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

JOHN COLGAN,¹ HANNAH EN HUI YUAN,² ETTALY KARA FRANKE,² AND JEREMY LUBAN^{1,2*}

*Departments of Microbiology¹ and Medicine,² College of Physicians and Surgeons of
Columbia University, New York, New York 10032*

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The cellular peptidyl-prolyl isomerase cyclophilin A (CyPA) is incorporated into human immunodeficiency virus type 1 (HIV-1) virions via direct contacts with the HIV-1 Gag polyprotein. Disruption of the Gag-CyPA interaction leads to the production of HIV-1 particles lacking CyPA; these virions are noninfectious, indicating that contacts between CyPA and Gag are necessary for HIV-1 replication. Here, we have used the yeast two-hybrid system in conjunction with an *in vitro* binding assay to identify the minimal domain of Gag required for binding to CyPA. Analysis of a panel of *gag* deletion mutants in the two-hybrid system indicated that a region spanning the central portion of the capsid (CA) domain was sufficient for interactions with CyPA, but discrepancies between results obtained in different fusion protein contexts suggested that multimerization of Gag might also be necessary for binding to CyPA. Consistent with a requirement for multimerization, the binding of Gag to CyPA *in vitro* required a region within the nucleocapsid (NC) domain shown previously to be important for Gag self-association. Substitution of a heterologous dimerization motif for the region from NC also promoted specific binding to CyPA, confirming that interactions with CyPA are dependent on Gag multimerization. Fusion of the heterologous dimerization motif to a 100-amino-acid domain from CA was sufficient for binding to CyPA *in vitro*. These results define the minimal CyPA-binding domain within Gag and provide insight into the mechanism by which CyPA is incorporated into HIV-1 virions.

The *gag* gene of human immunodeficiency virus type 1 (HIV-1) and other retroviruses encodes a polyprotein that carries out numerous functions during virion assembly and in the infection of a new host cell (for reviews, see references 33 and 67). A key step toward a more detailed understanding of the role that Gag plays in the retroviral life cycle is the identification of cellular proteins necessary for Gag function. However, aside from the observation that the association of Gag with the cell membrane depends on cotranslational modification by a cellular *N*-myristoyltransferase (8, 28, 53, 54), very little is known about the involvement of host cell proteins during phases of the viral life cycle requiring Gag function.

Using a yeast genetic screen designed to detect protein-protein interactions, it was found that a class of cellular proteins known as cyclophilins (CyPs) interact specifically with the HIV-1 Gag polyprotein (42). Originally identified as the cellular target for the immunosuppressive drug cyclosporin A (CsA) (29), the CyPs are a family of peptidyl-prolyl isomerases that are found in organisms ranging from bacteria to humans (65). Members of the CyP family are localized to various compartments within eukaryotic cells and are expressed in all tissues in metazoans (22, 59). Although their role within cells remains poorly understood, it is thought that the CyPs facilitate the correct folding and trafficking of proteins (16, 24, 59). This idea is supported by studies demonstrating that CyPs can increase the rate of protein folding *in vitro* by catalyzing the isomerization of peptidyl-prolyl bonds (17, 21, 45, 57) and by experiments showing that the presence of CsA, which inhibits peptidyl-prolyl isomerase activity, disrupts the folding of sev-

eral proteins *in vivo* (39, 60). In addition to accelerating the rate of protein folding, CyPs can also increase the yield of properly folded proteins *in vitro*, presumably by preventing the aggregation of folding intermediates that arise during renaturation (21). This finding suggests that CyPs can function as molecular chaperones within cells, an idea supported by studies showing that a CyP homolog is required for the transport of rhodopsin through the endoplasmic reticulum in *Drosophila melanogaster* (3, 11) and by experiments demonstrating that expression of CyP is heat inducible and facilitates the survival of yeast cells exposed to heat stress (62).

Studies aimed at determining the function of the Gag-CyP interaction have shown that CyPA, the major cytosolic form of the CyP family, is incorporated into HIV-1 virions via contacts with Gag (20, 63). Drugs or mutations in Gag that disrupt the Gag-CyPA interaction *in vitro* (20, 42) can block incorporation of CyPA into virions (20, 63). These same compounds or mutations also inhibit the replication of HIV-1 in tissue culture (4, 20, 35, 55, 63, 66), indicating that the Gag-CyPA interaction plays a critical role at some point in the HIV-1 life cycle. In contrast, other closely related retroviruses do not incorporate CyPA and are not inhibited by compounds which block the Gag-CyP interaction (6a, 20, 63). Although CyPA incorporation is not essential for HIV-1 virion assembly, the presence of CyPA within virions is required for some event following receptor binding but preceding reverse transcription (6, 63).

Determining the region of Gag that interacts with CyPA should lead to a better understanding of how CyPA is incorporated into virions and might also provide further insight into the function(s) of CyPA during the HIV-1 life cycle. Also, because HIV-1 Gag is one of only a few proteins known to interact with CyPA, characterization of the interaction between these two proteins might increase our understanding of the role of the CyPs within cells.

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

Here, we define the minimal region of Gag required for specific interactions with CyPA. We demonstrate that the CyPA-binding domain in Gag is a discrete domain spanning the central portion of the capsid (CA) domain and show that incorporation of CyPA into virions is dependent on homomeric interactions between molecules of Gag.

MATERIALS AND METHODS

Bacteria, yeast, and transformations. All plasmid DNAs were propagated in *Escherichia coli* DH5 α . Glutathione *S*-transferase (GST) fusion proteins and HIV-1 Gag protein derivatives were expressed in DH5 α . *E. coli* BMH 71-18 *mutS* was used for site-directed mutagenesis. *E. coli* MH-6 (*leuB600 pyrF::Tn5 lacX74 hsdR galU galK*), provided by Aaron Mitchell, was used to recover plasmids containing the *LEU2* gene from *Saccharomyces cerevisiae*. *S. cerevisiae* GGY1::171 (*MAT α leu2-3, 112 his3-200 met-tyr1 ura3-52 ade2 gal4Dgal80D URA3::GAL1-lacZ*) (26), carrying a *GAL1-lacZ* fusion gene integrated into the chromosome, was transformed by a modified lithium acetate method (25). Yeast transformants were screened for β -galactosidase (β -Gal) activity by replica plating colonies onto nitrocellulose filters (7); filters were frozen at -70°C for 20 min and then soaked in buffer containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) for up to 15 h at 30°C .

Cloned DNAs and plasmids. Nucleotide positions in *gag* are relative to the 5' edge of the 5' long terminal repeat in the HIV-1 proviral clone HXB2C. To create pBMT0, all polylinker sequences in pBSII-KS⁻ (Stratagene) except those extending from the *Bam*HI site through the *Sal*I site were removed by standard methods. This plasmid was digested with *Bam*HI and *Sal*I and ligated to a fragment containing HIV-1 *gag* sequences from pGAL4DB-HG (41), generating pBMT0. pBMT0-P1144 was constructed by digesting pBMT0 with *Nde*I and *Bgl*II and replacing the wild-type *gag* coding sequences with the corresponding *gag* sequences from pT7HG(pro+)-P1144 (44), which contains a 12-bp *Xho*I linker (5'-CTCGAGCTCGAG-3') inserted at nucleotide 1144.

Yeast expression plasmids encoding all *gag* 3' deletion (3' Δ) mutants except 3' Δ 1686 have been described elsewhere (19). Mutant 3' Δ 1686 was created by digesting pBMT0 with *Age*I and *Sal*I, treating the plasmid with Klenow enzyme, and then recircularizing it, which regenerates the *Sal*I site at the 3' end of the *gag* sequence. The *Bam*HI-*Sal*I fragment from pBMT0-3' Δ 1686 was then used to replace wild-type *gag* sequences in pGAL4DB-HG. The plasmid expressing mutant 3' Δ 1686 as a fusion with the GAL4 activation domain was created by inserting the *Bam*HI-*Sal*I fragment from pBMT0-3' Δ 1686 between the *Bam*HI and *Sal*I sites in pGADNOT (42). The 5' Δ 1320 mutation was obtained by screening libraries of random deletion mutants, using the two-hybrid system as described below. Plasmids encoding *gag* sequences from positions 1320 to 1686 fused in frame to the GAL4 DNA-binding or activation domain were constructed by replacing the *Spe*I-*Sal*I fragment in pGAL4DB-HG-5' Δ 1320 and pGAD-5' Δ 1320 with the 175-bp *Spe*I-*Sal*I fragment from BMT0-3' Δ 1686. pGAL4DB-CyPA was constructed by inserting the *Eco*RI fragment from clone 2-1, which encodes CyPA (42), into the *Eco*RI site of pMA424 (15). The resulting plasmid expresses CyPA as a fusion with the GAL4 DNA-binding domain.

pBS-HA was constructed by inserting a 45-bp synthetic oligonucleotide duplex (5'-CTAGTGCCACCATGGGTACCCATACGATGTTCAGATTACGCTG-3' hybridized to 5'-GATCCAGCGTAATCTGGAACATCGTATGGGTAACCATGGGTGGCA-3') encoding a start codon plus the HA1 epitope (YYP-DVDPYA) from the hemagglutinin (HA) protein of influenza virus (47) between the *Spe*I and *Bam*HI sites of pBSII-KS⁻. Sequences encoding the start codon and epitope tag were fused to *gag* sequences by ligating the 1.6-kb *Nde*I (Klenow enzyme treated)-*Sal*I fragment from pBMT0 to the 3.0-kb *Bam*HI (Klenow enzyme treated)-*Sal*I fragment from pBS-HA. The resulting plasmid, pBS-HA-Gag, was digested with *Nco*I (which cuts at the 5' end of the synthetic oligonucleotide duplex) and *Sal*I, producing a 1.6-kb fragment. This fragment was inserted between the *Nco*I and *Sal*I sites in the bacterial expression plasmid pSE420 (Invitrogen) to create pSE-HA-Gag. The epitope tag was fused to *gag* sequences beginning at nucleotide 1320 by ligating the 1-kb *Sma*I-*Sal*I fragment from pGAL4DB-HG-5' Δ 1320 to the 3.0-kb *Bam*HI (Klenow enzyme treated)-*Sal*I fragment from pBS-HA. The resulting plasmid was digested with *Nco*I and *Sal*I, producing a 1.0-kb fragment. This fragment was inserted between the *Nco*I and *Sal*I sites in pSE420, creating pSE-HA-Gag-5' Δ 1320. pSE-HA-Gag-5' Δ 1320-3' Δ 2093 was constructed using pBMT0-3' Δ 2093, which contains an *Xba*I linker (catalog no. 1062; New England Biolabs, Beverly, Mass.) encoding stop codons in all three frames inserted into the *Bgl*II site of pBMT0 as described previously (19). The 880-bp *Pst*I-*Sal*I fragment pBMT0-3' Δ 2093 was ligated to the 4.7-kb *Pst*I-*Sal*I fragment from pSE-HA-Gag-5' Δ 1320, creating pSE-HA-Gag-5' Δ 1320-3' Δ 2093. pSE-HA-Gag-5' Δ 1320-3' Δ 2007 was created by ligating the 880-bp *Pst*I-*Sal*I fragment from pBMT0-3' Δ 2007 (which contains an *Xba*I linker inserted into the *Apa*I site of Gag) to the 4.7-kb *Pst*I-*Sal*I fragment from pSE-HA-Gag-5' Δ 1320.

To create plasmids encoding regions of Gag fused to the GCN4 leucine zipper, a 78-bp *Xho*I-*Hind*III fragment from plasmid pMEXCRP2/GCN4LZ (kindly provided by P. Johnson) was inserted between the *Xho*I and *Hind*III sites of pSE420, generating pSE-GCN4LZ. To create pSE-HA-Gag-5' Δ 1320-3' Δ 1681-GCN4, the 3.4-kb *Xho*I (Klenow enzyme treated)-*Apa*I fragment from pSE-

GCN4LZ was ligated to the 1.4-kb *Acc*I (Klenow enzyme treated)-*Apa*I fragment from pSE-HA-Gag-5' Δ 1320. Deletion mutants ending at nucleotides 1654, 1619, 1584, and 1559 were generated by *Bal* 31 digestion (see below), and *Bam*HI-*Sal*I fragments containing these endpoints were inserted into pBSII-KS⁻, creating pBS-3' Δ 1654, -3' Δ 1619, -3' Δ 1584, and -3' Δ 1559. pSE-HA-Gag-5' Δ 1320-3' Δ 1654-GCN4 and pSE-HA-Gag-5' Δ 1320-3' Δ 1619-GCN4 were created by ligating the 245-bp *Sal*I (Klenow enzyme treated)-*Pst*I fragment from pBS-3' Δ 1654 or the 210-bp *Sal*I (Klenow enzyme treated)-*Pst*I fragment from pBS-3' Δ 1619 to the 1.3-kb *Ecl*136II-*Bgl*II fragment from pSE-GCN4LZ and the 3.4-kb *Pst*I-*Bgl*II fragment from pSE-HA-Gag-5' Δ 1320. pSE-HA-Gag-5' Δ 1320-3' Δ 1584-GCN4 was created by ligating the 175-bp *Sal*I (Klenow enzyme treated)-*Pst*I fragment from pBS-3' Δ 1584 to the 1.3-kb *Sac*I (Klenow enzyme treated)-*Bgl*II fragment from pSE-GCN4LZ and the 3.4-kb *Pst*I-*Bgl*II fragment from pSE-HA-Gag-5' Δ 1320. Similarly, pSE-HA-Gag-5' Δ 1320-3' Δ 1559-GCN4 was created by ligating the 151-bp *Sal*I (Klenow enzyme treated)-*Pst*I fragment from pBS-3' Δ 1559 to the 1.3-kb *Xho*I (Klenow enzyme treated)-*Bgl*II fragment from pSE-GCN4LZ and the 3.4-kb *Pst*I-*Bgl*II fragment from pSE-HA-Gag-5' Δ 1320.

The GST expression constructs pGST-CyPA and pGST-Gag have been described elsewhere (5, 42). pGST-Gag-GCN4 was created by ligating the 3.3-kb *Mlu*I-*Dra*I fragment from pGST-Gag to the 3.1-kb *Sac*I (Klenow enzyme treated)-*Mlu*I fragment from pSE-GCN4LZ.

Site-directed mutagenesis. Missense mutations were created by using a Trans-former site-directed mutagenesis kit (Clontech). The GCN4 leucine zipper mutant L17D was created by using the oligonucleotide 5'-CACTTGGAAATGAGGTGGCCAGAAAGAAAGAAATTAGTTGGCGAACGC-3'. The *gag* mutants P181A, P254A, P255A, and P257A were created using the oligonucleotides 5'-CATGGTGTAAATCCTGAGCGGTGGCTCCTTC-3', 5'-CCTACTGGGATCGGTGATCATTTATGTCATCC-3', 5'-CCTACTGGGATGGCCGGATTATTTGTCATCC-3', and 5'-CTCCTACTGCGATCGGTGGATTATTTG-3'. The *gag* mutants P217A, G221A, P222A, P225A, and P231A have been described elsewhere (6, 20). The identities of all point mutations were confirmed by dideoxynucleotide DNA sequence analysis.

Construction of *Bal* 31 deletion mutant libraries. To create 5' deletion (5' Δ) mutants, 10 μg of pBMT0-P1144 was linearized with *Xho*I, extracted with phenol-chloroform followed by chloroform, and then ethanol precipitated. The linear DNA was incubated with 10 U of *Bal* 31 exonuclease (New England Biolabs) in 600 mM NaCl-20 mM Tris-HCl (pH 8.0)-12 mM CaCl₂-12 mM MgCl₂-1 mM EDTA in a final volume of 100 μl . Aliquots (25 μl) were removed at the indicated time points, and the exonuclease was inactivated by the addition of EDTA to 0.67 mM. Each aliquot was extracted with phenol-chloroform followed by chloroform and then ethanol precipitated. The products from each time point were ligated to phosphorylated *Bam*HI linkers (catalog no. 1071; New England Biolabs) and then digested with *Bam*HI and *Sal*I. The resulting fragments were separated by agarose gel electrophoresis, and truncated *gag* fragments were purified and ligated to pGAD-M-PMV (19) digested with *Bam*HI and *Sal*I. This resulted in the substitution of HIV-1 *gag* fragments for the Mason-Pfizer monkey virus *gag* sequence in pGAD-M-PMV. Bacterial transformants arising from each ligation were pooled, and plasmid DNA from each pool was then used to transform yeast cells. Plasmids from selected positive clones were isolated as described previously (61) and recovered by transformation into bacteria. *Bam*HI-*Sal*I fragments from the recovered plasmids were inserted into pBSII-KS⁻ and subjected to dideoxynucleotide DNA sequence analysis using a synthetic oligonucleotide complementary to the T7 phage promoter present in pBSII-KS⁻.

In vitro binding experiments. Bacterial lysates containing recombinant proteins were prepared as described previously (42). Binding experiments were performed in a 200- μl reaction volume. Bacterial lysates were incubated together in binding buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5% glycerol) for 1 h at 4°C on a Nutator (Becton Dickinson, Parsippany, N.J.). Twenty microliters of a 50% (vol/vol) slurry of glutathione-agarose beads (Sigma), prepared as described previously (42), was added, and the mixture was incubated for 30 min at 4°C on a Nutator. Glutathione-agarose beads were pelleted by a 5-s spin in a microcentrifuge and then washed three times with 200 μl of binding buffer. Beads were resuspended in 35 μl of 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer (56), boiled for 5 min, and then pelleted. Aliquots (8 μl) of supernatant were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Gels were either stained with Coomassie blue or processed for Western blot (immunoblot) analysis.

Antibodies and Western blot analysis. A murine monoclonal antibody (12CA5 [14]) raised against the nine-amino-acid HA1 epitope from the influenza virus HA protein was purchased from Berkeley Antibody Company, Berkeley, Calif. A murine monoclonal antibody raised against p24 was purchased from Dupont. Western blot analysis was performed essentially as described previously (42).

RESULTS

Identification of the N-terminal boundary of the CyPA-binding domain within HIV-1 Gag. Previous analysis of a panel of selected *gag* deletion mutants indicated that the CyPA-binding domain was encoded by sequences downstream of position

FIG. 1. Strategy used to create libraries of deletion mutants. (A) A plasmid containing a cDNA encoding the protein of interest is linearized at a unique restriction site (RS). RE, restriction enzyme. (B) The linear DNA is digested with *Bal* 31 exonuclease for 5, 10, 20, or 30 min. (C) Following removal of the exonuclease, linkers encoding a restriction site are ligated to the digestion products. The resulting products are then incubated with two different restriction enzymes. One enzyme cuts within the added linker sequence, and the other cuts at the 3' end of the cDNA. (D) Fragments liberated by restriction enzyme digestion are isolated and inserted into a yeast expression vector so that the encoded protein is expressed as a fusion with the GAL4 activation domain. (E) Pools of expression plasmids from each time point are cotransformed into yeast cells with a plasmid expressing an interacting protein fused to the GAL4 DNA-binding domain, and the resulting transformants are assayed for β -Gal activity as described previously (42).

1184, the last nucleotide of matrix (MA) coding sequence (42). To determine the 5' boundary of sequences encoding the CyPA-binding domain more precisely, we used the yeast two-hybrid system (15) to screen libraries of randomly generated *gag* deletion mutants for clones encoding proteins retaining CyPA-binding activity. The strategy used to create the libraries is presented schematically in Fig. 1. To generate deletions, a Gag-encoding plasmid (pBMT0-P1144) was linearized at a restriction site 40 nucleotides upstream of the beginning of CA coding sequence and then exposed to *Bal* 31 exonuclease for 5, 10, 20, or 30 min. Restriction digest analysis confirmed that the average size of the deletions increased with longer exposure to *Bal* 31 (Table 1). Digestion products from each time point were then inserted into a yeast expression plasmid so that the encoded Gag proteins were expressed as fusions with the GAL4 activation domain.

Plasmid pools from each time point were cotransformed with a plasmid (pGAL4DB-CyPA) encoding the GAL4 DNA-

TABLE 1. Characterization of HIV-1 *gag* 5' Δ mutant libraries

Duration (min) of <i>Bal</i> 31 treatment	Avg no. of base pairs deleted ^a	% of clones retaining the ability to interact with CyPA in the two-hybrid system ^b
5	150	70
10	250	30
20	300	2.0
30	350	0.2

^a Estimated by restriction enzyme digestion of pooled plasmid DNA.

^b Determined by assaying colonies for β -Gal activity following transformation with the indicated *gag* deletion library plus an expression plasmid encoding CyPA fused to the GAL4 DNA-binding domain.

binding domain fused to CyPA. Plasmids were transformed into *S. cerevisiae* GGY1::171, which contains an integrated copy of the *lacZ* gene downstream of an upstream activation sequence recognized by the GAL4 DNA-binding domain. Several thousand transformants from each time point library were replica plated and assayed for β -Gal activity (Table 1). The majority of transformants (70%) from the 5-min library retained activity, indicating that most of the clones generated at this time point either escaped digestion by *Bal* 31 or retained sequences encoding the CyPA-binding domain. In contrast, a significantly lower percentage of transformants from the other libraries retained β -Gal activity, and the number of positives observed at each time point decreased in proportion to the length of exposure to *Bal* 31. For example, 30% of the clones in the 10-min library displayed β -Gal activity, while only 0.2% of the clones in the 30-min library retained activity. These results indicate that the majority of clones generated at the longer time points either lack insert, contain an insert that is not in the proper reading frame, or contain an insert that lacks sequences encoding some part of the CyPA-binding domain.

We next set out to identify clones that contained the largest deletions but which retained sequences encoding the CyPA-binding domain. A total of 4 blue colonies were observed in the 30-min library, and these plus an additional 32 blue colonies from the 20-min library were chosen for further analysis. Plasmid DNA from each colony was recovered, and the extent of deletion was assessed by restriction enzyme digestion. Twelve plasmids harboring the largest deletions were identified, and the encoded proteins were retested for the ability to interact with CyPA in the two-hybrid system. All 12 clones produced β -Gal activity when cotransformed with a plasmid expressing the GAL4 DNA-binding domain fused to CyPA but not with a plasmid expressing only the GAL4 DNA-binding domain, confirming that the *lacZ* expression observed was dependent on the presence of CyPA.

To determine the extent of deletion in each clone, all 12 plasmids were subjected to DNA sequence analysis (Table 2). This analysis showed that the largest deletion (5' Δ 1320) retained sequences starting at nucleotide 1320 and encoded a protein beginning with the glycine found at position 178 in Gag, which is equivalent to the residue 46 in CA. Subsequent analysis of plasmids recovered from colonies scoring negative for *lacZ* expression in the initial screen showed that plasmids containing deletions beyond position 1320 were present in the 30-min library but that the Gag proteins encoded by these plasmids were unable to interact with CyPA in the two-hybrid system (data not shown). These results suggest that deletions extending beyond nucleotide 1320 remove sequences encoding part of the CyPA-binding domain.

In the library screen, mutant 5' Δ 1320 was expressed as a fusion with the GAL4 activation domain. To confirm that this

FIG. 2. Interactions between CyPA and truncated Gag derivatives in the two-hybrid system. The full-length Gag polypeptide is depicted schematically at the top. Vertical bars represent cleavage sites recognized by the viral protease. The identities of the major cleavage products are indicated. The Gag derivatives tested for the ability to interact with CyPA are depicted schematically on the left; gray bars represent sequences retained by each mutant. The columns at the right show the β -Gal activity produced by yeast cells cotransformed with a plasmid expressing the indicated Gag deletion mutant plus a plasmid expressing the appropriate GAL4-CyPA fusion protein. β -Gal activity produced by the resulting transformants was scored as strong (+), trace (+/-), or absent (-). N.D., not determined. The first column shows the β -Gal activity observed when the Gag mutants were expressed as fusions with the GAL4 DNA-binding domain and tested for the ability to interact with CyPA fused to the GAL4 activation domain. The second column shows the β -Gal activity observed when the Gag mutants were expressed as fusions with the GAL4 activation domain and tested for the ability to interact with CyPA fused to the GAL4 DNA-binding domain.

Gag derivative could interact with CyPA regardless of fusion protein context, we constructed a plasmid expressing mutant 5'Δ1320 fused to the GAL4 DNA-binding domain and then tested this protein for the ability to interact with CyPA fused to the GAL4 activation domain (Fig. 2). Deletion mutants 5'Δ906 and 5'Δ1590 were also tested in both fusion protein contexts, providing a positive and a negative control, respectively, for CyPA-binding activity. These experiments showed that mutant 5'Δ1320 retained the ability to interact with CyPA when fused to the GAL4 DNA-binding domain. As expected from previous analysis (42), mutant 5'Δ906 interacted with CyPA in either fusion protein context, while 5'Δ1590 failed to show any CyPA-binding activity. Together, these results indicate that the glycine at position 178 in Gag represents the N-terminal boundary of the CyPA-binding domain.

Assessment of Gag C-terminal truncation mutants for CyPA-binding activity. Having identified the 5' boundary of sequences encoding the CyPA-binding domain, we next attempted to define the 3' boundary by using the two-hybrid system. As observed previously (42), when fused to the GAL4 DNA-binding domain, mutants 3'Δ2093, 3'Δ2007, 3'Δ1906, and 3'Δ1787 were able to interact with CyPA (Fig. 2). Likewise, mutant 3'Δ1686 retained the ability to interact with CyPA when fused to the GAL4 DNA-binding domain. As expected from previous analysis (42), mutant 3'Δ1509 failed to interact with CyPA when fused to the GAL4 DNA-binding domain. These results indicate that the region of Gag encoded by sequences downstream of nucleotide 1686 is dispensable for interactions with CyPA in the two-hybrid system.

We next tested whether a gag double mutant beginning at

TABLE 2. N-terminal amino acid sequence of truncated Gag derivatives retaining CyPA-binding activity

Construct ^a	No. of independent clones isolated	N-terminal amino acid sequence
5'-Δ1221	3	QAISPRTLNAWVKVVEEKAFSPEVIMFSALESEGATPQDLNTM...
5'-Δ1223	1	AISPRTLNAWVKVVEEKAFSPEVIMFSALESEGATPQDLNTM...
5'-Δ1250	2	WVKVVEEKAFSPEVIMFSALESEGATPQDLNTM...
5'-Δ1290	1	VIMFSALESEGATPQDLNTM...
5'-Δ1295	1	PMFSALESEGATPQDLNTM...
5'-Δ1320	4	GATPQDLNTM...
Wild-type Gag		PIVQNIQQQM [↑] VHQAISPRTLNAWVKVVEEKAFSPEVIMFSALESEGATPQDLNTM...

^a Nucleotide positions in 5'Δ mutants are relative to the 5' edge of the 5' long terminal repeat in the HIV proviral clone HXB2C.

nucleotide 1320 and ending at nucleotide 1686 encoded a protein that was capable of interacting with CyPA. Like mutants 5'Δ1320 and 3'Δ1686, double mutant 5'Δ1320-3'Δ1686 retained the ability to interact with CyPA when fused to the GAL4 DNA-binding domain (Fig. 2). This result suggests that the region of Gag encoded by nucleotides 1320 to 1686 is sufficient for interactions with CyPA in the two-hybrid system.

In contrast to the foregoing results, when the 3'Δ mutants and the double mutant were tested as fusions with the GAL4 activation domain, only mutant 3'Δ2093 fully retained the ability to interact with CyPA (Fig. 2). Under the same conditions, mutant 3'Δ2007 displayed only slight activity, while the remainder of the 3'Δ mutants or the double mutant had no detectable activity (Fig. 2). The lack of interaction observed with mutants 3'Δ2007, 3'Δ1906, and 3'Δ1787 was not due to protein instability, as previous experiments demonstrated that these proteins accumulate in yeast cells (19). Thus, Gag mutants which terminate prematurely upstream of nucleotide 2093 are not able to interact with CyPA when fused to the GAL4 activation domain.

One explanation for the disparate results obtained when the 3'Δ mutants and the double mutant were tested in different fusion protein contexts might be that Gag must dimerize in order to interact with CyPA and that the GAL4 DNA-binding domain, which binds to DNA as a dimer (9), substitutes for a dimerization function located at the C terminus of the full-length Gag protein that is absent from all the 3'Δ mutants except 3'Δ2093. Previous studies have indicated that nucleotides 2007 to 2093 encode a domain required for Gag-Gag interactions (19). Therefore, it is possible that mutant 3'Δ2093 (or full-length Gag) binds CyPA when fused to the GAL4 activation domain because it retains this region, while the remaining 3'Δ mutants fail to interact with CyPA because they lack the ability to dimerize. We describe below a series of experiments that address the importance of Gag multimerization for CyPA binding.

Multimerization of Gag is required for binding to CyPA in vitro. To examine the possibility that dimerization or multimerization of Gag is required for CyPA binding, we used a previously described protein binding assay (42) to test some of the Gag mutants described above not only for the ability to interact with CyPA but also for the ability to interact with Gag itself. In this assay, lysates from bacteria expressing mutant Gag proteins are incubated with bacterial lysates containing either GST, GST-CyPA, or GST-Gag. Following incubation, glutathione-agarose beads are added to recover the GST proteins. After extensive washing, any complexes between the Gag derivative and the added GST protein are eluted from the beads by boiling, and aliquots of the eluate are subjected to SDS-PAGE. A profile of the proteins eluted from the beads following a typical binding experiment is shown in Fig. 3A. The resolved proteins are then transferred to nitrocellulose, and any bound Gag protein is detected by Western blotting.

To make use of the binding assay, we created bacterial expression plasmids encoding mutants 5'Δ1320 and 5'Δ1320-3'Δ1686. To detect mutant 5'Δ1320 by Western blotting without detecting the GST-Gag fusion, the expression plasmid encoding 5'Δ1320 was constructed so that the nine-amino-acid HA epitope tag was fused to the N terminus of Gag sequences. An expression plasmid encoding the full-length Gag protein fused to the epitope tag (HA-Gag) was also created. Bacterial lysates containing these proteins were then prepared so that the Gag derivatives could be tested for the ability to interact with Gag or CyPA in vitro. As expected from previous studies (42), HA-Gag bound to GST-CyPA but not to GST (Fig. 3B). HA-Gag was also able to interact with GST-Gag (Fig. 3B),

indicating that full-length Gag is able to form homomultimers under the conditions tested.

Consistent with the results of the two-hybrid system, HA-Gag5'Δ1320 was able to bind to GST-CyPA in vitro and could also interact with GST-Gag (Fig. 3C). These results confirm that mutant 5'Δ1320 is capable of binding to CyPA and to full-length Gag. In contrast, mutant 5'Δ1320-3'Δ1686 failed to bind to either GST-CyPA or GST-Gag (Fig. 3D). These results suggest that the Gag domain encoded by nucleotides 1320 to 1686 is not sufficient for interactions with CyPA, perhaps because this fragment is unable to self-associate.

As described above, the region encoded by nucleotides 2007 to 2093 was essential for interactions with CyPA when the 3'Δ mutants were tested as GAL4 activation domain fusions in the two-hybrid system (Fig. 2). To determine whether this region was required for binding to either CyPA or Gag in vitro, we constructed bacterial expression plasmids containing *gag* nucleotides 1320 to 2093 or 1320 to 2007 fused in frame to sequences encoding the epitope tag. Nucleotide 1320, rather than nucleotide 789, was used as the start of Gag coding sequence in both plasmids because the results shown in Fig. 3C indicate that the region encoded by sequences upstream of nucleotide 1320 is not required for CyPA-binding activity. Each of the Gag mutants was expressed in bacteria and tested for the ability to interact with GST-CyPA or GST-Gag in vitro (Fig. 4). HA-Gag5'Δ1320-3'Δ2093 was able to interact with GST-CyPA and was also capable of binding to GST-Gag (Fig. 4A). In contrast, HA-Gag5'Δ1320-3'Δ2007 failed to interact with either GST-CyPA or GST-Gag (Fig. 4B). These results indicate that nucleotides 2007 to 2093 encode a domain capable of mediating Gag-Gag interactions in vitro, which is consistent with findings from a previous study (19). Furthermore, these results are consistent with the idea that multimerization of Gag is necessary for binding to CyPA.

Fusion of a heterologous dimerization motif to Gag sequences restores specific binding to CyPA in vitro. To test the hypothesis that Gag self-association is necessary for interactions with CyPA, we next determined whether fusion of a heterologous dimerization motif to a Gag mutant lacking detectable Gag-binding activity would promote interactions with CyPA in vitro. To do this, we created a bacterial expression plasmid containing *gag* nucleotides 1320 to 1686 fused in frame at the 3' end to sequences encoding a 24-amino-acid leucine zipper motif found in the yeast transcription factor GCN4. We chose to use the GCN4 leucine zipper because previous studies showed that this domain can form stable dimers in solution (48) and can mediate dimerization in the context of a chimeric protein (32). The expression plasmid also contained sequences encoding the epitope tag fused in frame to the 5' end of the *gag* sequences.

HA-Gag5'Δ1320-3'Δ1681-GCN4 protein was expressed in bacteria and then tested for the ability to interact with GST-CyPA, GST-Gag, or a GST-Gag-GCN4 leucine zipper fusion protein in vitro (Fig. 5). This latter GST derivative was used to test whether the leucine zipper could mediate dimer formation when fused to a region from Gag. In striking contrast to Gag5'Δ1320-3'Δ1686, HA-Gag5'Δ1320-3'Δ1681-GCN4 was able to interact specifically with GST-CyPA (Fig. 5B). As expected from the results shown in Fig. 3D, HA-Gag5'Δ1320-3'Δ1681-GCN4 failed to interact with GST-Gag. However, the HA-Gag-GCN4 chimera was able to bind to GST-Gag-GCN4, indicating that the fusion protein can dimerize as a result of the presence of the leucine zipper.

We next confirmed that the dimerization function provided by the leucine zipper was essential for binding to CyPA. Previous studies demonstrated that substitution of aspartate for

FIG. 3. The region of Gag encoded by nucleotides 1320 to 1686 is not sufficient for binding to CyPA *in vitro*. Bacterial lysates containing GST, GST-CyPA, or GST-Gag (depicted schematically in panel A) were incubated with lysates containing the indicated Gag derivatives (depicted schematically in the appropriate panel). GST proteins plus any associated Gag protein were then collected with glutathione-agarose beads. Following extensive washing, bound proteins were eluted and analyzed by SDS-PAGE followed by Western blotting. (A) Gel stained with Coomassie blue R-250 showing the GST proteins recovered from a typical binding reaction. (B to D) Western blots showing the amount of full-length HIV-1 HA-Gag, HA-Gag 5'Δ1320, or Gag 5'Δ1320-3'Δ1686 retained by each of the indicated GST proteins. Lanes marked INPUT show 10% of the total Gag protein included in each binding reaction. All binding reactions and Western blot analyses were performed as described in Materials and Methods. Western blots shown in panels B and C were probed with antibodies raised against the HA epitope; the Western blot shown in panel D was probed with antibodies raised against the HIV-1 CA protein, which recognize both the Gag 5'Δ1320-3'Δ1686 protein and the GST-Gag fusion protein.

the leucine at position 26 in the zipper prevents dimer formation (32). This single amino acid change was therefore introduced into the HA-Gag-GCN4 coding region, and the mutant protein was expressed and tested for the ability to bind to GST-CyPA, GST-Gag, or GST-Gag-GCN4 (Fig. 5C). Unlike HA-Gag5'Δ1320-3'Δ1681-GCN4, the HA-Gag-GCN4 leucine zipper mutant failed to bind to either GST-CyPA or GST-Gag-GCN4, indicating that dimerization as mediated by the leucine zipper was indeed required for binding to CyPA.

To confirm that the HA-Gag-GCN4 chimera bound to CyPA through the same contacts as full-length Gag, we used genetic and pharmacologic tools to characterize the binding properties of the fusion protein. Previous studies showed that the Gag-CyPA interaction is blocked by the immunosuppressive drug CsA (42). When increasing concentrations of CsA were added to binding reactions, interactions between HA-Gag5'Δ1320-3'Δ1681-GCN4 and GST-CyPA were strongly inhibited (Fig. 6A). As seen with full-length Gag (42), the slope

of the binding inhibition curve was very steep, with complete inhibition observed at 1 μM CsA. Addition of CsA had no effect on the binding of HA-Gag5'Δ1320-3'Δ1681-GCN4 to GST-Gag-GCN4 (Fig. 6A), indicating that the drug did not inhibit dimerization of the Gag-GCN4 fusion protein.

Previous analysis showed that substitution of an alanine for a single proline (amino acid 222) in full-length Gag prevents binding to CyPA (20). Sequences from positions 1320 to 1681 encompass the codon for Pro-222, and when the proline-to-alanine mutation (P222A) was introduced into the HA-Gag-GCN4 chimera, binding to GST-CyPA was disrupted (Fig. 6B). In contrast, substitution of an alanine in place of Pro-222 did not affect interactions with GST-Gag-GCN4, indicating that this mutation does not disrupt dimerization. Similar results were obtained when the glycine at position 221 was replaced with alanine (G221A) (Fig. 6C), a mutation that also prevents interactions between full-length Gag and CyPA (6). Together, these results demonstrate that dimerization of Gag is required

FIG. 4. CyPA-binding activity of Gag in vitro requires the ability to form Gag-Gag homomultimers. The HA-Gag derivatives tested are depicted schematically above the appropriate blot. Western blots show the amount of HA-Gag 5'Δ1320-3'Δ2093 (A) or HA-Gag 5'Δ1320-3'Δ2007 (B) retained by each of the indicated GST proteins. Binding reactions and Western blots were performed as described in the legend to Fig. 3. Western blots were probed with antibodies that recognize the HA epitope.

for interactions with CyPA and indicate that a homodimer consisting of the region encoded by nucleotides 1320 to 1681 can recognize CyPA in a manner identical to that of the full-length Gag polypeptide.

Definition of the minimal CyPA-binding domain within Gag. Having confirmed that the binding of Gag to CyPA requires dimerization, we set out to identify the 3' boundary of sequences encoding the CyPA-binding domain. To do this, we generated random 3' deletions by *Bal* 31 digestion starting at nucleotide 1686 and then inserted the digestion products into a yeast expression plasmid so that the encoded mutants could be screened for the ability to interact with CyPA when fused to the GAL4 DNA-binding domain. Upon transformation into yeast cells, however, most of the clones gave rise to *lacZ* expression in the absence of a plasmid encoding CyPA fused to the GAL4 activation domain, indicating that most of the deletion mutants encoded proteins that spontaneously activate transcription. Because of this problem, we were unable to use the two-hybrid system to define the 3' boundary of sequences encoding the CyPA-binding domain.

As an alternative approach, plasmid DNA was isolated from randomly chosen transformants and screened by restriction digest to identify clones harboring deletions extending past nucleotide 1686. Several plasmids encoding 3'Δ mutants, including mutants ending at positions 1654, 1619, 1584, and 1559, were obtained. To test these mutants for CyPA-binding activity, bacterial expression plasmids containing *gag* sequences starting at nucleotide 1320 and ending at these nucleotides were created. Each plasmid was constructed so that sequences encoding the epitope tag and the GCN4 leucine zipper were fused in frame to the 5' and 3' ends, respectively, of *gag* coding sequences.

Each of the fusion proteins was expressed in bacteria and tested for the ability to interact with GST-CyPA, GST-Gag, or GST-Gag-GCN4 in vitro (Fig. 7). This analysis showed that HA-Gag5'Δ1320-3'Δ1619-GCN4 was able to interact with both GST-CyPA and GST-Gag-GCN4 but not with GST-Gag (Fig. 7A). Similarly, the HA-Gag-GCN4 fusion protein terminating at nucleotide 1654 also retained CyPA-binding activity (data not shown). As observed with full-length Gag or the larger Gag-GCN4 fusion protein described above, the binding

of HA-Gag5'Δ1320-3'Δ1619-GCN4 to CyPA was disrupted by CsA or the P222A mutation (data not shown).

In contrast to these mutants, HA-Gag5'Δ1320-3'Δ1584-GCN4 failed to bind to GST-CyPA or GST-Gag but retained the ability to interact with GST-Gag-GCN4 (Fig. 7B). Likewise, the HA-Gag-GCN4 fusion protein terminating at nucleotide 1559 did not bind to CyPA (data not shown). These results show that the C-terminal boundary of the CyPA-binding domain is encoded by sequences between nucleotides 1584 and 1619 and indicate that the 100-amino-acid domain encoded by *gag* sequences from nucleotides 1320 to 1619 is sufficient for CyPA binding when dimerized.

Only one of eight prolines within the minimal CyPA-binding domain is essential for interactions with CyPA. Because CyPA is a peptidyl-prolyl isomerase, we next tested the importance of each of the eight prolines within the minimal CyPA-binding domain of Gag for binding to CyPA. Figure 8 depicts the location and amino acid sequence of the CyPA-binding domain within the Gag polyprotein. Also shown are individual amino acid changes that have been introduced into this region thus far and the effects of these mutations on the binding of full-length Gag to CyPA. As might be expected, the glycine and proline residues at positions 221 and 222, respectively, that we had previously shown to be critical for the Gag-CyPA interaction (6, 20) are located in the center of the 100-amino-acid sequence. In contrast to Pro-222, substitution of alanine for any of the other seven proline residues found within the minimal domain did not affect the Gag-CyPA interaction (reference 20 and data not shown). These results demonstrate that the only proline residue necessary for binding to the peptidyl-prolyl isomerase CyPA lies at position 222 in Gag.

DISCUSSION

Previous studies have demonstrated that the cellular peptidyl-prolyl isomerase CyPA is incorporated into HIV-1 particles via contacts with the Gag polyprotein and that the presence of CyPA within virions is necessary for infectivity (6, 20, 63). The experiments shown here demonstrate that a region of Gag spanning the central portion of the CA domain encodes all information necessary for specific interactions with CyPA and that only one of eight prolines within this region is recognized by the peptidyl-prolyl isomerase. In addition, the experiments presented here show that Gag must multimerize in order to interact with CyPA, indicating that CyPA is incorporated into HIV-1 virions during a phase of virion assembly in which Gag monomers are able to self-associate.

Our results indicate that a 100-amino-acid region located roughly in the center of CA encodes the CyPA-binding domain within HIV-1 Gag. This finding is consistent with our previous studies demonstrating that the HIV-1 CA protein alone can bind to CyPA in vitro and that linker insertion mutations within sequences encoding this region disrupt interactions between Gag and CyPA in the two-hybrid system (42). Our results are also in agreement with virological studies showing that missense mutations or short deletions within this region of the Gag polyprotein disrupt the incorporation of CyPA into virions (6, 20, 63). Moreover, our results showing that CsA or mutations at positions 221 and 222 in Gag inhibit interactions between the 100-amino-acid domain and CyPA in a manner identical to that seen with the full-length Gag strongly suggest that the minimal CyPA-binding domain recognizes CyPA through the same amino acid contacts made by full-length Gag.

In addition to CyPA, the HIV-1 Gag polyprotein mediates the incorporation of several other elements into virions, including viral genomic RNA (1, 5, 12, 27, 43), the Gag-Pol

FIG. 5. Fusion of a heterologous dimerization motif to Gag residues encoded by nucleotides 1320 to 1686 restores specific binding to CyPA. (A) The GST proteins used in the binding reactions are depicted schematically on the left. The gel at the right shows the GST proteins recovered from a typical binding reaction. Proteins were visualized by staining with Coomassie blue R-250. The GCN4 leucine zipper is a 24-amino-acid dimerization motif found naturally in the yeast transcription factor GCN4. (B and C) Western blots showing the amount of HA-Gag 5'Δ1320-3'Δ1681-GCN4 or HA-Gag 5'Δ1320-3'Δ1681-GCN4 leucine zipper motif (LZM) retained by each of the indicated GST proteins. The HA-Gag-GCN4 fusion proteins tested are depicted schematically above the appropriate blot. L→D represents the single amino acid change introduced into the leucine zipper that disrupts dimer formation. All binding reactions and Western blot analyses were performed as described in the legend to Fig. 3. Western blots were probed with antibodies that recognize the HA epitope.

precursor (49, 58), the viral envelope protein (13, 68), and the viral accessory protein Vpr (37, 38, 40, 50). Our results show that the CyPA-binding domain does not overlap any region in Gag that is known to interact with these elements and that this domain can recognize CyPA in the absence of these other regions. These observations, coupled with the previous demonstration that expression of Gag alone is sufficient for the production of virus-like particles containing high levels of CyPA (20), indicate that viral elements other than Gag are not necessary for specific incorporation of CyPA into virions. This conclusion is very similar to that drawn from studies concerning Vpr, which showed that the p6 domain of Gag is the only viral component required for incorporation of this protein into virions (37, 38, 40, 50).

Although the process of retroviral assembly has been studied intensively, relatively little is known about how the correct stoichiometry between virion components is established during particle formation. Our results showing that HIV-1 Gag must dimerize in order to interact with CyPA suggest that the molar ratio between these two proteins within virions is regulated at

least in part by the multimerization state of Gag within a host cell. The idea that Gag interacts with CyPA as a multimer is consistent with previous studies showing that the molar ratio between Gag and CyPA within virions is roughly 10 to 1 (20, 63). Although this proportion is significantly higher than the 2-to-1 ratio suggested by the results presented here, it seems plausible that at least some of the numerous intra- and intermolecular interactions that Gag mediates during the complex process of virion assembly are not reproduced in the systems that we have used to define the CyPA-binding domain and that these interactions play some role in regulating the stoichiometry between Gag and CyPA within virions.

While it is not known precisely where or when Gag begins to self-associate within the cell, it has been established that HIV-1 is a C-type virus, meaning that HIV-1 Gag multimerizes and forms detectable particle-like structures only upon association with the host cell membrane (23, 33). The fact that HIV-1 follows this type of morphogenic pathway, combined with the demonstration here that interactions with CyPA are dependent on Gag multimerization, suggests that incorporation of

FIG. 6. The Gag-GCN4 fusion protein binds CyPA through the same contacts as full-length Gag. (A) The Western blot at the left shows the amount of HA-Gag 5'Δ1320-3'Δ1681-GCN4 retained by GST-CyPA in the presence of the indicated concentration of CsA. The Western blot at the right shows the amount of HA-Gag 5'Δ1320-3'Δ1681-GCN4 retained by GST-Gag-GCN4 in the absence or presence of CsA (5 μM). (B and C) Western blots showing the amount of HA-Gag 5'Δ1320-3'Δ1681-GCN4 P222A or HA-Gag 5'Δ1320-3'Δ1681-GCN4 G221A retained by each of the indicated GST proteins. The HA-Gag-GCN4 fusion proteins tested are depicted schematically above the appropriate blot. The amino acid changes introduced are shown below the appropriate schematic. All experiments were performed as described in the legend to Fig. 3. Western blots were probed with antibodies that recognize the HA epitope.

CyPA into HIV-1 virions may be a relatively late event in particle formation. Although we cannot rule out that Gag interacts with CyPA before associating with the plasma membrane, previous studies have shown that mutations or drugs which disrupt the Gag-CyPA interaction have no gross effect on particle formation (6, 20, 63), which implies that the Gag-CyPA interaction does not facilitate the correct synthesis and/or transport of nascent Gag molecules within the cell. Likewise, addition of CyPA has no effect on the ability of Gag to self-associate in vitro (10), suggesting that Gag-CyPA interaction is not essential for some early event in virion assembly.

Because CyPA can increase the rate of protein folding and also prevent the aggregation of folding intermediates (17, 21, 45, 57), it seems likely that interactions with CyPA are required for the proper folding and transport of numerous cellular proteins. Although the results presented here define a natural CyPA-binding motif, a search of the GenBank database (National Center for Biotechnology Information) using the

BLAST algorithm (2) failed to identify proteins other than Gag that contain a region displaying any significant homology to the 100-amino-acid domain defined here. This suggests that interactions between CyPs and other proteins within the cell either involve other types of binding domains or occur independently of any obvious conserved structural motif. Support for this latter possibility comes from biochemical studies showing that the CyPA-catalyzed isomerization of peptidyl-prolyl bonds within model tetrapeptide substrates is not strongly influenced by the identity of the residue preceding proline (30, 31). In contrast to these findings, the stable interaction between HIV-1 Gag and CyPA is highly dependent on the glycine preceding the proline at position 222, which suggests that interactions between Gag and CyPA may not be representative of the types of contacts that occur between CyPs and other proteins within cells.

It remains unclear precisely what types of intermolecular contacts mediate the binding of Gag to CyPA. However, the

FIG. 7. Definition of the minimal CyPA-binding domain in HIV-1 Gag. The HA-Gag-GCN4 fusion proteins tested are depicted schematically above the appropriate blot. Western blots show the amount of HA-Gag 5'Δ1320-3'Δ1619-GCN4 (A) or HA-Gag 5'Δ1320-3'Δ1584-GCN4 (B) retained by each of the indicated GST proteins. Experiments were performed as described in the legend to Fig. 3. Western blots were probed with antibodies that recognize the HA epitope.

facts that the Gag-CyPA interaction is inhibited by CsA (42) and that the CA domain of Gag is specifically recognized by a monoclonal antibody raised against CsA (18) suggest that these two molecules resemble each other structurally and therefore may interact with CyPA through very similar contacts. Nuclear magnetic resonance studies have shown that CsA binds to the active site of CyPA by mimicking the structure of a proline-containing peptide and that the bond within CsA that corresponds to the peptidyl-prolyl bond is able to rotate freely, thereby facilitating a tight association between the two molecules (64). The formation of a similar complex between Gag and CyPA would explain the need for a glycine preceding the proline at position 222, since this amino acid would allow the peptidyl-prolyl bond more rotational freedom than would be seen with any other residue at this position (52).

It also remains unclear whether an isolated Gag dimer interacts with a multimeric form of CyPA or a single molecule of this protein. While some X-ray diffraction studies have suggested that CyPA does not self-associate in solution (36) and that CyPA can bind to CsA as a monomer (46), others have shown that CyPA can interact with itself when either CsA or a tetrapeptide substrate is bound within the isomerase active site

(34, 51). These findings raise the possibility that contacts between the CyPA-binding domain of Gag and the isomerase active site also induce dimerization of CyPA and allow for the formation of a symmetrical complex that contains two molecules of each protein. Although we have not ruled out this possibility, the fact that there is considerably less CyPA than Gag within virions seems to argue against this idea.

Alternatively, a single Gag dimer may interact with a monomeric form of CyPA, with each molecule of Gag recognizing a different region of the enzyme. Support for such an idea comes from other X-ray diffraction studies concerning the CyPA-CsA complex, which showed that CsA can interact with either the isomerase active site of CyPA or a region located on the other side of the protein (51). As suggested above, if the CyPA-binding domain of Gag forms a structure that closely resembles CsA, then one molecule within the Gag dimer may interact with this region while the other makes contacts with the active site of CyPA, resulting in the formation of an asymmetrical complex containing two molecules of Gag bound to a single molecule of CyPA. This hypothesis would explain why adding increasing concentrations of CsA to binding reactions containing Gag and CyPA produces a sigmoidal inhibition

FIG. 8. Amino acid sequence of the minimal CyPA-binding domain in Gag. The location of the minimal CyPA-binding domain within HIV-1 Gag is depicted schematically. The amino acid sequence of this domain is shown in the exploded view between the dashed lines. Individual amino acid changes introduced into the HIV-1 Gag polypeptide are denoted by the arrows below the sequence; + signifies that the indicated mutation has no detectable effect on the binding of full-length Gag to GST-CyPA in vitro, and - signifies that the indicated mutation abolishes the binding of full-length Gag to GST-CyPA in vitro.

curve (42), which indicates that the formation of the Gag-CyPA complex requires contacts with at least two sites on CyPA that are also recognized by Csa.

Aside from allowing the comparisons described above, the demonstration that a relatively short domain from Gag is capable of specifically recognizing CyPA when dimerized should make it possible to determine the structure of this region, either alone or complexed with CyPA, by nuclear magnetic resonance spectroscopy or X-ray diffraction. This analysis, in turn, should provide insight into the role that CyPA plays in the HIV-1 life cycle and might aid in the development of other compounds that block the Gag-CyPA interaction and inhibit viral replication.

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