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Torc-Dependent Creb Activity is Regulated by Glucose in Beta Cells

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TORC-DEPENDENT CREB ACTIVITY IS REGULATED BY GLUCOSE IN BETA
CELLS

By

Deidre Jansson

A thesis submitted to the Faculty of Graduate and Post-doctoral Studies, University of
Ottawa, in partial fulfillment of the requirements for the degree of

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ABSTRACT

Our bodies go through cycles of feeding and fasting, and in order to control blood glucose levels, will respond by releasing hormones such as glucagon and insulin. After feeding, insulin is released from the pancreatic beta cells to reduce blood glucose levels. When our bodies are unable to do this the result is hyperglycemia, leading to diabetes, of which there are two types. Type 1 diabetes is an autoimmune disease where the body's T cells attack and destroy the insulin producing beta cells, rendering these patients insulin-dependent. Type 2 diabetes however, results from chronic high blood glucose levels. This will lead to insulin resistance and eventually beta cell apoptosis.

Recently it was observed that the transcription factor cAMP Response Element Binding (CREB) protein is required for beta cell survival. Expression of a dominant-negative CREB under the control of the beta cell specific rat insulin promoter leads to progressive reduction of beta cell mass and diabetes in mice. Interestingly, the same signals that lead to insulin secretion, cAMP and Ca^{2+} , are required for activation of CREB.

Transducers of Regulated CREB activity (TORCs) were recently identified as CREB coactivators that are responsible for the synergistic activation of CREB target genes by cAMP and Ca^{2+} . Under resting conditions phosphorylated TORCs are sequestered in the cytoplasm by 14-3-3 proteins. After stimulation with cAMP and Ca^{2+} TORCs become dephosphorylated and enter the nucleus, where they bind to the bZIP domain of CREB to activate transcription. In the absence of these stimuli, TORC2 is phosphorylated by the salt inducible kinase 2 (SIK2) at S171. Treatment of 293 cells with cAMP leads to the phosphorylation of SIK2 and therefore the dissociation of this

kinase from TORC2. Additionally, the calcium responsive phosphatase calcineurin (Cn) in response to calcium stimuli dephosphorylates TORC2 leading to dissociation of 14-3-3 proteins and hence TORC2 nuclear entry.

Interestingly while 293 cells require no more than a cAMP stimulus for TORC2 nuclear entry, beta cells remain in the cytoplasm following the same treatment. Additionally, only after treatment with cAMP and calcium does TORC2 lose 14-3-3 binding and enter the nucleus of beta cells to activate CREB. My hypothesis was that there remained additional regulatory phosphorylation site(s) that governed TORC2:14-3-3 binding and therefore nuclear entry of TORC2 in beta cells.

Work in this thesis reveals that S275 is the remaining phosphorylation site that governs 14-3-3 binding and is regulated by glucose/ Ca^{2+} stimuli in beta cells. This evidence provides an explanation for the synergistic activation of TORC2 and therefore CREB in response to cAMP and Ca^{2+} . Additionally the potential kinase responsible for phosphorylation of this site was identified to be MARK2, a member of the AMPK family of kinases.

I have identified an additional phosphorylation site in TORC2, S275 that is involved in 14-3-3 binding. This serine, in addition to S171 must be dephosphorylated to induce TORC2 nuclear entry and subsequent CREB activation. I was also able to show that the phosphorylation of this site is responsive to glucose in beta cells and in primary mouse islets.

The goal of this thesis was to identify these remaining sites in order to uncover the regulatory mechanisms of TORC2-dependent CREB activation in response to cAMP and Ca^{2+} in the insulin producing beta cells.

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DEDICATION

I would like to dedicate this work to my wonderful parents, whose love and support made me believe I could do anything I ever wanted.

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LIST OF ABBREVIATIONS

A-CREB	Dominant Negative CREB
Akt	Protein Kinase B
AMP	Adenosine MonoPhosphate
AMPK	Adenosine MonoPhosphate-activated Protein Kinase
ATF-1	Activating Transcription Factor-1
ATP	Adenosine TriPhosphate
BAD	Cell Death Mediator
BDNF	Brain Derived Neurotrophic Factor
bZIP	Basic Leucine Zipper Domain
cAMP	cyclic-Adenosine MonoPhosphate
CREB	cAMP Response Element Binding protein
CREM	cAMP Response Element Modulator
CsA	Cyclosporin A
DNA	DeoxyriboNucleic Acid
EGF	Epidermal Growth Factor
Ex-4	Exendin-4
FOXO	Forkhead Box Transcription Factors “O”-regulated by PI3K/Akt pathway
FCS	Fetal Calf Serum
FSK	Forskolin
G6Pase	Glucose-6-Phosphatase
GIP	Glucose-dependent Insulinotropic Peptide
GLP1	Glucagon-Like Peptide-1
GLUT-2	Glucose Transporter Type 2
GLUT-4	Glucose Transporter Type 4
GST	Glutathione S Transferase
HIT-T15	Hamster Insulinoma Cell Line
IP3	Inositol-1,4,5-triPhosphate
IRS	Insulin Receptor Substrate
KID	Kinase Inducible Domain
LIRKO	Liver specific Insulin Receptor KnockOut
MAPKAP-K2	Mitogen Activated Protein Kinase-Activated Protein Kinase 2
MARK	MAP/microtubule Affinity Regulating Kinase
MIN6	Mouse Insulinoma Cell Line 6
MSK1	Mitogen and Stress-activated protein Kinase-1
NES	Nuclear Export Sequence
NLS	Nuclear Localization Sequence
NFAT	Nuclear Factor of Activated T-cells
NMDA	N-Methyl-D-Aspartic acid
PDX-1	Pancreas Duodenum Homeobox-1
PEPCK	PhosphoenolPyruvate CarboxyKinase
PGC-1 α	Peroxisome proliferator-activated Receptor –gamma Coactivator-1 alpha
PI3K	PhosphotidylInositol 3-Kinase

PKA	Protein Kinase A
PKB	Protein Kinase B
Q1, Q2	Glutamine-rich Domains
RIP	Rat Insulin Promotor
RSK	Ribosomal S6Kinase
SIK	Salt-Inducible protein Kinase
TAF _{II} 130	TATA-box binding protein Associated Factor
TFIID	Transcription Factor II D
TGFβ	Transforming Growth Factor Beta
TORC	Transducer of Regulated CREB activity

LITERATURE REVIEW

Glucose Metabolism

Glucose is a source of energy that the body requires to carry out everyday activities. Human bodies respond to cycles of feeding and fasting cues by releasing hormones to maintain blood glucose homeostasis at a concentration ranging from 4-6mM. Hormones such as insulin and glucagon are released from the islets of Langerhans to mediate this homeostasis (1). These hormones respond to feeding cues from the beta cell and fasting cues from the alpha cell respectively (2). After feeding, glucose enters the beta cell through the glucose receptor GLUT-2 (3) and is oxidized, leading to membrane depolarization through the closure of ATP-dependent K⁺ channels (4). This membrane depolarization induces opening of the voltage sensitive L-type Ca²⁺ channels and subsequent calcium influx from extracellular as well as intracellular stores (1). The extracellular influx of calcium is required for insulin secretion as blockage of voltage gated channels with hydroxydipyrindines blocks insulin secretion (5). Intracellular calcium is stored in the endoplasmic reticulum and released upon binding of inositol 1,4,5-triphosphate (IP3) to its receptor (6). Interestingly, insulin secretion itself triggers transcription of the insulin gene for the second peak of insulin secretion (7).

An additional response to feeding involves incretin hormones glucagon-like peptide-1 (GLP1) and glucose-dependent insulinotropic peptide (GIP) being secreted from the small intestine. Through binding receptors on the beta cell membrane these two proteins are able to activate adenylate cyclase leading to the increase in levels of cAMP

(8). The combination of these two signals, Ca^{2+} and cAMP, results in insulin being secreted from the beta cells of the pancreas, (Figure 1-1).

Insulin binds to the insulin receptor located in the cell membranes of adipose and muscle tissues. Binding of the substrate induces one β -subunit tyrosine kinase domain of the receptor to trans-autophosphorylate the flanking subunit activating the kinase (9). The commonly known substrates of this receptor are the insulin receptor substrate (IRS) family members (IRS1 and IRS2), that upon phosphorylation, will in turn activate phosphatidylinositol 3-kinase (PI3K). The product of PI3K, phosphatidylinositol-3,4,5-triphosphate will recruit and activate protein kinase B (PKB) and Akt protein kinases. The result of this signaling pathway being activated is translocation of the glucose transporter-4 (GLUT-4) to the cell membrane to promote glucose uptake (10), (Figure 1-1).

Under fasting conditions the liver releases glucose into the bloodstream from glycogen stores in response to glucagon signals from the alpha cells in the pancreas. This glucose can also come from non-carbohydrate stores such as alanine, and glycerol through the process of gluconeogenesis. This process is regulated by the enzymes glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (11). The expression of these genes in the liver is regulated by peroxisome proliferator-activated receptor- γ , coactivator-1 α (PGC-1 α) (12), which is in turn regulated by the cAMP response element binding protein (CREB) (13). Glucagon stimulates gluconeogenesis in times of fasting through cAMP signaling and CREB (13). Insulin, on the other hand has been shown to down-regulate PGC-1 α as the liver-specific insulin-receptor knockout mouse (LIRKO) expresses increased levels of this protein.

Figure 1-1 Schematic of signaling required for insulin secretion.

Glucose oxidation results in increased ATP concentration in the beta cell. Elevations in the ATP:ADP ratio in the cell closes the K^+ channels leading to depolarization of the cell membrane. Depolarization then leads to influx of extracellular Ca^{2+} and stimulation of insulin secretion. In addition, a rise in cAMP concentration triggered by the binding of GLP1 to its receptor on the beta cell membrane, leads to PKA activation and subsequent insulin secretion.

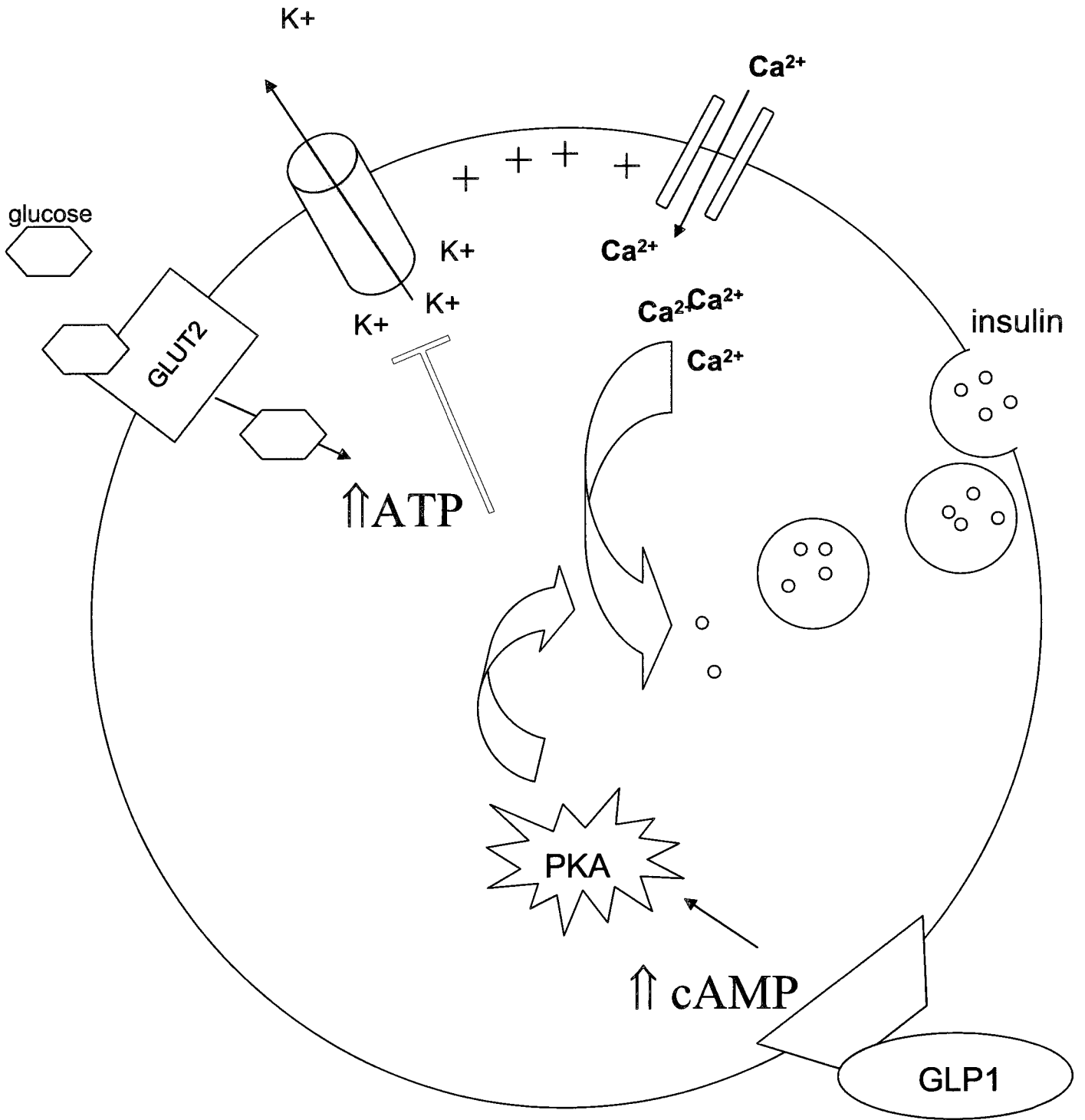
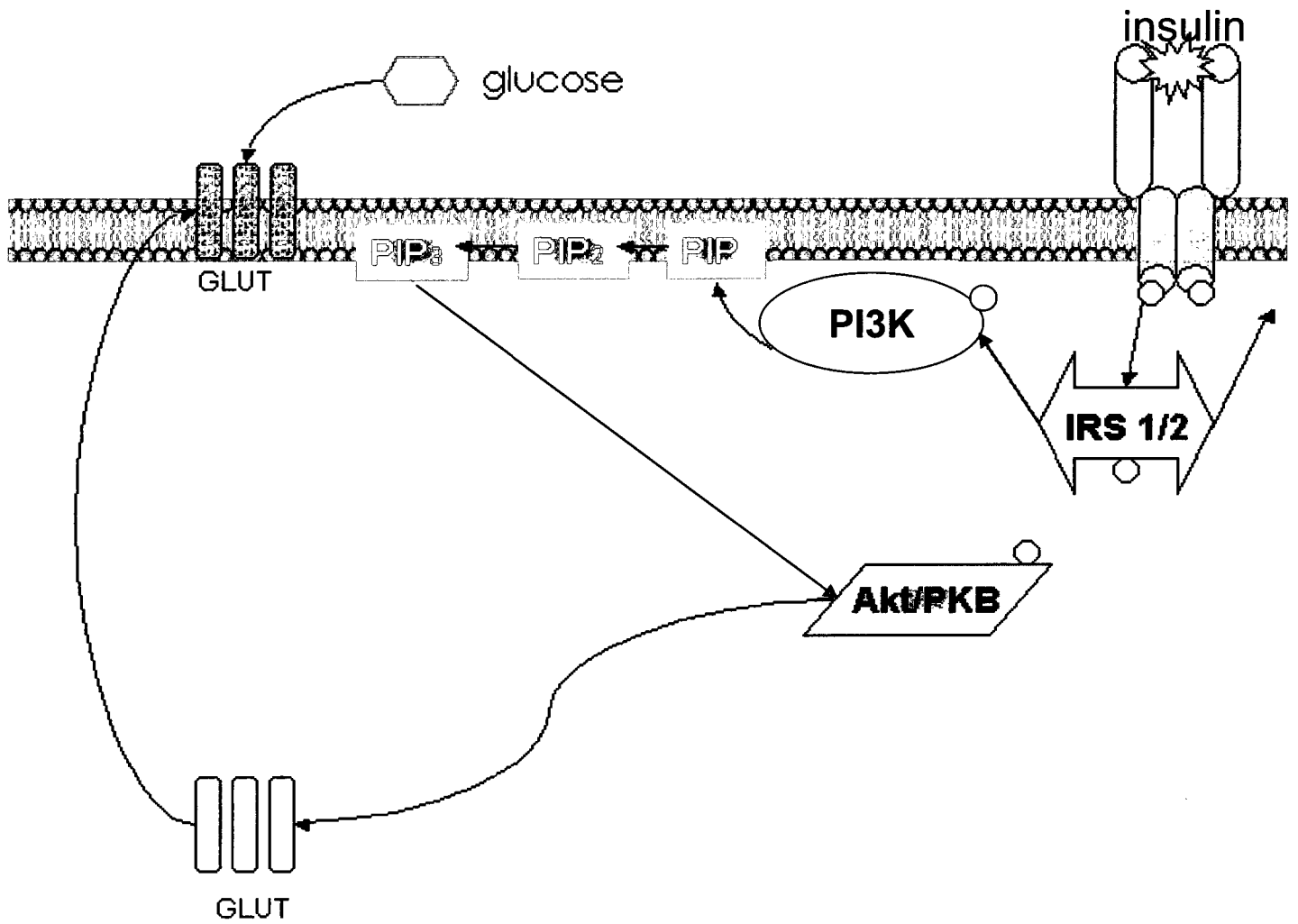


Figure 1-2 Model of insulin action.

Insulin binds to the insulin receptor in the cell membrane and activates PI3K. The product of PI3K, phosphatidylinositol-3,4,5-triphosphate activates Akt to initiate the translocation of the glucose transporter and glucose entry into the cell.



In addition this mouse shows increased blood glucose levels due to the inability of insulin to shut down glucose production (14). Insulin has also been implicated in down-regulation of gluconeogenesis through the Forkhead protein FoxO1, which also regulates PGC-1 α (15). In response to insulin in the liver, activated Akt inhibits FoxO1 by phosphorylation leading to decreased PGC-1 α (15, 16).

Diabetes

The inability to maintain glucose homeostasis results in diabetes. Type 1 diabetes is the result of an autoimmune dysfunction where the host's immune cells attack and destroy the beta cells and accounts for 10% of all diabetes cases (17). For a long time the only treatment available to these patients has been an injection the hormone insulin itself. However, there has been much research into various treatments such as gene therapy to target the immune system (18), islet transplantation (19) and β -cell regeneration (20).

Often following a transplant immunosuppressant drugs are used to inhibit the host's immune system attacking the new tissues. Two drugs that are commonly used are Cyclosporin A (CsA) and Tacrolimus, however, treatment can result in diabetes (21). The mechanism of action of these drugs is the inhibition of the serine/threonine phosphatase calcineurin (Cn), essentially blocking early T-cell activation by blocking transcription factors NFAT and CREB (22, 23). In fact, studies show that treatment of islets with CsA results in decreased insulin gene transcription (24) and apoptosis (25, 26). Additionally CsA is able to block cAMP and Ca²⁺ induced CREB-dependent insulin gene transcription (27).

Type 2 diabetes develops from overnutrition and chronic high glucose levels. This phenotype often leads to insulin resistance and later insulin deficiency caused by beta cell loss through apoptosis (28). Interestingly, beta-cell mass can increase during insulin resistance (29), mediated by the response of IRS2 to increased glucose levels (30, 31). However, the principle difference between insulin resistance and insulin deficiency is the rate of apoptosis of the beta cell. Particularly, beta cell loss exceeding 60% results in insulin deficiency and diabetes (32). At this time, the mechanism by which hyperglycemia causes beta cell death is not clear, but several possibilities have surfaced. Glucotoxicity is calcium concentration-dependent, and results in increased gene expression of hexokinase-1, glucose-6-phosphatase, and the CREB antagonist CREM, while decreasing the expression of insulin, GLUT-2, glucokinase and calcium channel genes (33). Interestingly chronic high glucose abolishes phosphorylated CREB and even causes a decrease in CREB protein in the cell (34).

CREB

Structure and Function

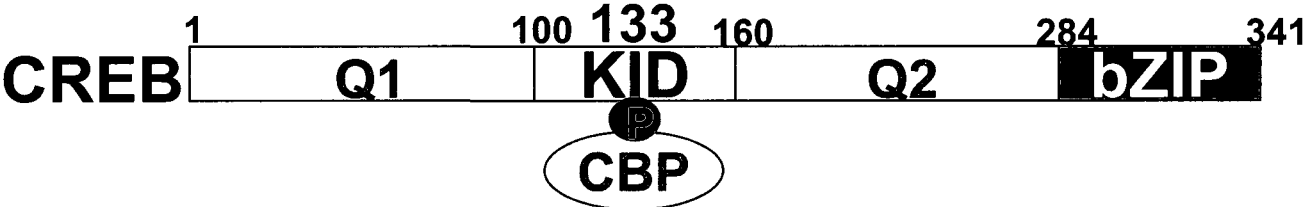
The cAMP response element (CRE) binding protein is a member of a transcription factor family that include CRE modulator (CREM) and the activating transcription factor-1 (ATF-1) that bind to cAMP response element containing promoters to activate transcription (35). The structure of CREB includes a kinase inducible domain (KID) (36), flanked by two glutamine rich domains (Q1 and Q2), and a bZIP domain located at the carboxy terminus (37) that is required for transcriptional activity (38) (see Figure 1-3). CREB is phosphorylated in the KID at Serine 133 by

protein kinase A in response to increased cAMP in the cell (39). In basal conditions PKA resides in the cell as a heterotetramer consisting of two regulatory (R) and two catalytic (C) subunits (40). Upon cyclic AMP binding to the regulatory subunits the catalytic units are released from inhibition and the protein becomes active (40). CREB will bind to DNA as a dimer through its bZIP domains to activate transcription (41).

Phosphorylation by PKA at S133 promotes recruitment of the histone acetyl transferase CREB binding protein (CBP/p300) (42) and the glutamine-rich Q2 domain enhances the interaction of CREB with TAF_{II}130/135 (43). As its name indicates CREB transcriptional activation is initiated in response to cAMP and will bind to cAMP responsive elements (TGACGTCA (44, 45)). Interestingly, a study published in 2005 had shown that CREB was found in DNA complexes of about 3000 genes (46), that's about 20% of the protein population. It is therefore not surprising that CREB has also been implicated in the regulation of many of those genes such as those involved in metabolism, transcription, neurotransmission, cell cycle/DNA repair, growth factors, reproduction/development, and cell structure (47). In addition, CREB transcriptional activity can be stimulated by a number of other stimuli and environmental factors such as EGF, testosterone, growth hormone, oxytocin, dopamine receptors, NMDA receptors, TGF β , hydrogen peroxide, phospholipids, pain, Ca²⁺, and glucose (48). As S133 phosphorylation is thought to be the key element in CREB activation, many kinases in addition to PKA have been shown to do this *in vitro* and *in vivo*. For example, pp90 ribosomal S6 kinase (RSK) (49), MAPKAP-K2 (50), and MSK1 (51) were shown to

Figure 1-3 The Structure of CREB indicating motifs and regulatory phosphorylation site.

Q1 and Q2 are hydrophobic glutamine-rich sequences, important for TAF_{II}130 binding. The KID is the kinase inducible domain, which is responsible for binding CBP/p300. It also harbors S133, which when phosphorylated, promotes binding of CBP and p300. The bZIP domain binds DNA and is required for transcription and is responsible for the CRE-site specificity.



directly phosphorylate S133 after stimulation with nerve growth factor. There is also much evidence that CREB activation is regulated by calcium signals in the cell. The specific kinases involved in phosphorylating S133 in response to increased intracellular Ca^{2+} concentration are the calcium/calmodulin dependent kinases I, II, and IV, and of course PKA (47).

CREB in Beta Cells

In response to calcium signals CREB has been shown to regulate transcription of brain-derived neurotrophic factor (BDNF), which is important in survival and differentiation of neurons (52). However, of particular interest in our lab is how CREB regulates genes involved in beta cell survival. Recently, CREB had been implicated in the transcription of the insulin receptor substrate genes (IRS); which are known to be crucial to growth, differentiation, metabolism and survival of beta cells (53). *Irs2* knockout mice show reduced beta cell mass and a reduction in insulin content leading to diabetes at 10 weeks (54). Indeed the importance of this gene was demonstrated in human islets as overexpression of *Irs2* was able to block the induction of apoptosis in response to chronic high glucose (55). The possible mechanism for this is that Akt, the survival kinase downstream of IRS2 promotes cell survival by phosphorylating the cell death mediator BAD and inhibiting apoptosis (56). This is quite interesting clinically as beta cell loss induced by chronic high glucose is thought to be one of the key factors leading to type 2 diabetes (57, 58).

Jhala et al., while working with mice expressing a dominant negative CREB (A-CREB) under the control the β -cell specific rat insulin promotor (RIP) revealed that although these mice are indistinguishable from control littermates at birth, the transgenic

mice later develop hyperglycemia leading to overt diabetes at 12-14 weeks due to an insulin deficiency (59). Further investigation of this phenotype showed that although staining for insulin, Glut-2 and PDX-1 was comparable, there was a 70% decrease in beta cell mass of RIP A-CREB mice. The indication that CREB is required for β -cell survival came from evidence of activation of the apoptotic program such as staining for cleaved caspase-3 and caspase-6 (59). Electron micrograph analysis also revealed nuclear condensation and fragmentation in the β -cells of 8-week old RIP A-CREB mice. Gene profiling studies were carried out to determine how CREB regulates survival of these cells and uncovered that IRS-2 was highly regulated by cAMP and A-CREB in MIN6 cells (59). Consistent with CREB binding to cAMP response elements, the IRS-2 promoter contains a half CRE site and is able to bind CREB in MIN6 cells, demonstrating that CREB regulates transcription of IRS-2 directly (59). The requirement for CREB in beta cell survival was confirmed by a separate group several years later in human islets. Induction of human islets with dominant negative and inactive (S133 to Ala) CREB mutants resulted in increased caspase-3 and 9 activity (60).

TORC

Structure and Function

Recently, a family of co-activators: Transducers of Regulated CREB activity (TORC), were characterized by Conkright et al. in a screen of the Mammalian Genome Consortium encompassing 1/3 of the genome (61). This lab was screening for proteins that affected the activity of a CRE-containing EVX-1 luciferase reporter vector. There are three TORC proteins TORC1, 2 and 3 which are 32% homologous and are

ubiquitously expressed at low levels in all tissues (Figure 1-4). However, TORC1 was found to be abundant in certain regions of the brain, and increased TORC2 and TORC3 levels were found in T and B lymphocytes (61). TORC2, our protein of interest has an N-terminal coiled-coil domain, a nuclear localizing sequence at aa 56-144, two nuclear export sequences (NES1 and NES2) between aa 145-320, and a C-terminal transactivation domain (Figure 1-5) (62). TORCs were shown to activate CREB via its bZIP domain, and interestingly, this activation was independent of CREBs S133 phosphorylation status. Further investigation of the mechanism of TORC/CREB interaction revealed that TORC tetramerizes and binds through its highly conserved N-terminal coiled coil domain to the CREB dimer (61). The specific amino acid on CREB required for the CREB:TORC interaction was identified as R314, as mutating this to alanine abolished the activating effect of TORC on CREB target gene expression (62). TORCs were named as coactivators because they do not bind DNA directly, but through their interaction with bZIP domain of CREB are able to enhance its interaction with TAF_{II}130 component of the TFIID complex (61, 63).

Calcium and cAMP signals appear to converge on CREB to promote a synergistic activation of transcription. Inhibition of calcium influx with nifedipine, an L-type calcium antagonist, blocked induction of CREB activity by glucose in MIN6 cells (mouse insulinoma cell line) (62). However, treatment of these cells with cyclosporine A (CsA) an inhibitor of the calcium responsive Ser/Thr phosphatase, calcineurin, blocked CREB target gene induction by both calcium and cAMP agonists.

Figure 1-4 TORC amino acid alignment and conservation.

TORCs 1,2 and 3 amino acid sequence in mice (TORC1, TORC2, TORC3), zebrafish (zfTORC), and drosophila (dTORC). Conserved regions are shaded and regions such as the CREB binding domain, the regulatory region and the transactivation domain are boxed in red.

Figure 1-5 Structure of TORC indicating motifs and identified phosphorylation sites.

Abbreviations are as follows: CBD (CREB binding domain), NES (nuclear export sequence), Ser/Pro (serine and proline rich region), TAD (transactivation domain). The red circles represent the 12 previously identified possible regulatory phosphorylation sites.

TORC2

CBD

NLS

NES1

NES2

SER/PRO

TAD



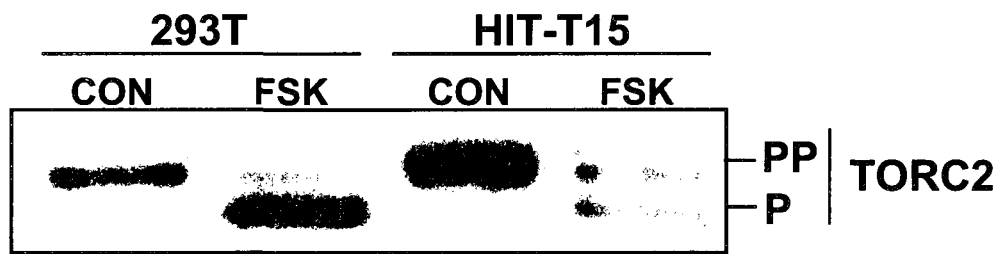
Further investigation of the mechanism of this activation revealed that the effect of the two stimuli together on CREB S133 phosphorylation were no more than when added individually. Additionally, CsA did not alter the phosphorylation status of S133 in response to either stimuli, indicating that there is an additional regulatory mechanism controlling the synergism of calcium and cAMP stimuli on CREB activation (62).

TORC2 knockdown studies in beta cells revealed that TORC2 is the protein responsible for the cooperative action of calcium and cAMP on CREB (62).

The ability of TORC2 to bind CREB is dependent on its cellular localization as it must bind CREB in the nucleus. Under resting conditions in either HIT-T15 (hamster insulinoma beta cell line) or MIN6 (mouse insulinoma beta cell line) cells TORC2 resides in the cytoplasm and only relocates to the nucleus after both KCl and forskolin (FSK), treatment, Ca^{2+} and cAMP agonists respectively. Interestingly, TORC3 contains a single amino acid variant in its nuclear export sequence resulting in constitutive nuclear localization as well as increased CREB target gene activation (62). Similar to several transcription factors such as the FOXO family members (64), TORC nuclear localization is controlled by its phosphorylation status at specific amino acid residues. In congruence with this data, TORCs phosphorylation status is also regulated by cAMP signals in 293 cells indicated by a single faster migrating species on a western blot after treatment with cAMP stimuli compared to a doublet under resting conditions. Interestingly, the same treatment in beta cells results in only partial TORC2 dephosphorylation as seen on a western blot (Figure 1-6). Additionally both TORC2 nuclear entry and dephosphorylation is inhibited after treatment with CsA. Sreaton et al. identified S171 as the amino acid dephosphorylated by cAMP signals in the beta cell line HIT-T15.

Figure 1-6 TORC2 is differentially phosphorylated in beta cells.

Western blot analysis of endogenous TORC2 in HEK 293T and HIT-T15 cells with or without treatment of forskolin (FSK) for 0.5 hours. Membrane was probed with anti TORC2 from rabbit. After FSK treatment in 293 cells TORC2 is predominantly dephosphorylated as indicated by the lower band shift, however after the same treatment in beta cells, TORC2 remains approximately 50% phosphorylated.

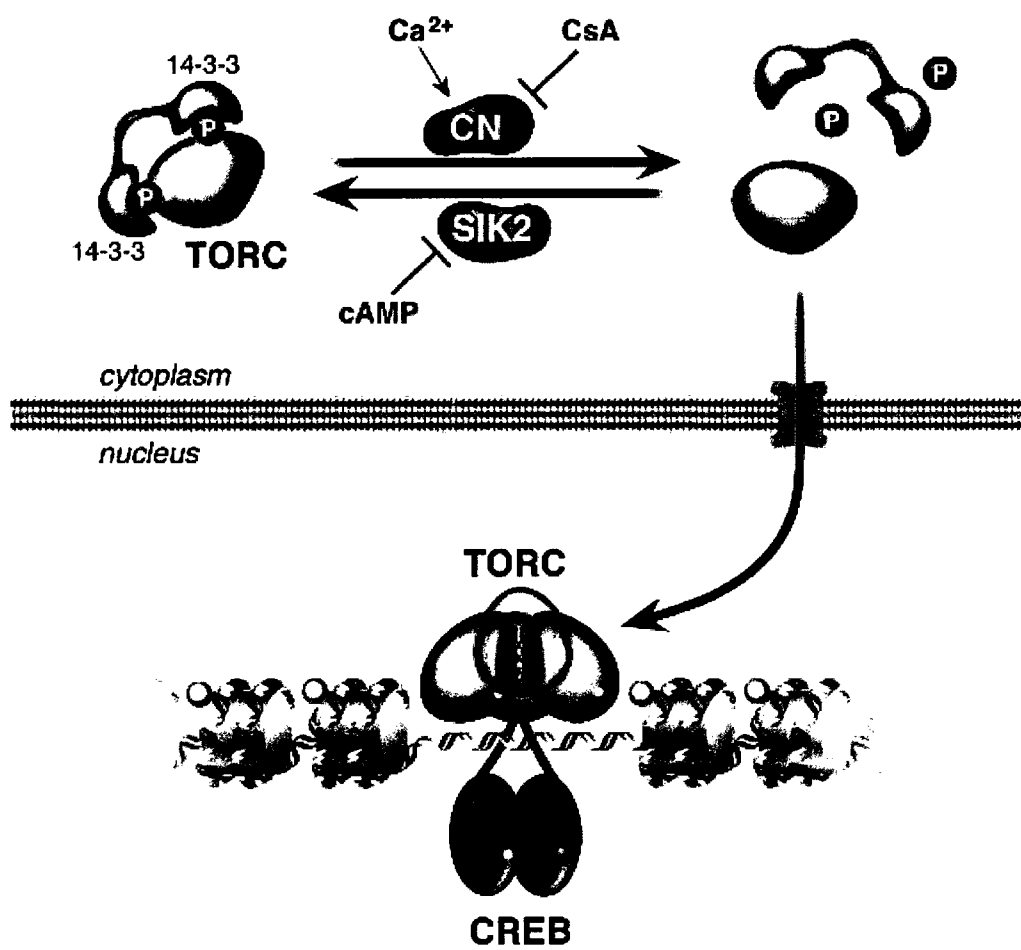


Interestingly 14-3-3 proteins were recovered in immunoprecipitates of TORC2 from HIT-T15 cells and shown to bind specifically to phospho-S171 and sequester TORC2 in the cytoplasm under resting conditions (62). 14-3-3s are a family of dimeric proteins that bind specifically to phospho-serine and threonine motifs (RSXpSXP and RXXXpSXP) and have important roles in cell functions such as signal transduction and apoptosis (65). The hypothesis of how these proteins inhibit the nuclear localization of TORC2 is that by binding to pS171 they may alter the conformation of TORC2 so as to disguise the NLS, as has been shown with class II histone deacetylases (66). Curiously, mutation of S171 to alanine, decreased but did not abolish 14-3-3 binding indicating that although pS171 is sufficient for binding, it is not necessary.

The calcium responsive phosphatase Calcineurin (Cn) was also retrieved in immunoprecipitates of TORC2 and shown to dephosphorylate TORC2. This would explain the ability of CsA to inhibit de-phosphorylation (as it inhibits Calcineurin) and therefore nuclear entry and CREB target gene activation by TORC2. Investigation into the kinase that phosphorylates the regulatory S171 site uncovered the salt-inducible kinase 2 (SIK2). SIK2 contains an N-terminal serine/threonine protein kinase domain, a ubiquitin associate motif, and a C-terminal protein kinase A phosphorylation site (67). A member of the AMP-activated protein kinase (AMPK) family of kinases, SIK2 associates with wild-type but not the S171A mutant of TORC2 in the cytoplasm and becomes dissociated after phosphorylation by PKA, a kinase downstream of the cAMP pathway (62). Moreover, over-expression of SIK2 increases 14-3-3 binding to TORC2 as well as inhibits TORC2 nuclear localization. The model of TORC-dependent CREB activation is depicted in Figure 1-7.

Figure 1-7 Model of TORC regulation and CREB activation.

TORC2 is sequestered in the cytoplasm of resting cells by 14-3-3 proteins that bind specifically to phosphorylated TORC2. Once stimulated with cAMP and Ca²⁺ TORC2 becomes dephosphorylated and enters the nucleus and activates CREB-dependent gene transcription. In response to Ca²⁺, the phosphatase Calcineurin (Cn) dephosphorylates TORC2, and this is hindered by treatment with cyclosporine A (CsA). Additionally, cAMP inhibits TORC2 phosphorylation at S171 by the salt-inducible kinase (SIK2) through activation of PKA.



TORC in Neurons

Interestingly, TORC1, the most abundant TORC in the brain, is also regulated by cAMP and Ca^{2+} stimuli in neurons (68). Neurons and beta cells share a dependence on intercellular calcium concentrations for cell membrane depolarization that leads to secretion of neurotransmitters or insulin respectively. TORC1 is required for maintenance of late phase long term potentiation in the CA1 neurons of the hippocampus (69), confirming the importance of these proteins downstream of the second messengers calcium and cAMP. Additionally, TORC1 is essential for CREB-dependent transcription in response to cAMP and Ca^{2+} stimuli in cortical neurons (68).

TORC in other Metabolic Tissues

The involvement of TORCs in metabolism is not confined to the beta cells and neurons, however, TORC2 is very important in glucose metabolism in the hepatocytes of the liver. Glucagon released from the alpha cells in the pancreas will trigger cAMP signaling in the liver leading to activation of CREB target genes (47). Disruption of CREB activity in hepatocytes leads to hypoglycemia and a reduction in gluconeogenesis, revealing the requirement for CREB in gluconeogenic gene transcription (13). As previously stated, CREB regulates the transcription of several gluconeogenic genes such as PGC-1 α , phosphoenolpyruvate carboxy kinase (PEPCK) and glucose-6 phosphatase (G6Pase) (13), and this is dependent upon TORC2 (70). In hepatocytes both SIK1 expression and AMPK activation block TORC2 nuclear entry in a S171 dependent mechanism. AMPK consists of three subunits, an α catalytic subunit, and two regulatory subunits, β and γ . AMPK is activated by binding of AMP to the γ -subunit which

activates the kinase activity of the α -subunit. This association with AMP promotes phosphorylation of Thr172 which is required for the kinase activity (71). Metformin, a drug that has been used for over 10 years to treat symptoms of type 2 diabetes has been linked to AMPK and TORC2. Metformin has the ability to reduce blood glucose levels through an unknown mechanism of AMPK activation in insulin resistant states (72). In support of AMPKs involvement in lowering blood glucose levels, deletion of its upstream kinase LKB1 leads to AMPK inactivation, and hyperglycemia due to deregulated TORC2 dependent gluconeogenic gene expression (73). Fasting conditions require TORC2 in hepatocytes for gluconeogenesis when blood glucose levels are low, however how is this activity of TORC2 turned off after feeding? Very recently Dentin et al., showed that in re-feeding conditions insulin is responsible for the hepatic TORC2 re-phosphorylation by SIK2 promoting cytoplasmic translocation and its subsequent proteosomal degradation (74). It has also been shown that TORC2 enhances IRS2 gene expression in hepatocytes and this leads to attenuation of the gluconeogenic program, limiting glucose output during fasting (75).

Rationale and Outline for the Thesis Project

Since the identification of TORC2 as a CREB co-activator in beta cells, its importance in metabolism has become ever more apparent. Deletion or inhibition of CREB in beta cells results in increased apoptosis and decreased anti-apoptotic signaling (60), revealing the necessity of CREB for survival of these cells. In addition, deletion of TORC2 in beta cells abolishes CREB activation in response to the cAMP agonist, Forskolin (61). TORC2 provided the mechanism of the cooperative action of cAMP and Ca^{2+} on CREB activity. With this knowledge in mind, our lab made an important observation: in 293 cells TORC2 required only a cAMP stimulus to enter the nucleus and activate CREB, however, in the glucose responsive beta cells, both cAMP and Ca^{2+} signals were required for this very same action. Indeed previous work has shown that 14-3-3 proteins will bind to phosphorylated TORC2 in the absence of these signals and that treatment of 293 cells with cAMP results in the dephosphorylation of S171 and subsequent nuclear entry of TORC2. However, following treatment of beta cells with Forskolin, or mutation of the cAMP responsive S171 to Ala, TORC2 retained 14-3-3 binding and remained in the cytoplasm. Only treatment of both cAMP and Ca^{2+} resulted in dephosphorylation (as shown by a faster migrating species on a western blot), nuclear entry of TORC2 and CREB activation. This led me to the hypothesis that there was an additional regulatory phosphorylation site besides S171 on TORC2 that governed 14-3-3 binding and hence nuclear entry. Identification of this site and its regulation could provide insight into beta cell survival and function.

MATERIALS AND METHODS

Cell Culture

HIT-T15 (hamster insulinoma beta cell line) cells were maintained in 50% optimem (GIBCO), 50% Dulbecco's minimum essential medium (DMEM), with heat inactivated 5% fetal calf serum (FCS) and 1U/ml penicillin and 1ug/ml streptomycin (P/S) (Fisher). HIT cells were used between passages 60-85.

MIN6 (mouse insulinoma cell line) cells were maintained in DMEM with 10% FCS and penicillin and streptomycin with 0.8% β -mercapto-ethanol. MIN6 cells were used between passages 20-40.

Human embryonic kidney (HEK) 293A cells were maintained in DMEM with 10% FCS and P/S.

All cells were kept at 37°C with 5% CO₂, and were passaged at 70-100% confluency.

Cell Treatments

Starvation conditions were done in KREBs ringer buffer (KRB) (128.8mM NaCl, 4.8mM KCl, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 2.5mM CaCl₂, 5mM NaCO₃, 10mM HEPES and 0.1% BSA), pH 7, for 2 half-hour intervals. When cells were pre-treated with cyclosporine A (CsA) 1 μ M (Calbiochem), they were incubated in KRB for 0.5 hours, then rinsed with new KRB and treated with CsA for 1 hour and then stimulated as indicated. Where indicated cells were equilibrated in KRB for 0.5 hours, rinsed with new KRB and treated with CsA 1 hour, then treated with 2.75mM glucose to starve for 1 hour, then stimulated accordingly. Stimulation of glucose was with 20mM, unless otherwise

stated. Forskolin and Exendin-4 (SIGMA) were used at 10 μ M and 10nM respectively, and KCl treatment was used at 45mM. For western and immunofluorescence data stimulation was for 0.5 hours, for reporter data stimulation was for 4-6 hours.

Western Blot Analysis

Cells were rinsed 2-3X with phosphate buffered saline (PBS) and lysed with 1X lysis buffer (62.5mM TRIS-HCl pH 6.8, 10mM glycerol, 2%SDS, 0.1% bromophenol blue, DTT 50mM) and run on a discontinuous polyacrylamide gel with a lower resolving gel (8% acrylamide mixture (BIO RAD), 380mM Tris-HCl pH 8.8, 0.1% SDS and 0.1% ammonium persulfate, TEMED) and an upper stacking gel (4% acrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, and TEMED). Gels were run for 1 hour at a constant voltage of 150V. Gels were transferred to a 0.45 μ m PVDF membrane (Millipore) for 1 hour and 20 minutes at 100V using the wet method. Membranes were blocked for 1 hour at room temperature or overnight at 4°C in 5% milk in TBST (20mM TRIS-HCl pH 7.5, 125mM NaCl, 0.1 % Tween).

Far Western Analysis

Far Western protein analysis was performed as described for westerns. Cells were transfected with Flag-tagged proteins and cell lysates were then immunoprecipitated as described below and analyzed using an SDS-PAGE. However, instead of using a primary antibody to probe the membrane, purified recombinant GST-14-3-3 proteins (6 μ g/ml) in 5% milk in TBST was used for 1hour at room temperature. Following this incubation, membrane was probed with mouse anti-GST for 1hour at room temperature.

Following rinsing it was then probed with a secondary antibody against mouse IgG and developed as a regular western blot.

Protein Purification

Recombinant 14-3-3 proteins were purified from XL1-Blue bacteria. Bacteria were inoculated with AMP-resistant XL1-Blue that had been transformed with pGEX - GST-14-3-3. Bacteria were induced at an OD of 0.6-0.8 at room temperature in a shaker with 0.2mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4-6 hours. Cultures were centrifuged at 5000g, 10 minutes at 4°C and resuspended in 10mls ice-cold PBS. They were centrifuged again at 5000g, 10 minutes at room temperature and lysed with 10ml lysis buffer (50mM TRIS-HCl pH 8.0, 200mM NaCl, 1mM EDTA, 1mM DTT, and 2mM phenylmethylsulfonylfluoride (PMSF)). Lysates were the sonicated, 1% Triton X-100 was added, sonicated again, and centrifuged at 13,000g for 10 minutes at 4°C. Glutathione sepharose beads (GE Healthcare) rinsed in PBS were added as a 1:1 slurry (200 μ l) to the supernatant. Samples were mixed by inversion for 2minutes at room temperature and centrifuged at 300g for 1 minute. Once beads were rinsed, 5 μ l was taken and analyzed by SDS-PAGE and coomassie to confirm presence of GST-14-3-3 protein. Proteins were eluted from a BIO RAD column with 20mM L-glutathione (SIGMA) pH 8.5. Protein concentration was measured using the BIO RAD Bradford Assay with Bovine Serum Albumin as a standard.

Antibodies

Membranes were probed with primary antibodies 1 hour at room temperature or overnight at 4°C. Membranes were rinsed 3-6X for 1 hour in TBST. Secondary antibodies were used at 1:5000 (anti-mouse and anti-rabbit from goat, BIO RAD) for 30 minutes-1 hour. The following primary antibodies were used: rabbit anti TORC2 1:2000 and rabbit anti-phospho S171A (amino acids 161-181 of mouse TORC2) were generated as described in (61), rabbit anti-phospho Ser 275 TORC2 100ng/ml (Covance), anti-acetyl-CoA carboxylase (ACC), anti-phospho ACC, anti-AMPK, anti-phospho AMPK and anti PARP, all from rabbit 1:1000 (Cell Signaling), anti-cleaved caspase-3 1:500 (Cell Signaling), anti-GST-GFP (raised in rabbits against GST-GFP fusion protein (61)), mouse anti-FLAG M2 1:2000 (SIGMA), Alexa Fluor[®] 488 donkey anti-mouse 1:1000 (Invitrogen).

Plasmids

The following pcDNA FLAG-TORC2 plasmids (T2 WT, T2 S70A, T2 S171A, T2 S275A, T2 369A, T2 1-240, T2 389-692), as well as pTarget LUC vector containing the EVX promoter region, pBluescript, RSV-βgal and pGEX-GST-14-3-3 plasmids were described previously (62). Other TORC2 constructs (T2 S171A + S275A (T2 S275A backbone), S70A + S171A (S70A backbone, S127A + S171A + S275, S171A + S238A + S275A, S171A + S245A + S275A (S171A + S275A backbone) were made using a quick change PCR protocol with the following oligonucleotides:

mT2-S127A/f TACCCCGCCACATTGACgcTTCTCCATATAGCCCTGCC

mT2-S127A/r GGCAGGGCTATATGGAGAAgcGTCAATGTGGCGGGGTA

mT2 S238A/f	GATGCTAAGAAGCTGTCCgCATCTTCCTCTCGACCTCGA
mT2 S238A/r	TCGAGGTCGAGAGGAAGATGcGGACAGCTTCTTAGCATC
mT2 S245A/f	TCTTCCTCTCGACCTCGAgCCTGTGAAGTCCCTGGAATC
mT2 S245A/r	GATTCCAGGGACTTCACAGGcTCGAGGTCGAGAGGAAGA
mT2 S171A/f	CTTAACAGGACAAGCGCTGACTCTGCTCTTCACAC
mT2 S171A/r	GTGTGAAGAGCAGAGTCAGCGCTTGTCTGTTAAG

Plasmids were sequenced to confirm presence of the mutations and expression was confirmed in 293T cells. T2 1-321 and T2 1-389 WT, and S171A, S275, and S171A + S275 constructs were made using standard cloning techniques with oligonucleotides for the T2 Not forward (start) 321 or 389 reverse using the WT or mutant backbones made previously. These were also sequenced and expression was confirmed in 293T cells.

Transfections

Plasmids were mixed with optimem according to table 1. Lipofectamine 2000 (L2000) (Invitrogen) was added to Optimem, after 5 minutes this mix was added to DNA/Optimem tubes. After 20 minutes this was added directly onto cells. HIT-T15 and MIN6 cells were incubated for 40 hours, and cell media was changed at approximately 20 hours. 293A and 293T cells were incubated for 24 hours.

Cell numbers are given for a transfection with a 40-hour turnaround time (~20% fewer cells).

Plate	Surface Area (cm ²)	293T	MIN6 or HIT	TOTAL (ul) Optimem/well [DNA + L2000]	L2000 per well (ul)	TOTAL DNA/well (ug)	Total medium volume
96-well	0.32	50,000	80,000	10	0.1	0.1	100 ul
48-well	0.95	1.4 X 10 ⁵	2.4 X 10 ⁵	25	0.38	0.15	250 ul

24-well	1.9	2.8×10^5	4×10^5	50	0.75	0.25	500 ul
12-well	3.8	5.7×10^5	8×10^5	100	1.5	0.4	1 ml
6-well	9.5	1.25×10^6	2.4×10^6	200	3	1	2 ml

Cell numbers are given for a transfection with a 24-hour turnaround time.

Plate	Surface Area (cm ²)	MIN6 or HIT	TOTAL (ul) Optimem/well [DNA + L2000]	L2000 per well (ul)	TOTAL DNA/well (ug)	Total medium volume
48-well	0.95	2.0×10^5	25	0.38	0.15	250 ul
24-well	1.9	3.2×10^5	50	0.75	0.25	500 ul
12-well	3.8	6.4×10^5	100	1.5	0.4	1 ml
6-well	9.5	1.8×10^6	200	3	1	2 ml

Immunoprecipitation

Transfected cells were rinsed with PBS and lysed with Lysis buffer (25mM TRIS-HCl pH 7.5, 150mM NaCl, 50mM NaF, 0.5mM EDTA pH 8, 0.5% Triton-X 100, 5mM β -glycerophosphate, with fresh 1mM DTT, 1mM PMSF, 1mM NaVO₃) and scraped into eppendorf tubes. Lysates were sonicated and centrifuged for 20 minutes at 4°C at 13,000rpm. Lysates were then transferred to a new tube and 2% was reserved as an “input”. Beads (2 μ g/ml anti-Flag M2 agarose from mouse SIGMA) rinsed 3X with lysis buffer were added to lysates (15 μ l of 1:1 beads:lysis buffer/sample) and placed on a rotator for 1 hour at 4°C. Beads were then rinsed 3X with lysis buffer. After the third rinse, all buffer was removed with a 27gauge syringe and 50 μ l of 1X SDS lysis buffer with DTT was added and samples were analyzed by SDS-PAGE.

Immunofluorescence

Transfected (at 24 hours post-transfection) or non-transfected cells were trypsinized and plated onto poly-L lysine coated coverslips in 6-well plates. At 40 hours post-transfection cells were treated as indicated and rinsed with PBS, then fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Fixative was removed and cells were then rinsed 3X for 5 minutes each with 0.1M fresh glycine in PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 2 minutes then rinsed with PBS. Cells were then blocked with 3% BSA in PBS for 1 hour on a shaker. Slides were placed cells down onto 75 μ l of primary antibody (concentrations as described for western) on parafilm for 1 hour at room temp. Slides were rinsed 3X with PBS then probed with secondary antibody (AlexaFluor[®] 488) 40 minutes at room temperature. Slides were again rinsed 3X and placed onto slides with Vectashield (Vector Laboratories) that contains DAPI stain and sealed with nail polish.

CREB Reporter Assay

Cells were transfected in triplicate into 24-well plates. 40 hours post-transfection cells were treated as indicated and lysed with 100 μ l extract buffer (25mM Gly-Gly (SIGMA), 15mM MgSO₄, 1% Triton X-100, 1mM DTT) then incubated at room temperature for 10 minutes. Lysates were transferred to a 96-well v-bottom plate and centrifuged for 10 minutes at room temperature at 3500rpm. The supernatant was then used to measure luciferase and β gal activity. 10 μ l of supernatant was transferred to an opaque 96-well plate and 90 μ l of assay buffer (80mM Gly-Gly, 12.5mM MgSO₄, 16.3mM KPO₄, 2mM ATP and 2mM DTT) was added. The plate was placed in the

BioTek Synergy 2 plate reader equipped with Gen5 software and 100 μ l of 0.1mM luciferin potassium salt (BD Bioscience) in Gly-Gly buffer (1M Gly-Gly (Sigma), 15mM MgSO₄) was added by the dispenser and luminescence was measured. The β gal assay required 50 μ l of lysate with 50 μ l of 2X β gal buffer (1.33mg/ml ortho-Nitrophenyl- β -galactoside (ONPG) (SIGMA) 100mM β -mercaptoethanol, 2mM MgCl₂, 40mM NaHPO₄ pH 7.3). Activity was measured at 420nm with the same plate reader. Luciferase data was then normalized using β gal activity.

Kinase Assay

Min6 cells were seeded and transfected in a 6-well tissue culture dish. Cells were transfected with a DNA:cell ratio (1 μ g DNA/well) according to the table above and incubated at 37°C for 24 hours. Cells were harvested with 100 μ l of lysis buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 50mM NaF, 0.5mM EDTA pH 8, 0.5% Triton-X 100, 5mM β -glycerophosphate, with fresh 1mM DTT, 1mM PMSF, 1mM NaVO₃) and incubated for 1 hour on ice. Lysates were sonicated and transferred to a 96-well V-bottom plate and centrifuged for 10 minutes at 3,000rpm at 4°C. Supernatants were then transferred to a glutathione coated plate, sealed and incubated for 2 hours on ice to allow binding of the GST-tagged plamids to the plate. Plates were rinsed 3 times with 100 μ l of lysis buffer, then rinsed once with 50 μ l of kinase buffer (20mM Tris pH 7.5, 5mM β -glycerophosphate, 0.2mM NaVO₄). The peptide substrate in kinase buffer was added to the plates along with 330 μ M MgCl₂, 1 μ M ATP and γ ³²-ATP. This was covered and incubated in a hybridization oven at 30°C for 20 minutes. The reaction was transferred back to ice and 2X lysis buffer (1X=62.5mM TRIS-HCl pH 6.8, 10mM glycerol,

2%SDS, 0.1% bromophenol blue, DTT 50mM) was added. This mixture was then run on a 12% polyacrylamide gel and exposed to film at -80°C. Performed by Accalia Fu (Screaton lab).

Islet Extraction and Treatment

All animal protocols were approved by and carried out according to the Canadian Council for Animal Care (CCAC). C57/Bl6 mice between 3 and 4 months were anesthetized with isoflurane and euthanized by cervical dislocation. Islets were isolated as previously reported (76) with modification. The pancreatic duct was cannulated with 3mls of 0.7 mg/mL collagenase from *Clostridium histolyticum* type XI (Sigma) in Hanks Buffered Saline Solution (HBSS) with 1mM MgCl₂, 5mM D-glucose, pH 7.4. Once the pancreas was injected, it was removed and placed into 50mL conical with remaining buffer on ice. Pancreata were digested at 37°C for 9-12min and vortexed. Islets were rinsed 7-10X, 4 minutes each with Hanks Buffered Saline Solution (HBSS) with 1mM MgCl₂, 5mM D-glucose, pH 7.4, 1mM CaCl₂ added. Islets were then manually purified from petri dishes twice and placed into RPMI media overnight at 37°C in petri dishes. Following overnight incubation the islets were counted and split as evenly as possible into treatment groups into eppendorf tubes. Approximately 80-100 islets were used for each treatment. Islets were starved in KRB for 2 30 minute rinses at 37°C. They were then treated as indicated (Exendin-4, glucose or both) for 1hr at 37°C. Islets were lysed by removing all but 20µl of buffer, and adding 20µl 2X SDS lysis buffer (125mM Tris-HCl pH 6.8, 20mM glycerol, 4%SDS, 0.1% bromophenol blue, DTT 50mM) and analyzed by SDS-PAGE.

TORC2 knockdown in islets

The TORC2 knockdown virus was made by Tassnim Moradipour, an honours student in the Sreaton lab in 2007. Three 21-nucleotide regions of the mouse TORC2 cDNA were selected using algorithms from the Invitrogen website. 5' and 3' complementary oligomers to these regions were ordered and reconstituted to 200mM. Touchdown PCR was performed on each set of oligomers in order to anneal the strands. Each set of shRNAs were cloned into pENTR/U6 RNAi entry vector and transformed into Top10 competent *E. coli* and DNA was obtained from minipreps. Hairpins were tested by co-transfection into HEK293T cells with a pTARGET-luc-mTorc2 reporter vector. Sequences used to make the hairpins are as follows with the underlined region targeting TORC2:

```
mT2_shrna_1787_bottom AAA AGG AAT CTG GAG CAA TTC AAC ATT CGT GTT GAA TTG CTC  
                        CAG ATT CC  
mT2_shrna_1787_top   CAC CGG AAT CTG GAG CAA TTC AAC ACG AAT GTT GAA TTG CTC  
                        CAG ATT CC
```

Preparation of the adenovirus was made by recombination of the pENTR/U6-hairpin with pAd/BLOCK-iT™-DEST RNAi Gateway® Vector (Invitrogen) according manufacturers instructions. Plasmids were then transformed into DH5α competent *E. coli*. Viral amplification was done in HEK293A cells. Cells were transfected the day following seeding with 2ug of pAd/BLOCKiT-hairpin per 60mm tissue culture dish using Lipofectamine 2000 (Invitrogen). Cells were overlaid with 5% agar in PBS:low serum tissue culture media in 1:3 ratio. Upon plaque formation, plaques were picked and

freeze-thawed three times and stored at -80°C . The virus was propagated in HEK293T cells using the plaques that were picked and dead cells were collected. Viral concentrations were approximated using a known GFP expressing adenovirus. Islets were extracted and previously described, however, immediately following purification islets were infected with the T2i virus at 2.6×10^9 pfu/mL for three days and harvested.

RESULTS

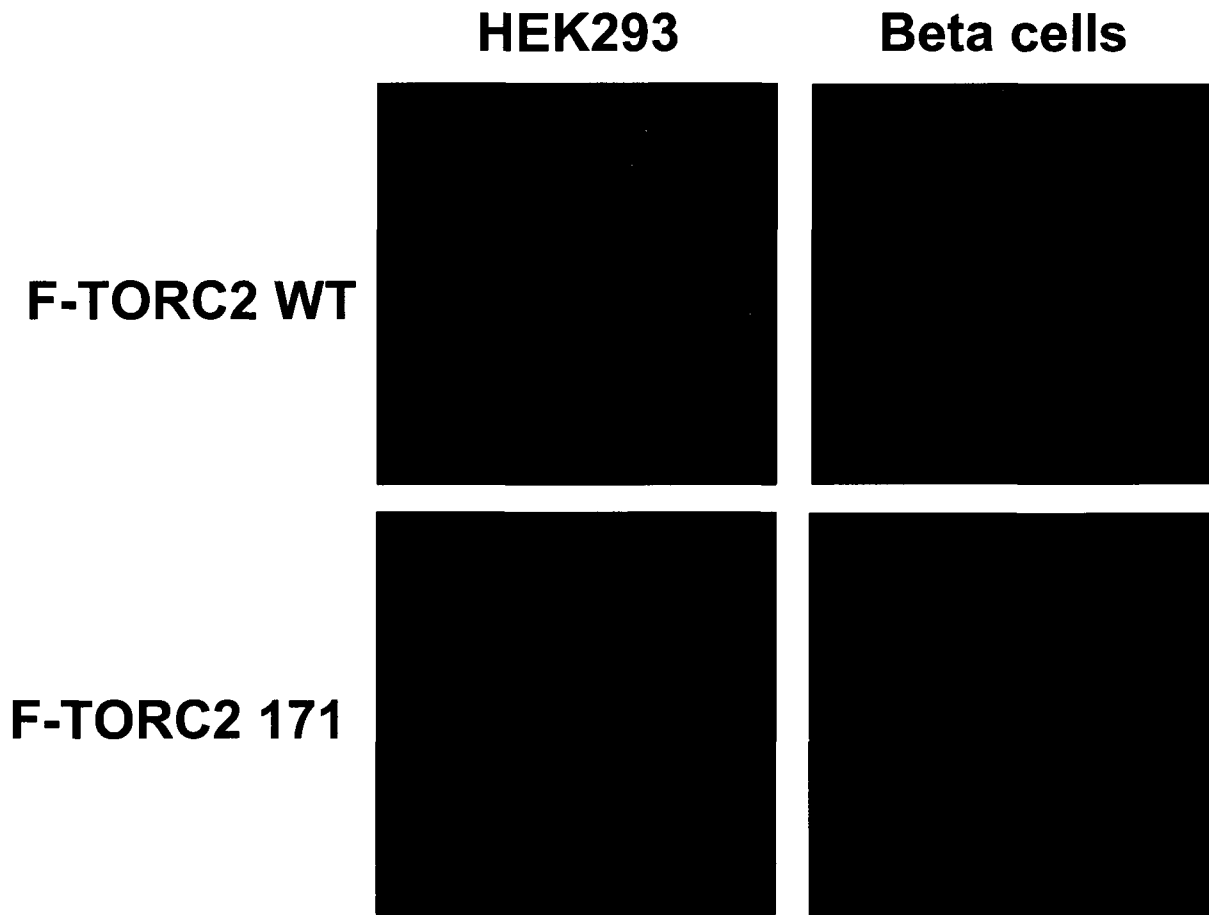
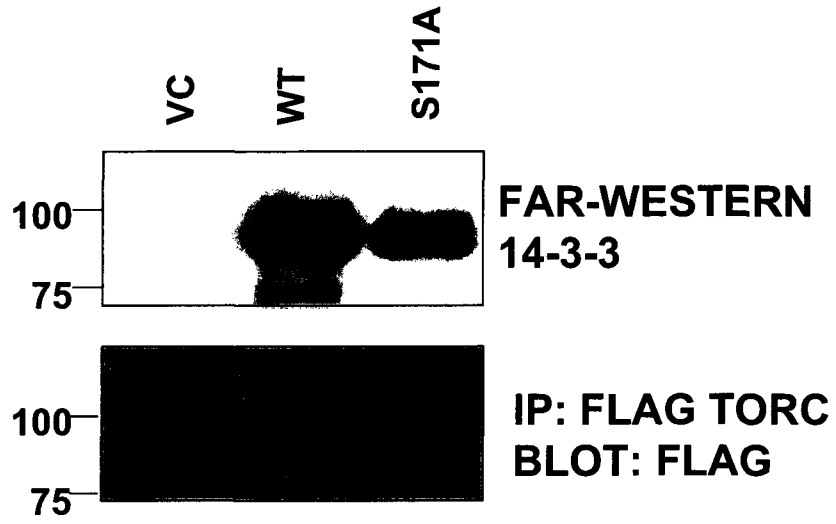
Identification of the Remaining 14-3-3 Binding Site on TORC2

Previous data showed that mutation of serine 171 to alanine of TORC2 resulted in reduced but not complete loss of 14-3-3 binding by far western analysis and co-immunoprecipitation experiments in the beta cell line HIT-T15 (62). I examined the ability of recombinant 14-3-3 proteins to bind both the wild-type and S171A mutant of TORC2 by transfecting FLAG-TORC2 wild-type and the S171A mutant into HIT-T15 cells. Cell lysates were run on an 8% SDS-PAGE and after transferring the proteins to a PVDF membrane it was probed with recombinant GST-tagged 14-3-3 proteins to examine direct binding. I was able to confirm by far western analysis in HIT-T15 cells that the S171A mutant bound less 14-3-3 protein compared to wild-type TORC2, (Figure 3-1, top). In addition, as 14-3-3 binding regulates TORC2 cellular localization I sought to observe the localization of the S171 mutant in comparison to wild-type TORC2 in beta cells. Again to confirm previous data I performed immunofluorescence staining for FLAG in 293A and HIT-T15 cells transfected with either the wild-type or the S171 mutant of FLAG-tagged TORC2. In regular media, although wild-type TORC2 resides in the cytoplasm in both cell types, in 293A cells the S171A mutant resides in the nucleus, and in beta cells this mutant is located in the cytoplasm (Figure 3-1, bottom).

Figure 3-1 TORC2 is differentially regulated in beta cells.

Top: Far western analysis of HIT-T15 cells transfected with FLAG-tagged TORC2 constructs. TORC2 was immunoprecipitated from cell lysates and analyzed using SDS-PAGE. Blots were probed with recombinant GST-14-3-3 protein. 14-3-3 binding to wild-type and S171A mutant TORC2 is shown. Total FLAG protein also shown.

Bottom: FLAG-tagged TORC2 was transfected into HIT-T15 (beta cells) and HEK 293A cells. Cells were grown in regular cell media, fixed and stained for FLAG. Bar indicates 25 μ m. The nuclear entry of the Serine 171 to Alanine (S171A) mutant of TORC2 in HEK 293 cells and the cytoplasmic localization of TORC2 in HIT-T15 cells is shown.



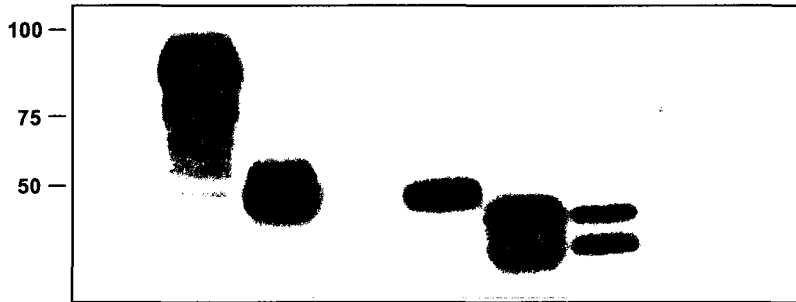
These experiments indicated that TORC2:14-3-3 binding and localization were differentially regulated in beta cells. Since it has been shown previously that TORC2:14-3-3 binding is regulated by phosphorylation, it is possible that TORC2 phosphorylation is also differentially regulated in beta cells.

In order to identify other possible site(s), I used a far western approach to narrow down the region of TORC2 required for 14-3-3 binding. Using a series of FLAG-tagged N-terminal and C-terminal deletions I was able to narrow down the region of TORC2 required for 14-3-3 binding to the first 321 amino acids. As seen in figure 3-1, wild-type TORC2 was able to bind the recombinant 14-3-3 proteins, as were the deletion mutants consisting of amino acids 1-389, and 1-321 (figure 3-2, lanes 2, 3 and 6 respectively). Interestingly 14-3-3 binding was completely abolished when amino acids 1-389 were deleted (Figure 3-2 lane 4). The smallest fragment of TORC2 to retain 14-3-3 binding even in the presence of the S171A mutation enabled me to identify amino acids 1-321 of TORC2 as being required for 14-3-3 binding (Figure 3-2, lanes 6 and 7). I also noticed a loss of 14-3-3 binding when deleting the first 240 amino acids of TORC2 (data not shown), however, this could have been a result of this region being the coiled-coil domain (61), and removing it could severely alter the proper folding of the TORC2 protein thus rendering the result inconclusive. For this reason I continued my search for the phosphorylation site in the region between amino acids 1-321. I also confirmed that this binding was specific to phosphorylated TORC as when I treated the cell lysate with calf intestinal phosphatase (CIP) to remove the phosphate group from TORC2, binding was lost (Figure 3-2, lanes 8 and 9).

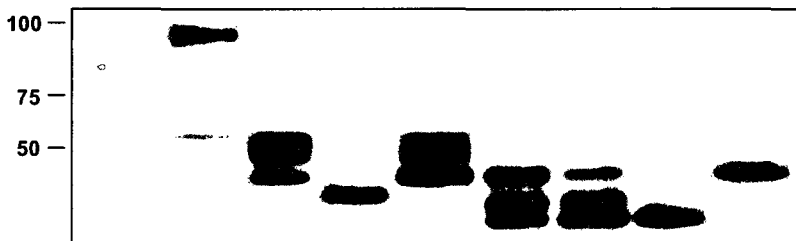
Figure 3-2 Amino acids 1-389 in TORC2 are required for 14-3-3 binding in beta cells.

Far western analysis of HIT-T15 cells were transfected with FLAG-TORC2 wild-type (wt), C-terminal and N-terminal deletions and Serine→Alanine mutants (CON is pcDNA FLAG vector alone, numbers designate amino acids present in FLAG-tagged construct, S171A indicates that Serine 171 is mutated to Alanine, + indicates treatment with calf intestinal phosphatase (CIP)). FLAG proteins were immunoprecipitated and analyzed SDS-PAGE. The membrane was then probed with recombinant GST-tagged 14-3-3 proteins to examine binding. The smallest region to retain 14-3-3 binding is amino acids 1-321, indicating that this region is required for the interaction between TORC2 and 14-3-3.

FLAG T2:	CON	WT	1-389	389-692	1-389 S171A	1-321		1-389	
CIP:	-	-	-	-	-	WT	171	171	171
						-	-	+	+



**FAR-WESTERN
14-3-3**



**IP: FLAG TORC
BLOT: FLAG**

The previous experiments served to narrow-down the region containing the remaining regulatory phosphorylation site(s) in TORC2 to the first 321 amino acids. In order to identify specifically the functional phosphorylation site we examined the amino acid sequences surrounding potential Serines within the region 1-321 of TORC2.

Additionally, I hypothesized that this regulatory site would be important in survival of the beta cell, as such I focused on regions that were highly conserved throughout TORC1, TORC2 and TORC3 and found several possible 14-3-3 binding sites that conformed to the 14-3-3 binding sites R/KXXpSXP or RKXXXpSXP where X is any amino acid and pS is phosphorylated Serine (77), (Figure 3-3). I expected that if the regulatory phosphorylation site was mutated, and could not be phosphorylated, then 14-3-3 binding would be lost on a far western. Using the 1-321, and 1-389 TORC2 plasmid backbones I used quickchange PCR to create Ser→Ala mutants of the Serines in question. I transfected these new FLAG-tagged TORC2 mutants into HIT-T15 cells and analyzed the lysates using SDS-PAGE. The proteins were transferred to a PVDF membrane and after blocking in milk were probed with the recombinant GST-tagged 14-3-3 proteins to determine binding. In the context of either the first 321, or 389 amino acids both the S171A and S275A mutation resulted in a decrease in 14-3-3 binding (Figure 3-4, lanes 3,4,7 and 8). Additionally, the double mutant S171A and S275A had a co-operative effect and abolished 14-3-3 binding completely (Figure 3-4, lane 5 and 9). This data could only be functionally relevant if confirmed in the full length TORC2 protein, therefore I repeated the experiment by transfecting with the full-length Flag-tagged TORC2 proteins into HIT-T15 cells and examined 14-3-3 binding.

Figure 3-3 Consensus binding sequence of 14-3-3 proteins in conserved TORC domains.

14-3-3 proteins have 2 consensus binding sequences: mode 1 and mode 2. The conserved regions in TORCs 1, 2 and 3 are listed that conform to this sequence. The Serines in bold in TORC2 were mutated to alanine to investigate 14-3-3 binding. The + signs indicate that the mutation did reduce 14-3-3 binding in far western analysis. The – sign indicates no significant difference from the wild-type.

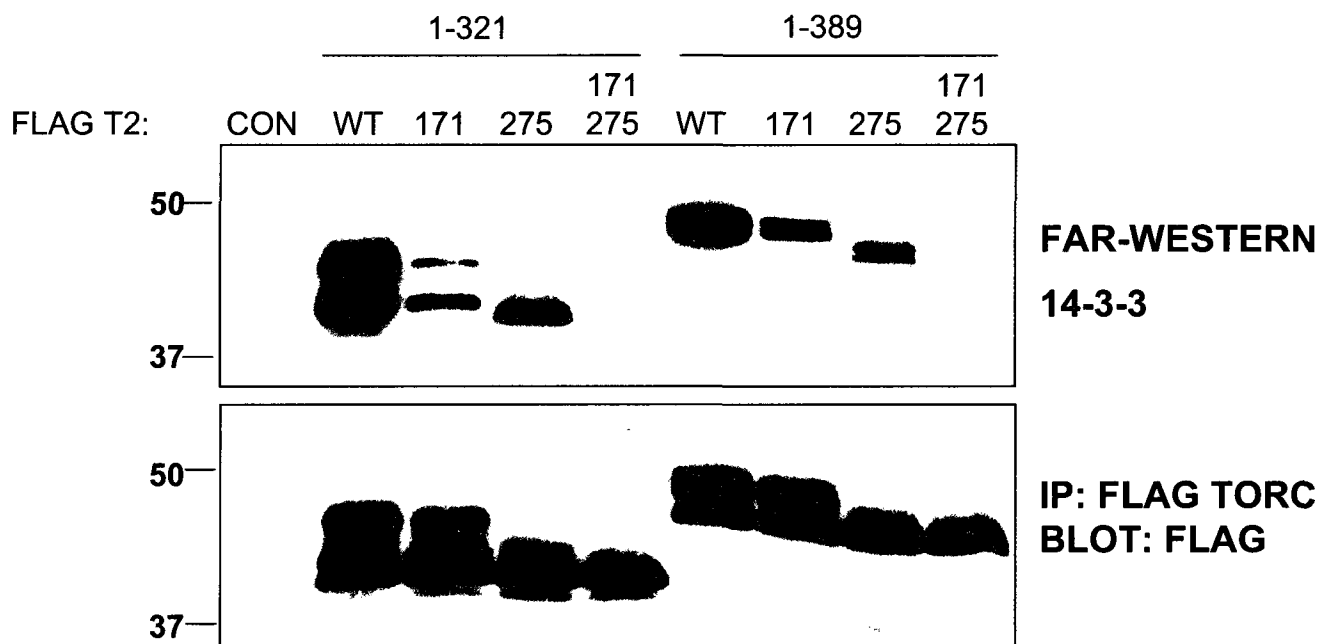
Mode 1 RXX-[pS/pT]XP
 Mode 2 RXXX[pS/pT]XP

14-3-3
 binding

S171	T1: W R R T N S D S A L H	+
	T2: L N R T S S D S A L H	
	T3: L N R T N S D S A L H	
S70	T1: Q Y Y G G S L P N V N	-
	T2: S H Y G G S L P N V N	
	T3: Q Y H G G S L P N V S	
S127	T1: G R Q A D S C P Y G T	-
	T2: T R H I D S S P Y S P	
	T3: G R Q F D G S A F G A	
S238	T1: T K K T G S R P K S C	-
	T2: A K K L S S S S R P	
	T3: T K E I Q S L S G R P	
S245	T1: S S R P R S C E V P G	-
	T2: S S R P R S C E V P G	
	T3: S G R P R S C D V G G	
S275	T1: H N T G G S L P D L T	+
	T2: M N T G G S L P D L T	
	T3: L N T G G S L P D L T	

Figure 3-4 Mutation of S171 and S275 to A abolishes 14-3-3 binding in the context of the first 389 amino acids.

Far western analysis of HIT-T15 cells transfected with C-terminal deletions (1-321, and 1-389 are amino acids present) of FLAG-tagged TORC2 wild type and Serine → Alanine mutants (S171A, S275A and S171A+S275A). FLAG-tagged proteins were immunoprecipitated and run on SDS-PAGE. Membranes were probed with GST-tagged recombinant 14-3-3 proteins to examine binding. Membranes were also probed for FLAG as a control of loading. In the context of either of the deletion mutants mutation of either S171 or S275 to Alanine reduced binding, however, mutating the both two amino acids together eliminated binding of 14-3-3. This experiment was done twice.



This result was interesting as 14-3-3 binding was reduced by the mutation of S171, S275 and the double S171 and S275 mutant (Figure 3-5, lanes 4, 5 and 6), however it was not lost completely as was expected. Further mutation of Serines 127, 238 and 245 in the background of the double S171 and S275 mutant did not further reduce the binding of 14-3-3 proteins (Figure 3-5, lanes 7, 8, 9 and 10). I was able to identify S275 as a possible regulatory phosphorylation site, affecting TORC2:14-3-3 binding, however, the full length double S171A and S275A mutant retained 14-3-3 binding. This led me to believe that there remained an additional phosphorylation site(s) that regulated 14-3-3 binding. For this reason I examined the effect of the S369A mutant, a residue that was identified as a phosphorylation site previously (62). I repeated the previous experiment where HIT-T15 cells were transfected with FLAG-tagged wild-type, S171A, S275A, S171A and S275A, S369A and the S171A, S275A and S369A triple mutant to examine 14-3-3 binding. Once again, the single mutants, as well as the double (S171A and S275A) mutant resulted in reduced 14-3-3 binding (Figure 3-6, lanes 3,4 and 5). Interestingly, the S369A mutant reduced but did not abolish 14-3-3 binding (Figure 3-6, lane 6). However, it was only the triple mutant with S171A, S275A, and S369A that completely lost 14-3-3 binding (Figure 3-6, lane 7).

Figure 3-5 Full-length mutant TORC2 (S171A + S275A) retains 14-3-3 binding.

Far western analysis of HIT-T15 cells transfected with full-length FLAG-tagged TORC2 wild type and Serine→ Alanine mutants (amino acid mutated is indicated by number). FLAG-tagged proteins were immunoprecipitated and analyzed using SDS-PAGE. Membranes were probed with GST-tagged recombinant 14-3-3 proteins to examine binding. Membranes were also probed for FLAG as a control of loading. 14-3-3 binding is reduced in the single mutants S171A, S275A as well as the double mutant, S171A + S275A. Binding is not significantly reduced by additional Serine→Alanine mutations to the double mutant, compared to the double mutant. This experiment was repeated at least three times.

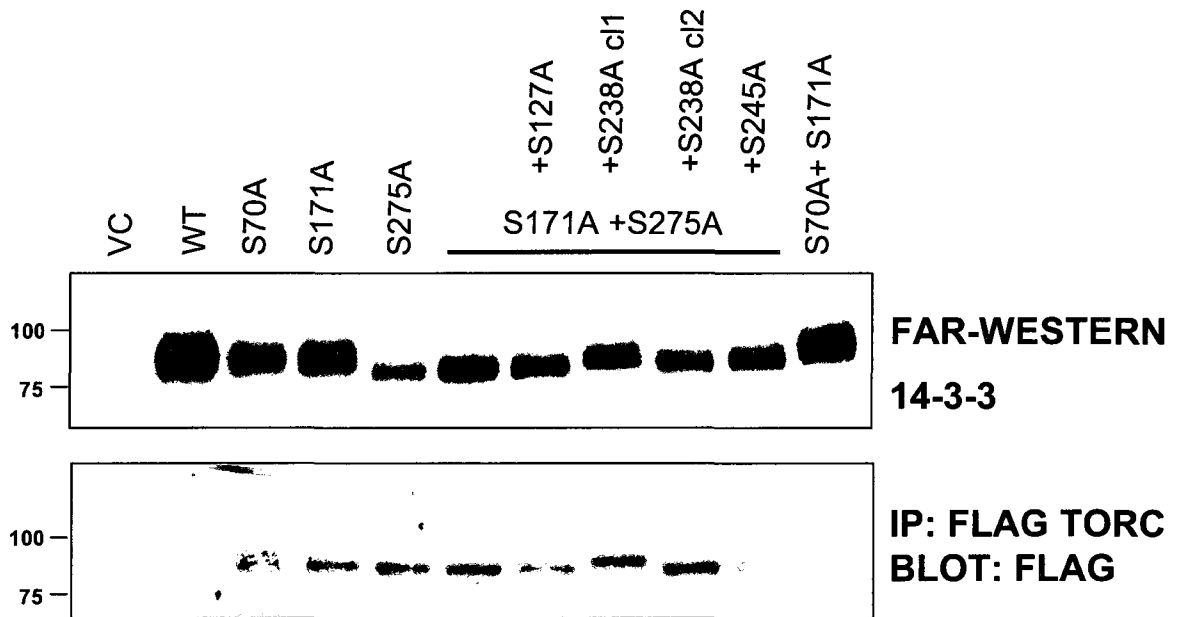
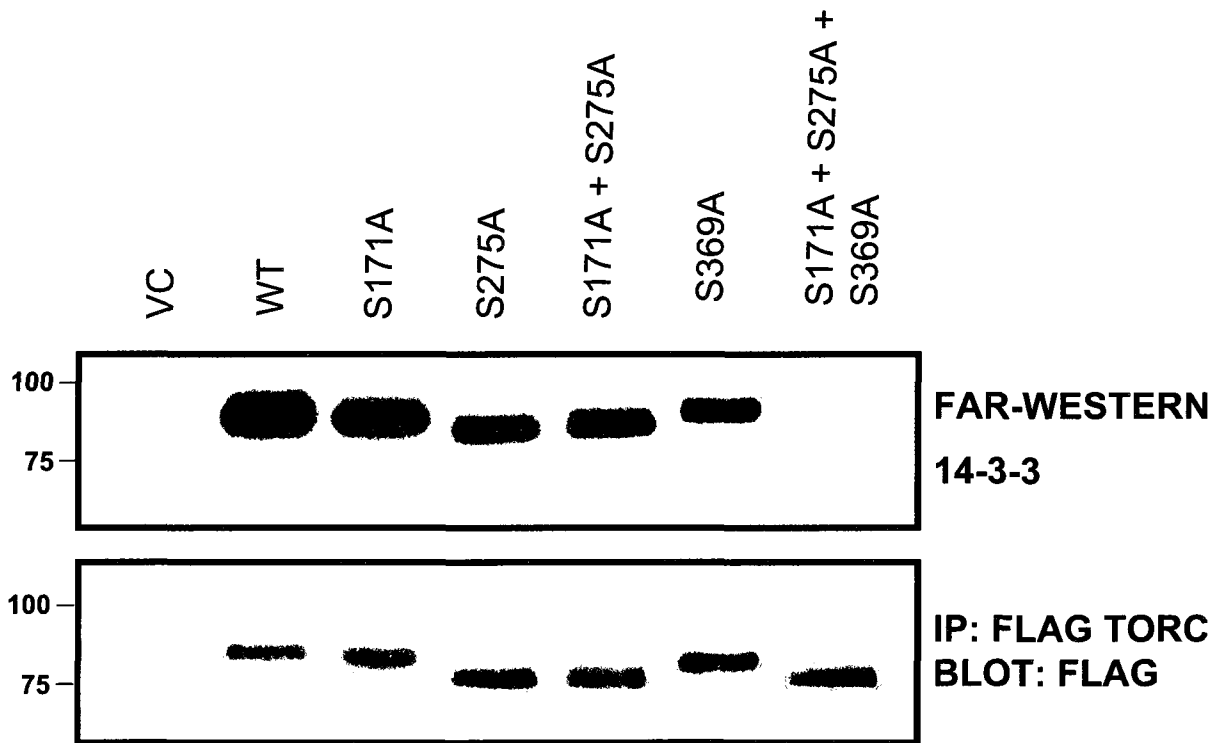


Figure 3-6 Full-length TORC2 requires mutation of S171, S275 and S369 to abolish 14-3-3 binding in beta cells.

Far western analysis of HIT-T15 cells transfected with full-length FLAG-tagged TORC2 wild type and Serine → Alanine mutants (amino acid mutated is indicated by number). FLAG-tagged proteins were immunoprecipitated and analyzed using SDS-PAGE. Membranes were probed with GST-tagged recombinant 14-3-3 proteins to examine binding. Membranes were also probed for FLAG as a control of loading. 14-3-3 binding is reduced in the single mutants S171A, S275A, S369A as well as the double mutant, S171A + S275A. However, 14-3-3 no longer binds the full-length protein when all three Serines (S171+S275+S369) are mutated to Alanine.



Localization of TORC2 Mutants

The model thus far indicates that TORC2 dephosphorylation and subsequent release from 14-3-3 proteins are required for its nuclear entry and subsequent CREB co-activation. As such, TORC2 dephosphorylation is thought to be a result of cAMP and Ca²⁺ signals. I hypothesized that the mutant that was unable to bind 14-3-3 proteins would enter the nucleus without a glucose or cAMP stimulus. In order to investigate this, I performed an immunofluorescence assay to localize TORC2 in beta cells. I transfected FLAG-tagged wild-type TORC2, along with the mutants S171A, S275A, and the double mutant (S171A + S275A) into HIT-T15 cells. This experiment was first done in regular cell media, untreated. Cells were fixed and immunofluorescence was performed against FLAG, and the results were visualized with a fluorescence microscope. I had observed that the double mutant (S171A + S275A) was localized primarily to the nucleus, while all other TORC2 constructs resided in the cytoplasm (data not shown). However, since the real question was whether this double mutant was able to enter the nucleus without stimulation, and the cell media used in this case contained 20mM glucose, it was necessary to repeat the experiment in the absence of glucose. The same experiment was repeated and prior to fixation, the cells were starved for 1 hour in KREBs Ringer Buffer (KRB) without glucose. Quantification of the localization of TORC2 as either nuclear, cytoplasmic or both showed that the double mutant (S171A and S275A) was over 90% in the nucleus in the absence of glucose or cAMP stimuli (Figure 3-7, bottom). In addition there was no significant difference between the wild-type, or either of the single mutants (S171A or S275A).

Figure 3-7 Identification of a constitutively nuclear TORC2.

HIT-T15 cells were transfected with FLAG-TORC2 wild-type and Serine→Alanine mutants.

Top: Cells were starved of glucose for 2X 0.5 hours in KRB then fixed. Cells were stained for FLAG and visualized with a fluorescence microscope. WT is wild-type TORC2, the numbers indicate the Serine residue mutated to Alanine, (Green indicates TORC2, blue indicates DAPI stain, bar indicates 10µm).

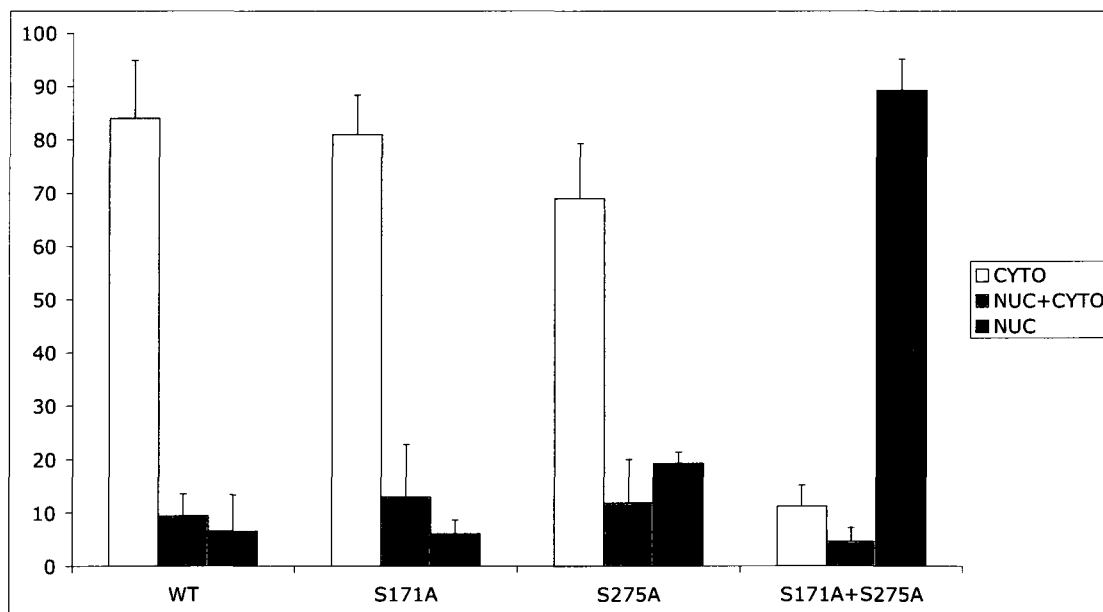
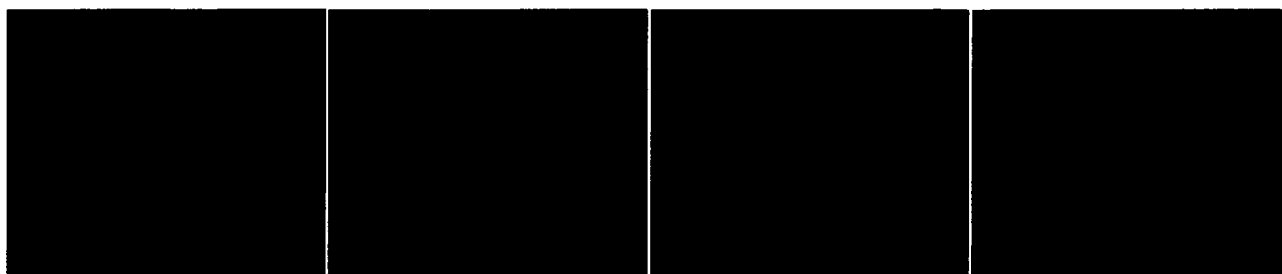
Bottom: Cells were quantified as cytoplasmic (cyto), nuclear (nuc), or both (nuc + cyto) with a total of 300 cells counted per transfection and experiment was done three times. Error bars indicate +/- standard deviation. The double mutant TORC2 S171a + S275A resides over 80% in the nucleus under glucose starved conditions.

WT

171

275

171+275



The far-western data had revealed that the triple mutant (S171A + S275A + S369A) had been the only mutant unable to bind 14-3-3 proteins, yet it appeared not to be necessary for TORC2 nuclear entry as there was no significant difference in localization between it and the double mutant (Figure 3-8, top). This result could indicate that the phosphorylation of this specific amino acid might be required for 14-3-3 binding, however not involved in TORC2 nuclear localization.

Previously TORC2 was shown to be responsible for the co-operative effect of cAMP and Ca^{2+} signals on CREB activation (62). The S171 site had been reported to be responsive to cAMP signaling in HEK293T cells by becoming dephosphorylated resulting in localization of TORC2 to the nucleus. These previous experiments had also shown that beta cells required both a cAMP and a glucose stimulus for nuclear entry. In order to elucidate the signaling mechanism governing the dephosphorylation of both S171 and S275 in beta cells I transfected both single mutants (S171A and S275A) into HIT-T15 cells. I was interested in the localization of these mutants in the presence of either cAMP or Ca^{2+} stimuli. To tease apart the signaling pathways involved in the regulation of these phosphorylation sites I quantified the localization of either single mutant in response to cAMP, or Ca^{2+} with KRB as an unstimulated control. The results revealed that the nuclear localization of the S171A mutant increased 5-fold in response to Forskolin (cAMP stimulus) compared to the control (Figure 3-8, bottom). Interestingly this mutant was localized in the nucleus approximately 80% of the time in response to glucose (Ca^{2+} stimulus). A similar, yet opposite result was seen for the S275A mutant; in the absence of stimulus this TORC2 mutant was found primarily in the cytoplasm.

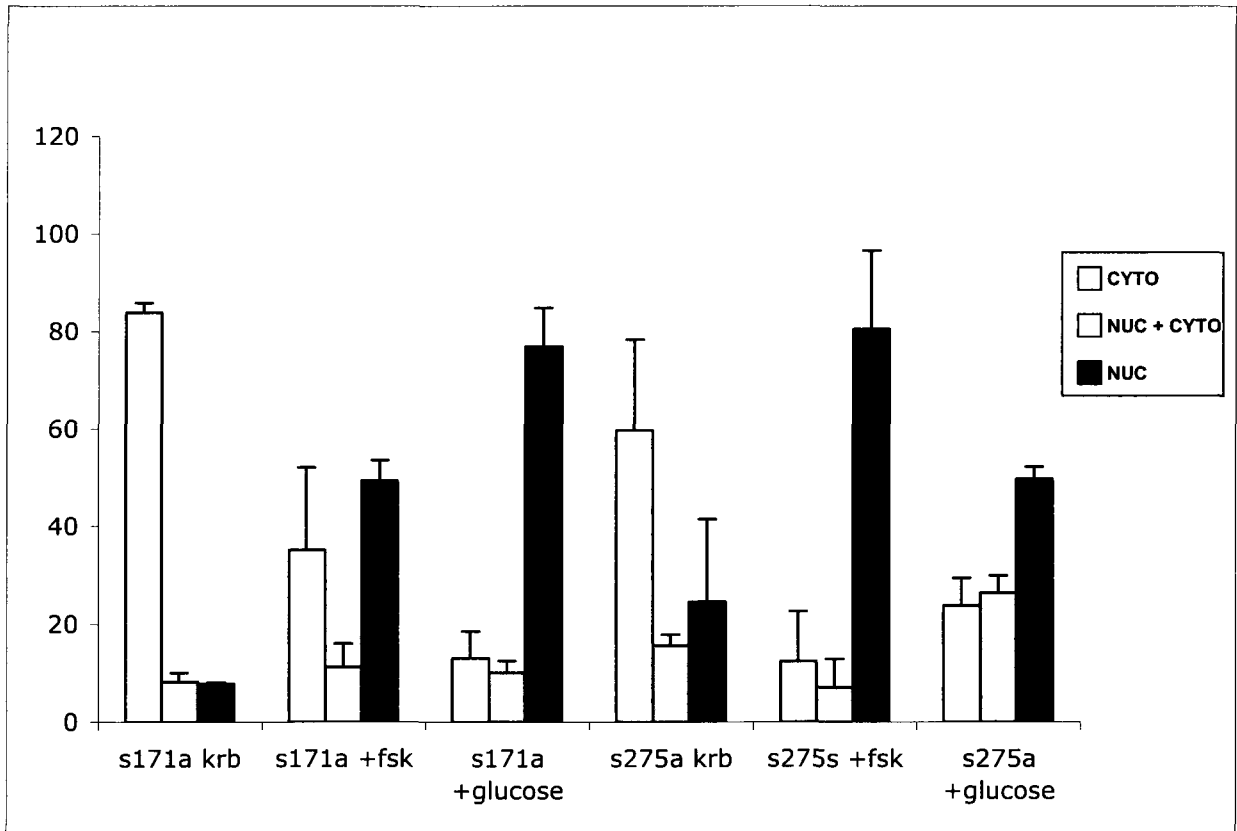
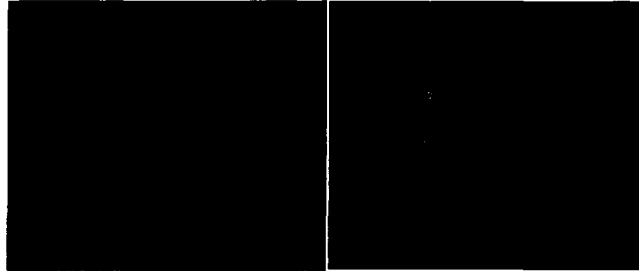
Figure 3-8 S369 does not effect TORC2 nuclear localization.

Top: HIT-T15 cells were transfected with Flag-TORC2 Serine→Alanine mutants. Numbers indicate the Serine → Alanine mutation. Cells were starved of glucose for 2 X 0.5 hours in KRB then fixed and stained for FLAG. (Green indicates TORC2, blue indicates DAPI stain, bar indicates 10µm). This experiment was repeated at least three times.

Bottom: HIT-T15 cells were transfected with FLAG-TORC2 S171A or S275A and starved for 2 X 0.5 hours, then treated with either Forskolin (10µM) or 20mM glucose for 0.5 hours. Cells were quantified as cytoplasmic (cyto), nuclear (nuc), or both (nuc + cyto) with a total of 300 cells counted per transfection and experiment was done three times. Error bars indicate standard deviation.

369

171+275+369



Upon treatment with glucose this S275A mutant relocated to the nucleus approximately 50% of the time, and this number increased to over 80% with Forskolin treatment. This result would indicate that both phosphorylation sites respond primarily to one stimulus, S171 to cAMP and S275 to Ca²⁺. However, there was an increase of nuclear localization with both mutants in response to both cAMP and Ca²⁺, which indicates that there may be a certain level of cross-talk between these two signals.

Constitutively Nuclear TORC Activates CREB without Stimulation

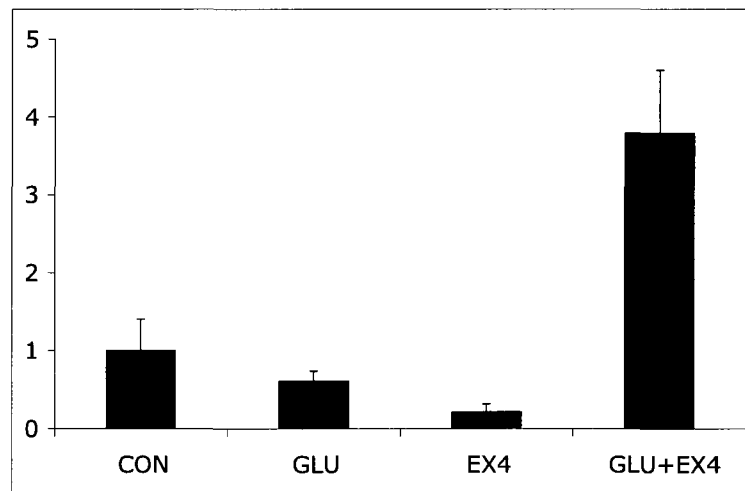
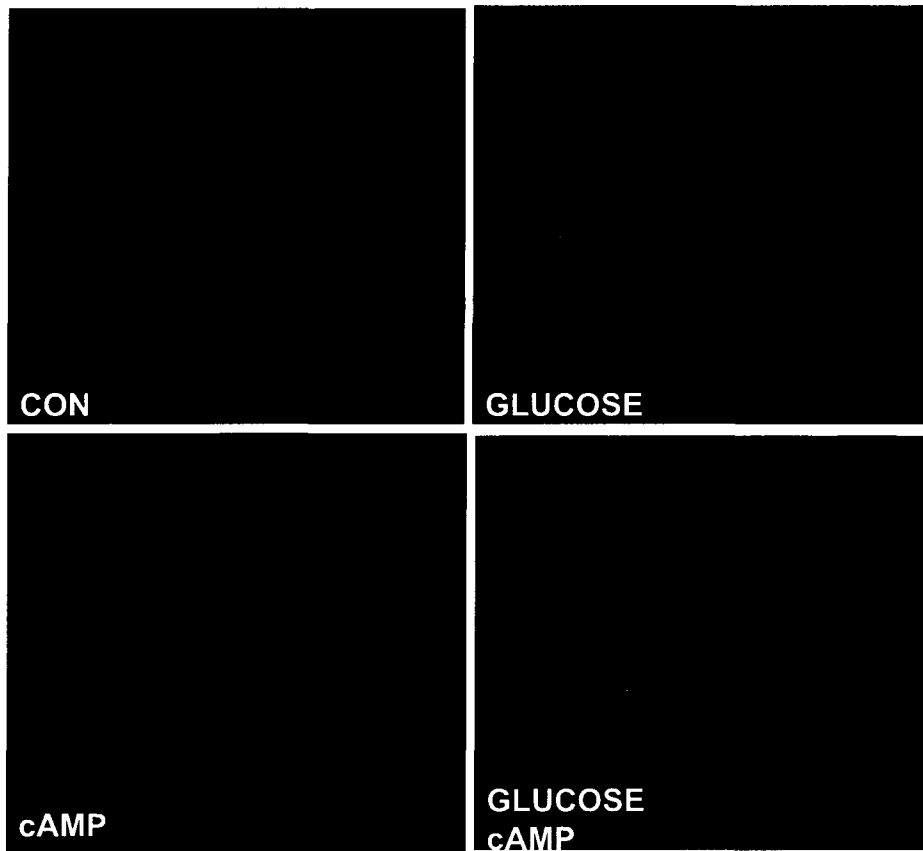
Beta cells require two signals; cAMP and Ca^{2+} to fully activate CREB-dependent transcription (62). These signals are triggered in the body from gut hormones GLP-1 and glucose respectively. Previously to induce Ca^{2+} signaling I would treat cells with KCl, which depolarizes the cell and leads to Ca^{2+} influx. However, physiologically, it is glucose that performs this function in the beta cells of the body. We wanted to determine if endogenous TORC2 responded to this physiological stimulus in beta cells by relocating to the nucleus and activating CREB. HIT-T15 cells were starved of glucose, then treated with either Forskolin (cAMP stimulus) or glucose, or the two together. The cells were then fixed and stained for endogenous TORC2. Andy Ng in the lab was able to show that in HIT-T15 cells neither glucose or cAMP alone were able to cause TORC2 nuclear relocation. However, in the presence of both stimuli, endogenous TORC2 is found in the nucleus where it can activate CREB dependent transcription (Figure 3-9, top).

Additionally as CREB requires TORC2 nuclear entry in response to cAMP and Ca^{2+} stimuli, if glucose and cAMP can relocate endogenous TORC2 to the nucleus in beta cells it should be able to activate CREB-dependent transcription. Indeed data from Chantal Depatie showed that levels of CREB target gene mRNA in MIN6 cells increased only with both Exendin-4 (cAMP agonist) and glucose stimuli (Figure 3-9, bottom). Previously published data indicates that the Ca^{2+} responsive phosphatase Cn, is able to dephosphorylate TORC2 in response to a depolarizing stimulus (62).

Figure 3-9 TORC2 requires cAMP and glucose stimuli for nuclear entry and CREB target gene activation.

Top: HIT-T15 cells were starved for 2 X 0.5 hours in KRB then treated with Exendin-4 (cAMP stimulus) (10nM) and/or glucose (20mM) for 0.5 hours then fixed. Cells were stained with TORC2 antibody from rabbit, and Alexa Fluor® 488-conjugated anti-rabbit secondary antibody. TORC2 is only able to enter the nucleus following stimulation with both glucose and cAMP. Bar indicates 50µm (Experiment done by Andy Ng).

Bottom: Quantitative PCR analysis of CREB target gene, NR4A2 in mouse islets. Following isolation from C57Bl6 mice, islets were starved in KRB for 2 X 0.5 hours, then treated with Exendin-4 (10nM) and/or glucose (20mM). Histogram reveals a synergistic activation of CREB in response to glucose and cAMP. (Experiment done by Chantal Depatie).



In order to investigate this and confirm TORC2 in the glucose responsive pathway, a CREB reporter assay was done to measure the ability of endogenous TORC2 to activate a CREB reporter gene in the presence of the phosphatase inhibitor cyclosporine A (CsA). To confirm that the Calcineurin inhibitor CsA was able to inhibit glucose induced CREB activity HIT-T15 cells were transfected with CRE-luciferase to measure CREB activity. After 40 hours half of the cells were pre-treated with CsA and then were treated with forskolin and/or glucose. Figure 3-10 reveals that CsA is able to inhibit CREB activity induced by cAMP and glucose. These experiments provided me with sufficient evidence that TORC2 is responsive to glucose and cAMP in beta cells.

I have been able to demonstrate that there are two amino acids (S171 and S275) that are responsible for TORC2 nuclear localization, and there are two signals (cAMP and glucose) that are responsible for TORC2 nuclear localization and CREB activation. Furthermore mutation of these two serines abolishes the necessity of cAMP or glucose for TORC2 nuclear entry. The hypothesis that follows is that the mutant that could enter the nucleus sans stimuli, could potentially also activate CREB. In order to test this, I used a reporter assay to test the ability of the double mutant to activate a CRE-luciferase construct. I co-transfected into HIT-T15 cells FLAG-tagged wild-type as well as Serine→Alanine mutants (S171A, S275A and the double mutant S171A+S275A) with the CRE-luciferase plasmid and RSV-β-galactosidase to normalize. The reporter assay revealed that in glucose starved conditions while transfection of the single mutants S171A or S275A increase reporter activity approximately 20- and 10-fold compared to wild-type respectively and transfection of the double mutant (S171A+S275A) was able to activate the CREB reporter 60-fold compared to wild-type TORC2 (Figure 3-11, top).

Figure 3-10 The synergistic activation of CREB is dependent on Calcineurin (Cn).

CREB reporter data measuring luciferase activity of a CRE-luciferase reporter gene in HIT-T15 cells. Cells were starved in KRB for 0.5 hours, then treated with Forskolin (10 μ M) and/ or glucose (20mM) for 4-6 hours. Histogram depicts fold activity of the CREB reporter gene in comparison to untreated. Black bars indicate pre-treatment of 2X 1 hour with the Calcineurin inhibitor, Cyclosporin A (1 μ M). Data is a representation of the experiment done twice, error bars are +/- standard deviation from samples performed in triplicate.

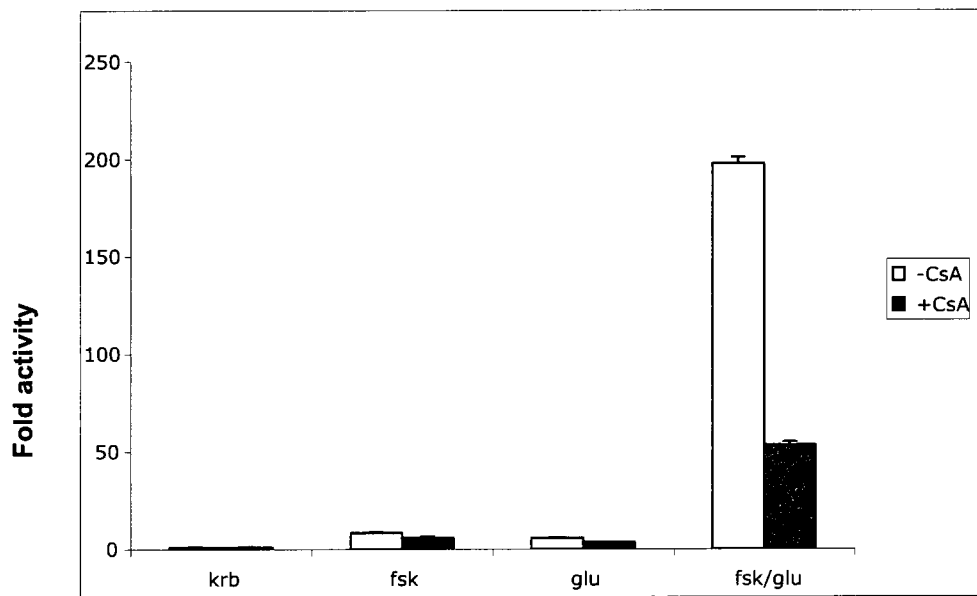
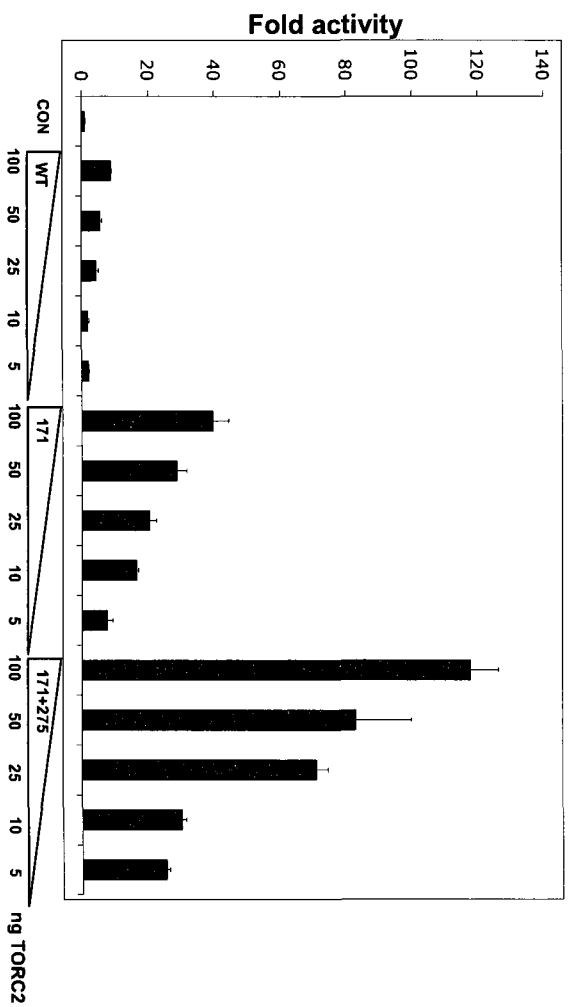
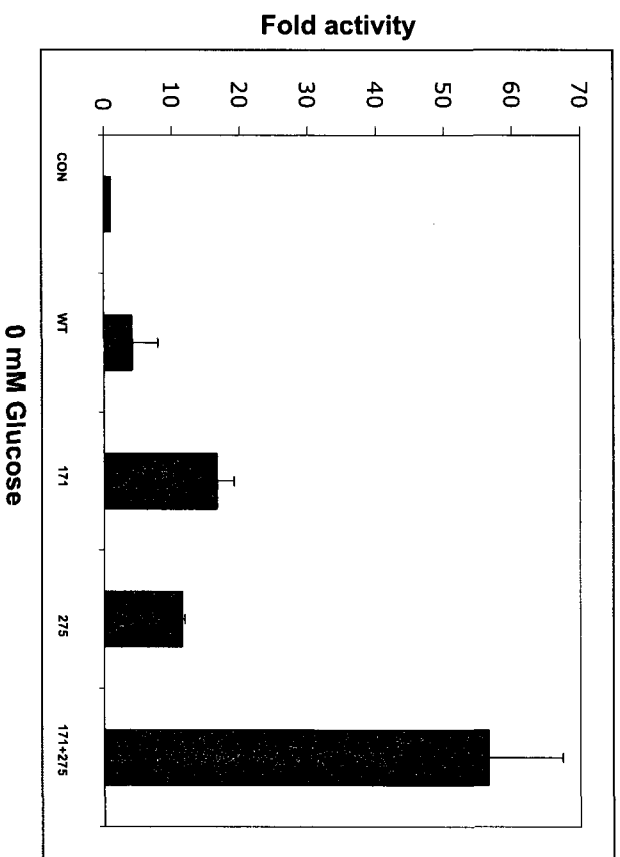


Figure 3-11 The TORC2 double mutant S171A + S275A is constitutively active in beta cells.

CREB reporter data measuring luciferase activity of a CRE-luciferase reporter gene in HIT-T15 cells co-transfected with FLAG-TORC2 wild-type and mutants (numbers indicate Serine→ Alanine mutations). Data is a representation of experiments done three times, error bars are +/- standard deviation from samples done in triplicate.

Top: Fold activity of luciferase of TORC2 constructs compared to vector alone in HIT-T15 cells starved 4-6 hrs with KRB.

Bottom: Fold activity of luciferase of titrated TORC2 constructs compared to vector alone in HIT-T15 cells starved 4-6 hrs with KRB. CREB reporter activity is dependent on TORC2 concentration.

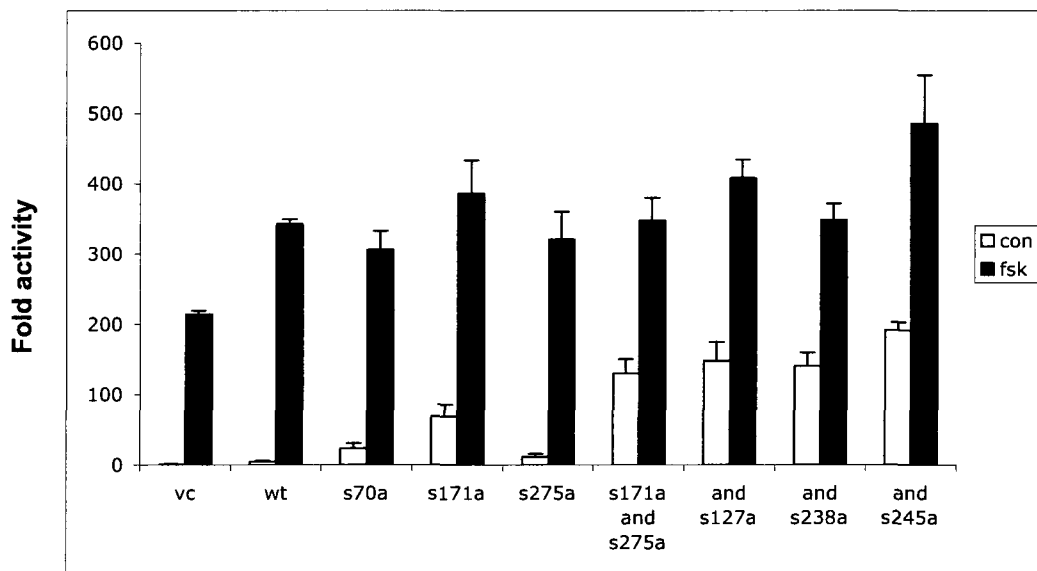


At first glance this does appear to be quite a substantial increase in activity, however, in reference to Sreaton *et al.* 2004, which had shown a 300-fold activation it would appear that there was still some necessity for cAMP and/or Ca²⁺ stimuli for full CREB activation. For this reason I repeated the reporter experiment titrating in increasing amounts of TORC2 to determine if the CREB activation was somewhat dependent upon concentration of TORC2 plasmid transfected. The dependence of CREB activity on concentration of TORC2 was confirmed in a plasmid concentration dependent manner in HIT-T15 cells (Figure 3-11, bottom).

TORC2 localization data had revealed that mutation of S171 and S275 was sufficient to relocate TORC2 to the nucleus in beta cells. However, the far-western analysis showed that mutation of S171+S275+S369 to alanine was required to lose 14-3-3 binding. This would indicate that perhaps S369 played a role in the nucleus binding to 14-3-3, and it was unclear whether that was related to CREB activity. Therefore I tested the ability of the single mutant S369A, as well as the triple mutants S171A+S275A+(S369A or S127A or S238A or S245A) to activate a CREB reporter gene. Reporter data indicated no significant difference of these triple mutants in CREB activity compared to the double mutant (Figure 3-12). This result does not mean that these amino acids do not play a role in TORC2 function, it might simply be that they are involved in another pathway of TORC2 that does not involve CREB.

Figure 3-12 Additional mutations are not significantly different from the double mutant in CREB activation.

CREB reporter data measuring luciferase activity of a CRE-luciferase reporter gene in 293T cells co-transfected with FLAG-TORC2 wild-type and mutants (numbers indicate Serine→ Alanine mutations). Histogram depicts fold activity in comparison to vector alone in regular cell media. Black bars indicate treatment with forskolin for 4-6 hours. Data is a representation of experiments done three times, error bars are +/- standard deviation from samples done in triplicate.



Phosphorylation of S275 on TORC2 is Regulated by Glucose in Beta Cells

Screaton et al. had shown that the phosphorylation status of S171 in TORC2 was regulated by cAMP signaling (62). Once TORC2 was dephosphorylated, 14-3-3 proteins would dissociate and allow TORC2 to enter the nucleus. The TORC2 mutant S275→A that could no longer be phosphorylated, in addition to the S171A mutation reduced 14-3-3 binding, allowed TORC2 nuclear entry and CREB target gene activation. Additionally, often the phosphorylation status of proteins can be detected by their migration on SDS-PAGE. We noticed that TORC2 had a lower migrating species in response to glucose (Figure 3-13, top, lanes 2 and 3, experiment done by Rob Screaton), and this same shift was seen when S275 was mutated to Ala (Figure 3-13, bottom, lane 4). To confirm that it was indeed the phosphorylation status of S275 that was regulated by glucose in beta cells we generated a phospho-specific antibody against S275 of TORC2. I performed a western blot analysis of MIN6 cells in glucose starved media or treated with 20mM glucose for 30 minutes and examined phospho-275 levels. I observed that phospho-275 levels decrease with glucose treatment, but not cAMP treatment (Figure 3-14, top, lane 2 and 3 respectively). Additionally a time-course experiment in HIT-T15 cells showed that P275 levels begin to decrease after only 5-10 minutes of glucose treatment (Figure 3-14, bottom, lane 4). Pre-treatment of these cells with cyclosporine A (a known calcineurin inhibitor) restored phospho-S275 levels to control levels (in starved media) as seen in Figure 3-15, top, lane 6, indicating that glucose treatment activates the phosphatase calcineurin to dephosphorylate TORC2 at S275.

Figure 3-13 The shift in TORC2 in response to glucose corresponds to the shift of the S275A mutation.

Top: Western blot analysis of endogenous TORC2 in MIN6 cells in normal growth medium (NM) or starved of glucose in KRB for 2X 0.5 hours (-Glucose) and treated with glucose (20mM) and/or Exendin-4 (10nM) for 0.5 hours. Upper band indicates phosphorylated TORC2. (Experiment done by Rob Screatton).

Bottom: Western blot analysis of HIT-T15 cells transfected with FLAG-TORC2 wild-type and S→A mutants in regular cell media. Membrane was probed for FLAG. Presence of S275→A mutation reveals a lower migrating species, consistent with shift following glucose treatment.

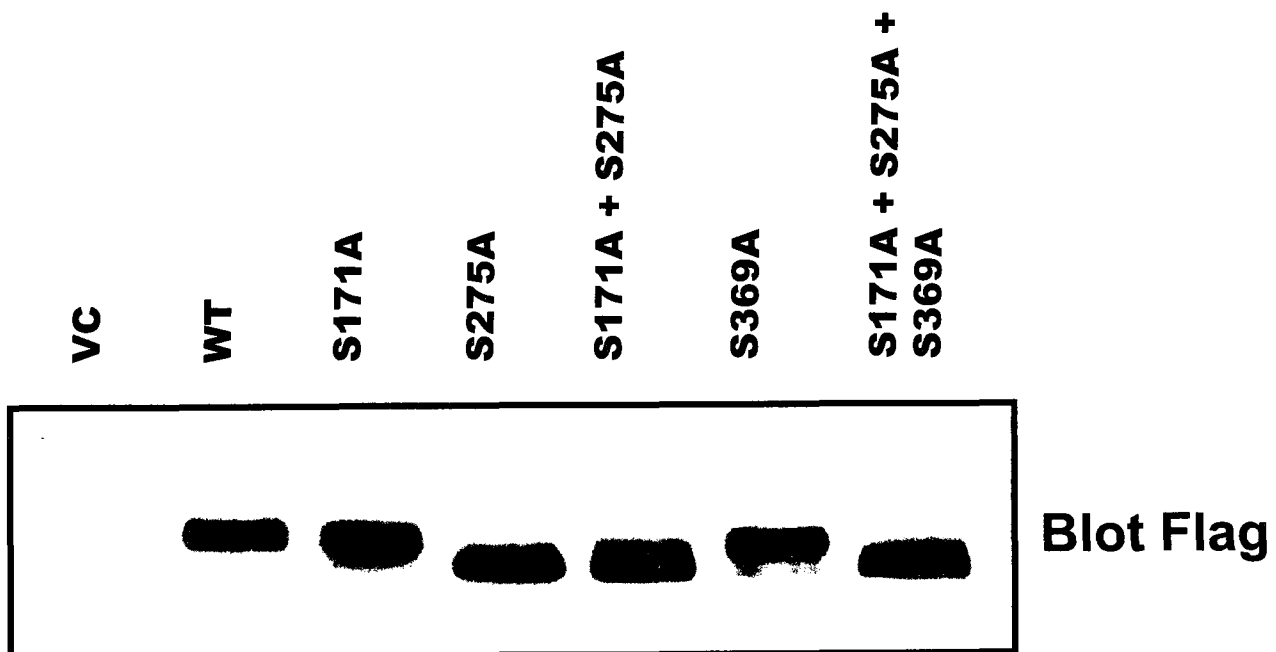
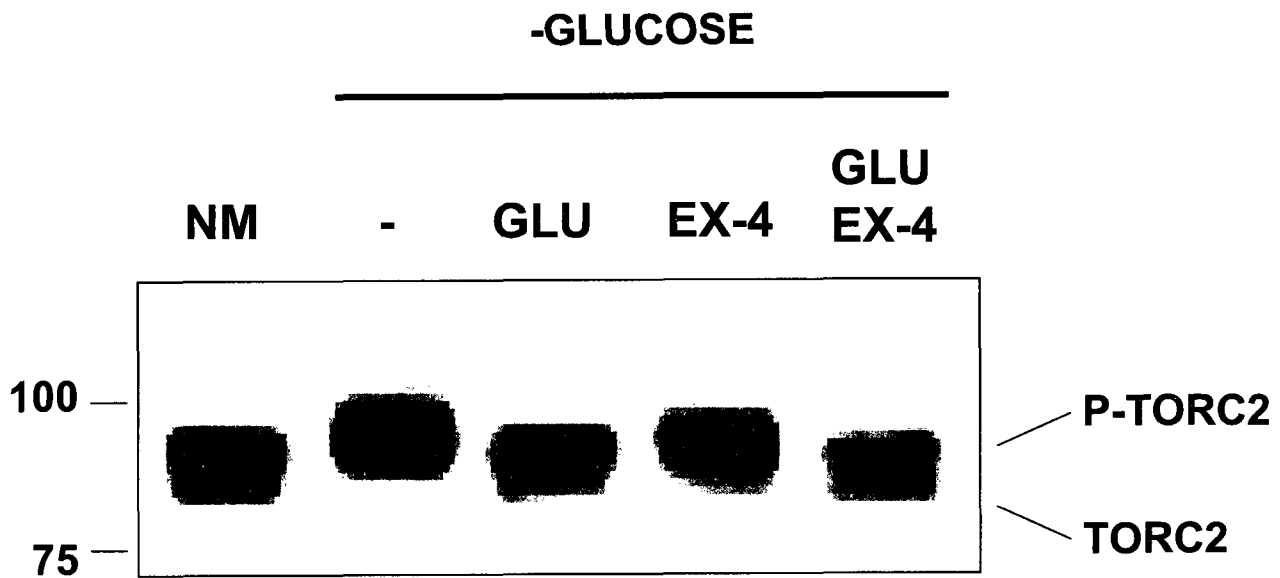


Figure 3-14 S275 phosphorylation responds to glucose in a time-dependent manner.

Top: Western blot analysis of endogenous TORC2 in MIN6 cells. Cells were starved of glucose in KRB for 2X 0.5 hours, then treated with glucose (20mM) and/or Exendin-4 (10nM). Phospho-275 TORC2 antibody reveals reduced P275 in response to glucose. Bottom: Western blot analysis of endogenous P275 in response to glucose (20mM) in HIT-T15 cells. Reduced P275 is observed after only 5 minutes of glucose treatment.

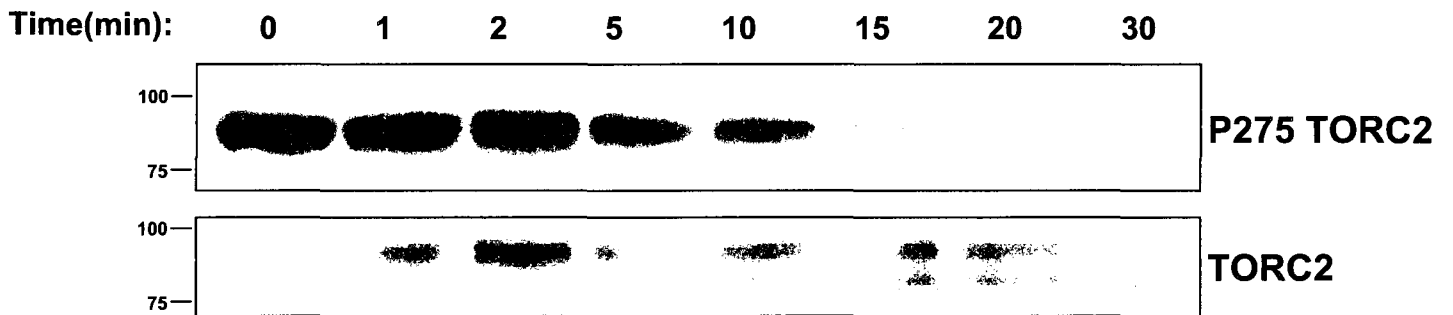
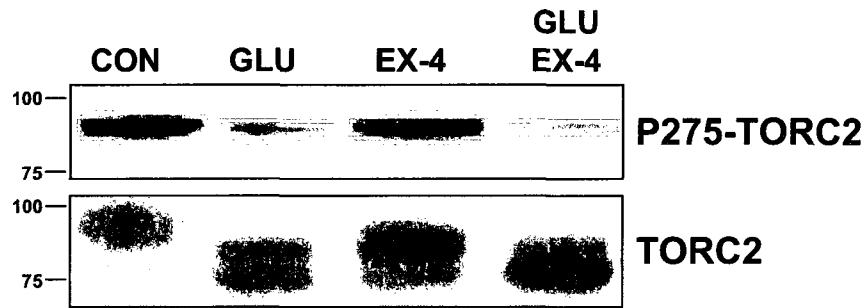
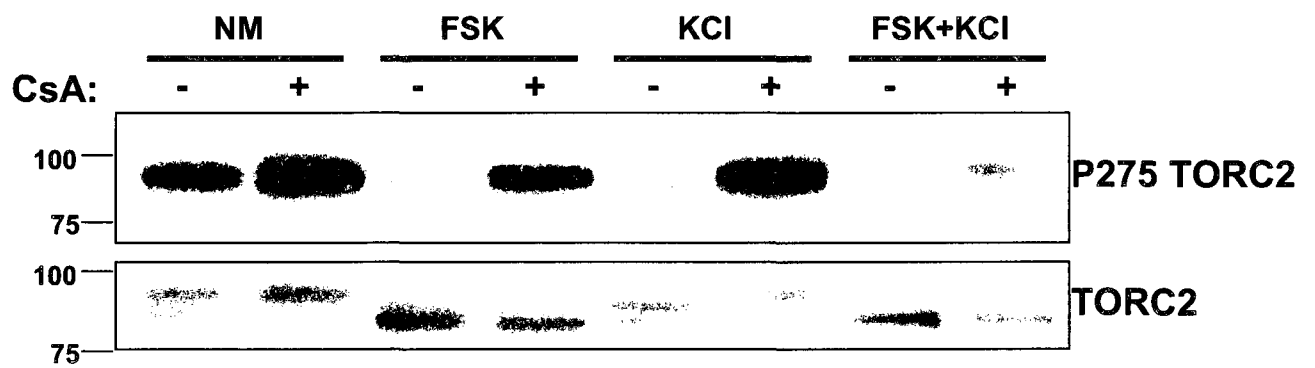
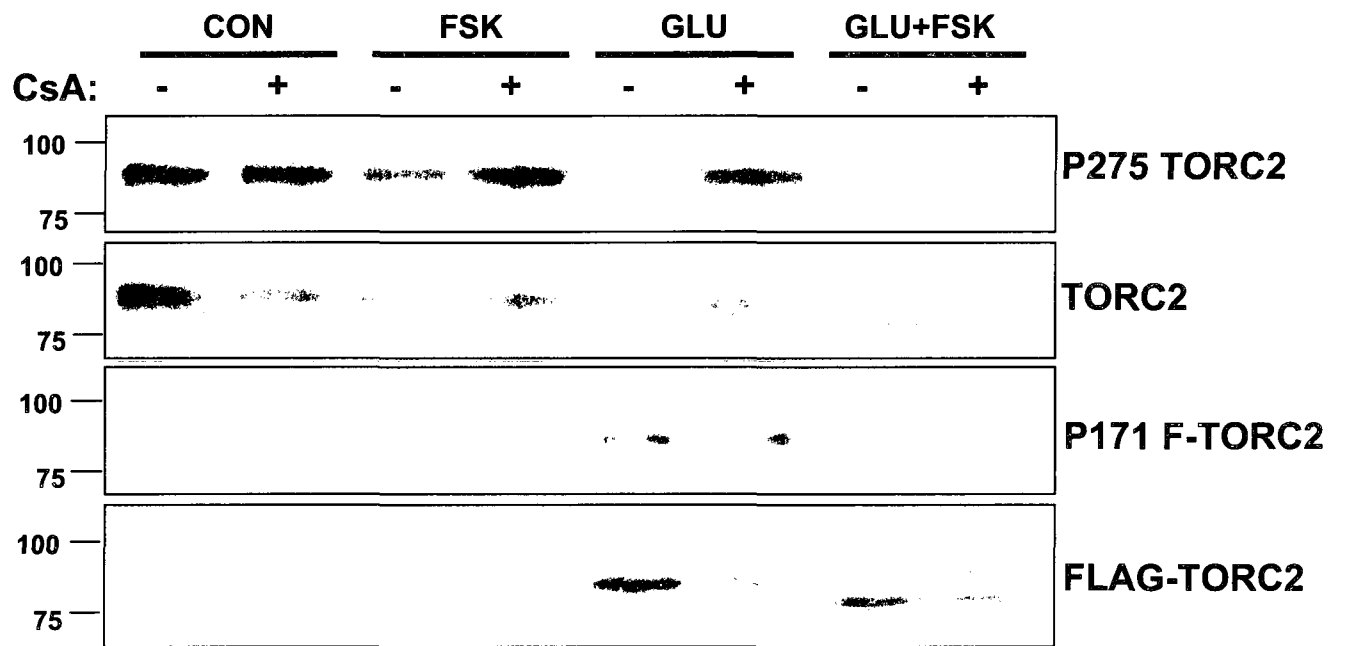


Figure 3-15 P275 responds to glucose and P171 responds to cAMP.

Top: Western blot analysis of endogenous Phospho-275 TORC2 in HIT-T15 cells. Cells were starved of glucose for 2 X 0.5 hours in KRB and treated with FSK (10 μ M) and/or glucose (20mM) for 0.5 hours. Probing with a phospho-specific antibody to S275 or S171 reveals a reduction in P275 levels in response to glucose only, and a reduction in P171 levels in response to cAMP only. Treatment with (+) or without (-) cyclosporin A (1 μ M) shows a rescue of P275 levels but not P171 levels indicating that P275 is regulated by calcineurin.

Bottom: Western blot analysis of endogenous Phospho-275 levels in HIT-T15 cells in regular growth media. Cells treated with forskolin (10 μ M) or a depolarizing stimulus (KCl, 45mM) with (+) or without (-) cyclosporine A (1 μ M) treatment. P275 blot reveals a decrease in P275 levels in response to both cAMP and Ca²⁺ stimuli.

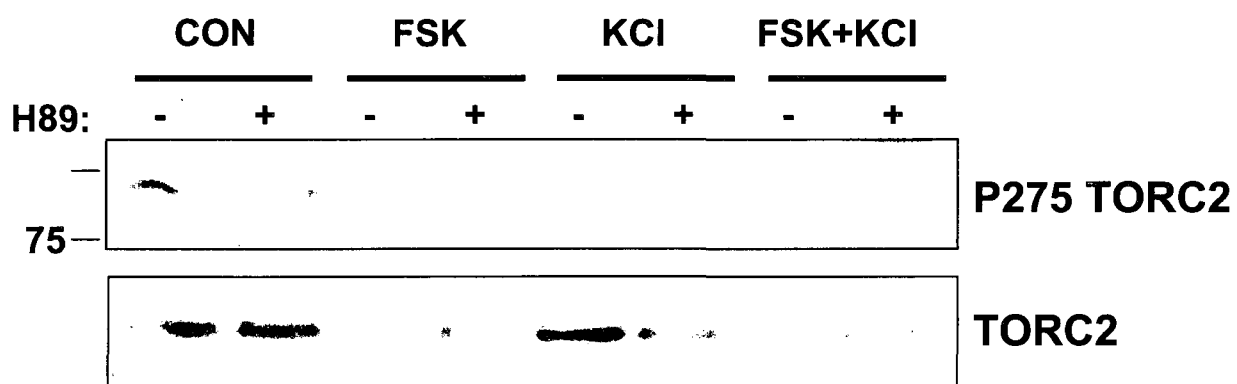
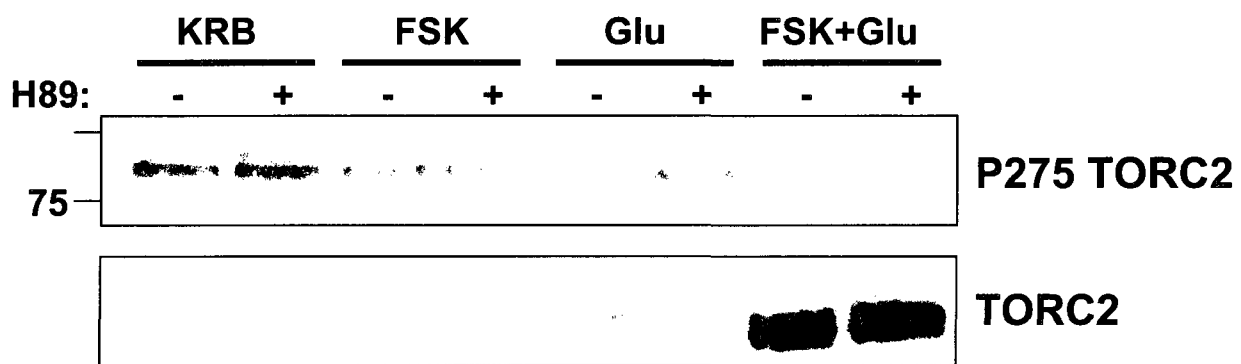


To further delineate the regulation of TORC2 at the phosphorylation level I performed a western blot analysis of phospho-S171 levels with a phospho-specific antibody for S171 and revealed that P171 levels decrease with forskolin treatment but not glucose (Figure 3-15, top, lanes 3-6). Whereas forskolin treatment had no effect on P275 status in the absence of glucose (Figure 3-15, top, lane 3), this treatment did cause a decrease in P275 levels in regular cell media containing 20mM glucose (Figure 3-15, bottom, lane 3). Furthermore, while pre-treatment of cells with cyclosporine A rescued S275 phosphorylation, it had no effect on P171 levels. Data up to this point indicated that cAMP could affect phospho-S275 levels, therefore to confirm I examined the effect of the PKA inhibitor on P275 levels. PKA is a kinase that phosphorylates SIK2 in the presence of cAMP, inhibiting the phosphorylation of S171 (62). Hence, if the inhibitor of PKA (H89) affects P275 levels, then cAMP does achieve some level of cross talk in this pathway. Western blot analysis of P275 levels in response to H89 showed no difference in the absence of glucose (Figure 3-16, top, lanes 3 and 4). However, H89 treatment did show a rescue of S275 phosphorylation when glucose was present (Figure 3-16, top, lanes 5-8) and in regular growth media (Figure 3-16, bottom, lanes 3-8). This data would indicate the phosphorylation status of S171 responds primarily to cAMP, and S275 responds to glucose. However S275 dephosphorylation can be augmented by treatment with a cAMP stimulus signifying the importance of both cAMP and Ca^{2+} signals in TORC2 nuclear entry and CREB activation

Figure 3-16 The PKA inhibitor H89 rescues P275 dephosphorylation in the presence of glucose.

Top: Western blot analysis of endogenous P275 TORC2 in HIT-T15 cells. Cells were starved of glucose and treated with forskolin (10 μ M) and/or glucose (20mM) with (+) or without (-) pretreatment of PKA inhibitor H89. In the absence of glucose H89 has no affect on P275 levels.

Bottom: Western blot analysis of endogenous P275 TORC2 in HIT-T15 cells. CON indicates regular growth medium, cells were treated with forskolin (10 μ M) and/or KCl (45mM) with (+) or without (-) pretreatment of H89. Glucose is required for H89 rescue of P275 levels.



To demonstrate the biological importance of this glucose regulated phosphorylation site in primary cells, I isolated mouse islets from C57Bl6 mice. Islets were starved in Krebs Ringer Buffer (KRB) (zero glucose) and stimulated with glucose, forskolin or both. Half of the samples were also pretreated with cyclosporine A and P275 levels were examined by western blot (Figure 3-17). Consistent with the beta cell line data, P275 levels are unchanged with Exendin-4 treatment but are reduced in response to glucose in islets. Additionally the phosphorylation levels of S275 are rescued with pre-treatment of cyclosporine A.

The necessity for CREB for islet survival has already been established (59). CREB activity has also been shown to be dependent on TORC2 in MIN6 cells (62). To tie this data together I set out to confirm the requirement of TORC2 for islet survival. In order to determine the necessity of TORC2 in islets I infected islets with an adenovirus expressing a TORC2 short-hairpin. Western blot analysis of the lysates revealed elevated levels of cleaved caspase-3 and cleaved PARP in the samples where TORC2 was knocked down (Figure 3-18). This would indicate that there is increased cell death in the absence of TORC2 in islets, resulting from hindered survival. This data further demonstrates that TORC2 is a critical component in beta cell survival.

Figure 3-17 Phospho-275 is regulated by glucose and Calcineurin in islets.

Top: Western blot analysis of primary islets starved of glucose for 2 x 0.5 hours, and treated with Exendin-4 (10nM) and/or glucose (20mM) reveals a decrease in P275 levels in response to glucose. Bottom: Western blot analysis of primary islets starved of glucose for 0.5 hours, treated with (+) or without (-) cyclosporine A (1 μ M) for 1hour, followed by Exendin-4 (10nM) and glucose (20mM) treatment. The loss of P275 with Exendin-4 and glucose treatment is rescued by treatment with cyclosporine A.

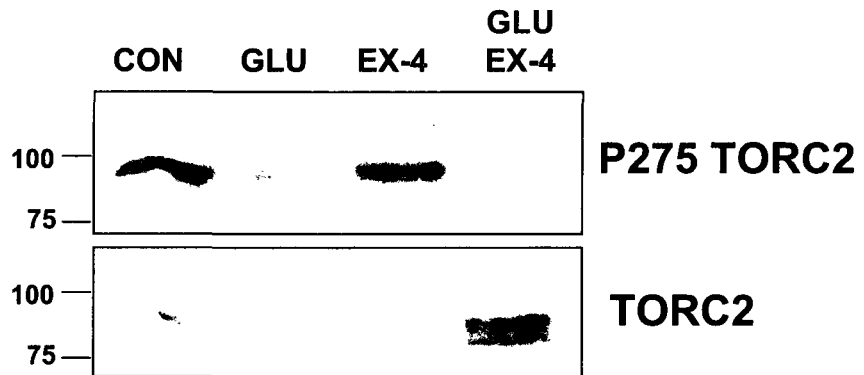
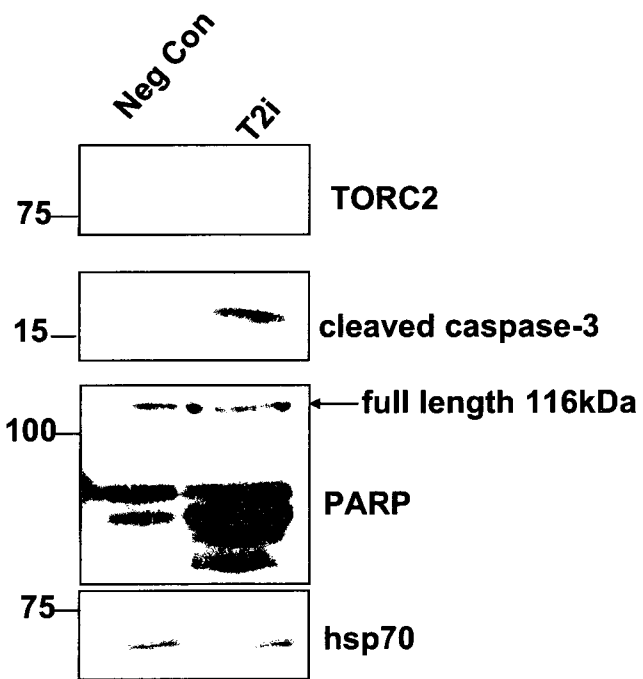


Figure 3-18 Knockdown of TORC2 activates apoptotic signaling in primary mouse islets.

Western blot analysis of primary mouse islets infected with adenovirus expressing a control short-hairpin or a hairpin targeting TORC2. Membrane was probed with anti-cleaved caspase-3 and PARP, both indicators of apoptotic signaling. An approximately 70% reduction in TORC2 in islets increases cleaved caspase-3 and cleaved PARP levels.

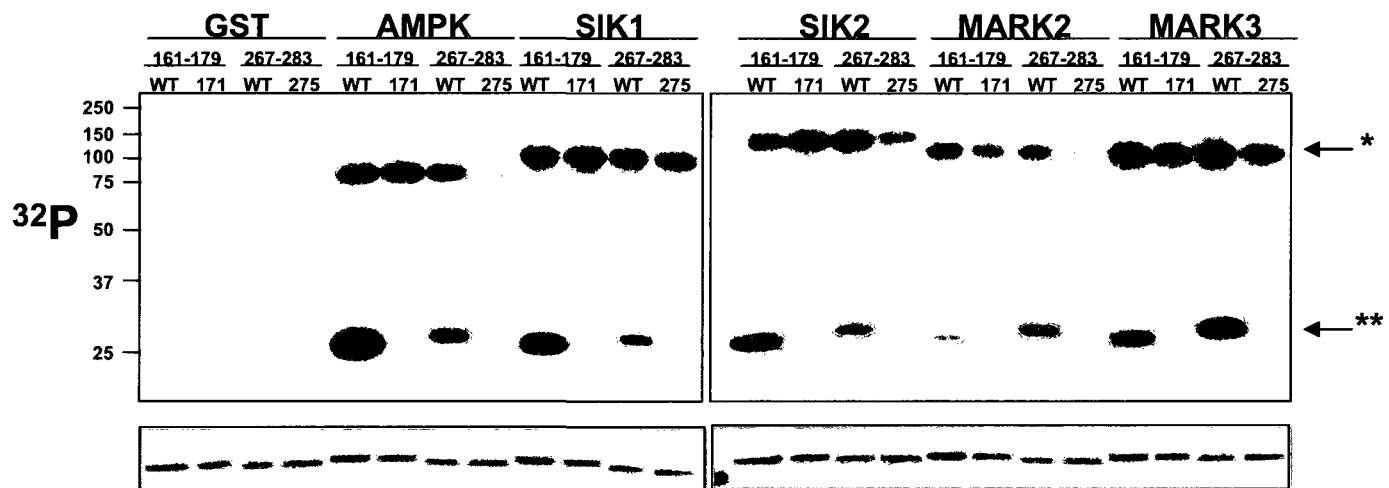


S275 on TORC2 is Phosphorylated by the AMPK family of Kinases

Previously published data had shown that S171 was phosphorylated by SIK2 and in this phosphorylated state TORC2 is retained in the cytoplasm. Moreover, expression of a consistently active SIK2 was enough to retain TORC2 in the cytoplasm and reduce CREB activity even after forskolin treatment (62). We sought out to determine the signaling pathway that regulated the phosphorylation of S275 and therefore needed to discover the kinase that phosphorylated S275. We performed a kinase screen using 180 recombinant GST-kinases transfected into 293T cells. The *in vitro* kinase assay using a peptide corresponding to the region of S275 revealed that MARK2 and MARK3 were able to phosphorylate S275 (78). As the MARKs are members of the AMPK family of kinases, Accalia Fu in the lab did an *in vitro* kinase assay testing the ability of AMPK, SIK1, SIK2, MARK2 and MARK3 to phosphorylate peptides corresponding to regions surrounding S275 and S171. Interestingly the *in vitro* kinase assay using GST-purified kinases from MIN6 cells revealed that all were able to phosphorylate both substrates (Figure 3-19).

Figure 3-19 The AMPK family of kinases phosphorylate TORC2 S275.

Top: In vitro kinase assay measuring ability of purified GST-kinases AMPK α 1, SIK1, SIK2, MARK2, and MARK3 to phosphorylate peptides corresponding S171 and S275 (TORC2 aa 161-179 and TORC2 aa 267-283, both wild-type and S \rightarrow A mutants). MIN6 cells were transfected with GST-tagged kinases and purified to use in an *in vitro* kinase assay * indicates TORC2 phosphorylation and ** indicates autophosphorylation of the kinase.. Experiments done by Accalia Fu.



The fact that these kinases can phosphorylate both sites does not necessarily mean that it will happen in vivo. In order to test the effects of these kinases on TORC2-dependent CREB activity in cells I co-transfected into HIT-T15 cells TORC2 wt or S→A mutants with the individual kinases to measure CREB reporter activity. The results revealed that both SIK1 and SIK2 were able to inhibit wild-type and mutant TORC2 (data not shown). However, MARK3 and to our surprise, constitutively active AMPK (AMPK-CA) were not able to inhibit CREB activity. MARK2 expression did inhibit wild-type TORC2 and the S171A mutant, but not S275A or the double S171A + S275A mutant (Figure 3-20). AMPK is known to be the energy sensor of the cell, however my results show that it does not affect TORC2 S275-dependent CREB activity. This is interesting because S275 is regulated by glucose. Therefore to confirm that AMPK is not affecting the phosphorylation status of S275 I performed western blot analysis of HIT-T15 cells under starved or glucose treated conditions. Phospho-AMPK levels confirmed that AMPK is phosphorylated, and activated in low glucose (Figure 3-21), which is why we were surprised when it did not effect CREB activity. To confirm that indeed AMPK is not acting on this phosphorylation site to inhibit TORC2 I treated MIN6 cells with increasing amounts of and activator of AMPK, AICAR and blotted for P275. Figure 3-21, bottom, shows that although phosphorylation levels of the AMPK substrate acetyl-CoA carboxylase increase, P275 levels do not.

Figure 3-20 MARK2 inhibits TORC-dependent CREB activity in beta cells.

Top: CREB reporter assay in HIT-T15 cells co-transfecting TORC2 wt and S→A mutants with vector or constitutively active AMPK. AMPK does not significantly affect CREB activity. Bottom: CREB reporter assay in HIT-T15 cells co-transfecting TORC2 wild-type and S→A mutants with vector alone or MARK2 or MARK3 kinases. MARK2 is able to inhibit TORC2 wild-type, and single mutants S171A and S275A, but not the double mutant S171A + S275A.

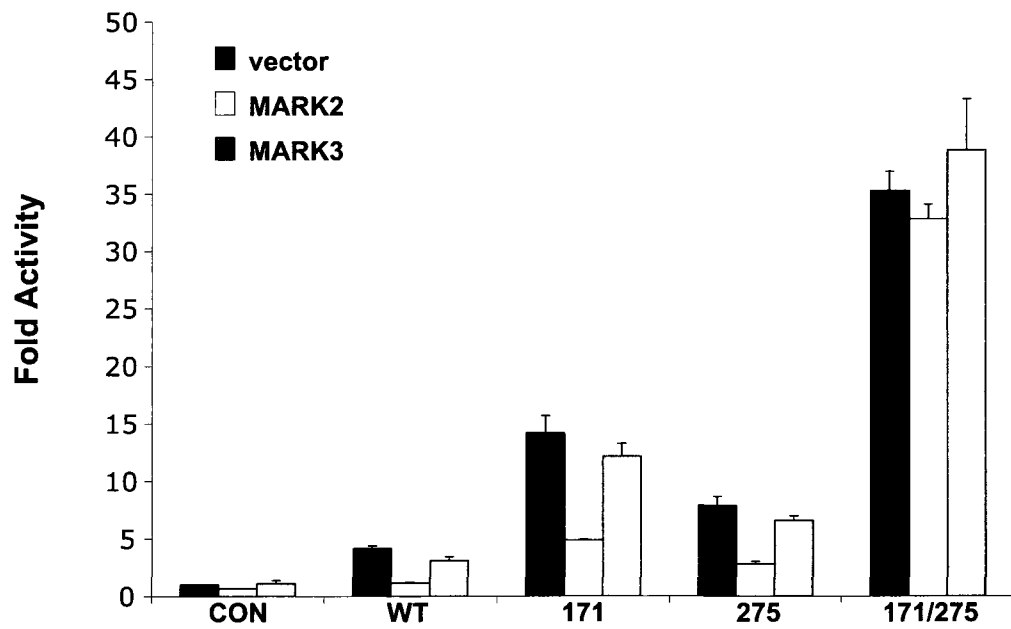
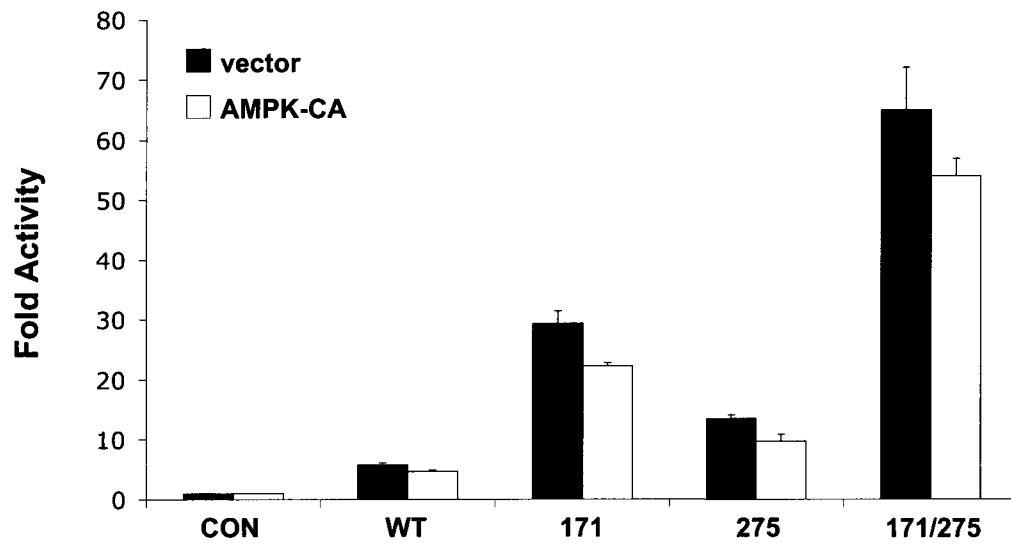
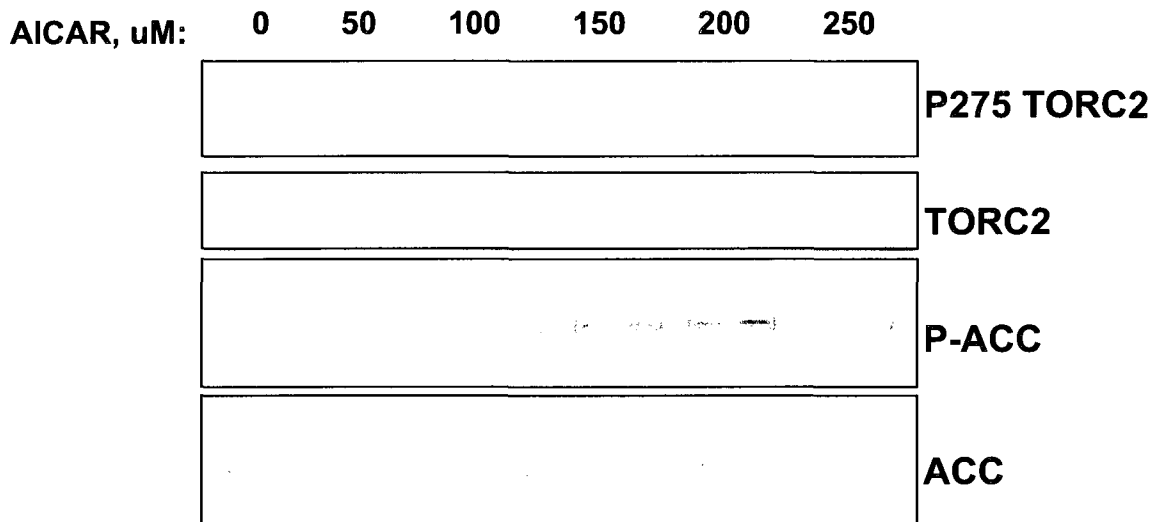
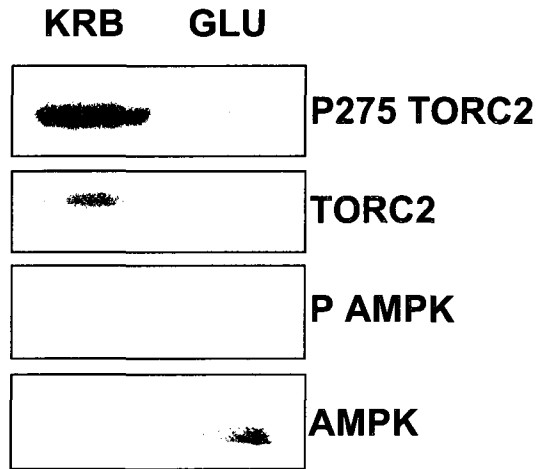


Figure 3-21 AMPK does not affect TORC2 S275 phosphorylation.

Top: Western blot analysis of endogenous P275 TORC2, and P-AMPK and AMPK levels of HIT-T15 cells starved with KRB 2 X 0.5 hours with or without treatment of 20mM glucose. Bottom: Western blot analysis of MIN6 cells treated with AICAR 1hr blotted for P275 and TORC2 as well as phospho-acetyl CoA carboxylase (PACC) and ACC. Although AMPK is regulated by glucose, as is P275, AMPK does not affect P275 levels.



Chapter 4

DISCUSSION

Scientists are beginning to discover the importance of TORC2 in the metabolic tissues of the body that respond to feeding and fasting cues. Recent data reveals the importance of TORC2 in hepatocytes, its regulation by insulin (74) and as a glucose sensor (79). TORC1 has also been shown recently to be involved in leptin and Stat-3 signaling of the hypothalamus, controlling weight gain and fertility (80). I was interested in delineating the pathway of TORC2 activity in the beta cells of the pancreas and have made some significant discoveries.

Recently TORC2 was shown to be required for CREB activity in beta cells. It was my intention to identify the regulatory domains and specific amino acids responsible for CREB activation. TORC2, and therefore CREB, are both responsive to the second messengers, cAMP and Ca^{2+} , which are interestingly the same signals that are required for insulin secretion. Indeed delineating the mechanism of this activation, specifically in beta cells, is metabolically and medically important.

It was previously shown that TORC is responsible for the synergistic activation of CREB by cAMP and Ca^{2+} stimuli. My data has revealed that these two stimuli act on two separate amino acid residues in the signaling cascade leading to CREB target gene activation (see Figure 4-1). In non-glucose responsive cells such as HEK293T, cAMP causes the dephosphorylation of S171 on TORC2 resulting in its release by 14-3-3 proteins followed by its nuclear entry. I have shown that in beta cells, cAMP stimulation is required but insufficient for TORC2 nuclear entry. In beta cells, glucose is required for dephosphorylation of S275, and this in combination with cAMP will produce nuclear

localization of TORC2. In addition, S275 can become dephosphorylated after cAMP treatment but only in the presence of glucose, re-iterating the dependence of the regulatory site on glucose. It is interesting that in 293 cells, S275 and S171 are phosphorylated, but both are dephosphorylated following cAMP treatment (Andy Ng, unpublished data 2008). One explanation that can be offered for this is perhaps beta cells have adapted to regulate the phosphorylation of S275 by glucose specifically. This would be logical because their response to glucose is crucial to the metabolism of the organism. As the amino acid sequence surrounding these two sites is conserved in TORC1, 2 and 3 from zebrafish to humans, it would be fascinating to see how these sites are regulated in other metabolic tissues such as hepatocytes, muscle, and adipose tissues. Recent evidence has shown that in hepatocytes, S70 of TORC2 is responsive to glucose and when this serine is mutated to aspartate to mimic a phosphorylated residue, it loses the glucose response (79). I had tested this residue for its involvement in TORC2 nuclear localization and CREB activation. Curiously I had found that the mutation of this Serine to Alanine made no difference in 14-3-3 binding or CREB activity. This is yet another example of differential signaling of the same protein (TORC2) in two separate metabolic tissues. Specifically it would be interesting to determine the regulation and role of this phosphorylation site in neurons, which are constantly responding to signals cued by Ca^{2+} influx.

I had also observed that although mutation of S171 and S275 to alanine in the context of truncated TORC2 abolished 14-3-3 protein binding, the full length protein required the additional mutation of S369 to Alanine to inhibit 14-3-3 binding. With this result the hypothesis was that mutation of all three Serines would be required to result in

constitutively nuclear TORC2; however, this was not the case. The double mutant S171 + S275 was over 90% nuclear in the absence of glucose or cAMP. The additional mutation of S369 was not significantly different. This poses the question: what is the regulatory purpose of this amino acid binding to 14-3-3s? The fact that the amino acid sequence surrounding S369 is not conserved suggests its role is evolutionarily novel. As mutation of S369 makes no difference in localization of TORC2 or CREB activity, perhaps it is important in another aspect of TORC function that is beyond its CREB coactivator role. Additionally, we have no knowledge of the signals that control S369 phosphorylation. Is this site imperative to metabolic processes, or something completely different such as cell survival, differentiation, insulin secretion?

Our lab identified MARK2 as the kinase that phosphorylates S275 on TORC2. The proposed model of the signaling pathway leading to TORC2 activation in beta cells to date is shown in Figure 4-1. Overexpression of this kinase in HIT-T15 cells inhibits TORC2-dependent CREB activity in a reporter assay. Interestingly, co-expression of TORC2 in the presence or absence of MARK2 in regular glucose conditions showed no difference in TORC2 localization (data not shown). This result could indicate that MARK2 activity itself is not regulated, that it is the phosphatase Cn alone that is regulated and it is a balance of MARK2 kinase activity versus the phosphatase activity that changes in response to glucose, instead of an on/off switch in the cell. Additionally SIK1 and SIK2 were able to inhibit CREB-induced activity in all TORC2 mutants. We were quite confused by this result, if cAMP does not primarily affect S275, then why, when S171 is mutated do the SIKs play a role? Perhaps the reason is because the kinases are being overexpressed in these cells, therefore become less specific. The better

experiment to match the CREB reporter would be a knock-down of these kinases in the presence of the mutant TORCs to determine CREB inhibition. I was in the process of attempting to knock-down MARK2 in HIT-T15 and MIN6 cells, however was unsuccessful. Ultimately if possible islets would be the ideal cells in which to perform these experiments, however due to the difficulty in culturing islets for long periods, and their low transfection efficiency, using cell lines is the preferred method for now.

Figure 4-1 Model of TORC2 activation.

A novel glucose responsive phosphorylation site in TORC2 has been identified. Serine 275 is regulated by glucose in beta cells while cAMP controls serine 171 phosphorylation. De-phosphorylation of these two sites leads to the synergistic TORC2 dependent CREB target gene transcription.

cAMP



PKA



SIK2



S171

Glucose



Ca²⁺



Cn



MARK2



S275

TORC2



As we did not have a reliable antibody for MARK2, I had transfected esiRNAs targeting MARK2 into HIT-T15 and MIN6 cells and measured CREB target gene expression by PCR as a preliminary trial. I did not see any difference in the mRNA levels when expressing these esiRNA constructs. The reason for this could have been that the constructs did not work, or that the MARK2 protein has a long half life and the timeline I was using was simply too short. Additionally, there are 4 MARK proteins, and there may be redundancy between the different isoforms. However, this explanation is doubtful because recently transgenic MARK2 whole knock-out mice were made and these mice had presented with a fascinating phenotype. The MARK2 null mice were leaner, with increased metabolism, and insulin hypersensitivity (81). This is an indication that we are indeed on the right track, however the phenotype of the MARK2 knockout mice may be misleading. This knockout is throughout the entire mouse and as I have shown you, TORC2 is differentially regulated in different metabolic tissues. Therefore, to see the true metabolic effect of the TORC2:MARK2 signaling cascade, MARK2 must be removed from one tissue at a time.

AMPK is known as the energy sensing kinase that controls glucose and lipid metabolism (82). Therefore, it appeared logical when it was identified as a potential kinase to phosphorylate S275. Indeed AMPK inhibits TORC2-dependent CREB activity in hepatocytes by phosphorylation of S171 (70). However, my research revealed that it was not involved in the phosphorylation status of S275 of TORC2 in beta cells. This leads to the conclusion that TORC2 is activated in two separate tissues by the same second messenger under opposing conditions.

In hepatocytes TORC2 is dephosphorylated under fasting conditions to activate gluconeogenic gene transcription by glucagon via the cAMP pathway to increase blood glucose levels (12). In beta cells TORC2 is dephosphorylated in feeding conditions by GLP-1 via the cAMP pathway to decrease blood glucose levels by enhancing survival and proliferation of beta cells (62). The ability of TORC2 to respond to opposite metabolic conditions in the liver and the pancreas via the same mechanism is fascinating and further investigation into this could prove quite valuable in the understanding of human metabolism.

Although several new treatment options for diabetes have arisen in the past few years, there remains a common hurdle to overcome: maintaining beta cell survival. Studies of islet transplants have revealed that the immunosuppressant, cyclosporine A (CsA) or Tacrolimus, could be causing apoptosis of the beta cells. The desired mechanism of action of CsA is as a Calcineurin inhibitor, inhibiting T-cell activation and cytokine gene expression (21). However, this drug has shown adverse effects such as post transplant diabetes mellitus (PTDM) in renal transplant patients (21). *In vitro* studies show that prolonged CsA treatment results in decreased insulin secretion and insulin content in the cells. In addition, *in vivo* studies show cyclosporine-induced beta cell degranulation, vacuolization, and a 50% decrease in mRNA content rat islets (83). The possible mechanism for this may be that CsA inhibits the activity of the phosphatase calcineurin (Cn) (84). Calcineurin activity is required for membrane depolarization-induced CREB activity (85) and promotes beta cell survival and proliferation (59). The signaling pathways that lead to CREB activation are not completely understood, and as such, the work in our lab will delineate the role TORCs play in beta cell survival.

The future of this research is ongoing in Dr. Sreaton's lab. Of great importance is the effect of SIK2 or MARK2 knock down on beta cell survival and CREB activity. A transgenic mouse model of a SIK2 or MARK2 inducible knock-out specifically in the beta cells of the pancreas is being generated. The hypothesis would be that these mice could maintain lower glucose levels even under a high fat diet as their beta cells would be constantly proliferating and surviving, therefore producing insulin. Perhaps inhibitors of these kinases could target the pancreas of diabetic patients to promote survival of the beta cells and hence insulin secretion. The current study identified a specific amino acid in TORC2 that is regulated by glucose in the beta cells of the pancreas. This contributes significantly to our current understanding of the regulatory mechanisms involved in human metabolism and maintenance of homeostasis.

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Curriculum Vitae

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Academic Background and Training

Master's	Biochemistry: Human Molecular Genetics	Master's of Science	University of Ottawa	CANADA	05/2007- 05/2009
Bachelor's, Honours	Forensic Biology	Bachelor of Science	Laurentian University	CANADA	09/2002- 04/2006

Technical Experience

MSc. Thesis Project: TORC2 Regulation in Beta Cells of the Pancreas
Objective: to investigate the regulation of the CREB coactivator TORC2. Used methods such as protein manipulation, expression and purification, western blot analysis, gene regulation and expression as well as cloning techniques, immunofluorescence, maintaining and treating cell cultures, kinase assays with radioactive materials and islet isolation from mice.

BSc. Preliminary experience with forensic techniques such as blood spatter analysis, fingerprinting, entomology, biological sample examination as introduced in course/laboratory work.

Work Experience

Senior Research Technician	University of Ottawa Cellular and Molecular Medicine	Examining the role of NFκB in breast cancer using mouse and cell culture models	10/2008-05/2009
Student Research Assistant	University of Calgary Biochemistry & Molecular Biology	Investigating telomerase activity and relation to ING proteins in cancer cells	08/2006 - 12/2006
Summer Student Research Assistant	Laurentian University of Sudbury Chemistry and Biochemistry	Design of a telomerase inhibitor via solid- phase protein synthesis	05/2005 - 08/2005

Laboratory Teaching Assistant	Laurentian University of Sudbury Chemistry and Biochemistry	Monitored and conducted marking and teaching assistance in 2 nd year biochemistry course	09/2004 - 12/2004
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Publications:

Jansson, D., Ng, A.C., Fu, A., Depatie, C., Al Azzabi, M., and Screatton, R. Glucose Controls CREB Activity in Islet Cells via Regulated Phosphorylation of TORC2. *Proc Natl Acad Sci U S A*, 105(28), 10161 (2008).

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Volunteer Experience and Extracurricular Activities:

Volunteer for Habitat for Humanity 2001-2008.

Volunteer for Actua Science Camp for Kids as a Mentor.

Also participated in the Terry Fox Run and the Run for the Cure.

I enjoy working part-time for, and playing recreational soccer, ultimate frisbee and floor hockey with the Ottawa Sport and Social Club.

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