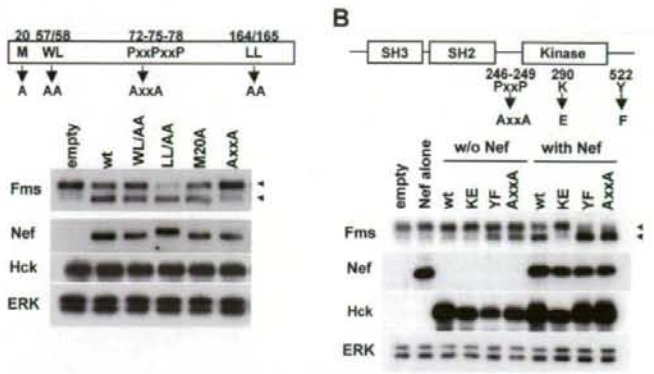

Figure 4. Activation of Hck by Nef is essential but not sufficient for accumulation of gp130Fms. (A) The Nef mutants used (M20A, WL/AA, AxxA, and LL/AA) are schematically shown. All the constructs are CD8-Nef chimeras. 293 cells stably expressing Fms were cotransfected with wild-type Hck and the plasmid indicated, and then analyzed for the expression of Fms, Nef, Hck, or ERK by Western blotting. (B) Schematic representations of Hck and the mutants used. KE is the kinase-dead form, whereas AxxA and YF are the constitutive-active forms.[14] 293 cells stably expressing Fms were transfected with empty vectors (empty), Nef plasmid (Nef), or the indicated Hck plasmid ("w/o Nef" lanes), or cotransfected with wild-type Nef and the indicated Hck plasmid ("with Nef" lanes). Then, the transfected cells were analyzed for the expression of Fms, Nef, Hck, or ERK by Western blotting. (A,B) The ERK blot is a loading control. The ◀ indicate the position of gp150Fms or gp130Fms.

or Hck alone. In a significant proportion of the cells coexpressing Nef/Hck, intense staining of Fms was detected in a perinuclear compartment (Figure 3A top Nef/Hck panel), which largely overlapped the signal for GM130, a marker for the Golgi apparatus[44] (Figure 3A bottom panels) and that for Vti1a, another Golgi marker[45] (Figure S4). Such intense staining of Fms in the perinuclear compartment was also detected in a few cells transfected with Nef alone or Hck alone (Figure 4A; Figure S4), which overlapped the signal for GM130 (Figure S4). Thus, it was highly likely that gp130Fms appeared in the coexpressing cells predominantly localized to the Golgi. As the N-glycosylation of many glycoproteins including Fms is known to be intimately linked with intracellular trafficking,[46-50] we then analyzed the state of the N-glycosylation of gp130Fms. For this purpose, we used 2 lectins, WGA and Con A, which recognize sialic acid and mannose, respectively.[39] As shown (Figure 3B), both gp150Fms and gp130Fms bound to Con A, whereas only gp150Fms bound to WGA, indicating that gp150Fms was modified with both mannose and sialic acid, but gp130Fms was not modified with sialic acid. Indeed, gp150Fms and gp130Fms showed similar electrophoretic mobility following the complete digestion of oligosaccharide groups by PNGase F, whereas only gp130Fms was sensitive to Endo-H, which selectively cleaves high-mannose type oligosaccharides (Figure 3C). These results suggested that the difference in their sizes was due to a difference in the N-glycosylation. Given that nascent Fms polypeptides are modified initially with mannose at the endoplasmic reticulum and terminally with sialic acid at the Golgi,[46-49] our results strongly suggested that the Nef/Hck-dependent accumulation of gp130Fms at the Golgi was due to the perturbation of intracellular N-glycosylation and/or trafficking of nascent Fms.

Down-regulation of Fms by Nef is dependent on activation of Hck and spatial regulation of active Hck

We next attempted to clarify the role of Hck in the down-regulation of Fms expression by Nef. Initially, we examined whether the direct interaction with Hck (Figure S5) was required for the function of Nef, using Nef mutants. As shown (Figure 4A), the AxxA mutant defective in the interaction with Hck[12] failed to down-regulate Fms, that is, the decrease of gp150Fms and the concomitant increase of gp130Fms. In contrast, the other 3 mutants still down-regulated Fms (Figure 4A). The WL/AA and LL/AA mutants, and the M20A mutant were shown to be defective in the down-regulation of CD4 and MHC I, respectively,[24,37] which was confirmed in our experimental system (data not shown). These results suggested that the down-regulation of Fms by Nef was mechanistically different from that of CD4 or MHC I, and dependent on the direct interaction with Hck. Thus, we next examined whether the activation of Hck by Nef was necessary and sufficient for the down-regulation of Fms, using Hck mutants. As shown (Figure 4B), Nef failed to down-regulate Fms when cotransfected with the kinase-dead KE mutant, but almost completely down-regulated Fms when cotransfected with the YF or AxxA mutant, both of which were the constitutive-active form. However, it should be noted that the transfection of these constitutive-active forms of Hck alone was not necessarily sufficient to achieve the full down-regulation of Fms (Figure 4B, see YF, AxxA, wt + Nef, YF + Nef, and AxxA + Nef lanes). These results clearly indicated that the activation of Hck was necessary but not sufficient for the Nef/Hck-induced down-regulation of Fms.

It has been shown that Nef distributes to the Golgi as well as the plasma membrane.[22,24] Indeed, intense signal of the CD8-Nef chimera was detected in the perinuclear compartment, which overlapped the signal for GM130 (Figure 5A). Thus, it was possible that the activation of Hck at the Golgi or the recruitment of the active Hck to the Golgi was another factor necessary for Nef to down-regulate Fms. To explore this possibility, we examined whether the active Hck in the Nef-expressing cells indeed localized to the Golgi and its existence at the Golgi correlated with the down-regulation of Fms. To detect the active Hck, we stained cells with the antibody specific for Hck phosphorylated at Tyr411, which was the major autophosphorylation site.[14] As shown (Figure 5B), an intense signal for the active Hck was indeed detected in the perinuclear compartment, in cells coexpressing Nef and wild-type Hck but not in cells expressing wild-type Hck alone (top panels), which largely overlapped the signal for GM130 (middle panels). Such colocalization of Nef and active Hck in the perinuclear compartment was also observed in macrophages nucleofected with the CD8-Nef plasmid (Figure S6). Moreover, the constitutive-active AxxA Hck tended to localize to the perinuclear compartment when expressed alone, and almost exclusively localized to the perinuclear compartment when coexpressed with Nef (Figure 5B bottom panels). Thus, the degree to which the active Hck accumulated at the Golgi correlated well with the observed down-regulation of Fms (Figure 4B). Taken together, these results suggest that the novel function of Nef (ie, the down-regulation of Fms expression by perturbing the maturation/trafficking of nascent Fms) is dependent on both the activation of Hck and the spatial regulation of the active Hck.

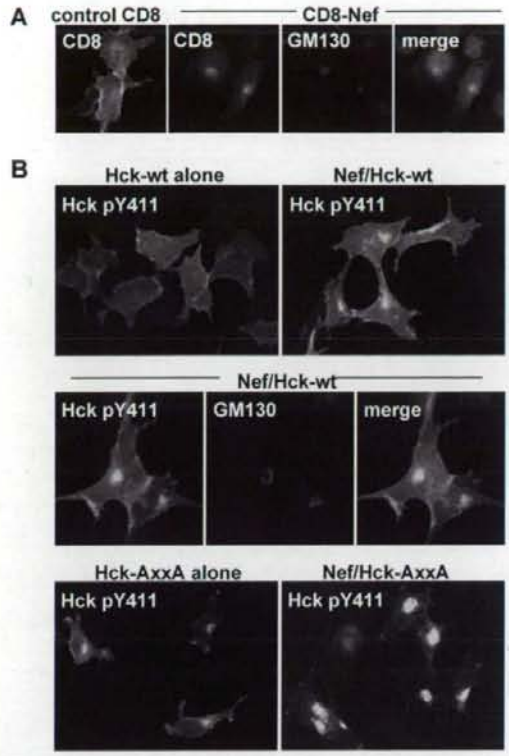

Figure 5. Nef induces Golgi localization of active Hck. (A) Parental 293 cells were transfected with the control CD8 plasmid or CD8-Nef plasmid, and then stained with anti-CD8 antibody (green), anti-GM130 antibody (red), or DAPI (blue). (B) In the top panels, parental 293 cells were transfected with wild-type Hck, or cotransfected with wild-type Hck and Nef, and then stained with the antibody specific for active Hck (ie, Hck phosphorylated at Tyr411). In the middle panels, parental 293 cells cotransfected with wild-type Hck and Nef were costained with anti-Hck pTyr411 antibody (green), anti-GM130 antibody (red), and DAPI (blue). In the bottom panels, parental 293 cells were transfected with the constitutive-active AxxA Hck (see Figure 4B), or cotransfected with the AxxA Hck and wild-type Nef, and then stained with anti-Hck pTyr411 antibody. See "Western blotting, flow cytometry, and immunofluorescence with 293 cells" for image acquisition information.

Discussion

In this study, we showed for the first time that Nef down-regulated the expression of Fms (Figures 1,2). The down-regulation was due to perturbation of the intracellular trafficking of nascent Fms (Figure 3), and likely to be a cause of the inhibitory effect of Nef on the activity of M-CSF because neither the activity of GM-CSF nor the cell surface expression of GM-CSF receptors was inhibited by Nef (Figure 1). Importantly, the present study strongly suggested that the down-regulation of Fms expression by Nef was due to a previously unreported mechanism that depended on both the activation of Hck and the aberrant spatial regulation of the active Hck (Figures 4,5).

The Nef-induced down-regulation of Fms was obviously mechanistically different from that of CD4 or MHC I in its dependence on Hck (Figures 2A,3A)[6,7,20-23] but appeared to resemble that of HFE. The Nef-induced down-regulation of HFE was abolished by either a mutation in the PxxP motifs of Nef or the overexpression of the dominant-negative Hck.[24] However, how Hck was involved in the Nef-induced down-regulation of HFE remains to be analyzed.[24] Interestingly, the YxxA motif in the cytoplasmic tail of HFE (^{342}YVLA) was shown to be required for Nef to down-regulate HFE.[24] The tyrosine-based YxxA motif was conserved in the kinase domain of Fms (^{873}YQMA, GenBank accession number P07333). However, when coexpressed with Hck, Nef also down-regulated a Fms mutant lacking the motif prepared by introducing the stop codon at ^{873}Y (data not shown). Thus, the mechanism for the Nef/Hck-induced down-regulation of Fms was likely to be somewhat different from that of HFE. Our earlier experiment revealed that gp130Fms was tyrosine phosphorylated in cells coexpressing Nef and Hck.[26] However, the ligand-independent tyrosine phosphorylation of Fms was not a direct cause of the down-regulation of Fms, because Nef also down-regulated a Fms mutant lacking the entire intracellular region when coexpressed with Hck (Figure S7).

The Nef/Hck-induced down-regulation of Fms was associated with the accumulation of the immature Fms at the Golgi (Figure 3). The experiment with Hck mutants clearly demonstrated that the activation of Hck was indispensable for the down-regulation of Fms (Figure 4B). The finding that Nef failed to down-regulate Fms when coexpressed with Lyn or Fgr (data not shown) further supported the conclusion, because Hck was the only Src kinase activated by Nef among Src kinases highly expressed in macrophages (ie, Hck, Lyn, and Fgr).[13-16] However, to our surprise, the activation of Hck was not the sole determinant of the down-regulation of Fms, because the expression of the constitutive-active Hck (YF or AxxA) alone was insufficient to fully achieve the down-regulation (Figure 4B). Our finding that the degree to which the active Hck accumulated at the Golgi correlated well with that of the down-regulation of Fms (Figures 4B,5B) strongly suggested that Nef down-regulated Fms through both the activation of Hck and the accumulation of the active Hck at the Golgi. The idea may answer why Hck, the downstream effector molecule important for the Fms signaling pathways,[38,50-53] is involved in the down-regulation of Fms by Nef.

A significant pool of Nef has been shown to localize to the Golgi.[22,24] Indeed, the CD8-Nef chimera used in this study localized to the Golgi as well as the plasma membrane (Figure 5A). This was not due to the fusion of the region of CD8 to the N-terminus of Nef, because the Nef-EGFP chimera, in which EGFP was fused to the C-terminus of Nef, also localized to the Golgi (data not shown). Thus, it was likely that the interaction with the Golgi-resident Nef or the recruitment of the active Hck led to the accumulation of the active Hck at the Golgi. However, it is unclear how this accumulation leads to a block of the intracellular trafficking of Fms in the same compartment. A plausible possibility might be direct interaction of the active Hck with Fms at the Golgi. Indeed, our earlier coimmunoprecipitation experiment revealed the formation of a molecular complex between Hck and Fms.[26] Meanwhile, it is known that the tyrosine located in the juxtamembrane domain of Fms (Y561 in human and Y559 in murine) serves as a binding site for Src kinases including Hck when the residue is autophosphorylated.[51-54] However, when coexpressed with Hck, Nef also down-regulated a Fms mutant in which the tyrosine residue was replaced with phenylalanine (data not shown). Thus, the active Hck at the Golgi may interact with Fms via unidentified site(s) or form complexes with Fms indirectly. Another possibility might be an alteration of the Golgi structure caused by the accumulation of the active Hck at the compartment. Recent studies revealed that Src kinases including Hck were present on the Golgi membrane as well as the plasma membrane.[55-57] The importance of the Golgi-localized Src kinases for the maintenance of the Golgi structure was clearly demonstrated by the finding that SYF

fibroblasts lacking the 3 ubiquitous Src kinases (Src, Yes, and Fyn) exhibited an aberrant morphology of the Golgi with collapsed stacks and bloated cisternae.[58] Interestingly, it was also demonstrated that the exogenous expression of the constitutive-active Src (E378G) in the SYF cells affected the distribution of some if not all Golgi-specific proteins.[58] Thus, it is possible that the accumulation of the active Hck affects the structure of the Golgi and thereby perturbs the trafficking of Fms.

A study with HIV-1 transgenic mice has clearly proved the importance of the interaction of Nef with Hck in macrophages for the development of AIDS.[11] Nevertheless, the functional consequences of the Nef/Hck interaction are not fully understood. The activation of Hck induced by the direct interaction with Nef is basically thought to cause the activation of macrophages, which may favor the replication of HIV-1. Indeed, Komuro et al demonstrated that the expression of Hck at a high level in macrophages correlated well with high titer replication of HIV-1.[59] Moreover, Briggs et al raised the possibility that the Nef-Hck interaction caused the activation of the Stat3 transcription factor, thereby mimicking the signaling pathway of the GM-CSF receptor.[25] However, the present study revealed that the Nef/Hck interaction also played a negative role: the molecular interaction caused the down-regulation of Fms and inhibition of the activity of M-CSF, which is likely to be due to the aberrant spatial regulation of the active Hck. The differential modulation of the activities of GM-CSF and M-CSF by Nef may alter the profile of production of cytokine/chemokines in HIV-1–infected macrophages, contributing to the development of AIDS. Future studies will clarify whether small compounds specifically targeting the Nef-Hck interaction prevent the progression of the disease. Moreover, a detailed mechanistic analysis of the unique function of Nef will help us to understand how Fms and Src kinases tightly regulate the signaling pathways and functions of macrophages.

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Authorship

Contribution: M.H. and S.S. were responsible for the overall experimental work and design; Y.Y. and H.A., for DNA cloning; R.H., for Western blotting; H.H., for flow cytometry; N.S., for immunofluorescence; K.M. and S.O., for project planning and data analysis.

M.H. and S.S. contributed equally to this study.

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MDM2 is a novel E3 ligase for HIV-1 Vif

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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, ΔRF, which is inactive for the ubiquitination activity of MDM2 [24]. Ectopic expression of MDM2 suppressed cellular Vif levels, but the expression of ΔRF 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-

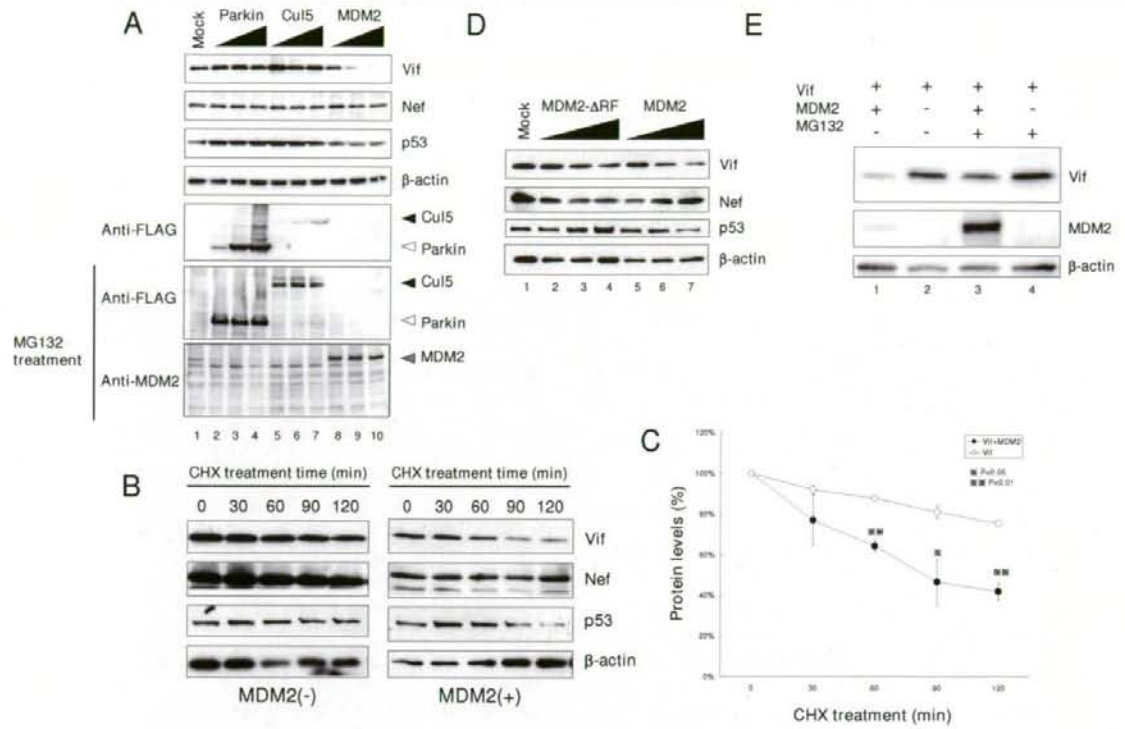

Figure 1

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

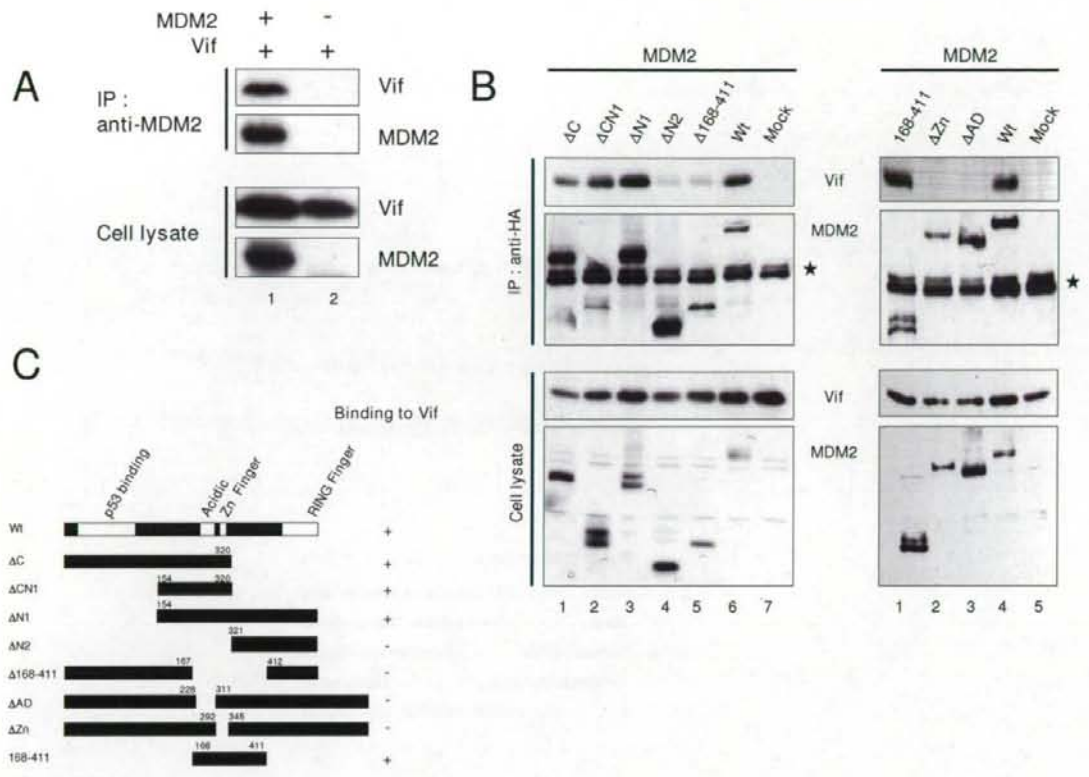

Figure 2

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 thenimmunoprecipitation. (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 ubiqutination 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

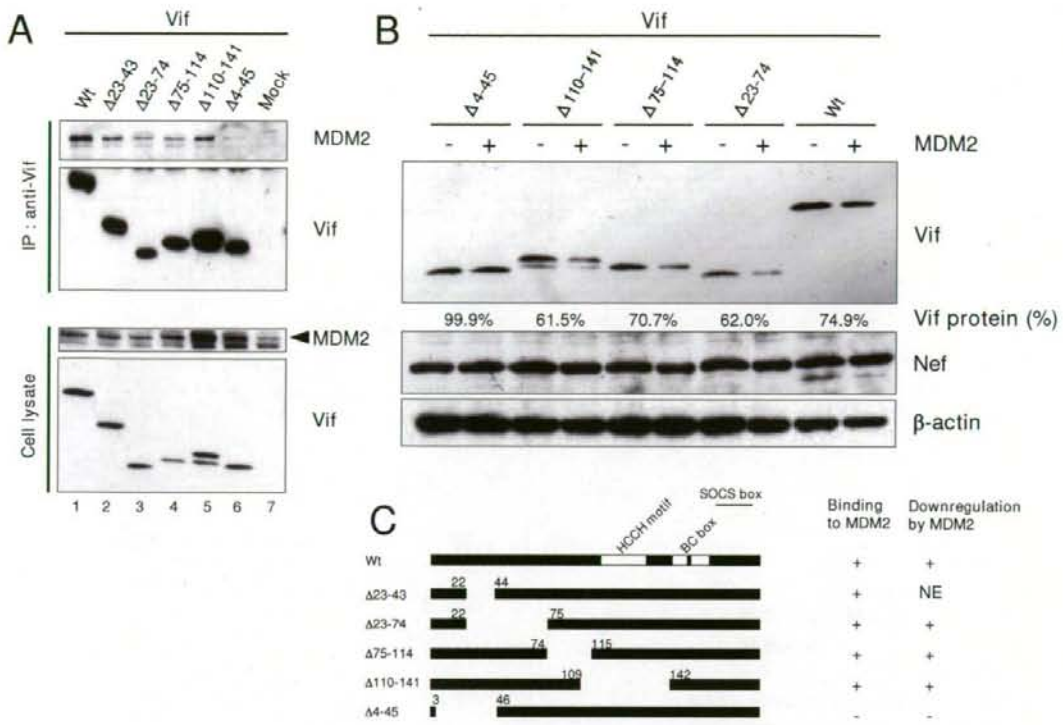

Figure 3

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 ubiqutin, 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 overexpres-

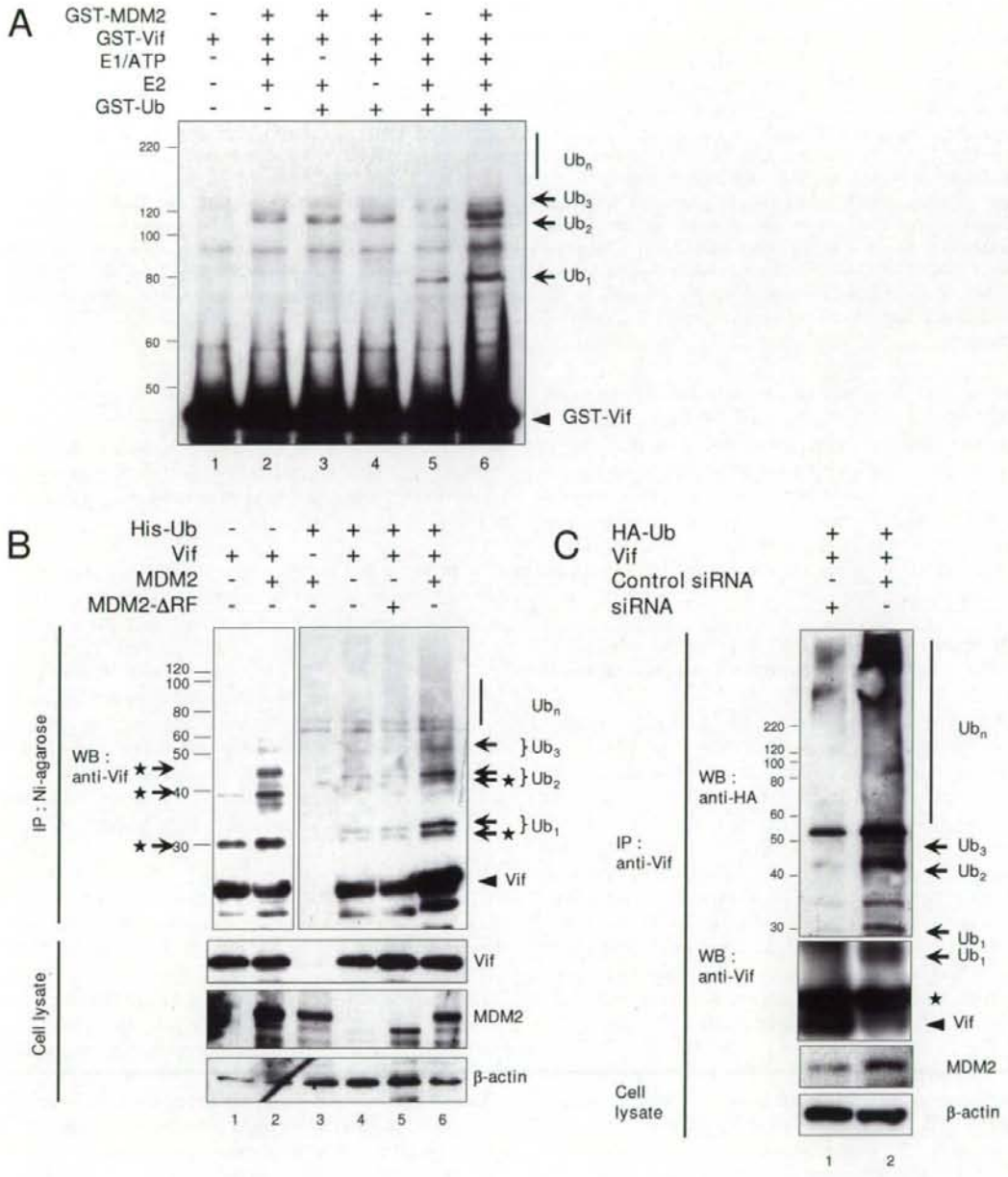

Figure 4 *(see legend on next page)*

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 ΔRF 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 ubiqutination 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 ΔVif 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 proliferating 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 ubiquination of A3G but indirectly through Vif ubiqutination 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 Vif (Fig. 5E), whereas a Cul5-Vif complex

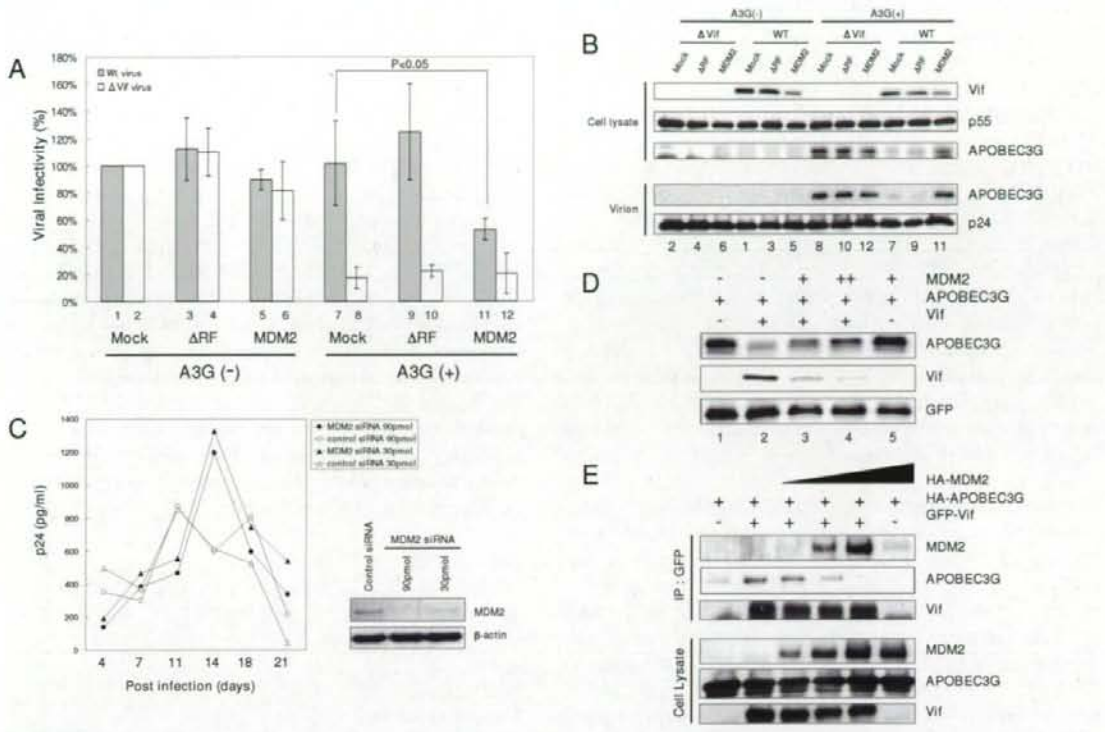

Figure 5

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 ΔVif viruses were produced from HEK293T cells transfected with expression vectors for MDM2 Wt and a ΔRF 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 ΔRF 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 regulateing 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 deomain 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/Δenv-Luc (WT) and pNL43/ΔenvΔvif-Luc (ΔVif) [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 $MgCl_2$/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 $MgCl_2$/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 immunoblottimg 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-$1_{JR\text{-}FL}$ 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, SI., and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KItoh, 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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MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

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ABSTRACT

More than 170 million people worldwide are chronically infected by HCV, which is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. Although animal models of viral hepatitis are a prerequisite for the evaluation of antiviral and vaccine efficacy, the restricted host range of HCV has hampered the development of a suitable small animal model of HCV infection. Use of the chimpanzee, the only animal known to be susceptible to HCV infection, is limited by ethical and financial restrictions. In this regard GBV-B, being closely related to HCV, appears to be a promising non-human surrogate model for the study of HCV infection. This review describes the characteristic of GBV-B infection of New World monkeys, and discusses current issues concerning the GBV-B model and its future directions.

Key words GBV-B, HCV, hepatitis C, monkey.

INTRODUCTION

Since HCV was identified as a major causative agent for non-A, non-B hepatitis in 1989 by Choo *et al.* (1), it has become evident that HCV is disseminated worldwide and is carried by an estimated more than 170 million people (2). In most advanced nations, the prevalence of HCV infection is roughly 1–2% and further dissemination is suppressed. By contrast, among developing countries the number of HCV-infected patients is still increasing due to iatrogenic exposure, including blood transfusion from unscreened donors and reuse or inappropriate sterilization of contaminated medical equipment, and injecting drug use (3). After HCV exposure, about 70% of individuals who exhibit acute infection progress to chronic liver disease, and many of these patients develop hepatic cirrhosis and hepatocellular carcinoma (2). Currently, the only treatment available for patients with chronic HCV infections is combination therapy with pegylated interferon and ribavirin. As the standard therapy is effective in only approximately 50% of patients with chronic HCV hepatitis, the other half of affected patients are still threatened by poor prognosis (4). It is therefore urgent to develop more effective therapeutics for HCV infection. At the same time, prophylactic vaccines are indispensable for prevention of further spread of HCV in developing countries, including reduction of the risk to health care workers of occupational transmission.

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

Research in infectious diseases will never progress without animal models. Because conventional small animals are not susceptible to HCV infection due to its limited host range, development of an effective prophylactic vaccine, as well as unveiling of the molecular mechanism of viral pathogenesis, has been hampered. Nonetheless, decades

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List of Abbreviations: ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; GBV-B, GB virus-B; GE, genome equivalents; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency viruses; SIVmac, SIV derived from rhesus macaques.

of struggle have resulted in a number of animal models for HCV infection and hepatitis C.

Recently, a number of rodent models have been developed (for a review of this topic see reference 5). Rodent models, which permit HCV replication and involve transplantation of human hepatocytes, include immunotorelated fetal rat (transplantation of human hepatoma cell line Huh7 into newborn rats after prior injection of the same cells into pregnant females) (6) and immunodeficient mice such as trimera mice (7, 8) and uPA mice (9–11). These rodent models are highly useful for evaluating the efficacy of antiviral drugs and neutralizing monoclonal antibodies. In addition, a number of HCV transgenic mice have been developed. These enable direct characterization of the effects of expression of HCV genes on liver injury (5). These small animal models do not require costly facility for primates as mentioned later. While having a number of merits as mentioned above, these rodent models still have some limitations. For example the former models are not suitable for investigation of the pathogenesis of hepatitis C and the development of effective vaccine strategies, while in the latter models the proteins of interest are usually over-expressed as compared with natural HCV infection, and the integration site of the transgene may have an influence on the outcome of the study.

The chimpanzee model is the most straightforward since this animal can be experimentally infected with HCV. One third of HCV-inoculated chimpanzees develop chronic infection, while infection resolves in the remainder after an acute phase lasting 2–3 months, indicating that the chronicity rate in chimpanzees is somewhat lower than in humans (12). The chimpanzee model has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination. However, irrespective of its benefits, many obstacles need to be overcome in order to use this model. For example in many countries it is illegal to employ the chimpanzee as an experimental animal, primarily due to ethical, (and secondly to financial), reasons. In fact, in 2004 the Dutch government decided to stop all research with chimpanzees at the biomedical primate research center in Rijswijk, Netherlands. As this was the only primate center in Europe where chimpanzees were used for biomedical research, this decision made chimpanzees unavailable as experimental animals in Europe. It is still possible to employ chimpanzees for biomedical research in some other countries, including the USA. However, the National Center for Research Resources of the National Institute of Health in the USA has recently decided not to continue to breed chimpanzees for research (13). It is estimated that the existing chimpanzees in the National Center for Research Resources will die within 30 years.

AIDS-related research has been one of the major purposes for using chimpanzees. However, due to the reasons mentioned above, as well as the endangered status of chimpanzees, nowadays AIDS scientists mainly make use of macaque monkeys infected with SIV as a non-human primate surrogate AIDS model (14, 15). The macaque/SIV model is useful since SIV is highly related to HIV-1, and induces AIDS-like diseases that are comparable to those of humans infected with HIV-1. Taking this into consideration, an alternative surrogate model which employs New World monkeys infected with GBV-B may be promising for future HCV/hepatitis C research.

GBV-B AS A NON-HUMAN PRIMATE SURROGATE MODEL OF HCV INFECTION

Among viruses so far known, GBV-B is the most closely related to HCV. However, due to a lack of epidemiological information as discussed below, GBV-B has been tentatively classified in the Hepacivirus genus of the Flavivirus family. Originally, Deinhardt *et al.* (16) found that some tamarins (genus *Saguinus*) developed hepatitis after inoculation with an inoculum obtained from a surgeon with the initials GB who had contracted hepatitis. After 11 passages in tamarins they obtained serum including GB agent(s), and were then able to achieve molecular cloning of GBV-A and GBV-B as flavivirus-like genomes (17) and to demonstrate GBV-B as an agent which could cause hepatitis in tamarins (18). Although it was unclear whether GBV-B originated from the GB inoculum or the tamarins themselves, later animal studies demonstrated that GBV-B is infectious for tamarins but not chimpanzees (19) and reciprocally that HCV is infectious for chimpanzees but not tamarins (20). These findings led to the retrospective conclusion that at least one of the tamarins employed for the *in vivo* passage study was persistently infected with GBV-B, and therefore GBV-B is probably a virus that originated in tamarins (20). However, GBV-B has not so far been isolated from additional tamarins, probably due to limited epidemiological analyses. Thus the natural host(s) and prevalence of GBV-B are yet to be determined.

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

Previous data have shown that a number of New World monkeys (parvorder *Platyrrhini*) including tamarins, the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*) are susceptible to GBV-B infection, as summarized in Table 1, although in tamarins peak concentrations of viruses in plasma are higher (10^7–10^{10} GE/ml) than in other monkeys (10^5–10^8 GE/ml) (21–29). In general, in any monkey species viremia

Table 1 Summary of characteristics of acute GBV-B infection in monkeys

Monkeys permissive of experimental infection	Tamarins (Genus *Saguinus*) Common marmoset (*Callithrix jacchus*) Owl monkey (*Aotus trivirgatus*)
Appearance of viremia	1–2 weeks post infection
Peak levels of viremia	Tamarins; 10^7–10^{10} GE/ml Marmoset and owl monkey; 10^5–10^8 GE/ml
Peak ALT levels	Approximately 200–500 IU/ml
Duration of viremia	2–3 months
Timing of seroconversion	A couple of weeks before clearance of viremia

persists for 2–3 months and is followed by clearance. GBV-B-infected monkeys with viremia usually develop self-resolving subacute hepatitis, as indicated by increases in the concentrations of serum enzymes such as ALT, gamma-glutamyltranspeptidase, and isocitrate dehydrogenase. Pathologically, degeneration and apoptosis of hepatocytes, as well as disruption and dilation of sinusoids, have been observed in the livers of GBV-B-infected tamarins with higher viremia and ALT activity (29). It is possible that GBV-B-specific CTL may cause the liver damage. However, a recent study reported that CTL are induced at a late stage of subacute GBV-B infection, and are inversely correlated with reduction in viremia (30). Since liver damage is usually found very early (1–2 weeks) after infection, when specific CTL are not observed, it is likely that viral replication in the hepatocytes leads directly to the early onset of cytopathic effects, while lower numbers of CTL may also contribute to cytotoxicity.

The clearance of viremia in the acute phase of GBV-B infection should require an effective antiviral immune response. In particular, in both GBV-B and HCV intrahepatic CTL appear to play a major role in viral clearance (30, 31). In addition, secondary GBV-B infection after clearance of the primary viremia induces a strong T cell response, leading to virtual absence of viremia, indicating that efficient memory is a key to protection from chronic viral infection (30, 32). In pre-immune chimpanzees antibody-mediated depletion of either CD4 or CD8 T lymphocytes affects their ability to control viral replication, resulting in prolonged viremia, demonstrating essential roles for both CD4 and CD8 memory in protection from viral persistence (33, 34).

On the other hand, the significance of humoral immunity in controlling GBV-B replication is still unclear. It is reasonable to assume that neutralizing antibodies also play important roles in the clearance of subacute viremia and protection from viral persistence. In the case of HCV, in one well characterized single-source outbreak of hepatitis C, viral clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection, while chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in this phase. Patients with resolution of infection were shown to exhibit broader cross-neutralizing activity of antibodies in the early phase of infection (35). In one chronic HCV patient who was followed up for 30 years, it has also been shown that HCV continuously escaped the host's immune system by repeated mutational changes, resulting in loss of recognition of the HCV envelope glycoproteins by antibodies (36). The fact that the sequences of envelope glycoprotein and specificity of neutralizing antibody change over time suggests that neutralizing antibodies exert selective pressure on HCV evolution. Thus, although neutralizing antibodies (and/or CTL) are not necessarily capable of controlling chronic viral infection, frequent escape from the antibodies needs so called fitness cost, resulting in the partial suppression of viral loads. Indeed, HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (37).

Although features of the subacute phase of GBV-B infection are similar to that of HCV, a major defect of GBV-B infection as a surrogate model for HCV is that it is difficult to chronically infect monkeys. While as many as 70% of humans with HCV infection become chronically infected, only approximately a third of chimpanzees do so (2, 12). By contrast, only a few cases regarding chronic GBV-B infection have been reported so far. The best example was a case of a tamarin persistently infected with GBV-B (24); the monkey exhibited acute mild hepatitis with viremia (peak level; $\sim 10^9$ GE/ml), which reduced to a set point level (less than 10^4 GE/ml) at 16 weeks post infection, followed by a gradual increase in viremia which reached $>10^7$ GE/ml at 112 weeks post infection, along with a significant ALT increase. However, the viremia suddenly declined thereafter and became undetectable, in association with a reduction in antibody titer, and subsequent *in vivo* passage of virus obtained from the tamarin failed to reproduce persistent infection in other tamarins (24). In addition, immunosuppression of a GBV-B-infected tamarin by FK506 treatment, or infection of GBV-B with deletion of poly(U) tract in the 3′ UTR, reportedly resulted in relatively long-term persistent infection of GBV-B for up to 46 and 90 weeks, respectively (23, 27). These results indicate that GBV-B may have the potential for establishing chronic infection.

Furthermore, our recent study has demonstrated that among four common marmosets infected with GBV-B derived from a molecular clone pGBB (21), two developed long-term chronic infection for up to three years, with recurrent viremia in which plasma viral RNA levels fluctuated between undetectable and 10^5 GE/ml, which is equivalent to the case of chimpanzees chronically infected with

HCV (Iwasaki *et al.*, manuscript in preparation). Notably, the induction of antiviral antibody response as measured by anti-Core and -NS3 antibodies was delayed in both cases, followed by a gradual increase, and then sustained high antibody titers. This was in contrast with an abrupt and transient increase at the end of periods of subacute viremia in marmosets and tamarins with viral clearance. Whether a delayed antibody response is associated with persistent GBV-B infection remains to be determined.

Taken together, these findings indicate the similarity between HCV and GBV-B in regard to their ability to induce chronic infection, and also shed light on the further potential of GBV-B as a surrogate model for HCV.

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

Although many questions are still to be addressed, accumulating evidence from extensive studies to date has greatly advanced the usefulness of the GBV-B as a surrogate model for HCV. The GBV-B model may be applicable for evaluating the feasibility and safety of anti-HCV vaccines employing novel viral vectors and gene therapy which creates RNA interference. For example, in a recent pilot study we showed that systemic administration of cationic liposome-encapsulated small interfering RNA to marmosets resulted in efficient regulation of GBV-B replication, indicating the usefulness of the surrogate model for proving the feasibility of RNA interference technology for future clinical application (38). This GBV-B model will also be helpful in identifying the virological and immunological factors which determine whether the outcome is acute resolving or chronic infection. While the GBV-B model appears to be valuable, development of an HCV/GBV-B chimeric virus would greatly expand the utility of the surrogate model, since it would enable us to directly evaluate antiviral vaccines and chemicals for HCV as a preclinical study. Rijnbrand *et al.* have reported that a chimeric GBV-B with 5′ untranslated region from HCV is infectious and causes hepatitis in tamarins (39). As recently demonstrated by Chevalier *et al.* (40), this will be a good model for evaluating the potential of small interfering RNA specific to HCV genome for future clinical application.

In regard to this, we may refer to an elegant precedent in the case of the macaques AIDS model. SIVmac is well known to efficiently infect, and result in the development of AIDS in macaques. Furthermore HIV-1, of which only 7% of the entire genome is derived from SIVmac, has been demonstrated to overcome the host range of authentic HIV-1, and to acquire the ability to productively infect macaque cells (41, 42). Instead of endangered chimpanzees, tamarins/marmosets which can be chronically infected with an HCV/GBV-B chimera (hopefully capable of inducing chronic hepatitis) should be the next generation of a promising non-human primate surrogate model for HCV infection, one which is similar to the macaques AIDS model. Whatever animals are used for pre-clinical study, it is important to keep in mind that results obtained from monkey models using either GBV-B or HCV/GBV-B chimera (as well as SIV or HIV/SIV chimera) may not necessarily be applicable to humans, because of potential differences in the molecular structure and/or mechanism by which antivirals and/or viral and host proteins function. Further characterization and understanding of the molecular biology and immunology of virus-host interactions will help in developing novel antiviral strategies.

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