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Mode of Action of SDZ NIM 811, a Nonimmunosuppressive Cyclosporin A Analog with Activity against Human Immunodeficiency Virus (HIV) Type 1: Interference with HIV Protein-Cyclophilin A Interactions

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Cyclosporins, in particular the nonimmunosuppressive derivative SDZ NIM 811, exhibit potent anti-human immunodeficiency virus type 1 (HIV-1) activity in vitro. SDZ NIM 811 interferes at two stages of the viral replication cycle: (i) translocation of the preintegration complex to the nucleus and (ii) production of infectious virus particles. Immunosuppressive activity is not correlated with anti-HIV-1 activity of cyclosporins. However, binding to cyclophilin A, the major cellular receptor protein of cyclosporins, is a prerequisite for HIV inhibition: all structural changes of the cyclosporin A molecule leading to loss of affinity to cyclophilin abolished the antiviral effect. Cyclosporin derivatives did not interact directly with HIV-1 proteins; cyclophilin was the only detectable receptor protein for antivirally active cyclosporins. There is no evidence that inhibition of HIV occurs via a gain of function of cyclophilin in the presence of cyclosporins: the complex of cyclophilin A with SDZ NIM 811 does not bind to calcineurin or to any other viral or cellular proteins under conditions in which calcineurin binding to the cyclophilin A-cyclosporin A complex is easily detectable. Thus, the loss of function caused by binding of cyclosporins to cyclophilin seems to be sufficient for the anti-HIV effect. Cyclophilin A was demonstrated to bind to HIV-1 p24^{gag}, and the formation of complexes was blocked by cyclosporins with 50% inhibitory concentrations of about 0.7 μM. HIV-2 and simian immunodeficiency virus are only weakly or not at all inhibited by cyclosporins. For *gag*-encoded proteins derived from HIV-1, HIV-2, or simian immunodeficiency virus particles, cyclophilin-binding capacity correlated with sensitivity of the viruses to inhibition by cyclosporins. Cyclophilin A also binds to HIV-1 proteins other than *gag*-encoded proteins, namely, p17^{gag}, Nef, Vif, and gp120^{env}; the biological significance of these interactions is questionable. We conclude that HIV-1 Gag-cyclophilin A interaction may be essential in HIV-1 replication, and interference with this interaction may be the molecular basis for the antiviral activity of cyclosporins.

Infection with the human immunodeficiency virus (HIV) and its sequel, AIDS, remain a health threat of global significance. Because of the limitations of currently available therapies, an extensive search for new anti-HIV agents is ongoing. Two classes of potential drugs may be distinguished: (i) compounds designed to interfere directly with the life cycle of HIV, e.g., by inhibiting a viral enzyme or regulatory protein; and (ii) agents intended to modulate immune responses to influence the pathogenesis of disease caused by HIV. From the second group of agents, the immunosuppressive drug cyclosporin A (CsA; Fig. 1) has been proposed to exert a beneficial effect in disease caused by HIV (1), since it would block T4 lymphocyte activation, which is required for HIV replication (39, 48, 49) and would thus act as an indirect antiviral drug. In addition, it would inhibit steps of the autoimmune process which lead to killing of T4 lymphocytes (16, 19, 40) and would counteract HIV-induced apoptotic death of T4 cells (15).

With this proposal in mind and subsequent to a report by Wainberg et al. (45) on anti-HIV activity of CsA, we evaluated this drug and more than 200 derivatives for inhibition of HIV type 1 (HIV-1) replication. Furthermore, the activity of the compounds in an interleukin 2 (IL-2) reporter gene assay as an indicator of their immunosuppressive potential was assessed. Surprisingly, antiviral potency did not correlate with the immunosuppressive potential of the compounds (33): nonimmunosuppressive CsA derivatives were equal or even superior in anti-HIV-1 activity to immunosuppressive ones. [Me-Ile^4]cyclosporin (SDZ NIM 811) (Fig. 1) is a representative of a group of 4-substituted cyclosporins that are devoid of immunosuppressive activity and that exhibit potent and selective anti-HIV-1 activity in cell culture (33). The antiviral efficacy of SDZ NIM 811 was demonstrated in various cell lines, primary T4 lymphocytes, and primary monocytes. This compound inhibited replication of several HIV-1 clinical isolates from different geographic locations in primary cells with 50% inhibitory concentrations (IC_{50}s) of 0.011 to 0.057 μg/ml, while cytostatic effects in proliferating cell lines were detected at 4 to 6 μg/ml. HIV-2 and simian immunodeficiency virus (SIV) were only weakly or not at all inhibited by cyclosporin derivatives.

Evaluation of SDZ NIM 811 in comparison to the immunosuppressants CsA and FK506 in primary T4 lymphocytes clearly demonstrated that the mechanism of antiviral action of cyclosporins is not prevention of T4 cell activation (33). CsA has, in addition to its immunosuppressive activity, a direct antiviral effect by a mechanism which it presumably shares with SDZ NIM 811; this direct antiviral effect is not exerted by the immunosuppressant FK506.

The mechanism of action of SDZ NIM 811 is clearly different from those of other anti-HIV agents described so far. We identified two stages in the viral life cycle where this compound

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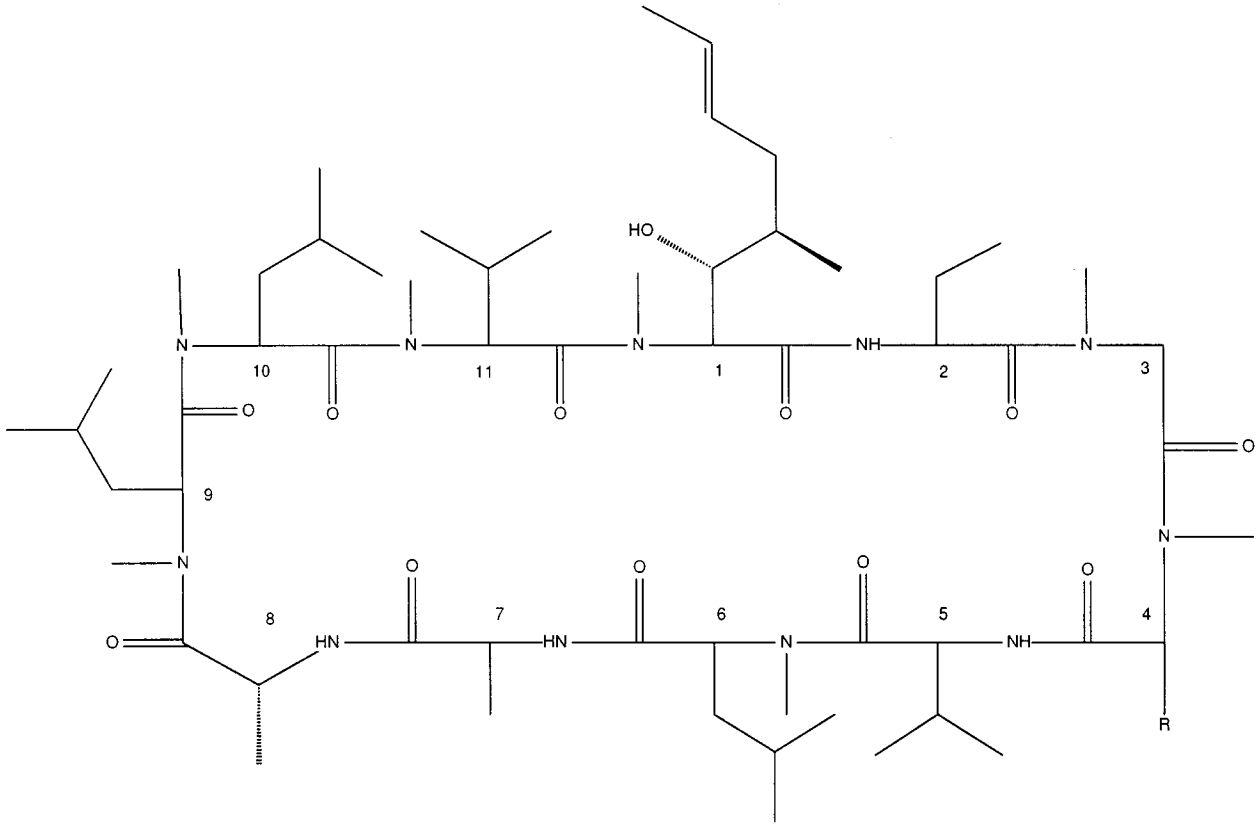

FIG. 1. Structural formula of cyclosporins. When R is CH_2-$CH(CH_3)_2$, the compound is CsA; when R is $CH(CH_3)(CH_2$-$CH_3)$, the compound is [MeIle^4]cyclosporin (= SDZ NIM 811).

interferes. (i) During establishment of infection, it prevents formation of circular HIV DNA and integration of viral DNA into the host genome, while not reducing the amount of unintegrated cDNA (38). (ii) At a late stage of virus replication, SDZ NIM 811 leads to a dose-dependent reduction of infectivity of shedded virus particles while not inhibiting viral antigen expression (33, 38).

To understand the molecular bases for these effects, we investigated the interactions of cyclosporins with viral and cellular proteins. The major intracellular receptor protein for cyclosporins in T cells is cyclophilin A (CypA) (18, 35). Luban et al. (24) suggested a possible connection between this protein and HIV replication: CypA was demonstrated to bind to the p55^{gag} protein of HIV-1, and the formation of this complex was inhibited by CsA. Subsequently, it was proposed that Cyp, which is a peptidyl prolyl *cis-trans* isomerase, might be required for unfolding of the Gag protein and thus be essential for HIV replication (21). Cyclosporins are potent inhibitors of the isomerase activity of Cyp and therefore could interfere with this function.

Here, we present evidence that the antiviral activity of cyclosporins is correlated with their ability to bind to human T-cell CypA; no direct interactions between HIV proteins and cyclosporins were detected. Furthermore, we studied the interactions of CypA with HIV-1 proteins and the inhibition of these interactions by SDZ NIM 811 and other cyclosporins. We found that not only the HIV-1 *gag*-encoded proteins p24 and p55 but also other viral proteins form a complex with Cyp; the formation of these complexes in all cases was inhibited by cyclosporins. On the other hand, HIV-2 or SIV Gag proteins derived from viral particles showed much less affinity for CypA than HIV-1 Gag proteins. The relevance of these findings with respect to the mode of action of antiviral cyclosporins is discussed.

MATERIALS AND METHODS

Cyclosporins. Compounds I, III, V, and VI are natural fungal metabolites; the production of compound VI has been described elsewhere (43). Compounds IV and IX are semisynthetic derivatives. Compound IX is a metabolite described previously (26). The remaining compounds are synthetic analogs of CsA prepared by the same strategy used for the synthesis of CsA (47). The HIV protease inhibitor SDZ 284-269, a close analog of compound I (37), was synthesized at Sandoz Forschungsinstitut, Vienna, Austria; its synthesis will be described elsewhere (36a).

Viruses. HIV-1 strain IIIB and HIV-2 strains ROD and EHO have been described elsewhere (7, 30, 32). HIV-1 strain D370 was isolated in 1989 from a patient in San Diego, Calif.; HIV-2 MS was isolated from a patient from the Ivory Coast (20). SIV_{mac251} was isolated from macaques (8).

Cytopathic effect inhibition assay. The cytopathic effect inhibition assay procedure described by Pauwels et al. (29) was used with minor modifications. The human T-cell leukemia virus type I-transformed cell line MT4, which was previously shown to be highly permissive for HIV infection, was used as the target cell. Inhibition of HIV-1 strain IIIB-induced cytopathic effect was determined by measuring the viability of both HIV- and mock-infected cells. Viability was assessed spectrophotometrically via in situ reaction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide. Virus-infected and uninfected cultures without test compounds were included as controls, as were uninfected cells treated with test compounds. The virus was adsorbed to a cell suspension containing 10^6 cells per ml at 37°C for 1 h. Then, the infected cells were added to microtiter plates containing the test compounds to give 10^5 cells per ml. Thus, compounds were added postadsorption. The IC_{50}s reported here are the means of at least five determinations.

Antiviral activity assays in MT4 cells and primary T4 lymphocytes. HIV-1 was absorbed to MT4 cells (28), then the inoculum was removed, and the infected cells were added to tissue culture plates containing the test compounds at the

appropriate concentrations. Virus inoculum was adjusted to give an exponential increase of p24 antigen concentration in the supernatants of infected cells up to day 4 postinfection. At days 3 and 4 postinfection, the supernatants were analyzed for p24 antigen concentration by enzyme-linked immunosorbent assays (ELISA) (Coulter). IC_{50}s were calculated by comparing p24 antigen concentrations in supernatants of treated, infected cells with those of untreated, infected cells.

Primary T4 lymphocytes were purified from human spleens obtained from healthy donors by using a commercial kit (Lympho-Kwik) as described recently (10). Preparations obtained by this procedure contained 60 to 80% CD4-positive cells as analyzed by fluorescence-activated cell sorting. Cells were stimulated with phytohemagglutinin, infected with HIV-1, and incubated in medium containing IL-2. Test compound was added after stimulation and virus adsorption. Every 3 to 4 days, half of the supernatant of the infected cultures was removed and replaced by fresh medium containing IL-2 and the test compound at the particular concentration. The concentration of viral p24 antigen was determined in the supernatants by ELISA (Coulter). IC_{50}s were calculated by comparing p24 antigen concentrations in supernatants of treated, infected cells with those of untreated, infected cells at days postinfection, when p24 production was increasing exponentially.

Biotinylation of CypA. Two microliters of a 200 mM solution of *N*-hydroxysuccinimidyl-biotin (Boehringer-Mannheim) in dimethyl sulfoxide (DMSO) was added to 75 μg of human T-cell CypA (recombinant protein purified from *Escherichia coli*; unpublished) in 100 μl of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.4], 100 mM NaCl). The mixture was left at room temperature for a total of 2 h; after 0.5 and 1 h, additional 2-μl aliquots of activated biotin were added. Finally, 5 μl of 1 M Tris-HCl, pH 7.8, was added to stop the reaction. The slightly turbid mixture was then applied to a Sephadex G-25 column (Pharmacia), equilibrated with buffer A, which separated the biotinylated protein from the reagent.

Solid-phase cyclosporin-Cyp binding assay. A competitive solid-phase enzyme assay was adapted from the assay of Quesniaux et al. (31) to study the binding of cyclosporin derivatives to CypA. Briefly, a [D-Lys^8]cyclosporin derivative was coupled to bovine serum albumin (BSA) and was used to coat a polyvinyl microtiter plate (2 μg/ml in phosphate-buffered saline [PBS] [pH 7.4] for 2 h at 37°C). After saturation of the plate with 2% BSA in PBS (1 h at 37°C) and washing the plate once with PBS containing 0.05% Tween 20 and three times with PBS, biotinylated Cyp (100 ng/ml) was added, and the plate was incubated overnight at 4°C (in 1% BSA–PBS). After the plate was washed, bound biotinylated Cyp was detected with streptavidin coupled to alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc.; 1:6,000 in 1% BSA–PBS, 2 h at 37°C). The A_{405} was measured after hydrolysis of *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer [pH 9.6] for 1 to 2 h at 37°C). The interaction between Cyp and the cyclosporin derivatives free in solution was measured by competition. Cyclosporin derivatives (1 mg/ml ethanolic solution) were added to the Cyp solutions (at 1:100 dilution, 8.3 μM), and further 10-fold dilutions were made directly in the microtiter plate. After incubation overnight at 4°C, unbound Cyp was removed and the assay was continued as described above. For cyclosporin analogs, the competitive potential calculated as the percent inhibition of the control reaction between Cyp and coated cyclosporin in the absence of inhibitor (10 replicates per plate). The IC_{50}s for the cyclosporin derivatives were compared with the IC_{50} for CsA included in triplicate in each microtiter plate.

Assay for immunosuppression. IL-2 promoter activation upon T-cell stimulation was determined in a Jurkat cell line containing the β-galactosidase gene as a reporter under IL-2 promoter control (2).

Assays of HIV-1 enzymes. HIV-1-specific enzymes were assayed in the presence or absence of cyclosporins according to the following published procedures: reverse transcriptase (17), integrase (4), and protease (3). Cyclosporins were added to the assay buffers from stock solutions in DMSO to a final concentration of up to 50 μM and a final DMSO content of 5%.

Gel filtration assay for SDZ NIM 811-protein binding. The gel filtration assay was performed in a manner analogous to the procedure described previously (18). Test proteins (HIV proteins or CypA, each at a concentration of 2 μM) were incubated in PBS for 1 h at room temperature with SDZ NIM 811 radiolabeled by tritiation of the double bond in position 1 ([Mebmt-δ,ε,ζ,η-^{3}H]dihydro-[MeIle^4]cyclosporin (T. Moenius, Sandoz, Basel) at a concentration of 1 μg/ml. Mixtures were then applied to a column of Sephadex LH-20 in PBS which retards unbound cyclosporin while protein-cyclosporin complexes are expected at the elution front. Eluate fractions were analyzed by liquid scintillation counting.

Cross-linking studies with a radiolabeled cyclosporin derivative. A derivative containing a photoreactive diazirine group linked to the cyclosporin ring via a spacer ([(4-diazo-2,2,2-trifluoro-ethyl)benzoyl]γ-aminobutanoyl-*O*-D-serine^8-cyclosporin) (12) was radiolabeled by tritiation of the double bond in position 1 (T. Moenius, Sandoz, Basel). This compound was incubated at a concentration of 1 μg/ml with the test proteins, each at a concentration of 2 μM, for 30 min at room temperature. Then the mixture was exposed to UV light from a type IL-350-M UV source (254 nm; Bachhofer, Reutlingen, Germany) at a distance of 10 cm on ice for 5 to 20 min. Aliquots of the mixture were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography.

Trapping of proteins from cell lysates by GST-Cyp–cyclosporin complexes. A tissue extract from bovine brain was prepared as described previously (23). Its cytosolic fraction was incubated with glutathione *S*-transferase (GST)-Cyp (obtained as described previously [23]) in the presence or absence of CsA or SDZ NIM 811. After incubation, GST-Cyp and the proteins that eventually adsorbed to it were bound to glutathione Sepharose, the beads were washed and eluted, and the eluate was subjected to SDS-PAGE, followed by silver staining.

Analogous experiments with lysates of either MT4 cells or chronically HIV-1 IIIB-infected Jurkat cells were performed. Here, cells were metabolically labeled with [^{35}S]methionine for 16 h in the presence or absence of SDZ NIM 811, harvested, and lysed in a hypotonic buffer (20 mM Tris-HCl [pH 6.8], 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5% [vol/vol] glycerol). Clarified lysates were used for incubations with GST-Cyp and cyclosporins as described above.

Cyp isomerase activity. The assay of the rotamase activity of CypA was performed as described previously (11). The concentration of Cyp was 2.5 nM; cyclosporins were added at various concentrations from stock solutions in ethanol.

Production of HIV-1 p24^{gag} and p17^{gag}. The region of the HIV-1 *gag* gene encoding the matrix (MA) and the capsid (CA) proteins (nucleotides 792 to 1876) was amplified from the genome of HIV-1 strain HXB2 by PCR with oligonucleotides 5′-TACGATGACGGATCCGGTGCGAGAGCGTCAGTATTAAGCGGG-3′ and 5′-TACGATGACGTCGACTTACAAAACTCTTGCCTTATGGCC-3′ as primers. The resulting DNA fragment was cleaved with *Bam*HI and *Sal*I and ligated with appropriately cleaved pGEX-4T-1 (Pharmacia). This construct, containing the p17-p24 region at the 3′ end of the GST gene, was then used for transformation of *E. coli* DH1.

For production of the GST-p17-p24 fusion protein, bacteria were cultivated in Luria-Bertani broth containing 100 μg of ampicillin per ml and expression was induced with 1 mM isopropylthiogalactoside (IPTG) in mid-log phase. At 3 h postinduction, bacteria were harvested and washed with PBS. Ten milliliters of buffer A (50 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 1 mg of lysozyme per ml) per gram of bacterial cell paste was added to the bacteria. After 30 min on ice, the suspension was sonicated at 100 W with a Branson sonifier (three times for 20 s each time). The clarified supernatant was applied to a glutathione Sepharose column (Pharmacia) equilibrated with PBS. The column was washed with 5 volumes of PBS and then eluted with 50 mM Tris-HCl (pH 8.0)–20 mM glutathione. The eluate contained the GST-Gag fusion protein with a purity of >95%, as judged by SDS-PAGE.

The eluate was dialyzed against 5 mM morpholinoethanesulfonic acid (MES) (pH 6.5)–5 mM dithiothreitol. Cleavage between the MA and CA proteins was then performed by HIV-1 protease (purified as previously described [3]). Two micrograms of protease was used per milligram of fusion protein, and cleavage was allowed to proceed for 1 h at 37°C. By adjusting the pH to 8.0 with Tris base, the protease was inactivated. The mixture was then passed through a glutathione Sepharose column as described above to remove GST-p17. HIV-1 protease was removed from the flowthrough by binding it subsequently to pepstatin agarose (Sigma).

To obtain the p17 protein, the GST-p17 fusion protein retained on the last glutathione Sepharose column was eluted with 20 mM glutathione in 50 mM Tris-HCl, pH 8.0. Thrombin (30 U/mg of protein) was used to cleave between GST and p17. After 1 h at 37°C, the mixture was dialyzed against PBS and was then applied to a glutathione Sepharose column to remove the GST protein. The eluate contained the p17 protein.

Both p17 and p24 were analyzed by N-terminal sequencing and were shown to have correct N termini. According to SDS-PAGE with silver staining and reversed-phase high-pressure liquid chromatography, they were >95% pure. Immunodetection of p17^{gag} was achieved by using a monoclonal antiserum bought from DuPont NEN. Polyclonal antibodies against p24^{gag} were raised in rabbits at Sandoz Forschungsinstitut.

Production of HIV-1 and HIV-2 p55^{gag} protein. The *gag* genes of HIV-1 (nucleotides 789 to 2291) and HIV-2 (547 to 2112) were amplified from HIV-1 strain HXB2 and HIV-2 ROD genomes by PCR. The primers for HIV-1 were 5′-TACGATGACGGATCCGGTGCGAGAGCGTCAGTATTAAGCGGG-3′ and 5′-TACGATGACGTCGACTTACAAAACTCTTGCCTTATGGCC-3′, and the primers for HIV-2 were 5′-TACGATGACCATATGGGCGCGAGAAACTCCGTCTTGAGAGGG-3′ and 5′-TACGATGACGGATCCGACGATGACGATAAGGGCGCGAGAAACTCCGTCTTGAGAGGG-3′. The resulting DNA fragments were cloned directly into plasmid pCR (Invitrogen) and cleaved out off the resulting constructs with *Nde*I and *Bam*HI. Fragments then were ligated to appropriately cleaved plasmid pET9c (42). Recombinant plasmids were isolated from *E. coli* HB101 and then were used for transformation of *E. coli* BL21 (DE3)pLysS (41).

For protein expression, the recombinant bacteria were grown at 37°C in shaken flasks in Luria-Bertani medium containing both kanamycin and chloramphenicol at 50 μg/ml. Expression was induced at an optical density at 600 nm of 0.6 to 0.8 with 5 mM IPTG. At 3 h postinduction, bacteria were harvested and then lysed in a buffer containing Nonidet P-40 and subjected to five freeze-thaw cycles and sonication as described previously (24). Following ultracentrifugation (100,000 × *g*, 30 min, 4°C), the supernatants were stored at −70°C and used without further purification for Cyp binding assays. As judged from Coomassie blue-stained SDS-polyacrylamide gels, the amounts of HIV-1 and HIV-2 Gag

proteins in these crude preparations were similar. In anti-p24 or anti-p26 immunoblots of the preparations, a major band at 55 kDa is observed, but several smaller bands are also observed. These bands have been demonstrated to be products of C-terminal degradation by *E. coli* proteases in the case of the HIV-1 Gag protein (24).

Other viral proteins. HIV-1 gp41^{env}, gp120^{env}, Vif, and Tat, HIV-2 p26^{gag}, and SIV p28^{gag} as well as polyclonal antibodies against these proteins were purchased from ABT, London, United Kingdom. Recombinant HIV-1 reverse transcriptase (17), HIV-1 protease (3), and HIV-1 integrase (4) were purified as described previously; polyclonal antibodies against these enzymes were generated in rabbits at Sandoz Forschungsinstitut. The HIV-1 Nef protein and rabbit anti-Nef antiserum were kindly supplied by G. Werner, and recombinant HIV-1 Rev and anti-Rev antiserum were kindly supplied by J. Hauber (G. Werner and J. Hauber, both at Sandoz Forschungsinstitut).

Analysis of Gag proteins from virus particles. Chronically HIV-1 IIIB-infected Jurkat cells isolated in our laboratories from a de novo HIV-1 IIIB infection of Jurkat cells or chronically HIV-1 IIIB, HIV-2 EHO, or SIV-infected HUT78 cells, isolated from de novo infection of HUT78 cells, were suspended at 10^5 cells per ml in fresh medium, containing either the protease inhibitor SDZ 284-269 at various concentrations or no inhibitor (control). After 48 h of incubation at 37°C, the cells were removed by centrifugation and the clarified supernatants were submitted to ultracentrifugation (160,000 × *g*, 1 h at 4°C). The pellet from 8 ml of supernatant containing HIV-1 particles was dissolved in 100 μl of buffer B (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 5 mM dithiothreitol, 0.5% Nonidet P-40, 5% [wt/vol] glycerol). This solution was used for the Cyp-protein binding assays described below.

Cyp-protein binding assays. For Cyp-protein binding assays, either a GST-human T-cell CypA fusion protein (produced in *E. coli* and purified as described previously [23]) or biotinylated CypA (see above) at a concentration of 0.18 μM was used. Cyp was incubated with the various test proteins in buffer B in a total volume of 100 μl. After 1 h at room temperature, 25 μl of a 50% (vol/vol) suspension of either glutathione or avidin Sepharose (Pharmacia) was added. The mixture was rotated end-over-end for 1 h. After centrifugation, the gel was washed three times with buffer B. Finally, the gel was eluted with 25 μl of buffer C (0.13 M Tris-HCl [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.5% bromophenol blue) by heating to 95°C for 5 min. After centrifugation, an aliquot of the supernatant was subjected to SDS-PAGE; gels containing 12.5% acrylamide were used in all instances except for gp120 for which 7.5% acrylamide gels were used. Gels were run on a Pharmacia Phast system device and then were subjected to blotting to nitrocellulose sheets. Proteins were visualized by immunostaining using the appropriate first antibody and a species-specific peroxidase-conjugated second antibody. Detection of reactive protein bands was achieved by using the enhanced chemiluminescence kit from Amersham which allows visualization of bands on X-ray films. For molecular weight markers, colored proteins (Rainbow Markers, Amersham) which were visible on the nitrocellulose after blotting were used.

For GST-Cyp–protein complexes, elution of specifically bound proteins from the gel could also be achieved with 20 mM glutathione in 50 mM Tris-HCl, pH 8.0 instead of the SDS-containing buffer.

Inhibition of HIV protein-Cyp complex formation by cyclosporins. The Cyp-protein binding assay was performed in the presence of cyclosporins by adding the test compound from a stock solution in DMSO to the incubation buffer prior to the addition of biotinylated Cyp and the appropriate protein. The maximal concentrations of cyclosporins were 20 μM, and the final DMSO content was 5%.

For determination of IC_{50}s, films of immunoblots were scanned by densitometry. Data were plotted and IC_{50}s were calculated with the program Grafit.

Nondenaturing gel electrophoresis. To analyze the interaction between Cyp and p17, CypA (11.2 μM) was incubated with different concentrations of recombinant p17 (2 to 50 μM). The mixture was then subjected to native PAGE (precast Clean Gels [Pharmacia]; 10% acrylamide, 2% *N,N′*-methylene bisacrylamide; swelling buffer consists of 45 mM Tris-borate [pH 7.6], 1 mM EDTA, and 10% glycerol; anode buffer consists of 175 mM Tris-HCl [pH 8.9]; cathode buffer consists of 567 mM glycine; 10 min of 300 V and then 3 h of 900 V) as described previously (25). Proteins were visualized by silver staining.

RESULTS

Correlation of anti-HIV-1 activity of cyclosporins with Cyp binding. We tested about 200 derivatives of CsA (Fig. 1) for inhibition of HIV-1-induced cytopathic effect in MT4 cells. The capacities of these compounds to bind to human T-cell CypA were assessed in a competitive solid-phase binding assay. A representative selection of the results is shown in Table 1. CsA (compound I) inhibits HIV-1 strain IIIB with an IC_{50} of 470 nM. The cyclosporin derivatives which bind to Cyp with an affinity comparable to that of CsA (compounds II to IX, relative IC_{50}s of 0.5 to 2.6), also show anti-HIV activity with IC_{50}s ranging from 50 to 560 nM. An exception to this rule is [D-Lys^8]cyclosporin (compound VIII) which does not inhibit virus replication, while binding to Cyp in the cell-free assay. This result can be explained by an over 20-fold-reduced cellular uptake of this derivative compared with that of CsA (unpublished results). All modifications of the CsA molecule which led to a marked reduction of Cyp-binding capacity (relative IC_{50} of >10) yielded molecules devoid of anti-HIV activity (no inhibition at 5 μM, relative IC_{50} of >10). These modifications are either within the binding site of Cyp for CsA (residues 1, 2, 3, 10, and 11 [31]) or lead to a distortion of the CsA skeleton (e.g., compound XIII).

TABLE 1. Activity against HIV-1, Cyp binding, and immunosuppression of 15 CsA analogs

Compound[a]	Relative IC_{50}[b]		
	Antiviral activity[c]	Cyp binding[d]	Immunosuppression[e]
I (CsA)	1	1	1
II ([8′-Hydroxy-MeBmt^1]Cs)	1.2	2.20	11
III ([Thr^2]Cs)	1.1	1.4	1
IV ([*O*-Acetyl-D-MeSer^3]Cs)	0.23	0.75	2.4
V ([γ-Hydroxy-MeLeu^4]Cs)	0.72	1	110
VI ([MeIle^4]Cs)	0.35	0.59	>1,700
VII ([MeVal^4]Cs)	0.11	0.54	>2,500
VIII ([D-Lys^8]Cs)	>10	2.6	150
IX ([γ-Hydroxy-MeLeu^9]Cs)	0.63	1.5	12
X ([*O*-Acetyl-MeBmt^1]Cs)	>10	>97	270
XI ([Pro^3]Cs)	>10	140	348
XII ([Thio-MeLeu^4]Cs)	>10	30	ND[f]
XIII ([Leu^{5}Pro^6]Cs)	>10	20	>2,700
XIV ([γ-Hydroxy-Leu^9]Cs)	>10	15	62
XV ([MeAla^{10}]Cs)	>10	26	15

[a] Cs, cyclosporin; Bmt, (4*R*-4-[(*E*)-2-butenyl]-4-methyl-L-threonine; Abu, L-aminobutyric acid. Me indicates N methylation of the amino acids.
[b] Relative IC_{50} is IC_{50} of analog divided by IC_{50} of CsA.
[c] Measured by inhibition of HIV-1 strain IIIB-induced cytopathic effect in MT4 cells.
[d] Binding to human T-cell CypA as determined relative to CsA in a competition assay.
[e] Immunosuppression measured relative to CsA in an IL-2 reporter gene assay in Jurkat cells.
[f] ND, not done.

In addition, the immunosuppressive activities of the cyclosporin analogs were measured in an IL-2 reporter gene assay (2). Table 1 demonstrates that antiviral activity does not correlate with the immunosuppressive potential of the compounds. In particular, derivatives carrying substitutions in the 4-position of the cyclosporin molecule, like [MeVal^4]cyclosporin, [γ-hydroxy-MeLeu^4]cyclosporin, and [MeIle^4]cyclosporin (= SDZ NIM 811 [Fig. 1]), have no or very poor immunosuppressive activity but are potent inhibitors of HIV-1 replication exhibiting IC_{50}s of 50, 338, and 164 nM, respectively. Therefore, we conclude that binding to Cyp, but not immunosuppressive activity, correlates with anti-HIV activity of the cyclosporin analogs.

Lack of direct interactions of cyclosporins with HIV proteins. We addressed the possibility that inhibition of HIV-1 by cyclosporins could be mediated by direct interactions of the compounds with viral proteins. (i) Enzyme activity of HIV-1 reverse transcriptase, proteinase, and integrase was measured in the presence or absence of CsA or SDZ NIM 811; no inhibition by the cyclosporins was observed at concentrations up to 50 μM. (ii) In the Sephadex LH-20 gel filtration assay described by Handschumacher et al. (18), we did not observe comigration of radiolabeled dihydro-SDZ NIM 811 with

HIV-1 p24^{gag}, p17^{gag}, or gp120^{env}; as expected, we could detect binding of the derivative to CypA in a control experiment. (iii) In another attempt to observe binding of cyclosporins to these viral proteins, we incubated them with a photoreactive and radiolabeled cyclosporin derivative (12) and irradiated the mixture with UV light; no reaction with the HIV proteins was detectable while Cyp was covalently labeled in a control experiment (data not shown). Thus, no evidence for direct binding of cyclosporins to viral proteins was obtained.

Lack of interaction of Cyp-SDZ NIM 811 complex with viral or cellular proteins. The correlation of antiviral activity of cyclosporins with their capacity to bind to Cyp and the lack of direct interactions of the compounds with viral proteins point to Cyp as the relevant receptor protein. Cyp by itself might have an important function during HIV replication which would be blocked by cyclosporins. Alternatively, analogous the immunosuppressive mechanism of action of cyclosporins, where the Cyp-CsA complex binds to calcineurin, a protein phosphatase involved in immune regulation (23), the complex between Cyp and cyclosporin might interfere at some stage of the viral life cycle. In this case, Cyp would gain a new function by the presence of cyclosporin rather than lose its function.

In an experiment similar to that designed for trapping calcineurin out of cell homogenates (23), we incubated GST-Cyp–SDZ NIM 811 complex with brain homogenate and then passed the mixture through a glutathione Sepharose column to trap any proteins bound to the complex. No proteins specifically binding to the Cyp-SDZ NIM 811 complex were detected in the eluate (not shown); in a control reaction using GST-Cyp–CsA complex, calcineurin A and B as well as calmodulin were detected by the method of Liu et al. (23). By using the same method but replacing the brain homogenate by lysates of either uninfected MT4 cells or chronically HIV-1 IIIB-infected Jurkat cells (in each case, proteins were metabolically radiolabeled with [^{35}S]methionine), we were unable to detect a protein which would specifically bind to the Cyp-SDZ NIM 811 complex (data not shown).

Furthermore, the complexes of Cyp with either CsA or SDZ NIM 811 did not inhibit the activity of the viral enzymes reverse transcriptase, protease, and integrase at concentrations of the complexes of up to 50 μM. Thus, no evidence for a cellular or viral protein capable of forming a ternary complex with Cyp-SDZ NIM 811 was obtained.

We also tested whether the nonimmunosuppressive derivative SDZ NIM 811 would inhibit the rotamase activity of Cyp. Indeed, the *cis-trans* prolyl isomerase activity was inhibited in the assay described by Fischer et al. (11) with IC_{50}s of 40 nM for both SDZ NIM 811 and CsA.

Interaction of CypA with HIV-1 CA protein. It has been reported that CypA binds to the HIV-1 *gag*-encoded proteins p55 and p24 (24). We confirmed this result by using incubation of GST-Cyp with the HIV-1 CA protein p24, followed by addition of glutathione Sepharose, elution of bound proteins with SDS-containing buffer, and finally analysis of the eluate by immunoblotting with anti-p24 antibodies. Alternatively, a 10 mM solution of glutathione also eluted the GST-Cyp–Gag protein complex from the solid support (not shown).

In further experiments we replaced the GST-Cyp by biotinylated Cyp. This proved advantageous, since it excluded any interaction of proteins with the GST portion of GST-Cyp which we had detected repeatedly with various proteins. Avidin Sepharose was then used to trap complexes of the biotinylated Cyp with proteins. Bound proteins were eluted with SDS buffer.

As shown in Fig. 2, p24^{gag} bound to biotinylated Cyp. This was true for p24 produced in *E. coli* (Fig. 2A) and for the genuine CA protein p24^{gag} from a lysate of HIV-1 virus particles (Fig. 2B), produced by chronically infected Jurkat cells.

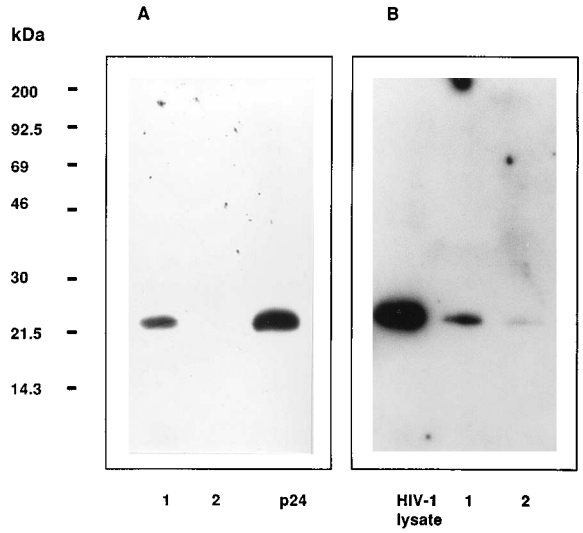

FIG. 2. Binding of HIV-1 CA protein (p24^{gag}) to biotinylated CypA. Recombinant p24 (0.4 μM) (A) or lysate of HIV-1 core particles (B) was incubated with (lanes 1) or without (lanes 2) biotinylated CypA (0.18 μM). Complexes were trapped on avidin Sepharose and eluted with SDS-containing buffer; the eluate was subjected to SDS-PAGE, followed by immunoblotting with anti-p24 antibodies.

We incubated biotinylated CypA with increasing concentrations of recombinant HIV-1 CA protein and measured the relative amounts of complex by densitometry (Fig. 3). Half-maximal binding was observed at concentrations of approximately 100 nM of CA protein.

Inhibition of CypA-CA interaction by SDZ NIM 811. It had been reported previously (24) that the binding of HIV-1 CA to Cyp is inhibited by CsA. We found that nonimmunosuppressive cyclosporin derivatives like SDZ NIM 811, also block the formation of complexes. From the experiments depicted in Fig. 4, an IC_{50} of 0.65 ± 0.05 μM was estimated. In fact, all cyclosporin derivatives which bound to Cyp with affinities similar to that of CsA (compounds I to IX in Table 1) inhibited the formation of complexes with p24 at comparable concentrations, while compounds that do not bind to Cyp (compounds X to XVI) were inactive (data not shown).

Virus specificity of inhibition by SDZ NIM 811 and of Cyp-CA protein interaction. Cyclosporin derivatives were found to be generally less potent inhibitors of HIV-2 replication than of HIV-1. In particular, the inhibitory activity of SDZ NIM 811 against HIV-1 and HIV-2 replication in the MT4 cell line and in primary T4 lymphocytes is demonstrated in Table 2. Obviously, HIV-2 strains are inhibited only at concentrations 5 to 10 times higher than those for HIV-1 strains. Furthermore, SDZ NIM 811 proved to be not inhibitory to SIV replication in CEMX174 cells (42).

If interference with Cyp-Gag interaction is indeed relevant to the mode of antiviral action of cyclosporin derivatives, differences between the behavior of Gag proteins from HIV-1, HIV-2, and SIV in the Cyp binding assay are expected. To address the specificity of binding of HIV-1 CA protein to CypA, we performed experiments with the corresponding proteins from HIV-2 and SIV produced in *E. coli*. Surprisingly, these CA proteins also bind to CypA (Fig. 5A and B). Furthermore, the Gag precursor proteins of HIV-1 and HIV-2 produced in *E. coli* both form complexes with Cyp (Fig. 5C and D).

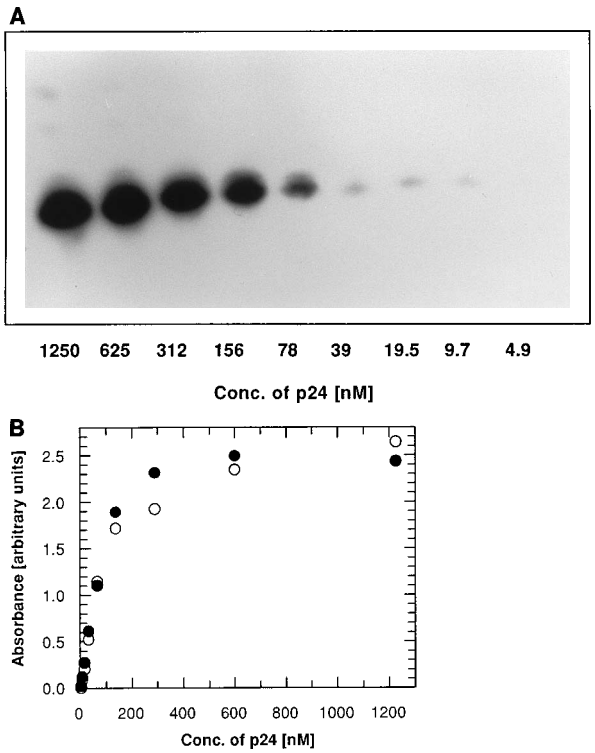

FIG. 3. Affinity of binding of HIV-1 CA protein to human T-cell CypA. (A) Immunoblot with anti-p24 antibodies. (B) Plot of densitometric absorbance units versus total p24 (data from two independent experiments [○ and ●] shown). Biotinylated CypA (0.18 μM) was incubated with increasing concentrations (Conc.) of HIV-1 CA protein ($p24^{gag}$). Complexes were trapped with avidin beads, eluted, and subjected to SDS-PAGE. CA protein was visualized by immunoblotting and was quantified by densitometry.

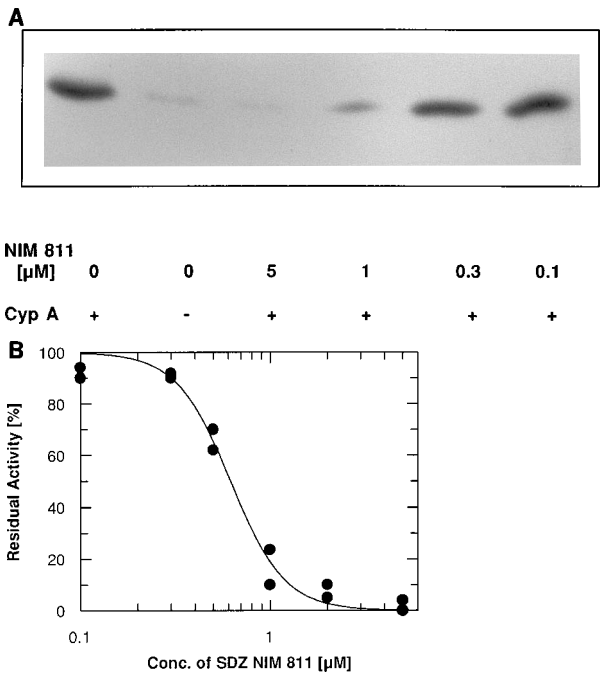

FIG. 4. Inhibition of binding of $p24^{gag}$ complex to biotinylated CypA by SDZ NIM 811. (A) Immunoblot of eluate from avidin Sepharose. (B) Inhibition curve calculated from densitometric scans of immunoblots of three independent experiments similar to that shown in panel A. HIV-1 p24 (0.4 μM) was incubated with (+) or without (−) biotinylated CypA (0.18 μM) in the presence of different concentrations (Conc.) of SDZ NIM 811 (NIM 811).

We determined whether the affinities between Cyp and the three CA proteins are of the same order of magnitude. In Fig. 6, a competition experiment with HIV-1 and HIV-2 CA is depicted: approximately 50% inhibition of binding of HIV-1 CA is observed at equimolar concentrations of both CA proteins. A competition experiment with HIV-1 and SIV CA yielded a similar result (not shown). Thus, the recombinant CA proteins of HIV-1, HIV-2, and SIV bind to CypA with the same affinity. Also, the complexes of Cyp with CA proteins of HIV-2 and SIV are disrupted by CsA or SDZ NIM 811 at concentrations similar to that needed in the case of HIV-1 CA (IC_{50} = 0.8 ± 0.2 μM). In addition, we compared binding of recombinant HIV-1 and HIV-2 Gag precursor proteins to Cyp in competition experiments: both proteins bound with similar affinities (data not shown).

Finally, we analyzed the genuine Gag proteins from lysates of HIV-1, HIV-2, or SIV virus particles in the Cyp binding assay. The virus particles were produced by HUT78 cells, chronically infected by either HIV-1, HIV-2, or SIV. To obtain mature or immature Gag protein, the cells were incubated in the absence or presence of an HIV or SIV protease inhibitor. A dose-dependent inhibition of Gag cleavage was observed, leading to increasing accumulation of Gag precursor proteins as monitored by SDS-PAGE and immunoblotting (data not shown). After ultracentrifugation of cell culture supernatants, the levels of Gag protein in the concentrated virus particle lysate were determined by ELISA using antibodies specific for either HIV-1 or HIV-2 or SIV. For the Cyp binding assay, approximately equal concentrations of HIV-1, HIV-2, or SIV Gag protein were incubated with biotinylated Cyp.

In contrast to the results obtained with Gag proteins produced in *E. coli*, a clear difference was observed between HIV-1 and HIV-2 or SIV Gag proteins from lysates of virus particles (Fig. 7). While HIV-1 Gag protein bound effectively to Cyp, almost no binding could be detected with HIV-2 and SIV Gag proteins. This difference in affinity to Cyp is estimated to be at least 50-fold. Both the immature (Fig. 7) and mature (not shown) Gag proteins derived from HIV-2 and SIV virions bound only poorly to CypA under conditions where strong binding with HIV-1 Gag proteins was observed.

Interaction of CypA with further viral proteins. We had observed that $p24^{gag}$ from a lysate of HIV-1 core particles can bind to Cyp (Fig. 2). The same samples used in Fig. 2, lanes 3 and 4, i.e., eluates from avidin Sepharose following incubation of HIV-1 lysate with and without biotinylated Cyp, were subjected to immunoblotting using monoclonal antibodies against the MA protein $p17^{gag}$ of HIV-1. This experiment revealed

TABLE 2. Activity of SDZ NIM 811 against HIV-1 and HIV-2 strains

Cell type	Virus strain	IC_{50}[a] (μg/ml)
MT4	HIV-1 IIIB	0.048
	HIV-2 ROD	0.21
	HIV-2 EHO	0.38
T4 lymphocytes	HIV-1 D370	0.016
	HIV-2 MS	0.19

[a] The concentrations of compound reducing p24 antigen in the supernatants of infected cultures by 50% are shown. For the MT4 cell line, p24 was measured at day 3 postinfection; for primary T4 lymphocytes, p24 was determined at two to four time points postinfection when antigen concentration in the untreated control culture increased exponentially.

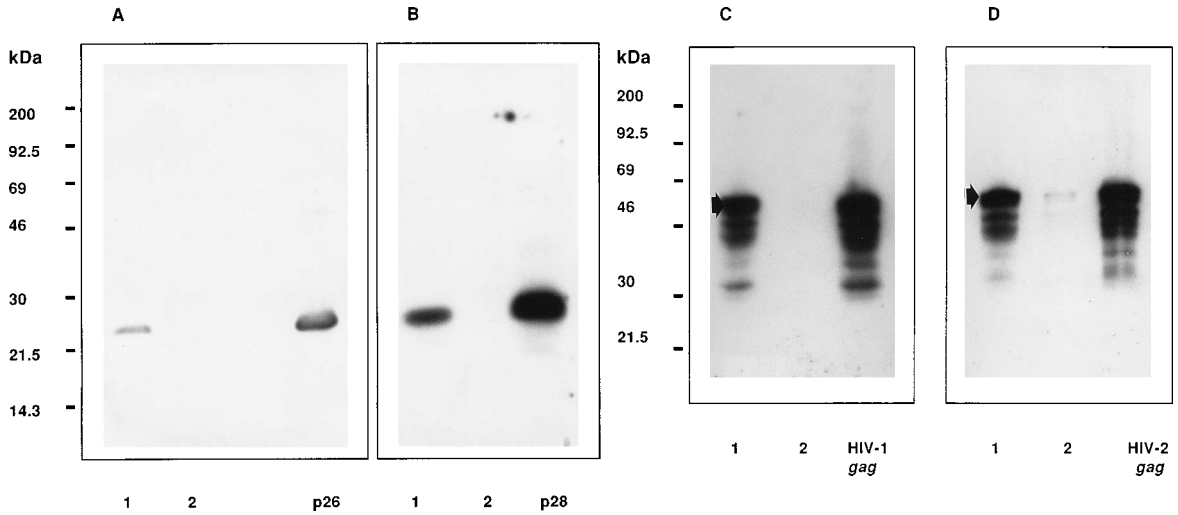

FIG. 5. Binding of recombinant Gag proteins to CypA. (A) p26^{gag} of HIV-2; (B) p28^{gag} of SIV; (C) HIV-1 Gag precursor; (D) HIV-2 Gag precursor. Proteins were incubated with (lanes 1) or without (lanes 2) biotinylated CypA (0.18 μM), and complexes were bound to avidin Sepharose and eluted with SDS-containing buffer. p26^{gag} and p28^{gag} were added as pure proteins (0.28 μM); HIV-1 and HIV-2 Gag precursor proteins were added from clarified lysates of recombinant *E. coli*. Immunoblots were developed with antibodies against HIV-1 p24, HIV-2 p26, or SIV p28. The positions of full-length precursor are indicated by arrows.

that the MA protein contained in the core particles had also bound to biotinylated CypA (Fig. 8A).

To further confirm this result, we prepared recombinant p17 expressed in *E. coli*; this protein differs from the natural one by two additional amino acids (Gly-Ser) at the N terminus and the lack of myristoylation. We repeated the Cyp binding assay with this material: as is evident from Fig. 8B, binding of recombinant p17 to CypA also occurs. In addition, a gel shift analysis in nondenaturing polyacrylamide gels was performed with unmodified Cyp and recombinant p17. Two additional bands of higher negative charge appeared on the gel with increasing concentrations of p17, obviously corresponding to Cyp-p17 complexes (not shown).

We were prompted by this result to investigate whether other HIV-1 proteins, too, would bind to biotinylated Cyp. In fact, in addition to the MA and CA portions of p55^{gag}, we also observed binding of gp120^{env}, Nef, and Vif (Fig. 9). A number of other viral proteins, however, namely, p7^{gag}, gp41^{env}, Rev, Tat, protease, reverse transcriptase, and integrase did not bind to CypA (Table 3).

To get an idea about the relevance of these interactions, the relative affinities of the proteins to CypA in comparison to HIV-1 CA were assessed by competition experiments similar to that depicted in Fig. 6: incubations contained a constant concentration of CA protein (0.1 μM), 0 to 2 μM competitor protein, and 0.18 μM biotinylated Cyp. We found that binding of the MA protein p17^{gag} and Nef to Cyp was at least 20-fold weaker than that of CA. On the other hand, an only twofold molar excess of Vif was able to displace approximately 50% of p24 from the complex with CypA, indicating that both proteins bind with similar affinities. Furthermore, gp120^{env} exhibited approximately three times higher affinity to Cyp than p24^{gag}, since at a concentration of 30 nM of gp120, about 50% of p24 were displaced from the complex.

We also tested whether complex formation of all Cyp-binding viral proteins would be inhibited by cyclosporins. We found that SDZ NIM 811 at a concentration of 20 μM also displaced p17^{gag}, gp120^{env}, Vif, and Nef from CypA (Table 3).

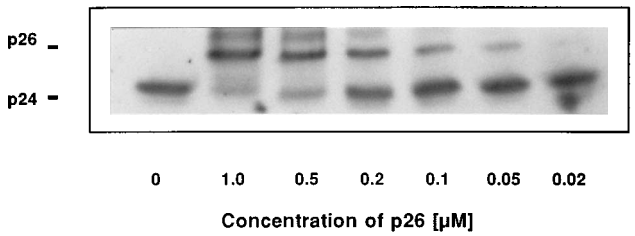

FIG. 6. Competition of recombinant HIV-1 and HIV-2 Gag protein binding to Cyp. Biotinylated CypA (0.18 μM) was incubated with HIV-1 p24^{gag} (0.17 μM) and different concentrations of HIV-2 p26^{gag}, and complexes were bound to avidin Sepharose and eluted with SDS-containing buffer. Immunoblots were developed with antibodies against HIV-1 p24 which cross-react with HIV-2 p26.

DISCUSSION

We had observed previously that nonimmunosuppressive cyclosporins, in particular SDZ NIM 811, exhibit potent anti-HIV-1 activity in vitro (33). The mechanism of action of cyclosporins is clearly not prevention of T4 cell activation (33) but is different from those of all other anti-HIV agents described so far. Recently we demonstrated that SDZ NIM 811 interferes at two different stages of the viral replication cycle: (i) translocation of the preintegration complex to the nucleus (38) and (ii) production of infectious virus particles (33, 38). To understand the molecular bases of these effects, here we investigated the interactions between antiviral cyclosporins and Cyp, between Cyp and viral proteins, and the influence of cyclosporins on these latter interactions.

The data presented in Table 1 clearly demonstrate that immunosuppressive activity is not a requirement for anti-HIV-1 activity of cyclosporins. Immunosuppression in these experiments was measured as inhibitory potency of cyclosporins in an IL-2 reporter gene assay; similar results were obtained when the compounds were tested in the mixed-lymphocyte reaction (not shown). Further evidence that SDZ NIM 811 is devoid of immunosuppressive activity was derived from the following experiments (33). (i) The compound showed no activity in the localized graft-versus-host model in rats. (ii) In the in vivo antibody response to sheep erythrocytes, assessed by determination of splenic plaque-forming cells in rats, SDZ NIM 811

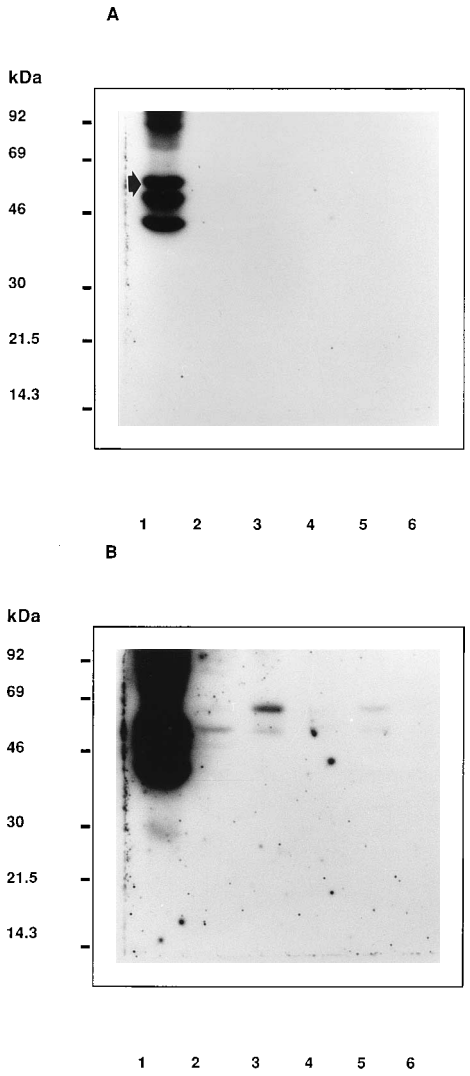

FIG. 7. Binding of HIV-1, HIV-2, and SIV Gag precursor proteins from virus particles to Cyp. Lysates of immature virus particles produced in the presence of 1 μg of the protease inhibitor SDZ 284-269 per ml were incubated with or without biotinylated CypA (0.18 μM). Approximately 9 ng of Gag protein detected by specific ELISA (Coulter) of each virus type were incubated with CypA in a volume of 100 μl. Complexes were trapped on avidin Sepharose and eluted with SDS-containing buffer. The eluate was subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were developed with antibodies against HIV-1 p24 which cross-react with the HIV-2 and SIV Gag proteins; reactivity is approximately three to five times weaker with the HIV-2 or SIV proteins than with the HIV-1 protein. Three bands corresponding to full-length (arrow) and partly cleaved short HIV-1 Gag protein are detected. The immunoblot was exposed for 1 (A) or 20 (B) min. Lanes 1, HIV-1 particles incubated with Cyp; lanes 2, HIV-1 particles alone; lane 3, HIV-2 particles incubated with Cyp; lane 4, HIV-2 particles alone; lane 5, SIV particles incubated with Cyp; lane 6, SIV particles alone.

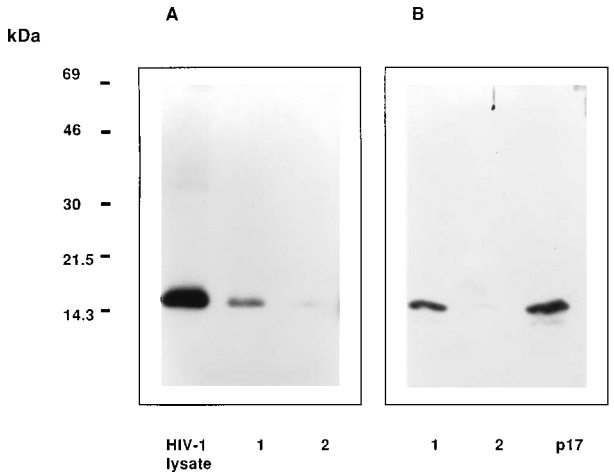

FIG. 8. Binding of HIV-1 MA protein ($p17^{gag}$) to human T-cell CypA. Lysate of HIV particles (A) or recombinant p17 (6.9 μM) (B) was incubated with (lanes 1) or without (lanes 2) biotinylated CypA (0.18 μM). Complexes were trapped on avidin Sepharose and eluted with SDS-containing buffer; the eluate was subjected to SDS-PAGE, followed by immunoblotting with anti-p17 antibodies.

did not significantly change the number of plaques per spleen. (iii) In a model of orthotopic kidney allogeneic transplantation, SDZ NIM 811 did not prevent graft rejection. Thus, while immunosuppressive drugs like CsA and FK506 are capable of blocking HIV replication via prevention of T-cell activation (1, 33), a different mechanism must account for the ability of immunosuppressive and nonimmunosuppressive cyclosporins to inhibit HIV in T-cells after activation in monocytes or in cell lines.

A correlation is evident between the ability of cyclosporins to bind to their major cellular receptor protein, CypA, and their antiviral activity (Table 1). Binding to CypA obviously is a prerequisite for HIV inhibition, since all structural changes of the CsA molecule leading to loss of affinity to Cyp abolish the antiviral effect. Among the compounds tested, we find some exceptions to this rule. However, these derivatives are pro-drugs, e.g., [2-*O*-acetyl-Thr^2]cyclosporin does not bind to CypA but is deacylated in cells to compound III (34) and, consequently, is both immunosuppressive and antivirally active in cellular assays (not shown).

Binding to Cyp seems to be necessary but not sufficient for antiviral activity of cyclosporins: in addition to binding to Cyp in vitro, a derivative must penetrate cells to reach the Cyp located in the cytoplasm. Therefore, positively charged compounds like compound VIII (Table 1) and certain negatively charged phosphate and phosphonate derivatives have no antiviral activity (unpublished results).

We did not find any indication that cyclosporin derivatives interact directly with HIV-1 proteins. They do not inhibit HIV-1 enzymes and they do not bind to Gag and Env proteins, as evidenced by gel filtration and UV cross-linking experiments. Cyp proved to be the only detectable receptor protein for the antiviral cyclosporins.

The mode of action of the immunosuppressive activity of CsA is understood in considerable detail (for a review, see reference 46): the compound does not exert its action by inhibiting the rotamase activity of Cyp, but its binding enables Cyp to form a ternary complex with the protein phosphatase calcineurin. This interaction leads to modulation of the response of T cells to immune stimuli on the transcriptional level. There is no evidence that inhibition of HIV occurs via a similar gain of function of Cyp in the presence of cyclosporins: the complex of CypA with SDZ NIM 811 does not bind to calcineurin or to any other viral or cellular proteins under conditions where calcineurin binding to the CypA-CsA complex is detectable. We conclude that Cyp itself plays an essential role in HIV replication and that binding of cyclosporins leads to a loss of function of Cyp. This function could well be the rotamase activity which might be required at some stage of the viral life cycle.

Luban et al. (24) demonstrated that the HIV-1 Gag proteins

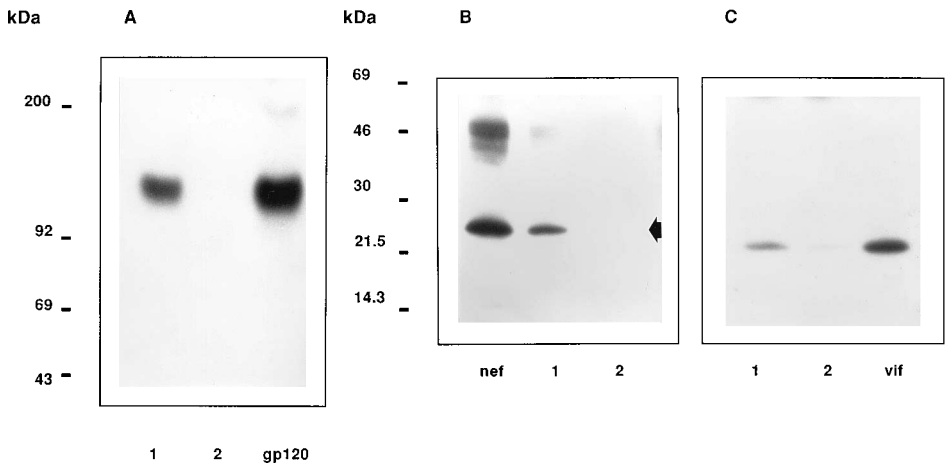

FIG. 9. Binding of HIV-1 gp120^{env}, Nef, and Vif to biotinylated CypA. (A) gp120^{env} (0.17 μM); (B) Nef (0.4 μM; position indicated by arrow); (C) Vif (0.28 μM). Proteins were incubated with (lanes 1) or without (lanes 2) biotinylated CypA (0.18 μM). Complexes were trapped on avidin Sepharose and eluted with SDS buffer; the eluate was subjected to SDS-PAGE, followed by immunoblotting with antibodies against the proteins.

bind to CypA. We confirmed this result using recombinant proteins as well as virus particle-derived Gag proteins. Since efficient binding is observed at submicromolar concentrations of both p24 and CypA, the protein-protein interaction can be assumed to be effective and relevant also under physiological conditions. The formation of complexes is in fact inhibited by those cyclosporin derivatives, both immunosuppressive and nonimmunosuppressive ones, which bind to Cyp. It is plausible to assume that interaction of CypA with HIV-1^{gag} is essential for viral replication. Cyp could act as a proline isomerase assisting in the folding or unfolding of Gag proteins (as proposed in reference 21), or it may function in cellular transport of Gag, since protein shuttling is also a potential role of cyclophilins (46). Gag proteins, in particular p17^{gag}, may play a role, e.g., in translocation of the preintegration complex to the nuclear compartment (6). Incorrect folding of Gag or lack of Cyp in the virus particles may also be the explanation why HIV particles released from cells in the presence of SDZ NIM 811 are noninfectious while retaining apparently normal amounts of processed Gag proteins (33, 38, 42).

We found that HIV-2 and SIV capsid proteins produced in *E. coli* bind to CypA with an affinity similar to that of the corresponding HIV-1 protein. Furthermore, recombinant HIV-1 and HIV-2 Gag precursor proteins bind to CypA with similar affinities. All these interactions can be inhibited by SDZ NIM 811. It appeared from these data that there is no correlation between binding of Gag from different virus types to CypA in vitro and the sensitivity to inhibition by cyclosporins of the viruses in cells. These findings were surprising in the light of two other observations: (i) HIV-2 is inhibited by SDZ NIM 811 and other derivatives at least 5 to 10 times less efficiently than HIV-1 (Table 2), while SIV is not inhibited at all (42); (ii) CypA is incorporated into virions of HIV-1, but not into HIV-2 or SIV particles (42; our unpublished results), and packaging of Cyp into the HIV-1 virions is dependent on Gag protein (42). Thus, incorporation of Cyp in virus particles seemed to correlate with sensitivity to inhibition by cyclosporins (42).

TABLE 3. Binding of HIV-1 proteins to human T-cell CypA

Protein	Binding to biotinylated CypA[a]	
	In the absence of 20 μM SDZ NIM 811	In the presence of 20 μM SDZ NIM 811
p24^{gag}	+	−
p17^{gag}	+	−
gp120^{env}	+	−
Nef	+	−
Vif	+	−
p7^{gag}	−	NA
gp41^{env}	−	NA
Rev	−	NA
Tat	−	NA
Protease	−	NA
Reverse transcriptase	−	NA
Integrase	−	NA

[a] Viral proteins were incubated with biotinylated CypA in the presence or absence of SDZ NIM 811, then avidin Sepharose was added, and bound proteins were eluted with SDS-containing buffer. The eluate was analyzed by SDS-PAGE and subsequent immunoblotting. +, binding; −, no binding; NA, not applicable.

In contrast to the results obtained with recombinant proteins, genuine Gag proteins of HIV-2 and SIV derived from virus particles, both mature and immature, barely bound to CypA, while HIV-1 Gag proteins showed strong binding under comparable conditions. Obviously, for Gag proteins derived from HIV-1, HIV-2, or SIV particles, Cyp binding correlates with sensitivity of the viruses to inhibition by cyclosporins. Gag proteins from *E. coli* folded differently than genuine ones may be the reason for the different behavior in the Cyp binding assay. This finding clearly deserves further investigation. Also, more insight into the affinities, association kinetics, and structural requirements of Gag protein-Cyp complexes has to be obtained to correlate the observations made in cellular test systems with in vitro data from biochemical assays.

We have observed that CypA also binds to HIV-1 proteins other than Gag. Interaction with the matrix protein p17^{gag} and with Nef is at least 20-fold weaker than that with p24^{gag}. It is therefore questionable if these interactions are relevant. Binding of p17^{gag} to GST-CypA was not observed by Luban et al. (24) in the *Saccharomyces cerevisiae* two-hybrid system which may indicate that this cellular assay is less sensitive than the in vitro binding assay.

Vif binds to CypA with an affinity similar to that of Vif to p24^{gag}; however, the Vif protein is dispensable for HIV-1 replication in some cell lines (5, 14, 36) where cyclosporins are also antivirally active (e.g., in the Jurkat T-cell line). On the other hand, the phenotype of Vif-negative mutants of HIV-1 is

reminiscent to that of HIV-1 virions assembled in the presence of SDZ NIM 811. Virus particles are formed and show wild-type amounts of Gag, Env, reverse transcriptase, and genomic RNA, but they are not infectious since they are impaired in their ability to complete synthesis of proviral DNA upon de novo infection (44). The relevance of this parallel situation remains to be evaluated by further experiments.

$gp120^{env}$ binds even tighter to Cyp than does $p24^{gag}$; however, since both infectious particle formation and a step of viral replication after reverse transcription are inhibited by cyclosporins, gp120 would have to enter the cell to have an effect on these events.

In our opinion, it is not too surprising that a variety of HIV-1 proteins bind to Cyp since many Cyp-protein interactions have been reported, few of which may be of biological importance (see, e.g., references 9, 11, 13, and 22). These interactions may simply be based upon interaction of proline residues located in surface-exposed loops with the catalytic site of the prolyl *cis-trans* isomerase activity of Cyp. Even Cyp-protein interactions involving nonproline sequences have been demonstrated to occur (27) but without any obvious biological relevance.

In summary, we propose that interference with HIV-1 Gag-CypA interaction may be the molecular basis for the antiviral activity of nonimmunosuppressive and immunosuppressive cyclosporins. The results that (i) cyclosporins interfere with translocation of the preintegration complex to the nucleus, (ii) viral particles produced in the presence of cyclosporins are less infectious, and (iii) these particles contain less Cyp may be explained by the following hypothesis: Cyp might play an essential role in early steps of the viral replication cycle, e.g., by inducing a conformational change in p24, which may cause removal of this protein from the preintegration complex, rendering this complex able to enter the nucleus. This function would be performed only by Cyp molecules carried within the virus core particle. Lack of incorporation of Cyp into virus particles in the presence of cyclosporins would lead to a defect in early processes. The presence of cyclosporins during the early replication steps of intact entering viruses would also block the function of the particle-associated Cyp.

We feel, however, that the final proof that Gag-Cyp interaction is not just accidental but is essential during HIV-1 replication is still lacking. Such evidence could be obtained in one or both of the following ways: (i) HIV-1 should not be able to produce infectious progeny in CypA-deficient T cells; (ii) HIV-1 strains resistant to inhibition by cyclosporins in primary T cells should carry mutations in the *gag* region coding for p24; it is questionable, however, if these mutants would be viable. Experiments in these directions are in progress in our laboratories. They should help not only in the further elucidation of the mode of action of SDZ NIM 811 but will hopefully lead to novel generations of drugs based on the understanding of the mechanism of HIV inhibition by cyclosporins.

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