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A Noncanonical mu-1A-Binding Motif in the N Terminus of HIV-1 Nef Determines Its Ability To Downregulate Major Histocompatibility Complex Class I in T Lymphocytes

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Downregulation of major histocompatibility complex class I (MHC-I) by HIV-1 Nef protein is indispensable for evasion of protective immunity by HIV-1. Though it has been suggested that the N-terminal region of Nef contributes to the function by associating with a mu-1A subunit of adaptor protein 1, the structural basis of the interaction between Nef and mu-1A remains elusive. We found that a tripartite hydrophobic motif (Trp13/Val16/Met20) in the N terminus of Nef was required for the MHC-I downregulation. Importantly, the motif functioned as a noncanonical mu-1A-binding motif for the interaction with the tyrosine motif-binding site of the mu-1A subunit. Our findings will help understanding of how HIV-1 evades the antiviral immune response by selectively redirecting the cellular protein trafficking system.

Nef is an *N*-myristoylated protein of 27 to 35 kDa conserved among primate lentiviruses, and the expression of human immunodeficiency virus type 1 (HIV-1) Nef contributes to the progression of AIDS (4). One of the multiple functions of Nef is the downregulation of major histocompatibility complex class I (MHC-I) (41). MHC-I downregulation by Nef has been shown to protect infected cells from cytotoxic T lymphocyte (CTL) killing (9, 36, 44, 50), contributing to viral persistence *in vivo* (44). However, the molecular mechanism by which Nef downregulates MHC-I is not fully understood.

Previous reports have shown that several functional residues in Nef contribute to MHC-I downregulation. For instance, deletion of N-terminal residues 17 to 26 abolishes the Nef function (27). In particular, Met20 was found to be indispensable for MHC-1 downmodulation (2). In addition, residues 62 to 65 of Nef are critical for the ability of Nef to target MHC-I (37, 39). The residues reportedly function as a binding site for phosphofurin acidic cluster sorting protein 1 (PACS-1) (10), although this conclusion is still controversial (5, 24). Finally, the $_{69}$-PxxP-$_{78}$ region in the core domain, which is associated with its ligand molecules having an SH3 domain, is required for the function (17). Nef does not affect MHC-I synthesis and transport to the endoplasmic reticulum and the *cis* Golgi apparatus (41); hence, Nef is thought to act on MHC-1 as it traffics from the trans-Golgi network (TGN) to the plasma membrane or in the recycling pathway. The mu-1A subunit of adapter protein complex 1 (AP-1) has been shown to act as an essential factor in MHC-I downregulation (23, 40), and hypothetical models that involve complexes of Nef, mu-1A, and MHC-I have been proposed (29, 49). However, the structural basis for Nef interaction with mu-1A remains to be elucidated. In this study, we further examined the role of Met 20 in the N terminus of Nef and found that a conserved tripartite hydrophobic motif composed of Trp13 and Val16 as well as Met20 acted as a novel motif for the interaction with the tyrosine motif-binding site of the mu-1A subunit.

MATERIALS AND METHODS

Plasmid constructs. The plasmids encoding HIV-1 proviral genomes containing *nef* gene mutants were designed based on pNL4-3 (1). The Nef(−), M20A, M20R (2), Δmyr, R mutant (48), and Δ62-68 mutant (39) were described previously. The mutant Nef(−) lacks expression of Nef because of an alteration of the first ATG codon to ACC. Met20 was replaced with Ala and Arg for M20A and M20R, respectively. Δmyr lacks the signal for myristoylation by Glu-to-Ala substitution. The R mutant replaced four instances of Arg at residues 17, 19, 21, and 22 with Ala. Δ62-68 deleted residues 62 to 67.

To generate substitution mutants, we digested *env*-defective variant pNL43-K1 (7) with BamHI and XhoI, and the fragment encoding a portion of the *nef* gene was subcloned into pGEM-7zf (Promega, Japan). Based on this subcloning plasmid, we generated substitution mutants by site-directed mutagenesis using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and the following primers: for G12A, 5′-GGTCAAAGAGT AGTGTGATTGCGTGGCCAGCTG-3′ and 5′-CAGCTGGCCACGCAA TCACACTACTCTTTGACC-3′; for G12E, 5′-GGTCAAAGAGTAGTGT GATTGAGTGGCCAGCTG-3′ and 5′-CAGCTGGCCACTCAATCACA CTACTCTTTGACC-3′; for G12R, 5′-GGTCAAAGAGTAGTGTGATTC GCTGGCCAGCTG-3′ and 5′-CAGCTGGCCAGCGAATCACACTACT CTTTGACC-3′; for W13A, 5′-GTGATTGGCGCCCCTGCTGTAAGGG AAAG-3′ and 5′-CTTTCCCTTACAGCAGGGGCGCCAATCAC-3′; for W13Y, 5′-GTAGTGTGATTGGATATCCTGCTGTAAGGGAAAG-3′ and 5′-CTTTCCCTTACAGCAGGATATCCAATCACGCTGC-3′; for V16A, 5′-GTGATTGGATGGCCAGCTGCGAGGGAAAGAATGAG-3′ and 5′-CTCATTCTTTCCCTCGCAGCTGGCCATCCAATCAC-3′; for V16E, 5′-GTGATTGGATGGCCAGCTGAGAGGGAAAGAATGAG-3′ and 5′-CTCATTCTTTCCCTCTCAGCTGGCCATCCAATCAC-3′; for

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V16R, 5′-GTGATTGGATGGCCAGCTCGGAGGGAAAGAATGAG-3′ and 5′-CTCATTCTTTCCCTCCGAGCTGGCCATCCAATCAC-3′; for E18A, 5′-GATTGGATGGCCAGCTGTAAGGGCCAGAATGAG-3′ and 5′-CTCATTCTGGCCCTTACAGCTGGCCATCCAATC-3′; for E18D, 5′-GATTGGATGGCCAGCTGTAAGGGACAGAATGAG-3′ and 5′-CTCATTCTGTCCCTTACAGCTGGCCATCCAATC-3′; and for E18R, 5′-GATTGGATGGCCAGCTGTAAGGCGGAGAATGAG-3′ and 5′-CTCATTCTCCGCCTTACAGCTGGCCCATCCAATC-3′.

All constructs described above were verified by nucleotide sequencing with a BigDye Terminator cycle sequencing kit (version 1.1) and a Genetic Analyzer (ABI PRISM 3100; Applied Biosystems, Foster City, CA). The A-MLV-Env expression plasmid SA-A-MLV vector (34) and the VSV-G expression construct pCMV-G (51) were used for the production of pseudotyped viruses by cotransfection with pNL4-3 or its variants as previously described (3).

Cell culture. 293T cells were cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and antibiotics. CEM-GFP cells contain HIV-1 long-terminal-repeat-driven green fluorescence protein (GFP) cDNA, and GFP expression is inducible by Tat (14). The cells and Jurkat cells were maintained with RPMI 1640 with 10% fetal bovine serum and antibiotics.

Antibodies. In this study, we used the following antibodies: a rabbit polyclonal anti-Nef antibody (2949) and an AIDS-patient serum (provided by the AIDS Research and Reference Reagent Program, NIH), an RPE-cy5-conjugated or a nonlabeled anti-CD4 monoclonal antibody (MAb) (MT310; Dako, Japan), anti-HLA-ABC MAbs (B9.12.1, Beckman Coulter, Fullerton, CA; W6/32, ebioscience, San Diego, CA), an allophycocyanin (APC)-conjugated goat anti-mouse Ig (BD Bioscience, San Jose, CA), an anti-gamma-adaptin MAb (100/3; Sigma), an anti-NeuN MAb (A60; Millipore, Hercules, CA), and a rabbit anti-mu-1A antibody (Sigma) (28).

Transfection and preparation of viruses, cell lysates, and viral lysates and Western blotting. 293T cells were transfected with pNL4-3 or its *nef* mutants by the use of Lipofectamine 2000 (Invitrogen, San Diego, CA). Transfected 293T cells (2×10^7) and culture supernatants were harvested at 48 h posttransfection. The culture supernatants were filtered (0.45-μm-pore-size filter), quantified for p24 capsid antigen levels by enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, Buffalo, NY), and stored at −80°C. The cells were washed with phosphate-buffered saline (PBS) twice and resuspended in PBS with protease inhibitor cocktails (Complete Mini; Roche, Mannheim, Germany) and lysed with 2× sample buffer for 5 min at 95°C. For harvesting viral particles, the virus supernatants were ultracentrifuged through a cushion of 20% (wt/vol) sucrose–PBS at 110,000 × *g* for 60 min at 4°C and then lysed with 2× sample buffer for 5 min at 95°C. The protocol of Western blotting was based on a previously described method (3). To detect the Nef and p24 proteins in cell lysates and viral particles, we used anti-Nef polyclonal antibody and AIDS-patient serum, respectively. Immunoreactive proteins were visualized using chemiluminescence (Immobilon; Millipore).

Downregulation assay. CEM-GFP cells (5×10^5) were infected with viruses by spinoculation at 30°C and 1,200 × *g* for 2 h as previously described (30). The cells were immediately transferred to the T25 flask with fresh medium and cultured at 37°C. At 48 h after infection, the cells were incubated with an RPE-cy5-conjugated anti-CD4 MAb or an anti-HLA-ABC MAb, followed by treatment with an APC-conjugated anti-mouse Ig at 4°C for 30 min. The fluorescence intensity for GFP and MHC-I was detected by a FACSCalibur flow cytometer (BD Bioscience).

Immunofluorescence staining. At 24 h after infection, HIV-1-infected cells were transferred to chamber slides. Cells were incubated at 37°C for 30 min and centrifuged at 600 rpm for 5 min at room temperature. Cells were fixed with 3% paraformaldehyde at room temperature for 15 min, washed once with PBS, and incubated with a primary antibody solution overnight at 4°C. We subjected the following primary antibodies to double staining: an anti-CD4 MAb (MT310) (1:1,000), an anti-HLA-ABC MAb (W6/32) (1:5,000), and an anti-Nef polyclonal antibody (1:5,000). After one wash with PBS, cells were incubated with an Alexa 555-conjugated anti-rabbit IgG antibody or an Alexa 488-conjugated anti-mouse IgG antibody for 1 h at 4°C. After two washes with PBS and one wash with distilled water, we mounted samples on soft-mount solution (Wako). We subjected the following antibodies to triple staining: an anti-Nef MAb (1:500) labeled with a secondary antibody (Alexa 405) (1:500), stained using a Zenon labeling kit (Invitrogen); an anti-mu-1A polyclonal antibody (1:10,000); and an anti-HLA-ABC MAb (W6/32) (1:5,000). The samples were examined with a Digital Eclipse C1 confocal microscope (Nikon, Kanagawa, Japan).

Visualization of Nef N terminus based on the NMR model. Molecular structures were analyzed based on the nuclear magnetic resonance (NMR) model of the myristoylated Nef N terminus (Protein Data Bank [PDB] code 1QA5) (15). Structures were visualized using PyMol (W. L. DeLano; http://pymol.org), and hydrophobic accessible surface areas were calculated with Surface Racer (45).

Molecular modeling of the mu-1A subunit, the N-terminal region of Nef, and the complex of mu-1A with the N-terminal region of Nef. We constructed structural models of a human mu-1A protein with homology modeling, using two crystal structures of a rat AP-2 mu-2 subunit (PDB codes 1BW8 and 1HES) (32, 33) as the modeling templates, with the BioInfoBank Meta Server (http://meta.bioinfo.pl/). Homology modeling of mu-1A and structural models of the wild type (WT) and four mutants of the HIV-1 Nef N terminus (residues 9 to 26, with PDB code 1QA5 used as a modeling template) (15) was done using the SwissModel server (http://swissmodel.expasy.org/workspace/). The mu-1A model was subjected to docking with Nef N-terminal models by the use of a ASEDock module (Ryoka Systems Inc., Tokyo, Japan) (16) operated in the Molecular Operating Environment (MOE). The precision of docking results determined with the ASEDock module is generally equivalent to the experimental error value (i.e., a few angstroms); a result of dTTP docking with the ASEDock at the catalytic site in a reverse transcriptase (RT) closed configuration had a root-mean-square deviation of about 1.6 Å compared to that determined by X-ray crystallography (1RTD), which was a range within the resolution of the crystal structure (8). Potential sites of binding of mu-1A to the Nef peptides were searched with SiteFinder module and selected on the basis of analogy to the interaction between mu-2 and the YxxΦ motif (32, 33). Upon reviewing the results of the simulations, we considered the conformational flexibilities of peptide and side chains of mu-1A. Conformations of both main and side chains of the Nef peptides were randomly searched, and the Nef peptide models were automatically arranged in the binding cleft of mu-1A. Energy minimization of the peptide–mu-1A complex was performed under conditions in which the side chain atoms in mu-1A and all atoms in the peptide were not constrained but the main chain atoms in the mu-1A were tethered with 100 kcal/mol/Å^2. The AMBER ff99 force field (47) and the generalized Born/volume integral (GB/VI) implicit solvent model (22) were applied for the modeling. The three-dimensional (3D) structures were thermodynamically optimized by energy minimization using the MOE package and the same force field. A physically unacceptable local structure of the optimized 3D model was further assessed and refined on the basis of Ramachandran plot evaluation using the MOE package and 3D structure evaluation with Verify3D software (http://nihserver.mbi.ucla.edu/Verify_3D/) (26).

MD simulation. To estimate the binding affinity of a Nef N-terminal region to the mu-1A subunit, we performed molecular dynamics (MD) simulations and the subsequent binding energy calculations with AMBER 9 software (35). For the top-ranked model of the docking simulation of mu-1A and each Nef, a 1.5-ns (10^{-9} s) MD simulation was initiated at 300°K (26.85°C) with a time step of 1.0 fs (10^{-15} s). The AMBER ff99SB force field (19) and the GB implicit solvent model (IGB = 5) (31) were applied for potential energy calculations. The cutoff for long-distance-interaction energy was set at 15.0 Å, and the SHAKE algorithm was applied for bonds concerning hydrogen atoms. Subsequently, using 500 tra-

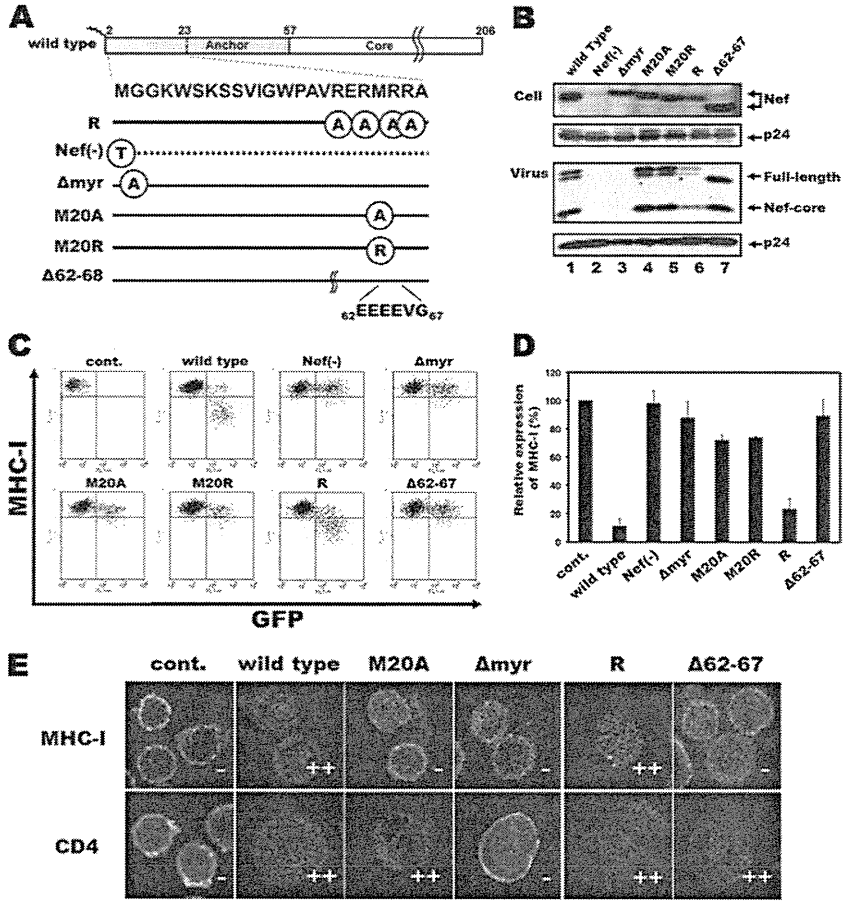

FIG 1 The basic cluster in the amino-terminal region did not contribute to the Nef function of MHC-I downregulation. (A) Schematic diagram of Nef mutants in the N-terminal region. Amino acid numbers are given above the box. The N-terminal myristoyl moiety is depicted as a zigzag line. (B) Detection of the cellular expression and viral packaging of WT and mutant Nef proteins. Upper panels, anti-Nef antibody; lower panels, AIDS-patient serum. (C) Analysis of the MHC-I downregulation by Nef mutants. CEM-GFP cells were infected with equal amounts of viruses. The cells were treated with an anti-HLA-ABC MAb, followed by staining with an APC-conjugated anti-mouse Ig. GFP-positive cells, i.e., HIV-1-infected cells, were analyzed for fluorescence intensity by flow cytometry. cont., uninfected cells. (D) The levels of MHC-I expression in the GFP-positive population. Data are shown as percentages of the MHC-I expression on the cell surface in comparison with the control as 100%. The ratios of all samples were calculated using the means of fluorescence intensity data. (E) Analysis of MHC-I and CD4 downregulation by immunofluorescence staining. At 48 h after infection, CEM-GFP cells were immunostained with MHC-I, CD4, and Nef antibodies. Green, MHC-I or CD4; red, Nef.

jectories during 1.0 to 1.5 simulations, we estimated approximated values of binding energy (ΔGb) between mu-1A and each Nef by the molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) method (21). The AMBER ff99SB force field and the PBSA methods (25) were applied for potential energy calculations. The PBSA method is more time-consuming but more accurate than the GB method. The cutoff value was applied for calculations of long-distance-interaction energy.

Coimmunoprecipitation. For immunoprecipitation, we used an anti-gamma-adaptin MAb, which was reported previously to coimmunoprecipitate the mu-1A subunit (11), or an anti-NeuN MAb, which expresses only in neural cells, as a negative control. At 20 h after HIV-1 infection, Jurkat cells were incubated with 20 mM NH_4Cl for 4 h. It was confirmed that this treatment did not influence the levels of MHC-I downregulation in the infected Jurkat cells. We harvested and lysed the cells with lysis buffer (1% digitonin, 150 mM NaCl, 50 mM Tris-HCl [pH 7.0], 1 mM $CaCl_2$, 1 mM $MgCl_2$, Complete Mini) for 20 min on ice. The lysates were centrifuged at 6,000 rpm for 1 min at 4°C, and the supernatants were recovered and precleared with 50 μl of Dynabeads (Invitrogen) for 1 h at 4°C. Also, 50 μl of beads and antibodies were mixed and incubated for 90 min at 4°C to form the bead-Ig complexes. The complexes were washed once with the lysis buffer, the precleared supernatant was added, and the mixture was incubated for 90 min at 4°C for immunoreactions. After the immunoreactions, the beads were washed 4 times with lysis buffer. The immunoprecipitated materials were eluted with 40 μl of 0.1% citrate at room temperature, 2× sample buffer was added, and the mixture was boiled for 5 min at 95°C. The samples were analyzed by Western blotting. Input controls were 1/50 of the volume of the immunoprecipitated protein. Consistent results were obtained from three independent experiments.

RESULTS

Analysis of the importance of the N-terminal region of Nef for MHC-I downregulation. A basic cluster (Arg17, -19, -21, and -22) is relatively conserved among Nef proteins of various HIV-1 subtypes. It has been shown that the basic cluster supports plasma membrane binding of myristoylated proteins by contributing electrostatic interactions with negatively charged acidic phospholipids such as phosphatidylserine and phosphatidylinositol at the cytosolic surface of the plasma membrane (38). We therefore hypothesized that Met20 and the basic cluster could cooperatively contribute to the MHC-I downregulation. To evaluate this possibility, we analyzed the function of a Nef variant referred to as the R mutant in which the Arg residues were replaced by Ala (Fig. 1A).

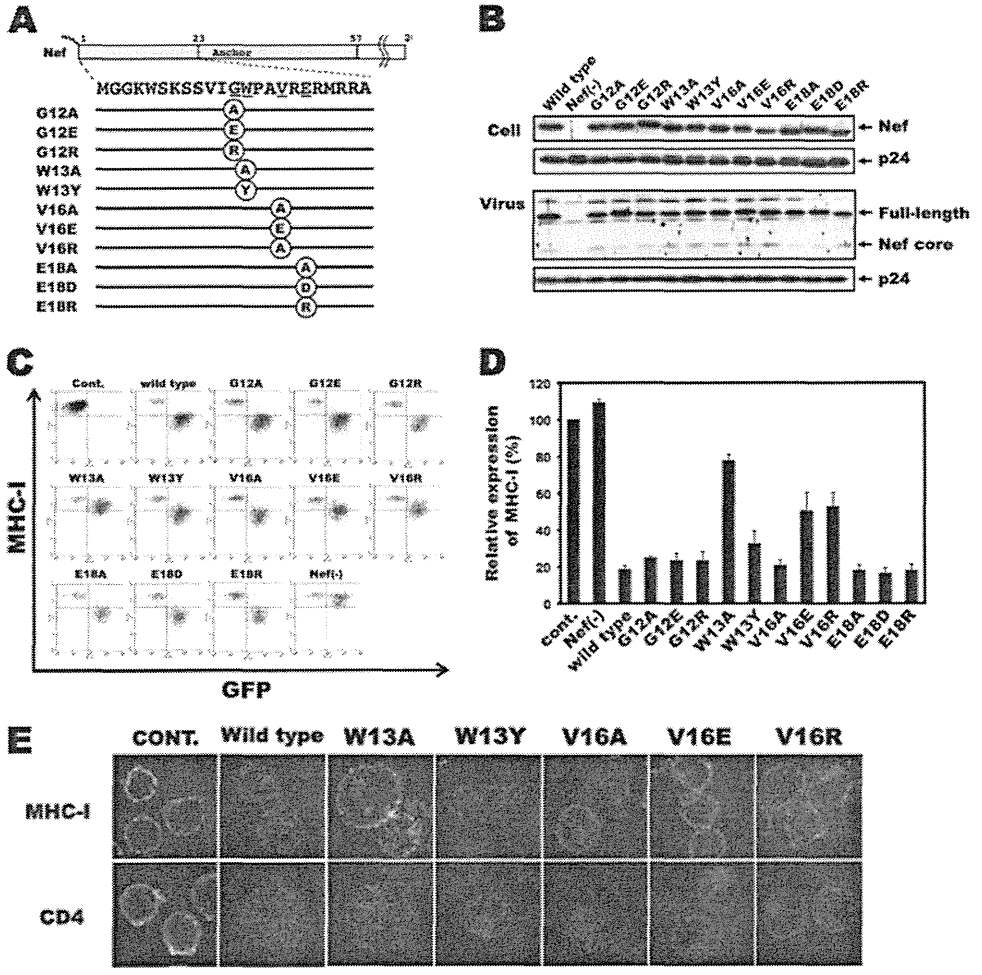

FIG 2 Conserved hydrophobic residues in the N terminus of Nef contribute to the function of MHC-I downregulation. (A) Schematic diagram of substitution mutants in the N terminus of Nef. Amino acid numbers are given above the box. (B) The cellular expression and viral packaging of WT Nef and Nef mutants. Nef and p24 CA protein were detected by Western blotting. (C) Flow cytometric analysis of the MHC-I downregulation by Nef. CEM-GFP cells were infected with the viruses as indicated and stained for MHC-I at 48 h after infection as described for Fig. 1. Cont., uninfected cells. (D) The level of MHC-I expression in the GFP-positive subpopulation. Data shown are percentages of the MHC-I expression on the cell surface in comparison with the uninfected control (cont.) as 100%. The ratios of all samples were calculated as described in the Fig. 1D legend. (E) Analysis of MHC-I and CD4 localization by immunofluorescence staining. Green, MHC-I or CD4; red, Nef.

First, we determined the expression of Nef mutants and confirmed that WT Nef and its variants were intracellularly expressed at comparable levels. Virion packaging was less efficient in the R mutant (Fig. 1B), as previously shown (48). Next, we analyzed the effect of the mutations on the MHC-I downregulation. WT Nef clearly downregulated MHC-I on the surface of the HIV-1-infected T cells, while the Nef(−), M20A, M20R, and Δ62-68 variants lost the ability to downregulate MHC-I (Fig. 1C, D, and E). Contrary to our expectations, the R mutant retained an ability to downregulate MHC-I nearly equivalent to that of the WT (Fig. 1C, D, and E). In addition, the ability of the R mutant as well as the M20A, M20R, and Δ62-68 variants to downregulate CD4 was comparable to that seen with WT Nef (Fig. 1E). These results indicate that the reduced ability of the R mutant to incorporate into virions, which may be due to inefficient association with acidic phospholipids of the plasma membrane via electrostatic interactions as described previously (6), had no detectable effect on the MHC-I-regulatory function of Nef.

The N-terminal region of Nef has a number of conserved amino acid residues other than the basic cluster. It is conceivable that, in addition to Met20, they could cooperatively contribute to the Nef activity. In order to examine this possibility, we selected four residues as candidates, i.e., Gly12, Trp13, Val16, and Glu18, which are adjacent to Met20 and are relatively conserved among various HIV-1 subtypes. These residues were replaced with residues of a different electric charge or length of side chain (Fig. 2A). It was confirmed that the characteristics of expression as well as virion incorporation of these mutants were comparable (Fig. 2B). Interestingly, replacement of Trp13 by Ala but not by Tyr and of Val16 by Glu or Arg but not by Ala affected the Nef effect (Fig. 2C, D, and E). All these mutants were comparable to WT Nef in their ability to downregulate CD4 (Fig. 2E). Moreover, it was confirmed that double (W13A/V16R) and triple (W13A/V16R/M20A) substitution mutants of Nef also lost the ability to downregulate MHC-I but remained active with respect to CD4 (data not shown). Taken together, our data indicated that, in addition

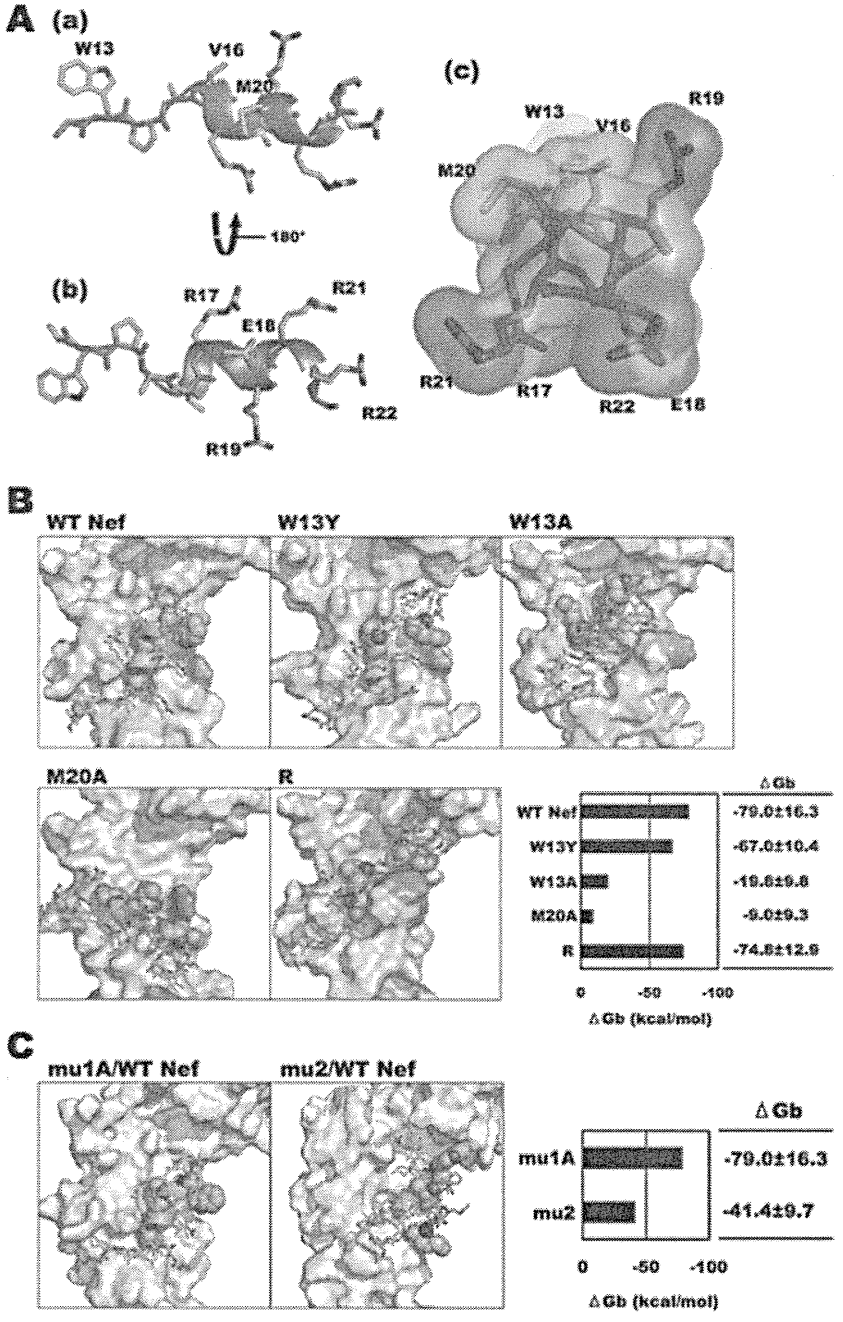

FIG 3 The three amino acids may form the site of binding to mu-1A, as shown by simulation *in silico*. (A) Molecular structure showing residues 13 to 23 of Nef taken from the NMR structure of myristoylated HIV-1 Nef residues 1 to 56 (PDB code 1QA5). Atoms are indicated as follows: red, oxygen atoms; blue, nitrogen atoms; green, carbon atoms; yellow, sulfur atoms; gray, main chain atoms (N, Cα, C, O). The helix conformation is indicated by ribbon representations. (a) and (b) represent side views rotated 180°. (c) illustrates a front view obtained by rotating (a) 90° around its short axis. (B) Final structures of each MD simulation. Atoms of mu-1A are represented by white surfaces, and atoms of Nef are shown in green. The 13th, 16th, and 20th amino acids in the Nef are highlighted with sphere representations. The Phe18 and Trp240 in mu-1A are highlighted in orange. Calculated binding energies between mu-1A and Nef mutants are indicated. (C) Summary of binding mode of mu-1A or mu-2 with WT Nef and calculated binding energies between mu-1A/mu-2 and Nef mutants.

to Met20, the Trp13 and Val16 residues in Nef were critical for the MHC-I downregulation.

The significance of the tripartite residues for MHC-I downregulation. In order to get insights into the structural properties of the tripartite Trp13, Val16, and Met20 (WVM) motif in Nef, we projected our results onto the NMR structure of the myristoylated Nef N terminus (PDB entry 1QA5) (15). Residues Val16 and Met20 were located on the same side of the N-terminal α-helix of Nef. In contrast, Arg17, -19, -21, and -22 residues were located on the opposite side of the α-helix, thus creating a basic surface. Trp13 was positioned immediately upstream of the N-terminal helix, with its hydrophobic side chain pointing in the same direction as those of Val16 and Met20 (Fig. 3A). Together, Trp13, Val16, and Met20 formed a hydrophobic surface of about 375 Å^2.

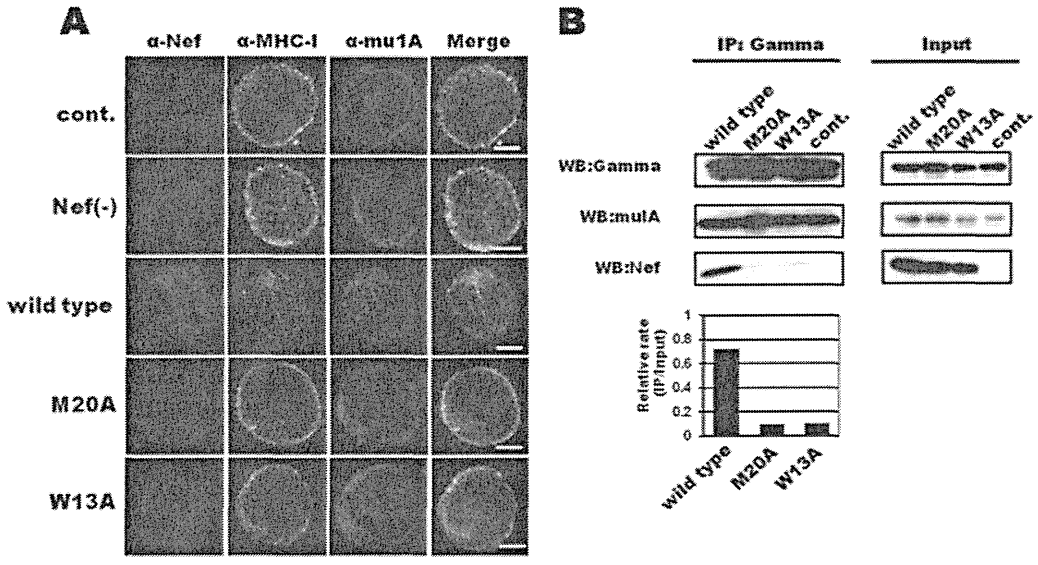

FIG 4 Two amino acids at the N terminus of Nef contribute to binding with mu-1A. (A) Immunofluorescence staining. Cells were stained with Nef, MHC-I, and mu-1A antibodies and analyzed by confocal microscopy. Blue, Nef; green, MHC-I; red, mu-1A. Bars, 10 μm. (B) Analysis of interaction of Nef proteins with mu-1A by coimmunoprecipitation. Jurkat cells infected with viruses were lysed at 24 h after infection and immunoprecipitated with an anti-gamma-adaptin MAb. The samples were detected by Western blotting using Nef and mu-1A antibodies. Immunoprecipitated samples of uninfected cells are shown as controls. Input controls were 1/50 of the volume of the immunoprecipitated protein.

These results may explain why the basic cluster adjacent to the Met20 comprised of Arg17, -19, -21, and -22 is dispensable for MHC-1 downregulation.

The mu-1A subunit of AP-1 has been shown to recognize binding motifs in the cytoplasmic tail of cargo proteins and binds them with the binding pockets. One of sorting motifs is an acidic dileucine motif (ED]xxxL[L), and the other is a tyrosine motif (YxxΦ; Φ represents bulky hydrophobic amino acids, which can be phenylalanine, isoleucine, leucine, methionine, or valine) (6a). Considering our finding that the replacement of Trp13 by Tyr but not Ala maintained the Nef effect on MHC-1 downregulation (Fig. 2), we hypothesized that Trp13 and Val16 could act as a noncanonical mu-1A-binding motif. To test this, we undertook docking simulations in terms of the interaction between the tyrosine motif-binding site of the mu-1A subunit and the N-terminal region of Nef. Based on the NMR structures of these proteins, we simulated the docking of the N-terminal region of Nef with mu-1A (Fig. 3B). An aromatic ring of the Trp13 of WT Nef was specifically placed in the front face of two aromatic rings of Phe18 and/or Trp 240 in mu-1A, contributing to hydrophobic interactions with the Nef N-terminal region. Protruding side chains of Met20 and Val16 of WT Nef were specifically embedded in a bowl shape of the hydrophobic cavity in the binding surface of mu-1A. In contrast, side chains of Arg17, -19, -21, and -22 residues were positioned in the direction opposite the mu-1A-binding surface. In addition, these hydrophobic interactions were well preserved in W13Y and R but not in W13A and M20A. Consequently, the binding configurations of these molecules were similar in the WT, W13Y, and R but not in W13A and M20A (Fig. 3B).

We then evaluated the influence of mutations on the binding free energy, ΔG_b, which indicates the binding strength between Nef and mu1A (Fig. 3B). The ΔG_bs of W13Y and the R mutant were nearly equal to that of the WT, whereas those of W13A and M20A were much lower, consistent with interaction modes of these variants in the structural models. These structural data were consistent with the results from the functional analyses presented in Fig. 2 and support our hypothesis that Trp13 and Val16 can form a noncanonical mu-1A-binding motif. Of note, the results from the interaction model showed that the hydrophobic motif of the WT Nef fitted only poorly onto mu-2, the subunit of AP-2, because of a poor fit of Met20 onto the mu-2-binding surface. Consequently, the ΔG_b between the N-terminal region of Nef and mu-2 was much lower than that of Nef and mu-1A (Fig. 3C). These results may explain why Met20 is critical for the selective binding of Nef to mu-1A, and the result is consistent with our finding that the N terminus of Nef is important for the downregulation of MHC-I but not of CD4.

The results of the computer simulation strongly suggest that the tripartite WVM motif contributes to the interaction of Nef with mu-1A. We further analyzed the intracellular localization in order to ascertain results from the docking simulation analyses. Immunofluorescence staining demonstrated that WT Nef but not M20A or W13A significantly colocalized with MHC-I and mu-1A and reduced the surface expression of MHC-I (Fig. 4A). Moreover, we sought to confirm the interaction of Nef with mu-1A by coimmunoprecipitation (Fig. 4B). Comparable levels of gamma and mu-1A subunits of the adapter complex as well as Nef were detected among the samples harvested from the Jurkat cells expressing WT Nef and its variants. Importantly, an anti-gamma adaptin antibody coprecipitated mu-1A irrespective of the expression of Nef due to the formation of a hemicomplex as subunits of AP-1, and the antibody also coprecipitated WT Nef but not the M20A and W13A mutants.

DISCUSSION

In the present study, we addressed the question of how the amino acids in the N terminus of Nef functioned for MHC-I downregu-

lation. We show here for the first time that Nef specifically interacts with the mu-1A subunit of AP-1 through the WVM domain and that Trp13 and Val16, in addition to Met20, are involved as a novel motif, which was evident from the present data obtained from functional, biochemical, structural, and *in silico* analyses.

We show here that the hydrophobic motif composed of Trp13, Val16, and Met20 interacts with mu-1A at the tyrosine motif-binding site. It is notable that Trp13 is highly conserved among HIV-1 subtypes whereas Val16 and Met20 are not necessarily conserved and V16I and M20I substitutions are also observed. In accordance with this point of view, we constructed structural models of mu-1A docked to the Nef N-terminal peptides containing a W13F, W13K, W13I, V16I, or M20I substitution. The models suggest that these peptides exert binding configurations comparable to that of wild-type Nef peptide, except for M20I, which appears to exhibit a substantial influence compared with the others (data not shown). Importantly, HIV-1 subtype C and simian immunodeficiency virus cpz (SIVcpz) Nef clones with the M20I substitution have been demonstrated to be functional in terms of the ability to downregulate MHC-I (18, 43, 46). Taken together, our results indicate that the hydrophobic patch interacts with mu-1A at the YXXΦ-binding site in a noncanonical manner and that the substitutions may not affect the MHC-I-regulatory function, which in turn implies that the highly conserved Trp13 could play a critical role for another function in Nef. These issues remain to be further elucidated.

The unique positioning of Met20 in the tripartite hydrophobic motif compared with the canonical tyrosine motif indicates that it is a critical residue to compensate for the absence of Tyr at position 13 and to selectively interact with mu-1A. Consistent with this notion, mu-2 showed much lower free binding energy than mu-1A due to the absence of an appropriate pocket structure such as is required for the interaction with the side chain of Met20 (Fig. 3C). It has been reported that Nef downregulates both MHC-I and CD4 through interaction with AP-1 and AP-2, respectively, and that a dileucine motif and a diacidic motif in the C terminus of Nef interact with the mu-2 subunit of AP-2 (12), while the molecular basis for the selective interaction of Nef with AP-1 and AP-2 remains to be elucidated. The present data showing that the hydrophobic motif in the N terminus of Nef specifically interacts with mu-1A of AP-1 explain the molecular mechanism for the differential effects of Nef on MHC-I and CD4.

Collins's group demonstrated that M20 was critical for the association of Nef with mu-1 as well as MHC-I downregulation (40, 49), which is consistent with our present results. In contrast, Singh et al. indicated in their report that M20 had little or no effect on the binding of Nef with mu-1 (42). This discrepancy may be due to differences in the assay systems used for detection of binding of Nef and mu-1, i.e., we as well as Collins's group employed T lymphocytes expressing Nef in a coimmunoprecipitation study as well as an MHC-I downregulation assay, whereas Singh et al. used an MHC-I-CD-Nef chimera and mu-1 expressed in *Escherichia coli* in an *in vitro* binding assay. We believe that the former approach should better represent the physiological machinery by which Nef exerts intrinsic activity.

In this study, we have found that the N-terminal region around the Met20 residue in Nef includes two separable functional motifs: an Arg-rich basic cluster and a noncanonical mu-1A-binding motif. Albeit overlapping in the primary structure, the two motifs were separable on the 3D structure of this region, as shown in Fig. 3. Previous reports have shown that the basic cluster supports plasma membrane binding of the myristoylated protein, including Src, MARCKS, and HIV-1 Gag, by contributing electrostatic interactions with negatively charged acidic phospholipids such as phosphatidylserine and phosphatidylinositol at the cytosolic surface of the plasma membrane (38) and also that the *N*-myristoylation is essential for the Nef-mediated biological activities, including MHC-I downregulation (2). We therefore hypothesized that Met20, together with the basic cluster, could play a critical role for the MHC-I downregulation. However, contrary to our assumption, the basic cluster was dispensable for the ability of Nef to downregulate MHC-I (Fig. 1), suggesting that that ability is independent of the association of Nef with phospholipids. Since Nef protein tends to localize to both the plasma membrane and perinuclear compartment (13), it is still possible that the myristoyl moiety could contribute to the MHC-I downregulation by way of association with the TGN or endosomes. This is in agreement with a previous report showing that the assembly of a complex that includes Nef, MHC-I, and AP-1 early in the secretory pathway is important for the MHC-I downregulation (20).

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ORIGINAL PAPER

Lineage-specific evolution of T-cell immunoglobulin and mucin domain 1 gene in the primates

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Abstract T-cell immunoglobulin domain and mucin domain containing protein 1 (TIM1), also known as a cellular receptor for hepatitis A virus (HAVCR1) or a molecule induced by ischemic injury in the kidney (KIM1), is involved in the regulation of immune responses. We investigated a natural selection history of *TIM1* by comparative sequencing analysis in 24 different primates. It was found that *TIM1* had become a pseudogene in multiple lineages of the New World monkey. We also investigated T cell lines originated from four different New World monkey species and confirmed that *TIM1* was not expressed at the mRNA level. On the other hand, there were ten amino acid sites in the Ig domain of TIM1 in the other primates, which were suggested to be under positive natural selection. In addition, mucin domain of TIM1 was highly polymorphic in the Old World monkeys, which might be under balanced selection. These data suggested that *TIM1* underwent a lineage-specific evolutionary pathway in the primates.

Keywords Natural selection · Molecular evolution · Pseudogene · TIM1 · Primate

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Introduction

Comparative genomics is a useful tool for understanding the gene function from the view point of evolution. It has recently been reported that genes involved in regulation of immune system may have undergone the control of positive selection (Gibbs et al. 2007; Kosiol et al. 2008). The accelerated evolution may be due to a direct consequence of complex selection pressure exerted by infectious reagents including microbes and viruses (Barreiro and Quintana-Murci 2010). The known cases include genes of defensin family, which play crucial roles in antibacterial activity (Hollox and Armour 2008), and genes of APOBEC family, which are known to function as specific inhibitors against the infection of human immunodeficiency virus-1 (HIV-1) (Sawyer et al. 2004).

We previously performed a comparative genome analysis of primates and reported that genes encoding the immunoglobulin superfamily (IgSF) were classified into 11 functional categories based on the Gene Ontology (GO) database. The IgSF genes in three functional categories, immune system process (GO:0002376), defense response (GO:0006952), and multi-organism process (GO:0051704), had more chance to be under the positive natural selection than the IgSF genes in the other categories (Ohtani et al. 2011). In our previous comparative genome analysis, we focused on the orthologous

IgSF genes that appeared to be functional in all of human, chimpanzee, orangutan, rhesus macaque, and common marmoset. In other words, we excluded several genes of which an ortholog was considered to be non-functional, i.e., deleted gene, grossly rearranged gene, or pseudogene, in any of the five primate species. Such a lineage-specific destruction of IgSF genes, especially those involved in the immune response, may be interesting in view of the natural selection occurred during the evolution of primates. One of the excluded genes in our previous analysis was a gene for T-cell Ig domain and mucin domain containing protein 1 (TIM1), which was suggested to be a pseudogene due to an insertion in the common marmoset, while it should be functional in the other primates. TIM1 tightly linked to immune system, playing an important role in generation and/or maintenance of the balance between T helper 1 (Th1) cells and T helper 2 (Th2) cells, and it is up-regulated in Th2 cells after activation and interacts with its ligand expressed on antigen-presenting cells (de Souza and Kane 2006). *TIM1* can be found in the non-primate mammals including mouse and rat. However, *TIM1* orthologs are not found in the non-mammalian vertebrates such as chiken and zebrafish, implying that it might be involved in the mammalian-specific function. In addition, it was reported that *TIM1* is highly polymorphic in humans, but quite less polymorphic in chimpanzees, especially around the mucin domain (Nakajima et al. 2005). These observations suggested a unique evolutionary feature of *TIM1* in the primates.

In human, *TIM1* located on chromosome 5 at band q33 contains two distinct domains (Ig domain and mucin domain) (Khademi et al. 2004). It is known that TIM1 is a cellular receptor for hepatitis A virus (HAVCR1) in human (Feigelstock et al. 1998). TIM1 is also known to be induced in the kidney by ischemic injury and is called as kidney injury molecule 1 (KIM1) (Ichimura et al. 1998). It has been reported that *TIM1* polymorphisms are associated with various immune-related diseases and infectious diseases, including asthma, allergic rhinitis, atopic dermatitis, multiple sclerosis, type 1 diabetes, rheumatoid arthritis, AIDS, and cerebral malaria (Khademi et al. 2004; Kuchroo et al. 2003; McIntire et al. 2004; Meyers et al. 2005b; Su et al. 2008; Wichukchinda et al. 2010). These data implied that variations in *TIM1* might have been more or less selected during the evolution of humans.

In the present study, we determined nucleotide sequences of exons or equivalent regions of *TIM1* from 24 different primate species, including eight hominoids, six Old World monkeys, nine New World monkeys, and one prosimian, to investigate an evolutionary history of *TIM1*.

Materials and methods

Subjects

DNA samples from 24 primate species including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), western gorilla (*Gorilla gorilla*), Bornean orangutan (*Pongo pygmaeus*), western black-crested gibbon (*Nomascus concolor*), lar gibbon (*Hylobates lar*), siamang (*Symphalangus syndactylus*), rhesus macaque (*Macaca mulatta*), long-tailed macaque (*Macaca fascicularis*), Hamadryas baboon (*Papio hamadryas*), mantled Guereza colobus (*Colobus guereza*), dusky leaf monkey (*Trachypithecus obscurus*), silver leaf monkey (*Trachypithecus cristatus*), Geoffroy's spider monkey (*Ateles geoffroyi*), white-fronted spider monkey (*Ateles belzebuth*), tufted capuchin (*Cebus apella*), common squirrel monkey (*Saimiri sciureus*), white-lipped tamarin (*Saguinus labiatus*), golden-handed tamarin (*Saguinus midas*), cotton-top tamarin (*Saguinus oedipus*), golden lion tamarin (*Leonthopithecus rosalia),* common marmoset (*Callithrix jacchus*), and Sunda slow loris (*Nycticebus coucang*) were the subjects.

Polymerase chain reaction (PCR) and sequencing analysis

Sequence information for homologous regions to the coding regions of human *TIM1* was obtained from 24 primate species by direct sequencing of PCR products from the genomic DNA samples. Primers used for PCR and direct sequencing were designed by referring the human, chimpanzee, rhesus macaque, common marmoset gene sequences, and whole-genome shotgun sequences from prosimians deposited in the UCSC Genome Browser and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supplemental Table S1). PCR condition was composed of a denaturing step (94 °C for 2 min), 35 cycles of chain reaction (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min), and a final extention step (72 °C for 5 min). The PCR products were then purified and sequenced by the BigDye Terminator cycling system using an ABI3130x automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Editing and assembly of sequences were done by using SEQUENCHER (Gene Codes, Ann Arbor, MI, USA). When sequence variations (heterozygous sequences) in a specific species were detected, the sequences which were more conserved among 24 primate species were considered as ancestral sequences and used for statistical analyses. The *TIM1* sequences determined in this study were deposited in DNA Data Bank of Japan (DDBJ) (Supplemental Table S2).

Expression analysis of *TIM1*

Total RNA was extracted from a human T cell line, Jurkat, and four different T cell lines originated from the New World monkeys, HSF-10 (tufted capuchin), HSQ-115 (common squirrel monkey), HST-3 (white-lipped tamarin) (Akari et al., manuscript in preparation), and HSCj-109 (common marmoset) (Hohjoh et al. 2009), by using RNAiso (TaKaRa Bio Inc., Shiga, Japan). Extracted RNAs (500 ng) were subjected to reverse transcription (RT) by using RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan). Aliquots of RT products were used for the expression analysis of *TIM1*. Primers for PCR were designed in the highly conserved regions of *TIM1* among human and the New World monkeys (Supplemental Table S3). PCR condition was the same as that described in the previous section.

Diversity of TIM1 mucin domain in the Old World monkeys

Nucleotide sequences for the mucin domain from eight samples of rhesus macaques were determined by sequencing of PCR products, which were cloned into pT7Blue Blunt vector and transformed Nova Blue Single Competent cells using the Perfectly Blunt cloning kit (Novagen Inc., Madison, WI). Colony PCR was used to identify positive clones, and at least 20 positive clones from each sample were subsequently sequenced as described previously. We also examined length variations of exon 4 from 16 rhesus macaques and 10 crab-eating macaques by direct sequencing of the PCR products.

Statistical analyses

We used both Bn-Bs program and PAML program as described previously (Ohtani et al. 2011). In brief, the Bn-Bs program was used to investigate the presence of branch-specific positive selection (the branch model). The Bn-Bs program estimates the values of non-synonymous substitution rate (dn) and synonymous substitution rate (ds) based on the modified Nei–Gojobori method, where a phylogenetic tree is given (Zhang et al. 1998). The value of ω, an abbreviation for the value of dn/ds, is a criterion of natural selective pressure acting on the gene, and the modified Nei–Gojobori method has been used for estimating non-synonymous/synonymous substitution rates (Nei and Gojobori 1986). Statistical significance of the difference between dn and ds was examined by *Z*-test (Chatterjee et al. 2009). An ordinary least-squares method was used to estimate branch lengths and variances for *Z*-test. The least-squares method gives estimates for evolutionary distances among the analyzed sequences (Rzhetsky and Nei 1993). The PAML program version 4.7 was used to investigate the presence of site-specific positive selection (the site model). The site model treats ω allowing the variance among codons (Yang 2005; Yang and Nielsen 2000), and the following null and alternative models were implemented in the site model: M0 (null), M1a (nearly neutral), M2a (positive selection) (Wong et al. 2004), M3 (discrete), M7 (beta), and M8 (beta and ω) (Yang and Nielsen 2000). The likelihood ratio tests (LRT) of three pairwise comparisons, i.e., comparisons of M1a vs. M2a, M1a vs. M3, and M7 vs. M8, determined whether particular models would provide a significantly better fit. When the LRT suggested positive selection, the Bayes empirical Bayes (BEB) method was used to detect the sites under the positive selection (Yang et al. 2005). To investigate a possible selection operated on exon 4 region of *TIM1* alleles in rhesus macaques, we calculated Tajima's *D* (Tajima 1989; Tamura et al. 2011).

Results

TIM1 is non-functional in several lineages of New World monkey

TIM1 is a member of *TIM* gene family composing of *TIM1*, *TIM3*, and *TIM4*, in the human genome (Khademi et al. 2004). In the previous comparative genome analysis, we searched for orthologous genes for human *TIM1*, *TIM3*, and *TIM4* in the genome of chimpanzee, orangutan, rhesus macaque, and common marmoset by using the UCSC/MULTIZ alignment program. It was found that there was an insertion of 205 bp in exon 2-equivalent region in the common marmoset gene, which would generate multiple frameshift/nonsense mutations in the coding sequence and/or destroy the splicing junction.

To confirm the presence of deleterious insertion in *TIM1* in the genome of common marmoset and possibly in other primate genomes, we determined nucleotide sequences for exons or equivalent regions of *TIM1* from 24 primate species including human, chimpanzee, orangutan, rhesus macaque, and common marmoset. For this purpose, we designed primers by referring the known *TIM1* sequences (Supplemental Table 1). The sequencing analysis of the genomic gene for *TIM1* revealed the deleterious insertions of 206–212 bp in several New World monkeys, i.e., golden lion tamarin (212 bp), cotton-top tamarin (206 bp), white-lipped tamarin (207 bp), and golden-handed tamarin (210 bp) (Supplemental Figure S1). It was speculated that the insertion had been occurred within a sequence stretch of 13 bp, AGCCTCATCCTAC, corresponding to codons 9–13, because these sequences were repeated and flanked the insertion in the genomes of common marmoset and cotton-top tamarin, and there were a few substitutions in this sequence stretch from the other New World monkeys (Supplemental Figure S1). The inserted sequences belong to the

LINE/L1 (L1PA7) repeat, which contain a poly A stretch at one end in a reverse orientation to the TIM1 coding sequences, and homologous sequences can be found as multiple copies in the marmoset genome. On the other hand, we found nucleotide substitutions in exon 3-equivalent regions, which resulted in termination mutations in three other New World monkeys not carrying the insertion, common squirrel monkey (S84X), tufted capuchin (V22X), and Geoffroy's spider monkey (C36X) (Fig. 1). Among the New World monkey species investigated in this study, only the white-fronted spider monkey appeared to carry a functional gene for TIM1.

To investigate whether *TIM1* was non-functional in the New World monkey lineages, we performed RT-PCR analysis of mRNA expression in T cell lines originated from human, tufted capuchin, squirrel monkey, white-lipped tamarin, and common marmoset. As illustrated in Fig. 2a, two pairs of primers were used in the RT-PCR analysis, where forward primers were designed in exon 3 and junction of exon 5–exon 6, while reverse primers were designed in exon 9 and junction of exon 5–exon 6 (Supplemental Table S3). The *TIM1* expression was confirmed in the human T cell line, but could not be detected in the T cell lines from the New World monkeys carrying either the insertions (common marmoset and white-lipped tamarin) or the nonsense mutations (common squirrel monkey and tufted capuchin) (Fig. 2). No expression of *TIM3* and *TIM4* was observed in the T cell lines from human and the New World monkeys (data not shown).

Positive selection sites of *TIM1* in the primates

In the other primate species than the New World monkey, *TIM1* appeared to be functional, and there were many substitutions. When we calculated the dn and ds values in each primate lineage, it was found that the dn values were higher

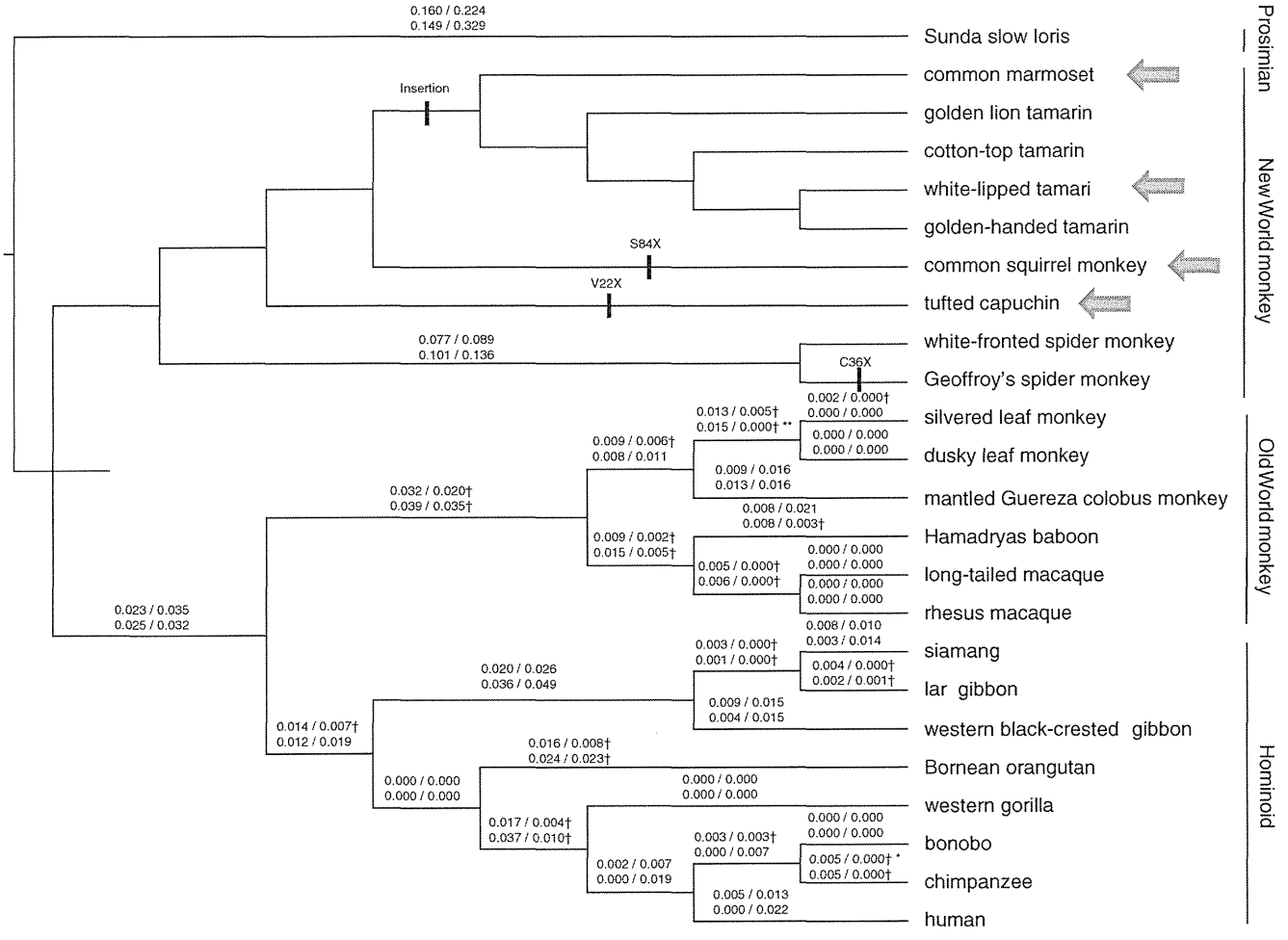

Fig. 1 Phylogenetic trees for TIM1 in the primate evolution. Values above branches indicate estimated values of dn and ds per lineage by using the Bn-Bs program. Upper values are for the entire coding region, while lower values are for the Ig domain. *Daggers* indicate that the dn value was higher than the ds value. *Asterisks* indicate that there is a significant difference between the dn and ds values ($^{**}p<0.01$; $^{*}p<0.05$; *Z*-test). *Vertical lines* indicate that *TIM1* had become pseudogene in the specific lineage. *Arrows* indicate the species for which the mRNA expression of *TIM1* in T cell line was investigated

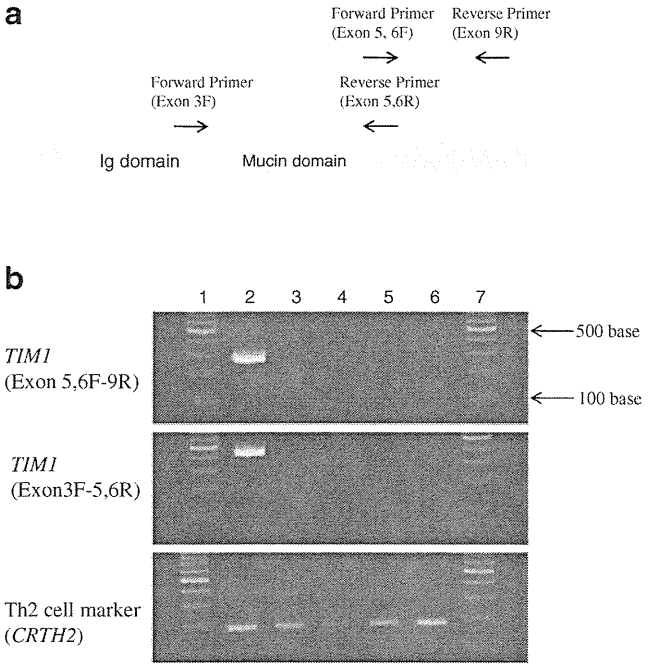

Fig. 2 Expression of *TIM1* in T cell lines. **a** TIM1 cDNA is schematically shown. *Arrows* indicate the regions of PCR primers which were designed in the highly conserved coding regions of *TIM1* among human and New World monkeys. **b** From *left* to *right*: *lane 1* 100-bp ladder size marker, *lane 2* Jurkat (human), *lane 3* HSF-10 (tufted capuchin), *lane 4* HSQ-115 (common squirrel monkey), *lane 5* HST-3 (white-lipped tamarin), *lane 6* HSCj-109 (common marmoset), and *lane 7* 100-bp ladder size marker. A marker gene (*CRTH2*) was used as a positive control of gene expression because it is known to be expressed in T cells especially in Th2-type cells

than the ds values in several lineages, especially in the Old World monkeys (Fig. 1). Sequence alignment of TIM1 at the amino acid (AA) level in the primates is shown in Fig. 3. To identify possible target sites for positive selection, we analyzed the TIM1 sequences by the BEB method. Sixteen TIM1 sequences from primate species, human, chimpanzee, bonobo, western gorilla, Bornean orangutan, western black-crested gibbon, lar gibbon, siamang, rhesus macaque, long-tailed macaque, Hamadryas baboon, mantled Guereza colobus, dusky leaf monkey, silver leaf monkey, white-fronted spider monkey, and Sunda slow loris, were used in the statistical test. It was revealed that 14 AA sites, at 23, 25, 30, 37, 51, 54, 58, 59, 72, 93, 102,120, 125, and 288 positions equivalent to the human TIM1, were the positively selected sites. These 14 AA sites were highly variable among the 16 primate sequences, e.g., AA site at 23 was lysine in hominoid; asparagine in rhesus macaque, long-tailed macaque, Hamadryas baboon, and mantled Guereza colobus; tyrosine in dusky leaf monkey and silver leaf monkey; serine in white-fronted spider monkey; and glutamine in Sunda slow loris. Most of the positive selection sites (10/14: 71.4 %) were found in the Ig domain (Fig. 3a, b).

Diversity of TIM1 in the Old World monkey

As shown in Fig. 3, a large number of deletion/insertion events were observed in the mucin domain (Fig. 3b, c). It was reported that human TIM1 exhibited a high degree of amino-acid variability in the mucin domain (Nakajima et al. 2005). Because the mucin domain of TIM1 encoded by exon 4 might be under the positive selection in the Old World monkey (Supplemental Table S4), we investigated the diversity of TIM1 in rhesus macaques by determining nucleotide sequences for the mucin domain from eight samples (16 haplotypes). As shown in Fig. 4, a high level of sequence diversity with multiple insertion/deletion of 18-bp sequences, A(T/C)GACAAC(G/A)(A/G)C(T/C)CT(T/G)CCA forming a part of AA stretch Thr-Thr-Thr-Thr-Leu-Pro (TTTTLP), was observed in the mucin domain of rhesus TIM1. We then investigated a possible selection by using the Bn-Bs program, but no statistically significant data were obtained, presumably because the compared sequences were not long enough to give a definite conclusion. However, when we calculated Tajima's *D* for these *TIM1* alleles, a value of 0.607 was obtained, which suggested a balanced selection of polymorphisms in the mucin domain of TIM1 in the Old World monkey.

On the other hand, because the diversity of mucin domain in the rhesus macaques could be detectable as a length diversity of exon 4, we examined length variations of *TIM1* exon 4 in additional samples of rhesus and long-tailed macaques. It was found that the length polymorphism was due to the repeat number polymorphisms or insertion/deletion polymorphisms of 18-bp unit and its components of 3- and 6-bp repeats (Supplemental Table S5).

Discussion

In this study, we investigated sequence diversity in the protein coding exons of *TIM1* from various species by direct sequencing method. Although there were a few sites with heterozygous sequences, we used "evolutionary conserved" sequences obtained from each sample in the statistical analyses so that the substitutions were underestimated in this study. Even though there was an underestimation, we demonstrated that the Ig domain of TIM1 has been under the positive selection during the course of primate evolution. Another interesting finding was that TIM1 has undergone pseudogene evolution in the New World monkey. It was suggested that the generation of pseudogene had occurred several times over the New World monkey lineages, by the insertion of deleterious sequences or base substitutions leading to a termination codon. Similar natural selection pattern was reported for type 1 vomeronasal receptors (*V1RL*), in which the pseudogene generation had independently

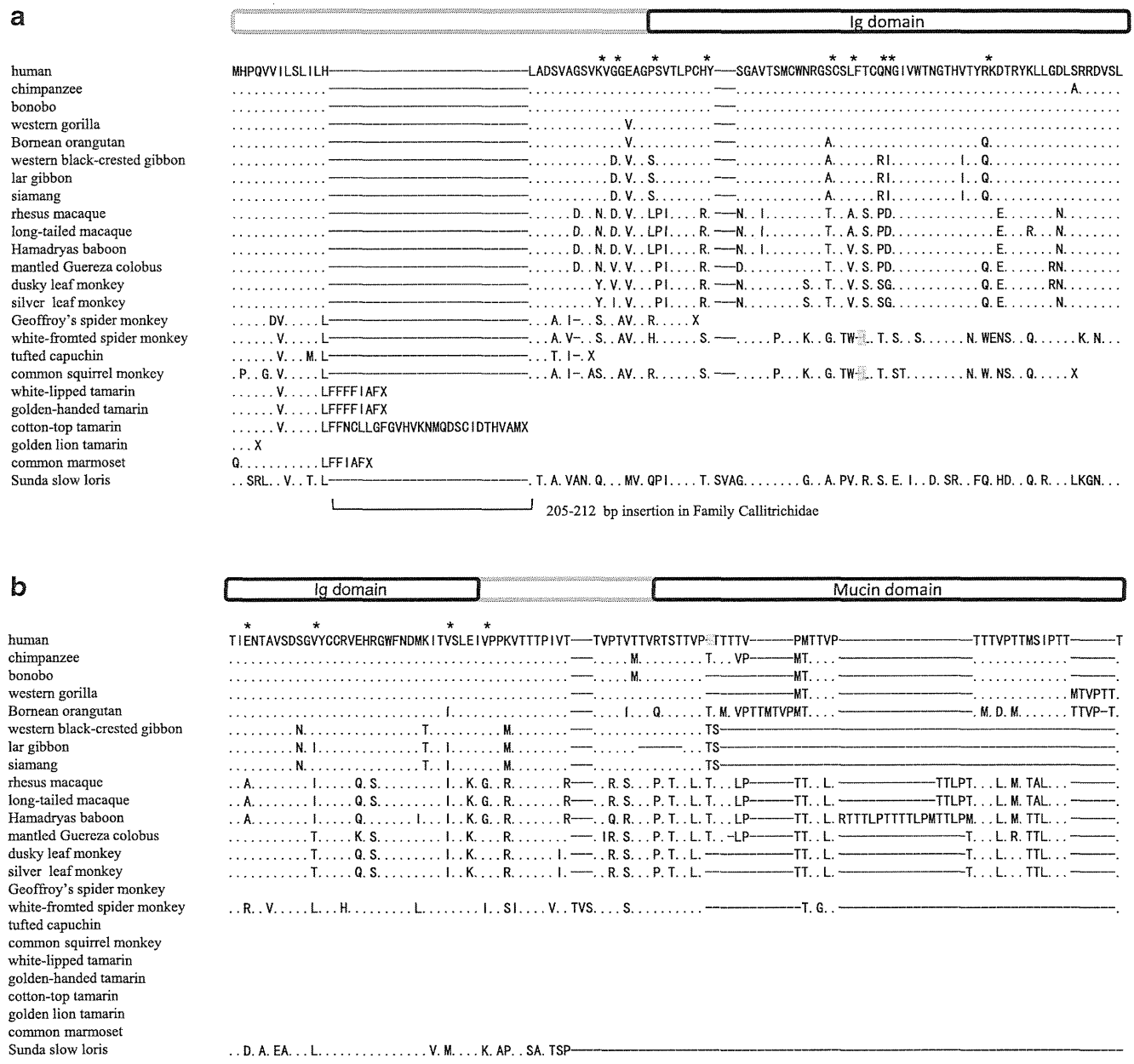

Fig. 3 Alignments of TIM1 amino acid sequences from 24 primate species. *Dots* indicate identities to the human reference sequence, while *dashes* indicate alignment gaps. *Asterisks* indicate AA sites identified as being under the significant positive selection ($p<0.05$). **a**, **b**, **c**, and **d** represent quarter parts of TIM1 from N-terminus to C-terminus. Ig and mucin domains are schematically indicated

occurred in the human and other primate lineages, implying that the function of *VIRL* might be highly lineage specific (Mundy and Cook 2003).

TIM1 plays an important role in generation and/or maintenance of the balance between Th1 and Th2 cells (Su et al. 2008). It is known that a natural ligand for TIM1 is T-cell Ig domain and mucin domain containing protein 4 (TIM4) (Meyers et al. 2005a), and the interaction of TIM1 with TIM4 plays a crucial role in sustaining the polarization status of Th2 cells (Khademi et al. 2004; Mariat et al. 2005). Because TIM1 expressed on the surface of Th2 cells regulated the immune response by modulating cytokine production in mammals, the Th1/Th2 balance might be skewed or affected by the lack of TIM1 in the most lineages of New World monkey. Previous study demonstrated that expression of Th1-type cytokines, IFN-γ and TNF-β, was considerably lower than that of Th2-type cytokines, IL-4 and IL-10, in a New World monkey, owl monkey (Pico de Coana et al. 2004). This observation may support the disturbance of Th1/Th2 balance in the owl monkey lacking TIM1, although we cannot exclude a possibility that some other molecules than TIM1 might regulate Th1/Th2 balance

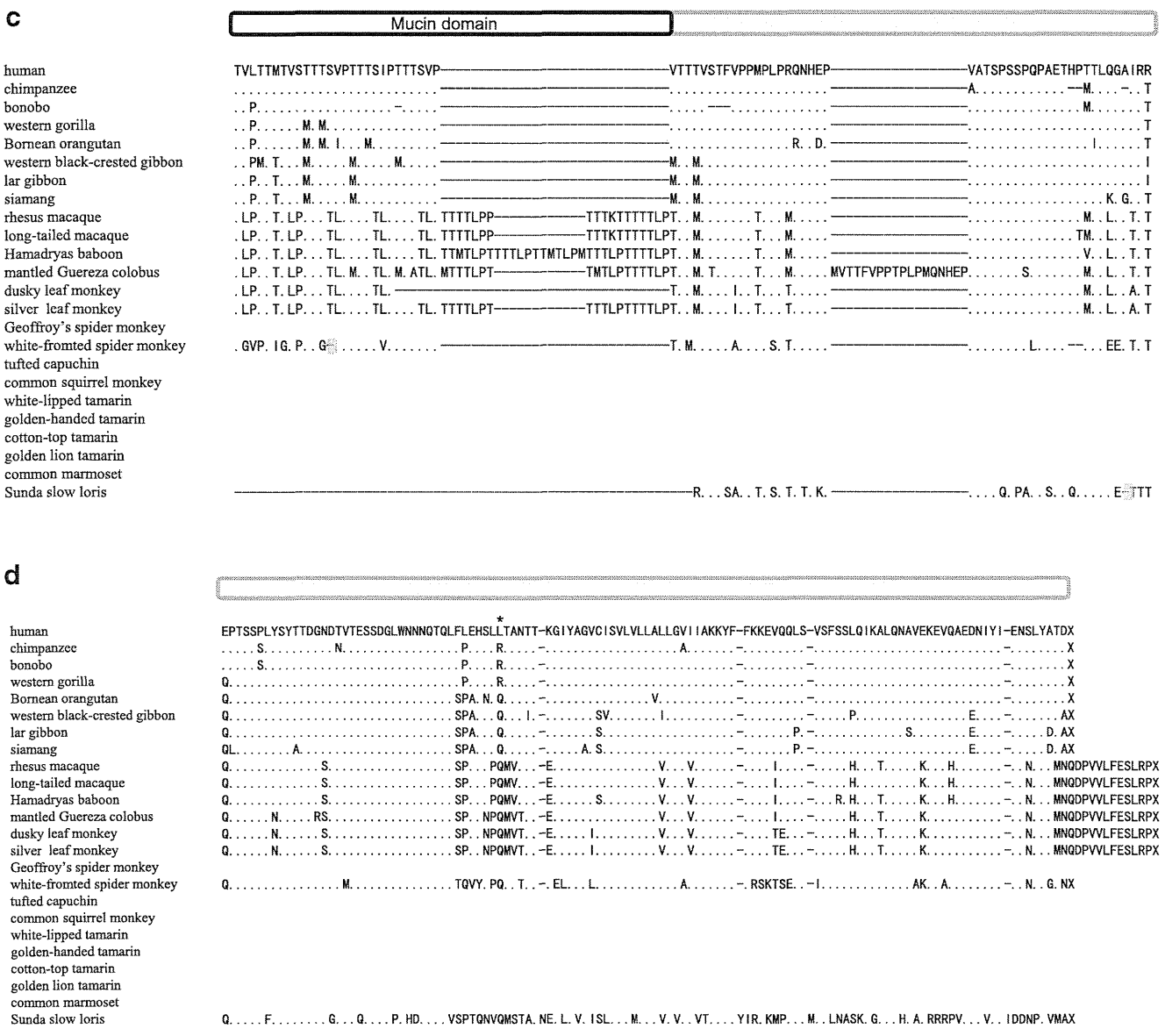

Fig. 3 (continued)

in the primates including the TIM1-deficient New World monkey. It is interesting to note that there is a *TIM1*-like gene on chromosome 13 in the marmoset genome, which might replace the function of *TIM1*. However, this TIM1-like gene lacks introns and contains several in-frame stop codons, when it is aligned with human *TIM1* (Supplemental Figure S2).

It is known that TIM1 is a cellular receptor for hepatitis A virus (HAV) in human (Feigelstock et al. 1998). The results in this study suggested that the New World monkey lineages

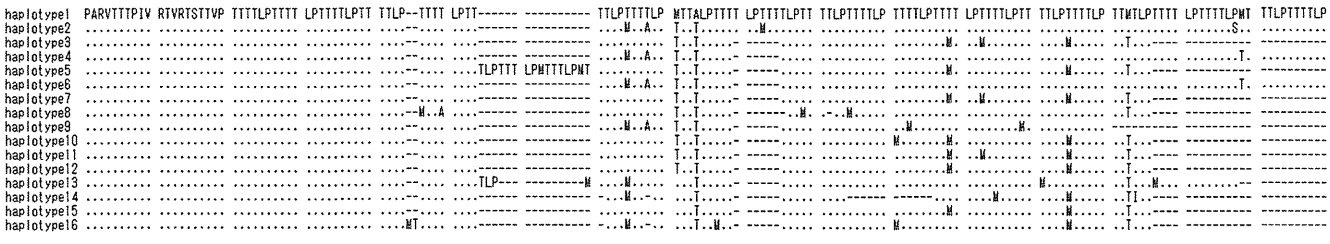

Fig. 4 Diversity of the mucin domain in rhesus macaques. *Dots* indicate identities to the haplotype 1 sequence, while *dashes* indicate alignment gaps

might lack a cellular receptor for HAV, TIM1. However, because the New World monkey is susceptible to HAV infection (Mathiesen et al. 1980), further studies are needed to clarify or find other cellular receptors for HAV in the New World monkey and a cause or reason of pseudogene generation, which had occurred in several lineages of the New World monkey. In addition, it has been reported that TIM1 polymorphisms are associated with resistance to autoimmune diseases including multiple sclerosis, which are associated with the imbalance between Th1 and Th2 cells (Khademi et al. 2004). Nevertheless, common marmosets are used as an animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (Uccelli et al. 2003). Thus, susceptibilities of the New World monkey to the autoimmune diseases should be investigated in relation to the non-functional TIM1.

In this study, 14 AA sites of TIM1 were identified as positively selected sites in the evolutional course of primates other than the New World monkey. It was suggested that the Ig domain of TIM1 was a binding site for HAV (Feigelstock et al. 1998). In addition, structural conformation of the mucin domain is required for the efficient viral entry (McIntire et al. 2003). In this study, TIM1 was considered to be under the significant positive natural selection in the Ig domain, prompting us to investigate the three dimensional (3D) structures of the Ig domains using SWISS-MODEL, an Automated Comparative Protein Modeling Server (http://swissmodel.expasy.org/SWISS-MODEL.html) (Bordoli et al. 2009). As shown in Fig. 5, it was suggested that most of the target sites for the positive selection accumulated on the surface of Ig domain. These observations support a hypothesis that the evolution of TIM1 in the primates might be driven by exogenous pathogens. The positively selected sites in the Ig domain of TIM1 in the primates other than the New World monkey and a high level diversity in the mucin domain of TIM1 in the Old World monkey might be a direct consequence of a selection pressure exerted by HAV. However, because TIM1 polymorphisms are also associated with other infectious diseases including HIV/AIDS and cerebral malaria, further functional studies are required to clarify the mechanism of natural selection at specific sites of Ig and mucin domains of TIM1. For example, we are now investigating whether the TIM1 repeat polymorphism would influence the production level of neutralizing antibodies against challenging Simian Immunodeficiency Virus (SIV) in experimental models of SIV vaccination in rhesus macaques (Sugimoto et al. 2010; Ishii et al. 2012; Nomura et al. 2012).

Because TIM1 is known to interact with TIM4 and both Ig and mucin domains of TIM1 are involved in this interaction (Meyers et al. 2005a), one might speculate a co-evolution of TIM1 and TIM4. It should be noted here that human TIM family includes three members, TIM1, TIM3, and TIM4, while mouse TIM family includes eight members, TIM1 toTIM8. Although we searched for orthologs of mouse TIM2, TIM5, TIM6, TIM7, and TIM8 in the common marmoset genome by using Blat program (http://genome.ucsc.edu/cgi-bin/hgBlat), we could not detect any orthologous genes. Therefore, TIM family in the New World monkey consists of only two functional members, TIM3 and TIM4. Then, we investigated a possible evolutionary selection of TIM3 and TIM4. However, no significant positive selection appeared to operate on the evolution of TIM3 and TIM4 in the primates (Supplemental Table S6). A marginal and non-significant positive selection for TIM4 in chimpanzee was observed, but it may not correlate with co-evolution of TIM1 and TIM4, because the mucin domain of TIM1 is virtually non-polymorphic in chimpanzee (Nakajima et al. 2005). Nevertheless, the observations in this study suggest that the diversity of TIM family is widely ranged among mammalian species. It may be of interest to investigate whether the binding affinity of TIM1 and TIM4 would be affected by the TIM1 variations in future experiments. On the other hand, it may be noteworthy that TIM1, TIM3, and TIM4 can independently serve as receptors for phosphatidylserine to mediate uptake of apoptotic cells (Kobayashi et al. 2007; Freeman et al. 2010), implying that their cooperation would be dispensable in some functional aspects.

In conclusion, we investigated the molecular evolution of TIM1 in 24 primate species. TIM1 had become pseudogenes in most lineages of the New World monkey, while it

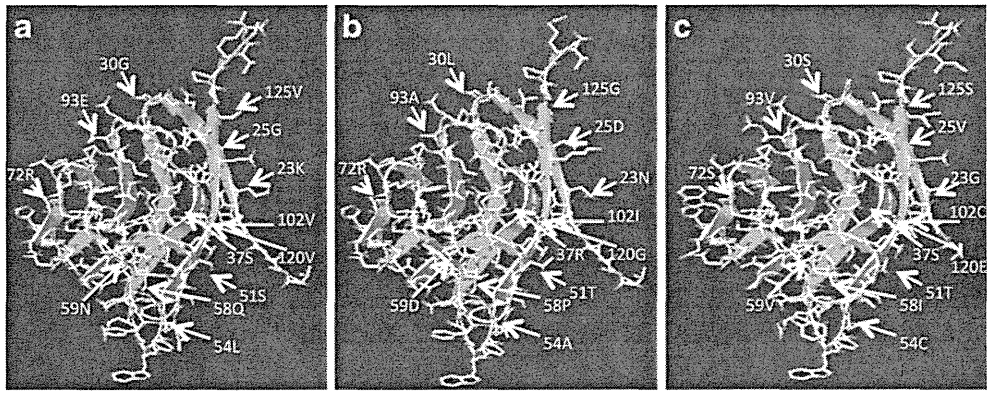

Fig. 5 Three-dimensional structures of TIM1 modeled by SWISS-MODEL. *Arrows* indicate AA sites identified as being under the positive selection by using the BEB method in the PAML program. **a** human TIM1, **b** rhesus macaque TIM1, **c** long-haired spider monkey TIM1

was under the positive selection in the other primates, especially in the Old World monkey. TIM1 might undergo a selection pressure exerted by infectious disease and autoimmune disease.

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ORIGINAL RESEARCH ARTICLE
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Allele frequency of antiretroviral host factor TRIMCyp in wild-caught cynomolgus macaques (*Macaca fascicularis*)

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A recent study showed that the frequency of an antiretroviral factor *TRIM5* gene-derived isoform, TRIMCyp, in cynomolgus macaques (*Macaca fascicularis*) varies widely according to the particular habitat examined. However, whether the findings actually reflect the prevalence of TRIMCyp in wild cynomolgus macaques is still uncertain because the previous data were obtained with captive monkeys in breeding and rearing facilities. Here, we characterized the *TRIM5* gene in cynomolgus macaques captured in the wild, and found that the frequency of the TRIMCyp allele was comparable to those in captive monkeys. This suggests that the previous results with captive monkeys do indeed reflect the natural allele frequency and that breeding and rearing facilities may not affect the frequency of *TRIM5* alleles. Interestingly, the prevalence of a minor haplotype of TRIMCyp in wild macaques from the Philippines was significantly lower than in captive ones, suggesting that it is advantageous for wild monkeys to possess the major haplotype of TRIMCyp. Overall, our results add to our understanding of the geographic and genetic prevalence of cynomolgus macaque TRIMCyp.

Keywords: cynomolgus monkey, TRIM5α, TRIMCyp, genetic diversity, host factor

INTRODUCTION

In 2004, TRIM5-Cyclophilin A (CypA) chimeric protein, referred to as TRIMCyp, was first identified in owl monkeys (*Aotus trivirgatus*), which belongs to New World monkeys (NWMs) (Sayah et al., 2004). The discovery of TRIMCyp in owl monkeys explains the novel post-entry restriction of human immunodeficiency virus type 1 (HIV-1), which is uniquely seen in owl monkey-derived cells but not in other NWM-derived cells. Owl monkey TRIMCyp is derived from LINE-1-mediated retrotransposition of CypA cDNA into the region between *TRIM5* exons 7 and 8. On the other hand, the strong post-entry restriction of HIV-1 in Old World monkey (OWM)-derived cells was thought to be dependent on a TRIM5α-mediated mechanism (Stremlau et al., 2004; Nakayama and Shioda, 2010). Interestingly, among OWMs, pig-tailed macaques (*Macaca nemestrina*; hereafter denoted as PMs) uniquely show higher susceptibility to HIV-1 infection when compared with other OWMs (Agy et al., 1992). However, the mechanism underlying this higher susceptibility was unclear. Thereafter, it was found that PMs exclusively have the TRIMCyp genotype, which is a strong genetic determinant of their susceptibility to HIV-1 infection (Liao et al., 2007; Brennan et al., 2008; Virgen et al., 2008). Subsequently, TRIMCyp was also discovered in rhesus macaques (*Macaca mulatta*; hereafter denoted as RMs) and cynomolgus macaques (*Macaca fascicularis*; hereafter denoted as CMs) (Brennan et al., 2008; Newman et al., 2008; Wilson et al., 2008).

TRIMCyp is an alternatively spliced isoform of the *TRIM5* gene in which the PRYSPRY domain of TRIM5α is replaced with a retrotransposed *CypA* gene. Unlike owl monkey TRIMCyp, the *CypA* gene in OWM TRIMCyp, is inserted in the 3′-untranslated region (UTR) of the *TRIM5* gene. The retrotransposition of the CypA sequence is concomitant with a single nucleotide polymorphism (SNP) at the exon 7 splice acceptor site; this leads to skipping of exons 7 and 8 encoding the PRYSPRY domain and splicing to the inserted *CypA* gene (Johnson and Sawyer, 2009). Thus, the presence or absence of the CypA sequence in the 3′ UTR leads to expression of TRIMCyp or TRIM5α (Nakayama and Shioda, 2012).

Current data suggest that PMs exclusively express TRIMCyp and not TRIM5α. In the case of RMs, the frequency of TRIMCyp in Indian RM was approximately 25%, while it was not found in the Chinese RM population (Wilson et al., 2008). In addition, we observed that the frequency of TRIMCyp in Burmese RM was approximately 10% (unpublished data), suggesting a geographical deviation in the frequency of RM TRIMCyp. In the case of CM, we and other groups reported that TRIMCyp is present at higher frequency when compared with RM (De Groot et al., 2011; Dietrich et al., 2011; Saito et al., 2012). Interestingly, we and other groups found a geographical deviation in the frequency of TRIMCyp in CM (Dietrich et al., 2011; Berry et al., 2012; Saito et al., 2012). In particular, we showed that the frequency of TRIMCyp in the Philippine population was higher than that in Indonesian and Malaysian populations. Dietrich et al. also reported that the frequency of TRIMCyp in the Philippine population was higher than that in Indonesia, Indochina, and Mauritian populations (Dietrich et al., 2011). Moreover, they