The Homeodomain Protein IDX-1 Increases After an Early Burst of Proliferation During Pancreatic Regeneration

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Islet duodenal homeobox 1 (IDX-1/IPF-1/STF-1/PDX-1), a homeodomain protein that transactivates the insulin promoter, has been shown by targeted gene ablation to be required for pancreatic development. After 90% pancreatectomy (Px), the adult pancreas regenerates in a process recapitulating embryonic development, starting with a burst of proliferation in the epithelium of the common pancreatic duct. In this model, IDX-1 mRNA was detected by semiquantitative reverse transcription–polymerase chain reaction in total RNA from isolated common pancreatic ducts at levels 10% of those of isolated islets. The IDX-1 mRNA levels were not significantly different for common pancreatic ducts of Px, sham Px, and unoperated rats and did not change with time after surgery. By immunoblot analysis, IDX-1 protein was only faintly detected in these ducts 1 and 7 days after Px or sham Px but was easily detected at 2 and 3 days after Px. Similarly, IDX-1 immunostaining was barely detectable in sham or unoperated ducts but was strong in ducts at 2–3 days after Px. The increase of IDX-1 immunostaining followed that of BrdU incorporation (proliferation). These results indicate a posttranscriptional regulation of the IDX-1 expression in ducts. In addition, islets isolated 3–7 d after Px showed higher IDX-1 protein expression than control islets. Thus, in pancreatic regeneration IDX-1 is upregulated in newly divided ductal cells as well as in islets. The timing of enhanced expression of IDX-1 implies that IDX-1 is not important in the initiation of regeneration but may be involved in the differentiation of ductal cells to β-cells.
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BrdU, 5-bromo-2′-deoxyuridine; DIC, differential interference contrast; FITC, fluorescein isothiocyanate; IDX-1, islet duodenal homeobox 1; PBSS, phosphate-buffered saline and NaCl; Px, partial pancreatectomy; RT-PCR, reverse transcription–polymerase chain reaction.

Islet duodenal homeobox 1 (IDX-1/IPF-1/STF-1), a transcription factor (1–3) sometimes designated PDX-1, is homologous to a *Xenopus* endoderm–specific homeodomain protein, XIHbox 8; both proteins transactivate insulin gene expression through conserved enhancer elements (1,4). IDX-1 regulates insulin gene transcription via the A-elements (FLAT or CT boxes) of the insulin gene and may be involved in the glucose-mediated upregulation of insulin gene expression (5–7).

IDX-1 is found in the mouse primitive gut as early as the stage of 13 somites (E8.5), about a day before endocrine gene expression (glucagon and insulin) and 1.5 days before the first morphologic appearance of pancreas organogenesis (1,8,9). Subsequently, IDX-1 is transiently present in the fetal duodenum and in pancreatic exocrine, endocrine, and ductal cells, but for the most part, it disappears by late fetal development (E15.5). In adults, it is present in islets (mainly the β-cells) and in the duodenum (3). The crucial role of IDX-1 in pancreas development was observed in IDX-1–null ("knockout") mice (10,11). The homozygous null animals lack pancreatic development even though the duodenum, gut, spleen, and liver appeared grossly normal. Furthermore, only a few insulin-positive cells could be detected in these animals, which had high blood glucose levels and died within a few hours of birth (12).

During normal pancreatic development in the mouse, a dorsal bud forms from the primitive gut on E10, followed by a ventral bud (13,14). The epithelial cells of these buds are presumably pluripotent pancreatic cells that proliferate to form branching ductules that differentiate to a fully developed pancreas with mature ductal, exocrine, and endocrine elements (14). During islet development, pluripotent islet cells are believed to develop into one of the four cell types after passing through a transient phase in which two or more of the hormone genes are coexpressed in the same cell (8,15,16). Such coexpression is also found in cloned cell lines derived from islet cell tumors (17,18).

We have previously found that after 90% partial pancreatectomy (Px) in the adult rat, there is substantial regeneration of both endocrine and exocrine pancreas (19–22). This process involves two pathways: *1*) proliferation and differentiation of the ductal epithelium and *2*) replication of preexisting differentiated exocrine and endocrine cells. The proliferation starts in the common pancreatic duct, followed by formation of new branches of the ductal tree. Morphologically,

focal zones of proliferating, branching ducts and ductules appear, and these go on to differentiate into mature ducts, acini, and islets of Langerhans. In this mode of regeneration, the pancreas recapitulates embryonic development, giving rise to whole new pancreatic lobes containing normal exocrine and endocrine elements (22).

In this study, we investigated the role of IDX-1 in pancreatic regeneration in adult rats using the Px model. The levels of IDX-1 protein increased in ducts and islets 2–3 days after Px, following the initiation of ductal cell proliferation. Thus, during regeneration of the adult rat pancreas, IDX-1 expression is most prominently enhanced during the period of differentiation that follows an earlier stage of cell proliferation.

RESEARCH DESIGN AND METHODS

Partial pancreatectomy. Partial (90%) pancreatectomy was performed on 4- to 5-week-old Sprague-Dawley rats (Taconic, Germantown, NY). The pancreas was removed by gentle abrasion with cotton applicators, leaving the major blood vessels supplying other organs intact (19). The remnant (residual pancreas) was anatomically well defined as the tissue within 1–2 mm of the common pancreatic duct that extends to the first part of the duodenum. The sham surgery was an identical procedure except that the pancreatic tissue was lightly rubbed between fingertips instead of being removed. At 7 and 14 days after surgery, blood samples were taken from snipped tails and plasma glucose levels were measured on a Beckman Glucose Analyzer II. Pancreatic remnants were excised under overdose anesthesia at 1, 2, 3, 7, and 14 days after Px or sham Px. Some animals (2–3 each of unoperated rats and rats 18 h and 1, 2, and 3 days after Px) were injected intraperitoneally with 100 mg/kg body wt 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) 6 h before being killed. BrdU is a thymidine analog that is incorporated in newly synthesized DNA and thus labels replicating cells (23).

As previously described (19), for 1–2 days after Px, there was no change in body weight; after that, weight gain was similar for Px and sham Px rats. After 90% Px, plasma glucose levels after morning feeding are in the mildly hyperglycemic range; in these animals, plasma glucose was 150–160 mg/dl at 6–7 days and 200–250 mg/dl at 14 days after Px.

Islet and duct isolation. Islets of Langerhans were isolated from remnants by collagenase digestion followed by islet separation using a Histopaque (Sigma) gradient with subsequent hand-picking (24). Using a modified islet isolation method, the common pancreatic ducts were isolated by injecting 1 ml M199 medium containing 1.5 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) into the parenchyma of the pancreatic remnant and incubating it at 37°C for 40 min with intermittent shaking. The tissue was then washed 3 times with M199 medium containing neonatal calf serum, and the common pancreatic ducts (one per animal) were hand-picked and cleaned of contaminating tissue (stromal, islet, exocrine, and blood vessels) under a dissecting microscope. Such isolated ducts, when embedded and sectioned as 1-µm plastic sections, showed that the epithelium was contaminated by only a small amount of stroma (mainly extracellular matrix with very few fibroblasts); less than 10% of the original stroma (originally ~100 µm thick) remained, and this was "caught" between the evaginations characteristically seen in these ducts.

Gene expression measurement by semiquantitative reverse transcription–polymerase chain reaction. Total RNA was prepared from isolated common pancreatic ducts by modification of the guanidine thiocyanate method (25). Isolated ducts from individual rats were homogenized in guanidine thiocyanate, then 1/10 volume sodium acetate, equal volume phenol, and 1/5 volume chloroform/isoamyl (25:1) were added, and the mixture was incubated on ice for 15 min and centrifuged at 10,000*g* for 20 min at 4°C. The aqueous phase was removed and precipitated with an equal volume of isopropanol and 1 µl glycogen overnight at –20°C. Another 20-min 10,000*g* centrifugation yielded a pellet that was resuspended in equal volumes of guanidine and isopropanol, reprecipitated for 1 h at 4°C, and spun at 10,000*g* for 20 min at 4°C. The resultant pellet was washed with 75% ethanol and air-dried. RNA was resuspended in diethyl pyrocarbonate water, quantified by spectrophotometer, and qualified by 1% agarose gel electrophoresis.

For cDNA synthesis, 500 ng total RNA was denatured at 85°C for 3 min and quickly chilled on ice, added to the reverse transcriptase (RT) mixture containing 1× Superscript 1st Strand Buffer (Gibco/BRL, Gaithersburg, MD), 40 U RNAsin (Promega, Madison, WI), 10 mmol/l DTT (Gibco/BRL), 50 ng random hexamers (Gibco/BRL), 1 mmol/l dNTPs (Gibco/BRL), and 100 U Superscript RT (Gibco/BRL) to a total volume of 25 µl, and covered with a drop of mineral oil. Tubes were put into the thermal cycler, heated to 25°C for 10 min, 42°C for 60 min, and 95°C for 10 min, and brought to 4°C. After cDNA synthesis, samples were diluted with 50 µl H_2O.

Polymerase chain reaction (PCR) was carried out in 50-µL reactions using 4.5 µL of the diluted cDNA reaction product (corresponding to 30 ng RNA equivalent) as template mixed with 45.5 µL of PCR mix (1× Taq buffer [Promega], 1.5 mmol/l $MgCl_2$ [Promega], 10 pm primers [Genosys, The Woodlands, TX], 160 µmol/l cold dNTPs [Gibco/BRL], 5 U Taq polymerase [Gibco/BRL], and 2.5 µCi [α^{32}P]dCTP [NEN, Boston, MA]). Primers were as follows: IDX, 5′-CGG ACA TCT CCC CAT ACG; 3′-AAA GGG AGA TGA ACG CGG (which yield a 169-bp PCR product); and internal control cyclophilin, 5′-AAC CCC ACC GTG TTC TTC 3′-TGC CTT CTT TCA CCT TCC C (which yield a 400-bp PCR product). The thermal cycling protocol began with a denaturing step of 95°C for 5 min, then 20 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min, and finished with 72°C for 10 min. Unincorporated nucleotides were removed using G-50 columns (Probequant; Pharmacia, Piscataway, NJ). Reaction products were separated on a 6% polyacrylamide gel in Tris borate EDTA buffer, dried, and exposed overnight to a phosphorimage screen. Screens were scanned using a Molecular Dynamics Storm Phosphorimager, and reaction products were quantitated with ImageQuant software (Molecular Dynamics, Sunnydale, CA), using the rectangle mode/average intensity subtracting background average intensity. Results are calculated as a percentage of internal standard and presented as mean ± SE.

Reaction conditions were standardized so as to observe linear amplification of PCR products (for both IDX-1 and cyclophilin) for different amounts of cDNA (10–80 ng RNA equivalent) and cycle numbers (18–22 cycles). To circumvent any problems caused by differential amplification of cDNA between different sets of samples, cDNA from two samples were included in each set as an internal control. In a given set, PCR amplifications were performed either on cDNAs from multiple samples (Px and sham Px) of the same time points or Px and sham Px samples of each time point, with no effect on the final results. RNAs from pancreatic islets and muscle were used as positive and negative controls, respectively, for IDX-1 mRNA. Other controls included in this study were PCR amplification of RT-negative reaction product for each sample; comparison of expression of cyclophilin with other control genes such as α-tubulin to confirm that its expression was not altered under experimental conditions; and use of an additional set of nonoverlapping *idx-1* primers with α-tubulin as the internal control, which gave similar results.

Protein expression by Western blot. Protein extracts were prepared by SDS lysis (26) followed by sonication. For ducts, samples were from individual animals: four rats were used for each of sham Px, unoperated, and 1-day Px groups; two rats for 2- and 7-day Px; and six rats for 3-day Px. For islets, there were two pooled samples, each from four rats, for the 3-day Px group; one pooled sample from four rats for sham Px; one pooled sample from six rats for 7-day Px; and two pooled samples, each from six rats, for 14-day Px. Protein content of whole-cell lysates was measured by the BCA protein assay kit (Pierce, Rockford, IL). Total protein was fractionated on SDS-10% polyacrylamide gels and electroblotted onto nitrocellulose membranes (Amersham, Arlington Heights, IL). The membranes were incubated with rabbit anti-rat IDX-1 antibody (1:20,000), either with Hm-65 directed against a fusion protein containing part of the IDX-1 homeodomain (3) or with a newer Hm-253 directed against the synthetic COOH-terminal peptide (C)SPQPSSIAPLRPQEPR conjugated to keyhole limpet hemocyanin. The secondary antibody was donkey anti-rabbit antiserum conjugated to horseradish peroxidase (1:4,000) (Amersham). Immunoreactive proteins were visualized using the enhanced chemiluminescence system (Amersham).

Immunolocalization. For paraffin sections, remnant tissue from 18-h and 1-, 2-, 3-, and 7-day Px and sham Px rats (2–4 rats in each group) was fixed for 2 h at room temperature in freshly prepared 4% formaldehyde in 0.1 mol/l phosphate buffer and processed for embedding. For frozen sections, remnant tissue from 2-, 3-, 5-, and 7-day Px and sham Px rats (2–4 rats in each group) was fresh frozen in chilled isopentane and stored at –70°C. Immediately before staining, frozen sections (10 µm) were fixed in –20°C acetone. Paraffin sections (5–7 µm) were deparaffinized and microwaved in citrate buffer (3 times for 5 min) for antigen retrieval (27). Sections from all blocks were then rinsed in Tris-saline, incubated for 10–20 min in 0.3% Triton X-100 (Fisher) with 1% lamb serum (Gibco BRL), and after a second rinse, incubated in rabbit blocking serum (Vector, Burlingame, CA). Immunostaining for BrdU was performed using a Cell Proliferation Kit (Amersham). Sections were incubated for 60 min at room temperature with a mouse monoclonal antibody anti-BrdU (1:25), washed with PBSS (pH 7.4, phosphate-buffered saline with added 0.9% NaCl), incubated with peroxidase-linked sheep anti-mouse Ig, and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) plus a substrate/intensifier containing hydrogen peroxide and nickel chloride/cobalt chloride. The sections were then washed with 0.01 mmol/l HCl, rinsed with distilled water, incubated with 5% normal donkey serum (Jackson Immunochemicals, West Grove, PA) for 30 min at room temperature, and incubated overnight at 4°C with IDX-1 antibody (Hm-253, dilution 1:500 to 1:5,000). After rinsing with PBSS, blocking for nonspecific binding of streptavidin (Vector Labs blocking kit) was done for 30 min. After washing, sections were incubated 1 h at room temperature with donkey biotinylated anti-rab-

bit IgG (1:400, Jackson Immunochemicals) as the secondary antibody followed by streptavidin-conjugated fluorescein isothiocyanate (FITC) (1:400, Jackson Immunochemicals) for 1 h at room temperature. After extensive rinsing, slides were mounted with DABCO glycerol antifading mounting medium. Controls of nonspecific serum of appropriate species gave no cross-reactions.

Images of double-stained sections were taken on Zeiss LSM 410 microscope as overlaid images in differential interference contrast (DIC) for BrdU peroxidase staining and with fluorescence filters for FITC. Sections from all the time points were stained and photographed in parallel and at the same settings. Adobe Photoshop was used to make final figures; again, all manipulations were done in parallel.

RESULTS

Discrepancy of expression of IDX-1 mRNA and protein in pancreatic ducts after partial pancreatectomy. We have previously shown that after partial pancreatectomy, there is an expansion of the duct epithelium and its subsequent differentiation into new lobes of pancreas, comprising both endocrine and exocrine tissues. Since this process recapitulates embryonic development and *idx-1* is expressed in the duct epithelium during development, we first examined *idx-1* gene expression in the isolated common pancreatic ducts, those ducts in which ductal proliferation is initiated after Px. By semiquantitative RT-PCR using cyclophilin as an internal control, we found that IDX-1 mRNA was expressed equally in the common pancreatic ducts of Px and sham Px rats (Fig. 1*A*–*C*). Surprisingly, its expression did not change at 18 h or 1, 2, 3, or 7 days after Px or sham Px (Fig. 1*B*). The level of *idx-1* expression in these ducts was approximately 10 times lower than in isolated islets (Fig. 1*C*).

The lack of change in IDX-1 mRNA expression after partial pancreatectomy contrasted with the changes of IDX-1 protein levels in both isolated common pancreatic ducts and islets. As measured by Western blot analysis, the expression of 42-kDa IDX-1 protein was only faintly detected in ducts from sham Px rats or from animals 1 and 7 days after Px but was increased at 2 and 3 days after Px (Fig. 2*A*). Densitometric measurements show that the IDX-1 protein levels of sham Px and 1- and 7-day Px were not different from each other, but those of 2- and 3-day Px were more than double those of sham Px or unoperated rats (Fig. 2*B*). Even though there is some biological variation in the time of peak expression, all eight samples, each from a different animal, from 2- and 3-day Px were increased compared with sham Px or unoperated rats (Fig. 2*A* and *C*). Other proteins (~65 kDa at all time points, ~80 kDa at day 7 only) were recognized only by the Hm-65 antibody (against part of the IDX-1 homeodomain) and not by the more specific Hm-253 antibody (against IDX-1 COOH-terminal peptide) (Fig. 2*D*); these bands of higher molecular weight may correspond to other cross-reacting homeodomain proteins.

In islets, IDX-1 protein expression was also enhanced after Px. By densitometry of Western blot analysis of two samples of each group, IDX-1 protein levels were increased almost fivefold in islets 3 days after Px compared with sham Px rats. This high IDX-1 protein expression gradually decreased during the subsequent days (Fig. 3*A*), and at 14 days after Px, IDX-1 protein levels were comparable to those of sham Px rats (Fig. 3*B*).

IDX-1 protein increases in ductal cells after replication as seen by immunostaining. The localization of IDX-1 protein in the regenerating pancreas was similar for frozen and paraffin sections and for immunoperoxidase and immunofluorescence. To correlate the expression of IDX-1 with the duct proliferation described previously, paraffin sections were double-immunostained for IDX-1 and incorporated BrdU. While the islets had IDX-1 staining at all time points, little to no IDX-1 staining was detected in the epithelium of the common pancreatic duct from unoperated or sham Px animals (Fig. 4*A*); these ducts are quiescent, with little to no BrdU incorporation. At 18 h after Px, a few ductal epithelial cells showed BrdU incorporation (Fig. 4*B* and *C*), but only some of them also have IDX-1 staining. Most (45 of 55) single BrdU$^+$ cells were IDX-1$^-$, while all obvious daughter cells were BrdU$^+$ IDX-1$^+$ (Fig. 4*D*–*F*). One day after Px, the time of peak proliferation of the common pancreatic duct epithelium, most of the ductal epithelial cells (both luminal and within the blind evaginations [Fig. 4*F*]) are BrdU$^+$ IDX-1$^-$ or BrdU$^+$ IDX-1$^+$,

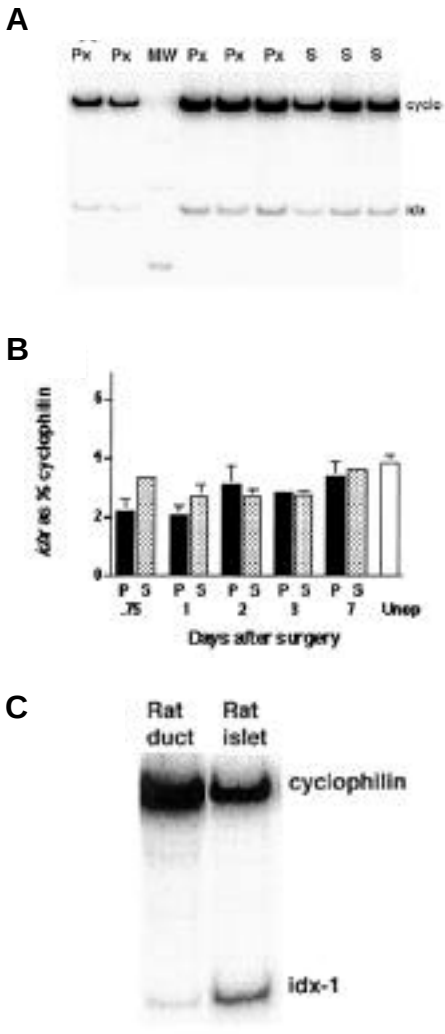

FIG. 1. IDX-1 mRNA in isolated common pancreatic ducts after Px. *A*: Representative gel of RT-PCR with primers to cyclophilin (400 bp) and *idx-1* (169 bp) using RNA from isolated common pancreatic duct of individual rats at 3 days after either Px (P) or sham Px (S). MW, lane of molecular markers. *B*: Graph of the quantification of gels (as in *A*) of sets of samples (three to five independent samples) from different time points after surgery using the values for cyclophilin as internal control. During the week after surgery, there was no difference in the expression of IDX-1 mRNA (2–3% of cyclophilin mRNA) in the common pancreatic ducts of Px, sham Px, or unoperated rats. *C*: Representative gel comparing IDX-1 mRNA in duct and islets isolated from unoperated rats. In islets, the IDX-1 mRNA was 26.3 ± 1.6% of cyclophilin mRNA.

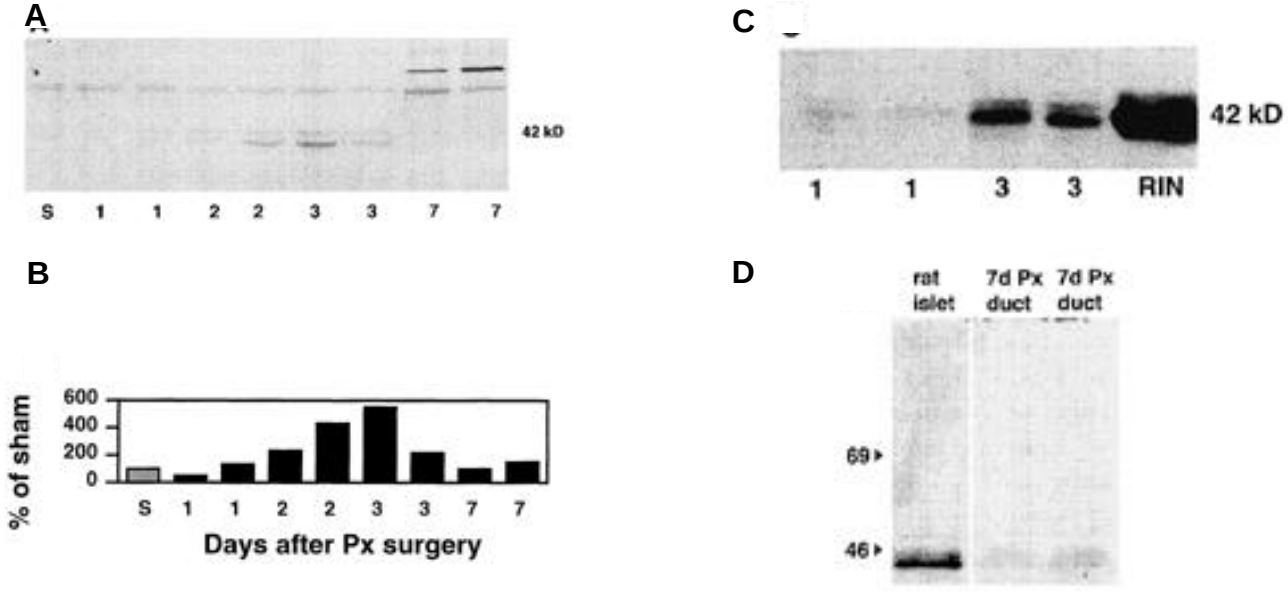

FIG. 2. IDX-1 protein in isolated common pancreatic ducts after Px. *A*: Representative Western blot analysis of protein (100 µg) from isolated common pancreatic ducts shows that IDX-1 protein (42 kDa) levels are increased at 2 and 3 days after Px compared with 1 and 7 days after Px (P) and sham Px (1 day after surgery) (S). As seen in the two samples (each from a different animal), for each time point after Px, there is some biological variation in the time of peak expression. *B*: Corresponding densitometric readings presented as percentage of sham value. *C*: The increase in IDX-1 protein between 1 and 3 days is shown again in this Western blot of additional independent samples, here with RIN 1027-B2 cells as positive control. *D*: When Hm-253 antibody was used with two samples from 7-day ducts and rat islet as positive control, the ~65- and ~80-kDa bands were not seen, suggesting that they are probably other cross-reacting homeodomain proteins recognized only by anti–IDX-1 antibody (Hm-65) directed against part of the IDX-1 homeodomain.

whereas a few $BrdU^-$ $IDX\text{-}1^+$ cells can be seen in the luminal epithelium (Fig. 4*G*). In contrast, at 3 days after Px, few cells of the common pancreatic duct epithelium are $BrdU^+$ but most are stained for IDX-1 protein (Fig. 4*H*); this pattern was found in all sections from ducts 3 days after Px. As we showed previously, proliferation of the main ducts lags behind that of the common pancreatic ducts, such that at 1 day after Px (Fig. 5*A*) only a few cells in the main duct are $BrdU^+$; of those, only occasional cells are also $IDX\text{-}1^+$. At 3 days after Px, however, the ducts are again quiescent (little or no $BrdU^+$) but are strongly $IDX\text{-}1^+$ (Fig. 5*B*). At 5 to 7 days after Px, few positive (BrdU or IDX-1) staining nuclei are seen in the common and main pancreatic ducts (data not shown). In the terminal branches of the newly expanded ductal tree (the focal areas of regeneration) the more proximal (larger) ductules contained many $IDX\text{-}1^+$ and few $BrdU^+$ cells, and the most distal (smallest and still proliferating) ductules had more $BrdU^+$ and fewer $IDX\text{-}1^+$ nuclei (Fig. 6*A* and *B*).

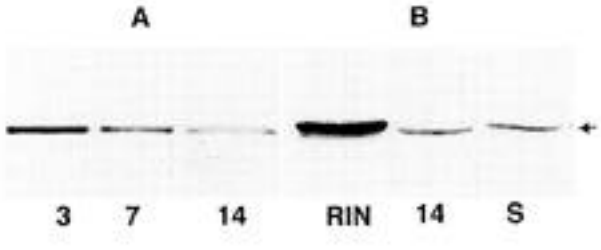

FIG. 3. IDX-1 protein in isolated islets after Px. *A*: Western blot analysis of protein (63 µg) from islets isolated from pancreatic remnants after Px showed increased IDX-1 protein levels 3 days after Px, gradually decreasing at 7 to 14 days; the sham sample (not shown) was barely detectable at this exposure. *B*: With more protein (100 µg), different exposure, and different samples, the IDX-1 protein levels at 14 days after Px and sham (S) were seen to be comparable. RIN 1027-B2 cell line (RIN) is used as positive control. Hm-253 antibody used.

DISCUSSION

During embryonic development, pluripotent cells of the primary pancreatic buds differentiate into specific pancreatic cell types such as ductal, exocrine, or one of the four islet cell types (13–15). The specificity of such differentiation depends on transcription factors that activate sets of genes which determine the terminal phenotype of the cell. For example, the differentiation of adipoblasts into mature adipocytes is critically dependent on an early phase of cell proliferation and requires the expression of the transcription factor C/EBP-α; after the proliferative phase is completed, C/EBP-α is sufficient for the differentiation (28,29). Similarly IDX-1, a pancreatic transcription factor, has been shown to be necessary for fetal pancreatic development (10,11) and also for activation of key genes in β-cells such as insulin, GLUT2, IAPP, and GK (6,30–35). In this study, we have examined IDX-1 expression in relation to pancreas regeneration after partial pancreatectomy. We show that in adult pancreatic regeneration, the IDX-1 protein level is markedly upregulated but that its mRNA is unchanged.

Reports of IDX-1 in pancreatic ducts have been variable. All pancreatic ductal cells express IDX-1 in early pancreatic development (until E15.5), but subsequently only islet hormone–positive cells and a few ductal cells were found to have IDX-1 (9,10,12). In the initial report of IDX-1 (3), some adult rat duct cells were reported positive for IDX-1, but others have reported that only islet cells are IDX-1–positive in adult pancreas (4). Low levels of IDX-1 mRNA, however, as reported

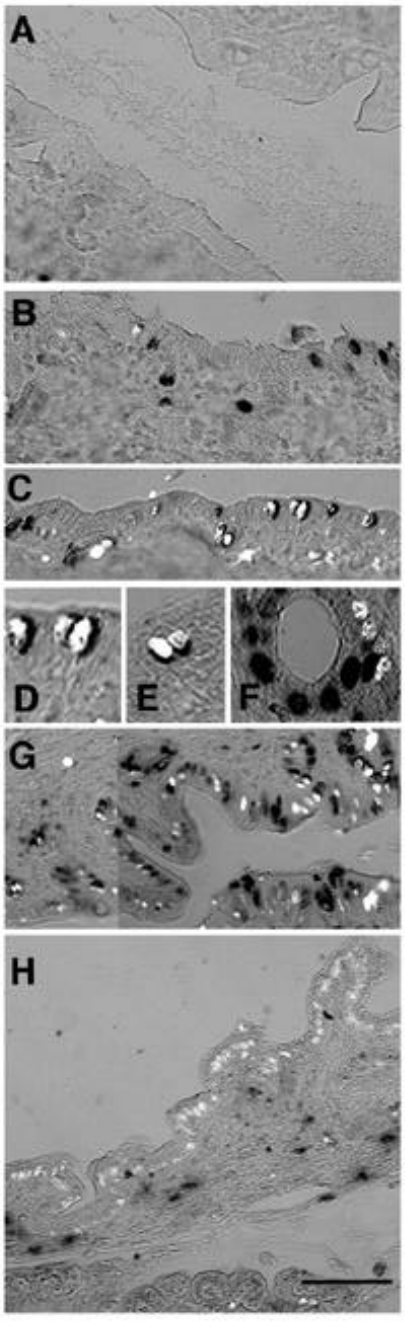

FIG. 4. Immunolocalization of IDX-1 expression in the common pancreatic duct after Px. The common pancreatic duct has simple columnar epithelium with numerous blind evaginations; primary branches from these ducts are main ducts that have simple cuboidal epithelium. *A*: Common pancreatic duct from unoperated control rat showing little to no staining for either IDX-1 or incorporated BrdU; these ducts are quiescent and differentiated as ducts. *B* and *C*: Sections of similar ducts 18 h after Px showing the variability in the amount of proliferation seen at that time. Only a few cells have incorporated BrdU (black) during the 6 h before death; IDX staining (white) is seen mainly in those daughter cells that had incorporated the BrdU. *D* and *E*: At higher magnification, examples of the double-staining daughter cells at 18 h after Px. *F*: Both $BrdU^+$ IDX^- cells (left) and $BrdU^+$ IDX^+ cells (right) in an evagination from a duct 24 h after Px. *G*: By 24 h after Px, many more cells in the common pancreatic duct are $BrdU^+$ IDX^- or $BrdU^+$ IDX^+; a few cells that are only IDX^+ can be seen in the luminal epithelium. *H*: By 3 days after Px, most of the epithelial cells in these ducts are quiescent again (without BrdU) but are positive for IDX-1. Along the bottom edge, a few IDX-1–positive centroacinar cells are visible. *A*, *B*, *C*, *G*, and *H*: magnification bar = 50 µm; *D*, *E*, and *F*: twice that magnification.

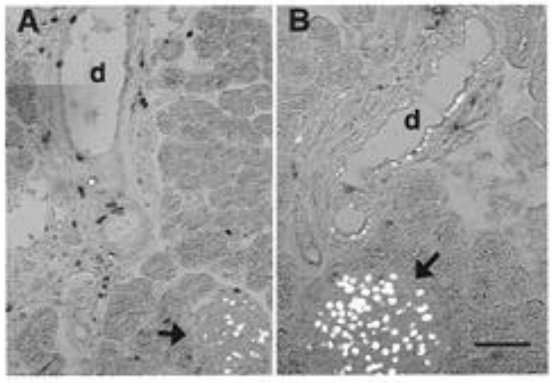

FIG. 5. Immunolocalization of IDX-1 and BrdU in the main ducts and islet after Px. *A*: One day after Px, only a few epithelial cells of the main ducts (d) incorporate BrdU, and even fewer express IDX-1; compare with the IDX-1 staining in the islet in the lower right hand corner (arrowhead). *B*: We have shown an increase in replication in these ducts between 24 and 48 h (22); here, at 3 days after Px, these ducts (d) are once again quiescent, with little BrdU incorporation but with most cells expressing IDX-1 protein. The IDX-1 staining in the islet (arrowhead, lower left corner) is more intense than in the islet in *A*; this was a consistent finding. Magnification bar = 50 µm.

by β-galactosidase in transgenic mice with constructs of *idx-1* promoter-Lac Z, have been detected in some adult exocrine cells (11) and in most cells of the larger pancreatic ducts (36). We have used the sensitive radioactive semiquantitative RT-PCR method to show IDX-1 mRNA in adult pancreatic ducts, albeit at levels 10% of that of islets. Our finding that there were no discernible changes in mRNA in contrast to large changes in protein expression suggests the regulation of IDX-1 expression in ducts at the posttranscriptional level.

Increased IDX-1 protein was seen following proliferation in the epithelium throughout the ductal tree. During the period of 24–36 h after Px, the epithelium of the common pancreatic duct has a burst of proliferation seen as a transient threefold

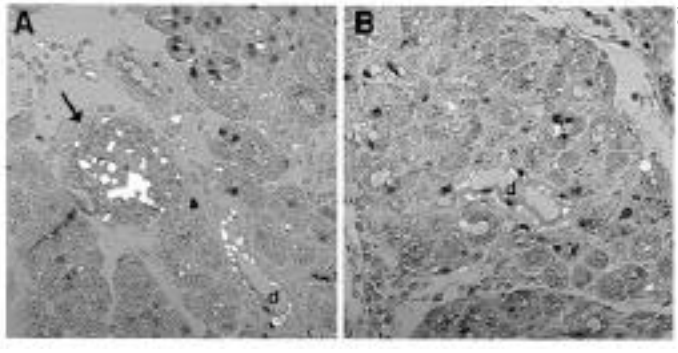

FIG. 6. Immunolocalization of IDX-1 expression in the focal regions of regeneration after Px. From 3 days after Px, in the terminal branches of the newly expanded ductal tree (the focal areas of regeneration), the more proximal (larger) ductules (d) contained many $IDX\text{-}1^+$ cells and few $BrdU^+$, and the most distal (smallest and still proliferating) ductules had more $BrdU^+$ and fewer $IDX\text{-}1^+$ nuclei. *A*: The small ductules of the focal region (upper right corner) have numerous $BrdU^+$ cells but few $IDX\text{-}1^+$ in contrast with both the islet and the larger ductule (d) with few $BrdU^+$ and many $IDX\text{-}1^+$ cells. Pre-existing exocrine tissue with a few $BrdU^+$ cells is seen in the lower left corner. *B*: In other focal regions (here the whole field), the small terminal ductules have reduced proliferation and more cells express IDX-1. Magnification bar = 50 µm.

increase of BrdU incorporation; by 48 h after Px, the BrdU incorporation in this duct has returned to basal levels (22). Yet in the 1st day after Px, little increase of IDX-1 protein levels was found in the common pancreatic duct by either Western blot or immunostaining. IDX-1 protein levels were only increased at 2–3 days after Px and returned to basal levels at 7–14 days after Px. The double immunostaining showed that initially the cells that incorporated BrdU did not express detectable levels of IDX-1 protein. Our detection methods have thresholds that are not necessarily zero, so we cannot rule out that there are very few molecules of IDX-1 present. In individual cells, BrdU incorporation preceded the expression of IDX-1 protein, but IDX-1 remained, at least transiently, in the duct cells after replication. At 3 days after Px, when both immunostaining and protein levels of IDX-1 were markedly increased in these ducts, BrdU incorporation was low, indicating that the cells were now quiescent. The same pattern, that of increased IDX-1 expression only after proliferation, was also seen further out the ductal tree in the main ducts and distal smaller ductules. This lag suggests that IDX-1 protein must have a role other than initiation of regeneration and proliferation.

An intriguing finding was that BrdU was incorporated in cells before IDX-1 was detectable but that all obvious $BrdU^+$ daughter cells expressed IDX-1. Furthermore, this expression was transient. This turning on and then off of IDX-1 protein expression resembles what is seen in embryonic ducts. Most early embryonic duct cells express IDX-1 protein, but it is mostly lost in ducts in the days before birth (9). It is important to remember that mature duct cells are both quiescent and have a differentiated phenotype with specific functions not seen in pancreatic exocrine or endocrine cells (37). Perhaps with the differentiation into a mature duct phenotype there is repression of the IDX-1 protein expression, and conversely, with replication, a transient loss of this differentiation/repression. The regulation of IDX-1 protein expression may occur at the level of translational inhibition or at the level of protein stability. Translational inhibition is an important regulatory mechanism for controlling expression of genes during early development, in response to various stresses as well as for maintenance of metabolic homeostasis; such inhibition can be mediated by a regulatable repressor (38–42). Since differentiation is such a complex event, any regulatable repressor factor may affect the translation of many different proteins in a cascade, and the effect on IDX-1 may only be indirect. IDX-1 protein may or may not be causative in the dedifferentiation; indeed, IDX-1 may only be a marker of this state. Our data on the expansion, dedifferentiation, and subsequent redifferentiation of duct cells are similar to a recent report of the hepatocyte as a "facultative stem cell" (43). We hypothesize that the $IDX\text{-}1^+$ duct cells that regain their pluripotency transiently could be considered the true precursor cells of the adult pancreas, or its facultative stem cells. Further experiments will be needed to answer these questions.

This Px animal model becomes a model of diabetes after several weeks of moderate, but chronic, hyperglycemia. Previously, we have shown that 4 weeks after Px, the islet IDX-1 protein levels of moderately hyperglycemic rats were dramatically reduced to about 20% of levels in islets from sham Px rats (44,45). Using the same semiquantitative RT-PCR protocols, we have found that in islets isolated 4 weeks after Px the IDX-1 mRNA is also reduced to about 50% of control (46). In the present study, we examined the expression of IDX-1 in islets during the regenerative phase immediately after Px and found that IDX-1 protein is also increased transiently in the islets. While IDX-1 protein was abundant in islets of the sham Px or unoperated rats, its substantial increase after Px parallels that of the replication of differentiated β-cells. Before 3 days after Px there is little replication in the islets. At 3 days, both the mitotic index of β-cells within pre-existing islets (20) and the IDX-1 protein levels in islets are enhanced fivefold and are still somewhat elevated at 7 days after Px. These data suggest that IDX-1 may be regulated during replication of differentiated β-cells. In Px animals, mild hyperglycemia develops only by 3–4 days after the surgery and is maintained thereafter. Since IDX-1 expression and activity can be regulated by glucose (5,7,47–54), the observed changes in IDX-1 levels in islets may be due in part to regulation of IDX-1 in response to a change in blood glucose levels. However, it would be difficult to attribute the fivefold increase in IDX-1 protein in islets to the initial mild hyperglycemia in Px animals. The initiation of IDX-1 expression in pancreatic ducts appears to be independent of glucose, since the increase precedes the hyperglycemia after Px.

We demonstrate here that the transcription factor IDX-1 is upregulated during pancreatic regeneration in adult rats both in the pancreatic ducts and in the islets. Even in the same model, however, the regulation of IDX-1 protein in the pancreatic ducts and pancreatic islets is different. Within the adult ducts, IDX-1 protein is expressed transiently at detectable levels only after replication, and its levels are not related to hyperglycemia. Islets, however, even when quiescent, express 10 times more IDX-1 than ducts, and their expression of IDX-1 is enhanced at least for the 1st week. In the pancreatic ducts, it is clear that enhanced IDX-1 protein expression occurs without change in RNA, suggesting that its regulation must be at a posttranscriptional level. The timing of its enhanced expression implies that IDX-1 is not important in the initiation of regeneration.

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