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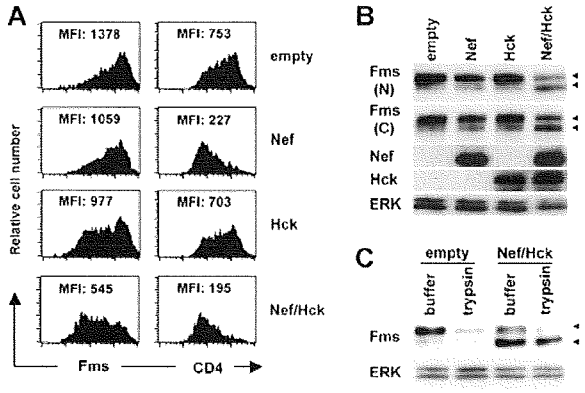

Figure 2. Nef reduces surface expression of Fms in 293 cells and increases intracellular gp130Fms, in the presence of Hck. (A) In the left panels, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef (CD8-Nef) or Hck (IRES-EGFP), and then stained with PE-labeled anti-Fms. In the right panels, 293 cells stably expressing CD4 were transfected with the indicated plasmids and stained with PE-labeled anti-CD4. These cells were costained with APC-labeled anti-CD8, and the data for cells positive for both CD8 and EGFP are shown. The MFI of the expression of Fms or CD4 is indicated. (B) As in panel A, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef or Hck. Total cell lysate was prepared and subjected to Western blotting with antibodies against the N-terminal portion of Fms (N), the C-terminal portion of Fms (C), Hck, Nef, or ERK. (C) 293 cells stably expressing Fms were cotransfected with Nef and Hck (Nef/Hck), or transfected with empty vectors (empty), and then treated with trypsin or control buffer. Total cell lysate was prepared and subjected to Western blotting with antibodies against the C-terminal portion of Fms or ERK. (B,C) The ERK blot is a loading control. The ◀ indicate the position of gp150Fms or gp130Fms.

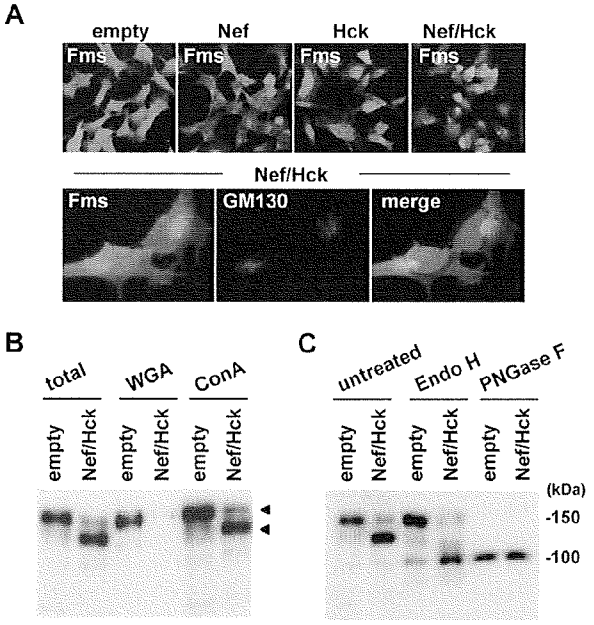

Figure 3. gp130Fms appearing in 293 cells coexpressing Nef/Hck is Golgi-localized underglycosylated Fms. (A) 293 cells stably expressing Fms were transfected with the control CD8 plasmid (empty) or CD8-Nef plasmid (Nef). Similarly, 293 cells stably coexpressing Fms and Hck were transfected with the control CD8 plasmid (Hck) or CD8-Nef plasmid (Nef/Hck). These cells were stained with anti-Fms antibody (top panels). In the bottom panels, 293 cells stably coexpressing Fms and Hck were transfected with CD8-Nef and costained with anti-Fms antibody (green), anti-GM130 antibody (red), and DAPI (blue). (B) 293 cells stably expressing Fms were cotransfected with Nef and Hck (Nef/Hck), or transfected with empty vectors (empty). The total cell lysate was subjected to Fms Western blotting directly (total) or after pull down with WGA-agarose or Con A–agarose. The arrowheads indicate the position of gp150Fms or gp130Fms. (C) Total cell lysate prepared as in panel B was subjected to Fms Western blotting directly (untreated) or after treatment with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F).

The novel function of Nef was further confirmed by nucleofecting Nef into human primary macrophages (Figure 1C,D). The purity of the macrophage preparations was usually more than 95% and 85% when assessed by the expression of CD14 and Fms, respectively (Figure S3). We used the CD8-Nef plasmid encoding Nef fused to the extracellular/transmembrane regions of CD8[34] to identify Nef-positive macrophages. The nucleofection of the control CD8 plasmid encoding only those regions of CD8 did not affect the expression of Fms (Figure 1C "control CD8" panels). In contrast, in the CD8-Nef–nucleofected macrophages, the Fms^{high} population was reduced as the expression of CD8-Nef increased (Figure 1C "CD8-Nef" panels). Such down-regulation of Fms as well as CD4 in the CD8-Nef^{high} population was reproducibly observed with macrophages derived from different donors (Figure 1D). The supernatant obtained from macrophages nucleofected with the CD8-Nef plasmid did not affect the level of Fms in TF-1-fms cells (data not shown), suggesting that production of M-CSF, if any occurred, was not involved in the Nef-induced down-regulation of Fms in macrophages.

Down-regulation of Fms by Nef is Hck-dependent and due to inhibition of intracellular maturation/trafficking of Fms

As both TF-1-fms cells[26] and macrophages[16] endogenously expressed Hck, it was possible that Hck was involved in the down-regulation of Fms caused by Nef. To examine this possibility and clarify the molecular mechanisms by which Nef down-regulated Fms, we next performed a transfection experiment using human 293 cells. As shown (Figure 2A left panels), the cotransfection of Nef and Hck markedly reduced the surface expression of Fms, although the transfection of Nef alone or Hck alone was effective to a certain degree. This was in contrast with the finding that the transfection of Nef alone was almost sufficient to reduce the surface expression of CD4 (Figure 2A right panels). The reduced surface expression of Fms was confirmed by Western blotting. As shown (Figure 2B), the amount of Fms species with a molecular weight of 150 kDa (gp150Fms, upper arrowhead) in the cells coexpressing Nef/Hck was obviously less than that in cells expressing Nef alone or Hck alone. Indeed, gp150Fms was the cell surface form of Fms, because the treatment of the cell surface with trypsin resulted in the loss of gp150Fms (Figure 2C). The trypsin-resistant gp150Fms might represent an intracellular pool of mature Fms that would be rapidly inserted into the plasma membrane. Interestingly, in parallel with the decrease in the expression of gp150Fms, an increase in the expression of a lower molecular weight species (130 kDa, lower arrowheads) was observed in the cells coexpressing Nef/Hck (Figure 2C). The 130-kDa species was a Fms-related product, because the 2 antibodies against the different portions of Fms (the N-terminus and C-terminus) detected the species (herein referred to as gp130Fms). In contrast to gp150Fms, gp130Fms was an intracellular form of Fms, because it was unaffected by the trypsin treatment (Figure 2C). Thus, the down-regulation of Fms observed in TF-1-fms-Nef-ER cells and macrophages was reproducible in 293 cells cotransfected with Nef and Hck, and associated with the increase of the intracellular gp130Fms.

To further characterize the intracellular gp130Fms that appeared in the cells coexpressing Nef/Hck, we next performed immunofluorescence microscopy. As shown (Figure 3A top panels; Figure S4 top panels), the pattern of Fms staining in the coexpressing cells was quite different from that in cells expressing Nef alone

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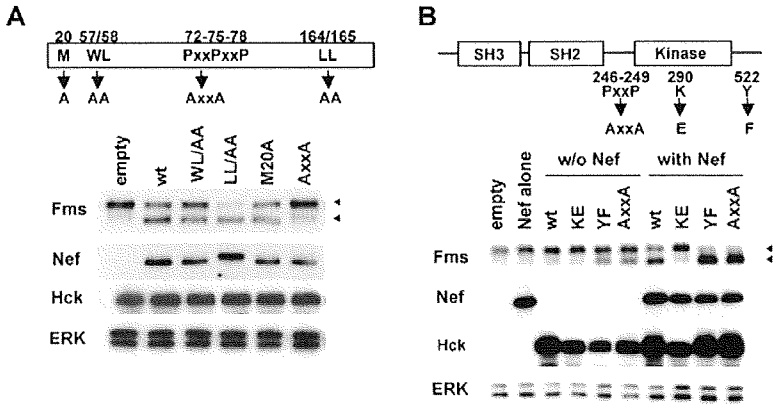

Figure 4. Activation of Hck by Nef is essential but not sufficient for accumulation of gp130Fms. (A) The Nef mutants used (M20A, WL/AA, AxxA, and LL/AA) are schematically shown. All the constructs are CD8-Nef chimeras. 293 cells stably expressing Fms were cotransfected with wild-type Hck and the plasmid 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.

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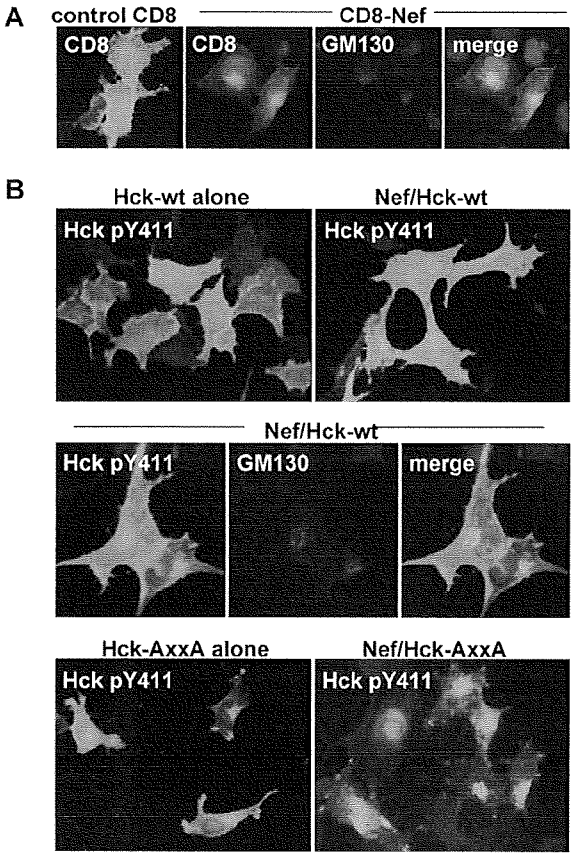

Figure 5. Nef induces Golgi localization of active Hck. (A) Parental 293 cells were transfected with the control CD8 plasmid or CD8-Nef plasmid, and then stained with anti-CD8 antibody (green), anti-GM130 antibody (red), or DAPI (blue). (B) In the top panels, parental 293 cells were transfected with 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

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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.

Acknowledgments

We thank Y. Endo for secretarial assistance.

This work was supported in part by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan (S.O., S.S.).

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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Immunogenetics (2008) 60:727–735
DOI 10.1007/s00251-008-0332-0

ORIGINAL PAPER

Natural selection in the *TLR*-related genes in the course of primate evolution

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Received: 4 June 2008 / Accepted: 4 September 2008 / Published online: 23 September 2008
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Abstract The innate immune system constitutes the front line of host defense against pathogens. Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. Here, we provide evidence that the TLR-related genes have come under natural selection pressure in the course of primate evolution. We compared the nucleotide sequences of 16 TLR-related genes, including *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14*, among seven primate species. Analysis of the non-synonymous/synonymous substitution ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. The genomic segments encoding the intracellular Toll/interleukin 1 receptor domains, which exhibited lower rates of non-synonymous substitution, have undergone purifying selection. In contrast, *TLR4*, which carried a high proportion of non-synonymous substitutions in the part of extracellular domain spanning 200 amino acids, was found to have been the suggestive target of positive Darwinian selection in primate evolution. However, sequence analyses from 25 primate species, including eight hominoids, six Old World monkeys, eight New World monkeys, and three prosimians, showed no evidence that the pressure of positive Darwinian selection has shaped the pattern of sequence variations in *TLR4* among New World monkeys and prosimians.

Keywords Toll-like receptor · Natural selection · Primate evolution

Electronic supplementary material The online version of this article (doi:10.1007/s00251-008-0332-0) contains supplementary material, which is available to authorized users.

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Introduction

Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains with varying numbers of leucine-rich-repeat motifs (LRR) and a cytoplasmic signaling domain termed the Toll/interleukin 1 receptor (TIR) domain (Akira et al. 2006; Bowie and O'Neill 2000). Different TLRs recognize a variety of

pathogen-associated molecules, including lipids and nucleic acids, and all TLRs transduce signals through TIR domains to activate immune cells (Akira et al. 2006; Bowie and O'Neill 2000). Stimulation with TLR ligands recruits adaptor proteins such as Myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor molecule 1 (TICAM1), and TICAM2, all of which also have a TIR domain, to the cytoplasmic portion of the TLRs and activate signaling cascades to produce proinflammatory cytokines and chemokines (Akira et al. 2006; Bowie and O'Neill 2000).

Viral, bacterial, and parasitic infections have been postulated to be among the strongest selective pressures on primate evolution. It is also widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis* bacilli and HIV-1, are different among primate species (Isaza 2003; Stremlau et al. 2004). Given that TLRs play crucial roles in the innate immune system, the intriguing hypothesis that TLRs have emerged under the intense pressure of natural selection in the course of primate evolution is rising. Actually, it is suggested that *TLR1*, *TLR6*, and *TLR10* have come under particular natural selection pressures in the human population, because the sequence variations of these three genes display considerable geographical diversity in the British population (Wellcome Trust Case Control Consortium 2007). Moreover, it has been reported that natural selection has acted on *TLR4* in humans, since excess of rare non-synonymous polymorphisms in *TLR4* are observed in humans (Smirnova et al. 2001).

To investigate the natural selection hypothesis, we analyzed the nucleotide sequences of 16 TLR-related genes, including ten *TLRs* (TLR1–10), four genes linked to signal transduction (*MYD88*, *TILAP*, *TICAM1*, and *TICAM2*), and two genes linked to *TLR4* (*MD2* and *CD14*) in primates. MD2 and CD14 are key molecules of the LPS signaling through TLR4 (Poltorak et al. 1998; Shimazu et al. 1999; Nagai et al. 2002). Our study shows that the genomic segments encoding the intracellular TIR domains have undergone purifying selection and that the extracellular domain of *TLR4* has been the suggestive target of positive Darwinian selection in the course of primate evolution. We concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in primate evolution.

Materials and methods

DNA sequences

DNA samples from 25 primates, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Hylobates syndactylus*), crab-eating macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), hamadryas baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), silvered lutong (*Trachypithecus cristatus*), dusky lutong (*Trachypithecus obscurus*), common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), red-handed tamarin (*Saguinus midas*), lion tamarin (*Leontopithecus rosalia*), common squirrel monkey (*Saimiri sciureus*), tufted capuchin (*Cebus apella*), long-haired spider monkey (*Ateles belzebuth*), and Central American spider monkey (*Ateles geoffroyi*), tarsiers (*Tarsius* spp.), lesser galago (*Galago senegalensis*), and ring-tailed lemur (*Lemur catta*) were analyzed. Overlapping primer sets covering all coding exons of 16 genes including the *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were designed on the basis of size and overlap of PCR amplicons. Genomic DNA was subjected to PCR amplification followed by sequencing using the BigDye Terminator cycling system. Sequencing analysis was performed in an ABI3130x automated DNA sequencer (Applied Biosystems).

Statistical analysis

Sequence alignments were performed by the Clustal X program (Thompson et al. 1997). All values for Ka, Ks, Ka/Ks, %GC, and Codon Bias Index (CBI) were evaluated by DnaSP (Rozas et al. 2003). The Bn–Bs program (Zhang et al. 1998) was applied to evaluate the Ka/Ks ratio in individual branches of the primate phylogenetic tree. We studied positive Darwinian selection for the target region of TLR4 by using the MEGA version 4.0 program (Tamura et al. 2007). Ancestral amino acid sequence was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989).

Results

The nucleotide sequences of tn *TLRs* (TLR1–10), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were determined among seven primates, including human, chimpanzee, bonobo, gorilla, orangutan, crab-eating macaque, and rhesus macaque. All sequences were newly determined in the study, and all accession numbers were shown in Table S1. The lengths of the deduced coding sequences for each gene differed among the seven primates, as summarized in Table 1.

To evaluate the non-synonymous/synonymous substitution ratio, we applied the Bn–Bs program (Zhang et al. 1998). This program uses a modified Nei–Gojobori method (Nei and Gojobori 1986) to estimate pairwise synonymous

Table 1 The non-synonymous and synonymous substitution ratio for 16 TLR-related genes among seven primates

Gene	Chromosome (Human)	Size of coding region (bp)	Entire coding sequence			TIR domain			Non-TIR region		
			Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs
TLR1	4p14	2,358	0.041	0.095	0.429	0.019	0.103	0.164	0.046	0.093	0.491
TLR2	4q32	2,352, 2,349[Orangutan]	0.025	0.086	0.290	0.009	0.058	0.157	0.028	0.091	0.311
TLR3	4q35	2,712	0.032	0.121	0.267	0.018	0.070	0.259	0.035	0.130	0.270
TLR4	9q32–33	2,517, 2,490[Orangutan], 2,478[Crab-eating, Rhesus]	0.038	0.085	0.447	0.006	0.118	0.053	0.045	0.079	0.566
TLR5	1q41–42	2,574	0.030	0.108	0.282	0.018	0.173	0.105	0.032	0.097	0.339
TLR6	4p14	2,388, 2,385[Bonobo]	0.030	0.120	0.240	0.021	0.199	0.105	0.031	0.105	0.293
TLR7	Xp22.3–p22.2	3,147	0.014	0.069	0.202	0.003	0.087	0.034	0.016	0.066	0.236
TLR8	Xp22.3–p22.2	3,123, 3,120[Crab-eating, Rhesus]	0.020	0.095	0.209	0.003	0.056	0.054	0.023	0.101	0.224
TLR9	3p21.3	3,096	0.029	0.153	0.187	0.003	0.202	0.016	0.032	0.145	0.224
TLR10	4p14	2,433	0.024	0.106	0.228	0.018	0.045	0.402	0.026	0.118	0.216
MYD88	3p22–p21.3	888	0.009	0.096	0.094	0.000	0.087	0.000	0.017	0.102	0.165
TIRAP	11q23–q24	663, 660[Crab-eating, Rhesus]	0.035	0.164	0.216	0.032	0.254	0.126	0.037	0.110	0.341
TICAM1	19p13.3	2,139[a]	0.039	0.171	0.227	0.007	0.163	0.041	0.046	0.172	0.269
TICAM2	5q23.1	705	0.020	0.119	0.167	0.000	0.132	0.000	0.033	0.110	0.300
MD2	8q21.11	480	0.015	0.054	0.269	–	–	–	–	–	–
CD14	5q31.1	1,125	0.013	0.040	0.332	–	–	–	–	–	–

[a] *TICAM1* has a CCT(Pro)-repeat variation

and non-synonymous distances among the sequences and then estimates the branch lengths in terms of synonymous (bs) and non-synonymous substitutions (bn) per site by using the ordinary least-squares method, while the tree topology is given. Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages. When the value of Σbn and Σbs and the ratio of Σbn/Σbs were evaluated for the entire coding sequences from each gene, there was no evidence to support that these genes have come under the pressure of positive natural selection. All of the values of the Σbn/Σbs ratio from the analyzed genes were much lower than 1.0, which suggested that these genes have been under the pressure of negative selection (Table 1).

To identify the genomic segments, which have undergone natural selection, a sliding window plot analysis (600-bp window with 30-bp steps) was performed throughout these genes. Analysis of the Σbn/Σbs ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. Three candidate segments, where the pressure of negative or positive natural selection might have operated, were identified in *TLR7*, *MYD88*, and *TLR4* (Fig. 1a).

Two target segments showed little non-synonymous nucleotide difference among the seven primates (Fig. 1a and Supplementary material, Fig. S1). One was located at the coding segment encoding the C-terminal of TLR7 and the other at the segment encoding the C-terminal of MYD88, both of which encode the TIR domain (Fig. 1a). Phylogenetic comparisons from 14 human sequences encoding TIR domains reveal no obvious similarity between *TLR7* and *MYD88* (Fig. 2a). We then evaluated the Σbn/Σbs ratios for the TIR domains for 14 genes with TIR domains. The sizes of the genomic segments encoding TIR domains [average 393 bp (249–426 bp)] were smaller than the window size (600 bp) used in our analysis so that our window analysis would underestimate the Σbn/Σbs ratios for TIR domains. The values of Σbn and Σbn/Σbs ratio for TIR domains displayed lower values when compared with those of the non-TIR coding sequences except for Σbn/Σbs ratio from *TLR10* (Table 1 and Fig. 2b). In particular, *TLR7*, *TLR8*, *TLR9*, *MYD88*, and *TICAM2* have much lower values for Σbn at the TIR domains. Taken together, it is suggested that the TIR domains have been under the control of negative/purifying selection.

On the other hand, sequence comparisons among the seven primates support the positive Darwinian selection at the extracellular domain of TLR4, for which the Σbn/Σbs ratios were much higher than 1.0 (the highest value in the 600-bp window is 2.37, with a statistical significance in *Z* test; Zhang et al. 1998, Tamura et al. 2007; *Z* score 2.16; *p* value <0.01; Fig. 1). Among analyzed windows fro TLR-related genes, *TLR4* and *TICAM1* have extreme high values of Σbn. However, the windows of *TICAM1* harboring high values of Σbn also have the high value of Σbs. The high values of %GC seem to be associated with the high-nucleotide substitution rate in *TICAM1* (Fig. 1a and b). A lower value of CBI was also correlated with a lower synonymous nucleotide substitution rate (data not shown).

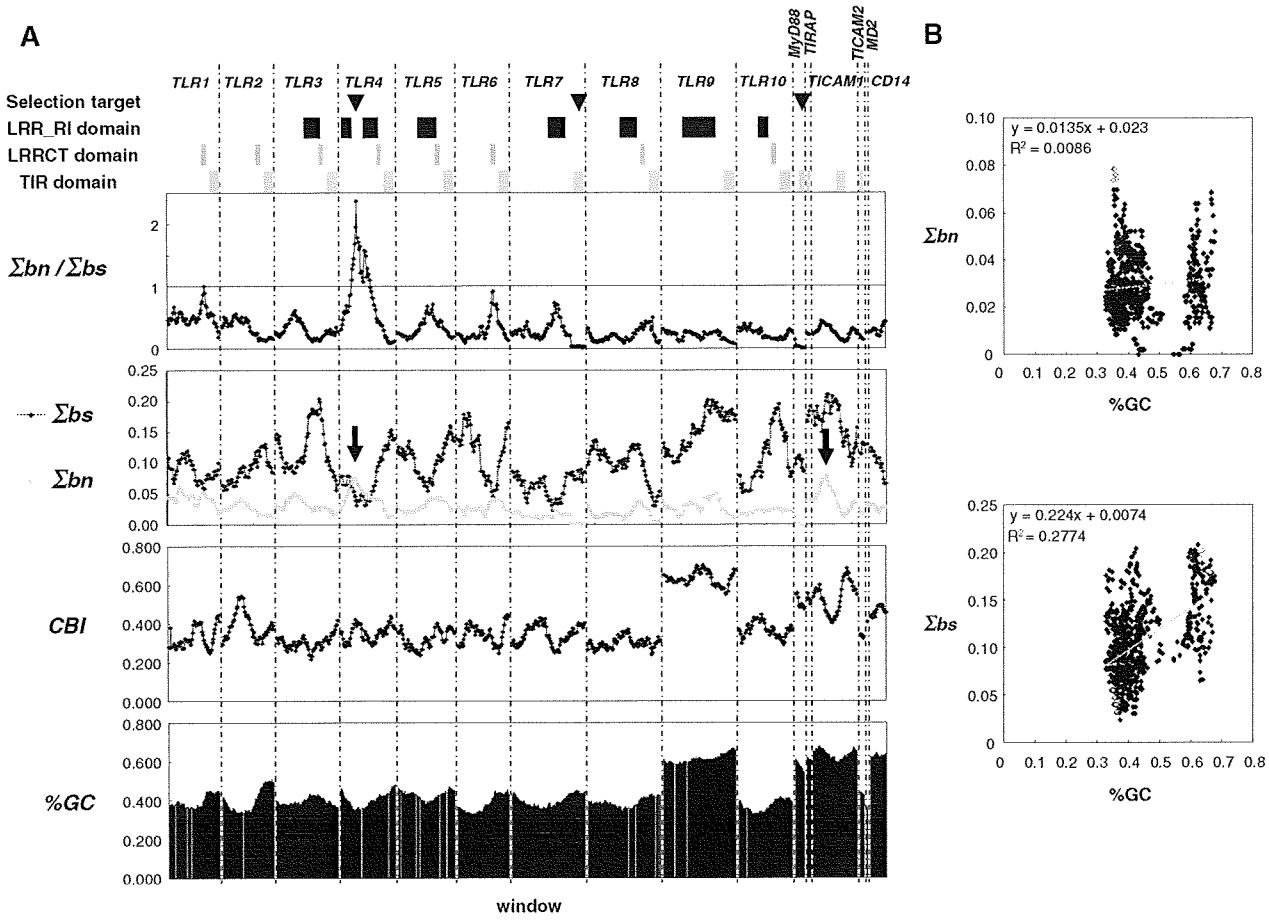

Fig. 1 **a** The values of Σbn/Σbs, Σbn, Σbs, CBI, and %GC based on the sliding window plot analysis for the TLR-related gene (600-bp window with 30-bp steps). The *arrow heads* indicate the candidate segments for the pressure of positive or negative natural selection. The *arrows* indicate that *TLR4* and *TICAM1* have extreme high values of Σbn among analyzed windows. CBI is a measure of the deviation from the equal use of synonymous codons, which indicates the extent to which a gene uses a subset of optimal codons (Bennetzen and Hall 1982). Three conserved domain structures, *LRR_RI* (leucine-rich repeats, ribonuclease inhibitor-like subfamily), *LRRCT* (leucine-rich repeat C-terminal domain), and *TIR* (Toll/interleukin-1 receptor homology domain), are referred from CD-search (Marchler-Bauer and Bryant 2004). **b** Pairwise comparisons between Σbn and %GC and between Σbs and %GC. All values of Σbn, Σbs, and %GC were based on the sliding window plot analysis for the TLR-related gene. *TLR4* (*white lozenge*) and *TICAM1* (*gray lozenge*) have several windows with extreme high values of Σbn

In the window of *TLR4* harboring the highest value of Σbn, its level of CBI (0.398) was almost equivalent to the average level among analyzed windows (0.387±0.114).

The estimated values of bn and bs of each lineage at *TLR4* target region were shown in Fig. 3. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs. These lineages have a relatively low value of bs; however, the values of bn were much larger than the estimated value of bs for entire TLR4 coding sequences in each lineage except for orangutan lineage (Supplementary material, Fig. S1). These lines of evidence suggested that the extracellular domain of TLR4 has been the possible target of positive Darwinian selection in the course of primate evolution.

To evaluate this finding further, we determined the sequences of a ~600 bp *TLR4* target region from additional 18 primates, including three gibbons (black gibbon, white-handed gibbon, and siamang), four Old World monkeys (hamadryas baboon, black and white colobus, silvered lutong, and dusky lutong), eight New World monkeys (common marmoset, cotton-top tamarin, red-handed tamarin, lion tamarin, common squirrel monkey, tufted capuchin, long-haired spider monkey, and Central American spider monkey), and three prosimians (tarsiers, lesser galago, ring-tailed lemur). Each of target sequences from three gibbons, two lutongs, and two tamarins is identical so that a total of 21 sequences were advanced to further analyses.

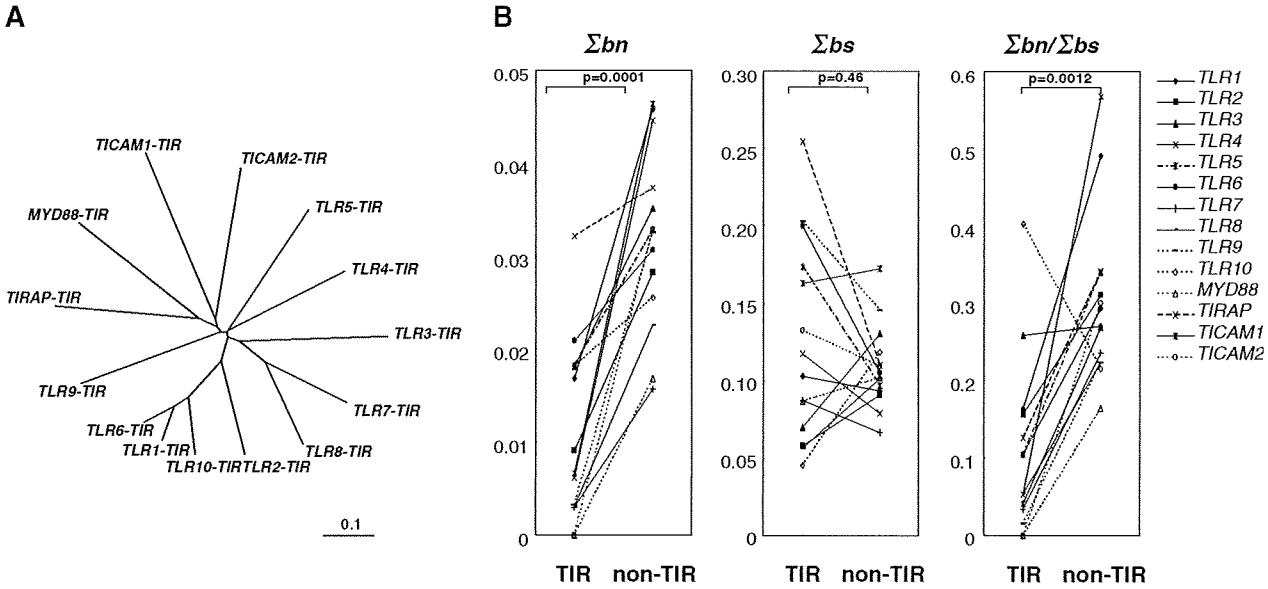

Fig. 2 **a** A neighbor-joining phylogenetic tree for 14 human sequences encoding TIR domains. Alignment and neighbor-joining tree for sequences was inferred using the Clustal X (Thompson et al. 1997) and TreeView programs. **b** The values of Σbn, Σbs, and Σbn/Σbs for the TIR domains and non-TIR coding regions of 14 genes, including *TLR*s (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, and *TICAM2*. Statistical significance was tested with the Wilcoxon matched-pairs signed-ranks test

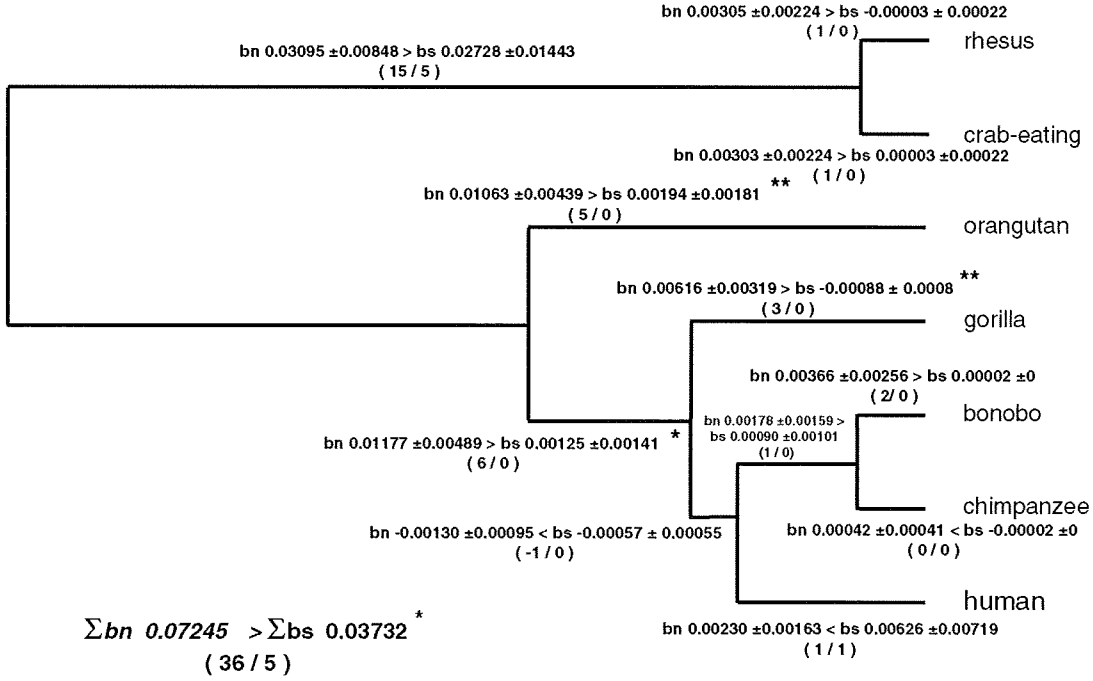

Fig. 3 Phylogenetic tree and the value of bn and bs for the selection target in the extracellular domain of TLR4 among seven primate species. We applied the Bn–Bs program (Zhang et al. 1998) to evaluate the value of bn and bs in individual branches of the primate phylogenetic tree (the values were shown with standard errors). Numbers of non-synonymous/synonymous substitutions for each branch are shown in *parentheses*. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs (*$p<0.05$ and **$p<0.01$ in Z test)

The estimated values of bn are larger than those of bs in several lineages among hominoids and Old World monkey, which is much evident in the lineages among hominoids. These lineages have a relatively low value of bs. On the other hand, the most estimated value of bn in the lineages among New World monkeys and prosimians are lower than those of bs (Fig. 3a, Supplementary material, Table S2; Fig. 4). Similar findings were shown in the analyses of synonymous (Ks) and non-synonymous nucleotide substitution rate (Ka) for the pairing of the 21 primate sequences at the target region of *TLR4* are shown in Fig. 3b and Supplementary material, Table S1. The Ka/Ks ratios from the comparisons among hominoids and Old World monkeys were much higher than 1.0; however, all values from the comparisons among New World monkeys and prosimians were under 1.0.

Ancestral amino acid sequence for 21 primates was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989; Fig. 5). Among 210 amino acids from TLR4 target region, less than 50% of

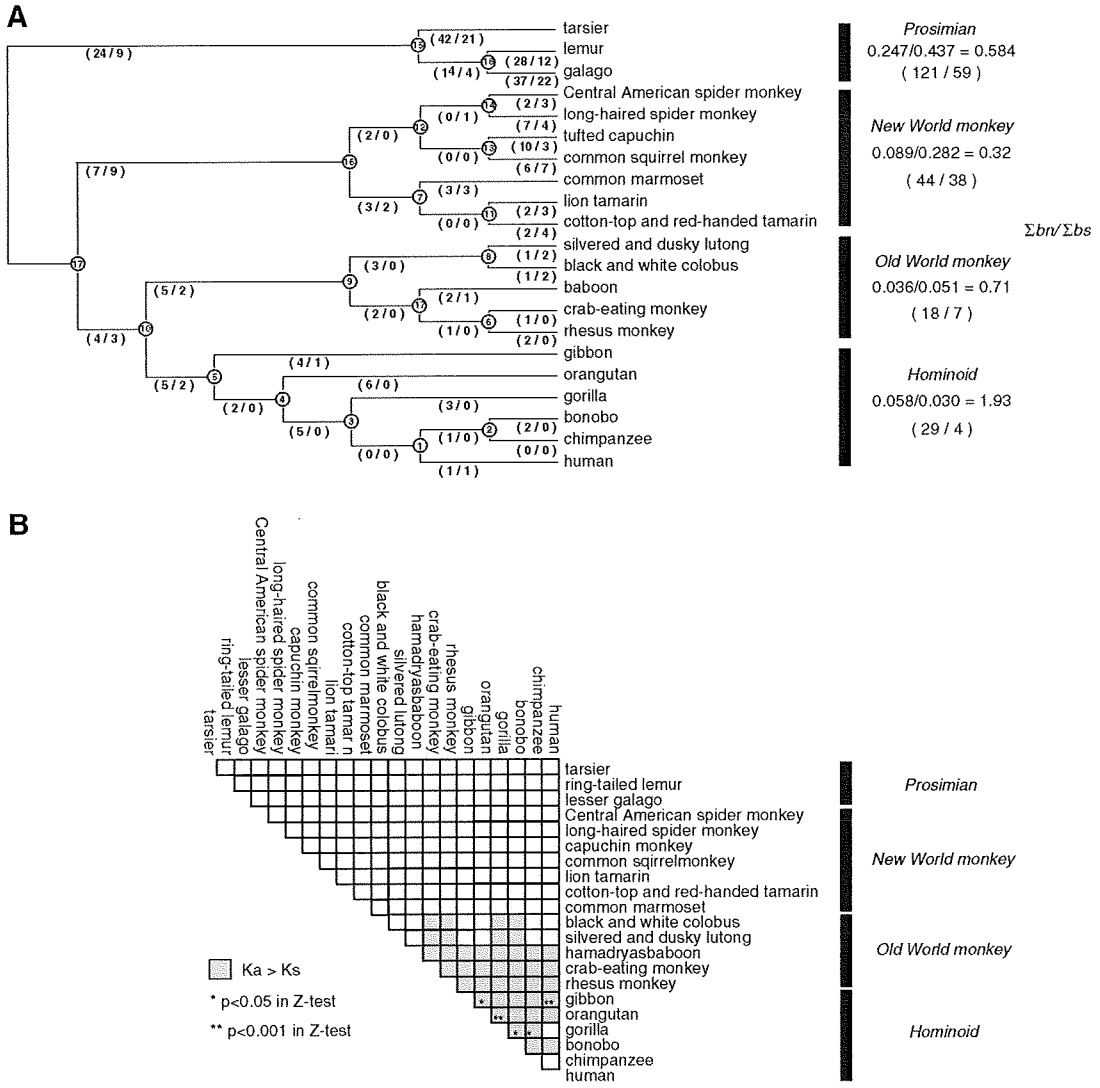

Fig. 4 **a** Phylogenetic tree and the nucleotide substitutions for the extracellular domain of TLR4 from 21 sequences. We applied the Bn–Bs program (Zhang et al. 1998) to evaluate the values of bn and bs in individual branches of the primate phylogenetic tree. *Numbers in parentheses* indicate the number of nucleotide substitutions at non-synonymous/synonymous sites. In several lineages, the value of bn is larger than that of bs (Table S3). Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages from hominoids, Old World monkeys, and New World monkeys. Branches are not scaled. *Numbered circles* are used to specify individual branches and are referenced in Supplementary material, Table S3. **b** Ka/Ks ratios for all comparisons among 21 TLR4 target sequences. The values of Ka/Ks for 627-bp target sequences were evaluated, because the comparisons among 21 sequences showed that a 3-bp deletion were observed in sequences from lesser galago (*G. senegalensis*) and ring-tailed lemur (*L. catta*)

```
Ancestral aa      DLS?N?IQNIY?KDLQVLHQ??LLNLSLDLSLNPIBFIQPGAFK?IRLHKLTLR?NFDSLNVMKTCIQGLAGLEVHRLVLGEFRNERN?EDFDKSALEGLCNLTI
                          *   *  *                 **          *       **                    **         *   *  *
Human             ...S.K..S..CT..R....MP............MN........E.........N.............................G.L.K..............
Chimpanzee        ...S.K..S..CT..R....MP............MN........E.........N.............................G.L.K..............
Bonobo            ...S.K..S..CT..R....MP............MN........E.........N.............................E.L.K..............
Gorilla           ...S.K..S..CT..R....MP............MT........E.........N....................R..........G.L.K..............
Orangutan         ...S.K..S..C........MP...........AMN........E.........NS...................H.........K.L.K..T...........
Gibbon            ...S.K..S..C........MP............MN........E.S.......N.............................G.L.E..............

Rhesus            ...S.N.....C........MP.S...........N........E.........S...D...........................L.E....S.........
Crab-eating       ...S.N.....C........MP.S...........N........E.........S...D...........................L.E....S.........
Baboon            ...S.N.....C........MP.P...........N........E.........S...D...........................L.E..............
Lutong            ...S.N.....C........MP.P...........N........E.........S...G.........................Q..L.E..............
Colobus           ...S.N.....C........MP.P...........N........E.........S...G.........................Q..L.E..............

Marmoset          ...S.N.....CQ.......MP.............N........E...R.....N............R....................I..............S.
Tamarin           ...S.N.....C........MP.............N........E.........N...............T................I..............S.
Lion Tamarin      ...S.K..S..C........MP.............N........E...R.....N.............................I..............S.
Squirrel          ...S.N.....C........MP.............N........E.........N......A........................I................
Capuchin          ...S.N.....C........MP.............N........E...R.....N............H...................I................
Long-hair Spider  ...S.N.....C........MP.............N.......QE.S.R.....N.....H........................I................
CA-spider         ...S.N.....C........MP.............N........E.........N.............................I................

Galago            ...N.N....SH........VPSH...........D.....S..G...RE....S.....S.......N..............K...YVK.I.Q...........
Lemur             ...K.N.....HE..H....NSS............D.....S..G.........S....V.I..................W.......YVK.............A.
Tarsier           ...N.K.....H.......PIS.I.......W...D...S....D.........S..N.M.E...........K..QF..........M....I.V.N.......

                                   ↓
Ancestral aa      ?EFRLA?LDDYLBD??DLFNCLANVS??SLVS?N??R?E??S?NFRWQ?LELVNCKFEQFPTL?L?SLKR??FTANKGGN?F?EV?LPSLEFLDLSRNGLSFKGC
                          *           *           ** *      *       *   *     *           * *
Human             E.....Y..Y..D.II......T...SF....VTIE.VKDF.Y..G..H........G.....K.K....LT..S.....A.S..D...................
Chimpanzee        E.....Y..Y..D.II......T...SF....VTIKSVKDF.Y..G..H........G.....K.K....LT..S.....A.S..D...................
Bonobo            E.....Y..Y..D.II......T...SF....VTIKSVKDF.Y..G..H....K...G.....K.K....LT..S.....A.S..D...................
Gorilla           E.....Y..Y..D.II......T...SF....VTIE.VKDF.Y..G..H........G.....K.K....LT..S.....A.S..D...................
Orangutan         E.....Y..Y..D.II..........SF....VTIKSVKDF.Y..G..H........G.....E.K....LT........A.S..D...................
Gibbon            E.....Y..H..D.II..........SF....VTIK.V.DF.Y..G..H........G.....N.K....LT....R...A.S..D...................

Rhesus            E....TY..Y..DNII..........SF....VSIK.V.DF.Y..R..H..............E.E....LT........A.S..D...................
Crab-eating       E....TY..C..DNII..........SF....V.IK.V.DF.Y..R..H..............E.K....LT........A.S..D...................
Baboon            E....TY..Y..DNII........A.SF....V.IK.V.DF.Y..R..H..............E.E....LT........A.S..D...................
Lutong            E.....Y..Y..NNII........I.SF....VTIK.V.DF.Y..R..H..............E.E....LT........A.S..D...................
Colobus           E.....Y..Y..NNII..........SF....VTIK.V.DF.Y..R..H..............E.E....LT.I......A.S..D...................

Marmoset          K.....H...FPD.II..........SF....VYIK.I.DF.Y..R..H............P.E.K....LT........P.S..D...................
Tamarin           K.....H...FPD.II..........SF....VYIK.I.DF.Y..R..H............P.K.K....LT........P.S..D...................
Lion Tamarin      K.....H...FPD.II..........SF....VYIK.I.DF.Y..R..H............P.K.K....LT....N...P.S..D...................
Squirrel          N.....Y...F.D.II..........SF...NVHIK.V.DF.Y..R..H......V.Q...P.K.K....LT......R.H.S..D...................
Capuchin          K.....Y..NFPD.II......V...SF..L.VYIK.V.DF.Y..R..H......I.Q...P.K.K....LT.SK...R.H.A..D...................
Long-hair Spider  K.....H...FPD.II..........SF....VYIK.V.DF.Y..R..H......V.Q...P.K.K....LT......R.P.S..D...................
CA-spider         K.....H..NFPD.II..........SF....VYIK.V.DF.Y..R..H......I.....P.E.K....LT......R.H.S..D...................

Galago            E.....H...T.QGA-E.LH..E...AI...SLDLS.LKWPYK..K.KS...ID.........E.FY...FI....R..NT.IK.K..D..Y....K....YM..
Lemur             E..S..N.EES.K.A-...H..T...AI...SLDLNYLKGFYN.YG.RS..............E.F..E.FIL.H..D.LT.A..E.....Y..I.K........
Tarsier           K...F.C.YHDFS.AT..........TI..IHL.LD.L.GLPN.VR..S....T..L......EIP....FI...H.S.YT.A.FK..............N....
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Fig. 5 Alignment of TLR4 target amino acid sequences of 21 primate species. *Dots* indicate identity to the ancestral sequence predicted by PROTPARS program in PHYLIP (Tamura et al. 2007), and *dashes* indicate a gap. *Asterisks* indicate amino acids which had been substituted since the emergence of hominoids. *Arrow* indicates the position corresponding to human mutation, D299G. The protein sequences are given by the *one-letter code* and consistent with the IUB standard abbreviations. *Question marks* in the ancestral amino acid sequence indicate the amino acid of which ancestral sequence was not determined by PROTPARS program (Felsenstein 1989)

amino acids [42.9% (90/210)] were conserved among 21 primate species, and at least 24 amino acids were replaced since the emergence of hominoids.

Discussion

Several lines of evidence suggested that genes linked to immune and defense systems are good candidate genes under the pressure of positive natural selection. However, most of the TLR-related genes have come under negative natural selection in the course of primate evolution. Our study suggested that the genomic segments encoding the intracellular TIR domains have undergone strong purifying selection and that the extracellular domain of TLR4 has been a single target of positive Darwinian selection among the TLR-related genes. We have no strong evidence that the extracellular domains of other TLRs, which recognize a variety of pathogen-associated molecules, has been under the pressure of natural selection in the course of primate evolution.

The TIR domains are key components in TLR signal transduction. In particular, an adaptor protein, MYD88, is tightly linked to all of the TLR signaling pathways except for TLR3 (Akira et al. 2006; Bowie and O'Neill 2000). This may be the reason for that the amino acid sequences have not been altered in the course of primate evolution. Most of mutations arising at the TIR domains would in all likelihood have been deleterious and thus reduced the fitness in the primates who harbored such changes.

Among the TLRs, *TLR7*, *TLR8*, and *TLR9* have much lower values of Σbn at the TIR domains. These three genes recognize nucleic acids in the innate immune system and are categorized into highly related subfamilies based on phylogenetic comparison (Akira et al. 2006). TLR7 and TLR8 are known to recognize single-strand RNA molecules

and the natural ligand of TLR9 is CpG motif-containing DNA (Akira et al. 2006; Heil et al. 2004). *TLR7* and *TLR8* are also mapped on chromosome X in the human, chimpanzee, and rhesus macaque genome, which might be linked to the reduced Σbn for these two genes in primate evolution, because reduced divergence has been observed in chromosome X when a comparison was performed on the human and rhesus macaque genome (Rhesus Macaque Genome Sequencing and Analysis Consortium 2007).

On the other hand, it is likely that the part of the extracellular domain of TLR4 has been under the pressure of positive natural selection in the several lineages since the emergence of Catarrhina. The values of bn were higher than those of bs in the several lineages. In particular, the values of bn in the lineages of gorilla and the lineage just after the split of ancestors of four great apes (gorilla, bonobo, chimpanzee, and human) were much larger than the estimated value of bs for entire TLR4 coding sequences. However, we cannot rule out the possibility that chance variation in the value of bs, rather than positive selection, accounted for the observed pattern (Hughes and Friedman 2008), because these lineages have a relatively low value of bs.

This suggestive *TLR4* target region, which encodes the extracellular domain next to the domain with LRR, has been reported to be hypervariable and to contribute to species-specific recognition of several molecules such as taxol, a lipid IVa, and LPS (Smirnova et al. 2000; Hajjar et al. 2002; Lien et al. 2000). Our study showed that the positions of amino acid replacements since the emergence of Catarrhina, which might be linked to species-specific recognition of LPS, are widely distributed in the TLR4 selection target. This target region also has been reported to be linked to the susceptibility to LPS in humans (Arbour et al. 2000). A missense human mutation D299G, in which an aspartic acid is replaced by a glycine at the 299 amino acid position of human TLR4, is associated with a blunted response to LPS and increased susceptibility to Gram-negative bacterial infections. An aspartic acid at the 299 amino acid position of human TLR4 is highly conserved among great apes and gibbon, whereas an aspartic acid has been replaced by a glycine in the lineage of Old World Monkeys (Fig. 5). These results indicated that the sensitivity to a certain type of LPS might differ between great apes and Old World Monkeys.

Given that TLR4 recognizes a wide variety of ligands such as LPS and viral envelope proteins, the differences in the species-specific susceptibility to infectious disease might have been linked to natural selection pressure. It is widely accepted that the susceptibility to infectious pathogens differ among primates. For example, Asian Old World monkeys are highly susceptible to infection with *M. tuberculosis* bacilli, while New World monkeys appear to be fairly resistant (Isaza 2003). Furthermore, the species-specific restrictions operating on HIV-1 infection are well known. Humans as well as chimpanzees but not New and Old World monkeys are susceptible to HIV-1 (Stremlau et al. 2004). Though we are not aware of the exact pathogens, it is highly possible that wide-spread pathogens might have been linked to the selective pressure for TLR4.

In this study, we evaluated the molecular evolution of TLR-related genes in primates and concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in the course of primate evolution, but positive selection pressure has been limited across the TLR family.

Acknowledgments This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and research grants from the Ministry of Health, Labor and Welfare, Japan, the Japan Health Science Foundation, and the program of Founding Research Centers for Emerging and Reemerging Infection Disease supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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MDM2 is a novel E3 ligase for HIV-1 Vif

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Published: 7 January 2009

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

Received: 16 September 2008
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, 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-

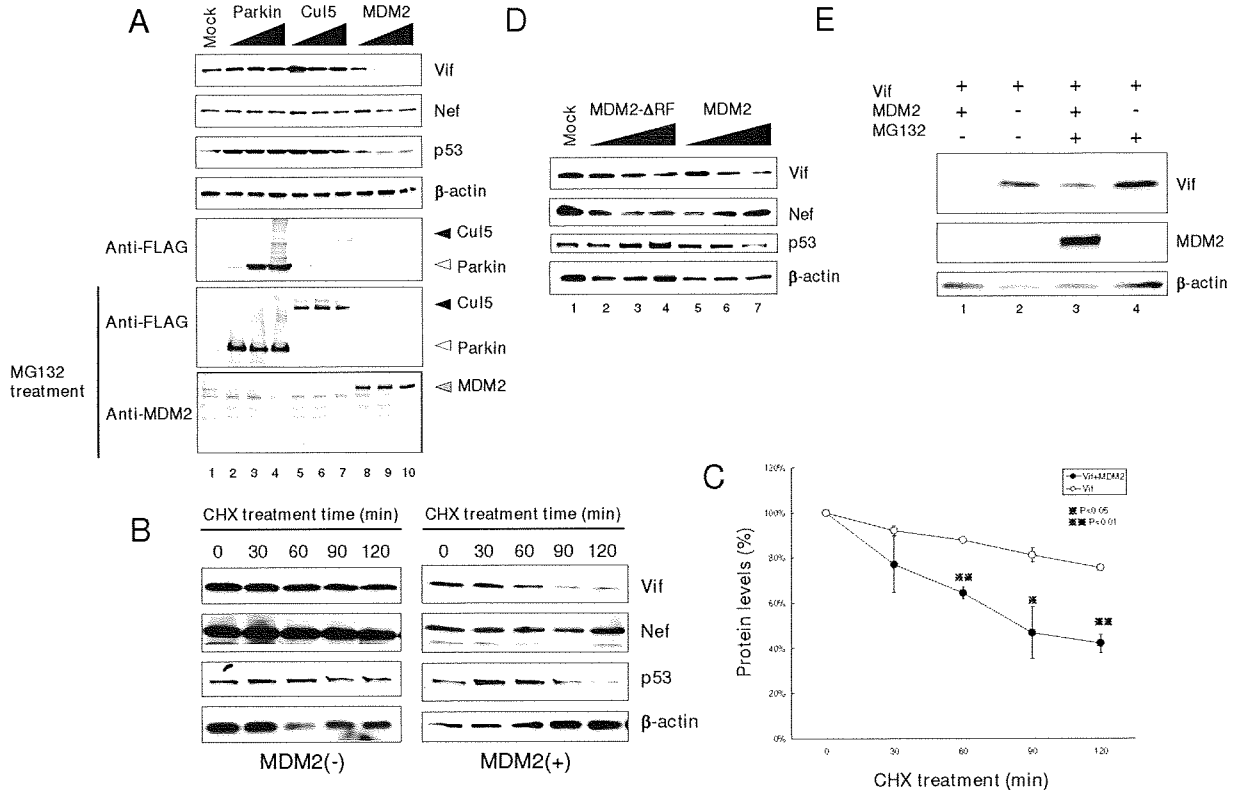

Figure 1

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

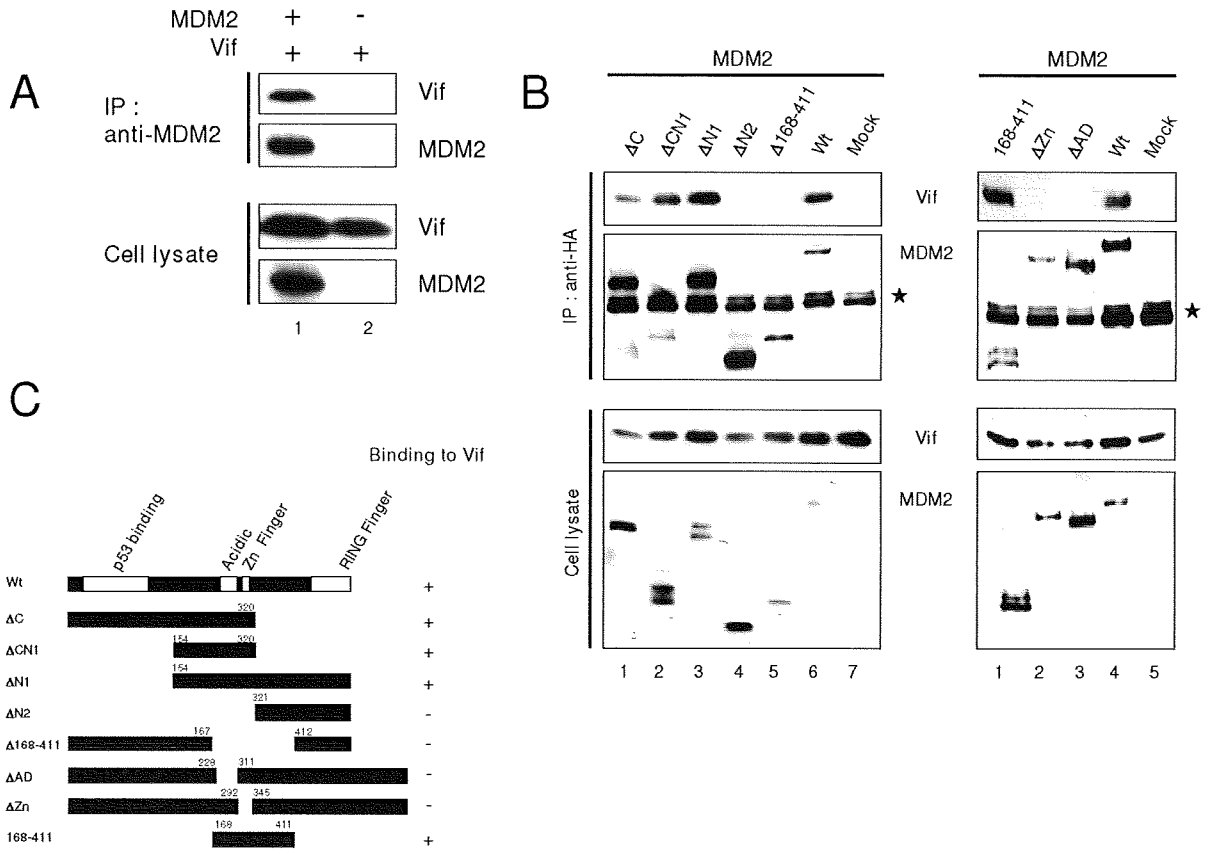

Figure 2
MDM2 bound Vif in its central domain. (A) Immunoprecipitation assays revealed the interaction of MDM2 with Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 and Vif and treated with MG132 for 6 hrs prior to harvest. Cell lysates were immunoprecipitated with anti-MDM2 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

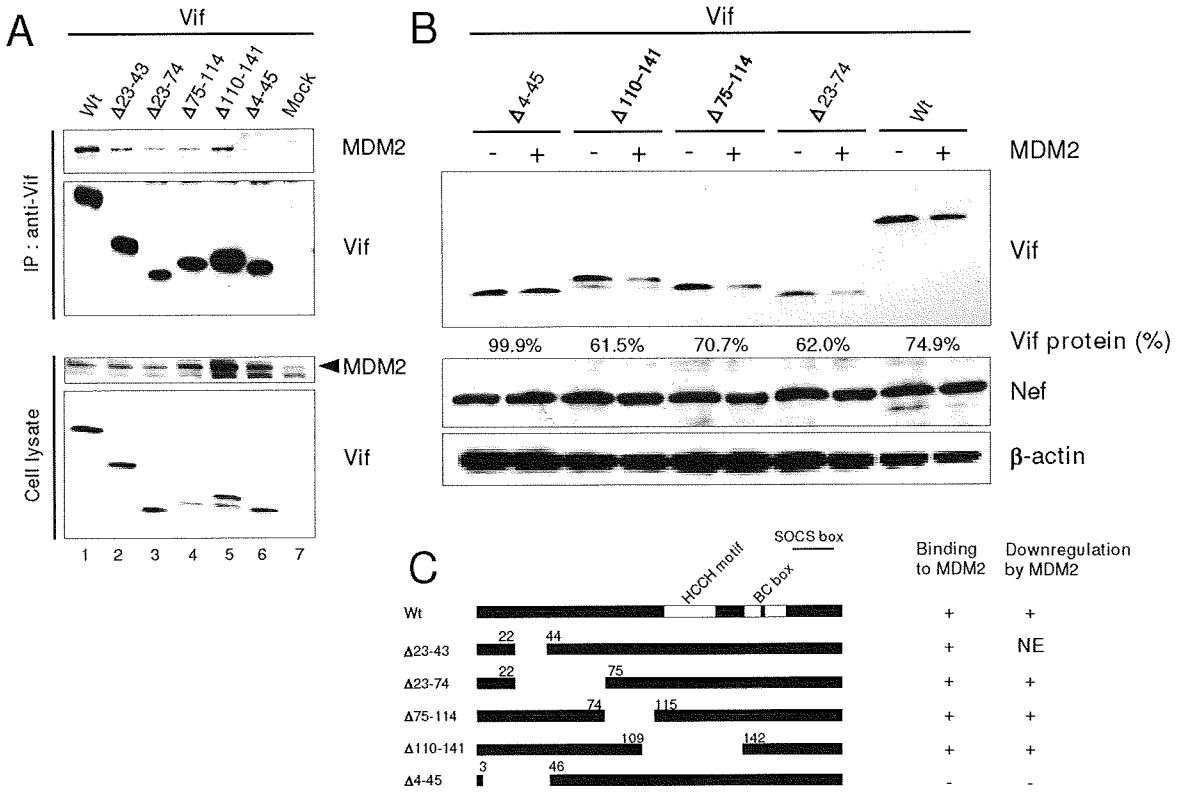

Figure 3

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-

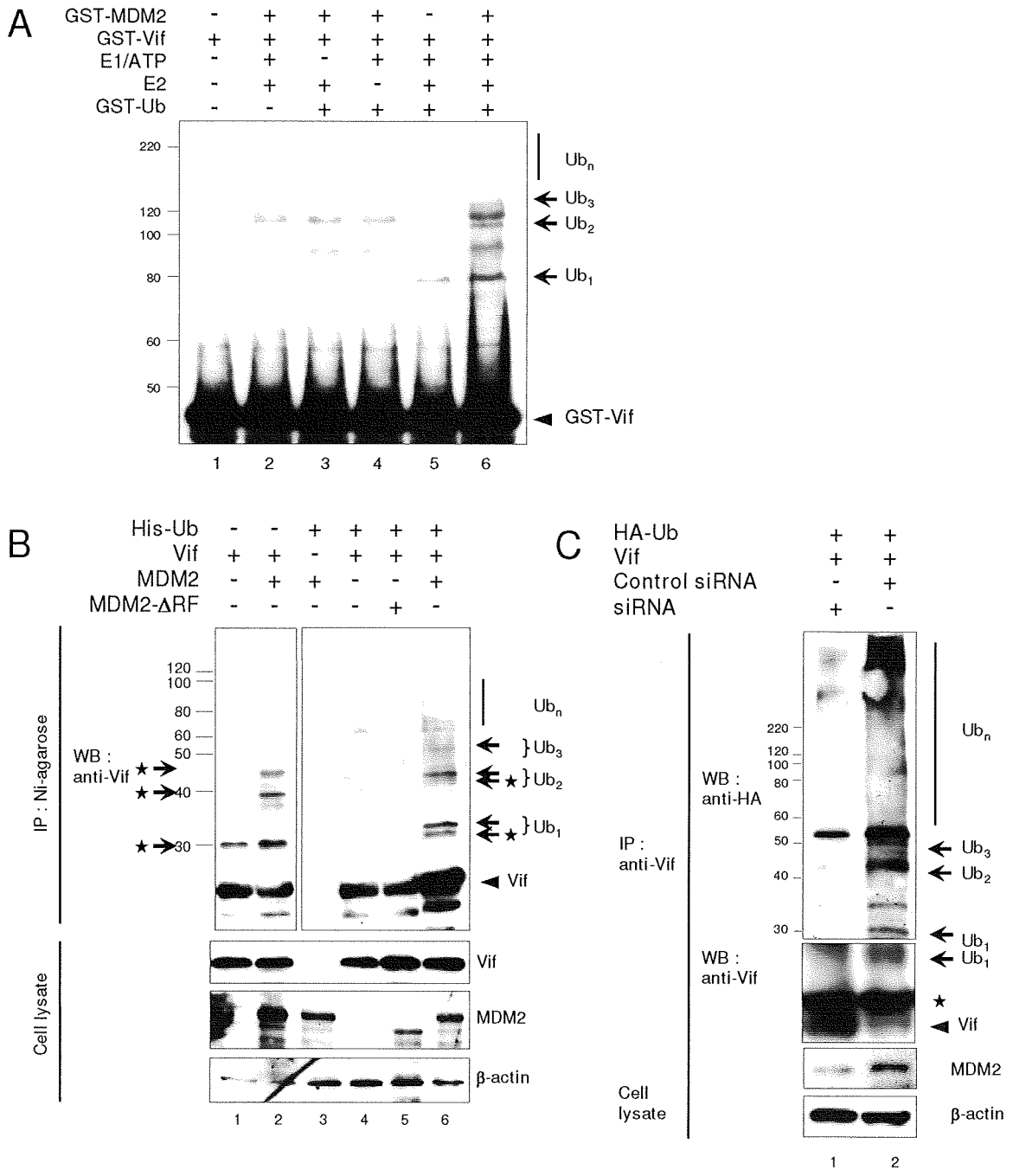

Figure 4 *(see legend on next page)*