Cj-Htt protein is detectable with the antibodies (Figs. 3A–C). The 350 kDa mouse Htt protein was detected by the MAB2166 antibody (Fig. 3A), but hardly with the other antibodies (Figs. 3B and C). This may be caused by possibly low expression level of mouse Htt in the brain tissue, and/or by difference in the epitope sequences between the common marmoset and mouse Htt proteins. Other than the 350 kDa band, a few bands migrated faster than the 350 kDa band were also observed. They may be degradation products of the Htt protein, and different cells and/or species might have different degradation of the protein. To clarify these, further studies need to be carried out.

In addition to the Htt protein, we also examined the expression of amyloid precursor protein (App) with the 22C11 antibody, which can recognize the same amino acid sequence at positions 66–81 of either the human or mouse App. As a result, the App signal was able to be detected in either the common marmoset or mouse brain tissue, but not in the common marmoset immortalized peripheral blood mononuclear cells (PBMCs) (Fig. 3D), suggesting little or no expression of App in PBMCs and availability of the antibody for detection of the Cj-App protein.

3.3. Gene silencing against the *Cj-Htt* gene

To verify the data presented here and tools for the detection of *Cj-Htt*, we carried out gene silencing against the expression of endogenous *Cj-Htt* by means of RNA interference (RNAi), and assessed the knockdown potency of designed siRNA targeting *Cj-Htt* using the tools evaluated above. Based on a previous study where a competent siRNA duplex, siRNA-HDexon 1, conferring strong inhibition against the expression of the human *Htt* gene was used (Liu et al., 2003), we chemically synthesized a siRNA duplex, cjHtt-1 siRNA, corresponding to the siRNA-HDexon 1 duplex; note that there is one nucleotide change between cjHtt-1 siRNA and siRNA-HDexon 1 (Fig. 4A).

To examine the effect of the siRNA duplex on gene silencing, we constructed a reporter plasmid carrying the 5' terminal region of

Cj-Htt cDNA linked with the GFP reporter gene (the 5'*Cj-Htt*-GFP fusion gene). The reporter plasmid and the siRNA were cotransfected into mouse Neuro2a cells, and the expression of the 5'*Cj-Htt*-GFP fusion gene was examined by a fluorescent microscope. As shown in Fig. 4B, the data indicated that the cjHtt-1 siRNA duplex was able to induce strong RNAi activity against the fusion gene expression.

Next, we introduced the cjHtt-1 siRNA duplex into immortalized common marmoset mononuclear cells by means of electroporation, and two days after transfection, the expression levels of the endogenous *Cj-Htt* mRNA and protein were examined by RT-real time PCR and Western blotting, respectively. As shown in Fig. 5, the results consistently indicated that *Cj-Htt* mRNA (A and B) and protein (C) levels markedly decreased in the presence of the cjHtt-1 siRNA duplex, i.e., potent RNAi knockdown against the endogenous *Cj-Htt* gene was induced by the siRNA duplex. Finally, the data presented here also indicate that proper detection of the newly identified *Cj-Htt* gene and its products can be performed by means of the methods and tools assessed in this study.

In conclusion, we described for the first time the common marmoset *Htt* gene, and also detection methods and tools for the gene and its gene products. The data presented here may assist us in promoting a non-human primate HD model with the common marmoset.

Acknowledgments

We would like to thank Dr. R.C. Desrosiers for kindly providing HS viruses. We also thank Drs. K. Nakamura, T. Kabuta, M. Suzuki, and C. Konya for their helpful advice and discussion. Finally, we would like to thank Dr I. Kanazawa for his encouragement and helpful advice. This work was supported by research grants from the Ministry of Health, Labour and Welfare of Japan.

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Research

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MDM2 is a novel E3 ligase for HIV-1 Vif

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Published: 7 January 2009

Received: 16 September 2008

Retrovirology 2009, 6:1 doi:10.1186/1742-4690-6-1

Accepted: 7 January 2009

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

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Abstract

The human immunodeficiency virus type 1 (HIV-1) Vif plays a crucial role in the viral life cycle by antagonizing a host restriction factor APOBEC3G (A3G). Vif interacts with A3G and induces its polyubiquitination and subsequent degradation via the formation of active ubiquitin ligase (E3) complex with Cullin5-ElonginB/C. Although Vif itself is also ubiquitinated and degraded rapidly in infected cells, precise roles and mechanisms of Vif ubiquitination are largely unknown. Here we report that MDM2, known as an E3 ligase for p53, is a novel E3 ligase for Vif and induces polyubiquitination and degradation of Vif. We also show the mechanisms by which MDM2 only targets Vif, but not A3G that binds to Vif. MDM2 reduces cellular Vif levels and reversely increases A3G levels, because the interaction between MDM2 and Vif precludes A3G from binding to Vif. Furthermore, we demonstrate that MDM2 negatively regulates HIV-1 replication in non-permissive target cells through Vif degradation. These data suggest that MDM2 is a regulator of HIV-1 replication and might be a novel therapeutic target for anti-HIV-1 drug.

Background

Host restriction factors protect hosts from viruses, whereas viruses evade these proteins to replicate more efficiently in host cells. The interplay between the host restriction factors and viral proteins is therefore very important for regulating viral replication [1,2]. A3G (Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) is a newly identified anti-HIV-1 host factor [3], which belongs to the APOBEC superfamily of cytidine deaminases, consisting of APOBEC1, APOBEC2, AID (activation-induced cytidine deaminase), APOBEC3(A-H), and APOBEC4 [4]. A3G is incorporated into HIV-1 virions and inhibits HIV-1 replication by inducing G-to-A hypermutation in viral cDNA during reverse transcription [5-8]. HIV-1 Vif counteracts A3G by targeting it for proteasomal degradation, thus supporting HIV-1 replication in non-permissive target cells [9-11]. Vif forms a ubiquitin ligase (E3) complex with Cullin5 (Cul5), Elongin B, and Elongin C and functions as a substrate recognition subunit of this complex to induce ubiquitination and subsequent degradation of A3G [12,13]. Vif also counteracts several APOBEC3 proteins including APOBEC3F (A3F) [14,15]. These observations reconcile the long-standing mystery of why Vif function is necessary for HIV-1 to infect non-permissive cells. On the other hand, it has been shown that intracellular levels of Vif are maintained relatively low by ubiquitination in virus-producing cells [16-18]. Although several groups have reported E3 ligases important for Vif ubiquitination [17,18], the precise roles and mechanisms of Vif ubiquitination remain unclear. Here we demonstrate that MDM2 is a novel E3 ligase for Vif and that it induces ubiquitination and degradation of Vif, thereby regulating HIV-1 replication.

Results

MDM2 downregulates cellular Vif levels by inducing its degradation in a proteasome-dependent manner

To investigate the biological roles and molecular mechanisms of Vif ubiquitination, we tried to identify a novel E3 ligase that may be involved in the ubiquitination of Vif. During a search for Vif-interacting proteins in the HIV, Human Protein Interaction Database of National Institute for Allergy & Infectious Diseases <http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/>, we were struck by a protein called Gankyrin (proteasome 26S subunit, non-ATPase, 10 (PSMD10)). We first examined the biological effects of Gankyrin, but could not detect a downregulation of Vif (data not shown). As we previously reported that Gankyrin itself doesn't have an enzymatic activity and that it rather enhances the E3 ligase activity of MDM2 on p53 ubiquitination and degradation as a co-factor [19], we tested the possibility that MDM2 plays an important role in Vif ubiquitination as a novel E3 ligase. We examined the effect of several E3 ligases including

MDM2 (a RING finger type E3 that mediates p53 ubiquitination and degradation [20]), Cul5 (another RING finger type E3 that forms a complex with Vif and is reported to induce Vif ubiquitination [17,21]), and Parkin (another RING finger type E3) on cellular Vif levels (Fig. 1A). HEK293T cells were transfected with a subgenomic expression vector pNL-A1 that expressed all HIV-1 proteins except for *gag* and *pol* products [22], together with the expression plasmids for these E3 ligases. We found that the ectopic expression of MDM2 downregulated the cellular levels of Vif as well as p53 in transfected cells in a dose-dependent manner (Fig. 1A, lanes 8-10), whereas Parkin and Cul5 did not affect their cellular levels (lanes 2-4 and 5-7, respectively), even though the latter proteins were expressed more than MDM2. Our results are discrepant with previous reports that demonstrated Cul5 induced Vif ubiquitination and degradation [17,23]. We assume that overexpression of Cul5 alone is insufficient to induce Vif degradation, because other E3 components are not overexpressed. Ectopic expression of MDM2 did not affect cellular levels of another viral protein such as Nef, suggesting that MDM2 specifically downregulated Vif levels; this result also excluded the possibility that MDM2 affected the transcriptional activity of the HIV-1 LTR.

Because it is well known that MDM2 regulates p53 levels by modulating its protein stability, we next examined the protein stability of Vif with the ectopic expression of MDM2. HEK293T cells were transfected with pNL-A1 with or without a MDM2 expression vector and treated with cycloheximide 21 hrs after transfection. After cycloheximide treatment, cellular levels of Vif decreased by 60% in MDM2-transfected cells and by 20% in control cells, respectively (Fig. 1B & 1C), indicating that Vif decayed much faster when MDM2 was overexpressed. The stability profile of Vif protein was similar to that of p53 (Fig. 1B). However, in our hands, the half-life of Vif protein was longer than those shown in previous studies from several laboratories. We interpret that this difference is attributable to divergent methods used in the studies which employed radioisotopes or cycloheximide. Thus, our findings suggest that MDM2 affects the stability of Vif protein similar to its effect on p53. We also examined the stability of Vif in MDM2^{-/-} MEF cells. Vif decayed much faster in p53^{-/-} MEF cells than in p53^{-/-}MDM2^{-/-} double knock-out (DKO) MEF cells (Additional file 1), suggesting that endogenous MDM2 can also influence the stability of Vif. We then tested a RING finger domain-deleted MDM2 mutant, Δ RF, which is inactive for the ubiquitination activity of MDM2 [24]. Ectopic expression of MDM2 suppressed cellular Vif levels, but the expression of Δ RF did not (Fig. 1D). This result suggests that ubiquitination of Vif by MDM2 is involved in the downregulation of cellular Vif levels. We further treated transfected cells with a proteasome inhibitor MG132 to see whether the down-

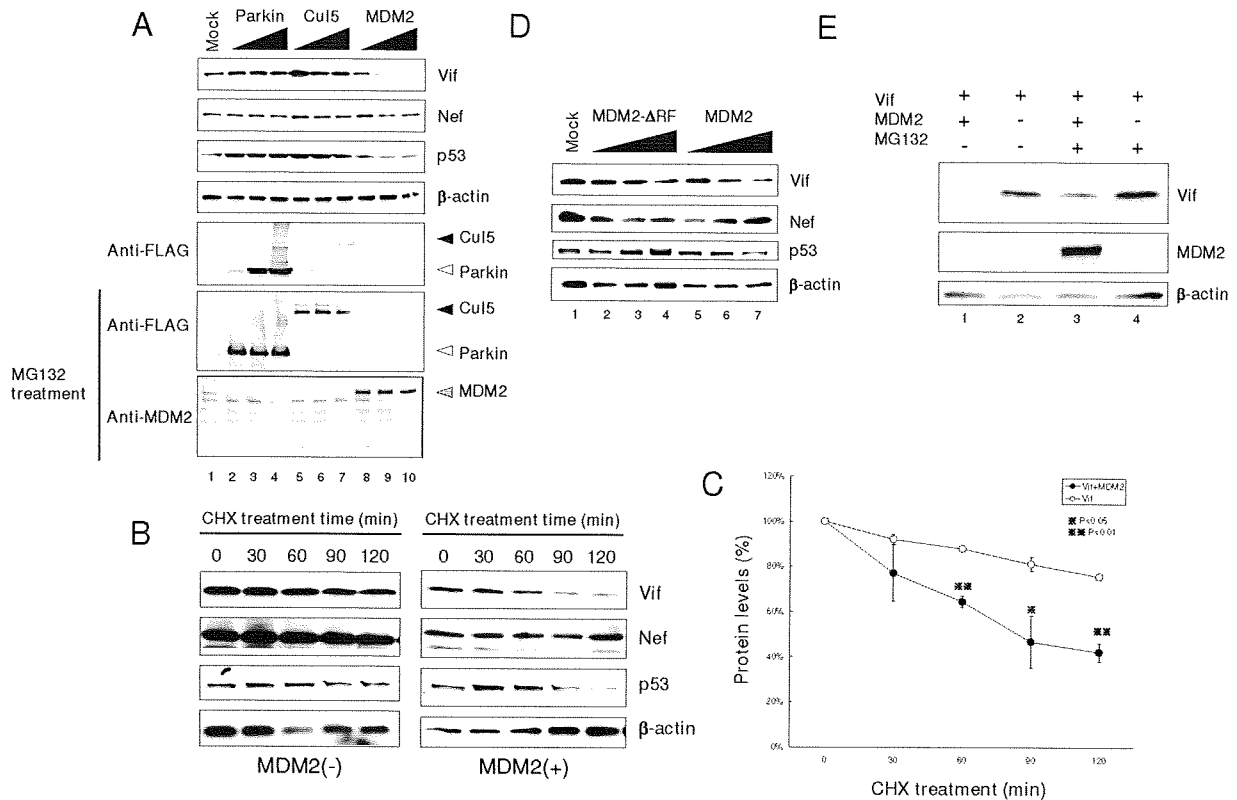


Figure 1

MDM2 downregulated cellular Vif levels in a proteasome dependent manner. (A) MDM2 reduced cellular levels of Vif as well as p53, but not that of Nef. HEK293T cells were cotransfected with expression vectors for the indicated E3 ligases and a subgenomic HIV-1 expression vector pNL-A1. Cell lysates were subjected to immunoblotting with the indicated Abs. We could not detect the expression of FLAG-MDM2 without MG132 treatment, because of a rapid degradation of MDM2. MG132 treatment enabled us to detect expression of MDM2 only with anti-MDM2 Ab, but not with anti-FLAG mAb. (B) Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX)(80 μg/ml) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs. (C) The amounts of Vif and Nef were quantified by densitometry, and Vif protein levels were calculated using Nef protein levels as normalizing loading controls and presented as percentage values relative to that without CHX treatment set as 100%. Values are presented as averages of three independent experiments. (D) MDM2 downregulated Vif, but a ΔRF mutant did not. HEK293T cells were cotransfected with expression vectors for MDM2 and the mutant together with pNL-A1, and cell lysates were subjected to immunoblotting with the indicated Abs. (E) p53^{-/-}MDM2^{-/-} DKO-MEF cells were cotransfected with expression vectors for MDM2 and Vif, and treated with 10 μM MG132 for 6 hrs, and cell lysates were subjected to immunoblotting with the indicated Abs.

regulation of Vif by MDM2 was proteasome-dependent. Treatment with MG132 clearly restored the cellular Vif level that was downregulated by MDM2 (Fig. 1E, top panel, lane 3 as compared with lane 1), supporting that the MDM2-mediated downregulation of Vif was proteasome-dependent. Taken together, we concluded that MDM2 downregulates cellular Vif level by inducing its degradation in a proteasome-dependent manner.

MDM2 specifically binds and downregulates Vif

To further investigate the molecular link between MDM2 and Vif, we next examined the physical interaction of MDM2 with Vif. Immunoprecipitation assays showed that Vif was co-precipitated with MDM2 (Fig. 2A). Glutathione S-transferase (GST) pull-down assays showed that MDM2 was found in GST-Vif-bound, but not GST-bound, material (data not shown). Using a series of MDM2 deletion mutants, we determined that the central region of MDM2 (amino acids 168–320) was necessary for Vif binding (Fig. 2B, left panel & 2C). To more precisely

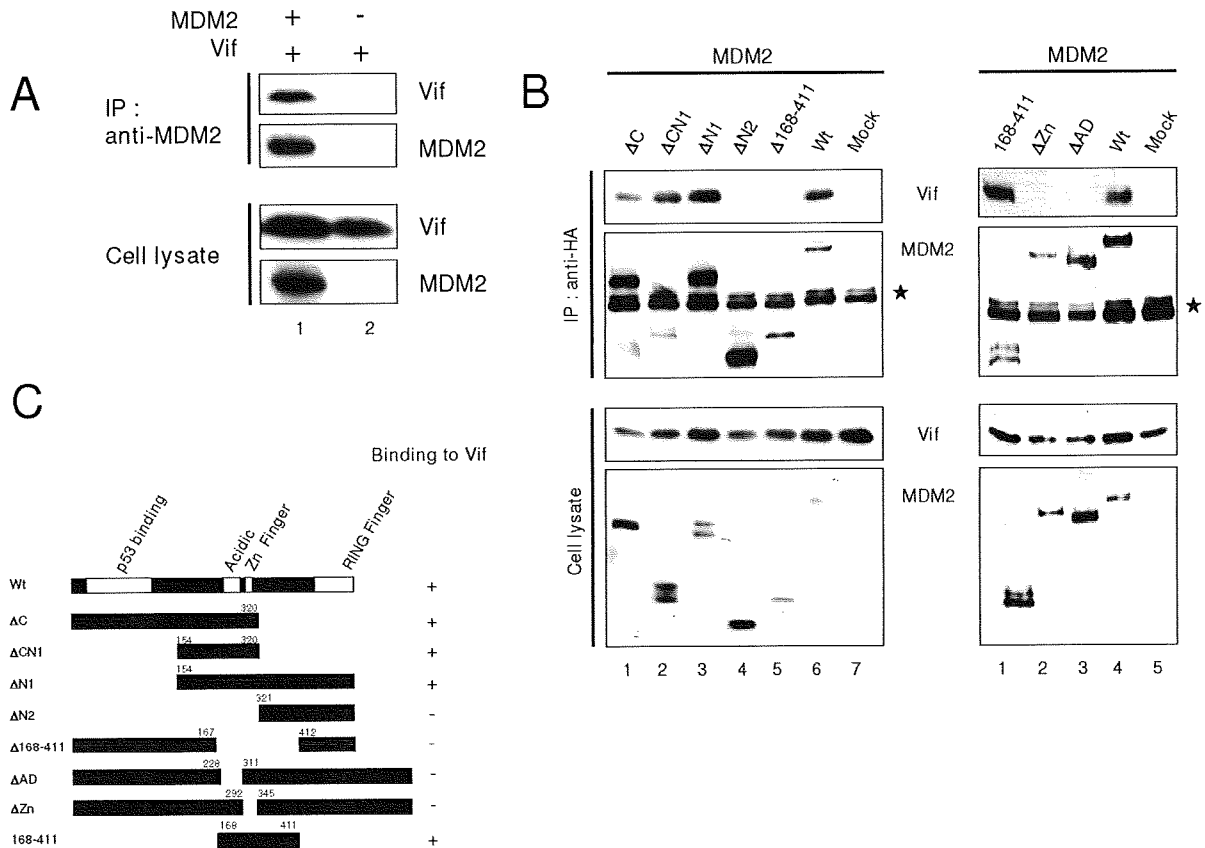


Figure 2
MDM2 bound Vif in its central domain. (A) Immunoprecipitation assays revealed the interaction of MDM2 with Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 and Vif and treated with MG132 for 6 hrs prior to harvest. Cell lysates were immunoprecipitated with anti-MDM2 mAb followed by immunoblotting with the indicated Abs (upper two panels). Cell lysates were also subjected to immunoblotting with the indicated Abs (lower two panels). (B) The interaction domain of MDM2 with Vif. HEK293T cells were cotransfected with expression vectors for HA-tagged MDM2 wild type (Wt) and mutants together with pNL-A1, and cell lysates were immunoprecipitated with anti-HA mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin heavy chains from the immunoprecipitation. (C) Schematics of MDM2 mutants binding to Vif are shown.

determine a Vif-binding domain, we further tested mutants deleted in a Zn Finger domain (ΔZn) or in an acidic domain (ΔAD). Neither mutant could bind Vif, whereas the mutant containing amino acids 168–411 was able to bind Vif, suggesting that both domains are necessary and that the central domain is sufficient for Vif binding (Fig. 2B, right panel & 2C). Additionally, using a series of Vif deletion mutants, we also found that the N-terminal region of Vif (amino acids 4–22) is needed for MDM2 binding (Fig. 3A & 3C). Furthermore, we examined the MDM2-mediated downregulation of Vif mutants. MDM2 was able to efficiently downregulate cellular levels of the

MDM2-binding Vif mutants but not that of an MDM2-non binding mutant, Δ4–45 (Fig. 3B). Collectively, these results indicated that the Vif-MDM2 interaction is required for MDM2-mediated downregulation of Vif (Fig. 3C).

MDM2 induces ubiquitination of Vif

Since we found that MDM2 bound Vif and promoted its degradation via a proteasomal pathway, we next examined whether MDM2 is involved in the polyubiquitination of Vif. *In vitro* ubiquitination assays revealed that bacterially expressed GST-MDM2 was able to induce the

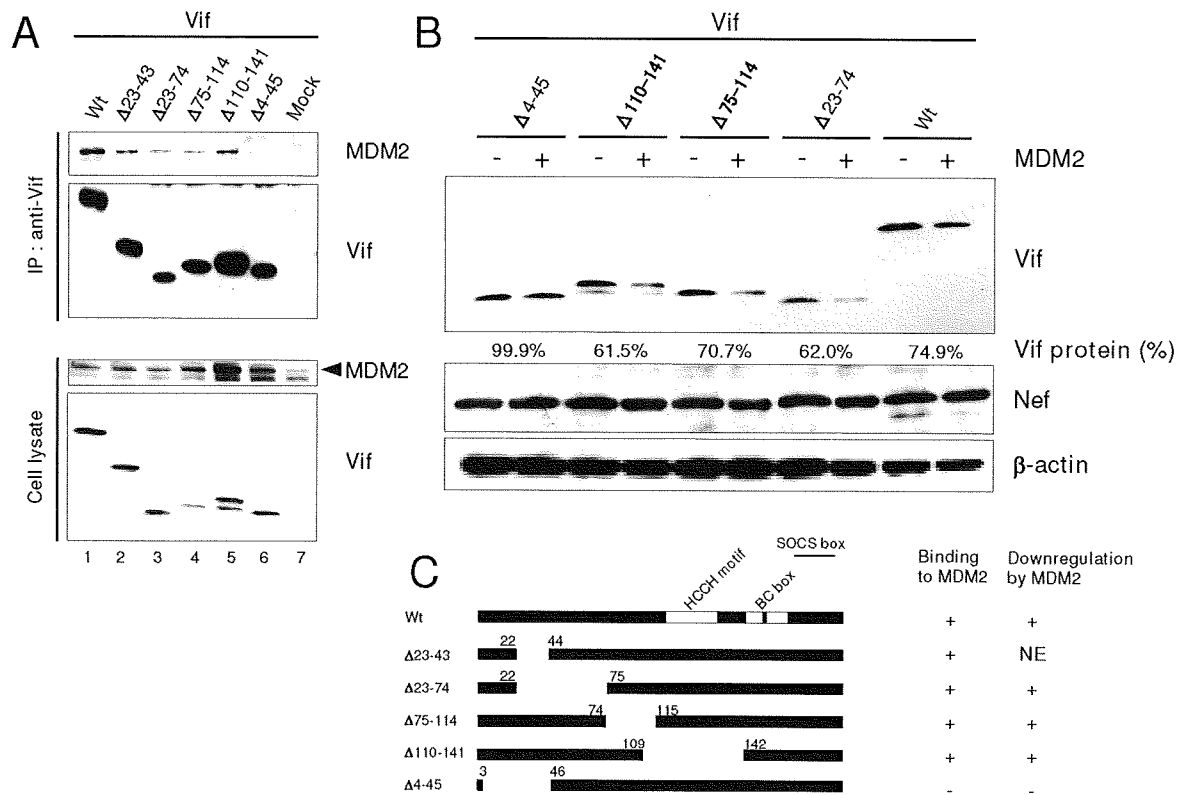


Figure 3
MDM2 specifically bound and downregulated Vif. (A) The interaction domain of Vif with MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants together with pCMV/HA-MDM2, and cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Arrowhead indicates MDM2. (B) The downregulation of Vif protein by MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants with or without pCMV/HA-MDM2, and cell lysates were subjected to immunoblotting with the indicated Abs. The amounts of Vif were quantified by densitometry and shown as the protein ratio relative to that without expression of MDM2. (C) Schematics of Vif mutants bound by and downregulated by MDM2. NE: not examined.

polyubiquitination of purified GST-Vif protein *in vitro* (Fig. 4A). The ubiquitination of Vif by MDM2 was specific, as the omission of ubiquitin, E1, E2, or MDM2 prevented Vif-ubiquitination as shown in our previous experiments [13]. We also performed *in vitro* ubiquitination assays using immunopurified MDM2 and Cul5. Immunopurified MDM2 was able to induce ubiquitination of Vif *in vitro* to the same extent as Cul5 (Additional file 2, part A), while it could not ubiquitinate the N-terminal Vif deletion mutant Δ22 that was defective for binding MDM2 (Additional file 2, part B). These findings suggest that the interaction with MDM2 is important for Vif ubiquitination. We performed *in vivo* ubiquitination assays to further investigate the importance of MDM2 in Vif ubiquitination. Lysates of cells co-expressing Vif, either with an

MDM2 wild type (Wt) or a ΔRF mutant, and His-tagged Ubiquitin (His-Ub) were analyzed for the presence of ubiquitinated Vif conjugates (Fig. 4B). Unfortunately, we detected a Vif band that non-specifically bound to Ni-NTA agarose (arrowhead) due to its nature as a sticky protein. Overexpression of MDM2 induced a ladder detected by anti-Vif Ab, even in the absence of His-Ub (lane 2), suggesting that this ladder represented Vif protein polyubiquitinated with endogenous Ub (arrows with asterisk). Furthermore, in the presence of His-Ub, we detected a doublet of ladder which presumably represented Vif protein polyubiquitinated with endogenous and His-tagged Ub (arrows with asterisk and arrows, respectively). We also obtained similar results using a UbiQapture™-Q Kit (data not shown). We thus concluded that the overexpress-

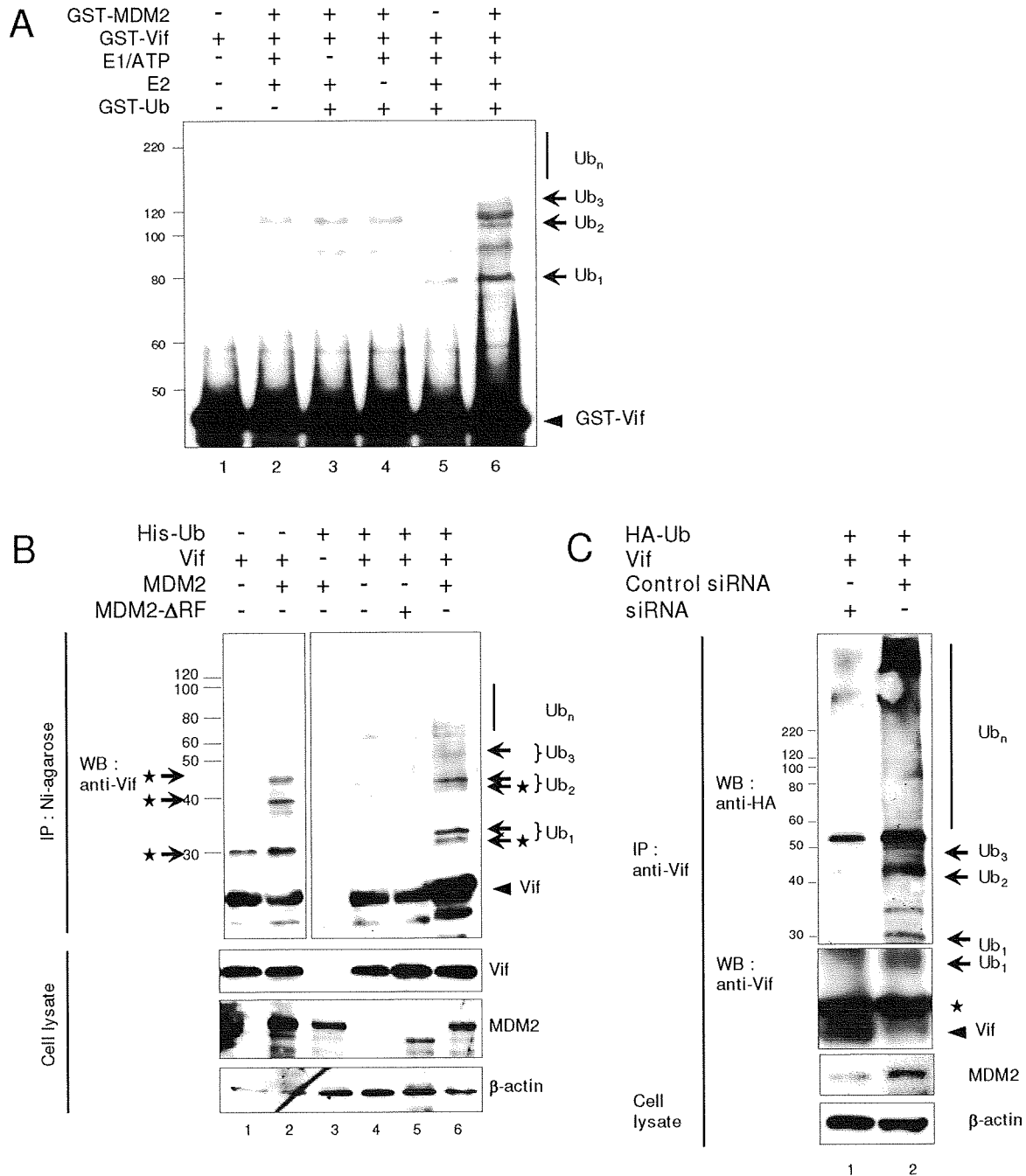


Figure 4 (see legend on next page)

Figure 4 (see previous page)

MDM2 induced the polyubiquitination of Vif *in vitro* and *in vivo*. (A) GST-MDM2 induced the polyubiquitination of Vif *in vitro*. Bacterially expressed GST-Vif was subjected to *in vitro* ubiquitination assays. The reaction was performed in the presence or absence of E1, E2, GST-MDM2, and GST-Ubiquitin as indicated. Reactions were subjected to immunoblotting with anti-Vif mAb. Arrows indicate GST-ubiquitin-conjugated Vif. (B) Overexpressed MDM2 induced the polyubiquitination of Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 Wt and a Δ RF mutant together with expression vectors for Vif and His-Ubiquitin (His-Ub) as indicated. Cells were treated with MG132 for 6 hrs, and cell lysates were precipitated with Ni-NTA agarose beads followed by immunoblotting with the indicated Abs. Since Vif naturally bound to Ni-NTA agarose, we detected a Vif band itself (arrowhead), whereas no signal was detected in cells lacking Vif (lane 3). Arrows indicate His-Ub-conjugated Vif. Arrows with asterisk indicate Vif conjugated with endogenous ubiquitin. (C) Transduction of siRNA reduced cellular levels of endogenous MDM2 and polyubiquitination of Vif. HEK293T cells were cotransfected with expression vectors for MDM2 siRNA and control siRNA together with expression vectors for Vif and HA-Ubiquitin (HA-Ub). Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin light chains from the immunoprecipitation.

sion of exogenous MDM2 efficiently induced polyubiquitination of Vif *in vivo*. Furthermore, the knock-down of endogenous MDM2 expression by introduction of MDM2-specific short interfering RNA (siRNA) resulted in a significant reduction in the amount of polyubiquitinated Vif, commensurate with the extent of reduced MDM2 expression (Fig. 4C). Collectively, these data indicated that MDM2 mediates polyubiquitination of Vif both *in vitro* and *in vivo*.

MDM2 negatively regulates HIV-1 replication in non-permissive cells through ubiquitination and degradation of Vif

Next, we examined the effect of MDM2 on HIV-1 replication. In a single round infection assay (Fig. 5A), in the absence of A3G, viral replication was not affected by expression of MDM2 and/or Vif (lanes 1–6). In contrast, in the presence of A3G in a non-permissive cell setting, without the expression of MDM2, the wild type virus could replicate but the Δ Vif virus could not, as previously reported (lanes 7 & 8) [3,8]. Co-expression of MDM2 reduced the cellular level of Vif (Fig. 5B, upper panel, lanes 5 & 11), resulting in the increased virion incorporation of A3G (Fig. 5B, 2nd lower panel, lane 11 as compared with lanes 7) and the greater suppression of viral replication (Fig. 5A, lane 11 as compared with lane 7).

We also tested the effect of MDM2 on HIV-1 replication in the presence of A3F. MDM2 suppressed viral replication in the presence of A3F, similar to results shown for A3G (Additional file 3). These data indicated that the MDM2-mediated Vif downregulation led to upregulated cellular A3G and A3F levels in producer cells, resulting in less infectious HIV-1 virions produced. Since MDM2 was previously reported to upregulate HIV-1 transcription by ubiquitination of Tat, we further examined HIV-1 replication in macrophages knocked down for MDM2 (Fig. 5C). We chose terminally differentiated macrophages as the target, because the knockdown of MDM2 is lethal for pro-

liferating cells. HIV-1 replicated more efficiently in macrophages transfected with MDM2 siRNA than in control siRNA-transfected macrophages. These data indicated that MDM2 negatively regulated HIV-1 replication in non-permissive target cells through the ubiquitination and degradation of Vif.

To obtain further insights into the mechanisms why our MDM2 system did not induce the ubiquitination of A3G which was bound to Vif, we tested the expression levels and the binding affinity of A3G to Vif in transfected cells. Co-expression of MDM2 reduced the cellular levels of Vif and inversely increased the A3G levels in a dose dependent manner (Fig. 5D). Immunoprecipitation assays revealed that the co-expression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner (Fig. 5E). These data suggest that the interaction between MDM2 and Vif precludes A3G from binding to Vif.

Discussion

In this study, we report that MDM2 is a novel E3 ligase for HIV-1 Vif. MDM2 physically interacts with Vif and functions as an E3 ligase for Vif to induce its polyubiquitination and proteasomal degradation. Several E3 ligases including Cul5 [17], Nedd4, and AIP4 [18], have been reported to induce Vif ubiquitination, and the roles of Cul5 for Vif ubiquitination and degradation are especially well documented. Dang et al. have recently reported that Cul5 induces A3G degradation not by direct ubiquitination of A3G but indirectly through Vif ubiquitination and that polyubiquitinated Vif might serve as a vehicle to transport A3G into proteasomes for degradation [23]. In this manuscript, we show that MDM2 only targets Vif for degradation but not A3G, although MDM2 and Cul5 both induce Vif ubiquitination (Additional file 2, part A). MDM2 reduced cellular Vif levels and inversely increased A3G levels (Fig. 5B & 5D), unlike Cul5. One possible explanation is that the binding of MDM2 to Vif precluded A3G from binding to Vif (Fig. 5E), whereas a Cul5-Vif complex

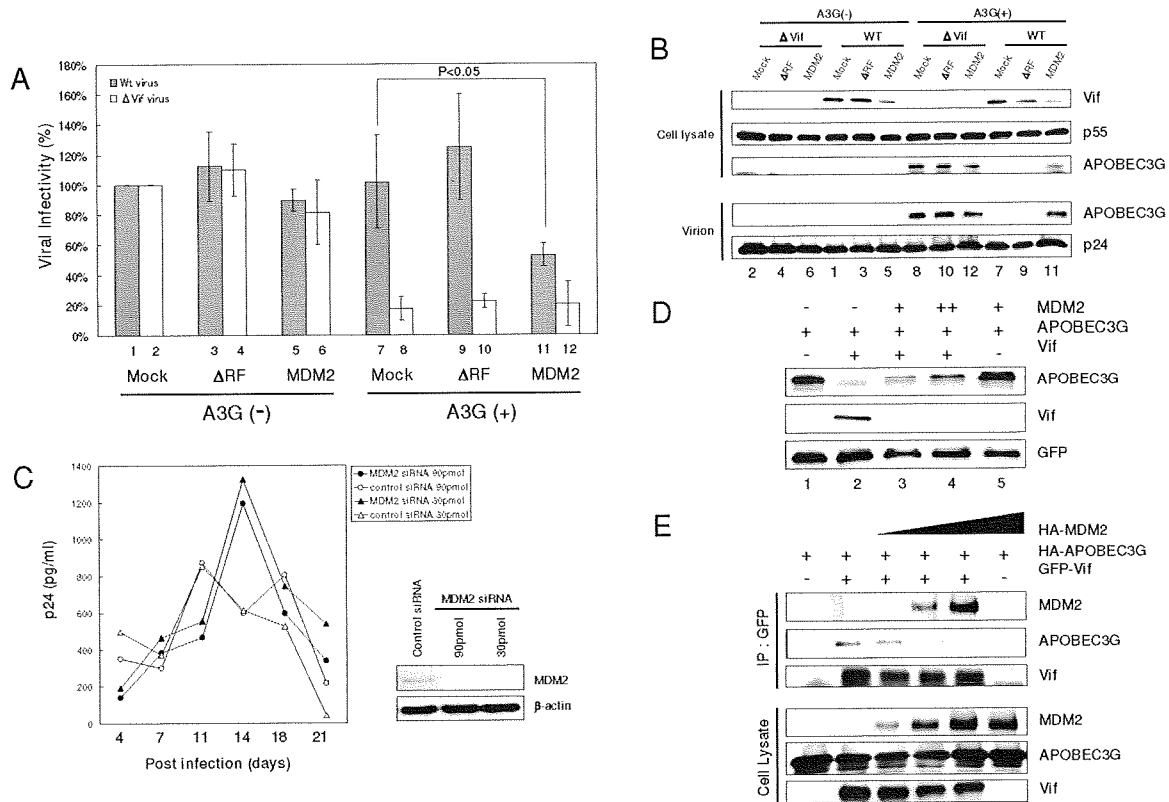


Figure 5
MDM2 negatively regulated HIV-1 replication in non-permissive cells through the degradation of Vif. (A) The overexpression of MDM2 inhibited HIV-1 replication in the presence of A3G. NL-43 Wt and Δ Vif viruses were produced from HEK293T cells transfected with expression vectors for MDM2 Wt and a Δ RF mutant in the presence or absence of A3G. The viral infectivity was examined using M8166 cells. Values are presented as averages of more than 3 independent experiments. (B) MDM2 reduced cellular levels of Vif, resulting in more incorporation of A3G into HIV-1 virions. Immunoblotting for cell lysates (upper 3 panels) and precipitated virions (lower 2 panels) was performed with the indicated Abs. Lane numbers correspond to those in Fig. 4A. (C) HIV-1 replication in macrophages transfected with MDM2- and control-siRNA. MDM were transfected with MDM2- and control-siRNA and challenged with R5 HIV-1_{JR-FL} (left panel). Cell lysates were subjected to immunoblotting with the indicated antibodies (right panels). (D) Coexpression of MDM2 reduced cellular levels of Vif and inversely increased A3G levels in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, Vif, GFP, and MDM2 as indicated. Cell lysates were subjected to immunoblotting with the indicated Abs. (E) Immunoprecipitation assays revealed that the coexpression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, GFP-Vif, and MDM2 as indicated. Cell lysates were immunoprecipitated with anti-GFP mAb followed by immunoblotting with the indicated Abs.

can bind A3G to form a ternary complex. MDM2 binds the N-terminal region of Vif which does not overlap with, but is close to the A3G/A3F binding domain [25]. This binding might affect the interaction of Vif with A3G and/or A3F. Furthermore, the evidence that an MDM2 Δ RF mutant failed to protect A3G indicated that the ubiquitination and degradation of Vif is necessary to protect A3G and A3F from Vif. These findings suggest that different E3 ligases might play different roles in Vif ubiquitination. Further studies on the different roles of Vif ubiquitination

by different E3 ligases and their virological significance should be investigated.

We demonstrate that MDM2 negatively regulated HIV-1 replication through Vif degradation. Through the degradation of target proteins (p53, pRB, etc), MDM2 can exert profound physiological effects on the regulation of cell cycle, cell proliferation, DNA repairs and other processes. To our knowledge, this is the first report to show that MDM2 plays an important role in viral replication

through the degradation of viral proteins. Recently, MDM2 was also reported to ubiquitinate HIV-1 Tat protein and activate its transcriptional activity in a non-proteolytic manner [26]. Our experiment using MDM2 knockdown macrophages showed that HIV-1 replication in these macrophages was more efficient than in control siRNA-transfected macrophages. These data are consistent with MDM2 negatively regulating HIV-1 replication through Vif ubiquitination (Fig. 5C). However, the replication efficiency of HIV-1 in MDM2 knockdown macrophages was only 2-fold higher and was slower than in control siRNA-transfected macrophages. This suggests the possibilities that the ubiquitination of Tat might work as a positive regulatory factor at an earlier phase of infection and that MDM2 might be involved in both positive and negative regulation of HIV-1 replication at different stages. Further studies on the detailed effect of MDM2 on HIV-1 replication are needed.

We also demonstrated that Vif can bind MDM2 directly. We also mapped the interaction domain of MDM2 with Vif to amino acids 168–320 which is located in its central acidic and Zn finger domains. This central domain is different from the primary p53-binding site of MDM2 which is located in its N-terminal region; however, this central domain was recently reported as a second p53-binding site and was shown to be important for the regulation of p53 stability [27-30] (Fig. 2B & 2C). Interestingly, several proteins including p300, p14^{ARF}, and pRB bind to the central domain of MDM2 and regulate the stability and function of p53 via MDM2 [28,31]. Thus, it is possible that Vif might affect the stability and function of p53. Indeed, we confirmed that Vif can stabilize p53 (*Izumi et al., unpublished data*), which could explain why the effect of MDM2 on p53 degradation was weaker than that on Vif as shown in Fig. 1A. A further study is under way to elucidate this new function of Vif (*Izumi et al., HIV-1 Vif induces G2 cell cycle arrest via the p53 pathway, unpublished*).

Finally, expanding evidence suggests that the ubiquitination system plays important roles in many aspects of HIV-1 replication including the degradation of A3G by Vif [9-11], the degradation of CD4 by Vpu [32], HIV-1 viral budding [33], Tat-mediated transactivation [26], and Vpr-induced G2 cell cycle arrest [34,35]. The functional linkage between Vif and MDM2 also suggests that ubiquitin processes such as the A3G/Vif interplay is highly complex. It is obvious that HIV-1 replication in target CD4+ T cells is strongly affected by the interplay of these proteins. From the viral point of view, this interplay might give an advantage to HIV-1 replication. One possibility is that MDM2 regulates cellular Vif levels appropriately, such as not to affect viral replication [36] but just enough to antagonize A3G. Recent studies suggest that the G-to-A mutations induced by A3G may not be the mechanism by

which A3G restricts or controls viral replication [37] and that a partially effective Vif inhibitor may actually accelerate the evolution of drug resistance and immune escape [38]. The inhibitory activity of MDM2 toward Vif could be partially effective and therefore could lead to viral evolution of drug resistance and immune escape. More recently, Nathans et al. have reported a small molecule that specifically antagonizes Vif function and inhibits viral replication by targeting the A3G/Vif axis. This compound enhances Vif degradation only in the presence of A3G, but does not induce A3G degradation and rather stabilizes A3G. They suggested the possibility of a new proteolytic enzyme for Vif degradation and that their new compound interferes with Vif interaction with a host protein in a Vif-A3G-host protein complex, thereby making Vif less stable. The precise biological significance of this Vif-A3G-host protein complex requires future elucidation. Nevertheless, modification or intervention of such Vif-A3G-host protein interplay could lead to the development of new therapeutic strategies for HIV-1 infection.

Conclusion

MDM2 is a novel E3 ligase for Vif which induces the polyubiquitination and degradation of Vif to negatively regulate HIV-1 replication.

Methods

Plasmid constructs

Expression vectors for hemagglutinin (HA)- or FLAG-tagged MDM2, pCMV4/HA-MDM2 or pCMV4/FLAG-MDM2, and their mutants were constructed as previously described [19]. An expression vector for HA-tagged human APOBEC3G, pcDNA3/HA-hA3G [39], and HIV-1 reporter plasmids, pNL43/ Δ env-Luc (WT) and pNL43/ Δ env Δ vif-Luc (Δ Vif) [8], were constructed as previously described. Expression vectors for FLAG-tagged Parkin and Cul5 (pcDNA3/FLAG-Parkin and pcDNA3/FLAG-Cul5, respectively) were constructed by the PCR method. Complementary DNA for HIV-1 Vif was also cloned into pDON-AI (TAKARA BIO INC.) and pDON/EGFP for expression of Vif and EGFP-fused Vif (EGFP-Vif). The subgenomic expression vector pNL-A1, which expresses all HIV-1 proteins except for *gag* and *pol* products, and its mutants expressing Vif deletion mutants were kind gifts from Dr. K. Strebel [22].

Co-immunoprecipitation assays

We performed an immunoprecipitation assay for protein-protein interaction *in vivo*, as described previously [8]. HEK293T cells were cotransfected with pCMV4/HA-MDM2 and pNL-A1 by the calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH7.4/150 mM NaCl/1 mM MgCl₂/0.5% TritonX-100/10% Glycerol) and complexes were immunoprecipitated with anti-MDM2 monoclonal antibody

(mAb) (SMP-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Ab-1, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) and Protein A-Sepharose beads (Amersham Biosciences Corp.) at 4°C. The beads were washed with RIPA buffer (50 mM Tris-HCl pH8.0/150 mM NaCl/1% Triton-X 100/0.1% SDS/0.1% DOC) and analyzed by immunoblotting with anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) [40] or anti-HA mAb (12CA5). To map the regions of MDM2 necessary for binding to Vif, HEK293T cells were cotransfected with expression vectors for a series of MDM2 deletion mutants together with pNL-A1. Complexes were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb. To map the regions of Vif necessary for binding to MDM2, HEK293T cells were cotransfected with expression vectors for a series of Vif deletion mutants together with pCMV4/HA-MDM2. Complexes were immunoprecipitated with anti-Vif mAb and analyzed by immunoblotting with anti-MDM2 mAb. In all these experiments, transfected cells were treated with MG132 for 6 hrs prior to harvesting in order to stabilize both Vif and MDM2; otherwise we could not detect the expression of MDM2 because of its rapid degradation, as seen in Fig. 1A.

In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl/2 mM ATP/5 mM MgCl₂/2 μM DTT) with E1 (200 ng), E2(Ubc5c)(150 ng), and GST-tagged ubiquitin (GST-Ub) (10 μg) as described previously [13]. MDM2 and Vif were expressed as GST-fusion proteins in Escherichia coli strain DH5α and BL21, respectively. The reactions were incubated at 30°C for 90 min. The samples were subjected to immunoblotting with anti-Vif mAb to detect GST-ubiquitin conjugated Vif.

For *in vivo* ubiquitination assays, HEK 293T cells were cotransfected with plasmids expressing Vif, FLAG-MDM2 or its mutants, and His-tagged ubiquitin (His-Ub) as indicated. Cells were treated with 10 μM MG132 for 6 hrs prior to harvesting. Forty-eight hours post transfection, cell lysates were affinity-purified with Ni-NTA-agarose beads (Invitrogen corporation, Carlsbad, CA) and analyzed by immunoblotting with anti-Vif mAb.

For production of RNAi within the cells, we used the pSuper vector as described previously [19]. pSuper-MDM2-1 contained the 19 nt derived from the *mdm2* cDNA (nt 404–422) as the target sequence. Double-stranded RNA containing scrambled 19 nt was used as a control. HEK293T cells were transfected with pSuper plasmids together with plasmids expressing Vif and HA-Ub. Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with anti-HA mAb.

Single round infection assays with HIV-1 luciferase reporter virus

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/Δenv-Luc (Wt) or pNL43/ΔenvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or an expression vector for MDM2 or a mutant in the presence or absence of pcDNA3/hA3G by calcium phosphate as previously described [8]. The reporter viruses were adjusted according to p24 values and used to infect M8166 target cells. Productive infection was measured by luciferase activity and values were presented as percent infectivity relative to the value of each virus without the expression of hA3G.

Knockdown of MDM2 in macrophages and replication assays

Monocyte-derived macrophages (MDM) were cultured for 7 days from CD14+ monocytes isolated from the peripheral blood of an HIV-1-negative healthy individual. Electroporation with Stealth Select RNAi for MDM2 or Control (Invitrogen Corporation) was performed using the Nucleofector machine (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's instructions. Twenty four hours after transfection, MDM were challenged with R5 HIV-1_{JR-FL} at multiplicity of infection of 0.1 at 37°C for 3 hrs. The cells were cultured from day 4 to 21 after infection, and the concentration of p24 antigen in the supernatant was measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit (ZeptMetrix, Buffalo, NY).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI designed research, performed research, contributed vital new reagents, analyzed data, and wrote the paper. ATK designed research, analyzed data, wrote the paper, and organized the research. KS, KIo, and MM prepared the materials and performed a part of the research. KIwai, HK, TS, MT, SL, and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KIttoh, and JF designed the research, contributed vital new reagents, and analyzed the data. TU analyzed the data, drafted the paper, and organized the research.

Additional material

Additional file 1

Supplementary figure 1 – the stability of Vif protein in p53^{-/-} MEF and p53^{-/-}MDM2^{-/-} MEF cells. MEF cells were transfected with pDON/Vif or pcDNA3/HA-A3G. Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs.

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Additional file 2

Supplementary figure 2 – immunopurified MDM2 induced the polyubiquitination of Vif in vitro. (A) MDM2 as well as Cul5 induced the polyubiquitination of Vif. HEK293T cells were transfected with expression vectors for His-MDM2 and His-Cul5. His-tagged proteins were purified using Ni-NTA agarose and subjected to in vitro ubiquitination assays as described in a legend to Fig. 4A. Reactions were subjected to immunoblotting with anti-Vif Ab. Arrows indicate GST-Ub-conjugated Vif. Asterisks indicate non-specific bands associated with GST-Vif protein recognized by anti-Vif Ab, as they are seen in lanes 1 and 3. (B) MDM2 induced the polyubiquitination of Vif Wt but not that of Δ22 that was defective for binding MDM2. Filled asterisks indicate non-specific bands associated with GST-Vif protein, while white asterisks indicate those associated with GST-Vif Δ22.

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Additional file 3

Supplementary figure 3 – the overexpression of MDM2 inhibited HIV-1 replication in the presence of A3F. Single round infection assays were performed in the presence or absence of A3F as described in a legend to Fig. 5A. Values are presented as averages of more than 3 independent experiments.

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Acknowledgements

We thank Drs. K. Strebel for the pNL-A1 plasmid and its derivative mutants, D. P. Lane for p53^{-/-}MDM2^{-/-}DKO-MEF, and M. Malim for the anti-Vif mAb (#319) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This study was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, from the Ministry of Health, Labour and Welfare, Japan, from the Naito Foundation, and from Mitsubishi Pharma Research Foundation.

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Efficient inhibition of SDF-1 α -mediated chemotaxis and HIV-1 infection by novel CXCR4 antagonists

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(Received November 11, 2008/Revised December 25, 2008/Accepted December 25, 2008/Online publication February 24, 2009)

CXC chemokine receptor-4, the receptor for stromal cell-derived factor-1 α as well as human immunodeficiency virus type 1, belongs to the chemokine receptor family and has been shown to play a critical role in directing the migration of cancer cells to sites of metastasis as well as human immunodeficiency virus type 1 infection. We had previously reported that a duodenally absorbable CXC chemokine receptor-4 antagonist, KRH-1636, showed a potent anti-human immunodeficiency virus type 1 activity both *in vivo* and *in vitro*. In this study, we initially examined the effect of the compound and its derivatives on stromal cell-derived factor-1 α -mediated chemotaxis of cancer cells in order to evaluate if they could be applicable as a novel inhibitor of cancer metastasis. We found that both KRH-2731 and KRH-3955 were highly potent antagonists of stromal cell-derived factor-1 α -mediated chemotaxis, i.e. the derivatives exhibited 50% effective concentrations of less than 10 nM, for more than 1000-fold efficacy improvement over the prototype KRH-1636. We further demonstrated the greater anti-human immunodeficiency virus type 1 efficacy of the derivatives compared with the original KRH-1636. Taken together, the KRH-1636 derivatives KRH-2731 and KRH-3955 may be promising as a novel inhibitory drug for cancer metastasis as well as for human immunodeficiency virus type 1 infection. (*Cancer Sci* 2009; 100: 778–781)

Chemokines are secretory proteins with a molecular weight of about 8–14 kDa, and are generally alkaline and heparin-bound. The small chemokine proteins are classified into four highly conserved groups, i.e. CXC, CC, C, and CX3C (X indicates the number of amino acids between the cysteine residues) on the basis of the position of the first two cysteines that are adjacent to the amino terminus.⁽¹⁾ An established role for several members of the CXC and CC chemokine families is to provide directional cues for the movement of leukocytes in development, homeostasis, and inflammation.⁽²⁾ At the time of the movement of leukocytes, chemokine concentration gradually increases at the inflammatory site because the chemoattractants released from the luminal surface of the endothelium, the inflammatory site of the lymphocyte, are rapidly diluted and swept downstream by blood flow. Leukocytes in the mainstream of blood flow may make contact with the endothelium via a group of molecules called selectins,⁽³⁾ and may then roll along the endothelial surface.

The cell surface molecule CXC chemokine receptor-4 (CXCR4) is a 7-transmembrane-spanning, G-protein-coupled receptor for the CXC chemokine stromal cell-derived factor-1 α (SDF-1 α)/pre-B-cell growth stimulating factor (PBSF)/CXCL12.⁽²⁾ The open reading frame of the *CXCR4* gene encodes a peptide of 352 amino acids and is interrupted by one intron in the region encoding the N-terminal segment.⁽⁴⁾

CXCR4 is a receptor for the SDF-1 α . SDF-1 α interacts with CXCR4 to play a variety of physiological roles: B-cell formation in liver and bone marrow at the fetal stage, homing of bone marrow cells in the developmental process, formation of the interventricular septum, regulation of movement of the cerebellum

granule cell in neurogenesis, and large vasculogenesis that nourishes the gastrointestinal tract.⁽²⁾ Since both CXCR4 and SDF-1 α knockout mice do not survive, the interaction between these molecules is essential in the developmental process.^(5–7) It has been reported recently that CXCR7 binds with high affinity to SDF-1 α and to interferon-inducible T-cell α -chemoattractant (I-TAC, also known as CXCL11).⁽⁸⁾ However, unlike other chemokine receptors, ligand activation of CXCR7 induces neither Ca²⁺ mobilization nor cell migration.⁽⁸⁾

CXCR4 is also shown to be one of the coreceptors for human immunodeficiency virus type 1 (HIV-1).⁽⁹⁾ Entry of HIV-1 into target cells involves interactions of the viral envelope protein (Env) with CD4 and a coreceptor, mainly either CXCR4 for T-cell-tropic HIV-1,^(10,11) or CCR5 for macrophage-tropic HIV-1.^(12,13) In acute HIV-1 infection, primarily macrophage-tropic strains are involved in transmission of the virus, whereas T-cell-tropic strains emerge later and are associated with the rapid progression to AIDS.⁽⁹⁾

Importantly, cancer cells originating from the pancreas, brain, breast, prostate, kidney, ovaries, thyroid, and malignant melanoma express CXCR4; however, normal tissues scarcely express CXCR4. Increasing CXCR4 promotes metastasis of these tumor cells toward SDF-1 α -expressing organs including the lungs, liver, lymph nodes, bone marrow, and adrenal glands.^(14–17) Further, interaction between CXCR4 and SDF-1 α promotes progression of chronic and acute lymphocytic leukemia,⁽³⁾ and exacerbation of chronic rheumatoid arthritis.⁽¹⁸⁾

We previously reported that a duodenally absorbable CXCR4 antagonist, KRH-1636, competitively blocked the association of the Env protein of HIV-1 with CXCR4 both *in vivo* and *in vitro* as well as the interaction of SDF-1 α with CXCR4.⁽¹⁹⁾ We therefore hypothesized that KRH-1636 could be a promising chemical for offering protection from both cancer metastases induced by SDF-1 α and from CXCR4-tropic HIV-1 infection. In order to assess this possibility, we sought to evaluate whether the CXCR4 antagonist KRH-1636 and its derivatives could potentially inhibit SDF-1 α -mediated chemotaxis of cancer cells as well as HIV-1 infection.

Materials and Methods

Reagents. SDF-1 α (R&D systems, Minneapolis, MN, USA) was dissolved in phosphate-buffered saline (PBS) at 1 μ M. KRH-1636,⁽¹⁹⁾ and its derivatives KRH-2731, -3148, and -3955 were synthesized at Kureha Chemical Industry (Tokyo, Japan). These

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Abbreviations: CXCR4, CXC chemokine receptor-4; DMSO, dimethyl sulfoxide; EC₅₀, 50% effective concentration; Env, envelope protein; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type 1; mAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline; PBSF, pre-B-cell growth stimulating factor; PE, phycoerythrin; SDF-1 α , stromal cell derived factor-1 α .

compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1%.

Cell culture. Jurkat and its subline Jurkat E6-1 were used in this study. The cells were cultured in a complete medium (CM) composed of RPMI-1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1% 2-Mercaptoethanol at 37°C in a humidified environment with a 5% CO₂ atmosphere.

Fluorescence-activated cell sorter (FACS) analysis. Expression of CXCR4 and CD4 on Jurkat cells was measured by flow cytometry. The cells were suspended at 1×10^5 cells/mL in PBS containing 1% FCS. The cells were reacted with phycoerythrin (PE)-labeled mouse monoclonal antibodies (mAbs) to human CXCR4 (12G5; eBioscience, San Diego, USA) and CD4 (Leu3a; Becton Dickinson, Tokyo, Japan) as a positive control at 4°C for 1 h. The treated cells were washed and fixed with 1% formalin in PBS. Fluorescence of the stained cells was detected by a FACSCalibur (Becton Dickinson), followed by the analysis of fluorescence intensity by CellQuest software (Becton Dickinson).

Cytotoxic assay. Jurkat cells were treated with CXCR4 antagonists at 37°C for 1 h. The cells were harvested and resuspended in a 96-well plate. The viability of the treated cells was measured using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Chemotaxis assay. Cellular chemotaxis was investigated using a 24-well culture plate with 8-µm-pore filters (Transwell; Corning, Tokyo, Japan). Jurkat cells were washed three times in a FCS-free medium and suspended at 3×10^6 cells/mL in RPMI-1640 containing 0.1% bovine serum albumin (control medium). The control medium (0.2 mL) containing 3×10^5 cells was added to the upper well; the control medium (0.6 mL) with or without SDF-1α (100 ng/mL) or CXCR4 antagonists (10 µM) was added to the lower well. The culture plate was incubated for 3 h at 37°C; thereafter, the cells in the upper or lower well were then harvested and resuspended in a 96-well plate. The number of cells in each well was measured using a Cell Counting Kit-8. Optical density (OD) (455 nm/650 nm) values were measured on a microplate reader. The chemotaxis index was calculated as follows: [(OD of treated cells in the lower well – OD of control medium in the lower well)/(OD in sum of the lower and upper wells – OD of control medium in the lower well)] × 100.

For evaluating the inhibitory effect of the CXCR4 antagonists on chemotaxis, cells were pretreated with CXCR4 antagonists at 37°C for 1 h, followed by the chemotaxis assay as stated above.

Anti-HIV-1 assay. Human peripheral blood mononuclear cells, which were activated with immobilized anti-CD3 mouse mAb in RPMI-1640 medium supplemented with 10% FCS for 3 days, were infected with NL4-3 at a multiplicity of infection of 0.001. After 3 h of adsorption, the cells were washed, and cultured in CM supplemented with recombinant human interleukin-2 (50 U/mL), in the presence or absence of the test compounds. Amounts of HIV-1 capsid (p24) antigen produced in the culture supernatants were measured by an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY, USA) 7–10 days after infection.

Results

The initial purpose of this study was to evaluate whether a series of CXCR4 antagonists could inhibit cancer metastasis, which is promoted by the interaction between SDF-1α and CXCR4. In order to evaluate the antagonistic effect of the compounds, we sought to develop an assay system for quantitatively detecting SDF-1α-mediated chemotaxis induced by the interaction. In this experiment, we employed CD4⁺ leukemic cell line Jurkat as a CXCR4⁺ indicator.⁽²⁰⁾ Since Jurkat sublines have different characteristics, we compared CXCR4 expression in the original Jurkat cells and its subline E6-1 by using flow cytometry. As expected, CXCR4 expression was comparable in both cell lines, while CD4 expression was greater in the Jurkat cells (Fig. 1a).

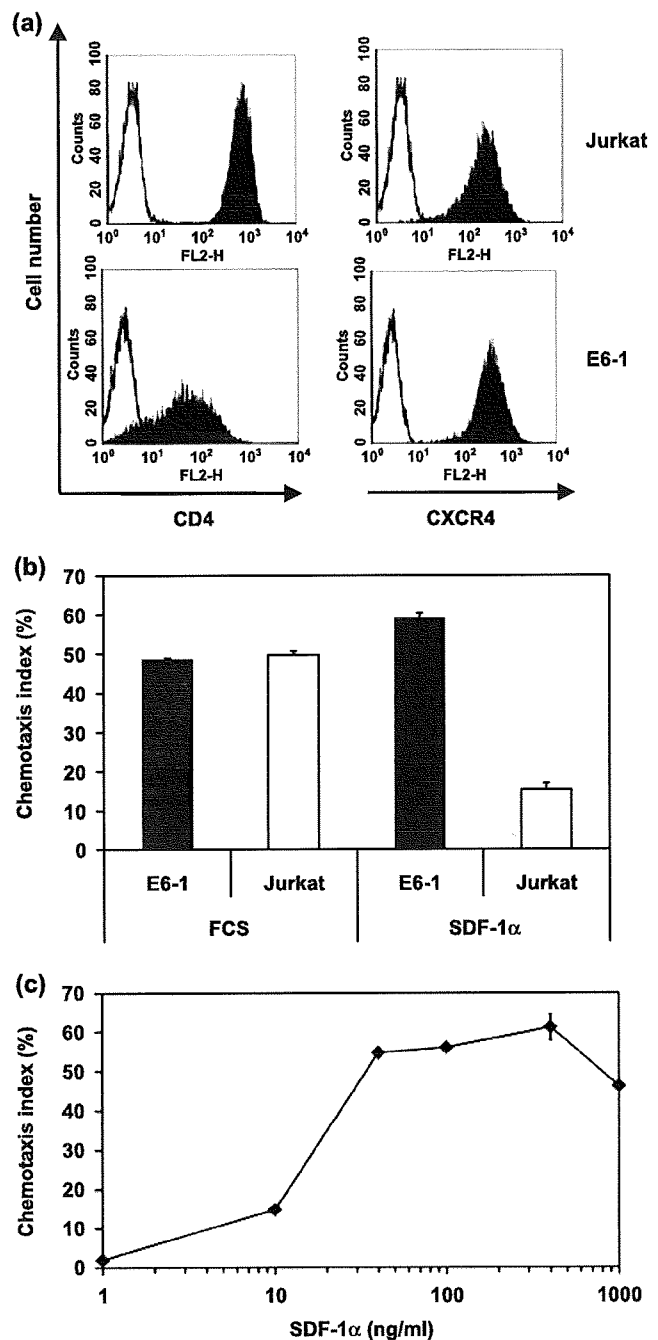


Fig. 1. A quantitative assay system for stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. (a) Evaluation of CD4 and CXCR4 expression on Jurkat and its subline E6-1. The cells were stained with phycoerythrin-labeled anti-CXCR4 or anti-CD4 mouse monoclonal antibodies. Open and closed lines indicate fluorescence of the control and stained cells, respectively. (b) Effect of SDF-1α on chemotaxis of Jurkat and its subline E6-1. The cell lines were incubated with the control medium including 400 ng/mL of SDF-1α or 10% fetal calf serum (FCS) for 24 h at 37°C. The results are shown as a chemotaxis index and standard deviation. The calculation of the chemotaxis index is described in 'Materials and Methods'. (c) Dose-dependent effect of SDF-1α on the chemotaxis of E6-1 cells. Increasing amounts of SDF-1α were treated with E6-1 cells for 3 h and the levels of migration to the lower well are indicated as a chemotaxis index.

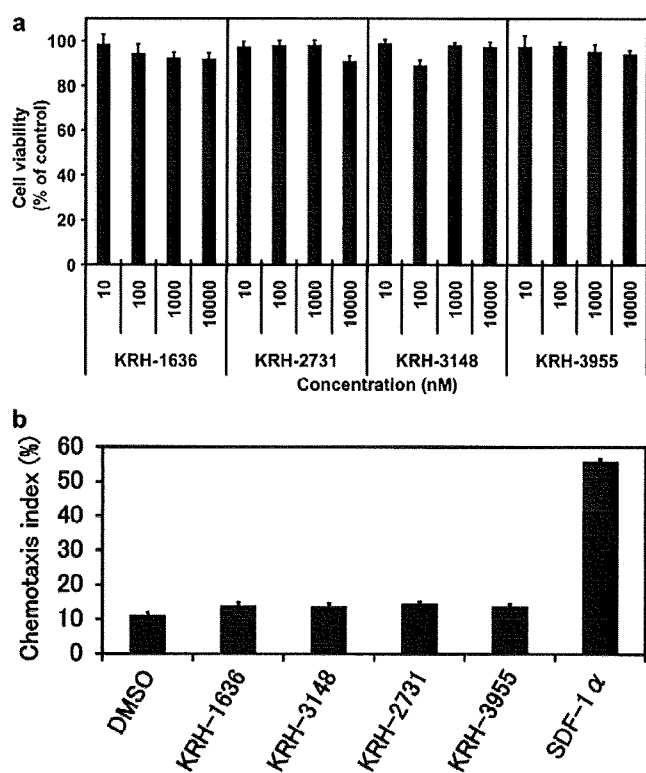


Fig. 2. CXCR4 chemokine receptor-4 (CXCR4) antagonists exhibited neither cytotoxic nor agonistic effects. (a) Increasing amounts of CXCR4 antagonists were examined for their cytotoxic effect on E6-1 cells. (b) CXCR4 antagonists (10 μ M) or stromal cell-derived factor-1 α (SDF-1 α) (100 ng/mL) were added to the lower wells in a chemotaxis assay and were incubated at 37°C for 3 h. The treated E6-1 cells were evaluated for the chemotaxis index. DMSO, dimethyl sulfoxide.

Next, the two cell lines were analyzed for SDF-1 α -mediated chemotaxis activity; after 24 h of incubation, about 30% of both Jurkat and E6-1 migrated to the lower wells in the presence of the control medium. Since the value was the background for this chemotaxis assay, we subtracted this value from the subsequent experiments. We decided to use 400 ng/mL of SDF-1 α for the chemotaxis assay as previously described by Liang *et al.*⁽²¹⁾ It was found that SDF-1 α induced a four-fold increase in the migration efficiency of E6-1 cells compared to the original Jurkat cells (Fig. 1b). Therefore, we decided to use E6-1 cells for the subsequent experiments.

Next, we attempted to optimize the experimental conditions for the SDF-1 α -mediated chemotaxis assay. The chemotaxis index plateaued at approximately 60% after 3 h incubation of E6-1 cells with 400 ng/mL of SDF-1 α (data not shown). We then examined the effect of increasing concentration of SDF-1 α on the chemotaxis index and found that the level of chemotaxis was augmented in a dose-dependent manner and plateaued when more than 40 ng/mL of SDF-1 α was used (Fig. 1c). Accordingly, the optimal condition for the chemotaxis assay in subsequent experiments was 100 ng/mL of SDF-1 α for a 3h incubation period.

Next, we analyzed the cytotoxicity of CXCR4 antagonists to E6-1 cells. As indicated in Figure 2(a), the CXCR4 antagonists were not cytotoxic for E6-1 cells at a 10 μ M concentration. To ascertain the possibility of these antagonists also exhibiting agonistic activities, we examined the chemotaxis activity of the antagonists. We observed that 100 ng/mL SDF-1 α efficiently induced migration of E6-1; however, none of antagonists induced migration even at 10 μ M (Fig. 2b). This indicated that the CXCR4 antagonists did not possess agonistic properties.

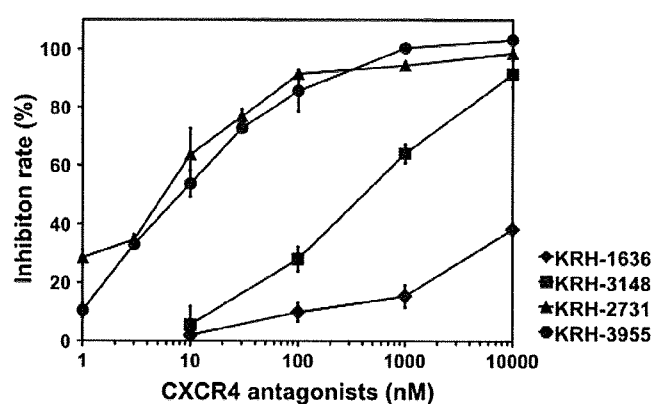


Fig. 3. Dose-dependent effect of CXCR4 chemokine receptor-4 (CXCR4) antagonists on inhibition of stromal cell-derived factor-1 α (SDF-1 α)-mediated chemotaxis. E6-1 cells were pretreated with each concentration of CXCR4 antagonists at 37°C for 1 h, followed by incubation with 100 ng/mL of SDF-1 α for 3 h. The cells were evaluated for the chemotaxis index. The inhibition rate was calculated as the percentage inhibition of chemotaxis by the antagonists.

Table 1. Inhibitory effects of CXCR4 antagonists on SDF-1 α -mediated chemotaxis and HIV-1 infection

CXCR4 antagonists	CXCR4 (EC ₅₀ , nM)	
	Chemotaxis	HIV-1
KRH-1636	>10 000	42
KRH-3148	396.7	4
KRH-2731	9.2	0.9
KRH-3955	5.3	1

The effect of CXCR4 antagonists on the chemotaxis was investigated under the same conditions as described above. The prototype antagonist KRH-1636 inhibited the SDF-1 α -mediated chemotaxis up to approximately 40% at a maximal concentration (10 μ M). By contrast, KRH-3148 almost completely inhibited the chemotaxis at the maximal concentration; moreover, KRH-2731 and KRH-3955 showed the maximum inhibition rate even at 1 μ M (Fig. 3). In order to quantitatively compare these efficacies, 50% effective concentration (EC₅₀) was calculated (Table 1). The results from this study clearly showed that KRH-2731 and KRH-3955 were effective at >1000-fold as compared with KRH-1636.

We further evaluated the effect of the compounds on HIV-1 infection. Anti-HIV-1 activities in nM of KRH-1636, KRH-3148, KRH-2731, and KRH-3955, which were shown as EC₅₀, were 42, 4, 0.9, and 1, respectively (Table 1). The efficacy of the antagonists was highly correlated with their inhibitory effects on HIV-1 infection by interrupting the association of the Env with CXCR4. Interestingly, inhibition of chemotaxis by KRH-1636 and KRH-3148 was relatively lower than that of HIV-1 infection compared with KRH-2731 and KRH-3955. The difference may be because action sites of KRH-2731 or KRH-3955 against CXCR4 are somewhat different from those of KRH-3148 (Sei Kumakura, unpublished data). In summary, these results demonstrate that both KRH-2731 and KRH-3955 are capable of efficiently inhibiting SDF-1 α -mediated chemotaxis as well as infection of T cell-tropic HIV-1.

Discussion

The present study demonstrated that the novel CXCR4 antagonists efficiently inhibited SDF-1 α -mediated chemotaxis as well as

infection of T cell-tropic HIV-1. Two compounds KRH-2731 and KRH-3955 were found to be highly potent inhibitors for both efficacies without any cytotoxicity or agonistic activity, indicating that they may be promising as anti-cancer metastasis and anti-HIV-1 drugs. In particular, both KRH-2731 and KRH-3955 efficiently inhibited calcium signaling induced by SDF-1 α at a concentration of 10 nM, while KRH-3148 and KRH-1636 inhibited at 100 nM and at greater than 10 μ M, respectively (Sei Kumakura *et al.*, unpublished results). This indicated that their antagonistic effects were highly correlated with their abilities to inhibit chemotaxis and HIV-1 infection.

While the Jurkat cell line expressed a smaller but almost comparable level of CXCR4 compared with E6-1 cells (Fig. 1a), their migration levels in the presence of SDF-1 α were quite different (Fig. 1b). It is possible that the original Jurkat cells express non-functional CXCR4 with regard to signal transduction that is required for chemotaxis.

Tumor cells from various types of human cancers of epithelial, mesenchymal, and hematopoietic origins express high levels of CXCR4.^(14,16) The interaction of SDF-1 α with its receptor CXCR4 contributes to metastasis of breast cancer as well as a number of other malignancies in the lung, brain, and prostate. Furthermore, patients with cancers expressing high levels of CXCR4 have more extensive metastasis at lymph nodes compared with low CXCR4-expressing ones.⁽²²⁾ On this basis, the efficient CXCR4 antagonists demonstrated in this study may be highly valuable for the regulation of cancer metastasis. In fact, a synthetic peptide against CXCR4 efficiently inhibited metastasis of breast cancer in a mouse model,⁽²¹⁾ thus providing support to our notion. However, a hurdle remains for the delivery of the

peptide inhibitor to the primary focus of cancer in patients, thus impeding the clinical application of the inhibitor. In this regard, our low molecular weight CXCR4 antagonists are promising because they are non-cytotoxic and can be administered orally. In fact, KRH-3955 showed oral bioavailability of 25.6% in rats and its oral administration blocked X4 HIV-1 replication in the human peripheral blood lymphocytes and in severe combined immunodeficiency mouse system (Tsutomu Murakami *et al.*, manuscript in preparation). It is notable that AMD3100, another small non-peptide CXCR4 antagonist, has been shown to inhibit metastasis of cancer cells *in vitro* and *in vivo*.^(23,24) Moreover, our preliminary data suggested that injection of the breast cancer cell line MDA-231 produced a huge tumor at the inoculated site as well as aggressive metastasis in the lungs of mice, and that our compounds partially inhibited both the primary tumor growth and the metastasis (data not shown).

In conclusion, CXCR4 antagonists, which can be orally administered, are promising agents for SDF-1 α -mediated metastasis of cancer cells and also for the treatment and prophylaxis of a number of diseases related to the interaction between CXCR4 and SDF-1 α , the best example of which would be an anti-HIV-1 drug.

Acknowledgments

We thank M. Kannagi and S. Yamaoka, Tokyo Medical and Dental University, for critical discussions. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology; the Ministry of Health, Labor and Welfare; and Human Health Science of Japan.

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