The design of artificial retroviral restriction factors

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Abstract

In addition to the ability to bind the retroviral capsid protein, the retroviral restriction factors Fv1, Trim5α and Trim5–CypA share the common property of containing sequences that promote self-association. Otherwise Fv1 and Trim5α appear unrelated. Mutational analyses showed that restriction was invariably lost when changes designed to disrupt the sequences responsible for multimerization were introduced. A novel restriction protein could be obtained by substituting sequences from the self-associating domain of Fv1 for the Trim5 sequences in Trim5–CypA. Similarly, a fusion protein containing cyclophilin A joined to arfaptin2, a protein known to form extended dimers, was also shown to restrict HIV-1. Hence, multimerization of a capsid-binding domain could be the common minimum design feature for capsid-dependent retroviral restriction factors. However, not all domains that promote multimerization can substitute for the N-terminal domains of Fv1 and Trim5α. Moreover, only CypA can provide a capsid-binding site with different N-terminal domains. It is suggested that the spatial relationship between the multiple target binding sites may be important for restriction.

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Introduction

Retroviruses are restricted by a number of cellular factors. Some of these act during the early phase of the lifecycle and block the virus in a capsid (CA)-dependent manner (Stoye, 2002). The prototypic restriction factor Fv1 was first described in the early 1970’s as a genetic locus in mice that prevents infection by certain strains of murine leukemia virus (MLV) (Lilly, 1970). There are two major alleles of Fv1: Fv1n restricts the B-tropic MLV while Fv1b restricts the N-tropic strain (Hartley et al., 1970). A single amino acid change at residue 110 in CA can alter the susceptibility to restriction, suggesting a direct interaction between virus CA and restriction factor (Kozak and Chakraborti, 1996). Fv1 has been cloned and is related to the Gag gene of an endogenous retrovirus, HERV-L (Best et al., 1996).

More recently, restriction of N-tropic MLV has also been reported in non-murine cells (Towers et al., 2000). In addition, infection by HIV-1 is blocked in certain primate cells in a similar way (Besnier et al., 2002; Cowan et al., 2002). The primate restriction factor is Trim5α (Stremlau et al., 2004), a cellular protein that bears no apparent similarity to Fv1. Instead, it belongs to a large family of Trim proteins containing the tripartite motifs RING, B-Box and Coiled coil (RBCC) (Reymond et al., 2001). In addition to the RBCC, Trim5α also possesses a C-terminal B30.2 domain. Studies of a series of chimeric proteins made between the human Trim5α, which does not restrict HIV-1 well, and rhesus Trim5α, which does, have revealed that the specificity determinants of restriction lie in the B30.2 domain (Perez-Caballero et al., 2005; Stremlau et al., 2005; Yap et al., 2005). Indeed, the residues in this domain that influenced restriction were found to be under positive selection, suggesting that the gene might have evolved to protect against retroviral infection (Sawyer et al., 2005).

In owl monkeys, the B30.2 domain of Trim5α has been replaced with cyclophilin A (CypA) through a retrotranspositional insertion of a CypA cDNA between exons 7 and 8 of the Trim5 locus (Nisole et al., 2004; Sayah et al., 2004). The resulting fusion protein restricts HIV-1 in a CypA-dependent manner; a non-binding mutant of CA, G89V, is not restricted. In addition, restriction can also be abolished by treatment with
cyclosporin A, which prevents the binding of CypA to CA (Luban et al., 1993). This suggested that in Trim5CypA, CypA had replaced the function of the B30.2 domain in recognizing and binding HIV-1 CA. The RBCC of Trim5CypA can also be replaced with the RBCC from other members of the Trim family, in particular, Trim1, Trim18 and Trim19 (Li et al., 2006a; Yap et al., 2006; Zhang et al., 2006). These results suggested that the RBCC of other Trim family members can provide any function required for restriction. To examine the role(s) of the RBCC domain we have now performed an analysis of the function of the N-terminal domain and Trim5α in retrovirus restriction and used the information obtained to design two artificial restriction factors.

Results

The Trim5α coiled coil motif is essential for restriction

We have previously found that the B30.2 domain of Trim5α could be replaced by CypA for HIV-1 restriction (Yap et al., 2005). In addition, the RBCC domain of other Trim proteins is interchangeable with that of Trim5CypA in blocking HIV-1 restriction (Yap et al., 2006a; Yap et al., 2006; Zhang et al., 2006). These proteins do not restrict HIV-1 but their CypA derivatives do so in a manner that is dependent on the ability to recognize the CypA binding loop of CA. This result suggested that the RBCC of other Trim family members can provide any function required for restriction. To examine the role(s) of the RBCC domain we have now performed an analysis of the function of the N-terminal domain and Trim5α in retrovirus restriction and used the information obtained to design two artificial restriction factors.

The N-terminal domain of Fv1 can replace the RBCC in Trim5CypA restriction of HIV-1

We next set out to test the idea that it might be possible to generate an artificial restriction factor by combining any multimerization domain with a CA-binding domain. These initial attempts were unsuccessful. Hybrid molecules generated by fusing glutathione-S-transferase (dimer (Vargo et al., 2004)), Rad50 (dimer (Hopfner et al., 2002)) or α-tubulin (higher multimer (Nogales and Wang, 2006)) to CypA did not restrict HIV (Fig. 2).

Postulating that the separation of the CA binding domains might play an important role in determining the restriction potential of the hybrid molecule we next tested fusions between the N-terminal domain of the Fv1 protein and CypA. Fv1 is a gene in mice that confers resistance to MLV (Lilly, 1970). Like Trim5α, Fv1 restriction is CA-dependent (Kozak and Chakraborti, 1996) and saturable (Duran-Troise et al., 1977). Functional analyses of Fv1 suggested that it consists of 2 domains separated by a flexible linker (Bishop et al., 2001). Initial studies indicated that the N-terminal domain is involved in localization while
### Table A

<table>
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<th>B-MLV</th>
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### Figure C

- WT, DelR, DelB, DelCC, DelRB, C15,18A, C97A
- RhTrim5
- RhTSC

- Western Blot Analysis
the C-terminal domain contains specificity determinants and multimerization signals (Yap and Stoye, 2003). More recent studies have shown that the N-terminal domain of Fv1 is also capable of multimerization, adopting an extended dimer conformation when expressed in Escherichia coli or baculovirus vectors (Bishop et al., 2006). In addition, the COILS program of EMNet predicts a coiled coil motif in the N-terminal domain of Fv1 between residues 82 to 116 with greater than 99% probability.

To test whether the N-terminal domain of Fv1 can replace that of Trim5, different lengths of the 5’ end of the Fv1 gene were joined to the CypA encoding region of OMKTrim5CypA and the resulting fusion proteins were tested for their ability to restrict HIV-1. Cells transduced with CypA alone did not restrict HIV-1 (Fig. 3A). By contrast, fusion proteins that contained at least the first 158 residues of Fv1 fused to CypA were found to restrict HIV-1 but not the non-binding mutant G89V. Fv1(1–138)CypA and Fv1(1–148)CypA, did not show restriction. However, protein studies revealed that these constructs show little if any stable expression (Fig. 3B). These results suggested that the first 158 residues in Fv1 contained a function that was performed by the RBCC of Trim proteins in HIV-1 restriction and provide compelling evidence that the N-terminal domain of Fv1 can functionally replace the RBCC domain in Trim5CypA-mediated HIV restriction, implying that dimeric binding of CypA to HIV-1 can block virus replication.

**Fusion of dimer-forming Arfaptin2 to CypA results in the restriction of HIV-1**

To examine the generality of this observation we fused CypA, to Arfaptin2, a protein that had previously been shown to form strong dimers (Tarricone et al., 2001). Arfaptin2 mediates cross-talk between Rac and Arf GTPases (D’Souza-Schorey et al., 1997). There have been no previous reports of any link between this protein and the retroviral infection cycle and it does not restrict HIV-1 (Fig. 4A). The structure of arfaptin2 has been solved and was found to contain a BAR domain that forms a crescent shaped dimer (Tarricone et al., 2001). The C-terminus of each monomer resided in the middle of the crescent while the N-terminus was unstructured. We reasoned that fusing CypA to the C-terminus of Arfaptin2 might either disrupt the dimer formation or hinder binding to CA. Instead, a fusion protein was constructed by fusing the entire Arfaptin2 reading frame to the C-terminus of CypA. Restriction assays showed that CypA–Arfaptin2 (1–341) restricted HIV-1 but not the non-binding mutant G89V, confirming that dimerization of a CA-binding domain was sufficient to bring about restriction (Fig. 4A). The structure of the first 117 residues of Arfaptin2 remains unknown (Tarricone et al., 2001). To investigate if this region was contributing to the restriction, 2 other constructs were made containing different lengths of Arfaptin2. While CypA–Arfaptin2 (60–341), which was missing half of the N-terminal region, could still restrict HIV-1 (Fig. 4A), this ability was lost when the first 117 residues of Arfaptin2 was removed in CypA–Arfaptin2 (118–341), although the latter construct contained an intact BAR domain. This suggested that a linker region between the dimerization domain and the CA-binding domain was important for restriction.

Arfaptin2 has been shown to associate with the trans Golgi network (Peter et al., 2004). Since Fv1 is associated with tubules of the trans Golgi network (Yap and Stoye, 2003), it seemed possible that the restriction of CypA–Arfaptin2 was due to a re-targeting of the CA-binding motif to the trans Golgi network. To test this possibility, two double-point mutants of CypA–Arfaptin2 were made, K226E, Q227E and R232E, R239E. These mutants had been previously shown to abolish localization to the trans Golgi network while retaining structural integrity (Peter et al., 2004). Localization studies confirmed that Arfaptin2 and CypA–Arfaptin2 localize to the trans Golgi

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**Fig. 1. Functional analyses of the RBCC domain.** (A) Substitution mutants. (B) Deletion mutants. A schematic representation of the mutation is shown on the left while effects on the restriction of different viruses are shown on the right. Restriction assays were performed by FACS analyses of HT1080 cells to determine the ratio of percentage infected Trim positive cells to percentage infected Trim negative cells. A ratio that is less than 0.3 is scored as restriction while a ratio greater than 0.7 is taken as no restriction. Values between 0.3 and 0.7 are interpreted as partial restriction. Ratios indicating restriction are boxed. (C) Analysis of protein expression. Cells transduced with the restriction factor constructs were lysed and restriction factor expression levels analyzed by Western blotting using an anti-Trim5α antibody.
network while the K226E, Q227E (Fig. 4B) and R232E, R239E (not shown) mutants were evenly distributed through the cytoplasm. The non-localizing mutants still retained the ability to block HIV-1, suggesting that the dimerization feature of this protein and not the localization signal was important for HIV-1 restriction.

To confirm the restriction properties of Fv1CypA and CypA–Arfaptin2, we introduced these genes into vectors encoding neomycin resistance, established permanent cell cultures by selection with G418 followed by HIV-1 titration (Fig. 5). Fv1(1–158)CypA restricted HIV-1 almost as efficiently as rhT5C, but CypA–Arfaptin2, although showing greater than five fold inhibition was significantly less efficient. Robust inhibition by rhT5CdeltaBOX was also confirmed in this experiment, although this was somewhat less potent than rhT5C. Conversely, rhTrim5αdeltaBOX appeared not to inhibit HIV-1 infection at all giving titres slightly higher than the negative control.

Restriction properties of Fv1CypA and CypA–Arfaptin2

To help understand the mechanism of action of Fv1CypA and CypA–Arfaptin2, the stage of the viral life cycle that they blocked was determined by quantifying the products of reverse transcription and 2-LTR circles. Cells transduced with the restriction factors were infected with equivalent amounts of HIV-1 and total DNA was isolated 7 h or 24 h following infection. Quantitative PCR was performed to detect products of reverse transcription and 2-LTR circles, respectively. As described previously, there was less reverse transcription product detected for cells expressing OMK Trim5CypA (Yap et al., 2006) (Fig. 6). CypA–Arfaptin2 did not seem to reduce the amount of reverse transcription product compared to the negative control. Fv1CypA, however, resulted in an intermediate level of reverse transcription. Correspondingly, there were fewer 2-LTR circles detected in cells expressing OMK Trim5CypA (Fig. 6B). Fv1CypA also seemed to reduce the number of 2-LTR circles while CypA–Arfaptin2 did not have an effect. Taken together, these results suggested that Fv1CypA could reduce the process of reverse transcription, although not as efficiently as OMK Trim5CypA. However, it did reduce the amount of 2-LTR circles, which are an indication of nuclear translocation (Brown, 1997), at least as well as Trim5CypA. CypA–Arfaptin2, on the other hand did not affect either reverse transcription or nuclear entry. Hence, the novel restriction factors blocked different stages of the viral lifecycle, with Fv1CypA possibly acting at more than one stage.

Analysis of the multimerization status of Fv1Cyp and CypA–Arfaptin2 constructs

Fv1(20–200) and Arfaptin2 are dimers (Bishop et al., 2006; Tarricone et al., 2001). Although it seemed extremely unlikely that fusion to CypA would alter the multimerization status of these proteins it seemed important to exclude this formal possibility by co-precipitation and molecular weight determination experiments. In the first series of experiments HA-tagged CypA fusions were co-transfected with a variety of untagged Fv1 or Arfaptin2 constructs. Lysates from these cells were precipitated with anti-HA antibody and analyzed by Western blotting using antibodies to Fv1 or Arfaptin2 (Figs. 7A, B). Immunoprecipitation of the untagged proteins was only seen in the presence of HA-tagged protein implying the presence of dimers or higher-order multimers with both Fv1(1–158)CypA and CypA–Arfaptin2.

To examine the extent of multimerization we analyzed purified Fv1(1–200)CypA by size exclusion chromatography using multi-angle laser light scattering (Wen et al., 1996), a method yielding an estimate of absolute molecular weight. The chromatogram recorded by the differential refractometer and that recorded from the intensity of scattered light yield coincident peaks (Fig. 8A) indicating uniformity of the protein species. Molecular weight analysis gave a value of 81 kDa (Fig. 8B), exactly twice the size of the formula molecular weight for Fv1(1–200)CypA implying that this protein is indeed a dimer.

Discussion

Functional analyses of the RBCC belonging to rhTrim5α and rhT5C have revealed that the coiled coil motif is essential...
for restriction activity. A similar motif is present in Fv1 within the N-terminal domain of Fv1. Although there is no sequence homology between Fv1 and Trim5α, they both restrict retroviruses in a CA-dependent manner. In the Trim proteins, the coiled coil has been implicated in protein–protein interactions (Reymond et al., 2001) and has been reported to mediate homomultimerization leading to the formation of trimeric Trim5 (Mische et al., 2005). The importance of multimerization is supported by the present observations that novel factors that restrict HIV-1 can be created by fusing a binding domain, CypA, to a multimerization domain, either from Fv1 or from the totally unrelated Arfaptin2.

Multimerization of the restriction factor could contribute to its antiviral activity in several ways. It could serve to increase the avidity of the factor for CA through promoting the attachment of several binding domains simultaneously in a manner analogous to the binding of sialic acid residues by influenza hemagglutinin (Skehel and Wiley, 2000). Thus, monomeric CypA binds HIV-1 CA relatively weakly with a dissociation constant in the low μM range (Howard et al., 2003; Yoo et al., 1997). However, simultaneous binding of two or three joined CypA domains to a lattice of CA targets would give rise to an apparent $K_d$ corresponding to essentially irreversible binding. The failure to detect direct binding of Fv1 or Trim5α to unpolymerized CA from MLV or HIV-1 might reflect a monomer binding affinity say in the high μM or low mM range. Such binding might have limited consequences unless multiple interactions took place. In such a way tight binding might occur, securely anchoring biochemical signals, for example specifying interactions with the proteasome (Wu et al., 2006), on the restriction factor to its target, even when the restriction factor is present at very low concentrations within the cell.

Multimerization could also have more direct effects on restriction by cross-linking several subunits of CA on the surface of the viral core very soon after entry into the cytoplasm. The binding of several CA subunits might interfere with the controlled disassembly (Forshey et al., 2002) that is required for reverse transcription resulting in the absence of early reverse transcriptase products. Alternatively, the cross-linked CA subunits might prevent a reorganization of the viral core, a process that could be required to allow formation of a more compact structure that could pass through the nuclear pore. This could account for the reduction in the 2-LTR circle formation, which is taken as an indication of nuclear entry. Finally, cross-linking of the CA molecules that remain associated with the translocated pre-integration complex could interfere with the steps that are involved in integration. This could

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Fig. 4. Restriction of HIV-1 by CypA–Arfaptin2. (A) Schematic representations of the fusion proteins and the mutant derivatives are shown on the left and the restriction data indicated on the right. Restriction assays were performed as described for Fig. 1. Values representing restriction are boxed. (B) Immunofluorescence analyses. Arfaptin2 and CypA–Arfaptin2 were detected with anti-Por1 followed by TRITC-conjugated anti-goat IgG and are shown in red. Nuclei were stained with DAPI and shown in blue.
account for the very late block by restriction factors such as CypA–Arfaptin.

Despite having no homology at the sequence level, Fv1 and Trim5α seem to share a similar structural organization. Both have a multimORIZATION domain that is followed by a binding domain. Some of these domains are interchangeable like the RBCC and Fv1 coiled coil in the case of fusions to CypA. On the other hand, we have observed that fusion proteins containing the N-terminal domain of Fv1 fused to the B30.2 domain of rhTrim5α and the RBCC of rhTrim5α fused to the C-terminal domain of Fv1 had no restriction activity (data not shown). However, novel restriction factors like CypA–Arfaptin2 can be constructed based on this organization. In the case of CypA–Arfaptin2, the binding domain precedes the multimORIZATION domain at the sequence level. Thus, structurally, the organization would still be analogous to Trim5α and Fv1 with binding domains linked by an extended multimORIZATION domain. However, the nature of the multimORIZATION domain can differ; while the Trim5 RBCC domain forms a trimer (Mische et al., 2005), the N-terminal domain of Fv1 (Bishop et al., 2006) and the Arfaptin 2 (Tarricone et al., 2001) molecule are dimers. Hence, while trimerization as seen with Trim5α and Trim5CypA is not an absolute requirement for restriction, the ability to multimetrize plays an essential role. It would be interesting to study the effects of different states of multimORIZATION on the stage of block in the viral life cycle. A trimer, for example, might be more efficient at holding the core together and preventing disassembly, thereby blocking early while a dimer might not hinder disassembly but could remain bound to the CA on the disassembled core to affect steps in trafficking or integration.

Multimerization is clearly an integral part of retroviral restriction (Javanbakht et al., 2005; Perez-Caballero et al., 2005). However, the ability to multimetrize does not seem to be sufficient for retroviral restriction. This is highlighted by the fact that not all domains that promote multimORIZATION could
restrict HIV-1 in fusion with CypA. For example, fusions of CypA to GST, Rad50 or α-tubulin did not result in restriction of HIV-1. It is not clear what features these proteins lack that are common to Fv1 and Arfaptin2. Fv1 is a restriction factor and might conceivably possess some as yet unidentified motif necessary for restriction but it seems unlikely that this feature would be shared with Arfaptin2. Rather, it is tempting to suggest that the dimensions of the multimerization domain may play an important role. Arfaptin2, which is able to provide the multimerization function to restrict, contains an extended Bar domain which separates the two CypA domains by about 140 Å (Tarricone et al., 2001). The N-terminal domain of Fv1 containing the coiled coil can also provide this function. Although the predicted coiled coil was only between residues 82 and 116, at least the first 158 residues of Fv1 were required for restriction. This region of Fv1 was recently reported to form an extended dimer resembling the arfaptin2 dimer (Bishop et al., 2006). Hence, it is possible that there is a minimal length requirement for the multimerization domain. In addition, linker regions between the multimerization and binding domains could also be important, as observed in Fv1 and CypA–Arfaptin2. Indeed, recent studies have shown that the linker region between the coiled coil and B30.2 domain is also required for trimerization (Javanbakht et al., 2006), indicating the possibility of the interaction domain extending beyond the predicted coiled coil. In the context of the hexameric arrangement of CA on the surface of the core (Li et al., 2000; Mortuza et al., 2004), the distance between adjacent CA molecules is likely to be too small to allow binding of an extended multimer of restriction factor to adjacent CA molecules. Perhaps restriction requires binding to CA molecules positioned in different hexameric rings of the core.

The precise roles of the RING and B-Box motifs in restriction remain to be elucidated. Previous studies have shown that deletion of either region leads to a significant loss of restriction activity suggesting that both regions may supply some effector functions required for activity (Diaz-Griffero et al., 2006; Javanbakht et al., 2005; Perez-Caballero et al., 2005; Stremlau et al., 2004). However, some of our data, most strikingly with rhT5CdeltaBOX, are inconsistent with this interpretation. Possibly very subtle differences in the construction of the different expression constructs are responsible.
Perhaps more likely are complications resulting from differences in expression level. It is noteworthy that essentially all mutational studies so far reported in this area have been performed using transduced or transfected constructs. This results in very much higher levels of protein expression than for naturally expressed Trim5α, T5C or Fv1. Distinguishing effects resulting from over-expression from those caused by the introduced mutations remains a major challenge for all who work in this field. Nevertheless, it is clear that the RING and B-Box are entirely dispensable in the contexts of Fv1(1–158) CypA and CypA–Arfaptin2 (1–341) as expressed here. Thus if an effector function is absolutely required for restriction, it must be supplied by some other protein motif. Confounding, or perhaps suggesting a solution to, this problem are observations that different restriction factors can act at different stages in the viral life cycle, perhaps acting in different ways (Yap et al., 2006).

Indeed, individual factors, for example Trim5α, Trim5CypA and, possibly, Fv1(1–158)CypA (see Fig. 6) can show effects at different stages (Anderson et al., 2006; Wu et al., 2006). Untangling the different mechanisms of restriction remains a formidable task.

Materials and methods

Cell lines and virus production

All cells were maintained in Dulbecco’s modified Eagle’s media containing 10% fetal calf serum and 1% penicillin/streptomycin. Viruses were produced by transient transfection of 293T cells as previously described (Soneoka et al., 1995). All viruses were pseudotyped with VSVG expressed from the plasmid pCZVSVG (Bock et al., 2000). The EGFP marker was expressed from vectors pCSGW (Bainbridge et al., 2001) and pLNCG (Yap et al., 2004) for HIV-1 and MLV respectively. HIV-1 Gag–pol was produced from either p8.91 (wild type) (Zufferey et al., 1997) or pG89V (cyclophilin A non-binding mutant) (Yap et al., 2004). All restriction genes were expressed from derivatives of pLGatewayIEYFP (Yap et al., 2005) or pLGatewaySN that was delivered using the Moloney gag–pol from pHIT60.

Plasmid constructs

The retroviral vector, pLGatewaySN was constructed by inserting the Gateway Reading Frame A (Invitrogen) fragment into the HpaI site of pLXSN.

RBBC point mutants of rhTrim5 and rhT5C were made by site-directed mutagenesis of plasmids pLRhTrim5IEYFP and pLRhT5CIEYFP as described previously (Yap et al., 2005). Deletion mutants were constructed by overlapping, two-stage PCR using the primers listed in Table 1. The gene to be deleted was amplified in 2 fragments: the first before the deleted section, with primer pair DeltaRINGRev, DeltaBoxRev or DeltaCoilRev and Trim5F, and the second after the deletion with primer pair Trim5Rev or CypARev and DeltaRINGF, DeltaBoxF or DeltaCoilF. The 2 fragments were then joined by amplifying with Trim5F and Trim5Rev or CypARev. The previously introduced mutations remained a major challenge for all who work in this field. Nevertheless, it is clear that the RING and B-Box are entirely dispensable in the contexts of Fv1(1–158)
OMKT5C with forward primer CypAF and reverse primers CypArfRev1, CypArfRev2 or CypArfRev3 was joined to the product from the amplification of pGEXArfaptin2 with forward primers CypArfF1, CypArfF2, CypArfF3 and reverse primer Arfaptin2Rev with primers CypAF and Arfaptin2Rev to produce the CypA–Arfaptin2 fusions. Arfaptin2 was amplified from pGEXArfaptin2 with primer pairs Arfaptin2F and Arfaptin2Rev. All final PCR products were cloned into TopoDENTR vector (Invitrogen) before insertion into pLGate-way or IYFP pLGatewaySN using LR clonase (Invitrogen) and sequencing. Various constructs were retrofitted with a HA tag by PCR using the original forward primer plus HACypARev (CTAAGCGTAATCTGGAACATCGTATGGGTATTCGAGTTGTCCACAGTCAGCAATGG) or HAarfRev (CTAAGCGTAATCTGGAACATCGTATGGGTACTGCTCCTCTAGGCAGAGGTTTC), before cloning into the TopoDENTR and reinsertion into the Gateway vector. Hybrid molecules fusing GST, Rad50 or α-tubulin to CypA were generated similarly (details available on request).

**Restriction assays**

Restriction assays were routinely performed using FACS analysis of HT1080 cells to determine the ratio of the percentage of restriction factor expressing cells that were infected to that of non-expressing cells by two-color FACS analysis as previously described (Bock et al., 2000; Yap et al., 2004). A ratio of less than 0.3 was taken as strong restriction while a ratio that was more than 0.7 was taken as no restriction. Ratios between 0.3 and 0.7 were taken to indicate partial restriction. Alternatively, HT1080 cells were transduced with restriction genes cloned in pLGatewaySN. The cells were selected on G418 (1 mg/ml) for 2 weeks before seeding in 12-well plates at a density of $5 \times 10^4$ cells per well. The cells were then challenged with different quantities of HIV-1 carrying the GFP marker and the percentage of infected cells assessed by FACS 3 days post-infection.

**Western blot analyses**

Cells were seeded in 12 well plates to a density of $5 \times 10^4$ cells per well 24 h pre-transduction. The cells were transduced with retroviral vectors carrying the restriction gene so the 70% of the cells were transduced as determined by FACS analyses of the EYFP marker. The transduced cells were plated in 6 cm dishes and grown to confluency before lysing with buffer containing 1% NP40. Bradford assays were performed to determine the total protein concentration of the cell lysates and 25 μg of total protein was loaded in each lane of a SDS–polyacrylamide gel. The separated bands were transferred onto a PVDF membrane which was blocked overnight in PBS containing 0.1% Tween and 5% milk. Primary and secondary antibody binding were performed in PBS containing 0.1% Tween and 5% milk with four washes of 5 min each in PBS containing 0.1% Tween and 0.5% milk. Goat anti-Trim5α (AbCam) and Rabbit anti-Fv1 (Bishop et al., 2001) were used at dilutions of 1:1000, while HRP–anti-goat (Vector laboratories) and HRP–anti-rabbit (Pierce) were used at dilutions of 1:1000 and 1:5000, respectively. Detection was performed using the ECL reagent from Amersham.

**Co-immunoprecipitation**

Fv1(1–158)CypAHA or CypArfaptinHA were co-expressed with untagged proteins in 293T cells, which were seeded in 6 cm
dishes at a density of $2 \times 10^6$ cells per dish 24 h before transfection. The cells were transfected with 10 μg of each plasmid. In dishes where only one factor was transfected, the empty vector pLgatewayIRESYFP was used to make the total amount of DNA up to 20 μg. Cell lysates were harvested 48 h post-transfection and used immediately for immunoprecipitation. Immunoprecipitation was performed using the anti-HA immunoprecipitation kit from Sigma. The transfected cells were lysed with 550 μl of 1× IP buffer each. A final addition of 1 mM IPTG to a mid-log culture.

**Protein purification**

The DNA sequence coding for Fv1(1–200)CypA was cloned into a pET22b expression vector (Novagen) to produce an N-terminal hexa-histidine fusion protein. The nucleotide sequence of the expression clone was verified by automated DNA sequencing and the protein expressed in E. coli BL21 (DE3) by addition of 1 mM IPTG to a mid-log culture.

Cells were lysed by sonication and Fv1(1–200)CypA was purified from clarified crude cell extracts using immobilised metal ion affinity (Ni-NTA) and gel filtration chromatography (Superdex 200). The purity of preparations was monitored by SDS–PAGE and the protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient derived by summing the contributions from tyrosine and tryptophan residues: Fv1(1–200)CypA, ε$_{280}$ = 40,700.

**Quantitative PCR**

Virus stocks were treated with RNase-free DNase (Promega) at 10 units/ml for 1 h before infecting TE671 cells expressing different restriction factors. Total cellular DNA was extracted using the DNeasy kit from Qiagen at 7 and 24 h post-infection for detection of reverse transcription products and 2-LTR circles, respectively. The extracted DNA was quantified with a spectrophotometer and 150 ng was used per reaction for detecting RT products while 500 ng was used for detecting 2-LTR circles and integrated provirus. As previously described (Yap et al., 2006), primer pairs HIVEF/HIVER and 2LTRF/2LTRR were used to detect early RT product RU5 and 2-LTR circles respectively in a 25 μl reaction containing 12.5 μl Sybergreen mix (Abgene) and 70 nm of each primer. The reactions were performed in the ABI Prism 7000 sequence detection system from AB Applied Biosystems, using a program that consisted of an initial incubation at 50 °C for 2 min followed by 95 °C for 15 min before 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

**Immunofluorescence analyses**

Cells were plated in 12-well plates at a density of $5 \times 10^4$ 24 h prior to transduction with retroviral vectors carrying the different restriction genes. The transduced cells were seeded onto coverslips in 12-well plates 2 days post-transduction and allowed to adhere for 24 h before fixing for 15 min with 4% parafomaldehyde in PBS. This was followed by permeabilizing with 0.2% Triton X in PBS for 15 min and one wash with PBS. Blocking in 1% BSA in PBS was performed for 20 min before binding of primary antibody in PBS containing 1% BSA for 1 h. Anti-Por1 (Santa Cruz) was used at a dilution of 1:200. The cells were washed 3 times in PBS containing 1% BSA for 5 min each before proceeding to secondary antibody binding for 1 h with Texas Red-conjugated anti-rabbit antisera (Santa Cruz) at a dilution of 1:400. Coverslips were washed 3 times with PBS before mounting on a slide. Microscopy was performed using a using a Deltavision Olympus IX70 inverted microscope through a 100× objective lens with Softworx image acquisition software.
Multi-angle laser light scattering

The solution molecular weight of Fv1(1–200)CypA was determined using on line multi-angle laser light scattering coupled with size exclusion chromatography. Samples of Ni affinity-purified Fv1(1–200)CypA (200 μg) were applied to a Superdex 200 10/300 GL column equilibrated in 20 mM Tris–HCl, 150 mM NaCl 1 mM TCEP at a flow rate of 0.5 ml/min. The column was mounted on a Jasco HPLC controlled by the Chrompass software package. The Scattered light intensity of the column eluent was recorded at sixteen angles using a DAWN–HELEOS multi-angle laser light scattering detector (Wyatt Technology Corp., Santa Barbara, CA). The Protein concentration of the eluent was determined from the refractive index change (dn/dc=0.186) using an OPTILAB–rEX differential refractometer equipped with a Peltier temperature-regulated flow cell, maintained at 25 °C (Wyatt Technology Corp., Santa Barbara, CA). The wavelength of the laser in the DAWN–HELEOS and the light source in the OPTILAB–rEX was 658 nm. The weight-averaged molecular weight of material contained in chromatographic peaks was determined using the ASTRA software version 5.1 (Wyatt Technology Corp., Santa Barbara, CA). Briefly, at 1-s intervals throughout the elution of the Fv1-cyp peak the scattered light intensities together with the corresponding protein concentration were used to construct Debye plots (KC/Rθ vs. sin^2(θ/2)). The weight-averaged molecular weight was then calculated at each point in the chromatogram from the intercept of an individual plot. An overall average molecular weight and polydispersity term was calculated by combining and averaging the data from the individual measurements.

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