

Cytokine Induction of Fas Gene Expression in Insulin-Producing Cells Requires the Transcription Factors NF- κ B and C/EBP

Martine I. Darville and Décio L. Eizirik

Fas-mediated cell death may play a role in the autoimmune destruction of pancreatic β -cells in type 1 diabetes. β -Cells do not express Fas under physiological conditions, but Fas mRNA and protein are induced in cytokine-exposed mouse and human islets, rendering the β -cells susceptible to Fas ligand-induced apoptosis. The aim of the present study was to investigate the molecular regulation of Fas by cytokines in rat β -cells and in insulin-producing RINm5F cells. Fas mRNA expression was increased 15-fold in fluorescence-activated cell sorting-purified rat β -cells exposed to interleukin (IL)-1 β , whereas γ -interferon had no effect. Transfection experiments of rat Fas promoter-luciferase reporter constructs into purified rat β -cells and RINm5F insulinoma cells identified an IL-1 β -responsive region between nucleotides -223 and -54. Inactivation of two adjacent NF- κ B and C/EBP sites in this region abolished IL-1 β -induced Fas promoter activity in RINm5F cells. Binding of NF- κ B and C/EBP factors to their respective sites was confirmed by gel shift assays. In cotransfection experiments, NF- κ B p65 transactivated the Fas promoter. NF- κ B p50 and C/EBP β overexpression had no effect by themselves on the Fas promoter activity, but when cotransfected with p65, each factor inhibited transactivation by p65. These results suggest a critical role for NF- κ B and C/EBP factors in cytokine-regulation of Fas expression in insulin-producing cells. *Diabetes* 50:1741-1748, 2001

Aptosis is the main mode of β -cell death in diabetes-prone NOD mice (1-3). In this species, β -cell apoptosis precedes massive T-cell infiltration, suggesting a role for inflammatory mediators produced by early infiltrating cells, such as macrophages and dendritic cells (2,3). In line with this possibility, expression of the cytokines interleukin (IL)-1, γ -interferon (IFN- γ), and the inducible form of nitric oxide

synthase (iNOS) have been detected in the pancreas of prediabetic NOD mice (4-6). Furthermore, in vitro exposure of rodent and human islet cells to cytokines leads to β -cell death mostly by apoptosis (7-9). Cytokines may induce direct deleterious effects on β -cells via generation of toxic oxygen free radicals and nitric oxide (NO) (6,10,11). In addition to these effects, cytokines induce a complex up- and downregulation of multiple genes in β -cells (12). One of the genes upregulated by cytokines in rodent and human islets is the proapoptotic receptor Fas (9,13-16).

Fas (CD95, APO-1) is a cell-surface receptor belonging to the nerve growth factor/tumor necrosis factor (TNF) receptor family, whereas its ligand, FasL, belongs to the TNF family (17,18). FasL is expressed in activated T-cells and macrophages, whereas Fas may be expressed in several target cells. Interaction between FasL and Fas leads to apoptotic death of Fas-expressing cells (18). Normal pancreatic β -cells do not express Fas (19). However, Fas expression is induced in β -cells from diabetic NOD mice (19). The role for Fas in β -cell destruction in NOD mice remains controversial. The inability of Fas-deficient NOD-*lpr/lpr* mice to develop diabetes may be due to dysregulation of the adaptive immune system in these mice (20,21). On the other hand, NOD-*lpr/lpr* mice bearing the *scid* mutation (which eliminates enhanced FasL-mediated T-cell lytic activity) have reduced the incidence of diabetes after adoptive transfer of diabetogenic NOD spleen cells (22). Moreover, Fas-deficient NOD mice with CD4⁺ T-cells expressing the highly diabetogenic β -cell-specific 4.1-T-cell receptor are protected from diabetes (16), and neutralization of FasL prevented syngeneic islet graft destruction in diabetic NOD mice (23). Fas expression is induced in vitro in NOD islet cells by IL-1 β , IFN- γ , or a combination of IL-1 β , IFN- γ , and TNF- α (19), and in normal mouse islet cells by IL-1 β alone (13,24). These IL-1 β -treated islet cells undergo apoptosis when exposed to an agonist anti-Fas monoclonal antibody (13). Fas expression is also induced in human β -cells exposed to IL-1 β (14), and it has been suggested that IL-1 β -induced Fas expression is mediated by NO production (14). This role for NO has not, however, been reproduced by other investigators (15,24). Moreover, we have recently observed that cytokines induce Fas mRNA expression in islets isolated from iNOS knockout mice (9) and in human islets exposed to IL-1 β and IFN- γ in the presence of iNOS blockers (9,12), excluding a major role for NO in this phenomenon. The potential role of β -cell Fas expression

From the Gene Expression Unit, Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium.

Address correspondence and reprint requests to Dr. Martine I. Darville, Gene Expression Unit, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090-Brussels, Belgium. E-mail: mdarv@mebo.vub.ac.be.

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ANOVA, analysis of variance; CMV, control vector; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; ICAM-1, intercellular adhesion molecule-1; IFN- γ , γ -interferon; IL, interleukin; iNOS, inducible form of nitric oxide synthase; L-MA, N^G-methyl-L-arginine; NO, nitric oxide; PCR, polymerase chain reaction; RT, reverse transcriptase; TNF, tumor necrosis factor.

for the pathogenesis of type 1 diabetes was highlighted by a recent study by Moriwaki et al. (25). Using pancreatic biopsy specimens obtained from 13 recent-onset diabetic patients, it was shown that Fas expression is upregulated in pancreatic β -cells of inflamed islets, whereas FasL is present in CD8⁺ cells, macrophages, and CD4⁺ cells infiltrating the islets (25). This makes Fas an attractive target for genetic interventions to protect β -cells against immune-mediated death in early type 1 diabetes. For this purpose, it is crucial to understand the molecular mechanisms involved in cytokine-induced Fas expression in β -cells.

Fas upregulation by IFN- γ is impaired in human cell lines deficient in STAT1 (26). The use of RelA(p65)-deficient mouse embryonic fibroblasts demonstrated that NF- κ B activation is required for maximal induction of Fas expression by TNF- α (or lipopolysaccharide) + IFN- γ (27). The rat Fas promoter has been sequenced and shown to contain potential C/EBP and NF- κ B binding sites (28). Unfortunately, there are no available data on cytokine regulation of rat Fas promoter activity in β -cells or in other cell types.

In the present study, we investigated the transcriptional regulation of the rat Fas gene by IL-1 β and IFN- γ . This was performed by transfection of Fas promoter-luciferase reporter constructs in fluorescence-activated cell sorting (FACS)-purified primary β -cells and in insulin-producing RINm5F cells and by mutational analysis, gel shift assays, and cotransfection experiments. The data obtained indicate that the transcription factors NF- κ B and C/EBP are required for IL-1 β -induced Fas expression in insulin-producing cells.

RESEARCH DESIGN AND METHODS

Plasmid constructs and mutagenesis. The Fas promoter region was isolated by polymerase chain reaction (PCR) using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) on 0.5 μ g of rat genomic DNA prepared from liver as described (29). The primers used were based on the published rat Fas sequence (28). The forward primer (position -798 to -781) was 5'-CGTCTAGACAGCTCTTCTGGTCAACA with an added *Xba*I site, and the reverse primer (position +108 to +126) was 5'-CGGGATCCTAGACTGCCTCTGGGTGT with an added *Bam*HI site. The amplified product was cloned into pBluescript KS (Stratagene, La Jolla, CA). Two clones were sequenced and shown to contain small and identical differences with the published sequence (28) so that nucleotide -798 was renumbered -811. The presently cloned Fas sequence was deposited in the GenBank database under accession number AF308477. The promoter region (-811 to +126) and its 5' deleted fragments were isolated from pBluescript by digestion with *Bam*HI (3' end) and with the following restriction enzymes for the 5' end: *Xba*I (-811), *Eco*RI (-223), or *Nci*I (-54), filled in with the Klenow fragment of DNA polymerase I. The fragments were cloned into the *Sma*I-*Bgl*II sites of pGL3-Basic (Promega, Madison, WI) to obtain the pFas luc constructs. Mutations for the NF- κ B and C/EBP sites at positions -142 and -130, respectively, were generated on pFas-811 luc by PCR (30) using the Expand High Fidelity PCR system. The pairs of primers were as follows: 5'-ATCAAGCCCTGCTGGGGCCT and 5'-ATCCTGGGCGTTCACAGTC for deletion of nucleotides -143 to -124, 5'-ATCAAGCCCTGCTGGGGCCT and 5'-TGCATAAATGGCATTATGCATCTGGGCGC for mutation in the NF- κ B site, and 5'-GGGGAATGCCCATTTCTAGATCAAGCCCTGCTTG and 5'-ATCCTGGGCGTTCACAGTC for mutation in the C/EBP site (mutated bases are in italics). All mutations were confirmed by sequencing. Expression vectors for NF- κ B p65 (CMV-p65), NF- κ B p50 (CMV-p50), C/EBP β (CMV-C/EBP β), and control vector (CMV) were provided by H. Liu and R.M. Pope (31).

Cell culture, transfection, and luciferase assay. RINm5F insulinoma cells were cultured in RPMI-1640 medium with Glutamax-1 (Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum. Rat β -cells were FACS purified from islets isolated from male Wistar rats, as previously described (32). The rats were housed according to the guidelines of the Belgian Regulation for Animal Care, and the experimental protocol was

approved by the Ethics Committee for Animal Experiments of the Vrije Universiteit Brussel. These preparations contained >95% β -cells and were cultured in HAM's F-10 medium supplemented with 10 mmol/l glucose, 2 mmol/l glutamine, 0.5% bovine serum albumin, and 50 μ mol/l 3-isobutyl-1-methylxanthine (IBMX). The presence of IBMX at this concentration preserves β -cell survival in culture (33) with minimal effects on cAMP formation in the absence of adenylate cyclase activators (34). RINm5F and primary β -cells were cotransfected with the luciferase test plasmids and with pRL-CMV (Promega), as an internal control, by lipofection using lipofectAMINE (Life Technologies, Gaithersburg, MD) as previously described (35). The following modifications were introduced for β -cell transfections: 4×10^4 cells were seeded in 96-well plates and incubated for 3 h with 0.05 μ g test plasmid, 0.005 μ g pRL-CMV, 0.5 μ g PLUS Reagent, and 0.25 μ l lipofectAMINE. The day after transfection, the cells were exposed for 16 h to 50 units/ml (for RINm5F cells) or 30 units/ml (for β -cells) of recombinant human IL-1 β (provided by Dr C.W. Reynolds, National Cancer Institute, Bethesda, MD) and/or 1,000 units/ml recombinant mouse IFN- γ (Life Technologies). The concentrations of cytokines selected for the experiments were based on previous data from our group (35-38). In cotransfection experiments, RINm5F cells were transfected with 0.1 μ g test plasmid, 0.005 μ g pRL-CMV, and the indicated amounts of expression vectors. The total amount of DNA was kept at 0.2 μ g by adding the control vector CMV. The cells were harvested 24 h after transfection. Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega). Values obtained for the promoterless vector pGL3-Basic were 1 and 0.6 light units in RINm5F and β -cells, respectively. The transfection efficiency of primary β -cells was 10-20%. Test values were corrected for the luciferase activity value of the internal control pRL-CMV and expressed as relative luciferase activity.

Electrophoretic mobility shift assays. Nuclear extracts were prepared from RINm5F cells as described (39). Nuclear proteins (4-5 μ g) were preincubated with 1 μ g poly(dIdC) in 20 μ l containing 10 mmol/l HEPES (pH 7.9), 50 mmol/l KCl, 5 mmol/l MgCl₂, 0.05 mmol/l EDTA, 0.5 mmol/l dithiothreitol, and 10% glycerol for 10 min at 0°C before addition of 50 molar excess of competing oligonucleotide, as indicated, and radiolabeled probe (30,000 cpm). The incubation was continued for 20 min at 0°C for NF- κ B electrophoretic mobility shift assays (EMSA) or at room temperature for C/EBP EMSAs. Where indicated, 1 μ l of antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was added for 20 min at room temperature. The samples were electrophoresed on 5% polyacrylamide gels in 25 mmol/l Tris, 25 mmol/l boric acid, and 0.5 mmol/l EDTA for NF- κ B EMSAs or in 1 \times Tris-glycine-EDTA buffer for C/EBP EMSAs. Oligonucleotides for EMSAs were as follows (upper strand shown): Fas NF- κ B (NF)-C/EBP, 5'-agGATGGGGAATGCCCATTTATGCAATCAAG; Fas NF- κ B (NF)-C/EBPmut, 5'-agGATGGGGAATGCCCATTTCTAGATCAAG; Fas C/EBP, 5'-agCCATTTATGCAATCAAG; NF- κ B consensus (cons), 5'-agCTTCAGAGGGGACTTTCCGAGA; C/EBP consensus (cons), 5'-agCAGATTGCGCAATCTGCA. The lowercase letters represent added nucleotides to allow end-labeling with the Klenow fragment of DNA polymerase I.

Reverse transcriptase-PCR analysis. Reverse transcriptase (RT)-PCR was performed on poly(A)⁺ RNA as described (35). Primers for Fas were 5'-GAATGCAAGGGACTGATAGC (forward primer) and 5'-TGGTTCGTGTGCAAGGCTC (reverse primer). The PCR for Fas and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (as in 35) mRNA detection was performed with 34 and 28 cycles, respectively. These numbers of cycles were selected to allow cDNA amplifications in the linear range.

Western blot analysis. Nuclear extracts were prepared from 10⁶ RINm5F cells transfected with the expression vectors for p65, p50, or C/EBP β alone, or for p65 in combination with p50 or C/EBP β at a one-to-two ratio, as indicated above. Proteins were fractionated by SDS-PAGE and transferred on nitrocellulose. The filters were incubated with anti-p65, anti-p50, or anti-C/EBP β antibodies and then with a secondary antibody (peroxidase-labeled anti-IgG). Detection was performed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Statistical analysis. Results are given as means \pm SE. Multiple comparisons were performed by analysis of variance (ANOVA), followed by group comparisons using the Student's paired *t* test with correction of the *P* values for multiple comparisons by the Bonferroni method.

RESULTS

Cytokine regulation of Fas mRNA expression in rat β -cells. To determine which cytokine is the main inducer of Fas expression in rat β -cells, FACS-purified rat β -cells were exposed for 6 or 24 h to IL-1 β , IFN- γ , or a combination of both cytokines, and Fas mRNA content was determined by RT-PCR. Unstimulated cells expressed low

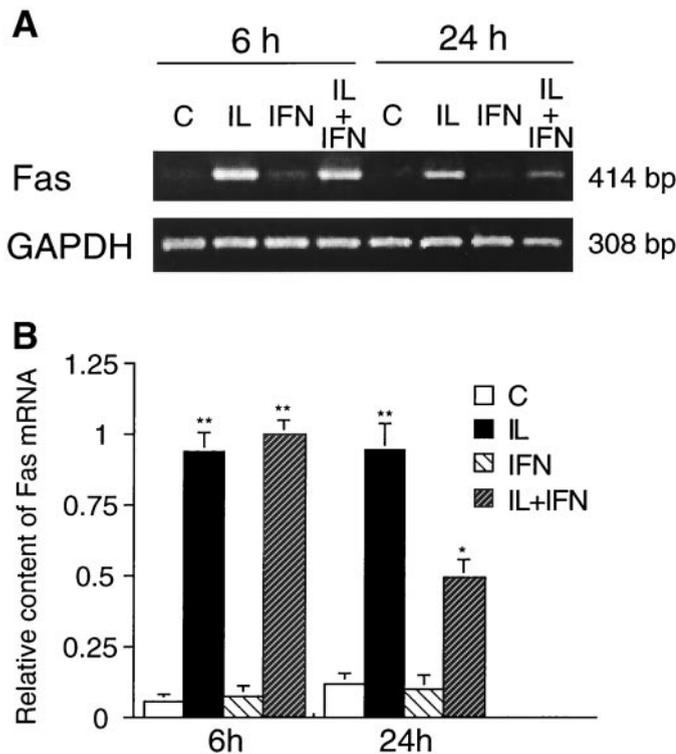


FIG. 1. Cytokine regulation of Fas mRNA expression in rat β -cells. Rat β -cells were cultured for 6 or 24 h in control medium (C, no cytokine added) or with IL-1 β (30 U/ml), IFN- γ (1,000 U/ml), or IL-1 β + IFN- γ . Fas and GAPDH mRNA content was analyzed by RT-PCR. **A:** A representative experiment is shown. **B:** Shown are the relative Fas mRNA contents expressed as optical densities, corrected for GAPDH. The results are the means \pm SE of three to five independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control (ANOVA).

levels of Fas mRNA (Fig. 1). Fas mRNA expression was induced 15-fold in cells exposed to IL-1 β for 6 h, and this increase was not potentiated by IFN- γ . Increased Fas mRNA expression was maintained over a period of 24 h (Fig. 1). To determine the role of NO in Fas expression in rat β -cells, FACS-purified rat β -cells were treated for 24 h with IL-1 β in the presence or absence of N^G -methyl-L-arginine ([L-MA] 1 mmol/l), an inhibitor of iNOS activity. The optical density values for Fas mRNA content, corrected for GAPDH mRNA content, were comparable in the presence or absence of L-MA (control, 0.22 ± 0.04 ; L-MA, 0.05 ± 0.05 ; IL-1 β , 0.79 ± 0.06 ; IL-1 β + L-MA, 1.1 ± 0.17 ; $n = 3$), showing that the iNOS blocker did not prevent Fas mRNA induction. This result confirms previous observations in mouse (9,15) and human islets (9) that cytokine-induced Fas expression is independent of NO. On the basis of these results, the subsequent experiments on promoter regulation were performed in the presence of IL-1 β alone.

Identification of the cytokine-responsive element in the Fas promoter. To delineate the Fas promoter region responding to IL-1 β , the promoter fragment from nucleotides -811 to +123 relative to the transcription start site and its 5' deletants (Fig. 2A) were cloned into the luciferase reporter plasmid pGL3 and transfected into FACS-purified β -cells treated with IL-1 β or left untreated. In untreated cells, all constructs have a low basal activity (Fig. 2B) consistent with the lack of TATA box or Sp1 binding sites in the rat Fas promoter. In IL-1 β -treated cells, the luciferase activity of the constructs pFas-811 luc

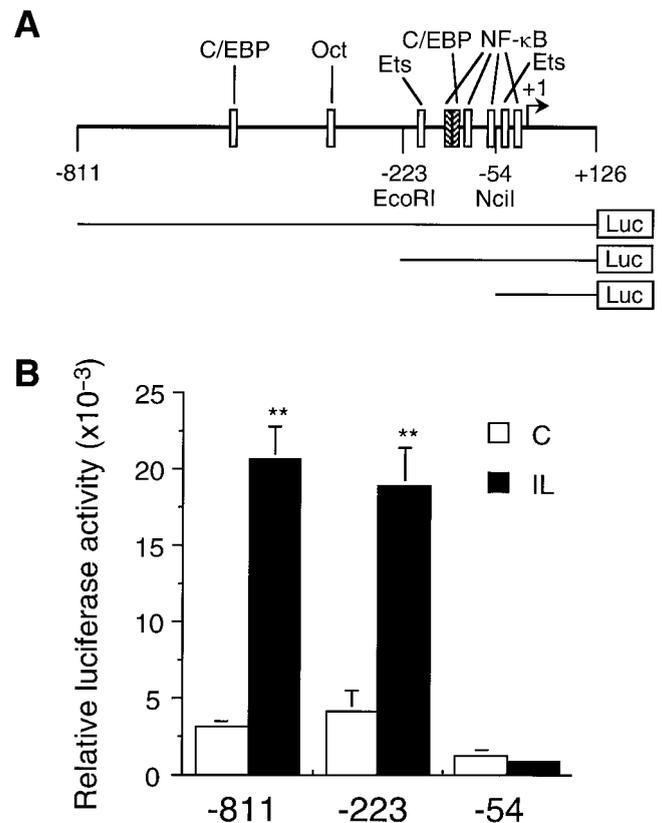


FIG. 2. Localization of the cytokine-responsive elements in the Fas promoter in rat β -cells. **A:** Schematic representation of the rat Fas promoter. Putative binding sites for transcription factors are indicated by boxes. Hatched boxes represent binding sites that were mutated in this study. The promoter fragments cloned upstream of the luciferase reporter gene are indicated in the lower part of the figure. **B:** β -Cells were transfected with the 5' deleted promoter constructs shown in A and left untreated (control [C]) or treated with IL-1 β for 16 h. Relative luciferase activity values are the means \pm SE of five independent experiments. The relative luciferase activity value of the promoterless pGL3 vector is 0.0008. ** $P < 0.01$ vs. respective control (ANOVA).

and pFas-223 luc was induced more than sevenfold, whereas pFas-54 luc was not responsive to IL-1 β (Fig. 2B). These results suggest that the element or elements responding to IL-1 β are located between nucleotides -223 and -54 and rule out a role for the potential upstream C/EBP and Oct binding sites (Fig. 2A). The same experiments were reproduced in an insulinoma cell line, RINm5F, with similar results as those observed in β -cells (Fig. 3A). Hence (and due to the large number of cells required), subsequent experiments to identify the regulatory elements in the Fas promoter were performed in RINm5F cells.

The region between nucleotides -223 and -54 contains three potential NF- κ B binding sites, a C/EBP binding site, and a binding site for Ets factors (Fig. 2A). Contribution of the Ets binding site located at position -196 to the promoter activity was not significant because a construct containing the promoter region up to -153, from which the Ets site is absent, was still responsive to IL-1 β (data not shown). NF- κ B and C/EBP synergize for transcriptional activation of many proinflammatory genes through cooperative binding (40,41) or direct interaction (42,43). Thus, we chose to mutate the adjacent NF- κ B and C/EBP sites (Fig. 2A). A pFas-811 luc construct with nucleotides

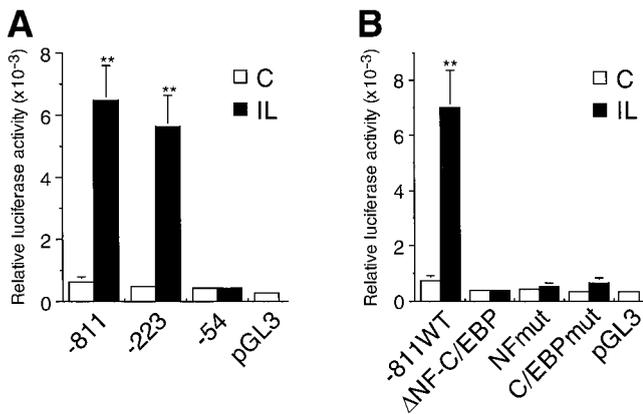


FIG. 3. Localization of the cytokine-responsive elements in the Fas promoter in RINm5F cells. **A:** The cells were transfected with the 5' deleted promoter constructs shown in Fig. 2A and left untreated (control [C]) or treated with IL-1β for 16 h. Relative luciferase activity values are the means ± SE of four independent experiments. ***P* < 0.01 vs. respective control (ANOVA). **B:** The cells were transfected with the promoterless pGL3 vector, the wild-type (WT) pFas-811 luc, or the same construct with deletion of the NF-κB and C/EBP sites indicated in Fig. 2A (ΔNF-C/EBP), or with either the NF-κB site mutated (NFmut) or the C/EBP site mutated (C/EBPmut). The cells were treated as in A. Relative luciferase activity values are the means ± SE of three independent experiments. ***P* < 0.01 vs. respective control (ANOVA).

-143 to -124 deleted, eliminating both binding sites (ΔNF-C/EBP), completely lost responsiveness to IL-1β (Fig. 3B). Similarly, pFas-811 luc, with either the NF-κB (NFmut) or the C/EBP site mutated (C/EBPmut), was unable to respond to IL-1β (Fig. 3B). These results indicate that both binding sites are required for IL-1β induction of the Fas promoter.

NF-κB and C/EBP binding to the Fas promoter. To identify the factors binding to the IL-1β-responsive element, we performed EMSAs using a probe spanning the NF-κB and C/EBP sites from nucleotides -148 to -116 (probe Fas NF-C/EBP), and nuclear extracts from RINm5F cells untreated or exposed to IL-1β for 30 min and 4 h. A major complex *a* and a minor complex *b* were induced in cells treated with IL-1β, with a higher binding activity at 30 min (Fig. 4A). Complex *a* was similarly observed when the C/EBP site was mutated in the probe (probe Fas NF-C/EBPmut, Fig. 4A). Complex *b* was more clearly detected when using the Fas NF-C/EBPmut probe. This finding may be due to the absence of a nonspecific complex, observed with the wild-type probe, which interferes with the visualization of complex *b*. These results are consistent with NF-κB binding. Indeed, we have previously shown that binding activity of NF-κB is maximal at 30 min in IL-1β-exposed RINm5F cells (38). The nature of complexes *a* and *b* was further characterized by competition and supershift experiments. The two complexes were specifically competed by an excess of unlabeled probe (Fig. 4B, lane 2) and by a consensus NF-κB oligonucleotide (lanes 4 and 13) but not by an unrelated oligonucleotide (lane 5). An antibody directed against the NF-κB family member p65 supershifted complex *a* (lanes 6 and 14), whereas an antibody directed against another NF-κB family member, p50, affected both complexes *a* and *b* (lanes 10 and 16), suggesting that complex *a* contains p65 homodimers and/or heterodimers of p65 and p50 and that complex *b* contains homodimers of p50. With use of the Fas NF-C/EBP probe, no complex could be competed by a consensus C/EBP oligonucleotide (Fig. 4B, lane 3) and antibodies

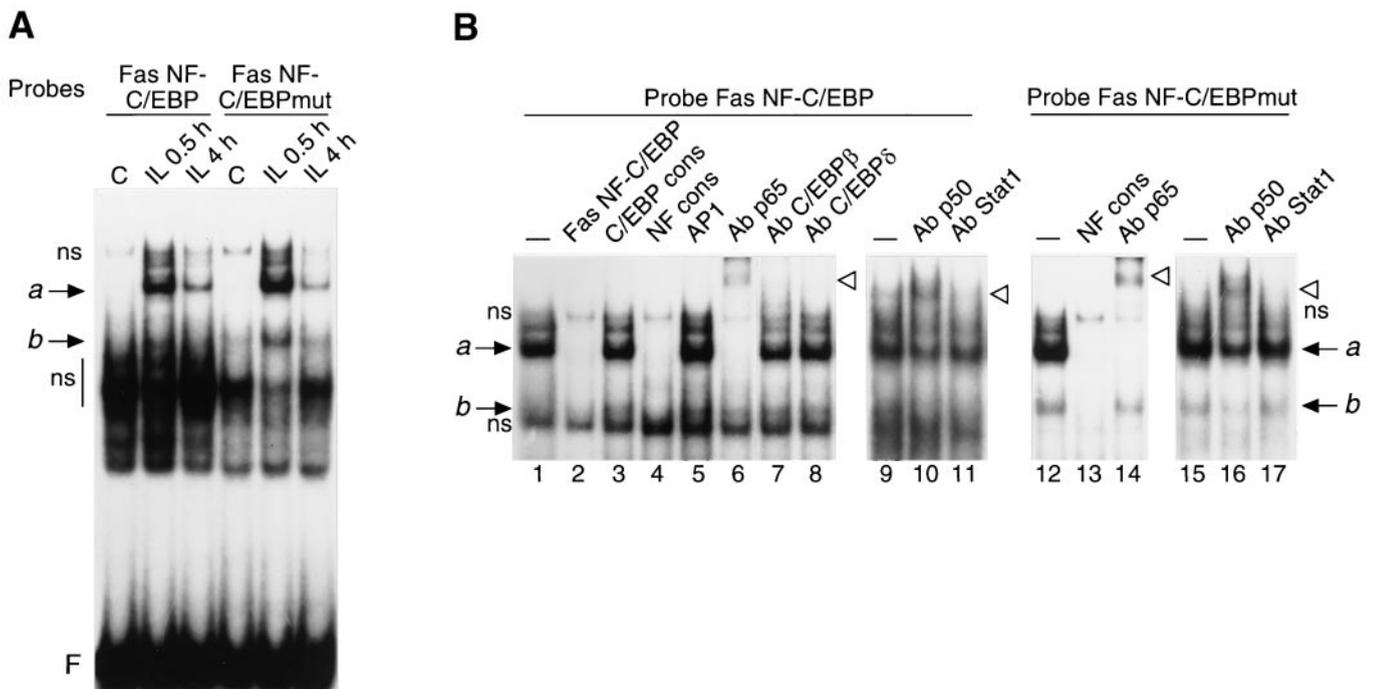


FIG. 4. NF-κB binding to the Fas promoter. **A:** EMSAs were performed in electrophoresis buffer Tris-borate with an oligonucleotide spanning from nucleotides -148 to -116 and containing the NF-κB and C/EBP sites (probe Fas NF-C/EBP) or with the C/EBP site mutated (probe Fas NF-C/EBPmut), and with nuclear extracts from RINm5F cells left untreated (control [C]) or treated with IL-1β for 30 min or 4 h. Arrows *a* and *b* indicate specific complexes; ns, nonspecific complexes; F, free probe. **B:** EMSAs were performed with the same probes as in A and with nuclear extracts from RINm5F cells treated with IL-1β for 30 min and with competing oligonucleotides (lanes 2–5 and 13) or specific antibodies (lanes 6–8, 10, 11, 14, 16, and 17) added to the reaction as indicated above the lanes. Arrowheads indicate supershifted complexes. The figures are representative of three similar experiments.

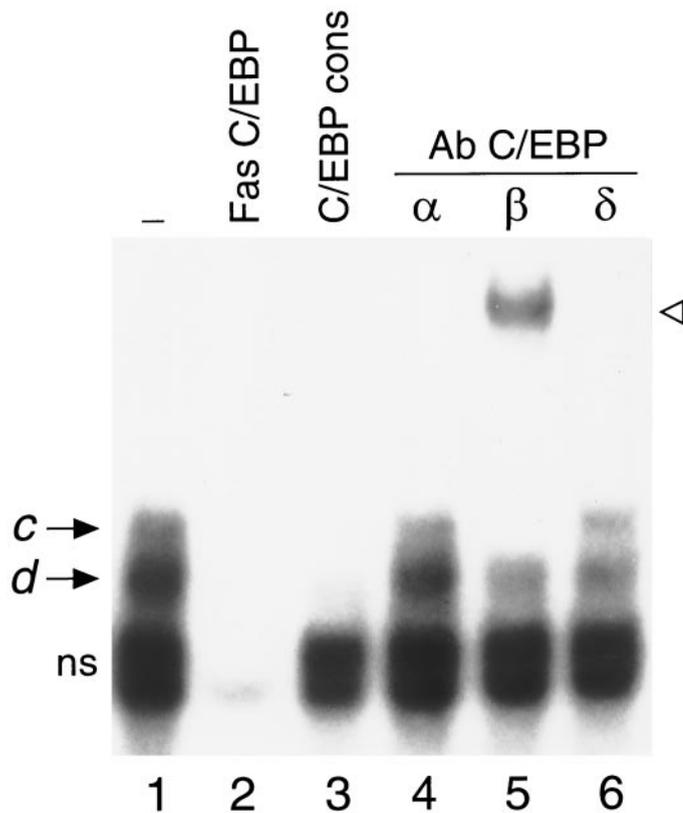


FIG. 5. C/EBP binding to the Fas promoter. EMSAs were performed in electrophoresis buffer Tris-glycine with an oligonucleotide spanning from nucleotides -134 to -116 and containing the C/EBP site (Fas C/EBP), using nuclear extracts from RINm5F cells treated with IL- 1β for 2 h. Competing oligonucleotides (lanes 2 and 3) or specific antibodies (lanes 4–6) were added to the reaction as indicated above the lanes. Arrows *c* and *d* indicate specific complexes; ns, nonspecific complexes; \triangle , supershifted complexes. The figure is representative of two similar experiments.

against C/EBP family members, C/EBP β and $-\delta$, had no effect (lanes 7 and 8), indicating that C/EBP did not bind the probe and that NF- κ B/C/EBP heteromers did not interact with the probe under these experimental conditions. Similarly, no complex could be detected when a probe spanning the C/EBP site from nucleotides -134 to -116 (probe Fas C/EBP) was used (data not shown). This was unexpected because mutation of the C/EBP site abolished IL- 1β induction of the promoter activity (Fig. 3B), suggesting that this site is functional. To further clarify this issue, we performed EMSAs, changing the electrophoresis buffer Tris-borate to a buffer of higher ionic strength, Tris-glycine (44). Under these conditions, however, two complexes (*c* and *d*) were similarly formed with the probe Fas C/EBP in untreated cells and in cells treated with IL- 1β for 30 min up to 8 h (Fig. 5 and data not shown). These two complexes were specifically competed by the unlabeled probe (Fig. 5, lane 2) and by a consensus C/EBP oligonucleotide (lane 3). Antibodies directed against C/EBP family members were used in supershift experiments to further characterize the complexes. An antibody directed against C/EBP α had no effect (lane 4), whereas an antibody directed against C/EBP β altered the mobility of both complexes (lane 5), and an antibody directed against C/EBP δ disrupted complex *d* (lane 6). This suggests the presence of C/EBP β in complex *c* and

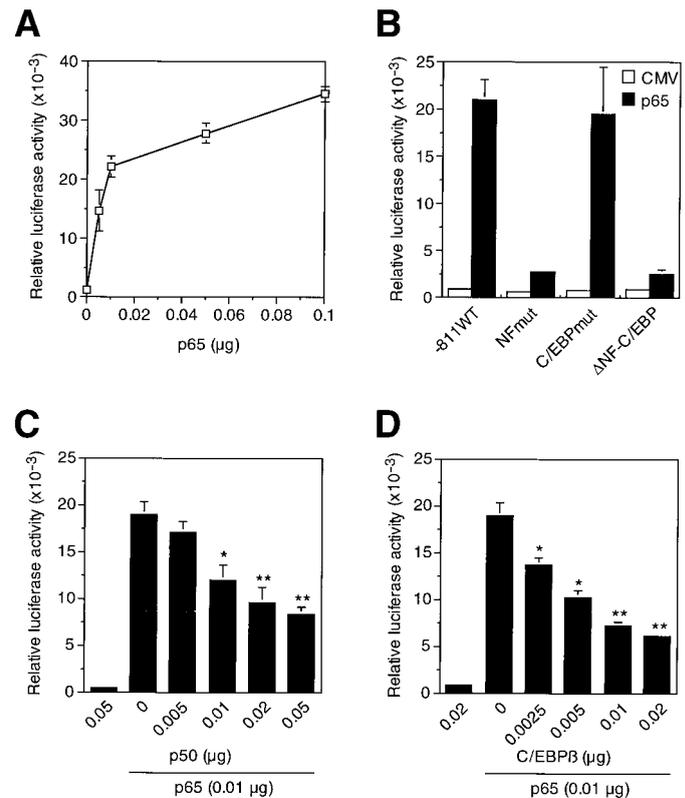


FIG. 6. Effect of overexpression of NF- κ B p65, NF- κ B p50, and C/EBP on Fas promoter activity. **A:** RINm5F cells were cotransfected with the wild-type pFas-811 luc and 0.005–0.1 μ g of the expression vector CMV-p65. The amount of DNA transfected was kept constant by adding the control vector CMV. Relative luciferase activity values are the means \pm SE of four independent experiments. **B:** The wild-type (WT) pFas-811 luc or the same construct with deletion of the NF- κ B and C/EBP sites indicated in Fig. 2A (Δ NF-C/EBP), or with either the NF- κ B site mutated (NFmut) or the C/EBP site mutated (C/EBPmut), were cotransfected with 0.01 μ g of CMV-p65 or the control plasmid CMV. Relative luciferase activity values are the means \pm SE of three independent experiments. **C** and **D:** The wild-type pFas-811 luc was cotransfected with 0.01 μ g of CMV-p65 and/or increasing amounts of CMV-p50 from 0.005–0.05 μ g (in **C**) or increasing amounts of CMV-C/EBP β from 0.0025 to 0.02 μ g (in **D**). Relative luciferase activity values are the means \pm SE of four independent experiments. * P < 0.05, ** P < 0.01 vs. p65 alone (paired *t* test).

the presence of C/EBP β and $-\delta$ in complex *d*. These experimental conditions, however, were not adequate for NF- κ B detection either with the Fas NF-C/EBP probe or a NF- κ B consensus probe (data not shown).

Regulation of the Fas promoter by NF- κ B p65, NF- κ B p50, and C/EBP β . To evaluate whether NF- κ B and C/EBP factors are able to transactivate the Fas promoter, we performed cotransfection experiments with Fas promoter constructs and expression vectors for NF- κ B p65, NF- κ B p50, and C/EBP β . First, the ability of p65 to activate the wild-type pFas-811 luc construct and its mutants was examined. NF- κ B p65 transactivated the Fas promoter in a dose-dependent manner (Fig. 6A). At the suboptimal dose (0.01 μ g), p65 induced the activity of the promoter construct with the C/EBP site mutated (C/EBPmut) similarly as the wild-type construct (Fig. 6B). The activity of the promoter constructs with either the NF- κ B site mutated (NFmut) or the NF- κ B and C/EBP sites deleted (Δ NF-C/EBP) was marginally induced compared with the wild-type construct (Fig. 6B), indicating that p65 induction is specific and that the NF- κ B site at position -142 is responsi-

ble for induction of the Fas promoter activity by NF- κ B. To determine the effect of overexpression of p65 together with p50 on Fas promoter activity, the two expression vectors were cotransfected, keeping p65 constant and increasing the amount of p50. Overexpression of p50 alone had no effect on the wild-type promoter activity (Fig. 6C). However, when cotransfected with p65, it inhibited the p65-induced promoter activity in a dose-dependent manner (Fig. 6C).

Overexpression of C/EBP β transfected at doses up to 0.02 μ g had no effect on the activity of pFas-811 luc (Fig. 6D). At higher doses (up to 0.1 μ g), C/EBP β induced the luciferase activity in a nonspecific way since the activity of the promoterless vector pGL3 was also increased (data not shown). When cotransfected at increasing amounts (up to 0.02 μ g) with a constant amount of p65, C/EBP β inhibited the p65-induced promoter activity in a dose-dependent manner (Fig. 6D). Western blot analysis for p65, p50, and C/EBP β showed efficient nuclear expression of the different proteins (Fig. 7). Moreover, the expression of p65 was not affected by the expression of p50 or C/EBP β (Fig. 7).

DISCUSSION

The Fas pathway may contribute to β -cell death in type 1 diabetes. Fas expression is induced by cytokines on β -cells and renders them susceptible to apoptosis mediated by FasL expressed on islet-infiltrating T-cells and macrophages (13,15,16,25). The mechanism or mechanisms by which Fas gene expression is induced by cytokines in β -cells or in other cell types remain to be investigated.

In the present study, we show that Fas mRNA expression is induced by IL-1 β alone in rat β -cells, whereas IFN- γ has no effect. We identified the transcription factors NF- κ B and C/EBP as key regulators of rat Fas promoter activity. First, mutation of either the NF- κ B binding site located at -142 bp or the C/EBP binding site located at -130 bp abolished IL-1 β induction of Fas promoter activity. Second, we detected by EMSAs and supershift experiments the binding of NF- κ B p65 and p50 factors as well as C/EBP β and δ factors to their respective sites. Third, in cotransfection experiments, NF- κ B p65 transactivated the Fas promoter. Coexpression of NF- κ B p50 or C/EBP β with p65 resulted in a dose-dependent inhibition of the p65-induced Fas promoter activity.

Interactions between the members of the NF- κ B family have been characterized in detail (45). Thus, p65/p50 heterodimers, the most common dimers, and p65 homodimers are activators of gene transcription, whereas p50 homodimers, which lack a transactivation domain, are transcriptionally inactive (46). Our cotransfection experiments with p65 and p50 did not show synergistic activation by the two factors but resulted instead in inhibition of p65-driven Fas promoter activity. This suggests that p65 homodimers, and not p65/p50 heterodimers, are the main activators of Fas gene transcription. Similar observations were made for the regulation of monocyte chemoattractant protein-1 promoter in mesangial cells (47) and fibroblasts (48), and intercellular adhesion molecule-1 (ICAM-1) promoter in astrocytes (49). p50 Homodimers may be formed at higher p50:p65 ratios and function as repressor of Fas promoter activity by interfering with p65 binding. In

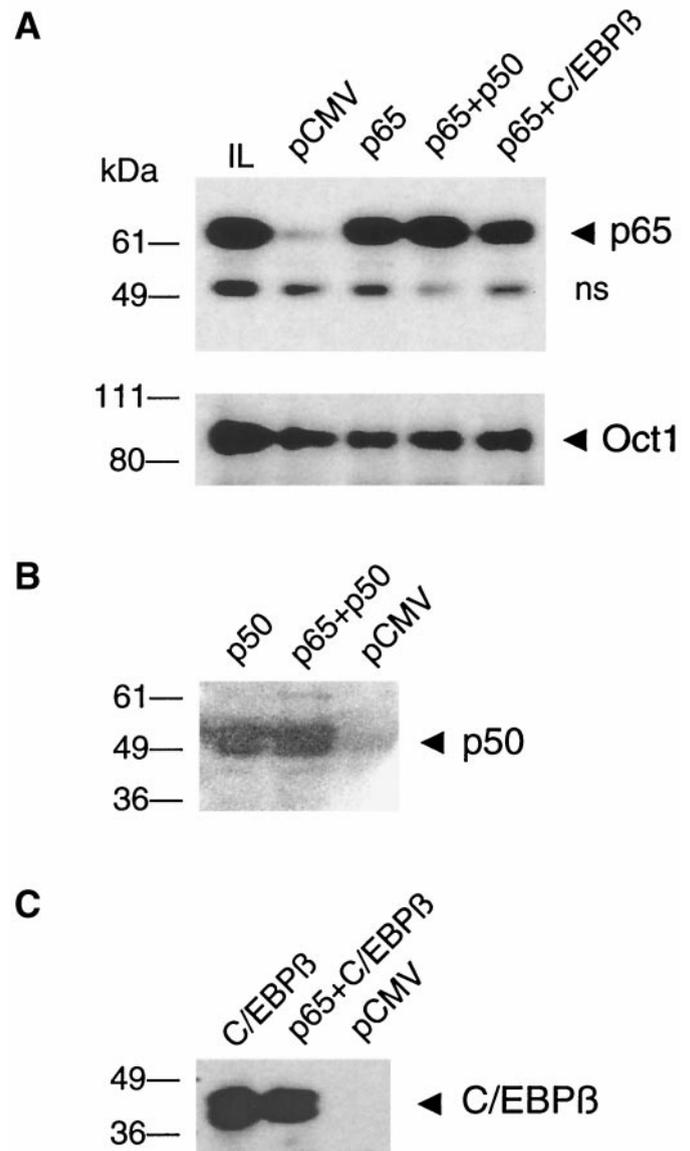


FIG. 7. Transient overexpression of NF- κ B p65, NF- κ B p50, and C/EBP β in RINm5F cells. Cells were transfected with the expression vectors for p65, p50, or C/EBP β alone, or for p65 in combination with p50 or C/EBP β at a one-to-two ratio, or with the empty vector pCMV, as indicated above the lanes. Nuclear proteins were fractionated by SDS-PAGE and immunoblotted with antibodies specific for p65 or Oct-1 (used as control for protein loading) in A; p50 in B; and C/EBP β in C. In A, untransfected cells exposed to IL-1 β for 30 min (IL) were used as a positive control. Arrowheads indicate the expressed proteins. ns, A nonspecific band. The figure is representative of two similar experiments.

line with this possibility, we observed by EMSAs that p50 homodimers may bind to the NF- κ B site of the promoter (Fig. 4).

Cooperative binding and physical association between NF- κ B and C/EBP proteins have been described as mechanisms for synergistic transactivation of proinflammatory (40–43) and viral genes (50). This was not observed in the present experiments. Both factors bound independently to their respective sites, and no NF- κ B-C/EBP heteromers were detected in EMSAs. This could, however, be due to the different experimental conditions required to detect NF- κ B and C/EBP binding. There was no synergistic activation of the Fas promoter when NF- κ B p65 and

C/EBP β were coexpressed in transfection studies. Instead, overexpression of C/EBP β had an inhibitory effect on p65-induced promoter activity. These findings are similar to those observed for the transcriptional regulation of ICAM-1 in astrocytes (49) and of TNF- α in macrophages A(31). The transactivation capacity of C/EBP β is enhanced by phosphorylation of its activation domain (51). Thus, it cannot be excluded that IL-1 β induces posttranslational modification of C/EBP β , preventing the presently observed inhibitory effect of C/EBP β on Fas promoter activity. Furthermore, cofactors may be required for functional synergism between NF- κ B and C/EBP on the Fas promoter. For example, CREB-binding protein/p300 are required for maximal transcriptional activity of E-selectin promoter by NF- κ B and ATF-2/c-Jun (52) and IL-6 promoter by NF- κ B, C/EBP, CREB, and AP-1 (53). High-mobility group I(Y) proteins are required for proper regulation of the IL-2R α gene by NF- κ B and E1f-1 (54) and of the IFN- β gene by NF- κ B, ATF-2/c-Jun, and IRF (55).

In conclusion, we have demonstrated for the first time that NF- κ B plays a critical role in the regulation by cytokines of the rat Fas promoter in insulin-producing cells. This novel information provides the rationale to target NF- κ B activation, by genetic intervention, in future attempts to prevent β -cell destruction in animal models of type 1 diabetes and allograft rejection.

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REFERENCES

- Kurrer MO, Pakala SV, Hanson HL, Katz JD: β cell apoptosis in T cell-mediated autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:213–218, 1997
- O'Brien BA, Harmon BV, Cameron DP, Allan DJ: Apoptosis is the mode of β -cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. *Diabetes* 46:750–757, 1997
- Mauricio D, Mandrup-Poulsen T: Apoptosis and the pathogenesis of IDDM: a question of life and death. *Diabetes* 47:1357–1363, 1998
- Rothe H, Faust A, Schade U, Kleeman R, Bosse G, Hibino T, Martin S, Kolb H: Cyclophosphamide treatment of female non-obese diabetic mice causes enhanced expression of inducible nitric oxide synthase and interferon- γ , but not interleukin-4. *Diabetologia* 37:1154–1158, 1994
- Rabinovitch A, Suarez-Pinzon WL, Sorenson O, Bleackley RC: Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: identification of iNOS-expressing cells and relationships to cytokines expressed in the islets. *Endocrinology* 137:2093–2099, 1996
- Eizirik DL, Pavlovic D: Is there a role for nitric oxide in β -cell dysfunction and damage in IDDM? *Diabetes Metab Rev* 13:293–308, 1997
- Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL: Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138:2610–2614, 1997
- Hoorens A: *Distinction Between Necrosis and Apoptosis of Pancreatic Beta Cells*. PhD thesis. Brussels, Belgium, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, 1999
- Liu D, Pavlovic D, Flodström M, Sandler S, Eizirik DL: Cytokines induce apoptosis in β -cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS $^{-/-}$). *Diabetes* 49:1116–1122, 2000
- Mandrup-Poulsen T: The role of interleukin-1 in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetologia* 39:1005–1029, 1996
- Rabinovitch A: An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetes Metab Rev* 14:129–151, 1998
- Eizirik DL, Darville MI: β -cell apoptosis and defense mechanisms: lessons from type 1 diabetes mellitus. *Diabetes* 50 (Suppl. 1):S64–S69, 2001
- Yamada K, Takane-Gyotoku N, Yuan X, Ichikawa F, Inada C, Nonaka K: Mouse islet cell lysis mediated by IL-1-induced Fas. *Diabetologia* 39:1306–1312, 1996
- Stassi G, Maria RD, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C, Trucco M: Nitric oxide primes pancreatic beta cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J Exp Med* 186:1193–1200, 1997
- Zumsteg U, Frigerio S, Holländer GA: Nitric oxide production and Fas surface expression mediate two independent pathways of cytokine-induced murine β -cell damage. *Diabetes* 49:39–47, 2000
- Amrani A, Verdaguer J, Thiessen S, Bou S, Santamaria P: IL-1 α , IL-1 β , and IFN- γ mark β -cells for Fas-dependent destruction by diabetogenic CD4 $^{+}$ T lymphocytes. *J Clin Invest* 105:459–468, 2000
- Nagata S, Golstein P: The Fas death factor. *Science* 267:1449–1456, 1995
- Nagata S: Apoptosis by death factor. *Cell* 88:355–365, 1997
- Suarez-Pinzon W, Sorenson O, Bleackley JF, Elliot JF, Rajotte RV, Rabinovitch A: β -cell destruction in NOD mice correlates with Fas (CD95) expression on β -cells and proinflammatory cytokine expression in islets. *Diabetes* 48:21–28, 1999
- Allison J, Strasser A: Mechanisms of β cell death in diabetes: a minor role for CD95. *Proc Natl Acad Sci U S A* 95:13818–13822, 1998
- Kim S, Kim KA, Hwang DY, Lee TH, Kayagaki N, Yagita H, Lee MS: Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J Immunol* 164:2931–2936, 2000
- Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, Matis LA, Wang Y: Significant role for Fas in the pathogenesis of autoimmune diabetes. *J Immunol* 164: 2523–2532, 2000
- Suarez-Pinzon WL, Power RF, Rabinovitch A: Fas ligand-mediated mechanisms are involved in autoimmune destruction of islet beta cells in non-obese diabetic mice. *Diabetologia* 43:1149–1156, 2000
- Thomas HE, Darwiche R, Corbett JA, Kay TW: Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *J Immunol* 163:1562–1569, 1999
- Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, Iwahashi H, Nakajima H, Namba M, Nagata S, Hanafusa T, Matsuzawa Y: Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset type I diabetes mellitus. *Diabetologia* 42:1332–1340, 1999
- Xu X, Fu XY, Plate J, Chong ASF: IFN- γ induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 58:2832–2837, 1998
- Ouaz F, Li M, Beg AA: A critical role for the RelA subunit of nuclear factor κ B in regulation of multiple immune-response genes and in Fas-induced cell death. *J Exp Med* 189:999–1004, 1999
- Kimura K, Asami K, Yamamoto M: Structure of the promoter for the rat Fas antigen gene. *Biochim Biophys Acta* 1352:238–242, 1997
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: *Current Protocols in Molecular Biology*. New York, Wiley & Sons, 1994, p. 2.2.1–2.2.3
- Coolidge CJ, Patton JG: Run-around PCR: a novel way to create duplications using polymerase chain reaction. *BioTechniques* 18:763–764, 1995
- Liu H, Sidiropoulos P, Guobin S, Pagliari LJ, Birrer MJ, Stein B, Anrather J, Pope RM: TNF- α gene expression in macrophages: regulation by NF- κ B is independent of c-jun or C/EBP β . *J Immunol* 164:4277–4285, 2000
- Pipeleers DG, in't Veld PA, Van De Winckel M, Maes E, Schuit FC, Gepts W: A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806–816, 1985
- Ling Z, Hannaert JC, Pipeleers D: Effect of nutrients, hormones and serum on survival of rat islet β cells in culture. *Diabetologia* 37:15–20, 1994
- Schuit FC, Pipeleers DG: Regulation of adenosine 3', 5'-monophosphate levels in the pancreatic β cell. *Endocrinology* 117:834–840, 1985
- Darville MI, Eizirik DL: Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* 41:1101–1108, 1998
- Bedoya FJ, Flodström M, Eizirik DL: Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide synthase mRNA, but not superoxide dismutase mRNA, in insulin-producing cells. *Biochem Biophys Res Commun* 210: 816–822, 1995
- Flodström M, Eizirik DL: Interferon- γ -induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology* 138:2747–2753, 1997
- Darville MI, Ho Y-S, Eizirik DL: NF- κ B is required for cytokine-induced

- manganese superoxide dismutase expression in insulin-producing cells. *Endocrinology* 141:153–162, 2000
39. Schreiber E, Matthias P, Müller MM, Schaffner W: Rapid detection of octamer binding proteins with “mini-extracts,” prepared from a small number of cells. *Nucl Acids Res* 17:6419, 1989
 40. Stein B, Baldwin AS: Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF- κ B. *Mol Cell Biol* 13:7191–7198, 1993
 41. Catron KM, Brickwood JR, Shang C, Li Y, Shannon MF, Parks TP: Cooperative binding and synergistic activation by RelA and C/EBP β on the intercellular adhesion molecule-1 promoter. *Cell Growth Differ* 9:949–959, 1998
 42. Stein B, Cogswell PC, Baldwin AS: Functional and physical associations between NF- κ B and C/EBP family members: a Rel domain-bZIP interaction. *Mol Cell Biol* 13:3964–3974, 1993
 43. Ray A, Hannink M, Ray BK: Concerted participation of NF- κ B and C/EBP heteromer in lipopolysaccharide induction of serum amyloid A gene expression in liver. *J Biol Chem* 270:7365–7374, 1995
 44. Plevy SE, Gemberling JHM, Hsu S, Dorner AJ, Smale ST: Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol* 17:4572–4588, 1997
 45. Baldwin AS: The NF- κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 14:649–686, 1996
 46. Ballard DW, Dixon EP, Peffer NJ, Bogerd H, Doerre S, Stein B, Greene WC: The 65-kDa subunit of human NF- κ B functions as a potent transcriptional activator and a target for ν -Rel-mediated repression. *Proc Natl Acad Sci U S A* 89:1875–1879, 1992
 47. Stylianou E, Nie M, Ueda A, Zhao L: c-Rel and p65 trans-activate the monocyte chemoattractant protein-1 gene in interleukin-1 stimulated mesangial cells. *Kidney Int* 56:873–882, 1999
 48. Ping D, Boekhoudt GH, Rogers EM, Boss JM: Nuclear factor- κ B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. *J Immunol* 162:727–734, 1999
 49. Lee SJ, Hou J, Benveniste EN: Transcriptional regulation of intercellular adhesion molecule-1 in astrocytes involves NF- κ B and C/EBP isoforms. *J Neuroimmunol* 92:196–207, 1998
 50. Ruocco MR, Chen X, Ambrosino C, Dragonetti E, Liu W, Mallardo M, De Falco G, Palmieri C, Franzoso G, Quinto I, Venuta S, Scala G: Regulation of HIV-1 long terminal repeats by interaction of C/EBP (NF-IL6) and NF- κ B/Rel transcription factors. *J Biol Chem* 271:22479–22486, 1996
 51. Trautwein C, Caelles C, van der Geer P, Hunter T, Karin M, Chojkier M: Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* 364:544–547, 1993
 52. Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T: CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci U S A* 94:2927–2932, 1997
 53. Vanden Berghe W, De Bosscher K, Boone E, Plaisance S, Haegeman G: The nuclear factor- κ B engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *J Biol Chem* 274:32091–32098, 1999
 54. John S, Reeves RB, Lin JX, Child R, Leiden JM, Thompson CB, Leonard WJ: Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. *Mol Cell Biol* 15:1786–1796, 1995
 55. Yie J, Merika M, Munshi N, Chen G, Thanos D: The role of HMG I(Y) in the assembly and function of the IFN- β enhanceosome. *EMBO J* 18:3074–3089, 1999