CRTC2 Is Required for β-Cell Function and Proliferation

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Previous work in insulinoma cell lines has established that calcineurin plays a critical role in the activation of cAMP-responsive element binding protein (Creb), a key transcription factor required for β-cell function and survival, by dephosphorylating the Creb coactivator Creb-regulated transcription coactivator (Crtc)2 at 2 regulatory sites, Ser171 and Ser275. Here, we report that Crtc2 is essential both for glucose-stimulated insulin secretion and cell survival in the β-cell. Endogenous Crtc2 activation is achieved via increasing glucose levels to the physiological feeding range, indicating that Crtc2 is a sensor that couples ambient glucose concentrations to Creb activity in the β-cell. Immunosuppressant drugs such as cyclosporin A and tacrolimus that target the protein phosphatase calcineurin are commonly administered after organ transplantation. Chronic use is associated with reduced insulin secretion and new onset diabetes, suggestive of pancreatic β-cell dysfunction. Importantly, we show that overexpression of a Crtc2 mutant rendered constitutively active by introduction of nonphosphorylatable alanine residues at Ser171 and Ser275 permits Creb target gene activation under conditions when calcineurin is inhibited. Taken together, these data suggest that promoting Crtc2-Creb activity is required for β-cell function and proliferation and promoting this pathway could ameliorate symptoms of new onset diabetes after transplantation. (*Endocrinology* 154: 2308–2317, 2013)

Loss of functional β-cell mass underlies both type 1 and type 2 diabetes, with recovery ultimately requiring the restoration of normal β-cell glucose responsiveness along with the replenishment of β-cell mass. Islet transplantation is a promising strategy for restoring β-cell mass in patients with type 1 diabetes; it involves the transplantation of donor islets into the portal vein coupled with treatment with immunosuppressants tacrolimus (FK506), rapamycin/sirolimus, and neutralizing IL-2 receptor monoclonal antibody to prevent graft immune rejection (1, 2). Although the initial results of clinical trials have been promising, 90% of patients become insulin dependent within 5 years after transplantation (3).

Mounting evidence supports the idea that immunosuppressant regimens cause unwanted impairment of β-cell function, contribute to new onset diabetes after transplantation and impair efficacy of islet transplantation (3). First, exposure of islets to FK506 and sirolimus/rapamycin impairs β-cell function and survival (4–8). Second, FK506 and cyclosporin A (CsA) inhibit the phosphatase calcineurin and reduce insulin mRNA levels, content, and secretion and impair β-cell survival in β-cell lines, rat islets, and human islets (4, 6–11). Furthermore, restoration of glucose regulation in diabetic NOD-SCID mice through transplantation of human islets was impaired by FK506 treatment (8). Rapamycin, an mammalian target of rapamycin kinase inhibitor, blocks protein translation and attenuates insulin secretion in rat and human islets (12, 13). Combined treatment of rapamycin and FK506, the same treatment used in the Edmonton Protocol, exacerbates

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received October 29, 2012. Accepted May 9, 2013.
First Published Online May 15, 2013

Abbreviations: BrdU, bromo deoxyuridine; Creb, cAMP-responsive element binding protein; CRTC, Creb-regulated transcription coactivator; Crtc2-DM, Crtc2 double mutant; CsA, cyclosporin A; Ex-4, Exendin-4; FCS, fetal calf serum; FK506, tacrolimus; GFP, green fluorescent protein; GSIS, glucose-stimulated insulin secretion; Irs2, insulin receptor substrate 2; KRB, Krebs-Ringer buffer; NFAT, nuclear factor of activated T-cells; P, phospho; QPCR, quantitative PCR; shRNA, short hairpin RNA.

these negative effects on β-cell regeneration and survival (7, 8, 14). Last, and perhaps most significant, is the onset of diabetes and hyperglycemia observed in immunosuppressed patients after transplantation, with reported incidences ranging from 2% to 53%, a bona fide translational concern (15–17).

Genetic ablation of the regulatory subunit of calcineurin also supports its role in pancreatic β-cell development, maintenance of β-cell mass, insulin secretion, and glucoregulation that have been linked to impaired nuclear factor of activated T-cells (NFAT) activation (18–20). However, impaired β-cell function and survival observed in the presence of immunosuppressants could be due to dysregulation of additional calcineurin targets. cAMP-responsive element binding protein (Creb) activation is dependent on calcineurin and induces a variety of transcriptional programs involved in energy balance and glucose homeostasis in adipose tissue, skeletal muscle, brain, liver, and the β-cell (21). Disruption of Creb activity in the islet results in severe diabetes and glucose intolerance due to impaired β-cell survival and proliferation (22, 23). Promotion of Creb activity by transgenic expression of constitutively active Creb binding protein promotes β-cell proliferation and mass but impairs glucose-stimulated insulin secretion (GSIS) in isolated islets (24). Thus precise regulation of Creb activity characterized by transient activation and inactivation may be crucial for the maintenance of functional β-cell mass.

Glucose and incretin hormones promote β-cell fitness and growth and are important stimuli for preparation of the β-cell for subsequent challenges (25, 26). In the β-cell, Creb is activated in response to these feeding cues, which involves the signal-dependent activation of the Creb coactivators Creb-regulated transcription coactivators (CRTCs) (formerly transducers of regulated creb activity) (27–29). Creb activation leads to the induction of an expression profile that contributes to the maintenance of β-cell function and survival, including the insulin receptor adaptor insulin receptor substrate 2 (Irs2) (22). Although they are thought to operate via independent pathways, there is evidence for cross talk between calcium and cAMP signaling (30). Indeed, cAMP signaling triggers calcium signaling events via the inositol 1,4,5-triphosphate receptor in response to glucagon in hepatocytes and activates gluconeogenesis via Crtc2-Creb (31). Because Crtc2 activation is dependent on dephosphorylation by calcineurin, this suggests that immunosuppressants may lead to β-cell dysfunction or even death via impairment of Creb activation (28, 29). These observations prompted us to further evaluate the role and regulation of Crtc2 in the β-cell and evaluate the impact of immunosuppressants on the Crtc2-Creb pathway.

Research Design and Methods

Islet isolation

Mouse islets were harvested from male FVB/n mice of at least 8 weeks of age as described (32). Islets were picked manually using a dissection microscope. A total of 75-100 islets were used for analysis of Crtc2 phosphorylation and mRNA analysis of Creb targets.

Tissue culture

MIN6 cells (passage 25–35) were cultured in DME plus 10% fetal calf serum (FCS) plus antibiotics containing 100μM β-mercaptoethanol. Short hairpin RNA (shRNA) targeting Crtc2 were packaged in 293T cells cultured in 10-cm dishes using pLKO.1, pCMV8.3, and pDMG vectors. Seed sequences of shRNAs targeting Crtc2 were as follows: shRNA 1, GACCCATACTATGACCCATTT; shRNA 2, GAGGACTCATTCCGTAGTGAT; shRNA 3, AGCAAGGTGTAGAGGGAAATC; and shRNA 4, GATGCTAAAGTCCCTGCTATT. Virus was concentrated 100× by ultracentrifugation at 28 000*g* for 2 hours with a lower 20% sucrose layer. Virus was resuspended at 4°C overnight and stored in aliquots at −80 for use; 1.25×10^5 MIN6 cells were infected with 1 μL of concentrated lentivirus for 72 hours before analysis. For overexpression, Crtc2-GFP constructs were cloned into the pLDpuro vector (33); 15 μL of concentrated lentivirus were used to infect 1.25×10^5 MIN6 cells. Islets were cultured in RPMI 1640 plus 10% FCS plus antibiotics. Mouse islets were dispersed by trypsin digestion, seeded in poly-D lysine coated plates, and allowed to attach before infection.

GSIS

Assays in MIN6 cells and isolated islets were performed as described (32). Briefly, insulin levels were determined in triplicate after either a 1-hour incubation in Krebs-Ringer buffer (KRB) with 1mM or 20mM glucose (MIN6) or a 30-minute incubation in 2.8mM or 16.7mM glucose (islets). Whole islets were sonicated for insulin content and DNA quantitation using Quant-iT PicoGreen kit (Invitrogen, Carlsbad, California). All insulin measurements were performed by homogenous time resolved fluorescence analysis (CisBio, Bedford, Massachusetts) and normalized to DNA. Insulin content was determined from acid-ethanol extracts.

Cell treatments for phosphorylation analysis of Crtc2

Cells were starved in KRB with 1mM glucose for 1 hour and then either starved for an additional hour or treated with 10nM Exendin-4 (Ex-4) and 20mM glucose alone or together for 30 minutes before extraction with Laemmli sample buffer. For islet experiments, 75 islets per condition were treated for 1 hour with 2.8mM (low) and 16.7mM (high) glucose with or without Ex-4. Where applicable, CsA (500nM), FK506 (10nM), or vehicle (ethanol and dimethyl sulfoxide, respectively) was included throughout the duration of the starvation and treatment incubations.

Quantitative PCR (QPCR)

MIN6 cells were cultured in 5mM glucose in DME plus 10% FCS plus antibiotics containing 100 μM β-mercaptoethanol for

16 hours before incubation in 1mM glucose in KRB or 20mM glucose and 10nM Ex-4 for 4 hours. RNA was harvested using RNeasy kit (QIAGEN, Valencia, California). cDNA was prepared from 750 ng of RNA in a reaction containing 1mM deoxyribonucleotide triphosphate, 50-μg/mL oligo homo-oligomeric deoxythymine, 18 nucleotides in length, 10mM dithiothreitol, 1× first-strand buffer, and SuperScript II Reverse Transcriptase (Invitrogen). QPCR was performed using QuantiTech SYBR Green PCR kits (QIAGEN) and using an Eppendorf Mastercycler ep gradient S thermocycler (Eppendorf, Hamburg, Germany). Oligo sequences were as follows: nuclear receptor subfamily 4, group a, member 2 forward, cgacctctccggcctttta and reverse, cgccgaaatcgttgtcagta; Irs2 forward, agctttgattggctgtcctggag and reverse, tggcgatatagttgaggccgttgt; and 36B4 (an alternative name for the gene now called ribosomal protein, large, P0 [Rplp0]) forward, ccacgaaatctccagaggcac and reverse, atgatcagcccgaaggagaagg. Transcriptional changes in Creb target genes were normalized to 36B4.

Western blotting

Western blot analysis has been described elsewhere (28, 29). Phospho (P)Ser171 antiserum was affinity purified before use.

Fluorescence imaging

MIN6 cells were infected with Crtc2-green fluorescent protein (GFP) lentivirus for 72 hours before treatment and fixation for image analysis using an Opera automated confocal microscope. Localization analysis of nuclear and cytoplasmic intensity of GFP signal was determined on a single-cell basis averaged within 40 fields/well (20-50 cells/field) and 3 wells/condition and quantitated using an Acapella algorithm run on Columbus software (PerkinElmer, Waltham, Massachusetts). The contrast ratio (nuclear-cytoplasmic to nuclear+cytoplasmic) was normalized to 200-ng/mL leptomycin B-treated MIN6 cells expressing wild-type Crtc2-GFP for 1 hour, which was considered to be completely nuclear. Endogenous Crtc2 staining was performed using Crtc2 primary antibody (Bethyl Laboratories, Montgomery, Texas) at 1:200 and rabbit Alexa Fluor 488 secondary (1:1000). After 72 hours of knockdown, MIN6 cells were incubated with 1-μg/mL bromo deoxyuridine (BrdU) in media for 14 hours, fixed, permeabilized with 0.1% triton, and treated with 2N HCl for 30 minutes at room temperature. Cells were rinsed with 0.1M borate buffer (pH 8.5) before staining for BrdU (1:500) (Sigma, St Louis, Missouri) and antimouse Alexa Fluor 488 secondary antibody. eBioscience antimouse antigen Ki67-conjugated to Alexa Fluor 570 at 1:1000 was used (eBiosciences, Inc., San Diego, California). Condensed nuclei were identified based on small size and bright intensity using Columbus software.

Statistical analysis

All studies were performed in at least 3 independent experiments. Results are shown as mean ± SEM of representative data unless stated in figure legend to have been pooled across experiments. Two-tailed, unpaired Student's *t* tests were used to determine statistical significance. $P < .05$ was considered significant.

Results

Crtc2 is required for β-cell function and proliferation

To evaluate whether impaired Crtc2-Creb activation contributes to β-cell dysfunction, we examined the functional consequence of acute loss of Crtc2 in the β-cell using RNA interference. Knockdown of Crtc2 in MIN6 mouse insulinoma cells using 4 lentiviral shRNAs targeting different regions of the Crtc2 mRNA reduced insulin secretion in response to glucose by at least 50% of that seen in control cells (Figure 1A). Direct depolarization of the cell membrane with KCl restored insulin release in Crtc2 knockdown cells, suggesting that the defect in insulin secretion is due to impaired responsiveness to glucose and not synthesis of insulin or the secretory machinery per se (Figure 1B). Consistent with this, insulin content in MIN6 cells lacking Crtc2 was either unchanged or elevated when targeted with 3 out of 4 shRNAs compared with control cells (Supplemental Figure 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Knockdown of Crtc2 reduced cell numbers and proportion of Ki67- and BrdU-positive cells with all shRNAs tested (Figure 1, C and D, and Supplemental Figure 1C). Prolonged knockdown with 2 shRNAs targeting Crtc2 (>72 h) also resulted in caspase 3 cleavage (Figure 1F). However, we observed less than 10% condensed apoptotic nuclei with all shRNAs at 72 and 96 hours (Figure 1D and Supplemental Figure 1D), suggesting that the major determinant of reduced cell number in Crtc2 knockdown cells is impaired proliferation. To confirm these effects of Crtc2 in primary cells, we infected dispersed mouse islets with lenti-Crtc2 shRNAs for 96 hours and observed a 30% reduction in GSIS in the absence of Crtc2 (Figure 1G). Crtc2 knockdown in mouse islet cells did not reduce insulin content, supporting that Crtc2 is specifically required for glucose-responsive insulin secretion and not insulin synthesis (Supplemental Figure 1B). We anticipate that the difference in severity of the MIN6 and mouse islet secretion phenotype is owing to less efficient knockdown of Crtc2. Supporting this notion, Crtc2 knockdown evaluated by Western blot analysis was less complete in dispersed mouse islets (70%) (Figure 1H) compared with MIN6 cells (90%) (Figure 1F), whereas infection efficiency was comparable between cells (>90%) (data not shown). Taken together, these data demonstrate that Crtc2 is required for the maintenance of functional β-cells.

Glucose and cAMP signaling induce Crtc2-Creb activation in β-cells

Calcium and cAMP stimuli are known to promote Crtc2 nuclear entry and enhance Creb activity in MIN6

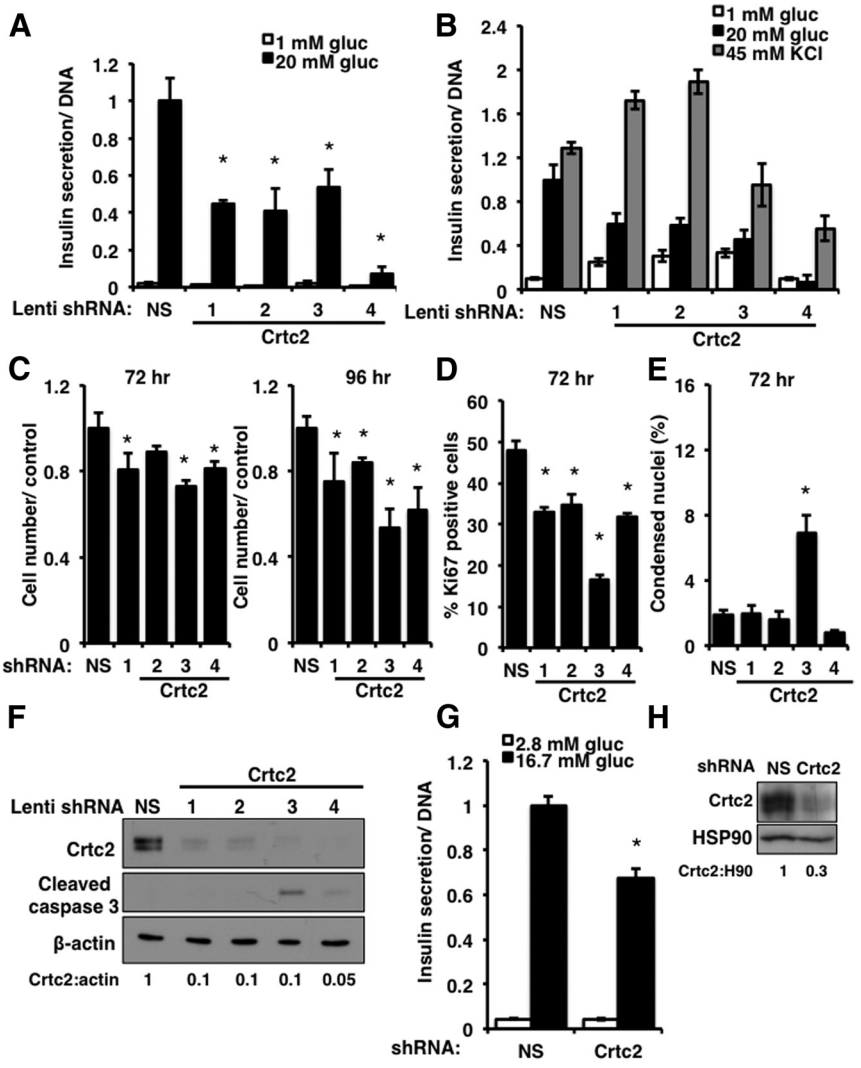

Figure 1. Crtc2 is required for β-cell function and survival. (A) Loss of Crtc2 inhibits GSIS in MIN6 cells. Effect of 4 independent shRNAs targeting Crtc2 on insulin secretion in response to 1mM or 20mM glucose treatment is shown. NS, nonspecific shRNA control. *, $P < .05$ compared with NS control +20mM glucose. (B) Insulin secretion is restored by membrane depolarization with 45mM KCl in MIN6 cells lacking Crtc2. Effect of 4 independent shRNAs targeting Crtc2 on insulin secretion in response to 1mM or 20mM glucose or 45mM KCl is shown. NS, nonspecific shRNA control. In panels A and B, insulin secretion values were normalized to DNA content and expressed relative to high-glucose control cell levels. (C) Loss of Crtc2 reduces cell number. Cell number was determined using a Cellomics Vti automated microscope. Data are presented normalized to cell number in NS control shRNA-infected cells. (Left panel) 72 hours after gene silencing. (Right panel) 96 hours after gene silencing. *, $P < .05$ compared with NS control. (D) Loss of Crtc2 reduces the proportion of Ki67-positive MIN6 cells after 72 hours of knockdown as determined using Cellomics Vti automated microscope. *, $P < .05$ compared with NS control. (E) The percentage of condensed nuclei in MIN6 cells after 72 hours of knockdown. Condensed nuclei were identified on the basis of small size and bright intensity using Columbus software and were expressed as percentage of total cells. *, $P < .05$ compared with NS control. (F) Crtc2 knockdown with 2 Crtc2 shRNAs (3 and 4) leads to production of cleaved caspase 3. Quantitation of Crtc2:actin in control NS and Crtc2-shRNA lentivirus-infected cells is shown below. (G) Insulin secretion is impaired in isolated mouse islets lacking Crtc2. Effect of Crtc2 knockdown on glucose-stimulated (16.7mM) insulin secretion compared with control NS-infected cells is shown. (H) Western blotting showing knockdown of Crtc2 in mouse islets used for GSIS experiments. Quantitation of knockdown is shown at bottom. Insulin secretion values were normalized to DNA content and expressed relative to high-glucose control cell levels. *, $P < .05$. Data shown are either average ± SEM of 3 independent experiments (A–E and G) or representative data from 3 independent experiments (F and H). HSP, heat shock protein; gluc, glucose.

and HIT-T15 insulinoma cells (28, 29). To confirm these findings in a more physiologically relevant setting, we used the glucagon-like peptide 1 agonist Ex-4 to stimulate cAMP signaling and quantitatively monitored Crtc2 localization with an automated confocal microscope. To monitor signal-dependent changes in Crtc2 subcellular localization, we infected MIN6 cells with lentivirus expressing a Crtc2-GFP construct. After treatment with either glucose or the physiological glucagon-like peptide 1 receptor agonist Ex-4, Crtc2-GFP displayed partial nuclear localization that was enhanced with combined treatment (Figure 2, A and B). Staining of endogenous Crtc2 protein in MIN6 cells confirmed these results (Supplemental Figure 2, A and B). These data confirm that cAMP and glucose regulate Crtc2 nuclear entry in the β-cell (29) but suggest that Crtc2 can partially respond to glucose or cAMP stimuli on its own. To evaluate the extent to which Crtc2 contributes to cAMP and glucose-dependent Creb transcription, we performed QPCR analysis of endogenous Creb target gene levels in MIN6 cells treated with glucose and Ex-4 after infection with a lentivirus expressing control or Crtc2 shRNA. Glucose and Ex-4 alone or together increased mRNA levels of Nr4a2 and Irs2 (Figure 2, C and D), consistent with previous findings (28, 29). In MIN6 cells lacking Crtc2, induction of Nr4a2 and Irs2 mRNA in response to glucose alone or glucose and Ex-4 was blocked by 60% and 30% compared with control cells, respectively, whereas the mRNA and protein levels induced by Ex-4 alone were unaffected in the absence of Crtc2 (Figure 2, D and E). Surprisingly, IRS-2 protein accumulation was only significantly blocked by knockdown of Crtc2 in MIN6 cells after treatment with glucose and Ex-4 together (Figure 2E, quantitated in F). These data demonstrate for the first time that induction of endogenous Creb target

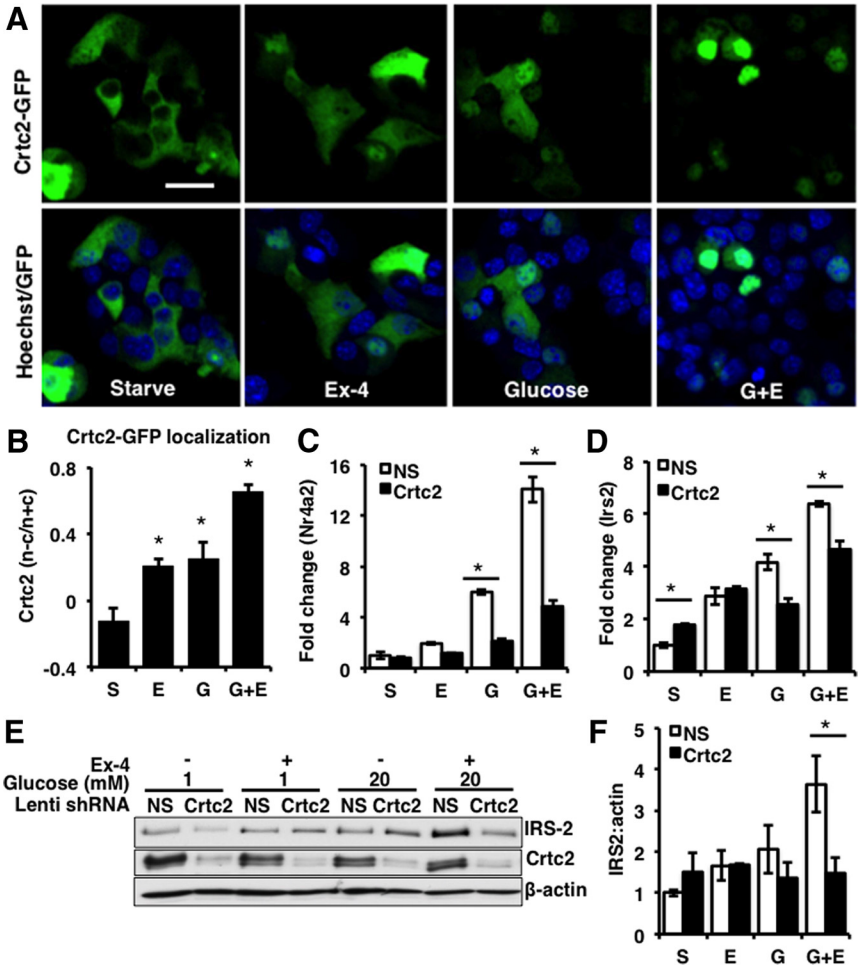

Figure 2. Crtc2 is required for glucose and cAMP activation of Creb in the β-cell. (A) Fluorescence images of Crtc2-GFP MIN6 cells treated with 20mM glucose and 10nM Ex-4 alone or together (G+E). (Bottom row) Merge of Crtc2-GFP image with nuclei stained with Hoechst. (B) Histogram showing localization of Crtc2-GFP in MIN6 cells expressed as a contrast ratio (nuclear intensity-cytoplasmic intensity to total intensity). The contrast ratio for each condition is normalized to the contrast ratio for Crtc2-GFP signal in cells treated for 1 hour with the nuclear export inhibitor leptomycin to cause nuclear accumulation of Crtc2. Cells were pretreated for 1 hour in 1mM glucose before treatment as indicated. S, starve, 1mM glucose; E, 10nM Ex-4; G, 20mM glucose; G+E, 20mM glucose and 10nM Ex-4 for 1 hour. (C and D) Knockdown of Crtc2 reduces glucose and Ex-4-induced Creb-regulated transcription of (C) Nr4a2 and (D) Irs2. Histograms show level of Creb target gene mRNA normalized to 36B4 and expressed relative to control basal levels as measured by QPCR analysis. (E) Crtc2 is required for induction of Irs2 protein in response to glucose and Ex-4 in MIN6 cells. Western blottings showing levels of Irs2 and Crtc2 protein in MIN6 cells in the presence of control (NS) and Crtc2 lenti-shRNA. Treatment with 1mM or 20mM glucose and 10nM Ex-4 is shown. (F) Histogram showing induction of IRS2 protein induction in MIN6 cells. *, $P < .05$. Data shown are either average ± SEM of 3 independent experiments (B–D and F) or representative data from 3 independent experiments (A and E).

genes in the β-cell is dependent on glucose/cAMP activation of Crtc2.

Crtc2 dephosphorylation is stimulated by glucose and requires calcineurin

In the β-cell, it is established that Crtc2 is dephosphorylated in response to glucose and cAMP at Ser275 and Ser171, respectively, which stimulates nuclear entry of Crtc2 after loss of phosphorylation-dependent binding to 14-3-3 proteins (29). To confirm these findings in islets, we generated a PSer171 antibody to monitor phosphorylation of endogenous Crtc2. We first confirmed the specificity of the P171 and P275 antibodies by Western blot analysis using GFP-tagged Crtc2 Ser to Ala P-mutants (Supplemental Figure 3, A and B). We next examined the impact of a gradient of glucose concentrations to further characterize the novel glucose regulation of Crtc2 by monitoring endogenous Crtc2 phosphorylation in MIN6 cells and mouse islets. Crtc2 is dephosphorylated at both sites in response to glucose in MIN6 cells and primary mouse islets at glucose concentrations at or above 7.5mM, which correlates to the switch from fasted to fed state glucose levels (Figure 3, A and B, quantified in Supplemental Figure 3, C and D). These data suggest a role of Crtc2 in physiological glucose sensing. We then monitored Crtc2 phosphorylation in extracts of cells that were starved for 1 hour in low glucose and then treated for 30 minutes with glucose and Ex-4. In the absence of glucose, Ex-4 treatment does not reduce phosphorylation of endogenous Crtc2 at Ser171, as observed previously using overexpressed FLAG-tagged Crtc2 (Figure 3, C and D, and Supplemental Figure 3, E and F) (29). Crtc2 dephosphorylation at Ser171 and Ser275 was blocked by treatment with calcineurin inhibitors in MIN6 cells and isolated mouse islets, suggesting that the sites are coregulated and that Crtc2 activation is dependent on calcineurin (Figure 3, C and D, and Supplemental Figures 3, E and F, and 4). In contrast, Creb phosphorylation at Ser133 was unaffected by treatment with CsA, suggesting that impaired Creb activity in the presence of CsA is not due to changes in Creb phosphorylation (Figure 3, C and D). The calcineurin inhibitor FK506 impaired glucose-dependent nuclear entry of Crtc2 in MIN6 cells (Figure 3E) and dispersed mouse islets (Figure 3F), suggesting that phosphorylation-dependent localization of endogenous Crtc2 is dependent on calcineurin.

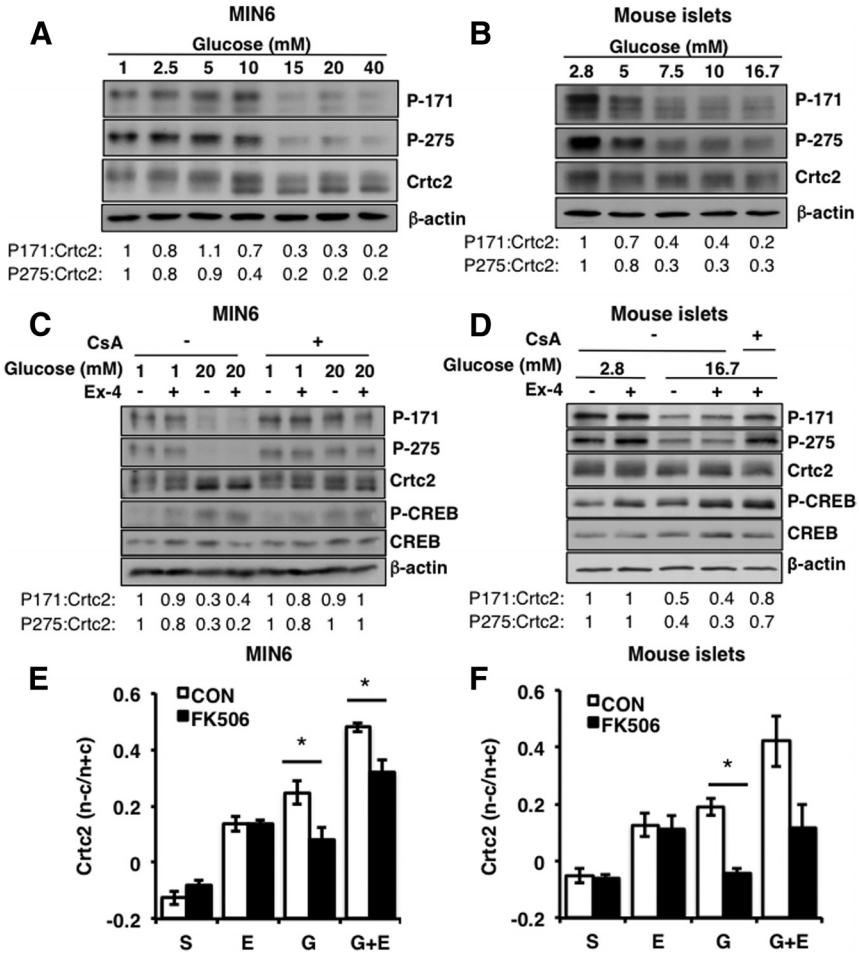

Figure 3. Crtc2 dephosphorylation is stimulated by glucose and requires calcineurin. (A) Western blottings showing levels of PSer171, PSer275, and total Crtc2 protein in MIN6 cells cultured in the indicated glucose concentrations. The stoichiometry of phosphorylation is indicated below the blots. β-Actin blot is shown as a control. Western blottings are representative of 3 independent experiments. (B) Crtc2 is dephosphorylated in response to glucose in primary mouse islets and is activated within a physiological range of glucose. Western blottings showing levels of PSer171, PSer275, and total Crtc2 protein in mouse islets treated with glucose concentrations over the physiological range. The stoichiometry of phosphorylation is indicated below the blots. β-Actin blot is shown as a control. Western blottings are representative of 3 independent experiments. (C and D) CsA inhibits Crtc2 dephosphorylation. Western blottings showing effect of treatment with calcineurin inhibitor CsA of levels of PSer171 and PSer275 in MIN6 cells (C) and isolated mouse islets (D). Levels of P-Creb and total Creb protein are shown. CsA treatment does not affect CREB phosphorylation. The stoichiometry of phosphorylation is indicated below the blots. β-Actin blot is shown as a control. Western blottings are representative of 3 independent experiments. (E and F) Glucose-dependent nuclear relocalization of endogenous Crtc2 protein is dependent on calcineurin. Histograms showing localization of Crtc2-GFP in (E) MIN6 cells and (F) mouse islets expressed as a contrast ratio = (nuclear intensity − cytoplasmic intensity) / total intensity. The contrast ratio for each condition is normalized to the contrast ratio for Crtc2-GFP signal in cells treated for 1 hour with the nuclear export inhibitor leptomycin to cause nuclear accumulation of Crtc2. MIN6 cells and mouse islets were treated with 1mM glucose KRB or 2.8mM glucose KRB for 1 hour before 1-hour treatment with glucose (G) and Ex-4 (E) alone or together (G+E) in KRB. Effect of calcineurin inhibitor FK506 is shown. Data shown represent the average ± SEM from 3 independent experiments. *, $P < .05$; CON, control.

β-Cell function and Creb-regulated transcription require calcineurin

To evaluate the implication of immunosuppression on β-cell function, we treated MIN6 cells and isolated islets with FK506 and CsA for 3 and 5 days, respectively, and performed GSIS assays. Insulin secretion was reduced 40%–50% in both MIN6 cells and isolated mouse islets treated with increasing doses of FK506 and CsA (Figure 4, A and B). Western blot analysis indicated that induction of IRS2 protein, both in response to glucose and with Ex-4 cotreatment, was also reduced by 50% by FK506 and CsA (Figure 4C). Consistent with this, signal-dependent activation of the Creb target genes Nr4A2 and Irs2 in MIN6 cells and mouse islets was reduced by 25%–75% in the presence of FK506 (Figure 4, D and E). Moreover, CsA treatment reduced Nr4a2 mRNA levels in both MIN6 cells and islets (Figure 4, F and G).

Constitutively active Crtc2 maintains Creb activity in the presence of calcineurin inhibitors

Previous work demonstrated that mutation of both Ser171 and Ser275 to alanine (Crtc2 double mutant [Crtc2-DM]) resulted in constitutive nuclear localization of Crtc2 and elicited signal-independent Creb activity in luciferase-based transcription assay (29). Because Crtc2-DM cannot be inhibited by phosphorylation, we sought to evaluate the extent to which Crtc2-DM could sustain Creb target gene activation when calcineurin is inhibited. To address this, we generated lentiviruses expressing GFP-tagged Crtc2 P-mutant cDNAs and monitored Crtc2 localization in infected cells. Immunofluorescence analysis revealed that mutation of the individual phosphorylation sites (Ser171A and Ser275A) caused partial nuclear entry of Crtc2, whereas Crtc2-DM was almost completely nuclear (Figure 5, A and B). Overexpression of Crtc2-DM induced basal mRNA levels of Nr4a2 in MIN6 cells and dispersed mouse islets (Supplemental Figure 5A and Figure 5C). Furthermore, Crtc2-DM maintained mRNA levels of Nr4a2 and IRS2 at higher levels in the presence of CsA for 24 hours when compared with GFP and wild-type Crtc2-expressing

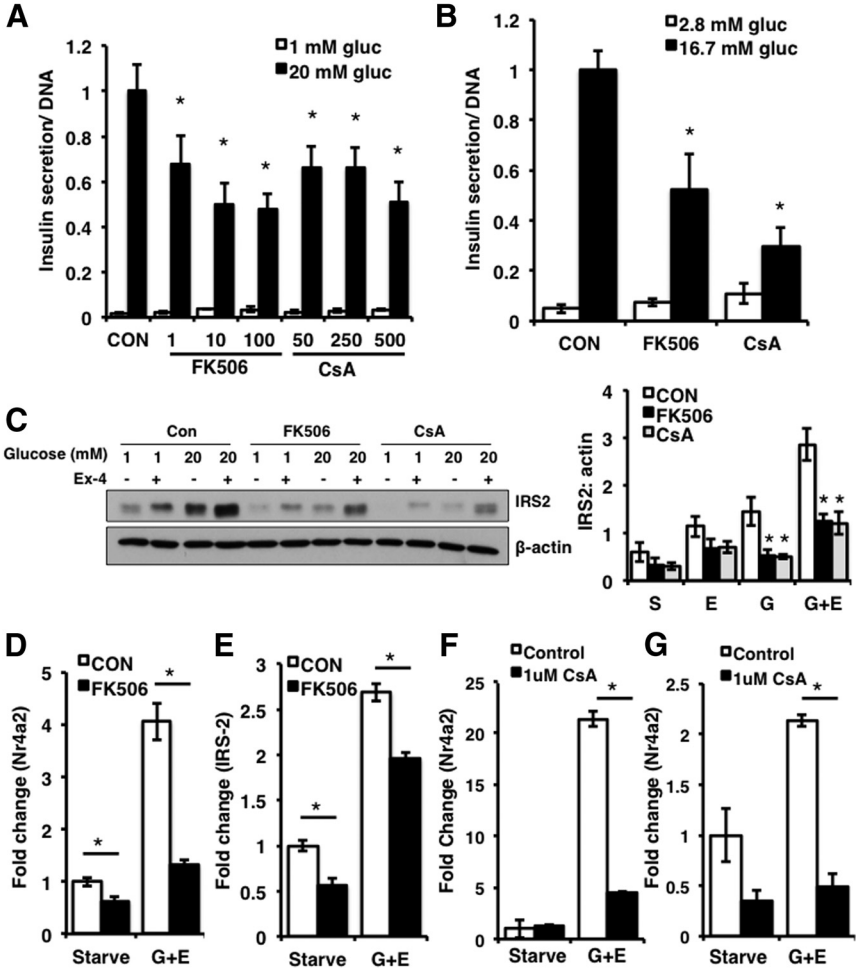

Figure 4. β-Cell function and Creb-regulated transcription is impaired by calcineurin-inhibiting immunosuppressants. (A) Histogram showing insulin secretion in low (1mM) and high (20mM) glucose in MIN6 cells treated with FK506 or CsA at the indicated concentrations. Insulin secretion values were normalized to DNA content and expressed relative to high-glucose control cell levels. Data shown are average ± SEM from 3 independent experiments. (B) Histogram showing insulin secretion from isolated mouse islets cultured in low (2.8mM) and high (16.7mM) glucose, with our without calcineurin inhibitors FK506 or CsA for 3 and 5 days, respectively. Insulin secretion values were normalized to DNA content and expressed relative to high-glucose control cell levels. Data shown are average ± SEM from 3 independent experiments. (C) Induction of IRS2 protein in response to glucose and Ex-4 is impaired by calcineurin-inhibiting immunosuppressants CsA and FK506 in MIN6 cells. Western blottings showing IRS2 protein levels in the presence of FK506 and CsA in MIN6 cells cultured in low (1mM) or high (20mM) glucose. Histogram showing level of IRS2 protein in response to indicated treatment is shown at right. Data are averages ± SEM from 3 independent experiments. (D and E) Histograms showing QPCR analysis of induction of Nr4a2 and Irs2 in isolated mouse islets in response to glucose and Ex-4 (G+E) in the presence of FK506. (F and G) Histograms showing QPCR analysis of induction of Nr4a2 and Irs2 in isolated mouse islets in response to glucose and Ex-4 (G+E) in the presence of CsA. Data shown are average ± SEM from 3 independent experiments. *, $P < .05$; CON, control.

cells. Protein levels of Irs2 were also induced by Crtc2-DM and maintained in the presence of calcineurin inhibitors in MIN6 cells (Figure 5F and Supplemental Figure 5B). We observed that Crtc2-DM overexpression impaired insulin secretion owing to a 50% reduction in insulin content (data not shown), consistent with previous results (24). We next generated tet-inducible Crtc2 constructs to examine whether acute expression of Crtc2-DM could promote insulin secretion when calcineurin is inhibited. Thirty-six hours after induction with doxycycline, Crtc2-DM expression resulted in a statistically significant increase in glucose-stimulate insulin secretion in MIN6 cells in the presence of FK506, an effect not seen in control GFP and Crtc2-wt cells (Figure 5G). We conclude that nuclear Crtc2 promotes β-cell function in the presence of calcineurin inhibitors but prolonged expression of constitutively nuclear Crtc2 has negative effects on insulin content. These data suggest that impaired Creb-regulated transcription and insulin secretion in the presence of immunosuppressants is in part due to inhibition of Crtc2 in the β-cell.

Discussion

The role of the Crtc-Creb pathway in regulating energy balance at both the cellular and organismal level is becoming increasingly evident (reviewed in Ref. 21). Whole-body Crtc2 knockout mice show reduced glucose output from the liver due to increased insulin sensitivity and reduced gluconeogenesis (34, 35). Although the effect of loss of Crtc2 in the pancreatic islet was not evaluated in either of these studies, our data demonstrate that Crtc2 is important for β-cell function in cell lines and in the isolated islet. β-Cell-specific ablation of Crtc2 will be necessary to clarify its role in whole-body glucose metabolism.

The net impact of pharmacological calcineurin inhibition on glucose metabolism is still unclear. In healthy humans, short-term treatment with FK506 improves glucose clearance, potentially due to increased insulin sensitivity or a reduction in glucose output from the liver through inhibition of Creb (Ref. 36 and data not shown). We show for the first time that the diabetogenic effect of chronic calcineurin-based immunosuppression is therefore likely due to functional failure of the β-cell compartment to meet insulin requirements when Crtc2 is chronically inhibited.

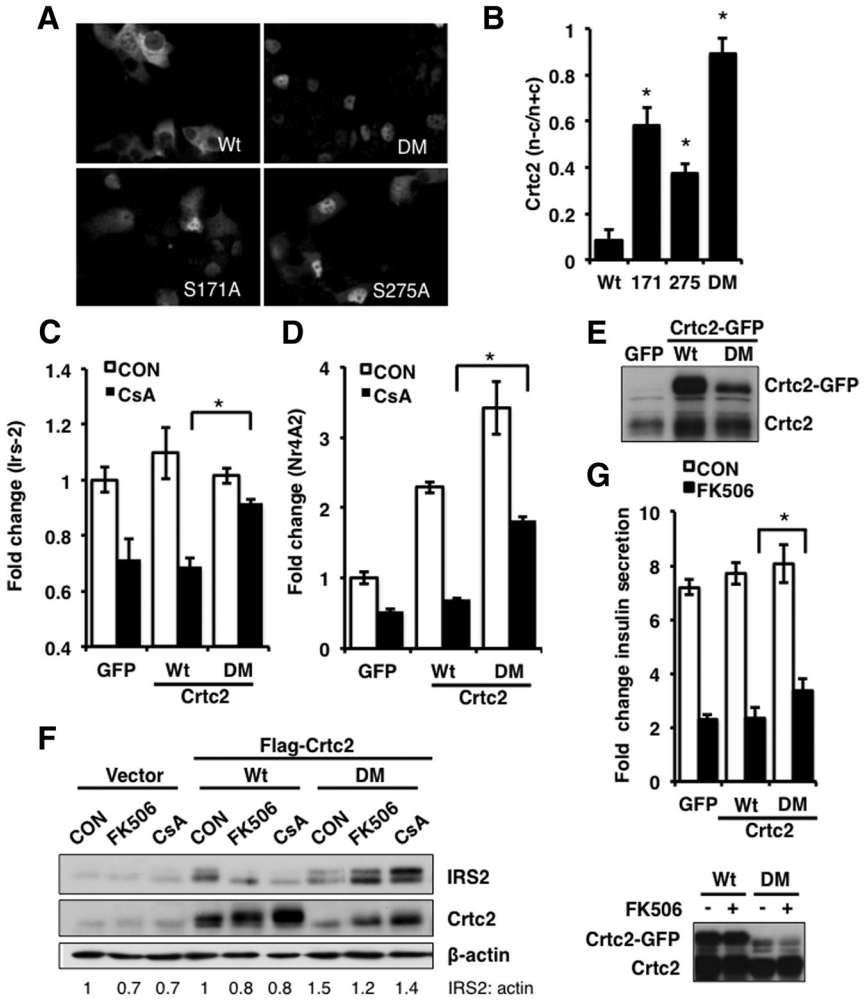

Figure 5. (A) Fluorescence micrographs showing subcellular localization of Crtc2-GFP fusion proteins in MIN6 cells. Effect of mutation of Ser171 and Ser275 to Ala alone or together (double mutant [DM]) is shown. Note that Crtc2 P-mutants at Ser171, Ser275, and Ser171,275A (DM) are more nuclear than Crtc2-wt in low-glucose conditions. (B) Histogram showing Crtc2 localization expressed as a contrast ratio (nuclear intensity-cytoplasmic intensity to total intensity) normalized to Crtc2-wt-infected cells treated with leptomycin for 1 hour to cause nuclear accumulation of Crtc2. (C) Histogram showing QPCR analysis of Irs2 mRNA in MIN6 cells expression control GFP alone, Crtc2-wt-GFP, or Crtc2-DM-GFP. Effect of CsA treatment is shown. (D) Histogram showing QPCR analysis of Nr4a2 mRNA in MIN6 cells expression control GFP alone, Crtc2-wt-GFP, or Crtc2-DM-GFP. Effect of CsA treatment is shown. (E) Western blotting showing levels of Crtc2-GFP fusion proteins in MIN6 cells used for gene expression analyses shown in C and D. (F) Western blotting showing IRS2 and Crtc2 protein levels in the presence of FK506 and CsA in MIN6 cells expressing Crtc2-wt or Crtc2-DM. β-Actin blot is shown as loading control. Fold change in Irs2 protein level is shown underneath blots. (G) Histogram showing fold change in insulin secretion (insulin secreted in high glucose/insulin secreted in low glucose) in MIN6 cells expressing Crtc2-wt and Crtc2-DM. Control GFP cells are shown as control. Data shown are average ± SEM from 3 independent experiments. *, $P < .05$; CON, control; Wt, wild-type.

The impact of altered calcineurin activity on β-cell function and survival has largely been attributed to its most well-established substrate NFAT (19). In addition, NFAT has recently been shown to regulate glucose-dependent Irs2 induction in rat islets (20). Our data suggests that alterations of calcineurin activity can also affect β-cell function and Irs2 expression through regulation of Crtc2-Creb. Modulation of calcineurin or Creb activity in the β-cell has profound effects on glucose metabolism through altered β-cell function and survival. Interestingly, both ablation (18, 19) and promotion (37) of calcineurin activity in the β-cell results in diabetes in mice. β-Cell-specific ablation of Creb activity caused severe diabetes (22, 23), and although whole-body metabolism was not documented, constitutive Creb activity impaired β-cell function despite promotion of β-cell proliferation and mass (24). These data suggest that finely tuned regulation of calcineurin and Creb may be crucial for the β-cell. Taken together, our data suggest that inappropriate inactivation of the Crtc2-Creb pathway may underlie the loss of functionality and survival seen in with immunosuppression and may represent a new target for therapeutic intervention in new onset diabetes after transplantation and potentially other immunosuppression-related deficiencies.

The requirement for precise regulation of signaling pathways is exemplified by glucose stimulation in the β-cell. Glucose stimulation has been shown to promote human and rodent β-cell replication and promote restoration of normal glycaemia in diabetic mice transplanted with human islets (38). On the other hand, chronic hyperglycemia results in impaired β-cell function and death. Indeed, a recent study demonstrated that chronic hyperglycemia caused prolonged Creb activation, induction of inducible cAMP early repressor and subsequent reduction of insulin, neurogenic differentiation and sulfonylurea receptor 1 expression, effects that were not observed under moderate glucose conditions in which Creb activation was transient (39). Our data demonstrate that Crtc2 phosphorylation in islets is dynamically regulated by fluctuations in glucose concentrations around fasting and feeding, suggesting that Crtc2 is a glucose sensor for Creb activation and could contribute to dysregulated Creb activation in such hyperglycemic settings.

In the β-cell, cross talk between calcium and cAMP signaling coordinates transcriptional responses and pro-

vides a redundancy to ensure β-cell responsiveness. Our study supports previous findings that Crtc2-Creb activation is regulated by both signaling pathways (28, 29) in primary β-cells and highlights the feasibility of alternative strategies to circumvent the requirement of calcineurin for Creb activity. For instance, although activation of calcineurin is predominately regulated by calcium signaling, promotion of cAMP signaling promotes human β-cell survival when calcineurin is inhibited (40). Given that glucose and cAMP signaling are essential stimuli that regulate β-cell growth and function, failure to activate the cAMP/calcium-regulated gene expression program may prevent β-cell adaptation to increased demand, particularly in hyperglycemic conditions. Further dissection of the role of Crtc2:Creb pathway in β-cell biology will lead to better understanding of why grafted β-cells have impaired long-term survival and may highlight the need to improve current immunosuppression therapies used after transplantation.

Acknowledgments

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This work was supported by the Canadian Institutes of Health Research Operating Grant MOP#97772 (to R.A.S.). R.A.S. holds the Canada Research Chair in Apoptotic Signaling. C.E.E. was supported by an Ontario graduate scholarship. A.F. was supported by a studentship from the Canadian Diabetes Association.

Disclosure Summary: The authors have nothing to disclose.

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