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Sanglifehrin A, a Novel Cyclophilin-Binding Immunosuppressant, Inhibits IL-2-Dependent T Cell Proliferation at the G1 Phase of the Cell Cycle

Ling-Hua Zhang* and Jun O. Liu†‡§

Sanglifehrin A (SFA) is a novel immunosuppressive natural product that binds to cyclophilin but is structurally distinct from cyclosporin A (CsA). We have investigated the cellular and molecular mechanisms of the action of SFA in T lymphocytes. We show that SFA inhibits T cell proliferation induced by IL-2 with an IC50 of 200 nM. Distinct from CsA, which also binds to cyclophilin, SFA does not affect calcium-dependent IL-2 production, although SFA enhanced IL-2 gene transcription in the same cells. SFA blocks T cell proliferation induced by IL-2 in G1, with no appreciable effect on IL-2 receptor expression in a manner similar to that of the immunosuppressant rapamycin. Unlike rapamycin, however, SFA has no effect on the phosphorylation or enzymatic activity of p70S6 kinase, distinguishing SFA from rapamycin in their mode of action. SFA inhibits hyperphosphorylation of Rb and the activity of cyclin E-cyclin-dependent kinase 2 on IL-2 signaling. These results suggest that SFA has a novel mode of action in comparison with CsA, FK506, and rapamycin, and that its use as a molecular probe may lead to the discovery of a novel target involved in T cell activation.


Immunosuppressive agents that bind immunophilins, cyclosporin A (CsA), FK506, and rapamycin, have not only revolutionized the field of organ transplantation (1–4) but also offered useful molecular tools for dissecting intracellular signal transduction pathways involved in T cell activation (5–12). CsA and FK506 bind to their respective immunophilin receptors, cyclophilin and FKBP (13–15). The resulting complexes bind to and inhibit the phosphatase activity of a common target, the calcium-dependent protein phosphatase calcineurin (16–21). Although FK506 and rapamycin share the same immunophilin receptor, i.e., FKBP, the FKBP-FK506 and FKBP-rapamycin complexes bind to distinct cellular targets and interfere with T cell activation at distinct sites (22, 23). The FKBP-rapamycin complex binds to the phosphatidylinositol-3 kinase homologue FKBP-rapamycin-associated protein (FRAP)/RAFT1/mTOR, inhibiting growth factor-mediated cell proliferation at the G1 phase of the cell cycle (24–28). The distinct mechanisms of action of the FKBP-FK506 and FKBP-rapamycin complexes raised the possibility that there may exist cyclophilin ligands that block T cell activation with a mechanism distinct from that of CsA but similar to that of rapamycin.

Using a cyclophilin-binding assay, Sanglier, Fehr, and colleagues at Novartis isolated a family of structurally related immunosuppressive natural products from a fermentation broth of the actinomycete Streptomyces flavolues and named them sanglifehrins (29, 30). The chemical architecture of sanglifehrin A (SFA) is novel and distinct from that of CsA (Fig. 1), making it an attractive synthetic target (31–33). In mixed lymphocyte reactions, SFA is ~10-fold less potent than CsA. In contrast, SFA binds to cyclophilin A with an affinity that is 20-fold higher than that of CsA (29). The cyclophilin-SFA complex does not interact with calcineurin, indicating that SFA is likely to interfere with T cell activation at a site distinct from that of CsA.

To gain insight into the molecular mechanism of action of sanglifehrins, we investigated the effect of SFA on calcium-dependent cytokine production and IL-2-dependent T cell proliferation. We show that SFA has no effect on calcium-dependent IL-2 production, even though it stimulated IL-2 transcription. Rather, it inhibits IL-2-dependent T cell proliferation. Unlike rapamycin, the IL-2-dependent activation of p70S6 kinase (p70S6k) is not affected by SFA. We further demonstrate that SFA blocks cell cycle progression of T cells at G1-S transition. This is likely due to the inhibition of cyclin E-cyclin-dependent kinase (Cdk2) activity by SFA. Thus, the immunosuppressive activity of SFA can be attributed to its ability to inhibit G1 cell cycle kinetics. SFA appears to be a novel member of the immunophilin-binding ligand family that possesses a distinct mechanism of action from CsA, FK506, and rapamycin.

Materials and Methods

**Materials**

SFA was provided by Dr. Richard Sedrani from Novartis Pharma (Basel, Switzerland). PMA, ionomycin, RNase A (type III-A), ATP, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]Thymidine, [γ-32P]ATP, and ECL reagents were from NEN Life Science Products (Boston, MA). Recombinant mouse IL-2 (rIL-2, 2×10^5 U/ml) was purchased from PharMingen (San Diego, CA). Purified polyclonal rabbit anti-p70S6k Ab, anti-cyclin E, anti-Cdk2, anti-actin Abs, protein G/A-Sepharose, p70S6k substrate peptide, and protein kinase C inhibitor peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific IL-2 primers were purchased from Clontech Laboratories (Palo Alto, CA); the Titan One Tube RT-PCR System was from Roche Molecular Biochemicals (Indianapolis, IN). The IL-2 ELISA kit was obtained from Genzyme Diagnostics (Cambridge, MA). The NF-κB, NFAF, and IL-2 luciferase reporter genes were obtained from Dr. Michael Karin (34).
Sanglieförnin A (SFA)

**FIGURE 1.** Chemical structure of SFA.

**Cell lines**

The IL-2-dependent T cell lines, CTLL-2 and HT-2, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and kindly provided by Dr. Herman Eisen (Massachusetts Institute of Technology Center for Cancer Research, Cambridge, MA), respectively. Both cell lines were maintained in RPMI 1640 supplemented with rmIL-2 (50 U/ml), 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 6 mM HEPES. Jurkat T cells, also from ATCC, were maintained in complete RPMI 1640.

**Cell proliferation assays**

Lymphocyte proliferative assays were conducted as previously described with slight modifications (35). Briefly, cells of midlog phase were plated in 96-well microtiter plates at 2 × 10⁴/well in the presence or absence of 50 U/ml rmIL-2 and indicated reagents and incubated at 37°C in 5% CO₂ for 18 h. [³H]Thymidine (1 μCi/well) was added, and incubation was continued for an additional 6 h. Cells were harvested onto glass fiber mats for scintillation counting. IC₅₀ was defined as the drug concentration required to inhibit proliferative responses of cells by 50%, and calculated from linear regression analysis of plotted values.

**Cell cycle analysis**

CTLL-2 cells were incubated in the presence or absence of SFA. Cells were pelleted at 200 × g, fixed in 1 ml cold 70% ethanol in PBS at 4°C for 1 h, and washed twice with PBS. RNAse (100 μl; 1 mg/ml in PBS), 20 μl propidium iodide (2.5 mg/ml in PBS), and 0.88 ml PBS were added to the pellet cells, and the cell suspensions were incubated in the dark for 15 min and kept at 4°C until FACS analyses. The propidium iodide fluorescence of individual nuclei was determined using a Becton Dickinson FACScan with an excitation wavelength of 488 nm and an emission wavelength at 675 nm. Cell cycle distribution was analyzed with CellQuest version 3.1 acquisition software and the ModFit LT version 2.0 program.

**RT-PCR**

The Titan One Tube RT-PCR System (Boehringer, Indianapolis, IN) was used to detect IL-2 mRNA. Total RNA (1 μg) from control and drug-treated cells was reverse transcribed to synthesize the cDNA strand. Amplification was performed using upstream and downstream primers specific for human IL-2 or β-actin. The sequences of the primers are: 5'-CATTG CACTAAGTTCTGACCTTGTCA-3' (IL-2, 5'-primer); 5'-CTGTTGATAT TGCTGATAAGTCCCTG-3' (IL-2, 3'-primer); 5'-GTGGGGCGCTCT AGGCACCAA-3' (β-actin, 5'-primer); 5'-CTCTTGTGATGCAGCG AATTTC-3' (β-actin, 3'-primer). PCR conditions were 94°C for 30 s (denaturation), 55°C for 30 s (annealing), and 68°C for 45 s (elongation), for a total of 25 cycles. Earlier studies had shown a good correlation between template input and intensity of amplified fragments. Control reactions included total RNA without reverse transcriptase or without template. PCR products were analyzed using 1.8% agarose and visualized by staining with ethidium bromide.

**Western blot analysis**

CTLL-2 cells were treated with test agents and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 10 mg/ml leupeptin, and 10 mg/ml aprotonin. The amount of protein in each sample was determined by Bradford assay. Equal amounts of lysates were subjected to SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride membrane. After blocking with 5% nonfat milk in PBS-0.1% Tween 20 at room temperature for 1 h, the membrane was probed with Abs. PhosphoPlus p70(S6k) (Thr389, Thr421), Ser252, Ab, and Rb Abs were used to probe the phosphorylation states of p70(S6k) and Rb, according to the manufacturer’s instructions (New England Biolabs, Beverly, MA).

**Immunoprecipitation and kinase assay**

CTLL-2 cells were harvested from complete medium, washed with PBS, resuspended, and cultured in basal medium (~ IL-2). SFA or control solutions were added for an indicated time in the presence or absence of IL-2 (50 U/ml). Cells were harvested and lysed, and debris was removed by centrifugation. Each sample containing 250 μg proteins was incubated with 4 μg of Abs specific for p70(S6k), cyclin E, or Cdk2 at a shaker at 4°C for 4 h, followed by centrifugation of 30 μl protein GA-Sepharose. The mixtures were incubated for an additional hour. The protein GA-Sepharose beads were separated by centrifugation, washed with ice-cold lysis buffer with and without cold kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂). For p70(S6k) kinase activity, pellets were mixed with 30 μl kinase buffer containing 25 μM p70(S6k) substrate, 500 nM concentrations each of protein kinase A and protein kinase C inhibitor peptides, 50 μM ATP, and 5 μCi [γ-³²P]ATP (specific activity, 3000 Ci/mmol) on ice. The samples were incubated at 30°C for 15 min and transferred to ice before 30 μl 10% TCA were added. Samples were centrifuged at 13,000 rpm at 4°C for 10 min, washed twice with 0.1% acetic acid and transferred to scintillation vials for counting in a Beckman LS1801 counter (Beckman Instruments, Fullerton, CA). For Cdk activity, immunoprecipitates were mixed in 50 μl ice-cold kinase buffer supplemented with 2 μg histone HI, 50 μM ATP, and 5 μCi [γ-³²P]ATP. The reaction mixtures were incubated at 30°C for 15 min and quenched by boiling in SDS-PAGE sample loading buffer. Samples were resolved by 12% SDS-PAGE. The gel was dried and analyzed by autoradiography.

**Results**

Effects of SFA on IL-2 production and IL-2 promoter activation

SFA has been reported to inhibit mixed lymphocyte reactions without affecting the activation of an IL-2 reporter gene (29). We were therefore surprised to observe that SFA stimulated the IL-2 reporter gene in response to stimulation by PMA and ionomycin. We therefore examined the level of IL-2 message stimulated by PMA and ionomycin in Jurkat T cells (Fig. 2A). We subsequently examined the level of IL-2 message stimulated by PMA and ionomycin in the absence or presence of increasing concentrations of SFA (Fig. 2B). A dose-dependent enhancement of IL-2 mRNA accumulation was observed. When the levels of secreted IL-2 in the supernatant were determined, SFA had little effect on the amounts of IL-2 protein produced (Fig. 2C). The disparity between the IL-2 mRNA and IL-2 protein levels on treatment with SFA suggests that SFA has a negative effect on IL-2 production. To determine whether SFA has an effect on the secretion of IL-2 protein along the secretory pathway, intracellular IL-2 levels were
measured in the presence and absence of SFA by in situ immunostaining of IL-2 protein followed by FACS analysis. No additional IL-2 was found to accumulate intracellularly on treatment with SFA (data not shown).

To further examine the enhancement of SFA on IL-2 transcription as judged by both the IL-2 reporter gene and IL-2 mRNA, we determined the effect of SFA on the activation of NFAT and NF-κB, two major transcription factors responsible for the activation of the IL-2 promoter. Although SFA alone had little effect on the NFAT reporter gene activity, it significantly stimulated the NF-κB reporter gene activation (Fig. 2A). This stimulation was seen whether cells were further stimulated with PMA or ionomycin. Despite the unique effect of SFA on NF-κB, the normal level of IL-2 production ruled out the possibility that its immunosuppressive activity arose directly from its effect on IL-2.

**SFA inhibits the proliferation of T cells in response to IL-2 stimulation**

Full activation of T cells requires two successive signaling pathways, one mediated by the TCR leading to production of IL-2 and expression of CD25 that forms part of the high affinity IL-2 receptor. Because SFA did not affect IL-2 production, we examined whether it had any effect on IL-2 receptor-mediated T cell proliferation. For this study, we used the murine CTL line CTLL-2 as a model system. CTLL-2 exhibits strict dependence on exogenous IL-2 for maintenance of viability and continuous proliferation in culture. Treatment of CTLL-2 cells with SFA led to a dose-dependent inhibition of cell proliferation as measured by DNA synthesis, with an IC₅₀ of 200 nM (Fig. 3A). In comparison, rapamycin is much more potent than SFA in inhibiting T cell proliferation, with an IC₅₀ of ~5 nM in the same assay (data not shown). The inhibition of CTLL-2 proliferation by SFA was not due to cytotoxicity as the cells remained viable at the highest concentration of SFA used, as determined by trypan blue exclusion (Fig. 3A). At 1000 nM, SFA reduced IL-2-stimulated [³H]thymidine incorporation by nearly 70% over the entire range of IL-2 concentrations tested (Fig. 3B). To determine whether SFA inhibited an early or late step relative to the activation of IL-2 receptor, CTLL-2 cells were exposed to SFA (500 nM) at various times after addition of IL-2. The IL-2-dependent DNA synthesis remained sensitive to SFA for at least 12 h after the initial stimulation with IL-2 (Fig. 3C). This result suggested that SFA interfered with an event that was relatively distal to the activation of IL-2 receptor signaling. Similar results were also obtained for the IL-2-dependent T cell line HT-2 (data not shown).

**SFA affects a distinct pathway from rapamycin**

Given that SFA inhibited IL-2-dependent T cell proliferation, we examined whether this inhibition is mediated through some known components of the IL-2 receptor signaling pathway. We began by determining whether SFA affected surface expression of IL-2Rα, -β, or -γ chains, the down regulation of which might account for the inhibition by SFA. FACS analysis for IL-2Rα, -β, and -γ chains revealed no change in the expression level of IL-2Rα on treatment of CTLL-2 cells with SFA (data not shown), ruling out the possibility that SFA blocks IL-2 signaling by inhibiting expression of the IL-2 receptor.

Next, we investigated whether SFA affects some known downstream mediators of the IL-2R signaling pathway. The molecular target for the FKBP-rapamycin complex was first identified in yeast as two related proteins containing phosphatidylinositol-3 kinase homology domains, TOR1 and TOR2 (24, 37–39). The corresponding mammalian homologues were subsequently identified and named RAFT1/FRAP/mTOR (25–28). One of the downstream targets of RAFT1/FRAP/mTOR was identified as p70s6k, a mitogen-activated serine/threonine protein kinase that is required for cell growth and G₁ cell cycle progression (40–43). Treatment of T cells and other rapamycin-sensitive cell types leads to inhibition of
phosphorylation and activation of p70\textsuperscript{s6k}. We determined p70\textsuperscript{s6k} activity in CTLL-2 cells in response to IL-2 stimulation in the presence and absence of SFA. As shown in Fig. 4A, treatment of CTLL-2 cells with IL-2 led to activation of p70\textsuperscript{s6k} activity as early as 30 min after stimulation. As expected, the activation and phosphorylation of p70\textsuperscript{s6k} is potently inhibited by rapamycin. In contrast, SFA had no effect on p70\textsuperscript{s6k} activation in response to IL-2 stimulation for either 30 or 180 min in CTLL-2 cells at the highest dose used (1000 nM; Fig. 4A). SFA also failed to inhibit p70\textsuperscript{s6k} phosphorylation in CTLL-2 cells in response to IL-2 for up to 12 h (Fig. 4B). Thus, inhibition of IL-2-dependent T cell proliferation by SFA is mediated by a different pathway from that affected by rapamycin.

SFA blocks T proliferation at the G\textsubscript{1} phase of the cell cycle

Because SFA inhibited IL-2-dependent T cell proliferation, we determined whether this inhibition impinges on a specific phase of the cell cycle. Thus, CTLL-2 cells were synchronized by incubation in basal medium containing no IL-2 for 14 h. As a result of CTLL-2 cells with IL-2 led to activation of p70\textsuperscript{s6k} activity as early as 30 min after stimulation. As expected, the activation and phosphorylation of p70\textsuperscript{s6k} is potently inhibited by rapamycin. In contrast, SFA had no effect on p70\textsuperscript{s6k} activation in response to IL-2 stimulation for either 30 or 180 min in CTLL-2 cells at the highest dose used (1000 nM; Fig. 4A). SFA also failed to inhibit p70\textsuperscript{s6k} phosphorylation in CTLL-2 cells in response to IL-2 for up to 12 h (Fig. 4B). Thus, inhibition of IL-2-dependent T cell proliferation by SFA is mediated by a different pathway from that affected by rapamycin.

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FIGURE 5. Effect of SFA on cell cycle progression of T cells. A. CTLL-2 cells were cultured in basal medium in the absence of IL-2 for 14 h. The cells were exposed to the indicated stimuli, and cell cycle distributions were determined. a. Control cells cultured in basal medium for 14 h in the absence of IL-2; b, c, and d. The IL-2-deprived cells were restimulated for 14 h with 50 U/ml IL-2 in the presence of DMSO, SFA (1 µM), or rapamycin (10 nM). B. Effect of SFA on IL-2-dependent cell cycle progression. The IL-2-deprived CTLL-2 cells were restimulated with 50 U/ml IL-2 in the presence or absence of SFA (1 µM) for indicated amounts of time and then collected for cell cycle analysis.
IL-2 deprivation, >90% of cells are accumulated in G1 at the end of the incubation (Fig. 5A, a). The cells were subsequently stimulated with IL-2 for 12 h in the presence or absence of 1 μM SFA. Stimulation with IL-2 led to a synchronous entry into the S phase and a concomitant decrease in cells in G1 to ~27% (Fig. 5A, b). Treatment of cells with SFA, however, significantly blocked the S phase entry with ~78% of cells remaining in G1 (Fig. 5A, c). As a control, rapamycin also inhibited S phase entry of IL-2-deprived cells after restimulation with IL-2 (Fig. 5A, Panel d). As shown in Fig. 5B, the IL-2-dependent CTLT-2 cells required 11–12 h to traverse the G1 phase after restimulation with IL-2. The cell cycle blocking effect of SFA was most pronounced at 15 h when the cell cycle was followed over time.

**SFA inhibits hyperphosphorylation of Rb and cyclin E-Cdk2 activity**

One of the common events in G1-S transition is the phosphorylation of Rb by Cdkks. Starvation of CTLT-2 cells of IL-2 led to an accumulation of hypophosphorylated form of Rb as judged by its gel mobility shift. Restimulation of arrested CTLT-2 cells by IL-2 led to hyperphosphorylation of Rb (Fig. 6). Treatment of cells with SFA inhibited Rb hyperphosphorylation in response to IL-2. We then examined whether SFA affected cyclin E-Cdk2 and cyclin D-Cdk4/6 activity, which are known to be responsible for Rb phosphorylation in G1. Although cyclin D-Cdk4/6 activity was not affected, that of cyclin E-Cdk2, as measured by phosphorylation of histone H1, was inhibited by SFA in a dose-dependent manner (Fig. 7A). To gain insight into the upstream regulatory steps that may be affected by SFA, we performed a Western blot to determine the levels of cyclin E and Cdk2 in the presence of SFA. The expression of cyclin E and Cdk2 was not affected by SFA (Fig. 7B), indicating that the inhibition of cyclin E-Cdk2 activity by SFA did not result from a decrease in expression of either cyclin E or Cdk2. In addition, we have also examined the effect of SFA on the level of p27, which has been previously shown to be stabilized by rapamycin (44). No change in p27 concentration was seen when CTLT-2 cells were treated with SFA (data not shown), further distinguishing SFA from rapamycin in their modes of action.

**Discussion**

The immunophilin ligands, CsA, FK506, and rapamycin, have found widespread use in the clinic as immunosuppressive drugs. Their potential in treating immune disorders other than graft rejection, however, is limited in large part by their side effects including nephrotoxicity and neurotoxicity (45). Despite recent developments of new immunosuppressants, there remains an unmet need for less toxic and more widely applicable immunosuppressive agents. As new members of the immunophilin-binding ligand family, sanglifehrins have the potential of becoming useful immunosuppressants.

**FIGURE 6.** Effect of SFA on phosphorylation of Rb protein. IL-2-deprived CTLT-2 cells were restimulated with 50 U/ml IL-2 in the presence or absence of SFA for 14 h. Detergent-soluble cellular extracts (20 μg protein) were prepared, subjected to SDS-PAGE, and immunoblotted with anti-Rb Abs.

**FIGURE 7.** Effect of SFA on cyclin E-dependent kinase activity and on expression of cyclin E and Cdk2. A, Detergent-soluble cellular extracts (250 μg) were used for immunoprecipitation with anti-cyclin E Abs. Immunoprecipitates were assayed for associated histone H1 kinase activity with [γ-32P]ATP as a cosubstrate. B, IL-2-deprived CTLT-2 cells were restimulated with 50 U/ml IL-2 in the presence or absence of SFA for the indicated amounts of time. Detergent-soluble cellular extracts (20 μg protein) were prepared and immunoblotted with anti-cyclin E, anti-Cdk2 or anti-actin Abs.

CsA, FK506, and rapamycin belong to a unique family of immunosuppressive drugs the biological activities of which are manifested through their recruitment of abundant cytosolic immunophilin receptors, forming ligand-protein complexes that inhibit the ultimate targets. Like CsA, SFA binds cyclophilin. It is tempting to speculate that SFA may have a mode of action similar to that of CsA; the cyclophilin-SFA complex may target a different protein, in that it does not affect calcineurin (29). It remains to be seen, however, whether the immunosuppressive activity is mediated by or independent of cyclophilin binding.

We have shown that SFA has no effect on IL-2 production in response to TCR agonists such as PMA and ionomycin, suggesting that the immunosuppressive activity of SFA is mediated through a distinct target from that of CsA (Fig. 2C). In addition to IL-2 secretion, we also examined the effect of SFA on IL-2 transcription. Surprisingly, SFA was found to stimulate accumulation of the IL-2 mRNA in a dose-dependent manner, suggesting that SFA may either stimulate IL-2 transcription or stabilize the IL-2 mRNA (Fig. 2B). The fact that SFA stimulates the IL-2 reporter gene suggests that the observed accumulation of IL-2 mRNA is due mainly to the enhanced transcription of IL-2 by SFA. These observations may be explained by the high affinity of SFA for cyclophilin and the lack of effect of the cyclophilin-SFA complex on calcineurin. Cyclophilin is a highly abundant and basic protein. It is capable of interacting with calcineurin with low affinity in the absence of CsA both in vitro and in vivo in yeast (46). Thus, endogenous cyclophilin serves as a low affinity inhibitor of calcineurin. Binding of SFA to endogenous cyclophilin is likely to relieve this inhibition, leading to higher calcineurin activity in the presence of SFA. The resulted increase in calcineurin activity may be responsible for the enhanced transcription of IL-2. Consistent with this idea, SFA was found to enhance the reporter gene activity of NFAT and NF-κB, both of which are dependent on calcineurin activation. SFA alone was capable of activating NF-κB reporter.
but not that of NFAT (Fig. 2A), which may also contribute to the elevated activation of IL-2 transcription.

It is somewhat paradoxical that SFA enhances IL-2 transcription without affecting IL-2 protein secretion. This paradox may be explained by the binding of SFA to cyclophilin as well. It has been shown that the cyclophilin homologues in Drosophila, NinaA, is involved in the secretion of rhodopsin (47). It has also been reported that cyclophilin B is present in the ER and appears to play a role in the secretory pathway as a protein chaperone (48, 49). It is thus possible that IL-2 secretion and folding in the ER may require the proline isomerase activity of cyclophilin B. Inhibition of cyclophilin B by SFA may thus have an inhibitory effect on IL-2 secretion. Alternatively, it is possible that SFA has an inhibitory effect on IL-2 translation or IL-2 protein stability before or during its transport through the secretory pathway. To distinguish between these two possibilities, we determined the intracellular level of IL-2 upon stimulation of T cells by PMA and ionomycin in the presence of SFA. There was no change of intracellular IL-2 level in the presence of SFA, ruling out the possibility that SFA blocks IL-2 trafficking as a consequence of its association with cyclophilin B. This result also suggests that SFA may inhibit IL-2 mRNA translation.

Having ruled out the possibility that SFA inhibits IL-2 production, we turned to the IL-2-dependent T cell proliferation. Using the CTL-2 cell as a model system, we found that IL-2-dependent CTL-2 proliferation is significantly inhibited by SFA in a dose- and time-dependent manner. This result placed the site of action of SFA within the same stage of T cell activation as that for rapamycin. We thus examined the effect of SFA on the activity of p70S6K, a mitogen-activated protein kinase which is inhibited by rapamycin and is known to play an important role in G1-S transition (50). Unlike rapamycin, however, SFA had no effect on p70S6K kinase activity in CTL-2 cells. Thus, the target for SFA either lies downstream from RPT1/FK506/mTOR or is part of a pathway that is distinct from that for RPT1/FK506/mTOR.

Similar to rapamycin, SFA also blocks IL-2-dependent cell cycle progression at the G1-S transition as evidenced by the accumulation of cells in G1 in the presence of SFA. Among the events involved in controlling G1 progression, we found that SFA inhibited the hyperphosphorylation of Rb by cyclin E-Cdk2. When tested in vitro, SFA had no effect on cyclin E-Cdk2 activity, suggesting that the effect of SFA on cyclin E-Cdk2 is indirect and that the target for SFA lies upstream of cyclin E-Cdk2. The identification of the direct target for SFA may throw new light on the regulation of cell cycle progression in T cells.

**Note Added in Proof.** A manuscript describing SFA and its biochemical characterization was submitted along with this paper and will appear soon (51).

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**References**


