Molecular Recognition in the HIV-1 Capsid/Cyclophilin A Complex

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The HIV-1 capsid protein (CA) makes an essential interaction with the human peptidyl prolyl isomerase, cyclophilin A (CypA), that results in packaging of CypA into the virion at a CA to CypA stoichiometry of ~10:1. The 231 amino acid residue capsid protein is composed of an amino-terminal CypA binding domain (1 to 151; CA151) and a carboxyl-terminal dimerization domain (151 to 231). We find that CypA binds dimeric CA and monomeric CA151 with identical intrinsic affinities (Kd = 16(±4) μM). This result demonstrates that capsid dimerization and cyclophilin A binding are not thermodynamically coupled and suggests that the substoichiometric ratio of CypA in the HIV-1 virion results from the intrinsic stability of the CA/CypA complex. In the known co-crystal structure of the CA151/CypA complex, CypA binding is mediated exclusively by an exposed capsid loop that spans residues Pro85 to Pro93. The energetic contributions to CypA binding were quantified for each residue in this loop, and the results demonstrate that the Gly89-Pro90 dipeptide is the primary cyclophilin A recognition motif, with Pro85, Val86, His87, Ala88, and Pro93 also making energetically favorable contacts. These studies reveal that the active site of CypA, which can catalyze the isomerization of proline residues in vitro, also functions as a sequence-specific, protein-binding motif in HIV-1 replication.

Keywords: human immunodeficiency virus; capsid; cyclophilin; binding; chaperone

Introduction

The HIV-1 Gag polyprotein dictates both viral assembly and the packaging of essential factors into the virion (for reviews, see Wills & Craven, 1991; Gelderblom et al., 1992; Hunter, 1994; Kruhllsch, 1996). It is now known that at least one of these essential packaged factors is a cellular protein, the abundant cytoplasmic proline isomerase, cyclophilin A (CypA: Luban et al., 1993; Franke et al., 1994b; Thali et al., 1994). CypA binds to the central “capsid” domain of Gag and is thereby packaged at a virion stoichiometry of ~2000 CA: ~200 CypA (Franke et al., 1994b; Thali et al., 1994; Franke & Luban, 1995; Ott et al., 1995). As the immature virion buds, the 55 kDa Gag polyprotein (residues 1 to 499) is cleaved by the viral protease to produce three new proteins, matrix (MA, residues 1 to 132), capsid (CA, 133 to 363) and nucleocapsid (NC, 378 to 432), as well as three smaller polypeptides (p6, p2 and p1: Di Marzo Veronese et al., 1988; Henderson et al., 1992). Following cleavage, the capsid protein rearranges into the characteristic cone-shaped core structure that surrounds the RNA genome at the center of the virion. CypA then appears to exert its essential function on capsid immediately following the infection of a new cell, possibly helping to disassemble the capsid core structure (Braaten et al., 1996a,c; Gamble et al., 1996).

Cyclophilin A is the founding member of what is now a large family of proteins that are ubiquitous in both prokaryotes and eukaryotes (Handschumacher et al., 1984; Fischer et al., 1989; Takahashi et al., 1989; Schreiber & Crabtree, 1992;
Stamnes et al., 1992; Walsh et al., 1992; Kunz & Hall, 1993). CypA is an abundant cytoplasmic protein, but other cyclophilin family members exhibit tissue-specific and organelle-specific expression patterns, and function as subdomains of larger proteins. Various cyclophilin family members have been reported to play roles in such diverse and important cellular functions as the heat-shock response (Sykes et al., 1993), cell-surface recognition (Anderson et al., 1993; Friedman et al., 1993), protein folding and trafficking (Colley et al., 1991; Lodish & Kong, 1991; Helekar et al., 1994; Ferreira et al., 1996), lactate metabolism (Davis et al., 1992), and various intracellular signaling pathways (Xu et al., 1992; Ratajczak et al., 1993; Bram & Crabtree, 1994; Cardenas et al., 1994; Chang & Lindquist, 1994; Duina et al., 1996; Freeman et al., 1996; Weisman et al., 1996).

Despite extensive study, it is not yet clear precisely what cyclophilins do in the cell. The broad spectrum of biological pathways in which they appear suggests a role for cyclophilins in protein folding and, indeed, two distinct roles for cyclophilins in the folding process have been proposed. First, the rotamase activity of cyclophilins may allow them to play a general role in accelerating protein folding. Proline isomerization is the rate-limiting step for the folding of many proteins in vitro (e.g. see Brandts et al., 1975; Schmid & Baldwin, 1978; Jackson & Ferscht, 1991; Schmid, 1993) and CypA accelerates the overall folding rates of a number of such proteins (reviewed by Schmid, 1993). A second role for cyclophilins in protein folding is suggested by the fact that CypA can act as a molecular chaperone in vitro, increasing the yield of correctly folded protein by suppressing off-pathway folding reactions and preventing the aggregation of proteins in their unfolded states (Freskård et al., 1992; Lilie et al., 1993; Freeman et al., 1996). Intriguingly, this chaperone activity appears independent of the enzyme's rotamase activity. Although the rotamase and chaperone activities of cyclophilins have been well documented in vitro, the roles of these two activities in vivo are not fully understood.

A second major unanswered question in cyclophilin biology is whether or not cyclophilins typically bind specifically to their target proteins. On the one hand, if cyclophilins normally function as general prolyl isomerases, they might be expected to exhibit little sequence specificity. This model is consistent with the reported lack of CypA substrate discrimination across a series of different proline-containing model peptide sequences (Harrison & Stein, 1990). However, at least a subset of cyclophilins seem to bind specifically to target proteins in order to perform their essential functions. The best-characterized example of this is NinaA, a cyclophilin of the Drosophila eye, which forms a specific complex with rhodopsin 1 (Rh1: Schneuwly et al., 1989; Shieh et al., 1989; Baker et al., 1994). NinaA is essential for translocation of Rh1 from the endoplasmic reticulum, through the secretory pathway, and into the rhabdomere where phototransduction occurs (Colley et al., 1991). Although the Rh1/NinaA interaction is not understood in detail, loss of function mutations cluster about the ninaA active site, suggesting that the active site mediates Rh1 binding (Ondek et al., 1992). Other examples in which cyclophilins appear to form specific complexes with their target proteins include CypB binding to the calcium-signaling protein CAML (Bram & Crabtree, 1994), and CypC binding to the cell-surface protein CyCAP (Friedman et al., 1993). It therefore appears that sequence-specific protein binding may prove a recurring theme in cyclophilin function.

In addition to its importance in HIV-1 replication, the HIV-1 CA/CypA interaction may serve as a paradigm for understanding how cyclophilins achieve sequence-specific protein binding. Like NinaA, the CypA active site appears to participate in capsid binding, since CA/CypA complex formation is competitively inhibited by the rotamase inhibitor cyclosporine and its analogues (Luban et al., 1993; Franke et al., 1994b; Thali et al., 1994; Billlich et al., 1995). Moreover, mutation of a single capsid proline residue, Pro90, abolishes CypA binding, packaging, and viral replication (Franke et al., 1994b). In principle, however, binding specificity in the CA/CypA complex could either be achieved entirely within the CypA binding site or through additional protein-protein interactions that extend beyond the active site. The latter model is supported by the report that high-affinity cyclophilin A binding requires dimerization of the target Gag (or capsid) protein (Colgan et al., 1996). This observation suggests either that capsid dimerization creates a high-affinity cyclophilin A binding surface or that distal CypA-CypA interactions allow CypA to bind cooperatively to the capsid dimer.

Several recent experiments suggest an alternative model, however, in which the CypA active site itself discriminates between proline residues embedded in different local sequence contexts. For example, Luban and co-workers have shown that HIV-1 CA residue Gly89, as well as Pro90, is essential for CypA binding and viral packaging (Braaten et al., 1996b). Similarly, our recent CA151/CypA co-crystal structure revealed that capsid residues 85 to 93 make a series of favorable contacts in the CypA active site, with no other significant CypA-CA or CypA-CypA interaction (Gamble et al., 1996).

In order to determine precisely how cyclophilin A recognizes the HIV-1 capsid protein, we have examined various aspects of CA/CypA complex formation in solution. These experiments reveal the thermodynamics and energetic determinants of cyclophilin A binding, and demonstrate that the cyclophilin A active site itself acts as a sequence-specific, protein-binding motif.
Results

Recombinant capsid proteins

Primary sequences and labeling schemes for the HIV-1 capsid and human cyclophilin A proteins are shown in Figure 1(a) and (b), respectively. The crystallographically defined interaction between cyclophilin A and the amino-terminal domain of the capsid is shown in Figure 1(c) (Gamble et al., 1996). As shown in Figure 2, efficient systems have been developed for expressing and purifying large quantities of the full-length HIV-1 capsid protein (CA; Figure 2(a)), the amino-terminal capsid domain (CA151; Figure 2(b)), and human cyclophilin A (CypA; Figure 2(c)). Both capsid proteins are ideally suited for biophysical and structural studies, since they are highly soluble (>50 mg/ml) and can be efficiently isolated in high yields (>30 mg/l Escherichia coli culture).

Self-association of capsid proteins

Several groups have reported that HIV-1 capsid proteins dimerize in vitro (Ehrlich et al., 1992, 1994; Rosé et al., 1992; Brooks et al., 1994). Our full-length structure of the CypA protein (Ke et al., 1991) is shown above the primary sequence. Residues involved in capsid binding are underlined (Gamble et al., 1996). (c) Ribbon representation of the CA151/CypA complex.
recombinant capsid protein (25.6 kDa) is also predominantly dimeric at a concentration of 200 μM as analyzed by dynamic light-scattering ($M_{sol} = 51(\pm 2)$ kDa). The CA monomer-dimer equilibrium was quantified by analytical ultracentrifugation, yielding an estimated dissociation constant of $18(\pm 1)$ μM (Figure 3(a)). This value agrees well with previous measurements of the capsid $K_d$, which ranged between 10 and 30 μM (Rosé et al., 1992; Brooks et al., 1994).

In order to test the influence of capsid dimerization on cyclophilin A binding, it was necessary to create a monomeric capsid protein. Partial proteolysis and NMR structural studies have indicated that capsid residues 1 to 151 (CA151) form an independently folded domain that is monomeric at low millimolar concentrations (Gitti et al., 1996). This observation was confirmed by equilibrium sedimentation and dynamic light-scattering analyses of CA151. Sedimentation distributions of three different CA151 concentrations were globally fit to a simple monomer model (Figure 3(b)), yielding a shape-independent estimated particle mass of $16.4(\pm 0.3)$ kDa, in agreement with the calculated mass of the CA151 monomer (16.7 kDa). Similarly, CA151 appeared monomeric as measured by dynamic light-scattering ($M_{sol} = 18.1(\pm 0.1)$ kDa; 158 μM).

**Figure 3.** Equilibrium sedimentation analyses of the oligomeric states of HIV-1 CA and CA151: (a) CA exhibits a monomer-dimer equilibrium. The Figure shows raw sedimentation data, the optimized global fit, and residuals (above) for sedimentation of a 9.8 μM sample of CA. The global fit was performed using equilibrium sedimentation data from initial protein concentrations of 5.6, 9.8 and 15.8 μM, yielding an estimated dissociation constant, $K_d = 1.8(\pm 0.1) \times 10^{-5}$ M. The reported error is the 95% confidence level, the goodness of fit (GOF) was 1.67, and the fit exhibited small, random residuals. (b) CA151 is monomeric. The Figure shows the raw sedimentation data, optimized global fit, and residuals (above) for a 14.9 μM sample of CA151. The estimated protein mass is $16.4(\pm 0.3)$ kDa, in good agreement with the mass of the CA151 monomer (16.7 kDa). The global fit was performed using equilibrium sedimentation data from initial protein concentrations of 7.2, 14.9 and 34.3 μM. The GOF is 1.46 and the fit exhibited small, random residuals.

**Figure 4.** Isothermal titration microcalorimetric analyses of the cyclophilin A/capsid interaction. (a) Titration of CA151 into CypA. Heat released at 20°C when 632 μM CA151 was titrated in 20 μl aliquots into 1.296 ml 59 μM CypA. (b) Integrated binding isotherm of the CA151/CypA titration and experimental fit to a single-site model. The best fit (shown) yielded parameters of $n = 1.13(\pm 0.05)$, $K_d = 1.6(\pm 0.4) \times 10^{-5}$ M$^{-1}$, $\Delta H = -9.5(\pm 0.6) \pm$ kcal/mol, $\chi^2 = 9.0$. (c) Titration of CypA into CA. The titration was as described for (a) except that 661 μM CypA was titrated into 59 μM CA. (d) Integrated binding isotherm of the CA/CypA titration and experimental fit to a single-site model. The best fit (shown) yielded parameters of $n = 1.01(\pm 0.04)$, $K_d = 1.6(\pm 0.3) \times 10^{-5}$ M$^{-1}$, $\Delta H = -9.5(\pm 0.6)$ kcal/mol, $\chi^2 = 7.1$.

**Calorimetric quantification of the capsid/cyclophilin A interaction**

Isothermal titration microcalorimetry (ITC) was used to measure dissociation constants for the CA/CypA and CA151/CypA complexes. This method has the advantage of allowing rigorous quantification of the stoichiometry, energy and enthalpy of complex formation in solution, but requires milligram quantities of pure proteins (Brandts et al., 1990; Bundle & Sigurskjold, 1994; Fisher & Singh, 1995). The titration of CA151 into cyclophilin A is shown in Figure 4(a). Raw calorimetric data were corrected by subtraction of the appropriate blank titrations (negligible), and the integrated binding isotherm (Figure 4(b)) was successfully fit to a single-site binding model, yielding a CA151 to CypA binding ratio of $1.13(\pm 0.05)$, $K_d = 16(\pm 4)$ μM, and $\Delta H = -9.5(\pm 0.6)$ kcal/mol.

The analogous titration of cyclophilin A into full-length capsid protein (CA) is shown in Figure 4(c). Raw titration data were again corrected by subtracting the appropriate blank titrations. In this case the blank titration of buffer into capsid resulted in significant heat absorbance, presumably owing to dissociation of dimeric CA upon dilution. Nevertheless, the corrected binding isotherm (Figure 4(d)) again fit a single-site binding model, yielding a CypA to CA stoichiometry of $1.01(\pm 0.04)$, $K_d = 16(\pm 3)$ μM, and $\Delta H = -9.5(\pm 0.6)$ kcal/mol. These experiments de-
demonstrate that capsid dimerization and CypA binding are not coupled equilibria, since the measured stoichiometry, equilibrium binding constants and enthalpies for CypA binding to CA151 and CA are indistinguishable, even though the CA/CypA titration was performed at an initial capsid concentration of 59 μM, where >50% of the CA molecules were dimeric.

The large quantities of pure proteins required for ITC measurements made this method impractical for a comprehensive mutational analysis of the CA/CypA interaction. Surface plasmon resonance (SPR) spectroscopic methods were therefore also used to quantify the interaction. SPR has distinct advantages over ITC in speed, sensitivity and scale, but requires independent validation to ensure that binding equilibria are not altered by surface effects (Jönsson et al., 1991; Malmqvist, 1993; Morton et al., 1995; O’Shannessy & Winzor, 1996). Initial experiments examined the binding of pure, monomeric CA151 to a pure, immobilized glutathione-S-transferase-CypA fusion protein (GST-CypA). GST-CypA was captured on the sensor surface by an anti-GST antibody covalently linked to the carboxymethyl dextran layer (Jönsson et al., 1991), providing a homogeneous surface of unperturbed CypA binding sites. CA151 binding was then quantified by changes in the surface refractive index. A correction for refractive index changes due to bulk solvent and non-specific background binding was performed simultaneously in a separate flow-cell derivatized with the anti-GST antibody binding to GST alone.

As shown in Figure 5(a), binding responses were highly reproducible in repeated measurements across a concentration range of 0 to 57 μM CA151. The sensorgrams revealed that equilibrium was achieved rapidly. Furthermore, the binding responses returned to baseline levels very quickly after washing with buffer, indicating that the complex has a very high dissociation rate. The magnitudes of equilibrium binding responses at different CA151 concentrations were used to determine the dissociation constant for CA151/CypA complex by non-linear least-squares fitting to a simple one-to-one interaction model. Equivalent dissociation constants were obtained for measurements made at two different CypA surface derivatization levels and the data from the two surfaces were averaged to give the normalized binding data shown in Figure 5(c). The fit provides an estimated dissociation constant of 18(±2) μM, in good agreement with the CA151/CypA dissociation constant measured under the same conditions by isothermal titration microcalorimetry (see Table 1).

Subsequent experiments measured CypA binding to the intact capsid protein. In this case, the GST-CA fusion protein was captured directly from the soluble fraction of crude E. coli extracts following induction of protein expression. Capture of GST-CA directly onto the sensor surface was highly specific, since non-specific protein binding in control extracts lacking GST-CA was negligible (not shown). CypA dissociation constants were again obtained by fitting binding data (Figure 5(b)) from two different CA surface derivatization levels to a single-site binding model (Figure 5(c)). Nine independent repetitions of this experiment yielded a mean dissociation constant of 15 μM, with a standard deviation of ±5 μM (Table 1). The excellent agreement between all of the ITC and SPR measurements validates the SPR method, and further supports the conclusion that the carboxyl-terminal dimerization domain of HIV-1 CA does not influence the affinity of CypA binding.
Table 1. Measured dissociation constants for capsid/cyclophilin A complexes

<table>
<thead>
<tr>
<th>Protein interaction</th>
<th>Physical measurement</th>
<th>pH</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CypA/CA</td>
<td>ITC</td>
<td>6.5</td>
<td>16(4)b</td>
</tr>
<tr>
<td>CA151/CypA</td>
<td>ITC</td>
<td>6.5</td>
<td>16(3)</td>
</tr>
<tr>
<td>GST-CA/CypA</td>
<td>SPR</td>
<td>6.5</td>
<td>15 ± 5a</td>
</tr>
<tr>
<td>GST-CypA/CA151</td>
<td>SPR</td>
<td>6.5</td>
<td>18(2)</td>
</tr>
<tr>
<td>GST-MA-CA/CypA*</td>
<td>SPR</td>
<td>6.5</td>
<td>12(1)</td>
</tr>
<tr>
<td>GST-CA-NC-p6/CypAf</td>
<td>SPR</td>
<td>6.5</td>
<td>14(2)</td>
</tr>
<tr>
<td>GST-CypA/CA151</td>
<td>SPR</td>
<td>5.5</td>
<td>42(2)</td>
</tr>
<tr>
<td>GST-CypA/CA151</td>
<td>SPR</td>
<td>6.5</td>
<td>18(2)</td>
</tr>
<tr>
<td>GST-CypA/CA151</td>
<td>SPR</td>
<td>7.5</td>
<td>24(1)</td>
</tr>
<tr>
<td>GST-CypA/CA151</td>
<td>SPR</td>
<td>8.5</td>
<td>35(2)</td>
</tr>
</tbody>
</table>

* Isothermal titration calorimetry was performed as described in Materials and Methods.

Numbers in parentheses represent estimated errors of one standard deviation in the final digit and were derived from the fits of a single binding experiment.

Surface plasmon resonance spectroscopy was performed as described in Materials and Methods.

Value is the average of nine independent measurements made on different days with different protein preparations. The error is the standard deviation in the repeated experiments. In these experiments, the GST-CA protein was affinity-purified directly from soluble expression extracts onto an anti-GST mAb-derivatized sensor chip. Proteins used in all other experiments were purified to homogeneity prior to use in the binding assays.

GST-MA-CA consists of the GST protein fused to HIV-1 Gag residues 1 to 363.

GST-CA-NC-p6 consists of the GST protein fused to HIV-1 Gag residues 133 to 499. Note that this protein exhibited several degradation products in addition to the full-length protein when purified by glutathione affinity chromatography and analyzed by SDS-PAGE.

Importance of other Gag domains for CypA binding

Since cyclophilin A is initially packaged by the HIV-1 Gag polyprotein, we considered the possibility that the amino-terminal matrix or the carboxyl-terminal nucleocapsid domains of Gag might participate in CypA binding. CypA binding to immobilized GST-MA-CA (Gag residues 1 to 363) and to GST-CA-NC-p6 (Gag residues 133 to 432) was therefore examined. As shown in Table 1, measured dissociation constants for CypA binding were 12(±1) µM (MA-CA) and 14(±2) µM (CA-NC). In a parallel control experiment, the measured dissociation constant for the CA/CypA complex was 12.0(±0.4) µM. We therefore conclude that the CypA binding affinity is not significantly affected by Gag residues outside the central capsid domain.

Mutational analysis of the cyclophilin A binding site on HIV-1 CA

The biochemical experiments described above, together with the CA151/CypA co-crystal structure (Gamble et al., 1996), indicate that CA/CypA complex formation is mediated entirely by the contiguous capsid loop sequence Pro85 to Pro93 binding in the active site of cyclophilin A (see Figures 1(c) and 6(a)). To determine the relative importance of individual capsid residues in sequence-specific cyclophilin A binding, each CA binding loop residue was mutated and the effect of the mutation on CypA binding energy was measured (Table 2 and Figure 6(b)). Mutations were made in the context of the full-length capsid protein, and affinity binding measurements were performed by SPR spectroscopy as described above. To keep the mutagenesis as unbiased as possible, each CA loop residue was initially mutated to alanine, except for Ala88 and Ala92, which were mutated to both Gly and Val. Additional point mutations were subsequently used to test specific aspects of CypA recognition. Overall, the mutational analysis revealed that capsid residues Gly89 and Pro90 are the key determinants for cyclophilin A recognition, while the flanking residues Pro85, Val86, His87, Ala88, Ala92 and Pro93 also make favorable contributions to the affinity of the CA/CypA complex. As described below in detail, these results are in excellent agreement with the CA151/CypA co-crystal structure.

The CA151/CypA co-crystal structure indicated that CA Pro90 should be a major determinant of CypA binding, because the proline ring is buried in a deep hydrophobic pocket formed by CypA residues Phe60, Met61, Phe113 and Leu122. Analogous binding of proline residues has been observed in a number of cyclophilin A peptide complexes (Kallen & Walkinshaw, 1992; Ke et al., 1993; Zhao & Ke, 1996a,b). Detailed examination of the structure suggested that an Ala90 side-chain could be accommodated in the CypA binding pocket, albeit with the loss of favorable binding interactions at the Pro90 C' and C" positions, whereas any larger side-chain should clash with the sides of the proline binding pocket. For example, a β-branched side-chain at CA residue 90 is predicted to clash with the CypA Leu122 side-chain. Consistent with this analysis, mutation of CA Pro90 to Ala reduced the CypA binding affinity by 2.1 kcal/mol, while mutation of Pro90 to Val resulted in immeasurably low (>3 kcal/mol reduced) CypA binding affinity.

The mutational analyses further revealed that CA Gly89 is also a major determinant of CypA recognition, since mutation of Gly89 to Ala or Val reduced the CypA binding by 2.0 and 2.9 kcal/mol, respectively. The CA151/CypA co-crystal structure shows that Gly89 contributes to CypA binding affinity in two different ways. First, Gly89 adopts phi/psi angles (148°/155°) that are less favored for non-glycine residues. Moreover, the lack of a side-chain at Gly89 allows this residue to fit snugly against the CypA active site, whereas a β-carbon atom at this position would exhibit steric clash with CypA Arg55 N°2 and Gln63 O°1. The unusual torsion angles and tight fit of Gly89 in the CypA active site appear critically important, because they allow the Gly89-Pro90 peptide bond to adopt an unprecedented trans conformation. This is in contrast to all previously characterized model peptide complexes with CypA, in which the peptide pro-
line residue was always in the cis conformation (Kallen & Walkinshaw, 1992; Ke et al., 1993; Kakalis & Armitage, 1994; Zhao & Ke, 1996a,b). The trans-Pro90 conformation, in turn, orients the preceding capsid residues down into the CypA active site groove, where they make a series of favorable contacts (Figure 6(a)). The structure therefore clearly rationalizes why adding steric bulk to the Gly89 side-chain is energetically unfavorable.

A third determinant of CypA binding specificity is His87, which contributes to CypA binding by forming a van der Waals interaction (CA His87 Cε...
Table 2. Cyclophilin A binding to mutant HIV-1 capsid proteins

<table>
<thead>
<tr>
<th>Capsid mutation</th>
<th>CypA binding affinity (Kd, µM)</th>
<th>Fold decreasea</th>
</tr>
</thead>
<tbody>
<tr>
<td>P85A</td>
<td>75(2)b</td>
<td>3.0</td>
</tr>
<tr>
<td>V86A</td>
<td>64(3)</td>
<td>2.7</td>
</tr>
<tr>
<td>H87A</td>
<td>66(2)</td>
<td>4.8</td>
</tr>
<tr>
<td>H87Q</td>
<td>62(6)</td>
<td>4.8</td>
</tr>
<tr>
<td>H87R</td>
<td>193(13)</td>
<td>8</td>
</tr>
<tr>
<td>A88V</td>
<td>44(4)</td>
<td>3.4</td>
</tr>
<tr>
<td>A88G</td>
<td>106(1)</td>
<td>4.4</td>
</tr>
<tr>
<td>G89A</td>
<td>400(30)</td>
<td>31</td>
</tr>
<tr>
<td>G89V</td>
<td>2000(850)</td>
<td>154</td>
</tr>
<tr>
<td>P90A</td>
<td>470(120)</td>
<td>36</td>
</tr>
<tr>
<td>P90V</td>
<td>1800(200)</td>
<td>&gt;150</td>
</tr>
<tr>
<td>I91A</td>
<td>18 ± 10d</td>
<td>1.2</td>
</tr>
<tr>
<td>I91V</td>
<td>12(2)</td>
<td>1.0</td>
</tr>
<tr>
<td>A92G</td>
<td>32(2)</td>
<td>2.3</td>
</tr>
<tr>
<td>A92V</td>
<td>22(3)</td>
<td>1.7</td>
</tr>
<tr>
<td>P93A</td>
<td>38(1)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

All measurements were made by surface plasmon resonance spectroscopy. GST-CA proteins were affinity-purified directly from soluble expression extracts onto anti-GST mAb-derivatized sensor chips. Binding experiments were subsequently performed with pure CypA in the mobile phase.

a Fold decrease is the decrease in CypA binding affinity relative to a control wild-type GST-CA/CypA binding experiment(s) performed in parallel. Kd measurements for nine independent control reactions varied over a range of 15±(±5) µM.

b Numbers in parentheses represent estimated errors of one standard deviation in the final digit(s) and were derived from the fits of a single binding experiment.

c Binding of CypA to the immobilized GST-CA P90V mutant was too low to quantify accurately.

d Kd for three independent measurements with error given at one standard deviation.

HIV-1 Capsid/Cyclophilin A Interaction

Table 2. Cyclophilin A binding to mutant HIV-1 capsid proteins

The group O viruses are apparently unique amongst HIV-1 strains, in that they package cyclophilin A, but do not require the protein to replicate (Braaten et al., 1996b). Moreover, the CypA binding loop sequences of the group O viruses, while retaining the central GlyPro binding dipeptide sequence, are otherwise significantly diverged from the far more prevalent group M viruses. For example, the residues surrounding the CypA binding loop in the group O, HIV-1MVPS180 capsid protein (GTHPPAGLPQGQm) differs from the group M consensus sequence (LHPVHAGPIAPGQMmm) at seven different positions, five of which occur within the CypA binding loop itself. It was therefore of interest to quantify how tightly CypA binds to the HIV-1MVPS180 loop sequence.
This was done by creating and expressing a chimeric GST-CA protein that contained the HIV-1MVP5180 binding loop sequence in the context of an otherwise wild-type GST-CA₈₃₋₄₃ capsid protein. The chimeric capsid protein was immobilized and cyclophilin A binding was quantified by SPR spectroscopy as described above.

Although CypA bound somewhat less tightly to the chimeric capsid protein (K_d = 42(±1) μM, Table 2) as compared to the wild-type CA₈₃₋₄₃ protein, the increase in dissociation constant was modest (2.5-fold). This result demonstrates that although sequences surrounding the central Gly-Pro sequence can and do modulate CypA binding, the cyclophilin A active site can tolerate significant sequence variability without dramatic loss of binding affinity. Indeed, it is even possible that while some of the changes in the HIV-1MVP5180 binding loop sequence presumably reduce CypA binding affinity (e.g. His87 in HIV-1MVP5180 to Ala87 in HIV-1MVP5180 capsid), other changes in the loop sequence may compensate by actually increasing the binding energy.

Discussion

Capsid dimerization

Equilibrium sedimentation analyses demonstrate that the carboxyl-terminal 80 amino acid residues of the HIV-1 capsid protein (residues 152 to 231) are essential for full affinity dimerization. Together with previous proteolytic mapping and NMR studies (Gitti et al., 1996), these experiments demonstrate that the HIV-1 capsid protein is composed of two domains, an amino-terminal core domain that binds cyclophilin A, and a carboxyl-terminal assembly domain that mediates the highest affinity capsid-capsid interaction. Division of capsid into two distinct functional domains is supported by genetic studies that reveal that deleterious mutations in the carboxyl-terminal third of the protein generally prevent Gag assembly and particle release (Jowett et al., 1992; Hong & Boulanger, 1993; von Poblotzki et al., 1993; Chazal et al., 1994; Dorfman et al., 1994; Franke et al., 1994a; Mammano et al., 1994; Zhao et al., 1994; Carrière et al., 1995; Reicin et al., 1995; Srivivasakumar et al., 1995; Zhang et al., 1996). In contrast, deleterious mutations in the amino-terminal two-thirds of capsid typically give rise to assembled, but non-infectious virions that frequently fail to form the mature capsid core structure (Wang & Barklis, 1993; Dorfman et al., 1994; Reicin et al., 1995).

Two different co-crystal structures of HIV-1 capsid proteins bound to other proteins have recently been reported (Gamble et al., 1996; Momany et al., 1996). In the structure of CA₁₅₁ bound to CypA (Gamble et al., 1996), the capsid fragment is missing residues 152 to 231 and therefore cannot reconstitute the entire dimer interface formed by the intact capsid protein. Consistent with this model, we have recently crystallized and determined the structure of the carboxyl-terminal domain of capsid (CA₁₅₁₋₂₃₁; unpublished results). As expected, CA₁₅₁₋₂₃₁ forms a dimer.

The crystal structure of full-length HIV-1 CA in complex with an anti-CA Fab fragment has been reported (Momany et al., 1996). In this structure, the carboxyl-terminal domains of adjacent CA molecules are distant from one another and lack defined electron density. We therefore conclude that antibody binding has disrupted the high-affinity CA-CA interface formed between the carboxyl-terminal domains, and that this crystal lattice is dominated by Fab packing interactions. This interpretation is consistent with the fact that identical crystalline lattices form in both the presence and absence of the capsid protein.

Viral packaging of cyclophilin A

Our binding measurements have revealed the stoichiometry and thermodynamics of the cyclophilin A/capsid interaction. Formation of the CA/CypA complex is enthalpically driven, with a dissociation constant, K_d, of 16 μM. Although cyclophilin A is initially packaged by the intact Gag polyprotein, fusing the capsid domain to either the amino-terminal matrix domain or the carboxyl-terminal NC and p6 domains has no effect on CypA binding affinity. The simplest interpretation of these data is that the capsid domain alone mediates CypA binding (although the experiments do not formally rule out the possibility that CypA binding could somehow be modulated by contacts created in the assembled Gag particle that are not mimicked in our fusion proteins).

The micromolar dissociation constant observed for the CA/CypA complex suggests that simple mass action may determine the substoichiometric level of viral CypA packaging. Given a K_d for the CA/CypA complex of 16 μM, the ~10:1 ratio of capsid to cyclophilin A in the HIV-1 virion would be achieved at a free cytoplasmic cyclophilin A protein concentration of 1.6 μM. Although intracellular levels of cyclophilin A have not been quantified precisely, a micromolar CypA concentration appears reasonable, since the protein is highly abundant (Kolitsky et al., 1986) and approximately 3 μM concentrations of cyclosporine or its analogues are required to achieve full stoichiometric inhibition (Franke et al., 1994a; Thali et al., 1994).

Our experiments demonstrate that capsid dimerization and cyclophilin A binding are thermodynamically independent. This conclusion contradicts a recent report that Gag (or capsid) dimerization is required for CypA binding (Colgan et al., 1996). A possible explanation for this discrepancy is that the previous investigations examined capsid binding to immobilized GST-CypA. In this configuration, dimerization of Gag (or capsid) molecules would increase the apparent cyclophilin binding affinity if the capsid dimers bound bivalently to immobilized CypA (the chelate effect). In contrast, our microcalorimetry experiments were performed in solution.
HIV-1 replication.
inhibit viral packaging of cyclophilin A and block more than 44-fold, and might also be expected to it appears that the Gly-Pro dipeptide, rather than interactions along the CypA active site cleft. Hence moreover, there is no significant CypA-CypA interaction in the co-crystal structure that would account for cooperative binding of CypA to the CA dimer. (Gamble et al., 1996). Taken together, all of these data indicate that cyclophilin A packaging is mediated entirely by contacts between the CypA active site and the exposed capsid loop sequence spanning residues Pro85 to Pro93.

Molecular recognition in the CA/CypA complex

Our mutational analyses reveal that the cyclophilin A active site can strongly discriminate between proline residues in different sequence contexts. The key to sequence-specific binding in the CA/CypA complex is the presence of a glycine residue (Gly89) preceding the target proline residue (Pro90). Glycine apparently allows the CypA active site to accommodate the CA Pro90 peptide bond in a trans conformation, thereby directing the capsid target sequence to form a series of favorable interactions along the CypA active site cleft. Hence it appears that the Gly-Pro dipeptide, rather than proline alone, forms the fundamental recognition site for cyclophilin A. The importance of this recognition motif for HIV-1 replication has been demonstrated by Luban and co-workers, who have shown that mutation of either Gly89 or Pro90 to Ala blocks both cyclophilin A packaging and viral replication (Franke et al., 1994b; Braaten et al., 1996a). Our mutational analyses demonstrate that sequences flanking the Gly-Pro binding motif can also contribute significantly to CypA recognition. For example, we find that the CA Ala88 to Gly mutation decreases the CypA binding affinity more than 44-fold, and might also be expected to inhibit viral packaging of cyclophilin A and block HIV-1 replication.

Cyclophilin A and HIV-1 replication

Although the precise role of cyclophilin A in HIV-1 replication remains uncertain, the protein appears to perform its essential function early in the viral life-cycle (Steinkasserer et al., 1995; Braaten et al., 1996a). Luban and co-workers have reported that CypA-deficient virions assemble, mature and fuse with their target cells normally, but that the virus fails to initiate reverse transcription (Braaten et al., 1996a). This does not seem to reflect a direct effect of CypA on reverse transcriptase, since the endogenous reverse transcription activity in detergent-permeabilized, CypA-deficient virions is normal (Braaten et al., 1996a). Instead, genetic analyses indicate that the capsid is both the CypA binding partner and the functional target of CypA (Aberham et al., 1996; Braaten et al., 1996a; Dorfman & Göttlinger, 1996). These observations lead to the hypothesis that cyclophilin A may act to facilitate rearrangement or uncoating of the capsid core structure upon infection.

Although cyclophilin A binding does not affect formation of the highest affinity CA dimer interface, CypA could still affect higher order capsid assembly or disassembly by altering one of the other, lower affinity CA-CA interfaces involving the amino-terminal capsid domain. In the CA151/CypA co-crystal structure, CA151 molecules associate via two distinct interfaces to form long planar strips. We envision that the surface of the viral capsid core could assemble by side to side packing of these capsid strips. In the crystal, close packing of the capsid strips is prevented by stoichiometric binding of cyclophilin A molecules along the outer edges of the strips. In the virion, however, the substoichiometric levels of CypA could simply serve to destabilize the cooperative interactions between associated capsid strips. Thus, we hypothesize that the function of cyclophilin A in HIV-1 replication may be to weaken the capsid core and thereby promote its dissociation upon infection.

Other cyclophilins

Our studies demonstrate that the CypA active site binds specifically to the HIV-1 capsid protein, providing the first well-characterized biological function for a cyclophilin active site. This observation again raises the possibility, first suggested by Schreiber & Crabtree (1992), that proline isomerization may not be the primary function of cyclophilins in vivo. As has been pointed out by Harrison & Stein (1990), the modest k_cat/K_M for cyclophilin A binding to small model peptides suggests that rate enhancements for non-specific proline isomerization will be quite modest at physiological CypA concentrations. Instead, it appears that other cyclophilins may function in analogy to the model that we propose for CypA in HIV-1, i.e. by binding target proteins with some level of sequence discrimination and functioning by modulating or preventing protein-protein interactions. This model does not, of course rule out the possibility that proline isomerase activity may also be important in vivo.

Since the cyclophilin active site is largely conserved in eukaryotic cyclophilins (Stamnes et al., 1992), we speculate that Gly-Pro dipeptides will form the basic recognition motif for other cyclophilins. We envisage that different cyclophilins may discriminate between their target proteins by mak-
ing complementary interactions with sequences flanking the Gly-Pro recognition element. The Gly-Pro motif forms a convenient binding “handle”, since this particular dipeptide is very likely to reside in an exposed loop in a folded target protein. Indeed, since Gly-Pro sequences are likely to end up in exposed loops and not in integral secondary structures, it even seems possible that some cyclophilins may act as chaperones by transiently associating with the Gly-Pro sequences of folding proteins and promoting their intramolecular folding by shielding one face of the unfolded protein against intermolecular aggregation.

In summary, we have demonstrated that the cyclophilin A active site mediates sequence-specific binding of the HIV-1 capsid protein, recognizing an exposed capsid loop sequence that spans residues Pro85 to Pro93. The capsid dipeptide, Gly89-Pro90, forms the core recognition motif, with adjacent residues making a series of additional energetically significant interactions along the CypA active site groove. We speculate that the HIV-1 CA/CypA interaction may serve as a paradigm for sequence-specific protein recognition within the cyclophilin family, with Gly-Pro forming a general cyclophilin recognition motif.

Materials and Methods

HIV-1 capsid protein expression and purification

Procedures used to express and purify the intact (CA) and truncated (CA151) capsid proteins were similar and are therefore described in detail only for the full-length protein. The HIV-1NL4-3 capsid gene was amplified from proviral pNL4-3 DNA (Adachi et al., 1986; Myers et al., 1995) using the polymerase chain reaction (PCR). PCR primers were designed to introduce Ndel and BamHI cloning sites, a stop codon, and optimized E. coli codons for the first three amino acid residues (Sambrook et al., 1989). The amplified gene was subcloned into the phage T7 RNA polymerase-based expression vector, pET3a (Studier et al., 1990) and the resulting plasmid (WISP93-73) was confirmed by DNA sequencing. The expression vector for the truncated CA151 protein (WISP95-69) was constructed by introducing oligonucleotides encoding a stop codon immediately following CA amino acid codon 151 and subcloning the coding region into pET11a.

Protein expression levels and fully purified samples of the HIV-1 capsid and CA151 proteins are shown in Figure 2(a) and (b), respectively. BL21 (DE3) E. coli cells harboring the expression plasmids were grown in LB medium and capsid protein expression was induced late in log-phase growth (A_{600}=0.8; lane 1) by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM (1 mM for the pET11a expression constructs). Soluble capsid protein accumulated to very high levels during the four hour incubation period (Figure 2(a), lane 2). All steps in the protein purification were performed at 4°C. The cells were lysed in a French press and the supernatant sonicated to reduce viscosity. Insoluble material was removed by centrifugation at 40,000 g for one hour and the CA protein was concentrated by precipitation from 20% saturated ammonium sulfate. CA was redissolved in a buffer of 25 mM KMorps (pH 6.9)/5 mM β-mercaptoethanol and purified by cation-exchange chromatography on S-Sepharose (Pharmacia). The protein eluted at 300 mM NaCl during a 250 ml 0 to 1 M linear salt gradient. This procedure typically yielded >30 mg of pure capsid protein per liter of E. coli culture. The recombinant protein was characterized by N-terminal amino acid sequencing (NH$_2$-P-I-V), amino acid analysis, and electrospray mass spectrometry ($M_{obs}=25,603$ g/mol, $M_{calc}=25,602(±2)$ g/mol). A non-native N-terminal methionine residue was quantitatively (>97%) removed during protein expression and the primary sequence of the purified recombinant protein was therefore identical with the authentic HIV-1 capsid protein. The protein exhibits a single major isofrom in isoelectric focusing experiments (pI=7.05). CA151 was also processed during expression to produce a native N terminus, as analyzed by N-terminal amino acid sequencing and mass spectrometry ($M_{obs}=16,701$ g/mol, $M_{calc}=16,700(±4)$ g/mol).

DNA encoding HIV-1NL4-3 CA (encoding Gag sequences residues 133 to 363, MA-CA (1 to 363), and CA-NC (133 to 499) was also subcloned into a pGEX2T (Pharmacia) vector that was modified to contain an in-frame Ndel restriction site immediately downstream of the glutathione-S-transferase gene (WISP94-18). The CA construct was subsequently used as a template for PCR-based or MutA-Gen Phagemid (Biorad) mutagenesis (Kunkel et al., 1987), to produce the 17 mutant capsid proteins used to examine the determinants of CypA binding specificity.

Human cyclophilin A expression and purification

The human CypA gene was amplified in a PCR from a HeLa cDNA pool (Stratagene). The amplified DNA fragment was subcloned into the Ndel/BamHI sites of pET3a (Studier et al., 1990) to create WISP94-1 (encoding native CypA) and into the same sites in WISP94-18 to create WISP94-57 (encoding GST-CypA). The cloned human CypA genes were sequenced and corresponded exactly to the published cDNA sequence of human T-cell CypA (Haendler et al., 1987).

The induction and purification of recombinant CypA is shown in Figure 2(c). CypA expression was induced late in log phase (lane 1) and the protein was allowed to accumulate for four hours (lane 2). Soluble human CypA was purified to homogeneity by sequential ammonium sulfate fractionation, cation-exchange and hydrophobic chromatographies following a published procedure (Liu et al., 1990). The purified protein was isolated in reasonable yields (typically ~25 mg/l cells) and was characterized by N-terminal amino acid sequencing (NH$_2$(M)IVNP), amino acid analysis, and mass spectrometry ($M_{obs}=18,013$ g/mol; $M_{calc}=18,012(±1)$ g/mol). Approximately 15% of the protein molecules were missing their N-terminal methionine residue ($M=17,881(±1)$ g/mol), in good agreement with a previous report (Liu et al., 1990).

The GST-CypA fusion protein produced from the expression vector WISP94-57 was purified by glutathione affinity chromatography on G-Sepharose (Pharmacia). The protein eluted at a glutathione concentration of ~6 mM from a linear gradient of 0 to 10 mM glutathione in 100 ml of 50 mM Tris-Cl (pH 8.0). The protein was subsequently dialyzed extensively to remove bound glutathione and purified to homogeneity by cation-exchange chromatography on S-Sepharose (Pharmacia). The protein eluted at 400 mM NaCl from a linear gradient of 0
Dynamic light-scattering spectroscopy

Dynamic light-scattering was performed to determine the oligomeric states of the CA and CA151 proteins using a Dynapro-801 light-scattering instrument (Protein Solutions Inc.). Proteins were dissolved in 10 mM potassium phosphate (pH 6.5), 2 mM β-mercaptoethanol at concentrations of 200 μM CA and 158 μM CA151. At least eight measurements were made and averaged for each sample. Protein molecular masses were estimated by assuming globular protein structures and errors are reported as the standard deviation between the independent measurements.

Equilibrium sedimentation

Analytical ultracentrifugation was used to quantify the oligomerization of CA and CA151. Centrifugation experiments were performed on a Beckman Optima XL-A ultracentrifuge operating at rotor speeds of 20,000 (CA) or 25,000 (CA151) rpm. The proteins were centrifuged at 20°C in 25 mM sodium phosphate (pH 6.5), 100 mM NaCl, 2 mM β-mercaptoethanol. Absorbance profiles were measured at 280 nm and were collected every four hours until protein distribution had reached equilibrium. After 24 hours, eight scans along the cell radius were collected with a step size of 0.003 cm, averaged, and corrected for background absorbance against a buffer blank.

Equilibrium distributions were fit to the single homogeneous species model or to a monomer-dimer model as described (McRorie & Voelker, 1993), using the Microcal Origin non-linear least-squares fitting algorithm provided within the XL-A data analysis software. A solvent density of 1.0052 g ml⁻¹ was used and partial specific volume estimates of 0.737 ml g⁻¹ (CA) and 0.740 ml g⁻¹ (CA151) were derived from the amino acid compositions of the two proteins (Laue et al., 1992).

Titration calorimetry

Isothermal titration calorimetry (ITC) was performed at 20.0°C using a MicroCal Omega titration calorimeter. Two different titrations were performed: (1) 632 μM CA151 was titrated into 59 μM CypA; and (2) 661 μM CypA was titrated into 59 μM CA. Each titration consisted of 15 injections of 20 μl, made every five minutes, into a reaction cell volume of 1.296 ml. All proteins were dissolved in buffered solutions of 100 mM KCl, 25 mM sodium phosphate (pH 6.5), 2 mM β-mercaptoethanol. Reaction heats were baseline-corrected manually using the Origin TC software, corrected by subtraction of the appropriate blank titrations, and integrated. Thermodynamic parameters for the binding reactions were derived by fitting the corrected binding isotherms to single-site binding models as described (Wiseman et al., 1989), with stoichiometries (n), enthalpies (AH°), and equilibrium dissociation constants (Kd) allowed to float during nonlinear least-squares fits of the data. Titrations were performed under conditions where the product of the binding constant and the reservoir macromolecule concentration (c) was approximately 4, and can therefore be expected to provide reasonable estimates of the binding equilibria.

Surface plasmon resonance measurements

SPR measurements were performed on a BIACORE 2000 biosensor, with CM5 research-grade chips, NHS/EDC coupling reagents, ethanalamine, anti-GST monoclonal antibody and P20 from Biacore AB (Uppsala, Sweden). The binding assays were performed by capturing either GST-CypA or GST-CA onto the chip surfaces and measuring the interaction with either CA151 or CypA in solution. A monoclonal antibody against GST (anti-GST) was immobilized on the sensor surface using a standard amine coupling procedure (Löfås & Johnsson, 1990). The carboxymethyl dextran surface was activated with a seven minute injection of a mixture of NHS/EDC (each at 0.1 M in water). The antibody was immobilized at the same level (~5000 RU) on different flow-cells by exposing the activated surfaces to a 10 μg/ml sample in 10 mM sodium acetate (pH 5.0) for 300 seconds. After the coupling step, the remaining activated groups were blocked with a seven minute wash of 1 M ethanolamine (pH 8.2), followed by several ten second washes with glycine (pH 2.0) to remove non-covalently bound protein.

All subsequent binding experiments were performed in buffer containing 100 mM sodium chloride, 25 mM potassium phosphate (pH 6.5), 1 mM β-mercaptoethanol, and 0.005% (v/v) P20 at pH 6.5, unless otherwise indicated. Purified GST-CypA was captured onto anti-GST surfaces at two different densities by injecting supernatant containing GST-CypA for varying lengths of time at a flow-rate of 5 μl/minute. Equal quantities of recombinant GST alone were captured onto control surfaces. To perform the kinetic measurements for CA151 binding, the flow-path was changed to include all four flow-cells and the flow-rate was increased to 50 μl/minute: 22 μl of CA151 at concentrations of 57, 19, 6.3 and 2.1 μM along with a buffer blank were injected over all four flow-cells in series. Since the response returned rapidly to baseline, no regeneration step was required between CA151 injections. Each binding experiment was repeated two or three times in random order. Analogous experiments were performed at pH 5.5, 7.5 and 8.5.

Similar binding experiments were performed for the GST-CA constructs by capturing these proteins onto the sensor surface and monitoring the interaction with CypA in solution. In this case, native and mutant GST-CA proteins were captured directly from crude E. coli supernatants. To examine CypA binding, 30 μl of CypA solution was injected at concentrations of 114, 38, 12 and 4.2 μM along with a buffer blank. Each CypA injection was repeated three times in random order over two different densities of GST-CA. Control experiments were performed using a recombinant GST to ensure that CypA did not interact with this protein or the anti-GST antibody on the sensor surface.

To analyze the interactions recorded on the biosensor, the raw response data collected from the individual flow-cells were baseline-corrected by subtracting the average of the response over 60 seconds prior to sample injection. To correct for refractive index change and non-specific binding, the response collected over a blank surface was subtracted from the flow-cell containing the GST-fusion protein. The response at equilibrium, which represents the level of CA/CypA complex formation, was determined by averaging the signal obtained over five to ten seconds after the start of the injection. Equilibrium dissociation constants (Kd) for each interaction were determined from non-linear least-squares curve fitting of the data.
to a single-site binding model as described in the BIACORE manual.

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References


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