Available online at www.sciencedirect.com
ScienceDirect
Virology 367 (2007) 19–29
VIROLOGY
www.elsevier.com/locate/yviro

The ability of multimerized cyclophilin A to restrict retrovirus infection

Hassan Javanbakht [a], Felipe Diaz-Griffero [a], Wen Yuan [a], Darwin F. Yeung [a], Xing Li [a], Byeongwoon Song [a], Joseph Sodroski [a,b,*]

[a] Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Division of AIDS, Harvard Medical School, Boston, MA 02115, USA
[b] Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA

Received 10 January 2007; returned to author for revision 2 February 2007; accepted 13 April 2007
Available online 15 June 2007

Abstract

In owl monkeys, the typical retroviral restriction factor of primates, TRIM5α, is replaced by TRIMCyp. TRIMCyp consists of the TRIM5 RING, B-box 2 and coiled-coil domains, as well as the intervening linker regions, fused with cyclophilin A. TRIMCyp restricts infection of retroviruses, such as human immunodeficiency virus (HIV-1) and feline immunodeficiency virus (FIV), with capsids that can bind cyclophilin A. The TRIM5 coiled coil promotes the trimerization of TRIMCyp. Here we show that cyclophilin A that is oligomeric as a result of fusion with a heterologous multimer exhibits substantial antiretroviral activity. The addition of the TRIM5 RING, B-box 2 and Linker 2 to oligomeric cyclophilin A generated a protein with antiretroviral activity approaching that of wild-type TRIMCyp. Multimerization increased the binding of cyclophilin A to the HIV-1 capsid, promoting accelerated uncoating of the capsid and restriction of infection.

Keywords: Cyclophilin A; HIV-1; FIV; Retroviral capsid; Coiled-coil domain; Multimerization; Restriction; TRIM5; TRIMCyp

Introduction

TRIM5α mediates early, post-entry blocks to the infection of cells by retroviruses, including human immunodeficiency virus (HIV-1) (Stremlau et al., 2004; Perron et al., 2004; Hatziioannou et al., 2004; Keckesova et al., 2004; Yap et al., 2004). TRIM5α is a member of a family of proteins that contain a *tri*partite *m*otif, hence the designation TRIM (Reymond et al., 2001). The tripartite motif includes a *R*ING domain, *B*-box 2 domain and coiled-coil (*cc*) domain; TRIM proteins have also been called RBCC proteins. TRIM proteins exhibit the propensity to form cytoplasmic or nuclear bodies (Reymond et al., 2001). Many cytoplasmic TRIM proteins contain a C-terminal B30.2 or SPRY domain. The coiled-coil domain of TRIM5 is necessary for multimerization (Javanbakht et al., 2005; Perez-Caballero et al., 2005a). TRIM5α proteins from different species have been shown to form trimers (Mische et al., 2005). Changes in the rhesus monkey TRIM5α ($TRIM5\alpha_{rh}$) coiled coil or adjacent linker 2 (L2) region can disrupt trimerization (Javanbakht et al., 2006). TRIM5α variants that restrict HIV-1 infection specifically interact with HIV-1 capsid–nucleocapsid (CA–NC) complexes assembled *in vitro* (Stremlau et al., 2006a; Li et al., 2006). An intact B30.2/SPRY domain, which is thought to contact the capsid directly, and the ability to trimerize are required for efficient TRIM5α interaction with HIV-1 CA–NC complexes (Javanbakht et al., 2006; Stremlau et al., 2006a). Trimerization may allow TRIM5α to interact with sites on the surface lattice of the retroviral capsid that exhibit trimeric pseudosymmetry (Mische et al., 2005). Significant gains in avidity would accrue to the interactions of two oligomeric complexes with compatible symmetry. Thus, TRIM5α trimerization contributes to avidity for the retroviral capsid and to the ability to restrict virus infection (Javanbakht et al., 2006; Stremlau et al., 2006a).

In owl monkeys, *TRIMCyp*, which is the result of a retrotransposition event, replaced the *TRIM5* gene (Nisole et al., 2004; Ribeiro et al., 2005; Sayah et al., 2004). The encoded TRIMCyp protein contains the RBCC domains of TRIM5 fused to cyclophilin A. TRIMCyp can restrict some retroviruses, in-

* Corresponding author. Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, 44 Binney Street, JFB 824, Boston, MA 02115, USA. Fax: +1 617 632 4338.
E-mail address: joseph_sodroski@dfci.harvard.edu (J. Sodroski).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.04.034

cluding HIV-1, by virtue of a specific recognition of the viral capsid protein by the cyclophilin A moiety (Nisole et al., 2004; Sayah et al., 2004; Towers et al., 2003). Robust TRIMCyp-mediated restriction involves at least two functions: (1) capsid binding, which occurs most efficiently for trimeric TRIMCyp proteins that retain the coiled-coil and cyclophilin A domains, and (2) an effector function that depends upon the B-box 2 domain (Diaz-Griffero et al., 2006). Although the cyclophilin A moiety of TRIMCyp interacts at low affinity with the monomeric HIV-1 capsid protein, trimerization of TRIMCyp mediated by its coiled coil contributes to the interaction with the processed multimeric capsid (Diaz-Griffero et al., 2006). Here we investigate the structural requirements for cyclophilin A to function as a retroviral restriction factor. We created cyclophilin A fusion proteins containing coiled coils derived from the GCN4 transcription factor. GCN4 coiled coils were chosen because they are structurally well characterized, can assume different oligomerization states depending on previously defined amino acid changes and are derived from a protein unrelated to TRIM proteins (Harbury et al., 1993; Lumb and Kim, 1995; Weissenhorn et al., 1997). We show that a multimeric cyclophilin A protein can partially restrict retrovirus infection. The efficient interaction of multimeric cyclophilin A with the retroviral capsid complex promotes capsid uncoating and the retrovirus-restricting ability of these multimers. The addition of the TRIM5 RING, B-box 2 and Linker 2 (L2) regions to oligomeric cyclophilin A generated a restriction factor with a potency approaching that of TRIMCyp.

Results

Construction of multimeric cyclophilin A

To investigate the effect of multimerization on cyclophilin A (CypA), we constructed chimeric proteins containing elements of TRIMCyp, including the CypA moiety, fused with GCN4 variants that form either trimers or dimers (Harbury et al., 1993; Lumb and Kim, 1995; Weissenhorn et al., 1997). The following proteins were designed: (1) RBGCN4TriL2Cyp, in which the coiled-coil domain of TRIMCyp has been replaced by a heterologous trimeric coiled coil derived by modification of the GCN4 transcription factor (Harbury et al., 1993; Lumb and Kim, 1995; Weissenhorn et al., 1997); (2) RBGCN4TriCyp, a construct in which the coiled coil and Linker 2 regions of TRIMCyp are replaced by the trimeric GCN4 coiled-coil; (3) GCN4TriL2Cyp, which consists of a trimeric GCN4 motif fused at the N-terminus of the Linker 2 (L2) region of TRIMCyp; (4) GCN4TriCyp, which consists of a trimeric GCN4 motif fused at the N-terminus of the cyclophilin A domain of TRIMCyp; (5) GCN4DiCyp, which contains a dimeric GCN4 motif at the N-terminus of the cyclophilin A domain of TRIMCyp; and (6) CypA, the monomeric cyclophilin A moiety of the TRIMCyp protein (Fig. 1A).

All of the proteins, as well as rhesus monkey TRIM5α (TRIM5α_{rh}) as a control, were expressed stably in Cf2Th canine thymocytes. The CypA, TRIMCyp, TRIM5α and GCN4 fusion proteins have an influenza hemagglutinin (HA) epitope tag at their carboxyl termini. All of the proteins were expressed at least as well as the wild-type TRIMCyp proteins; CypA was expressed at a level higher than those observed for the fusion proteins and wild-type TRIMCyp (Fig. 1B).

To examine the oligomerization state of the CypA and the GCN4 fusion proteins, cross-linking with increasing concentrations of ethylene glycol-bis(succinimidyl succinate) (EGS) was employed. CypA exhibited only monomers, even at high concentrations of cross-linker (Fig. 2A). The GCN4DiCyp protein efficiently formed dimers. Cross-linked dimers of the GCN4TriCyp protein were evident; trimers and higher order forms of GCN4TriCyp were less efficiently cross-linked. RBGCN4TriCyp exhibited trimers and higher order multimers after cross-linking. GCN4DiCyp and GCN4TriL2Cyp exhibited evidence of dimer formation (Fig. 2B). The RBGCN4TriL2Cyp exhibited trimeric and higher order forms after cross-linking. The wild-type TRIMCyp protein cross-linked into trimers. Thus, all of the fusion proteins oligomerize.

We examined the subcellular localization of TRIMCyp, CypA, GCN4DiCyp and the fusion proteins by staining cells expressing these proteins with an antibody directed against the HA epitope tag. As previously demonstrated (Diaz-Griffero et al., 2006; Perez-Caballero et al., 2005b), TRIMCyp formed cytoplasmic bodies and also exhibited diffuse cytoplasmic staining (Fig. 2C). In contrast to the wild-type TRIMCyp protein, the CypA protein exhibited a diffuse pattern of expression throughout the cells. The intracellular localization of RBGCN4-TriL2Cyp was very similar to that of wild-type TRIMCyp. Diffuse intracellular localization, in some cases with nuclear exclusion, was seen for RBGCN4TriCyp, GCN4TriL2Cyp, GCN4TriCyp and GCN4DiCyp. The intensity of the staining reflected the protein expression level (see Fig. 1B). These results indicate that artificial multimerization is compatible with cytoplasmic localization of the CypA protein.

The ability of multimeric cyclophilin A protein to restrict retrovirus infection

We examined the ability of the cyclophilin A fusion proteins to inhibit infection by retroviruses that are sensitive to TRIMCyp restriction (Diaz-Griffero et al., 2006). Cf2Th canine thymocytes expressing TRIM5α_{rh}, TRIMCyp, CypA, RBGCN 4-TriL2Cyp, RBGCN4TriCyp, GCN4TriL2Cyp, GCN4TriCyp and GCN4DiCyp or transduced with the empty LPCX vector were challenged with VSV G glycoprotein-pseudotyped recombinant retroviruses (HIV-1-GFP and FIV-GFP) expressing green fluorescent protein (GFP). As an indicator of successful infection, GFP-positive cells were scored (Fig. 3A). Cells expressing TRIMCyp and TRIM5α_{rh} proteins potently resisted HIV-1 infection, whereas cells expressing CypA were infected at least as well as the control cells transduced with the empty LPCX vector. Cf2Th cells expressing GCN4TriCyp and GCN 4-DiCyp exhibited a partial block to HIV-1 infection. RBG CN4TriCyp inhibited HIV-1 infection less efficiently than either GCN4TriCyp or GCN4DiCyp. However, Cf2Th cells expressing GCN4TriL2-Cyp and RBGCN4TriL2Cyp restricted HIV-1 more potently

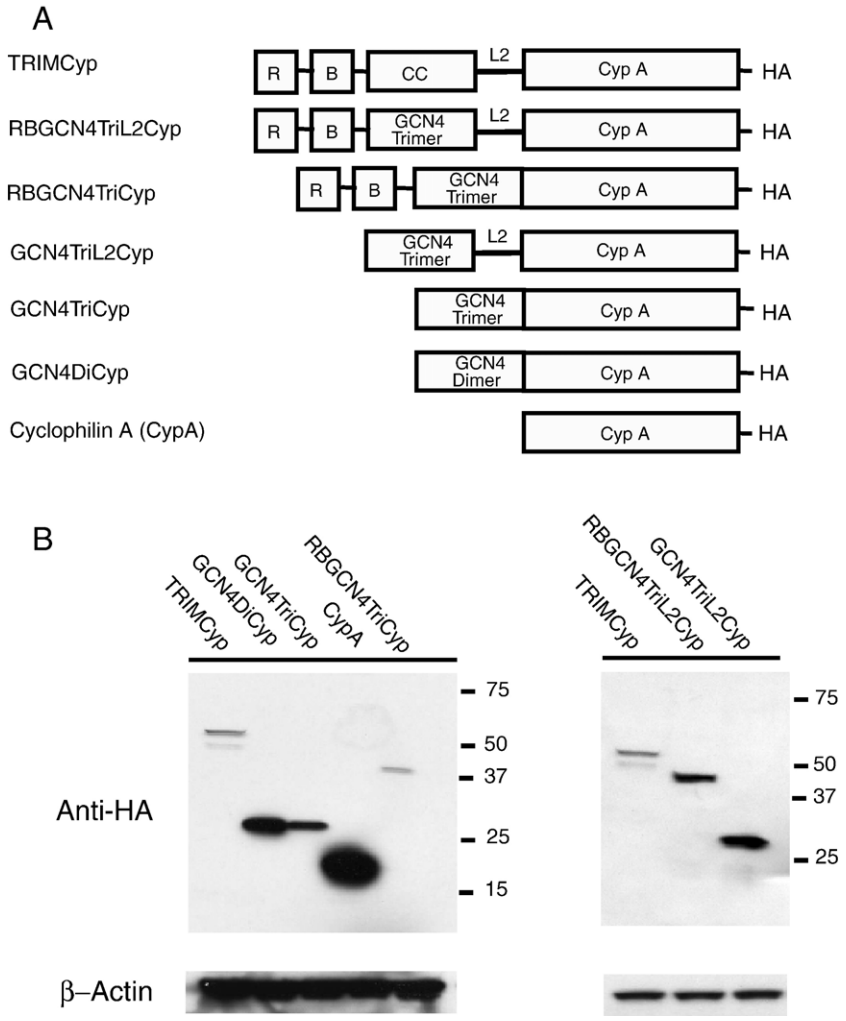

Fig. 1. Structure and expression of wild-type cyclophilin A and oligomeric cyclophilin A proteins. (A) The wild-type owl monkey cyclophilin A protein is depicted, aligned with the fusion proteins containing different oligomerization domains. The RING (R), B-box 2 (B), coiled-coil (CC) and Linker 2 (L2) regions are derived from TRIMCyp. The GCN4 trimer and dimer sequences (Harbury et al., 1993; Lumb and Kim, 1995) are shown. The C termini of the proteins contain hemagglutinin (HA) epitope tags. (B) Cf2Th cells were transduced with pLPCX vectors expressing the indicated proteins. Cell lysates were Western blotted and probed with antibodies directed against either the HA epitope tag (*top panels*) or β-actin (*bottom panels*).

than cells expressing GCN4TriCyp. Thus, although cyclophilin A fusion with the heterologous dimeric or trimeric GCN4 motifs was sufficient for some level of anti-HIV-1 activity, the addition of the RING/B-box2 and Linker 2 (L2) sequences of TRIMCyp increased the potency of the HIV-1 restriction.

The susceptibility of the Cf2Th cells expressing the various constructs to infection by recombinant FIV-GFP is shown in Fig. 3B. The wild-type TRIM5α_{rh} and TRIMCyp proteins potently restricted FIV infection. The Cf2Th cells expressing CypA were slightly more susceptible to FIV infection than the control cells transduced with the empty LPCX vector. All of the multimeric cyclophilin proteins restricted FIV-GFP infection with an efficiency at least as great as that seen for HIV-1 infection. A similar rank order of potency of the multimeric proteins was observed for the two viruses; for example, RBGCN4TriL2Cyp and GCN4TriL2Cyp exhibited the most potent activity against both HIV-1 and FIV infections. Thus, FIV-GFP infection is susceptible to restriction by multimeric forms of cyclophilin A.

The capsids of murine leukemia viruses, N-MLV and B-MLV, are not known to bind cyclophilin A and thus these viruses are not sensitive to TRIMCyp restriction (Perron et al., 2004; Yap et al., 2004; Keckesova et al., 2004; Hatziioannou et al., 2004; Nisole et al., 2004). To examine whether the antiretroviral activity of the multimeric GCN4–CypA fusion proteins is specific, we challenged Cf2Th cells expressing these proteins with N-MLV-GFP and B-MLV-GFP. Cells expressing TRIM5α_{rh} or TRIMCyp were tested in parallel. Although N-MLV infection is potently restricted by TRIM5α_{hu}, TRIM5α_{rh} only partially restricts N-MLV infection (Hatziioannou et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). As expected, partial inhibition of N-MLV infection was observed in Cf2Th cells expressing TRIM5α_{rh} (Fig. 3C). Neither TRIMCyp nor the GCN4–CypA fusion proteins inhibited N-MLV infection. None of the proteins tested affected the efficiency of B-MLV infection (Fig. 3D). Apparently, multimerization of CypA promotes inhibitory activity against retroviruses like HIV-1 and FIV that bind CypA (Diaz-Griffero et al.,

2006; Lin and Emerman, 2006), and not against viruses that do not bind CypA.

Multimeric CypA-mediated restriction requires CypA-capsid interaction

To investigate whether the ligand-binding capability of the cyclophilin A moiety in the fusion proteins contributes to the observed retroviral restriction, we challenged Cf2Th cells expressing the fusion proteins with HIV-1-GFP and FIV-GFP in the presence of cyclosporine A. Cyclosporine A has been shown to bind cyclophilin A and inhibit its binding to HIV-1 and FIV capsid proteins (Bosco and Kern, 2004; Diaz-Griffero et al., 2006; Luban et al., 1993; Lin and Emerman, 2006). Fig. 4A shows that Cf2Th cells expressing TRIMCyp and GCN4–CypA fusion proteins were infected by HIV-1 at least as efficiently as the control cells transduced with the empty LPCX vector. By contrast, TRIM5α_{rh} retained most of its anti-HIV-1 activity in presence of cyclosporine A, as expected (Stremlau et al., 2006b). Similar results were observed for FIV infection (Fig. 4B). We conclude that retroviral restriction by the multimeric CypA constructs requires a CypA domain capable of interacting with its ligand, presumably the retroviral capsid in this case.

The ability of multimeric cyclophilin A constructs to bind HIV-1 CA–NC complexes

TRIM5α_{rh} and TRIMCyp gain significant avidity for the retroviral capsid by virtue of the trimeric state (Diaz-Griffero et al., 2006; Javanbakht et al., 2006). TRIM5α_{rh} trimerization has been suggested to facilitate multivalent association with the HIV-1 capsid (Mische et al., 2005). To investigate the potential contribution of multimerization to the interaction of cyclophilin A with the HIV-1 capsid, we utilized a recently established capsid-binding assay (Stremlau et al., 2006a) to evaluate the multimeric cyclophilin A proteins. In this assay, HIV-1 capsid-like complexes are assembled *in vitro* from purified HIV-1 capsid–nucleocapsid (CA–NC) proteins (Ganser et al., 1999). After incubation with lysates from cells expressing a restriction factor, the CA–NC complexes are allowed to sediment through 70% sucrose cushions. The restriction factor co-sediments through the sucrose cushion only if it associates with the HIV-1

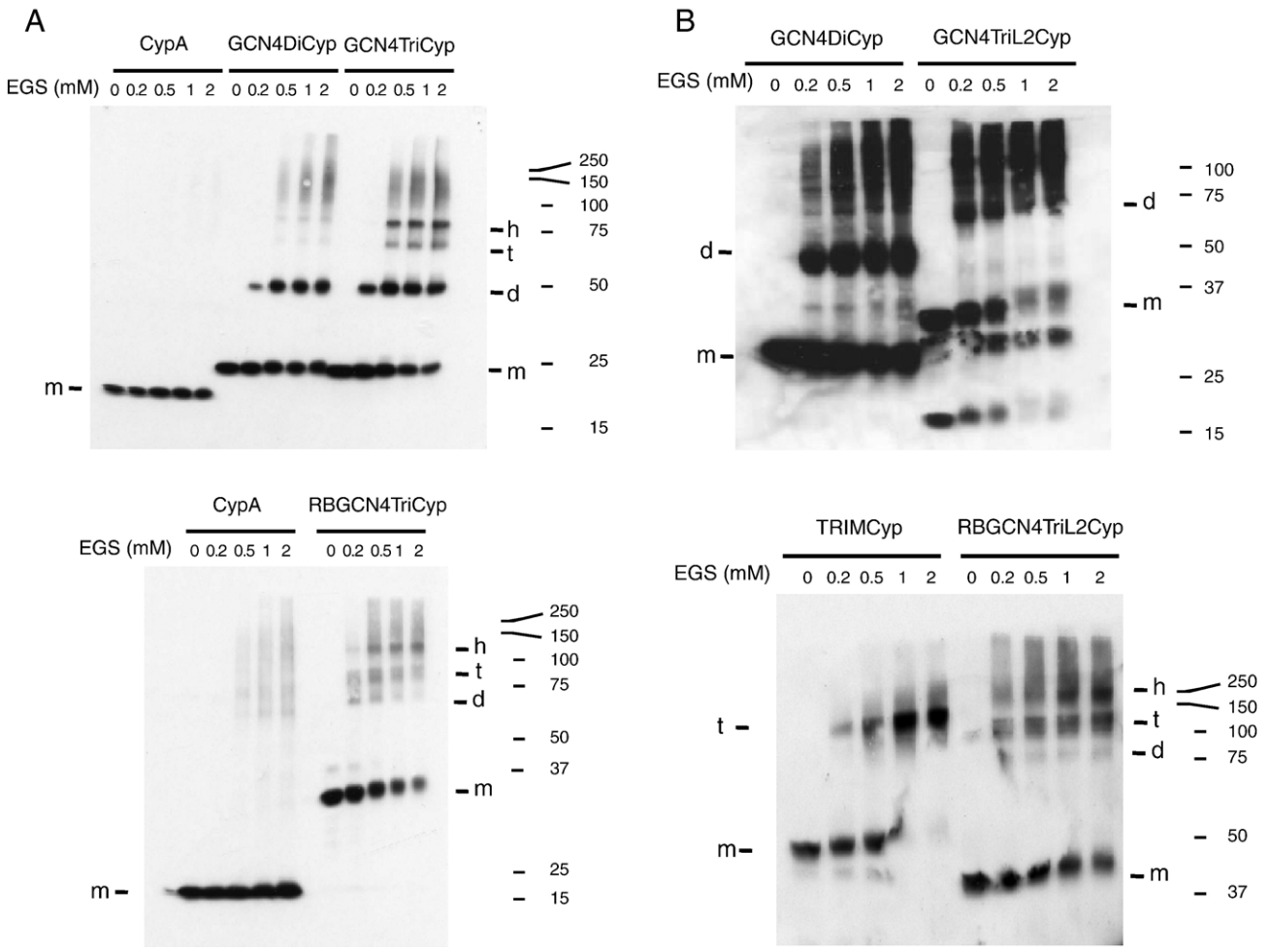

Fig. 2. Oligomerization states of CypA, TRIMCyp and GCN4–CypA fusion proteins. (A, B) Lysates from 293T cells expressing the indicated proteins were cross-linked with increasing concentrations of EGS and were subjected to Western blotting with an anti-HA antibody. Monomeric (m), dimeric (d), trimeric (t) and higher order (h) forms of the proteins are indicated. (C) Cf2Th cells stably expressing the indicated HA-tagged proteins were fixed and stained using an FITC-conjugated anti-HA antibody, as described in Materials and methods. Representative confocal microscopic images of the Cf2Th cells expressing the indicated proteins are shown.

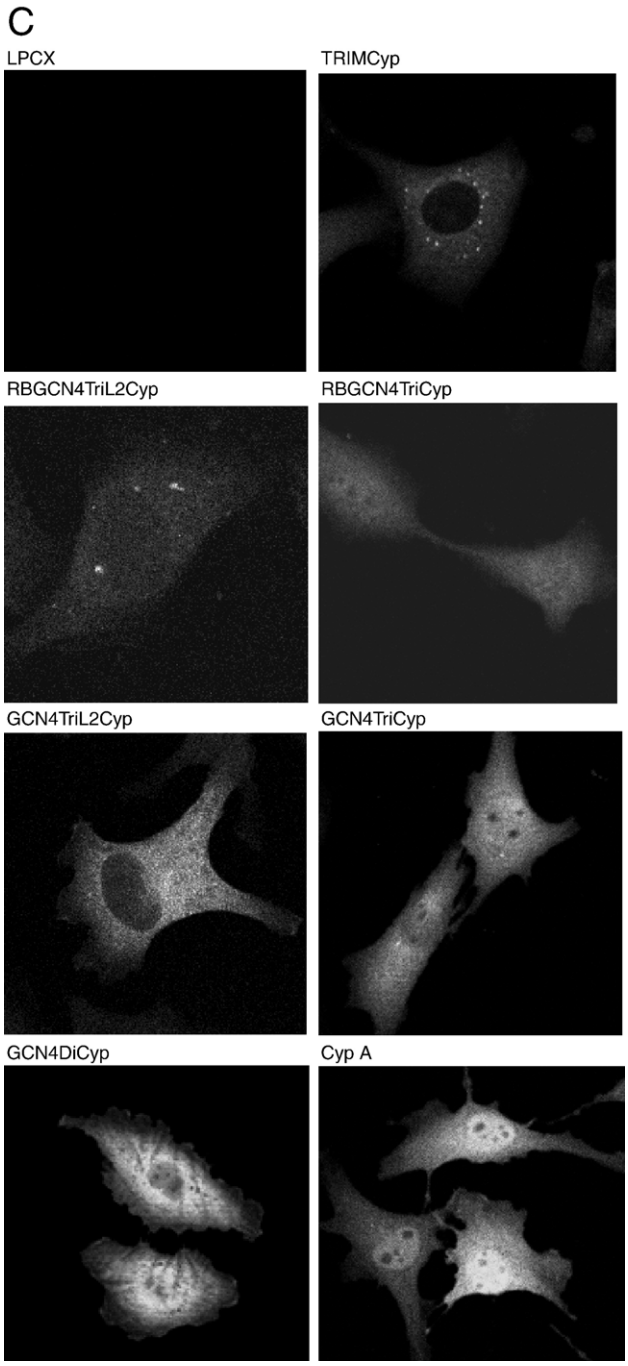

Fig. 2 (*continued*).

CA–NC complexes (Stremlau et al., 2006a). As expected (Stremlau et al., 2006a; Diaz-Griffero et al., 2006), TRIMCyp interacted efficiently with the HIV-1 CA–NC complexes (Fig. 5). Cyclophilin A is known to interact with the HIV-1 capsid protein monomer (Braaten et al., 1997; Franke et al., 1994; Bosco and Kern, 2004), but the affinity of this interaction is low (Yoo et al., 1997). Consistent with previous results (Diaz-Griffero et al., 2006), the CypA interaction with the HIV-1 CA–NC complexes was weak (compare Input and Bound in Fig. 5). By contrast, all of the multimeric GCN4–CypA fusion proteins that we tested exhibited a higher affinity for HIV-1 CA–NC complexes. These data suggest that multimerization increases the avidity of CypA fusion proteins for HIV-1 capsid complexes.

Disruption of the oligomerization of a multimeric CypA construct

To confirm that multimerization of CypA was responsible for the observed increases in HIV-1 capsid binding and restriction, we replaced three of the isoleucine residues important for trimerization of the GCN4 moiety in GCN4TriCyp with hydrophilic residues. The GCN4TriCyp protein with isoleucines 15, 18 and 22 of trimeric GCN4 converted to serines is designated MutGCN4TriCyp. The ability of the GCN4TriCyp protein to form oligomers was dramatically reduced by the modification of GCN4 isoleucine residues (Fig. 6A). The MutGCN4TriCyp protein did not detectably bind HIV-1 CA–NC complexes (Fig. 6B) nor restrict HIV-1 infection (Fig. 6C). We conclude that multimerization of CypA is important for efficient binding to the HIV-1 capsid and HIV-1 inhibition.

Effect of a multimeric CypA protein on cytosolic HIV-1 capsids

Previous studies have suggested that retroviral capsids undergo accelerated uncoating after they enter the cytosol of cells expressing a restricting TRIM5α protein (Stremlau et al., 2006a; Perron et al., 2007). To investigate whether TRIMCyp or the RBGCN4TriL2Cyp protein, which both efficiently restrict HIV-1 infection, might affect HIV-1 capsid stability, we examined the fate of the HIV-1 capsid in cells expressing these proteins. Control cells transduced with the empty LPCX vector and cells expressing TRIMCyp or RBGCN4TriL2Cyp were incubated with recombinant HIV-1-GFP pseudotyped with the VSV G glycoprotein or, as a negative control, lacking envelope glycoproteins. In parallel, a mutant (G89A) HIV-1-GFP virus, which is resistant to TRIMCyp restriction (Towers et al., 2003; Sayah et al., 2004), was studied. Sixteen hours after virus-cell incubation, the cells were lysed and the cell lysates analyzed for particulate and soluble HIV-1 p24 capsid protein. No cytosolic HIV-1 capsid protein was detected in cells incubated with viruses lacking envelope glycoproteins, as expected (Fig. 7). Particulate capsid proteins from the wild-type and G89A HIV-1-GFP viruses were detected in the control LPCX cells. The amounts of particulate wild-type HIV-1 capsid protein in the cytosol of cells expressing TRIMCyp and RBGCN4TriL2Cyp were significantly decreased. By contrast, the levels of particulate G89A mutant HIV-1 capsid protein were comparable in the control LPCX-transduced cells and the cells expressing the TRIMCyp and RBGCN4TriL2Cyp proteins. We conclude that expression of the TRIMCyp and RBGCN4TriL2Cyp proteins leads to decreases in the amounts of particulate, cytosolic HIV-1 capsids in the target cells.

Discussion

In this study, we investigated the effect of multimerization on the ability of cyclophilin A to restrict retroviruses. We show that multimerization of cyclophilin A is sufficient to allow some

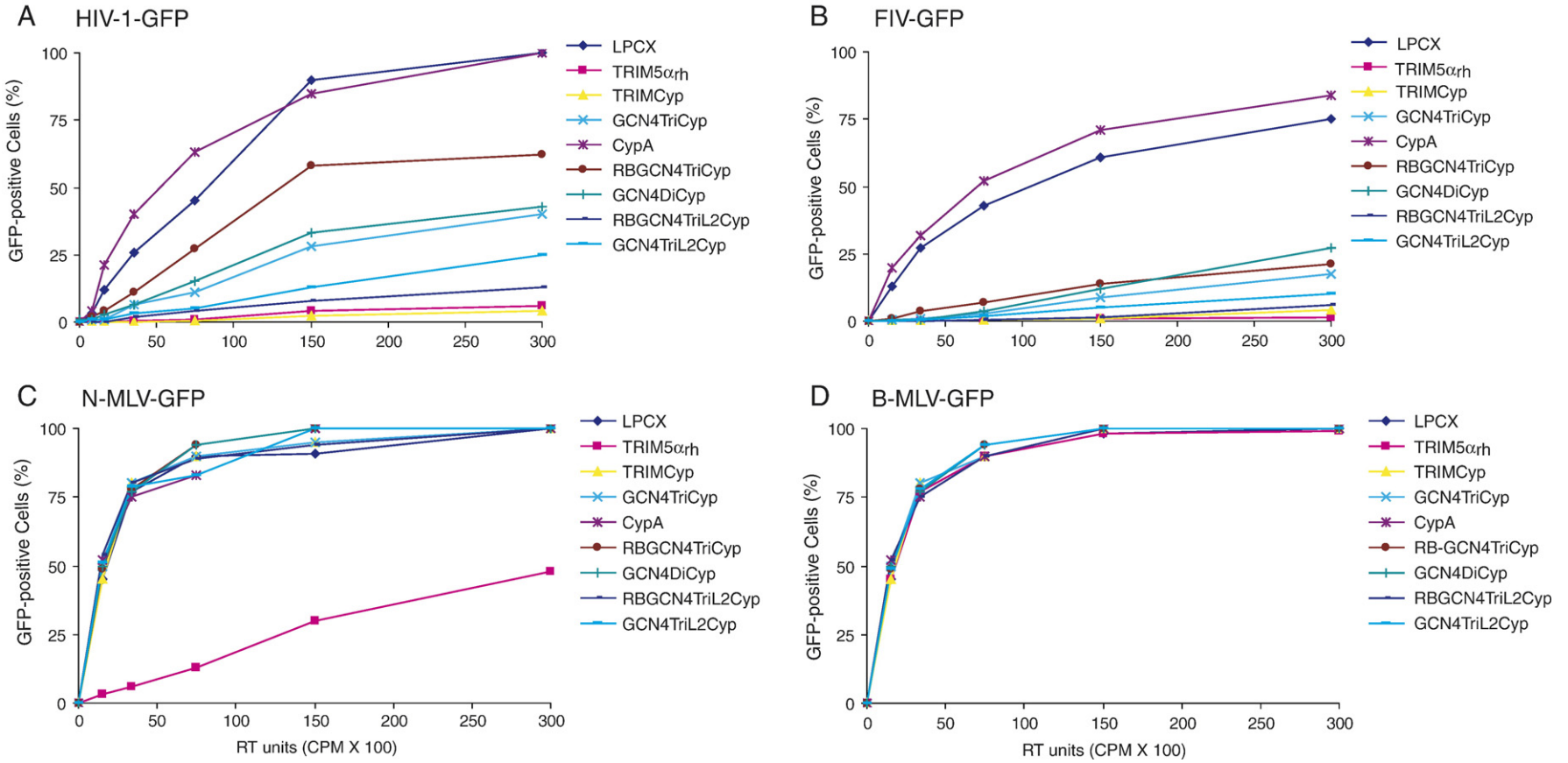

Fig. 3. Effect of expression of CypA and fusion proteins on retroviral infection. Cf2Th cells expressing wild-type TRIM5α_{rh}, TRIMCyp and variants of the cyclophilin A proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of HIV-1-GFP (A), FIV-GFP (B), N-tropic murine leukemia virus (N-MLV) (C) or B-MLV (D). Infected, GFP-positive cells were counted by FACS. The results of a typical experiment are shown. Similar results were obtained in three independent experiments.

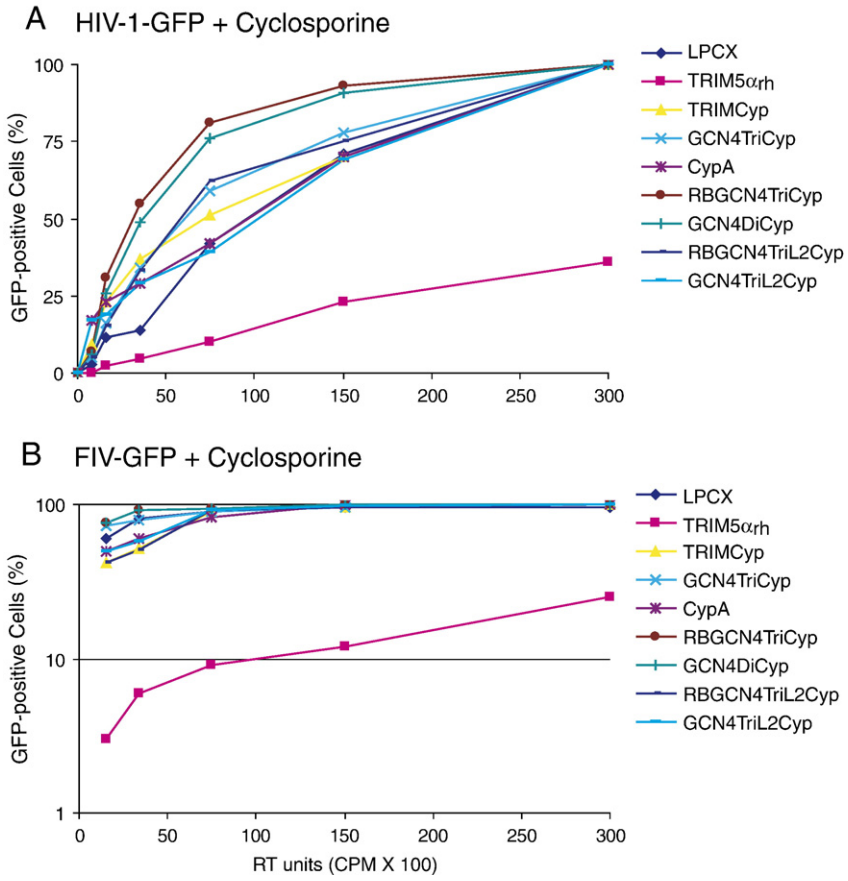

Fig. 4. CypA interaction with retroviral capsids is required for restriction activity. Cf2Th cells expressing the wild-type TRIM5α_{rh}, TRIMCyp and variants of CypA proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of HIV-1-GFP (A) and FIV-GFP (B) in presence of 5 mM cyclosporine A. Infected, GFP-positive cells were counted by FACS. The results of a typical experiment are shown. Similar results were obtained in three independent experiments.

level of inhibition of particular retroviruses. Both HIV-1 and FIV are restricted by TRIMCyp in a manner inhibitable by cyclosporine A (Diaz-Griffero et al., 2006; Luban et al., 1993). The inhibitory activity likely depends upon the interaction of the CypA moiety with the retroviral capsid. Although cyclophilin A is known to interact with both the unprocessed HIV-1 Gag polyprotein and the processed capsid protein with low affinity (Franke et al., 1994; Thali et al., 1994; Gamble et al., 1996; Yoo et al., 1997), we show here that the ability of multimeric cyclophilin A to restrict retroviruses correlates with an increased avidity for the processed and assembled capsid. Multimerization of CypA likely increases avidity for the assembled retroviral capsid rather than creating a new mode of binding. In support of this assertion, GCN4TriCypA and GCN4DiCypA restrict the same subset of retroviruses (HIV-1, FIV) as TRIMCyp. Moreover, the ability of the GCN4–CypA fusion proteins to restrict these retroviruses is sensitive to cyclosporine A.

Although both TRIM5α and TRIMCyp are naturally trimeric (Mische et al., 2005; Diaz-Griffero et al., 2006), we did not observe significant differences between the ability of GCN4TriCyp and GCN4DiCyp to restrict retroviruses. GCN4TriCypA and GCN4DiCyp were designed to form trimers and dimers, respectively. Trimeric restriction factors may have an avidity advantage in interacting with the pseudosymmetric trimeric structures on the assembled retroviral capsid (Mische et al., 2005). One explanation for the functional equivalence of GCN4TriCyp and GCN4DiCyp could be that the GCN4TriCyp protein only inefficiently forms trimers or that the trimers formed are unstable.

Multimerization apparently promotes retrovirus restriction by increasing the association of CypA moieties and the viral capsid. This implies that an increased binding of CypA to the incoming capsid may be detrimental to infection. Indeed, instances have been reported where an abundance of CypA in the target cell appears to inhibit HIV-1 infection (Yin et al., 1998; Hatziioannou et al., 2005; Diaz-Griffero et al., 2006). In addition, qualitative differences in capsid binding by CypA might be promoted by multimerization. For example, the order in which the capsid proteins are bound by CypA might influence whether an enhancement or an inhibition of viral replicative events ensues.

The potent HIV-1 restriction mediated by wild-type TRIMCyp and the RBGCN4TriL2Cyp proteins was accompanied by a decrease in particulate HIV-1 capsids in the cytosol. These results are consistent with a growing body of evidence supporting accelerated uncoating of the retroviral capsid as a mechanism of TRIM5α-mediated restriction (Stremlau et al., 2006a; Perron et al., 2007). That CypA oligomerization might also lead to retrovirus restriction by accelerating capsid disassembly implies

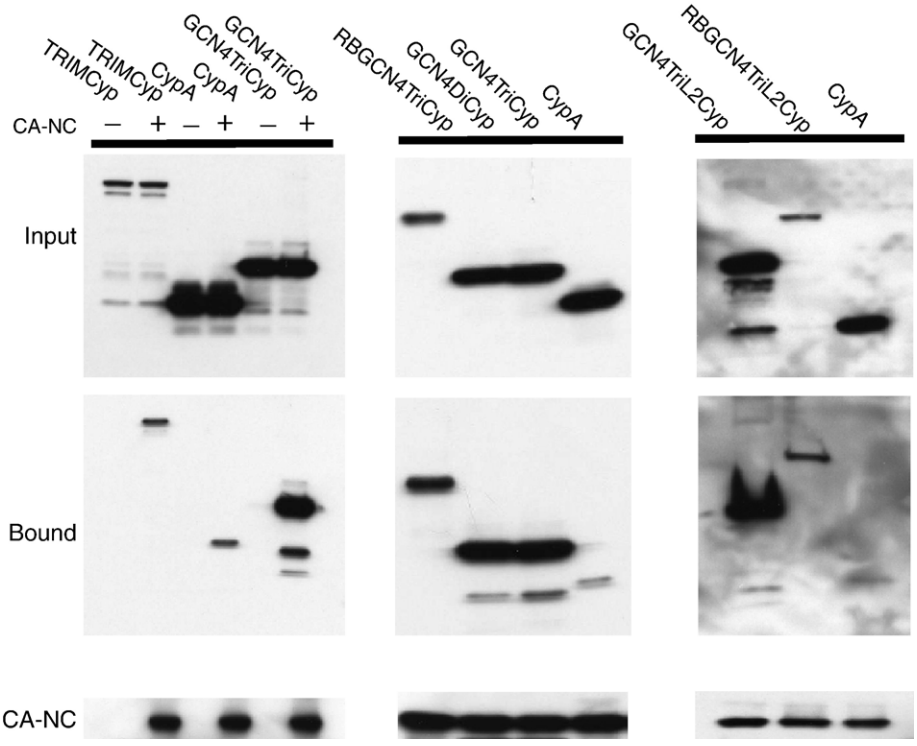

Fig. 5. Specific association of TRIMCyp and CypA variants with HIV-1 CA–NC complexes. In vitro assembled CA–NC complexes (Ganser et al., 1999) were mixed with 293T lysates containing TRIMCyp, cyclophilin A or the indicated cyclophilin A variants and layered onto 70% sucrose cushions before centrifugation. In some experiments (left panel), CA–NC complexes were not added, serving as a negative control. Immediately prior to mixing, an aliquot of the cell lysate was removed and blotted with α-HA antibodies to determine the steady-state expression levels of the different cyclophilin A variants (Input). After centrifugation, the pellet was resuspended in SDS sample buffer and analyzed by Western blotting using an anti-HA antibody (for Bound protein) or an anti-p24 antibody (to detect CA–NC).

that CypA itself may promote uncoating, presumably through its prolyl isomerase activity. In this model, multimerization of CypA results in an increased efficiency of capsid binding and augments the consequences of CypA-capsid association. Such a model explains why multimerization of the TRIM5α B30.2(SPRY) domain is apparently insufficient for retrovirus restriction. For example, TRIM5α proteins with certain B-box 2 domain alterations trimerize and bind retroviral capsids efficiently, yet are devoid of restricting activity (Javanbakht et al., 2005; Diaz-Griffero et al., 2006; Perez-Caballero et al., 2005a; Stremlau et al., 2006a; Diaz-Griffero et al., submitted for publication). Of interest, expression of these B-box 2 mutants of TRIM5α in target cells does not result in accelerated uncoating of the capsids of retroviruses infecting those cells (Perron et al., 2007; Diaz-Griffero et al., submitted for publication). Thus, a B-box 2 domain function appears to be essential for TRIM5α-mediated capsid disassembly and viral restriction, perhaps by recruiting cellular cofactors. By contrast, RING and B-box 2 functions are not absolutely essential for TRIMCyp-mediated retroviral restriction, although they contribute to the efficiency of the restriction. A capsid-uncoating/restriction activity intrinsic to the CypA domain itself would explain the different dependencies of TRIM5α and CypA-based restriction factors on the B-box 2 domain and associated cellular cofactors.

The RBGCN4TriCyp and GCN4TriCyp proteins, although able to restrict infection by HIV-1 and FIV, did so less efficiently than TRIMCyp. The addition of the Linker 2 (L2) region to these proteins augmented their restricting ability for both HIV-1 and FIV. The Linker 2 region plays a major role in trimerization of TRIM5α(Javanbakht et al., 2006) and may modulate the spatial relationship of the CypA moiety with the remainder of the TRIMCyp protein. The phenotypes with respect to restriction enhancement for the RING/B-box 2 and L2 additions were interdependent, consistent with such a structural interaction. The precise role played by the RING and B-box 2 domains and the Linker 2 region in retrovirus restriction by TRIMCyp and TRIM5α is a subject for further investigation.

Materials and methods

Plasmid construction

The plasmids expressing the wild-type and mutant TRIMCyp proteins were constructed using PCR-directed mutagenesis. The TRIMCyp cDNA was PCR-amplified (Stremlau et al., 2004) and digested with *Eco*RI and *Cla*I, sites that were introduced by each of the PCR primers. These fragments were cloned into the *Eco*RI and *Cla*I sites of pLPCX (Stratagene). The trimeric GCN4 protein sequence reads as follows: MKQIEDKIEEIESKIKKIENEIARIKKLIGEG. The MutGCN4TriCyp protein has an altered GCN4 sequence: MKQIEDKIEEIESK*S*KK*S*ENE*S*ARIKKLIGEG.

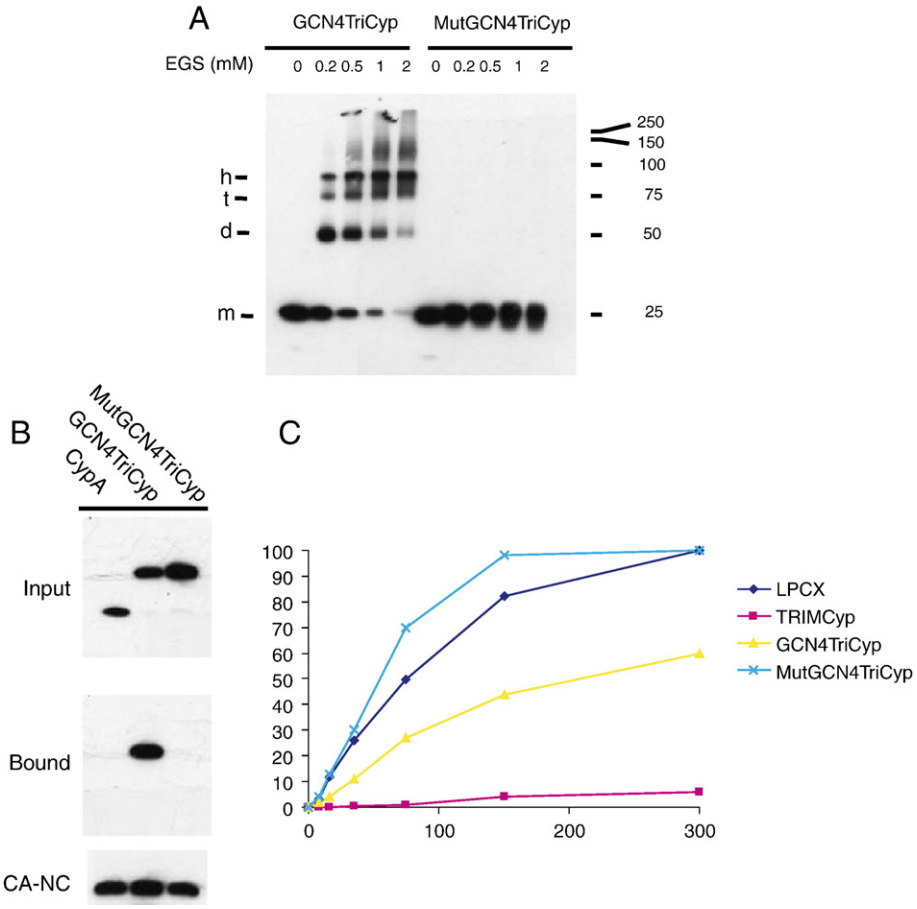

Fig. 6. Effects of disruption of oligomerization of the GCN4TriCyp protein. (A) The oligomerization of the GCN4TriCyp and Mut GCN4TriCyp proteins was studied by EGS cross-linking and SDS–PAGE. Monomeric (m), dimeric (d), trimeric (t) and higher order (h) forms of the proteins are indicated. (B) Cell lysates containing the indicated proteins were incubated with HIV-1 CA–NC complexes. The mixtures were pelleted through 70% sucrose cushions. The amount of each protein in the input lysate and the CA–NC-bound pellet is shown. The amount of HIV-1 CA–NC protein detected in the pellet is shown in the bottom row. (C) Cf2Th cells expressing the indicated proteins or control LPCX-transduced cells were exposed to recombinant HIV-1-GFP. The percentage of GFP-positive cells at 72 h after virus-cell incubation is shown. The experiment was repeated with comparable results.

Creation of cells stably expressing TRIMCyp variants

Retroviral vectors encoding wild-type or mutant TRIMCyp proteins were created using the pLPCX plasmid (Stremlau et al., 2004). The LPCX vectors contain only the amino-acid-coding sequence of the TRIMCyp variants. Recombinant viruses were produced in 293T cells by cotransfecting the pLPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Yee et al., 1994). The resulting virus particles were used to transduce approximately 1×10^6 Cf2Th cells in the presence of 5 μg/ml polybrene. Cells were selected in 5 μg/ml puromycin (Sigma).

Infection with viruses expressing green fluorescent protein (GFP)

Recombinant HIV-1, FIV, N-MLV and B-MLV expressing GFP were prepared as described (Stremlau et al., 2004). HIV-1 viral stocks were quantified by measuring reverse transcriptase (RT) activity. For infections, 3×10^4 Cf2Th cells seeded in 24-well plates were incubated in the presence of virus for 24 h. Cells were washed and returned to culture for 48 h and then subjected to FACS analysis with a FACScan (Becton Dickinson).

Capsid-binding assay

Purification of the HIV-1 CA–NC protein expressed in *E. coli* was carried out as previously described (Ganser et al., 1999). High-molecular-weight HIV-1 capsid complexes were assembled using 300 μM CA–NC protein and 60 μM $(TG)_{50}$ DNA oligonucleotide in a volume of 100 microliters of 50 mM Tris–HCl (pH 8.0) and 500 mM NaCl. The reaction was allowed to proceed overnight at 4 °C, and the assembled CA–NC complexes were stored at 4 °C until needed.

For a source of TRIMCyp and GCN4–CypA fusion proteins, 10^6 293T cells seeded in a 6-well dish were transfected with 1 μg of the appropriate pLPCX plasmid, using Lipofectamine 2000. Forty-eight hours later, the cells were harvested in PBS containing 5 mM EDTA and resuspended in 250 μl of hypotonic lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 1 mM

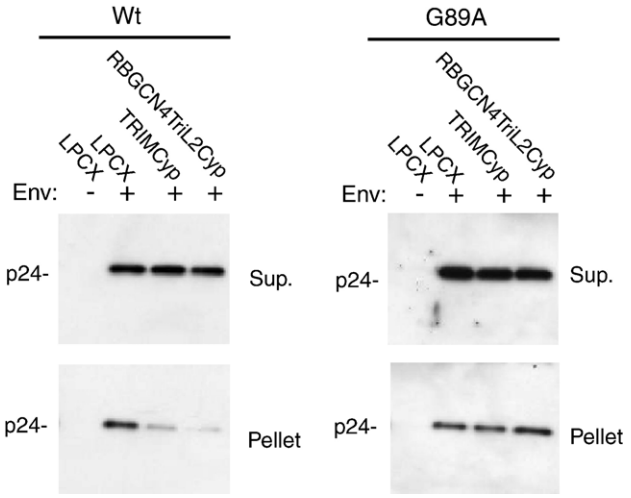

Fig. 7. Fate of the wild-type and G89A HIV-1 capsids in target cells expressing TRIMCyp and RBGCN4TriL2Cyp. VSV G-pseudotyped, wild-type (wt) HIV-1-GFP and mutant HIV-1-GFP containing a G89A capsid change were incubated with Cf2Th cells expressing TRIMCyp or RBGCN4TriL2Cyp, or control cells transduced with the empty LPCX vector. In parallel, LPCX-transduced cells were exposed to these viruses lacking envelope glycoproteins (Env^-). Sixteen hours later, the cells were lysed and the cell lysates were analyzed on 50% sucrose cushions, as described (Stremlau et al., 2006a). The HIV-1 p24 capsid protein detected in the particulate fraction (Pellet) and supernatant (Sup.) is shown.

EDTA) and placed on ice for 15 min. The cells were lysed by using a 2-ml Dounce homogenizer (Pestle B, 15 strokes) and the cell debris removed by centrifugation at 4 °C for 10 min at maximum speed (14,000×*g*) in an Eppendorf microfuge. Fifty microliters of lysate was saved for assessment of the input amount of TRIMCyp or CypA derivative in the assay. Two-hundred microliters of the cleared cell lysate were combined with 5 μl of HIV-1 CA–NC complexes from the assembly reaction and the concentration of NaCl adjusted to 150 mM. The mixture was incubated for 1 h at room temperature with gentle mixing. After incubation, the mixture was layered onto a 2-ml 70% sucrose cushion (prepared in 1× PBS) and centrifuged at 110,000×*g* for 1 h at 4 °C in a Beckman SW55Ti rotor. The pellet was resuspended in 50 μl of 1× SDS sample buffer and subjected to SDS–PAGE and Western blotting.

Protein analysis

Cellular proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% NP-40; 2 mg of aprotinin/ml; 2 mg of leupeptin/ml; 1 mg of pepstatin A/ml; 100 mg of phenylmethylsulfonyl fluoride/ml). The cell lysates were analyzed by SDS–PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Pharmacia Biotech). Detection of protein by Western blotting utilized monoclonal antibodies that are specifically reactive with the HA (Roche) or V5 (Invitrogen) epitope tags, and monoclonal antibodies to β-actin (Sigma). Detection of proteins was performed by enhanced chemiluminescence (NEN Life Sciences Products), using the following secondary antibodies obtained from Amersham Pharmacia Biotech: anti-mouse (β-actin) and anti-rat (for HA).

Cross-linking

The HA-tagged proteins were expressed transiently in 293T cells or stably in Cf2Th cells. Cells were washed in phosphate-buffered saline (PBS) and lysed in NP40 lysis buffer (0.5% Nonidet P40 (NP40), 1× protease inhibitor (complete EDTA-free, Roche Diagnostics) in PBS) for 45 min at 4 °C. Lysates were centrifuged at 14,000×*g* for 15 min at 4 °C. The cleared lysates were not stored or frozen but rather were directly cross-linked. Approximately 100–200 μl of cleared lysates were diluted with PBS/1 mM EDTA to a final volume of 400 μl. Lysates were cross-linked with varying concentrations (up to 2 mM) of ethylene glycol-bis(succinimidyl succinate) (EGS) for 30 min at room temperature and centrifuged briefly in a table-top centrifuge. The reaction mix was quenched with 0.1 M Tris–HCl, pH 7.5, and briefly centrifuged. The cleared, cross-linked lysates were precipitated with the anti-HA antibody HA.11 (Covance) and protein A-Sepharose beads (Amersham) for 2 h at 4 °C; final volumes for the immunoprecipitation were greater than 700 μl. The beads were washed four times with NP40 wash buffer (10 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.5% NP40) and boiled in LDS sample buffer (106 mM Tris–HCl, 141 mM Tris Base, pH 8.5, 0.51 mM EDTA, 10% glycerol, 2% LDS, 0.22 mM SERVA Blue G250, 0.175 mM phenol red; Invitrogen) with a final concentration of 1.2% β-mercaptoethanol (β-ME) for 10 min. Precipitated proteins were separated on 8% or 12% Tris–glycine gels, transferred to a PVDF membrane and detected with the horseradish peroxidase-conjugated 3F10 anti-HA antibody (Roche Diagnostics) and the ECL Plus Western Blotting Detection System (Amersham).

Immunofluorescence confocal microscopy

Cf2Th cells expressing the HA epitope-tagged TRIMCyp, CypA or variant CypA proteins were grown overnight on Lab-Tek II Chamber Slides (Nalge Nunc International). Following fixation for 15 min in Cytofix/Cytoperm (BD Biosciences) and permeabilization for 15 min in Perm/Wash (BD Biosciences), the cells were incubated for 1 h with rat anti-HA 3F10 antibody (Roche). The cells were then incubated with anti-rat IgG conjugated with FITC (Santa Cruz) to detect the expression of the TRIMCyp or CypA variants and were examined by confocal fluorescence microscopy.

Fate-of-capsid assay

The assay to examine the fate of HIV-1 capsids in the cytosol of control and TRIMCyp-expressing cells was performed as described by Stremlau et al., 2006a.

Acknowledgments

We thank Ms. Yvette McLaughlin and Elizabeth Carpelan for manuscript preparation. This work was supported by grants

from the National Institutes of Health (AI063987, HL54785 and a Center for AIDS Research Award AI28691), the International AIDS Vaccine Initiative, the Bristol-Myers Squibb Foundation, The William A. Haseltine Foundation for the Arts and Sciences and the late William F. McCarty-Cooper. H.J. was supported by a fellowship from the Canadian Institutes of Health Research.

References

Bosco, D.A., Kern, D., 2004. Catalysis and binding of cyclophilin A with different HIV-1 capsid constructs. Biochemistry 43 (20), 6110–6119.

Braaten, D., Ansari, H., Luban, J., 1997. The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. J. Virol. 71 (3), 2107–2113.

Diaz-Griffero, F., Vandegraaff, N., Li, Y., McGee-Estrada, K., Stremlau, M., Welikala, S., Si, Z., Engelman, A., Sodroski, J., 2006. Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. Virology 351 (2), 404–419.

Diaz-Griffero, F., Kar, A., Perron, M., Xiang, S.-H., Javanbakht, H., Li, X., Sodroski, J., submitted for publication. Modulation of retroviral restriction and proteasome inhibitor-resistant turnover by changes in the TRIM5 α B-box 2 domain, manuscript submitted.

Franke, E.K., Yuan, H.E., Luban, J., 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature 372 (6504), 359–362.

Gamble, T.R., Vajdos, F.F., Yoo, S., Worthylake, D.K., Houseweart, M., Sundquist, W.I., Hill, C.P., 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell 87, 1285–1294.

Ganser, B.K., Li, S., Klishko, V.Y., Finch, J.T., Sundquist, W.I., 1999. Assembly and analysis of conical models for the HIV-1 core. Science 283 (5398), 80–83.

Harbury, P.B., Zhang, T., Kim, P.S., Alber, T., 1993. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science 262 (5138), 1401–1407.

Hatziioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., Bieniasz, P.D., 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. Proc. Natl. Acad. Sci. U. S. A. 101 (29), 10774–10779.

Hatziioannou, T., Perez-Caballero, D., Cowan, S., Bieniasz, P.D., 2005. Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. J. Virol. 79 (1), 176–183.

Javanbakht, H., Diaz-Griffero, F., Stremlau, M., Si, Z., Sodroski, J., 2005. The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5alpha. J. Biol. Chem. 280 (29), 26933–26940.

Javanbakht, H., Yuan, W., Yeung, D.F., Song, B., Diaz-Griffero, F., Li, Y., Li, X., Stremlau, M., Sodroski, J., 2006. Characterization of TRIM5alpha trimerization and its contribution to human immunodeficiency virus capsid binding. Virology.

Keckesova, Z., Ylinen, L.M., Towers, G.J., 2004. The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. Proc. Natal. Acad. Sci. U. S. A. 101 (29), 10780–10785.

Li, Y., Li, X., Stremlau, M., Lee, M., Sodroski, J., 2006. Removal of arginine 332 allows human TRIM5α to bind human immunodeficiency virus (HIV-1) capsids and to restrict infection. J. Virol. 80, 6738–6744.

Lin, T.Y., Emerman, M., 2006. Cyclophilin A interacts with diverse lentiviral capsids. Retrovirology 3, 70.

Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., Goff, S.P., 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell 73 (6), 1067–1078.

Lumb, K.J., Kim, P.S., 1995. A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. Biochemistry 34 (27), 8642–8648.

Mische, C.C., Javanbakht, H., Song, B., Diaz-Griffero, F., Stremlau, M., Strack, B., Si, Z., Sodroski, J., 2005. Retroviral restriction factor TRIM5alpha is a trimer. J. Virol. 79 (22), 14446–14450.

Nisole, S., Lynch, C., Stoye, J.P., Yap, M.W., 2004. A trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. Proc. Natl. Acad. Sci. U. S. A. 101, 13318–13324.

Perez-Caballero, D., Hatziioannou, T., Yang, A., Cowan, S., Bieniasz, P.D., 2005a. Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. J. Virol. 79 (14), 8969–8978.

Perez-Caballero, D., Hatziioannou, T., Zhang, F., Cowan, S., Bieniasz, P.D., 2005b. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. J. Virol. 79 (24), 15567–15572.

Perron, M.J., Stremlau, M., Song, B., Ulm, W., Mulligan, R.C., Sodroski, J., 2004. TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc. Natl. Acad. Sci. U.S.A. 101 (32), 11827–11832.

Perron, M.J., Stremlau, M., Lee, M., Javanbakht, H., Song, B., Sodroski, J., 2007. The human TRIM5α restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. J. Virol. 81, 2138–2148.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., Guffanti, A., Minucci, S., Pelicci, P.G., Ballabio, A., 2001. The tripartite motif family identifies cell compartments. EMBO J. 20 (9), 2140–2151.

Ribeiro, J.P., Menezes, A.N., Moreira, M.A., Bonvicino, C.R., Seuanez, H.N., Soares, M.A., 2005. Evolution of cyclophilin A and TRIMCyp retrotransposition in New World primates. J. Virol. 79 (23), 14998–15003.

Sayah, D.M., Sokolskaja, E., Berthoux, L., Luban, J., 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature 430 (6999), 569–573.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427 (6977), 848–853 [see comment].

Stremlau, M., Perron, M., Lee, M., Yuan, L., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D., Sundquist, W.I., Sodroski, J., 2006a. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5α restriction factor. Proc. Natl. Acad. Sci. U.S.A. 103, 5514–5519.

Stremlau, M., Song, B., Javanbakht, H., Sodroski, J., 2006b. Cyclophilin A: an auxiliary but not necessary cofactor for TRIM5α restriction of HIV-1. Virology 351, 120.

Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C., Sodroski, J., Göttlinger, H., 1994. Functional association of cyclophilin A with HIV-1 virions. Nature 372, 363–365.

Towers, G.J., Hatziioannou, T., Cowan, S., Goff, S.P., Luban, J., Bieniasz, P.D., 2003. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat. Med. 9 (9), 1138–1143.

Weissenhorn, W., Calder, L.J., Dessen, A., Laue, T., Skehel, J.J., Wiley, D.C., 1997. Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 94 (12), 6065–6069.

Yap, M.W., Nisole, S., Lynch, C., Stoye, J.P., 2004. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 101 (29), 10786–10791 [see comment].

Yee, J.K., Friedmann, T., Burns, J.C., 1994. Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol 43 (Pt. A), 99–112.

Yin, L., Braaten, D., Luban, J., 1998. Human immunodeficiency virus type 1 replication is modulated by host cyclophilin A expression levels. J. Virol. 72 (8), 6430–6436.

Yoo, S., Myszka, D.G., Yeh, C., McMurray, M., Hill, C.P., Sundquist, W.I., 1997. Molecular recognition in the HIV-1 capsid/cyclophilin A complex. J. Mol. Biol. 269, 780–795.