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COMMENTARY

OBSERVATIONS ON THE MECHANISM OF ACTION OF FK-506

A PHARMACOLOGIC PROBE OF LYMPHOCYTE SIGNAL TRANSDUCTION

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The lymphocyte is an extraordinarily complicated cell. Activation of B and T cells through their respective antigen receptors leads to a cascade of biochemical events which result in the production of mediators (i.e. lymphokines) and their differentiation into effector cells (e.g. generation of immunoglobulin-secreting cells and cytotoxic lymphocytes). From one perspective, the lymphocyte can serve as an excellent model system for understanding complex eukaryotic signal transduction processes. At another level, modulation of immune responsiveness holds the key to effective therapy of organ transplantation rejection and control of a variety of autoimmune diseases, such as juvenile diabetes and rheumatoid arthritis.

The modern era of transplantation began nearly 10 years ago with the first clinical trials of cyclosporin A (CsA†). Its efficacy in organ and bone marrow transplantation and in autoimmune disease states has demonstrated the value of nonspecific immunosuppressive therapy in these clinical situations. During those 10 years, the effect of CsA on a variety of activation and differentiation events in the lymphocyte was documented extensively. Despite significant advances in our knowledge of the biochemical events that lead to lymphocyte signal transduction and lymphokine gene expression during that period of time, few insights into the mechanism of action of CsA were forthcoming.

The search for safer and more effective immunosuppressants has led, more recently, to the discovery of FK-506 [1]. Although they are structurally unrelated (see Fig. 1), CsA and FK-506 share several mechanistic similarities at the cellular and molecular levels, and discussion of these surprising observations forms the basis of this review. While many compounds, such as corticosteroids and anti-metabolites, can inhibit a lymphocyte proliferative response *in vitro* and serve as potent immunosuppressives *in vivo*, the selectivity of CsA and FK-506 for certain types of activation events clearly sets them apart from these other agents. On one level, the insights into the mechanism of action of CsA and FK-506 may provide the basis for the discovery of other novel immunosuppressive agents. In addition, these molecules have served as pharmacologic probes to define a subset of Ca^{2+}-associated signal transduction pathways that appear to be most prominent in lymphocytes but may exist in other cells as well. Combined with the characterization of the major intracellular receptors for CsA and FK-506, these immunosuppressive agents may provide us with important tools to understand some of the biochemical events needed to translate a signal from the extracellular membrane to the nucleus.

FK-506: *Discovery and* in vivo *pharmacology*

FK-506 was discovered in 1985 by scientists at the Fujisawa Pharmaceutical Company in a fermentation broth of a soil organism, *Streptomyces tsukubaensis* [1, 2]. Several other microorganisms were also identified that produce FK-506 or structurally related macrolides [3, 4], but since FK-506 is the most active in this series of molecules, it has been studied most extensively. The first published studies demonstrated that FK-506 is a potent inhibitor of murine or human T-lymphocyte proliferation, with an ED_{50} of approximately 0.1 nM, close to 100 times more active than CsA [2, 5–7]. The selectivity of FK-506 was quickly shown to be quite similar to that of CsA with respect to its ability to inhibit lymphokine production by activated T cells, while having no effect on bone marrow colony formation or lymphokine-dependent proliferation [2, 5–8].

FK-506 has been studied in a wide variety of models of experimental transplanation and autoimmunity. This area has already been reviewed recently and will not be covered exhaustively here [9]. In general, FK-506 is effective in blocking allograft rejection in a number of species and can be used at doses 10–100 times lower than the dose of CsA required in the same models [10–15]. In the rat,

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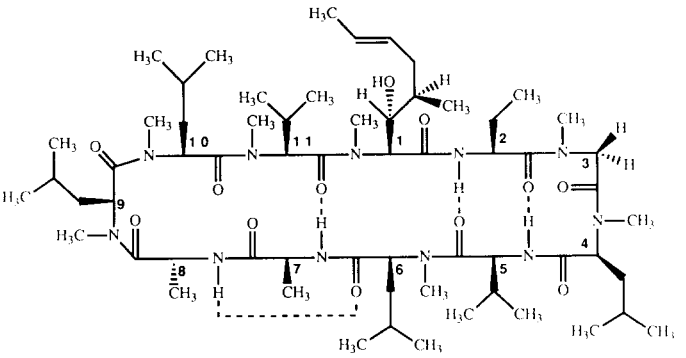
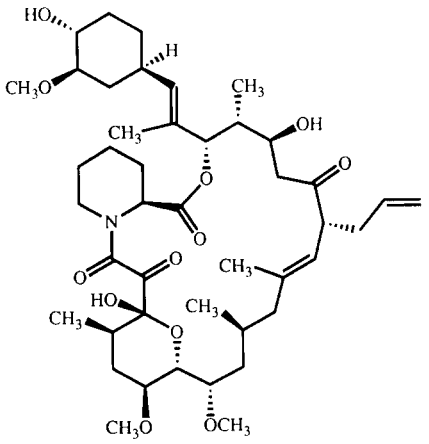
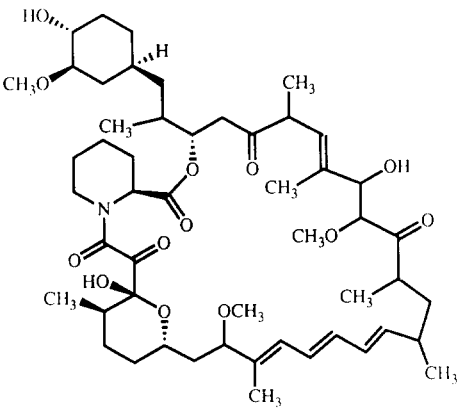
† Abbreviations: CsA, cyclosporin A; FKBP, FK-506 binding protein; TNF, tumor necrosis factor; NF-AT, nuclear factor of activated T cells; LPS, lipopolysaccharide; TIMP, tissue inhibitor of metalloproteases; and PMA, phorbol myristate acetate.



Cyclosporin A



FK 506



Rapamycin

Fig. 1. Structures of cyclosporin A, FK-506, and rapamycin.

FK-506 prolongs allograft survival following transplantation of heart, liver and skin [10, 12]. Transplantation experiments in the dog have been somewhat more controversial, since some groups suggest that dose-limiting toxicities (see below) compromise the effectiveness of therapy [16], whereas other groups report much greater success [12, 14]. On the other hand, FK-506 is able to prevent renal allograft rejection in the baboon with minimal side-effects [14, 15]. With respect to autoimmune diseases, beneficial effects of FK-506 have been observed in rat models of experimental autoimmune encephalomyelitis, experimental autoimmune uveoretinitis, collagen-induced arthritis, and two strains of spontaneously autoimmune mice, MRL/lpr and (NZB × NZW)F_1 mice [17–21].

The toxicity of FK-506 in dogs has been until quite recently the major impediment to the clinical development of this compound. While there is little evidence of clinical or pathological changes in rodents or primates treated with prolonged courses of FK-506 at therapeutic doses, dogs experience severe side-effects at these dosage levels [12, 14, 16]. The most dramatic side-effects in dogs relate to the gastrointestinal tract where emesis, weight loss and intussusception have been observed frequently. In addition, myocardial degeneration and arteritis, as well as lymphocytic infiltration in other organs, has been seen.

Clinical trials were begun in February, 1989, at the University of Pittsburgh, focusing primarily on liver transplantation [22]. The rationale for this decision was 2-fold: first, the severe toxicity observed in dogs was not demonstrated in primates and, therefore, may not translate to humans; second, the failure rate of liver allografts remains unacceptably high with conventional immunosuppressive therapy. The Pittsburgh group has reported remarkable success in their early trials [22]. FK-506 was used first in recipients under conventional immunosuppression who had rejection, nephrotoxicity, or both. This salvage therapy was successful in seven of the ten attempts. Subsequently, patients who underwent fresh orthotopic liver transplantation were given FK-506 plus low-dose steroids from the outset. None of these first six patients had rejection, and serious side-effects were not encountered. Thus, it appears likely that clinical evaluation of FK-506 will be extended

to other areas of organ transplantation, and it may be a significant improvement over CsA.

Effects of FK-506 on lymphocyte activation

Lymphokine gene expression. T-cell activation occurs following a specific recognition event via the T-cell receptor/CD-3 complex combined with additional signals provided by accessory cells and/or cytokines (for a recent review, see Ref. 23). A complex series of biochemical events ensue, including phosphoinositide turnover, activation of protein kinase C, a rise in intracellular Ca^{2+} and phosphorylation of proteins on serine, threonine and tyrosine residues. There is no evidence that CsA or FK-506 affects these early events associated with T-cell receptor recognition [24, 25]. These biochemical processes lead to the coordinate expression of a set of gene products critical for lymphocyte growth and differentiation, and a great deal of attention has been focused on these events.

It is well known that one of the principal sites of the immunosuppressive action of CsA is inhibition of the expression of a discrete set of lymphokine genes at the transcriptional level. This phenomenon has been demonstrated for CsA, primarily for the IL-2 gene, in heterogeneous T-cell populations [26], on cloned T-cell lines [27], and by *in situ* hybridization both *in vitro* [28] and *in vivo* [29]. Recently, Tocci *et al.* [30] documented that FK-506 inhibits induction of the same set of lymphokine genes, including IL-2, IL-3, IL-4, GM-CSF, TNFα, and interferon-γ. Furthermore, nuclear run-off transcription studies reveal that both FK-506 and CsA directly inhibit transcription of the IL-2 gene. A more detailed molecular picture has emerged from the work of Crabtree and coworkers, who have identified several regions of the IL-2 promoter critical for IL-2 gene expression [31]. CsA was found to specifically inhibit the appearance of DNA binding activity (as assayed by gel retardation) of one such region, termed NF-AT, and it abolished the ability of the NF-AT binding site to activate a linked reporter gene in transfected mitogen-stimulated T lymphocytes [32]. Recent experiments with FK-506 reveal remarkably similar results.* Thus, at every level at which lymphokine gene regulation has been examined, the effects of FK-506 and CsA are virtually indistinguishable.

There are several experimental systems that can be used to document the selectivity of FK-506 and CsA with respect to gene transcription. First, FK-506, like CsA, did not affect expression of the mRNAs for IL-1α or IL-1β in LPS-activated human monocytes or of stromelysin, collagenase or TIMP in IL-1-treated synovial fibroblasts [30]. Second, transcription of other genes expressed following T-cell activation, such as *c-fos*, the transferrin receptor and, in particular, the IL-2 receptor α chain, were not inhibited by these compounds [30]. Another experiment that demonstrates this selectivity is the observation that inhibition of T-cell proliferation in cultures treated with CsA or FK-506 can be reversed by addition of recombinant IL-2 [8]. The finding suggests that, with the exception of IL-2 or similar growth factors, all other gene products and pathways remain intact in cells treated with these immunosuppressive agents.

FK-506 and CsA define a subset of Ca^{2+}-associated signal transduction pathways in lymphocytes. The data reviewed in the preceding section support the widely held notion that the principal site of action of FK-506 and CsA is at the level of lymphokine gene regulation. While it is reasonable to conclude that inhibition of lymphokine production is the critical event leading to the immunosuppressive effects of these agents *in vitro*, we contend that they do not act *directly* at the transcriptional level. Instead, we hypothesize that these compounds act at an earlier biochemical step in certain types of signal transduction pathways that may, indeed, predominate in the cascade leading to lymphokine production. Three areas of investigation strengthen this view: (1) studies on non-lymphokine gene regulation; (2) the lack of effects of FK-506 and CsA on certain lymphocyte activation pathways; and (3) the effect of the immunosuppressants on processes that do not involve transcriptional regulation.

FK-506 and CsA have been shown to modulate the expression of non-lymphokine genes in several model systems. For example, both compounds inhibit induction of *c-myc* mRNA in mitogen-stimulated human T cells [30]. A more interesting finding is that of Zipfel *et al.* [33], who have identified a number of gene products that are induced following cellular activation of lymphocytes and/or fibroblasts. Some of the induced genes expressed by both cell types are sensitive to inhibition by CsA in T cells but not in fibroblasts. Therefore, it is the mode of signal transduction, and not the gene *per se*, that determines whether these immunosuppressants can exert their effect.

Several experimental systems have been identified in which the ability of FK-506 and CsA to inhibit lymphocyte signal transduction events was dependent on the mode of cellular activation. In general, only activation pathways that cause a measurable rise in intracellular Ca^{2+} were CsA/FK-506 sensitive. For example, CsA and FK-506 inhibit IL-2 production when human lymphocytes are activated via the CD3/T-cell receptor complex but not when the same cells are triggered via the CD28 pathway [34, 35]. In murine B cells the compounds inhibited Ia expression induced by ionomycin but not that triggered by IL-4 (Wicker LS, Nichols E and Sigal NH, unpublished observations). Using a murine T-cell clone that produces IL-4 upon activation, Dumont *et al.* [8] showed that activation of this line with a combination of the phorbol ester PMA + IL-1 is not sensitive to CsA or FK-506, whereas induction of IL-4 expression with PMA + the Ca^{2+} ionophore ionomycin is inhibited by the drugs. Thus, transcription of gene products that are typically sensitive to the immunosuppressive agents can be rendered resistant when an alternative activation pathway is utilized. In contrast to activation via the T-cell receptor/CD-3 complex or via addition of ionomycin, which induce a measurable rise in intracellular Ca^{2+} and have a requirement for extracellular Ca^{2+}, these alternative pathways do not have a similar

* Crabtree G, personal communication, cited with permission.

Table 1. Comparison of biochemical properties of FKBP and cyclophilin

Properties	FKBP	Cyclophilin
Molecular weight	11,000–12,000	17,000
Heat stability	+	−
Trypsin stability (1 hr, 37°)	+	−
Cytosolic localization	+	+
Abundance (% total cytoplasmic protein)	0.2%	0.1–0.4%
Presence in all cells	+	+
Phylogenetic conservation	+	+
Peptidyl-prolyl isomerase activity	+	+

requirement for extracellular Ca^{2+} and induce little rise in intracellular Ca^{2+}.

A third line of evidence that supports consideration of FK-506 and CsA as probes for a subset of Ca^{2+}-associated signal transduction pathways in lymphocytes is their effect on events that are unlikely to involve transcriptional regulation. A good example of this phenomenon in T cells is the ability of CsA to inhibit granule exocytosis of cytotoxic lymphocytes triggered by antibodies to the T-cell receptor/CD-3 complex [36, 37]. A similar observation has been made recently in neutrophils, where degranulation induced by a Ca^{2+} ionophore was blocked by FK-506 and CsA, whereas degranulation by several inflammatory mediators was unaffected (Forrest MJ and Sigal NH, unpublished observations). It is relevant to the preceding discussion that only the ionophore-mediated pathway has a requirement for extracellular Ca^{2+}. Other non-transcriptional processes that have been shown to be inhibited by CsA include (1) induction of IL-3 by a Ca^{2+}-dependent signal transduction pathway in mast cells [37, 38], which is controlled at the post-transcriptional level [39]; and (2) anti-IgE-induced mediator release from human basophils [40].

The ability of FK-506 and CsA to inhibit signal transduction events in non-lymphoid cells raises the question as to why these drugs appear to selectively block immune responsiveness *in vivo*. One possibility is that the Ca^{2+}-associated pathways in the lymphocyte are absolutely essential for cellular activation, whereas those in other cell types only serve accessory functions. This notion is consistent with the view that recognition of antigen via the T-cell receptor is a critical step in IL-2 gene expression; in contrast, the pathways blocked by CsA and FK-506 in neutrophils, mast cells and basophils may not be the primary ones that lead to mediator release.

The observation that CsA and FK-506 do not inhibit the early biochemical events associated with T-cell receptor recognition, combined with the discussion in the preceding paragraphs, suggest that the site of action of these immunosuppressive agents is likely to be distal to receptor/membrane-associated events but proximal to the transcriptional factors *per se*. Identification of the biochemical steps critical to the action of these immunosuppressants, discussed in the following section, must take into account these observations. The studies described in this review underscore the complexity of these intermediate steps in the transduction process and point out the utility of FK-506 and CsA as pharmacologic probes for these events.

Biochemical site of action of FK-506

Proline isomerases—The major intracellular receptors for FK-506 and CsA. A number of proteins have been hypothesized to mediate the immunosuppressive activity of CsA. Cyclophilin, discovered by Handschumacher *et al.* [41] in 1984, is a ubiquitous and abundant cytosolic protein that is highly conserved among eukaryotic organisms [42] and is responsible for the uptake of CsA into the cell. Several observations have strengthened the hypothesis that cyclophilin is involved in the mechanism of action of CsA. First, the specificity of cyclophilin binding was examined with a limited set of cyclosporin analogs, and, within this narrow series, immunosuppressive activity was found to correlate with cyclophilin binding [43]. Second, cyclophilin was discovered recently to catalyze the *cis-trans* isomerization of peptidyl-proline bonds, and CsA was shown to inhibit this enzymatic activity [44, 45]. The discovery that the *Drosophila nina*A mutant, which is defective in the conversion of opsin to rhodopsin, has a mutation in a cyclophilin-like gene [46, 47] illustrates how this molecule may be involved in a signal transduction pathway.

The remarkable similarities between FK-506 and CsA carry over to the biochemical level, as summarized in Table 1. Siekierka *et al.* [48] first showed that a radiolabeled analog of FK-506 readily accumulates in the cytosol of the T-cell lymphoma line JURKAT in a temperature-dependent manner. The protein responsible for this accumulation is similar to cyclophilin in cytosolic location and abundance, but is slightly smaller in molecular weight (11–12 kD versus 18 kD for cyclophilin), is heat stable and does not bind CsA. Subsequently, both Siekierka *et al.* [49] and Harding *et al.* [50] purified this protein to homogeneity. Like cyclophilin, the FK-506 binding protein (FKBP) possesses peptidyl-prolyl *cis-trans* isomerase (PPIase) activity which is inhibited by its immunosuppressive ligand but not by CsA. Furthermore, FK-506 binds to FKBP with a K_d of 0.4 to 0.8 nM, as estimated from Scatchard analysis of direct binding or competition binding data [49]. The affinity measurements are consistent with the potency of FK-506 in cellular assays and support the notion that FKBP may mediate the immunosuppressive activity of this molecule.

More recently, FKBPs from human JURKAT

cells, bovine thymus and yeast were isolated and characterized.* The three proteins exhibited identical molecular weights and immunological cross-reactivity in Western blot analysis using an antiserum to bovine FKBP. N-Terminal amino acid sequence comparisons indicate that the human and bovine FKBPs differ by a single amino acid at position 49, while yeast FKBP exhibits 50% exact homology and 76% homology if conserved amino acid changes are considered. From the N-terminal amino acid sequences accumulated to date, there is no apparent sequence homology to cyclophilin or any other protein in the NBRF database. Furthermore, all three proteins have PPIase activity. Once again, these data mirror observations previously made with cyclophilin, which reveal it to be a phylogenetically highly conserved protein found in abundance in all cell types and tissues [42].

The finding that FKBP, like cyclophilin, possesses PPIase activity provides evidence that these enzymes may play a critical role in lymphocyte signal transduction. While the molecular mechanism that might involve such isomerases in lymphocyte activation remains to be discovered, the fact that both FKBP and cyclophilin are PPIases (inhibitable by their immunosuppressive ligands) must be viewed as more than a fortuitous coincidence. We and others have speculated that FKBP and cyclophilin catalyze a critical proline isomerization step(s) in components of the Ca^{2+}-associated signal transduction pathways described in the preceding section. In this model, the activity of signal transduction elements, such as transcriptional regulatory factors, protein kinases or ion channels, could be "conformationally" regulated by peptidyl-prolyl bond isomerization. FK-506 and CsA, as potent inhibitors of their respective PPIases, would selectively block events catalyzed by these proteins.

There are two issues that immediately arise regarding the hypothesized role of PPIases in signal transduction. The first is the paradox between the ubiquitous nature of these proteins and the relative selectivity of FK-506 and CsA for only certain cell types. Perhaps the tissue selectivity is due to the presence of cell-specific PPIase substrates in lymphocytes and related cells. The second question relates to why the cell requires at least two PPIases for protein folding, particularly since FKBP is only 1/20 as active as cyclophilin in catalyzing the *cis-trans* isomerization of the peptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. This may imply that FKBP and cyclophilin themselves may have unique substrate specificities that allow them to play distinct roles in the signal transduction process. Indeed, using a series of synthetic peptides of the general structure *N*-succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide, the two enzymes were found to have unique substrate specificities, with FKBP having a preference for hydrophobic residues at the substituted position [51]. When the preferred substrate is used, FKBP is similar to cyclophilin in enzymatic activity.

Does inhibition of PPIase mediate the immunosuppressive activity of FK-506 and CsA? The preceding section presents a series of correlative observations to suggest a critical role for FKBP and cyclophilin in the mechanism of action of FK-506 and CsA, but no biochemical or genetic data exist to provide a direct link. Thus, although it is clear that the proteins are the major cytosolic receptors for FK-506 and CsA and are responsible for the accumulation of the compounds within the cell, we have no formal proof of whether FKBP and cyclophilin are the mediators of the immunosuppressive activities of the drug. In particular, questions relating to the relationship between inhibition of PPIase and immunosuppression remain. Some of these issues have been addressed by experiments with rapamycin (Fig. 1), a macrolide antibiotic structurally related to FK-506.

Rapamycin was discovered over a decade ago, and, although first described as an anti-fungal agent, it was shown subsequently to have immunosuppressive activity *in vivo* [52]. Recently, Dumont *et al.* [8] discovered that while rapamycin inhibits T-cell proliferation with a potency similar to that of FK-506, this suppressive activity clearly differs from that exerted by FK-506 and CsA. Rapamycin appears to lack the selectivity for suppression of Ca^{2+}-associated signal transduction pathways in the lymphocyte and does not block accumulation of IL-2 mRNA induced by any mode of activation. The data suggested that rapamycin may inhibit lymphocyte proliferation through the disruption of the signals provided by lymphokine growth factors, such as IL-2. Moreover, using cellular readouts that were differentially sensitive to inhibition by FK-506 or rapamycin, Dumont *et al.* [53] also have shown that FK-506 and rapamycin act as reciprocal antagonists. It is noteworthy that rapamycin is unable to reverse the immunosuppressive activity of CsA. Evidence that rapamycin and FK-506 may share a common receptor first came from experiments in which rapamycin was shown to block the uptake of radiolabeled FK-506 into a murine T-cell line. This has been confirmed in binding studies with purified FKBP (Siekierka JJ, unpublished observations). Rapamycin interacted with FKBP with an affinity similar to that of FK-506 and was equipotent to FK-506 in blocking the PPIase activity of FKBP (but not cyclophilin) (Siekierka JJ, unpublished observations). These results strongly imply that inhibition of the enzymatic activity of FKBP does not directly result in suppression of the Ca^{2+}-associated signal transduction pathways and IL-2 gene expression that have been linked to the immunosuppressive effects of FK-506. Indeed, the evidence points to a much more subtle/complex role for these enzymes than previously hypothesized.

What alternatives to a central role for FKBP might be envisioned? First, other proteins, perhaps evolutionarily related to FKBP, may be involved in the mechanism of action of FK-506. A molecule genetically related to FKBP and perhaps expressed in a lymphocyte-specific fashion could account for the selectivity of FK-506 for signal transduction pathways in only certain cell types. A second hypothesis suggests that binding to FKBP and PPIase inhibition

* Siekierka JJ, Wiederrecht G, Greulich H, Boulton D, Hung SHY, Cryan J and Sigal NH, *J. Biol. Chem.*, in press.

are necessary but not sufficient for immunosuppression. Thus, FKBP, through its PPIase activity, may alter the conformation of FK-506 in such a way that it can now bind to a second "acceptor" molecule, which actually mediates the immunosuppressive effect of FK-506. For example, rapamycin may interact appropriately with FKBP but fail to bind to the acceptor protein critical for the IL-2 gene expression pathway. Third, FKBP, in addition to its role as a PPIase involved in protein folding, may be a subunit of a larger holoenzyme complex, the function of which is important in lymphocyte activation but only distantly related to its known PPIase activity. Disulfide isomerase, an enzyme important in protein folding, has been documented to function as a subunit in enzymatic activities unrelated to its "housekeeping" role [54].

Concluding observations

Since its discovery in 1985, information concerning the mechanism of action of FK-506 at the cellular, molecular and biochemical levels has grown enormously. FK-506 has been shown to be remarkably similar to CsA in its site of action with respect to its ability to inhibit lymphokine production at the level of transcriptional initiation and in its selectivity for a subset of signal transduction pathways that are associated with a rise in intracellular Ca^{2+}. In addition, FK-506 and CsA interact with distinct, abundant, highly-conserved, cytosolic proteins, both of which possess related PPIase activities. This flood of new information, unfortunately, has not been met, as yet, by an equivalent level of understanding of these observations. Indeed, in many respects, our current level of knowledge has raised many more questions than have been answered.

Among the questions to be addressed, the first relates to whether these major cytosolic proteins and their enzymatic activities are critically involved in mediating the immunosuppressive activity of the drugs. Such issues may be approached through genetic techniques, such as the generation of CsA-resistant yeast by Tropschug and coworkers [55], and through identification of physiologic substrates for FKBP and cyclophilin. Second, we must better understand what role these small proteins play in signal transduction. In this regard it will be important to identify the Ca^{2+}-associated events that are FK-506/CsA sensitive and establish a link between these biochemical processes and the enzymological observations defined to date. Finally, we must ask whether the toxicities observed in clinical and animal studies with CsA and FK-506 may be due to inhibition of PPIase-dependent pathways in other cell types. It is hoped that the answers to such questions will lead to a better understanding of lymphocyte signal transduction and to the development of more effective and safer immunosuppressive agents.

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