0014-4754/93/490533-06$1.50 + 0.20

Interleukin-8 is a Cyclosporin A binding protein

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Received 20 April 1993; accepted 26 April 1993

Abstract. Inflammatory immune reactions occur during transplant rejections and autoimmune diseases. Such reactions are mediated by cytokines, including interleukin-8 (IL-8). Cyclosporin A (CsA) exerts immunosuppressive activities[1,2] by binding to immunoregulatory proteins termed cyclophilins[3]. The anti-inflammatory effects of CsA are still not fully understood. Searching for novel neutrophil-activating proteins, we observed that an antiserum against human recombinant Interleukin-8 (IL-8) cross-reacted with cyclophilins in Western blots. Furthermore, native IL-8 was found to specifically bind CsA, whereas biologically inactive analogs of CsA were not bound by IL-8. Putative binding sites for CsA on IL-8 could be identified on the basis of structural similarities between IL-8 and cyclophilin. However, IL-8 lacks peptidyl-prolyl-isomerase (PPlase) enzyme activity, which is regarded as a characteristic of cyclophilins[4,5,6]. We conclude that the specific binding of CsA to IL-8 may explain some of the anti-inflammatory effects of CsA. IL-8 may be a novel member of the cyclophilins lacking PPlase activity.
Key words. Cyclosporin A; cyclophilin; immunophilin; Interleukin-8.

The scientific importance and the clinical use of Cyclosporin A (CsA) are steadily expanding. The latter now includes protection of transplants against rejection, treatment of autoimmune diseases (e.g. rheumatoid arthritis), and chronic inflammatory diseases (e.g. psoriasis)[2]. The immunosuppressive activity of CsA is due to its inhibition of cytokine production in T cells.[7] However, the anti-inflammatory action of CsA is still not fully understood[7], e.g. CsA inhibits psoriatic inflammation without suppressing IL-8 production in the skin[8]. Also, neither the known inhibition of peptidyl-prolyl-isomerase (PPlase) activity of cyclophilins by CsA nor the effect exerted by the complex of CsA and cyclophilin with calcineurin resulting in inhibition of the phosphatase activity of calcineurin explains the anti-inflammatory effects of CsA[4,7,9,10].

Recent research indicates that IL-8[11–13] is a major pro-inflammatory cytokine. IL-8 induces neutrophil chemotaxis, activation, degranulation and the production of superoxide anions from leukocytes (for review see refs. 14, 15, 16). Monocytes, granulocytes, lymphocytes and endothelial cells as well as synovial cells and chondrocytes release IL-8 upon stimulation with LPS, IL-1 or TNFα. The production appears to be regulated in a complex manner within the cytokine network[4,17,18–22]. IL-8 is found in generalized and localized inflammatory reactions. High levels of IL-8 are found in the serum of patients with sepsis[23], in synovial fluid from patients with rheumatoid arthritis[24–26] and in the skin of patients suffering from psoriasis[8,27,28]. IL-8 and cyclophilins are known to be chemoattractants for leukocytes[29,30] and this activity of cyclophilin is inhibited by CsA[29,30]. Here we report that IL-8 and cyclophilins, in addition to the fact that both are chemoattractants for leukocytes, do have similar tertiary structures and do share the property of being CsA binding proteins.

Materials and methods

Reagents and antisera. Human recombinant IL-8 (8 kDa, 72 amino acids, CY-19) and anti-IL-8 antisera were purchased from Biosource, Camarillo, CA. CsA and [D-MeVal11]CsA were kindly provided by Sandoz, Basle and rapamycin by Wyeth-Ayerst, Princeton. Tritiated CsA (^{3}H-CsA, 12 μg/ml; 0.1 mCi/ml) was purchased from Amersham, Buckinghamshire. The antiserum against denatured pig kidney cyclophilins (see below) was raised in a rabbit (As-Cyp-den).

Cyclophilins. Purification of cytosolic cyclophilin from U937 cells and of 17 kDa and 19 kDa cyclophilins from pig kidney was done as previously described[31]. Cell homogenates from U937 cells or pig kidney were precipitated with ammonium sulfate (60%), dialyzed against 10 mM Tricine pH 8.5 and applied to cation exchange chromatography (Mono S, Pharmacia). After gel filtration on Sephadex G 75 (Pharmacia), pooled active fractions were applied to affinity chromatography on Fractogel TSK AF blue (Merck). An additional gel filtration on Superdex 75 (Pharmacia) yielded pure cyclophilin.

Immunoprecipitation. For immunoprecipitation U937 cells were lysed by sonication in H-buffer (50 mM HEPES

pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride. The lysate was clarified by centrifugation at 18 000 rpm for 30 min. After preclearing by incubation with an excess of proteinA-Sepharose (Pharmacia) cyclophilin was immunoprecipitated from these extracts using a 1:150 dilution of anti-IL-8 or As-Cyp-den antisera. The optimized ratio for immunoprecipitation was 100 µl of anti-IL-8 or As-Cyp-den antisera and 100 µl proteinA-Sepharose beads per lysate prepared from approximately 10^9 U937 cells. Sepharose beads were washed three times in RIPA (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA). Then Sepharose beads were boiled for 5 min in Laemmli sample buffer and immunoprecipitated protein was analyzed by SDS-Page and silver staining.

Western blots. Approximately 5 µg of cyclophilin purified from U937 cells (18 kDa), a mixture of 17 kDa and 19 kDa cyclophilin purified from pig kidney, or IL-8 were loaded on a SDS-polyacrylamide gel. Proteins were mixed with sample buffer, without or with SDS- and heat-denaturation. After separation, proteins were transferred to nitrocellulose paper and unspecific binding to nitrocellulose was blocked with 3% powdered fat-free milk for 1 hr at room temperature. Anti-IL-8 anti-serum (1:500) or As-Cyp-den (1:500) anti-serum was added to the blot and incubated for 6 h at room temperature. The blot was developed with horseradish peroxidase-conjugated goat anti-rabbit immunglobulin (Sigma) by incubation overnight and staining with chloronaphthol (Sigma).

LH-20 minicolumn assay. CsA binding was studied with ^{3}H-CsA and IL-8 using the Sephadex LH-20 (Pharmacia) column method previously described[3]. Briefly, mini columns (2 ml) were pre-equilibrated in Tris buffer (20 mM, pH 7.8). Samples for assay were diluted to 90 µl with Tris buffer containing 7% newborn calf serum (Gibco) in small glass test tubes. After the addition of 10 µl of ^{3}H-CsA in 40% ethanol, the samples were mixed and applied to the LH-20 column. 50 µl of fractions (0.5 ml) were assayed for radioactivity in 2 ml liquid scintillation cocktail (Packard) diluted with 0.95 ml Tris-buffer.

Results and discussion

Investigating the release of both IL-8 and cyclophilins from macrophages, we observed that commercially available polyclonal antibodies against IL-8 as well as our own antisera raised in rabbits against denatured cyclophilins from pig kidney (AS-Cyp-den) immunoprecipitated an 18 kDa protein from homogenates of U937 cells (fig. 1A, lanes 1, 2). This protein displays the same relative gel mobility as cyclophilin purified from

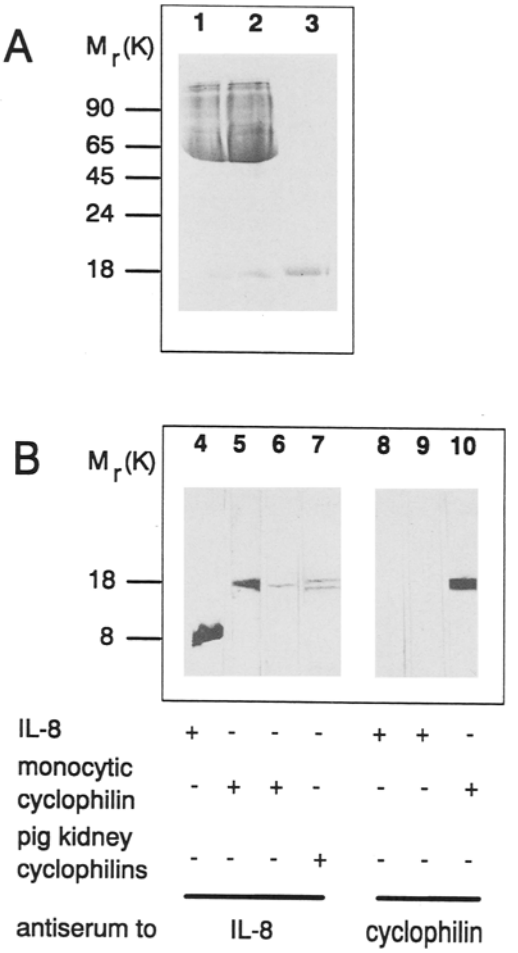

Figure 1. Immunological cross-reactivity of IL-8 and cyclophilins. A. Cyclophilins were immunoprecipitated with anti-IL-8 (lane 1) or AS-Cyp-den (lane 2) from a cell lysate of the human monocytic cell line U937. Immunoprecipitates from cell lysates (lanes 1, 2) and purified cytosolic U937 cyclophilin (lane 3) were analyzed by polyacrylamide gel electrophoresis and silver staining.
B. Cyclophilins and IL-8 were stained with antisera against IL-8 (lane 4–7) or cyclophilin (lanes 8–10) in Western blots.

U937 cells (fig. 1a, lane 3). The anti-IL-8 and the AS-Cyp-den antisera were used to further examine the epitope specificity of the observed cross-reactivity in Western blots. Figure 1B shows that the anti-IL-8 antiserum stains IL-8 (lane 4) and U937 cyclophilin (lane 5). Staining is less pronounced when U937 cyclophilin is denatured by heat and SDS (lane 6). In addition, 17 and 19 kDa cyclophilins from pig kidney are stained by IL-8 antiserum (lane 7). Affinity purified anti-IL-8 antiserum (Biosource, Camarillo) gave identical results (data not shown). As-Cyp-den antiserum reacted with U937 cyclophilin (lane 10) but showed no cross-reactivity with IL-8 under native (lane 8) or denatured (lane 9) conditions. The detection of cyclophilins by IL-8 antisera in Western blots indicates the presence of similar or identical epitope(s) on both, IL-8 and cyclophilins.
Further proof that a similarity of IL-8 and cyclophilins exists was obtained from binding studies using the clas-

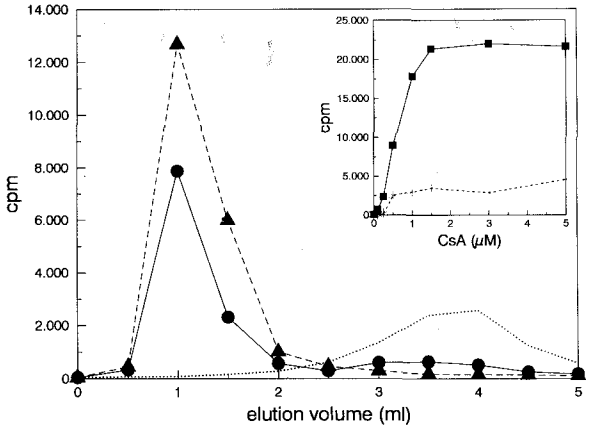

Figure 2. IL-8 binds Cyclosporin A. CsA binding was studied with ^{3}H-CsA and IL-8 using the Sephadex LH-20 column method. Data shown reflect elution of ^{3}H-CsA alone (dotted line) or after complex formation with either 10 μg human monocytic IL-8 (filled circles) or 2.5 μg of cyclophilin (filled triangles) purified from U937 cells. In the experiment shown in the inset the concentration of IL-8 was kept constant at 5 μM whereas the concentration of ^{3}H-CsA added was varied. Binding of ^{3}H-CsA to IL-8 appears not to occur in a stoichiometric 1:1 ratio, because saturation of 5 μM IL-8 is reached at 1.5 μM of CsA (filled squares). Based on protein concentration (reconfirmed by Bradford protein assay) and on the assumption that the IL-8 preparation used was fully active, the binding ratio of IL-8 to CsA approximates 4:1. The binding activity of IL-8 for ^{3}H-CsA was fully destroyed after preincubation of IL-8 with 0.1 M dithiothreitol in 20 mM Tris (pH 7.2) for 30 min at 37 °C (broken line). All binding experiments were repeated at least three times.

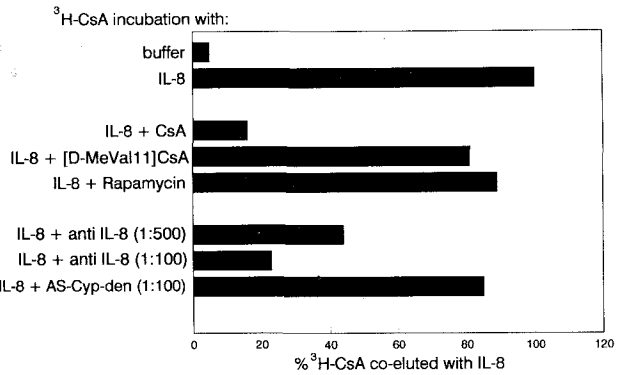

Figure 3. Characteristics of the interaction between IL-8 and CsA. CsA binding to IL-8 was measured as described in the legend of Figure 2. To block binding of CsA to IL-8, 10 μg IL-8 were pre-incubated with different compounds (5 μM) for 30 min at room temperature, then ^{3}H-CsA was added and the mixture was applied on an LH-20 column. Data are shown as % binding activity (co-elution) of ^{3}H-CsA with IL-8 alone. All experiments were performed in triplicate; the SD was always <5%.

sical LH-20 mini column assay[3]. Native (but not heat-denatured) IL-8 from monocytes and ^{3}H-CsA applied to an LH-20 column eluted together (fig. 2), as would be expected for a cyclophilin-type protein. Binding of ^{3}H-CsA to IL-8 was found to be saturable, with an apparent IC_{50} of 0.85 μM (fig. 2, inset), comparable to the binding constant of *E. coli* cyclophilin (1 μM). Furthermore, reducing IL-8 with 0.1 M dithiothreitol abolished the binding capacity of IL-8 for ^{3}H-CsA (fig. 2, inset). The latter observation may be explained by the structural characteristics of IL-8.

The structure of IL-8 in solution, as deduced by NMR and X-ray crystallographic studies, unambiguously corresponds to a dimeric molecule[32–34]. The IL-8 dimer is primarily stabilized by hydrogen bonds between the first β-strands (residues 23–29) of each IL-8 molecule and in addition by side-chain interactions. There are two intramolecular disulfide bridges, between Cys-7 and Cys-34 and between Cys-9 and Cys-50. These interactions are believed to play an important role in stabilizing the biologically active conformation[32, 33]. On the basis of this information and the similarity of the tertiary structures of cyclophilins and IL-8 (see below and fig. 4) we reasoned that only native, dimeric IL-8 should bind CsA, and denatured IL-8 should not.

The binding of CsA to IL-8 demonstrated by the co-elution of ^{3}H-CsA with IL-8 from the LH-20 column could be inhibited by preincubation with unlabeled CsA, but not by non-immunosuppressive analogs such as [D-MeVal11]CsA, which are known not to bind to cyclophilins (fig. 3). No binding between IL-8 and rapamycin, a specific inhibitor of the PPIase activity of the FK-506 binding protein, could be observed (fig. 3). The binding of IL-8 to ^{3}H-CsA could, however, be inhibited by preincubation of IL-8 with anti-IL-8 antisera.

Cyclophilins are also characterized by their PPIase activity (for review see ref. 9). Consequently, we investigated whether IL-8 also exhibits PPIase activity[31]. PPIase activity was tested using five substrates (succinyl-Ala-Xaa-Pro-Phe-P-nitroanilide with Xaa = Ala, Ser; Phe; Glu; Lys). IL-8 (up to 5 mM) was added to the reaction mixture containing substrate (25 mM) in 35 mM HEPES pH 7.8 at 10 °C. Addition of chymotrypsin (100 mg/ml) was used to start the reaction. In contrast to cyclophilins, IL-8 did not show any PPIase activity on these substrates (data not shown). This might indicate that IL-8 is a PPIase which differs in substrate specificity from known PPIases or that IL-8 is not a PPIase. The latter appears more likely since the primary sequence met-ala-asn-ala-gly necessary for PPIase activity in human cyclophilins (reviewed in ref. 9) corresponds to cys-ala-asn-thr-glu in IL-8.

Our results indicate a close similarity or even partial identity between well known cyclophilins and IL-8. Both are chemoattractants, both bind CsA, but not rapamycin, and show a specific cross-reactivity with anti-IL-8 antisera (table). It is tempting to speculate that (some) cyclophilins are cytokines or that cytokines such as IL-8 belong to the cyclophilin family (table). This view is further supported by tertiary structure data. For example, there is a striking similarity between the three-dimensional structures of human cyclophilin A[35–38] and the dimer of IL-8[32–34] (fig. 4A). Each struc-

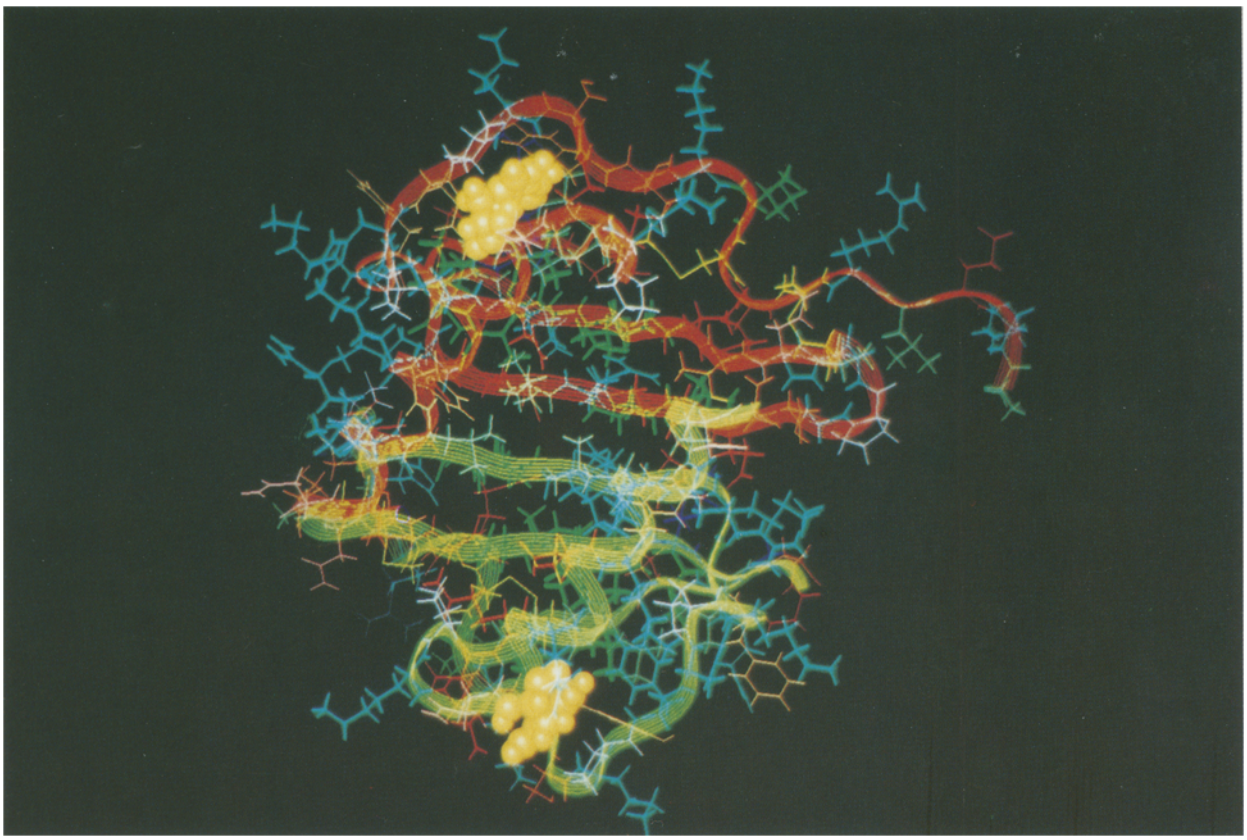

Fig. 4A

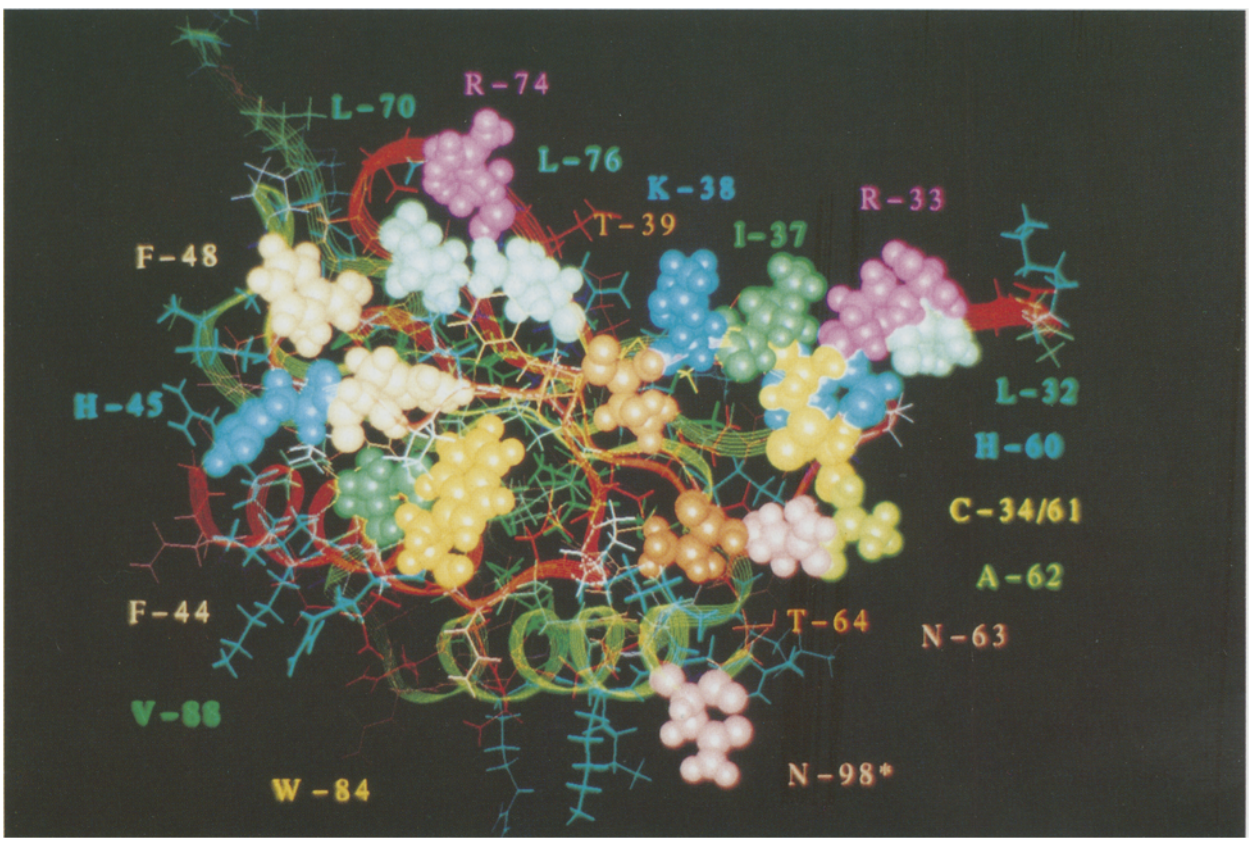
R-74
L-70
L-76
K-38
R-33
T-39
I-37
F-48
H-45
L-32
H-60
C-34/61
A-62
F-44
T-64
N-63
V-88
W-84
N-98*

Fig. 4B

Comparison of the properties of IL-8 and cyclophilins

Properties	IL-8	Cyclophilins
Binding of CsA	yes	yes
Binding of rapamycin	no	no
PPIase activity	(no)	yes
Detection of antisera against IL-8	yes	yes
Structural similarity (antiparallel β-sheet, two α-helices, exposed tryptophan)	yes	yes
Chemoattractant for neutrophils	yes	yes

ture exhibits a prominent plane (bent for cyclophilins β-barrel) built from antiparallel β-sheets and two α-helices, both of which are located above the β-sheets but without direct contact between the helices, thus allowing free access from on top of the β-sheets and between the α-helices, as from the opposite site of the plane[32, 36]. A conserved tryptophan at position 121 in human cyclophilins is important for CsA binding (for review see ref. 9). IL-8 also contains a tryptophan (position 84) which is exposed at the surface (fig. 4A) and may fulfill a similar function with respect to CsA binding of IL-8. Furthermore, the residues of human cyclophilin A defining the CsA binding site[37, 38] appear to have their counterparts in the dimer of IL-8 (fig. 4B). Further structural and functional analyses of IL-8 will be required to address the importance of the similarities between IL-8 and 'classical' cyclophilins in immune and inflammatory reactions in detail. IL-8 appears not to be a PPIase, but its effect on calcineurin or other regulatory phosphatases remains to be determined. Already, our observation may shed some light on the as yet poorly understood anti-inflammatory effects of CsA.

Acknowledgements. We thank R. Bang and U. Krummrei for help with some experiments, B. Layh and E. Yassin for technical assistance, and T. Hall for stimulating discussions. This research was supported by a grant from the DFG (SFB263).

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Figure 4. Putative binding sites for CsA on IL-8.
A. A ribbon plot of IL-8 depicts its dimeric nature as determined by Clore et al.[32] by NMR of IL-8 in solution. The two chains of IL-8 are shown (chain A in red and chain B in green). Data from the Brookhaven National Laboratory database[39] were used to build up the dimer. Highlighted as point of reference is residue W-84 (numbering according to the IL-8 sequence including the signal peptide stored with the accession number P10145 in Swissprot protein database[40] which corresponds to W-57 in Brookhaven database entry 1 IL-8, compare fig. 4B). The orientation of the molecule was chosen to show the similarity of the three dimensional structures of IL-8 with cyclophilins (compare for example ref. 36).
B. Two putative binding sites for CsA on IL-8 could be identified on the basis of similarities of type and location of amino acids between IL-8 and cyclophilin. Recently, binding of CsA in cyclophilin was shown by Theriault et al.[37] and Pflügl et al.[38] to involve directly (in clockwise direction) contact residues H-126, F-113, F-60, A-101, N-102, A-103, T-73, R-33, I-57, and Q-63. For the presentation of the putative bindings sites of CsA on the IL-8 dimer a view of IL-8 from above W-84 on the A-chain was chosen. One site includes amino acids H-45 (shown in blue on the left), F-48, L-70, R-74, L-76, W-84, V-88, and F-44. This site comprises a hydrophobic area of IL-8. The other site is similarly hydrophobic and includes H-60 (shown in blue on the right), C-34, C-61, A-62, N-63, T64, I-37, R-33, and L-32. The participation of some amino acids in the proximity like A-96* (not highlighted for reasons of clarity in the figure shown) or N-98* (both on chain B) in the binding of CsA would require large structural rearrangements and thus these amino acids cannot be expected to be in direct contact with CsA. For similar reasons, involvement of T-39 and possibly K-38 in binding of CsA can be ruled out.

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