Chemistry & Biology *13*, 99–107, January 2006 DOI 10.1016/j.chembiol.2005.10.017

Rapamycin Analogs with Differential Binding Specificity Permit Orthogonal Control of Protein Activity

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Summary

Controlling protein dimerization with small molecules has broad application to the study of protein function. Rapamycin has two binding surfaces: one that binds to FKBP12 and the other to the Frb domain of mTor/FRAP, directing their dimerization. Rapamycin is a potent cell growth inhibitor, but chemical modification of the surface contacting Frb alleviates this effect. Productive interactions with Frb-fused proteins can be restored by mutation of Frb to accommodate the rapamycin analog (a rapalog). We have quantitatively assessed the interaction between rapalogs functionalized at C16 and C20 and a panel of Frb mutants. Several drug-Frb mutant combinations have different and nonoverlapping specificities. These Frb-rapalog partners permit the selective control of different Frb fusion proteins without crossreaction. The orthogonal control of multiple target proteins broadens the capabilities of chemical induction of dimerization to regulate biologic processes.

Introduction

Conditional regulation of gene activity greatly facilitates the analysis of a gene's function by isolating a loss or gain of function to discrete periods of time. Several techniques achieve conditional regulation, but temporal control of gene function at the protein level with small molecules can greatly enhance the kinetics of experimental regulation relative to strategies that rely on gene deletion, transcription, or inhibitory RNAs.

We have developed an alternative approach to conditional protein regulation that combines the specificity possible with genetic techniques with the experimental tractability of pharmaceutical intervention. This system uses one class of small molecules to govern the activity of a broad number of protein targets made sensitive to the drug by fusion to a drug binding protein domain. It is based on the biologic principle that proximity commonly regulates protein activity [1] and that chemical induction of homo- or heterodimerization with small molecules allows experimental control over the biochemical activity of the target protein [2, 3]. The macrolides rapamycin and FK506 act by inducing the heterodimerization of cellular proteins. Each drug binds with high affinity to the FKBP12 protein, creating a drug-protein complex that subsequently binds and inactivates mTOR/FRAP and calcineurin, respectively [4–6]. The *F*KBP-*r*apamycin *b*inding (Frb) domain of mTOR has been defined [7] and applied as an isolated 89 amino acid protein moiety that can be fused to a protein of interest. Rapamycin can then induce the approximation of Frb fusions to FKBP12 or proteins fused with FKBP12 [1, 8–10]. Several applications for this technology have been devised, including the dimerization and activation of cell surface receptors that control cell growth, differentiation, and death [2, 11, 12], subcellular localization of proteins [13–17], and recruitment of transcriptional control units to DNA binding proteins [8, 9, 18, 19]. More recently, a technique has been devised in which latent protein targets fused to an unstable variant of Frb are stabilized by the drug-directed recruitment of FKBP [10].

Rapamycin has several properties of an ideal dimerizer: it has a high affinity ($K_D < 1$ nM) for Frb when bound to FKBP12, and is highly specific for the Frb domain of mTor. Rapamycin is an effective therapeutic immunosuppressant with a favorable pharmacokinetic and pharmacodynamic profile in mammals; however, rapamycin is a potent teratogen, discouraging its use for whole animal protein dimerization studies [20, 21]. In fact, treatment with rapamycin during mouse embryogenesis gives the same embryonic lethal phenotype as the mouse mTor mutant, *flat-top* [20] supporting the single-target specificity of rapamycin. Modification of the surface of rapamycin contacting Frb [22] can prevent binding to mTor and mitigate the inhibitory effects of rapamycin [10, 23]. Compensatory mutation of the Frb domain to form a surface that accommodates the "bumped" rapamycin restores dimerizing interactions only with the Frb mutant and not to the endogenous mTor protein [23]. Reformation of the binding interaction with the rapamycin analog or "rapalog" C20-methallylrapamycin (C20-Marap) was selected in a triple mutant Frb termed Frb*.

In our recent studies, we have created Frb* fusions with two mouse genes, *GSK-3β* and *Pax6*, by homologous recombination of *Frb** coding sequences at the target genes ([10] and J.H. Bayle et al., submitted). In each case, the *Frb** fusion allele produces an unstable protein with a loss-of-function phenotype in vivo, while treatment with C20-Marap recruits FKBP and stabilizes the latent GSK-3βFrb* or Frb*Pax6 protein to rescue protein function. The rapid and reversible stabilization of Frb*-tagged proteins has the potential to be a powerful conditional allele system applicable to broad classes of cytosolic proteins [10]. However the pharmacokinetics of C20-Marap in vivo are poor.

To improve the technology for whole animal studies, we wished to find rapalogs that also have specificity for Frb* or other mutant Frb domains. Our first goal was to improve the understanding of the efficient binding of C20-Marap with Frb* relative to Frb at the primary structural level. We then screened new variant Frbs against a group of rapalogs to find new drug-mutant combinations that may have improved specificity or pharmacokinetics. As a result, we have built an array of Frb mutants that have separate but overlapping specificities for

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rapalogs derived at C20 or C16. The discrete specificity of individual drug-mutant combinations permits the development of dimerizer strategies that operate in an independent or orthogonal manner in the same cell environment. Each mutant was also assessed for its contribution to the stability of the Frb domain to determine if the altered protein conformations underlying drug selectivity were also responsible for destabilization of Frb. These results are reported by J.E. Gestwicki et al. (submitted).

Results

The minimal Frb domain comprises amino acids 2025–2114 of human mTor. Frb* is a triple mutant resulting in the following amino acid substitutions in the 89 amino acid four-helical Frb bundle: Lys2095 to Pro, Thr2098 to Leu, and Trp2101 to Phe. To understand the basis for the specificity of C20-Marap for Frb* over wild-type Frb, we have systematically analyzed the contribution of each of the three substituted amino acids in Frb* by making single and double mutant combinations from the wild-type. The nomenclature for these mutants is in a three letter code representing the amino acids positions that vary in Frb*, with the wild-type denoted as KTW for Lys2095, Thr2098, and Trp2101. Frb* is denoted as PLF in this system to represent the Pro, Leu, and Phe substitutions at the corresponding positions.

Binding of rapamycin and C20-Marap was assessed in a rapalog-dependent transcriptional switch in which three copies of human FKBP12 were fused to the yeast GAL4 DNA binding domain and the mutant (or wild-type) Frb domain was fused to the potent transcriptional activation domain from the herpes simplex virus VP16 protein [8]. Coexpression of these recombinant constructs in COS1 cells, together with a secreted bacterial alkaline phosphatase (SeAP) reporter that contained Gal4-specific DNA binding elements, promoted SeAP expression only in the presence of a rapalog capable of interacting with FKBP and the Frb variant (Figure 1A). Transfected cells were split into identical pools and dosed in triplicate or quadruplicate with increased concentrations of rapamycin or C20-Marap. With the wild-type Frb domain (KTW) fused to VP16, increasing rapamycin or rapalog concentrations stimulated SeAP activity with a sigmoid dose dependence curve (Figure 1B). The half-maximal activity (EC_{50}) was 0.95 nM for rapamycin-FKBP binding to KTW. This EC_{50} was close to the documented binding constant for rapamycin-FKBP to the wild-type Frb [7], demonstrating that the transcriptional switch provided a good indicator of the strength of drug-FKBP binding to the Frb domain. In the same assay with the wild-type Frb (KTW), C20-Marap was able to stimulate reporter activity only at high drug concentrations, and maximal reporter activity could not be attained (Figure 1B). In contrast, the triple mutant Frb(PLF) gave strong reporter activity at low concentrations of C20-Marap (Figure1C). This finding indicates that the lack of interaction of C20-Marap with KTW was not due to impaired interaction with FKBP (which was common to both assays), but rather a steric prevention of interaction between the side chains of KTW with methallyl-bumped drug. The steric bulk on C20-Marap was accommodated by the amino acid substitutions on PLF, while binding of rapamycin itself was not affected (Figure 1C). These results are in agreement with those previously reported [10, 23].

The three amino acid substitutions in PLF confer specificity for C20-Marap though it was unknown whether all three mutations were required. To assess the importance of each substituted amino acid to the accommodation of the methallyl bump, we tested double mutants that contained combinations of the substitutions in PLF. Reversion to Trp2101 yielded a Frb double mutant, termed PLW. This mutant reduced C20-Marap binding (Figure 1D), while reversion at either of the other positions (Thr2098 in PTF or Lys2095 in KLF) had less or no effect on C20-Marap interaction (Figures 1E and 1F). We found that rapamycin had a higher EC_{50} for the PTF construct relative to PLF or KTW, while C20-Marap bound PTF and PLF with equal efficiency. These results indicate that the most important site of steric inhibition was amino acid 2101, and a principal source of drug specificity was likely the plasticity afforded the phenyl of Phe at this position to accommodate the methallyl on C20-Marap. This proposal was supported by the relatively efficient interaction of C20-Marap with the single mutant KTF in which only Trp2101 was modified from wild-type (Figure 2D).

The C20-Marap-PLF combination is ideal for in vivo studies, as C20-Marap does not bind effectively to the wild-type Frb domain of mTor; however, C20-Marap is more unstable in animal serum than rapamycin and other rapalogs. Therefore, we tested other rapalogs derivatized at the interacting surface with Frb for specificity to Frb mutants at Trp2101 and Thr2098. Interestingly, we observed a specificity for one derivative, C16-(S)-butylsulfonamidorapamycin (C16-BSrap) opposite to that of C20-Marap (Figure 2). C16-BSrap bound efficiently to the wild-type Frb (KTW), but not to PLF or the single mutant KTF, indicating that the Trp to Phe variation was the source of the different specificity between these two rapalogs. The parent drug, rapamycin, was capable of binding to each mutant.

This observation led us to develop and screen a group of rapalogs substituted at C16 or C20, positions directly opposing helix 4 of Frb, for specific interactions with Frb mutants. Some rapalogs added steric bulk at these positions with a planar aromatic group or with groups that have a wider conformational space, while other modifications were predicted to add flexibility to rapamycin. The chemicals were synthesized for this study or procured from Ariad Pharmaceuticals (Cambridge, MA) or Novartis AG (Basel, Switzerland). One interesting rapalog was synthesized by acid-mediated production of a carbocation at C16 that was quenched with 3-methylindole to produce C16-(S)-3-methylindolerapamycin (C16-iRap). This rapalog had an Frb specificity profile that differed from both C16-BSrap and C20-Marap. Substitution of Phe for Trp2101 prevented binding of C16-iRap only in the context of Thr at position 2098 (Figure 3). Substitution to Leu 2098 in the KLF double mutant reversed the steric block in KTF and permitted binding of C16-iRap with an EC_{50} similar to KLW. Similarly, Thr2098 was permissible only in the context of Trp at 2101.

The number of mutants screened against the rapalogs described above was expanded to include combinations

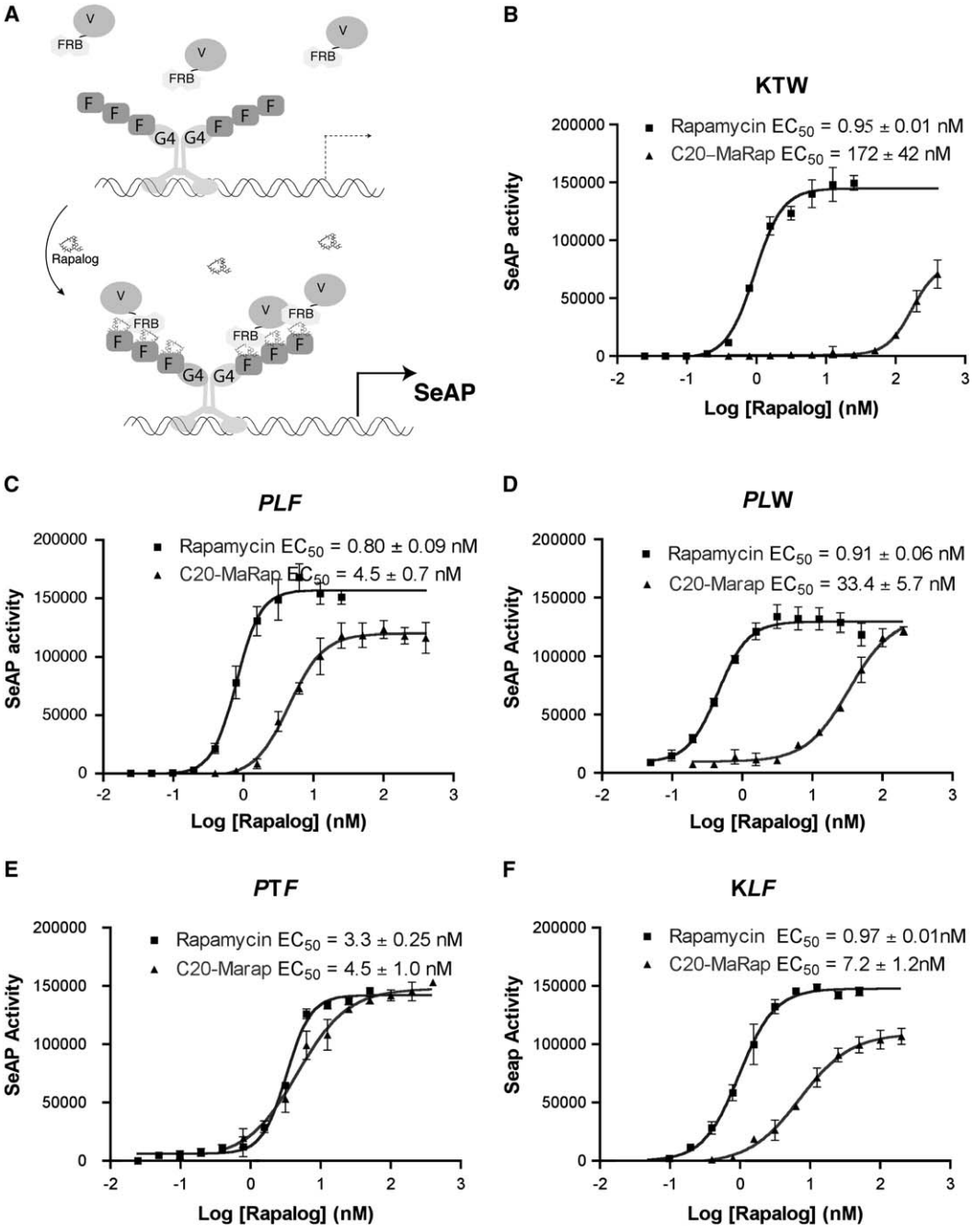

Figure 1. C20-Methallylrapamycin Binds Specifically to a Mutant Frb Domain Containing Phe at Amino Acid 2101

(A) Schematic diagram of rapamycin- or rapalog-directed transcription switch. FKBP12 is fused to the yeast Gal4 DNA binding domain, which is tethered to reporter plasmid DNA containing Gal4-specific DNA recognition elements. In the absence of drug, transcription of secreted bacterial alkaline phosphatase is low. Inclusion of rapalog recruits Frb fused to a VP16 transcriptional activation domain to the promoter and SeAP transcription is stimulated.

(B–F) SeAP activity in transfected COS cells containing the transcriptional switch and stimulated with increasing concentrations of rapamycin (squares) or C20-methallylrapamycin (C20-Marap; triangles). Frb-VP16 constructs are denoted above the graphs in single-letter amino acid code indicating positions 2095, 2098, and 2101 with (B) utilizing Lys2095, Thr2098, and Trp2101 (the wild-type) and (C) Pro2095, Leu2098, and Phe 2101 (aka Frb*). Residues mutated from the wild-type are red and italicized.

Error bars represent ± 1 SD.

with alanine, proline, or threonine substituted for Lys2095, and additional substitutions at Thr2098 were also screened. The results of this screen standardized between experiments with the EC_{50} of rapamycin against Frb KTW are presented in Table 1. Several of these mutants, notably those with Phe at amino acid 2101 and Thr at 2098, had a reduced affinity for rapamycin (compare KTF with KTW or KLF; similarly, compare PLF with PTF) and, in some cases, appeared to bind effectively to other drugs (compare C20-Marap and rapamycin on

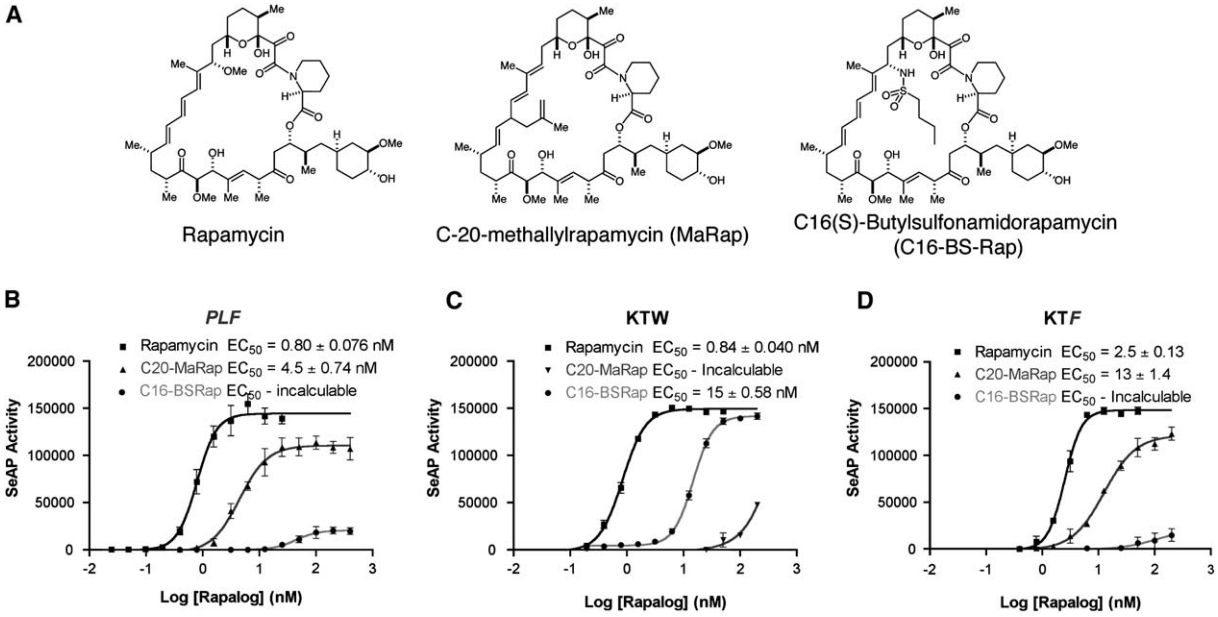

Figure 2. Discrete Frb Binding Specificities Are Conferred by Modification of Rapamycin

(A) Chemical structures of rapamycin, C20-Marap and C16-(S)-butylsulfonamidorapamycin (BS-Rap).
(B–D) SeAP reporter activity in the rapalog-dependent transcriptional switch plotted at increasing concentrations of rapamycin (squares), C20-Marap (triangles), or C16-BSrap (circles) with the Frb variant PLF (B), KTW (C) or KTF (D).
Error bars represent ± 1 SD.

PTF [Figure 1E]). In general, variation at Lys2095 had little effect on drug binding. C16-BSrap and C16-AiRap bound poorly to all mutants with Phe at 2101 despite the context of mutations at the other positions. C16-(S)-7-methylindolerapamycin (AP21967, also denoted C16-AiRap in this study), another indole derivative at C16, but with the indole inverted relative to the linkage of C16-iRap, does not interact efficiently with wild-type Frb (KTW), and has an intermediate binding for mutants with Leu 2095 only if Trp2101 remains wild-type (the

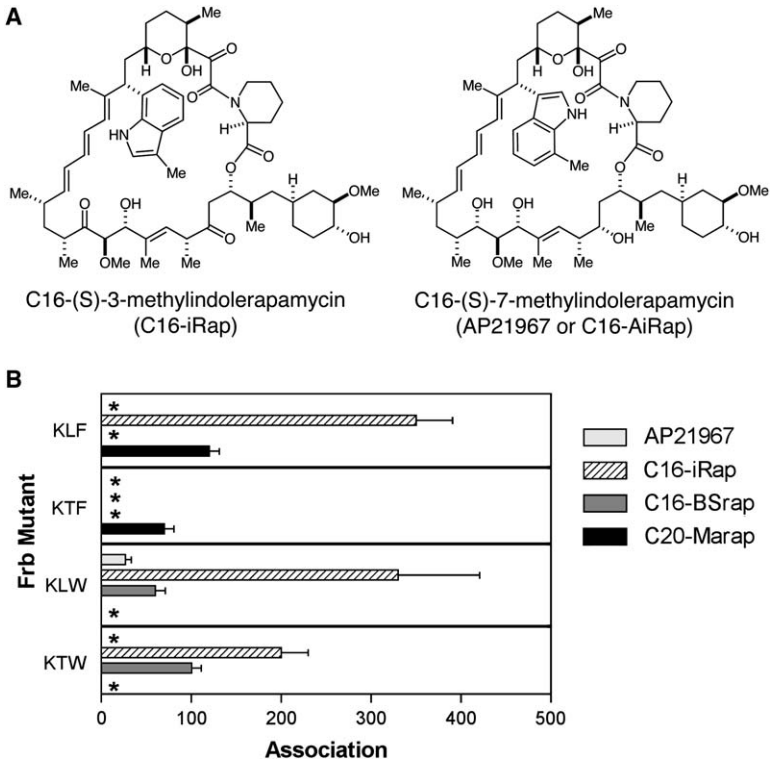

Figure 3. Binding Specificity of Bumped Rapalogs Depends on Variation at Frb Amino Acids 2098 and 2101

(A) Chemical structures of C16-(S)-3-methylindolerapamycin (C16-iRap) and C16-(S)-7-methylindolerapamycin (AP21967/C16-AiRap).
(B) SeAP reporter assays were performed with Frb variants KTW, KLW, KTF, and KLF with increasing concentrations of C20-Marap (red), C16-BSrap (orange), C16-iRap (green), and AP21967 (black). Results are plotted as the inverse of the EC_{50} for the drug-Frb variant combination in megamolar scale. Low (or zero) values thereby indicate a high EC_{50}. *Interaction too weak to be calculated.
Error bars represent ± 1 SD.

Table 1. EC_{50} of Rapamycin and Four Rapalogs for Frb Variants Annotated According to the Amino Acids at Positions 2095, 2098, and 2101

Mutant Frb	Rapamycin	C20-Marap	C16-BSrap	C16-iRap	AP21967 (C16-AiRap)
KTW	0.45	—[a]	2.7	2.3	—[a]
PLF	0.80	4.5	—[a]	6.1	—[a]
KLW	3.2	52	16	1.2	37
PLW	5.0	30	26	13	26
TLW	2.1	34	7.4	4.6	19
ALW	1.7	34	7.5	4.3	19
PTF	3.3	4.5	—[a]	—[a]	ND
ATF	2.7	15	—[a]	—[a]	ND
TTF	3.0	13	—[a]	—[a]	ND
KLF	0.93	4.5	—[a]	6.1	—[a]
PLF	0.8	4.5	—[a]	6.4	—[a]
TLF	1.6	8.5	—[a]	4.3	—[a]
ALF	1.6	1.0	—[a]	12	—[a]
KTF	1.8	9.3	—[a]	—[a]	—[a]
KHF	1.5	6.5	—[a]	14	ND
KFF	2.2	15	—[a]	—[a]	ND
KLF	0.93	4.5	—[a]	12	—[a]

Values are measured in the rapalog-dependent transcriptional switch and restandardized for transfection efficiency between experiments according to the EC_{50} of rapamycin against KTW or PLF. ND, not determined.

[a] A half-maximal concentration greater than 150 nM that cannot be determined accurately.

"LW" combination) (Table 1 and Figure 3). C20-Marap had a similar EC_{50} as AP21967 if Thr2098 was changed to Leu in the context of Trp at 2101 (compare KTW with KLW), but the binding was dramatically improved if Trp2101 was changed to Phe. While little specific effect on drug binding was observed if Lys2095 was mutated, variation at this position could influence the stability of the Frb domain in combination with substitutions at Thr2098 (J.E. Gestwicki et al., submitted).

Orthogonal Rapalog-Dependent Systems

Drug-dependent protein dimerization has many applications for the control of protein function in vivo, including the inducible stabilization of targets fused to an unstable Frb mutant, the ligand-directed activation of signal transduction, the selective control of transcription, and the subcellular localization of target proteins. The specificity of rapalogs with different derivative groups for interaction with individual Frb mutants provided a foundation to develop multiple rapalog-dependent systems that share components but can be regulated independently.

To demonstrate the possibilities and efficacy of orthogonal regulation, we attempted to regulate the subcellular compartmentalization of a single target protein differentially using specific rapalogs. GSK-3β is a kinase and a key regulator of multiple intracellular signaling pathways that is normally localized both in the nucleus and cytoplasm. GSK-3β was fused with FKBP12 and coexpressed with two different Frb mutants tagged with orthogonal subcellular targeting signals. Frb(TLW) was fused to an 11 amino acid nuclear export sequence derived from the Rev protein of human immunodeficiency virus to produce TLW-exp, and KTF was fused to two copies of the 7 amino acid nuclear localization sequence from SV40 Large T antigen.

In the absence of drug, GSK-3β was observed both in the cytoplasm and nucleus (Figure 4A). C20-Marap binds efficiently to KTF, but inefficiently to the TLW mutant. Addition of 10 nM C20-Marap resulted in localization of GSK-3β to the nucleus due to dimerization of the GSK-3β-FKBP target, with KTF-Imp in preference to TLW-Exp (Figure 4C). Stimulation with C16-BSRap had the opposite targeting effect, and GSK-3β-FKBP was rapidly localized to the cytoplasm by selective recruitment of the KTW-Exp (Figure 4B). Interestingly, the majority of cells incubated with rapamycin also localized GSK-3β-FKBP to the cytoplasm (Figure 4D).

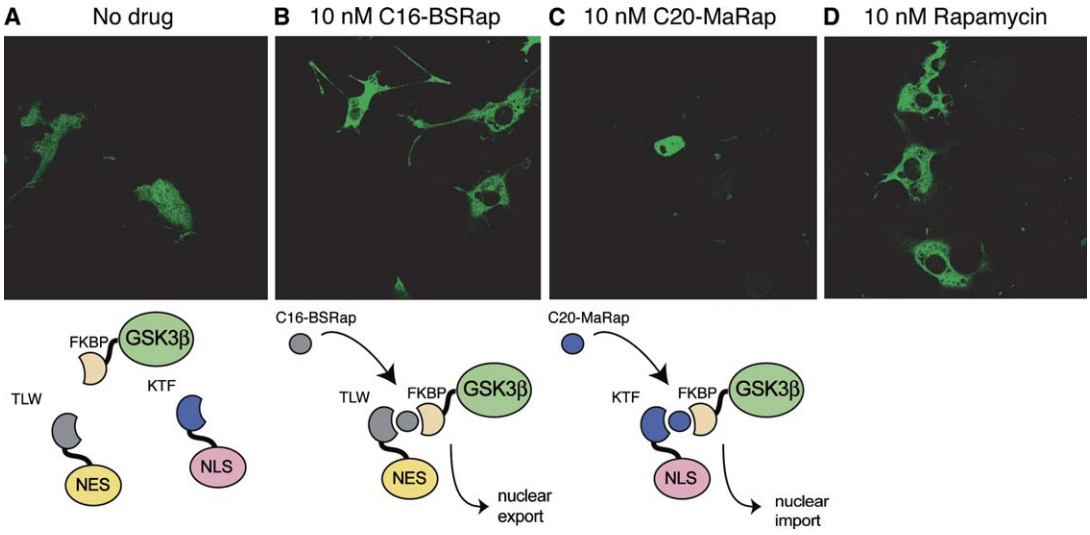

Figure 4. Orthogonal Control of GSK-3β Subcellular Localization with Specific Rapalogs

FKBP-GSK-3β-GFP is coexpressed with Frb(TLW)-NES and Frb(KTF)-NLS. GFP is visualized in transiently transfected COS1 cells without stimulation where GSK-3β is localized to both cell compartments (A) or with 1 hr stimulation with (B) 10 nM C16-BSrap, (C) 10 nM C20-Marap, or (D) 10 nM rapamycin. Illustrations beneath each figure depict the drug-selective recruitment of either TLW-exp or KTF-imp to FKBP-GSK-3β to direct nuclear import or export.

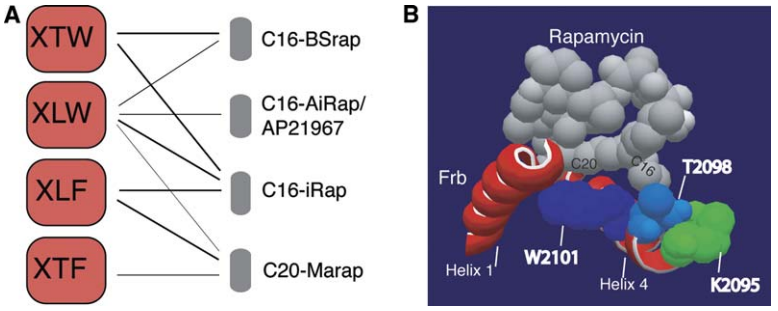

Figure 5. Summary of Alterations at Amino Acids 2098 and 2101 in Frb that Underlie the Specificity of Given Rapalogs

(A) Different Frb varients are listed in illustated form with "X" representing amino acid 2095. The relative strength of interaction with the rapalogs listed on the right is indicated by the thickness of the line.

(B) Ribbon diagram of the crystal structure of the Frb-rapamycin complex [22] with the amino acids altered in this study space-filled as indicated along with rapamycin (gray).

Discussion

Our identification of the amino acids critical to the specificity of C20-Marap for Frb*/PLF has uncovered new specific drug-protein interactions that will broaden the capabilities of induced dimerization to regulate biologic function. Specifically, we found that amino acids 2098 and 2101 underlie the binding specificity of rapalogs derivitized at C16 and C20. Substitution with phenylalanine at 2101 permits strong binding of C20-Marap, while this substitution prevents binding of the C16 derivatives. If threonine 2098 is replaced with leucine, binding of C16-AiRap/AP21967 and C20-Marap is enhanced and C16-iRap binding interactions are reformed in the context of Trp at 2101. Thus, each of the rapalogs has a unique, partially overlapping, and occasionally opposite binding specificity for Frb mutant combinations at 2098 and 2101: C20-Marap with "TF" and "LF," C16-BSrap with "TW" and "LW," C16-iRap with "TW," "LW" and "LF," and C16-AiRap/AP21967 with "LW" exclusively (Figure 5A).

In the crystal structure of FKBP-rap in complex with the Frb domain, Trp2101 is partially buried between helix 4 and helix 1 of the Frb four-helix bundle. The planar aromatic surface of the indole side chain opposes the coordinated triene between C17 and C22 of rapamycin [22]. It appears that the steric bulk of the methallyl group of C20-Marap directed into the indole ring can be accommodated by substitution of the indole for the phenyl ring of Phe. The methallyl addition at C20 also breaks the conjugation of the triene and may permit greater torsional flexibility in the drug as well as adding bulk. It is not known how the altered rapamycin conformation may contribute to the drug's specificity; however, the increased flexibility of the rapamycin backbone may contribute to the drug's instability in animal serum.

The different binding specificities of C16-iRap and C16-AiRap/AP21967 are particularly interesting, as each rapalog has a planar indole substitution in the same stereoconformation but with a different linkage to C16. Unlike other rapalogs with bulky additions at C16, C16-iRap will bind to Frb mutants with a Trp2101-to-Phe substitution, but only if Leu is at position 2098. The indole ring in either orientation points into the interface with helix 4 of the Frb domain in close proximity to amino acids 2098 and 2101. There is relatively little bulk added by substituting Phe for Trp, and it appears confounding that substitution of Leu, with a bulky side chain, for Thr at 2098 should accommodate C16 bumps on rapamycin. It is possible that these substitutions accommodate the bumps by altering the secondary structure of helix four slightly. Supporting this notion, the $\Delta\Delta G$ of the Frb domain is greatest when Leu is substituted for Thr at 2098 (J.E. Gestwicki et al., submitted).

These studies are part of our development of rapalog-directed dimerization as a method for conditional protein regulation in vivo. We chose to make discrete site-directed mutations that were primarily reversions of the Frb*(PLF) triple mutant to refine our understanding of the basis for the specificity of C20-Marap for PLF over the wild-type (KTF) and determine if this specificity could be separated from the structural instability of PLF. A further screen of randomly generated mutations, such as a recursive analysis in mammalian cells [23] or yeast [24], will likely yield mutant Frbs with enhanced affinity for the individual rapalogs described here and therefore enhanced specificity for orthogonal regulation of Frb-tagged proteins.

The Frb mutants described in this article have been examined for their thermal stability relative to wild-type (KTW) and their ability to be stabilized in vivo in the presence of rapamycin (J.E. Gestwicki et al., submitted). Importantly, the combinations of amino acid substitutions that destabilize Frb, and therefore lead to the greatest degree of induced stabilization, can be separated from those that determine drug specificity. This has important consequences for the utility of rapalogs in conditional systems that act in a "drug-on" fashion, such as the drug induction of a latent, destabilized Frb fusion, versus a "drug-off" system, such as the drug-targeted mislocalization of an active nuclear protein to the cytoplasm. It should be noted that saturation of a target protein with the drug-targeting element is likely to be required to give productive mislocalization.

The overlapping (and in some cases unique) specificity of individual rapalogs for different Frb mutants broadens the possibilities for the conditional regulation of protein function with rapalog-directed dimerization (Figure 6). Multiple target proteins can be coexpressed as fusions with different Frb variants to permit their coordinated, orthogonal regulation. As an example, two transcription factors can be expressed, one fused with KTW and the other with KTF with the exporter FKBP-NES also expressed (Figure 6A). In the presence of C16-BSrap, only the KTW-fused target is exported from the cell nucleus, while C20-Marap will induce the export of the KTF-fused target.

Like most chemically regulated biologic systems, the rapalog-induced effect is reversed after treatment is discontinued or replaced with a competitor. This property can be utilized selectively by treatment with a drug that binds to both Frb mutants to produce a response, and

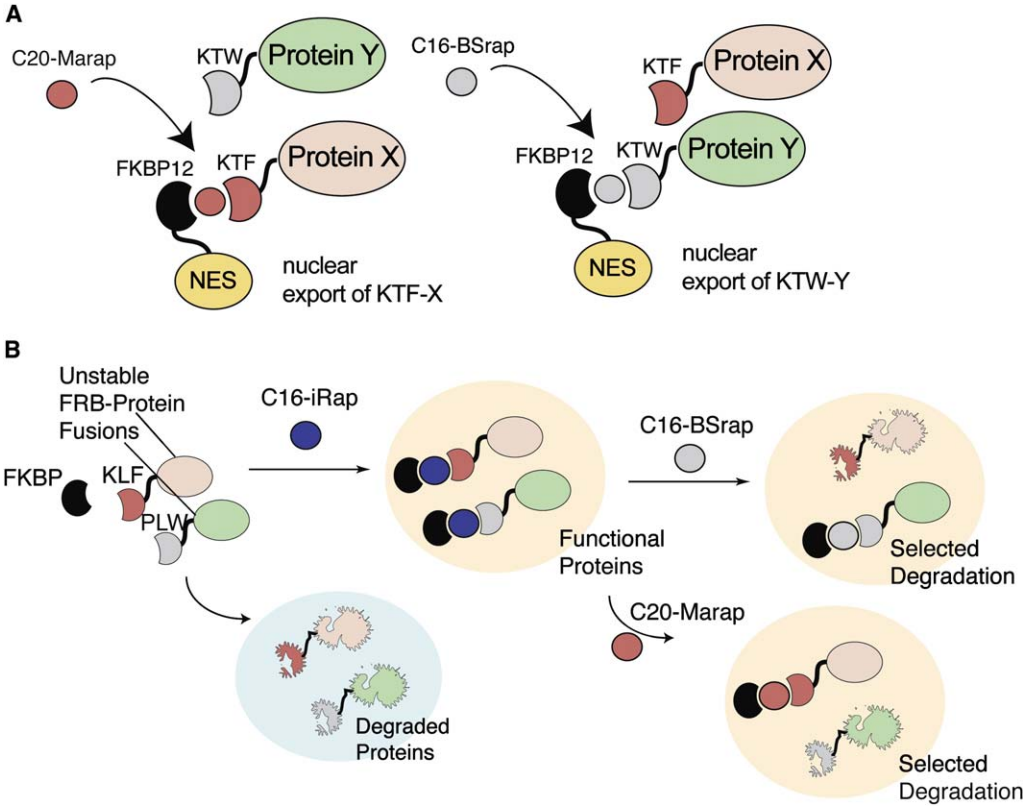

Figure 6. Schematics of Applications for Orthogonal Control of Protein Function with Rapalogs

(A) Selective recruitment of FKBP-NES "exporter" protein to Frb-tagged fusions with specific rapalogs and Frb variants. C20-Marap selectively exports an Frb(KTF)-protein target (left), while BS-rap selectively exports a Frb(KTW)-protein fusion (right).

(B) Selective stabilization of protein targets fused to unstable Frb variants. Fusion of protein targets to Frb (KLF) or Frb (PLW) leads to their degradation. Both Frb variants can interact and be stabilized by C16-iRap by recruitment of FKBP. Replacement of C16-iRap with C20-Marap (upper) or C16-BSrap permits the continued stabilization of only the target capable of maintaining specific interaction with the Frb variant and the rapalog.

later switching to a drug that only interacts with only one of the Frb fusions. As an example (Figure 6B), one protein is fused to the partially unstable Frb PLW and the other with KLF. In the presence of C16-iRap, both fusions are stabilized and active because this drug recruits FKBP to each mutant. When C16-iRap is washed out and replaced with C16-BSrap, FKBP-BSrap is no longer recruited to the KLF fusion to degrade while the PLW fusion remains stabilized. Similarly, switching from C16-iRap to C20-Marap selectively retains stabilized KLF fusions.

An alternative use for orthogonal rapalogs in regulation is the differential regulation of a single target protein fused to FKBP by the specific recruitment of one of multiple Frb-tagged regulatory domains. In an example from this study, the kinase GSK-3β, which is localized both in the cytoplasm and the nucleus, is fused with FKBP and selectively recruited to or exported from the nucleus with rapalog-specific Frb mutants fused to a nuclear localization sequence or nuclear export sequence, respectively. Such systems can be useful to parse nuclear functions from cytoplasmic functions for proteins that have roles in multiple signaling pathways. Other examples in which a "one target, multiple effect" strategy can be employed include transcriptional regulation, in which the DNA binding domain of a transcription factor of interest is stripped of its domains that influence transcription and fused with FKBP. Selective recruitment of an activation domain or a repression domain (or both, in sequence) with specific rapalogs gives the experimentalist greater control of the timing of subsequent biochemical effects of stimulated or repressed transcription [18, 19]. Similarly, recruitment of kinases and phosphatases to nodes of signaling activity can facilitate subsequent proteomic analysis of the effect of this recruitment. We expect that the available technologies based on chemical induction of dimerization can be significantly broadened by the use of combinations of Frb fusions and rapalogs, both for the characterization of biologic responses and biochemical effects.

Significance

Earlier studies [10, 23] found that drugs bumped at the C20 position of rapamycin could alleviate the inhibitory effects of rapamycin and permit chemical induction of dimerization in vivo. The triple mutant selected to accommodate the bump on rapamycin (PLF) destabilizes the Frb domain and transfers this instability to proteins fused to Frb [10], an effect reversed by rapalog recruitment of FKBP. The drug-specific and destabilizing properties of PLF could have been the result of a structural "loosening" of Frb that permits

the accommodation of bulky groups in a more flexible protein surface. The results of this study do not favor such a model, as other rapalogs bumped along the Frb binding surface do not bind to PLF, but instead interact with other Frb mutants. This finding supports a lock-and-key model for rapalog-Frb mutant binding with destabilization a separate consequence. In fact, some mutants that interact with C20-Marap are not destabilizing to Frb (J.E. Gestwicki et al., submitted). The specificity of mutant Frb domains for different rapalogs permits the orthogonal regulation of multiple Frb-fused target proteins. Several schemes are presented to take advantage of this improvement of dimerizer technology. Perhaps the most important advantage of this advancement to biologists is the coordinated experimental regulation of multiple proteins in a biological pathway. Determining order in a pathway such as a signal transduction cascade is performed genetically through tests of epistasis. In this test, mutants with distinct phenotypes are crossed, and the mutant whose phenotype is evident is determined to be downstream in a pathway. These powerful experiments require that mutants, both loss- or gain-of-function or at least with distinguishable phenotypes, exist—a tall order in mammalian systems. Chemical regulation of one Frb-tagged protein in combination or discord with another will facilitate the assignment of order in biological pathways through a sort of chemical epistasis.

Experimental Procedures

Chemistry

NMR spectra were recorded on a Varian UI-500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) and AM-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) spectrometers. Chemical shifts are reported in ppm from tetramethylsilane using the solvent resonance as an internal standard (dimethylsulfoxide, 2.49 ppm in ^{1}H NMR and 39.5 ppm in ^{13}C NMR). Infrared spectra were recorded on a Perkin Elmer Spectrum BX FTIR System. Mass spectral data were acquired at the Stanford University Vincent Coates Foundation Mass Spectrometry Laboratory. Dichloromethane was distilled over calcium hydride prior to use. All other materials were used without purification. The syntheses of C16-alkoxy rapamycin analogs followed the method of Clardy and Holt [25].

C16-(S)-3-methylindolerapamycin

To a flame-dried and argon-cooled 25 ml round-bottom flask was added rapamycin (50.3 mg, 55.0 μmol), 3-methylindole (14.4 mg, 110.0 μmol), and CH_2Cl_2 (5.0 ml). This solution was cooled to −40ºC and trifluoroacetic acid (17.0 μl, 220.0 μmol) was added. After 5 hr at −40ºC, 8 ml ethyl acetate and 8 ml brine were added. The solution was warmed to room temperature, the layers separated, and the organic layer dried with sodium sulfate. After concentration under reduced pressure, the crude material was chromatographed using a mobile phase gradient of 1:1 hexanes:ethyl actetate to 2:3 hexanes:ethyl acetate to yield 55.1 mg (99%) of the desired product. Purity was demonstrated by HPLC using a methanol:water:acetonitrile (36:13:1) mobile phase, a Waters XTerra phenyl 5 μm column at 50ºC, and monitoring at 278 nm. The C16-(*S*) stereochemistry was assigned by comparison of the C22 chemical shift in the ^{1}H NMR spectra of the product with rapamycin and C16-(*R*)-trimethoxyphenyl rapamycin [25, 26]. Only the *trans*-conformer is described in the ^{1}H NMR characterization. IR (film) 3418 s, 2931 min, 1717 s, 1652 s, 1456 min, 1386 w, 990 w, 737m. ^{1}H NMR (400 MHz, DMSO-d_6, 25ºC) 10.03 (s, 1H), 7.36 (d, 1H, J = 8 Hz), 7.25 (d, 1H, J = 8 Hz), 6.98 (t, 1H, J = 7 Hz), 6.91 (t, 1H, J = 7 Hz), 6.44–6.08 (m, 5H), 5.43 (dd, 1H, J = 10, 15 Hz), 5.25 (d, 1H, J = 4 Hz), 5.20 (d, 1H, J = 10 Hz), 5.05 (m, 1H), 4.91 (d, 1H, J = 6 Hz), 4.59 (d, 1H, J = 4 Hz), 4.09 (m, 1H), 3.98 (d, 1H, J = 4 Hz), 3.66 (m, 1H), 3.32–3.29 (m, 7H), 3.18–3.16 (m, 3H), 2.92–2.78 (m, 2H), 2.46–2.38 (m, 2H), 2.26–2.06 (m, 3H), 2.14 (s, 3H), 2.04–1.50 (m, 13H), 1.46–1.06 (m, 12H), 1.04–0.80 (m, 15H), 0.74–0.56 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6, 25ºC) δ210.4, 208.1, 199.0, 169.2, 167.1, 140.3, 138.1, 137.2, 135.4, 135.2, 130.8, 129.0, 128.4, 125.1, 122. 7, 120.1, 117.9, 117.5, 110.5, 106.6, 98.9, 85.6, 83.8, 75.6, 73.3, 73.3, 66.9, 57.0, 56.8, 51.1, 44.8, 43.6, 38.5, 38.1, 35.3, 34.9, 33.5, 32.9, 32.6, 31.5, 31.4, 29.0, 26.6, 26.1, 24.5, 21.8, 20.4, 17.4, 16.0, 15.5, 14.7, 13.7, 13.2, 8.6. HRMS calculated for $C_{59}H_{85}N_2O_{12}$ (M+H): 1013.6103 amu, found (ESI) 1013.6096 amu.

Synthesis of C20-methallylrapamycin is described in Stankunas et al. [10]. C16-(S)Butylsulfonamidorapamycin (AP23050) and C16-(S)-7-methylindolerapamycin (AP21967) were generously provided by Drs. Tim Clackson and Victor Rivera at Ariad Pharmaceuticals.

Mutagenesis

All *Frb* mutants were prepared by the quikchange mutagenesis method (Stratagene, San Diego, CA) using the protocols provided by the manufacturer. Briefly, PCR reactions containing the parent plasmid pBJ5-Frb-VP16-HA or pBJ5-Frb*-VP16-HA and overlapping oligonucleotides containing the relevant base changes were performed with Pfu polymerase (Stratagene). PCR reactions were digested with the methylation-sensitive restriction endonuclease Dpn1 to eliminate the parent plasmid and the reaction was transformed in XL10-Gold Ultracompetent *E. coli* (Stratagene). All mutants were confirmed by sequencing.

SeAP Assay

Drug efficacy was determined in a three-plasmid transcriptional switch reporter assay. ~10^6 COS1 cells were coelectroporated with 2 μg of the DNA binding construct pBJ5-Gal4-FKBP(3), 2 μg pBJ5-Frb-VP16 (or the relevant mutant Frb) and 2 μg of the Gal4-SeAP reporter. Electroporation details are available upon request. Cells were immediately aliquoted to 96-well plates with flat bottoms. After 24 hr incubation media was supplemented with rapalog in triplicate and in serial 2-fold dilutions. Following 24 hr further incubation, cells with media were wrapped carefully in plastic wrap and heated to 65ºC for two hours to inactivate endogenous phosphatases. 50 μl of media was transferred to a black 96-well plate (Costar 3915) and supplemented with 50 μl 1mM Methylumbelliferrylphosphate (Sigma, St. Louis, MO) in 1 M Diethanolamine (pH 10.0 with carbonate). After 16 hr incubation at 37ºC, SeAP activity was measured by fluorescence with a Spectamax M2 fluorometer (Molecular Devices, Sunnyvale, CA) with transmission set at 355 nM and emission at 460 nM. Data were analyzed with Softmax Pro (Molecular Devices), Microsoft Excel, and Prism 3 (GraphPad Software).

Transfections and Staining

COS1 cells were electroporated with 1 μg pS-FKBP-GSK-3β-GFP, 1 μg of the "exporter" pBJ5-Frb(TLW)-Exp-HA containing a nuclear export sequence derived from the rev protein of human immunodeficiency virus and 3 μg of the "importer" pBJ5-Frb(KTF)-Imp with a nuclear localization sequence from SV40 Large T antigen. Cells were plated on autoclaved coverslips and incubated for 36 hr, followed by 1 hr of rapalog treatment (10 nM). Cells were fixed for 10 min in 4% paraformaldeyde/PBS at 4ºC and mounted for microscopy with DAPI to visualize the nucleus. Microscopy was performed with a Leica confocal microscope and Leica software.

Acknowledgments

These studies were supported by grants from the NIH (AI060037) to G.R.C. and (GM 068589) to T.J.W. and the Howard Hughes Medical Institute. J.G. is a Helen Hay Whitney fellow. We thank Penny Bruggemann for administrative support. We wish to thank Tim Clackson and Victor Rivera of Ariad Pharmaceuticals for generously providing several rapalogs, including AP21967 and AP23050. We wish to thank Jim Havrenak and David Baker (University of Washington) for important insights into Frb and rapamycin contact points at the atomic level that stimulated the direction of this study.

Received: May 12, 2005
Revised: September 29, 2005
Accepted: October 20, 2005
Published: January 20, 2006

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., 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. U.S.A. *93*, 4604–4607.
4. 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.
5. 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.
6. Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35–43.
7. 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. U.S.A. *92*, 4947–4951.
8. 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.
9. Rivera, V.M., Clackson, T., Natesan, S., Pollock, S., 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.
10. Stankunas, K., Bayle, J.H., Gestwicki, J.E., Lin, Y.M., Wandless, T.J., and Crabtree, G.R. (2003). Conditional protein alleles using knockin mice and a chemical inducer of dimerization. Mol. Cell *12*, 1615–1624.
11. Spencer, D.M., Belshaw, P.J., Chen, L., Ho, S.N., Randazzo, F., Crabtree, G.R., and Schreiber, S.L. (1996). Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Curr. Biol. *6*, 839–847.
12. 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.
13. 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.
14. 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.
15. Holsinger, L.J., Spencer, D.M., Austin, D.J., Schreiber, S.L., and Crabtree, G.R. (1995). Signal transduction in T lymphocytes using a conditional allele of Sos. Proc. Natl. Acad. Sci. U.S.A. *92*, 9810–9814.
16. Klemm, J.D., Beals, C.R., and Crabtree, G.R. (1997). Rapid targeting of nuclear proteins to the cytoplasm. Curr. Biol. *7*, 638–644.
17. Pecot, M.Y., and Malhotra, V. (2004). Golgi membranes remain segregated from the endoplasmic reticulum during mitosis in mammalian cells. Cell *116*, 99–107.
18. 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.
19. Biggar, S.R., and Crabtree, G.R. (2001). Cell signaling can direct either binary or graded transcriptional responses. EMBO J. *20*, 3167–3176.
20. Hentges, K.E., Sirry, B., Gingeras, A.C., Sarbassov, D., Sonenberg, N., Sabatini, D., and Peterson, A.S. (2001). FRAP/mTOR is required for proliferation and patterning during embryonic development in the mouse. Proc. Natl. Acad. Sci. USA *98*, 13796–13801.
21. Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.
22. Choi, J., Chen, J., Schreiber, S.L., and Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science *273*, 239–242.
23. 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.
24. Clemons, P.A., Gladstone, B.G., Seth, A., Chao, E.D., Foley, M.A., and Schreiber, S.L. (2002). Synthesis of calcineurin-resistant derivatives of FK506 and selection of compensatory receptors. Chem. Biol. *9*, 49–61.
25. Luengo, J.I., Yamashita, D.S., Dunnington, D., Beck, A.K., Rozamus, L.W., Yen, H., Bossard, M.J., Levy, M.A., Hand, A., Newman-Tarr, T., et al. (1995). Structure-activity studies of rapamycin analogs: evidence that the C-7 methoxy group is part of the effector domain and positioned at the FKBP12-FRAP interface. Chem. Biol. *2*, 471–481.
26. Kessler, H., Haessner, R., and Schuler, W. (1993). Structure of rapamycin: an NMR and molecular-dynamics investigation. Helv. Chim. Acta *76*, 117–130.