Pancreatic β-Cell-specific Repression of Insulin Gene Transcription by CCAAT/Enhancer-binding Protein β

INHIBITORY INTERACTIONS WITH BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR E47*

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Chronic exposure of β-cells to supraphysiologic glucose concentrations results in decreased insulin gene transcription. Here we identify the basic leucine zipper transcription factor, CCAAT/Enhancer-binding protein β (C/EBPβ), as a repressor of insulin gene transcription in conditions of supraphysiological glucose levels. C/EBPβ is expressed in primary rat islets. Moreover, after exposure to high glucose concentrations the β-cell lines HIT-T15 and INS-1 express increased levels of C/EBPβ. The rat insulin I gene promoter contains a consensus binding motif for C/EBPβ (CEB box) that binds C/EBPβ. In non-β-cells C/EBPβ stimulates the activity of the rat insulin I gene promoter through the CEB box. Paradoxically, in β-cells C/EBPβ inhibits transcription, directed by the promoter of the rat insulin I gene by direct protein-protein interaction with a heptad leucine repeat sequence within activation domain 2 of the basic helix-loop-helix transcription factor E47. This interaction leads to the inhibition of both dimerization and DNA binding of E47 to the E-elements of the insulin promoter, thereby reducing functionally the transactivation potential of E47 on insulin gene transcription. We suggest that the induction of C/EBPβ in pancreatic β-cells by chronically elevated glucose levels may contribute to the impaired insulin secretion in severe type II diabetes mellitus.

Insulin is a hormone essential for the control of mammalian glucose homeostasis and is produced predominantly in pancreatic β-cells of adult animals (1). The expression of the insulin gene occurs to a large extent at the level of transcription. Control elements residing in the 5’ 350-base pair sequence flanking exon 1 of the rat insulin I gene are sufficient to direct β-cell-specific expression (2) (Fig. 1A). Arrays of A and E elements1 (Far-FLAT, Nir-P1) constitute symmetrical enhancers that cooperatively account for >90% of the transcriptional activity of the insulin gene promoter (3). The E elements are recognition motifs for transcription factors in the basic helix-loop-helix (bHLH)2 family, such as E12 and E47, which activate the insulin promoter in close synergism with A element binding homeobox transcription factors, such as IDX-1.

Chronic hyperglycemia may contribute to the pancreatic β-cell dysfunction observed in patients with type II diabetes, a phenomenon attributed to the concept of glucose toxicity (4). Studies using in vivo animal models and in vitro β-cell lines have demonstrated that a reduction of insulin gene transcription by glucose toxicity is associated with the loss of transactivator proteins such as IDX-1/IPF-1/STF-1 and RIPA3h1-binding protein (5–10). Because insulin gene transcription is both positively and negatively regulated, we sought to identify repressors that might also mediate the effects of glucose toxicity on insulin gene transcription. In this report we describe CCAAT/Enhancer binding protein-β (C/EBPβ) as a glucose-induced repressor of insulin gene transcription.

C/EBPs are a family of transcription factors that regulate genes of the acute phase response, cell growth, differentiation, and the expression of cell type-specific genes (11–16). The C/EBPs consist of the activators C/EBP α, β, γ, δ, and ε and the repressors CHOP, LIP, and C/EBP-30; the latter two repressors arise by alternative downstream translation of the mRNAs (17). The C/EBPs bind to DNA exclusively as dimers and contain a conserved C-terminal basic region-leucine zipper domain that is characterized by a DNA-contacting basic region linked to a leucine zipper dimerization motif (18). They bind preferentially to a consensus DNA sequence T(T/G)NNGNAA(T/G) (19, 20). The founding member of the family of C/EBP transcription factors, C/EBPα, is expressed during terminal differentiation of cells such as adipocytes (13) and keratinocytes. C/EBPβ is abundant in liver, is expressed in response to stress-activated signaling pathways, and activates the expression of genes involved in the acute phase response such as cytokine genes. It has been shown that the expression of C/EBPβ transactivates the transcription of genes encoding the insulin receptor and glucose transporter-2 (21, 22), suggesting that C/EBPβ may play an important role in glucose homeostasis and the metabolic stress associated with diabetes mellitus. The promoters of both the rat insulin I and II gene, as well as the human insulin gene, contain sequence elements that closely resemble the insulin gene promoter.

1 Throughout this report, the revised nomenclature of the cis-acting elements of the insulin promoters (65) is used, and the former indications are given in parentheses.

2 The abbreviations used are: bHLH, basic helix-loop-helix; IDX-1, islet-duodenum-homeobox factor; RIPE, rat insulin II promoter element; BETA-2, β-cell E-box transactivator 2; C/EBPs and -β, CCAAT/Enhancer-binding protein α and β, respectively; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; bp, base pair(s); CRE, cAMP response element; CREB, CRE-binding protein; AD1 and AD2, activation domains 1 and 2, respectively.
Repression of the Insulin Gene by C/EBPβ

EXPERIMENTAL PROCEDURES

Reagents—DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim; radioactive compounds were from NEN Life Science Products; β-luciferin-potassium was from the Analytical Luminescence Laboratory (San Diego, CA); RPMI 1640 and DMEM medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Nucleotides were obtained from Pharmacia Biotech Inc. All other reagents were purchased from Sigma.

Cell Culture—The pancreatic β-cell line HIT-T15 (27) at passage 64 and COS-7 cells were purchased from the ATCC. Ins-1 (28) cells at passage 99 were a gift from Dr. Clae B. Wellleim (University of Geneva, Switzerland). HIT-T15 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) with 10% FBS at 37 °C in a 5% CO2, 95% air atmosphere as described (7). Ins-1 cells were grown in RPMI 1640 medium with 10% FBS, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, and 10 mM HEPES as reported (28). Both cell lines were passaged weekly. For the model of long-term exposure, HIT cells were cultured from passage 64 to passage 80 in 11.1 mM glucose or 5.6 mM medium (Life Technologies, Inc.) with 10% FBS at 37 °C in a 5% CO2, 95% air atmosphere.

DNA-Protein Binding Assays—Electrophoretic mobility shift assays (EMSAs) were performed as described (34), using bacterially expressed C/EBPβ or nuclear extracts prepared from HIT-T15, βTC-6, and Ins-1 cells (37). The oligonucleotides used for EMSA are as follows: CEB oligonucleotide, 5'-GATCTGAGGGGCTAAGTGTATTTCAAACTTGGCTTAGTGTAGTG-3' (antisense strand); CEB mutated oligonucleotide, 5'-GATCTGAGGGGCTAAGTGTATTTCAAACTTGGCTTAGTGTAGTG-3'; consensus CAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTC-3'; CRE oligonucleotide, 5'-GATCTTAGTGTAGTGACGTCTATAGGCG-3'; FLAT oligonucleotide, 5'-GATCCTCATCAGGGCTATGCGCCCTTTGTTAATAATCTAATTACCCTAGGTC-3'; CRE-mut oligonucleotide, 5'-GATCTCAGGGCTAAGTGTATTTCAAACTTGGCTTAGTGTAGTG-3'.

Gottschalk et al.

Male Sprague-Dawley rats (150–200 g) were anesthetized with 100 mg/kg intraperitoneal pentobarbital sodium. Islets were isolated from the pancreas using an adaptation for rat islets of the method of Gotthel et al. (32). Briefly, after cannulation of the common bile duct and instillation of 10 ml of a prewarmed (37°C) solution containing 1 mg/ml Collagenase P and 0.5 mg/ml DNase I, the pancreas was removed and digested for 30 min at 37°C in a shaking water bath followed by dilution and washing of the digest and hand picking of the released islets under a dissecting microscope. Liver nuclei were prepared by the method of Gorski et al. (33).

Antiserum and Western Immunoblot—Polyclonal rabbit antiserum for C/EBPβ and E47 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The IDX-1 antiserum was described previously (34). The IDX-1 antisera was described previously (34). Western immunoblot analysis was performed on nuclear extracts prepared from the cell lines according to standard techniques (35). Extracts of pancreatic islet whole cells and liver nuclei were prepared by lysing isolated rat islets and liver nuclei in SDS-PAGE sample buffer (36). In each lane, a sample containing 100 μg of protein was loaded.

Purification of Proteins Expressed in Bacteria—A C/EBPβ protein fragment containing the basic region-leucine zipper binding domain that is present at the carboxyl terminal methionine residue of the BamHI-XhoI site of the pRSET-A vector (Invitrogen Inc., Carlsbad, CA). The protein was transcribed and translated (32) and purified with a nickel-chelating resin column and eluted by a pH gradient.

DNA-Protein Binding Assays—Electrophoretic mobility shift assays (EMSAs) were performed as described (34), using bacterially expressed C/EBPβ or nuclear extracts prepared from HIT-T15, βTC-6, and Ins-1

3 J. Ferrer, unpublished results.
Repression of the Insulin Gene by C/EBPβ

RESULTS

Potential C/EBPβ-binding Sites in the Promoters of the Rat and Human Insulin Genes—Inspection of the promoters of the rat insulin I, rat insulin II, and human insulin genes reveals nucleotide sequence elements that resemble the consensus motif that binds the C/EBP family of transcription factors (Fig. 1, A and B).

Expression of C/EBPβ in Rat Islets and Cultured β-Cell Lines and Regulation by High Glucose Levels—Isolated rat pancreatic islets and several β- and non-β-cell lines were assayed for C/EBPβ expression by Western immunoblot using liver nuclei extracts as a positive control, since C/EBPβ was originally defined as the liver-enriched activator protein, LAP (14). The 32-kDa C/EBPβ protein was detected in isolated rat islet whole cell extracts (Fig. 2A), although the antisera also recognized a more abundant protein with an apparent molecular mass of 42 kDa. C/EBPβ was also detected in the nuclear extracts from Ins-1, βTC-6, and HIT-T15 cells, which are islet β-cell lines derived from rat, mouse, and hamster, respectively.

To determine whether the expression of C/EBPβ is altered in β-cells during chronic or short term exposure to supraphysiologic concentrations of glucose, we used standard in vitro glucose desensitization models (6, 30). HIT-T15 cells were serially passaged in RPMI 1640 medium containing 11.1 mM glucose or 0.8 mM glucose for 16 weeks. Since the EC50 for glucose-stimulated insulin secretion is left-shifted to 1 mM in HIT-T15 cells rather than about 8 mM in normal islets (50), 11.1 mM glucose was chosen as a supraphysiologic concentration, and 0.8 mM was considered a physiological concentration of glucose for the HIT-T15 cells. As shown in Fig. 2B, the expression of IDX-1 decreased from week 4 to 16, confirming the published observations (9). In contrast, the expression of C/EBPβ was markedly enhanced from week 8 to 16. These observations indicate that the level of C/EBPβ in HIT-T15 cells is up-regulated by prolonged exposure to high glucose concentrations and that C/EBPβ might serve as a repressor of insulin gene transcription. The increased expression of C/EBPβ in HIT-T15 cells after chronic exposure to high glucose was prevented by culturing HIT-T15 cells in the RPMI 1640 medium containing 0.8 mM glucose (Fig. 2B). To validate the in vitro long term model and to show an effect of high glucose concentrations on the regulation of insulin secretion, we measured the insulin concentration in the culture medium in response to increasing glucose concentrations in HIT-T15 cells at different passages in 2-h static incubation intervals. Whereas HIT-T15 cells cultured in high glucose displayed a passage-dependent decrease in glucose-responsive insulin secretion (50 ± 7.7% after 16 weeks in 11.1 mM glucose), no such decrease was seen in cells cultured in low glucose (data not shown). The findings of long term high glucose exposure on C/EBPβ expression were confirmed using Ins-1 cells, which respond to glucose similarly to isolated islets. C/EBPβ expression was enhanced in Ins-1 cells cultured at 25 mM but not 5.6 mM glucose, whereas IDX-1 levels were decreased in Ins-1 cells cultured at 25 but not at 5.6 mM glucose (data not shown).

To more precisely test the regulation of C/EBPβ, we sought to examine the role of supraphysiologic glucose concentrations in a short term model, which has recently been used to examine IDX-1 expression in response to high glucose concentrations (30). Exposure of Ins-1 cells to 25 mM glucose for 72 h and then reversing the high glucose concentration back to normal resulted in a marked up-regulation of C/EBPβ after 24 h. The up-regulation of C/EBPβ was reversible by a subsequent 24-h period in 5.6 mM glucose (Fig. 2C). As a control, the expression of IDX-1 was examined to confirm the down-regulation of IDX-1 protein in this system described previously. We also measured the insulin content of the cells to validate the effect of high glucose on this parameter. A decrease in insulin content during the 72-h high glucose period was observed (from 98 ± 4.3 milliunits/mg protein before, to 37.4 ± 6.1 milliunits/mg protein after 72 h in 25 mM glucose), which was partially reversible by the subsequent culture period of 24 h in low glucose (59.4 ± 6.2 milliunits/mg protein) (data not shown).

Bacterially Expressed C/EBPβ Binds to Insulin Promoter Sequences—Mapping of the C/EBPβ-binding sites within the
Repression of the Insulin Gene by C/EBPβ

rat insulin I promoter was carried out using DNase I protection footprinting assays on a 280-base pair fragment of the 5’-flanking region of the rat insulin I gene (nucleotides −280 to +1) labeled on the coding strand. Incubation of this labeled fragment of DNA with bacterially expressed C/EBPβ resulted in three regions of DNase I protection and additional hypersensitive sites, indicating that C/EBPβ interacts with specific sequence regions in the promoter (Fig. 3A). Counting from the transcription initiation site (+1), the first protected region from nucleotide −70 to −86 contains the previously characterized A1 (P1) element (between nucleotides −64 and −85). The second region, from nucleotide −107 to −121, corresponds to the E1 (Nir) box (between nucleotides −104 and −112). DNase I hypersensitive sites flank both the A1 (P1) and E1 (Nir) elements. The third region, from nucleotide −126 to −147, however, contains the CEB box and has not been described previously. Notably, the bacterially expressed C/EBPβ also gave a hypersensitive digestion pattern at the boundaries of the A3/4 (FLAT) element (between nucleotides −207 and −227), suggesting that C/EBPβ may distort the DNA in this region without completely protecting the FLAT element from DNase I digestion. These data confirm that C/EBPβ binds to the CEB box and suggest that the recombinant protein also binds to the A1 (P1) element and the E1 (Nir) box and may interact with the A3/4 (FLAT) element. C/EBPβ does not appear to bind to the rat insulin I gene cAMP-response element (CRE, −184TGACGTCAT−174), although the rat insulin I gene promoter CRE contains a nearly canonical TGACGTTC core sequence for binding of C/EBPβs and cAMP-response element-binding protein (CREB).

To examine whether the bacterially expressed C/EBPβ binds to the insulin promoter sequence element (CEB box), predicted to contain the consensus binding site (Fig. 1A), EMSAs with oligonucleotides corresponding to this region were performed (Fig. 3B). C/EBPβ forms DNA-protein complexes with a 51-bp oligonucleotide probe comprising the CEB box of the rat insulin I promoter. Binding of C/EBPβ to this element was also demonstrated by using the corresponding sequences of the rat insulin II and using the human insulin gene promoters as probes (data not shown). These data indicate that the bacterially expressed C/EBPβ binds to the CEB boxes within the promoters of these three insulin genes as predicted by sequence comparison (Fig. 1B).

To assess the relative affinity of C/EBPβ interaction with the A1 (P1) element, the E1 (Nir) box, the CEB box, and the A3/4 (FLAT) element, EMSA was performed using the CEB probe in the presence or absence of 30- or 300-fold excesses of the unlabeled oligonucleotides containing the CEB box, mutated CEB box, P1 element, Nir box, or FLAT element. The oligonucleotides containing the Nir box, the P1 element, and the FLAT element competed with the CEB probe for binding to the bacterially expressed C/EBPβ, but with at least 10-fold less efficiency than the unlabeled oligonucleotide containing the CEB box (Fig. 3B). These data suggest that C/EBPβ interacts with the P1, Nir, and FLAT elements with a relatively lower efficiency compared with the CEB box.

Endogenously Expressed C/EBPβ in HIT-T15 Cells Binds to the CEB Box—EMSAs were used to characterize the DNA-binding properties of endogenously expressed C/EBPβ in HIT-T15 cells (Fig. 3C). The DNA probe was the CEB consensus oligonucleotide. Binding reactions were performed with or without the addition of C/EBPβ antisem or preimmune serum. One of the slowest migrating complexes for the CEB box probe (lane 2) was disrupted by the addition of C/EBPβ antisem (lane 4) but not by the preimmune serum (lane 3). Moreover, the C/EBPβ antisem resulted in the appearance of

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**Fig. 2. Expression of C/EBPβ in rat islets and β-cell lines and regulation by glucose.** Western immunoblot analysis of C/EBPβ and IDX-1 in pancreatic islet whole cell extracts (WCE) and β-cell nuclear extracts (NE). A, expression in three β-cell lines (HIT-T15, βTC-6, Ins-1), rat liver nuclei, and rat pancreatic islets. B, HIT-T15 cells were cultured for up to 16 weeks in high (11.1 mM) or low (0.8 mM) glucose. C, Ins-1 cells, cultured in 5.6 mM glucose, were exposed to 25 mM glucose for up to 72 h. In the culture shown in the right lane (72/24 rev.), the concentration of glucose was lowered to 5.6 mM for 24 h after the 72-h incubation in 25 mM glucose. The bar graphs in B and C represent means ± S.E. (n = 3).
a slower migrating “supershifted” complex (lane 4). These findings imply that the supershifted band in the presence of C/EBPα antiserum represents the complex of C/EBPα and CEB box probe and possibly additional proteins.

The binding specificities of the protein complex that binds to the probe containing the CEB box were examined by EMSA under conditions of competition with unlabeled oligonucleotides containing the wild type and mutated CEB box (Fig. 3C). The addition to the binding reaction of a 30- or 300-fold unlabeled wild type oligonucleotide containing the CEB box resulted in the abolishment of the C/EBPα complex (Fig. 3C, lanes 7 and 8). In contrast, the unlabeled oligonucleotide containing the mutated CEB box (from 5'-ageTGTAAAT-3' to 5'-ageCTGCCG-3') was a much weaker competitor for the binding to the labeled CEB box probe (Fig. 3C, lanes 10 and 11). These observations suggest that the endogenously expressed C/EBPα in HIT-T15 cells binds specifically to the CEB box.

**Transactivation of the Rat Insulin I Gene Promoter by C/EBPα in Non-β-cells—**Because HeLa and BHK-21 cells lack certain β-cell-specific transcription factors required for the expression of the insulin gene, such as IDX-1 and BETA-2 (51, 52), they have been widely used to assess the transactivation of the insulin gene by recombinant proteins. The effect of C/EBPα on the rat insulin I gene promoter was examined by cotransfection experiments using HeLa and BHK-21 cells with a C/EBPα expression vector (pcDNA I) and a reporter construct containing portions of the rat insulin I gene 5'-flanking region in the plasmid pXP2 (−2410INS-LUC). The empty expression vector for C/EBPα, pcDNA I, had no significant effect on 2410INS-LUC expression. In addition, C/EBPα did not affect the truncated thymidine kinase promoter of herpes simplex virus (−81 to +52 bp, pTKS1-LUC) that lacks a C/EBPα-binding site. To examine whether the transactivation of −2410INS-LUC by C/EBPα was mediated through interactions with the CEB box, nucleotide...
substitution mutations were introduced into this site in the rat insulin I promoter from $^{125}$agcTGTAAT to $^{133}$agcCT-GCCG (CEB mutation). The same mutations of CEB that were introduced into the oligonucleotide probes of the gel shift assays depicted in Fig. 3A resulted in a marked decrease in the binding affinity of C/EBPb. Mutation of the CEB box significantly reduced the transactivation by C/EBPb on the rat insulin I gene promoter (from 22- to 2-fold), suggesting that C/EBPb transactivates the rat insulin I gene promoter mainly through the newly identified CEB box in non-ß-cells (Fig. 4A). Other gene promoters containing the C/EBPb-binding sites, such as an angiotensinogen promoter (APRE-LUC), glucagon promoter (−350Glu-LUC), rat IDX-1 promoter (−190IDX-LUC), human ß-cell sulfonylurea receptor promoter (−180SUR-LUC), rat insulin II promoter CAT reporter (−410INS-II-CAT), and 5’-deletion mutant luciferase reporter constructs of the rat insulin I promoter from −410 to −90 are shown.

Repression of the Rat Insulin I Gene Promoter by C/EBPb in ß-Cells—To examine whether C/EBPb also transactivates the
Repression of the Insulin Gene by C/EBPβ

28355

rat insulin I gene promoter in β-cells, the C/EBPβ expression vector and −410INS-LUC were co-transfected into HIT-T15 cells. Surprisingly, C/EBPβ markedly inhibited the rat insulin I gene promoter activity without affecting the 81 bp of the thymidine kinase promoter (pTKS1-LUC) (Fig. 4C). In contrast, both the APRE-LUC and glucagon promoter (−350Gluc-LUC) were activated by C/EBPβ in HIT-T15 cells, with 23.8 ± 2.1- and 2.3 ± 0.1-fold stimulation, and the promoters of IDX-1 and SUR were stimulated 5.8 ± 0.2 and 2.1 ± 0.3-fold, respectively (Fig. 4D). These findings indicate that the C/EBPβ-mediated repression of the rat insulin I gene promoter activity in HIT-T15 cells is unique to the rat insulin I gene inasmuch as C/EBPβ activates the glucagon, APRE, IDX-1, and SUR promoters in HIT-T15 cells. C/EBPs also inhibited the rat insulin I gene promoter activity in HIT-T15 cells (data not shown). The rat insulin II gene promoter (−410INS-II-CAT), which also contains a putative C/EBPβ-binding site (Fig. 1B), was also inhibited by C/EBPβ (Fig. 4E). In contrast to the transactivation of the rat insulin I gene promoter in the non-β HeLa and BHK cells, mutation of the CEB box within the 410-bp rat insulin I gene promoter did not affect the inhibition of the mutated rat insulin I gene promoter activity by C/EBPβ in HIT-T15 cells (Fig. 4C, CEB-Mutation). These observations suggest that C/EBPβ inhibits the rat insulin I gene promoter through cis-elements or their corresponding transcription factors other than the CEB box and its binding proteins. Similar results were obtained from co-transfection experiments using βTC-6 and Ins-1 cells (data not shown).

Localization of the DNA Sequences Important for C/EBPβ Repression of Rat Insulin I Gene Promoter Activity in β-Cells—The DNA sequences within the rat insulin I 5′-flanking region essential for the C/EBPβ-mediated repression of promoter activity was investigated by introducing a series of 5′-deletions into a rat insulin I promoter-LUC fusion gene (Fig. 4F). Deletion of the DNA sequence between bp −410 and −282 did not affect the C/EBPβ-mediated inhibition of the rat insulin I gene promoter activity in HIT-T15 cells, indicating that this region is not important for the C/EBPβ action. Subsequent deletions suggest that there are two regions critical for the negative regulation of the rat insulin I gene promoter activity by C/EBPβ. The removal of the sequence between −282 and −190 that contains the E2 (Far) box and the A3/4 (FLAT) element resulted in a decreased basal promoter activity but also in a significantly decreased repression by C/EBPβ. The involvement of the sequence proximal to bp −120, which contains the E1 (Nir) box and the A1 (P1) element, in C/EBPβ-mediated repression of the rat insulin I gene promoter activity was also suggested, although not unequivocally established, because basal promoter activity dropped to background levels upon deletion of the E1 (Nir) element. More important, however, the deletion of the promoter region between −190 and −120, which contains the CEB box, did not completely abolish C/EBPβ-mediated inhibition of the rat insulin I gene promoter, suggesting that this element is not exclusively mediating the C/EBPβ effects on this promoter in pancreatic β-cells. However, deletion to −90, which eliminates the E1 (Nir) element eliminates inhibition by C/EBPβ. The results of these experiments further more suggest that the rat insulin I gene promoter contains multiple negative regulatory DNA elements or their corresponding DNA-binding protein factors as targets for the C/EBPβ-mediated repression in β-cells. The co-transfection of the deletion constructs and the C/EBPβ expression vector into βTC-6 cells gave similar results (data not shown).

Interaction of C/EBPβ with Basic Helix-Loop-Helix Transcription Factors—Because deletion or mutation of the CEB box from an insulin-promoter-reporter plasmid did not abolish repression of reporter gene activity by C/EBPβ in β-cells (but did abolish activation in non-β-cells), we reasoned that the molecular mechanism for the inhibition of insulin promoter activity in β-cells may be different from direct binding to the CEB box. One possibility is an inhibitory interaction of C/EBPβ with activating transcription factors of the insulin gene. One family of transcription factors that has been shown to transactivate the insulin gene promoter consists of bHLH proteins, such as E12/E47 and BETA-2. We examined by co-immunoprecipitation experiments whether C/EBPβ would interact with the bHLH factor E47. Indeed, endogenous E47 immunoreactivity, co-migrating with E47-protein expressed in COS cells (Fig. 5A, lane 2), was readily co-immunoprecipitated from nuclear extracts of the β-cell line Ins-1 using an antiserum directed against C/EBPβ (Fig. 5A, lane 4), providing evidence for a direct protein-protein interaction of these two transcription factors. This molecular interaction was further confirmed in a β-cell-independent cell system by co-immunoprecipitation of E47 with C/EBPβ antiserum from extracts of COS-7 cells, transfected with expression plasmids for C/EBPβ and E47 and labeled in vivo with [35S]methionine for visualization of the immunocomplexes by autoradiography (Fig. 5B, upper panel). No specific immune complexes were precipitated from cells, transfected with empty vectors (Mock, lanes 1 and 2). After transfection with E47 expression plasmid alone, the expressed E47 protein could be immunoprecipitated with the E47 antiserum (lane 4), but not with the C/EBPβ antiserum (lane 3). Only when both C/EBPβ and E47 were expressed in COS-7 cells, co-immunoprecipitation of C/EBPβ and E47 proteins was accomplished by C/EBPβ antiserum (lane 5). Of note, C/EBPβ could not be co-immunoprecipitated with the antiserum directed to E47 (Fig. 5B, lane 6, and Fig. 5A, lane 3), a finding we attribute to a possible masking effect of the antigenic epitope in E47 upon interaction with C/EBPβ. The specificity of the C/EBPβ antiserum is demonstrated by immunoprecipitating only C/EBPβ protein from extracts of COS-7 cells, transfected with the C/EBPβ expression vector only (Fig. 5B, lanes 7 and 8). The identity of the bands in the autoradiography was confirmed by Western immunoblotting with specific antisera for C/EBPβ and E47 (Fig. 5B, lower panels).

Interference of C/EBPβ with the Leucine Zipper Domain of E47—The bHLH proteins of the E2A family, E12 and E47, contain two structurally and functionally distinct transactivation domains, AD1 and AD2 (55). In contrast to AD1, which is equally active in all cells, the AD2 transcriptional activation domain functions preferentially in pancreatic β-cells. Analysis of the amino acid sequence of E47 reveals a heptad leucine repeat within AD2, which is conserved in the E47 proteins of different animal species. Therefore, we reasoned that an interaction of the leucine zipper transactivation factor C/EBPβ with the leucine repeat within AD2 of E47 could underlie the demonstrated physical interaction of both proteins. To further characterize the molecular basis of this interaction, we introduced two point mutations into the leucine repeat domain of E47, changing the first two leucines to phenylalanes (Fig. 5C) and yielding the protein E47-LZ−. In vitro translated and labeled proteins of C/EBPβ, E47, and E47-LZ− were subjected to immuno precipitation, and immune complexes were visualized by autoradiography after fractionation by SDS-PAGE (Fig. 5D, top). Aliquots of the reticulocyte lysates before immunoprecipitation were included as controls in the electrophoresis (Fig. 5D, lanes 1–3). In reactions containing only single proteins, both wild type and E47-LZ− proteins were immunoprecipitated by the E47 antiserum (lanes 4 and 5), and in vitro translated C/EBPβ protein was precipitated by C/EBPβ antiserum (lane 6). When mixed together, E47 antiserum precipitated again...
only wild type E47 (lane 7) or mutated E47 (lane 9) alone. Both wild type E47 and C/EBPβ, however, were co-immunoprecipitated by the C/EBPβ antiserum (lane 8). In contrast, only C/EBPβ alone was precipitated by the C/EBPβ antiserum when used with the mutated E47-LZ2 (lane 10). These findings provide evidence for the notion that C/EBPβ may interact with E47 via the leucine repeat within AD2. This protein-protein interaction was also confirmed by an antiserum-independent assay, namely the GST pull down procedure. Labeled and in vitro translated wild type E47 specifically bound to GST-C/EBPβ (Fig. 5E, lane 4). In contrast, the E47-LZ2 mutant protein showed no interaction with the GST-C/EBPβ fusion protein (lane 5). GST-C/EBPβ was able to precipitate both wild type E47 and C/EBPβ (lane 7) but not mutant E47-LZ2 together with C/EBPβ (lane 8). C/EBPβ was included in the reactions in lanes 6–8 to ensure that the GST-C/EBPβ was functional (that it dimerized with C/EBPβ).

Inhibition of E47 Binding Activity by Interaction with C/EBPβ—To determine whether interactions of C/EBPβ with E47 affect the binding activity of the E47 homodimer to the E-box DNA response elements within the insulin promoter, we tested the binding of in vitro translated proteins in electrophoretic mobility shift assays on the E1 elements Nir and RIPE3 of the rat insulin I and II promoters, respectively (Fig. 6, A and B). On both elements, the specific complex containing the E47 homodimer (lanes 1), as determined by competition
with an excess of cold probe (lanes 2) and antiserum supershift analysis (lanes 3), is either diminished (Nir) or displaced (RIPE3) by the addition of C/EBPb (lanes 4). In contrast, the E47-LZ2 mutant protein, although capable of forming a binding complex on the DNA elements (lanes 5), is resistant to interference by C/EBPb (lanes 8). This observation further supports the involvement of the leucine repeat sequence within activation domain 2 of E47 in the interaction with C/EBPb.

**Functional Inhibition of the Transcriptional Activation Potential of E47 by C/EBPb**—The question whether the protein-protein interaction between C/EBPb and E47 would also lead to a functional inhibition of the transactivation potential of E47 was assessed by transient transfection into HIT-T15 cells of expression plasmids, encoding fusion proteins of the yeast Gal4-DNA binding domain with either AD1 or AD2 of E47, together with a luciferase reporter construct containing a multimerized Gal4-binding site (GBS) linked to 59 bp of the angiotensinogen gene promoter (Fig. 7). The Gal4 constructs containing AD1 and AD2 were equally active in transactivating the luciferase reporter gene in HeLa cells (not shown), whereas the AD2 construct (Gal4-DBD:E47-(329–436)) was significantly more active than the AD1 construct (Gal4-DBD:E47-(1–99)) in HIT-T15 cells. Cotransfection of an expression plasmid for C/EBPb repressed the activity of Gal4-DBD:E47-(329–436) (AD2) on the luciferase reporter gene, leaving the transactivation potential of Gal4-DBD:E47-(1–99) (AD1) unaffected (Fig. 7). These findings provide evidence for a functional inhibitory interaction of C/EBPb with the AD2 domain of E47 leading to a reduced transactivation potential of E47.

**DISCUSSION**

Chronic hyperglycemia in patients with type II diabetes mellitus may contribute to defective glucose-induced insulin secretion, a phenomenon that has been attributed to glucose toxicity (4). After culture in high glucose concentrations for 7 days, human islets contain markedly reduced insulin content, a change that can be partially reversed by subsequent culture in lower glucose concentrations (56). Using immortalized β-cell lines, it was found that chronic exposure to supraphysiologic glucose concentrations is associated with decreased insulin gene transcription and decreased expression of the insulin gene transactivators IDX-1 and RIPE3b1-binding proteins (5–9). It was recently reported, however, that the chronic glucotoxic alterations of insulin gene expression in the pancreatic β-cell line HIT-T15 are only partially reversible upon subsequent lowering of the high glucose levels, although the expression of IDX-1 and RIPE3b1 factors was readily restored (57). These findings imply that an additional inhibitory factor, which reg-
ulates insulin gene transcription under these conditions, may be involved. In this study, we have identified C/EBPβ as a transrepressor of insulin gene transcription, which is up-regulated by supraphysiological glucose levels in pancreatic β-cell lines. A high affinity binding site for C/EBPβ in the rat insulin I gene promoter, the CEB box, and several relatively lower affinity sites, namely the A1(P1), the E1(Nir) box, and the A3/4(FLAT) elements, were identified. DNase I footprint analysis using recombinant C/EBPβ indicates that C/EBPβ binds to the CEB box, the Nir box, and the P1 element and may interact with the FLAT element. DNA-protein binding assays using short oligonucleotides indicated that the CEB box is the high affinity binding site, whereas the other sites interact with C/EBPβ with at least a 10-fold lower affinity compared with the CEB box. Although C/EBPβ is capable of binding to the CREs of the phosphoenolpyruvate carboxykinase and somatostatin genes (58, 59), it did not interact with the rat insulin I gene CRE as indicated by both DNase I footprint analysis and EMSA. C/EBPα was shown not to bind to the glucagon CRE (53, 58), suggesting that the CRE alone is not sufficient for the binding of C/EBP proteins, and the flanking sequences may play a critical role for the converged binding of C/EBP proteins and C/EBPα.

Previous work has demonstrated that the E2(Far) box and the A3/4(FLAT) element, and their counterparts located proximal to the transcription initiation site, termed the E1(Nir) box and the A1(P1) element, are the most important cis-acting DNA elements required for rat insulin I gene expression. The E2A gene products, E12, E47, and/or E2–5, bind to the Far and Nir boxes and activate the rat insulin I gene promoter synergistically with the homeodomain proteins IDX-1(IPF-1/STF-1/PDX-1), HNF-1α, and Lmx-1 (52, 54, 60), which bind to the FLAT and P1 elements. Although C/EBPα activates the rat insulin I gene promoter in non-β-cells through binding to the newly identified CEB box, this interaction does not mediate the repression of the rat insulin I promoter by C/EBPβ in β-cells. Our studies indicate that C/EBPβ inhibits rat insulin I gene transcription through physical and functional interaction with the basic helix-loop-helix protein, E47.

The bHLH protein family of transcription factors is divided into three classes according to their DNA-binding properties, structural features, and tissue distribution (61). Factors of the E2A family (E12, E47, E2–5) are ubiquitously expressed members of class A. Class A factors of the E2A family are exemplified by the major β-cell nuclear binding complex (insulin enhancer factor, IEF) of the rat insulin I and human insulin gene promoters (62). Furthermore, the class B bHLH factor BETA-2 is expressed specifically in pancreatic β- and α-cells and is reported to heterodimerize with E2A proteins on the RIPE3 element of the rat insulin II promoter, an interaction that is believed to contribute to the tissue specific expression of the insulin II gene (51). Importantly, more than 90% of the overall activity of the rat insulin I promoter activity is attributable to the synergistic transactivation by E2A proteins and homeobox factors (IDX-1, Lmx-1) (3). Thus, it is conceivable that molecular interference of C/EBPβ with E47 disrupts not only the homo- or heterodimerization of the bHLH factors themselves but also their synergistic transactivation with homeodomain proteins (Fig. 8).

Two transactivation domains (AD1 and AD2) have been identified in E47. The AD2 subdomain contains a heptad leucine repeat sequence. Mutation of this “leucine zipper” altered the transcriptional activity of E47. Interestingly, AD1 functions in a wide variety of tissues and cells, whereas the expression of AD2 activity is largely restricted to pancreatic β-cells, supporting a potentially important role for the AD2 domain in regulating gene transcription in β-cells. In addition to the ability of the AD2 domain of E47 to contribute to transactivation, we uncovered evidence that the leucine repeat serves as a domain for a direct protein-protein interaction with C/EBPβ, mutations of two of the leucines abrogated this interaction. Furthermore, C/EBPβ inhibited binding of the E47 homodimer to E-box-containing elements within the rat insulin I and II promoters. Whether this is due to the formation of a classical leucine zipper dimerization between C/EBPβ and E47 has not been unequivocally established by our studies. A report showing an inhibition of insulin gene transcription by the leucine zipper transcription factor c-Jun via targeting of the AD2 of E47 in β-cells (48, 63) parallels our findings in part. c-Jun functionally inhibited the transactivation potential of the AD2 of E47, but in contrast to our observations with C/EBPβ, c-Jun did not appear to physically interact with the AD2 of E47. Therefore, it is conceivable that leucine zipper transcription factors of different families may interact by different mechanisms with the AD2 of E47.

Recently, it has been suggested that E2A factors are not required for insulin gene transcription, based on the observation that mice with a targeted disruption of the E2A gene do not appear to have abnormalities in the morphology of the endocrine pancreas and do not develop overt diabetes (64). Because the basal and stimulated, as well as the tissue-specific, expression of the insulin gene is regulated in a complex manner, however, the absolute contribution of different bHLH proteins to insulin promoter activity within different animal species is largely unknown. E2A gene products represent only one subfamily of bHLH factors, and other ubiquitously expressed members could substitute for E2A in the mouse gene knockout model. This notion is further supported by the observation that BETA-2 knockout mice do develop diabetes (66), a circumstance that may be attributable to the tissue-specific action of this bHLH transcription factor in mice, and the E47/BETA-2 heterodimer may also be a target for C/EBPβ-mediated repression of insulin II gene activity. Furthermore, it remains to be determined whether or not E47/BETA-2 heterodimers also bind to and activate E-box-containing elements within the rat insulin I promoter, a finding that would further support the
Repression of the Insulin Gene by C/EBPβ

28359

importance of C/EBPβ as a glucose-induced repressor of insulin gene transcription.

In conclusion, C/EBPβ may serve as a transcription factor mediating the dysregulation of insulin gene expression under conditions of sustained supraphysiological glucose concentrations. In fact, we have extended our studies toward examination of the expression of C/EBPβ in pancreatic β-cells in animal models of diabetes mellitus, with preliminary results implying an involvement of this factor in the pathophysiology of glucotoxic alterations during the development and progression of this disease.5

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