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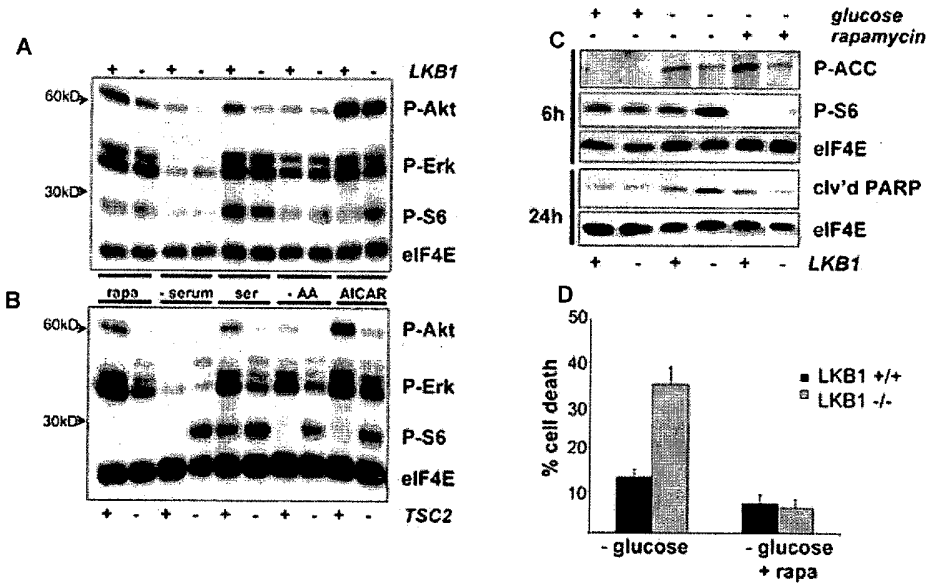

(57) Abstract: The invention relates to inhibition of mTOR, p70S6K1 or Rheb kinase activity for treating disorders including diabetes. The invention also relates to screening for agents that inhibit the activities of mTOR, p70S6K1 or Rheb proteins, which are useful in the treatment of diabetes, as well as preparing compounds for treatment of diabetes.

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METHODS FOR IDENTIFYING DIABETES THERAPEUTICS

Related Applications

This application claims the benefit under 35 U.S.C. § 119(e) of United States provisional patent application serial number 60/600,193, filed August 10, 2004, the contents of which are incorporated herein by reference in their entirety.

Government Support

This invention was made in part with government support under grant number GM56203 (5R01) from the National Institutes of Health (NIH). The government may have certain rights in this invention.

Field of the Invention

The invention relates to inhibition of mTOR, p70S6K1 or Rheb activity for treating disorders including diabetes. The invention also relates to screening for agents that inhibit the activities of mTOR, p70S6K1 or Rheb proteins, which are useful in the treatment of diabetes, as well as preparing compounds for treatment of diabetes.

Background of the Invention

An estimated 15.7 million Americans have diabetes, and individuals with adult-onset, type 2, diabetes represent 90 to 95 percent of all diabetics. Almost one-third of all diabetics in the U.S. are unaware that they have the disorder, and undetected and uncontrolled diabetes can have serious side effects, such as blindness, heart disease, nerve disease, and kidney disease.

In addition to the increased clinical risks, type 2 diabetes may also result in a reduced quality of life for the affected individual. Because type 2 diabetes is a major disorder in current society, which has serious health and life quality consequences, improved methods of treatment are needed to provide better therapy of diabetes with fewer and/or lesser side effects.

Impaired energy metabolism is a primary defect in type 2 diabetes (Rutter et al., *Biochem J.* 375:1-16, 2003). AMP-activated protein kinase (AMPK) is a highly conserved sensor of cellular energy status found in all eukaryotic cells (Hardie, et al., *FEBS Letters* 546:1113-1120, 2003). Recent studies have indicated that AMPK is a critical regulator of

leptin-induced fatty acid metabolism and glucose uptake in skeletal muscle (Minokoshi, et al., *Nature* 415:339-343, 2002; Mu, et al., *Mol. Cell.* 7:1085-1094, 2001). AMPK is activated by stimuli that increase ATP consumption or inhibit ATP production in mammalian cells. Such stimuli include pathological stresses such as oxidative damage, osmotic shock, hypoxia, and glucose deprivation, as well as physiological stimuli such as exercise, contraction, and hormones including leptin and adiponectin in skeletal muscle (Hardie et al., 2003). AMPK is the primary regulator of the cellular response to lowered ATP levels in cultured cells. Accordingly, phosphorylation of its downstream targets results in the up-regulation of ATP-producing catabolic pathways and the downregulation of ATP-consuming processes.

The LKB1 serine/threonine kinase (e.g., GenBank accession number AAC15742) has been demonstrated to be essential to activation of AMPK (Shaw et al., *Proc. Natl. Acad. Sci. USA* 101(10):3329-3335, 2004). LKB1 inactivation is the genetic basis of Peutz-Jeghers syndrome, a familial colorectal polyp disorder in which patients are also predisposed to early onset cancers at in other tissues (Yoo, et al., *Nat. Rev. Cancer* 2, 529-535,2002).

The protein TOR (target of rapamycin) senses and integrates nutrient and growth factor signals, including the signal produced by AMPK. One mechanism by which this may occur is that activated AMPK can phosphorylate the tumor suppressor protein tuberous sclerosis 2 (TSC2, or tuberin), increasing the inhibition of mTOR (Inoki et al., *Cell*, 115:577–590, 2003).

TSC2 is a GTPase activating protein (GAP) for Rheb (Ras homolog enriched in brain) protein. Rheb is a member of the Ras superfamily GTP-binding proteins that is conserved from yeast to human; Rheb proteins bind and hydrolyze GTP. Activated Rheb-GTP activates mTOR (Inoki et al., *Genes Dev.* 17(15):1829-1834, 2003).

One current theory holds that disregulated TOR function may contribute to the development of type 2 diabetes (Fingar and Blenis, *Oncogene* 23:3151-3171, 2004). Knockout mice that are deficient in S6K1, which is downstream of TOR, develop type 2 diabetes (Shima et al., *EMBO J.*, 17:6649–6659, 1998). The S6K1-deficient mice are diabetic due to an impaired ability to produce insulin as a result of decreased pancreatic β-cell size (Pende et al., *Nature*, 408:994–997, 2000). Thus, it appears that reduced signaling downstream of TOR can result in diabetes.

Summary of the Invention

It has now been discovered, surprisingly, that inhibition of the mTOR pathway, including p70S6K1, mTOR and/or Rheb is useful for treating diabetes by increasing insulin signaling, thus resulting in an enhancement of insulin responsiveness.

According to one aspect of the invention, methods for identifying compounds useful as pharmacological agents for the treatment of diabetes are provided. The methods include contacting a p70S6K1 polypeptide with a candidate pharmacological agent, and determining the activity of the contacted p70S6K1 polypeptide. A decrease in the activity of the p70S6K1 polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the p70S6K1 polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes. In certain embodiments, the methods also include determining an second amount of activity of the p70S6K1 polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the p70S6K1 polypeptide as the control amount of activity.

Preferably the activity of the p70S6K1 polypeptide is kinase activity, which preferably is measured by phosphorylation of ribosomal S6 protein or a peptide comprising the phosphorylation site of ribosomal S6 protein. In some of the methods provided, the p70S6K1 polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent. In other embodiments, the assay is an *in vitro* assay.

According to another aspect of the invention, methods for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness are provided. The methods include administering to a subject in need of such treatment an effective amount of an agent that decreases the activity of p70S6K1 polypeptide in the subject, as a treatment for the disorder. Preferably the disorder is type 2 diabetes.

In certain embodiments, the agent decreases the kinase activity of p70S6K1 polypeptide. Preferred agents that decrease the kinase activity of p70S6K1 polypeptide include N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89), 1-(5-isoquinolinesulfonyl)-1H-hexahydro-1,4-diazepine (HA-1077, fasudil), an analog or derivative thereof, a salt thereof, or a solvate thereof.

In other embodiments, the agent decreases the amount of p70S6K1 polypeptide. Preferred agents that decrease the amount of p70S6K1 polypeptide include siRNA/RNAi molecules and antisense nucleic acid molecules.

According to still another aspect of the invention, methods for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness are provided. The methods include identifying a compound that decreases activity of a p70S6K1 polypeptide and formulating the compound for administration to a subject in need of such treatment. In some embodiments the disorder is type 2 diabetes. Preferably the compound that decreases activity of a p70S6K1 polypeptide is identified by any of the foregoing screening methods.

In another aspect of the invention, methods for identifying compounds useful as pharmacological agents for the treatment of diabetes are provided. The methods include contacting a mTOR polypeptide with a candidate pharmacological agent, and determining the activity of the contacted mTOR polypeptide. A decrease in the activity of the mTOR polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the mTOR polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes. In certain embodiments, the methods also include determining an second amount of activity of the mTOR polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the mTOR polypeptide as the control amount of activity.

Preferably the activity of the mTOR polypeptide is kinase activity; preferably the kinase activity of the mTOR polypeptide is measured by phosphorylation of p70S6K1, eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), or a peptide comprising the phosphorylation site of p70S6K1 or 4E-BP1. In some of the methods provided, the mTOR polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent. In other embodiments, the assay is an *in vitro* assay.

According to another aspect of the invention, methods for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness are provided. The methods include administering to a subject in need of such treatment an effective amount of an agent that decreases the activity of mTOR polypeptide in the subject, as a treatment for the disorder. Preferably the disorder is type 2 diabetes.

In some embodiments, the agent decreases the kinase activity of mTOR polypeptide. Preferably the agent that decreases the kinase activity of mTOR polypeptide is rapamycin (sirolimus), a rapamycin analog or derivative, a salt thereof, or a solvate thereof. Preferred rapamycin analogs or derivatives include everolimus (RAD-001), or rapamycin 42-ester with 3-hydroxy-2- (hydroxymethyl)-2- methylpropionic acid (CCI-779).

In other embodiments, the agent decreases the amount of mTOR polypeptide. Preferably the agent that decreases the amount of mTOR polypeptide is a siRNA/RNAi molecule or an antisense nucleic acid molecule.

According to yet another aspect of the invention, methods for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness are provided. The methods include identifying a compound that decreases activity of a mTOR polypeptide and formulating the compound for administration to a subject in need of such treatment. Preferably the disorder is type 2 diabetes. Preferably the compound that decreases activity of a mTOR polypeptide is identified by any of the foregoing screening methods.

According to another aspect of the invention, methods for identifying compounds useful as pharmacological agents for the treatment of diabetes are provided. The methods include contacting a Rheb polypeptide with a candidate pharmacological agent, and determining the activity of the contacted Rheb polypeptide. A decrease in the activity of the Rheb polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the Rheb polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes. Preferably the methods also include determining an second amount of activity of the Rheb polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the Rheb polypeptide as the control amount of activity.

In certain embodiments, the activity of the Rheb polypeptide is GTP binding activity. In those embodiments, it is preferred that the GTP binding activity of the Rheb polypeptide is measured by binding of detectably labeled non-hydrolyzable analog of GTP. Preferably the detectably labeled non-hydrolyzable analog of GTP is [^{35}S]GTPγS.

In certain embodiments, the activity of the Rheb polypeptide is GTPase activity. In those embodiments, it is preferred that the GTPase activity of the Rheb polypeptide is measured by hydrolysis of detectably labeled GTP. Preferably detectably labeled GTP is [α-^{32}P]GTP.

In some of the methods provided, the Rheb polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent. In other embodiments, the assay is an *in vitro* assay.

According to still another aspect of the invention, methods for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness are provided. The methods include administering to a subject in need of such treatment an

effective amount of an agent that decreases the activity of Rheb polypeptide in the subject, as a treatment for the disorder. Preferably the disorder is type 2 diabetes.

The agent, in some embodiments, decreases the GTPase activity and/or the GTP binding activity of Rheb polypeptide. In other embodiments, the agent decreases the amount of Rheb polypeptide. Preferably the agent that decreases the amount of Rheb polypeptide is a siRNA/RNAi molecule or an antisense nucleic acid molecule.

In still other embodiments, the agent that decreases the activity of Rheb polypeptide is a farnesyl transferase inhibitor, an analog or derivative thereof, a salt thereof, or a solvate thereof.. Preferred farnesyl transferase inhibitor include FTI 277, Zarnestra® (tipifarnib, R115777), RPR-130401 or lonafarnib (SCH66336).

According to still another aspect of the invention, methods for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness are provide. The methods include identifying a compound that decreases activity of a Rheb polypeptide and formulating the compound for administration to a subject in need of such treatment. Preferably the disorder is type 2 diabetes. Preferably the compound that decreases activity of a Rheb polypeptide is identified by any of the foregoing screening methods.

In another aspect, the invention provides for use of the foregoing agents, compounds and molecules in the preparation of medicaments, particularly medicaments for the treatment of reduced insulin responsiveness, e.g., type 2 diabetes.

These and other aspects of the invention are described further below.

Brief Description of the Figure

Figure 1 shows the correlation of increased mTOR/p70S6K1 signaling and decreased insuling signaling.

Detailed Description of the Invention

We examined the effect of loss of LKB1, the upstream AMPK activating kinase (Shaw et al. (2004), Proc. Natl. Acad. Sci. USA *101*, 3329-3335), on mTOR (mammalian TOR) and phosphoinositide 3-kinase (PI3K)/Akt signaling. We observed that in primary mouse embryonic fibroblasts lacking LKB1 or TSC2 there is a consistent and direct correlation with increased mTOR signaling and decreased PI3K/Akt signaling. In addition, prolonged inhibition of mTOR with 24 hour rapamycin treatment rescues glucose starvation

induced cell death suggesting that inhibition of mTOR results in a restoration of Akt and PI3K pro-survival signaling.

We hypothesize that these results in the LKB1-deficient cells may explain the therapeutic benefit of metformin and glitazone compounds (e.g. rosiglitazone) which are two widely prescribed Type 2 diabetes treatments. Both metformin and glitazones act to activate AMPK and thus inhibit mTOR signaling as we show for other AMPK agonists (e.g., 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). By inhibiting mTOR, they relieve the negative feedback from mTOR to PI3K/Akt, enhancing insulin sensitivity. This has a broad implication for diabetes therapeutics.

Loss of insulin/Akt signaling is a hallmark feature of type 2 diabetes and therapeutic restoration of insulin/Akt signaling is one of the chief goals of current therapeutic aims. From our results, we predict that inhibition of mTOR signaling, for example inhibition of mTOR itself, inhibition of p70S6K1 activity or inhibition of Rheb activity, results in a concomitant restoration of insulin/Akt signaling. Based on this, we suggest screening for direct chemical inhibitors of mTOR, p70S6K1 or Rheb to identify potential therapeutic compounds. Such compounds are useful in the treatment of type 2 diabetes or any clinical conditions resulting from a loss of insulin responsiveness.

Currently available type 2 diabetes therapeutics have not been molecularly or biochemically targeted in their development and the reason for their therapeutic success, however limited, is not known. These compounds may work therapeutically by indirectly inhibiting mTOR through AMPK activation. If so, then direct inhibitors of mTOR, p70S6K1 or Rheb signaling are expected to be more potent in their therapeutic potential. By directly inhibiting mTOR, p70S6K1 or Rheb signaling, one might achieve the highest possible restoration of insulin signaling in diabetic tissue, resulting in more potent, long lasting therapeutics.

Determination of the catalytic activity of mTOR or p70S6K1 polypeptides for diagnostic, prognostic, and therapeutic purposes is an aspect of the invention. The catalytic activity of a mTOR or p70S6K1 polypeptide may be determined and candidate pharmaceutical agents can be tested for their ability to decrease the mTOR or p70S6K1 catalytic activity. The determination that a compound decreases the mTOR or p70S6K1 catalytic activity indicates that the compound may be useful as an agent to treat disorders involving a loss of insulin responsiveness, such as type 2 diabetes. For example, a mTOR or p70S6K1 polypeptide may be contacted with a substrate of the polypeptide and the catalytic

activity of the mTOR or p70S6K1 monitored and determined, then the mTOR or p70S6K1 polypeptide may be contacted with a candidate agent and the polypeptide's catalytic activity determined upon contact with the substrate. Such assays may be cell-based assays or *in vitro* assays and may also be useful to monitor effects of *in vivo* administration of inhibitors of mTOR or p70S6K1 catalytic activity in cells or animals, including humans. *In vitro* assays can be, for example, polypeptide-based assays. Likewise, inhibition of Rheb GTPase and/or GTP binding activity can be used in screening and diagnostic methods. GTPase activity and GTP binding activity can be assayed using standard methods that are well known in the art. For example, GTPase activity can be determined by hydrolysis of detectably labeled GTP, e.g.; [α-^{32}P] GTP. GTP binding can be determined, for example, by binding of a detectable non-hydrolyzable GTP analog, e.g., [^{35}S]GTPγS. See for example, Tabancay et al., *J. Biol. Chem.* 278(41):39921-39930, 2003.

The invention also relates in part to assays used to determine the activity of a mTOR, p70S6K1 or Rheb polypeptide, e.g., for screening inhibitors of these polypeptides. The mTOR, p70S6K1 or Rheb polypeptide may be attached to a surface and then contacted with a substrate molecule and the level of catalytic activity of the mTOR, p70S6K1 or Rheb polypeptide or fragment thereof can be monitored and quantitated using standard methods. The aforementioned assays are not intended to be limiting. Assays for catalytic activity may also be done with the components in solution, using various art-recognized detection methods, and/or other kinase assay methods known to one of ordinary skill in the art, some of which are described herein below. Typically these will be kinase assays as are well known in the art; certain examplary methods are provided in the Examples below.

The invention further provides efficient methods of identifying pharmacological agents or lead compounds for agents useful for decreasing mTOR, p70S6K1 or Rheb activity. Generally, the screening methods involve assaying for compounds that decrease (i.e., inhibit) phosphorylation of a substrate. Such methods are adaptable to automated, high throughput screening of compounds.

A wide variety of assays for pharmacological agents are provided, including labeled *in vitro* kinase phosphorylation assays, cell-based phosphorylation assays, assays for determining affinity of interacting proteins (e.g., immunoprecipitations, two-hybrid assays) etc. For example, in vitro kinase phosphorylation assays are used to rapidly examine the effect of candidate pharmacological agents on the phosphorylation of a substrate by, for

example, mTOR or p70S6K1 or a fragment thereof. GTP binding and/or GTPase assays can be used to determine the activity of Rheb.

The candidate pharmacological agents can be derived from, for example, combinatorial peptide libraries, small molecule libraries, or natural product libraries.

In general, substrates used in the assay methods of the invention are added to an assay mixture as an isolated molecule. For use with mTOR, a preferred substrate is p70S6K1 or eIF4E-binding protein 1 (4E-BP1), although mTOR autophosphorylation also can be detected. For p70S6K1, a preferred substrate is S6 ribosomal protein. Still other substrates for the kinases will be known to one of ordinary skill in the art. The assay mixture can include detectable phosphate compounds (e.g. ^{32}P or ^{33}P), so that protein substrates phosphorylated by mTOR or p70S6K1 are readily detectable. Alternatively, mTOR or p70S6K1 activity on a substrate can be measured using other detectable means such as antibody capture of specific phosphorylated polypeptides, chromatographic means, etc. Similarly, GTP binding and/or GTPase activity of Rheb protein can be assessed using standard methods in the art.

A typical assay mixture for measuring kinase activity includes a protein substrate of the kinase being assayed (e.g., mTOR or p70S6K1), or a peptide having a phosphorylation site motif of the substrate and a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection.

Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides (e.g., kinase sites), and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the

like. Where the agent is a nucleic acid (i.e., aptamer), the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated.

mTOR, p70S6K1 or Rheb inhibitors also can be designed using rational structure-based methods such as the methods described in PCT/US98/10876 and references described therein.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous methods are available and knowns to one of ordinary skill in the art for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, random or non-random peptide libraries, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily be modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein activity. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, mTOR or p70S6K1 phosphorylates a polypeptide at a certain level or Rheb binds GTP (i.e., control level). The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the presence or absence of phosphorylation of a substrate, or binding of a substrate, is detected by any convenient method available to the user. For cell free assays, a separation step may be used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximum signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific binding or interaction such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected using any convenient method. For example, phosphorylation produces a directly or indirectly detectable product, e.g., phosphorylated substrate. In other assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, fluorescence, optical or electron density, etc). or indirect detection (e.g., epitope tag such as the FLAG, V5 or *myc* epitopes, an enzyme tag such as horseradish peroxidase or luciferase, a transcription product, etc.). The label may be bound to a substrate, to the proteins employed in the assays, or to the candidate pharmacological agent. Phosphorylated substrates also can be assayed using IMAP technology (Molecular Devices, Sunnyvale, CA), that uses the specific binding of metal (MIII) coordination complexes to phosphate groups at high salt concentration and a fluorescence polarization readout.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly

detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

Thus the present invention includes automated drug screening assays for identifying compositions having the ability to increase phosphorylation of a substrate directly or indirectly. The automated methods preferably are carried out in an apparatus which is capable of delivering a reagent solution to a plurality of predetermined compartments of a vessel and measuring the change in a detectable molecule in the predetermined compartments. Exemplary methods include the following steps. First, a divided vessel is provided that has one or more compartments which contain a substrate which, when exposed to mTOR or p70S6K1, has a detectable change. The mTOR or p70S6K1 can be in a cell in the compartment, in solution, or immobilized within the compartment. Next, one or more predetermined compartments are aligned with a predetermined position (e.g., aligned with a fluid outlet of an automatic pipette) and an aliquot of a solution containing a compound or mixture of compounds being tested for its ability to decrease mTOR or p70S6K1 kinase activity is delivered to the predetermined compartment(s) with an automatic pipette. The substrate also can be added with the compounds or following the addition of the compounds. Finally, detectable signal; emitted by the substrate is measured for a predetermined amount of time, preferably by aligning the cell-containing compartment with a detector. Preferably, the signal also measured prior to adding the compounds to the compartments, to establish e.g., background and/or baseline (control) values. For competition assays, the compounds can be added with or after addition of a substrate or inhibitor to the mTOR or p70S6K1 polypeptide-containing compartments. One of ordinary skill in the art can readily determine the appropriate order of addition of the assay components for particular assays. Similar assays for GTP binding and/or GTPase activity of Rheb can be performed in a similar fashion.

At a suitable time after addition of the reaction components, the plate is moved, if necessary, so that assay wells are positioned for measurement of signal. Because a change in the signal may begin within the first few seconds after addition of test compounds, it is desirable to align the assay well with the signal detector as quickly as possible, with times of about two seconds or less being desirable. In preferred embodiments of the invention, where the apparatus is configured for detection through the bottom of the well(s) and compounds are added from above the well(s), readings may be taken substantially continuously, since the plate does not need to be moved for addition of reagent. The well and detector device should

remain aligned for a predetermined period of time suitable to measure and record the change in signal.

The apparatus of the present invention is programmable to begin the steps of an assay sequence in a predetermined first well (or rows or columns of wells) and proceed sequentially down the columns and across the rows of the plate in a predetermined route through well number n. It is preferred that the data from replicate wells treated with the same compound are collected and recorded (e.g., stored in the memory of a computer) for calculation of signal.

To accomplish rapid compound addition and rapid reading of the response, the detector can be modified by fitting an automatic pipetter and developing a software program to accomplish precise computer control over both the detector and the automatic pipetter. By integrating the combination of the fluorometer and the automatic pipetter and using a microcomputer to control the commands to the detector and automatic pipetter, the delay time between reagent addition and detector reading can be significantly reduced. Moreover, both greater reproducibility and higher signal-to-noise ratios can be achieved as compared to manual addition of reagent because the computer repeats the process precisely time after time. Moreover, this arrangement permits a plurality of assays to be conducted concurrently without operator intervention. Thus, with automatic delivery of reagent followed by multiple signal measurements, reliability of the assays as well as the number of assays that can be performed per day are advantageously increased.

Similar assays can be used to identify compounds that increase mTOR, p70S6K1 or Rheb activity, which may be useful as controls or in preparing animal models of disease.

Inhibitors of mTOR, p70S6K1 or Rheb polypeptide activity (e.g., kinase activity, GTPase activity or GTP binding activity) identified by the methods described herein are useful to treat diseases or conditions that result from increased or excessive mTOR, p70S6K1 or Rheb polypeptide activity, including disorders characterized by loss of (or reduced) insulin responsiveness (e.g., type 2 diabetes). For treatment of such conditions, an effective amount of a mTOR, p70S6K1 or Rheb polypeptide inhibitor is administered to a subject.

As used herein a "control" may be a predetermined value, which can take a variety of forms. It can be a single cut-off value, such as a median or mean. It can be established based upon comparative groups, such as in groups having normal amounts of circulating insulin and groups having abnormal amounts of circulating insulin. Another example of comparative groups would be groups having a particular disease, condition or symptoms and groups

without the disease, condition or symptoms, such as individuals diagnosed with type 2 diabetes mellitus and individuals free of type 2 diabetes mellitus. Another comparative group would be a group with a family history of a condition and a group without such a family history. The predetermined value can be arranged, for example, where a tested population is divided equally (or unequally) into groups, such as a low-risk group, a medium-risk group and a high-risk group or into quandrants or quintiles.

The predetermined value, of course, will depend upon the particular population selected. For example, an apparently healthy population will have a different 'normal' range than will a population which is known to have a condition related to increased mTOR, p70S6K1 or Rheb activity, or a disorder characterized by a loss of insulin responsiveness. Accordingly, the predetermined value selected may take into account the category in which an individual falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of ordinary skill in the art. By abnormal is meant statistically significantly different relative to a selected control. Typically the control will be based on apparently healthy normal individuals in an appropriate age bracket.

In some embodiments, a control sample is from a cell, tissue, or subject that does not have a disorder associated with mTOR, p70S6K1 or Rheb activity or a disorder characterized by a loss of insulin responsiveness. In other embodiments the control sample is a sample that is untreated with a candidate agent. For example, an effect of a candidate agent may be determined by determining the catalytic activity of a mTOR, p70S6K1 or Rheb polypeptide (e.g., kinase activity, GTPase or GTP binding activity) in advance of contacting the mTOR, p70S6K1 or Rheb polypeptide with the agent, and again after contacting the mTOR, p70S6K1 or Rheb polypeptide with the agent, in which case, the initial level of catalytic activity or binding determined may serve as a control level against which the post-contact level of catalytic or binding activity may be compared. In such assays, the source of the mTOR, p70S6K1 or Rheb polypeptide may be a biological sample from a subject known to be free of a disorder characterized by a loss of insulin responsiveness (e.g., type 2 diabetes mellitus) or may be a sample from a cell or tissue from a subject with a known disorder characterized by a loss of insulin responsiveness, and in each case the before-contact determination of catalytic activity may be the control for the after-contact determination of catalytic activity.

Examples of methods for obtaining the biological sample from the tissue biopsy of a subject include aspiration, gross apportioning of a mass, microdissection, laser-based

microdissection, or other art-known cell-separation methods. Fluid biological samples are taken from a subject using standard methods well known in the art.

Because of the variability of the cell types in tissue biopsy material, and the variability in sensitivity of the diagnostic methods used, the sample size required for analysis may range from 1, 10, 50, 100, 200, 300, 500, 1000, 5000, 10,000, to 50,000 or more cells. The appropriate sample size may be determined based on the cellular composition and condition of the biopsy and the standard preparative steps for this determination and subsequent isolation of fractions of the sample (e.g., polypeptides, nucleic acids) for use in the invention are well known to one of ordinary skill in the art. An example of this, although not intended to be limiting, is that in some instances a sample from the biopsy may be sufficient for assessment of RNA expression without amplification, but in other instances the lack of suitable cells in a small biopsy region may require use of RNA conversion and/or amplification methods or other methods to enhance resolution of the nucleic acid molecules. Such methods, which allow use of limited biopsy materials, are well known to those of ordinary skill in the art and include, but are not limited to: direct RNA amplification, reverse transcription of RNA to cDNA, real-time RT-PCR, amplification of cDNA, or the generation of radiolabeled nucleic acids.

The surprising discovery that inhibition of mTOR results in a restoration of Akt and PI3K signaling indicates that inhibitors of mTOR, p70S6K1 or Rheb relieve the negative feedback from mTOR to PI3K/Akt, thereby enhancing insulin sensitivity. Thus inhibitors of mTOR, p70S6K1 or Rheb are useful in the treatment of type 2 diabetes and other disorders in which a loss of insulin responsiveness is involved. In particular, methods for treating such disorders are provided by the invention, in which mTOR or p70S6K1 activity is decreased, which decreases phosphorylation of the respective substrates of these kinases. Activity of mTOR or p70S6K1, as used herein in these contexts, means kinase activity. Methods for treating such disorders in which Rheb activity is decreased are also provided by the invention which decreases activity of mTOR and downstream proteins. Activity of Rheb in this context means GTPase and/or GTP binding activity.

Any method for decreasing mTOR, p70S6K1 or Rheb activity will be useful in the treatment of the aforementioned disorders characterized by loss of insulin responsiveness (e.g., type 2 diabetes). mTOR, p70S6K1 or Rheb activity can be decreased by pharmacological inhibitors of the enzyme activity or the expression of the proteins. Preferred inhibitors are those that bind directly to the mTOR, p70S6K1 or Rheb proteins. Most

preferred are inhibitors of the p70S6K1 protein, which are expected to have greater selectivity.

In accordance with the invention, known inhibitors of mTOR that can be used include rapamycin (sirolimus), and rapamycin analogs and derivatives such as everolimus (RAD-001), rapamycin 42-ester with 3-hydroxy-2- (hydroxymethyl)-2- methylpropionic acid (CCI-779) (see also Dutcher, *Curr. Oncol. Rep.* 6(2):111-115, 2004; Huang and Houghton, *Curr. Opin. Investig. Drugs* 3(2):295-304, 2002; and US patents 5,362,718; 5,780,462; 5,922,730; 5,955,457; 6,015,809; 6,117,863; 6,399,625; 6,399,626; 6,432,973; 6,440,991; 6,677,357; and 6,680,330). Known inhibitors of p70S6K1 include N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89), 1-(5-isoquinolinesulfonyl)-1H-hexahydro-1,4-diazepine (HA-1077, fasudil). Known inhibitors of Rheb include farnesyl transferase inhibitors, e.g., FTI 277, Zarnestra® (tipifarnib, R115777), RPR-130401, lonafarnib (SCH66336).

Agents that decrease the expression of mTOR, p70S6K1 or Rheb (e.g., by decreasing expression of the endogenous gene) also can be used for these purposes. Such agents include antisense nucleic acid molecules and siRNA or RNAi molecules.

One set of embodiments of the aforementioned compositions and methods include the use of antisense molecules or nucleic acid molecules that reduce expression of genes via RNA interference (RNAi or siRNA). One example of the use of antisense, RNAi or siRNA in the methods of the invention is their use to decrease the level of expression of one or more ceramide biosynthetic pathway enzymes. The antisense oligonucleotides, RNAi, or siRNA nucleic acid molecules used for this purpose may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art-recognized methods, which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In some embodiments of the invention, the antisense or siRNA oligonucleotides also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways, which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside

linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars that are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus, modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acid molecules encoding proteins of the invention, together with pharmaceutically acceptable carriers.

The use of RNA interference or "RNAi" involves the use of double-stranded RNA (dsRNA) to block gene expression. (see: Sui, G, et al, *Proc Natl. Acad. Sci U.S.A.* 99:5515-5520,2002). Methods of applying RNAi strategies in embodiments of the invention would be understood by one of ordinary skill in the art.

Methods in which small interfering RNA (siRNA) molecules are used to reduce the expression of mTOR, p70S6K1 or Rheb may be used. In one aspect, a cell is contacted with a siRNA molecule to produce RNA interference (RNAi) that reduces expression of one or more of the aforementioned genes. The siRNA molecule is directed against nucleic acids coding for the relevant polypeptide (e.g. RNA transcripts including untranslated and translated regions). Well known methods such as Western blotting can be used for determining the level of protein expression and Northern blotting or RT-PCR can be used for determining the level of mRNA transcript of the targeted gene.

As used herein, a "siRNA molecule" is a double stranded RNA molecule (dsRNA) consisting of a sense and an antisense strand. The antisense strand of the siRNA molecule is a complement of the sense strand (Tuschl, T. et al., 1999, *Genes & Dev.*, 13:3191-3197; Elbashir, S.M. et al., 2001, *EMBO J.*, 20:6877-6888; incorporated herein by reference). In one embodiment the last nucleotide at the 3' end of the antisense strand may be any

nucleotide and is not required to be complementary to the region of the target gene. The siRNA molecule preferably is 19-23 nucleotides in length and may form a hairpin structure. In one preferred embodiment the siRNA molecule includes a two nucleotide 3' overhang on the sense strand. In a second preferred embodiment the two nucleotide overhang is thymidine-thymidine (TT). The siRNA molecule corresponds to at least a portion of a target gene. In one embodiment the siRNA molecule corresponds to a region selected from a cDNA target gene beginning between 50 to 100 nucleotides downstream of the start codon. In a preferred embodiment the first nucleotide of the siRNA molecule is a purine.

The siRNA molecules can be plasmid-based. In a preferred method, a polypeptide encoding sequence of one of the aforementioned genes is amplified using the well known technique of polymerase chain reaction (PCR). The use of the entire polypeptide encoding sequence is not necessary; as is well known in the art, a portion of the polypeptide encoding sequence is sufficient for RNA interference. The PCR fragment is inserted into a vector using routine techniques well known to those of skill in the art. Combinations of the foregoing can be expressed from a single vector or from multiple vectors introduced into cells.

In one aspect of the invention, a mammalian vector comprising any of the nucleotide coding sequences of the invention is provided. The mammalian vectors include but are not limited to the pSUPER RNAi vectors (Brummelkamp, T.R. et al., 2002, Science, 296:550-553, incorporated herein by reference). In one embodiment, a nucleotide coding sequence can be inserted into the mammalian vector using restriction sites, creating a stem-loop structure. In a second embodiment, the mammalian vector may comprise the polymerase-III H1-RNA gene promoter. The polymerase-III H1-RNA promoter produces a RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines (T5) in a row. The cleavage of the transcript at the termination site occurs after the second uridine and yields a transcript resembling the ends of synthetic siRNAs containing two 3' overhanging T or U nucleotides. The antisense strand of the siRNA molecule hybridizes to the corresponding region of the mRNA of the target gene.

Preferred systems for mRNA expression in mammalian cells are those such as pSUPER RNAi system as described in Brummelkamp et al. (2002, Science, 296:550-553). Other examples include but are not limited to pSUPER.neo, pSUPER.neo+gfp, pSUPER.puro, BLOCK-iT T7-TOPO linker, pcDNA1.2/V5-GW/lacZ, pENTR/U6, pLenti6-

GW/U6-laminshrna, and pLenti6/BLOCK-iT-DEST. These vectors are available from suppliers such as Invitrogen, and one of skill in the art would be able to obtain and use them.

Treatment for a disorder resulting from loss of insulin responsiveness may include, but is not limited to, dietetic therapy and pharmaceutical therapy. In some embodiments, treatment may include administration of a pharmaceutical agent that decreases mTOR, p70S6K1 or Rheb activity. The inhibitors of mTOR, p70S6K1 or Rheb activity can be administered in conjunction with other pharmaceutical agents known for treatment of such disorders. For example, in the treatment of type 2 diabetes, other therapeutics such as insulin sensitizers, insulin secretagogues, insulin, and the like, can be administered in conjunction (simultaneously or sequentially) with therapeutics that decrease mTOR, p70S6K1 or Rheb activity or expression.

As used herein, a subject is preferably a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat, or rodent. In all embodiments, human subjects are preferred. In some embodiments, the subject is suspected of having a disorder associated with abnormally increased mTOR, p70S6K1 or Rheb activity (relative to healthy individuals), which results in or results from a loss of insulin responsiveness, such as type II diabetes.

Methods for identifying subjects having or suspected of having a disorder associated with abnormally increased mTOR, p70S6K1 or Rheb activity or a disorder characterized by a loss of insulin responsiveness include, but are not limited to: physical examination, analysis of a subject's medical history, analysis of a subject's family medical history, blood tests, visual exam, mean body mass assessment, and/or weight assessment. Diagnostic methods for identifying subjects having or suspected of having a disorder characterized by a loss of insulin responsiveness are well-known to those of skill in the medical arts, although not necessarily with respect to increased mTOR, p70S6K1 or Rheb activity.

As used herein, a biological sample includes, but is not limited to: tissue, cells, or body fluid (e.g., blood or lymph node fluid). The fluid sample may include cells and/or fluid. The tissue and cells may be obtained from a subject or may be grown in culture (e.g., from a cell line). In some embodiments of the invention, the biological sample is a control sample.

The amino acid sequences identified herein as mTOR, p70S6K1 or Rheb polypeptides, and the nucleotide sequences encoding them, are sequences deposited in databases such as GenBank (e.g., M60724 (p70S6K1), L34075 (human TOR), Z29677 (Rheb)). The use of these known mTOR, p70S6K1 or Rheb sequences in pharmaceutical screening assays, determination of pharmaceutical agents, and diagnostic assays for disorders

resulting from loss of insulin responsiveness, e.g., type 2 diabetes, as described herein is novel. Homologs, alleles, orthologs and other variants of the mTOR, p70S6K1 or Rheb nucleic acid sequences and polypeptides sequences can also be used, as appropriate, as will be known to one of ordinary skill in the art. In general, homologs, alleles and other variants typically will share at least 90% nucleotide identity and/or at least 95% amino acid identity to the sequences of a mTOR, p70S6K1 or Rheb nucleic acid and polypeptide, respectively, in some instances will share at least 95% nucleotide identity and/or at least 97% amino acid identity, and in other instances will share at least 97% nucleotide identity and/or at least 99% amino acid identity. The homology can be calculated using various, publicly available software tools developed by NCBI (Bethesda, Maryland) that can be obtained through the Internet or using a variety of commercially available softward packages. Exemplary tools include the BLAST system available from the website of the National Center for Biotechnology Information (NCBI) at the National Institutes of Health.

The identification herein of mTOR, p70S6K1 or Rheb polypeptides as involved in physiological disorders involving a loss of insulin responsiveness also permits the artisan to diagnose a disorder characterized by expression of mTOR, p70S6K1 or Rheb polypeptides, and characterized preferably by an alteration in functional activity of the mTOR, p70S6K1 or Rheb polypeptides.

Thus the invention also includes methods to monitor the onset, progression, or regression of a disorder associated with increased or excessive mTOR, p70S6K1 or Rheb activity in a subject by, for example, obtaining samples at sequential times from a subject and assaying such samples for the level of expression of mTOR, p70S6K1 or Rheb nucleic acid molecules, the level of expression of mTOR, p70S6K1 or Rheb polypeptide molecules, and/or the level of activity of a mTOR, p70S6K1 or Rheb polypeptide (including kinase activity). A subject may be suspected of having a disorder associated with increased or excessive mTOR, p70S6K1 or Rheb activity or may be believed not to have such a disorder and in the latter case, the sample expression or activity level may serve as a control for comparison with subsequent samples.

Onset of a condition is the initiation of the changes associated with the condition in a subject. Such changes may be evidenced by physiological symptoms, or may be clinically asymptomatic. For example, the onset of a disorder associated with increased or excessive mTOR, p70S6K1 or Rheb activity may be followed by a period during which there may be physiological changes in the subject, even though clinical symptoms may not be evident at

that time. The progression of a condition follows onset and is the advancement of the physiological elements of the condition, which may or may not be marked by an increase in clinical symptoms. Onset and progression are similar in that both represent an increase in the characteristics of a disorder (e.g., expression or activity of mTOR, p70S6K1 or Rheb molecules), in a cell or subject, onset represents the beginning of this disorder and progression represents the worsening of a preexisting condition. In contrast to onset and progression, regression of a condition is a decrease in physiological characteristics of the condition, perhaps with a parallel reduction in symptoms, and may result from a treatment or may be a natural reversal in the condition.

A marker for disorders associated with increased or excessive mTOR, p70S6K1 or Rheb activity may be the level or amount of catalytic activity of a mTOR or p70S6K1 polypeptide or the GTPase and/or GTP binding activity of Rheb, the level or amount of specific phosphorylation of a mTOR or p70S6K1 substrate, or the level of expression of a mTOR, p70S6K1 or Rheb nucleic acid or polypeptide.

The invention also involves the use of agents such as polypeptides that bind to mTOR, p70S6K1 or Rheb polypeptides or substrates of such polypeptides. Such binding agents can be used, for example, in screening assays to detect the presence or absence of mTOR, p70S6K1 or Rheb polypeptides or their substrates and in purification protocols to isolate mTOR, p70S6K1 or Rheb, their substrates or complexes of mTOR, p70S6K1 or Rheb polypeptides and their substrates.

The invention, therefore, embraces peptide binding agents which, for example, can be antibodies or fragments of antibodies having the ability to selectively bind to mTOR, p70S6K1 or Rheb polypeptides or their substrates. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology. As used herein, mTOR, p70S6K1 or Rheb antibodies, are antibodies that specifically bind to mTOR, p70S6K1 or Rheb polypeptides, respectively.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated

an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (HAMA) responses when administered to humans.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which

the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to mTOR, p70S6K1 or Rheb polypeptides, their substrates and complexes of both mTOR, p70S6K1 or Rheb polypeptides and their substrates. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the mTOR, p70S6K1 or Rheb polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the mTOR, p70S6K1 or Rheb polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the mTOR, p70S6K1 or Rheb polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the mTOR, p70S6K1 or Rheb polypeptides.

The invention also relates in part to methods of treating disorders associated with abnormally increased mTOR, p70S6K1 or Rheb activity, which include a loss of insulin responsiveness, such as type 2 diabetes. An "effective amount" of a drug therapy is an amount of an agent that decreases mTOR, p70S6K1 or Rheb activity that alone, or together

with further doses, produces the desired response, e.g. reduction of symptoms of type 2 diabetes, or other disease associated with a loss of insulin responsiveness.

In the case of treating a particular disease or condition the desired response is inhibiting the progression of the disease or condition. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine diagnostic methods known to one of ordinary skill in the art for any particular disease. The desired response to treatment of the disease or condition also can be delaying the onset or even preventing the onset of the disease or condition, or reversing the physiological effects of the disease.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the agent that decreases mTOR, p70S6K1 or Rheb activity (alone or in combination with other therapeutic agents) be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The pharmaceutical compositions used in the foregoing methods preferably are sterile and contain an effective amount of an agent that decreases mTOR, p70S6K1 or Rheb activity for producing the desired response in a unit of weight or volume suitable for administration to a patient.

The doses of an agent that decreases mTOR, p70S6K1 or Rheb activity administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

Various modes of administration will be known to one of ordinary skill in the art which effectively deliver the agent that decreases mTOR, p70S6K1 or Rheb activity to a desired tissue, cell or bodily fluid. Administration includes: topical, intravenous, oral, intracavity, intrathecal, intrasynovial, buccal, sublingual, intranasal, transdermal, intravitreal, subcutaneous, intramuscular and intradermal administration. The invention is not limited by the particular modes of administration disclosed herein. Standard references in the art (e.g., *Remington's Pharmaceutical Sciences*, 18th edition, 1990) provide modes of administration and formulations for delivery of various pharmaceutical preparations and formulations in pharmaceutical carriers. Other protocols which are useful for the administration of agent that decreases mTOR, p70S6K1 or Rheb activity will be known to one of ordinary skill in the art, in which the dose amount, schedule of administration, sites of administration, mode of administration (e.g., intra-organ) and the like vary from those presented herein.

Administration to mammals other than humans of agents that decrease mTOR, p70S6K1 or Rheb activity, e.g. for testing purposes or veterinary therapeutic purposes, is carried out under substantially the same conditions as described above. It will be understood by one of ordinary skill in the art that this invention is applicable to both human and animal diseases that can be treated by an agent that decreases mTOR, p70S6K1 or Rheb activity. Thus this invention is intended to be used in husbandry and veterinary medicine as well as in human therapeutics.

In general, a therapeutically effective amount of an agent that decreases mTOR, p70S6K1 or Rheb activity typically varies from about 0.01 ng/kg to about 1000 μg/kg, preferably from about 0.1 ng/kg to about 200 μg/kg and most preferably from about 0.2 ng/kg to about 20 μg/kg, in one or more dose administrations daily, for one or more days. Lesser or greater amounts may be found to be therapeutically effective and thus also are useful in accordance with the invention. For treatments of disorders, it is preferred that doses of agents that decrease mTOR, p70S6K1 or Rheb activity are formulated and administered in doses between 0.2mg to 5000mg of the agent that decreases mTOR, p70S6K1 or Rheb activity. More preferably, an effective amount will be in the range from about 0.5mg to 500mg of the agent that increases mTOR, p70S6K1 or Rheb activity, according to any standard procedure in the art. Administration of agents that decrease mTOR, p70S6K1 or Rheb activity compositions to mammals other than humans, e.g. for testing purposes or veterinary therapeutic purposes, is carried out under substantially the same conditions as described above.

The pharmaceutical preparations of the invention may be administered alone or in conjunction with standard treatment(s) of disorders associated with alterations in mTOR, p70S6K1 or Rheb activity, such as type 2 diabetes or other disorders associated with a loss (i.e., a reduction or complete loss) of insulin responsiveness. For example, treatment for type 2 diabetes with a pharmaceutical agent of the invention, may be undertaken in parallel with treatments for diabetes that is known and practiced in the art. For example, such treatments may include, but are not limited to administration of metformin, pioglitazone, and/or rosiglitazone. Other known treatments for type 2 diabetes include pharmaceutical agents that increases insulin release, which may include, but are not limited to sulfonylureas, nateglinide and repaglinide. In some treatment methods, sulfonylureas include, but are not limited to glibenclamide (glyburide), gliclazide and glimepiride. In some embodiments of the invention, insulin may be administered to the subject, in conjunction with the treatment methods of the invention.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts. Preferred components of the composition are described above in conjunction with the description of the agent that decreases mTOR, p70S6K1 or Rheb activity of the invention.

An agent that decreases mTOR, p70S6K1 or Rheb activity may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the

active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the agent that decreases mTOR, p70S6K1 or Rheb activity, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, as described above, including: acetate, phosphate, citrate, glycine, borate, carbonate, bicarbonate, hydroxide (and other bases) and pharmaceutically acceptable salts of the foregoing compounds.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise an agent that decreases mTOR, p70S6K1 or Rheb activity. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA.

A long-term sustained release implant also may be used for administration of the pharmaceutical agent composition. "Long-term" release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well known to those of ordinary skill in the art and include some of the release systems described above. Such implants can be particularly useful in treating conditions characterized by insufficient decreases mTOR, p70S6K1 or Rheb activity by placing the implant near portions of a subject affected by such activity, thereby effecting localized, high doses of the compounds of the invention.

The invention also includes kits for assaying the activity level of a mTOR, p70S6K1 or Rheb polypeptide (e.g., kinase activity, GTPase activity or GTP binding activity) for determining whether test compounds decrease the mTOR, p70S6K1 or Rheb polypeptide activity. One example of such a kit of the invention is a kit that provides components necessary to determine the activity level of a mTOR, p70S6K1 or Rheb polypeptide of the invention using a kinase assay. The components can include an appropriate substrate molecule as well as necessary cofactors and other components (e.g., buffers, radioactive molecules).

Another example of a kit of the invention, is a kit that provides components necessary to determine the level of expression of a mTOR, p70S6K1 or Rheb nucleic acid molecule of the invention. Such components may include, primers useful for amplification of a mTOR, p70S6K1 or Rheb nucleic acid molecule and/or other chemicals for PCR amplification.

The foregoing kits can include instructions or other printed material on how to use the various components of the kits for diagnostic purposes or for compound screening purposes.

Examples

Summary

LKB1 is a serine/threonine kinase that directly phosphorylates Thr172 of AMPKα in vitro and activates its kinase activity (Hawley et al. (2003) J. Biol. *2*, 28; Woods et al. (2003) Curr. Biol. *13*, 2004-2008; Shaw et al. (2004), Proc. Natl. Acad. Sci. USA *101*, 3329-3335). LKB1 and AMPK activate TSC2/tuberin in response to cellular energy stress, negatively regulating signaling by mTOR, a serine /threonine kinase that regulates protein synthesis and cell growth in all eukaryotes (Inoki et al. (2003), Cell *115*, 577-590). Akt activates mTOR signaling by directly phosphorylating and inactivating the TSC2 tumor suppressor, also

named tuberin (Manning et. al. (2002), Molecular Cell *10*, 151-162; Inoki et al. (2002) Nature Cell Biology *4*, 648-657). mTOR activity in turn inhibits Akt/PI3K activity via negative feedback.

p70S6K1 is activated downstream of mTOR and insulin signaling and is a critical modulator of cell growth in mammalian cells (Fingar and Blenis (2004) Oncogene *23*,3151-3171). We show that LKB1-deficient MEFs are hypersensitive to apoptosis induced by glucose deprivation, and rapamycin, an inhibitor of mTOR, rescues this apoptosis. We further demonstrate that LKB1-deficient MEFs exhibit defective AMPK activation and mTOR inhibition following glucose deprivation. Prolonged treatment in glucose-free media leads to apoptosis, as indicated by the extent of caspase-mediated PARP cleavage in total cell lysates. Therefore, inhibition of mTOR or its downstream substrate p70S6K1 may restore insulin sensitivity by enhancing Akt/PI3K function.

Materials

Antibodies

Anti-phospho-AMPK (T172), anti-phospho-ACC, anti-phospho-p70S6K1 (T389), anti-phospho-p70S6K1 (T421/S424), anti-phospho ribosomal protein S6 (S235/236), anti-ribosomal protein S6, anti-eIF4E, anti-phospho-Akt (S473), anti-phospho-ERK (T202/Y204), anti-4E-BP1, and cleaved PARP (mouse-specific) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-LKB1 antiserum was previously described (Bardeesy et al., Nature 2002, Sept 12; 419(6903) 127-8). Anti-FLAG antibodies (M2 monoclonal and FLAG polyclonal) were from Sigma Aldrich Corporation (St. Louis, MO). Anti-HA probe polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Chemicals

AICAR was obtained from Toronto Research Chemicals (Downsview, ON, Canada). Glucose-free media, D-PBS (containing 1 g/l D-glucose) and dialyzed serum was from Invitrogen (Carlsbad, CA). Rapamycin was from EMD Biosciences (San Diego, CA). 7-methyl GTP sepharose was obtained from Amersham Biosciences (Piscataway, NJ).

Vector constructs

Constructs used: FLAG-wild-type LKB1. FLAG kinase-dead (K781) LKB1 and FLAG-tuberin were described previously (Shaw et al. (2004), Proc. Natl. Acad. Sci. USA

101, 3329-3335, Manning et. al. (2002), Molecular Cell *10*, 151-162)

Mice

Littermate-derived *LKB1*$^{+/+}$ and$^{-/-}$ mouse embryo fibroblasts (MEFs) were prepared as described previously (Bardeesy et al., Nature (2002), Sept 12; *419*(6903) 127-8). *TSC2*$^{-/-}$ *p53*$^{-/-}$ littermate MEFs were obtained from Dr. D. Kwiatkowski (Harvard Medical School, Boston, MA) as described previously (Zhang, et. al., Journal of Clinical Investigation (2003) Oct; *112*(8): 1223-33).

Example 1: LKB1- and TSC2-deficient MEFs show similar aberrant deregulation of intracellular signaling in response to specific environmental stresses

We have previously made the observation that AMPK is required for LKB1-mediated inhibition of mTOR signaling. Furthermore, this inhibition occurs through tuberin/TSC2 and involves inhibition of p70S6K1 activity via mTOR. We set out to determine whether other known stresses that inhibit mTOR activity might also function through LKB1/AMPK.

Littermate-matched LKB1- or TSC2-deficient MEFs were compared to wild-type MEFs in their response to a number of stress stimuli known to inhibit mTOR signaling. mTOR signaling was examined by immunoblotting for phospho-Thr389 p70S6K1 (P-p70S6K1) using a specific antibody (anti-phospho-p70S6K1 (T389) or anti-phospho-p70S6K1 (T421/S424)) in accordance with the manufacturer's instructions. Activation of Akt and ERK was examined using specific activation state phospho-specific antibodies (Ser473 for Akt, Thr202/Tyr204 for ERK) in accordance with the manufacturer's instructions. eIF4E was used as a loading control.

All MEFs were serum-deprived in DMEM alone for 24 hours. Indicated samples were then placed in fresh media containing 10% serum, with or without 20nm rapamycin or 2 mM AICAR, all for 90 minutes. Amino acid deprivation was performed by placing the serum-starved cells into D-PBS with 10% dialyzed serum for 90 min.

When treated with AICAR, an AMP mimetic capable of activating LKB1 signaling, *LKB1*$^{-/-}$ cells failed to downregulate p70S6K1 activity. In *TSC2*$^{-/-}$ cells, p70S6K1 was not inhibited in response to any of the stimuli applied (Figures 1A and 1B). Neither ERK nor Akt activation was enhanced in LKB1-deficient cells following any of the cellular stresses applied.

We next examined whether *LKB1*$^{-/-}$ and *TSC2*$^{-/-}$ MEFs share similar biological

properties. Both cell types have been previously shown to undergo apoptosis in response to AMPK activation (Shaw et al. (2004), Proc. Natl. Acad. Sci. USA *101*, 3329-3335; Inoki et al. (2003), Cell *115*, 577-590) and that this response can be rescued by concurrent treatment with rapamycin. We therefore investigated whether glucose deprivation would also selectively lead to the apoptosis of LKB1-deficient MEFs and whether this effect could be rescued by rapamycin treatment.

First, we characterized that AMPK signaling and mTOR was aberrant in *LKB1*$^{-/-}$ cells following glucose deprivation (Figure 1C). Cells were placed in normal media, glucose-free media with 10% dialyzed serum, or glucose-free media with 10% dialyzed serum containing 20 mM rapamycin for 6 or 24 hours as indicated. As expected, in the *LKB1*$^{-/-}$ cells, phospho-S6 was elevated and phospho-ACC was decreased, indicating a defect in AMPK signaling in these cells following glucose deprivation (Shaw et al. (2004) Cancer Cell *6*, 91-99). Using caspase activation—as detected by PARP cleavage in lysates—as a measure of apoptosis (Shaw et al. (2004), Proc. Natl. Acad. Sci. USA *101*, 3329-3335; Inoki et al. (2003), Cell *115*, 577-590), we observed that *LKB1*$^{-/-}$ MEFs show elevated sensitivity to apoptosis induced by glucose withdrawal as compared to their wild-type counterparts, and that rapamycin potently inhibited this apoptotic phenotype (Figure 1C). The PARP cleavage in lysates was mirrored in direct assays of *LKB1*$^{-/-}$ MEFs to glucose deprivation-induced cell death and the suppression of this effect by rapamycin (Figure 1D).

These data reveal a consistent and direct correlation with increased mTOR/p70S6K1 signaling and decreased PI3K signaling, and suggest that inhibition of mTOR results in a restoration of Akt and PI3K pro-survival signaling. Direct inhibition of substrate p70S6K1 may provide a more potent means of restoring insulin signaling via Akt/PI3K.

Example 2: High throughput cell-based assay to identify inhibitors of p70S6K1

A high throughput cell-based assay is performed by screening a cell line using a phospho-specific antibody for phospho-S6 (of which several are commercially available, e.g. Cell Signaling Technology, Beverly, MA). Cells (e.g. HEK293 cells, NIH3T3 fibroblasts or 3T3L1 adipocytes) are plated in full serum in 96 or 384 well plates, candidate inhibitor compounds added and then screened by immunocytochemistry for phospho-S6. Candidate inhibitor compounds that inhibit p70S6K1 kinase activity directly or by affecting a protein upstream in the pathway are those that diminish the phospho-S6 signal. Whether such compounds directly bind and inhibit p70S6K1 is confirmed, for example, using an *in vitro*

assay as described in Example 3.

Example 3: High throughput *in vitro* assay to identify inhibitors of p70S6K1

A more direct assay is to screen directly for p70S6K1 inhibitors *in vitro*. This is a biochemical assay in which kinase activity is assayed. A variety of detectable molecules for determining the kinase activity of p70S6K1 are available. To increase the quantitative and high throughput nature without needing radioactivity, one can use fluorescence polarization to quantify the amount of activity of the kinase of interest (e.g. p70S6K1). A flourescein-conjugated peptide substrate for p70S6K1 (such those derived from its optimal consensus sequence in the ribosomal S6 protein) is mixed with p70S6K1 with or without inhibitor to form an assay mixture. In this assay, a fluorescently labeled peptide substrate is phosphorylated by the kinase and captured on metal-derivatized nanoparticles ("IMAP", Molecular Devices Corporation, Sunnyvale, CA). At high salt concentration, trivalent metal cations bind and capture phosphorylated peptides. The amount of flouresceinated, phosphorylated peptide bound is measured by an increase in the fluorescence polarization signal caused by a decrease in the molecular mobility of the bound product. (Turek-etienne, et al. (2003) Assay Drug Dev Technol., Aug;*1*(4):545-53).

It is preferable to determine that candidate inhibitors are specific for p70S6K1 and not related kinases such as the Rsk or Msk family of kinases. To test for specificity, the assays can be repeated (or run in parallel) with additional kinases, such as those in the Rsk, Msk and/or PKA families of kinases. Specificity need not mean that the compound has activity on p70S6K1 and no activity on other kinases. For example it has been reported that the compound H89 inhibits p70S6K1 with a IC50 of 80 nM while it inhibits MSK1 at 120 nM and PKA at 135 nM (Davies et al., Biochemical Journal (2000), *351*, 95-105). Similar assays are performed for other kinases of interest, e.g., mTOR.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here. Each reference cited herein is incorporated by reference in its entirety.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

We claim:

Claims

1. A method for identifying compounds useful as pharmacological agents for the treatment of diabetes, comprising

contacting a p70S6K1 polypeptide with a candidate pharmacological agent, and

determining the activity of the contacted p70S6K1 polypeptide, wherein a decrease in the activity of the p70S6K1 polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the p70S6K1 polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes.

2. The method of claim 1, further comprising determining an second amount of activity of the p70S6K1 polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the p70S6K1 polypeptide as the control amount of activity.

3. The method of claim 1, wherein the activity of the p70S6K1 polypeptide is kinase activity.

4. The method of claim 3, wherein the kinase activity of the p70S6K1 polypeptide is measured by phosphorylation of ribosomal S6 protein or a peptide comprising the phosphorylation site of ribosomal S6 protein.

5. The method of claim 1, wherein the p70S6K1 polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent.

6. A method for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness comprising:

administering to a subject in need of such treatment an effective amount of an agent that decreases the activity of p70S6K1 polypeptide in the subject, as a treatment for the disorder.

7. The method of claim 6, wherein the disorder is type 2 diabetes.

8. The method of claim 6, wherein the agent decreases the kinase activity of p70S6K1 polypeptide.

9. The method of claim 8, wherein the agent that decreases the kinase activity of p70S6K1 polypeptide is N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89), 1-(5-isoquinolinesulfonyl)-1H-hexahydro-1,4-diazepine (HA-1077, fasudil), an analog or derivative thereof, a salt thereof, or a solvate thereof.

10. The method of claim 6, wherein the agent decreases the amount of p70S6K1 polypeptide.

11. The method of claim 10, wherein the agent that decreases the amount of p70S6K1 polypeptide is a siRNA/RNAi molecule or an antisense nucleic acid molecule.

12. A method for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness, comprising

identifying a compound that decreases activity of a p70S6K1 polypeptide and

formulating the compound for administration to a subject in need of such treatment.

13. The method of claim 12, wherein the disorder is type 2 diabetes.

14. The method of claim 12, wherein the compound that decreases activity of a p70S6K1 polypeptide is identified by the method of any of claims 1-5.

15. A method for identifying compounds useful as pharmacological agents for the treatment of diabetes, comprising

contacting a mTOR polypeptide with a candidate pharmacological agent, and

determining the activity of the contacted mTOR polypeptide, wherein a decrease in the activity of the mTOR polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the mTOR polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes.

16. The method of claim 15, further comprising determining an second amount of activity of the mTOR polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the mTOR polypeptide as the control amount of activity.

17. The method of claim 15, wherein the activity of the mTOR polypeptide is kinase activity.

18. The method of claim 17, wherein the kinase activity of the mTOR polypeptide is measured by phosphorylation of p70S6K1, eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), or a peptide comprising the phosphorylation site of p70S6K1 or 4E-BP1.

19. The method of claim 15, wherein the mTOR polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent.

20. A method for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness comprising:

administering to a subject in need of such treatment an effective amount of an agent that decreases the activity of mTOR polypeptide in the subject, as a treatment for the disorder.

21. The method of claim 20, wherein the disorder is type 2 diabetes.

22. The method of claim 20, wherein the agent decreases the kinase activity of mTOR polypeptide.

23. The method of claim 22, wherein the agent that decreases the kinase activity of mTOR polypeptide is rapamycin (sirolimus), a rapamycin analog or derivative, a salt thereof, or a solvate thereof.

24. The method of claim 23, wherein the rapamycin analog or derivative is everolimus (RAD-001), or rapamycin 42-ester with 3-hydroxy-2- (hydroxymethyl)-2- methylpropionic acid (CCI-779).

25. The method of claim 20, wherein the agent decreases the amount of mTOR polypeptide.

26. The method of claim 25, wherein the agent that decreases the amount of mTOR polypeptide is a siRNA/RNAi molecule or an antisense nucleic acid molecule.

27. A method for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness, comprising

identifying a compound that decreases activity of a mTOR polypeptide and

formulating the compound for administration to a subject in need of such treatment.

28. The method of claim 27, wherein the disorder is type 2 diabetes.

29. The method of claim 27, wherein the compound that decreases activity of a mTOR polypeptide is identified by the method of any of claims 15-19.

30. A method for identifying compounds useful as pharmacological agents for the treatment of diabetes, comprising

contacting a Rheb polypeptide with a candidate pharmacological agent, and

determining the activity of the contacted Rheb polypeptide, wherein a decrease in the activity of the Rheb polypeptide contacted with the candidate pharmacological agent relative to a control amount of activity of the Rheb polypeptide is an indication that the candidate pharmacological agent is useful in the treatment of diabetes.

31. The method of claim 30, further comprising determining an second amount of activity of the Rheb polypeptide in the absence of the candidate pharmacological agent, and using the second amount of activity of the Rheb polypeptide as the control amount of activity.

32. The method of claim 30, wherein the activity of the Rheb polypeptide is GTP binding activity.

33. The method of claim 32, wherein the GTP binding activity of the Rheb polypeptide is measured by binding of detectably labeled non-hydrolyzable analog of GTP.

34. The method of claim 33, wherein the detectably labeled non-hydrolyzable analog of GTP is [^{35}S]GTPγS.

35. The method of claim 30, wherein the activity of the Rheb polypeptide is GTPase activity.

36. The method of claim 35, wherein the GTPase activity of the Rheb polypeptide is measured by hydrolysis of detectably labeled GTP.

37. The method of claim 36, wherein the detectably labeled GTP is [α-^{32}P]GTP.

38. The method of claim 30, wherein the Rheb polypeptide is contained within a cell, and the cell is contacted with the candidate pharmacological agent.

39. A method for treating a subject having or suspected of having disorder characterized by reduced insulin responsiveness comprising:

administering to a subject in need of such treatment an effective amount of an agent that decreases the activity of Rheb polypeptide in the subject, as a treatment for the disorder.

40. The method of claim 39, wherein the disorder is type 2 diabetes.

41. The method of claim 39, wherein the agent decreases the GTPase activity of Rheb polypeptide.

42. The method of claim 39, wherein the agent decreases the GTP binding activity of Rheb polypeptide.

43. The method of claim 39, wherein the agent decreases the amount of Rheb polypeptide.

44. The method of claim 43, wherein the agent that decreases the amount of Rheb polypeptide is a siRNA/RNAi molecule or an antisense nucleic acid molecule.

45. The method of claim 39, wherein the agent that decreases the activity of Rheb polypeptide is a farnesyl transferase inhibitor, an analog or derivative thereof, a salt thereof, or a solvate thereof..

46. The method of claim 45, wherein the farnesyl transferase inhibitor is FTI 277, Zarnestra® (tipifarnib, R115777), RPR-130401 or lonafarnib (SCH66336), an analog or derivative thereof, a salt thereof, or a solvate thereof.

47. A method for preparing a drug for the treatment of a disorder characterized by reduced insulin responsiveness, comprising

identifying a compound that decreases activity of a Rheb polypeptide and

formulating the compound for administration to a subject in need of such treatment.

48. The method of claim 47, wherein the disorder is type 2 diabetes.

49. The method of claim 47, wherein the compound that decreases activity of a Rheb polypeptide is identified by the method of any of claims 30-38.

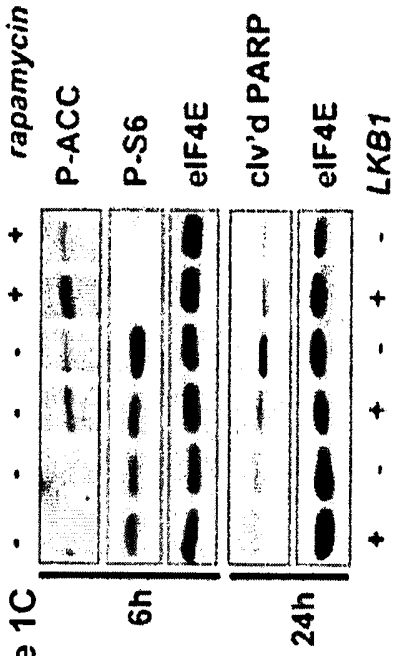

Figure 1C

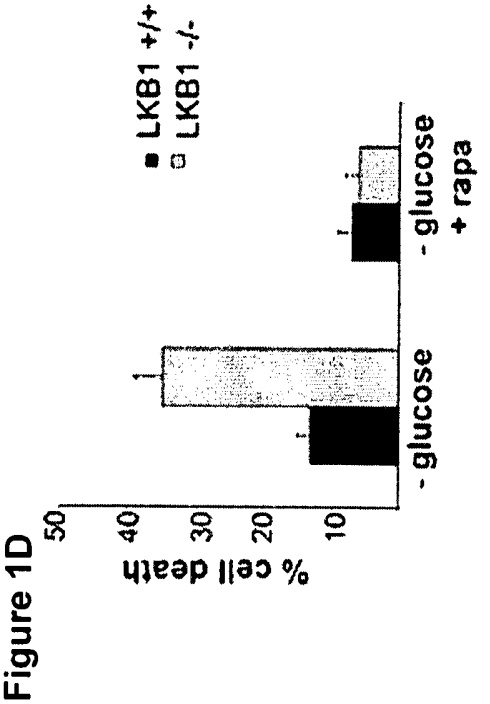

Figure 1D

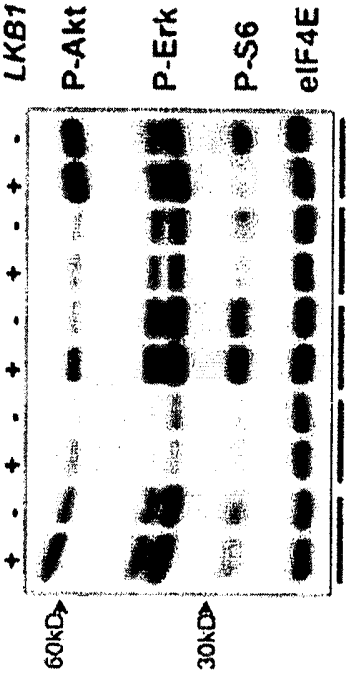

Figure 1A

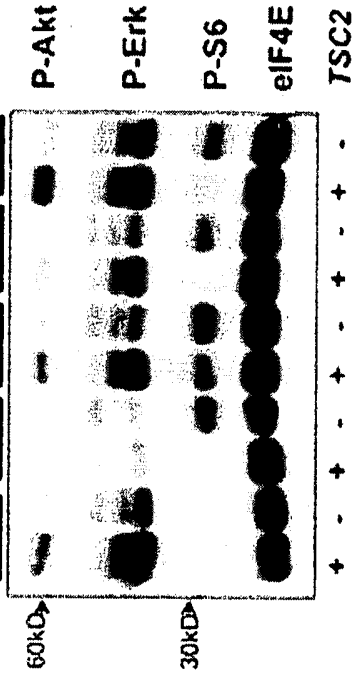

Figure 1B