



## The impact of $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II on insulin gene expression in MIN6 cells

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### ABSTRACT

$\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) is expressed in insulin-secreting  $\beta$  cells. However, the effects of CaMKII on insulin synthesis are unknown. Although Ser133 phosphorylation of cyclic AMP-responsive element-binding protein (CREB) typically increases CREB transcriptional activity, CaMKII phosphorylates CREB at Ser142 and at Ser133 to exert a dominant inhibitory effect. Our objective was to characterize the role of CaMKII in insulin gene expression. In MIN6 cells, insulin gene promoter activity was significantly down-regulated by wild-type (WT) CaMKII $\delta$ 2, but was significantly upregulated after small interfering RNA (siRNA) knockdown of CaMKII $\delta$  expression. These results were independent of glucose concentrations and membrane depolarization. Insulin mRNA levels were also decreased by WT CaMKII $\delta$ 2 and increased by CaMKII $\delta$  siRNA. Downregulation of insulin gene promoter activity by WT CaMKII $\delta$ 2 was partly mediated via cyclic AMP-responsive element 2 (CRE2). WT CaMKII $\delta$ 2 significantly increased CREB phosphorylation at Ser142 and significantly decreased binding to CREB binding protein (CBP), whereas kinase dead CaMKII $\delta$ 2 did not. Our results indicate that CaMKII $\delta$ 2 downregulates insulin gene expression by Ser142 phosphorylation of CREB and reducing binding of CREB to CBP.

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### 1. Introduction

$\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) is a multi-functional serine/threonine protein kinase. It belongs to a family of 81 proteins in humans that play important role in cellular signaling via  $\text{Ca}^{2+}$  signal transmission [1]. In mammals, CaMKII is expressed by four closely related genes,  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , each of which produces mRNA that can be alternatively spliced to generate ~30 CaMKII variants. The major activators of CaMKII are  $\text{Ca}^{2+}$  and calmodulin [2]. Increases in intracellular  $\text{Ca}^{2+}$  cause the formation of a complex between  $\text{Ca}^{2+}$  and calmodulin. This complex then binds to the regulatory domain of CaMKII, inducing autophosphorylation of Thr287 in CaMKII $\beta$ ,  $\gamma$  and  $\delta$ , or Thr286 in CaMKII $\alpha$ , which activates CaMKII protein kinase activity. Activated CaMKII phosphorylates numerous target proteins and is involved in various cellular functions including trafficking, docking, fusion of secretory

granules, regulation of gene expression, modulation of ion channel function, and intracellular signal transduction [3–6].

Several CaMKII isoforms, including CaMKII  $\beta$ ,  $\delta$ , and  $\gamma$ , have been identified in rat pancreatic islets [7,8] and insulinoma cell lines [3,9], although CaMKII $\alpha$  mRNA expression was low in islet  $\beta$  cells [10]. Intracellular  $\text{Ca}^{2+}$  plays a critical role in glucose-induced insulin secretion in  $\beta$  cells [11] as it induces  $\text{Ca}^{2+}$ /calmodulin complex formation, followed by auto-phosphorylation and activation of CaMKII [12,13]. CaMKII $\delta$ 2 is involved in glucose-induced insulin secretion, possibly by phosphorylating synapsin I [14], a critical step in insulin granule exocytosis in MIN6 cells.

Insulin synthesis in  $\beta$  cells is important for maintaining glucose homeostasis. Transcriptional control of the insulin gene is modulated by *cis*-acting regulatory sequences located from the transcriptional start site to –400 bp upstream. Cyclic AMP (cAMP) responsive elements (CREs) bind to a broad array of closely related members of the basic leucine zipper domain cAMP-responsive element (CRE) binding protein (CREB)/activating transcription factor (ATF) family [15]. Wu et al. [4] previously reported dominant inhibitory effects of CaMKII on CREB. CaMKIV and protein kinase A (PKA) stimulate CRE-dependent gene expression by phosphorylating

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CREB at Ser133. Under these conditions, CREB exists as a dimer on CREs in its target genes and binds to CREB binding protein (CBP), which activates gene transcription. CaMKII phosphorylates CREB at Ser133 and Ser142. Phosphorylation at Ser142 prevents CREB dimerization on DNA and binding with CBP, thus exerting a dominant inhibitory effect [4]. Because CaMKII is a major CaMK isoform in  $\beta$  cells [15], we hypothesized that CaMKII is activated by hyperglycemia-induced  $\text{Ca}^{2+}$  influx, followed by inhibition of CREB and insulin gene transcription. Therefore, in this study, we investigated the effects of CaMKII on CREB phosphorylation and insulin gene promoter activity.

## 2. Materials and methods

### 2.1. Cells and reagents

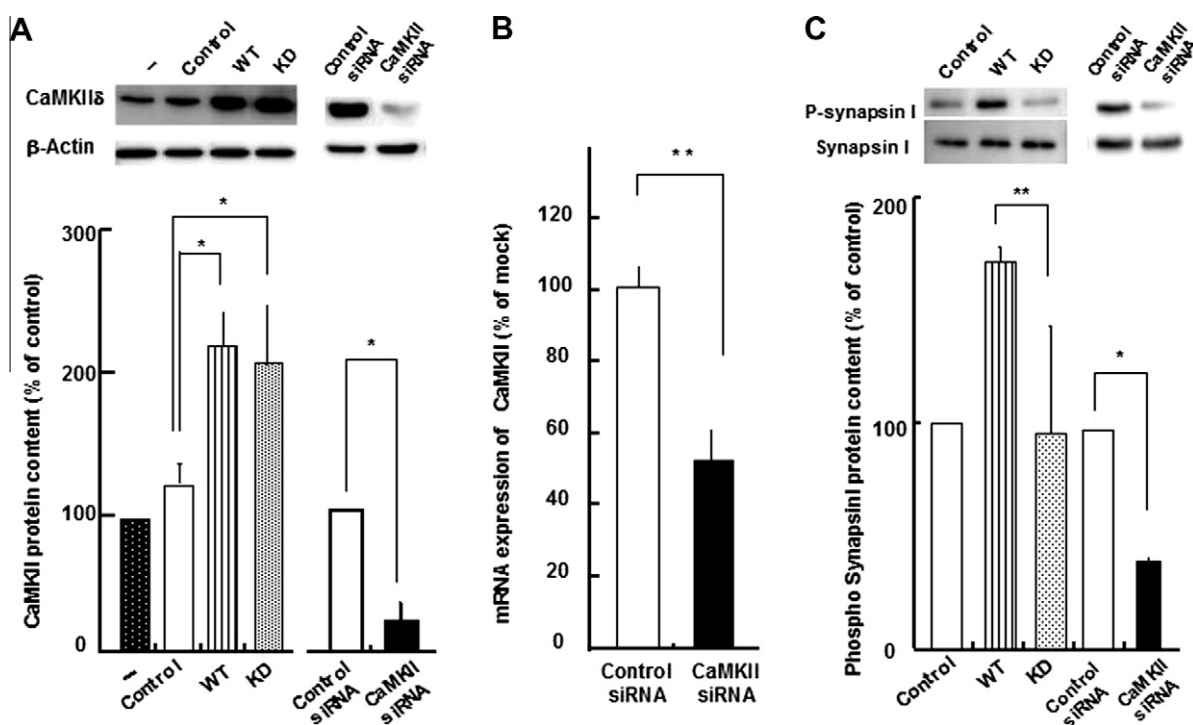
MIN6 cells were a kind gift from Dr. Susumu Seino (Division of Cellular and Molecular Medicine, Kobe University, Graduate School of Medicine, Kobe, Japan) and were cultured under standard conditions in DMEM supplemented with 25 mM glucose and 15% heat-inactivated FBS. The anti-phospho-Ser142-CREB antibody was provided by Dr. Michael E. Greenberg (Harvard Medical School, Boston, MA). Mouse monoclonal and rabbit polyclonal anti-phospho-Ser133-CREB antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-CREB and mouse monoclonal anti-CBP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-synapsin I and rabbit polyclonal anti-phospho-Ser603-synapsin I antibodies were purchased from Millipore (Merck Millipore, MA). The rabbit polyclonal anti-CaMKII $\delta$  antibody was purchased from Trans Genic Inc. (Kumamoto, Japan).

### 2.2. Expression and reporter plasmid construction

pCAGGSneo expression vectors carrying WT and kinase dead (KD) forms of CaMKII $\delta$  were provided by Dr. Yusuke Takeuchi (Tohoku University; Supplemental Fig. 1) [16]. Promoter activity of the human insulin gene was analyzed using pGL3, a low background promoterless luciferase reporter vector [17]. The phINS356LUC plasmid contained the –356 to +14 portion of the human insulin gene promoter. CRE2 and CRE1/2 site mutations were generated using a GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) with the respective primers (Supplemental Table 1). Plasmids mutated at CRE1 and CRE1/2 were designated phINS356m2LUC and phINS356m1/2LUC, respectively.

### 2.3. Transient transfection and luciferase reporter assays

MIN6 cells ( $5 \times 10^5$  cells/well) were seeded in 35 mm or six-well plates for plasmid transfection using HilyMax reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cells were incubated for 3 h with 1 ml of serum-free transfection cocktail containing 1–2  $\mu\text{g}$  WT or KD CaMKII $\delta$  expression and luciferase vectors. Whole cell protein extracts were prepared 48 h later. Then, cells were cultured DMEM supplemented with 15% FBS plus either 25 mM glucose, 3 mM glucose or 30 mM KCl, for 12 h before the luciferase assay. Firefly and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions.



**Fig. 1.** Expression of WT and KD CaMKII $\delta$ 2. (A) Effects of WT and KD CaMKII $\delta$ 2 overexpression and siRNA knock down of CaMKII $\delta$  in MIN6 cells. After transfection with the WT and KD CaMKII $\delta$ 2 vectors or CaMKII $\delta$  siRNA, the lysates of MIN6 cells were subjected to western blot analysis using an anti-CaMKII $\delta$ 2 antibody. Upper panel: representative western blots. Lower panel: densitometric analysis of the western blot (mean  $\pm$  SE,  $n = 8$ ); \* $p < 0.01$  versus basal levels. (B) mRNA expression of CaMKII $\delta$  in MIN6 cells transfected with CaMKII $\delta$  siRNA. Values are means  $\pm$  SE ( $n = 8$ ). \*\* $p < 0.001$  versus control siRNA. (C) CaMKII kinase activity evaluated by phosphorylation of Synapsin I, a CaMKII-specific substrate. MIN6 without vector, white dotted bar; control vector, white bar; WT CaMKII $\delta$ 2 vector, striped bar; KD CaMKII $\delta$ 2 vector, black dotted bar; control siRNA, gray bar; CaMKII $\delta$  siRNA, black bar. Values are means  $\pm$  SE ( $n = 8$ ). \* $p < 0.05$  and \*\* $p < 0.001$  versus control (analysis of variance and Fisher's protected least significant difference test).

## 2.4. RNA interference

Small interfering RNA (siRNA) against mouse CaMKII $\delta$ , ON-TARGETplus SMARTpool siRNA (J-040821-05-0005), and negative control ON-TARGETplus siCONTROL non-targeting pool (D-001810, siNC) were purchased from Dharmacon (Denver, CO). Approximately  $1 \times 10^6$  MIN6 cells were microporated with 60 nmol of siRNA according to the manufacturer's instructions (LMS Co. Ltd., Tokyo, Japan) and tested 24 h later.

## 2.5. Immunoprecipitation

MIN6 cells were transfected with pCAGGSneo plasmid alone, WT or KD CaMKII $\delta$ 2 expression plasmids. Immunoprecipitation was performed as previously described [16]. The eluate was then subjected to western blot analysis.

## 2.6. Immunoblotting

The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Western blotting was performed as previously described [18].

## 2.7. Real-time qRT-PCR

qRT-PCR was performed as previously reported [18] using primers for the human insulin gene (Supplemental Table 2). Insulin

mRNA expression levels were quantified after normalization for  $\beta$ -actin mRNA levels. A melting curve analysis was performed to assess PCR product specificities.

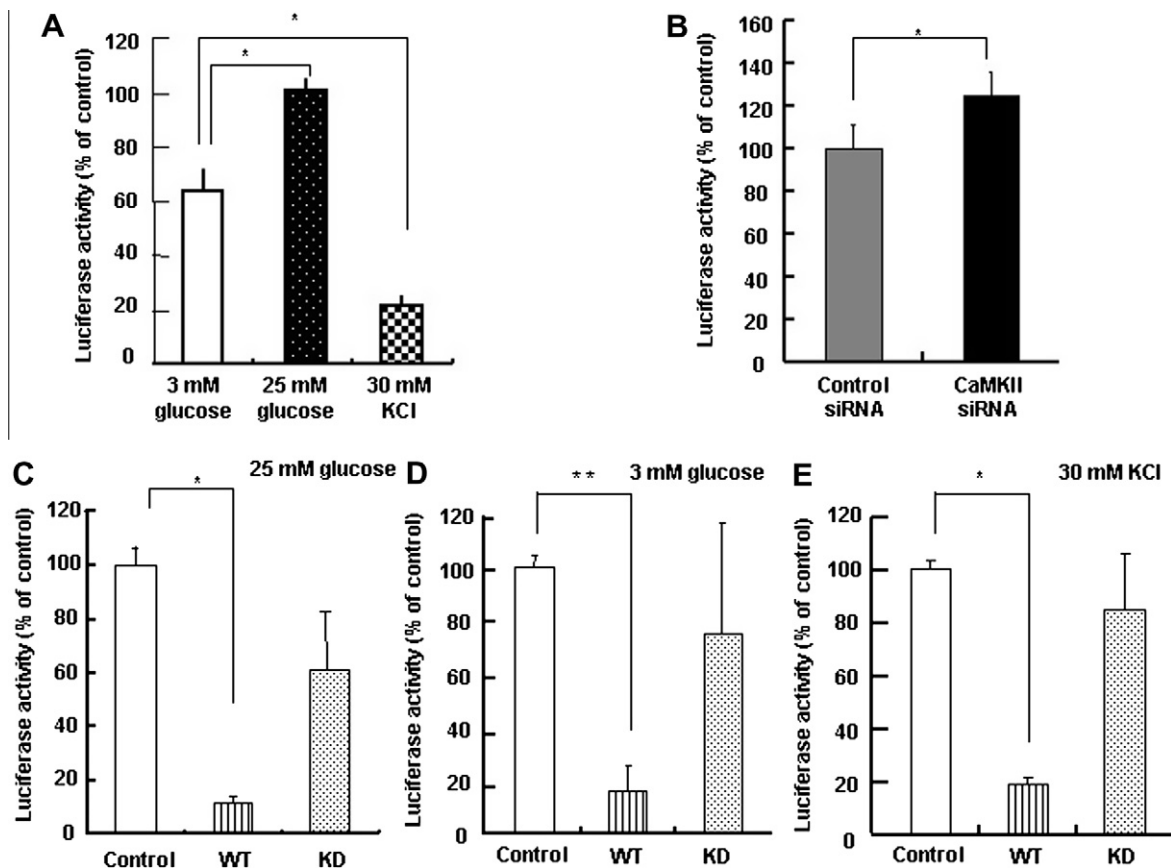
## 2.8. Statistical analysis

Data are expressed as means  $\pm$  standard error of the mean from at least three experiments. Data were statistically analyzed using unpaired two-tailed *t* tests for two groups, while differences among more than two groups were assessed by analysis of variance and Fisher's protected least significant difference test. Values of *p* < 0.05 were considered statistically significant.

## 3. Results

### 3.1. Effects of WT or KD CaMKII $\delta$ 2 overexpression and CaMKII $\delta$ siRNA in MIN6 cells

MIN6 cells were transiently transfected with pCAGGSneo carrying either WT or KD CaMKII $\delta$ 2. In KD CaMKII $\delta$ 2, Lys43 was substituted with Ala, which inhibited the ATP binding and kinase activities of CaMKII $\delta$ 2 [19] (Supplemental Fig. 1). WT and KD CaMKII $\delta$ 2 overexpression and siRNA-induced knockdown of CaMKII $\delta$  expression in MIN6 cells were confirmed by western blot analysis and qRT-PCR. WT and KD CaMKII $\delta$ 2 overexpression increased protein expression by  $\sim$ 2-fold compared with the expression of endogenous CaMKII $\delta$ 2 (Fig. 1A). CaMKII $\delta$  siRNA significantly



**Fig. 2.** Effects of CaMKII $\delta$ 2 overexpression or CaMKII $\delta$  knock down on human insulin gene promoter activity. (A) Insulin promoter activity in MIN6 cells transfected with control vector and incubated in 3 mM glucose (white bar), 25 mM glucose (white dotted bar), or 30 mM KCl (checked bar). Values are means  $\pm$  SE (*n* = 8). \**p* < 0.05 between the indicated groups. (B) Effects of CaMKII siRNA transfection on insulin promoter activity in MIN6 cells. Control siRNA, gray bar; CaMKII siRNA, black bar. Values are means  $\pm$  SE (*n* = 8). \**p* < 0.001 between the indicated groups. (C–E) Effects of WT or KD CaMKII $\delta$ 2 overexpression on insulin gene promoter activity after incubation in 25 mM glucose (C), 3 mM glucose (D), or 30 mM KCl (E). Control vector, white bar; WT CaMKII $\delta$ 2 vector, striped bar; KD CaMKII $\delta$ 2 vector, black dotted bar. Values are means  $\pm$  SE (*n* = 12). \**p* < 0.05 and \*\**p* < 0.001 versus control vector.

reduced CaMKII expression at the protein (Fig. 1A) and mRNA (Fig. 1B) levels. To evaluate CaMKII kinase activity, we performed western blot analysis using anti-phospho-Ser603-synapsin I antibodies. This test revealed that phosphorylation of synapsin I was significantly increased by WT CaMKII $\delta$ 2 and decreased by CaMKII $\delta$  siRNA (Fig. 1C).

### 3.2. Effects of CaMKII $\delta$ 2 on insulin gene promoter activity in MIN6 cells

We performed luciferase assays using MIN6 cells cultured in DMEM supplemented with 3 mM glucose, 25 mM glucose, or 30 mM KCl (Fig. 2A). Insulin promoter activity was significantly increased in cells cultured in 25 mM glucose and significantly decreased in those cultured in 30 mM KCl compared with that in cells cultured in 3 mM glucose. We then examined the effects of WT CaMKII $\delta$ 2, KD CaMKII $\delta$ 2, and CaMKII $\delta$  siRNA on insulin gene promoter activity. Insulin promoter activity was significantly increased by suppressing CaMKII expression with CaMKII $\delta$  siRNA (Fig. 2B). WT CaMKII $\delta$ 2 overexpression significantly decreased insulin gene promoter activity to  $10.2 \pm 1.5\%$  of the control, whereas KD CaMKII $\delta$ 2 overexpression did not affect promoter activity (Fig. 2C). Next, we evaluated the effects of glucose or KCl-induced membrane depolarization on insulin gene promoter activity regulated by CaMKII. The effects of WT CaMKII $\delta$ 2 and KD CaMKII $\delta$ 2 on insulin gene promoter activity were generally unchanged in 25 and 3 mM glucose and 30 mM KCl ( $10.2 \pm 3.0\%$ ,  $17.1 \pm 10.4\%$ , and  $19.5 \pm 2.2\%$ , respectively, for WT;  $59.8 \pm 23.8\%$ ,  $75.6 \pm 41.7\%$ , and  $84.5 \pm 21.9\%$ , respectively, for KD; Fig. 2C–E).

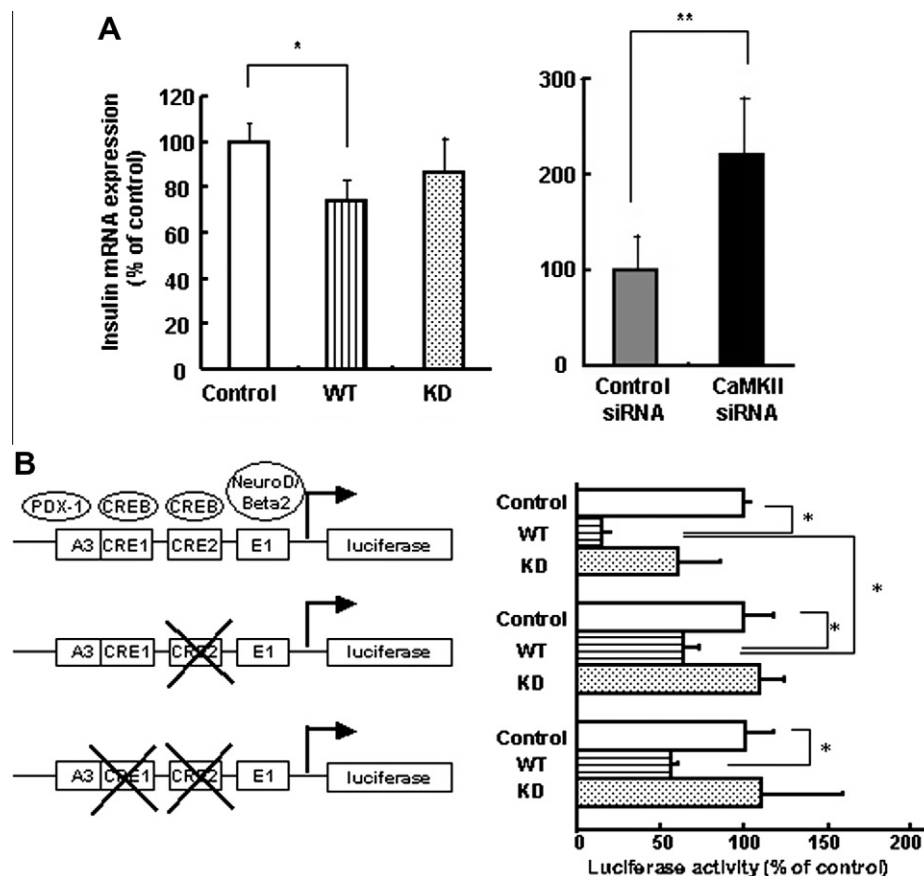
These results suggest that insulin gene promoter activity was downregulated by CaMKII $\delta$ 2 independent of glucose concentration and membrane depolarization.

### 3.3. Effects of CaMKII $\delta$ 2 overexpression or knock-down on insulin mRNA levels in MIN6 cells

To determine whether the changes in insulin gene promoter activity also affected insulin mRNA levels, we measured insulin mRNA levels in MIN6 cells. Insulin mRNA was significantly decreased by  $73.3 \pm 16.0\%$  ( $p < 0.05$ ) by WT CaMKII $\delta$ 2, but was significantly increased by  $221.4 \pm 26.7\%$  ( $p < 0.01$ ) by CaMKII $\delta$  siRNA (Fig. 3A). These observations further confirm that insulin gene promoter activity is downregulated by activation of CaMKII $\delta$ 2.

### 3.4. Identification of cis-acting regulatory sequences in the insulin gene promoter

To assess whether CREs in the human insulin gene promoter were involved in the downregulation of insulin gene promoter activity by WT CaMKII $\delta$ 2 overexpression, we performed luciferase assays using the insulin gene promoter bearing mutations in CREB binding sites, CRE1 and 2 (CRE1/2), and CRE2 (Supplemental Fig. 2). As shown in Fig. 3B, a CRE2 mutation partially but significantly rescued the inhibitory effect of WT CaMKII $\delta$ 2 overexpression on insulin gene promoter activity (from  $10.2 \pm 1.5\%$  to  $76.8 \pm 10.3\%$ ), whereas mutations of CRE1/2 did not have additional effects (from  $10.2 \pm 1.5\%$  to  $52.8 \pm 0.1\%$ ). These data suggest that



**Fig. 3.** (A) Effects of WT or KD CaMKII $\delta$ 2 expression vectors or CaMKII $\delta$  siRNA overexpression on insulin mRNA levels in MIN6 cells. MIN6 cells were transfected with the indicated vector or CaMKII $\delta$  siRNA, and lysates subjected to quantitative RT-PCR. Control vector, white bar; WT CaMKII $\delta$ 2 vector, striped bar; KD CaMKII $\delta$ 2 vector, black dotted bar; control siRNA, gray bar; CaMKII $\delta$  siRNA, black bar. Values are means  $\pm$  SE ( $n = 10$ ). \* $p < 0.01$  and \*\* $p < 0.001$  versus control. (B) Effects of CRE mutations on insulin gene promoter activity. MIN6 cells were transfected with a pGL3 luciferase vector containing the CRE-mutated human insulin gene promoter and each vector. Control vector, white bar; WT CaMKII $\delta$ 2 vector, striped bar; KD CaMKII $\delta$ 2 vector, black dotted ba. Values are means  $\pm$  SE ( $n = 14$ ). \* $p < 0.01$  between the indicated groups.



CaMKII $\delta$ -induced downregulation of human insulin gene promoter activity is mediated by CRE2 but not by CRE1.

### 3.5. Effects of CaMKII $\delta$ on Ser142 phosphorylation of CREB and binding to CBP in MIN6 cells

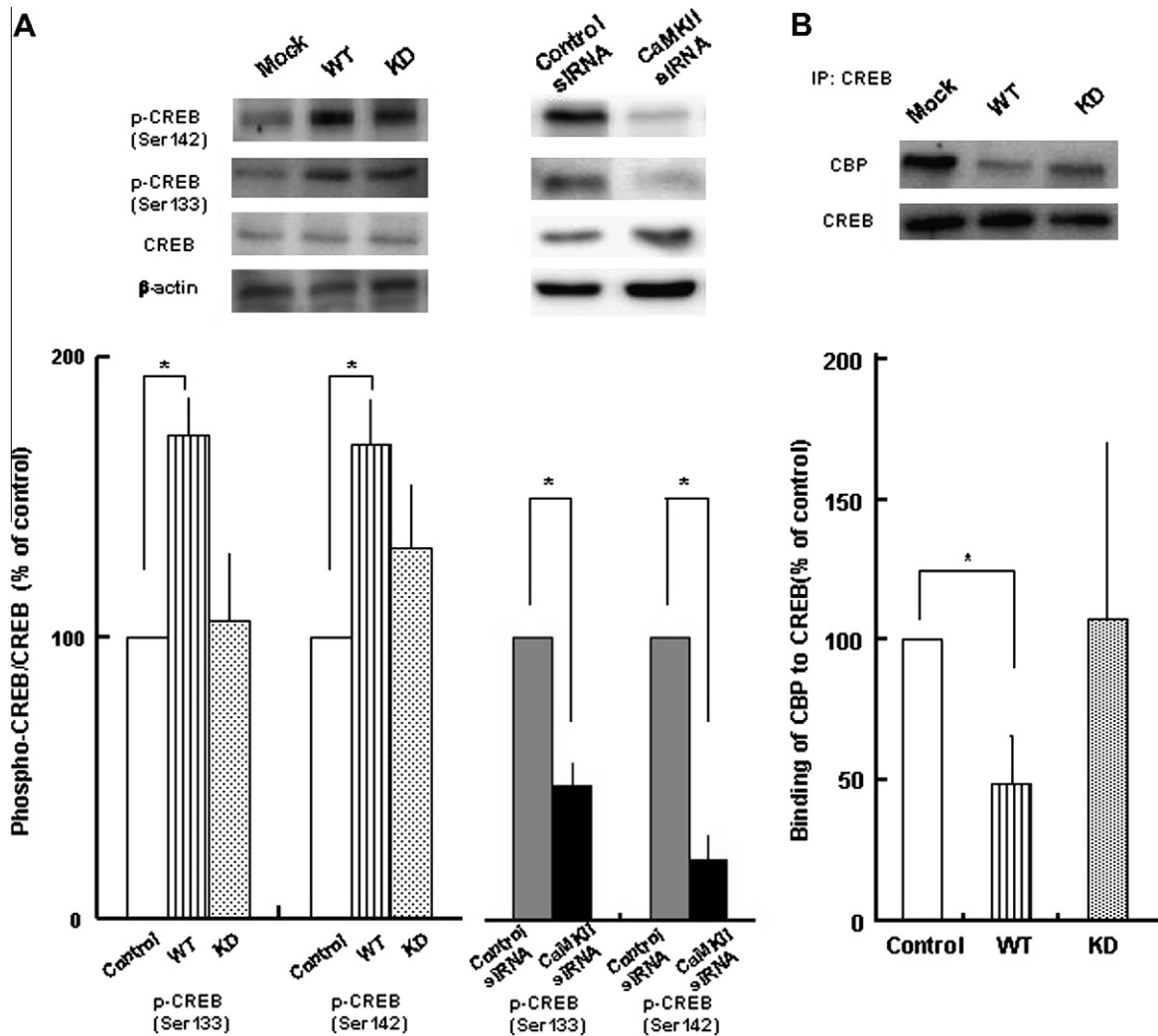
Because downregulation of insulin gene promoter activity by CaMKII $\delta$  was partially rescued by a CRE2 mutation, we hypothesized that Ser142 of CREB was phosphorylated by CaMKII $\delta$ , which might decrease binding of CREB to CBP and suppression of CREB-mediated transcription. To test this hypothesis, we performed western blot analysis using an anti-phospho-Ser142-CREB antibody. While CREB phosphorylation at Ser142 and Ser133 was significantly increased by WT CaMKII $\delta$  (Ser142:  $168.4 \pm 16.3\%$ ; Ser133:  $172.5 \pm 12.6\%$ ), CaMKII $\delta$  siRNA markedly decreased phosphorylation of CREB at Ser142 and Ser133 (Ser142:  $21.4 \pm 8.7\%$ ; Ser133:  $47.3 \pm 8.1\%$ ; Fig. 4A). To confirm that CaMKII $\delta$  regulates insulin gene transcription by preventing CREB binding to CBP, we performed immunoprecipitation followed by western blot analysis using anti-CBP and anti-CREB antibodies (Fig. 4B). CREB binding to CBP was significantly decreased by WT CaMKII $\delta$  ( $48.3 \pm 5.9\%$ ),

but not by KD CaMKII $\delta$  ( $107.9 \pm 62.7\%$ ). Taken together, these data indicate that CaMKII $\delta$  inhibits CREB binding to CBP by phosphorylating CREB at Ser142, which downregulates insulin gene promoter activity.

## 4. Discussion

In this study, we demonstrated that CaMKII $\delta$  inhibited insulin gene promoter activity in MIN6 cells. Several studies have described the role of CaMKII $\delta$  in  $\text{Ca}^{2+}$ -dependent insulin secretion [3,9,10,13,20–22]. However, the role of CaMKII $\delta$  in insulin gene transcription has not been thoroughly investigated. In MIN6 cells, we demonstrated that insulin gene promoter activity and insulin mRNA expression were decreased by WT CaMKII $\delta$  and increased by CaMKII $\delta$  siRNA.

Insulin gene transcription is regulated by increases in extracellular glucose and  $\text{Ca}^{2+}$  levels, nutrient availability, and hormone signaling [23]. In MIN6 cells, we found that insulin promoter activity was activated by 25 mM glucose and suppressed by 30 mM KCl compared with that in 3 mM glucose. We hypothesized that KCl may enhance the inhibitory effects of WT CaMKII $\delta$  overexpression



**Fig. 4.** Effects of CaMKII $\delta$  overexpression or CaMKII $\delta$  siRNA on phosphorylation of CREB at Ser142 and Ser133, and binding to CBP. (A) Upper panel: representative western blot analysis of MIN6 cells transfected with WT or KD CaMKII $\delta$  and CaMKII $\delta$  siRNA using anti-phospho-Ser142 and -Ser133-CREB antibodies. Lower panel: densitometric analysis of the western blot for Ser142 phosphorylation. (B) Upper panel: immunoprecipitation and western blot analysis. MIN6 cell lysates were immunoprecipitated with an anti-CREB antibody followed by immunoblotting with anti-CBP and anti-CREB antibodies. Lower panel: densitometric analysis of the western blot for CBP. Data are normalized for the expression of  $\beta$ -actin in the same sample and are expressed as a percentage of the control value. Control vector, white bar; WT CaMKII $\delta$  vector, striped bar; KD CaMKII $\delta$  vector, black dotted bar; control siRNA, gray bar; CaMKII $\delta$  siRNA, black bar. Values are means  $\pm$  SE ( $n = 8$ ). \* $p < 0.01$  versus control.

on insulin gene promoter activity because  $\beta$  cell stimulation with insulin secretagogues, such as glucose, tolbutamide, and high  $K^+$ , enhanced CaMKII activity [3,22]. However, high glucose and  $K^+$  did not affect CaMKII $\delta$ -mediated downregulation of insulin gene promoter activity (Fig. 2C–E), suggesting that the inhibitory effects of CaMKII $\delta$  are independent of glucose and membrane depolarization.

CaMKII inhibits the transcriptional activity of various genes with CREs in the promoter to a greater degree than the stimulatory effects of PKA [21] and CaMKIV [24–26]. Only primates possess CRE1–4 in the insulin gene, but the CRE2 sequence corresponds to a single CRE in other mammals, including rodents [27]. Our results suggest that CRE2, but not CRE1, is the primary target of CaMKII $\delta$ -mediated downregulation of insulin gene promoter. The most likely explanation for this observation is that CRE1 overlaps with an A3 site (Supplemental Fig. 2). The A3 site is a binding site for pancreatic and duodenal homeobox gene-1 (PDX-1), which is mainly expressed in  $\beta$  cells and is required for pancreatic development and glucose-induced insulin gene transcription [28]. Overlapping of the A3 site with CRE1 might result in occupation of CRE1 by PDX-1, thereby preventing other transcription factors from binding to this region.

A mechanism for CaMKII-mediated down-regulation of CRE-dependent genes expression has been proposed. CREB phosphorylation at Ser142 inhibits CREB dimerization, and reduces binding of CREB to CBP [4,29]. CaMKII-induced phosphorylation of CREB at Ser133 and Ser142 does not stimulate CREB-dependent gene transcription [24,25]. This mechanism is consistent with our current results.

We found that CRE1/2 and CRE2 mutations partially rescued the downregulation of insulin gene promoter activity caused by CaMKII $\delta$ . The insulin gene promoter is organized in a complex arrangement of discrete *cis*-acting sequence motifs, and its activity is controlled by various mechanisms involving several *trans*-activating factors, including CREB, PDX-1, and NeuroD. PDX-1 promotes insulin gene transcription in a glucose-dependent manner [30]. CaMKII mediates phosphorylation of NeuroD at Ser336 in primary neurons [31]. Therefore, the partial rescue of insulin gene promoter activity mediated by CRE1/2 and CRE2 mutations might be due to interactions between CaMKII $\delta$  and other transcriptional factors, including PDX-1 and/or NeuroD.

Osterhoff et al. [32] demonstrated that insulin gene transcription was downregulated by CaMKII $\delta$  inhibition and upregulated by CaMKII $\delta$  overexpression. They proposed that the mechanism involved CaMKII $\delta$  in the activation of PDX-1, and that CaMKII $\delta$  did not directly phosphorylate the transcription factors. However, our study showed that insulin promoter activity was increased by CaMKII $\delta$  siRNA and decreased by phosphorylation of CREB at Ser142 as a result of WT CaMKII $\delta$  overexpression. The cause of this discrepancy is unknown, but may be due to differences in experimental methods including the use of antisense-nucleotides.

Although WT CaMKII $\delta$  overexpression sufficiently affected insulin gene promoter activity, KD CaMKII $\delta$  did not affect insulin gene promoter activity. We used a KD form of CaMKII $\delta$  that prevents ATP binding [19]. Wu et al. reported that CaMKII modifies CREB at Ser142, which inhibited CREB dimerization and binding to CBP, and that CREB phosphorylated at Ser142 has a dominant inhibitory effect [4]. KD CaMKII $\delta$  expression might be insufficient to inhibit Ser142 phosphorylation of CREB caused by endogenous CaMKII.

Some patients with type 2 diabetes show increases in blood glucose levels after long-term sulfonylurea therapy [33]. This phenomenon, known as secondary sulfonylurea failure, is thought to be a result of progressive  $\beta$  cell functional loss over the course of type 2 diabetes [34]. In  $\beta$  cells, prolonged hyperglycemia and long-term use of sulfonylurea may induce chronic  $Ca^{2+}$  influx fol-

lowed by prolonged CaMKII activation. Therefore, we propose that downregulation of insulin gene transcription by CaMKII overexpression may be involved in decreased insulin synthesis caused by sustained hyperglycemia and/or long-term use of sulfonylurea. Further studies are needed to examine the effects of CaMKII on glucose toxicity and secondary sulfonylurea failure.

In conclusion, our study showed that CaMKII $\delta$  regulates the insulin gene promoter. CaMKII $\delta$  enhanced CREB phosphorylation at Ser133 and Ser142, which might inhibit CREB binding to CBP, and downregulation of insulin gene promoter activity. Therefore, CaMKII $\delta$  may play an important role in insulin gene expression and the pathogenesis of diabetes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2012.04.091>.

## References

- [1] G. Manning, D.B. Whyte, R. Martinez, et al., The protein kinase complement of the human genome, *Science* 298 (2002) 1912–1934.
- [2] M.T. Swilius, M.N. Waxham,  $Ca^{2+}$ /calmodulin-dependent protein kinases, *Cell Mol. Life Sci.* 65 (2008) 2637–2657.
- [3] K. Matsumoto, K. Fukunaga, J. Miyazaki, et al.,  $Ca^{2+}$ /calmodulin-dependent protein kinase II and synapsin I-like protein in mouse insulinoma MIN6 cells, *Endocrinology* 136 (1995) 3784–3793.
- [4] X. Wu, C.T. McMurray, Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein, *J. Biol. Chem.* 276 (2001) 1735–1741.
- [5] O. Uchi, J.K. Komukai, Y. Kusakari, et al.,  $\alpha 1$ -adrenoceptor stimulation potentiates L-type  $Ca^{2+}$  current through  $Ca^{2+}$ /calmodulin-dependent PK II (CaMKII) activation in rat ventricular myocytes, *Proc. Natl. Acad. Sci. USA* 102 (2005) 9400–9405.
- [6] A. Menegon, C. Verderio, C. Leoni, et al., Spatial and temporal regulation of  $Ca^{2+}$ /calmodulin-dependent protein kinase II activity in developing neurons, *J. Neurosci.* 22 (2002) 7016–7026.
- [7] I. Niki, K. Okazaki, M. Saitoh, et al., Presence and possible involvement of  $Ca^{2+}$ /calmodulin-dependent protein kinases in insulin release from the rat pancreatic beta cell, *Biochem. Biophys. Res. Commun.* 191 (1993) 255–261.
- [8] V. Urquidí, S.J. Ashcroft, A novel pancreatic beta-cell isoform of calcium/calmodulin-dependent protein kinase II (beta 3 isoform) contains a proline-rich tandem repeat in the association domain, *FEBS Lett.* 358 (1995) 23–26.
- [9] M. Mohlig, S. Wolter, P. Mayer, et al., Insulinoma cells contain an isoform of  $Ca^{2+}$ /calmodulin-dependent protein kinase II delta associated with insulin secretion vesicles, *Endocrinology* 138 (1997) 2577–2584.
- [10] H. Rochlitz, A. Voigt, B. Lankat-Buttgereit, et al., Cloning and quantitative determination of the human  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaMK II) isoforms in human beta cells, *Diabetologia* 43 (2000) 465–473.
- [11] A. Tegenholm, E. Gylfe, Oscillatory control of insulin secretion, *Mol. Cell Endocrinol.* 297 (2009) 58–72.
- [12] P. Rellos, A.C. Pike, F.H. Niesen, et al., Structure of the CaMKII $\delta$ /calmodulin complex reveals the molecular mechanism of CaMKII kinase activation, *PLoS Biol.* 8 (2010) e1000426.
- [13] R.A. Easom, CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis, *Diabetes* 48 (1999) 675–684.
- [14] H. Yamamoto, K. Matsumoto, E. Araki, et al., New aspects of neurotransmitter release and exocytosis: involvement of  $Ca^{2+}$ /calmodulin-dependent phosphorylation of synapsin I in insulin exocytosis, *J. Pharmacol. Sci.* 93 (2003) 30–34.
- [15] K. Ohneda, H. Ee, M. German, Regulation of insulin gene transcription, *Semin. Cell Dev. Biol.* 11 (2000) 227–233.
- [16] H. Tabuchi, H. Yamamoto, K. Matsumoto, et al., Regulation of insulin secretion by overexpression of  $Ca^{2+}$ /calmodulin-dependent protein kinase II in insulinoma MIN6 cells, *Endocrinology* 141 (2000) 2350–2360.

- [17] N. Furukawa, T. Shirotani, E. Araki, et al., Possible involvement of atypical protein kinase C (PKC) in glucose-sensitive expression of the human insulin gene: DNA-binding activity and transcriptional activity of pancreatic and duodenal homeobox gene-1 (PDX-1) are enhanced via calphostin C-sensitive but phorbol 12-myristate 13-acetate (PMA) and Go 6976-insensitive pathway, *Endocr. J.* 46 (1999) 43–58.
- [18] H. Oiso, N. Furukawa, M. Suefuji, et al., The role of class I histone deacetylase (HDAC) on gluconeogenesis in liver, *Biochem. Biophys. Res. Commun.* 404 (2011) 166–172.
- [19] R.C. Rich, H. Schulman, Substrate-directed function of calmodulin in autophosphorylation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II, *J. Biol. Chem.* 273 (1998) 28424–28429.
- [20] H.S. Bhatt, B.P. Conner, G. Prasanna, et al., Dependence of insulin secretion from permeabilized pancreatic beta-cells on the activation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II. A re-evaluation of inhibitor studies, *Biochem. Pharmacol.* 60 (2000) 1655–1663.
- [21] K.A. Krueger, E.I. Ings, A.M. Brun, et al., Site-specific phosphorylation of synapsin I by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in pancreatic betaTC3 cells: synapsin I is not associated with insulin secretory granules, *Diabetes* 48 (1999) 499–506.
- [22] N. Inagaki, T. Maekawa, T. Sudo, et al., C-Jun represses the human insulin promoter activity that depends on multiple cAMP response elements, *Proc. Natl. Acad. Sci. USA* 89 (1992) 1045–1049.
- [23] K. Docherty, W.M. Macfarlane, M.L. Read, et al., Regulation of insulin gene transcription by nutrients, *Biochem. Soc. Trans.* 24 (1996) 368–372.
- [24] G.A. Gonzalez, M.R. Montminy, Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133, *Cell* 59 (1989) 675–680.
- [25] P. Sun, L. Lou, R.A. Maurer, Regulation of activating transcription factor-1 and the cAMP response element-binding protein by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases type I, II, and IV, *J. Biol. Chem.* 271 (1996) 3066–3073.
- [26] J. Kasahara, K. Fukunaga, E. Miyamoto, Differential effects of a calcineurin inhibitor on glutamate-induced phosphorylation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases in cultured rat hippocampal neurons, *J. Biol. Chem.* 274 (1999) 9061–9067.
- [27] C.W. Hay, K. Docherty, Comparative analysis of insulin gene promoters: implications for diabetes research, *Diabetes* 55 (2006) 3201–3213.
- [28] H. Hui, R. Perfetti, Pancreas duodenum homeobox-1 regulates pancreas development during embryogenesis and islet cell function in adulthood, *Eur. J. Endocrinol.* 146 (2002) 129–141.
- [29] B. Mayr, M. Montminy, Transcriptional regulation by the phosphorylation-dependent factor CREB, *Nat. Rev. Mol. Cell Biol.* 2 (2001) 599–609.
- [30] A.L. Mosley, J.A. Corbett, S. Özcan, Glucose regulation of insulin gene expression requires the recruitment of p300 by the beta-cell-specific transcription factor Pdx-1, *Mol. Endocrinol.* 18 (2004) 2279–2290.
- [31] B. Gaudilliere, Y. Konishi, N. de la Iglesia, et al., CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis, *Neuron* 41 (2004) 229–241.
- [32] M. Osterhoff, M. Mohlig, M. Schwanstecher, et al.,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II delta2 regulates gene expression of insulin in INS-1 rat insulinoma cells, *Cell Calcium* 33 (2003) 175–184.
- [33] D.R. Matthews, C.A. Cull, I.M. Stratton, et al., UKPDS 26: sulphonylurea failure in non-insulin-dependent diabetic patients over six years. UK Prospective Diabetes Study (UKPDS) Group, *Diabet. Med.* 15 (1998) 297–303.
- [34] A.A. Riedel, H. Heien, J. Wogen, et al., Loss of glycemic control in patients with type 2 diabetes mellitus who were receiving initial metformin, sulfonylurea, or thiazolidinedione monotherapy, *Pharmacotherapy* 27 (2007) 1102–1110.