

Ectopic expression of the beta-cell specific transcription factor Pdx1 inhibits glucagon gene transcription

B. Ritz-Laser¹, B. R. Gauthier¹, A. Estreicher¹, A. Mamin¹, T. Brun¹, F. Ris², P. Salmon³, P. A. Halban², D. Trono³, J. Philippe¹

¹ Diabetes Unit, University Hospital Geneva, Geneva, Switzerland

² Jeantet Research Laboratories, Geneva, Switzerland

³ Department of Microbiology, Centre Médical Universitaire, Geneva, Switzerland

Abstract

Aims/hypothesis. The transcription factor Pdx1 is required for the development and differentiation of all pancreatic cells. Beta-cell specific inactivation of *Pdx1* in developing or adult mice leads to an increase in glucagon-expressing cells, suggesting that absence of Pdx1 could favour glucagon gene expression by a default mechanism.

Method. We investigated the inhibitory role of Pdx1 on glucagon gene expression in vitro. The glucagonoma cell line InR1G9 was transduced with a *Pdx1*-encoding lentiviral vector and insulin and glucagon mRNA levels were analysed by northern blot and real-time PCR. To understand the mechanism by which Pdx1 inhibits glucagon gene expression, we studied its effect on glucagon promoter activity in non-islet cells using transient transfections and gel-shift analysis.

Results. In glucagonoma cells transduced with a *Pdx1*-encoding lentiviral vector, insulin gene expression was induced while glucagon mRNA levels were reduced by

50 to 60%. In the heterologous cell line BHK-21, Pdx1 inhibited by 60 to 80% the activation of the α -cell specific element G1 conferred by Pax-6 and/or Cdx-2/3. Although Pdx1 could bind three AT-rich motifs within G1, two of which are binding sites for Pax-6 and Cdx-2/3, the affinity of Pdx1 for G1 was much lower as compared to Pax-6. In addition, Pdx1 inhibited Pax-6 mediated activation through G3, to which Pdx1 was unable to bind. Moreover, a mutation impairing DNA binding of Pdx1 had no effect on its inhibition on Cdx-2/3. Since Pdx1 interacts directly with Pax-6 and Cdx-2/3 forming heterodimers, we suggest that Pdx1 inhibits glucagon gene transcription through protein to protein interactions with Pax-6 and Cdx-2/3.

Conclusion/interpretation. Cell-specific expression of the glucagon gene can only occur when *Pdx1* expression extinguishes from the early α cell precursor. [Diabetologia (2003) 46:810–821]

Keywords Endocrine pancreas, Pdx1, Pax-6, Cdx-2/3, glucagon, transcriptional regulation, α -cell.

The mammalian pancreas arises as two evaginations, the dorsal and ventral pancreatic anlage, from the embryonic endoderm in the region of the future duode-

num. Determination of the pancreatic fate of the dorsal anlage requires signals from the notochord repressing endodermal Sonic hedgehog expression, whereas the ventral anlage forms independently from the notochord [1, 2]. In the mouse, the first two transcription factors expressed in the prospective pancreatic endoderm are *HB9* and *Pdx1*, which are detected at the 8 to 10 somite stage, respectively (embryonic day (E) 8–8.5) [3, 4, 5]; both factors are transiently expressed in the entire pancreatic anlage and are crucial for its development. Mice deficient in *HB9* selectively lack the dorsal pancreas due to a defect in specification of the pancreatic epithelium, whereas the ventral pancreas develops and exhibits more subtle defects in beta-cell differentiation and islet organization [3, 4]. Inacti-

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Corresponding author: B. Ritz-Laser PhD, Diabetes Unit, University Hospital Geneva, 24, rue Micheli-du-Crest, 1211 Geneva 14, Switzerland

E-mail: Beate.Laser@medecine.unige.ch

Abbreviations: E, embryonic day; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; PD, paired domain; HD, homeodomain; PDHD, paired-linker-homeodomain.

vation of *Pdx1* results in pancreatic agenesis through a failure in proliferation of the pancreatic epithelium as well as in the branching and differentiation of the pancreatic buds [5, 6]. The widespread expression of *HB9* and *Pdx1* in the embryonic pancreas declines at E9.5–10.5 (*HB9*) and E10.5 (*Pdx1*) and they are later restricted to beta cells (*HB9*) or beta and δ cells (*Pdx1*). Similarly, the homeobox gene *Isl-1* is transiently expressed in the entire dorsal bud and later restricted to pancreatic endocrine cells; mice deficient in *Isl-1* lack the dorsal exocrine pancreas and have no differentiated islet cells [7, 8, 9]. Another transcription factor expressed very early (E9) during development in discrete cells of the pancreatic epithelium is *Pax-6*, a member of the paired-homeobox family. *Pax-6* is crucial for the differentiation of glucagon-producing α cells inasmuch as *Pax-6* homozygous mutant mice have no or few α cells [10, 11]. In contrast, inactivation of *Pax-4*, another paired homeobox gene, results in the selective lack of beta and δ cells; mice lacking both *Pax-6* and *Pax-4* have no endocrine cells [12].

Several lines of evidence suggest that *Pdx1* plays a key role not only in islet cell differentiation but also in maintaining the differentiated state and control of islet-hormone gene expression. Pdx1 is a major transactivator of the insulin and somatostatin genes through its synergistic interaction with E47/Beta2 and Pax6 or Pbx/Prep1, respectively [13, 14, 15, 16, 17, 18]. Beta-cell specific inactivation of *Pdx1* in developing mice resulted in a decrease in beta-cells with a concomitant 2.5-fold increase in Glu+ cells and to coexpression of insulin and glucagon in 22% of cells suggesting that α -cell differentiation and glucagon gene expression are favored by the absence of *Pdx1* in vivo [19]. Furthermore, transient inhibition of *Pdx1* in pancreatic beta cells of adult mice by antisense RNA expression using a Tet-On system led to reduction of *Pdx1* dependent beta-cell specific gene expression and to a striking increase in the number of glucagon gene expressing cells that were homogeneously distributed within the islet [20]. Similarly, we could recently show in a Tet-On system in insulinoma cells that functional inactivation of *Pdx1* resulted in differentiation of insulin-producing beta cells into glucagon-producing α -cells [21].

In this work, we aimed to complement the studies of *Pdx1* function in beta cells by analysing the effect of ectopic *Pdx1* expression in α cells. Since α -cell specificity is mainly characterized by glucagon gene expression, we studied *Pdx1* effects on glucagon gene transcription in glucagonoma (InR1G9) cells by Lentiviral transduction and on glucagon promoter activity in non-islet cells. We show a new potential function of *Pdx1* as mediator of cell-specific expression of the glucagon gene through inhibition of transcription.

Materials and methods

Cell culture and DNA transfection. The glucagon-producing hamster InR1G9 [22] and mouse α TC1 [23], the insulin-producing hamster HIT-T15 [24], the non-islet Syrian baby hamster kidney (BHK-21) and human embryonic kidney (HEK) cell lines were grown in RPMI 1640 (Seromed; Basel, Switzerland) supplemented with 5% heat-inactivated fetal calf serum and 5% heat-inactivated newborn calf serum, 2 mmol/l glutamine, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin. BHK-21 and HEK cells were transfected by the calcium phosphate precipitation technique [25] using 10–15 μ g of total plasmid DNA per 10-cm petri dish. One μ g of pSV2A PAP, a plasmid containing the human placental alkaline phosphatase gene, driven by the simian virus 40 (SV40) early promoter was added to monitor transfection efficiency [26]. Transfection of InR1G9 cells was done using the DEAE-dextran method as described previously [27]. cDNAs for the hamster *Cdx-2/3* and *Pdx1* (M.S. German, University of California, San Francisco, Calif., USA), rat *Isl-1* (D. Drucker, University of Toronto, Toronto, Canada), mouse *Meis2* (N. Copeland, National Cancer Institute, Frederick, USA), and quail *Pax-6* (S. Saule, Institut Curie, Orsay, France), were cloned in the expression vector pSG5 (Stratagene). In experiments using variable quantities of expression vectors coding for *Pdx1*, *Pax-6*, or *Cdx-2/3*, total amount of DNA was kept constant by adding appropriate amounts of the empty vector pSG5. Reporter plasmids consisted of the CAT reporter gene driven by different fragments of the rat glucagon gene promoter (-292GluCAT, -138GluCAT, G3-138GluCAT, G3-31GluCAT [28] or the rat insulin I gene promoter (-410InsCAT, comprising 410 bp of the 5' flanking sequence and 49 bp of exon 1 and intron 1 of the rat insulin I gene) [29]. Data are presented as fold stimulation of the CAT activity obtained with the reporter plasmid alone and are the means \pm SEM of at least three experiments.

Transduction of InR1G9 cells with a lentiviral vector encoding hPDX1. A Human *PDX1* cDNA (*hPDX1*), kindly provided by V. Schwitzgebel (University Hospital Geneva, Geneva, Switzerland), was subcloned into pBluescript (Stratagene) and then inserted into an optimized HIV-based vector (pWPT, kindly provided by M. Wiznerowicz, Faculty of Medecine, Geneva, Switzerland), generating the pWPT-PDX transfer plasmid. Map and sequence are available at <http://www.medecine.unige.ch/~salmon/pWPT-PDX.html>. Stocks of pWPT-PDX lentiviral vectors were produced using transient cotransfection of 293T cells with pWPT-PDX, R8.91 (second generation HIV-packaging plasmid [30], and pMDG (VSV envelope-expression plasmid) as described previously [31]. Vectors were concentrated by ultracentrifugation and resuspended in serum-free medium (CellGro SCGM, CellGenix, Freiburg, Germany) as described previously [32]. Vector stocks were stored at -70°C and titres were determined by enzymatic assay of HIV reverse transcriptase. For hPDX1 transduction, 3×10^4 INR1G9 cells were transduced in 50 μ l of RPMI 1640 10% FCS with 2.5×10^4 pWPT-PDX transducing units (as determined by RT assay). After 24 h, cells were washed twice with RPMI 10% FCS and cultured for further 72 h before testing for *Pdx1* expression by immunocytochemistry using anti-Pdx1 antibodies kindly provided by C. V. E. Wright (Vanderbilt Medical Center, Nashville, Tenn., USA). RNA was isolated for northern blotting using Trizol (Invitrogen) and whole-cell protein extracts were prepared for EMSA. Northern blots were sequentially hybridized with random labelled complementary DNA (cDNA) probes for rat glucagon and proinsulin I and an 18S ribosomal (rRNA) oligonucleotide probe (5'-GCCGTCCTC-TTAATCATGGCCTCAGTTCC). Hybridization signals of the

pWPT-PDX transduced InR1G9 cells were quantified using a PhosphoImager (Molecular Dynamics) and expressed as glucagon mRNA/18S rRNA ratio relative to the control cells.

Quantitative RT-PCR. Reverse transcription was done by using 2 µg of total RNA isolated from HIT-T15 and from non-infected or lentivirus-infected InR1G9 cells, random hexanucleotide primers and Superscript II reverse transcriptase (Invitrogen). One tenth of the resulting cDNA was used for real-time PCR in the LightCycler instrument (Roche) using the QuantiTect SYBR Green PCR kit (Qiagen) as recommended by the provider. The following primers were used: insulin sense 5'-tcttctacacaccaagacc, antisense 5'-gtccacaatgccagcttc; glucagon sense 5'-gatcattccagcttccag, antisense 5'-ctgtaagggtcccttcagc; 28S rRNA sense 5'-tagccaatgcctctcatc, antisense 5'-acctctcatgtcttcc.

Chloramphenicol acetyltransferase (CAT) and protein assays. Cell extracts were prepared 48 h after transfection and analysed for CAT and alkaline phosphatase activities as described previously [28]. Quantification of acetylated and non-acetylated forms was done with a PhosphorImager (Molecular Dynamics). A minimum of three independent transfections was done; each of them carried out in duplicate.

Electrophoretic Mobility Shift Assays (EMSA). Nuclear extracts from InR1G9, HIT-T15 or BHK-21 cells transfected with *Pax-6* or *Pdx1* expression plasmids or the empty vector pSG5 were prepared according to [33]. EMSAs were carried out as described previously [34] adding first 8 to 28 µg (8 µg in standard reactions) of nuclear extracts and subsequently labelled oligonucleotides containing the rat glucagon gene G1 (G1-56, G1-52, G1-54, G1-50) or G3 (5'-GCTGAAGTA-GTTTTTCACGCCTGACTGAGATTGAAGGGTGTATTTC) sequence [35, 36], or the A3/A4 element of the rat insulin I gene (InsA3/A4, 5'-GATCTTGTTAATAATCTAATTACCCT-AGAACAATTATTAGATTAATGG) [37]. In competition experiments, labelled and cold oligonucleotides were added simultaneously and when specific antibodies were used, they were added after half of the incubation time. Antisera were generously provided by H. Edlund (anti-Pdx1; University of Umea, Umea, Sweden), S. Saule (anti-Pax-6 homeodomain, serum no. 13; Institut Curie, Orsay, France), D. Melloul (anti full-length Pdx1; Hebrew University Hadassah Medical Center, Jerusalem, Israel), and M.S. German (anti-Cdx-2/3; University of California, San Francisco, Calif., USA).

Mutagenesis of Pdx1. In vitro mutagenesis was done by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) according to the manufacturer's protocol using the following primers for the I192Q mutation of *Pdx1*: forward 5'-gagacacatacaagcagtgtgtccaaaacc and reverse 5'-ggttttggaaccactgcttgatgtctc.

GST fusion proteins and GST-precipitation. Hamster Pax-6 GST fusion proteins have been described [36]. GST fusion proteins were expressed in *E. coli* and purified according to the manufacturer recommendations (Pharmacia). L-[³⁵S]methionine-labelled Pdx1 was generated in vitro using the TNT wheat germ extract system (Promega) and GST-precipitations were done as described previously [36].

Data analysis. Data are presented as means ± SE and statistical significance was tested by analysis of variance and Student's *t* test where applicable. The threshold for statistical significance was a *p* value of less than 0.05 (* and ** indicate statistical significance with *p*<0.05 and *p*<0.01, respectively).

Results

Lentiviral transduction of Pdx1 in InR1G9 cells inhibits transcription of the endogenous glucagon gene. To assess the effect of ectopic *Pdx1* expression in glucagon-producing cells, we transduced the hamster glucagonoma cell line InR1G9 with a lentiviral vector coding for *hPdx1*. After 4 days, expression of Pdx1 in two independent transductions of InR1G9 cells was confirmed by immunocytochemistry and EMSA in 90% of the cells (data not shown). Northern blot and real-time PCR analysis showed that *Pdx1* repressed endogenous glucagon mRNA levels by 50% and induced the endogenous insulin gene in the glucagonoma cell line (Fig. 1).

Pdx1 inhibits transcriptional activation of the glucagon gene promoter by Pax-6 and Cdx-2/3. To investigate the mechanisms by which *Pdx1* decreases glucagon mRNA levels, we analysed its effect on transactivation of glucagon gene reporter constructs in the non-islet cell line BHK-21. The paired-homeodomain protein Pax-6, which is essential for the development of α cells, acts as a major transactivator of the glucagon gene by its interaction with the α-cell-specific promoter element G1 and the enhancer G3 [10, 11, 36]. In addition, the homeodomain protein Cdx-2/3 transactivates the glucagon gene promoter independently through G1 and acts synergistically with Pax-6 [36, 38]. Since Pdx1 has been reported to interact with both Pax-6 and Cdx-2/3 in vitro [15, 38], we investigated the effects of Pdx1 on Pax-6 and Cdx-2/3 induced activation of glucagon gene promoter constructs. Overexpression of *Pdx1* resulted in a dose-dependent inhibition, up to 90%, of Pax-6 mediated transcriptional stimulation whether G1 (-138GluCAT), G3 (G3-31GluCAT), or both G1 and G3 (G3-138GluCAT) were present in the reporter construct (Fig. 2). Pdx1 is thus able to inhibit Pax-6 mediated activation of glucagon gene expression through both the G1 and G3 elements in BHK-21 cells. In addition to its inhibitory action on Pax-6, Pdx1 reduced the Cdx-2/3 and Pax6/Cdx-2/3-mediated activations of G1 (-138GluCAT) by 65% and 85%, respectively (Fig. 2C). Control cotransfections with *Isl-1* [39] and *Meis2* [40] had no effect on Pax-6 or Cdx-2/3 induced transcriptional activation (Fig. 2C).

When *Pdx1* was transfected in BHK-21 cells in the absence of Pax-6 and Cdx-2/3, CAT activity driven by -138Glu was stimulated (Fig. 2C, Fig. 3). This effect was dose-dependent and quantitatively similar to the activation observed with the rat insulin I promoter construct -410InsCAT (Fig. 3). However, the maximal 4.6-fold stimulation of the glucagon gene promoter by *Pdx1* was weak when compared to that obtained with Pax-6 or Pax6/Cdx-2/3.

Pdx1 binds to the G1 element. The G1 element of the glucagon gene promoter contains three AT-rich se-

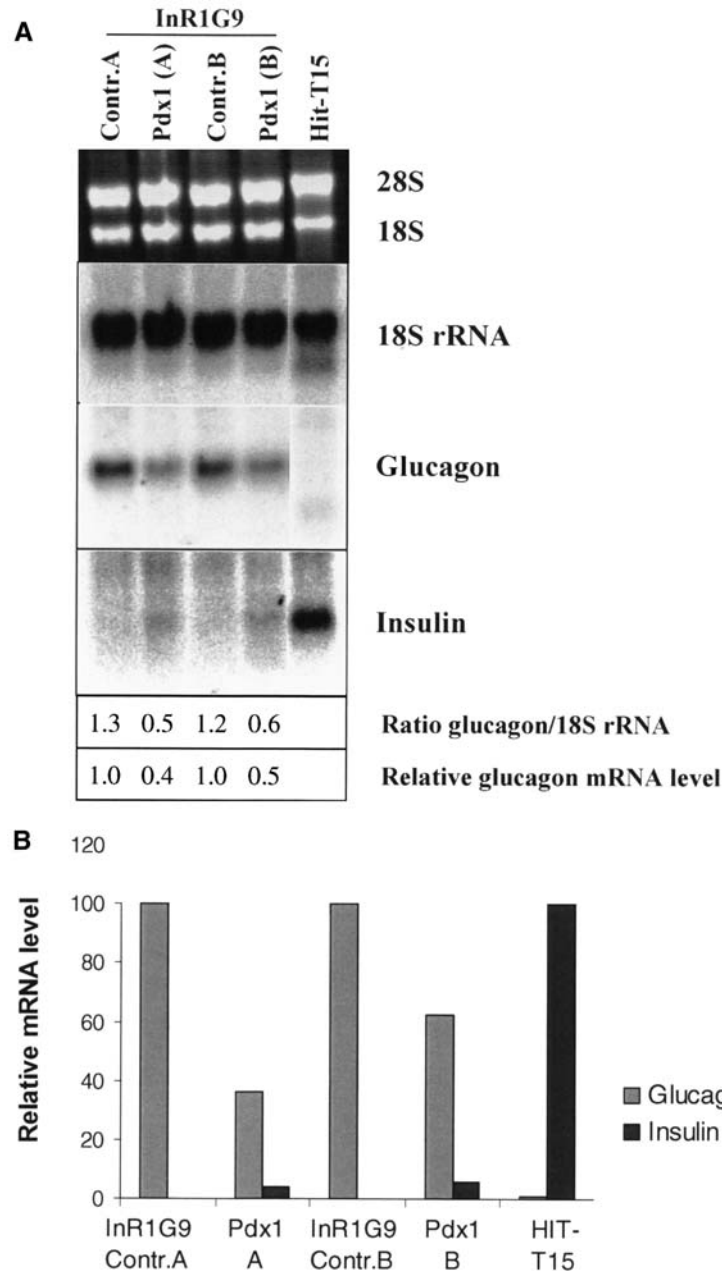


Fig. 1A, B. Pdx1 induces insulin mRNA and reduces endogenous glucagon mRNA levels in glucagonoma cells. Northern blot (A) and Real-time RT-PCR (B) analysis of glucagon-producing InR1G9 cells (contr. A, contr. B), from two experiments of unselected InR1G9 cellpools transduced with a lentiviral vector coding for *hPdx1* (Pdx1 A, Pdx1 B), and Hit-T15 cells. The proportion of infected cells was roughly 90% (immunocytochemistry), but Pdx1 infected cellpool A showed a slightly stronger Pdx1 protein complex in EMSA that cellpool B. (A) Northern blot sequentially hybridized with probes for insulin, glucagon and 18S rRNA. (B) Quantitative RT-PCR showing that *Pdx1* reduces glucagon mRNA levels to roughly 36% and 62% of wild-type levels (cellpool A and B, respectively). Whereas insulin mRNA was undetectable in noninfected InR1G9 cells by Northern blot (A), quantitative RT-PCR provided evidence of as little as 0.2% of insulin mRNA levels found in HIT-T15 cells. Pdx1 infection increased insulin mRNA levels by 21 and 27-fold reaching 4% and 6% (cellpool A and B, respectively) of HIT-T15 insulin mRNA levels

quences, two of which form a nearly perfect direct repeat with binding sites for the homeodomain containing proteins Pax-6 and Cdx-2/3 (Fig. 4A) [35, 36, 38, 41]. Since *Pdx1* is a member of the *Antennapedia* family of homeodomain proteins [42] and able to transactivate -138Glu in the absence of Pax-6 and Cdx-2/3, we tested whether Pdx1 could bind to the glucagon gene promoter. With glucagon-producing InR1G9 cell extracts, at least three protein complexes are formed on G1-56 comprising the direct repeat element within G1; Pax-6 as a monomer, Pax-6/Cdx-2/3 as a heterodimer, and HNF3 β [36, 43] (Fig. 4A,B, G1-56). Using nuclear extracts from the insulin-producing cell line HIT-T15, which contains Pdx1, an additional complex was detected. This band co-migrated with a complex formed in BHK-21 cells overexpressing *Pdx1* and was recognized by anti-Pdx1 antibodies.

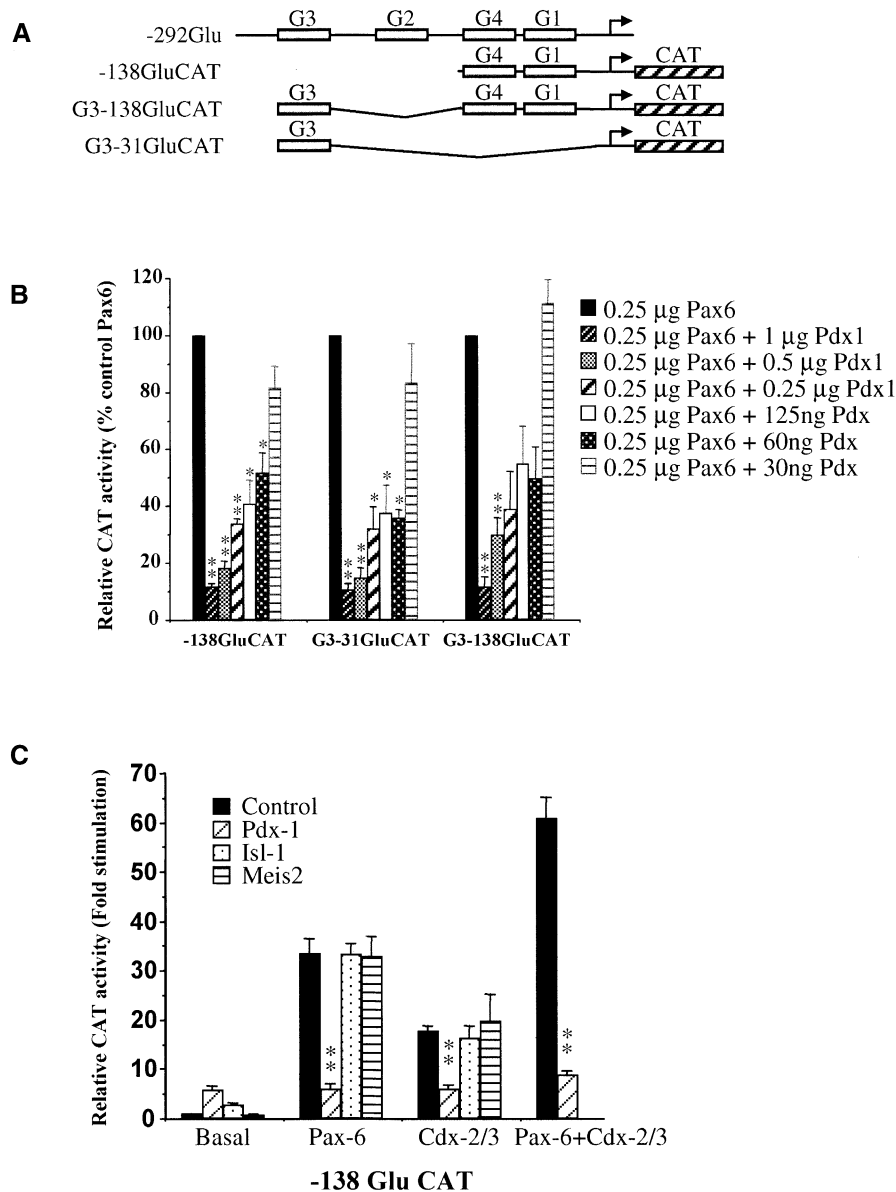


Fig. 2A–C. *Pdx1* interferes with transcriptional activation of the glucagon gene promoter by *Pax-6* and *Cdx-2/3*. (A) Schematic representation of glucagon reporter gene constructs used in this study. Characterized cis-acting control elements are designated as G1 to G4. (B) Dose-dependent inhibition of the *Pax-6*-mediated activation of the glucagon gene elements G1 (-138GluCAT) and/or G3 by *Pdx1* in BHK-21 cells. (C) To assess their effect on *Pax-6* and/or *Cdx-2/3* mediated activation of the glucagon gene promoter, BHK-21 cells were cotransfected with 1 µg of *Pdx1*, *Pax-4*, *Meis2*, and/or *Isl-1* expression vectors, 250 ng of expression vectors containing the *Pax-6* and/or *Cdx-2/3* cDNAs and 10 µg of -138GluCAT

Pdx1 is thus able to interact with the G1 element. To localize the binding site of *Pdx1* within G1, we used oligonucleotides G1–54, G1–52, and G1–50 representing the three AT-rich elements. *Pdx1* bound to all these sites (Fig. 4B) and is thus capable of interacting with three AT-rich motifs within the first 100 bp of the glucagon gene promoter.

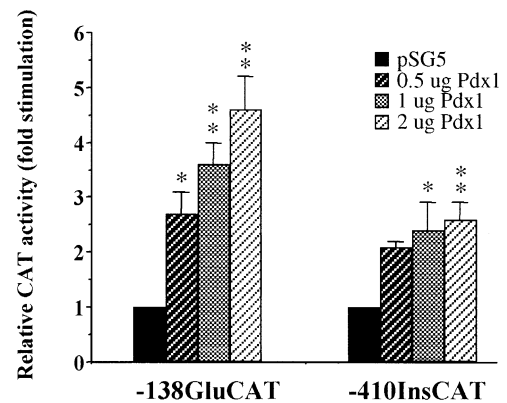


Fig. 3. Dose-dependent activation of the glucagon gene element G1 by *Pdx1*. BHK-21 cells were cotransfected with 10 µg of the indicated reporter plasmids and increasing amounts of *Pdx1* expression vectors

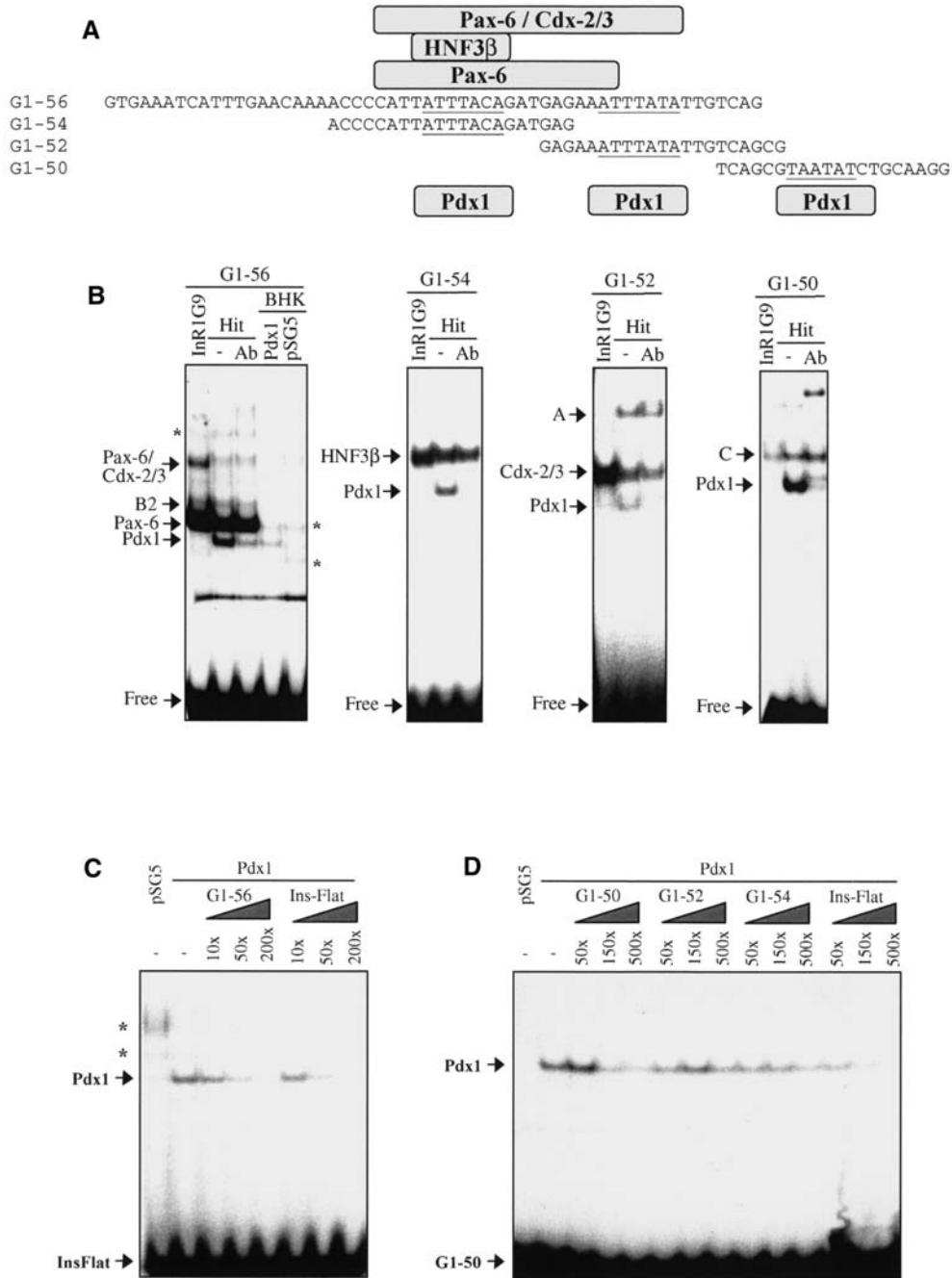


Fig. 4A–D. Pdx1 binds to three sites within the glucagon gene G1 element. (A) Schematic diagram illustrating oligonucleotides for the glucagon gene G1 element and protein complexes formed on G1. The AT-rich elements are underlined. (B) EMSA using nuclear extracts from InR1G9, Hit-T15, or BHK-21 cells overexpressing *Pdx1*. Pdx1 was able to bind the three AT-rich elements contained within G1. Ab indicates the addition of anti-Pdx1 antibodies. A and C represent yet unidentified protein-DNA complexes. (C, D) Nuclear extracts of BHK-21 cells overexpressing *Pdx1* were incubated with the InsA3/A4 or G1-50 oligonucleotide and the indicated excess of cold competitor oligonucleotides. Bold arrowheads and asterisks indicate specific and non-specific complexes, respectively

To assess the relative affinity of Pdx1 for G1 compared to the A3/A4 of the rat insulin I gene, a known Pdx1 binding site, we carried out EMSA competition experiments. A similar molar excess of cold G1-56 and InsA3/A4 oligonucleotides competed for the Pdx1 complex formed with InsA3/A4 indicating that Pdx1 binds to both elements with roughly the same affinity (Fig. 4C). These data are in agreement with the equivalent transactivation of the glucagon and insulin gene promoters by *Pdx1*. When the individual glucagon gene binding sites were used for EMSA, the most proximal motif G1-50 displayed a slightly higher affinity for Pdx1 compared to G1-52 and G1-54 located within G1-56 but lower compared to InsA3/A4 (Fig. 4D).

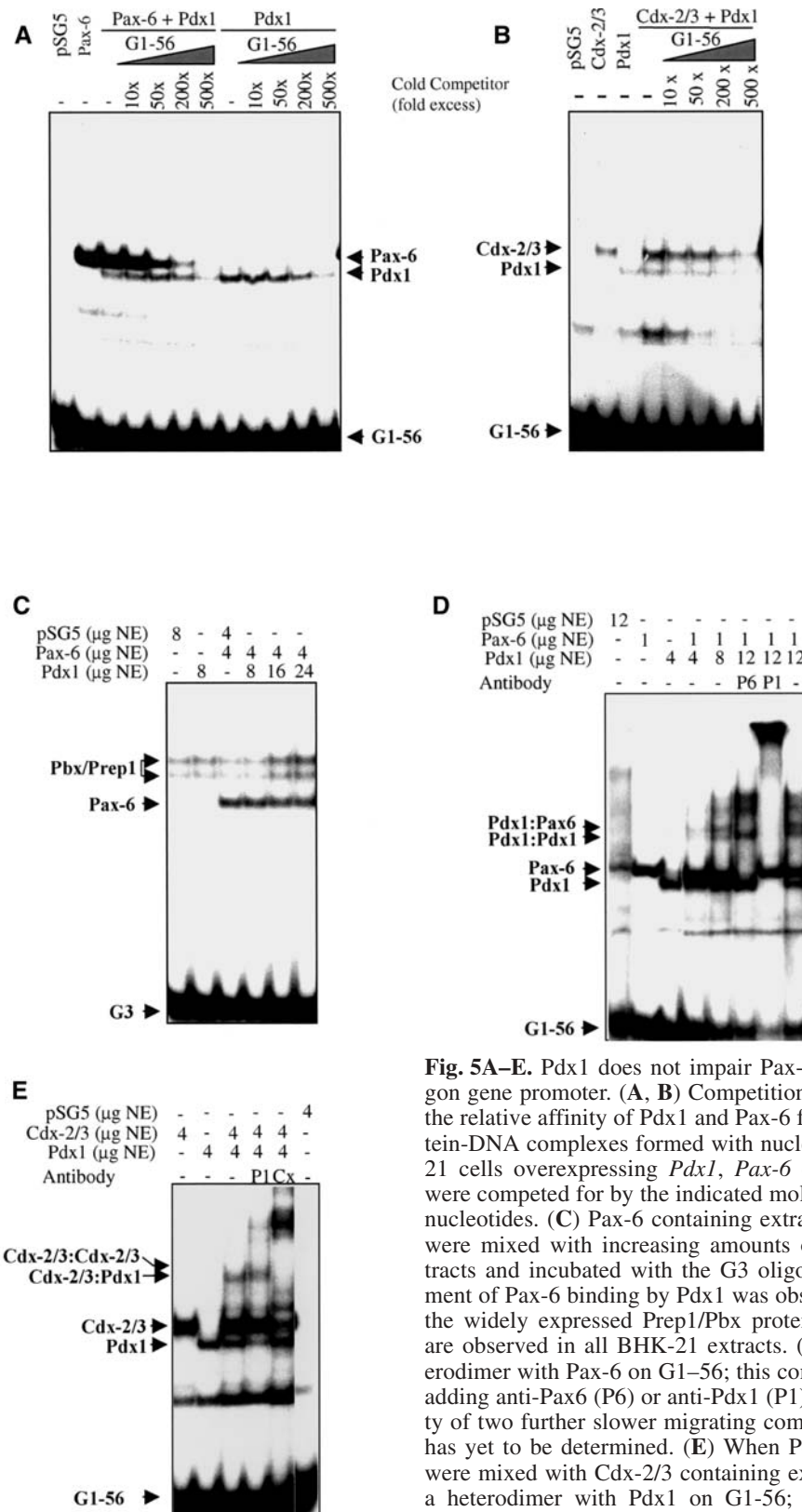


Fig. 5A–E. Pdx1 does not impair Pax-6 binding to the glucagon gene promoter. (**A, B**) Competition experiments analysing the relative affinity of Pdx1 and Pax-6 for the G1 element. Protein-DNA complexes formed with nuclear extracts from BHK-21 cells overexpressing *Pdx1*, *Pax-6* or *Cdx-2/3* and G1-56 were competed for by the indicated molar excess of cold oligonucleotides. (**C**) Pax-6 containing extracts from BHK-21 cells were mixed with increasing amounts of Pdx1 containing extracts and incubated with the G3 oligonucleotide. No impairment of Pax-6 binding by Pdx1 was observed. Heterodimers of the widely expressed Prep1/Pbx proteins formed on G3 [44] are observed in all BHK-21 extracts. (**D**) Pdx1 formed a heterodimer with Pax-6 on G1-56; this complex was displaced by adding anti-Pax6 (P6) or anti-Pdx1 (P1) antibodies. The identity of two further slower migrating complexes containing Pdx1 has yet to be determined. (**E**) When Pdx1 containing extracts were mixed with Cdx-2/3 containing extracts, Cdx-2/3 formed a heterodimer with Pdx1 on G1-56; this complex was displaced by adding anti-Cdx-2/3 antibodies. Note that when anti-Pdx1 antibodies were used, the Cdx-2/3-Pdx1 heterodimer was displaced and a Cdx-2/3 homodimer with only slightly slower migration than Cdx-2/3-Pdx1 was formed

Pdx1 forms heterodimers with Pax-6 and Cdx-2/3 on G1. To investigate the mechanism by which Pdx1 affects the Pax-6 and Cdx-2/3 mediated activation of the glucagon gene, we assessed whether Pdx1 could affect Pax-6 and Cdx-2/3 binding to DNA *in vitro*. When BHK-21 nuclear extracts containing Pax-6 were mixed with Pdx1-containing extracts, Pdx1 formed a weaker complex on G1-56 as compared with the binding reaction without Pax-6 (Fig. 5A). Furthermore, competition with cold G1-56 showed a better affinity of Pax-6 compared with Pdx1 for G1. By contrast with Pax-6, Cdx-2/3 and Pdx1 displayed similar affinity for G1-56 (Fig. 5B).

Alternatively to a mechanism of direct competition for the binding site, Pdx1 could interact with Pax-6 and/or Cdx-2/3 and either form a heterodimer or impair DNA binding by protein to protein interaction. We therefore analysed the effect of adding various amounts of Pdx1 on Pax-6 binding to G3 and G1. Pdx1 overexpressed in BHK-21 cells did not interact with G3, only heterodimers of the ubiquitous proteins Pbx and Prep1 [44] were observed (Fig. 5C). Pax-6 formed a complex with G3 that was not affected by the addition of increasing amounts of Pdx1-containing extracts (Fig. 5C). In contrast to G3, Pdx1 bound as a monomer and a homodimer to G1 (Fig. 5D). In addition, a Pax6:Pdx1 heterodimer was formed that was recognized by both anti-Pdx1 and anti-Pax-6 antibodies, thus confirming the protein to protein interaction of both transcription factors (Fig. 5D). When Pdx1 and Cdx-2/3 containing extracts were mixed, both proteins bound as monomers and Pdx1 did not impair Cdx-2/3 binding. In addition, both proteins formed a heterodimer that was recognized by anti-Cdx-2/3 and anti-Pdx1 antibodies (Fig. 5E). Note that when anti-Pdx1 antibodies were used, the Cdx-2/3-Pdx1 heterodimer was displaced by a Cdx-2/3 homodimer with only slightly slower migration than Cdx-2/3-Pdx1. We therefore conclude that Pdx1 could exert its transcriptional inhibition on the glucagon gene promoter through protein to protein interaction with Pax-6 and Cdx-2/3 that could change their conformation and interfere with their transcriptional properties. Furthermore, inhibition of Pax-6 mediated activation could also in part be mediated by competition of binding to the G1 but not the G3 element.

Pdx1 interacts with the homeodomain of Pax-6. To test for direct physical interactions of Pdx1 and Pax-6, we did GST precipitation assays using GST fusion proteins containing either the Pax-6 paired domain (PD), homeodomain (HD) or paired-linker-homeodomain (PDHD) and ³⁵S-labelled Pdx1. Protein to protein interactions with Pdx1 were observed with fusion proteins comprising the Pax-6 HD (10% of the input), whereas precipitation of Pdx1 with the Pax-6 PD was weak. However, adding the PD and linker domain to the Pax-6 HD increased interaction to 17% (Fig. 6).

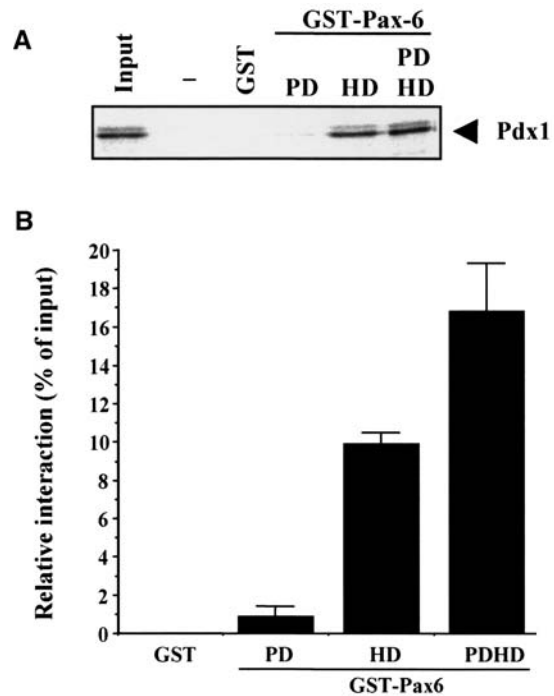


Fig. 6A, B. Pdx1 interacts with the HD of Pax-6. (A) GST-precipitation assay using 10 μ g of GST alone, GST-Pax-6 paired (PD), homeo (HD) or paired-linker-homeodomain (PDHD), fusion proteins immobilized on sepharose beads and *in vitro* synthesized, ³⁵S-labelled Pdx1. Lane input, 10% of the respective *in vitro* translation reaction used for protein to protein interaction. (B) Quantification of protein interactions shown in (A) as the precipitation rates relative to the respective input. Data are presented as the mean \pm SEM of at least three experiments

These data show that Pdx1 interacts with Pax-6 in the absence of DNA and that major contacts are mediated by the Pax-6 homeodomain although the linker domain and/or PD might be required for full interactions.

Inhibition of Pax-6 and Cdx-2/3 mediated activation is independent of Pdx1 DNA binding. To further test our hypothesis that Pdx1 represses Pax-6 and Cdx-2/3 mediated activation of the glucagon gene promoter through protein to protein interactions, we mutated *Pdx1* in an amino acid (I192Q) of the DNA recognition helix within the homeodomain that has been reported to abolish binding to the A3/A4 element of the rat insulin I gene promoter [45]. This mutant Pdx1 protein was produced at equivalent levels in fibroblast cells and in *in vitro* translation assays (Fig. 7A), and its binding activity to the G1 element was reduced by more than 90% (Fig. 7B).

In cotransfection assays, a quantitatively similar repression of Cdx-2/3 was observed with the Pdx1 wt and mutant I192Q protein (Fig. 7C) confirming that the inhibition of Cdx-2/3 mediated transcriptional activation is independent of Pdx1 DNA binding and rather occurs through protein to protein interactions. In contrast, repression of Pax-6 mediated activation was attenuated by the Pdx1 mutation (78% and 54%

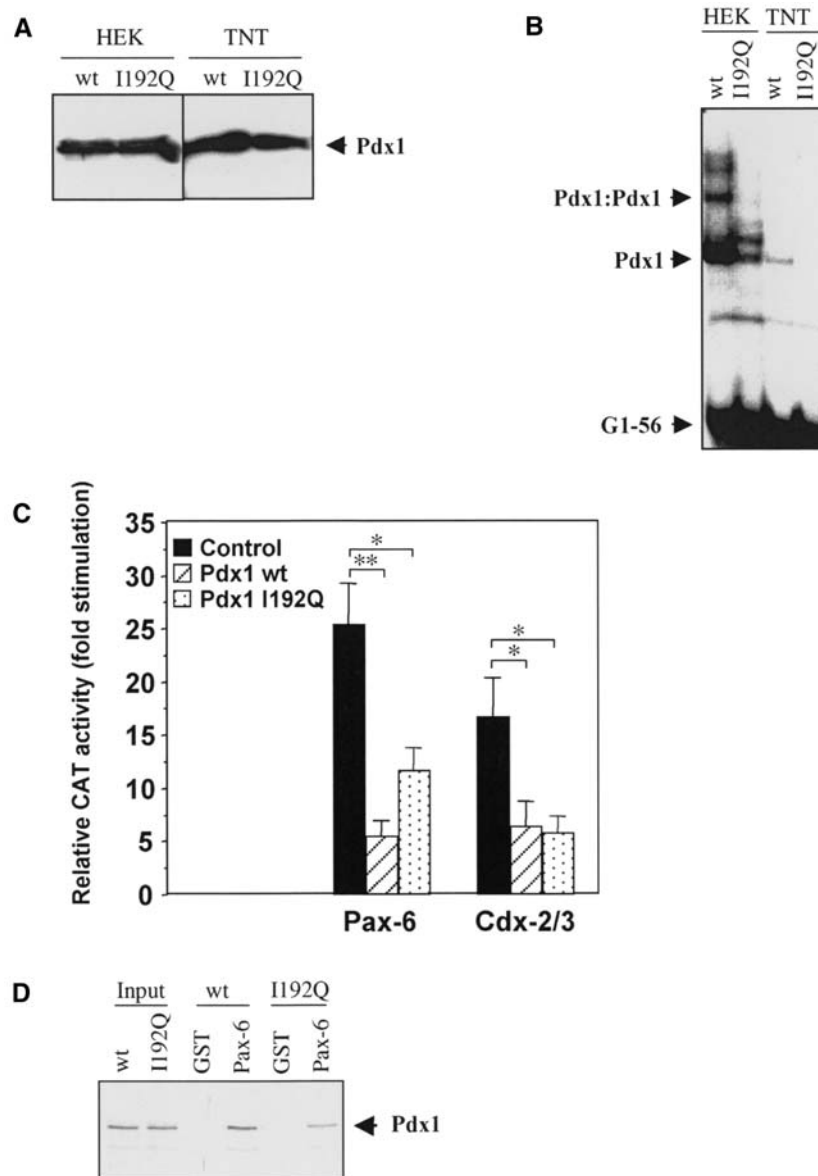


Fig. 7A–D. Mutant Pdx1 protein impaired in DNA binding still repressed transcriptional activation of the glucagon gene promoter by Pax6 and Cdx-2/3. **(A)** Western blot analysis showing similar synthesis of wild-type (wt) and mutant (I192Q) Pdx1 in HEK cells and in in vitro transcription/translation (TNT). **(B)** EMSA analysis showing 90% reduced binding activity of Pdx1 I192Q to G1 as compared to Pdx1 wt. **(C)** To assess the effect of mutant *Pdx1* on *Pax-6* and *Cdx-2/3* mediated activation of the glucagon gene promoter, BHK-21 cells were cotransfected with 1 μ g of wt *Pdx* or *Pdx1* I192Q expression vectors and 250 ng of expression vectors containing the *Pax-6* or *Cdx-2/3* cDNAs as well as 10 μ g of -138GluCAT. **(D)** GST-precipitation assay using 10 μ g of GST alone or GST-Pax-6 paired-linker-homeodomain fusion protein immobilized on sepharose beads and in vitro synthesized, 35 S-labelled Pdx1 wt or I192Q. Lanes input, 10% of the respective in vitro translation reactions used for protein to protein interaction

by wt and I192Q Pdx1, respectively). We therefore tested by GST pull-down whether the I192Q mutation affected interactions between Pdx1 and Pax-6. Protein to protein interactions of both transcription factors were reduced by approximately 65% indicating that the Pdx1 homeodomain is critical for protein interactions with Pax-6 (Fig. 7D). Since the reduced repressor activity of Pdx1 I192Q on Pax-6 corresponds quantitatively with the decreased protein to protein interaction of both factors, we suggest that Pdx1 could exert its repressor activity primarily through protein to protein interaction. Our data cannot, however, rule out a contribution of DNA binding competition to its inhibitory effect on Pax-6.

Discussion

We show that *Pdx1* is able to act as a repressor of the endogenous glucagon gene in InR1G9 glucagonoma

cells while simultaneously inducing insulin gene transcription. Similarly, *Pdx1*-induction of insulin and islet-amyloid-polypeptide gene expression has been shown in stable clones of glucagon-producing AN697 and α TC1 cells, but in these studies, no effect on glucagon gene expression has been observed [46, 47]. Although we have no clear explanation for this discrepancy, our recent results showing de-repression of the glucagon gene through overexpression of a dominant-negative Pdx1 protein in insulinoma cells [21] and in vivo data showing de-repression of glucagon gene expression by functional inactivation of *Pdx1* in beta cells of developing and adult mice [19, 20] support our findings. However, these studies do not discriminate between direct effects of *Pdx1* on glucagon gene expression and indirect effects mediated by the transcriptional network of the beta cell. Overexpressing *Pdx1* in glucagonoma cells allowed us to assess the role of Pdx1 on the relative expression of the insulin and glucagon genes in the absence of other beta-cell specific factors and demonstrates the critical direct role of Pdx1 in the cell's ability to transcribe these genes.

The inhibitory effect of Pdx1 on glucagon gene expression is mediated in vitro by impairing Pax-6 and Cdx-2/3 driven activation of the G3 and G1 promoter elements. Pdx1 did not interact directly with G3 and although it bound to three AT-rich elements within G1, two of which are binding sites for Pax-6 and Cdx-2/3, its relative affinity was lower as compared to Pax-6. Pdx1 is subject to post-translational modifications such as phosphorylation altering its subcellular localization [48]. We cannot exclude that these modifications could enhance Pdx1 binding to the G1 element in vivo. However, the DNA binding deficient Pdx1 mutant I92Q repressed Cdx-2/3 mediated activation quantitatively similarly to the wild-type protein and although Pax-6 driven activation was reduced with Pdx1 I92Q, protein-to-protein interactions with Pax-6 were equally affected. Moreover, since Pdx1 repressed Pax-6 mediated activation also through G3, a paired box binding site, we suggest that the major molecular mechanism of transcriptional inhibition by Pdx1 could be through protein to protein interaction although we cannot exclude a contribution of DNA binding competition to its inhibitory effect on Pax-6.

Two mechanisms might thus explain the repression of transcriptional activation by Pdx1; (i) through direct interactions with Pax-6 and Cdx-2/3, Pdx1 could change their conformations thereby impairing contacts to coactivator proteins or favour contacts with corepressors. We show by GST precipitation assays protein to protein interactions between Pdx1 and Pax-6 that are mediated in part by the Pax-6 homeodomain and the capacity of Pdx1 to form a heterodimer with both Pax-6 and Cdx-2/3. Similarly, Pdx1 has also been shown to interact with Cdx-2/3 in solution [49] thus

potentially neutralizing Cdx-2/3 function independent of DNA binding. In the same study, Pdx1 was able to inhibit the Cdx-2/3 mediated activation of the sucrose-isomaltase gene in heterologous cells. (ii) Alternatively, free Pdx1 might efficiently compete for common coactivators and impair their interaction with DNA-bound transcription factors thus blocking transcriptional activation. In this regard it is interesting that overexpression of Pdx1 in insulin-producing cells has recently been shown to repress insulin promoter activity [50]. Similarly, an excess of Pdx1 impaired its synergy with E47 on the insulin mini-enhancer in non-islet cells [16]. One common cofactor linking specific transcription factors to the basal transcription machinery, CBP/p300, is able to physically interact with Pax-6, Cdx-2/3, and Pdx1 [38, 51]. We therefore cotransfected BHK-21 cells with increasing amounts of *CBP* or *p300*; however, this complementation with transcriptional coactivators failed to compensate for the transcriptional repression by Pdx1. Pax-6 contains an unusually long transactivation domain that has been reported to directly interact with the TATA binding protein within TFIID (TBP) [52]. Pdx1 could thus impair Pax-6 interactions with TBP or alternatively, act by disturbing a multifactorial transcription complex by altering the ratio of its free components.

Pdx1 has been implicated in the tissue-specific activation of different genes through cell type and promoter context dependent protein to protein interactions. In acinar cells, Pdx1 associates with Pbx1 and Meis2 on the B element of the elastase I gene [40] and endocrine-specific complexes consist of Pdx1/Pbx, Pdx1/Pax-6 or E47/Beta2/Pdx1 on the somatostatin and insulin gene promoter, respectively [13, 14, 15, 16, 17]. These protein contacts allow for cooperative DNA binding and synergistic transcriptional activation. We show a new potential function of Pdx1 as mediator of cell-specific expression of the glucagon gene through its inhibition of Pax-6 and Cdx-2/3 mediated transcription.

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