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COMMENTARY

CYCLOSPORIN BINDING PROTEINS

IDENTIFICATION, DISTRIBUTION, FUNCTION AND RELATION TO FK BINDING PROTEINS

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Cyclosporin A (CsA†) is a cyclic undecapeptide (Fig. 1), which has immunosuppressive properties [1]. It has been used successfully to prevent allograft rejection and to treat several autoimmune diseases [2]. Molecular studies on its mode of action have revealed that CsA prevents T-lymphocyte activation at the level of cytokine gene transcription [reviewed in Refs. 3–5].

Recent investigations with macrolide immunosuppressants showed that FK506 has the same effect on cytokine gene transcription as CsA, thereby inhibiting T-lymphocyte activation [6–10]. These findings provoked investigations at the molecular level designed to identify possible pathways common for both immunosuppressants. Such studies may provide important insights into the control of cytokine gene activation. Since both immunosuppressants cause renal dysfunction, common threads to immunosuppression and nephrotoxicity may also be elucidated.

This article reviews the cellular cyclosporin binding proteins, especially the cyclophilin (CPH) family and the related FK-binding proteins, and is restricted to mammalian proteins. The function of these binding proteins, also known as immunophilins [11], and the targeting of the drug–immunophilin complexes to the phosphatase calcineurin will be discussed.

Cellular uptake of CsA

Specific, saturable and reversible binding was shown on murine and human mononuclear blood leukocytes by means of a [^{3}H]CsA derivative. From kinetic and equilibrium binding studies, an affinity constant of 10^{-7} M (K_d) and about 10^6 bound molecules per cell were calculated [12]. Furthermore, CsA uptake was independent of active cellular metabolism [13]. Investigations over a broader concentration range in erythrocytes and several nucleated cells revealed two components of cell binding [3] (Fig. 2): a saturable, cytosolic binding at low CsA concentrations and a non-saturable, non-specific partitioning into the membrane at higher CsA concentrations. These findings, together with evidence for CsA accumulation within the cell, suggested the existence of an intracellular binding protein [14].

The cyclophilin family

The discovery of cyclophilin A (CPH-A), an 18 kDa protein which specifically binds CsA, was a seminal contribution [15] that opened many avenues of further research. The amino acid sequence of CPH-A was apparently not related to any known protein [16]. However, it was later established that CPH-A was homologous to a propyl-peptidyl *cis-trans*-isomerase, also known as rotamase [17, 18]. This provided a new perspective on the mode of CsA action as active cyclosporins were shown to bind to CPH-A [19] and inhibit its rotamase activity. Thus, it was speculated that the folding of a protein factor central to T-lymphocyte activation might be rate limiting.

The specificity of cyclosporin–CPH-A binding was investigated by LH-20 column [16], a competitive solid phase enzyme-linked immunosorbent assay [20, 21] and photoaffinity labeling [22]. Binding to CPH-A correlated with the immunosuppressive activity of cyclosporin analogues. Amino acids 1, 2, and 10 and 11 of the CsA molecule were found to be essential for cyclophilin binding. Subtle changes in these residues reduced both the affinity for CPH-A and the *in vitro* immunosuppressive activity [21, 23].

The synthesis of a photoaffinity-labeled cyclosporin analogue allowed the identification of several additional CsA binding proteins [22, 24, 25]. In the T-cell line Jurkat, labeled proteins of 21, 25, 40 and 60 kDa were identified [11]. The labeled proteins at 21 and 25 kDa are identical with CPH-A and CPH-B (Fig. 3). CPH-B is a second CsA binding protein, which has an endoplasmic reticulum retention signal [26–28].

Two new members of this family were also identified: CPH-C, which reportedly has a restricted tissue distribution [29], and CPH-D [30]. These latter proteins are less abundant than CPH-A and have a molecular mass of approximately 22 kDa;

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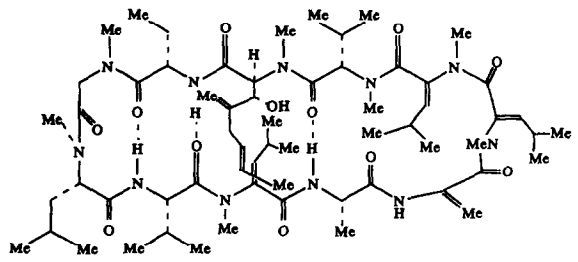
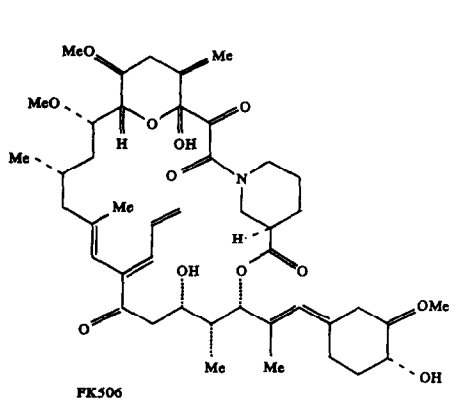
† Abbreviations: CsA, cyclosporin A; CPH, cyclophilin; NF-AT, nuclear factor of activated T-cells; and NF-ATc, cytoplasmic component of the nuclear factor present in activated T-cells.





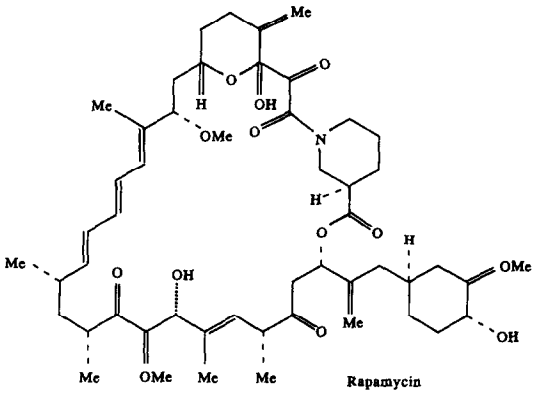


Fig. 1. Chemical structures of immunosuppressants binding to the protein family of immunophilins.

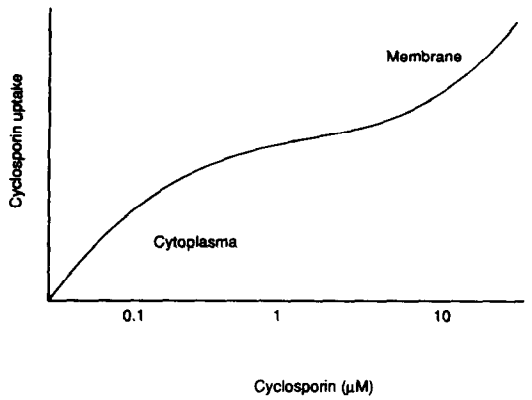


Fig. 2. Biphasic cellular uptake of [^{3}H]CsA. Saturable, cytosolic binding at low concentrations and non-saturable membrane binding at high concentration. Reproduced with permission from *Pharmacol Rev* **41**: 407–422, 1989. Copyright (1989) American Society for Pharmacology and Experimental Therapeutics [Ref. 3].

thus, they were not distinguishable from CPH-B by photoaffinity labeling.

Kieffer *et al.* [31] purified a 40 kDa protein (CPH-40) by affinity chromatography; the partial sequence analysis of this protein showed homology with CPH-A. CPH-40 antiserum did not cross-react with CPH-A in immunoblot analysis. Another 45 kDa CsA binding protein, which is phosphorylated, was reported but not characterized further [32].

All the members of the CPH family have rotamase (prolyl-peptidyl *cis-trans*-isomerase) activity, which is inhibited by CsA [30, 33]. CPH-A exhibits the highest specific activity and is most sensitive to CsA inhibition. Macrolide-derived immunosuppressants, however, do not affect this CPH rotamase activity.

All CPHs, with the possible exception of CPH-C, are found in both lymphoid and non-lymphoid tissues at high abundance. The subcellular localization of CPH proteins has been investigated by biochemical cell fractionation studies and immunoelectron microscopy: CPH-A and -B were found in both the cytosol and nucleus, although a specific association with organelle structures was not apparent [34–36]. CPH-B and -D possess a membrane localization signal and are found in the endoplasmic reticulum membrane fraction.

A secreted homologue of CPH-B was found in milk and was called SCYLP [37]. Further homologous CPHs were identified and cloned in many species including rat, mouse, *Drosophila*, *Neurospora crassa*, yeast and bacteria [38–46]. Sherry *et al.* [47] reported an 18 kDa protein secreted by murine macrophages after lipopolysaccharide stimulation that has both inflammatory and chemotactic activities, and a high degree of homology to CPH-A.

A comparison of the aligned sequences of human CPH-A and -B (Fig. 4) allows the delineation of

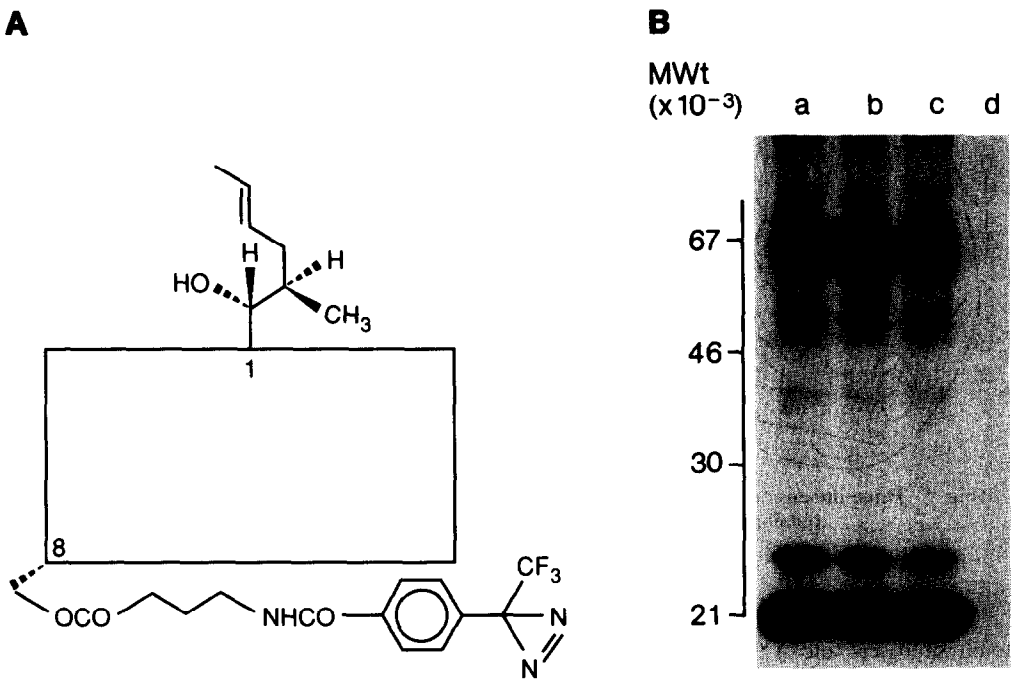


Fig. 3. *In vivo* detection of CsA receptor proteins. (A) Structure of photoaffinity label derivative. (B) Cell cultures of the human T-cell line Jurkat were incubated with titrated photoaffinity label-probe. After UV cross-linking, the labeled cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the proteins detected by fluorantoradiography (a). The specificity of binding is defined by competition with 10× molar excess CsA (d) or by lack of competition by inactive CsH (b) or FK506 (c).

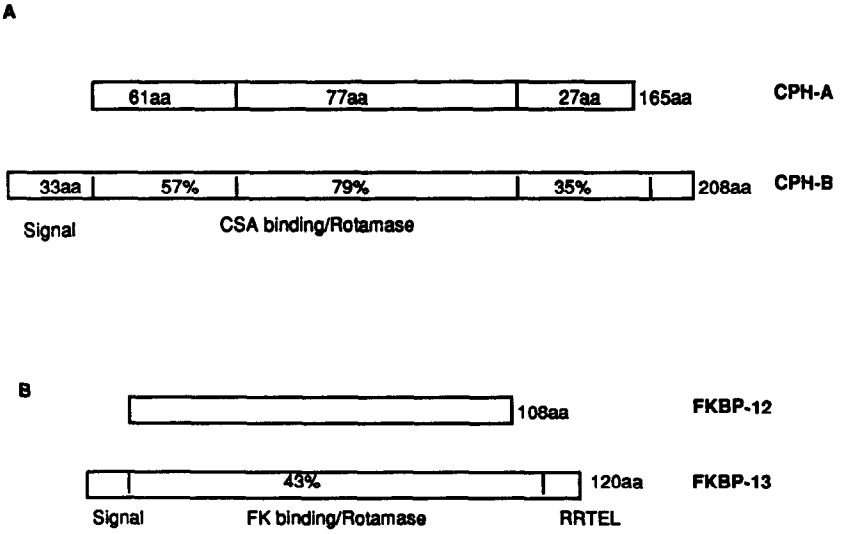


Fig. 4. General domain structures of the members of the cyclophilin and FKBP family. (A) Cyclophilin (CPH-A and -B). (B) FKBP-12 and -13.

several domains (I–V): The first domain (I) consisting of 33 amino acids (aa) is found only in CPH-B and contains a signal sequence. The second domain of 61 aa (II) and the third domain of 77 aa (III) have 57 and 79% homology, respectively, and are the most conserved regions. They contain the binding and catalytic sites. Domains IV and V have unique aa sequences of 27 and 10 aa, respectively [28].

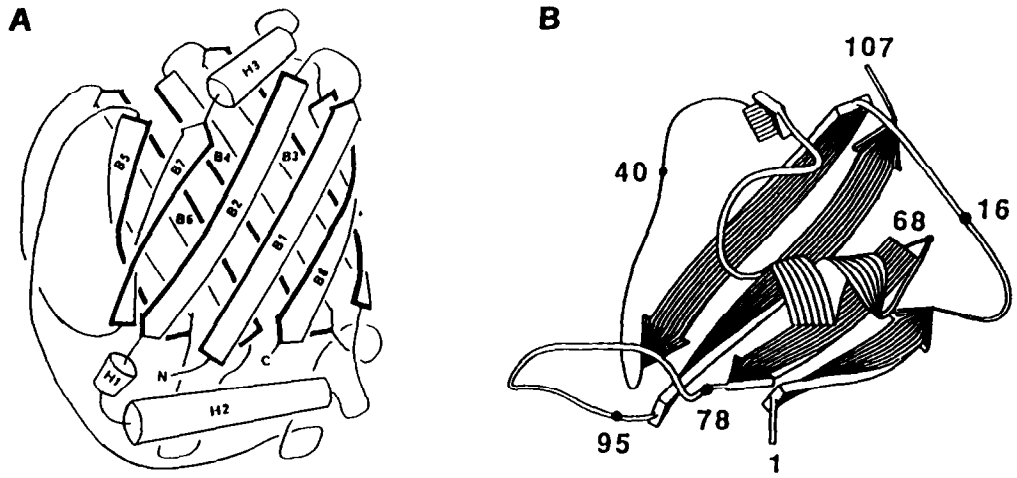


Fig. 5. Three-dimensional structure of human CPH-A (A) and of human FKBP (B). The data were obtained by X-ray and NMR analyses according to Refs. 51 and 54.

The physiological role of CPH-A and its relatives in the absence of CsA is largely unknown. One protein with homology to CPH-B for which a function has been described is the ninaA protein of the retina in *Drosophila* [46]. On the other hand the murine homolog of CPH-A was shown recently to have proinflammatory and chemotactic properties [47]. The relative abundance and high conservation suggest, however, an important role for the normal cell function [15, 34, 43, 48]. The search for an endogenous ligand of CPH has been hitherto unsuccessful.

Two potential mechanisms are postulated for CPH-A in CsA-mediated immunosuppression: the initial uptake and intracellular concentration of active cyclosporins at cytosolic and/or nuclear sites [14] followed by the inhibition of the enzymatic activity of CPH-A.

However, the role of the rotamase inhibition has been questioned, since the IL-2 gene transcription is fully inhibited at just 1% occupancy of CPH-A. Thus, the rotamase model linking the inhibition of the cellular rotamase to inhibition of gene transcription is rather unlikely (see below).

The complex of CsA and cyclophilin was investigated by X-ray and NMR techniques [49–53]. CPH-A exhibits a β barrel shape with a radius of 17 Å. The main structural elements are two perpendicular four-stranded β-sheets and two well defined α helices (Fig. 5A). Most of the hydrophobic side chains are packed in a hydrophobic core. Other hydrophobic residues occur in the contact region between the two helices, the β-sheets and in the CsA binding site. Replacement of the two cysteines (Cys 62 and 115) by alanine affected neither CsA binding nor rotamase activity. The sole tryptophan residue (Trp 121), however, is necessary for binding [55].

The macrolide binding protein, FKBP (see below), has no significant homology with CPH-A (Fig. 5B). Common three-dimensional surface structures which could preexist or be induced by CsA or FK506 on their respective immunophilins are presently an area of intense research.

Other CsA binding proteins

CsA was found to bind to several other proteins in the membrane, cytosol and nucleus (Table 1; for a review see Ref. 3). In the membrane, binding to the gp170 glycoprotein, the Na^+-D-glucose-cotransporter and the bile acid transporter has been shown. The gp170 protein functions as a non-specific ATP-dependent membrane transporter. Overexpression of gp170, found typically in tumor cells, results in active export of chemotherapeutic drugs, a phenomenon known as multi-drug resistance. CsA binds [24] and inhibits this transporter, a property which has been clinically explored [56]. CsA does not inhibit the Na^+-D-glucose-cotransporter CsA [57]. CsA might bind to a protein associated with the bile acid transporter [58]. Binding to these proteins is rather non-specific in that it does not discriminate between immunosuppressively active and inactive derivatives [3].

In addition to the CPH proteins, other cytosolic proteins were reported to bind CsA. Calmodulin [59] and ornithine decarboxylase were considered to be direct targets of cyclosporin; however, these findings were not supported by further experiments [60, 61]. Binding proteins of 40 and 60 kDa [22] have been observed and may contain the phosphatase calcineurin (see below).

That photoaffinity labeling of nuclear fractions and especially of purified nuclear factors yielded no conclusive results (Ryffel B, unpublished results) may indicate that the main site of action of CsA is located in the cytosol.

Recently, Cacalano *et al.* [62] demonstrated a specific membrane binding for CsA. The authors synthesized polyvalent macromolecular CsA derivatives, which although not able to enter the cell, are still capable of blocking T-cell activation. Furthermore, the macromolecular CsA induced agglutination of lymphoid, but not of HeLa cells,

Table 1. Proposed cellular targets of CsA

Cellular localization and target molecule	Affinity (K_d, M^{-1})	Specificity of binding
Membrane		
T cell receptors, MHC class II		?
Bile acid transporter		No
IL-IR		?
Lysolecithin acyltransferase		?
Prolactin receptor		Yes
170 Glycoprotein (gp 170)	$\sim 10^{-7}$	No
Cytosol		
Ornithine decarboxylase		?
Calmodulin	10^{-6}	No
Cyclophilin	10^{-8}	Yes
Nucleus		
Calmodulin	10^{-5}	No
Cyclophilin	10^{-8}	Yes
Transcription factors		?

and the agglutination was neutralized by anti-CsA monoclonal antibodies [63]. Based on their results the authors postulated the existence of functional membrane receptors, which have yet to be molecularly identified. However, similar hypotheses were formulated previously, but the experimental evidence has been difficult to provide [12].

FK-binding proteins join the family of immunophillins

The macrolide immunosuppressants are a structurally distinct family of immunosuppressants, comprised of FK506 and rapamycin. Both FK506 and rapamycin bind to a 12 kDa cytosolic protein, FKBP-12 [37, 64–67]. While FK506 inhibits cytokine gene transcription in a manner identical to that of CsA, rapamycin has a completely different mode of action [46]. FKBP-12 has no homology to any known protein, but has a rotamase activity comparable to that of CPHs. The rotamase activity of FKBP-12 is inhibited by FK506 and rapamycin, but is not affected by CsA. Since both FK506 and rapamycin have similar inhibitory effects on FKBP-12, inhibition of the rotamase cannot explain the different action of the two drugs.

Additional members of the family with binding specificity for macrolide immunosuppressants were sought (Table 2). FKBP-13, a membrane form like CPH-B, was reported by Jin *et al.* [74]. FKBP-13 has properties similar to those of the cytosolic form, FKBP-12, that is it binds to both FK506 and rapamycin.

The discovery of FKBP-25 proved to be interesting in that it appears to bind rapamycin selectively [75]. The 101 aa of the N-terminus of FKBP-25 is unrelated to the FKBP proteins and consists of an α helix. The C-terminus 114 aa is homologous to FKBP-12, except for a nuclear targeting sequence. The selective inhibition of the S6-kinase pathway by rapamycin [77] but not by FK506 or CsA, in lieu of the selectivity of rapamycin for FKBP-25 and its special structural features, leads to the suggestion that FKBP-25 may be important in the signalling of this pathway. Finally, FKBP-59, binding both rapamycin and FK506, was identified and shown to be related to and associated with heat shock proteins and the corticosteroid receptor [76].

It is important to note that there is no sequence homology between members of the cyclophillin and FKBP families. The general structures of cyclophilin and FKBP are compared in Fig. 4 [54, 70–72].

FKBP-13 has an N-terminal signal peptide of 21 amino acids and contains a potential endoplasmic reticulum retention signal (Arg-Thr-Glu-Leu) at the C-terminus [74]. The homology with FKBP-12 is 43% at the amino acid level. The conserved residues (92 amino acid C-terminal sequence) contain the drug-binding and rotamase activity site. The three-dimensional structure was investigated by NMR and X-ray crystallography. The main structural elements are a five-stranded antiparallel β-sheet which wraps around a short helix without any similarity to CPH-A.

FK506 binds in a shallow cavity between the α helix and the β-sheet, half of the ligand being buried in the receptor protein. The binding site is composed of conserved aromatic residues [for review see Ref. 4].

Inhibition of rotamase activity cannot explain immunosuppression

Although inhibition of the rotamase activity of the CPHs was fairly well correlated with the immunosuppressive activity of the cyclosporin derivatives [10], such correlation was not found with the macrolide immunosuppressants. Experimental evidence, contradicting an important role for the rotamase activity, is summarized:

1. The fact that CsA and FK506 have no cross-inhibitory effects on each other's rotamase renders their role in immunosuppression questionable. Inhibition of the rotamase activity occurs at much higher concentrations than that needed for inhibition of cytokine gene transcription.
2. In addition, CsA and FK506 block only one

Table 2. Properties of CsA and FK506 binding proteins (immunophilins)

Name	Molecular weight (kDa)	Homology (%)	Location*	Rotamase activity	References
Cyclophilins					
CPH-A	18		c	+	3, 10, 11, 13, 15–18, 20, 23, 33, 38–42, 55, 68
CPH-B	22	64	m	+	13, 26–28, 33, 44, 68, 69
CPH-C	23	?	?	+	29, 30
CPH-D	22	72	m	+	30
CPH-40	40	?	c	+	31
CPH-45	45	?			32
FKBPs					
FKBP-12	12		c	+	54, 70, 64–67, 71–73
FKBP-13	13	60	m	+	74
FKBP-25	25	40	n	+	75
FKBP-59	59		n?	+	76

* Cellular location: membrane (m), cytosol (c), and nuclear (n).

rotamase, e.g. CPH-A or FKBP, leaving the other rotamase fully functional. However, either drug is sufficient to block T-lymphocyte activation.

3. Rotamases have unknown substrate specificities and their *in vivo* role is poorly defined. Although rotamase activity may enhance protein folding [78], no misfolding is known to occur in the absence of rotamase.
4. That analogues of FK506, e.g. 506BD (a non-immunosuppressive derivative) and rapamycin (an active immunosuppressant with a different mode of action), both bind FKBP and inhibit its rotamase activity suggests that rotamase inhibition is not important in macrolide-induced immunosuppression [79, 80].
5. Based on physico-chemical considerations, the proline binding capability of the immunophilins is probably the basis of the measured rotamase activity, which in fact can be explained alone by the proline binding property [4, 81].

Based on these arguments and the fact that the rotamases have no absolute substrate specificity, it was suggested that the immunophillins have a "dominant" function; binding of the drug to the cognate immunophilin may thus result in a gain of function [11]. Proline binding by immunophilins may be an important property for the association with common target proteins.

Calcineurin as a common target of drug–immunophilin complex

These many arguments render the rotamase model improbable; therefore, other molecular targets explaining the identical effects of these immunosuppressants were sought. As shown in Fig. 6, further analysis of photoaffinity-labeled Jurkat cells by immunoblotting revealed that the 60 kDa binding protein is most likely calcineurin. Calcineurin is readily detected in T-lymphocytes and thus may have a function in T-cell activation.

Using immunophilin affinity columns, two groups independently demonstrated that the phosphatase calcineurin forms a complex with the drug and immunophilin [29, 84]. The complex was only formed in the presence of the drug and cognate immunophilin together with calmodulin and calcium. Rapamycin bound to FKBP did not form a complex with calcineurin. The immunosuppressants CsA and FK506 in the presence of their specific immunophilins and calcium inhibit calcineurin phosphatase activity.

Calcineurin consists of a catalytic (A) and regulatory domain (B). Calcineurin B has a molecular weight of 19 kDa and has high homology to calmodulin. Calcineurin A, the 61 kDa subunit, contains the catalytic domain of the serine-threonine phosphatase. Calcineurin occurs ubiquitously throughout the body and is a highly conserved protein. Two isozymes of calcineurin A (types I and II) have been cloned and are formed by alternative splicing events [85]. The C-terminus contains an inhibitory domain and an adjacent calmodulin binding domain, which are rapidly removed by limited proteolysis. The central part of the protein, being resistant to proteolysis, harbors the catalytic domains and is identical for the two isozymes of calcineurin. This region shows extended similarities with the catalytic subunits of protein phosphatases 1 and 2B, which define a distinct family of protein phosphatases. The 40 aa N-terminal fragment, which is specific for calcineurin, contains 11 successive prolines, possibly important for the binding to CPH-A/B or FKBP.

Preliminary results show that CsA binds to the catalytic domain of calcineurin A (Ryffel B, unpublished observation). The specificity of CsA binding to calcineurin A is presently under investigation.

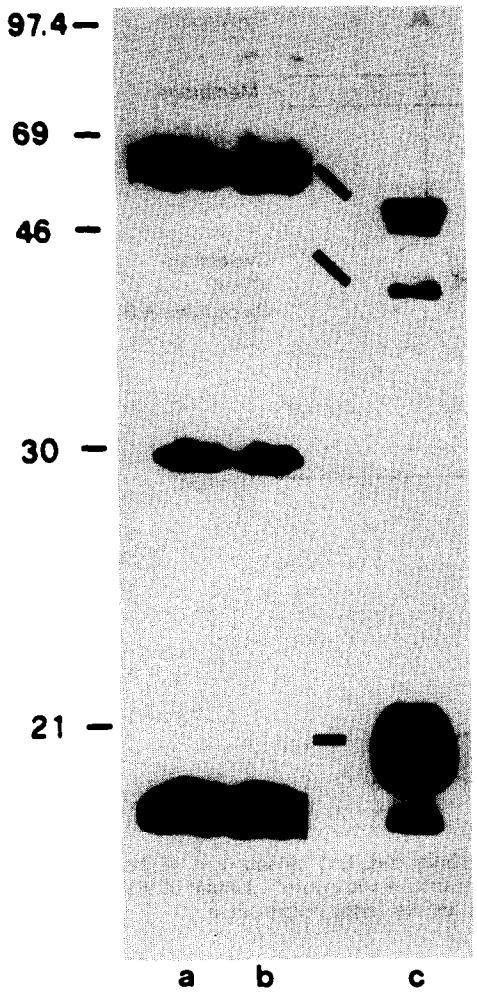


Fig. 6. Immunoblot analysis of calcineurin isolated from Jurkat cells. The cells were photoaffinity labeled with CsA. Both calcineurin and CsA antibodies [82, 83] identified proteins at 30 and 60 kDa. Jurkat cells were untreated (a), or were incubated with PL-CS followed by UV cross-linking (b, c). Shown are immunoblots with antiserum to calcineurin (a,b) or monoclonal antibody to CsA (c).

Role of calcineurin in T-cell activation: Role for IL-2 gene transcription

Calcineurin is abundant in lymphoid cells [86]. An immunoblot of Jurkat cell lysate with calcineurin antiserum revealed proteins of 16 and 61 kDa (calcineurin A and B), and probably a proteolytic fragment of 28 kDa (Fig. 6). Fruman *et al.* [87] demonstrated calcineurin phosphatase activity in lysates from Jurkat T-cells. Both CsA and FK506 specifically inhibited cellular calcineurin activity at drug concentrations that inhibit IL-2 synthesis in activated T-cells, while rapamycin had no effect. These findings taken together suggest that calcineurin plays a role in T-cell activation. Swanson *et al.* [88] compared the capabilities of CPH-A and -B in modulating calcineurin phosphatase activity and showed that CPH-B was more potent that CPH-A.

Another approach selected to investigate the role of calcineurin in T-cell activation was the cotransfection of an IL-2 promotor linked reporter gene construct together with murine calcineurin A into Jurkat cells. As expected, the overexpression of calcineurin caused relative resistance to the immunosuppressants, thus necessitating higher concentrations in order to achieve the same immunosuppressive effect [89, 90]. These results implicate calcineurin as a component of the T-cell receptor signal transduction pathway. Furthermore, the results provide biological evidence to support the hypothesis that the interaction of the drug–immunophilin complex with calcineurin is essential for CsA- or FK506-mediated immunosuppression.

Based on the presently available data the following mechanisms on the mode of action of CsA and FK506 may be formulated (Fig. 7) [4, 89–92].

CsA and FK506 bind specifically to their cognate immunophilins permitting local intracellular drug accumulation and targeting of the relevant effector protein, putatively calcineurin A. The immunophilin–drug complex inhibits the calcineurin phosphatase activity in a dose-dependent fashion. The cytoplasmic component of the nuclear factor present in activated T-cells (NF-ATc) was proposed as the specific substrate of the drug–immunophilin–calcineurin complex. It is hypothesized that dephosphorylation of NF-ATc is necessary for its nuclear translocation and the formation of a functional NF-AT complex [91, 92]. Binding of the NF-AT complex to the promotor region initiates IL-2 gene transcription. Thus, by blocking the phosphatase activity of calcineurin, CsA and FK506 may inhibit the nuclear translocation of NF-ATc and block IL-2 gene transcription. Dephosphorylation was shown to control the nuclear translocation of a transcription factor in yeast [93].

Rapamycin, although binding to the same immunophilin as FK506, FKBP-12, neither binds nor inhibits calcineurin phosphatase and has no effect on IL-2 gene transcription. By contrast, rapamycin inhibits the activation process at a later stage, e.g. the IL-2 receptor-induced entry into S-phase and subsequent T-cell proliferation [79, 80, 94]. Kuo *et al.* [77] presented evidence that IL-2 selectively induces the phosphorylation and activation of p70 S6 kinase. Rapamycin, but not FK506 or CsA, completely and rapidly inhibits IL-2-induced phosphorylation and activation of p70 S6 kinase at immunosuppressive concentrations. The selective blockade of the p70 S6 kinase activation by rapamycin implicates this signalling pathway in the regulation of T-cell entry into S phase. In addition, Chung *et al.* [95] showed that rapamycin blocked phosphorylation and activation of p70 S6 kinase in a variety of animal cells of non-lymphoid origin.

These studies demonstrate that a growth factor-induced signalling event, not merely restricted to T cells, may impinge upon rapamycin through the induction of a blockade of entry into the S-phase.

Role of immunophilins and calcineurin in renal dysfunction

Despite its high selectivity for lymphocytes, CsA causes renal dysfunction. At immunosuppressive doses a reduction of the glomerular filtration rate

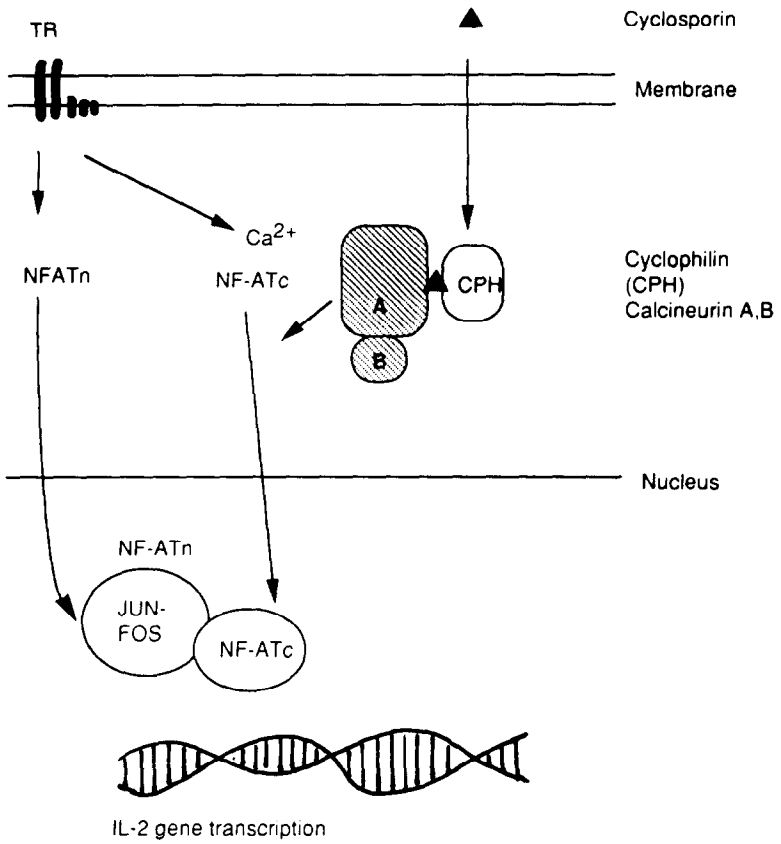


Fig. 7. Schematic representation of the drug–immunophilin complex, binding and inhibiting the calcineurin phosphatase and thereby inhibiting the translocation of the cytosolic subunit of the nuclear factor of T-cell activation (NF-ATc). See text for further explanation.

(GFR) and slight arterial hypertension have been observed [96]. The present knowledge on the pathogenesis of this renal dysfunction is summarized in Fig. 8. CsA may have direct effects on renal mesangial cells resulting in the reduction of the GFR. In addition, endothelial cells and smooth muscle cells, especially of the afferent arterioles, may respond directly to CsA and release further vasoactive mediators. At higher concentrations, the tubular epithelial cells themselves may develop degenerative changes.

The abundance of CPH-A and -B as well as of calcineurin, at least as determined by Western blots, does not differ between drug-sensitive (lymphocytes and kidney) and drug-resistant organs. Friedman and Weissman [29] have claimed that CYP-C occurs only in the immune system and the kidney; CYP-C could therefore be the protein explaining the relative tissue specificity of CsA, e.g. immune system and the kidney. However, these findings have yet to be confirmed by other investigators.

Attempts have been made by several groups to correlate the abilities of several CsA derivatives to bind cyclophilin with their immunosuppressive and toxic activities *in vivo* [10]; however, presently available *in vitro* data do not confirm such a relationshi The formation of drug–immunophilin complex with calcineurin is very likely in renal target cells, although these complexes have not been demonstrated yet *in vivo*.

A further challenging question is the substrate specificity of the renal calcineurin–immunophilin complex and whether it differs from that of lymphocytes. Possible candidate substrates include the suspected effector peptides of toxicity or the factors upstream leading to mature effectors. Presently, peptides considered as important for the development of nephrotoxicity are endothelin, renin and transforming growth factor-β.

Despite initial enthusiastic reports, FK506 was shown to cause a form of nephrotoxicity similar to that of CsA in controlled clinical trials. The related macrolide rapamycin with its different mode of immunosuppression induction is devoid of nephrotoxic side effects (Ryffel B, unpublished observation). One possible explanation is that rapamycin neither binds to calcineurin nor inhibits its phosphatase activation. These findings suggest that the immunophilin–drug–calcineurin complex may indeed be involved in the development of the nephrotoxicity of both CsA and FK506.

Summary and perspectives

In the last few years rapid progress has been made

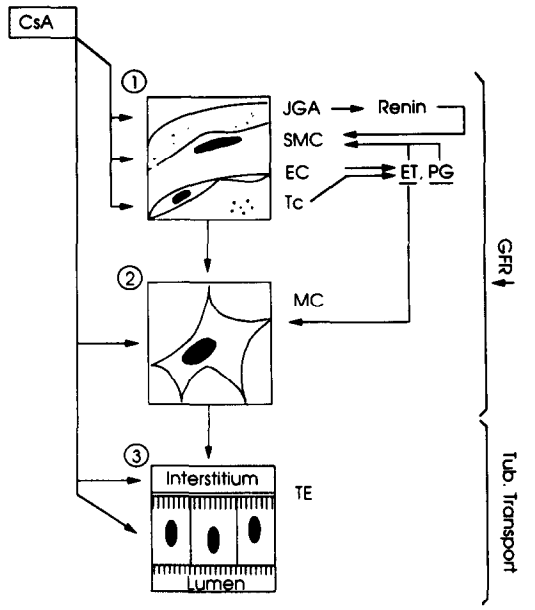


Fig. 8. Possible role of calcineurin in cyclosporin nephrotoxicity. The cellular targets of cyclosporin comprise: (1) components of the glomerulus (juxtaglomerular apparatus, JGA); endothelial cells (EC), smooth muscle cells (SMC), and thrombocytes (Tc); (2) mesangium cells (MC); and (3) tubular epithelium (TE)-interstitial cells. Targeting of the immunophilin–drug complex to calcineurin results in increased synthesis and release of renin, endothelin (ET) and prostaglandin (PG), thereby reducing the glomerular filtration rate (GFR). See text for further explanation.

in the understanding of the control of T-lymphocyte activation, fostered by the introduction of several novel tools of investigation: the peptide CsA and the macrolide immunosuppressants FK506 and rapamycin. The initial step enabling this progress was the discovery by Borel and coworkers [1, 97] of the immunosuppressive properties of the fungal-derived undecapeptide CsA. The initial observations by Borel, e.g. high selectivity for the immune system, reversible mode of action and absence of myelotoxicity, were not only confirmed by many scientists, but allowed the development of a clinically invaluable immunosuppressant.

The discovery that CsA inhibits the transcription of the IL-2 gene along with other cytokine genes after T-cell activation stimulated investigation into the regulatory regions of the IL-2 enhancer and nuclear transcription factors. Several sites within the IL-2 enhancer region which are affected by CsA have been identified [4, 7, 9]. The sites most sensitive to CsA are the binding sites for NF-AT and OAP (octamer-associated proteins).

NF-AT (nuclear factor of activated T-cells) is only expressed in activated T-cells and is composed of at least two components; an inducible nuclear component (NF-ATn) and a constitutively expressed cytoplasmic component (NF-ATc). NF-ATn was shown recently to be identical to the AP-1 complex comprising the heterodimer of JUN and FOS protein [98]. The formation of the AP-1 complex was shown repeatedly to be CsA resistant. NF-ATc is a preformed cytosolic protein (of about 80 kDa), which is translocated upon T-cell activation. Flanagan *et al.* [91] made the important observation that CsA and FK506 inhibit the nuclear translocation of NF-ATc, thus preventing the formation of a functional NF-AT complex necessary for binding on the IL-2 gene enhancer. An important question, which has not yet been answered, is how CsA inhibits the nuclear translocation of NF-ATc. Answers may be found in the identification of molecular targets of CsA within the cell. The discovery of the cytosolic CsA receptor by Handschumacher *et al.* [15] initiated much fruitful research resuting in the identification of several binding proteins for CsA and macrolides, collectively termed immunophilins [11]. An intriguing feature of the immunophilins is their common rotamase activity.

Although the specific binding property of immunophilins allowing intracellular drug accumulation is not questioned, the relationship of the inhibition of their rotamase activity to the immunosuppressive effect is debatable. New perspectives were opened by the discovery that the drug–immunophilin complex binds calcineurin, a ubiquitous phosphatase that is inhibited by CsA and FK506.

This discovery clearly stimulated a search for tissue specific substrates. Thus far no hard facts are available, but it is hypothesized that activation of calcineurin may be important for the translocation of NF-ATc and thus IL-2 gene transcription.

While many research groups are focusing on the search for calcineurin substrates, other proteins should be considered as targets to which the drug–immunophilin complex may bind, and thus regulate its function. The association of FKBP with the glucocorticosteroid receptor and the calcium release channel (ryanodine receptor) in skeletal muscle [99] are such examples.

An important area of further research is the tissue specificity of members of the immunophilin family and calcineurin isoforms. Thus far only CPH-C is thought to be tissue specific, i.e. expressed in lymphocyte and kidney. The role of CPH and calcineurin in the development of nephrotoxicity is presently unknown. A rapid development in the molecular understanding of the factors responsible for nephrotoxicity is expected in the near future.

The selective inhibition of the S6-kinase by rapamycin together with the selective receptor FKBP25 is also an area that may provide new insights in the biochemical mechanisms of the cytokine signalling pathways.

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