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Molecular Recognition of Immunophilins and Immunophilin-Ligand Complexes

Stuart L. Schreiber,* Jun Liu, Mark W. Albers, Michael K. Rosen,
Robert F. Standaert, Thomas J. Wandless, Patricia K. Somers

Department of Chemistry, Harvard University
12 Oxford Street, Cambridge, Massachusetts 02138

Abstract Immunophilin-ligand complexes have been used to identify a previously unknown step in Ca^{2+}-dependent signal transduction pathways. This Report, which we dedicate to **Professor Harry H. Wasserman**, describes structural and mechanistic aspects of immunophilin research.

In addition to their important medical applications, the immunosuppressants FK506, cyclosporin A, and rapamycin have proven to be valuable reagents for studying intracellular signal transduction mechanisms.[1] Signal transduction refers to the process by which extracellular molecules are able to influence intracellular processes. In

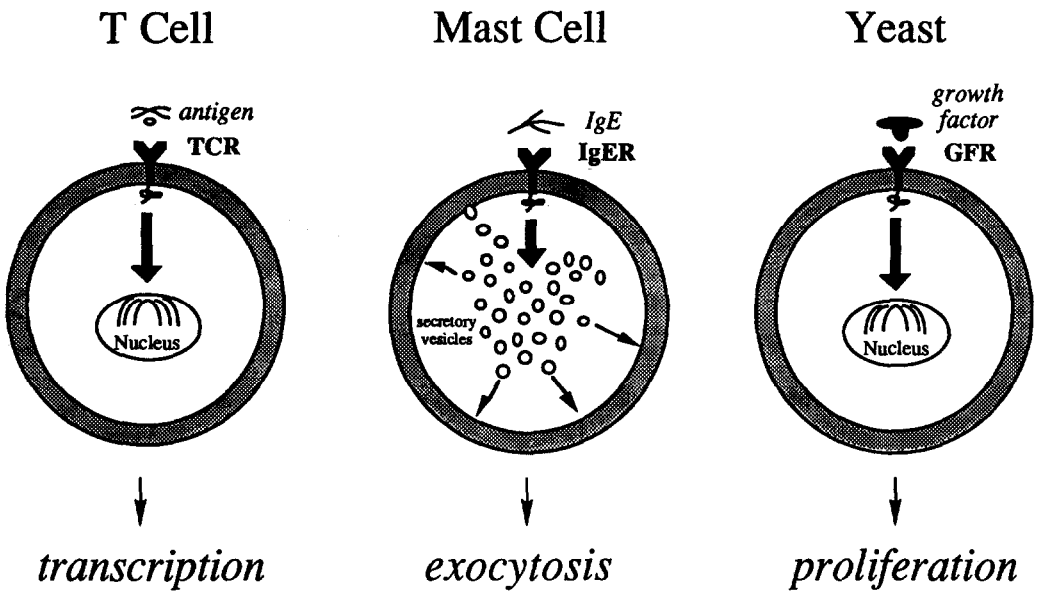


Figure 1 Three cellular systems that are sensitive to immunophilin-ligand complexes.

recent years, much progress has been achieved in our understanding of the mechanisms of signaling at the membrane and within the nucleus of the cell. In contrast, very little is known about the mechanisms by which signals are transduced through the cytoplasm of the cell, which has been referred to as the "black box" of the signal transduction pathway.

Three systems that are sensitive to these immunosuppressants and thus have been studied in some detail are depicted in Figure 1. Transcription of specific genes following stimulation of the T-cell receptor has been studied in the T-cell[2,3,4] whereas the exocytosis of secretory vesicles following stimulation of the IgE receptor has been investigated in the mast cell.[5] In addition, several events, including death and proliferation, have been examined in yeast.[6,7] These investigations have utilized complexes of immunophilins (immunosuppressant binding proteins) and their immunosuppressive ligands (Figure 2), which interfere with signal transduction pathways in the cytoplasm of the cell, following early membrane-associated events. Therefore, they serve as a window for the events that constitute cytoplasmic signaling. In this Report we describe the structural basis of immunophilin-ligand complexation and the identification of target molecules of these complexes.

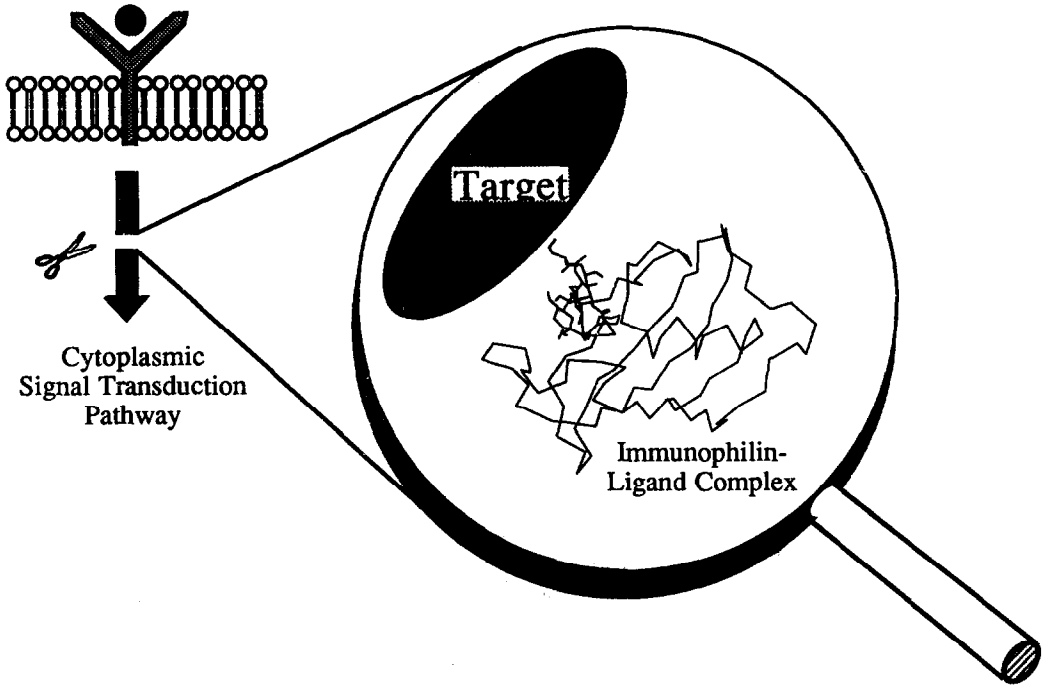


Figure 2 Immunophilin-ligand complexes serve as probes of cytoplasmic signaling mechanisms.

The immunophilin ligands that have been utilized in our studies are shown in Figure 3. These include the cyclic peptide, cyclosporin A (CsA), the macrolides FK506 and rapamycin, and the nonnatural immunophilin ligand, 506BD.[8] There exist two families of immunophilins: the cyclophilins[9], which bind CsA, and the FKBPs[10,11], which bind FK506 and rapamycin (Figure 4). Many of the immunophilins have catalytic properties. These enzymes catalyze the interconversion of the *cis*- and *trans*-rotamers of a peptidyl-prolyl amide bond in peptide and protein substrates. These rotamase enzymes are potently inhibited by their cognate immunosuppressive ligand(s). Although a number of cyclophilin and FKBP family members have recently been characterized[5,10,12], the focus here will be on the predominant cyclophilin, cyclophilin A, and the predominant FKBP, FKBP12.

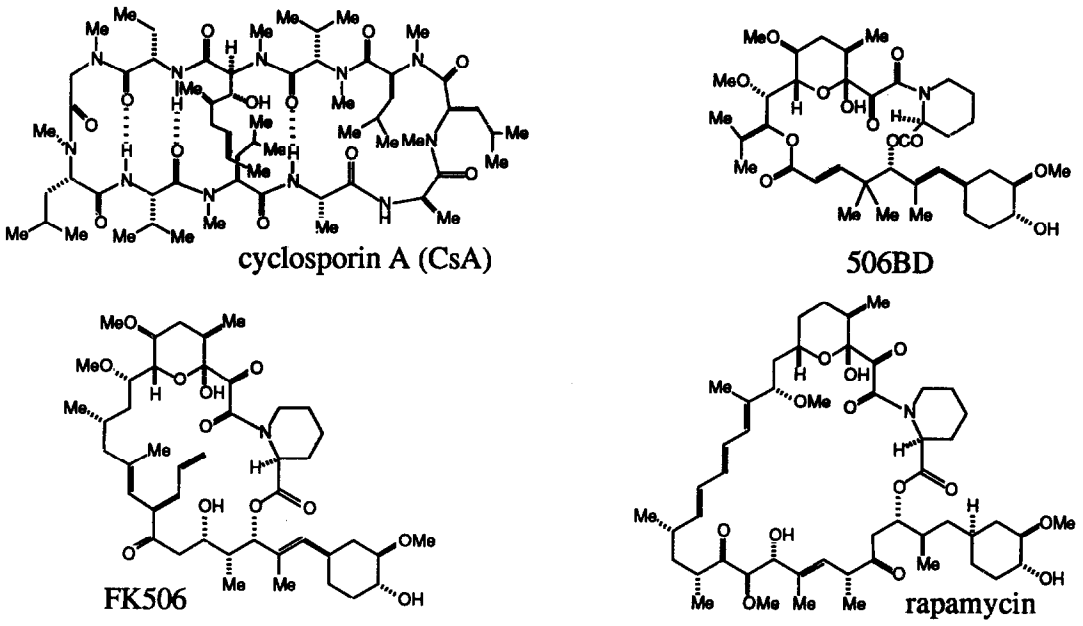
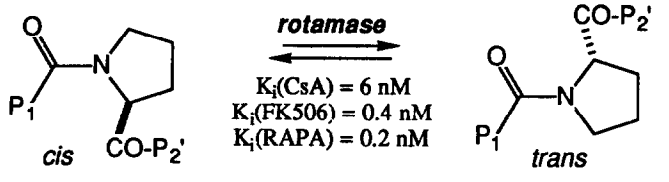


Figure 3 Structures of high-affinity immunophilin-ligands.

Important similarities and differences exist in the actions of these immunosuppressants (Figure 5). Despite their dissimilar structures, CsA and FK506 interfere with a common set of signaling pathways.[2,4,5] These are Ca^{2+}-dependent pathways that emanate from, for example, the T cell receptor in T lymophcytes and the IgE receptor in mast cells. Evidence has been gathered that suggests CsA and FK506 interfere with the same step in these signaling pathways and by a similar mechanism. Rapamycin, despite its structural similarity with FK506, interferes with a distinct set of Ca^{2+}-independent signaling pathways. These include pathways emanating from lymphokine receptors, such as the interleukin-2 receptor in T cells, and growth factor receptors in hepatocytes.[13]



cyclophilins

CyP A *Science* **226**, 544 (1984).
CyP B *PNAS* **88**, 1903 (1991).
CyP C *Cell* **66**, 798 (1991).
ninaA *Cell* **65**, 219 (1991).

FKBPs

FKBP12 *Nature* **341**, 755 (1989) *Ibid.*, p. 758.
FKBP13 *PNAS* **88**, 6677 (1991).
FKBP25 A. Galat *et al.*, submitted.
FKBP60 *J. Am. Chem. Soc.* **113**, 758 (1991).

Fig 4 Two families of immunophilins. The K_i value for CsA corresponds to a measurement with cyclophilin A whereas the K_i values for FK506 and rapamycin correspond measurements with FKBP12.

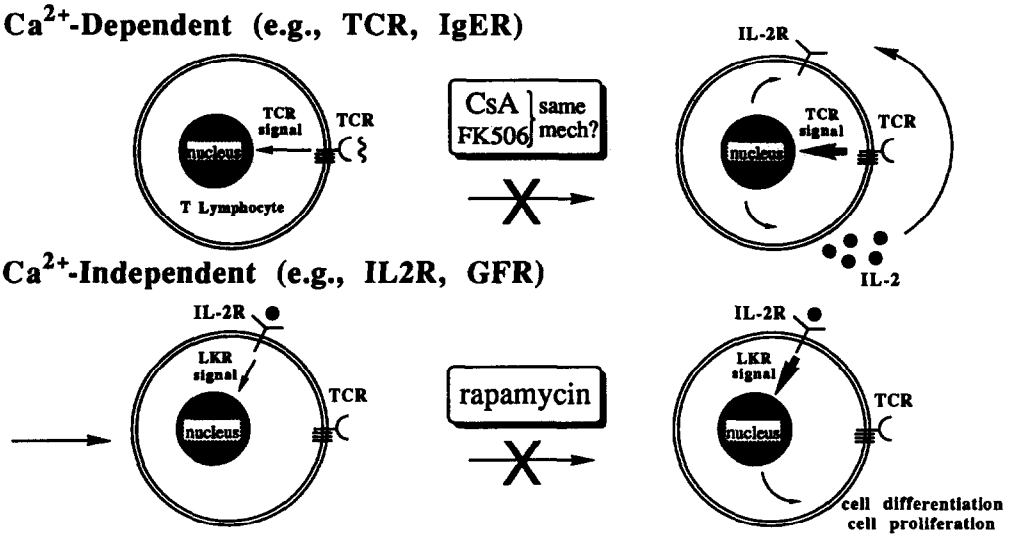


Figure 5 FK506 and CsA inhibit Ca^{2+}-dependent signaling pathways whereas rapamycin interferes with Ca^{2+}-independent signaling pathways.

The rotamase activity of immunophilins led to an initial model of drug action whereby drug binding to the immunophilin produces a loss of function (Figure 6). Rotamases were envisioned as key components of signaling pathways, and their inhibition by drug binding would arrest the signal. In the past several years much evidence has accumulated that argues against the "rotamase" model of drug action.[3,4,5,8] Instead, it appears that the binding of drug to immunophilin results in a gain of function.[1] In the "active-complex" model of drug action, complexes of cyclophilin-CsA and FKBP-FK506 interfere with Ca^{2+}-dependent signaling pathways and the complex of FKBP-rapamycin interferes with Ca^{2+}-independent signaling pathways. A provocative aspect of this model is that the same FKBP could be responsible for forming two inhibitory complexes with different biological activities. (It will

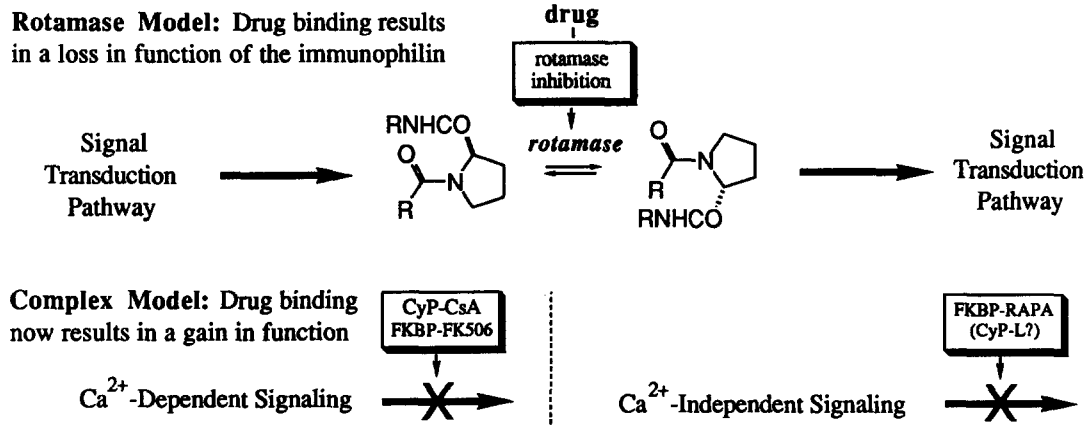


Figure 6 Immunophilin-ligand complexation results in a gain of function.

be interesting to learn if a cyclophilin ligand exists that forms an inhibitory complex akin to the FKBP-rapamycin complex.) The active-complex model does not specify a role for unligated immunophilin--this is an active area of investigation. A rationale for the ability of FKBP to give rise to two distinct inhibitory complexes was suggested by the molecular structures of FK506 and rapamycin (Figure 7).[1] The common structural elements found within these macrolides were suggested to constitute FKBP-binding domains. Following binding to the presenting molecule, FKBP, the different effector elements (depicted in the gray circles) of FK506 and rapamycin were envisioned as being responsible for the selectivity exhibited by the immunosuppressants. Evidence in support of this view was provided by experiments centered on a molecule we have named 506BD, which stands for "the FKBP binding domain of FK506".[8] These studies were instrumental in distinguishing between the two biological models shown in Figure 6.

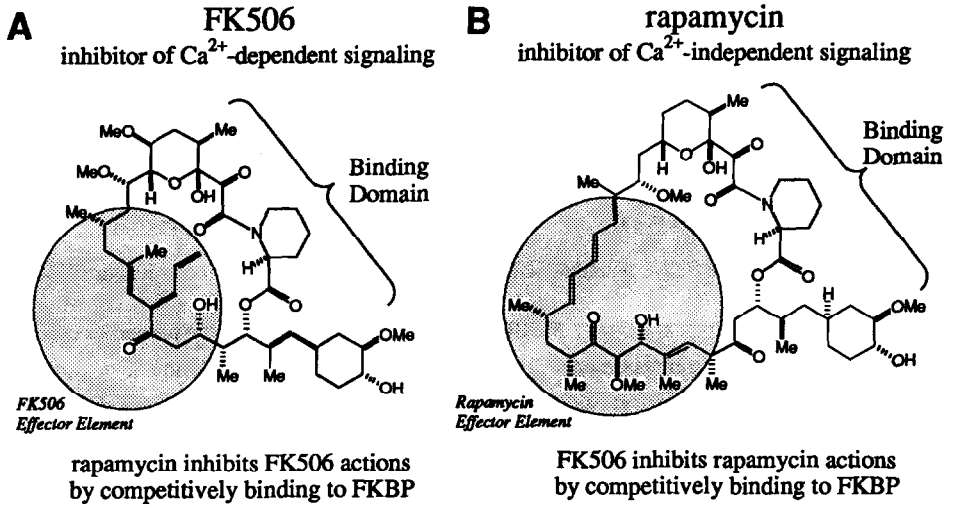


Figure 7 FK506 and rapamycin are comprised of common FKBP-binding domains and distinct effector elements (shown in the shaded circles).

506BD was designed to contain the structural elements in FK506 and rapamycin that are responsible for binding to FKBP, yet to lack the effector elements of either agent. This was achieved by bridging two atoms in FK506 that are at the intersection of the binding and effector domains (Figure 8). The indicated *trans*-enoate was added in place of the allyl-substituted effector loop found in FK506. According to the active-complex model, 506BD should be able to block the ability of FK506 (but not CsA) to inhibit Ca^{2+}-dependent signaling pathways and rapamycin to inhibit Ca^{2+}-independent signaling pathways, by competing with FK506 and rapamycin for binding to FKBP. Since 506BD lacks the effector elements of either macrolide, the active-complex model predicts that 506BD would not interfere with either type of signal transmission pathway. A very different outcome would result if the rotamase model was operative--the ability of 506BD to bind to FKBP should result in the inhibition of rotamase activity and, thus, of signal transmission. 506BD was synthesized (longest linear sequence = 27 steps) and found to be a potent inhibitor of the rotamase activity of FKBP (inhibitory constant K_i = 5 nM).[8] The analysis of the cell signaling inhibitory properties of 506BD and its effects on CsA, FK506, and rapamycin led to a clear

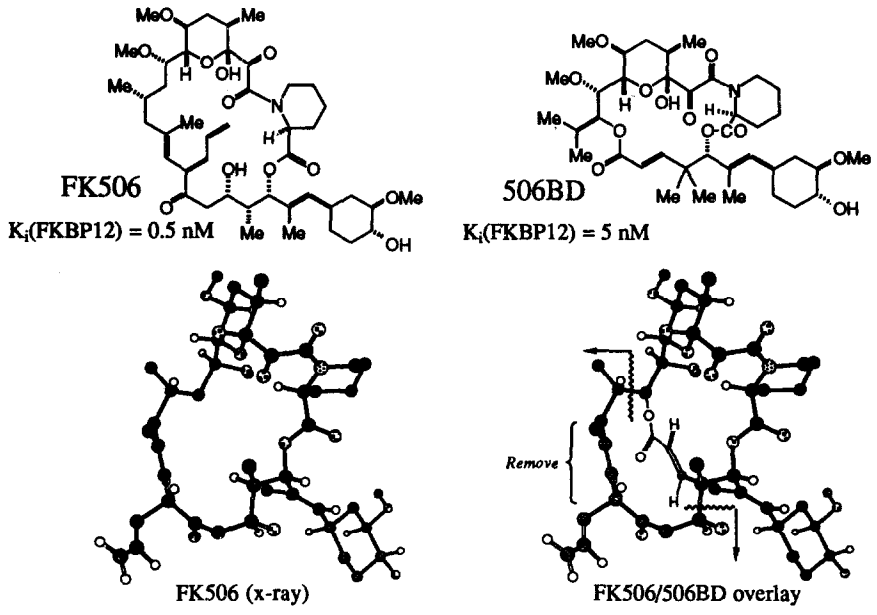


Figure 8 A *trans*-enoate in the FKBP ligand 506BD was used to replace the effector element of FK506.

distinction between the two biological models. 506BD was found to exhibit each of the properties described above that are supportive of the active-complex model. Following studies of 506BD in T lymphocytes[8], these findings were extended in investigations of exocytosis in mast cells.[5] Further evidence in support of the active-complex model has been gathered in experiments in lower eukaryotes as well. G. Livi and co-workers[6] showed that the deletion of FKBP12 from yeast, which are normally sensitive to nanomolar concentrations of rapamycin, results in a rapamycin-resistant strain (Figure 9). Thus, rapamycin is intrinsically inactive in yeast and is best viewed as a prodrug. It is only after the FKBP-rapamycin complex forms, which occurs in the mutant strain following expression of yeast or human FKBP12 and treatment with rapamycin, that the actions of rapamycin are seen. Related findings have been obtained with cyclophilin-CsA by M. Tropschug and co-workers[14] and FKBP-FK506 by M. N. Hall and co-workers.[7]

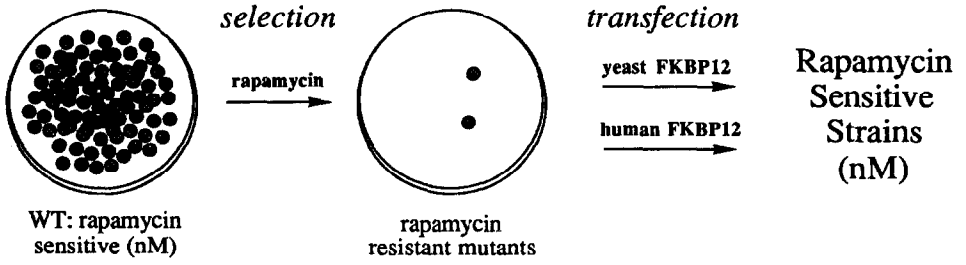


Figure 9 The "active-complex" model is operative in *Saccharomyces cerevisiae*, where it has been shown that FKBP12 mediates the actions of rapamycin.

The diagram in Figure 10 summarizes our current thinking in this area. The actions of CsA and FK506 are mediated by distinct receptors (immunophilins), a cyclophilin and an FKBP, respectively. The roles of individual cyclophilin and FKBP family members have not yet been defined in most instances. The differing effects of rapamycin and 506BD on the actions of CsA and FK506 support a role for the individual immunophilin-drug complexes. Because rapamycin and 506BD can compete with FK506 for binding to FKBP, they block the actions of FK506 in T cells and mast cells. As rapamycin and 506BD do not bind to cyclophilin, they do not block CsA's actions. Aside from their different sensitivities to rapamycin and 506BD, CsA and FK506 have nearly identical biological properties. This would suggest that they eventually act upon a common target, which might be associated with the Ca^{2+}-dependent signaling pathways in T cells and mast cells. One possibility is that the two immunophilin-drug complexes interact directly with the common target. Other properties expected of this hypothetical molecule are that it should not interact with immunophilin or drug alone, or with the complex of FKBP-rapamycin. This latter complex should interact with a distinct target(s) associated with Ca^{2+}-independent signaling pathways.

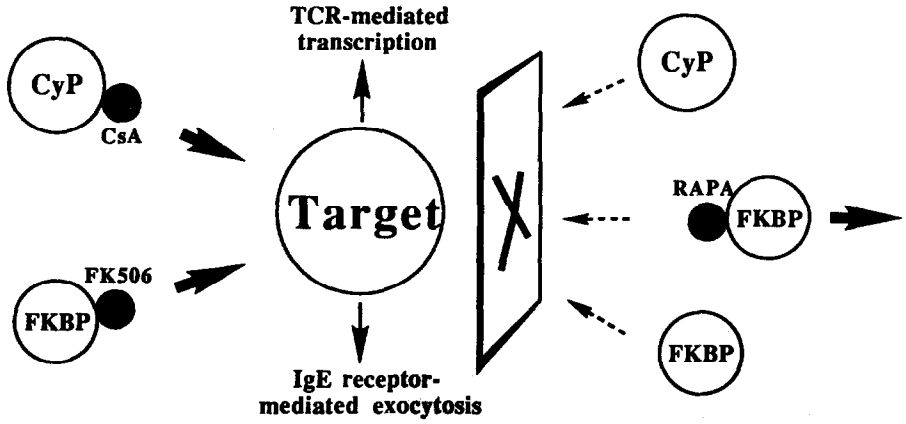


Figure 10 Mechanistic studies suggest that the cyclophilin-CsA and FKBP-FK506 complexes act upon a common target molecule. One possibility is that this target binds directly to the two immunophilin-drug complexes (but not drug or immunophilin alone, or the FKBP-rapamycin complex, which interacts with a distinct target that is associated with Ca^{2+}-independent signaling pathways). The indicated target may be involved in a step in signal transduction that is common to Ca^{2+}-dependent signaling pathways that include those emanating from the T cell receptor in T cells and the IgE receptor in mast cells.

To test this possibility, several reagents for use in affinity chromatography were prepared (Figure 11). The fusion proteins glutathione-S-transferase-FKBP12 and glutathione-S-transferase-cyclophilin A were expressed in *E. coli* in our laboratory[15], while the cyclophilin C fusion protein was provided to us by Jeff Friedman and Irv Weissman at Stanford.[16] These researchers had found that a 77 kDa protein from a stromal cell line associates with cyclophilin C and that a 55 kDa protein associates with the cyclophilin C-CsA complex. We found that the cyclophilin-CsA and FKBP-FK506 complexes retain the complex of the Ca^{2+}, calmodulin-dependent serine/threonine protein phosphatase calcineurin (also named PP2B; this heterodimeric enzyme is comprised of a 61kDa chain (calcineurin-A) and a 19 kDa chain (calcineurin-B)) and calmodulin when treated with calf thymus and

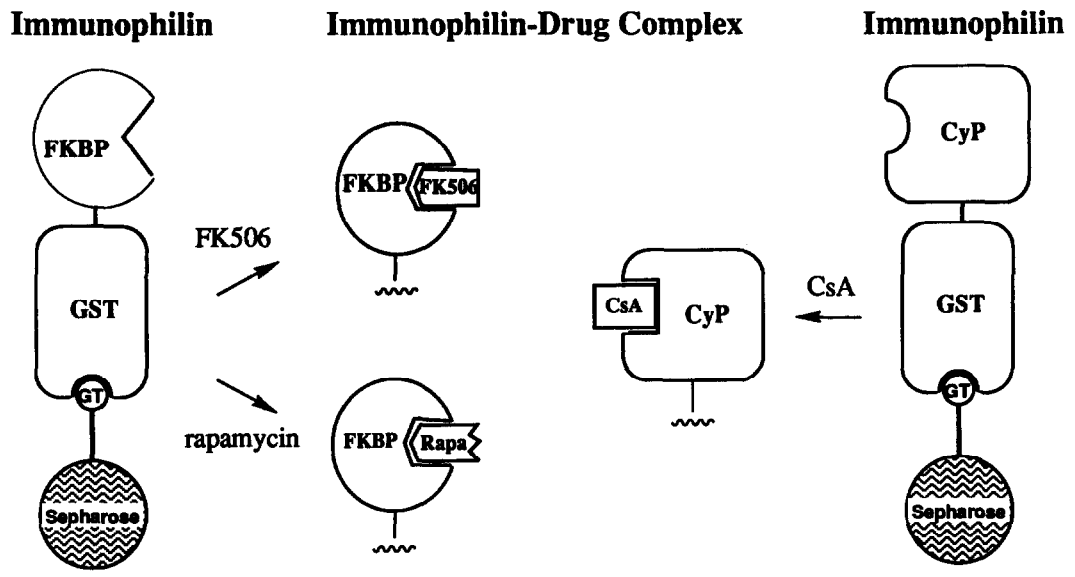


Figure 11 Five affinity reagents that were used in experiments aimed at identifying the common target of cyclophilin-CsA and FKBP-FK506 complexes.

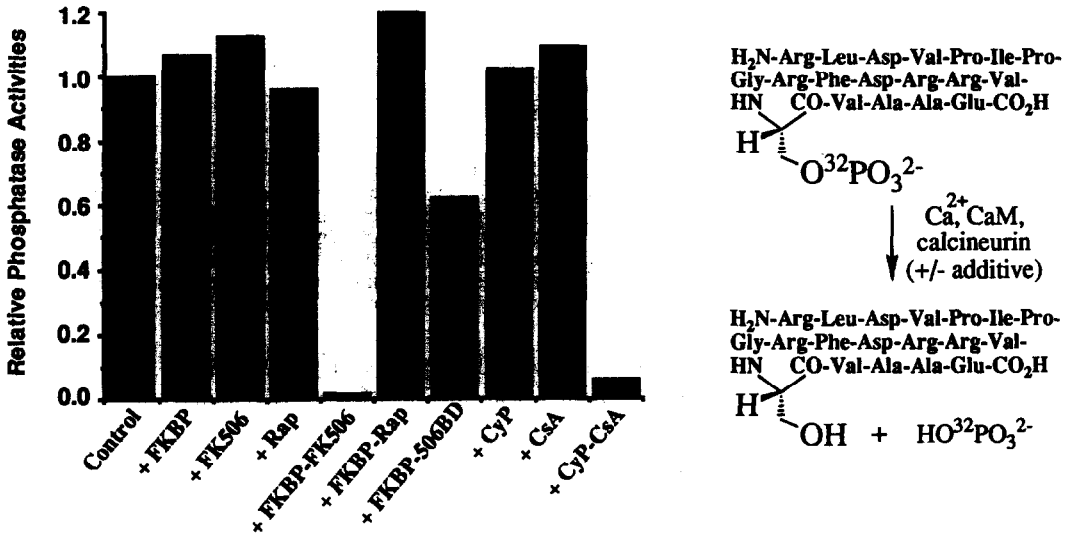


Figure 12 Specific immunophilin-drug complexes inhibit the phosphatase activity of the Ca^{2+}, calmodulin-dependent protein phosphatase calcineurin (PP2B) when assayed with a phosphopeptide substrate. Conditions are described in reference 15.

calf brain tissue extracts. Soluble cyclophilin-CsA and FKBP-FK506 complexes bind competitively to calcineurin, indicating their binding sites are similar or overlapping. In accord with a role for calcineurin as the cellular target of both CsA and FK506, neither unligated immunophilins or drugs nor the FKBP-rapamycin complex bind to the phosphatase. Specificity is clearly evident in a study of the effects of various combinations of drugs and immunophilins on the phosphatase activity of calcineurin when assayed with a labeled phosphopeptide substrate (Figure 12).[15] The specificity for calcineurin has been verified in studies of immunophilins and their drug complexes with three additional cytoplasmic serine/threonine protein phosphatases, PP1, PP2A, and PP2C (Unpublished results in collaboration with Philip Cohen and Carol Mackintosh). Although our experiments are limited to *in vitro* protein binding and enzyme inhibition assays, the remarkable interactions between immunophilin-drug complexes and calcineurin (Figure 13) suggest that it is an excellent candidate for the physiologically relevant

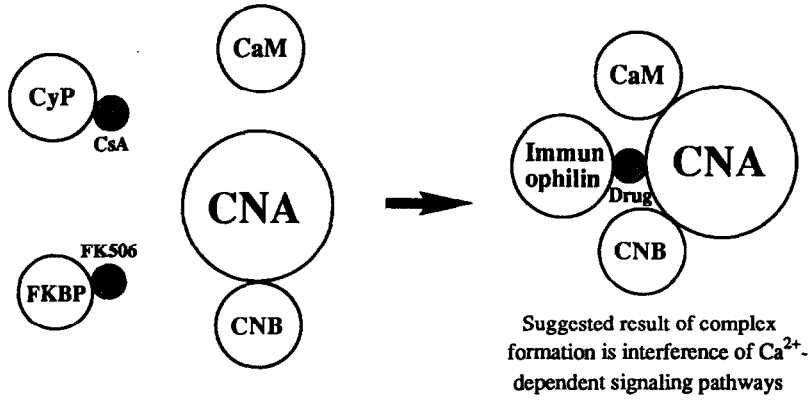


Figure 13 Two immunophilin-drug complexes bind with high affinity to calcineurin-calmodulin to form pentameric complexes. Based upon our *in vitro* biochemical experiments, we hypothesize that the protein phosphatase calcineurin is the physiological target of cyclophilin-CsA and FKBP-FK506 complexes.

common target of CsA and FK506. Several plausible models are now under consideration that invoke a role for this calcium-dependent phosphatase in signal transmission pathways characterized by an initial rise in intracellular Ca^{2+} levels. To distinguish among these, it will be necessary to define the cellular substrates of calcineurin. Recent findings by Crabtree and co-workers suggest that the function of the cytosolic component of the transcription factor NF-AT (NF-AT_c)[17] may be either directly or indirectly linked to the cellular actions of calcineurin. A general mechanistic overview is provided in Figure 14. Activation of the T cell receptor (TCR) in T cells or the IgE receptor (IgER) in mast cells leads to a rise in the concentration of intracellular Ca^{2+}. We propose this results in the activation of calcineurin, which either directly or indirectly activates transcription factors, such as NFAT and OAP, that bind to the enhancer of the interleukin-2 (IL-2) gene. Whereas cyclophilin-CsA and FKBP-FK506 modulate the phosphatase activity of calcineurin, FKBP-rapamycin appears to modulate the activity of a yet-to-be identified target molecule that is a component of Ca^{2+}-independent signaling pathways.

CsA and FK506 therefore behave as a "molecular glue"--they bring together two normally non-interacting proteins, their cognate immunophilin and the protein phosphatase calcineurin. The structural dissimilarity between the immunosuppressants and the unrelated sequences of cyclophilins and FKBPs make this result all the more remarkable. In order to understand the basis for molecular recognition within these pentameric complexes (Figure

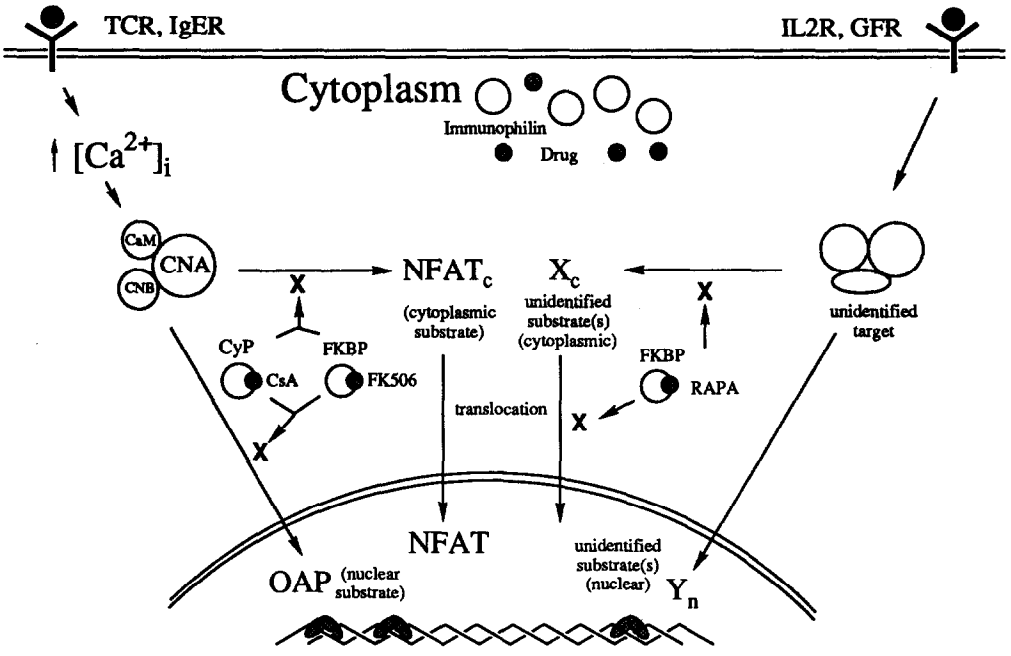


Figure 14 A mechanistic scheme for the actions of immunophilin-drug complexes on Ca^{2+}-dependent and Ca^{2+}-independent signal transduction pathways.

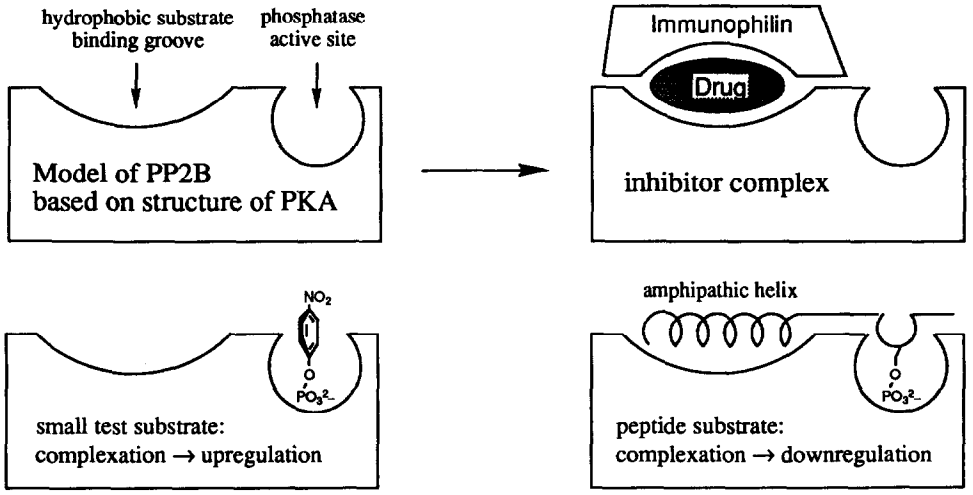


Figure 15 The ability of immunophilin-drug complexes to increase the rate of calcineurin-catalyzed dephosphorylation of a small test reagent while potently inhibiting the dephosphorylation of a phosphopeptide substrate suggests that inhibitor binding occurs to a substrate binding site, which is adjacent to the enzyme active site.

13), detailed structural analyses will be required. A clue relating to the general nature of immunophilin-drug/calcineurin complexation has been obtained from additional experiments with phosphorylated substrates of calcineurin. We find that a specific effect of the cyclophilin-CsA and FKBP-FK506 complexes on the phosphatase activity of calcineurin is also seen with the small, nonpeptide substrate *p*-nitrophenyl phosphate. In this case, however, the immunophilin-drug complexes accelerate the rate of dephosphorylation. We interpret these results with the structural model shown in Figure 15. In analogy to the structure of the protein kinase PKA[18], calcineurin may contain a hydrophobic substrate binding groove adjacent to the enzyme active site. The x-ray structure of a PKA-peptide inhibitor complex reveals that the peptide binds to the substrate-binding groove through hydrophobic contacts with an N-terminal amphipathic helix, whereas its unstructured C-terminus occupies the active site. We suggest the immunophilin-drug complexes bind to a related specificity-determining site, as opposed to the active site, on calcineurin. Since the small reagent presumably interacts only with active site residues on calcineurin, it is able to co-exist with the immunophilin-drug complex.

Although we have not achieved a detailed understanding of the structure of pentameric complexes, new insights have been gained into immunophilin-ligand complexation, particularly as it pertains to FKBP12. A stereo view of the solution conformation of human FKBP12, which we determined by protein NMR methods[19,20], is provided in Figure 16. The protein is seen to consist of a twisted (right-handed) and strongly curled five-stranded antiparallel β-sheet that surrounds a short α-helix. The drug binding site consists of an array of highly conserved aromatic residues. FK506 binds to this site through contacts with the structural elements that are common to both FK506 and rapamycin (what was correctly conjectured to be the FKBP-binding domain of FK506). These structural insights were made possible by our ongoing collaboration with Professor Jon Clardy (Cornell University, Chemistry Department). The Clardy group has succeeded in determining the high resolution x-ray structures of both the human FKBP12-FK506[21] and human FKBP12-rapamycin[22] complexes. The stereo view of the former structure (Figure 17) reveals the composite surface that serves as the calcineurin-binding element of the complex. As was anticipated, the effector element of FK506 is exposed to the aqueous environment in the x-ray structure, and therefore is likely to be intimately involved in the binding to calcineurin. Whereas a detailed comparison of unbound and bound FKBP is still ongoing, the effect of FKBP-binding on the conformation of FK506 was immediately apparent. The conformational changes in the x-ray structures of the unbound (Scheme 18)[23] and bound (Scheme 19)[21] drug are manifold. A similar observation has recently been recorded in studies of the conformation of CsA when bound to cyclophilin A.[24,25] As is readily apparent, there is little relationship between the unbound (Figure 20)[26] and bound (Figure 21)[24,25] conformations of CsA. In light of their competitive binding to calcineurin, it will be important to compare the structures of immunophilin-drug complexes once the structure of the cyclophilin-CsA complex has been determined.

In summary, the natural products CsA, FK506, and rapamycin are prodrugs (or proinhibitors). Binding to their cognate immunophilin *in vivo* induces a gain of function and results in the formation of the true drug (inhibitory) entity. In the case of cyclophilin-CsA and FKBP-FK506, this gain is suggested to correspond to a modulation of the phosphatase activity of calcineurin (PP2B), which occurs through the formation of pentameric complexes.

In the future, a number of important issues will be addressed. Some of the most pressing questions are listed:

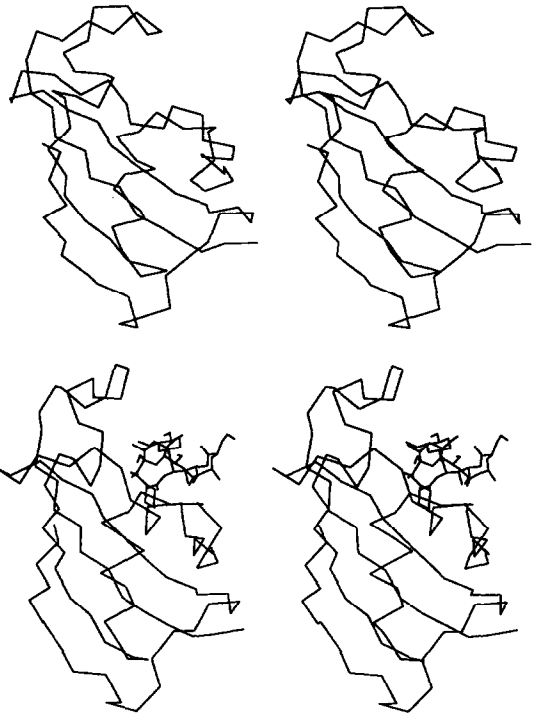


Figure 16 Stereo view of the solution conformation of human FKBP12 determined by NOE-restrained MD.[19]

Figure 17 Stereo view of the X-ray structure of the human FKBP12-FK506 complex determined at 1.7 Å resolution.[20]

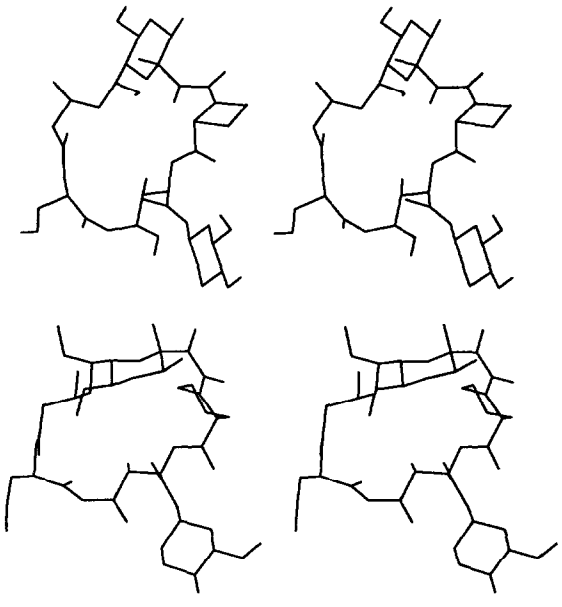


Figure 18 Stereo view of the conformation of unbound FK506.

Figure 19 Stereo view of the conformation of bound FK506.

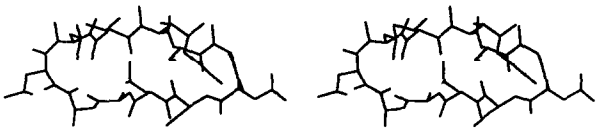


Figure 20 Stereo view of the conformation of unbound CsA.[26]

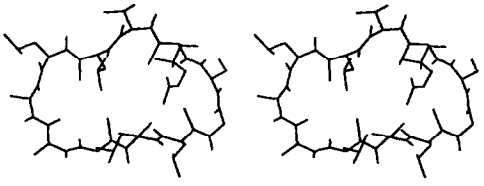


Figure 21 Stereo view of the conformation of bound CsA.[24,25]

1. Is calcineurin the physiologically relevant target of the actions of CsA and FK506?
2. What is the cellular role of calcineurin (e.g. in the $G_0 \rightarrow G_1$ transformation of the cell cycle)?
3. What are the cellular substrates of calcineurin? How close are we to identifying each of the molecules associated with the black box of a signal transduction pathway?
4. What is the target of the FKBP-rapamycin complex? (Homologous protein phosphatase family members exist that act, like rapamycin, in G_1.)
5. Are the prodrugs mimics of an endogenous cellular regulator that also bridges immunophilins and calcineurin?
6. What is the structural basis for pentamer formation? Can this knowledge be put to use in structure-based drug design efforts?
7. Can calcineurin be used as a tool for the discovery of new immunosuppressants that modulate phosphatase activity by directly binding to calcineurin?

These questions illustrate a number of exciting challenges that will keep those of us involved in this field of endeavor quite busy for some time to come. With a continuation of the current pace of immunophilin research, it may not take long to obtain answers to many of these questions.[27]

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27. Transcripts of related lectures are scheduled for publication elsewhere: (a) Transplantation Proceedings of the First International Congress on FK506, and (b) The Robert A. Welch Foundation Conference on Chemical Research XXXV. Chemistry at the Frontiers of Medicine.