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The Human Immunodeficiency Virus Type 1 Capsid p2 Domain Confers Sensitivity to the Cyclophilin-Binding Drug SDZ NIM 811

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Human immunodeficiency virus type 1 (HIV-1) specifically incorporates the host cell peptidyl-prolyl isomerase cyclophilin A into virions via contacts with the capsid (CA) domain of the Gag polyprotein Pr55^{gag}. The immunosuppressant drug cyclosporin A and the nonimmunosuppressive cyclosporin A analog SDZ NIM 811 bind to cyclophilin A and inhibit its incorporation into HIV-1 virions. Both drugs inhibit the virion association of cyclophilin A and the replication of HIV-1 with a similar dose dependence. In contrast, these compounds are inactive against other primate lentiviruses which do not incorporate cyclophilin A, such as simian immunodeficiency virus (SIV). To locate determinants which confer sensitivity to SDZ NIM 811, we generated chimeric proviruses between HIV-1 and SIV$_{mac}$. A hybrid SIV$_{mac}$ which has the CA-p2 domain of the Gag polyprotein replaced by the corresponding domain from HIV-1 replicated in an established CD4$^+$ cell line and in human but not macaque peripheral blood mononuclear cells. The transfer of the HIV-1 CA-p2 domain to SIV$_{mac}$ led to the efficient incorporation of cyclophilin A, and SDZ NIM 811 effectively inhibited both the virion association of cyclophilin A and the spread of the hybrid virus in infected cultures. We conclude that the HIV-1 CA-p2 domain contains determinants which confer the necessity to interact with cyclophilin A for efficient virus replication. Furthermore, our data show that the CA-p2 domain can play a crucial role in species tropism.

The internal structural proteins of human immunodeficiency virus type 1 (HIV-1) enter the virion as part of the Gag polyprotein Pr55^{gag}, which is subsequently cleaved by the viral protease. The Gag cleavage products found in the mature virion include the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins, which are common to all retroviruses, and a peptide (p6^{gag}) which is found only in primate lentiviruses (20). While Pr55^{gag} can form virus-like particles in the absence of other virally encoded proteins, the formation of infectious virions requires the inclusion of the viral glycoproteins, replicative enzymes, and genomic viral RNA (21). In addition, unlike other retroviruses, HIV-1 needs to interact with the host cell protein cyclophilin A to form fully infectious virions (15, 45). A consequence of this interaction is the incorporation of cyclophilin A into HIV-1 virions (15, 30, 45).

Cyclophilin A is an abundant cytosolic member of a ubiquitous family of peptidyl-prolyl *cis-trans* isomerases, termed the cyclophilins, which share a high affinity for the immunosuppressant cyclosporin A (CsA) (13, 18, 44, 46). Protein receptors for the immunosuppressive drugs FK506 and rapamycin form a second family of peptidyl-prolyl *cis-trans* isomerases, termed the FK506-binding proteins (FKBPs), which are structurally unrelated to cyclophilins (19, 40). While the isomerase activity of cyclophilins and FKBPs (collectively termed immunophilins) is potently inhibited by their respective ligands, immunosuppression does not result from the inhibition of this enzymatic activity (4, 41). Rather, CsA-cyclophilin and FK506–FKBP complexes form composite surfaces which interact with and inhibit the enzymatic activity of calcineurin, a phosphatase required for T-cell activation (10, 26, 29, 37). CsA analogs such as SDZ NIM 811 ([Melle^4]cyclosporin), which inhibit the peptidyl-prolyl isomerase activity of cyclophilin A in vitro yet lack immunosuppressive activity in vivo, because the drug-cyclophilin complex does not bind to calcineurin, have been described (35, 41).

Relatively little is known about the physiologic roles of immunophilins in the absence of immunosuppressive drugs. Several reports show that immunophilins can assist protein folding in vitro and in vivo by catalyzing the otherwise slow *cis*/*trans* isomerization of proline imidic peptide bonds (24, 28, 34, 36, 43). Furthermore, in vitro refolding experiments with carbonic anhydrase indicate that cyclophilin A can act as a true chaperone by transiently binding to early-folding intermediates (16). Immunophilins are also known to form more stable complexes with other proteins. For instance, the *Drosophila* cyclophilin homolog NinaA, which is required for the transport of the visual pigment molecule rhodopsin 1 from the endoplasmic reticulum, forms a stable complex with its target protein in vivo (2). FKBP12 stably associates with the ryanodine receptor and with the related inositol 1,4,5-trisphosphate receptor, and dissociation of FKBP12 from the Ca^{2+} channels formed by these two proteins alters channel conductance (6, 9). Furthermore, cyclophilins A and B were shown to stably interact with the HIV-1 Gag polyprotein in vitro (27).

We and others recently demonstrated that the HIV-1 Gag-cyclophilin A interaction is functionally relevant for HIV-1 replication (15, 45). Cyclophilin A is specifically incorporated into HIV-1 virions via an interaction with Pr55^{gag} but is not found in other retroviral particles (15, 45). We showed that the incorporation of cyclophilin A into HIV-1 virions is inhibited by CsA and by the nonimmunosuppressive CsA analog SDZ NIM 811 in a dose-dependent manner (45). Drug-induced reductions in virion-associated cyclophilin A levels are accompanied by significant reductions in virion infectivity (45). In

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contrast, SDZ NIM 811 is inactive against the simian immunodeficiency virus SIV_{mac}, a related primate immunodeficiency virus that does not incorporate cyclophilin A (35, 42, 45). These results indicate that the specific anti-HIV-1 activity of cyclosporins is a direct consequence of their effect on the $Pr55^{gag}$-cyclophilin A interaction.

The virion association of cyclophilin A is mediated by a specific interaction with the CA domain of $Pr55^{gag}$ (45). Within the HIV-1 CA domain, determinants that are specifically required for the Gag-cyclophilin A interaction in vitro have been localized to a 30-amino-acid region that contains a conserved array of proline residues (15). The substitution of a single proline in this array disrupted the in vitro interaction with cyclophilin A, the incorporation of cyclophilin A into virions, and viral replication (15).

The critical role of a proline residue in the interaction with the peptidyl-prolyl isomerase suggested that the CA domain may not only serve as a vehicle which carries cyclophilin A into the virion but may also account for the unique dependence of HIV-1 on the interaction with cyclophilin A. To explore this possibility, we generated *gag* chimeras between HIV-1 and SIV_{mac}. We report here that the transfer of the HIV-1 CA-p2 domain to SIV_{mac} is sufficient to transfer both the ability to incorporate cyclophilin A and sensitivity to SDZ NIM 811. These findings imply that some aspect of HIV-1 CA protein function is uniquely dependent on the interaction with cyclophilin A. We also report that the transfer of the HIV-1 CA-p2 domain to SIV_{mac} allows viral replication in human but not macaque peripheral blood mononuclear cells (PBMC), indicating that the CA protein plays an important role in viral tropism.

MATERIALS AND METHODS

Plasmids. The parental HIV-1 proviral construct used in this study, HXBH10, is a vpu^+ variant of the infectious HXB2 proviral clone (17). HXBH10/R^+ is a vpr^+ variant of HXBH10, which was generated by removing a single-base insertion (nucleotide [nt] 5773) in the *vpr* gene of HXBH10. HXBH10-PR^-, which was used to express the HIV-1 Gag polyprotein $Pr55^{gag}$, is identical to HXBH10 except for a point mutation that inactivates the HIV-1 protease (17). To obtain the chimeric HIV/SIV-CA Gag polyprotein expression construct HXBH10(SIV-CA)-PR^-, the HIV-1 CA-p2 coding region in HXBH10-PR^- (nt 1185 to 1919) was replaced by the corresponding region from $SIV_{mac}239$ (nt 1458 to 2195). To express the parental $SIV_{mac}239$ Gag polyprotein, a fragment containing the *gag* gene of $SIV_{mac}239$ (nt 1080 to 3034) was inserted into HXBH10 between nt 637 and 5228, yielding HXBH10/SIV^{gag} (45). The chimeric SIV/HIV-CA Gag polyprotein expression construct HXBH10/SIV(HIV-CA)^{gag} was then obtained by replacing the CA-p2 coding region of the SIV_{mac} *gag* gene (nt 1458 to 2195) in HXBH10/SIV^{gag} by the corresponding sequence from HIV-1_{HXB2} (nt 1185 to 1919). The transferred HIV-1 CA-p2 coding sequence contained a translationally silent mutation (G to A) at nt 1442 to eliminate the *Sph*I site in the HIV-1_{HXB2} CA coding region. The HIV-1 and SIV_{mac} CA-p2 sequences were transferred by using blunt-end cloning sites, which were generated at the domain boundaries by site-directed mutagenesis (Fig. 1). The introduced cloning sites either did not alter the nucleotide sequence of the fused *gag* coding regions or generated only translationally silent mutations. The cloning sites for the construction of the HIV/SIV-CA *gag* gene were generated with oligonucleotides 5′-GCCAAAATT ACGTAATAGTGCAGAAC-3′ (*Sna*BI site at the HIV-1 MA-CA boundary), 5′-GAGGAGGAAATAGGCCTGTACAACAAA-3′ (*Stu*I site at the SIV_{mac} MA-CA boundary), 5′-CCTTTTGCAGCTGCCCAACAGAG-3′ (*Pvu*II site at the SIV_{mac} p2-NC boundary), and 5′-TCAGCTACCATGGCCATGCAGAGA GGC-3′ (*Msc*I site at the HIV-1 p2-NC boundary). The oligonucleotides used for the construction of the reciprocal SIV/HIV-CA *gag* gene were 5′-GAGGAAAT TACGTAGTACAACAA-3′ (*Sna*BI site at the SIV_{mac} MA-CA boundary), 5′-GTCAGCCAAAATAGGCCTATAGTGC-3′ (*Stu*I site at the HIV-1 MA-CA boundary), 5′-GCTACCATAATGGCCAAGAGAGGCAAT-3′ (*Msc*I site at the HIV-1 p2-NC boundary), 5′-CCTTTTGCAGGAGCTCAACAGAGG-3′ (*Ecl*136II site at the SIV_{mac} p2-NC boundary).

For virus replication studies, the chimeric HIV/SIV-CA *gag* gene was inserted into the infectious HXBH10 proviral construct in place of the HIV-1 *gag* gene, yielding HXBH10(SIV-CA). Full-length wild-type $SIV_{mac}239$ proviral DNA was obtained by ligating the two SIV_{mac} segments contained on p239SpSp5′ and p239SpE3′ (23). The chimeric SIV/HIV-CA *gag* gene was inserted into p239SpSp5′ in place of the SIV_{mac} *gag* gene, yielding p239SpSp5′(HIV-CA).

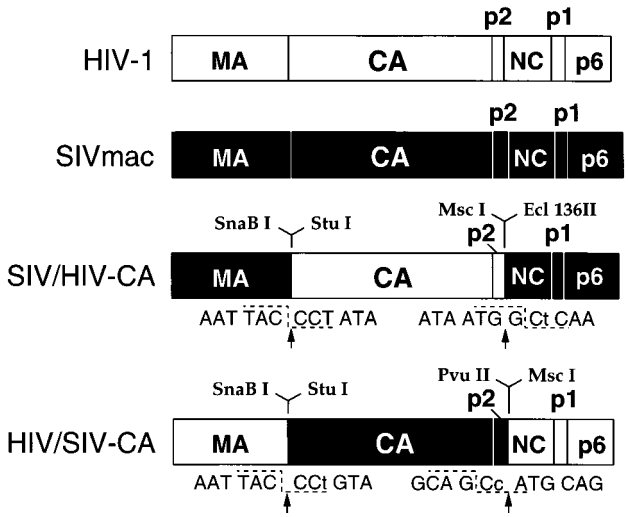

FIG. 1. Domain organization of the parental and chimeric *gag* gene products. Coding sequences for HIV-1 domains (open boxes) and SIV_{mac} domains (solid boxes) were fused without altering the predicted amino acid sequences of individual domains. The codons for the P2 to P2′ positions of proposed chimeric cleavage sites are shown, and domain boundaries are indicated by arrows. The ends of DNA fragments that were joined by blunt-end ligation are separated by dashed lines. The fragments were obtained by cleavage at the indicated restriction enzyme sites which were generated by site-directed mutagenesis. Translationally silent nucleotide substitutions introduced to generate these restriction sites are indicated by lowercase letters.

Full-length $SIV_{mac}239$(HIV-CA) proviral DNA was then obtained by ligating p239SpSp5′(HIV-CA) and p239SpE3′.

Viral protein analysis. HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and transfected with proviral DNA by a calcium phosphate precipitation technique as described previously (12). The cultures were metabolically labeled with [^{35}S]methionine (50 μCi/ml) from 48 to 60 h posttransfection. Virions released into the supernatant were pelleted through a 20% sucrose cushion (in phosphate-buffered saline) for 90 min at 4°C and 27,000 rpm in a Beckman SW 41 rotor. Pelleted viral particles were lysed in RIPA buffer (140 mM NaCl, 8 mM Na_2HPO_4/2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]) and analyzed either directly by electrophoresis through SDS–12.5% polyacrylamide gels or by immunoprecipitation with a rabbit anti-human cyclophilin A antiserum prior to electrophoresis (45).

Virus replication studies. CEMx174 cells (5×10^6) were transfected by the DEAE-dextran method as described previously (25) with either 5 μg of the full-length proviral construct HXBH10(SIV-CA) or 5 μg of p239SpSp5′ or p239SpSp5′(HIV-CA) ligated to 5 μg of p239SpE3′ at a common SpH I site. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, and the reverse transcriptase activity in the culture supernatants was measured daily as described previously (25). Virus-containing supernatants were harvested at day 10, passed through 0.45-μm syringe filters, and stored at −80°C. Thawed supernatant containing 4×10^4 cpm of reverse transcriptase activity was used to infect 8×10^6 fresh CEMx174 cells or PBMC. The cells were exposed to virus-containing supernatant for 1 h, and the cultures were then split and maintained in the absence or presence of SDZ NIM 811 at 0.5 μM. PBMC were isolated from the blood of healthy humans or rhesus macaques by separation on Ficoll-Hypaque. Rhesus macaque PBMC were obtained from the New England Regional Primate Research Center, Southborough, Mass. Human PBMC were treated with 1 μg of phytohemagglutinin (Murex Diagnostics Ltd., Dartfield, United Kingdom) per ml for 4 days, washed free of lectin, and maintained in RPMI 1640 medium supplemented with 15% fetal calf serum and 5% human T-STIM without PHA, an interleukin-2-containing human lymphocyte-conditioned medium from Collaborative Biomedical Products, Bedford, Mass. Rhesus macaque PBMC were stimulated by treatment with 10 μg of concanavalin A (type IV; Sigma) per ml for 4 days, washed, and resuspended in RPMI 1640 medium containing 15% fetal calf serum and 20 U of interleukin-2 (Collaborative Biomedical Products) per ml.

RESULTS

Construction of chimeric *gag* genes. To study the role of the HIV-1 CA protein in the Gag-cyclophilin A interaction, chi-

meric *gag* genes containing HIV-1 and SIV_{mac} sequences were created. SIV_{mac} was chosen as a parent because it is closely related to HIV-1 but does not incorporate cyclophilin A and is unaffected by cyclophilin-binding drugs (45). Furthermore, it has been demonstrated that replication-competent HIV-1/SIV_{mac} chimeras can be generated (25, 38). Previous studies had suggested, however, that HIV-1/SIV_{mac} constructs that encode chimeric Gag domains are more likely to be replication defective than are constructs that contain the entire *gag* gene from a single parent (38). Therefore, in an attempt to improve the chances of obtaining functional chimeric *gag* genes, we precisely replaced sequences between Gag polyprotein domain boundaries as defined by proteolytic cleavage sites.

Recent findings indicate the biological significance of the presence of multiple CA protein species with different C termini in mature avian retroviruses (31, 32). It has been noted that the CA/NC junctions of these avian viruses and of HIV-1 show a limited degree of sequence similarity (32), suggesting that mature HIV-1 and SIV_{mac} virions may also contain functionally important CA protein species which include sequences from the spacer region between the CA and NC domains in the Gag precursor. We therefore decided to exchange the CA domain together with the p2 spacer peptide. The HIV-1 CA and p2 coding region was replaced by the corresponding region from SIV_{mac} by using blunt-end cloning sites, which were created by site-directed mutagenesis. The mutagenic primers were designed so that the predicted amino acid sequence of the fused Gag domains remained unaltered. The resulting HIV/SIV-CA *gag* gene (Fig. 1) contains two translationally silent mutations but is otherwise identical in nucleotide sequence to the *gag* genes of either HIV-1_{HXB2} or $SIV_{mac}239$. The reciprocal SIV/HIV-CA *gag* gene (Fig. 1) harbors the HIV-1 CA and p2 coding region in an SIV_{mac} background and contains a single translationally silent mutation.

Viral particle formation by chimeric *gag* gene constructs. We first determined whether the chimeric *gag* genes retained the ability to direct the production of viral particles. The chimeric *gag* genes were initially expressed in the absence of a functional viral protease to prevent Gag polyprotein processing and thereby simplify a comparison of virion-associated proteins. To express the HIV/SIV-CA *gag* gene product as an uncleaved polyprotein, the HXBH10(SIV-CA)-PR^- proviral construct, which encodes an inactive HIV-1 protease, was made. The reciprocal SIV/HIV-CA *gag* gene was inserted into the HIV-1 proviral clone HXBH10 in place of the HIV-1 *gag* and *pol* genes. An analogous construct that expresses the authentic SIV_{mac} Gag polyprotein has been described previously (45).

The proviral DNAs harboring the parental or chimeric *gag* genes were transfected into HeLa cells, and [^{35}S]methionine-labeled particulate material released into the supernatant was pelleted through 20% sucrose, solubilized in RIPA buffer, and analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. The HIV/SIV-CA and SIV/HIV-CA *gag* gene constructs yielded predominant bands with electrophoretic mobilities that corresponded well to the expected sizes of the chimeric Gag polyproteins (Fig. 2A). The intensities of these bands indicated that the chimeric SIV/HIV-CA *gag* gene construct produced particles as least as efficiently as did the constructs which expressed the parental HIV-1 and SIV_{mac} Gag polyproteins (Fig. 2A, lanes 1 to 3). Particle formation by the HIV/SIV-CA *gag* gene construct was slightly less efficient (lane 4).

To examine whether appropriate Gag polyprotein processing occurred in the presence of a functional protease, the HXBH10(SIV-CA) proviral construct was transfected into HeLa cells. Chimeric virions released into the supernatant contained a prominent protein of approximately 27 kDa which was not present in authentic HIV-1 virions (Fig. 2C), as expected if the 27-kDa SIV_{mac} CA protein was correctly liberated from the chimeric HIV/SIV-CA Gag polyprotein. However, additional prominent bands of approximately 28 and 24 kDa were obtained, suggesting inefficient processing at the SIV_{mac} CA/p2 cleavage site as well as some aberrant processing of the SIV_{mac} CA protein. Processing of the chimeric Gag polyprotein encoded by the reciprocal $SIV_{mac}239$(HIV-CA) provirus could not be analyzed in HeLa cells, because proviral DNA generated from genomic halves by ligation did not yield sufficient amounts of virions in these cells.

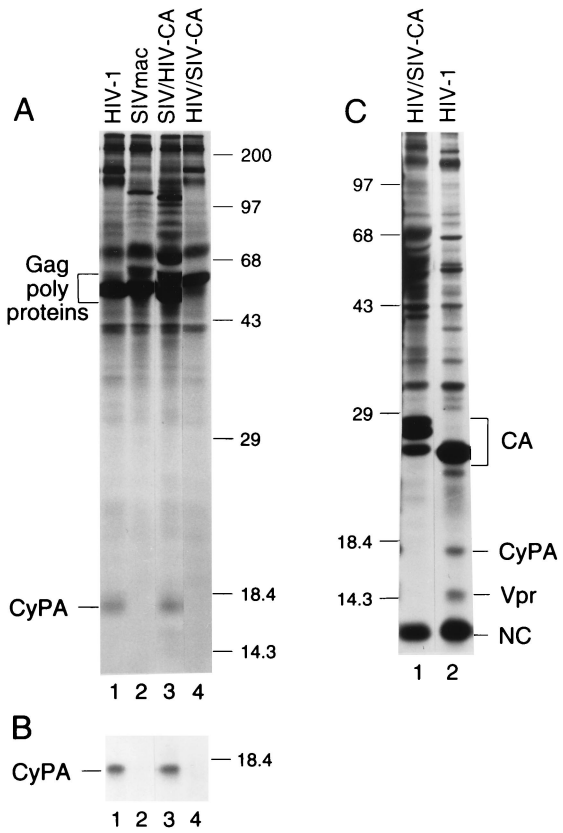

FIG. 2. Particle formation by chimeric *gag* gene constructs and incorporation of cyclophilin A (CyPA). The *gag* genes expressed are indicated above each lane. (A and B) HeLa cells were transfected with the HIV-1 Gag polyprotein expression construct HXBH10-PR^- (lanes 1), the SIV_{mac} Gag polyprotein expression construct HXBH10/SIV^{gag} (lanes 2), or the chimeric Gag polyprotein expression constructs HXBH10/SIV(HIV-CA)^{gag} (lanes 3) and HXBH10(SIV-CA)-PR^- (lane 4). (C) HeLa cells were transfected with proviral constructs encoding a functional viral protease. Lanes: 1, HXBH10(SIV-CA); 2, HXBH10/R^+, a *vpr*$^+$ variant of HIV-1_{HXB2}. [^{35}S]methionine-labeled particulate material released into the supernatant was pelleted through 20% sucrose and disrupted in RIPA buffer. Aliquots were either analyzed directly by SDS-PAGE and autoradiography (A or C) or immunoprecipitated with rabbit anti-cyclophilin A serum prior to SDS-PAGE (B).

HIV-1 CA-p2 domain-mediated incorporation of cyclophilin A and inhibition by SDZ NIM 811. As expected, viral particles formed by the HIV-1 Gag polyprotein Pr55^{gag} contained a protein of 18 kDa (Fig. 2A, lane 1), which could be immunoprecipitated by an antiserum directed against human cyclophilin A (Fig. 2B, lane 1). While cyclophilin A was not detectable in particles formed by the Gag polyprotein of SIV_{mac} (Fig. 2A and B, lanes 2), the lysate of particles formed by the SIV/

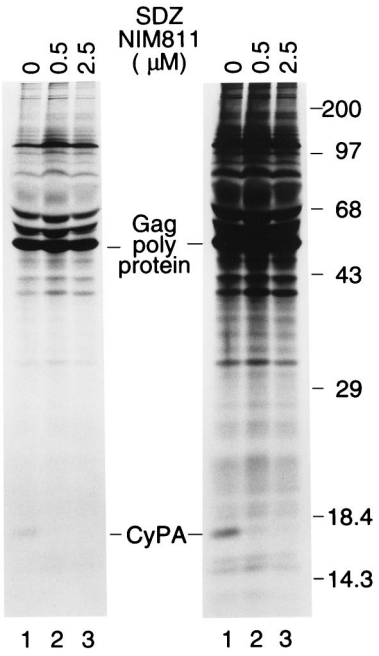

FIG. 3. Effect of SDZ NIM 811 on the association of cyclophilin A (CyPA) with chimeric viral particles. HeLa cells were transfected with the chimeric Gag polyprotein expression construct HXBH10/SIV(HIV-CA)^{gag}. Metabolic labeling with [^{35}S]methionine from 48 to 60 h posttransfection was performed in the presence of the indicated concentrations of SDZ NIM 811 dissolved in dimethyl sulfoxide or of dimethyl sulfoxide alone. Viral particles released during the labeling period were pelleted through 20% sucrose cushions, disrupted in RIPA buffer, and directly analyzed by SDS-PAGE and autoradiography. Two different exposures are shown to facilitate a comparison of the amounts of particle-associated Gag polyprotein and cyclophilin A.

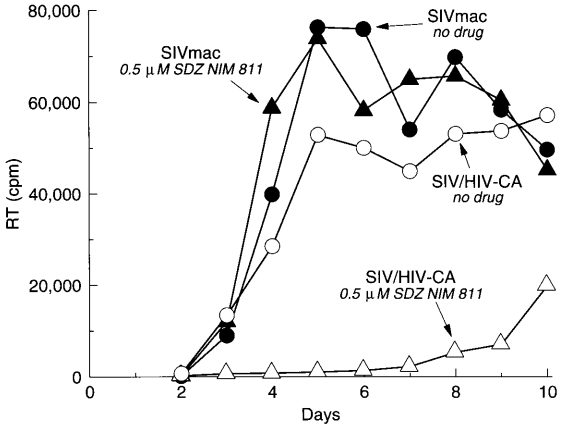

FIG. 4. Comparison of the effect of SDZ NIM 811 on the replication of the parental SIV$_{mac}$239 and the chimeric SIV$_{mac}$239(HIV-CA). Fresh CEMx174 cells were exposed for 1 h to equivalent amounts of wild-type or chimeric virus based on reverse transcriptase (RT) activity and then split and maintained in the absence of drug or in the presence of 0.5 μM SDZ NIM 811. Viral replication was monitored daily by measuring particle-associated reverse transcriptase activity in the culture supernatants.

HIV-CA Gag polyprotein contained cyclophilin A at levels that were comparable to those found in HIV-1 virion lysates (Fig. 2A and B, lanes 3). In contrast, particles formed by the HIV/SIV-CA Gag polyprotein lacked cyclophilin A (Fig. 2A and B, lanes 4). Thus, cyclophilin A incorporation correlated with the presence of the HIV-1 CA-p2 domain.

We previously demonstrated that the incorporation of cyclophilin A into HIV-1 virions is inhibited by CsA and by the nonimmunosuppressive CsA analog SDZ NIM 811 (45). As shown in Fig. 3, SDZ NIM 811 also inhibited the incorporation of cyclophilin A into particles produced by the SIV/HIV-CA Gag polyprotein. The amounts of pelletable Gag polyprotein released were similar in the presence and absence of SDZ NIM 811, indicating that the drug had no effect on the assembly or release of the chimeric particles even at 2.5 μM. However, a substantial reduction in the amount of cyclophilin A associated with the particulate fraction was already apparent at 0.5 μM. The inhibitory effect of the drug on the incorporation of cyclophilin A into the chimeric particles was similar in magnitude to that previously observed for authentic HIV-1 virions (45).

HIV-1 CA-p2 domain confers sensitivity to SDZ NIM 811. Both HIV-1 and SIV$_{mac}$ replicate well in the human B-T hybrid lymphoblastoid cell line CEMx174 (45). To determine whether the CA-p2 domains of HIV-1 and SIV$_{mac}$ are functionally exchangeable, CEMx174 cells were transfected with full-length HIV-1 and SIV$_{mac}$ proviral DNAs harboring the chimeric HIV/SIV-CA and SIV/HIV-CA *gag* genes, respectively. Virus replication was monitored by measuring particle-associated reverse transcriptase activity in the culture supernatants. Transfection of the HXBH10(SIV-CA) construct, which contains a full-length HIV-1 provirus with an SIV$_{mac}$ CA-p2 domain, did not result in detectable virus replication over a 2-month observation period (data not shown). However, significant levels of reverse transcriptase activity were detectable by day 10 in cultures transfected with the reciprocal SIV$_{mac}$239(HIV-CA) construct, which represents a full-length SIV$_{mac}$ provirus with an HIV-1 CA-p2 domain, and by day 5 in cultures transfected with wild-type SIV$_{mac}$239 proviral DNA (data not shown).

The replication kinetics of the parental SIV$_{mac}$239 and the chimeric SIV$_{mac}$239(HIV-CA) were also compared in experiments in which CEMx174 cells were infected with virus stocks. Supernatants from transfected CEMx174 cell cultures were collected on day 10 and incubated with fresh CEMx174 cells after normalization for reverse transcriptase activity. While the parental SIV$_{mac}$239 virus yielded somewhat higher peak levels of reverse transcriptase activity, the SIV$_{mac}$239(HIV-CA) chimera reproducibly exhibited essentially similar replication kinetics under these conditions (Fig. 4).

We previously reported that SDZ NIM 811 inhibits the replication of HIV-1 but is inactive against SIV$_{mac}$, which does not incorporate cyclophilin A (45). Since the SIV$_{mac}$239(HIV-CA) chimera replicated well in CEMx174 cells, it was possible to determine whether the transfer of the HIV-1 CA-p2 domain to SIV$_{mac}$, which led to the incorporation of cyclophilin A, also altered its sensitivity to SDZ NIM 811. Remarkably, the spread of the SIV$_{mac}$239(HIV-CA) chimera in an infected culture of CEMx174 cells was markedly inhibited by SDZ NIM 811 at concentrations as low as 0.5 μM (Fig. 4). In contrast, as expected, the spread of the parental SIV$_{mac}$239 was unaffected (Fig. 4). SDZ NIM 811 inhibited the replication of the SIV$_{mac}$239(HIV-CA) chimera at least as effectively as that of HIV-1 under the same culture conditions (45). Thus, the transfer of the HIV-1 CA-p2 domain to SIV$_{mac}$ was sufficient to transfer full sensitivity to SDZ NIM 811.

Transfer of the HIV-1 CA-p2 domain to SIV$_{mac}$ allows replication in human but not macaque PBMC. The ability of the SIV$_{mac}$239(HIV-CA) chimera to productively infect primary human or macaque PBMC was examined with the same virus stock used to infect the established CEMx174 cell line. The parental SIV$_{mac}$ replicates efficiently in both human and *Ma-*

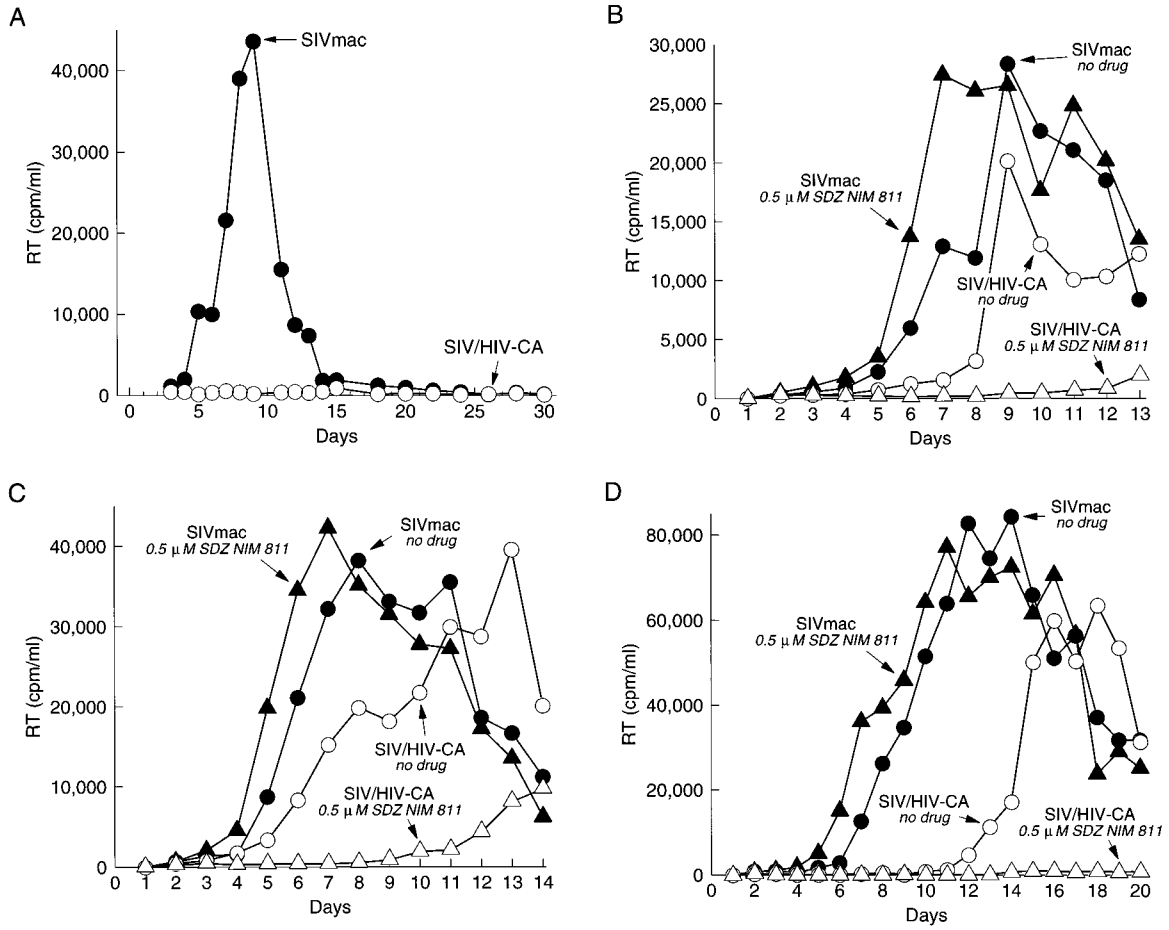

FIG. 5. Comparison of the ability of $SIV_{mac}239$ and $SIV_{mac}239$(HIV-CA) to replicate in macaque and human PBMC. Stimulated macaque PBMC (A) or human PBMC from three different donors (B to D) were exposed for 1 h to equivalent amounts of wild-type or chimeric virus, and virus replication was monitored daily by measuring reverse transcriptase (RT) activity in the culture supernatants. Human PBMC were split after infection and kept in the absence of drug or in the presence of 0.5 μM SDZ NIM 811.

caca mulatta PBMC, whereas HIV-1 does not productively infect *M. mulatta* PBMC (1, 38, 39). We assessed the ability of the $SIV_{mac}239$(HIV-CA) chimera to replicate in stimulated PBMC from three healthy rhesus macaques. While PBMC from each animal supported the replication of $SIV_{mac}239$, as evidenced by a rise in reverse transcriptase activity following infection, the $SIV_{mac}239$(HIV-CA) chimera showed no sign of replication in PBMC from any of these monkeys (Fig. 5A and data not shown). In contrast, PBMC from three human donors were susceptible to infection both with the parental $SIV_{mac}239$ virus and with the $SIV_{mac}239$(HIV-CA) chimera (Fig. 5B to D). The chimera replicated somewhat more slowly than the parental virus did, in particular in PBMC from donor 3, but eventually produced reverse transcriptase levels that approached those of $SIV_{mac}239$. In all cases, $SIV_{mac}239$ was unaffected by SDZ NIM 811 at 0.5 μM, whereas the replication of the $SIV_{mac}239$(HIV-CA) chimera was inhibited at the same concentration of drug (Fig. 5B to D).

DISCUSSION

HIV-1 differs from other retroviruses, even from closely related primate lentiviruses, in its ability to incorporate cyclophilin A (15, 30, 45), which is accompanied by a unique sensitivity to drugs that inhibit the virion association of cyclophilin A (3, 5, 15, 35, 45). Previous studies have shown that the incorporation of cyclophilin A is mediated by the CA domain of the HIV-1 Gag polyprotein $Pr55^{gag}$ (45). However, it remained to be determined whether the HIV-1 CA domain merely provides a docking site which allows cyclophilin A to gain access to the virion or whether the requirement for the interaction is also specified by CA sequences. Using a virus replication system in which the functional relevance of the incorporation of cyclophilin A can be assessed, we provide evidence strongly supporting the latter possibility.

Our results show that SIV_{mac}, upon replacement of its CA-p2 domain with HIV-1 sequences, acquires a phenotype which resembles that of HIV-1 both with respect to cyclophilin A incorporation and with respect to sensitivity to a drug that inhibits the incorporation of cyclophilin A. The nonimmunosuppressive CsA analog SDZ NIM 811, which interferes with the incorporation of cyclophilin A into HIV-1 virions (45), inhibits the replication of HIV-1 but is inactive against the related SIV_{mac}, which does not incorporate cyclophilin A (35, 45). To further explore the relationship between sequences required for cyclophilin A incorporation and determinants that confer sensitivity to cyclosporins, we exchanged CA and p2 coding sequences between HIV-1 and SIV_{mac}. The CA-p2 domain of SIV_{mac} was not functional in the context of HIV-1, perhaps because the chimeric Gag polyprotein was not pro-

cessed correctly by the HIV-1 protease. However, the reciprocal construct replicated well in CEMx174 cells, permitting an analysis of its sensitivity to SDZ NIM 811. The replacement of the CA-p2 domain of SIV_{mac} with the corresponding domain from HIV-1 led to the efficient incorporation of cyclophilin A into virions, which could be effectively inhibited by SDZ NIM 811. Remarkably, SDZ NIM 811 also inhibited the spread of the chimeric virus in newly infected cultures whereas the spread of the parental SIV_{mac} was unaffected. These findings provide compelling evidence that the specific anti-HIV-1 effect of cyclosporins is a direct consequence of their inhibitory effect on the interaction between HIV-1 CA sequences and cyclophilin A. Furthermore, these results imply that HIV-1 CA sequences not only mediate the interaction but in addition specify its necessity for efficient virus replication and are thus primarily responsible for the striking differences between closely related primate lentiviruses in their reliance on cyclophilin A.

The Gag-cyclophilin interaction is not required for Gag-directed particle assembly or release, since HIV-1 particle formation is not affected by SDZ NIM 811 (45). Also, HIV-1 Gag mutants that fail to interact with cyclophilin A retain the ability to efficiently produce viral particles (15, 45). Since cyclophilin A is a component of the HIV-1 virion, its major role in the viral life cycle may be played after the completion of particle assembly, such as during formation of the mature core structure or during uncoating. While HIV-1 virions produced in the presence of SDZ NIM 811 appear to have a normal morphology (20a), cyclophilin A could nevertheless play a subtle role in the assembly of the viral nucleoprotein core. In any case, an involvement of cyclophilin A in some aspect of core function is strongly suggested by the crucial role of CA, which forms the shell of the core, in viral sensitivity to SDZ NIM 811.

How might cyclophilin A affect CA protein function? Although the peptidyl-prolyl isomerase activity of cyclophilin A points to a catalytic role in the initial folding of the CA domain within the context of $Pr55^{gag}$, its binding to mature HIV-1 CA protein (27) suggests a role at a later stage. It was recently reported that the HIV-1 CA protein and CsA display a common epitope detected by a monoclonal antibody (14). The antibody recognizes *N*-methyl leucines 9 and 10 of the cyclic undecapeptide CsA (7). Interestingly, the peptide bond between these two residues isomerizes from *cis* to *trans* upon binding to cyclophilin (22, 47), and it has been proposed that binding of cyclophilin A to HIV-1 Gag may induce similar structural transformations (14). A novel role for an immunophilin in a stable complex which does not depend on its peptidyl-prolyl isomerase activity was recently reported for FKBP12 bound to the inositol 1,4,5-trisphosphate receptor. In this case, the immunophilin appears to act as an adaptor protein which anchors the phosphatase calcineurin to an appropriate substrate, thereby ensuring preferential modulation of a specific target (8). It remains to be addressed whether the peptidyl-prolyl isomerase activity of cyclophilin A is required for its effect on HIV-1 replication.

Interestingly, the SIV_{mac}239(HIV-CA) chimera, despite being mostly SIV_{mac}, displayed a host range which resembled that of HIV-1 rather than that of SIV_{mac}. The chimera replicated efficiently in a human cell line and in human PBMC but failed to productively infect macaque PBMC. The exceptionally high conservation of cyclophilins would suggest that the failure of the chimera to replicate in macaque PBMC is not related to its dependence on cyclophilin A. It seems more likely that the HIV-1 CA protein lacks specific determinants which are required for growth in macaque but not human PBMC. Our results are consistent with earlier studies of HIV-1/SIV_{mac} chimeras, which showed that HIV-1 sequences encompassing the 3′ half of the long terminal repeat as well as *gag* and *pol* restrict viral replication in macaque PBMC (38, 39). Certain strains of murine leukemia viruses carry host range determinants which also map to the CA protein (11). In restrictive cells, the efficiency of integration of the viral genomic DNA into the host cell genome is reduced (33), suggesting that the CA protein may play an important role in early stages of the retroviral replication cycle. It will be of interest to determine which stage of the replication cycle of the SIV_{mac}239(HIV-CA) chimera is restricted in macaque PBMC.

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