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Ligand-Regulated Peptides: A General Approach for Modulating Protein-Peptide Interactions with Small Molecules

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Summary

We engineered a novel *li*gand-*r*egulated *p*eptide (LiRP) system where the binding activity of intracellular peptides is controlled by a cell-permeable small molecule. In the absence of ligand, peptides expressed as fusions in an FKBP-peptide-FRB-GST LiRP scaffold protein are free to interact with target proteins. In the presence of the ligand rapamycin, or the nonimmunosuppressive rapamycin derivative AP23102, the scaffold protein undergoes a conformational change that prevents the interaction of the peptide with the target protein. The modular design of the scaffold enables the creation of LiRPs through rational design or selection from combinatorial peptide libraries. Using these methods, we identified LiRPs that interact with three independent targets: retinoblastoma protein, c-Src, and the AMP-activated protein kinase. The LiRP system should provide a general method to temporally and spatially regulate protein function in cells and organisms.

Introduction

With the sequence of the human genome complete, a major challenge of life science research is the determination of the cellular functions of the predicted proteins. Indeed, this challenge is further complicated because many genes encode multiple protein domains with distinct functions and/or multiple mRNA splice variants. Biologists and chemists have developed a broad set of experimental tools to elucidate the functions of proteins and other macromolecules in cells and organisms. These approaches offer distinct advantages/disadvantages and can be differentiated by their scope of application, specificity, timescale of regulation, dose dependence, and reversibility.

Genetic approaches are the most common methods to identify the functions of proteins. Genetic mutation targets a single allele and provides absolute specificity. However, there are disadvantages to this approach: limitation to organisms that are tractable to genetic protocols; difficulty in achieving temporal control of inhibition; potential compensation by functionally related proteins; difficulty in identifying mutations that inhibit protein subdomains when little is known about the structure/function of a target; and embryonic lethality when gene function is studied in whole organisms [1, 2]. An additional genetic approach that has gained widespread use is RNA interference (RNAi) [3]. RNAi allows for specific inhibition of a target protein from knowledge of its gene sequence alone. However, the functions of protein subdomains cannot be directly investigated. In addition, temporal control of target inhibition depends on the timescale of mRNA and protein degradation and typically ranges between hours and days.

The chemical modulation of protein function with small molecules has a long history in the discipline of pharmacology. More recently, the field of chemical genetics [4] has sought to systematically identify modulators of target protein function by screening libraries of small molecules. Particular advantages of the small-molecule approach are precise temporal control of target function (onset of small-molecule action ranges from seconds to minutes), control of the level of modulation by varying the dose, reversibility of modulation by removal of the compound, and ease of use in a variety of experimental systems. The small-molecule approach has limitations in specificity and scope, because families of related proteins are often influenced, and many targets such as protein-protein interactions are not readily regulated by small molecules.

An additional approach to study protein function involves the use of genetically encoded macromolecules [5–7]. Nucleic acids or polypeptides that bind targets with high affinity and specificity can be selected from combinatorial libraries. These "aptamers" can be expressed and used to modulate the function of their targets inside cells. Indeed, peptide aptamers expressed as fusions to stable globular proteins have had widespread use in this regard. Additionally, peptide aptamers can be directed to virtually any compartment within a cell using the appropriate trafficking signals. The aptamer approach allows the activity of individual protein domains to be studied when little is known about their structure/function. However, a disadvantage of the aptamer approach is that it lacks the exquisite temporal control provided by small molecules.

An emerging theme for solutions to these problems is to combine the advantages of small molecules and genetically encoded macromolecules. A number of different combined chemical and genetic approaches have already been published: the regulated association of engineered proteins with chemical inducers of dimerization [8, 9]; the design of noncovalent [10–15] and covalent [16–18] ligands specific for engineered or mutant [19, 20] proteins; systems for ligand-regulated expression [21–28], ligand-regulated deaggregation [29], ligand-regulated RNA aptamers [30], riboswitches [31, 32], and allosteric ribozymes [32, 33]; and ligand-induced covalent complex formation [34]. These approaches provide the additional advantage that the activity of the small molecule is restricted to the subset of cells or tissues expressing the genetically encoded effectors.

Here, we report the design of a novel *li*gand-*r*egulated *p*eptide (LiRP) system for the regulation of peptide/target

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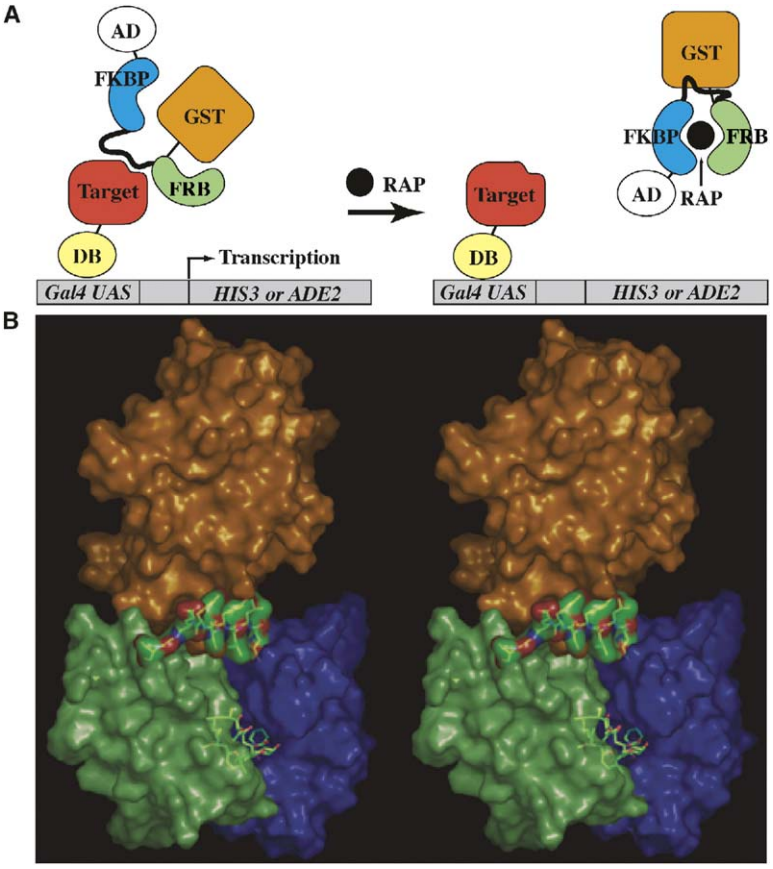

Figure 1. LiRP Identification and Rapamycin-Induced Conformational Change

(A) Schematic representation of the YTH system used to identify LiRPs. Target proteins ("baits") are expressed as fusions to the Gal4 DNA binding domain (DB; yellow). The LiRP scaffold presenting various targeting peptides ("preys") are expressed as fusions to the Gal4 activation domain (AD; white). Bait and prey interaction promotes transcription of *GAL1-HIS3* and *GAL2-ADE2* reporter genes that promote cell growth on media lacking histidine and adenine, respectively. Rapamycin promotes a conformational change in the LiRP scaffold that prevents interaction of prey peptides with bait proteins and transcription of reporter genes. FKBP (blue); FRB (green); GST (orange); RAP, rapamycin (black).

(B) Molecular model of the LiRP system. Divergent stereo pair showing FKBP (blue surface), FRB (green surface), GST (orange surface), ELLYCYEGS peptide (sticks, colored by element with surface), and rapamycin (sticks, colored by element). This model was created and energy minimized using Sybyl molecular modeling software (Tripos corporation), and the image was rendered in PyMOL (W.L. DeLano, "The PyMOL Molecular Graphics System" [2002]; http://www.pymol.org).

protein interactions. A tripartite scaffold protein enables the cell-permeable small molecule rapamycin to regulate the binding interactions of peptides with target proteins. The overall scaffold protein contains three protein domains: FK506 binding protein 12 (FKBP) [35, 36]; FKBP-rapamycin binding domain [37] (FRB) from the target of rapamycin protein [35, 36, 38, 39] (FRAP, mTOR, RAFT); and glutathione S-transferase (GST) (Figure 1). Peptides presented in the context of the FKBP-peptide-FRB-GST scaffold protein are free to interact with target proteins in the absence of rapamycin. In the presence of rapamycin, a complex forms between FKBP, rapamycin, and FRB [39]. This complex limits the conformational freedom of the peptide, preventing interactions with the target protein. In addition, the larger GST protein domain acts to sterically occlude access of the peptide to the target protein. The LiRP system should provide a unique tool for studying dynamic intracellular processes by controlling the binding activity of peptide regulators of target protein function. As a result, it represents a novel addition to a set of approaches that combine the benefits of small molecules with the benefits of macromolecular recognition. Moreover, the LiRP system should provide unique advantages over these systems for the study of protein function.

Results

Rational Design of the LiRP Scaffold Protein

We based our scaffold protein on the trimeric interaction of FKBP, rapamycin, and the FRB domain [39]. By generating an FKBP-peptide-FRB fusion protein, we reasoned that binding rapamycin would cause a reduction in the conformational freedom of the peptide linker. In addition, we observed that the C terminus of the FRB domain was in close proximity to the peptide linker and might be fused to the large GST protein domain to physically occlude access to the peptide (Figure 1). We hypothesized that conformational constraint and/or steric occlusion would prevent interaction of the peptide linker with the target protein. Overall, the LiRP scaffold has the following primary sequence: HA epitope tag-hFKBP12 (amino acids 2–108)-EL-peptide-GS-hFRB (amino acids 2021–2112 of mTOR, T2098L)-EF-GST (amino acids 2–218 of *S. japonicum* GST). The T2098L mutation was included in the FRB domain to allow conformational switching with both rapamycin and nonimmunosuppressive derivatives such as AP23102 [40]. We utilized the yeast two-hybrid (YTH) system to screen rationally designed LiRPs or to select LiRPs from a combinatorial peptide library (Figure 1A).

Rational Design of a LiRP Targeting Retinoblastoma Protein

We chose human retinoblastoma protein (hRb) as an initial target for the LiRP system. hRb has a highly conserved surface cleft that binds to peptides that contain the sequence LXCXE [41]. A YTH bait plasmid was constructed that encodes a Gal4 DNA binding domain (DB) fusion to hRb (DB-hRb). DNA encoding LYCYE-containing peptides of varying length from the HPV16 E7 onco-

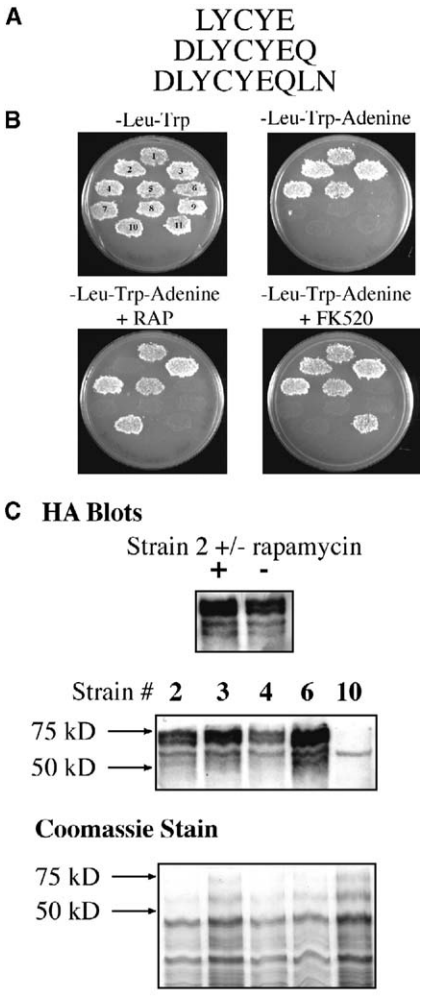

Figure 2. A Rationally Designed LiRP Targeting hRb

(A) Primary sequence for each of the three E7 peptides displayed by the FKBP-peptide-FRB-GST scaffold protein (Scaff-peptide).

(B) YTH results. Yeast strains were replica plated onto experimental plates: SC-Leu-Trp, SC-Leu-Trp-Adenine, SC-Leu-Trp-Adenine + 1 μM rapamycin, and SC-Leu-Trp-Adenine + 1 μM FK520. Each strain is numbered and expresses the following pairs of fusion proteins: strain 1, DB-FKBP/full-length Gal4 transcription factor; strain 2, DB-hRb/Scaff-LYCYE; strain 3, DB-hRb/Scaff-DLYCYEQ; strain 4, DB-hRb/Scaff-DLYCYEQLN; strain 5, DB-hRb/Scaff (No GST)-LYCYE; strain 6, DB-hRb/Scaff-SS; strain 7, empty bait vector/Scaff-LYCYE; strain 8, empty bait vector/Scaff-DLYCYEQ; strain 9, empty bait vector/Scaff-DLYCYEQLN; strain 10, DB-FKBP/AD-FRB; strain 11, DB-calcineurin A/AD-FKBP.

(C) LiRP steady-state expression levels. (Upper panel) Anti-HA Western blot for strain 2 grown in the presence or absence of 1 μM rapamycin. (Middle panel) Anti-HA Western blot for strains grown in the absence of rapamycin. Strain 10 lacks the expression of an HA-tagged fusion protein and serves as a negative control. (Lower panel) Coomassie-stained gel for strains grown in the absence of rapamycin. Equivalent amounts of total protein per lane as for identical samples run in the middle panel.

protein (Figure 2A) was cloned into a YTH prey plasmid that encodes a Gal4 activation domain (AD) fusion to the LiRP scaffold protein (AD-HA-FKBP-E7 peptide-FRB-GST). Bait and prey plasmids were cotransformed into YTH strain PJ69-4A (*tor2-1 Δfpr1*), and strains expressing both bait and prey fusion proteins were selected on SC-Leu-Trp media. These strains were replica plated onto SC-Leu-Trp-Adenine ± 1 μM rapamycin plates to assay for rapamycin-regulated activation of the *GAL2-ADE2* reporter gene. In addition, an SC-Leu-Trp-Adenine plate containing 1 μM of the small molecule FK520 was used as a negative control. Like rapamycin, FK520 binds with high affinity to FKBP [42]. However, unlike rapamycin, this complex binds to calcineurin and not to FRB [43]. E7 peptides of all three lengths interacted with the hRb bait when presented from the LiRP scaffold (Figure 2B; strains 2, 3, and 4). In each case, expression of DB-hRb was required for reporter gene activation (Figure 2B; strains 7, 8, and 9). The LiRP scaffold itself did not interact with DB-hRb (Figure 2B; strain 6). Interestingly, the LYCYE peptide alone showed a rapamycin-dependent growth inhibition (Figure 2B; strain 2). In contrast, the LiRP scaffold presenting LYCYE-containing peptides only 2 and 4 amino acids greater in length did not display rapamycin-dependent growth inhibition (Figure 2B; strains 3 and 4). An identical scaffold protein displaying the LYCYE peptide but lacking the GST moiety (AD-HA-FKBP-LYCYE-FRB) did not show rapamycin-dependent growth inhibition (Figure 2B; strain 5). Therefore, for the LYCYE sequence, the potential conformational constraint provided by FKBP-rapamycin-FRB complex formation alone appears insufficient, and the steric occlusion provided by GST is required for the rapamycin-dependent phenotype. When FK520 was substituted for rapamycin, ligand-dependent growth inhibition was not observed for the LiRP scaffold presenting the LYCYE peptide (Figure 2B; strain 2). Strains expressing either DB-calcineurin A/AD-FKBP or DB-FKBP/AD-FRB, included as positive controls for ligand function, displayed anticipated phenotypes: reporter genes were activated in these strains upon formation of calcineurin A-FK520-FKBP or FKBP-rapamycin-FRB complexes, respectively (Figure 2B; strains 10 and 11). Importantly, an anti-HA Western blot using cells grown in the presence or absence of rapamycin showed no significant change in expression levels of the LiRP scaffold displaying the LYCYE peptide (Figure 2C, upper panel). This result shows that the mechanism of rapamycin regulation is not due to a rapamycin-induced decrease in the expression level of the LiRP scaffold. In addition, approximately equal amounts of fusion protein are detected for each of the strains expressing E7 peptides displayed from the LiRP scaffold (Figure 2C, lower panel) showing that the rapamycin-regulated phenotype for E7 peptides of varying length is not the result of differences in expression levels.

Rational Design of LiRPs Targeting c-Src SH3

We chose the mouse c-Src SH3 domain as a second target protein. The SH3 domain of c-Src binds to two classes of polyproline type II helices in opposite orientations: class I peptides with a consensus sequence of RXLPPLP and class II peptides with a consensus sequence of XPPLPXR [44]. A YTH bait plasmid was constructed that encodes the protein DB-c-Src that includes both SH3 and SH2 domains. This fusion protein was shown to interact with a known c-Src SH3 binding protein fused to AD, AD-p130Cas (Figure 3B; strain 11) [45]. DNA encoding the peptides RALPPLP (class I peptide) and PPLPPR (class II peptide) was cloned into a

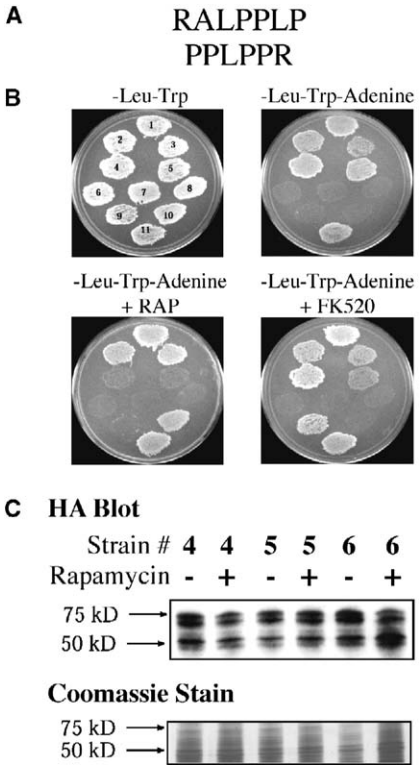

Figure 3. Rationally Designed LiRPs Targeting c-Src SH3

(A) Primary sequence for class I and class II peptides displayed by the FKBP-peptide-FRB-GST scaffold protein (Scaff-peptide).

(B) YTH results. Yeast strains were replica plated onto experimental plates: SC-Leu-Trp, SC-Leu-Trp-Adenine, SC-Leu-Trp-Adenine + 1μM rapamycin, and SC-Leu-Trp-Adenine + 1 μM FK520. Each strain is numbered and expresses the following pairs of fusion proteins: strain 1, DB-FKBP/full-length Gal4 transcription factor; strain 2, DB-SrcN/Scaff (No GST)-RALPPLP; strain 3, DB-SrcN/Scaff (No GST)-PPLPPR; strain 4, DB-SrcN/Scaff-RALPPLP; strain 5, DB-SrcN/Scaff-PPLPPR; strain 6, DB-SrcN/Scaff; strain 7, empty bait vector/Scaff-PPLPPR; strain 8, empty bait vector/Scaff-RALPPLP; strain 9, DB-CnA/AD-FKBP; strain 10, DB-FKBP/AD-FRB; strain 11, DB-SrcN/AD-Cas.

(C) Anti-HA Western blots. Strains 4, 5, and 6 grown in the presence or absence of 1 μM rapamycin.

YTH prey plasmid that encodes an AD fusion to the LiRP scaffold (AD-HA-FKBP-class I/II peptide-FRB-GST) (Figure 3A). Bait and prey plasmids were cotransformed into YTH strain PJ69-4A (*tor2-1 Δfpr1*), and strains expressing both bait and prey fusion proteins were selected on SC-Leu-Trp media. Strains were replica plated onto SC-Leu-Trp-Adenine ± 1 μM rapamycin plates to assay for rapamycin-regulated activation of the *GAL2-ADE2* reporter gene or ±1 μM FK520 as a negative control. Both the class I and class II peptides interacted with the c-Src SH3 domain when presented from the LiRP scaffold (Figure 3B; strains 4 and 5). In each case, expression of DB-c-Src was required for reporter gene activation (Figure 3B; strains 7 and 8). The LiRP scaffold itself did not interact with DB-c-Src (Figure 3B; strain 6). In the presence of rapamycin, both class I and class II peptides presented from the LiRP scaffold showed rapamycin-dependent growth inhibition (Figure 3B; strains 4 and 5). An identical scaffold protein displaying the class I and class II peptides but lacking GST (AD-HA-FKBP-class I/II peptide-FRB) did not show rapamycin-dependent growth inhibition (Figure 3B; strains 2 and 3), demonstrating again that the steric bulk of GST is necessary for the rapamycin-dependent growth inhibition. Ligand-dependent growth inhibition was not observed for the LiRP scaffold presenting class I/II peptides when FK520 was substituted for rapamycin (Figure 3B; strains 4 and 5). Importantly, anti-HA Western blots for strains grown in the presence or absence of rapamycin display no significant change in expression levels for class I and class II peptides presented from the LiRP scaffold (Figure 3C). This result shows that the mechanism of rapamycin regulation is not due to a ligand-induced decrease in expression level of the LiRP scaffolds.

Dose-Response Experiments with Rationally Designed LiRPs

We further characterized ligand-mediated growth inhibition in the YTH system by varying the amount of rapamycin or the nonimmunosuppressive rapamycin derivative AP23102 (Ariad Pharmaceuticals) in Sc-Leu-Trp-Adenine liquid culture (Figure 4). In all cases, growth inhibition increased with increasing concentrations of ligand. As expected, no growth inhibition was detected in the presence of rapamycin or AP23102 for a control strain expressing DB-c-Src/AD-p130Cas fusion proteins.

Identification of LiRPs from a Combinatorial Peptide Library

We chose hRb and the mouse AMP-activated protein kinase α1 subunit (AMPK-α1) as targets for selection of LiRPs from a randomized peptide library. A YTH bait plasmid was constructed that encodes a DB fusion to AMPK-α1 (DB-AMPK α1). A plasmid DNA library was synthesized that encodes seven random amino acid residues linking the FKBP and FRB domains of the LiRP scaffold. The plasmid DNA library was constructed by cloning 5′-GAGCTC (SacI site)-(NNS; N = A, G, T, C, S = G, C)$_7$-GGATCC (BamHI site)-3′ dsDNA library fragments into vector pPrey-Scaffold encoding the AD-HA-FKBP-EL(SacI site)-peptide-GS(BamHI site)-FRB-GST fusion protein. The plasmid DNA library consisted of ~7.5 × 10^7 independent members, where ~52% possessed library inserts of unit length. The remaining ~48% of library members either had inserts greater than unit length (resulting from ligation of multiple library inserts), had no insert, or possessed cloning artifacts that removed portions of FKBP, FRB, and/or GST. Table 1 and Table 2 list the insert sequences and rapamycin-regulated phenotypes for library members that interact with hRb and AMPK-α1 bait proteins, respectively.

In selections targeting hRb, seven colonies were identified from a total of 1.02 × 10^6 transformants. Table 1 lists the sequences of seven unique clones confirmed to have a positive YTH interaction after retransformation into YTH strain PJ69-4A (*tor2-1 Δfpr1*). All seven clones contained the LXCXE sequence motif. Five of the seven clones were identified as rapamycin-regulated LiRPs. For all seven clones, the register of the

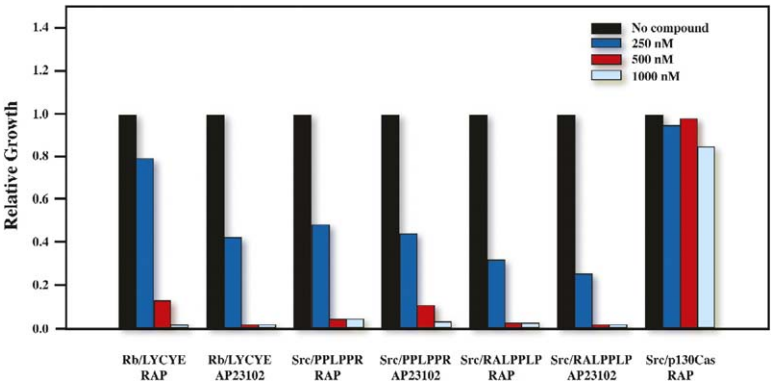

Figure 4. YTH Dose Response with Rapamycin and AP23102

YTH strains were grown in SC-Leu-Trp-Adenine liquid culture in the presence of 0, 250 nM, 500 nM, or 1 μM rapamycin or the nonimmunosuppressive rapamycin analog AP23102. Turbidity (OD_{600}) was measured at a representative time point as the no compound cultures approached saturation (OD_{600}= 6–10). The known interaction pair p130Cas/c-Src is included to show that rapamycin has little effect at the concentrations tested.

LXCXE motif begins at either the first or second randomized amino acid residue. Both LiRP and non-LiRP clones are represented in both registers. For example, the sequences LRCTEEV and SLLCREK were rapamycin regulated, whereas the sequences LYCWEEL and NLYCTEN interacted with DB-hRb in the presence and absence of rapamycin. These results demonstrate that the regulation of peptide-target protein interactions with rapamycin can be sensitive to subtle changes in peptide sequence, possibly due to sequence-dependent peptide conformations in the rapamycin bound LiRP scaffold.

In selections targeting AMPK-α1, forty interacting colonies were identified from 1.23×10^6 transformants. Table 2 lists the sequences of fifteen unique clones that were confirmed for a positive YTH interaction following retransformation into YTH strain PJ69-4A (*tor2-1 Δfpr1*). All fifteen clones showed rapamycin-regulated interaction with AMPK-α1. Each clone encoded a similar peptide sequence with the consensus motif (K/R) R(Q/M)RXXX.

Discussion

We have identified LiRPs that target hRb, c-Src, and AMPK-α1 by rational design or selection from a random peptide library. Our results suggest that LiRPs can be identified for a wide variety of target proteins. In cases where peptide sequences are known to interact with target proteins, these sequences or sequence variants can be tested for functionality in the LiRP system. Alternatively, if peptides are unknown or nonfunctional, it is possible to identify LiRPs from random peptide libraries. For example, a LiRP with the sequence SLLCREK was identified from a screen of a random peptide library after previous efforts suggested that peptides similar to DLYCYEQ would lack rapamycin regulation (Figure 2). In addition, a screen of a random peptide library for LiRPs that target AMPK-α1 identified 15 rapamycin-regulated clones with the consensus sequence (K/R)R (Q/M)RXXX. To our knowledge, this sequence motif does not match peptide sequences known to bind AMPK, and we are currently investigating its biological relevance. Together, these results demonstrate that novel LiRPs can be identified by screening random peptide libraries. Moreover, this approach may prove successful when known peptides for target proteins are unavailable or rational design efforts have previously failed.

Table 1. LiRPs Targeting hRb Selected from the AD-HA-FKBP-$(X)_7$-FRB-GST Combinatorial Peptide Library

Clone ID	Sequence	LiRP Activity
Rb-1-1	LRCTEEV	+
Rb-1-2	LYCWEEL	–
Rb-1-3	NLYCTEN	–
Rb-2-1	LFCDEARGSCIGLTYS	+
Rb-2-2	LECEEAI	+
Rb-2-3	LMCMEED	+
Rb-2-4	SLLCREK	+

The LXCXE motif is present in all clones (underlined). LiRP activity was determined by replica plating strains onto SC-Leu-Trp-Adenine ± 1 μM rapamycin plates. +, robust growth in the absence of rapamycin with no detectable growth in the presence of rapamycin; –, robust growth in the presence or absence of rapamycin. Clone Rb-2-1 is the result of aberrant ligation of two DNA library inserts into the same plasmid DNA vector.

Table 2. LiRPs Targeting AMPK-α1 Selected from the AD-HA-FKBP-$(X)_7$-FRB-GST Combinatorial Peptide Library

Clone ID	Sequence	LiRP Activity
α1-1	YRQRDKF	+
α1-2	RRQRFMFGSCIGLTYS	+
α1-3	RRMWPWS	+
α1-4	RRMRPCR	+
α1-5	RRQCRHL	+
α1-6	RRQSHWS	+
α1-7	KRMRTWK	+
α1-8	KRQRWCY	+
α1-9	KRQRDFT	+
α1-10	KRQTLWW	+
α1-11	RRCRERT	+
α1-12	QRMRDRS	+
α1-13	RHQSWWH	+
α1-14	KRMRSSV	+
α1-15	RMMRSRT	+

LiRP activity was determined by replica plating strains onto SC-Leu-Trp-Adenine ± 1 μM rapamycin plates. +, robust growth in the absence of rapamycin with no detectable growth in the presence of rapamycin; –, robust growth in the presence or absence of rapamycin. Clone α1-2 is the result of aberrant ligation of two DNA library inserts into the same plasmid DNA vector.

The suitability of a particular peptide-target protein combination for the LiRP system will likely depend on the structural characteristics of the interaction. For the rationally designed LiRPs presented here, the peptides bind to shallow pockets on the surface of the target protein in extended conformations: the LYCYE motif adopts a β-strand-like structure; and the c-Src SH3 peptides adopt polyproline helices [41, 44]. These peptide-target protein interactions represent challenging examples for regulation through conformational constraint alone. However, the combination of conformational constraint and steric occlusion was able to impart rapamycin regulation to these peptides. Short peptides of five to seven amino acid residues are likely sufficient to bind to the shallow clefts typical of enzyme active sites. The isolation of LiRPs Rb-2-1 and α1-2 (Tables 1 and 2), each containing an irregular peptide insert 16 amino acids in length, suggests that the LiRP scaffold has the potential to regulate longer peptides. Ongoing investigations using peptide libraries greater than seven amino acid residues will identify if longer peptides can be regulated as LiRPs. In addition, we are investigating the use of other steric occlusion domains and domain-domain fusion junctions to potentially generalize the LiRP scaffold to longer peptides. If longer peptides can be used, they may provide enhanced affinity/specificity for interaction with target proteins.

Our data support a mechanism of rapamycin-induced conformational change of the LiRP scaffold protein and steric occlusion mediated by GST (Figure 1). In all cases tested, GST was a required component of the scaffold, indicating the importance of steric occlusion for regulating these peptide-protein interactions. The steady-state expression levels of rationally designed LiRPs did not detectably change in the presence or absence of rapamycin (Figures 2C and 3C), indicating that a rapamycin-induced decrease in expression levels is not the mechanism of rapamycin-mediated growth inhibition. Moreover, introduction of the S2035T mutation into FRB, which blocks interaction of FKBP-rapamycin and FRB [37], abolishes LiRP activity when introduced into the LYCYE-LiRP (data not shown). Figure 1B illustrates a model of the LiRP scaffold showing a single molecule of GST. However, GST is a dimeric enzyme [46], and the dimer interface of GST in the LiRP scaffold is solvent exposed. Therefore, the potential exists for heterodimerization of the LiRP scaffold with endogenous GST or homodimerization between two scaffold molecules. In either case, molecular modeling suggests that dimerization would only slightly increase the steric bulk in the vicinity of the peptide. The functional consequence of these potential dimerization events is currently unknown.

The LiRP system should be useful for investigations of target protein function in mammalian cells. Peptides presented from the LiRP scaffold can be expressed inside cells and are free to interact with target proteins. Addition of rapamycin promotes a rapid, dose-dependent, and potentially reversible decrease in the LiRP-target protein interaction. However, the immunosuppressive properties of rapamycin may potentially complicate the interpretation of experimental results in experimental applications. Fortunately, nonimmunosuppressive rapamycin analogs have been developed that possess good pharmacological properties, are physiologically inert, and can induce binding interactions between FKBP and a T2098L mutant of FRB [47, 48]. The LiRP scaffold incorporates the T2098L FRB mutation, and we demonstrated ligand regulation with the nonimmunosuppressive rapamycin analog AP23102 (Figure 4). Nonimmunosuppressive rapamycin analogs should allow the use of the LiRP system in cells and whole organisms without the complicating effects of mTOR inhibition.

The LiRP system provides potential advantages compared to similar approaches for regulating target protein function. The LiRP system is functionally similar to the *li*gand-regulated *R*NA *a*ptamer (LiRA) approach published by Vuyisich and Beal [30]. The LiRP and LiRA systems allow expression of transgenes encoding macromolecules that can potentially interact with target proteins with high affinity/specificity, and both offer the potential rapid relief of target protein interaction upon addition of a small-molecule ligand (switch-off of macromolecule binding). Both systems offer advantages similar to peptide and RNA aptamers [5–7]: transgene expression precludes the need for mutagenesis of endogenous transcription cassettes, a requirement of other approaches [8–29, 31–34]; the activity of target protein subdomains can be regulated while leaving the activity of other subdomains intact, which is difficult or impossible to achieve with other approaches [3, 31–33]. In addition, the use of small molecules to regulate the binding activity of macromolecules should allow temporal control of target protein activity on timescales markedly faster than related approaches [21–29, 31, 33, 34]. Thus, both systems offer the potential to investigate target protein function in dynamic cellular processes (e.g., signal transduction, etc.). The overall scope of both the LiRP and LiRA systems could be extended by variants where macromolecules are triggered to bind to target proteins by the addition of a cell-permeable small molecule (switch-on of macromolecule binding). Indeed, it may be possible to switch on LiRP-target protein interactions by displacing rapamycin from the closed complex with high-affinity ligands that bind FKBP and induce the open conformation of the scaffold. As compared with the LiRA system, the LiRP system offers a number of unique advantages: the ability to rationally design LiRPs without the need for selection from combinatorial libraries using known peptide ligands for target proteins; optimized YTH reagents for the facile selection of LiRPs from combinatorial libraries; the availability of physiologically inert, cell-permeable ligands; potentially higher steady-state expression levels of LiRP fusion proteins compared to RNA aptamers [6, 32], an important parameter for achieving potent target inhibition; the potential to direct LiRP activity to subcellular compartments with protein trafficking signals; and the functionality of peptides for modulating protein/protein interactions. As these approaches are further developed, it is likely that each will have particular advantages and find complementary applications in biological investigations.

Significance

New experimental approaches for regulating the activity of proteins will allow novel insight into their cel-

lular functions. We have developed the ligand-regulated peptide (LiRP) system as a general approach for the control of intracellular peptide binding activity by the addition of a cell-permeable small molecule. LiRPs will likely combine the advantages of peptides (broad target applicability and binding specificity) and small molecules (rapid onset of action, dose dependence, and reversibility) for the study of target protein function. As a result, LiRPs will likely enable new experimental approaches for the investigation of dynamic cellular processes. In addition, the LiRP system provides unique advantages compared with similar approaches, including the ability to rationally design LiRPs using known peptide sequences and the use of physiologically inert, cell-permeable ligands. In summary, the rational design or selection of LiRPs from random peptide libraries should allow the temporal (timing of ligand addition) and spatial (tissue and cell compartment restriction) control of a broad range of macromolecule interactions.

Experimental Procedures

Isolation of Rapamycin and FK520

Rapamycin-producing strain *Streptomyces hygroscopicus* subsp. *hygroscopicus* (ATCC #29253) and FK520-producing strain *Streptomyces hygroscopicus* subsp. *ascomyceticus* (ATCC #55087) spores were produced on ISP2 agar media in petri dishes at 30°C for 14 days. Glycerol (20%) was added to the petri dishes to prepare a spore suspension, which was stored at –80°C. Production media (chemically defined media 4 [49] for rapamycin, liquid ISP2 media for FK520) were inoculated with spore suspension and grown with shaking at 25°C for 5–7 days. The method for purification of FK520 and rapamycin was adapted from Kino et al. [50]. The mycelium was collected after centrifugation, and either FK520 or rapamycin was extracted with methanol. This crude mixture was concentrated, and organic compounds were extracted with ethyl acetate and washed with brine. This crude extract was further purified via silica column chromatography, using a gradient of 1:1 to 3:1 ethylacetate:hexane.

YTH Reagents

We have constructed a unique set of YTH reagents to assay the activity of various peptides in the LiRP system. We generated a derivative of YTH strain PJ69-4A [51] for use in experiments that include both rapamycin and FKBPs. Rapamycin binds endogenous FKBPs in yeast, leading to inhibition of the TOR1 and TOR2 proteins, followed by G1 phase cell cycle arrest [38]. We have introduced *tor2-1* and *Δfpr1* alleles into the genome of PJ69-4A to nullify the effects of rapamycin [38, 52]. Deletion of *FPR1*, the major FKBP in yeast, reduces intracellular competition for binding to rapamycin, thus facilitating its participation in the YTH interaction. The *TOR2* mutation renders the yeast target of rapamycin insensitive to the growth-arresting effects of rapamycin, precluding the possibility of complementing the *Δfpr1* allele when expressing FKBP fusion proteins [53].

Strain Generation

S. cerevisiae strain PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*) genomic DNA was used as PCR template with primers 1/2 and 3/4 (for a list of primers, see the Supplemental Data available with this article online). PCR products were digested with Xba1 and ligated. The ligation product was used as template for a second round of PCR using primers 1/3. The resulting 519 bp product encodes a portion of TOR2 from *S. cerevisiae* (GenBank accession number NC_001143, locus tag YKL203C) with S1975R point mutation. In addition, a silent mutation generating an Xba1 site was introduced at nucleotide 5913. Gel-purified 519 bp fragment was transformed into *S. cerevisiae* strain PJ69-4A and plated onto YPD + 100 ng/ml rapamycin. Rapamycin-resistant colonies were streaked onto YPD + 100ng/ml rapamycin plates for individual colonies. Individual colonies were grown in YPD followed by isolation of genomic DNA. PJ69-4A (*tor2-1*) strains were identified by PCR amplifying from genomic DNA using primers 5/6 and digesting the product with Xba1. PCR with primers 7/8 and plasmid pFA6a-kanMX4 generated the kanMX4 disruption cassette [54] flanked on both 5′ and 3′ termini with 45 bp of sequence surrounding the *S. cerevisiae FPR1* ORF (GenBank accession number NC_001146, locus tag YNL135C). This fragment was transformed into PJ69-4A (*tor2-1*) followed by plating onto YPD + 200 mg/l G418. G418 resistant colonies were streaked onto YPD + 200 mg/l G418 plates for individual colonies. Individual colonies were grown in YPD followed by isolation of genomic DNA. PJ69-4A (*tor2-1 Δfpr1*) strains were identified by PCR amplifying from genomic DNA using primers 9/10/11 and screening for a 915 bp product. PJ69-4A (*tor2-1 Δfpr1*) strains were confirmed by sequencing PCR products from genomic DNA. PCR with primers 9/11 generated a product that was gel purified and sequenced with primer 12. PCR with primers 13/14 generated a product that was gel purified and sequenced with primer 15. PCR with primers 5/6 generated a product that was gel purified and sequenced with primer 16. The integrity of the recombination junctions and the presence of the *tor2-1* allele were confirmed by analyzing sequence data.

Plasmids

hFKBP12 (GenBank accession number NM_000801; residues 2–108), hFRB (GenBank accession number NM_004958; residues 2023–2112), and hRb A + B domains (GenBank accession number M28419; residues 380–785) were amplified using a human B cell cDNA library as template. PCR with primers 17/18 generated a product that was cloned into the Nde1/EcoR1 sites of plasmid pGBKT7 (Clontech) to give plasmid pBait-12. PCR with primers 19/20 generated a product that was cloned into the Spe1/EcoR1 sites of plasmid pGADGH (Clontech) to give plasmid pPrey-FRB. PCR with primers 21/22 generated a product that was cloned into the EcoR1/BamH1 sites of pGBKT7 to give pBait-hRb. PCR with primers 23/24 and 25/20 generated products that were used in SOE PCR and cloned into the Spe1/EcoR1 sites of plasmid pGADGH to give plasmid pPrey-12-FRB-2023. PCR with primers 26/20 using plasmid pPrey-12-FRB-2023 as template generated a product that was cloned into the BamH1/EcoR1 sites of plasmid pPrey-12-FRB-2023 to give plasmid pPrey-12-FRB-2021. Quickchange mutagenesis with primers 27/28 on plasmid pPrey-12-FRB-2021 generated plasmid pPrey-12-FRB. PCR with primers 29/30 and 31/32 using plasmid pPrey-12-FRB or plasmid pGEX2TK (Pharmacia), respectively, as template generated products cloned into the Spe1/Xho1 sites of plasmid pGADGH to give plasmid pPrey-Scaffold. Plasmid pPrey-Scaffold encodes an AD-HA epitope tag-hFKBP12 (amino acids 2–108)-ELSSGS-hFRB (amino acids 2021–2112, T2098L)-EF-GST (amino acids 2–218 of *S. japonicum* GST) fusion protein. Primers 33/34, 35/36, or 37/38 were annealed and extended using Klenow DNA polymerase. Products were cloned into Sac1/BamH1 sites of plasmid pPrey-Scaffold to give plasmids pPrey-12-LYCYE-FRB-GST, pPrey-12-DLYCYEQ-FRB-GST, and pPrey-12-DLYCY-EQLN-FRB-GST, respectively. PCR with primers 33 and 20 using plasmid pPrey-12-LYCYE-FRB-GST as template generated a product that was cloned into the SacI/EcoRI sites of plasmid pPrey-12-FRB to give plasmid pPrey-12-LYCYE-FRB. PCR with primers 39/23 generated a product that was cloned into the Spe1/EcoR1 sites of plasmid pGADGH to give plasmid pPrey-12. Mouse c-Src (GenBank accession number BC039953, amino acids 1–250) was amplified from the plasmid pmc-Src-CMV using primers 40/41, and the resulting PCR fragment was digested with EcoR1/Sal1. This insert was ligated into EcoR1/Sal1-cut pGBKT7 to generate the plasmid pDB-SrcN. Mouse p130Cas (GenBank accession number U28151) was PCR amplified using primers 42/43, and the resulting PCR fragment was digested with BamH1/EcoR1. The insert was ligated to BamH1/EcoR1-cut pGAD-GH to produce plasmid pAD-Cas. Primers 44/45 and 46/47 were annealed and extended using Klenow DNA polymerase. Products were digested with Sac1/BamH1 and cloned into plasmid pPrey-12-FRB to generate plasmids pPrey-12-RALPPLP-FRB and pPrey-12-PPLPPR-FRB. Spe1/BamH1 fragments from these two plasmids were cloned into plasmid pPrey-Scaffold to generate plasmids pPrey-12-RALPPLP-FRB-GST and pPrey-12-PPLPPR-FRB-GST. PCR with primers 48/

49 generated a product that was cloned into the NcoI/SmaI sites of plasmid pGBKT7 to generate plasmid pBait-α1 (mouse AMPK-α1; GenBank accession number AY885266).

YTH Experiments

Bait plasmids contain the *TRP1* selection marker. Prey plasmids contain the *LEU2* selection marker. Bait and prey plasmids were transformed into strain PJ69-4A (*tor2-1 Δfpr1*) and plated onto SC-Leu-Trp media. Plasmid-containing strains were patched onto SC-Leu-Trp followed by replica plating onto SC-Leu-Trp-Adenine ± 1 μM rapamycin or SC-Leu-Trp-Adenine ± 1 μM FK520. Replica plates were allowed to incubate 2–3 days prior to image acquisition. Liquid culture assays were performed to determine the dose response of YTH interactions. Test strains were grown overnight in SC-Leu-Trp media in the presence of 1 μM rapamycin to prevent expression of reporter genes. Separate flasks with SC-Leu-Trp-Adenine ± rapamycin or rapamycin analog AP23102 (Ariad Pharmaceuticals) at varying concentrations were inoculated to OD_{600} ~0.05. Growth was monitored by determining OD_{600} at multiple time points until saturation of the culture.

Western Blots

Strains containing bait and prey plasmids were grown overnight in SC-Leu-Trp to OD_{600} ~0.8–1.1. An equivalent number of cells were pelleted for each culture (4 OD_{600} units). Cell pellets were resuspended in 100 μl sample buffer (100 mM Tris-HCl pH = 6.8, 4% w/v SDS, 2% v/v β-mercaptoethanol, 0.001% w/v bromophenol blue, 20% glycerol) and ~50 μl of 0.5 mm glass beads. Cell suspension was vortexed for 1 min followed by incubating for 5 min at 100°C. Ten microliter sample/lane was resolved using 12% SDS-PAGE. Gels were blotted onto 0.45 μm nitrocellulose membrane or stained using Coomassie Brilliant Blue R-250. Nitrocellulose membrane was probed with mouse anti-HA monoclonal antibody (Sigma) followed by goat anti-mouse alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was resolved using chemiluminescent detection (Pierce).

Plasmid DNA Library Synthesis

Plasmid DNA library synthesis followed published protocols [55]. Briefly, duplex DNA library insert was synthesized by annealing oligonucleotides 5′-GTG CAT CGG GTT GAG CTC $(NNS)_7$ GGA TCC ACT GTA GGT CAC-3′ (N = 25% A, 25% G, 25% C, 25% T; S = 50% G, 50% C) and 5′-GTG ACC TAC AGT GGA TCC-3′ (IDT) followed by primer extension using Klenow DNA polymerase in the presence of dNTPs. Duplex DNA library insert was digested with SacI/BamHI and gel purified. Plasmid vector pPrey-Scaffold was digested with SacI/BamHI, CIAP treated, and gel purified. DNA library insert and vector were ligated using T4 DNA ligase and desalted using gel filtration (Centri-Sep, Princeton separations). Plasmid DNA library was transformed into *E. coli* strain MC1061 using ten independent electroporations (20 kV/cm; 200 Ω; 25 μF). After recovery in SOC media for 1 hr at 37°C, independent electroporations were pooled in 1 liter LB + AMP (50 μg/ml). Aliquots of the pooled electroporations were serially diluted followed by plating onto LB + AMP (50 μg/ml) to titer the overall number of transformants. Pooled electroporations were grown overnight in 1 liter LB + AMP (50 μg/ml) followed by large-scale plasmid purification (Promega wizard megaprep kit). Overall, ~7.5×10^7 independent transformants were amplified to give ~1.5 mg of plasmid DNA library. Seventeen independent transformants were chosen at random for DNA sequencing. Library members contained the desired insert (~52%), inserts greater than unit length (~11%; resulting from ligation of multiple library inserts), no insert (~17%), or clones missing portions of FKBP, FRB, and/or GST (~17%) likely resulting from restriction enzyme star activity.

Combinatorial Peptide Library Selection Experiments

Large-scale transformations of YTH strain PJ69-4A (*tor2-1 Δfpr1*) followed published protocols [56]. Briefly, 20–60 μg of plasmid DNA library was transformed into YTH strain PJ69-4A (*tor2-1 Δfpr1*) containing various bait plasmids. Serially diluted aliquots of the initial transformation were plated onto SC-Leu-Trp plates to determine the overall number of yeast transformants. The remaining amount of the initial transformation was plated onto SC-Leu-Trp-His + 3 mM 3-amino-1,2,4-triazole (3-AT; Sigma) plates and incubated at 30°C for 1 week. Colonies showing a His^+ phenotype were counted, and either all or a subset of colonies were patched onto SC-Leu-Trp plates. Patches from SC-Leu-Trp plates were replica plated onto SC-Leu-Trp-Adenine ± 1 μM rapamycin or SC-Leu-Trp plates. Library plasmids were rescued from His^+ colonies following published protocols [57]. Rescued library plasmids were sequenced followed by retransformation into YTH strain PJ69-4A (*tor2-1 Δfpr1*) to confirm plasmid linkage of phenotypes.

Supplemental Data

The Supplemental Data include a list of primers and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/7/847/DC1/.

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