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Chemistry

Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen

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ABSTRACT **The natural product rapamycin has been used to provide temporal and quantitative control of gene expression in animals through its ability to interact with two proteins simultaneously. A shortcoming of this approach is that rapamycin is an inhibitor of cell proliferation, the result of binding to FKBP12–rapamycin-associated protein (FRAP). To overcome this limitation, nontoxic derivatives of rapamycin bearing bulky substituents at its C16-position were synthesized, each in a single step. The isosteric isopropoxy and methallyl substituents with the nonnatural C16-configuration abolish both binding to FRAP and inhibition of T cell proliferation. Binding proteins for these derivatives were identified from libraries of cDNAs encoding mutants of the FKBP12–rapamycin-binding (FRB) domain of FRAP by using a mammalian three-hybrid transcription assay. Targeting of the mutations was guided by the structure of the FKBP12-rapamycin–FRB ternary complex. Three compensatory mutations in the FRB domain, all along one face of an α-helix in a rapamycin-binding pocket, were identified that together restore binding of the rapamycin derivatives. Using this mutant FRB domain, one of the nontoxic rapamycin derivatives induced targeted gene expression in Jurkat T cells with an EC_{50} below 10 nM. Another derivative was used to recruit a cytosolic protein to the plasma membrane, mimicking a process involved in many signaling pathways.**

A general technique for regulating the proximity and orientation of proteins in cells has been developed (1). Small molecule chemical inducers of dimerization (CIDs), which bind two proteins simultaneously have been used to activate cell surface receptors [T cell (2, 3) and Fas (4, 5) receptors], intracellular proteins [Src (6), SOS (7), and Raf (8, 9)], and transcriptional activators (10–12), and to translocate intracellular proteins to the plasma membrane (6–8, 10) and nucleus (10). CIDs have also been used to induce frog mesoderm tissue in animal pole explants (J. Yang, K. Symes, M. Mercola, and S.L.S., unpublished results), to activate cell-specifically a cell-death signaling pathway in mice (4), and to regulate *in vivo* the production of human growth hormone in mice (11).

The immunosuppressant rapamycin (**1**) is a naturally occurring CID, interacting with FK506-binding protein (FKBP12; refs. 13 and 14) and FKBP12–rapamycin-associated protein [FRAP (15), also named RAFT (16)] simultaneously. The binary FKBP12–rapamycin complex interacts with an 11-kDa domain of FRAP (17), termed the FKBP12–rapamycin-binding (FRB) domain, and inhibits the kinase activity of FRAP (18). Inhibition of FRAP blocks signaling to $pp70^{S6K}$ and 4E-BP, thereby preventing growth factor-mediated increases in the rate of translation of specific mRNA transcripts and G1 cell cycle progression (19). Obstruction of this pathway in T lymphocytes blocks interleukin 2 receptor signaling, which is thought to be the basis for the immunosuppressive actions of rapamycin.

The ability of rapamycin to bind FKBP12 and the FRB domain simultaneously has been exploited to control gene transcription (Fig. 1*A*) (11, 12). Fusion of a DNA-binding domain to FKBP12 and a transcriptional activation domain to an FRB domain leads to rapamycin-dependent recruitment of the activation domain to the DNA-binding domain, resulting in a functional transcriptional activator. Target genes containing several tandem upstream binding sites for the FKBP12 fusion protein can be induced to high levels with this system in mice (11) and in cell culture (12).

CIDs have also been used to control signal transduction events other than gene expression (Fig. 1*B*). For example, FK1012 (2, 3) (a homodimerizer) and FKCsA (10) (a heterodimerizer) have been used as adaptors to induce the membrane recruitment and activation of cytosolic signaling proteins, in analogy to the function of the adaptor proteins Grb2 (20) and the p85 regulatory subunit of PI3K (21). Fusion proteins containing single or multiple copies of FKBP12 can be targeted to the plasma membrane by addition of the 14 amino acid myristoylation sequence of v-src to their N termini, as demonstrated by immunocytochemistry (3) and subcellular fractionation (I. Graef, L. J. Holsinger, S. T. Diver, S.L.S., and G. R. Crabtree, unpublished results) experiments. Coexpression of these membrane-docking proteins with cytosolic signaling proteins fused to FKBP12 or cyclophilin leads to FK1012- or FKCsA-dependent membrane recruitment of the cytosolic proteins. Several signaling pathways have been activated using this approach (6–8, 10).

Using rapamycin to activate cellular events, however, can be complicated by its native biological activity. Altering the rapamycin structure to weaken binding to either FKBP12 or FRAP mitigates its cellular toxicity (15). The x-ray structure of an FKBP12–rapamycin–FRB ternary complex reveals those interactions critical for assembly of the complex (22). Modification of the C16-methoxy group of rapamycin, which points directly toward the FRB domain, can lead to loss of FRAP binding (23). Inversion of the C16-stereochemistry without a change in conformation would create a high-energy interaction between the C16-side chain and the tetrahydropyran ring of rapamycin reminiscent of a syn-pentane interaction (Fig. 2*B*). Based on the crystal structure of C16-(R)-thiomethyl rapamycin bound to FKBP12 alone (23) the modified rapamycin derivatives would be expected to undergo a series of bond rotations to alleviate this strain, resulting in a multi-angstrom displacement of the triene in the FRB-binding pocket. This structural distortion abolishes binding to the FRB domain, thereby eliminating the ligand's immunosuppressive and toxic properties.

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Abbreviations: CID, chemical inducer of dimerization; FRAP, FKBP12–rapamycin-associated protein; FRB, FKBP12–rapamycin binding; SEAP, secreted alkaline phosphatase; UAS, upstream activation sequence.

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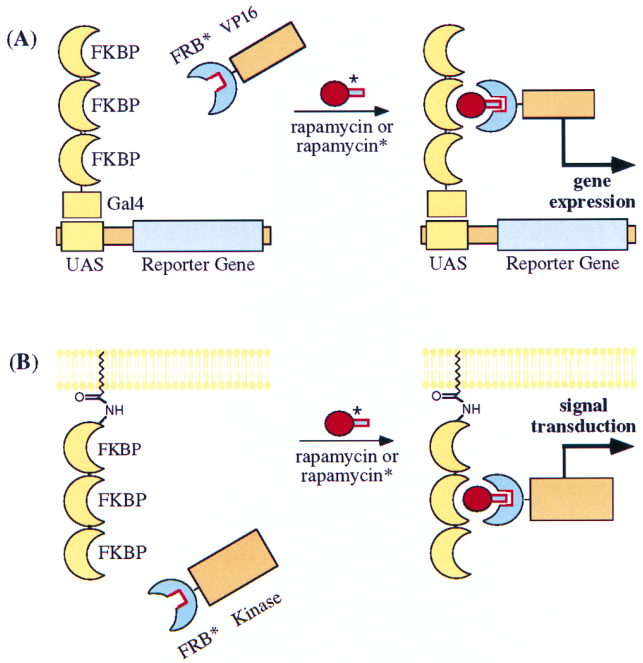


FIG. 1. Conditional gene activation and membrane recruitment with nontoxic derivatives of rapamycin. (*A*) Cartoon of rapamycin*-induced recruitment of FRB* to Gal4-FKBP3. (*B*) Cartoon of conditional membrane recruitment of cytosolic FRB* domains to myristoylated FKBP3.

High-affinity receptors for nontoxic rapamycins are required to use these ligands as CIDs. A rational approach to receptor design (24) was avoided since stereochemical modification of rapamycin at C16 yields a global structural change in the rapamycin derivative, and the consequences of such a change in terms of interactions with FRB residues cannot be easily predicted. Instead, a structure-based selection approach was used in which targeted residues in the rapamycin-binding pocket of the FRB domain were randomized to all 20 amino acids ("positional libraries"). Gain-of-function mutations in the FRB domain that restore binding to the C16-rapamycin derivatives were then selected from these libraries using a mammalian three-hybrid screen. Novel receptor–ligand complexes identified in this way were found to be effective in controlling gene expression and protein translocation without interfering with FRAP-mediated cell cycle progression.

MATERIALS AND METHODS

DNA Constructs. The constructs MF3, Gal4-FKBP3, and upstream activation sequence (UAS)-SEAP have been described (10). All FRB constructs incorporate residues 2025–2114 of FRAP. Gal4 DNA-binding domain includes residues 1–147 and the VP16 activation domain includes residues 413–490. The FRB–kinase fusion consists of residues 1967–2549 of FRAP. FRB* is defined as the triple mutation K2095P, T2098L, and W2101F.

Construction of FRB Libraries. Oligonucleotide primers containing random sequences at specific codons were used to generate FRB libraries by PCR. These PCR products were ligated into a VP16 fusion vector, digested with a restriction endonuclease to remove background ligation products, and electroporated into XL-1 blue cells. Transformants were selected directly in 100 ml of 2xYT media containing ampicillin. Each library contained over 1,000 distinct members, as estimated by streaking 5% of the electroporation mixture on Luria–Bertani agar containing ampicillin.

Synthesis of 16-Alkoxy-Substituted Rapamycins 2–7. For this synthesis the method of Luengo *et al.* (25) was used. Rapamycin (20 mg) was dissolved in 3 ml of the appropriate alcohol in the presence of five equivalents of *p*-TsOH. After 5 hr, thin-layer chromatography indicated complete conversion to the mixture of C16-epimers. The reaction mixture was concentrated, diluted with CH_2Cl_2 (5 ml), partitioned with saturated $NaHCO_3$ (aq), washed once with brine, and dried (Na_2SO_4). Chromatography (SiO_2, 5 g in 3:1 EA-hex) afforded the two C16-diastereomers in good yield. For some derivatives, further purification was accomplished by HPLC on silica gel (Rainin Instruments Microsorb 80–120-C5, 3:1 EA-hex).

Synthesis of 16-Methallyl Rapamycins 9R and 9S. A modification of the allylation method reported by Luengo *et al.* (25) was used. Rapamycin (24 mg, 26 μmol) was added in 3 ml of CH_2Cl_2 to an oven-dried 10-ml round bottom flask equipped with rubber septum, nitrogen inlet, and magnetic stirring bar. After the flask was cooled to −40°C, methallyltrimethylsilane (50 μl, 290 μmol, 11 eq.) was added, followed by dropwise addition of a 0.1 M BF_3-OEt_2 in benzene (0.11 ml, 110 μmol, 4.2 eq.). The reaction was stirred until rapamycin was consumed, as indicated by thin-layer chromatography, poured onto saturated $NaHCO_3$ (aq), washed once each with saturated $NaHCO_3$ (aq) and brine, and then dried (Na_2SO_4) to give 24 mg of a crude white solid. The two C16-diastereomers composed 83% (20 mg) of this mixture, as determined by HPLC. Reversed-phase chromatography [retention time (r.t.)/min: 23.7 (**9S**), 24.2 (**9R**), Vydac C18 column, 70/30 0.1% trifluoroacetic acid (aq.)/CH_3CN to 10/90 0.1% trifluoroacetic acid (aq.)/CH_3CN over 30 min at 70°C] afforded pure **9S** (r.t. 23.7 min) and **9R** (r.t. 24.2 min), which were identified by fast atom bombardment-mass spectroscopy and NMR spectroscopy (see *Results*).

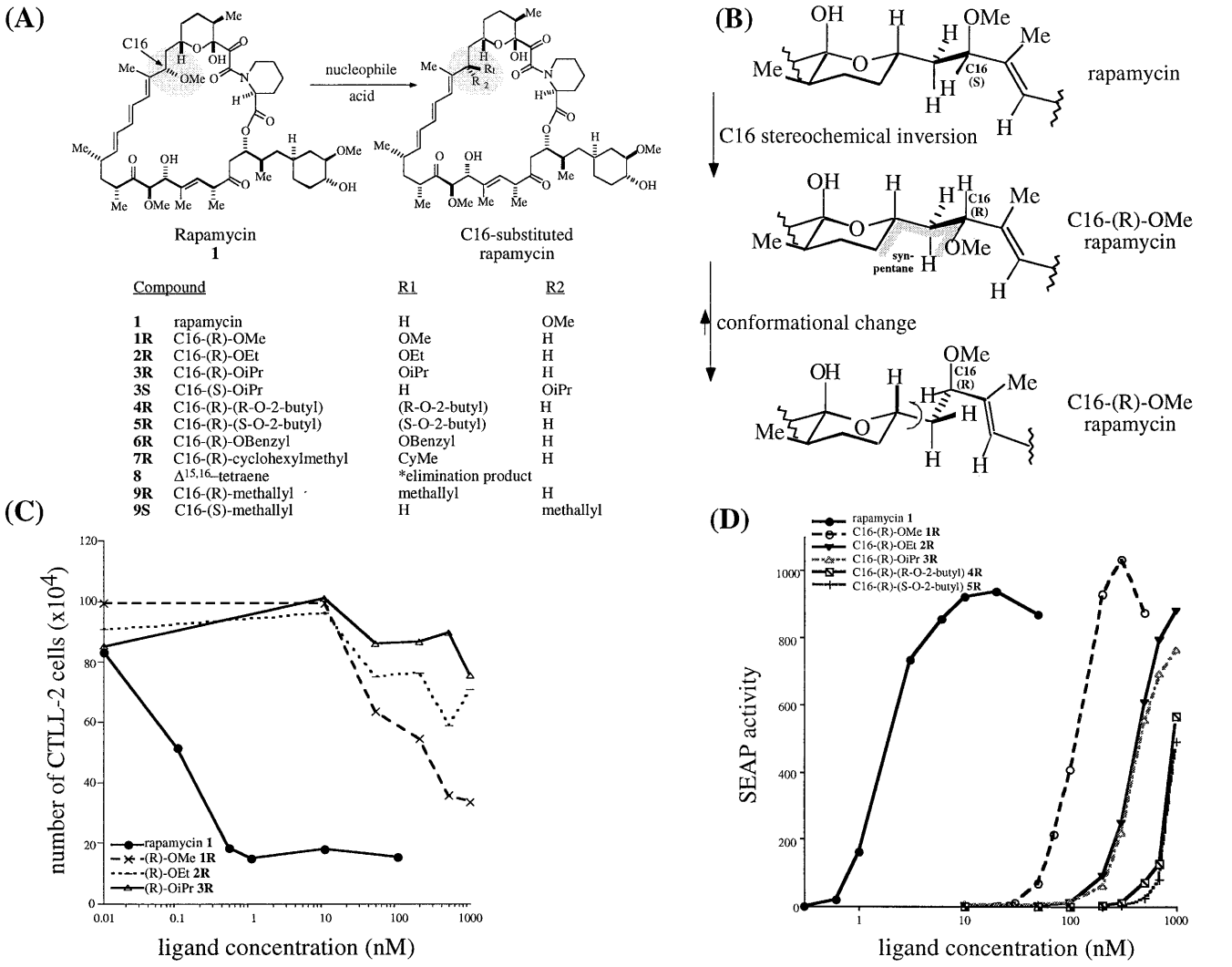
RESULTS

Conditional Gene Activation with Rapamycin. A cellular system allowing conditional gene activation with derivatives of rapamycin was developed in analogy to an earlier study (11) (Fig. 1*A*). Fusion constructs were prepared encoding the Gal4 DNA-binding domain linked to three tandem copies of FKBP12 (Gal4-FKBP3) and the VP16 activation domain linked to a single FRB domain (FRB-VP16). A SEAP reporter was used bearing five upstream Gal4-binding sites (UAS sites) and a minimal interleukin 2 basic promoter. (This reporter is referred to as UAS-SEAP.) Coexpression of these three constructs in T-antigen-transformed Jurkat cells leads to rapamycin-dependent activation of the SEAP reporter gene. Rapamycin stimulates SEAP activity 300- to 400-fold over background phosphatase levels from untransfected cells with an EC_{50} of 2–5 nM (Fig. 2*D*). Omitting any of the constructs or rapamycin results in the absence of any phosphatase activity above background levels (data not shown).

Synthesis and Evaluation of Nontoxic Rapamycin Derivatives. As described by Luengo *et al.* (23, 25), treatment of rapamycin with *p*-toluenesulfonic acid in the presence of various alcohols leads to S_N1 solvolysis of the C16-methoxy group. C16-(S)- and (R)-diastereomers are produced in approximately a 2:1 ratio and subsequently purified by HPLC. Compounds **1R** to **7R** of Fig. 2*A* were synthesized with this method.

The growth inhibitory properties of rapamycin derivatives **1R-5R** were assayed by monitoring their effects on CTLL-2 cell division. CTLL-2 cells are interleukin 2-dependent and highly sensitive to rapamycin ($IC_{50} < 1$ nM). However, CTLL-2 cell growth is not inhibited by similar concentrations of the C16-(R)-alkoxy diastereomers; the IC_{50}s for these compounds are significantly higher (Fig. 2*C*).

The C16-(R)-alkoxy rapamycins are also impaired in their ability to induce expression from the UAS-SEAP reporter in



Compound		R1	R2
1	rapamycin	H	OMe
1R	C16-(R)-OMe	OMe	H
2R	C16-(R)-OEt	OEt	H
3R	C16-(R)-OiPr	OiPr	H
3S	C16-(S)-OiPr	H	OiPr
4R	C16-(R)-(R-O-2-butyl)	(R-O-2-butyl)	H
5R	C16-(R)-(S-O-2-butyl)	(S-O-2-butyl)	H
6R	C16-(R)-OBenzyl	OBenzyl	H
7R	C16-(R)-cyclohexylmethyl	CyMe	H
8	$\Delta^{15,16}$-tetraene	*elimination product	
9R	C16-(R)-methallyl	methallyl	H
9S	C16-(S)-methallyl	H	methallyl

FIG. 2. Structure and activity of modified rapamycins. (*A*) Chemical structure of rapamycin and C16-substituted derivatives. (*B*) Conformational change induced in rapamycin by stereochemical inversion of C16. (*Top*) The local conformation surrounding C16 in rapamycin. (*Middle*) The "syn-pentane" conformation introduced by stereochemical inversion at C16. (*Bottom*) The rotations known to occur in C16-invertants that alleviate the syn-pentane strain. (*C*) Inhibition of CTLL-2 cell growth with rapamycin derivatives. CTLL-2 cells were grown to a density of 10^5 cells/ml in RPMI medium 1640 supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin, 20 mM Hepes (pH 7.4), sodium pyruvate (220 mg/liter), 50 μM 2-mercaptoethanol, and interleukin 2 (20 units/ml). Two hundred and fifty microliters of these cells were seeded in 8-well plates with 250 μl of media containing two times the appropriate ligand concentration. After 72 hr, cells were counted on a hematocytometer. (*D*) Transcriptional activation with rapamycin and C16-rapamycin derivatives. T-antigen-expressing Jurkat T cells were transfected with 1 μg of SEAP reporter, 1 μg of Gal4-FKBP3, and 4 μg of FRB-VP16. SEAP assays were performed as described (10).

cells cotransfected with Gal4-FKBP3 and FRB-VP16. As shown in Fig. 2*D*, the EC_{50}s of the C16-(R)-alkoxy derivatives are higher than those reported for rapamycin or the other (S)-alkoxy compounds (data not shown). Stereochemical modification at C16, while having no effect on the ability of rapamycin to bind to FKBP12 (23), significantly diminishes its ability to bind to and inhibit FRAP.

Selection of Receptors for Nontoxic Rapamycin Derivatives. A mammalian three-hybrid screen (Fig. 3) (10, 26) was used to identify compensatory mutations of FRB that restore binding to the structurally modified ligands in Fig. 2*A*. Positional libraries were constructed having individual sites of the FRB domain randomized to any of the 20 amino acids; the codon to be randomized was replaced with a 32-permutation mixture represented by the base pairs (GATC, GATC, and GC). Excluding A and T from the third base pair removed stop codons from the library and increased the proportion of less frequently represented amino acids. These libraries were then fused to the VP16 activation domain and sequenced to verify randomization at the indicated sites.

To screen these positional libraries, we used the mammalian transcription system already described, with Gal4-FKBP3 serving as the bait and the UAS-SEAP gene functioning as the reporter. T-antigen expressing Jurkat T cells were cotransfected with these two constructs and either wild-type FRB-VP16 or a positional library FRB-VP16 fusion. The cells were then treated with various concentrations of the modified rapamycins and assayed for SEAP production. As described earlier, wild-type FRB-VP16 requires high concentrations of the C16-(R)-alkoxy rapamycins to activate SEAP production. If a positional library contained members with improved affinity for these compounds, that library would require lower concentrations of the compounds to induce SEAP production. The amplitude of the SEAP signal should correlate with the percentage of proteins able to bind at a specific ligand concentration. Since 300- to 400-fold activation is typically achieved in the rapamycin-based transcription assay, a positional library containing only one protein that binds a modified rapamycin would activate transcription about 15- to 20-fold, a response well above background. When a library showed

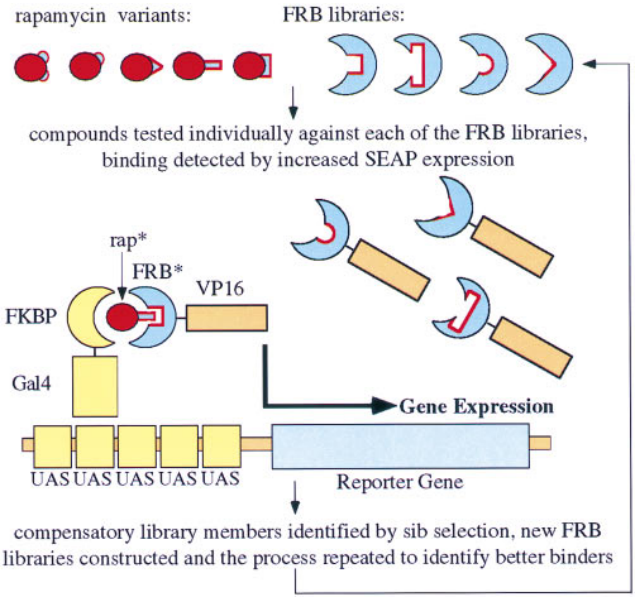


FIG. 3. A mammalian three-hybrid-based assay used to discover novel receptor–ligand complexes. Rapamycin derivatives were screened individually against FRB libraries. Compensatory binding mutations were detected by improved SEAP synthesis relative to wild type at low ligand concentrations. Those libraries with compensatory mutations were then deconvoluted and the compensatory clones were sequenced. Additional libraries were constructed in the context of early compensatory mutations and the process was repeated.

improved responsiveness to a nontoxic ligand, the active library member was determined by a sib-selection deconvolution scheme. Bacteria containing the positional library DNA were streaked onto Luria–Bertani–ampicillin plates and individual colonies, each containing one library member, were used to inoculate overnight cultures. These overnight cultures were grouped into several pools and transcription assays were performed with DNA purified from the pools. Members of those pools that showed improved responsiveness to a modified rapamycin were then tested individually in the transcription assay. The clones that activated transcription with the lowest concentrations of modified rapamycin were identified by DNA sequencing. Once a compensatory mutation was identified, new libraries were generated in the context of that mutation; double mutations were then selected that improved binding relative to the single mutation.

Using this technique, positional libraries were constructed at residues of the FRB domain in the rapamycin-binding site, as determined by analysis of the crystal structure of the FKBP12–rapamycin–FRB complex (Fig. 4). These libraries were screened for improved responsiveness to any of the nontoxic derivatives **1R-8**. After three sequential rounds of selection, a triple mutant FRB (termed FRB*) with the mutations T2098L, W2101F, and K2095P was identified that restored binding to one of the least toxic derivatives, C16-(R)-OiPr rapamycin (**3R**). Cotransfection of FRB*-VP16, Gal4-FKBP3, and UAS-SEAP leads to **3R**-dependent activation of the SEAP reporter gene with an EC_{50} below 10 nM. This ligand concentration is greater than two orders of magnitude below that required for inhibition of CTLL-2 cell division (Fig. 5)

Structure-Based Design of a More Readily Purified and Stable Compound That Binds Selectively to FRB*. During attempts to scale up the synthesis of **3R**, we encountered difficulties associated with its instability and with its tendency to coelute with the C16-diastereomer **3S** and trace quantities of unreacted rapamycin, both potent inhibitors of cell division. We therefore synthesized an isosteric variant of **3R**, compound **9R,** which has a carbon–carbon bond at C16 in place of the

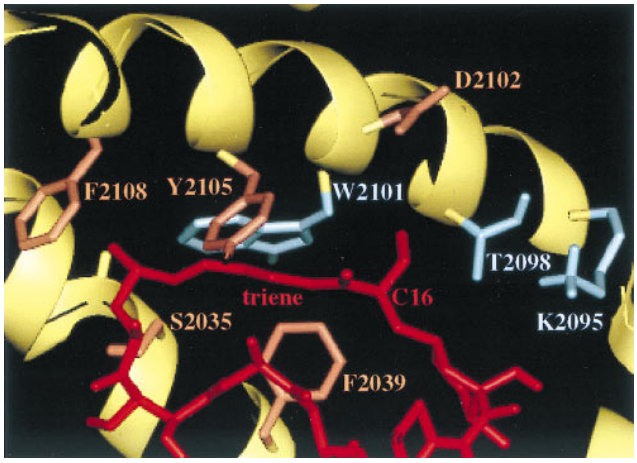
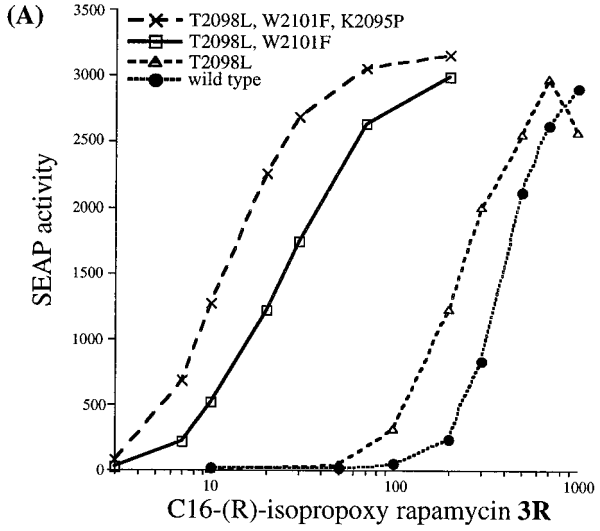


FIG. 4. X-ray structure of the FRB–rapamycin interface. Rapamycin is red and the FRB protein backbone is yellow. Some of the FRB residues selected for mutation are indicated as blue and brown; residues that led to compensatory binding to C16-(R)-isopropoxy rapamycin **3R** are blue. In the first round of selection, libraries were constructed at positions F2039, T2098, W2101, D2102, Y2105, and F2108. In the context of T2098L or T2098I, libraries were constructed at S2035, F2039, K2095, W2101, and D2102 for the second round of screening. For the third generation of libraries, positions L2031, A2034, S2035, Y2038, F2039, M2047, L2051, K2095, D2102, Y2104, Y2105, and F2108 were randomized in the context of T2098L, W2101F or T2098L, W2101L.

acid-labile carbon–oxygen bond in rapamycin and **3R**. Treatment of rapamycin with excess boron trifluoride etherate in the presence of methallyltrimethylsilane afforded **9R** and **9S** in approximately a 2:1 ratio. The predominant and desired **9R** diastereomer was purified by reverse-phase HPLC without elimination. The configuration at C16 was assigned based on a comparison of diagnostic ^1H resonances in the NMR spectra of **9R** and **9S** with those of other C16-rapamycin derivatives reported by Luengo *et al.* (25).

9R behaves similarly to **3R** in the transcription assay, in that it binds with high affinity to FRB*, but with low affinity to wild-type FRB (Fig. 5*B*). **9R** is also nontoxic to CTLL-2 cells ($IC_{50} > 1\ \mu M$) at concentrations required to induce gene expression. Due to its stability and ease of preparation, compound **9R** is the favored one among those reported here for use in future experiments involving inducible protein association.

Conditional Membrane Recruitment with a Nontoxic Rapamycin. To show the generality of using FRB* and **9R** (rap*) to regulate protein associations, we used this receptor–ligand pair to recruit a cytosolic protein to another protein docked at the membrane through an N-terminal myristoyl fatty acid (Fig. 1*B*). Previous work has shown that one or three tandem FKBP12s (MF1 or MF3) can be directed to the plasma membrane by addition of the first 14 amino acids of the v-src protein (2, 3), encoding a myristoyl-based membrane localization sequence. We used a coimmunoprecipitation assay to demonstrate that cytosolic proteins consisting of either the FRB or FRB* domains fused to the C-terminal kinase domain of FRAP (termed FRB kinase and FRB* kinase, respectively) can be recruited in a ligand-dependent manner to the plasma membrane of cells coexpressing MF3. A hemagglutinin tag was added to MF3 and a Flag epitope tag was added to FRB kinase or FRB* kinase. Cells coexpressing these constructs were treated with rapamycin or **9R** (Fig. 6, rap*), lysed, and immunoprecipitated with anti-hemagglutinin antibody. An anti-Flag Western blot was then obtained using the immunoprecipitates to analyze protein associations. Treatment of the cells with 20 nM rapamycin leads to highly effective recruit-



(B)

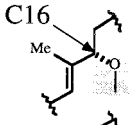
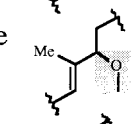
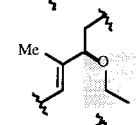
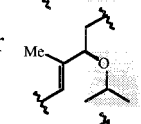
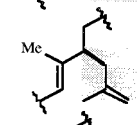
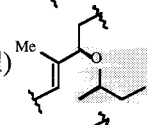
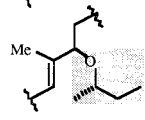
compound	structure	EC50 with FRB(nM)	EC50 with FRB*(nM)
rapamycin **1**		2-5	2-5
C16-(R)-OMe **1R**		100-300	70-100
C16-(R)-OEt **2R**		400-1000	20-30
C16-(R)-OiPr **3R**		400-1000	7-10
C16-(R)-methallyl **9R**		400-1000	10-20
C16-(R)-(R-O-2-butyl) **4R**		>1000	100-300
C16-(R)-(S-O-2-butyl) **5R**		>1000	70-100

FIG. 5. Inducible gene expression with novel receptor–ligand complexes. (*A*) Stepwise recovery of binding to C16-(R)-isopropoxy rapamycin **3R**. The dose-response curves correspond to the mutations with greatest affinity for **3R** identified after each round of selection. (*B*) The ability of different modified rapamycins to activate transcription with wild-type FRB-VP16 and FRB*-VP16. The **3R** and **9R** compounds both bind strongly to FRB* and can be used to activate transcription, but bind poorly to wild-type FRB and are thus nontoxic.

ment of wild-type FRB kinase, but treatment with 20 nM **9R** or no ligand do not. However, the FRB* kinase harboring the K2095P, T2098L, and W2101F mutations is recruited by either 20 nM rapamycin or 20 nM **9R**. The FRB* kinase does not coassociate with myristoylated FKBP12 in the absence of ligand.

DISCUSSION

A general way to regulate protein associations with nonimmunosuppressive rapamycin derivatives has been developed.

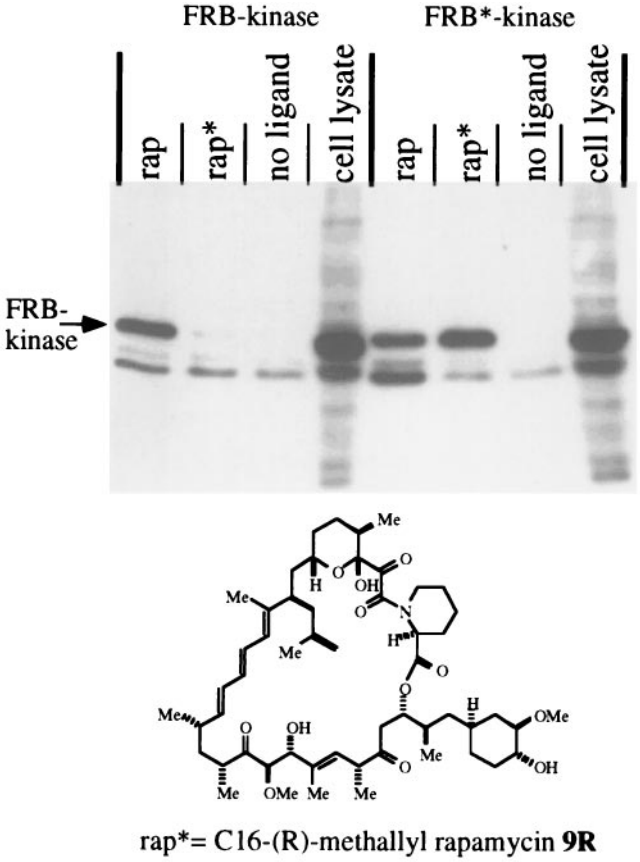


FIG. 6. Coimmunoprecipitation assay demonstrating conditional membrane recruitment with a nontoxic rapamycin derivative, rap* (**9R**). T-antigen expressing Jurkat cells were cotransfected with 0.5 μg of MF3 and 4 μg of FRB kinase (lanes 1–4) or FRB* kinase (lanes 5–8). After 48 hr, the cells were incubated for 30 min with ligand [20 nM rapamycin, lanes 1 and 5; 20 nM C16-(R)-methallyl rapamycin (rap*), lanes 2 and 6; no ligand, lanes 3 and 7], washed with cold PBS, and lysed in PINT buffer (18) (0.3% Triton X). Aliquots were removed for the cell lysate (lanes 4 and 8), and the remainder was pelleted at 14,000 rpm for 10 min to separate the nuclear fraction, and immunoprecipitated with anti-hemagglutinin antibody and protein A-conjugated to beads. The immunoprecipitates were washed extensively with PINT buffer and the bound proteins were eluted with soluble peptide corresponding to the Flu epitope. The eluted proteins were separated by SDS/PAGE and detected by Western blot analysis with anti-Flag M2 antibody. The structure of rap* (**9R**) is shown below the Western blot.

Ligands that are unable to bind to the rapamycin target FRAP were synthesized, and compensatory mutations in the FRB domain of FRAP that restore binding to these compounds were selected in a mammalian three-hybrid screen. These novel ligand–receptor complexes were used to induce the expression of a reporter gene and the membrane recruitment of a cytosolic protein.

In previous studies, compensatory modifications to cyclophilin and its ligand cyclosporin were prepared using site-directed mutagenesis and chemical synthesis, respectively (24). The modifications were designed based on an analysis of the x-ray structure of a cyclophilin–cyclosporin complex. The result was a cyclosporin variant (CsA*) that fails to bind wild-type cyclophilin, but that binds a triple mutant of cyclophilin (Cyp*) with greater affinity than that of the native complex. In this study, we used the x-ray structure of the FKBP12–rapamycin–FRB ternary complex to identify interacting residues on rapamycin and FRAP that could be altered to create novel receptor–ligand complexes. Several rapamycin derivatives were synthesized in which the C16-stereochemistry

was inverted and the C16-side chain enlarged. These compounds neither bind the FRB domain nor inhibit FRAP function, as demonstrated in the FRB-mediated transcription and CTLL-2 cell inhibition assays, respectively. Since the structural distortion induced by altering the stereochemistry at C16 affects the interactions of rapamycin with several FRB residues, the "rational" approach to designing compensatory mutations used with cyclophilin–cyclosporin may have proved difficult. Instead, a rapid library-based mammalian three-hybrid screen was developed to find a binding solution. The assay involved mutagenizing several positions that surround the C16-substituent on the rapamycin framework in multiple rounds, deconvoluting at each round to identify mutations that allowed C16-modified rapamycins to activate transcription at increasingly lower concentrations.

Three FRB mutations that restore binding to **9R** (rap*; this methallyl derivative of rapamycin is envisioned as the preferred CID for future experimentation based on its ease of synthesis and purification, its chemical stability, and its lack of toxicity and anticipated stability *in vivo*) and **3R** were identified (the triple mutant is referred to as FRB*). These mutations all lie on one face of an α-helix in the rapamycin-binding pocket of FRB. The mutations T2098L and K2095P may help accommodate the structural distortion created by triene rotation and, based upon the results summarized in Fig. 5*B*, the mutation W2101F appears to create a specificity pocket for C16-side chains similar in size to a leucine residue, such as the isopropoxy and methallyl moieties. These mutations provide a receptor still suited for binding to unmodified rapamycin, as evidenced by the transcription and membrane recruitment assays.

The rap* compound has been used to control gene expression and protein subcellular localization *in vivo*. Inducible gene expression systems, including the rapamycin (11)-, tetracycline (27)-, RU486 (28)-, and ecdysone-based (29) systems have been successfully applied to animal studies. For example, in transgenic mice, the tissue-specific expression of calcium–calmodulin-dependent kinase II has provided insights into the mechanisms of memory formation (30), the controlled production of cre-recombinase has been used for the inducible ablation of genes (31), and the regulated expression of human growth hormone has led to small molecule-dependent control of animal body size (11, 28). Spatial, temporal, and quantitative control of gene expression will provide a mechanism both to study the function of specific genes *in vivo* and to control the concentration levels of therapeutic proteins in the context of gene therapy.

Inducible membrane recruitment of cytosolic signaling molecules using CIDs is sufficient to activate a variety of pathways, including those propagating both growth-promoting (J. Yang and S.L.S., unpublished results) and death-promoting signals (10). We have demonstrated that a cytosolic FRB* fusion protein containing a kinase domain can be recruited to a myristoylated docking protein at nontoxic ligand concentrations. By fusing FRB* to various signaling proteins, these proteins should be recruited to the plasma membrane and possibly other organelles and sites in the cell, including the nucleus (10). The ease of synthesis and purification of rap* (**9R**), combined with its ability to permeate many cells (including yeast cells) and tissues, and its inability to bind to and inhibit endogenous FRAP, suggests that rap* will be a useful reagent for inducing the association of two target proteins, both in tissue culture and in transgenic animals.

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