Effect of cyclosporin A on the replication cycle of human immunodeficiency virus type 1 derived from H9 and Molt-4 producer cells

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The effect of cyclosporin A (CsA) on the replication of human immunodeficiency virus type 1 (HIV-1) was studied. CsA treatment inhibited virus production in chronically infected H9 and Molt-4 cells. CsA treatment of HeLaCD4-LTR/β-gal cells or extracellular viruses also inhibited infection (IC50 1 μg/ml). The intracellular CsA-binding molecule cyclophilin A was detected in HIV-1 derived from chronically infected H9 cells, but it was present at a substantially lower level in HIV-1 derived from chronically infected Molt-4 cells. The low level of cyclophilin A in viral particles derived from Molt-4 cells correlated well with their substantially lower infectivity as assayed on HeLaCD4-LTR/β-gal cells. CsA treatment of infected cells showed a dose-dependent reduction of cyclophilin A incorporation into virions; the amount of cyclophilin A incorporation was found to be dependent on the producer cell type.

Cyclosporin A (CsA) is a potent immunosuppressive agent capable of preventing tissue rejection in organ transplantation (for review see Walsh et al., 1992). The intracellular CsA-binding protein cyclophilin A (CyPA) is a member of a family of proteins called immunophilins. The CsA-CyPA complex blocks the calcium-calmodulin-dependent phosphatase, calcineurin, that is an essential component involved in the expression of the IL-2 cytokine (Walsh et al., 1992). CyPA is a cytoplasmic enzyme that has peptidyl-prolyl cis-trans isomerase (PPIase) activity.

The isomerase activity of cyclophilins of various sources catalyses the otherwise slow isomerization of amino acid-proline peptide bonds and can accelerate the refolding of proline-containing peptides in vitro and in vivo. This effect is blocked by CsA (for reviews see Gething & Sambrook, 1992; Schmid et al., 1993). Proline-rich regions are known to be of special significance in chain conformation and protein folding (MacArthur & Thornton, 1991) and many viral proteins, including retroviral proteins, are known to contain segments rich in proline repeats (Versteegen et al., 1982; Oroszlan & Gilden, 1985; MacArthur & Thornton, 1991; Franke et al., 1994).

In a previous report on the specificity of human immunodeficiency virus type 1 (HIV-1) proteinase (PR) based on kinetic and modelling studies using oligopeptide substrates we suggested that cis-trans isomerization of proline residues at PR cleavage sites in Gag proteins may be involved in virus maturation (Tózsér et al., 1992). Proline residues have a relatively high probability of forming the cis isomer, rather than the trans isomer, of the preceding peptide bond (MacArthur & Thornton, 1991). Molecular modelling indicated that the trans, rather than the cis, isomer provided a better fit into the substrate binding pocket (Tózsér et al., 1992). Immuno-philins may convert the uncleavable cis form of the substrate to the readily cleavable trans isomer.

Based on these findings, we have considered that the CyPA inhibitor CsA may have an effect on HIV-1 infection. To study the possible effect of CsA on Gag processing in virions, 35S-radiolabelled HIV-1 virus lysates obtained from CsA-treated chronically infected H9 cells were normalized for an equal radioactive count, immunoprecipitated with HIV-1 p24 antibody, then subjected to SDS-PAGE and autoradiography. Our results suggest that treatment of chronically infected H9/HIV-1 cells with increasing concentrations of CsA results in a decrease of HIV-1 p24, but does not result in an attendant increase of unprocessed HIV-1 Pr55 precursor polyprotein (Fig. 1).

The effect of CsA on virus production in chronically infected H9/HIV-1 and Molt-4/HIV-1 cells was also studied. The presence of CsA caused a dose-dependent decrease of virus production (as measured by reverse transcriptase activity in cell supernatants) with an apparent IC50...
Virus lysates were prepared by the addition of 200 μl of cold lysis buffer were washed, pelleted and resuspended in growth medium containing incubated for 45 min, then labelled for 45 min in 2 μCi of methionine-free RPMI 1640 containing 1% FCS. The cells were cleared with preimmune goat serum, then immunoprecipitated with anti-HIV-1 p24 serum and Protein A-Sepharose CL-4 overnight at 4 °C. The immunocomplexes were washed three times with lysis buffer and once with deionized water. Then they were suspended in electrophoresis sample buffer (100 mM-Tris-HCl pH 6.8, 200 mM-dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and analysed by SDS-PAGE. After electrophoresis, the gels were prepared for autoradiography as described by Bonner & Laskey (1974). Lane 1, 0 μg/ml CsA; lane 2, 5 μg/ml CsA; lane 3, 10 μg/ml CsA; lane 4, 20 μg/ml CsA and lane 5, 14C-labelled molecular mass markers.

Fig. 1. Immunoprecipitation of 35S-methionine labelled viral lysates from H9/HIV-1pNL cells. H9/HIV-1pNL cells (10⁶) were pelleted and resuspended in methionine-deficient RPMI 1640 containing 1% FCS. The cells were incubated for 45 min, then labelled for 45 min in 2 μl of methionine-free RPMI 1640 containing 200 μCi [35S]methionine (> 800 Ci/mM). The cells were washed, pelleted and resuspended in growth medium containing medium containing 0–20 μg/ml CsA and incubated for 48 h. Viral supernatants were purified by centrifugation at 170000 g for 1 h through a 20% sucrose cushion. Virus lysates were prepared by the addition of 200 μl of cold lysis buffer (0.05 M-Tris-HCl pH 8.3, 0.15 M-NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 0.02% sodium azide). After 1 h at 4 °C, the lysates were clarified by centrifugation and an equal aliquot (50000 c.p.m.) of each was used for immune precipitation. The lysates were cleared with preimmune goat serum, then immunoprecipitated with anti-HIV-1 p24 serum and Protein A-Sepharose CL-4 overnight at 4 °C. The immunocomplexes were washed three times with lysis buffer and once with deionized water. Then they were suspended in electrophoresis sample buffer (100 mM-Tris-HCl pH 6.8, 200 mM-dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and analysed by SDS-PAGE. After electrophoresis, the gels were prepared for autoradiography as described by Bonner & Laskey (1974). Lane 1, 0 μg/ml CsA; lane 2, 5 μg/ml CsA; lane 3, 10 μg/ml CsA; lane 4, 20 μg/ml CsA and lane 5, 14C-labelled molecular mass markers.

value of 4–6 μg/ml, depending on the cell type (not shown). These data are similar to results previously reported for Molt-4 cells chronically infected with HIV-1NDK (Karpas et al., 1992). CsA treatment itself did not affect the level of CD4 expression of uninfected or HIV-1-infected H9 cells (data not shown); this is also consistent with the results previously reported for Molt-4 cells (Karpas et al., 1992). The CsA-CyPA complex could decrease virus production in chronically infected cells by inhibiting T cell activation, a prerequisite for HIV-1 infection (Klatzmann & Gluckman, 1986), by inhibiting the nuclear translocation of NF-AT required for IL-2 transcription (Flanagan et al., 1991). Since the HIV-1 LTR also contains an NF-AT binding site (Shaw et al., 1988), the transcription of viral genes could be affected as well.

Luban et al. (1993) reported that CyPA specifically binds to the HIV-1 Gag polyprotein Pr55Gag at the N-terminal region of the p24 protein via the -Tyr-Pro- cleavage site, where isomerization is thought to play a role in maturation (Tózsér et al., 1992), and CsA disrupts this interaction. This region contains a proline array (PX₄PX₂PX₄P) that is conserved among HIV-1 isolates and may be required for HIV-1 Gag binding to cyclophilin, since mutation of a single proline in Gag not only disrupts the Gag-cyclophilin interaction in vitro but also cyclophilin incorporation into virions, and virus replication (Franke et al., 1994; Thali et al., 1994).

To study the effect of CsA on the early phase of the virus life cycle, a multinuclear activation galactosidase infectivity (MAGI) assay was used (Kimpiton & Emerman, 1992). Treatment of HeLaCD4-LTR/β-gal cells with CsA 18 h before infection decreased their susceptibility to virus infection, with an IC₅₀ of 1 μg/ml and an IC₉₀ of 20 μg/ml (Fig. 2). Similar dose-dependent inhibition of infection was found, with an IC₅₀ value of 1 μg/ml when virus derived from chronically infected H9/HIV-1pNL cells was pretreated with CsA and used to infect HeLaCD4-LTR/β-gal cells (Fig. 2). These experiments show that CsA affects the early phase of the virus life cycle by acting on steps preceding integration. Our data are also supported by the results of Wang & Barklis (1993) who found that an HIV-1 mutant lacking the 56 amino acid region of Gag to which cyclophilins bind is fully competent in virus assembly and virion formation, but the resulting virions are not infectious. Recent studies conducted by Billich et al. (1995) and Steinkasner et al. (1995) have shown that CsA and SDZ NIM811, a nonimmunosuppressive CsA analogue, inhibited the formation of 2-LTR circles when HIV-1-infected MT4 cells were studied for a single cycle of virus replication. Dose-dependent inhibition of 2-LTR circle formation by SDZ NIM811 was shown to affect the kinetics of viral DNA synthesis and, specifically, nuclear localization of preintegration complexes. These studies demonstrated that, unlike the CsA-CyPA complex, SDZ NIM811-CyPA did not bind calcineurin;
Fig. 3. CyPA content and infectivity of HIV-1IB derived from chronically infected H9 and Molt-4 cells treated with CsA. The cells were incubated for 4 days before virus isolation in the absence and presence of CsA. Cell culture supernatants of untreated and CsA-treated cells were clarified by centrifugation, passed through a 0.45 µm filter and pelleted through a 20% sucrose cushion. Virus preparations were adjusted to the same p24 content determined by ELISA (Nagy et al., 1994). Lysates of equal amount of viruses derived from CsA-treated chronically infected H9/HIV-1IB (a) and Molt-4/HIV-1IB (b) cells were separated on a 10% tricine-SDS gel and immunoblotted using a rabbit anti-CyPA antibody (Affinity BioReagents) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine and 4-nitro blue tetrazolium chloride as substrates (a, b, upper panels). After recording the results, the membranes were treated with 2 M-glycine pH 2.8, 0.5 M-NaCl for 5 min, neutralized with 1 M-Tris-HCl pH 8.0, blocked, incubated overnight with a polyclonal goat anti-HIV-1 p24 antibody, followed by incubation with horseradish peroxidase-conjugated anti-goat IgG (H + L) antibody, then developed with 3,3'-diaminobenzidine (Bio-Rad) as substrate (a, b, lower panels). The amounts of CsA (µg/ml) used in cell pretreatments are indicated above the lanes. Equal amounts of virus preparations from the chronically infected H9/HIV-1IB (c) and Molt-4/HIV-1IB (d) cells were added to HeLaCD4-LTR/LacZ indicator cells to determine HIV-1 infectivity by the MAGI assay (Kimpton & Emerman, 1992).

Therefore, the anti-HIV-1 effect is not due to inhibition of T cell activation.

The mechanism of the effect of CsA on the early phase of virus replication is not known. The capsid entering the cell contains three retroviral enzymes and structural proteins. In vitro experiments with the retroviral proteinase and reverse transcriptase showed that CsA (up to 50 µg/ml concentration) had no effect on these enzymatic activities (data not shown). It is more likely that CsA acts by dissociating CyPA and p24 (Luban et al., 1993). Because the HIV-1 preintegration complex does not seem to contain the capsid protein (Farnet & Haseltine, 1991; Bukrinsky et al., 1993), the removal of the capsid protein during or after reverse transcription could be a necessary step for transport of the virus to the nucleus and subsequent integration. CyPA attached to the capsid protein might be involved in such a removal.

Similar to reports from other laboratories, CyPA was readily detectable in HIV-1 lysates derived from H9 cells. It was detected, by Western blot analysis, as an 18 kDa doublet (Fig. 3a, upper panel) and could be reduced to 50% with
approximately 1 μg/ml CsA, which is in good agreement with the IC₅₀ value determined from the MAGI assay. This suggests that the association of CsA with the virion-associated CyPA could be responsible for the early-phase effect of CsA. In contrast to the findings with H9 cells, a much lower level of CyPA was detected in virions derived from chronically infected Molt-4 cells, even in the absence of CsA treatment (Fig. 3b, upper panel) and it was completely undetectable in virions derived from cells preincubated with 10 μg/ml CsA. Although the viral protein content of these samples was normalized in the p24 antigen capture assay, the amount of viral protein in the samples was indicated by reprobing the membranes with anti-HIV-1 p24 antibody (Fig. 3a, b, lower panels). This blot analysis also suggests that CsA does not have an effect on Gag processing since no accumulation of the polyprotein precursor form is seen. However, the effect of CsA on peptidyl-prolyl isomerization could not be excluded because cis-trans isomerization could also occur, albeit more slowly, in the absence of PPIases.

To determine the infectivity of virions produced by treated and untreated cells, cell-free virus was normalized for p24 content and used in the MAGI assay (Fig. 3c, d). The virions derived from Molt-4 cells were approximately threefold less infectious, based on equal p24 amounts and reverse transcriptase activity, than those obtained from H9 cells. The infectivity of virions derived from CsA-treated cells was decreased.

Billich et al. (1995) showed that CyPA can bind recombinant Gag proteins of HIV-1, HIV-2 and simian immunodeficiency virus produced in E. coli with equal affinities; however, the virion-derived proteins showed dramatic differences in binding to CyPA. Only the proteins of HIV-1 purified from virions were found to have a strong affinity for CyPA. The binding of virion-derived Gag proteins correlated with the sensitivity of the viruses toward inhibition by cyclosporins (Billich et al., 1995). The difference in folding of the proteins from E. coli, compared with those derived from virions, could be responsible for their different behaviour in the CyPA binding assay. Our results suggest that not only the type of virus, but also the producing cell line, could be responsible for the different CyPA-binding capability of retroviruses, possibly due to altered folding and/or post-translational modification.

As exemplified in a recent report (Weber & Galpin, 1995), the combined effects of the selective growth inhibition of HIV-1-infected cells and inhibition of the early phase of the retroviral cycle could make CsA a promising anti-HIV-1 drug.

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