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Caffeine Targets TOR Complex I and Provides Evidence for a Regulatory Link between the FRB and Kinase Domains of Tor1p*

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The target of rapamycin (TOR) kinase is an important regulator of growth in eukaryotic cells. In budding yeast, Tor1p and Tor2p function as part of two distinct protein complexes, TORC1 and TORC2, where TORC1 is specifically inhibited by the antibiotic rapamycin. Significant insight into TORC1 function has been obtained using rapamycin as a specific small molecule inhibitor of TOR activity. Here we show that caffeine acts as a distinct and novel small molecule inhibitor of TORC1: (i) deleting components specific to TORC1 but not TORC2 renders cells hypersensitive to caffeine; (ii) rapamycin and caffeine display remarkably similar effects on global gene expression; and (iii) mutations were isolated in Tor1p, a component specific to TORC1, that confers significant caffeine resistance both *in vivo* and *in vitro*. Strongest resistance requires two simultaneous mutations in *TOR1*, the first at either one of two highly conserved positions within the FRB (rapamycin binding) domain and a second at a highly conserved position within the ATP binding pocket of the kinase domain. Biochemical and genetic analyses of these mutant forms of Tor1p support a model wherein functional interactions between the FRB and kinase domains, as well as between the FRB domain and the TORC1 component Kog1p, regulate TOR activity as well as contribute to the mechanism of caffeine resistance.

Rapamycin is an immunosuppressive and anti-proliferative antibiotic that targets the TOR[2] kinase, a member of the phosphatidylinositol 3-kinase-like kinase family of protein kinases (1). TOR is a large (~280 kDa) protein and assembles into distinct membrane-associated protein complexes, termed TOR complex 1 (TORC1) and TORC2, in yeast as well as in higher eukaryotes (1). In yeast, TORC1 contains Tor1p or Tor2p as well as several additional proteins, including Kog1p, Lst8p, and Tco89p (2, 3). TORC1 mediates a multitude of rapamycin-sensitive activities related to cell growth, including control of translation, gene expression, and protein trafficking and stability (1, 2). TORC2 contains Tor2p as well as Lst8p, Avo1p-Avo3p, and Bit61p (2–5), and its activity is required for polarized cell growth and cytoskeletal organization (1, 2). TORC2 does not interact with nor is inhibited by rapamycin (1, 2). In higher eukaryotes, TORC1 consists of mammalian TOR (mTOR), mLST8/GβL (the ortholog of Lst8p), and Raptor (the ortholog of Kog1p), whereas TORC2 consists of mTOR, mLST8/GβL, and mAVO3/Rictor (the ortholog of Avo3p) (1, 6–9).

Much of what we know about TORC1 has come from the use of rapamycin as a specific small molecule inhibitor of TOR activity (1). Rapamycin, in conjunction with the highly conserved prolyl-isomerase FKBP, binds to the FRB domain of TOR located immediately N-terminal to its kinase domain (1, 10). A single amino acid change at a highly conserved serine residue within the FRB domain is sufficient to prevent binding of the rapamycin-FKBP complex to TOR and confer rapamycin resistance both *in vivo* and *in vitro* (1, 10–16). How rapamycin affects TORC1 activity remains unclear, although a number of *in vitro* studies have shown that the rapamycin-FKBP complex impairs TOR kinase activity (12, 17–21). Alternatively, it has been proposed that rapamycin prevents binding of a regulatory partner, for example Raptor, to mTOR that is essential for its activity (1, 7, 22–24). Interestingly, rapamycin-resistant mutant forms of mTOR have been shown to possess reduced kinase activity, prompting the suggestion that the FRB domain may somehow itself be involved in substrate recognition (19).

In addition to rapamycin, a number of other pharmacological agents have been shown to affect TOR, particularly in mammalian cells, including members of the methylxanthine family of compounds such as caffeine (25–27). Caffeine affects a diverse array of cellular processes related to cell growth, DNA metabolism, and cell cycle progression, most likely by acting as a low affinity ATP analog (28, 29). Both caffeine and the related compound theophylline have been shown to inhibit phosphorylation of mTOR-dependent substrates both *in vitro* as well as *in vivo* (25–27). Caffeine has not been used widely as a tool for probing mTOR function, however, potentially due to the pleiotropic behavior of this compound as well as the fact that it interacts with mTOR with relatively low affinity, *i.e.* in the submillimolar range (25–27).

In *Saccharomyces cerevisiae*, increased sensitivity to caffeine has been correlated with defects in the "cell integrity pathway," whereby cell well and/or plasma membrane stability is monitored in response to osmotic or thermal stress (30, 31). Several signaling pathways have been implicated in cell integrity main-

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[2] The abbreviations used are: TOR, target of rapamycin; mTOR, mammalian TOR; SCD, synthetic complete dextrose; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; GEO, NCBI Gene Expression Omnibus; PHASI, phosphorylated heat and acid stable protein regulated by insulin.

TABLE 1
Strains of *S. cerevisiae* used for this study

Strain	Description	Reference/source
W303a	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+*	50
W303α	*MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+*	50
S288c	*Matα gal2 mal*	Botstein laboratory
MY1384	Σ;2000 series, *Mata*	Microbia
PLY254	W303a, except *tor1*::*His3MX6*	3
PLY332	W303a, except *tco89*::*His3MX6*	3
JK9-3da	*Mata leu2-3,112 ura3-52 rme1 trp1 his4 can1-100 GAL*$^+$ *HMLa*	13
3H11-1c	JK9-3da, except *TOR1-1*	13
JH12-17b	JK9-3da, except *TOR2-1*	13
PLY297	JK9-3da, except *tor1*::*His3MX6*	3
PLY344	JK9-3da, except *tco89*::*His3MX6*	3
PLY365	W303a/α, except *tor1*::*TRP1 tco89*:: *His3MX6* [pNB100]	3
PLY366	W303a/α, except *TOR1/tor1*:: *His3MX6*	3
PLY369	W303a/α, except *TCO89/tco89*::*His3MX6*	This study
PLY475	W303a/α, except *AVO1/avo1*::*His3MX6*	This study
PLY477	W303a/α, except *AVO3/avo3*::*His3MX6*	This study
PLY479	W303a/α, except *KOG1/kog1*::*His3MX6*	This study
PLY315	W303a/α, except *LST8/lst8*::*His3MX6*	This study
PLY314	W303a/α, except *TOR2/tor2*::*His3MX6*	This study
PLY671	W303a, except *KOG1-MYC:TRP1*	This study
PLY673	W303a, except *LST8-MYC:TRP1*	This study
PLY675	W303a, except *TCO89-MYC:TRP1*	This study

TABLE 2
Plasmids used this study

Plasmid	Description	Reference/source
pNB100	*TOR2* in Ycplac33 (*URA3*)	51
pJK4	*TOR2* in Ycplac111 (*LEU2*)	51
pRS315	*LEU2*, CEN/ARS	52
pYDF23	*LEU2*, CEN/ARS *TOR1-1*	16
pPL132	*LEU2*, CEN/ARS *HA3-TOR1*	This study
pPL155	pPL132, *HA3-TOR1* A1957V mutation	This study
pPL156	pPL132, *HA3-TOR1* I1954V mutation	This study
pPL157	pPL132, *HA3-TOR1* W2176R mutation	This study
pPL158	pPL132, *HA3-TOR1* I1954V/W2176R mutations	This study
pPL159	pPL132, *HA3-TOR1* A1957V/W2176R mutations	This study
pPL164	pPL132, *HA3-TOR1* 1a1 mutant from PCR mutagenesis	This study
pPL165	pPL132, *HA3-TOR1* 1a7 mutant from PCR mutagenesis	This study

tenance, however, the relevant targets for caffeine have not yet been identified (30, 31). In this regard, we as well as others have reported that mutation of distinct components of TORC1 lead to defects in cellular integrity, including increased sensitivity to caffeine (3, 32). Independently, a large scale screen of yeast deletion mutants for altered drug sensitivities also identified TORC1 mutants as displaying increased caffeine sensitivity (33). Here we have pursued these observations and present evidence that TORC1 is indeed a significant target for caffeine in yeast. As part of these studies, we have identified mutations within the FRB and kinase domains of Tor1p that together confer significant levels of caffeine resistance. Characterization of these mutants provides independent evidence that the FRB domain is an important regulator of TOR kinase function.

MATERIALS AND METHODS

Strains, Media, and General Methods—Strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Cells were cultured in YPD (2% yeast extract, 1% peptone, and 2% dextrose) or synthetic complete dextrose (SCD) medium (0.8% yeast nitrogen base without amino acids, pH 5.5, 2% dextrose) supplemented with amino acids as described (34). Rapamycin (Sigma) was dissolved in Me_2SO, and caffeine (Sigma) was dissolved in H_2O; both compounds were added to liquid cultures and agar plates at the final concentrations indicated in the text and figure legends. Yeast transformations were performed using a lithium acetate procedure (35).

Construction of Yeast Strains—All gene deletions strains were constructed by replacing an entire open reading frame with a selectable maker following transformation of a linear fragment of DNA constructed by PCR. Specifically, we used *His3MX6* as a selectable marker from plasmid pFA6a-His3MX6 (36) and used forward and reverse primers that contained 50 bp corresponding to the 5′- or 3′-ends of the target open reading frame, followed by 20 bp corresponding to the 5′- or 3′-ends of the marker gene. All disruptions were confirmed by PCR using different primers that could detect specific integration events for each linear DNA fragment. Kog1p, Tco89p, and Lst8p were each tagged at their C termini with multiple copies of the Myc epitope using a PCR-based gene-tagging method as described previously (4).

Plasmid Construction—Plasmid pPL132 was constructed in multiple steps. The starting plasmid was pYDF23, which carries the *TOR1-1* allele under control of its native promoter (16). We first introduced sequences corresponding to three copies of the HA epitope (*HA3*) at the N terminus of the coding region of *TOR1-1*. This was accomplished by PCR amplification using genomic DNA from strain PLY298 (3) that expresses *HA3-TOR1* to generate a linear fragment of DNA that encodes the promoter region of *TOR1* followed by the N terminus of *TOR1* fused to *HA3*. This fragment was used in a co-transformation along with gapped pYDF23 to generate an intact *HA3*-tagged *TOR1-1* gene. Next, the FRB region of this plasmid was

removed by digestion with BglII and NdeI followed by transformation of strain JK9-3da to recover the wild-type FRB sequences by gap repair. The success of each step was verified by restriction digestion, PCR, and DNA sequence analysis. The functionality of pPL132 was demonstrated by its ability to rescue cell integrity defects of strain PLY254 carrying a *tor1* deletion in the W303a background (3).

Mutagenesis of pPL132—Hydroxylamine mutagenesis of pPL132 was carried out essentially as described (37). Briefly, 10 μg of plasmid was incubated at 75 °C in 1 M hydroxylamine for ~15 min, followed by transformation of W303a cells. Cells were allowed to recover for 6 h in SCD minus leucine media and were then plated on SCD minus leucine agar plates containing 9 mM caffeine and then incubated for several days at 30 °C. Control transformations onto plates lacking caffeine demonstrated that $\sim 3 \times 10^5$ cells were transformed using this procedure. Plasmids were recovered from several colonies that appeared on caffeine-containing plates and were used to transform fresh W303a cells, followed by re-plating on 9 mM caffeine plates. Three plasmids that passed this criteria where subjected to a combination of Gap-repair and fragment exchange reactions, where in each case the caffeine-resistant phenotype was localized to an NcoI-BsiWI fragment that spanned the FRB and kinase domains of *TOR1*. This region was sequenced, and all three plasmids were found to possess nucleotide changes predicted to create an A1957V mutation in *TOR1*.

Error-prone PCR mutagenesis of pPL132 was carried out essentially as described (38) with several modifications. A 1583-bp fragment spanning the NcoI and BsiWI sites in TOR1 was amplified using pPL132 DNA as template. Mutagenic PCR conditions used 5 mM $MnCl_2$, 2 mM each dCTP, dGTP, and dTTP, and 1 mM dATP. A 1383-bp fragment was excised from pPL132 by digestion with NcoI and BsiWI, and the resulting linearized plasmid vector was mixed with the PCR product in a 1:30 ratio and used to co-transform W303a cells. Following recovery in SCD minus leucine media, cells were plated directly onto SCD minus leucine agar plates containing 9 mM caffeine. Control transformations onto plates lacking caffeine demonstrated that $\sim 3.5 \times 10^5$ cells were transformed using this procedure. Following several days of growth at 30 °C, plasmids were rescued from colonies that appeared on caffeine-containing plates, re-tested by transformation of fresh W303a cells, and then ultimately sequenced. Site-directed mutagenesis was carried out using a QuikChange XL II kit (Stratagene) and pPL132 DNA as a template, following instructions provided by the manufacturer.

Gene Expression Studies—Northern blot analysis was performed as described (39). A 10-ml culture was grown to mid log phase (A_{600} = 0.5) and harvested by centrifugation, and total RNA was extracted by performing a hot phenol method. 20 μg of total RNA was loaded onto a 1.5% agarose, 6.9% formaldehyde gel and run in 1× E buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 0.5 mM EDTA). The RNA was transferred overnight onto a Duralon-UV membrane (Stratagene, La Jolla, CA) and probed overnight at 65 °C in Church hybridization buffer (0.5 M $NaPO_4$, pH 7.2, 7% SDS, 1 mM EDTA). Membranes were washed in 2× sodium saline citrate (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) buffer, exposed to a phosphorimaging screen, and analyzed using a Storm 860 imaging system and software provided by the manufacturer (Amersham Biosciences). DNA templates for the Northern probes were made as described previously (39). Relative levels of specified mRNAs were normalized to actin (*ACT1*) mRNA.

For gene expression analysis using DNA microarrays, 250-ml cultures were harvested for each sample, poly(A) mRNA was isolated, and fluorescently labeled cDNAs were prepared essentially as described (40). Arrays were scanned using a GenePix 4000a microarray scanner (Axon Instruments, Inc.) and analyzed using GenePix Pro 3.0 provided by the manufacturer. Data were uploaded to an AMAD microarray data base using a Linux operating system, and analyzed using software Cluster 3.0 and Java Treeview (all publicly available at derisilab.ucsf.edu/microarray/index.html). All microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) and are available through GEO Series accession numbers GSE4584 and GSE4586.

Immune Complex Kinase Assay and Co-immunoprecipitations—The protocol for kinase assays using PHAS-I as a substrate was developed based on previous reports (5, 19, 21) with several modifications based on our immunoprecipitation conditions for TORC1 (3). Two liters of each strain was grown in SCD minus leucine media to mid log phase (0.5 A_{600}/ml) at 30 °C. Cells were washed with sterile H_2O, pelleted again, and resuspended in yeast extract buffer (YEB, 50 mM HEPES-KOH, pH 7.1, 100 mM β-glycerol phosphate, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 10% glycerol, 0.25% Tween 20, and 150 mM KCl). Cells were again pelleted, resuspended in 2 ml of YEB containing protease inhibitors (mixture tablet, Roche Applied Science), 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride and frozen dropwise by transfer pipette into liquid nitrogen. The cell pellet was then transferred to a pre-chilled mortar and pestle containing liquid nitrogen and ground into a fine powder (~150 strokes followed by the addition of liquid nitrogen, repeated three times). After the liquid nitrogen boiled off, the powder that remained was transferred to 1.5-ml microcentrifuge tubes and thawed. All of the following steps were performed at 4 °C unless stated otherwise. Extracts were then centrifuged twice at 20,000 × *g* for 20 min, transferring the supernatant to a new tube each time. 500 μl of each extract was then transferred into new tubes and pre-cleared by binding to 25 μl of protein A-coupled beads (Amersham Biosciences) for 45 min. 5 μl of affinity-purified anti-HA polyclonal antibody (3) and 25 μl of fresh protein A-coupled beads were incubated together at room temperature for 1 h and were added to each tube of cleared extract. Extract-bead mixtures were then incubated for 3 h at 4 °C with end-over-end rotation. Following this incubation, beads were washed five times with 1 ml of YEB. To each tube of beads, 56 μl of kinase buffer (YEB containing protease inhibitors (mixture tablet, Roche Applied Science)), 2 mM dithiothreitol, 4 mM $MnCl_2$, and 1.5 μg of PHAS-I (Stratagene) substrate was added. Reactions were started by adding 4 μl of ATP mix (1.5 mM ATP, 2.5 μCi/μl [γ-^{32}P]ATP, 3000 Ci/mmol, Amersham Biosciences). Where indicated, caffeine was added at final concentrations listed in the text and figure legends prior to addition of ATP. Samples were incubated at 30 °C for 15 min,

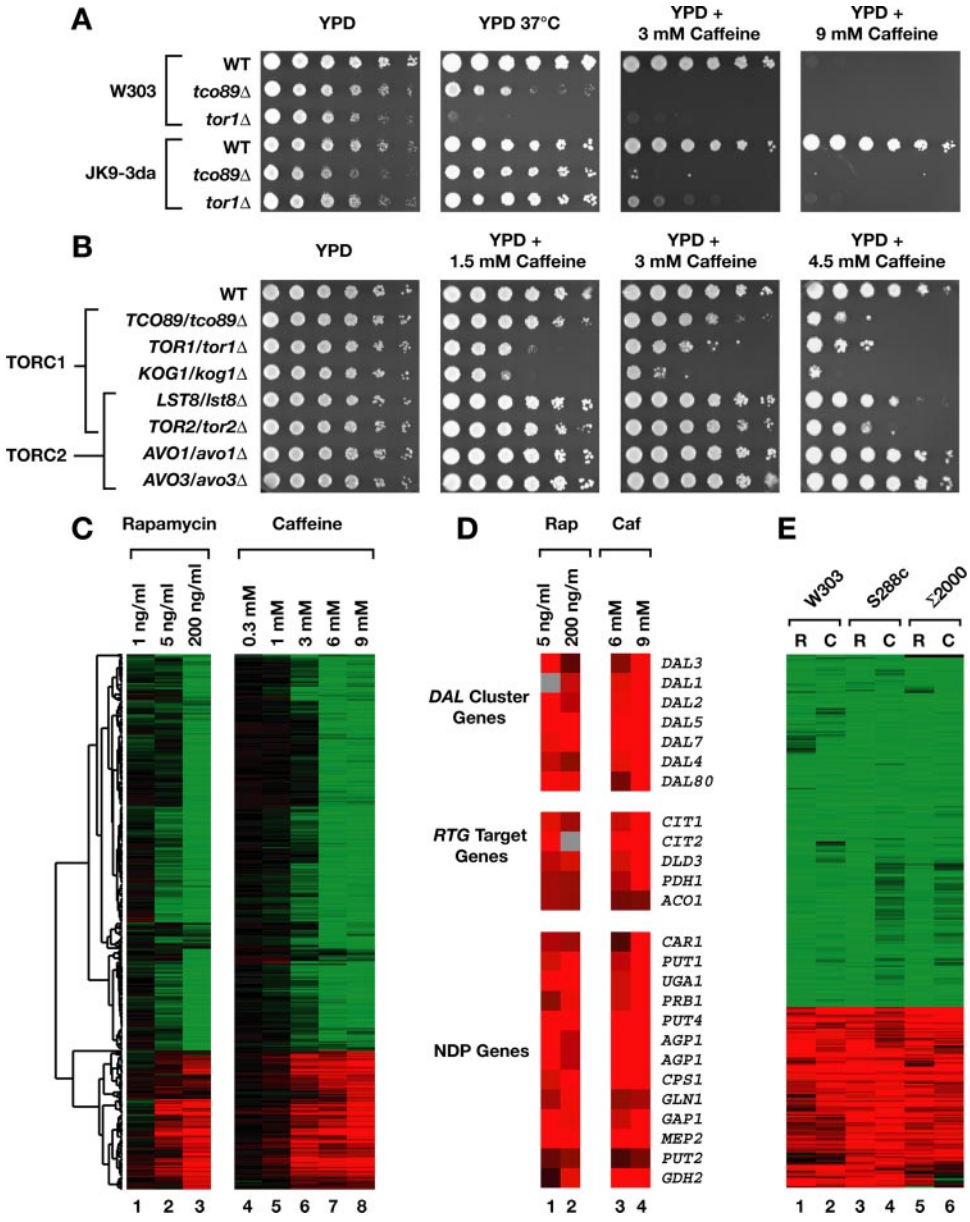

FIGURE 1. **TORC1 is a target for caffeine in yeast.** *A*, wild-type (*WT*), *tor1*Δ, and *tco89*Δ cells derived from W303a and JK9-3da strain backgrounds were grown to mid log phase (A_{600} = 0.5), washed with sterile water, serially diluted, and plated onto a YPD agar plate or YPD agar plates that contained caffeine at the concentrations indicated and incubated at 30 °C for 3 days. Also shown are cells plated onto a YPD agar plate and incubated at 37 °C for ~2 days. *B*, wild-type and heterozygous diploid mutants, where each mutant was deleted for one copy of an indicated TORC1 and/or TORC2 component, all in the W303a/α background, were grown as described in *A*, plated onto YPD agar plates that contained the indicated concentration of caffeine, and incubated at 30 °C for 3 days. *C*, comparison of effects of rapamycin and caffeine on global gene expression, where untreated W303a cells were compared with cells treated with an indicated concentration of caffeine or rapamycin. Shown are results of hierarchical cluster analysis of genes whose expression is affected 4-fold or greater (log scale = 10) in at least one array. *D*, a subset of data from *C* showing *DAL* cluster genes, *RTG* target genes, and NDP genes (40–43) whose expression is increased following treatment with rapamycin or caffeine. *E*, response of three different strain backgrounds (W303, S288c, and Σ2000) to rapamycin *versus* caffeine. Shown are results of hierarchical cluster analysis of genes whose expression is affected 4-fold or greater (log scale = 10) in at least three arrays, where untreated cells were compared with cells treated with 9 mM caffeine (denoted as "*C*") or 200 ng/ml rapamycin (denoted as "*R*"). In *C* and *D*, cells were grown in YPD to mid log phase (A_{600} = 0.5) and treated with rapamycin or caffeine, as indicated, for 30 min. All data for *C* and *D* are available under GEO accession number GSE4584.

and reactions were then stopped by addition of 15 μl of 4× SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE on a 7.5%/12.5% discontinuous gel. The top of the gel was used for immunoblotting of Tor1p, and the bottom was stained with Coomassie Blue, exposed to a phosphorimaging screen, and analyzed using a Storm 860 imaging system and software provided by the manufacturer (Amersham Biosciences). Control experiments demonstrated that no background labeling of PHAS-I occurred in extracts prepared from *tor1*Δ cells that carried an empty plasmid vector (*i.e.* in the absence of *HA3-TOR1*), demonstrating the specificity of this system. Co-immunoprecipitation experiments were carried out as described previously (3, 4).

RESULTS

TORC1 Is a Target for Caffeine in Yeast—We reported recently that cells singly deleted for genes encoding two non-essential TORC1-specific components, Tor1p or Tco89p, display a number of cell integrity-related phenotypes, including temperature sensitive (*ts*) growth, hypersensitivity to the cell wall binding agent calcofluor white, and hypersensitivity to caffeine (3) (Fig. 1*A*). Both the *ts* and calcofluor white-sensitive phenotypes were restricted to the W303a strain background that contains a mutant allele of the *SSD1* gene, termed *ssd1-d*, which is known to sensitize mutants for cell integrity defects (3) (Fig. 1*A*). By contrast, here we have found that hypersensitivity to caffeine was observed for *tor1*Δ and *tco89*Δ mutants in both the W303a and JK9-3da strain backgrounds, where the latter possesses a wild-type *SSD1* gene, suggesting caffeine has a more direct influence on TORC1 (Fig. 1*A*).

Consistent with this conclusion, a recent high throughput analysis of yeast heterozygous diploid mutants for altered drug sensitivities also identified mutants in three TORC1 genes, *TOR1*, *KOG1*, and *TCO89*, as being caffeine-hypersensitive (33) (note that this study refers to *TCO89* using the obsolete name *SHD7*). Importantly, this study was conducted using strains derived from the S288c strain background, which carries a functional *SSD1* allele (33).

We tested the generality of these findings by constructing diploid strains that contained single gene disruptions of indi-

TABLE 3

Caffeine-resistant phenotypes of *TOR1* mutants

W303a (WT) and PLY254 (*tor1*Δ) were transformed with the indicated plasmids and grown on SCD minus leucine plates that contained the indicated concentrations of caffeine. Following 2–4 days of growth, colony sizes were scored as follows: +++, strong growth; ++, moderate growth; +/−, weak growth; 0, no growth.

	Caffeine (mM)				
	0	3	6	9	12
WT					
pRS315	+++	+++	+/−	0	0
PPL132 (*HA3-TOR1*)	+++	+++	+	0	0
PPL155 (*HA3-TOR1* A1957V)	+++	+++	+++	++	0
PPL156 (*HA3-TOR1* I1954V)	+++	+++	+++	++	0
PPL157 (*HA3-TOR1* W2176R)	+++	+++	+	0	0
PPL158 (*HA3-TOR1* I1954V/W2176R)	+++	+++	+++	+++	++
PPL159 (*HA3-TOR1* A1957V/W2176R)	+++	+++	+++	+++	++
***tor1*Δ**					
pRS315	+++	0	0	0	0
PPL132 (*HA3-TOR1*)	+++	++	+/−	0	0
PPL155 (*HA3-TOR1* A1957V)	+++	+++	+++	++	0
0PPL156 (*HA3-TOR1* I1954V)	+++	+++	+++	++	0
PPL157 (*HA3-TOR1* W2176R)	+++	++	+/−	0	0
PPL158 (*HA3-TOR1* I1954V/W2176R)	+++	+++	+++	+++	++
PPL159 (*HA3-TOR1* A1957V/W2176R)	+++	+++	+++	+++	++

vidual components of TORC1 and/or TORC2 and then examined these mutants for sensitivity to caffeine. Consistent with previous findings (33), deletion of components specific to TORC1 (*e.g. TOR1*, *KOG1*, and *TCO89*) resulted in the greatest sensitivity to caffeine (Fig. 1*B*). In addition, we found that deletion of components that are shared between TORC1 and TORC2 (*e.g. TOR2* and *LST8*) also displayed hypersensitivity but at a higher concentration of caffeine (Fig. 1*B*). By contrast, deletion of components specific to TORC2 (*e.g. AVO1* and *AVO3*) showed no increased sensitivity to caffeine, compared with wild-type cells (Fig. 1*B* and data not shown).

Treating yeast cells with rapamycin results in widespread and characteristic changes in gene expression (1, 40–43). We reasoned that, if caffeine targets TORC1, then treating cells with this drug might elicit effects similar to rapamycin with respect to these transcriptional readouts. Accordingly, we examined global transcriptional changes when wild-type W303 cells were grown in rich media (YPD) and treated with either rapamycin or caffeine (Fig. 1*C*). Indeed, remarkable overlap was observed with respect to transcriptional changes caused by treatment with either drug (Fig. 1*C*). Importantly, these similarities included not only repression of r-protein gene expression but also included increased expression of distinct nitrogen-regulated gene clusters, for example *RTG* target, *DAL*, and nitrogen discrimination pathway genes, that together are characteristic of the cellular response to rapamycin (Fig. 1*D*) (40–43). A survey of three different wild-type strain backgrounds confirmed the overall similarity of the transcriptional response to treatment with rapamycin and caffeine (Fig. 1*E*). A number of strain-specific differences were also observed for each drug, indicating that genetic factors in addition to TORC1 contribute to the precise response of cells to rapamycin as well as caffeine (Fig. 1*E*). This observation is consistent with the finding that different wild-type strain backgrounds display unique basal sensitivities to both rapamycin as well as caffeine (3) (Fig. 1*A*).

Isolation of Caffeine-resistant Alleles of TOR1—We reasoned that, if TORC1 was a target for caffeine, then it might be possible to isolate mutants in *TOR1* that afforded increased resistance to the drug. In a preliminary experiment, we transformed wild-type W303a cells with a hydroxylamine-treated plasmid that expressed an epitope-tagged *TOR1* gene (*HA3-TOR1*) and grew the resulting transformed cells on agar plates containing 9 mM caffeine, a concentration that was completely inhibitory for growth for this strain background (see "Materials and Methods"). Three independently derived, plasmid-dependent caffeine-resistant colonies were obtained, and subsequent analyses revealed that each *TOR1* gene possessed a mutation that was predicted to create an alanine to valine substitution at position 1957 within the FRB domain of Tor1p. Site-directed mutagenesis was used to confirm that this A1957V single amino acid substitution was necessary as well as sufficient for growth of cells in the presence of 9 mM caffeine (Table 3).

Based on these results, we mutagenized the FRB as well as adjacent kinase domains using error-prone PCR in an effort to generate additional caffeine-resistant *TOR1* alleles (see "Materials and Methods"). Six independent *TOR1* mutants were isolated that allowed W303a cells to grow on agar plates containing up to 12 mM caffeine, two of which are shown in Fig. 2*A*. None of these mutants conferred increased resistance to a number of other drugs tested, including doxycycline, chlorpromazine, camptothecin, cycloheximide, and hygromycin B, suggesting they were specific for resistance to caffeine.[3] Sequence analysis revealed that, although each mutant contained a number of different predicted amino acid substitutions, all shared similar substitutions at two specific positions: (i) a change at position isoleucine 1954 within the FRB domain to either valine (two isolates) or threonine (four isolates); (ii) a change at position tryptophan 2174 within the kinase domain to either arginine (five isolates) or cysteine (one isolate) (Fig. 2*B*). Site-directed mutagenesis was used to demonstrate that a combination of two mutations, I1954V and W2176R, was both necessary as well as sufficient for growth in the presence of 12 mM caffeine (Table 3). Each caffeine-resistant allele conferred similar levels of resistance in both wild-type as well as *tor1*Δ cells, where in the latter strain the sole source of *TOR1* was plasmid-encoded (Table 3; see below). We note that no mutants were isolated that afforded growth of W303a wild-type

[3] A. Reinke, J. C.-Y. Chen, S. Aronova, and T. Powers, unpublished results.

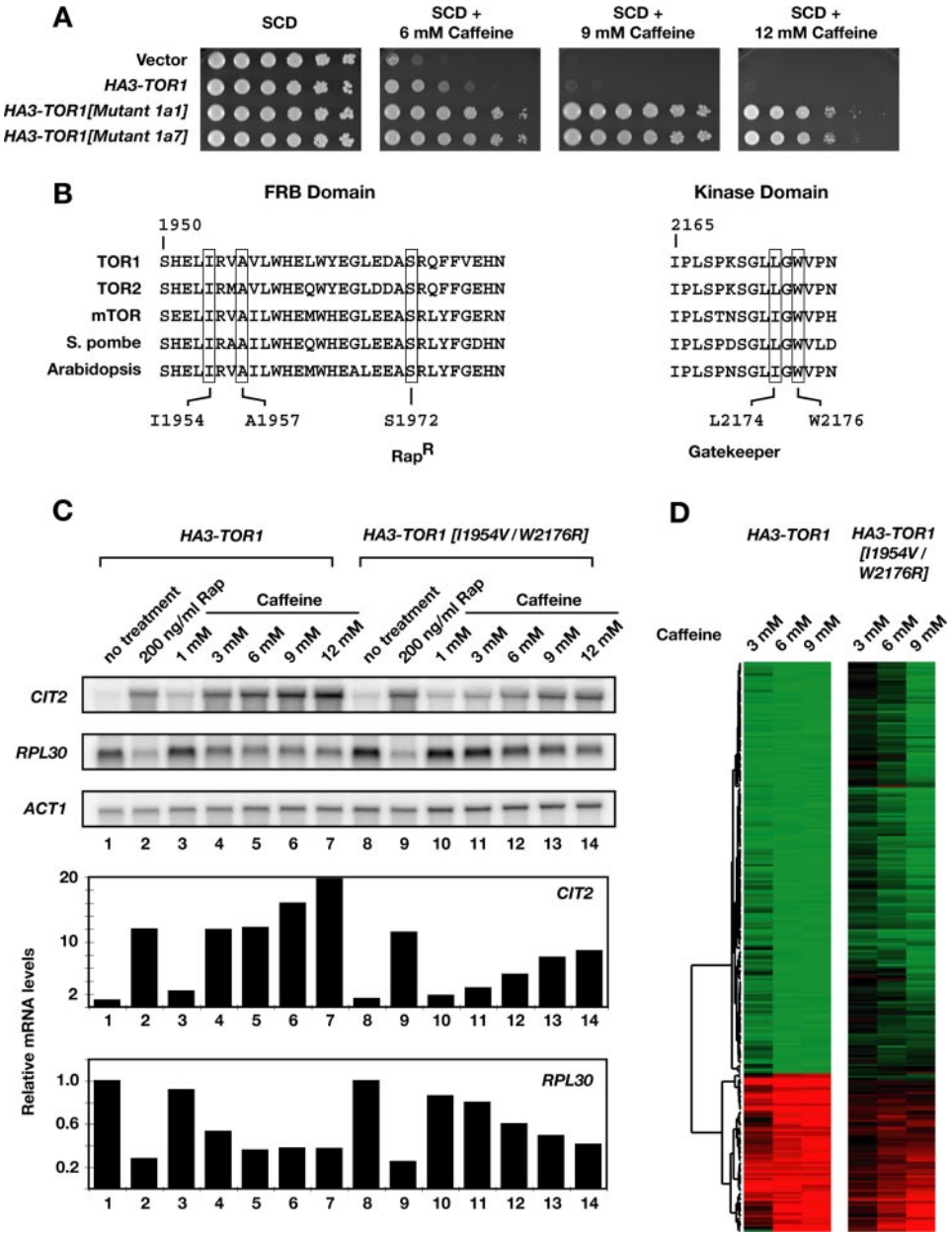

FIGURE 2. **Identification and characterization of caffeine-resistant *TOR1* mutants.** *A*, WT (W303a) cells were transformed with a control plasmid (pRS315) or plasmids that expressed wild-type *HA3-TOR1* (pPL132) or either one of two caffeine-resistant *TOR1* mutants, *HA3-TOR1-1a1* or *HA3-TOR1-1a7* (pPL164 and pPL165, respectively). Cells were grown in SCD media lacking leucine at 30 °C to mid log phase, serial dilutions were made, and cells were plated onto SCD minus leucine agar plates that contained the indicated concentrations of caffeine. Photographs of plates were taken following ~3 days of growth. *B*, alignments of segments of the FRB and kinase domains of selected TOR homologues showing the location of amino acids identified in this study where mutations result in caffeine resistance. Also shown is position Ser-1972 within the FRB domain, which is the site of mutations that confer rapamycin resistance, as well as position Leu-2174 within the kinase domain, which corresponds to the "gatekeeper" position as defined by Shokat and co-workers (45). *Numbering* of amino acids is according to Tor1p. *C*, Northern blot analysis of *tor1*Δ (PLY254) cells transformed with plasmids expressing either *HA3-TOR1* (pPL132) or *HA3-TOR1* (I1954V/W2176R) (pPL158). Cells were grown to mid log phase in SCD minus leucine media at 30 °C and treated with rapamycin or caffeine at the indicated concentrations for 30 min. Cells were harvested, and total RNA was isolated and used for Northern blot analysis, probing for the specified mRNAs. *D*, effects of caffeine on global gene expression in *tor1*Δ (PLY254) cells expressing either *HA3-TOR1* or *HA3-TOR1* (I1954V/W2176R). Shown are results of hierarchical cluster analysis, based on genes whose expression was affected 4-fold or greater (log scale = 10) in cells expressing *HA3-TOR1* treated with 6 and 9 mM caffeine. Data for *D* are available under GEO accession number GSE4586.

or *tor1*Δ cells on agar plates containing a concentration of caffeine higher than 12 mM, suggesting we had encountered one or more unknown constraints by this approach.

Interestingly, we observed that the I1954V and W2176R double mutant enabled *tor1*Δ cells derived from the JK9-3da background to grow on concentrations of caffeine up to 20 mM (data not shown). This finding is consistent with our above conclusion that genetic factors in addition to TORC1 contribute to the detailed response of cells to caffeine; however, as also stated above, these genetic factors are likely to be distinct from the *SSD1* gene.

We compared the effects of caffeine on gene expression in cells expressing the *HA3-TOR1* (I1954V/W2176R) double mutant *versus* wild-type *HA3-TOR1*. Unless stated otherwise, for this and all remaining experiments, we used a *tor1*Δ strain so that the sole source of *TOR1* would be plasmid-encoded. Northern blot analysis was used to examine expression of two representative target genes, *CIT2* and *RPL30*, which are negatively and positively regulated by TORC1, respectively (39, 42) (Fig. 2*C*). We observed a significant difference in the response of these genes to caffeine within the two strains, where a higher concentration of drug was required to elicit a comparable response in cells expressing *TOR1* (I1954V/W2176R) (Fig. 2*C*). Microarray analysis of global gene expression patterns confirmed that, for essentially all genes affected by caffeine, a significantly higher dose of drug was also required to show a comparable change in expression in cells expressing mutant *versus* wild-type *TOR1* (Fig. 2*D*). By contrast, no change in response to rapamycin was observed for these strains with respect to *CIT2* or *RPL30* expression (Fig. 2*C*, compare *lanes 2* and *9*), consistent with our finding that the *TOR1* (I1954V/W2176R) mutant does not confer rapamycin resistance (see below).

Functional Dissection of Tor1p FRB and Kinase Domain Mutations—We sought to explore further the molecular basis for the caffeine-resistant phenotypes observed for both the *TOR1* (I1954V/W2176R) double as well as *TOR1* (A1957V) single mutants described above. Toward this end, we constructed versions of

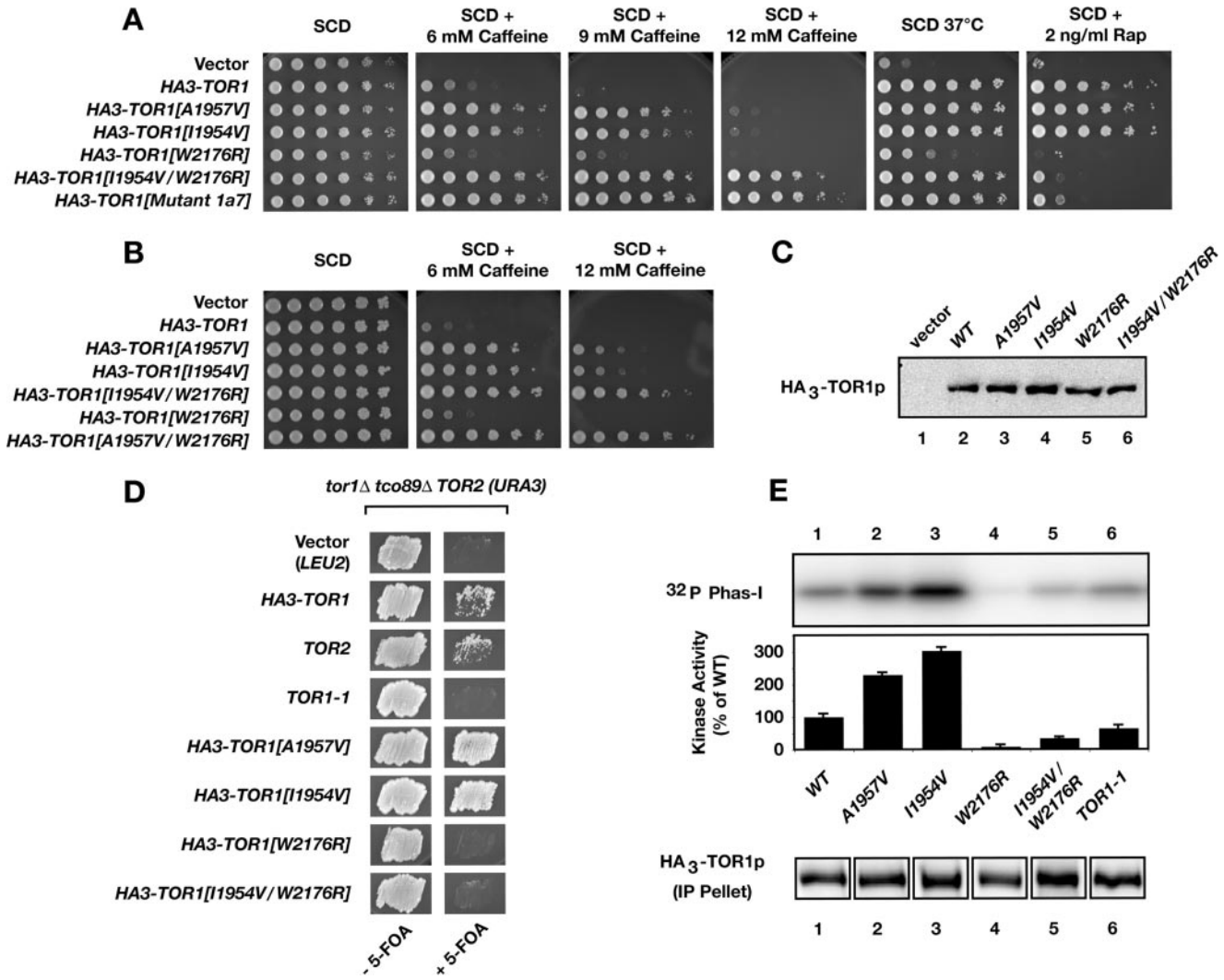

FIGURE 3. **Molecular basis for caffeine resistance by identified *TOR1* mutants.** *A* and *B*, *tor1*Δ (PLY254) cells were transformed with a control plasmid (pRS315) or plasmids that expressed wild-type *HA3-TOR1* (pPL132) or mutant *HA3-TOR1* alleles as indicated. Cells were grown in SCD minus leucine media to mid log phase at 30 °C, serially diluted, and plated out on SCD minus leucine agar plates that contained the indicated concentrations of caffeine or rapamycin. All plates were incubated at 30 °C, except for the indicated plate that was incubated at 37 °C. Photographs of plates were taken following ~3 days of growth. *C*, Western blot analysis of HA_3-Tor1p expressed in strains used in *A*. Cells were grown in SCD minus leucine media to mid log phase at 30 °C, harvested, and processed for Western blot analysis as described (3). *D*, testing the ability of different *TOR1* alleles to suppress the synthetic lethality of *tor1*Δ *tco89*Δ cells. Strain PLY365 was transformed with a control plasmid (pRS315) or plasmids that expressed *TOR2* (pJK4), wild-type *HA3-TOR1* (pPL132), or mutant *HA3-TOR1* alleles as indicated. Equivalent amounts of cells were then patched onto SCD minus leucine agar plates that either lacked or contained 1 mM 5-fluororotic acid to counter select for the *TOR2 URA3* plasmid (pNB100) present in PLY365. In *E*: *Upper panels*, results of immune complex kinase assays where Tor1p kinase activity is measured from *tor1*Δ (PLY254) cells that expressed the indicated alleles of *TOR1*. The blot is a representative experiment showing the amount of ^{32}P incorporated into PHAS-I, and the graph shows the average of three independent experiments, where *error bars* denote the standard deviation of the mean. *Lower panels*, Western blot analysis of extracts depicts levels of Tor1p present in each kinase assay.

TOR1 that expressed the I1954V FRB domain and W2176R kinase domain mutations singly, and we constructed a double mutant that combined the A1957V FRB and W2176R kinase domain mutations, and then examined the phenotypes of these mutants under a variety of conditions (Fig. 3, *A* and *B*). We observed that both the I1954V and A1957V FRB domain single mutants afforded a comparable level of caffeine resistance, where cells expressing these alleles grew at 9 mM but not 12 mM caffeine (Fig. 3*A*). Interestingly, the W2176R kinase domain mutation in combination with the A1957V FRB mutation also allowed growth at 12 mM caffeine, demonstrating that this kinase domain mutation could be combined with either of the two FRB domain mutations to confer increased caffeine resistance (Fig. 3*B*). By contrast, the W2176R kinase domain on its own conferred no caffeine resistance and, moreover, displayed both temperature-sensitive growth as well as rapamycin hypersensitivity, compared with wild-type *HA3-TOR1*, suggesting that this mutation impaired Tor1p function (Fig. 3*A*). Interestingly, the I1954V/W2176R double mutant was not temperature-sensitive, but it did display rapamycin hypersensitivity (Fig. 3*A*). These differences in phenotypes are unlikely to be explained by differences in protein stability, because Western blot analysis of cell extracts demonstrated that each mutant produced comparable steady-state levels of Tor1p (Fig. 3*C*).

We tested further the functionality of the FRB and kinase domain mutants by examining their ability to suppress the synthetic lethality of a *tor1*Δ *tco89*Δ double mutant (3). As reported previously (3), a wild-type copy of *TOR2* carried on a *LEU2*

plasmid allows *tor1*Δ *tco89*Δ cells to drop a *TOR2 URA3* plasmid, as judged by the appearance of colonies following counter selection against the *URA3* gene on agar plates containing 5-fluororotic acid (Fig. 3*D*). Similarly, we observed that the *HA3-TOR1 LEU2* (pPL132) plasmid also allowed for production of 5-fluororotic acid-resistant colonies with equal efficiency as the *TOR2 LEU2* plasmid (Fig. 3*D*). By contrast, neither the W2176R kinase domain single mutant nor the I1954V/W2176R double mutant allowed production of 5-fluororotic acid-resistant colonies, consistent with their impaired phenotypes described above. Remarkably, for both single FRB mutants, I1954V and A1957V, reproducibly more 5-fluororotic acid-resistant colonies were obtained in this assay, suggesting these alleles allowed *tor1*Δ *tco89*Δ cells to drop the *TOR2 URA3* plasmid with greater efficiency than wild-type *TOR1* or *TOR2* (Fig. 3*D*).

To gain insight into the molecular basis for these observed phenotypic differences, we examined the kinase activity of wild-type as well as several mutant HA_3-Tor1p proteins using an immune complex kinase assay that employed mammalian PHAS-I protein as a model substrate (5, 19, 21; see "Materials and Methods"). Western blot analysis confirmed that an equivalent amount of Tor1p protein was used in each assay (Fig. 3*E*, *bottom panels*). We observed that the W2176R kinase domain mutant had severely diminished activity, in comparison to wild-type Tor1p (Fig. 3*E*, *upper panels*, compare *lanes 1* and *4*). By contrast, the I1954V/W2176R double mutant displayed an intermediate activity, where it was improved relative to the kinase domain mutation alone yet possessed ~30% of the activity of wild-type (Fig. 3*E*, *upper panels*, compare *lanes 1*, *4*, and *5*). Remarkably, both of the single FRB domain mutations displayed a significant (~2- to 3-fold) increase in kinase activity compared with wild-type (Fig. 3*E*, *upper panels*, compare *lane 1* with *lanes 2* and *3*). Thus, in general there was remarkable agreement in terms of the *in vivo* phenotypes observed for these mutants and their *in vitro* kinase activity.

To extend these results further, we asked whether the behavior of the FRB and/or kinase domain mutations correlated with differences in the integrity of TORC1 by performing co-immunoprecipitation experiments. For this, we introduced plasmids that expressed wild-type *HA3-TOR1*, the single I1954V FRB domain mutation, the single W2176R kinase domain mutation, or an untagged control vector, into strains expressing Myc-epitope-tagged versions of Kog1p, Lst8p, or Tco89p. Extracts were prepared from these strains and used for immunoprecipitations using anti-HA antibody directed against HA_3-Tor1p, followed by Western blotting using anti-Myc antibody to assess the degree of co-precipitation of the different TORC1 components. We performed these experiments in strains expressing endogenous Tor1p, reasoning that competition for assembly with wild-type protein might reveal subtle differences.

Indeed, we observed that the I1954V FRB mutant afforded a subtle but reproducible increase in the amount of co-precipitating Kog1p-Myc, compared with wild-type HA_3-Tor1p (Fig. 4, *A* and *B*). By contrast, no significant differences were observed between wild-type and the FRB domain mutant with respect to interactions with Lst8p-Myc or Tco89p-Myc (data not shown). Similarly, no significant differences were observed for interactions between the W2176R kinase domain mutant and any of the TORC1 partners (Fig. 4, *A* and *B*, and data not shown). Thus, we conclude that the overall stability of TORC1 is not significantly impacted by either of these Tor1p mutations; rather, the activating I1954V FRB domain mutation results in only subtle modulation of interactions between Tor1p and Kog1p.

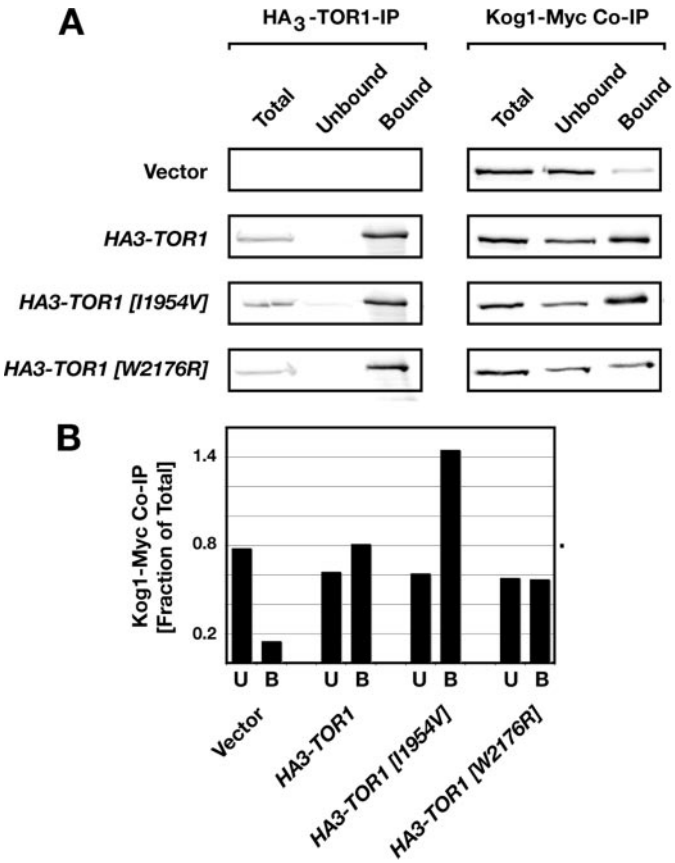

FIGURE 4. **An activating FRB domain mutation influences interactions between Tor1p and Kog1p.** *A*, immunoprecipitation (*IP*) of HA_3-Tor1p (*left panels*) and co-immunoprecipitation of Kog1p-Myc (*right panels*) from extracts prepared from strain PLY671 expressing the indicated plasmids. *B*, quantification of data, where levels of Kog1p-Myc present in unbound (*U*) and bound (*B*) fractions are expressed as a fraction of the total amount of Kog1p-Myc present in each reaction. Similar results were observed in two independent sets of experiments.

Rapamycin-resistant TOR Alleles Display Several Impaired Phenotypes—We sought to determine whether caffeine resistance was in general associated with mutations within the FRB domain by examining the behavior of the S1972R rapamycin-resistant *TOR1-1* allele. Toward this end, we transformed *tor1*Δ cells with plasmids that expressed *TOR1*, *TOR1-1*, or a control vector. We observed that, although the *TOR1-1* plasmid conferred an expected level of rapamycin resistance, this mutant was hypersensitive to caffeine, relative to wild-type *TOR1* (Fig. 5*A*). Similar results were also obtained using rapamycin-resistant strains in the JK9-3da background that expressed chromosomal *TOR1-1* or *TOR2-1* mutant genes (Fig. 5*B*). Two other observations indicated the *TOR1-1* allele encodes a Tor1p protein with impaired activity. First, we observed that this allele was unable to suppress the synthetic lethality of the *tor1*Δ

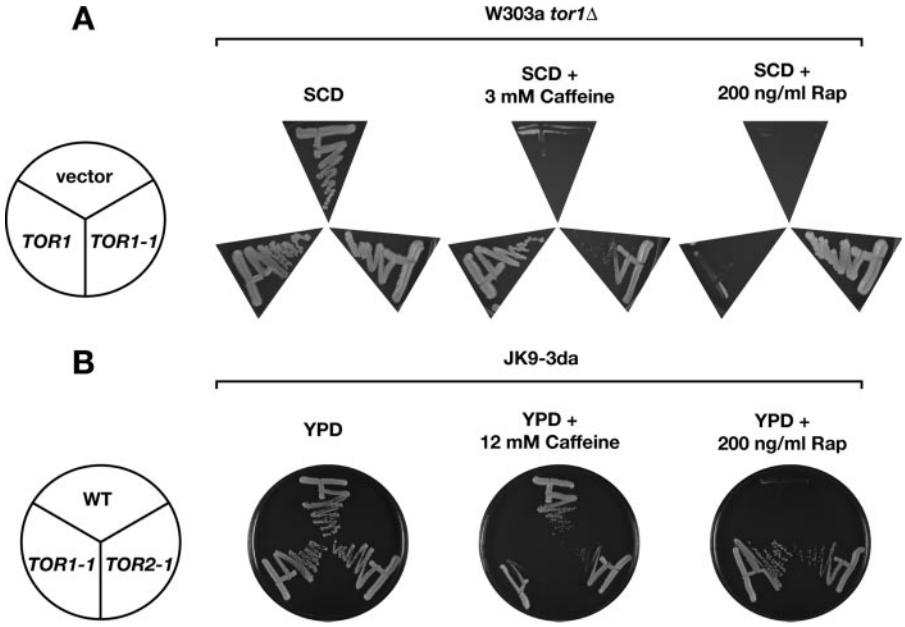

FIGURE 5. **Rapamycin-resistant alleles of TOR are hypersensitive to caffeine.** *A*, *tor1*Δ (PLY254) cells were transformed with a control plasmid (pRS315) or plasmids that expressed wild-type *TOR1* (pPL132) or *TOR1-1* (pYDF23) and streaked out onto SCD minus leucine agar plates, which contained the indicated concentrations of caffeine or rapamycin. *B*, wild-type (JK9-3da), *TOR1-1* (3H11-1c), and *TOR2–1* (JH12-17b) cells were streaked out onto YPD agar plates containing the indicated concentrations of caffeine or rapamycin. For *A* and *B*, plates were photographed following incubation at 30 °C for ~3 days. Note that different concentrations of caffeine were used in *A* and *B* due to differences in basal sensitivities of W303a *versus* JK9-3da.

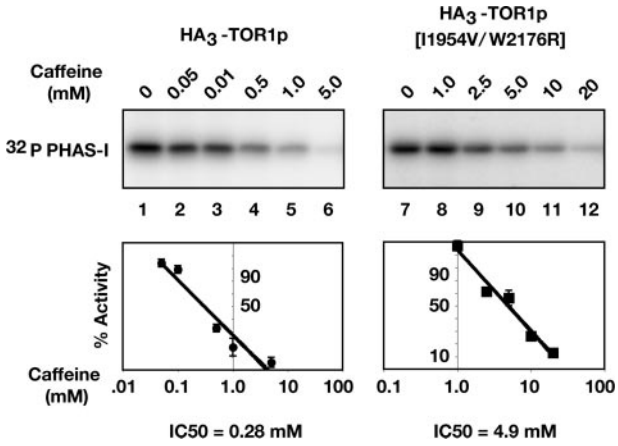

FIGURE 6. **Caffeine-resistant mutations in Tor1p affect interactions with caffeine *in vitro*.** Immune complex kinase assay shows activities of wild-type HA_3-Tor1p and HA_3-Tor1p (I1954V/W2176R) in the presence of the indicated concentrations of caffeine. *Blots* show representative samples of the amount of ^{32}P incorporated into PHAS-I, and the *graph* depicts the average of three independent experiments, where *error bars* denote ±S.D. Data were fit using linear regression to calculate an IC_{50} by caffeine for each kinase (26). Note that the exposure of the two blots has been adjusted so that the intensities of samples without caffeine appear equivalent for both WT HA_3-Tor1p and HA_3-Tor1p (I1954V/W2176R), to compensate for the reduced activity of the mutant, and has no effect on calculation of IC_{50} values.

*tco89*Δ double mutant, in contrast to wild-type *TOR1* (Fig. 3*D*). Second, Tor1-1p displayed reduced *in vitro* kinase activity relative to wild-type Tor1p in the immune complex kinase assay (Fig. 3*E*). Together these results demonstrate that adjacent mutations within the FRB domain lead to different phenotypes and suggest that this domain modulates Tor1p activity in distinct ways. This conclusion is also consistent with our finding that the caffeine-resistant FRB domain mutations do not on their own confer increased rapamycin resistance (Fig. 3*A* and data not shown).

Tor1p (I1954V/W2176R) Double Mutant Confers Caffeine Resistance in Vitro—To begin to test for physical interactions between Tor1p and caffeine, we used the immune complex kinase assay described above to determine whether caffeine inhibited Tor1p kinase activity, and, if so, whether this inhibition was influenced by the I1954V and W2176R mutations. We observed that caffeine inhibited the kinase activity of HA_3-Tor1p with an IC_{50} of 0.28 mM (Fig. 6, *left panels*), in very good agreement with the reported IC_{50} of ~0.4 mM caffeine for inhibition of mTOR (26). By contrast, the IC_{50} for inhibition of HA_3-Tor1p (I1954V/W2176R) was increased ~17-fold to 4.9 mM (Fig. 6, *right panels*). Based upon these results we conclude that together the I1954V and W2176R mutations in Tor1p result in impaired interactions with caffeine *in vitro* that is likely to contribute, at least in part, to caffeine-resistant growth of cells expressing this mutant *in vivo*.

FRB and Kinase Domain Mutations Likely Act Exclusively in cis—Both TORC1 as well as TORC2 have been proposed to function as dimers (1, 5). In addition, genetic evidence from *Drosophila* indicates that TOR in higher eukaryotes may also function as a multimer (44). Given these findings, we were interested in determining whether the I1954V FRB/W2176R kinase domain mutations function strictly within the same *TOR1* gene product to confer high levels of caffeine resistance or whether they might also function in *trans*. Toward this end, we introduced the I1954V and W2176R mutations into cells on separate *TOR1* CEN/ARS plasmids and asked whether these cells were able to grow on agar plates containing 12 mM caffeine. Caffeine-resistant colonies were indeed obtained, however, sequence analysis of plasmids retrieved from these cells revealed that in every case growth at 12 mM caffeine correlated with a recombination event between the plasmids such that both mutations were expressed in the context of a single *TOR1* gene (data not shown). Based on this observation, we conclude that these mutations are likely to act strictly within the context of a single Tor1p protein to confer highest levels of caffeine resistance.

DISCUSSION

We have demonstrated that TORC1-dependent signaling represents an important target for caffeine in yeast. Our analysis of caffeine-resistant *TOR1* alleles has been particularly informative in that it demonstrates the close correspondence between the effects of rapamycin and caffeine on global gene expression may be directly attributable to inhibition of TORC1. Of potentially greater importance, however, is our finding that

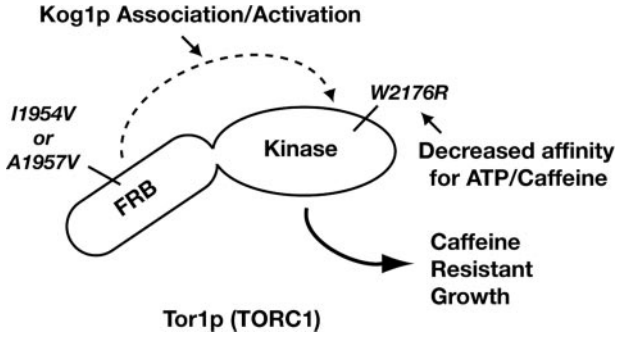

FIGURE 7. **Working model for mechanism of caffeine resistance for Tor1p mutants.** Model shows possible interactions between FRB and kinase domain mutations that together contribute to highest levels of caffeine resistance, which may also involve interactions with Kog1p. For clarity, only the FRB and kinase domains of Tor1p are shown. See text for details.

caffeine-resistant mutations within the FRB domain result in increased Tor1p function, as defined both by a number of *in vivo* phenotypes as well as by their *in vitro* kinase activity. Together with our finding that the rapamycin-resistant FRB domain mutation instead causes impaired Tor1p function, as has been shown previously for mTOR (19), these results underscore the importance of the FRB domain as an important modulator of TOR activity.

We can begin to account for the caffeine resistance conferred by the *TOR1* I1954V/W2176R as well as A1957V/W2176R double mutants by the working model presented in Fig. 7. According to this model, the W2176R kinase domain mutation impairs interactions with both caffeine as well as ATP, resulting in a Tor1p with severely impaired kinase activity. This suggestion is consistent with the proximity of Trp-2176 to Leu-2174, where the latter amino acid corresponds to the so-called "gatekeeper" identified recently by Shokat and co-workers (45) as likely being located within the ATP binding pocket and involved in nucleotide recognition within the phosphatidylinositol 3-kinase and PIKK protein kinase families. When the kinase domain mutation is combined with either one of the kinase-activating FRB mutations, however, the resulting double mutants now possess sufficient kinase activity to both support adequate Tor1p function as well as display significant caffeine resistance. By themselves, the FRB domain mutants are able to provide limited caffeine resistance, presumably due to their increased kinase activity. Consistent with this interpretation is the fact that the S1972R rapamycin-resistant *TOR1-1* allele possesses diminished kinase activity and is hypersensitive to caffeine.

Our results also begin to address the molecular basis by which caffeine-resistant mutations within the FRB domain influence Tor1p kinase function with the finding that the I1954V mutation results in increased association between Tor1p and its partner Kog1p. This result is consistent with previous findings for mTOR that the FRB domain is an important regulatory point for interactions with Raptor and, moreover, that Raptor is an important regulator of mTORC1 activity (6–8). The relationship between the FRB domain of Tor1p and Raptor/Kog1p is likely to be complicated, however, because available evidence suggests Raptor is unlikely to interact directly with the FRB domain of mTOR (7). We point out that our findings do not rule out the possibility that direct interactions between the FRB and kinase domains may additionally account the mutant phenotypes described here. The development of a more sensitive *in vitro* reconstituted system will be important for addressing these questions, as well as for exploring the exact mode of inhibition of TORC1 by caffeine.

It is likely that caffeine will turn out to interact with other components in yeast as well, including Tor2p within the context of TORC2. For example, an allele of *TOR2* has been described that is hypersensitive to caffeine and that impairs receptor-mediated endocytosis by what appears to be a TORC2-specific pathway (46). Similarly, temperature-sensitive mutations have been identified in the TORC2-specific component *AVO3* that also display caffeine hypersensitivity (47).[3] These findings are consistent with the fact that TORC2 is also strongly linked to cell integrity maintenance (5, 30). It is therefore unclear why we did not observe increased sensitivity to caffeine in *AVO1/avo1*Δ or *AVO3/avo3*Δ heterozygous mutants compared with wild type (Fig. 1*A*). One possibility is that TORC2 is less sensitive to changes in the dosage of these TORC2-specific genes compared with components of TORC1, at least with respect to this phenotype. A prediction of these findings is that it should be possible to isolate caffeine-resistant alleles of *TOR2*. Indeed, in a preliminary study we have obtained *TOR2* mutants that confer limited caffeine resistance.[3] Interestingly, these mutations appear to map to a distinct region of the kinase domain of *TOR2*. Whether these alleles affect TORC1 and/or TORC2 readouts remains to be determined.

In addition to mTOR, caffeine has been shown to inhibit the *in vitro* kinase activity of a number of other mammalian PIKKs, including ATM, ATR, and DNA-PK (26, 48, 49). Inhibition of ATM and ATR by caffeine has been correlated with the ability of the drug to suppress DNA damage-induced cell cycle checkpoints (26). This correlation has been questioned recently, however, following observations that several substrates of ATM and/or ATR are either not affected or instead become hyperphosphorylated following caffeine treatment *in vivo* (28, 29). Thus, the target for caffeine with respect to DNA damage-induced cell cycle arrest apparently remains to be identified (29). By contrast, there is a better correlation between the *in vitro* and *in vivo* effects of caffeine on mTOR-dependent substrates (19, 25). Together with our results presented here, we believe that inhibition by caffeine is likely to represent a common feature of TOR signaling. Here it is relevant that the *TOR1* mutations identified in this study correspond to highly conserved amino acids within TOR proteins from across the phylogenetic spectrum (Fig. 2*B*). Thus, it should be possible to identify and/or construct caffeine-resistant *TOR* alleles in other organisms as well. Such an approach may prove helpful in distinguishing the effects of caffeine on TOR-dependent *versus* TOR-independent readouts in other systems.

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