Degradation of cAMP-Responsive Element–Binding Protein by the Ubiquitin-Proteasome Pathway Contributes to Glucotoxicity in β-Cells and Human Pancreatic Islets

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OBJECTIVE—In type 2 diabetes, chronic hyperglycemia is detrimental to β-cells, causing apoptosis and impaired insulin secretion. The transcription factor cAMP-responsive element–binding protein (CREB) is crucial for β-cell survival and function. We investigated whether prolonged exposure of β-cells to high glucose affects the functional integrity of CREB.

RESEARCH DESIGN AND METHODS—INS-1E cells and rat and human islets were used. Gene expression was analyzed by RT-PCR and Western blotting. Apoptosis was detected by cleaved caspase-3 emergence, DNA fragmentation, and electron microscopy.

RESULTS—Chronic exposure of INS-1E cells and rat and human islets to high glucose resulted in decreased CREB protein expression, phosphorylation, and transcriptional activity associated with apoptosis and impaired β-cell function. High-glucose treatment increased CREB polyubiquitination, while treatment of INS-1E cells with the proteasome inhibitor MG-132 prevented the decrease in CREB content. The emergence of apoptosis in INS-1E cells with decreased CREB protein expression knocked down by small interfering RNA suggested that loss of CREB protein content induced by high glucose contributes to β-cell apoptosis. Loading INS-1E cells or human islets with a cell-permeable peptide mimicking the proteasomal targeting sequence of CREB blocked CREB degradation and protected INS-1E cells and human islets from apoptosis induced by high glucose. The insulin secretion in response to glucose and the insulin content were preserved in human islets exposed to high glucose and loaded with the peptide.

CONCLUSIONS—These studies demonstrate that the CREB degradation by the ubiquitin-proteasome pathway contributes to β-cell dysfunction and death upon glucotoxicity and provide new insight into the cellular mechanisms of glucotoxicity. *Diabetes* **58:1105–1115, 2009**

The deterioration of β-cell function and survival caused by chronic exposure to supraphysiological concentrations of glucose is termed glucotoxicity (1–6). Evidence (1–8) supports the notion that increased β-cell death by apoptosis is associated with the onset of type 2 diabetes. Given the pivotal role of β-cell mass in determining whether an individual will progress to type 2 diabetes, there is growing interest in studies aimed at understanding the mechanisms that control life and death of β-cells (1–6).

The cAMP-responsive element–binding protein (CREB) is a transcription factor that binds to the cAMP response element within the promoter region of many genes. This transcription factor is essential for glucose homeostasis and β-cell survival (9–11). Mice that are deficient in CREB activity, caused by expression of a dominant-negative A-CREB transgene in β-cells, develop diabetes secondary to β-cell apoptosis (9). Glucose and glucagon-like peptide (GLP)-1 activate CREB by phosphorylation at residue serine 133 via calcium and cAMP pathways to regulate the expression of β-cell genes, such as the antiapoptotic B-cell lymphoma 2 (*bcl-2*) and insulin receptor substrate-2 (*IRS-2*) genes (9,10). CREB overexpression in MIN6 cells leads to protection against cytokine-induced apoptosis (10), while dominant-negative mutant forms of CREB induce apoptosis and decrease the antiapoptotic action of growth factors in human islets (11). Pathways that regulate CREB phosphorylation have been intensively studied, while those controlling CREB expression in normal or pathophysiological situations within β-cells have received little attention. We reported that the extracellular signal–regulated kinases (ERKs)1/2 signaling cascade controls the phosphorylation and protein level of CREB and that this signaling network plays a key role in β-cell survival (12).

CREB activity is essential for β-cell survival (9–11). However, we hypothesize that intracellular concentrations of CREB may be critical for β-cell survival and function and can be regulated at the level of protein stability/degradation. Within its primary structure, CREB presents a specific sequence known to be a potential proteasomal targeting sequence. The presence of a functional proteasome pathway in β-cells (13–17), which has been reported to be activated by high glucose and to degrade proteins such as MafA and IRS-2 (13,14), prompted us to examine whether chronic exposure of β-cells to supraphysiological

A

IB: Cleaved caspase-3

IB: β-actin

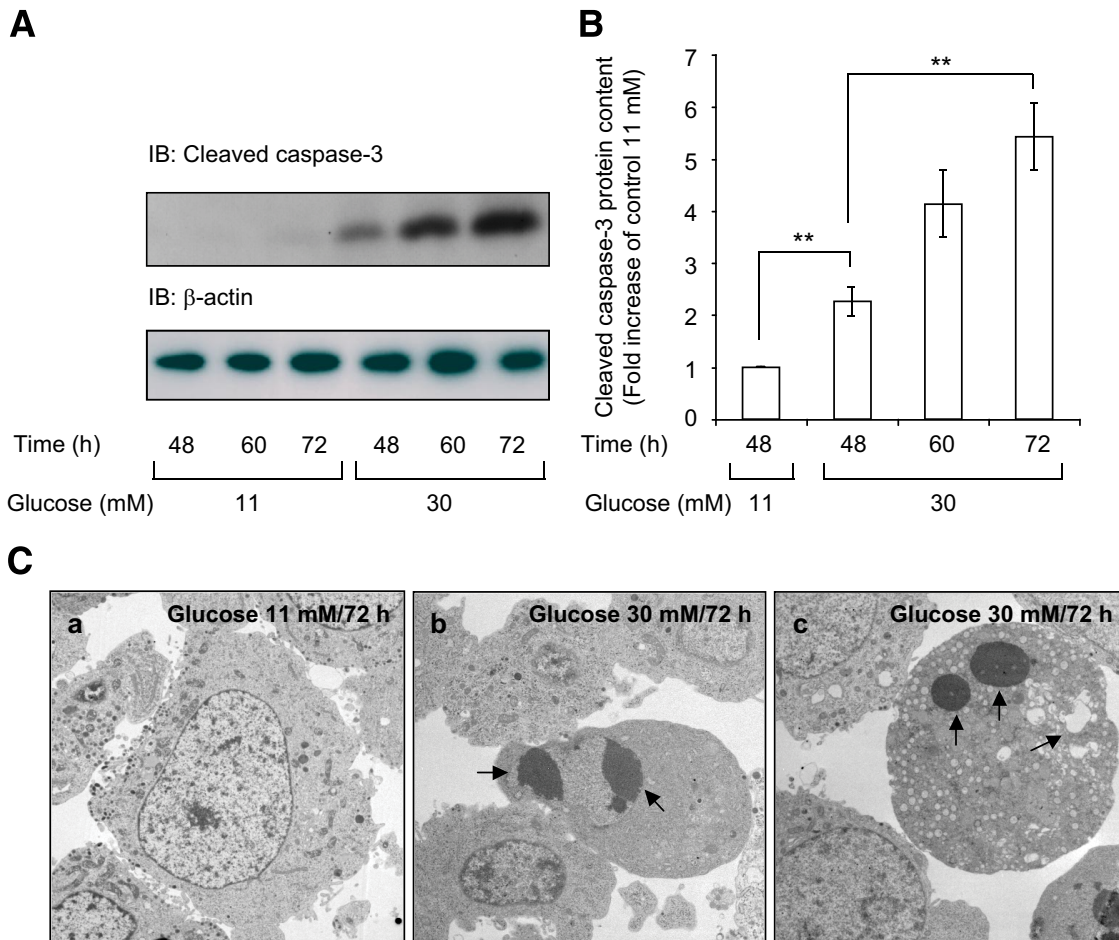| Time (h)	48	60	72	48	60	72
Glucose (mM)		11			30	

B

C

FIG. 1. Apoptosis in INS-1E cells exposed to high-glucose treatment. High glucose concentration led to a time-dependent increase in cleaved caspase-3. Levels of β-actin are shown as loading control. *A:* Typical autoradiographs representative of four experiments are shown. *B:* Graph representing cleaved caspase-3 levels deduced from quantitative results of four representative experiments obtained from analyses using a Java-based image processing program (Image J), developed by Wayne Rasband (National Institutes of Health). *C:* INS-1E cells cultured for 72 h in 30 mmol/l glucose presented a series of morphological changes at distinct phases of apoptotic process (indicated by arrows in b and c). These ultrastructures of apoptosis were not detected in INS-1E cells cultured for 72 h in 11 mmol/l glucose (a). Quantification of apoptotic cells was performed on printed micrographs obtained from three independent experiments. The percentage of apoptotic cells versus normal cells was evaluated based on the described morphological apoptotic characteristics. At least 150 cells were counted for each condition. The result was expressed as percent of total cells. (A high-quality digital representation of this figure is available in the online issue.)

concentrations of glucose adversely affects levels of CREB mRNA and protein expression.

RESEARCH DESIGN AND METHODS

Materials and reagents. Anti-CREB, anti–cleaved caspase-3, anti-bcl2, and anti-ubiquitin antibodies were from Cell Signaling Technology (New England Biolabs, Beverly, MA). Anti–phosphoserine133-CREB antibody was obtained from Upstate (Charlottesville, VA). Anti–activating transcription factor (ATF)-1 and anti-glucokinase antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–β-actin antibody was obtained from Sigma (St. Louis, MO). Culture media and FCS were purchased from Life Technologies (Life Technologies, Grand Island, NY). MG-132 was purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma (St. Louis, MO).

Cell culture, electron microscopy, and isolation of rat pancreatic islets. The rat β-cell line INS-1E was provided by Dr. P. Maechler (Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, Geneva, Switzerland) (18). Electron microscopy was performed as previously described (12). Islets were isolated from fed male Wistar rats (Charles River, L'Arbresle, France) (aged 11–14 weeks) weighing 280–320 g (12). Batches of 100–200 islets were cultured in RPMI-1640 medium containing 11 or 30 mmol/l glucose, 5% BSA, 100 UI/ml penicillin, and 100 mg/ml streptomycin.

Diabetic Goto-Kakizaki rats. Ten- to 12-week-old male nondiabetic Wistar and diabetic Goto-Kakizaki (GK) rats were maintained at a constant temperature (21°C–23°C) with a 12:12-h light:dark cycle. Food and water were available ad libitum. The characteristics of the nonobese GK rat model of type 2 diabetes maintained in the colony at the University of Paris 7 have been

described previously (19). Islets were isolated at the University of Paris 7 as previously described (20). Groups of 300–500 Wistar or GK islets were stored at −80°C until studied. All rats used in this study were treated in accordance with European Community guidelines, and the local institution approved the experimentation.

Human islets processing, DNA fragmentation, and evaluation of insulin content and secretion. Human pancreata were harvested in three brain-dead nonobese nondiabetic donors (ages 25, 54, and 67 years) in agreement with French regulations and the local institutional ethical committee. Human islets were isolated at the Institut National de la Santé et de la Recherche Médicale (INSERM) U859 (Lille, France) according to a slightly modified version (21) of the automated method (22). All experiments on human islets were performed at INSERM U859 (Lille, France). Islets were cultured in CMRL-1066 supplemented with 0.625% BSA, 100 UI/ml penicillin, and 100 mg/ml streptomycin and containing 5.5 or 30 mmol/l glucose. Apoptosis was evaluated by determination of histone-complexed DNA fragments (mono- and oligonucleosomes) with the Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany).

Insulin content was measured by immunoradiometric assay (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) after insulin extraction of islets (3×40 islet equivalents [IEs] per condition) with 2 mol/l acetic acid containing 0.2% BSA. For insulin release experiments, islets (5×40 IE) were preincubated for 30 min in RPMI medium containing 10% newborn calf serum and 2.8 mmol/l glucose and subjected to two successive 1-h incubations with 2.8 (basal) and 20 (stimulation) mmol/l glucose. The stimulation index was defined as the ratio of stimulated to basal insulin secretion.
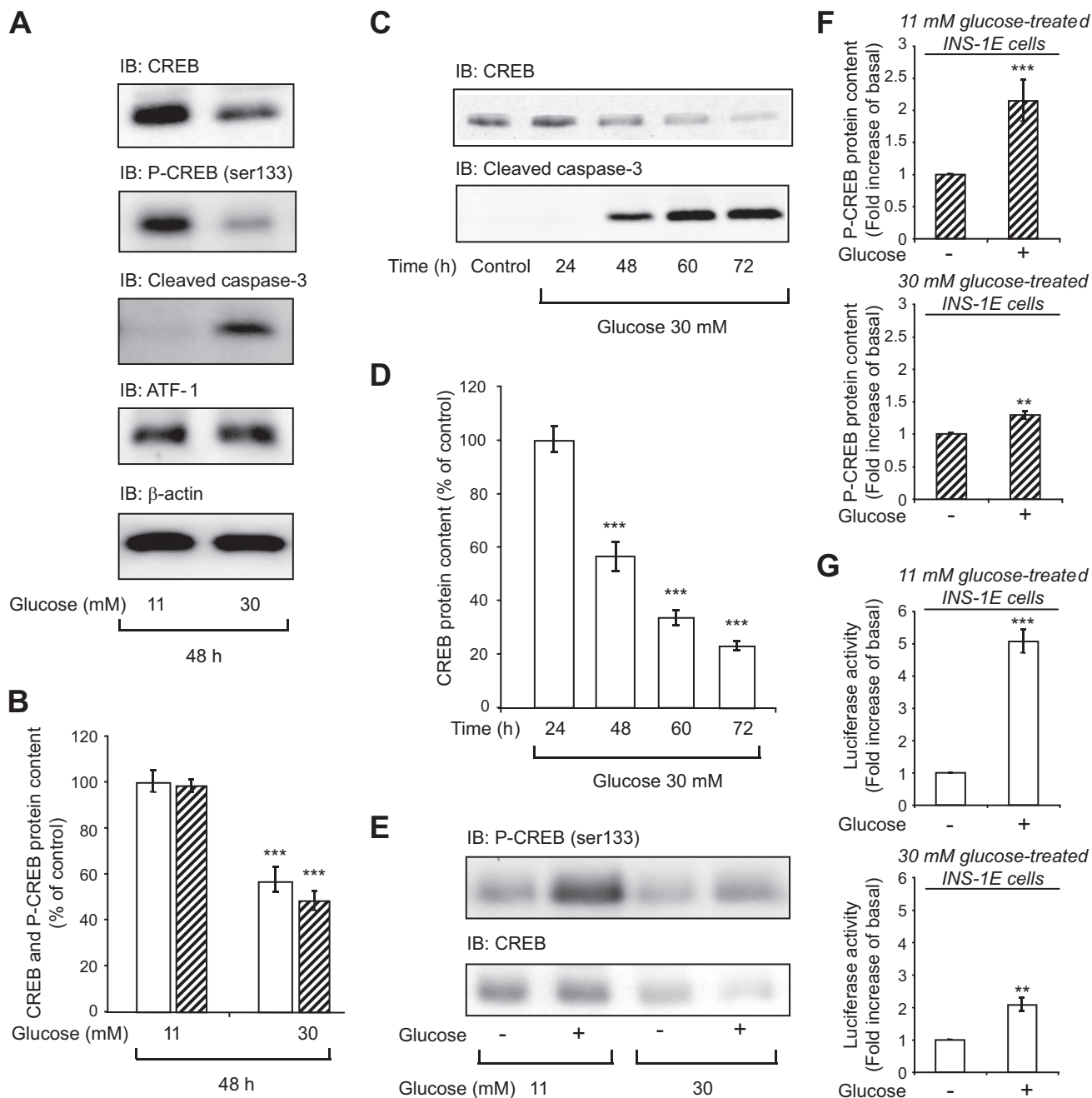
FIG. 2. CREB expression, phosphorylation, and transcriptional activity in INS-1E cells exposed to high glucose. *A*: Levels of CREB, P-CREB (ser133), cleaved caspase-3, ATF-1, and β-actin detected by Western blotting in INS-1E cells cultured for 48 h in 11 or 30 mmol/l glucose. *C*: Levels of CREB in INS-1E cells cultured in 30 mmol/l glucose for various times as indicated or in INS-1E cultured in regular cultured medium (control). As control, levels of cleaved caspase-3 in INS-1E cells cultured in 30 mmol/l glucose for various times or in INS-1E cultured in regular cultured medium (control) is also shown. Typical autoradiographs representative of 5 to 10 experiments are shown. Graphs representing CREB protein expression (*B* and *D*) and CREB phosphorylation levels (*B*) deduced from quantitative results of 5–10 representative experiments obtained from analyses using Image J. ☐, CREB; ▨, P-CREB (serine 133). *E*: Levels of phospho-CREB and CREB detected by Western blotting in INS-1E cells cultured for 48 h in 11 or 30 mmol/l glucose and then stimulated with 10 mmol/l glucose for 15 min. *F*: The graph represents levels of phosphorylation of CREB deduced from quantitative results of 6–10 representative experiments, obtained using Image J. *G*: Measurement of luciferase activity in INS-1E cells transiently cotransfected for 6 h with pFR-Luc and pFA2-CREB, cultured for 48 h in 11 or 30 mmol/l glucose and then stimulated with 10 mmol/l glucose for 4 h.

Measurement of CREB activity, quantitative real-time PCR, and CREB small interfering RNA in INS-1E cells. CREB activity was measured using transient transfection with a combination of pFR-Luc reporter plasmid containing Gal4 response elements (2.7 μg) and the fusion transactivator plasmid pFA2-CREB (0.3 μg), in which the transactivation domain of CREB is linked to DNA binding domain of Gal4 (Stratagene, La Jolla, CA), as previously described (10). Quantitative real-time PCR (qRT-PCR) was performed using LightCycler technology (Roche, Meylan, France) as previously described (12). For the CREB small interfering RNA (siRNA) experiment, 20- to 25-nucleotide stealth-prevalidated siRNA duplexes designed for rat CREB

were used (Invitrogen, Carlsbad, CA). INS-1E cells were seeded in 12-well plates in culture medium without antibiotics and grown overnight to reach 30% confluency. The next day, lipofectAMINE2000-siRNA complexes were prepared according to the manufacturer's instructions. Three different CREB siRNA duplexes were tested at final concentrations of 25 (0.12 μg), 50 (0.24 μg), or 100 nmol/l (0.48 μg) in each well. Cells were transfected with CREB siRNA or control siRNA (which corresponds to a nontargeting 20- to 25-nucleotide siRNA designed as negative control) for 6 h before switching to fresh culture medium including antibiotics. Seventy-two hours after transfection, cells were lysed.

Western blotting and immunoprecipitation. Following incubation, cells, rat, or human islets were lysed for 30 min at 4°C in a lysis buffer as described (12,23). All samples were normalized for protein content by a Bradford assay, and equal amounts of proteins (25–50 μg of protein/lane) were analyzed (12,24,25). For immunoprecipitation, supernatants were incubated with anti-CREB antibody. Immunocomplexes were precipitated from the supernatant with protein A/G plus agarose, washed three times with ice-cold lysis buffer, boiled in Laemmli's sample buffer, and resolved by SDS-PAGE (25). Proteins were visualized by chemiluminescence detection (Pierce, Rockford, IL).

HIV-tat peptides treatment. Peptides were synthesized by Eurogentec (Seraing, Belgium) and added on INS-1E cells for 48 h or on human islets for 72 and 120 h (final concentration 10 μmol/l). Equal volume of the solvent-carrier DMSO was incubated with cells or islets as a control. To monitor peptide delivery, INS-1E cells were grown on glass coverslips for 3 days and incubated for 20 h without (a) or with (b) biotinylated HIV-tat peptide. Cells were fixed in methanol at 4°C and permeabilized in 0.25% Triton X-100. Cells were then incubated for 2 h with Texas red–conjugated avidin (1:100 dilution) in PBS containing 0.25% Triton X-100. Coverslips were mounted using a polyvinyl alcohol medium and observed with a Leitz DMRB microscope equipped for epifluorescence (Leica, Solms, Germany).

Statistical analysis. Results are expressed as the means ± SE for n independent experiments. Differences between results were analyzed by using Student's t test or ANOVA followed by the Fisher's least-significant difference test using Statview 4.1 software (Abacus, Berkeley, CA). A P value of <0.05 was considered significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

RESULTS

Chronic exposure of INS-1E cells to high glucose concentration induces apoptosis. Levels of cleaved caspase-3, a key executioner and marker of apoptosis (26), gradually increased between 48 and 72 h in cells exposed to 30 mmol/l glucose. Exposure of INS-1E cells to 11 mmol/l glucose for 48, 60, and 72 h preserved cells from apoptosis since no emergence of cleaved caspase-3 was observed (Fig. 1*A* and *B*).

Morphological characteristics of apoptosis were observed by electron microscopy several hours following cleavage of caspase-3. INS-1E cells exposed to 30 mmol/l glucose for 72 h presented morphological changes of apoptotic process, such as margination of chromatin toward the nuclear membrane (Fig. 1*C*, b, *arrows*) or swelling of cytoplasm and fragmentation of condensed nuclear chromatin into round spheres characteristic of a later apoptotic phase (Fig. 1*C*, c, *arrows*). Enough microphotographs were acquired to count ~30% of INS-1E cells with apoptotic characteristics. No apoptotic features were observed in INS-1E cells cultured in 11 mmol/l glucose for 72 h (Fig. 1*C*, a).

Chronic exposure of INS-1E cells to high glucose concentration decreases CREB expression and function. Treatment of INS-1E cells with 30 mmol/l glucose for 48 h led to a 42 ± 5% decrease in CREB protein content, while the CREB-like transcription factor ATF-1 remained unaffected (Fig. 2*A* and *B*). Full activation of CREB requires phosphorylation at serine 133 (27). The serine 133 phosphorylation of CREB decreased by 51 ± 5% in cells exposed to 30 mmol/l glucose for 48 h (Fig. 2*A* and *B*). CREB levels markedly declined with high-glucose treatment reaching minimal levels by 72 h (Fig. 2*C* and *D*).

We measured CREB activity in response to acute glucose stimulation in high-glucose–treated INS-1E cells. We assessed the serine 133 phosphorylation of CREB in cell lysates and carried out transient transfection assays with two plasmids that measure specifically the promoter activity mediated by the transactivation domain of CREB (10). In cells exposed to high glucose for 48 h, glucose-stimulated CREB phosphorylation at serine 133 was almost completely abolished (Fig. 2*E* and *F*) and glucose-
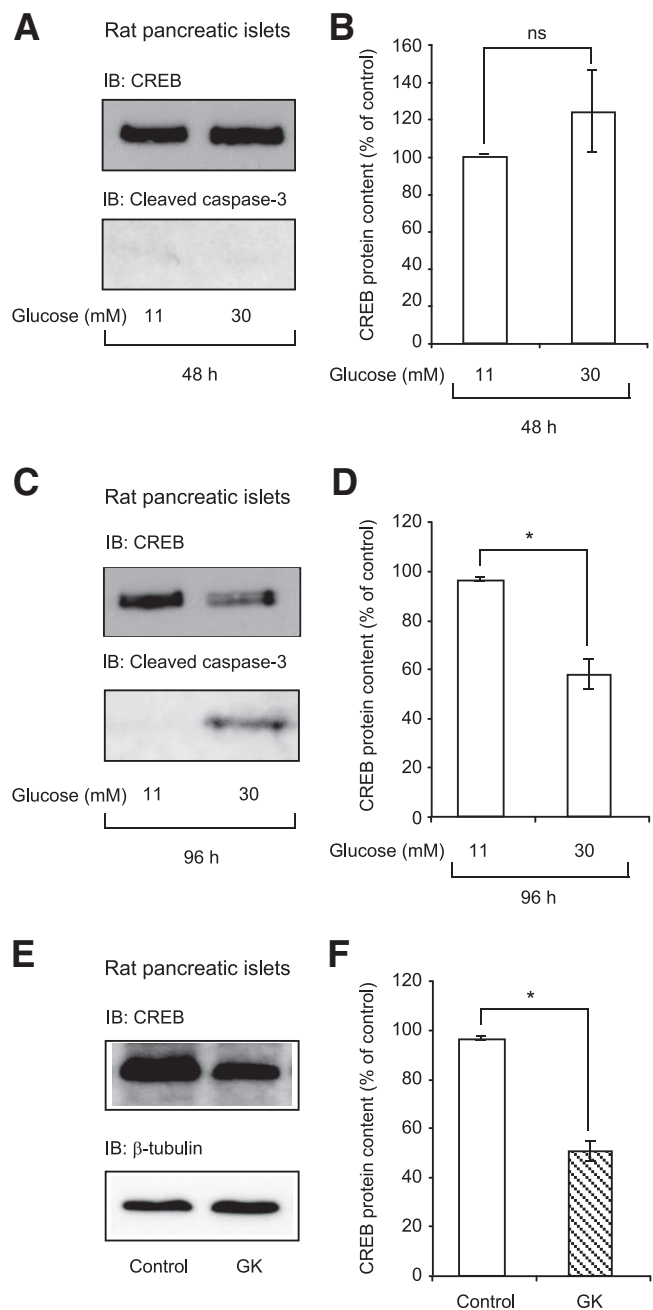
FIG. 3. CREB protein expression in isolated rat islets exposed to high-glucose concentration and in isolated GK rat islets. *A*: Levels of CREB and cleaved caspase-3 detected by Western blotting in rat islets cultured for 48 h in 11 or 30 mmol/l glucose. *B*: Results are quantified using Image J (ns, not significant). *C*: Levels of CREB and cleaved caspase-3 detected by Western blotting in rat islets cultured for 96 h in 11 or 30 mmol/l glucose. *D*: Results are quantified using Image J. The most representative blots obtained from three independent experiments are shown. *E*: Levels of CREB detected by Western blotting in control and GK rat islets. Levels of β-tubulin are shown as loading control. The most representative blots obtained from three independent experiments are shown. *F*: Results are quantified using Image J.

induced CREB reporter activity was drastically decreased (Fig. 2*G*).

High-glucose exposure affects CREB protein expression in rat islets. Following isolation, islets were cultured in 11 mmol/l glucose for 24 h and exposed to 11 or 30 mmol/l glucose for 48 or 96 h. While a 48-h exposure of islets to high glucose had no effect on CREB protein
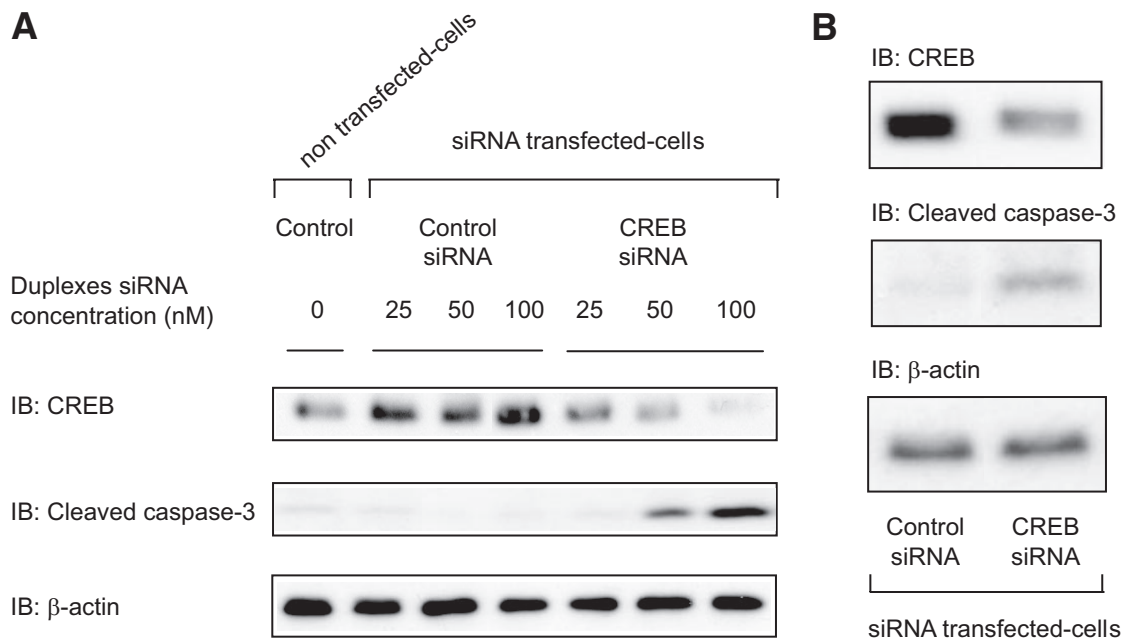
FIG. 4. Loss of CREB protein expression by siRNA induces apoptosis in INS-1E cells. *A*: Dose response of control siRNA or CREB siRNA on CREB and cleaved caspase-3 protein levels. *B*: Levels of CREB and cleaved caspase-3 in control siRNA or in CREB siRNA (50 nmol/l)-transfected cells. *A* and *B*: Specificity of the siRNA approach was ascertained using similarly sized CREB-related siRNA duplexes (siRNA control) that failed to induce any change in the expression of β-actin used as internal and loading control. Typical autoradiographs representative of three independent experiments are shown.

expression (Fig. 3*A* and *B*), a 96-h exposure of islets to high glucose induced a 43 ± 6% reduction in CREB protein expression associated with the emergence of cleaved caspase-3 (Fig. 3*C* and *D*).

We verified whether hyperglycemia leads to CREB protein degradation in vivo. We used the GK rat model, one of the best-characterized animal models of spontaneous type 2 diabetes (19). Notably, we found a significant (~47%) reduction in CREB protein expression in islets isolated from GK rats, demonstrating that hyperglycemia leads to CREB degradation in vivo (Fig. 3*E* and *F*).

Loss of CREB protein expression leads to β-cell apoptosis. To evaluate whether CREB downregulation might play a role in the progression of β-cell apoptosis induced by the high-glucose exposure, the expression of CREB was silenced by siRNA to the same extent as observed in INS-1E cells exposed to high glucose for 48 h (i.e., ~50% decrease in CREB protein content). Transfection of INS-1E cells using 25, 50, and 100 nmol/l of CREB siRNA resulted in ~20, ~50, and ~85% knockdown of CREB protein content, respectively, and gradual emergence of cleaved caspase-3 (two- and fourfold increase for 50 and 100 nmol/l CREB siRNA, respectively) (Fig. 4*A*). An ~50% loss of cellular CREB protein content, similar to the level of CREB observed following 48 h of high-glucose exposure, causes the emergence of cleaved caspase-3 (Fig. 4*B*).

High-glucose–induced CREB degradation is mediated by the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway plays an important role in the degradation of regulatory proteins, including transcription factors (28). Examination of the rat primary structure of CREB revealed a specific sequence known to be a potential proteasomal targeting sequence (29,30). This motif belongs to a homologous consensus sequence family (DSUXXS, where D is aspartic acid, S is serine, U is a hydrophobic amino acid, and X is any amino acid) (29). The CREB sequence (DSVTDS) is closely located to lysine

residue(s) and is found between residues D^{116} and S^{121} within a 60-residue region of CREB called kinase-inducible domain (KID) (Fig. 5*A*) (27). The DSVTDS sequence does not exist within other proteins including CREB-related ATF transcription factors and is conserved in the human primary structure of CREB (ref. 30 and S.C., S.D., unpublished observations). While CREB protein content was clearly decreased (Fig. 2*A* and B), we found that CREB mRNA levels were not altered in INS-1E exposed for 48 h to high glucose (Fig. 5*B*), indicating that the decreased CREB protein expression occurs most probably at a posttranscriptional level.

Proteins are targeted for proteasome-mediated degradation by covalent attachment of multiple moieties of ubiquitin to lysine residue (28,29). We next sought to establish whether CREB becomes polyubiquitinated upon 48 h of high-glucose treatment. CREB was immunoprecipitated from cell lysates and then subjected to Western blotting for the detection of ubiquitin. The blot shows an increase in polyubiquitinated CREB at 48 h of high-glucose exposure (Fig. 5*C*).

We evaluated the levels of CREB content in INS-1E cells exposed to high glucose for 48 h and treated for the last 12 h with or without the proteasome inhibitor MG-132. The lowest concentration of MG-132 (150 nmol/l) and a 12-h treatment were used to avoid cytotoxicity (31). Treatment of cells with MG-132 totally prevented CREB protein degradation induced by the high-glucose exposure and partially preserved CREB phosphorylation at serine 133 (Fig. 5*D*).

Depending on the cell type, inhibition of the proteasome using MG-132 leads to cell survival or apoptosis (32,33). We verified whether inhibition of the proteasome activity with MG-132 prevented glucotoxicity. Although treatment of cells with MG-132 prevented CREB protein degradation induced by the high glucose (Fig. 5*D*), this treatment did not prevent apoptosis of cells exposed to 30 mmol/l glucose but, rather, increased cleaved caspase-3 levels. This treatment also increased cleaved caspase-3 levels in
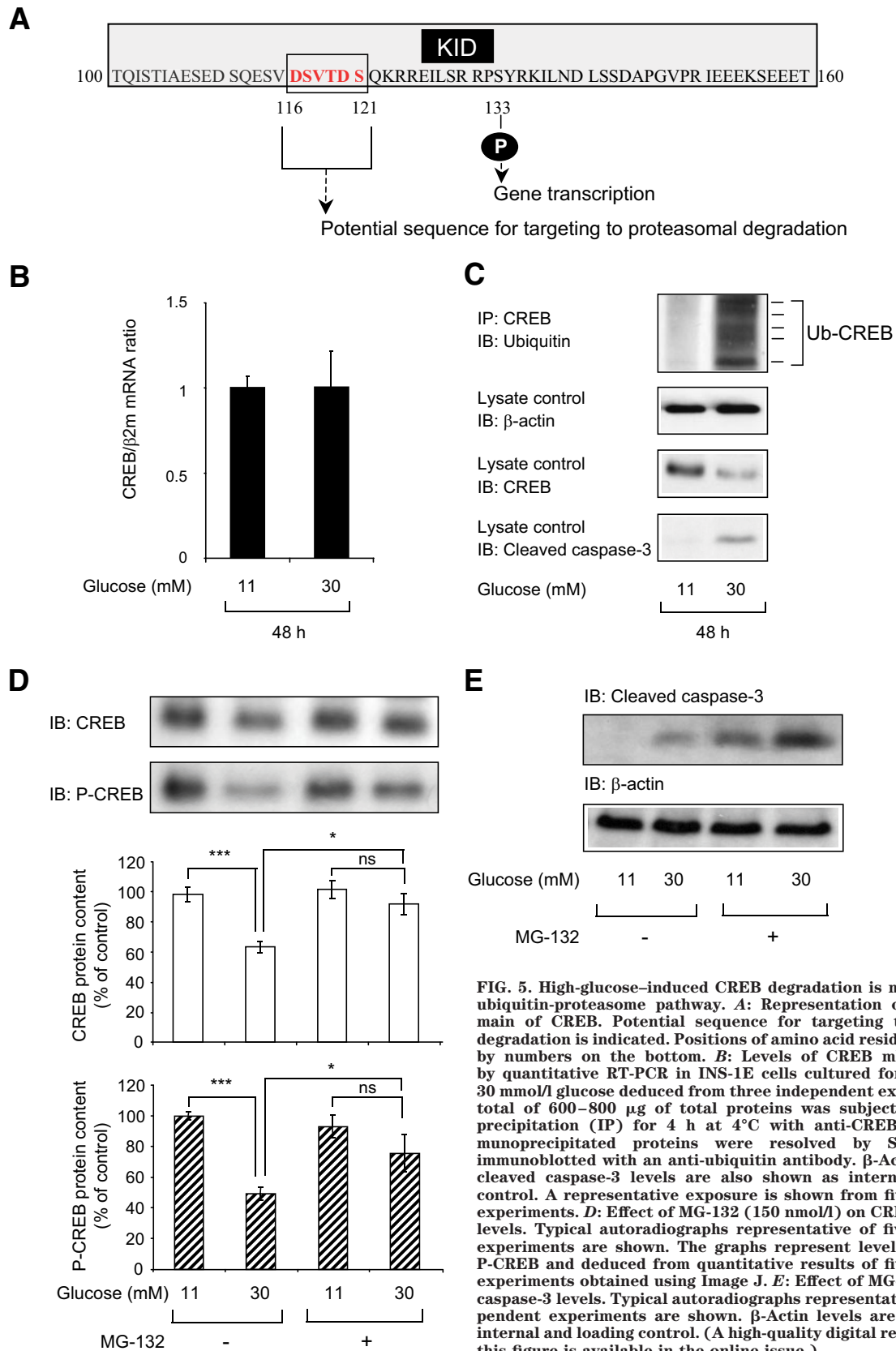
A

B

Glucose (mM) 11   30

48 h

C

IP: CREB
IB: Ubiquitin

Ub-CREB

Lysate control
IB: β-actin

Lysate control
IB: CREB

Lysate control
IB: Cleaved caspase-3

Glucose (mM)   11   30

48 h

D

IB: CREB

IB: P-CREB

E

IB: Cleaved caspase-3

IB: β-actin

Glucose (mM)   11   30   11   30

MG-132   –   +

FIG. 5. High-glucose–induced CREB degradation is mediated by the ubiquitin-proteasome pathway. *A*: Representation of the KID domain of CREB. Potential sequence for targeting to proteasomal degradation is indicated. Positions of amino acid residues are marked by numbers on the bottom. *B*: Levels of CREB mRNA evaluated by quantitative RT-PCR in INS-1E cells cultured for 48 h in 11 or 30 mmol/l glucose deduced from three independent experiments. *C*: A total of 600–800 µg of total proteins was subjected to immunoprecipitation (IP) for 4 h at 4°C with anti-CREB antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with an anti-ubiquitin antibody. β-Actin, CREB, and cleaved caspase-3 levels are also shown as internal and loading control. A representative exposure is shown from five independent experiments. *D*: Effect of MG-132 (150 nmol/l) on CREB and P-CREB levels. Typical autoradiographs representative of five independent experiments are shown. The graphs represent levels of CREB and P-CREB and deduced from quantitative results of five independent experiments obtained using Image J. *E*: Effect of MG-132 on cleaved caspase-3 levels. Typical autoradiographs representative of five independent experiments are shown. β-Actin levels are also shown as internal and loading control. (A high-quality digital representation of this figure is available in the online issue.)

cells exposed to 11 mmol/l glucose (Fig. 5*E*). Thus, it is likely that inhibition of the proteasome in β-cells blocks not only the degradation of key survival factors, such as CREB, but also the degradation of proapoptotic factors and damaged, misfolded, or misassembled proteins, which favors the accumulation of numerous deleterious signals for β-cell survival reinforcing the emergence of apoptosis. These results further suggest that pharmacological blockade of the proteasome does not protect β-cells against the apoptosis induced by high glucose. Hence, using a protein

TABLE 1
Sequences of synthetic peptides

HIV-tat peptide	Sequences
Scr: scrambled peptide control	H2N-<u>YGRKKRRQRRRG</u>*STKVDSQRDV*-COOH
Tat-CREB: CREB targeting sequence peptide	H2N-<u>YGRKKRRQRRRG</u>**VDSVTDSQKR**-COOH
	P P
2P tat-CREB: 2P CREB targeting sequence peptide	H2N-<u>YGRKKRRQRRRG</u>**VDSVTDSQKR**-COOH

HIV-tat peptide sequence is underlined, scrambled peptide is in italics, and CREB targeting sequence is in bold.

transduction technology, we tested whether blocking CREB polyubiquitination and its proteasome degradation may protect β-cells against the deleterious effects of hyperglycemia. **Peptide mimicking the CREB proteasomal targeting sequence inhibits high-glucose–mediated CREB degradation and protects INS-1E cells from apoptosis.** The potential role of the DSVTDS sequence in targeting CREB to the proteasome for degradation was investigated. INS-1E cells were exposed to high glucose and loaded with synthesized cell-permeable peptide mimicking the CREB proteasomal targeting sequence. Protein transduction domains (PTDs), such as the small PTD from the tat protein

of HIV-1, allow the efficient delivery of proteins and peptides into cells through the plasma membrane (34). A scrambled peptide and a 10–amino acid peptide derived from the CREB proteasomal targeting sequence were covalently linked at their NH$_2$-terminus to a 12–amino acid carrier peptide derived from the HIV-tat sequence (Table 1). To monitor peptide delivery, INS-1E cells were incubated with HIV-tat peptides conjugated with biotin. Texas red–conjugated avidin incubation reveals that the peptides entered the cells and were localized mostly in the cytoplasm as well as in the nucleus and in the nucleolus (Fig. 6*A*).

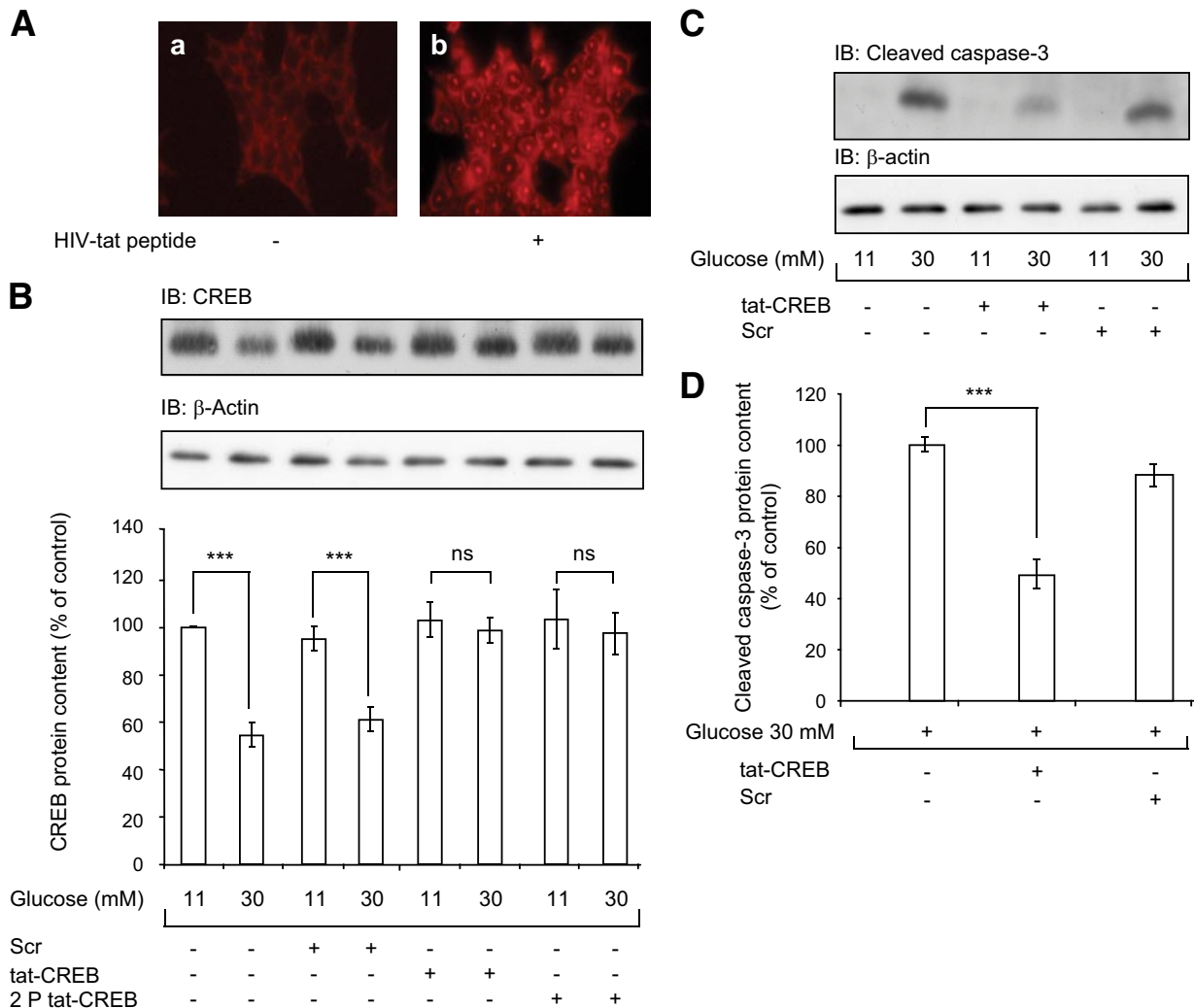Although the scrambled peptide (Scr) had no effect on

FIG. 6. CREB proteasomal targeting sequence peptide inhibits high-glucose–mediated CREB degradation and apoptosis in INS-1E cells. *A*: Localization of HIV-tat peptides in cells. CREB protein levels in INS-1E cells loaded or not with HIV-tat peptides for 48 h (ns, not significant). *B*: As a control, levels of β-actin were unchanged by the peptide loading. *C*: Cleaved caspase-3 levels in INS-1E cells loaded or not with HIV-tat peptides for 48 h. Typical autoradiographs representative of five to six independent experiments are shown. The graphs represent levels of CREB (*B*) or cleaved caspase-3 (*D*) and deduced from quantitative results of five to six representative experiments obtained using Image J. (A high-quality digital representation of this figure is available in the online issue.)
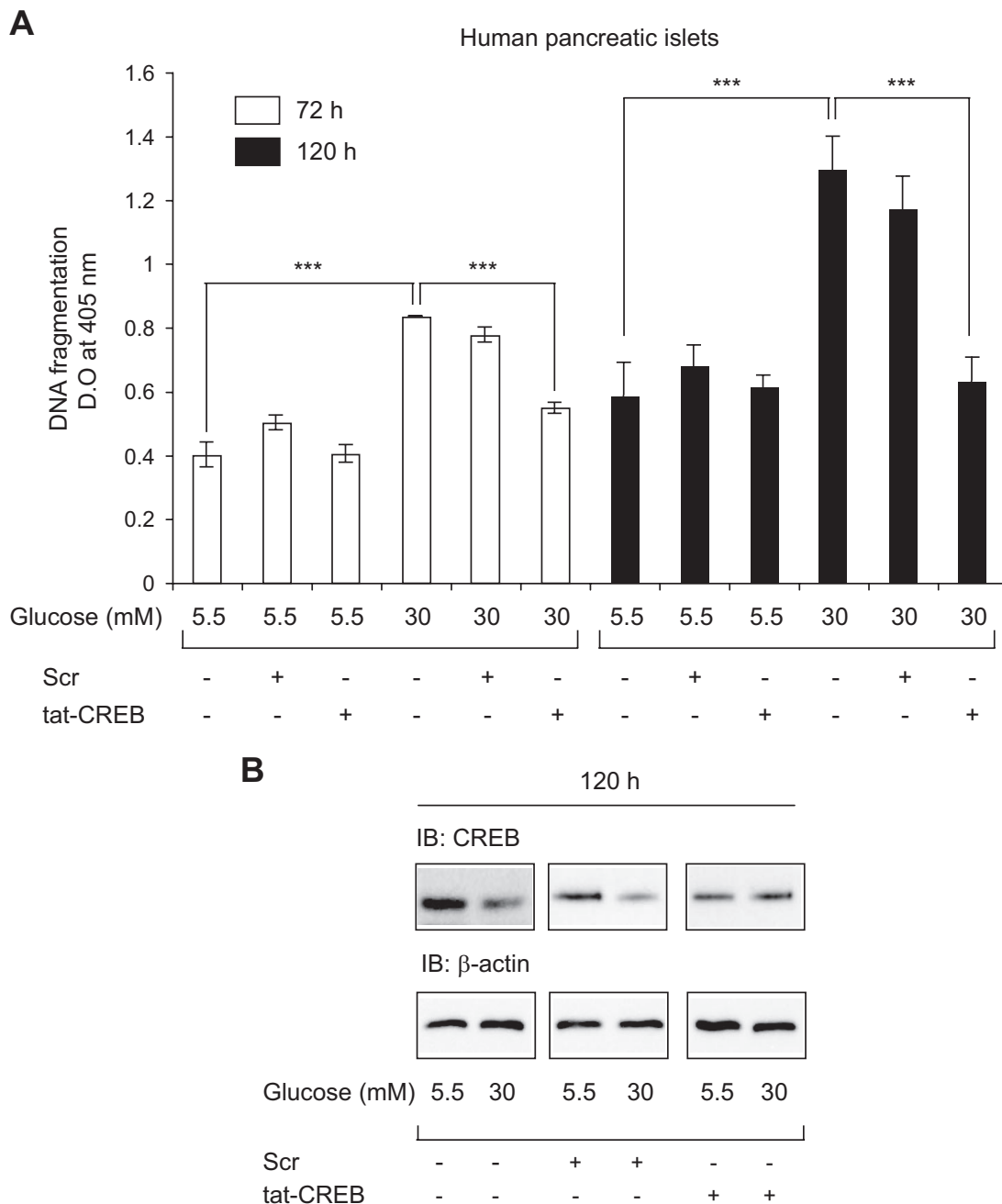
A

Human pancreatic islets

B

FIG. 7. CREB proteasomal targeting sequence peptide protects human islets from glucotoxicity-induced apoptosis, insulin content decrease, and insulin secretion failure. Human islets were incubated for 72 or 120 h in culture medium containing 5.5 or 30 mmol/l glucose added or not with HIV-tat peptide (tat-CREB) or scrambled peptide control (Scr). Assays were performed on islets from three different pancreases. *A*: DNA fragmentation assay. *B*: Determination of CREB and β-actin levels analyzed by Western blotting in human islets exposed to 5.5 or 30 mmol/l glucose and loaded or not with tat-CREB or Scr peptides for 120 h. The most representative blots obtained from three independent experiments are shown. *C*: Insulin content. *D*: Stimulation index defined as the ratio of stimulated (20 mmol/l glucose) to basal (2.8 mmol/l glucose) insulin secretion. *E*: Levels of bcl-2, glucokinase, and β-actin detected by Western blotting in INS-1E cells cultured for 48 h in 11 or 30 mmol/l glucose and loaded or not with HIV-tat peptides. Typical autoradiographs representative of three independent experiments are shown. The graphs represent levels of bcl-2 or glucokinase deduced from quantitative results of three representative experiments obtained using Image J.

CREB downregulation, the CREB proteasomal targeting sequence HIV-tat peptide (tat-CREB) totally blocked CREB degradation induced by high glucose, indicating that the DSVTDS sequence plays a key role in targeting CREB to the proteasome (Fig. 6*B*). Because we noticed the presence of phosphorylable serine residues within the proteasomal targeting sequence of CREB, we synthesized a double serine phosphorylated CREB proteasomal targeting sequence HIV-tat peptide (2P tat-CREB) (Table 1). Interestingly, loading INS-1E with the 2P tat-CREB peptide inhibited the CREB

downregulation (Fig. 6*B*), suggesting that high-glucose–induced phosphorylation of the DSVTDS sequence also plays a role for the targeting of CREB to proteasomal degradation.

To further assess the hypothesis that the lack of CREB observed following high-glucose exposure contributes to the emergence of β-cell apoptosis, we measured the levels of cleaved caspase-3 in INS-1E cells loaded with the CREB proteasomal targeting sequence peptide and displaying a complete preservation of CREB protein expression (Fig. 6*B*). Notably, emergence of cleaved caspase-3 induced by high
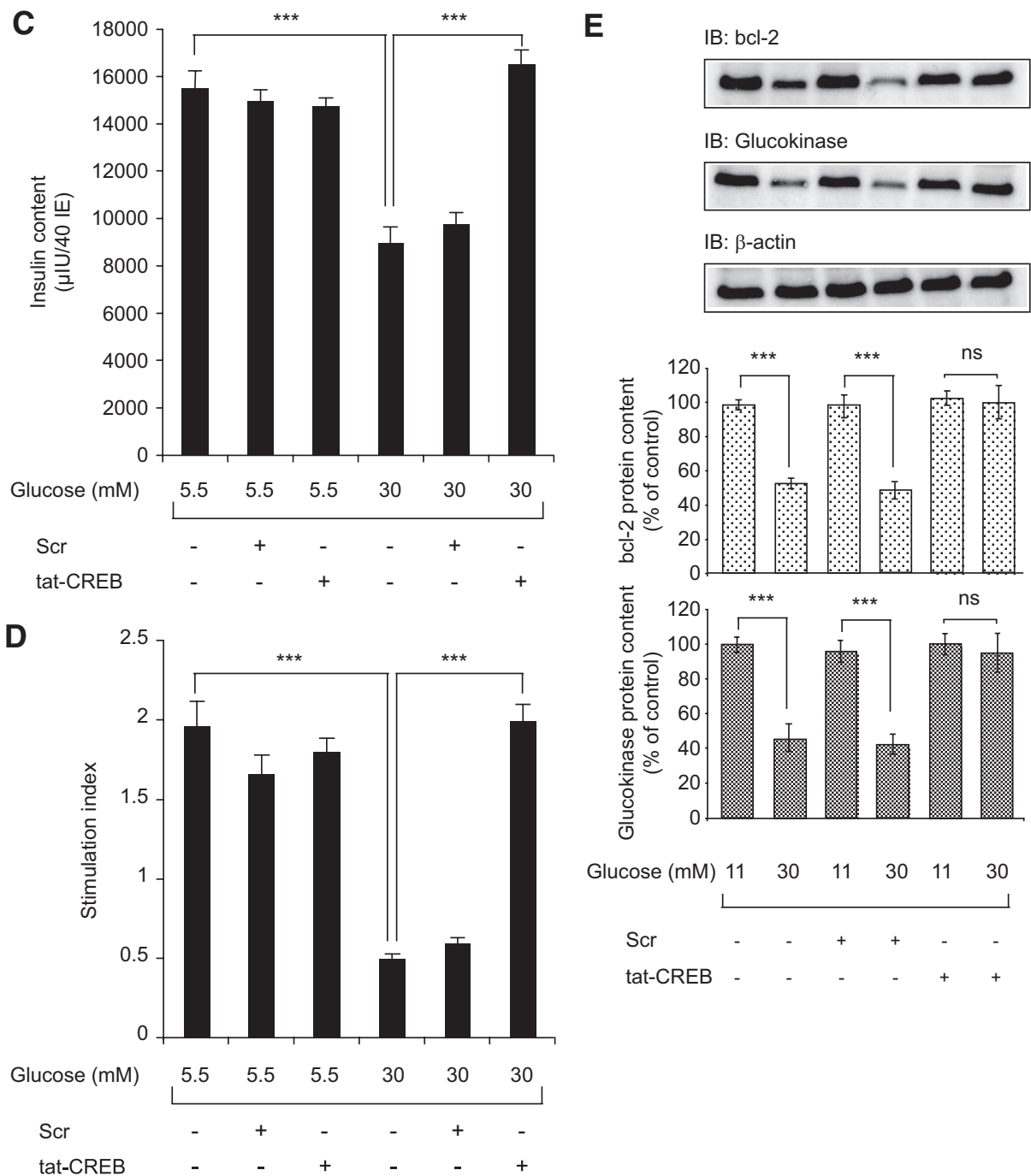
C

D

E

FIG. 7. Continued.

glucose was significantly reduced, by $51 \pm 5\%$, in cells loaded with the CREB proteasomal targeting sequence peptide (Fig. 6*C* and *D*).

CREB proteasomal targeting sequence peptide protects human islets from glucotoxicity-induced apoptosis, insulin content decrease, and insulin secretion failure. Studies reported that HIV-tat peptides transduced practically 100% of the islet cell population and that the delivery was even highly effective in cells located in the inner core region of the human islets (35,36). We investigated whether loading cultured human islets with the CREB proteasomal targeting sequence peptide protects them from apoptosis induced by chronic high-glucose exposure over time. Human islets were exposed to culture

medium containing 5.5 or 30 mmol/l glucose for 72 or 120 h, and DNA fragmentation, which reflects apoptosis (37), was determined. DNA fragmentation gradually increased between 72 and 120 h in islets exposed to 30 mmol/l glucose compared with islets exposed to 5.5 mmol/l glucose (Fig. 7*A*). Loading human islets with the CREB proteasomal targeting sequence peptide blocked by ~ 62 and $\sim 100\%$ the DNA fragmentation induced by a 72 or 120 h exposure to high-glucose concentration, respectively, indicating that the peptide is very efficient in protecting human islets from the deleterious apoptotic effect of glucotoxicity (Fig. 7*A*).

We determined CREB protein expression in human islets chronically exposed to high glucose and loaded with

the CREB proteasomal targeting sequence peptide. A 120-h exposure of human islets to high glucose induced a drastic reduction in CREB protein content (Fig. 7*B*). The Scr peptide had no effect on CREB downregulation, whereas the tat-CREB peptide totally blocked CREB degradation in human islets exposed to high glucose (Fig. 7*B*).

Chronic high-glucose exposure of β-cells deteriorates not only β-cell survival but also insulin secretion and insulin transcription (1–8). Indeed, we observed that insulin content and insulin secretion in response to glucose (stimulation index in the graph) were significantly decreased by 42 and 74%, respectively, in human islets exposed for 120 h to a high-glucose concentration (30 mmol/l) (Fig. 7*C* and *D*). Notably, we observed that loading human islets with the CREB proteasomal targeting sequence peptide totally prevented glucotoxicity-induced insulin content decrease and insulin secretion failure (Fig. 7*C* and *D*), indicating that the human islets loaded with the peptide are viable and functional and protected against the deleterious effects of glucotoxicity.

These observations suggest that the preservation of CREB expression in β-cells is essential for survival but also favors the maintenance of an efficient glucose sensing. Notably, we found that loading INS-1E cells with the CREB proteasomal targeting sequence peptide totally prevented high-glucose–induced downregulation of the antiapoptotic protein bcl-2 and of the glucose sensor glucokinase (38) (Fig. 7*E*).

DISCUSSION

Here, we report that high-glucose treatment of β-cells increased CREB polyubiquitination and that inhibition of the proteasome activity or loading β-cells with a peptide mimicking the CREB proteasomal targeting sequence blocked high-glucose–mediated downregulation of CREB. A large family of ubiquitin protein ligases recognize specific motifs in protein substrates, allowing polyubiquitination to proceed. Modifications of these motifs, such as serine phosphorylation induced by protein kinases, may render them susceptible to recognition by the ubiquitin ligases (28,29). Hence, it is likely that some high-glucose–induced modifications of the CREB proteasomal targeting sequence and/or high-glucose–activated serine kinase(s) allows a specific ubiquitin ligase or a specific enzymatic complex to act on CREB for polyubiquitination with subsequent proteasomal degradation. Regarding the data obtained with the nonphosphorylated and the double-phosphorylated CREB proteasomal targeting sequence, it can be hypothesized that upon high-glucose exposure, the DSVTDS sequence of CREB is specifically recognized by a protein complex allowing the recruitment of one (or two) CREB kinase(s) (which remain to be identified) and the phosphorylation of the serines within the sequence. The polyubiquitination apparatus recognizes this phosphorylated degradation signal. This triggers the polyubiquitination of nearby lysine(s) and the subsequent proteasomal degradation. Since chronic oxidative stress has been proposed to be a central mechanism for glucotoxicity in β-cells (5,39), it is possible that the oxidative stress generated by chronic exposure to high glucose plays a role in the recruitment of the molecular mechanism(s) targeting CREB to the proteasome pathway for degradation. Moreover, it has been reported that the association of small ubiquitin-related modifier-1 (SUMO1) with CREB stabilizes and promotes nuclear localization of CREB (40). Since a potential role of SUMO in β-cells has recently emerged (41,42), it is now also of interest to investigate whether SUMOylation may play a role in CREB degradation and CREB-targeted degradation process.

Chronic hyperglycemia is now well-known to have severe adverse effects on β-cell survival and function as a reduction of insulin secretion in response to glucose and insulin stores termed glucotoxicity (1–8). Hence, the preservation of a functional β-cell mass has become the major point of research in type 2 diabetes, and the future therapy of type 2 diabetes aims at protecting the β-cell from apoptotic death (1–6). Notably, we found that the insulin secretion in response to glucose and the insulin content were totally preserved in human islets exposed to high glucose and loaded with the CREB proteasomal targeting sequence peptide. This suggests that the preservation of CREB protein expression in β-cells is essential for survival and also favors the maintenance of an efficient glucose sensing, exocytosis machinery, and insulin gene transcription. In line with this, we found that protecting CREB from degradation by loading INS-1E cells with the CREB proteasomal targeting sequence peptide totally prevented high-glucose–induced downregulation of the antiapoptotic protein bcl-2 and glucokinase. The *bcl-2* gene is transcriptionally regulated by CREB (10). However, whether the glucokinase gene is transcriptionally regulated directly or indirectly by CREB remains to be elucidated. With respect to the role of chronic oxidative stress in β-cell death, it can be further hypothesized that CREB may also control the expression of key enzymes that counteract the emergence of oxidative stress.

Preventing apoptosis of β-cells also holds promise to improve islet transplantation outcomes as a treatment of type 1 as well as type 2 diabetes (21,22,43). Following transplantation, islets undergo drastic apoptosis that limits their function and long-term islet graft survival (21,22,44). Among the detrimental effects, transplanted islets have to fight against the hyperglycemic excursions of the recipient (44). Because of its ability to protect the viability and the glucose sensitivity of human islets exposed to high glucose, the CREB proteasomal targeting sequence peptide could be an asset in the preparation of islets for transplantation.

In conclusion, we report that chronic exposure of β-cells to high-glucose concentrations decreased CREB protein expression due to ubiquitin-proteasome–mediated degradation. Protection of CREB from degradation in β-cells chronically exposed to high-glucose concentration not only favors β-cell survival but also preserves glucose sensing, insulin gene transcription, and insulin secretion. Thus, our data demonstrate that CREB protein expression levels within the β-cells are essential for the control of a functional β-cell mass. These studies also illustrate that a specific blockade of CREB targeting to proteasomal degradation may be therapeutically useful to protect β-cells from the deleterious effects of chronic hyperglycemia.

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