Original Article

The Cell Cycle Inhibitory Protein p21^{cip} Is Not Essential for Maintaining β-Cell Cycle Arrest or β-Cell Function In Vivo

Irene Cozar-Castellano, Marcia Haught, and Andrew F. Stewart

p21^{cip1}, a regulatory molecule upstream of the $G_{1/0}$ checkpoint, is increased in β-cells in response to mitogenic stimulation. Whereas p21^{cip1} can variably stimulate or inhibit cell cycle progression, in vitro studies suggest that p21^{cip1} acts as an inhibitor in the pancreatic β-cell. To determine the functional role of p21^{cip1} in vivo, we studied p21-null mice. Surprisingly, islet mass, β-cell replication rates, and function were normal in p21-null mice. We next attempted to drive β-cell replication in p21-null mice by crossing them with rat insulin II promoter–murine PL-1 (islet-targeted placental lactogen transgenic) mice. Even with this added replicative stimulus of PL, p21-null islets showed no additional stimulation. A $G_{1/S}$ proteome scan demonstrated that p21^{cip1} loss was not associated with compensatory increases in other cell cycle inhibitors (pRb, p107, p130, p16, p19, and p27), although mild increases in p57 were apparent. Surprisingly, p18, which had been anticipated to increase, was markedly decreased. In summary, isolated p21^{cip1} loss, as for pRb, p53, p18, and p27 and other inhibitors, results in normal β-cell development and function, either because it is not essential or because its function is subserved or complimented by another protein. These studies underscore marked inhibitory pressure and the complexity and plasticity of inhibitory pathways that restrain β-cell replication. *Diabetes* 55: 3271–3278, 2006

The rate of pancreatic β-cell replication is extremely slow. For example, Finegood et al. (1) report that 3% of rodent β-cells replicate every 24 h. Kushner and colleagues (2) have recently reported that in older mice, this rate is even slower, in the range of 0.07% of β-cells per 24 h. Butler et al. (3) have reported that only 0.04% of adult human β-cells stain for the cell cycle progression marker Ki-67. Using similar techniques, Kassem et al. (4) have suggested that <1% of adult human β-cells stain for Ki-67, although this rate may be higher in embryonic (4–6%) and neonatal (2%) life. Recently, using lineage tracing methods, Dor et al. (5) have demonstrated that although β-cell replication may be slow, it is nonetheless the principal mechanism of self-renewal for β-cells.

β-Cells can be coaxed to replicate more quickly by a variety of maneuvers and physiological stimuli, including subtotal pancreatectomy (5), pregnancy (6), obesity (7), glucose infusion (8), and others. In addition, several growth factors have been reported to induce β-cell replication, exemplified by insulin (9), hepatocyte growth factor (HGF) (10), placental lactogen (PL) (11), prolactin (PRL) (6), and glucagon-like peptide-1 (7–36) (12). Downstream intracellular signaling pathways implicated in β-cell replication include insulin receptor substrate-2 (13), the protein kinase C system (14), the mitogen-activated kinase system (15), the JAK2-STAT5 pathway (16), and the phosphatidylinositol 3-kinase pathway (17). Ultimately, all of these growth factors and signaling pathways must converge on the basic cell cycle replicative machinery.

Interest in basic cell cycle control mechanisms as they relate to the β-cell has increased recently, with the demonstration that cell cycle control molecules, which have been studied intensively in other systems, are also highly relevant to the control of β-cell replication (rev. in 18–20). For example, Georgia and Bhushan (21) and Kushner et al. (22) have demonstrated that cyclin D_2 is essential for normal β-cell replication. Friedrichsen et al. (23) have demonstrated that cyclin D_2 is downstream of the PRL, JAK2-STAT5 signaling pathway. Tsutsui et al. (24) and Rane et al. (25) have shown that cdk-4 is essential for β-cell replication, and Bernal-Mizrachi and colleagues (17) have demonstrated that cdk-4 is regulated by the phosphatidylinositol 3-kinase–protein kinase B (PKB)/Akt pathway (17). We have demonstrated that phosphorylation of the retinoblastoma protein pRb using cdk-4 and cyclin D_1 markedly accelerates rodent and human β-cell replication (26).

Cell cycle progression is negatively controlled by two classes of cell cycle inhibitors known as the INK4s (specifically p15, p16, p18, and p19) and the CIPs (also known as the KIPs or WAFs, specifically p21, p27, and p57) (18). Although these have been studied extensively in cancer and developmental biology, they have only recently come to attention in the β-cell. Recently, we have catalogued the cell cycle proteins that oversee the $G_{1/S}$ transition in the murine islet and have found that each of the seven INKs and CIPs are present (27). This has been supported by Bernal-Mizrachi and colleagues (17) and Karnik et al. (28). Karnik has reported that the increased β-cell replication

From the Division of Endocrinology, The University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Irene Cozar-Castellano, PhD, Division of Endocrinology, BST E-1140, University of Pittsburgh School of Medicine, 200 Lothrop St., Pittsburgh, PA 15213. E-mail: cozari@dom.pitt.edu.

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BrdU, bromodeoxyuridine; HGF, hepatocyte growth factor; MEF, mouse embryonic fibroblast; PKB, protein kinase B; PL, placental lactogen; PRL, prolactin; RIP, rat insulin II promoter.

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characteristic of the multiple endocrine neoplasia type 1 syndrome is associated with the specific loss of p18 and p27 (28). Franklin et al. (29) and Uchida et al. (7) have reported that p27 loss is associated with only a mild increase in β-cell mass under basal conditions in mice, but β-cell replication and mass increase remarkably in response to high-fat feeding or leptin receptor loss (7). p57 loss has been associated with the focal form of human persistent hyperinsulinemic hypoglycemia of infancy (30).

p21 is the third member of the CIP/KIP family. It is generally regarded as a cell cycle inhibitor, inhibiting cdk-2 kinase activity (31,32). However, in some systems and in some settings, it can also serve to accelerate cell cycle progression, because it is required for cdk-4/cdk-6/cyclin D complex assembly and for their nuclear import (31,32). It also has additional functions, including binding to and inhibiting the activity of proliferating cell nuclear antigen, which in turn is required for DNA polymerase activity (33).

In the course of cataloging cell cycle proteins in the murine islets, we found that p21 was profoundly and uniquely upregulated by a number of factors that cause cell cycle progression (27). These included HGF, parathyroid hormone–related protein, PL, PRL, cdk-4, and cyclin D_1. Bernal-Mizrachi and colleagues (17) have recently reported that p21 is markedly induced in the islet by activation of the PKB/Akt pathway. We have also found that p21 is a cytosolic protein under basal conditions but moves into the nuclear compartment with cell cycle activation (27). Finally, in functional terms, we demonstrated that in vitro, murine islets derived from p21-null mice displayed markedly increased DNA synthesis in response to both PRL and HGF (27). These findings are consistent with p21 serving in the β-cell as a cell cycle inhibitor. The goal of the current studies was to determine the function of p21 in the β-cell in vivo.

Here, we report that p21 loss in vivo results in little, if any, β-cell, islet, or metabolic phenotype. Moreover, crossing p21-null mice with rat insulin II promoter (RIP)-mPL1 mice that overexpress PL1 in the pancreatic islet results in no additional β-cell replication beyond that encountered in the RIP-mPL1 mice. Finally, we demonstrate that p21 loss is associated with, and presumably complimented by, subtle but apparently sufficient changes in other INK4 and CIP/KIP family members. These findings support the concept that the β-cell is under great pressure to remain in $G_{1/0}$ arrest and that removal of a single $G_{1/S}$ inhibitor, p21, is unable to relieve this inhibitory pressure.

RESEARCH DESIGN AND METHODS

Mouse models. p21-null mice (34) and controls were purchased from The Jackson Laboratories (Bar Harbor, ME) and are on a SV129/C57Bl6 background. RIP-mPL1 mice are transgenic mice in which the RIP was used to overexpress the murine PL-1 cDNA in the pancreatic β-cell. These mice have been described in detail previously (11) and are on a C57Bl6/CD-1/SJL background. Compound transgenic/knockout mice that overexpress PL1 in the islet, but globally lack p21, were generated by crossing the above two types of mice. The background of these mice is mixed, but all controls (normal, RIP-mPL-1, and p21-null mice) were F2 generation mice derived from the same crosses. Equivalent numbers of males and females were used. Genotyping was done using PCR of tail DNA as described previously (11). All of these studies were approved in advance by and were in compliance with the University of Pittsburgh Institutional Animal Care and Use Committee.

Glucose, insulin, intraperitoneal glucose tolerance test, and insulin tolerance test. Blood glucose, plasma insulin, intraperitoneal glucose tolerance testing, and insulin tolerance testing were all performed exactly as described in detail previously (10,11,26,27).

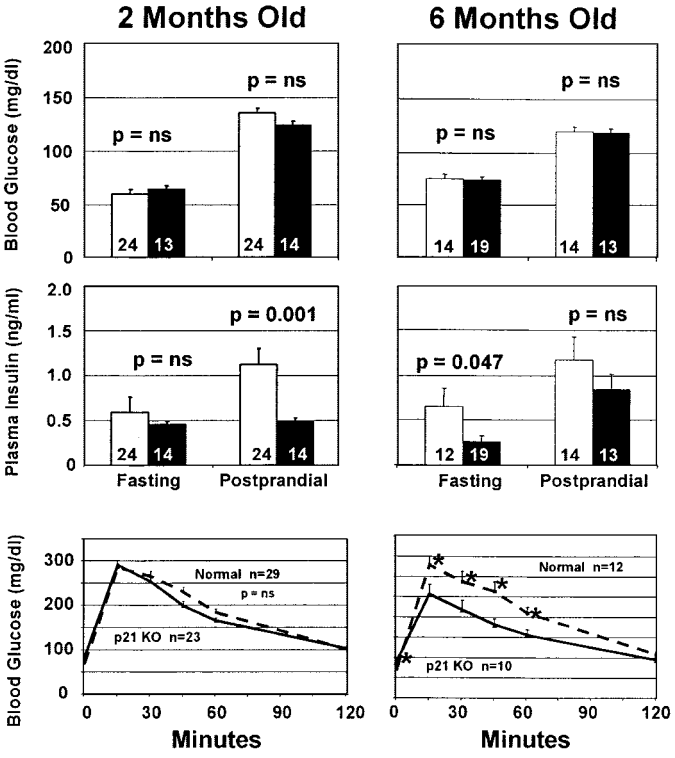

FIG. 1. Glucose homeostasis in p21^{cip1} knockout mice at 2 and 6 months of age. □, normal controls; ■, p21 knockout mice. The numbers within the bars in the *top panels* represent the numbers of animals studied. The *bottom panels* represent intraperitoneal glucose tolerance tests, performed as described in RESEARCH DESIGN AND METHODS. The error bars represent SE. See text for details.

Pancreatic histology, immunohistochemistry, and histomorphometry. Pancreas specimens were removed, fixed in Bouin's solution, embedded, sectioned, and stained precisely as described previously (10,11,26,27). Histomorphometry was performed and quantitated using a computerized quantitative histomorphometric system described previously (10,11,26,27). Proliferation was assessed using bromodeoxyuridine (BrdU) incorporation and cell death/nuclear pyknosis by propidium iodide staining as described previously (10,11,26,27).

Murine islet isolation and $G_{1/S}$ proteome analysis. Islets were isolated from the normal, transgenic, knockout, and compound knockout/transgenic mice as described in detail previously (10,11,26,27). Extracts were prepared and immunoblotted using the antisera and techniques described in detail previously (27), with the exception that the p57 antiserum used previously was replaced by that from GeneTex (San Antonio, TX), and the p15 antiserum was from Neomarkers (Fremont, CA).

Statistics. One-way ANOVA for repeated measures, Student's unpaired two-tailed *t* tests, and area-under-the-curve analyses were used as indicated, and *P* values <0.05 were considered significant.

RESULTS

Glucose homeostasis in the p21-null mouse. Because p21 restrains β-cell proliferation in vitro (27), we anticipated that p21-null mice would display islet hyperplasia and resultant hyperinsulinemia and hypoglycemia. Surprisingly, this proved not to be the case. As can be seen in Fig. 1, *left*, fasting and postprandial blood glucose values were indistinguishable in the normal and p21-null mice at 2 months of age. In contrast to our expectation, insulin levels were actually lower in the p21-null mice than in the normal littermates. Glucose tolerance testing was indistinguishable in the p21 mice and their controls.

Reasoning that it might require a period of months before an islet phenotype was observable in response to p21 loss, we repeated these studies at 6 months of age. As

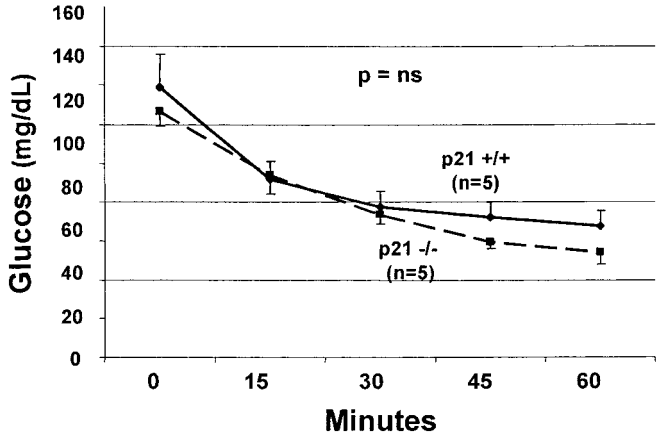

FIG. 2. Insulin tolerance testing in p21 knockout mice compared with normal controls. Animals were 6 months old. The error bars represent SE. There were no statistical differences either using Student's *t* test or area-under-the-curve analysis. See text for details.

can be seen in Fig. 1, *right*, fasting and postprandial glucoses were not different between p21-null and control mice. Once again, and in contrast to expectations, insulin concentrations were actually reduced in the p21-null mice, and these differences were statistically significant. In contrast to the findings at 2 months, by 6 months, p21-null mice were also clearly more glucose tolerant than controls, and this difference was highly statistically significant.

The findings described above suggested that p21-null mice are abnormally sensitive to insulin. To document this, we performed insulin tolerance testing on control and p21-null mice at 6 months of age. Although there appeared to be a trend toward increased insulin sensitivity (Fig. 2), this did not achieve statistical significance.

Islet histology and histomorphometry in the p21-null mouse. We also had anticipated that p21-null mice would display islet hyperplasia. As can be seen in Fig. 3*A*, *top*, this also proved unfounded. Quantitative islet histomorphometry revealed that islet number and islet mass were identical between the p21-null mice and controls, and this was true at 2 months and at 6 months of age. Fig. 3*A*, *bottom*, shows BrdU incorporation in insulin-positive cells, a measure of β-cell replication. As can be seen from Fig. 3*A*, although at both ages, it appeared that there might be a trend toward increased proliferation, there was no statistical difference between normal compared with the p21-null mice at either age. Fig. 3*B*, *top*, illustrates the BrdU incorporation in the two genotypes. Figure 3*B*, *bottom*, also demonstrates that cell death rates, as assessed by nuclear pyknosis using propidium iodide staining, did not appear different in p21-null mice versus controls. Quantitation of cell death rates using propidium iodide staining of pyknotic DNA in β-cells revealed that β-cell death rates (means ± SE) were 0.53 ± 0.23% in the normal controls versus 0.38 ± 0.06% in the p21KO animals. These differences were not significant.

Generation of RIP-mPL1 × p21-null mice. Reasoning that the addition of a potent β-cell mitogen in the setting of p21 loss might unmask a latent propensity toward increased β-cell replication, we generated mice in which p21 was absent and in which the potent β-cell mitogen, PL1, was simultaneously overexpressed in the β-cell. Figure 4*A* displays p21 and PL1 immunoblots on extracts of islets from each of these mice and confirms that p21 is absent in the islets of p21-null mice and that PL1 is overexpressed in RIP-mPL1 mice.

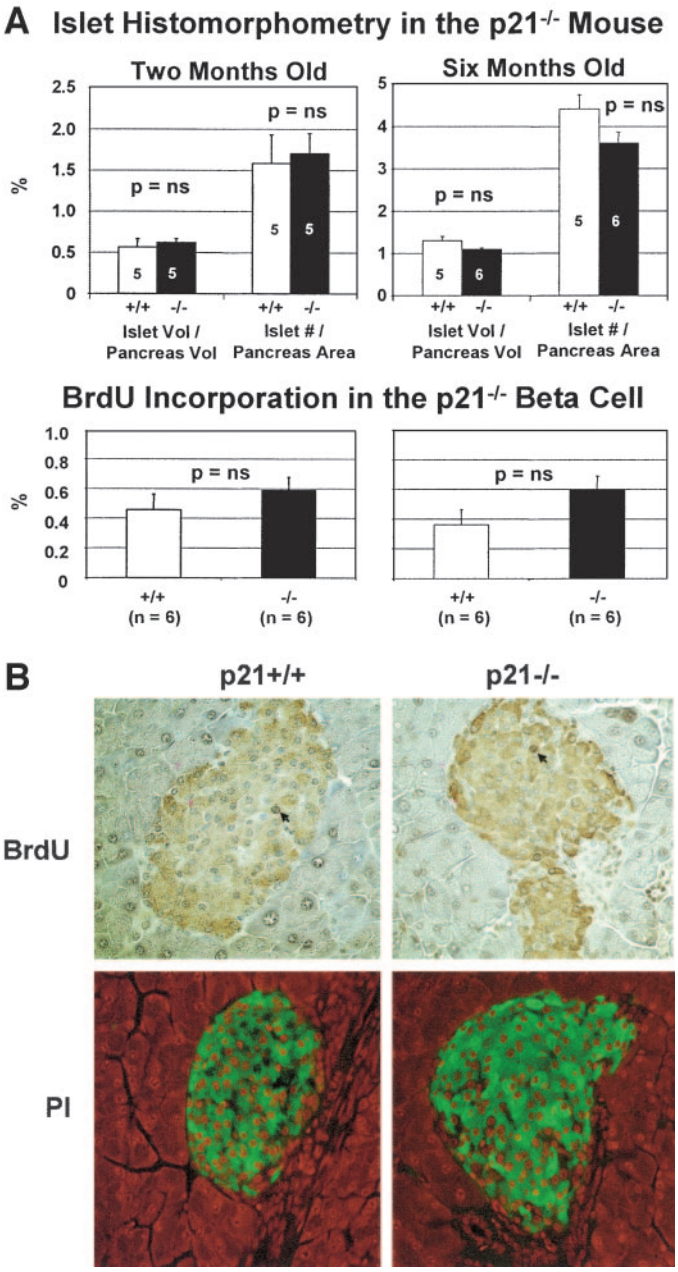

FIG. 3. Islet histomorphometry and β-cell proliferation and death rates. *A*: The *top panels* display islet histomorphometry (islet mass as a function of total pancreatic area, and islet number per square millimeter of islet area). The *bottom panels* represent β-cell replication rates as assessed using BrdU labeling. For the BrdU experiments, one pancreas slide was counted for each of five to six animals, and 500–1,000 β-cells were counted per slide. Statistical analysis was performed using Student's two-tailed unpaired *t* test. The numbers within the bars represent the numbers of animals studied. *B*: Representative examples of BrdU staining in normal controls and p21 knockout mice (*top*) and comparable and very low levels of apoptosis as assessed by nuclear pyknosis in both types of islets (*bottom*). In the *bottom panels*, red represents propidium iodide (PI), and green represents insulin staining. For the propidium iodide quantitation, three slides from three different animals were counted, and a minimum of 500–1,000 β-cells were counted from each animal. See text for details.

Glucose homeostasis in RIP-mPL1 × p21-null mice. We had previously demonstrated that RIP-mPL1 mice display both fasting and postprandial hypoglycemia compared with control littermates on a C57Bl6/CD-1/SJL background (11). Figure 4*B* demonstrates that this remains true on the mixed CD-1/C57Bl6/SV129 background generated in

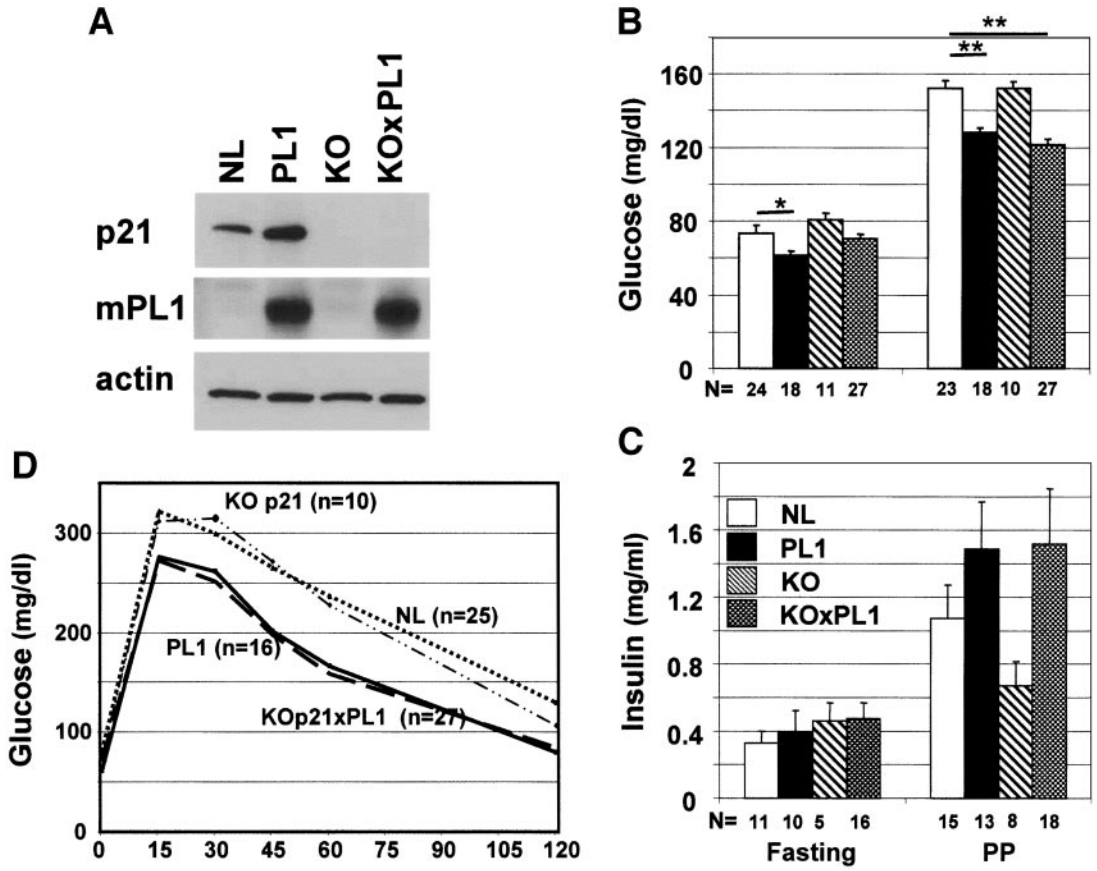

FIG. 4. Metabolic effects of overexpression of mPL1 in the p21 knockout islet. *A*: Representative Western blots demonstrating that p21 is absent in p21 knockout islets, and mPL1 is overexpressed in RIP-mPL1 islets. *B*: Fasting and postprandial glucose values in the four genotypes. □, normal control littermates; ■, RIP-mPL1 mice; ▧, p21 knockout mice; ▨, p21-deficient mPL1-overexpressing mice. The numbers below the bars represent the numbers of animals studied. The error bars represent SE. *C*: Insulin values in the same animals. *D*: Intraperitoneal glucose tolerance testing in the four groups.

the current studies. The animals used were 2–3 months old. However, as can be seen from Fig. 4*B*, p21 loss alone on a mixed CD-1/C57Bl6/SV129 background, as was seen in Fig. 1 on the SV129/C57Bl6 background, again did not cause hypoglycemia nor did it exacerbate the hypoglycemia of the RIP-mPL1 mice. Figure 4*C* shows that as previously reported on the C57Bl6/CD-1/SJL background (11), RIP-mPL1 mice on the new mixed background also display inappropriate hyperinsulinemia in the face of relative hypoglycemia. However, p21 loss has little additional effect on circulating insulin concentrations: p21 loss, as in Fig. 1, once again is associated with a reduction, instead of the anticipated increase, in circulating insulin. Finally, Fig. 4*D* demonstrates that whereas RIP-mPL1 mice display enhanced glucose tolerance, p21 loss confers no additional effect.

Quantitative islet histomorphometry and β-cell proliferation in the RIP-mPL1 × p21-null mouse. Figure 5 shows representative insulin-stained sections of whole pancreata from the RIP-mPL1 × p21-null mice and the three controls. As can be seen from the Fig. 5 and concordant with results shown in Fig. 3*A* on a C57Bl6/SV129 background, p21 loss on this mixed C57Bl6/SV129/CD-1 background results in no increase in islet mass, size, or number. As reported previously on the C57Bl6/SJL/CD-1 background (11), the RIP-mPL1 mice on this new mixed background once again display increases in islet number, size, and overall mass. However, removal of p21 resulted in no additional increment in islet number, size, or overall mass. These observations are confirmed quantitatively and statistically in Fig. 6*A*; p21 loss has no additional effect on islet number or overall β-cell mass.

β-Cell proliferation was assessed in each of the four genotypes and is displayed in Fig. 6*B*. As reported previously on the C57Bl6/CD-1/SJL background (11), PL1 overexpression approximately doubles β-cell replication rates. In contrast to our hypothesis and in contrast to events in isolated islets (27), the loss of p21 in the setting of PL overexpression in vivo resulted in no additional increment in β-cell replication rates. As observed on the SV129/C57Bl6 background (Fig. 3*A*, *bottom*), loss of p21 on this mixed background may have been associated with a trend toward increased β-cell replication, although once again, this was nonsignificant.

Control of the $G_{1/S}$ checkpoint in the islets of p21-null and RIP-mPL-1 × p21-null mice. Because β-cell replication rates did not further increase in RIP-mPL1 islets lacking p21 compared with RIP-mPL1 islets, we wondered whether additional cell cycle inhibitory molecules might compensate for p21 loss. We therefore comprehensively surveyed the molecules that control the $G_{1/S}$ checkpoint in islets derived from each of the four genotypes. As can be seen in Fig. 7*A*, there were no apparent differences among the four genotypes in the three pocket proteins, pRb, p107, or p130.

Figure 7*B* reveals no differences in cyclins D_1 or D_3 nor in cdk-4. Cyclin D_2 appears to be increased in response to PL1 overexpression, although this difference did not

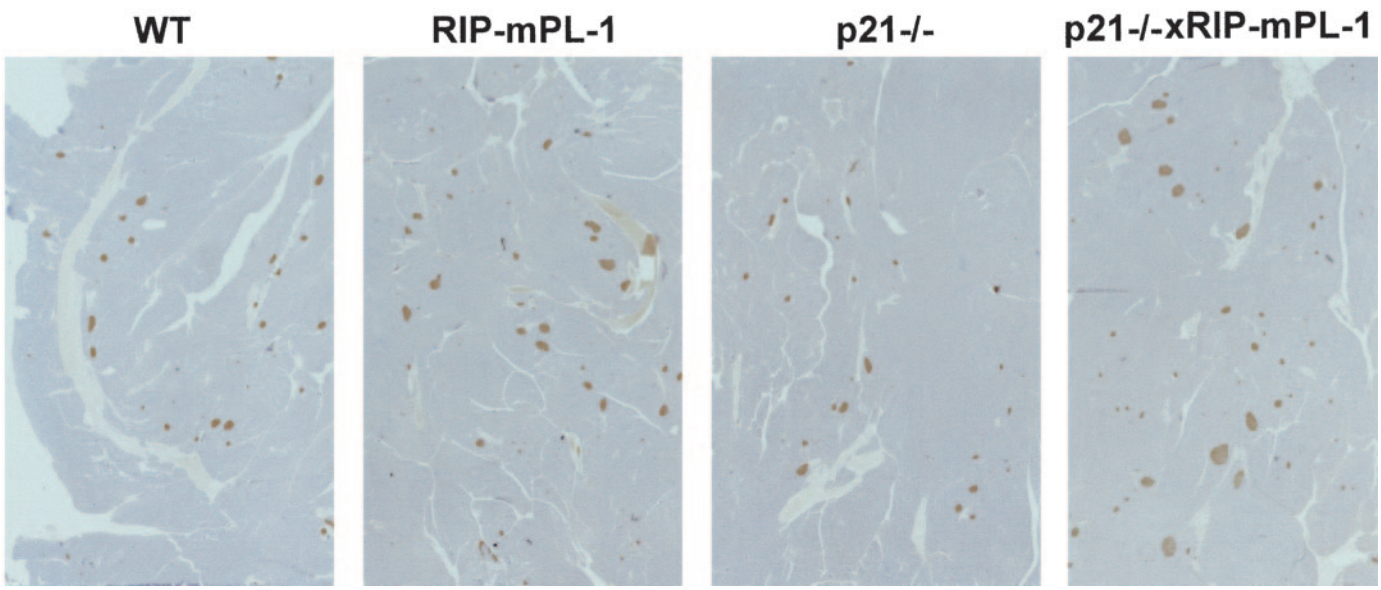

FIG. 5. Islet immunohistochemistry in the four genotypes. Brown represents insulin, and gray-blue represents the exocrine pancreas.

achieve statistical significance as assessed using densitometric analysis. As we have reported previously (27), cdk-6 is not present in the murine islet. Cyclin A, cyclin E, and cdk-2 are not altered among the four genotypes (Fig. 7*C*).

Figure 7*D* and *E* display a number of remarkable findings among the members of the INK4 and CIP/KIP families. First, as in Fig. 4, the p21-null mouse islet contains no measurable p21, as expected. Second, as can be seen in Fig. 4, p21 loss is associated with marked reductions in p18. Thus, p21-null islets are actually functionally null for two cell cycle inhibitors, p18 and p21, yet β-cell proliferation rates are not measurably increased. Third, PL1 overexpression markedly upregulates p18 in the p21-null islets, restoring it to normal levels. Fourth, p57, the third CIP/KIP family member and presumed β-cell inhibitor of replication (30), consistently appears to be increased by a factor of 20% in p21-null islets, as assessed in four separate experiments, although this change did not reach statistical significance by quantitative densitometry of scanned immunoblots. Fifth, efforts to accelerate β-cell replication by PL1 overexpression were associated with a substantial reduction in p57. And sixth, p27, a p21 homolog and family member that appears to be so sensitive to increases in fat intake (7) and so critical to restraining β-cell replication in the multiple endocrine neoplasia type 1 syndrome (28) and in leptin receptor deficiency (7), is completely uninfluenced by p21 loss or PL1 overexpression.

DISCUSSION

These studies make the surprising observation that although p21 may be upregulated in a variety of situations associated with β-cell replication (17,27) and although it may be a potent inhibitor of β-cell cycle progression in vitro (27), it is nonessential for restraint of β-cell replication in vivo, even in response to a potent β-cell mitogen such as PL1. In addition, although p21 and p27 are members of the same family and both are expressed in the β-cell, they seem to respond to very different mitogenic and metabolic signals. Finally, whereas p21 loss is associated with no apparent compensation by p27, the third member of the CIP/KIP family, p57, is consistently mildly upregulated in the islet by p21 loss.

In a more general sense, these studies are particularly instructive. First, comprehensive analysis of the molecules that regulate the $G_{1/S}$ transition reveal a remarkable complexity of cell cycle control in the islet (Fig. 8). Second, they reveal a remarkable plasticity, with removal of one inhibitor apparently being fully compensated by replacement in protein/and or function by other inhibitors. Third, they underscore the importance of inhibitory control in maintaining β-cell cycle arrest. Fourth, they reveal the complexity of responses to cell cycle activation by growth factors such as PL1. And fifth, these observations suggest specific cell cycle inhibitory molecules as key targets for further study.

One important theme that arises from these studies is the complexity of cell cycle control and the complexity of the response of the β-cell to mitogens. Thus, although it is tempting to think of growth factors as driving cell cycle progression in a monovalent or single pathway kind of mechanism, it is more likely that growth factors can and do influence cell cycle progression in many ways. For example, PL and PRL likely accelerate cell cycle progression by increases in cyclin D_2 (23), as well as by decreases in p57 (here) and other CIP/KIPs-INKs. Similarly, as recently demonstrated by Bernal-Mizrachi and colleagues (17), the PKB/Akt pathway leads to increases in multiple cell cycle proteins (cyclins D_1 and D_2) and reductions in others (p57), all of which have the net effect of increasing cdk-4 activity and relieving cell cycle arrest. At the same time, we (27) and Bernal-Mizrachi and colleagues (17) have shown that other inhibitory cell cycle proteins, such as p21, are activated by growth factors and by PKB/Akt in what would seem to be an attempt to slow cell cycle progression. The net effect of these changes is cautious β-cell replication.

Another principle derived from these studies and those of others is the importance, and perhaps dominance, of inhibitory tone of cell cycle control in the β-cell. As discussed above, β-cells have long been characterized as being unable to replicate. Now it is apparent that every

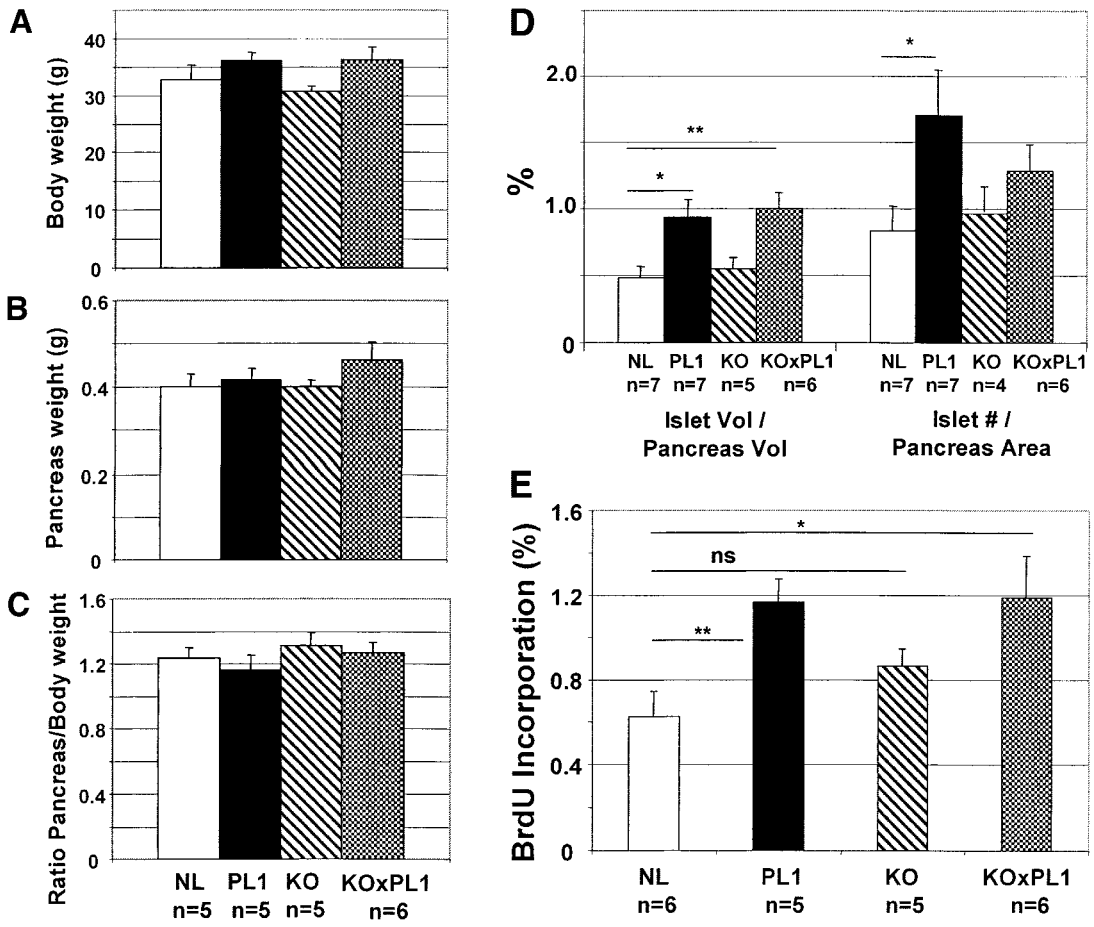

FIG. 6. Islet histomorphometry and proliferation rates in the four genotypes. *A*: Body weight in the four genotypes. *B*: pancreas weights in the four genotypes. *C*: Ratio of pancreas weight to body weight. There were no differences among the four genotypes with respect to body weight or pancreas size. *D*: Islet number and islet area in the four genotypes. *E*: BrdU proliferation rates in the four genotypes. □, normal control littermates; ■, RIP-mPL1 mice; ▧, p21 knockout mice; ▩, the p21-deficient mPL11-overexpressing mice. The numbers below the bars represent the numbers of animals studied. For the BrdU experiments, one pancreas slide was counted for each of five to six animals, and 500–1,000 β-cells were counted per slide. Statistical analysis was performed using Student's two-tailed unpaired *t* test. The error bars represent SE. See text for details.

single $G_{1/S}$ inhibitory molecule is present in the β-cell. Moreover, we have learned that removal of a single inhibitor (specific examples are pRb [35–37], p53 [35,36], p18 [29], p27 [7,29], and now p21) has little effect on the restraint of cell cycle control. In contrast, removal of two or more specific cell cycle inhibitors (examples are pRb in combination with p53 [35,36], p18 in combination with p27 [29], or removal of menin, which results in combined p18 and p27 loss [28]) regularly do lead to loss of β-cell $G_{0/1}$ arrest. These considerations prompt the questions as to which specific combinations of cell cycle inhibitors are sufficient to release β-cells from cell cycle arrest. This should be an important consideration for further studies.

One obvious question that arises from these studies is if p21 is a β-cell cycle inhibitor, then can the INK4s compensate and replace the missing p21 function? As shown in Fig. 7 and deduced from Fig. 8, p18 is an unlikely candidate because p21 loss is accompanied by marked reductions in p18. p21 function, in theory, could be replaced by p15, p16, or p19. p15, p16, and p19 knockout mice have been prepared and are viable. These mice are susceptible to a variety of tumors, but glucose homeostasis and islet mass and β-cell proliferation have not been reported (38,39). We believe that these are attractive candidates for further study, either alone or in combination with p21 loss.

A second obvious question is whether the remaining CIP/KIPs, p27 or p57, could compensate for p21 loss. Regarding p27, although there is no apparent change in the absolute levels of p27 in islets, it remains formally possible that p27 could compensate, for example by shifting from a predominantly cytosolic location to a nuclear location, and thereby compensate for p21 loss. This kind of potential compensation can best be explored using mouse genetic models of combined p21 plus p27 loss. p21 × p27 double knockout mice have been prepared and are viable (40,41) and display marked obesity and insulin resistance, but no islet or insulin secretory phenotype has been described. These studies may also suggest that p21 is not required for assembly of cdk-4/cyclin D complexes.

Regarding p57, Kassem et al. (30) have demonstrated that p57 is lost in persistent hyperinsulinemic hypoglycemia of infancy and may contribute to the increase in β-cell mass in these children. Thus, the increase in p57 in the islets of p21-null mice would be expected to restrain proliferation, and its decline with the addition of PL1 would support the notion that p57 may act as an inhibitor of β-cell proliferation, and PL1 could act, at least in part, by reducing p57 levels. Again, p57-null mice have been prepared but display embryonic lethality. Once again, no islet phenotype has been reported in p57-null mice (42).

A third obvious question is if p21 loss is associated with increased responsiveness to growth factors in vitro (27),

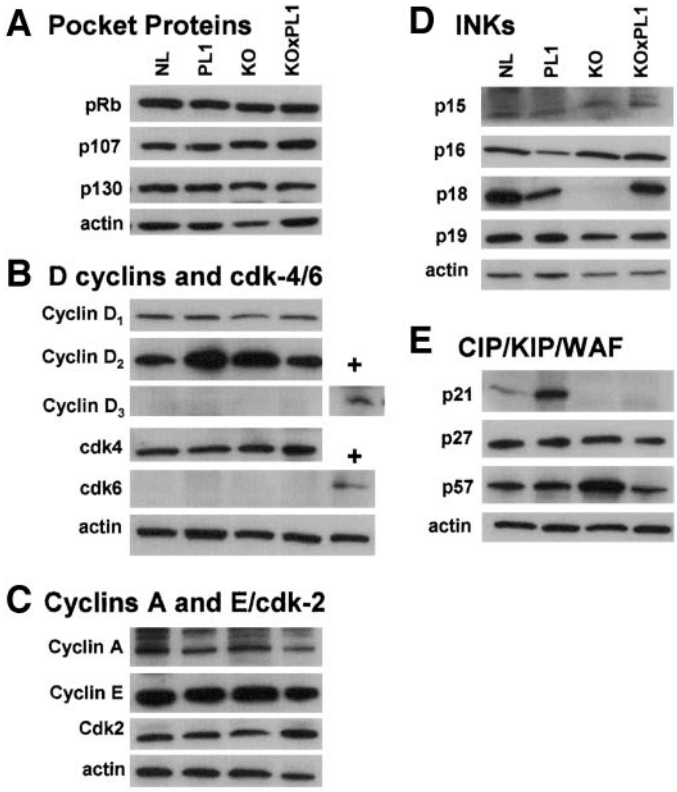

FIG. 7. *A–E*: Immunoblotting of $G_{1/S}$ checkpoint control proteins. Each Western blot is representative of three to six immunoblots. Actin immunoblotting was performed for each blot, but only representative actin immunoblots are shown. +, positive control for cyclin D3 and for cdk-6, which was mouse intestine.

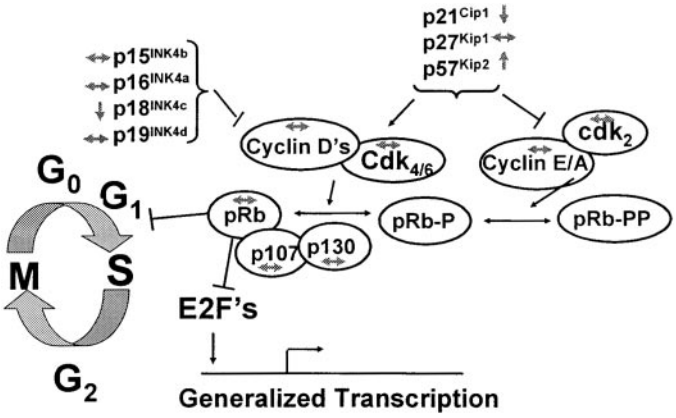

FIG. 8. A schematic demonstrating the responses of $G_{1/S}$ checkpoint control proteins to loss of p21. See text for details.

why did this not occur in vivo? The most likely explanation for these findings is that in a brief in vitro experiment in which growth factors are added for 48 h (27), there is inadequate time for other members of the CIP/KIP-INK families (or possibly other as yet unidentified proteins) to compensate and restrain proliferation. In contrast, in germline (lifelong) p21 loss in vivo, even in the setting of (lifelong) PL1 overexpression, there is more than ample time for one or more inhibitory proteins to replace the lost p21 function. This kind of time-dependent compensation has been demonstrated beautifully by Sage et al. (43) for pRb in mouse embryonic fibroblasts (MEFs): whereas germline (i.e., chronic) Rb loss per se has no adverse effects on maintaining cell cycle arrest in MEFs, "acute" removal of pRb from $pRb^{lox/lox}$ MEFs using infection with a Cre recombinase-expressing adenovirus resulted in prompt cell cycle acceleration and failure of $G_{1/0}$ arrest. Cell cycle control was regained over ensuing days with the recruitment/replacement of other cell cycle inhibitors.

These studies together with our prior studies (27) and those of Bernal-Mizrachi and colleagues (17) highlight a difference between p21 and p27, two putatively similar members of the CIP/KIP family. p21 appears to be reproducibly increased by growth factors and agents that drive mitogenesis. These include growth factors (HGF, PL, and parathyroid hormone–related protein) (27), mitogenic signaling molecules (PKB/AKt) (17), and cell cycle progression molecules, such as cdk-4 and cyclin D_1 (26). In contrast to the increase in p21, these conditions are not associated with changes in p27 (17,27). Conversely, high-fat feeding in normal mice (7) or Zucker fatty rats (44) and leptin receptor loss (7), all "metabolic" stresses to the islet, are associated with increments in p27 without changes in p21. Moreover, loss of the menin protein is associated with losses of p18 and p27 without changes in p21 (28). Collectively, these observations might suggest that whereas both p21 and p27 are important for β-cell cycle control and may compliment for each others' loss, they subserve fundamentally different functions, with p21 responding to more mitogenic stimuli and p27 responding to more metabolic stimuli. Documenting whether such a difference is real will require additional studies.

One final curious and thus far unexplained observation is the finding of euglycemia and improved glucose tolerance in the p21-null mice in the setting of reduced circulating insulin levels. We were not able to document enhanced insulin sensitivity in our mice using insulin tolerance testing, but speculate that such increased peripheral insulin sensitivity would be present if sought using more sensitive and rigorous techniques such as the hyperglycemic glucose clamps. Interestingly, and in contrast to our results, a report by Naaz et al. (41) reported that p21-null mice display mild increases in fat stores, and this was associated with insulin resistance. In contrast, adiposity was not a part of the original description of p21-null mice. These findings may suggest that global p21 loss in all tissues, including, for example, liver, fat, and skeletal muscle, alters responsiveness to insulin and suggest that β-cell–specific disruption of the p21 gene may be of interest to remove confounding effects on the β-cell of global p21 deficiency. Collectively, because these studies were presumably performed on mice of different genetic backgrounds, these observations may suggest that p21 may affect insulin sensitivity in a strain-dependent manner.

In summary, these studies demonstrate that solitary loss of a single cell cycle inhibitor, in this instance p21, is unable to free β-cells from $G_{1/0}$ arrest even under the challenge of mitogenic stimulation by PL. This failure of the loss of a single cell cycle inhibitor to relieve $G_{1/0}$ repression has been demonstrated previously for pRb, p18, p27, and p53 and is now extended to p21. In marked contrast, loss of two or more specific cell cycle inhibitors, for example pRb plus p53 or p18 plus p27, results in robust β-cell cycle proliferation and increments in islet mass. From a teleological standpoint, one must wonder why the β-cell is under such impressive and duplicative restraint. From a therapeutic standpoint, these studies make it clear that multiple cell cycle inhibitors need to be inactivated or removed to permit cell cycle progression in the pancreatic β-cell.

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