Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 332 (2005) 304–310
BBRC
www.elsevier.com/locate/ybbrc

# Characterization of the cloned full-length and a truncated human target of rapamycin: Activity, specificity, and enzyme inhibition as studied by a high capacity assay

Lourdes Toral-Barza [a], Wei-Guo Zhang [a], Craig Lamison [b], James LaRocque [b], James Gibbons [a], Ker Yu [a,*]

[a] Department of Discovery Oncology, Wyeth Research, Pearl River, NY 10965, USA
[b] Department of Chemical and Screening Sciences, Wyeth Research, Pearl River, NY 10965, USA

Received 13 April 2005
Available online 30 April 2005

**Abstract**

The mammalian target of rapamycin (mTOR/TOR) is implicated in cancer and other human disorders and thus an important target for therapeutic intervention. To study human TOR in vitro, we have produced in large scale both the full-length TOR (289 kDa) and a truncated TOR (132 kDa) from HEK293 cells. Both enzymes demonstrated a robust and specific catalytic activity towards the physiological substrate proteins, p70 S6 ribosomal protein kinase 1 (p70S6K1) and eIF4E binding protein 1 (4EBP1), as measured by phosphor-specific antibodies in Western blotting. We developed a high capacity dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) for analysis of kinetic parameters. The Michaelis constant ($K_m$) values of TOR for ATP and the His6-S6K substrate were shown to be 50 and 0.8 μM, respectively. Dose–response and inhibition mechanisms of several known inhibitors, the rapamycin–FKBP12 complex, wortmannin and LY294002, were also studied in DELFIA. Our data indicate that TOR exhibits kinetic features of those shared by traditional serine/threonine kinases and demonstrate the feasibility for TOR enzyme screen in searching for new inhibitors.


*Keywords:* TOR; Rapamycin; PIKK; Kinase inhibitor; Anticancer; High-throughput screening

In mammalian cells, TOR is a major component in the PI3K/AKT/TOR signaling pathways. The amino acid sequence and cellular function of the TOR proteins are highly conserved across the species [1,2]. TOR contributes to cell growth and survival by controlling cellular protein translation in response to growth factors, nutritional signals, and energy supply [1,2]. The catalytic domain of TOR displays a high homology to the phosphatidylinositol-3 kinase (PI3K) related kinase (PIKK) family that includes PI3K, DNA-PK, ATM, and ATR [3]. It has been shown that the TOR proteins isolated from mammalian cells could directly phosphorylate the translation components p70S6K1 and 4EBP1 [4–6].

The TOR signaling pathway is widely implicated in cancer and has emerged as an important target for therapeutic intervention (reviewed in [7,8]). The rapamycin analogs such as CCI-779 have shown promising efficacy in preclinical cancer models as well as in ongoing human trials [7,8]. Thus, the biological importance and validity of TOR as a drug target have made it a high priority target for novel inhibitors. To date, however, high capacity enzyme assays for TOR have not been reported. Due to its large molecular weight, and the technical and

* Corresponding author. Fax: +1 845 602 5557.
*E-mail address:* yuk@wyeth.com (K. Yu).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.117

resource challenges in production and storage of TOR enzyme, the existing assays [4–6,9] employ a fresh immunoprecipitation of TOR from cell lysates followed by immune-complex kinase assay. Most assays also require the use of radioactive ATP or Western blotting for detection of substrate phosphorylation. Consequently, in vitro studies of the enzyme kinetic parameters and inhibitor mechanisms have been very limited and are unavailable in the literature. These same challenges also limited efforts in searching for novel inhibitors. In this report, we have expressed, purified, and characterized active human TOR full-length and a truncated enzyme. We demonstrate the utility of a sensitive, high capacity DELFIA format of TOR in searching for novel small molecule inhibitors.

## Materials and methods

*Chemicals.* All general chemical reagents used for buffers and assays were purchased from Sigma unless otherwise specified. Wortmannin was obtained from Calbiochem. Rapamycin was obtained from the Wyeth Chemical and Pharmaceutical Development.

*cDNA cloning and plasmids.* The human TOR cDNA was cloned from human placenta Quick cDNA (Cat # 637208, BD-Clontech). The entire cDNA was sequenced and confirmed to be identical to the previous report [10]. The full-length TOR and the region corresponding to amino acid 1360–2549 [11] were inserted into the *Eco*RI site of p-FLAG-CMV-2 (Sigma) to generate the Flag-tagged full-length FLAG-TOR(FL) and the truncated FLAG-TOR(3.5), respectively. The His6-tagged p70S6K1 (His6-S6K) construct was made by inserting a fragment of human p70S6K1 (amino acids 332–415) [5] into the *Bam*HI and *Eco*RI site of the bacterial vector pET-32a(+) (Novagen) to generate a fusion of additional 165 amino acids to the S6K1 fragment. This expression system offers multiple tags for purification and detection, as well as a greatly enhanced solubility to the expressed protein.

*Expression and purification of human TOR and His6-S6K.* All cell culture media, supplements, and transfection reagents were obtained from Invitrogen. HEK293 (ATCC) cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μg/mL penicillin, 50 μg/mL streptomycin, and 1 mM glutamine. Plasmid DNA (50 μg per 150-mm culture plate) of the p-FLAG-TOR(FL) and p-FLAG-TOR(3.5) was transiently transfected into HEK293 using Lipofectamine 2000. Cells were processed 48 h later with all steps performed at 4 °C. Cells were washed with phosphate-buffered saline (PBS) and scraped off the plate in 1.5 mL lysis buffer (20 mM Tris–HCl (pH 7.5), 100 mM KCl, 20 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 0.25 mM $Na_3VO_4$, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 nM okadaic acid, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μM microcystin LR). The cell lysate was then sonicated, incubated for 30 min with gentle shaking, and then cleared by centrifugation for 30 min at 14,000*g* using a Beckman J2-HS centrifuge. The clear lysate was collected and stored at −80 °C. FLAG-TOR proteins were isolated using anti-FLAG M2 affinity gel (Sigma) by batch absorption method. The previously frozen lysate was thawed at 4 °C, added to a pre-equilibrated anti-FLAG M2 gel (1 mL affinity beads per 10 mL lysate), and incubated for 3 h with gentle shaking. The immune-complexes were washed sequentially with lysis buffer, lysis buffer plus 500 mM KCl, and the kinase buffer wash (10 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate, and 0.5 μM microcystin LR). FLAG-TOR enzyme was eluted by 200 μg/mL FLAG peptide (Sigma) in kinase buffer wash supplemented with 0.1% β-mercaptoethanol, 0.03% BRIJ, and 10% glycerol. Eluted proteins were quickly frozen on dry ice and stored in −80 °C.

Bacterial culture with pET-His6-S6K was grown in 37 °C and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h before cell pellets were collected. The His6-S6K fusion protein was purified following the protocol provided by the manufacturer for batch purification under native conditions using Ni–NTA Superflow resin (Qiagen). A His6-tagged human FKBP12 construct [12] was similarly expressed and purified.

Protein concentrations were determined by the Bradford method (Biorad) using bovine serum albumin (BSA) as standard. To assess for purity, eluted FLAG-TOR proteins were resolved in a 3–8% Tris–acetate NuPAGE gel, and the His6-S6K in a 10% Bis–Tris NuPAGE gel (Invitrogen). Gels were either directly stained with Coomassie blue or transferred to nitrocellulose for Western blotting (see below).

*Immune-complex TOR kinase assay and Western blotting.* The endogenous TOR of LNCap cell lysate was immunoprecipitated by anti-FRAP/TOR (N-19, Santa Cruz). Cell lysate (1.0 mg) was mixed with 4 μg antibody coupled to protein-G/A agarose in 1 mL lysis buffer. The immune-complexes were washed sequentially with lysis buffer, lysis buffer plus 500 mM KCl, and kinase buffer wash. The immune-complexes were subjected to kinase reaction for 30 min at 30 °C in a final volume of 50 μL containing 10 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate, and 0.5 μM microcystin LR, 1 mM DTT, 10 mM $MnCl_2$, 100 μM ATP, 1 μg His6-S6K or 1 μg His6-4EBP1 (Stratagene). Kinase reactions (immune-complex and purified enzymes) were terminated by NuPAGE LDS sample buffer and resolved in a 4–12% NuPAGE Bis-Tris gel for Western blotting with anti-P(T389)-p70S6K and anti-P(T46)-4EBP1 (Cell Signaling), anti-FRAP/TOR (N-19, Santa Cruz), anti-FLAG M2 (Sigma), and anti-His6 (Clone His-1, Sigma). In the radioactive assay, 10 μCi [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, Amersham) and 100 μM cold ATP were used. $^{32}$P-labeled products were resolved by SDS–PAGE and subjected to autoradiogram to Kodak X-ray films.

*DELFIA format of purified FLAG-TOR.* The routine assays with purified FLAG-TOR (FL and 3.5) were performed in 96-well plates as follows. Enzymes were first diluted in kinase assay buffer (10 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate, 10 mM $MnCl_2$, 0.5 mM DTT, 0.25 μM microcystin LR, and 100 μg/mL BSA). To each well, 12 μL of the diluted enzyme was mixed briefly with 0.5 μL test inhibitor or control vehicle dimethyl sulfoxide (DMSO). The kinase reaction was initiated by adding 12.5 μL kinase assay buffer containing ATP and His6-S6K to give a final reaction volume of 25 μL containing 800 ng/mL FLAG-TOR, 100 μM ATP, and 1.25 μM His6-S6K. The reaction plate was incubated for 2 h (linear at 1–6 h) at room temperature with gentle shaking and then terminated by adding 25 μL Stop buffer (20 mM Hepes (pH 7.4), 20 mM EDTA, and 20 mM EGTA).

The DELFIA detection of the phosphorylated (Thr-389) His6-S6K was performed at room temperature using a monoclonal anti-P(T389)-p70S6K antibody (1A5, Cell Signaling) labeled with Europium-N1-ITC (Eu) (10.4 Eu per antibody, PerkinElmer). The DELFIA buffer and Enhancement solution were purchased from PerkinElmer. Forty-five microliters of the terminated kinase reaction mixture was transferred to a MaxiSorp plate (Nunc) containing 55 μL PBS. The His6-S6K was allowed to attach for 2 h after which the wells were aspirated and washed once with PBS. One hundred microliters of DELFIA buffer with 40 ng/mL Eu-P(T389)-S6K antibody was added. The antibody binding was continued for 1 h with gentle agitation. The wells were then aspirated and washed four times with PBS containing 0.05% Tween 20 (PBST). One hundred microliters of DELFIA Enhancement solution was added to each well and the plates were read in a PerkinElmer Victor model plate reader. Data obtained were used to calculate enzymatic activity and enzyme inhibition by potential inhibitors.

## Results and discussion

### *Activity and specificity of purified FLAG-TOR in substrate phosphorylation*

We cloned human TOR cDNA and expressed both the FLAG-tagged full-length and the truncated TOR, FLAG-TOR(FL) and FLAG-TOR(3.5), in HEK293 cells (Fig. 1A). The 132 kDa FLAG-TOR(3.5) is similar to the previously described 1362C mTOR fragment [11] that retains the FAT, FRB (FKBP12–rapamycin binding) and the C-terminal kinase domains. Purification by FLAG-M2 affinity gel yielded about 50–100 μg enzyme per 150-mm culture dish. Fig. 1B shows a Coomassie blue staining of the purified FLAG-TOR proteins. When run with the SeeBlue Plus2 pre-stained molecular weight marker standard (Invitrogen), the FLAG-TOR(FL) and FLAG-TOR(3.5) each migrated as single band around 210 and 111 kDa, respectively. Western blots (not shown) confirmed the reactivity of both purified proteins to anti-FLAG and anti-TOR antibodies. The His6-S6K fragment expressed from the pET-32a(+) has a calculated molecular mass of 30 kDa and was visualized around 32 kDa (Fig. 1C), and it also reacted to anti-His6 (not shown).

To determine the activity and specificity of FLAG-TOR enzymes, we assayed substrate phosphorylation by Western blotting. It was previously established that TOR immune-complex could directly phosphorylate p70S6K1 at Thr-389 and 4EBP1 at Thr-46/37 [4,5]. As shown in Fig. 2A, the FLAG-TOR(FL) can efficiently phosphorylate His6-S6K at Thr-389 and His6-4EBP1 at Thr-46, as measured by the strong reactivity to the anti-P(T389) and anti-P(T46) antibodies. Interestingly, the truncated FLAG-TOR(3.5) showed a similarly robust phosphorylation to the substrates (Fig. 2A). Judging from the intensity of phosphor-signals, both TOR enzymes appear to possess a comparable activity with FLAG-TOR(3.5) being slightly more active. It was also apparent that both enzymes had identical activity in the presence of 0.1 and 1 mM ATP. The FLAG-TOR(3.5) was also assayed with radioactive ATP. In Fig. 2B, [$^{32}$P]ATP was incorporated into His6-4EBP1 and the truncated TOR itself in a TOR-dependent manner. These data indicate that the truncated TOR is capable of both autophosphorylation and substrate phosphorylation.

### *Development of a sensitive and high capacity DELFIA*

We employed an Eu-P(T389)-S6K antibody to design a DELFIA format as illustrated in Fig. 3A. In this assay format, both the FLAG-TOR(FL) and FLAG-TOR(3.5) demonstrated a specific and dose-dependent phosphorylation of His6-S6K measured as DELFIA signal (Fig. 3B). As with the result obtained by Western blotting,

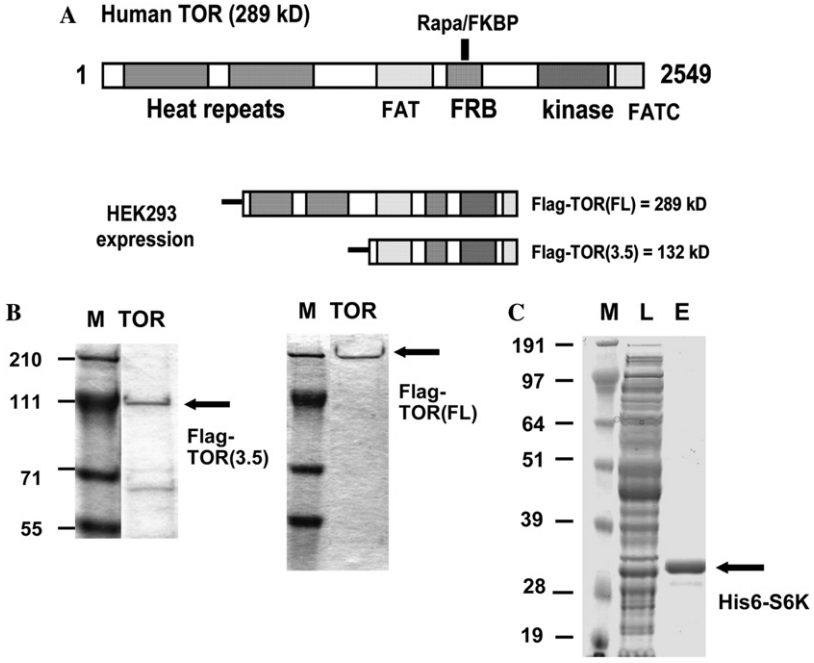


Fig. 1. Expression and purification of recombinant human TOR and His6-S6K. (A) Schematic presentation of human TOR showing the HEAT repeats, FAT, FRB domains, and the C-terminus kinase catalytic domain. The FLAG-TOR(FL) and FLAG-TOR(3.5) were transiently expressed in HEK293 cells and purified. (B) Equal amounts of purified proteins were resolved in a 3–8% Tris–acetate NuPAGE gel and stained with Coomassie blue. M, molecular marker; TOR, FLAG-TOR(FL) or FLAG-TOR(3.5) as indicated by arrows. (C) The purified bacterially expressed His6-S6K fragment was resolved in a 10% Bis-Tris NuPAGE gel and stained with Coomassie blue. L, total cell lysate; E, eluted protein.

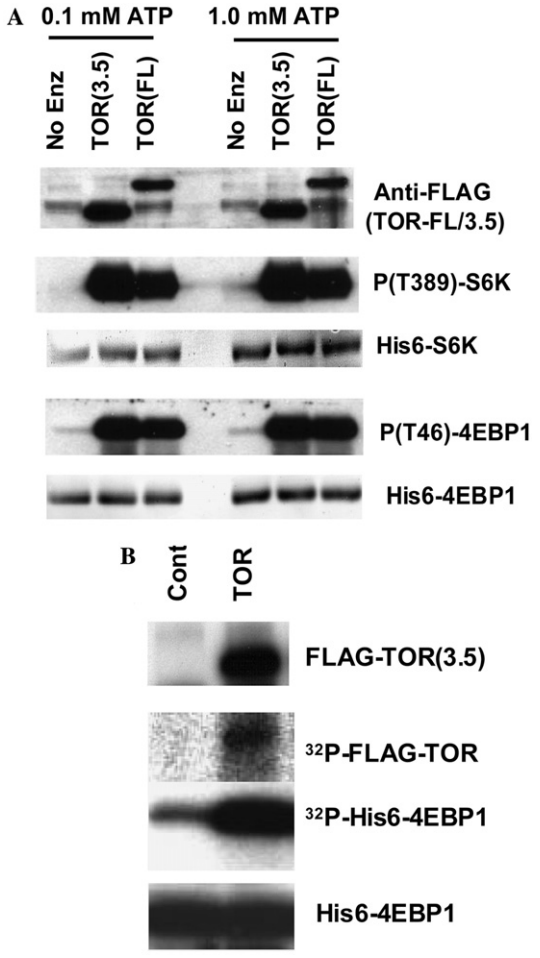


Fig. 2. Catalytic activity and specificity of FLAG-TOR(FL) and FLAG-TOR(3.5) in substrate phosphorylation. (A) Purified FLAG-TOR(FL) and FLAG-TOR(3.5) (100 ng) were assayed to phosphorylate His6-S6K (1 μg) or His6-4EBP1 (1 μg) in 25 μL with 0.1 or 1 mM ATP. The assay products were resolved in 10% Bis-Tris NuPAGE gels. The blots were stained with Ponceau S for total His6-S6K and His6-4EBP1, and were then probed with anti-FLAG, anti-P(T389)-p70S6K, and anti-P(T46)-4EBP1. (B) FLAG-TOR(3.5) was assayed in a radioactive assay using His6-4EBP1 and [γ-$^{32}$P]ATP. Assay products were resolved in a 4–20% Tris–glycine SDS–PAGE, processed, and detected separately by autoradiogram and by Western blot with anti-FLAG antibody.

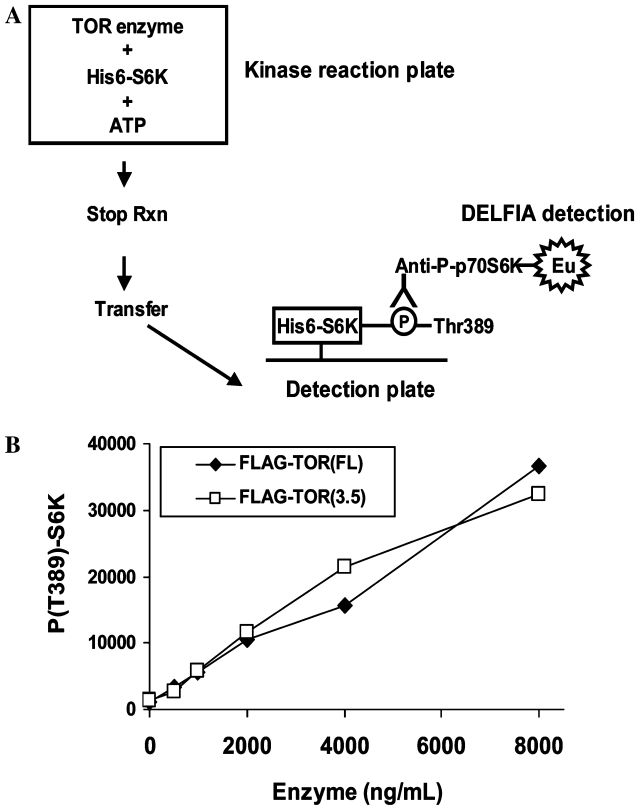


Fig. 3. A microtiter plate DELFIA of TOR. (A) Schematic presentation of the TOR kinase reaction and the DELFIA format detection. (B) Kinase reaction (25 μL per well) containing 100 μM ATP, 1.25 μM His6-S6K substrate, and 500–8000 ng/mL of FLAG-TOR(FL) or FLAG-TOR(3.5) were carried out in a 96-well plate for 1 h. Reactions were terminated and transferred to a MaxiSorp plate for capturing His6-S6K. DELFIA detection of the phosphorylated His6-S6K by the Eu-P(T389)-S6K antibody was performed as illustrated in (A) and described in Materials and methods.

the truncated FLAG-TOR(3.5) had a nearly identical activity compared to the FLAG-TOR(FL). Because of its reduced size and it being less cumbersome in production, our subsequent efforts focused on FLAG-TOR(3.5). Assay optimization was achieved by a number of factors such as buffer type, dose titration of the enzyme, substrate, ATP, and assay time course. We observed that while the purified TOR was relatively stable when stored in −80 °C, enzyme activity diminished substantially when diluted and kept in assay buffer for 2 h (Fig. 4A). Inclusion of DTT and BSA in the enzyme dilution buffer greatly stabilized the enzyme even when the diluted enzyme was left at room temperature for up to 2 h (Figs. 4A and B). To determine the time course of the kinase reaction, standard reaction mixtures were incubated and terminated at various time points. The data in Fig. 4C indicated that the TOR kinase activity was linear for at least 4–6 h. In consideration of optimal assay signal and time management, we adopted 2 h as standard assay time.

*Substrate and ATP dependence*

We determined the Michaelis constant ($K_m$) values for the substrate His6-S6K and ATP by incubating FLAG-TOR(3.5) with various concentrations of His6-S6K and ATP. Figs. 5A and B show the representative Michaelis–Menten plots of FLAG-TOR(3.5) enzyme rate as a function of increasing His6-S6K concentration and a constant ATP concentration or vice versa. The apparent $K_m$ for substrate His6-S6K was calculated to be around 0.8 μM (Fig. 5A). The apparent $K_m$ for ATP was around 50 μM (Fig. 5B). This value for ATP is significantly lower than a previously reported value of 1 mM ATP [13]. To further clarify this discrepancy

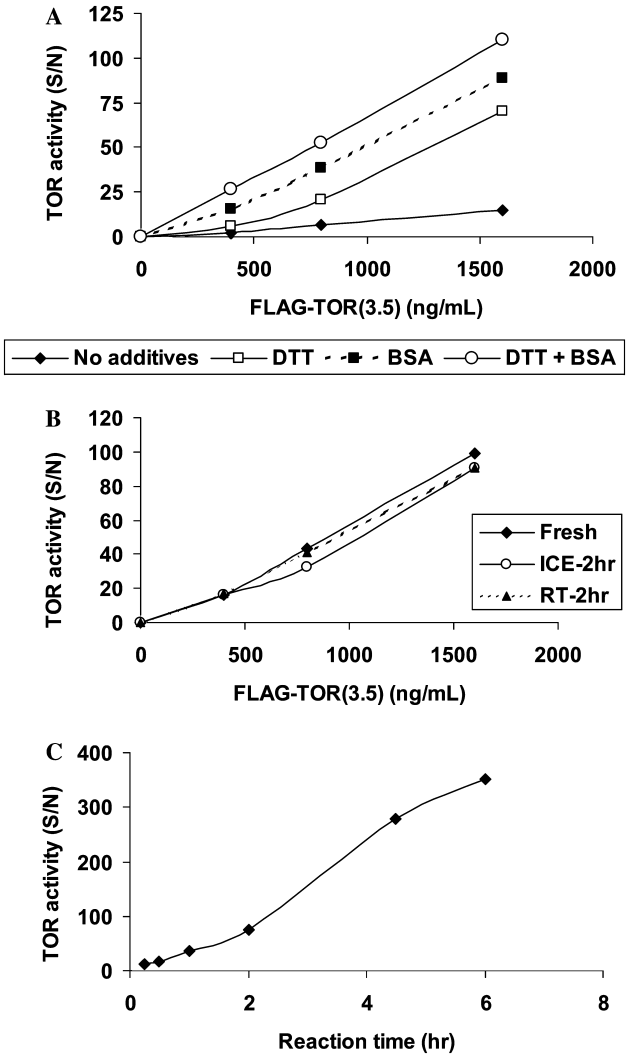


Fig. 4. Enzyme stability and assay time course. (A) FLAG-TOR(3.5) was diluted in the kinase assay buffer, or diluted in the assay buffer with 0.5 mM DTT, or 100 μg/mL BSA, or DTT plus BSA. The diluted enzymes were left at room temperature for 1 h before being assayed (2 h assay). (B) FLAG-TOR(3.5) was diluted in assay buffer containing 0.5 mM DTT and 100 μg/mL BSA, and either assayed immediately (fresh) or allowed to sit for 2 h at room temperature (RT-2 h), or on ice (ICE-2 h) before being assayed (2 h assay). (C) The optimal assay was run using 800 ng/mL FLAG-TOR(3.5) and 1.25 μM His6-S6K. The reaction was stopped at the indicated time points and detected as in Fig. 3. The assay response was linear for 4–6 h.

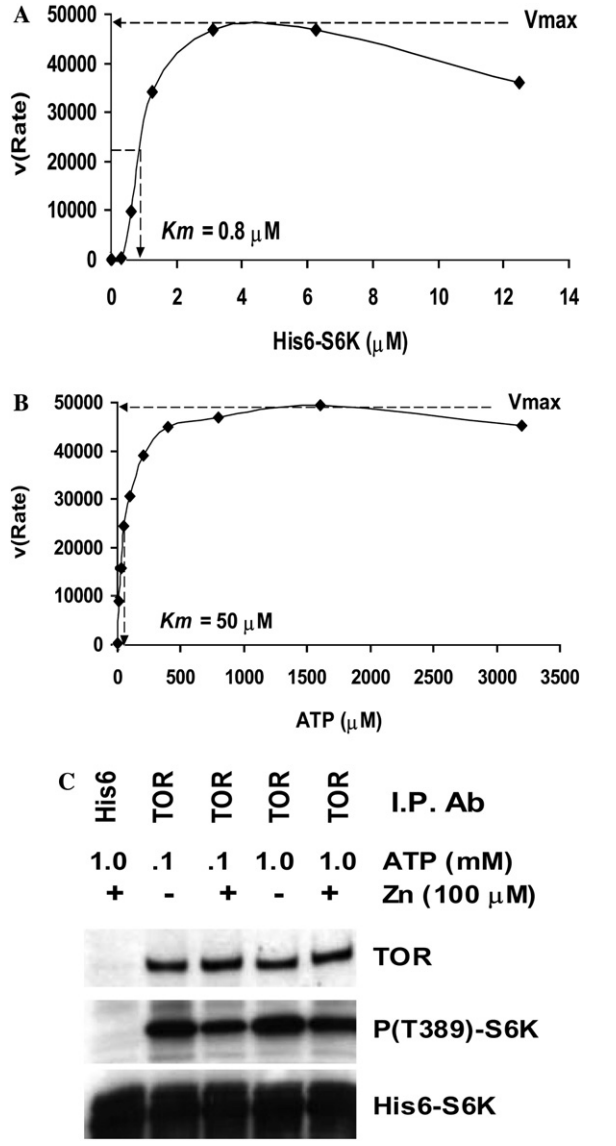


Fig. 5. Determination of TOR $K_m$ values for His6-S6K and ATP. (A) The enzyme rate (V) was measured in assay with various amounts of His6-S6K and a constant 200 μM ATP. (B) The enzyme rate ($V$) was measured with varying amounts of ATP and a constant 4 μM His6-S6K. Both assays were run using 800 ng/mL FLAG-TOR(3.5), terminated, and detected by DELFIA as in Fig. 3. $K_m$ values were calculated from the $X$-axis intercept that coincided with the 1/2 $V_{max}$ point. The apparent $K_m$ of TOR(3.5) for substrate His6-S6K was calculated to be 0.8 μM. The apparent $K_m$ of TOR(3.5) for ATP was calculated to be 50 μM. The data in (A,B) are representative graphs of at least three assays. (C) The endogenous TOR immunoprecipitated from LNCap cell lysate was assayed in an immune-complex kinase assay with 0.1 or 1 mM ATP. The reactions were terminated and analyzed by Western blot with anti-TOR, anti-P(T389)-p70S6K, and anti-His6 antibodies.

and because the previous result [13] was generated by an immune-complex assay of a full-length TOR, we conducted a similar assay using the endogenous full-length TOR immunoprecipitated from LNCap cells. The immune-complexes were assayed with 0.1 or 1 mM ATP, with or without zinc supplement. As shown in Fig. 5C, the TOR-dependent Thr-389 phosphorylation of His6-S6K was similar in 0.1 and 1 mM ATP, and addition of zinc reduced activity slightly. Thus, our data in Figs. 5B and C and in Fig. 2A demonstrated that both the full-length and the truncated TOR exhibited apparent ATP $K_m$ values no greater than 50 μM. Although our result is substantially different from the report [13], the kinetic parameters of TOR determined in our assays are well within the $K_m$ ranges of ATP and substrate reported for traditional serine/threonine kinases [17].

*TOR enzyme inhibition*

Once we have established an optimal DELFIA, we assessed the effects of known inhibitors of PI3K and TOR, wortmannin and LY294002 [6]. TOR activity was measured in the presence of various doses of wortmannin, LY294002, and staurosporine. In Fig. 6A, both wortmannin and LY294002 dose dependently inhibited TOR activity with $IC_{50}$ (the concentration that confers 50% inhibition) values of 0.2 and 1.5 μM, respectively. In contrast, the general kinase inhibitor staurosporine was largely inactive at the doses up to 3 μM (Fig. 6A). The rapamycin–FKBP12 complex inhibits TOR by binding to the FRB domain [12]. Some reports suggest that rapamycin may inhibit TOR substrate phosphorylation by activation of PP2A phosphatase rather than inhibition of the catalytic activity of TOR [14,15]. In the assay shown in Fig. 6B, while rapamycin alone had little effect, the rapamycin–FKBP12 complex exhibited a highly potent and dose-dependent inhibition of TOR catalytic activity with an $IC_{50}$ value of 2 nM. Thus, although the cellular mechanism of TOR inhibition remains complex, our data support a direct inhibition of TOR catalytic activity by the rapamycin–FKBP12 complex at the doses that confer inhibition of TOR signaling in cells.

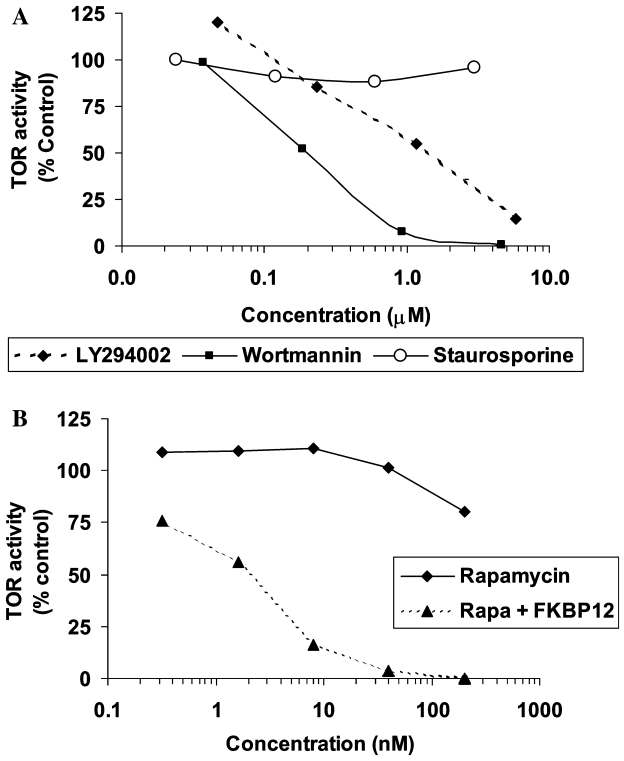


Fig. 6. Inhibition of FLAG-TOR(3.5) enzymatic activity by known inhibitors. (A) Wortmannin, LY294002, staurosporine or (B) rapamycin, rapamycin plus FKBP12. Inhibitors or vehicle DMSO were first added to the enzyme mixture. The kinase reactions were initiated with the addition of substrate mixture with His6-S6K and ATP. The assays were continued for 2 h and detected as in Fig. 3. The TOR activity in the presence of various concentrations of inhibitor was expressed as percent of control activity relative to the DMSO vehicle treatment. The inhibitor concentration that confers 50% reduction of TOR activity is termed as $IC_{50}$.

*Mechanism and mode of inhibition*

The PI3K inhibition mechanisms by wortmannin and LY294002 were previously elucidated by the co-crystallization studies with PI3Kγ [16]. On the basis of high homology in the active site between PI3K and TOR, these compounds may inhibit TOR by similar binding mechanisms. We employed the DELFIA to validate the inhibition kinetics. In Fig. 7A, TOR inhibition $IC_{50}$ values by wortmannin and LY294002 were

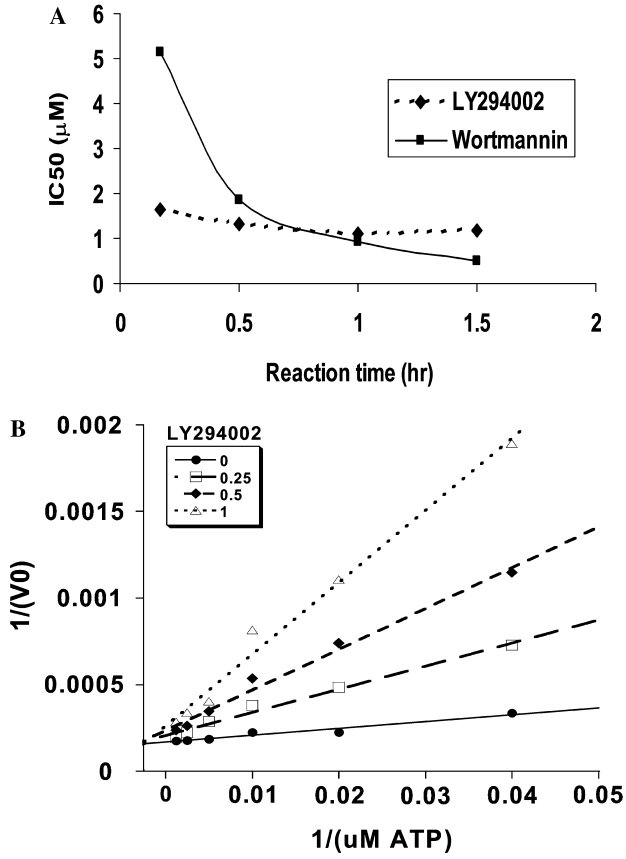


Fig. 7. Inhibition kinetics of wortmannin and LY294002. (A) The inhibitors were first added to the substrate/ATP mixture and the reactions were immediately initiated by adding the enzyme mixture. The assays were terminated at various time points during the incubation period for $IC_{50}$ determination. $IC_{50}$ values are plotted against reaction times. (B) LY294002 was tested in an ATP matrix competition assay. Various doses of the compound were added to the substrate/ATP mixture and the reactions were immediately initiated by adding the enzyme mixture. The assays were terminated in 20 min and detected as in Fig. 3. The initial enzyme rate ($V_0$) against 0, 0.25, 0.5, and 1.0 μg/mL LY294002 at various concentrations of ATP was measured to generate the double-reciprocal plot.

determined after various assay times. LY294002 achieved nearly identical $IC_{50}$ values at all assay time points indicating time independence, a feature of reversible inhibitor. In contrast, wortmannin exhibited a slow but steady time-dependent decrease in $IC_{50}$ consistent with it being an irreversible inhibitor. LY294002 was also tested in an inhibitor versus ATP matrix assay. The initial enzyme rates ($V_0$) were determined at various doses of LY294002 in the presence of various concentrations of ATP. In Fig. 7B, the double-reciprocal plots obtained in the absence or with various doses of LY294002 showed a family of lines with a common intercept on the $1/(V_0)$ axis indicating that the TOR inhibition by LY294002 was ATP competitive. Similar experiments on wortmannin indicated that it was also ATP competitive (not shown). In the PI3K$\gamma$ co-crystallization study [16], wortmannin and LY294002 were each shown to bind to the ATP pocket. Further, wortmannin covalently modifies a critical Lys-833 of the active site of PI3K$\gamma$ resulting in an irreversible inhibition. Thus, our data in Figs. 7A and B collectively indicate that both LY294002 and wortmannin inhibit TOR through most likely the same mechanisms as previously elucidated for PI3K. These data also established a general utility of the TOR DELFIA in future studies of inhibition mechanisms of novel inhibitors.

## Conclusions

Recombinant human TOR enzymes have been successfully produced and purified from HEK293 cells. A sensitive and high capacity DELFIA for TOR kinase inhibitors was developed and validated. This assay gave us a valuable tool to screen the vast Wyeth collection of compounds and extracts for identification of novel inhibitors of human TOR.

## Acknowledgments

We thank Adam Pitkin, Ann Stephenson for technical assistance, and Dr. Kim Arndt for helpful discussion.

## References

[1] T. Schmelzle, M.N. Hall, TOR, a central controller of cell growth, Cell 103 (2000) 253–562.
[2] D.C. Fingar, J. Blenis, Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression, Oncogene 23 (2004) 3151–3171.
[3] C.T. Keith, S.L. Schreiber, PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints, Science 270 (1995) 50–51.
[4] G.J. Brunn, C.C. Hudson, A. Sekulic, J.M. Williams, H. Hosoi, P.J. Houghton, J.C. Lawrence Jr., R.T. Abraham, Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin, Science 277 (1997) 99–101.
[5] P.E. Burnett, R.K. Barrow, N.A. Cohen, S.H. Snyder, D.M. Sabatini, RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1, Proc. Natl. Acad. Sci. USA 95 (1998) 1432–1437.
[6] G.J. Brunn, J. Williams, C. Sabers, G. Wiederrecht, J.C. Lawrence Jr., R.T. Abraham, Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002, EMBO J. 15 (1996) 5256–5267.
[7] M.A. Bjornsti, P.J. Houghton, The TOR pathway: a target for cancer therapy, Nat. Rev. Cancer 4 (2004) 335–348.
[8] K. Inoki, M.N. Corradetti, K.L. Guan, Dysregulation of the TSC–mTOR pathway in human disease, Nat. Genet. 37 (2005) 19–24.
[9] G.G. Chiang, R.T. Abraham, Determination of the catalytic activities of mTOR and other members of the phosphoinositide-3-kinase-related kinase family, Methods Mol. Biol. 28 (2004) 125–141.
[10] E.J. Brown, M.W. Albers, T.B. Shin, K. Ichikawa, C.T. Keith, W.S. Lane, Schreiber, A mammalian protein targeted by G1-arresting rapamycin–receptor complex, Nature 369 (1994) 756–758.
[11] M. Vilella-Bach, P. Nuzzi, Y. Fang, J. Chen, The FKBP12–rapamycin-binding domain is required for FKBP12–rapamycin-associated protein kinase activity and G1 progression, J. Biol. Chem. 274 (1999) 4266–4272.
[12] J. Choi, J. Chen, S.L. Schreiber, J. Clardy, Structure of the FKBP12–rapamycin complex interacting with the binding domain of human FRAP, Science 273 (1996) 239–242.
[13] P.B. Dennis, A. Jaeschke, M. Saitoh, B. Fowler, S.C. Kozma, G. Thomas, Mammalian TOR: a homeostatic ATP sensor, Science 294 (2001) 1102–1105.
[14] R.T. Peterson, B.N. Desai, J.S. Hardwick, S.L. Schreiber, Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12–rapamycinassociated protein, Proc. Natl. Acad. Sci. USA 96 (1999) 4438–4442.
[15] R.T. Peterson, P.A. Beal, M.J. Comb, S.L. Schreiber, FKBP12–rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions, J. Biol. Chem. 275 (2000) 7416–7423.
[16] E.H. Walker, M.E. Pacold, O. Perisic, L. Stephens, P.T. Hawkins, M.P. Wymann, R.L. Williams, Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine, Mol. Cell 6 (2000) 909–919.
[17] A.M. Edelman, D.K. Blumenthal, E.G. Krebs, Protein serine/threonine kinases, Annu. Rev. Biochem. 56 (1987) 567–613.