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A Molecular Determinant of Human Immunodeficiency Virus Particle Assembly Located in Matrix Antigen p17

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We report single-point mutations that are located in the matrix protein domain of the *gag* gene of human immunodeficiency virus type 1 and that prevent Gag particle formation. We show that mutations of p17 that abolish human immunodeficiency virus particle assembly also prevent the dimerization of p17 protein, as measured directly by a protein-protein binding assay. In the three-dimensional structure of p17, mutations that abolish dimerization are located in a single alpha helix that forms part of a fingerlike projection from one side of the molecule. Peptides derived from this region of p17 also reduce the level of p17 dimer when they are added to p17-expressing cells and compete for p17 self-association when present in protein-protein binding assays. We propose that the dimerization of the Gag precursor that occurs by the interdigitation of alpha helices on adjacent matrix molecules is a key stage in virion assembly and that the prevention of such an interaction is the molecular basis of particle misassembly.

The *gag* gene of human immunodeficiency virus type 1 (HIV-1) encodes a single open reading frame of 55 kDa. The initial translated product, $Pr55^{Gag}$, assembles to form a near-spherical immature HIV particle which, upon cleavage by the virion-associated protease, undergoes morphological change to produce mature, infectious virus particles with a conical rather than spherical internal morphology (46). The $Pr55^{Gag}$ protein is made up of three distinct subdomains, the matrix domain (p17), the capsid domain (p24), and the nucleocapsid domain (p15), each of which is released from the precursor by the action of the virion protease (43). The change in the morphology of the virion core that occurs during maturation is thought to result from the realignment of released Gag domains within the virus particle (11, 46). In accordance with this hypothesis, $Pr55^{Gag}$ antigen expressed in the absence of the virion protease assembles to form HIV-like particles that are arrested in the immature, spherical form (5, 12, 20–23, 30, 35, 37–39). Sequences involved in the protein-protein interactions that lead to particle assembly have been mapped throughout the molecule. The capsid domain of Gag has been shown to have properties of self-assembly following expression and purification (7, 13, 31), and in addition, deletion mutations within the capsid domain in the context of $Pr55^{Gag}$ more often than not disrupt Gag particle formation (19, 27, 36, 42, 44). By contrast, the carboxy-terminal p15 domain appears to be largely dispensable for particle assembly (20, 22, 37), although specific signals for the release of virions from the surface of cells and some signals for self-assembly have been mapped to p6 (14) and p7 (1, 4, 45), respectively. Mutational analysis of the matrix domain has shown it to contain signals for nuclear localization (2), transport to the plasma membrane (8, 9, 48), and the coincorporation of envelope antigen (47) in addition to signals for particle assembly (9). Genetic evidence also suggests a role for the interaction of matrix domains in assembly (6, 27). To date, however, direct experimental evidence for p17 oligomerization in the assembly of the Gag particle has not been presented. Here we show that single-point mutations that abolish Gag particle assembly also prevent p17 dimerization, as measured directly by far-Western blotting. Dimerization is also prevented when peptides derived from the same region of p17 are added to cells expressing p17. We interpret these results and the mutations previously reported to prevent particle assembly (4, 9) in light of the structure of p17 (28, 29).

Results and Discussion. To assess the role of sequence changes in the p17 domain of $Pr55^{Gag}$ on particle assembly, four point mutations (Cys-57→Ser, Leu-64→Ala, Leu-78→Ala, and Cys-87→Ser) were introduced into the *gag* gene and expressed in insect cells with recombinant baculoviruses. When the wild-type *gag* gene is expressed in this system, Gag viruslike particles (VLP), which closely resemble the early budding form of HIV, assemble and bud from the cell plasma membrane. The ability of each mutant form of Gag to express VLP was monitored by Western blot analysis of the infected cell supernatant and electron microscopy of the infected cell surface. The single-amino-acid changes Cys-57→Ser and Leu-64→Ala led to the absence of antigen from the supernatant at 2 days postinfection (data not shown) and prevented VLP formation and instead produced an electron-dense layer below the plasma membrane (Fig. 1) typical of the VLP-negative phenotypes previously reported for Gag mutants (3, 22, 37, 38). The mutations Leu-78→Ala and Cys-87→Ser had no discernible effect on VLP formation, as revealed by Western blots of the antigen present in the supernatant or by electron microscopy (Fig. 1).

To assess the role of protein-protein interactions within the p17 domain in VLP assembly, each p17 mutation was recloned into the expression vector pGEX2T and expressed as a fusion protein with glutathione–*S*-transferase (GST) to allow single-step purification of the GST-matrix fusion protein (40). Following growth and induction, each mutant gave rise to a GST-p17 fusion protein of the predicted molecular mass (43 kDa) which could be either purified as the 43-kDa fusion protein by elution from immobilized glutathione or cleaved with throm-

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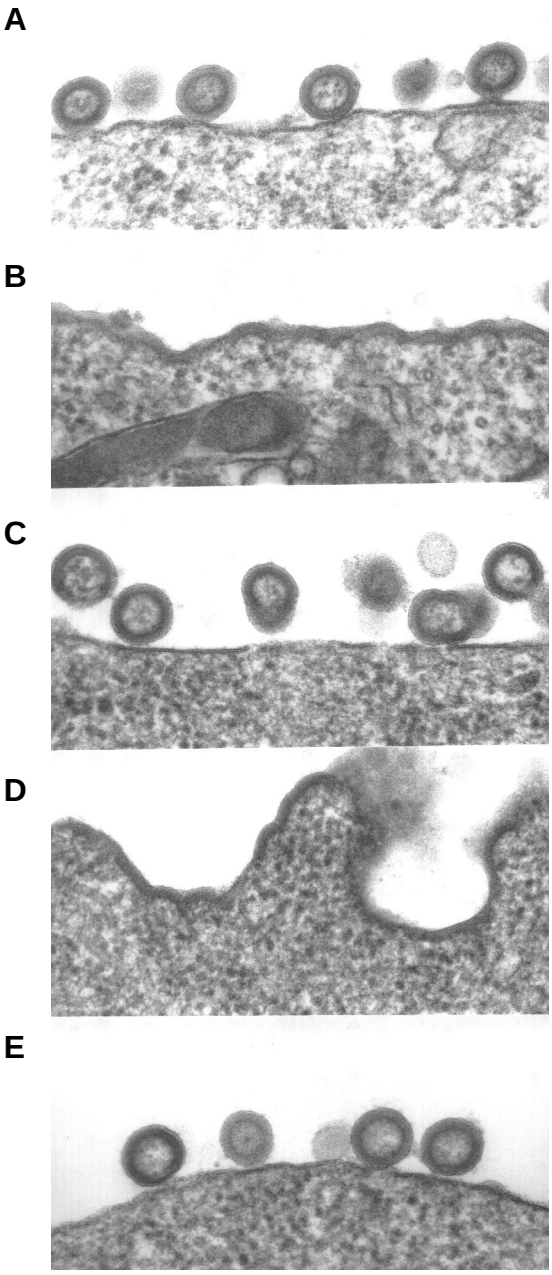

FIG. 1. Analysis of the effects of point mutations in the p17 domain on VLP formation. Site-directed mutagenesis of the *gag* gene and the generation of recombinant baculoviruses were done by standard procedures (24, 25). *Spodoptera frugiperda* (Sf9) cells were infected at a multiplicity of infection of 5 with recombinant baculoviruses expressing wild-type or mutant $Pr55^{Gag}$ and fixed at 2 days postinfection for conventional Araldite embedding and sectioning as described previously (16, 22). Sections were examined with a Philips CM12 electron microscope operating at 80 kV. The samples are wild-type $Pr55^{Gag}$ (A), Cys-57→Ser (B), Cys-87→Ser (C), Leu-64→Ala (D), and Leu-78→Ala (E). The electron-dense material under the plasma membrane in samples of Cys-87→Ser and Leu-78→Ala is morphologically similar to that produced by deletions in p24 that inactivate particle assembly in this system (49).

bin while bound to glutathione resin to release only the p17 domain (Fig. 2). With these molecules, far-Western blotting (15, 18) was used to assess the ability of each mutant p17 to bind to wild-type p17 and to itself in the form of a GST fusion protein. Cleaved mutant p17 antigens were resolved on polyacrylamide gels, transferred to polyvinylidene difluoride membranes for renaturation, and incubated with either wild-type GST-p17 or each mutant GST-p17. Protein-protein interactions were detected by probing the membrane with an anti-GST serum. Probing each cleaved antigen with wild-type GST-p17 showed that mutant p17 (Leu-78→Ala and Cys-87→Ser) bound GST-p17 as well as wild-type p17 (Fig. 3; compare lanes 3 and 5 with lane 1) but that neither the Cys-57→Ser mutant p17 nor the Leu-64→Ala mutant p17 bound GST-p17 (Fig. 3, lanes 2 and 4). Probing the blot with GST only gave no signal (Fig. 3). Similarly, when each cleaved mutant antigen was probed with itself in the form of a GST-tagged fusion protein, positive binding was observed with Leu-78→Ala and Cys-87→Ser while neither Cys-57→Ser nor Leu-64→Ala showed the ability to self-associate (Fig. 4). Thus, single-amino-acid substitutions that abolish VLP formation also prevent the self-

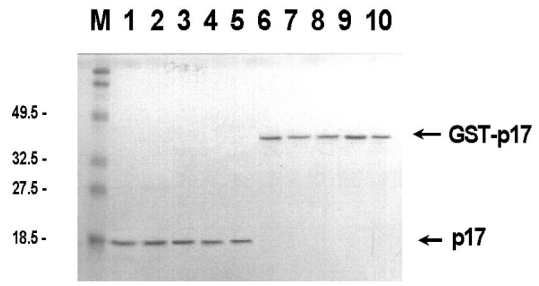

FIG. 2. Expression and purification of the GST-p17 proteins. The p17 coding region of wild-type Gag or each of the mutants described was recovered by PCR with oligonucleotides p17F (5′-CGCGGGATCCATGGGTGCGAGAGCGTCAGT-3′) and p17R (5′-CGCGGAATTCAGTAATTTTGGCTGACCTGACTG-3′). The amplified fragments were cleaved with *Bam*HI and *Eco*RI (underlined in the sequence) and cloned into the *Escherichia coli* expression vector pGEX2T (40). All mutants were expressed equally well and were soluble following sonication of induced cell extracts. GST fusion proteins were purified as described previously (40) or cleaved in situ by incubation of the glutathione bead-fusion protein mixture with thrombin as described elsewhere (10). Following cleavage, the supernatant, containing only p17, was desalted and concentrated with a Molcut II (Millipore) with a molecular mass cutoff of 5 kDa. Purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 14% polyacrylamide gels and stained with Coomassie blue. Lane 1, wild-type p17; lane 2, p17C57S; lane 3, p17C87S; lane 4, L64A; lane 5, L78A; lane 6, wild-type GST-p17; lane 7, GST-p17C57S; lane 8, GST-p17C87S; lane 9, GST-p17L64A; lane 10, GST-p17L78A. Molecular mass markers (lane M) are from Bio-Rad and are indicated on the left in kilodaltons. The positions of p17 and GST-p17 are indicated.

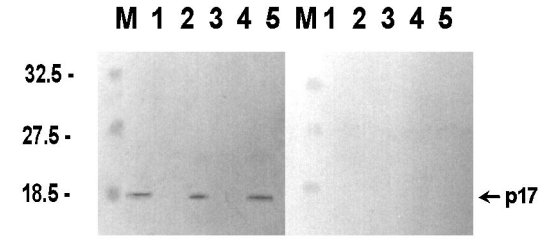

FIG. 3. Far-Western blotting of each p17 mutant for the ability to interact with the p17 wild type. Purified p17 antigen (5 μg) (as shown in Fig. 2, lanes 1 to 5) was resolved by SDS-PAGE on 14% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated overnight with 20 to 30 μg of purified GST-p17 (Fig. 2, lane 6) or GST only in a total volume of 3 ml under conditions described previously (18), except that the entire procedure was carried out at 4°C in a cold room. After the membrane was washed, bound GST fusion protein was detected with a guinea pig anti-GST serum and an alkaline phosphatase conjugate. Lane M, markers (the numbers on the left are kilodaltons); lane 1, wild-type p17; lane 2, p17C57S; lane 3, p17C87S; lane 4, p17L64A; lane 5, p17L78A. The lanes on the left were probed with GST-p17, while those on the right were probed with GST only. The position of p17 as identified by the stained gel is indicated.

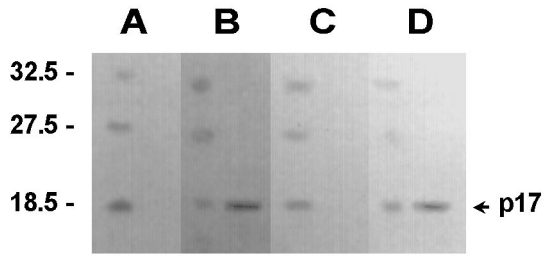

FIG. 4. Far-Western blots of mutant p17 proteins, showing their ability to self-interact with their GST counterparts. The procedure was the same as that described in the legend for Fig. 2, except that membranes for only two gel lanes were prepared for each mutant, one lane for markers and one for purified p17 mutant. Each membrane was incubated with the homologous p17 mutant in the form of its counterpart GST fusion protein before being processed as described previously. Markers in the left-hand lanes of each panel are indicated (in kilodaltons). Panel A, p17C57S with GST-p17C57S; panel B, p17C87S with GST-p17C87S; panel C, p17L64A with GST-p17L64A; panel D, p17L78A with GST-p17L78A. The position of p17 is indicated.

assembly of p17 antigen at least to the level of dimer, as measured by this direct protein-protein binding assay.

The recently determined three-dimensional structures of p17 (28, 29) show a molecule with four main helices, and the mutations Cys-57→Ser and Leu-64→Ala are located in helix B. Other p17 mutations noted to prevent HIV particle assembly also uniquely map to helix B (4, 9, 47), which has an amphipathic nature and is exposed to the solvent. On the assumption that the orientation of the p17 molecule suggested by the structure is correct, (29), which would be consistent with direct measurements of the width of the $Pr55^{Gag}$ monomer by high-resolution electron microscopy analysis (33), helix B forms the understrand of a fingerlike projection from the side of the molecule when viewed from the top (Fig. 5). As it has been suggested that Gag monomers pack by interaction at their sides (33), we suggest that helix B of p17 is involved in the protein-protein interactions that lead to VLP formation, which is consistent with the phenotypes that we and others have observed for mutations in this region of the molecule. A peptide spanning amino acids 47 to 59 of the p17 domain of HIV-1 Gag has been shown to reduce the level of infectious virus when added to HIV-1-infected cultures and, significantly, to give rise to HIV particles with an aberrant morphology (34). Residues 47 to 59 also derive, in part, from p17 helix B, and the model of Gag assembly suggested here might indicate that helix B-derived peptides competitively inhibit the dimerization of p17 by interaction with helix B. To examine this possibility, a recombinant baculovirus was constructed to express wild-

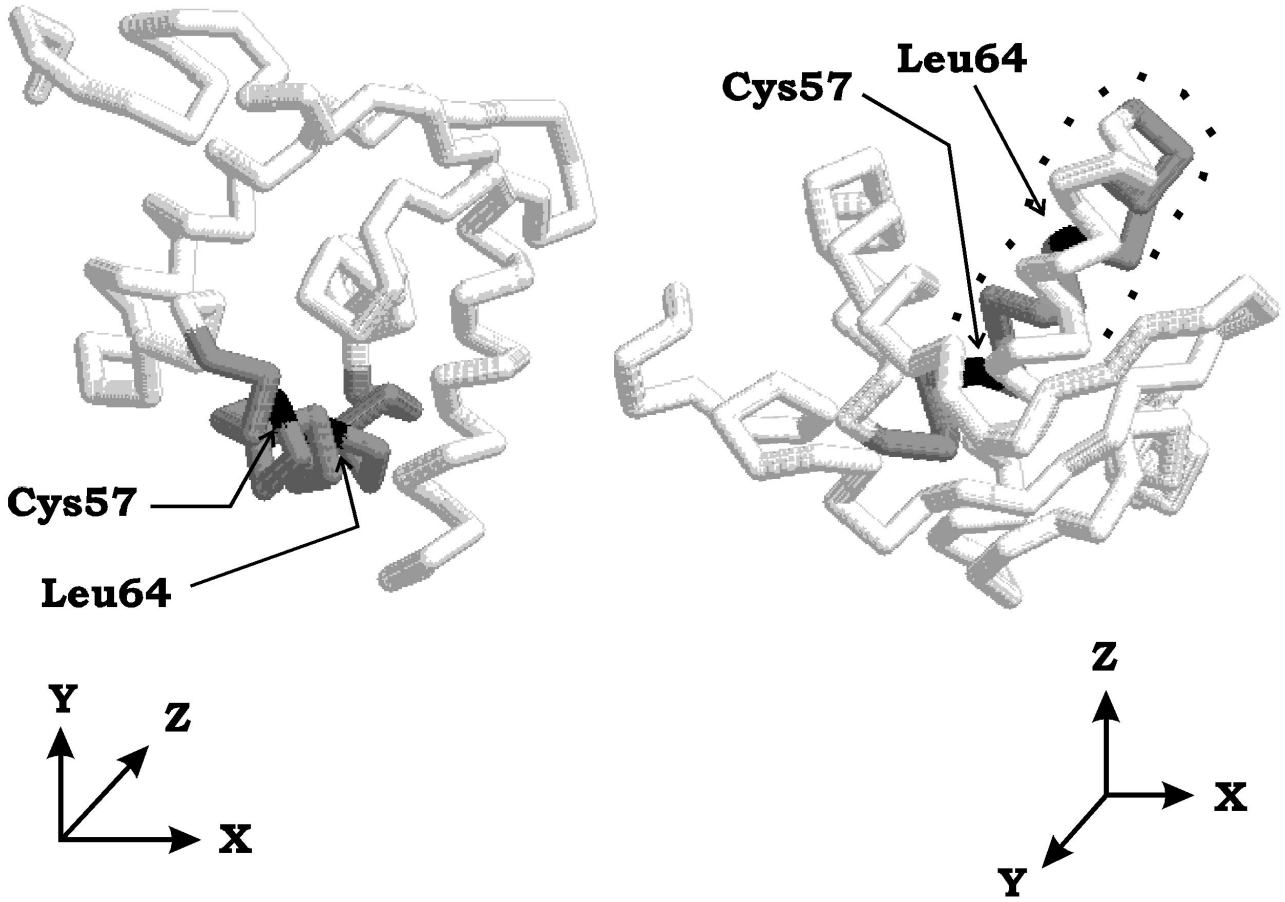

FIG. 5. Three-dimensional structure of the p17 domain of $Pr55^{Gag}$, after Matthews et al. (29). The structures were rendered from the p17 coordinates with the program RasMol (V2.5 [Roger Sayle]) running on a Silicon Graphics IRIS workstation. On the left is a view of the molecule from the side, with the amino terminus at the top left and the carboxy terminus at lower right. There are four main helices in the protein, and the side view has been centered on the long axis through helix C. Helix B (residues 53 to 68) is shaded, and the mutated residues discussed in this study are indicated. On the right is a rendering of the same molecule rotated 90 degrees out of the page and viewed from the top down the long axis through helix D. The locations of the mutations described in the text are shown. Helix B is shaded, and the fingerlike projection mentioned in the text is indicated by the dotted line.

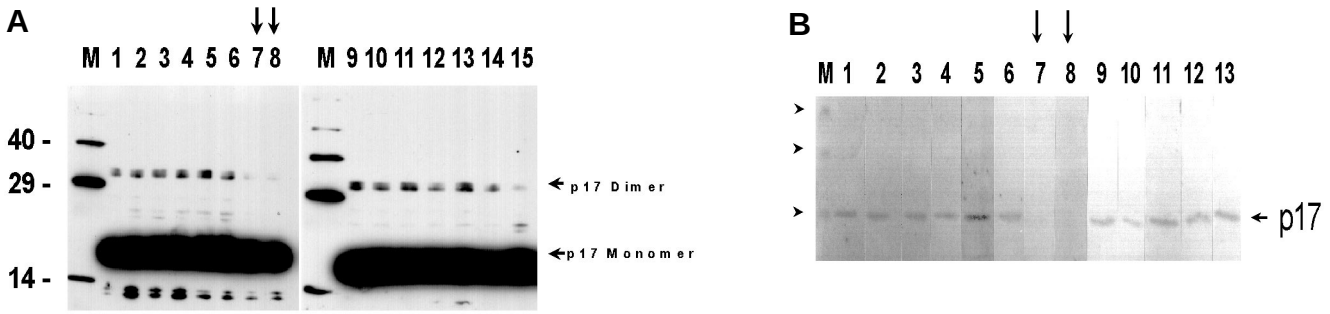

FIG. 6. Peptide inhibition of p17 dimer formation. (A) The p17 coding region was recloned from pGEX2T-p17 to the baculovirus transfer vector pAcCL29-1 (26) and used to generate a recombinant baculovirus expressing high levels of p17. An overlapping set of 13 peptides (15-mers overlapping by 5 residues) representing the entire coding region of p17 was obtained from the Medical Research Council AIDS reagent repository (17), and each peptide was dissolved in water at 1 mg/ml. *S. frugiperda* cells were infected with p17 recombinant baculovirus in media supplemented with each peptide at 200 μg/ml (34). At 2 days postinfection, total cell lysates were prepared by direct resuspension of the infected cell pellet in 3% SDS–5% β-mercaptoethanol–50 mM Tris HCl (pH 9). The lysates were vortexed exhaustively to reduce viscosity but were not heated before being resolved by SDS-PAGE on 14% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blotted with a p17 monoclonal antibody and an anti-mouse peroxidase conjugate. The blot was developed by BM chemiluminescence (Boehringer) and overexposed to reveal the trace amounts of p17 dimer discussed in the text. No higher-order multimers were seen, but at this level of exposure, some breakdown products of the p17 dimer are observed at molecular masses of around 20 kDa, and some breakdown of the p17 monomer is also apparent below 17 kDa. Peptide 7 (amino acids 61 to 75) and peptide 8 (amino acids 71 to 85) (indicated by arrows) specifically reduced the level of p17 dimer on the blots. Lanes 14 and 15 contain wild-type p17 with no peptide added and p17Cys-57→Ser, respectively. The level of dimer formed under these conditions by p17Cys-57→Ser is reduced in accordance with the far-Western data of Fig. 3. Prestained marker sizes are indicated on the left (in kilodaltons). (B) Peptide competition for p17 interaction in vitro. Far-Western blots of purified p17 antigen with GST-p17 in the presence of the overlapping set of p17 peptides are shown. The blots were prepared with purified cleaved p17 antigen as described in the legend to Fig. 3 and cut into strips. Each strip was incubated sequentially with one of the p17 peptides at 10 μg/ml followed by GST-p17 at 30 μg/ml. After that, the blots were washed and developed as described in the legend to Fig. 3. Binding of GST-p17 was reduced when peptides 7 and 8 were present in competition (indicated by arrows) but was not reduced in the presence of any other peptide tested. All the peptides were soluble at the concentrations used. The marker bars on the left of the figure indicate 32.5, 27.5, and 18.5 kDa.

type p17, and the oligomeric state of the antigen was assessed by Western blotting after expression in the presence of an overlapping set of 13 peptides spanning the entire p17 coding region. Expression of wild-type p17 gave rise to substantial levels of p17 monomer as a doublet, representing myristoylated and nonmyristoylated forms of the protein (data not shown). In addition, a low level of a 34-kDa reactive doublet was discernible on Western blots of infected cell extracts (Fig. 6). The level of the 34-kDa doublet varied with the treatment of the sample prior to electrophoresis. The doublet was not observed when samples were boiled in loading buffer for 15 min prior to application to the gel but was routinely detected if cell lysates were not heated prior to their application. Moreover, elution of the immune-reactive 34-kDa bands from polyvinylidene difluoride membranes followed by boiling of the sample in sample buffer and reelectrophoresis gave rise to the subsequent detection of p17 on Western blots. The monoclonal antibody used for the identification of p17 on far-Western blots showed no cross-reaction on uninfected or baculovirus-infected controls (data not shown), and together, these data are consistent with the notion of the 34-kDa doublet as the dimer of myristoylated and nonmyristoylated forms of expressed p17. No higher-molecular-weight bands were observed under these conditions. The p17 dimer was specifically reduced when p17 was expressed in the presence of helix B-derived peptides 7 and 8 (Fig. 6A). To confirm these results, peptides 1 to 13 were also used as agents for competition during far-Western assays of wild-type p17 interactions. GST-p17 binding to cleaved p17 (as in Fig. 3) was reduced in the presence of peptides 7 and 8 but not in the presence of any other peptide tested (Fig. 6B). Together, these data are consistent with the notion of a molecular contact between Gag monomers that involves helix B of the p17 domain and that can be disrupted by synthetic peptides competing for the site of interaction.

Two of the mutations described here (Cys-57→Ser and Cys-87→Ser) have also been reported in the context of an HIV-1 provirus (9). In those experiments, Cys-57→Ser caused the complete absence of virus replication despite normal levels of Gag Pr55 expression, which is consistent with a block in particle assembly, while Cys-87→Ser caused a transient reduction in virus replication that recovered after a lag of about 1 week. As Cys-87→Ser did not affect particle production in the recombinant baculovirus system used here, p17 Cys-87 is unlikely to be involved directly in Gag-Gag interactions but may be involved in other aspects of infectious particle production, such as cellular routing, protein processing, or the early events of infection. The clustering of Cys-57→Ser and Leu-64→Ala and, more generally, a range of particle-negative genotypes (amino acids 55 to 59 [9] and 41 to 68 [4]) on a single helix of the p17 structure is indicative of a discrete locus essential for Gag particle assembly. Interestingly, particle-negative mutations in the 41-to-68 region of p17 are strongly *trans*-dominant when coexpressed with wild-type p55 (4), suggesting a dead end for the assembly of Gag p17 mixed mutant–wild-type molecules.

The interdigitation of helices on adjacent molecules is a reoccurring theme in many patterns of virus architecture examined to date (41), and it is clear that other sequences in Gag, such as those mapped in the p24 domain (7) or nucleocapsid domain (1) in addition to those in p17 helix B, contribute to the complete Gag oligomerization process. The architecture of the HIV capsid has been suggested to be icosahedral, with Gag monomers forming hexomeric and pentameric ring structures that are the repeating units of the icosahedral shell (33). Conceivably, dimers of Gag maybe formed by the interlocking of p17 domains with the higher-order oligomerization effected by sequences in the carboxy-terminal domains of p24 or the nucleocapsid. Such an arrangement of Gag molecules would also allow for the rearrangement of Gag domains that occurs at virus maturation. At that point, upon cleavage by the virion-associated protease, the carboxy-terminal Gag domains p24 and p15 realign, but p17 remains in an icosahedral arrangement which is detectable in the mature virion (32).

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