Original Article

ERK1/2 Control Phosphorylation and Protein Level of cAMP-Responsive Element–Binding Protein

A Key Role in Glucose-Mediated Pancreatic β-Cell Survival

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cAMP-responsive element–binding protein (CREB) is required for β-cell survival by regulating expression of crucial genes such as *bcl-2* and *IRS-2*. Using MIN6 cells and isolated rat pancreatic islets, we investigated the signaling pathway that controls phosphorylation and protein level of CREB. We observed that 10 mmol/l glucose–induced CREB phosphorylation was totally inhibited by the protein kinase A (PKA) inhibitor H89 (2 μmol/l) and reduced by 50% with the extracellular signal–regulated kinase (ERK)1/2 inhibitor PD98059 (20 μmol/l). This indicates that ERK1/2, reported to be located downstream of PKA, participates in the PKA-mediated CREB phosphorylation elicited by glucose. In ERK1/2-downregulated MIN6 cells by siRNA, glucose-stimulated CREB phosphorylation was highly reduced and CREB protein content was decreased by 60%. In MIN6 cells and islets cultured for 24–48 h in optimal glucose concentration (10 mmol/l), which promotes survival, blockade of ERK1/2 activity with PD98059 caused a significant decrease in CREB protein level, whereas CREB mRNA remained unaffected (measured by real-time quantitative PCR). This was associated with loss of *bcl-2* mRNA and protein contents, caspase-3 activation, and emergence of ultrastructural apoptotic features detected by electron microscopy. Our results indicate that ERK1 and -2 control the phosphorylation and protein level of CREB and play a key role in glucose-mediated pancreatic β-cell survival.
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Glucose utilization and oxidation within the pancreatic β-cells lead to ATP production and subsequent closure of the ATP-dependent potassium channel, eliciting the entry of calcium through the opening of L-type voltage-dependent calcium channels (1–3). The calcium entry induced by glucose stimulates insulin secretion and activates various kinases in the β-cells, including protein kinase A (PKA) and p44/42 mitogen-activated protein kinases (also called extracellular signal–regulated kinase [ERK]1/2) (4–9). The cAMP-PKA pathway controls the exocytosis of insulin granules and the expression of numerous β-cell genes (10–12). The ERK1/2 pathway is organized hierarchically into three-tiered modules. ERK1 and -2 are phosphorylated on Thr202 and Tyr204 by ERK1/2 kinases (MEK1/2), which are phosphorylated and activated by ERK1/2 kinases kinases (Raf isoforms) (13,14). In the β-cell nucleus, glucose-activated ERK1 and -2 phosphorylate and induce the functional activity of the transcription factors *Beta2* and *PDX-1*, resulting in a cumulative transactivation of the insulin gene promoter (15). ERK1/2 activity was also found in the cytoplasm of glucose-stimulated β-cells, leading to the phosphorylation of proteins implicated in exocytosis of insulin granules such as synapsin I (16). Cross talk between the cAMP-PKA and ERK1/2 signaling pathways in β-cells has been reported. Indeed, we and others have shown that glucose-induced calcium entry leads to cAMP-induced activation of PKA, which acts upstream of MEK1/2 to mediate ERK1/2 phosphorylation (4,16).

Recently, it has been reported that glucose-induced calcium entry also activates (by phosphorylation) the transcription factor cAMP-responsive element–binding protein (CREB) and its paralog, ATF-1, at a single site corresponding to serine 133 in CREB (17). Phosphorylation of serine 133 is required for CREB-mediated transcription (18). CREB has been reported to be required in glucose homeostasis and β-cell survival by regulating the expression of the genes *bcl-2* and *IRS-2* (17,19). Mice deficient in CREB activity in β-cells develop diabetes secondary to β-cell apoptosis (17), whereas overexpression of wild-type CREB in MIN6 cells protects against cytokine-induced apoptosis (19). These reports point to CREB as an important target for strategies aimed at improving the survival of β-cells (17,19).

β-Cell survival is both positively and negatively regulated in response to the glucose conditions to which pancreatic β-cells are exposed. Exposure of rat islets or INS-1 or MIN6 β-cells to 6–10 mmol/l glucose for minutes to hours results in the inhibition of apoptosis and thus maintenance of survival (20,21). In contrast, chronic exposure of rat islets or β-cells to 11–30 mmol/l glucose results in β-cell dysfunction and death, a concept known as glucotoxicity (22,23). To date, the action mechanism of glucose on β-cell survival has not been identified. In the

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CREB, cAMP-responsive element–binding protein; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal–regulated kinase; GLP-1, glucagon-like peptide-1; PKA, protein kinase A; siRNA, small interfering RNA.

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TABLE 1
Sequence of primer used in quantitative RT-PCR analysis of MIN6 cells

Gene	Accession no.	Product size (bp)	Forward primer	Reverse primer
Creb1	NM_133828	194	GCAACCAAGTTGTTGTTCA	TCTACGACATTCTCTTGCT
Bcl-2	NM_009741	194	CTTAGAAAATACAGCATTGCGGAG	GGATGTGCTTTGCATTCTTGG
B2m	NM_009735	198	CGCCTCACATTGAAATCC	CTCGATCCCAGTAGACG

All sequences are shown from 5′ to 3′.

present report, we investigated the pathway that controls phosphorylation and protein level of CREB. Our studies show that ERK1 and -2 control the phosphorylation and protein level of CREB and play a key role in glucose-mediated β-cell survival.

RESEARCH DESIGN AND METHODS

Anti–phospho-ERK1/2 (which selectively recognizes the doubly phosphorylated active forms of these kinases), anti-CREB, caspase-3, and cleaved caspase-3 antibodies were purchased from Cell Signaling (New England Biolabs, Beverly, MA). Phosphorylated CREB levels in MIN6 cells were detected with anti–phospho-CREB antibody (which detects CREB when phosphorylated at serine 133 and the phosphorylated form of the CREB-related protein, ATF-1) obtained from Cell Signaling. To detect phosphorylated CREB within isolated islets, anti–phospho-CREB antibody (Upstate, Charlottesville, VA) (which does not recognize CREB-related proteins) was used. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 media, and FCS were purchased from Life Technologies (Gibco BRL, Grand Island, NY). PD98059 was purchased from Calbiochem (La Jolla, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation of pancreatic rat islets. Male Wistar rats (Iffa-Credo, l'Arbresle, France) had free access to laboratory chow and water. They were housed, handled, and killed according to the rules of the CNRS Animal Care and Use Committee. Islets were isolated from fed male rats weighing 280–320 g on the day of killing by collagenase digestion (24), followed by Ficoll gradient separation.

Culture of MIN6 cells. MIN6 cells (passage 18–25) were cultured at 37°C in 5% CO_2 in DMEM containing 25 mmol/l glucose and 15% FCS as described (16,25). For apoptosis studies, cells were cultured for 48 h at 37°C in 5% CO_2 in serum-free DMEM containing various glucose concentrations ranging from 2.8 to 25 mmol/l. Exposure of MIN6 cells for 48 h to serum-free DMEM containing 25 mmol/l glucose was used as control for survival (26).

Western blotting. Before stimulation, MIN6 cells plated in six-well dishes were stabilized for 2 h at 37°C in glucose-free HEPES-balanced Krebs-Ringer bicarbonate buffer (16). Batches of 100–200 islets were stabilized for 2 h in Krebs-Ringer bicarbonate buffer containing 1.4 mmol/l glucose and 1g/l BSA or cultured for 12, 24, or 48 h at 37°C in 5% CO_2 in RPMI-1640 medium containing 10 mmol/l glucose and 5 g/l BSA. After stimulation or culture, MIN6 cells or islets were lysed for 30 min at 4°C in a solubilizing buffer and equal amounts of lysate proteins (25–50 μg protein/lane) were analyzed by Western blot (16,27). Visualization and quantification of the bands were obtained using a Kodak Image Station 2000R system (Eastman Kodak, Rochester, NY).

ERK2 siRNA in MIN6 cells. ERK2 expression was silenced in MIN6 cells as described (16), using mouse 20- to 25-nucleotide prevalidated small interfering RNA (siRNA) duplexes (Santa Cruz Biotechnology). The day before transfection, MIN6 cells were seeded in 12-well plates in regular growth medium but without antibiotics and grown overnight to reach 40–50% confluency. The day of the experiment, transfectant-siRNA complexes were prepared according to the manufacturer's instructions. The final concentration of siRNA is 25 nmol/l in each well (0.239 μg). siRNA transfection reagent is a highly efficient reagent for siRNA delivery with minimal cellular damage (ref. sc-29528; Santa Cruz Biotechnology). Transfectant-siRNA complexes were then added dropwise while gently rocking the 12-well plates. MIN6 cells were transfected with ERK2 siRNA or control siRNA (which corresponds to a nontargeting 20- to 25-nucleotide siRNA designed as a negative control) for 6 h at 37°C before switching to fresh growth medium including antibiotics; 24 or 48 h after transfection, cells were stimulated and lysed.

Real-time quantitative PCR. Total RNA was extracted from MIN6 cells using TRIZOL reagent (Invitrogen, Grand Island, NY). Real-time quantitative PCR was performed using LightCycler technology (Roche, Meylan, France). A specific set of primers was chosen to obtain a product close to 100 bp (Table 1). cDNA was obtained from 2 μg total RNA with SuperScript II RNase H^- Reverse Transcriptase and 0.5 μg Random Primer hexamer (Invitrogen). cDNA was diluted 1:36, and 2 μl was used as template for quantification. PCR was performed using a 10× LightCycler Faststart DNA Master Sybr Green I mix (Roche), 5 pmol/μl of each primer, and 2.5 mmol/l $MgCl_2$. After an 8-min denaturation at 95°C, 45 cycles were performed: 95°C for 5 s, 64°C for 10 s, and 72°C for 6 s. As control, a melting curve was performed after each amplification. A standard curve was made with serial dilutions (H_2O; 1:6, 1:36, 1:216, 1:1,296, and 1:7,776) from a control cDNA sample to evaluate the efficiency of the primers and to relatively quantify the expression level of each sample. All measurements were normalized using the β2 microglobulin (*β2m*) gene, a well-known housekeeping gene (28) that did not vary and was the best control compared with others in our experiments.

Electron microscopy. MIN6 cells or batches of 100–200 islets were immersed overnight at 4°C in a solution of 3.5% glutaraldehyde in phosphate buffer (0.1 mol/l, pH 7.4), rinsed, and postfixed in a 2% osmic acid for 1 h at room temperature. Cells or islets were then dehydrated in a graded series of ethanol solutions (30–100%) and embedded in Spurr's resine. Thin sections (85 nm; Leica-Reichert Ultracut E) were collected at different levels of each block. Sections were counterstained with uranyl acetate (1.5% in EtOH 70%) and lead citrate and observed using a Hitachi 7100 transmission electron microscope at the Centre Régional d'Imagerie Cellulaire de Montpellier (France).

Nuclear staining. MIN6 cells were prepared as described (27) and incubated in 4′,6-diamidino-2-phenylindole dihydrochloride hydrate. Apoptotic cells were identified by the presence of condensed nuclei stained in blue (29) and visualized using an epifluorescence microscope (Optiphot-2; Nikon).

Statistical analysis. Results are expressed as the means ± SE. Differences between results were analyzed by using Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Phosphorylation of CREB by glucose is mediated through a PKA-ERK1/2 signaling network in MIN6 cells. We first confirmed that treatment of glucose-starved MIN6 cells with glucose (10 mmol/l) stimulated rapid yet transient phosphorylation of CREB at serine 133, with maximal effect observed at 5–10 min and a return to basal by 20–30 min (Fig. 1*A*), as reported (17).

The complete suppression of glucose-induced CREB phosphorylation by a noncytotoxic treatment with a low concentration (2 μmol/l) of the PKA-specific inhibitor H89 indicates that glucose-stimulated phosphorylation of CREB requires PKA activation (Fig. 1*B* and *C*). The specificity of the H89 treatment was ascertained by the fact that it had no effect on EGF-induced ERK1/2 phosphorylation (data not shown).

Since we and others reported that H89 inhibited glucose-induced ERK1/2 activation, indicating that ERK1 and -2 are located downstream of PKA (4,16), we evaluated the role of ERK1/2 on CREB phosphorylation. We used PD98059, a selective inhibitor of MEK1 and -2 (the kinases upstream of ERK1/2). When used at 20 μmol/l, PD98059 completely blocks the phosphorylation of ERK1/2 induced by glucose (16). As shown in Fig. 1*D* and *E*, PD98059 treatment suppressed glucose-induced CREB phosphorylation by 50–55%. No other protein kinase has been found to be inhibited or activated by PD98059 at a concentration (≤25 μmol/l) that prevents activation of the ERK1/2 cascade (30). Moreover, to rule out the possibility that PD98059 may act as a moderate metabolic poison on

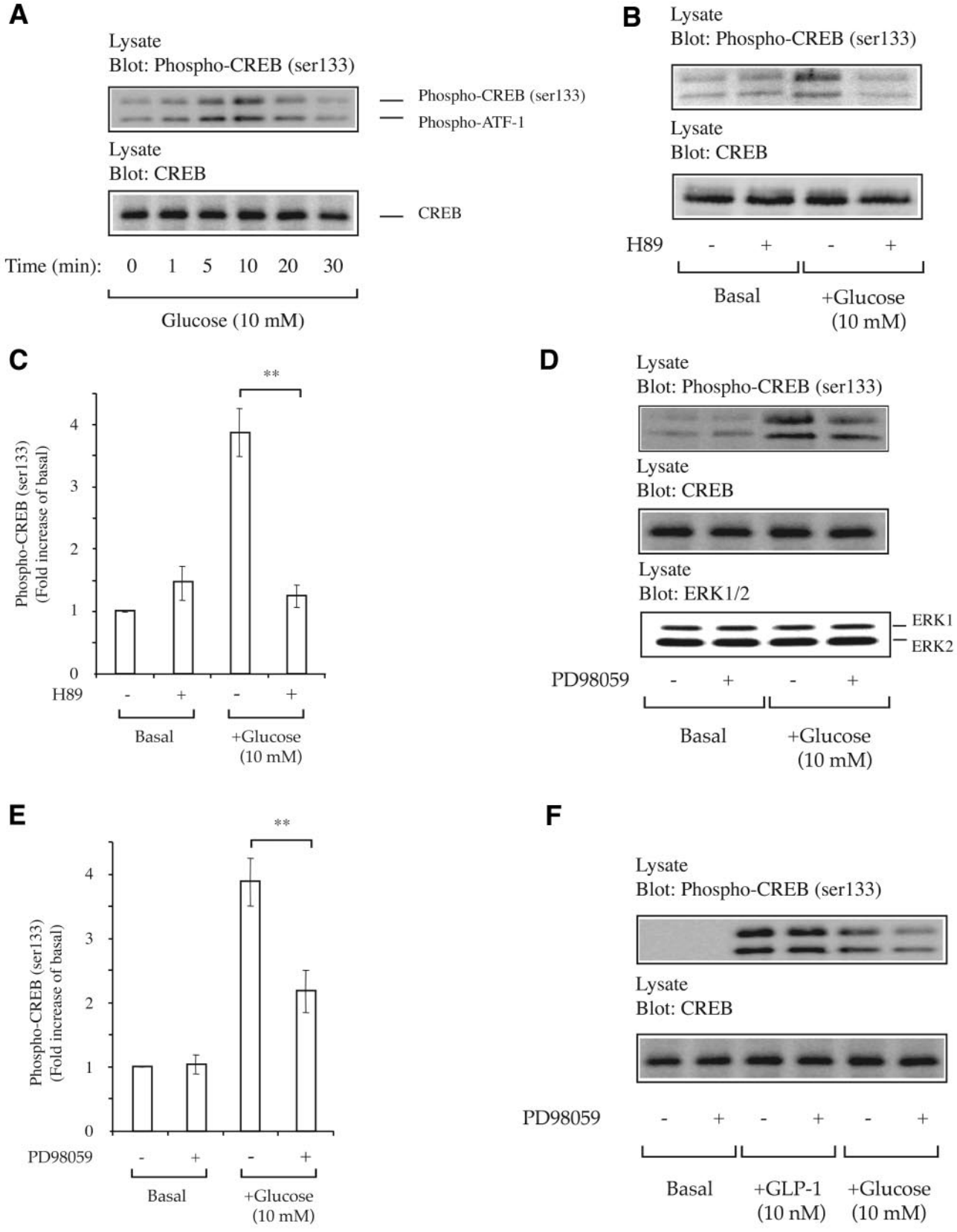


FIG. 1. Mechanisms of glucose-induced CREB phosphorylation in MIN6 cells. Phospho-CREB levels in cells stimulated with glucose for various times (*A*), treated (or not) with H89 (2 μmol/l) or PD98059 (20 μmol/l), and stimulated with glucose (*B*, *D*, and *F*) or GLP-1 (*F*) for 10 min. H89 or PD98059 was added (or not) during the 2-h stabilization period and maintained during stimulation. Exposures (*A*, *B*, *D*, and *F*) and means ± SE of five experiments (*C* and *E*). ***P*<0.01 vs. glucose-induced CREB phosphorylation. ser133, serine 133.

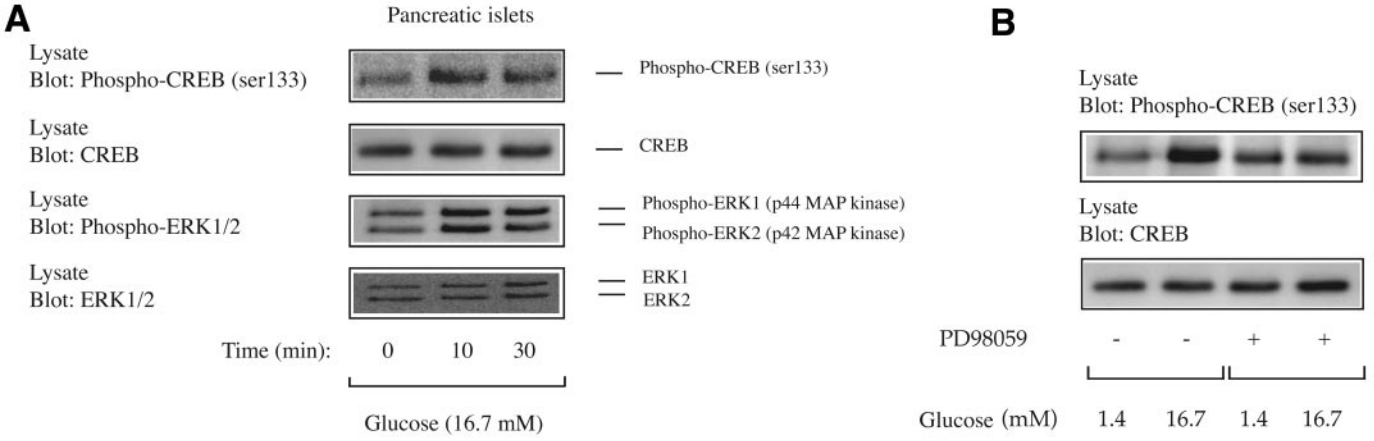


FIG. 2. CREB phosphorylation in pancreatic islets. *A*: Phospho-CREB and phospho-ERK1/2 levels in islets stimulated with glucose. *B*: Phospho-CREB levels in islets treated (or not) with PD98059 (20 μmol/l) and stimulated with glucose for 10 min. PD98059 was added (or not) during the 2-h stabilization period and maintained during stimulation. Exposures of three experiments are shown. ser133, serine 133.

glycolysis or Krebs cycle, we previously verified that PD98059 (20 μmol/l) treatment did not inhibit the calcium influx induced by glucose (16).

Exposure of MIN6 cells to glucagon-like peptide-1 (GLP-1) also promoted CREB phosphorylation (Fig. 1*F*), as reported (17). However, addition of PD98059 partially blocked glucose-induced CREB phosphorylation but had no effect on GLP-1 stimulation (Fig. 1*F*). These data are consistent with a previous report indicating that glucose and GLP-1 activate CREB via distinct pathways in β-cells (17). Together, these results demonstrate that CREB phosphorylation induced by glucose requires PKA activity and that ERK1 and -2, located downstream of PKA (4,16), cooperate with PKA to mediate part of the PKA-mediated CREB phosphorylation elicited by glucose.

Glucose induces CREB phosphorylation through ERK1/2 in islets of Langerhans. To confirm, in a more physiological model, the involvement of ERK1/2 in the glucose-induced CREB phosphorylation evidenced in MIN6 cells, we used isolated rat islets of Langerhans. Treatment of islets with glucose (16.7 mmol/l) stimulated phosphorylation of ERK1/2 and CREB in the same temporal pattern, with maximal effect observed at 10 min and decrease at 20–30 min (Fig. 2*A*). Notably, a PD98059 treatment, which eliminates the ERK1/2 phosphorylation elicited by glucose in pancreatic islets (16), inhibited the CREB phosphorylation (50–55%) (Fig. 2*B*), as observed in MIN6 cells.

Disrupting ERK1/2 activity in MIN6 cells by siRNA causes impaired function and decreased protein level of CREB. We performed an siRNA knockdown strategy to silence the expression of ERK1/2 proteins in MIN6 cells. We previously observed that transfection of MIN6 cells using modest amounts of ERK2 siRNA (50 nmol/l) resulted in 100% knockdown of both ERK1 and -2 and led to MIN6 cell mortality 48–60 h after transfection (16). To avoid drastic cell death, we used lower concentration of ERK2 siRNA duplexes (25 nmol/l) and investigated ERK1/2 activity and CREB function 24 h after transfection. ERK1 and -2 protein levels and phosphorylation in response to glucose were decreased by 60–80% in ERK2 siRNA–transfected MIN6 cells compared with nontransfected or control siRNA-transfected MIN6 cells (Fig. 3). The specificity of our siRNA approach was ascertained using other similarly sized ERK1/2-related siRNA duplexes (siRNA control), which failed to induce any change in the expression of any of proteins studied or using β-actin as internal and loading control (Fig. 3).

We found that glucose-stimulated CREB phosphorylation was highly reduced in ERK2 siRNA-transfected cells. Notably, we verified the protein level of CREB in ERK2 siRNA–transfected cells and found that it was decreased by ~60% (Fig. 3). The impaired protein level of CREB was even higher (>75%) 48 h after transfection (data not shown). However, these cells were not viable. It should be noted that in ERK1/2-downregulated MIN6 cells by siRNA, glucose-stimulated CREB phosphorylation was severely blocked, whereas the PD98059 treatment suppressed glucose-stimulated CREB phosphorylation by 50–55% (Fig. 1*D* and *E*). This greater inhibition of CREB phosphorylation observed in ERK1/2-downregulated MIN6 more likely results from the downregulation of the kinases (ERK1 and -2), which participate in two concomitant events: phosphorylation and control of protein level of CREB (Figs. 1*D* and 3).

ERK1 and -2 are essential to promote survival of MIN6 cells. Prolonged culture of β-cells in suboptimal glucose concentrations (≤5 mmol/l) induces apoptosis, whereas glucose (from 6 to 10 mmol/l) promotes their survival by activating synthesis of proteins that suppress the constitutive apoptotic program (20,21). We cultured MIN6 cells in optimal glucose concentration (10 mmol/l) and tested the hypothesis of whether disruption of ERK1/2 activity, using PD98059 treatment, causes downregulation of CREB and subsequent emergence of apoptosis. Apoptosis was detected by evaluating nuclear condensation (29) and levels of 17-kDa cleaved caspase-3, a key executioner and marker of apoptosis (31,32). We measured in parallel the disappearance of the 35-kDa inactive full-length form of caspase-3.

Exposure of MIN6 cells to serum-free DMEM containing 25 mmol/l glucose preserved cells from apoptosis until 48 h, as reported (26), since no change in caspase-3 content was observed compared with cells maintained in regular culture medium (control) (Fig. 4*A*). Culture in serum-free DMEM containing 10 mmol/l glucose also protected MIN6 cells from apoptosis, since caspase-3 content remained unaffected until 48 h (Fig. 4*A*) and for a prolonged time thereafter (>48 h) (data not shown). In contrast, exposure of MIN6 cells to low glucose concentration (2.8 mmol/l) for 48 h led to apoptosis, decreased caspase-3 content, and increased cleaved caspase-3 (Fig.

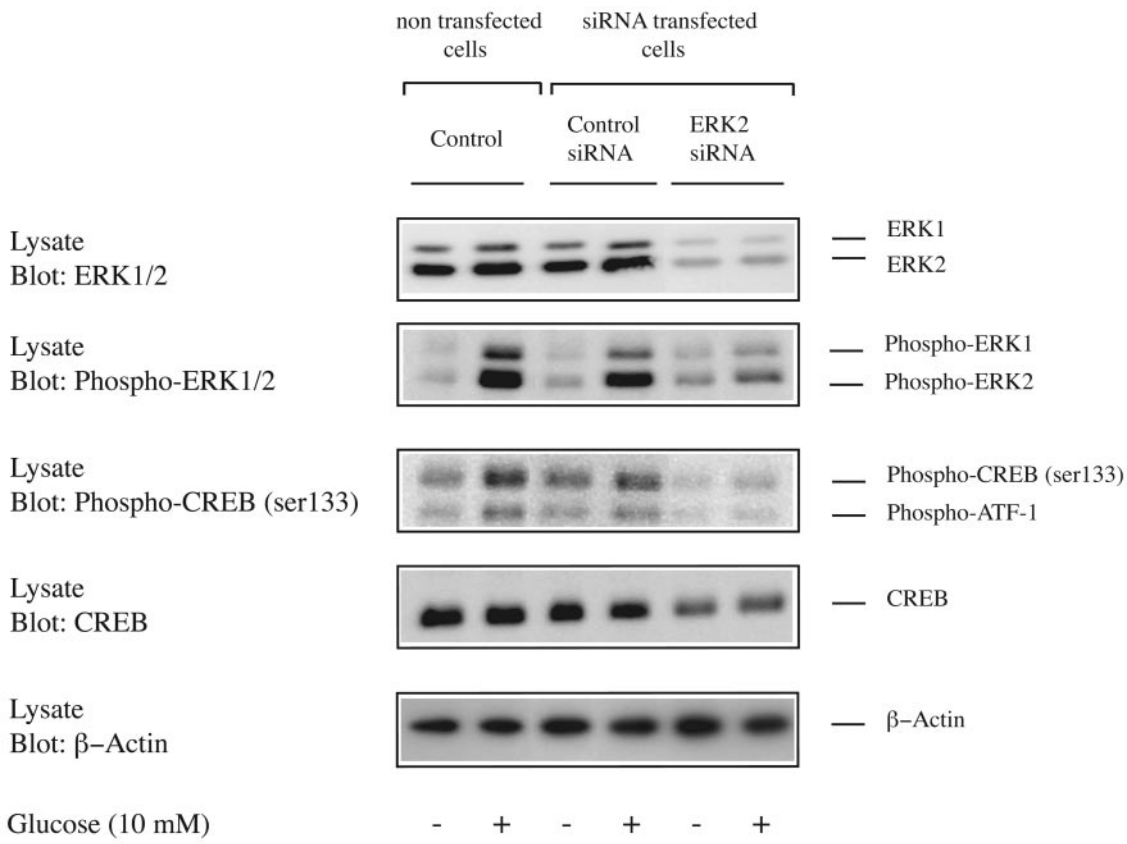


FIG. 3. CREB phosphorylation and protein content in ERK2 siRNA–transfected MIN6 cells. Phospho-ERK1/2, ERK1/2, phospho-CREB, CREB, and β-actin levels in nontransfected cells (control), siRNA control, or ERK2 siRNA–transfected cells after a 10-min stimulation with glucose. Exposures of five experiments are shown. ser133, serine 133.

4*A* and *B*). These observations are consistent with previous reports (20,21) demonstrating that the viability of cultured β-cells and isolated islets depends on the prevailing glucose concentration, with an optimal glucose concentration for rodent β-cell survival of ~10 mmol/l.

We performed CREB immunoblots of lysates from MIN6 cells exposed for 48 h to 10 mmol/l glucose and treated or not treated with a low concentration of PD98059. PD98059 treatment induced a 85% decrease in CREB protein level, while the expression of β-actin remained unaffected (Fig. 4*C* and *D*). To determine whether ERK1/2 control CREB expression in MIN6 cells at transcriptional and/or translational level(s), we developed a real-time quantitative PCR using a LightCycler system to evaluate changes in CREB mRNA (Table 1). Although CREB protein level was decreased by 85%, CREB mRNA levels were the same with or without PD98059 treatment (Fig. 4*E*).

The antiapoptotic gene *bcl-2* is transcriptionally upregulated by CREB and mediates survival effects of CREB in β-cells (33–35). Interestingly, in PD98059-treated CREB-downregulated MIN6 cells, significant decreases in both *bcl-2* protein and mRNA levels were observed (45 and 50%, respectively) (Fig. 5*A* and *B*). The observation that the loss of *bcl-2* expression is not complete could be explained by the fact that *bcl-2* is not exclusively transcriptionally upregulated by the ERK1/2-CREB signaling network and may be regulated through additional pathways. Since decreased *bcl-2* expression favors the release of caspase activators from mitochondria, leading to the cleavage of caspase-3, we evaluated the emergence of apoptosis in PD98059-treated CREB-downregulated MIN6 cells. Decreased *bcl-2* expression was associated with loss of caspase-3 content by 45% and appearance of cleaved caspase-3 (Fig. 5*A* and *C*).

We also examined the presence of apoptotic cells by nuclear staining and electron microscopy. In control MIN6 cells exposed for 48 h to 10 mmol/l glucose, no apoptotic cells were observed (Fig. 6*A* and *B*, *a*). In contrast, treatment with PD98059 for 48 h clearly induced apoptosis in MIN6 cells, since 5–10% of cells presented nuclear condensation (arrows in Fig. 6*A*, *b*) and typical ultrastructural features of apoptosis. According to studies on identification of apoptosis in β-cells by electron microscopy (20), we observed that the MIN6 cells that enter apoptosis undergo a series of morphological changes at distinct phases of apoptotic process, as follows: margination of chromatin toward the nuclear membrane characteristic of early apoptotic phase (Fig. 6*B*, *b*), condensation of chromatin and swelling of cytoplasm (Fig. 6*B*, *c*), or fragmentation of condensed nuclear chromatin into round spheres characteristic of late apoptotic phase (Fig. 6*B*, *d*).

ERK1/2 control CREB expression and survival in islets of Langerhans. We first evaluated ERK1/2, CREB, caspase-3, and cleaved caspase-3 protein levels in islets freshly isolated (control) or cultured for 12 h in an optimal concentration of 10 mmol/l glucose (20,21). ERK1/2 protein levels were dramatically decreased in islets 12 h after isolation compared with freshly isolated islets. In parallel, a decrease in CREB protein level was associated with a significant decrease in the inactive form of caspase-3 and emergence of cleaved caspase-3 (Fig. 7*A*). This confirms that isolation procedure exposes the islets to ischemic,

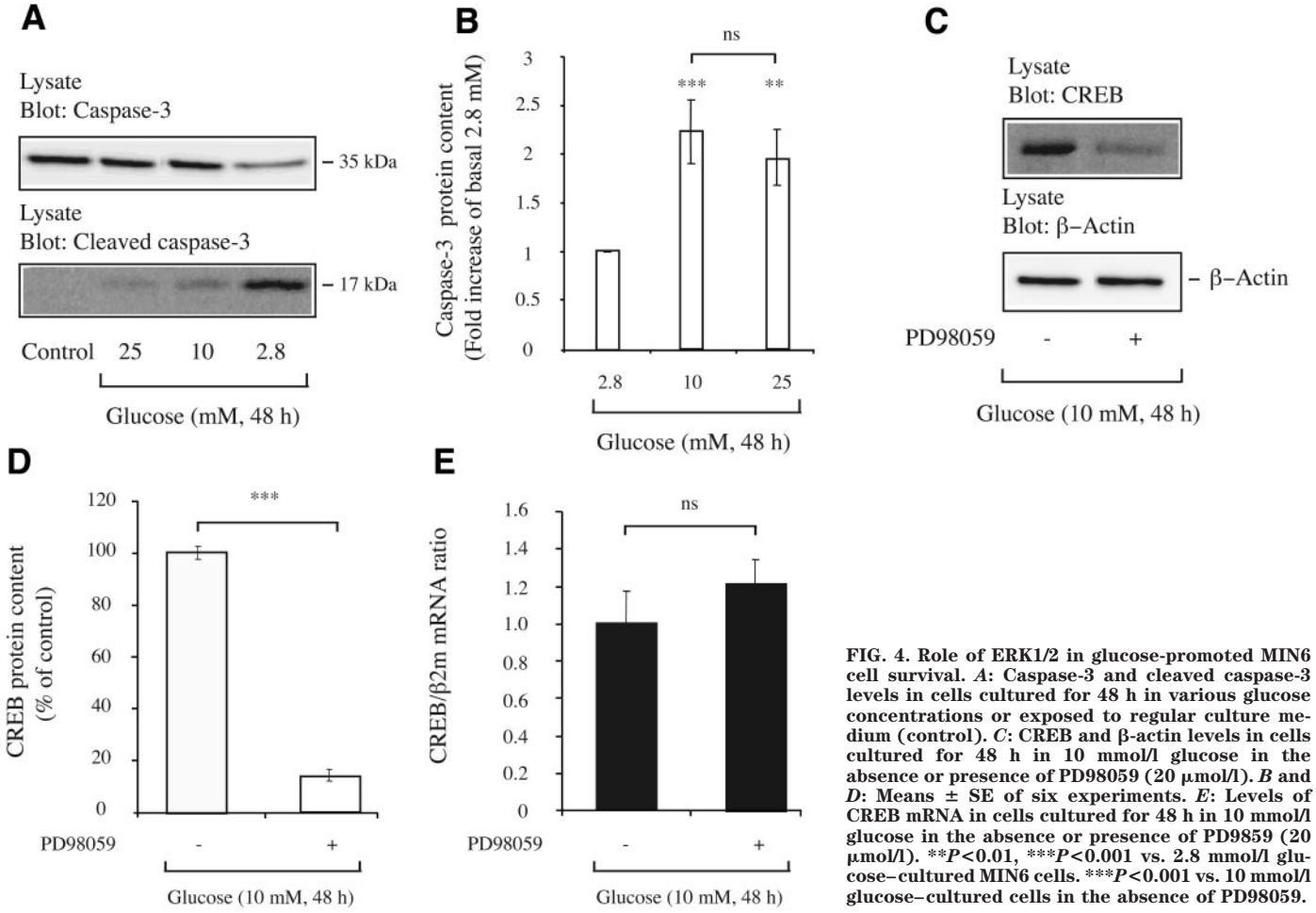


FIG. 4. Role of ERK1/2 in glucose-promoted MIN6 cell survival. *A*: Caspase-3 and cleaved caspase-3 levels in cells cultured for 48 h in various glucose concentrations or exposed to regular culture medium (control). *C*: CREB and β-actin levels in cells cultured for 48 h in 10 mmol/l glucose in the absence or presence of PD98059 (20 μmol/l). *B* and *D*: Means ± SE of six experiments. *E*: Levels of CREB mRNA in cells cultured for 48 h in 10 mmol/l glucose in the absence or presence of PD9859 (20 μmol/l). **$P<0.01$, ***$P<0.001$ vs. 2.8 mmol/l glucose–cultured MIN6 cells. ***$P<0.001$ vs. 10 mmol/l glucose–cultured cells in the absence of PD98059.

osmotic, and mechanical stresses leading to apoptosis (36,37). The decrease of ERK1/2 and CREB could be an important regulator of cell death in islets during the hours following the isolation procedure.

Batches of 100–200 islets were then exposed to 10 mmol/l glucose for an additional 24- or 48-h period. After a 24-h culture, expression of the proteins ERK1/2, CREB, and inactive caspase-3 was restored, although not completely, compared with control islets and a very low level of cleaved caspase-3 was detected (Fig. 7*A*). This confirms that the rate of apoptosis is decreased by prolonged culture of islets (>12 h after isolation) in 10 mmol/l glucose (20,21).

We next addressed whether ERK1 and -2 control the protein level of CREB in islets. First, CREB protein content was measured in islets after a 24-h culture in the presence of 10 mmol/l glucose, with or without PD98059. PD98059 treatment induced a significant 35% decrease in CREB protein level, and a less CREB-phosphorylated form was noticed (Fig. 7*B* and *C*). However, very low and similar levels of cleaved caspase-3 were detected in control untreated (Fig. 7*A*) and PD98059-treated (data not shown) islets. These results raise the possibility that a short period of PD98059 treatment (24 h) and a 35% loss of CREB protein content are not sufficient to engage the cells in an expressed and clearly detectable apoptotic program. Confirming this, very few morphological characteristics of apoptosis in β-cells from islets were observed in both experimental situations.

Since it is well known that emergence of cleaved caspase-3 and subsequent morphological ultrastrutural characteristics of apoptosis are observed several hours after dysfunction of survival signaling pathways, the contents of the proteins CREB and cleaved caspase-3 were measured in islets after a 48-h culture in the presence of 10 mmol/l glucose, with or without PD98059. In PD98059-treated islets, CREB protein level was decreased by 50%. This was associated with emergence of cleaved caspase-3 and a significant decrease in the inactive form of caspase-3 (Fig. 7*D*). As shown in Fig. 7*E*, compared with control untreated β-cells visually selected by the presence of typical insulin-containing granules according to ultrastructural description (38) (Fig. 7*E*, *a*), exposure to PD98059 induced apoptosis in insulin-containing β-cells; 5–10% presented typical ultrastructural features of apoptosis. Numerous laminated membranous structures with the aspect of myelin figures (myelinoid bodies, also called "fingerprints"), typical of early events of cell death (20), were observed in cells treated with PD98059 (Fig. 7*E*, *b* and *c*). It should be noted that we also observed poorly granulated β-cells showing morphological changes characteristic of end-stage apoptosis evidenced by cytoplasmic vacuolation and chromatin condensation ((Fig. 7*E*, *d*).

DISCUSSION

Here, we have shown that ERK1 and -2 control the phosphorylation and protein level of CREB and play a key role in glucose-mediated pancreatic β-cell survival.

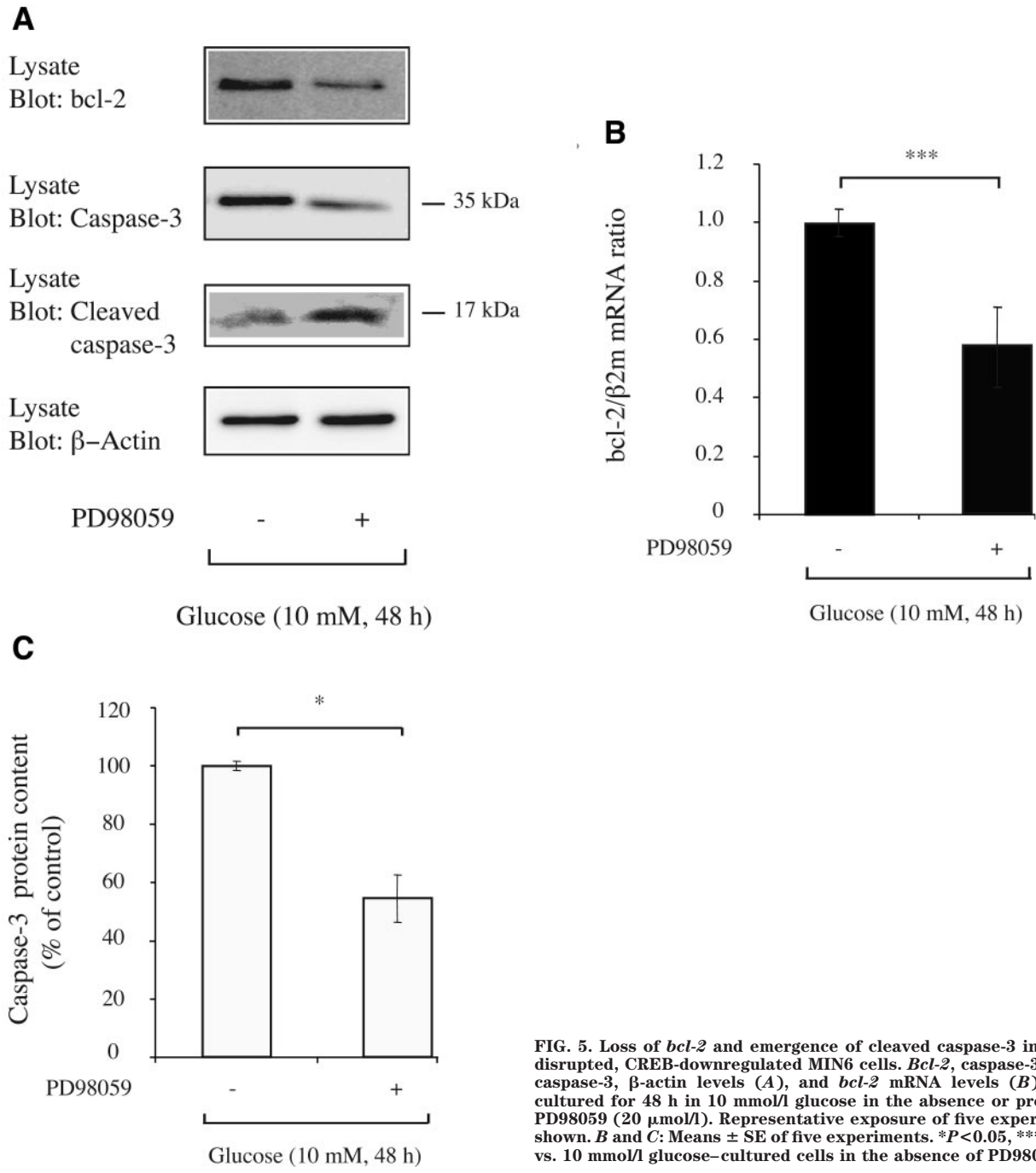


FIG. 5. Loss of *bcl-2* and emergence of cleaved caspase-3 in ERK1/2-disrupted, CREB-downregulated MIN6 cells. *Bcl-2*, caspase-3, cleaved caspase-3, β-actin levels (*A*), and *bcl-2* mRNA levels (*B*) in cells cultured for 48 h in 10 mmol/l glucose in the absence or presence of PD98059 (20 μmol/l). Representative exposure of five experiments is shown. *B* and *C*: Means ± SE of five experiments. **P* < 0.05, ****P* < 0.001 vs. 10 mmol/l glucose–cultured cells in the absence of PD98059.

Phosphorylation of CREB at serine 133 is required to initiate transcription; this covalent modification was initially attributed to PKA (18). However, studies have established that several kinases can phosphorylate CREB at the same serine 133 site (18). In β-cells, membrane depolarization induced by glucose leads to the opening of voltage-dependent calcium channels in the plasma membrane, resulting in influx of extracellular calcium. Inside the cells, calcium activates many kinases, such as PKA (4–9). Activated PKA can translocate to the nucleus, where it directly phosphorylates CREB at serine 133 (18). Activated PKA can also mediates ERK1/2 phosphorylation (4,16). ERK1/2 cannot directly phosphorylate CREB. However, ERK1/2 can activate (by phosphorylation) members of the pp90rsk family of protein kinases (RSK1–3), which in turn translocate to the nucleus, where they directly phosphorylate CREB at serine 133 (18). Here, we report that CREB phosphorylation induced by glucose requires PKA activity. ERK1 and -2, located downstream of PKA (4,16), cooperate with PKA and are responsible for half of the PKA-mediated CREB phosphorylation elicited by glucose. To achieve stoichiometric CREB phosphorylation, it is possible that more than one CREB kinase is required.

The duration of serine 133 phosphorylation determines the efficacy with which CREB induces gene expression (18,39). In both MIN6 cells and islets, we observed that glucose induces transient phosphorylation of CREB such that serine 133 is already dephosphorylated within 30 min. This phosphorylation duration has been reported to be efficient for gene transcription (18,39). In β-cells, CREB

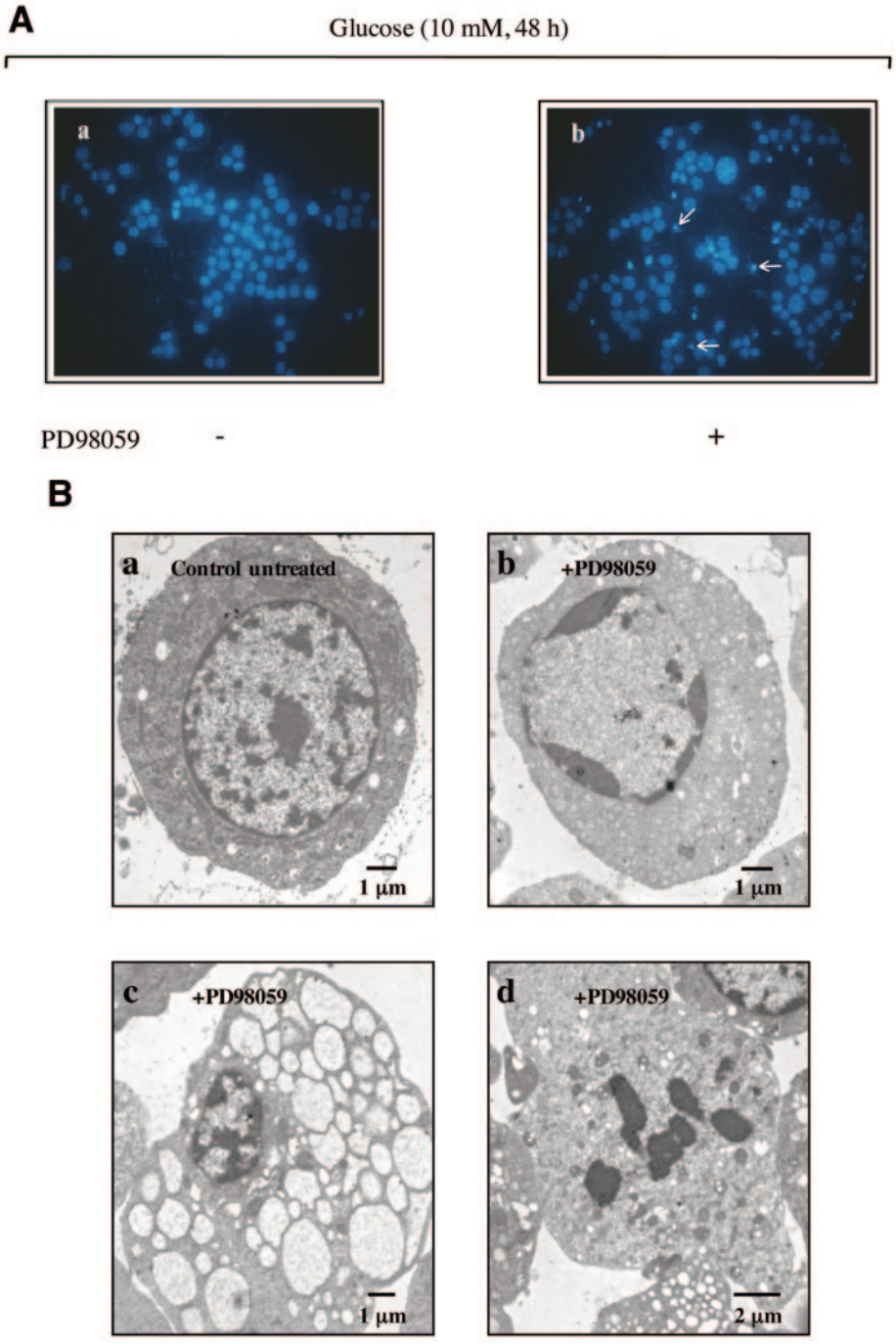
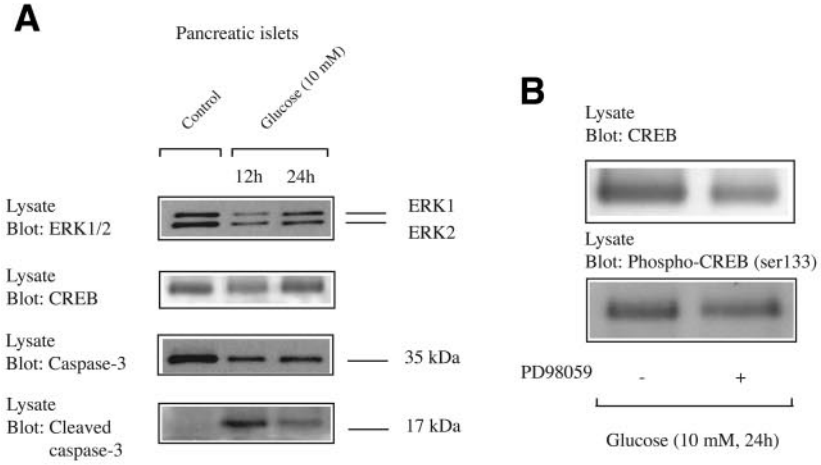


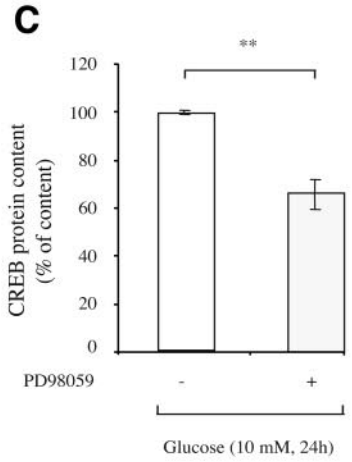
FIG. 6. Ultrastructural features of apoptosis in ERK1/2-disrupted, CREB-downregulated MIN6 cells. Nuclear staining (*A*) and electron microscopy (*B*) of cells cultured for 48 h in 10 mmol/l glucose in the absence or presence of PD98059 (20 μmol/l).

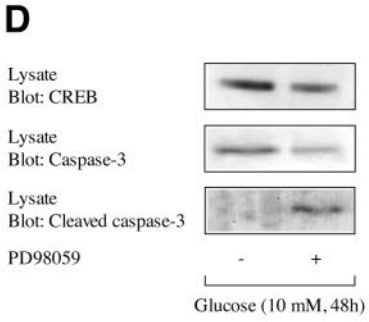
controls the expression of the genes *bcl-2* and *IRS-2* (17,19,33). It remains to be established whether this transient CREB phosphorylation closely parallels the appearance of *bcl-2* or *IRS-2* transcription in β-cells induced by glucose stimulation.

Importantly, by disruption of ERK1/2 expression using an ERK2 siRNA strategy in MIN6 cells, we confirmed that ERK1 and -2 regulate phosphorylation of CREB at serine 133 and found that these kinases are also required for CREB protein level. Blockade of ERK1/2 activity in both MIN6 cells and isolated islets cultured in the presence of optimal glucose concentration for β-cell survival leads to impaired CREB protein level, loss of *bcl-2* content, and emergence of molecular and morphological apoptotic features. From these results, we can conclude that ERK1 and -2, by (at least) controlling function and expression of CREB, play a key role in glucose-mediated β-cell survival. However, we cannot exclude the possibility that ERK1 and -2 also control other molecular targets involved in protection against apoptosis.

It must be noted that although CREB protein level was decreased by 85% in PD98059-treated MIN6 cells for 48 h, CREB mRNA levels were the same with or without PD98059 treatment. This indicates that ERK1/2 activity regulates CREB protein level, most probably at the post-transcriptional level. ERK1/2 activity might regulate phosphorylation of translation factors such as eIF-4E (translation initiation factor 4E) and/or 4E-BP1 (eIF-4E–binding protein 1, also known as PHAS-1) (40–43), which are implicated in CREB mRNA translation. We cannot







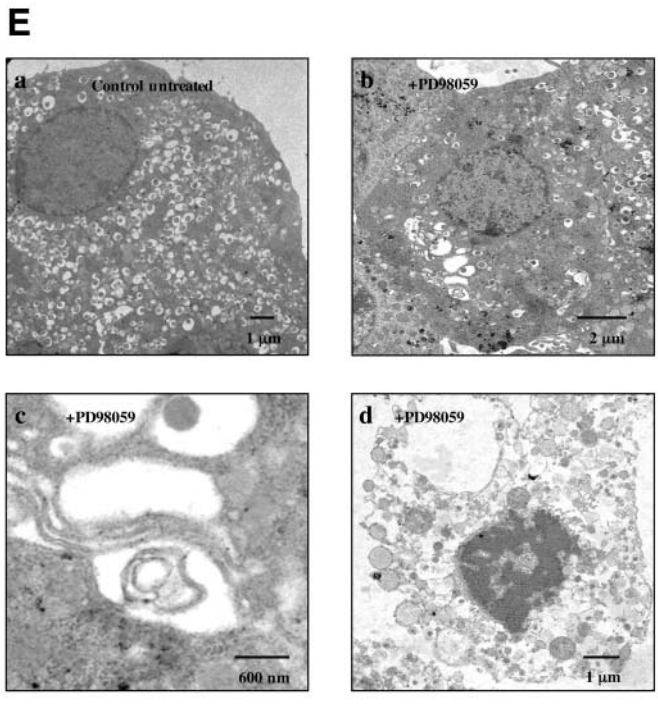


FIG. 7. Role of ERK1/2 in glucose-promoted islet survival. *A*: ERK1/2, CREB, caspase-3, or cleaved caspase-3 levels in freshly isolated islets (control) or in islets cultured in 10 mmol/l glucose for 12 h following isolation or cultured for an additional 24-h period. *B* and *D*: CREB, phospho-CREB, caspase-3, or cleaved caspase-3 levels in 10 mmol/l glucose–cultured islets for 24 (*B*) or 48 h (*D*) in the absence or presence of PD98059 (20 μmol/l). Exposures (*A*, *B*, and *D*) and means ± SE of four experiments (*C*). *E*: Electron microscopy of islets cultured in 10 mmol/l glucose for 48 h in the absence or presence of PD98059. **$P<0.01$ vs. 10 mmol/l glucose–cultured islets in the absence of PD98059.

exclude another possibility that ERK1/2 activity controls CREB protein degradation within the β-cells.

Although glucose promotes inhibition of apoptotic program in β-cells in the 6- to 10-mmol/l concentration range, it is now demonstrated that chronic high glucose (11–30 mmol/l) exerts deleterious effects on β-cell function and induces apoptosis (22,23). It will be of interest to investigate the chronic effects of high glucose on the functional integrity of ERK1/2 and CREB. As an example, ERK1/2 activation by interleukin-1β has been reported to be a

mediator of the long-term deleterious effect of high glucose on β-cell function (44).

Enhancing survival or preventing apoptosis of β-cells holds promise for improving islet transplantation outcomes as a treatment for type 1 diabetes and for protecting against deleterious effects of hyperglycemia in type 2 diabetes (45,46). Our work identifies some new molecular events that promote β-cell survival. Here, we ascribe a new role to ERK1/2 in the control of phosphorylation and protein level of CREB and report the importance of ERK1/2 in glucose-mediated β-cell survival. These results indicate that the ERK1/2-CREB network is a novel target for strategies aimed at improving the survival of β-cells.

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