Role of the Basic Helix-Loop-Helix Transcription Factor p48 in the Differentiation Phenotype of Exocrine Pancreas Cancer Cells

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Abstract
The majority of human pancreatic adenocarcinomas display a ductal phenotype; experimental studies indicate that tumors with this phenotype can arise from both acinar and ductal cells. In normal pancreas acinar cells, the pancreas transcription factor 1 transcriptional complex is required for gene expression. Pancreas transcription factor 1 is a heterooligomer of pancreas-specific (p48) and ubiquitous (p75/E2A and p64/HEB) basic helix-loop-helix proteins. We have examined the role of p48 in the phenotype of azaserine-induced rat DSL6 tumors and cancers of the human exocrine pancreas. Serially transplanted acinar DSL6 tumors express p48 whereas DSL6-derived cell lines, and the tumors induced by them, display a ductal phenotype and lack p48. In human pancreas cancer cell lines and tissues, p48 is present in acinar tumors but not in ductal tumors. Transfection of ductal pancreas cancers with p48 cDNA did not activate the expression of amylase nor a reporter gene under the control of the rat elastase promoter. In some cell lines, p48 was detected in the nucleus whereas in others it was cytoplasmic, as in one human acinar tumor. Together with prior work, our findings indicate that p48 is associated with the acinar phenotype of exocrine pancreas cancers and it is necessary, but not sufficient, for the expression of the acinar phenotype.

Introduction
The majority of tumors arising in human pancreas derive from the exocrine tissue and are classified as “ductal-type” on the basis of histological appearance (1). Our laboratory and others have shown that these tumors express molecular markers characteristic of normal ductal cells (2). Among them are cytokeratin polypeptides 7, 19, and 4 (3–5), MUC3 and MUC5B mucins (5–7), dipeptidylpeptidase IV (8), and the cystic fibrosis transmembrane regulator (9). In addition, these tumors lack expression of acinar markers, although occasional reports have described such expression (10). At the ultrastructural level, cell lines derived from human pancreas cancers resemble ductal cells and lack the main features of acinar cells, i.e., zymogen granules (2, 11).

The target cell for carcinogenesis in the human pancreas, leading to the common ductal-type adenocarcinoma, is not known. Several candidates have been proposed (discussed in Refs. 12 and 13): (a) a multipotential stem cell presumably located in the pancreatic ducts; (b) acinar cells that lose their differentiated properties and acquire a ductal-type phenotype representing either dedifferentiation or transdifferentiation; (c) islet-derived cells that acquire a ductal phenotype; and (d) pancreatic ductal cells. The elucidation of this issue is hampered by the fact that it is not possible to follow histological changes in the human pancreas sequentially. Nevertheless, several observations suggest that pancreatic ductal cells are the target for carcinogens in humans: (a) severe dysplasia, a general hallmark of high risk for neoplasia, is rarely observed in acinar cells, even in tissue from patients with pancreas cancer (14); (b) ductal cell hyperplasia, flat or papillary, can often be identified in tissue from patients with pancreas cancer (15). However, similar histological changes can also be identified in tissue from patients without cancer, in particular in old individuals and in patients with chronic pancreatitis (15, 16); and (c) recent evidence shows that some of the genetic lesions that are associated with pancreas cancer can also be detected in putative preneoplastic lesions, such as flat and papillary ductal cell hyperplasia. For example, mutations in codon 12 of K-ras occur in a high proportion of pancreas cancers (17) and have also been reported in ductal cell hyperplasia associated with chronic pancreatitis and pancreas cancer (18, 19). Such evidence may not be considered strong because the mutation found in the tumor often, but not always, corresponds to the mutation found in putative preneoplastic lesions (18–21) and because K-ras mutations are restricted to a few codons, thus increasing the likelihood that the presence of the same mutation in putative preneoplastic lesions and in tumor from a given patient may be attributable to chance. A stronger evidence comes from the study by Moskaluk et al. (21), showing that in two cases with pancreas cancer, putative
differentiation of exocrine pancreatic tumors

...transcriptional complex designated PTF1, which binds to a bipartite site in the A element of the regulatory region of these genes (28) and whose activity closely parallels the expression of pancreas-specific gene products during development (29). PTF1 is constituted by three different bHLH transcription factors: p64 and p75 (30), which are ubiquitous, and p48 whose expression is restricted to the pancreas (31). p64 is the product of REB, and p75 is the product of E2A (32). Although p48 and p64 bind to DNA, p75 is involved in the transport of the p48/p64 heterodimer to the nucleus (33). The bHLH region of p48 shares sequence homology with myoD (31), a transcription factor that specifies activation of myocyte differentiation in nonmuscle cells (34). Such homology, together with the finding that p48 expression is restricted to the exocrine pancreas, suggests that p48 may be involved in the activation of an acinar differentiation program. However, acinar gene expression also requires the contribution of other tissue-restricted transcription factors such as HNF-3β (35) and Pdx-1 (36).

In this study, we have addressed two questions: (a) whether the phenotype of rat and human exocrine pancreatic tumors is related to the expression of p48; and (b) whether p48 is able to instruct an acinar differentiation program in pancreas cancer cells displaying a ductal phenotype. Our findings, together with previous work (31), provide evidence that p48 is necessary, but not sufficient, for the activation of the acinar phenotype in ductal cells, even when they are derived from cells with acinar differentiation potential. Furthermore, we show that under certain circumstances, p48 is retained in the cytoplasm, thus losing its ability to form the PTF1 complex and contribute to the activation of acinar genes.

results

the azaserine-induced rat pancreas cancer model.

Azaserine induces focal acinar cell hyperplasia and acinar cell tumors in rats. Serially transplanted acinar tumors induced by azaserine maintain acinar features (Fig. 1A). Pettengill et al. (26) have shown that when these tumors are placed in culture, loss of acinar features and acquisition of ductal characteristics take place. In this way, two independent cell lines have been established, DSL6A and DSL6B from a transplanted tumor. When these cells are injected s.c. or orthotopically into male Lewis rats, ductal-like tumors develop that are morphologically very similar to the common ductal adenocarcinoma occurring in humans (Fig. 1, C and E). At the ultrastructural level, the serially transplanted tumors have acinar features and contain cells with electron-dense secretion granules (Fig. 1B), whereas the tumors induced by DSL6A and DSL6B cell lines display epithelial features but lack exocrine secretion granules (Fig. 1, D and F).

expression of the ptf1 complex components in azaserine-induced rat pancreatic tumors and derived cell lines.

The PTF1 complex has been shown to be involved in the activation of expression of acinar cell-specific genes (28–31). Therefore, we set out to examine the expression of PTF1 components in the tumors and cell lines described above. To this end, we raised an antiserum against the pancreas-specific component p48.

production of p48-specific, affinity-purified rabbit antibodies.

Rabbit polyclonal serum against rat p48 produced in Escherichia coli was first preabsorbed with nuclear extracts from a mixture of rat tissues (see "Materials and Methods") and subsequently purified by affinity chromatography with His-tagged rat p48. Fig. 2A shows the reactivity of the affinity-purified immunoglobulin fraction with nuclear extracts from AR42J cells and nuclear extracts from a variety of normal rat tissues. A band of Mr ~48,000 was specifically identified in AR42J cells and in normal pancreas tissue. There was no reactivity with nuclear or cytoplasmic extracts from nonpancreatic tissues. To confirm the specificity of the antiserum, immunohistochemical assays on frozen sections of normal rat and human tissues were performed (Fig. 2B). A
nuclear pattern of staining was identified in the majority of acinar cells though the intensity of staining showed some variation from cell to cell. Acinar staining could be inhibited by preincubation of the antibody with purified p48. All ductal cells were consistently unreactive with anti-p48 antibodies; double labeling with antibodies detecting p48 and antibodies detecting cytokeratins 7 and 19, which are restricted to centroacinar and ductal cells in the pancreas (3–5), revealed no coexpression (data not shown). p48 was not detected in islet cells (Fig. 2B). Apart from the pancreas, there was no reactivity with a large panel of normal tissues: esophagus, stomach, small bowel, colon, gallbladder, liver, breast, ovary, cervix, larynx, trachea, lung, kidney, and thyroid, indicating that p48 is selectively expressed in normal acinar cells. Similar results were obtained using normal rat and human tissues.

Expression of PTF1 Complex Components in Azaserine-induced Rat Pancreatic Tumors. Expression of p48 transcripts was analyzed by RT-PCR; p48 was detected using Western blotting and immunohistochemistry. AR42J cultured cells, displaying acinar features, and normal rat pancreas tissue were used as controls.

p48 mRNA was detected in AR42J cells but not in ARIP, DSL6A, or DSL6B cells. p48 transcripts were detected in the tumors that were serially transplanted and in those induced by DSL6B cells but not in tumors induced by DSL6A cells (Fig. 3A). p48 protein was exclusively detected in serially transplanted tumors (Fig. 3B). Amylase was detected only whenever p48 protein was also detectable; in tumors from DSL6B cells, low levels of p48 mRNA were present, but no p48 protein was detected by western blotting (Fig. 3B). Using immunohistochemistry, p48 was detected in the nucleus of cancer cells in serially transplanted tumors, and amylase was weakly detected, mainly in luminal areas (Fig. 4, A and B). By contrast, neither p48 nor amylase were detected in tumors induced by cultured DSL6A or DSL6B cells (Fig. 4, C–F). Altogether, these results indicate that loss of acinar features in cultured tumor cells and in tumor tissues is associated with the loss of expression of p48.

To determine whether the other components of the PTF1 complex were expressed in DSL6 cells and tumors, RT-PCR was used. Transcripts of E2A and REB were detected in all cell lines and tumor tissues examined (Fig. 3A), indicating that lack of expression of these ubiquitous bHLH transcription factors does not occur during the loss of the acinar phenotype.

Expression of the PTF1 Components in Human Pancreas Tumors. To examine p48 transcript expression in human pancreas tissue and cultured cells, a partial cDNA encoding the bHLH domain was isolated by RT-PCR using degenerate oligonucleotides. PCR products were cloned, and both strands of DNA were sequenced. The comparison of the cDNA sequence coding for the bHLH region of rat and human p48 is shown in Fig. 5. The deduced amino acid sequence in this region is identical for rat and human p48, and there is 79% nucleotide identity; all differences between the two sequences correspond to third nucleotides of codons.

Expression of p48 in a panel of human pancreas cancer cell lines and in normal pancreas tissue was analyzed by RT-PCR, Western blotting, and immunohistochemistry (Fig. 6). p48 transcripts were detected in normal pancreas tissue.
but were undetectable, using a nested PCR, in all human pancreas cancer cell lines displaying a ductal phenotype examined (n = 8; Fig. 6A). In agreement with these findings, p48 was not detected in cell lysates of cultured pancreas cancer cells (Fig. 6B). Using immunohistochemistry on frozen tissue sections, p48 was absent from all ductal pancreas adenocarcinomas examined (n = 18; Fig. 7B). By contrast, anti-p48 antibodies showed strong reactivity with the three acinar pancreatic tumors examined. In two cases, classified as “predominantly acinar-glandular” and “predominantly solid,” all cells showed nuclear staining (Fig. 7C); in one additional tumor, classified as “mixed acinar-solid,” p48 was detected exclusively in the cytoplasm of 30% of tumor cells, and it was detected in the nucleus of a small proportion of tumor cells (<5%; Fig. 7D). In these tumors, amylase expression was analyzed using immunohistochemistry. In the aci-
nar-glandular tumor, strong expression of amylase was observed; by contrast, in the mixed acinar-solid tumor, amylase was detected in a very low proportion of cells (5%; data not shown). Anti-p48 antibodies did not react with any of the nonpancreatic tumors analyzed (Table 1).

The expression of the other components of the PTF1 transcription complex in cell lines derived from ductal-type pancreas cancers was examined by RT-PCR. The two isoforms of E2A, E12 and E47, and HEB were detected in all cell lines examined (Fig. 6C). Therefore, the only component whose expression is absent in ductal pancreatic tumors is p48.

Is Transfection of p48 cDNA into Ductal-like Cells Able to Induce an Acinar Phenotype? The DSL6 cells and tumors provide an excellent model to analyze whether p48 can activate an acinar differentiation program because the ductal-like DSL6A and DSL6B cells are derived from an acinar cell tumor. A light hematoxylin counterstaining was performed not to mask nuclear staining with anti-p48 antibodies.

Fig. 4. Immunohistochemical analysis of the expression of p48 and amylase in DSL6-derived tumor tissues; a light hematoxylin counterstaining was performed not to mask nuclear staining with anti-p48 antibodies. A, C, and E, p48; B, D, and F, amylase; A and B, serially transplanted DSL6 tumor; C and D, DSL6A-induced tumor; E and F, DSL6B-induced tumor. A and B, ×100; C–F, ×200.

Fig. 5. Alignment of the sequences of the bHLH region of human and rat p48 cDNA. A 79% nucleotide and 100% amino acid identity is observed. H, human; R, rat.
Differentiation of Exocrine Pancreatic Tumors

Fig. 6. p48 expression in human pancreas cancer cell lines displaying a ductal phenotype. Panel A, RT-PCR analysis of p48 transcripts in pancreas cancer cell lines. K-ras transcript amplification is shown as a control of RNA quality and amount. Lane 1, AsPC-1; Lane 2, Capan-2; Lane 3, HPAF; Lane 4, SK-PC-1; Lane 5, IMIM-PC-1; Lane 6, IMIM-PC-2; Lane 7, MZPC-1; Lane 8, RWP-1. Panel B, Western blotting analysis of p48 expression in pancreas cancer cells (IMIM-PC-2 and IMIM-PC-1) and in control colon cancer cells (HT-29). Normal human pancreas tissue (NPT) and AR42J cells are shown as positive controls. C, control for protein loading. Panel C, RT-PCR analysis of the expression of HEB and E2A transcripts in human pancreas cancer cells. Results are shown for the two variants of E2A, E47 and E12.

Discussion

In this work, we show that down-regulation of p48 was associated with the change of an azaserine-induced acinar cell carcinoma to a ductal phenotype and that human ductal-type pancreas cancers lack p48 expression. By contrast, expression of the other two members of the exocrine pancreas-specific transcription complex PTF1 is maintained both in rat and human ductal tumors. These results indicate that p48 is selectively absent from ductal-type tumors.

Until recently, p48 was thought to participate exclusively in exocrine pancreas differentiation. However, inactivation of the p48 gene in mice by homologous recombination leads to an anaplastic phenotype characterized by the lack of an exocrine pancreas and the presence of a reduced number of individual endocrine cells in the spleen (38). These findings indicate that p48 plays a role both early and late in pancreas development and differentiation. However, little is known about the genetic mechanisms regulating the expression of p48. The p48 promoter has been cloned and sequenced, but its activity is not restricted to pancreatic cells, and putative tissue-specific control elements have not been identified in as much as 10 kb of 5′-flanking region (39). Several explanations can account for the finding that, upon in vitro culture, DLS6 cells displaying an acinar phenotype lose p48 expression: culture may select for populations lacking p48 expression and having some growth advantage; alternatively, the maintenance of p48 expression may require cellular interactions with the mesenchyme or with neural cells that cannot be maintained in vitro. In support of the latter hypothesis is the observation that cultures of normal human exocrine pancreas undergo an acinar-to-ductal phenotypic switch in vitro that is also accompanied by the loss of expression of p48 (40, 41). In these cells, a selective outgrowth of cells lacking p48 cannot be proposed because normal pancreatic cells undergo limited replication in vitro (40). There is extensive evidence that mesenchymal-epithelial interactions play a major role in development (42) and differentiation (43). For example, the LIM-homeodomain protein Isl-1 is expressed in all postmitotic islet cells as well as in mesenchymal cells surrounding the dorsal but not the ventral evagination of the gut endoderm during development. Inactivation of Isl-1 in the mouse results in the lack of Isl-1-expressing dorsal mesenchyme, lack of exocrine cell differentiation in the dorsal but
not in the ventral pancreatic bud, and lack of endocrine cells in all of the pancreas. In vitro reconstitution experiments have shown that the wild-type pancreatic mesenchyme can support the normal development of the dorsal exocrine pancreas from Isl-1²/² mice (43). A search for regulatory motifs in the 10 kb upstream of the p48 gene has failed to provide clues about tissue-specific transcription factors/complexes that may be involved in its regulation, although a putative Pdx-1 binding site has been identified (39). The molecular mechanisms underlying the down-regulation of p48 gene expression in tumors remain unknown, although at least in the human tumors, the p48 gene is generally retained.⁴

Our findings support the contention that p48 is necessary, but not sufficient, for the activation of acinar genes in pancreatic cells. This conclusion, which is also supported by in vitro studies of promoter regulation (35, 36) and by transfection of an antisense construct of p48 cDNA in AR42J cells (31), is drawn from our transfection studies using both rat and human pancreas cancer cells. AR42J cells, which spontaneously express amylase and other acinar genes (25), are equipped with the transcription factor machinery necessary for their expression, and transfection with p48 cDNA is accompanied by enhanced expression both in transient and stable assays (this work and data not shown). By contrast, transfection of p48 cDNA into other pancreatic cells did not result in the activation of the expression of amylase nor a reporter construct containing the A element of the elastase promoter, indicating lack of formation of an active PTF1 complex. Two types of mechanisms seem to contribute to the lack of effects of p48 overexpression. In DSL6A and RWP-1 cells, p48 was undetectable in the nucleus. p75, encoded by the ubiquitously expressed E2A gene, has been shown to be responsible for the nuclear import of the p48/p64 complex (33); however, E2A is expressed in both cells. Similarly, the REB/HEB gene is also expressed in both lines. Thus, the molecular basis of the cytoplasmic accumulation of p48 remains to be elucidated. Id proteins, initially identified as inhibitors of differentiation of muscle and lymphoid cells (44, 45), are candidates to play a role in the subcellular distribution of p48. Id proteins, of which four members have been described thus far, contain an HLH domain that enables their dimerization with bHLH factors but lack the basic domain that allows DNA binding, therefore sequestering bHLH-type transcription factors and acting in a dominant-negative fashion (44–46). Id1 has been directly implicated in the suppression of mammary cell differentiation (47). There is currently no direct evidence that Id proteins interact with p48 nor that they can block its transcriptional activity. In addition to them, other unidentified proteins might interact with p48 and retain it in the cytoplasm.

The pathophysiological relevance of the subcellular distribution of p48 is underlined by our findings in human acinar

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Table 1  Immunohistochemical analysis of p48 expression in human adenocarcinomas

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Positive/Total tested</th>
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<tbody>
<tr>
<td>Pancreas</td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>0/17</td>
</tr>
<tr>
<td>Cystadenocarcinoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Acinar</td>
<td>3/3</td>
</tr>
<tr>
<td>Colon</td>
<td>0/5</td>
</tr>
<tr>
<td>Breast</td>
<td>0/2</td>
</tr>
<tr>
<td>Lung</td>
<td>0/2</td>
</tr>
<tr>
<td>Biliary tract</td>
<td>0/2</td>
</tr>
<tr>
<td>Ovary</td>
<td>0/1</td>
</tr>
</tbody>
</table>

Fig. 7. p48 expression in human pancreatic tissues using immunoperoxidase; tissues are lightly counterstained with hematoxylin. A, normal pancreas; B, ductal-type adenocarcinoma; C, acinar-type adenocarcinoma showing homogeneous nuclear staining; D, acinar-type adenocarcinoma showing heterogeneous, predominantly cytoplasmic staining. T, tumor cells. Arrowheads, areas of cytoplasmic distribution of p48. ×200.
tumors. Unlike normal acinar cells, one acinar tumor showed p48 expression exclusively in the cytoplasm. Furthermore, preliminary data indicate that p48 preferentially accumulates in the cytoplasm of acinar/ductular complexes in areas of chronic pancreatitis associated with pancreas cancer. The abnormal subcellular distribution of p48 might contribute to the disregulation of acinar gene expression and pancreatic insufficiency.

In ARIP cells, p48 did localize to the nucleus, but it did not activate acinar gene expression nor a reporter construct for the PTF1 complex. It has been shown that expression of acinar enzymes in AR42J cells requires the expression of the winged helix-loop-helix factor HNF-3β (35). Indeed, ARIP cells express low levels of HNF-3β. Because transfection efficiency for these cells is very low, we have not been able to conclusively show the precise role of HNF-3β by cotransfecting its cDNA with that of p48. Other mechanisms may account for the lack of activation of acinar genes. For example, the histone acetyltransferase PCAF and p300/CBP promote both MyoD-dependent transcription and myogenic differentiation in muscle cells (48).

It is currently not known whether p48 may play a role in tumor progression. Acinar-to-ductal conversion has been demonstrated in other tumors of the exocrine pancreas in rodents, most notably the Ela-myc transgenic mouse. In these animals, acinar tumors progress to develop a ductal phenotype similar to that of the majority of human exocrine pancreas cancers (27). It is conceivable that loss of acinar features and p48 expression might favor tumor progression, although this possibility has not been examined in detail. Studies on the role of the bHLH transcription factor MyoD in muscle differentiation support such contention. MyoD induces the expression of the cyclin-dependent kinase inhibitor p21 at the transcriptional level, leading to an arrest in G1 that allows the activation of a cell differentiation program in normal cells (49–51). Such effects are abrogated in a high proportion of tumor cells (52), despite that MyoD is expressed in the majority of rhabdomyosarcomas (53, 54). Furthermore, pRb favors both cell cycle exit and the expression of muscle-specific genes by cooperating with Myo-D, whereas in the absence of pRb, myogenic transcription factors are inactive (55).

Finally, p48 is an excellent marker of the acinar cell differentiation in the pancreas. Although its selectivity is unlikely to be of clinical interest in the setting of human exocrine pancreatic cancers because acinar tumors are rare, the study of the expression, subcellular distribution, and transcriptional activity of p48 should shed light on the molecular mechanisms involved in the diseases of the exocrine pancreas.

Materials and Methods

Cells and Tissues. The following human pancreas cancer cell lines were used: AsPC-1, Capan-2, and HPAF cells, obtained from the American Type Culture Collection (Manassas, VA); SK-PC-1, IMIM-PC-1, and IMIM-
Table 2  PCR primers and conditions used for the detection of transcripts encoding for transcription factors

<table>
<thead>
<tr>
<th>Rat</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Annealing (°C)</th>
<th>Cycles (n)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p48</td>
<td>TGGACGTCTATAACGACC</td>
<td>GCAGAACAGATCTTCCGGTTC</td>
<td>55</td>
<td>37</td>
<td>714</td>
</tr>
<tr>
<td>E2A</td>
<td>CATCCACGCTCTCCGAGTCA</td>
<td>TCTGCCACTGTAGTGACCTCC</td>
<td>55</td>
<td>37</td>
<td>339</td>
</tr>
<tr>
<td>REB</td>
<td>CTCTAGACCTTACAAGGAAAG</td>
<td>GGAAGATGTGAGGGCGCGCAA</td>
<td>55</td>
<td>37</td>
<td>427</td>
</tr>
<tr>
<td>p–Actin</td>
<td>CATGAAGACCTTATGCAA</td>
<td>AGCCAGGCGCAAAATGTCTCAT</td>
<td>58</td>
<td>27</td>
<td>473</td>
</tr>
</tbody>
</table>

PC-2 cells, established in our laboratory (11), MZPC-1 cells, obtained from A. Knuht (Nordwestern Krankenhaus, Frankfurt, Germany); and RWP-1 cells, obtained from N. Vaysse (INSERM U151, Toulouse, France). AR42J and ARIP rat pancreas cancer cells (24) were obtained from N. Vaysse and O. Hagenbühl (ESREC, Lausanne, Switzerland), respectively. HT-29 colon cancer cells were from A. Zweibaum (INSERM U505, Paris, France). Conditions for culture of human pancreas cancer cells have been reported elsewhere (11). AR42J cells were seeded at approximately 1.5 × 10^5 cells/cm², cultured using DMEM supplemented with heat-inactivated fetal bovine serum (10%), 1-glutamine, nonessential amino acids, penicillin, and streptomycin, and passed at weekly intervals. ARIP cells were cultured similarly except that they were seeded at a 10-fold lower density.

DSL6A and DSL6B cell lines were obtained as described by Pettengill et al. (26). DSL6A and DSL6B cells were cultured in Waymouth's MB 752/1 medium supplemented with heat-inactivated fetal bovine serum (10%), 1-glutamine, nonessential amino acids, penicillin, and streptomycin, and passed at weekly intervals.

To obtain grafted tumors, cryopreserved pieces of the DSL6 tumor or in vitro cultured DSL6A and DSL6B cells (2 × 10^6) were injected s.c. into male Lewis rats. Tumor development was monitored weekly, and tumors of 1–2 cm diameter were surgically excised for analysis.

Normal human tissues were obtained from surgical samples except for normal pancreas, which came from organ donors; tumor tissues were obtained from surgery performed at Hospital del Mar, Barcelona, Spain, except for the three acinar tumors which were kindly provided by Dr. A. Scarpa (Istituto di Anatomia e Istologia Patologia, Facoltà di Medicina e Chirurgia, Verona, Italy).

Electron Microscopy. Tissue fragments of approximately 1–2 mm³ were fixed with 2% glutaraldehyde in PBS, postfixed with osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 resin. Thin sections were obtained using a ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Philips 301 electron microscope.

Antibodies. To generate p48-specific polyclonal antibodies, rat p48 cDNA was cloned in pBUC and used to transform BL21 strain bacteria. Recombinant colonies were individually analyzed. A positive colony was selected and induced with isopropyl-1-thio-

Western Blotting. Normal rat tissues were immediately frozen at −80°C. To prepare nuclear extracts, tissues were rapidly thawed and homogenized with a Dounce homogenizer in 50 mM Tris (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5 mM DTT, and 0.3 M sucrose containing a cocktail of protease inhibitors, filtered through a gauze, and separated through a 0.9 M sucrose cushion by centrifugation at 7000 rpm for 15 min at 4°C. The supernatant was recentrifuged under the same conditions to obtain cytoplasmic extracts. The pellet was resuspended in the homogenization buffer, brought to 0.3 sucrose and 0.2% NP40, homogenized, and recentrifuged through a sucrose cushion as described above. The pellet, considered the nuclear extract, was used for Western blotting after lysing and clearing by centrifugation. An aliquot of 40 µg was fractionated by 10% SDS-PAGE, transferred to nitrocellulose filters, and incubated with Western blotting blocking buffer (Tris-buffered saline containing 5% skim milk and 0.05% Tween 20). After incubating with specific antibodies (anti-p48 or anti-amyrase, 0.5 µg/mL in Western blotting blocking buffer), filters were washed and incubated with peroxidase-labeled goat antirabbit immunoglobulin (Dakopatts, Glostrup, Denmark), and reactions were developed with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Cultured cells were lysed in 25 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 1% SDS containing a protease inhibitor cocktail; lysates were boiled for 15 min and cleared, and protein concentration was determined. Proteins (50 µg) were fractionated by SDS-PAGE, and Western blotting was carried out as described above.

Immunohistochemistry and Immunocytochemistry. Fresh tissues were immediately frozen in isopentane cooled at −80°C. Five micron sections were fixed for 10 min with 4% paraformaldehyde, incubated for 15 min with H₂O₂ to block endogenous peroxidase, and washed with PBS. After blocking with 1% BSA, 0.1% saponin, and 0.1% Triton X-100 in PBS (blocking buffer) for 30 min, sections were incubated with primary antibody diluted in the same buffer. After washing with PBS, sections were incubated with biotin-conjugated goat antirabbit immunoglobulin (Dakopatts, Glostrup, Denmark), and reactions were developed using diamobenzidine as a chromogen. Affinity-purified rabbit anti-p48 antibodies were used at 1–5 µg/mL. Anti-amyrase antibodies were used at 10 µg/mL, and normal rabbit serum diluted to contain a comparable concentration of IgG was used as control.

Immunocytochemical assays were performed on cells fixed with 4% paraformaldehyde, permeabilized with blocking buffer, and incubated 10 (Bio-Rad, Richmond, CA). Anti-p48 antibodies present in the fraction that did not bind to this matrix were subsequently affinity-purified on a Ni-NTA matrix to which His-tagged p48 had been previously bound following the manufacturer’s recommendations (Qiagen, Valencia, CA). Bound antibodies were eluted with 3.5 M MgCl₂; their reactivity with recombinant p48 and specificity were analyzed by Western blotting as described below. This antibody preparation recognizes exclusively a protein of Mₐ ~48000 in nuclear extracts from rat or human pancreas tissue. Rabbit polyclonal antibodies detecting human and rodent amyrase were purchased from Sigma Chemical Co. (St. Louis, MO).
with antibodies as described above. For these experiments, an irrelevant mouse monoclonal antibody (B12, to detect dextran) was used as control.

RNA Expression Analysis. RNA from tissues and cultured cells was isolated using guanidinium isothiocyanate as described by Chomczynski and Sacchi (56). For RT-PCR, 4 μg of DNase-treated total RNA were used to synthesize cDNA using a mixture of oligo-d(T) and random hexamers. An aliquot of the cDNA products (1:16) was used for PCR using amplification conditions optimized for each primer pair. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The primers and PCR conditions used are shown in Table 2. The identity of the amplified products was confirmed by digestion with restriction enzymes or by direct sequencing.

To analyze human p48 mRNA expression, a partial cDNA encompassing the region coding for the conserved bHLH domain was obtained. Degenerate oligonucleotides flanking the conserved bHLH region were used to amplify oligo-d(T)-primed cDNA from normal human pancreas tissue: forward and reverse primers corresponded to the amino acid sequences ANVRER and YINFL, respectively. After amplification, a PCR product of the expected size was eluted from an agarose gel after electrophoresis and cloned in pGEMT using manufacturer’s recommendations, and positive colonies were isolated. Three independent clones were studied, and both strands of the insert DNA were sequenced using an Applied Biosystems A310 equipment. The sequence of the three clones was identical. This sequence has been submitted to GenBank with accession number AF181999.

Transient Transfection and Reporter Gene Expression Assays. Rat and human pancreatic cells (6 × 10^5) were seeded on six-well plastic plates (Costar, Cambridge, MA) or on sterile coverslips. Twenty-four h later, cells were transfected with 1 μg of linearized plasmid, either with empty vector (pcDNA3) or with the same vector containing the full-length rat p48 cDNA (pcDNA3.p48) using Lipofectamine (Lipofectamine Plus Reagent; Life Technologies, Inc., Gaithersburg, MD), following the manufacturer’s instructions. Cells were harvested at different time points to determine the optimal time for analysis; in general, assays were performed 48 h after transfection. For Western blotting and immunocytochemical procedures, cell lysates were prepared as described above. To determine the transcriptional activity of the PTF1 complex, cells were cotransfected with one of the two plasmids described above (0.2 μg) plus 0.2 μg of a plasmid, kindly provided by G. Swift and R. MacDonald (University of Texas Southwestern Medical Center, Dallas, TX), containing the hGH cDNA downstream from a 6-mer of the A element from the rat elastase promoter (6A26Elp.hGH; Ref. 37) and 0.1 μg of plasmid pGL2-control vector (Promega Corp., Madison, WI), containing the luciferase cDNA, to normalize for transfection efficiency. For these experiments, 10^5 cells were seeded in wells of 24-well plates (Costar) in duplicates, and transfection was performed 24 h later using the Lipofectamine Plus reagent. Forty-eight h later, hGH activity was measured in the culture medium using a RIA (Nichols Institute Diagnostics, San Juan Capistrano, CA), and amylase activity was determined using the AMYL commercial kit (Boehringer Mannheim, Mannheim, Germany). Cells were lysed and processed for luciferase activity assays according to the manufacturer’s recommendations (Promega).

Acknowledgments

We thank the investigators mentioned in the text for providing cells and reagents, O. Hagenbühle for the rat p48 cDNA and for many valuable discussions, and C. Balaguk, E. Battle, M. Garrido, J. Lloreta, P. Navarro, T. Palomero, and the members of the Unitat de Biologia Cel·lular i Molecular, Institut Municipal d’Investigació Mèdica, for valuable contributions.

References


bHLH protein p48, a DNA-binding subunit of PTF1, acts as a morphogen.


