

The Hydrophobic Pocket of Cyclophilin Is the Binding Site for the Human Immunodeficiency Virus Type 1 Gag Polyprotein

DOUGLAS BRAATEN,¹ HUSAMUDDIN ANSARI,² AND JEREMY LUBAN^{1,3*}

Department of Microbiology,¹ Integrated Program in Cellular, Molecular, and Biophysical Studies,² and Department of Medicine,³ College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received 26 September 1996/Accepted 9 December 1996

Completion of an early step in the human immunodeficiency virus type 1 (HIV-1) life cycle requires incorporation into virions of the cellular peptidyl-prolyl isomerase cyclophilin A (CyPA) by the Gag polyprotein. Elucidation of the biochemical role of CyPA would be aided by a detailed analysis of the genetic requirements for the formation of the Gag-CyPA complex; previous experiments have demonstrated the requirement for a critical proline and the immediately preceding glycine, located within the capsid domain of Gag, but nothing is known about the necessary CyPA residues. Cyclophilins possess a hydrophobic pocket where proline-containing peptide substrates and the immunosuppressive drug cyclosporine A bind. In this study, we engineered five CyPA mutations, each of which alters a residue that contributes to the hydrophobic pocket. Compared with the wild-type protein, all of the mutants drastically reduced CyPA binding to HIV-1 Gag and similarly inhibited CyPA incorporation into virions. In addition, we demonstrated that previously reported differences between the Gag-binding properties of CyPA and CyPB are due to adventitious association involving residues in the signal sequence of CyPB and that the core domain of CyPB interacts with Gag in a fashion which is indistinguishable from that of CyPA. These studies indicate that, as with other proline-containing peptides or cyclosporine A, HIV-1 Gag directly contacts residues in the hydrophobic pocket of CyPA.

Late in the infectious cycle of human immunodeficiency virus type 1 (HIV-1), the Gag polyprotein binds to the cytosolic protein cyclophilin A (CyPA); by this association, CyPA is incorporated into virions, where it is required for the subsequent round of viral replication (15, 31). The exact role of CyPA is unknown, but its incorporation into virions is necessary for a capsid (CA) function—perhaps virion uncoating—occurring after membrane fusion and before reverse transcription (3, 4).

As an initial step towards understanding the biochemical role of CyPA in HIV-1 replication, the genetic requirements for Gag's interaction with CyPA have been examined, both in vitro and in the context of infectious virus. CyPA-binding activity localizes to a proline-rich stretch of amino acids located in the middle of the CA domain of Gag (4, 8, 15, 24, 31). P222, a residue within this domain, and the preceding residue, G221, are critical for binding (4), a fact that is particularly intriguing considering CyPA's ability to catalyze the isomerization of peptidyl-prolyl bonds (13). Consistent with the genetic data is the recently solved three-dimensional structure of HIV-1 CA, in which the proline-rich domain forms an exposed loop which would be accessible for binding to CyPA (17).

Though a great deal has been learned about the CA residues necessary for binding to CyPA, nothing is known about the CyPA sequences required for binding to CA. Since members of the CyP family of proteins are intracellular targets of the clinically important immunosuppressive drug cyclosporine A (CsA) (18, 29), structural studies with these proteins have been extensive (10, 11, 20, 22, 23, 33). CyPs consist of an eight-strand beta-barrel with a hydrophobic pocket opening to one side of the hydrophobic core composed of residues from four of the eight beta-strands and a loop of aromatic residues; CsA

and proline-containing peptides contact residues within the hydrophobic pocket. Since CsA inhibits the Gag-CyPA interaction (15, 24, 31), Gag too may bind to the hydrophobic pocket of CyPA.

In addition to binding to CyPA, the Gag polyprotein of HIV-1 also binds to another member of the CyP family, CyPB (24), but there are important differences between CyPA and CyPB: CyPB binds to Gag polyproteins containing the CA mutation G221A or P222A, whereas CyPA does not (4, 15), and CyPB remains bound to the HIV-1 Gag polyprotein at a 10-fold greater concentration of CsA than is necessary to block the CyPA-CA interaction (24). In addition to these differences, CyPB, unlike CyPA, binds to the Gag polyprotein of simian immunodeficiency virus of macaque strain 239 (SIV_{MAC239}) (15). There are two possible explanations for these data. If Gag interacts with the hydrophobic pocket of CyP proteins, CyPB has different substrate-binding requirements than does CyPA. Alternatively, the difference in the observed binding properties may be due to the fact that CyPB possesses a 33-amino-acid hydrophobic leader peptide not found in CyPA (19); it is possible that the in vitro binding properties of CyPB are due to adventitious association by hydrophobic residues in this region.

To address the question of sequence requirements for CyP binding to Gag, we present in this paper data on the effects of specific mutations in the hydrophobic pocket of CyPA on binding to the HIV-1 Gag polyprotein. Since the mutated residues are conserved between the hydrophobic pockets of both CyPA and CyPB, any effects seen will address the general question of which amino acids are necessary for CyP binding to Gag. Additional data presented will address the binding properties of CyPB. We have engineered a CyPB protein which lacks the leader sequence to test if this truncated version of CyPB has binding properties similar to those of CyPA. Together, the data presented for both CyPA and CyPB provide a sketch of the primary sequence requirements for association of CyPs with the Gag polyprotein of HIV-1.

* Corresponding author. Mailing address: Department of Microbiology, College of Physicians & Surgeons, Columbia University, 701 W. 168th St., New York, NY 10032. Phone: (212) 305-8706. Fax: (212) 305-8706. E-mail: Luban@cuccfa.ccc.columbia.edu.

MATERIALS AND METHODS

Plasmids and mutagenesis. Cloned DNAs were propagated in *Escherichia coli* DH5 α by standard methods (28). *E. coli* BMH 71-18 *mutS* was used for site-directed mutagenesis.

Mutations were created by using the Transformer site-directed mutagenesis kit (Clontech) and a cDNA clone of human CyPA in pBS-KSII $^-$ (Stratagene). First, missense mutations that eliminated the two internal *Nco*I sites in the cDNA of CyPA, at nucleotides 310 and 439, were made to simplify subsequent cloning steps into bacterial and eukaryotic expression vectors. The *Nco*I site at nucleotide 310 was changed to a *Clai*I site with the oligonucleotide 5'-GCATTTGCC ATCGATAAGATGCCAG-3'; primer 5'-GCGCTCCATCGCTCCAC-3' was used to eliminate the other *Nco*I site at nucleotide 439. The resulting plasmid was used as a template to create the following missense mutations in the hydrophobic pocket of CyPA: 5'-CCCTGGAATGATTTCGCTGAAAGCAGG-3' for H54Q, 5'-CCCTGGAATAATCGCGTGAAGCAG-3' for R55A, 5'-CCTGACACATGGCCCTGGAATAATTC-3' for F60A, 5'-GGCAGTGCATATGGCAAAC TGGGAACC-3' for F113A, and 5'-CAAACACCACCTGCTTGCATC-3' for H126Q. The sequence of each mutant was confirmed by standard methods (28).

Primers were designed to amplify the region of CyPB containing both the sequences homologous to CyPA (the core domain) and the C-terminal domain unique to CyB: 5'-CGGCATGTGTCACCGTCAGGGTG-3' and 5'-GCGTAG TCTGGGACGTCGTATGG-3'. The PCR conditions were as follows. A 20- μ l reaction volume consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl $_2$, 0.001% (wt/vol) gelatin, 0.2 mM each deoxynucleoside triphosphate, 10 pmol of each oligonucleotide, 50 ng of a human cDNA clone of CyPB as the template, and 0.5 U of Ampli-Taq DNA polymerase. The sample was amplified for 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min in a Perkin Elmer 2400 machine. The 541-bp PCR product (Δ CyPB) was cloned into pBS-KSII $^-$ (Stratagene) by utilizing restriction sites created during the PCR. *Nco*I at the 5' end and *Eco*RI at the 3' end, and was subsequently sequenced.

Proteins containing the designed point mutations in the hydrophobic pocket of CyPA were expressed as glutathione *S*-transferase (GST) fusion proteins by cloning the CyP cDNAs into a version of the bacterial expression vector pGEX-2TK (Promega) modified to contain an *Nco*I site. The appropriate vector and insert fragments (*Nco*I-*Eco*RI fragments) were isolated and ligated in low-melting-point agarose (SeaPlaque LMA, from FMC). Likewise, to make the GST- Δ CyPB expression construct, the *Nco*I-*Eco*RI PCR fragment was cloned directly into modified pGEX-2TK.

Mutant CyPA proteins were expressed in mammalian cells as influenza virus hemagglutinin (HA) epitope fusions (12) by using the SR α promoter (30). SR α expression constructs of the CyPA point mutants and of Δ CyPB were cloned by digesting the pGEX-2TK constructs with *Bam*HI, filling in the ends with Klenow, and then digesting the constructs with *Eco*RI to obtain the CyP insert fragments. The insert fragments were ligated to a vector fragment from pIX156 (5), which was produced by digestion with *Xho*I, filling in of the ends with Klenow, and then digestion with *Eco*RI. All of the SR α constructs were cloned such that proteins expressed from them had the HA epitope fused in frame to the carboxy-terminal end; this enabled subsequent identification of the proteins with a monoclonal antibody to the HA epitope.

HIV-1 Gag numbering is with respect to the amino terminus of the Gag polyprotein. The HIV-1 Gag polyprotein bacterial expression plasmid pT7HG (pro $^-$) has been described previously (25), as have the Gag mutants P222A and G221A (4, 15), and the SIV_{MAC239} Pr55^{gag} expression construct (15). pSR α 55 is a Rev-independent expression construct for gag sequences (15).

In vitro binding experiments. In vitro binding experiments were performed as described previously (7). Briefly, equivalent amounts of GST fusion proteins, produced in *E. coli* DH5 α , were adsorbed to glutathione-agarose beads at 4°C, after which unbound protein was removed by washing with TK buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM CaCl $_2$, 2 mM MgCl $_2$, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5% glycerol). The loaded beads were then incubated with bacterially produced Gag lysates at 4°C for 30 min. After three subsequent washes of the beads in TK buffer, the beads were pelleted a final time and the bound protein complexes were dissolved in 2 \times sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl [pH 6.8], 1.7 M β -mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were either stained with Coomassie blue or processed for Western blotting as described below.

CsA was obtained from Sandoz Pharmaceuticals Corporation (East Hanover, N.J.) and dissolved in ethanol to make a 1.25 mM stock solution prior to addition to the binding reactions. In drug competition experiments, CsA was mixed with the recombinant proteins at the indicated concentrations.

Cell culture, transfections, and virion production and purification. Human fibroblast 293T cells were maintained in Dulbecco modified Eagle medium (DMEM)-F12 (1:1) supplemented with 10% fetal bovine serum. Proteins were expressed transiently by calcium phosphate transfection of 10 μ g of each expression construct with the Mammalian Cell Transfection Kit (Specialty Media, Lavellette, N.J.). Supernatants and cell lysates were harvested 48 h posttransfection. For virion preparation, 7.5 ml of supernatant collected from 293T cells were passed through a 0.45- μ m-pore-size filter to remove cellular debris. The filtrate was layered onto 2 ml of 25% sucrose in TNE (10 mM Tris-HCl [pH 7.5], 100

mM NaCl, 1 mM EDTA) and centrifuged at 80,000 \times *g* for 2 h in a Beckman SW41 rotor. The virion-containing pellet was resuspended in 30 μ l of 2 \times SDS sample buffer for Western blotting. For cell lysates, cells were first washed with 1 \times phosphate-buffered saline and then 1 ml of lysis buffer A (50 mM NaCl, 25 mM Tris-HCl [pH 8.0], 1% Triton X-100, 1% sodium deoxycholate, 10 μ g of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) was added and the cells were allowed to lyse for 5 min on ice. The lysate was scraped, transferred to Eppendorf tubes, and then spun at 14,000 \times *g* in an Eppendorf microcentrifuge for 20 min at 4°C. A 40- μ l volume of supernatant was mixed with an equal volume of 2 \times SDS sample buffer and then boiled for 5 min before SDS-PAGE loading.

Western blot analysis. After size separation by SDS-PAGE, proteins were transferred to either nitrocellulose membranes (Schleicher & Schuell) or polyvinylidene difluoride transfer membranes (PolyScreen; Du Pont/NEN) with a miniblotting apparatus (Bio-Rad, Hercules, Calif.). The following primary antibodies were used: a murine anti-HIV-1 p24 monoclonal antibody from Du Pont/NEN (NEA-9306); a murine anti-HIV-2 core protein monoclonal antibody from the National Institutes of Health AIDS Research and Reference Program (catalog no. 740); a rabbit anti-CyPA antibody which recognizes both wild-type CyPA and HA-tagged CyPA, from Affinity BioReagents, Heshanic Station, N.J. (catalog no. PA3-021); and monoclonal antibody 12CA5, which recognizes the HA epitope, purchased from Berkeley Antibody Company, Berkeley, Calif. Binding of these antibodies was detected after incubation with the appropriate peroxidase-coupled secondary antibody with the Renaissance chemiluminescence kit (Du Pont/NEN).

RESULTS

Mutations in the hydrophobic pocket of CyPA disrupt binding to the HIV-1 Gag polyprotein. Previous studies have identified the amino acids comprising the hydrophobic pocket of CyPA, where proline-containing peptides and CsA bind (10, 11, 20, 22, 23, 33). Additional biochemical studies using engineered hydrophobic pocket mutants demonstrated specific effects on peptidyl-prolyl isomerase activity and binding of CsA (34). Our aim was to duplicate these mutations and to determine if any of them affect the previously demonstrated property of CyPA to bind to HIV-1 Gag polyprotein Pr55^{gag} (24).

Mutations were engineered in the human CyPA cDNA and subcloned into a vector for expression as GST fusion proteins in bacteria (Fig. 1). Crude preparations of each of the GST-CyPA fusion proteins were incubated with bacterially produced HIV-1 protein Pr55^{gag} in the presence of glutathione-agarose beads; proteins retained by the beads were subjected to SDS-PAGE (Fig. 2). A Coomassie blue-stained gel showed that for all of the CyPA point mutants equal amounts of GST fusion proteins were retained by the glutathione-agarose beads (Fig. 2A), as demonstrated by the major high-molecular-weight band in lanes 2 to 7; the minor band in each lane is most likely a proteolytic cleavage product of the GST peptide from the fusion proteins. Pr55^{gag} bound to wild-type CyPA, (apparent from the higher-molecular-weight bands seen in lane 2) but binding to any of the mutant proteins was not detectable by Coomassie blue staining.

Western blotting was performed on the samples from the same binding experiment by using a monoclonal antibody to HIV-1 CA. In agreement with the results from the Coomassie blue-stained gel, Pr55^{gag} was retained by wild-type CyPA but not by the mutant CyPAs (Fig. 2B). The only exception revealed by the greater sensitivity of the Western blot was weak binding of Pr55^{gag} to F113A-CyPA, though by serially diluting the wild-type CyPA-Gag complexes (data not shown), we estimated that even this mutation reduced CyPA's affinity for Gag 10-fold. The results of the binding experiments with these CyPA mutants indicate that the Gag-binding site on CyPA is contained within the hydrophobic pocket.

Hydrophobic pocket mutations abolish CyPA packaging into virions. We next determined if the in vitro binding results could be extended to a more physiologically relevant condition. Previous analysis of total protein from purified HIV-1 virions demonstrated that CyPA is specifically packaged into virion

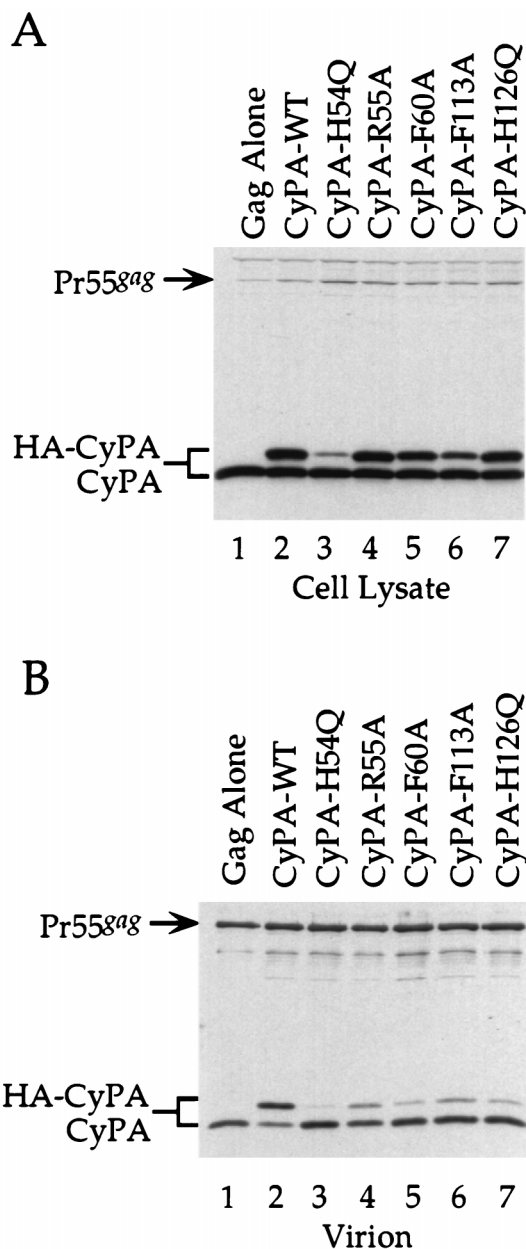


FIG. 3. Mutations within the hydrophobic pocket inhibit CyPA incorporation into virions. 293T cells were cotransfected with pSR α 55 and each of the indicated CyPA mutant expression constructs. Total cell lysates (A) and virion proteins purified from the supernatant (B) were subjected to SDS-PAGE and Western blot analysis with both anti-HIV-1 CA and anti-CyPA monoclonal antibodies. Lane 1 shows samples from cells transfected with pSR α 55 alone. The position of migration of the HIV-1 Gag polyprotein (Pr55^{gag}) is indicated on the left. The positions of migration of endogenous CyPA and lower-mobility HA-CyPA are indicated on the left. WT, wild type.

levels of the transfected mutant CyPAs expressed in cells, was then compared to the same value obtained for the transfected wild-type CyPA. Virion incorporation for each of the mutant CyPAs was 10 to 100 times less efficient than for the wild-type HA-CyPA (Fig. 3B, lanes 3 to 7). In agreement with the *in vitro* binding data, the magnitude of the reduction was the least with CyPA-F113A. The results from these experiments demonstrate that mutations within the hydrophobic pocket of CyPA affect its ability to be incorporated into virions. That

these mutations also abolish the CyPA-Gag interaction, as demonstrated by the *in vitro* binding experiments described above, is consistent with the model of CyPA's incorporation into virions via specific association with the Gag polyprotein.

Some mutant CyPA was detectable in the virion preparations. We believe that this was due to the imperfection of virion purification methods and not to Gag-independent mechanisms of incorporation. In fact, small quantities of CyPA pelleted through 35% sucrose cushions in the absence of *gag* expression (data not shown). This observation is in agreement with previous reports that multiply banded virion preparations may be contaminated with cellular debris (26).

Deletion of the N-terminal leader peptide of CyPB alters its binding properties *in vitro*. Previous *in vitro* binding experiments have demonstrated that CyPB binds to both HIV-1 Pr55^{gag} and the Gag polyprotein of SIV_{MAC239} Pr57^{gag}; these data contrast with those obtained with CyPA, which does not bind to SIV_{MAC239} Pr57^{gag} (8, 15, 24). We found this difference puzzling, considering the strong sequence homology between these two CyPs and, in particular, the conservation of the hydrophobic pocket residues (Fig. 1). Aside from scattered sequence differences found between the conserved core domains of the two proteins, CyPB is distinguished from CyPA by its 33-amino-acid leader peptide and a small carboxy-terminal peptide of 10 amino acids (19).

To determine if the leader peptide has an effect on the binding properties of CyPB, the core domain of 510 amino acids plus the C-terminal 10 amino acids was cloned into a bacterial expression vector to produce a GST fusion protein (GST- Δ CyPB). The binding properties of GST- Δ CyPB were tested with respect to both the HIV-1 and SIV_{MAC239} Gag polyproteins by mixing GST- Δ CyPB or GST-CyPA with either bacterially produced Pr55^{gag} of HIV or Pr57^{gag} of SIV_{MAC239} in the presence of glutathione-agarose beads; adsorbed protein complexes were purified and then subjected to SDS-PAGE (Fig. 4). In addition, GST-CyPB was mixed with Pr57^{gag} of SIV_{MAC239}. A Coomassie blue-stained SDS-polyacrylamide gel (Fig. 4A) showed that comparable amounts of HIV-1 Pr55^{gag} were retained by GST- Δ CyPB and GST-CyPA and that SIV_{MAC239} Pr57^{gag} bound to GST-CyPB but not to GST- Δ CyPB or to GST-CyPA (Fig. 4A, lanes 5 to 8).

Western blotting with a monoclonal antibody that recognizes both Pr55^{gag} of HIV-1 and Pr57^{gag} of SIV_{MAC239} was performed on the same complexes (Fig. 4B). The results parallel exactly those obtained by Coomassie blue staining. HIV-1 Gag bound to CyPA and Δ CyPB (Fig. 4B, lanes 3 and 4, respectively), but SIV_{MAC239} Gag bound only to full-length CyPB (Fig. 4B, lane 7). These results indicate that HIV-1 Gag interacts with the core of CyPB; SIV_{MAC239} Gag does not bind to the core of CyPB but to the hydrophobic leader peptide of this protein, calling into question the biological significance of this particular interaction.

It has been shown previously that HIV-1 Gag mutants G221A and P222A interfere with the CyPA-Gag association but have no effect on the CyPB-Gag association (4, 15). To further assess the binding characteristics of Δ CyPB, *in vitro* experiments were performed to compare the binding properties of Δ CyPB and CyPB with respect to these HIV-1 Gag mutants. GST- Δ CyPB or GST-CyPB was mixed with equivalent amounts of bacterially produced mutant Gag proteins. Protein complexes adsorbed to glutathione-agarose beads were purified and separated by SDS-PAGE. Coomassie blue staining demonstrated that equal amounts of GST- Δ CyPB and GST-CyPB bound to the beads (Fig. 5A). Western blotting on an identical gel, using the monoclonal antibody to HIV-1 CA, demonstrated that both mutant proteins bound to GST-CyPB

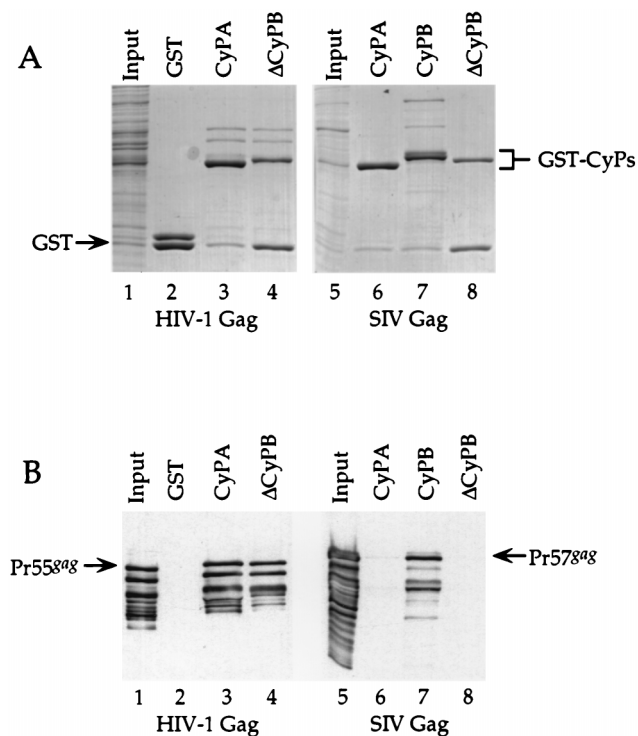


FIG. 4. Δ CyPB binds to HIV-1 Gag but not to SIV_{MAC239} Gag. Lysates of bacterially expressed HIV-1 Gag (lanes 1 to 4) or SIV_{MAC239} Gag (lanes 5 to 8) were mixed with lysates from bacteria expressing either GST or GST fused to the indicated CyPs. Complexes retained by glutathione-agarose beads were subjected to SDS-PAGE and then visualized by Coomassie blue staining (A) or processed for Western blot analysis by using a monoclonal antibody which recognizes the Gag polyproteins of both HIV-1 and SIV_{MAC239} (B). Lanes 1 and 5 show 10% of the Gag lysates used in the binding experiments. The positions of migration of GST and the HIV-1 Pr55^{gag} and SIV_{MAC239} Pr57^{gag} polyproteins are indicated on the sides. The positions of migration of the different GST-CyP fusion proteins are also indicated.

(Fig. 5B, lanes 2 and 4), which is consistent with what has been seen previously with CyPB (15). In contrast, neither Gag mutant bound to GST- Δ CyPB (Fig. 5B, lanes 1 and 3). These results again demonstrate the similarity of the binding properties of CyPA and Δ CyPB.

Δ CyPB/Gag and CyPA/Gag interactions are inhibited by the same concentration of CsA. The ability of CsA to interfere with the CyPA-Gag interaction is well documented (2, 4, 14, 15, 21, 24, 27, 31, 32). It has also been demonstrated that the CyPB-Gag interaction is much less sensitive to disruption by CsA than is the CyPA-Gag interaction (24). Figure 6 shows a Western blot analysis of protein complexes obtained from a binding experiment in which GST- Δ CyPB (Fig. 6A) and GST-CyPB (Fig. 6B) were compared with respect to binding to HIV-1 Gag in the presence of increasing concentrations of CsA. The results show that CyPB remained bound to HIV-1 Gag even at over 10 μ M CsA (Fig. 6A, lanes 1 to 6), which is consistent with what has been reported previously. In contrast, binding of Δ CyPB to Gag was blocked at CsA concentrations between 0.3 and 1.0 μ M (Fig. 6B, lane 3), the same range of concentrations at which the CyPA-Gag interaction is blocked under identical binding conditions (24). This indicates that the strength of the interaction between HIV-1 Gag and the core of either CyPA or CyPB cannot be distinguished in this assay.

Δ CyPB is incorporated into virions in a manner analogous to that of CyPA. Since CyPB is localized to the secretory pathway by its leader peptide, we expected that it would not be

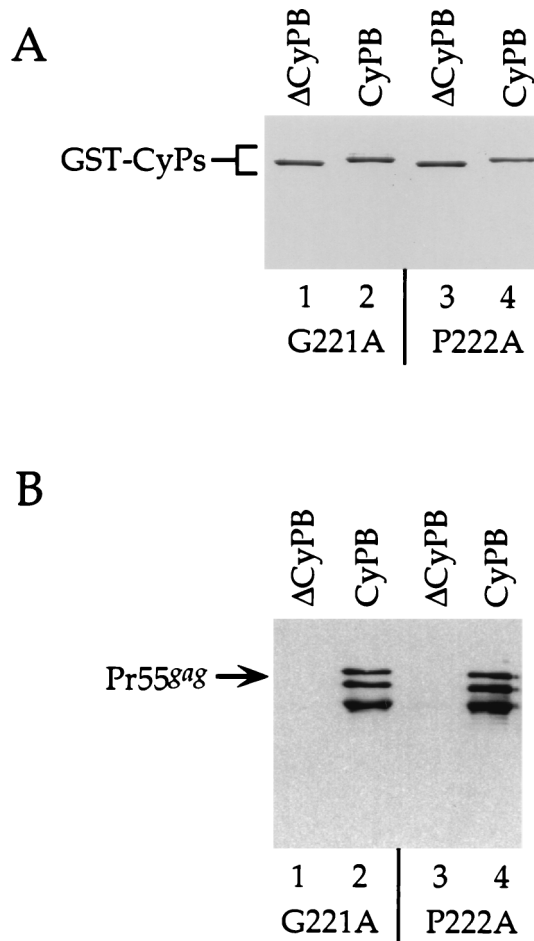


FIG. 5. Δ CyPB does not bind to HIV-1 Gag mutants. Lysate of bacteria expressing HIV-1 Gag mutant G221A (lanes 1 and 2) or P222A (lanes 3 and 4) was mixed with lysate from bacteria expressing GST fused to the indicated CyPs. Complexes retained by glutathione-agarose beads were subjected to SDS-PAGE and then visualized by Coomassie blue staining (A) or processed for Western blotting by using an anti-CA monoclonal antibody (B). G221A and P222A are mutations within the proline-rich region of Gag that block the binding of CyPA. The position of migration of the full-length HIV-1 Gag polyprotein (Pr55^{gag}) is indicated on the left. The positions of migration of the different GST-CyP fusion proteins are also indicated.

incorporated into virions despite its ability to bind to HIV-1 Gag *in vitro*. Since Δ CyPB lacks the leader peptide—and therefore would not be fated for localization to the secretory pathway—we determined if Δ CyPB itself contains sequences necessary and sufficient for incorporation into virions. pSR α Δ CyPB, pSR α CyPB, and pSR α CyPA were each cotransfected with pSR α 55 into 293T cells. Figure 7 shows Western blotting results obtained with cell lysates (Fig. 7A) and purified virion particles (Fig. 7B) produced from transfected cells; blots were probed by using both anti-HIV-1 CA and anti-HA monoclonal antibodies. In the lysates, each of the CyPs was expressed at about the same steady-state level (Fig. 7A). In the virion preparations, CyPA and Δ CyPB were detected (Fig. 7B, lanes 1 and 2) but CyPB was not (lane 3). Thus, like CyPA, Δ CyPB is specifically incorporated into virions. These results, together with those of the *in vitro* binding experiments, indicate that the sequences necessary for incorporation into HIV-1 virions are conserved between CyPA and CyPB but that CyPB

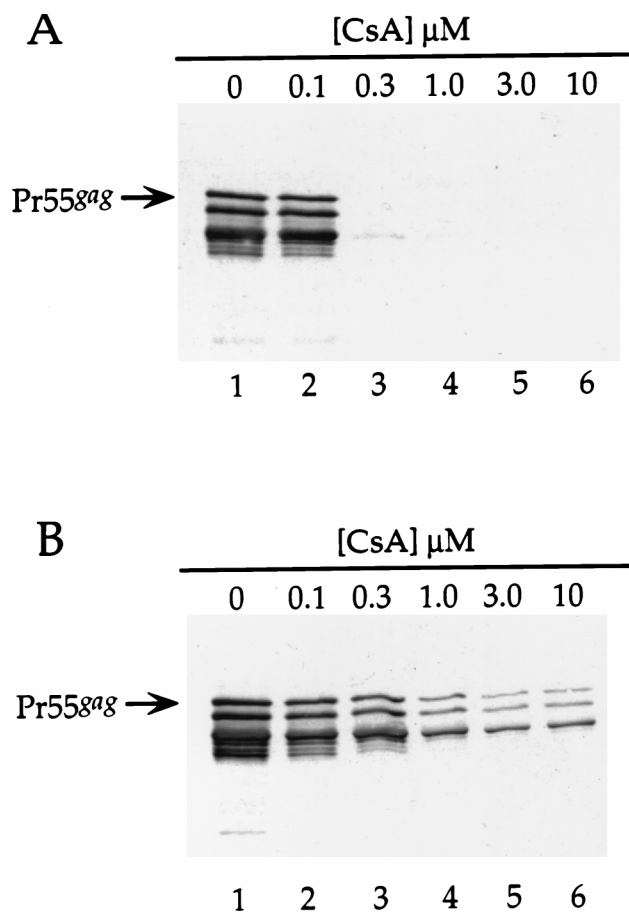


FIG. 6. Inhibition of Δ CyPB binding to HIV-1 Gag by CsA. Lysate of bacteria expressing HIV-1 Gag was mixed with lysates from bacteria expressing GST fused to Δ CyPB (A) or to CyPB (B) in the presence of the indicated concentrations of CsA. Complexes retained by glutathione-agarose beads were visualized by Western blotting with an anti-HIV-1 CA monoclonal antibody. The position of migration of the full-length HIV-1 Gag polyprotein (Pr55^{gag}) is indicated on the left.

is not normally incorporated into virions because it is sequestered in the cellular secretory apparatus.

DISCUSSION

The importance of understanding the primary sequence requirements of CyP binding to the Gag protein of HIV-1 is evident from the fact that HIV-1 virions depleted of CyPA are not infectious (4, 15, 31). The infectivity of these virions is blocked prior to reverse transcription but after membrane fusion (4). Genetic information about the Gag-CyPA interaction will undoubtedly prove critical for determining the exact biochemical role of CyPA in HIV-1 replication. For example, it has already been determined that HIV-1 dependence upon CyPA maps to residues in CA that are distinct from, though immediately adjacent to, residues required for CyPA binding (3). This information suggests that CyPA is required for HIV-1 uncoating, the process by which CA separates from the remainder of the viral nucleoprotein complex following entry into a new target cell (6, 9, 16). The identification of a specific Gag peptidyl-prolyl bond (G221-P222) that is required for CyPA binding suggests that CA undergoes *cis-trans* isomerization at this position as a result of the interaction; consistent with this possibility is the finding that of the 12 prolines ob-

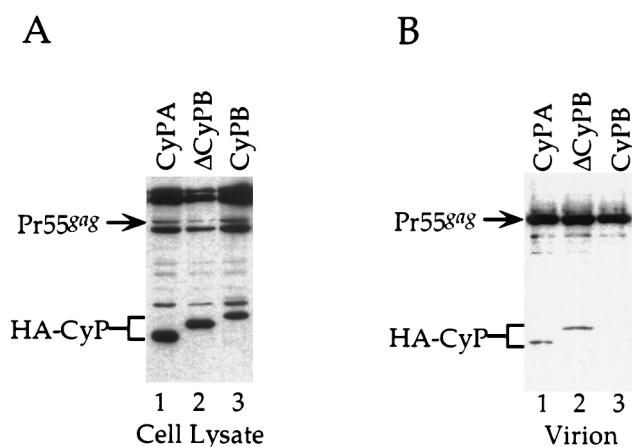


FIG. 7. Incorporation of Δ CyPB into HIV-1 virions. 293T cells were cotransfected with pSR α 55 and either pSR α CyPA, pSR α Δ CyPB, or pSR α CyPB. (A) Total cell lysate from each transfection was subjected to Western blotting with both anti-HIV-1 CA and anti-HA monoclonal antibodies. (B) Virions produced from the same transfections were subjected to Western blotting with the same two antibodies as in panel A. The positions of migration of the HIV-1 Gag polyprotein (Pr55^{gag}) and the different HA-CyPs are indicated.

served in the CA structure, only P222 exists in both *cis* and *trans* conformations (17). On the other hand, the stable interaction that exists between these two proteins suggests that, as has been proposed for *Drosophila* CyP NinaA (1), the primary function of CyPA in HIV-1 replication may be as a chaperone of CA uncoating, which simply exhibits peptidyl-prolyl isomerase activity as a result of its hydrophobic proline-binding site (29).

To extend the genetic analysis further, in this study we demonstrated that specific mutations within the hydrophobic pocket of CyPA disrupt binding to HIV-1 Gag. The CyPA mutations selected for study here have been characterized previously: structural studies have demonstrated that these residues form the hydrophobic pocket where proline-containing peptides and CsA bind (10, 11, 20, 22, 23, 33), and biochemical studies have confirmed that these residues are important for binding to CsA and for peptidyl-prolyl isomerase activity (34). Our results here, indicating that HIV-1 Gag binds to the hydrophobic pocket of CyPA, agree nicely with the previous demonstration that Gag residues G221 and P222 are required for the interaction (4, 15) and lend further support to the idea that CA is a substrate of the CyPA isomerase. The CyPA mutagenesis data presented here are also in agreement with the previous demonstration that CsA acts as a competitive inhibitor of the Gag-CyPA interaction (24), an activity due to CsA's ability to bind to the hydrophobic pocket of CyPA.

By using a truncated form of CyPB, we have shown that the leader peptide is responsible for the observed difference between the binding profiles of CyPB and CyPA with respect to Gag proteins encoded by different lentiviruses. Until now, it was possible to suggest that, because of its different binding property with respect to Gag of SIV_{MAC239}, CyPB's active site in fact had very different substrate requirements than that of CyPA. The data presented here discount this possibility. That Δ CyPB binds like CyPA to all of the substrate Gag proteins tested here strongly suggests that the same regions of Δ CyPB and CyPA are involved in the interaction; this is especially likely since the critical amino acids comprising the active site of CyPA are completely conserved in CyPB.

Together, the results presented above provide a profile of the primary sequence requirements for CyP binding to HIV-1

Gag: a region homologous to the hydrophobic pocket of CyPA is necessary and sufficient for the interaction; this is true whether or not this region is in the context of CyPA itself, as evidenced by the conservation of the core sequences in CyPB. Additional genetic and structural studies are needed to determine the precise biochemical requirements for the interaction between CyPs and HIV-1 Gag.

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REFERENCES

- Baker, E. K., N. J. Colley, and C. S. Zuker. 1994. The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *EMBO J.* **13**:4886–4895.
- Bartz, S. R., E. Hohenwarter, M.-K. Hu, D. H. Rich, and M. Malkovsky. 1995. Inhibition of human immunodeficiency virus replication by nonimmunosuppressive analogs of cyclosporin A. *Proc. Natl. Acad. Sci. USA* **92**:5381–5385.
- Braaten, D., C. Aberham, E. K. Franke, L. Yin, W. Phares, and J. Luban. 1996. Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. *J. Virol.* **70**:5170–5176.
- Braaten, D., E. K. Franke, and J. Luban. 1996. Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 prior to the initiation of reverse transcription. *J. Virol.* **70**:3551–3560.
- Bram, R. J., D. T. Hung, P. K. Martin, S. L. Schreiber, and G. R. Crabtree. 1993. Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. *Mol. Cell. Biol.* **13**:4760–4769.
- Bukrinsky, M. L., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson. 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl. Acad. Sci. USA* **90**:6125–6129.
- Colgan, J., H. E. H. Yuan, E. K. Franke, and J. Luban. 1996. Binding of the human immunodeficiency virus type 1 Gag polyprotein to cyclophilin A is mediated by the central region of capsid and requires Gag dimerization. *J. Virol.* **70**:4299–4310.
- Dorfman, T., and H. G. Göttinger. 1996. The human immunodeficiency virus type 1 capsid p2 domain confers sensitivity to the cyclophilin-binding drug SDZ NIM 811. *J. Virol.* **70**:5751–5757.
- Farnet, C. M., and W. A. Haseltine. 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.* **65**:1910–1915.
- Fesik, S. W., R. T. Gampe, H. L. Eaton, G. Gemmecker, E. T. Olejniczak, P. Neri, T. F. Holzman, D. A. Egan, R. Edalji, R. Simmer, R. Helfrich, J. Hochlowski, and M. Jackson. 1991. NMR studies of [¹³C] cyclosporin A bound to cyclophilin: bound conformation and portions of cyclosporin involved in binding. *Biochemistry* **30**:6574–6583.
- Fesik, S. W., R. T. Gampe, T. F. Holzman, D. A. Egan, R. Edalji, J. R. Luly, R. Simmer, R. Helfrich, V. Kishore, and D. H. Rich. 1990. Isotope-edited NMR of cyclosporin A bound to cyclophilin: evidence for a trans 9,10 amide bond. *Science* **250**:1406–1409.
- Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell. Biol.* **8**:2159–2165.
- Fischer, G., B. Wittmann-Liebold, K. Lang, T. Kiefhaber, and F. X. Schmid. 1989. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* **337**:476–478.
- Franke, E. K., and J. Luban. 1996. Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag-cyclophilin A interaction. *Virology* **222**:279–282.
- Franke, E. K., H. E. H. Yuan, and J. Luban. 1994. Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* **372**:359–362.
- Gallay, P., S. Swingle, C. Aiken, and D. Trono. 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell* **80**:379–388.
- Gitti, R. K., B. M. Lee, J. Walker, M. F. Summers, S. Yoo, and W. I. Sundquist. 1996. Structure of the amino-terminal core domain of the HIV-1 capsid protein. *Science* **273**:231–235.
- Handschumacher, R., M. Harding, J. Rice, and R. Drugge. 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**:544–547.
- Hasel, K. W., J. R. Glass, M. Godbout, and J. G. Sutcliffe. 1991. An endoplasmic reticulum-specific cyclophilin. *Mol. Cell. Biol.* **11**:3484–3491.
- Kallen, J., C. Spitzfaden, M. G. M. Zurini, G. Wider, H. Widmer, K. Wuthrich, and M. D. Walkinshaw. 1991. Structure of human cyclophilin and its binding site for cyclosporin A determined by X-ray crystallography and NMR spectroscopy. *Nature* **353**:276–279.
- Karpas, A., M. Lowdell, S. Jacobson, and F. Hill. 1992. Inhibition of human immunodeficiency virus and growth of infected T cells by the immunosuppressive drugs cyclosporin A and FK506. *Proc. Natl. Acad. Sci. USA* **89**:8351–8355.
- Ke, H. 1992. Similarities and differences between human cyclophilin A and other β -barrel structures. Structural refinements at 1.63 Å resolution. *J. Mol. Biol.* **228**:539–550.
- Ke, H., L. D. Zydowsky, J. Liu, and C. T. Walsh. 1991. Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution. *Proc. Natl. Acad. Sci. USA* **88**:9483–9487.
- Luban, J., K. A. Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff. 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73**:1067–1078.
- Luban, J., and S. Goff. 1991. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 Gag polyprotein. *J. Virol.* **65**:3203–3212.
- Ott, D. E., L. V. Coren, D. G. Johnson, R. C. Sowder, L. O. Arthur, and L. E. Henderson. 1995. Analysis and localization of cyclophilin A found in virions of human immunodeficiency virus type 1 MN strain. *AIDS Res. Hum. Retroviruses* **11**:1003–1006.
- Rosenwirth, B., A. Billich, R. Datema, P. Donatsch, F. Hammerschmid, R. Harrison, P. Hiestand, H. Jaksche, P. Mayer, P. Peichl, V. Quesniaux, F. Schatz, H.-J. Schuurman, R. Traber, R. Wenger, B. Wolf, G. Zenke, and M. Zurini. 1994. Inhibition of HIV-1 replication by SDZ NIM 811, a non-immunosuppressive cyclosporin A analogue. *Antimicrob. Agents Chemother.* **38**:1763–1772.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schreiber, S. L., and G. R. Crabtree. 1992. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13**:136–142.
- Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai. 1988. Sr alpha promoter: an efficient and versatile mammalian cDNA expression system. *Mol. Cell. Biol.* **8**:466–472.
- Thali, M., A. A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Göttinger. 1994. Functional association of cyclophilin A with HIV-1 virions. *Nature* **372**:363–365.
- Wainberg, M., A. Dascal, N. Blain, L. Fitz-Gibbon, F. Boulterice, K. Numazaki, and M. Tremblay. 1988. The effect of cyclosporine A on infection of susceptible cells by human immunodeficiency virus type 1. *Blood* **72**:1904–1910.
- Weber, C., G. Wider, B. von Freyberg, R. Traber, W. Braun, H. Widmer, and K. Wuthrich. 1991. The NMR structure of cyclosporin in aqueous solution. *Biochemistry* **30**:6563–6574.
- Zydowsky, L. D., F. A. Etzkorn, H. Y. Chang, S. B. Ferguson, L. A. Stolz, S. I. Ho, and C. T. Walsh. 1992. Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. *Protein Sci.* **1**:1092–1099.