Involvement of PDX-1 in activation of human insulin gene transcription

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Abstract

Islet β cell-specific transcription of the insulin gene is mediated through the binding of the islet-enriched PDX-1, BETa2, and MafA transcription factors to conserved 5′-flanking region regulatory elements. However, additional non-conserved sequences within this region are also significant in regulating expression. Thus, PDX-1 binds to and activates the GG2 element located between nucleotides -145 and -140 of the human gene, while the corresponding, but non-identical, site in the rodent insulin genes are negatively regulated by the Nkx2-2 transcription factor. Here, we show that despite binding PDX-1 approximately 20-fold less effectively than the conserved insulin A3 and A1 sites in gel mobility shift assays, human GG2 appears to be more important for the activation of transfected human insulin enhancer-driven reporter constructs in β cell lines. Furthermore, functional interaction analysis in non-islet cell lines demonstrated that PDX-1 binding to GG2, A1, and A3 contributes to synergistic activation of insulin gene expression with MafA. Our analysis also illustrated the requirement of poorly conserved human sequences between -293 and -251 in mediating activity through the more upstream A3 binding site. Collectively these experiments have revealed distinct features in control of the human and rodent insulin genes by PDX-1, processes that may be involved in regulating insulin expression under both normal and diabetic conditions in humans.


Introduction

Insulin is exclusively synthesized in and released from the β cells of pancreatic islets. Cell-type-restricted expression is conferred at the transcriptional level by a unique combination of regulators enriched in β cells (Stein 2001). Transgenic studies in mice first demonstrated that approximately 350 base pairs (bp) upstream of the transcription start site of the non-allelic rat insulin I and II (rat I and II) genes mediate β cell-specific expression (Hanahan 1985, Dandoy-Dron et al. 1991). These regions are 76% identical and display roughly 60% identity to the corresponding 5′-flanking sequence of the human insulin gene, suggesting functional conservation between species. Indeed, the bases located between -353 and +1 of the human gene were subsequently found to be sufficient to drive cell-type selective transgene activity in vivo (Fromont-Racine et al. 1990). The ability of this region to restrict expression to β cells has been demonstrated recurrently in transfection assays using transformed cell lines (Walker et al. 1983, Edlund T et al. 1985, Boam et al. 1990, Ahlgren et al. 1998, Edlund H 1998). Furthermore, this proximal 350 bp control region is sufficient to confer responsiveness of insulin-driven reporter genes in both transfected islets (German et al. 1990, Odagiri et al. 1996) and cell lines (Sharma & Stein 1994, Sharma et al. 1995) to glucose, the most important metabolic effector of β cell function. Altogether, these studies clearly demonstrated the importance of sequences proximal to the insulin promoter in regulating β cell-specific and glucose-responsive expression.

Both cell-restricted and glucose-inducible expression of mammalian insulin genes appear to be controlled, at least in part, by the effects of islet-enriched transcriptional activators binding conserved C, E, and A elements (Stein 2001). These elements reside between -350 to -90 bp, a region with β cell-specific enhancer-like properties (Edlund T et al. 1985, Fromont-Racine et al. 1990). The C1 element, located between bp -125 and -116 of the human gene, binds the basic leucine-zipper factor MafA (Matsuoka et al. 2003), while E1 (bp -111/-102) is a site for regulation by a heterodimer of ubiquitous E47 and the islet-enriched BETa2 basic helix-loop-helix containing proteins (Naya et al. 1995), and A1 (bp -83/-75) and A3 (bp -216/-207) are critical sites of control by the PDX-1 homeodomain protein (Ohlsson et al. 1993, Petersen et al. 1994). While these elements appear to be principal contributors to cell-exclusive and glucose-responsive activation of insulin gene transcription, additional species-specific cis-acting sequences have also been implicated...
in regulation (Boam et al. 1990, Sander et al. 1998, Okita et al. 1999, Bartoo-Shifman et al. 2002, Oetjen et al. 2003). For example, the human insulin GG2 element located between bp -145 and -140 is a site of positive regulation by PDX-1, despite lacking a characteristic homeodomain factor TAAT core binding motif (Le Lay et al. 2004). In contrast, the corresponding GG2 element of the rat and mouse insulin genes are negatively regulated by the islet-enriched factor Nkx2·2, with species-selective binding resulting from differences at nucleotides -144 and -141 in the human gene (Cissell et al. 2003). The human insulin gene also possesses a unique glucose- and islet cell-specific control region spanning bp -292 to -243, termed the Z region (Sander et al. 1998), which is poorly conserved in the rat and mouse genes.

In the present study, we first compared the contribution of PDX-1 binding at GG2 relative to the conserved A1 and A3 sites in human insulin gene activation. Although GG2 displayed a lower PDX-1 binding affinity in gel shift assays, it was more critical to human insulin activation in β cell transfection assays. GG2 also contributed to synergistic activation by PDX-1 and MafA. Moreover, the ability of PDX-1 to activate through the human A3 element was found to be dependent upon upstream sequences from bp -293 to -251, an area of poor sequence homology spanning the unique human Z control domain. These studies have revealed distinctions in control of the human and rodent insulin genes by PDX-1, features that may be of importance in vivo.

**Materials and Methods**

**DNA constructs**

The human insulin enhancer-driven luciferase (Luc) reporter containing wild-type (WT) 5′ control sequences from -251 to +10 (-251 Luc WT) has been described previously (Sharma et al. 1995). The human insulin (Ins) A1 (-83/-75 bp), GG2 (-145/-140), and A3 (-216/-207 bp) elements were mutated in -251 Luc using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with the following primers: A1 mutant (mut) (5′-TTGCCCACCCAGCCCTCTTGAGGGCCA GCGGCAGGGGTTG-5′), GG2 mut (5′-TATGGCTTTGGCTGCTCTACAG-3′), and A3 mut (5′-TACAGTTGGAGGTCTTCAGG-3′). Mutated sequences are underlined. Human insulin gene also possesses a unique glucose- and islet cell-specific control region spanning bp -292 to -243, termed the Z region (Sander et al. 1998), which is poorly conserved in the rat and mouse genes.

**Gel mobility shift assays**

Nuclear extracts were prepared from cell lines using procedures previously described by Schreiber et al. (1989). DNA binding analysis was performed with double-stranded oligonucleotides to human Ins A1 (5′-ACCCCA GCCCTAATGCGCAGGCGG-3′), human Ins GG2 (5′-CCAGCACCAGGGAATGTCGGGAA-3′), human Ins A3 (5′-GTGTTAACCTCTAATGCGGCCG-3′) and Nkx2·2 consensus (GGT TTGACTTTGGG) (Watada et al. 2000). The annealed A1 oligonucleotide was labeled with [γ-32P] ATP using T4 polynucleotide kinase.

Standard binding reactions (25 µl total volume) were conducted at 4 °C for 30 min with 10 µg nuclear extract and 400 fmole of radiolabeled probe in binding buffer containing 10 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol, and 1 µg poly(dI-dC). Competition assays were performed under the same conditions, except that an excess of competitor DNA was included in the reaction along with the probe prior to the addition of protein. Protein–DNA complexes were resolved on 5% non-denaturing polyacrylamide gels run in 1 × TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA) at room temperature. Gels were then dried and binding complexes were visualized by autoradiography and quantitated by densitometric scanning.

**Cell culture and transfections**

Monolayer cultures of βTC-3 (mouse) and HeLa (human) cell lines were maintained as described previously.


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Regulation of human insulin gene by PDX-1

Results

GG2 element binds PDX-1 less effectively than conserved A1 and A3

PDX-1 activates human GG2, but not the corresponding element in the rodent insulin I or II genes (Cissell et al. 2003, Le Lay et al. 2004). Interestingly, the human site lacks the TAAT core motif commonly associated with homeodomain protein binding. To directly compare the efficacy with which this non-conserved, atypical site binds PDX-1 relative to the conserved and canonical human A1 and A3 elements, competition analyses in gel mobility shift assays were performed. GG2 was less capable of competing for PDX-1 binding to the A1 probe than A1 and A3 in βTC-3 nuclear extracts (Fig. 1A). Quantitation of the PDX-1 binding complex revealed that, respectively, A1 and A3 bound approximately 15- and 25-fold better than GG2 (Fig. 1B). Furthermore, the Nkx2-2 consensus sequence, used during the biochemical isolation and identification of PDX-1 as the GG2 site activator (Le Lay et al. 2004), was found to compete for PDX-1 binding nearly 10-fold more effectively than GG2, despite itself lacking a TAAT core (Fig. 1). Collectively, these results demonstrated the ability of PDX-1 to bind a variety of A/T-rich sequences in vitro.

PDX-1 binding at GG2 is critical to human insulin expression in β cells

Although the exact mechanism(s) involved in mediating synergistic transcriptional activation of the insulin gene by PDX-1, MafA, and BETA 2 is unclear, functional interactions between these factors is important in control. For example, although PDX-1 alone is a poor stimulator of insulin enhancer-driven reporter expression, high-level co-operative activation is observed upon co-transfection with MafA in non-β cells (Zhao et al. 2005).

To compare the contribution of GG2, A1, and A3 to PDX-1/MafA-mediated activation of the human insulin gene, expression constructs for PDX-1 and MafA were co-transfected into HeLa cells with WT and PDX-1 binding-deficient site mutant versions of -251 Luc, a luciferase reporter driven by the nucleotides between -251 and +10 of the human insulin gene. As expected, none of the reporters significantly responded to PDX-1, but each was activated similarly by MafA alone (Fig. 2). Synergistic stimulation of -251 WT Luc was observed when PDX-1 and MafA were combined, however, the level of activation was attenuated by each of the PDX-1 site mutants (Fig. 2). These results indicated that PDX-1 binding to GG2 is involved in potentiating transcription of the human insulin gene.

To assess the relative importance of the GG2, A1, and A3 PDX-1 binding sites to human insulin gene expression in β cells, the WT and mutant versions of -251 Luc were transfected into βTC-3 cells. The GG2 mutant was approximately 75% less active than WT, while A1 mutant activity was only reduced by 55% despite binding PDX-1 much more effectively in gel shift assays (Fig. 3). In contrast, mutation of the high-affinity A3 element resulted in slightly enhanced activity compared with the WT construct (Fig. 3). These results indicate that PDX-1 binding to GG2 is critical to human insulin gene transcription in islet β cells.

Z-Region sequences mediate PDX-1 activation of the human insulin gene

It was surprising that the A3 mutant did not reduce -251 Luc activity, since preventing PDX-1 binding at this site severely diminishes activation of reporter constructs driven by either the corresponding sequences of the rat II insulin gene (-238 Luc) (Fig. 3), or human insulin control sequences extending to bp -342 (Petersen et al. 1994). Interestingly, the -342 to -252 region of human is not well conserved with rodent insulin (~50% identical), yet contains elements that contribute to β cell-selective and glucose-responsive activation of human insulin gene expression (Pino et al. 2005), including the Z control region between bp -292 and -243 (Sander et al. 1998). Consistent with a role in transcriptional activation, human insulin enhancer-driven reporters containing control sequences extending to nucleotides -342 and -293 were approximately 4- to 5-fold more active than -251 Luc (Fig. 4).

The effects of the PDX-1 site mutants on the extended -342 Luc and -293 Luc constructs were also measured in transfected βTC-3 cells and compared with their impact on -251 Luc activity. The A1 and GG2 mutations reduced -342 Luc and -293 Luc activity even more dramatically than in -251 Luc (Fig. 4C), suggesting that PDX-1 regulation is influenced by functional interactions with both distal and proximal control factors. Additionally, extending the control sequence to bp -293 and bp -342

(2005). The day before transfection, βTC-3 (10⁶) and HeLa (0-2 × 10⁵) cells were transferred into 35 mm wells. The lipofectamine reagent was used to introduce pcDNA3-MafA (0-25 µg), pcDNA3-PDX-1 (0-25 µg), pcDNA3 control (0-25 µg), WT or mut Insulin-Luc (0-25 µg), pHRL-TK (10 ng), pTK-CAT (1 µg), WT or mut Insulin-CAT (1 µg), and pRSV-Luc (0-5 µg). Dual luciferase (Promega), CAT (Nordeen et al. 1987), and luciferase (De Wet et al. 1987) enzymatic assays were performed on cell extracts prepared 40 to 48 hr after transfection. The Luc activity from pHRL-TK and pRSV-Luc served as controls for transfection efficiency in dual luciferase and CAT assays, respectively. The statistical significance of normalized Luc and CAT activities were determined by Student’s t-test. Each experiment was carried out three times with two independently isolated DNA preparations.
restored the sensitivity of these reporters to the mutation of A3 that prevents PDX-1 binding and activation at that site (Fig. 4C). Furthermore, the activity of the A3 mutants was reduced to a similar level (~45% of WT; Fig. 4C), specifically implicating the involvement of factors associated with Z region in modulating PDX-1 activation through the A3 site. To test this proposal, the region spanning bp -293 to -192, which contains both the Z region and the A3 site, was ligated upstream of the herpes virus thymidine kinase (TK) promoter in the pTK-CAT reporter vector (A3/Z TK-CAT). When transfected into TC-3 cells, A3/Z TK-CAT was approximately 2-fold more active than the empty TK-CAT vector (Fig. 5). Significantly, bp -293/-192 activation was impaired upon mutating the A3 site, demonstrating the involvement of PDX-1 in expression of the reporter (Fig. 5). These results not only indicate that PDX-1 is important in Z region-mediated activation of the human insulin gene, but it also likely reveals another fundamental difference in how PDX-1 activates human versus rodent insulin gene expression.

**Discussion**

PDX-1 is a key co-ordinator of processes involved in islet β function and pancreas development (Ahlgren et al. 1996). This factor was first isolated and has been principally characterized in the context of its integral role in insulin gene transcription. Thus, PDX-1 alone is capable of activating endogenous insulin expression in non-β cells (Serup et al. 1996, Watada et al. 1996), while siRNA knockdown of PDX-1 in β cell lines or primary islets decreases insulin mRNA levels (Iype et al. 2005).
PDX-1 also regulates glucose-responsive activation of the insulin gene (Petersen et al. 1994, Marshak et al. 1996). Although it is unclear how glucose stimulates PDX-1-mediated activation, interactions with other regulatory proteins are likely to be influenced by post-translational modifications of this factor. In the current study, we have shown the importance to human insulin transcription of PDX-1 binding to the novel GG2 site, and illustrated how communication between conserved A3/PDX-1 and the human Z-region impacts upon expression. These results also exemplify how PDX-1 activates human insulin differently from the more extensively characterized rodent genes, which may be of significance in control under normal and diabetic conditions in humans.

PDX-1 binds very effectively to the canonical insulin A1 and A3 elements in gel shift assays (Ohlsson et al. 1993, Peshavaria et al. 1994, Petersen et al. 1994), but can also interact with sites lacking the TAAT motif commonly associated with homeodomain protein binding, like human GG2 (GGAAAT) and the Nkx2·2 consensus (TTAAGT) (Le Lay et al. 2004). Our competition studies indicate that PDX-1 binds to A1 or A3 roughly 15- and 25-fold better than GG2, but only 2- to 3-fold more effectively that the Nkx2·2 site (Fig. 1). These findings most likely reflect the involvement of non-core flanking sequences in DNA binding recognition as proposed by Liberzon et al. (2004), and affirms that PDX-1 binding in vitro is not absolutely dependent on a TAAT core sequence. Unfortunately, it is not possible to directly determine if PDX-1 binds within the GG2 region of the human insulin gene in vivo due to the unavailability of human islet β cells. No suitable, well-characterized human β cell lines have been developed for this type of analysis, while the use of human islets is impractical, largely due to short supply. Furthermore, the method of choice for this type of study is the chromatin immunoprecipitation assay, which can detect PDX-1 binding within the endogenous mouse insulin genes (presumably to conserved A1 and A3) (Cissell et al. 2003), but is unable to distinguish occupancy of A3 from A1 (or GG2 in humans) because of their close proximity.

Despite binding to PDX-1 relatively poorly in vitro, a dysfunction mutation in GG2 was more detrimental to
human insulin gene enhancer-driven reporter activity than the A1 and A3 mutants in transfected β cells. Indeed, others have found that homeodomain-containing proteins function in a very precise and specific manner in vivo in spite of relatively low binding specificity and affinity in vitro (Mann 1995, Biggin & McGinnis 1997). One explanation for this apparent paradox is that target specificity is conferred through co-operation with other regulatory factors. This model is consistent with studies indicating that direct interactions between PDX-1, BETA2/E47 and MafA are important for maximal insulin gene expression (Peers et al. 1994, Qiu et al. 2002, Zhao et al. 2005). Our data showing that mutations in each of the PDX-1 binding sites within the human insulin control region negatively impact synergistic activation mediated by PDX-1 and MafA support this proposal. However, PDX-1 and MafA were recently found to only synergistically activate rodent, and not human, insulin enhancer-driven reporter expression (Docherty et al. 2005), a dissimilarity due possibly to differences in the human control sequences driving reporter expression and/or variations in the assayed cell lines.

The importance of PDX-1 interactions with other human insulin activator factors was also observed upon comparing mutant site-responsiveness of -342 Luc and -293 Luc to -251 Luc. Most strikingly, mutation of A3 did not have a detrimental impact on -251 Luc activity, a result that contrasts with PDX-1 regulation by the corresponding rodent insulin control region, wherein activity is compromised upon mutation (Peshavaria et al. 1994) (e.g. see -238 Luc in Fig. 2). A3 mutant sensitivity was restored by Z control region sequences located between nucleotides -293 and -251, an area poorly conserved with rodents. In fact, A3-dependent regulation was not only observed in the -342 Luc and -293 Luc constructs, but also in activation by the isolated Z/A3 region (Fig. 5).

Interestingly, GG2 and A1 mutant expression was more...
severely affected in the -342 Luc and -293 Luc backgrounds than in -251 Luc in β cells (Fig. 4C), implying that interactions between PDX-1 and factors binding within the distinctive Z region are fundamental to human insulin transcription. The activity of the Z region appears to be regulated by several distinct control elements, including a glucose-responsive factor unique to islets (Sander et al. 1998). Unfortunately, the factors controlling expression from this region have not been identified despite the presence of potential E box and palindromic regulatory elements (Sander et al. 1998, Pino et al. 2005).

Despite the awareness that deregulation of insulin gene expression contributes to diabetes in humans, a disproportionate focus has been directed toward using rodent insulin genes as the model of mammalian insulin transcription. The observed sequence homology between the human and rodent insulin regulatory regions (~60%) has led to the misconception that their mechanisms of control are also conserved, when in fact the arrangement of cis-acting elements are quite distinct between species (Read et al. 1993, Clark et al. 1995, Le Lay et al. 2004, Matsuoka et al. 2004, Neve et al. 2005). In addition, a polymorphic region unique to the human insulin gene termed the VNTR (variable number of tandem repeats), which extends 5′ from bp -360, has been mapped as a susceptibility locus for Type I diabetes (Bennett et al. 1995). In light of these dissimilarities, our studies emphasize novel ways the human and rodent insulin genes are regulated by PDX-1, a factor whose relationship to pancreas formation and β cell function in humans is well documented (Macfarlane et al. 1999). Work such as this underscores unique regulatory features of the human insulin gene, and provides insight into how β cell function is regulated under normal and diabetic conditions.

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