A Chemical Genetic Screen for mTOR Pathway Inhibitors Based on 4E-BP-Dependent Nuclear Accumulation of eIF4E

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DOI 10.1016/j.chembiol.2009.11.010

SUMMARY

The signal transduction pathway wherein mTOR regulates cellular growth and proliferation is an active target for drug discovery. The search for new mTOR inhibitors has recently yielded a handful of promising compounds that hold therapeutic potential. This search has been limited by the lack of a high-throughput assay to monitor the phosphorylation of a direct rapamycin-sensitive mTOR substrate in cells. Here we describe a novel cell-based chemical genetic screen useful for efficiently monitoring mTOR signaling to 4E-BPs in response to stimuli. The screen is based on the nuclear accumulation of eIF4E, which occurs in a 4E-BP-dependent manner specifically upon inhibition of mTOR signaling. Using this assay in a small-scale screen, we have identified several compounds not previously known to inhibit mTOR signaling, demonstrating that this method can be adapted to larger screens.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a key integrator of multiple intracellular and extracellular cues regulating cell growth and proliferation. Enhanced progrowth mTOR signaling is often seen in cancer resulting from mutations of upstream regulators and proliferation. Enhanced progrowth mTOR signaling is often induced by multiple intracellular and extracellular cues regulating cell growth. Here we describe a novel cell-based chemical genetic screen useful for efficiently monitoring mTOR signaling to 4E-BPs in response to stimuli. The screen is based on the nuclear accumulation of eIF4E, which occurs in a 4E-BP-dependent manner specifically upon inhibition of mTOR signaling. Using this assay in a small-scale screen, we have identified several compounds not previously known to inhibit mTOR signaling, demonstrating that this method can be adapted to larger screens.
A screen wherein phosphorylation of known mTOR substrates is used as an endpoint is restricted by the availability of suitable antibodies to such phosphoproteins. The phosphoprotein biomarker for mTORC1 signaling, rpS6, also reflects inhibition of S6K1 and non-mTOR inputs to this kinase, whereas antibodies recognizing direct substrates of mTOR (S6K1 and 4E-BP1) have largely proven unsuitable for immunostaining experiments (Engelman et al., 2008; Guerin et al., 2009). Alternative approaches are thus required to monitor mTOR substrate phosphorylation in vivo.

Eukaryotic initiation factor (eIF) 4E, the mRNA 5' cap-binding protein, is a key regulator of mRNA translation in the cytoplasm, and the activity of eIF4E is repressed by 4E-BPs. Binding of the 4E-BPs to eIF4E is controlled by the mTOR-dependent phosphorylation of 4E-BPs (Gingras et al., 1998; Pause et al., 1994) such that the hypophosphorylated forms of 4E-BPs bind to eIF4E and prevent interaction of eIF4E with eIF4G, thus impairing cap-dependent translation (Haghighat et al., 1995). Conversely, in cells with strong mTOR signaling, 4E-BPs become hyperphosphorylated, releasing eIF4E from 4E-BPs for interaction with eIF4G and assembly into the eIF4F complex. Whereas eIF4E is predominantly cytoplasmic in mammalian cells, nuclear eIF4E has been observed using biochemical fractionation and immunofluorescence analyses (Dostie et al., 2000; Lang et al., 1994; Lejbakowicz et al., 1992), and serum starvation induces an accumulation of eIF4E within the nucleus (Oh et al., 2007). This finding first appeared counterintuitive, because this treatment increases the binding of 4E-BP1 to eIF4E, and biochemical fractionation experiments had previously led to the conclusion that 4E-BP1 localization is restricted to the cytoplasm (Kim and Chen, 2000; Kleijn et al., 2002; Zhang et al., 2002). Using a rabbit monoclonal antibody suitable for immunostaining, we recently showed that 4E-BP1 is present in the nuclei in addition to the cytoplasm of mammalian cells (Rong et al., 2008). Furthermore, we also demonstrated that 4E-BPs are required for the nuclear accumulation of eIF4E seen following serum-starvation and/or rapamycin-treatment: conditions that suppress mTOR signaling (Rong et al., 2008).

The above-described mTOR signaling-modulated and 4E-BP-dependent alteration in eIF4E subcellular localization forms the basis for a novel screening approach. In this report, we demonstrate that cytoplasmic-to-nuclear ratio of eIF4E is a powerful read-out for mTOR signaling and describe a novel cell-based chemical genetic screen that analyzes chemical compound libraries across genetically distinct cell lines. The identification of novel mTOR pathway inhibitory compounds using our small-scale screen may provide new insights into the regulation of mTORC1 versus mTORC2 signaling. It also provides a proof-of-principle that larger screens using this method are possible, both in screening for novel mTOR inhibitory compounds and for counter screens in the search for specific inhibitors of other kinases, particularly PIKKs.

RESULTS

mTOR Pathway Inhibition Is Required for Nuclear Accumulation of eIF4E: Dependence on 4E-BPs

To evaluate the potential of eIF4E localization as a specific marker for mTOR activity we assessed eIF4E localization using immunofluorescence after treatment with known PI3K-mTOR pathway inhibitors (LY294002, wortmannin, rapamycin, and PI-103) or inhibitors of other signaling pathways: U0126, SB203580, and JNK inhibitor II (Bain et al., 2007). Screening for agents that induce a 4E-BP-dependent increase in nuclear eIF4E was performed using both wild-type murine embryonic fibroblasts (MEFs) and 4E-BP1, 4E-BP2 double-knockout (4E-BP1/2 DKO) MEFs, which serve as a negative control. Nuclear accumulation of eIF4E occurred in wild-type, but not in 4E-BP1/2 DKO MEFs, treated with PI3K-mTOR pathway inhibitors (Figure 1 and data not shown). Therefore, inhibition of mTOR signaling resulting in dephosphorylation of nuclear 4E-BPs is necessary for nuclear accumulation of eIF4E. Importantly, the inhibitors of other signaling pathways failed to induce nuclear eIF4E, demonstrating that this readout is specific to mTOR inhibition. Parallel western blot analyses were performed and demonstrated a strong correlation between inhibition of mTOR signaling, dephosphorylation of 4E-BP1, and nuclear accumulation of eIF4E in wild-type but not in 4E-BP1/2 DKO cells (Figure 1). Thus, using immunofluorescence to study eIF4E localization is a powerful assay for in vivo mTOR activity.

Primary Screening to Identify Candidate mTOR Inhibitory Compounds

A screen of 3584 compounds was performed using wild-type MEFs to test the assay for the identification of potentially novel mTOR inhibitors. Chemical libraries used include Prestwick Chemical (1120 structurally diverse marketed drugs), Biomol (361 natural products), Sigma LOPAC (885 pharmacologically active organic compounds), and Microsource Discovery (1218 structurally diverse compounds). For each plate of the 96-well plate-based screen, wells were each treated with one of 80 compounds, while 16 wells were used as negative (dimethyl sulfoxide [DMSO]) and positive (rapamycin) controls. eIF4E immunofluorescent intensity was scored within nuclei as defined by DAPI staining or within a cytoplasmic compartment as defined by a ring outside the nuclei (Figure 2A). For each plate, rapamycin controls were confirmed to induce nuclear accumulation of eIF4E (representative images and values are shown in Figure 2B). Because some apparently toxic compounds dramatically altered cell number, cell morphology, and cell size (Figure 2C and data not shown), these compounds were eliminated prior to data analysis (see Experimental Procedures). Some of these compounds induced significant nuclear accumulation of eIF4E (see Figure S1A available online), and may warrant further characterization. Transformed, normalized and standardized (see Experimental Procedures) cytoplasmic-to-nuclear ratio was used to identify potential mTOR pathway inhibitory compounds. Forty top-scoring compounds were randomly chosen and assessed for their ability to induce dephosphorylation of 4E-BP1 as observed by western blot analysis. Notably, only 20% of the compounds induced a significant decrease in 4E-BP1 phosphorylation, emphasizing the need for secondary screening (Figure S1B).

To directly compare this screening method with immunofluorescence-based monitoring of rpS6 phosphorylation as a read-out of mTOR signaling, IC50 values were calculated for the PI3K-mTOR inhibitor LY294002 using both assays and found
Figure 1. Inhibition of mTOR Signaling Causes 4E-BP-Dependent Nuclear Accumulation of eIF4E

(A) Immunofluorescence analysis of eIF4E localization in response to treatment with rapamycin (100 nM), U0126 (20 μM), or PI-103 (2 μM) reveals that inhibition of the mTOR-PI3K pathway specifically induces nuclear accumulation of eIF4E in wild-type and not 4E-BP1/2 DKO cells.

(B) Western blot analysis demonstrates 4E-BP1 dephosphorylation, as evidenced by accelerated SDS-PAGE mobility and use of phospho-4E-BP1 (Thr37/46) antibody, correlates strongly with nuclear eIF4E. S6K1 (Thr389) and ERK1/2 (Thr202/Tyr204) serve as controls for inhibitor function and specificity.
to be similar (Figure 3). Maximal inhibition of phospho-rpS6 immunofluorescence intensity and maximal induction of nuclear eIF4E were both observed at a concentration of 50 μM, consistent with previous work demonstrating a greater than 99% inhibition of PI3K at this dose (Vlahos et al., 1994).

**Chemical Genetic Screening to Identify mTOR Pathway Inhibitors**

The one hundred top-scoring candidate compounds from the primary screen above were subjected to a secondary chemical genetic screen. Because mTOR pathway inhibition-induced nuclear accumulation of eIF4E is dependent on 4E-BPs (Rong et al., 2008), 4E-BP1/2 DKO MEFs were used to eliminate false-positive compounds. As an added control, a homogeneous clonal population of 4E-BP1/2 DKO MEFs rescued by stable expression of HA-4E-BP1 was also included in this secondary screen. Immunofluorescence and western blot characterization of this cell line confirmed that HA-4E-BP1 expression was homogeneous (Figure 4A) and comparable (about one-half) to that of 4E-BP1 in wild-type cells (Figure 4B). Importantly, exogenous HA-tagged 4E-BP1 induced the alteration of eIF4E subcellular localization phenotype in response to mTOR signaling perturbation (Figure 4A).

As expected, the rapamycin control screening wells displayed significantly reduced (p < 0.001) eIF4E cytoplasmic-to-nuclear ratio relative to DMSO controls in wild-type but not 4E-BP1/2 DKO cells, and this phenotype was rescued by expression of exogenous HA-4E-BP1 (Figure 4C). Data representing transformed and normalized eIF4E subcellular localization and cytoplasmic compartment size were used as input parameters in a principal components analysis (PCA, see Experimental Procedures). A clear pattern that separated DMSO and rapamycin controls emerged, and only a handful of experimental compounds clustered with rapamycin (Figure 5A). Thus, this method was used to score the compounds, and the top 15 were identified as putative candidates. The individual effects on eIF4E localization across the three cell lines are displayed for the 15 putative candidates and 42 marginally scoring compounds (Figure 5B). As an inadvertent internal positive control, 1 of the 15 identified compounds was rapamycin. The probability of randomly choosing rapamycin as 1 of 15 top-scoring compounds is less than 0.5%. Other identified compounds known to impact signaling pathways include phorbol esters (12-deoxyphorbol 13-phenylacetate 20-acetate and 12-deoxyphorbol 13-acetate) and a non-phorbol ester PKC activator, mezerein. It is not surprising that these, as PKC activators, might reduce 4E-BP1 phosphorylation, because an often-studied phorbol ester/PKC activator, PMA, shows this activity (Guan et al., 2007; Hizli et al., 2006). It should be noted, however, that PMA increases mTOR signaling in some cell lines by inducing p90RSK-dependent inactivation of the mTOR suppressor, TSC2 (Roux et al., 2004).
Identified Compounds Inhibit mTORC1 Signaling

A confirmatory immunofluorescence analysis of cells treated with putative candidates using rpS6 phosphorylation as a different marker for mTOR signaling revealed that cells treated with all but one of the compounds (vinblastine sulfate salt) displayed dramatically reduced S6 ribosomal protein phosphorylation, while four moderately scoring compounds showed no significant reduction in S6 phosphorylation (Figure S2A). Western blot analyses confirmed that five compounds (rapamycin, CP107H6, gingerol, fumagillin, and himbacine) dramatically inhibit, five compounds (12-deoxyphorbol 13-phenylacetate, fumonisin B2, e-64-C, ebelactone B, and 12-deoxyphorbol 13-acetate) moderately inhibit, and three compounds (wedelactone, mezerein, and aphidicolin) fail to significantly inhibit mTORC1 signaling to 4E-BP1 and rpS6 in wild-type MEFs and in HeLa S3 cells under the conditions used for the screen (Figure 6A). Further analyses were performed to assess the specificity of seven promising mTORC1 inhibitory compounds using ERK1/2 phosphorylation as a read-out for serum-induced signaling (Figure 6B). Of the tested compounds, ebelactone B, fumagillin, fumonisin B2, and e-64-c failed to block ERK1/2 phosphorylation. To assess the reproducibility of the findings reported here, the four compounds able to inhibit mTOR signaling but not ERK1/2 phosphorylation were purchased, and western blot analysis confirmed that all significantly reduced phosphorylation of 4E-BP1 and S6K1 (Figure S2B).

DISCUSSION

The development of cell-based assays and high-throughput screens for inhibitors of mTOR signaling is intensively pursued. The screen described in this report is particularly powerful and highly adaptable to high-throughput screening because it utilizes a readily monitored change in eIF4E subcellular localization and genetically modified cell lines to measure the in vivo interaction of eIF4E with 4E-BP1 upon suppression of mTOR signaling. Although fluorescence resonance energy transfer (FRET) has been successfully demonstrated in vitro as a means to monitor the interaction of eIF4E with 4E-BP1 (Kimball and Horetsky, 2001), no in vivo counterpart has been described. The use of fluorophore-labeled eIF4E could certainly reduce the manpower required for the eIF4E translocation screen we describe, although the subcellular localization of fluorophore-tagged eIF4E may not recapitulate that of endogenous eIF4E (Kedersha et al., 2005).

This report describes a novel screening method for mTOR pathway activity and by no means represents a comprehensive search for novel mTOR inhibitory compounds. Even within
the compound libraries analyzed, mTOR pathway inhibitory compounds will have been missed. A number of compounds were eliminated from candidacy for causing gross morphological or cell number alterations at a concentration of 50 μM. It is possible that some of these could function as excellent, highly potent mTOR pathway inhibitors at lower concentrations. Indeed, the PI3K inhibitors (LY294002 and wortmannin) were both present in the compound libraries screened, but only wortmannin was carried on to the secondary screen. Ultimately, however, both were removed from candidacy due to the aforementioned cell number and cytoplasmic compartment area filters, presumably due to toxicity at the chosen concentration and treatment time. It is noteworthy, however, that rapamycin even at 50 μM (2000 times the standard concentration) was identified as one of the 15 best scoring compounds in this screen. Presumably, this is a reflection of the nontoxicity of this natural product across a broad range of concentrations. Indeed, the bulk of compounds identified as inducing “rapamycin-like” changes in eIF4E subcellular localization in the secondary, chemical genetic screen described above are also natural products.

Although none of the identified compounds was comparable to rapamycin in its ability to specifically inhibit mTOR signaling across a broad range of concentrations, multiple compounds did significantly inhibit mTORC1 signaling under the conditions used for the screen. Although the scope of this study was not aimed at determining the molecular link between each compound and mTOR signaling, known targets of four promising mTORC1 pathway inhibitory compounds identified here merit discussion. Ebelactone B, for example, is a known inhibitor of membrane esterases that may regulate PP2A function (Kowluru et al., 1996; Tan and Rando, 1992). It is also noteworthy that the known PIKK inhibitors wortmannin and LY294002 are also lactones (Konaklieva and Plotkin, 2005), and the lactone oxygen of wortmannin appears to be crucial for competition with ATP (Walker et al., 2000). Fumagillin is known to inhibit methionine aminopeptidase 2 (MetAP2) (Griffith et al., 1997), a potential Ras GTPase activating protein (Xu et al., 1990). Identification of fumagillin as an mTOR pathway inhibitory agent suggests MetAP2 activity may feed into mTOR signaling. Indeed, MetAP2 is upregulated in neurofibromatosis 1 (NF1)–associated glioma, which is characterized by hyperactive mTOR signaling (Dasgupta et al., 2005a), and proliferation of NF1−/− cells is blocked with fumagillin or rapamycin (Dasgupta et al., 2005b). Fumonisin B2, an inhibitor of sphinganine N-acyltransferase (ceramide synthase) (Wang et al., 1991), was also identified and may suggest a link between sphingolipid metabolism and mTOR signaling. Finally, the calpain inhibitor E-64-c also reduced mTOR signaling, which is unexpected in light of previous evidence that calpain itself is inhibitory to Akt and mTOR signaling (Smith and Dodd, 2007).

SIGNIFICANCE

The mammalian target of rapamycin (mTOR) has proven to be a therapeutically beneficial drug target due to its...
phylogenetically conserved role of integrating intracellular and extracellular signals to modulate cellular growth and proliferation. In this report we provide strong proof-of-principle data illustrating the power and usefulness of a novel cell-based assay for compounds that impact mTOR signaling. The readout for this screen is the nuclear accumulation of the mRNA 5' cap binding protein eIF4E, which we demonstrate to occur specifically upon inhibition of mTOR-dependent phosphorylation of 4E-BPs. This chemical genetic screen makes use of genetically modified cell lines, thus reducing the possibility of false positives, including autofluorescent compounds. Given the ongoing efforts toward development of mTOR and PIKK inhibitors, this screen can directly be used with much larger chemical libraries to identify novel classes of mTOR pathway inhibitory molecules. The compounds identified in our small-scale screen as specifically inhibiting mTORC1 signaling to 4E-BP1 and S6K1 provide new information regarding the regulation of mTOR signaling and may form the basis of novel strategies to target this important regulator of cell growth and proliferation.

**EXPERIMENTAL PROCEDURES**

The wild-type and 4E-BP1−/−; 4E-BP2−/− MEFs have been previously described, as has the pBABE-puro-HA-4E-BP1 rescue cell line (Rong et al., 2008). The subcellular localization of eIF4E was monitored by indirect immunofluorescence (anti-mouse IgG Alexa647, 1:200, Invitrogen) colocalization with DAPI (200 ng/ml)-stained nuclei, wherein cells were washed with cold phosphate-buffered saline (PBS), prior to fixation with formaldehyde (3.7% in PBS, 37°C, 10 min) and permeabilization with methanol (100%, −20°C) followed by blocking in bovine serum albumin (BSA) (2% in PBS) and overnight incubation with eIF4E mAb (1:400 in 2% BSA, BD Transduction Labs). Commercially available compound libraries (Prestwick, Biomol, MicroSource Discovery, and Sigma LOPAC) were used for screening at 50 μM for 5 hr. This and similar drug concentrations have previously been shown to be optimal for inducing nuclear accumulation of the transcription factor FOXO1A using high content screening (Haney, 2008; Kau et al., 2003), and hence was chosen as the starting concentration for subsequent screening experiments. Additional compounds including PI-103, JNK inhibitor II (EMD Biosciences), LY294002, Wortmannin, U0126 (Sigma-Aldrich), Rapamycin, Swainsonine, Fumagillin, E-64-c, Fumonisin B2, and Ebelactone B (Biomol) were purchased separately for controls and validation screening.

For all screening experiments, cytoplasmic-to-nuclear eIF4E intensity ratios (MetaXpress® Translocation-Enhanced) for each well of 96-well plates were collected, as were compartment size and cell number values. All data analysis was performed in R (www.r-project.org). For both the primary and the
supplemental data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00401-3.

Acknowledgments

This research was supported by a National Cancer Institute of Canada Grant (to N.S.) and by a CIHR Team Grant (CTP-79858) on the Molecular Basis of Translational Control of Memory Formation (to J.P.). We thank Xiaofeng Wang for technical assistance with screening experiments and the McGill Life Sciences Complex Imaging Facility for assistance with confocal microscopy. M.L. and R.S. were supported by McGill-CIHR Chemical Biology Studentships. M.L. is a Research Student of the Terry Fox Foundation (Award #700029).

Received: August 6, 2009
Accepted: November 13, 2009
Published: December 23, 2009

References


figure 6. Western Blot-Based Characterization of Top-Scoring Compounds

(A) The majority of top-scoring compounds (50 µM, 5 hr) significantly reduce 4E-BP1 and rps6 phosphorylation in wild-type MEF’s (upper) and in HeLa S3 cells (lower). The phorbol esters 12-deoxyphorbol 13-phenylacetate (12-DP 13-PA) and 12-deoxyphorbol 13-acetate (12-DP 13-A) induce intermediate effects.

(B) Western blot assessment of the impact of these compounds (2 µM, 1 hr) on serum-stimulated (20%, 30 min) ERK phosphorylation.

Secondary screen, data associated with compounds causing extreme changes in compartment size (<75% mean) or cell number (<25% mean) were discarded and did not participate in data transformation and normalization. For the first screen, cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and did not participate in data transformation and normalization. For the first screen, cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjected to normalization plate by plate. The resulting data set (three cell types, both cytoplasmic-to-nuclear eIF4E intensity ratios were log2 transformed and subjecte
mTOR-Dependent eIF4E Translocation Screen


