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Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells

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The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein binds most members of the cyclophilin family of peptidyl-prolyl isomerases. Of 15 known human cyclophilins, cyclophilin A (CypA) has been the focus of investigation because it was detected in HIV-1 virions. To determine whether CypA promotes HIV-1 replication, we deleted the gene encoding CypA (*PPIA*) in human CD4$^+$ T cells by homologous recombination. HIV-1 replication in *PPIA*$^{-/-}$ cells was decreased and not inhibited further by cyclosporin or *gag* mutations that disrupt Gag's interaction with cyclophilins, indicating that no other cyclophilin family members promote HIV-1 replication. The defective replication phenotype was specific for wild-type HIV-1 since HIV-2/SIV isolates, as well as HIV-1 bearing a *gag* mutation that confers cyclosporin resistance, replicated the same in *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells. Stable re-expression of CypA in *PPIA*$^{-/-}$ cells restored HIV-1 replication to an extent that correlated with steady-state levels of CypA. Finally, virions from *PPIA*$^{-/-}$ cells possessed no obvious biochemical abnormalities but were less infectious than virions from wild-type cells. These data formally demonstrate that CypA regulates the infectivity of HIV-1 virions.

Keywords: cyclophilin/cyclosporin/Gag/human immunodeficiency virus/retrovirus

Introduction

Human immunodeficiency virus type 1 (HIV-1) *gag* encodes proteins that play roles in practically every step of the virus life cycle (Freed, 1998). The Gag polyprotein orchestrates the formation and release of enveloped virions from infected cells. Concurrent with budding of nascent virions, the Gag polyprotein is cleaved by the viral protease to produce, among other products, matrix, which lines the virion envelope; capsid, which forms the virion core; and nucleocapsid, which coats viral genomic RNA. To initiate infection, the mature virion binds to cell surface receptors on a susceptible target cell and fuses its membrane with the cell's plasma membrane. A viral ribonucleoprotein complex then enters the target cell cytoplasm where *gag*-encoded proteins participate in reverse transcription, nuclear transport and establishment of the provirus.

Since viruses are obligate intracellular parasites, host–cell factors might be required for any of the HIV-1 *gag*-encoded functions mentioned above. Attempts to identify these putative factors have revealed a number of Gag-interacting proteins, including actin (Rey *et al*., 1996; Liu *et al*., 1999; Wilk *et al*., 1999; Ott *et al*., 2000), ubiquitin (Ott *et al*., 1998), calmodulin (Radding *et al*., 2000), the motor protein KIF-4 (Tang *et al*., 1999), the nuclear transporter karyopherin-α (Gallay *et al*., 1996; Agostini *et al*., 2000), translation elongation factor 1-α (Cimarelli and Luban, 1999), translation initiation factor 2 (Wilson *et al*., 1999), the HO3 histidyl-tRNA synthetase (Lama and Trono, 1998) and a human member of the trithorax/polycomb group of proteins (Peytavi *et al*., 1999). Convincing evidence that any of these factors are required for HIV-1 replication has remained elusive. In contrast, it is instructive to consider studies that exploited CD4-negative or chemokine receptor-negative cells to demonstrate that these cell surface proteins are required for viral entry (Maddon *et al*., 1986; Feng *et al*., 1996). To date, cells that do not express the Gag-binding factors have yet to be identified or generated.

In addition to the factors mentioned above, members of the large family of proteins known as the cyclophilins (Table I) were found to bind to HIV-1 Gag in one of the first reported two-hybrid screens for a cDNA encoding an interacting protein (Luban *et al*., 1993). Cyclophilins were discovered originally because of their high affinity for cyclosporin (Handschumacher *et al*., 1984), an immunosuppressive drug used to prevent allograft rejection. The clinical effect of the drug is not thought to result from inhibition of a cyclophilin function. Instead, immunosuppression results when the cyclophilin–cyclosporin complex binds and inhibits calcineurin (Friedman and Weissman, 1991; Liu *et al*., 1991b), a calcium-dependent, serine-threonine phosphatase required for transcriptional activation of many cytokine genes in stimulated T cells. Considering the immunosuppression associated with HIV-1 infection, cyclophilins seemed intriguing Gag-binding partners. Gag and cyclosporin were later found to compete for the same binding site on cyclophilin (Gamble *et al*., 1996; Braaten *et al*., 1997; Dorfman *et al*., 1997), but the Gag–cyclophilin complex does not interact with calcineurin (Luban *et al*., 1993).

Cyclophilins are defined by a conserved sequence of ~150 amino acids that form an eight-stranded β-barrel with a hydrophobic pocket that serves as the binding site for cyclosporin and HIV-1 Gag (Ke *et al*., 1991; Mikol *et al*., 1993; Gamble *et al*., 1996). Some cyclophilins, such as CypA, consist of just this core domain. In other cases, the cyclophilin domain is embedded within a more complex protein. Proteins containing a cyclophilin domain have been implicated in a number of cellular processes, including protein secretion, mitochondrial function, RNA

Table I. The known human cyclophilins

Protein name	Protein size	Subcellular localization	Binds to HIV-1 Gag[a]	HUGO gene name	GenBank accession Nos
CypA	18 kDa	cytoplasm	yes	*PPIA*	X52851 Y00052
CypB, SCYLP	18–20 kDa	ER and secretory pathway	yes	*PPIB*	M60857 M63573 M60457
CypC	18 kDa	secretory pathway	yes	*PPIC*	S71018
CypF, Cyp3	18–22 kDa	mitochondrial inner membrane	ND	*PPIF*	M80254
PPIL1, CypM	18 kDa	cytoplasm	ND	*PPIL1*	AF090992
USA-CyP, Cyp20	20 kDa	nucleus; human U4/U6 snRNP-associated protein	ND	*USA-CYP*	AF016371
CypE, Cyp33A	40 kDa	RNA-binding nuclear protein	ND	*PPIE*	AF042385 AF104013
Cyp33B	shorter isoform of 33A	RNA-binding nuclear protein	ND	*PPIE*	AF042386
Cyp40	40 kDa	cytoplasm and nucleus; associated with steroid receptors	yes	*PPID*	L11667 D63861
Serologically defined colon cancer antigen 10	50 kDa	nucleus	ND	*SDCCAG10*	AF039692
Cyp60	60 kDa	nucleus; interacts with the proteinase inhibitor elgin C	yes	*PPIL2*	U37219
KIAA0073, Hal539-CyP	60 kDa	unknown	ND	*KIAA0073*	D38552
CARS-CyP, Clk-associating RS cyclophilin	89 kDa	associated with the nuclear matrix and splicing factors	yes	*CYP*	U40763 X99717
NK-TRCyP	89 kDa	associated with the nuclear matrix and splicing factors	yes	*NKTR*	L04288
RAN-BP2, NUP-358	358 kDa	associated with the nuclear pore, cytoplasmic face	no	*RAN-BP2*	L41840 D42063

[a]HIV-1 Gag binding to GST fusions with CypA, B or C as previously described (Franke *et al*., 1994); identical methods were used to determine binding to GST fusions with the cyclophilin domains from Cyp40, 60, CARS, NK-TR and RAN-BP2/NUP-358. ND, not determined.

processing and transcriptional regulation (Colgan *et al*., 2000), but the exact biochemical function in cells of the core cyclophilin domain is unknown. One function is, presumably, the maintenance of proper protein conformation, since cyclophilins catalyze the *cis–trans* interconversion of peptide bonds N-terminal to proline, an activity that has been shown to stimulate the rate of refolding of model proteins *in vitro* (Fischer *et al*., 1989; Takahashi *et al*., 1989). It has been suspected, therefore, that cyclophilins regulate the conformation of HIV-1 Gag (Luban *et al*., 1993).

Following the discovery of the Gag–cyclophilin interaction, CypA in particular was found to be a constituent of the HIV-1 virion (Franke *et al*., 1994; Thali *et al*., 1994). Additional studies have attempted to provide evidence of a functional role for CypA, either early in infection of susceptible cells (Steinkasserer *et al*., 1995; Braaten *et al*., 1996b; Sherry *et al*., 1998; Saphire *et al*., 1999) or in the assembly of HIV-1 virions (Agresta and Carter, 1997; Streblow *et al*., 1998; Bristow *et al*., 1999). These studies relied largely on the use of mutations in *gag* or competitive inhibitors such as cyclosporin to block the Gag–cyclophilin interaction; however, neither of these experimental conditions abrogates the interaction completely and both potentially can cause pleiotropic effects. A third confounding issue is the number and abundance of cyclophilins in mammalian cells; at present there are 15 known human cyclophilins (Table I) and nearly all that have been tested are capable of binding HIV-1 Gag (Luban *et al*., 1993; Franke *et al*., 1994). Thus, it has not been possible to determine conclusively which cyclophilin family members, if any, promote HIV-1 replication.

As a means to address these issues, we produced *PPIA*$^{-/-}$ Jurkat T-cell lines by homologous recombination. These cell lines have enabled us formally to demonstrate that CypA is required for wild-type replication kinetics of HIV-1 and, more specifically, for the infectivity of HIV-1 virions. We also present data indicating that, with regard to replication of HIV-1, none of the 14 other known cyclophilins substitutes functionally for CypA in *PPIA*$^{-/-}$ Jurkat cells.

Results

Gene targeting of PPIA by homologous recombination

A cell line lacking *PPIA* expression would be an ideal reagent with which to study the functional role of CypA for HIV-1. Initially, we screened 10 cell lines and 40 primary tumors by northern blotting for the absence of *PPIA* expression; none was found (data not shown). We therefore set out to produce a *PPIA*$^{-/-}$ cell line.

Production of *PPIA*$^{-/-}$ cells proceeded first by obtaining genomic clones of the human *PPIA* locus, which was complicated by the fact that multiple reverse transcribed *PPIA* pseudogenes are present in the genome (Haendler and Hofer, 1990). Two PCR primer sets, designed to amplify distinct regions of the functional *PPIA* genomic locus, were thus used to screen a human foreskin fibroblast P1 phage library. Three P1 clones were obtained, one of which was used for subsequent cloning and for fluorescence *in situ* hybridization (FISH) on metaphase spreads of two human CD4$^+$ T-cell lines to determine the *PPIA* copy number (Braaten *et al*., 1996d). The Jurkat T-cell line was determined to be diploid for *PPIA*, and the H9 T-cell line tetraploid (data not shown). To produce *PPIA*$^{-/-}$ Jurkat cells, then, two consecutive rounds of gene targeting would be required.

The promoter and all but the last exon of both *PPIA* alleles (~5 kb of contiguous genomic DNA) in Jurkat cells

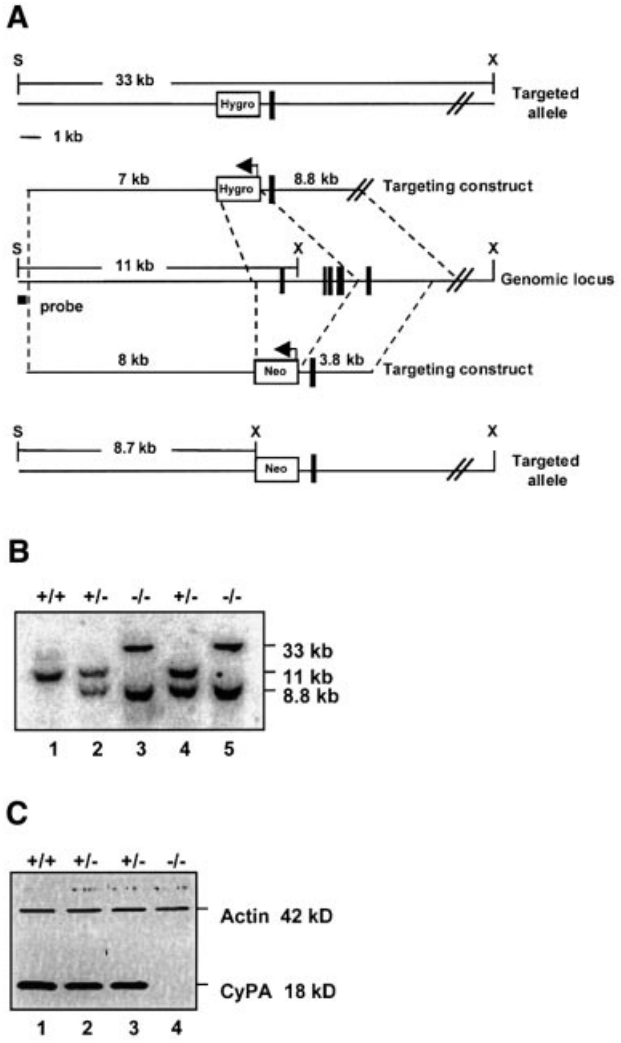

Fig. 1. Gene targeting of the *PPIA* locus by homologous recombination. (**A**) Strategy for deleting all but the fifth exon of both copies of *PPIA* in Jurkat T cells by two consecutive rounds of gene targeting. Linearized targeting plasmids are shown above and below a schematic of the genomic locus. 'Neo', neomycin (G418) resistance gene; 'Hygro', hygromycin resistance gene; S, *Sac*I; X, *Xba*I. (**B**) Southern blot hybridization of $PPIA^{+/+}$, $PPIA^{+/-}$ and $PPIA^{-/-}$ cell lines; total cellular DNAs were digested with both *Sac*I and *Xba*I and probed with the 5′ probe (shown in A). (**C**) Western blot of total cell lysates from $PPIA^{+/+}$, $PPIA^{+/-}$ and $PPIA^{-/-}$ cell lines, probed with polyclonal anti-actin and anti-CypA antibodies.

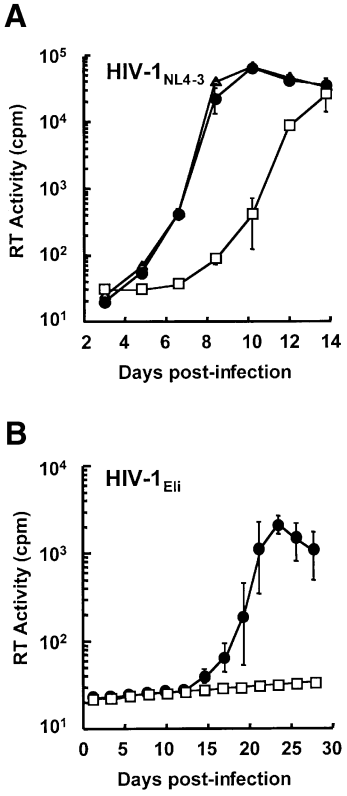

Fig. 2. HIV-1 replication is decreased in $PPIA^{-/-}$ Jurkat T cells. (**A**) Replication of HIV-1_{NL4-3} in $PPIA^{+/+}$ (filled circles), $PPIA^{+/-}$ (shaded triangles) and $PPIA^{-/-}$ (open squares) cell lines, demonstrating delayed kinetics in $PPIA^{-/-}$ cells. (**B**) Replication of HIV-1_{Eli} in $PPIA^{+/+}$ (filled circles) and $PPIA^{-/-}$ (open squares) cell lines, demonstrating an even greater defect in replication than HIV-1_{NL4-3}.

was deleted by homologous recombination using two targeting plasmids encoding different selectable markers (Figure 1A). Five $PPIA^{+/-}$ and two $PPIA^{-/-}$ clones were produced at a targeting efficiency of ~1 in 350 drug-resistant cultures. The recombinant clones were screened by Southern blot hybridization with a *PPIA* locus-specific probe (Figure 1B) and by western blotting total cell lysates probed with a polyclonal antibody raised against CypA (Figure 1C). These experiments demonstrated that homologous recombination had occurred specifically at the functional *PPIA* gene and that CypA-null cell lines had been produced.

Initially, the growth rate of $PPIA^{+/-}$ and $PPIA^{-/-}$ cell lines was compared with that of $PPIA^{+/+}$ cells by measuring incorporation of [^{3}H]thymidine; no differences were observed (data not shown). The cell lines were also compared for cell surface expression of CD4 and CXCR4, which are required for both attachment and entry of HIV-1 into Jurkat T cells; again, no significant differences were observed between the cell lines in these assays (data not shown).

Decreased replication of HIV-1 in $PPIA^{-/-}$ Jurkat T cells

We next compared the $PPIA^{+/+}$, $PPIA^{+/-}$ and $PPIA^{-/-}$ cell lines for replication of HIV-1_{NL4-3} (Figure 2A). In $PPIA^{+/+}$ and $PPIA^{+/-}$ cells, replication of HIV-1_{NL4-3} was the same, with the onset of exponential kinetics by day 3 and the peak of virus production at day 9; since the relative amount of CypA was similar in the two cell lines (Figure 1C), we had expected the kinetic profiles to be the same. In contrast to $PPIA^{+/+}$ and $PPIA^{+/-}$ cells, in $PPIA^{-/-}$ cells the onset of exponential growth of HIV-1_{NL4-3} was significantly delayed, by between 4 and 5 days (Figure 2A).

To determine whether decreased HIV-1 replication in $PPIA^{-/-}$ cells was peculiar to the laboratory-adapted viral strain HIV-1_{NL4-3}, we tested HIV-1_{Eli}, an isolate that was cloned directly from peripheral blood mononuclear cells of an infected individual (Peden *et al*., 1991). Replication of HIV-1_{Eli} in $PPIA^{-/-}$ cells was, if anything, even more decreased than that of HIV-1_{NL4-3} (Figure 2B).

The observation that reverse transcriptase activity eventually accumulated to wild-type level in the medium

of *PPIA*$^{-/-}$ cells infected with HIV-1_{NL4-3} could be explained by the appearance of a viral clone bearing a gain-of-function mutation resulting in replication that is independent of CypA. Such 'CypA-independent' mutants of HIV-1 have in fact been selected by serial passage of HIV-1 in the presence of a competitive inhibitor of the Gag–cyclophilin interaction (Aberham *et al*., 1996; Braaten *et al*., 1996a). To test this possibility, samples of virus taken from the peak of infection of *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells (days 9 and 14, respectively) with HIV-1_{NL4-3} were used to infect fresh *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells; the replication kinetics in the re-infection were the same as in the initial experiment (data not shown). In addition, RT–PCR sequencing of the region of *gag* that encodes residues required for binding to (Gamble *et al*., 1996) and which determines the functional dependence on (Aberham *et al*., 1996; Braaten *et al*., 1996a) CypA was performed on virion RNA isolated from the peak of infection. No mutations were observed (data not shown). Thus, neither re-initiated infections nor the results of RT–PCR sequencing indicated that virus had been produced with a gain-of-function mutation.

Replication of HIV-$1_{NL4-3/A224E}$ and HIV-2/SIV_{SM} is not defective in PPIA$^{-/-}$ cells

A possible explanation for the decreased replication of HIV-1 in the *PPIA*$^{-/-}$ Jurkat clones is that the cells are non-permissive for replication of any virus, irrespective of the viruses' functional reliance on CypA. To rule out this possibility, we tested *PPIA*$^{-/-}$ cells for replication of HIV-$1_{NL4-3/A224E}$, one of the CypA-independent HIV-1 mutants that was selected by serial passage of virus in the presence of an analog of cyclosporin (Aberham *et al*., 1996; Braaten *et al*., 1996a). HIV-$1_{NL4-3/A224E}$ replicated very similarly in both *PPIA*$^{-/-}$ and *PPIA*$^{+/+}$ cells (Figure 3A). We also tested *PPIA*$^{-/-}$ cells for replication of HIV-2_{77618}, HIV-2_{GB122}, HIV-2_{7312A} and $SIV_{SMpbj1.9}$ (Dewhurst *et al*., 1990; Owen *et al*., 1998), viruses related to HIV-1 but that have no known functional interaction with cyclophilins (Franke *et al*., 1994; Thali *et al*., 1994; Braaten *et al*., 1996c). All of the HIV-2/SIV_{SM} viruses replicated the same in *PPIA*$^{-/-}$ and *PPIA*$^{+/+}$ cells (Figure 3B and C; data not shown). These data demonstrating that the *PPIA*$^{-/-}$ Jurkat clones are fully permissive for the replication of viruses closely related to wild-type HIV-1 are consistent with the interpretation that the reduced replication seen for HIV-1 is a specific consequence of the engineered mutation.

Replication of HIV-1 in PPIA$^{-/-}$ cells is not inhibited further by conditions that abrogate the Gag–cyclophilin interaction

Given our finding that CypA regulates but is not essential for replication of HIV-1_{NL4-3}, and given that many cyclophilin family members bind to Gag (Table I) (Luban *et al*., 1993; Franke *et al*., 1994), it was possible that cyclophilins other than CypA might promote HIV-1 replication in the *PPIA*$^{-/-}$ Jurkat cells. Cyclosporin, an immunosuppressive compound with high affinity for the hydrophobic pocket of most cyclophilins, competitively inhibits HIV-1 Gag binding to cyclophilin family members (Luban *et al*., 1993; Franke *et al*., 1994; Rosenwirth *et al*., 1994; Thali *et al*., 1994; Steinkasserer *et al*., 1995; Braaten *et al*., 1996b, 1997; Franke and Luban, 1996), thereby effectively inhibiting HIV-1 replication. Thus, to determine whether HIV-1 replication in *PPIA*$^{-/-}$ cells is dependent upon other cyclophilin family members, we compared *PPIA*$^{-/-}$ and *PPIA*$^{+/+}$ cells for replication of HIV-1_{NL4-3} in the presence of methyl-Ile4-cyclosporin, an even more effective inhibitor of HIV-1 replication than the parent compound cyclosporin (Rosenwirth *et al*., 1994; Thali *et al*., 1994; Franke and Luban, 1996). In *PPIA*$^{+/+}$ cells, 2.5 μM methyl-Ile4-cyclosporin inhibited replication of HIV-1_{NL4-3} between 100- and 1000-fold compared with no drug (Figure 4A); under the same conditions, replication of HIV-2 was unaffected by the drug (Figure 4B), consistent with what has been published previously (Thali *et al*., 1994). In contrast, in *PPIA*$^{-/-}$ cells, 2.5 μM methyl-Ile4-cyclosporin inhibited replication of HIV-1_{NL4-3} <10-fold compared with no drug (Figure 4C) and, as in *PPIA*$^{+/+}$ cells, replication of HIV-2 was unaffected by the drug (Figure 4D). Importantly, the magnitude and kinetics of HIV-1 replication in the presence of 2.5 μM methyl-Ile4-cyclosporin were very similar in *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells (compare Figure 4A and C).

In addition to abrogation by competitive inhibitor, interaction between HIV-1 Gag and cyclophilin family members is significantly diminished by particular *gag* mutations, e.g. G221A and P222A (Franke *et al*., 1994; Braaten *et al*., 1996b, 1997). Viruses bearing these mutations, HIV-$1_{NL4-3/G221A}$ and HIV-$1_{NL4-3/P222A}$, would thus be predicted to replicate poorly, but any replication capacity that they exhibited would be cyclophilin independent. If these viruses are independent of cyclophilins, their replication should be the same in

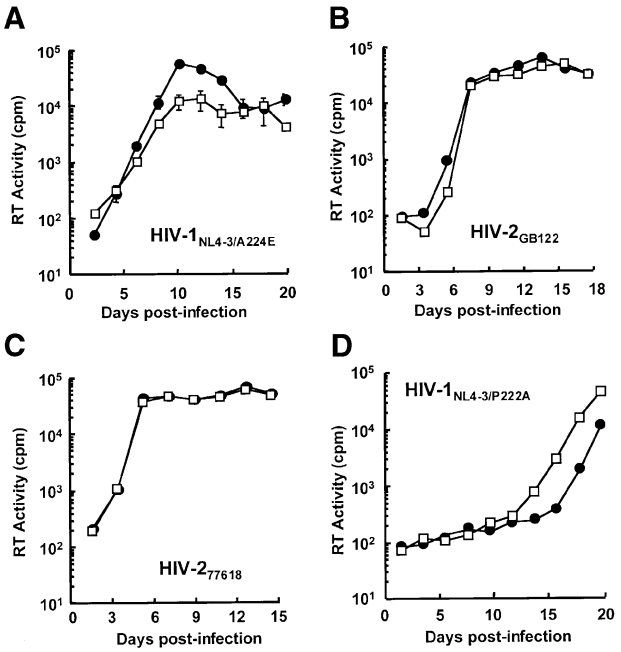

Fig. 3. The decreased replication phenotype in *PPIA*$^{-/-}$ Jurkat T cells is specific to wild-type HIV-1. Replication of HIV-$1_{NL4-3/A224E}$ (**A**), HIV-2_{GB122} (**B**), HIV-2_{77618} (**C**) or HIV-$1_{NL4-3/P222A}$ (**D**) is nearly the same in *PPIA*$^{+/+}$ (filled circles) and *PPIA*$^{-/-}$ cells (open squares), indicating that replication of these viruses is independent of CypA.

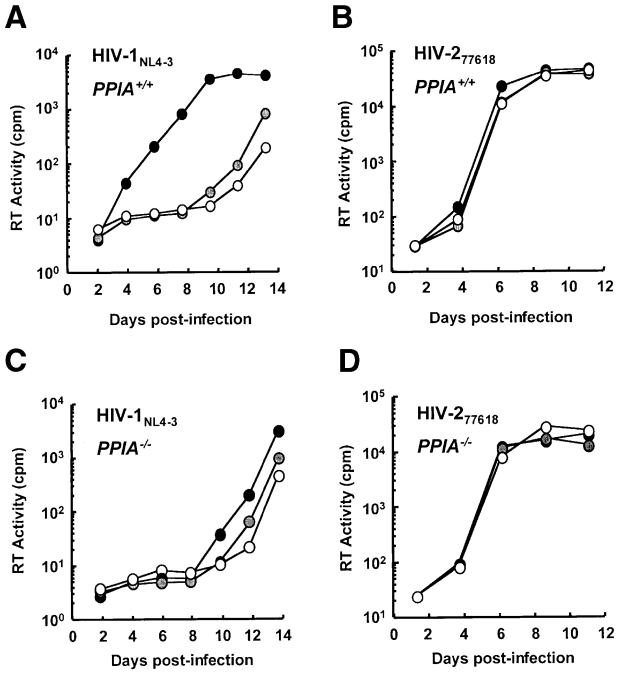

Fig. 4. HIV-1 replication in *PPIA*$^{-/-}$ cells is insensitive to a competitive inhibitor of the Gag–cyclophilin interaction. Effect of methyl-Ile4-cyclosporin on replication of HIV-1_{NL4-3} (**A**) and HIV-2_{77618} (**B**) in *PPIA*$^{+/+}$ cells. Effect of methyl-Ile4-cyclosporin on replication of HIV-1_{NL4-3} (**C**) and HIV-2_{77618} (**D**) in *PPIA*$^{-/-}$ cells. No drug (filled symbols), 1.25 μM (shaded symbols) and 2.5 μM (open symbols) methyl-Ile4-cyclosporin.

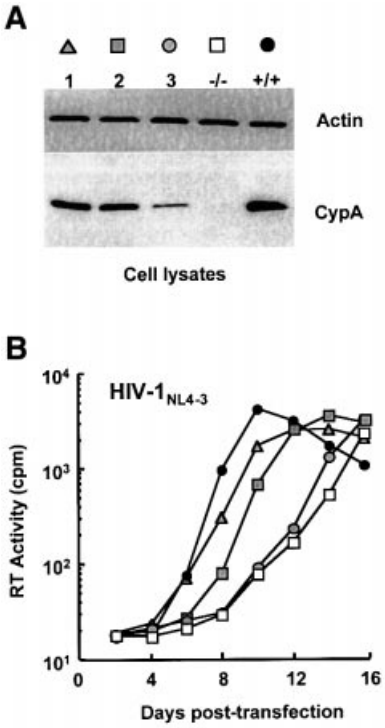

Fig. 5. HIV-1 replication kinetics depend on the level of CypA expression. (**A**) Western blot of total cell lysates showing the relative level of CypA in the cell lines. (**B**) Replication of HIV-1_{NL4-3} in *PPIA*$^{+/+}$ cells (filled circles), in three cell lines derived from *PPIA*$^{-/-}$ cells stably re-expressing CypA (shaded symbols) and in *PPIA*$^{-/-}$ cells (open squares). Higher CypA expression levels correlate with more rapid replication kinetics.

PPIA$^{+/+}$ and *PPIA*$^{-/-}$ cells. These predictions were confirmed, as replication of HIV-$1_{NL4-3/G221A}$ and HIV-$1_{NL4-3/P222A}$ in *PPIA*$^{+/+}$ cells was similar to that of wild-type HIV-1_{NL4-3} in *PPIA*$^{-/-}$ cells, but abrogated no further in *PPIA*$^{-/-}$ cells (Figure 3D and data not shown).

Taken together, the results with methyl-Ile4-cyclosporin and with the *gag* mutant viruses are consistent with the conclusion that no cyclophilins other than CypA promote HIV-1 replication.

Rescue of HIV-1 replication kinetics in PPIA$^{-/-}$ *cells by re-introduction of CypA*

If the absence of CypA expression is the sole cause of the defective replication of HIV-1 in *PPIA*$^{-/-}$ Jurkat cells, re-expression of CypA in the cells should restore the replication kinetics to wild type. To test this, *PPIA*$^{-/-}$ cells were transfected with a CypA expression plasmid, and cell lines expressing CypA were derived by limit-dilution cloning; western blot analysis confirmed the presence of CypA in individual clones (Figure 5A). The clones were screened for replication of HIV-2 to eliminate those with general defects unrelated to CypA; three clones not defective in this assay were then tested for replication of HIV-1. Compared with the parent *PPIA*$^{-/-}$ cells, clones re-expressing CypA demonstrated faster replication kinetics of HIV-1_{NL4-3} (Figure 5B); similar results were obtained with HIV-1_{Eli} as well as with another set of clones re-expressing CypA that had been generated with the second of the two *PPIA*$^{-/-}$ cell lines (data not shown). Importantly, the degree to which the replication kinetics was restored to wild type correlated with the level of CypA expression in the individual clones.

HIV-1 virions produced by PPIA$^{-/-}$ *cells are less infectious than virions from PPIA*$^{+/+}$ *cells*

Previous work has demonstrated that conditions which block CypA binding to Gag, such as treating infected cells with cyclosporin or creating specific point mutations in *gag*, render virions defective at an early step of the viral life cycle before the start of reverse transcription (Braaten *et al*., 1996b). These studies found no defects in the RNA or protein content, or in endogenous reverse transcriptase activity, of CypA-deficient virions (Braaten *et al*., 1996b; Grattinger *et al*., 1999). We performed metabolic labeling and pulse–chase analysis of HIV-1 proteins produced by *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells and observed no significant differences between the cell lines in terms of Gag translation efficiency, protein stability or polyprotein processing (data not shown). In addition, we analyzed, by western blotting, protein constituents of virions (e.g. capsid, matrix, Nef and gp160/120) produced by *PPIA*$^{-/-}$ cells and, except for the absence of CypA, detected no differences from virions produced by *PPIA*$^{+/+}$ cells.

We next measured the relative infectivity of virions produced by *PPIA*$^{-/-}$ cells in a single round of infection. Wild-type and 'CypA-null' virions were harvested from cultures of *PPIA*$^{+/+}$ cells and two *PPIA*$^{-/-}$ clones after 6 and 10 days, respectively, of infection with wild-type HIV-1_{NL4-3}. Similarly, a stock of HIV-$1_{NL4-3/G221A}$ was

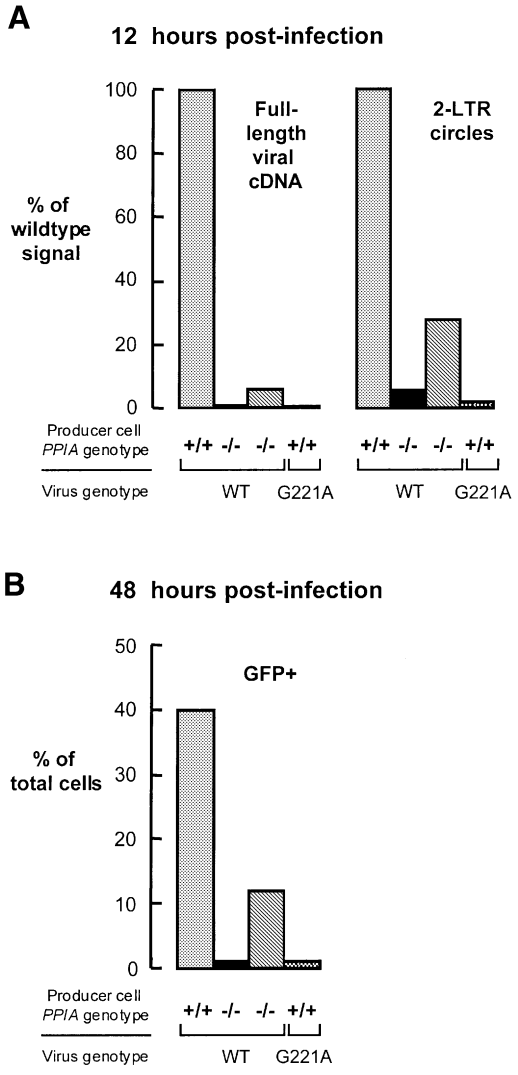

Fig. 6. Virions produced from *PPIA*$^{-/-}$ cells are less infectious than those produced from *PPIA*$^{+/+}$ cells. Virus stocks (wild-type HIV-1_{NL4-3} and HIV-$1_{NL4-3/G221A}$) were produced in either *PPIA*$^{+/+}$ or *PPIA*$^{-/-}$ cells (as indicated) and used to infect equal numbers of GHOST cells. (**A**) Low molecular weight DNA was isolated 12 h post-infection. Real-time PCR was performed to quantitate full-length viral cDNA and 2-LTR circles. The relative levels of products (percentage of wild-type signal) are shown. (**B**) The percentage of GFP$^+$ cells (of total cells) was determined by flow cytometry 48 h post-infection.

produced from *PPIA*$^{+/+}$ cells; this virus bears one of the *gag* mutations that significantly reduces binding to and virion incorporation of CypA. All virions produced from the *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ infections were purified and normalized by exogenous reverse transcriptase assay. GHOST cells (Morner *et al*., 1999), a CD4$^+$/CCR5$^+$/CXCR4$^+$ human osteosarcoma cell line containing an LTR-*gfp* reporter gene, were infected with the virus stocks and then processed either for the presence of reverse transcriptase products by real-time PCR (12 h post-infection) or for expression of green fluorescent protein (GFP) by flow cytometry (48 h post-infection).

Both full-length viral cDNA (indicative of completion of reverse transcription) and 2-LTR circles (indicative of nuclear translocation of the pre-integration complex) were measured by real-time PCR. As a percentage of product measured for wild-type HIV-1_{NL4-3} produced in *PPIA*$^{+/+}$ cells, both full-length cDNA and 2-LTR circles for wild-type HIV-1_{NL4-3} produced in the two *PPIA*$^{-/-}$ cell lines were significantly reduced (Figure 6A). The apparent difference in magnitude seen in virus produced by the two *PPIA*$^{-/-}$ clones appears not to be a fixed property of the cell lines, as experimental variation in this range was observed. As expected from previous work (Braaten *et al*., 1996b), full-length cDNA and 2-LTR circles were reduced for HIV-$1_{NL4-3/G221A}$ as well (Figure 6A).

The GHOST cells were also screened by flow cytometry for expression of GFP, which, in these cells, is indicative of HIV-1 integration. Relative to the 40% GFP-positive cells for wild-type HIV-1_{NL4-3} produced in *PPIA*$^{+/+}$ cells (Figure 6B), the number of GFP-positive cells for wild-type HIV-1_{NL4-3} produced in the two *PPIA*$^{-/-}$ cell lines was between 4- and 10-fold lower (the same difference as that for 2-LTR circles), similar to the percentage of GFP-positive cells for HIV-$1_{NL4-3/G221A}$ produced in *PPIA*$^{+/+}$ cells. These results indicate that virions produced by *PPIA*$^{-/-}$ cells are defective at some early step of the virus life cycle before, or concurrent with, reverse transcription.

Discussion

We have produced *PPIA*$^{-/-}$ cell lines by homologous recombination in the widely used Jurkat T-cell line. As reagents for studying the biology of HIV-1, these cells have enabled us to address several issues concerning the role of cyclophilins in the replication of HIV-1.

CypA is required for wild-type HIV-1 replication kinetics

Since we were unable to identify CypA-null conditions with which to study HIV-1 replication, demonstration of the functional relevance of CypA necessitated the generation of *PPIA*$^{-/-}$ cells by homologous recombination in a human T-cell line. Our targeting efficiency was low, but within the range previously reported for somatic cell targeting with non-isogenic DNA (Sedivy *et al*., 1999). Once the *PPIA*$^{-/-}$ cells were generated, HIV-1 replication was then rescued with a CypA expression plasmid; this 'rescue experiment', in which CypA was formally proven to be a determinant of HIV-1 replication kinetics, is analagous in outline to studies proving that CD4 and chemokine receptors are essential viral entry factors (Maddon *et al*., 1986; Feng *et al*., 1996). Other than the host *N*-myristoyltransferase, a protein that catalyzes a co-translational modification required for Gag polyprotein targeting to the inner face of the plasma membrane (Göttlinger *et al*., 1989; Bryant and Ratner, 1990), CypA is perhaps the first Gag-associated host factor that has been clearly demonstrated to play a significant role in HIV-1 replication.

Replication of primary isolate HIV-1_{Eli} was found to be even more decreased in *PPIA*$^{-/-}$ cells than was replication of the laboratory-adapted strain HIV-1_{NL4-3}. This suggests the intriguing possibility that primary isolates of HIV-1 as a group are more functionally dependent on CypA than are laboratory-adapted strains, which in turn may be indicative of a greater importance of CypA for HIV-1 replication kinetics *in vivo* than *in vitro*. This would not be

unprecedented since a number of HIV-1 factors appear more important *in vivo* than in tissue culture. As but one example, *vpr* is selected against in tissue culture but selected for *in vivo* (Goh *et al*., 1998). As is the case with *vpr*, Gag's interaction with CypA might also contribute indirectly to viral replication *in vivo* via effects on antiviral immunity, although the studies here do not address this possibility.

CypA regulates HIV-1 virion infectivity

We have detected no significant biochemical abnormalities in virions produced from the *PPIA*$^{-/-}$ cells. These results are in keeping with previous studies in which the Gag–cyclophilin interaction was disrupted using *gag* mutations or cyclosporin (Franke *et al*., 1994; Thali *et al*., 1994; Braaten *et al*., 1996b; Saphire *et al*., 1999). We have also confirmed that virions produced by *PPIA*$^{-/-}$ cells are defective at an early stage of the virus life cycle. These data do not, however, pinpoint exactly where CypA is acting. Virions produced under conditions that block the Gag–CypA interaction exhibit normal endogenous reverse transcriptase activity *in vitro* (Braaten *et al*., 1996b), yet it is still possible that virion-associated CypA directly modifies the reverse transcription machinery in a manner that is only evident during entry into a susceptible target cell. Virion-associated CypA might be required during the process of virion uncoating (Luban, 1996), or for virion binding or fusion to target cells (Saphire *et al*., 1999). In addition, the data do not rule out the possibility that the defect observed before the start of reverse transcription is a consequence of CypA deficiency during the preceding virion assembly process.

HIV-1 Gag is one of a small group of proteins that have been shown to bind CypA. These proteins include Hsp90 (Nadeau *et al*., 1993), the transcription factor YY1 (Yang *et al*., 1995), the antioxidant protein Aop1 (Jaschke *et al*., 1998) and members of the Sin3–Rpd3 histone deacetylase complex (Arevalo-Rodriguez *et al*., 2000). Whether peptidyl-prolyl bonds in Gag or any of these proteins undergo functionally significant, CypA-dependent isomerization is unknown. Unfortunately, genetic analysis of this question has provided no simple answer in that all mutants disruptive of CypA's peptidyl-prolyl isomerase activity also disrupt Gag-binding activity (Braaten *et al*., 1997; Dorfman *et al*., 1997). Deciphering the biochemical role of CypA in HIV-1 replication may simply require a detailed understanding of the conformational changes undergone by *gag*-encoded proteins from the point of translation until viral entry.

Other cyclophilin family members do not substitute for CypA in promoting HIV-1 replication

The availability of *PPIA*$^{-/-}$ cell lines has also provided the opportunity to assess the contribution to HIV-1 replication of cyclophilin family members other than CypA. Based on the conservation of amino acid residues shown to be required for CypA binding to cyclosporin (Zydowsky *et al*., 1992), most of the other cyclophilins would be expected to have the same relative affinity for cyclosporin, and thus would be blocked by the drug from binding to HIV-1 Gag. Thus, if one of these cyclophilins were capable of substituting functionally for CypA, replication of HIV-1 in *PPIA*$^{-/-}$ cells should be significantly inhibited in the presence of methyl-Ile4-cyclosporin, as it is in *PPIA*$^{+/+}$ cells. However, it is not, which indicates that no other cyclophilin substitutes for CypA in HIV-1 replication. Consistent with this conclusion is our finding that HIV-$1_{NL4\text{-}3/G221A}$ and HIV-$1_{NL4\text{-}3/P222A}$, viruses bearing mutations that disrupt Gag binding to all cyclophilins, replicate as well as wild-type virus in *PPIA*$^{-/-}$ cells.

A few of the known human cyclophilins, Cyp40, Cyp60, CARS-CyP and NK-TRCyP (Table I), lack a tryptophan corresponding to Trp121 of CypA and thus would possibly be less sensitive to cyclosporin and its analogs (Liu and Walsh, 1990; Bossard *et al*., 1991; Liu *et al*., 1991a; Zydowsky *et al*., 1992); consequently, one of these cyclophilins might interact with HIV-1 Gag in the presence of the drugs. Even if this does occur, the replication data presented here demonstrate that such a cyclophilin is not functionally equivalent to CypA.

CypA is not essential for viability of human T cells

Finally, despite being present at micromolar concentrations (Koletsky *et al*., 1986), CypA is not essential for viability or growth of the human T-cell lines described here. Similar results have been obtained with disruption of CypA homologs in bacteria, budding yeast, *Neurospora* and mouse (Tropschug *et al*., 1989; Herrler *et al*., 1994; Colgan *et al*., 2000). CypA thus appears to be universally dispensable for protein folding in cells, perhaps due to functional redundancy with other cyclophilin family members or other protein families that exhibit peptidyl-prolyl isomerase activity, such as the FK-binding proteins and parvulins (Heitman, 1997). The viability of a yeast strain harboring null mutations in all eight cyclophilin genes and in all four FK-binding protein genes argues against the latter explanation (Dolinski *et al*., 1997). Our studies do not address whether CypA is required for more specialized functions such as cytokine production or antigen processing.

The fact that CypA is not essential for Jurkat T cells, and that HIV-1 replication kinetics appear to be modulated by CypA expression levels (Figure 5), has important clinical implications. Perhaps analogously to the case of the Δ32 allele of the HIV co-receptor CCR5 (Liu *et al*., 1996), alleles of *PPIA* that result in low or no expression of CypA might be compatible with life, and might protect individuals who carry them from HIV-1 transmission or from progression to AIDS. Our findings also suggest that CypA is a viable cellular target for novel anti-HIV-1 therapeutics.

Materials and methods

Targeting constructs

Primers were designed to amplify across *PPIA* (Haendler and Hofer, 1990) exons 3 and 4 (5′-GCTCTGAGCACTGGAGAGAAAGGATTTGG-3′ and 5′-CACCAGTGCCATTATGGCGTGTGAAGTC-3′) and a region 5′ of the core promoter (5′-GGAATGCGGACGCAAGGCTGCTC-3′ and 5′-GGAATTCGCAAAACGGCACGAGCCTGGCCTCC-3′). These primers were used to screen a human foreskin fibroblast, P1 phage library (Genome Systems, St Louis, MO). Three P1 clones were obtained from the screen, each with ~80–90 kb of contiguous genomic DNA inclusive of the *PPIA* locus. One clone (Genome Systems control #4149) was used for constructing the 'Neo' and 'Hygro' targeting plasmids (Figure 1).

Gene targeting by homologous recombination

Linearized targeting constructs were electroporated into 10^7 Jurkat cells (ATCC #TIB 152), and grown in RPMI with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Two rounds of electroporation and selection were required to obtain *PPIA*$^{-/-}$ cells. *PPIA*$^{+/+}$ cells were electroporated with the Neo construct, followed by selection in G418 (700 μg/ml), to produce *PPIA*$^{+/-}$ cell lines; *PPIA*$^{+/-}$ clones were electroporated with the Hygro targeting construct, followed by selection in both hygromycin (400 μg/ml) and G418 (700 μg/ml), to produce *PPIA*$^{-/-}$ cell lines. After a given electroporation, cells were allowed to recover for 36 h in fresh medium and then viable cells were aliquoted in medium plus antibiotics into 96-well, round bottom plates (Nunc) at a concentration of cells that would result in cultures growing in 30% (or less) of wells of a given 96-well plate. Replica plates of cultures were stored at –70°C in RPMI with 20% FBS, penicillin/streptomycin and 10% dimethylsulfoxide (DMSO; Sigma).

Stable cell lines re-expressing CypA were derived from limit-dilution cloning in 1.5 mg/ml puromycin (Sigma) of *PPIA*$^{-/-}$ cells electroporated with the linearized plasmids pEF1αCypA (10 μg) and pPGK-puromycin (2 μg).

Southern blot hybridization

Drug-resistant clones were screened for homologous recombination by Southern blot hybridization of total cellular DNAs made directly in 96-well plates. Cell cultures were centrifuged (500 r.p.m.) for 2 min, washed with phosphate-buffered saline (PBS) and lysed in 50 μl of 10 mM NaCl, 10 mM Tris pH 7.5, 20 mM EDTA, 0.5% sarcosyl and 1 mg/ml freshly added proteinase K; the 96-well plates were then placed at 50°C for 18 h. DNAs were precipitated by adding 15 μl of 5 M NaCl and 100 μl of absolute ethanol per well; the precipitates were pelleted by centrifugation at 4000 r.p.m. for 15 min, washed three times with 70% ethanol and then air-dried. Digestion cocktail [40 μl per well of 50 mM potassium acetate, 20 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), 0.1 mM spermidine, 10 U of *Xba*I and 10 U of *Sac*I] was added and the plates were incubated at 37°C for 18–24 h. Digested DNAs were separated in 0.7% agarose gels and then transferred to a Genescreen Plus membrane (NEN). Membranes were incubated with a probe specific for the *PPIA* locus but not contained within either targeting plasmid. The probe was labeled by random hexamer primer synthesis (Megaprime labeling kit, Amersham). Hybridizing fragments were visualized by a PhosphorImager (Molecular Dynamics).

Virology

Virus stocks for infections were produced in 293T cells [grown in Dulbecco's modified Eagle's medium (DMEM)/F12, 10% FBS and penicillin/streptomycin] by transient transfection (Braaten *et al*., 1996b) of the following supercoiled proviral DNAs: HIV-1_{NL4-3} (Adachi *et al*., 1986), HIV-$1_{NL4-3/A224E}$ (Aberham *et al*., 1996; Braaten *et al*., 1996a), HIV-$1_{NL4-3/P222A}$, HIV-$1_{NL4-3/G221A}$ (Franke *et al*., 1994; Braaten *et al*., 1996b) and HIV-1_{Eli} (Peden *et al*., 1991). Virus stocks from *PPIA*$^{+/+}$ and *PPIA*$^{-/-}$ cells were collected at the peak of infection for each cell line and purified by ultracentrafugation, as described (Braaten *et al*., 1996b). All virus stocks were normalized by exogenous reverse transcriptase assay (Braaten *et al*., 1996b). Stocks of primary isolates HIV-2_{77618}, HIV-2_{GB122}, HIV-2_{7312A} (Owen *et al*., 1998) and $SIV_{SMpbj1.9}$ (Dewhurst *et al*., 1990) were normalized for p27 by enzyme-linked immunosorbent assay (ELISA). Infections of Jurkat cells were initiated either by incubation of cells with virus stocks or by transfection of proviral DNA into cells with DEAE–dextran, as described (Braaten *et al*., 1996c). Replication kinetics of all viruses were monitored by exogenous reverse transcriptase assay (Braaten *et al*., 1996b). Drug inhibition studies were performed as previously described (Franke and Luban, 1996).

Single-round infectivity assays

GHOST cells (NIH AIDS Research & Reference Reagent Program, Catalog #3943) were incubated with virus stocks for 2 h, as described (Braaten *et al*., 1996b), and then infections were allowed to proceed for the times indicated. Low molecular weight DNA preps were made (Hirt, 1967), and real-time PCR was performed for full-length viral cDNA and 2-LTR circles (Cimarelli *et al*., 2000). To detect GFP expression, 48 h post-infection GHOST cells were trypsinized, washed with PBS, resuspended in 2% paraformaldehyde and then incubated at 4°C for 24 h. The number of GFP-positive cells was measured by flow cytometry (FACScalibur, Becton Dickinson).

Antibodies and western blot analysis

Proteins were separated in 12% SDS–polyacrylamide gels and then transferred to PVDF membranes (NEN Life Sciences). The membranes were incubated with anti-CyPA (Affinity Bioreagents) and anti-actin (Sigma) primary antibodies and secondary horseradish peroxidase-coupled anti-rabbit Ig antibody (Promega). The secondary antibody was visualized with a luminol reagent (Renaissance®, from Dupont NEN).

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References

Aberham,C., Weber,S. and Phares,W. (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.

Adachi,A., Gendelman,H.E., Koenig,S., Folks,T., Willey,R., Rabson,A. and Martin,M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.*, **59**, 284–291.

Agostini,I., Popov,S., Li,J., Dubrovsky,L., Hao,T. and Bukrinsky,M. (2000) Heat-shock protein 70 can replace viral protein R of HIV-1 during nuclear import of the viral preintegration complex. *Exp. Cell Res.*, **259**, 398–403.

Agresta,B.E. and Carter,C.A. (1997) Cyclophilin A-induced alterations of human immunodeficiency virus type 1 CA protein *in vitro*. *J. Virol.*, **71**, 6921–6927.

Arevalo-Rodriguez,M., Cardenas,M.E., Wu,X., Hanes,S.D. and Heitman,J. (2000) Cyclophilin A and Ess1 interact with and regulate silencing by the Sin3–Rpd3 histone deacetylase. *EMBO J.*, **19**, 3739–3749.

Bossard,M.J., Koser,P.L., Brandt,M., Bergsma,D.J. and Levy,M.A. (1991) A single Trp121 to Ala121 mutation in human cyclophilin alters cyclosporin A affinity and peptidyl-prolyl isomerase activity. *Biochem. Biophys. Res. Commun.*, **176**, 1142–1148.

Braaten,D., Aberham,C., Franke,E.K., Yin,L., Phares,W. and Luban,J. (1996a) Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. *J. Virol.*, **70**, 5170–5176.

Braaten,D., Franke,E.K. and Luban,J. (1996b) Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. *J. Virol.*, **70**, 3551–3560.

Braaten,D., Franke,E.K. and Luban,J. (1996c) Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV(CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. *J. Virol.*, **70**, 4220–4227.

Braaten,D., Wellington,S., Warburton,D. and Luban,J. (1996d) Assignment of cyclophilin A (PPIA) to human chromosome band 7p13 by *in situ* hybridization. *Cytogenet. Cell Genet.*, **74**, 262.

Braaten,D., Ansari,H. and Luban,J. (1997) The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. *J. Virol.*, **71**, 2107–2113.

Bristow,R., Byrne,J., Squirell,J., Trencher,H., Carter,T., Rodgers,B., Saman,E. and Duncan,J. (1999) Human cyclophilin has a significantly higher affinity for HIV-1 recombinant p55 than p24. *J. AIDS Hum. Retrovirol.*, **20**, 334–336.

Bryant,M. and Ratner,L. (1990) Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl Acad. Sci. USA*, **87**, 523–527.

Cimarelli,A. and Luban,J. (1999) Translation elongation factor 1-α interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. *J. Virol.*, **73**, 5388–5401.

Cimarelli,A., Sandin,S., Hoglund,S. and Luban,J. (2000) Rescue of multiple viral functions by a second-site suppressor of a human

immunodeficiency virus type 1 nucleocapsid mutation. *J. Virol.*, **74**, 4273–4283.

Colgan,J., Asmal,M. and Luban,J. (2000) Isolation, characterization and targeted disruption of mouse *ppia*: cyclophilin A is not essential for mammalian cell viability. *Genomics*, **68**, 167–178.

Dewhurst,S., Embretson,J.E., Anderson,D.C., Mullins,J.I. and Fultz,P.N. (1990) Sequence analysis and acute pathogenicity of molecularly cloned SIVSMM-PBj14. *Nature*, **345**, 636–640.

Dolinski,K., Muir,S., Cardenas,M. and Heitman,J. (1997) All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **94**, 13093–13098.

Dorfman,T., Weimann,A., Borsetti,A., Walsh,C.T. and Gottlinger,H.G. (1997) Active-site residues of cyclophilin A are crucial for its incorporation into human immunodeficiency virus type 1 virions. *J. Virol.*, **71**, 7110–7113.

Feng,Y., Broder,C.C., Kennedy,P.E. and Berger,E.A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, **272**, 872–877.

Fischer,G., Wittmann-Liebold,B., Lang,K., Kiefhaber,T. and Schmid,F.X. (1989) Cyclophilin and peptidyl-prolyl *cis–trans* isomerase are probably identical proteins. *Nature*, **337**, 476–478.

Franke,E.K. and Luban,J. (1996) Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag–cyclophilin A interaction. *Virology*, **222**, 279–282.

Franke,E.K., Yuan,H.E. and Luban,J. (1994) Specific incorporation of cyclophilin A into HIV-1 virions. *Nature*, **372**, 359–362.

Freed,E.O. (1998) HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology*, **251**, 1–15.

Friedman,J. and Weissman,I. (1991) Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. *Cell*, **66**, 799–806.

Gallay,P., Stitt,V., Mundy,C., Oettinger,M. and Trono,D. (1996) Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J. Virol.*, **70**, 1027–1032.

Gamble,T.R., Vajdos,F.F., Yoo,S., Worthylake,D.K., Houseweart,M., Sundquist,W.I. and Hill,C.P. (1996) Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. *Cell*, **87**, 1285–1294.

Goh,W.C., Rogel,M.E., Kinsey,C.M., Michael,S.F., Fultz,P.N., Nowak,M.A., Hahn,B.H. and Emerman,M. (1998) HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr *in vivo*. *Nature Med.*, **4**, 65–71.

Göttlinger,H.G., Sodroski,J.G. and Haseltine,W.A. (1989) Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl Acad. Sci. USA*, **86**, 5781–5785.

Grattinger,M., Hohenberg,H., Thomas,D., Wilk,T., Muller,B. and Krausslich,H.G. (1999) *In vitro* assembly properties of wild-type and cyclophilin-binding defective human immunodeficiency virus capsid proteins in the presence and absence of cyclophilin A. *Virology*, **257**, 247–260.

Haendler,B. and Hofer,E. (1990) Characterization of the human cyclophilin gene and of related processed pseudogenes. *Eur. J. Biochem.*, **190**, 477–482.

Handschumacher,R., Harding,M., Rice,J. and Drugge,R. (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science*, **226**, 544–547.

Heitman,J. (1997) Peptidyl-prolyl isomerases: an overview of the cyclophilin, FKBP and parvulin families. In Gething,M.-J. (ed.), *Guidebook to Molecular Chaperones and Protein-Folding Catalysts.* Oxford University Press, Oxford, UK, pp. 359–367.

Herrler,M., Bang,H. and Marahiel,M.A. (1994) Cloning and characterization of *ppiB*, a *Bacillus subtilis* gene which encodes a cyclosporin A-sensitive peptidyl-prolyl *cis–trans* isomerase. *Mol. Microbiol.*, **11**, 1073–1083.

Hirt,B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.*, **26**, 365–369.

Jaschke,A., Mi,H. and Tropschug,M. (1998) Human T cell cyclophilin18 binds to thiol-specific antioxidant protein Aop1 and stimulates its activity. *J. Mol. Biol.*, **277**, 763–769.

Ke,H.M., Zydowsky,L.D., Liu,J. and Walsh,C.T. (1991) Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution. *Proc. Natl Acad. Sci. USA*, **88**, 9483–9487.

Koletsky,A.J., Harding,M.W. and Handschumacher,R.E. (1986) Cyclophilin: distribution and variant properties in normal and neoplastic tissues. *J. Immunol.*, **137**, 1054–1059.

Lama,J. and Trono,D. (1998) Human immunodeficiency virus type 1 matrix protein interacts with cellular protein HO3. *J. Virol.*, **72**, 1671–1676.

Liu,B., Dai,R., Tian,C.J., Dawson,L., Gorelick,R. and Yu,X.F. (1999) Interaction of the human immunodeficiency virus type 1 nucleocapsid with actin. *J. Virol.*, **73**, 2901–2908.

Liu,J. and Walsh,C.T. (1990) Peptidyl-prolyl *cis–trans*-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proc. Natl Acad. Sci. USA*, **87**, 4028–4032.

Liu,J., Chen,C.M. and Walsh,C.T. (1991a) Human and *Escherichia coli* cyclophilins: sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue. *Biochemistry*, **30**, 2306–2310.

Liu,J., Farmer,J.D.,Jr, Lane,W.S., Friedman,J., Weissman,I. and Schreiber,S.L. (1991b) Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. *Cell*, **66**, 807–815.

Liu,R. *et al.* (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell*, **86**, 367–377.

Luban,J. (1996) Absconding with the chaperone: essential cyclophilin–Gag interaction in HIV-1 virions. *Cell*, **87**, 1157–1159.

Luban,J., Bossolt,K.L., Franke,E.K., Kalpana,G.V. and Goff,S.P. (1993) Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell*, **73**, 1067–1078.

Maddon,P.J., Dalgleish,A.G., McDougal,J.S., Clapham,P.R., Weiss,R.A. and Axel,R. (1986) The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*, **47**, 333–348.

Mikol,V., Kallen,J., Pflugl,G. and Walkinshaw,M.D. (1993) X-ray structure of a monomeric cyclophilin A–cyclosporin A crystal complex at 2.1 Å resolution. *J. Mol. Biol.*, **234**, 1119–1130.

Morner,A., Bjorndal,A., Albert,J., Kewalramani,V.N., Littman,D.R., Inoue,R., Thorstensson,R., Fenyo,E.M. and Bjorling,E. (1999) Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. *J. Virol.*, **73**, 2343–2349.

Nadeau,K., Das,A. and Walsh,C.T. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl-prolyl isomerases. *J. Biol. Chem.*, **268**, 1479–1487.

Ott,D.E. *et al.* (1998) Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. *J. Virol.*, **72**, 2962–2968.

Ott,D.E. *et al.* (2000) Actin-binding cellular proteins inside human immunodeficiency virus type 1. *Virology*, **266**, 42–51.

Owen,S.M., Ellenberger,D., Rayfield,M., Wiktor,S., Michel,P., Grieco,M.H., Gao,F., Hahn,B.H. and Lal,R.B. (1998) Genetically divergent strains of human immunodeficiency virus type 2 use multiple coreceptors for viral entry. *J. Virol.*, **72**, 5425–5432.

Peden,K., Emerman,M. and Montagnier,L. (1991) Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL and HIV-1ELI. *Virology*, **185**, 661–672.

Peytavi,R., Hong,S.S., Gay,B., d'Angeac,A.D., Selig,L., Benichou,S., Benarous,R. and Boulanger,P. (1999) HEED, the product of the human homolog of the murine *eed* gene, binds to the matrix protein of HIV-1. *J. Biol. Chem.*, **274**, 1635–1645.

Radding,W., Williams,J.P., McKenna,M.A., Tummala,R., Hunter,E., Tytler,E.M. and McDonald,J.M. (2000) Calmodulin and HIV type 1: interactions with *gag* and *gag* products. *AIDS Res. Hum. Retroviruses*, **16**, 1519–1525.

Rey,O., Canon,J. and Krogstad,P. (1996) HIV-1 Gag protein associates with F-actin present in microfilaments. *Virology*, **220**, 530–534.

Rosenwirth,B. *et al.* (1994) Inhibition of human immunodeficiency virus type 1 replication by SDZ NIM 811, a nonimmunosuppressive cyclosporine analog. *Antimicrob. Agents Chemother.*, **38**, 1763–1772.

Saphire,A.C., Bobardt,M.D. and Gallay,P.A. (1999) Host cyclophilin A mediates HIV-1 attachment to target cells via heparans. *EMBO J.*, **18**, 6771–6785.

Sedivy,J., Vogelstein,B., Liber,H., Hendrickson,E. and Rosmarin,A. (1999) Gene targeting in human cells without isogenic DNA. *Science*, **283**, 9.

Sherry,B. *et al.* (1998) Role of cyclophilin A in the uptake of HIV-1 by macrophages and T lymphocytes. *Proc. Natl Acad. Sci. USA*, **95**, 1758–1763.

Steinkasserer,A. *et al.* (1995) Mode of action of SDZ NIM 811, a

nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus type 1 (HIV-1): interference with early and late events in HIV-1 replication. *J. Virol.*, **69**, 814–824.

Streblow,D.N., Kitabwalla,M., Malkovsky,M. and Pauza,C.D. (1998) Cyclophilin A modulates processing of human immunodeficiency virus type 1 p55Gag: mechanism for antiviral effects of cyclosporin A. *Virology*, **245**, 197–202.

Takahashi,N., Hayano,T. and Suzuki,M. (1989) Peptidyl-prolyl *cis–trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature*, **337**, 473–475.

Tang,Y., Winkler,U., Freed,E.O., Torrey,T.A., Kim,W., Li,H., Goff,S.P. and Morse,H.C.,III (1999) Cellular motor protein KIF-4 associates with retroviral Gag. *J. Virol.*, **73**, 10508–10513.

Thali,M., Bukovsky,A., Kondo,E., Rosenwirth,B., Walsh,C.T., Sodroski,J. and Gottlinger,H.G. (1994) Functional association of cyclophilin A with HIV-1 virions. *Nature*, **372**, 363–365.

Tropschug,M., Barthelmess,I.B. and Neupert,W. (1989) Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. *Nature*, **342**, 953–955.

Wilk,T., Gowen,B. and Fuller,S.D. (1999) Actin associates with the nucleocapsid domain of the human immunodeficiency virus Gag polyprotein. *J. Virol.*, **73**, 1931–1940.

Wilson,S.A. *et al*. (1999) Cloning and characterization of hIF2, a human homologue of bacterial translation initiation factor 2, and its interaction with HIV-1 matrix. *Biochem. J.*, **342**, 97–103.

Yang,W.M., Inouye,C.J. and Seto,E. (1995) Cyclophilin A and FKBP12 interact with YY1 and alter its transcriptional activity. *J. Biol. Chem.*, **270**, 15187–15193.

Zydowsky,L.D., Etzkorn,F.A., Chang,H.Y., Ferguson,S.B., Stolz,L.A., Ho,S.I. and Walsh,C.T. (1992) Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. *Protein Sci.*, **1**, 1092–1099.

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