Protein Kinase C-ζ Activation Markedly Enhances β-Cell Proliferation

An Essential Role in Growth Factor–Mediated β-Cell Mitogenesis

Rupangi C. Vasavada,[1] Lin Wang,[1] Yuichi Fujinaka,[1] Karen K. Takane,[1] Taylor C. Rosa,[1] Jose M.D. Mellado-Gil,[1] Peter A. Friedman,[2] and Adolfo Garcia-Ocaña[1]

OBJECTIVE—Diabetes results from a deficiency of functional β-cells. Previous studies have identified hepatocyte growth factor (HGF) and parathyroid hormone–related protein (PTHrP) as two potent β-cell mitogens. The objective of this study is to determine *1*) whether HGF and PTHrP have additive/synergistic effects on β-cell growth and proliferation; *2*) the signaling pathways through which these growth factors mediate β-cell mitogenesis; and *3*) whether activation of this/these signaling pathway(s) enhances human β-cell replication.

RESEARCH DESIGN AND METHODS—We generated and phenotypically analyzed doubly transgenic mice overexpressing PTHrP and HGF in the β-cell. INS-1 and primary mouse and human islet cells were used to identify mitogenic signaling pathways activated by HGF and/or PTHrP.

RESULTS—Combined overexpression of HGF and PTHrP in the β-cell of doubly transgenic mice did not result in additive/synergistic effects on β-cell growth and proliferation, suggesting potential cross-talk between signaling pathways activated by both growth factors. Examination of these signaling pathways in INS-1 cells revealed atypical protein kinase C (PKC) as a novel intracellular target activated by both HGF and PTHrP in β-cells. Knockdown of PKCζ, but not PKCι/λ, expression using specific small-interfering RNAs blocked growth factor–induced INS-1 cell proliferation. Furthermore, adenovirus-mediated delivery of kinase-dead PKCζ completely inhibited β-cell proliferation in primary islet cells overexpressing PTHrP and/or HGF. Finally, adenovirus-mediated delivery of constitutively active PKCζ in mouse and human primary islet cells significantly enhanced β-cell proliferation.

CONCLUSIONS—PKCζ is essential for PTHrP- and HGF-induced β-cell proliferation. PKCζ activation could be useful in therapeutic strategies for expanding β-cell mass in vitro and in vivo. ***Diabetes*** **56:2732–2743, 2007**

Diabetes occurs when a decrease in the number of functional β-cells negatively affects the insulin supply required for glucose homeostasis. Renewal of β-cells in normal adult mice occurs mainly through proliferation of preexisting β-cells, suggesting that adult pancreatic β-cells retain a significant proliferative capacity (1). β-Cell proliferation can be stimulated in vitro and/or in vivo by several growth factors, including insulin, IGFs, glucagon-like peptide 1 (GLP-1), lactogens, parathyroid hormone–related protein (PTHrP), and hepatocyte growth factor (HGF) (2).

PTHrP, required for normal growth, survival, and differentiation of a number of tissues, is expressed in every tissue, including human and rodent islet cells (2,3). The PTH1 receptor (PTH1R), a seven transmembrane G-protein–coupled receptor for the NH_2-terminal region of PTHrP (4), colocalizes with β-cells in rodent islets (5). In vitro studies have demonstrated that PTHrP is mitogenic, antiapoptotic, and insulinotropic for rodent β-cells (2,6,7). HGF is a mitogenic, antiapoptotic, morphogenic, and angiogenic factor in cells expressing the tyrosine kinase receptor, c-Met (8). In the pancreas, c-Met is expressed in mouse ductal cells and rodent and human β-cells (9,10). In vitro studies have shown that HGF is a mitogen for rodent and human β-cells (2,9,11). To determine the effect of PTHrP and HGF in the β-cell in vivo, we previously generated transgenic mice overexpressing PTHrP or HGF in the β-cell through the use of the rat insulin II promoter (RIP). Both RIP-PTHrP and RIP-HGF transgenic mice display β-cell hyperplasia and improved glucose homeostasis (12–15).

Current therapeutic approaches for tissue regeneration include combinatorial use of growth factors that synergistically/additively interact to stimulate tissue regrowth. In the pancreas, synergistic/additive interactions between transforming growth factor-α/epidermal growth factor and gastrin can further enhance β-cell mass, ameliorating diabetes in rodent studies (16,17). In the search for growth factor combinations that could further increase β-cell proliferation and mass, we crossed RIP-PTHrP and RIP-HGF mice to generate doubly transgenic mice. Interestingly, simultaneous in vivo overexpression of both growth factors in the β-cell of doubly transgenic mice did not

From the [1]Department of Medicine, Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; and the [2]Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Adolfo Garcia-Ocaña, PhD, Division of Endocrinology, University of Pittsburgh, 200 Lothrop St., BST-E1156, Pittsburgh, PA 15261. E-mail: ocana@dom.pitt.edu.

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Adv-CA-PKCζ, adenovirus containing the cDNA of constitutively active PKCζ; Adv-KD-PKCζ, adenovirus containing the cDNA of kinase-dead PKCζ; Adv-LacZ, adenovirus containing the cDNA of β-galactosidase; BrdU, 5-bromo-2′-deoxyuridine; DAG, diacylglycerol; DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; GLP-1, glucagon-like peptide 1; HGF, hepatocyte growth factor; MOI, multiplicity of infection; PDK-1, phosphoinositide-dependent kinase 1; PI, phosphatidylinositol; PKC, protein kinase C; PTH1R, parathyroid hormone 1 receptor; PTHrP, parathyroid hormone–related protein; RIP, rat insulin II promoter; siRNA, small-interfering RNA.

result in additive/synergistic effects on β-cell proliferation and islet hyperplasia compared with single-transgenic siblings. These studies suggested that cross-talk between intracellular signals and/or a common rate-limiting target in the signaling cascades activated by PTHrP and HGF might prevent further enhancement in β-cell proliferation in doubly transgenic mice. Analysis of mitogenic signaling pathways activated by both HGF and PTHrP in the β-cell revealed that both growth factors converge on the atypical protein kinase C (PKC)ζ to drive β-cell proliferation.

The PKC family of proteins belongs to the protein kinase A, protein kinase G, and PKC (AGC) Ser/Thr protein kinases. It has been subdivided into three major groups, depending on their cofactor requirements: classical PKCs (α, β, and γ), which are Ca^{2+}/diacylglycerol (DAG) dependent; novel PKCs (δ, ε, η, and θ), which are Ca^{2+} independent but DAG dependent; and atypical PKCs (ζ and ι/λ), which are both Ca^{2+} independent and DAG independent (18). PKCζ, a relatively novel downstream target of phosphatidylinositol (PI) 3-kinase–phosphoinositide-dependent kinase-1 (PDK-1) activation, is critical for mitogenic signal transduction in a variety of cell types, including fibroblasts, glial cells, and oocytes (19–21). Interestingly, in the present studies, we have found that PKCζ activation is essential for HGF- and PTHrP-induced β-cell proliferation. Furthermore, constitutive activation of PKCζ enhances mouse and human β-cell replication in vitro. These studies, therefore, define an important role for PKCζ as a mediator of, and potential therapeutic target for, β-cell proliferation.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. The generation of RIP-PTHrP and RIP-HGF transgenic mice has been previously described (12,14). Transgenic lines with the highest transgene expression in RIP-PTHrP mice (C57B16XSJLXSencar), line 1799 (12), and RIP-HGF mice (CD-1), line 51 (14), were bred to obtain four mouse genotypes: normal, single-transgenic RIP-PTHrP or RIP-HGF, and doubly transgenic mice. Only littermates (3–6 months old) from the first generation obtained from cross-breeding of heterozygote RIP-HGF and RIP-PTHrP transgenic mice in their original backgrounds were used for these studies. All studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

Murine islet RNA isolation and RT-PCR. Mouse islets were isolated, RNA was extracted, and semiquantitative RT-PCR (35 cycles) was performed with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) and primers for HGF, PTHrP, c-met, PTH1R, and actin with actin competimers (Ambion, Austin, TX), as previously described (5,10).

Pancreas immunohistochemistry and histomorphometry. Pancreata were removed, fixed overnight in Bouin's solution, and embedded in paraffin. Immunostaining for insulin, HGF, PTHrP, and 5-bromo-2′-deoxyuridine (BrdU) was performed as previously reported (12,14). Histomorphometric analysis on insulin-stained sections was performed using the MetaMorph Imaging System Analysis software package (Universal Imaging, Downingtown, PA) (10).

BrdU incorporation in vivo. In vivo β-cell replication was determined by BrdU incorporation in mice injected intraperitoneally with BrdU (10 μl/g body wt) (Cell Proliferation kit; Amersham Pharmacia Biotech) and killed 6 h later. Pancreatic sections were costained for insulin and BrdU (10,14). At least 1,000 β-cell nuclei were counted per pancreas in a blinded fashion.

[^{3}H]thymidine incorporation in INS-1 cells. Rat insulinoma (INS-1) cells were seeded in 48-well plates at 4×10^4 cells/well in RPMI 1640 containing 11 mmol/l D-glucose and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, and 50 μmol/l β-mercaptoethanol (culture medium). Twenty-four hours later, cells were serum-depleted with medium containing 2 mmol/l D-glucose. After an additional 24 h, cells were re-fed with fresh medium without FBS and containing 2 or 20 mmol/l D-glucose, different agents (HGF, PTHrP, and signaling inhibitors), and [^{3}H]thymidine (0.5 μCi/well; Amersham Pharmacia Biotech). Wortmannin, PD98059 (Cell Signaling Technology, Danvers, MA), Gö6983, rottlerin, and the myristolated atypical PKCζ peptide inhibitor (Biomol International, Plymouth Meeting, PA) were added 30 min before HGF, PTHrP, HGF plus PTHrP, or vehicle. [^{3}H]thymidine incorporation was stopped 18–20 h later; radioactivity was corrected for protein levels measured by the Bradford method (22). Results are expressed as percentage of counts per minute incorporated per microgram of protein in control cells (100%).

Western blots. Total cell and islet extracts (30 μg) and INS-1 cell nuclear extracts (50 μg) were analyzed by immunoblot, and quantitative densitometry of digitalized blots was analyzed statistically (22,23). Primary antibodies for phospho-Ser729–PKCε, PKCζ, PKCι/λ (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-Thr638/641–PKCα/βII, phospho-Thr505–PKCδ, phospho-Thr410/403–PKCζ/ι/λ, Akt, phospho-Ser473-Akt, extracellular signal–regulated kinase (ERK)1/2, phospho-ERK1/2, and hemagglutinin (Cell Signaling Technology) were used at the dilutions indicated by the manufacturer.

PKCζ and PKCι/λ small-interfering RNA in INS-1 cells. INS-1 cells were seeded in six-well plates at 4×10^5 cells/well and kept in culture medium without antibiotics for 24 h. Then, rat PKCζ small-interfering RNA (siRNA), rat PKCι/λ siRNA, and control siRNA (scrambled sequences siRNA) (Dharmacon, Chicago, IL) oligonucleotides were diluted in OptiMEM containing Lipofectamine (Invitrogen, Carlsbad, CA), mixed gently, incubated at room temperature for 30 min, and added to the cells. After 6 h at 37°C, transfection medium was removed, and fresh culture medium was added for 24 h. Cells were then re-plated in 24-well plates and subsequently serum-depleted for [^{3}H]thymidine incorporation studies or Western blot analysis, as described above.

Generation of adenoviruses. Adenovirus containing the cDNA of constitutively active PKCζ (Adv-CA-PKCζ), kinase-dead PKCζ (Adv-KD-PKCζ), or β-galactosidase (Adv-LacZ) were prepared as previously described (24,25). The Adv-LacZ was supplied by Dr. Christopher Newgard (Duke University, Durham, NC). The constitutively active PKCζ and kinase-dead PKCζ cDNAs were provided by Dr. Alex Toker (Harvard Medical School, Boston, MA). The constitutively active PKCζ contains the NH_2-terminal c-Src myristoylation signal fused to PKCζ, which directs PKCζ to the plasma membrane leading to its constitutive activation. The kinase-dead PKCζ (K281W) contains a mutation in Lys-281 essential for its kinase activity, and it has been widely used to inhibit endogenous PKCζ activity (26). Both forms of PKCζ contain a hemagglutinin tag for monitoring expression. Adenovirus concentration was determined by optical density at 260 nm and by plaque assay.

BrdU incorporation in human and mouse primary islet cell cultures. Human islets were provided by the ICR Basic Science Islet Distribution Program. Single islet cell cultures were prepared by trypsinizing human or mouse islets for 8–10 min at 37°C. Trypsinization was stopped with RPMI containing 5 mmol/l D-glucose, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin; cells were plated on 12-mm glass coverslips, placed in 24-well plates, and incubated at 37°C for 2 h to allow cells to attach to the glass surface (27). Islet cells were simultaneously transduced with purified adenovirus at a multiplicity of infection (MOI) of 100 plaque-forming units per cell or left uninfected. Subsequently, complete medium was added, and cells were incubated for 24 h. Thereafter, mouse or human islet cells were serum-depleted overnight with RPMI medium containing 2 or 5 mmol/l D-glucose, respectively. BrdU (1:1,000 dilution) was then added for 18 h before cells were rinsed with PBS and subsequently fixed in 2% paraformaldehyde for 30 min at room temperature. To analyze β-cell proliferation rates, BrdU and insulin immunofluorescent staining was performed using an anti-insulin antibody (Zymed, South San Francisco, CA) at 1:50 dilution and an anti-BrdU antibody (Amersham Pharmacia Biotech) at 1:5 dilution (28). Visualization of staining was achieved using fluorescein isothiocyanate–conjugated anti–guinea pig IgG and tetramethylrhodamine isothiocyanate–conjugated rabbit anti-mouse IgG secondary antibodies (Sigma), respectively. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Analysis of transduction rates in primary islet cells was performed by hemagglutinin and insulin staining (28).

Statistical analysis. Data are expressed as the means ± SE. Statistical significance was determined by unpaired two-tailed Student's *t* test. Statistical significance was considered at $P < 0.05$.

RESULTS

Doubly transgenic mice do not display further improvement in glucose homeostasis compared with single-transgenic mice. Crossing the original RIP-PTHrP and RIP-HGF mice resulted in offspring of four distinct genotypes: normal, single-transgenic RIP-PTHrP, single-transgenic RIP-HGF, and doubly transgenic mice containing both PTHrP and HGF transgenes. Doubly transgenic mouse islets displayed increased HGF and PTHrP mRNA

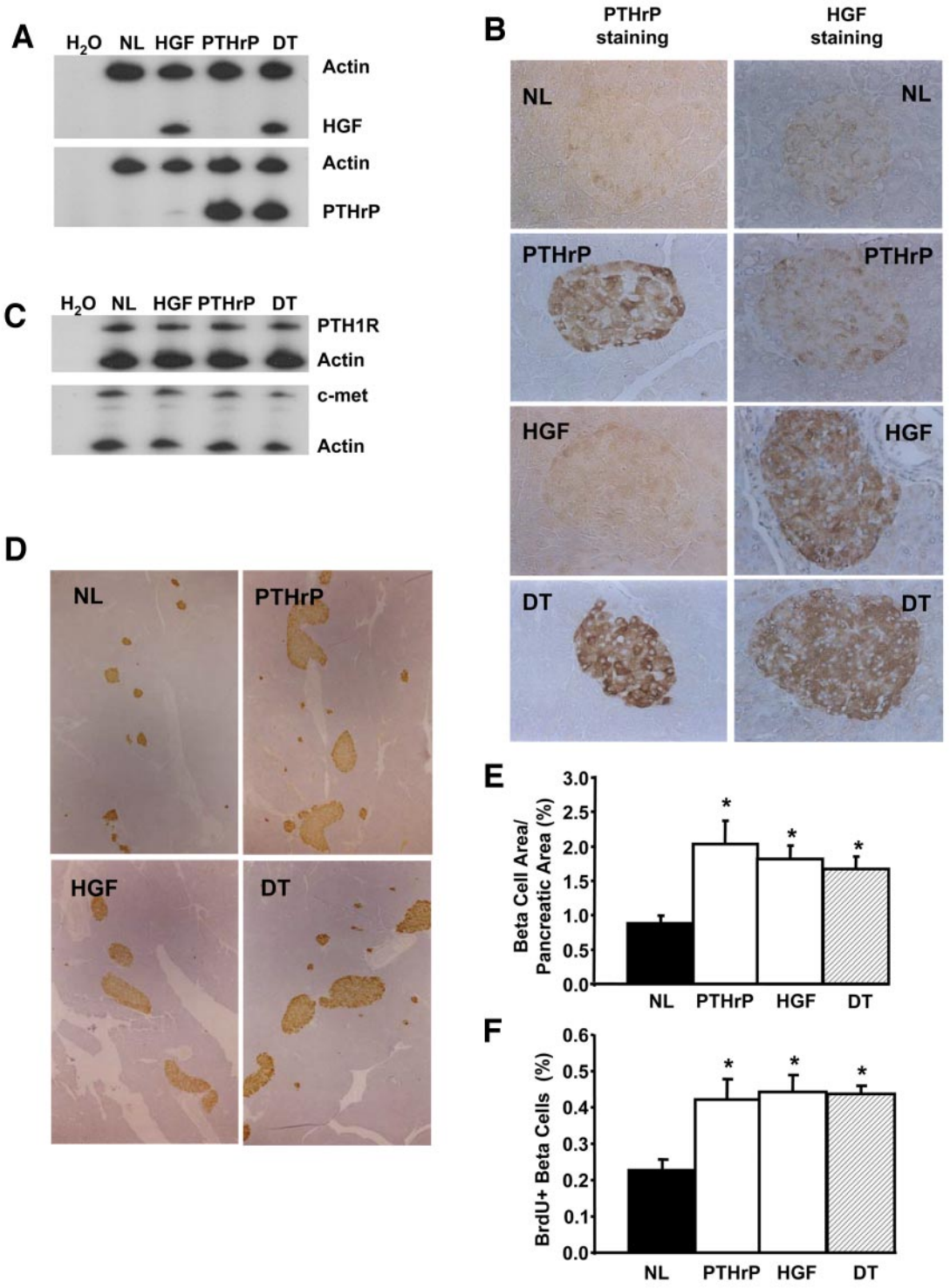

FIG. 1. Effect of PTHrP and/or HGF overexpression on β-cell growth and proliferation in single-transgenic HGF or PTHrP and doubly transgenic compared with normal (NL) mice. *A*: Semiquantitative RT-PCR measuring PTHrP and HGF mRNA in mouse islets. H_2O represents the negative control without RNA. *B*: Immunohistochemical staining of mouse pancreatic sections with antibodies to HGF or PTHrP. *C*: PTH1R and c-met mRNA expression was measured as in *A*. *D*: Low-magnification photomicrographs of insulin-stained pancreatic sections. *E* and *F*: Quantitative histomorphometric analysis of β-cell area per pancreatic area and BrdU incorporation in β-cells. Values are means ± SE of five to seven pancreata each. *$P < 0.05$ vs. NL. (Please see http://dx.doi.org/10.2337/db07-0461 for a high-quality digital representation of this figure.)

and protein expression compared with normal mouse islets, and this increase was similar to that observed in single-transgenic mouse islets (Fig. 1*A* and *B*). PTHrP and/or HGF overexpression in transgenic islets did not significantly alter PTH1R and c-met mRNA expression, as assessed by semiquantitative RT-PCR (Fig. 1*C*).

Single-transgenic RIP-PTHrP and RIP-HGF mice and doubly transgenic mice displayed significantly lower blood glucose, relative hyperinsulinemia, enhanced glucose tolerance, and superior glucose-stimulated insulin secretion in isolated islets compared with normal mice. However, doubly transgenic mice did not exhibit further enhance-

ment in these phenotypic characteristics compared with the single-transgenic mice (Supplemental Fig. 1, which is detailed in the online appendix [available at http://dx.doi.org/10.2337/db07-0461]).

Doubly transgenic mice do not display further increased β-cell mass and proliferation compared with single-transgenic mice. Insulin immunostaining of pancreatic sections showed that both single-transgenic and doubly transgenic mice have markedly enlarged islets compared with normal littermates (Fig. 1*D*). Quantitative histomorphometric analysis of these pancreatic sections revealed that β-cell mass (assessed as β-cell area per pancreatic area) (Fig. 1*E*) and islet number (not shown) were significantly increased in single-transgenic mice and in doubly transgenic mice compared with normal littermates. Interestingly, neither β-cell area per pancreatic area nor islet number showed a further increase in doubly transgenic mice relative to the single-transgenic littermates, indicating that simultaneous overexpression of HGF and PTHrP in the β-cell does not induce additive/synergistic effects on β-cell mass.

Analysis of in vivo BrdU incorporation in β-cells showed that doubly transgenic mice had a significant increase in β-cell proliferation rates compared with normal littermates. However, it was not significantly different compared with single-transgenic mice (Fig. 1*F*). Taken together, these results indicate that HGF and PTHrP overexpression in the β-cell of doubly transgenic mice does not further enhance β-cell growth or function compared with overexpression of each growth factor alone.

PTHrP and HGF increase INS-1 cell replication. To examine the mitogenic signaling pathways activated by PTHrP and HGF, we initiated studies on INS-1 cells. HGF and PTHrP(1–36) increased INS-1 cell replication in a dose-dependent manner under low glucose conditions, as assessed by [^{3}H]thymidine incorporation (Fig. 2*A*). Interestingly, the mitogenic effects of both peptides were nonadditive with the effect of 20 mmol/l glucose (Fig. 2*B*). Furthermore, combined addition of PTHrP and HGF did not induce additive proliferative effects (Fig. 2*A* and *B*), similar to observations in doubly transgenic β-cells in vivo (Fig. 1*F*). Taken together, these results indicate that INS-1 cells may serve as a useful model system for studying the intracellular mechanisms and the cross-talk underlying the nonadditive proliferative effects of these growth factors in β-cells.

HGF and PTHrP phosphorylate novel PKCs δ and ε and atypical PKCζ/ι/λ in INS-1 cells. We next explored which signaling pathways were phosphorylated and/or activated by PTHrP and HGF in INS-1 cells. No changes in cAMP or intracellular Ca^{2+} levels were observed in INS-1 cells treated with PTHrP(1–36) or HGF (Supplemental Fig. 2). We then analyzed which types of PKC are present and susceptible to phosphorylation by addition of PTHrP(1–36) and/or HGF in INS-1 cells, using Western blot analysis of representative phosphorylated PKCs: PKCα/βII (classical), PKCδ and -ε (novel), and PKCζ and -ι/λ (atypical). HGF, PTHrP(1–36), and the combination of PTHrP plus HGF did not significantly change the phosphorylation levels of PKCα/βII (Fig. 3*A*; Supplemental Fig. 3). In contrast to the classical PKCs, HGF and PTHrP(1–36) alone or in combination significantly increased PKCδ, PKCε, and PKCζ/ι/λ phosphorylation (Fig. 3*A*; Supplemental Fig. 3). Furthermore, nuclear extracts of INS-1 cells incubated with HGF, PTHrP(1–36), or the combination also displayed significantly increased levels of phospho-PKCζ/ι/λ (Fig. 3*B*; Supplemental Fig. 3) similar to that induced by GLP-1, previously shown to increase the presence of phospho-PKCζ/ι/λ in INS-1 cell nuclei (23).

Next, we analyzed whether PTHrP(1–36), HGF, or the combination could activate Akt and ERK1/2 in INS-1 cells. As expected, HGF significantly increased ERK1/2 and Akt phosphorylation. However, PTHrP(1–36) did not significantly activate either intracellular target, nor did it alter HGF-induced activation of these signaling proteins (Fig. 3*C* and *D*; Supplemental Fig. 3).

PI 3-kinase and PKC are involved in HGF- and PTHrP-mediated INS-1 cell proliferation. To examine the role of these signaling pathways in growth factor–mediated INS-1 cell proliferation, we used pharmacological inhibitors. The PI 3-kinase inhibitor, wortmannin (100 nmol/l), and the pan-PKC inhibitor, Gö6983 (200 nmol/l) (29), completely abolished HGF-, PTHrP(1–36)-, and HGF plus PTHrP–induced proliferative effects. These results indicate that activation of PI 3-kinase and some PKC isoform is essential for HGF- and PTHrP-induced INS-1 cell proliferation (Fig. 4*A*). In addition, the ERK1/2 inhibitor, PD98059 (10 μmol/l), did not alter PTHrP- or HGF-mediated INS-1 cell proliferation but significantly enhanced the proliferative effects of these two factors in combination. This result suggests that ERK1/2 activation mediated by HGF did not participate in HGF-induced proliferation and might possibly have a negative effect on PTHrP-mediated INS-1 cell replication.

Activation of PKCζ/ι/λ mediates HGF- and PTHrP-induced INS-1 cell proliferation. We next analyzed whether the novel PKCδ or the atypical PKCζ/ι/λ, both of which are phosphorylated by HGF and PTHrP (Fig. 3*A*), mediate HGF- and PTHrP-induced proliferation in INS-1 cells. As shown in Fig. 4*B*, 10 μmol/l rottlerin, the novel PKCδ inhibitor, did not alter PTHrP-mediated proliferation. Interestingly, rottlerin significantly enhanced the mitogenic effect of HGF and HGF plus PTHrP (Fig. 4*B*). Most importantly, the cell-permeable atypical PKCζ/ι/λ inhibitor completely abolished HGF-, PTHrP-, and HGF plus PTHrP–mediated INS-1 cell proliferation (Fig. 4*B*). Collectively, these results suggest that sequential activation of PI 3-kinase and atypical PKCs are essential for both HGF- and PTHrP-mediated mitogenic effects in INS-1 cells. Interestingly, atypical PKCζ/ι/λ phosphorylation induced by PTHrP, HGF, and PTHrP plus HGF was significantly inhibited in INS-1 cells treated with 100 nmol/l wortmannin (Supplemental Fig. 4).

siRNA-mediated downregulation of PKCζ but not PKCι/λ impairs HGF- and PTHrP-mediated INS-1 cell proliferation. Analysis of the presence of the two atypical PKC isoforms, PKCζ and PKCι/λ, by Western blot using specific antibodies for each isoform revealed that both are expressed in rodent and human islets and in rodent insulinoma lines, including INS-1 cells (Fig. 5*A*). We therefore analyzed the effect of specifically downregulating PKCζ or PKCι/λ expression on PTHrP- and HGF-mediated INS-1 cell proliferation using specific siRNA oligonucleotides. As shown in Fig. 5*B* and *C*, 100 nmol/l PKCζ siRNA oligonucleotides reduced 70–80% PKCζ expression without affecting PKCι/λ levels. Conversely, 50 nmol/l PKCι/λ siRNA oligonucleotides reduced 70–80% PKCι/λ expression without affecting PKCζ levels (Fig. 5*D* and *E*). Importantly, downregulation of PKCζ completely blunted the mitogenic effect of HGF, PTHrP, and HGF plus PTHrP (Fig. 5*F*). This effect was specific to PKCζ, because analogous downregulation of PKCι/λ did not affect prolifera-

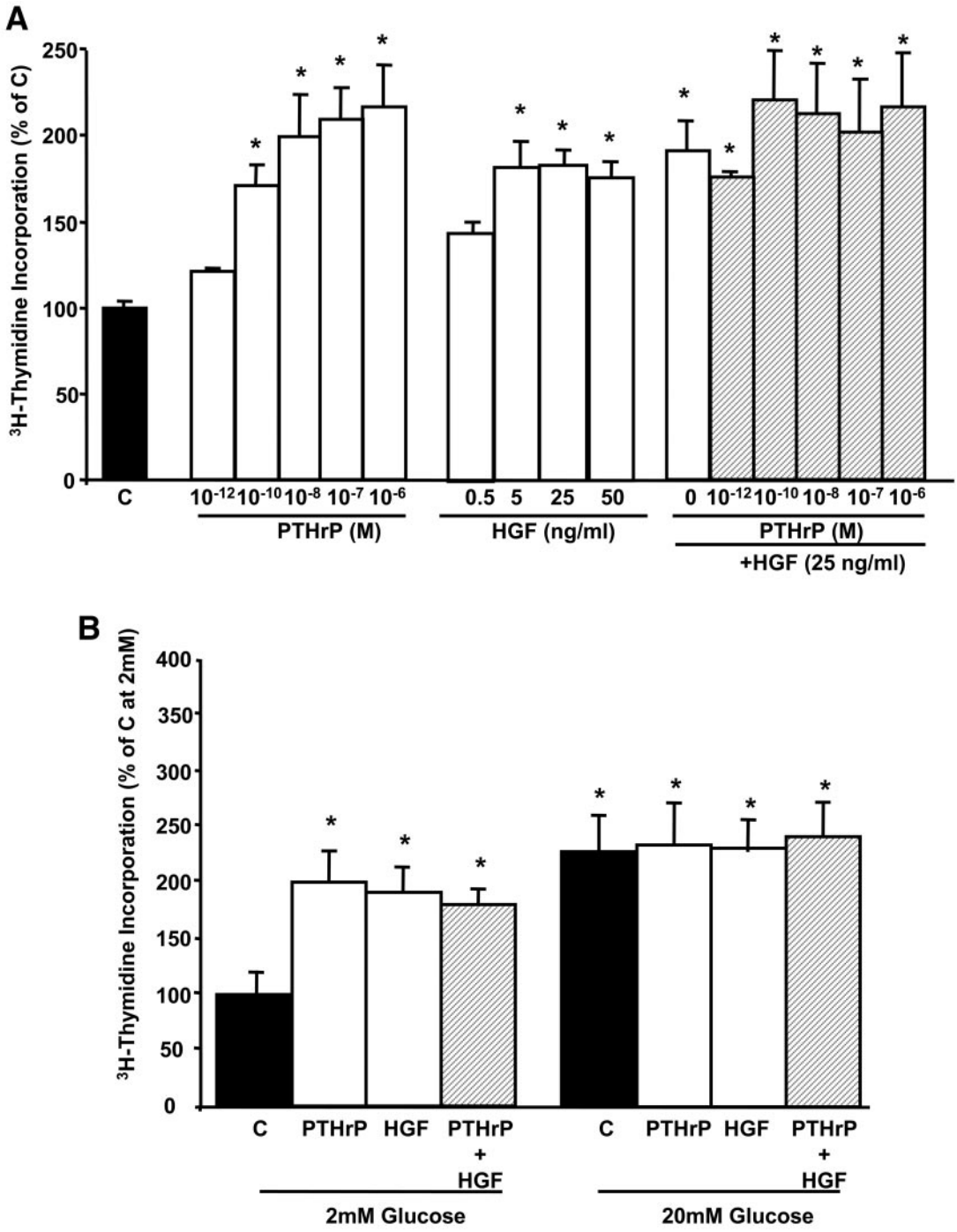

FIG. 2. HGF and PTHrP(1–36) increase INS-1 cell proliferation measured by [^{3}H]thymidine incorporation: absence of additive/synergistic effects. *A*: Proliferation was measured with different human PTHrP(1–36) and/or human HGF concentrations at 2 mmol/l glucose. *B*: Effect of 100 nmol/l PTHrP(1–36) and/or 25 ng/ml HGF at 2 and 20 mmol/l glucose. Results are expressed as percentage of control at 2 mmol/l glucose (C, 100%). Values are means ± SE of four experiments in triplicate. **P* < 0.05 vs. C at 2 mmol/l glucose.

tion mediated by these growth factors (Fig. 5*G*). Interestingly, downregulation of PKCζ or PKCι/λ did not decrease basal (2 mmol/l glucose) INS-1 cell proliferation. Collectively, these results indicate that HGF- and PTHrP-mediated INS-1 cell proliferation, but not basal proliferation, requires normal PKCζ expression.

Adenovirus-mediated transfer of kinase-dead PKCζ into HGF- and/or PTHrP-overexpressing primary mouse β-cells diminishes replication rates in vitro. The results described so far indicate that PKCζ is important for the mitogenic effects of HGF and PTHrP in INS-1 cells, but they do not provide any insight as to whether this occurs in normal primary β-cells. Therefore, we developed an in vitro assay for β-cell replication in primary islet cell cultures from single-transgenic RIP-PTHrP and RIP-HGF mice and doubly transgenic mice. As shown in Fig. 6*A*, the number of BrdU-positive nuclei in insulin-positive cells was increased in all transgenic compared with normal islet cell cultures. Quantitation of BrdU incorporation was markedly and significantly increased in RIP-HGF, RIP-PTHrP, and doubly transgenic mouse β-cells compared with normal β-cells (Fig. 6*B*). These results indicate that

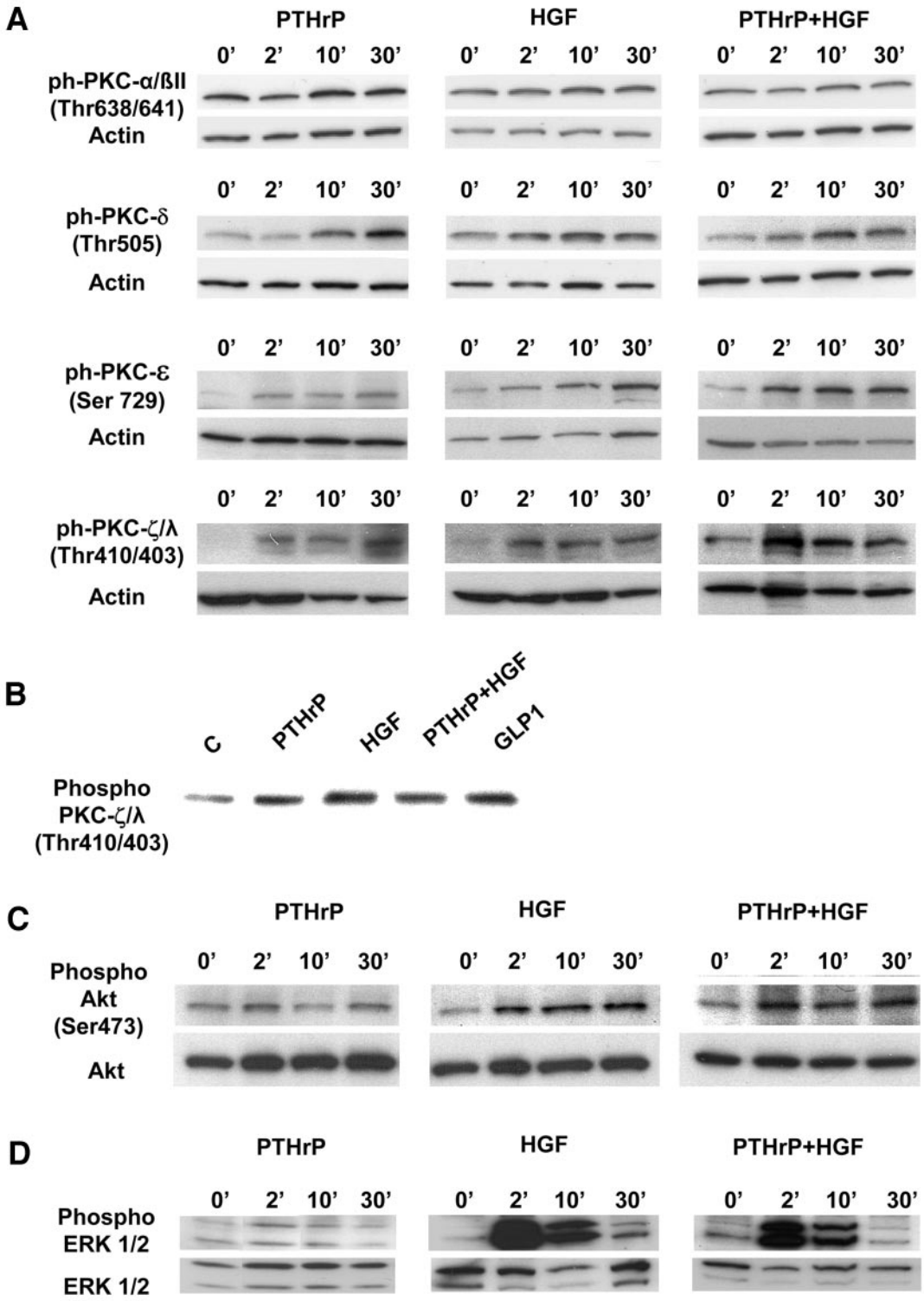

FIG. 3. Signaling pathway activation in INS-1 cells treated with 100 nmol/l human PTHrP(1–36) and/or 25 ng/ml human HGF at 2 mmol/l glucose by Western blot. *A*: Phosphorylation levels of PKCα/βII (Thr638/641), PKCδ (Thr505), PKCε (Ser729), and PKCζ/ι/λ (Thr410/403) in INS-1 cells treated for different time periods with HGF and/or PTHrP. *B*: Levels of phospho-Thr410/403–PKCζ/ι/λ in nuclear extracts of INS-1 cells treated for 5 min with vehicle (C), 100 nmol/l GLP-1, PTHrP, and/or HGF. Gel-loading equivalence was confirmed by staining of the membranes with Ponceau's solution. *C* and *D*: Phosphorylation levels of Akt (Ser473) and ERK1/2 in INS-1 cells treated as in *A*. In all cases, at least three experiments were performed with similar results.

transgenic mouse β-cells in culture maintain the increased proliferation status observed in vivo (Fig. 1*F*).

We next analyzed the effect of adenovirus-mediated transfer of a kinase-inactive PKCζ mutant (Adv-KD-PKCζ) on BrdU incorporation rates in β-cells from RIP-PTHrP, RIP-HGF, and doubly transgenic mice in culture. As shown in Fig. 6*C* and *D*, transduction of normal mouse islet cells with Adv-KD-PKCζ resulted in an 80–90% transduction rate and a marked increase in PKCζ expression levels, as assessed by both hemagglutinin tag immunofluorescence

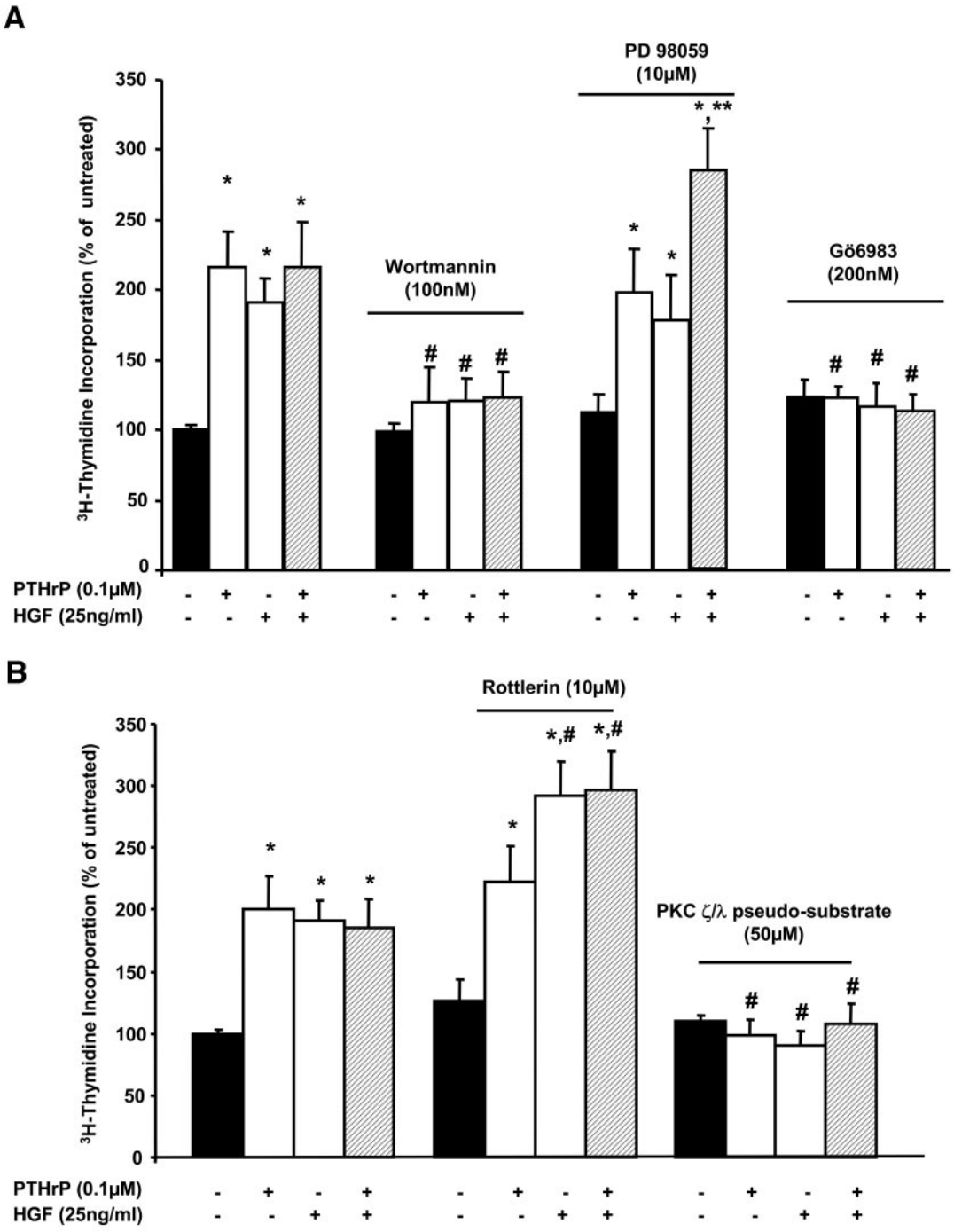

FIG. 4. Effect of 100 nmol/l wortmannin, 10 μmol/l PD98059, or 200 nmol/l Gö6983 (*A*) and 10 μmol/l rottlerin or 50 μmol/l cell-permeable PKCζ pseudosubstrate (*B*) on PTHrP-, HGF-, and PTHrP plus HGF–mediated INS-1 cell proliferation at 2 mmol/l glucose measured by [^{3}H]thymidine incorporation. Values are means ± SE of four experiments in triplicate. *$P < 0.05$ vs. vehicle control; #$P < 0.05$ vs. the corresponding peptides with no inhibitors; **$P < 0.05$ vs. individual peptides in the presence of PD98059.

and Western blot, respectively. Interestingly, transduction with kinase-dead PKCζ did not induce a decrease in basal (2 mmol/l glucose) β-cell proliferation in normal cells compared with uninfected or Adv-LacZ–transduced islet cell cultures (Fig. 6*E*). Most importantly, however, the increase in BrdU incorporation observed in RIP-PTHrP, RIP-HGF, and doubly transgenic mouse β-cells was eliminated when transduced with Adv-KD-PKCζ (Fig. 6*E*), indicating that proliferation of single-transgenic and doubly transgenic mouse β-cells requires PKCζ activity.

Adenovirus-mediated expression of constitutively active PKCζ in mouse and human islet cells in culture increases β-cell proliferation. Collectively, the knockdown and inhibitor studies indicate that PKCζ is a key mediator of HGF- and PTHrP-induced β-cell proliferation. To understand whether constitutively activated PKCζ could independently drive β-cell proliferation, we next used adenovirus-mediated transfer of constitutively active PKCζ (Adv-CA-PKCζ) in mouse and human primary islet cell cultures. Transduction of mouse islet cells with

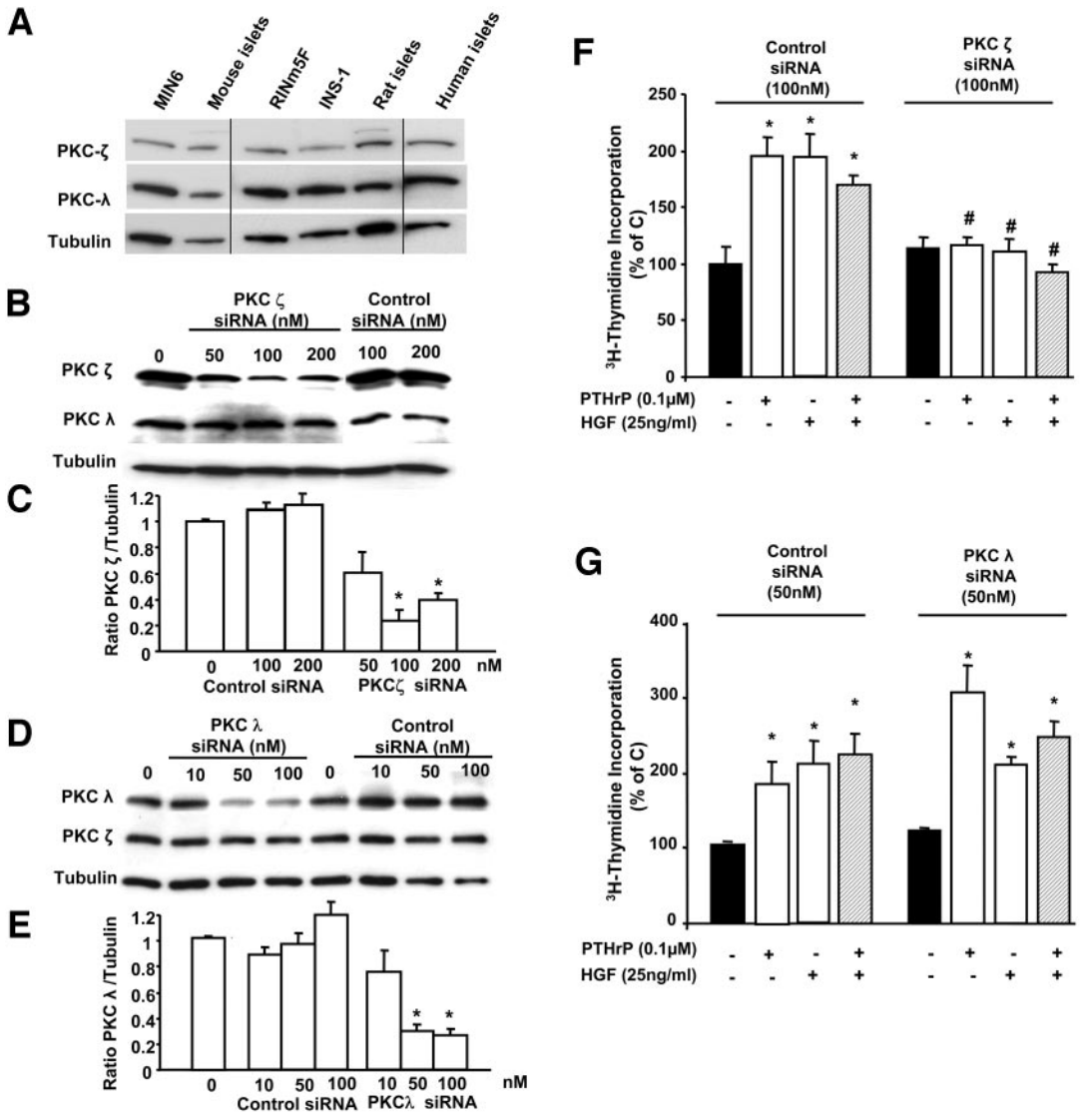

FIG. 5. Effect of siRNA-mediated downregulation of PKCζ and PKCι/λ on INS-1 cell proliferation induced by PTHrP(1–36) and/or HGF. *A*: Representative Western blot of PKCζ and PKCι/λ expression in mouse, rat, and human islets and three rodent insulinoma cell lines ($n = 3$). *B* and *D*: Representative Western blot of PKCζ and PKCι/λ expression in INS-1 cells 72 h after transfection with control, PKCζ (*B*), or PKCι/λ (*D*) siRNAs. Quantitation of PKCζ (*C*) and PKCι/λ (*E*) expression in INS-1 cells transfected with control, PKCζ, or PKCι/λ siRNAs ($n = 3$). *$P < 0.05$ vs. same dose of control siRNA. INS-1 cell proliferation measured by [^{3}H]thymidine incorporation in PKCζ (*F*) and PKCι/λ (*G*) siRNA-transfected cells treated with vehicle, 100 nmol/l human PTHrP(1–36), and/or 25 ng/ml human HGF ($n = 3$, each in triplicate). *$P < 0.05$ vs. corresponding control; #$P < 0.05$ vs. their corresponding peptide-treated cells transfected with control siRNA.

Adv-CA-PKCζ resulted in an 80–90% transduction rate and a marked increase in PKCζ expression and phosphorylation, as assessed by immunofluorescence (Fig. 6*C*) and Western blot (Fig. 7*A*), respectively. As shown in Fig. 7*B*, BrdU incorporation significantly increased (2.5-fold) in mouse β-cells transduced with Adv-CA-PKCζ compared with uninfected or Adv-LacZ–transduced β-cells. Most importantly, human islet cells in culture transduced with Adv-CA-PKCζ also displayed a significant three- to fourfold increase in β-cell proliferation compared with uninfected or Adv-LacZ–transduced cells (Fig. 7*C*). These results indicate that constitutive activation of PKCζ increases mouse and human β-cell proliferation in vitro.

DISCUSSION

These studies demonstrate a novel and critical role for PKCζ in regulating both rodent and human β-cell proliferation. They also demonstrate that both PTHrP and HGF act via the PI 3-kinase/PKCζ pathway to induce β-cell replication. Because the PI 3-kinase signaling pathway has been implicated in β-cell proliferation mediated by several growth factors and nutrients, such as glucose (11,30–34), and because PKCζ has been implicated in GLP-1–mediated INS-1 cell replication (24), it is likely that PI 3-kinase/PKCζ is a central pathway that controls growth factor– and glucose-mediated β-cell replication.

Contrary to our expectations, doubly transgenic mice overexpressing both PTHrP and HGF in the β-cell did not display further increased β-cell growth and proliferation compared with single-transgenic mouse counterparts. This led us to hypothesize that PTHrP and HGF might activate a common downstream signaling pathway in the β-cell and/or that negative cross-talk occurs among intracellular signals activated by these growth factors. Others had previously shown that PTHrP- and HGF-mediated proliferative effects are dependent on PKC activation in rat

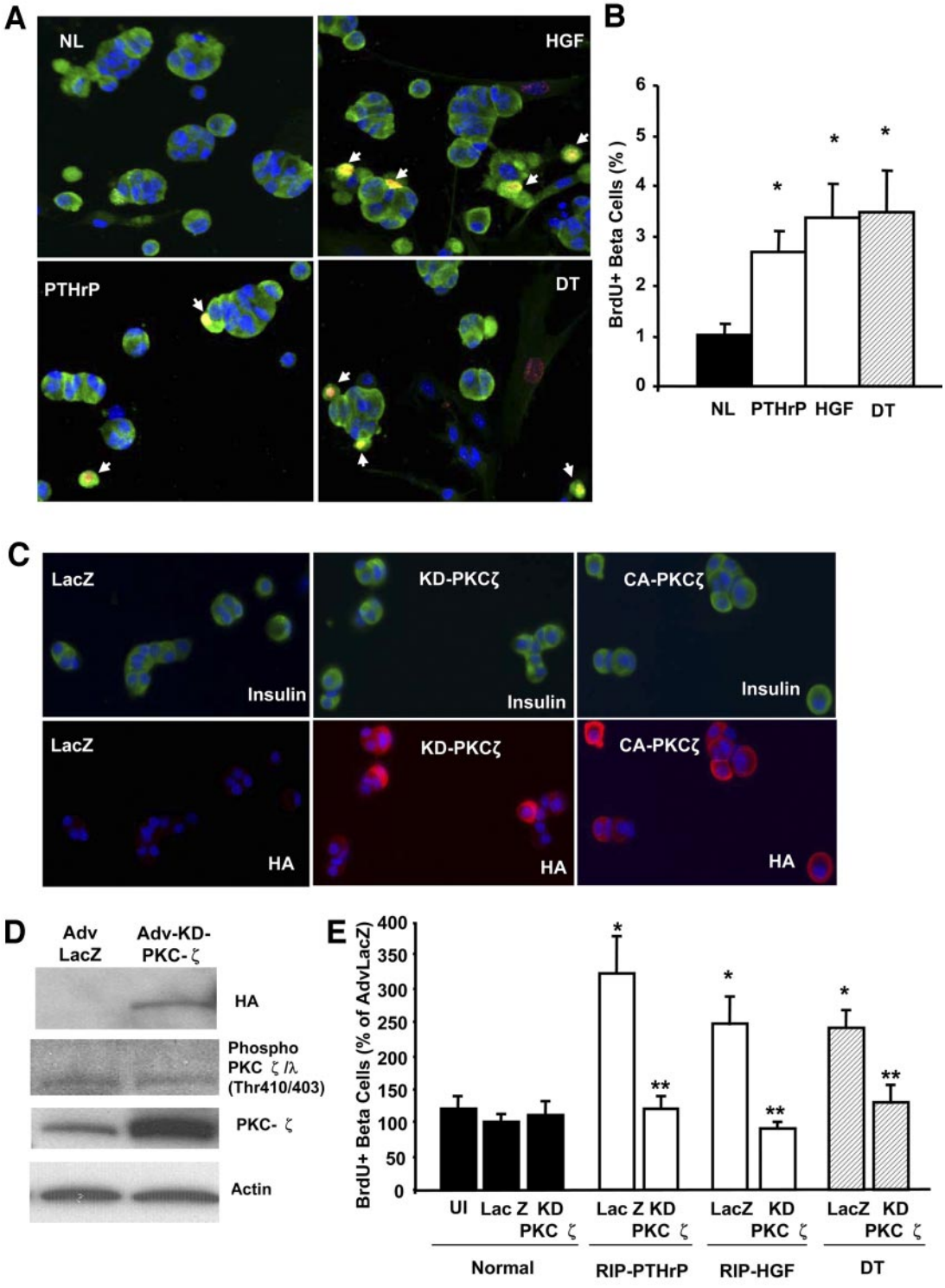

FIG. 6. Kinase-dead PKCζ expression inhibits single-transgenic PTHrP or HGF and doubly transgenic mouse β-cell proliferation. *A*: Representative photomicrographs of immunofluorescent staining for insulin (green), BrdU (red/orange), and DAPI (blue) in mouse primary islet cell cultures. Arrows indicate insulin-positive, BrdU-positive cells. *B*: Quantitation of percent BrdU-positive and insulin-positive cells in primary islet cell cultures from the four types of mice. $n = 4$ mice of each genotype; 2–3 islet cultures per mouse. *$P < 0.05$ vs. NL. *C*: Normal mouse primary islet cells 48 h after transduction with 100 MOI of Adv-LacZ, Adv-KD-PKCζ, or Adv-CA-PKCζ, stained for insulin (green), hemagglutinin (red), and DAPI (blue). *D*: Representative Western blot of hemagglutinin tag, phospho-Thr410/403–PKCζ/ι/λ, PKCζ, and actin expression in mouse islet extracts 48 h after transduction with 250 MOI of Adv-LacZ or Adv-KD-PKCζ ($n = 3$). *E*: Quantitation of percent BrdU-positive and insulin-positive cells in mouse primary islet cell cultures from the four types of mice transduced with 100 MOI of Adv-LacZ or Adv-KD-PKCζ. Results are expressed as percentage of Adv-LacZ–transduced NL β-cells (0.90 ± 0.20%). $n = 4$ mice of each genotype; 2 islet cultures per mouse per condition. *$P < 0.05$ vs. NL-Adv-LacZ; **$P < 0.05$ vs. single-transgenic or doubly transgenic mouse cells transduced with Adv-LacZ. (Please see http://dx.doi.org/10.2337/db07-0461 for a high-quality digital representation of this figure.)

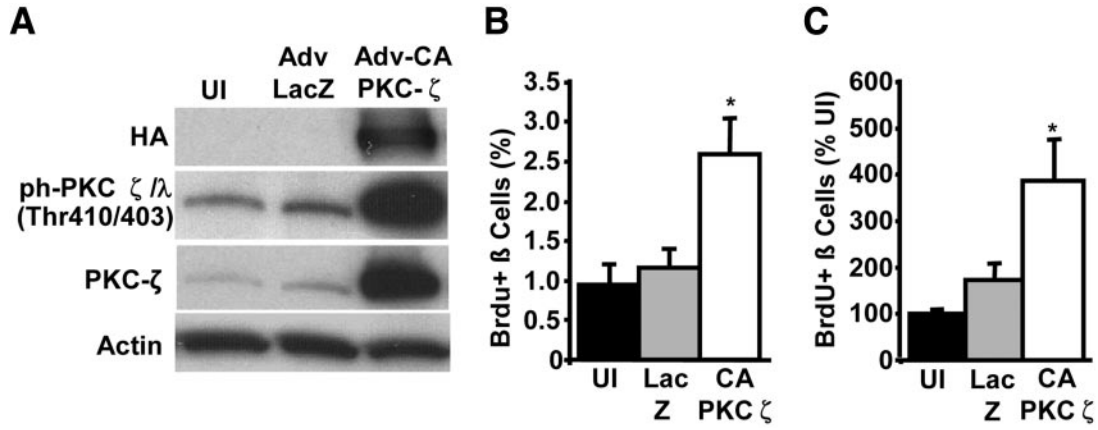

FIG. 7. Constitutively active PKCζ expression increases mouse and human β-cell proliferation. *A*: Representative Western blot of HA, phospho-Thr410/403–PKCζ/ι/λ, PKCζ, and actin expression in mouse islet extracts 48 h after transduction with 250 MOI of Adv-LacZ or Adv-CA-PKCζ ($n = 3$). *B* and *C*: Percentage of BrdU-positive and insulin-positive cells in primary islet cell cultures uninfected (UI) or transduced with 100 MOI of Adv-LacZ or Adv-CA-PKCζ in normal mouse islet cells (*B*) ($n = 4$ mice; 2 wells per condition; *$P < 0.05$ vs. uninfected and LacZ) and in human primary islet cell cultures (*C*) ($n = 3$ human islet preparations; 3 wells per condition per islet preparation) in which results are expressed as percentage of uninfected β-cells (0.66 ± 0.1%) (*$P < 0.05$ vs. UI and LacZ).

islets and insulinoma cells, respectively (6,11). Furthermore, PTHrP can activate the PI 3-kinase/Akt pathway in human osteoblast–derived osteosarcoma cells (35), as does HGF in INS-1 and mouse islet cells (11,24). However, whether PTHrP activates the PI 3-kinase/Akt pathway in the β-cell was unknown. Finally, activation of tyrosine kinase receptors has been reported to uncouple G-protein–coupled receptors from downstream signaling targets (36), suggesting potential negative cross-talk between these pathways.

Based on these observations, we decided to analyze in detail the signaling pathways stimulated by these two growth factors in the β-cell and to assess their impact on HGF- and PTHrP-mediated β-cell proliferation. We used INS-1 cells, because HGF and PTHrP each stimulate their proliferation, and the combination of both growth factors did not further enhance mitogenesis, mirroring the in vivo results in doubly transgenic mice. Of the seven signaling targets studied, i.e., cAMP, intracellular Ca^{2+}, the three PKC subtypes, Akt, and ERK1/2, only the novel PKCs δ and ε and atypical PKCζ/ι/λ were activated/phosphorylated by both PTHrP and HGF. Moreover, the level of activation/phosphorylation was not further enhanced by the combination of both growth factors. Importantly, the PI 3-kinase inhibitor, wortmannin, or the general PKC inhibitor, Gö6983, completely blocked PTHrP- and HGF-mediated INS-1 cell proliferation. Because PKCs can be activated by PI 3-kinase–PDK-1 (18,19), these results suggest that PTHrP and HGF induce INS-1 cell proliferation through the sequential activation of PI 3-kinase/PDK-1/PKCs (Fig. 8).

Recent studies have shown that PKCζ is critical for mitogenic signal transduction acting either as a kinase or as an adaptor protein in fibroblasts, oocytes, HeLa, HEK293, and glial cells (20,21,37). PKCζ is expressed in rodent islets and insulinoma cells, where it has been shown to be activated by glucose and GLP-1 (23,38–42). Buteau et al. (23) have shown that PKCζ activation is essential for GLP-1–mediated INS-1 cell proliferation. Furthermore, PKCζ overexpression enhances IGF-1–, insulin-, and serum-induced proliferation in RIN1046-38 cells (43). Here, we show that siRNA-mediated downregulation of PKCζ completely eliminated PTHrP- and HGF-induced proliferation in INS-1 cells. This result is important because it reveals that PKCζ is a novel and essential intracellular target for HGF- and PTHrP-mediated INS-1 cell proliferation. However, it does not address whether this is the case for the mitogenic effects of HGF and PTHrP in primary islet cell cultures or in vivo. Importantly, we observed that adenovirus-mediated expression of kinase-dead PKCζ in primary islet cells from single-transgenic PTHrP, single-transgenic HGF, or doubly transgenic mice fully blocked PTHrP- and/or HGF-mediated proliferation in β-cells. This suggests that not only the presence but the activation of PKCζ is needed for HGF and PTHrP to induce their mitogenic effects in primary β-cells (Fig. 8). On the other hand, basal (2 mmol/l glucose) β-cell proliferation was not affected by PKCζ downregulation or kinase-dead overexpression in INS-1 and mouse primary β-cells, respectively. Taken together, these studies indicate that activation of PKCζ by growth factors, such as PTHrP, HGF, or GLP-1, is an essential intracellular signaling requirement for growth factor–induced β-cell proliferation.

PKCι/λ is normally expressed in rodent islets and insu-

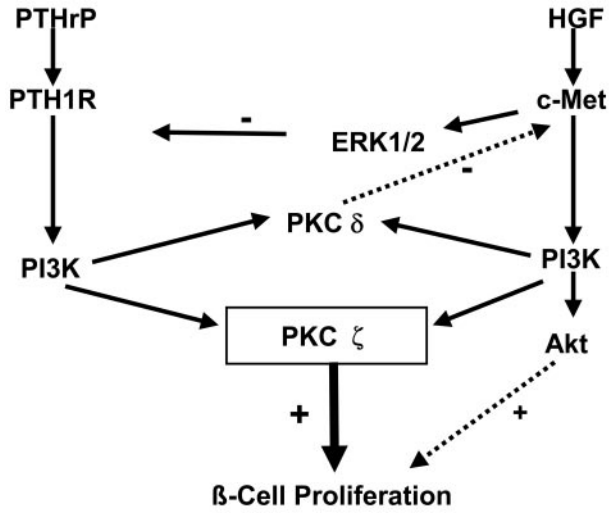

FIG. 8. Model for the mitogenic signaling pathways induced by PTHrP and HGF in the β-cell. Broken arrows denote potential regulatory pathways. PI3K, PI 3-kinase.

linoma cells and, as shown here, is also present in human islets (44,45). β-Cell–specific knockout mice for PKCι/λ display decreased glucose tolerance, hypoinsulinemia, and decreased glucose-stimulated insulin secretion without alterations in β-cell mass, suggesting that PKCι/λ plays a role in the regulation of β-cell function but is dispensable for β-cell proliferation (45). Similarly, in the current studies, siRNA-mediated downregulation of PKCι/λ did not affect basal, PTHrP-, HGF-, or PTHrP plus HGF–mediated INS-1 cell proliferation. Collectively, these observations suggest that atypical PKCι/λ may be important for normal β-cell function but not for basal- or growth factor–mediated β-cell proliferation.

Incubation of INS-1 cells with the novel PKCδ inhibitor, rottlerin, significantly enhanced the mitogenic effect of HGF but did not have any consequence on PTHrP-mediated INS-1 cell proliferation. Activation of PKCδ has been shown to be a negative regulator of β-cell proliferation induced by IGF-1 and glucose (46). Furthermore, PKCδ activation has also been shown to attenuate cellular responsiveness to HGF in A549 cells (47). Collectively, these results suggest that PKCδ may negatively regulate HGF-mediated β-cell proliferation (Fig. 8). However, because rottlerin inhibits other kinases with half-maximal inhibitory concentration values below the dose used in these studies (48), other technical approaches will be needed to determine whether repression of PKCδ further enhances growth factor–mediated β-cell proliferation.

Because growth factor–induced PKCζ activation enhanced β-cell proliferation, we wondered whether constitutive activation of PKCζ in mouse and human β-cells could induce an increase in basal cell proliferation. Previously, Buteau et al. (23) demonstrated that adenovirus-mediated expression of constitutively active PKCζ increases INS-1 cell proliferation. As shown in the present studies, constitutive activation of PKCζ markedly and significantly increased BrdU incorporation in normal mouse β-cells. Most importantly, constitutive activation of PKCζ markedly and significantly increased BrdU incorporation in human β-cells. This result suggests that approaches to increase the activation of PKCζ in human β-cells may be of value for future therapeutic strategies aimed at increasing human β-cell proliferation.

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