Cytoplasmic Body Component TRIM5α Requires Lipid-enriched Microdomains for Efficient HIV-1 Restriction*

TRIM5α is a member of the tripartite motif (TRIM) family of proteins and affects both early and late phases of the retroviral life cycle. Although TRIM5α multimerizes to form cytoplasmic bodies, which are thought to play an important role in viral restriction, the identity of TRIM5α-containing cytoplasmic bodies remains elusive. To better understand TRIM5α cytoplasmic body constituents and the cellular proteins that could be involved in the TRIM5α-mediated antiviral activities, we sought TRIM5α-binding factors. We identified a lipid microdomain protein flotillin-1/Reggie-2 as an interacting partner of TRIM5α via co-immunoprecipitation. Immunohistochemistry studies confirmed the co-localization of rhesus monkey TRIM5α (TRIM5arh) cytoplasmic bodies with flotillin-1/Reggie-2. Caveolin-1, another lipid microdomain-associated protein, also co-localized with TRIM5α cytoplasmic bodies. Intriguingly, disruption of cellular cholesterol by cycloheximide perturbed TRIM5α cytoplasmic body formation. Furthermore, lipid starvation partially relieved the endogenous post-entry restriction of HIV-1 infection, which could be subsequently restored by lipid repletion. These observations indicate the involvement of cellular lipids in TRIM5α-mediated antiviral activities. Given that many viruses utilize cellular lipid microdomains for viral entry and assembly, it is plausible that lipid-enriched domains provide microenvironments where TRIM5α recognizes retroviral components.

Received for publication, June 24, 2010, and in revised form, August 30, 2010. Published, JBC Papers in Press, September 1, 2010, DOI 10.1074/jbc.M110.158188

Seiga Ohmine1, Ryuta Sakuma1,2, Toshie Sakuma3, Tayaramma Thatava4, Gonzalo P. Solis5, and Yasuhiro Ikeda1,3

From the 1Department of Molecular Virology, Tokyo Medical and Dental University of Konstanz, D-78457 Konstanz, Germany

* This work was supported, in whole or in part, by National Institutes of Health Grant 1R56AI074363-01A1 (to Y. I.). This work was also supported by the Mayo Foundation (to Y. I.).
1 Recipient of a Siebens Ph.D. training fellowship.
2 Present address: Dept. of Molecular Virology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.
3 To whom correspondence should be addressed: 200 First St. SW, Rochester, MN 55905. Tel.: 507-538-0153; Fax: 507-266-2122; E-mail: ikeda.yasuhiro@mayo.edu.

© 2010 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
down did not have remarkable effects on TRIM5αrh-mediated cellular restriction; however, depletion of cellular lipids and cholesterol disrupted TRIM5α cytoplasmic body formation and impaired the TRIM5αrh-mediated post-entry restriction. Subsequent lipid repletion completely restored the TRIM5αrh-mediated post-entry restriction of HIV-1. Similar effects were observed in the late restriction upon lipid depletion. Thus, our results demonstrate that target cell lipids play a critical role in the cytoplasmic body formation and antiviral activities of TRIM5α.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T, FRhK4, TE671, and GHOST(3)R3/X4/R5 (25) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. For generation of rhesus monkey FRhK4 cells stably expressing HA- or mCherry-tagged TRIM5αrh, FRhK4 cells were transduced by a retroviral vector and selected under the presence of puromycin. Resulting puromycin-resistant cells were named FRhK4T5αHA and FRhK4T5αCh cells, respectively.

**Plasmids**—C-terminally HA-tagged TRIM5αrh-expressing plasmid pH-T5α and codon-optimized HIV-1 GagPol expression construct pH-G/P was described previously (16). pLPCX-TRIM5αrh (4), which was kindly provided by Dr. Joseph Sodroski, was used to generate pH-T5αCh, where the C-terminal end of TRIM5αrh was tagged with a red fluorescent protein mCherry. The HIV-1 molecular clones pNL4-3 (26) and pSIVMac1A11 (27) were used to generate HIV-1 and SIV4 viruses, respectively. A Reggie-2 expression plasmid was used to express flotillin-1/Reggie-2 protein (28).

**Confocal Microscopy Analysis**—FRhK4T5αCh cells were seeded into LabTek II 8-well chamber slides (Nunc, Rochester, NY) at ~1.5 × 10⁶ cells/ml. 48 h later, cells were fixed in 4% paraformaldehyde, permeabilized on ice using 0.1% saponin, and then blocked with 5% fetal bovine serum in PBS for 30 min at room temperature. Primary antibodies (rat anti-HA, 1:250, Roche Applied Science; mouse anti-caveolin-1, 1:250, BD Transduction Laboratories; rabbit anti-EEA1, 1:250, Cell Signaling Technology (Danvers, MA); mouse anti-flotillin-1/Reggie-2, 1:250, BD Transduction Laboratories; mouse anti-HSP70, 1:250, Cell Signaling Technology; rabbit anti-Rab7, 1:250, Cell Signaling Technology; and mouse anti-tubulin, 1:250, Sigma) and secondary FITC-conjugated antibodies were used (Texas red-conjugated donkey anti-rat IgG, 1:250, Jackson ImmunoResearch, West Grove, PA; FITC-conjugated goat anti-mouse IgG, 1:250, Roche Applied Science; and FITC-conjugated donkey anti-rabbit IgG 1:250, Jackson ImmunoResearch). Images were obtained using the Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Generation of co-localization coefficients were performed with the Zeiss LSM 510 META software. Co-localization coefficients were calculated as the sum of intensities of co-localizing pixels in channels 1 and 2, as compared with the overall sum of pixel intensities in the channel. A value of 0 corresponds to no co-localization, and 1 indicates all pixels to be co-localized. Scale bars of 20 μm were also generated by the Zeiss LSM 510 META software.

**Immunoprecipitation**—293T cells were co-transfected with 4.0 μg of pH-T5α and 0.5 μg of pNL4-3 in 15-cm culture plates. 40 h post-transfection, cells were treated with 40 μM MG115 for 6 h (Sigma). Producer cells were washed with ice-cold PBS, and cell lysates were harvested in 1.0 ml of RIPA lysis buffer. Cell debris was removed by centrifugation, and the supernatant was kept for analysis. Immunoprecipitation of HA-tagged TRIM5α proteins was performed using agarose beads conjugated with mouse anti-HA antibody (Sigma) and were incubated at 4 °C overnight with rotation. The beads were washed at least 20 times with RIPA buffer and pelleted. Immunoprecipitated proteins were eluted off the HA-agarose beads through a 30-min room temperature incubation with 100 μg of HA peptide (Sigma), according to the manufacturer’s instructions. Immunoprecipitation of flotillin-1/Reggie-2 was performed using monoclonal antibody against human flotillin-1/Reggie-2 protein (BD Transduction Laboratories). To remove any non-specific binding of proteins, the antibody against flotillin-1/Reggie-2 was added to the supernatant at a 1:250 concentration after a 30 min rotation with protein G beads (Sigma) and rotated overnight at 4 °C. After more than 20 washes with RIPA buffer, the protein-antibody complex was pulled down using protein G beads. Laemmli sample buffer with β-mercaptoethanol was directly added to the beads, and protein samples were heat-denatured for immunoblot analysis.

**Immunoblotting**—Proteins were subjected to SDS-PAGE in a 4–15% Tris–HCl gel and then transferred onto a polyvinyliden fluoride membrane at 0.7 mA/cm² for 40 min. Membranes were blocked in 5% milk PBS overnight prior to application of antibodies. Antibodies were used in the following concentrations: rat anti-HA (1:1000, Roche Applied Science), mouse anti-flotillin-1/Reggie-2 (1:1000), and mouse anti-β-actin (1:3000, Sigma). Peroxidase-conjugated secondary antibodies (goat anti-rat IgG, goat anti-mouse IgG, and goat anti-rabbit IgG, Thermo Scientific, Waltham, MA) were used at a 1:2000 concentration.

**Generation of Flotillin-1/Reggie-2 and TRIM5αrh Knockdown Cell Lines**—Lentiviral vectors were generated by transfecting pLKO.1-based lentiviral vectors carrying shRNA sequences against flotillin-1/Reggie-2 (RHS4533NM-005803, Open Biosystems) or TRIM5αrh-specific shRNA pLKO-H4 (RHS3979-9574739, Open Biosystems), along with an HIV-1 packaging plasmid, pCMV-R8.91, and a vesicular stomatitis virus glycoprotein G (VSV-G) expressing plasmid, pMD.G (29) (kind gifts from Dr. Didier Trono), into 293T cells using FuGENE 6. 48–72 h later, viral supernatant was collected and filtered through a 0.45-μm syringe filter and concentrated by ultracentrifugation at 21,500 rpm for 2 h at 4 °C using an SW-41 rotor (Beckman Instruments). Following infection, stably transduced cells were selected in the presence of 3 μg/ml puromycin.

**RT-PCR**—Total cellular RNA was harvested with TRIzol reagent (Invitrogen), and 5 μg of total RNA was used to synthesize the first strand DNA with an oligo(dT) primer (SuperScript III first-

---

4 The abbreviations used are: SIV, simian immunodeficiency virus; LPDS, lipoprotein-deficient serum; 20H(p)CD, 2-hydroxypropyl-β-cyclodextrin; CD, cyclodextrin; VSV-G, vesicular stomatitis virus glycoprotein G.

---

Involvement of Cellular Lipids in TRIM5α Restriction
Involvement of Cellular Lipids in TRIM5α Restriction

strand synthesis system, Invitrogen). One-tenth of the reverse transcription products was used to amplify TRIM5αr-specific cDNA with primers 5'-GCAGTTAGGTCATTGATGGGA-GGTGCCCA-3' and 5'-ACGCTGACTGTAATTGATAAGCTTGGTGAGCACAGA-GTCAAG-3. Flotillin-1/Reggie-2 primers were used to 5'-GTTTACA-CCTGCCATGGGGTCCCCATCTCAG-3' and 5'-GGGCCCTTCTTACGTCTAATCTCCTGTC-3'. α-Tubulin primer set used in these experiments was described previously (30).

**RESULTS**

**Lipid Microdomain-associated Protein Flotillin-1/Reggie-2 Co-immunoprecipitates with TRIM5αr**—To gain an insight into the identity of TRIM5α cytoplasmic bodies where TRIM5αr appears to interact with incoming HIV-1 capsid (13, 23), we examined cellular factors that may associate with TRIM5αr. 293T cells were transfected with a plasmid expressing a C-terminally HA-tagged TRIM5αr with or without pNL4-3. Cell lysates were immunoprecipitated with anti-HA antibody as described under “Experimental Procedures.” Eluted proteins were then separated in a 4–15% Tris-HCl gradient gel and silver-stained for protein visualization. A representative silver stain is shown (Fig. 1A). Prominent bands and the corresponding areas in the control lane were excised and analyzed using tandem MS. Bands B, F, and H appeared more prominently following longer development times (data not shown). Peptide assignments from the tandem MS spectra were accepted if they could be established at greater than 99.9% probability as specified by the PeptideProphet algorithm. Keratin was excluded as it is known as a common contaminant in proteomic analysis. The identified proteins are listed in Fig. 1B. Of the several proteins identified, flotillin-1/Reggie-2, α-tubulin, and heat shock protein 70 (HSP70) were further analyzed as their peptides were identified at least nine times (Fig. 1B). HIV-1 p24 was not detected upon TRIM5αr immunoprecipitation (data not shown). To verify the HA immunoprecipitation results, we used an anti-flotillin-1/Reggie-2 monoclonal antibody to immunoprecipitate endogenous flotillin-1/Reggie-2 in TRIM5αr- and/or NL4-3-expressing 293T cells. Although endogenous flotillin-1/Reggie-2 was below detectable levels via immunoblot analysis, flotillin-1/Reggie-2 immunoprecipitation enriched flotillin-1/Reggie-2 signals (Fig. 1C). Furthermore, we were able to detect co-immunoprecipitated TRIM5αr-HA upon flotillin-1/Reggie-2 pulldown (Fig. 1C). We could not confirm the bindings of TRIM5αr with α-tubulin or HSP70 by using antibodies against respective proteins (data not shown).

Flotillin-1/Reggie-2 and Caveolin-1 Co-localizes with TRIM5αr in Cytoplasmic Bodies—First, we retrovirally transduced rhesus monkey FRhK4 cells to express C-terminally mCherry-tagged TRIM5αr (TRIM5αrCh, Fig. 2A). After selection in the presence of puromycin, we obtained the bulk population of FRhK4 cells that stably express mCherry-tagged TRIM5αr, termed FRhK4T5αCh cells. TRIM5αr-mCherry signals were observed predominantly as discrete cytoplasmic bodies in FRhK4T5αCh cells (Fig. 2, B and D→G). To test whether flotillin-1/Reggie-2 co-localizes with TRIM5αr in rhesus monkey FRhK4 cells, FRhK4T5αCh cells were immunostained for endogenous flotillin-1/Reggie-2 and analyzed under confocal microscope. Flotillin-1/Reggie-2 signals were found on the cell membrane as well as in cytosol as discrete cytoplasmic bodies. Cytoplasmic flotillin-1/Reggie-2 signals frequently co-localized with the TRIM5αr-mCherry signals (co-localization coefficient = 0.593) (Fig. 2B).

To rule out the possibility that the C-terminal mCherry tag affects the TRIM5αr subcellular localization, FRhK4T5αrHA cells, stably expressing C-terminally HA-tagged TRIM5αr, were also generated. FRhK4T5αrHA cells were immunostained with anti-HA and anti-flotillin-1 primary antibodies and visualized using secondary antibodies conjugated with Texas Red and FITC, respectively. Both TRIM5αr and flotillin-1/Reggie-2 formed cytoplasmic bodies, and the co-localization coeffi...
Involvement of Cellular Lipids in TRIM5α Restriction

coefficients between the signals were nearly identical to those observed in the FRhK4T5αCh cells (Fig. 2C, co-localization coefficient = 0.598).

Because flotillin-1/Reggie-2 is a well established lipid microdomain marker, we further assessed the possible localization of TRIM5αrh in lipid microdomains. We used another lipid microdomain marker caveolin-1, a protein that is the primary constituent of cellular caveolae (34), for the co-localization study. When FRhK4T5αCh cells were stained with anti-caveolin-1 antibody (Fig. 2D), prominent co-localization signals were observed between TRIM5αrh-mCherry and caveolin-1 at cytoplasmic bodies (co-localization coefficient = 0.267). TRIM5αrh was not co-localized to the cell membrane with flotillin-1/Reggie-2 (Fig. 2B) or caveolin-1 (data not shown) signals. Untransduced parental FRhK4 cells showed similar flotillin-1/Reggie-2 and caveolin-1 staining patterns, suggesting that overexpression of TRIM5αrh did not affect endogenous flotillin-1/Reggie-2 or caveolin-1 subcellular localization (data not shown). When antibodies against early (EEA1) and late endosomes (Rab7) were used, both signals did not prominently co-localize with TRIM5αrh-mCherry in FRhK4 cells (Fig. 2, E and F, co-localization coefficient = 0.091 and 0.099, respectively). α-Tubulin and HSP70, which were co-immunoprecipitated with TRIM5αrh (Fig. 1B), did not show clear co-localization signals with TRIM5αrh-mCherry in FRhK4T5αCh cells (Fig. 2, G and H). Our results therefore suggest the localization of TRIM5αrh cytoplasmic bodies with proteins associated with subcellular lipid microdomains.

Flotillin-1/Reggie-2 Knockdown Does Not Affect the TRIM5αrh-mediated Restriction of HIV-1 in FRhK4 or 293T Cells—Flotillin-1/Reggie-2 is a lipid-associated protein that has been identified in many subcellular microdomains, including endosomes, phagosomes, plasma membrane, trans-Golgi network, and lipid droplets (35–38). Flotillin-1/Reggie-2 up-regulation is observed in peripheral blood mononuclear cells upon treatment with HIV-1 components, suggesting flotillin-1/Reggie-2 to be a part of the cellular response to viral infection (39). To clarify the role of flotillin-1/Reggie-2 in the TRIM5αrh-mediated restriction of HIV-1 infection and production, FRhK4 cells were transduced by lentiviral vectors carrying flotillin-1/Reggie-2-specific shRNA sequences, FLOT17–19 and -21 or an empty vector, pLKO.1. After puromycin selection, we obtained stable shRNA-expressing FRhK4 cells. Stable flotillin-1/Reggie-2 knockdown was verified by immunoblotting in the cells expressing shRNA FLOT17–19, although stable introduction of shRNA FLOT21 did not efficiently knock down the flotillin-1/Reggie-2 expression (Fig. 3A). When the cells were infected with a VSV-G-pseudotyped, GFP-expressing HIV-1 vector, no apparent differences in HIV-1 transduction efficiency were observed between control and flotillin-1/Reggie-2 knockdown cells (Fig. 3B). These observations suggest no role of flotillin-1/Reggie-2 in the post-entry restriction of HIV-1 in FRhK4 cells.

Next, to assess the influence of flotillin-1/Reggie-2 knockdown on the TRIM5α-mediated late restriction, the shRNA-carrying FRhK4 cells were transfected with an HIV-1 infectious molecular clone, pNL4-3, and HIV-1 production was monitored. No remarkable difference in TRIM5αrh-mediated restriction of HIV-1 production was observed upon flotillin-1/Reggie-2 knockdown (Fig. 3C). We also tested the effects of flotillin-1/Reggie-2 knockdown on the late restriction in TRIM5αrh-overexpressing 293T cells. 293T cells were co-transfected with the flotillin-1/Reggie-2-specific shRNA-expressing lentiviral vector plasmids along with the HIV-1

**FIGURE 1. Identification of potential TRIM5αrh-interacting proteins.** A, 293T cells were transfected with an HA-tagged TRIM5αrh-expressing plasmid with or without proviral HIV-1 plasmid pNL4-3. TRIM5αrh was immunoprecipitated using anti-HA-agarose beads. Proteins were eluted off the beads as described under “Experimental Procedures” and were subjected to SDS-PAGE. Silver-stained bands that were excised and analyzed by MS/MS analysis. Identifications were accepted if they could be established at greater than 99.9% probability, as specified by the Peptide Prophet algorithm, and contained at least nine identified peptides. Letters correspond to the band identified in the silver-stained gel in A. No HIV-1 Gag was detected in the samples. C, endogenous flotillin-1/Reggie-2 was immunoprecipitated from 293T cells expressing TRIM5αrh (T5αrh) with or without NL4-3. Anti-HA antibody was used to detect HA-tagged TRIM5αrh, although anti-flotillin-1 antibody was used to detect flotillin-1/Reggie-2. Arrows indicate appropriate protein size.
Involvement of Cellular Lipids in TRIM5α Restriction

Infected clones and TRIM5αr-expressing plasmids. TRIM5αr-mediated restriction of HIV-1 production remained unaffected by transient flotillin-1/Reggie-2 knockdown (Fig. 3D). In addition, flotillin-1/Reggie-2 overexpression in the presence of TRIM5αr did not enhance the late restriction activity in 293T cells (Fig. 3E). We therefore concluded that flotillin-1/Reggie-2 is not required for the TRIM5αr-mediated late restriction.

Disruption of Cellular Cholesterol Alters TRIM5αr Subcellular Localization—Because lipid microdomains proteins flotillin-1/Reggie-2 and caveolin-1 co-localized with TRIM5αr cytoplasmic bodies, we further examined whether disruption of cellular lipid microdomains will have any effect on TRIM5α subcellular localization. Treatment with 10 mM 2OHpβCD, which depletes cellular cholesterol, altered the localization of TRIM5α from discrete cytoplasmic bodies (95.6% cells with >10 discrete cytoplasmic bodies) to more diffuse cytoplasmic localization (61.4% cells with >10 discrete cytoplasmic bodies) (Fig. 4). 2OHpβCD treatment also reduced flotillin-1/Reggie-2 cytoplasmic body formation (from 88.79 to 23.97% with >10 discrete cytoplasmic bodies). Accordingly, co-localized signals between TRIM5αr and flotillin-1/Reggie-2 were decreased, as untreated control cells showed more prominent co-localization (co-localization coefficient = 0.619) than 2OHpβCD-treated cells (co-localization coefficient = 0.172) (Fig. 4). These observations implicate a role for cellular cholesterol in the formation of TRIM5α cytoplasmic bodies.

Lipid Starvation Increases HIV-1 Vector Infectivity in FRhK4 Cells—The disruption of TRIM5αr cytoplasmic bodies through cholesterol depletion suggested that cellular lipids may play an important role in the TRIM5α-mediated restrictions. To address the possible role in the restriction, we examined the susceptibility of target cells upon serum starvation and depletion conditions. Because VSV-G-pseudotyped viruses are largely unaffected by lipid depletion (40–42), we challenged target cells with VSV-G-pseudotyped HIV-1 vectors. After incubation in 5% LPDS-supplemented media for 48 h, FRhK4 cells were treated with mock (LPDS) or 10 mM 2OHpβCD (LPDS + CD) for 45 min, followed by GFP-expressing HIV-1 vector infection (Fig. 5A). Intriguingly, LPDS and LPDS + CD-treated FRhK4 cells showed increased permissivity to HIV-1 by 10-fold when compared with control (Fig. 5A). When LPDS + CD-treated cells were lipid-repleted with excess LDL or water-soluble cholesterol (data not shown).

Identical experimental conditions were applied to TE671 cells, in which infection of N-MLV, but not NB-MLV, is strongly blocked by human TRIM5α at the post-entry stage. LPDS- and LPDS + CD-treated cells showed a 60-fold increase in N-MLV titers, although lipid repletion partially reduced N-MLV infectivity to that of control TE671 cells (Fig. 5B). In contrast, NB-MLV infectivity was not strongly affected by the lipid starvation/depletion and the following lipid repletion. These data suggest that target cell lipid status plays a role in the cellular restriction against incoming HIV-1 and N-MLV.

Lipid Modulation Affects TRIM5αr-mediated Post-entry Restriction of HIV-1—Target cell lipid starvation and cholesterol depletion rendered FRhK4 and TE671 cells permissive to HIV-1- or N-MLV-based vectors. To address the involvement of TRIM5α in this observation, we generated stable TRIM5α knockdown cell lines and examined the effects of lipid modula.
Involvement of Cellular Lipids in TRIM5α Restriction

FIGURE 4. Treatment with 2OHp/CD disrupts TRIM5αrh cytoplasmic body formation. Immediately after treating FRhK4T5αrh cells with 10 mM 2OHp/CD for 60 min, cells were fixed and stained against flotillin-1/Reggie-2 (green). Representative images are shown, and percentages represent the proportion of TRIM5αrh cytoplasmic bodies/cell and flotillin-1/Reggie-2 cytoplasmic bodies/cell, and merge panels show the co-localization coefficient between TRIM5αrh and flotillin-1/Reggie-2 signals. Nuclei were counterstained with DAPI (blue). Bars, 20 μm.

FIGURE 5. Lipid depletion modulates the infectivity of HIV-1 and N-MLV vectors in FRhK4 cells and TE671 cells. Cells were cultured in 10% FBS McCoy's media (Ct), 5% lipoprotein-deficient serum supplemented McCoy's media (LPDS), 5% LPDS McCoy's media and 10 mM 2OHp/CD treatment (LPDS + CD), or 5% LPDS McCoy's media and 10 mM 2OHp/CD treatment, followed by 10% FBS McCoy's media (Repletion). A, FRhK4 cells were infected with increasing amounts of VSV-G pseudotyped HIV-1 GFP vector or SIVMAC GFP vector, and GFP-expressing cells were quantified using flow cytometry 48–72 h post-infection. Data are representative of three independent experiments. B, TE671 cells were infected with increasing amounts of VSV-G pseudotyped N-MLV GFP vector or NB-MLV GFP vector, and GFP-expressing cells were quantified using flow cytometry 48–72 h post-infection. Data are representative of three independent experiments.
Involvement of Cellular Lipids in TRIM5α Restriction

FIGURE 6. Treatment with 2OHβCD shows little effects on the post-entry restriction in TRIM5αrhm knockdown FRhK4 cells. A, total RNA was harvested from control FRhK4 cells and single cell clones from the LKO.1- or LKO.1-H4-transduced FRhK4 cells (H4.2, H4.4, and H4.6). Levels of TRIM5α mRNA in FRhK4 clones were analyzed by RT-PCR (upper panel), along with α-tubulin mRNA as a control (lower panel). B, serially diluted RT products (equivalent to 0.5, 0.25, 0.125, 0.063, and 0.031 μg of RNA samples) were used to amplify the TRIM5αrhm-specific transcript. C, parental FRhK4 cells, LKO.1, H4.2, H4.4, and H4.6 were infected with GFP-carrying HIV-1 vectors. Parental FRhK4, LKO.1, and H4.2 cells were infected with SiVmac vectors. 3 days post-infection, the GFP-positive populations were analyzed by flow cytometry. D, LKO.1 or H4.2 cells were cultured in 10% FBS McCoy’s media (CT), 5% LPDS McCoy’s media (LPDS), 5% LPDS McCoy’s media, and 10 mM 2OHβCD treatment (LPDS + CD) or 5% LPDS McCoy’s media and 10 mM 2OHβCD treatment, followed by 10% FBS McCoy’s media (Repletion). LKO.1 or H4.2 cells were infected with increasing amounts of HIV-1 GFP vector, and GFP-expressing cells were quantified using flow cytometry 48–72 h post-infection. Data are representative of three independent experiments.

under lipid starvation conditions (Figs. 5A and 6D), where LPDS and LPDS + CD treatment relieved the viral restriction by 10-fold, and lipid repletion restored viral infectivity to that of controls (Fig. 6D). In contrast, the H4.2 clone did not show little differences in permissivity against HIV-1 upon lipid depletion (Fig. 6D). Two other TRIM5α knockdown clones also showed similar results (data not shown). These observations indicate that modulation of cellular lipids affect HIV-1 infectivity only in the presence of TRIM5αrhm.

Lipid Modulation Does Not Affect TRIM5αrhm Expression—To rule out the possibility that lipid modulation affects TRIM5αrhm expression in the target cells, we examined the levels of HA-tagged TRIM5αrhm expression upon lipid modulation using FRhK4T5αHA cells. Under conditions where TRIM5αrhm-mediated restriction activities were disrupted, TRIM5αrhm protein expression remained largely unchanged (Fig. 7A). When mRNA levels of endogenous TRIM5α were examined using FRhK4 cells under these same conditions, lipid depletion showed no notable effects on TRIM5α mRNA levels (Fig. 7B). These data suggest that lipid depletion conditions do not affect TRIM5α expression. We therefore concluded that cellular lipids support the TRIM5αrhm-mediated antiviral activity against HIV-1 infection.

Depletion of Cellular Cholesterol Abrogates the TRIM5αrhm-mediated Late Restriction in 293T Cells—We next examined whether depletion of cellular cholesterol affects the TRIM5αrhm-mediated late restriction activity in FRhK4 and 293T cells. When the influence of cholesterol depletion was examined in FRhK4 cells, 2OHβCD treatment increased HIV-1 production up to 3-fold (Fig. 8A). Moreover, when HIV-1 NL4-3 virions were generated in 293T cells transiently expressing TRIM5αrhm, 2OHβCD treatment increased HIV-1 titers by up to 20-fold (Fig. 8B). In the absence of TRIM5αrhm, 2OHβCD-mediated cholesterol depletion did not affect HIV-1 production in 293T cells (Fig. 8B). When SiVmac, which is resistant to TRIM5αrhm-mediated late restriction (16), was produced in the presence or absence of TRIM5αrhm, 2OHβCD showed no notable effects (Fig. 8B). Similar to the effects of 2OHβCD treatment on the protein expression of FRhK4T5αHA cells (Fig. 7A), treatment of 293T cells transiently expressing TRIM5αrhm with 10 mM 2OHβCD did not show any remarkable differences in protein expression (Fig. 8C). Similar to our observations during the post-entry restriction, these observations indicate that efficient TRIM5αrhm-mediated late restriction requires cellular cholesterol.
Involvement of Cellular Lipids in TRIM5α Restriction

HIV-1 restriction factor TRIM5α resides in host cell cytoplasmic bodies, where TRIM5α is thought to interact with viral components during viral infection (13, 23). TRIM5α also forms cytoplasmic bodies during the late restriction (16, 17); however, the details of TRIM5α cytoplasmic body constituents, as well as the ensuing mechanisms of TRIM5α-mediated restriction, remain poorly defined. Here, we demonstrated that TRIM5αr mediates antiviral activities. Knockdown of flotillin-1/Reggie-2 expression, however, showed no remarkable effects in TRIM5αr-mediated antiviral activities. Knockdown of flotillin-1/Reggie-2 expression, however, showed no remarkable effects in TRIM5αr-mediated antiviral activities (Fig. 3, A–C). The TRIM5αr-mediated late restriction of HIV-1 was not affected by knockdown or overexpression of flotillin-1/Reggie-2 (Fig. 3, D and E). Although it remains possible that other cellular factor(s), such as flotillin-2/Reggie-1, masked the effects of flotillin-1/Reggie-2 knockdown by compensating for reduced flotillin-1/Reggie-2 functions (28, 43, 49), our data indicate no role of flotillin-1/Reggie-2 in TRIM5αr-mediated restrictions.

Although flotillin-1/Reggie-2 unlikely plays a role in TRIM5αr restrictions, our results strongly suggest that TRIM5αr cytoplasmic bodies are lipid-enriched. Lipid-enriched cellular microdomains are known to be multifunctional. They are involved in the transport of intracellular lipids (46), the converging of proteosomal and autophagic degradation pathways (50–52), and the removal and storage of excess proteins from cellular compartments (53). For example, aberrant or overexpression of caveolin-1, -2, or -3, which are involved in many functions ranging from endocytosis to signal transduction, leads to the sequestering of these proteins to these lipid bodies (34, 54, 55). This raises the concern over the TRIM5αr localization in lipid bodies as an artifact due to stably expressed TRIM5α. In this regard, the recent article by Kim et al. (56) is notable. The authors used a biochemical analysis and revealed the presence of endogenous human TRIM5α in lipid-enriched microdomains from human fibroblast cell lysates (56). These data rule out the possible artifacts due to sequestering of an overexpressed TRIM5α protein in lipid microdomains. In the course of this study, Hwang et al. (57) reported that TRIM5αr was present in detergent-insoluble fractions when expressed in 293T cells, which further support our current findings.

Our immunoprecipitation and tandem MS analysis also identified α-tubulin as a potential TRIM5αr binding partner (Fig. 1, A and B). However, no prominent co-localization

altered TRIM5αr cytoplasmic body formation and impaired the endogenous post-entry restriction activity against HIV-1 in FRhK4 cells. Lipid modulation also impaired the TRIM5αr-mediated late restriction activities against HIV-1. Our data therefore demonstrated the importance of cellular lipids in TRIM5αr cytoplasmic body formation and efficient TRIM5αr-mediated antiviral activities.

Our biochemical analysis identified membrane raft protein flotillin-1/Reggie-2 as a potential TRIM5α binding partner (Fig. 1, A–C). Although flotillin-1/Reggie-2 is most often reported as a membrane raft protein, it has also been reported to localize to cytoplasmic lipid bodies (43–46). Consistent with these reports, immunohistochemistry analysis of mCherry- or HA-tagged TRIM5αr protein demonstrated that flotillin-1/Reggie-2 co-localized with TRIM5αr as cytoplasmic bodies (Fig. 2B). Flotillin-1/Reggie-2 is up-regulated in peripheral blood mononuclear cells upon HIV-1 infection, suggesting flotillin-1/Reggie-2 as a part of the cellular response to viral infection (39). HIV-1 assembly also takes place in subcellular microdomains that are lipid-enriched (47, 48). Because flotillin-1/Reggie-2 was identified as an intracellular TRIM5α binding partner, we reasoned that flotillin-1/Reggie-2 might play a role in TRIM5αr-mediated antiviral activities. Knockdown of flotillin-1/Reggie-2 expression, however, showed no remarkable effects in TRIM5αr-mediated post-entry restriction activities (Fig. 3, A–C). The TRIM5αr-mediated late restriction of HIV-1 was not affected by knockdown or overexpression of flotillin-1/Reggie-2 (Fig. 3, D and E). Although it remains possible that other cellular factor(s), such as flotillin-2/Reggie-1, masked the effects of flotillin-1/Reggie-2 knockdown by compensating for reduced flotillin-1/Reggie-2 functions (28, 43, 49), our data indicated no role of flotillin-1/Reggie-2 in TRIM5αr-mediated restrictions.
between TRIM5αrh and α-tubulin was observed (Fig. 2F). Because α-tubulin is found to be tightly associated with flotillin-1/Reggie-2 in membrane raft domains from rat brain homogenates (58), α-tubulin may have been pulled down along with flotillin-1/Reggie-2. The identification of HSP70 (Fig. 1B), one of the best characterized molecular chaperones proteins (59), as a potential binding partner was intriguing, because HSP70 is specifically incorporated into primate lentiviral virions (60). Although we were unable to see prominent co-localization of HSP70 with TRIM5αrh cytoplasmic bodies in FRhK4 cells (Fig. 2G), a previous study identified partial HSP70 co-localization with TRIM5αrh at distinct cytoplasmic bodies in HeLa cells (61). Because HSP70 can be translocated to lipid bodies upon stimulation (62), the discrepancy between our data and the reported HSP70 co-localization data may be partially explained by the differences in cell types and/or the dynamicity of subcellular lipid compartments.

Lipids and lipid-associated proteins are reported to be involved in the initial stages of the HIV-1 life cycle. For instance, both primary HIV-1 receptor CD4 (40, 63) and HIV-1 co-receptor CCR5 (64) reside on host cell membrane raft domains. Del Real et al. (65) reported that initial HIV-1 entry into host cells is crippled when the primary CD4 receptor is translocated to non-raft domains on the plasma membrane. To avoid the possible influence of lipid depletion on HIV-1 and MLV entry, we here used VSV-G pseudotyped viruses, which are largely unaffected by lipid depletion (40–42). Lipid starvation resulted in reduced post-entry restriction against HIV-1 (Fig. 5A) and N-MLV (Fig. 5B), which could be restored by subsequent lipid repletion (Fig. 5, A and B). Lipid starvation/depletion conditions did not affect HIV-1 permisivity in TRIM5αrh-knockdown FRhK4 cells, which indicate the increase in permisivity observed upon lipid modulation is dependent on TRIM5α expression. Because lipid modulation affected TRIM5α cytoplasmic body formation without affecting TRIM5α expression (Figs. 4, 7A, and 8C), it is plausible that lipid depletion may disrupt TRIM5α compartmentalization with viral components in lipid microdomains, thereby reducing the availability of TRIM5α during the restrictions. Given that the proteosomal degradation pathway is involved in the initial TRIM5α-mediated post-entry restriction step (13, 14), these events may occur in lipid microdomains, leaving the possibility that lipid depletion disrupts the ensuing proteosomal degradation pathways in the TRIM5α-mediated restriction. Further studies of the target-cell lipid biology would likely elucidate the details of cellular lipid involvement in the TRIM5α-mediated restrictions.

The association of TRIM5α with subcellular lipid microdomains may not be surprising, as several cellular restriction activities against lentiviruses occur at lipid-enriched microdomains (66–69). Involvement of cellular lipids in the post-entry restriction of HIV has been reported as Lv2 activity, where a yet-to-be-identified restriction factor determines the cellular tropism of two related HIV type 2 (HIV-2) isolates (66–68). Lv2 restriction is determined by the viral capsid and envelope protein sequences, and Lv2 lentiviral restriction activity is determined by the route of viral entry; the restriction is only prominent after the restriction-sensitive viral core reached a subcellular compartment where Lv2 activity is accessible (67, 68). Although Lv2 restriction appears to be independent of human TRIM5α-mediated post-entry restriction (66), these observations indicate the involvement of cellular lipids in the post-entry restriction of lentiviruses.

Cellular lipids are also required for HIV-1 Vpu to counteract CD317/BST-2/tetherin (69). Previous reports suggest that these lipid microdomains can act as scaffolds or platforms for protein–protein interactions and protein compartmentalization (70, 71). Similar to our observations with VSV-G pseudotyped vectors, several reports suggest that endocytic routes of viral entry into target cells are dependent on cellular cholesterol but are independent of flotillin-1/Reggie-2, caveolin-1, or clathrin (72, 73). Thus, lipid microdomains may provide subcellular microenvironments where antiviral factors, such as TRIM5α, Lv2, and CD317/BST-2/tetherin, can recognize and/or sense viral components. Elucidating the roles of cellular lipids and lipid-associated proteins in TRIM5α-mediated antiviral activities may result in a better understanding of the underlying mechanisms of TRIM5α restrictions, ultimately leading to novel antiviral strategies.

Acknowledgments—We thank Drs. R. Cattaneo, B. F. Horazdovsky, S. J. Russell, E. M. Poeschla, R. D. Single, R. E. Pagano, and A. Ono for helpful discussions. The following reagents were obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health: HIV-1 p24 monoclonal antibodies 183-H12-5C from Dr. B. Chesbro and K. Wehrly; AG3.0 from Dr. J. Allan; pNL4-3 from Dr. M. Martin, and GHOST(3)R3/X4/R5 cells from Drs. V. N. KewalRamani and D. R. Littman.

REFERENCES

1. Perron, M. J., Stremlau, M., Song, B., Uln, W., Mulligan, R. C., and Sodroski, J. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 11827–11832
2. Hatziioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., and Bieniasz, P. D. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10774–10779
3. Yap, M. W., Niso, S., Lynch, C., and Stoye, J. P. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10786–10791
4. Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P., and Sodroski, J. (2004) Nature 427, 848–853
5. Perez-Caballero, D., Hatziioannou, T., Zhang, F., Cowan, S., and Bieniasz, P. D. (2005) J. Virol. 79, 15567–15572
6. Song, B., Javanbakht, H., Perron, M., Park, D. H., Stremlau, M., and Sodroski, J. (2005) J. Virol. 79, 3930–3937
7. Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D. J., Sundquist, W. I., and Sodroski, J. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 5514–5519
8. Yap, M. W., Niso, S., and Stoye, J. P. (2005) Curr. Biol. 15, 73–78
9. Keckesova, Z., Yilnen, L. M., and Towers, G. J. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10780–10785
10. Diaz-Griffero, F., Vandegraft, N., Li, Y., McGee-Estrada, K., Stremlau, M., Welikala, S., Si, Z., Engelman, A., and Sodroski, J. (2006) Virology 351, 404–419
11. Javanbakht, H., Diaz-Griffero, F., Stremlau, M., Si, Z., and Sodroski, J. (2005) J. Biol. Chem. 280, 26933–26940
12. Song, B., Diaz-Griffero, F., Park, D. H., Rogers, T., Stremlau, M., and Sodroski, J. (2005) Virology 343, 201–211
13. Campbell, E. M., Perez, O., Anderson, J. L., and Hope, T. J. (2008) J. Cell Biol. 180, 549–561
14. Rold, C. J., and Aiken, C. (2008) PLoS Pathog. 4, e1000074
15. Zhang, X., Kondo, M., Chen, J., Miyoshi, H., Suzuki, H., Ohashi, T., and Shida, H. (2010) Microbes Infect. 10, 768–777
