Functional Evidence for the Involvement of Microtubules and Dynein Motor Complexes in TRIM5α-Mediated Restriction of Retroviruses

Paulina Pawlica, Valerie Le Sage, Nolwenn Poccardi, Michel J. Tremblay, Andrew J. Mouland and Lionel Berthoux

Published Ahead of Print 5 March 2014.

Updated information and services can be found at:
http://jvi.asm.org/content/88/10/5661

These include:

REFERENCES
This article cites 84 articles, 47 of which can be accessed free at: http://jvi.asm.org/content/88/10/5661#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Functional Evidence for the Involvement of Microtubules and Dynein Motor Complexes in TRIM5α-Mediated Restriction of Retroviruses

Paulina Pawlica, a Valentine Le Sage, b Nolwenn Poccardi, a Michel J. Tremblay, c Andrew J. Mouland, b Lionel Berthoux a

Laboratory of Retrovirology, Department of Medical Biology and BioMed Group, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada; HIV-1 RNA Trafficking Laboratory, Lady Davis Institute at the Jewish General Hospital and Department of Medicine, McGill University, Montréal, Québec, Canada; Centre de Recherche en Infectiologie, Centre de Recherche du CHU de l’Université Laval, Québec, Canada.

ABSTRACT

The tripartite motif (TRIM) family of proteins includes the TRIM5α antiretroviral restriction factor. TRIM5α from many Old World and some New World monkeys can restrict the human immunodeficiency virus type 1 (HIV-1), while human TRIM5α restricts N-tropic murine leukemia virus (N-MLV). TRIM5α forms highly dynamic cytoplasmic bodies (CBs) that associate with and translocate on microtubules. However, the functional involvement of microtubules or other cytoskeleton-associated factors in the viral restriction process had not been shown. Here, we demonstrate the dependency of TRIM5α-mediated restriction on microtubule-mediated transport. Pharmacological disruption of the microtubule network using nocodazole or disabling it using paclitaxel (originally named taxol) decreased the restriction of N-MLV and HIV-1 by human or simian alleles of TRIM5α, respectively. In addition, pharmacological inhibition of dynein motor complexes using erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and small interfering RNA-mediated depletion of the dynein heavy chain (DHC) similarly decreased TRIM5α-mediated restriction. The loss in restriction resulting from either the disassembly of microtubules or the disruption of dynein motor activity was seen for both endogenous and overexpressed TRIM5α and was not due to differences in protein stability or cell viability. Both nocodazole treatment and DHC depletion interfered with the dynamics of TRIM5α CBs, increasing their size and altering their intracellular localization. In addition, nocodazole, paclitaxel, and DHC depletion were all found to increase the stability of HIV-1 cores in infected cells, providing an alternative explanation for the decreased restriction. In conclusion, association with microtubules and the translocation activity of dynein motor complexes are required to achieve efficient restriction by TRIM5α.

IMPORTANCE

The primate innate cellular defenses against infection by retroviruses include a protein named TRIM5α, belonging to the family of restriction factors. TRIM5α is present in the cytoplasm, where it can intercept incoming retroviruses shortly after their entry. How TRIM5α manages to be present at the appropriate subcytoplasmic location to interact with its target is unknown. We hypothesized that TRIM5α, either as a soluble protein or a high-molecular-weight complex (the cytoplasmic body), is transported within the cytoplasm by a molecular motor called the dynein complex, which is known to interact with and move along microtubules. Our results show that destructuring microtubules or crippling their function decreased the capacity of human or simian TRIM5α to restrict their retroviral targets. Inhibiting dynein motor activity, or reducing the expression of a key component of this complex, similarly affected TRIM5α-mediated restriction. Thus, we have identified specific cytoskeleton structures involved in innate antiretroviral defenses.

Members of the tripartite motif (TRIM) family of proteins have been described to exhibit antiviral properties (1–4). The best-known member is TRIM5α, first characterized as a factor from rhesus macaque (rhTRIM5α) that potently inhibits human immunodeficiency virus 1 (HIV-1) (5). Other TRIM5α orthologs from some New World and Old World monkeys also provide protection against HIV-1 infection (6–8). Human TRIM5α (huTRIM5α), while not having the ability to restrict HIV-1, protects against N-tropic murine leukemia virus (N-MLV) and equine infectious anemia virus (6, 7, 9–11). Expression of TRIM5α is induced by type 1 interferons, supporting their role as innate immunity effectors (12, 13). In addition, TRIM5α acts as an innate sensor of retroviral infections, triggering an antiviral signaling pathway that can lead to interferon production (14, 15).

Retroviral restriction is initiated by specific recognition of the N-terminal domain of incoming retroviral capsid (CA) proteins by the B30.2/PRYSPRY domain of TRIM5α (16, 17). TRIM5α binds to intact CA cores rather than monomeric CA proteins (18) and, as a result of this interaction, replication is impaired by several effector mechanisms (reviewed in references 19, 20, and 21). Thus far, two major TRIM5α-mediated blocks have been described. The first one is accelerated disassembly of the retroviral CA core accompanied by a decrease in amounts of reverse transcription products (17, 22–24). As a consequence of the disassembly induced by TRIM5α, core components such as the viral RNA and integrase are solubilized or degraded (25). This restriction effector mechanism also involves the degradation of TRIM5α by the proteasome in the presence of the restricted virus (26). Accordingly, treatment with proteasome inhibitors restores seem
ingly normal disassembly of the viral core and rescues the production of viral cDNA (25, 27, 28). However, proteasome inhibition does not fully rescue infectivity in restrictive conditions, pointing to the existence of a second, proteasome-independent restriction mechanism. The precise mechanism of this second block is still unclear, but access of the viral DNA to the nucleus is inhibited (27, 29). This could be related to the “sequestration” of incoming retroviruses in cytoplasmic bodies (CBs) formed by TRIM5α proteins (30, 31).

TRIM5α contains a coiled-coil domain responsible for protein dimerization and a B-box domain important for the higher-order organized states that probably promote the formation of CBs (32, 33). CBs were described as dynamic structures constantly associating and disassociating with each other, exchanging TRIM5α proteins with a pool of proteins diffused in the cytoplasm (34), and their size depends on the level of TRIM5α expression (35). The role of CBs in retroviral restriction is still unclear, and some reports refute their relevance in this process (35, 36). Indeed, no CBs have been detected at endogenous TRIM5α expression levels, and it is possible that some observed CBs are artifacts stemming from protein overexpression (36). On the other hand, TRIM5α CBs were found to colocalize with ubiquitin (30), proteasomal subunits (31, 37), and p62/Sequestosome-1 (38). p62 is an important adaptor protein with a role in cell signaling and protein degradation (reviewed in references 39 and 40). In addition, TRIM5α proteins form bodies that enclose incoming restriction-sensitive viruses and closely resemble preexisting CBs (30). Collectively, these observations suggest that TRIM5α CBs are relevant to restriction mechanisms.

The microtubule network, a component of the cellular cytoskeleton, is made of highly dynamic filaments built of tubulin α/β heterodimers and plays multiple roles in the cell, including intracellular transport, organelle positioning, and cell division (41). Microtubules provide platforms for molecular motors, which enable active transport through the dense cytoplasm of the cell. The dynein motor complex (reviewed in reference 42) is a microtubule-associated molecular motor that transports various cellular cargos toward the microtubule-organizing center (MTOC) at the minus-end of microtubules. The MTOC is found in the vicinity of the nucleus except during cell division. Several viruses, including HIV-1, were described as recruiting dynein motor complexes for their transport during the early stages of their replication (reviewed in references 43, 44, and 45). TRIM5α CBs also associate with microtubules, and their movements along these filaments have been observed (34). However, a functional role for this association has not been demonstrated, nor have the molecular motors responsible for TRIM5α movement been identified. We sought here to determine whether the integrity of microtubules was functionally important for restriction to occur. In addition, we investigated the role of the dynein motor in this process. Using pharmacological and genetic approaches coupled with imaging analyses, we provide evidence that both microtubules and dynein motor activity are important for the restriction process mediated by TRIM5α.

**MATERIALS AND METHODS**

**Cells, pharmaceuticals, and antibodies.** Human embryonic kidney 293T cells, human epithelial carcinoma HeLa cells, human U373-derived MAGI cells, rhesus macaque kidney FRHK-4 cells, and feline renal CRFK cells were maintained in Dulbecco modified Eagle medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum and antibiotics at 37°C and 5% CO₂. All cell culture reagents were from HyClone (Thermo Scientific, Logan, UT). HeLa cells stably expressing FLAG-tagged TRIM5 proteins were generated by retroviral transduction as described previously (22, 46). Nodocaldoze, paclitaxel (originally named taxol), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), phenazine methosulfate (PMS), heparin sodium salt, and cycloheximide were provided by Sigma (St. Louis, MO). Anti-dynein heavy chain (anti-DHC) rabbit polyclonal antibodies were obtained from Santa Cruz (Dallas, TX). The horseradish peroxidase (HRP)-conjugated mouse anti-actin antibody was from Sigma. Capsid (CA; p24) was detected by using a mouse monoclonal antibody (clone 183) from the AIDS Research and Reference Reagent Program. The FLAG epitope was detected using the M2 mouse monoclonal antibody (Sigma) or the M2 rabbit polyclonal antibody from Cell Signaling (Danvers, MA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies used as secondary antibodies in Western blots were all from Santa Cruz.

**Plasmid DNAs and retroviral vectors production.** The plasmid encoding a green fluorescent protein (GFP)-tagged version of α-tubulin (47) was a gift from Ali Saib. To produce viral vectors, 10-cm culture dishes of subconfluent HEK293T cells were cotransfected using polyethylenimine (25 kDa; Polyscience) with the appropriate plasmids as follows. For wild-type (WT) or the G89V CA mutant of HIV-1, we used 10 μg of pTRIP_CMV-GFP, 10 μg of WT or G89V pΔR8.9, and 5 μg of pMD-G. For N-MLV-GFP and B-MLV-GFP, we used 10 μg of pCNCG, 5 μg of pMD-G and 10 μg of pCIGN (N-MLV_GFP) or pCIG3B (B-MLV-GFP). For HIV-1NL43-GFP, we used 10 μg of pNL-GFP and 5 μg of pMD-G. For SIVmac-GFP, we used 10 μg of pSIV_bac-GFP and 5 μg of pMD-G (22, 48–54). pNL4.3-INRES-GFP (55) encodes a version of the HIV-1 NL4-3 strain (56) expressing GFP in addition to the viral proteins and was a gift from David N. Levy. Production of the corresponding virus (HIV-1NL4.3-INRES-GFP) was done by transfecting 10 μg of the plasmid in subconfluent HEK293T cells in a 75-cm flask. Media were changed at 16 h posttransfection, and virus-containing supernatants were collected after an additional 32 h of culture. All viral stocks were clarified by centrifugation for 5 min at a relative centrifugal force (RCF) of 400. All viral vectors were titrated in permissive CRFK cell using GFP expression as a marker of successful transduction. In some experiments, reverse transcriptase activity was measured on virus stocks using the EnzChek kit (Molecular Probes) according to the manufacturer’s instructions.

**Viral challenges, pharmacological treatments, and RNA interference.** Cells were seeded in 24-well plates at 10⁵ cells/well (HeLa, CRFK, and MAGI cells) or 5 × 10⁴ cells/well (FRHK-4 cells) and were challenged the next day with various retroviral vectors (HIV-1NL4.3-INRES-GFP, HIV-1NL4.3-INRES, SIVmac-GFP, N-MLV_GFP, or B-MLV_GFP) or a replication-competent retrovirus (HIV-1NL4.3-INRES-GFP). When applicable, cells were pretreated for 15 min with nocodazole, paclitaxel, or EHNA, and infections were then performed in the presence of these drugs. Media were changed after 16 h, and at 48 h postinfection the cells were treated with trypsin and fixed in 2% formaldehyde (Fisher Scientific) in phosphate-buffered saline (PBS). The percentages of GFP-positive cells were then determined by analyzing 1 × 10⁴ to 3 × 10⁴ cells on a FC500 MPL cytometer (Beckman Coulter) using the CXP software. For the siRNA treatments, 2 × 10⁵ cells were plated in each 3.5-cm of a six-well plate and transfectected with 40 nM small interfering RNA (siRNA) using DharmaFECT 1 (Dharmacon). The siRNA against the heavy chain of dynein (DHC) has been previously described (57) and targets the sequence 5'-GATCGGAGATT. Control siRNA (purchased from Dharmacon) was designed to target a luciferase sequence (5'-CGTAGGCGGAAATCTTCGAT) absent in the human genome. At 48 h posttransfection, the cells were seeded in 24-well plates and were challenged the next day with retroviral vectors as described above.

**Stability assay.** A total of 10⁶ HeLa cells stably expressing FLAG-rHTRIM5α were seeded in six-well plates 1 day prior to the experiment.
For the siRNA treatments, cells were transfected 48 h before seeding, as described above. Cells were pretreated for 1 h with 100 µg of cycloheximide/ml and then treated with the indicated drugs without removing cycloheximide and harvested at the indicated time points. Drug concentrations were as follows: nocodazole, 0.1 µM; paclitaxel, 0.1 µM; and EHNA, 600 µM. Cells were lysed in cold stability buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with Complete protease inhibitor cocktail (Roche, Bale, Switzerland) and processed for Western blotting. rhTRIM5α was detected by using the anti-FLAG rabbit polyclonal antibody.

Viability assay. A total of 1 × 10^4 HeLa cells or 5 × 10^5 FRHK-4 cells were seeded per well in 96-well plates. The next day, the cells were washed with PBS, and the wells were repleted with 100 µl of DMEM without phenol red supplemented with serum and serial dilutions of the tested drugs. After 16 h of incubation, 100 µl of the PBS solution containing 50 µg of XTT and 6 µg of PMS was added. The cells were incubated 2 to 4 h at 37°C, and then the absorbance was read at 490 nm on a Synergy HT (Bio-Tek) plate reader and corrected for background values determined on blank samples.

Fate-of-capsid assay. To analyze postentry capsid disassembly, a protocol adapted from Perron et al. (7) was used as described earlier by Keckesova et al. (7). Briefly, 3 × 10^5 HeLa cells seeded in 10-cm dishes were infected with HIV-1_GFP at a multiplicity of infection (MOI) of 0.1, as calculated on the permissive control cells. Then, 2 h later, virus-containing supernatants were removed, and the cells were rinsed once in PBS, followed by a gentle trypsinization treatment (a 1:1 trypsin-PBS mixture for 10 s at room temperature). Fresh medium containing the appropriate drugs was then added, and cells were incubated at 37°C at 5% CO₂ for an additional 4 h. The cells were then treated with trypsin and resuspended in ice-cold lysis buffer (100 mM Tris-HCl [pH 8.0], 0.4 mM KCl, 2 mM EDTA, Roche’s Complete protease inhibitor) and disrupted with a Dounce homogenizer. Whole-cell lysate (WCL) samples were collected at this point. In order to remove cell debris and nuclei, lysates were centrifuged for 5 min at an RCF of 1,000 at 4°C and then layered on top of a 50% sucrose cushion prepared in STE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Particulate viral cores were sedimented by ultracentrifugation in a Sorval WX Ultra 100 ultracentrifuge at 175,000 × g for 2 h at 4°C. Pellets were resuspended in denaturing gel loading buffer and processed for CA Western blotting, together with whole-cell lysates. The postcentrifugation supernatants were collected from the fraction above the sucrose cushion, excluding the sucrose-supernatant interface.

Immunofluorescence (IF) microscopy. For the analysis of the localization of LAMP-1 and microtubules, 2 × 10^5 cells (HeLa) or 1 × 10^5 cells (FRHK-4) were seeded on glass coverslips placed in 3.5-cm wells. For siRNA treatments, cells were transfected 48 h prior to seeding as described above. For the cells expressing GFP-tubulin, 2 µg of the plasmid were transfected per well 24 h prior to seeding. The day after seeding, cell were fixed (siRNA treatment) or incubated for 2 h with drugs (nocodazole, paclitaxel, and EHNA) and then fixed. Fixation was done for 10 min in prewarmed 4% formaldehyde-DMEM at 37°C, followed by three washes with ice-cold PBS. The cells were then permeabilized by treatment with 0.1% Triton X-100 – 0.1 mM sodium citrate for 1 to 2 min on ice. The cells were then washed again three times with PBS and treated with 10% normal goat serum (Sigma) containing 0.3 M glycine (Sigma) for 30 min at room temperature. This was followed by a 4-h incubation with a murine antibody against the FLAG epitope diluted 1:400 in PBS containing 10% normal goat serum. The cells were washed five times and fluorescently stained with the Alexa Fluor 594-conjugated goat anti-mouse antibody (Molecular Probes) at a 1:200 dilution. The cells were washed five times in PBS before mounting them in Vectashield (Vector Laboratories, Burlington, Ontario, Canada). Hoechst 33342 (0.8 µg/ml; Molecular Probes) was added, along with the penultimate PBS wash, to visualize the DNA. Z-stacks were acquired on an AxioObserver microscope (Zeiss, Toronto, Ontario, Canada) equipped with the Apotome module, and median Z-stacks were retained for analysis. For the analysis of TRIM5α CB sizes, FLAG foci in a given cell were manually outlined in the AxioVision software for calculation of the surface. A minimum of 100 to 214 CBs from 10 randomly chosen cells were included in the analysis. For the analysis of TRIM5α CB localization, the cell’s edge was outlined, and for each FLAG focus in a given cell, we measured the closest distance to the nuclear membrane and the closest distance to the plasma membrane using AxioVision. A minimum of 85 and up to 440 CBs from a minimum of five randomly chosen cells were included in the analysis. To avoid human bias, CBs size and localization analyses were performed blindly by students not otherwise involved in this project using image files that had coded names. For the analysis of TRIM5α microtubule colocalization, images were acquired by using the Apotome in raw data mode to allow for subsequent deconvolution, which was done using the AxioVision software (Carl-Zeiss).

RESULTS

Nocodazole and paclitaxel treatments rescue the infectivity of retroviruses restricted by endogenous TRIM5α. In order to determine whether the microtubule network has a functional role in retroviral restrictions mediated by TRIM5α, we used nocodazole and paclitaxel, pharmacological agents that prevent the polymerization of microtubules (58) or block their dynamics by preventing disassembly (59), respectively. First, we tested the effect of nocodazole and paclitaxel on the restriction of HIV-1 or N-MLV at multiple drug concentrations (Fig. 1). In this set of experiments, we infected human HeLa cells with N-MLV_GFP, a huTRIM5α-sensitive N-tropic MLV vector expressing GFP and, as a control, its restriction-insensitive B-tropic counterpart (B-MLV). Similarly, we infected rhesus macaque FRHK-4 cells with a rHTRIM5α-sensitive HIV-1 vector expressing GFP or, as a control, with SIVmac_GFP, a rHTRIM5α-insensitive simian immunodeficiency virus (SIV) strain mac239-based vector also expressing GFP. These infections were accomplished using virus doses that had been previously determined to result in 0.1 to 1% infected (GFP-positive) cells in the absence of drug. In these experimental conditions, we found that both nocodazole and paclitaxel in-
interference with the microtubule network. Paclitaxel treatments of nocodazolized HeLa cells (Fig. 1) indicated that disruption of microtubules by nocodazole or paclitaxel increased HIV-1NL43-GFP infection by up to 8.5-fold (Fig. 1B). In these cells, nocodazole was seen to promote the formation of abnormal microtubule bundles (65), a phenotype that was observed in both HeLa and FRhK-4 cell lines (Fig. 2D). Collectively, the data in Fig. 1 and 2 show that disrupting the microtubule network by nocodazole or paclitaxel treatments relative to the magnitude of the restriction itself. These results also did not allow us to analyze whether the effect seen was dependent on the MOI used. Therefore, we performed virus dose-dependent experiments using fixed drug concentrations. We used nocodazole and paclitaxel concentrations that corresponded to their peak of antirestriction activity as determined in Fig. 1. When the virus amounts used were normalized according to the infectivity in nonrestrictive CRFK cells (CRFK infectious units [IU]), we found N-MLV<sub>GFP</sub> to be to be ~500-fold less infectious than B-MLV<sub>GFP</sub> in HeLa cells (Fig. 2A), reflecting the expected level of N-MLV restriction by endogenous huTRIM5α in these cells (7). Permissiveness to the huTRIM5α-insensitive B-MLV vector was not significantly affected by either nocodazole or paclitaxel treatments, regardless of the virus amounts used (Fig. 2A). In contrast, N-MLV infectivity was increased by up to 65-fold after nocodazole treatment and by up to 71-fold after paclitaxel treatment (Fig. 2A). In other words, nocodazole and paclitaxel reduced restriction of N-MLV to a level of only ~20-fold in HeLa cells. In these cells, the effects of nocodazole and paclitaxel on N-MLV were greatest when a relatively small amount of virus was used (5 CRFK IU), whereas there was only an ~7-fold increase in N-MLV infection at MOIs 10-fold higher (Fig. 2A). These observations probably reflect the fact that saturation of endogenous huTRIM5α by large amounts of N-MLV capsids partly suppresses restriction in the absence of pharmacological treatment.

As expected, HIV-1NL43-GFP was strongly restricted (~1,000-fold) relative to SIV<sub>mac</sub>-GFP in macaque FRhK-4 cells, when the two viruses were normalized according to their infectious titers in CRFK cells (Fig. 2B) (49, 61). Nocodazole and paclitaxel had no effect on infection by SIV<sub>mac</sub>-GFP regardless of the amount of virus used (Fig. 2B). In contrast, both nocodazole and paclitaxel increased infection by HIV-1NL43-GFP in these cells by 16- to 17-fold at low MOIs. When the virus dose used was >100 CRFK IU, the enhancing effects of nocodazole and paclitaxel was larger, which again was probably due to saturation of TRIM5α by incoming capsids. The cyclophilin A (CypA) binding loop of CA is a major determinant of HIV-1 sensitivity to restriction by TRIM5α (62), and the CA-G89V mutant, which abrogates CypA binding (63), is known to be less susceptible to restriction by rhTRIM5α (49, 64). Thus, we hypothesized that nocodazole and paclitaxel treatments would have a smaller enhancing effect on CA-G89V HIV-1 compared to its WT counterpart. Indeed, we found that in subsaturating conditions (<200 CRFK IU), nocodazole and paclitaxel increased WT HIV-1<sub>CMV-GFP</sub> infection by ~10-fold (5.6- to 16-fold) and ~7.5-fold (4.7- to 10.5-fold), respectively (Fig. 2C). In contrast, nocodazole and paclitaxel increased the infectivity of the mutant virus by only ~3.7-fold (2.3- to 5.0-fold) and ~3.4-fold (2.0- to 5.7-fold), respectively (Fig. 2C).

In order to verify that nocodazole and paclitaxel had the expected effect on the microtubule network in the cell lines used, we transfected a construct expressing a GFP–α-tubulin fusion in HeLa and FRhK-4 cells and then treated the cells with the same concentrations of nocodazole and paclitaxel as those used in Fig. 2A to C. The cells were then processed for IF analysis (Fig. 2D). Nocodazole prevents polymerization of microtubules (58) and, consequently, microtubules appeared shortened and/or disassociated; most of the signal was diffuse and distributed throughout the cytoplasm (Fig. 2D). Paclitaxel, on the other hand, binds to microtubule polymers to prevent their disassembly (59), resulting in the formation of abnormal microtubule bundles (65), a phenotype that was observed in both HeLa and FRhK-4 cell lines (Fig. 2D). Collectively, the data in Fig. 1 and 2 show that disrupting the microtubule network...
dynamics (either assembly or disassembly) of microtubules results in a decrease of restriction by endogenous TRIM5α without inhibiting it completely. Interestingly, when drug concentrations were optimized, we observed that nocodazole and paclitaxel had very similar effects on N-MLV or HIV-1 (Fig. 2A to C), suggesting that disruption of the microtubule network inhibited TRIM5α regardless of the drug’s mechanism of action.

Pharmacological inhibition of dynein function rescues the infectivity of TRIM5α-restricted retroviruses. TRIM5α CBs are associated with microtubules, and their movements within the cytoplasm seem to be at least partly dependent upon them (34). However, the molecular motors driving TRIM5α CBs movements along microtubules are not known. We hypothesized that dynein played a role in TRIM5α localization and contributed to its antiretroviral activity. We first infected HeLa cells with N- or B-MLV_GFP viral vectors in the presence of increasing concentrations of EHNA (Fig. 3A), a drug that inhibits the ATPase activity associated with the heavy chain of axonemal and cytoplasmic dyneins (66, 67). In the presence of EHNA, N-MLV_GFP infectivity increased by up to 22-fold compared to the vehicle control, while the EHNA treatment caused a drug concentration-dependent decrease in permissiveness to B-MLV (Fig. 3A). In macaque FRhK-4 cells, EHNA increased permissiveness to HIV-1NL43-GFP by up to 18.5-fold in a drug concentration-dependent fashion (Fig. 3B). In contrast, EHNA slightly decreased (<2-fold) infection by the restriction-insensitive SIVmac_GFP. We then performed the reverse experiments, infecting the cells at a fixed EHNA concentration but using multiple MOIs. Viruses were equalized as before, based on their titers in the nonrestrictive CRFK cells. In HeLa cells, treatment with 600 μM EHNA had no effect on B-MLV_GFP infectivity, but it increased permissiveness to N-MLV_GFP infection by up to 22-fold, depending on the MOI (Fig. 3C). In FRhK-4 cells (Fig. 3D), EHNA (1.2 mM) enhanced HIV-1NL43-GFP infection at sub-saturating MOIs (∼100 CRFK IU) by 7.5-fold on average (3.2- to 13.5-fold). In contrast, EHNA caused a reduction in SIVmac_GFP infectivity to ∼0.4-fold in the untreated control (Fig. 3D). Thus, the magnitude of HIV-1 enhancement by EHNA in FRhK-4 cells is probably underestimated in this experiment due to the negative effect of the drug on infectivity, as seen with SIVmac_GFP. In order to verify that the EHNA treatments indeed affected dynein function, we analyzed the subcellular distribution of LAMP-1, a marker for late endosomes (68). Impairment of dynein function causes a shift of late endosomes toward the cell periphery, as previously described by us (57) and others (69). Cells were stained for LAMP-1 after treatment or not with 600 μM (HeLa) or 1.2 mM (FRhK-4) of EHNA. We then counted the number of cells exhibiting a juxtanuclear localization of LAMP-1 versus the ones with peripheral localization (Fig. 3E). In the dimethyl sulfoxide-treated

![FIG 2](http://jvi.asm.org/figures/2014/08/10/005665/f2a.png) Pharmacological disruption of microtubules decreases endogenous TRIM5α-mediated retroviral restriction. (A to C) Effect of nocodazole (noc) and paclitaxel (txl) on restriction. Human HeLa cells (A) or macaque FRhK-4 cells (B and C) were infected with multiples doses of N-MLV_GFP, B-MLV_GFP, HIV-1NL43-GFP, or SIVmac_GFP as indicated. In panel C, FRhK-4 cells were infected with WT HIV-1CMV-GFP or with the CA-G89V mutant of this vector. Infections were performed for 16 h and in the absence of drug or in the presence of either nocodazole or paclitaxel. Nocodazole was used at 0.1 μM in HeLa cells and at 6 μM in FRhK-4 cells, and paclitaxel was used at 0.1 μM in HeLa cells and at 2 μM in FRhK-4 cells. The x axis in each graph represents the amounts of virus used expressed in infectious units (IU) based on infectious titers calculated for each virus in permisive feline CRFK cells. Infected (GFP-expressing) cells were detected by flow cytometry at 2 days postinfection. (D) IF microscopy analysis of microtubules in treated cells. HeLa and FRhK-4 cells were transfected with GFP-tubulin and 2 days later were subjected to 2-h drug treatments using the concentrations described above and then fixed. GFP fluorescence was observed by IF microscopy, along with DNA, which was stained using DAPI (blue staining). taxol, paclitaxel. The scale bars on the images represent 10 μm. A representative image from each condition is presented.
control cells, the localization of LAMP-1 was predominantly juxtanuclear. Specifically, 92% ± 2.1% (the standard deviation) HeLa cells and 87% ± 1.5% FRhK-4 cells had juxtanuclear LAMP-1. As exemplified in Fig. 3E, EHNA treatment caused significant decreases in juxtanuclear LAMP-1, which represented 11% ± 4.6% and 8.6% ± 1.7% of total LAMP-1 in HeLa cells and FRhK-4 cells, respectively. In conclusion, EHNA can rescue both N-MLV and HIV-1 from restriction by different orthologs of endogenously expressed TRIM5 but does not totally block restriction.

Depletion of DHC counteracts TRIM5α-mediated retroviral restriction. In order to directly test the hypothesis that dynein function is important for TRIM5α-mediated restriction, we depleted dynein heavy chain (DHC) by transfection of an siRNA, as described previously (57). Knocking down DHC is known to disrupt all dynein-mediated transport activities, and it can also affect the assembly of microtubules by inhibiting the anterograde transport of microtubule complexes (70). HeLa cells were transfected with a siRNA targeting DHC or with an irrelevant siRNA targeting luciferase (Luc). Knockdown of DHC was efficient (Fig. 4A), resulting in an 85.4% ± 6.7% reduction in protein levels, as estimated by Western blotting in four independent experiments. In addition, DHC depletion caused a redistribution of LAMP-1 from juxtanuclear to peripheral (Fig. 4B), similar to what we had previously published (57). Specifically, 86.6% of HeLa cells transfected with the control siRNA had juxtanuclear staining compared to 35.5% for the cells transfected with the siRNA targeting DHC. As shown in Fig. 4C, DHC knockdown caused a significant increase in HeLa permissiveness to N-MLV GFP infection at all MOIs examined (4.6-fold on average; range, 4.0- to 6.0-fold), while having no effect on infection by B-MLV GFP. Thus, dynein motor complexes are involved in the restriction of N-MLV by endogenous huTRIM5α. Since dynein’s transport function is dependent on microtubules, we predicted that combining pharmacological disruption of microtubules and DHC depletion would have nonadditive effects. Therefore, we analyzed the permissiveness to infection by N-MLV GFP and B-MLV GFP of cells depleted or not for DHC, in the presence of nocodazole or paclitaxel. As
shown in Fig. 4D, nocodazole alone increased N-MLV infection by an average of 23-fold (range, 11- to 34-fold), while dual treatment with nocodazole and DHC siRNAs resulted in a slightly smaller increase in N-MLV GFP infectivity (13-fold on average; range, 8- to 19-fold). Treatment with paclitaxel resulted in an average increase of 16.7-fold (range, 13- to 24-fold) in permissiveness to N-MLV GFP, and the enhancement effect was only slightly bigger (23-fold; range, 18- to 28-fold) when DHC depletion was combined with paclitaxel treatment (Fig. 4E). Altogether, the results in Fig. 4D and E show that disruption of the microtubule network and DHC depletion had non-additive effects on the restriction of N-MLV by endogenous huTRIM5α.

Inhibition of HIV-1 restriction by overexpressed rhTRIM5α in human cells is counteracted by depletion of DHC or microtubule disruption. The experimental results shown in Fig. 1 to 4 indicate that restriction of HIV-1 or N-MLV by endogenous huTRIM5α or rhTRIM5α cells is partly suppressed by DHC knockdown or by pharmacological disruption of microtubules using nocodazole or paclitaxel. We therefore decided to determine whether these interventions would also inhibit HIV-1 restriction in cells in which exogenous FLAG-tagged rhTRIM5α was overexpressed. rhTRIM5α was expressed in HeLa cells through retroviral transfer, and nontransduced cells were eliminated by puromycin treatment. We infected the transduced cells with the restriction-sensitive HIV-1 vectors HIV-1CMV-GFP and HIV-1NL4-3-GFP, as well as the rhTRIM5α-insensitive SIVmac-GFP (Fig. 5A). The two vectors used differ in that HIV-1CMV-GFP does not carry the viral products Vpr, Nef, Vif, and Vpu, and no viral proteins are expressed following integration (71). In contrast, HIV-1NL4-3-GFP encodes all HIV-1 proteins with the exception of Env and Nef, and viral proteins are expressed in infected cells (72). Restriction was observed for both viruses in HeLa-rhTRIM5α cells, compared to SIVmac-GFP and after normalization of viral stocks according to their titers on CRFK cells (Fig. 5A). Specifically, HIV-1CMV-GFP and HIV-1NL4-3-GFP were restricted ~40-fold and between ~48-
and 78-fold, respectively. Nocodazole and paclitaxel increased permissiveness to HIV-1-CMV-GFP by 10.1-fold and 9.8-fold, on average, and they increased permissiveness to HIV-1NL43-GFP by averages of 17.8- and 15.6-fold, respectively (Fig. 5A). None of the drug treatments had a significant effect on infection by SIVmac-GFP although paclitaxel seemed to increase HIV-1 infectivity at relatively low MOIs and decrease it at relatively high MOIs (Fig. 5A). Next, we used FLAG-rhTRIM5α-transduced HeLa cells transfected with the control (luciferase-targeting) siRNA or with the siRNA targeting DHC (Fig. 5B). In this experiment, restriction of HIV-1NL43-GFP by rhTRIM5α was particularly high (>1,000-fold). We found that cells depleted of DHC were more permissive to infection by HIV-1NL43-GFP than cells transfected with the control siRNA (an 11.1-fold increase on average). In contrast, DHC knockdown slightly decreased infection by the control SIVmac-GFP vector (Fig. 5B).

In the next set of experiments (Fig. 5C to F), we compared the effect of nocodazole, paclitaxel, and DHC depletion on the permissiveness to HIV-1 of cells transduced with FLAG-rhTRIM5α or with a nonrestrictive control (huTRIM5α). The expression levels of huTRIM5α and rhTRIM5α were found to be comparable (Fig. 5F). Cells were infected at an MOI leading to ca. 1% infected cells in the absence of treatment, as in Fig. 1. The addition of nocodazole increased the permissiveness of HeLa-rhTRIM5α cells to HIV-1-CMV-GFP by (5.3 ± 0.21)-fold (Fig. 5C), while it had a much smaller effect on HeLa cells transduced with the "empty" vector (1.5 ± 0.04)-fold or HeLa cells transduced with huTRIM5α (1.46 ± 0.10)-fold. Likewise, treating the cells with paclitaxel increased permissiveness to HIV-1-CMV-GFP by (3.9 ± 0.33)-fold (Fig. 5D), while it slightly decreased infection of HeLa vector and HeLa-huTRIM5α cells [(0.67 ± 0.13)-fold and (0.63 ± 0.12)-fold, respectively]. Depleting DHC similarly increased permissiveness to HIV-1-CMV-GFP in cells expressing rhTRIM5α by (4.2 ± 0.38)-fold while having no effect in cells expressing the human ortholog (Fig. 5E). In conclusion, microtubule disruption with nocodazole and paclitaxel treatment and DHC depletion specifically inhibited the restriction of HIV-1 by exogenously expressed rhTRIM5α in human cells.

Nocodazole and paclitaxel inhibit TRIM5α-mediated restriction of an HIV-1 vector bearing autologous envelope proteins. The experiments shown in Fig. 1 to 5 were all performed using vesicular stomatitis virus protein G (VSV G)-pseudotyped vectors. TRIM5α-mediated restriction is not known to be affected by the mode of virus entry; nonetheless, we decided to verify that
were retrovirally transduced to express huTRIM5α expression levels. The amounts of virus used were adjusted to obtain 0.25 μg/ml. Cells were infected in triplicates with single doses of HIV-1NL43-IRES-GFP. The amounts of virus used were adjusted to obtain 0.01% of infected cells in the absence of the drug and yield 0.022% ± 0.005% infected MAGI [vector] cells, 0.16% ± 0.03% infected MAGI [huT5α] cells, and 0.043% ± 0.006% infected MAGI [rhT5α] cells. Supernatants were replaced with fresh medium containing heparin at 16 h postinfection in order to prevent re-infections. Infected (GFP-positive) cells were detected by flow cytometry 2 days postinfection, and the results are presented as the fold changes in the percentages of infected cells relative to the relevant untreated controls (relative change in infectivity). * and **, P = 0.0134 and P = 0.0068, respectively (Student t test). (B) Western blot analysis of FLAG-huTRIM5α and FLAG-rhTRIM5α expression in stably transduced MAGI cells. Actin was analyzed as a loading control.

nocodazole and paclitaxel would inhibit the TRIM5α-mediated restriction of a virus bearing its autologous envelope. For that, we used a fully infectious HIV-1 clone (HIV-1NL43-IRES-GFP) encoding GFP in addition to all of the other viral proteins. Human MAGI cells (73), which are U373 (glioblastoma) cells stably expressing the HIV-1 receptors and thus permissive for HIV-1 infection, were retrovirally transduced to express huTRIM5α or rhTRIM5α. rhTRIM5α was expressed at higher levels compared to huTRIM5α (Fig. 6B), which may reflect intrinsic differences in expression levels of the two proteins, since similar observations were made in other cellular contexts (22, 46). HIV-1 was restricted ~41-fold in the cells transduced with rhTRIM5α compared to control ("empty" vector-transduced) cells while, as expected, it was not restricted in cells transduced with huTRIM5α (data not shown). Cells transduced with either of the two TRIM5α orthologs or transduced with the empty vector were then infected with HIV-1NL43-IRES-GFP using amounts of the virus leading to ca. 0.1% infected cells and in the presence or absence of drug treatment. As shown Fig. 6A, nocodazole and paclitaxel increased the capacity of HIV-1 to infect cells transduced with rhTRIM5α by (6.1 ± 1.7)-fold and (12.1 ± 4.5)-fold, respectively. The drugs had no significant effect on HIV-1 infectivity in the nonrestrictive control cells. Thus, integrity of the microtubule network is important for the efficient restriction by TRIM5α regardless of the mechanism of virus entry.

Loss of restriction is not caused by decreased TRIM5α stability or by decreased cellular viability. Next, we investigated a possible impact of the treatments on the turnover/stability of TRIM5α. HeLa cells stably expressing FLAG-rhTRIM5α were either subjected to treatment with vehicle, nocodazole, paclitaxel, or EHNA or transfected with DHC or control siRNAs, using the same conditions as described above. De novo mRNA translation was inhibited using cycloheximide, and we monitored the decrease in protein levels for TRIM5α and for actin as a control (Fig. 7A and B). We found that the turnover of TRIM5α was not affected by the various treatments used. EHNA slightly decreased TRIM5α levels at a single time point (1 h of cycloheximide treatment), while DHC depletion also slightly decreased TRIM5α levels at 3 and 4.5 h of cycloheximide treatment (Fig. 7A). However, the results derived from three experiments revealed that these effects were not statistically significant (Fig. 7B).

Nocodazole, paclitaxel, and EHNA disrupt essential cellular functions, and it was thus important to ensure that the loss of restriction observed in our experimental conditions was not an artifact caused by gross cytotoxic effects. In all our infectivity assays, drugs are added for only 16 h and then removed, since only the first hours of infection are relevant to mechanisms of TRIM5α-mediated restriction. Thus, we examined cellular viability after 16-h treatments of HeLa and FRhK-4 cells with increasing concentrations of nocodazole, paclitaxel, and EHNA (Fig. 7C). Viability was monitored using the XTT assay, a colorimetric method to measure cellular metabolic activity, in particular the activity of dehydrogenase enzymes (74). Both nocodazole and paclitaxel caused a progressive but relatively modest loss in viability, obviously reflecting a concentration-dependent inhibition of cell division (75, 76). At the relatively low concentrations used for infectivity assays, nocodazole caused an ~19% decrease in the viability of HeLa cells and an ~32% decrease in the viability of FRhK-4 cells (Fig. 7C). Similarly, paclitaxel caused an ~23% decrease in the viability of HeLa cells and an ~20% decrease in the viability of FRhK-4 cells at the concentrations used in our restriction assays (Fig. 7C). In both cases, the concentrations used were well below the threshold at which the decrease in viability becomes sharper, probably reflecting the occurrence of cytotoxic mechanisms in addition to the inhibition of cell division. Based on our XTT data, we would estimate these cytotoxic effects to take place at concentrations greater than ~30 μM for nocodazole and ~20 μM for paclitaxel. We obtained a different pattern for EHNA: in both HeLa and FRhK-4 cells, viability decreased slowly to reach 85 to 90% of the control levels at a 1 mM concentration of the drug, but the drop in viability was much sharper at higher concentrations, and viability was fully lost at ~5 mM (Fig. 7C). At the concentrations used in our infectivity assays (600 μM in HeLa cells, 1.2 mM in FRhK-4 cells), EHNA caused an ~12% decrease in viability in HeLa cells and an 8% decrease in FRhK-4 cells (Fig. 7C). Thus, even though these concentrations are close to the concentrations at which viability starts declining sharply, cellular viability is still at the level of untreated cells, and it is unlikely that the effects of EHNA on restriction result from its cytotoxicity.

DHC depletion, nocodazole treatment, and paclitaxel treatment stabilize postentry HIV-1 core. A characteristic effect of TRIM5α-mediated postentry retroviral restriction is the increase in the CA core disassembly of restriction-sensitive retroviruses, i.e., a decrease in core stability. We used the well-established fate-of-capsid assay (17, 22, 77) to analyze the impact of nocodazole treatment, paclitaxel treatment, and DHC depletion on TRIM5α-mediated core disassembly. HeLa cells expressing rhTRIM5α or mock transduced with the empty vector were exposed to HIV-
In the presence or the absence of nocodazole, paclitaxel, or DHC siRNA. The efficiency of DHC knockdown was the same as in Fig. 4A (not shown). Cells were lysed and core-associated (pelletable) CA was isolated by ultracentrifugation through a sucrose cushion. The relative amounts of core-associated, soluble (postcentrifugation supernatant-associated), and total (whole-cell lysate [WCL]) CAp24 were assessed by densitometry of Western blots from three independent experiments (Fig. 8). The amounts of pelletable CA recovered were estimated relative to the total CA or relative to the soluble CA. As a control, cells were infected with a vector devoid of envelope proteins and thus incompetent for entry. Both virus preparations were normalized using a reverse transcription assay to ensure equal virus input. In the absence of the VSV G envelope, no or little CA signal was detectable in the infected cells, confirming that the CA detected was not associated with unfused viral particles (Fig. 8). As expected and in the absence of treatment, the relative amounts of pelletable CA cores were reduced in cells expressing rhTRIM5α, compared to the control cells transduced with the empty vector (Fig. 8). Specifically, the pellet/WCL CA ratios were reduced by 85.9% ± 9.0%, 70.4% ± 14.0%, and 75.7% ± 23.0%, respectively, in the experimental results shown in Fig. 8A, B, and C. Similarly, the pellet/supernatant CA ratio was reduced by 85.0% ± 12.7%, 72.2% ± 11.5%, and 88.4% ± 9.1% in cells expressing rhTRIM5α (Fig. 8A to C, right panels, respectively).

We observed that all of the treatments used had a stabilizing effect on postentry CA cores, both in permissive (control) cells and in restrictive (TRIM5α-expressing) cells (Fig. 8A to C). However, the increase in stability mediated by DHC depletion and paclitaxel treatment was greater in rhTRIM5α-expressing cells than in control cells, a finding consistent with the ability of these treatments to rescue infection. Specifically, DHC depletion in-
creased the pellet/WCL CA ratio by 1.28-fold in control cells, whereas this increase was by 3.10-fold in rhTRIM5α-expressing cells (Fig. 8A). Similarly, DHC depletion increased the pellet/supernatant CA ratio by 2.11-fold in control cells and by 5.78-fold in rhTRIM5α-expressing cells (Fig. 8A). Paclitaxel treatment increased the pellet/WCL CA ratio by 1.61-fold in control cells, while the corresponding increase in rhTRIM5α-expressing cells was 2.52-fold. Similarly, paclitaxel increased the pellet/supernatant CA ratio by 1.46-fold in control cells and by 3.87-fold in rhTRIM5α-expressing cells (Fig. 8B). We obtained different effects for nocodazole, since treatment with this drug sharply increased CA core stability both in permissive and in restrictive conditions (Fig. 8C). Specifically, nocodazole increased the pellet/WCL CA ratio by 3.94-fold in control cells and by 2.83-fold in rhTRIM5α-expressing cells. Similarly, the pellet/supernatant CA ratio was increased by 3.77-fold in control cells and by about the same amount (4.05-fold) in rhTRIM5α-expressing cells treated with this drug (Fig. 8C). Therefore, disrupting the microtubule network or the DHC motor has a stabilizing effect on postentry HIV-1 cores, which may decrease their susceptibility to TRIM5α-mediated restriction. In addition, the magnitude of this stabilizing effect was greater in cells expressing rhTRIM5α under treatment by paclitaxel or DHC siRNAs, which is consistent with these interventions specifically increasing HIV-1 infectivity in restrictive conditions. Nocodazole treatment increased postentry HIV-1 core stability equally in permissive and restrictive conditions, but its effect in permissive conditions was much stronger (3.94-fold) compared to DHC depletion (1.28-fold) or paclitaxel treatment (1.61-fold), perhaps masking the specific effect on restriction (see Discussion).
Effect of nocodazole treatment and DHC depletion on the size and localization of TRIM5 CBs. The capacity of nocodazole treatment and DHC depletion to reduce TRIM5α-mediated restriction could stem from effects on the dynamics of TRIM5α, which would affect the size and/or localization of CBs. HeLa cells stably expressing FLAG-tagged rhTRIM5α were treated with nocodazole or depleted for DHC and then stained for FLAG (Fig. 9A and B). The settings were adjusted to reveal CBs but not the diffuse FLAG signal. The size (area) of CBs and their relative distances to the nucleus and to the “edge of cell” (Fig. 9D) were calculated as detailed in Materials and Methods. Nocodazole treatment and DHC knockdown increased the average size of the CBs (Fig. 9C) and decreased their relative distances to the nucleus and to the “edge of cell” (Fig. 9D).
rhTRIM5α CBs by 33.9% ± 7.8% and by 52.6% ± 8.9%, respectively (Fig. 9C). In addition, rhTRIM5α CBs were found to be 31.5% ± 3.7% closer to the nucleus after nocodazole treatment (Fig. 9D). In contrast, DHC depletion caused a significant rhTRIM5α CBs localization shift (14.2% ± 2.8%) toward the plasma membrane (Fig. 9D). The results presented in Fig. 9 show that the microtubule network is important for the dynamics of TRIM5α CBs. Depletion of DHC and disruption of microtubules can have distinct effects on TRIM5α CBs, but the increased peripheral localization of CBs seen upon DHC depletion suggests that dynein motor complexes are responsible for their retrograde transport. To test more directly whether dynein is involved in the association of TRIM5α CBs with microtubules, we cotransfected HeLa cells stably expressing FLAG-rhTRIM5α with siRNAs targeting either DHC or an irrelevant control and with a plasmid expressing GFP–α-tubulin and then stained for FLAG (Fig. 9E and F). Images were acquired with the Apotome system and deconvolved to facilitate analysis, and colocalization was quantified from multiple randomly chosen cells. The depletion of DHC resulted in a modest (23% ± 0.3%) and yet statistically significant decrease in the colocalization of rhTRIM5α CBs and microtubules (Fig. 9G). Altogether, these results suggest that dynein motor complexes are involved in the association of TRIM5α CBs with microtubules and in the retrograde transport of either TRIM5α CBs.

**DISCUSSION**

In this study, we examined the importance of microtubules and of the microtubule-associated dynein motor complexes in the restriction of HIV-1 and N-MLV by TRIM5α. Pharmacological and siRNA-mediated depletion studies clearly show that the capacity of TRIM5α to efficiently restrict incoming retroviruses is dependent on intact microtubules and on a functioning dynein motor complex (Figs. 1 to 6). This was true regardless of (i) the retrovirus and TRIM5α ortholog studied, (ii) whether TRIM5α was expressed at endogenous levels or overexpressed through stable transduction, and (iii) the mechanism of virus entry. Nocodazole and paclitaxel, two drugs that disrupt the structure of the microtubule network through different mechanisms, had very similar, sometimes identical effects on retroviral restriction levels (Figs. 2 and 4). DHC depletion generally had a smaller effect on restriction than treatment with nociocazole or paclitaxel (Fig. 4A), which may be due to the observed incomplete knockdown (Fig. 4A). However, DHC depletion did not synergistically inhibit TRIM5α in the presence of either nocodazole (Fig. 4D) or paclitaxel (Fig. 4E), implying that these treatments all affected the same pathway. Finally, the observed impact of microtubules or dynein disruption on virus restriction was not caused by gross differences in expression levels or TRIM5α stability (Fig. 7A and B) and was not a putative artifact stemming from cytotoxicity (Fig. 7C).

Destabilization of the viral CA core upon interaction with TRIM5α is a hallmark of restriction and occurs in the hours following virus entry. We observed that DHC depletion, nocodazole treatment, and paclitaxel treatment all increased the relative amounts of pelletable CA, both in permissive and in restrictive conditions. This indicates that these treatments increase the stability of postentry HIV-1 CA cores. To our knowledge, this effect of interventions altering the cytoskeleton on HIV-1 disassembly had never been investigated before. Although the mechanism behind HIV-1 postentry CA core stabilization is unclear, it could contribute to making HIV-1 partly resistant to TRIM5α by countering the destabilizing effect of TRIM5α (78). The increase in the relative amounts of core-associated CA induced by DHC depletion or paclitaxel treatment was greater in cells expressing rhTRIM5α compared to control cells (Figs. 8A and B). This is consistent with these treatments specifically increasing HIV-1 infectivity in restrictive conditions. The results with nocodazole were more ambiguous, since this drug strongly increased the relative levels of core-associated CA (a 3- to 4-fold increase) both in permissive and in restrictive conditions (Fig. 8C). This apparent lack of specificity of nocodazole in this assay might be due to compound effects of the drug. For instance, nocodazole could increase the apparent stability in permissive conditions through an additional mechanism that does not take place in restrictive conditions, hence masking the restriction-specific effect that is seen with DHC siRNAs or paclitaxel. Consistent with this, nocodazole increased the relative amounts of core-associated CA in permissive cells about twice as much as DHC depletion or paclitaxel treatment did.

Nocodazole treatment increased the size of TRIM5α CBs and caused them to be positioned closer to the nucleus (Fig. 9), which is consistent with previous observations by Diaz-Griffero et al. (79). These data suggest that nocodazole interferes with the dynamics of TRIM5α CBs, perhaps by preventing the exchange of TRIM5α proteins between diffuse cytoplasmic population and CB-associated population (34). DHC depletion increased the average size of rhTRIM5α CBs (Fig. 9C) but also caused CBs to accumulate toward the cell periphery (Fig. 9D). In addition, there was a modest but significant decrease in the association between microtubules and rhTRIM5α (Fig. 9G). Altogether, these results suggest that the dynein motor translocates rhTRIM5α CBs on microtubules and is at least partly responsible for their retrograde transport. When DHC is depleted, rhTRIM5α CBs may shift toward the periphery of cells due to interactions with other molecular motors, such as the reverse polarity motor kinesin, which performs anterograde transport and can transport the same cargos as dynein does (80). Alternatively, DHC depletion could enhance the association of rhTRIM5α with late endosomes, as was observed previously for components of the HIV-1 ribonucleoprotein such as Gag and the genomic RNA (57). Nocodazole treatment and DHC depletion showed distinct phenotypes with regard to TRIM5α-mediated CA core disassembly (Fig. 8) and TRIM5α CBs localization (Fig. 9D), and yet their effects on restriction are clearly not additive (Fig. 4). Thus, it is likely that these phenotypic differences simply reflect different mechanisms of action by which the two treatments inhibit a single pathway.

Perhaps surprisingly, the various interventions used in the present study generally had little effect on the transduction of HIV-1, SIVmac, and MLV vectors in permissive conditions. Nocodazole was originally reported to modestly inhibit the early stages of HIV-1 infectivity in MAGI cells (81). However, other studies demonstrated a lack of HIV-1 sensitivity to this drug in human embryo kidney 293T cells (82) and in CEM T cells (83). At first glance, such a lack of inhibition by nocodazole contradicts a role for microtubules in HIV-1 postentry intracellular transport (84). However, the existence of a population of nocodazole-resistant microtubules, which are less dynamic, has been described (85), raising the possibility that HIV-1 uses these specific microtubules for its transport. Likewise, depleting DHC had a small effect on the infection of human permissive HeLa cells by an SIV-
mac vector (Fig. 5B), and no effect was seen on the infection of Hela cells by B-MLV (Fig. 4C) or HIV-1 (Fig. 5E) vectors. To the best of our knowledge, the only published evidence of a functional role for dynein motor complexes in HIV-1 postentry transport consisted in the injection of an antibody interfering with dynein motor function, leading to an ~50% decrease in HIV-1 movement toward the nucleus (84); however, this observation was not supported by infectivity data. Clearly, the precise mechanism of retroviral capsids transport toward the nucleus is yet to be determined (45). Regardless, our results show that disruption of the microtubule network mediates this step in the HIV life cycle toward the nucleus (84); however, this observation was not supported by infectivity data (45). Regardless, our results show that disruption of the microtubule network mediates this step in the HIV life cycle toward the nucleus (84); however, this observation was not supported by infectivity data.

References


Role of Cytoskeleton in TRIM5α Function


29. Berthoux L, Sebastian S, Sokoloska E, Luban J. 2005. Cyclophilin A is required for TRIM5α-mediated resistance to HIV-1 in Old World mon-


