Maturation-induced Conformational Changes of HIV-1 Capsid Protein and Identification of Two High Affinity Sites for Cyclophilins in the C-terminal Domain*

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Viral incorporation of cyclophilin A (CyPA) during the assembly of human immunodeficiency virus type-1 (HIV-1) is crucial for efficient viral replication. CyPA binds to the previously identified Gly-Pro-Pro site of the capsid protein p24, but its role remained unclear. Here we report two new interaction sites between cyclophilins and p24. Both are located in the C-terminal domain of p24 around Gly-Pro157 and Gly-Pro224. Peptides corresponding to these regions showed higher affinities (Kd < 0.3 μM) for both CyPA and cyclophilin B than the best peptide derived from the Gly-Pro-Pro site (Kd = 8 μM) and thus revealed new sequence motifs flanking Gly-Pro that are important for tight interaction of peptide ligands with cyclophilins. Between CyPA and an immature (unprocessed) form of p24, a Kd of ~8 μM was measured, which corresponded with the Kd of the best of the Gly-Pro-Pro peptides, indicating an association via this site. Processing of immature p24 by the viral protease, yielding mature p24, elicited a conformational change in its C-terminal domain that was signaled by the covalently attached fluorescence label acrylodon. Consequently, CyPA and cyclophilin B bound with much higher affinities (~0.6 and 0.25 μM) to the new, i.e. maturation-generated sites. Since this domain is essential for p24 oligomerization and capsid cone formation, CyPA bound to the new sites might impair the regularity of the capsid cone and thus facilitate in vivo core disassembly after host infection.

The cyclophilins represent a ubiquitous family of proteins that are abundant in nearly every cell type and are highly conserved through evolution (1). The main representative of the cyclophilins in mammals is the cytosolic 18-kDa CyPA. The closely related CyPB (21 kDa) contains a N-terminal signal sequence that directs this isoform to the endoplasmic reticulum. A characteristic property of cyclophilins is their peptidyl-prolyl cis-trans isomerase (PPIase) activity and their ability to bind cyclosporin A (CsA) that inhibits the PPIase activity. In T cells, the CsA-CyPA complex associates with and inhibits the Ca²⁺-dependent protein phosphatase calcineurin, resulting in immunosuppression. Natural endogenous ligands of cyclophilins with similar actions as CsA are unknown, and the cellular functions of cyclophilins are still a matter of investigation. A new important property of cyclophilins was found in the human immunodeficiency virus life cycle (2, 3) when a specific association of CyPA with the Gag polyprotein p55Gag of HIV-1 (4) was detected.

During or after budding of immature viral particles out of the host cell, the noninfectious virions with a spherical core consisting of Gag proteins are transformed to infectious virions and undergo a dramatic structural rearrangement to an icosahedral shape (5). In the course of this maturation, the pol-encoded HIV protease processes the Gag polyprotein precursor into three major proteins and three smaller polypeptides (6, 7). The capsid protein p24, consisting of a N-terminal and a C-terminal domain, condenses to form the conical core structure surrounding the viral genome. One CyPA molecule per 10 Gag molecules is incorporated into nascent virions, amounting to a total of ~200 molecules/virion (2, 3). CyPB was shown to form in vitro similar complexes with the Gag polyprotein as CyPA but was not found in virions in vivo (2). The interaction with the Gag polyprotein and p24 is mediated by the active site of CyPA and is suppressed in the presence of CsA or SDZ NIM811 (3, 8), a natural nonimmunosuppressive CsA analog that also binds to CyPA and inhibits its PPIase activity. CsA prevents the incorporation of CyPA but inhibits neither the assembly of viral components nor the production of morphologically intact virions (9). However, virions lacking CyPA were found to be considerably reduced in their propagation (10). Single point mutations in the p24 domain of the Gag polyprotein precursor revealed a region with an array of four proline residues to be involved in binding to CyPA (2). Recently, the crystal structure of CyPA complexed with a domain of p24 encompassing the N-terminal two-thirds (residues 1–151) showed binding of CyPA to the proline-rich segment that forms a flexible surface-exposed loop in the mainly a-helical p24 fragment (11). The contact region on the p24 loop is restricted to a relatively small area with Gly-Pro-Pro as the central element buried in the active site of CyPA. This Gly-Pro motif seems to be a prerequisite for stable association. Only if glycine precedes a proline residue is the peptide stretch able to snuggle deeply into the active site of CyPA.

So far, the role of CyPA in the interaction with HIV-1 p24 is not clear. CyPA might act as PPIase and catalyze a cis-trans isomerization in the p24 peptide backbone or might function as chaperone by binding the capsid protein in a conformational prone for propagation, i.e. by destabilizing the capsid cone for postentry disassembly (10). Such an action of CyPA, however,...
could not be expected to result solely from binding to the site on the loop around Gly-Pro\textsuperscript{30} in the N-terminal domain. Very recently, it was reported that CyPA disrupts high order p24 complexes, most probably by interfering with p24-p24 interactions (12). As the assembly and disassembly of p24 occur both in the same compartment of the host cell, it remained a puzzle how this CyPA-p24 (Gly-Pro\textsuperscript{30}) interaction should initiate capsid disassembly without already affecting viral assembly.

In the present investigation, we have identified two additional CyPA-binding sites in the C-terminal domain of mature p24 capsid protein. Both sites show higher affinities for CyPA and CyPB than the Gly-Pro\textsuperscript{30} site of p24. These two high affinity binding sites seem to be accessible for CyPA only in the mature p24 capsid protein, i.e. after processing of immature p24 by the HIV-1 protease. A hypothetical model of the function of CyPA in the disassembly of the capsid cone is proposed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant immature HIV-1 capsid protein was obtained from D. Sizmann (Hoffmann-La Roche, Basel) in 6 M guanidine HCl and was efficiently refolded by an established method (13, 14) into a correctly folded soluble protein. Folded immature capsid protein was analyzed by CD spectroscopy with regard to secondary structure composition. The obtained CD spectra were comparable with that reported for native HIV-1 capsid (13, 420) and immature p24 (2.1 or 0.1 mM) or to labeled immature p24 (1.0 mM) in 20 mM sodium phosphate, pH 7.4, 60 mM NaCl, and 60 mM KCl. After each addition, the mixture was incubated until equilibrium had been reached before recording the spectra. In order to calculate \( K_v \) values, the change in relative fluorescence was fitted (SigmaPlot, Jandel Corp.) according to the following equation: \[ \Delta F = (1 - 10^{-C_{420} \cdot K_v}) \cdot (1 - 10^{-C_{420} \cdot 1}) \cdot (1 - 10^{-C_{420} \cdot 1}) \] where \( \Delta F \) is the relative fluorescence change, \( P \) is the total concentration of p24, \( F \) is the total concentration of CyP, and \( K_v \) is the dissociation equilibrium constant. \( K_v \) values of independent experiments were within the indicated calculated S.D. values (Table I).

**PPIase Activity Measurement**—PPIase activity was measured at 4 °C essentially as recently described (17) with the standard chymotrypsin-coupled assay (18). The assay mixture (0.08 nM NaCl, 0.05 mM Heps, pH 8; final volume 200 μl) contained 50 μM of succinyl-Ala-Leu-Pro-Phe-\textsuperscript{14}nitroanilide (Bachem) and was incubated for at least 10 min at 4 °C. The reaction was started by the addition of chymotrypsin (60 μM) and monitored at a 390 nm with a Hewlett-Packard 5405A UV/VIS-spectrophotometer. For competitive inhibition experiments, recombinant human CyPA (1–5 nm) or CyPB (8–21 nM) was preincubated with or without the corresponding p24 component in the presence of 0.5 mM bovine serum albumin for 1 h at room temperature prior to chymotrypsin addition. In the case of peptide p214–228, the assay mixture additionally contained 250 μM dithiothreitol. The apparent reaction rate constant \( k_{app} \) was obtained by analyzing the exponential phase (50–500 s) of the curve according to a pseudo-first order reaction. The rate of the spontaneous, uncatalyzed isomerization \( k_{isom} \) (0.0036 ± 0.0002 s\(^{-1}\), at 4 °C) was subtracted from \( k_{app} \). The percentage inhibition was calculated in relation to the PPIase activity measured in the absence of competitors. In general, each measurement was performed twice, and the average was calculated. Obtained data were fitted according to the equation for competitive inhibition: percentage inhibition = \( [(100 \times 1) \times (1 + IC_{50})] \), where \( IC_{50} \) is the total concentration of the inhibitor and \( S/K_v \) corresponds to \( [K_v \times (1 + (S/K_v))] \). \( K_v \) is the inhibition constant of the competitor, \( S \) is the concentration of the PPIase substrate used in the standard assay, and \( K_v \) is the Michaelis-Menten constant of the substrate. Since \( S/K_v \) was approximately 100 for the used PPIase substrate, the \( IC_{50} \) values are reflecting \( K_v \) values.

**Preparation of Mature p24**—In order to obtain mature p24, folded immature p24 protein was incubated with 1/100 (w/w) of recombinant HIV-1 protease in 30 mM MES, pH 5.5, 200 mM NaCl, 1% (w/v) glycerol, and 1 mM EDTA at 37 °C. The reaction was monitored by analyzing aliquots with SDS-polyacrylamide gel electrophoresis and silver staining (Phast gels, Amersham Pharmacia Biotech). Usually, processing was complete after 4–6 h. The reaction was stopped by adjusting the pH to 8.0. Subsequently, 100 μM EDTA and 5 μM phenylmethanesulfonyl fluoride was added, and the sample was dialyzed against 3 mM Tris-HCl, pH 8.0, 10 mM CaCl\(_2\), and 1 mM guanidine HCl for 1 h at 37 °C. The product was analyzed by liquid chromatography-mass spectrometry (LC-MS) linked to an API III electrospray ionization (ESI) mass spectrometer; Photon Technology International). Excitation was set to 370 nm (3-nm bandwidth), and emission was recorded from 400 to 600 nm (3-nm bandwidth). In order to measure dissociation constants of the complexes between the cyclophilins and mature or immature p24, CyPA or CyPB were added to the reaction mixture (25,579.5 Da for mature p24, 13,420 Da for immature p24) in 20 mM sodium phosphate, pH 7.4, 60 mM NaCl, and 60 mM KCl. After each addition, the mixture was incubated until equilibrium had been reached before recording the spectra. In order to calculate \( K_v \) values, the change in relative fluorescence was fitted according to the following equation: \[ \Delta F = (1 - 10^{-C_{420} \cdot K_v}) \cdot (1 - 10^{-C_{420} \cdot 1}) \cdot (1 - 10^{-C_{420} \cdot 1}) \] where \( \Delta F \) is the relative fluorescence change, \( P \) is the total concentration of p24, \( F \) is the total concentration of CyP, and \( K_v \) is the dissociation equilibrium constant. \( K_v \) values of independent experiments were within the indicated calculated S.D. values (Table I).

**Fluorescence Measurements**—Folded immature p24 (10 μM) was dialyzed against 10 mM HEPES, pH 7.0, and 10 μM dithiothreitol and then incubated with acrylodan (6-acryloyl-2-methyl-5-(N,N-dimethylaminonaphthalene, 10-fold molar excess over sulfhydryl groups; Molecular Probes Europe BV) in 50 mM HEPES, pH 7.0, 5% (v/v) acetonitril and 0.5 μM guanidine HCl (a concentration at which native p24 is still stable according to Ref. 14) for 2 h at room temperature in the dark on a rotary device. The reaction mixture subsequently was loaded onto a PD10 gel filtration column (Amersham Pharmacia Biotech) and eluted with 50 mM HEPES, pH 7.0, 5% (v/v) acetonitril, and 0.2 mM guanidine HCl. Protein (p24)-containing fractions were pooled; dialyzed against 2 mM sodium phosphate, pH 7.4, 6 mM NaCl, 6 mM KCl, and 1% (v/v) acetonitril; and vacuum-concentrated to a concentration of 250 μM. Mass and UV spectroscopy of the product revealed only monomabeled p24 (most probably at Cy5 by Edman degradation; see Ref. 16). With 0.3 mol of acrylodan incorporated per mol of p24. In order to obtain acrylodan-labeled mature p24, labeled immature p24 was treated with HIV-1 protease as described before and proven by mass spectroscopy to be correctly processed.

Fluorescence measurements were performed at 25 °C with a spectrofluorimeter (model A1010 equipped with FELIX fluorescence analysis software; Photon Technology International). Excitation was set to 370 nm (3-nm bandwidth), and emission was recorded from 400 to 600 nm (3-nm bandwidth). In order to measure dissociation constants of the complexes between the cyclophilins and mature or immature p24, CyPA or CyPB were added to the reaction mixture (25,579.5 Da for mature p24, 13,420 Da for immature p24) in 20 mM sodium phosphate, pH 7.4, 60 mM NaCl, and 60 mM KCl. After each addition, the mixture was incubated until equilibrium had been reached before recording the spectra. In order to calculate \( K_v \) values, the change in relative fluorescence was fitted according to the following equation: \[ \Delta F = (1 - 10^{-C_{420} \cdot K_v}) \cdot (1 - 10^{-C_{420} \cdot 1}) \cdot (1 - 10^{-C_{420} \cdot 1}) \] where \( \Delta F \) is the relative fluorescence change, \( P \) is the total concentration of p24, \( F \) is the total concentration of CyP, and \( K_v \) is the dissociation equilibrium constant. \( K_v \) values of independent experiments were within the indicated calculated S.D. values (Table I).
amino acid sequence of mature p24 (Fig. 1) reactive cysteine residue at position 218 of p24 (see Ref. 16 and of an intramolecular disulfide bond between Cys198 and Cys218 figure p24 (see “Experimental Procedures”). Although formation on ice. After 30 min, the reaction was stopped by the addition of 2 M solution of HPLC-purified peptide in 200 mM sodium phosphate, pH 7.0, terminally acetylated. quantified by amino acid analysis. Fragment ion mass spectrum of the bound immature p24. The presence of a

Fig. 1. Scheme of immature HIV-1 capsid protein p24 (A) and amino acid sequence of mature p24 (B). Immature p24 protein consists of the entire p24 capsid protein (residues 1–231, B) flanked N-terminally by a stretch of 25 amino acids (aa) including a His tag, and C-terminally by 20 amino acids. Similar to the genuine Gag precursor, the N-terminal extension contained 13 specific amino acid residues from the matrix protein p17, whereas the C-terminal extension contained 12 specific residues from p24, a spacer peptide linking the C terminus of p24 to the nucleocapsid protein p7 in the original Gag polyprotein. The cleavage sites recognized by the HIV-1 protease are indicated by arrows. The numbering of the amino acids (B) refers to the mature p24 capsid protein, and the MHR is indicated by shading. The positions of the CyPA-binding fragments I–V (Fig. 4F) within p24 are indicated. The Gly-Pro sequence elements (boldface type) are supposed to be the central CyPA-binding segments of the peptides.

Waters) and proved to be correct by mass spectrometry and quantitative amino acid analysis.

For acetylation of p(153–172), acetic anhydride (3-fold molar excess, dissolved in 20 mM acetic acid) was added dropwise to a well stirred solution of HPLC-purified peptide in 200 mM sodium phosphate, pH 7.0, on ice. After 30 min, the reaction was stopped by the addition of 2 M hydroxylamine HCl in 0.5 M Tris-HCl, pH 9.6. The product was purified on a C4-reversed phase HPLC column (4.6 × 220 mm, Brownlee) and quantified by amino acid analysis. Fragment ion mass spectrum of the triply charged peptide ion proved the peptide to be exclusively N-terminally acetylated.

RESULTS

Interaction of CyPA and CyPB with p24—The presence of a reactive cysteine residue at position 218 of p24 (see Ref. 16 and Fig. 1B) was utilized to introduce the sulfhydryl-specific, environmentally sensitive fluorophore acrylodan (19) into immature p24 (see “Experimental Procedures”). Although formation of an intramolecular disulfide bond between Cys196 and Cys218 has been observed in a truncated p24 encompassing the C-terminal domain (20), such a disulfide bond could not be shown under physiological conditions (16) and is not expected, since the Gag precursor folds and oligomerizes in the reducing environment of the host cell. Acrylodan-labeled immature p24 exhibited maximal fluorescence emission at 498 nm when excited at 370 nm (Fig. 2, C and D). Upon the addition of CyPA, the fluorescence of labeled immature p24 increased in intensity and underwent a blue shift. A dissociation equilibrium constant, $K_d$, of 8.2 μM (Table I) was calculated from the binding curve (Fig. 2C). In contrast, the fluorescence intensity of labeled immature p24 decreased upon the addition of CyPB (Fig. 2D), and a $K_d$ of 0.9 μM (Table I) was determined. The observed changes in relative fluorescence of labeled immature p24 upon the addition of CyPA or -B were abolished in the presence of CsA, yielding the same fluorescence spectrum as that of unbound immature p24.

In order to determine the binding characteristics of mature p24, folded immature p24 was correctly processed to mature p24 by the HIV-1 protease, which is also responsible for in vivo processing of the Gag polyprotein. The fluorescence spectra of unliganded acrylodan-labeled mature p24 exhibited maximal emission at 508 nm (Fig. 2, A and B). The fluorescence decreased in intensity upon the addition of CyPA or CyPB without a perceivable shift in $\lambda_{max}$. The $K_d$ values for the CyPA-p24 complex and for the CyPB-p24 were calculated from their respective binding curves (Fig. 2, A and B; Table I). The contribution of the acrylodan label to the binding of p24 to CyP was negligible, as judged by acrylodan-labeled -mercaptoethanol that did not show any change in fluorescence intensity upon CyP addition. Again, upon the addition of CsA to the mixture of CyP and p24, the fluorescence spectrum was the same as that of unbound p24. Thus, CsA prevented also the interactions of CyPA and CyPB with labeled mature p24, demonstrating specific binding of labeled p24 in the active site of the cyclophilins.

PPase Inhibition of CyP by Immature and Mature p24—To verify the binding properties obtained with the fluorimetric measurements, the inhibitory effect of immature p24 on the PPIase activity of CyPA and CyPB was determined. Immature p24 inhibited the PPIase activity of CyPB ($K_i = 1.2 \mu M$; Fig. 3) more efficiently than that of CyPA ($K_i = 8.5 \mu M$). Both $K_i$ values were in excellent agreement with the fluorimetrically determined $K_d$ values.

Due to the presence of a His tag in immature p24 (Fig. 1), preformed complexes between CyPA and immature p24 could be precipitated with Ni²⁺-agarose and quantified by densitometric analysis of silver-stained protein bands after SDS-polyacrylamide gel electrophoresis. The calculated apparent disso-
PPIase activity of CyPA and CyPB each by im-

...ucces in which relative low concentrations have been ex-


timated Kd value of ~19 µM obtained from experiments in which relative low concentrations have been used (0.25 µM p24 and 25 nM to 2.5 µM CyPA) is, as expected (due to a certain dissociation during the procedure), higher (~2-fold) than the Kd and K values (Table I). CsA efficiently disrupted the complex (i.e. no CyPA was co-precipitated with immature p24 in the presence of CsA).

Mature p24 was, in contrast to immature p24, susceptible to cleavage by chymotrypsin (used in high concentrations in the standard PPIase assay, see "Experimental Procedures"). Most of it was found to be degraded upon analysis by SDS-polyacrylamide gel electrophoresis at the end of the assay. Nevertheless, the PPIase activity of CyPA and CyPB was effectively inhibited at lower concentrations of mature p24 than of immature p24 (Fig. 3). In the case of CyPB, nanomolar concentrations of mature p24 already inhibited part of the PPIase activity of CyPB, but complete inhibition was achieved only at higher concentrations (>10 µM). Therefore, the fluorimetrically determined Kd values seem to reflect the true binding affinities of mature p24 (Table I).

Mapping the CyPA-binding Sites on the p24 Capsid Protein—
The higher affinity of mature p24 as compared with immature p24 with cyclophilins indicates that additional binding sites on p24 become accessible after the precursor is processed by the HIV-1 protease. To map potential CyPA-binding sites on HIV-1 p24, chymotryptic fragments of recombinant p24 were incubated with human CyPA and subjected to a fast size exclusion chromatography (Fig. 4). CyPA-bound peptide segments of p24 co-eluted together with CyPA in the void volume. CyPA-con-


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taining fractions were analyzed by reversed-phase HPLC (Fig. 4, D–F). Five CyPA-associated p24 peptides (I–V) were found and identified by mass spectrometry. Peptide I corresponded to the four proline residue-containing sequence 81–117, which encompasses the known Gly-Pro motif of p24 (Fig. 1B). The molecular mass of peptide II mapped to C-terminal fragment 187–227 of mature p24 containing a Gly-Pro sequence element. Peptides III, IV, and V, consisting of residues 152–185, 130–164, and 153–187, respectively, overlap in the major homology region (MHR) of p24 (21), all having two proline residues and a Gly-Pro sequence element.

To confirm the binding of the proteolytic p24 fragments to CyPA, the same procedure was performed with cyanogen bromide-fragmented p24. Two CyPA-bound peptides were subjected to N-terminal amino acid sequence analysis. The obtained N-terminal sequences YXPTSLIDIKQ and TAXQGVG, where X denotes an unidentified amino acid residue, corresponded to cyanogen bromide-generated fragments 145–185 and 216–235, respectively (Fig. 1). Both peptides thus matched the two new binding sites on p24 found with the chymotryptic fragments.

PPIase Inhibition of CyP by p24 Peptide Segments—Synthetic peptides, corresponding to the chymotryptic fragments with Gly-Pro as the central binding motif, were used to confirm and to define the new CyP-binding sites on p24. The binding properties of these p24 peptides were examined by means of their inhibitory effect on the PPIase activity of CyPA and CyPB. Peptide p(87–101), corresponding to a segment within peptide I, inhibited the PPIase activity of CyPA with a Kd of 170
The elimination of the positive charge by biotinylation of the N terminus improved the binding 6-fold (Table I), whereas biotin itself did not affect the PPIase activity in concentrations of up to 0.2 mM. Additional amino acid residues preceding His87 contributed to better binding of peptide I (K_i of 8.3 μM), which encompasses the entire sequence of the surface-exposed loop between helices 4 and 5 of p24 (Fig. 5A; Table I). Peptide I was purified from the chymotrypsin digest of p24 in amounts sufficient for the inhibition studies.

The N-acetylated synthetic MHR peptide p(153–172) was shown to inhibit the PPIase activity of CyPA and CyPB already at lower concentrations (Fig. 5B) compared with peptide I (Fig. 5A). The experimental data displayed a complex inhibition behavior due to the fragmentation of the Phe-containing MHR peptide during the time of activity measurement by chymotrypsin, which was present in high concentrations in the standard PPIase assay. Apparently, the inhibition curves reflect a heterogeneous and dynamic inhibition of the PPIase activities by a mixture of truncated MHR peptides, whose individual affinities will be lower than that of the original MHR peptide. Thus, only IC_{50} values of 12 and 27 μM for CyPA and CyPB, respectively, could be determined. The effective concentrations of intact MHR peptide must be much lower than the indicated concentrations (Fig. 5B). The observed partial inhibition at initial concentrations of the peptide below 0.1 μM, however, shows that intact MHR peptides are able to bind to the active site of both cyclophilins at very low concentrations. This suggests the MHR peptide to be a high affinity ligand for CyPA (K_i ≤ 1 μM) if residues following Phe^{861} are not cleaved off by chymotrypsin. The fact that complexes of CyPA with chymotryptic or cyanogen bromide fragments of p24 encompassing the MHR could be isolated (Fig. 4F) corroborates this conclusion.

Peptide p(214–228), corresponding to the C-terminal binding site on p24, displayed a homogeneous competitive inhibition of PPIase activity and seems to have the highest affinity toward CyPA (K_i of 0.35 μM) and CyPB (K_i of 0.29 μM) among the three identified p24 binding sites (Fig. 5C; Table I). The presence of a cysteine residue (Cys^{216}) allowed us to label this peptide with acrylodan (for details, see Ref. 22) and to measure its interactions with CyPA and CyPB. The calculated K_i values 0.40 and 0.29 μM, respectively, corresponded excellently with the K_i values determined by the competitive PPIase assay (Table I), emphasizing the equivalence of the different methods applied.

**DISCUSSION**

The results presented in this study indicate that HIV-1 capsid protein p24 contains, in total, three sites that are able to interact specifically with CyPA and CyPB. One binding site, the low affinity site, matches to the loop around Gly-Pro^{80}, which has been previously proposed on the basis of mutation experiments (2) and verified by crystallographic analysis of the complex of CyPA with CA_{151}, a truncated p24 encompassing the N-terminal domain (11). The reported K_i of ~16 μM for the CA_{151}-CyPA complex (23) is comparable with the present value of 8 μM for peptide I (Table I). The two additional binding sites, one within the MHR and another at the very C terminus of mature p24, show higher affinities and were not known so far. Both of them contain the Gly-Pro motif, which was shown to be the central element and a prerequisite for binding to CyPA (11, 24). A homology among the Gly-Pro adjacent amino acid residues of the three binding sites is not evident. The more than 20-fold higher affinity of CyPA for the C-terminal peptide than for the Gly-Pro^{80} peptides might be attributed to the presence of an additional Gly and of positive charged amino acid residues in the segment following Gly-Pro^{224} (Table I). A homologous peptide containing the sequence SIHIGPRAF and which fulfills the above criteria was shown to bind to CyPA with a K_d...
of 0.33 μm and to CyPB with a $K_\text{D}$ of 0.19 μm (22). Basic residues, C-terminal of Gly-Pro, are also found in the MHR peptide p153–172, which binds more tightly to the cyclophilins than the Gly-Pro$^{90}$ peptides if the residues following Phe$^{161}$ are still present (see “Results”). The crystallographic studies of the low affinity binding site revealed that the Gly-Pro$^{90}$ adjacent residues predominantly interact with CyPA by their backbone atoms (11, 25). The present results with the two high affinity binding sites, however, demonstrate significant contributions of GP-flanking side chains to the binding to cyclophilins.

Whereas complex formation of CyPA and CyPB with the p24 peptides was comparable, substantial differences in affinity were found with mature and immature p24. The immature protein form has, like the unprocessed Gag polyprotein precursor, an N- and C-terminal extension (Fig. 1) that seems to be important for proper assembly of the initial virion core shell (26–29) and thus probably also for its proper conformation. The binding affinity of CyPA for immature p24 ($K_\text{D} = 8.3$ μm) is identical with that for the Gly-Pro$^{90}$ site. These results suggest that the binding of CyPA to immature p24 occurs through the Gly-Pro$^{90}$ site, as proposed previously (2, 11, 23, 30). In contrast to CyPA, the affinity of CyPB for immature p24 is 8-fold higher ($K_\text{D} = 1$ μm) indicating a complex formation via a different binding site on p24, most likely the MHR. Evidence for a different binding site for CyPB on immature (i.e., unprocessed) p24, compared with CyPA, is given by the different fluorescence changes upon the addition of the cyclophilins (Fig. 2, C and D) but also by the observation that two Gag mutants, which had either Gly$^{80}$ or Pro$^{90}$ exchanged for Ala, lost their ability to bind CyPA but were still able to associate with CyPB (2, 9). The stronger interaction of the Gag polyprotein with CyPB than with CyPA explains why 10-fold higher concentrations of CsA are needed to disrupt this complex (4).

Processing the immature p24 protein to the mature p24 form of the capsid protein by HIV-1 protease increased the affinity 13- and 4-fold for CyPA and CyPB, respectively. The agreement of the $K_\text{D}$ values for mature p24 with that for peptide p214–228 suggests the C-terminal binding site to be responsible for the high affinity interaction of the mature p24 form with the cyclophilins and not the Gly-Pro$^{90}$ site. The fluorescence intensity of acrylodan-labeled immature p24 increased upon CyPA binding, resulting in a $K_\text{D}$ of 8.2 μm, whereas that of labeled mature p24, carrying the environmentally sensitive label at the same site, decreased upon the addition of already low concentrations of CyPA. This observation indicates different primary interaction sites of immature and mature p24 with CyPA and thus different conformations of the two p24 forms. The induced accessibility of the C-terminal binding site(s) on maturation has to be caused by a conformational rearrangement in the C-terminal domain of the capsid protein or by the loss of N-terminal and C-terminal extensions of immature p24. The observed processing-induced change in the fluorescence spectrum of unliganded p24 (Fig. 2, A and B versus C and D) indicates a structural rearrangement, particularly in the C-terminal domain where the label is attached (see “Experimental Procedures”). A maturation-induced accessibility of the MHR-located binding site for CyPA is also indicated by the comparable estimated $K_\text{D}$ for the MHR peptide p153–172 (see discussion under “Results”) and for the mature p24 protein. The recently reported structure of the C-terminal domain of p24 (20) suggests that both high affinity binding sites are accessible for CyPA. Structural data of the C-terminal 11 amino acids, including Gly$^{223}$, Pro$^{224}$, however, could not be collected, suggesting a rather flexible structure. Thus, this part of p24 is very likely to be as well suited as the free peptide to fit into the active site of CyPA and CyPB as predicted from the present study.
might be accessible to cellular cyclophilins after infection only, if the regularity and stability of the structure of the capsid cone is impaired, by virus-intrinsic CyPA molecules. In some cases, destabilizing mutations in p24 (31, 32) might dispense the virus from incorporation of CyPA. Intracellular cyclophilins and possibly other components might then facilitate complete disassembly. If the capsid cone is not destabilized, initiation of the disassembly process will be difficult but might be possible in cells with high concentrations of intracellular cyclophilins, as was recently observed (33).

In conclusion, the present finding of two high affinity binding sites for CyPA and CyPB within the C-terminal domain of HIV-1 p24 revealed not only sequence motifs of Gly-Pro flanking regions that are important for tight binding of peptide ligands to the cyclophilins but also a feasible model for the cyclophilin-capsid protein interactions in the HIV-1 life cycle (Fig. 6). CyPA is incorporated into the virions by binding to the Gly-Pro" site (N-terminal domain of p24) of the Gag polypeptide precursor. Processing of Gag proteins by the viral protease induces a conformational change and generates high affinity binding sites in the C-terminal domain of p24 that will attract CyPA, and this complex could destabilize the capsid cone for disassembly. In view of the observed binding of CyPA to a site in the MHR of p24, which is well conserved in HIV-1, HIV-2, and possibly other components might then facilitate complete virus from incorporation of CyPA. Intracellular cyclophilins destabilizing mutations in p24 (31, 32) might dispense the regularity and stability of the structure of the capsid cone if the regularity and stability of the structure of the capsid cone is impaired, by virus-intrinsic CyPA molecules. In some cases, destabilizing mutations in p24 (31, 32) might dispense the virus from incorporation of CyPA. Intracellular cyclophilins and possibly other components might then facilitate complete disassembly. If the capsid cone is not destabilized, initiation of the disassembly process will be difficult but might be possible in cells with high concentrations of intracellular cyclophilins, as was recently observed (33).

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