Original Article

Akt Induces β-Cell Proliferation by Regulating Cyclin D1, Cyclin D2, and p21 Levels and Cyclin-Dependent Kinase-4 Activity

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Proliferation is the major component for maintenance of β-cell mass in adult animals. Activation of phosphoinositide 3-kinase/Akt-kinase pathway is a critical regulator of β-cell mass. Pancreatic β-cell overexpression of constitutively active Akt in mice (*caAkt^{Tg}*) resulted in marked expansion of β-cell mass by increase in β-cell proliferation and size. The current studies provide new insights into the molecular mechanisms involved in β-cell proliferation by Akt. Proliferation of β-cells in *caAkt^{Tg}* was associated with increased cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 (cdk4) activity. To determine the role of cdk4 in β-cell proliferation induced by Akt, we generated *caAkt^{Tg}* mice that were homozygous, heterozygous, or nullizygous for *cdk4*. The results of these studies showed that deletion of one *cdk4* allele significantly reduced β-cell expansion in *caAkt^{Tg}* mice by decreased proliferation. *CaAkt^{Tg}* mice deficient in cdk4 developed β-cell failure and diabetes. These experiments suggest that Akt induces β-cell proliferation in a cdk4-dependent manner by regulation of cyclin D1, cyclin D2, and p21 levels. These data also indicate that alteration in levels of these cell cycle components could affect the maintenance of β-cell mass in basal states and the adaptation of β-cells to pathological states resulting in diabetes. *Diabetes* 55:318–325, 2006

The defects that result in diabetes are diverse, but pancreatic β-cell failure is a major component of this disease (1,2). Pancreatic β-cell mass results from a dynamic balance of neogenesis, proliferation, cell size, and apoptosis (3). Recent data using cell lineage tracing experiments suggest that replication is a major component involved in maintenance of β-cell mass in adult animals (4). The molecular mechanisms involved in regulation of β-cell mass are unknown. Several lines of evidence suggest that activation of phosphoinositide 3-kinase (PI 3-kinase) signaling pathway plays critical roles in regulation of β-cell mass and function (5–10).

One of the major targets of PI 3-kinase is the serine-threonine kinase Akt, the cellular homolog of the viral oncogene v-Akt (11). Akt is a convergent point of several growth signals induced by growth factors, insulin, and gut incretins. Transgenic mice overexpressing a constitutively active form of Akt in islet β-cells (*caAkt^{Tg}*) have provided further evidence for the role of this kinase in islet physiology (12,13). These mice exhibit striking increases in β-cell mass, proliferation, neogenesis, and cell size. Akt regulates cell cycle progression by alterations in levels and subcellular distribution of components of the cell cycle machinery (14). The molecules and the mechanisms involved in the regulation of cell cycle by growth signals mediated by Akt in pancreatic β-cells are ill defined.

Insight into the mechanisms involved in the regulation of cell cycle in β-cells have been provided by experiments in cyclin-dependent kinase-4 (cdk4)-deficient mice. These mice exhibited growth retardation and diabetes due to a reduction in β-cell mass during the first 6 weeks of age (15,16). In contrast, mice expressing a mutant cdk4 that cannot bind the inhibitors of cdk4 (INK4s) of the cell cycle displayed pancreatic hyperplasia due to abnormal proliferation of β-cells (15). Moreover, overexpression of cdk4 by adenoviral transfer in human islets increases proliferation in vitro (17). The role for cyclins D2 and D1 in β-cells was demonstrated by reduced β-cell mass and hyperglycemia in mice deficient for these G_1 components (18,19). These results establish the cyclin-cdk4 complex as an essential regulator of islet β-cell proliferation and are consistent with the hypothesis that regulation of cell cycle progression is essential for normal β-cell mass and function in adult mice.

The current studies attempt to elucidate the mechanisms involved in regulation of β-cell mass by the PI 3-kinase/Akt kinase signaling pathway. To study the molecular mechanisms involved in the proliferative response of β-cells induced by Akt, we have assessed expression levels of components that regulate cdk4 activity in *caAkt^{Tg}* mice and generated *caAkt^{Tg}* mice lacking one or two *cdk4* alleles. Moreover, the results of these experiments suggest that Akt induces β-cell proliferation in a cdk4-dependent manner and that increased cyclin D1, cyclin D2, and p21 levels are associated with proliferative responses induced by activation of the PI 3-kinase/Akt pathway in β-cells.

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BrdU, bromodeoxyuridine; cdk4, cyclin-dependent kinase-4; FOXO1, forkhead box factor 1; GSK, glycogen synthase kinase; INK4, inhibitor of cdk4; PI 3-kinase, phosphoinositide 3-kinase.

RESEARCH DESIGN AND METHODS

Generation of *caAkt^{Tg}/cdk4* intercrosses. Mice overexpressing constitutively active Akt1 in β-cells (*caAkt^{Tg}*) are in C57/B6 background and have been described previously (13). The *cdk4*-deficient mice had been described elsewhere and are in the CJ-7 background (16). Littermates from the cross between *caAkt^{Tg}/cdk4$^{+/-}$* and *cdk4$^{+/-}$* mice were used for these experiments. Control and experimental animals were on comparably mixed background. Experiments were performed in males, but females exhibited a similar phenotype. All procedures were performed in accordance with the Washington University Animal Studies Committee.

Western blotting and cdk4 activity. The following antibodies were used: cyclin D1, p27, p57, phospho-forkhead box factor 1 (phospho-Foxo1) (Ser256), glycogen synthase kinase (GSK)3β, phospho-GSK3 (Ser9), and phospho-Rb (Ser780) (Cell Signaling, Beverly, MA). Antibodies for p21 and p18 were from Santa Cruz Biotechnology (Santa Cruz, CA), and cyclin D2 and cdk4 were obtained from Abcam (Cambridge, MA). Protein obtained from islets (50 μg) (~100 islets) was used for each experiment. Membranes containing islet lysates were incubated for 24 h with primary antibodies at the dilutions recommended by the manufacturer. Immunoblotting experiments were performed at least three times in duplicate. Scanning densitometry of protein bands was determined by pixel intensity using NIH Image J software (v1.31 freely available at http://rsb.info.nih. gov/ij/index.html) and normalized against that of actin.

Cdk4 kinase assays were carried out based on protocols described previously (20). Pooled islets from five mice were lysed in lysis buffer, and protein concentration was determined using DC Protein Assay kit (Bio-Rad, Hercules, CA). For each sample, 300 μg total protein was immunoprecipitated using 1 μg anti-cdk4 (C-22; Santa Cruz Biotechnology) and 50 μl protein G Sepharose beads (Sigma Aldrich, St. Louis, MO). The final kinase reaction was carried out in 50 mmol/l HEPES, pH 7.5, 10 mmol/l MgCl2, 1 mmol/l dithiothreitol, 2.5 mmol/l EGTA, 10 mmol/l glycerophosphate, 0.1 mmol/l Na_2VO_4, 1 mmol/l NaF, 5 μmol/l ATP, 6 mCi per reaction of [γ-^{32}P]ATP (Amersham Biosciences), and GST-Rb 769-921 (Santa Cruz Biotechnology). Samples were incubated at 30°C for 30 min and separated by polyacrylamide gel electrophoresis. The amount of ^{32}P-labeled GST-Rb was evaluated by autoradiography and quantified using PhosphorImager and ImageQuant (Molecular Dynamics) analysis.

Quantitative RT-PCR. Total RNA was purified from at least four mice using RNeasy (Qiagen, Valencia, CA). cDNA was synthesized using random hexamers and reverse transcribed with Superscript II (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Applied Biosystems, Foster City, CA) for insulin and amylase, and *Taq*-man reagent was used for other genes as described using the ABI 7000 sequence detection system (Applied Biosystems) (21). To detect the degree of exocrine contamination, we determined the abundance of amylase and insulin in islet samples as previously described (22). Islet preparations from transgenic and nontransgenic mice with a similar degree of contamination were used for the experiments. For comparison of transcript levels between samples, relative quantification of steady-state mRNA levels of a gene across multiple samples was obtained. A standard curve of cycle thresholds for serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of 18S ribosomal RNA, which were obtained from a similar standard curve. All PCRs were performed in triplicate. SE of the quantity of transcript normalized to the amount of 18S ribosomal RNA was calculated from a formula with consideration of error propagation. When gene expression levels of two conditions were compared, the ratio was expressed with SE calculated from the same formula. Specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis of PCR products. Sequences of primers used in this study are as follows. Mouse insulin: forward, 5′-CCACA AAGATGCTGTTTGAC-3′, and reverse, 5′-CCCTTAGTGACCAGCTATAA-3′; and amylase: forward, 5′-TTGCCAAGGAATGTGAGCGAT-3′, and reverse, 5′-CCAAGGTCTTGATGGGTTATGAA-3′. Primers from cell cycle components were purchased from Applied Biosystems with reference numbers p21 (Mm 00432448), p27 (Mm 00438167), p57 (Mm 00438170), Cdk4 (Mm 01624002), Ccnd1 (Mm 00432359), Ccnd2 (Mm 00438071), Ccnd3 (Mm 01273583), p15 (Mm 00483241), p16 (Mm 00494449), p18 (Mm 00483243), and p19 (Mm 00486943).

Immunohistochemistry and immunofluorescence. Pancreatic tissue was fixed overnight in 3.7% formalin solution and embedded in paraffin using standard techniques. Immunostaining for insulin was done as described previously (13). Immunofluorescence for phospho-GSK3β (Ser9) (Cell Signaling) and insulin was performed as described previously (13).

Islet morphometry and proliferation analysis. Pancreata obtained from 8- to 10-week-old mice were used for morphometry and immunohistochemistry. The β-cell mass was calculated by point counting morphometry from five insulin-stained sections (5 μm) separated by 200 μm using the BQ Classic98 MR software package (Bioquant, Nashville, TN) as described previously (13). Pancreata from neonates were obtained during the first 12 h of life. Proliferation was assessed in insulin- and bromodeoxyuridine (BrdU)-stained sections from mice injected with BrdU for 6 h as previously described (13). At least 900 insulin-stained cells were counted for each animal.

Metabolic studies. Fasting blood samples were obtained after overnight fasting from the tail vein. Fed glucose measurements were performed early in the morning between 9:00 and 10:00 A.M. All of the metabolic studies were performed in male mice. Glucose was measured on whole blood using AccuChek II glucometer (Roche Diagnostics, Indianapolis, IN). Plasma insulin levels were determined using Rat insulin ELISA kit (Crystal Chem, Chicago, IL). Glucose tolerance tests were performed in 12-h–fasted animals by injecting glucose (2 mg/g) intraperitoneally as described previously (13).

Islet isolation. Islet isolation was accomplished by collagenase digestion as described previously (13). After isolation, islets were hand picked and lysed in lysis buffer (Cell Signaling).

Statistical analysis. All values are expressed as means ± SE. Paired Student's *t* test was used for all other comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Signaling pathways involved in regulation of proliferation in *caAkt^{Tg}* mice. To begin to elucidate the mechanisms implicated in regulation of β-cell proliferation by Akt, we focused on downstream signaling pathways associated with progression through G_1 phase and entry into S phase. Inhibition of GSK3β activity by phosphorylation in Ser9 is one of the potential mechanisms for cell cycle regulation by Akt. To assess the status of GSK3 activity in vivo, we performed immunofluorescence staining using an anti-phosho Ser9 antibody. Low levels of phospho-Ser9 GSK3β staining were observed in nontransgenic *cdk4$^{+/+}$* mice (Fig. 1*A*). In contrast, islets from *caAkt^{Tg}* mice exhibited striking immunostaining for phospho-Ser9 GSK3β (Fig. 1*A*). GSK3β phosphorylation was increased (2.3 ± 0.1, $P < 0.05$) in islet lysates from *caAkt^{Tg}/cdk4$^{+/+}$*, confirming the results obtained by immunofluorescence staining (Fig. 1*B*). GSK3β can modulate cell cycle by regulating cyclin D levels. Cyclins D1 and D2 but not D3 protein levels were markedly increased in *caAkt^{Tg}/cdk4$^{+/+}$* mice (4.1 ± 1 and 1.9 ± 0.1, respectively, $P < 0.05$) (Fig. 1*C–E*). Levels for Cdk4 were not different between *caAkt^{Tg}/cdk4$^{+/+}$* and nontransgenic *cdk4$^{+/+}$* (Fig. 1*F*). These results suggest that Akt regulates cell cycle in β-cells at least in part by regulation of GSK3β activity and cyclin D1 and D2 levels.

The FOXO1 is inactivated by Akt through direct phosphorylation, resulting in translocation to the cytoplasm and degradation by proteosomes (23,24). Akt-induced inactivation of Foxo1 results in transcriptional inhibition of the p27Kip1 promoter. To determine whether Akt regulates β-cell proliferation by phosphorylation of Foxo1 and p27Kip levels, we performed immunoblotting using islet lysates from *caAkt^{Tg}* and nontransgenic *cdk4$^{+/+}$*. As shown in Fig. 2*A*, a significant increase in Foxo1 phosphorylation at Ser256 specifically seen in slower migrating bands was obtained in islet lysates from *caAkt^{Tg}/cdk4$^{+/+}$* mice. Interestingly, p27Kip levels were not different between *caAkt^{Tg}/cdk4$^{+/+}$* and nontransgenic *cdk4$^{+/+}$* (Fig. 2*B*).

Another mechanism involved in induction of proliferation by Akt is the regulation of p21, p57, and the INK family of inhibitors. Akt can regulate p21 levels directly by phosphorylation of Ser146 or indirectly by inhibition of p21 ubiquination mediated by GSK3β phosphorylation of Thr57 (25–29). Levels of p21 were significantly increased

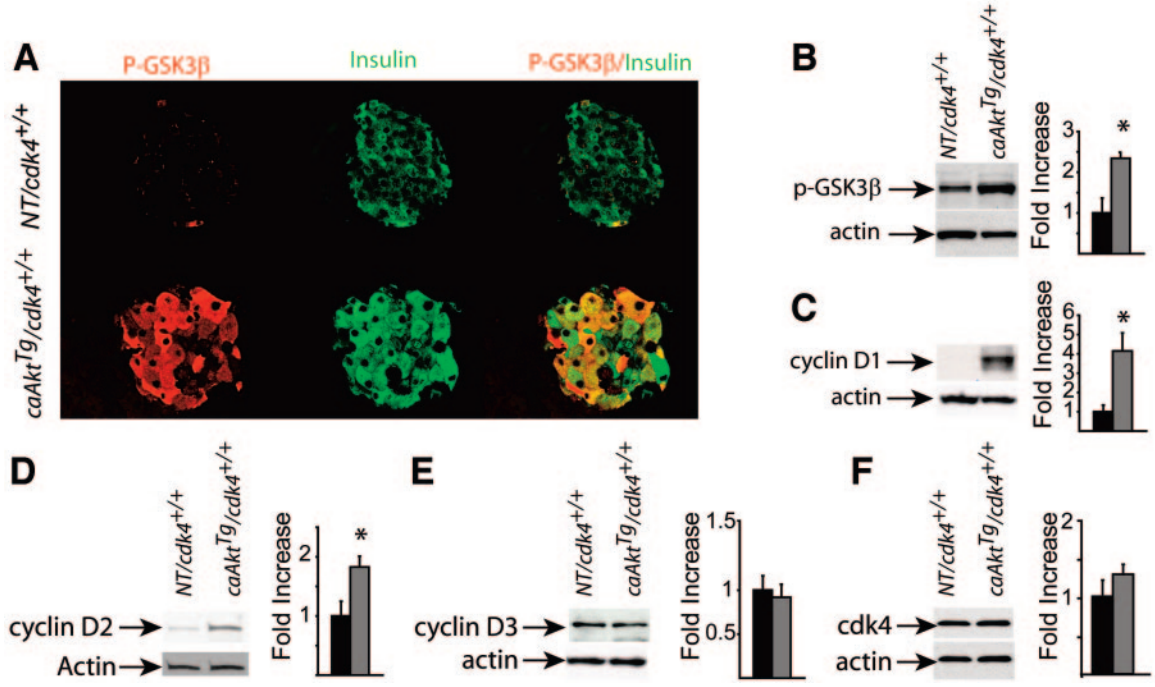

FIG. 1. Assessment of GSK3β phosphorylation cyclin D1, D2, and D3 levels and cdk4 levels in islets from *caAkt*^{Tg} and nontransgenic mice. *A*: Immunofluorescence staining for phospho-GSK3β (Ser9) (red), insulin (green), and double staining in pancreatic sections from *caAkt*^{Tg}/*cdk4*$^{+/+}$ and nontransgenic *cdk4*$^{+/+}$ mice. *B*: Total pancreatic islet lysates from *caAkt*^{Tg} and nontransgenic mice were immunoblotted with antibodies against phospho-GSK3β (Ser9) or actin. Protein levels for cyclins D1 (*C*), D2 (*D*), and D3 (*E*) and cdk4 (*F*) were assessed in islet lysates from *caAkt*^{Tg}/*cdk4*$^{+/+}$and nontransgenic *cdk4*$^{+/+}$ mice. Immunoblotting for actin was used as loading control. The figures are representative blots of at least three experiments in duplicate ($n \geq 6$). *$P < 0.05$.

in *caAkt*^{Tg} mice when compared with those of nontransgenic *cdk4*$^{+/+}$ (Fig. 2*C*). Analysis of p57 expression showed decreased levels in *caAkt*^{Tg} mice (Fig. 2*C*). Levels for p18 in *caAkt*^{Tg}/*cdk4*$^{+/+}$ islets were not significantly different than those of nontransgenic *cdk4*$^{+/+}$ (Fig. 2*E*). Levels for p16, 15, and 19 were not detectable by immunoblotting using islet lysates. These observations suggest that Akt regulates p21 and p57 but not p27 protein levels in islet β-cells.

Islet mRNA expression for G_1 components. Regulation of cell cycle by Akt could result from transcriptional regulation of cell cycle genes. To determine whether the changes in protein levels observed in Figs. 1*A* and 2 are dependent on transcription, we performed real-time PCR in islets from *caAkt*^{Tg} and nontransgenic *cdk4*$^{+/+}$ (Fig. 3). In contrast to protein levels, cyclin D1 and D2 mRNA were reduced in *caAkt*^{Tg} mice when compared with nontransgenic *cdk4*$^{+/+}$ (Fig. 3). Reduction of cyclin D3, cdk4, and

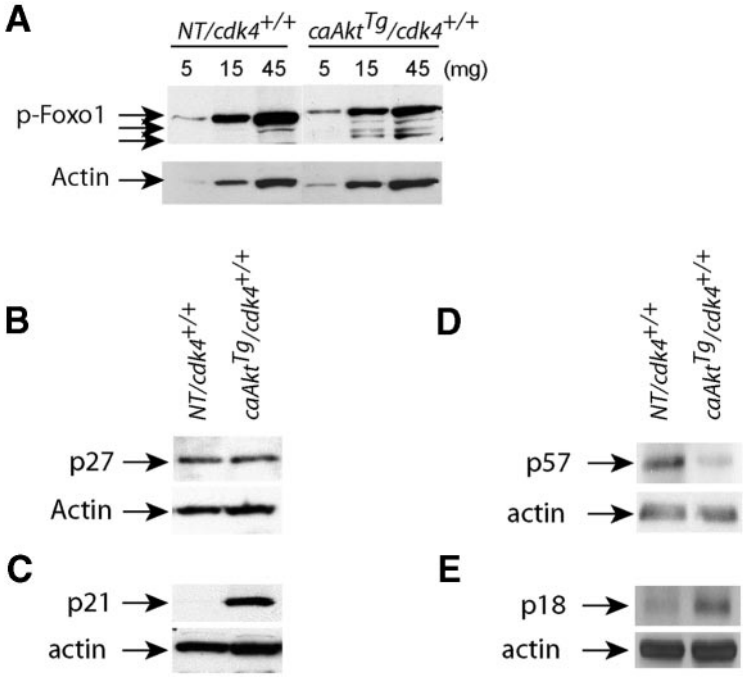

FIG. 2. Protein levels for phospho-Foxo1, p27, p21, p57, and p18 in islets lysates from *caAkt*^{Tg} and nontransgenic *cdk4*$^{+/+}$ mice. Immunoblotting for phospho-Foxo (*A*), p27 (*B*), p21 (*C*), p57 (*D*), and p18 using islet lysates from 4-month-old *caAkt*^{Tg}/*cdk4*$^{+/+}$ and nontransgenic *cdk4*$^{+/+}$ mice. Immunoblotting for actin was used as loading control. The figures are representative blots of at least three experiments in duplicate ($n \geq 6$).

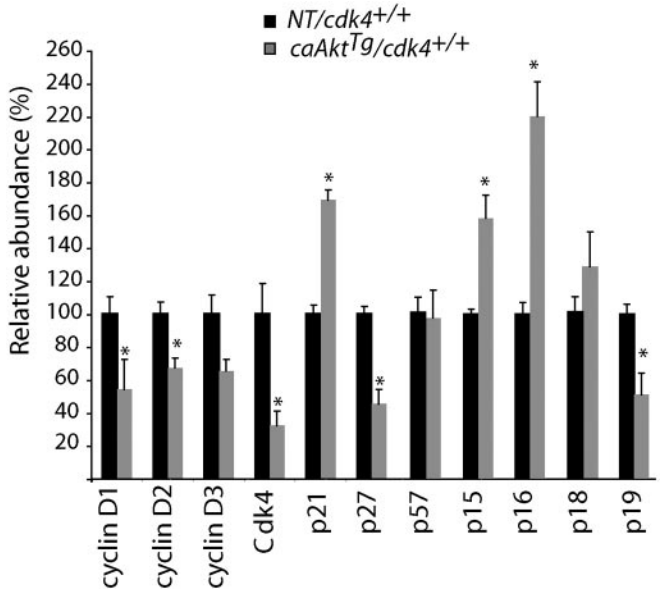

FIG. 3. Measurement of mRNA levels in islets from *caAkt*^{Tg} and nontransgenic *cdk4*$^{+/+}$ mice. TaqMan RT-PCR for cyclins D1, D2, and D3; cdk4; cip/kip; and INK inhibitors using islets from 4-month-old *caAkt*^{Tg} and nontransgenic *cdk4*$^{+/+}$ males (n = 4) as described in RESEARCH DESIGN AND METHODS. Data are normalized to 18S ribosomal RNA gene expression and expressed as means ± SE. *P < 0.05.

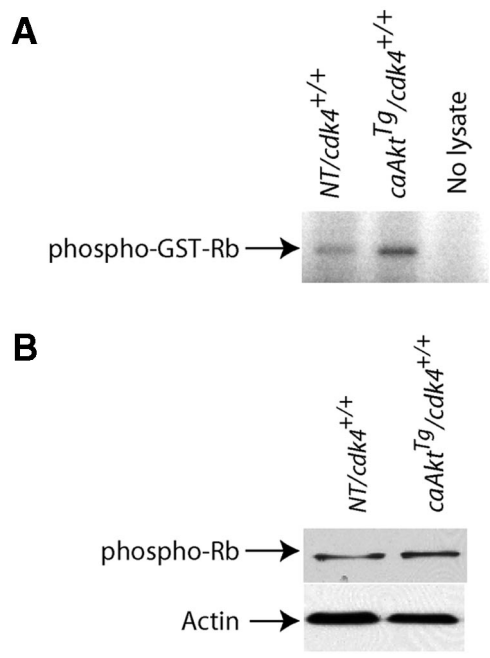

FIG. 4. Assessment of cdk4 activity in islets from *caAkt*^{Tg} and nontransgenic *cdk4*$^{+/+}$ mice. *A*: Activity of cdk4 by in vitro kinase assay using islet lysates obtained from pooled islets from four to six mice using recombinant GST-Rb (769-921) as substrate. *B*: Immunoblotting for phospho-Rb (Ser780) in islet lysates from *caAkt*^{Tg}/*cdk4*$^{+/+}$ and nontransgenic *cdk4*$^{+/+}$. Data are representative of at least two independent experiments in duplicate.

p27 mRNA levels were also observed (Fig. 3). Consistent with the changes in protein, increased p21 mRNA levels were observed in islets from *caAkt*^{Tg} mice. Increased expression of the INK cell cycle inhibitors p15 and p16 but not p18 was obtained in islets from *caAkt*^{Tg} mice (Fig. 3). Interestingly, levels for p19 mRNA were reduced in *caAkt*^{Tg} mice, suggesting that Akt differentially regulates the different components of the INK4 family of cell cycle inhibitors. Taken together, these observations suggest that Akt regulates the level of cyclin D1, cyclin D2, p21, and p57 levels in β-cells primarily by translational regulation and/or protein stability.

Assessment of cdk4 activity in *caAkt*^{Tg}. To determine whether the changes in G_1 components were associated with cdk4 activity, we performed in vitro kinase activity assays using recombinant GST-Rb (769-921) as substrate (20). This exogenous substrate contains the phosphorylation site for cdk4. The incorporation of radioactive phosphate to this substrate is proportional to cdk4 activity in the immunoprecipitate. As shown in Fig. 4*A*, phosphorylation of recombinant GST-Rb was increased in islet lysates from *caAkt*^{Tg}, suggesting that cdk4 was activated by Akt signaling in these islets (Fig. 4*A*). No activity was observed in the absence of lysates (control). Similar results were obtained in MIN6 cells expressing constitutively active Akt (data not shown). Phosphorylation of Ser780 in pRb protein is mediated by the cyclin D1/cdk4 complex (30). A significant increase in endogenous Rb phosphorylation at Ser780 was observed in islets from *caAkt*^{Tg} mice, suggesting that cdk4 activity is augmented (Fig. 4*B*) ($P < 0.05$). Taken together, the results from these experiments strongly indicate that Akt regulates cdk4 activity in β-cells. To determine whether overexpression of caAkt in β-cells induces proliferation solely by regulation of cdk4 activity, we generated *caAkt*^{Tg} mice that were homozygous, heterozygous, or nullizygous for CDK4.

Assessment of growth in *caAkt*^{Tg}/*cdk4* intercross. Decreased growth was observed in *cdk4*$^{-/-}$ mice as described previously and shown in Fig. 5 (16). This growth retardation was not altered by overexpressing Akt in β-cells (Fig. 5). *CaAkt*^{Tg} mice exhibited comparable weight gain with nontransgenic *cdk4*$^{+/+}$ mice. Interestingly, *cdk4*$^{+/-}$ mice had a significant decrease in weight after 30 days of life, and this alteration was corrected by overexpression of caAkt in pancreatic β-cells (Fig. 5).

Metabolic assessment of *caAkt*^{Tg}/*cdk4* intercross. To determine whether decreased *cdk4* levels induced abnormalities in glucose metabolism in *caAkt*^{Tg} mice, blood samples from 6- to 8-week-old mice were analyzed in the fasted state and in the fed state. After 16 h of fasting, no

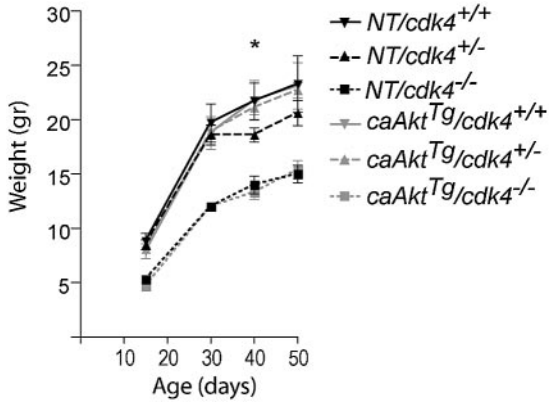

FIG. 5. Growth curves in *caAkt*^{Tg} and *cdk4*$^{+/-}$ intercross. Body weights were measured in the morning in males at the indicated ages of nontransgenic *cdk4*$^{+/+}$ (n = 6), nontransgenic *cdk4*$^{+/-}$ (n = 17), nontransgenic *cdk4*$^{-/-}$ (n = 6), *caAkt*^{Tg}/*cdk4*$^{+/+}$ (n = 9), *caAkt*^{Tg}/*cdk4*$^{+/-}$ (n = 22), and *caAkt*^{Tg}/*cdk4*$^{-/-}$ (n = 5) mice. Data are means ± SE. *P < 0.05, nontransgenic *cdk4*$^{+/-}$ vs. *caAkt*^{Tg}/*cdk4*$^{+/-}$.

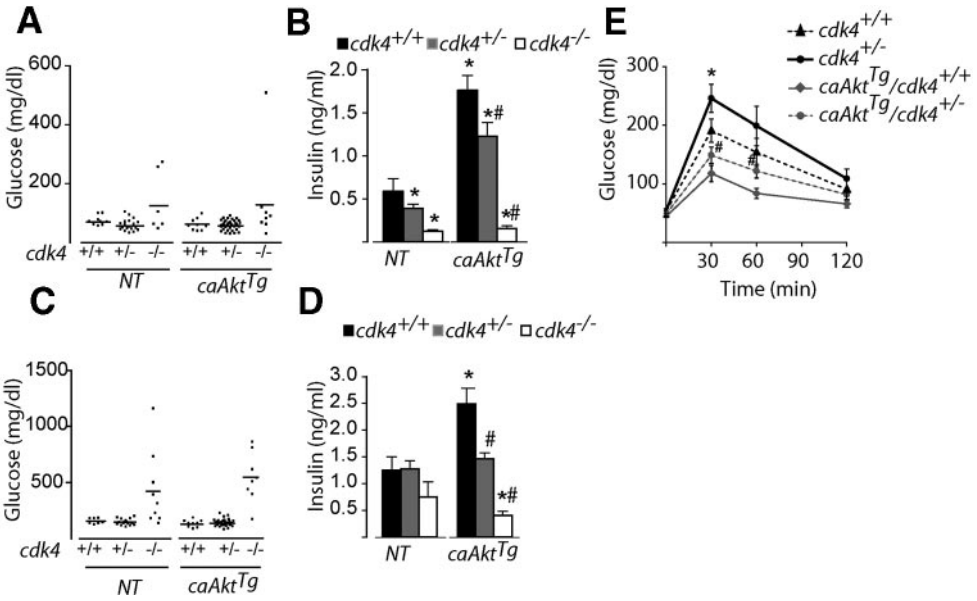

FIG. 6. Assessment of carbohydrate metabolism in *caAkt^{Tg}* and *cdk4$^{+/-}$* intercross. *A*: Fasting measurements were performed on 16-h–fasted 6- to 8-week-old mice. Glucose levels in nontransgenics (NT) or caAkt^{Tg} containing two alleles ($^{+/+}$), containing one allele ($^{+/-}$), or deficient ($^{-/-}$) in *cdk4*. *B*: Insulin levels obtained in the same group of mice. *C*: Assessment of the carbohydrate metabolism in the fed state was performed early in the morning in 6- to 8-week-old mice. Glucose levels in nontransgenics (NT) or caAkt^{Tg} containing two alleles ($^{+/+}$), containing one allele ($^{+/-}$) or deficient ($^{-/-}$) in *cdk4*. *D*: Insulin levels were measured in the same group of mice. *E*: Intraperitoneal glucose tolerance tests were performed using 2 g/kg body wt in 4-month-old mice after overnight fasting (n = 10). Data are means ± SE (*P < 0.05 when compared with nontransgenic cdk4$^{+/+}$; #P < 0.05 when compared with *caAkt^{Tg}/cdk4$^{+/+}$*) ($n \geq 4$).

differences were observed between nontransgenic *cdk4$^{+/-}$* and nontransgenic *cdk4$^{+/+}$* (Fig. 6*A*). The majority of the nontransgenic *cdk4$^{-/-}$* exhibited normal glucose after prolonged fasting (Fig. 6*A*). In the *caAkt^{Tg}* mice, normal glucose was detected in transgenic mice with one or two cdk4 alleles (Fig. 6*A*). Similar to nontransgenic *cdk4$^{-/-}$*, hyperglycemia was detected in some *caAkt^{Tg}* mice deficient in cdk4 (Fig. 6*A*). We then assessed insulin levels in the fasting state (Fig. 6*B*). Insulin levels were reduced in nontransgenic *cdk4$^{+/-}$* and nontransgenic *cdk4$^{-/-}$* mice when compared with nontransgenic *cdk4$^{+/+}$* mice (Fig. 6*B*, $P < 0.05$). Serum insulin values were increased in *caAkt^{Tg}/cdk4$^{+/+}$* related to nontransgenic *cdk4$^{+/+}$*, and these levels were decreased in absence of one or two cdk4 alleles (Fig. 6*B*). No differences in serum insulin levels were observed between nontransgenic and *caAkt^{Tg}* mice deficient in cdk4 (Fig. 6*B*). The results of these experiments indicate that fasting serum insulin are reduced in *caAkt^{Tg}* mice lacking one or deficient for cdk4.

In the fed state, glucose levels in *cdk4$^{+/-}$* did not differ from those of *cdk4$^{+/+}$* mice (Fig. 6*C*). The majority of *cdk4$^{-/-}$* mice exhibited a diabetic phenotype as previously described (Fig. 6*C*, $P < 0.05$) (15,16). In *caAkt^{Tg}* mice, glucose levels in *caAkt^{Tg}/cdk4$^{+/+}$* and *caAkt^{Tg}/cdk4$^{+/-}$* were comparable and not different than those of nontransgenic *cdk4$^{+/+}$* (Fig. 6*C*). Overexpression of Akt in β-cells did not alter fed glucoses in *cdk4$^{-/-}$* mice (Fig. 6*C*, $P < 0.05$). Assessment of insulin levels in the nontransgenic mice showed similar levels in *cdk4$^{+/-}$* and *cdk4$^{+/+}$* mice (Fig. 6*D*). Although there was tendency to lower insulin levels in *cdk4$^{-/-}$* mice, this was not statistically significant; but considering the magnitude of hyperglycemia in the fed state (Fig. 6*C*), these insulin measurements are abnormally low. In *caAkt^{Tg}* mice, insulin levels were increased twofold in *caAkt^{Tg}/cdk4$^{+/+}$* mice. A significant reduction in serum insulin was observed in *caAkt^{Tg}* lacking one *cdk4* allele, and these levels were not different than those found in the nontransgenic *cdk4$^{+/+}$* mice (Fig. 6*D*). *caAkt^{Tg}* deficient in *cdk4* exhibited a marked reduction in fed serum insulin levels, and these were significantly lower than those in nontransgenic *cdk4$^{+/+}$* (Fig. 6*D*). The results of these experiments suggest that overexpressing Akt in β-cells does not rescue the diabetic phenotype observed in *cdk4$^{-/-}$* mice and that hyperinsulinemia found in *caAkt^{Tg}* mice is normalized in absence of one cdk4 allele and is strikingly reduced in mice deficient in *cdk4*.

To assess glucose tolerance in mice that were not deficient in cdk4, we performed intraperitoneal glucose tolerance tests in 4- to 6-month-old mice. As shown in Fig. 6*A*, fasting glucose was similar between the different genotypes after a 12 h fast. Blood glucose was elevated 30 min after glucose injection in nontransgenic *cdk4$^{+/-}$* mice when compared with that of nontransgenic *cdk4$^{+/+}$* mice (Fig. 6*E*, $P < 0.05$). As described previously, *caAkt^{Tg}* exhibited improved glucose tolerance at 30 and 60 min after glucose injection (Fig. 6*E*). Glucose tolerance in *caAkt^{Tg}* mice lacking one allele of cdk4 was not different than that of the nontransgenic *cdk4$^{+/+}$* at 30 and 60 min after glucose injection (Fig. 6*E*). The results of these experiments suggest that a 50% reduction in cdk4 levels can affect glucose tolerance in nontransgenic mice and normalize the improved glucose tolerance in *caAkt^{Tg}* mice.

Overexpression of caAkt induces increase in β-cell mass and proliferation in a *cdk4*-dependent mechanism. The previous studies suggest that metabolic changes in *caAkt^{Tg}* were affected by decreased cdk4 levels and suggest that changes in β-cell mass could be responsible for these observations. Histology of pancreas and quantitation of β-cell mass were then assessed by islet morphometry. In adult pancreata from nontransgenic mice, *cdk4$^{+/-}$* exhibited comparable β-cell area with that of *cdk4$^{+/+}$* mice (Fig. 7 and quantitative analysis in Fig. 8*A*). A significant reduction in β-cell area was observed in *cdk4$^{-/-}$* mice (Fig. 7 and quantitative analysis in Fig. 8*A*). The severe decrease in islet area in cdk4$^{-/-}$ mice resulted from reduction in mean islet size (Table 1). Compared with nontransgenic *cdk4$^{+/+}$*, overexpression of the caAkt was associated with 8- to 10-fold elevation in β-cell area due to increased number, size, and islet density (Fig. 7 and quantitative analysis in Fig. 8*A*; Table 1). The increase in β-cell area observed in *caAkt^{Tg}* was decreased in *caAkt^{Tg}/cdk$^{+-/-}$* mice mainly by reduction in the number of islets, mean islet size, and density (Fig. 6 and quantitative analysis in Fig. 7*A*; Table 1). Interestingly, pancreata from *caAkt^{Tg}/cdk4$^{-/-}$* had almost undetectable β-cells, and the islet number, islet size, and islet density were not altered

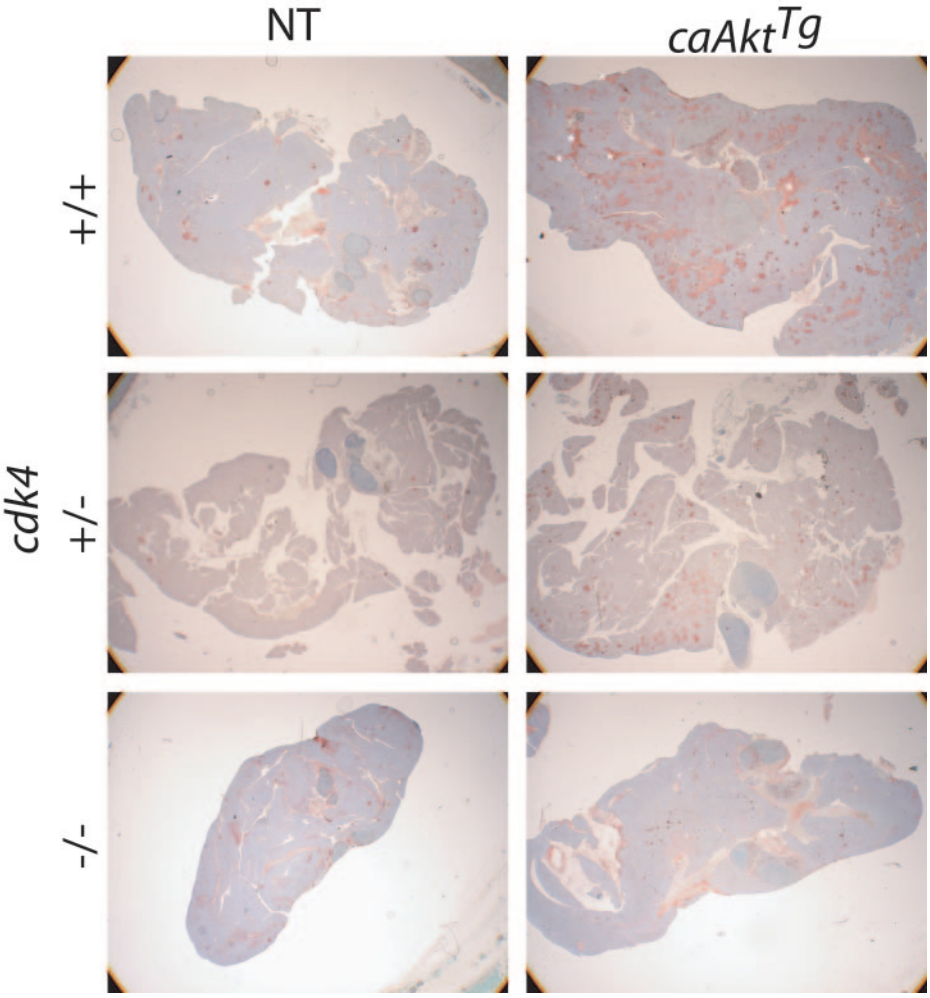

FIG. 7. Determination of pancreatic morphology among 4-month-old *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross. Representative pancreatic morphology from mice with different genotypes stained for insulin (red) and counterstained with hematoxylin (blue). Background staining is shown with asterisks.

by overexpression of caAkt when compared with nontransgenic *cdk4*$^{-/-}$ (Fig. 7 and quantitative analysis in Fig. 8*A*; Table 1). Analysis of proliferation by BrdU incorporation demonstrated that *caAkt*^{Tg}/*cdk4*$^{+/+}$ mice exhibited a ninefold increase in proliferation when compared with nontransgenic *cdk4*$^{+/+}$ mice (Fig. 8*B*). The proliferative response in *caAkt*^{Tg}/*cdk4*$^{+/+}$ mice was reduced by 70% in *caAkt*^{Tg}/*cdk4*$^{+/-}$ (Fig. 8*B*). Interestingly, proliferation in *caAkt*^{Tg}/*cdk4*$^{+/-}$ was still higher than that observed in nontransgenic *cdk4*$^{+/+}$ (Fig. 8*B*). No significant difference was observed between nontransgenic *cdk4*$^{+/+}$ and nontransgenic *cdk4*$^{+/-}$ mice (Fig. 8*B*). The results of these

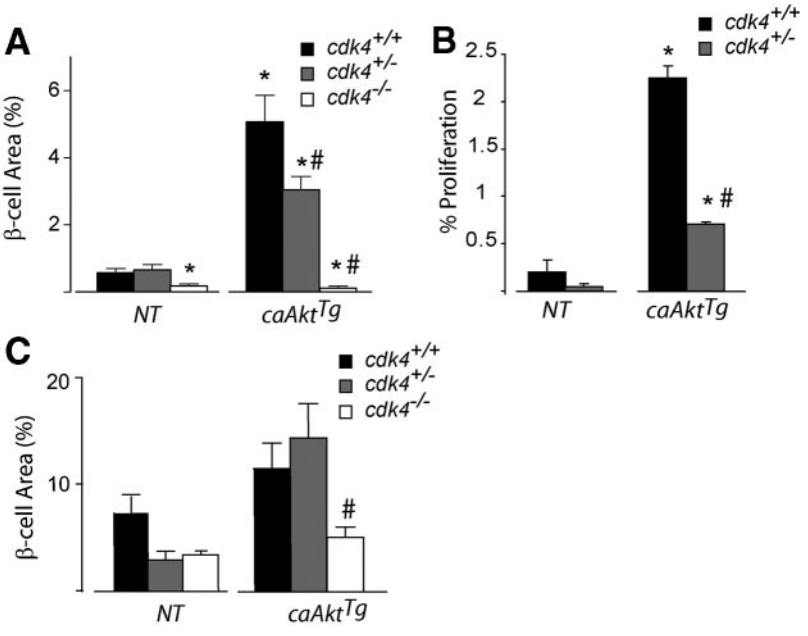

FIG. 8. Determination of islet morphometry and proliferation among 4-month-old *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross. *A*: β-Cell area of 4-month-old progeny from *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross ($n \geq 4$). *B*: BrdU incorporation in *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross ($n = 3$). Cdk4$^{-/-}$ mice not included because of the severely reduced β-cell mass. *C*: β-Cell area in neonatal pancreas from *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross was obtained during the first 16 h of life. Data are means ± SE (P* < 0.05 when compared with nontransgenic *cdk4*$^{+/+}$; #*P* < 0.05 when compared with *caAkt*^{Tg}/*cdk4*$^{+/+}$).**

TABLE 1
Islet morphometry in 4-month-old *cdk4*$^{+/+}$ *caAkt*^{Tg} intercross

	Islet number	Mean islet size ($\mu m^2 \times 10^3$/islet)	Islet density (islet/μm^2)
Nontransgenic *cdk4*$^{+/+}$	219 ± 14	4.0 ± 1	1.3 ± 0.1
Nontransgenic *cdk4*$^{+/-}$	256 ± 31	4.6 ± 0.7	1.2 ± 0.1
Nontransgenic *cdk4*$^{-/-}$	187 ± 53	1.1 ± 0.3*	1.4 ± 0.4
caAkt^{Tg}/*cdk4*$^{+/+}$	2,864 ± 92*	5.6 ± 0.3*	8.9 ± 0.9*
caAkt^{Tg}/*cdk4*$^{+/-}$	2,155 ± 185*†	4.4 ± 0.2†	6.9 ± 0.6*†
caAkt^{Tg}/*cdk4*$^{-/-}$	203 ± 92†	0.6 ± 0.1*†	1.3 ± 0.6†

Data are means ± SE. *$P < 0.05$ vs. nontransgenic *cdk4*$^{+/+}$; †$P < 0.05$ vs. *caAkt*^{Tg}/*cdk4*$^{+/+}$.

experiments suggest that overexpression of caAkt in β-cells induces β-cell mass by increased proliferation in a cdk4-dependent mechanism.

Assessment of islet mass in the neonatal period. To determine whether Akt regulates β-cell mass during development and whether this effect is mediated through cdk4, we then measured β-cell mass and insulin levels in the neonatal period (Fig. 8*C*). In nontransgenic mice, a lower but not significant decrease in β-cell mass was observed in *cdk4*$^{+/-}$ and *cdk4*$^{-/-}$ neonates (Fig. 8*C*). In *caAkt*^{Tg} mice, a tendency to higher β-cell mass was found in *caAkt*^{Tg}/*cdk4*$^{+/+}$ and *caAkt*^{Tg}/*cdk4*$^{+/-}$ when compared with nontransgenic *cdk4*$^{+/+}$ mice (Fig. 8*C*). Similar β-cell mass was found between *caAkt*^{Tg} mice with one or two alleles of cdk4 (Fig. 8*C*). A 2.5-fold reduction in β-cell mass was observed in *caAkt*^{Tg} deficient in cdk4 when compared with *caAkt*^{Tg}/*cdk4*$^{+/+}$ or *caAkt*^{Tg}/*cdk4*$^{+/-}$ mice (Fig. 8*C*, $P < 0.05$). These findings suggest that overexpression of caAkt in β-cells increases β-cell mass in embryonic stages in a cdk4-dependent manner. Another conclusion derived from these experiments is that cdk4 levels can regulate β-cell mass during development, and this effect is more apparent in conditions of increased proliferation.

DISCUSSION

Genetic lineage tracing experiments in β-cells suggest that proliferation is the major factor in maintenance of β-cells in vivo. The PI 3-kinase/Akt pathway has been shown to be critical in regulation of β-cell mass and function. The results of the current studies serve to elucidate the mechanisms involved in the regulation in vivo of β-cell proliferation by Akt. The current novel findings suggest that Akt induces β-cell proliferation by modulation of cyclin D1, cyclin D2, p21, and p57 levels and that the changes in these G_1 components result in activation of cdk4. The increase in cell cycle components results primarily from increased translational efficiency and/or protein stability. The observations derived from the genetic experiments indicate that proliferative signals induced by Akt are mediated in a cdk4-dependent manner. These experiments imply that deregulation of cell cycle components such as cyclin D1, cyclin D2, and p21 could be an important component for adaptive proliferative responses of β-cells to insulin resistance and to the resistance to experimental diabetes.

The Akt pathway is the primary mediator of PI 3-kinase–initiated signaling and has a number of downstream substrates that may contribute to cell cycle progression. The results of the current experiments show that β-cell proliferation induced by Akt was associated with phosphorylation of GSK3β (Fig. 1*A* and *B*) and increased cyclin D1 and D2 levels. The unchanged mRNA expression for these cyclins suggests that the alteration occurs at the level of translation or protein degradation. These findings are in agreement with recent studies suggesting that cyclins D2 and D1 are critical for maintenance of β-cell mass (18,19). Evidence for augmented replication of human islets in vivo by overexpression of cyclin D1 and cdk4 demonstrates that manipulation of Akt signaling have potential applications given the current need to expand human β-cells for transplantation (17).

The activity of cdk4 is regulated in part by the association to D cyclins and negatively regulated by the INK4s and the CIP/KIP inhibitors. In the present report, mRNA levels of the INK4s p15 and p16 but not p18 and P19 were elevated (Fig. 3). The function of these cell cycle inhibitors in the phenotype was partially determined because we were able to detect protein levels only for p18 by Western blotting using protein lysates from 110 islets (50–60 μg). Assessment of the CIP/KIP inhibitors showed that inactivation of Foxo1 by Akt phosphorylation was associated to decreased p27 mRNA but not protein levels, suggesting that posttranslation mechanisms are also important to maintain p27 levels. The normal levels of p27 protein in islets from *caAkt*^{Tg} mice imply that this is not a major factor for cell cycle progression induced by Akt. Decreased levels for p57 were observed in *caAkt*^{Tg} mice, suggesting that Akt regulates distal events in cell cycle progression. In contrast to p27, these data showed increased p21 levels induced by Akt. The function of p21 in cell cycle is complex, and new evidence suggests that the main function during cell cycle progression appears to be ensuring appropriate cdk4 activation and cell survival (31–35). The role of p21 in the proliferative phenotype is unclear, but it is possible that the increase in the number cdk4 complex proteins (cyclin D1, D2, and p21) stabilizes and prevents p21 degradation.

Several reports suggest that cdk4 is necessary to maintain postnatal β-cell proliferation (15,16,36). The marked decrease in β-cell mass found in *caAkt*^{Tg}/*cdk4*$^{+/-}$ and *caAkt*^{Tg}/*cdk4*$^{-/-}$ together with the kinase assays indicate that cdk4 activity is the final step in the regulation of cell cycle by Akt. These data also suggest that in differentiated β-cells, there are not cdk4-independent pathways for β-cells to enter the cell cycle even in conditions of extreme activation of growth signals like Akt. In contrast to the results in adult mice, the β-cell mass changes in *caAkt*^{Tg}/*cdk4*$^{-/-}$ neonates (Fig. 8*C*) suggest that cdk4 can be limiting during embryogenesis in conditions of increased growth signals, i.e., gestational diabetes.

The current studies elucidate some of the mechanism that regulates cdk4, allowing β-cells to enter the cell cycle. The identification of cdk4 and cyclins D1 and D2 as major molecules regulating islet growth and survival by Akt may lead to development of pharmacological agents to enhance β-cell mass for the treatment of diabetes, transplantation success, and protection against autoimmune destruction.

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