Pdx1 Level Defines Pancreatic Gene Expression Pattern and Cell Lineage Differentiation*

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Haiyan Wang‡§, Pierre Maecler‡, Beate Ritz-Laser¶, Kerstin A. Hagenfeldt‡,
Hisamitsu Ishihara‡, Jacques Philippe‡, and Claes B. Wollheim‡§

From the ‡Division of Clinical Biochemistry and the ¶Diabetes Unit, Department of Internal Medicine,
Geneva University Medical Center, CH-1211 Geneva 4, Switzerland

The absence of Pdx1 and the expression of brain-4 distinguish α-cells from other pancreatic endocrine cell lineages. To define the transcription factor responsible for pancreatic cell differentiation, we employed the reverse tetracycline-dependent transactivator system in INS-I cell-derived subclones INSrβ and INSrβ to achieve tightly controlled and conditional expression of wild type Pdx1 or its dominant-negative mutant, as well as brain-4. INSrβ cells express not only insulin but also glucagon and brain-4, while INSrβ cells express only insulin. Overexpression of Pdx1 eliminated glucagon mRNA and protein in INSrβ cells and promoted the expression of β-cell-specific genes in INSrβ cells. Induction of dominant-negative Pdx1 in INSrβ cells resulted in differentiation of insulin-producing β-cells into glucagon-containing α-cells without altering brain4 expression. Loss of Pdx1 function alone in INSrβ cells, which do not express endogenous brain-4 and glucagon, was also sufficient to abolish the expression of genes restricted to β-cells and to cause α-cell differentiation. In contrast, induction of brain-4 in INSrβ cells initiated detectable expression of glucagon but did not affect β-cell-specific gene expression. In conclusion, Pdx1 confers the expression of pancreatic β-cell-specific genes, such as genes encoding insulin, islet amyloid polypeptide, Glut2, and Nkx6.1. Pdx1 defines pancreatic cell lineage differentiation. Loss of Pdx1 function rather than expression of brain4 is a prerequisite for α-cell differentiation.

The pancreatic islets of Langerhans are composed of four different endocrine cell types: glucagon- (α), insulin- (β), somatostatin- (δ), and pancreatic polypeptide-producing cells (1). These four endocrine cell lineages are differentiated from a common neurogenin3-expressing precursor (2–6). Targeted disruption of mouse genes encoding various transcription factors has demonstrated their importance in the control of islet cell development and differentiation. The homozygous deletion of the Pdx1/Ipf1/Id1x1/Sft1 gene in mice (7, 8) and in a patient (9) causes pancreas agenesis. In neuroD-null mice, the endocrine (α, β, and δ) cell mass is markedly reduced (10). In Isl1-deficient mice, the dorsal pancreatic mesenchyme and four hormone-producing endocrine cell lineages are deleted (11). Homozygous disruption of the Nkx2.2 gene in mice results in reduced α and pancreatic polypeptide cell mass as well as defective β-cell differentiation (12). Pax6 is indispensable for α-cell development (13, 14), whereas homozygous Pax4-null mice lack β- and δ-cells (15). Nkx6.1 is required for mature β-cell differentiation and β-cell neogenesis during the secondary transition (16).

The β-cell-specific inactivation of the Pdx1 gene in mice has revealed that Pdx1 is required for maintaining the β-cell phenotype by positively regulating insulin expression and by repressing glucagon expression (17). Furthermore, brain4 has been suggested to confer the pancreatic α-cell specificity (18). The absence of Pdx1 and the expression of brain4 are characteristics of the mature islet α-cell lineage (5, 6, 8, 18–20). Nkx6.1 is exclusively expressed in islet β-cells after embryonic day 13 (17, 21, 22) and is required for mature β-cell differentiation (16). Pax4, which is expressed only transiently in the fetal pancreas and functions as a transcriptional repressor of the glucagon promoter, plays a significant role in the development and differentiation of islet β-cell lineage (15, 23–25). It has been demonstrated that Pdx1 binds to the promoters of the Nkx6.1 and Pax4, and Pdx1 is also necessary for Nkx6.1 expression (17, 25, 26). However, the correlation of Pdx1 function with the expression of brain4 and Pax4 in the transcriptional hierarchy has not been well defined.

The pluripotent property of islet tumor cells has been described and used as a model to study islet cell differentiation and to identify islet cell-specific transcription factors (18, 20, 21, 27–29). INS-1 cells, which express endogenous pancreatic transcription factors Pdx1, neuroD, Isl1, Pax4, Pax6, Nkx2.2, Nkx6.1, HNF1α, and HNF4α (30) and display differentiation plasticity, would be suitable for elucidating the function of transcription factors in islet cell differentiation. The present study was designed to define the role of Pdx1 and brain4 in the regulation of pancreatic cell lineage differentiation using INS-1-derived stable cell lines expressing Pdx1, its dominant-negative mutant (DNPdx1), or brain4 under the control of the reverse tetracycline-dependent transactivator (rtTA) (31). The established INS-1 subclones would also allow us to pinpoint the Pdx1-specific downstream target genes.

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‡ To whom correspondence may be addressed: Division de Biochimie Clinique et de Diabétoogie Expérimentale, Département de Médecine Interne, Center Médical Universitaire, CH-1211 Geneva 4, Switzerland. Tel.: 41 22 702 5548; Fax: 41 22 702 5543; E-mail: Haiyan.Wang@medicine.unige.ch or Claes.Wollheim@medicine.unige.ch.

1 The abbreviations used are: DN, dominant-negative; rtTA, reverse tetracycline-dependent transactivator; FCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; IAPP, islet amyloid polypeptide; DOX, doxycycline.
**EXPERIMENTAL PROCEDURES**

**Cloning of the Rat Brain4 cDNA, Construction of Plasmids, and Generation of Stable Cell Lines**—The rat insulinoma INS-1 cell line-derived stable clones were cultured in RPMI 1640 in 11.2 mM glucose (30), unless otherwise indicated. The first-step stable clones INS<sub>b</sub> and INS<sub>b</sub>, which express the reverse tetracycline-dependent transactivator, were reported previously as INS-r<sub>b</sub> and INS-r<sub>b</sub>, respectively (32). Plasmids used in the secondary stable transfection were constructed by subcloning the cDNAs encoding the mouse Pdx1 (kindly supplied by Dr. H. Edlund, Umea, Sweden), its dominant-negative mutant (DN-Pdx1), and the rat brain 4 into the expression vector pUHD10–3 (a kind gift from Dr. H. Bujard). DN-Pdx1 (truncated mutation lacking the first 79 amino acids) was PCR-amplified from Pdx1 using the following primers: 5'-ccagctgctcggtagctcggcctctgg-3' and 5'-ccagctgcagtcgctcgcggcctctgg-3'. The PCR fragment was subcloned into modified pCDNA3.1myc (Invitrogen) and sequenced.

**Immunoblot and Immunofluorescence**—Immunoblotting procedures were performed as described previously using enhanced chemiluminescence (Pierce) for detection (30). The dilution for antibody against Pdx1 C terminus (a kind gift from Dr. H. Edlund) was 1:5,000.

For immunofluorescence cells were grown on polyornithine-treated glass cover slips for 1 day prior to 4 days of treatment with 500 ng/ml doxycycline. The cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% bovine serum albumin. The preparation was then blocked with phosphate-buffered saline-bovine serum albumin before incubating with the first antibodies mouse monoclonal anti-human insulin (1:1,000 dilution; from Sigma) and rabbit polyclonal anti-porcine glucagon (1:2,500 dilution) (33), followed by the second antibody labeling. The resultant immunofluorescence was viewed using a Zeiss laser scan confocal 410 microscope (Zurich, Switzerland).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts from INS-1 cells grown in culture medium with or without 500 ng/ml doxycycline for 24 h and from BHK-21 cells transfected with various expression plasmids were prepared according to Schreiber et al. (34). The following double-stranded oligonucleotides were used as probes: the rat insulin I FLAT element 5'-GATCTTGTTAATAATCTAATTACC-3' (35, 36) and the rat glucagon G1 element (37). EMSA procedures including conditions for probe labeling, binding reactions, unlabeled probe competition, and antibody supershift were performed as previously reported (37). Polyclonal antibodies raised against Pdx1 and Pax-6 were generously provided by H. Edlund and A. Saud, respectively.

**Cell Extract Fractionation**—Cells in 10-cm diameter dishes were cultured with or without 500 ng/ml doxycycline for 24 h at 2.5, 6, 12, and 24 mM glucose. After washing twice with ice-cold phosphate-buffered saline, the cells were suspended and allowed to swell for 15 min at 4 °C in 400 μl of hypotonic buffer composed of 20 mM Tris (pH 7.4), 5 mM EDTA, 2 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. After 3 cycles of freeze-thaw, the cytosolic proteins (supernatant) were separated from the nuclear fraction (pellet) by centrifugation. The two resulting fractions were denatured and analyzed by SDS-PAGE. The isolated rat islets but not detected in parental INS-1 cells (Fig. 1). Somatostatin mRNA was abundantly expressed in INS<sub>b</sub>, which represents a hybrid of α- and β-cells, were used for the present study. As shown in Fig. 1, INS-r<sub>b</sub> and INS-1 cells express predominantly the β-cell-specific markers insulin and IAPP. In contrast, INS<sub>b</sub> cells express not only insulin and IAPP but also glucagon and brain-4 (Fig. 1). Somatostatin mRNA was abundantly expressed in freshly isolated islets but not detected in parental INS-1 cells or derived clones (Fig. 1). Pdx1 mRNA was present in both INS<sub>b</sub> and INS<sub>b</sub> lines (Fig. 1).

**RESULTS**

**Characterization of INS-1-derived Subclones Expressing the rtTA and Establishment of Secondary Stable Lines Overexpressing Pdx1, DN-Pdx1, or Brain-4**—The rat insulinoma INS-1 cells were used as the parental line for stable transfection of an expression plasmid encoding the rtTA (31, 32). A clone termed INS<sub>b</sub>, which maintains the INS-1 β-cell phenotype, and another clone called INS<sub>b</sub>, which represents a hybrid of α- and β-cells, were used for the present study. As shown in Fig. 1, INS<sub>r</sub>b and INS-1 cells express predominantly the β-cell-specific markers insulin and IAPP. In contrast, INS<sub>b</sub> cells express not only insulin and IAPP but also glucagon and brain-4 (Fig. 1). Somatostatin mRNA was abundantly expressed in freshly isolated islets but not detected in parental INS-1 cells or derived clones (Fig. 1). Pdx1 mRNA was present in both INS<sub>b</sub> and INS<sub>b</sub> lines (Fig. 1).

To study whether the production of glucagon in INS<sub>b</sub> cells is correlated with the expression of brain-4, we generated an INS<sub>b</sub> derived stable cell line expressing brain-4 in a doxycycline-inducible manner. The cDNA encoding brain-4 was obtained by reverse transcription-PCR using RNA from freshly isolated rat islets, cloned into pGEM-T vector and sequenced. To delineate the Pdx1-specific target genes and the Pdx1-regulated islet cell lineage differentiation, we also established both INS<sub>b</sub>- and INS<sub>b</sub>-derived stable clones expressing Pdx1 or DN-Pdx1 under the control of rtTA. DN-Pdx1 represents the epitope Myc-tagged truncated Pdx1 mutant protein lacking the N-terminal transactivation domain (the first 79 amino acids).
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(40). The clones, named rβ-brain4-119, rβ-Pdx1-6, rβ-DN-Pdx1-28, rβ-Pdx1-21, and rαβ-DN-Pdx1-59, which expressed the transgenes at the highest level after induction and showed only background expression under non-induced conditions, were selected for the present study.

Both Pdx1 and DN-Pdx1 Proteins Translocated from the Cytoplasm to the Nucleus in a Glucose-dependent Manner but Did Not Change Molecular Mass—It has been reported that glucose stimulates translocation of Pdx1 from the cytoplasm or nuclear periphery to the nucleus in human islets and MIN6 cells (41–43). Macfarlane et al. (41) also demonstrated that the Pdx1 translocation is concomitant with a shift in molecular mass from 31 to 46 kDa, whereas Rafiq et al. (42) reported no such change. We investigated the intracellular location of both endogenous Pdx1 and induced Pdx1 using cytoplastic and nuclear fractions prepared from rαβ-Pdx1-21 cells incubated in 2.5, 6, 12, and 24 mM glucose, respectively, for 24 h (Fig. 2A). The majority of Pdx1 protein was present in the cytosolic fraction when rαβ-Pdx1-21 cells were maintained at 2.5 mM extracellular glucose concentration; however, in 6 mM glucose, Pdx1 predominantly translocated to the nuclear fraction (Fig. 2A). Similar results were obtained using the rβ-Pdx1-6 line (data not shown). The 31-kDa form of Pdx1 described by Macfarlane et al. (41) was not detected by Western blotting of the cytoplasmic fraction (Fig. 2A) or whole cell extracts (data not shown) from the INS-1-derived clones. Nevertheless, the endogenous Pdx1 protein in Min6-m9 cells (44) also translocated from the cytoplasm to the nucleus in a glucose-dependent manner but did not change molecular mass (Fig. 2B). However, there is a clear difference in glucose responsiveness in INS-1-derived cells compared with MIN6-m9 cells, because Pdx1 translocation was maximally stimulated at glucose concentrations between 2.5 and 6 mM in INS-1-derived clones (Fig. 2A), whereas glucose-regulated Pdx1 translocation occurred over the full range from 2.5 to 24 mM glucose in MIN6-m9 cells (Fig. 2B). The induced DN-Pdx1 mutant protein lacking the N-terminal transactivation domain translocated in a way similar to endogenous Pdx1 in both rαβ-DN-Pdx1-59 (Fig. 2C) and rβ-DN-Pdx1-28 (Fig. 2D) cells, in agreement with the identification of the nuclear localization signal in the Pdx1 homeodomain (45).

It is noteworthy that the glucose responsiveness, in terms of both Pdx1 translocation and insulin secretion in INSrαβ-derived cells, was equivalent to that in INSrβ-derived cells (Fig. 2A, C, and D and Table 1). The right shift of the glucose-induced Pdx1 translocation in MIN6-m9 cells (Fig. 2B) corresponds to the right shift of the glucose-stimulated insulin secretion in these cells (44) relative to INS-1-derived cells (Fig. 2A, C, and D and Table 1). Thus, half-maximal insulin secretion in MIN6-m9 cells was observed at 15 mM glucose (44), whereas the value is 6 mM in the INS-1-derived clones (Table 1). Whether or not there is a direct correlation between the glucose-induced Pdx1 translocation and the glucose-stimulated insulin secretion remains to be established.

Induction of Pdx1 Eliminates Glucagon Expression in INSrαβ Cells and Promotes β-Cell-Specific Gene Expression in INSrβ Cells—Quantitative Northern blotting was employed to study the impact of induction of Pdx1 on the expression of INS-1 mRNAs at extracellular glucose concentrations of 2.5, 6, 12, and 24 mM. As demonstrated in Fig. 3A, glucagon mRNA expression in INSrαβ cells was no longer detectable after induction of Pdx1 for 48 h, whereas brain4 mRNA remained constant. Overexpression of Pdx1 in rαβ-Pdx1-21 cells slightly raised the mRNA level of Nkx6.1 but had no significant effect on the mRNA expression of Nkx2.2, Pax4, Pax6, Isl1, and β2/NeuroD (Fig. 1A). We also performed EMSA experiments with a probe corresponding to the rat insulin I FLAT element, which contains the Pdx1-binding site (35, 36). Using nuclear extracts from rαβ-Pdx1-21 cells cultured with or without 500 ng/ml doxycycline for 24 h, we found that induction of Pdx1 resulted in a dramatic increase (>20-fold) in its binding activity to the rat insulin promoter (data not shown). Unexpectedly, we did not see a concomitant rise in the mRNA levels of insulin or IAPP (Fig. 3A). Interestingly, the insulin content in rαβ-Pdx1-21 cells was increased by more than 2-fold (~Dox, 33.65 ± 1.37 ng/mg or protein versus +Dox, 74.55 ± 17.23 ng/mg of protein; p < 0.005) after 4 days induction of Pdx1, despite the fact that the insulin mRNA was reduced by 32.6% under the same condition. In contrast, the glucagon content in rαβ-
Pdx1-21 cells dropped by 94% (−Dox, 8.09 ± 1.51 ng/mg of protein versus +Dox, 0.48 ± 0.11 ng/mg of protein; p < 0.001) after induction of Pdx1 for 4 days. Consistently, the immunofluorescence double staining of rβ-DN-Pdx1-21 cells showed that the glucagon expression was no longer detectable, whereas the insulin level was concomitantly increased after doxycycline induction for 4 days (Fig. 3B).

As shown in Fig. 3C, the graded overexpression of Pdx1 in rβ-DN-Pdx1-6 cells caused a stepwise increase in the expression of Nkx6.1 mRNA. The expression of another β-cell-specific marker, Glut2, was also slightly increased by induction of Pdx1 (Fig. 3C). Overexpression of Pdx1 alone is not sufficient to raise the endogenous insulin and IAPP mRNA levels (Fig. 3C). However, overexpression of Pdx1 may facilitate the biosynthesis of insulin or the formation (or maturation) of insulin granules, because doxycycline treatment for 4 days also significantly increased the insulin content in rβ-DN-Pdx1-6 cells without altering insulin mRNA expression.

Loss of Pdx1 Function Converts INSrβα and INSrβ Cells into Glucagon-predominant α-Cells Independent of Brain4 Expression—We predicted that DN-Pdx1, which lacks the transactivation domain but preserves an intact DNA-binding domain (40), would exert its dominant-negative function by competing with endogenous Pdx1 for binding to the cognate site in the rat insulin promoter. Indeed, our EMSA experiments using nuclear extracts from rβ-DN-Pdx1-59 cells showed that the binding activity of endogenous Pdx1 to the rat insulin I FLAT element was abolished over 90% after 24 h of induction of DN-Pdx1 (data not shown). The expression of β-cell-specific genes encoding insulin, IAPP, Glut2, and Nkx6.1 was drastically reduced but not completely eliminated after treatment of rβ-DN-Pdx1-59 cells with doxycycline for 4 days (Fig. 4A). Concomitantly, the glucagon transcript level in these cells increased 5- and 10-fold after induction of DN-Pdx1 for 2 and 4 days, respectively, whereas brain4 mRNA expression was not altered (Fig. 4A). Similarly, immunofluorescence confocal microscopy studies in rβ-DN-Pdx1-59 cells showed that the insulin-staining cells were lost by 90%, and the glucagon-staining cells accounted for over 90% of the whole cell population after treatment with doxycycline for 4 days (Fig. 4B). Accordingly, the insulin content in these cells decreased by 72% (48.15 ± 8.81 versus 13.68 ± 1.56 ng/mg of protein; p < 0.001), whereas the glucagon content increased over 2-fold (35.77 + 7.69 versus 75.07 + 6.12 ng/mg of protein; p < 0.001). Induction of DN-Pdx1 had no significant effect on the mRNA expression of β2/NeuroD, Pax4, Pax6, Nkx2.2, and Isl-1 (Fig. 4A), suggesting that these pancreatic transcription factors are not Pdx1-specific target genes.

We also performed quantitative Northern blot analysis to investigate the consequences of DN-Pdx1 induction on the gene expression patterns of rβ-DN-Pdx1-28 cells that do not express detectable endogenous brain4. As shown in Fig. 4C, the expression of islet β-cell-specific markers, insulin, IAPP, Glut2, and Nkx6.1 in rβ-DN-Pdx1-28 cells was dramatically decreased in a time-dependent manner (Fig. 4C). In contrast, glucagon mRNA

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expression in rβ-DN-Pdx1-28 cells (Fig. 4C) rose from an undetectable background to a level comparable with that of fully induced rαβ-DN-Pdx1-59 cells (Fig. 4A), whereas brain4 mRNA remained undetectable (data not shown) after induction of DN-Pdx1. These results demonstrate that dominant-negative suppression of Pdx1 function alone is sufficient to differentiate the insulin-predominant β-cell lineage to the glucagon-predominant α-cell phenotype. The brain4 expression is not required for this effect, although there is a time delay in glucagon gene expression comparing rβ-DN-Pdx1-28 (Fig. 4C) with rαβ-DN-Pdx1-59 cells (Fig. 4A).

Although it has been proposed that Pdx1 regulates the human glucokinase promoter activity (46), we found that the endogenous rat glucokinase mRNA expression was unresponsive to Pdx1 function in rβ-DN-Pdx1-6 (Fig. 3C), rβ-DN-Pdx1-28 (Fig. 4C), rαβ-Pdx1-21 (Fig. 3A), and rαβ-DN-Pdx1-59 (Fig. 4A) cells.

**Induction of Brain4 Is Sufficient for Initiating a Detectable Level of Glucagon Expression in INS-rβ Cells but Not Mature α-Cell Differentiation**—To investigate whether loss of Pdx1 function is necessary for α-cell differentiation, we examined the gene expression patterns in rβ-brain4-119 cells using Northern blot analysis. After induction of brain4 at an extremely high level for 4 days, glucagon mRNA expression was initiated to a detectable level (Fig. 5) that is, however, 10–20-fold lower compared with that of rβ-DN-Pdx1-28 (Fig. 4C) and rαβ-DN-Pdx1-59 (Fig. 4A) cells under similar conditions. Unlike induction of DN-Pdx1, however, forced expression of brain4 did not alter the mRNA levels of islet β-cell-specific genes encoding insulin, IAPP, Nkx6.1, and Glut2 (Fig. 5). Pdx1 is not the only a detectable level of glucagon expression but did not suppress the β-cell-specific gene expression. Northern blot analysis of gene expression in rβ-brain4-119 cells induced with 500 ng/ml doxycycline and cultured first in normal (11.2 mM) glucose medium for 4 days and then incubated further for 8 h at the indicated glucose concentrations. 20 μg of total RNA samples were analyzed by hybridizing with the indicated cDNA probes. GK, glucokinase.

**FIG. 5.** Induction of brain4 in the rβ-brain4-119 clone triggered only a detectable level of glucagon expression but did not suppress the β-cell-specific gene expression. Northern blot analysis of gene expression in rβ-brain4-119 cells induced with 500 ng/ml doxycycline and cultured first in normal (11.2 mM) glucose medium for 4 days and then incubated further for 8 h at the indicated glucose concentrations. 20 μg of total RNA samples were analyzed by hybridizing with the indicated cDNA probes. GK, glucokinase.
To explore the possible mechanism underlying the repressive function of Pdx1 on glucagon gene promoter activity, we performed EMSA experiments using nuclear extracts from rβ-Pdx1-21 and rβ-DN-Pdx1-59 cells and the G1 element of the rat glucagon promoter that confers α cell specificity (37). As shown in Fig. 6A, Pdx1 overexpressed in rβ-Pdx1-21 cells formed a complex on G1 that migrated similarly to the paired homeodomain protein Pax-6, a major G1-binding factor. Similarly, DN-Pdx1 from doxycycline-induced rβ-DN-Pdx1-59 cells interacted with the G1 element, and anti-Pdx1 antibodies specifically recognized both Pdx1 protein complexes (Fig. 6A). To assess the affinity of DN-Pdx1 and Pax-6 for G1, we performed EMSA competition experiments. When BHK-21 nuclear extracts containing Pax-6 were mixed with DN-Pdx-1-containing extracts, Pdx1 formed a weaker complex on G1-56 as compared with the individual binding reaction (Fig. 6B). Furthermore, a 10 times greater excess of cold G1-56 was required to compete for DN-Pdx1 than for Pax-6, indicating a better affinity of Pax-6 for G1 as compared with DN-Pdx1. It has previously been shown that Pdx1 has a lower affinity for G1 when compared with Pax-6 but an affinity similar to that of Cdx-2/3, a homeodomain protein interacting synergistically with Pax-6 on G1.3 Similarly, Cdx-2/3 and DN-Pdx1 displayed comparable affinity for G1 (Fig. 6C). However, because Pdx1 and DN-Pdx1 had opposite effects on glucagon promoter activity, it is unlikely that Pdx1 exerts transcriptional repression through its binding to the G1 element. Its ability to interact with other pancreatic transcription factors at the protein level could be an alternative explanation, because we found that Pdx1 suppressed the transactivation of Pax6 through protein–protein interaction independent of DNA binding.3

**DISCUSSION**

Establishment of an in Vitro Model of Islet Cell Lineage Differentiation—Overexpression of a transcription factor, CCAAT/enhancer-binding protein β, in a pancreatic tumor cell line has recently been demonstrated to provoke the transdifferentiation toward the hepatocyte phenotype (47). In fact, the differentiation potential and the multihormonal phenotype of pancreatic precursor cells nor mature differentiated mono-hormone-producing islet cells have been known for a long time (27, 28). We found that rat insulinoma INS-1 cells also display high plasticity of differentiation, and we could actually detect by reverse transcription-PCR (data not shown) the mRNAs of four islet cell specificity (37). As with other pancreatic transcription factors at the protein level have previously been shown that Pdx1 has a lower affinity for G1 when compared with Pax-6 but an affinity similar to that of Cdx-2/3, a homeodomain protein interacting synergistically with Pax-6 on G1.3 Similarly, Cdx-2/3 and DN-Pdx1 displayed comparable affinity for G1 (Fig. 6C). However, because Pdx1 and DN-Pdx1 had opposite effects on glucagon promoter activity, it is unlikely that Pdx1 exerts transcriptional repression through its binding to the G1 element. Its ability to interact with other pancreatic transcription factors at the protein level could be an alternative explanation, because we found that Pdx1 expressed in BHK-21 cells overexpressing DN-Pdx1, Pax-6 or Cdx-2/3 competed for the indicated molar excess of cold G1-56 oligonucleotides.

**FIG. 6.** Pdx1 and DN-Pdx1 bind to the glucagon gene promoter element G1. A, EMSA using nuclear extracts from rβ-Pdx1-21 and rβ-DN-Pdx1-59 cells incubated for 24 h in the absence or presence of 500 ng/ml doxycycline. Both Pdx1 and DN-Pdx1 were able to bind G1. Pax6 and Pax1 indicate the addition of anti-Pax-6 and anti-Pdx1 antibodies, respectively. B and C, competition experiments analyzing the relative affinity of DN-Pdx1 and Pax-6 or Cdx-2/3 for the G1 element. Protein–DNA complexes formed with nuclear extracts from BHK-21 cells overexpressing DN-Pdx1, Pax-6, or Cdx-2/3 were competed for by the indicated molar excess of cold G1-56 oligonucleotides.

Glucose Regulates the Cellular Translocation of Pdx1 Protein

[^3]: B. Ritz-Laser, submitted for publication.
without Altering Its Molecular Mass—The present study supports the previous reports that glucose stimulates translocation of Pdx1 from the cytoplasm or nuclear periphery to the nuclei (41, 42). In addition, we found that the majority of Pdx1 protein is located in the nucleus at physiological concentrations of glucose (6 mM). During starvation associated with hypoglycemia, Pdx1 would be transported out of the nuclei, which may prevent the β-cell from producing excessive insulin. Although Macfarlane et al. (41) reported a shift in the molecular mass of Pdx1 (from 31 to 46 kDa) concomitant with the glucose-stimulated Pdx1 translocation, our Western blotting data demonstrated that Pdx1 protein migrated at a constant molecular mass of 46 kDa irrespective of the glucose concentration. Our results are in agreement with another study by Rafiq et al. (42).

The nuclear localization signal of Pdx1 has been identified as part of the homeodomain (43). This explains why the translocation of DN-Pdx1 lacking the N-terminal transactivation do-part of the homeodomain (45). This explains why the translocation of DN-Pdx1 lacking the N-terminal transactivation do-part of the homeodomain (45). This explains why the translocation of DN-Pdx1 lacking the N-terminal transactivation do-part of the homeodomain (45). This explains why the translocation of DN-Pdx1 lacking the N-terminal transactivation do-part of the homeodomain (45). This explains why the translocation of DN-Pdx1 lacking the N-terminal transactivation do-part of the homeodomain (45).

Pdx1 Paradox—Pdx1 exerts paradoxical effects on insulin mRNA expression and insulin promoter activity in insulin-producing cells and non-β-cells (40, 50–56). The ectopic expression of Pdx1 in non-β-cells resulted in the induction of insulin generation (29, 50) or transactivation of insulin promoter activity (40, 51–55). Consistent with previous studies in insulin-producing β-cells (52, 56), we demonstrated that overexpression of Pdx1 did not increase, but even reduced, the insulin mRNA levels in INS-1-derived subclones. It has been hypothesized that the suppressive effect of Pdx1 in β-cells is due to cooperative interactions between Pdx1 and other transcription factors (53–57). The present study showed that overexpression of Pdx1 in INS-1 cells also raised the mRNA level of the β-cell-restricted transcription factor Nkx6.1, known as a potent transcriptional repressor of the intact insulin promoter (22, 25). The negative feedback mechanism of Nkx6.1 provides an alternative explanation for the inhibitory effect of Pdx1 in β-cells.

The relevance of the increase of Nkx6.1 may also explain the paradoxical effect of Pdx1 on glucagon promoter activity. We showed that induction of Pdx1 in INSραβ cells dramatically suppressed the glucagon promoter activity and eventually eliminated the expression of glucagon mRNA and protein. In contrast, the ectopic expression of Pdx1 in αTC1 cells, predominantly expressing glucagon, did not inhibit glucagon gene transcription. Nkx6.1 is expressed in the insulin-producing β-cell lines, including INS-1-derived subclones, but not in the α-cell lines such as αTC1 cells (21, 25). It is therefore likely that Nkx6.1, induced by Pdx1 (17, 25, and the present study), may indeed function as a potent transcriptional repressor (22) and contribute to the inhibitory effect of Pdx1 on glucagon gene transcription. However, this hypothesis cannot explain why targeted disruption of mouse Nkx6.1 gene did not cause increased α-cell mass (16). Although we found that Pdx1 is capable of binding the glucagon G1 element, the binding activity is not required for the suppressive function of Pdx1 on the glucagon promoter. Protein-protein interactions with other transcription factors including Pax6 and Pax4 could also contribute to the transcriptional repression of glucagon. Pax4, a transcriptional repressor of the glucagon promoter (23, 24), is essential for pancreatic β-cell development (15) and is expressed transiently in the differentiating fetal β-cells but not in the differentiated adult β-cells (25). Although we found that Pdx1 alone did not have any significant effect on the Pax4 mRNA expression, we cannot rule out the possibility that Pdx1 suppresses glucagon promoter activity through synergistic interaction with Pax4.

Overexpression of Pdx1 Raises Cellular Insulin Content without Increasing Insulin mRNA Expression—Another intriguing function of Pdx1 is the increase in cellular insulin content, without affecting insulin mRNA levels. We therefore suggest that Pdx1 may also regulate the biosynthesis of insulin or the formation or maturation of insulin granules.

Pdx1 Is Required for Maintaining Insulin Expression—The ectopic expression of Pdx1 alone in glucagonoma cells in vitro (29) and in mouse hepatocytes in vivo (50) resulted in induction of insulin production, suggesting that Pdx1 is a master gene in the regulation of insulin expression. We demonstrated here that dominant-negative suppression of Pdx1 function in INS-1 cells caused a drastic reduction, but not complete elimination, of the insulin mRNA expression, insulin immunofluorescence staining, and cellular insulin content. Our results are in agreement with the previous in vivo study of Ahlgren et al. (17), who showed that β-cell-specific inactivation of the mouse Pdx1 gene resulted in up to 90% reduction in the pancreatic insulin content. We thus conclude that Pdx1 is required for maintaining insulin expression.

Loss of Pdx1 Function rather than Expression of Brain4 is the Prerequisite for α-Cell Differentiation—The absence of Pdx1 and the specific expression of brain4 are the hallmarks of differentiated α-cells. The present study provided profound evidence that loss of Pdx1 function rather than expression of brain4 is the determining factor for α-cell differentiation. We showed that induction of DN-Pdx1 in INSραβ and INSρβ cells not only markedly repressed insulin expression but also caused pronounced expression of glucagon. We also performed immunofluorescence double labeling to show that dominant-negative suppression of Pdx1 function converted the insulin-producing β-cell lineage to the α-cell-dominated phenotype. In contrast, induction of brain4 in INSρβ cells initiated only detectable amounts of glucagon, without inhibiting the β-cell-specific gene pattern. The expression of endogenous brain4 in INSραβ cells may partially explain the glucagon production.

The β-cell-specific disruption of Pdx1 in vivo shifted the β/α cell ratio from 5:1 to 1:1 (17), which seems moderate in comparison to the 90% conversion of β-cells to α-cells in our study. The discrepancy may be due to the slower proliferation and neogenesis or to less differentiation plasticity of islet β-cells in vivo.

Pdx1-specific Patterns of Gene Expression—We found that the β-cell genes encoding insulin, IAPP, Glut2, and Nkx6.1 are Pdx1-specific target genes. Dominant-negative suppression of Pdx1 function drastically and selectively reduced the expression of these mRNA species. Although it has been reported that Pdx1 binds to the Pax4 promoter (26), we showed that Pdx1 alone is not sufficient to regulate Pax4 mRNA expression. Glucokinase expression is not restricted to islet β-cells although it is less abundant in α-cells (58). We demonstrated that the mRNA expression of glucokinase is completely unresponsive to Pdx1 regulation. This concurs with a previous study on transgenic mice (17). These results contrast with the earlier claims that Pdx1 regulates the glucokinase gene (46).

Conclusion—We conclude that Pdx1 is required for maintaining the expression of β-cell-specific genes and β-cell lineage differentiation. Loss of Pdx1 function rather than expression of brain4 is the prerequisite for α-cell differentiation.

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