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Diverse HIV viruses are targeted by a conformationally dynamic antiviral

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Abstract

Rhesus TRIMCyp (RhTC) is a potent antiviral that inhibits the replication of diverse HIV viruses. Here we show that it has acquired the ability to target multiple viruses by evolving an active site that interconverts between multiple conformations. Mutations that have relieved active site constraints allow RhTC to dynamically sample conformational space, including radically different conformers that are used to target both HIV-1 and HIV-2 viruses. Introduction of a reversible constraint into RhTC allows specificity to be switched between a single conformation specific for HIV-1 and a dynamic ensemble that targets multiple viruses. These results illustrate how conformational diversity can be used to expand the target diversity of innate immune receptors and help explain the efficiency of antiviral recognition.

Introduction

A successful immune response depends upon successful pathogen detection. This is a significant challenge as the diversity of pathogen molecules is almost limitless. Furthermore, successful detection places the pathogen under selection pressure to evolve a variant molecule that escapes recognition. The result is a constant evolutionary struggle between host and pathogen as each continually adapts to achieve an advantage. However, pathogens have an apparent advantage over their hosts in that they evolve far more rapidly. Moreover, innate antiviral proteins like TRIM5α[1] and TRIMCyp[2, 3] that directly bind and restrict the replication of lentiviruses like HIV have a fixed specificity within the lifetime of an individual. How these proteins target rapidly evolving and diverse viruses is unclear.

Recently, we described the structure of Rhesus macaque TRIMCyp (RhTC) and showed how two mutations in the Cyp domain have remodelled its active site to allow it to bind HIV-2[4]. However, RhTC has been shown to target and restrict multiple diverse lentiviruses in addition to HIV-2, including HIV-1 O group, SIVagm and FIV[5]. If the active site has been remodelled to bind HIV-2 how does RhTC target these other viruses? The ancestor of the cyclophilin domain in RhTC, CypA, has a single conformation and can

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The atomic coordinates and structure factors have been deposited in the Protein Data Bank, <http://www.rcsb.org> (PDB ID codes to be added in proofs).

only functionally interact with HIV-1 and not HIV-2. We therefore reasoned that the mutations in RhTC may have made its active site conformationally dynamic, such that instead of adopting a single conformation specific to HIV-2, it isomerises between multiple conformations that can target multiple viruses.

Using a combination of X-ray crystallography, NMR dynamics, isothermal titration calorimetry and protein engineering studies we show here that the active site of RhTC is in a state of conformational flux, allowing it to rapidly sample multiple conformations. Furthermore, the data suggest that these multiple conformations are functional and are used by RhTC in order to target multiple viruses. Finally, we have incorporated a reversible constraint into RhTC that allows us to switch it between a single conformation that can only target HIV-1 and a dynamic state that can target multiple viruses. These results illustrate how conformational diversity can be exploited by hosts to target multiple viral strains.

Results

RhTC targets multiple viruses by adopting radically different conformations

RhTC evolved from a duplicated cyclophilin A (CypA) gene that retrotransposed into the TRIM5 locus[3]. CypA efficiently binds to the capsid of HIV-1 but not HIV-2, yet RhTC restricts both viruses. The cyclophilin domain of RhTC (unless stated otherwise, this will subsequently be referred to as “RhTC”) has two mutations with respect to CypA: D66N and R69H. These confer binding to the HIV-2 capsid (CA) protein whilst maintaining binding to HIV-1 O-group CA[4]. Previously we have shown that these two mutations result in a new active site conformation, however, it is not clear how this new conformation gives rise to HIV-2 binding nor how binding to HIV-1 O group is simultaneously maintained, especially given the shift away from the conformation found in CypA that is known to be required.

HIV capsids have been shown to interact with CypA utilizing residues solely within the cyclophilin-binding loop[6]. Previous work has also shown that binding can be recapitulated by a linear peptide consisting purely of cyclophilin-binding loop[7], and that binding and specificity can be transferred by transplanting the cyclophilin-binding loop from one virus onto the capsid of another[8, 9]. To understand the structural basis of RhTC dual targeting, we determined crystal structures of RhTC in complex with the N-terminal domain of an HIV-1 O-group CA chimaera (HIV-1 CA_{O-loop}) and with the cyclophilin-binding loop of HIV-2 CA (Fig. 1). In the RhTC:HIV-2 complex, the active site of RhTC adopts a very similar conformation to the previously solved structure of free RhTC (Fig. 1b, PDB ID code 2WLW). In particular, loop₆₆₋₇₄ forms an extended open β -zipper structure that differs dramatically from its conformation in CypA. This conformation is the result of very large main chain rearrangements of >15 Å. The extension of loop₆₆₋₇₄ has allowed the rearrangement of key residues with respect to CypA, such as Arg55 that can now make a stacking interaction with the Pro86-Gly87 peptide bond in HIV-2 CA.

Next, we compared the conformation of RhTC in the HIV-2 complex to its conformation in the HIV-1 O group complex. There are significant differences in the conformation of RhTC between the two complexes. In particular, in the O Group complex, RhTC does not have an extended loop₆₆₋₇₄ β -zipper structure but instead the loop doubles-back after residue F67 (Fig. 1b). In this respect, RhTC in the HIV-1 O-group complex is more similar to the closed conformation of CypA in its complex with the same O-group capsid (1M9D) (Fig. 1b)[10]. However, there is evidence that loop₆₆₋₇₄ in RhTC is more flexible than in CypA. The B-factors for RhTC loop₆₆₋₇₄ are higher than in the scaffold and residues 70 and 71 have been omitted due to incomplete density (Supplementary Figures 1 & 2). We therefore hypothesised that the reason why RhTC can bind both HIV-2 and HIV-1 O group viruses is that the two mutations, D66N and R69H, may have increased the flexibility of loop₆₆₋₇₄.

unconstraining it from a single fixed conformation. Furthermore, we reasoned that the crystal structures may be showing just two of a large number of possible RhTC conformations.

The RhTC binding loop is highly mobile

In order to test our hypothesis that loop₆₆₋₇₄ of RhTC has evolved to become conformationally dynamic, we examined its structure in solution using ^{15}N backbone relaxation NMR experiments and compared it to CypA (Fig. 2a). ^{15}N longitudinal relaxation (T_1), ^{15}N transverse relaxation (T_2) and heteronuclear cross-relaxation $\{^1\text{H}\}^{15}\text{N}$ NOE experiments allow the assessment of protein dynamics on a residue-by-residue basis. The backbone dynamic properties of CypA have been studied in detail [11], and our data for CypA and RhTC are in broad agreement with previous studies. Based on averaged ^{15}N T_2 relaxation times and $\{^1\text{H}\}^{15}\text{N}$ NOE ratios for residues in defined secondary structure elements, both proteins have similar overall tumbling rates and internal dynamics (Fig. 2a). However, the two point mutations D66N and R69H result in dramatic differences within loop₆₆₋₇₄. In CypA, loop₆₆₋₇₄ has decreased apparent ^{15}N T_2 relaxation times as a result of conformational exchange contributions on a micro- to millisecond time-scale (Fig. 2a) [12]. Chemical exchange does not contribute to the intrinsic backbone dynamics and is therefore not observed in the $\{^1\text{H}\}^{15}\text{N}$ NOE data (Fig. 2a). Analysis of the free CypA crystal structure reveals that loop₆₆₋₇₄ is tethered at each end by a double-glycine motif (GGxxxxxxxGG), whilst the loop itself is tightly constrained by a network of hydrogen bond interactions mediated by the side chain of Asp66 (Fig. 2b). This suggests that the origin of slow conformational exchange processes in CypA is segmental motions of loop₆₆₋₇₄ as it pivots around the double-glycine repeats.

In contrast to CypA, the ^{15}N T_2 relaxation times for loop₆₆₋₇₄ in RhTC are substantially increased (Fig. 2a, bottom right), indicating high flexibility in particular for residues Thr68, His70, Asn71 and Gly74. This coincides with a decrease in $\{^1\text{H}\}^{15}\text{N}$ NOE ratios (Fig. 2a), giving values associated with highly dynamic regions [13]. Both experiments reveal a striking change in the mobility of loop₆₆₋₇₄ from slow segmental motion on a micro- to millisecond time-scale to fast quasi-individual mobility on a picosecond time-scale. The residues (68-74) that have undergone this change in timescale of motion are those whose main chain atoms form a hydrogen bond network with the side-chain of Asp66 in CypA. (Fig. 2a) However, the RhTC crystal structure shows that, due to mutation of Asp66 to Asn66, this hydrogen bond network is lost and residues 68-74 are no longer centrally coordinated or constrained to move as a single unit (Figs. 2b and 2c). Together, the NMR and crystallographic data indicate that a loss of loop constraints in RhTC has led to a gain of dynamics.

To further study how loop dynamics have been altered in RhTC, we employed relaxation dispersion methods to understand and define the time scale of loop residue motions. Relaxation dispersion measurements dissect contributions to the apparent transverse relaxation rates $R_{2\text{app}}$ ($1/T_{2\text{app}}$) into two components: the intrinsic transverse relaxation rate ($R_{2\text{intr}}$) as determined by the overall tumbling of a protein and its fast internal (picosecond) mobility and the chemical exchange contribution (R_{ex}), which is a measure of exchange between two or multiple states on a time scale slower than the overall correlation time (t_c) of the protein, (typically on a μs -ms time scale). The application of increasing spinlock fields ν_{CPMG} during a constant time T_2 experiment leads to an attenuation of chemical exchange contributions, which in the ideal case of an infinitely strong spinlock field provides a measure of the intrinsic $R_{2\text{intr}}$ rates. The spin lock dependent rate of attenuation is therefore a direct measure of the time scale of exchange processes present.

In agreement with published data[12], we find that CypA residues 67, 68, 72, 74 and 77 but not 76 and 78 undergo conformational exchange (Fig. 3 & Supplementary Table 1). Global fitting using NESSY results in exchange values (k_{ex}) similar to those published for group II residues, (65-84)[14]. No attenuation is observed for the corresponding residues in RhTC, although the residues in the stem region at either end of the loop (62-66 and 79-80) have similar dynamic properties. The differences in loop₆₆₋₇₄ dynamics between CypA and RhTC are therefore confined to specific residues at the loop apex that are constrained in CypA and unconstrained in RhTC (Fig. 2a&b and Fig. 3). The heteronuclear $\{^1H\}^{15}N$ NOE and T_2 data indicate that these residues undergo fast picoseconds reorientations, however the possibility that this is superposed on an underlying large scale loop motion with a slow time scale cannot be excluded. The existence of simultaneous slow and fast conformational motion has recently been demonstrated by Henzler-Wildman *et al*[14]. The fact that RhTC has both the flexible double glycine ‘hinge’ found in CypA and additional freedom of motion supplied by the D66N mutation would support a similar conclusion.

Viral specificity can be fixed by stabilizing a specific RhTC conformation

The data shows that RhTC has evolved mutations that have made its active site dynamic. Next, we investigated whether this property of dynamics enables RhTC to bind multiple viruses. To test this, we determined whether different conformations confer binding to HIV-1 O group and HIV-2 and whether by constraining loop₆₆₋₇₄ into a single conformation we could make RhTC specific to a single virus. A ‘conformational trap’ strategy was used to lock RhTC into a single conformation via a disulphide-bridge. RhTC contains a Cys residue at position 52, which is in close proximity to His70, when loop₆₆₋₇₄ adopts a closed conformation as observed in CypA and the RhTC:HIV-1 O group complex (Ca-Ca separation of ~ 7.5 Å). Conversely, in the open conformation seen in the RhTC:HIV-2 complex these two residues remain >18 Å apart. Taking advantage of this difference, we mutated His70 to Cys so that if the closed conformation is populated in the free state it would become trapped via a disulphide bridge, preventing isomerisation to the open conformation. We then tested our prediction that locking the closed conformation in place would prevent HIV-2 CA binding but maintain binding to HIV-1 O-group CA by isothermal titration calorimetry (ITC) and x-ray crystallography (Fig. 4). ITC revealed that RhTC H70C has lost the ability to bind HIV-2 and can only bind HIV-1 O group capsid. The structure of RhTC H70C in complex with HIV-1 O group capsid confirms that this is because loop₆₆₋₇₄ has been trapped in a closed conformation through the formation of the predicted disulphide bond between C52 and C70. The structure also gives independent confirmation that it is indeed the closed loop conformation that binds HIV-1 O group, as suggested by the RhTC:O group structure. Fixing the closed loop conformation in place through the disulphide bridge has inhibited the flexibility of loop₆₆₋₇₄, such that the loop B-factors are now similar to the rest of the protein scaffold (Supplementary Figure 1). Moreover, residues 70 and 71 for which there was incomplete density in the RhTC:O group structure can now clearly be seen. The combination of ITC and structure strongly suggest that a closed loop conformation is used to bind HIV-1 O group and that an inability to adopt an open conformation, as seen in the RhTC:HIV-2 complex, prevents binding to HIV-2.

To confirm that it is the inability to adopt an open conformation that prevents RhTC H70C from binding HIV-2, we repeated the ITC binding experiments in the presence of DTT. As can be seen in Figure 4b, upon addition of DTT, binding to HIV-1 O group is maintained and binding to HIV-2 is recovered. To ensure that addition of DTT has reduced the disulphide bridge and that recovery of HIV-2 binding is associated with the ability to adopt an open conformation, we solved the structure of free RhTC H70C in the presence of DTT. The structure shows that DTT reverses the disulphide bridge and that an open conformation is now adopted, explaining why binding is now seen by ITC (Fig 4). Together with the

NMR data, this suggests that RhTC is structurally dynamic and capable of adopting functionally distinct conformations.

The NMR data suggest that RhTC does not simply isomerise between open and closed conformations but is highly dynamic, sampling a large number of conformers of which these are merely two extremes. To demonstrate that RhTC can sample other conformers than the open and closed states, we investigated its binding to the cyclic 11-residue non-ribosomal peptide cyclosporin A (CsA). Determination of the crystal structure of RhTC in complex with CsA, revealed a third active site conformation (Supplementary Figure 3). This structure shows that RhTC is not in equilibrium between two fixed conformations but, together with the NMR, suggests it dynamically samples a range of states. RhTC may access a large volume of conformational space that encompasses radically different open and closed states, but it also adopts conformations inbetween (Supplementary Figure 3). Furthermore, these different states may be functionally relevant and expand binding specificity.

An interesting question arising from the evolution of conformational dynamics in RhTC is whether the increased flexibility is penalized by loss of affinity due to the reduction of conformational entropy upon binding. To address this, we compared the thermodynamics of HIV-1 CA_{O-loop} binding to RhTC with RhTC/H70C. It might be expected that binding to RhTC (and RhTC/H70C in the presence of reducing agent) would occur with a weaker affinity and be more entropically unfavourable due to the greater flexibility in loop₆₆₋₇₄. However, our ITC data show that both affinity and change in entropy (ΔS) are similar for HIV-1 CA_{O-loop} binding to RhTC ($K_d = 38.5 \mu\text{M}$; $T\Delta S = 1.98 \text{ kcal.mol}^{-1}$), RhTC/H70C (in the presence of DTT; $61.3 \mu\text{M}$; $1.59 \text{ kcal.mol}^{-1}$) and RhTC/H70C (in the absence of DTT; $47.8 \mu\text{M}$; $2.12 \text{ kcal.mol}^{-1}$) (Supplementary Fig. 4a and Fig. 4c). Thus, whilst there is a theoretical cost to binding affinity associated with increased flexibility, we have no evidence of this in our system. Nonetheless, as ITC measures the total entropy change of the system it is possible that the entropic cost associated with stabilizing a single protein conformation is offset by the release of solvent molecules.

RhTC targets multiple viruses except HIV-1 M-group

To investigate the extent of RhTC specificity we examined its interaction with CA proteins from other diverse lentiviruses. Using ITC, we found that RhTC binds feline immunodeficiency virus (FIV) CA and lion lentivirus (LLV) CA (Supplementary Figs. 4b and 4c), in addition to HIV-1 O-group CA and HIV-2 CA. RhTC binding is conserved to these viruses despite the fact they have very different cyclophilin-binding loops, both in terms of length and amino acid composition (Fig. 5a). RhTC is therefore an effective innate antiviral, capable of binding multiple, diverse targets despite the fact that it is encoded by a single gene. Interestingly, RhTC is unable to bind or target HIV-1 M-group viruses, the strain responsible for the current world-wide AIDS pandemic[15].

We attempted to explain loss of binding to RhTC using our combined structural, NMR and ITC data. Comparison of the RhTC:O group complex with the previously solved CypA:M group[6]complex shows that the rigid loop₆₆₋₇₄ conformation found in the CypA structures is not present in RhTC. The rigid conformation of loop₆₆₋₇₄ is critical for HIV-1 M binding as correct positioning of the Gly72 peptide oxygen is required for formation of a hydrogen bond with the main chain nitrogen at capsid position 88. The loss of this interaction in RhTC may explain why it cannot bind M group virus (Fig. 5b). Conversely, it is possible that RhTC still binds HIV-1 O group because whilst the hydrogen bond interaction is lost a compensating interaction is made. The more relaxed loop₆₆₋₇₄ conformation in RhTC results in rearrangement of Thr73, generating a new interaction with capsid through hydrophobic burial of the methionine side chain at position 88. Crucially, with a highly conserved alanine at position 88, HIV-1 M-group escapes this interaction. Mutation of Ala88 to Met or Val is

sufficient to make HIV-1 M-group susceptible to RhTC whereas the reciprocal mutation allows HIV-1 O-group to escape [4]. Thus, the same mutation, D66N, which imparts conformational dynamics on RhTC through weakening of the hydrogen bond network holding loop₆₆₋₇₄ in place may also be responsible for loss of binding to M group virus.

Discussion

Our data suggest that, during its evolution, RhTC has acquired mutations that have conferred a property of conformational dynamics. This property allows RhTC to isomerise between radically different active conformations that are used to target different HIV viruses. Upon constraining RhTC into a single conformation only one virus can be bound. Releasing RhTC constraints recovers conformational diversity and concomitantly multiviral binding. Thus, whilst previous work had suggested that RhTC has evolved a new active site, we have found it has actually evolved an active site capable of many different conformations. This conformational plasticity appears to be an intrinsic property as an open conformation can be formed in the absence of HIV-2 whilst a closed conformation must be populated in the free state in order for it to be fixed in the disulphide bridge experiment. Such an interpretation is supported by the NMR data, which shows that residues within the loop are highly mobile. Of course this does not rule out that induced fit also plays a role and it is likely that upon binding of capsid some alteration in the RhTC structure occurs.

Viruses are known to evolve extremely rapidly and as a consequence evade recognition by the immune system. This presents two fundamental problems for the innate immune response. First, it must be able to deal with many different strains of the same virus. Second, it must have a way of countering the rapid emergence of new strains. The adaptive immune system solves both problems genetically by recombining receptors from a library of gene fragments and somatically evolving them during an infection. In contrast, innate effector proteins are not produced by random recombination and their evolution, being generational, is orders of magnitude slower. Our findings suggest that innate antivirals can augment their lack of genetic diversity with diversity at the level of protein structure. RhTC uses its conformational diversity to expand the repertoire of viruses it can target. Furthermore, the fact that RhTC can target multiple viruses suggests that there can be selection pressure by more than one virus at any one time. The fact that some viruses such as HIV-1 M group are not susceptible suggests that there is also selection pressure on the virus to escape. We cannot know the identity of the viruses that have placed RhTC under selection in evolutionary history and it is therefore intriguing that whilst M group viruses escape RhTC they have not been found in studied macaque populations.

Our data illustrates how conformational diversity can be used to expand antiviral function. Future work is required to determine whether this strategy is employed by other innate antivirals, such as TRIM5 α . TRIM5 α binds to viral capsids using its PRYSPRY domain, a β -sandwich fold with a binding site comprised of six flexible loops. We speculate that adoption of alternative loop conformations may explain why TRIM5 α is able to restrict diverse viruses including lentiviruses and gamma retroviruses[1, 16-18], as well as beta retroviruses[19], and even very distantly related spumaviruses[20]. Conformational diversity of TRIM5 α may complement the mechanism by which this and related antivirals directly contact the viral capsid. Recent data has shown that TRIM5 α forms a lattice across the capsid, suggesting that apparent affinity may be increased through avidity[21]. This manner of binding could compensate for the entropic cost associated with the stabilisation of a single conformer upon ligand binding. In a general context, our data provides an example of how a single gene does not necessarily give rise to a single protein with a single conformation, but rather multiple conformations, each of which can bind a different target.

Methods

Protein expression and purification

Macaca mulatta TRIMCyp cyclophilin domain (RhTC; residues 1-165 (corresponding to TRIMCyp residues 304-468); NCBI accession number ABX56710.1); HIV-1 M-group (NL4-3) N-terminal capsid domain (HIV-1 M-group CA; residues 1-146 (Gag residues 133-278); AAB60571.1); and HIV-2 N-terminal capsid domain (HIV-2 CA; residues 1-144 (Gag residues 136-279); AAB00763.1) were cloned as NdeI/MluI fragments in pOPT. HIV-1 M-group (NL4-3) N-terminal capsid domain harbouring the HIV-1 O-group (MVP-5180) capsid cyclophilin-binding loop (HIV-1 CA_{O-loop}; residues 1-78, 101-146 (NL4-3), residues 79-100 (MVP-5180; AAA44859.1)) was cloned as an NdeI/HindIII fragment in pOPT. FIV N-terminal capsid domain (FIV CA; residues 1-139 (Gag residues 136-274); AAU12277.1) and LLV N-terminal capsid domain (LLV CA; residues 1-143; strain B 458, [22]) were cloned as NdeI/MluI fragments in pOPTH. Site-directed mutagenesis was carried out using the QuikChange methodology.

Unlabelled proteins were expressed in *Escherichia coli* C41(DE3) cells in 2TY medium. ¹⁵N- and ¹⁵N/¹³C-labelled proteins were expressed in K-MOPS minimal medium supplemented with [¹⁵N]NH₄Cl and D- [¹³C₆]glucose as appropriate. RhTC and HIV CA proteins were purified by ammonium sulfate precipitation, followed by sequential use of cation exchange and size exclusion chromatography. FIV CA and LLV CA proteins were purified by sequential use of Ni²⁺-affinity and size exclusion chromatography.

Crystallization and structure determination

Crystals were grown in the conditions detailed in Supplementary Table 2 by using the vapour diffusion method at 17°C. Cryoprotection was accomplished as described in Supplementary Table 2. Crystals were cryocooled by plunging into liquid nitrogen, and X-ray data were collected at 100 K using a nitrogen stream. Data were collected at Beamline ID23-1 of the ESRF (Grenoble, France), Beamline I03 of the Diamond Light Source (Didcot, United Kingdom) and on an in-house rotating anode X-ray generator. Data were processed using MOSFLM[23] and the CCP4 suite[24]. All structures were determined by molecular replacement using Phaser[25]. The structures of the *Macaca mulatta* TRIMCyp cyclophilin domain (PDB ID code 2WLW)[4] and the HIV-1 M-group N-terminal capsid domain (PDB ID code 1AK4)[6] were used as search models. Residues 65-75 (cyclophilin) and 78-101 (capsid) were omitted from the search models. Model building was performed using Coot[26] and refinement was carried out using REFMAC5[27]. Model validation was performed using MolProbity[28]. Ramachandran analysis indicates the final models contain: 97.8, 2.2 and 0.0% (RhTC:HIV-1 O); 98.2, 1.8 and 0.0% (RhTC:HIV-2); 96.8, 3.2 and 0.0% (RhTC:CsA); 98.2, 1.8 and 0.0% (RhTC/H70C:HIV-1 O); and 97.2, 2.8 and 0.0% (RhTC/H70C) of residues in favoured, allowed and disallowed regions, respectively. The structures of RhTC:HIV-1 O, RhTC:CsA and RhTC/H70C:HIV-1 O contain multiple copies of the RhTC:ligand complexes per asymmetric unit. These exhibit differing qualities of electron density for loop₆₆₋₇₄, and as a consequence we have omitted some sections of loop₆₆₋₇₄ in those chains where the electron density is ambiguous. We have based our analyses on the chains containing complete loop₆₆₋₇₄ models. Data collection and refinement statistics are shown in Supplementary Table 2. Figures were prepared using PyMOL.

Uniform isotope labeling

CypA and RhTC were expressed in DE3 bacteria from pOPTH plasmids in K-MOPS buffer supplemented with 20 mM ¹⁵NH₄Cl as the sole nitrogen source, 4 mM pH 8.0 KPOi, 0.1 % sodium ampicillin salt, vitamins and 0.4 % glucose. The bacterial culture was grown to 0.500-0.700 OD₆₀₀ at 37 °C and induced with Isopropyl- β-D-1-thiogalactopyranoside

(IPTG). The proteins were expressed at 20 °C overnight and then purified using Ni-affinity chromatography and gel-filtration chromatography.

Backbone resonance assignments

Backbone resonances for CypA and RhTC were assigned based on standard triple resonance experiments namely HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO acquired on a Bruker Avance 700MHz spectrometer equipped with a triple resonance cryoprobe at 298K.

¹⁵N relaxation data

Backbone ¹⁵N relaxation data – T₁, T₂ and heteronuclear {¹H}¹⁵N NOE – were acquired as pseudo-3D experiments on a Bruker Avance III 600MHz spectrometer with TCI cryoprobe at 298K with sample concentrations of 500 μM for RhTC and 170 μM for CypA. The T₂ relaxation delays were set to 8ms, 17ms, 34ms, 51ms, 68ms, 85ms, 102ms, 119ms, 136ms, 153ms and 204ms. The T₁ relaxation delays were set to 10ms, 20ms, 40ms, 80ms, 120ms, 160ms, 320ms, 640ms, 1280ms, 1600ms and 2000ms. Recovery delays in all experiments were set to 5s. For an error estimation, the initial data points in the T₁ and T₂ experiments as well as the heteronuclear {¹H}¹⁵N NOE data were acquired in duplicates.

For an initial estimation of contributions from chemical exchange processes, CPMG based T₂ relaxation experiments were acquired on a Bruker DRX 500MHz spectrometer at 298 K with refocusing delays of 450us and 900us, respectively. T₂ relaxation delays were set to 16ms, 31ms, 47ms, 63ms, 78ms, 94ms, 110ms, 141ms, 157ms, 204ms and 235ms.

CPMG relaxation dispersion experiments were acquired with fresh NMR samples at final concentrations of 500 and 170 μM for RhTC and CypA, respectively in a 50 mM KPO_i buffer, pH 6.5 with 2 mM DTT. Pseudo-3D experiments were acquired on a Bruker Avance III 600MHz spectrometer with TCI cryoprobe at 298 K using standard Bruker pulse sequences in an interleaved manner with a constant time delay of 40 ms. By varying the interpulse delays 11 different spinlock fields between 50 and 1000 Hz were applied.

All datasets were processed in Topspin 3.0 (Bruker, Karlsruhe), the intensities were extracted with Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco) and the analysis was performed in NESSY[29]. Model selection for the exchange process was determined by the AICc (Akaike's Information Criteria Corrected for small sample size) model selection protocol in NESSY except for residue 71 in RhTC where no exchange was assumed. Further details are described in the Supplementary Material.

Isothermal titration calorimetry

Samples were dialysed against potassium phosphate (50 mM, pH 7.4), NaCl (100 mM) and DTT (1 mM), unless stated otherwise. DTT concentrations were adjusted to 0 mM or 5 mM for experiments probing the effect of redox potential on RhTC/H70C. Protein concentrations were determined by absorbance at 280 nm. ITC experiments were carried out using a MicroCal iTC200 calorimeter. Experiments were conducted by titrating capsid (syringe) into cyclophilin (cell) at 10°C, unless stated otherwise. Data were analysed using MicroCal Origin 7.0 implementing a simple one set of binding sites model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

RhTC binding to HIV CA proteins utilizing diverse active site conformations. **(a)** RhTC in complex with HIV-1 CA_{O-loop}. RhTC is shown as a surface representation in grey, with the dynamic active site loop (residues 66-74) highlighted in yellow. HIV-1 CA_{O-loop} is shown in orange, with the monomer as a cartoon representation. The complexed structures of RhTC with HIV-1 CA_{O-loop} and HIV-2 are compared, with the interacting residues from the cyclophilin binding loops shown as ball-and-stick representations in orange. **(b)** The two RhTC complexed structures HIV-1 (cyan) and HIV-2 (orange) are shown superposed together with the free structures of RhTC (wheat) and CypA (green; PDB ID code 2CPL). The capsid of HIV-1 is shown in gray for comparison. The RhTC:HIV-2 complexed structure is similar to free RhTC whereas the RhTC:HIV-1 structure is similar to free CypA. A ball-and-stick representation of the superposed active site loop residues 64-75 from the two RhTC complexed structures is shown. The two structures show completely divergent loop conformations beginning at residue D66 and continuing until G75.

Figure 2.

NMR analysis of CypA and RhTC backbone mobility. **(a)** The top panels show T_1 relaxation times for CypA (left) and RhTC (right). The middle panels show heteronuclear $\{^1\text{H}\}^{15}\text{N}$ NOE ratios for 500MHz (gray circles) and 600MHz ^1H resonance frequencies (black circles). The bottom panels show ^{15}N T_2 relaxation data for 500MHz (black circles) and 600MHz ^1H resonance frequencies (gray squares). In all panels, active site loop residues 66-74 are highlighted in yellow. **(b)** Detail of the CypA crystal structure (PDB ID code 2CPL) illustrating how the loop conformation is constrained as a result of hydrogen bonds mediated by D66 (highlighted in yellow). The double-glycine 'hinges' are highlighted in red. **(c)** RhTC has lost the hydrogen bond network mediated by residue 66 as a result of its mutation to asparagine. The loop main chains **(b and c)** are shown as ball-and-stick representations.

Figure 3.

Relaxation dispersion profiles for loop residues in CypA (top panel) and RhTC (bottom panel). The CPMG field dependency of residues F67, T68 and N71 in CypA indicates contributions from μs -ms conformational exchange in contrast to the corresponding loop residues in RhTC. A superposition of CypA (yellow) and RhTC (gray) free structures is shown below, showing divergence in loop residues between 67-73. Note that residues F67, T68 and N71, which undergo exchange in CypA but not RhTC, are those residues which are coordinated by a hydrogen bond network in CypA but not RhTC.

Figure 4.

An engineered redox-sensitive switch controls the dynamic nature of RhTC. **(a)** Crystal structure of RhTC/H70C, locked in a closed conformation, in complex with HIV-1 CA_{O-loop}. **(b)** RhTC/H70C closed (left) and open (right) conformations are both observed following the addition of reducing agent, but in the absence of ligand, as alternate conformations in the same crystal structure. The RhTC loops and Cys52, and residues 87-92 of HIV-1 CA_{O-loop} **(a and b)** are shown as ball-and-stick representations in yellow and orange, respectively. **(c)** ITC studies of the interaction between RhTC/H70C and HIV-1 CA_{O-loop} (left) and HIV-2 CA (right), in the presence and absence of reducing agent (DTT). Affinities for O group in the presence and absence of DTT are 48 and 61 μM respectively and 130 μM for HIV-2 in the presence of DTT. There is no measurable binding to HIV-2 in the absence of DTT.

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