Protein Kinase B Activity Is Sufficient to Mimic the Effect of Insulin on Glucagon Gene Transcription*

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Insulin inhibits glucagon gene transcription, and insulin deficiency is associated with hyperglucagonemia that contributes to hyperglycemia in diabetes mellitus. However, the insulin signaling pathway to the glucagon gene is unknown. Protein kinase B (PKB) is a key regulator of insulin signaling and glucose homeostasis. Impaired PKB function leads to insulin resistance and diabetes mellitus. Therefore, the role of PKB in the regulation of glucagon gene transcription was investigated. After transient transfections of glucagon promoter-reporter genes into a glucagon-producing islet cell line, the use of kinase inhibitors indicated that the inhibition of glucagon gene transcription by insulin depends on phosphatidylinositol (3) kinase. Furthermore, insulin caused a PI 3-kinase-dependent phosphorylation and activation of PKB in this cell line as revealed by phospho-immunoblotting and kinase assays. Overexpression of constitutively active PKB mimicked the effect of insulin on glucagon gene transcription. Both insulin and PKB responsiveness of the glucagon promoter were abolished when the binding sites for the transcription factor Pax6 within the G1 and G3 promoter elements were mutated. Recruitment of Pax6 or its potential coactivator, the CREB-binding protein (CBP), to G1 and G3 by using the GAL4 system restored both insulin and PKB responsiveness. These data suggest that insulin inhibits glucagon gene transcription by signaling via PI 3-kinase and PKB, with the transcription factor Pax6 and its potential coactivator CBP being critical components of the targeted promoter-specific nucleoprotein complex. The present data emphasize the importance of PKB in insulin signaling and glucose homeostasis by defining the glucagon gene as a novel target gene for PKB.

The pancreatic islet hormones insulin and glucagon are biologic antagonists in the regulation of blood glucose concentration. Insulin is known to increase peripheral glucose uptake and oppose hepatic glucose production. In contrast, glucagon balances the effect of insulin on blood glucose levels by increasing hepatic glucose production and opposing hepatic glucose storage (1–3). As the glucagon-producing α-cells are mainly located in the peripheral regions of the islets of Langerhans surrounding the insulin-producing β-cells, they are exposed to high concentrations of insulin (1–3). Acting directly on α-cells, insulin inhibits glucagon secretion as well as glucagon gene transcription (4). The inhibition of glucagon gene transcription by insulin depends on the paired domain transcription factor Pax6, which binds to the G1 and G3 element within the glucagon gene promoter (5). Pax6 binding to the G1 element within the glucagon promoter also confers responsiveness of the glucagon gene to thiazolidinediones, a novel class of insulin-sensitizing drugs (6). The paracrine inhibition of glucagon gene transcription by insulin is an important mechanism in the regulation of blood glucose concentrations. Consequently, in diabetic patients a relative hyperglucagonemia has been described that contributes to hyperglycemia in these patients (1). The elevated glucagon levels may be explained by the loss of insulin-mediated inhibition of glucagon synthesis and secretion.

Numerous signaling pathways downstream of the insulin receptor tyrosine kinase have been suggested to contribute to gene regulation by insulin, including PKC1 (7), p70 S6 kinase (8), mammalian targets of rapamycin (8, 9), PI 3-kinase/PKB (10–15), glycogen synthase kinase-3 (16), a PI 3-kinase/ERK1/2 pathway (7), and a Ras/ERK/p90 ribosomal S6 kinase (RSK) pathway (17). There is strong evidence suggesting that activation of the PI 3-kinase/PKB pathway is required for the maintenance of normal glucose homeostasis. The serine/threonine kinase PKB (also called Akt) is known to be activated by phosphorylation in a PI 3-kinase-dependent manner (18, 19) and to mediate biological effects of insulin such as stimulation of GLUT4-dependent glucose transport, glycogen synthesis, and protein synthesis or the suppression of hepatic gluconeogenesis (20). Suppression of hepatic gluconeogenesis has also been suggested to be achieved, in part, by PKB-dependent repression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase catalytic subunit gene expression (10, 13), although this conclusion is controversial (14, 21, 22). In addition, activation of PKB has been found to be a central mechanism in the insulin-dependent regulation of target genes like the IGFBP-1 gene (12), the GLUT1 gene (23), or the fatty acid synthase gene (24). Consistent with this view, impaired PKB

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1 The abbreviations used are: PKC, protein kinase C; CRE, cAMP-responsive element; CREB, CRE-binding protein; CBP, CREB-binding protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IGFBP-1, insulin-like growth factor-binding protein 1; IRE, insulin-responsive element; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/ERK kinase; PI, phosphatidylinositol; PISCES, pancreatic islet cell-specific enhancer sequence; PKB, protein kinase B.
function has been found to cause insulin resistance and diabetes mellitus in animal models and humans (25, 26).

Although the paracrine effect of insulin on the glucagon-producing α-cells within the endocrine pancreas is a crucial regulator of glucagon gene transcription (4, 5) and, furthermore, although an impaired inhibition of glucagon gene transcription by insulin is involved in the pathogenesis of the relative hyperglucagonemia and consecutive hyperglycemia in diabetic patients (1, 27), the signal transduction mechanisms linking the insulin receptor to the promoter of the glucagon gene have not been characterized yet. Given the essential role of PKB in maintaining glucose homeostasis, we investigated the potential role of PKB in the insulin-dependent repression of glucagon gene transcription. We found the effect of insulin on the promoter of the glucagon gene to be mediated via the activation of PI 3-kinase. At the same time, insulin activated, in a PI 3-kinase-dependent manner, PKB, which could mimic the effect of insulin. Furthermore, the transcription factor Pax6 and the coactivator CBP were found to be critically involved in the effect of insulin. Furthermore, the transcription factor Pax6 in a PI 3-kinase-dependent manner, PKB, which could mimic activation of PI 3-kinase. At the same time, insulin activated, of PKB in maintaining glucose homeostasis, we investigated gene have not been characterized yet. Given the essential role diabetic patients (1, 27), the signal transduction mechanisms

EXPERIMENTAL PROCEDURES

Materials—Insulin was from Serva (Heidelberg, Germany), and a stock solution (10 μM) was prepared in 0.9% saline containing 2 mg/ml bovine serum albumin. Genistein, Ly 294002, Ro-31-8220, PD 98059, and rapamycin were purchased from Sigma-Aldrich. Controls received the solvent only.

Plasmid Constructs—The plasmids -350GluLuc (28), -136GluLuc, 3xGluCRE/136GluLuc, 4xG3A-136GluLuc, 4xG2-136GluLuc, pT81Luc, 4xG3A/T81/Luc, -350/5matG1/G3/Luc, pGAL4-CPF, pGAL4-Pax6, pGAL4-VP16, pmyr-PKB, pPKB-K179M (29), p70 S6 kinase (1 M rapamycin, final concentration). The luciferase activities are expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± S.E. of three independent experiments, each done in duplicate. * p < 0.05 (Student’s t test).

RESULTS

Insulin Inhibits Glucagon Gene Transcription through Activation of PI 3-Kinase—A 350-base pair fragment of the rat glucagon gene promoter was shown to confer tissue-specific gene expression (33) and to mediate the response of the promoter to cAMP, calcium, PKC, and insulin (5, 28, 34–39). To investigate the signaling mechanisms involved in the regulation of the glucagon promoter by insulin, a reporter gene construct containing 350 base pairs of the 5′-flanking region of the rat glucagon gene (construct -350GluLuc) was transiently transfected into InR1-G9 cells (3 μg), and the blots were probed with an antibody directed against the influenza hemagglutinin protein (sc-805, Santa Cruz Biotechnology, Santa Cruz, California).

Cell Culture and Transfection of DNA—The glucagon-producing pancreatic islet cell line InR1-G9 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (28). Cells were trypsinized and transfected in suspension by the diethylaminoethyl-dextran method (28) with 2 μg of reporter gene plasmid and the indicated amount of expression vector per 6-cm dish. Cotransfections were carried out with a constant amount of DNA, which was maintained by adding the Bluescript vector (Stratagene). In all experiments, 0.5 μg of cytomegalovirus-GFP (plasmid pCMV-GFPp) per 6-cm dish were cotransfected to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from luciferase/GFP ratios). Twenty-four hours after transfection, cells were incubated in serum-free RPMI 1640 medium containing 0.5% bovine serum albumin and antibiotics as described above. When indicated, cells were treated with insulin (10 nM) for 23 h before harvest. Kinase inhibitors were added, when indicated, 0.5 h before insulin. Cell extracts (28) were prepared 48 h after transfection. The luciferase assay was performed as described previously (28). GFP was measured in the cell extracts using the FluoroCount microscope fluorometer (Packard).

PKB Kinase Assay—The assay was carried out as described previously (31, 32). In brief, cells were lysed with ice-cold lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM Nα,Nα,Nα,Nα diethyldithioctoil, and 100 μg/ml streptomycin). The protein concentration in the cell lysates was determined using a BCA kit (Pierce). Twenty micrograms of protein per lane were analyzed by Western blotting. Detection of PKB, (phospho-serine 473 PKB) was performed with specific antibodies (Cell Signaling). Nitrocellulose membranes were controlled for equal loading and transfer by staining with Ponceau S. After incubation with the appropriate first antibody and peroxidase-coupled secondary antibody, the signal was visualized by enhanced chemiluminescence. For immunoblotting of myr-PKB and myr-PKB-K179M (Fig. 3C), expression vectors encoding the hemagglutinin-tagged proteins were transfected into InR1-G9 cells (3 μg), and the blots were probed with an antibody directed against the influenza hemagglutinin protein (sc-805, Santa Cruz Biotechnology, Santa Cruz, California).

Immunoblots—The protein concentration in the cell lysates was determined using a BCA kit (Pierce). Twenty micrograms of protein per
glucagon gene is inhibited by insulin. Furthermore, these data suggest that both tyrosine kinase and PI 3-kinase activity, but not PKC, MEK, or p70 S6 kinase activity, are required to mediate this effect. Class 1a PI 3-kinase consists of a p110 catalytic subunit and a p85 or p55 regulatory subunit that possess two SH2 domains that interact with tyrosine-phosphorylated Tyr(P)-Met-Xaa-Met and Tyr(P)-Xaa-Xaa-Met motifs in insulin receptor substrate proteins (40, 41). Therefore, our data suggest that the proximal insulin-signaling pathway to the glucagon gene may involve the recruitment and activation of PI 3-kinase.

Insulin Leads to a PI 3-Kinase-dependent Phosphorylation and Activation of PKB in InR1-G9 Cells—PI 3-kinase may transmit multiple signals, including the activation of PKB (41). PI 3-kinase catalyzes the phosphorylation of phosphoinositides on the 3 position to produce phosphatidylinositol 3-phosphates. The formation of phosphatidylinositol 3,4,5-triphosphate is thought to recruit PKB through its pleckstrin homology domain to the plasma membrane, thereby inducing a conformational change in PKB that allows its activation by phosphorylation at two residues (Thr-308 and Ser-473) (41-43). Because the above results suggest that the inhibition of glucagon gene transcription by insulin is mediated by PI 3-kinase (Fig. 1) and because PKB is a known downstream target of PI 3-kinase signaling, we next tested the potential involvement of PKB. To study a potential role of PKB in the regulation of glucagon gene transcription, we first studied the effect of insulin on PKB activity in glucagon-producing pancreatic islet cells (Fig. 2). As demonstrated by phosphoserine-specific immunoblot, insulin induced the phosphorylation of PKB on Ser-473 in glucagon-producing pancreatic islet cells (Fig. 2A, top). This phosphorylation is required for the activation of PKB (42). To test whether insulin increases PKB activity in InR1-G9 cells, PKB was precipitated with specific antibodies, and the amount of enzymatic activity in the precipitates was then determined via the use of a synthetic peptide based on the sequence of the PKB phosphorylation site in glycogen synthase kinase-3. As shown in Fig. 2A (bottom), insulin markedly stimulated PKB activity in glucagon-producing islet cells. Stimulation of PKB activity by insulin was dose- and time-dependent (Fig. 2, A and C). Maximum stimulation of PKB activity was at ~10-100 nM insulin, resulting in a 13-fold increase of PKB activity over basal activity (Fig. 2A). The EC50 value of insulin for the stimulation of PKB activity was at ~1 nM (Fig. 2A), corresponding to the reported IC50 value of insulin for inhibition of glucagon gene transcription (0.5 nM) (5). In addition, these data relate well to the intra-islet situation in vivo, where α-cells are exposed to insulin concentrations ranging from 1 to 100 nM (4). When a PI 3-kinase inhibitor (100 μM Ly 294002) was added, the activation of PKB by insulin was completely abolished (Fig. 2B). Time course studies demonstrated an activation of PKB by insulin within minutes (Fig. 2C) with a sustained response (>24 h (~30% of maximum activity; data not shown), thereby providing further support for the idea that activation of PKB is involved in the insulin-dependent regulation of glucagon gene transcription in InR1-G9 cells. These results demonstrate that treatment with insulin results in a PI 3-kinase-dependent activation of PKB in the InR1-G9 cell line.

PKB Mimics the Effect of Insulin on the Glucagon Gene Promoter—Because the results obtained to date suggest that insulin both inhibits glucagon gene transcription and activates PKB activity via PI 3-kinase in pancreatic islet cells, we investigated whether activated PKB is sufficient to mimic the effect of insulin on the glucagon gene promoter. Co-expression of a constitutively active version of PKB (construct myr-PKB) (44) in InR1-G9 cells was found to inhibit the transcriptional activity of the glucagon reporter gene (Fig. 3A). The extent of the inhibition of glucagon gene transcription by myr-PKB (by 45%) was similar to the inhibition by insulin (Fig. 3A). In contrast, a catalytically inactive PKB mutant bearing a point mutation in position 179 of the kinase domain (construct PKB-K179M) (29) did not inhibit glucagon gene transcription (Fig. 3A). Also, a second kinase-dead form of PKB (myr-PKB-K179M), which is identical to the constitutively active myr-PKB apart from the single amino acid change in the kinase-dead form myr-PKB-K179M, did not inhibit glucagon gene transcription (Fig. 3B). The lack of effect of myr-PKB-K179M was not due to the decreased protein expression of the catalytically inactive mutant PKB protein (Fig. 3C), indicating that the effect of PKB on the glucagon promoter is specific and due to its kinase activity.
PKB, like Insulin, Targets Several Control Elements within the Glucagon Gene Promoter—The rat glucagon gene promoter contains the enhancer-like elements G2 and G3 as well as a CRE (45). The truncated glucagon gene promoter (136 base pairs) containing the proximal promoter elements G1 and G4 exhibits low transcriptional activity but is essential for proper enhancer function (45). A previous study has shown that insulin does not inhibit glucagon gene transcription through a single IRE (5); insulin responsiveness of the glucagon gene is rather conferred by interactions between a proximal promoter and more distal enhancer-like elements involving the paired domain transcription factor Pax6 (5). This finding suggests that insulin may target arrays of transcription factors at the coactivator level (5). Consistent with this view, the ligation of several enhancer-like elements (CRE, G3, G2) onto an insulin-nonresponsive minimal glucagon promoter (136 base pairs) resulted in the generation of insulin-responsive fusion genes (5). As shown in Fig. 4A, PKB, like insulin, can repress the expression of these CRE-, G3A-, and G2-glucagon minimal promoter constructs. The truncated glucagon promoter (-136GluLuc) was not responsive to PKB (Fig. 4A). The kinase-dead PKB mutant myr-PKB-K179M had no effect (not shown). When compared with -136GluLuc (100 ± 2%), basal activity of the constructs was 179 ± 8% (3xCRE-136GluLuc), 11,110 ± 397% (4xG3A-136GluLuc), and 422 ± 48% (4xG2-136GluLuc) (n = 6 each), as has been reported previously (5). These results indicate that PKB, like insulin, targets several control elements within the glucagon gene promoter.

It has been shown previously that G3A, but not the CRE and G2 elements, confers insulin responsiveness to a heterologous minimal thymidine kinase promoter (5). As shown in Fig. 4B, PKB, like insulin, inhibited the transcriptional activity of this G3A-thymidine kinase promoter construct. The kinase-dead PKB mutant, myr-PKB-K179M, had no effect (not shown). In contrast to G3A, three copies of the glucagon CRE or four copies of the G2 element did not confer PKB responsiveness to this heterologous promoter (not shown). The fact that G3A confers insulin and PKB responsiveness suggests that sequence motifs of this enhancer-like element may play a specific role in the inhibition by PKB of glucagon gene transcription. G3A contains the domain A of G3 (from -262 to -247) with the PISCES (pancreatic islet cell-specific enhancer sequence) motif (45), which is also present in the proximal promoter element G1 (45).

Inhibition of Glucagon Gene Transcription by Insulin and PKB Depends on the PISCES Motifs within the Glucagon Promoter Involving Pax6 and CBP—Within the glucagon gene promoter the PISCES motifs in G1 and G3 were shown to be essential regulatory elements in islet β-cell-specific activation of the glucagon gene and its inhibition by thiazolidinedione oral antidiabetic drugs (5, 6, 45). Furthermore, the PISCES motifs are required for the inhibition of glucagon gene transcription by insulin (5). To investigate whether the effect of PKB is conferred by the same regulatory elements as the effect of insulin, we used a mutated glucagon promoter with PISCES in G3 and G1 being converted into GAL4 binding sites (construct -350(mutG1/G3)GluLuc) (Fig. 5A). We found that mutation of the PISCES motifs in the glucagon promoter abolished the responsiveness of the promoter both to insulin as well as to PKB (Fig. 5B). Basal activity of -350(mutG1/G3)GluLuc was low (6 ± 1% of wild type) but well detectable, as has been reported previously (5). Furthermore, the lack of inhibition by insulin and PKB of -350(mutG1/G3)GluLuc was not just secondary to low basal activity, because internal deletion of the G2 element decreased basal activity of the glucagon promoter to a similar degree but did not abolish the inhibition by insulin (5).
and PKB (data not shown). Thus, the present data show that the inhibition of glucagon gene transcription by PKB depends on the PISCES motifs as does the inhibition by insulin. Together with the above data, this suggests that PKB is a downstream effector of the insulin signal to the glucagon gene.

The PISCES motif is known to serve as a binding site for the PISKES motif as does the inhibition by insulin. To determine whether PKB is a downstream effector of the insulin signal to the glucagon gene, we transfected the glucagon promoter with a GAL4-VP16 expression vector. As shown in Fig. 5A, the basal activity of the glucagon promoter was completely restored when CBP was recruited to the mutated PISCES motifs by cotransfection of a GAL4-CBP expression vector (Fig. 5D). Cotransfection of this amount of GAL4-CBP expression vector raised basal activity of -350(mutG1/G3)GluLuc 17.4-fold (n = 6), thus achieving approximate wild-type activity (5). The cotransfection of kinase-dead forms of PKB, PKB-K179M (Fig. 5D) and myr-PKB-K179M (not shown), did not inhibit CBP-driven glucagon promoter activity. In contrast to CBP, the related cofactor p300 did not confer insulin and PKB responsiveness to the glucagon promoter (Fig. 5E). Taken together, these data indicate that activated PKB is sufficient to mimic the effect of insulin on glucagon gene transcription. In addition, the data provide strong evidence that insulin acts on the G1 and G3 elements within the glucagon gene promoter via a mechanism involving the integrated action of PKB, Pax6, and the coactivator CBP.

DISCUSSION

Insulin is well known to inhibit the transcription of the glucagon gene in pancreatic islet α-cells, which is an important physiological mechanism involved in the complex regulatory process of glucose homeostasis (1, 5, 48). Thus, an impaired suppression of glucagon synthesis and secretion, as is typically found in patients with diabetes mellitus, may contribute to hyperglycemia. Consequently, it has been shown, that the inhibition of glucagon secretion reduces fasting hyperglycemia in diabetic animals and humans (27, 49). Therefore, the regulation of glucagon gene expression and the signaling mechanisms involved in this process are of particular interest because they are potential targets for novel pharmacological approaches for the treatment of diabetes. However, the signaling mechanism integrating the response of the glucagon gene promoter to insulin has not been characterized.

Recent studies emphasize the central role of PKB in regulating glucose metabolism. Both animal models using gene targeting approaches as well as human genetic studies have revealed that an impaired function of PKB is sufficient to result in a state of insulin resistance and diabetes mellitus (25, 26). Given the importance of PKB in insulin signaling and glucose homeostasis, we focused on the characterization of PKB in mediating the effect of insulin on glucagon gene transcription. Analysis of the proximal steps of insulin signaling by the use of pharmacological inhibitors revealed that the effect of insulin on the glucagon gene promoter is independent of MEK, PKC, and p70 S6 kinase but involves a process of tyrosine phosphorylation and activation of PI 3-kinase. In addition, our results demonstrate that insulin both inhibits glucagon gene transcription and activates PKB through PI 3-kinase. Furthermore, constitutively active PKB was found to mimic the inhibitory effect of insulin on the glucagon gene promoter, therefore sug-
FIG. 5. The PISCES motif of the G1 and G3 elements within the glucagon gene promoter is required for both insulin and PKB responsiveness of the glucagon gene involving Pax6 and CBP. 

A, structural organization of the 350-bp glucagon gene promoter construct (-350GluLuc; top) and the glucagon gene promoter construct with mutation of the PISCES motifs within G1 and G3 into GAL4 binding sites (-350(mutG1/G3)GluLuc; bottom). Pax6 is known to bind to the PISCES motifs within G1 and G3. B, the PISCES motifs within G1 and G3 are required for the effect of insulin and PKB on glucagon promoter activity. InR1-G9-cells were transfected with the plasmid -350(mutG1/G3)GluLuc with or without 2 μg of the construct myr-PKB. The cells were kept in the presence or absence (black bar) of 10 nM insulin for 23 h before harvest. Luciferase activity is expressed as the percentage of the mean value of the activity measured in the controls (no insulin, no myr-PKB). Insulin and PKB had no statistically significant effect on the mutated glucagon promoter activity (Student’s t test). C, Pax6 binding to the G1 and G3 elements restores insulin and PKB responsiveness of the glucagon gene promoter. InR1-G9 cells were transfected with -350(mutG1/G3)GluLuc together with 50 ng of the expression vector encoding the fusion protein GAL4-Pax6 and either a 2-μg control plasmid (black bar) or an expression vector encoding either myr-PKB or PKB-K179M, respectively. The cells were treated with insulin (10 nM) or left untreated. D, CBP recruited to the glucagon promoter restores insulin and PKB responsiveness of the mutated glucagon reporter gene construct. The reporter gene -350(mutG1/G3)GluLuc was transfected into InR1-G9 cells together with an expression vector (2 μg) encoding the fusion protein GAL4-CBP in combination with expression vectors encoding myr-PKB, PKB-K179M, or an empty vector as a control (black bar). The cells were treated with insulin (10 nM) or left untreated. E, GAL4-p300 does not restore the insulin and PKB response. The reporter gene plasmid -350(mutG1/G3)GluLuc was transfected into InR1-G9 cells together, as indicated, with expression vectors encoding GAL4-p300 (0.1 μg), GAL4-CBP (2 μg), and myr-PKB (2 μg). The cells were treated with insulin (10 nM) or left untreated. In each experiment, the mean luciferase activity in the respective controls (no insulin, no myr-PKB; black bars) was set at 100%. Values are means ± S.E. of three independent experiments, each done in duplicate or triplicate. * , p < 0.05 (Student’s t test).
gesting that insulin exerts this response through activation of PI 3-kinase and PKB.

It has been suggested that PKB regulates the transcription of insulin target genes such as GLUT-1 (23), fatty acid synthase (24), IGFBP-1 (12), phosphoenolpyruvate carboxykinase (13), and glucose-6-phosphatase (10), although for the latter two genes the reports are controversial (14, 21, 22). Analysis of the promoters of the IGFBP-1 and phosphoenolpyruvate carboxykinase genes has provided evidence for the critical involvement of a so-called consensus IRE (T/G/A/TTT) and transcription factors of the Foxo-family in this process (50). Other IREs have also been identified such as the serum response element and binding sites for transcription factors like AP-1, SREBP-1c, and the liver X-activated receptor (5, 50). However, the promoters of various insulin target genes, including the glucagon gene, clearly lack defined IREs (5, 51, 52). Therefore, one current concept of insulin action on gene transcription suggests that insulin may target a composition of transcription factors and coactivators independently of a particular IRE (51, 52). Mapping studies of the glucagon promoter have indicated that it is not a single DNA element that confers the insulin response to this gene but interactions between the proximal promoter element G1 and more distal enhancer-like elements, including G3 (5). Both of these control elements contain a so-called PISCES motif that has been shown to serve as a binding site for the paired domain transcription factor Pax6 (46, 47). Pax6 is a crucial regulator of glucagon gene transcription, conferring responsiveness to both insulin as well as the peroxisome proliferator-activated receptor γ to the glucagon gene (5, 6). In this study we found that the PISCES motif in G1 and G3 is essential also for the effect of PKB on the glucagon promoter. In addition, targeting Pax6 to the PISCES motif in G1 and G3 completely restored both the insulin as well as the PKB responsiveness of the glucagon promoter. Taking into consideration that Pax6 contains a putative phosphorylation site for PKB, RXRXX(S/T), within its paired-domain, it is tempting to speculate that PKB may act on the glucagon promoter by directly phosphorylating Pax6. However, using in vitro phosphorylation assays and Western blots, we did not find evidence for a direct phosphorylation of Pax6 by PKB (data not shown). Therefore, these data suggest that Pax6 is regulated by PKB through an indirect mechanism.

The coactivator proteins CBP and p300 are known to interact with sequence-specific transcription factors and components of the basal transcription machinery and are thought to be general integrators of the transcriptional process (53). Consistent with the view that, in many cases, insulin may regulate gene transcription independently of a particular IRE but may rather target arrays of interacting transcription factors at the activator level (51, 52, 54, 55), effects of insulin on CBP/p300 function have been described recently (5, 56–59). However, whether insulin alters CBP function positively, negatively, or not at all depends on the recruiting transcription factors involved as well as on the promoter context (5, 56–59). The C-terminal transactivation domain of Pax6 is proline/serine/threonine-rich and can bind to CBP/p300 (60, 61), suggesting that recruitment of CBP may be important for the distinct function of Pax6 at the glucagon promoter. Indeed, the transcriptional activity conferred by CBP (as a GAL4-CBP fusion protein) to the glucagon promoter is inhibited by insulin (5). The present study now demonstrates that PKB, like insulin, also suppresses the transcriptional activity conferred by CBP to the glucagon promoter. Similarly, insulin and PKB have been found to inhibit the activity of the transcription factor CREBPβ through CBP at the IGFBP-1 promoter in HepG2 hepatoma cells (56). However, this effect of insulin and PKB on the IGFBP-1 promoter seems to be mediated through a PKB phosphorylation site of CBP that is conserved in p300 (Ser-1834) (56), whereas insulin and PKB responsiveness was conferred to the glucagon promoter by CBP but not p300 (this study). Interestingly, CBP is phosphorylated by insulin-induced signaling at Ser-436 in serum-starved 293T cells (59). This residue is not conserved in p300 (59). The significance of CBP-Ser-436 in the inhibition of glucagon gene transcription by insulin and PKB remains to be defined. When taken together, the present study suggests that Pax6 and CBP may be essential components of a glucagon promoter-specific nucleo-protein complex that integrates the activities of proximal promoter elements and more distal enhancer-like elements and the function of which is sensitive to insulin through PKB.

Several lines of evidence indicate that insulin acts not only on pancreatic islet α-cells to inhibit glucagon gene transcription but also acts, in an autocrine manner, on β-cells to stimulate insulin gene transcription (8, 62). This finding suggests an important functional role for the insulin receptor in glucose sensing by the pancreatic β-cell and proposes that defects in insulin signaling at the levels of both the α- and the β-cell may contribute to the observed alterations in glucagon and insulin secretion in type II diabetes mellitus. Data have been presented that show that secreted insulin acts via β-cell insulin receptors and up-regulates insulin gene transcription by signaling through the PI 3-kinase/p70 S6 kinase and calcium/calcmodulin-dependent protein kinase pathways (8). By suggesting that insulin inhibits glucagon gene transcription through PKB, the data of the present study indicate that insulin regulates the glucagon and insulin genes through distinct signaling pathways in pancreatic islet α- and β-cells, respectively.

In conclusion, our results demonstrate that activation of PKB is sufficient to mimic the effect of insulin on the glucagon promoter and suggest that the inhibition of glucagon gene transcription by insulin is mediated via activation of PI 3-kinase and PKB. Furthermore, we propose a model according to which the effect of insulin and PKB on the promoter of the glucagon gene essentially requires the targeting of the transcriptional coactivator CBP and the paired domain transcription factor Pax6 to the PISCES motifs within the glucagon promoter. The present data emphasize the importance of PKB in insulin signaling and glucose homeostasis by defining the glucagon gene as a novel target gene for PKB.

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