METHOD FOR SCREENING COMPOUNDS FOR THOSE THAT MODULATE TRANSUCERS OF REGULATED CREB ACTIVITY

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Field of Classification Search None
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References Cited

OTHER PUBLICATIONS


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ABSTRACT

In accordance with the present invention, it has been discovered that glucose and incretin hormones promote pancreatic islet cell survival via the calcium and cAMP dependent induction, respectively, of the transcription factor CREB. Specifically, a signaling module has been identified which mediates cooperative effects of calcium and cAMP on islet cell gene expression by stimulating the dephosphorylation and nuclear entry of TORC2, a cytoplasmic CREB coactivator. The module comprises a cAMP regulated snf1-like kinase called SIK2 and the calcium regulated phosphatase calcineurin, both of which associate with TORC2 in the cytoplasm. TORC2 is repressed under basal conditions through a phosphorylation dependent interaction with 14-3-3 proteins. cAMP and calcium signals stimulate CREB target gene expression via complementary effects on TORC2 dephosphorylation; cAMP disrupts TORC2-associated activity of SIK2 or regulated family members, whereas calcium induces TORC2 dephosphorylation via calcineurin. These findings provide a novel mechanism by which CREB activates cellular gene expression, depending on nutrient and energy status, and facilitate development of assays to identify compounds which modulate the role of TORCs. In accordance with the present invention, it has been discovered that fasting and energy-sensing pathways regulate the gluconeogenic program in liver by modulating the nuclear entry of a transcriptional coactivator called Transducer of Regulated CREB Activity 2 (TORC2). Hepatic TORC2 over-expression induces fasting hyperglycemia, whereas knockdown of TORC2 leads to fasting hypoglycemia and silencing of the gluconeogenic program. Since a majority of individuals with Type II diabetes exhibit fasting hyperglycemia due to elevated hepatic gluconeogenesis, compounds that enhance TORC2 phosphorylation will find use as therapeutic agents in this setting.

4 Claims, 16 Drawing Sheets
OTHER PUBLICATIONS


**FIGURE 15**

Table $^{32}$P-incorporation into GST-fusion peptides by SIK kinase domain

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<th>Protein (Target residue)</th>
<th>Accession No.</th>
<th>Peptide$^\text{1)}$</th>
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SIK consensus

L-x-R-S/T-x-S-x-x-x-L

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1) bold letters indicate residues conserved in the AMPK consensus [H-(B-X or X-B)-X-X-S-X-X-X-H].
2) relative $^{32}$P-incorporation compared with Syntide2; I, >80%; II, 80-20%; III, <20%; IV, undetectable.
3) synthetic substrates of AMPK, SAMS peptide and modified peptides.
4) amino acid substitution.
FIGURE 16
FIGURE 17
FIGURE 18
FIGURE 19
**FIGURE 20**

- **A**
  - Graph showing the expression levels of different genotypes (GST, WT, S171A, SAMS) under control (CON) and AMP treatment.

- **B**
  - Bar graph showing the effect of FSK and AICAR on phosphorylation of TORC2 and TORC5.

- **C**
  - Western blots comparing wild-type (wt) TORC2, S171A TORC2, CON, FSK, FSK + AICAR, FSK + SIK1 conditions.

- **D**
  - Bar graph showing mRNA levels of PEPCK and PGC-1α under different conditions.

- **E**
  - Schematic diagram illustrating the AMPK pathway and its relationship to Gluconeogenesis.
METHOD FOR SCREENING COMPOUNDS FOR THOSE THAT MODULATE TRANSDUCERS OF REGULATED CREB ACTIVITY

RELATED APPLICATION

This application claims priority from U.S. Patent Application No. 06/608,407 filed Apr. 4, 2005, the entire contents of which is hereby incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to methods for screening compounds to determine if such compounds are capable of enhancing islet cell activity and/or survival, capable of promoting CREB-mediated gene expression in islet cells, capable of effecting transport of Transducers of Regulated CREB (TORCs) from the cytoplasm into the nucleus of an islet cell, capable of effecting interactions between TORCs and member(s) of the 14-3-3 family of proteins, and the like. In additional aspects, the present invention relates to methods for enhancing islet cell activity and/or survival.

BACKGROUND OF THE INVENTION

Under feeding conditions, elevations in blood glucose and circulating incretin hormones such as glucagon like peptide-1 (GLP-1) stimulate islet survival in part via the activation of the transcription factor CREB (Jhala et al., 2003). Elevations in glucose stimulate insulin secretion and islet cell gene expression via closure of KATP channels and subsequent influx of calcium through activated L-type calcium channels (Newgard and McGarry, 1995). In contrast, GLP-1 has been found to promote islet cell survival and proliferation by activation of the cAMP pathway (Hui et al., 2003). Transgenic mice expressing a dominant negative CREB polypeptide in islets develop diabetes with apoptosis of insulin producing beta cells due in part to reduced expression of IRS2, a direct target of CREB activity (Jhala et al., 2003).

cAMP promotes the expression of cellular genes by triggering the PKA mediated phosphorylation of CREB at Ser133 (Gonzalez and Mostiminy, 1989). Phosphorylation of CREB at Ser133 in turn stimulates gene expression by enhancing recruitment of the histone acetylase coactivator paralogs CHP and P300 (Arias et al., 1994; Chiria et al., 1993; Kwon et al., 1994). The structure of the CREB:CBP complex, using relevant interaction domains, called KID and KIX, respectively, reveals that phospho (Ser133) forms direct contacts with residues in KIX that account for half of the free energy of complex formation (Parker et al., 1998; Radhakrishnan et al., 1997). Binding of KID to KIX also promotes a random coil to helix transition in KID that favors formation of hydrophobic contacts with residues lining a shallow groove in KIX.

In addition to cAMP, CREB is Ser 33 phosphorylated in response to a number of stimuli, including growth factors, shear stress, and UV light (Mayr and Mostiminy, 2001). A number of these stimuli, however, are incapable of promoting target gene activation via CREB per se due in part to secondary phosphorylation of CREB at inhibitory sites. Neuronal depolarization triggers phosphorylation of CREB not only at Ser133 but also at Ser142 and Ser143 (Kornhauser et al., 2002), for example, and these modifications destabilize the CREB:CBP complex by electrostatic repulsion (Kornhauser et al., 2002; Parker et al., 1998).

The ability of calcium signals to promote CREB-dependent transcription while apparently blocking CBP recruitment, at least via the KID domain, is indicative of the potential presence of other coactivators that either mitigate these effects or function independently of CBP/P300. The involvement of a distinct CREB coactivator in promoting calcium dependent gene expression is further indicated by studies in which addition of calcineurin antagonists is observed to block calcium-stimulated CREB activity without affecting levels of CREB Ser133 phosphorylation (Schwaninger et al., 1995). The identification of such putative coactivator(s), however, remains elusive.

Although the KID domain in CREB is thought to mediate target gene activation in response to most extracellular stimuli, other regions, most notably the bZIP DNA binding/dimerization domain, have also been implicated in this process. In previous studies using GAL4-CREB fusion proteins to define domain requirements for transcriptional activation, for example, both KID and bZIP domains were found to contribute importantly to cAMP and KCl responsiveness (Bonni et al., 1995a; Sheng et al., 1991). These results are also indicative of the involvement of additional cofactors that promote cAMP and calcium dependent transcription through an interaction with the CREB bZIP domain. Consistent with the ability of this region to recruit components of the transcriptional apparatus, the CREB bZIP domain has been found to act as a potent repressor of numerous transcription factors when over-expressed in various cells (Lemaignre et al., 1993).

Accordingly, there is a need in the art for methods to identify compounds that modulate the above-described interactions. Such compounds will find use in a variety of applications, such as, for example, enhancing islet cell activity and/or survival, promoting CREB-mediated gene expression in islet cells, effecting transport of Transducers of Regulated CREB (TORCs) from the cytoplasm into the nucleus of islet cells, effecting interactions between TORCs and member(s) of the 14-3-3 family of proteins, and the like.

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that glucose and incretin hormones promote pancreatic islet cell survival via the calcium and cAMP dependent induction, respectively, of the transcription factor CREB. Specifically, a signaling module has been identified which mediates cooperative effects of calcium and cAMP on islet cell gene expression by stimulating the dephosphorylation and nuclear entry of TORC2, a cytoplasmic CREB coactivator. The module comprises a cAMP regulated sucrose-non-fermenting-1 (srt11)-like protein kinase called SIK2 (and related family members) and the calcium regulated phosphatase calcineurin, both of which associate with TORC2 in the cytoplasm. TORC2 is repressed under basal conditions through a phosphorylation dependent interaction with 14-3-3 proteins. cAMP and calcium signals stimulate CREB target gene expression via complementary effects on TORC2 dephosphorylation; cAMP disrupts the TORC2-associated activity of SIK2 and related family members, whereas calcium induces TORC2 dephosphorylation via calcineurin. The results described herein establish that glucose and incretin hormones exert synergistic effects on CREB activity and islet cell survival by targeting a signaling module that contains TORC2-associated kinase and phosphatase activities, respectively.

In recent high-throughput expression screens to identify novel modulators of CREB activity, a family of CREB coactivators, referred to as Transducers of Regulated CREB activi-
ity (TORCs) have been characterized (see, for example, Conkright et al., 2003a; and lourgenko et al., 2003). The three exemplary TORC family members identified thus far share a highly conserved N-terminal coiled-coil domain that mediates a direct association with the bZIP domain of CREB. The present disclosure establishes that TORC2 is a cytoplasmic co-factor that translocates to the nucleus in response to cAMP and calcium signals where it modulates CREB target gene expression. TORC2 shuttling activity is regulated by associated protein kinase (e.g., SIK2 or related family members) and phosphatase (e.g., calcineurin) activities that modulate levels of TORC phosphorylation. These findings provide a novel mechanism by which CREB activates cellular gene expression, depending on nutrient and energy status, and facilitate development of assays to identify compounds which modulate the role of TORCs.

Mammals achieve energy balance by modulating hepatic glucose output depending on nutritional status (Saltiel and Kahn, 2001). Elevations in glucagon during fasting trigger the gluconeogenic program, for example, via cAMP responsive factor CREB (Herzig et al., 2001). By contrast, exercise and other stressors that deplete cellular ATP levels inhibit gluconeogenesis via the AMP kinase pathway, although the underlying mechanism has remained elusive (Kahn et al., 2005). In accordance with the present invention, it has been discovered that fasting and energy-sensing pathways regulate the gluconeogenic program in liver by modulating the nuclear entry of a transcriptional coactivator called Transducer of Regulated CREB Activity 2 (TORC2) (see, for example, Conkright et al., 2003a; lourgenko et al., 2003; Screaton et al., 2004; and Bittinger et al., 2004). Thus, under feeding conditions, TORC2 is sequestered in the cytoplasm via phosphorylation at Ser171 (see Screaton et al., 2004).

Glucagon administration promotes rapid Ser171 dephosphorylation, nuclear translocation, and recruitment of TORC2 to gluconeogenic promoters in liver. Hepatic TORC2 over-expression induces fasting hyperglycemia, whereas knockdown of TORC2 leads to fasting hypoglycemia and silencing of the gluconeogenic program. Following prolonged exposure to glucagon, TORC2 activity is attenuated by a negative feedback loop involving the CREB mediated induction of SIK1, a Ser/Thr kinase that phosphorylates TORC2 at Ser171 (Screaton et al., 2004; and Kato et al., 2004). Knockdown of SIK1 enhances TORC2 activity on gluconeogenic genes, whereas SIK1 over-expression silences the gluconeogenic program and promotes fasting hypoglycemia in mice. Similarly, induction of the AMPK (AMP-activated protein kinase) pathway with an AMP analog inhibits TORC2 activity on gluconeogenic genes; these effects are rescued by expression of phosphorylation-defective Ser171 Ala TORC2. Since a majority of individuals with Type II diabetes exhibit fasting hyperglycemia due to elevated hepatic gluconeogenesis, compounds that enhance TORC2 phosphorylation will find use as therapeutic agents in this setting.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-1E demonstrate that the CREB bZIP domain is required for cooperativity between cAMP and calcium signals. Specifically, FIG. 1A summarizes the results of a transient transfection assay of HIT insulinoma cells transfected with the CREB-dependent EVX-1 luciferase reporter. Cells were treated with KCI (45 mM) and/or forskolin (10 nM) for six hours as indicated. The effect of treatment with the calcineurin inhibitor, Cyclosporine A (CsA, 5 FM) is shown.

FIG. 1B presents quantitative PCR (Q-PCR) analysis of the CREB target gene NRA42 in MIN6 insulinoma cells exposed to glucose (20 mM) and/or exenatide (10 nM). The effect of the calcium channel antagonist, nifedipine, and CsA is shown.

FIG. 1C is a Western blot assay of phospho (Ser133)CREB levels in HIT cells following treatment with KCI (45 mM), forskolin (10 nM), or both together for 30 minutes. The effect of CsA treatment on levels of phospho (Ser133)CREB is indicated.

FIG. 1D illustrates the effect of calcium and cAMP agonists on CREB-CBP complex formation. A mammalian two-hybrid assay was carried out employing HIT cells transfected with GAL4-KID and KIX-VP16 expression plasmids. The luciferase activity from cells co-transfected with GAL4 luciferase reporter is shown. Cells were treated with forskolin and KCI (6 hours), alone and in combination, as indicated.

FIG. 1E illustrates the effect of KCI and forskolin on the activity of full-length GAL4-CREB and truncated GAL4-CREB AbZIP polypeptides lacking the bZIP domain (amino acid residues 284-341) in transfected HIT cells. Treatment with CsA is shown.

FIGS. 2A-2D demonstrate that TORC2 is recruited to the promoter and mediates CREB target gene activation in response to cAMP and calcium signals. Specifically, FIG. 2A summarizes the results of a transient assay of HIT cells co-transfected with CREB-dependent EVX-1 luciferase reporter and TORC2 expression vector, or control empty vector. Cells were treated with forskolin or KCI as indicated. The effect of CsA (5 mM) is shown.

FIG. 2B illustrates the effect of TORC2 knockdown on induction of EVX-1 reporter in response to cAMP. The left panel illustrates a transient assay of HEK293T cells co-transfected with non-specific or TORC2 RNAi plasmid plus EVX-1 reporter. The right panel illustrates the rescue of activity with TORC3 expression plasmid shown. The inset presents a Western blot assay of endogenous TORC2 protein levels in TORC2 RNAi or non-specific RNAi transfected cells.

FIG. 2C summarizes the results of transient transfection assays of HIT cells expressing wild-type and mutant GAL4-CREB polypeptides that are defective in either CBP (M1: Ser133 Ala) or TORC2 (M2: Arg314 Ala) binding. Comparable expression of wild-type and mutant GAL4-CREB polypeptides was confirmed by Western blot assay (not shown). The effect of forskolin (F) and KCI (K), either alone or in combination, on co-transfected GAL4 luciferase reporter is shown. Treatment with CsA is indicated.

FIG. 2D presents the results of chromatin immunoprecipitation assay of HEK293T cells using CREB, CBP, and TORC2 (T2) specific antisera. PCR amplification of the cAMP responsive NRA42 promoter (5') and 3' flanking region fragments from CREB, CBP, and TORC2 immunoprecipitates is shown. The effect of forskolin treatment (30 min) is indicated. Linearity of the PCR assay with decreasing DNA input levels is also indicated.

FIGS. 3A-3D demonstrate that cAMP promotes cytoplasmic to nuclear translocation of TORC2. Specifically, FIG. 3A presents fluorescence microscopic analysis of Flag-tagged TORC2 in human ATBYH fibroblasts treated with forskolin (10 nM) or control vehicle for 30 minutes as indicated.

FIG. 3B illustrates the effect of exportin inhibitor leptomycin B (LMB) on nuclear targeting of TORC2 in transfected ATBYH fibroblasts. DAPI staining is shown alongside to indicate nuclei.

FIG. 3C, top panel, illustrates the effect of Tyr282 to Phe mutagenesis in NESP on TORC3 localization in control and
forskolin treated A7431 cells. Wild-type TORC3 staining in control cells is also shown. FIG. 3C, bottom panel, presents amino acid sequence alignments for NES1 and NES2 motifs in TORC1, TORC2, and TORC3, relative to consensus NES motif shown below (SEQ ID: NOS 10:16, respectively, in order of appearance. Residue Tyr282 in TORC3 is highlighted.

FIG. 3D, top panel, presents a comparison of basal TORC2 (T2) and TORC3 (T3) activities in HEK293T cells co-transfected with EVX-1 reporter. Comparable expression of TORC2 and TORC3 proteins was confirmed by Western blot analysis (not shown). The effect of N-terminal arc myristilation tag on TORC2 and TORC3 activities is shown. FIG. 3D, bottom panel, demonstrates the effect of adding the Ty282Ph mutation on basal TORC3 activity, relative to empty vector control in HIT cells co-transfected with EVX-1 reporter.

FIGS. 4A-4C demonstrate that cAMP and calcium promote TORC2 dephosphorylation and nuclear entry. Specifically, FIG. 4A, left panel, presents a Western blot of nuclear (N) and cytoplasmic (C) fractions from HEK293T cells transfected with Flag-tagged TORC2 (FLAG-T2) or empty vector (etv). Compare the mobility of TORC2 immunoreactive bands. Endogenous CREB immunoreactivity is shown to verify the efficient fractionation of cytoplasmic and nuclear extracts. FIG. 4A, right panel, illustrates the effect of calf intestinal alkaline phosphatase (CIP) treatment on the electrophoretic mobility of cytoplasmic TORC2.

FIG. 4B illustrates the effect of co-stimulation with forskolin and KCl (5-30 minutes) on endogenous TORC2 phosphorylation in HIT cells. Treatment with CaA is indicated.

FIG. 4C, top panel presents an SDS-PAGE analysis of 32P-labeled Flag-TOR2 immunoprecipitates from transfected HIT cells incubated with inorganic 32P. Control cells transfected with empty vector (vec) are also indicated. Treatment with forskolin (F), KCl (K), or forskolin plus KCl are shown. The effect of CaA is indicated. FIG. 4C, middle panel, presents a Western blot assay of immunoprecipitated 32P-labeled TORC2 protein from top panel. FIG. 4C, bottom panel, illustrates the relative levels of TORC2 phosphorylation for each treatment, normalized to the immunoprecipitated TORC2 protein levels shown.

FIGS. 5A-5D illustrate that 14-3-3 proteins associate with and repress TORC activity. Specifically, FIG. 5A, top panel, presents the results of a co-immunoprecipitation assay of Flag-TORC1 with endogenous 14-3-3 proteins in HEK293T cells. The effect of forskolin (F) treatment on the TORC1: 14-3-3 association is shown. The relative effect of Ser/Thr phosphatase PP1/PP2A (okadaic acid; OA) or CaA on TORC1: 14-3-3 interaction is indicated. FIG. 5A, bottom left panel, is a Western blot assay of 14-3-3 proteins recovered from TORC control anti-gut immunoprecipitates, illustrating the effect of forskolin treatment on endogenous TORC: 14-3-3 complexes in PC12 and HEK293T cells. Input levels of 14-3-3 and TORC proteins are shown. Comparable recovery of endogenous TORC proteins from TORC immunoprecipitates is indicated. FIG. 5A, bottom right panel, illustrates the kinetics of TORC2 dissociation from endogenous 14-3-3 proteins in response to forskolin treatment of HEK293T cells. The levels of Flag-tagged TORC 2 (FLAG-T2) recovered from flag immunoprecipitates at each time point is shown below.

FIG. 5B summarizes the results of co-immunoprecipitation assays using truncated TORC2 polypeptides to define the 14-3-3 interaction site. The deletion endpoints in TORC2 are shown.

FIG. 5C illustrates the effect of 14-3-3 beta over-expression on EVX-1 reporter activity in HEK293T cells co-transfected with wild-type TORC2, 14-3-3 interaction defective (A56-547) TORC2, or empty vector. FIG. 5D demonstrates that TORC2 binds to calcineurin. A pull-down assay of GST-calcineurin A (amino acid residues i-347) with 35S-labeled wildtype and mutant TORC2 polypeptides containing internal deletions are indicated. FIGS. 6A-6D demonstrate that SIK2 (or related family members), snf1-like kinase, associates with and phosphorylates TORC2. Specifically, FIG. 6A, demonstrates that TORC2 associates with a cytoplasmic protein kinase activity. In vitro kinase assays of endogenous TORC (1P:TORC) and transfected Flag-tagged TORC2 (1P:FLAG) immunoprecipitates were prepared from nuclear or cytoplasmic fractions of control and forskolin treated cells as shown. 32P-labeled bands (left) and corresponding protein levels by Western blot assay (right) are indicated.

FIG. 6B summarizes immunoprecipitation assays of HEK293T cells co-transfected with expression vectors for SIK2 and Flag-tagged TORC2. Western blot assays of SIK2 recovered from Flag immunoprecipitates using anti-SIK2 antiserum are shown. FIG. 6C identifies phosphopeptides recovered from Flag-tagged TORC2 immunoprecipitates identified by MSMS analysis (SEQ ID: NOS: 17-27, respectively, in order of appearance). Amino acid endpoints and phosphorylated residues are indicated. Consensus SIK2 phosphorylation site shown: β- hydrophobic; B- basic; X- any amino acid.

FIG. 6D demonstrates that SIK2 phosphorylates TORC2 at Ser171. An in vitro kinase assay of wild-type and Ser171Ala GST TORC2 polypeptides (amino acid residues 162-179) was conducted using purified SIK2 as indicated. The effect of SIK2 (or related family members) on phosphorylation of GST alone or GST-estradiol 2 containing a consensus SIK2 phosphorylation site (PLARTLSVGLPDKK; SEQ ID NO:1) is shown. Input levels of individual GST proteins (CBH) are shown below.

FIGS. 7A-7C demonstrate that TORC2 is retained in the cytoplasm under basal conditions via SIK2 (or related family members)-dependent phosphorylation at Ser171. Specifically, FIG. 7A presents a Western blot analysis of total and phospho (Ser587)-SIK2 levels in COS-7 cells transfected with wild-type and PKA phosphorylation defective Ser587Ala mutant SIK2 expression vector. The effect of forskolin treatment is shown.

FIG. 7B, left panel, presents a comparison of wild-type, PKA phosphorylation defective (Ser587Ala), and kinase-inactive (Lys49Met) SIK2 constructs on EVX-1 reporter activity in control and forskolin treated HEK293T cells. FIG. 7A, right panel, illustrates the effect of wild-type and Ser171 Ala mutant TORC2 polypeptides on EVX-1 reporter activity in control and forskolin stimulated HEK293T cells.

FIG. 7C, top panels, illustrate the effect of SIK2 (or related family members) on TORC2 subcellular localization. Immunofluorescence microscopy is presented for A7YH1 cells transfected with Flagtagged TORC2 plus wild-type or PKA phosphorylation defective SIK2 (Ser587Ala) as indicated. DAPI staining is shown below each panel. FIG. 7C, bottom panels, illustrate the effect of Ser171 Ala mutagenesis on cellular localization of TORC2. Treatment with forskolin is indicated.

FIG. 8 presents a model for cooperative induction of cellular genes in response to cAMP and calcium signals in insulin producing beta cells. SIK2 (and related family members) promotes Ser171 phosphorylation and association of TORC2 with 14-3-3 proteins. cAMP and calcium agonists activate
TORC2 via cooperative effects on TORC2 dephosphorylation and nuclear entry. cAMP inhibits TORC2-associated activity of SIK2 and related family members, whereas calcium signals promote calcineurin dependent dephosphorylation of TORC2. Nuclear TORC2 stimulates CREB activity via an interaction with the BZIP domain of CREB. In parallel, cAMP stimulates phosphorylation of CREB at Ser133 and recruitment of the histone acetylase CBP to the promoter.

Figure 9 illustrates the time course (in minutes) of CREB Ser133 phosphorylation in HEK cells following treatment with forskolin (10 μM) or KCl (45 mM). The effect of cyclosporine A (CsA; 5 μM) is shown. Immunoreactive bands correspond to phospho (Ser133) CREB (top) and phospho (Ser63) ATF1 (bottom).

Figure 10 presents the characterization of a TORC interaction defective mutant CREB polypeptide. A pull-down assay of 32P-labeled TORC1 was carried out with wild-type and mutant GST CREB bZIP (amino acid residues 284-341) polypeptides. The effect of alanine substitutions at polar residues in the leucine zipper domain on TORC binding are shown. Binding of TORC to itself via N-terminal coiled-coil domain (TORC1-I29) is shown for comparison. Coomassie stained gel showing comparable input levels of GST-CREB bZIP proteins was used for the pull-down assays.

Figure 11 presents the results of transient assays of HEK293T cells transfected with flag-tagged wild-type, Arg314Ala (R314A), and Glu319Ala (E319A) CREB plus TORC1 expression vector. Luciferase activity obtained from co-transfected EVX-1 reporter plasmid is shown. Comparable binding of wild-type and mutant Arg314Ala (R314A) CREB to the CRE was verified by gel mobility shift assay (not shown).

Figure 12 indicates the relative importance of CREB:CBP and CREB:TORC complexes for transcriptional activation in response to Camp and calcium signals in PC12 cells. Transient assay was conducted with PC12 cells co-transfected with wild-type, CBP interaction defective (M1: Ser33Ahn), TORC interaction defective (M2: Arg314Ahn), or CBP and TORC defective (M1/M2) GAL4-CREB expression vectors as indicated. The activity was determined for cotransfected GAL4 luciferase reporter in cells treated with forskolin (10 μM), KCl (45 mM), or forskolin plus KCl for four hours as shown.

Figure 13, top panels, illustrates the cellular localization of endogenous TORC2 proteins in ATYBI cells using TORC specific antiserum. The effect of forskolin (10 μM, 30 minutes) and leptomycin B (LMB, 10 ng/ml, 2 hr) treatment is shown. DAPI staining is shown below to visualize the nuclei. Figure 13, bottom panel, illustrates the effect of LMB treatment on TORC1 localization in ATYBI cells transfected with flag-tagged TORC1 expression vector. TORC1 localization was followed using anti-flag antiserum. DAPI staining is shown alongside.

Figure 14 identifies nuclear localization (NLS) and nuclear export (NES) signals in TORC. Summary in the right margin thereof shows the predominant cellular localization (Nuclear, Cytoplasmic;C) of TORC2 polypeptides fused to green fluorescent protein (GFP). TORC2 amino acid endpoints for each fusion protein shown.

Figure 15 provides the characterization of optimal phosphorylation sites for SIK2. Potential SIK2 substrates identified in database search were tested by in vitro kinase assay with purified SIK2. The table shows the substrates tested and the relative stoichiometry of phosphorylation. The optimal motif for SIK2 mediated phosphorylation is shown. Figure 15 discloses SEQ ID NOS: 1 and 28-35, respectively, in order of appearance.

Figures 16A-16E illustrate the effect of fasting and feeding signals on activation of CREB:TORC and CREB:CBP pathways in liver. Specifically, Figure 16A presents an immunohistochemical analysis of CREB and phospho (Ser133) CREB staining on liver sections from mice 10 minutes following intraperitoneal (IP) injection with insulin, glucagon, or vehicle (PBS). DAPI staining is shown to highlight nuclei.

Figure 16B presents a Western blot analysis of phospho (Ser133) CREB and CREB levels in liver extracts prepared from the same three treatment groups as described above. Figure 16C presents an immunohistochemical analysis of TORC2 localization in liver sections from mice 10 minutes following the same intraperitoneal (IP) injections as described above with respect to Figure 16A. DAPI staining is shown to highlight nuclei.

Figure 16D presents a Western blot assay of HA-TORC2 immunoprecipitates prepared from whole liver extracts of treatment groups described above with anti-HA antiserum. Western blotting with phospho (Ser171) specific and non-discriminating TORC2 antiserum is shown. The top band in the glucagon treated sample corresponds to Ser171-phosphorylated (PTORC2).

Figure 16E presents the results of a Chromatin Immunoprecipitation (ChIP) assay of liver extracts from mice 10 minutes following IP glucagon or insulin administration. The recruitment of TORC2 to CREB target genes (PEPCK, G6Pase) in liver is demonstrated. FDPS, CREB target gene is induced only in the fed state (FDPS) in liver. Levels of TORC2 recruitment to each gene were determined by Q-PCR analysis.

Figures 17A-17F demonstrate that TORC2 is required for hepatic gluconeogenesis during fasting. Specifically, Figure 17A illustrates the effect of TORC2 on induction of gluconeogenic genes (PEPCK, PGC-1α, G6Pase) by FSK (10 μM, 2 hr) in cultured primary rat hepatocytes infected with TORC2 or control GFP adenovirus as indicated.

Figure 17B demonstrates that TORC2 stimulates gluconeogenic genes via CREB. The effect of dominant negative A-CREB (AC) adenovirus on G6Pase mRNA levels is illustrated in cells co-infected with GFP or TORC2 adenovirus and treated with FSK as indicated.

Figure 17C summarizes the effect of TORC2 on glucose output from primary rat hepatocytes in response to fasting and feeding signals. Cells were infected with TORC1, TORC2, or control GFP adenovirus and then treated with FSK plus dexamethasone (FSK/DEX) for four hours. The effect of insulin on glucose output is shown.

Figure 17D summarizes the effect of TORC2 over-expression on fasting glucose metabolism. Blood glucose levels in control (GFP) and TORC2 adenovirus infected mice is shown, taken sequentially after 7 and 24 hours of fasting (n=3). Below the graph presented in Figure 17D, a Western blot analysis of liver extracts from control and TORC2 adenovirus infected mice is presented, showing the relative levels of endogenous and adenovirus expressed HA-TORC2 (denoted by an arrow).

Figure 17E summarizes the effect of acute TORC2 knockdown on fasting glucose levels in mice (n=3). Mice were infected with TORC2 RNAi or unspecific (US) RNAi adenovirus. The inset presents a Western blot assay of TORC2 levels in primary hepatocytes infected with unspecific on TORC2 RNAi adenovirus.

Figure 17F presents a Q-PCR analysis of hepatic mRNAs from TORC2 deficient (TORC2 RNAi) and control (US) mice. The levels of gluconeogenic (PEPCK, PGC-1α, G6Pase, PC) and mitochondrial (Cox4, Cyt-C) gene expressions are shown.
FIGS. 18A-18H illustrate that the induction of the AMP kinase family member SIK1 by CREB during fasting attenuates the gluconeogenic program. Specifically, FIG. 18A illustrates the time course of PEPCK gene expression in primary rat hepatocytes following exposure to FSK. mRNA levels were determined by Q-PCR analysis.

FIG. 18B illustrates the effect of CHX pretreatment on induction of gluconeogenic genes (PEPCK, PGC-1α, G6Pase) by glucagon in primary rat hepatocytes. Relative mRNA levels are shown.

FIG. 18D demonstrates the effect of fasting on mRNA levels for SIK1, SIK2, and SIK3 genes in liver by Q-PCR analysis (n=3).

FIG. 18E presents a Western blot analysis of SIK1 protein levels in whole liver extracts from mice under ad libitum, fasting, or refed conditions. CREB levels are shown for comparison.

FIG. 18F summarizes the effect of FSK treatment on SIK1 mRNA levels in primary rat hepatocytes. Cells were infected with control (GFP) or dominant negative A-CREB adenovirus as indicated.

FIG. 18G summarizes the effect of co-transfected PKA, dominant negative A-CREB, or control (empty) expression vector on SIK1 luciferase reporter activity in transiently transfected HepG2 hepatocytes.

FIG. 18H, top, provides a schematic of a SIK1 promoter, showing the presence of two CRE sites at positions indicated relative to the transcriptional start site. FIG. 18H, bottom, presents the results of a Chromatin Immunoprecipitation (ChIP) assay of CREB immunoprecipitates prepared from SV40-transformed mouse hepatocytes, showing recovery of SIK1 promoter or negative control ACT B promoter, which lacks consensus CREB binding site. Genomic DNA input (In) levels (1%) are shown.

FIGS. 19A-19F demonstrate that SIKs inhibit hepatic gluconeogenesis via phosphorylation of TORC2 at Ser171. Specifically, FIG. 19A, top, presents a Western blot showing the effect of US and SIK1 RNAi adenoviruses on the levels of SIK1 protein in rat hepatocytes. FIG. 19B, bottom, presents a Western blot assay with nondiscriminating TORC2 antisera, showing the effect of SIK1 RNAi adenovirus on TORC2 dephosphorylation (compare P-TORC2 and TORC2 bands) following exposure of primary rat hepatocytes to glucagon (Glu). The arrow identifies phosphorylated TORC2.

FIG. 19E and 19F demonstrate the role of Ser171 in TORC2 for SIK1 inhibition of gluconeogenic genes. Specifically, the effect of SIK1 on the induction of gluconeogenic PGC-1α (see FIG. 19E) and PEPCK (see FIG. 19F) genes by wild-type and Ser171 Ala TORC2 is illustrated in primary rat hepatocytes.

FIGS. 20A-20E demonstrate that the energy sensing AMPK (AMP-activated protein kinase) pathway regulates TORC2 activity in liver. Specifically, FIG. 20A illustrates the relative phosphorylation of wild-type or Ser171 Ala mutant recombinant GST-TORC2 (161-181) compared to GST only or optimal AMPK peptide substrate (SAM5) by activated AMPK in vitro. Addition of AMP to reactions is shown. The relative incorporation of γ−32P-ATP by each substrate is indicated.

FIG. 20F demonstrates the effect of the AMP analog AICAR (1 mM) on phosphorylation of endogenous TORC2 in primary rat hepatocytes. Cells were exposed to AICAR and FSK for 30 minutes. Lower mobility bands (arrow) indicate phosphorylated TORC2 polypeptides.

FIG. 20C illustrates the effect of AICAR and SIK1 on cellular localization of wild-type or Ser171 Ala TORC2 in primary rat hepatocytes. Cells were infected with adenoviruses for HA-tagged wild-type or mutant TORC2 and SIK1 as indicated. Infected cells were exposed to FSK and AICAR for 30 minutes. TORC2 localization was examined with anti-HA antiserum. Cells were counterstained with DAPI to visualize nuclei.

FIG. 20D summarizes the effect of AICAR on expression of gluconeogenic genes (PEPCK, PGC-1α) in primary rat hepatocytes. Cells infected with mutant Ser171Ala TORC2 (171) adenovirus indicated. Treatment with AICAR and FSK is shown.

FIG. 20E illustrates an auto-regulatory loop which controls gluconeogenic gene expression in liver. Activation of the cAMP pathway by glucagon triggers expression of the gluconeogenic program via acute Ser171 dephosphorylation and activation of TORC2. At late times after glucagon stimulation TORC2 activity is attenuated via CREB-mediated induction of SIK1, which in turn rephosphorylates TORC2 at Ser171. In response to AICAR depletion, TORC2 activity is also inhibited by AMPK (AMP-activated protein kinase) mediated phosphorylation at Ser171.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of enhancing islet cell activity and/or survival. Invention methods comprise determining the effect of test compound on one or more of:

- the transport of a Transducer Of Regulated CREB (TORC) from the cytoplasm into the nucleus of an islet cell,
- the kinase activity of a snf1-like kinase,
- the interaction between a Transducer Of Regulated CREB (TORC) and a member of the 14-3-3 family of proteins, or
- the level of phosphorylation of a Transducer Of Regulated CREB (TORC), wherein one or more of the following, in the presence of test compound, is indicative of a compound which is capable of enhancing islet cell activity and/or survival: enhanced transport of TORC from the cytoplasm into the nucleus of an islet cell, increased kinase activity of the snf1-like kinase, disruption of the interaction between TORC and a member of the 14-3-3 family of proteins, or a reduction in the level of phosphorylation of TORC.

In accordance with one embodiment of the present invention, there are provided methods of screening test compounds
to determine if such compounds are capable of enhancing islet cell activity and/or survival. Invention methods comprise determining the effect of test compound on the transport of a Transducer Of Regulated CREB (TORC) from the cytoplasm into the nucleus of an islet cell, wherein enhanced transport of TORC from the cytoplasm into the nucleus of an islet cell in the presence of test compound is indicative of a compound which is capable of enhancing islet cell activity and/or survival.

As employed herein, “islet cell activity” refers to the role islet cells play in the regulation of blood glucose levels. For example, beta islet cells produce and secrete insulin, which acts to decrease blood glucose levels by mediated cell absorption of glucose. Failure (e.g., cell death) of beta islet cells (and hence, the ability to produce insulin) has been implicated, for example, in the development of type II diabetes in obese individuals. Similarly, in type I diabetes, beta islet cells are depleted by autoimmune attack thereon. In contrast to the role of beta islet cells, alpha islet cells secrete glucagon, which increases blood glucose levels by stimulation of cellular production and release of glucagon.

As employed herein, “survival” of islet cells refers to the continued viability of islet cells whether in native host tissue, or upon transplantation, the ability of islet cells to resist the factors which lead to cell death (and hence type II diabetes), the ability of islet cells to resist the factors which lead to autoimmune attack thereon, and the like.

As readily recognized by those of skill in the art, a number of Transducers Of Regulated CREB (TORCs) are known. Indeed, several isofoms have been identified (e.g., TORC1, TORC2, TORC3, and the like). TORCs have been shown to accumulate in the nucleus in response to increased intracellular calcium or cAMP, contributing to activation of CRE-dependent transcription.

In accordance with another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of enhancing islet cell activity and/or survival. Invention methods comprise determining the effect of test compound on the kinase activity of a serine/threonine kinase (sIK). Inactivation of the sIK kinase in the presence of said test compound is indicative of a compound which is capable of enhancing islet cell activity and/or survival.

As readily recognized by those of skill in the art, a number of sIK-like kinases are known, e.g., SIK1, SIK2, SIK3, and the like. These SIK proteins are serine/threonine kinases and are members of an AMP-activated protein kinase family. Salt-inducible kinase 1 (SIK1) was first isolated from the adrenal glands of rats on a high salt diet. SIK1 is primarily expressed in rat adrenal gland and may play a role in regulating steroidogenic gene expression. SIK2 and SIK3 were subsequently cloned and exhibit adiopose-specific and ubiquitous expression, respectively. SIK2 phosphorylates cytoplasmic TORC at Ser71, thereby mediating the phosphate-dependent interaction of TORC and 14-3-3. This results in a repression of TORC translocation to the nucleus and a decrease in TORC-mediated CREB activity.

In accordance with still another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of enhancing islet cell activity and/or survival. Invention methods comprise determining the effect of test compound on the interaction between a Transducer Of Regulated CREB (TORC) and a member of the 14-3-3 family of proteins, wherein disruption of the interaction between TORC and a member of the 14-3-3 family of proteins in the presence of test compound is indicative of a compound which is capable of enhancing islet cell activity and/or survival.

As readily recognized by those of skill in the art, 14-3-3 proteins comprise a family of phosphoserine/phosphothreonine binding proteins. At least seven isoforms of 14-3-3 (β, γ, ε, σ, ξ, τ, and η) have been described in mammalian cells. 14-3-3 proteins form homodimers and heterodimers capable of interacting with other cellular proteins. The phosphoserine/phosphothreonine-binding activity of 14-3-3 enables these molecules to interact with a wide variety of other cellular proteins and thereby contribute to the regulation of a number of important cellular processes (e.g., metabolism, apoptosis, cell cycle control, and the like). 14-3-3 proteins repress TORC translocation to the nucleus via a phosphate-dependent interaction with TORC. Disruption of such interactions (e.g., via dephosphorylation of TORC) allows TORC to translocate to the nucleus and increase TORC-mediated CREB activity.

In accordance with a further embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of enhancing islet cell activity and/or survival. Invention methods comprise determining the effect of test compound on the level of phosphorylation of a Transducer Of Regulated CREB (TORC), wherein a reduction in the level of phosphorylation of TORC in the presence of test compound is indicative of a compound which is capable of enhancing islet cell activity and/or survival.

There are a variety of positions on the TORCs which can be phosphorylated, e.g., at a position comparable to Ser71 of TORC2, and the like.

In accordance with a further embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of promoting CREB-mediated gene expression in islet cells. Invention methods comprise determining the effect of test compound on one or more of the transport of a Transducer Of Regulated CREB (TORC) from the cytoplasm into the nucleus of an islet cell, the kinase activity of a sIK-like kinase, the interaction between a Transducer Of Regulated CREB (TORC) and a member of the 14-3-3 family of proteins, or the level of phosphorylation of a Transducer Of Regulated CREB (TORC), wherein one or more of the following, in the presence of test compound, is indicative of a compound which is capable of promoting CREB-mediated gene expression in islet cells:

- enhanced transport of TORC from the cytoplasm into the nucleus of an islet cell, reduced kinase activity of the sIK-like kinase, disruption of the interaction between TORC and a member of the 14-3-3 family of proteins, or
- a reduction in the level of phosphorylation of TORC.

In accordance with still another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of promoting CREB-mediated gene expression in islet cells. Invention methods comprise determining the effect of test compound on the transport of a Transducer Of Regulated CREB (TORC) from the cytoplasm into the nucleus of an islet cell, wherein enhanced transport of TORC from the cytoplasm into the nucleus of an islet cell in the presence of test compound is indicative of a compound which is capable of promoting CREB-mediated gene expression in islet cells.
In accordance with yet another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of
promoting CREB-mediated gene expression in islet cells. Invention methods comprise determining the effect of test compound on the kinase activity of a snf1-like kinase, wherein reduced kinase activity of the snf1-like kinase in the presence of said test compound is indicative of a compound which is capable of promoting CREB-mediated gene expression in islet cells.

In accordance with another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of
promoting CREB-mediated gene expression in islet cells. Invention methods comprise determining the effect of test compound on the interaction between a Transducer Of Regulated
CREB (TORC) and a member of the 14-3-3 family of proteins, wherein disruption of the interaction between TORC and a member of the 14-3-3 family of proteins in the presence of test compound is indicative of a compound which is capable of promoting CREB-mediated gene expression in islet cells.

In accordance with still another embodiment of the present invention, there are provided methods of screening test compounds to determine if such compounds are capable of
promoting CREB-mediated gene expression in islet cells. Invention methods comprise determining the effect of test compound on the level of phosphorylation of a Transducer Of Regulated CREB (TORC), wherein reduction in the level of phosphorylation of TORC in the presence of test compound is indicative of a compound which is capable of promoting CREB-mediated gene expression in islet cells.

In accordance with yet another embodiment of the present invention, there are provided methods of screening test compounds to determine whether such compounds effect the
kinase activity of an snf1-like kinase. Invention methods comprise determining the effect of test compound on the kinase activity of an snf1-like kinase.

As readily recognized by those of skill in the art, a test
compound which effects the interaction between TORC and a member of the 14-3-3 family of proteins can either enhance or disrupt such interaction.

In accordance with still another embodiment of the present invention, there are provided methods of screening test compounds to determine whether such compounds effect the level of phosphorylation of a Transducer Of Regulated CREB (TORC). Invention methods comprise determining the effect of test compound on the level of phosphorylation of TORC.

As readily recognized by those of skill in the art, a test
compound which effects the level of phosphorylation of TORC can either enhance or reduce the level of phosphorylation of TORC.

In accordance with a further embodiment of the present invention, there are provided compounds identified by any of the above-described methods.

In accordance with yet another embodiment of the present invention, there are provided methods for enhancing islet cell activity and/or survival, said method comprising enhancing the transport of a Transducer Of Regulated CREB (TORC) from the cytoplasm into the nucleus of an islet cell.

As readily recognized by those of skill in the art, transport of TORC from the cytoplasm into the nucleus of an islet cell can be enhanced in a variety of ways, e.g., by blocking activation of SIK2 (or related family members), by disrupting the interaction between TORC and a member of the 14-3-3 family of proteins, by dephosphorylating TORC, and the like. As readily recognized by those of skill in the art, this can be accomplished in a variety of ways, e.g., by administering an effective amount of a compound identified by any of the above-described methods to a subject in need thereof.

Described herein is a new pathway that operates in parallel with the histone acetylase coactivators CBP/P300 to activate cellular genes in response to cAMP and calcium signals (see FIG. 8). Despite their cooperative effects on cellular gene expression, calcium and cAMP do not promote either CREB Ser133 phosphorylation or CREB:CBP complex formation in a synergistic fashion. Indeed, knockin mice with point mutations in the KIX domain of CBP and P300 that disrupt the CREB interaction display only modest changes in cAMP responsiveness (Kasper et al., 2002), consistent with the existence of a distinct pathway that also mediates cellular gene activation via CREB.

The CREB:TORC pathway is activated in response to extracellular signals; it does not require Ser133 phosphorylation but rather operates through the CREB bZIP domain, a region which has been found to contribute functionally to cAMP and calcium signaling in excitable cells (Boni et al., 1995b; Sheng et al., 1991). The importance of this domain for target gene activation may explain in part why CREB homodimerizes selectively with related family members (CREB1, ATF1, CREM) and not with other bZIP proteins (Newman and Keating, 2003).

TORC's fulfill a number of criteria for coactivators that mediate cooperativity between cAMP and calcium signals. First, they stimulate CREB activity through a bZIP domain interaction; point mutants that disrupt this interaction compromise cellular responses to both cAMP and calcium agonists. Indeed, TORC2 is recruited to the promoter in a signal-dependent manner, and knockdown of TORC2 disrupts induction of CREB target genes in response to cAMP.

Disrupting the CREB:CBP interaction by mutagenesis of the Ser133 phosphoacceptor site in CREB compromises cAMP inducibility, but has no effect on either the costimulatory actions of cAMP and calcium second messengers nor on
the ability of CaA to block this cooperativity. The importance of the bZIP domain but not phosphoserine(Ser133) for calcium dependent transcription may explain in part why CREB remains active in this setting even though calcium signals trigger its phosphorylation at sites (Ser142 and 143) that are inhibitory for the CREB(CBP) interaction (Kornhauser et al., 2002).

Without wishing to be bound by any theory, the putative mechanism underlying TORC activation is reminiscent of the cytoplasmic family of NFAT transcription factors (Crabtree and Olson, 2002; Hogan et al., 2003). Indeed, TORCs and NFATs are both maintained in the cytoplasm under basal conditions via phosphorylation at sites that promote an interaction with 14-3-3 proteins. Both sets of proteins are dephosphorylated in response to calcium signals via a direct association with the calcium dependent phosphatase calcineurin. Indeed, the calcineurin binding site in TORC contains a sequence (TORC2: amino acid 248-PGNNDFESEQ ID NO:2) that resembles the consensus calcineurin interaction motif identified for NFATs (PXIXIIIESEQ ID NO:3) (Aranburu et al., 1998), although the importance of this site for the TORC: calcineurin interaction was not addressed. Like NFATs, binding sites for calcineurin and 14-3-3 proteins on TORC appear to cluster within a regulatory domain that also contains nuclear import and export signals. The Ser171 SIK2 phosphorylation site in TORC2 is part of a potential mode 2 binding site for 14-3-3 (RXXXPSPXPSEQ ID NO:4). Based on its proximity to TORC2 NLS motifs (aa 1-147), Ser171 is believed to function a gatekeeper function, masking NLS motifs from the import machinery in a manner comparable to NFAT (Okamura et al., 2000) and other proteins. It is of note that TORC2 is also phosphorylated at numerous additional sites in addition to Ser171, and these may also contribute to TORC2 regulation.

cAMP and calcium promote TORC2 dephosphorylation cooperatively in beta cells via their effects on TORC2 associated Ser/Thr kinase and phosphatase activities. Treatment with the calcineurin inhibitor CsA reverses the effects of cAMP and calcium on TORC dephosphorylation and CREB target gene activation. Conversely, cAMP promotes TORC activation by disrupting the TORC associated ser/thr protein kinase SIK2 (and related family members).

SIK2 is a member of a larger family of at least 13 AMPK (AMP-activated protein kinase) related kinases that includes 3 SIKs (SIK1, SIK2, SIK3), 4 MARKs (MARK-1, MARK-2, MARK-3, PAR1A, MARK-4) as well as AMPK-α1 and AMPK-α2. The AMPK related family of kinases is activated through phosphorylation by LKB1, a tumor suppressor that is mutated in Peutz-Jeghers syndrome (PJS), a familial disorder characterized by multiple colon polyps and an increased incidence of colon and other cancers (Carling, 2004; Lizzano et al., 2004; Shaw et al., 2004; Woods et al., 2003). LKB1 phosphorylates AMPK family members, including SIK2, at a conserved Thr in the T-loop that stimulates their kinase activity more than 50-fold (Carling, 2004). Loss of LKB1 would be predicted to activate CREB target gene expression by reducing levels of SIK2 (or related family member)-dependent TORC phosphorylation. Remarkably, TORC1 has been described as a part of a fusion protein with the Notch co-activator mastermind like 2 (MAML2) that arises from a chromosomal translocation in mucoepidermoid carcinomas of the salivary and bronchial glands (Enlund et al., 2004; Tonon et al., 2003). Notably the TORC1-MAML2 fusion gene contains the CREB binding domain of TORC1 (aa 1-42) but lacks the central TORC regulatory domain that is phosphorylated by SIK2 (or related family members). Correspondingly, the TORC-MAML2 fusion gene is constitutively nuclear and displays high basal activity on CREB target genes (Conkright et al., 2003a; Tonon et al., 2003). The results presented herein are consistent with the proposed mechanism whereby the loss of LKB-1 activity in PJS similarly promotes oncogenic transformation in part by activating the CREB:TORC pathway.

Elevations in circulating glucose and GIP-P1 promote islet survival in part through their cooperative effects on CREB activity. Calcineurin inhibitors such as FK506 and CsA have been found to cause β cell failure and diabetes with high frequency in organ transplant recipients receiving chronic immunosuppressive therapy (Al-Uzairi et al., 2001; Filler et al., 2000). Based on their ability to interfere with glucose and incretin signaling to TORC, these calcineurin inhibitors may promote islet cell death in part by blocking CREB target gene activation. In this regard, the development of specific SIK antagonists is expected to improve islet cell function and offer useful therapy for insulin resistant individuals.

The effects of SIK2 and related family members on cellular gene regulation by CREB is likely to extend to other electrically excitable tissues. Both SIKs and TORCs are highly expressed in the brain, for example, an area where CREB appears to act within higher order processes such as learning and memory. Targeted disruption of SIK and TORC genes in these and other tissues will enable one to mediate responses to extracellular signals.

The invention will now be described in greater detail with reference to the following non-limiting examples. See also Scearson et al., 2004 and Koo et al., 2005, the entire contents of each of which is hereby incorporated by reference.

EXAMPLES

Chemicals:
LMB and Exendin-4 (Sigma, Saint Louis, Mo.) were used at 10 ng/ml and 10 mM, respectively. Nifedipine (10 μM), Cyclosporine A (CsA, 5 μM) and okadaic acid, (OA, 100 nM) were from Calbiochem (San Diego, Calif.). 32P orthophosphate in 0.02 N HCl was from ICN.

Cell Culture:
HEK293T cells were cultured in DMEM+10% FBS+pene/strep. HIT-T15 cells were cultured in F12/K medium with 10% HBS+2.5% FBS+pene/strep. MIN6 were cultured in DMEM with 10% FBS+pene/strep and 50 μM beta-mercaptoethanol. Quantitative PCR:
Cells were treated with cAMP agonist ( Forskolin, 10 μM) and KCl (45 mM) or vehicle control. For glucose experiments, MIN6 cells were starved overnight in DMEM containing 2.75 mM glucose plus 16 mM mannitol and 10% FBS. The next day the medium was changed for the indicated times+ inhibitor. Total RNA from treated MIN6 or HIT cells was extracted using an RNeasy mini-kit (Qiagen, Valencia, Calif.). 500 ng-1 μg of total RNA was used for generating cDNA with Superscript II enzyme (Invitrogen, Carlsbad, Calif.). cDNAs were analyzed by quantitative PCR using SYBR green PCR kit and an ABIPRISM 7700 Sequence detector (Perkin Elmer, Foster City, Calif.). All PCR data for CREB target NR4A2 (Conkright et al., 2003b) was normalized to ribosomal L32 or 36B4 expression in the corresponding sample.

Antisense, Western Blot Analysis, Immunoprecipitation and GST-Pulldown:
Rabbit pan-TORC (raised against 1-42 of human TORC1) and TORC2 selective rabbit polyclonal antiserum (raised against amino acids 454-607 of mouse TORC2) were gener-
ated as previously described (Conkright et al., 2003a). Whole cell protein was extracted from cultured HEK293T, HIT, and MIN6 cells in Laemmli SDS sample buffer. Alternatively, whole cell protein was extracted from liver tissue or primary hepatocytes in SDS-urea-lysis buffer. 10-20 μg of protein was separated by 8, 10 or 4-20% SDS-PAGE and transferred to PVDF membrane (Millipore Corp., Bedford, Mass.). Alternatively, 50-100 μg of protein was loaded onto a 6% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.). Chromatin immunoprecipitation and co-immunoprecipitation experiments were performed as described (Conkright et al., 2003a).

Western blot analyses were conducted on whole cell, cytoplasmic and nuclear extracts with the following rabbit polyclonal antisera: phospho (Ser133) specific affinity purified 5322 and phospho (Ser133) specific antibody from Rockland Immunoclonal, Gilbertsville, Pa.), non-discriminating CREB (244), phospho (Ser171) CREB, TORC2, pan-TORC, SIK1 and SIK2, 14-3-3 and Hsp90 antibodies were from Santa Cruz (Santa Cruz, Calif.), FLAG-M2 (Sigma) and GAL4 (Santa Cruz) monoclonal antibodies were used at 1:2000 and 1:1000 dilutions, respectively. For quantitative western blotting, goat anti-mouse-800 nm fluorescent conjugate to detect FLAG-M2 was used at 1:40,000 dilution according to manufacturer’s instructions (Lico) prior to analysis using the Odyssey detection system. GST-pull downs were performed as described (Asharian et al., 2001).

For immunoprecipitation, whole cell protein was extracted from liver tissue, primary hepatocytes, or mouse hep cIc7 in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM β-glycerol phosphate, 1 mM Na3VO4, 1 mM NaF and 1 mM DTT). Immunoprecipitation of HA-TORC2 was performed according to manufacturer’s instructions.

DNA Plasmid Constructs:

Site-specific mutant TORC and CREB cDNAs were generated using the Quickchange protocol (Stratagene, La Jolla, Calif.) or by PCR-mediated restriction fragment recombination and then verified by sequencing. Protein expression of these constructs was evaluated by Western blot and immunofluorescence analysis using anti-Flag or anti-GAL4 antibodies. TORC2 RNAi plasmid has been described (Conkright et al., 2003a).

SIK1 promoter sequences containing residues -425 to -76 were amplified by PCR from mouse genomic DNA and inserted into pXP2 Luc reporter vector to generate mSIK1 (-425+76) Luc construct. PEPPCK (--549/+49) Luc reporter, AOX Luc reporter, PGC-1 expression construct, and non-specific control p-U6-US construct have been described previously (see Koo et al., 2004). pU6-RNAi plasmids were generated as described previously. The coding sequence from 468 to 488 (GGGGCCAGTTGTTAGACTGCC; SEQ ID NO:5) was used for targeting mouse TORC2, and the coding sequence from 355 to 376 (GGGGCCATTGAGTACACAGG; SEQ ID NO:6) was used for targeting mouse nRT SIK1.

Transient Reporter Assays:

HEK293T, HIT or PC 12 cells were co-transfected with EVX or GAL4-LUC reporter plasmid, RSV-beta galactosidase and FLAG-TORC and/or SIK2 expression plasmids overnight using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.). DNA/lipid complexes were washed the next day and extracts prepared 24 h (293T and PC 12) or 40 hr (HITs) after transfection. 45 mM KCl and/or 10 μM forskolin were added 6 hr prior to harvest and 7 hr after Ca2+ treatment where indicated. Luciferase values were normalized to β-gal activity from co-transfected RSV-beta galactosidase activity in the corresponding sample.

In Vivo and In Vivo Phosphorylation Assays:

HIT and MIN6 insulinoma cells were transfected with Flag-tagged TORC1, TORC2, or TORC3 expression vector. In vivo: after 4 hr, transfected cells were incubated with phosphate-free Dulbecco’s modified Eagle medium containing 10% dialyzed serum and 1 μCi of [32P]orthophosphate/ml. After 4 hr, cells were washed with ice cold Tris-buffered saline, and harvested in either boiling SDS or RIPA lysis buffer (0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl) containing a cocktail of phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium fluoride, 1 mM sodium vanadate plus 20 μg RNase A and 1 μM DTT). Samples were diluted to IX RIPA (for boiling SDS samples) and immunoprecipitated after preclearing with Staph A using anti-Flag agarose (Sigma). In vitro: FLAG-TORC immune complexes were collected on Protein A-agarose, extensively washed and then subjected to a kinase assay by incubation with 50 μCi γ-ATP and 10 μM unlabeled ATP at 37° C. For 30 minutes in 50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM KCl. The reaction was washed once with reaction buffer and stopped by boiling in 2X Laemmli sample buffer.

Indirect Immunofluorescence:

Human ATBYF fibroblasts on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min. For staining of transient transfecants, cells were fixed 40 hr after transfection. Fixative was quenched with 0.1 M glycine prior to permeabilization with 0.1% TX-100/BSA for 2 min. Nonspecific sites were blocked with 3% BSA/BSA prior to staining with anti-FLAG M2 monoclonal antibody (1:2000) or anti-pan TORC antiseraum (1:1000) in 3% BSA/BSA. Antigens were revealed by incubation with donkey anti-mouse or anti-rabbit Alexa 488- or Cy3-conjugated secondary antibody (Molecular Probes, Eugene, Ore., Jackson Immunoreagents) prior to mounting in Vectashield with DAPI counterstain (Vector Labs). All steps were performed at 25° C.

Mass Spectrometry and Phosphorylation Site Determination:

Proteins resuspended in 8 M Urea, 100 mM Tris pH 8.5 were subjected to reduction and alkylation using 5 mM TCEP (Sigma) and 10 mM IAM (Sigma) respectively. Protein mixtures were then divided into three equal aliquots and diluted to 2 M Urea (4 M for Subtilisin) followed by digestion with 0.01 μg/μl Trypsin (Promega). 0.005 μg/μl Elastase and 0.005 μg/μl Subtilisin (Roche) (MacCoss et al., 2002). The resulting peptide mixture was then analyzed by a 7 step MudPIT analysis essentially as described (Link et al., 1999), except proteins were displaced from the SCX to the RP column using the following salt step gradients (1%0% (2) to 10% (3) to 25% (4) 25-50% (5) 50-65% (6) 65-80% and (7) 80-100% of Buffer-A to Buffer-C. Peptides were eluted from the RP column into the mass spectrometer using a linear gradient of 15-55% of Buffer-A to Buffer-B. Mobile-phase buffers were, for Buffer-A: 95% H2O, 5% acetonitrile, 0.1% formic acid; for Buffer-B, 20% H2O, 80% acetonitrile, 0.1% formic acid; for Buffer-C, 500 mM NH4OAc, 5% acetonitrile, and 0.1% formic acid. Tandem mass spectra were searched against the most recent versions of the predicted rat, mouse, and human proteins, to which common contaminants, such as keratin and trypsin, were added using a modified version of the PEP-PROB algorithm. Search results were filtered and grouped using DTASelect (Tabb et al., 2002). For phosphorylation analysis a subset database was generated which contained only the pro-
proteins that were identified. The MS/MS data were then re-
ssearched against this subset database for the modification of
480 on Ser/Thr/Tyr. The spectra containing the prominent
98-Da neutral loss were also searched against the subset
database by using a modified version of SEQUEST that con-
siders the unique MS/MS fragmentation patterns of phospho-
ylated Ser and Thr containing peptides (MacCoss et al.,
2002). Only phosphorylation sites that were matched by mul-
tiple tandem mass spectra representing sequences of different
molecular weights (from the non-specific enzymes used in
the digest) were called matches. Tandem mass spectra
matched to phosphorylated peptides were manually vali-
dated.

Culture of Primary Hepatocytes:
Rat primary hepatocytes were prepared from 200-300g of
Sprague-Dawley rats by collagenase perfusion method as
described previously (see Koo et al., 2004). 1x10^6 cells
were plated in 6 well plates with medium 199 (Invitrogen,
Carlsbad, Calif.) supplemented by 10% FBS, 5 units/ml penicillin,
10µg/ml streptomycin, and 10 nM dexamethasone for 3-6
hours. After attachment, cells were infected with adenovi-
rus for 16 hours. Subsequently, cells were maintained
in medium 199 without FBS and dexamethasone for 24 hours
(for cDNA expression adenoviruses) or 48 hours (for RNAi
adenoviruses) and 10M forskolin or 100 nM glucagon for 2
hours unless noted otherwise. For experiments with
cycloheximide, 10 µg/ml cycloheximide was added 30 minutes
prior to the addition of 100 nM glucagon.

Transfection Assays:
Human hepatoma HepG2 cells were maintained with
Ham’s F12 medium supplemented with 10% FBS (Invitro-
gen, Carlsbad, Calif.). For transfection, Eugene 6 reagent
(Roche Applied Science) was used according to the manufac-
turer’s instructions. Each transfection was performed with 100
ng of luciferase construct, 50 ng of beta galactosidase expres-
sion plasmid and 15 ng of expression vector for TORC2, 50
ng of expression vector for CREB, 50ng of expression vec-
tor for SIK1, or 50 ng of expression vector for PKA catalytic
subunit. If necessary, the empty pCDNA3 vector (Invitrogen,
Carlsbad, Calif.) was used for adding a constant amount for
each transfection. 48 hours post transfection, cells were
treated with either DMSO or 10M Forskolin (Sigma, St.
Louis, Mo.) for 4 hours and were harvested for luciferase
assays. The luciferase activity was normalized by beta galac-
tosidase activity. For transfection with AOX reporter con-
structs, 100 ng of luciferase construct, 50 ng of beta galac-
tosidase expression plasmid and 5 or 15 ng of expression vector
for PGC-1α or 50 ng of expression vector for SIK1
were used.

Recombinant Adenoviruses:
Adenoviruses expressing GFP only and unoccupied control
were described previously (see Koo et al., 2004). Adenovi-
rous for SIK1, SIK2, as well as wild-type and Ser171 Ala
TORC2 expression were generated by homologous recombi-
nation between adenovirus bacTORC2, SIK1 or SIK2 were
generated by homologous recombination between adenovi-
rous backbone vector pAD-Easy and linearized transfer vector
pAD-Track that contains mouse TORC2 cDNA, rat SIK1
cDNA or mouse SIK2 cDNA sequences, respectively. Adenovi-
ruses for TORC2 RNAi and SIK1 RNAi were generated
by homologous recombination between adenovirus backbone
vector pAD-Easy and linearized transfer vector pAD-
Track that contains U6-TORC2 RNAi or U6-SIK1 RNAi
sequences, respectively. The virus contained the cDNA that
express GFP under the control of CMV promoter for moni-
toring the infection efficiency. For animal experiments,
viruses were purified by CsCl method and dialyzed against
PBS buffer containing 10% glycerol before the injection.

Animal Experiments:
Mice 7-week-old C57Bl/6 mice were purchased from Har-
lan and maintained in regular chow under the 12-h light-dark
cycle. 0.5x10^6 plaque-forming units per recombinant adenovir-
us was delivered by a systemic tail vein injection to mice
that were anesthetized with Iso-Flurane. For measuring fast-
ing blood glucose level, animals were fasted for 16 hours with
free access to water. Blood glucose was monitored at the end
of each fasting period for 5 to 9 days. Liver tissues were
collected at the end of experiments and immediately frozen
in liquid nitrogen. Western blots were performed with GFP anti-
body to check the relative infection. For the localization of
TORC2 in liver, mice with adenovirus for mouse TORC2
were injected intraperitoneally with either 5 Ag/kg body
weight of glucagon, 1 unit/kg body weight of insulin on PBS.

Metabolites:
Blood glucose level was monitored from tail vain blood
using an automatic glucose monitor (One Touch, Lifescan,
 Fremont, Calif.). Plasma insulin levels were determined
using a commercial insulin enzyme-linked immunosorbent
assay kit (ALPCO Diagnostics, Windham, N.H.).

Immunostaining:
Formalin-fixed, paraffin-embedded liver sections (5 µm)
were deparaffinized in two changes of xylenes and hydrated
to H2O by successive 5-min washes in 100% ethanol, 90%
ethanol, 70% ethanol, and distilled H2O. Antigen unmasking
was performed by microwaving slides for 10 min in 1 mM
EDTA. After cooling to room temperature, slides were rinsed
twice in PBS. Slides were then incubated with PBS and 5%
normal donkey serum for 20 min. After incubation, slides
were incubated with the following rabbit antibodies for 60
min at room temperature: antiTORC2 (1:1600), anti-CREB
(244, 1:600) and anti-pCREB (5322, 1:500). After antibody
incubation and extensive washes, slides were incubated with
donkey anti-rabbit Cy3 at 1:600 dilutions for 45 min at room
temperature. Slides were then washed three times in PBS
and mounted with coverslips with the use of Vectashield
mounting media containing 4',6-diamidino-2-phenylindole (DAPI).

Chromatin IP:
Mice were injected intraperitoneally with either 5 µg/kg
body weight of glucagon or 1 unit/kg body weight of insulin
for 10 minutes. Subsequently, nuclear isolation, chromatin
crosslinking and ChIP assays were performed as described
(see Scearson et al., 2004). Precipitated DNA fragments were
analyzed by quantitative polymerase chain reaction (Q-PCR)
amplification using primers directed against the mouse
promoters listed in figure legends. For analysis of mouse SIK1
promoter, SV40-transformed mouse hepatocytes were grown
to 90% confluence, and used for ChIP assays as described
(see Scearson et al., 2004). SV40-transformed mouse hepatocytes
were grown to 90% confluence, and ChIP assays were
performed. Precipitated DNA fragments were analyzed by poly-
merase chain reaction (PCR) amplification using primers
directed against the mouse SIK1 promoter or beta actin cod-
ing region as negative control.

In Vitro Kinase Assay:
Recombinant GST-TORC2 (161 -181) wild-type and
Ser171 Ala proteins were phosphorylated in vitro with 100
µM purified AMPK (AMP-activated protein kinase) (Upstate
Biotech) ±300 µM AMP according to the manufacturer’s
instructions. Following 15 min. incubation with γ-32P-ATP,
relative $^{32}$P incorporation was measured by scintillation. GST substrates and SAMS peptide were used at 3 μM in each reaction.

Example 1

A Calcineurin Sensitive Cofactor Promotes Cooperation between cAMP and Calcium Pathways

Hamster insulinoma (HT) cells were employed to test the relative effects of cAMP and calcium signals on CREB activity. Opening of voltage-sensitive L-type calcium channels in response to KCl depolarization induced the CREB-dependent EVX-1 promoter (Conkright et al., 2003b) 5-fold in HT cells, whereas addition of cAMP agonist stimulated the reporter 10-fold (see Fig. 1A). Co-stimulation with both forskolin and KCl stimulated CREB activity 90-fold, demonstrating the cooperative effects of cAMP and calcium pathways on CREB target gene expression in these cells. Pre-treatment with the calcineurin inhibitor cyclosporine A (CsA) blocked cooperativity between cAMP and calcium pathways, indicating that calcineurin performs an important role in modulating CREB activity. Consistent with the effects of calcium channel activity on the EVX-1 promoter, treatment with high glucose (20 mM) induced the expression of the endogenous CREB target gene N4A2 5-fold in MIN6 insulinoma cells; and co-stimulation with glucose plus exendin-4, an analogue of the incretin hormone GLP-1, enhanced N4A2 mRNA levels 35-fold (see Fig. 1B). In keeping with the importance of calcium entry for CREB target gene activation, treatment with the calcium channel inhibitor nifedipine blocked the effect of glucose on N4A2 mRNA accumulation. Cooperative induction of N4A2 mRNA levels by glucose and exendin-4 was also calcineurin-dependent; addition of CsA disrupted the increase in N4A2 mRNA gene expression by both stimuli (see Fig. 1B).

The importance of Ser133 phosphorylation for CREB activation in response to cAMP and calcium signals (Shaywitz and Greenberg, 1999) prompted examination of the extent to which this site mediates cooperativity between both pathways. Treatment with forskolin and KCl increased levels of CREB Ser133 phosphorylation in HT cells (see Figs. 1C and 9); co-stimulation with forskolin plus KCl had no additional effect on phosphorylation (Ser133) CREB levels relative to forskolin alone. Despite its ability to inhibit CREB activity in cells stimulated with cAMP and calcium agonists, CsA had no effect either on the stoichiometry or kinetics of CREB Ser133 phosphorylation in response to these signals (see Figs. 1C and 9). These results suggest that calcium and cAMP act synergistically on a calcineurin regulated component which is distinct from the CREB Ser133 phospho-acceptor site.

The recruitment of CBP/P300 to the promoter is thought to be a common pathway for activation of cellular genes via CREB in response to various stimuli (Goodman and Smolik, 2000). To compare the effects of calcium and cAMP pathways in promoting the CREB-CBP interaction, mammalian two-hybrid assays were performed using GAL4-KID and KIX-VP16 expression vectors. Addition of forskolin to HIT cells stimulated the KID-KIX interaction 20-fold by GAL4 luciferase reporter assay; but KCl depolarization had no effect in this regard, despite its ability to induce comparable levels of Ser133 phosphorylation (see Fig. 1D). Addition of forskolin with KCl rescued KID-KIX complex formation, albeit at similar levels to forskolin alone (see Fig. 1D). These results indicate that the cooperativity between calcium and cAMP signals on CREB target gene expression is not reflected at the level of the KID-KIX interaction.

Previous reports suggesting that the CREB bZIP domain contributes to target gene activation by CREB (Bonnin et al., 1995a) prompted testing of the importance of this domain in mediating cooperativity between cAMP and calcium signals. In transient assays of HIT cells using a GAL4-CREB polyepitope containing the GAL4 DNA binding domain fused to CREB, KC1 and forskolin induced GAL4 reporter activity 2 and 5-fold individually but 22-fold when added in combination; and these stimulatory effects were potently inhibited by CsA (see Fig. 1E). By contrast, a truncated GAL4-CREBBZIP polyepitope lacking the C-terminal bZIP domain (aa. 284-341) in CREB showed only a modest response to forskolin and no induction by KCl. Notably, the GAL4-CREBBZIP polyepitope elicited no cooperativity between cAMP and calcium agonists, revealing the importance of the bZIP domain for this effect.

Example 2

TORCs Promote CREB Activation in Response to cAMP and Calcium Signals

The involvement of the CREB bZIP domain in mediating cooperativity between cAMP and calcium signals prompted examination of the role of TORCs, a family of CREB coactivators which bind directly to the bZIP, in this process. The TORC family comprises three members (TORC1, TORC2, TORC3) each of which contains highly conserved N-terminal CREB binding (aa. 1-42 in TORC1) and C-terminal trans-activation (aa. 517-634) domains (Conkright et al., 2005a; lourgenko et al., 2003). Over-expression of TORC2 induced basal EVX-1 promoter activity 20-30 fold; and treatment with cAMP or calcium channel agonist further potentiated the reporter 25-fold and 30-fold, respectively, suggesting that TORC2 is regulated by both signals (see Fig. 2A). Consistent with the notion that TORC2 mediates CREB target gene expression via a calcineurin dependent mechanism, addition of CsA disrupted TORC2 activity in HIT cells treated with KC1 (see Fig. 2A).

To determine whether TORC2 is necessary for cAMP dependent induction of CREB target genes, knockdown experiments were performed in HEK293T cells for their high transfection efficiency relative to HIT cells. Consistent with its ability to potentiate CREB activity in response to cAMP and calcium agonists, TORC2 was observed to facilitate target gene activation; knockdown of TORC2 expression with a TORC2 RNAi construct reduced TORC2 protein levels and disrupted cAMP dependent induction of the EVX-1 promoter relative to a non-specific RNAi plasmid (Conkright et al., 2003a) (see Fig. 2B, left panel). Expression of TORC3, which is not recognized by the TORC2 RNAi construct, rescued induction of the EVX-1 promoter by cAMP agonist (see Fig. 2B, right panel).

To further evaluate the role of TORCs in CREB activation, a screen was conducted in efforts to identify TORC interaction-defective CREB polyepitopes. Based on previous data showing that the CREB-bZIP domain mediates complex formation with TORC (Conkright et al., 2003a), basic and leucine zipper regions were tested separately in pull down assays; it was determined that the leucine zipper motif alone was sufficient for binding to TORC.

Mutagenesis studies on charged residues within the leucine zipper were then carried out due to the sensitivity of the CREB-TORC complex to high salt disruption. Out of five independent mutants tested, only one, Arg314A1a, showed
substantially no TORC binding activity (see FIG. 10). Interestingly, Arg314 is conserved amongst CREB family members (CREB1, ATF1, CREM), consistent with the suggestion of a potential role for this residue in CREB dependent transcription. By contrast with its potent activity on wild-type CREB protein, TORC1 had no effect on the EVX-1 reporter in cells expressing the CREB Arg314 Ala mutant, demonstrating the importance of this amino acid for CREB activation via TORC1 (see FIG. 11).

To compare the relative contributions of CREB-CBP and CREB-TORC complexes for cellular gene induction, transient assays of HT1 cells were performed with GAL4-CREB polypeptides containing point mutations that disrupt interactions with CBP (M1: Ser133 Ala), or TORC (M2: Arg314 Ala). By contrast with the wild-type GAL4-CREB polypeptide, the CBP interaction defective M1 mutant was less responsive to cAMP (10-fold vs. 2.5 fold), but remained sensitive to cooperative effects of cAMP and KCl as well as to the inhibitory effects of CsA (see FIG. 2C). Mutation of Arg314 in GAL4-CREB (M2) not only disrupted cAMP inducibility, but also compromised cooperativity between cAMP and KCl (see FIG. 2C). Similar results were noted in other electrically excitable cells including PC12 pheochromocytoma cells (see FIG. 12), consistent with the hypothesis that TORCs function in a variety of cellular contexts.

The apparent requirement of CBP and TORC complexes for cellular gene activation via CREB prompted examination of whether these proteins are recruited to the promoter by chromatin immunoprecipitation (ChIP) assay. This analysis was conducted in HEK293T cells due to the absence of hammer genomic sequence data for CREB target genes. In ChIP assays on the endogenous CREB target gene NR4A2 (Conkright et al., 2008b), it was found that CREB occupied the promoter both under basal conditions and in response to cAMP treatment (see FIG. 2D). CBP and TORC2 were absent from the NR4A2 promoter under basal conditions; following treatment with forskolin for 30 minutes, both were recruited to the promoter but not to a control 3′ end fragment of the NR4A2 gene. These results are consistent with functional data from both TORC2 knockdown and CREB transgenic analyses (see FIGS. 2B and 2C) demonstrating the role of TORC2 as a signal-dependent CREB coactivator.

Example 3

TORCs Migrate to the Nucleus in Response to cAMP

Immunofluorescence studies were performed to determine the mechanism by which cAMP triggers recruitment of TORC2 to the promoter. The relative absence of cytoplasm in HIT cells for microscopic analysis of TORC localization prompted the use of human ATY1 fibroblasts. Flag-tagged and endogenous TORC2 proteins were largely confined to the cytoplasm of ATY1 cells under basal conditions; and treatment with cAMP agonist promoted nuclear accumulation of TORC2 within 30 minutes (see FIGS. 3A and 13). Treatment with the exportin inhibitor leptomycin B (LMB) strongly enhanced nuclear accumulation of both endogenous and transfected TORC1 and TORC2 within 30 to 60 minutes, indicating that these processes likely cycle in and out of the nucleus in the absence of cellular stimuli (see FIGS. 3B and 13).

To characterize regions in TORC that promote either cytoplasmic or nuclear accumulation, cellular fluorescence assays were performed on truncated TORC1 polypeptides fused to green fluorescent protein (GFP). Nuclear localizing activities (NLS1 and NLS2) were detected near the N-terminus of TORC1 (aa. 1-147), whereas nuclear export sequences (NES1, NES2) were located within the central Ser/Pro rich domain from aa. 148-290 (see FIG. 14). Both NLS and NES motifs were well conserved within the three TORC family members, and mutagenesis of individual leucines in NES1 and NES2 promoted nuclear accumulation of TORCs.

TORC3 contains a Phe to Tyr282 substitution in NES1, which would be predicted to disrupt export activity (see FIG. 3C, bottom). Indeed, TORC3 was localized exclusively in the nucleus of both control and cAMP stimulated cells (see FIG. 3C, top); consistent with its nuclear location, wild-type TORC3 was far more active in potentiating CREB activity under basal conditions relative to TORC2 (see FIG. 3D). Conversely, addition of an N-terminal src myristilation signal targeted TORC3 to the cytoplasm and rendered the protein transcriptionally inactive (see FIG. 3D). Likewise, mutagenesis of Tyr282 in TORC3 to Phe promoted cytoplasmic accumulation of the protein and repressed basal TORC3 activity.

cAMP treatment triggered translocation of mutant TORC3 Tyr282Phe to the nucleus, consistent with the notion that cAMP stimulates nuclear entry of TORC proteins (see FIG. 3C).

Example 4

TORC is Dephosphorylated in Response to cAMP and Calcium Signals

To determine the mechanism by which extracellular signals regulate TORC2 nuclear entry, the biochemical properties of TORC2 were compared in nuclear and cytoplasmic fractions of HEK293T cells. Flag-tagged TORC2 appeared as a 85 kD doublet in cytoplasmic fractions and as a single fast migrating species in nuclear extracts (see FIG. 4A, left panel). Indeed, treatment with calf intestinal alkaline phosphatase (CIP) transformed the cytoplasmic TORC2 doublet into a single faster migrating species, consistent with the belief that TORC2 undergoes dephosphorylation upon nuclear entry (see FIG. 4A, right panel).

To determine whether TORC2 phosphorylation is regulated in response to calcium and cAMP signals, Western blot assays were performed on endogenous TORC2 protein in HIT cells. Consistent with results using the Flag-tagged protein, endogenous TORC2 also appeared as two closely migrating bands in total extracts (see FIG. 4B). Co-stimulation with KCl plus forskolin promoted extensive TORC2 dephosphorylation; and pre-treatment with CsA partially reversed this effect, consistent with the belief that calcineurin promotes TORC2 dephosphorylation in response to these inducers (see FIG. 4B).

To evaluate the status of TORC2 phosphorylation directly, 32P-labeling experiments were performed. SDS-PAGE analysis of flag-tagged TORC2 immunoprecipitates from HIT cells revealed that in vivo 32P-labeled TORC2 was dephosphorylated within 10 minutes after treatment with forskolin or KCl (see FIG. 4C). Co-stimulation with forskolin and KCl reduced phospho-TORC2 levels further; these effects were blocked by co-treatment with CsA, consistent with the belief that calcineurin promotes TORC2 dephosphorylation in this setting (see FIG. 4C). TORC2 appears to be phosphorylated exclusively on serine by phospho-amino acid analysis; and two dimensional tryptic mapping of 32P-labeled
TORC2 reveals at least seven spots of comparable intensity, indicating that TORC2 is extensively phosphorylated at numerous sites.

Example 5
TORCs Associate with 14-3-3 Proteins

To clarify the mechanism by which TORC phosphorylation promotes its cytoplasmic retention, proteomic analyses were performed to search for TORC interacting proteins. Immunoprecipitates of TORC1 and TORC2 were prepared from stable cell lines expressing Flag-tagged versions of either protein. Both TORCs were found to interact strongly with multiple members of the 14-3-3 family of proteins (e.g., 92.5% coverage for 14-3-3E). 14-3-3 proteins have been found to bind a number of regulatory proteins, most notably CDC25A, forkhead, and NFAT family members, and to inhibit their biological function (Brunet et al., 1999; Chen et al., 2003; Chow and Davis, 2000; Durocher et al., 2000).

Co-immunoprecipitation studies were performed to confirm the proteomic results and to explore the potential role of 14-3-3 proteins in regulating TORC activity. Endogenous 14-3-3 proteins were recovered from immunoprecipitates of Flag-TORC1 and Flag-TORC2 expressing cells as well as from immunoprecipitates of endogenous TORC proteins from HEK293T and PC12 cells (see FIG. 5A). Consistent with its ability to promote TORC translocation to the nucleus, forskolin treatment disrupted both transfected Flag-TORC1 14-3-3 (see FIG. 5A, top panel) and endogenous TORC: 14-3-3 interactions (see FIG. 5A, bottom left panel). The kinetics of TORC2: 14-3-3 dissociation parallel the time course for TORC2 dephosphorylation and nuclear entry in response to AMP agonist; in co-immunoprecipitation studies of Flag-TORC2 and 14-3-3 proteins, the TORC2:14-3-3 interaction was diminished by about half within 10 minutes of forskolin treatment and was maximally reduced after 30 to 60 minutes (see FIG. 5A, bottom right panel).

The general importance of Ser/Thr phosphorylation for association with 14-3-3 family members prompted examination of whether the inhibitory effects of CsA on TORC activity correlate with changes in TORC: 14-3-3 binding. CsA treatment greatly enhanced complex formation under basal and cAMP stimulated conditions, whereas the protein phosphatase PP1/PPIA inhibitor okadasic acid (OA) had no effect on this interaction. These data are consistent with the belief that calcineurin mediates dissociation of TORC: 14-3-3 complexes in response to calcium signals by dephosphorylation of TORCs (see FIG. 5A).

Co-immunoprecipitation studies were performed on mutant TORC polyproteins to identify regions in TORC2 that mediate the 14-3-3 interaction. The central Ser/Pro rich domain in TORC2 (aa. 56-547) appeared important in this regard; relative to other mutant TORC2 polyproteins, no endogenous 14-3-3 proteins were recovered from immunoprecipitates of mutant TORC2 (5A6-547) (see FIG. 5B). Consistent with its proposed role in promoting cytoplasmic retention of TORC proteins, over-expressed 14-3-3 beta inhibited basal EVX-1 reporter activity in HEK293T cells transfected with a TORC2 expression vector. By contrast, 14-3-3 beta over-expression had no effect on the activity of the TORC2 (5A6-547) protein, demonstrating the importance of the TORC: 14-3-3 interaction for repression of CREB target genes (see FIG. 5C).

The ability of CsA to block TORC2 dephosphorylation and to enhance the 14-3-3 interaction prompted an evaluation of the role of calcineurin in this process. Remarkably, calcinin A and B subunits were identified in proteomic analyses of Flag-tagged TORC1 (10.4% peptide coverage) and TORC2 (7.9% peptide coverage) immunoprecipitates. TORC2 appears to bind to calcineurin A directly; in pull-down assays 35S-labeled TORC2 was efficiently precipitated with GST-calcineurin A (aa. 1-347) but not GST beads (see FIG. 5D, bottom panel). The 14-3-3 interaction-defective TORC2 (Δ56-547) protein, lacking the central regulatory domain, was also unable to associate with calcineurin, demonstrating the importance of this region in TORC2 for signal dependent modulation.

Example 6
TORC2 Associates with SIK2, a snf1 Related Kinase

The high levels of cytoplasmic TORC2 phosphorylation on serine under basal conditions prompted testing to determine whether TORC2 associates with a Ser/Thr kinase activity. Both endogenous and over-expressed TORC2 were readily phosphorylated by in vitro kinase assay of immunoprecipitates prepared from cytoplasmic (C) but not from nuclear (N) fractions of HEK293T cells (see FIG. 6A). TORC2 associated kinase activity was potently inhibited by treatment with forskolin (compare intensities of 85KD TORC2 bands), consistent with one of two explanations, i.e., that PKA either reduces the activity of the kinase or disrupts the TORC2: kinase complex. Two dimensional phospho-tryptic mapping studies of 35P-labeled flag-tagged TORC2 revealed a single major spot following in vitro kinase assay of TORC2 immunoprecipitates, consistent with the explanation that TORC2 associated kinase phosphorylates TORC2 at one principal site rather than at the multiple sites observed in vivo.

Proteomic analysis of Flag-TORC2 immunoprecipitates prepared from transfected HEK293T cells revealed the presence of the Salt Inducible Kinase 2 (SIK2: 6 peptides, 11.7% coverage), a member of the snf1 family of energy-sensing kinases previously found to inhibit transcription of cAMP responsive genes (Dei et al., 2002). Confirming this finding, SIK2 was readily detected in anti-flag immunoprecipitates prepared from 11 EVK293T cells co-transfected with SIK2 and Flag-tagged TORC2 expression vectors (see FIG. 6B). Moreover, TORC2 mobility was noticeably reduced in cells co-expressing SIK2, consistent with the suggestion that this kinase directly phosphorylates TORC2 (see FIG. 6B, compare lanes 3,4).

To identify sites on TORC2 that are phosphorylated by SIK2 and other cellular kinases, tandem mass spectrometric (MS/MS) analysis was performed of TORC2 phospho-peptides generated from TORC2 immunoprecipitates. Eleven phospho-peptides were recovered by this analysis; and most of these mapped to the central regulatory domain (see FIG. 6C). Remarkably, one TORC2 phospho-peptide corresponded to an optimal site for SIK2 phosphorylation (LXB (S/T)SXXXL. (SEQ ID NO:7); aa.166-LNRTSSDSL (SEQ ID NO:8) in TORC2 (see FIG. 15)). To determine whether Ser171 in TORC2 is indeed phosphorylated by SIK2 in vitro kinase assays were performed using a GST-TORC2 (aa 161-181) substrate. In 32P-labeling studies, SIK2 was found to phosphorylate wild-type but not Ser171 Ala mutant.
TORC2 in vitro, consistent with the suggestion that SIK2 phosphorylates TORC2 at a single site under basal conditions (see FIG. 6D).

Example 7
SIK2 Inhibits TORC2 Nuclear Translocation
cAMP has been reported to disrupt the inhibitory effects of SIK2 on CREB activity via the PKA mediated phosphorylation of SIK2 at Ser587 (Okamoto et al., 2004). Indeed, treatment with cAMP agonist induced phosphorylation of SIK2 at Ser587, by Western blot assay of GST-SIK2 expressing cells with phospho-Ser(587) specific antiseraum (see FIG. 7A). Consistent with its proposed role in regulating CREB activity, endogenous SIK2 was readily detected in H1T cell extracts by Western blot assay. To further explore the functional role of SIK2 in modulating CREB-dependent gene expression, transient assays were performed on HEK293T cells co-transfected with the EVX-1 reporter plasmid. Over-expression of wild-type SIK2 blocked reporter activity about 70% in cAMP stimulated cells; but kinase dead SIK2 (K49M) had no effect in this regard, demonstrating the importance of SIK2 catalytic activity for CREB inhibition (see FIG. 7B, left panel). PKA phosphorylation defective SIK2 (Ser587Ala) was far more potent in reducing CRE reporter activity, consistent with the belief that cAMP normally disrupts SIK2 activity in this context (see FIG. 7B, left panel).

To evaluate the effect of Ser171 phosphorylation by SIK2 on TORC2 activity, transient assays were performed on a Ser171Ala mutant TORC2 expression vector. Relative to the wild-type protein, TORC2 (Ser171Ala) was far more active in potentiating CREB activity under basal conditions, but displayed comparable activity to wild-type TORC2 following forskolin treatment (see FIG. 7B, right panel). These results are consistent with the suggestion that SIK2 dependent phosphorylation at Ser171 represses TORC2 under basal conditions; and that cAMP stimulates TORC2 activity by disrupting SIK2 mediated Ser171 phosphorylation.

The importance of TORC2 nuclear entry for target gene activation in response to cAMP prompted examination of the effect of SIK2 on TORC2 localization. Under basal conditions, flag-tagged TORC2 was localized to both nuclear and cytoplasmic compartments of A549 cells (see FIG. 7C). Over-expression of SIK2 efficiently blocked nuclear entry of TORC2 under basal conditions; and treatment with forskolin promoted nuclear entry of TORC2 in these cells, demonstrating the ability of PKA to overcome the inhibitory effects of SIK2 on TORC2 translocation. By contrast, TORC2 remained cytoplasmic even following cAMP treatment in cells expressing the PKA phosphorylation defective SIK2 (Ser587Ala), reinforcing the role of Ser587 for TORC2 activation. Phosphorylation of Ser171 by SIK2 appears important for cytoplasmic retention; mutant TORC2 (Ser171Ala) was targeted to the nucleus constitutively under both basal and cAMP stimulated conditions (see FIG. 7C). Taken together, these results are consistent with the proposal that the SIK2 mediated phosphorylation of TORC2 at a single site (Ser171) favors cytoplasmic retention of TORC2 and inhibition of CREB activity under basal conditions.

Example 8
TORC2, a Master Switch for Hepatic Gluconeogenesis
Under fasting conditions, pancreatic glucagon triggers the activation of catabolic programs in liver in part via the cAMP responsive factor CREB (see Herzig et al., 2001; Hall & Grauner, 1999; and Hanson & Reshef, 1997). CREB in turn stimulates gluconeogenesis and fatty acid oxidation genes by inducing expression of the nuclear hormone receptor coactivator PGC-1α (see Herzig et al., 2001 and Voon et al., 2004). Consistent with the above, mice deficient in PGC-1α display defects in hepatic gluconeogenesis and fatty acid oxidation (see Koo et al., 2004 and Lin et al., 2004).

Glucagon is thought to enhance CREB activity via the PKA mediated phosphorylation of CREB at Ser133, and this modification in turn stimulates target gene expression via the recruitment of the coactivator CBP to the promoter (see Chirvia et al., 1993 and Aries et al., 1994). Intraperitoneal (iP) administration of glucagon was found to promote CREB Ser133 phosphorylation in liver within 10 minutes by histochemical and Western blot analysis (see FIGS. 16A and 16B). Unexpectedly, iP insulin administration had comparable effects on Ser133 phosphorylation in the liver, arguing against a pivotal role for the CREB-CBP pathway in discriminating between fasting and feeding signals (see FIGS. 16A and 16B).

In addition to promoting CREB phosphorylation, cAMP has also been found to stimulate cellular gene expression via the dephosphorylation and nuclear entry of TORCs, a family of cytoplasmic coactivators that enhances cellular gene expression via an interaction with the CREB basic region/leucine zipper (bZIP) DNA binding domain (see Conkright et al., 2003a and Lourogenko et al., 2003). Thus, TORC2 activity was examined, as this family member was expressed at highest levels relative to TORC1 and TORC3 in liver as determined by quantitative PCR analysis. Hepatic TORC2 was localized primarily in the cytoplasm under ad libitum feeding conditions by immuno-histochemical analysis of liver sections (see FIG. 16C). iP glucagon administration induced translocation of TORC2 to liver nuclei within 10 minutes. Despite its ability to promote CREB Ser133 phosphorylation, insulin did not stimulate nuclear entry of TORC2, demonstrating the capacity of this coactivator to discriminate between fasting and feeding signals. Consistent with these dynamics, TORC2 was highly phosphorylated at Ser171 under ad libitum or insulin stimulated conditions, but was dephosphorylated following glucagon induction (see FIG. 16D). Moreover, in chromatin immunoprecipitation (ChIP) assays of liver tissue, glucagon, but not insulin, promoted recruitment of TORC2 to gluconeogenic genes (see FIG. 16E). Taken together, these results demonstrate that, by contrast with CREB Ser133 phosphorylation, TORC2 activity is selectively induced in response to fasting signals.

Based on its ability to translocate to the nucleus in response to glucagon, TORC2 would be expected to enhance gluconeogenic gene expression in a cAMP regulated manner. This proposal was tested by infecting primary rat hepatocytes with a TORC2 expressing adenovirus (Ad-TORC2). Ad-TORC2 had marginal effects on gluconeogenic genes (PGC-1α, PEPPCK, and glucose 6 phosphatase) under basal conditions but potentiated the entire program 10-fold following exposure to Forskolin (FSK) (see FIG. 17A). The effects of Ad-TORC2 on gluconeogenic gene expression were CREB dependent: expression of a dominant negative A-CREB polypeptide, which specifically inhibits binding of CREB but not other bZIP family members to DNA (see Ahn et al., 1998), disrupted Ad-TORC2 potentiation (see FIG. 17B).

The ability of TORC2 to modulate gluconeogenic gene expression in hepatocytes exposed to cAMP agonist is consistent with the proposal that this coactivator modulates glucose output from the liver in response to feeding signals. Expression of either Ad-TORC2 or its paralog TORC1 in
primary rat hepatocytes enhanced glucose output nearly 5-fold in cells exposed to FSK plus dexamethasone (see FIG. 17C); and glucose output from TGR2 over-expressing cells was disrupted by insulin treatment, demonstrating the ability of this coactivator to respond appropriately to both fasting and feeding signals. When expressed at levels comparable to the endogenous protein in liver, Ad-TGR2 promoted fasting hyperglycemia (see FIG. 17D). Levels of circulating insulin were elevated commensurately in Ad-TGR2 mice, indicating that the effects of this coactivator on hepatic glucose output are sufficient to trigger a counter-regulatory response.

Based on its ability to stimulate the gluconeogenic program and to promote hyperglycemia when over-expressed in mice, endogenous TGR2 is expected to regulate the response to fasting signals in liver. This expectation was tested employing a TGR2 RNAi construct that reduced expression of TGR2 proteins nearly 80% by Western blot assay (see FIG. 17E). Mice made acutely deficient in TGR2 by injection with the TGR2 RNAi adenovirus exhibited fasting hypoglycemia (60 mg/dl vs 100 mg/dl; see FIG. 17E); and mRNAs for gluconeogenic genes were reduced 3-fold on average relative to control littermates (see FIG. 17F).

Like other signaling pathways, cAMP stimulates gluconeogenic gene expression with burst-attenuation kinetics (see Sasaki et al., 1984). Following exposure of primary hepatocytes to Forskolin, PEPCK mRNA levels became maximal after 2 hours, returning to near baseline after 4 hours (see FIG. 18A). Consistent with this profile, TGR2 phosphorylation at Ser71 was strongly induced 3 hours after glucagon stimulations in primary rat hepatocytes (see FIG. 18B). Pre-treatment with protein synthesis inhibitor cycloheximide (CHX) blocked phosphorylation of TGR2 at Ser71 by glucagon at 3 hours, suggesting that fasting signals promote the synthesis of an activity which in turn feeds back to shut down the CREB:TORC pathway (see FIG. 18B). Consistent with this, CHX pre-treatment potentiated gluconeogenic gene expression in primary rat hepatocytes exposed to glucagon 2-3 fold (see FIG. 18C).

Based on the ability of CHX to disrupt TGR2 Ser71 phosphorylation, it was hypothesized that glucagon induces the expression of an inhibitory kinase during the attenuation period. In previous studies, the SIK family of AMP kinases has been found to associate with and to phosphorylate TGR2 at Ser71 (see Serleston et al., 2004), part of an optimal consensus site for AMPK (AMP-activated protein kinase) family members (LNRTSSDSAL; SEQ ID NO:9). Indeed, fasting induced SIK1 mRNA and protein levels in liver 4-fold relative to feeding conditions, whereas expression of other SIK family members (SIK2 and SIK3) was unaffected (see FIGS. 18D and 18E). Exposure of primary rat hepatocytes to FSK strongly induced SIK1 mRNA levels 20-fold, and these effects were disrupted by Ad-A-CREB (see FIG. 18F). Notably, FSK had no effect on mRNA levels for any of the 12 AMPK family members by gene profiling assay of primary mouse hepatocytes, indicating that these effects are indeed specific for SIK1.

Examination of the SIK1 gene promoter revealed two conserved cAMP responsive promoter elements in rat, mouse, and human orthologs, consistent with the proposal that SIK1 is a direct target for CREB induction. In transient assays of HepG2 hepatocytes, PKA stimulated SIK1 promoter activity about 20-fold; these effects were disrupted by co-expression of A-CREB (see FIG. 18G). Indeed, CREB was found to occupy the SIK1 promoter in ChIP assays of mouse hepatocytes, consistent with the proposed direct role for CREB in this process (see FIG. 18H). Having seen that glucagon triggers hepatic expression of SIK1 during fasting, it was next considered whether this kinase functions as part of an auto-regulatory loop in attenuating the gluconeogenic program. Using a SIK1 RNAi adenovirus that reduced SIK1 expression about 75%, it was found that glucagon stimulated TGR2 dephosphorylation to a far greater extent in SIK1 deficient mice compared to control cells (see FIG. 19A). Knockdown of SIK1 also enhanced gluconeogenic gene expression; mRNAs levels for PGC-1α were increased 70-fold in SIK1 deficient cells (see FIG. 19B). Conversely, over-expression of SIK1 induced Ser71 phosphorylation and blocked induction of the PEPCK promoter by TGR2 in hepatocytes exposed to FSK. However, SIK1 had no effect on the ability of PGC-1α to stimulate transcription from a PPARα target gene (Acyl CoA oxidase (AOX)), which is not consistent with the existence of a general inhibitory effect of this kinase on hepatocyte gene expression. The role of SIK1 on fasting glucose metabolism was further evaluated using Ad-SIK1. Relative to control littermates, mice injected with either Ad-SIK1 or Ad-SIK2 exhibited fasting hypoglycemia and reduced gluconeogenic gene expression (see FIGS. 19C and 19D). Although the ability of Ad-SIK1 to block hepatic glucose output in this setting could reflect an unanticipated induction of the insulin pathway, fasting insulin levels were actually lower in SIK1 or SIK2 expressing mice, compared to control mice; hepatic insulin signaling was comparable in primary hepatocytes infected with a SIK1 adenovirus, as revealed by Western blot assay of phospho-Ser473) Akt levels following 30 min exposure to insulin (100 nM).

To evaluate whether SIK1 inhibits the gluconeogenic program via TGR2 phosphorylation, an adenovirus expressing Ser71Ala TGR2 was prepared. Following injection into primary rat hepatocytes, adenoaviral wild-type and mutant Ser71Ala TGR2 polypeptides were expressed at comparable levels and had similar effects on PEPCK and PGC-1α gene expression (see FIGS. 19E and 19F). Ower-expression of SIK1 blocked wild-type TGR2 activity almost completely; but the mutant Ser71Ala TGR2 protein was refractory to SIK1 inhibition, demonstrating the importance of Ser71 phosphorylation for disruption of the gluconeogenic program by this kinase (see FIGS. 19E and 19F).

Activation of the AMPK (AMP-activated protein kinase) pathway in liver has been shown to block expression of gluconeogenic genes, although the relevant targets for this inhibition have remained elusive (see Lockhead et al., 2000 and Yamauchi et al., 2002). By contrast with SIK, ATP depletion appears to be uniquely sensed by AMPK, the founding member of this family (see Sakamoto et al., 2004; Lizcano et al., 2004; Bannjee et al., 2004; and Radzun et al., 2003). The presence of a consensus AMPK phosphorylation site (Ser71) that modulates TGR2 activity in liver prompted examination of whether AMPK inhibits gluconeogenesis via phosphorylation of TGR2 in response to ATP depletion. Activated AMPK phosphorylated wild-type, but not Ser71Ala mutant OGT-TGR2 (161-181) peptide in vitro (see FIG. 20A). Phosphorylation of TGR2 by AMPK was comparable to an optimal AMPK peptide substrate (SAMS (see Lizcano et al., 2004) and was induced by addition of AMP. Indeed, selective activation of cellular AMPK by exposure of primary hepatocytes to the AMP analogue 5-aminoimidazole-4-carboxamide riboside (AICAR) triggered robust phosphorylation of endogenous TGR2 even in the presence of FSK (see FIG. 20B). Based on its ability to promote Ser71 phosphorylation, AMPK might be expected to inhibit nuclear entry of TGR2 in cells exposed to FSK. Using primary rat hepatocytes
infected with adenoviruses expressing either wild-type or Ser171Ala mutant TORC2 polypeptides, it was found that FSK triggered translocation of wild-type TORC2 (see FIG. 20C). Confirming the pivotal role of Ser171 in this regard, mutant Ser171Ala TORC2 remained constitutively nuclear under both conditions. Treatment with AICAR inhibited nuclear entry of wild-type, but not Ser171Ala TORC2, in cells exposed to FSK; and adenoviral expression of SIK1 in these cells similarly blocked TORC2 nuclear entry in a Ser171 dependent manner (see FIG. 20C).

Activation of AMPK (AMP-activated protein kinase) by AICAR in primary hepatocytes to AICAR blocked induction of PEPCK and PGC-1α genes in response to FSK (see FIG. 20D). If the AMPK pathway inhibits the gluconeogenic program via TORC2, then a phosphorylation defective Ser171 Ala mutant TORC2 would be predicted to rescue the inhibitory effects of AICAR on these genes. Consistent with this prediction, mutant Ad-TORC (Ser171Ala) rescued expression of PEPCK and PGC-1α in the presence of AICAR inhibitor, demonstrating the importance of Ser171 in mediating inhibitory effects of AMPK on gluconeogenic genes (see FIG. 20D).

The results described herein are consistent with a mechanism of action whereby TORC2 functions as a master switch for modulation of gluconeogenic genes in response to nutritional and stress signals (see FIG. 20E). TORC2 activity is tightly regulated during fasting by SIK1, which forms part of an autoregulatory loop that attenuates the gluconeogenic program by phosphorylating TORC2 at Ser171 and promoting its export to the cytoplasm. TORC2 is additionally regulated by AMPK; activation of AMPK in response to an AMP analog disrupted hepatic gluconeogenic gene expression in part via Ser171 phosphorylation of TORC2. Indeed, a number of adipo-

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modification and improvements within the spirit and scope of the invention as set forth in the following claims.

All publications, patent applications, issued patents, and other documents referred to in this specification are herein incorporated by reference as if each individual publication, patent application, issued patent, or other document was specifically and individually indicated to be incorporated by reference in its entirety. Definitions that are contained in text incorporated by reference are excluded to the extent that they contradict definitions in this disclosure.

REFERENCES


Bergeron, R. et al. Effect of 5-aminosalicylate-4-carboxamido-


CAMP-dependent protein kinase-mediated activation of human cholesterol side chain cleavage cytochrome P450 promoter through the CREB basic leucine zipper domain. J Biol Chem 277, 15629-15637.


the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol Cell 6, 539-550.


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The invention claimed is:

1. A method of screening test compounds to determine whether such compounds affect the level of phosphorylation of a Transducer Of Regulated CREB (TORC), said method comprising determining the effect of test compound on the level of phosphorylation of TORC.

2. The method of claim 1 wherein said test compound enhances the level of phosphorylation of TORC.

3. The method of claim 1 wherein said test compound reduces the level of phosphorylation of TORC.

4. The method of claim 1 wherein said method is carried out in the further presence of calcineurin.