Original Article

Evaluation of β-Cell Replication in Mice Transgenic for Hepatocyte Growth Factor and Placental Lactogen

Comprehensive Characterization of the G_1/S Regulatory Proteins Reveals Unique Involvement of $p21^{cip}$

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We hypothesized that combined transgenic overexpression of hepatocyte growth factor (HGF) and placental lactogen in islets would lead to even greater increases in β-cell mass and replication than either growth factor alone. This did not occur, suggesting that β-cell replication is saturable or subject to molecular restraint. We therefore performed the first comprehensive G_1/S cell cycle survey in islets, cataloguing the broad range of kinases, cyclins, and kinase inhibitors that control the G_1/S transition in islets from normal, HGF, placental lactogen, and doubly transgenic mice. Many of the G_1/S checkpoint regulators (E2Fs; pRb; p107; p130; cyclins $D_{1,2,3}$, A, and E; cdk-2; cdk-4; p15; p16; p18; p19; p21; p27; MDM2; p53; c-Myc; and Egr-1) are present in the murine islet. Most of these proteins were unaltered by overexpression of HGF or placental lactogen, either alone or in combination. In contrast, $p21^{cip}$ was uniquely, dramatically, and reproducibly upregulated in placental lactogen and HGF islets. $p21^{cip}$ was also present in, and upregulated in, proliferating human islets, localizing specifically in β-cells and translocating to the nucleus on mitogenic stimulation. Homozygous $p21^{cip}$ loss releases islets from growth inhibition, markedly enhancing proliferation in response to HGF and placental lactogen. *Diabetes* **55:70–77, 2006**

Progress in pancreatic islet transplantation (1) and in immunosuppression directed against the autoimmunity of type 1 diabetes (2) has focused attention on a need to understand the cellular mechanisms that control β-cell replication. Surprisingly, little is known regarding the molecular control of cell cycle events in the β-cell, in part, because of a longstanding belief that β-cells cannot replicate and that, if induced to replicate, they inevitably would de-differentiate. In contrast, we (3–5) and others (6–10) have demonstrated that human and rodent β-cells can replicate and retain their differentiated phenotype in vivo. A recent study has suggested that replication of β-cells is the principal mechanism through which new β-cells are generated (6). Thus, a clear and comprehensive understanding of β-cell replication will be important for developing novel approaches to treating and preventing diabetes.

Important lessons regarding β-cell replication have been revealed from mouse genetic models. For example, combined knockout of the p53 and pRb tumor suppressor genes leads to pancreatic islet tumors (11,12). Similarly, overexpression of simian virus 40 viral large T-antigen also leads to islet hyperplasia, and ultimately to islet tumorigenesis (13). Cyclin D_1 is the most abundant oncogene in human insulinomas (14), and cyclin D_1 overexpression leads to β-cell hyperplasia in transgenic mice (15). Cyclin D_2 has been shown to be essential for achieving and maintaining β-cell mass (16,17) and deletion of E2F1 and E2F2 transcription factors results in β-cell failure (18,19). Knockout of cdk-4 results in β-cell failure and diabetic ketoacidosis (20,21), and overexpression of constitutively active cdk-4 results in β-cell hyperplasia and ultimately β-cell tumorigenesis (20). Adenoviral overexpression of cyclin D_1 and cdk-4 in human islets leads to an ~10-fold increase in the rate of β-cell replication, without any apparent loss of function (5). These observations are significant in aggregate, for they demonstrate that the cell cycle in the β-cell is accessible to study and manipulation. Importantly, because all of the molecules described above are involved in the progression or arrest of the cell cycle at the G_1/S checkpoint, they suggest that this is a particularly important site of control in the β-cell.

Because most or all of the molecules described above are present in all cells, it is not surprising that disturbing these molecules might disrupt cell cycle control in the β-cell. In contrast, the large group of cell cycle control molecules that lie upstream of these molecules, exemplified by the inhibitory kinase (INK), cyclin inhibitory proteins or kinase inhibitory proteins (CIP/KIP), p53, c-Myc, and others (rev. in 22–24), are less ubiquitous and often tissue-specific in their expression and/or function. Surprisingly, very little is known about their presence or function within the β-cell. Only one of these, $p27^{kip}$, has been
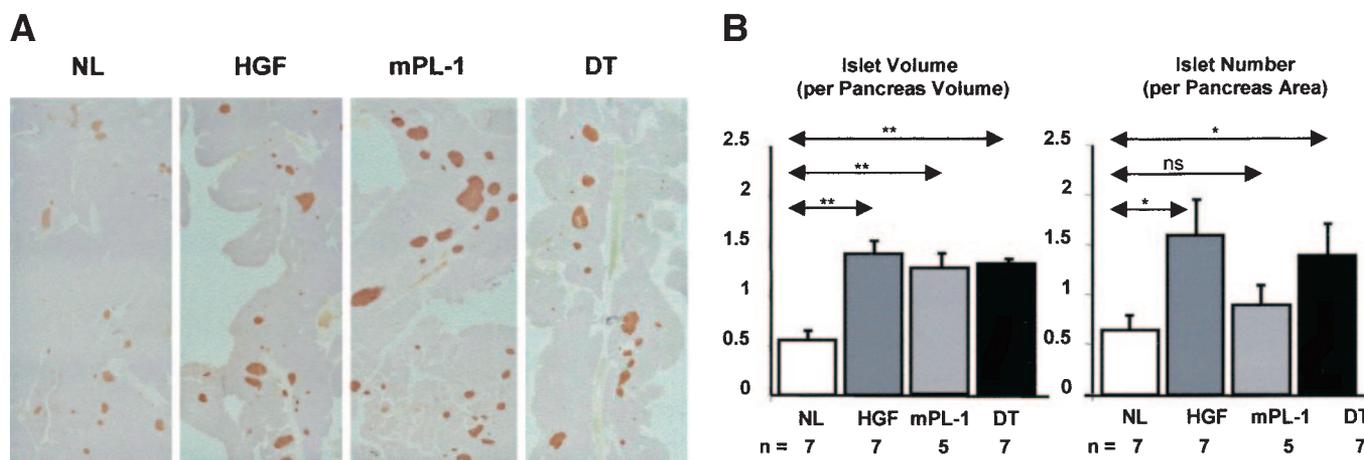
A

NL HGF mPL-1 DT

B

FIG. 1. *A*: Low-power photomicrographs of whole pancreatic insulin-stained sections from the four genotypes. Islet mass and islet size are increased in all three transgenic mouse types, but the double-transgenic mice are not different from their single-transgenic littermates. *B*: Quantitative pancreatic histomorphometry on pancreata of the four genotypes. As reported previously in the RIP-HGF and RIP-PL mice (3,4,26,27), islet mass is increased as shown. There is no further increase in either islet mass or number in the double-transgenic mice. *$P < 0.05$; **$P < 0.01$. n, the number of animals studied in each group.

implicated in control of the β-cell cycle, and this observation is very recent (25).

We have previously described three types of transgenic mice in which parathyroid hormone–related protein (PTHrP) (26), hepatocyte growth factor (HGF) (3,27), or placental lactogen (4,28) has been overexpressed in the β-cell under the control of the rat insulin promoter (RIP). Whereas many growth factors have been demonstrated to activate β-cell mitogenesis in vitro, these three RIP-PTHrP, RIP-placental lactogen (RIP-PL), and RIP-HGF mouse lines represent the only examples of growth factor–driven β-cell hyperplasia and insulin-mediated hypoglycemia. Because the RIP-PL and RIP-HGF mice have a marked increase in β-cell replication rates, and because HGF and placental lactogen signal through different pathways, we hypothesized that mice doubly transgenic for placental lactogen and HGF would display even greater increases in β-cell proliferation rates, β-cell mass, and overall β-cell function.

Here, we demonstrate that this hypothesis is incorrect, suggesting that intrinsic, rate-limiting controls exist that prevent unrestricted growth factor–driven β-cell expansion. To define these molecular controls, we have comprehensively catalogued the "pancreatic islet G_1/S proteome" for the first time.

RESEARCH DESIGN AND METHODS

Single-transgenic RIP-HGF and RIP-mPL1 transgenic mice that express HGF and mPL-1 under the control of the rat insulin II promoter (13) have been described in detail previously (3,4,26,27). RIP-HGF and RIP-PL mice were crossed to generate normal mice, mice singly transgenic for placental lactogen or HGF, and double-transgenic mice overexpressing HGF and placental lactogen in the pancreatic β-cell. All studies were performed on mice between 8 and 12 weeks of age, and mice were ~50% male and ~50% female. No significant differences were observed between males or females in these studies. The original backgrounds of the RIP-mPL-1 and RIP-HGF mice were CD-1, and the crosses thus remain on a CD-1 background. p21^{cip}-null mice (29) and controls on a C57B6 background were purchased from The Jackson Laboratories (Bar Harbor, ME). All procedures were approved by, and in compliance with, The University of Pittsburgh Institutional Animal Care and Use Committee.

Blood chemistries, intraperitoneal glucose tolerance testing, pancreatic histology and immunohistochemistry, quantitative pancreatic histomorphometry, and β-cell proliferation rate. These procedures were performed as described in detail previously (3–5,26,27) and in the figure legends. In vitro β-cell replication rates were assessed using tritiated thymi-

dine incorporation as described in detail previously (5). Briefly, p21−/− and normal mouse islets were freshly isolated and placed in RPMI medium containing 5 mmol/l glucose and 10% fetal bovine serum in 24-well plates, with 50 islets per well. HGF or prolactin was added 24 h later in the concentrations shown in the legend of Fig. 8 (for HGF, for both p21+/+ and p21−/−, 11 mmol/l glucose was used), and the islets were incubated for an additional 48 h. Tritiated thymidine (0.5 μCi/well) was added 24 h later in RPMI medium containing the growth factors but without fetal bovine serum, and the islets were allowed to incubate for an additional 24 h (for a total of 48 h with HGF or prolactin and 24 h with tritiated thymidine). The remainder of the procedure was as described previously (5).

Cyclin D_1 and cdk-4 adenoviruses. The adenoviral cyclin D_1 and cdk-4 studies were performed using adenoviruses, conditions, and reagents described in detail previously (5). Briefly, murine and human islets were transduced for 1 h with 500 multiplicity of infection of adenoviruses expressing cyclin D_1 and cdk-4 under the control of the cytomegalovirus promoter.

Islet isolation and semiquantitative RT-PCR. Mouse islets were isolated as described previously (3–5,26,27). Human islets were provided by the University of Pittsburgh Human Islet Isolation Facility as described in detail previously (5). Semiquantitative PCR was performed as described in detail previously (3–5,26,27) using the primer pairs shown in Supplemental Table 1, which is detailed in the online appendix (available at http://diabetes.diabetesjournals.org) (see table legend for full details).

Cell cycle protein immunoblots. Islet extracts were prepared using nuclear extraction buffer as described previously (5). Antisera and dilutions used are shown in Supplemental Table 2, which is detailed in the online appendix (available at http://diabetes.diabetesjournals.org). Each immunoblot in Figs. 4–7 was performed a minimum of three times on a minimum of three different islet preparations, and each was accompanied by an appropriate housekeeping protein control, tubulin, or actin. In cases in which minor changes appeared possible, the immunoblots were repeated for a total of five to eight times, and quantitative densitometry of digitalized blots was performed and analyzed statistically. Thus, the immunoblots and text descriptions are representative of multiple experiments. Because of space limitations, only representative immunoblots and action/tubulin controls are shown.

Statistics. For all studies, Student's two-tailed unpaired *t*-test was performed using Microsoft Excel. P values <0.05 were considered significant. Bars in the figures indicate SE.

RESULTS

Metabolic and histological characterization of mice singly and doubly transgenic for HGF and placental lactogen. Our original goal was to determine, by crossing RIP-PL with RIP-HGF mice, whether overexpression of both mitogenic growth factors in combination would lead to a further increase in islet mass and function. Supplemental Fig. 1, which is detailed in the online appendix, confirms, using RT-PCR, that expression of both trans-
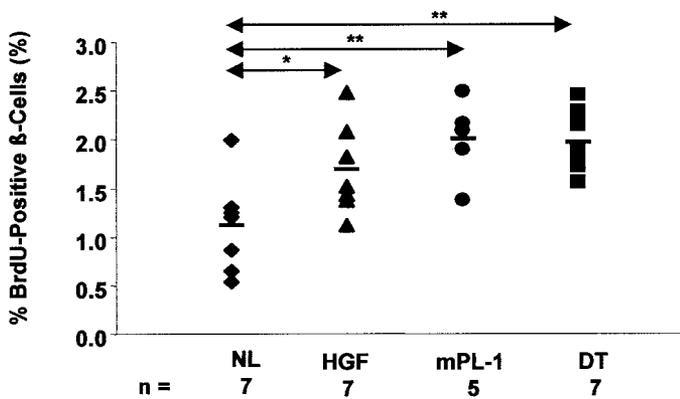
FIG. 2. 5-bromo-2′-deoxyuridine (BrdU) proliferation rates in islets from the four types of mice. β-Cell replication in vivo was quantified by BrdU incorporation as detailed previously (3–5,26,27). Briefly, mice were injected intraperitoneally with BrdU (Cell Proliferation kit; Amersham Pharmacia Biotech) and killed after 6 h. Pancreata were removed, fixed in Bouin's solution overnight, and then embedded, sectioned, deparaffinized, and costained using an anti-BrdU antibody (Amersham Pharmacia Biotech) and anti-insulin antibody (Zymed). After counterstaining with hematoxylin, the sections were counted in a blinded fashion. At least 500 islet nuclei were counted per section. The results are expressed as the percentage of BrdU-positive β-cells per total number of β-cell nuclei. Although the single transgenics display an increase in β-cell proliferation, this is not further increased in the double-transgenic islets.

genes occurs in islets of mice singly and doubly transgenic for placental lactogen and for HGF. Supplemental Fig. 2 of the online appendix shows fasting and postprandial glucose and insulin concentrations in the four mouse genotypes. The two single-transgenic lines, RIP-PL and RIP-HGF, reproduce their original phenotypes (3,4,26,27) in this second independent study. However, overexpression of placental lactogen and HGF in the double transgenics did not lead to an exaggeration either of fasting or postprandial hypoglycemia. Intraperitoneal glucose tolerance tests (Supplemental Fig. 3 of the online appendix) were also reflective of events described earlier (3,4,26,27): The RIP-HGF mice displayed mildly improved glucose tolerance, and the RIP-PL mice did not. Surprisingly, the double transgenics were significantly impaired compared with the normals and their single-transgenic littermates.

The RIP-HGF and RIP-PL mice displayed an obvious increase in β-cell mass (Fig. 1A), paralleling that described previously (3,4,26,27). However, the combined overexpression of the two growth factors had no additional effect on islet number or mass. This was confirmed using quan-

titative islet histomorphometry (Fig. 1B), which showed that islet cell volume was increased approximately three-fold in both single transgenics, as reported previously (3,4,26,27). However, there was no further increase in islet mass in their double-transgenic littermates. Similarly, islet number was increased in the RIP-HGF mice, but the combined overexpression of placental lactogen and HGF did not further increase islet number. Islet morphology was normal, although there appeared to be a redistribution of α-cells and somatostatin cells from the periphery into more central portions of the islet in the RIP-PL and double-transgenic mice (Supplemental Fig. 4, online appendix).

Prior studies using RIP-HGF and RIP-mPL1 mice have demonstrated that HGF and mPL1 increase rates of β-cell replication in vivo by a factor of ~2 (3,4,26,27). Figure 2 confirms these findings in this second study. However, mice doubly transgenic for placental lactogen and HGF did not display a further increase in β-cell replication rates.

Cataloguing the murine islet G$_1$/S cell cycle proteome. The failure of combined overexpression of two potent β-cell mitogenic growth factors to further increase β-cell replication or mass suggested that a regulatory system(s) may exist that represses cell cycle activity in response to excessive mitogenic stimuli. We therefore explored in a comprehensive fashion all of the key cell cycle regulatory molecules known to be involved in the G$_1$/S transition (Fig. 3). Because many, if not most, cell cycle regulatory proteins are known to be regulated at the posttranslational level, for example by ubiquitination and proteosamal degradation (22–24), we studied these molecules at the protein rather than RNA level.

The retinoblastoma protein, pRb, is the principal checkpoint protein of the G$_1$/S transition. Figure 4A demonstrates for the first time that pRb is present in murine islets and can be phosphorylated (inactivated) to phospho-pRb in murine islets by adenoviral overexpression of cyclin D$_1$ and cdk-4 (Fig. 4B). Surprisingly, pRb is not apparently phosphorylated by either HGF or placental lactogen alone or in combination. pRb is one member of a family of tumor suppressor proteins, the "pocket proteins," that includes pRb, p107, and p130. Figure 4A also demonstrates for the first time in any species that p107 and p130 are present in the islet. None of the pocket protein family members appear to be regulated by HGF, placental lactogen, or the combination.

Seven E2F transcription factors are believed to regulate the myriad genes required for G$_1$/S transition (Fig. 3).
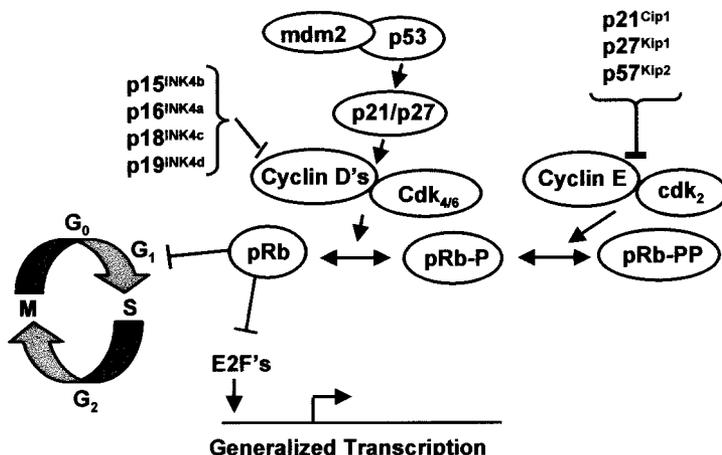

FIG. 3. A schematic representation of the molecular control of the cell cycle, with emphasis on the G$_1$/S checkpoint. pRb is the retinoblastoma protein, and in the figure, it represents all three pocket protein family members. pRb-P, phosphorylated pRb; pRb-PP, hyperphosphorylated pRb.
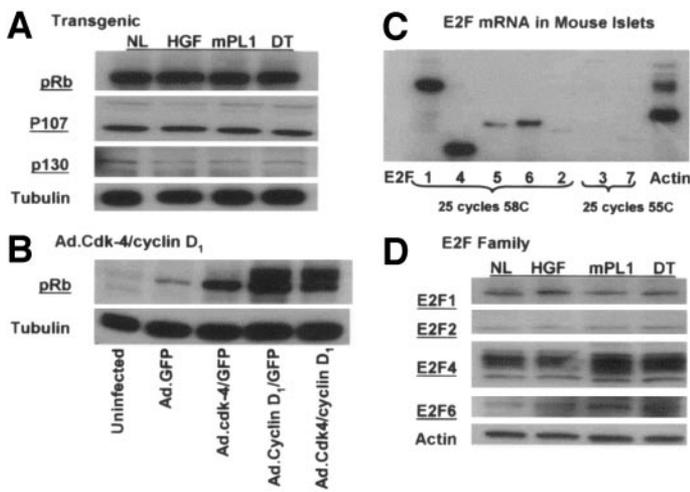
FIG. 4. Western blots of the cell cycle regulatory proteins. Islet extracts were resolved using 7.5, 10, or 15% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). For each of the figures shown, the Western blots were performed a minimum of three times on separate islet extracts and, in cases where there was a question of relative abundance, five to eight times. Below each panel, a representative control blot for actin or tubulin is shown. A: The three pocket protein family members in control and transgenic islets. pRb, p107, and p130 are present in the murine islet. B: Whereas pRb is not apparently phosphorylated in RIP-HGF or RIP-PL islets, it is clearly phosphorylated in response to forced overexpression of cdk-4 and cyclin D_1. For these experiments, adenovirus-expressing green fluorescent protein (GFP) as a negative control and cdk-4 and/or cyclin D_1 were incubated with islets for 1 h and then removed, and the islets were cultured for an additional 48 h, as reported previously for rat and human islets (5). Note that although pRb appears to be absent in uninfected islets in this photo, it is readily apparent on longer exposures: the exposure time was limited to allow clear visualization of the distinct pRb and ppRb bands. The ppRb band was confirmed to represent phospho-pRb by using phospho-pRb–specific antisera (Cell Signaling Technology, Beverly, MA). C: E2F family members. RT-PCR exploring the presence of the seven E2F family members in murine islets. E2Fs 1, 4, and 6 are most abundant. D: Western blots for E2Fs 1, 2, 4, and 6 in mice of the four genotypes. No differences are apparent.

Figure 4C demonstrates that mRNAs encoding the seven E2F proteins are expressed in the pancreatic islet. E2F1, a G_1/S transition agonist, and E2F4 and E2F6, G_1/S transition transcriptional repressors, are expressed most highly, whereas E2Fs 2, 3, 5, and 7 are expressed at lower levels.

We studied E2Fs 1, 2, 4, and 6 further, because they are expressed at the highest levels (E2Fs 1, 4, and 6) or have been implicated in β-cell function (E2Fs 1 and 2) (18,19). Figure 4D demonstrates that each of these four E2F proteins is present in the murine islet.

Figure 5A shows that all of the three members of the cyclin D family are present in the islets of CD-1 mice. Of particular note is cyclin D_3, which some authors have suggested is not abundant in the islet (16,17,30). This observation was confirmed by RT-PCR using two different sets of primers (not shown). Figure 5B demonstrates that cdk-4 is present in murine islets, whereas cdk-6 is completely absent. Figure 5C demonstrates that cyclin A, cyclin E, and cdk-2 are present in murine islet. Figure 5D demonstrates that all four members of the INK family of kinase inhibitors are present in the murine islet. Figure 5E demonstrates that p27 and p57, two members of the Cip-Kip-Waf family of kinase inhibitors, are also present in the murine islet. Figure 5F demonstrates the presence of c-Myc (assessed by RT-PCR in the absence of reliable antisera), MDM2, and Egr-1. p53 appears to be reproducibly increased in RIP-HGF islets. Importantly, none of the other proteins shown in Fig. 4 or 5 is differentially regulated by HGF, placental lactogen, or the combination.

$p21^{cip}$ is markedly upregulated in murine and human islets and traffics to the nucleus in response to mitogenic stimuli. Figure 6A reveals that $p21^{cip}$ is markedly, consistently, and very specifically upregulated in RIP-HGF and RIP-PL islets. Interestingly, although $p21^{cip}$ is upregulated in the RIP-PL and RIP-HGF islets, it is not further upregulated in the doubly transgenic HGF-PL islets. As shown in Fig. 6B, RIP-PTHrP islets, which also display increased β-cell replication (26,28), also demonstrate a marked increase in $p21^{cip}$ expression. Furthermore, $p21^{cip}$ is upregulated in mPL1 and double-transgenic RIP-PTHrP and RIP-mPL1 islets (28). Figure 6C demonstrates that $p21^{cip}$ expression is also increased in response to cell cycle activation by adenoviral overexpression of cyclin D_1 and cdk-4 (5). Figure 6D and E demonstrates that exogenous prolactin, a surrogate for placental lactogen, directly upregulates p21 protein expression in normal
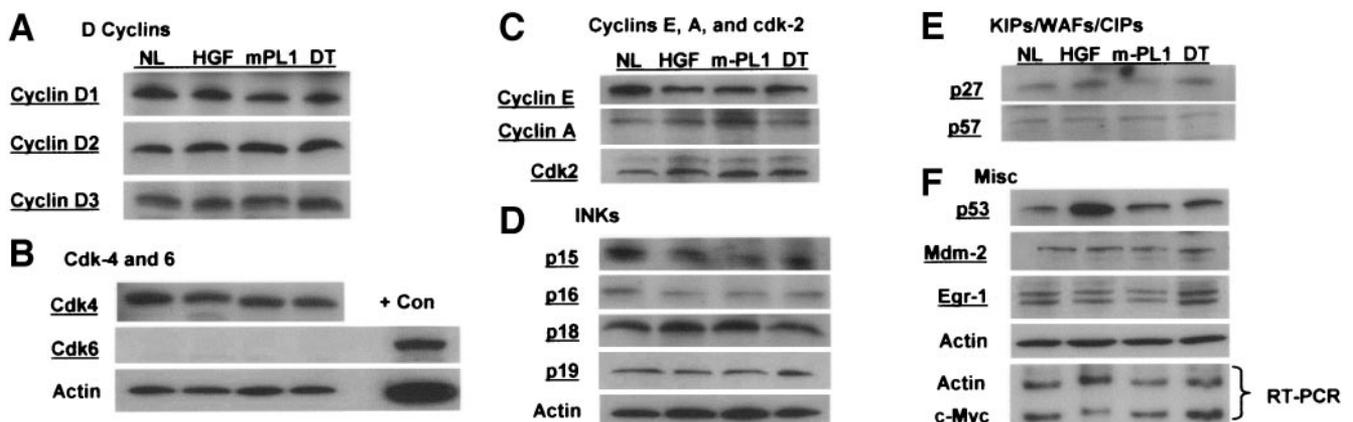

FIG. 5. Cyclins A, D, and E; their respective cdks; and upstream inhibitors. For each of the figures shown, Western blots were performed a minimum of three times on separate islet extracts and, in cases where there was a question of relative abundance, five to eight times. Below each panel, a representative control blot for actin is shown. A: The D cyclins. All three D cyclins are readily apparent in CD-1 islets. B: Cdk-4 and -6. Although murine islets contain abundant cdk-4, its homolog, cdk-6, is absent in murine islets. The positive control for cdk-6 is arterial smooth muscle. C: Cyclins A and E and cdk-2. All three members of this complex are present and unchanged among the four types of mice. D: The INK family. All four members are present and unchanged. E: The KIP/CIP/WAF family. Two of the three members of the CIP/KIP/WAF family, p27 and p57, are present and unaffected. F: Miscellaneous other cell cycle proteins. p53, MDM-2, Egr-1, and c-Myc are present in the murine islet. c-Myc was assessed using RT-PCR.
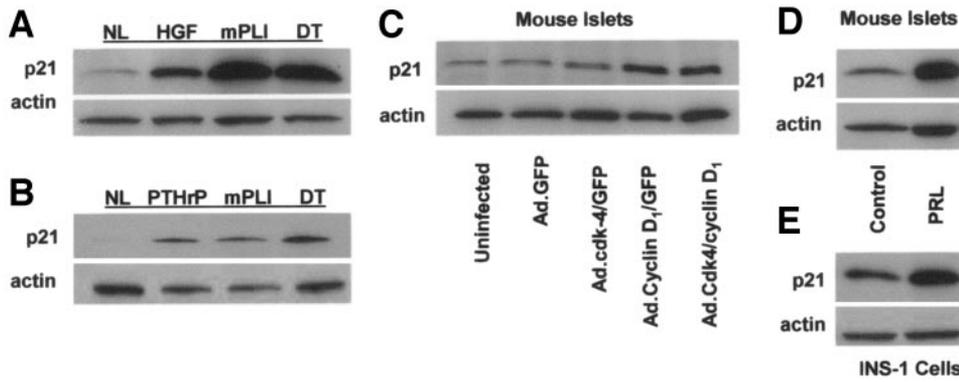
FIG. 6. p21^{cip} is upregulated in response to multiple different cell cycle activators in murine islets. *A*: p21^{cip} is upregulated in the islets of RIP-HGF, RIP-mPL-1, and double-transgenic mice, as compared with their normal littermates. *B*: p21^{cip} is also upregulated in the islets of RIP-PTHrP mice as well as in RIP-mPL-1 and RIP-mPL1/RIP-PTHrP double-transgenic mice (28), as compared with their normal littermates. *C*: Adenoviral cdk-4 and cyclin D$_1$ overexpression also leads to dramatic upregulation of p21^{cip} in CD-1 mouse islets compared with control or Ad.GFP-transduced human islets. *D*: Upregulation of p21^{cip} by exogenous administration of prolactin (200 ng/ml) versus control vehicle to normal CD-1 mouse islets for 4 days in culture. *E*: Upregulation of p21^{cip} by prolactin in the rat β-cell line, Ins-1, by prolactin (200 ng/ml) in culture.

isolated murine islets and in the rat insulinoma cell line, Ins-1.

All of the studies described thus far apply to murine islets. We wanted to determine whether these observations might apply to human islets. Figure 7*A* demonstrates that human islets express p21^{cip} protein as well and that it is dramatically increased in response to cell cycle activation by either cyclin D$_1$ alone or in combination with cdk-4 (5). p21^{cip} is present primarily in the cytoplasm of non-stimulated human β-cells but traffics into the nuclear compartment on mitogenic stimulation (Fig. 7*B*).

Loss of p21^{cip} results in increased islet cell proliferation responses to mitogenic stimuli. p21^{cip}-null mice are dwarf and tumor prone and develop renal failure by 6 months of age because of glomerulonephritis (29,31), complicating the evaluation of their phenotype in vivo. We therefore turned to islets isolated from these mice (29), stimulated in vitro with HGF or placental lactogen. As can be seen in Fig. 8, the addition of exogenous HGF or prolactin, a surrogate for placental lactogen, to normal murine islets had no effect on [^{3}H]thymidine incorporation. In contrast, when administered to p21^{cip}−/− islets, ^{3}H incorporation increased substantially. These observations suggest that p21^{cip} inhibits β-cell replication in response to β-cell mitogens.

DISCUSSION

These studies make six significant and novel observations: *1*) Combined overexpression in transgenic mice of two well-characterized and potent in vivo β-cell mitogens, placental lactogen and HGF, does not further enhance β-cell replication rates nor further enhance islet mass or function. *2*) We provide the first comprehensive catalog defining the cell cycle regulatory proteins that are likely to govern the G$_1$/S transition in the murine islet. This "catalog" or "G$_1$/S β-cell cycle proteome" has been characterized under basal and mitogenic conditions. *3*) p21^{cip} is markedly and uniquely subject to regulation by three different β-cell growth factors—HGF, placental lactogen, and PTHrP—as well as by a key G$_1$/S transition regulatory kinase complex, cyclin D$_1$/cdk-4. *4*) p21^{cip} traffics to the nucleus of β-cells under mitogenic stimulation. *5*) p21^{cip} serves to inhibit growth factor–driven replication in murine islets. *6*) These observations extend to human islets, where p21^{cip} is present, is upregulated, and translocates to the nucleus of human β-cells in association with activation of cell cycle progression.

The metabolic and histological findings in the RIP-PL and RIP-HGF mice confirm in an independent study that these two factors are potent and effective β-cell mitogens, increasing β-cell replication and aggregate β-cell mass in vivo, while retaining the differentiated functions of the β-cell. In contrast, the results of combined or simultaneous overexpression of placental lactogen and HGF were surprising: We had anticipated that because HGF and placental lactogen act via different signaling pathways—JAK2/STAT5 for placental lactogen (10,32) and phosphatidylinositol 3-kinase and mitogen-activated protein kinase
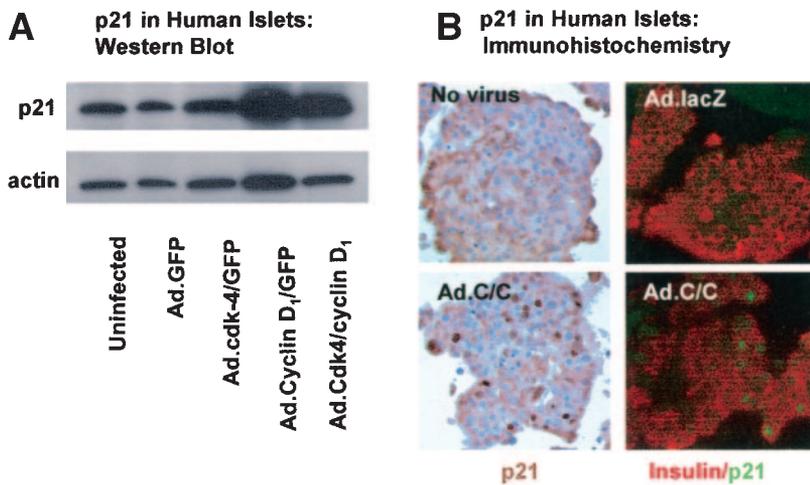

FIG. 7. p21^{cip} is upregulated in human islets in response to cdk-4/cyclin D$_1$ overexpression and translocates to the nucleus. *A*: Adenoviral cdk-4 and cyclin D$_1$ overexpression leads to dramatic upregulation of p21^{cip} in human islets compared with control or Ad.GFP-transduced human islets. *B*, *top left*: Immunoperoxidase staining. p21^{cip} is present in nonstimulated human islets in the cytoplasm of islet cells. *B*, *top right*: Immunoflourescent staining. p21^{cip} (green) colocalizes with insulin (red) in β-cells. *B*, *bottom panels*: Adenoviral cdk-4 and cyclin D$_1$ overexpression in human islets leads to translocation of p21^{cip} into the nucleus of islet cells (*left*), including β-cells (*right*).
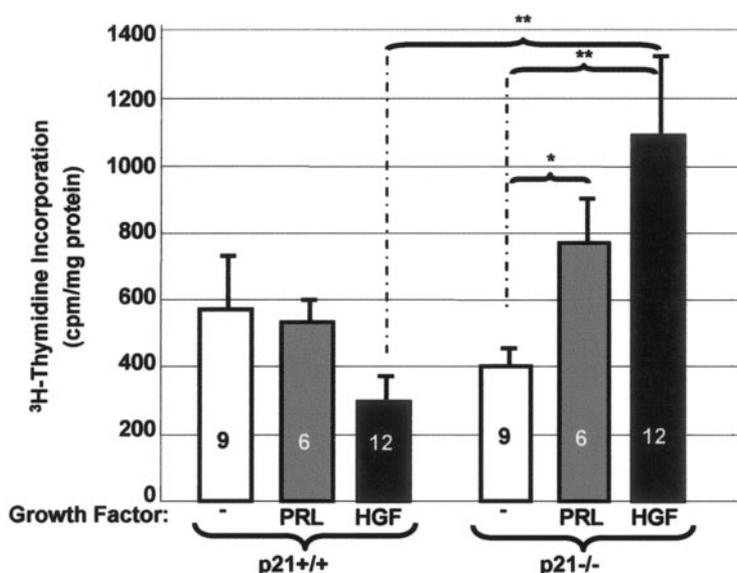
FIG. 8. [³H]thymidine incorporation into murine islets that contain or lack p21^{cip}. Prolactin (PRL) is used as a surrogate for placental lactogen. HGF (25 ng/ml) and PRL (200 ng/ml) fail to stimulate proliferation in normal adult murine islets. In contrast, identical treatment of p21^{cip}-null islets results in brisk activation of proliferation. The numbers within the bars indicate the number of wells used for each condition. Bars indicate SE. *$P < 0.05$; **$P < 0.01$.

for HGF (3,9)—they would have an additive or synergistic effect on islet mass. This lack of synergy is present in two additional double-transgenic systems: the cross of the RIP-HGF mouse with the RIP-PTHrP mouse (33) and the cross of the RIP-mPL-1 and RIP-PTHrP mouse (28). These findings suggest that intrinsic gatekeeper or rate-limiting pathway(s) may exist that prevent β-cell replication rates from exceeding certain predetermined limits.

We focused on the molecular control of the cell cycle, in part, because of our demonstration (3–5), as well as that of others (6–10), that β-cell can replicate and, in part, because of the essential absence of a "cell cycle roadmap" relating to the β-cell. Despite the extensive characterization of cell cycle regulatory mechanisms in fibroblasts, cancer cell lines, and developmental systems, relatively little is known about the molecular and cellular control of replication machinery in the β-cell. We selected the G_1/S transition point because, in general, growth factors activate cell cycle progression at G_1/S (22–24) and also because virtually all mouse genetic models had suggested that this was likely to be an important pathway for β-cell replication (11–21,25).

Under basal conditions, many, but not all, of the key G_1/S regulatory molecules are present in the murine pancreatic islet. For example, we show for the first time that pRb, the prototypical G_1/S gatekeeper in most systems, is present in the murine islet, as are its two pocket protein homologs, p107 and p130 (34,35). However, we see no evidence of pRb phosphorylation in these islets. E2F proteins mediate the transcriptional effects of pRb on downstream cell replication machinery. In the current study, we demonstrate for the first time that most E2F family members are present within the murine pancreatic islet and that, among these, E2Fs1 and 4 are most abundant.

All three members of the cyclin D family are present in the murine islet. This is significant because Martin et al. (31) and Kushner et al. (17) have suggested that cyclin D_3 is not abundant in the islet, and Georgia et al. (16) and Kushner et al. (17) indicate that null mutations for cyclin D_2 result in islet hypoplasia. All three of these prior studies were performed using C57B6 mice, whereas ours were performed using CD-1 mice. We recognize that the best current commercial antisera may cross-react among cyclin

D isoforms, and therefore we confirmed that cyclin D_3 mRNA is present in the CD-1 murine islet using RT-PCR with two different sets of primers (not shown). Thus, β-cell cycle control may prove to be species and strain specific. As with the cyclin D_3 immunoblot data shown in Fig. 5A, cyclin D_3 mRNA levels were not different in the islets of normal littermates compared with the RIP-HGF, RIP-mPL-1, or double-transgenic mice (not shown). We cannot exclude the possibility that a portion of the cyclin D_3 mRNA observed may be derived from contaminating exocrine or non–β-islet cells.

Null mutations for cdk-4 result in marked islet hypoplasia, hypofunction, and diabetic ketoacidosis (20,21). The studies reported here make it clear why: Surprisingly, murine islets specifically lack cdk-6, a functional analog of cdk-4. Thus, loss of cdk-4 in cells that already lack cdk-6 might be predicted to lead to islet failure.

Murine islets were shown to contain pRb, phospho-pRb, p107, p130, cyclins $D_{1,2,3}$, cyclin A, cyclin E, cdk-2, cdk-4, p15^{Ink}, p16^{Ink}, p18^{Ink}, p19^{Ink}, p27^{kip1}, p57^{cip2}, MDM2, Egr-1, and c-Myc. In contrast to our expectations, overexpression of HGF, placental lactogen, or the combination had essentially no effect on any of these cell cycle control molecules. This may reflect the fact that these molecules are not subject to regulation by growth factors in the islet, that their associations with one another and trafficking are more important than their amounts, or that increases or decreases in these proteins in a small subpopulation of proliferating β-cells are "diluted out" by the larger aggregate mass of G_0/G_1-arrested mass of β-cells. In contrast to all of these apparently "stable" cell cycle regulatory proteins, HGF, but not placental lactogen, reproducibly stimulated p53. Understanding the significance of this observation will require further study.

In contrast to the lack of change in the 20 molecules itemized above, p21^{cip} was dramatically and specifically upregulated in response to HGF, placental lactogen, PTHrP, and cyclin D_1 alone or in combination with cdk-4. Furthermore, p21^{cip} was upregulated by cell cycle activation not only in murine islets, but also in human islets. Importantly, this upregulation was observed to occur in the β-cell and to be associated with translocation from the cytoplasmic to the nuclear compartment. p21^{cip} has previously been shown to be present in rat and human β-cells

and in rat β-cell lines (36–38). However, it never has been implicated in β-cell cycle control. Kaneto et al. (36) have shown that p21^{cip} is upregulated in rat islets in response to oxidative stress induced by adding H_2O_2 and is also upregulated in isolated islets of obese Zucker diabetic rats. Forced overexpression of p21^{cip} led to a reduction in insulin content in rat islets, but no cell cycle studies were reported (36). Zhang et al. (37) have reported that induction of apoptosis in the insulinoma cell line RIN m5F leads to upregulation of p21^{cip}. Maitra et al. (38) reported that p21^{cip} mRNA is reduced in human insulinomas and that p21^{cip} is present in normal human β-cell nuclei but is downregulated in nuclei of human pancreatic endocrine tumors. Although no information regarding p21^{cip} involvement in β-cell replication was provided in any of these studies, they collectively suggest that p21^{cip} may be upregulated by β-cell injury or stress and that loss of p21^{cip} may be associated with islet tumorigenesis.

In some tissues and organs, p21^{cip} is an inhibitor of cell cycle progression, acting as a classical INK or KIP/CIP family member (22–24,39,40), whereas in other tissues or cells, it can serve as an agonist for cell cycle progression (31,41,42). Therefore, one might speculate that the increase in p21^{cip} expression in response to mitogenic stimuli serves as the rate-limiting factor, or the molecular "brakes," on β-cell cycle progression that we had hoped to identify. On the other hand, one might equally reasonably speculate that p21^{cip} is a mediator of cell cycle progression, or a molecular "accelerator," that augments β-cell cycle progression. The observation that p21^{cip}-null islets are more responsive to mitogenic stimulation by HGF and prolactin, used here as a surrogate for placental lactogen, supports the first possibility. We hypothesize, therefore, that the marked upregulation of p21^{cip} in the single and double-transgenic islets may prevent excessive β-cell replication in the single transgenics and that the exuberant β-cell replication and increase in islet mass we had anticipated might occur in the double transgenics.

The ability of p21^{cip}-null islets to proliferate basally and in response to growth factors indicates that one standard role of p21^{cip}—assembly of cdk-cyclin D and E complexes in the cytosol and use of the nuclear localization signal in p21^{cip} to transport these cdk-cyclin complexes into the nucleus where they can phosphorylate pRb—is not essential or is complemented by another protein that can serve the same function. One obvious candidate is p27^{kip}, which is believed to subserve the same function (22–25). p27^{kip} has recently been reported by Uchida et al. (25) to be critical to the reduction of β-cell mass and function in IRS-2−/− and leptin receptor–null mice. Interestingly, although p27^{kip} was upregulated in IRS-2−/− islets and trafficked to the nuclear compartment, there was no change in p21^{cip} in these "metabolically" abnormal islets. These events are the mirror image of those observed here, in which mitogenically challenged islets display upregulation and nuclear translocation of p21^{cip}, with no change in p27^{kip}. It seems possible that p27^{kip} may respond to metabolic signals such as glucose/lipotoxicity, whereas p21^{cip} may respond to mitogenic signals such as growth factors.

These studies have a number of limitations. First, as alluded to above, most cell cycle proteins shuttle between the nuclear and cytoplasmic compartments and within these compartments, shuttle among complexes with one another, yet we have provided no information on this trafficking of any molecule except for p21^{cip}. Further immunocytochemical and coimmunoprecipitation studies will be required to define the subcellular distributions and key interactions of the proteins shown in Fig. 3. Second, kinase activity and phosphorylation status are often more relevant to cell cycle activity than the absolute level of a particular cell cycle protein, yet the current studies do not provide information on kinase activity of the cdks. Third, growth factor–driven cell cycle protein changes likely occur exclusively or predominantly in a subset of cells in G_1 and S phases, but we have examined only whole islets. Thus, important single-cell changes in many of these proteins could be masked or diluted by a lack of change in the larger component of noncycling β-cells. Fourth, islets are complex mixtures of α-, β-, δ-, PP, ductal, endothelial, fibroblast, and other cells, and studies using immunoblots or whole islets may not reflect events in β-cells. Fifth, it is likely that other members of the G_1/S cell cycle proteome are present in the β-cell (for example, the DPs, the Ids), and additional yet unidentified cell cycle regulatory molecules are certain to be identified in the future. Sixth, these studies may have identified p21^{cip} as the molecular "β-cell brake" in HGF- and placental lactogen–stimulated cells, but they do not elucidate the cell cycle pathways that lead to increased β-cell proliferation. Thus, this is a preliminary "G_1/S proteome" database, and for all of the above reasons, it will be critical to build further upon this database, defining and refining the cellular and subcellular localization of key proteins, evaluating their phosphorylation status and kinase activity, and defining their intermolecular interactions and complexes and their functions. Finally, these studies have made no attempt to define the signaling pathways downstream of HGF and placental lactogen, such as phosphatidylinositol 3-kinase, mitogen-activated protein kinase, protein kinase C, JAK2-STAT5, etc., that lead to increases in p21^{cip}. Nor have we attempted here to define the cellular mechanisms responsible for p21^{cip} upregulation: transcription, translation, protein stability, etc. These are important goals of ongoing studies.

In summary, these studies confirm the potent islet growth factor activity of HGF and placental lactogen and suggest that growth factor–driven β-cell replication is likely intrinsically limited by molecular brakes. The studies define a preliminary β-cell G_1/S proteome and reveal a remarkable stability in cell cycle control molecules in response to HGF and placental lactogen. p21^{cip} is a novel, reproducible, readily measurable, highly selective, in vivo marker of cell cycle activation in the murine and human β-cell, and inhibits islet replication in response to growth factors. p21^{cip} likely represents the "molecular brakes" that prevent more exuberant proliferative responses to HGF and placental lactogen overexpression. p21^{cip} may be a therapeutic target for therapeutic manipulation of β-cell replication.

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