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Inhibition of vaccinia virus replication by cyclosporin A analogues correlates with their affinity for cellular cyclophilins

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The mechanism by which cyclosporin A (CsA) inhibits vaccinia virus (VV) replication is still unclear. The present study addresses the question of whether CsA-binding proteins named cyclophilins (Cyps) are involved in the anti-VV activity of CsA. Six CsA analogues were analysed, and their affinity for Cyps in VV-infected BSC-40 cells and their potency as inhibitors of VV replication were evaluated. It was demonstrated that analogues with strong Cyp-binding activity, such as CsC, CsG and [MeAla^{6}]CsA, also exhibit a strong antiviral effect. In contrast, drugs with low ([MeBm$_2$t^{1}]CsA and CsH) or no ([MeLeu^{11}]CsA) affinity for Cyps show poor or no antiviral activity. The data obtained suggest a correlation between the ability of CsA to block VV replication and Cyp binding activity, and indicate the involvement of Cyps in the VV replicative cycle. They also suggest that the anti-VV action of CsA may occur by a pathway distinct from that involved in the immunosuppressive effect of the drug.

Introduction

Vaccinia virus (VV) is the prototypic member of the family *Poxviridae*. The replicative cycle, despite its complexity, progresses in a co-ordinated way within the cytoplasm of infected cells. The control of gene expression is tightly regulated in cascade fashion, each step being strictly dependent on the success of the previous phase (reviewed by Moss, 1996). Several lines of evidence indicate a role for cellular components in the poxvirus growth cycle. It has recently been shown that viral intermediate-stage transcription factor 2 is a cellular protein (Rosales *et al.*, 1994). Therefore, it is possible that other cellular proteins might be involved in the virus replicative cycle.

Cyclosporin A (CsA) is a cyclic undecapeptide with strong immunosuppressive activity that stems from its ability to block early events in T-cell activation (Kronke *et al.*, 1984; Sigal & Dumont, 1992). The intracellular receptor for CsA consists of a family of remarkably conserved proteins termed cyclophilins (Cyps). The major isoform, CypA, is an 18 kDa cytoplasmic protein that binds with high affinity to CsA (Handschumacher *et al.*, 1984; Harding, 1991). The formation of the intracellular complex CsA–Cyp inhibits the peptidyl–prolyl *cis-trans* isomerase (PPIase) activity of Cyp (Takahashi *et al.*, 1989; Fischer *et al.*, 1989).

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The CsA–Cyp complex binds a third protein, calcineurin, a Ca^{2+}- and calmodulin-dependent phosphatase (Friedman & Weissman, 1991; Liu *et al.*, 1991). The formation of the triple complex blocks calcineurin activity (Liu *et al.*, 1992), and this inactivation leads to the transcriptional block of many genes involved in the launch of the immune response (McCaffrey *et al.*, 1993). Cyps are not the direct effectors of CsA immunosuppressive activity, yet they are abundant, widely distributed and well-conserved proteins, and isomerase activity is present in all isoforms (Harding, 1991). Recent studies have reported that Cyp isomerase activity may be involved in mediating cellular protein folding and trafficking (Gething & Sambrook, 1992; Fruman *et al.*, 1994). In this regard, CsA has proved to be of great potential as a molecular probe, assisting in the identification and characterization of pathways that involve Cyp activity.

It has been reported that CsA exerts an antiviral activity in several virus–host systems. It blocks progeny production in cells infected with vesicular stomatitis virus, murine cytomegalovirus (Gui *et al.*, 1983), herpes simplex virus (Vahlne *et al.*, 1992) and human immunodeficiency virus type 1 (HIV-1) (Wainberg *et al.*, 1988; Karpas *et al.*, 1992). Recently, we have reported the antiviral effect of CsA on VV replication, and showed that virus yield is inhibited by more than 97% after infection for 24 h in the presence of CsA. The analysis of protein synthesis revealed a reduction in both early and late expression. Viral DNA accumulation is also markedly inhibited in the presence of the drug (Damaso & Keller, 1994).

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Recent studies have reported that CypA associates with HIV Gag polyprotein. In the presence of CsA, this interaction is disrupted and infectivity of the virus progeny is reduced, indicating that CsA might inhibit HIV replication by binding to CypA (Luban *et al*., 1993; Franke *et al*., 1994; Tali *et al*., 1994). It has been suggested that CypA isomerase activity may be involved in the proper folding of Gag protein and that the presence of CsA would affect CypA action (Luban *et al*., 1993; Franke *et al*., 1994).

Although the anti-HIV activity of CsA is well-studied, the mechanism by which this drug exerts its antiviral effect on VV replication is still unclear. To address the issue of whether Cyps are involved in the antiviral activity of CsA on the VV life cycle, six CsA analogues were examined and an evaluation made of (1) their ability to bind Cyps in VV-infected cells, and (2) their effect on VV replication. Evidence is presented that the anti-VV activity of CsA analogues correlates with their affinity for Cyps, particularly CypA. Analogues that bind weakly to Cyps are also shown to be poor inhibitors of VV replication; on the other hand, CsA analogues with high affinity for Cyps exert a strong antiviral effect. The data presented here suggest that CsA might inhibit VV replication by binding to cyclophilin and blocking its isomerase activity, with a similar effect to that of CsA on the HIV life cycle. The relevance of these observations and the involvement of Cyps in the VV replicative cycle are discussed.

Methods

■ **Cells and virus.** BSC-40 cells (a monkey kidney cell line) were propagated at 37 °C in Dulbecco's modified Eagle's medium (Gibco) supplemented with 8% calf serum, 2% heat-inactivated foetal bovine serum (FBS), 500 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin. Experiments were carried out using VV (strain WR) that was routinely propagated and titred by plaque assay in BSC-40 cells.

■ **CsA and analogues.** CsA (Sandimmune) was a generous gift from Sandoz Pharmaceuticals. The analogues [MeLeu^{11}]CsA (L-665,142), [MeAla^{6}]CsA (L-665,890), and [MeBm$_2$t^{1}]CsA (L-674,184) were kindly provided by N. Sigal, Merck, Sharp & Dohme Research Laboratories, NJ, USA. CsG ([Nva^{2}]CsA), CsC ([Thr^{2}]CsA) and CsH ([D-MeVal^{11}]CsA) were kindly supplied by D. Römer, Sandoz Pharma, Basel, Switzerland. The analogue [D-8′-ornithine]CsA (L-669,429) (8′OCsA) was a generous gift from P. Durette, Merck, Sharp & Dohme Research Laboratories, NJ, USA. The drugs were dissolved in 100% DMSO and stored at 4 °C as 1000× concentrated stock solutions, except for [D-8′-ornithine]CsA which was dissolved in 100% methanol (2 mg/ml) and stored at 4 °C. Radiolabelled [MeBmt-β-^{3}H]CsA (^{3}H-CsA) was obtained from Amersham (6·2 Ci/mmol; 1 mCi/ml).

■ **Preparation of mock- and VV-infected cell extracts for CsA binding assays.** Confluent monolayers of BSC-40 cells (5×10^{6} cells per 100 mm dish) were infected at an m.o.i. of 7 p.f.u. per cell for 40 min at 37 °C in Puck's saline solution (0·02 mg/ml phenol red, 8 mg/ml NaCl, 0·4 mg/ml KCl, 1 mg/ml glucose, 0·35 mg/ml $NaHCO_3$, 4 mg/ml $MgCl_2$) containing 1% FBS. The inoculum was then removed and cells were washed and incubated with complete medium. This point was defined as zero time of infection. After 18 h, the monolayers were washed twice with PBS, scraped from the plates and centrifuged at 1000 ***g*** for 5 min at 4 °C. To prepare S100 extracts, the cells were resuspended in 20 mM potassium phosphate buffer pH 7·2 containing 1 mM aminoethyl benzenosulfonylfluoride (AEBSF; Calbiochem), and frozen at −70 °C. After thawing, the cells were disrupted by Dounce homogenization and the nuclear fraction was separated by centrifugation at 1800 ***g*** for 10 min at 4 °C. The supernatant was removed and centrifuged at 105 000 ***g*** for 90 min at 4 °C, using an SW 50.1 Beckman rotor. The supernatant (S100) was removed and aliquots were stored at −70 °C. To prepare S10 extracts, after the first centrifugation step the cells were resuspended in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 0·5% Triton X-100, 1 mM AEBSF pH 7·5) and placed on ice for 20 min. The post-mitochondrial fraction was obtained by centrifugation at 10 000 ***g*** for 10 min at 4 °C. The supernatant (S10) was removed and aliquots were stored at −70 °C. The protein concentration of S100 and S10 cell extracts was determined by the Bradford assay (Bradford, 1976).

■ **Sephadex LH-20 column assay.** To quantify the binding activity to Cyps, Sephadex LH-20 columns (Pharmacia) were used as previously described (Handschumacher *et al*., 1984). The reaction mixture (100 μl) contained 60 μg S100 cell extract, 7·5% FBS and 5 μCi/ml ^{3}H-CsA (1 μg/ml) in TBAz buffer (20 mM Tris–HCl pH 7·5, 5 mM β-mercaptoethanol, 0·033% sodium azide). The mixture was incubated for 20 min at room temperature and then applied onto a 10 × 0·5 cm column (2 ml) of Sephadex LH-20 pre-equilibrated in TBAz buffer. Fractions of 300 μl were eluted with TBAz buffer and collected in 2 ml Scintiverse (Fisher) for radioactivity determination. In competition experiments, each CsA analogue or cold CsA (50 μg/ml) was pre-incubated with the reaction mixture for 10 min at room temperature. The ^{3}H-CsA was then added and the reaction proceeded for an additional 20 min. In some experiments, only the first five fractions were collected in Scintiverse. The values in c.p.m. were summed and the results expressed as a percentage of the binding activity detected in extracts incubated with no drugs (control, 0·1% DMSO).

■ **Affinity chromatography and isolation of Cyps.** Affinity columns were prepared following the manufacturer's instructions for anhydrous coupling of the ligand to Affi-Gel 10 (Bio-Rad). Briefly, 2 ml Affi-Gel 10 were washed with 20 ml isopropanol, followed by 20 ml methanol. The resin was then resuspended in methanol (total volume of 4 ml) and mixed with 2 ml of 2·1 mg/ml 8′OCsA for 5 h at room temperature. After several washes with methanol to remove unbound 8′OCsA, the resin was incubated with 50 mM ethanolamine pH 8·0 for 16 h at room temperature to block unreacted groups (Harding & Handschumacher, 1988). The beads were washed first with methanol and then with TBAz buffer and kept at 4 °C. Control columns were prepared following the same procedure, except that CsA replaced 8′OCsA.

S10 cell extracts (250 μg) were incubated with each analogue or CsA (250 μg/ml) for 30 min at room temperature and the mixture was loaded onto affinity mini-columns (0·3 ml). Unbound proteins were recovered by washing the mini-columns with 3 volumes of TBAz buffer (the use of 7 volumes of TBAz produced the same results). Proteins specifically bound to the matrix were eluted by passing 500 μl CsA (1 mg/ml in 50% TBAz, 50% methanol) through the columns. Proteins in both fractions were precipitated with 4 volumes of cold acetone for 16 h at −20 °C, and recovered by centrifugation at 12 000 ***g*** for 35 min at 4 °C. The precipitates were resuspended in loading buffer (10% glycerol, 0·02% bromophenol blue, 100 mM DTT, 2% SDS) and proteins resolved on 15% SDS–PAGE (Laemmli, 1970).

■ **VV infection and drug treatment.** Confluent monolayers of BSC-40 cells grown in 6-well plates (5×10^{5} to 1×10^{6} cells per well) were infected with 200 p.f.u. per well for 2 h at 37 °C in Puck's saline containing 1% FBS. After this period (zero time of infection), cells were washed and incubated with complete medium containing 0·1% DMSO

(control) or one of the drugs (CsA or CsA analogues) at 15 μg/ml for 24 h at 37 °C. The monolayers were then prepared for virus titration and SDS–PAGE analysis following Western blot. Plaque number and virus yields were determined essentially as described elsewhere (Damaso & Keller, 1994). All measurements of VV production are expressed as the average of three experiments in duplicate. For SDS–PAGE and immunoblot analysis, monolayers were washed, resuspended in loading buffer and incubated at 95 °C for 10 min. Samples were loaded onto 15% SDS–polyacrylamide gels as described previously (Damaso & Moussatché, 1992).

■ **Antisera and immunoblot analysis.** Rabbit anti-CypA antibody was kindly provided by J. Luban, Columbia University, NY, USA. Total proteins of purified VV (strain WR) were solubilized in SDS and used for raising rabbit polyclonal antibody. Antiserum was generously prepared by S. Oliveira and W. Leon, Instituto de Microbiologia, UFRJ, Rio de Janeiro, Brazil.

After electrophoresis, the gels were soaked in transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol pH 8·6) for 20 min (Towbin *et al*., 1979) and the electrophoretic transfer to nitrocellulose membranes was carried out at 0·8 mA/cm² for 90 min using a Semiphor Semi-Dry Transfer unit (Hoefer Scientific). The gels were then submitted to Coomassie blue or silver staining and the blots were blocked with 5% non-fat dried milk in TBS-T (20 mM Tris–HCl, 150 mM NaCl, 0·05% Tween 20 pH 7·5) for 1 h. After three washes in TBS-T, the blots were incubated with agitation with primary antibody diluted 1:1000 in TBS-T for 2 h. The membranes were washed three times with TBS-T and incubated with goat anti-rabbit affinity purified IgG conjugated to alkaline phosphatase (1:3000; Bio-Rad) for 3 h. The immunoblots were washed twice with TBS-T and twice with TBS. Polypeptides were detected by incubation with 0·33 mg/ml nitro blue tetrazolium and 0·15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in AP buffer (100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$ pH 9·5). The reaction was stopped by soaking the blots in running water.

Results and Discussion

Binding activity of CsA analogues to Cyps in VV-infected cells

To gain insight into the mechanism by which CsA inhibits VV replication, the involvement of Cyps in the CsA antiviral effect was evaluated. The experimental strategy adopted was to examine a panel of six CsA analogues with different levels of affinity for Cyps ([MeLeu^{11}]CsA, [MeAla^{6}]CsA, [MeBm$_2$t^{1}]CsA, CsH, CsC and CsG) and determine their potency as inhibitors of VV replication. The binding activity of the analogues to Cyps was measured with a Sephadex LH-20 column assay (Handschumacher *et al*., 1984). Sephadex LH-20 is a weakly hydrophobic matrix in which CsA exhibits two distinct elution patterns: free CsA is retarded by the resin and is eluted slowly during the column washes. On the other hand, the binding of CsA to proteins of the cell extracts causes a shift in the CsA elution pattern and the drug is eluted rapidly in the first fractions collected. Fig. 1(A) shows the calibration of the Sephadex LH-20 columns using mock-infected cell extracts. The extracts, prepared as described in Methods, were incubated with ^{3}H-CsA for 20 min and loaded onto the columns. The binding of Cyps to ^{3}H-CsA causes the elution of ^{3}H-CsA in the first five fractions collected from the column. This peak was therefore used in the other experiments as a measurement of the CsA-binding activity present in cell extracts. As expected, free ^{3}H-CsA loaded in the absence of cellular proteins was eluted in later fractions. The incubation of the reaction mixture with an excess of cold CsA shifted the elution peak of ^{3}H-CsA that was retarded by the matrix (Fig. 1A).

To evaluate the binding activity of the CsA analogues, competition assays were performed with ^{3}H-CsA. Mock- and VV-infected cell extracts were incubated with one of the CsA analogues (50 μg/ml) for 10 min and then ^{3}H-CsA was added for an additional 20 min. The reaction mixtures were applied to the columns and the first five fractions were collected to determine the radioactivity, as described in Methods. The rationale behind this experiment was that in the presence of CsA analogues with a high affinity for Cyps, the formation of the Cyp–^{3}H-CsA complex should be reduced; this is reflected in a low c.p.m. detected in the first five fractions. On the other hand, analogues with a low affinity for Cyps should not severely affect the binding of ^{3}H-CsA to Cyps, resulting in high c.p.m. in the first peak. The results presented here show that CsA exhibited the highest Cyp-binding activity (Fig. 1B, C). CsG and [MeAla^{6}]CsA showed lower affinity than CsA, but still significantly inhibited the formation of the Cyp–^{3}H-CsA complex compared to the control, 0·1% DMSO, where no competition occurred. The analogues [MeBm$_2$t^{1}]CsA and CsH exhibited poor binding activity to Cyps compared to CsA, and [MeLeu^{11}]CsA showed no affinity at all under the conditions used. It was also observed that infection of the monkey kidney cell line BSC-40 with VV did not alter the level of affinity of each analogue for Cyps compared to mock-infected cells (Fig. 1B, C). However, the overall CsA-binding activity was higher in VV-infected cells than in mock-infected cells. Under conditions where no competition took place (control; 0·1% DMSO), 12386 ± 2081 (S.E.) c.p.m. was detected in mock-infected cells, compared to 21973 ± 3636 (S.E.) c.p.m. in VV-infected cells ($P < 0{\cdot}01$) and a similar ratio was observed with each of the analogues. The increased amount of Cyps in VV-infected cells is currently under investigation in this laboratory.

The next step was to analyse specific binding activity for the major cytosolic isoform CypA in VV-infected cells. Competition assays were performed using 8′OCsA/Affi-Gel affinity columns. The ligand is coupled to Affi-Gel matrix by its primary amino group which is not present in CsA molecules. Thus, we used the analogue 8′OCsA; it was suitable for our needs and also showed high affinity for Cyps, as do CsA and all derivatives with a substitution in residue 8 (Quesniaux *et al*., 1987). Since CsA does not have free amino groups, it was used to replace 8′OCsA in the preparation of control Affi-Gel columns, as described in Methods. VV-infected cell extracts were incubated with one of the analogues for 30 min and loaded onto 8′OCsA/Affi-Gel columns. Analogues with high affinity for CypA should compete with 8′OCsA, decreasing CypA retention by the resin and resulting in its release during

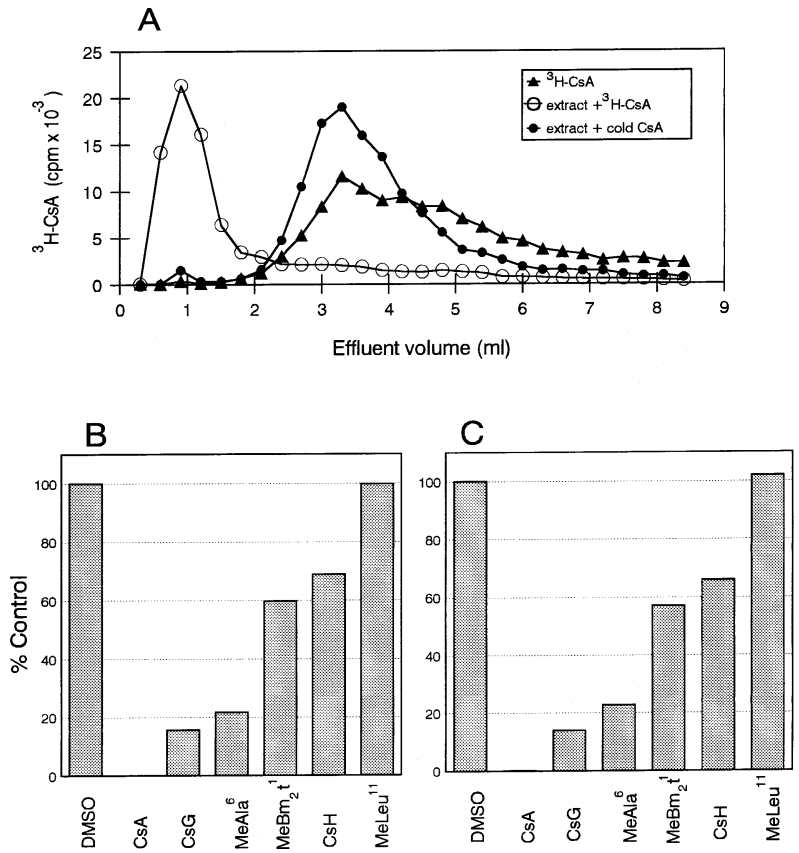

Fig. 1. Sephadex LH-20 column assay for binding activity to Cyps. BSC-40 cell extracts were incubated with ^{3}H-CsA in TBAz buffer for 20 min and loaded onto mini-columns (2 ml) of Sephadex LH-20. Fractions (0·3 ml) were eluted with TBAz buffer and radioactivity determined as described. Column calibration with mock-infected cell extracts is shown in (A). Extracts were pre-incubated with 0·1% DMSO, cold CsA or one of the CsA analogues (50 μg/ml) for 10 min followed by the addition of ^{3}H-CsA for 20 min. The mixture was applied to the columns and the first five fractions were collected and analysed for radioactivity. The values were summed and the results are expressed as a percentage of the activity detected in (B) mock- or (C) VV-infected cell extracts incubated with 0·1% DMSO.

the washing step. On the other hand, analogues with low affinity for CypA should not affect its retention by the column, and CypA would be recovered in the elution step with an excess of CsA. The fractions collected from the columns were processed as described in Methods for immunoblotting and detection with anti-CypA antibody. Fig. 2(A, B) shows the proteins retained by the affinity column and eluted with an excess of CsA. Fig. 2(C, D) shows data from a separate experiment where the fractions containing unbound proteins washed from the columns with TBAz buffer were collected. Fig. 2(A) presents the profile of bound proteins eluted with an excess of CsA. The gel was silver-stained after transfer to a filter for Western blot as shown in Fig. 2(B). One polypeptide of approximately 18 kDa was eluted from 8′OCsA/Affi-Gel columns (Fig. 2A, lanes 2–9) which corresponded to the major isoform CypA, as revealed by immunoblotting with specific antiserum (Fig. 2 B, lanes 2–9). No interactions between viral proteins or virus-induced proteins and 8′OCsA were detected, even with [^{35}S]methionine-labelled extracts (data not shown). As a control, infected cell extracts were incubated with 0·1% DMSO and loaded onto CsA/Affi-Gel columns. As expected, CypA was not retained by the matrix (Fig. 2A, B, lane 1) and was collected during the washing steps (Fig. 2D, lane 1). CypA did not bind to 8′OCsA/Affi-Gel matrix when extracts were incubated with the strong competitor CsA (Fig. 2B). As with CsA, CsC was observed to exhibit a high affinity for CypA. The incubation of CsC with the extract prevented CypA retention by the matrix (Fig. 2B), and the protein was released during the washing steps at levels comparable to those obtained during incubation of the extract with CsA (Fig. 2D). CsG and [MeAla^6]CsA bound to CypA with a reasonably high affinity, since only a small amount of CypA was retained by 8′OCsA/Affi-Gel (Fig. 2B) compared to the control (0·1% DMSO; Fig. 2B, lane 2). The analogue [MeLeu^{11}]CsA showed no binding activity, as observed in Fig. 1. CypA was not released during the washing step (Fig. 2D), and was retained by the matrix at levels similar to those detected when no analogues were incubated with the extract (Fig. 2B, compare lanes 2 and 9).

Taken together, the results obtained in the competition assays with ^{3}H-CsA and analysis of the specific binding to CypA enabled the order of Cyp-binding activity in infected cells to be defined as follows: [MeLeu^{11}]CsA < CsH < [MeBm$_2$t^1]CsA < [MeAla^6]CsA < CsG < CsC ⩽ CsA. A similar order of affinity for Cyps has been reported by other researchers using *in vitro* and *in vivo* T-cell systems (Quesniaux *et al.*, 1987; Durette *et al.*, 1988; Sigal *et al.*, 1991).

Effect of CsA analogues on VV replication

BSC-40 cell monolayers were infected with VV and, after adsorption, complete medium was added containing CsA or one of the analogues at 15 μg/ml. After 24 h, the monolayers

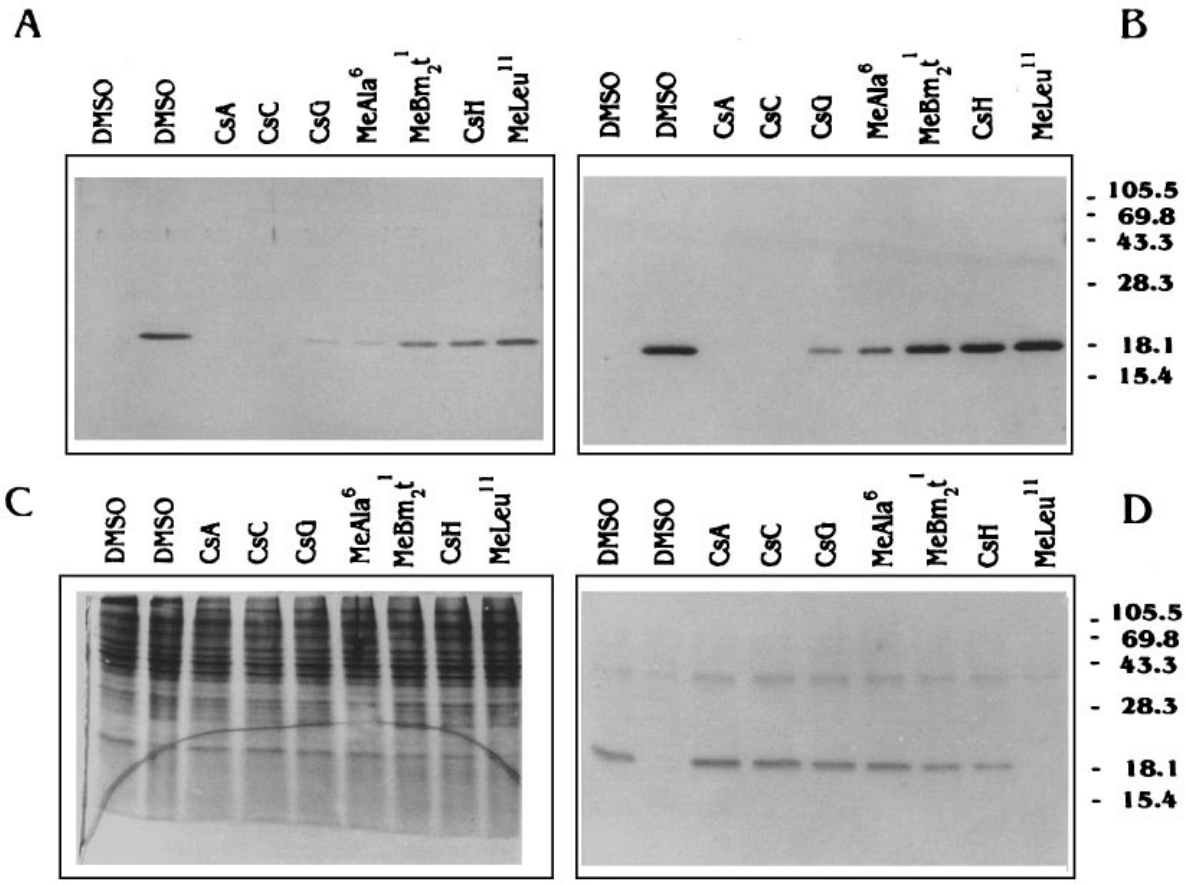

Fig. 2. Affinity columns for measurement of binding activity to CypA. VV-infected cell extracts were incubated with 0·1% DMSO, CsA or one of the CsA analogues (250 μg/ml) for 30 min and loaded onto 8′OCsA/Affi-Gel. Proteins retained by the columns were eluted with 1 mg/ml CsA (A, B) and, in a separate experiment, unbound proteins were collected by washes with TBAz buffer (C, D) as described. Proteins recovered from the columns were precipitated and prepared for 15% SDS–PAGE. After electrophoretic transfer the gels were stained with silver (A) or Coomassie blue (C) and the filters processed for immunological detection with anti-CypA antibody (B, D). The compounds incubated with the extracts are identified at the top of each lane. In lane 1, DMSO, refers to extracts incubated with 0·1% DMSO and loaded onto control columns, CsA/Affi-Gel. All other lanes designate extracts incubated with the referred compound and applied to 8′OCsA/Affi-Gel columns.

were collected for plaque assay or, in a parallel experiment, were stained for plaque number determination. As shown in Table 1, CsC exhibited the strongest inhibitory effect of all analogues on virus yield and plaque number. It blocked virus production by more than 98%, which is similar to the effect with CsA. The analogues CsG and [MeAla^6]CsA also demonstrated antiviral activity, but not as intensely as CsC; [MeBm$_2$t^1]CsA and CsH inhibited the production of infective particles by less than 40%; and [MeLeu^{11}]CsA presented no antiviral activity (Table 1). These results suggest that CsA analogues with a higher affinity for Cyps also have a stronger antiviral effect on VV production.

The cytotoxicity/viability of non-infected cells in the presence of the analogues was assessed by the method of trypan blue exclusion, and also by examining cell morphology by light microscopy. None of the analogues tested at 15 μg/ml for 24 h or 45 μg/ml for 7 h revealed signs of a toxic effect on BSC-40 monolayers (data not shown).

To further evaluate the antiviral action of CsA analogues on VV replication, we analysed (1) the accumulation of VV proteins, by immunoblotting using a polyclonal antibody raised against total proteins of purified virion; (2) the activity of *β*-galactosidase expressed under control of a late VV promoter of the recombinant virus vSC8 (Chakrabarti *et al.*, 1985; Damaso & Keller, 1994); and (3) the accumulation of viral DNA during infection (Damaso & Keller, 1994). The Western blot and *β*-galactosidase activity assays were performed at 24 h post-infection (p.i.), under similar conditions of cell infection and drug treatment to those described in Table 1. The effect of the analogues (45 μg/ml) on viral DNA accumulation in infected cells (m.o.i. of 7) was assayed at 7 h p.i. by slot blot hybridization, as described by Damaso & Keller (1994). The results of these experiments support the data in Table 1 on the effect of the six CsA analogues on virus production. The overall evaluation reveals a straight correlation between antiviral activity and affinity for Cyps.

Table 1. Effect of CsA analogues on VV yield and plaque number

BSC-40 cells infected as described in Methods were treated at zero time with 0·1% DMSO or one of the drugs at 15 μg/ml. At 24 h p.i., the monolayers were harvested for virus titration by plaque assay. In a parallel experiment, the cells were fixed and stained and the number of plaques was counted as described in Methods.

Compound (15 μg/ml)	Virus yield (p.f.u./ml)	Percentage inhibition (virus yield)*	Plaque number	Percentage inhibition (plaque number)*
0·1% DMSO	$8{\cdot}5 \times 10^5$	–	173	–
CsA	$3{\cdot}9 \times 10^3$	99·54	29	83·3
CsC	$1{\cdot}0 \times 10^4$	98·82	37	78·6
CsG	$1{\cdot}5 \times 10^5$	82·3	68	60·7
MeAla^6	$2{\cdot}23 \times 10^5$	73·7	70	59·5
MeBm$_2$t^1	$5{\cdot}6 \times 10^5$	34·1	129	25·4
CsH	$6{\cdot}0 \times 10^5$	29·4	130	24·8
MeLeu^{11}	$8{\cdot}6 \times 10^5$	0	168	0·03

* Values were calculated based on the following equation: percentage inhibition $= (1 - TA/TB) \times 100$, where TA represents total plaque or yield number in analogue-treated cells and TB represents total plaque or yield number in DMSO-treated cells.

Analogues with poor Cyp-binding activity, such as CsH and [MeBm$_2$t^1]CsA, affected VV production mildly (Table 1), and did not severely inhibit VV protein and DNA accumulation, or late *β*-galactosidase activity (data not shown). On the other hand, analogues with high affinity for Cyps, such as CsC, CsG and [MeAla^6]CsA, showed a similar effect to CsA, i.e. drastically inhibiting virus production (Table 1) and reducing the accumulation of viral DNA and proteins (data not shown). The compound [MeLeu^{11}]CsA did not bind to Cyps (Figs 1 and 2) and did not affect VV replication (Table 1; data not shown). Consequently the increasing order of antiviral potency and Cyp-binding activity were determined to be as follows: [MeLeu^{11}]CsA < CsH < [MeBm$_2$t^1]CsA < [MeAla^6]CsA < CsG < CsC ⩽ CsA. The poor antiviral activity (or lack of it in the case of [MeLeu^{11}]CsA) does not result from an inability of these analogues to penetrate the cells since they are able to cross the cellular membrane passively (Quesniaux *et al.*, 1987; Durette *et al.*, 1988; Sigal *et al.*, 1991). Therefore, the present results support the evidence that Cyps may be required in some stage(s) of the VV life cycle and that CsA exerts its anti-VV effect by binding to Cyps and impairing their activity.

The inhibition of Cyp activity by CsA could be responsible for impairment of the replication of several viruses (Gui *et al.*, 1983; Wainberg *et al.*, 1988; Vahlne *et al.*, 1992). Recently it has been reported that CypA interacts with HIV-1 Gag polyprotein and the complex is incorporated into the virions (Luban *et al.*, 1993; Franke *et al.*, 1994; Tali *et al.*, 1994). The disruption of this interaction by CsA or mutation in the *gag* gene prevents CypA incorporation into HIV-1 virions and reduces the infectivity of virus particles (Franke *et al.*, 1994; Tali *et al.*, 1994; Braaten *et al.*, 1996). The results of Billich *et al.* (1995) were similar to those presented here, demonstrating that [MeIle^4]CsA shows high affinity for Cyps and a strong anti-HIV-1 activity in infected cells. The studies of Bartz *et al.* (1995) strengthen this correlation, since [MeAla^6]CsA and [L-MeLeu(3-OH)^1]CsA also bind to Cyps with high affinity and inhibit HIV replication in chronically infected cells. In contrast, [D-MeVal^{11}, L-MeLeu(3-OH)^1]CsA does not bind to Cyps and is ineffective as an antiviral drug. The present results, together with the HIV-1 studies, suggest that Cyps are required during the virus life cycle and, consequently, replication is sensitive to CsA activity.

The data presented here indicate that the antiviral action of CsA occurs by a pathway distinct from that involved in immunosuppression. [MeAla^6]CsA shows high affinity for Cyps, but is a poor immunosuppressor and a weak inhibitor of calcineurin activity. [MeBm$_2$t^1]CsA is a strong immunosuppressor and severely inhibits calcineurin phosphatase action, but presents low binding activity for Cyps (Sigal *et al.*, 1991; Liu *et al.*, 1992; Nelson *et al.*, 1993). Nevertheless, it seems clear that other biological actions attributed to CsA correlate with its binding to Cyps and involve the inhibition of the PPIase activity, probably impairing a chaperone function. This suggests that PPIases are crucial for intracellular trafficking and protein folding (reviewed by Gething & Sambrook, 1992; Galat, 1993; Fruman *et al.*, 1994). These enzymes accelerate the folding of proteins *in vitro* and have the ability to bind proteins, acting as chaperones. Also, some PPIases are co-regulatory subunits of molecular complexes including the heat-shock proteins (HSPs) (Sykes *et al.*, 1993; Rassow *et al.*, 1995).

The role of other cellular chaperones in the VV life cycle has been investigated by other authors (Jindal & Young, 1992, Sedger *et al.*, 1996). Although VV causes a dramatic decrease in most cellular mRNA accumulation, it has been shown that

the level of HSP70 mRNAs is significantly increased in infected cells, together with a slight elevation in the level of HSP90 and HSP60 mRNAs (Jindal & Young, 1992). Additionally, a large fraction of cellular-induced HSP70 is associated with VV proteins and can co-sediment with viral proteins in a sucrose gradient. In contrast, in an HSP72-negative cell line or when HSP72 is overexpressed in cells, VV can replicate with similar virus yields (Sedger *et al*., 1996). To date, it is still not clear whether VV requires HSP70 or another chaperone to facilitate replication.

The data presented in this paper favour the hypothesis that Cyps could be required to mediate the proper folding of virus proteins, and that the inhibition of PPIase activity by CsA may be the biochemical basis of its antiviral effect. If this assumption is proved to be correct, these results indicate for the first time that the inhibition of a specific cellular protein with a known physiological role affects VV replication. Efforts to investigate the role of Cyps in the VV life cycle are in progress in this laboratory.

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