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Glucose stimulates the activation domain potential of the PDX-1 homeodomain transcription factor

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Abstract Glucose-stimulated expression of the insulin gene in β cells is mediated by the PDX-1 transcription factor. In this report, we show that stimulation results from effects on activation and DNA-binding potential. Thus, glucose specifically stimulated expression in MIN6 β cells from chimeras of PDX-1 and the GAL4 DNA-binding domain which spanned the N-terminal PDX-1 activation domain located between amino acids 1 to 79. GAL4:PDX activity was induced over physiological glucose concentrations and was also regulated by effectors of this response. The level of endogenous PDX-1 binding and phosphorylation were also induced under these conditions. We discuss how changes in PDX-1 phosphorylation may influence activity in glucose-treated β cells.

Key words: PDX-1; Glucose; Transcription; Insulin

1. Introduction

In higher eukaryotes, glucose homeostasis is primarily controlled through the concerted actions of glucagon and insulin released from pancreatic islet α and β cells, respectively. These hormones influence circulating blood glucose levels by effecting uptake and metabolism of this sugar in peripheral tissues [1]. Insulin acts by reducing glucose levels, whereas glucagon increases them. The primary physiological stimulus of insulin transcription, synthesis, and release in the β cells is glucose [2,3]. Metabolism of glucose in β cells is a requirement for these events [4]. The identification and characterization of the factors important in these processes provides insight into the control mechanisms important in glucose sensing, and perhaps, also, in the pathogenesis of islet dysfunction in non-insulin-dependent diabetes mellitus.

Glucose-responsive and pancreatic β cell-specific transcription of the insulin gene is mediated by *cis*-acting elements contained within the insulin enhancer region, which is located between nucleotides −340 to −91 relative to the transcription start site [3,5]. The *trans*-acting factors that bind to and activate A3- (−213 to −209 bp) [6–8], C1- (−115 to −107 bp) [9,10], and E1- (−100 to −91 bp) [9,11] driven expression appear to be important in regulating both of these responses. (The insulin *cis* elements are labeled in accordance with the nomenclature of German et al. [12].) Thus, mutations that decrease the binding affinity of the A3, C1, and E1 activators reduce both glucose-regulated and β cell-type-specific transcription from insulin gene reporter constructs. The PDX-1 homeodomain protein (also known as IPF1, STF-1, and IDX-1 [13–15]) appears to be the principal regulator of insulin A3 element-stimulated transcription [6,7]. The activator of E1 element-directed expression is a heterodimer, composed of proteins in the basic helix-loop-helix (bHLH) family that are enriched in islets (INSAF [16] or BETA2 [17,18]) and generally distributed (HEB [19] and E2A [20]). The C1 activator has not been isolated.

Little is known about how glucose regulates A3, C1, and E1 activator function in β cells. Previously, it had been shown that both C1- [9] and PDX-1- [8] binding activity were potentiated in glucose-stimulated cells. In contrast, there is little [21], or no increase [9] in E1 activator binding under these same conditions. The recent observation that the phosphorylation level of PDX-1 is induced in glucose-stimulated cells [22] suggests that activation of insulin transcription factor function may be mediated by posttranslational modification mechanisms (for review, see [23]) that influence either the binding and/or activation potential of the A3, C1, and E1 activators.

To begin to investigate this issue, we analyzed how PDX-1 activity was stimulated by glucose in MIN6 β cells and rat islets. Recent studies have shown that the transactivation domain of PDX-1 was contained within the N-terminal region sequences spanned by amino acids 1–79 [24]. Results obtained with chimeras between PDX-1 and the DNA-binding domain of the *Saccharomyces cerevisiae* transcription factor GAL4 revealed that PDX-1 transactivation domain activity was induced in glucose-stimulated β cells. Under these conditions, the level of phosphorylation and binding of PDX-1 were also induced. These results suggest that glucose influences both the binding and transactivation potential of PDX-1 in β cells. We discuss the possible importance of phosphorylation mechanisms in these activation processes.

2. Materials and methods

2.1. Cell lines and nuclear extract preparation

MIN6 β cells [25] were grown in DMEM containing 4.5 g glucose/l (GIBCO), 15% fetal calf serum (Sera-lab, Crawley Down, Sussex, UK), penicillin (100 U/ml), streptomycin (100 mg/ml) (GIBCO/BRL), and 50 μM β-mercaptoethanol. Nuclear extracts were prepared from 10^8 cells by modification of the procedure described in [26].

2.2. Transfections

A 60% confluent 6 cm^2 dish of MIN6 cells was transfected by the calcium co-precipitation method [27]. The islets were isolated from newborn rats [28] and left for 7 days in RPMI 1640 with 0.5% human serum to remove fibroblasts. The islets were then made into single cells by trypsinization, and single cells from 500 islets were transfected

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by Lipofectamine (Promega) in OPTIMEM (GIBCO). The DNA transfections contained a GAL4 expression construct, $(GAL4)_5$E1bCAT [29], and pGL2RSV luciferase (Promega). (The construction of each of the GAL:PDX wild-type and mutant constructs [24] and GAL4:VP16 [30] has been described.) The luciferase activity from pGL2RSV luciferase was used as a control. After transfection, the cells were grown in RPMI 1640 (10% FCS) containing either 3 or 20 mM glucose. The cells were harvested after 16 h, and luciferase (Promega) and chloramphenicol acetyltransferase (CAT) assays [27] performed. The CAT activity of each sample was normalized to the protein content (Biorad) of the extract.

2.3. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed using 5 μg of MIN6 nuclear extract and the human A3 element probe (5′ CCCCTGGTTAAGACTCTAAGGACCCGCTGG 3′) as described by Petersen et al. [6]. One μl of the preimmune or 1858 αPDX-1 antiserum was added prior to the addition of oligonucleotide. The 1858 αPDX-1 antiserum was raised against rat PDX-1 sequences spanning 221 to 284 [31].

2.4. Immunoblots

MIN6 and islet cells were incubated at 37°C in DME (Gibco) with 3 mM glucose for 90 min. ^{32}P-labeled inorganic phosphate (Amersham, final concentration 0.3 mCi/ml media) was then added and incubated at 37°C for 2 h in either 3 or 20 mM glucose. Whole cell extracts were prepared as described previously [32]. Two hundred μl of extract was incubated for 1 h with 2 μl of either the preimmune or 1858 αPDX-1 antiserum in lysis buffer (20 mM Imidazole-HCl pH 6.8, 100 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 0.2% Triton X-100, 10 mM NaF, 1 mM sodium vanadate, 1 mM sodium molybdate, 1 mM AEBSF). The PDX-1:Protein A Sepharose precipitated complexes were prepared as described previously [33] except that TBST buffer was exchanged with lysis buffer and resolved on a 10% SDS-polyacrylamide gel. The gel was dried and labeled protein bands visualized by autoradiography.

Immunoblots were performed as described [34] using 15 μg of nuclear extract that was resolved on a 10% SDS-polyacrylamide gel. αPDX-1 antiserum [31] or preimmune serum was used as primary antibody.

3. Results

3.1. Glucose stimulates the transactivation domain potential of PDX-1

To determine whether the transactivation domain function of PDX-1 was regulated by glucose, a series of PDX-1 mutants spanning the N-terminal activation domain were analyzed as fusion proteins to the DNA-binding domain of the *Saccharomyces cerevisiae* GAL4 transcription factor. The effect of glucose on herpes virus VP16 activation domain stimulated GAL4 expression and insulin enhancer-mediated activity was compared to the GAL4:PDX-1 response in these assays. The GAL4 fusion protein expression plasmids were cotransfected into MIN6 β cells and isolated rat islet cells along with a CAT reporter plasmid containing five GAL4 DNA-binding sites upstream of the E1b TATA. The transfected cells were then incubated in either 3 or 20 mM glucose containing medium for 16 h. Insulin secretion from MIN6 cells is stimulated under these conditions [25].

Each of the GAL4:PDX-1 fusion constructs was glucose-responsive (Fig. 1A and B). However, the GAL4:PDX-1 plasmids containing homeobox sequences (GAL4:PDX 1–284 and GAL4:PDX 1–220) were stimulated less effectively by glucose than the N-terminal PDX-1 fusion construct, GAL4:PDX 1–149 in MIN6 cells. Importantly, GAL4:PDX 1–149 was responsive within the same range of glucose concentrations as the insulin enhancer construct, rINS:CAT, in both MIN6 and islet cells (Fig. 1B and C). In contrast, GAL4:VP16 was only slightly responsive to glucose while the GAL4 cloning vector alone was unresponsive. These results suggested that glucose potentiated the transactivation capabilities of PDX-1 by altering transactivation domain-mediated stimulation.

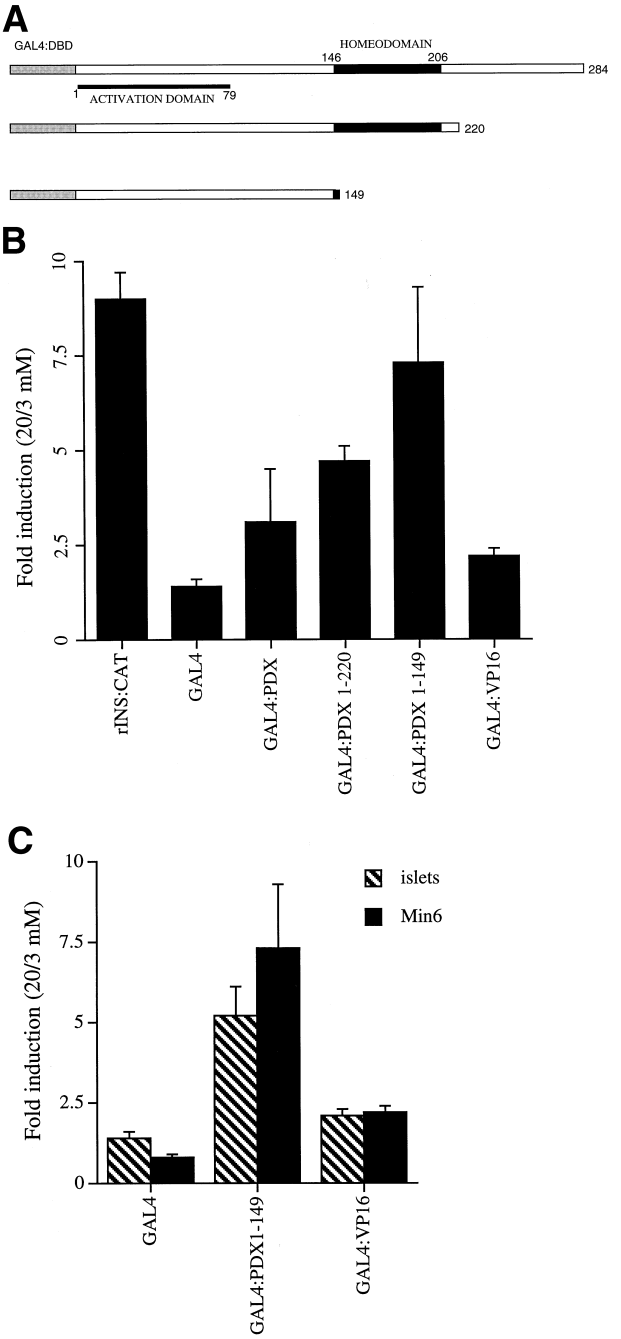

Fig. 1. Transactivation by PDX-1 is potentiated in glucose-stimulated MIN6 cells. A: Diagrammatic representation of the GAL4:PDX, GAL4:PDX 1–220, and GAL4:PDX 1–149 constructs. The activation and homeodomain regions span amino acids 1–79 and 146–206, respectively [24,38,43]. B: MIN6 cells and C: isolated rat islet cells were transfected with the GAL4 fusion vector, GAL4:PDX or GAL4:VP16 (2.5 μg) and $(GAL4)_5$E1bCAT (5 μg), or rINS:CAT (7.5 μg), along with pGL2 RSV luciferase (2.5 μg) (rINS:CAT contains 346 bp of the rat insulin I gene promoter fused to CAT [44]). The results are calculated as the ratio of the normalized CAT activity at 20 mM glucose divided by 3 mM ± S.E.M.

Previously it had been shown that glucose-treatment causes

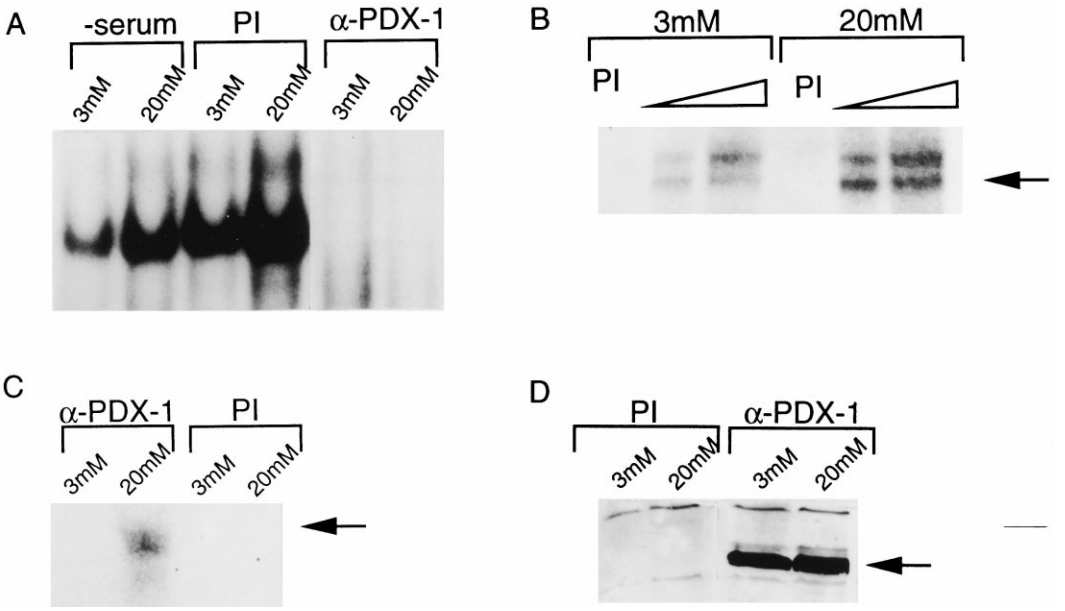

Fig. 2. PDX-1-binding and phosphorylation levels in glucose-stimulated MIN6 cells. A: PDX-1-binding activity in glucose-treated MIN6 cells. The EMSA was performed with nuclear extracts prepared from 3 or 20 mM glucose-treated MIN6 cells. The extracts were preincubated in the absence of serum (−serum), preimmune serum (PI), or αPDX-1 antiserum, and then binding activity determined using the human A3 element probe. B: PDX-1 phosphorylation level in glucose-treated MIN6 cells. The cells were grown in either 3 or 20 mM glucose plus ^{32}P-labeled inorganic phosphate. PDX-1 levels were determined by immunoprecipitation with increasing amounts of αPDX-1 antiserum and preimmune (PI) serum. The arrow denotes the position of PDX-1 in the blot. C: PDX-1 phosphorylation level in glucose-treated isolated islet cells. Same experiment as in B except that immunoprecipitation was carried out using the same amount of αPDX-1 antiserum and preimmune (PI) serum. D: The steady-state level of PDX-1 in glucose-treated MIN6 cells.

an increase in both the PDX-1-binding activity [8,22] and phosphorylation state [22] in islet β cells. We also found that the level of PDX-1 binding (Fig. 2A), as well as its phosphorylation level (Fig. 2B), increased in MIN6 cells grown under basal (3 mM) versus stimulating (20 mM) glucose concentrations. The state of phosphorylation also increased after glucose-treatment of rat islets (Fig. 2C). In contrast, these conditions did not influence the steady-state PDX-1 levels in MIN6 cells (Fig. 2D). Together, these data indicate that PDX-1 activation in glucose-stimulated β cells was meditated through changes in both the transactivation and DNA-binding potential of the protein.

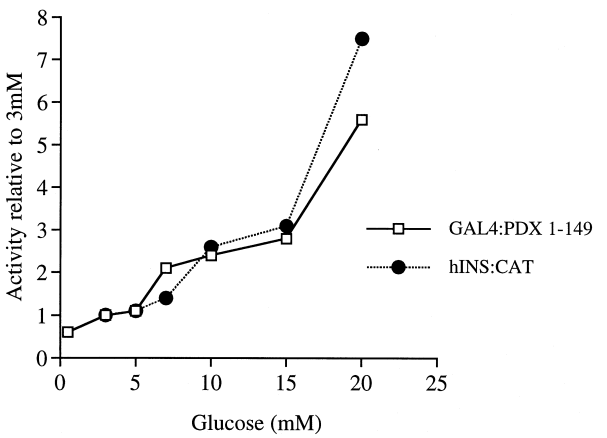

Fig. 3. GAL4:PDX 1–149 and hINS:CAT activity are co-regulated in glucose-treated MIN6 cells. MIN6 cells were transfected with hINS:CAT (7.5 μg), a CAT construct driven by 337 bp of the human insulin 5′-enhancer:promoter region [6], or GAL4:PDX 1–149 (2.5 μg) at the indicated glucose concentrations. The results shown are the mean of at least two experiments performed in duplicate.

3.2. Metabolic regulation of GAL4:PDX 1–149 activity

Metabolism of glucose in pancreatic β cells generates an intracellular signal(s) that results in increased insulin secretion, insulin biosynthesis, and insulin transcription [2,3,35]. We next examined whether GAL4:PDX 1–149 activity in MIN6 cells was regulated by physiological glucose concentrations, and by factors that can either substitute (i.e. mannose) or inhibit (i.e. 2-deoxy glucose) glucose metabolism. As a positive control, insulin enhancer-mediated activity (i.e. hINS:CAT) was analyzed in MIN6 cells transfected and treated in parallel with the GAL4:PDX 1–149 experimental samples.

Nearly superimposable glucose response curves were obtained from GAL4:PDX 1–149 and hINS:CAT (Fig. 3). The apparent half-maximal glucose concentration required for transcriptional activation was also similar to that of insulin secretion in islets ($EC^{50}S$ of 9 mM [36]), suggesting that the inductive signal(s) for these distinct responses are generated under similar glucose concentrations. The results of our effector studies suggest that GAL4:PDX 1–149 activity is also regulated by factors that influence insulin expression in islets. Thus, the non-metabolizable glucose analog, 2-deoxy glucose, inhibited both glucose-induced GAL4:PDX 1–149 and insulin enhancer-mediated activation to the same degree (Fig. 4). In addition, both of these glucose-sensitive activities were stimulated to a comparable level when the metabolizable glycolytic (i.e. mannose) cycle substrate was analyzed. These results provide additional evidence indicating that the activation poten-

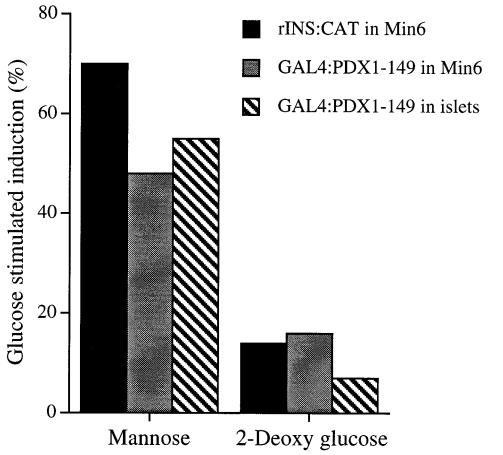

Fig. 4. Regulation of GAL4:PDX 1–149-mediated activation by effectors of glucose-stimulated expression. MIN6 cells and rat islets were transfected with GAL4:PDX 1–149 or rINS:CAT as described in Fig. 1B. The cells were grown in the presence of either 3 mM glucose, 20 mM glucose, 20 mM mannose or 20 mM 2-deoxy glucose. A representative experiment is shown. Results are given as the percentage of the glucose-stimulated expression.

tial of PDX-1 is an important functional determinant for glucose-induced transcription.

4. Discussion

The principal physiological regulator of insulin transcription in islet β cells is glucose. Previously it had been shown that the A3 element, which resides within the insulin enhancer region, mediates glucose-inducible transcription [6]. Furthermore, stimulation appeared to result from induced binding of the A3 activator, PDX-1 [8,22,37]. In this study, the molecular basis for activation was further defined. We have shown that glucose not only induces the transactivation potential of PDX-1 by effecting DNA binding, but also by stimulating transactivation domain function. Importantly, transcriptional activation by GAL:PDX responded to both physiological glucose concentrations as well as normal β cell effectors (i.e. mannose and 2-deoxy glucose). We conclude that modifications in both DNA-binding and transactivation capacity enable PDX-1 to mediate glucose-inducible insulin gene transcription in islet β cells.

Studies conducted in a number of different laboratories strongly indicate that PDX-1 is an important activator of the insulin gene [6,7,13,31,38,39]. The observation that the nuclei of essentially all insulin-producing islet β cells contain PDX-1, yet it's only present in a small subset of the other islet hormone-producing cells, also supports this hypothesis [31,38,40]. However, evidence showing that PDX-1 is not required for insulin gene transcription has come from the analysis of *pdx-1* mutant mice. Thus, even though pancreas formation is prevented in *pdx-1*$^{-/-}$ mice [41], insulin positive cells were transiently detected during embryonic development [42]. In addition, we recently found that insulin gene expression proceeded in the absence of PDX-1 in βTC-3 cells stably transfected with the PDX-1 transactivation mutant, ΔABC:PDX-1 [24] albeit with insulin gene transcription reduced by 75%. These results indicate that PDX-1 is not required for insulin gene transcription per se, but do contribute to the high transcriptional activity of the insulin gene in β cells. Since the function of PDX-1 in glucose-induced insulin gene expression would not have been discerned in these experimental systems we still strongly believe that this factor plays an essential regulatory role in this response.

In our analyses, the phosphorylation state of PDX-1 increased in parallel with its binding and activation potential in glucose-treated β cells. Unfortunately, the site(s) of modification within PDX-1 have not been identified.

It is our objective to understand the roles these factors play in PDX-1 function by analyzing how their interplay determines developmental and inducible transcriptional activation.

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