Cytokines are known to induce apoptosis of pancreatic β-cells. Impaired expression of the anti-apoptotic gene bcl-2 is one of the mechanisms involved. In this study, we identified a defect involving transcription factor cAMP-response element-binding protein (CREB) in the expression of bcl-2. Exposure of mouse pancreatic β-cell line, MIN6 cells, to cytokines (interleukin-1β, tumor necrosis factor-α, and interferon-γ) led to a significant (p < 0.01) decrease in Bcl-2 protein and mRNA levels. Cytokines decreased (58%) the activity of the bcl-2 promoter that contains a cAMP-response element (CRE) site. Similar decreases were seen with a luciferase reporter gene driven by tandem repeats of CRE and a CREB-specific Gal4-luciferase reporter, suggesting a defect at the level of CREB. The active phospho form (serine 133) of CREB diminished significantly (p < 0.01) in cells exposed to cytokines. Examination of signaling pathways upstream of CREB revealed a reduction in the active form of Akt. Cytokine-induced decrease of bcl-2 promoter activity was partially restored when cells were cotransfected with a constitutively active form of Akt. Several end points of cytokine action including decreases in phospho-CREB, phospho-Akt, and BCl-2 levels and activation of caspase-9 were observed in isolated β-cells. Overexpression of wild-type CREB in MIN6 cells by plasmid transfection and adenoviral infection led to protection against cytokine-induced apoptosis. Together, these results point to CREB as a novel target for strategies aimed at improving the survival of β-cells.

In type 1 diabetes, insulin-producing β-cells are selectively destroyed by a cellular autoimmune response. Proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ are released during this autoimmune response and are believed to be important mediators of β-cell destruction (1, 2). Elevated circulating levels of these cytokines have been reported in type 1 diabetic patients (3). In NOD mice and in BB rats, two genetic models for autoimmune diabetes, increased production of cytokines is observed (3). Antibodies or soluble receptors that neutralize cytokine action in these models prevent the development of diabetes (2, 4). Several studies have shown that the β-cell death induced by cytokines in type 1 diabetes is mainly through apoptosis (5, 6).

Cytokines are known to modulate the expression of several genes in β-cells (7, 8). In a recent study, Cardozo et al. (7) carried out a comprehensive analysis of genes that were modulated in β-cells exposed to IL-1β and IFN-γ. Genes involved in the β-cell functions were down-regulated, whereas genes associated with apoptosis were up-regulated. Apoptosis can result from a variety of intracellular events or extracellular pathways such as activation of death receptors. The Bcl-2 family of proteins is important for regulation of the intrinsic mitochondrial pathway of apoptosis (9). The family consists of pro-apoptotic (e.g. Bad, Bax, Bid, and Bim) and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xl). Bcl-2 is known to maintain the integrity of the mitochondrial membrane. When Bcl-2 heterodimerizes with pro-apoptotic proteins, cytochrome c is released from mitochondria into the cytosol. Cytochrome c binds to apoptotic protease-activating factor 1 (Apaf-1), leading to activation of caspase-9 and the intrinsic death pathway (10). The balance between these two groups of Bcl-2 family members determines the fate of cells exposed to apoptotic stimuli. Expression of bcl-2 is an important step in the regulation of cell survival (9). In transgenic mice overexpressing bcl-2, apoptotic cell death is significantly reduced (11). Previous studies have suggested that cytokine-induced apoptosis involves down-regulation of bcl-2. Decreased bcl-2 mRNA is observed during apoptotic cell death in β-cell lines and islets (12–14). Stable overexpression of bcl-2 in the insulin-producing β-cell lines RINm5F and βTC1 improves their survival when exposed to a combination of IL-1β, TNF-α, and IFN-γ (15, 16). Transfection of human islets with bcl-2 confers protection against cytokine-induced β-cell death.
cAMP-response element-binding protein (CREB) in the promoter region of several genes, including c-fos (TGACGTCA) in the promoter region of several genes, indicating CREB binds to the conserved palindrome sequence of transcription factors and is ubiquitously expressed (22). CREB is known to play an important role in cell growth factor-1-mediated regulation of bcl-2 promoter through a CRE site in the 5′-flanking region (18). In that study, CREB activation through phosphorylation resulted in induction of bcl-2 gene expression in B lymphocytes (18). Hypoxia-mediated induction of bcl-2 gene in neuronal cells has been shown to depend on cyclic AMP response element in its promoter (19). We have previously characterized insulin-like growth factor-1-mediated regulation of bcl-2 promoter through CREB in PC12 cells, a neuroendocrine cell line (20, 21). CREB mediates the proliferative effect of insulin-like growth, differentiation, and survival (24–26).

In the present study, we examined cytokine-mediated down-regulation of bcl-2 expression at the promoter level and analyzed the role of CREB in MIN6 cells, a mouse β-cell line, and isolated mouse islets. We present evidence to show that CREB-mediated gene expression is impaired in β-cells exposed to cytokines. Further, we demonstrate that overexpression of CREB by adenoviral gene transfer rescues β-cells from cytokine-induced apoptosis. Overexpression of mutant forms of CREB, on the other hand, results in increased sensitivity of β-cells to cytokine injury.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Adenovirus**—For the generation of recombinant adenoviruses by homologous recombination, cDNAs encoding full-length wild-type CREB and mutant CREBs (KCREB and MCREB) were first subcloned into HindIII and XbaI sites in the plasmid pACCMVpLpA, which encodes the left end of the adenovirus chromosome containing E1A gene and the 5′ half of the E1B gene replaced with cytomegalovirus major immediate early promoter, a multiple cloning site, and intron and polyadenylation sequences from SV40 (27). Plasmids containing the appropriate constructs in pACCMVpLpA were co-transfected with BsrBI-digested Ad5d5327 (wild-type TP complex in HEK 293 cells by the LipofectAMINE Plus method using 5 µg of the recombinant plasmid and ~0.2 µg of TP complex. After complete cytopathic effect was observed (7–10 days), cells were harvested, frozen, thawed to release virus, and used for plaque purification as described (21). After two steps of plaque purification, positive plaques were identified by Western analysis using FLAG and CREB antibody. Virus was propagated in HEK 293 cells and purified by CsCl gradient purification (28). MIN6 cells were infected with adenoviral β-gal, wild type CREB, KCREB or MCREB at a multiplicity of infection (m.o.i.) of 10–20/cell. Subsequent experiments were carried out after 24–72 h.

**Plasmids**—The different promoter constructs of the bcl-2 gene (CRE site-containing construct, −1640 to −1287; CRE mutated, −1640 to −1287; and truncated without CRE, −1526 to −1287) were linked to

![Figure 1](https://example.com/image1.png)

**Fig. 1.** Cytokine-induced down-regulation of bcl-2 expression. A, MIN6 cells were exposed to 1× and 2× mixtures of cytokines (1×: IL-1β (1 ng/ml), TNF-α (5 ng/ml), and IFN-γ (5 ng/ml)) for 48 h, and levels of different Bcl-2 members and active forms of caspases (3 and 9) were examined by immunoblot analysis. A representative blot of four is shown for each target. Blots were reprobed for β-actin. Band intensity was quantitated densitometrically. *, p < 0.01 compared with untreated control. B, MIN6 cells were exposed to a mixture of cytokines (Cyt mix) for 12 and 24 h. In another set of treatments for 48 h, individual cytokines (IL-1β (2 ng/ml), TNF-α (10 ng/ml), and IFN-γ (10 ng/ml) or cytokine mixture (Cyt mix; 1×) were used. Total RNA was isolated, and bcl-2 mRNA was measured by real-time quantitative RT-PCR and corrected for 18 S ribosomal RNA. Values are mean ± S.E. of four independent experiments carried out in triplicate. #, p < 0.05; *, p < 0.01; **, p < 0.001 versus untreated control (Con).
Cytokines decrease bcl-2 promoter activity in β-cells. A and B, MIN6 cells cultured in six-well (35-mm) plates were transfected with different promoter constructs linked to luciferase reporter as indicated (3 μg) and pRSV β-galactosidase (1 μg) along with 8 μl of LipofectAMINE 2000 reagent. C, MIN6 cells were transfected with 2 μg of bcl-2 promoter and 2 μg of CREB mutants (KCREB and MCREB) or vectors. After 6 h, the transfected cells were exposed to the following concentrations of cytokines: A, IL-1β (2 ng/ml), TNF-α (10 ng/ml), and IFN-γ (10 ng/ml) alone or at indicated combinations. B and C, cytokine mixture (1×) of IL-1β (1 ng/ml), TNF-α (5 ng/ml), and IFN-γ (5 ng/ml). After 36 h of treatment, cell lysates were prepared and assayed for luciferase and β-galactosidase. Values represent mean ± S.E. of four independent observations in triplicates. A, p < 0.05 (#) and p < 0.01 (*) when compared with untreated control (Con). B, p < 0.01 (*) compared with untreated; p < 0.01 (*) versus CRE control. C, p < 0.01 (*) compared with untreated; p < 0.01 (#) versus vector control.

Immunoblotting—Cells incubated under different conditions were washed with ice-cold PBS, and lysed with mammalian protein extraction reagent (M-PER™, Pierce) containing phospatidase and protease inhibitors. Protein samples (50 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were blocked with Tris-buffered saline plus Tween 20 (20 mM Tris-HCl [pH 7.9], 8.5% NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 1 h and exposed overnight at 4 °C to primary antibody in Tris-buffered saline plus Tween 20 containing 5.0% BSA. Antibodies specific for CREB, phospho-133-CREB, Bax, Bcl-2, Bcl-xL, Bcl-2 active cleaved forms of caspase-3 and caspase-9, Akt, phospho forms (serine 473 and threonine 303) of Akt, β-galactosidase, and β-actin were from Cell Signaling (Beverly, MA) and Sigma. Following treatment with primary antibodies, blots were exposed to secondary anti-rabbit IgG or anti-mouse IgG conjugated to alkaline phosphatase, developed with CDP-Star reagent (New England Biolabs, Beverly, MA), and exposed to x-ray film. Band intensities were analyzed densitometrically using a Fluor-S Multilmage and Quantity One software (Bio-Rad).

Real-time Quantitative RT-PCR—Total RNA was isolated from cytokine-treated MIN6 cells using TRIzol reagent (Invitrogen) and further purified by DNase digestion. The mRNA for bcl-2 was measured by real-time quantitative RT-PCR as described (21) using a PE Applied Biosystems Prism model 7700 sequence detection instrument (Applied Biosystems, Foster City, CA). For bcl-2, the sequences of forward and reverse primers (designed by Primer Express; PE Applied Biosystems, Foster City, CA) were 5′-TTGGAGATTTCTGTTGGAACT-3′ and 5′-GAGACAGCCAGATGGAAG-3′, respectively. The TagMan fluorogenic probe (PE Applied Biosystems) used was 5′-FAM-TGGGATGCCTTTGTGGAACT-3′. PCR reactions were performed in 20 μl of a reaction mixture consisting of 10 μl of 2X SYBR GREEN Master Mix (Applied Biosystems, Foster City, CA), 1 μl of 100 μg/ml DNAse-free RNase-free water, 2 μl of 10 mM forward and reverse primers, and 1 μl of cDNA (or 50 ng of total RNA). After an initial denaturation step of 5 min at 95 °C, 40 cycles were performed, with 15 sec at 95 °C and 1 min at 60 °C. The threshold cycle (CT) value was determined using the Sequence Detection System software (PE Applied Biosystems, Foster City, CA).

Transfection—Transient transfections in MIN6 cells were carried out using LipofectAMINE™2000 reagent (Invitrogen). Cells were cultured in six-well plates (35 mm) at ~70% confluence. Plasmids (4 μg) and LipofectAMINE™2000 reagent (8 μl), each diluted in 100 μl of Opti-MEM with reduced serum, were mixed at room temperature for 20 min and added to the cells. Transfection efficiency was normalized by in-
Cytokine-induced Down-regulation of CREB

RESULTS

Cytokine-mediated Down-regulation of bcl-2 Expression—Activation of caspase-9 is a marker for the mitochondrial intrinsic pathway of apoptosis and is determined by the balance between pro- and anti-apoptotic proteins of the Bcl-2 family. A panel of Bcl-2 family members was examined by immunoblot analysis in MIN6 cells after chronically (48 h) exposing them to 1× and 2× mixtures of cytokines (1×: 1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ). Quantitation of the bands by scanning densitometry corrected for β-actin levels revealed a significant decrease (1×: 42%; p < 0.01) in anti-apoptotic Bcl-2 content (Fig. 1A, upper right). The levels of Bcl-XL and the pro-apoptotic proteins Bad and Bax remained unaltered (Fig. 1A, left). An increase in activation of caspase-9 and caspase-3 was detected using antibodies specific for the active cleaved fragment of the respective proteases (Fig. 1A, lower right). The cytokine-mediated decrease of Bcl-2 level was seen at earlier time points as well before the activation of caspases 3 and 9. For example, after 12- and 24-h exposure to cytokines (2×), the Bcl-2 protein levels decreased by 26% (p < 0.05) and 35% (p < 0.01), respectively (not shown in Fig. 1A). Next we examined the bcl-2 mRNA levels by real-time quantitative RT-PCR using a TaqMan fluorogenic probe in MIN6 cells exposed to a mixture of cytokines (1×). After 12 and 24 h of exposure, the cytokines decreased the bcl-2 mRNA levels by 28% (p < 0.05) and 37% (p < 0.01), respectively (Fig. 1B). When the cells were exposed to individual cytokines or the mixture for a longer period of 48 h, there was a 39% decrease (p < 0.01) in cells exposed to IL-1β (2 ng/ml) alone, whereas TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) reduced the mRNA levels only moderately (23 and 25%), respectively (Fig. 1B). The mixture of all three cytokines (1×) at half the concentration used for individual treatment decreased bcl-2 mRNA levels by 58% (p < 0.001, Fig. 1B). These findings (Fig. 1, A and B) are consistent with earlier reports showing cytokine-mediated down-regulation of bcl-2 expression in β-cells (12–14).

Cytokines Decrease bcl-2 Promoter Activity in β-Cells—Having demonstrated the cytokine-mediated down-regulation of Bcl-2 protein and bcl-2 mRNA, next we examined their effect on bcl-2 promoter activity. We have previously characterized this promoter in relation to the positive role of CREB in neuronal cells (20). The objective of the next series of experiments was to determine whether bcl-2 promoter activity is affected by cytokines in MIN6 cells and, if so, whether CREB is involved. The cells were transiently transfected with a CRE site-containing bcl-2 promoter linked to a luciferase reporter gene and exposed to the cytokines alone at the concentrations used in 2× mixture. Among the individual cytokines, IL-1β alone decreased the promoter activity modestly by 26% (p < 0.05) (Fig. 2A). The effect of IL-1β was further enhanced by TNF-α (44%...
Cytokine-induced Down-regulation of CREB

Fig. 4. Cytokines decrease CREB phosphorylation in MIN6 cells. A and C, MIN6 cells cultured on 35-mm dishes were exposed to a mixture (1× and 2×) of cytokines (1×: IL-1β (1 ng/ml), TNF-α (5 ng/ml), and IFN-γ (5 ng/ml)) for 4 h (A) or 1× mixture for 12–48 h (C) and immunoblotted for phospho-CREB (serine-133). Blots were reprobed for CREB. A representative of 4 blots is shown. The intensities of bands in A and C were quantitated by scanning densitometry (Fluor-S Multi-Imager), and the results are presented in B and D, respectively. * p < 0.01 versus untreated control. E, cells were cultured on chamber slides in medium containing 0.01% or 10% FBS with or without cytokines for 6 h. After fixing and permeabilization, cells were exposed to phospho-CREB antibody followed by secondary antibody (anti-rabbit IgG-Cy3) and DAPI (2 μg/ml; nuclear staining). Upper panels show the PCREB (Cy3; red) images, and the lower panels show DAPI overlay for the nuclei.
Cytokine-induced Down-regulation of CREB

Our studies with bcl-2 promoter suggested that the transcription factor CREB could be the target of cytokine action in β-cells. To further characterize the down-regulation of CREB by cytokines, we analyzed the activation of this transcription factor in MIN6 cells exposed to cytokines. Full activation of CREB requires phosphorylation at serine 133 after binding to CRE. Immunoblot analysis of MIN6 cells exposed to cytokines for 4 h revealed a 40–60% decrease in phospho-CREB levels (Fig. 4, A and B). Next, the effect of cytokines on phospho-CREB and the total CREB protein levels were examined over a period of 12–48 h (Fig. 4, C and D). Significant decreases (p < 0.01) in phospho-CREB levels (48–68%) persisted over a period of 48 h. The total CREB protein level decreased by 45% (p < 0.01) after 48 h of exposure to cytokines. CREB promoter itself has CRE sites, and so down-regulation of CREB function can lead to impaired expression of CREB itself (34). The cytokine-mediated decrease in CREB phosphorylation was further confirmed by immunocytochemistry (Fig. 4E). Enhanced immunostaining with phosphoserine 133-specific antibody and Cy3 was seen in cells cultured in 10% serum medium, which decreased in the presence of cytokines. Quantitation of fluorescence intensity using Slide Book Application software indicated that cytokine-induced 64% decrease in PCREB-Cy3 level is comparable to the findings of immunoblot analysis. DAPI overlay (Fig. 4E, lower panel) confirmed the presence of CREB in the nucleus.

Cytokines Decrease the Active Form of Akt in MIN6 Cells—Cytokines are known to be phosphorylated by kinases activated by several upstream signaling pathways, such as the PI 3-kinase/PDK1/Akt pathway (21, 35). Akt/PKB is known to play an important role in growth and survival of β-cells (36). We hypothesized that cytokines could induce β-cell apoptosis by interfering with Akt-mediated activation of CREB. Activation of Akt was examined by immunoblot analysis using antibodies specific for the phospho forms of Akt. Significant decrease in phospho-Akt (threonine 308) levels was seen in cytokine-treated MIN6 cells (Fig. 5, A and B; p < 0.01). Similar decreases were detected using the antibody specific for serine 473 (results not shown). The involvement of the PI 3-kinase pathway in regulating bcl-2 promoter activity was demonstrated by the 44% decrease in reporter activity in promoter-transfected β-cells in terms of interactions between CREB and NF-κB.
cells when exposed to wortmannin, an inhibitor of PI 3-kinase (Fig. 5C). When the promoter construct was cotransfected along with ΔΔ88, a dominant-negative form of the regulatory p85 subunit of PI 3-kinase, or with kinase-dead PDK1, luciferase activity decreased by 37 and 56%, respectively (Fig. 5C). Inhibition of PI 3-kinase has been shown to decrease insulin-like growth factor-1-mediated protection of β-cells against cytokines (37, 38). This pathway is known to promote cell survival through multiple mechanisms (39, 40). Our results suggest that one such mechanism could be induction of bcl-2 promoter activity and appears to be a target of cytokine action. Next, we proceeded to examine whether the constitutively active form of Akt can overcome cytokine-induced down-regulation of bcl-2 promoter. As shown in Fig. 5D, the active Akt itself increased the basal promoter activity by 2.3-fold. Further, the inhibitory action of cytokines on bcl-2 promoter activity was partially overcome by the constitutively active form of Akt (Fig. 5D). Reporter activity was decreased modestly (17%) by cytokines in Akt-transfected cells compared with 60% decrease in cells without active Akt. This observation suggests that β-cells with the active form of Akt are more resistant to cytokine action. The partial restoration of bcl-2 promoter activity by Akt also suggested that other mechanisms are likely to be involved.

**Cytokine-mediated Down-regulation of CREB Function in Mouse Islets**—Our experiments described so far demonstrate that cytokines impair CREB activation by inhibiting activation of the upstream kinase Akt to result in down-regulated bcl-2 expression. These studies were carried out in MIN6 cells, a cell line derived from mouse β-cells. Next, we examined the critical end points of these findings in mouse islets. When the islets were chronically exposed to cytokines for 48 h, levels of phospho-CREB decreased by 47–56% (p < 0.01), whereas the CREB content did not change (Fig. 6, A and B). However, we had observed a decrease in CREB content in MIN6 cells under similar conditions (Fig. 4C) probably caused by differences in sensitivity to cytokines. In mouse islets, cytokines decreased (48–53%; p < 0.01) the phospho-Akt levels in relation to total Akt (Fig. 6A). Immunocytochemical analysis of Bcl-2 protein content and activation of caspase-9 in mouse islets exposed to cytokines for 48 h are shown in Fig. 6C. Marked decrease in fluorescent staining of Bcl-2 with Cy3 is seen in cytokine-treated islets. Quantitation of the fluorescent intensity using the Slide Book Application software revealed a mean decrease of 62%. This decrease led to activation of caspase-9, as detected using an antibody specific for the active cleaved fragment of caspase-9 (Fig. 6C). We also used an earlier time point of 24 h to examine the levels of phospho-CREB, Bcl-2, and active caspase-9 by immunoblot analysis. After 24 h of exposure to cytokines, there were significant decreases (p < 0.01) in CREB phosphorylation and Bcl-2 levels (Fig. 6D). However, there was no increase above the basal trace level of active caspase-9 at this time point, indicating that the effect of cytokines on bcl-2 expression is an earlier event (Fig. 6D).

**Overexpression of CREB Protects MIN6 Cells from Cytokines**—CREB is known to enhance the survival of several cell types including neurons (24). To determine the role of CREB in mediating survival of β-cells, we examined MIN6 cells transfected with a GFP-CREB construct. As seen in Fig. 7A, CREB-GFP localized in the nucleus of the cell (A3). Analysis of apoptosis in the GFP-CREB-transfected cells after chronic exposure to cytokines revealed a 70% decrease in β-cell death as compared with apoptosis in cells transfected with GFP alone (27 versus 8.3%) (Fig. 7B). The transfection efficiency in MIN6 cells being modest, we used an adenoviral gene transfer approach to...
overexpress CREB. In the first set of experiments, we characterized the expression of CREB by immunoblotting and immunocytochemical analysis. In MIN6 cells transduced with recombinant adenoviruses at an m.o.i. of 10 and 20, the active phospho form of CREB was up-regulated, as indicated by immunoblot analysis (Fig. 7C). Immunocytochemical analysis indicated a gene transfer efficiency of ∼75%, as indicated by the FLAG tag, as well as the active phospho form of overexpressed wild-type CREB (Fig. 7D). The culture conditions with serum-containing medium seem to be sufficient to maintain CREB in active phospho form. Next we examined CREB-mediated protection of β-cells from cytokine-induced apoptosis. Analysis of cells exposed to cytokines for 48 h demonstrated 9.3% of apoptosis in adenoviral CREB-transduced MIN6 cells as compared with 22.3% in cells transduced with the adenoviral control (58% reduction; Fig. 7E). When the infected MIN6 cells were exposed to cytokines for 72 h, significant (p < 0.01) protection by CREB was seen (33% in β-gal versus 14% in WTCREB). Thus, CREB appears to promote β-cell survival, as shown by two approaches to overexpress this transcription factor.

**Overexpression of Mutant CREB Increases Activation of Caspase-9**—To determine whether apoptosis is induced by down-regulation of CREB even in the absence of cytokines, MIN6 cells were infected (m.o.i. of 20) with adenovirus encoding CREB mutated at the DNA binding domain (KCREB), which sequesters endogenous CREB by heterodimerization. Increased expression of the flag tag of KCREB and β-gal (control) was seen in MIN6 by immunoblot analysis (Fig. 8A). Overexpression of the mutant CREB led to significant activation of caspase-9, a marker for the mitochondrial pathway of apoptosis when compared with cells infected with adenoviral β-gal (Fig. 8A). These cells were not exposed to cytokines. MIN6 cells infected with adenoviruses were further characterized by immunocytochemical analysis by double antibody staining with fluorogenic probes. Fluorescent staining of β-gal and the flag tag of KCREB with FITC (green) shows the efficient (70–80%) transfer of genes by this approach (Fig. 8B). Activation of caspase-9 was high in cells expressing KCREB as compared with β-gal virus-infected cells (Fig. 8B, red). Merging of the two images revealed random activation of caspase-9 in β-gal-overexpressing cells. For example, one arrow shows the overlap (upper; the color does not merge because of cytosolic localization of β-gal) and the other without overlap. On the other hand, significant overlap of KCREB and active caspase-9, giving orange color was seen as shown by the three arrows (Fig. 8B). In some cells, the intensities of KCREB (green) and active caspase-9 (red) do not match precisely. However, activation of caspase-9 is seen among KCREB-expressing cells in general after examining multiple fields. These results suggest that down-regulation of CREB in β-cells leads to stimulation of the mitochondrial pathway of apoptosis even in the absence of cytokines.
Adenoviral Transfer of Mutant Forms of CREB Enhances Cytokine-induced Apoptosis in MIN6 Cells—To determine whether CREB down-regulation renders β-cells more susceptible to cytokine-induced apoptosis, we overexpressed two mutant forms of CREB by adenoviral gene transfer. In addition to KCREB, the second mutant form used was MCREB. This construct is mutated at the phosphorylation site (S133A) and so cannot bind the coactivator CREB-binding protein. MIN6 cells infected for 24 h with KCREB or MCREB were exposed to cytokine mixture (1/1000: 1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ) for another 48 h. Activation of caspase-3 was used as a marker for apoptosis in these cells. Immunocytochemical analysis indicated that overexpression of either mutant form of CREB led to a 3-fold increase in the activation of caspase-3 as compared with β-gal-expressing cells (Fig. 9, A and B). In another set of the same experiment, apoptosis was quantitated by counting cells with condensed nuclei after staining with 33258 Hoechst dye (Sigma). Apoptosis was seen in 21% of β-gal-infected cells, whereas adenoviral transfer of the mutants KCREB and MCREB resulted in significantly increased (p < 0.01) susceptibility to injury (52.7 and 48% cell death, respectively) (Fig. 9C). These results suggest that survival of β-cells is compromised when CREB function is down-regulated, leading to enhanced cytokine-induced β-cell injury. Even in the absence of cytokines, adenoviral KCREB at a higher m.o.i. of 20 induced the activation of caspase-9 in the previous experiment (Fig. 8B).

DISCUSSION

The mechanism of cytokine-induced β-cell apoptosis in type 1 diabetes is not clearly understood. Cytokines have been shown modulate the expression of several genes, including transcription factors that are associated with β-cell function and death (7). In the present study, we demonstrate that cytokines impair the activity of bcl-2 promoter through down-regulation of the transcription factor CREB in mouse β-cells. The role of CREB in promoting β-cell survival has not been examined previously. We have now shown that overexpression of the CREB gene in a β-cell line leads to enhanced protection from cytokine-mediated cell death.

Cardozo et al. (7) in a recent study examined by microarray analysis >200 genes modulated by IL-1β and interferon-γ in rat β-cells. They observed that activation of NF-κB by these cytokines was pro-apoptotic in β-cells. Our present study examines the transcriptional regulation of bcl-2, an anti-apoptotic gene in relation to the transcription factor CREB. Overexpression of bcl-2 has been shown to rescue β-cells exposed to cytokines (15, 17). Cytokine-mediated β-cell apoptosis involves decreased expression of bcl-2 (12–14). In this study, we provide a transcriptional mechanism for these findings. We demon-
strate that cytokines decrease bcl-2 promoter activity in a transient transfection model. This anti-apoptotic gene bcl-2 is up-regulated by CREB in several cell types including β-cells (18, 20). Cytokine-induced decrease of bcl-2 expression in β-cells seems to involve defective CREB activation because they also inhibit a reporter driven by tandem repeats of CRE elements and a Gal4 reporter system specific for CREB. Under the same experimental conditions, cytokines activate a reporter gene driven by NF-κB-responsive elements. Interestingly the bcl-2 gene has been shown to be up-regulated by NF-κB in human prostate carcinoma cells (41). However, findings of our study and previous reports (12–14) show that bcl-2 expression is down-regulated by cytokines in β-cells. Further studies are needed to understand the interactions between NF-κB and CREB in the regulation of bcl-2 gene in β-cells.

The nuclear transcription factor CREB plays an important role in diverse cellular functions (25). Although CREB-mediated gene expression has been studied extensively in neurons, limited information is available regarding its role in β-cell function. Membrane depolarization and calcium influx in β-cells activate CREB through phosphorylation (42). Glucose-induced up-regulation of c-fos expression proceeds through activation of CREB (43). CREB and serum response factor also play a role in the transcriitional induction of egr-1, an early response gene (44). The 5′-flanking region of the rat insulin gene contains a CRE site, which appears to respond to ATF-2 or related CREB family members (45, 46). However, previous studies have not examined the role of CREB in promoting β-cell survival.

Cytokine-mediated down-regulation of CREB suggested that the signaling pathway leading to CREB activation could be impaired. After binding to CRE sites of responsive promoters, CREB needs to be phosphorylated at serine 133 so that it can bind to the coactivator CREB-binding protein. Initially, this covalent modification was attributed to cAMP-dependent protein kinase (47). However, subsequent studies have established that several kinases stimulated by growth factor-mediated signaling pathways can phosphorylate CREB at the same serine 133 site, leading to its activation (35, 48, 49). Du and Montminy (35) demonstrated that Akt, a downstream target of the PI 3-kinase pathway, stimulates CREB phosphorylation. Since then, we have shown that activation of Akt leads to up-regulation of bcl-2 expression through CREB in the neuronal cell line PC12 (21). Akt plays an important role in the regulation of β-cell function (50, 51), and transgenic overexpression of Akt in mouse β-cells leads to increased β-cell size and survival (36). Moreover, cytokines have been shown to decrease Akt activation (52, 53). One of the consequences of Akt down-regulation by cytokines seems to be decreased activation of CREB, leading to decreased bcl-2 expression.

In this study, cytokine-mediated down-regulation of CREB function was characterized by using a mixture of all three cytokines, IL-1β, TNF-α, and IFN-γ. When β-cells were exposed to individual cytokines, only modest effects on CREB were seen. Lymphoid infiltration of islets in type 1 diabetes leads to release of these cytokines. They induce apoptosis of β-cells through synergistic interactions (3). In addition to mu-
tual potentiation during intracellular signaling, one cytokine could also increase the production of another in β-cells. For example, IL-1β increases the production of TNF-α by β-cells thereby exaggerating cytotoxicity (54). IL-1β, synthesized as an inactive precursor, is cleaved and activated by interleukin-1-converting enzyme, the expression of which is induced by IFN-γ in pancreatic islets (55). Hence our findings are relevant to an in vivo condition where all these cytokines act in concert to induce β-cell apoptosis as in autoimmune diabetes.

Our present findings are directly relevant to type 1 diabetes, but the central mechanism involved also has potential implications in type 2 diabetes. Zucker diabetic rats, a model with gradual β-cell loss, exhibit decreased bcl-2 expression (56). Moreover, human pancreatic islets exposed to free fatty acids show a decrease in bcl-2 mRNA levels and activation of apoptosis (57). Inada et al. (58) reported an increase in the expression of several forms of CREB repressors such as ICER1, ICER Iy, CREM-17, and CREM-17X in pancreatic islets of type 2 diabetic Goto-Kakizaki rats. Finally, increased circulating TNF-α levels, which cause insulin resistance in type 2 diabetes (59), might also impair β-cell survival by the mechanism described in our present study.

Understanding the mechanism of islet death at the molecular level is essential for strategies aimed at preventing apoptosis in β-cells. Adenoviral transfer of the CREB gene into β-cells provides valuable reagents to modulate the PI 3-kinase pathway. Previous studies have shown that CREB plays a role in transcription factor CREB appears to be essential for improvement of transplantation outcomes. The findings of our present study suggest that the transcription factor CREB appears to be essential for improving survival of β-cells. Adenoviral transfer of the CREB gene into β-cells leads to enhanced survival after exposure to cytokines. Overexpression of mutant CREB (KCREB and MCREB) results increased susceptibility to cytokine-induced apoptosis. Previous studies have shown that CREB plays a role in β-cell function and survival. Together, our results suggest the usefulness of targeting the transcription factor CREB in efforts to improve β-cell function and survival in diabetes.

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Cytokine-mediated Down-regulation of the Transcription Factor cAMP-response Element-binding Protein in Pancreatic β-Cells
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