The Rapamycin-binding Domain of the Protein Kinase Mammalian Target of Rapamycin Is a Destabilizing Domain

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Rapamycin is an immunosuppressive drug that binds simultaneously to the 12-kDa FK506- and rapamycin-binding protein (FKBP12, or FKBP) and the FKBP-rapamycin binding (FRB) domain of the mammalian target of rapamycin (mTOR) kinase. The resulting ternary complex has been used to conditionally perturb protein function, and one such method involves perturbation of a protein of interest through its mislocalization. We synthesized two rapamycin derivatives that possess large substituents at the C-16 position within the FRB-binding interface, and these derivatives were screened against a library of FRB mutants using a three-hybrid assay in Saccharomyces cerevisiae. Several FRB mutants responded to one of the rapamycin derivatives, and twenty of these mutants were further characterized in mammalian cells. The mutants most responsive to the ligand were fuses to yellow fluorescent protein, and fluorescence levels were measured in the presence and absence of the ligand to determine stability of the fusion proteins. Wild-type and mutant FRB domains were expressed at low levels in the absence of the rapamycin derivative, and expression levels rose up to 10-fold upon treatment with ligand. The synthetic rapamycin derivatives were further analyzed using quantitative mass spectrometry, and one of the compounds was found to contain contaminants in the FRB domain. Furthermore, uncontaminated analogs retained the ability to inhibit mTOR, although with diminished potency relative to rapamycin. The ligand-dependent stability displayed by wild-type FRB and FRB mutants as well as the inhibitory potential and purity of the rapamycin derivatives should be considered as potentially confounding experimental variables when using these systems.

A common approach for studying complex biological processes is to perturb a protein of interest and observe subsequent changes to the biological system. The classical approach of disrupting a gene and studying the resultant phenotype is problematic for examining essential genes. As a result, several conditional techniques have been developed including the tetracycline/doxycycline and Cre recombinase/lox P systems, where control is exerted at the transcriptional level (1, 2). At the post-transcriptional level, RNA interference (RNAi) is becoming rapidly used as a method of gene silencing (3), but RNAi lacks tunability and does not affect existing protein levels. An ideal perturbation method would directly affect the protein with a cell-permeable probe that rapidly and reversibly targets a specific protein of interest.

Small molecules have been very useful as conditional perturbers; however, the lack of specificity of many of these reagents can make it difficult to unambiguously interpret results. One approach to ensure specificity is to chemically modify a small molecule with a large substituent, and this technique has been widely used to interrogate specific protein tyrosine kinases that possess a complementary cavity-forming mutation (4, 5). An alternative system has recently been developed using a cell-permeable small molecule to regulate the stability of specific proteins in a rapid, reversible, and dose-dependent manner (6).

A third approach for directly controlling protein function is by perturbing subcellular localization. Conditional regulation of protein localization has been achieved using the FKBP-rapamycin-FRB complex (7–12). With this method, a protein of interest is fused to either FKBP, the 12-kDa FK506- and rapamycin-binding protein or FRB, the FKBP-rapamycin binding domain of the protein kinase, mTOR. The other rapamycin-binding protein is modified to include a domain that regulates subcellular localization (e.g. nuclear export or plasma membrane-associated). The addition of rapamycin induces heterodimerization of the FRB and FKBP fusions and rapid formation of a ternary complex, which causes the protein of interest to be rapidly rerouted within the cell. One disadvantage of this system is that rapamycin has biological activity; it binds to endogenous mTOR kinase, inhibition of which may confound interpretation of the experiment (13, 14).

To engineer biologically silent ligands to induce dimerization, a “bump-hole” strategy has been pursued in which rapamycin derivatives were designed to possess bulky substitutents within the FRB/mTOR binding region. These derivatives bind poorly to mTOR, and thus are relatively nontoxic to cells, but can bind to FRB domains that contain compensatory cavity-forming mutations. One such derivative contains a methallyl

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addition at the C-20 position in rapamycin (MaRap) and binds a triple mutant of FRB (K2095P,T2098L,W2101F) designated FRB* (15). Fusing the essential gene encoding GSK-3β to FRB* and a nuclear export sequence to FKBP, the addition of MaRap should allow the formation of a ternary complex and a resultant ligand-dependent translocation of GSK-3β to the cytoplasm would be expected. However, in the absence of MaRap levels of GSK-3β expressed as a fusion to FRB* are significantly decreased with respect to the native protein, indicating that the FRB* domain confers instability (16).

To conditionally mislocalize a protein of interest in a rapid and reversible fashion, the protein domains that comprise the ternary complex should not be inherently unstable, and the small molecule should be easy to prepare and possess desirable pharmacokinetics in vivo. We set out to develop a conditional mislocalization system that would have these characteristics. To this end, we synthesized two rapamycin derivatives that possess large substituents at the C-16 position within the FRB* domain confers instability (16).

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FRB Library Generation — The FRB gene used for these studies encodes residues 2015–2114 of mTOR. Diversity in the FRB coding sequence and are described in Ref. 6. FRB22 as well as a FRB21/FRB22 double mutant. Three independent conditions were utilized to generate diversity within the FRB coding sequence and are described in Ref. 6.

Synthesis of Rapamycin Analogs — C-16-(R)-Trimethoxyphenyl rapamycin (TMOP-rap) and C-16-(S)-3-methylinadorapamycin (iRap) were synthesized as described previously (8, 10). AP21967 (C-16-(S)-7-methylinadorapamycin) was obtained from Ariad Pharmaceuticals, Inc., Cambridge, MA.

Cell Culture — All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated donor bovine serum (NIH3T3 cells), 10% heat-inactivated fetal bovine serum (FNX, 293T, and COS-1 cell lines) or 10% heat-inactivated horse serum (HEK293 cells).

Secreted Alkaline Phosphatase (SeAP) Reporter Assays — COS-1 cells were electroporated (7) with three vectors: pG5IL2SX to drive transcription of bacterial SeAP by five Gal4 DNA binding elements and a human interleukin-2 minimal promoter, pB75-GF3E, which expresses Gal4BD fused to three copies of FKBP, and pB75-FRBVE, which expresses an FRB fusion, wild-type or mutant, to the herpes simplex virus VP16 activation domain. Cells were treated as described previously (16, 21), and data were analyzed with Microsoft Excel and KaleidaGraph.

Transfections and Transductions — FRB mutants were cloned into the pBMN retroviral expression vector as N-terminal fusions to yellow fluorescent protein (YFP). Either the FNX ecotropic packaging cell line or 293T cells were transfected with the pBMN vectors using standard Lipofectamine 2000 protocols. For a transient transfection, 293T cells were cultured in growth medium for 24 h. To generate transduced NIH3T3 cells, viral supernatants were harvested from the FNX cells 48 h post-transfection, and NIH3T3 cells were incubated with the retroviral supernatants supplemented with 4 mg/ml polybrene.
for 4 h at 37 °C. Cells were cultured in growth medium for 24 h to allow for viral integration before analysis.

**Flow Cytometry with Mammalian Cells**—Cells were plated at 1 × 10⁶ cells/well of a 12-well plate and treated with iRap. For the stability assay, cells were treated with 1 μM iRap for 24 h and then harvested. For the dose-response assay, cells were treated with iRap (0–10 μM) and harvested at each time point. Cells were trypsinized from the plate, washed once with phosphate-buffered saline, and resuspended in 200 μl of phosphate-buffered saline. Cells were analyzed at the Stanford University FACS Facility, with 10,000 events collected for analysis.

**Immunoblotting for the mTOR Kinase Assay**—HEK293 cells were plated at 2–2.5 × 10⁵ cells/well of a 12-well plate and serum-starved for 24 h in Dulbecco’s modified Eagle’s medium only. Cells were mock-treated or treated with rapamycin (0.05–50 nM), iRap (0.5–500 nM), or AP21967 (0.5–500 nM) for 15 min at 37 °C. Serum was added to a final concentration of 20% for 30 min at 37 °C. Cells were lysed as described previously (22), and cell lysates were separated by SDS-PAGE. Resolved proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with a phosphospecific primary antibody against Thr-389 of p70 S6 kinase (catalog number 9205, Cell Signaling Technology, Inc., Danvers, MA). Data were analyzed using ImageQuant and KaleidaGraph.

### RESULTS

**Screening FRB Mutants in Yeast**—To identify FRB mutants capable of accommodating either TMOP-rap or iRap, we used a three-hybrid transcriptional switch assay in *S. cerevisiae*. In this assay, FKBP was fused to the Gal4 DNA-binding domain (Gal4BD), and FRB was fused to the Gal4 activation domain (Gal4AD). The addition of rapamycin led to the formation of the FKBP-rapamycin-FRB complex, reconstituting the Gal4 transcription factor. We engineered a yeast strain in which yeGFP was placed under the control of the GAL1/10 promoter, and reporter gene expression indicated successful formation of the ternary complex (Fig. 1A). The rapamycin derivatives TMOP-rap and iRap were synthesized with modifications at the C-16 position within the FRB-binding region to abrogate binding to the native target mTOR (Fig. 1B).

To accommodate the added steric bulk of the rapamycin analog, a compensatory cavity in FRB had to be introduced, and we used error-prone PCR and nucleotide analog mutagenesis to prepare a library of FRB mutants. The FRB library was designed to accommodate either TMOP-rap or iRap, we used a three-hybrid transcriptional switch assay in *S. cerevisiae*. In this assay, FKBP was fused to the Gal4 DNA-binding domain (Gal4BD), and FRB was fused to the Gal4 activation domain (Gal4AD). The addition of rapamycin led to the formation of the FKBP-rapamycin-FRB complex, reconstituting the Gal4 transcription factor. We engineered a yeast strain in which yeGFP was placed under the control of the GAL1/10 promoter, and reporter gene expression indicated successful formation of the ternary complex (Fig. 1A). The rapamycin derivatives TMOP-rap and iRap were synthesized with modifications at the C-16 position within the FRB-binding region to abrogate binding to the native target mTOR (Fig. 1B).

To accommodate the added steric bulk of the rapamycin analog, a compensatory cavity in FRB had to be introduced, and we used error-prone PCR and nucleotide analog mutagenesis to prepare a library of FRB mutants. The FRB library was designed and cloned into the destination vector by in vivo ligation, a process in which yeast cells ligated a library of PCR-derived inserts into a vector through homologous recombination. Successful recombination allowed cells to grow on selective dropout media. Transformed yeast were subjected to multiple rounds of FACS-based sorting, enriching for cells that expressed yeGFP.

A library containing ~10⁶ members was subjected to six rounds of sorting using flow cytometry. For each round, the transformed cells were induced with 5 μM TMOP-rap 16 h prior to sorting, and the fluorescent cells were collected and further cultured from 1 to 7 days between sorts. The library population contained ~2% positives upon initial analysis and was enriched to ~80% by the final sort. These fluorescent cells were collected and the plasmid DNA isolated and sequenced. Sequence analysis of 33 clones revealed only two mutations; the first was I2021T and the second involved a mutation of the stop codon to one encoding leucine. The construct encoding FRB possessed a second in-frame stop codon four residues beyond the original stop codon, thus the second mutant had a four-residue extension (Leu-Asp-Ser-Ser) at the C terminus.

To validate the screening method for selecting an FRB mutant that could bind TMOP-rap and to eliminate false positives, both FRB mutants in the pGAD-T7 vector were transformed into YSE2 and plated onto selective dropout media. Cells were treated with 5 μM TMOP-rap and analyzed by flow cytometry for expression of the reporter gene yeGFP. Surprisingly, the two FRB mutants responded weakly to TMOP-rap treatment, and the yeGFP expression levels did not reach those of the library population. To find an FRB mutant more respon-
sive to ligand treatment, a second library was generated in a similar method to the first. The two mutations derived from the first screen were included in the library generation, so the mixed FRB template included wild-type FRB, both single FRB mutants discovered from the first library and the corresponding double mutant. Additionally, another rapamycin derivative, iRap, was chosen for the screen.

A second FRB library containing 10^5 members was subjected to two rounds of sorting with flow cytometry. Transformed cells were treated with 1 μM iRap for 16 h prior to sorting, and the top 1% of cells were collected and grown in the absence of ligand for 48 h. These cells were then treated with iRap for 16 h prior to sorting. At this time, ~55% of the population was fluorescent (Fig. 2A). The top 1% of cells were collected, and the plasmid DNA isolated and sequenced. Sequence analysis of 54 clones revealed 29 new FRB mutants.

For validation, these FRB mutants were all individually transformed into YSE2 as described above. Two colonies expressing each mutant were treated with 1 μM iRap, and yeGFP expression levels were measured by flow cytometry. Fluorescence levels of the protein varied from barely above background to 6-fold higher (Fig. 2B). Twenty of these clones, all expressing high levels of yeGFP, were next tested in mammalian cells using a three-hybrid transcriptional switch.

Further Characterization of FRB Mutants in Mammalian Cells—COS-1 cells were co-transfected by electroporation with the vectors pG5IL2SX, pBJ5-GF3E, and pBJ5-FRBVE (encoding either wild-type or mutant sequences of FRB) (21). Mutant FRB sequences included the twenty clones from the iRap library as well as both of the mutants isolated from the first TMOP-rap-dependent screen. Supplemental table 1 lists the sequences of these 22 FRB mutants. Transfected cells were treated for 16 h in quadruplicate with rapamycin or iRap (0.5–500 nM). Fluorescence levels, representative of SeAP activity, were measured for all FRB constructs, and a representative sample is shown in Fig. 3. FRB mutants show varying response to iRap, and the EC_{50} values for the mutants range from 14.4 to 250 nM (Table 1). Additionally, iRap induces expression with an EC_{50} value of ~20 nM, indicating that both wtFRB and the FRB mutants isolated from the library screen respond similarly to iRap in this transcriptional switch assay.

Examining the Stability of FRB Mutants—An important characteristic for a conditional mislocalization system that operates in a rapid fashion is that all ligand-binding proteins are stable in the absence of ligand. This was not the case for the original MaRap-sensitive FRB mutant (16). To evaluate the stability of the FRB mutants isolated from the library screen, FRB mutants 2, 10, 11, and 19 as well as wtFRB were fused to the N terminus of YFP, and the constructs were expressed in mammalian cells. These four FRB mutants were chosen because they displayed the lowest EC_{50} values for iRap in the SeAP assay.

NIH3T3 cells were individually transduced with all five constructs and mock-treated or treated with 1 μM iRap 24 h prior to
FACS analysis. 293T cells were transiently transfected with only the wtFRB construct and treated as above. YFP fluorescence levels were measured in the presence and absence of iRap (Fig. 4A). In the absence of iRap, YFP fluorescence levels for the FRB constructs were only slightly above background, showing that the FRB proteins were unstable when expressed in mammalian cells. Moreover, as for FRB* treated with MaRap, the FRB proteins appeared to be stabilized when treated with iRap. A 10-fold increase in fluorescence was observed when cells stably expressing wtFRB fused to YFP were treated with iRap (Fig. 4A). Similar iRap-dependent stability was observed when measuring YFP expression levels for 293T cells transiently transfected with the wtFRB-YFP construct. All of the FRB mutants demonstrated similar increases in fluorescence (4–8-fold) upon treatment with iRap.

Characterization of FRB-induced Instability—To determine both the time required to achieve maximum fluorescence as well as the minimum dose required to reach this fluorescence level, a kinetic study of NIH3T3 cells stably expressing the wtFRB-YFP construct was carried out. Transduced cells were treated with various concentrations of iRap, and YFP fluorescence was monitored as a function of time (Fig. 4B). We observed that YFP fluorescence increased at approximately the same rate for the cell populations treated with 100 nM, 1 μM, and 10 μM iRap, whereas the cell population treated with 10 nM iRap showed decreased fluorescence levels at all time points. All cell populations achieved maximum fluorescence at 12 h, and levels dropped off slightly by 24 h, indicating that maximum fluorescence was reached by 12 h but not maintained in the absence of further dosing.

A dose-response assay was performed to determine the concentration of iRap required to stabilize the wtFRB-YFP fusion protein. NIH3T3 cells transduced with wtFRB-YFP were treated with iRap (0–1 μM) and YFP fluorescence levels measured. The cells achieved maximum fluorescence with 1 μM iRap and the EC_{50} value at ~10 nM (supplemental Fig. 2).

To confirm that YFP fluorescence levels correlate to protein expression levels, NIH3T3 cells transduced with the wtFRB-YFP construct and treated with 1 μM iRap for 24 h were harvested and immunoblotted with a primary antibody against YFP. The protein expression levels were consistent with fluorescence levels (data not shown).

**TABLE 1**

FRB mutants treated with iRap exhibit a range of EC_{50} values by the SeAP assay

Fluorescence data generated by the SeAP assay were fit and EC_{50} values derived from the curve fits. Data are presented as the mean value (±S.E.) for quadruplicate measurements. Identities of mutants are listed in supplemental Table S1.

<table>
<thead>
<tr>
<th>FRB mutant</th>
<th>EC_{50} (±S.E.)</th>
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<tr>
<td>WT FRB</td>
<td>20.5 (0.6)</td>
</tr>
<tr>
<td>FRB19</td>
<td>14.4 (0.4)</td>
</tr>
<tr>
<td>FRB22</td>
<td>18.4 (0.8)</td>
</tr>
<tr>
<td>FRB11</td>
<td>19.3 (1.1)</td>
</tr>
<tr>
<td>FRB10</td>
<td>20.4 (1.2)</td>
</tr>
<tr>
<td>FRB20</td>
<td>22.1 (2.0)</td>
</tr>
<tr>
<td>FRB14</td>
<td>23.0 (0.6)</td>
</tr>
<tr>
<td>FRB2</td>
<td>24.9 (2.1)</td>
</tr>
<tr>
<td>FRB21</td>
<td>26.0 (1.1)</td>
</tr>
<tr>
<td>FRB18</td>
<td>28.1 (2.0)</td>
</tr>
<tr>
<td>FRB1</td>
<td>29.7 (2.2)</td>
</tr>
<tr>
<td>FRB6</td>
<td>31.4 (1.1)</td>
</tr>
<tr>
<td>FRB17</td>
<td>32.6 (1.5)</td>
</tr>
<tr>
<td>FRB9</td>
<td>33.4 (2.1)</td>
</tr>
<tr>
<td>FRB4</td>
<td>39.0 (5.3)</td>
</tr>
<tr>
<td>FRB8</td>
<td>39.1 (2.3)</td>
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<tr>
<td>FRB5</td>
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<td>FRB16</td>
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<td>FRB13</td>
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<td>FRB3</td>
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<td>FRB7</td>
<td>88.8 (19.3)</td>
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<tr>
<td>FRB12</td>
<td>100 (55)</td>
</tr>
<tr>
<td>FRB15</td>
<td>250 (140)</td>
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</table>

**FIGURE 4.** Characterization of FRB mutants in mammalian cells. A, NIH3T3 cells were transduced with YFP, wtFRB-YFP or mutant FRB-YFP constructs, treated with 1 μM iRap for 24 h and assayed for YFP fluorescence by flow cytometry. 293T cells were transiently transfected with a wtFRB-YFP construct and treated as above. Data are presented as the mean fluorescence intensity (MFI) for each FRB construct with error bars for triplicate measurements. Black, NIH3T3 cells stably expressing wtFRB-YFP were treated with concentrations of iRap from 10 nM to 10 μM and YFP expression was monitored by flow cytometry for 24 h. Data are presented as the mean fluorescence intensity relative to the overall highest value. Experiment was performed in duplicate.

**mTOR Kinase Assays**—The experiments carried out hitherto utilized FRB, which is a relatively small domain within the
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mTOR kinase. To determine whether the rapamycin derivatives bound with the same affinity to mTOR as they bound to FRB, a series of S6 kinase assays was performed. The mTOR protein kinase phosphorylates p70 S6 kinase at threonine 389, so the ability of rapamycin and its derivatives to inhibit endogenous mTOR can be probed using a phosphospecific antibody for this residue. HEK293 cells were treated with rapamycin (0.05–50 nM), iRap (0.5–500 nM), or AP21967 (0.5–500 nM) for 15 min. Serum was added for 30 min and cell lysates were immunoblotted with a phosphospecific antibody against p70 S6 kinase T389. GAPDH serves as a loading control. A, representative blot showing levels of phosphorylated T389 of p70 S6 kinase in response to iRap treatment. The plus sign indicates the positive control lane (mock-treated cells) lane and the minus sign indicates the negative control lane (cells treated with 10 nM rapamycin). B, intensities of the signal from each sample were quantified with respect to the loading control and normalized to phosphorylation levels in untreated cells. Data were then plotted and fit to derive IC₅₀ values.

FIGURE 5. Inhibition of mTOR kinase activity. HEK293 cells serum-starved 24 h were mock-treated or treated with rapamycin (0.05–50 nM), iRap (0.5–500 nM), or AP21967 (0.5–500 nM) for 15 min. Serum was added for 30 min and cell lysates were immunoblotted with a phosphospecific antibody against p70 S6 kinase T389. GAPDH serves as a loading control. A, representative blot showing levels of phosphorylated T389 of p70 S6 kinase in response to iRap treatment. The plus sign indicates the positive control lane (mock-treated cells) lane and the minus sign indicates the negative control lane (cells treated with 10 nM rapamycin). B, intensities of the signal from each sample were quantified with respect to the loading control and normalized to phosphorylation levels in untreated cells. Data were then plotted and fit to derive IC₅₀ values.

DISCUSSION

We set out to discover a ternary complex comprising FKBP, an FRB mutant and a rapamycin derivative that could be used to conditionally mislocalize proteins to rapidly and reversibly probe their function. The FRB library screened with the rapamycin derivative iRap revealed twenty mutants that expressed yeGFP above background levels (Fig. 2B). In a mammalian three-hybrid system using the reporter gene SeAP, these FRB mutants exhibited a range of expression levels. Some mutants were slightly more potent than wtFRB (EC₅₀ values <20 nM), but most mutants were moderately weaker (EC₅₀ values >20 nM) (Table 1).

The iRap sample was pure by ¹H NMR analysis (95%), but we became concerned that trace quantities of rapamycin might be responsible for the behavior of some of the mutants isolated from the library screen. Upon analysis by quantitative mass spectrometry, we determined that the iRap sample contained ~2.6% rapamycin. Rapamycin was not detected (<0.1%) in the sample of AP21967. Our findings show that the bumped rapamycin analogs retain the ability to bind to wtFRB (Fig. 3) and full-length mTOR (Fig. 5), but trace quantities of rapamycin may improve the potency of the analogs. Given this fact, it was difficult to determine how much activity (i.e. isolation of mutants, activation of SeAP) is due to the rapamycin analog.

The fusion protein of wtFRB to YFP was examined in both stably transduced (NIH3T3) and transiently transfected (293T) cells, and the 10-fold increase in expression levels in the presence of iRap was consistent for both protocols. Additionally, wtFRB and the FRB mutant proteins were only partially rescued by ligand, as the fluorescence levels in the presence of iRap were still 1–2 orders of magnitude lower than a construct expressing YFP only. Thus, although the FRB proteins exhibited ligand-dependent stability, the degree of stabilization in the presence of ligand was modest relative to unfused YFP.

The destabilization behavior of FRB⁺ was originally attributed to the cavity-forming mutations, but it appeared that even the wild-type FRB protein was destabilizing. To create a technology to control protein function based on rapid subcellular localization, the ligand-binding proteins should be stable. Waiting 12–24 h for protein levels to rise largely removes the temporal advantage of ligand-induced mislocalization. Additionally, a mislocalization system built upon domains that display ligand-dependent stability may produce ambiguous results, as it would be difficult to ascertain how much of an observed phenotype is due to mislocalization versus changes in protein expression levels.

We found that, by monitoring fluorescence growth, NIH3T3 cells transduced with a wtFRB-YFP fusion and treated with 10 nM iRap reached 50% of the maximum value that the population treated with higher concentrations of iRap reached and had lower expression levels at every time point. Thus, when treating cells with a ligand at low concentrations, it is important to note that maximum protein expression levels are never achieved.

Upon examining the activity of rapamycin, iRap, and AP21967 in mTOR kinase assays, both iRap and AP21967 were found to inhibit mTOR with IC₅₀ values in the low nanomolar range (Fig. 5). Our studies to quantitate rapamycin levels in these samples revealed that rapamycin was not detected in AP21967 (~0.1%). Because the C-16 substituent of iRap was nearly identical to that of AP21967, this finding suggests that the ability of iRap to more potently inhibit mTOR (Fig. 5) or translocate fusion proteins (10) is likely due to traces of contaminating rapamycin.
In our search for a ternary complex that could be used to rapidly mislocalize proteins of interest, we have discovered that the FRB domain of the mTOR kinase is inherently unstable. When FRB is fused to the fluorescent protein YFP and studied in the presence and absence of ligand, the expression levels of the fusion protein increase from 4- to 10-fold for all of the FRB proteins examined. Both the ligand-dependent stability of FRB fusion proteins as well as the purity of rapamycin derivatives should be considered as potentially confounding variables when using rapamycin-based ligand-mediated colocalization systems.

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REFERENCES