Chemistry & Biology, Vol. 9, 49–61, January, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00085-6

Synthesis of Calcineurin-Resistant Derivatives of FK506 and Selection of Compensatory Receptors

Paul A. Clemons,[1,2] Brian G. Gladstone,[1,2] Abhinav Seth,[1,3] Elizabeth D. Chao,[1,3] Michael A. Foley,[4] and Stuart L. Schreiber[1,3,4,5]

[1] Howard Hughes Medical Institute at Harvard University

[2] Department of Molecular and Cellular Biology
Harvard University
7 Divinity Avenue
Cambridge, Massachusetts 02138

[3] Department of Chemistry and Chemical Biology
Harvard University
12 Oxford Street
Cambridge, Massachusetts 02138

[4] Institute of Chemistry and Cell Biology
Harvard Medical School
250 Longwood Avenue
Boston, Massachusetts 02115

Summary

We used olefin metathesis to synthesize C40 derivatives of FK506 and measured their ability, when complexed to FKBP12, to inhibit calcineurin's phosphatase activity. We identified modular dimerization domains (CABs) containing segments of the calcineurin A and B polypeptides. These CABs respond to FK506 both when overexpressed in mammalian cells and in yeast or mammalian three-hybrid assays. Using chemical genetic selection, we identified compensatory mutant CABs that respond to a calcineurin-resistant FK506 derivative at concentrations well below the response threshold for CABs containing only wild-type calcineurin sequence. These reagents provide a small molecule-protein combination orthogonal to existing dimerizer systems and may be used with existing systems to increase the complexity of induced-proximity experiments. This new use of the "bump-hole" strategy protects target cells from complications arising from the inhibition of endogenous calcineurin.

Introduction

Collectively termed *dimerizers* or chemical inducers of dimerization (CIDs) [1], small molecules that induce proximal relationships ("dimerization") between proteins include both semi-synthetic derivatives of natural agents [2–6] and small molecules prepared by total organic synthesis [7–9]. The rationale for the use of dimerizer systems to control signal transduction comes from the fact that they exploit a known *regulatory bias* of certain families of proteins. Namely, this bias is that protein function is controlled by regulated protein localization in vivo [1, 10]. The most widely used dimerization systems are based on natural and synthetic small molecules that bind to the 12 kDa FK506 binding protein (FKBP12) [2, 8, 11], and their use has become widespread in biological research. Early work in the preparation of homodimerizers focused on semisynthetic dimers of FK506 (collectively termed FK1012s) [2, 11]. Such homodimerizers are potent probes for certain systems but suffer (theoretically) from the formation of nonproductive interactions with endogenous FKBPs. Moreover, at high concentrations, these dimerizers may interfere directly with the cellular functions of even highly expressed endogenous proteins [12, 13]. Structure-based efforts to engineer fully synthetic FKBP12 ligands have met with much success [7, 14]. In general, using synthetic small molecules makes more tractable the problem of engineering small molecule-protein combinations that do not interfere with the cellular functions of endogenous FKBP12 [15–17]. In particular, α-keto-pipecolyl-amide derivatives [7] that selectively bind to a mutant allele (F36V) of FKBP12 have been engineered [9, 14]. Such FKBP12 "bump-hole" systems are extremely valuable models for molecular design and the general study of small molecule-protein interactions [18]. Indeed, this approach, originally developed in the context of exploring protein phosphatase function [19, 20], has recently been extended to the study of protein kinases [21, 22].

Dimerizers are also important tools in cell signaling research, particularly in cases where proximity effects are thought to dominate the regulatory logic of a given protein. In general, dimerization domains (e.g., FKBP12) are fused to a protein of interest to produce a conditional allele [2, 23], making some protein function dependent on the presence of a dimerizer. Used in this manner, dimerizer systems form a branch of reverse chemical genetics [24] (targeted modulation of protein function followed by a broad search for the resulting phenotype) and have been used directly to control membrane receptors [25], protein kinases [26, 27], death domains [3, 28], and transcription factors [29]. For a complete list of dimerizer references, see http://www.ariad.com/regulationkits/reg_ref1.html.

FK506 [2, 11], α-keto-pipecolyl-amides [8, 9], cyclosporin [3], and coumermycin [5] have been converted into homodimerizers, which are well-suited for circumstances in which the homodimerization of a single protein is sufficient to initiate a biological response. In a simple instance of a homodimerizer system, FK1012 was used to homodimerize the Bcl family member Bax [28]. In this case, fusion of Bax to FKBP12 yielded a single construct system that responds to FK1012 by forming a tripartite signaling complex (Figure 1A). In a slightly more complicated model, exemplified by the T cell receptor ζ chain [2, 30], tandem FKBP12 modules were appended to the receptor kinase domain. In this case, addition of the dimerizer promotes aggregation of the fusion protein (Figure 1B) and mimics the situation of antigen presentation and receptor capping in vivo. Homodimerizers have also been used in heterologous systems, in which two different proteins or domains are each fused separately to FKBP12 and then induced to

[5] Correspondence: sls@slsiris.harvard.edu

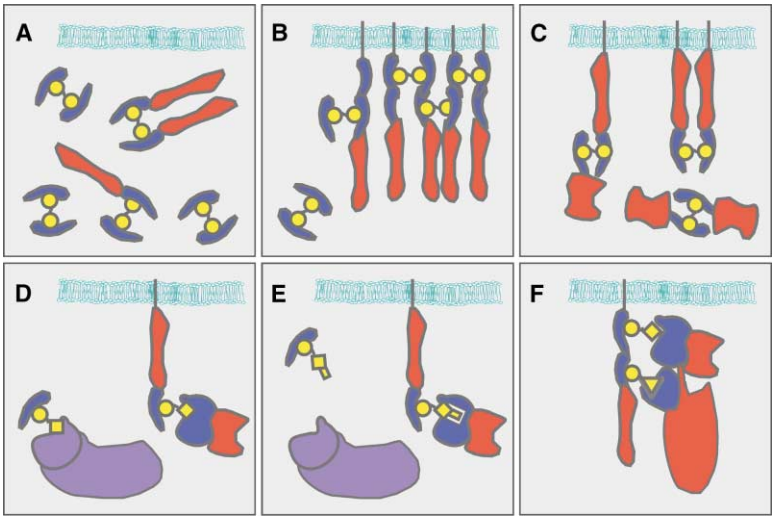

Figure 1. Conceptual Uses of Dimerizers to Induce Protein Proximity

Schematic representations include CIDs (yellow), dimerization domains (blue), effector domains (red), and endogenous signaling proteins (purple).

(A) Homodimerization for which a single-construct system consisting of an effector domain fused to a single dimerization domain was used. The panel illustrates unwanted interactions with endogenous dimerization domains (e.g., FKBP12).

(B) Oligomerization for which a single-construct system consisting of an effector domain fused to multiple dimerization domains was used. The panel illustrates unwanted interactions with endogenous dimerization domains.

(C) Dimerization of heterologous constructs containing the same dimerization domain, but different effector domains. The panel illustrates the formation of additional undesired complexes between engineered proteins.

(D) Heterodimerization of heterologous constructs containing different dimerization domains and different effector domains. The panel illustrates unwanted interaction with a CID-sensitive endogenous signaling protein (e.g., FRAP or calcineurin).

(E) Heterodimerization of heterologous constructs. A "bump-hole" strategy was used to protect endogenous signaling proteins.

(F) Formation of a multicomponent complex from two hemi-orthogonal heterodimerizers (e.g., rapamycin and FK506).

associate in a cellular context [2, 11]. An obvious problem with this approach is that it allows the formation of three different protein complexes (Figure 1C), *excluding* those containing endogenous FKBP12 (in the case of non-"bump-hole" systems) and higher-order complexes resulting from the use of tandem FKBP12 modules, as described above. For this reason, among others, heterodimerizers have been developed to effect the specific formation of the heterodimeric complex of interest [4, 6].

Early efforts to prepare heterodimerizers included the synthesis of FK-CsA, a semi-synthetic heterodimer of FK506 and cyclosporin A (CsA) [4]. More recently, heterodimerizer work has focused on dexamethasone-methotrexate [4, 6] and on the macrolide natural product, rapamycin. A natural heterodimerizer, rapamycin mediates the interaction of FKBP12 with the PIK-related kinase FRAP [31]. Conveniently, a small, stable domain of FRAP (residues 2025-2113), termed the FKBP12-rapamycin binding (FRB) domain [32], is competent to bind the FKBP12-rapamycin complex. However, the kinase activity of endogenous FRAP is potently modulated at relatively low concentrations of rapamycin [33], limiting the use of rapamycin itself as a dimerizer (Figure 1D). This limitation was circumvented by appending sterically bulky groups to rapamycin at the interface of its interaction with FRB. Specifically, C20-(*S*)-methallyl-rapamycin was shown to select for a mutant allele (termed FRB*; K2095P/T2098L/W2101F) over the "wild-type" FRB domain [34]. Thus, a FRAP-resistant rapamycin derivative, used in concert with both FKBP12 and FRB* fusion proteins, can elicit specific heterodimerization of two proteins (Figure 1E) without the side effect of endogenous FRAP inhibition.

Here we report the engineering of a novel heterodimerizer system based on FK506. Like rapamycin, FK506 is a natural dimerizer; it mediates the interaction of FKBP12 with the calcineurin A/B heterodimer [35]. As with FRAP [33], calcineurin activity is inhibited by the complex between FK506 and its immunophilin chaperone [35]. Preparation of the newly engineered dimerizer system proceeded in three stages: (1) synthesis and purification of calcineurin-resistant derivatives of FK506, (2) identification of a modular receptor for FKBP12-FK506 based on calcineurin protein sequence, and (3) chemical genetic selection of mutant proteins by the use of calcineurin-resistant derivatives of FK506 in a yeast three-hybrid assay. Because this new dimerizer system is hemi-orthogonal to that based on rapamycin (i.e., both compounds bind FKBP12), we envision that its future use in tandem with the rapamycin-based system will allow the specific and ordered assembly of multicomponent protein complexes ([36, 37]; Figure 1F).

Results

Preparation of Calcineurin-Resistant FK506 Derivatives by Olefin Metathesis

The major drawback of using FK506 (**1**) as a dimerizer to control cellular signal transduction is its natural biological activity. Calcineurin is a heterodimeric (A/B), calcium-dependent protein phosphatase necessary for the propagation of cytokine receptor signals in lymphocytes [38] and for synaptic vesicle recycling [39] and synaptic depotentiation in neurons [40]. The FKBP12-FK506 complex binds to calcineurin A/B and inhibits its phosphatase activity; the three-dimensional structure of this inhibitory quaternary complex has been determined at atomic resolution [41]. The terminal olefin (C39-C40) of FK506 protrudes into an interface between the calcineurin B binding helix (BBH) [41] of calcineurin A (CnA) and the third EF hand (a structural motif common to Ca^{2+} binding proteins [42]) of calcineurin B (CnB), making this an attractive moiety for derivatization (Figure 2A). Previous work to prepare FK1012s by olefin metathesis [11] revealed that trace amounts of C40-phenyl-FK506 (**3**) are produced as a by-product of the metathesis reaction (Figure 2B). This product is expected because ruthenium catalyst **2** is initially charged with benzylidene [43];

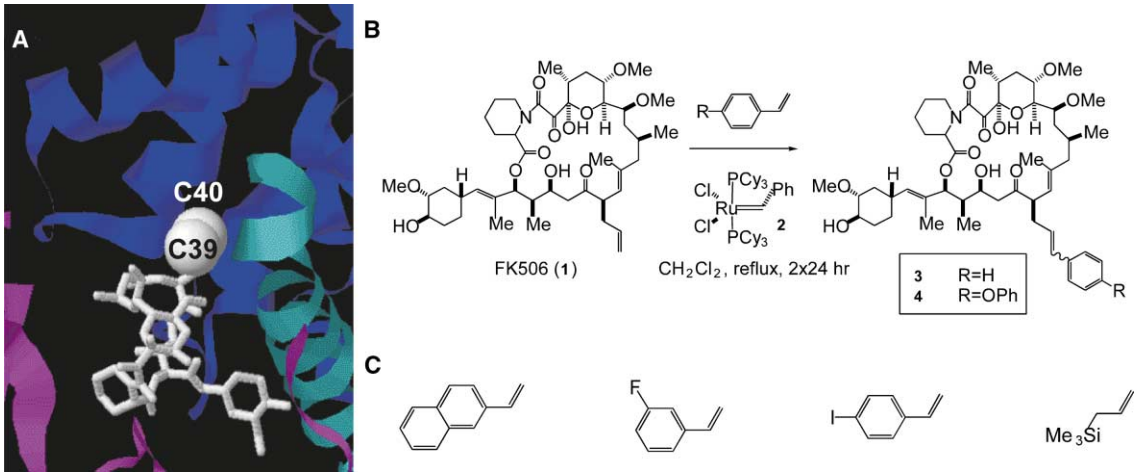

Figure 2. Derivatization of FK506 by Olefin Metathesis

(A) Close-up, based on crystallographic coordinates (Protein Databank: 1TCO), of the binding interface of FKBP12 (magenta), FK506 (white), CnA (green), and CnB (blue) [41]. The terminal olefin (C39-C40) of FK506 is shown in space-filling representation to illustrate its protrusion into the binding interface.

(B) Synthetic scheme for the olefin metathesis reaction used to prepare C40-phenyl-FK506 (**3**; C40-Ph) and C40-*p*-phenoxyphenyl-FK506 (**4**; C40-PhOPh).

(C) Additional olefin-containing compounds used to prepare C40-β-naphthyl-FK506 (C40-Nap), C40-*m*-fluorophenyl-FK506 (C40-FPh), C40-*p*-iodophenyl-FK506 (C40-IPh), C40-trimethylsilane (C40-TMS), by the same method. All compounds were tested against endogenous calcineurin (see Figure 3), after which only **3** and **4** were chosen for further study.

thus, the first catalytic cycle effectively results in the metathesis of styrene to FK506. We reasoned that the proportion of **3** to FK1012 could be increased by including additional styrene in the metathesis reaction, which indeed turned out to be the case. Using a similar strategy, we prepared C40-*p*-phenoxyphenyl-FK506 (**4**), and a series of additional C40-"bump" derivatives of FK506 by cross-metathesis of the appropriate olefin-containing starting material (Figure 2C).

The potency of **1** requires that any preparation of a calcineurin-resistant derivative be completely free of unreacted **1**. Thus, reaction optimization focused on the purity, rather than the yield, of C40 adducts. Because the efficiency of metatheses involving **1** is relatively poor [11], we used iterative rounds of separation by forward-phase (chloroform; $CHCl_3$) recycling HPLC to effect purification. In this method, eluent from the HPLC column is either reinjected onto the HPLC column or diverted to a fraction collector under operator control. Reinjection of eluate results in greater separation of peaks with each cycle of the instrument. Separation can be further augmented by "peak-shaving," in which overlapping shoulders from adjacent peaks are shunted to a waste fraction. We monitored the purification process in real-time by UV chart recording (254 nm). For both **3** and **4**, we determined the identity and purity of isolated product peaks by both FAB-MS and ^{1}H-NMR. For other derivatives, we isolated $<$1 mg of each compound, which we analyzed only for purity by FAB-MS (see Experimental Procedures), but which was nonetheless sufficient for preliminary biological assays.

To test the ability of C40 metathesis adducts to inhibit calcineurin function, we used a reporter gene assay that responds to **1** in a dose-dependent manner. The nuclear factor of activated T cells (NFAT) is a transcription factor whose nuclear localization is dependent on dephosphorylation by calcineurin [44]. Inhibition of calcineurin by FKBP12-FK506 prevents translocation of and transcriptional activation by NFAT. We transiently transfected Jurkat human lymphoma cells with a plasmid (NRE-AP) harboring a secreted alkaline phosphatase (AP) reporter gene downstream of twelve copies of an NFAT response element (NRE) [45]. In transfected cells, the combination of phorbol ester (PMA; phorbol-12-myristate-13-acetate) and ionomycin (Io) potently activates production of AP, as judged by incubation of supernatants with a fluorogenic substrate (MUP; 4-methylumbelliferylphosphate) [4, 34, 46]. We exposed transfected cells to varying concentrations of either **1** or one of the C40 derivatives after stimulation with PMA/Io. The results of this experiment (Figure 3) suggest that each derivative is less effective at inhibiting calcineurin activity than is **1**, as judged by the observed shifts in effective concentration for half-maximal effect (EC_{50}) on AP production. This result could be due to effects other than steric interference by C40 substituents; differences in solubility, cell permeability, or ability to bind FKBP12 could account for this observation. To control for these possibilities, we performed a cytoblot assay [47] in which **1**, **3**, or **4** was asked to suppress the ability of rapamycin to inhibit cell division by competing for binding to FKBP12 [48]. As both **3** and **4** (like **1**) were able to suppress the effects of rapamycin in this context (Brent R. Stockwell, P.A.C., and S.L.S., unpublished data), we are confident that both **3** and **4** are soluble, cell-permeable, and able to bind FKBP12.

Calcineurin Fusion Proteins and Dose-Dependent Transcriptional Activation

Active calcineurin phosphatase is composed of two polypeptides (CnA and CnB) [49], each of which contacts FKBP12-FK506 in the crystal structure of the quaternary complex (Figure 4A) [41]. To facilitate the use of

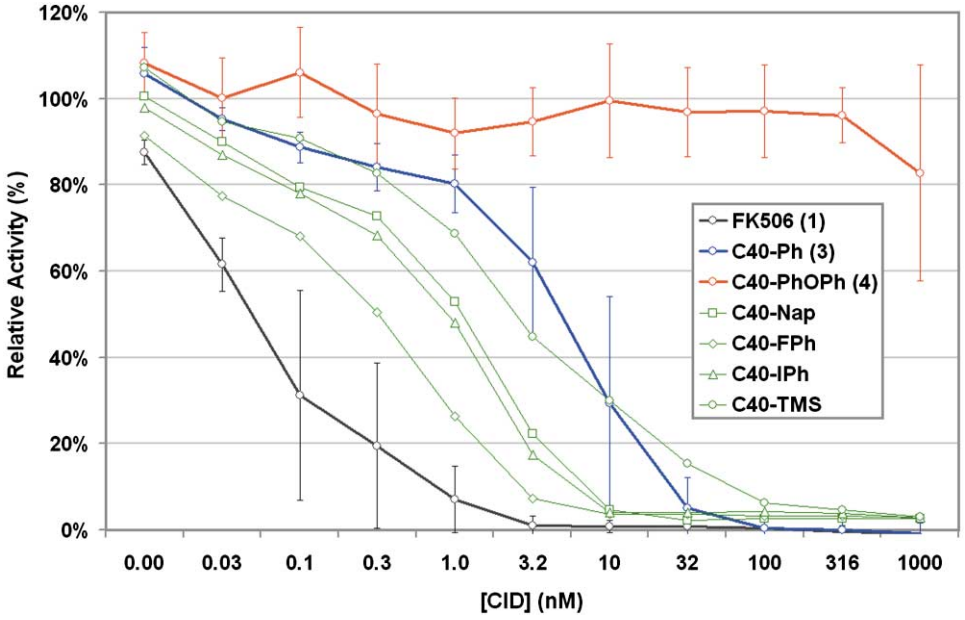

Figure 3. Effect of C40 Derivatization on Endogenous Calcineurin

Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells), stimulated with PMA/Io, and treated with serial dilutions of either FK506 (**1**) or the indicated C40 derivative. Abbreviations for CIDs are provided in the legend for Figure 2. Data are plotted as the average of either five [FK506 (**1**), C40-Ph (**3**), C40-PhOPh (**4**), C40-Nap] or three [C40-FPh, C40-IPh, C40-TMS] independent experiments, and error bars (where present) indicate the standard deviation of the mean.

calcineurin-derived modules for dimerizer research, we sought to prepare stable fusion proteins that were both competent to bind FKBP12-FK506 and expressed as a single polypeptide chain. The success of this approach owes to the fact that the C terminus of truncated CnA (residues 12–394) is close in space to the N terminus of CnB [41]. Guided by this observation, we prepared constructs encoding two single-chain fusion proteins, termed CABs for calcineurin A/B fusion proteins (Figure 4B). One construct includes both the phosphatase domain and BBH region [41] (residues 12–394) of CnA and is thus termed a functional CAB (fCAB; Figure 4C). A shorter construct consists of only the BBH region [41] of CnA (residues 340–394) fused to CnB; this construct encodes a relatively small polypeptide (230 amino acids) amenable for use as a modular CAB domain (mCAB; Figure 4D).

To test the ablility of fCAB to interact with FKBP12-FK506 in vivo, we exposed stimulated (PMA/Io) Jurkat cells cotransfected with NRE-AP and fCAB to varying concentrations of **1**. Not surprisingly, overexpression of fCAB (which contains the CnA phosphatase domain) completely suppressed the ability of **1** to inhibit AP production (Figure 5A). Because fCAB lacks both the calmodulin binding domain and the autoinhibitory domain (AID) [49, 50] of CnA, it is reasonable to expect that this construct would act as a dominant positive allele in the AP reporter assay. Specifically, fCAB exhibits deregulated phosphatase activity that, unlike endogenous calcineurin [49, 51], is independent of intracellular calcium concentration. Experimentally, this allowed us to distinguish reporter production due to transfected fCAB from that due to endogenous calcineurin. By omitting ionomycin from the assay, we deprived endogenous calcineurin of a stimulus necessary to activate NRE-AP. In this context, fCAB stimulates AP production in rough proportion to transfected DNA concentration, and this activation is sensitive both to **1** (Figure 5B) and **3** (Figure 5C). Thus, the 65 kDa fCAB protein (Figure 5D) retains calcineurin phosphatase activity and, more importantly, is sensitive to inhibition by FK506. Moreover, the EC_{50} relationship between **1** and **3**, established with endogenous calcineurin, is preserved in these experiments, suggesting that the C40 substituent of **3** is indeed responsible for reduced interaction with fCAB in a cellular context.

Because mCAB lacks phosphatase activity, we required another test to validate the ability of mCAB to interact in vivo with FKBP12-FK506. Following the lead of previous work on rapamycin [34, 52], we used a mammalian three-hybrid system [34, 52, 53] to study the interaction of mCAB with FKBP12-FK506. We fused mCAB to the transcriptional activation domain (AD) of the p65 subunit of human NF-κB (residues 361–551) [52]. The three-hybrid assay involves the recruitment of this mCAB-AD protein to DNA decorated with FKBP12 (Figure 6A). We chose as the reporter a construct containing the Gal4 response element (GRE) fused to AP (GRE-AP). For the bait, we chose a fusion of the Gal4 DNA binding domain (BD) to three tandem copies of FKBP12 (BD-F3) [4, 34]. We cotransfected cells with all three constructs and exposed them to varying concentrations of **1**, **3**, or **4**. For both derivatives, we observed a dose-dependent increase in AP production, though with EC_{50} values higher than that of **1** (Figure 6B). Once again, these results suggest a reduced ability of **3** or **4** to interact with mCAB. Interestingly, mCAB-AD expression levels increase with increasing concentration of DNA, whereas

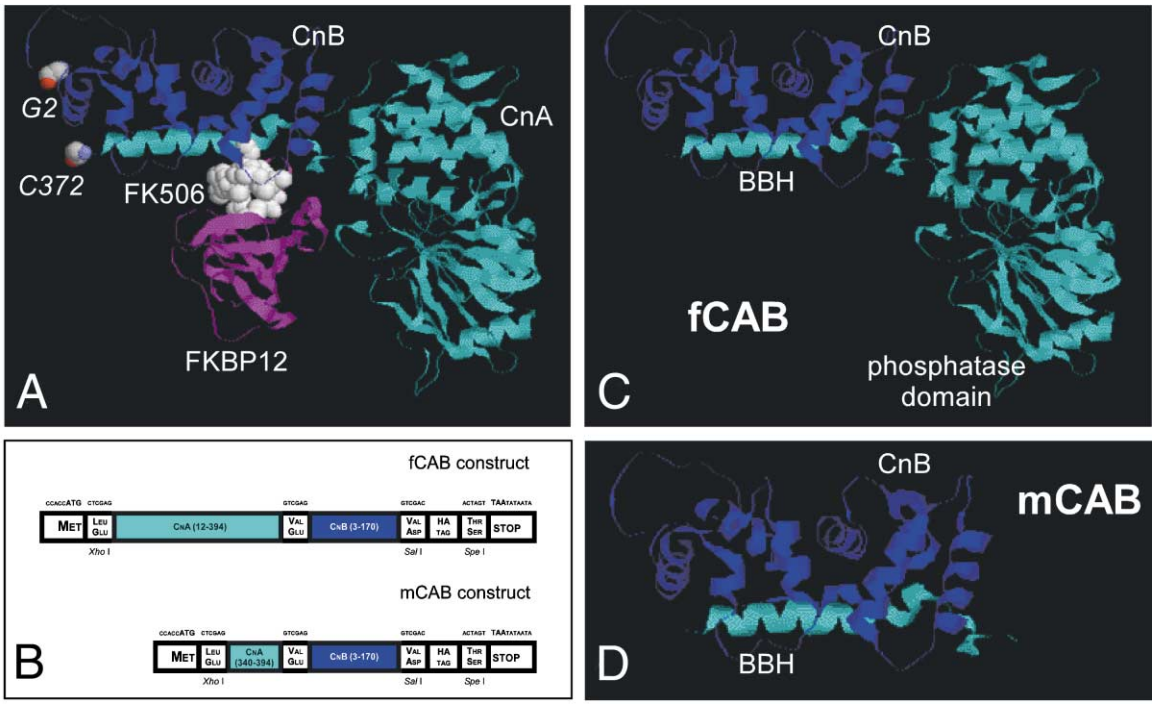

Figure 4. Preparation of Calcineurin A/B Fusion Proteins (CABs)

Representations of the FKBP12-FK506-calcineurin A/B complex are based on the same crystallographic coordinates and follow the same color scheme as in Figure 2.

(A) The C-terminal crystallographically resolved residue (Cys372) of CnA and the N-terminal residue (Gly2) of CnB are shown in space-filling representation to illustrate their proximity in space; 24 residues separate these positions in the single-chain CAB proteins.

(B) Schematic representations of the exact coding sequences of both functional CAB (fCAB) and modular CAB (mCAB) fusion proteins. In this study, we used human CnAα, which differs from bovine CnAα protein at only one residue (human, Ser59; bovine, Thr59) in the region of interest. Human and bovine CnB are identical at the amino acid level. Restriction sites within the open-reading frames encode the following amino acids (single-letter codes): XhoI (LE), SalI/XhoI fusion (VE), SalI (VD), and SpeI (TS).

(C) Crystallographically resolved residues present in the fCAB fusion protein.

(D) Crystallographically resolved residues present in the mCAB fusion protein.

AP activity decreases (Figure 6C), which probably reflects competition for cellular resources with the cotransfected reporter and bait constructs. Finally, we confirmed the physical association of mCAB with FKBP12-FK506 by coimmunoprecipitation (Figure 6D) of an FK506-dependent complex from cells cotransfected with mCAB-AD and a construct encoding epitope-tagged FKBP12 (Figure 6D).

To compare the mCAB dimerization system with a previously developed heterodimerizer system based on the FRB* domain [34], we directly compared mCAB-AD with FRB*-AD in parallel three-hybrid experiments. Jurkat cells were cotransfected with reporter, bait, and either mCAB-AD or FRB*-AD. In these experiments, cells were exposed to either rapamycin or FK506 at similar concentrations (Figure 6E). The data show that the response of mCAB to FK506 is comparable to that of the FRB* domain to rapamycin. To explore the use of multiple different dimerization domains in a single experiment, we took advantage of the fact that rapamycin and FK506 both bind the same primary target, namely FKBP12. Specifically, we cotransfected Jurkat cells with reporter, bait, and *both* mCAB-AD and FRB-AD. Under these circumstances, *either* FK506 *or* rapamycin was able to stimulate production of AP, and treatment with both CIDs resulted in the highest signal (Figure 6F). Although more extensive tests of such double dimerizer systems are warranted, we feel that these results are sufficient to demonstrate this aspect of the mCAB system.

Identification and Validation of Compensatory Mutants of mCAB

Much recent work in the redesign of small molecule-protein interfaces, particularly work involving FKBP12 and its ligands, has used a protein structure-based approach [7, 9, 14, 18, 54]. In contrast, we sought to extend the power of genetics to protein discovery by using a yeast three-hybrid selection strategy (Figure 7A) [53, 55, 56]. This system is analogous to the mammalian three-hybrid assay described above and elsewhere [34, 52], except that the reporter gene encodes a nutritional marker. By coupling yeast survival to small molecule-dependent protein-protein interaction, we can simultaneously query a larger proportion of sequence space. To implement this strategy, we fused mCAB to a BD from the bacterial transcription factor LexA [53] (mCAB-LexA) and fused FKBP12 to the transcriptional activator B42 [57] (FKBP-B42). Yeast cells were cotransformed with these two constructs and with a reporter gene en-

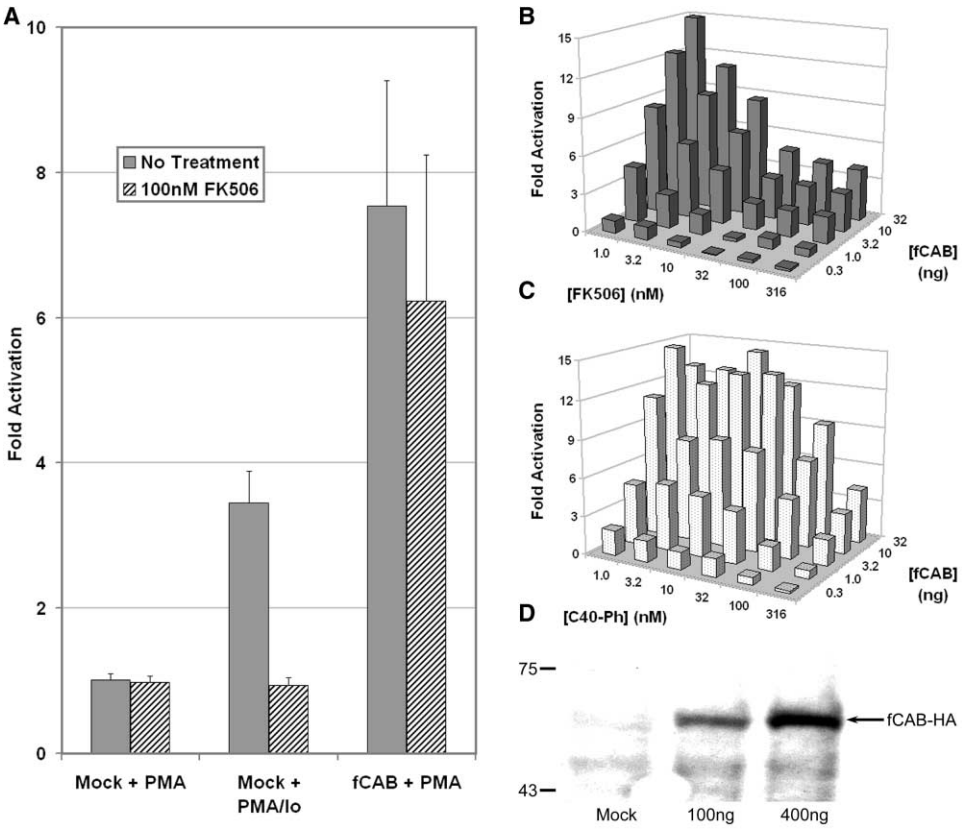

Figure 5. Activity and Inhibition of fCAB Protein

(A) Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells) and either empty pBJ5X vector or fCAB-HA (400 ng/10^6 cells), followed by treatment with PMA, Io, and FK506, as indicated, for 24 hr. Data are plotted as the average and standard deviation from either 24 (Mock + PMA) or six (Mock + PMA/Io, fCAB + PMA) independent experiments.

(B) Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells) and the indicated amount (per 10^6 cells) of fCAB-HA, followed by treatment with PMA and serial dilutions of FK506 for 24 hr. Data are plotted as the average of duplicate experiments.

(C) Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells) and the indicated amount (per 10^6 cells) of fCAB-HA, followed by treatment with PMA and serial dilutions of C40-Ph (**3**) for 24 hr. Data are plotted as the average of duplicate experiments.

(D) Jurkat cells were transfected with 0, 100, or 400 ng (per 10^6 cells) of fCAB-HA. After 24 hr, cleared cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with α-HA monoclonal antibody.

coding the nutritional markers *LEU2* and *URA3* under the control of a LexA-inducible promoter.

Our strategy for mutagenesis benefited from the previous identification of CnB residues contacting the FKBP12-FK506 complex [41]. In particular, the third EF hand of CnB contains several residues that contact the terminal olefin (C39-C40) of FK506 (Figure 7B). We prepared a three-position library of mutant mCAB alleles by randomizing the sequence LKMMV of CnB (residues 116–120) to XKMXX. This library was screened (as a fusion to LexA) in the yeast three-hybrid system on agar containing a low concentration (10 nM) of **4** at which "wild-type" mCAB-AD does not promote growth. We identified fourteen clones during the primary screen and retested each at varying concentrations of **1** or **4**. In particular, we identified one clone that restored the pattern of growth to that induced by "wild-type" mCAB-AD in combination with FK506 (Figure 7C). We chose **4** for yeast screening because of its performance in the calcineurin inhibition assay and the mammalian three-hybrid system. Specifically, **4** showed the most impaired ability, relative to **1**, both to inhibit endogenous calcineurin and to mediate AP production via mCAB-AD. For this reason, we deemed **4** our best "bump" compound and restricted our screen for a compensatory mutant "hole" in mCAB to **4**.

Sequence analysis of clones selected by yeast three-hybrid screening revealed a strong preference by **4** for the substitution M118G in CnB. This collection of clones allowed us to determine a consensus sequence, [L|*V*]KM*G*[*C*|*S*], for the mutated region (Figure 8A). To validate these results, we independently prepared several mutant alleles of mCAB-AD by site-directed mutagenesis. These alleles corresponded to CnB mutants (residues 116–120) LKM*G*V, LKM*GC*, LKM*GS*, *V*KM*GC*, and *V*KM*GS*. Each of these constructs was introduced into the mammalian three-hybrid system (as an AD fusion) to test whether any could restore binding to **4**. Of these proteins, the mutant mCAB^{VKMGC} (L116V/M119G/V120C; hereafter termed mCAB*) was best suited to restore binding, as judged by the most pronounced leftward shift in EC_{50} (Figure 8B). Interestingly, this protein had little effect on the dose-response to **3** in the same assay. Indeed, at modest concentrations of CID (30 nM),

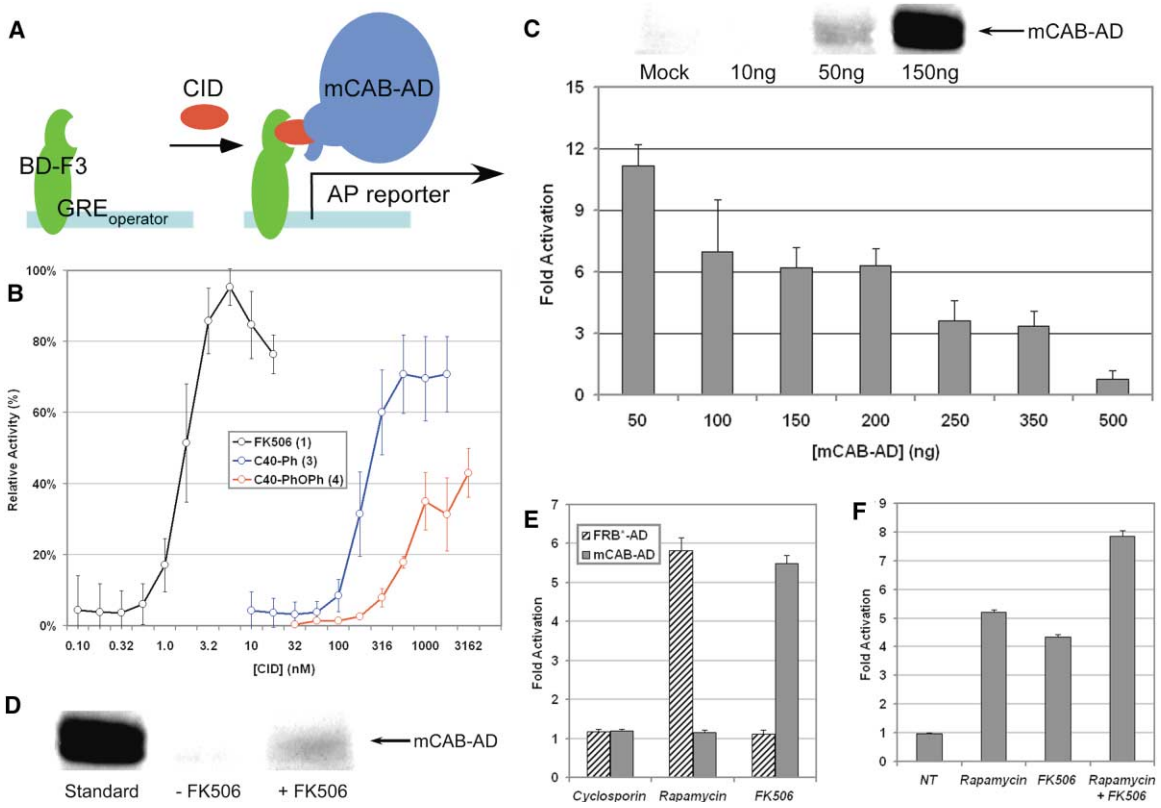

Figure 6. Activity of mCAB Protein in Mammalian Cells

(A) Mammalian three-hybrid system used in this study. Schematic representations include CID (red), bait protein (green), effector protein (blue), and DNA (cyan).

(B) Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells), BD-F3 bait (150 ng/10^6 cells), and mCAB-AD (50 ng/10^6 cells), followed by treatment with the indicated amount of **1**, **3**, or **4**, for 24 hr. Data are plotted as the average and standard deviation of either 12 [FK506 (**1**), C40-Ph (**3**)] or two [C40-PhOPh (**4**)] independent experiments.

(C) Jurkat cells were transfected as in (B) except that a variable amount (per 10^6 cells) of mCAB-AD DNA and a fixed concentration (100 nM) of FK506 were used. After 24 hr, one set of aliquots was lysed for separation by SDS-PAGE, transferred to nitrocellulose, and probed with α-FLAG monoclonal antibody while another identical set was tested for AP activity, which is plotted as the average and standard deviation of six independent experiments.

(D) Jurkat cells were transfected with mCAB-AD (100 ng/10^6 cells) and FKBP-HA (400 ng/10^6 cells) (see Experimental Procedures), followed by treatment with or without FK506 (200 nM) for 24 hr. α-HA immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with α-FLAG monoclonal antibody. The standard band is a cleared lysate from the 150 ng sample shown in (C).

(E) Jurkat cells were transfected with reporter and bait as in (B) and with either FRB*-AD or mCAB-AD (50 ng/10^6 cells), followed by treatment with CsA (1 μM), rapamycin (100 nM), or FK506 (200 nM) for 24 hr. AP activity is plotted as the average and standard deviation of six independent experiments.

(F) Jurkat cells were transfected with reporter and bait as in (B) and with *both* FRB*-AD and mCAB-AD (each at 50 ng/10^6 cells), followed by treatment with rapamycin (100 nM), FK506 (200 nM), or both, for 24 hr. AP activity is plotted as the average and standard deviation of four independent experiments.

AP production by the combination of **4** with mCAB*-AD exceeds that of **1** in combination with mCAB-AD (Figure 8C). To rule out the possiblity that mutant protein levels were responsible for this effect, we compared expression levels for mCAB-AD and mCAB*-AD by Western blot analysis (Figure 8D), which revealed comparable expression levels for the two proteins. These experiments confirm the result of the yeast three-hybrid selection and provide not only a new dimerizer system available for immediate use, but also a set of guidelines for its use in transfection experiments. Future studies of the interaction between FK506 (or its derivatives) and CAB proteins might shed light on the structural basis for binding in these novel small molecule-protein complexes.

Discussion

Resculpting of the small molecule-protein interface between FK506 and the calcineurin A/B heterodimer took place in three stages. First, we prepared calcineurin-resistant derivatives of FK506 by using crossed olefin metathesis to append steric bulk to the C39-C40 terminal olefin of FK506. We used a reporter gene assay to test the ability of such derivatives to inhibit endogenous calcineurin. Next, we prepared calcineurin A/B fusion proteins, termed CABs, to identify a binding module for the FKBP12-FK506 complex. The ability of CAB domains to bind FKBP12-FK506 was judged with two different chemical genetic approaches. We used ionomycin (Io)

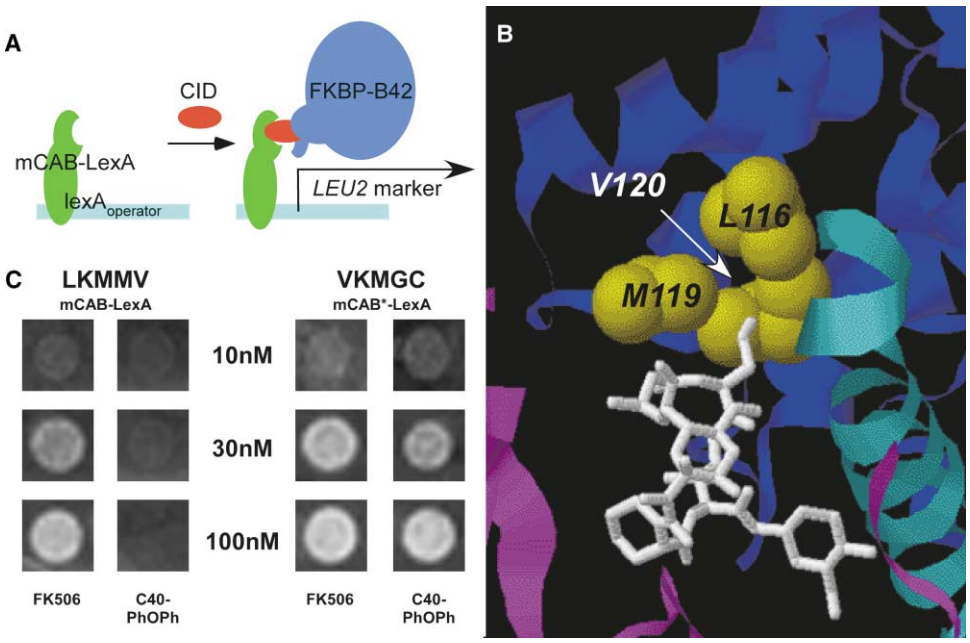

Figure 7. Selection of Mutant Proteins by the Yeast Three-Hybrid System

(A) Yeast three-hybrid system used in this study. Schematic representations include CID (red), bait protein (green), effector protein (blue), and DNA (cyan).

(B) Close-up of the interface between FK506 and CnB. The image is based on the same crystallographic coordinates and follows the same color scheme as that in Figure 2. The terminal olefin (C39-C40) of FK506 protrudes into a binding pocket formed by several amino acid side chains. Shown in space-filling representation (yellow) are the side chains of the three residues (CnB; L116, M119, V120) randomized by degenerate PCR mutagenesis to prepare the yeast three-hybrid library.

(C) After negative and positive selection (see Experimental Procedures), individual yeast clones were replica spotted on His/Trp/Leu-dropout agar containing the indicated concentration of FK506 or **4** and allowed to grow at 30°C for 72 hr before photography of the resulting colonies (approximately 5× magnification).

and FK506 to dissect the contributions of fCAB and endogenous calcineurin in an FK506-sensitive reporter gene assay. These experiments were made possible by the omission of the autoinhibitory domain (AID) of CnA [41, 49, 50] from the fCAB fusion protein, which renders this protein a calcium-independent source of calcineurin phosphatase activity. Thus, experiments conducted in the presence and absence of Io could be used to distinguish fCAB activity from endogenous calcineurin activity. Additionally, we used FK506 and derivatives as chemical activators of transcription in both yeast and mammalian three-hybrid systems. These experiments were inspired by the natural dimerizer property of FK506 [58], which allowed us rapidly to test mCAB constructs for docking activity (measured by recruitment of AD) in a cellular context.

In the mammalian three-hybrid system, we were able to control the interaction of three interacting molecules either by varying the dose of dimerizer or by changing the concentration of transfected DNA encoding the mCAB-AD fusion protein. Specifically, increasing the amount of mCAB*-AD results in an increased sensitivity of the system to C40-*p*-phenoxyphenyl-FK506 (P.A.C. and S.L.S., unpublished data). This fact has an important implication for the use of C40-*p*-phenoxyphenyl-FK506 and mCAB*-AD as a calcineurin-resistant dimerizer system. Because endogenous levels of calcineurin are relatively low, our results predict a broad dose window in which C40-*p*-phenoxyphenyl-FK506 could be used to recruit proteins containing an mCAB* domain without any effect on endogenous calcineurin. This relationship is analogous to the therapeutic index relating the efficacy and toxicity of a drug. The mCAB* domain, used in tandem with C40-*p*-phenoxyphenyl-FK506, provides an opportunity to elicit a dose response in cell culture without interfering with endogenous signaling pathways. Because C40-*p*-phenoxyphenyl-FK506 is unable to inhibit endogenous calcineurin, as judged by the use of an NFAT-responsive reporter, its use should be broadly applicable to dimerizer systems that use mCAB* as one of the dimerization domains. This property of the mCAB* system improves the chance that a particular signaling process can be studied in a dimerizer system.

One compelling potential feature of CABs as dimerization domains remains to be explored. Calcineurin is also the target of the cyclophilin-CsA complex [35]. Although the interaction of fCAB or mCAB with this complex remains to be tested, it is possible that some incarnation of a CAB protein could be engineered (or selected) to dock not only with FKBP12-FK506 but also with cyclophilin-CsA. Should this prove to be true, the CAB domain could assume broader roles in multicomponent dimerization systems because of its ability to dock with two different partners under the control of two different small molecules. If a CAB domain were prepared that could bind both complexes, one could imagine engineering cells or animals in which a fusion of CAB to some effector domain was present in the same cell as two other fusion

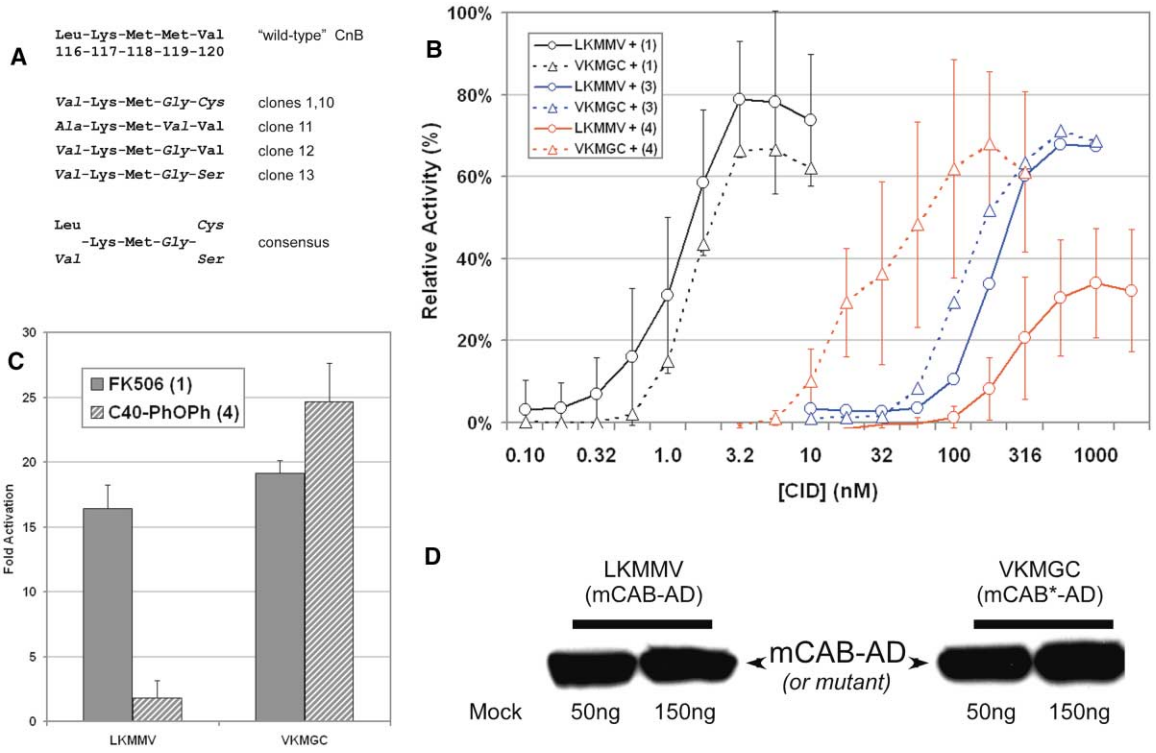

Figure 8. Validation of Selected Hits for C40-*p*-Phenoxyphenyl-FK506 (**4**) in Mammalian Cells

(A) Sequence of representative hits and consensus sequence for compensatory proteins.

(B) Jurkat cells were transfected with NRE-AP reporter (50 ng/10^6 cells), BD-F3 bait (150 ng/10^6 cells), and either mCAB-AD or mCAB*-AD (50 ng/10^6 cells), followed by treatment with the indicated amount of **1**, **3**, or **4**, for 24 hr. Data are plotted as the average of at least 4 independent experiments, and error bars (where present) indicate the standard deviation of the mean.

(C) Jurkat cells were transfected as in (B), then treated with either **1** or **4** (30 nM) for 48 hr. AP activity is plotted as the average and standard deviation of two independent experiments.

(D) Jurkat cells were transfected as in (B), except that a variable amount (per 10^6 cells) of mCAB-AD or mCAB*-AD DNA was used, as indicated. After 24 hr, cleared cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with α-HA monoclonal antibody.

proteins, one containing FKBP12 and one containing cyclophilin. In this scenario, the addition of FK506 or CsA would result in the formation of alternative complexes between mCAB and another dimerization domain. The use of tandem modular domains in this context would further enable the assembly of specific multicomponent complexes under the control of multiple dimerizers.

Finally, the advent of CAB domains themselves allows such multicomponent dimerization experiments. Both rapamycin and FK506 can now be used to recruit different modules (FRB and CAB, respectively) to fusion proteins containing FKBP12. As described above and elsewhere [1, 36, 37], both homo- and heterodimerizers have been used to exercise control over protein association under circumstances in which at most two different engineered proteins are specifically being induced to associate. Signal transduction in general, however, is characterized by the orderly formation of complex collections of proteins [59]. Docking domains based on novel small molecule-protein interactions extend the power of dimerizer methods to include multicomponent associations. For such studies, it is clearly desirable to have multiple small molecule-protein combinations that not only protect endogenous signaling molecules (e.g., FRAP and calcineurin) from inhibitory effects but also allow the ordered assembly of complexes containing more than two fusion proteins of interest.

Significance

We have used chemical genetic selection to discover a novel small molecule-protein combination based on the inhibitory complex between FKBP12-FK506 and the protein phosphatase calcineurin. This study used a combination of synthetic chemistry, molecular biology, fusion protein engineering, and a phenotypic, cell-based assay to modify components of an existing small molecule-protein complex. By combining these techniques, we were able to synthesize small molecules and screen them directly in a context that requires both solubility and cell permeability, rather than having to test for these properties in separate experiments. Guided by previous crystallographic work, we were able to select sites for both derivatization (of the small molecule) and mutagenesis (of the protein). Our use of a phenotypic assay, in this case the yeast three-hybrid assay, allowed the simultaneous query of a large proportion of sequence space for our mutant proteins and enabled the rapid identification of a novel receptor.

Experimental Procedures

Extraction of FK506 from Prograf Capsules

Prescription FK506 was purchased from CVS Pharmacy as Prograf capsules containing 5 mg FK506 each as a pharmaceutical preparation (Fujisawa). Capsules were opened manually, and bulk powder was crushed with a mortar and pestle before resuspension in 300 μL H_2O per mg of active ingredient. FK506 (**1**) was extracted by the addition of 700 μL ethyl acetate (EA) per mg to the aqueous suspension, which was then transferred to a separatory funnel. The initial EA fraction was set aside and pooled with three additional EA extracts of the aqueous suspension. The pooled EA fractions were back-extracted with an equal volume of H_2O until very little particulate matter was visible in the organic phase (at least three times). The organic phase was passed through Celite and a plug of glass wool, then dried over magnesium sulfate ($MgSO_4$). Purified FK506 was collected from the EA fraction by rotary evaporation, with recovery of 96% of the material (4.8 mg/capsule). The identity of **1** was confirmed at this stage by FAB-MS and ^{1}H-NMR.

Synthesis of C40 Derivatives of FK506

C40 derivatives of FK506 were prepared by a modification of the procedure reported by Diver and Schreiber for the synthesis of FK1012 by olefin metathesis [11]. FK506 (**1**) was added in dichloromethane (CH_2Cl_2) to an oven-dried 10 ml round-bottom flask equipped with a magnetic stir bar and rubber septum. Neat styrene or substituted styrene was added by pipettor; care was taken to pre-coat the pipet tip to ensure accurate volumetric transfer of the liquid reagent. In each reaction, 15 equivalents of styrene or substituted styrene was added, after which the septum was sealed and the vessel purged with Ar. Grubbs' catalyst (**2**; *bis*-(tricyclohexylphosphine)-ruthenium(IV) benzylidne dichloride) was obtained commercially (Strem) and stored under Ar after each use. Reaction mixtures were supplemented with 0.2 equivalents of **2** in a small portion of dry CH_2Cl_2 so that the final concentration of reagents was 10 mM FK506, 150 mM styrene or substituted styrene, and 2 mM catalyst. Reactions were allowed to proceed at reflux temperature (40°C) with stirring for 24 hr, at which time a second portion of **2** was added for an additional 24 hr. White precipitate (stilbene or substituted stilbene) was removed by passing crude reactions through Celite and glass wool. Extraction with EA, drying with $MgSO_4$, and rotary evaporation produced dark brown oils containing FK506, C40-substituted FK506, and FK1012, as judged by the presence of parent ion peaks in FAB-MS experiments. These mixtures were further purified by normal-phase HPLC in $CHCl_3$ as described below.

Purification of C40 Derivatives of FK506

Purification of C40 derivatives of FK506 was essential to the success of this study. Fortunately, our efforts at removing all traces of FK506 from reaction mixtures were aided by the fact that FK506 and its derivatives ionize very well by FAB-MS. We confirmed by spike-in experiments that as little as 0.05% contamination by FK506 is detectable as an M+Na parent ion peak by this sensitive measurement. Thus, we analyzed the HPLC fractions described below by FAB-MS and continued purification until *no* M + Na peak corresponding to FK506 was present in the spectrum. This allowed small quantities of HPLC eluate to be analyzed quickly and obviated the need to exchange the entire fraction into deuterated solvent between each purification step. Furthermore, this method ensures greater than 99.95% purity of the final products, which is far better than that obtained by conventional chromatography (monitored by TLC and NMR). We first chromatographed filtered reaction mixtures on an HPLC instrument bearing two tandem sizing columns (Japan Analytical Industries). After four cycles of injection, no trace of FK1012 was observed by FAB-MS in a mixture still containing both FK506 and C40 derivatives, which are relatively similar in mass. To separate FK506 from C40 derivatives, we used a recycling HPLC instrument bearing two tandem affinity columns (Japan Analytical Industries). After six to eight injection cycles with peak-shaving, we isolated the C40-phenyl (**3**) and C40-*p*-phenoxyphenyl (**4**) adducts, each in approximately 20% recovery. Furthermore, approximately 50% of the original mass of FK506 was recovered in both cases. These observations provide upper and lower limits for the yield of the reaction itself, which is difficult to determine exactly because of the loss of both FK506 and C40 adducts in shaved HPLC peaks. One additional four-cycle purification step (using the sizing columns) was required to remove all traces of FK506 (as judged by FAB-MS) and ruthenium contaminants (as judged by brown color). The resulting single HPLC peaks corresponding to both **3** and **4** were analyzed by ^{1}H-NMR, and each was assigned *(E)* double-bond geometry at C39-C40. We cannot precisely comment on the stereoselectivity of this reaction because the *(Z)* product may simply have separated poorly from FK506 and may have entered shaved peak fractions regardless of its relative abundance. In all, **3** and **4** were each subjected to three purification steps, totaling fourteen HPLC injection cycles, on two different instruments. Though the yield for these reactions is poor overall, the isolated products are completely free of FK506, which is essential for testing their activity in a cellular context.

DNA Constructs and PCR Mutagenesis

The constructs NRE-AP, GRE-AP, and BD-F3 have been described previously [4, 45, 46]. FRB*-AD was prepared (B.N. Desai, P. T.-X. Ngheim, P.A.C., and S.L.S., unpublished data) by PCR amplification of FRB* with a construct described by Liberles et al. as a template [34]; the encoded domain consists of mutant FRAP sequence corresponding to amino acid residues 2025–2114 in the full-length protein [32, 34, 52]. All new constructs for mammalian expression were prepared with pBJ5X (P.A.C. and S.L.S., unpublished results), a pBJ5 derivative [60] that places an open reading frame (ORF) upstream of an in-frame hemagglutinnin (HA) epitope tag [61] and downstream of a strong (SRα) mammalian promoter [60]. The pBJ5X plasmid contains a custom polylinker sequence (GCGGCCGCGGCC ACC*ATG*CTCGAG-ORF-GTCGAC-HA-ACTAGT*TAA*TATAATAGAA TTC) that allows for modular preparation of fusion proteins. Each of the XhoI, SalI, and SpeI restriction sites consists of six nucleotides in frame with the ORF encoding the inserted domain. Furthermore, because XhoI and SalI digests result in complementary overhangs, inserts excised from pBJ5X can be ligated directionally into an appropriately digested pBJ5X acceptor plasmid to generate constructs encoding either N- or C-terminal fusion proteins. In particular, constructs encoding the desired fragments of calcineurin were prepared by PCR amplification of nucleotide sequences corresponding to (1) residues 12–394 of human CnA, (2) residues 340–394 of human CnA, and (3) residues 3–170 of human CnB, then directional ligation into pBJ5X. fCAB was prepared by ligating the CnB sequence downstream of the long CnA sequence (encoding CnA 12–394), and mCAB was prepared by ligating the CnB sequence downstream of the short CnA sequence (encoding CnA 340–394). Fusion proteins to the AD of human p65 (residues 361–551) were prepared by PCR amplification of an XhoI-EcoRI fragment of p65, which was ligated directionally into similarly digested CAB constructs, resulting in loss of the HA tag (see below). Site-directed mutant CAB constructs were prepared by overlap extension PCR of CnB, with oligonucleotides encoding mutant protein sequence used as internal primers. Internal primer sequences at mutant codons was chosen based on mammalian codon preferences [62]. These mutant CnB fragments were subsequently used to generate fCAB or mCAB constructs exactly as described above. Construct identity was generally determined by restriction mapping and was subsequently confirmed for any segment introduced by PCR using DNA seqencing. All DNA destined for transfection into mammalian cells was further purified by phenol-$CHCl_3$ extraction and ethanol precipitation following purification from bacterial cells by the use of commercially available reagents (Promega).

Transfections, Immunoblots, and Immunoprecipitation

For experiments involving mammalian cells, mCAB-p65 was excised from pBJ5X (see above) and inserted into pBJ5E [32–34], which contains a similar polylinker (*ATG*-FLAG-GCGGCCGCGCTCGAG-ORF-GTCGAC*TAA*TATAATAGAATTC) encoding an N-terminal FLAG epitope tag instead of a C-terminal HA tag. All cell culture experiments were performed with "TAg" Jurkat cells, a human Jurkat T cell lymphoma line derivative that is stably transfected with SV40 large T antigen [63]. Cells were cultivated in RPMI-1640 medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum, 2 mM

L-glutamine, 100 units/ml penicillin, and 1 mg/ml streptomycin and were grown at 37°C and 5% CO_2 in a humidified incubator. Prior to transfection, cells were grown to a density of 10^6/ml and incubated with DNA for 10 min before electroporation with an ElectroCell Manipulator (BTX) at 250 V. Pulse lengths were controlled to 39–42 ms by using identically treated samples to optimize the capacitance (960–1040 μF). Cells were allowed to incubate for 10 min after electroporation before resuspension in cell culture media at 10^6 cells/mL. After 24 hr, cells were harvested by centrifugation for 10 min at 1000 rpm, resuspended in lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Triton X-100, 3.3% glycerol, 1 mM dithiothreitol, 2 μM leupeptin, and 2 μM aprotinin) for 10 min at 4°C, and centrifuged for 3 min at 3000 rpm to produce cleared lysates. Lysates were exposed to protein A/G-agarose (Sigma), preincubated for 30 min with α-HA monoclonal antibody (3F10; Gibco BRL), for 2 hr. Immunoprecipitates were washed thrice with lysis buffer and resuspended in lysis buffer containing sodium dodecyl sulfate (SDS) gel-loading buffer. When present, FK506 (200 nM) was maintained at the same concentration throughout the immunoprecipitation. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard protocols, and Western blots were probed with α-FLAG (M2; Sigma) or α-HA monoclonal antibody. Immunoreactive bands were detected with α-IgG secondary antibody coupled to horseradish peroxidase, and they were visualized by chemiluminescent detection (Amersham).

Mammalian Three-Hybrid Assays

"TAg" Jurkat cells were transiently transfected or cotransfected with pBJ5-based constructs (see figure legends) prepared as described above, aliquotted into 96-well microtiter plates, and treated for 24–48 hr with varying concentrations of FK506 or C40 derivatives. Assays for AP activity were performed by heating cultures (to inactivate endogenous phosphatase) for 100 min at 65–70°C, incubating aliquots of culture supernatant for 4–16 hr with an equal volume of assay buffer (2 M diethanolamine bicarbonate [pH 10] containing 120 mM MUP), and detecting fluorescent product by using either a Fluoroskan (Titertek) or SpectraMax (Gemini) microtiter plate reader. Optimized mammalian three-hybrid experiments include a 1:3:1 ratio of plasmids encoding GRE-AP, BD-F3, and mCAB-AD, respectively. Optimal conditions for electroporation were 250 ng DNA/10^6 Jurkat cells resuspended at 50 × 10^6 cells/ml. When present, PMA was added to cells at 50 ng/ml, and Io was added at 1 μM.

Yeast Three-Hybrid Screens

The mCAB-LexA construct used in the yeast three-hybrid system was prepared by fusing residues 340–370 of human CnA to residues 3–170 of CnB via a 7 amino acid linker (GGSGSGS) introduced by PCR as part of a study on linker length dependence in CAB proteins (B.G.G., A.S., and S.L.S., unpublished data). Library preparation used this construct as a starting point for site-directed mutagenesis using internal primer pairs fully degenerate at all three amino acid positions to be mutagenized. We introduced mCAB or the mutant library into pLexABD (Clontech) and introduced a single human FKBP12 module into pB42AD (Clontech) according to the manufacturer's protocol. The yeast strain YL(6.4)LU is a modification of EGY48 (Clontech) that has six copies of the LexA promotor upstream of *LEU2* and four copies of the LexA promoter upstream of *URA3*. YL(6.4)LU was transformed with both constructs by the lithium acetate method, and transformants were selected on His/Trp-dropout agar. To select against auto-activators, we replica-plated transformants on His/Trp-dropout agar containing 0.2% 5-fluoroorotic acid, 2% galactose, and 1% raffinose. Finally, true positives were selected on His/Trp/Leu-dropout agar containing FK506 (**1**) or C40-*p*-phenoxyphenyl-FK506 (**4**).

Acknowledgments

We thank the National Institute of General Medical Sciences (GM-52067) for the support of this research. We are deeply indebted to Professor Yoshii Kishi for the use of recycling HPLC capability and are especially grateful to Dr. Andrew Tyler for expert mass spectroscopy support. We thank Professor Jun Liu for the bicistronic plasmid pJA0815, encoding both CnA and CnB, and Professor Tom Maniatis for plasmid DNA encoding the p65 subunit of human NF-κB. The yeast strain YL(6.4)LU was graciously provided by Dr. Jing Huang. S.L.S. is an Investigator with the Howard Hughes Medical Institute at the Department of Chemistry and Chemical Biology, Harvard University.

Received: August 7, 2001
Accepted: October 2, 2001

References

1. Klemm, J.D., Schreiber, S.L., and Crabtree, G.R. (1998). Dimerization as a regulatory mechanism in signal transduction. Annu. Rev. Immunol. *16*, 569–592.
2. Spencer, D.M., Wandless, T.J., Schreiber, S.L., and Crabtree, G.R. (1993). Controlling signal transduction with synthetic ligands. Science *262*, 1019–1024.
3. Belshaw, P.J., Spencer, D.M., Crabtree, G.R., and Schreiber, S.L. (1996). Controlling programmed cell death with a cyclophilin-cyclosporin-based chemical inducer of dimerization. Chem. Biol. *3*, 731–738.
4. Belshaw, P.J., Ho, S.N., Crabtree, G.R., and Schreiber, S.L. (1996). Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. Proc. Natl. Acad. Sci. USA *93*, 4604–4607.
5. Farrar, M.A., Alberol, I., and Perlmutter, R.M. (1996). Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature *383*, 178–181.
6. Lin, H., Abida, W.M., Sauer, R.T., and Cornish, V.W. (2000). Dexamethasone-methotrexate: an efficient chemical inducer of protein dimerization in vivo. J. Am. Chem. Soc. *122*, 4247–4248.
7. Holt, D.A., Luengo, J.I., Yamashita, D.S., Oh, H.J., Konialian, A.L., Yen, H.K., Rozamus, L.W., Brandt, M., Bossard, M.J., Levy, M.A., et al. (1993). Design, synthesis, and kinetic evaluation of high-affinity fkbp ligands and the x-ray crystal structures of their complexes with fkbp12. J. Am. Chem. Soc. *115*, 9925–9938.
8. Amara, J.F., Clackson, T., Rivera, V.M., Guo, T., Keenan, T., Natesan, S., Pollock, R., Yang, W., Courage, N.L., Holt, D.A., et al. (1997). A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc. Natl. Acad. Sci. USA *94*, 10618–10623.
9. Keenan, T., Yaeger, D.R., Courage, N.L., Rollins, C.T., Pavone, M.E., Rivera, V.M., Yang, W., Guo, T., Amara, J.F., Clackson, T., et al. (1998). Synthesis and activity of bivalent FKBP12 ligands for the regulated dimerization of proteins. Bioorg. Med. Chem. *6*, 1309–1335.
10. Clemons, P.A. (1999). Design and discovery of protein dimerizers. Curr. Opin. Chem. Biol. *3*, 112–115.
11. Diver, S.T., and Schreiber, S.L. (1997). Single-step synthesis of cell-permeable protein dimerizers that activate signal transduction and gene expression. J. Am. Chem. Soc. *119*, 5106–5109.
12. Gold, B.G., Zeleny-Pooley, M., Wang, M.S., Chaturvedi, P., and Armistead, D.M. (1997). A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. Exp. Neurol. *147*, 269–278.
13. Terashima, A., Taniguchi, T., Nakai, M., Yasuda, M., Kawamata, T., and Tanaka, C. (2000). Rapamycin and FK506 induce long-term potentiation by pairing stimulation via an intracellular Ca(2+) signaling mechanism in rat hippocampal CA1 neurons. Neuropharmacology *39*, 1920–1928.
14. Clackson, T., Yang, W., Rozamus, L.W., Hatada, M., Amara, J.F., Rollins, C.T., Stevenson, L.F., Magari, S.R., Wood, S.A., Courage, N.L., et al. (1998). Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. Proc. Natl. Acad. Sci. USA *95*, 10437–10442.
15. Koide, M., Obata, K., Iio, A., Iida, M., Harayama, H., Yokota, M., and Tuan, R.S. (1997). Function of FK506 binding protein (FKBP) in chick embryonic cardiac development. Heart Vessels Suppl. *12*, 7–9.
16. Sabatini, D.M., Lai, M.M., and Snyder, S.H. (1997). Neural roles of immunophilins and their ligands. Mol. Neurobiol. *15*, 223–239.
17. Marx, S.O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., and Marks, A.R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (rya-

nodine receptor): defective regulation in failing hearts. Cell *101*, 365–376.
18. Clackson, T. (1998). Redesigning small molecule-protein interfaces. Curr. Opin. Struct. Biol. *8*, 451–458.
19. Belshaw, P.J., Schoepfer, J., Liu, K.M., and Schreiber, S.L. (1995). Rational design of orthogonal receptor-ligand combinations. Angew Chem. Int. Ed. *34*, 2129–2132.
20. Belshaw, P.J., and Schreiber, S.L. (1997). Cell-specific calcineurin inhibition by a modified cyclosporin. J. Am. Chem. Soc. *119*, 1805–1806.
21. Bishop, A.C., Shah, K., Liu, Y., Witucki, L., Kung, C., and Shokat, K.M. (1998). Design of allele-specific inhibitors to probe protein kinase signaling. Curr. Biol. *8*, 257–266.
22. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K.M. (1998). Engineering Src family protein kinases with unnatural nucleotide specificity. Chem. Biol. *5*, 91–101.
23. Spencer, D.M., Graef, I., Austin, D.J., Schreiber, S.L., and Crabtree, G.R. (1995). A general strategy for producing conditional alleles of Src-like tyrosine kinases. Proc. Natl. Acad. Sci. USA *92*, 9805–9809.
24. Schreiber, S.L. (1998). Chemical genetics resulting from a passion for synthetic organic chemistry. Bioorg. Med. Chem. *6*, 1127–1152.
25. Stockwell, B.R., and Schreiber, S.L. (1998). Probing the role of homomeric and heteromeric receptor interactions in TGF-beta signaling using small molecule dimerizers. Curr. Biol. *8*, 761–770.
26. Luo, Z., Tzivion, G., Belshaw, P.J., Vavvas, D., Marshall, M., and Avruch, J. (1996). Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature *383*, 181–185.
27. Farrar, M.A., Tian, J., and Perlmutter, R.M. (2000). Membrane localization of Raf assists engagement of downstream effectors. J. Biol. Chem. *275*, 31318–31324.
28. Gross, A., Jockel, J., Wei, M.C., and Korsmeyer, S.J. (1998). Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J. *17*, 3878–3885.
29. Ho, S.N., Biggar, S.R., Spencer, D.M., Schreiber, S.L., and Crabtree, G.R. (1996). Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature *382*, 822–826.
30. Pruschy, M.N., Spencer, D.M., Kapoor, T.M., Miyake, H., Crabtree, G.R., and Schreiber, S.L. (1994). Mechanistic studies of a signaling pathway activated by the organic dimerizer FK1012. Chem. Biol. *1*, 163–172.
31. Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756–758.
32. Chen, J., Zheng, X.F., Brown, E.J., and Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc. Natl. Acad. Sci. USA *92*, 4947–4951.
33. Brown, E.J., Beal, P.A., Keith, C.T., Chen, J., Shin, T.B., and Schreiber, S.L. (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo. Nature *377*, 441–446.
34. Liberles, S.D., Diver, S.T., Austin, D.J., and Schreiber, S.L. (1997). Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. Proc. Natl. Acad. Sci. USA *94*, 7825–7830.
35. Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP- FK506 complexes. Cell *66*, 807–815.
36. Muthuswamy, S.K., Gilman, M., and Brugge, J.S. (1999). Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol. Cell. Biol. *19*, 6845–6857.
37. Biggar, S.R., and Crabtree, G.R. (2000). Chemically regulated transcription factors reveal the persistence of repressor-resistant transcription after disrupting activator function. J. Biol. Chem. *275*, 25381–25390.
38. Clipstone, N.A., and Crabtree, G.R. (1992). Identification of calcineurin as a key signalling enzyme in T- lymphocyte activation. Nature *357*, 695–697.
39. Marks, B., and McMahon, H.T. (1998). Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. Curr. Biol. *8*, 740–749.
40. Zhuo, M., Zhang, W., Son, H., Mansuy, I., Sobel, R.A., Seidman, J., and Kandel, E.R. (1999). A selective role of calcineurin aalpha in synaptic depotentiation in hippocampus. Proc. Natl. Acad. Sci. USA *96*, 4650–4655.
41. Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., and Navia, M.A. (1995). X-ray structure of calcineurin inhibited by the immunophilin- immunosuppressant FKBP12–FK506 complex. Cell *82*, 507–522.
42. Persechini, A., Moncrief, N.D., and Kretsinger, R.H. (1989). The EF-hand family of calcium-modulated proteins. Trends Neurosci. *12*, 462–467.
43. Grubbs, R.H., and Chang, S. (1998). Recent advances in olefin metathesis and its application to organic synthesis. Tetrahedron *54*, 4413–4450.
44. Loh, C., Shaw, K.T., Carew, J., Viola, J.P., Luo, C., Perrino, B.A., and Rao, A. (1996). Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. J. Biol. Chem. *271*, 10884–10891.
45. Bram, R.J., Hung, D.T., Martin, P.K., Schreiber, S.L., and Crabtree, G.R. (1993). Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. Mol. Cell. Biol. *13*, 4760–4769.
46. Graef, I.A., Holsinger, L.J., Diver, S., Schreiber, S.L., and Crabtree, G.R. (1997). Proximity and orientation underlie signaling by the non-receptor tyrosine kinase ZAP70. EMBO J. *16*, 5618–5628.
47. Stockwell, B.R., Haggarty, S.J., and Schreiber, S.L. (1999). High-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications. Chem. Biol. *6*, 71–83.
48. Stockwell, B.R., and Schreiber, S.L. (1998). TGF-beta-signaling with small molecule FKBP12 antagonists that bind myristoylated FKBP12-TGF-beta type I receptor fusion proteins. Chem. Biol. *5*, 385–395.
49. Hemenway, C.S., and Heitman, J. (1999). Calcineurin. Structure, function, and inhibition. Cell Biochem. Biophys. *30*, 115–151.
50. Hashimoto, Y., Perrino, B.A., and Soderling, T.R. (1990). Identification of an autoinhibitory domain in calcineurin. J. Biol. Chem. *265*, 1924–1927.
51. Klee, C.B., Krinks, M.H., Manalan, A.S., Cohen, P., and Stewart, A.A. (1983). Isolation and characterization of bovine brain calcineurin: a calmodulin-stimulated protein phosphatase. Methods Enzymol. *102*, 227–244.
52. Rivera, V.M., Clackson, T., Natesan, S., Pollock, R., Amara, J.F., Keenan, T., Magari, S.R., Phillips, T., Courage, N.L., Cerasoli, F., Jr., et al. (1996). A humanized system for pharmacologic control of gene expression. Nat. Med. *2*, 1028–1032.
53. Licitra, E.J., and Liu, J.O. (1996). A three-hybrid system for detecting small ligand-protein receptor interactions. Proc. Natl. Acad. Sci. USA *93*, 12817–12821.
54. Yang, W., Rozamus, L.W., Narula, S., Rollins, C.T., Yuan, R., Andrade, L.J., Ram, M.K., Phillips, T.B., van Schravendijk, M.R., Dalgarno, D., et al. (2000). Investigating protein-ligand interactions with a mutant FKBP possessing a designed specificity pocket. J. Med. Chem. *43*, 1135–1142.
55. Luban, J., and Goff, S.P. (1995). The yeast two-hybrid system for studying protein-protein interactions. Curr. Opin. Biotechnol. *6*, 59–64.
56. Huang, J., and Schreiber, S.L. (1997). A yeast genetic system for selecting small molecule inhibitors of protein-protein interactions in nanodroplets. Proc. Natl. Acad. Sci. USA *94*, 13396–13401.
57. Ma, J., and Ptashne, M. (1987). A new class of yeast transcriptional activators. Cell *51*, 113–119.
58. Crabtree, G.R., and Schreiber, S.L. (1996). Three-part inventions: intracellular signaling and induced proximity. Trends Biochem. Sci. *21*, 418–422.
59. Heldin, C.H. (1995). Dimerization of cell surface receptors in signal transduction. Cell *80*, 213–223.

60. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988). SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. *8*, 466–472.
61. Pati, U.K. (1992). Novel vectors for expression of cDNA encoding epitope-tagged proteins in mammalian cells. Gene *114*, 285–288.
62. Sharp, P.M., and Li, W.H. (1987). The codon Adaptation Index—a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res. *15*, 1281–1295.
63. Northrop, J.P., Ullman, K.S., and Crabtree, G.R. (1993). Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. J. Biol. Chem. *268*, 2917–2923.