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Agenesis of Human Pancreas due to Decreased Half-Life of Insulin Promoter Factor 1

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Neonatal diabetes mellitus can be transient or permanent. The severe form of permanent neonatal diabetes mellitus can be associated with pancreas agenesis. Normal pancreas development is controlled by a cascade of transcription factors, where insulin promoter factor 1 (IPF1) plays a crucial role. Here, we describe two novel mutations in the IPF1 gene leading to pancreas agenesis. Direct sequence analysis of exons 1 and 2 of the IPF1 gene revealed two point mutations within the homeobox in exon 2. Genetic analysis of the parents showed that each mutation was inherited from one parent. Mutations localized in helixes 1 and 2, respectively, of the homeodomain, decreased the protein half-life significantly, leading to intracellular IPF1 levels of 36% and 27% of wild-type levels. Both mutant forms of IPF1 were normally translocated to the nucleus, and their DNA binding activity on different known target promoters was similar to that of the wild-type protein. However, transcriptional activity of both mutant IPF1 proteins, alone or in combination with HNF3β/Foxa2, Pbx1, or the heterodimer E47-β2 was reduced, findings accounted for by decreased IPF1 steady state levels and not by impaired protein-protein interactions. We conclude that the IPF1 level is critical for human pancreas formation. (*J Clin Endocrinol Metab* 88: 4398–4406, 2003)

THE PANCREAS ARISES from the endoderm as a dorsal and a ventral bud that fuse together to form a single organ (1). These morphological changes depend on sequential changes in gene expression. As shown in different mouse models, disruption of sequential gene expression events leads to the absence of the pancreas or to pancreatic malformations. The homeodomain protein insulin promoter factor 1 (IPF1), also known as pancreatic duodenal homeobox factor 1 (PDX1), is one of the most critical genes for pancreas development. The IPF1 gene consists of two exons coding for 283 amino acids (aa) (2, 3) with a predicted molecular weight of 46 and spans a region of about 6 kb on human chromosome 13 (13q12.1) (3). The structure of IPF1 comprises an N-terminal *trans*-activation domain, followed by a pentapeptide motif (FPWMK) interacting with Pbx1, and a homeodomain (aa 146–205) that contains three helixes and harbors a nuclear localization signal. IPF1 has been shown to be expressed at the foregut-midgut junction just before pancreas formation is initiated. Later it becomes restricted primarily to β-cells, where it regulates the expression of β-cell-specific genes (4). IPF1 is known to bind to the TAAT element of different target gene promoters, such as insulin, Pax4, Nkx6.1, heparin-binding epidermal growth factor (HB-EGF), and the IPF1 gene promoter itself, where it positively controls its own expression (5–8). Some of these genes are involved in pancreas development: Pax4 is critical for the differentiation of β- and δ-cells (9), Nkx6.1 is specifically required for β-cell formation (10), and HB-EGF is a growth factor positively regulated by IPF1 (7). Targeted disruption of the IPF1 gene in mice leads to agenesis of the pancreas and neonatal diabetes mellitus (ND) (11, 12).

Pancreatic agenesis is a rare disorder in humans. A single case of pancreatic agenesis related to a homozygous mutation of the IPF1 gene has been reported to date (13). This mutation led to a truncated, inactive protein. Here we report a compound heterozygous mutation of the IPF1 gene that resulted in pancreatic agenesis in a female infant. The infant was born at term, but was growth retarded, presumably due to the absence of insulin production *in utero*. Investigations of the functional characteristics of the two IPF1 proteins, mutated in helixes 1 and 2, respectively, of the homeodomain, revealed a decrease in protein half-life, leading to steady state protein levels at approximately 30% of IPF1 wild-type levels. This marked decrease in the IPF1 level may, in turn, prevent full activation of its own gene, leading to a further decrease in protein level and to pancreas agenesis as a consequence.

Abbreviations: aa, Amino acids; BHK, baby hamster kidney; CAT, chloramphenicol acetyl transferase; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; HB-EGF, heparin-binding epidermal growth factor; HEK, human embryonic kidney; IPF1, insulin promoter factor 1; ND, neonatal diabetes mellitus; PAP, placental-alkaline phosphatase; PDX1, pancreatic duodenal homeobox factor 1.

Materials and Methods

Analysis of patient DNA and mutagenesis

Genomic DNA was isolated from peripheral blood cells of the patient and both parents using the Genomic PrepBlood DNA Isolation Kit

(Amersham Pharmacia Biotech, Piscataway, NJ). PCR amplification of IPF1 was performed with the following primers: for exon 1: forward, 5′-CCATGAACGGCGAGGAGC-3′; and reverse, 5′-CAGGCTTACCT-GCCCACT-3′; for exon 2: forward, 5′-GCCCTGTGTCGCCCGCAG-3′; and reverse, 5′-TTGAAGCCCCTCAGCCAG-3′. Each amplification consisted of an initial denaturation step at 95 C for 15 min, followed by 30 cycles (94 C for 30 sec, 57 C for 30 sec, 72 C for 45 sec) with a final extension at 72 C for 10 min. The PCR fragments were directly sequenced with an ABI 3100 genetic analyzer (PE Applied Biosystems, Foster City, CA). IPF1 cDNA was obtained by RT-PCR (forward, 5′-acgaattcgGC-CATGAACGGCGA-3′; and reverse, 5′-acggatcctaTCGTGGTTCCT-GCG-3′) from human islet RNA isolated from whole pancreata obtained from multiorgan cadaver donors (20–65 yr) as described previously (14) and subcloned into pSG5 (Stratagene, Amsterdam, The Netherlands). *In vitro* mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol using following primers: for the E164D mutation: forward, 5′-GCT-AGAGCTGGAGAAGGATTTCCTATTCAACAAG-3′; and reverse, 5′-CTTGTTGAATAGGAAATCCTTCTCCAGCTCTAGC-3′; for the E178K mutation: forward, 5′-CGCGCCGGGTGAAGCTGGCTGTCATG-3′; and reverse, 5′-CATGACGCCAGCTTCACCCGGCGCG-3′.

Oral glucose tolerance test

The 75-g oral glucose tolerance test was carried out after a 10-h overnight fast. Subjects ingested 75 g glucose in 300 ml water over a period of less than 5 min. Blood samples were collected through an indwelling catheter before and at 30-min intervals after the glucose load over a 2-h period for determination of plasma glucose. Glucose concentrations were analyzed by the glucose oxidase method using the Vitros system (Ortho-Clinical Diagnostics, Rochester, NY).

Plasmids and reporter gene constructs

Chloramphenicol acetyl transferase (CAT) reporter constructs harboring specific *cis*-acting elements of the rat insulin I gene promoter (−410InsCAT) as well as the human IPF1 (PH1-TK-LUC) promoter were described previously (15, 16). The UE-A-TSEI-LUC reporter gene construct was a gift from B. Peers (17). Expression vectors harboring the hamster E47 and hamster β2 (also called NeuroD1) were provided by M. S. German (University of California, San Francisco, CA). Expression vectors containing HNF3β/Foxa2 and Pbx1 were gifts from R. Costa (University of Illinois, Chicago, IL) and M. Cleary (Stanford University School of Medicine, Stanford, CA), respectively.

Cell culture and transient transfections

The Syrian baby hamster kidney (BHK) 21, InRIG9 (18) and human embryonic kidney (HEK) 293T cell lines were grown and maintained in RPMI 1640 (Seromed, Basel, Switzerland); the NIH-3T3 cell line was grown in DMEM supplemented with 5% fetal calf serum, 5% newborn calf serum (Life Technologies, Basel, Switzerland), 100 U/ml penicillin (Seromed), 100 μg/ml streptomycin (Seromed), and 2 mM glutamine (Life Technologies, Basel, Switzerland). BHK21 and NIH-3T3 cells were transiently transfected using the calcium phosphate precipitation technique (19), InRIG9 cells were transfected by the diethylaminoethyl-dextran method (20). Each 10-cm petri dish received a precipitate containing 10 μg CAT gene reporter construct, 0.5 μg pSV$_2$PAP (internal control), and variable amounts of expression vectors for the various transcription factors (all cloned into pSG5). The final amount of DNA in each transfection was maintained constant by adding the empty expression vector pSG5. Cells were harvested 48 h posttransfection in 250 mM Tris-HCl and were disrupted by three freeze-thaw cycles. CAT and placental-alkaline phosphatase (PAP) activities were determined as previously described (21). PAP activity was used to standardize for transfection efficiency. The CAT/PAP activity values presented for each set of experiments correspond to the mean and SE of at least three individual transfections performed in duplicate. The luciferase assay was performed as previously described by adding to 30 μl cell extract 30 μl luciferase reagent (22).

Statistical analysis

Results are expressed as the mean ± SEM. Where indicated, the statistical significance of the differences between groups was estimated by *t* test.

Preparation of proteins and EMSA

Rat insulin A3/4 (23), human IPF1 PH1 (16), human HB-EGF TAAT4 (7), and rat somatostatin TSEII (17) probes were radioactively labeled by filling in the ends using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]deoxy-CTP. The probes were purified using the QIAquick nucleotide removal kit (Qiagen, Basel, Switzerland). Nuclear protein extracts enriched for IPF1, E164D, and E178K were obtained by transfecting BHK21 or HEK293T cells with 10 μg of various expression vectors. Cells were harvested 48 h post transfection, and whole cell and nuclear protein extracts were prepared as described previously (24). DNA binding assays and supershift using polyclonal anti-IPF1 antibody were performed as described previously (25).

Green fluorescent protein (GFP) fusion constructs and expression

The pEGFP-C1 plasmid (Clontech, Palo Alto, CA) was used to generate GFP-IPF1-WT, GFP-IPF1-E164D, and GFP-IPF1-E178K fusion constructs. Transfections of NIH cells were performed with the calcium phosphate precipitation method described above. One day after transfection the cells were analyzed by fluorescence microscopy.

Western blot assay

Nuclear fractions were isolated from transfected BHK21 and InRIG9 cells according to the protocol of Schreiber *et al.* (24). Approximately 17 μg of each protein extract were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred electrophoretically to polyvinylidene difluoride membranes. Immunoblotting was performed with polyclonal antibodies to IPF1 (1:10,000) directed against the whole protein or its C terminus and mouse antirabbit IgG antisera conjugated with horseradish peroxidase (1:2,000; Super Signal West Pico Trial Kit, Pierce Chemical Co., Rockford, IL). The protein loading was normalized for transfection efficiency by PAP activity. The experiment was repeated three times (n = 2 with extracts from BHK cells; n = 1 with extracts from InRIG9 cells).

Biosynthetic labeling

For IPF1 protein biosynthesis measurements, 36 h after transfection BHK21 cells were washed twice with PBS, shifted to RPMI 1640-modified medium (Invitrogen, San Diego, CA) depleted of methionine and cysteine, and complemented with 10% dialyzed fetal calf serum. After 30-min incubation, cells were labeled with 60 μCi/ml [^{35}S]methionine (Hartmann Analytic, Braunschweig, Germany) for 10 min at 37 C. Cells were then washed twice with PBS and harvested, and nuclear extracts were prepared in the presence of protease inhibitors as described by Antras *et al.* (26). For determination of IPF1 half-life, transfected BHK21 and InRIG9 cells were pulse-labeled as described above, except that the pulse was for 1 h in the presence of 60 μCi/ml [^{35}S]methionine. Cells were then washed twice with PBS and either lysed directly for nuclear extract preparation or returned to the normal culture medium containing 2 mM cold methionine and chased at 37 C for various times.

Immunoprecipitation and quantification of radioactive protein

For IPF1 immunoprecipitation, identical amounts of nuclear proteins were solubilized in 500 μl immunoprecipitation buffer containing 0.1% Triton X-100, 0.25% Nonidet P-40, 12 mM HEPES (pH 7.9), 100 mM KCl, 4 mM Tris-HCl (pH 8.0), 50 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin. The nuclear extracts were precleared with 40 μl protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 C, followed by centrifugation, and the supernatants were incubated with 1 μl antihuman full-length IPF1 antibody for 1 h with gentle

rotation. The antigen-antibody complexes were collected by the addition of 50 μl protein A-Sepharose for an additional 1-h incubation at 4 C. After five washings with immunoprecipitation buffer, they were resuspended in sodium dodecyl sulfate loading buffer and separated on 10% SDS-PAGE gels. Gels were then fixed, dried, and exposed to x-ray film. Quantitation of band intensity was performed by laser scanning densitometry.

In vitro protein-protein interaction assays

[^{35}S]Methionine-labeled E47, β2, HNF3β/Foxa2, and Pbx1 were produced using the TNT rabbit reticulocyte lysate-coupled transcription-translation system (Promega Corp., Madison, WI) according to the manufacturer's protocol. The labeled proteins were incubated with glutathione-*S*-transferase (GST), GST-IPF1, GST-E164D, or GST-E178K, and the binding reactions were treated as outlined by Ritz-Laser *et al.* (27).

Results

Description of the proband

The infant was born at term from nonconsanguineous parents and presented with intrauterine growth retardation [body weight, 2140 g (<P10); body length, 44 cm (<P10)]. At 12 d of life, glycemia of 854 mg/dl (47 mmol/liter) was observed, and the diagnosis of ND was made. Autoimmune antibodies were negative. The infant was treated with an insulin pump (1 U/kg·d); despite euglycemia, the infant did not gain weight, and exocrine pancreas insufficiency was diagnosed. Replacement therapy with pancreatic enzymes was initiated, and the infant developed normally thereafter. Imaging studies, including abdominal ultrasound and computed tomography scan, revealed no pancreas. The mother of the index case had gestational diabetes, and the infant's maternal uncle and grandmother suffer from type 2 diabetes mellitus, diagnosed at 39 and 45 yr of age, respectively. The family history for type 2 diabetes mellitus on the father's side was also positive (a paternal uncle diagnosed at age 45 yr as well as the paternal grandmother diagnosed at age 50 yr). The mother (36 yr old; body mass index, 22.3) and father (37 yr old; body mass index, 26.0) had high normal fasting blood sugar levels [101 mg/dl (5.6 mmol/liter) and 102 mg/dl (5.7 mmol/liter), respectively] and no glucose intolerance at this point (Table 1).

Analysis of the IPF1 gene

The IPF1 gene spans 852 bp in two exons. We therefore determined the nucleotide sequence of exons 1 and 2 by PCR amplification of the patient's genomic DNA, followed by direct sequencing. Comparison with the published sequence (AF035259 and AF035260) of the IPF1 gene revealed two missense mutations in exon 2 (AF035260: G319T, G359A). Both mutations lead to amino acid changes (E164D, E178K; Fig. 1). A similar procedure was carried out for both parents and showed that each mutation was inherited from one parent. The mutation (E164) was also present in the maternal uncle, who suffered from diabetes mellitus. The paternal mutation (E178K) created, in addition, a new restriction site (*Hph*1), which was used to confirm the mutation in the index case.

Mutations were localized in helixes 1 and 2, respectively, of the homeodomain at highly conserved sites, decreasing the likelihood that the point mutations represent simply a DNA sequence polymorphism. In addition, several groups have searched for mutations in the IPF1 gene, and differences in other polymorphisms and mutations have been published (13, 28), but neither the E164D nor the E178K have been described yet.

To test for any structural changes, we created a three-dimensional model of IPF1. Simulation of the above-described mutations, however, did not influence the predicted three-dimensional model or the predicted binding to DNA (data not shown).

TABLE 1. Oral glucose tolerance test results

Time (min)	Blood glucose [mg/dl (mmol/liter)]
Mother	
0	101 (5.6)
30	164 (9.1)
60	176 (9.8)
90	138 (7.7)
120	114 (6.3)
Father	
0	102 (5.7)
30	140 (7.8)
60	137 (7.6)
90	116 (6.4)
120	114 (6.3)

Transcriptional activation capacity of mutant IPF1 is reduced

To determine the functional impact of both mutations, we studied IPF1-mediated activation of the insulin and IPF1 promoters, two known target genes of IPF1, as a model system. In transient transfections in the nonislet BHK21 cell line, wild-type IPF1 increased transcriptional activity of the rat insulin I gene promoter (−410 InsCAT) in a dose-dependent manner up to 5.7-fold, as previously reported (8, 29–31). Conversely, both IPF1 mutations resulted in a significantly impaired induction of CAT activity (Fig. 2A). It has previously been shown that IPF1 can positively regulate its own expression by binding to the PH1 region of the human IPF1 promoter. The PH1 region (−2.809 to −2.655 kb) linked to the luciferase reporter gene driven by the TK promoter was used to assess the consequences of the two mutations of IPF1 (8). The PH1-LUC construct was cotransfected with increasing amounts of wild-type IPF1, E164D-IPF1, or E178K-IPF1 expression plasmids. As shown in Fig. 2B, both IPF1 mutations led to an impaired induction of luciferase compared with the wild type. We conclude from these results that both mutant IPF1 proteins lead to decreased transcriptional capacity on target genes such as the insulin and IPF1 genes.

Conserved DNA binding of mutated IPF1

A first step in the action of most transcription factors is binding to upstream regulatory elements within the promoter of target genes. Binding of IPF1 to different promoters depends on an intact homeodomain. *In vitro* studies point to an important role of IPF1 in the regulation of the insulin (4, 23, 32), IPF1 (8, 33), Pax4 (34), Nkx6.1 (6), and HB-EGF genes (7). All of these genes, except for the insulin gene, play an important role in endocrine pancreas development. Recently, IPF1 binding *in vivo* has been demonstrated by chromatin immunoprecipitation assays (35), confirming a strong inter-

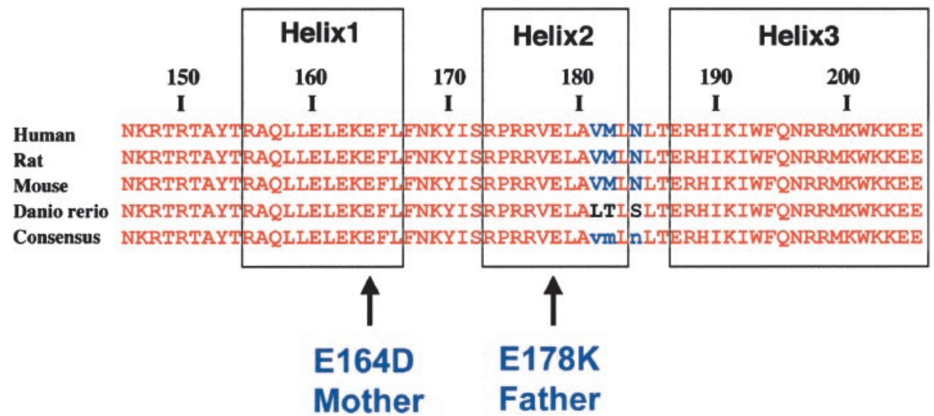

FIG. 1. Identification of two point mutations in the IPF1 gene. Two point mutations were found in the index patient, which both led to aa changes in helix 1 (E164D) and helix 2 (E178K), respectively, of the homeodomain of IPF1. Each parent was heterozygous for one of the two mutations. Sequence alignment of the homeodomain of human, rat, mouse, and *Danio rerio* PDX1/IPF1 shows the conservation of the mutated aa between species.

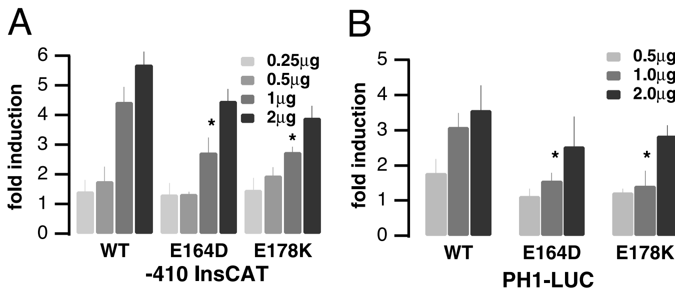

FIG. 2. Reduction of transcriptional activation capacity of mutant IPF1. The effects of IPF1 and its mutants on the rat insulin I promoter (−410InsCAT; A) and on the human IPF1 (PH1-LUC; B) promoters were determined. Transient transfection studies using BHK21 cells were performed with increasing amounts of wild-type and mutant IPF1. A, Data are presented as fold induction of basal CAT activity (reporter plasmid transfected along with an empty pSG5 expression vector; mean ± SEM; n = 4). *, $P < 0.05$ (significant reduction of transcriptional activation). B, Both mutants showed a significant reduction of transcriptional activation compared with wild-type when assessed by the effect on the IPF1 promoter (PH1-LUC). Data are presented as fold stimulation of basal LUC activity (reporter plasmid transfected along with an empty pSG5 expression vector; mean ± SEM; n = 3). *, $P < 0.05$ **, $P < 0.01$ (significant reduction of transcriptional activation).

action of IPF1 with the insulin, IPF1, and Pax4 promoters. As both of the proband's mutations were located within the homeodomain, we tested whether the reduced transcriptional activity could be due to decreased binding to target genes. We first tested binding of IPF1 and its mutants to the A3/4 element of the rat insulin promoter by EMSA studies, using nuclear protein extracts derived from BHK21 cells overexpressing IPF1 and its mutants (Fig. 3). As shown in Fig. 3A, binding of the IPF1 mutants was intact. The E178K mutant displayed a slightly slower migration, which was attributed to a change in charge (glutamic acid to lysine). To confirm that the migrating complexes indeed contained IPF1, we used specific anti-full-length IPF1 antisera (Fig. 3B).

To analyze the relative binding affinity of IPF1 and its mutants, gel-shift competition experiments were performed. Competition of the wild-type and mutant IPF1 complexes with cold oligonucleotides comprising IPF1 target sites of the rat insulin I, human IPF1, and human HB-EGF promoter was comparable, indicating very similar binding characteristics (Fig. 3C). Identical results were obtained for the human Pax4 and mouse Nkx6.1 promoters (results not shown). We conclude that both wild-type and mutant IPF1 proteins display similar binding affinities to the target DNA regulatory elements, and therefore decreased transcriptional capacity of the mutant IPF1 protein is not due to impaired DNA binding.

Mutant proteins translocate normally to the nucleus

It is well established that the homeodomain of IPF1 contains a nuclear localization signal (36). We used GFP fusion proteins of wild-type IPF1 and mutant forms to test whether the two mutations in the homeodomain of IPF1 affect nuclear translocation, which could possibly explain the observed impaired transcriptional capacity. Both mutant forms as well as the wild-type were, however, normally translocated to the nucleus of NIH-3T3 cells (Fig. 4, A–C), indicating that the nuclear import of the mutant IPF1s is not affected. Furthermore, when analyzing nuclear and cytoplasmic extracts of BHK21 cells transfected with the IPF1 cDNA, we found that approximately 90% of the wild-type and mutant IPF1 was nuclear as assessed by both Western blot and gel retardation assays (data not shown).

Decreased cellular levels of the overexpressed mutants compared with wild-type IPF1

During the course of our gel retardation experiments, we repeatedly noticed that the levels of the overexpressed mutant IPF1 proteins in BHK21 cells (whole cellular and nuclear extracts) were markedly lower than those of wild-type IPF1. We thus quantified the amount of overexpressed protein in BHK21 and InRIG9 cells by Western blot analysis. Using two different IPF1 antibodies, we found that the amount of both mutant proteins was reduced compared with wild-type levels (Fig. 4, D and E). In BHK cells using a C-terminal antibody, reductions to 22% (E164D) and 12% (E178K) compared with wild-type IPF1 levels were observed, whereas with a full-length antibody the reductions were 50% (E164D) and 36% (E178K), as quantified by densitometry and/or phosphorimager scans. Similar results were obtained in InRIG9 cells (data not shown).

To further investigate whether the reduced amount of mutant proteins was due to decreased biosynthesis or increased degradation, we determined the half-lives of these proteins in BHK21 cells. The half-life of wild-type IPF1 was 22 h, whereas that of E164D was reduced to 36% (8 h) of the wild-type value and E178K to 27% (6 h; Fig. 5, A and B). We

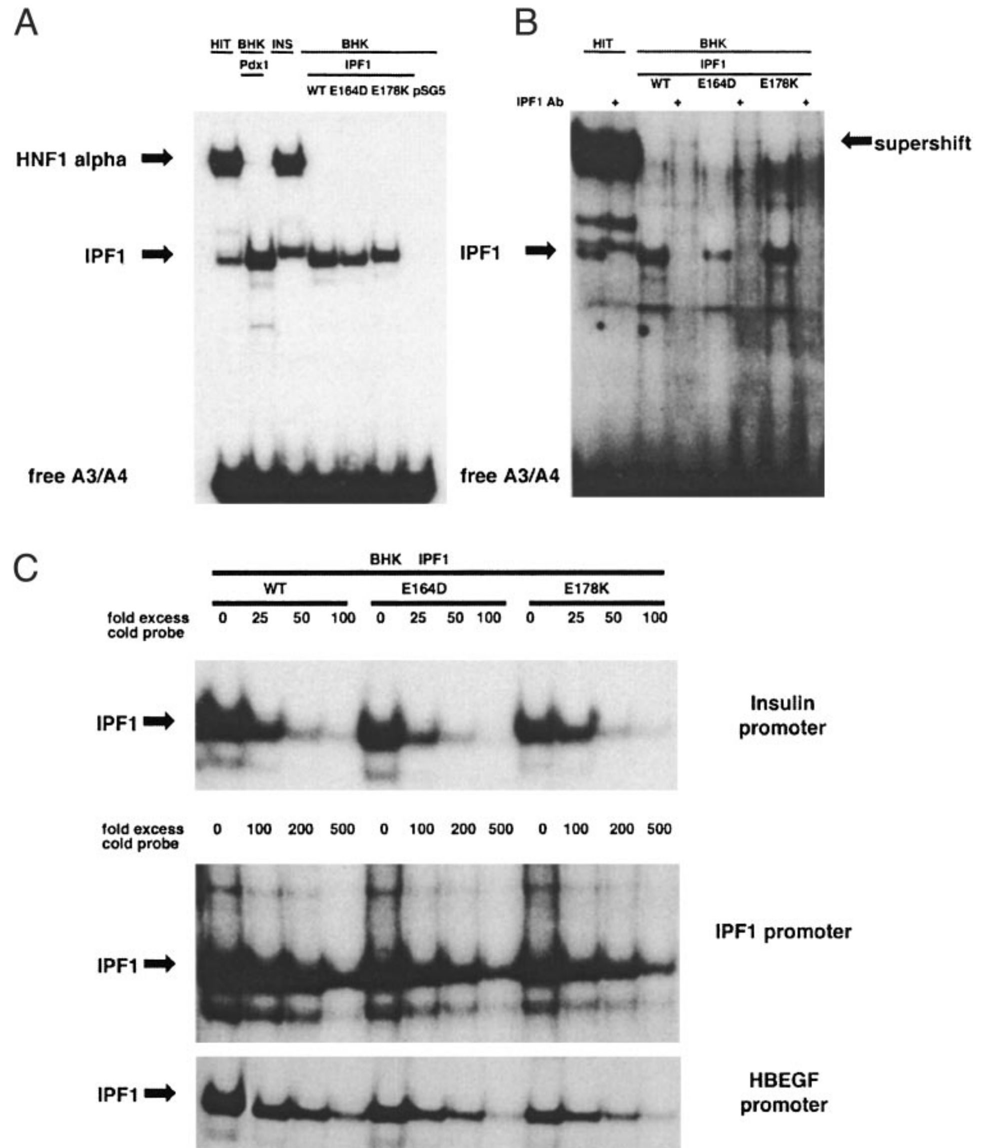

FIG. 3. Conserved DNA binding of mutated IPF1. EMSA analysis with wild-type and mutant IPF1 proteins and ^{32}P-labeled A3/4 element of the rat insulin gene promoter. A, Nuclear extracts (6 μg) of BHK21 cells expressing wild-type or mutated proteins were used. Nuclear extracts from the insulin-producing HIT and INS cells and transfected BHK21 cells with PDX1 cDNA served as positive controls. B, Nuclear extracts of BHK21 cells expressing the wild-type (8 μg) or the mutated proteins (12 μg for E164D and 18 μg for E178K) were used. C, EMSA analysis was performed using labeled oligonucleotides [A3/4 (insulin gene promoter), PH1 (IPF1 gene promoter) and TAAT4 (HB-EGF gene promoter)] and increasing fold molar excess (25- to 500-fold) of cold oligonucleotides comprising again the A3/4, PH1, and TAAT4 elements. For the competition experiments nuclear extracts of BHK21 cells expressing the wild-type (6 μg) or mutated (12 μg) proteins were used.

also determined the half-lives of IPF1 and its mutants in InRIG9 cells, a glucagon-producing cell line, to provide an endocrine cell environment. Again, the half-lives of both mutants were drastically reduced compared with that of wild-type IPF1 (32 h), to 6.5 h for the E164D mutant and to 3.3 h for the E178K mutant. By contrast, biosynthesis of mutant proteins quantified in three independent experiments was not different from that of the wild type (Fig. 5C).

Taken together, these results indicate that an increased degradation of mutant IPF1 proteins may lead to a reduction of IPF1 protein levels and ultimately to decreased transcriptional activity.

Functional interaction of mutant proteins with known partners

IPF1 is known to mediate multiple protein-protein interactions in the formation of transcriptional activation complexes on the insulin, IPF1, and somatostatin gene promoters (8, 17, 23, 31). Different deletions within the homeodomain of IPF1 have been shown to abolish binding of E47, indicating that helix 2 of the homeodomain is important for interaction with E47 (23). To establish whether the IPF1 mutants were capable of functionally interacting with the known partners of IPF1 on the A3/4 element of the insulin promoter, combinations of E47-β2 and wild-type or mutant IPF1 expression vectors were cotransfected in BHK21 cells along with the reporter construct −410InsCAT. A significant reduction of transcriptional activity was found with both mutants compared with the wild type (Fig. 6A); the reduction was, however, quantitatively similar to the decreased transcriptional activation conferred by the mutant IPF1 forms alone (Fig. 2).

We further investigated the functional interaction capacity of IPF1 and its mutants with HNF3β/Foxa2 on the PH1 element of the IPF1 promoter itself. An additive effect on luciferase activity compared with IPF1 alone was observed when wild-type IPF1 or the mutant forms were cotransfected together with HNF3β/Foxa2. However, although the mutant IPF1 did not reach the same transcriptional activity level as

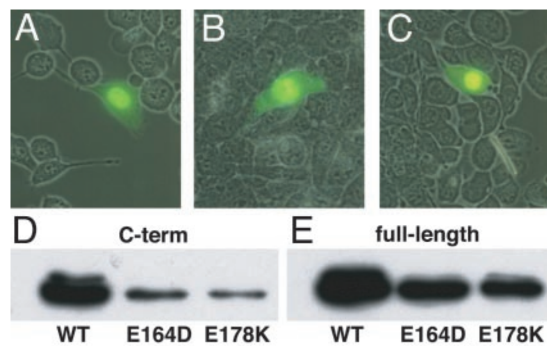

FIG. 4. Conserved nuclear translocation, but decreased amount of mutated protein. NIH-3T3 cells were transiently transfected with GFP fusion constructs of wild-type and mutant IPF1. Wild-type IPF1 (A), E164D (B), as well as E178K (C) IPF1 mutants were equally transferred to the nucleus. D and E, Western blot analysis of wild-type and mutant IPF1 proteins. The amounts of mutated IPF1 proteins were reduced compared with the wild-type as shown by Western blot analysis. E, Seventeen micrograms of nuclear extracts from transiently transfected BHK21 cells (with wild-type and mutant IPF1) were analyzed by electrophoresis and immunostained with either C-terminal (D) or full-length (E) IPF1 polyclonal antibodies. Using anti-C-terminal-specific PDX1 antibodies, reductions to 22% (E164D) and 12% (E178K) compared with wild-type IPF1 levels were observed; with a full-length antibody the reductions were 50% (E164D) and 36% (E178K).

the wild-type protein, the reduction was not statistically significant (Fig. 6B).

IPF1 also binds as a heterodimer with the ubiquitous homeodomain protein Pbx1 to target sites such as the UE-A-TSEI element of the somatostatin promoter. Furthermore, the IPF1-Pbx1 complex is required for expansion of pancreatic cell populations during pancreas development (37). We thus assessed the transcriptional activity conferred by the wild-type and mutant proteins along with Pbx1 on the UE-A-TSEI element of the somatostatin promoter. We observed an impaired *trans*-activation when the IPF1 mutant cDNAs were cotransfected with Pbx1 expression vector.

We also considered that decreased function of mutant IPF1 could be due to impaired interaction with p300/CBP-mediated potentiation of IPF1 action (38). The increase in the transcriptional activation capacity of mutant IPF1, however, was not different from that of the wild type when adding p300/CBP (data not shown) (39).

To further elucidate whether the decreased transcriptional activation capacity could be due to decreased protein-protein interactions, we performed GST pull-down assays between wild-type and mutant proteins and E47-β2, HNF3β/Foxa2, and Pbx1. As helix 2 of the homeodomain was previously shown to be involved in the interaction with E47-β2, we first tested protein-protein interactions of GST-IPF1 with the heterodimer E47-β2 (23). The signal obtained with radiolabeled E47-β2 and wild-type IPF1 was similar to that with IPF1-E178K and 30% higher compared toIPF1-E164D (Fig. 7A). However, when correcting for the input of the wild-type and mutant GST-IPF1 proteins by Western blot analysis (data not shown), interactions between E47-β2 and wild-type or mutant IPF1 were similar. Identical results were obtained when interactions with HNF3β/Foxa2 (Fig. 7B) or Pbx1 (Fig. 7C) with wild-type and mutant proteins were investigated. Indeed, when the obtained signal was corrected for the GST fusion protein input, the interaction between HNF3β/Foxa2 or Pbx1 and mutant IPF1 was quantitatively similar to that observed for wild-type IPF1 (data not shown). We also assessed whether heterodimer formation of IPF1 and its mutants were intact by EMSA analysis. As shown in Fig. 7D, heterodimers of mutant IPF1-Pbx1 as well as IPF1-HNF3β (data not shown) were similar to those observed with wild-type IPF1. We thus conclude that functional interactions of the mutant IPF1 with E47-β2, HNF3 β/Foxa2, or Pbx1 are not significantly impaired compared with those of the wild-type protein.

Our results indicate that the decreased transcriptional activity conferred by IPF1 mutants and interacting partners can be accounted for by decreased mutant IPF1 levels.

Discussion

Here we describe the first case of a compound heterozygous mutation of the IPF1 gene leading to pancreas agenesis and consequently neonatal diabetes mellitus. Extensive experimental support (11, 12, 40–44) has established IPF1 as a key regulator of pancreas development. In fact, inactivation of IPF1 in mice leads to a rudimentary pancreatic bud made of only a few glucagon-producing cells, which subsequently does not expand and eventually results in pancreas agenesis (11, 12). In humans, Stoffers *et al.* (13) have identified a homozygous mutation of the IPF1 gene in a female infant resulting in pancreatic agenesis. The single nucleotide deletion caused a frameshift and led to a truncated, inactive, IPF1 protein missing the entire homeodomain. In contrast, here we describe compound heterozygous mutations causing unstable IPF1 protein and leading to pancreas agenesis in a female infant.

Both IPF1 mutants led to a decrease in transcriptional activity of the insulin and IPF1 gene promoter constructs; however, the decrease was moderate. Furthermore, a dose-dependant increase in activity was observed with both wild-type and mutant IPF1; twice as much mutant cDNA is necessary to be transfected into BHK21 cells to obtain similar transcriptional activation compared with wild-type IPF1. These results suggested that the intranuclear levels of the mutant proteins could be the limiting factor to obtain quantitatively similar effects compared with the wild-type protein. The fact that similar results were observed for both mutant proteins despite different mutations also favored this hypothesis.

Transcriptional capacity of the mutant IPF1 was also investigated with its known partners, such as HNF3β/Foxa2, Pbx1, and the heterodimer E47-β2. A moderate decrease in transcriptional activity was observed with both mutant IPF1 proteins, again suggesting that protein levels, and not functional impairment, could be implicated.

We found a decreased half-life for both overexpressed mutant proteins in BHK21 and InRIG9 cells, a glucagon-producing cell line, compared with that of wild-type IPF1. In addition, markedly lower concentrations of IPF1 mutants were always obtained in nuclear extracts of BHK21 cells despite similar transfection efficiency with the wild-type and mutant IPF1 cDNA. By contrast, biosynthesis of both mutants was normal both in BHK21 cells and when assessed by

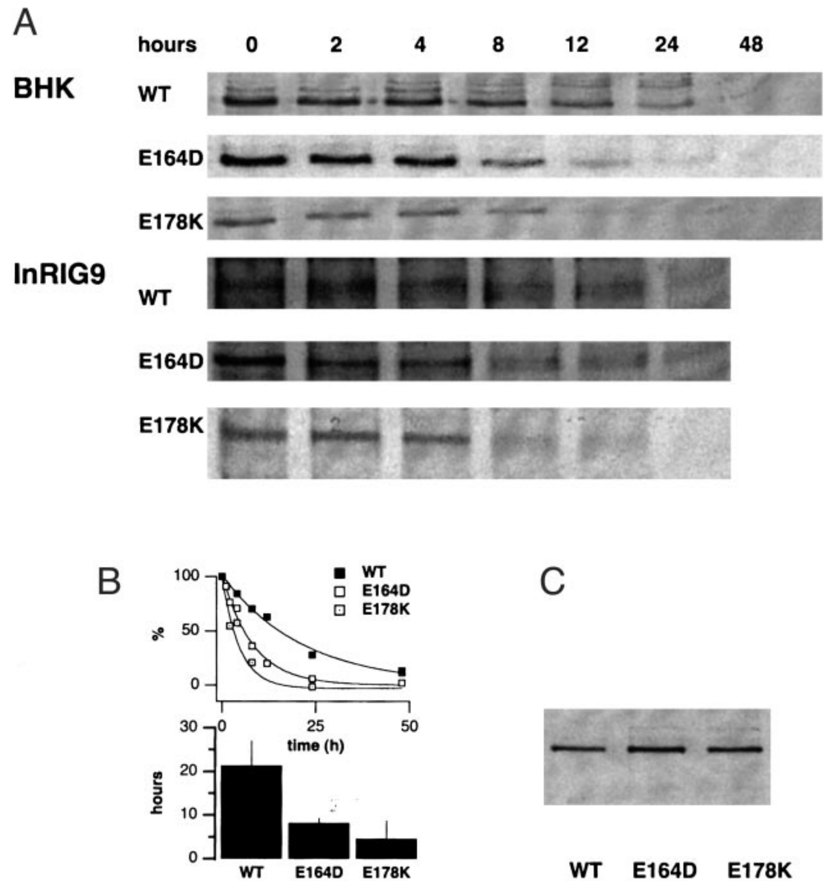

FIG. 5. Decreased amount of IPF1 protein due to increased degradation. A, BHK21 and InRIG9 cells were transfected with wild-type-IPF1, E164D-IPF1, and E178K-IPF1 cDNAs, respectively, pulse-labeled with [^{35}S]methionine for 1 h, and chased for up to 48 h. Immunoprecipitation of radioactive protein revealed faster degradation for both mutations. B, Gels were scanned, and calculations of half-lives were performed, setting the initial maximal radioactivity at 100%. Half-lives in BHK cells were calculated at 22 h for the wild-type protein, at 8 h for the E164D protein, and at 6 h for the E178K protein. The lines in the *upper panel* are calculated by exponential regression analysis. The *lower panel* shows a bar graph with the different half-lives and their confidence interval. In the InRIG9 cells similar results were obtained: 32 h for the wild-type protein, 6.5 h for the E164D mutant, and 3.3 h for the E178K mutant. C, Biosynthesis of wild-type and mutant IPF1 after labeling with [^{35}S]methionine for 10 min.

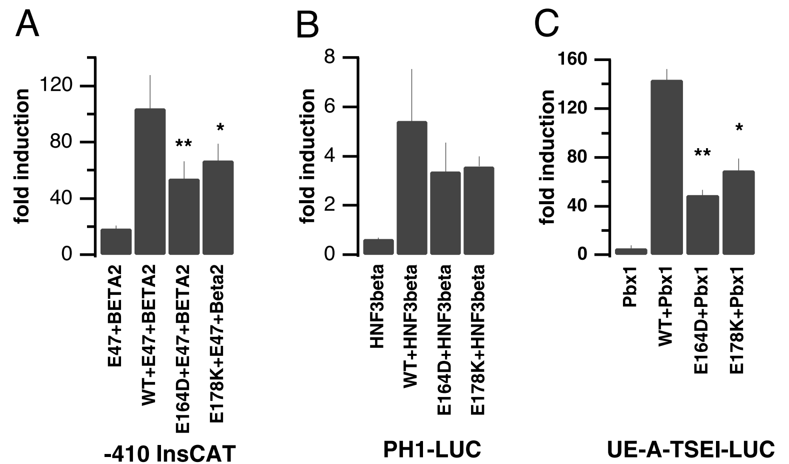

FIG. 6. Reduced transcriptional activation by mutant IPF1 in cotransfection experiments. Transient transfection studies using BHK21 cells were performed. A, Transient transfection with 0.25 μg wild-type and mutant IPF1 cDNAs, 0.25 μg E47 and β2 cDNAs, and 10 μg rat insulin gene promoter (n = 7). Data are presented as fold induction of basal CAT activity (reporter plasmid transfected along with an empty pSG5 expression vector). B, Transient transfection using 0.25 μg wild-type and mutant IPF1 as well as 0.25 μg HNF3β/Foxa2 cDNAs and 10 μg IPF1 gene promoter (PH1-LUC; n = 4). C, Transient transfection using again 0.25 μg wild-type and mutant IPF1 and 0.25 μg Pbx1 cDNAs on the UE-A-TSEI site of the somatostatin gene promoter (n = 4). Data are presented as fold stimulation of basal LUC activity (reporter plasmid transfected along with an empty pSG5 expression vector).

in vitro transcription translation assays. Our data thus indicate that both mutant IPF1 proteins have a short half-life, which markedly reduces their intracellular concentrations. Although our investigations of the function of IPF1, such as binding affinity for cognate DNA control elements, nuclear import, and interactions with other proteins, such as E47-β2, HNF3β/Foxa2, and Pbx1, have not identified any abnormality, we cannot rule out functional defects of the mutant IPF1, particularly as the mutations occur in the homeodomain at highly conserved sites.

Interestingly, we observed that IPF1 could also form homodimers, as seen on gel retardation assays. This is in concordance with the recent report that proteins from the homeodomain family are capable of forming homodimers (45). Homodimer formation of mutant IPF1 seemed to be decreased compared with that of wild-type IPF1. The biological significance of IPF1 homodimers has not been elucidated to date.

Our data clearly show that both mutations of IPF1 lead to a marked decrease in protein levels. This decrease may be further accentuated by the fact that IPF1 activates its own gene promoter, and thus in the presence of low levels of IPF1, the transcriptional activity of the IPF1 gene in the patient may be considerably reduced at a critical time of pancreas formation. These results suggest that the actual IPF1 expression

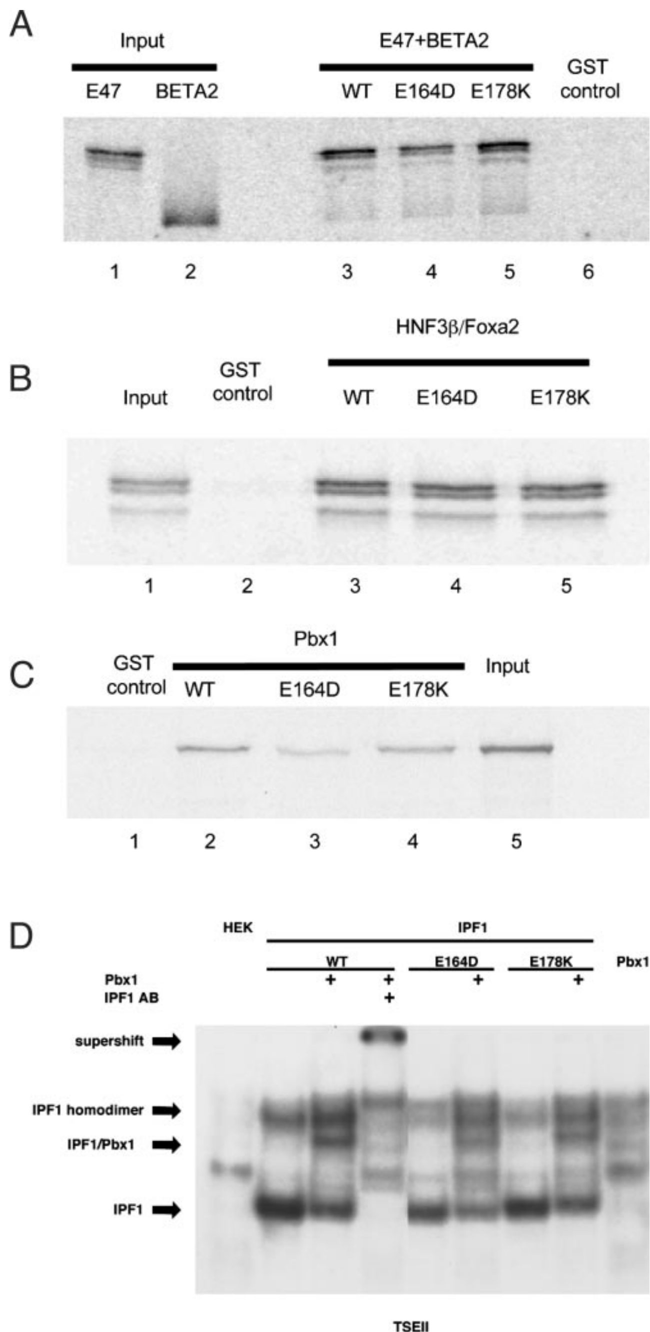

FIG. 7. Conserved protein-protein interaction of mutant IPF1. A, The interaction of IPF1 mutants E164D, E178K, and the heterodimer E47-β2 compared with the wild type is shown by GST pull-down assay. [^{35}S]Methionine-labeled E47-β2 was incubated with 10 μg bacterially expressed GST-IPF1WT (lane 3), GST-E164D (lane 4), GST-E178K (lane 5), and GST alone (lane 6). Lanes 1 and 2 show 1/10th of the input of E47 and β2, respectively. B, HNF3β/Foxa2 interaction with IPF1 wild-type and mutant proteins as analyzed by GST pull-down assay. [^{35}S]Methionine-labeled HNF3β/Foxa2 was incubated with 10 μg bacterially expressed GST-IPF1WT (lane 3), GST-E164D (lane 4), GST-E178K (lane 5), and GST alone (lane 2). C, GST pull-down assay showing the interaction between Pbx1 and IPF1 wild-type and its mutants. D, EMSA analysis with wild-type and mutant IPF1 proteins and the ^{32}P-labeled TSEII element of the somatostatin gene promoter. Nuclear extracts of wild-type (4 μg) and mutant (4 μg) IPF1 and Pbx1 (8 μg) from HEK293T cells were used. Antibody to IPF1 was able to supershift the heterodimer IPF1-Pbx1 as well as the homodimer IPF1.

level is critical for human pancreas formation. This hypothesis is further supported by the recent observation that only cells that express high levels of IPF1 emigrated from the epithelium into the mesenchyme and induced pancreatic bud formation, whereas cells expressing low levels of IPF1 remained within the epithelium during development (46).

Many diseases have been associated with mutations in transcription factors (47) that lead to haploinsufficiency, resulting in a reduction of the protein level, as in our case. The pathological mechanisms implicated in these diseases are mutations leading to an unstable protein, premature termination of translation, or a dominant negative effect.

Protein degradation is controlled by the ubiquitin-mediated system that degrades mutant proteins more rapidly than wild-type protein (48, 49). The ubiquitin system has been implicated in many human diseases, characterized by a change in the steady state level of a particular protein substrate. The diseases can be divided into two classes, resulting in either an accelerated, as in our case, or a decreased degradation rate of a particular protein. For example, an accelerated degradation of p53, which underlies the pathogenesis of human uterine cervical carcinoma, has been described (49). Recently it has been demonstrated that the ubiquitin pathway is involved in IPF1 degradation (50). It is therefore possible that the ubiquitin pathway is also involved in the increased turnover of both IPF1 mutants. Decreased IPF1 levels may also decrease the likelihood that target gene promoters are activated. In our case, the positive autoregulatory loop of IPF1 may then be disrupted. As for IPF1, several transcription factors may have a dual role, one during development and one in the differentiated state. In analogy to the mouse model, targeted disruption of IPF1 leads to the absence of the pancreas (11, 12), and tissue-specific disruption of IPF1 in the β-cell results in diabetes (4).

This suggests a threshold for IPF1 expression during development that was not achieved in the index case, resulting in pancreas agenesis. In adults, in contrast, a decreased IPF1 expression level may lead to diabetes (39). Indeed, several members of the family of the index case were already affected by diabetes mellitus; one diabetic family member could be analyzed and showed the E164D mutation. The parents had high normal fasting blood glucose levels in the absence of obesity, which may be associated with the single heterozygous mutation of the IPF1 gene. Indeed, heterozygous IPF1 mutations have also been linked to type 2 diabetes mellitus (28) and Pdx1$^{+/-}$ mice show an age-dependent decline of glucose tolerance due to increasing β cell apoptosis over time (51). It has also been noted that the severity of IPF1 mutations correlates with age of diabetes onset; therefore, mild partial IPF1 deficiency has the greatest effect late in life, but severe IPF1 deficiency leads to pancreas agenesis.

Taken together, a severely decreased level of IPF1 may lead to a developmental defect, such as pancreas agenesis, more subtle defects to derangements in glucose metabolism or diabetes. In conclusion, we propose that the absence of the pancreas in the index case may be explained by decreased IPF1 expression level during development, indicating that a sufficient level is crucial for human pancreas formation.

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