Glucose controls CREB activity in islet cells via regulated phosphorylation of TORC2

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CREB is a cAMP- and calcium-responsive transcriptional activator that is required for islet beta cell proliferation and survival. Glucose and incretin hormones elicit beta cell insulin secretion and promote synergistic CREB activity by inducing the nuclear relocalization of TORC2 (also known as Crtc2), a coactivator for CREB. In islet cells under basal conditions when CREB activity is low, TORC2 is phosphorylated and sequestered in the cytoplasm by 14-3-3 proteins. In response to feeding stimuli, TORC2 is dephosphorylated, enters the nucleus, and binds to CREB located at target gene promoters. The dephosphorylation of TORC2 at Ser-171 in response to cAMP is insufficient to account for the dynamics of TORC2 localization and CREB activity in islet cells. Here, we identify Ser-275 of TORC2 as a 14-3-3 binding site that is phosphorylated under low glucose conditions and which becomes dephosphorylated by calcineurin in response to glucose influx. Dephosphorylation of Ser-275 is essential for both glucose and cAMP-mediated activation of CREB in beta cells and islets. Using a cell-based screen of 180 human protein kinases, we identified MARK2, a member of the AMPK family of Ser/Thr kinases, as a Ser-275 kinase that blocks TORC2:CREB activity. Taken together, these data provide the mechanistic underpinning for how cAMP and glucose cooperatively promote a transcriptional program critical for islet cell survival, and identifies MARK2 as a potential target for diabetes treatment.

beta cell | kinase screening | MARK2 | cAMP | kinase

Loss of pancreatic beta cell function is the central feature of all forms of diabetes mellitus (1). Glucose is a critical stimulus for beta cell proliferation, and the capacity of the islet cell mass to expand (islet hyperplasia) in response to chronically elevated blood glucose, together with functional adaptive responses, permits secretion of sufficient insulin to meet demand in prediabetic patients (2). Failure of beta cells to proliferate and increase insulin output to meet increasing demand leads to type 2 diabetes. Although growth factor signaling pathways have been implicated in the process of beta cell expansion, the molecular details of how glucose promotes islet cell proliferation are still unclear (3, 4).

Insulin secretion is triggered by numerous metabolites, principal among which are glucose and incretin hormones (5). Beta cell glucose oxidation and consequent ATP production results in membrane depolarization, influx of extracellular calcium, and insulin release (6). Incretins promote the insulin secretory response primarily through activation of cAMP-dependent protein kinase A (PKA), promote cellular proliferation, increased beta cell mass, and resistance to apoptosis (7–14). In addition to insulin secretion, calcium and cAMP signaling events also synergistically elicit through cAMP response element binding protein (CREB) a transcriptional program that is thought to restore the beta cell to a metabolically fit state in preparation for the next round of feeding (15). CREB (and the related proteins CREM and ATF1) is the critical transcriptional activator that mediates cellular gene regulation in response to cAMP. CREB-dependent gene regulation is critical for governing islet cell proliferation and survival in vivo, because promoting CREB activity in islets via stabilization of the CREB/CBP complex increases beta cell mass and, conversely, disrupting CREB function in insulin-producing cells promotes apoptosis; glucose intolerance; and, eventually, diabetes in the mouse (16–18).

A distinct set of CREB coactivators, Transducers of Regulated CREB Activity (TORCs), are also activated by PKA; however, by a distinct mechanism whereby cAMP-PKA signaling promotes TORC relocalization from the cytoplasm to the nucleus where they bind to the DNA-binding/dimerization domain of CREB (19, 20). A growing body of evidence indicates that TORCs play a central role in signal integration to activate CREB in response to glucose and hormonal cues (21–23). TORC2 is negatively regulated by phosphorylation at Ser-171, which is a substrate for the salt-inducible kinases (SIKs) and AMP-activated protein kinase (AMPK) of the AMPK family (21, 23). Phospho-Ser-171 forms docking site for 14-3-3 proteins, phosphorylation-dependent allostere “chaperones” (24, 25) that, upon binding, mask an adjacent nuclear localization signal and thereby promote cytoplasmic accumulation of TORC2 (23). Upon receipt of extracellular cues that increase intracellular cAMP, and in some contexts calcium, TORC2 is released from 14-3-3 proteins and relocates to the nucleus (23, 25, 26). Although mutation of Ser-171 to Ala is sufficient to reduce 14-3-3-mediated cytoplasmic retention of TORC2, lowering the threshold for TORC2 nuclear entry and for TORC-dependent CREB target gene activation, dephosphorylation of Ser-171 alone in response to cAMP signals does not fully activate TORC2 in all cell types. In this study, we identify the remaining sites on TORC2 that mediate binding to 14-3-3 proteins, identify MARK2 as a TORC2 kinase, and delineate how glucose and cAMP signals converge on two of these sites to control TORC2 activity in islet cells.

Results

Identification of a Second Regulatory Phosphorylation Site on TORC2 in Islet Cells. CREB activity in islet cells is synergistically enhanced by cocomitigation with glucose and cAMP (23). In agreement with these data, we observed that treatment of glucose-starved MIN6 insulinoma cells with glucose and the incretin hormone exendin-4 (EX4, a cAMP agonist) provides 11-fold and 7-fold increases in mRNA for the CREB target genes NR4A2 and IRS2, respectively, in MIN6 insulinoma cells with glucose and the incretin hormone exendin-4 (EX4, a cAMP agonist) provides 11-fold and 7-fold increases in mRNA for the CREB target genes NR4A2 and IRS2, respectively, in MIN6 and MIN6C cells, compared with a 0-fold for EX4 alone (Fig. L4). Given that TORC2 nuclear relocalization underlies this synergy, and a phosphorylation defective Ser171Ala mutant of TORC2 isolated from HIT-T15 insulinoma cells retains 14-3-3 binding capacity (Fig. 1B and ref. 23), we sought to identify additional regulatory phosphorylation sites on TORC2 that bind to 14-3-3 proteins. To identify these site(s), we used a far-Western approach, using GST-tagged 14-3-3 in mRNA that mediate binding to 14-3-3 proteins, identifies MARK2 as a TORC2 kinase, and delineate how glucose and cAMP signaling converge on two of these sites to control TORC2 activity in islet cells.

The authors declare no conflict of interest.

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Serine 275 of TORC2 is a 14-3-3 binding site. (A) Treatment of glucose-starved (in KRB buffer for 1 h) MIN6 cells with 20 mM glucose (GLU) and 10 mM exendin-4 (EX4) elicits a synergistic increase in mRNA levels of CREB target genes NR4A2 and IRS2. (B) Far-Western analysis with truncated FLAG-TORC2 polypeptides 1–321 and 1–389 deletion constructs. Amino acid endpoints of the constructs are indicated. TORC2 polypeptides isolated from HIT-T15 cells were subjected to far-Western overlays with GST-14-3-3. 14-3-3:TORC2 complexes were detected with anti-GST antibody. 171 – Ser171Ala mutant. Treatment of the FLAG-TORC2 IPs with phosphatase is indicated (CIP). (Lower) Schematic of truncation and phosphorylation mutants used in B Upper and C. (D) 14-3-3 far-Western analysis with truncated FLAG-TORC2 polypeptides 1–321 and 1–389 containing point mutations at Ser-171 and -275 isolated from HIT-T15 cells. CON, vector control.

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**Fig. 1.** Serine 275 of TORC2 is a 14-3-3 binding site. (A) Treatment of glucose-starved (in KRB buffer for 1 h) MIN6 cells with 20 mM glucose (GLU) and 10 mM exendin-4 (EX4) elicits a synergistic increase in mRNA levels of CREB target genes NR4A2 and IRS2. (B) Far-Western analysis with FLAG-TORC2 WT (1-692) and deletion constructs. Amino acid endpoints of the constructs are indicated. TORC2 polypeptides isolated from HIT-T15 cells were subjected to far-Western overlays with GST-14-3-3. 14-3-3:TORC2 complexes were detected with anti-GST antibody. 171 – Ser171Ala mutant. Treatment of the FLAG-TORC2 IPs with phosphatase is indicated (CIP). (Lower) Schematic of truncation and phosphorylation mutants used in B Upper and C. (D) 14-3-3 far-Western analysis with truncated FLAG-TORC2 polypeptides 1–321 and 1–389 containing point mutations at Ser-171 and -275 isolated from HIT-T15 cells. CON, vector control.

**Fig. 2.** FLAG T2: CIP WT 1-321 1-389 1-396-983 1-321 1-389 IP: FLAG TORC2 BLOT: FLAG

A

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directly to 14-3-3 proteins. Whereas TORC2 WT and a C-terminal deletion (amino acid 1–389) of TORC2 both bound to 14-3-3, gross deletion of the N terminus (deletion of mutant amino acids 389–692) abrogated 14-3-3 binding (Fig. 1B, lane 4). Further truncation of TORC2 to amino acids 1–321 did not further reduce 14-3-3 binding. This residual binding required phosphorylation as treatment of the TORC2 immunoprecipitate with phosphatase (CIP) before SDS/PAGE abolished 14-3-3 binding (Fig. 1B, lanes 8 and 9). Importantly, whereas CIP treatment abolished 14-3-3:TORC2 complex formation, mutation on Ser-171 to Ala reduced but did not prevent 14-3-3 interaction with the N terminus of TORC2 (Fig. 1B, lane 7). Thus, we conclude that an additional TORC2 phosphorylation site for 14-3-3 interaction lies within amino acids 1–321.

Because Ser-171 is highly conserved from human to zebrafish in TORCs1–3 (19, 20, 23), we assumed that the additional regulatory site that mediates 14-3-3 binding would also be well conserved. TORC2 is exclusively phosphorylated on serine residues in HIT-T15 cells (23), so we selected five additional serine residues in TORC2 (Ser-70, Ser-127, Ser-238, Ser-245, and Ser-275) to evaluate as possible 14-3-3 interaction sites [supporting information (SI) Fig. S1], because they fit the following criteria: (i) reside in amino acids 1–321, (ii) conform to a consensus or near-consensus 14-3-3 binding site [mode 1 (R/KXXpSXP) and mode 2 (R/KXXX-pSXP)], in which pS is the phosphorylated serine, and X is any amino acid (24)], and (iii) highly conserved between human and mouse TORCs1 and 2. Given that TORC2:14-3-3 binding requires TORC2 to be phosphorylated, we expected to see that loss of critical phosphorylation sites would lead to a corresponding loss of 14-3-3 binding. In the far-Western screen. To test this, these five Ser residues were mutated to Ala to Mu in the 1–321 and 1–389 deletion constructs, and these new mutants were screened for 14-3-3 binding through far-Western analysis. Whereas mutation of either Ser 171 or Ser-275 significantly reduced 14-3-3 binding (Fig. 1C, lanes 3, 4, 7, and 8), mutation of these residues together abolished binding in the context of both 1–321 and 1–389 (Fig. 1C, lanes 5 and 9). Individual substitution of Ser-127, Ser-238, and Ser-245 for Ala did not appreciably reduce 14-3-3 binding (data not shown). In the context of the full-length TORC2 polypeptide, the mutation of Ser-171 and Ser-275 together were not sufficient to prevent 14-3-3 binding, indicating there was at least one more phosphorylation site on TORC2 that could serve to recruit 14-3-3 proteins (Fig. S2).

Because we identified Ser-369 in a tryptic peptide derived from TORC2 (23), we tested a triple Ser/Ala mutant of TORC2 in which Ser-171, 275, and 369 were all mutated to Ala in the far-Western assay it was unable to bind 14-3-3 proteins. We conclude that 14-3-3 proteins interact with TORC2 at Ser 171, 275, and 369.

Ser-171 and Ser-275 Control TORC Nuclear Localization and Activity in Islet Cells. If the idea is correct that 14-3-3:TORC complex formation prevents TORC nuclear entry, then a TORC2 mutant that cannot bind 14-3-3 proteins should relocalize to the nucleus of insulinoma cells in the absence of cAMP and calcium stimuli. To test whether such a correlation exists, we first determined the subcellular localization of full-length FLAG-TORC2 mutants harboring Ala mutations at Ser 171, 275, and 369 alone or in combination in unstimulated HIT-T15 cells. FLAG-TORC2 positive cells were scored as cytoplasmic (C), nuclear (N), or cytoplasmic and nuclear (C+N) (Fig. 24). Whereas TORC2 WT and all single Ser/Ala point mutants remained in the cytoplasm, we observed a pronounced nuclear relocalization of the Ser-171/275Ala mutant (~80% nuclear localization). Additional Ala substitutions at Ser-127, Ser-238, Ser-245, or Ser-369 in the FLAG-TORC2 Ser-171/275Ala background did not significantly increase its nuclear localization (data not shown). We conclude that the interaction of 14-3-3 proteins with TORC2 via Ser-171 and Ser-275 regulates TORC2 subcellular distribution and that the 14-3-3:Ser-369 complex governs an unknown function of TORC2.
to the nucleus, the same conditions that elicit a significant increase in CREB target gene levels in cells and in isolated islets (Figs. 1A and 2C). These data indicate that glucose cooperates with cAMP to induce TORC2 nuclear entry and activate CREB target genes. We predicted that a constitutively nuclear TORC2 should promote signal-independent CREB activity. To address this, we measured CREB activity induced by the TORC2 phosphorylation mutants in glucose-starved HIT-T15 cells, conditions used for insulin secretion studies. In glucose-starved (0 mM) conditions, we observed a >50-fold increase in CREB reporter activity in HIT-T15 cells when Ser 171 and Ser-275 were mutated together, compared with TORC2 WT (4-fold) or Ser 171 (16-fold) or Ser-275 alone (11-fold) (Fig. 2D). Western blot analysis indicated that all mutant proteins accumulated to equal levels as FLAG-TORC2 WT (Fig. S3). Taken together, these data indicate that glucose and cAMP promote TORC2 nuclear relocation and that constitutively nuclear TORC2 can promote CREB activity in the absence of stimuli.

Glucose Promotes Dephosphorylation of TORC2 at Ser-275 in Islet Cells. Because TORC2 nuclear entry is triggered by dephosphorylation of TORC2 and glucose is required for TORC2 nuclear relocation, we pursued the possibility that glucose treatment would promote TORC2 dephosphorylation. Western blot analysis of endogenous TORC2 in the glucose-responsive cell line MIN6 demonstrated that glucose starvation for 1 h led to rapid TORC2 phosphorylation (as measured by inhibition of mobility on SDS/PAGE) that could be reversed by 30-min stimulation with 20 mM glucose (Fig. 3A). Given the magnitude of the mobility shift that we observed here and in the FLAG-TORC2 mutants carrying a Ser275Ala mutation (Fig. 1C) and the involvement of Ser-275 in TORC2 subcellular localization, we predicted that the level of phosphoSer275 (P-Ser-275) would decrease upon glucose stimulation. To address this, we generated an antibody that recognizes P-Ser-275 on TORC2 and performed Western blot assays on protein extracts from glucose-starved MIN6 and HIT-T16 cells. The level of phosphorylation at Ser-275 decreased dramatically with 30-min treatment of MIN6 cells (Fig. 3B) and HIT-T15 cells (Fig. 3C) with 20 mM glucose, with a 50% reduction in P-Ser-275 levels observed after 5–10 min of treatment of HIT-T15 cells. In contrast, P-Ser-275 levels were unaffected by treatment with cAMP agonist, which specifically reduced the level of P-Ser-171. These data confirm that glucose alone can regulate P-Ser-275 levels. Furthermore, addition of CsA to HIT cultures before glucose stimulation completely blocked Ser-275 dephosphorylation (compare lanes 5 and 6 in Fig. 3D) but did not block dephosphorylation of Ser-171 (compare lanes 3 and 4 in Fig. 3D). Interestingly, we observed that the presence of glucose in the culture medium had a priming effect on cAMP-mediated dephosphorylation of Ser-275. In the absence of glucose, the effects of cAMP agonists (EX-4 or FSK) on global TORC2 phosphorylation (Fig. S5, compare lanes 6 and 10 and lanes 7 and 11) and on reducing P-Ser-275 levels specifically (Figs. 3 B–D) were negligible, yet these were both enhanced in the presence of glucose. To confirm these effects in primary islet cells, we treated purified glucose-starved islets with 20 mM glucose with or without 10 nM EX-4 and observed that TORC2 Ser-275 phosphorylation decreases after 1 h of glucose treatment, an effect that is enhanced by EX-4 (Fig. 3F). Taken together, these data indicate that in beta cells, in contrast to the phosphorylation status of Ser-171 (which responds primarily to cAMP signals (21, 23), that of Ser-275 is regulated by glucose and is mediated by calcineurin. Moreover, they demonstrate that dephosphorylation of Ser-275 is a critical event required for cAMP-mediated TORC2 activation in islet cells.
To identify a specific Ser-275 kinase, we provided a peptide sequence (268-PPMTGG51PDNLN-283) as substrate for the kinase assay. The results from the screen are presented in Fig. S6. Of the 180 library kinases, only MAP/microtubule affinity-regulating kinases (MARKs) 2 and 3 [also known as Par1b/EMK1 and Par1a/C-TAK1, respectively (28)], members of the AMPK family, phosphorylated the Ser-275-containing peptide. To confirm these results, we performed a secondary screen, using GST-MARKs 2 and 3, using the TORC2 268–283 peptide (Fig. 4B), and confirmed that Ser-275 is a MARK substrate. AMPK activity increases in cells under conditions of nutrient deprivation, including in beta cells starved for glucose (29). Given that MARKs and AMPK belong to the same protein kinase family, and that ambient glucose concentrations control P-Ser-275 levels on TORC2, we next tested the possibility that AMPK represents the TORC2 Ser-275 kinase. Consistent with a role for AMPK in phosphorylating TORC2 in beta cells, we detected an increase in AMPK P-Thr-172 levels in glucose-starved HIT-T15 cells (Fig. S7). However, expression of a constitutively active AMPK polypeptide (AMPK-CA) was unable to block TORC2-induced CREB activity in a CREB reporter assay in HIT-T15 cells (Fig. S8). Moreover, treatment of MIN6 cells with the AMPK agonist AICAR had no effect on P-Ser-275 levels, despite the observed increase in P-ACC levels, a known AMPK substrate (Fig. S9). However, MARK2 but not MARK3 blocked TORC2-induced CREB activity, supporting a role for MARK2 as a specific TORC2 inhibitor in cells (Fig. 4C). The constitutively nuclear TORC2 Ser-171/275Ala mutant was resistant to the inhibitory effects of MARK2. Taken together, our data indicate that MARK2 is a TORC2 Ser-275 kinase and that inhibition of TORC2:CREB activity by MARK2 involves phosphorylation of both Ser-171 and Ser-275.

Discussion

Induction of glucose-dependent insulin secretion by cAMP and calcium signals is the defining signaling event in islet beta cells. We show here that these signals also control CREB activity in islets by modulating the phosphorylation status of two residues of TORC2: Ser-171 (cAMP) and Ser-275 (glucose). In particular, we show that Ser-275 of TORC2 is a critical sensor of glucose/energy status in islet cells. We discuss here the roles of these key signaling pathways in the regulation of TORC2 phosphorylation and function of beta cells.

Two Signaling Pathways: Two Phosphorylation Sites. TORC2 nuclear entry is an essential event in signal-dependent activation of CREB target genes. Our data provide the mechanistic basis for the cooperativity of cAMP and glucose/calcium signaling for TORC nuclear relocation that is required for CREB activity in beta cells. Ser-171 of TORC2 is a critical site for negative regulation of TORC2 in beta cells and hepatocytes. In response to cAMP, PKA directly phosphorylates SIK2, leading to its dissociation from TORC2 and the dephosphorylation of TORC2 at Ser-171. In the absence of cAMP, glucose/calcium signals play a minor role in Ser-171 dephosphorylation (23). By contrast, glucose governs the phosphorylation status of Ser-275, which can be fine-tuned by cAMP signals. Ser-275 (unlike Ser-171) appears only to respond to cAMP in the presence of glucose, indicating that both the signals and protein complexes that regulate these two sites are different.

We developed the human protein kinase screening platform to permit unbiased identification of protein kinase:substrate pairs and approach allows for posttranslation modifications of the kinases required for their activity and provides an opportunity to study human protein kinase:substrate relationships, using extracellular triggers (growth factors, hormones, drugs, etc.) in their proper context. The screen permits simultaneous interrogation of kinase activities for their ability to phosphorylate a recombinant substrate harboring the phosphorylation site in question and is outlined in Fig. 4A.

TORC2 Ser-275 Is an AMPK Family Substrate. In an effort to identify the cellular machinery that governs Ser-275 phosphorylation, we sought to identify protein kinases that phosphorylate this residue. To this end, we generated a cDNA expression library of 180 GST-tagged human protein kinases (for a complete list, see Dataset 1) to permit expression and isolation of the kinases from mammalian cells for their use in parallel arrayed in vitro kinase assays.
Ser-369 does not conform to a consensus 14-3-3 binding site, and was mutated to Ala alone or in combination with Ser-171/275Ala. No functional consequence for TORC:CREB activity when Ser-369 TORC:14-3-3 complexes within the nucleus, perhaps in stabilizing regulatory TORC2 phosphorylation sites. This indicates that there is perhaps a function for nuclear TORC2 (Ser-171/275Ala) is still able to bind to 14-3-3atory TORC2 phosphorylation sites. It is curious that constitutively cells.

We have shown that the phosphorylation status of Ser-275 on TORC2 is modulated by extracellular glucose in islet cells. This indicates that the CREB pathway receives input from glucose via TORC2, providing a molecular link between glucose and a transcriptional program linked to beta cell proliferation and survival. The rapid kinetics of reduction in P-Ser-275 levels in response to glucose are consistent with known responses to cellular energy status, such as the increase in AMPK phosphorylation at Thr-172 (33). The sequence context surrounding Ser-171 and Ser-275 in TORC2 and alignment with consensus SIK1 (23) and AMPK (37) phosphorylation sites is shown at bottom. (C) CREB reporter assay in HIT-T15 cells showing inhibitory effect MARK2 on TORC2:CREB activity. Transfection with vector control (CON), Ser171Ala (171), Ser275Ala (275), and the Ser-171/275Ala mutant (171/275) is indicated. Assay was performed in the presence of glucose to maximize the potential inhibitory effects of the kinases.

facilitate the delineation of signal transduction pathways. Using this approach, we identified MARK2 as a Ser-275 kinase. Given the key role of AMPK in responding to increased energy demand, we were surprised to find that neither Ser-171 nor Ser-275 is regulated by AMPK in beta cells, as evidenced by the relative inefficiency with which Ser-275 is phosphorylated in vitro by AMPK, the absence of modulation of P-Ser-275 levels with AICAR treatment, and lack of inhibition of TORC2:CREB activity in the presence of activated AMPK. Our data indicate that TORC2 represents the sole target of MARK2 required for CREB inhibition and, moreover, that MARK2 inhibits TORC2 by phosphorylating both regulatory sites. MARK2 knockout animals display a metabolic phenotype (30) and learning and memory defects (31), areas in which CREB plays a central role (32). Future work should determine the relative importance of MARK2 in regulating TORC2:CREB activity in beta cells.

We have used a biochemical screen to identify additional regulatory TORC2 phosphorylation sites. It is curious that constitutively nuclear TORC2 (Ser-171/275Ala) is still able to bind to 14-3-3 proteins. This indicates that there is perhaps a function for TORC1:14-3-3 complexes within the nucleus, perhaps in stabilizing the transcriptionally active form of TORC. However, we observed no functional consequence for TORC:CREB activity when Ser-369 was mutated to Ala alone or in combination with Ser-171/275Ala. Ser-369 does not conform to a consensus 14-3-3 binding site, and the sequence context in which it is embedded bears no discernable homology with a site in TORC1 or TORC3.

TORC2 Activity Is Regulated by Glucose. We have shown that the phosphorylation status of Ser-275 on TORC2 is modulated by extracellular glucose in islet cells. This indicates that the CREB pathway receives input from glucose via TORC2, providing a molecular link between glucose and a transcriptional program linked to beta cell proliferation and survival. The rapid kinetics of reduction in P-Ser-275 levels in response to glucose are consistent with known responses to cellular energy status, such as the increase in AMPK phosphorylation at Thr-172 (33). The sequence context surrounding both Ser-171 and Ser-275 two sites is identical in all three mammalian TORCs, zebrafish, and fruit fly TORCs (19), indicating that both residues constitute critical regulatory sites. It will be of interest to examine the involvement of Ser-275 in the control of TORC2:CREB activity in other tissues. We attribute the accumulation of phosphate at Ser-275 in the presence of the immunosuppressant cyclosporin A to this site undergoing rounds of reversible phosphorylation catalyzed by calcineurin, even at steady state. CsA-induced hyperphosphorylation of Ser-275 was only observed in cells cultured in high glucose, supporting a role for glucose signaling in phosphate turnover at this site. Chronic treatment with calcineurin inhibitor contributes to new onset diabetes in patients receiving immunosuppression therapy (34, 35). Because CsA treatment prevented Ser-275 dephosphorylation, TORC2
nuclear entry, and CREB activation (effects that were obviated by constitutively active TORC2), we expect that inhibitors of TORC2 Ser-275 kinases, such as MARK2, may be used as adjuvant therapies for these patients.

Materials and Methods

Chemicals. Forskolin, exendin-4 and anti-FLAG agarose were from Sigma. Cyclosporin A was from Calbiochem.

Constricts. Recombinant TORC constructs were prepared by using standard PCR cloning techniques. Point mutations were generated with PhTuRbo and the QuikChange protocol (Stratagene). The presence of desired mutations was verified by sequencing.

Cell Culture. HEK293T were cultured in DME plus 10% FCS (HyClone). HIT-T15 cells were cultured in 1:1 DME plus 10% FCS:OPTI-MEM (Invitrogen). MIN6 cells were cultured in HEK293T medium supplemented with 50 mM beta-ME. For glucose starvation experiments, cells were incubated with Krebs-Ringer buffer plus 0.1% BSA lacking glucose (KRB) for 30 min, rinsed again with KRB, and incubated for a further 30 min (for a total of 1 h) before stimulation with 20 mM glucose and/or 10 nM E4A (final concentration). Identical results were obtained with cells cultured with 2.5 mM glucose before stimulation.

Antibodies and Immunoblotting. TORC2 antibodies were generated by the Covance custom antibody service. Anti-FLAG-M2 was from Sigma. Antibodies were used at 1–2 μg/ml for Western blot analysis and immunofluorescence as described in ref. 23. 14-3-3 far-Western assays were performed as described in ref. 23.

Recombinant TOR constructs were prepared by using standard PCR protocols. Identical results were obtained with cells cultured with 2.5 mM glucose before stimulation.

Kinase Library Screen. Full-length human protein kinase cDNA clones derived from the MGC/ORFeome (Open Biosystems, Invitrogen) were Gateway-recombined with pDEST27 vector (Invitrogen) to generate in-frame GST-kinase ORFs. Integrity of the GST proteins was verified by Western blot analysis. Plasmids encoding GST-kinases were transfected into 96-well plates and incubated for 4–6 h at 37°C before determination of luciferase and Reporter Assays.

14-3-3 far-Western assays were performed as described in ref. 23.

TORC2 antibodies were generated by the Covance custom antibody service. Anti-FLAG-M2 was from Sigma. Antibodies were used at 1–2 μg/ml for Western blot analysis and immunofluorescence as described in ref. 23.

Reporter Assays. HEK293T and HIT-T15 cells were transfected overnight in 24- or 48-well plates, using Lipofectamine 2000 (Invitrogen) and 150 ng (24-well plates) or 250 ng (48-well plates) of total plasmid per well. Twenty-four (HEK293T) and 40 (HIT-T15 and MIN6) hours after transfection, cells were treated with glucose and/or E4A for 4–6 h at 37°C prior to determination of luciferase and β-galactosidase activity as described in ref. 23.

Immunofluorescence Microscopy. For endogenous TORC2 detection, HIT-T15 cells were seeded onto poly(lysine)-coated coverslips. For FLAG-TORC2 detection, this was done 16 h after transfection. Cells were stimulated for 30 min with 10 μM forskolin and 45 mM KCl before fixation with 4% PFA. In some cases, cells were starved with KRB as before stimulation with glucose or CAMP agonist. Images were captured on a Nikon Axiovert inverted microscope with a ×63 oil-immersion lens with a CCD camera. For quantitation of subcellular localization, 200–300 cells per condition were scored for the presence of FLAG-TORC2 WT or phosphorylation mutant in the cytoplasm, nucleus, or in both compartments. TORC2 antibody for immunofluorescence is described in ref. 36.

Iset isolation. Animal work was approved by the University of Ottawa Animal Care Committee. Pancreata from anesthetized 6- to 12-week-old male C57/B16 mice were isolated by perfusion of the pancreatic bile duct with 0.7 ml/g (Sigma) in Hanks's buffered salt solution. Islets were purified from acinar tissue by two rounds of manual selection, using a dissecting microscope and cultured in RPMI medium containing 11 mM glucose, 10% FCS, 10 mM Hepes (pH 7.4), and 100 units/ml penicillin and 100 μg/ml streptomycin (growth medium) on bacterial dishes for 24 h before treatment.

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**Fig. S1.** Alignments of amino acid sequences from the three mammalian TORC proteins (T1, T2, T3) that flank candidate 14-3-3 binding sites. Consensus binding sites (Mode I, Mode II) for 14-3-3 cargo proteins are listed at the top. Plus signs indicate that the site mediates binding to 14-3-3s (i.e. a loss of binding for the Ser/Ala mutant at the indicated site was observed by far-Western analysis).
Fig. S2. 14-3-3 far-Western analysis with full-length FLAG-TORC2 harboring Ser/Ala substitutions at the indicated Ser residues.
Fig. S3. Western blot with anti-FLAG antibody showing protein levels of FLAG-TORC2 wt and phosphorylation site mutants. Vector only control (CON) and position of Ser/Ala mutants are indicated by numbers.
Fig. S4. CREB reporter assay in HIT-T15 cells showing activity of TORC2 mutants at steady state cultured in the presence of 20 mM glucose. Plasmid amount per well is indicated.
Fig. S5. Western blot with TORC2 antibody of extracts from HIT-T15 cell cultured in normal medium (NM) or glucose starved (–GLUCOSE) medium. Treatments are indicated.
Fig. S6. Autoradiograms of human protein library screen with Ser-275 peptide (amino acid 268–283 of mouse TORC2) and myelin basic protein (MBP) shown. Each lane represents the reaction products from a distinct kinase assay. MARK3 (Gel 1) and MARK2 (Gel 16), the only kinases to phosphorylate TORC2 268–283 peptide, are indicated.
Fig. S7. Western blot assay showing reduction of TORC2 P275 levels in glucose-treated HIT-T15 cells. Corresponding reduction in AMPK P-Thr-172 levels is also shown.
Fig. S8. CREB reporter assay in HIT-T15 cells showing lack of inhibition of TORC2:CREB activity by constitutively active AMPK (AMPK-CA). Transfection with vector control (CON), Ser171Ala (171), Ser275Ala (275), and the Ser-171/275Ala mutant (171/275) are indicated. Assay was performed in the presence of glucose to maximize the potential inhibitory effects of the kinases.
Fig. S9. Western blot assay showing effect of AMPK agonist AICAR on modulation of P-Ser-275 levels in HIT-T15 cells cultured in 20 mM glucose. Western blots depicting induction of phospho-ACC with increasing AICAR concentration and total ACC are shown.

Other Supporting Information Files

Dataset S1 (XLS)