

Identification of PCIF1, a POZ Domain Protein That Inhibits PDX-1 (MODY4) Transcriptional Activity

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Hox factors are evolutionarily conserved homeodomain-containing transcription factors that activate and repress gene expression in a precise temporally and spatially regulated manner during development and differentiation. Pancreatic-duodenal homeobox 1 (PDX-1) is a Hox-type protein that is a critical requirement for normal pancreas development and for proper differentiation of the endocrine pancreas. In humans, PDX-1 gene mutation causes pancreatic agenesis and early- and late-onset type 2 diabetes. PDX-1 consists of an N-terminal transactivation domain, a homeodomain responsible for DNA binding and nuclear localization, and a conserved C terminus that is mutated in human diabetes but whose function is poorly understood. We have identified a novel POZ domain protein, PDX-1 C terminus-interacting factor 1 (PCIF1)/SPOP, that interacts with PDX-1 both in vitro and in vivo. PCIF1 is localized to the nucleus in a speckled pattern, and coexpression of PDX-1 alters the subnuclear distribution of PCIF1. Functionally, PCIF1 inhibits PDX-1 transactivation of established target gene promoters in a specific and dose-dependent manner that requires critical amino acids in the PDX-1 C terminus. PCIF1 is expressed in adult pancreatic insulin-producing β cells, and overexpression of PCIF1 inhibits the rat insulin 1 and rat insulin 2 promoters in the MIN6 insulinoma β cell line. The coexpression of PCIF1 with PDX-1 in β cells and the ability of PCIF1 to repress PDX-1 transactivation suggest that modulation of PDX-1 function by PCIF1 may regulate normal β cell differentiation.

Hox factors are evolutionarily conserved homeodomain-containing transcription factors that are critical for the determination of the body plan during early development and for the regulation of key differentiation-specific genes that determine cellular specificity. Proper spatial and temporal expression of *Hox* factors is required to orchestrate anterior-posterior segmentation, limb development, and organogenesis. *Hox* proteins regulate gene transcription by a well-coordinated and tightly regulated balance of gene activation and repression. In particular, the addition and refinement of repression domains have contributed to the evolutionary diversification of body segmentation (15, 37).

Pancreatic-duodenal homeobox 1 (PDX-1; also known as IPF-1, IDX-1, STF-1, Xlhx8, GSF, and IUF-1) is a *Hox*-type homeodomain protein encoded by a gene in the *ParaHox* cluster that is expressed in the region of the developing foregut that later becomes the duodenum, distal stomach, and pancreas (5, 24, 28, 29, 32, 46). As development proceeds, high-level PDX-1 expression is eventually restricted to the hormone-producing β and δ cells of the fully developed endocrine pancreas, whereas lower levels of expression are detected in certain ductal cells, the majority of exocrine acinar cells, and epithelial cells and Brunner's glands in the distal stomach and proximal duodenum. In the differentiated endocrine pancreas, PDX-1 regulates the transcription of important target genes, including the insulin, somatostatin, and glucose transporter 2 genes (reviewed in reference 14).

Gene dosage has a marked influence on the phenotype of

Pdx-1 mutations. Homozygous disruption of the *Pdx-1* gene results in pancreatic agenesis in both mice and humans (23, 39), whereas heterozygous truncation and missense *Pdx-1* (*Ipf-1*) mutations are associated with both early (MODY4)- and late-onset forms of type 2 diabetes in humans (18, 26, 40). Heterozygous *Pdx1*^{+/-} mice exhibit glucose intolerance, impaired secretagogue-induced insulin secretion in vivo, abnormal islet architecture, increased islet apoptosis, and, in older mice, decreased β cell mass (4, 12, 22). Mice in which *Pdx-1* is specifically inactivated in islet β cells by Cre-LoxP recombination develop diabetes with decreased insulin and Glut 2 expression and an increased proportion of α cells in the islets (1). Recent mouse models allowing conditional regulation of *Pdx-1* expression also demonstrate the key role of proper levels of *Pdx-1* expression throughout embryonic development and adult life (19, 43).

PDX-1 is a 283-amino-acid (aa) protein with a central homeodomain flanked by proline-rich N- and C-terminal domains (24, 29). A transactivation domain maps to the N terminus in the context of insulin and somatostatin gene transcription, and deletion of the N terminus results in a dominant negative PDX-1 protein (25, 33). The functional synergy of PDX-1 with E box proteins, such as E12 and E47, to activate insulin gene transcription is mediated by the N terminus (33), and glucose augments PDX-1 transactivation via the N terminus as well (35). The coactivator CBP physically interacts with the N terminus (2). PDX-1 interacts with the homeoprotein PBX1 through a conserved pentapeptide motif, FPWMK, located N terminal to the homeodomain; this interaction is important for the proper proliferation and expansion of all of the differentiated cell types of the pancreas (13). Depending on the alternatively spliced isoform of PBX1 that is involved, core-

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pressors may be recruited to the PDX-1/PBX1 heterodimer complex (2). The PDX-1 homeodomain contains the DNA binding domain as well as an atypical nuclear localization signal (30).

Human mutations in the C-terminal region of PDX-1 are associated with the development of type 2 diabetes mellitus, emphasizing the functional importance of this region in vivo (18, 44). However, in contrast to the function of the N terminus and the homeodomain, that of the well-conserved C terminus is poorly understood. The available data support both repressive and activating roles for these sequences. C-terminal truncation of PDX-1 is associated with increased synergy with E47 on the insulin promoter (33). The PDX-1 C terminus also confers inhibition in the context of fusion to the heterologous Gal4 DNA binding domain (34, 38). In contrast, on the somatostatin TAAT1 enhancer, the PDX-1 C terminus may also contain a cryptic transactivation domain (25, 33).

To gain insight into the role of the PDX-1 C terminus, we investigated it as a protein-protein interaction domain by yeast two-hybrid analysis. We identified a nuclear POZ domain protein that we named PCIF1 (PDX-1 C-terminal interacting factor). PCIF1 is a nuclear protein that interacts with PDX-1 both in vitro and in vivo and inhibits PDX-1 transactivation. In the adult pancreas, PCIF1 is expressed in the insulin-producing β cells and in a subset of exocrine acinar and duct cells. PCIF1 is the first protein identified to interact with the C-terminal domain of PDX-1, and it is the first partner that represses PDX-1 transactivation. The coexpression of PCIF1 with PDX-1 in the pancreas suggests that modulation of PDX-1 function by PCIF1 may be required for normal pancreas development and/or differentiation.

MATERIALS AND METHODS

Yeast two-hybrid screen. A yeast two-hybrid screen was carried out using the mouse PDX-1 C terminus as bait. The region of the mouse PDX-1 containing aa 206 to 283 [mPDX-1(206-283)] was inserted into the yeast Gal4 DNA binding domain vector pGBKT7 (Clontech Laboratories, Inc., Palo Alto, Calif.). mPDX-1(206-283) pGBKT7 was transformed into *Saccharomyces cerevisiae* strain AH109, and expression was confirmed by Western blot analysis. A commercially obtained yeast two-hybrid cDNA library from an embryonic day 17 mouse (1.3×10^7 clones; Clontech Laboratories, Inc.) was screened by high-density mating with yeast cells containing the bait plasmid. The PCIF1 clone satisfied the following three criteria and was therefore further studied: it supported growth on plates under high-stringency conditions only in the presence of the PDX-1 bait; it strongly activated both α - and β -galactosidase reporter genes only in the presence of the PDX-1 bait; and finally, the interaction in yeast cells was confirmed by cotransformation of the purified prey and bait plasmids.

RNA isolation, RT-PCR, and creation of a PCIF1 eukaryotic expression vector. For reverse transcription (RT)-PCR, the amplification primers were designed based on the sequence of the prey plasmid insert (forward primer, 5'-CGG GTT GAG ATT AAC GAC-3'; reverse primer, 5'-GAC ACA GAG TAA AAG CTC-3'). Total RNA was isolated from mouse β TC3 insulinoma cells and from adult mouse pancreas (TRIZOL reagent; Invitrogen Corp., Carlsbad, Calif.) and reverse transcribed (Ready-To-Go T-Primed First Strand kit; Amersham Biosciences Corp., Piscataway, N.J.). Control PCR was performed on RT-negative samples to exclude genomic DNA contamination for each cDNA preparation.

Based on the high homology of the prey sequence with human SPOB (speckle-type POZ protein), PCR amplification primers were designed to isolate a full-length coding sequence of PCIF1 from adult mouse pancreas RNA (forward primer, 5'-AAG CTT AAA CTG GCG ATG-3'; reverse primer, 5'-CTC GAG AGT TAT TTA GTG-3'). The 1,122-nucleotide coding region and an N-terminal Flag epitope tag were cloned into the pcDNA3 eukaryotic expression vector (Invitrogen).

GST pull-down assays, coimmunoprecipitation, and Western blot analysis.

Glutathione *S*-transferase (GST) fusion vectors containing the mouse PDX-1 C terminus [mPDX-1(206-283) and mPDX-1(144-283)] were created in the bacterial expression vector pGEX-4T-1, expressed in BL21 bacteria, and purified using glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, N.J.). Five micrograms of purified GST or GST fusion proteins was incubated with 5 μ l of 35 S-labeled PCIF1 (TNT Quick coupled transcription and translation kit; Promega, Madison, Wis.) for 2 h at 4°C and washed five times before sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out.

For Western blot analysis, whole-cell lysates and nuclear extracts were prepared as previously described (41). For coimmunoprecipitations, cells were briefly sonicated in a mixture containing 0.5% Triton X-100, 50 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% sodium deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and protease inhibitor cocktail. The soluble fraction was immunoprecipitated overnight at 4°C with anti-PDX-1 antiserum or anti-PCIF1 antiserum. For endogenous protein interactions, lysates were prepared using NEHN buffer (150 mM NaCl, 1 mM EDTA, 25 mM HEPES [pH 7.9], 0.5% NP-40). Preimmune and immune PDX-1 and PCIF1 antisera were cross-linked to protein A-Sepharose simultaneously and under identical conditions. The PCIF1 antiserum used for immunoprecipitations was generated against a recombinant bacterially expressed GST-tumor necrosis factor (TNF) receptor-associated factor (TRAF) domain. Bound proteins were eluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer and analyzed by Western blotting.

Immunostaining. HeLa cells were transfected with expression vectors for PDX-1 and PCIF1 (Lipofectamine 2000; Invitrogen), split into plastic chamber slides, allowed to adhere for 24 h, fixed in 4% paraformaldehyde, and double stained for PDX-1 (at a 1:750 dilution; rabbit anti-rat PDX-1) (41) and the Flag epitope tag (mouse monoclonal anti-Flag at 5 μ g/ml; Sigma, St. Louis, Mo.). Secondary antisera were applied for 2 h at room temperature (a 1:1,500 dilution of Cy3 donkey anti-rabbit and a 1:100 dilution of Cy2 donkey anti-mouse; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.).

The PCIF1 antiserum utilized for staining is a peptide-specific rabbit polyclonal antiserum raised against the N terminus of PCIF1 (SRVSPPPPAEMSS GPC) and conjugated to keyhole limpet hemocyanin via the cysteine (Covance). The resulting N-terminal antiserum was affinity purified and used at a dilution of 1:500 for both paraffin-embedded and fixed frozen tissues. For light microscopy of paraffin-embedded sections of adult mouse pancreas, horseradish peroxidase-conjugated anti-rabbit secondary antiserum was used, the signal was visualized by incubation with diaminobenzidine, and a hematoxylin counterstain was applied. For immunofluorescence of adult mouse pancreas, fixed frozen tissues were permeabilized with Triton X-100 and methanol, costained with anti-PCIF1 and antisera directed against insulin (Linco), glucagon (SC-7780; Santa Cruz), or somatostatin (SC-7819; Santa Cruz), and visualized with Cy2- and Cy3-conjugated secondary antisera (Jackson ImmunoResearch). Images were captured with a Nikon E600 microscope equipped with a CoolSnap camera interfaced with IPLab software (Scanalytics, Inc.). The specificity of the PCIF1 signal was verified by using the same concentration of rabbit immunoglobulin G (IgG) as that of the primary antiserum, which gave no specific signal. Further, the PCIF1 signal was completely blocked by preincubation of the N-terminus-specific antiserum with the N-terminal peptide antigen but was unaffected by preincubation with a C-terminal PCIF1 peptide.

Transfections and reporter assays. HeLa cells and MIN6 cells seeded into six-well tissue culture dishes were transfected with 0.5 μ g of reporter plasmid, 0.25 μ g of each expression plasmid, 0.1 μ g of cytomegalovirus- β -galactosidase internal control plasmid, and empty expression vector plasmid to maintain a constant amount of DNA (Lipofectamine 2000; GIBCO). Cells were harvested 48 h after transfection and assayed for luciferase (luciferase assay kit; Promega) or chloramphenicol acetyltransferase (CAT) activity as previously described (41). All data were normalized to β -galactosidase activity. Transfection assays were performed in triplicate at least three times.

The reporter plasmids used were as follows: (TAAT1)₅-65 SMS-CAT (6), 5FF1CAT (16), G51BCAT (multimerized Gal4-responsive promoter), (μ E5+ μ E2+ μ E3)₄-TAAT-Luc (Igh enhancer reporter; gift of John Choi, Children's Hospital of Philadelphia), and rat INS1 CAT and rat INS2 CAT. The expression vectors used were as follows: pCMX-mPDX-1, which was created by inserting the mouse PDX-1 cDNA (gift of Christopher Wright, Vanderbilt University) into the expression vector pCMX (gift of Mitchell Lazar, University of Pennsylvania), pCMV5-human PDX-1, pBJ5-rat PDX-1, and pMIGR-E47 (gift of John Choi). For the Gal4 transactivation assays, we used the Gal4 DNA binding domain vector PM1 (Clontech) containing the following in-frame cDNAs: PDX-1 (41), PDX-1(1-138), PDX-1(1-210), PDX-1(1-238), BETA2, and E47 (gift of Tom Kadesch, University of Pennsylvania).

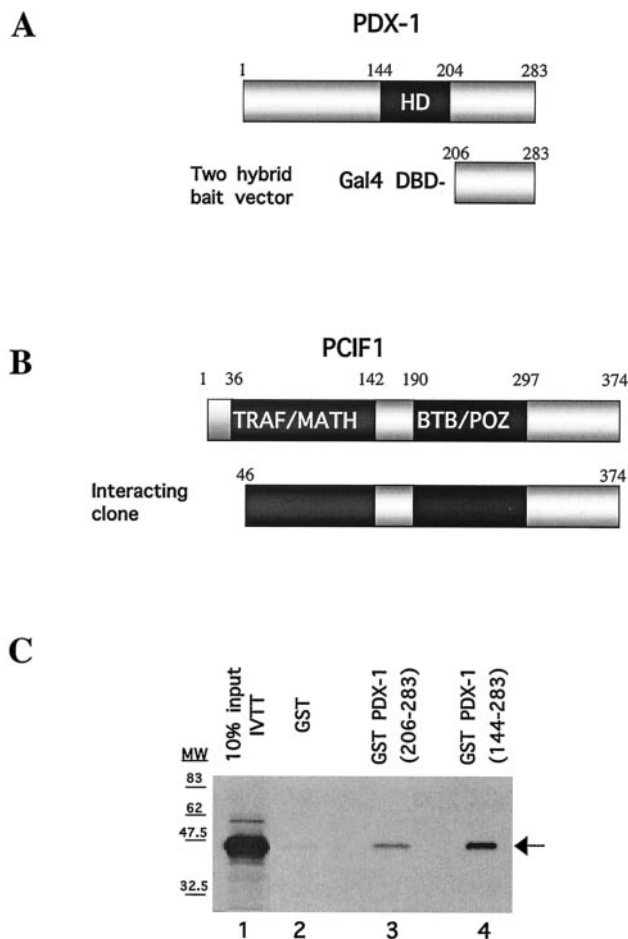


FIG. 1. Identification of PCIF1 in a yeast two-hybrid screen using the C terminus of PDX-1 as bait. (A) Diagram depicting the structure of PDX-1 and the region encoded in the bait vector. DBD, DNA binding domain. HD, homeodomain. (B) Diagram depicting the structure of PCIF1 and the portion encoded by the interacting clone. (C) GST pull-down assay demonstrating a specific interaction between the C terminus of PDX-1 and PCIF1. GST fusion proteins were incubated with in vitro-transcribed and -translated ^{35}S -labeled PCIF1. After washing, proteins bound to glutathione beads were analyzed by gel electrophoresis, dried, and exposed to film. Lanes: 1, 10% of the in vitro-transcribed and -translated product used in the assay; 2, GST; 3, GST-PDX-1(206-283); 4, GST-PDX-1(144-283). The position of ^{35}S -labeled PCIF1 is indicated by an arrow. IVTT, in vitro-transcribed and -translated product; MW, molecular weight (in thousands).

Nucleotide sequence accession number. The full-length coding sequence of PCIF1 isolated from adult mouse pancreas RNA has been deposited in GenBank under accession number AY538613.

RESULTS

Identification of PCIF1, a novel PDX-1 C terminus interacting factor. A yeast two-hybrid screen using the PDX-1 C terminus (aa 206 to 283) as bait yielded a clone that we have called PCIF1 (Fig. 1A and B). The physical interaction between PCIF1 and PDX-1 was confirmed in vitro by a GST pull-down assay (Fig. 1C). GST-PDX-1(206-283) and GST-PDX-1(144-283), but not GST alone, interacted weakly but specifically with ^{35}S -labeled in vitro-transcribed and -translated PCIF1. The interaction may be stabilized by the inclusion of

PDX-1 homeodomain sequences in the GST-PDX-1(144-283) fusion construct. By use of RT-PCR, the colocalization of PCIF1 mRNA with PDX-1 mRNA was demonstrated in adult mouse pancreas and in the mouse $\beta\text{TC-3}$ insulinoma cell line (data not shown). Following these initial observations, PCIF1 was further studied.

PCIF1 is the mouse homolog of human SPOP, a POZ domain protein of unknown function. A BLAST search of the PCIF1 clone identified its human homolog as SPOP, a widely expressed nuclear speckle-type protein of unknown function that was identified by expression cloning from a human HeLa cell cDNA library by use of the serum of a scleroderma patient (31). The PCIF1 gene encodes a protein of 374 aa that has a predicted molecular mass of 42 kDa and contains an N-terminal TRAF domain (aa 36 to 142) and a typical POZ domain (aa 190 to 297) (Fig. 1B). PCIF1 is evolutionarily conserved, sharing 100% amino acid identity with human SPOP and 78% amino acid identity with *Drosophila melanogaster* protein RE34508 (Fig. 2). The TRAF domain appears particularly well conserved. The portion of the PCIF1 gene present in the prey plasmid encodes aa 46 to 374, which includes a portion of the TRAF domain as well as the entire POZ and C-terminal domains (Fig. 1B). Thus, an intact TRAF domain does not appear to be necessary for the physical interaction of PCIF1 with PDX-1 in yeast cells.

PCIF1 is a nuclear protein. Upon examining the subcellular localization of PCIF1, we observed a speckled pattern of nuclear staining, similar to that reported for SPOP, in HeLa cells transfected with Flag-tagged PCIF1 (Fig. 3A) (31). When PCIF1 alone was transfected into HeLa cells, 92% of the labeled cells had a nuclear speckled pattern and 8% had a diffuse nuclear pattern of PCIF1 staining. Cotransfection of expression vectors for both PCIF1 and PDX-1 showed overlapping expression in the nuclei of transfected cells. Interestingly, the localization of PCIF1 to nuclear speckles was altered by the presence of PDX-1. HeLa cells cotransfected with both PDX-1 and PCIF1 had a predominantly diffuse pattern of staining (77% diffuse versus 23% speckled). In contrast, the staining pattern of PDX-1 was not affected by the overexpression of PCIF1 under these conditions. The nuclear localization of PCIF1 was confirmed by Western blot analysis of nuclear and cytoplasmic fractions of transfected HeLa cells (Fig. 3B). Thus, PDX-1 and PCIF1 were colocalized in the nucleus. The data also suggest that interaction with PDX-1 may modify the structure and/or other interactions of PCIF1, resulting in an altered subnuclear distribution. The ability of PDX-1 to alter the nuclear localization of PCIF1 further verifies that these proteins interact in vivo, and it suggests that there may be functional consequences of the interaction between PCIF1 and PDX-1.

PCIF1 interacts with PDX-1 in vivo. We next directly tested whether PCIF1 interacts with PDX-1 in vivo. We cotransfected HeLa cells with vectors expressing PDX-1 and PCIF1 tagged with Flag. Cell lysates were analyzed by immunoprecipitation with antisera directed against PDX-1 followed by Western blotting with Flag and PDX-1 antisera. Flag-PCIF1 was readily immunoprecipitated by PDX-1 antiserum only with coexpression of PDX-1 (Fig. 4A), indicating that PCIF1 can associate with PDX-1 in vivo.

We have also demonstrated the in vivo association of PDX-1

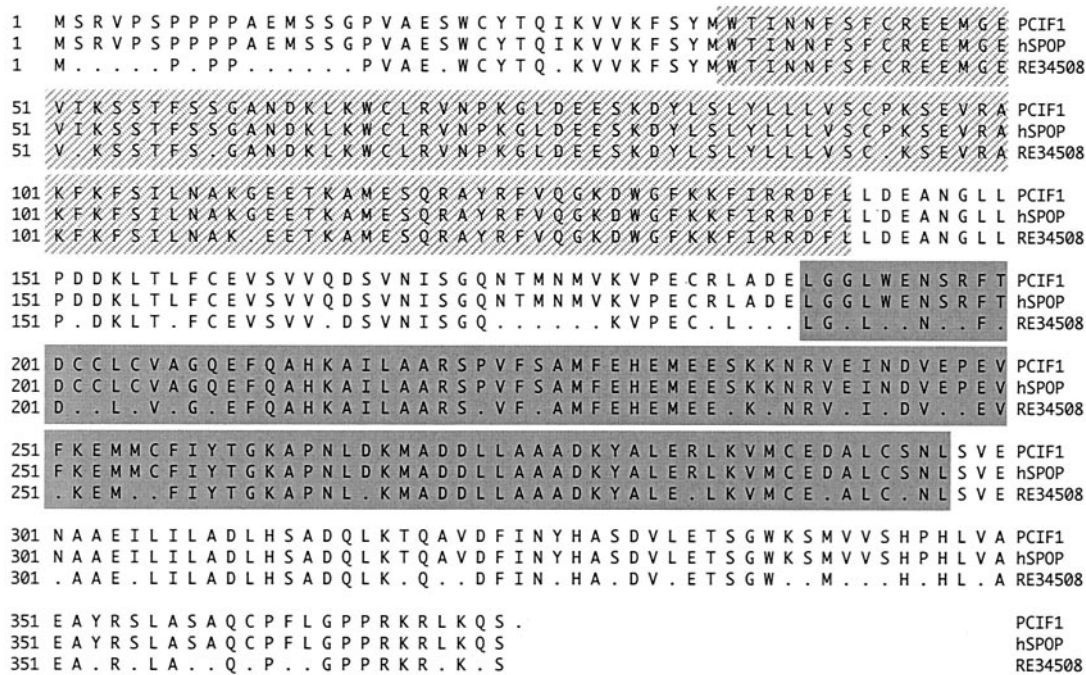


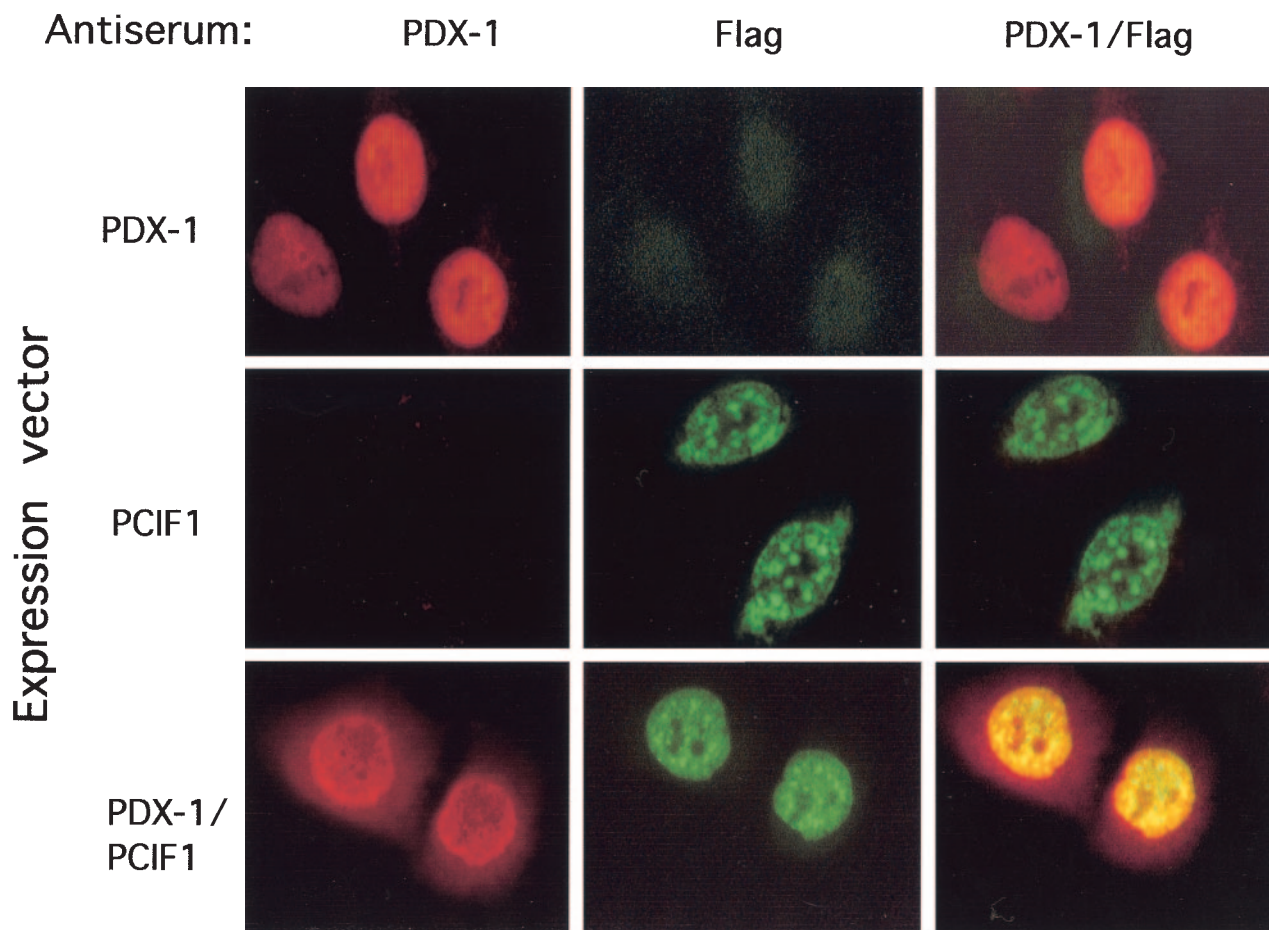
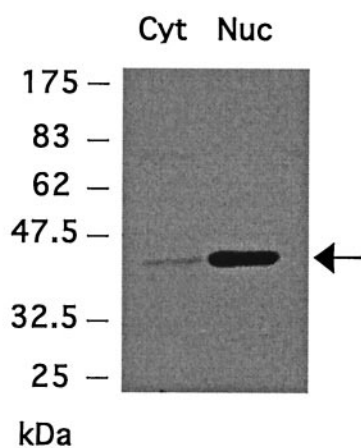
FIG. 2. PCIF1 is the mouse homolog of human SPOP, a novel nuclear speckle-type protein of unknown function. The amino acid sequence alignment of mouse PCIF1, human SPOP (hSPOP), and the *D. melanogaster* RE34508 protein product is shown. The location of the TRAF domain is indicated by hatching, and the location of the POZ domain is indicated by shading.

and endogenous PCIF1 protein in HeLa cell extracts. The level of PDX-1 expression in these transiently transfected HeLa cells approximates the endogenous level of PDX-1 in β insulinoma cell lines (Fig. 4D). As shown in Fig. 4B, PDX-1-specific antiserum was used to specifically immunoprecipitate PDX-1 (lane 2). This resulted in coimmunoprecipitation of endogenous PCIF1 (Fig. 4B, lane 6), whereas preimmune PDX-1 antiserum did not pull down PDX-1 or PCIF1 (lanes 4 and 8), demonstrating the specificity of the assay. Similarly, TRAF domain-specific anti-PCIF1 was used to specifically immunoprecipitate endogenous PCIF1 from HeLa cell extracts (data not shown), resulting in coimmunoprecipitation of PDX-1 (Fig. 4C, lane 2), whereas preimmune PCIF1 antiserum did not pull down PDX-1 (lane 1). We have also observed a reproducible but weak interaction between endogenous PCIF1 and PDX-1 in MIN6 and β TC-3 cells in which detection is limited by the lower abundance of PCIF1 in β insulinoma cells than in HeLa cells (not shown). Taken together, the GST pull-down assays and the interaction of overexpressed and endogenous proteins in eukaryotic cells indicate that PDX-1 interacts with PCIF1 in vitro and in vivo.

PCIF1 inhibits PDX-1 transactivation of target genes. To determine the functional consequence of PCIF1 interaction with PDX-1, we next analyzed the effect of PCIF1 on PDX-1 transactivation of two well-established PDX-1 target gene promoters. To this end, vectors expressing PCIF1 and PDX-1 were cotransfected into HeLa cells along with a CAT reporter gene driven by the somatostatin promoter element TAAT1. CAT

activity was then measured as a function of PCIF1 expression. PDX-1 strongly transactivated the somatostatin-enhancer promoter construct (Fig. 5) (25, 29). Transcriptional activation of this promoter by PDX-1 was significantly diminished by coexpression of PCIF1 (Fig. 5A). Western blot analysis revealed that PCIF1 coexpression did not affect PDX-1 expression levels, indicating that reduced PDX-1 expression did not explain the reduction in PDX-1 transactivation. Basal reporter gene expression was mildly diminished by PCIF1 in this assay but was entirely unaffected in two other PDX-1 transcriptional reporter assays (see below). Further, the inhibition of PDX-1 by PCIF1 was dose dependent, as demonstrated in Fig. 5B. A fourfold excess of PCIF1 expression plasmid led to nearly complete inhibition (~95%) of PDX-1 transactivation. In this latter experiment, a decrease in PDX-1 expression was evident with PCIF1 coexpression; however, the PDX-1 expression level did not vary further with increasing amounts of PCIF1 expression, in contrast to the progressive reduction in PDX-1 transactivation, again indicating that alterations in PDX-1 expression level do not account for the decrease in PDX-1 transactivation.

To determine whether PCIF1 can also modulate PDX-1 transactivation of the insulin promoter, we utilized a reporter containing the Far-FLAT minienhancer of the rat insulin promoter which contains adjacent A and E boxes that bind BETA2/E2A heterodimers and homeodomain factors, respectively (16). As shown in Fig. 6A, the strong synergistic activation of this enhancer by cotransfection of PDX-1 and the E2A

A**B**

gene product E47 was markedly inhibited by PCIF1 (~80%). This inhibition was specific for PDX-1, since PCIF1 did not inhibit the ability of E47 to transactivate the highly E47-responsive IgH enhancer reporter (μ E5+ μ E2+ μ E3)₄-TATA-Luc (6) (Fig. 6B). The specificity of PCIF1 for PDX-1 was

FIG. 3. The PCIF1 gene encodes a nuclear protein that colocalizes with PDX-1. (A) Double immunofluorescence staining of HeLa cells transfected with expression vectors encoding Flag-tagged PCIF1 (green) and PDX-1 (red). Expression vectors are indicated on the left, and antisera are indicated along the top. The right column shows the green and red images merged in Adobe Photoshop. (B) Western blot analysis of cytoplasmic (Cyt) and nuclear (Nuc) fractions prepared from HeLa cells transfected with Flag-tagged PCIF1. The position of PCIF1 is indicated by an arrow.

further demonstrated in a series of Gal4 fusion assays in which the coding regions of PDX-1, E47, and BETA2 were individually fused to the Gal4 DNA binding domain. The transactivation potential of the Gal4 fusions was assessed in the absence and presence of PCIF1. PCIF1 reduced PDX-1 transactivation in this assay by more than 90% but had no effect on the transactivation potential of E47 or BETA2 (Fig. 6C). Thus, PCIF1 specifically down-regulates the ability of PDX-1 to activate target genes. The data from this experiment also imply that inhibition of PDX-1 transactivation by PCIF1 does not simply occur by interference with the binding of PDX-1 to enhancer elements in target gene promoters, since activity in the Gal4 assay depends on the binding of the Gal4 DNA binding domain to the multiple upstream activation sequence

copies in the G51bCAT reporter and not on DNA binding by the PDX-1 homeodomain.

The functional interaction of PCIF1 with PDX-1 requires critical amino acids in the PDX-1 C terminus. The functional interaction of PCIF1 with PDX-1 is conserved during evolution. Mouse PCIF1 inhibited transactivation of mouse, rat, and human PDX-1 homologs to a similar extent (data not shown), indicating that PCIF1 interacts with a conserved motif in PDX-1. Since PCIF1 binds to the C terminus of PDX-1, we hypothesized that the C terminus would be required for repression of PDX-1 activity by PCIF1. Indeed, the ability of PCIF1 to inhibit transactivation was completely abolished with the deletion of the C terminus (Fig. 7A). In addition, PDX-1(1-210) exhibited decreased transactivation compared with that of full-length PDX-1, indicating that the C terminus of PDX-1 may subserve both transactivation and repression functions.

To map the region within the C terminus of PDX-1 that is required for PCIF1 inhibition, a series of truncation constructs were created as fusions with the Gal4 DNA binding domain. When only the C-terminal 45 aa were deleted from PDX-1 [Gal4-PDX-1(1-238)], PCIF1 still inhibited transactivation of the Gal4 reporter to the same degree as it did full-length Gal4-PDX-1 (Fig. 7B). Within the C terminus of PDX-1, aa 210 to 238 are highly conserved among human and rodent PDX-1 and the more distant *Xenopus* and zebra fish homologs of PDX-1. Remarkably, the deletion of aa 210 to 238 from Gal4-PDX-1(1-238) completely abolished repression by PCIF1. Western blot analysis indicated that this mutant was well expressed and appropriately localized to the nucleus (Fig. 7D). These data indicate that aa 210 to 238 of PDX-1 containing the proximal portion of the C terminus are essential for PCIF1 inhibition of PDX-1 transactivation. These functional data are in agreement with GST pull-down assays indicating that sequences in the C terminus of PDX-1 mediate the physical interaction with PCIF1 (Fig. 1C).

PCIF1 is expressed in pancreatic β cells and represses insulin promoter activity in MIN6 insulinoma cells. To determine whether endogenous PCIF1 is coexpressed with PDX-1 in vivo, we examined the adult tissue distribution of PCIF1 mRNA. As shown in Fig. 8A, PCIF1 mRNA is widely expressed but most abundant in the pancreas when normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA. When normalized to actin mRNA levels, the pancreas remains one of the two tissues that express the highest levels of PCIF1 mRNA (data not shown). These data are in agreement with the previously reported distribution of human SPOP mRNA (6). Next, we examined the distribution of PCIF1 in the pancreas by using affinity-purified N-terminal PCIF1 peptide-specific antiserum. Peptide immunoabsorption controls showed an appropriate blockade when the N-terminal peptide epitope was used and no effect when an unrelated C-terminal peptide epitope was used (data not shown). In the adult pancreas, PCIF1 was expressed in the islets and in a subset of acinar and duct cells (Fig. 8B), a pattern that closely parallels the well-established expression pattern of PDX-1. To confirm that PCIF1 is expressed in β cells, double immunofluorescence staining for PCIF1 and insulin was carried out. PCIF1 was expressed in the majority of β cells (Fig. 8C). Since PDX-1 is also expressed in the majority of β cells, this indicates that

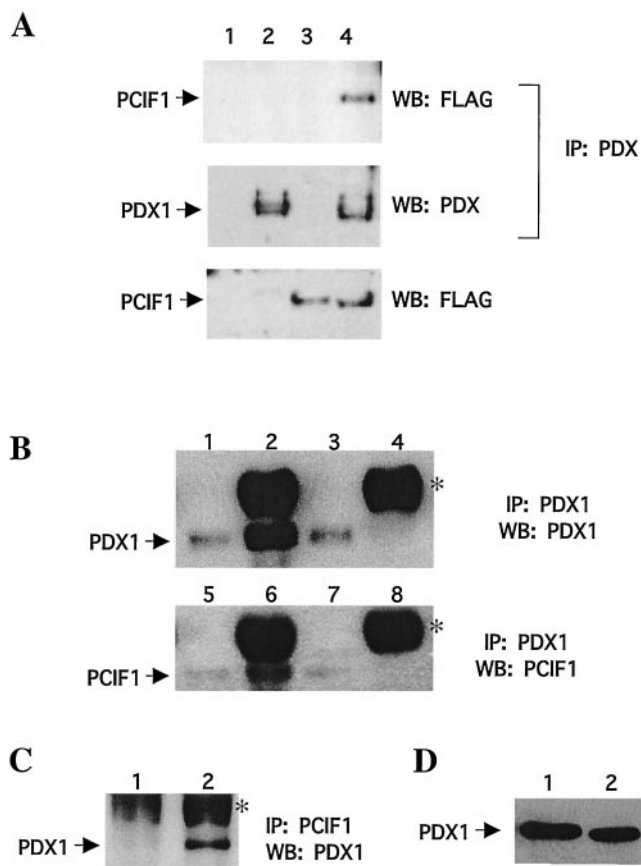


FIG. 4. Coimmunoprecipitation (IP) of PDX-1 and PCIF1 showing that PCIF1 and PDX-1 interact in vivo. (A) Lysates of transfected HeLa cells were immunoprecipitated with anti-PDX-1, and Western blot (WB) analyses for PDX-1 and Flag were performed. Lanes: 1, empty vectors; 2, PDX-1; 3, PCIF1; 4, PDX-1 and PCIF1. (B) Endogenous PCIF1 interacts with PDX-1 in transiently transfected HeLa cells. Lysates were prepared from HeLa cells transfected with 3.5-fold less PDX-1 expression vector (1 μ g/10-cm dish) than used for panel A, and immunoprecipitation of PDX-1 was carried out. Western blots for PDX-1 (lanes 1 to 4) and PCIF1 (lanes 5 to 8) are shown. Lanes 1, 3, 5, and 7, 0.5% of input extract; lanes 2 and 6, immunoprecipitation with anti-PDX-1; lanes 4 and 8, immunoprecipitation with preimmune serum. (C) HeLa cells were transfected as described for panel B, and lysates were immunoprecipitated with anti-PCIF1 (lane 2) or preimmune serum (lane 1) followed by Western blot analysis with anti-PDX-1. An asterisk indicates cross-reactivity of the secondary antiserum with the heavy chain of the immunoprecipitating antisera. (D) PDX-1 expression in HeLa cells transfected as described for panels B and C (lane 1) is similar to the endogenous PDX-1 expression level in mouse insulinoma MIN6 cells (lane 2) (15 μ g of protein loaded in each lane).

PCIF1 colocalizes with PDX-1 in β cells. Notably, endogenous PCIF1 expression appeared to be diffusely distributed in the nuclei of pancreatic β cells, in contrast to the highly speckled localization of overexpressed PCIF1 in HeLa cells. We could not determine whether PCIF1 and PDX-1 are colocalized in a subset of acinar cells because both detection reagents are rabbit polyclonal antisera.

We next determined whether PCIF1 also represses insulin gene transcription in β cells by using intact rat insulin 1 and 2 promoter reporters in mouse MIN6 insulinoma cells. As previously observed, the rat INS1 promoter had greater basal

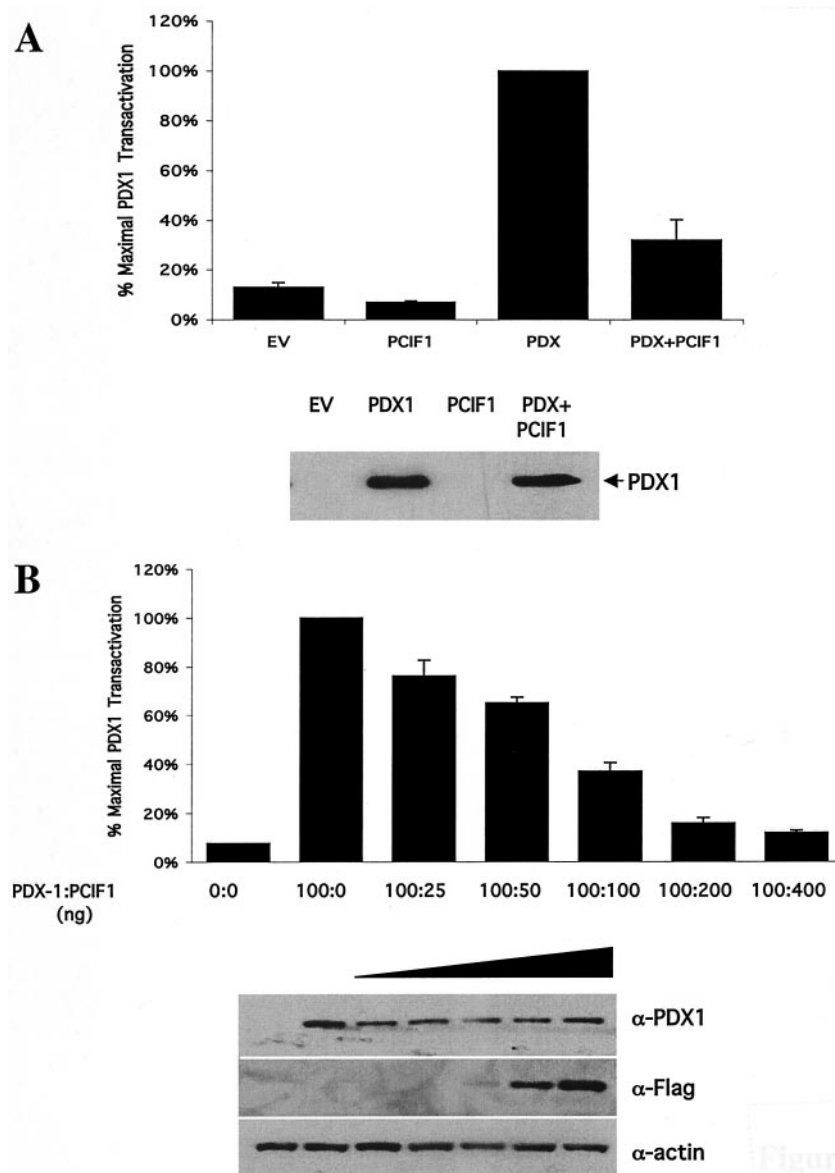


FIG. 5. PCIF1 inhibits PDX-1 transactivation of the somatostatin promoter in a dose-dependent manner. A Western blot showing the expression level of PDX-1 in the presence and absence of PCIF1 coexpression is shown beneath each graph. (A) HeLa cells transfected with expression vectors for Flag-PCIF1, PDX-1, and the corresponding empty vectors (EV) along with the PDX-1-responsive somatostatin promoter reporter (TAAT1)₅₋₆₅ SMS-CAT. (B) HeLa cells cotransfected with a constant amount of PDX-1 expression vector (100 ng) and increasing amounts of Flag-PCIF1 (0 to 400 ng) along with the (TAAT1)₅₋₆₅ SMS-CAT reporter. Activity was normalized for transfection efficiency according to internal-control β -galactosidase activity. Transactivation by PDX-1 alone was set at 100%.

activity than the rat INS2 promoter. We observed a dose-dependent inhibition of both reporters by PCIF1 (Fig. 8D). Similar results were observed in the β TC-3 insulinoma cell line (data not shown). Of note, the rat insulin 2 promoter was more sensitive to repression by PCIF1, which may be related to the presence of fewer homeodomain-binding A box elements and specifically the presence of only one A/E box enhancer (Nir-P1) in the rat insulin 2 promoter compared to the two enhancers (Nir-P1 and Far-FLAT) present in the rat insulin 1 promoter (17). These data indicate that PCIF1 is expressed in the islet β cells and that it can modulate insulin promoter activity in β cell lines.

DISCUSSION

Despite the extensive characterization of PDX-1 as a critical regulator of pancreas development and differentiated β cell function and as a human diabetes gene, the function of the conserved C terminus of PDX-1 has been elusive. In the present study, we demonstrate for the first time that the C terminus of PDX-1 serves as a protein-protein interaction domain that mediates the physical and functional association with a novel regulatory molecule, PCIF1.

PCIF1/SPOP has two intriguing functional domains: conserved N-terminal TRAF and C-terminal POZ domains. The

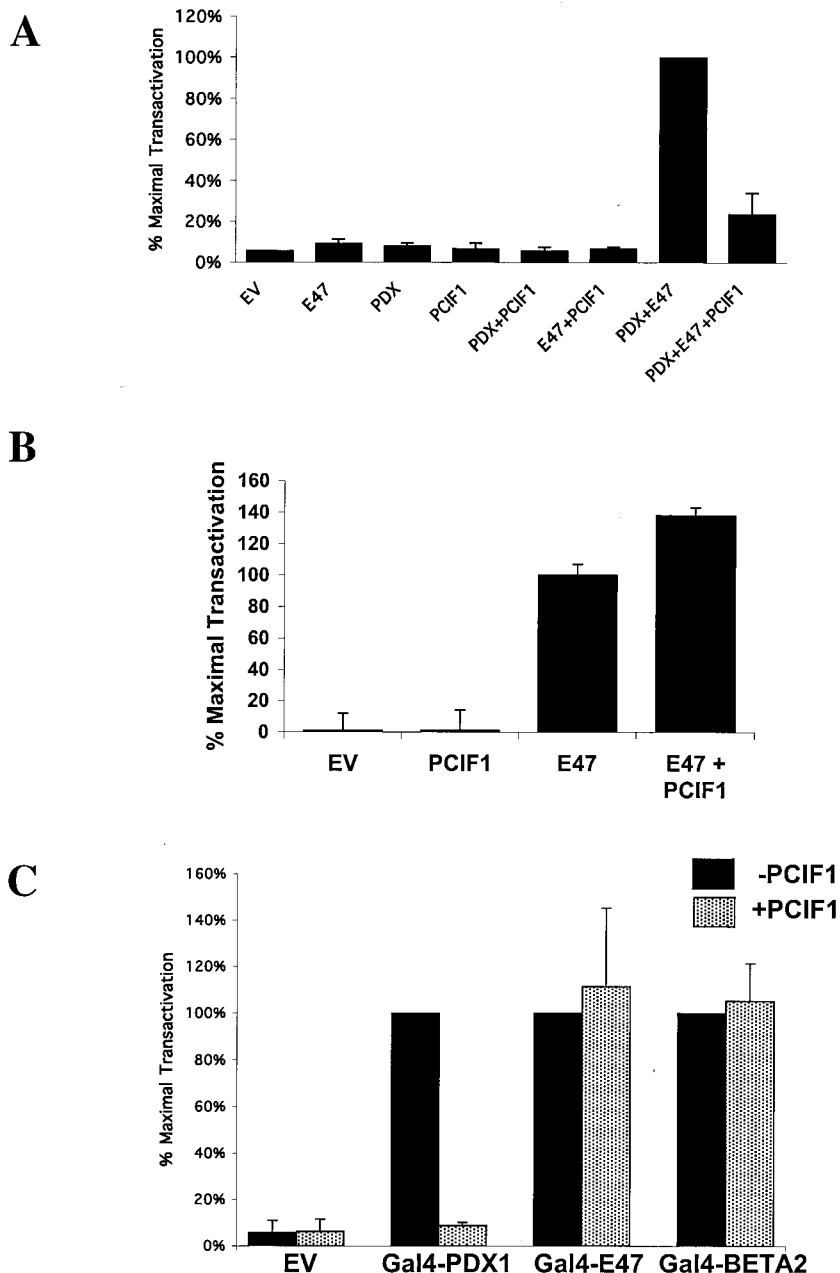


FIG. 6. PCIF1 specifically inhibits PDX-1 activation of the insulin promoter. (A) HeLa cells transfected with expression vectors for Flag-PCIF1, PDX-1, E47, and the corresponding empty vectors (EV) along with the rat insulin Far-FLAT minienhancer promoter reporter 5FF1CAT. Maximal synergistic transactivation by PDX-1 and E47 was set at 100%. (B) PCIF1 does not impair E47 transactivation. HeLa cells were cotransfected with pCMX-E47 and the E47-responsive reporter (μ E5+ μ E2+ μ E3)₄-TATA-Luc derived from an IgH promoter element. Data are presented as the percentage of maximal E47 transactivation in the absence of cotransfected PCIF1. (C) PCIF1 inhibition is specific for PDX-1. Gal4-PDX-1, Gal4-E47, and Gal4-BETA2 were cotransfected with EV (solid bar) or Flag-PCIF1 (shaded bar) along with the Gal4 reporter G51bCAT. The activity of each Gal4 fusion in the absence of PCIF1 was set at 100%. All reporter activities are normalized for transfection efficiency variation according to internal-control β -galactosidase activity.

POZ domain is an evolutionarily conserved protein-protein interaction motif found in diverse proteins, including transcription factors, oncogenic proteins, ion channel proteins, and some actin-associated proteins (7). The most well characterized POZ domain transcription factors are the human oncogene products PLZF (promyelocyte leukemia zinc finger) and BCL-6. PLZF translocations have been found in humans with

acute promyelocytic leukemia; these translocations result in the fusion of the PLZF POZ domain and two of its nine zinc fingers with the retinoic acid receptor α (RAR α) gene, which results in POZ domain-mediated recruitment of a histone deacetylase (HDAC) complex and resultant abnormal repression of RAR α target promoters (reviewed in reference 7). Recruitment of HDAC-corepressor complexes to the POZ

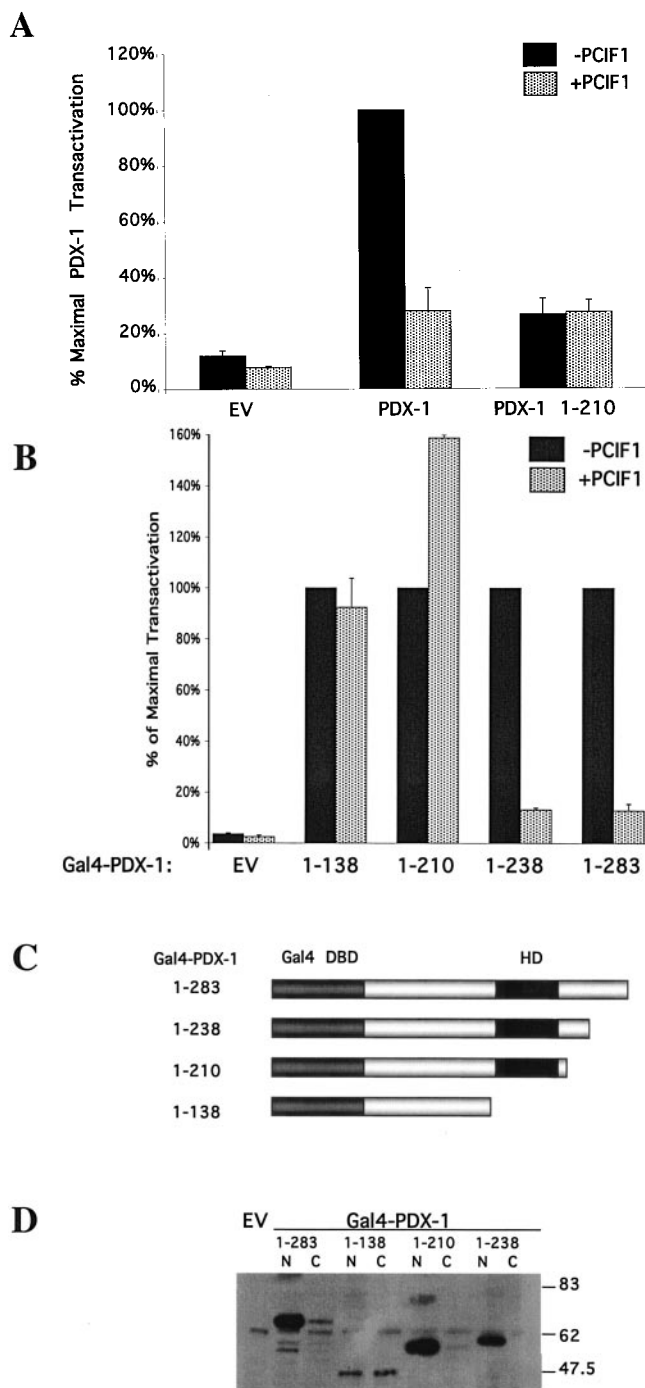


FIG. 7. PCIF1 inhibition of PDX-1 requires the C terminus of PDX-1. (A) HeLa cells transfected with expression vectors for Flag-PCIF1, PDX-1, PDX-1(1-210), and the corresponding empty vectors (EV) along with the reporter (TAAT1)₅₋₆₅ SMS-CAT are shown. Maximal transactivation by PDX-1 was set at 100%. (B) PCIF1 inhibition requires aa 210 to 238 of PDX-1. Gal4-PDX-1 or various C-terminal truncation mutants of PDX-1 fused to the Gal4 DNA binding domain were cotransfected with EV (solid bar) or Flag-tagged PCIF1 (shaded bar) along with the Gal4 reporter G51bCAT. The normalized CAT activity of each Gal4 fusion in the absence of PCIF1 was set at 100%. (C) Schematic depiction of the various Gal4-PDX-1 fusion constructs. (D) Western blot of cytoplasmic (C) and nuclear (N) extracts prepared from HeLa cells transfected with Gal4-PDX-1 fusion constructs. Visualization was with anti-PDX-1 number 251 directed against the N terminus. DBD, DNA binding domain; HD, homeodomain.

domain has been demonstrated for a number of other POZ domain transcription factors, including BCL-6, supporting this as a general mechanism for POZ-mediated transcriptional repression (8, 10, 11, 21, 45). These reports suggest that the likely mechanism of PCIF1/SPOP repression of PDX-1 target promoters is via recruitment of a corepressor complex. However, it is also important to note the existence of POZ domain factors that serve as transcriptional activators (27) and that repressive POZ domains that do not recruit the known corepressors and HDACs also exist (9).

The TRAF domain was originally defined as being in a family of adaptor proteins that bind to the cytosolic tail of TNF receptors (3, 48). TRAF domains are typically found at the C terminus, and they mediate both homotrimeric interactions and heterocomplex formation with TNF receptors, other adaptor proteins, kinases, and inhibitor-of-apoptosis homologs. The TRAF domain of PCIF1/SPOP is atypically located at the N terminus and does not self-associate, although it does interact weakly with the related domains from TRAF1 and TRAF6 (48). Recently, Takahashi et al. identified SPOP in a yeast two-hybrid screen for proteins that interact with the distinctive C-terminal domain of macroH2A1.2, a variant histone that is enriched in, although not exclusively localized to, the inactive X chromosome (42). Deletion of the TRAF domain from PCIF1/SPOP causes a relocation of SPOP from speckles to a more diffuse distribution within the nucleus, indicating a role in subnuclear localization (31; A. Liu and D. A. Stoffers, unpublished observations). It is intriguing to speculate that, in addition to the POZ domain, the TRAF domain of PCIF1/SPOP is also involved in mediating repression via effects on subnuclear localization and potentially through modification of chromatin structure.

Mining of the databases reveals at least eight close relatives of PCIF1/SPOP in the mouse genome (20; our unpublished observation). The POZ domain and C-terminal regions are particularly well conserved. The potential interaction of PDX-1 with other members of the PCIF1 gene family will be explored in future experiments. Notably, a *Caenorhabditis elegans* member of this family was described in two recent publications in which the *C. elegans* POZ and TRAF domain factor, MEL-26, was demonstrated to be a substrate-specific adaptor of the CUL-3 ubiquitin-ligase complex (36, 47). In this new model, the POZ domain of MEL-26 interacts with culin-3, a scaffold protein that mediates the interaction with an E3 ubiquitin ligase, while the TRAF domain is proposed to mediate interaction with substrates that are recruited to the CUL-3 complex for ubiquitination and subsequent degradation by the proteasome. In considering whether PCIF1/SPOP could regulate PDX-1 protein stability by enhancing ubiquitination and degradation, we have closely followed PDX-1 expression levels, and we have not observed a consistent alteration in PDX-1 molecular weight or expression level in the presence of PCIF1 (Fig. 5A). Based on our present data and on the plethora of reports implicating POZ domain factors in transcriptional repression, we believe that the functional interaction of PCIF1 with PDX-1 relates to the role of PCIF1 as a corepressor molecule.

The experimental data presented here support a model in which PCIF1/SPOP interacts with PDX-1 via a conserved sequence in the C terminus of PDX-1. The physical interaction

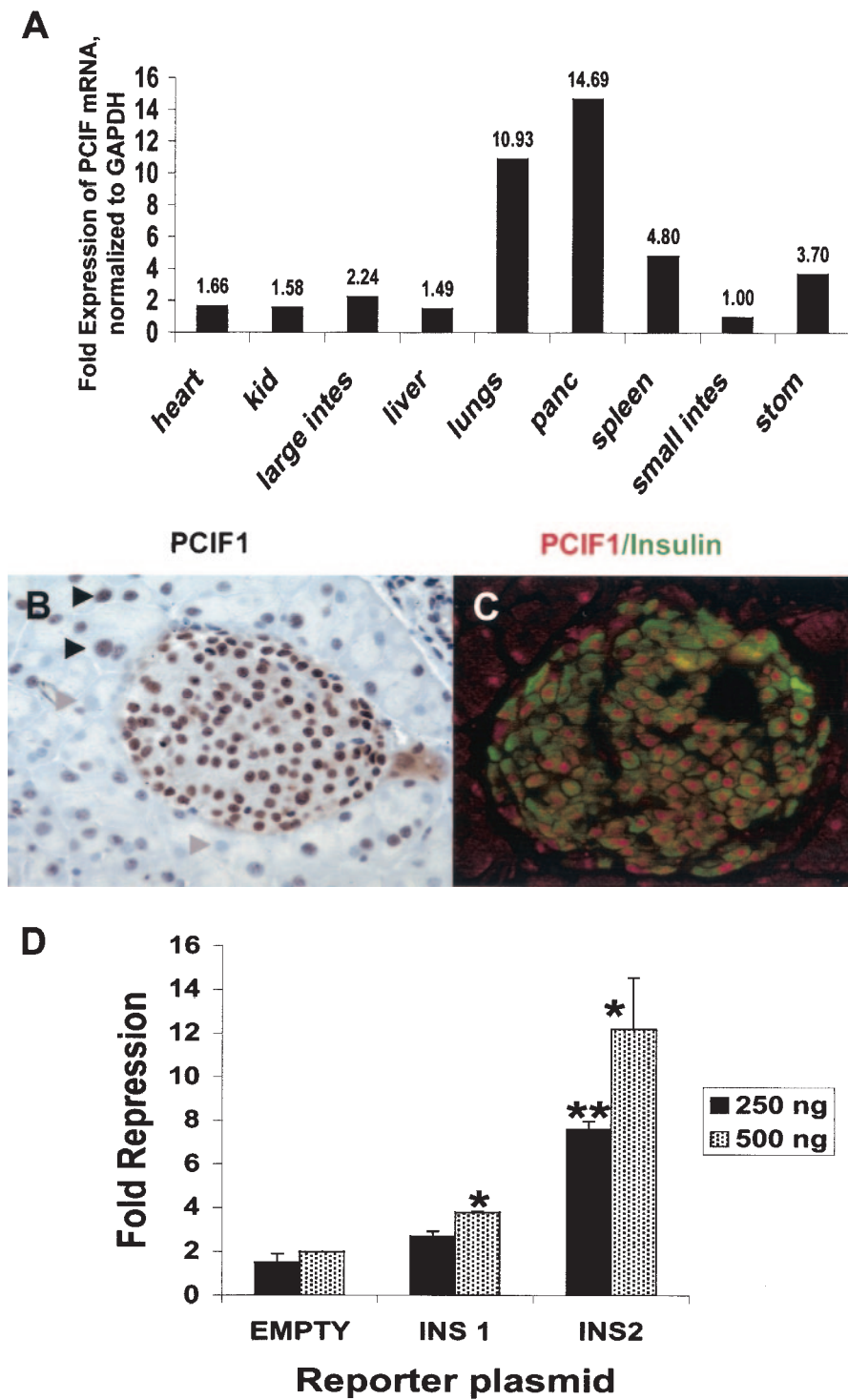


FIG. 8. PCIF1 is expressed in pancreatic β cells and represses insulin promoter activity in MIN6 cells. (A) Real-time PCR detection of PCIF1 reveals high expression in total pancreatic RNA. Total RNA from adult mouse tissues was isolated, and PCIF1 mRNA was quantitated by RT and real-time PCR detection. Data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The lowest expression, that for small intestine, was set to 1 for *n*-fold expression calculations. kid, kidney; intes, intestine; panc, pancreas; stom, stomach. (B) Immunohistochemistry of paraffin-embedded sections of adult mouse pancreas stained with anti-PCIF1. (C) Double immunofluorescence of fixed frozen sections of adult mouse pancreas for PCIF1 and insulin. (D) The insulin promoter reporters rat INS1 CAT and rat INS2 CAT were transfected into MIN6 cells in the presence of various amounts of PCIF1 expression vector (0, 250, and 500 ng). Data are normalized to internal-control β -galactosidase activity, and repression is expressed relative to 0 ng of PCIF1. *, $P < 0.05$; **, $P < 0.005$.

of PCIF1 with PDX-1 leads to repression of PDX-1 transactivation. PCIF1/SPOP is colocalized with PDX-1 in adult pancreatic β cell nuclei, and overexpression of PCIF1 inhibits the activity of the insulin promoter, a key PDX-1 target, in β cell lines. Thus, our identification and initial characterization of PCIF1 lead us to hypothesize that PCIF1 interacts with the C terminus of PDX-1 to recruit a repressor complex to the promoter of PDX-1 target genes, thereby influencing β cell development, growth, and/or function. It is also important to note that the broad expression domain of PCIF1/SPOP in extrapancreatic tissues compared to the more restricted domain of PDX-1 expression in foregut-derived structures suggests that PCIF1 is likely to have additional PDX-1-independent functions. Future studies will directly test these hypotheses. The modulation of PDX-1 function by PCIF1 may have application to potentially novel treatments of diabetes focused on β cell neogenesis from stem cells, transdifferentiation of β cells from other cell types, and gene therapy approaches to enhance β cell numbers and survival.

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