

Impaired Cyclic AMP-Dependent Phosphorylation Renders CREB a Repressor of C/EBP-Induced Transcription of the Somatostatin Gene in an Insulinoma Cell Line

MARIO VALLEJO,* MARILYN E. GOSSE, WILLARD BECKMAN, AND JOEL F. HABENER

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114

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Transcription factor CREB regulates cyclic AMP (cAMP)-dependent gene expression by binding to and activating transcription from cAMP response elements (CREs) in the promoters of target genes. The transcriptional transactivation functions of CREB are activated by its phosphorylation by cAMP-dependent protein kinase A (PKA). In studies of many different phenotypically distinct cells, the CRE of the somatostatin gene promoter is a prototype of a highly cAMP-responsive element regulated by CREB. We now report on a somatostatin-producing rat insulinoma cell line, RIN-1027-B2, in which transcription from the somatostatin gene promoter is paradoxically repressed by CREB. We find that CREB fails to transactivate a CRE-containing somatostatin-chloramphenicol acetyltransferase reporter even when coexpressed with the catalytic subunit of PKA. CAAT box/enhancer-binding protein β (C/EBP β) and C/EBP-related activating transcription factor bind to the CRE in the promoter of the somatostatin gene and transactivate transcription. CREB binds competitively with C/EBP β to the somatostatin CRE in vitro and represses C/EBP β -induced transcription of the CRE-containing somatostatin-chloramphenicol acetyltransferase reporter. The lack of CREB-mediated transcriptional stimulation is due to the presence of a heat-stable inhibitor of PKA that prevents activation of PKA and subsequent CREB phosphorylation in the nucleus. These findings indicate that dephosphorylated CREB is a negative regulator of C/EBP-activated transcription of the somatostatin gene promoter in RIN-1027-B2 cells.

Adaptive changes in the functional state of cells in response to various stimuli in the environment involve the activation of cellular second messenger pathways that cause changes in the transcription rates of target genes. Many extracellular signals are transmitted to the nucleus by pathways that selectively activate protein kinases and the resultant phosphorylation of transcription factors that in turn regulates the expression of specific genes (18). The cyclic AMP (cAMP)-dependent signal transduction pathway mediates transcriptional responses of many genes. The catalytic subunits of protein kinase A (PKA) released from the cytoplasmic inhibitory regulatory subunits by the actions of cAMP translocate to the nucleus, where they phosphorylate transcription factors such as CREB at specific serine residues, resulting in the activation of gene transcription (18, 27, 53).

CREB binds specifically to cAMP response elements (CREs) typified by the consensus palindromic sequence TGACGTC, present in the promoters of many genes, including the gene encoding the polypeptide hormone somatostatin, in which transcription rates are strongly regulated by cAMP (10, 20, 28, 35, 51). In particular, the CRE in the promoter of the somatostatin gene is highly responsive to cAMP stimulation and as such has been used extensively as a prototype CRE for the study of cAMP-dependent mechanisms of transcriptional regulation (9, 13, 19, 20, 28). These studies, however, have been carried out with heterologous cells that do not express the endogenous somatostatin gene, using transcription reporter

plasmids consisting of the somatostatin CRE driving heterologous promoters.

The expression of the somatostatin gene is restricted to neurons, thyroid C cells, D cells of the digestive tract, and D cells of the pancreatic islets of Langerhans (38). In the somatostatin-producing insulinoma cell line RIN-1027-B2 (B2 cells) (34), the somatostatin gene is regulated by several cell-specific elements that exert positive or negative control on gene transcription (36, 54). Earlier studies using B2 cells and various other cell lines and transfection assays clearly demonstrate a pivotal role of the CRE in the regulation of cell-specific expression of the somatostatin gene (2, 22, 36, 54). Despite the demonstration of an essential role for the CRE of the somatostatin gene in mediating cAMP-dependent stimulation of gene transcription in different cell types, cAMP does not stimulate transcription in B2 cells (32, 36, 54).

To analyze this apparent paradox, we examined the cellular mechanisms that might be responsible for the observed lack of response to cAMP. We find that CAAT box/enhancer-binding protein (C/EBP)-like CRE-binding proteins potently activate transcription of the somatostatin gene. CREB, one of the nuclear proteins bound by the CRE in B2 cells, is not phosphorylated by PKA because of the presence of a heat-stable kinase inhibitor. As a consequence, dephosphorylated CREB (dephospho-CREB) represses transcription of the somatostatin gene by competing with C/EBP proteins for binding to the CRE. We identify C/EBP-related activating transcription factor (C/ATF) (56) and C/EBP β (7, 60) as two of the major nuclear proteins that activate transcription of the somatostatin gene in a cAMP-independent manner.

* Corresponding author. Present address: Reproductive Endocrine Unit, BHX-5, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 726-5384. Fax: (617) 726-5357.

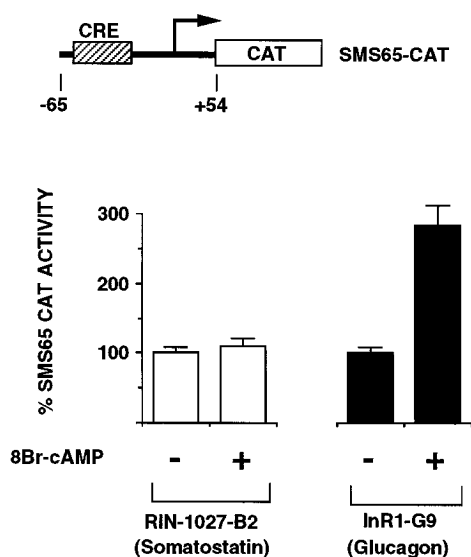


FIG. 1. Relative CAT activities obtained following transient transfections of somatostatin gene 5' deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 cells or glucagon-producing InR1-G9 cells cultured in the absence (-) or presence (+) of 1 mM 8-Br-cAMP for 12 h prior to harvesting. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT in the absence of 8-Br-cAMP stimulation. A schematic representation of the SMS65-CAT reporter plasmid is depicted at the top of the figure.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Radioactive compounds were obtained from Du Pont-New England Nuclear (Boston, Mass.). Nucleotides were purchased from Pharmacia-LKB (Piscataway, N.J.). Tissue culture media and reagents were obtained from Gibco-BRL (Grand Island, N.Y.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell lines. Rat islet somatostatin-producing B2 (34), hamster islet glucagon-producing InR1-G9 (49), mouse NIH 3T3 (ATCC CRL1658), and human choriocarcinoma JEG-3 (ATCC HTB36) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Antisera. CREB, C/EBP β , C/ATF, and PKA $_c$ (catalytic subunit of PKA) antisera are described elsewhere (15, 39, 56, 58). C/EBP α antiserum was raised by immunizing rabbits with a protein consisting of glutathione S-transferase (GST) fused to the amino-terminal portion of C/EBP α , excluding the basic region-leucine zipper (bZip) domain. C/EBP δ antiserum was a gift from S. L. McKnight (Tularik, Inc). Affinity-purified phosphorylated CREB (phospho-CREB) antibody was a gift from M. E. Greenberg (Harvard Medical School). Western immunoblots were carried out with a chemiluminescent detection system (ECL; Amersham), using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

Transfections and CAT assays. Islet cells were transfected by a modified DEAE-dextran procedure (54). JEG-3 cells were transfected by the calcium phosphate precipitation method (14). Chloramphenicol acetyltransferase (CAT) activity was measured by a solution assay (45) 48 h after transfection. All values are expressed as the means \pm standard errors of the means of at least three independent experiments carried out in duplicate.

GAL-CREB consists of rat CREB amino acids 1 to 261 fused to the carboxy terminus of GAL4 (amino acids 1 to 147). For construction of the plasmid encoding GAL-CREB, a PCR fragment with *Bam*HI and *Sac*I linkers was generated with specific primers that anneal to the corresponding sequences of a CREB cDNA template and ligated into the expression plasmid pSG424 (42) in frame with the sequence encoding GAL4 (amino acids 1 to 147), which corresponds to the DNA-binding domain of GAL4. The plasmid encoding GAL-C/EBP β was constructed in a similar way and contains the entire coding region of a C/EBP β cDNA. All other plasmids used in transfection studies have been described elsewhere.

DNA-protein binding assays. Electrophoretic mobility shift assays (EMSA) were carried out with nuclear extracts (44) in the presence of the protease inhibitors pepstatin A (1 mg/ml), leupeptin (10 mg/ml), aprotinin (10 mg/ml), and *p*-aminobenzamide (0.1 mM). Protein concentrations were determined by the Bio-Rad protein assay with bovine serum albumin as a standard. Synthetic complementary oligonucleotides with 5' GATC overhangs were annealed and labeled by a fill-in reaction, using [α - 32 P]dATP and Klenow enzyme. Binding

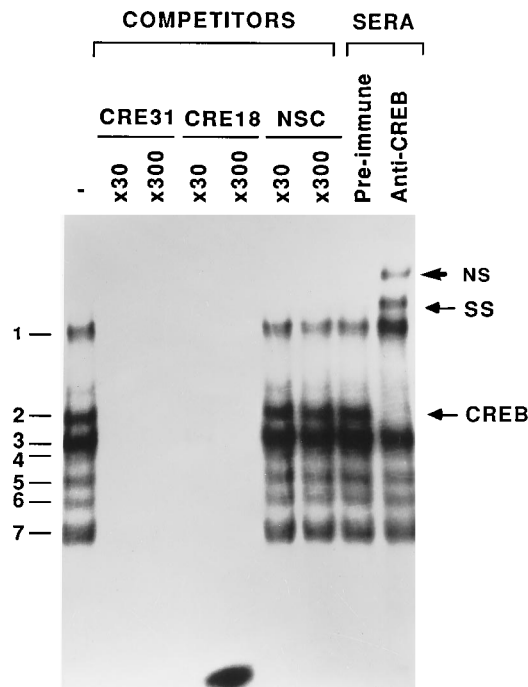
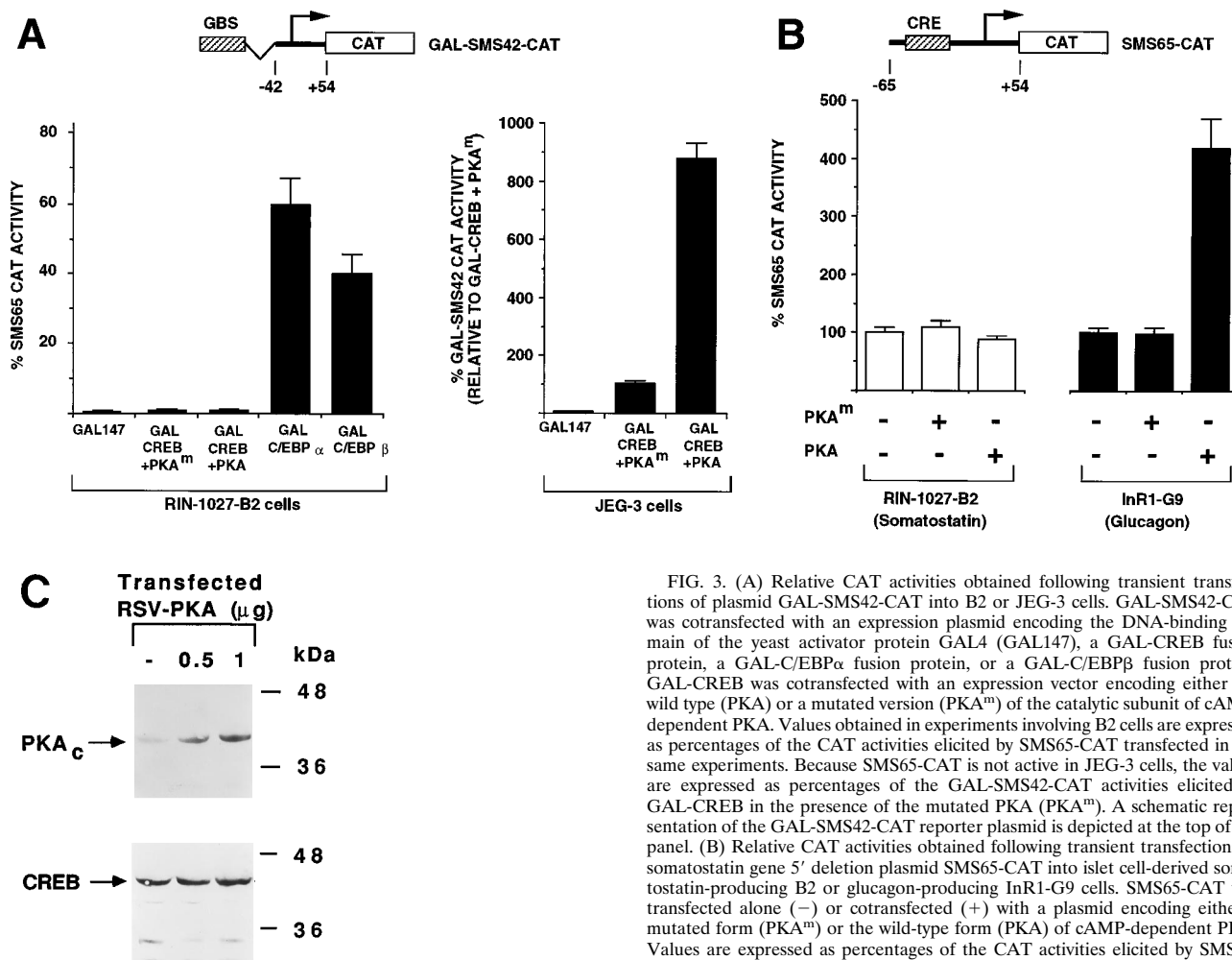


FIG. 2. EMSA of nuclear proteins from B2 cells bound to the somatostatin gene CRE. Competitions were with a 30- ($\times 30$) or 300-fold ($\times 300$) molar excess of unlabeled full-length somatostatin CRE oligonucleotide (CRE31), a truncated version spanning the core CRE and 5 flanking nucleotides at either side (CRE18), or a nonspecific competitor (NSC) oligonucleotide of unrelated sequence. A control (no competitor) (-) is shown. Antiserum supershift experiments were carried out by the addition to the binding reaction mixture of either anti-CREB specific or preimmune sera. Complex 2 supershifted (SS) by the CREB antiserum and a nonspecific band (NS) are shown. The positions of the complexes are shown to the left of the gel.

reactions were carried out in the presence of 2 μ g of poly(dI \cdot dC) and specific competitors, as indicated in the figures, using nuclear extracts (10 μ g of protein) incubated with 20,000 cpm of radiolabeled probe (approximately 6 to 10 fmol) in a total volume of 20 μ l containing 20 mM potassium phosphate (pH 7.9), 70 mM KCl, 1 mM dithiothreitol, 0.3 mM EDTA, and 10% glycerol. The sequences of the oligonucleotides used are as follows (coding strand): CRE31, 5'-GATCCGG CGCTCCTTGGCTGACGTCAGAGAGAGAGA-3'; CRE18, 5'-GATCCTT GGCTGACGTCAGAGAGAGA-3'; APRE-M6, 5'-GATCCACAGTTGTGATT TCACAACCTGACCAGA-3'; and NSC, 5'-GATCCCGGAGGACTGTCTCTCCGAGGACTGTCTCTCCGA-3'.

Bacterial expression of proteins. Synthesis of recombinant proteins was induced in *Escherichia coli* BL21(DE3)pLysS. CREB was produced from a plasmid consisting of the CREB cDNA placed under the control of the T7 polymerase promoter in the pET-3b prokaryotic expression vector (48, 55). C/EBP β was produced by the plasmid C/EBP β BD-RSET (gift from D. Ron, New York University) that contains a fragment of the C/EBP β cDNA spanning the carboxy-terminal domain that includes the bZip region, cloned into the prokaryotic expression vector pRSET-A (Invitrogen) that generates polyhistidine fusion proteins. Recombinant C/EBP β was purified with a nickel-chelate affinity resin.

Assay of cAMP-dependent protein kinase activity. Nuclei were prepared from B2, InR1-G9, JEG-3, or NIH 3T3 cell monolayers. Cells were scraped in phosphate-buffered saline and collected by centrifugation. The pellet was resuspended in 400 μ l of buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors and incubated in ice for 15 min. Nonidet P-40 was added to a final concentration of 0.06%, and then the cells were vortexed vigorously for 10 s. After centrifugation, the supernatant was used for determination of cytoplasmic PKA $_c$ activity. Pelleted nuclei were washed in the same buffer without Nonidet P-40. After the pellet was washed, it was sonicated in 100 μ l of the same buffer and centrifuged. PKA activity (41) in the supernatant was determined by incubating 50 μ g of protein (determined by the Bio-Rad protein assay) in a volume of 50 μ l containing 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 10 mM MgCl $_2$, 5 mM NaF, 0.25 mg of bovine serum albumin per ml, 0.02 mM [γ - 32 P]ATP, and 0.1 mg of Kemptide (Sigma) per ml, a PKA $_c$ -specific substrate. Reaction mixtures were incubated for



10 min at 37°C and spotted onto phosphocellulose filters (Whatman P-81). The filters were washed three times in 75 mM phosphoric acid, and radioactivity was determined by scintillation spectrometry. Values represent means \pm standard errors of the means of at least three independent experiments carried out in duplicate.

RESULTS

Transcription of the somatostatin gene in B2 cells is unresponsive to cAMP. Transient transfection studies in B2 cells using SMS65-CAT, a somatostatin-CAT reporter plasmid that contains the CRE as the only active *cis*-acting element (36, 54), showed no observable increase in the levels of CAT activity elicited by SMS65-CAT in response to 8-Br-cAMP (Fig. 1), a finding consistent with those observed earlier (32, 36). As a positive control, CAT activity elicited by SMS65-CAT transfected into another insulinoma cell line (InR1-G9) that produces glucagon is stimulated threefold by 8-Br-cAMP (Fig. 1). We sought to determine the basis for the defective cAMP response in B2 cells.

We considered the possibility that inhibition of the expression of CREB might explain the absence of a transcriptional response of the somatostatin gene to 8-Br-cAMP. We carried out gel EMSA with a synthetic oligonucleotide (CRE31) containing the somatostatin CRE (55) and found that several distinct protein-DNA complexes are present in B2 cells (Fig. 2). The formation of all the complexes detected by EMSA was

specifically inhibited by competition with unlabeled CRE oligonucleotides (Fig. 2). Preincubation of the binding reaction mixture with a CREB antiserum (R1090) (58) retarded the migration of protein-DNA complex 2, indicating that CREB is one of the proteins that bind to the somatostatin gene CRE in B2 cells (Fig. 2).

The possibility that the lack of cAMP-dependent transcriptional activity in B2 cells is due to inhibition of the binding of CREB to the CRE by other competing CRE-binding nuclear proteins was investigated by using GAL-CREB, an expression plasmid encoding a fusion protein in which the CRE-binding domain of CREB is replaced by the DNA-binding domain of the *Saccharomyces cerevisiae* transcriptional activator GAL4, that does not bind to any known mammalian gene promoter element. GAL-SMS42-CAT, a CAT reporter plasmid bearing a GAL4-binding site in place of the somatostatin gene CRE, was cotransfected into B2 cells together with expression plasmids encoding either the wild type or an enzymatically inactive

FIG. 3. (A) Relative CAT activities obtained following transient transfections of plasmid GAL-SMS42-CAT into B2 or JEG-3 cells. GAL-SMS42-CAT was cotransfected with an expression plasmid encoding the DNA-binding domain of the yeast activator protein GAL4 (GAL147), a GAL-CREB fusion protein, a GAL-C/EBP α fusion protein, or a GAL-C/EBP β fusion protein. GAL-CREB was cotransfected with an expression vector encoding either the wild type (PKA) or a mutated version (PKA^m) of the catalytic subunit of cAMP-dependent PKA. Values obtained in experiments involving B2 cells are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected in the same experiments. Because SMS65-CAT is not active in JEG-3 cells, the values are expressed as percentages of the GAL-SMS42-CAT activities elicited by GAL-CREB in the presence of the mutated PKA (PKA^m). A schematic representation of the GAL-SMS42-CAT reporter plasmid is depicted at the top of the panel. (B) Relative CAT activities obtained following transient transfections of somatostatin gene 5' deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 or glucagon-producing InR1-G9 cells. SMS65-CAT was transfected alone (-) or cotransfected (+) with a plasmid encoding either a mutated form (PKA^m) or the wild-type form (PKA) of cAMP-dependent PKA. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected alone. A schematic representation of the SMS65-CAT reporter plasmid is depicted at the top of the panel. (C) (Top) Western immunoblot showing the presence of PKA_c in B2 cell extracts before and after transfection of 0.5 or 1 μ g of a plasmid encoding PKA_c (RSV-PKA). -, no RSV-PKA. A short exposure (1 min) was used so that the observed increase in immunoreactivity stays within the linear range of detection on the film. Longer exposures (several minutes) revealed readily detectable levels of immunoreactive PKA_c in nontransfected B2 cells (not shown). (Bottom) Western immunoblot of the same extracts with R1090 CREB antiserum, showing the presence of immunoreactive CREB.

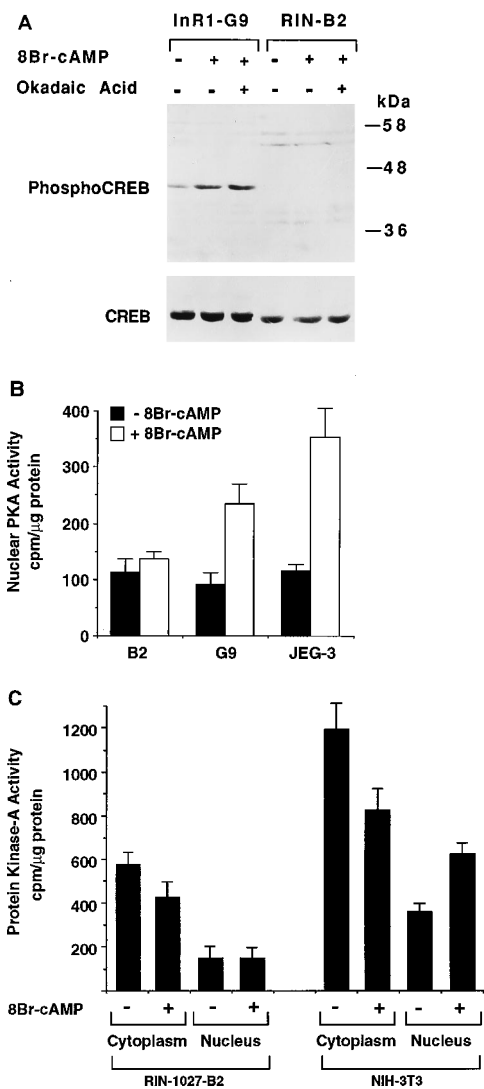


FIG. 4. (A) Western immunoblot carried out with extracts of islet glucagon-producing InR1-G9 or somatostatin-producing B2 cells, prepared before (–) or after (+) treatment of the cells for 30 min with 1 mM 8-Br-cAMP in the presence (+) or absence (–) of 100 nM okadaic acid. The results obtained with an antibody that recognizes specifically phospho-CREB (top) and the results obtained after processing the same extracts with the R1090 antiserum which does not discriminate between CREB and phospho-CREB, (bottom) are shown. (B) Relative PKA enzymatic activities determined for the nuclei of B2, InR1-G9 (G9), or JEG-3 cells prepared before or after treatment of the cells with 1 mM 8-Br-cAMP for 30 min. (C) Relative PKA enzymatic activities determined in the cytoplasm and nuclei of B2 or NIH 3T3 cells prepared before (–) or after (+) treatment of cells with 1 mM 8-Br-cAMP for 30 min. All reactions were carried out in the presence of 10 μ M cAMP to achieve maximum PKA activity.

mutant of the β isoform of the PKA_c (plasmids RSV-PKA and RSV-PKA^m, respectively) (25). No enhancement of CAT activity was observed when GAL-CREB was cotransfected with either mutated or wild-type PKA_c, indicating that the transactivational functions of CREB are impaired in B2 cells (Fig. 3A). As a positive control, the transactivation of GAL-SMS42-CAT is stimulated eightfold by GAL-CREB and PKA_c in JEG-3 choriocarcinoma cells (Fig. 3A). To determine whether B2 cells may provide a permissive environment for the expression of GAL-SMS42-CAT, GAL-SMS42-CAT was cotransfected with expression plasmids encoding fusion proteins consisting of the GAL4-binding domain and the transactivation

domains of transcription factor C/EBP α (33) or C/EBP β . GAL-SMS42-CAT is transactivated by both GAL-C/EBP α and GAL-C/EBP β in B2 cells (Fig. 3A). Thus, the failure of CREB to activate somatostatin gene transcription in B2 cells is not due to competitive interference with other CRE-binding proteins. Rather, the transactivation domain of CREB appears not to function, raising the possibility of a defect in the cAMP signaling pathway and consequent lack of phosphorylation of CREB by PKA.

The existence, however, of a defect within proximal steps in the cAMP-dependent signal transduction pathway in B2 cells appears unlikely, because hormonal stimulation of these cells rapidly increases levels of cAMP (11). Therefore, we examined the possibility that more-distal steps in the cAMP-dependent signaling pathway involving PKA itself may be defective. Transient transactivation studies using the somatostatin-CAT reporter plasmid SMS65-CAT and expression plasmids RSV-PKA^m and RSV-PKA were done in B2 cells compared with control InR1-G9 cells. In B2 cells, the levels of CAT activity were detectable, but cotransfection with the PKA_c expression plasmid (Fig. 3B) or with an expression plasmid encoding a mutated catalytic subunit of PKA that does not interact with the regulatory subunits (29) (data not shown) failed to increase activity further. In contrast, in InR1-G9 cells cotransfection of SMS65-CAT with RSV-PKA resulted in a fourfold increase in CAT activity (Fig. 3B). The activities of SMS65-CAT in B2 and InR1-G9 cells are similar (24% \pm 1.3% and 22% \pm 0.9%, respectively, relative to the activity of the control plasmid RSV-CAT transfected in each cell type). PKA_c is expressed by the plasmid RSV-PKA in transfected B2 cells as monitored by Western immunoblotting (Fig. 3C). CREB is readily detected by Western immunoblots of the extracts of B2 (Fig. 3C) and InR1-G9 (not shown) cells, using the CREB antiserum R1090, indicating that CREB is expressed equivalently in the two islet cell lines.

CREB is not phosphorylated by PKA in B2 cells. Because the transcriptional activity of CREB is critically dependent upon phosphorylation by cAMP-activated PKA (13), we investigated whether the failure of CREB to transactivate the somatostatin gene promoter may be due to defective phosphorylation by PKA. To determine whether CREB is phosphorylated by PKA in B2 cells, we treated cells with 8-Br-cAMP in the absence or presence of okadaic acid, a phosphatase inhibitor that inhibits dephosphorylation of CREB (16), and assayed for CREB phosphorylation by Western immunoblotting with an antibody specifically directed against phospho-CREB (12). Incubation of InR1-G9 cells with 8-Br-cAMP for 30 min results in the phosphorylation of CREB, further enhanced by okadaic acid (Fig. 4A). In contrast, no phospho-CREB is detected in extracts of similarly treated B2 cells, indicating a failure of cAMP stimulation to phosphorylate CREB (Fig. 4A). The amounts of CREB (phospho- and dephospho-) in B2 and InR1-G9 cells are comparable, as determined by immunoblotting using an antiserum (R1090) that detects both phospho- and dephospho-CREB equivalently (Fig. 4A).

Since Western immunoblot analyses showed that the amounts of the catalytic subunit of PKA in extracts of B2 and InR1-G9 cells are comparable (data not shown), the failure to detect phospho-CREB in B2 cells suggested that the catalytic activity of PKA may be defective. Because the activation of CREB by PKA phosphorylation occurs in the nucleus after cAMP-mediated translocation of PKA_c from the cytoplasm to the nucleus, we assayed directly for PKA_c catalytic activities in nuclear extracts prepared from B2 cells and compared them with the activities obtained in InR1-G9 and JEG-3 cells. Treat-

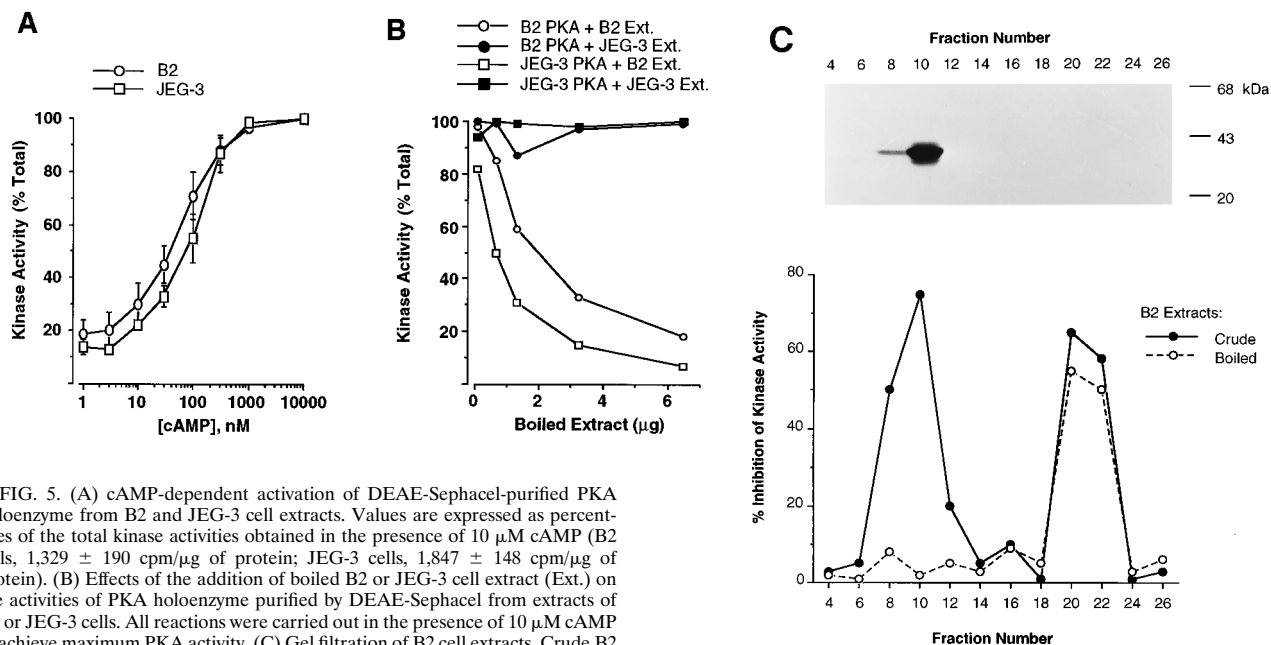


FIG. 5. (A) cAMP-dependent activation of DEAE-Sephacel-purified PKA holoenzyme from B2 and JEG-3 cell extracts. Values are expressed as percentages of the total kinase activities obtained in the presence of 10 μ M cAMP (B2 cells, 1,329 \pm 190 cpm/ μ g of protein; JEG-3 cells, 1,847 \pm 148 cpm/ μ g of protein). (B) Effects of the addition of boiled B2 or JEG-3 cell extract (Ext.) on the activities of PKA holoenzyme purified by DEAE-Sephacel from extracts of B2 or JEG-3 cells. All reactions were carried out in the presence of 10 μ M cAMP to achieve maximum PKA activity. (C) Gel filtration of B2 cell extracts. Crude B2 cell extracts were fractionated in a Superdex-75 column (Pharmacia), and fractions were collected. (Top) One aliquot of each fraction was labeled with 32 P-8-N₃-cAMP under UV radiation, resolved by SDS-PAGE, and exposed for autoradiography. This procedure allowed the identification of the photoaffinity-labeled regulatory subunit of PKA in fractions 8 and 10. (Bottom) Fractions from crude or boiled B2 cell extracts were assayed for PKA inhibitory activity with purified catalytic subunit (Promega). Experiments were run in triplicate and were repeated three times with similar results.

ment of control InR1-G9 and JEG-3 cells with 1 mM 8-Br-cAMP for 30 min resulted in 2.5- and 3.5-fold increases in the catalytic activity of PKA_c, respectively, whereas in B2 cells PKA_c catalytic activity did not increase in response to treatment with 8-Br-cAMP (Fig. 4B). Thus, a deficiency in nuclear PKA activity in B2 cells appears to be the cause of the lack of phosphorylation of CREB in response to 8-Br-cAMP.

We next compared the relative PKA_c activities in the cytoplasm and nuclei of B2 and NIH 3T3 cells. We chose NIH 3T3 as the control cells because phosphorylation of CREB after translocation of PKA_c from the cytoplasm to the nucleus occurs in these cells in response to cAMP stimulation (17). We determined PKA_c activity in the presence of 10 μ M cAMP to ensure that the regulatory and catalytic subunits of PKA are dissociated, so that the maximum activities in cytoplasm or nucleus are measured. As shown in Fig. 4C, treatment of NIH 3T3 cells with 1 mM 8-Br-cAMP for 30 min resulted in a decrease in the total catalytic activity of PKA_c in the cytoplasm and a concomitant increase in nuclear activity, reflecting the translocation of PKA_c into the nuclear compartment. The catalytic activity of PKA_c in the cytoplasm of untreated B2 cells was significantly lower than in untreated NIH 3T3 cells (Fig. 4C). Treatment of B2 cells with 8-Br-cAMP resulted in a decrease in the catalytic activity of PKA_c in the cytoplasm. However, no concomitant increase in PKA_c activity was observed in nuclear extracts of treated B2 cells (Fig. 4C). If B2 cells had a defect in translocation of PKA_c, a decrease in cytoplasmic catalytic activity should not have occurred. Therefore, the results of these experiments suggest the presence, predominantly in the nuclear compartment of B2 cells, of an inhibitor of PKA_c.

To explore this possibility in further detail, we subjected B2 cell extracts to ion-exchange chromatography on a DEAE-

Sephacel column (15) in an attempt to separate the PKA holoenzyme from the putative inhibitor. Prior to chromatography, PKA_c was readily detectable by Western immunoblotting of crude extracts of B2 cells, but its activity was not detectable even in the presence of 10 μ M cAMP (not shown). However, after ion-exchange chromatography, the addition of increasing amounts of cAMP to the fractions containing the purified PKA holoenzyme resulted in increased PKA_c activities in a concentration-dependent manner that was indistinguishable from that observed with similarly purified PKA holoenzyme from JEG-3 cells (Fig. 5A). Confirmation of direct binding of cAMP to the regulatory subunits of PKA was obtained by incubation of B2 cell extracts with 32 P-8-N₃-cAMP, followed by UV radiation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (47), which showed the specific radiolabeling of the regulatory subunits (data not shown). These experiments indicate that B2 cells have normal PKA holoenzymes that can be purified by ion-exchange chromatography and activated by cAMP through binding to the regulatory subunits. Next, we added increasing amounts of boiled B2 cell extracts to the purified PKA holoenzyme in the presence of 10 μ M cAMP, which resulted in inhibition of cAMP-induced PKA_c activities (Fig. 5B). This effect was not observed when boiled extracts of control JEG-3 cells were added (Fig. 5B).

To further characterize this inhibitory activity biochemically, we fractionated B2 cell extracts by gel filtration on a Superdex-75 column (Pharmacia) and assayed each fraction for inhibition of the catalytic activity of PKA_c (Promega) in vitro. When crude B2 cell extracts were used, two peaks of PKA_c inhibitory activity were observed (Fig. 5C). These activities were observed to coelute with molecular mass standards corresponding to 40 and 10 kDa, respectively. The first peak of inhibitory activity (fractions 8 to 10) was undetectable when boiled B2 cell extracts were used, but the activity of the second peak (fractions 20 to 22) was found to be heat stable (Fig. 5C). The heat instability and the approximate molecular mass of the activity present in the first peak suggested that the regulatory subunit of PKA may be responsible for this activity. To test this notion, aliquots of all column fractions were incubated with

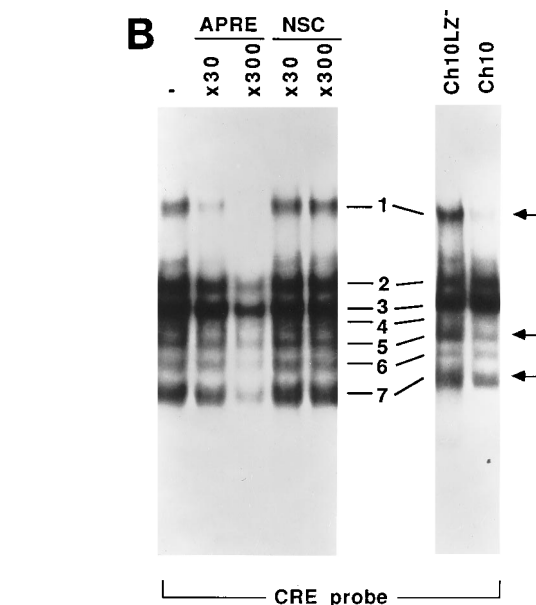
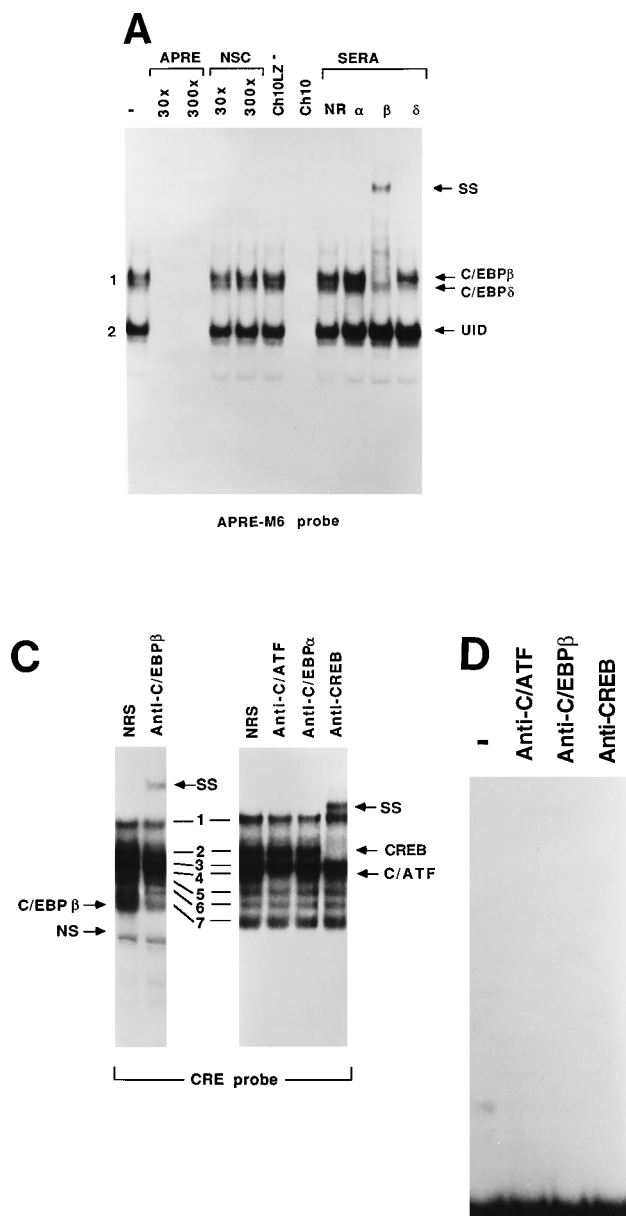


FIG. 6. (A) EMSA showing binding of nuclear proteins prepared from B2 cells to an oligonucleotide corresponding to the angiotensinogen gene acute-phase response element (APRE-M6). Nuclear extracts were incubated in the absence (-) or presence of competing APRE-M6 (APRE) or nonspecific competitor (NSC) oligonucleotides, recombinant full-length GST-CHOP-10 (Ch10) or a truncated version lacking the leucine zipper (Ch10LZ⁻), normal rabbit serum (NR), or a specific antiserum against C/EBP α (α), C/EBP β (β), or C/EBP δ (δ), respectively. Complexes corresponding to C/EBP β and C/EBP δ are indicated by arrows to the right of the gel. Lack of protein degradation after the addition of GST-CHOP-10 was assessed by SDS-PAGE (not shown). SS, supershifted band. UID, unidentified C/EBP-like complexes. The positions of complexes 1 and 2 are shown to the left of the gel. (B) EMSA showing binding of nuclear proteins from B2 cells to the somatostatin gene CRE in the absence (-) or presence (30- or 300-fold molar excess [$\times 30$ or $\times 300$, respectively]) of competing APRE-M6 (APRE) or nonspecific competitor (NSC) oligonucleotides, recombinant full-length GST-CHOP-10 (Ch10) or a truncated version lacking the leucine zipper (Ch10LZ⁻). Arrows indicate complexes that contain proteins whose binding to the CRE oligonucleotide is inhibited by GST-CHOP-10. The positions of the complexes are indicated by the numbers between the two gels. (C) EMSA showing binding of nuclear proteins from B2 cells to the somatostatin gene CRE in the presence of normal rabbit serum (NRS), or a specific antiserum against C/EBP β , C/ATF, C/EBP α , or CREB. Arrows indicate bands that are supershifted (SS) or inhibited in the presence of specific antisera. NS, nonspecific band. The positions of the complexes are indicated by the numbers between the two gels. (D) EMSA showing lack of binding of a specific antiserum against C/EBP β , C/ATF, or CREB to the somatostatin gene CRE.

³²P-8-N₃-cAMP, radiated with UV light, and resolved by SDS-PAGE (47). Photoaffinity labeling with ³²P-8-N₃-cAMP revealed the presence of regulatory subunits of PKA only in fractions 8 to 10 of crude B2 cell extracts (Fig. 5C). No incorporation of ³²P-8-N₃-cAMP was observed when photoaffinity labeling was carried out in the presence of 40 μ M cAMP (not shown), indicating specificity of binding of ³²P-8-N₃-cAMP to the regulatory subunits of PKA. Thus, these experiments indicate that in addition to regulatory subunits of PKA that normally inhibit the activity of PKA in the absence of cAMP stimulation, B2 cells contain a lower-molecular-weight, heat-stable inhibitor that inhibits PKA even in the presence of cAMP.

C/EBPs in B2 cells bind to the somatostatin gene CRE. The experiments described above suggested that proteins other than CREB bind the CRE and activate constitutive levels of transcription observed after transfection. Most CRE-binding proteins belong to the CREB/ATF family of transcription factors characterized by conserved bZip domains that mediate

DNA binding and protein dimerization (for a recent review, see reference 27). Proteins of the related but different C/EBP family of bZip transcription factors also bind to CRE sites (3, 30, 31, 56), but the functional significance of these bindings is unknown. Therefore, we examined the possibility that some of the somatostatin CRE-binding proteins detected by EMSA are related to C/EBP.

To determine whether C/EBP-like proteins are expressed in somatostatin-producing pancreatic islet cells, we tested whether an oligonucleotide probe corresponding to the angiotensinogen gene acute-phase response element (APRE-M6), a well-characterized C/EBP-binding site (5), binds to proteins present in the nuclear extracts of B2 cells. Two major sequence-specific DNA-protein complexes were detected with the APRE-M6 probe by EMSA (Fig. 6A). The addition of recombinant protein CHOP-10, an inhibitor of the binding of C/EBP to DNA (40), to the binding reaction mixture resulted in the inhibition of binding of protein complexes to the APRE-M6 probe, indicating that these complexes are com-

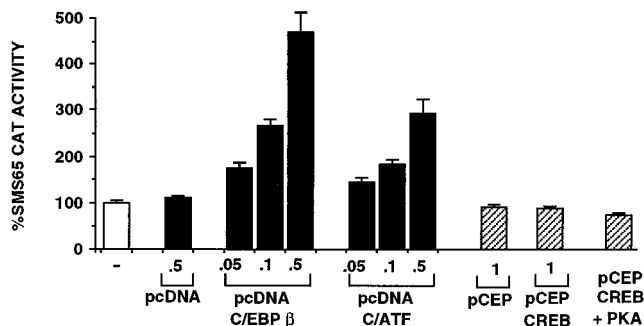


FIG. 7. Relative CAT activities obtained following transient transfections of somatostatin gene 5' deletion plasmid SMS65-CAT into islet cell-derived somatostatin-producing B2 cells. SMS65-CAT was transfected alone (-) or cotransfected with increasing amounts (in micrograms) of an expression plasmid (pcDNA or pCEP) encoding either C/EBP β , C/ATF, or CREB. Values are expressed as percentages of the CAT activities elicited by SMS65-CAT transfected alone.

posed of C/EBP-related proteins (Fig. 6A). Attenuation of the labeled complexes was observed after the addition of an amount of competitor oligonucleotide as low as the 10-fold molar ratio, indicating a relatively high binding affinity as well as specificity. Two members of the C/EBP family, C/EBP β and C/EBP δ , were identified as components of complex 1 by preincubation of the binding reaction mixtures with specific antisera to these transcription factors. The presence of these antisera resulted in the appearance of a supershifted complex (C/EBP β) and inhibition of binding of another protein complex (C/EBP δ), respectively (Fig. 6A). No C/EBP α was detected in these complexes with a specific C/EBP α antiserum (Fig. 6A). In addition, C/EBP α was undetectable in B2 cells by Western immunoblot and immunoprecipitation studies, but it was readily detectable after transfections of B2 cells with an expression vector encoding C/EBP α (data not shown).

C/EBP proteins in B2 cells are part of the protein complexes recognized by the somatostatin CRE, as shown by EMSA (Fig. 6B and C). The C/EBP-binding APRE-M6 oligonucleotide competed with CRE-bound complexes 1, 4, 6, and 7, whereas the addition of CHOP-10 to the binding reaction mixture eliminated complex 1 and attenuated the relative intensities of complexes 5 and 7 (Fig. 6B). C/EBP β antiserum supershifted complex 7 (Fig. 6C). No supershift was observed after the addition of C/EBP α (Fig. 6C) or C/EBP δ (data not shown) antiserum. Therefore, C/EBP β is present in B2 cells and binds to the somatostatin gene CRE.

We identified previously a new member of the CREB/ATF family of transcription factors, C/ATF, that dimerizes with C/EBP proteins and directs their binding to CRE sites (56). C/ATF antiserum attenuated EMSA complex 4, indicating that this complex contains C/ATF bound to the CRE (Fig. 6C). Because the C/ATF and C/EBP β antisera interfered with complexes with different electrophoretic mobilities, the complexes probably do not correspond to C/ATF-C/EBP β heterodimers (56). None of the antisera used in these experiments were found to bind to the CRE oligonucleotide probe (Fig. 6D). C/ATF in B2 cells was also detected by Western immunoblot analysis (data not shown).

C/EBP β and C/ATF activate and CREB represses transcription from the somatostatin CRE. Having established that C/EBP β and C/ATF in B2 cells bind the somatostatin gene CRE, we sought to determine whether these transcription factors transactivate the somatostatin gene promoter via the CRE site. Transient transactivation assays were done in B2 cells with

SMS65-CAT as a *cis*-reporter plasmid and different amounts of *trans*-expression plasmids encoding C/EBP β or C/ATF, resulting in dose-dependent increases of CAT activity (Fig. 7). Transactivation of SMS65-CAT by C/EBP β was more efficient than transactivation by C/ATF (Fig. 7) and was not modified by treatment of B2 cells with 8-Br-cAMP or by cotransfection with RSV-PKA (data not shown). When both C/ATF and C/EBP β expression vectors were cotransfected together with SMS65-CAT, additive effects were observed (data not shown). Cotransfection of SMS65-CAT with an expression plasmid encoding CREB did not increase CAT activity even after cotransfection with the RSV-PKA expression plasmid (Fig. 7).

Both C/EBP β and CREB bind to the CRE of the somatostatin gene, suggesting that they may compete for binding to the CRE. Thus, we examined by EMSA the binding of a truncated bacterially expressed C/EBP β (containing only the C-terminal 145 amino acids corresponding to the bZip domain) and a bacterially expressed full-length CREB to labeled somatostatin CRE oligonucleotide. Binding of both proteins to the CRE was abolished after the addition of similar amounts of excess CRE oligonucleotide (Fig. 8A), indicating that competition of C/EBP β and CREB for binding to the CRE can occur. We tested this notion directly by adding increasing amounts of CREB to a constant amount of C/EBP β bound to the CRE. Doing this resulted in a loss of the gel-shifting activity contributed by C/EBP β and an increase in the CREB gel-shifting activity (Fig. 8B). Experiments were also carried out by using the APRE-M6 oligonucleotide as the probe. CREB did not bind to the APRE-M6 probe, and no diminution of C/EBP β binding to the APRE-M6 was observed in the presence of CREB (Fig. 8B), demonstrating that the displacement of the binding of C/EBP β to the CRE by CREB is specific for the CRE sequence.

We next sought to determine whether displacement of C/EBP β from the CRE by an excess of dephospho-CREB would down-regulate somatostatin gene transcription. B2 cells were cotransfected with SMS65-CAT and a C/EBP β expression plasmid in the absence or presence of increasing amounts of an expression plasmid encoding CREB. C/EBP β -induced SMS65-CAT transcription decreased with increasing amounts of CREB expression plasmid (Fig. 8C). As a control, B2 cells were cotransfected with the reporter plasmid GAL-SMS42-CAT, an expression plasmid encoding a GAL-C/EBP β fusion protein, and increasing amounts of CREB expression plasmid. No significant decrease in GAL-SMS42-CAT activity was observed (Fig. 8D), indicating that the effect of CREB on C/EBP β -induced SMS65-CAT transcription is due to competition of C/EBP β by CREB bound to the CRE and not to sequestration of coactivating factors (squelching) or to interfering interactions between C/EBP β and CREB transactivation domains. These experiments indicate that in B2 cells CREB represses somatostatin gene expression by inhibiting by competition other transactivating transcription factors bound to the CRE and suggest that basal transcriptional levels of SMS65-CAT result from an equilibrium between C/EBP transactivators and dephospho-CREB repressor.

DISCUSSION

These studies demonstrate that transcription mediated by the somatostatin gene CRE in somatostatin-producing B2 cells is repressed by CREB because of a defect in the cAMP-dependent signaling pathway that inhibits phosphorylation of CREB by PKA. Somatostatin gene expression is responsive to cAMP stimulation in rat pancreatic islets (32). The islet B2 clonal cell line, however, used in this study was derived from a

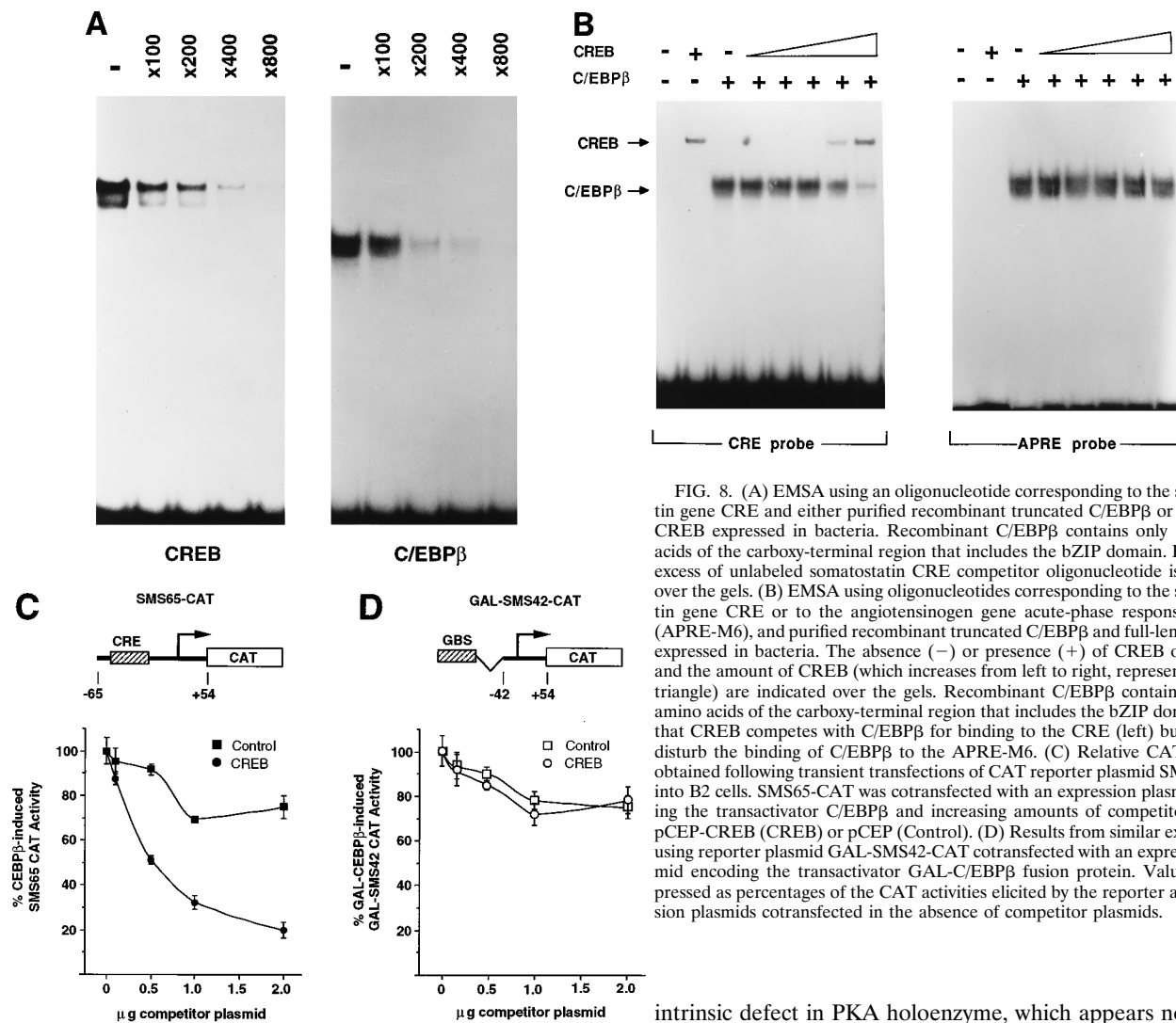


FIG. 8. (A) EMSA using an oligonucleotide corresponding to the somatostatin gene CRE and either purified recombinant truncated C/EBPβ or full-length CREB expressed in bacteria. Recombinant C/EBPβ contains only 147 amino acids of the carboxy-terminal region that includes the bZIP domain. Fold molar excess of unlabeled somatostatin CRE competitor oligonucleotide is indicated over the gels. (B) EMSA using oligonucleotides corresponding to the somatostatin gene CRE or to the angiotensinogen gene acute-phase response element (APRE-M6), and purified recombinant truncated C/EBPβ and full-length CREB expressed in bacteria. The absence (-) or presence (+) of CREB or C/EBPβ and the amount of CREB (which increases from left to right, represented by the triangle) are indicated over the gels. Recombinant C/EBPβ contains only 147 amino acids of the carboxy-terminal region that includes the bZIP domain. Note that CREB competes with C/EBPβ for binding to the CRE (left) but does not disturb the binding of C/EBPβ to the APRE-M6. (C) Relative CAT activities obtained following transient transfections of CAT reporter plasmid SMS65-CAT into B2 cells. SMS65-CAT was cotransfected with an expression plasmid encoding the transactivator C/EBPβ and increasing amounts of competitor plasmid pCEP-CREB (CREB) or pCEP (Control). (D) Results from similar experiments using reporter plasmid GAL-SMS42-CAT cotransfected with an expression plasmid encoding the transactivator GAL-C/EBPβ fusion protein. Values are expressed as percentages of the CAT activities elicited by the reporter and expression plasmids cotransfected in the absence of competitor plasmids.

radiation-induced insulinoma (34). The process of radiation-induced cellular transformation may have deregulated the expression of the somatostatin gene and perhaps other genes otherwise under the control of the cAMP signaling pathway. The state of relative dedifferentiation of the B2 cells may correspond to a stage of constitutive somatostatin gene expression that occurs during fetal islet development (1). Such a phenotypical switch from a cAMP-regulated pathway to a constitutive pathway of gene expression may be important for the maintenance of cell proliferation at particular times during development. Consistent with this view are the observations that somatostatin gene expression is not dependent on cAMP stimulation in at least two other pancreatic cell lines, RIN-1056-A (53a) and Tu6 cells, in which lack of cAMP-induced phosphorylation of CREB has been observed (22). In addition, in transfected F9 cells, cAMP inducibility of the somatostatin gene promoter appears to be dependent on the state of differentiation of the cells (24).

We observe that the absence of CREB phosphorylation is due to lack of cAMP-induced PKA catalytic activity in the nuclei of B2 cells. This lack of activation is not due to an

intrinsic defect in PKA holoenzyme, which appears normal in its capacity to bind cAMP and is activated by cAMP after purification from B2 cell extracts. In addition, it is unlikely that lack of activation of PKA results from sequestration of the catalytic subunit in a cytoplasmic compartment due to unusually high levels of regulatory subunit of PKA, because cotransfection of B2 cells with the SMS65-CAT reporter plasmid and an expression plasmid encoding a mutated catalytic subunit of PKA that does not interact with the regulatory subunits (29) does not result in increased levels of CAT activity. Rather, lack of activation of PKA appears to be due to the presence of a heat-stable inhibitor of PKA (PKI).

Our studies indicate that PKA inhibitors in B2 cells have important functions in transcriptional regulation of cAMP-dependent gene expression. Recently, molecular characterizations of cDNAs encoding at least three different PKI isoforms (PKI α , PKI β 1, and PKI β 2) have been reported (43, 57). Both PKI α and PKI β are expressed in pancreatic tissue (57), and it is not clear whether the PKI activity identified in B2 cells in this study corresponds to any of the known isoforms of PKI. Earlier biochemical studies revealed the presence of different size forms (between 15 and 4 kDa) of PKI in several tissues (43 and 57), but the exact number of PKI isoforms and their physiological roles remain obscure. We are currently carrying out experiments to determine unequivocally whether PKI from B2

cells corresponds to a new isoform in order to proceed to the molecular cloning of its cDNA.

Earlier studies showing that a mutated form of CREB with a serine-to-alanine substitution at position 133 (that is phosphorylated by PKA_c) is a negative transcriptional regulator of the *c-jun* proto-oncogene promoter (21) suggested that dephospho-CREB acts as a repressor of gene transcription. Our observations confirm this notion and underscore the critical dependency of the transactivation functions of CREB on phosphorylation by PKA (or calcium-dependent kinases [46]). Dephospho-CREB is a potent negative regulator of CRE-mediated transcription in B2 cells, in which the cAMP-PKA pathway is defective, which has implications for CREB-regulated gene transcription in cells in which this pathway is intact. It seems clear that the relative ratios of nuclear dephospho- and phospho-CREB determine the relative transcriptional transactivational potency of CREB. This supposition is consistent with the recently reported findings of Loriaux et al. (23), in which CREB dimers consisting of one phosphorylated monomer and one mutated unphosphorylated monomer, gives 50% of the transactivational activity of the wild-type phosphorylated dimer.

The lack of phosphorylation of CREB, however, does not fully explain the lack of basal activity of CREB, because CREB has transcriptional transactivation domains that act in the absence of phosphorylation by PKA (6, 22, 37). Therefore, an additional defect may be present in B2 cells, perhaps involving adapter proteins associated with the basal transcription machinery. The report of a CREB-binding protein that interacts with the kinase-inducible domain of CREB (8) suggests that interactions between CREB and adapter proteins may be important for the transcriptional transactivation of CREB. In addition, our studies do not strictly rule out the possibility that other factors such as hyperactive phosphatases or excess PKA regulatory subunits (4) may also contribute to lack of cAMP-induced transcriptional activity in B2 cells.

Recombinant C/EBP binds efficiently to CRE sequences in vitro (3, 31, 56). C/EBP β regulates phosphoenolpyruvate carboxykinase gene transcription by binding to several sites including an asymmetric CRE (30). We extend these observations by showing that at least two C/EBP proteins, C/EBP β and C/EBP δ , are expressed in somatostatin-producing B2 cells and bind the somatostatin gene CRE and that C/EBP β transactivates the somatostatin gene promoter. Our findings point to the existence of additional unidentified C/EBP-like proteins in B2 cells, as in other cell types (5, 40), that bind to the somatostatin CRE.

C/EBP α is typically expressed in cells upon reaching terminal differentiation (52). C/EBP β , however, is expressed in proliferating cells as an effector protein activated by intracellular signaling pathways (50, 59), including the cAMP-dependent pathway in PC12 cells (26). In our studies, however, C/EBP β activated transcription from the somatostatin gene CRE, but no further enhancement of this effect by 8-Br-cAMP or by cotransfection with RSV-PKA was observed. This lack of response to cAMP may be due to differences between PC12 and B2 cells or may be related to the defective phosphorylation of CREB by PKA.

In earlier studies (56), we described C/ATF, a bZip protein that mediates functional cross talk between transcription factors of both ATF and C/EBP families by forming heterodimers with C/EBP β . In this study, we find immunoreactive C/ATF in somatostatin-producing B2 cells. C/ATF regulates the expression of the somatostatin gene in B2 cells by binding to the CRE. However, the immunoreactive complex detected with the C/ATF antiserum by EMSA (complex 4) is different from

the immunoreactive complex detected with the C/EBP β antiserum (complex 7). Furthermore, only additive, not synergistic, interactions between C/ATF and C/EBP β were detected in cotransfection experiments with SMS65-CAT. Therefore, C/ATF and C/EBP β do not appear to interact in B2 cells, as they do in vitro or in transfected HepG2 cells (56).

In summary, we show that transcription from the somatostatin gene CRE in islet B2 cells is the result of a complex interaction between positive- and negative-acting bZip transcription factors, some of which remain to be identified. Further, these observations emphasize the critical importance of the availability of nuclear PKA_c activity in determining the balance between activation and repression of CRE-mediated transcription by phospho- and dephospho-CREB, respectively. Alterations in the cAMP second messenger signaling pathway and the existence of cross talk between different families of CRE-binding bZip transcription factors may have important functional consequences for developmental gene expression, switching from a regulated to constitutive pattern.

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