Molecular Mechanism of Immunosuppressive Agents

G. Baumann

THE cyclic peptide, cyclosporine (CyA, Sandimmune), isolated from the fungus, *Tolypocladium inflatum* Gams, was discovered 20 years ago, in January 1972.[1] Since then, it has revolutionized organ transplantation and is currently being tested for efficacy in the treatment of a number of autoimmune diseases, particularly psoriasis, rheumatoid arthritis, and nephrotic syndrome.[2] In addition to its use as a successful drug in clinical indications, CyA is also widely employed as an experimental tool for basic research, eg, into understanding signal transduction pathways and exploring novel possibilities for pharmacologic intervention.[3]

Early immunological studies revealed that CyA blocks activation of T cells and that this, in part, results from inhibition of transcription of lymphokines, most notably interleukin-2 (IL-2), the main growth factor for T cells.[4] The current concept of the mechanism of immunosuppression by CyA suggests that, by inhibiting IL-2 expression in T cells, CyA prevents helper T cells from orchestrating a response to foreign antigens. Because of the specific effects of CyA on lymphokine transcription in T cells, and the important role of T cells in graft rejection, research on the mechanism of action has focused mainly on the role of CyA in regulating gene expression in T lymphocytes. At least one of the intracellular targets for CyA has been identified and found to have an enzymatic activity. This high-affinity receptor for CyA belongs to the increasingly diverse family of general and tissue-specific cyclophilins.[5] All the members identified so far reveal peptidyl-prolyl *cis-trans* isomerase activity, catalysing the *cis-trans* isomerization of peptide bonds involving prolyl residues.[6] This enzymatic activity, which facilitates protein folding, is potently inhibited when the protein binds to CyA. A first line of evidence relating the biological effects of CyA to cyclophilin was obtained by comparing the ability of a large number of CyA analogues to bind to cyclophilin with their immunosuppressive activity in vitro.[7] All CyA analogues that showed a significant immunosuppressive activity also bound to cyclophilin. In addition, using a genetic approach involving the analysis of cyclophilin mutants, which do not bind to CyA, in lower eukaryotes (*Neurospora crassa, Saccharomyces cerevisiae*), Tropschug et al demonstrated that the cytotoxic effect of CyA was mediated by cyclophilin.[8]

More recently, there has been the discovery of an unrelated antibiotic product from *Streptomyces tsukubaensis*, with inhibitory effects on T-cell activation surprisingly similar to those produced by CyA.[9] This molecule, known as FK 506, is a macrolide that binds to a separate group of receptor proteins, termed FK-binding proteins (FKBPs).[10] Neither CyA nor FK 506 appears to crossreact with the receptors (ie, immunophilins) of the other. Since both drugs block the induction of cytokine gene transcription at the early stage of antigen-induced helper T-cell activation (G_0 to G_1 transition of cell cycle), it was assumed that T-cell activation required the separate activity of both immunophilins. This functional similarity was underscored by the findings that both cyclophilins and FKBPs were active as peptidyl-prolyl *cis-trans* isomerases, and that this activity was blocked by the binding of the appropriate drug. These observations suggested that immunosuppression resulted from improper folding of a transcription factor required for lymphokine mRNA expression. On the basis of this model, proline isomerase inhibition was believed to underlie the mechanism of T-cell inhibition by CyA and FK 506.

A major conceptional change came with data about yet another immunosuppressant, the macrolide, rapamycin, which was isolated from *Streptomyces hygroscopicus*. This compound inhibits T-cell activation at concentrations comparable to those of the structurally related FK 506, but with a mechanism that is strikingly different from that mediated by either FK 506 or CyA.[11] Rapamycin does not inhibit the transcription of early T-cell activation genes, including IL-2, but appears instead to block downstream events leading to T-cell proliferation, eg, the signal transduction pathway emanating from lymphokine receptors, such as the IL-2 receptor (G_1 to S phase transition). Rapamycin also binds to FKBP and inhibits the peptidyl-prolyl *cis-trans* isomerase activity, but it does not inhibit IL-2 transcription. This clearly demonstrates that the inhibition of the peptidyl-prolyl *cis-trans* isomerase activity of FKBP is insufficient per se to mediate the biological effect of FK 506.[12] In addition, it has been shown by competition experiments that FK 506 and rapamycin must bind to a common intracellular receptor, since rapamycin can act as a potent antagonist of FK 506.[13] The current model suggests that both drugs act via two regulatory domains: an immunophilin (FKBP)-binding domain which is shared between FK 506 and rapamycin, and an effector domain which is specific for both drugs.[12]

Strikingly, the same dual-domain concept has been

Ciclosporin FK-506 Rapamycin

Immunophilin:	Cyclophilin	FKBP	FKBP

Chemical structure — Immunophilin binding domain / Effector domain

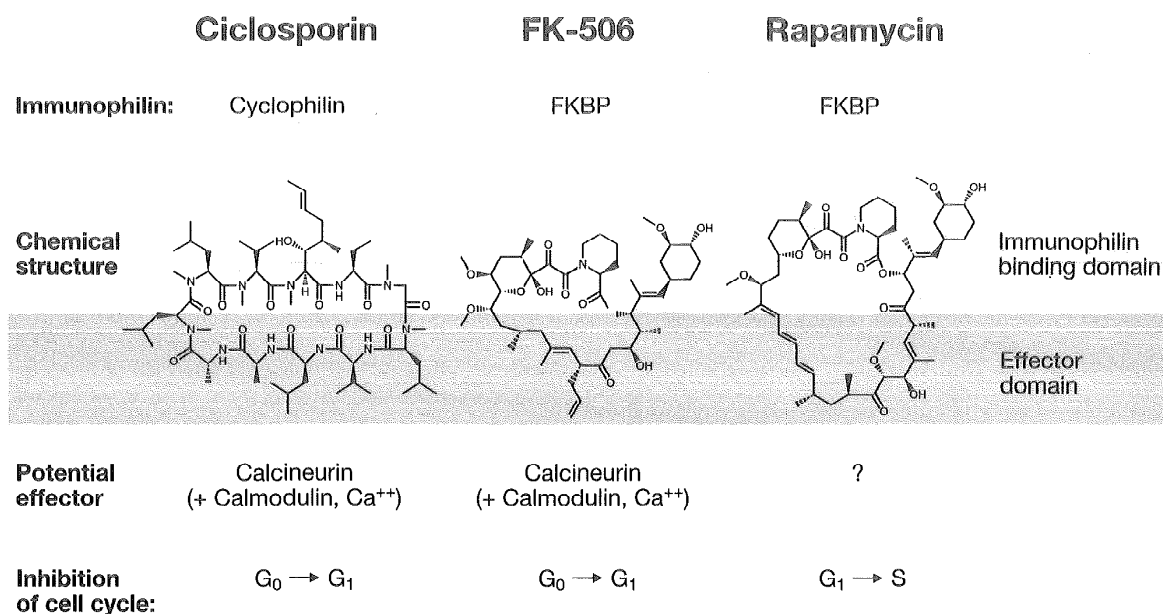| Potential effector	Calcineurin (+ Calmodulin, Ca^{++})	Calcineurin (+ Calmodulin, Ca^{++})	?
Inhibition of cell cycle:	$G_0 \to G_1$	$G_0 \to G_1$	$G_1 \to S$

Fig 1. Dual-domain concept for immunosuppressive compounds of microbial origin (CyA, FK 506, rapamycin).

shown to be valid for CyA (Fig 1). The binding site for cyclophilin has been mapped by immunochemical methods,[7] and was later confirmed by NMR analysis[14] and x-ray crystallography.[15] The definition of the effector site on the CyA molecule has been achieved by carefully selecting a small group of cell-permeable, cyclophilin-binding and *cis-trans* isomerase inhibitory CyA derivatives, which were found to be nonimmunosuppressive (G Zenke et al, in preparation). This small group of CyA analogues has been shown to reverse the immunosuppressive effect of CyA when added to T cells in a molar excess of CyA. Their inhibitory effect on the *cis-trans* isomerase activity of cyclophilin provides compelling evidence that, in analogy to FK 506 and FKBP, inhibition of this enzymatic activity is either irrelevant or at least insufficient for immunosuppression by CyA.[16] Binding of CyA or FK 506 to their respective immunophilins, however, is a prerequisite of their immunosuppressive potential. Most crucial for the activity of the compound is the effector site which, in the case of CyA, is comprised of those residues that are altered in the nonimmunosuppressive group of CyA derivatives competing for receptor binding. There remains the question of the extent to which CyA and FK 506, when bound to their receptors (immunophilins), exert their immunosuppressive activity by acting via their effector site on different primary targets or on different sites of a common primary target. This target is most probably a component of the signal transduction pathway which finally results in IL-2 transcription.

On the basis of this set of data, the teams of Schreiber[17] and Weissman[18] tested the notion that the drug-receptor complex was acting as a single effector. They used the immunophilin-drug complex as an affinity matrix to isolate cytoplasmic proteins which recognize specifically the dual complex. Surprisingly, complexes between CyA and cyclophilin and between FK 506 and FKBP bind to the same set of proteins. The two groups could demonstrate that, in the presence of Ca^{2+}, the immunophilin-drug complexes (but not cyclophilin, FKBP or FKBP-rapamycin) competitively bind directly to the serine-threonine phosphatase calcineurin, and only indirectly via calcineurin to calmodulin (Fig 2). As CyA and FK 506 exhibit specificity for activation pathways that induce an increase in intracellular Ca^{2+} concentrations, such as that mediated by the T-cell receptor, calcineurin may be involved in regulating the phosphorylation state of downstream components of this pathway. In this context, the intrinsic Ca^{2+}- and calmodulin-stimulated phosphatase activity of calcineurin is potently inhibited by CyA-cyclophilin and FK 506-FKBP complexes in vitro.

Biological studies should soon determine whether calcineurin is the relevant target of CyA and FK 506 in vivo and is thus a key molecule in the signal transduction pathway emanating from the T-cell receptor. Despite the fact that the effects of CyA and FK 506 are tissue-specific, their binding proteins, such as calcineurin, cyclophilin, and FKBP, are ubiquitous. This could be explained by their selective interactions with specific isoforms of calcineurin, by the existence of tissue-specific members of the immunophilin family, or of cell-specific calcineurin substrates. These substrates are most probably components of the signal transduction pathway which finally results in
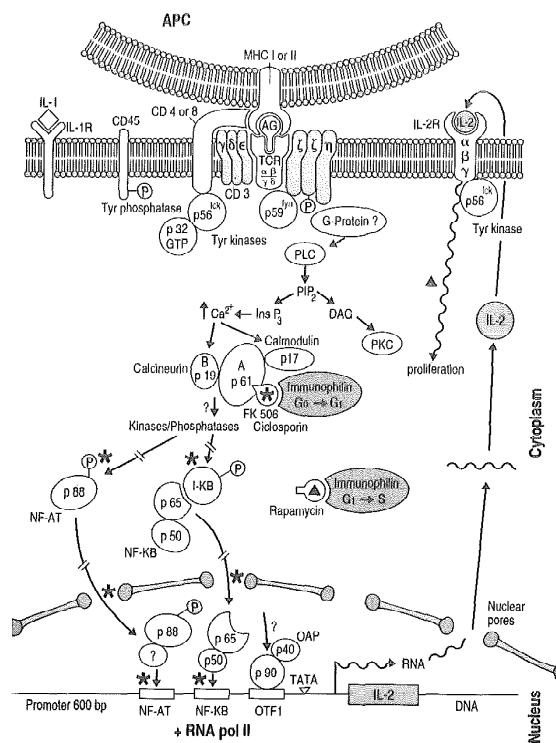
Fig 2. CyA (ciclosporin) and FK 506 both interfere, by binding to their respective immunophilins, with the function of intracellular molecules that transmit calcium-associated signals between the T-cell receptor (TCR) and the activation of lymphokine genes (IL-2) in the nucleus (for review, see Klausner and Samelson[25]). Transcriptional regulation of IL-2 gene expression is modulated by the combination of transcription factors (eg, NF-AT, NF-κB, OTF-1) interacting with their corresponding recognition sites at the IL-2 promoter. These DNA/protein complexes, together with RNA polymerase II (RNA pol II), result in the antigen-inducible transcription of IL-2. Potential intervention sites for the pentameric complex [calcineurin A (p61), B (p19), calmodulin (p17), immunophilin, drug], involving, eg, modification and translocation of antigen-inducible transcription factors [NF-AT (p88), NF-κB (p50, p65)], are indicated by asterisks. CyA and FK 506 interfere with the G_0 to G_1 transition of the cell cycle, whereas rapamycin interferes with the G_1 to S transition (indicated by a triangle). For details see text.

IL-2 transcription (Fig 2). Potential candidates, which are essential for IL-2 activation, are the antigen-inducible transcription factors NF-AT (*N*uclear *F*actor of *A*ctivated *T* cells)[19] and NF-κB (*N*uclear *F*actor of immunoglobulin κ light chain in *B* cells)[20] which were both reported as being affected in their IL-2 promoter binding activity by CyA and FK 506 but not by rapamycin.[21,22] In all probability, DNA binding of both transcription factors depends on protein modification and nuclear translocation of cytoplasmic precursors prior to their participation in the formation of a functional transcription complex. NF-κB in nonstimulated

cells is bound as an inactive precursor to its inhibitor I-κB. Upon T-cell activation, I-κB becomes phosphorylated and subsequently releases NF-κB from the cytoplasm into the nucleus.[23]

Crabtree et al recently demonstrated that NF-AT is formed when a signal from the antigen receptor induces a preexisting cytoplasmic subunit to translocate to the nucleus and combine with a newly synthesized nuclear subunit of NF-AT. CyA and FK 506 most probably block translocation by modification of the cytoplasmic component, without affecting synthesis of the nuclear subunit.[24]

In summary, recent data on the molecular mechanism of some immunosuppressive drugs provide strong support for the fascinating postulate that CyA and FK 506 work by binding to immunophilins and then, as a drug-immunophilin complex, inhibiting the calcium-activated protein phosphatase, calcineurin. This inhibition could result in an altered modification pattern of the cytoplasmic components of transcription factors, thereby disturbing their nuclear translocation, which is a prerequisite for proper IL-2 transcription. It looks as if, with the immunosuppressive microbial metabolites as molecular probes, the pieces of this complex signal transduction puzzle are starting to fit together! Once the details of the chain of events along the T-cell signaling pathways are known, the molecular structures involved will provide new tools to be used in the search for and the rational design of new and improved therapeutic agents.

ACKNOWLEDGMENTS

REFERENCES

1. Borel JF, Kis ZL: Transplant Proc 23:1867, 1991
2. Borel JF, Di Padova F, Mason J, et al: Pharmacol Rev 41:239, 1989
3. Hohman RJ, Hultsch T: New Biologist 2:663, 1990
4. Krönke M, Leonard WJ, Depper JM, et al: Proc Natl Acad Sci USA 81:5214, 1984
5. Handschumacher RE, Harding MW, Rice J, et al: Science 226:544, 1984
6. Fischer G, Wittmann LB, Lang K, et al: Nature 337:476, 1989
7. Quesniaux VFJ, Schreier MH, Wenger RM, et al: Transplant 46:23, 1988
8. Tropschug M, Barthelmess IB, Neupert W: Nature 342:953, 1989
9. Kino TH, Hatanaka H, Miyata S, et al: J Antibiot 40:1256, 1987
10. Siekierka JJ, Hung SHY, Poe M, et al: Nature 341:755, 1989
11. Dumont FJ, Staruch MJ, Koprak S, et al: J Immunol 144:251, 1990
12. Schreiber SL: Science 251:283, 1991
13. Dumont FJ, Melino MR, Staruch MJ, et al: J Immunol 144:1418, 1990

14. Weber G, von Freiberg B, Traber R, et al: Biochemistry 30:6563, 1991

15. Kallen J, Spitzfaden C, Zurini MGM, et al: Nature 353:276, 1991

16. Sigal NH, Dumont F, Durette P, et al: J Exp Med 173:619, 1991

17. Liu J, Farmer JD, Lane WS, et al: Cell 66:807, 1991

18. Friedman J, Weissman I: Cell 66:799, 1991

19. Emmel EA, Verweij CL, Durand DB, et al: Science 246: 1617, 1989

20. Lenardo MJ, Baltimore D: Cell 58:227, 1989

21. Mattila PS, Ullman KS, Fiering S, et al: EMBO J 9:4425, 1990

22. Baumann G, Geisse S, Sullivan M: New Biologist 3:270, 1991

23. Ghosh S, Baltimore D: Nature 344:678, 1990

24. Flanagan WM, Corthésy B, Bram RJ, et al: Nature 352:803, 1991

25. Klausner RD, Samelson LE: Cell 64:875, 1991