American Association for the Advancement of Science

SCIENCE

18 January 1991
Vol. 251 • Pages 241–348

American Association for the Advancement of Science

SCIENCE

ISSN 0036-8075
18 January 1991
Volume 251
Number 4991

■ **SCIENCE (ISSN 0036-8075) is published weekly on Friday, except the last week in December, by the American Association for the Advancement of Science, 1333 H Street, NW, Washington, DC 20005.** Second-class postage (publication No. 484460) paid at Washington, DC, and additional mailing offices. Domestic individual membership and subscription (51 issues): $82. Domestic institutional subscription (51 issues): $150. Foreign postage extra: Canada $46, other (surface mail) $46, air freight $90. First class, airmail, school-year, and student rates on request. Canadian GST Number: Pending. **Change of address**: allow 6 weeks, giving old and new addresses and 11-digit account number. **Postmaster**: Send change of address to *Science*, P.O. Box 1723, Riverton, NJ 08077. **Single copy sales**: $6.00 per issue prepaid includes surface postage; Guide to Biotechnology Products and Instruments, $20. Bulk rates on request. **Authorization to photocopy** material for internal or personal use under circumstances not falling within the fair use provisions of the Copyright Act is granted by AAAS to libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of $1 per copy plus $0.10 per page is paid directly to CCC, 27 Congress Street, Salem, Massachusetts 01970. The identification code for *Science* is 0036-8075/83 $1 + .10. *Science* is indexed in the *Reader's Guide to Periodical Literature* and in several specialized indexes.

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COVER Lakes and ponds on the arctic tundra with Itigaknit Mountain in the background, North Slope, Alaska. These aquatic ecosystems are continuously releasing carbon dioxide to the atmosphere. Much of the carbon originates in terrestrial environments, and accounting for this release substantially lowers the estimate of the worldwide arctic sink for atmospheric carbon dioxide. See page 298. [Photograph by George W. Kling]

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27. Funding for the El Paraíso research was provided by NSF grant BNS-83-03680, Ripon College Faculty Development Funds, and the Continental Coffee Products Company (a wholly owned subsidiary of Quaker Oats). The excavations were carried out under Credencial 038-83-DCIRBM, issued by the *Instituto Nacional de Cultura* of Peru. We thank A. A. Hunter (Missouri) who identified the squash seeds and A. Price, J. Atteberry, and L. Haubrich who helped in sorting and tallying data. Additional aid in processing the subsistence remains was given by N. Salazar and M. C. Rodríguez de Sandweiss in Peru. L. Salazar-Burger, assistant field director, was essential to the project. The *Centro de Investigaciones de Zonas Ardias* was our base of operations and analysis and we thank F. A. Engel and M. Vallejos and many other Peruvian collegues for support.

Chemistry and Biology of the Immunophilins and Their Immunosuppressive Ligands

STUART L. SCHREIBER

Cyclosporin A, FK506, and rapamycin are inhibitors of specific signal transduction pathways that lead to T lymphocyte activation. These immunosuppressive agents bind with high affinity to cytoplasmic receptors termed immunophilins (immunosuppressant binding proteins). Studies in this area have focused on the structural basis for the molecular recognition of immunosuppressants by immunophilins and the biological consequences of their interactions. Defining the biological roles of this emerging family of receptors and their ligands may illuminate the process of protein trafficking in cells and the mechanisms of signal transmission through the cytoplasm.

RESEARCH DURING THE PAST DECADE HAS CONTRIBUTED significantly to our knowledge of T lymphocyte function. The identification and functional analysis of T cell surface receptors (*1*) and nuclear transcription factors (*2*) have made these components of the signal transduction apparatus among the best understood in biology. This understanding is largely due to the use of probe reagents, such as monoclonal antibodies and radiolabeled nucleic acids, that have been developed for the study of surface and nuclear phenomena, respectively. However, the mechanisms for the transduction of signals through the cytoplasm, the "black box" of the signal transduction pathway, remain mysterious.

A family of natural products has emerged as probe reagents for cytoplasmic signaling mechanisms in the T lymphocyte. These small molecules are immunosuppressants that appear to exert their inhibitory actions distal to early membrane-associated events and proximal to nuclear processes. Studies on a family of immunosuppressant binding proteins, the immunophilins, have attempted to identify the structural requirements for high-affinity interactions between immunophilins and their immunosuppressive ligands and the biological consequences of the formation of immunophilin-ligand complexes. Although there is much to explore in this avenue of research, some general principles associated with the intermediary events of signal processing are emerging.

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The Immunosuppressants

Cyclosporin A (CsA), an inhibitor of T cell activation, is currently the favored therapeutic agent for prevention of graft rejection after organ and bone marrow transplantation, and it has been credited with initiating a revolution in clinical transplantation (*3–5*). The recently discovered compound FK506 inhibits T cell activation by mechanisms that are similar to those of CsA, but FK506 is 10 to 100 times as potent (*6*). FK506 has performed remarkably well in initial human clinical transplantation trials (*7, 8*), despite reports of toxic effects in animals (*6*). Rapamycin inhibits T cell activation at concentrations comparable to those of the structurally related FK506, yet with mechanisms that are strikingly different from those mediated by FK506, and thus CsA (*9*). Only CsA, FK506, and rapamycin have been used for the identification of members of the immunophilin class. A nonnatural ligand, 506BD (*10*), and analogs of CsA (*11–13*) have also provided insights into the inhibitory mechanisms of immunosuppressants. Many recently discovered immunosuppressive agents (*14*) with undefined mechanisms, such as

discodermolide (*15*) and deoxyspergualin (*16*), promise to reveal new facets of cytoplasmic signaling mechanisms (*17*) (Fig. 1).

The Immunophilins

The predominant CsA-binding protein in T lymphocytes is the soluble, cytosolic receptor cyclophilin (*18, 19*). Cyclophilin is an abundant and ubiquitous protein that is found in both prokaryotic and eukaryotic organisms. The major isoform of human cyclophilin has a mass of 17,737 daltons and an isoelectric point (pI) of 9.3. Two groups have independently reported that cyclophilin is identical to peptidyl-prolyl isomerase (*20, 21*), an enzyme that catalyzes the interconversion of the *cis*- and *trans*-rotamers of the peptidyl-prolyl amide bond of peptide and protein substrates, and this rotamase activity is potently inhibited by CsA.

Shortly after this discovery, the predominant FK506-binding protein in calf thymus, human spleen, and the T cell line Jurkat, termed FKBP, was isolated and characterized in two laboratories (*22, 23*). Like cyclophilin, FKBP was shown to have rotamase activity toward a peptide substrate. FK506 inhibits the rotamase activity of FKBP, but not of cyclophilin; likewise, CsA does not inhibit the rotamase activity of FKBP. The cloning (*24, 25*) and overexpression (*24*) of human recombinant FKBP and the cloning of an FKBP from *Neurospora crassa* (*26*) revealed that, despite their common enzymatic properties, FKBP and cyclophilin have dissimilar sequences. Human FKBP has a mass of 11,819 daltons and, like cyclophilin, is a basic (pI = 8.9) (*22, 24*), cytosolic protein (*27*). A prokaryotic organism, *Neisseria meningitidis*, was found to have an open reading frame that encodes an FKBP-like protein (*24*). More recently, FKBP was shown to be the predominant rapamycin-binding protein in yeast, calf thymus, and human T cells (Jurkat) (*28*). Rapamycin (dissociation constant K_d = 0.2 nM) has an even higher affinity for FKBP than does FK506 (K_d = 0.4 nM), and is also a potent inhibitor of FKBP's rotamase activity (inhibition constant K_i = 0.2 nM) (*29*).

Although cyclophilin and FKBP are the only well-characterized immunophilins, other members of this family are known to exist and are currently being investigated. For example, a CsA-binding phosphoprotein of relative molecular mass (M_r) 45,000 has been detected in Jurkat cells (*30*), and phosphoproteins of M_r 60,000 and 80,000 from this same cell line bind to both FK506 and rapamycin (*28*). The *ninaA* gene of *Drosophila* (*31, 32*) and a second cyclophilin-related gene in *Saccharomyces cerevisiae* (*33*) encode proteins that show high homology to cyclophilin. Several low molecular weight, basic proteins that are retained on CsA, FK506, or rapamycin affinity matrices have also been noted (*22, 28*). Partial sequence determination of FK506- and rapamycin-binding immunophilins of M_r 30,000 and M_r 13,000 has revealed that these molecules, together with FKBP, are members of a previously unknown family of immunophilins (*34*). Questions concerning the biological relevance, the rotamase activity, and the affinity to the cognate ligands of these low-abundance immunophilins should soon be answered.

Although the exact cellular concentrations of FKBP and cyclophilin are not known, both are abundant. Saturation binding in the cytosol of Jurkat cells was reported to occur at >5 nM ditritio-FK506 (*27*). As FKBP is the predominant cytosolic receptor for drug, this measurement is largely accounted for by FKBP, and thus the cytoplasmic concentration of FKBP may approach 5 nM. The high-affinity FKBP ligands FK506 and rapamycin, however, inhibit T cell proliferation at subnanomolar concentrations (median inhibition concentration IC_{50} ~0.5 nM) (*29, 35*). Therefore, inhibition of the rotamase activity of FKBP is very likely an insufficient requirement for mediating the actions of these drugs in T lymphocytes, because only a small fraction of the enzyme would be inhibited at effective drug concentrations. This point has been confirmed by mechanistic studies of FK506 and rapamycin (see below); likewise, investigations of CsA analogs support a similar conclusion regarding the rotamase activity of cyclophilin (*12*).

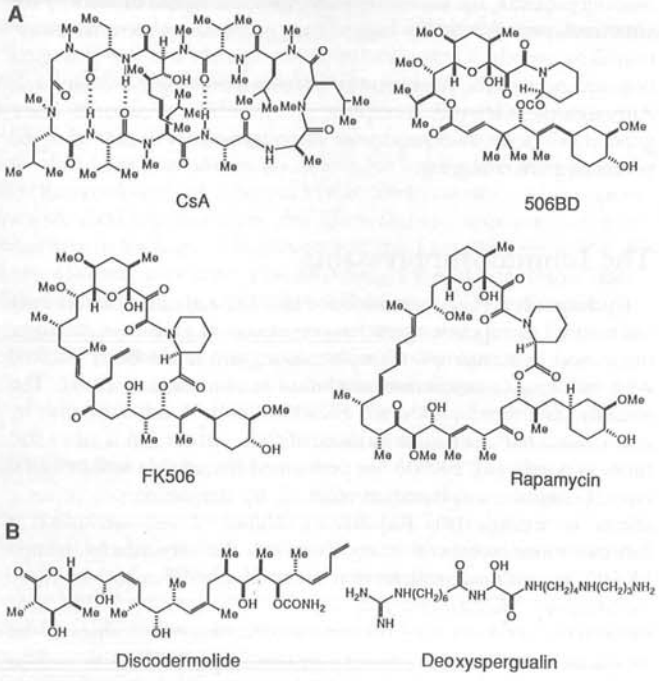

Fig. 1. Probe reagents of intracellular signaling pathways. (**A**) Recently investigated immunophilin ligands. (**B**) Immunosuppressive agents with unknown mechanisms of T cell inhibition. (Me, methyl.)

Molecular Recognition by the Immunophilins

The rotamase activity of these immunophilins and the ability of their immunosuppressive ligands to act as rotamase inhibitors provide an opportunity for exploration of the molecular basis for the high-affinity interactions that exist between them. Initial mechanistic studies of cyclophilin led to the suggestion that catalysis of the interconversion of *cis*- and *trans*-rotamers of a peptide substrate is achieved by the formation of a covalent bond to the carbonyl of the peptidyl-prolyl amide with a cysteine-derived thiol (*36*). Loss of amide resonance would be expected to lower the activation barrier to rotation about the amide C–N bond. Site-directed mutagenesis of human recombinant cyclophilin allowed the systematic replacement of all four cysteine residues in cyclophilin with alanine. Because all four mutants enzymes were fully active in the rotamase and binding assays, cysteine was ruled out as a participating residue in catalysis (*37*).

Additional mechanistic studies with both cyclophilin (*38*) and FKBP (*39*) strongly suggest that these enzymes catalyze rotamer interconversion by noncovalent stabilization of the twisted amide transition state for the noncatalyzed isomerization. The amide functionality exhibits a strong preference for a planar geometry, wherein the nitrogen lone pair is in conjugation with the carbonyl π-cloud. The energy cost of the twisted amide structure (Fig. 2A) is 15 to 20 kcal (*40*). The structural basis for cyclophilin and FKBP's ability to stabilize this transition-state structure must await further structural analyses of rotamase-peptide (or inhibitor) complexes.

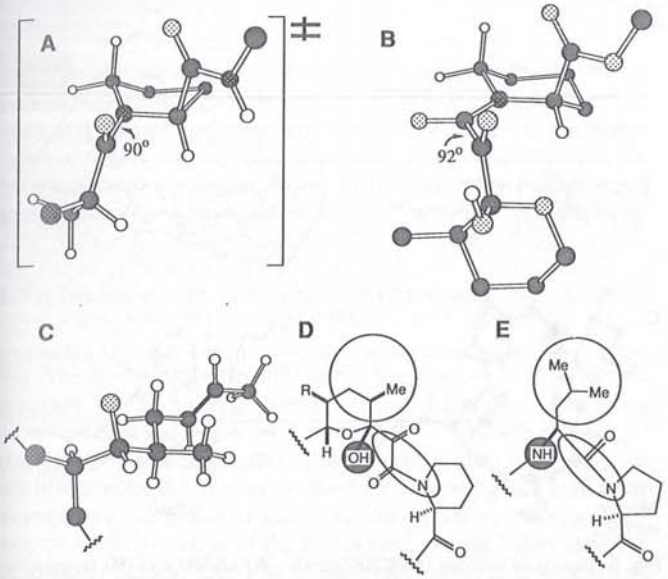

Fig. 2. (**A**) Model of the transition state structure of a twisted peptidyl-prolyl amide bond that is stabilized by the rotamase enzymes cyclophilin and FKBP. (**B**) Substructure of FK506 and (**C**) CsA (both from x-ray) that is proposed to mimic the twisted amide bond of a peptide substrate. (**D**) Substructure of FK506 (R = OMe) and rapamycin (R = H) proposed to mimic a twisted leucyl-prolyl amide bond of a peptide substrate. (**E**) Leucyl-prolyl fragment indicating structural similarities to immunosuppressant substructures.

However, the unusual structure of the immunophilin ligands and preliminary structural investigations of the immunophilin-ligand complex suggest a basis for their rotamase inhibitory properties. The total synthesis of a ^{13}C-labeled FK506 (*41*) provided a reagent to carry out ^{13}C nuclear magnetic resonance (NMR) studies of the FK506-FKBP complex (*42*). It was suggested that the ketone carbonyl adjacent to the homoprolyl amide bond of FK506 (Fig. 2B) and rapamycin is a mimic of the amide carbonyl of a peptide substrate. Thus, FK506 and rapamycin are transition-state analogs in that their ground-state geometry is similar to the transition-state structure of a peptide substrate (Fig. 2, A and B). Also, the side chain of the unusual amino acid *N*-methyl-butenylthreonine (MeBmt) of CsA, which is known to be essential for high-affinity binding of CsA to cyclophilin (*11, 12*), has structural similarity (Fig. 2C) to the aforementioned transition-state structure (Fig. 2A). This side chain may be a different type of surrogate for the twisted amide structure. In this regard, the α-branched hydroxyethylene substructure of CsA is reminiscent of the hydroxyethylene amide isostere found in aspartyl protease inhibitors such as pepstatin.

The analogy of the α-keto-homoprolyl grouping in FK506 and rapamycin to a twisted-amide bond of a peptide substrate was extended (*39*). A substrate containing a leucyl-prolyl dipeptide was found to be optimal for FKBP (*39, 43*). The structural similarities of FK506 and rapamycin to a twisted leucyl-amide bond (Fig. 2, D and E) suggest these agents may be transition-state analogs of a leucyl-(twisted amide)-prolyl peptide substrate for FKBP.

The Biological Function of Immunophilins

The complex series of events that comprises the T cell activation cascade transpires over several days (*2*). CsA, FK506, and rapamycin act within the first hours of the process (Fig. 3). Stimulation of the T cell receptor (TCR) by foreign antigen presented by a major histocompatibility (MHC) molecule on the surface of an antigen-presenting cell results in the activation of a TCR signal transmission pathway. The signal is transduced through the cytoplasm by an unknown mechanism and results in the activation of specific nuclear transcription factors, such as nuclear factor of activated T cells (NF-AT). These nuclear factors help to regulate the transcription of T cell activation genes, such as the gene of the lymphokine interleukin-2 (IL-2). Translation of the resultant message is followed by secretion of IL-2. CsA and FK506 are potent inhibitors of the TCR-mediated signal transduction pathway, as evidenced by their ability to inhibit the transcription of early T cell activation genes (*44*). CsA (*45*) and FK506 (*29, 46*), but not rapamycin, inhibit the binding of NF-AT to the IL-2 enhancer and inhibit transcriptional activation by NF-AT. CsA and FK506 also inhibit transcription mediated by AP-3 and Oct-1, and partially inhibit transcription mediated by NF-κB (*45, 46*). Another illustration involves the use of T cell hybridomas that undergo a suicidal event called apoptosis after stimulation of the TCR-CD3 complex. CsA and FK506, but not rapamycin, are potent inhibitors of apoptosis induced by an antibody to the TCR-CD3 complex (*29*).

T cell activation involves not only IL-2 secretion but also expression of the lymphokine receptor IL-2R on the surface of the cell. After the binding of IL-2 to IL-2R, a lymphokine receptor (LKR) signal transmission pathway is activated. Transduction of this signal again proceeds by an unknown mechanism through the cytoplasm and into the nucleus, where a different set of genes is transcribed. Whereas rapamycin, despite its structural similarity to FK506, has no effect on the production of IL-2, it potently inhibits the response of the T cell to IL-2 (*29, 35, 47*). Rapamycin thus appears to inhibit a later LKR-associated signaling pathway (Fig. 3). Because both rapamycin and FK506 are potent inhibitors of the rotamase activity of FKBP and inhibit distinct signaling pathways, these results support the suggestion that the inhibition of rotamase activity of FKBP is an insufficient requirement for mediating the actions of FK506 and rapamycin (*10, 29*).

In addition to their ability to inhibit different T cell activation events, rapamycin and FK506, but not CsA, have been shown to be mutually inhibitory in a variety of functional assays (*29, 47*). These results suggest a role for either a single immunophilin or separate immunophilins that share a common receptor site in mediating the actions of FK506 and rapamycin. Furthermore, rapamycin can distinguish the biological actions of FK506 and CsA, because it has no effect on the actions of CsA.

The mutual inhibition of FK506 and rapamycin was shown to be subject to a buffering action by FKBP (*29*). A concentration 10 to 100 times the effective drug concentration (IC_{50} ~ 0.5 nM) of

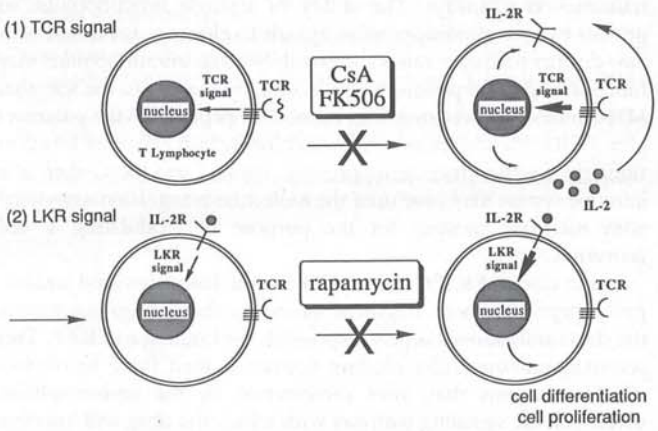

Fig. 3. Early events of the T cell activation cascade and the sites of inhibitory action by CsA, FK506, and rapamycin.

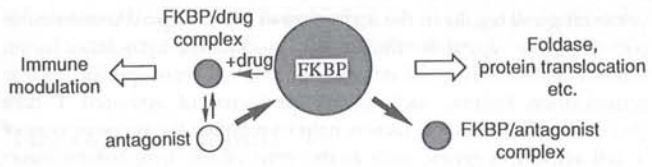

Fig. 4. Schematic illustrating the relative abundance of receptor and ligands. The abundant FKBP, which may have a cellular function as a foldase, is converted to an inhibitory complex on binding of the drug and buffers the actions of the antagonist.

either agent is required for inhibition of the actions of the other (*29, 47*). Thus, the abundant (~5 nM) uncomplexed immunophilin sequesters the antagonist. Only after the excess binding sites are occupied does the concentration of the antagonizing agent rise sufficiently to displace the drug effectively from its biological receptor. These findings also implicate the immunophilin-drug complex as the inhibitor of T cell activation. Although a role for FKBP as the mediator of the biological actions of rapamycin and FK506 has not been shown, the buffer effect of FKBP evident in the studies of reciprocal inhibition should also be operative with competing cellular receptors for these drugs. The low-abundance immunophilins must overcome the high abundance of FKBP and its high affinity for drug in order to compete effectively for binding (Fig. 4).

Invoking the immunophilin-drug complex as the biological effector addresses the issue of how the ubiquitous cyclophilin and FKBP could be involved in T cell activation. One possibility is that these proteins have a more general function, perhaps assisting in protein folding in vivo by acting as foldases. Only when the immunophilin combines with its immunosuppressive ligand does it inhibit T cell activation. The cellular immunophilin receptor (possibly FKBP), bound to either FK506 or rapamycin, may interact with different molecules in distinct pathways of T cell activation. According to this hypothesis, the specificity of the factors associated with different signaling pathways is determined by the precise geometry of the immunophilin-drug complex. Evidence has been presented (*48*) that the cyclophilin-CsA complex, and not CsA, is the agent responsible for the toxic actions of CsA in two lower eukaryotes. CsA-sensitive strains of *N. crassa* and *S. cerevisiae* were grown in the presence of CsA. Analysis of the CsA-resistant mutant strains that resulted revealed that either they no longer produced cyclophilin or, if they did, the cyclophilin of the mutant strains did not bind CsA (*48*).

The common biological receptor site implied by the mutual inhibition of FK506 and rapamycin suggests that the immunophilin may present multiple ligands to cytoplasmic components of signal transmission pathways. The ability of a single immunophilin to present two immunosuppressive ligands to effectors associated with two distinct pathways raises the possibility that immunophilins may function as general presenting molecules, by analogy to the way that MHC molecules present a large number of peptides to the polymorphic TCRs. If endogenous immunophilin ligands exist that function similarly to the immunosuppressive natural products, then the immune system may have used the molecular recognition associated with rotamase catalysis for the purpose of modulating T cell activation.

In the case of FK506 and rapamycin, the leucyl-(twisted amide)-prolyl peptidomimetic fragment shared by these drugs constitutes the structural element largely responsible for binding to FKBP. This common immunophilin binding domain is then fused to distinct effector elements that, after presentation by the immunophilin, determine the signaling pathway with which the drug will interfere (Fig. 5, A and B). This view of FK506 and rapamycin as dual domain agents was tested with an FKBP ligand designed to contain

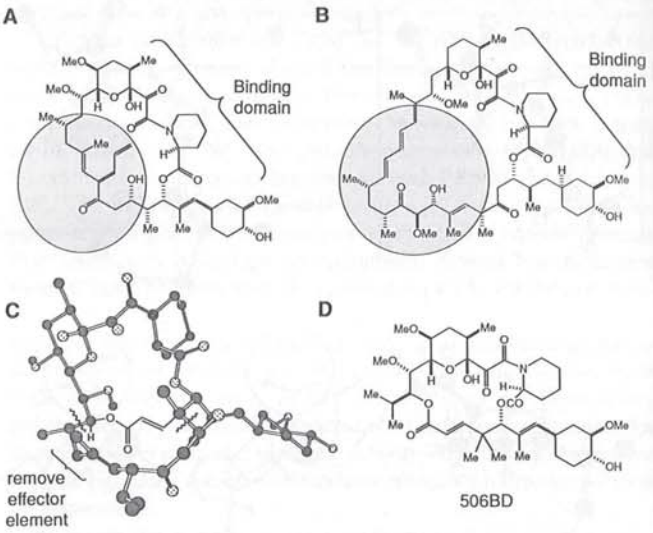

Fig. 5. Domainal analyses of FKBP ligands. (**A**) FK506 and (**B**) rapamycin binding domain and effector elements (shaded). (**C**) Structure of FK506 (x-ray) with enolate spacer drawn to illustrate scaffolding effect. (**D**) Removal of the outer loop of structure (C) results in 506BD, a high-affinity (K_i = 5 nM) ligand to FKBP.

the putative FKBP-binding domain of FK506 and rapamycin in the conformation found in the solid state of FK506 (Fig. 5C) (*10*). The resultant molecule, 506BD, was found to bind with high affinity (K_d = 20 nM) and to inhibit the rotamase activity (K_i = 5 nM) of FKBP potently (Fig. 5D). Because 506BD lacks the putative effector elements of either FK506 or rapamycin, it was not expected to inhibit either the TCR or LKR signaling pathways associated with T cell activation. Indeed, 506BD does not inhibit T cell activation by either mechanism, even at high concentrations. However, this immunophilin ligand inhibits the actions of both FK506 and rapamycin at concentrations that would be anticipated given the relative affinity of these agents to FKBP and the buffer effect (*10*). In addition to illustrating that the inhibition of the rotamase activity of FKBP is an insufficient requirement for mediating the actions of FK506 and rapamycin, these studies support the view that these immunosuppressants are composed of two domains, one important for binding to immunophilin (binding element) and one essential for biological action (effector element).

Future Prospects

The presence of cyclophilin and FKBP in many organisms suggests that these enzymes may have some general cellular function. The recent discoveries of proteins that assist in protein folding, unfolding, and translocation in vivo provide precedent for a similar function for rotamase enzymes (*49*). A role for an *Escherichia coli* cyclophilin in the secretory pathway was suggested after the discovery that it was localized in the periplasm (*50*). Similarly, the demonstration that an *N. crassa* FKBP catalyzed protein folding and the identification of mitochondrial forms of *N. crassa* cyclophilin and FKBP led to the suggestion that these immunophilins assist in the refolding of proteins that have traversed a biological membrane (*26*). The identification and characterization of new immunophilins will increase our understanding of these molecules. The structures of immunophilins and their drug complexes may prove illuminating, in regard to both enzyme mechanism and cell signaling inhibition.

Many questions remain unanswered concerning the mechanisms

of inhibition of T cell activation by these immunosuppressants and the role of their cytoplasmic binding proteins. To this end, an assessment of the relevance of individual immunophilins, such as cyclophilin, FKBP, and the low-abundance immunophilins, and their drug complexes, as well as the identification of their target factor or factors is under way. In the cases of CsA and FK506, it has been suggested that a component of the transcriptional apparatus that regulates IL-2 transcription is the target of the immunophilin-drug (CsA or FK506) complexes (*24*). These drugs potently inhibit the transcriptional activity of NF-AT but only partially inhibit its DNA binding activity (*29*). Thus, the target factor or factors may interact with NF-AT [and perhaps Oct-1, AP-3, and NF-κB (pleiotropism) (*51*)] and enhance their transcriptional activation properties. The requirement for protein synthesis before IL-2 gene transcription (*52, 53*) suggests that translation of a protein, perhaps an as yet unidentified activating factor, occurs before transcription of the IL-2 gene. This cytoplasmic activity (translation) is a potential site of action for the immunophilin-drug complex, which may act as a cytoplasmic anchoring protein (*54*). This putative activating factor may be a cellular analog of the herpes virus protein VP16, which is believed to bind to Oct-1 (a transcription factor also sensitive to drug) and thereby complete the formation of a preinitiation complex at the transcription start site (*55*).

Although research on these immunosuppressants has focused on T lymphocytes, the nephrotoxic properties (*8*), hepatotrophic effects (*56*), ability to inhibit apoptosis (*29*) and exocytosis (*57*), and actions in lower eukaryotes (*48*) of these agents indicate an alternate function in other cell types. It is possible that in each of these studies the immunophilin ligand is involved in the inhibition of protein trafficking. The varying effects of drug may be due to differences in the immunophilins found in different sources, or perhaps more likely, due to the interactions of the same inhibitory complexes with different protein targets. It is also important to identify potential endogenous ligands, possibly small-molecular regulatory agents, that these drugs may emulate.

The research outlined in this article illustrates an approach to the study of cellular, particularly cytoplasmic, phenomena. In this approach, modern techniques in chemistry and biology are melded so that the interactions of natural and synthetic ligands with their cellular receptors can be explored. Small molecules are used as probes, first for identification and isolation of relevant proteins in a system of interest and then for the development of a detailed understanding of the system through studies of structure and function. Such a chain of events has been initiated by a family of immunosuppressants, and other natural products might be useful as probes for a diverse set of events that includes protein trafficking and cytoskeletal dynamics. Although there is still much to be learned about cytoplasmic signaling mechanisms with use of immunophilin and immunosuppressant probes, the prospects for fundamental insights appear promising.

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58. I thank co-workers in my laboratory for contributions to the concepts outlined in this article, my collaborators S. J. Burakoff, B. E. Bierer, G. R. Crabtree, M. Karplus, G. L. Verdine, and C. T. Walsh for stimulating discussions, and the National Institute of General Medical Sciences (GM-38627) for financial support of immunophilin research.