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Cyclin D1 activation through ATF-2 in Reg-induced pancreatic β-cell regeneration

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Abstract ***Regenerating gene*** **product (Reg) is induced in pancreatic β-cells and acts as an autocrine/paracrine growth factor for regeneration via a cell surface Reg receptor. However, the manner by which Reg induces β-cell regeneration was unknown. In the present study, we found that Reg increased phospho-ATF-2, which binds to −57 to −52 of the cyclin D1 gene to activate the promoter. The Reg/ATF-2-induced cyclin D1 promoter activation was attenuated by PI(3)K inhibitors such as LY294002 and wortmannin. In *Reg* knockout mouse islets, the levels of phospho-ATF-2, cyclin D1, and phospho-Rb were greatly decreased. These results indicate that the Reg–Reg receptor system stimulates the PI(3)K/ATF-2/cyclin D1 signaling pathway to induce β-cell regeneration.**

Keywords: Reg; Pancreatic β-cell; Cyclin D1, ATF-2, PI(3)K; Knockout mouse

1. Introduction

Strategies for preventing β-cell destruction and influencing the replication and growth of the β-cell mass are important for the prevention and/or treatment of diabetes [1,2]. We have previously demonstrated the mechanism of β-cell damage and its prevention [1–3], and further found an autocrine/paracrine β-cell regeneration factor, *Reg* (*regenerating gene*) protein [4,5], that increases the β-cell replication [6–10]. *Reg* is induced in insulin-producing pancreatic β-cells by inflammatory stimulation such as by IL-6/glucocorticoids [11] and acts as an autocrine/paracrine growth factor for β-cell regeneration via a cell surface Reg receptor [12] to ameliorate experimental diabetes [6–8]. Recently, we produced *Reg* transgenic and knockout mice [2,8]. The islets from the transgenic mice expressing Reg in β-cells showed increased [^{3}H]thymidine incorporation, and the development of diabetes in the non-obese diabetic (NOD) mice carrying the *Reg* transgene was significantly retarded. On the other hand, the [^{3}H]thymidine incorporation in the islets from *Reg* knockout mice was decreased, and the islets of *Reg* knockout mice were significantly smaller than those of control wild mice when the goldthioglucose-induced hyperplasticity of islets was compared [8]. Therefore, adequate expression of *Reg* gene appears to be essential for the regeneration of β-cells. However, the Reg-induced intracellular signal transduction pathway in pancreatic β-cells has been elusive. In the present study, we found that Reg stimulates the PI(3)K/ATF-2/cyclin D1 signaling pathway to induce β-cell regeneration.

2. Materials and methods

2.1. Antibodies and reagents

[γ-^{32}P]ATP and ECL Western Blotting Detection System were purchased from Amersham Pharmacia Biotech, Buckinghamshire, England. G418, DMRIE-C and pCMV-SPORT β-gal from Invitrogen, Carlsbad, CA. PD98059 and wortmannin from Wako Pure Chemistry, Osaka, Japan, and LY294002 and SB203580 from Sigma (St. Louis, MO). KN-62, KN-93 and H-89 were from Seikagaku Corp., Tokyo, Japan, and K-252a from Calbiochem (San Diego, CA). Pica Gene Luminescence Kit was from Toyo Ink Mfg. Co., Ltd., Tokyo, Japan, and Aurora GAL-XE from ICN, Costa Mesa, CA. pGL3-Basic vector and pCIneo mammalian expression vector were from Promega, Madison, WI and pFR-Luc, pFA2-cJun, pFA2-Elk1, pFA2-CREB, pFA2-CHOP, pFA2-cFOS, pFA2-ATF2, pFC-dbd, pFC-MEKK, pFC-MEK1, pFC-MEK3 and pFC-PKA for PathDetect analyses from Stratagene, La Jolla, CA. PI(3)K expression vector, anti-PI(3)K p110α antibody for immunoprecipitation, and anti-poly-(ADP-ribose) polymerase (PARP) antibody were purchased from Upstate Biotechnology, Lake Placid, NY. The anti-phospho-ATF-2 (Thr-71) antibody kit, anti-Rb, anti-phospho-Rb (Ser-780, Ser-795, Ser-807/811) antibody kit, anti-SAPK/JNK, anti-p38 MAPK, and ATF-2 fusion protein were from Cell Signaling, Beverly, MA, and anti-cyclin D1, anti-ATF-2, anti-phospho-ATF-2 (Thr-71), anti-Sp3, anti-PI(3)K p110α, and protein A/G PLUS agarose were from Santa Cruz, Santa Cruz, CA. The ChIP-IT chromatin immunoprecipitation and shearing kit was purchased from Active Motif, Carlsbad, CA.

2.2. Mice and islet culture

Mice lacking Reg were generated by homologous recombination [8]. The mice used for each experiment were derived from ICR background and were from the same litter or the same family. All animals were maintained in a 12-h light/12-h dark photoperiod in a humidity- and temperature-controlled room (24 °C). Water and food were available ad libitum. The animals used in this study were treated in accordance with the Guiding Principles for Care and Use of Research Animals promulgated by Tohoku University Graduate School of Medicine, Sendai, Japan. The islet culture was carried out as described [6,8].

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After the initial recovery period in RPMI-1640 containing 10% FCS and 2.7 mM glucose for 24 h, islets were transferred to 96-well culture dishes in groups of 10 islets. The islets were cultured in RPMI-1640 containing 2% FCS and 2.7 mM glucose for 48 h.

2.3. Construction of reporter genes and expression vectors

The rat cyclin D1 promoter [13] (−539 to +39) was amplified by PCR and the 578-bp fragment was subcloned into pGL3-Basic vector (−539CD1LUC). The constructions, −60CD1LUC, −60mut1CD1LUC, and −60mut2CD1LUC, were created by PCR from −539CD1LUC with specific primers, and the region including the ATF-2 binding site was mutated from 5′-AGTAACGTCACTCGGACTAC-3′ to 5′-AGTATGGTCACTCGGACTAC-3′ (−60mut1CD1LUC) or to 5′-AGTAACCACACTCGGACTAC-3′ (−60mut2CD1LUC). The expression vectors encoding the wild type rat ATF-2 [14] (nucleotide residues 328–1830) and a dominant-negative mutant of the ATF-2 encoding amino acid residues 77–487 (nucleotide residues 567–1830) were constructed in pCIneo mammalian expression vector [15].

2.4. Cell culture and transfections

RINm5F β-cells [11,12], which were derived from a rat insulinoma, were in RPMI1640 supplemented with 10% FCS. Forty-eight hours before transfection, 1×10^5 cells were seeded into each well of a 24-well plate. Transfections were performed with DMRIE-C as described [11]. After 24 h incubation at 37 °C in RPMI1640 with 1% FCS, the medium of each well was replaced with fresh medium containing Reg (33 nM) and protein kinase inhibitors and incubated for a further 6 h. Luciferase activity was determined as described [11] using a Pica Gene Luminescence Kit. β-Galactosidase activity was determined using Aurora GAL-XE.

2.5. Immunoblot analyses

The whole cell or nuclear extract was subjected to SDS–polyacrylamide gel electrophoresis and electrotransferred to a PVDF membrane as described [16]. Western blotting was carried out as described previously [11,12,16,17].

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated and reverse-transcribed as described [11,17] using 1 μg RNA as a template. PCR was performed on each reverse-transcribed sample (1/20, 1 μl) for 35 and 30 cycles for cyclin D1 and glyceraldehydes-3phosphate dehydrogenase (GAPDH), respectively. The sequences of the primers for cyclin D1 cDNA (GenBank accession number NM171992) amplification were 5′-CGCGCCCTCCGTTTCTTACTTCA-3′ and 5′-AACTTCTCGGCAGTCAGGGGA-3′; for GAPDH cDNA amplification, they were as described [11].

2.7. Immunekinase assay

Forty μg of PI(3)K p110α expression vector was introduced into RINm5F cells (8×10^6 cells) as described [17]. After 48 h, cells were harvested in 0.2 ml of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet® P-40, 0.5% sodium deoxycholate, 1 tablet/50 ml of Complete™). The lysate was incubated on ice for 1 h, followed by centrifugation at $100\,000 \times g$ for 1 h. Immunoprecipitation was performed using either anti-PI(3)K p110α rabbit antibody or anti-CD38 rabbit antibody [18] and Protein A/G PLUS agarose. Immunoprecipitates were washed and subjected to in vitro ATF-2 kinase assay using ATF-2 fusion protein as a substrate. The reactions were done in a volume of 5 μl containing 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mM glycerol 2-phosphate, 2 mM DTT, and 50 ng/μl ATF-2 fusion protein at 30 °C. The reaction was terminated by the addition of SDS–polyacrylamide gel electrophoresis sample buffer, run on 12.5% SDS gels, transferred to PVDF membrane, and phospho-ATF-2 was detected by immunoblot analysis using anti-phospho-ATF-2 (Thr-71) antibody.

2.8. Oligodeoxyribonucleotides and electrophoretic mobility shift assay (EMSA)

The wild type ATF-2 site of the cyclin D1 promoter, CD1W, and its mutants, CD1M1 and CD1M2, were synthesized as complementary oligodeoxyribonucleotide strands for EMSA. The sequences of the individual oligonucleotides in the sense orientation were as follows: CD1W, 5′-AACAACAGTAACGTCACTCGGAC-3′ corresponding to nucleotides −66 to −44 of rat cyclin D1 promoter; CD1M1, 5′-AACAACAGTATGGTCACTCGGAC-3′; CD1M2, 5′-AACAACAGTAACCACACTCGGAC-3′. EMSA was performed using nuclear extracts as described previously [11]. Mobility shift assays were performed as described [11]. When competition experiments were conducted in the presence of a 100-fold molar excess of cold probe, an unlabeled competitor was added with the labeled probe. In the supershift assay, 2 μg of antibody for supershift grade were added prior to the addition of the ^{32}P-probe.

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using a ChIP-IT kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. Unless otherwise stated, all reagents, buffers, and supplies were included in the kit. Briefly, RINm5F cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were re-suspended in lysis buffer and incubated for 30 min on ice. The cells were homogenized and nuclei were re-suspended in shearing buffer and subjected to optimized ultrasonic disruption conditions to yield 100–400-bp DNA fragments. The chromatin was precleared with protein G beads and incubated (overnight at 4 °C) with 2 μg of anti-ATF-2 (Santa Cruz). Protein G beads were then added to the antibody/chromatin incubation mixtures and incubated for 1.5 h at 4 °C. After extensive washing, the immunoprecipitated DNA was removed from the beads in an elution buffer. To reverse cross-links and remove RNA, 5 M NaCl and RNase were added to the samples and incubated for 4 h at 65 °C. The samples then were treated with proteinase K for 2 h at 42 °C, and the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 95 °C for 3 min, 35 cycles of 95 °C for 20 s, 64 °C for 20 s, and 72 °C for 1 min) of the *cyclin D*1 promoter using specific forward (5′-TTCTCTGCCCGGCTTTGATCTC-3′) and reverse (5′-CTCTCTGCTACTGCGCCAACA-3′) primers designed to amplify the promoter region (−92 to +27 nucleotides in relation to the transcription start site). The PCR products were resolved by electrophoresis in a 2% agarose gel and visualized after ethidium bromide staining.

3. Results

3.1. Reg induced ATF-2 activation

To determine what intracellular signal transduction events are induced in pancreatic β-cells by Reg, we tested several signal transduction pathways in pancreatic β-cells by Reg stimulation using PathDetect in vivo *trans*-reporting systems and found that ATF-2, but not CREB, cJun, Elk1, CHOP or cFos, was activated by Reg stimulation (Table 1). The ATF-2 activation was also observed by the cotransfection of the Reg recep-

Table 1
Induction of ATF-2 dependent transcription by Reg[a]

	Relative luciferase activity (%)	
	No addition	Reg stimulation
ATF-2	100 ± 78.0	590 ± 38.1*
CREB	100 ± 26.5	129.4 ± 14.7
cJun	100 ± 73.7	71.1 ± 10.5
Elk1	100 ± 9.23	113.8 ± 38.5
CHOP	100 ± 6.88	136.4 ± 3.12
cFos	100 ± 21.38	78.3 ± 12.15

[a]RINm5F β-cells were cotransfected with pFA-ATF2, pFA2-CREB, pFA2-cJun, pFA2-Elk1, pFA-CHOP, pFA-cFos, together with the pFR-Luc reporter plasmids. Cells were also transfected with the pFC-dbd plasmid as the vector control. Cells were stimulated with Reg (33 nM). The results are means ± S.E. of three to six experiments.
*$P < 0.02$ (different from no addition).

tor expression plasmid instead of by Reg addition to the medium (Fig. 1A). Phosphorylation of ATF-2 at Thr-71 was increased by the stimulation of Reg (Fig. 1B). These results indicate that the Reg–Reg receptor system activates ATF-2, suggesting that genes under the control of ATF-2 play an important role in the cell cycle progression in pancreatic β-cells.

3.2. Reg-induced cyclin D1 activation through ATF-2

We investigated whether the target of ATF-2 is the cyclin D1 promoter in Reg-activated pancreatic β-cells by using a reporter plasmid containing the rat cyclin D1 promoter (−539 to +39 of GenBank AF148946; -539CD1LUC) in transiently transfected RINm5F β-cells, a rat insulinoma cell line. The cyclin D1 promoter activation by Reg addition to the culture medium was induced in a dose-dependent manner (Fig. 2A) and, in fact, the endogenous levels of cyclin D1 and cyclin D1 mRNA were increased by Reg treatment 2 h and 30 min later, respectively (Fig. 2B). Furthermore, the cotransfection of an ATF-2 expression vector with the cyclin D1 reporter construct also increased the activity of the cyclin D1 promoter (Fig. 2C). The addition of Reg to the medium of the cells which had been cotransfected with ATF-2 expression plasmid did not induce significantly more promoter activity than the ATF-2 cotransfection or Reg addition alone. The increases in the cyclin D1 promoter were almost the same as the increases in phospho-ATF-2 (Fig. 2C, lower panel). A deleted promoter (−60CD1LUC) containing the possible ATF-2 binding site (−57 to −52) still responded to the Reg addition as well as to the cotransfection of ATF-2 expression plasmid, but reporter mutants in which the ATF-2 site had been destroyed (−60mut1CD1LUC and −60mut2CD1LUC) showed no increase of promoter activity by either the Reg addition or the ATF-2 cotransfection (Fig. 2D). Electrophoretic mobility shift assays were then performed with nuclear extracts from RINm5F β-cells and ^{32}P-labeled double-strand oligonucleotide corresponding to the ATF-2 binding sequence of cyclin D1 promoter. As shown in Fig. 2E, Reg-stimulated RINm5F β-cell nuclear proteins formed a specific complex with the ATF-2 site, which could be blocked by a 100-fold excess of unlabeled probe. Addition of the antibody against ATF-2 resulted in the formation of a supershift, suggesting the presence of ATF-2 in the complex. This was further supported by the result that the addition of the antibody against phospho-ATF-2 at Thr-71 resulted in the formation of a supershift in the nuclear extract from Reg-stimulated RINm5F β-cells. The ATF-2 binding to the cyclin D1 promoter in response to Reg stimulation was further demonstrated by ChIP assay (Fig. 2F). The essential involvement of ATF-2 activation in the Reg-induced activation of the cyclin D1 promoter was further confirmed by the inhibition of the Reg-induced activation of the cyclin D1 promoter with the cotransfection of the dominant-negative ATF-2 expression plasmid (Fig. 2G).

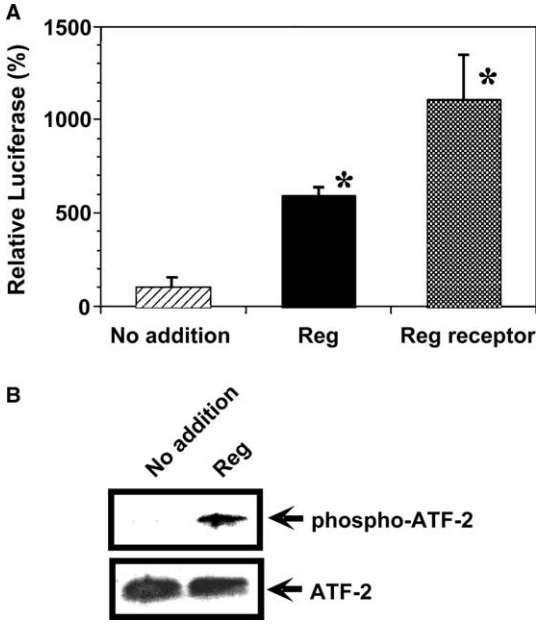

Fig. 1. Reg–Reg receptor signaling induces ATF-2 activation. (A) Overexpression of Reg receptor induces ATF-2 dependent transcription. RINm5F β-cells were cotransfected with pCIneo-Reg receptor expression plasmid [12] together with pFA-ATF2 and pFR-Luc plasmids. The results are means ± S.E. of three experiments. *, $P < 0.02$ (different from no addition). (B) Phosphorylation of ATF-2 at Thr-71 by Reg. RINm5F β-cell extracts were immunoblotted with a phospho-Thr-71-specific antibody for ATF-2, followed by reprobing with an anti-ATF-2 antibody to control for loading.

3.3. Involvement of the PI(3) kinase pathway in ATF-2 activation for cyclin promoter activity

To determine the intracellular signaling pathway(s) by which Reg induced the cyclin D1 promoter activity via the ATF-2 activation, several chemical inhibitors were employed. The addition of PI(3)K inhibitors LY294002 and wortmannin attenuated the Reg-induced ATF-2 phosphorylation/activation (Fig. 3A). Furthermore, the addition of PI(3)K inhibitors LY294002 and wortmannin to cells transfected with ATF-2 and the cyclin D1 promoter reporter showed attenuated ATF-2-induced cyclin D1 promoter activity (Fig. 3B) whereas SB203580, a p38 mitogen-activated protein (MAP) kinase inhibitor, PD98059, an inhibitor of the MAP kinase/ERK kinase-ERK pathway, KN-62 and KN-93, inhibitors of Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) II, K-252a, an inhibitor of CaM kinase IV, and the A-kinase inhibitor, H-89 showed a very weak (but statistically not significant) inhibitory effect on the cyclin D1 promoter activity in β-cells. In addition, overexpression of MEKK, MEK1, MEK3, and the catalytic subunit of PKA failed to increase the cyclin D1 promoter activity (not shown). The Reg-induced cyclin D1 promoter activity was actually inhibited by the addition of LY294002, whereas PD98059 scarcely reduced the activity (Fig. 3C). These results likely indicate that an LY294002-sensitive pathway involving PI(3)K mediates the signal transduction by Reg. Moreover, the immunoprecipitated PI(3)K phosphorylated ATF-2 in a time-dependent and dose-dependent fashion (Fig. 3D and E), indicating that PI(3)K directly phosphorylates ATF-2 to activate the cyclin D1 promoter.

3.4. Decreases in phospho-ATF-2, cyclin D1, and phospho-Rb in Reg knockout islets

We produced Reg-knockout mice by homologous recombination [8]. In $Reg^{-/-}$ mice, no Reg expression was detected in the pancreas and therefore no Reg was secreted into the islet culture medium [8]. We isolated pancreatic islets from $Reg^{+/+}$ and $Reg^{-/-}$ mice and compared their BrdU incorporation

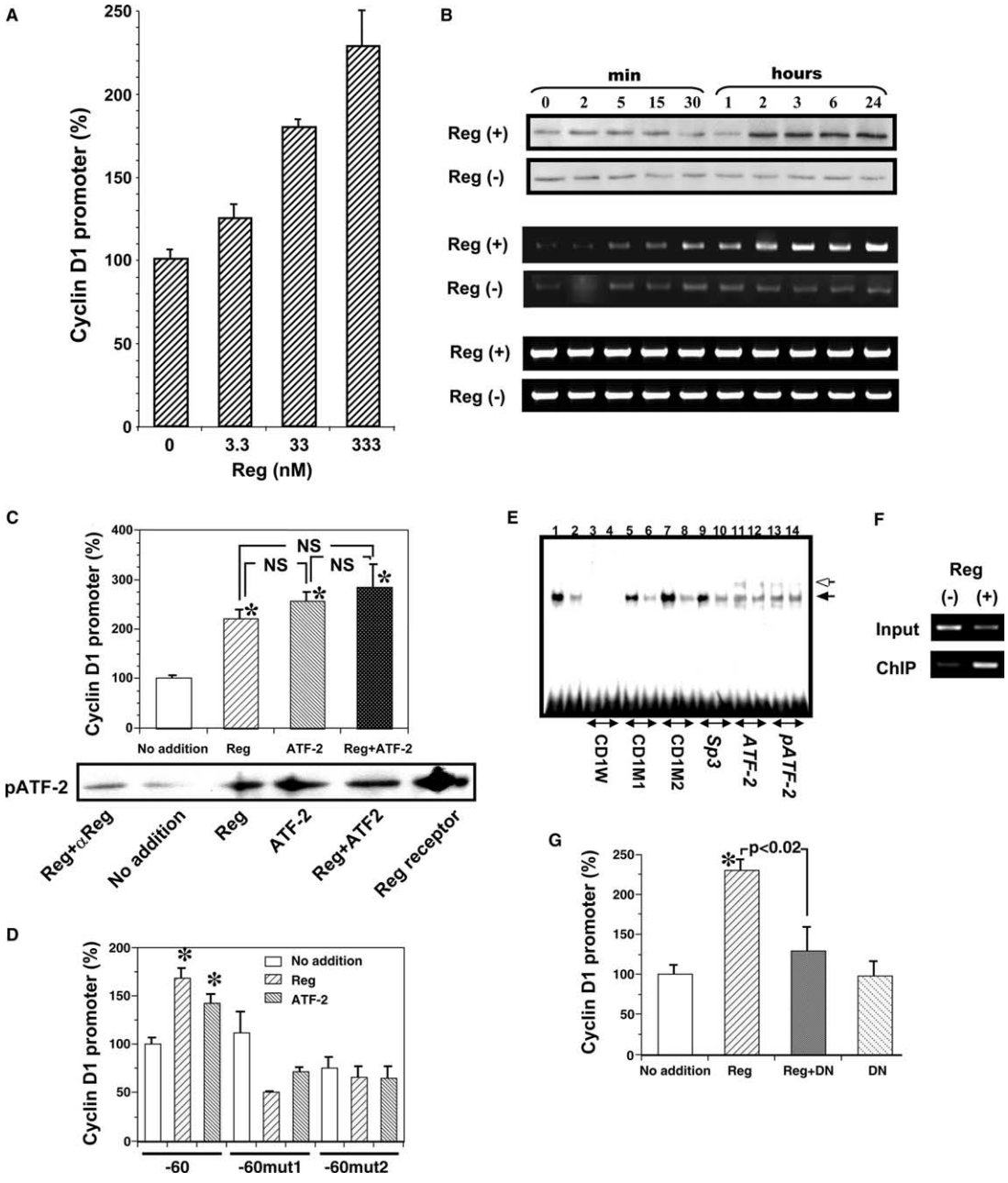

Fig. 2. Activation of the cyclin D1 promoter by ATF-2. (A) Reg induced the cyclin D1 promoter in a dose-dependent manner. RINm5F β-cells were transfected with −539CD1LUC and stimulated with increasing concentrations of Reg. The results are means ± S.E. of three experiments. (B) Induction of cyclin D1 by Reg treatment. RINm5F β-cells were treated with Reg (+) or without Reg (−) at 33 nM Reg. Cells were harvested at the indicated time after the stimulation. Upper panel shows immunoblot analysis of cyclin D1. Middle and lower panels show RT-PCR analyses of cyclin D1 and GAPDH, respectively. (C) Reg and ATF-2 induced the cyclin D1 promoter. RINm5F β-cells were transfected with −539CD1LUC and ATF-2 expression plasmid or with −539CD1LUC alone, and stimulated with Reg. The results are means ± S.E. of three to five experiments. *, $P < 0.01$ (different from no addition). NS, not significant. Phosphorylation status of ATF-2 by immunoblotting is also shown in the lower panel. Reg (33 nM) and anti-Reg monoclonal antibody (αReg; 5 μg/ml) [11] were added in the culture medium. Phosphorylation of ATF-2 by the addition of Reg was attenuated by the addition of anti-Reg monoclonal antibody, and Reg receptor overexpression without the addition of Reg increased the phosphorylation of ATF-2. (D) Localization of the ATF-2-dependent cyclin D1 promoter. RINm5F β-cells were cotransfected with reporter plasmids (−60CD1LUC, −60mut1CD1LUC or −60mut2CD1LUC) together with the ATF-2 expression plasmid. Cells were also transfected with either reporter plasmid alone and then stimulated with Reg. The results are the means ± S.E. of three to six experiments. *, $P < 0.01$ (different from no addition). (E) ATF-2 binds to the cyclin D1 promoter. Nuclear extracts from RINm5F β-cells were incubated with ^{32}P-labeled cyclin D1 promoter DNA. Nuclear extracts from Reg-treated cells were applied onto lanes 1, 3, 5, 7, 9, 11, and 13; those from untreated cells were applied onto lanes 2, 4, 6, 8, 10, 12, and 14. (F) ChIP assay showing Reg increases in ATF-2 binding to the cyclin D1 promoter. Nuclear proteins bound to genomic DNA in RINm5F cells were cross-linked and subjected to a ChIP assay using an antibody against ATF-2, and oligonucleotide primers to PCR amplify the cyclin D1 promoter. (G) ATF-2 activity is necessary for the activation of the cyclin D1 promoter by Reg. RINm5F β-cells were cotransfected with the −539CD1LUC reporter and either an empty expression vector or an expression vector for dominant-negative ATF-2 (DN). Cells were then stimulated with Reg (33 nM). The results are means ± S.E. of three to five experiments. *, $P < 0.01$ (different from no addition).

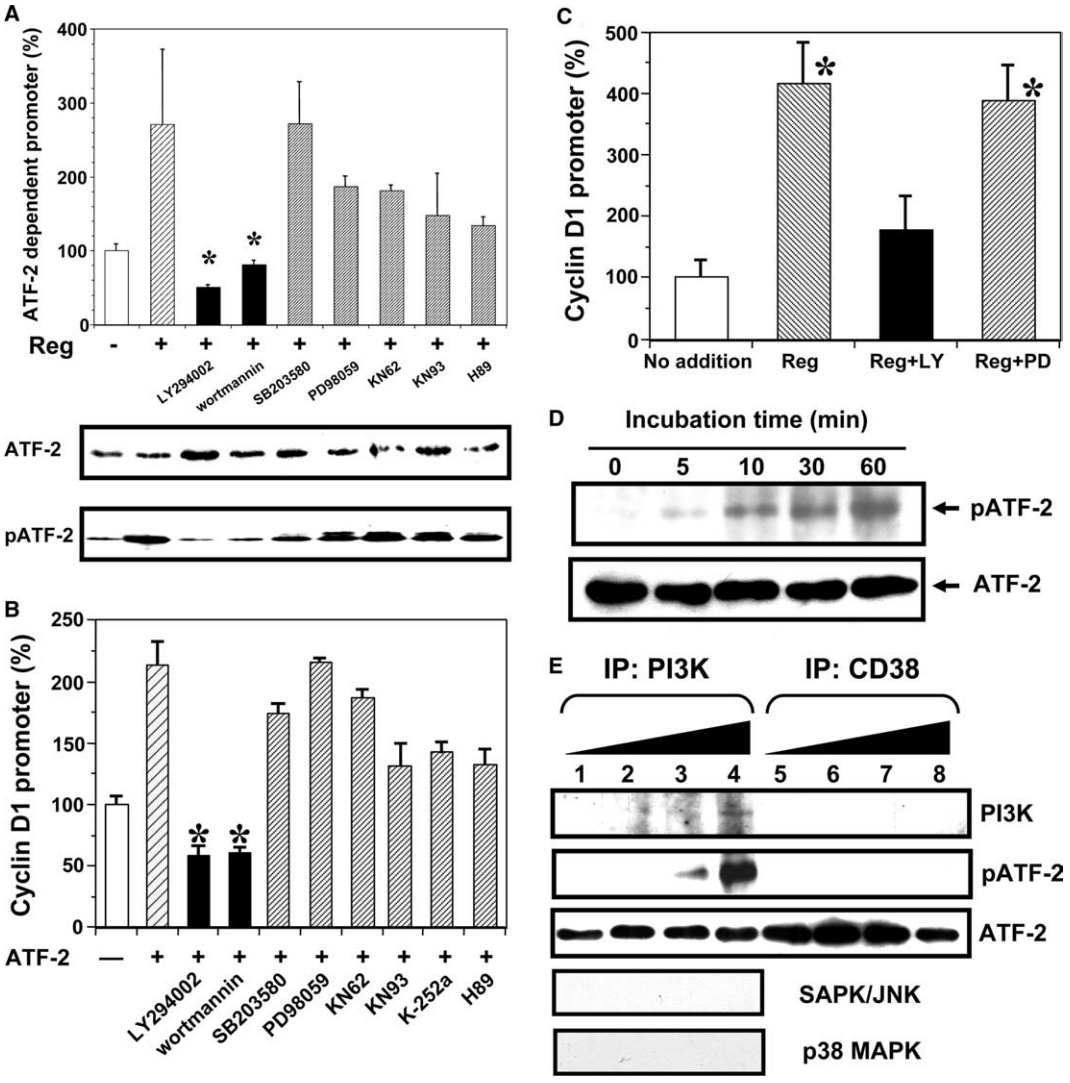

Fig. 3. Involvement of the PI(3)K pathway in ATF-2 activation for cyclin D1 promoter activity. The results of treatment with the MAP kinase/ERK kinase inhibitor PD98059 (10 μM), the PI(3)K inhibitors LY294002 (50 nM) and wortmannin (100 nM), the p38 MAP kinase inhibitor SB203580 (10 μM), the CaM kinase II inhibitors KN-62 (10 μM) and KN-93 (10 μM), the CaM kinase IV inhibitor K-252a (1 μM) and the A-kinase inhibitor H-89 (10 μM) were compared with that of DMSO vehicle treatment. (A) Attenuation of Reg-induced ATF activation by PI(3)K inhibitors. RINm5F β-cells were cotransfected with pFA-ATF2 and the pFR-Luc reporter plasmids. Cells were stimulated with Reg (33 nM). The results are means ± S.E. of three to six experiments. *, $P < 0.03$ (significantly decreased from the Reg activated promoter activity). The phosphorylation status of endogenous ATF-2 is also shown in the lower panel. The inhibitory effects of PD98059, KN62, KN93, and H89 were not statistically significant. (B) Attenuation of ATF-2-induced cyclin D1 promoter activity by PI(3)K inhibitors. RINm5F β-cells were cotransfected with −539CD1LUC reporter and ATF-2 expression vector. Protein kinase inhibitors were added to the culture medium. The results are means ± S.E. of three to five experiments. *, $P < 0.01$ (significantly decreased from the ATF-2 activated promoter activity). The inhibitory effects of PD98059, KN62, KN93, and H89 were not statistically significant. (C) Attenuation of Reg-induced cyclin D1 promoter activity by LY294002. RINm5F β-cells were stimulated by Reg. The results are means ± S.E. of three experiments. *, $P < 0.05$ (different from no addition). LY, LY294002. PD, PD98059. (D) Time-dependent phosphorylation of ATF-2 by immunoprecipitated PI(3)K. (E) Dose-dependent phosphorylation of ATF-2 by immunoprecipitated PI(3)K. Lanes 1 and 5, 0 μl; lanes 2 and 6, 1 μl; lanes 3 and 7, 2 μl; lanes 4 and 8, 4 μl of immunoprecipitates were used. Neither stress-activated protein kinase (SAPK)/cJun amino terminal kinase (JNK) nor p38 MAP kinase (MAPK) was detected in the immunoprecipitate by anti-PI(3)K p110.

and confirmed that the BrdU incorporation in $Reg^{-/-}$ islets without the addition of Reg in the culture medium was reduced compared to that in $Reg^{+/+}$ islets (data not shown). We then compared the levels of phospho-ATF-2, cyclin D1 protein, and phospho-Rb in homozygous Reg-deficient mouse islets with those in normal littermates to determine whether Reg induces the cyclin D1 gene for the cell cycle progression via the activation of PI(3)K/ATF-2 in $Reg^{+/+}$ and $Reg^{-/-}$ mouse islets (Fig. 4). $Reg^{+/+}$ islets secreted Reg in the culture medium and the phospho-Rb level in the islets was much higher than that in $Reg^{-/-}$ islets. As expected, both the levels of phospho-ATF-2 and cyclin D1 were decreased in islets from $Reg^{-/-}$ mice, whereas the levels of other proteins such as PARP [2,11] and CD38 [2,18] as controls were unchanged.

4. Discussion

The cyclin D1 gene encodes a regulatory subunit of a serine/threonine kinase that phosphorylates and inactivates the

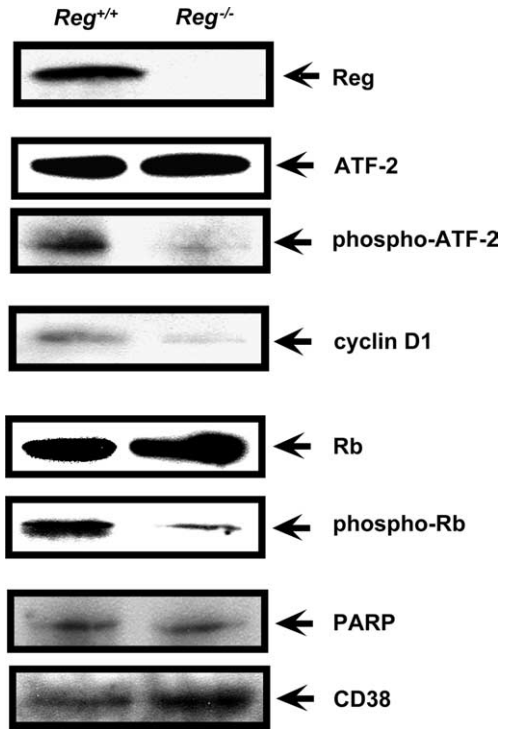

Fig. 4. Decreases in phospho-ATF-2, cyclin D1, and phospho-Rb in Reg-deficient islets. Islets were cultured without the addition of Reg. Total protein from islets isolated from $Reg^{+/+}$ or $Reg^{-/-}$ mice (20 μg) were analyzed by Western blot. Reg expression in culture medium (10 μl) was also analyzed.

retinoblastoma protein (pRb), allowing the release of sequestered transcription factors essential for the G1–S progression of the cell cycle [19–21]. An abundance of cyclin D1 has been shown to have a rate limiting effect on the cellular proliferation induced by diverse signaling pathways in a variety of mammalian cells [19–21]. The cyclin D1 gene expression appears to be essentially regulated at the transcription level and the transcriptional activation occurs in a cell type- and mitotic stimulation-specific manner [15,22,23]. Our results show conclusively that the activation of the cyclin D1 gene promoter in response to Reg stimulation is mediated by the PI(3)K/ATF-2 signal transduction pathway in pancreatic β-cells. The growth of pancreatic β-cells is determined by the number of β-cells entering the cell cycle rather than by changes in the rate of the cycle [1,2,4,8,24,25]. Reg, an autocrine/paracrine pancreatic β-cell regeneration factor, appears to stimulate the cell cycle progression [6–12]. Progression from the G1 to S phase of the cell cycle requires the activation of cyclin-dependent kinase (CDK) 4, and the CDK4 activation is controlled by the complex formation with its catalytic partner, cyclin D1. The expression of the cyclin D1 is controlled largely and perhaps entirely by extracellular signals. Reg activated the cyclin D1 promoter (Fig. 2) for the cell cycle progression, and ATF-2 is an essential transcription factor in the process of Reg-induced cyclin D1 promoter activation (Figs. 1 and 2). Reg receptor overexpression also activated ATF-2 and cyclin D1 promoter in a ligand-independent fashion (Figs. 1B and 2C), suggesting that oligomerization of Reg receptor can activate intracellular signaling events like Sky, MET, and CD30 receptors [26–28]. The PI(3)K activation is involved in the ATF-2 activation for cyclin D1 expression (Fig. 3). It has been reported that disruption of the CDK4 gene resulted in the development of insulin-deficient diabetes due to a reduction in the number of pancreatic β-cells, and that the expression of a mutant CDK4, which escaped from the inhibitory regulation of CDK4, caused islets to become hyperplastic [29]. The results of the CDK4 disruption well explain our results that the islets from the $Reg^{-/-}$ mice showed reduced BrdU incorporation (Fig. 4A), and that the islet hyperplasia induced by the gold-thioglucose treatment was attenuated by *Reg* gene disruption [8] because Reg induces a regulatory subunit of CDK4, cyclin D1, for the cell cycle progression. Reg was also reported to work as a growth factor for Schwann cells [30], motorneurons [31] and gastrointestinal epithelium [32,33], and cyclin D1 was suggested to be a key molecule for the regenerative growth of Schwann cells [34,35]. Therefore, the signal transduction mechanism in the Reg-induced regenerative growth of other tissues including Schwann cells may be the same as that of pancreatic β-cells. Further studies on tissue regeneration other than that of pancreatic β-cells using $Reg^{-/-}$ mice are in progress in our laboratory.

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