# Cyclophilin-A is a zinc-dependent DNA binding protein in macrophages

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Received 15 May 1995; revised version received 11 July 1995

**Abstract The association of cyclosporin A (CsA) immunosuppression with inhibition of transcription factor-dependent lymphokine gene activation formed the basis of our decision to investigate nuclear-associated Cyp isoforms. Immunofluorescence microscopy of mouse macrophages cell line with a monoclonal antibody mAb7F1 raised against CypA shows a co-localisation of CypA in the nucleus and in the cytosol. Nuclear CypA binds to DNA in a zinc ion-dependent manner, in contrast to recombinant CypB. Peptidyl-prolyl *cis/trans* isomerase (PPIase) activity of nuclear CypA is inhibited by zinc ions. The zinc inhibited CypA does not bind cyclosporin A (CsA). We suggest that nuclear Cyp in complex with zinc ions recognizes DNA sequences and is involved in transcription modulating processes.**

*Key words:* PPIase; Peptidyl-prolyl-*cis/trans*-isomerase; Cyclophilin; CsA; Zinc binding; DNA binding; Macrophage

## 1. Introduction

Immunophilins (Cyclophilins, Cyp's) and FK506 binding proteins, (FKBP) have been found as binding proteins of the immunosuppressive drugs cyclosporin A (CsA) and FK506 (see refs. in [1–3]). Immunophilin–drug complexes inhibit the production of interleukin-2 by inhibiting the calcium dependent phosphatase calcineurin [4]. In addition, immunophilins have a variety of more general functions [5,6,7,8], e.g. peptidyl-prolyl *cis/trans* isomerases (PPIases, EC 5.2.1.8) [9], and specific biological effects influencing cell activities in diseases [10–13]. It is likely that their divergent effects require alternative pathways of signal transduction.

Subgroups (A, B and C) of Cyp's have been identified and characterized in human macrophages and cell lines [14]. Under certain conditions cytoplasmic Cyp's may be released [15,16] and exert extracellular cytokine-like effects [17,18]. Moreover, FKBP have recently been found in the nucleus of Jurkat T cells [19,20].

Using specific antibodies for immunofluorescence investigations we found that CypA in contrast to CypB is localized in substantial amounts in the nucleus of mouse macrophages. The nuclear CypA binds DNA in a zinc-dependent manner. The specificity of the localization and zinc binding of CypA may suggest a functional role in the regulation of transcription processes.

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*Abbreviations:* Cyp, cyclophilin; CsA, cyclosporin A; HPLC, high performance liquid chromatography; Ig, immunglobulin; mAb(s), monoclonal antibody(-ies); PBS, phosphate-buffered saline; PPIase, peptidyl-prolyl *cis/trans*-isomerase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

## 2. Materials and methods

### 2.1. Cells and cell cultures

Mouse macrophage cell line H4-7 [21,22] were cultured in RPMI 1640 (Gibco), supplemented with 10% fetal calf serum (Gibco) plus 1% penicillin and streptomycin (Sigma), 140 μg/ml gentamycin (Biochrom, Berlin). For preparation of nuclei H4-7 cells were scraped off at a density of $1 \times 10^7$ cells per 100 mm Petri dish.

### 2.2. Preparation of nuclei

About $1 \times 10^9$ H4-7 cells were collected by centrifugation at $200 \times g$, washed in cold phosphate-buffered saline (PBS), and resuspended in 0.3 M SHM buffer (0.3 M sucrose, 25 mM HEPES, pH 7.2, 137 mM NaCl, 5 mM KCl, 0.7 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM $MgCl_2$). Cells were disrupted and centrifuged for cytosolic and nuclear fractions according to the standard procedures [23,24]. The preparations were assessed using the cytosolic enzyme LDH: this approach yielded > 98% purity [25].

### 2.3. Purification of the nuclear cyclophilin from H4-7 cells

The nuclear protein pellet was solubilized by ultrasound and loaded on a HiLoad 16/60 Superdex 75 Prep Grade gelfiltration column (Pharmacia) equilibrated with 20 mM HEPES (Sigma), pH 7.3, 0.1 M NaCl. The purification of Cyp was analyzed by measuring the PPIase activity as described [26]. Active fractions were pooled and dialyzed extensively against 10 mM Tricine, pH 8.0. The Cyp pool was applied to a TSK Affi-blue HR 5/5 column (Pharmacia) equilibrated with 10 mM Tricine, pH 8.0. Cyp eluted with a linear KCl gradient (0–500 mM) in the same buffer. The protein was further separated on a $C_4$-RP-HPLC chromatography column (Pharmacia, Sweden). After SDS-PAGE, the purified protein was transferred to PVDF membrane (Millipore) and subjected to amino acid sequence determination at Toplab (Munich) on a Beckman System Little Foot 3600 TD protein sequencer equipped with an on-line PTH derivatives system according to standard procedures.

### 2.4. Antibodies and immunological techniques

H4-7 macrophages, grown on coverslips, were washed three times in PBS and were fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature. After washing, the cells were permeabilized with 50% acetone/methanol at −20°C for 10 min. The fixed and permeabilized cells were first blocked with 10% FCS in PBS for 30 min. Then they were incubated overnight at 4°C with the monoclonal antibody mAb7F1 against CypA [27]. After washing, FITC-conjugated goat anti mouse IgG in PBS was applied to the cells for 2 h. For staining of DNA, Hoechst 33258 (Sigma) was added (final concentration of 5 μg/ml) together with the secondary antibodies. Finally, coverslips were washed, mounted in a solution of 10% (v/v) glycerol in PBS, sealed and observed under a Zeiss Axioskop microscope.

### 2.5. Western blotting

Samples to be analyzed were resuspended in Laemmli [28] sample buffer and subjected to 12% SDS-PAGE. Western blot analysis was performed as described [29] using anti-CypA mAb7F1 [27] and a polyclonal anti-sera against CypB [30].

### 2.6. Determination of PPIase activity and inhibition assays

The PPIase activity of cyclophilins was measured as described [26] using a Beckman DU 70 spectrophotometer with a thermostatted cell. For determination of the inhibition of the PPIase activity by $ZnCl_2$ (Merck) or other $Me^{2+}$ ions (stock solutions of $Me^{2+}$ salts were 100 mM)


*SSDI* 0014-5793(95)00815-2

the purified CypA was used. The reactivation of the PPIase activity with the specific zinc chelator 1,10-phenanthroline (Sigma) after inactivation with $Zn^{2+}$ was carried out as follows: a distinct concentration of purified Cyp was incubated with 1 mM $ZnCl_2$ for 15 min. Inhibition of the PPIase activity was controlled by the enzymatic test with the synthetic substrate succinyl-Ala-Ala-Pro-Phe-4-nitroanilide. Then 1 mM, 2 mM, 3 mM, or 5 mM 1,10-phenanthroline (stock solution 100 mM) were added, incubated on ice for 60 min, and PPIase activity was measured.

*2.7. DNA-binding assay and immuno-dot blot*

Purified nuclear CypA (about 200 μg protein) or recombinant CypB [30] was mixed with about 50 mg double-stranded native DNA-cellulose affinity matrix from calf thymus (Sigma; 4 mg of double-stranded DNA per 1 g of solid phase) in 20 mM HEPES, pH 7.3 (DNA binding buffer). Using a series of Eppendorf-tubes, different samples were prepared adding $ZnCl_2$, DTT, EDTA at various concentrations. Formation of complexes between protein and DNA-cellulose was allowed for 3 h under rocking at 4°C. After 3 times washing, CypA bound to DNA-cellulose was eluted with different amounts of NaCl in the DNA binding buffer. To visualize the binding of CypA to DNA-cellulose, an immunoblot with mAb7F1 was performed.

*2.8. 'South-Western blot' analysis*

Purified nuclear and cytosolic CypA were mixed with Laemmli sample buffer and not boiled. The proteins were separated in a 10% SDS-polyacrylamide gel (10 μg per lane), blotted onto a nitrocellulose sheet, and incubated for 16 h in renaturation buffer (20 mM Tris-HCl, pH 7.5, 1 mM $ZnCl_2$, 0.1% BSA) at 4°C. Total mouse macrophage genomic DNA was partially digested with *Sau*3AI (Gibco/BRL) to an average size of about 600 bp, labeled by Nick translation with [α-$^{32}$P]dCTP [31] and incubated with the blot in the reaction buffer (20 mM Tris-HCl, pH 7.5, 1 mM $ZnCl_2$) for 3 h at room temperature. Alternatively, the filter was incubated with mAb7F1 prior to probing with $^{32}$P-labeled DNA. The blot was washed five times, each for 10 min, in the reaction buffer at room temperature, dried, and autoradiographed.

An identical blot was processed as described above, except that the renaturation buffer and the reaction buffer contained 50 mM EDTA, 10 mM DTT in place of 1 mM $ZnCl_2$.

## 3. Results

*3.1. Detection of CypA in nuclei and cytosol of macrophages*

Cyclophilin isoforms have been detected in different cell organelles, e.g. endoplasmatic reticulum [32] and mitochondria [33]. We analyzed the cellular localization of Cyp's in the mouse macrophage cell line H4-7 by Western blot analysis using the anti-CypA mAb7F1 and an anti-CypB polyclonal antiserum. CypA was detected in the nuclear subfraction (up to 15% of total CypA content of H4-7 cells), in contrast to a major localization in the cytosolic subfraction. CypB is restricted to the cytosol (Fig. 1A). The nuclear and cytosolic preparations were

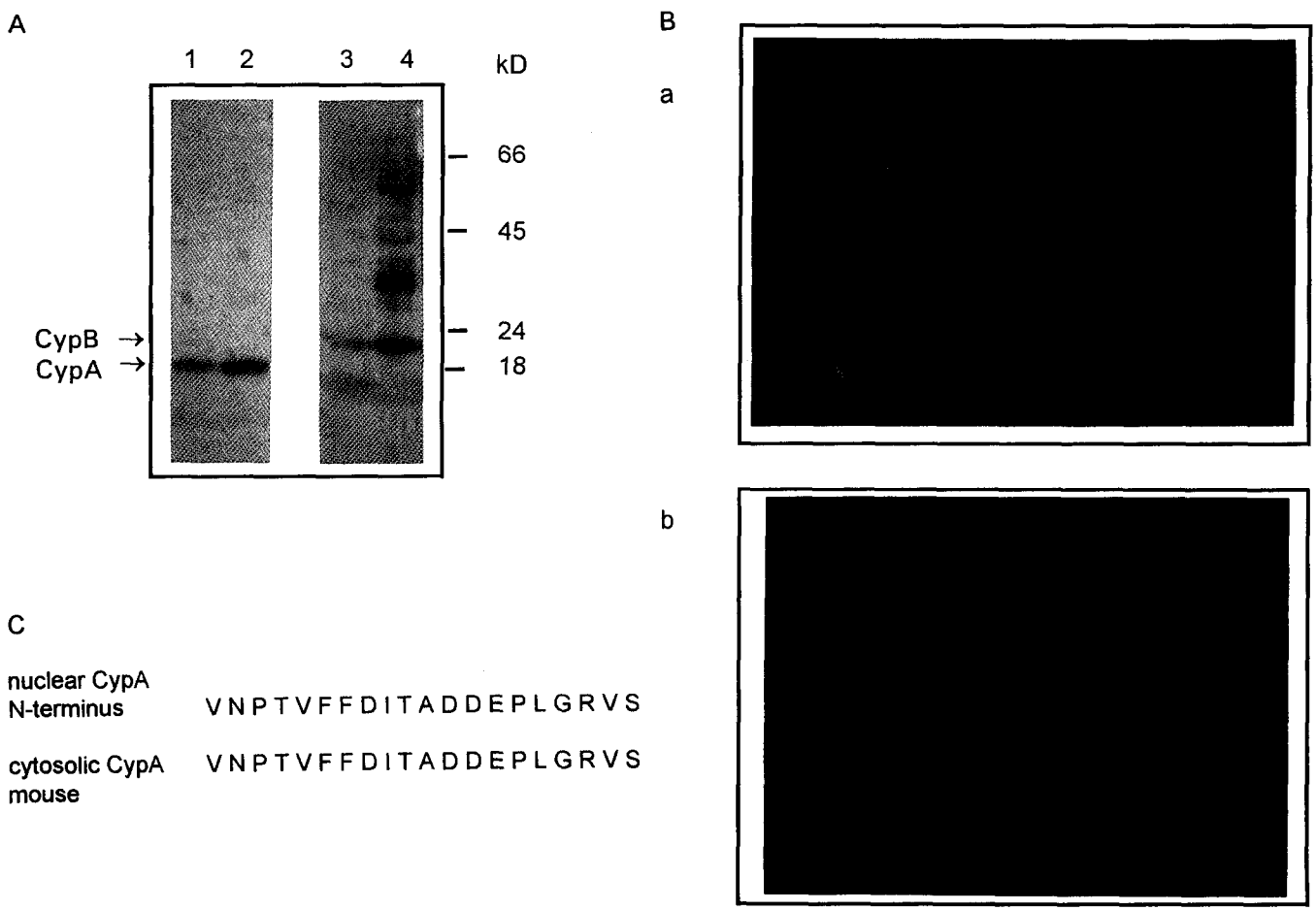


Fig. 1. (A) Localization of CypA to the nucleus by Western blot analysis. Nuclear (lanes 1, 3) and cytosolic lysates (lanes 2, 4) from mouse H4-7 macrophages were assessed by immunoblot using a monoclonal anti-CypA antibody mAb 7F1 (lanes 1, 2) and a polyclonal anti-CypB antiserum (lanes 3, 4). The position of molecular weight markers (Sigma) on the side of the gel. (B) Immunofluorescence localization of CypA by a double-label experiment using DNA-staining Hoechst dye 33258 (a) and monoclonal anti-CypA antibody 7F1 (b). Mouse macrophages were grown on coverslips, fixed with paraformaldehyde, permeabilized with aceton/methanol and incubated first with mAB 7F1 and then with FITC-conjugated anti-mouse IgG. Note staining of the nuclei and the cytoplasm with mAb 7F1. (C) N-Terminal amino acid sequence alignment of mouse nuclear and cloned cytosolic CypA (Swissprot accession number P17742).

confirmed by the cytosolic enzyme LDH. A simple cytosolic contamination of the nuclear subfraction was thereby excluded (data not shown).

Further attempts to demonstrate the nuclear localization of CypA were made by indirect fluorescence staining of fixed H4-7 cells with mAb7F1 and with Hoechst 33258 dye. Fig. 1B shows that CypA is located in the cytosol as well as in the nuclei. Staining of nuclear CypA correlates with DNA staining by Hoechst 33258 dye.

CypA could be isolated from the nuclear fraction by a sequence of gel filtration, affinity chromatography and subsequent HPLC reversed phase separation. We obtained an 18 kDa protein which shows PPIase activity and a high cross-reactivity with mAb7F1 in Western blot. Microsequencing of the N-terminus revealed a 100% identity of the nuclear Cyp (nCyp) with CypA (Fig. 1C; for comparison see [14]).

### 3.2. Zinc-dependent binding of CypA to DNA

The data presented above prompted us to investigate the biological function of CypA in the nuclei of H4-7 cells. Recently, a FKBP25 was described in the nuclei of Jurkat T cells that binds to DNA [19]. The finding that CypA localizes to the nucleus suggested that nCyp might also interact with DNA. To address this possibility, nCyp was incubated with native DNA-cellulose affinity matrix. Preincubation of nCyp with 1 mM $ZnCl_2$ resulted in DNA-cellulose binding (Fig. 2A). No binding of nCyp to DNA-cellulose was detected in an untreated sample or in the presence of EDTA/DTT. High salt concentrations (500 mM NaCl) were required to elute the protein from DNA-cellulose.

Further proof of a specific binding of nCyp to DNA was obtained by exposing nCyp from H4-7 cells to total genomic H4-7 DNA (partially digested with *Sau*3AI, labeled with [$\alpha$-$^{32}$P]dCTP) by means of a 'South-Western Blot' analysis. In the presence of $ZnCl_2$, the DNA bound to nCyp (Fig. 2B, lane A). There was no DNA binding to this protein when $ZnCl_2$ was displaced by DTT and EDTA (lane B). The identity of nCyp was confirmed by incubation with mAb7F1 prior to DNA probing to block the observed DNA binding (lane C). Specificity of CypA binding to DNA in the presence of $ZnCl_2$ was confirmed using recombinant CypB. Under these conditions CypB did not bind to DNA (lane D).

### 3.3. Functional changes of nCyp following zinc ion exposure

PPIase activity of nCyp was completely inhibited by the immunosuppressive drug, cyclosporin A (CsA), but not by FK506 (data not shown). This enzymatic function of nCyp in H4-7 cells is inhibited by zinc ions (Fig. 3A). After 30 min incubation with 1 mM $ZnCl_2$, nCyp has a residual activity of about 28% compared to untreated control. Further exposure reduces the activity to almost zero. The specific $Zn^{2+}$ chelator 1,10-phenanthroline was added to investigate the possibility of restoring enzyme activity. Nearly 90% of the starting activity was regained after 60 min treatment with 5 mM 1,10-phenanthrolin (Fig. 3B). Incubation of nCyp with $Ca^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$ had no effect on PPIase activity (Fig. 3A).

Additionally, $ZnCl_2$ interferes with the binding sites involved in CsA binding of nCyp [34], i.e. we analyzed the [$^3$H]CsA binding of nCyp after zinc treatment. Fig. 3C shows that zinc-inactivated nCyp loses the ability to bind CsA.

Finally, zinc ion binding of nCyp was analyzed by a metal ion affinity chromatography column. Salt was added to nCyp and starting buffer to exclude ion exchange effects with the affinity matrix. nCyp bound to the column and could be eluted by a SDS-containing buffer. Samples were analyzed by Western Blot with mAb7F1 (data not shown).

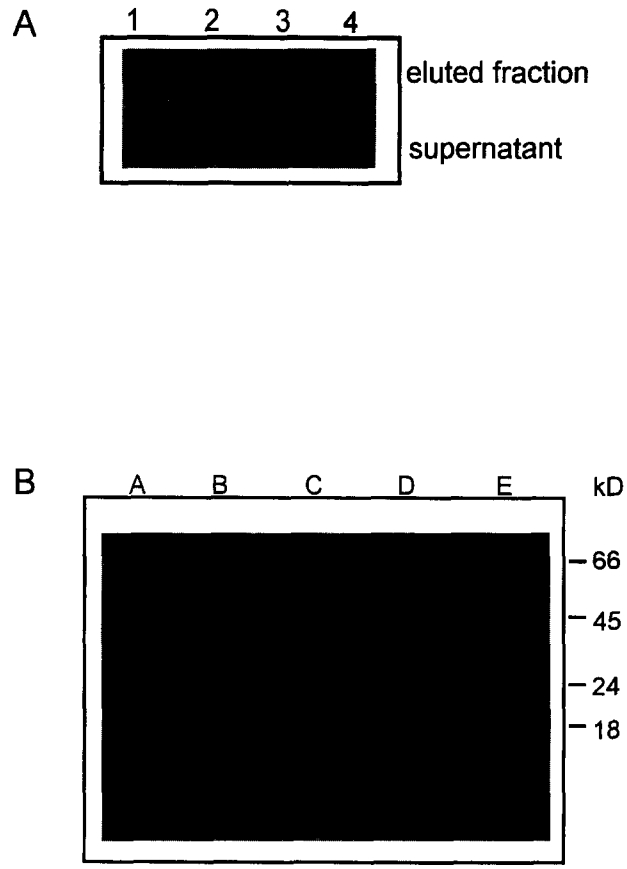


Fig. 2. CypA binding to DNA. (A) Dots showing either the supernatant of the nuclear CypA or eluted fractions from DNA-cellulose matrices with 500 mM NaCl. Nuclear CypA was preincubated in the presence of zinc ions with DNA-cellulose. The supernatant or eluted material were subjected to a dot blot and analysed by immunostaining with mAb 7F1. Lane 1 = nCyp in the absence of zinc; lane 2 = nCyp preincubated with 1 mM $ZnCl_2$; lane 3 = with 0.1 mM $ZnCl_2$; lane 4 = with 10 mM DTT, 50 mM EDTA. (B) South-Western blot of nCyp from macrophages analysed for binding of $^{32}$P-labeled fragmented genomic DNA. After separation in a 10% SDS-PAGE, proteins (5 $\mu$g) were transfered to a nitrocellulose filter and incubated in renaturation buffer. Blots were probed with $^{32}$P-labeled fragmented genomic DNA in the presence of either zinc ions (lane A) or EDTA and DTT (lane B). Lane C = preincubation with mAb 7F1 inhibits DNA binding. Note that purified CypB (lane D) does not bind to DNA in the presence of zinc ions. As a control in lane E, CypA was analysed in a Western blot with mAb 7F1.

## 4. Discussion

We identified two localizations of CypA in a mouse macrophage cell line: the cytosol (major fraction) and the nuclei. In addition, we demonstrated that the nuclear CypA is a zinc-dependent DNA binding protein exhibiting the well-known PPIase activity only in the absence of zinc ions.

The nCyp–zinc complex may bind to DNA unspecifically. In

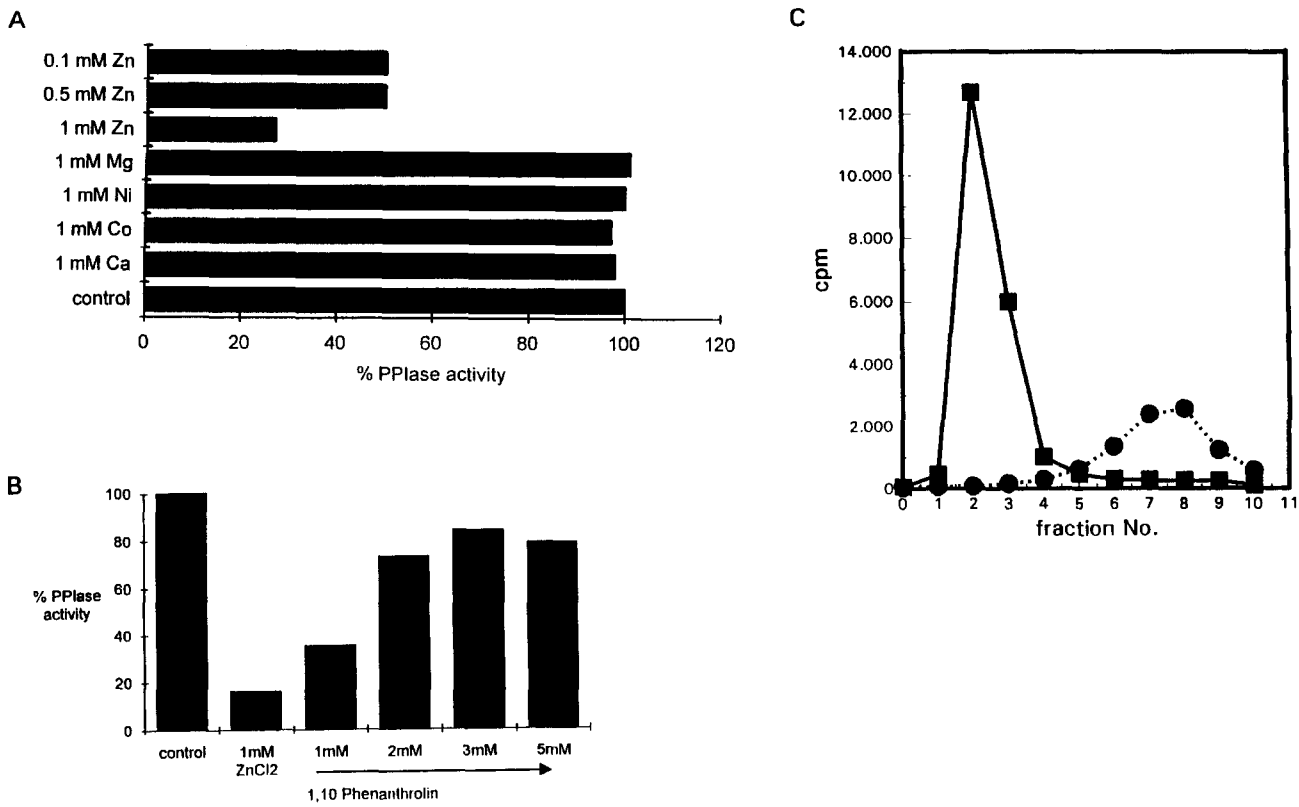


Fig. 3. Effect of $Zn^{2+}$ on PPIase activity and CsA-binding. (A) Different concentrations of zinc ions and other divalent cations were tested for their potency to inhibit PPIase activity of CypA. Bars represent the average of three experiments compared to untreated control (100%). (B) Reactivation of $Zn^{2+}$ inhibited nCyp and PPIase activity by 1,10-phenanthroline. PPIase activity of CypA was inhibited by preincubation with 1 mM $ZnCl_2$ for 30 min, then 1,10-phenanthroline in increasing concentrations was added. After 60 min samples were tested for PPIase activity. Controls are untreated samples incubated for the same time. Bars indicate PPIase activity in percent compared to control. (C) CypA preincubated with (●) or without 1 mM zinc ions (■) was analysed by the classical LH20 binding assay. The CypA–[$^3$H]CsA complex eluted in the first three fractions, in contrast to retarded free [$^3$H]CsA (fraction number > 10).

accordance with this assumption, we estimate the amount of DNA bound to nCyp/zinc rather high as indicated by the strong signal in the autoradiography of Fig. 2B. One may also speculate that the nCyp/zinc complex forms specific assemblies on target DNA elements together with eukaryotic transcription factors. Final proof for either assumption is lacking at present.

Some recent reports showed another family of immunosuppressive binding proteins, the FKBPs, to be located in the nuclear fraction, i.e. FKBP25 from T cells [19] and FKBP46 from insect cells [35]. Both proteins are characterized as DNA binding proteins, the specificity of the binding remaining speculative. Moreover, FKBP46 forms a protein–kinase complex involved in protein–protein interactions with basic chromatin proteins [35]. Also, Jin and Burakoff found FKBP25 associated with casein kinase II and nucleolin in nuclear extracts of murine T cells [20].

PPIase activity of nCyp is inhibited by zinc ions in a dose-dependent manner. Apparently, zinc binding induces conformational changes of nCyp. We assume the binding site of zinc to be not within the CsA binding pocket of nCyp according to the published data [34] and our computer modelling attempts (unpublished results).

The observed reactivation of zinc inhibited PPIase activity by 1,10-phenanthroline suggests that the mechanism of zinc inhibition is a reversible process rather than a simple denaturation. Under physiological conditions and in steady-state it seems possible that a (nucleus) fraction of nCyp complexes zinc, possibly together with DNA, whereas another (cytosolic) fraction remains enzymatically active, working as PPIase.

A recent report supports the assumption of nCyp binding to DNA indirectly [36]. It showed that CypA is involved in DNA fragmentation during apoptosis in rat thymocytes. The nuclease activity of CypA observed was found to be calcium dependent. Additionally, it was shown that recombinant CypA, CypB, and CypC digested DNA unspecifically in the presence of calcium in a radioactive gel nuclease assay. This nuclease activity can be stimulate by CsA [36]. By contrast, the nuclease activities of Cyp's are inhibited by zinc ions. We suggest the following model to explain the at a first glance contradictory observations: CypA can exist in two conformations — (i) a CsA binding; and (ii) a 'not binding' isoform. In the CsA-binding conformation, CypA shows PPIase and nuclease activity. The CsA-'not binding' conformation is induced by zinc ion binding and characterized by DNA-binding and a loss of PPIase and nuclease activity.

Therefore we assume that under normal growth conditions of mouse macrophages nCyp only binds to DNA in the presence of zinc ions, whilst during apoptosis zinc binding is lost and nuclease activity is induced. Further studies will be necessary to analyze a possible shift in metal ion binding and a

change in subcellular localization of CypA during apoptosis in macrophages.

Writing about zinc-mediated peptide loops interacting with nucleic acids, Luisi [37] postulated that zinc is compartmentalized in a way guaranteeing the presence of this metal ion at the right place and time. CypA with its ambivalent function as PPIase and zinc binding protein might be involved in this homeostasis.

*Acknowledgements:* We thank Dr. Kaever, Medizinische Hochschule, Hannover for the gift of H4-7 cells and Dr. Schumacher for providing the polyclonal antiserum. This work was supported by a grant from the DFG-SFB 263, TP B8; C2.

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