JOURNAL OF VIROLOGY, Aug. 1996, p. 5170–5176 Vol. 70, No. 8
0022-538X/96/$04.00+0

Cyclosporine A-Resistant Human Immunodeficiency Virus Type 1 Mutants Demonstrate that Gag Encodes the Functional Target of Cyclophilin A

DOUGLAS BRAATEN,[1] CLAUDIA ABERHAM,[2] ETTALY KARA FRANKE,[3] LEI YIN,[1] WILLIAM PHARES,[2] AND JEREMY LUBAN[1,3]*

Departments of Microbiology[1] and Medicine,[3] Columbia University College of Physicians and Surgeons, New York, New York 10032, and Sandoz Forschungsinstitut GmbH, A-1235 Vienna, Austria[2]

Received 5 March 1996/Accepted 17 May 1996

The cellular peptidyl-prolyl isomerase cyclophilin A is incorporated into human immunodeficiency virus type 1 virions via contacts with the proline-rich domain of the Gag polyprotein. Cyclosporine A and nonimmunosuppressive analogs bind with high affinity to cyclophilin A, compete with Gag for binding to cyclophilin A, and prevent incorporation of cyclophilin A into virions; in parallel with the disruption of cyclophilin A incorporation into virions, there is a linear reduction in the initiation of reverse transcription after infection of a T cell. Passage of human immunodeficiency virus type 1 in the presence of the drug selects one of two mutations, either of which alters the proline-rich domain of Gag and is sufficient to confer drug resistance on the cloned wild-type provirus. Neither mutation alters Gag's cyclophilin A-binding properties in vitro, and cyclophilin A incorporation into drug-resistant virions is effectively disrupted by cyclosporine A, indicating that the drug-resistant mutants do not require virion-associated cyclophilin A to initiate infection. That Gag's functional dependence on cyclophilin A can be differentiated genetically from its ability to bind cyclophilin A is further demonstrated by the rescue of a mutation precluding cyclophilin A packaging by a mutation conferring cyclosporine A resistance. These experiments demonstrate that, in addition to its ability to package cyclophilin A into virions, *gag* encodes the functional target of cyclophilin A.

The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein contains information sufficient for the assembly and release of enveloped virions (19, 40). It incorporates several viral elements into nascent virions, including viral genomic RNA (22), the envelope glycoprotein (10, 41), and *pol*-encoded enzymes (27, 34). As virions are released from the cell surface, the Gag polyprotein is cleaved by the viral protease to produce the matrix protein (MA), which lines the virion envelope; the capsid protein (CA), which forms the core of the virion; and the nucleocapsid protein (NC), which coats the genomic RNA. Following membrane fusion with a $CD4^+$ cell, the nucleoprotein core of the virion is released into the cell cytoplasm. The HIV-1 preintegration complex contains MA but no detectable CA (7, 11), and HIV-1 MA possesses a nuclear localization signal which appears to target the preintegration complex to the nucleus via the nuclear pore (6, 15).

During virion assembly, the cellular protein cyclophilin A (CyPA) is incorporated into HIV-1 virions via contacts with the proline-rich domain of the Gag polyprotein (13, 26, 37). The cyclophilins are a large family of peptidyl-prolyl isomerases which are thought to regulate protein folding (16, 18, 35). CyPA was originally discovered as a result of its high affinity for the immunosuppressive drug cyclosporine A (CsA) (17). A requirement for CyPA in a Gag folding pathway or in the intracellular targeting of a *gag*-encoded protein is suggested by the fact that *gag* mutations and CsA disrupt Gag binding to CyPA in vitro (23), block CyPA incorporation into virions (13, 37), and inhibit viral replication (4, 5, 20, 28, 37, 38). Virions rendered CyPA deficient by production in the presence of CsA or by mutations which disrupt the proline-rich domain of Gag possess no identifiable biochemical or structural deficits, but the infectivity of these virions is severely attenuated, with the block occurring early in the HIV-1 life cycle, at a step following receptor binding and membrane fusion but preceding reverse transcription (5, 13, 37). CyPA present in the target cell cannot rescue the infectivity of CyPA-deficient virions (5, 37), indicating that CyPA is required either during assembly or, most likely, within the virion itself.

Although the HIV-1 Gag polyprotein binds to CyPA and incorporates the cellular protein into virions, the substrate of this peptidyl-prolyl isomerase has not been identified. The substrate might be the Gag polyprotein or CA, the Gag polyprotein cleavage product which retains CyPA-binding activity (23). Alternatively, the CyPA substrate might be another viral or cellular protein which is present within the virion.

Passage of cloned HIV-1 in the presence of a CsA analog selected for mutant virus which is drug resistant. This phenotype is conferred by either of two point mutations, each of which alters the coding sequence of the proline-rich domain of Gag. Further characterization of the mutant virions indicates that they are able to replicate in a CyPA-independent fashion. These results demonstrate that a function encoded by wild-type HIV-1 *gag* requires CyPA.

MATERIALS AND METHODS

Plasmid DNAs. Most of the plasmid DNAs used were propagated in *Escherichia coli* DH5α by standard methods (31). Proviral DNAs were propagated in JM109 clone 3226 (Life Technologies, Inc., Gaithersburg, Md.) at 30°C to prevent unwanted deletions. Supercoiled plasmids were purified with the Plasmid Maxi kit (Qiagen, Chatsworth, Calif.).

HIV-1 Gag polyprotein bacterial expression plasmid pT7HG(pro$^-$) has been described previously (24). Human CyPA was expressed in bacteria by using a previously described glutathione *S*-transferase (GST) fusion protein expression construct (23). pNL4-3 is a plasmid containing a complete infectious clone of

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

HIV-1 (2). The retrovirus nucleotide sequence is numbered with respect to the 5′ edge of the 5′ long terminal repeat of the DNA provirus. The Gag amino acid sequence is numbered with respect to the amino terminus of the Gag polyprotein.

Drug-resistant viral stocks, cloning, and mutagenesis. Complete details of the drug-resistant virus selection and genetic characterization procedures used are described elsewhere (1). Briefly, viral stocks produced by infectious proviral clone HIV-1_{NL4-3} (2) were used to infect $CD4^+$ HeLa cells (21) in the presence of 2.5 μM (Me-Ile-4) CsA (28). Culture supernatant was passaged once per week onto fresh, uninfected cells for 6 weeks, at the end of which four independent viral stocks with altered drug sensitivity were obtained. Proviral DNA fragments were amplified by PCR from cells infected with mutant viral stocks after end point dilution. The fragments obtained were used to replace corresponding sequence fragments in wild-type provirus HIV-1_{NL4-3} by standard methods (31). Fragments encoding the proline-rich domain of Gag were found to confer altered drug sensitivity on otherwise wild-type provirus. Dideoxy sequencing of fragments encoding the proline-rich domain of Gag demonstrated that all four of the drug-resistant stocks contained proviruses with either a C-to-A transversion at nucleotide 1460 (encoding mutant A224E) or a G-to-A transition at nucleotide 1466 (encoding mutant G226D); proviruses containing either of these two point mutations, but otherwise wild type in sequence, possessed the drug-dependent phenotype in $CD4^+$ HeLa cells or CEM cells (1). These same proviruses were used in the experiments described here. To test the effect of these mutations on Gag binding to CyPA in vitro, *Pst*I-*Spe*I fragments (nucleotide positions 1415 to 1507) from the drug-resistant proviruses were subcloned into pT7HG (pro^-) to create the A224E and G226D versions of this plasmid.

HIV-1 *gag* mutants were constructed by using a *Pst*I-*Spe*I fragment from pNL4-3 (nucleotide positions 1415 to 1507) subcloned in pBluescript II KS^- (Stratagene Cloning Systems, La Jolla, Calif.) as a template and the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.). Mutant P222A was constructed by using a mutagenic oligonucleotide encoding a *Ban*I site to facilitate monitoring of the mutation (5′-TGGTGCAATGGCACCTGCATGCAC-3′). Double mutant P222A/A224E was constructed by using a mutagenic oligonucleotide encoding *Hin*P1 I and *Mfe*I sites (5′-GCCTGGCTCAATTGCGCCT GCATGC-3′). The sequence was confirmed by dideoxy sequencing with T7 and M13 reverse primers. Mutant sequences were transferred into pNL4-3 for expression within the context of a provirus.

Cell culture and DNA transfection. Human fibroblast 293T cells were maintained in Dulbecco modified Eagle medium-F12 (1:1) supplemented with 10% fetal calf serum. The Jurkat human lymphocyte cell line (39) was obtained from the National Institutes of Health AIDS Research and Reference Program and maintained in RPMI 1640 supplemented with 10% fetal calf serum.

Viral proteins were expressed transiently by calcium phosphate transfection of 10 μg of supercoiled proviral DNA into 293T cells with the Mammalian Cell Transfection Kit (Specialty Media, Lavellette, N.J.). Viral infections were initiated by using 10^7 Jurkat T cells, 2 μg of proviral DNA, and 250 μg of DEAE-dextran (Pharmacia Biotech Inc., Piscataway, N.J.) per ml in 1 ml of serum-free RPMI for 20 min at room temperature. Cells were washed in serum-free medium, resuspended in 3 ml of conditioned medium with 6 ml of fresh medium, and split into 3-ml cultures with CsA added at the concentrations indicated below. Every 2 days, supernatant was harvested and frozen, and cells were passaged. At the conclusion of the experiment, the stored samples were analyzed for reverse transcriptase (RT) activity as described below.

Exogenous RT assay. Cell culture supernatant 10 μl was added to 50 μl of RT cocktail [60 mM Tris-HCl (pH 8.0), 180 mM KCl, 6 mM $MgCl_2$, 6 mM dithiothreitol, 0.6 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.12% Triton X-100, 6 μg of oligo(dT) per ml, 12 μg of poly(rA) per ml, 0.05 mM [α-^{32}P]dTTP (800 Ci/mmol)] for 1 h at 37°C. A 2-μl volume was spotted onto DE-81 paper and washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (31). A PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) was used to quantitate the radioactivity incorporated.

Virion purification. A 5-ml volume of supernatant collected from 293T cells 48 hr posttransfection with infectious proviral constructs (approximately 10^4 infectious U/ml) was centrifuged at 1,000 rpm for 5 min and passed through a 0.45-μm-pore-size filter to remove cellular debris. The filtrate was layered onto a step gradient consisting of 2 ml of 25% sucrose over 2 ml of 45% sucrose in TNE (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA) and centrifuged at 80,000 × *g* for 2 h in a Beckman SW41 rotor. The virion-containing interface was harvested and diluted to 6 ml with TNE, and virions were pelleted at 80,000 × *g* for 2 h in an SW41 rotor. The pellet was resuspended in 50 μl of 2 × sodium dodecyl sulfate (SDS) sample buffer (31) for Western blotting (immunoblotting).

Recombinant protein expressed in bacteria. The GST-CyPA fusion protein expression plasmid was grown in *E. coli* DH5α. pT7HG-(pro^-), expressing native $Pr55^{gag}$ from the T7 promoter, was grown in JB-DE3, a *lon* mutant strain containing the T7 polymerase gene under the control of the *lac* mutant UV5 promoter (24). Protein was induced from bacterial expression plasmids with isopropyl-β-D-thiogalactopyranoside (IPTG) by standard methods (31). Three hours after induction, bacteria were pelleted, washed in TEK buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM KCl), and resuspended in lysis buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40). The suspended bacteria were frozen and thawed six times and sonicated on ice for 30 s with a Branson Sonifier 250 (Branson Sonic Power Company, Danbury, Conn.) at an output setting of 1 with a 30% duty cycle. Insoluble material was pelleted at 90,000 rpm for 10 min in a Beckman TL 100 ultracentrifuge. Supernatants were adjusted to 20% glycerol and stored at −70°C until use in binding assays.

In vitro binding of recombinant $Pr55^{gag}$ to GST-CyPA. Glutathione-agarose beads (Sigma Chemical Co., St. Louis, Mo.) were swollen overnight at 4°C in TEK buffer, washed three times in TEK buffer with 0.5% powdered milk, and stored at 4°C in binding 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). Typical binding reactions used crude bacterial lysates in a total volume of 200 μl of binding buffer containing approximately 2 μg of $Pr55^{gag}$ (approximately 0.2 μM) and 5 μg of GST-CyPA (approximately 0.5 μM). After incubation at 4°C on a Nutator (Becton Dickinson & Co., Parsippany, N.J.), 25 μl of 50% (vol/vol) glutathione-agarose beads in binding buffer was added and incubation was continued for another 30 min. The glutathione-agarose beads were collected with a 5-s pulse in a microcentrifuge and washed three times with 400 μl of binding buffer. Washed glutathione-agarose beads were resuspended in 25 μl of 2× SDS sample buffer (31), heated in boiling water for 5 min, and pelleted in a microcentrifuge. A 5-μl volume of the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Gels were stained with Coomassie blue to monitor recovery of the GST fusion proteins (data not shown) or processed for Western blot analysis as described below.

Antibodies and Western blot analysis. Proteins complexed with GST fusion proteins, or virion-associated proteins, were size separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes with the Bio-Rad mini-blotting apparatus (Bio-Rad, Hercules, Calif.). Murine monoclonal anti-HIV-1 p24 was obtained from Du Pont NEN (NEA-9306) and used as the primary antibody in all immunoblots. Rabbit anti-CyPA antibody, a gift from Louis Henderson (National Cancer Institute, Frederick, Md.), was used in combination with the anti-p24 antibody in immunoblots of virion-associated proteins. Binding of primary antibodies was detected with the appropriate peroxidase-coupled secondary antibody and the Renaissance chemiluminescence kit (Du Pont).

CsA. CsA was obtained from Sandoz Pharmaceuticals Corporation (East Hanover, N.J.). Prior to addition to tissue culture medium, or to the in vitro binding reactions, the drug powder was dissolved in ethanol to make stock solutions of less than 2 mM. All samples within a given experiment in which CsA was used received the same volume of ethanol, including the no-drug controls. In drug competition experiments, the GST-CyPA protein was incubated for 5 min with CsA in binding buffer prior to addition of the bacterial lysate containing $Pr55^{gag}$.

Analysis of virion-associated RNA. To check for the retention of engineered mutations in replicating virus, virion RNA was reverse transcribed and PCR amplified with *gag* primers and the products were digested with restriction enzymes diagnostic for the engineered mutations. To prepare virion RNA, cell culture supernatant from the peak of mutant virion production was normalized to the wild-type peak for RT. A 50-μl volume of each supernatant was then mixed with 500 μl of RNAzol B (Tel-Test, Inc., Friendswood, Tex.) and processed in accordance with the manufacturer's instructions. tRNA (60 μg) was added as a carrier prior to precipitation, and the RNA pellet was resuspended in 30 μl of water.

For cDNA synthesis, the virion RNA in 1 μl of each sample was denatured by heating in 10 μl of water and annealed to 25 pmol of an oligonucleotide primer complementary to nucleotides 1621 to 1647 of pNL4-3 (5′-GTCTTATGTCCA GAATGCTGGTAGGGCT-3′). The solution was brought to 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM MgCl–10 mM dithiothreitol–1 mM each deoxynucleoside triphosphate and heated at 42°C for 2 min. RNase H-free RT (200 U; Superscript II; Life Technologies, Inc.) was added, and the sample was incubated at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min.

A 2-μl volume of the reverse transcription reaction mixture was used as the template in a 20-μl reaction mixture containing 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 an oligonucleotide corresponding to pNL4-3 nucleotides 1213 to 1239 (5′-GGTACATCAGGCCATATCACCTAGAAC-3′) with 10 pmol of the oligonucleotide used for reverse transcription, and 0.5 U of AmpliTaq DNA polymerase. All reactions were carried out with a GeneAmp 2400 PCR system (Perkin-Elmer, Foster City, Calif.) for 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were size separated on agarose gels and visualized by ethidium bromide staining. The full-length product with all of the pNL4-3-derived viruses used here is 434 nucleotides. Restriction enzyme digestion of mutant P222A with *Ban*I reduces the product to 236- and 198-nucleotide fragments. Digestion of P222A/A224E with *Hin*P1 I or *Mfe*I reduces the product to fragments of 237 and 197 or 240 and 194 nucleotides, respectively.

RESULTS

Mutants A224E and G226D are sufficient to confer CsA resistance on HIV-1 in the Jurkat T-cell line. To determine if HIV-1 is capable of acquiring resistance to CsA and related

```
          205                                   241
Wild Type INEEAAEWDRLHPVHAGPIAPGQMREPRGSDIAGTTS
A224E     ------------------e---------------
G226D     --------------------d---------------
```

FIG. 1. CsA-resistant HIV-1 isolates contain either of two point mutations which alter the amino acid sequence of the proline-rich domain of Gag. The top line shows the amino acid sequence of the wild-type proline-rich domain of the HIV-1 Gag polyprotein. The underlined residues, G-221 and P-222, have previously been shown to be required for CyPA binding and for CyPA incorporation into virions (5, 13). Amino acid residues encoded by drug-resistant mutations are shown below those of the wild type. The amino acid altered by the point mutation (A-224→E or G-226→D) is indicated by a lowercase letter. Conserved residues are represented by dashes. Amino acid numbering is with respect to the amino terminus of the HIV-1_{NL4-3} (2) Gag polyprotein.

compounds, viral stocks produced by a cloned HIV-1 provirus were propagated in $CD4^+$ HeLa cells (21) in the presence of (Me-Ile-4)CsA. This compound is a nonimmunosuppressive CsA analog which, like the parent compound, disrupts the interaction between CyPA and HIV-1 Gag and inhibits HIV-1 replication (28, 37). Four viral stocks capable of replicating in the presence of (Me-Ile-4)CsA or CsA were obtained after 6 weeks of passage in tissue culture. Interestingly, these viral stocks were not only drug resistant but dependent upon the drug for replication in either $CD4^+$ HeLa cells or in the CEM T-lymphoblastoid cell line. Complete details of the selection of these viral stocks and a complete genetic characterization are presented elsewhere (1). Briefly, to identify mutations responsible for the drug-resistant phenotype, DNA fragments amplified from cells infected with the resistant viral stocks were used to replace corresponding fragments in the parental, wild-type provirus. Sequences encoding the proline-rich domain of Gag were associated with drug resistance; analysis of the primary structure of this region revealed that all drug-resistant stocks contained one of two point mutations, each of which encodes an alteration in the amino acid sequence of Gag: A-224→E or G-226→D (Fig. 1). Both mutations introduce a negatively charged amino acid within the domain of Gag previously shown to be required for CyPA binding and CyPA incorporation into virions (5, 13, 37).

To further characterize these mutants, the same HIV-1 proviral DNAs which had been shown to encode drug-dependent virus in $CD4^+$ HeLa cells or in CEM cells—wild type or containing single point mutations encoding A224E or G226D—were transfected into Jurkat T cells. Viral replication was monitored by measuring the accumulation of RT activity in the culture supernatant. The RT activity peak for the wild-type virus was observed at day 10 posttransfection (Fig. 2A). The two mutant viruses each produced an RT peak in Jurkat cells comparable in magnitude to that produced by the wild type, although in each case replication kinetics was delayed slightly: for A224E, the RT peak occurred at day 12 (Fig. 2B); for G226D, the peak occurred at day 14 (Fig. 2C). This result was in sharp contrast to the finding that these mutants were unable to replicate in $CD4^+$ HeLa cells or CEM cells in the absence of the drug (1). Similar results were obtained whether the viral infection was initiated by transfection of proviral DNA or by infection with exogenous virus and with proviral constructs containing a mutation (A-224→E or G-226→D) that had been engineered by site-directed mutagenesis (data not shown).

As previously reported (4, 20, 28, 37, 38), replication of wild-type HIV-1 was disrupted in a titratable fashion by increasing concentrations of CsA (Fig. 2A). CsA has a narrow therapeutic index, and the drug doses chosen were those which effectively inhibit HIV-1 replication without obvious toxicity to host cells (5). In contrast, CsA had minimal effects on the replication of mutant A224E or G226D (Fig. 2B and C). In fact, rather than inhibit viral replication, 0.5 μM CsA had a slight stimulatory effect on the replication of the mutant viruses; a similar stimulatory effect of CsA has been observed with non-HIV-1 primate lentiviruses that replicate independently of CyPA (5). These results indicate that each of the two mutations is sufficient to confer a CsA-resistant phenotype on otherwise wild-type cloned provirus in the Jurkat T-cell line.

A224E and G226D do not alter the CyPA-binding properties of the HIV-1 Gag polyprotein in vitro. Since CsA inhibits HIV-

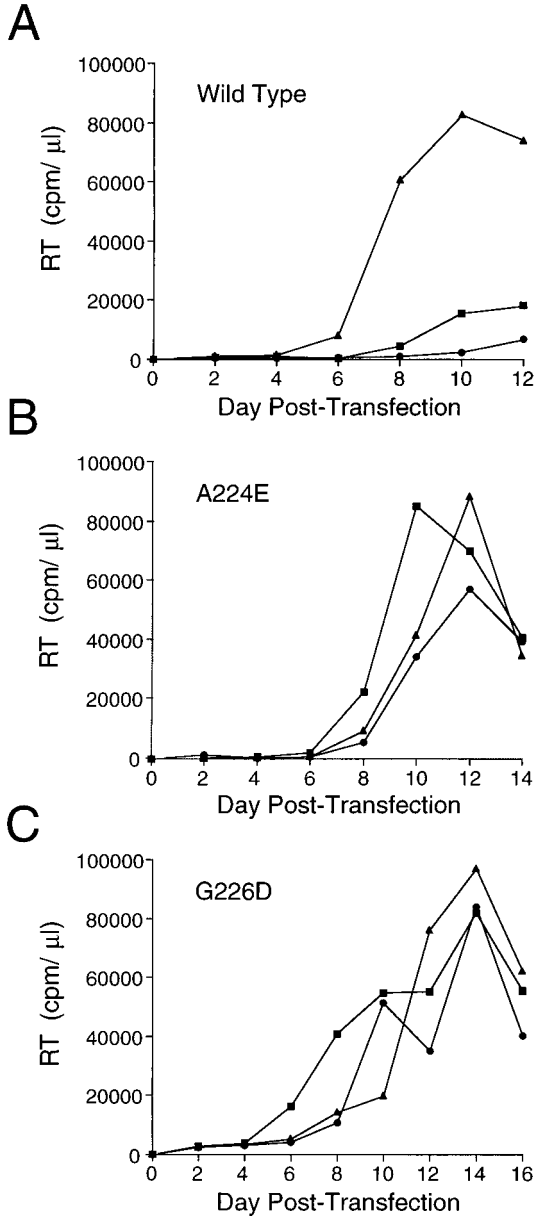

FIG. 2. Gag mutants A224E and G226D render HIV-1 CsA resistant. Viral replication kinetics in Jurkat T cells was measured following DEAE-dextran transfection of proviral DNAs from wild-type HIV-1_{NL4-3} (2) (A), mutant A224E (B), and mutant G226D (C). Virion accumulation in the culture supernatant is indicated by RT activity (ordinate) at the indicated times posttransfection of proviral DNAs (abscissa). CsA was dissolved in ethanol and maintained in tissue culture medium at the following concentrations: ▲, no drug; ■, 0.5 μM; ●, 2.5 μM. An equal volume of drug solvent was added to each culture, and the drug was replenished every 2 days for the duration of the experiments.

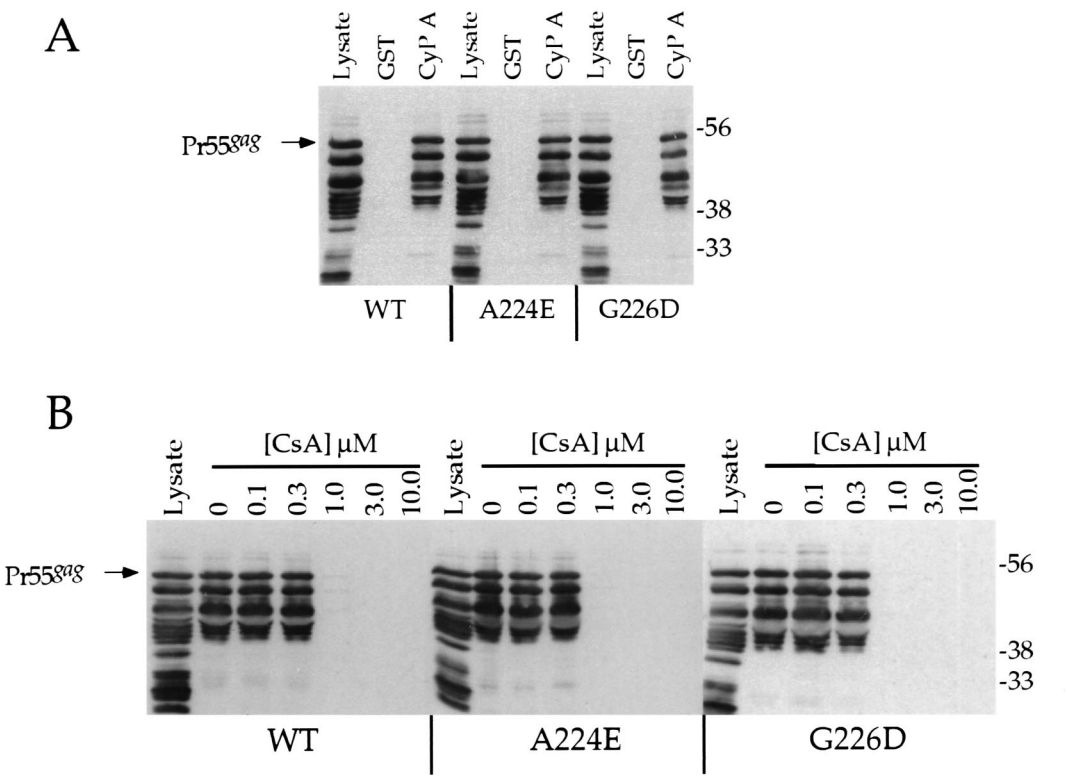

FIG. 3. HIV-1 Gag polyprotein mutants A224E and G226D bind specifically to CyPA in vitro with an affinity comparable to that of the wild-type protein. Lysate from bacteria expressing the indicated HIV-1 Gag polyprotein (wild type, A224E, or G226D) was mixed with lysate from bacteria expressing GST or GST fused to CyPA, as indicated (A), or with lysate from bacteria expressing GST fused to CyPA in the presence of the indicated concentrations of CsA (B). GST proteins were collected on glutathione-agarose beads. Bound protein was subjected to SDS-PAGE and visualized by Western blotting with a monoclonal anti-HIV-1 capsid antibody (Du Pont). In each segment of panel A, the total lysate from bacteria expressing the indicated Gag polyprotein is shown. The position of migration of $Pr55^{gag}$ is shown. WT, wild-type Gag polyprotein. The numbers on the right are molecular sizes in kilodaltons.

1 replication by competing with Gag for CyPA (13, 23, 37), A224E and G226D might confer CsA resistance by increasing the CyPA-binding affinity of the HIV-1 Gag polyprotein; this would render CsA less effective as a competitor. To determine if either A-224→E or G-226→D alters the CyPA-binding properties of the Gag polyprotein, the ability of each mutant protein to bind to CyPA was tested in vitro. A 90-nucleotide sequence fragment encoding each mutation was used to replace wild-type *gag* sequences in a previously characterized plasmid for expression of the HIV-1 Gag polyprotein in bacteria (24). As previously demonstrated (23), wild-type HIV-1 Gag polyprotein present in the total bacterial lysate was quantitatively recovered on glutathione-agarose beads to which GST-CyPA had been adsorbed (Fig. 3A). As indicated by the fraction of input protein bound, both mutant Gag proteins were capable of binding to CyPA to an extent similar to that of the wild-type protein (Fig. 3A). The specificity of binding of each protein was demonstrated by the fact that recovery of Gag was dependent upon the presence of the CyPA protein; no binding was detected with GST bound to glutathione agarose beads (Fig. 3A).

To assess the strength of binding of each mutant, we examined the effect of CsA on recovery of A224E or G226D by GST-CyPA. As previously demonstrated (23), CsA completely inhibited HIV-1 Gag binding to CyPA at drug concentrations equivalent to the concentration of CyPA in the binding reaction (Fig. 3B). The inhibition curve possesses a sigmoid character, consistent with the demonstrated requirement for cooperative interactions between Gag monomers (8). CsA completely inhibited the binding of Gag mutant A224E or G226D to CyPA at concentrations equivalent to those which blocked wild-type Gag binding (Fig. 3B). In addition, both mutants exhibited steep CsA inhibition curves identical to that observed with the wild-type protein (Fig. 3B). These experiments demonstrate that neither mutation alters the CyPA-binding properties of Gag to any significant extent.

CsA blocks CyPA incorporation into virions produced by HIV-1 mutants A224E and G226D. Next we examined the effect of A224E or G226D on the incorporation of CyPA into virions. Wild-type proviral DNA and mutants proviral DNA encoding A224E or G226D were transfected into 293T cells by calcium phosphate precipitation. At 48 h, virions were purified from the cell supernatant (see Materials and Methods for technical details) and subjected to SDS-PAGE and Western blotting with a combination of two primary antibodies; the first antibody recognizes HIV-1 Gag, and the second recognizes human CyPA.

The total virion yield for each mutant was equivalent to that of the wild type; this was demonstrated by virion-associated RT (data not shown) and by the intensity of the signal in the Western blot for particle-associated CA (Fig. 4). Purified virions produced by transfection of mutant provirus A224E or G226D also contained quantities of CyPA comparable to that found in virions produced by wild-type proviruses (Fig. 4).

To test for the effect of CsA on the incorporation of CyPA into virions, DNA transfections were performed with 2.5 μM CsA added to the tissue culture medium. This drug concentration was chosen because we had shown that it was sufficient to

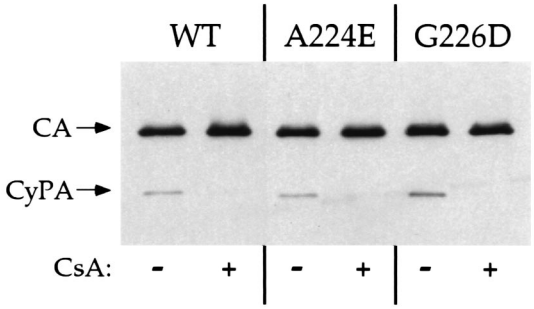

FIG. 4. Gag mutants A224E and G226D do not alter the CyPA-binding properties of HIV-1 Gag in virions. Immunoblot of virion protein purified from the supernatant of 293T cells transfected by calcium phosphate precipitation with the indicated proviral DNAs in the presence (+) or absence (−) of 2.5 μM CsA. Blots were probed with both a monoclonal anti-HIV-1 CA antibody and a rabbit anti-CyPA antibody (a gift from Louis Henderson). The positions of migration of CA and CyPA are shown. WT, wild type.

strongly inhibit the replication of wild-type virus but had only negligible effects on the replication of the mutant viruses (Fig. 2). When transfections were performed in the presence of 2.5 μM CsA, CyPA was undetectable in either wild-type or mutant virions (Fig. 4). Thus, with respect to CyPA binding in vitro and CyPA incorporation into virions, the biochemical properties of A224E or G226D are indistinguishable from those of the wild type. The drug-resistant phenotype is therefore explained not by increased CyPA-binding affinity on the part of the mutant Gags but by the ability of the mutants to convert virions to a CyPA-independent mode of infection.

CsA-resistant mutant A224E restores replication to a provirus harboring a mutation which precludes CyPA packaging. HIV-1 Gag mutant P222A is unable to bind or package CyPA (13); virions produced by this mutant have no obvious biochemical or structural defects, but their ability to initiate reverse transcription after infection of T cells is attenuated (5). If the replication defect of P222A resulted solely from disruption of CyPA packaging, then a mutation conferring the ability to replicate in the absence of CyPA should restore P222A infectivity. To test this idea, HIV-1 Gag double mutant P222A/A224E was constructed and its ability to replicate in Jurkat T cells was compared with those of the wild type and P222A. As previously reported, the replication of P222A was severely attenuated (Fig. 5A). In contrast, the P222A/A224E RT peak was comparable in magnitude to that of the wild type (Fig. 5A), indicating that A224E is able to rescue P222A when the two mutations are possessed by the same provirus.

Mutant P222A/A224E was examined further to determine if it retains characteristics of each of the single mutants. Like that of A224E (Fig. 2), P222A/P224E replication was slower than that of the wild type (Fig. 5A), as well as CsA resistant and slightly stimulated by drug (Fig. 5B). The single mutant P222A packages fivefold less CyPA than does the wild-type virus (5). CyPA packaging by P222A/A224E was reduced to the same extent as that by P222A, indicating that A224E did not restore CyPA-binding properties to P222A (Fig. 6). The phenotype of P222A/A224E, therefore, was a composite of the properties of each of the individual mutants.

Lastly, we determined if the RT peak observed following transfection of P222A/A224E proviral DNA was due to a replicating virus which retained both mutations, rather than contamination with wild-type virus or the appearance of a revertant which had lost either of the engineered mutations. Virion-associated RNA was isolated from supernatant containing the peak of RT (day 20 in Fig. 5A). This was used as the template in reverse transcription-PCR with primers specific for *gag* sequences surrounding the site of the engineered mutations. As with the wild-type virus, RNA isolated from P222A/A224E-derived virions gave the expected product of 434 bp (Fig. 7). No product was obtained when PCR was performed without prior reverse transcription (Fig. 7), indicating that the product was from RNA and not contaminating plasmid DNA. Similarly, no product was obtained when the template was pretreated with RNase A, whereas pretreatment with RQ1 DNase had no effect on product yield (data not shown). The oligonucleotides used to engineer the mutations incorporated restriction enzyme recognition sites, and digestion of the PCR products with these enzymes was used to monitor the presence of the mutations. Unlike the product obtained with wild-type-derived virus, the reverse transcription-PCR product produced with P222A/A224E-derived virion RNA as the template was cut to completion by both of the restriction enzymes used to monitor the mutations (Fig. 7). Dilution of the wild-type sample demonstrated that if the culture contained a revertant

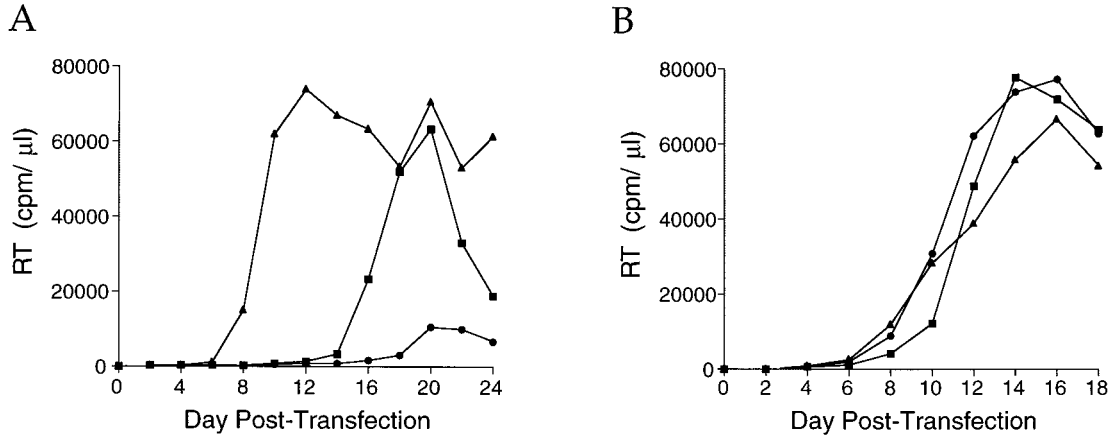

FIG. 5. Double mutant P222A/A224E is replication competent, indicating that A224E rescues the infectivity of a Gag mutant (P222A) which is unable to package CyPA. (A) Viral replication kinetics in Jurkat T cells following DEAE-dextran transfection of the following proviral DNAs: ▲, wild-type HIV-1_{NL4-3}; ●, mutant P222A; ■, double mutant P222A/A224E. (B) Viral replication kinetics in Jurkat T cells following DEAE-dextran transfection of double mutant P222A/A224E; CsA was maintained in the tissue culture medium at the following concentrations: ▲, no drug; ■, 0.5 μM; ●, 2.5 μM. Virion accumulation in the culture supernatant is indicated by RT activity (ordinate) at the indicated times posttransfection of proviral DNAs (abscissa).

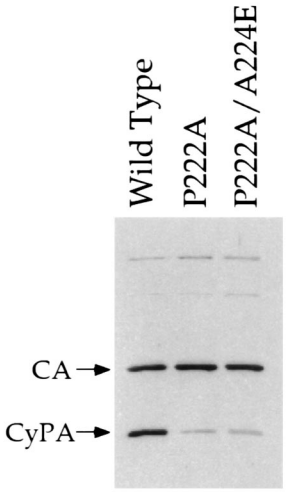

FIG. 6. Gag double mutant P222A/A224E is defective for CyPA packaging. Immunoblot of virion protein purified from the supernatant of 293T cells transfected by calcium phosphate precipitation with the indicated proviral DNAs as described in the legend to Fig. 4.

which had lost the mutations, the abundance of this virus was, at most, 1/30 of that of the double mutant. We conclude that A224E was able to rescue P222A, supporting our two contentions that A224E is capable of replicating in the absence of packaged CyPA and that the disruption of viral replication by P222A is a direct result of the mutant's inability to package CyPA.

DISCUSSION

The isolation of two *gag* point mutations, each of which removes HIV-1's dependence upon CyPA, demonstrates that in addition to encoding the CyPA-packaging function, *gag* encodes the functional target of CyPA. This result establishes HIV-1 *gag* as the second gene—the only other being a *Drosophila* rhodopsin (3, 9)—to encode a protein with a demonstrated functional requirement for an interaction with a cyclophilin family member.

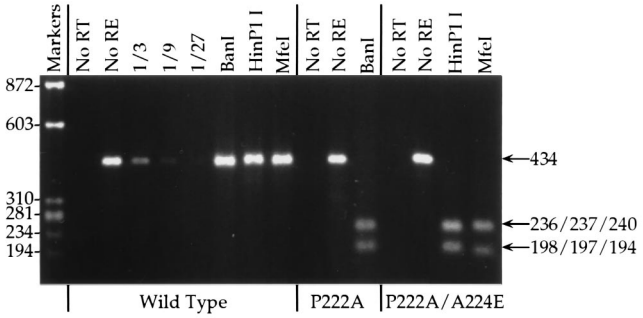

FIG. 7. Replicating virus initiated by transfection of P222A/A224E proviral DNA retains both mutations. Virion RNA from the wild type, mutant P222A, and mutant P222A/A224E RT peaks (day 20 from Fig. 5A) was purified and subjected to RT-PCR as described in Materials and Methods. Products were resolved on a 2% agarose gel and visualized by ethidium bromide staining. The wild-type sample was serially diluted by thirds, as indicated. Each sample was subjected to PCR without prior reverse transcription (No RT). Each PCR product is shown without digestion by restriction enzymes (No RE) and after digestion with the indicated restriction enzymes. Molecular size markers in base pairs are shown on the left. The expected sizes of the restriction enzyme digestion products (detailed in Materials and Methods) are shown on the right.

Although CyPA might be required during virion assembly, several observations suggest that CyPA plays an essential role subsequent to assembly, after proteolytic cleavage of the Gag polyprotein (5, 13, 37). First, significant quantities of CyPA are specifically incorporated into HIV-1 virions (molar ratio to Gag of roughly 1:10). Second, the absence of detectable biochemical or structural defects in virions rendered CyPA deficient by CsA or by *gag* mutations suggests that CyPA is dispensable for virion assembly. Third, disruption of CyPA packaging into virions is associated with a quantitative reduction in virion infectivity, with the block to replication occurring early in the virus life cycle, after membrane fusion but prior to reverse transcription (5, 13, 37). We propose that CA, the Gag polyprotein cleavage product which retains the proline-rich domain and, therefore, the ability to bind CyPA (5, 8, 23, 37), is the *gag* product most likely to be the functional target of CyPA; by introducing a negatively charged residue into the proline-rich domain, each CsA-resistant mutant might alter the structure of CA such that CyPA is no longer required for an early event in the HIV-1 life cycle, such as virion uncoating.

It remains to be determined if CyPA's peptidyl-prolyl isomerase activity (12, 14, 25, 33) is required for HIV-1 virion uncoating. Our previous observation that Gag residues G-221 and P-222 are required for binding to CyPA (5, 13) suggests that CyPA catalyzes the isomerization of the peptidyl-prolyl bond that joins G-221 to P-222. Alternatively, CyPA has also been demonstrated to function as a chaperone that prevents the aggregation of folding intermediates (3, 9, 14, 36); biochemical studies indicate that CA dissociates from the viral nucleoprotein complex during virion uncoating (7, 15), and CyPA might function as a chaperone of CA in HIV-1 virion uncoating, analogously to Hsp70, the ATPase which catalyzes the uncoating of clathrin from coated vesicles (29, 32).

The ease with which these CsA-resistant HIV-1 mutants were selected in tissue culture suggests that drugs which inhibit HIV-1 replication by blocking the Gag-CyPA interaction will select resistant mutants in vivo. Such drugs would therefore prove ineffective antiviral agents in HIV-1-infected individuals.

The observation that mutations A-224→E and G-226→D render the virus drug resistant in Jurkat cells, in contrast to the drug-dependent phenotype observed in $CD4^+$ HeLa cells or CEM cells, is somewhat surprising. The molecular basis for this phenotypic difference is unknown. The slight stimulatory effect on the replication of the mutants in Jurkat cells by the lower dose of CsA (Fig. 2) suggests that the cell type-specific phenotypic difference may only be relative and not an absolute genetic difference between these cell lines. Interestingly, a previous report noted that a two-amino-acid change in the proline-rich domain of HIV-1 Gag selectively altered the replication kinetics of the mutant virus in specific cell lines (30).

ACKNOWLEDGMENTS

We thank Louis Henderson for the anti-CyPA antibody. Jurkat clone E6-1, from American Type Culture Collection, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

This work was supported by grant AI 36199 from the National Institute of Allergy and Infectious Diseases to J.L. J.L. is an Irma T. Hirschl Scholar.

REFERENCES

1. **Aberham, C., S. Weber, and W. Phares.** 1996. Spontaneous mutations in the human immunodeficiency virus type 1 *gag* gene that affect viral replication in the presence of cyclosporins. J. Virol. **70:**3536–3544.
2. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an

infectious molecular clone. J. Virol. **59:**284–291.
3. **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.
4. **Bartz, S. R., E. Hohenwalter, 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.
5. **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.
6. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature (London) **365:**666–669.
7. **Bukrinsky, M. I., 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.
8. **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.
9. **Colley, N., E. Baker, M. Stamnes, and C. Zuker.** 1991. The cyclophilin homolog ninaA is required in the secretory pathway. Cell **67:**255–263.
10. **Dorfman, R., F. Mammano, W. A. Haseltine, and H. F. Göttlinger.** 1994. Role of the matrix protein in the virion incorporation of the human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **68:**1689–1696.
11. **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.
12. **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 (London) **337:**476–478.
13. **Franke, E. K., H. E. H. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature (London) **372:**359–362.
14. **Freskgard, P. O., N. Bergenhem, B. H. Jonsson, M. Svensson, and U. Carlsson.** 1992. Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. Science **258:**466–468.
15. **Gallay, P., S. Swingler, 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.
16. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature (London) **355:**33–45.
17. **Handschumacher, R., M. Harding, J. Rice, and R. Drugge.** 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science **226:**544–547.
18. **Heitman, J., N. R. Movva, and M. N. Hall.** 1992. Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. New Biol. **4:**448–460.
19. **Hunter, E.** 1994. Macromolecular interactions in the assembly of HIV and other retroviruses. Semin. Virol. **5:**71–83.
20. **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.
21. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:**2232–2239.
22. **Linial, M. L., and A. D. Miller.** 1990. Retroviral RNA packaging: sequence requirements and implications. Curr. Top. Microbiol. Immunol. **157:**125–152.
23. **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.
24. **Luban, J., and S. P. Goff.** 1991. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 *gag* polyprotein. J. Virol. **65:**3203–3212.
25. **Matouschek, A., S. Rospert, K. Schmid, B. S. Glick, and G. Schatz.** 1995. Cyclophilin catalyzes protein folding in yeast mitochondria. Proc. Natl. Acad. Sci. USA **92:**6319–6323.
26. **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.
27. **Park, J., and C. D. Morrow.** 1992. The nonmyristylated $Pr160^{gag\text{-}pol}$ polyprotein of human immunodeficiency virus type 1 interacts with $Pr55^{gag}$ and is incorporated into viruslike particles. J. Virol. **66:**6304–6313.
28. **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. Wolff, G. Zenke, and M. Zurini.** 1994. Inhibition of human immunodeficiency virus type 1 replication by SDZ NIM 811, a nonimmunosuppressive cyclosporine analog. Antimicrob. Agents Chemother. **38:**1763–1772.
29. **Rothman, J. E., and S. L. Schmid.** 1986. Enzymatic recycling of clathrin from coated vesicles. Cell **46:**5–9.
30. **Sakuragi, J.-I., H. Sakai, M. Kawamura, K. Tokunaga, S. Ueda, and A. Adachi.** 1995. Generation and characterization of a host cell-dependent gag gene mutant of human immunodeficiency virus type 1. Virology **212:**251–254.
31. **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.
32. **Schmid, S. L., and J. E. Rothman.** 1985. Enzymatic dissociation of clathrin cages in a two-stage process. J. Biol. Chem. **260:**10044–10049.
33. **Schonbrunner, E. R., S. Mayer, M. Tropschug, G. Fischer, N. Takahashi, and F. X. Schmid.** 1991. Catalysis of protein folding by cyclophilins from different species. J. Biol. Chem. **266:**3630–3635.
34. **Smith, A. J., N. Srinivasakumar, M.-L. Hammarskjöld, and D. Rekosh.** 1993. Requirements for incorporation of $Pr160^{gag\text{-}pol}$ from human immunodeficiency virus type 1 into virus-like particles. J. Virol. **67:**2266–2275.
35. **Stamnes, M. A., S. L. Rutherford, and C. S. Zuker.** 1992. Cyclophilins: a new family of proteins involved in intracellular folding. Trends Cell. Biol. **2:**272–276.
36. **Sykes, K., M.-J. Gething, and J. Sambrook.** 1993. Proline isomerases function during heat shock. Proc. Natl. Acad. Sci. USA **90:**5853–5857.
37. **Thali, M., A. A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Göttlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. Nature **372:**363–365.
38. **Wainberg, M., A. Dascal, N. Blain, L. Fitz-Gibbon, F. Boulerice, 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.
39. **Weiss, A., R. Wiskocil, and J. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two stimulus requirement for IL-2 production reflects events occurring at a pretranslational level. J. Immunol. **133:**123–128.
40. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639–654.
41. **Yu, X., X. Yuan, Z. Matsuda, T.-H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966–4971.