Induction of β-Cell Proliferation and Retinoblastoma Protein Phosphorylation in Rat and Human Islets Using Adenovirus-Mediated Transfer of Cyclin-Dependent Kinase-4 and Cyclin D_1

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The major regulator of the gap-1/synthesis phase (G_1/S) cell cycle checkpoint is the retinoblastoma protein (pRb), and this is regulated in part by the activities of cyclin-dependent kinase (cdk)-4 and the D cyclins. Surprisingly, given the potential importance of β-cell replication for islet replacement therapy, pRb presence, phosphorylation status, and function have not been explored in β-cells. Here, adenoviruses expressing cdk-4 and cyclin D_1 were used to explore rat and human pRb phosphorylation and β-cell cycle control. pRb is present in rat and human islets, and overexpression of cyclin D_1/cdk-4 led to strikingly enhanced pRb phosphorylation in both species. Combined overexpression of both cdk-4 and cyclin D_1 caused a threefold increase in [^{3}H]thymidine incorporation. This increase in proliferation was confirmed independently using insulin and bromodeoxyuridine immunohistochemistry, where human β-cell replication rates were increased 10-fold. Cdk-4 or cyclin D_1 overexpression did not adversely effect β-cell differentiation or function. The key cell cycle regulatory protein, pRb, can be harnessed to advantage using cyclin D_1/cdk-4 for the induction of human and rodent β-cell replication, enhancing replication without adversely affecting function or differentiation. This approach will allow detailed molecular study of the cellular mechanisms regulating the cell cycle in β-cells, β-cell lines, and stem cell–derived β-cells. *Diabetes* 53:149–159, 2004

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BrdU, bromodeoxyuridine; cdk, cyclin-dependent kinase; DAB, 3,3′-diaminobenzidine-tetra-hydrochloride; DMEM, Dulbecco's modified Eagle's medium; GK, glucokinase; GSIS, glucose-stimulated insulin secretion; Kir6.2, K^+ inward rectifier; HRP, horseradish peroxidase; MOI, multiplicity of infection; pRb, retinoblastoma protein; SUR-1, sulfonylurea receptor-1; TAg, T-antigen.

Although it is currently very clear that β-cells can replicate, albeit slowly, in vitro and in vivo, both basally and in response to a variety of maneuvers and stimuli (rev. in 1–3), the key components of the cell cycle machinery in the β-cell and the factors that regulate them are poorly understood at a molecular level. The retinoblastoma protein (pRb) (rev. in 4–6) is a key gap-1/synthesis phase (G_1/S) checkpoint gatekeeper. In its dephosphorylated (or active) state, pRb binds to the E2F family of cell cycle regulatory genes and leads to the transcriptional repression of downstream genes, with resultant cell cycle arrest. Conversely, when pRb is phosphorylated to form ppRb, it becomes inactive and releases E2Fs, removing transcriptional repression of critical cell cycle genes, and the cell cycle progresses. pRb can be phosphorylated by a number of kinases. These include cyclin-dependent kinase (cdk)-4 and -6, which form complexes with the D cyclins, as well as cyclin E and cdk-2, which also form a complex. Because of their importance, these cyclins and cdks are under tight regulatory control themselves. This regulation is principally inhibitory and is accomplished by inhibitory kinases or cyclin inhibitor proteins such as p16, p18, p21, p27, p53, p57, and others.

The majority of the information described above on pRb and ppRb has been obtained in human and animal cancers and fibroblasts (4–6). Surprisingly, given the current attention on β-cell replication, little is known regarding molecular control of the cell cycle in the β-cell. For example, we are unaware of any study examining either the presence of, or the phosphorylation status of, pRb in human or animal β-cells. On the other hand, there are some data that point to this pathway as being critical to the control of the cell cycle in β-cells. For example, SV-40 large T-antigen (TAg) is a transforming viral protein that interacts with p53 and pRb. TAg has been overexpressed in the β-cell of transgenic "RIP-TAg" mice and in cultured β-cell lines by Hanahan (7) and Efrat et al. (8–9), and increased β-cell replication resulted. Ultimately, autonomous β-cell tumors develop in RIP-TAg mice.

Homozygous disruption of the pRb gene in mice results in embryonic lethality (10). Heterozygous deletion results in adult animals characterized by the development of

multiple tumor types (10–12). Homozygous deletion of p53 also results in the development of a broad array of tumors, but in neither the pRb knockout nor the p53 knockout do β-cell tumors develop (10–12). Interestingly, however, when the pRb heterozygous +/− mice are crossed with the p53 homozygous −/− mice, one of the most prominent tumors to develop is insulinoma (11,12).

Chung et al. (13) reported that 43% of human islet cell tumors displayed upregulation of cyclin D_1. Rane et al. (14) and Tsutsui et al. (15) prepared cdk-4 knockout mice, anticipating a generalized cell proliferative defect. To their surprise, generalized disruption of cdk-4 leads to a very circumscribed phenotype: diabetic ketoacidosis associated with β-cell hypoplasia. Hypoplastic ovaries and testes were also observed, but no other abnormalities were detected. Conversely, Rane et al. (14) prepared a knock-in mouse in which generalized overexpression of a constitutively active form of cdk-4 was accomplished. These animals, too, had a very restricted phenotype, consisting most prominently of islet hyperplasia.

Franklin et al. (16) demonstrated that disruption of both p18 together with p27 results in β-cell hyperplasia, again suggesting that relief of repression of the pRb pathway leads to cell cycle progression in the β-cell. Kassem et al. (17) recently demonstrated that selective or tissue-specific loss of another G_1 cyclin inhibitor, $p57^{KIP2}$, is present in the proliferating β-cells of children with the focal variant of hyperinsulinism of infancy.

Collectively, these studies provide compelling evidence that the p53–cyclin D_1–cdk-4–pRb pathway may be central to the control of the cell cycle in β-cells, and they suggest that understanding the putative regulatory and potential therapeutic role of this pathway will be critical to understanding β-cell replication. On the other hand, these studies do not provide an integrated understanding of how the pathway operates or can be controlled. Moreover, with the exception of the reports by Chung et al. (13) and Kassem et al. (17), all of the above apply to mouse models and cell lines and therefore highlight the paucity of information available in human islets. Finally, as noted above, no evidence exists regarding the relatively straightforward question as to the existence, or the phosphorylation status, of pRb in rodent or human islets.

TABLE 1
PCR primers used for mRNA amplification

Gene name	Gene bank accession no.	5′ oligonucleotide	3′ oligonucleotide	Product size	Annealing temperature (C°)	Cycles	Actin P:C ratio
hCdk4	NM_000075	ATGGCTACCTCTCGATATGAGCCA	TCACTCCGGATTACCTTCATCCTT	912	65	24	2:8
hCyclin D_1	NM_053056	ATGGAACACCAGCTCCTGTGCTGC	TCAGATGTCCACGTCCCGCACGT	888	65	24	2:8
Lac Z	AJ308295	ACTATCCCGACCGCCTTACT	TAGCGGCTGATGTTGAACTG	172	58	24	2:8
mInsulin	X04725	CCCAGGCTTTTGTCAAACAGCA	CTCCAGTGCCAAGGTCTGAA	179–185	60	15	10:0
mGk	L38990	AAGGGAACAACATCGTAGGA	CATTGGCGGTCTTCATAGTA	130	56	25	2:8
rGlut2	J03145	TGGGTTCCTTCCAGTTCG	AGGCGTCTGGTGTCGTATG	183	55	20	3:7
mKir 6.2	AF037313	GGGCATTATCCCTGAGGAATAT	GAAGGACATGGTGAAAATGAGC	223	57	25	2:8
mSur-1	AF037296-AF037312	CCAAGGGAAGATTCAAATTCAA	GTCCTGTAGGATGATGGACAGG	261	58	25	2:8
rGlucagon	K02808	ACCTAGACTCCCGCCGTG	ATGTCTGCGCCCAAGTTC	245	58	20	10:0
hInsulin	AY138589	CTCTACCTAGTGTGCGGGGA	AGAGGGAGCAGATGCTGGTA	169	58	15	2:8
hGk	M90299	CACTGCTGAGATGCTCTTCG	CCACGACATTGTTCCCTTCT	197	57	24	1:9
hGlut2	J03810	GGAGTTGGCGCTGTAAACAT	AAACTCAGCCACCATGAACC	249	57	24	1:9
hKir 6.2	NM_000525	ATCATCGTCATCCTGGAAGG	GGTGTTGCCAAACTTGGAGT	162	58	25	2:8
hSur-1	AF087138	CTTCTCGCAGCTACGCTTCT	GCGTTCATCCACCAGTAGGT	216	57	24	2:8
hGlucagon	NM_002054	CATTCACAGGGCACATTCAC	CAGCTTGGCCTTCCAAATAA	190	57	24	2:8

RESEARCH DESIGN AND METHODS

Rat and human islet isolation. Rat islets were isolated according to the procedure of Ricordi and Rastellini (18) with minor modifications in accordance with and approved by the University of Pittsburgh institutional animal care and use committee. Human islets were provided by the University of Pittsburgh Human Islet Isolation Facility and were prepared according to Ricordi and Rastellini (18) as described in detail previously (19).

Immunohistochemistry and quantitative islet histomorphometry. Rat or human islets were embedded in paraffin after fixation in Bouin's solution. To place small numbers of rat or human islets in paraffin blocks in a fashion that would allow their identification and sectioning, islets were mixed into a slurry with Affi-Gel blue beads (BioRad, Richmond, CA) before embedding in paraffin. Serial 5-μm sections of the isolated islets were deparaffinized, rehydrated, quenched with 3% H_2O_2 in methanol for 1 min at room temperature, microwaved for 7 min, trypsinized for 10 min at room temperature, rinsed, and blocked with 2% goat serum. Primary antibody was incubated for 1 h at room temperature. The primary antibodies were diluted as follows: 1:10 anti-insulin (BioGenex Labs, San Ramon, CA), 1:40 anti–cytokeratin-19 (ck-19) (Novocastra/Vector Labs, Burlington, ON, Canada), and 1:5 anti-bromodeoxyuridine (BrdU; Amersham Pharmacia). Secondary antibodies (horseradish peroxidase [HRP]–linked) were incubated for 20 min at room temperature. The BrdU secondary antibody was developed with 3,3′-diaminobenzidine-

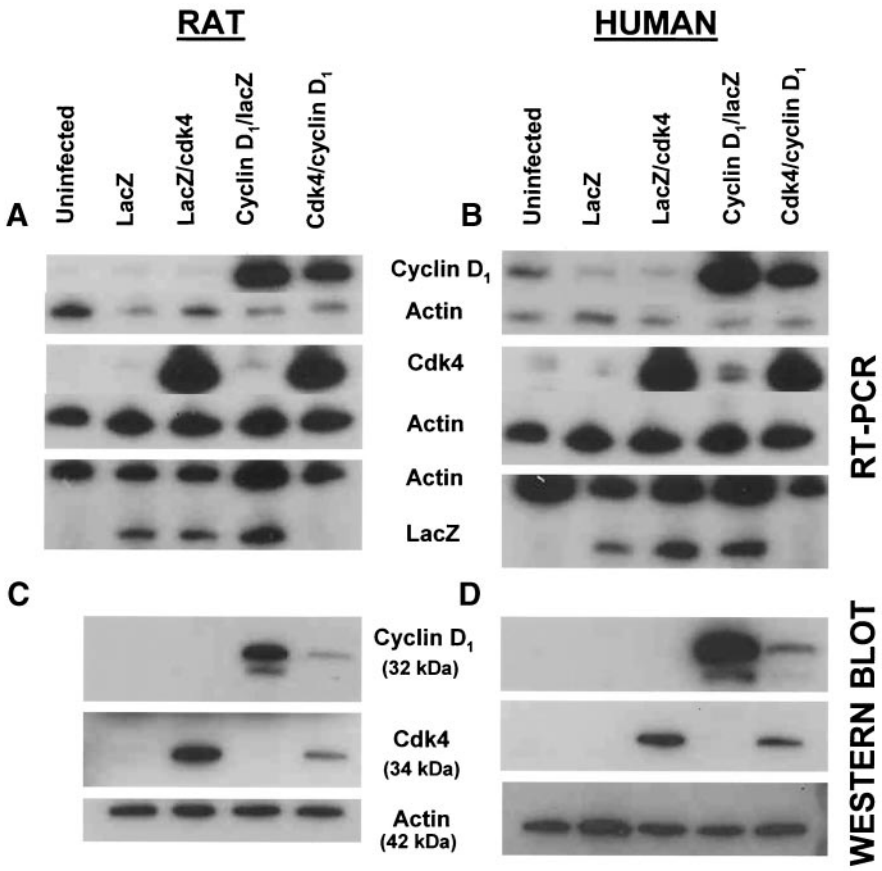

FIG. 1. *A* and *B*: Semiquantitative RT-PCR for cdk-4, cyclin D_1, and lacZ in rat islets (*A*) and human islets (*B*) transduced with the three adenoviruses. RT-PCR was performed with 1 μg of islet RNA. *C* and *D*: Immunoblots for cyclin D_1, cdk-4, and actin in rat (*C*) and human (*D*) islets transduced with the three viruses. For the lacZ and the cdk-4 and cyclin D_1 experiments, the total MOI was held constant at 500 MOI (200 MOI of Ad.cdk-4 and 300 MOI of Ad.lacZ; 200 MOI of Ad.lacZ and 300 MOI of Ad.cyclin D_1; and 200 MOI of Ad.cdk-4 and 300 MOI of Ad.cyclin D_1). Experiments were performed 96 and 48 h after transduction of rat and human islets, respectively.

tetra-hydrochloride (DAB) as substrate as well as $CoCl_2$ and $NiCl_2$. The secondary insulin HRP-linked antibody was visualized using only DAB. Sections were lightly counterstained using hematoxylin. Quantitative histomorphometry was performed in a blinded fashion as described previously (20,21).

Cell death assay. β-Cell death was assessed using nuclear pyknosis as described in detail previously (20,22). Briefly, fresh sections were cut from the same blocks used for human islet immunohistochemistry described in Figs. 3 and 4 and were costained for insulin (Zymed) and propidium iodide (Sigma). Pyknotic nuclei were counted and expressed a percent of total nuclei, and β-cells containing pyknotic nuclei were expressed as a function of total β-cells. Five human islet preparations were examined for five different conditions: control, Ad.lacZ, Ad.cdk-4, Ad.cyclin D_1, and the combination, with 500-2,000 nuclei being counted for each slide, for an average 1,000, depending on the number of islets present on a given slide.

Glucose-stimulated insulin secretion from isolated islets. Glucose-stimulated insulin secretion (GSIS) was performed under static conditions, as we have described in detail previously (20,21). Briefly, insulin release (radioimmunoassay; Linco Research, St. Louis, MO) was measured from isolated rat and human islets 24 h after infection with adenovirus. Groups of 10 islets were cultured at 2.8 or 5.5 mmol/l glucose for 20 min as described in Fig. 6, and then the medium was changed to 22 mmol/l glucose for 30 min. The medium was then removed, the islets extracted, and protein content measured. Results are expressed as the percentage of insulin released at high glucose (22 mmol/l) compared with basal glucose (2.8 or 5 mmol/l) concentrations.

Adenoviral cyclin D_1 and cdk-4 production and infection. Adenovirus (Ad.5) containing cdk-4 and cyclin D_1 were prepared according to methods we have previously described in detail (20), originally described by Becker et al. (23). The Ad.5 constructs were generously provided by Dr. Christopher Newgard (Duke University, Durham, NC). The human cyclin D_1 adenovirus was from Dr. Paul Robbins at the University of Pittsburgh, and the human cdk-4 cDNA was from Dr. Andrew Arnold at the University of Connecticut. Multiplicity of infection (MOI) was determined by both optical density at 260 nm (OD_{260}) and by plaque assay. MOI calculations assume 1,000 cells per islet. Rat and human islets were transduced with the adenoviruses as described in detail previously (20).

Western immunoblotting for cyclin D_1, cdk-4, and pRb. Islet extracts were prepared using nuclear extraction buffer, as we have described previously (20,21), and then resolved using 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Antisera for cyclin D_1 and cdk-4 were from Santa Cruz Biotechnology (Santa Cruz, CA), and antisera for pRb was from Pharmingen (San Diego, CA). The pRb antiserum recognizes both pRb and ppRb. Actin (antiserum was from Sigma, St. Louis, MO) was used as a control for sample loading.

Tritiated thymidine incorporation and BrdU labeling. Freshly isolated rat and human islets were exposed to purified adenovirus in 0.5 ml Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37°C and then rinsed with DMEM containing 1% penicillin and streptomycin without serum for 24 h at 37°C. Then, 1 μC_i of [^{3}H]thymidine (specific activity 83 Ci/mmol; Amersham Pharmacia, Piscataway, NJ) was added to the medium for an additional 24 h. To quantitate the amount of [^{3}H]thymidine incorporation, the islets were washed with ice-cold PBS and the proteins and DNA precipitated in ice-cold 10% trichloroacetic acid twice for 10 min on ice. The resulting precipitant was resuspended in 0.1 N NaOH at 60°C for 30 min and neutralized with 0.1 N HCl. Aliquots were counted in scintillation fluid and assayed for protein using the Bradford assay.

For BrdU labeling, a 1:1,000 dilution of BrdU (Cell Proliferation Kit; Amersham) was added to the culture medium instead of [^{3}H]thymidine for 18–20 h. The remainder of the procedure was performed as described above for [^{3}H]thymidine. The isolated islets were fixed in Bouin's solution for 2 h at room temperature and stored in neutral-buffered formalin before embedding in paraffin.

RT-PCR for cdk-4, cyclin D_1, GLUT-2, glucokinase, insulin, sulfonylurea receptor-1, K^+ inward rectifier 6.2, and glucagon. RNA was extracted using previously reported methods (20,21) from rat or human islets incubated 48 or 96 h after 1 h of exposure to adenovirus (500 MOI) containing lacZ,

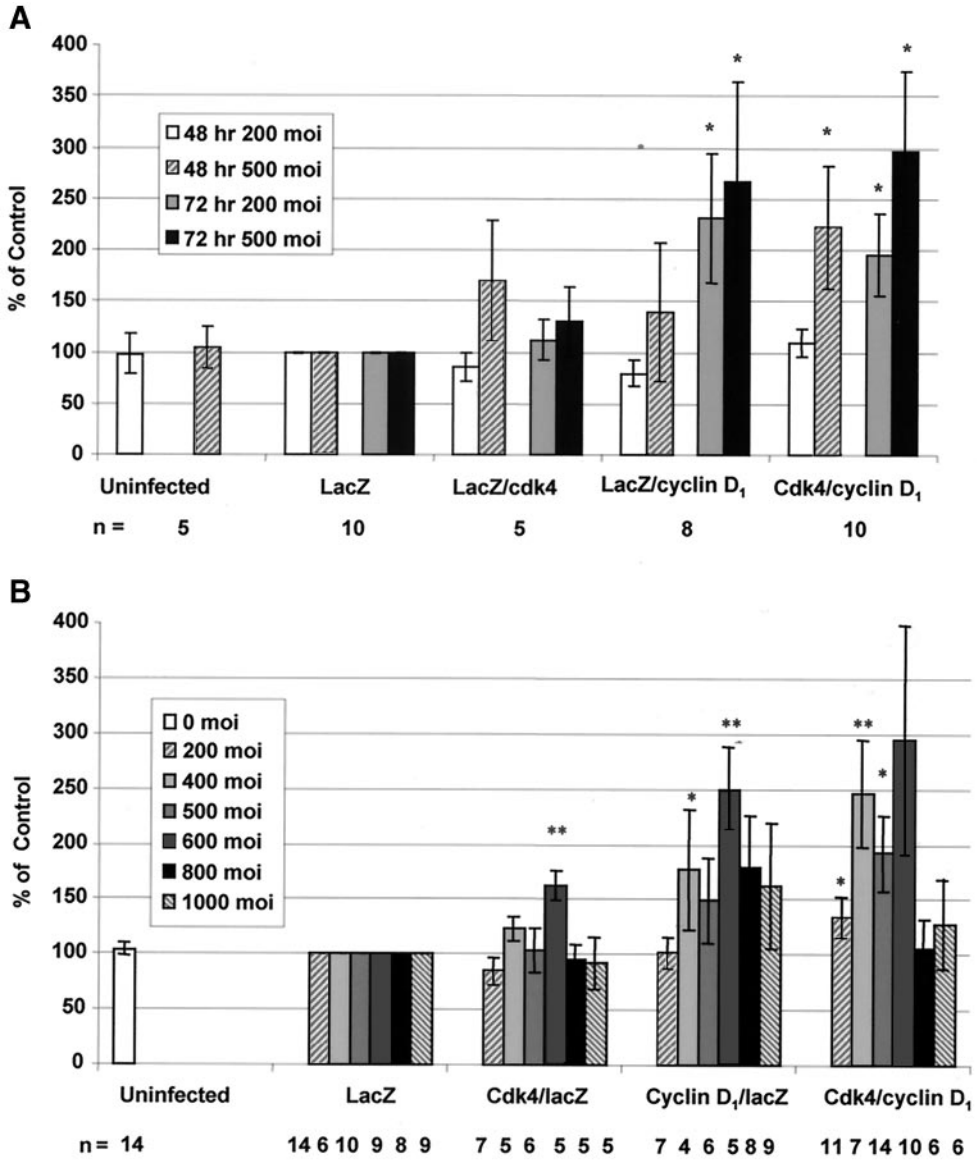

FIG. 2. [^{3}H]thymidine incorporation in isolated rat (*A*) and human (*B*) islets. Islets were transduced with lacZ, lacZ/cdk-4, lacZ/cyclin D_1, or cdk-4/cyclin D_1 adenoviruses at the indicated total MOI. Islets (50 islet equivalents [IEs] per well for rat and 100 IE per well for human, with 1 IE defined as 125 μm, as previously described) (20,21) were harvested 48–72 h postinfection (rats) or at 48 h (human), and during the last 24 h, the islets were incubated with [^{3}H]thymidine. Thymidine incorporation is expressed as a percent of the control Ad.lacZ-infected islets. The number of experiments performed (*n*) is shown, where 1 *n* equals one preparation of islets, each assayed in triplicate. *$P < 0.05$; **$P < 0.005$.

cyclin D_1, and/or cdk-4. RNA was reverse-transcribed, and semiquantitative PCR was performed using actin competimers, as previously described in detail (20,21). All experiments were performed using the linear portion of the PCR (20,21). The primers and PCR conditions used are shown in Table 1.

Statistical analysis. Bars in the figures indicate the standard error. For all studies, Student's two-tailed unpaired *t* test was performed using σ-STAT, as indicated in RESULTS and the figure legends. *P* values <0.05 were considered to be significant.

RESULTS

Adenoviruses containing cyclin D_1 and cdk-4 transduce rat and human islets. Initially, we were interested to know whether Ad.cyclinD_1, Ad.cdk-4, or both were able to transduce isolated islets. Figure 1 demonstrates, using RT-PCR (*A* and *B*) and Western immunoblotting (*C* and *D*), that the adenoviruses encoding cyclin D_1 and cdk-4 can effectively transduce rat and human islets. In these experiments, viruses were administered at a total MOI of 500 for 1 h, and islets were extracted for RNA or protein after 96 h. As shown in Fig. 1, both Ad.cdk-4 and Ad.cyclin D_1 led to increases in the expression of their respective mRNA and protein products. In each of three experiments using both rat and human islets, the combination Ad.cyclin D_1 and Ad.cdk-4 appeared to lead to lower steady-state levels of the corresponding protein than when they were introduced individually.

Cyclin D_1 and cdk-4 stimulate [^{3}H]thymidine in rat and human islets. As shown in Fig. 2*A*, the addition of Ad.lacZ and Ad.cdk-4 had no effect on [^{3}H]thymidine uptake in isolated rat or human islets with either 200 or 500 MOI at 48 or 72 h after transduction. In contrast, in rat islets Ad.cyclin D_1 stimulated [^{3}H]thymidine incorporation by two- to threefold after 72 h of exposure, but it had no effect at 48 h. The combined transduction with both cyclin

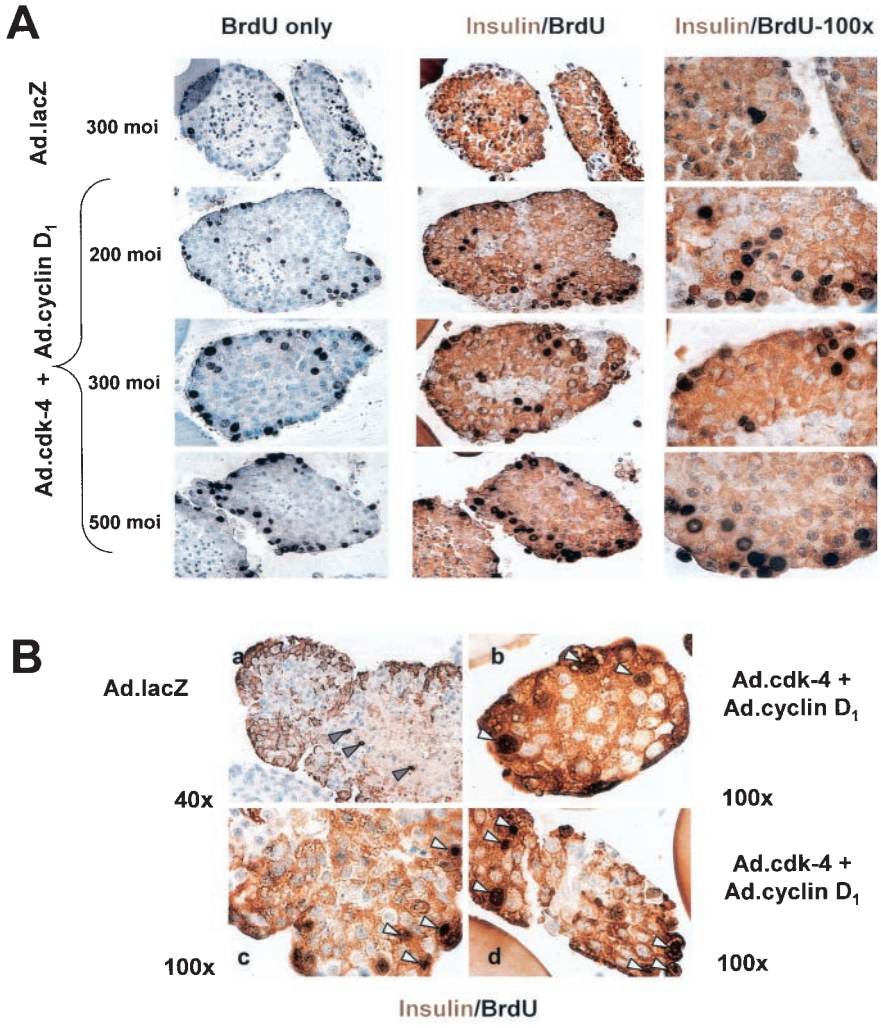

FIG. 3. BrdU and insulin staining in rat (*A*) and human (*B*) islets transduced with Ad .lacZ or Ad.cdk-4/Ad.cyclin D_1. In the rat studies, islets were transduced at the MOI indicated and fixed 72 h later. In the human studies, 300 MOI of lacZ and cdk-4/cyclin D_1 adenoviruses were used, and islets were fixed 72 h after transduction. The magnification is 100× unless otherwise indicated. The arrowheads in *B* indicate BrdU-positive nuclei.

D_1 and cdk-4 caused a 2.5-fold increase at 48 h and also led to a similar increase in [^{3}H]thymidine in rat islets at 72 h.

In human islets (Fig. 2*B*), in contrast to rat islets, overexpression of cdk-4 alone led to a statistically significant increase in [^{3}H]thymidine incorporation as compared with control islets. This could reflect the higher MOI of Ad.cdk-4 used in the human experiments (600 in human islets vs. 500 in rat islets), or it could reflect differences in human versus rat islets. As in rat islets, overexpression of either cyclin D_1 alone or combined with cdk-4 led to an approximate 2.5- to 3-fold increase in the rate of proliferation as assessed using [^{3}H]thymidine. Interestingly, MOI values >600–800 appeared to have deleterious effect on proliferation. Accordingly, 500 MOI was used for the studies that follow.

Cyclin D_1 and cdk-4 stimulate BrdU incorporation in rat and human islets. The studies described above suggest that delivery of cyclin D_1 and cdk-4 to islets stimulates islet cell proliferation, but they do not prove that the proliferation is occurring in β-cells. To define which cells were proliferating, we examined BrdU incorporation into isolated rat islets 48 h after transduction with the adenoviruses. As can be seen in Fig. 3*A* (*left column*), BrdU incorporation (dark black nuclei) is clearly more abundant in rat islets transduced with both Ad. cyclin D_1 and Ad. cdk-4 at MOI between 200 and 500 as compared with islets transduced with Ad.lacZ. As expected, because the adenoviruses infect the outer layer of islet cells most efficiently, the proliferation appears most robust in the outer layer of cells.

The middle and right columns of Fig. 3*A* show low- and high-power views, respectively, of serial sections of the islets shown in the left column stained with BrdU (black nuclei) and insulin (brown cytoplasm). As can be clearly seen, the majority of the cells in these rat islets are β-cells (insulin positive), and the BrdU incorporation is markedly increased in the β-cells of cyclin D_1- and cdk-4–exposed islets as compared with Ad.lacZ-treated islets. These studies document that the adenoviral delivery of cyclin D_1 and cdk-4 increases β-cell proliferation within isolated rat islets.

Human β-cells were also induced to replicate in adenovirally transduced islets. As can be seen in Fig. 3*B*, β-cells (insulin-positive cells) in Ad.lacZ-tranduced islets contained few BrdU-positive nuclei, whereas these were relatively abundant in cyclin D_1- and cdk-4–transduced islets. This is demonstrated quantitatively in Fig. 4. In Fig. 4*A*, the number of insulin- or CK-19–positive cells, expressed as a function of the total number of cells, is shown for each of the 10 human islet preparations used, and averages for these 10 preparations are shown in the two columns on the extreme right. This quantitative immunohistochemical

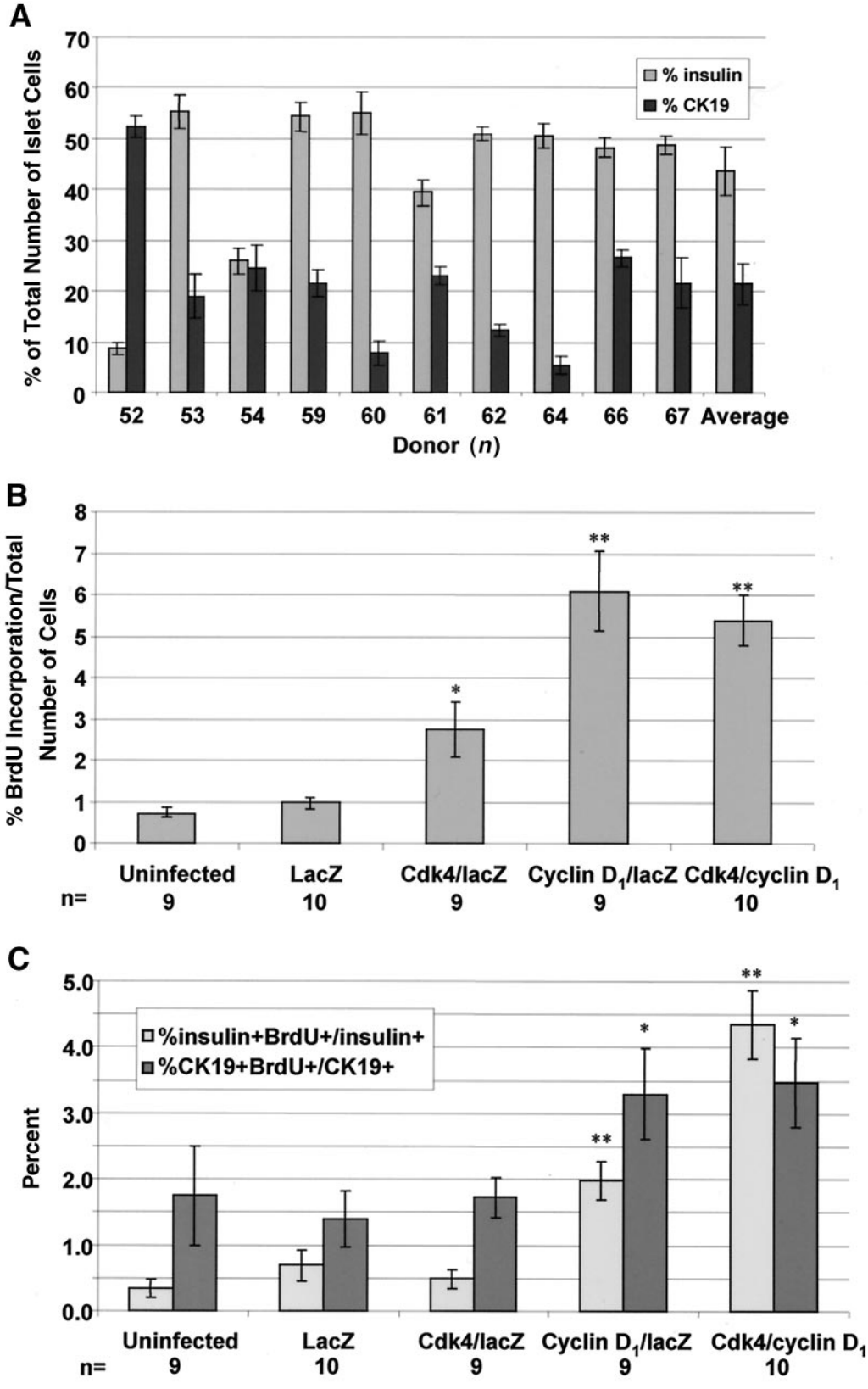

FIG. 4. Quantitation of BrdU incorporation into human islets. The number of cells scored in each experiment ranged from a minimum of 400 to a maximum of 5,000, depending on the number of islets contained per section. Results are means ± SE. *A*: The percentage of insulin and CK-19–positive cells as a function of the total number of cells in the 10 human islet preparations used. The last two columns represent the averages of the 10 islet preparations. *B*: The percentage of BrdU-positive nuclei per total islet cell number in adenovirus-transduced isolated human islets. *C*: The percentage of β (insulin positive) plus BrdU staining and ductal (CK-19) plus BrdU staining represented as a function of β or ductal cell subtypes, respectively. The transduction conditions are as in Fig. 3. *$P < 0.05$; **$P < 0.005$.

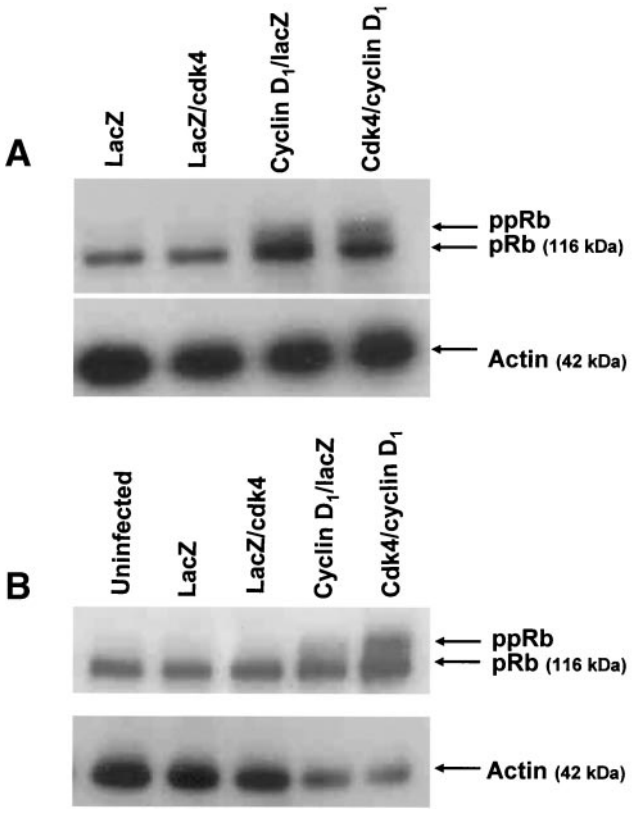

FIG. 5. Effect of cdk-4 and cyclin D_1 overexpression on pRb phosphorylation in rat (*A*) and human (*B*) islets. Ad.cdk-4 alone has no effect on pRb phosphorylation, whereas Ad.cyclin D_1 alone appears to have some ability to drive pRb phosphorylation. The combination of both cdk-4 and cyclin D_1 clearly converts pRb to its hyperphosphorylated form in both rat and human islets.

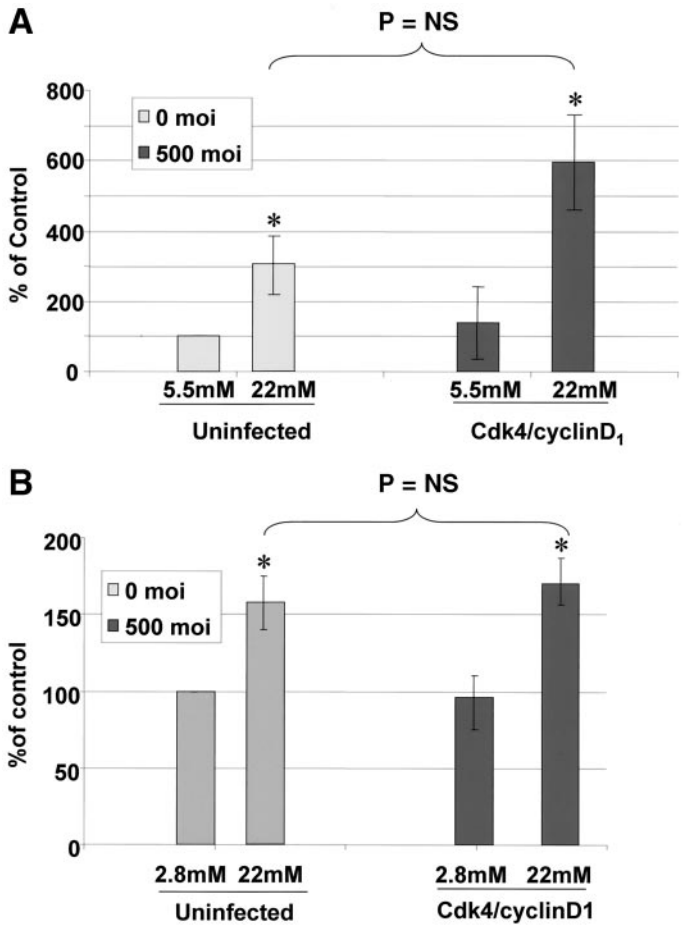

FIG. 6. *A*: GSIS in isolated rat islets at low (5.5 mmol/l) and high (22 mmol/l) glucose. *B*: GSIS in human islets at low (2.8 mmol/l) and high (22 mmol/l) glucose. Islets were either uninfected or transduced with the combination Ad.cdk-4/Ad.cyclin D_1 at 500 MOI. Results are shown as means ± SE of triplicate determinations in eight different preparations of rat islets and five of human islets. Ad.cyclin D_1/Ad.cdk-4 has no adverse effect on GSIS. *$P < 0.05$.

analysis indicated that β-cells and ductal cells in these isolated human islets constituted 43.7 ± 4.8 and 21.7 ± 4.1%, respectively (mean ± SE), of the cells within the 10 islet preparations.

The percent of BrdU-positive nuclei per total islet cell number for each of the virus additions as compared with controls is shown in Fig. 4*B*. As can be seen, cdk-4 alone increased total islet cell proliferation by approximately threefold, and cyclin D_1 alone or in combination with cdk-4 increased total islet cell proliferation by five- to sixfold. In Fig. 4*C*, BrdU immunostaining is displayed as a function of β (insulin-positive) and ductal (CK-19-positive) cell subtypes. No increase in β-cell proliferation was observed using cdk-4 alone, whereas an approximate 4-fold increase was seen in β-cells in islets transduced with cyclin D_1, and an approximate 10-fold increase in proliferation was observed in β-cells transduced using the combination of cdk-4 and cyclin D_1. With regard to CK-19 cells, cdk-4 alone had no effect, whereas cyclin D_1 or the cyclin D_1 plus cdk-4 combination stimulated proliferation by a factor of approximately twofold.

Cyclin D_1 and cdk-4 delivery increases pRb phosphorylation in rat and human islets. The phosphorylation status, indeed even the presence of pRb, in rat and human islets has not been described. As can be seen in Fig. 5*A*, uninfected (normal) and Ad.lacZ-transduced rat islets do contain pRb, but it is present in the un- or hypophosphorylated state (pRb). Ad.cdk-4 overexpression does not lead to increases in ppRb. In contrast, the exposure of isolated rat islets to either cyclin D_1 alone or combined with cdk-4 causes the phosphorylation of pRb to ppRb.

As can be seen in Fig. 5*B*, human islets also contain pRb. Ad.cdk-4 had no apparent effect on pRb phosphorylation. There was a suggestion of phosphorylation in the presence of cyclin D_1 overexpression, and phosphorylated ppRb was clearly present in human islets exposed to the combination of cdk-4 and cyclin D_1.

Adenovirus does not interfere with GSIS. Adenovirus can be toxic to cells. To help determine whether adenovirus at the MOI used had deleterious effects on the function of the islets, we examined GSIS in uninfected and cyclin D_1 plus cdk-4–transduced rat and human islets at 500 MOI. As can be seen in Figs. 6*A* and *B*, there was no difference between uninfected islets and cdk-4/cyclin D_1– transduced rat or human islets.

The effect of cdk-4 and cyclin D_1 on insulin, GLUT-2, glucokinase, sulfonylurea receptor-1, K^+ inward rectifier, and glucagon mRNA. Activation of cell proliferation in some systems results in de-differentiation. Accordingly, we examined the steady-state mRNA levels of the key β-cell molecules insulin, GLUT-2, glucokinase (GK), sulfonylurea receptor-1 (SUR-1), and K^+ inward rectifier 6.2 (Kir6.2), as well as glucagon, using semiquan-

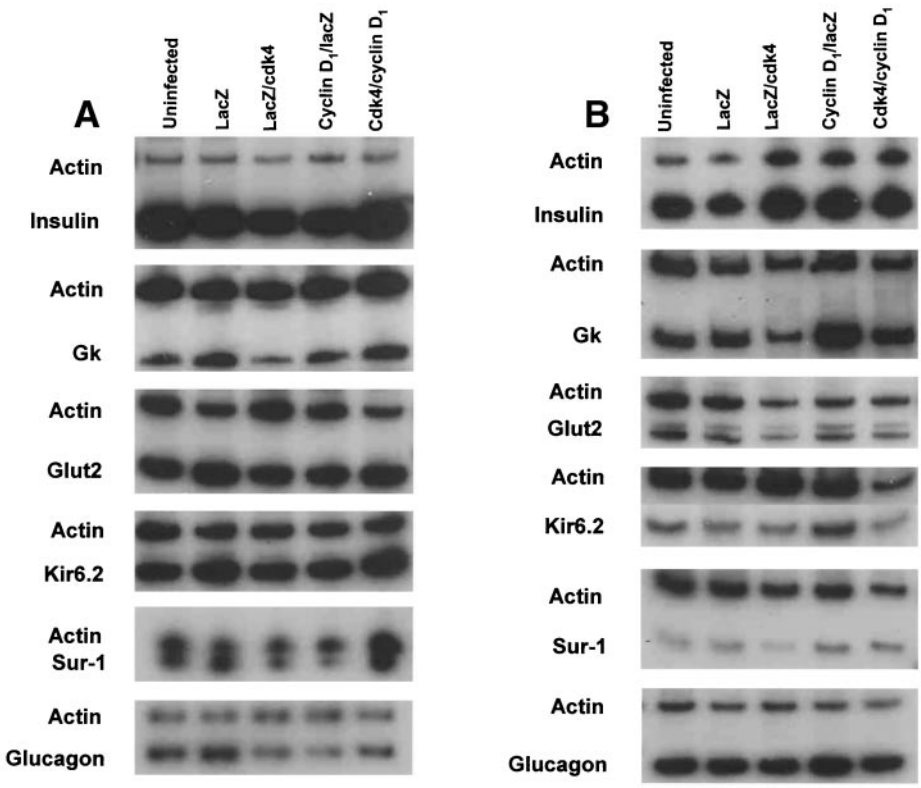

FIG. 7. The expression of islet differentiation markers in rat (*A*) and human (*B*) islets assessed using semiquantitative RT-PCR after transduction with Ad.cyclins. Expression of insulin, GK, GLUT-2, Kir6.2, SUR-1, and glucagon were measured in rat islets transduced with the adenoviruses shown above, using relative semiquantitative RT-PCR. β-Actin was the internal control.

titative RT-PCR. Representative data for rat and human islets are shown in Fig. 7. Although minor variations in some of these RNAs may be present, there does not appear to be a major downregulation of any of these RNAs.

The effect of cdk-4 and cyclin D_1 on islet cell death. Abnormal expression of cell cycle regulatory proteins activates cell death pathways in some systems. To determine whether the overexpression of cyclin D_1 or cdk-4 resulted in cell death in rat and/or human islets, we examined total islet cells and β-cells in five human islet preparations for nuclear pyknosis using propidium iodide staining. As shown in Fig. 8*A*, the rate of cell death is low, and there is no significant increase in the percent of either total islet cells or of β-cells in the cdk-4/cyclin D1–treated preparations as compared with the two control groups. A representative photomicrograph panel demonstrating each of the five conditions is shown in Fig. 8*B*.

DISCUSSION

In this report, we demonstrate for the first time that: *1*) the key cell cycle regulatory protein, pRb, is present in rat and human islets; *2*) it can be phosphorylated (i.e., inactivated) from the hypo- to hyperphosphorylated state in rat and human islets; *3*) this can be accomplished using a gene therapy approach with adenoviruses encoding cyclin D_1 and cdk-4; *4*) this results in 2- to 10-fold acceleration of the rate of replication of human and rat islet cells; and *5*) this occurs in β-cells as well as their CK-19–positive putative ductal cell precursors. Activation of the cell cycle has been documented in these experiments using three independent techniques: pRb phosphorylation, [^{3}H]thymidine incorporation, and BrdU immunohistochemistry.

It is important to emphasize that although ppRb phosphorylation should lead to cell cycle activation in β-cells, we have not proven in formal terms that the cell cycle activation is a result of ppRb phosphorylation. It is formally possible that other "pocket protein" family members such as p107 and p130 could be phosphorylated by cdk-4/cyclin D_1 and could mediate the effects of ppRb to activate E2F downstream pathways (4–6). Also, although we can manipulate pRb artificially using cdk-4/cyclin D_1, these observations shed no light on the physiological regulation of the β-cell cycle by potential upstream regulators of cyclin D_1/cdk-4, such as p16, p21, p27, and others. On the other hand, these results demonstrate that the pathway exists in β-cells and is experimentally accessible to study, including its potential physiological and pharmacological regulation by growth factors such as the placental lactogens, prolactin, hepatocyte growth factor, glucagon-like peptide, insulin-like growth factors, insulin, and other β-cell mitogens (1–3,24–29).

Interestingly, we found that CK-19–positive cells, which are felt to represent ductal cells and potentially β-cell precursors, could be induced to proliferate using cdk-4/cyclin D_1. This is of some interest, for it suggests that this technology might be useful therapeutically in expanding lines of β-cell precursors or expanding stem cell lines directed toward β-cell differentiation.

One surprise in the current study was that whereas the Ad.cdk-4 and Ad.cyclin D_1 viruses led to abundant expres-

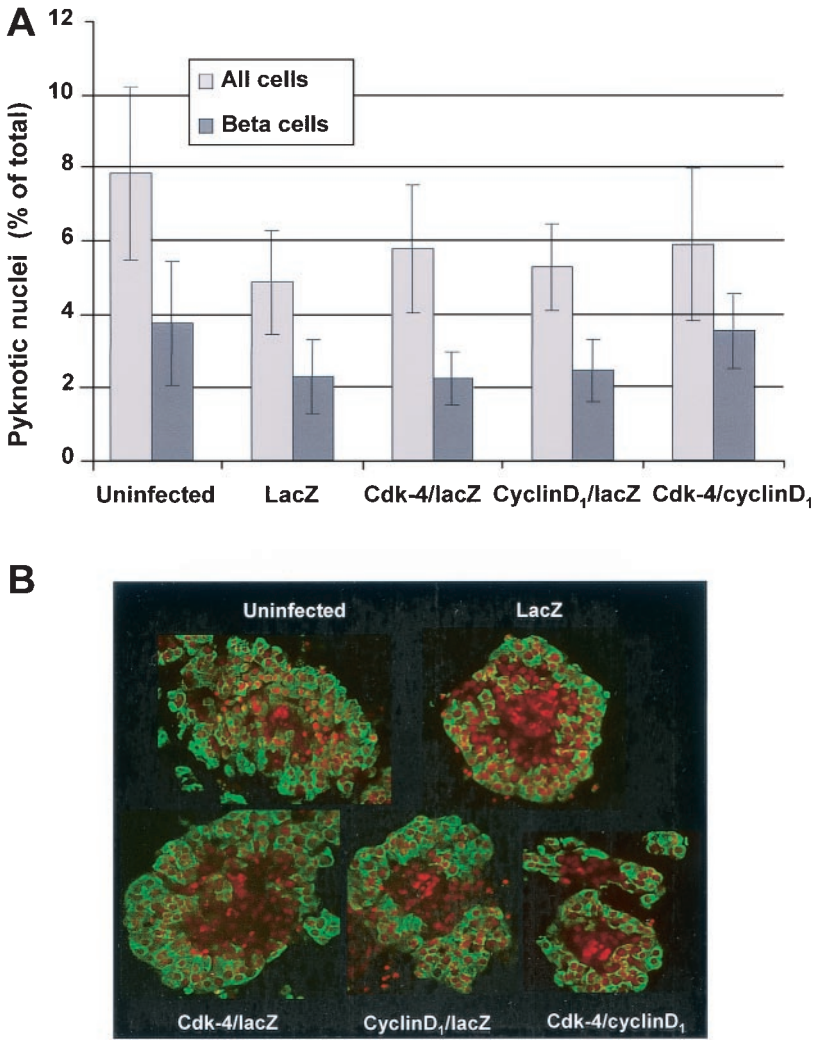

FIG. 8. Quantitation of cell death in human islets in response to Ad.cdk-4 and Ad.cyclin D_1. Islet sections from five human islet preparations used in Figs. 3 and 4 were stained using insulin (green) and propidium iodide (red), and total nuclei, pyknotic nuclei, β-cells, and β-cells containing pyknotic nuclei were quantitated. *A*: The percentage of total cells and of β-cells with nuclear pyknosis under each condition. Bars represent the standard error. *B*: Representative photomicrograph of each of the five conditions. There is no significant effect of Ad.cyclin overexpression on this measure of cell death.

sion of their corresponding proteins in isolated rat and human islets, when they were used in combination, the expression of both was attenuated. This was observed in three separate experiments using both rat and human islets. It cannot be caused by different total MOI values of the two viruses, for the total MOI was equal (500) in the rat and human experiments. We have not explored the molecular mechanisms which underlie this phenomenon, but we speculate that upregulation of one cyclin may accelerate the degradation of the other. Importantly, however, the residual amount of cdk-4 and/or cyclin D_1 was clearly sufficient to activate proliferation. The mechanism for the interactive downregulation of these two proteins needs to be examined further, but it suggests that there may be ways to further enhance both the level of expression of these two cyclins and their acceleration of β-cell proliferation.

In general, when one induces proliferation of a given cell type, one also induces de-differentiation. Thus, in the current context, it is important to note that despite the increased rate of proliferation, there is no evidence of de-differentiation of these islets. They retained their ability to sense glucose and secrete insulin, and they retained apparently normal levels of expression of key β-cell markers GLUT-2, glucokinase, insulin, SUR-1, and Kir6.2.

In some settings, overexpression of cyclins or cyclin-dependent kinases activates cell death pathways (4–6). If this were true for islets, the increase in proliferation observed could be balanced (or overbalanced) by an increase in cell death, with no net improvement (or a worsening) in islet mass. To begin to explore this possibility, we studied cell death in vitro in human islets overexpressing cdk-4/cyclin D_1 and found no measurable increase in either total islet cell death or β-cell death. Although these results are encouraging, we interpret them with caution, for rates of cell death are difficult to quantitate accurately, and in vitro observations may not predict in vivo events. We believe that the ultimate efficacy of cdk-4/cyclin D_1, and the balance of cell death versus cell replication, is best evaluated using an in vivo model, such as the transgenic or adenovirally transduced HGF models alluded to above (20,21,30).

Cyclin D_1 and cdk-4 are, or may be, oncogenes in some tissues (4–6). It is possible, therefore, that they alone or in combination could induce tumors in islets. Indeed, as noted above, cyclin D_1 has been associated with human insulinomas (13). On the other hand, as occurs with TAg, there is likely a requisite time of expression before which insulinoma does not occur (7–9). Thus, if necessary, one could engineer a vector to induce only temporary or transient expression so that early proliferation and engraftment could occur, but prolonged expression would not occur. Indeed, one of the negative features of the adenovirus is that it is nonintegrating and therefore permits only transient expression. In this setting, such a transient presence could serve not as a negative feature but as a positive attribute. Finally, whereas TAg is an oncogene in murine islets (7–9), transgenic life-long overexpression of cyclin D_1 in murine parathyroids results in parathyroid hyperplasia but not adenoma or carcinoma (31). Thus, although one must be vigilant for cdk-4/cyclin D_1–induced tumorigenesis, it is certainly possible that it may not occur. If it were to occur, it could possibly be resolved with appropriate regulation of the level and duration of expression.

In conclusion, we have demonstrated that cyclin D_1 alone or in combination with cdk-4 can induce β-cell proliferation in rat and human islets, that it is associated with phosphorylation of pRb, and that this approach is feasible in vitro as a method for increasing β-cell replication. Additional studies are required to address the function of the islets in vivo and the potential for tumorigenesis. If this approach proves to be efficacious in an in vivo model, it might potentially be applicable not only to isolated islets, but also to developing β-cell lines, to expanding populations of ductal cells or stem cell precursors of the β-cell, and to islets derived from xenograft sources, such as the pig or nonhuman primate. Finally, these studies document the accessibility of the molecular control of the cell cycle for study in rodent and human islets.

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