Exendin-4 Stimulation of Cyclin A2 in β-Cell Proliferation

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OBJECTIVE—β-Cell proliferation is an important mechanism underlying β-cell mass adaptation to metabolic demands. We have examined effects, in particular those mediated through intracellular cAMP signaling, of the incretin hormone analog exendin-4 on cell cycle regulation in β-cells.

RESEARCH DESIGN AND METHODS—Changes in islet protein levels of cyclins and of two critical cell cycle regulators cyclin kinase inhibitor p27 and S-phase kinase–associated protein 2 (Skp2) were assessed in mice treated with exendin-4 and in a mouse model with specific upregulation of nuclear cAMP signaling exhibiting increased β-cell proliferation (CBP-S436A mouse). Because cyclin A2 was stimulated by cAMP, we assessed the role of cylcin A2 in cell cycle progression in Min6 and isolated islet β-cells.

RESULTS—Mice treated with exendin-4 showed increased β-cell proliferation, elevated islet protein levels of cyclin A2 with unchanged D-type cyclins, elevated PDX-1 and Skp2 levels, and reduced p27 levels. Exendin-4 stimulated cyclin A2 promoter activity via the cAMP– cAMP response element binding protein pathway. CBP-S436A islets exhibited elevated cyclin A2, reduced p27, and no changes in D-type cyclins, PDX-1, or Skp2. In cultured islets, exendin-4 increased cyclin A2 and Skp2 and reduced p27. Cyclin A2 overexpression in primary islets increased proliferation and reduced p27. In Min6 cells, cyclin A2 knockdown prevented exendin-4–stimulated proliferation. PDX-1 knockdown reduced exendin-4–stimulated cAMP synthesis and cyclin A2 transcription.

CONCLUSIONS—Cyclin A2 is required for β-cell proliferation, exendin-4 stimulates cyclin A2 expression via the cAMP pathway, and exendin-4 stimulation of cAMP requires PDX-1. ***Diabetes* 57:2371–2381, 2008**

Pancreatic β-cell mass is dynamic and responds to variations in metabolic demand on insulin production. The inability of the endocrine pancreas to adapt to changing insulin demand (inadequate β-cell mass) is found both in type 1 and type 2 diabetes. Increasing β-cell mass by regeneration may ameliorate or correct both type 1 and 2 diabetes (1). Within the pancreas, β-cells regenerate predominantly by β-cell replication (2,3). In this context, insight into the mechanisms underlying β-cell proliferation and cell cycle regulation, may provide potential targets for therapy in situations of inadequate β-cell mass.

The incretin hormone glucagon-like peptide-1 (GLP-1) and its long-acting peptide analog exendin-4 stimulate β-cell proliferation in vitro and in vivo (4), leading to increased β-cell mass in rodents and amelioration of glucose metabolism in diabetic animal models and human diabetic subjects (4). Downstream effectors of the GLP-1 signaling to the cell nucleus include *1*) the epidermal growth factor receptor–phosphoinositol 3-kinase (PI 3-kinase)–protein kinase B (PKB/Akt)–forkhead box transcription factor O1 (FoxO1) pathway (5) and *2*) the cAMP–protein kinase A (PKA)– cAMP response element binding protein (CREB) pathway (4). Activation of the PI 3-kinase pathway results in phosphorylation and nuclear exclusion of FoxO1, thereby derepressing PDX-1 transcription (rev. in 6). Insulin and insulin-like growth factor stimulation of β-cell proliferation converge on the PKB/Akt-FoxO1-PDX-1 pathway (6,7). Transgenic β-cell–specific overexpression of phosphorylation-defective, nuclear FoxO1 results in reduced nuclear PDX-1, reduced β-cell number and islet size, and diminished β-cell proliferation response to exendin-4 (5,6). In addition, β-cell–specific deletion of PDX-1 in adult mice results in reduced response to GLP-1 effects on β-cell insulin secretion and proliferation (8), indicating that incretin hormone effects on β-cell proliferation require adequate PDX-1 levels. In contrast to the PI 3-kinase–mediated pathway, little is known on the specific effects of exendin-4 on cell cycle regulation in β-cells via the cAMP-CREB–signaling pathway (9).

We have interrogated exendin-4 and cAMP-CREB–mediated effects on cell cycle regulation in β-cells, taking advantage of the restricted expression of exendin-4 receptors, which—apart from some areas in the central nervous system—are found predominantly on pancreatic β-cells (4). In addition, we have used a mouse model with specific nuclear upregulation of cAMP-CREB–CREB binding protein (CBP) action and enhanced in vivo and in vitro β-cell proliferation (9) to specifically assess the role of cAMP-CREB–regulated transcription on cell cycle regulation. For exendin-4 effects on the cell cycle regulatory proteins, we have focused on cyclins (3,10) and S-phase kinase–associated protein 2 (Skp2) and p27 (11), which are critical regulators of cell cycle control in β-cells. In contrast to current understanding that only D-type cyclins respond to extracellular growth factors, we were surprised to find that in β-cells, cyclin A2 expression is upregulated by exendin-4 directly by cAMP-PKA-CREB-CBP signaling. Our in vivo studies indicate that upon exendin-4 stimulation of β-cells, cyclin A2 protein levels increase, whereas D-type cyclin levels do not change. In addition, exendin-4 treatment is accompanied by decreased p27 and concom-

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itant increase in Skp2 protein, which in cultured Min6 cells is stimulated by a PI 3-kinase–dependent pathway. Upregulation of cyclin A2 levels by lentiviral infection of primary murine β-cells is sufficient to increase proliferation activity. Furthermore, our studies suggest that in β-cells, homeodomain transcription factor PDX-1 expression is necessary for generating cAMP, which is required for exendin-4 effects on cyclin A2 expression via PKA-CREB-CBP.

RESEARCH DESIGN AND METHODS

Animal studies were approved in advance by the institutional animal care and use committee of Johns Hopkins University. All animal studies were conducted with mice ~2–3 months of age. C57Bl/6J were treated with exendin-4 in 10 nmol · kg^{-1} · day^{-1} i.p. PBS for 5 days or PBS only before islet isolation or tissue extraction. For β-cell apoptosis induction, 6-h–fasted mice were treated with 250 mg/kg streptozotocin (STZ; Sigma-Aldrich) in citrate buffer (pH 3) i.p. 24 h before pancreas harvest.

CBP-S436A knockin mice, which exhibit enhanced in vivo and in vitro β-cell proliferation (9,12), were back-crossed into C57Bl/6 background for six generations. CBP phosphorylation at serine 436 interferes with and reduces CREB/CBP interaction and reduces target gene transcription. Mutation of serine 436 to alanine (forming the mutant CBP-S436A) leads to enhanced basal and cAMP-stimulated transcription of CREB-responsive genes not suppressed by cellular events, which would result in CBP S436 phosphorylation (9,12). Homozygote and heterozygote CBP-S436A mice have similar islet morphometry (Supplemental Table 1, available in an online appendix at http://dx.doi.org/10.2337/db07-1541) (11,13) because of the dominant activating CBP-S436A mutation. Therefore, most studies herein were conducted on heterozygous (+/−) knockin mice and wild-type littermates.

Immunofluorescence histology and islet morphometry. Pancreata were fixed and stained, and islet morphometry was performed as previously described (9) and as summarized in the supplemental methods available in the online appendix.

Islet studies. Islets were isolated as previously described (9) and initially incubated in 24-well culture plates in RPMI-1640, 5 mmol/l glucose supplemented with 10% FCS, and 1% penicillin/streptomycin for 24 h before performing additional procedures. For β-cell apoptosis induction, islets were exposed to 1.5 mmol/l STZ for an additional 24 h before harvest (positive control of apoptosis). Islet exposure to 20 nmol/l exendin-4 was for 8 h after the initial time course studies, when islets were exposed for 0, 2, 4, 6, 8, and 12 h to PBS or exendin-4. cDNA synthesis from islet RNA and quantitative RT-PCR were performed using iScript One-tube RT-PCR with SYBR green on an iCycler (BioRad). Transcript levels were normalized to expression of housekeeping gene 36B4 in corresponding samples. PCR protocols and primers used for quantitative RT-PCR can be obtained on request. For lentiviral infection, freshly isolated islets were incubated in a microcentrifuge tube containing concentrated lentivirus (see supplemental methods), centrifuged at 2,000 rpm for 2 min, transferred to the a 96-well microtiter plate, and incubated at 37°C in 95% humidity and 5% CO_2. Western immunoblots were performed with 20–40 μg islets protein in radioimmunoprecipitation assay buffer using standard protocols. Information on antibodies used for immunoblots will be provided on request. Immunoblots for cyclin D subtypes were also performed using monoclonal antibodies (Neomarkers) that are specific for cyclin D1–D3 subtypes. Cyclin A1 was not examined because of its restricted expression to gonads (14). Parallel protein detection for actin was used to control for protein loading. Band intensity of interrogated protein was normalized to the corresponding actin intensity (BioRad Chemidoc XRS). Representative immunoblots are shown. Immunoblots were repeated at least four times.

Cells and transfections. Min6 cells (passages 25–34) were cultured as described previously (13). Plasmids used are summarized in the supplemental methods.

Chromatin-immunoprecipitation assay. Chromatin-immunoprecipitation (ChIP) assay was performed according to manufacturer's instructions (ChIP assay kit; Upstate) to detect binding of CREB and recruitment of CBP to the CRE containing regulatory elements in the murine insulin (positive control) and cyclin A2, D1, and D2 genes. ChIP was performed on DNA extracted from cultured MIN6 treated with or without exendin-4 as described above. Specific rabbit anti-CREB antibody (D. Ginty, Johns Hopkins University) or normal rabbit IgG antibody (Santa Cruz sc-2027) were used for immunoprecipitation. PCR was performed for 30 cycles of 30 s each of 94°C melting, 58°C annealing, and 72°C extension steps. An aliquot of the PCR product was fractionated on a 2% agarose gel containing ethidium bromide for UV visualization.

Small interfering RNA knockdown studies. Small interfering RNA (siRNA) (100 nmol/l for murine cyclin A2, 200 nmol/l for murine PDX-1, or corresponding amounts for scrambled siRNA; Ambion) were transfected using Fugene (Roche; 1 or 2.5 μl per well in a 24- or 6-well plate, respectively) according to the manufacturer's instructions. For siRNA-mediated knockdown in cultured primary islets, respective siRNA (400 nmol/l with Fugene 10 μl per well in 24-well culture plate) was added to the culture medium. Culture medium and siRNA were replaced every 12 h four times before islet harvest and further analysis. Primers for cyclin A2 (sense, 5′-GCUCAAGACUCGACGGGUtt-3′) and PDX-1 (sense, 5′-GGGAACUUAACCUAGGCGUtt-3′) effectively reduced cyclin A2 or PDX-1 (see below) levels as determined by immunoblot and were used in all subsequent studies, respectively.

cAMP measurements. cAMP measurements were performed on cultured Min6 and cultured islet extracts. Studies were performed in sextuplicate (Min6) or quadruplicate (cultured islets), and cAMP was measured by ELISA according to manufacturer's instructions (Assay Design).

Statistical analysis. Results are shown as averages and means ± SE. Calculations were conducted using either Prism 4 (Graph Pad) or Excel (Microsoft) software. Where appropriate, Student's *t* test or ANOVA was used to calculate differences between groups. A P value of <0.05 was considered significant and is indicated with an asterisk.

RESULTS

Exendin-4 treatment increases islet cyclin A2 and Skp2 protein levels. Of the islet cell population, 75–90% are β-cells. Furthermore, GLP-1 receptor is expressed primarily on β-cells within the pancreatic islet (4), and all β-cells participate equally in proliferation (2,3). Thus, changes in whole-islet protein extracts reflect mainly, if not exclusively, changes in β-cell protein levels (15).

Islet size increased by ~1.7-fold in exendin-4–treated C57Bl/6 mice, consistent with previous observations (16). This increase is due to β-cell hyperplasia and not hypertrophy because β-cell size did not change (Fig. 1*A*–*F*). Surprisingly, the D-type cyclin levels, which are considered to respond to extracellular growth stimuli, were not changed in exendin-4–treated islets. Exendin-4 treatment was associated with elevated PDX-1 (1.93 ± 0.20, $P < 0.05$) (as expected; 16), cyclin A2 (2.69 ± 0.17 vs. control, $P < 0.05$) and Skp2 (5.01 ± 0.98-fold vs. control, $P < 0.05$) protein levels (Fig. 2*A* and Table 1). Islet levels of p27 in the relatively young animals (8–12 weeks) were, as expected (17) low and decreased during exendin-4 treatment.

Nuclear cAMP-CREB upregulation stimulates cyclin A2. Because exendin-4 stimulates both the PI 3-kinase and cAMP pathways, we assessed specifically the in vivo effects of upregulated nuclear cAMP-CREB-CBP signaling (CBP-S436A knockin mouse) and increased β-cell mass and proliferation (9). CBP-S436A$^{+/-}$ mice exhibited increased β-cell Ki67 staining compared with wild-type littermates. This was paralleled by increased β-cell area without change in β-cell size (hyperplasia, not hypertrophy) (Fig. 1*G*–*L*) (9). In isolated islets, D-type cyclin protein levels were not different between wild-type and CBP-S436A$^{+/-}$ mice. In contrast, cyclin A2 levels were elevated by ~2.02 ± 0.20-fold ($P < 0.05$) in CBP-S436A mutants. p27 was low but detectable in control islets and below detectable levels in CBP-S436A islets. Skp2, FoxO1, and Pdx-1 levels were similar in wild-type and in CBP-S436A littermate islets (Fig. 2*B*; Table 1). Taken together, the findings in CBP-S436A mice suggest that β-cell activation of the cAMP-PKA-CREB-CBP pathway leads to cyclin A2 expression without changes in PDX-1, FoxO1, and Skp2.

Exendin-4 stimulates cyclin A2 and PDX-1 transcript in cultured islets. We next focused on the effects of exendin-4 on transcript and protein levels of cyclin A2, cyclin D2 (which among the D-type cyclins is considered

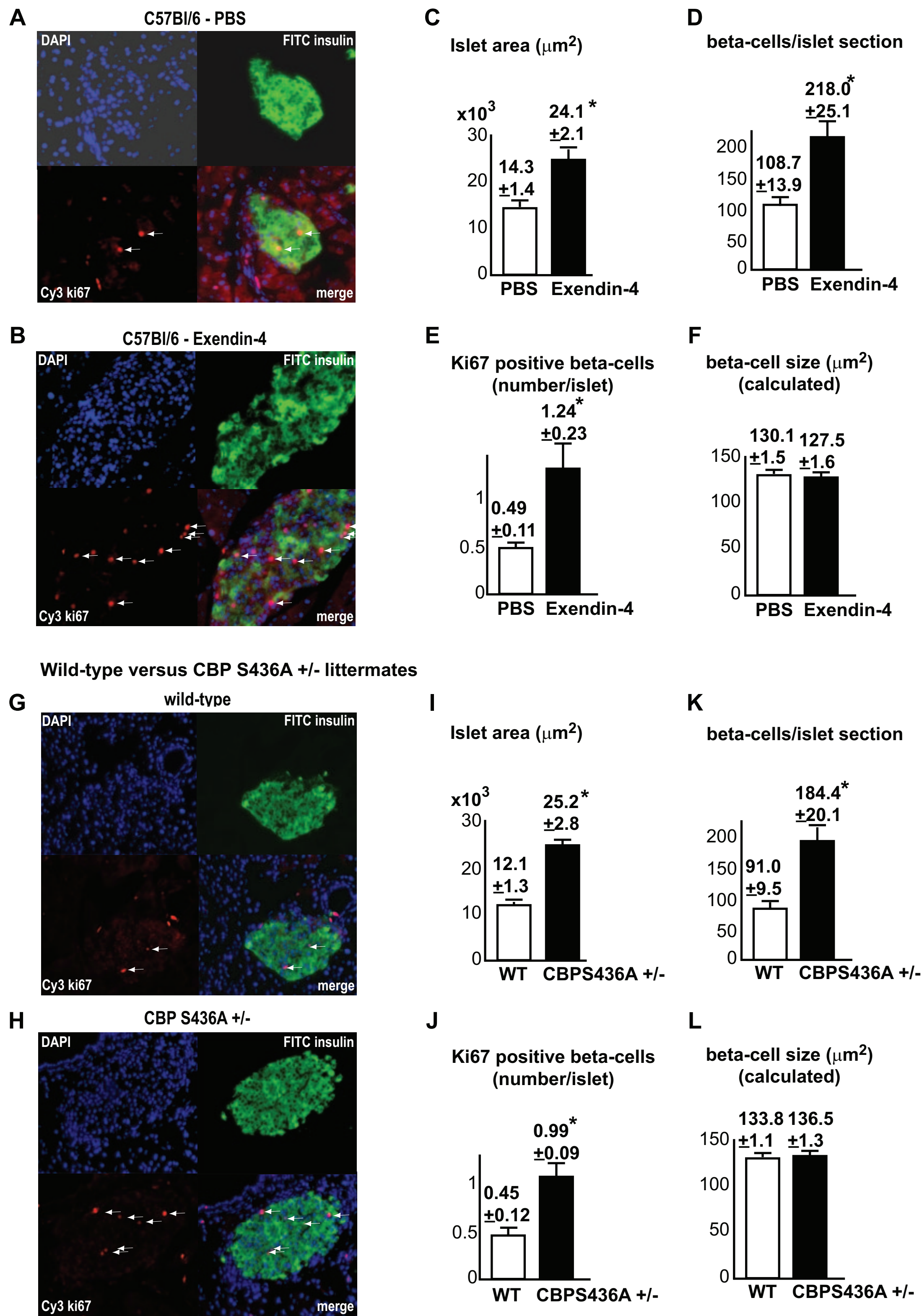

FIG. 1. Exendin-4 treatment and CBP-S436A$^{+/-}$ mutation result in increased β-cell proliferation and islet size. Representative fluorescence photomicrographs with pseudo-coloring of islet sections of C57Bl/6 littermates treated with either vehicle (PBS) (*A*) or exendin-4 (*B*) for 5 days and of wild-type (*G*) and CBP-S436A$^{+/-}$ (*H*) littermates. *A*, *B*, *G*, and *H* are partitioned as follows: *top left*, DAPI nuclear stain (blue); *top right*, fluorescein isothiocyanate (FITC) immunostain for insulin (green); *bottom left*, Cy3 immuno-stain for Ki67 (red); and *bottom right*, a merged image of the three preceding panels. Compared with controls (*A*), exendin-4–treated animals (*B*) exhibit increased islet area (*C*), β-cell proliferation as assessed by Ki67 in insulin-positive β-cells (*D*), and β-cells per islet (*E*) and unchanged calculated β-cell size (*F*). Similarly, compared with wild-type controls (*G*), CBP-S436A$^{+/-}$ mice (*H*) exhibit increased islet area (*I*), β-cell proliferation as assessed by Ki67 in insulin-positive β-cells (*J*), and β-cells per islet (*K*) and unchanged calculated β-cell size (*L*). Means ± SE in graphs are provided above the corresponding bars. *Significant difference from the corresponding control with $P < 0.05$. (Please see http://dx.doi.org/10.2337/db07-1541 for a high-quality digital representation of this figure.)

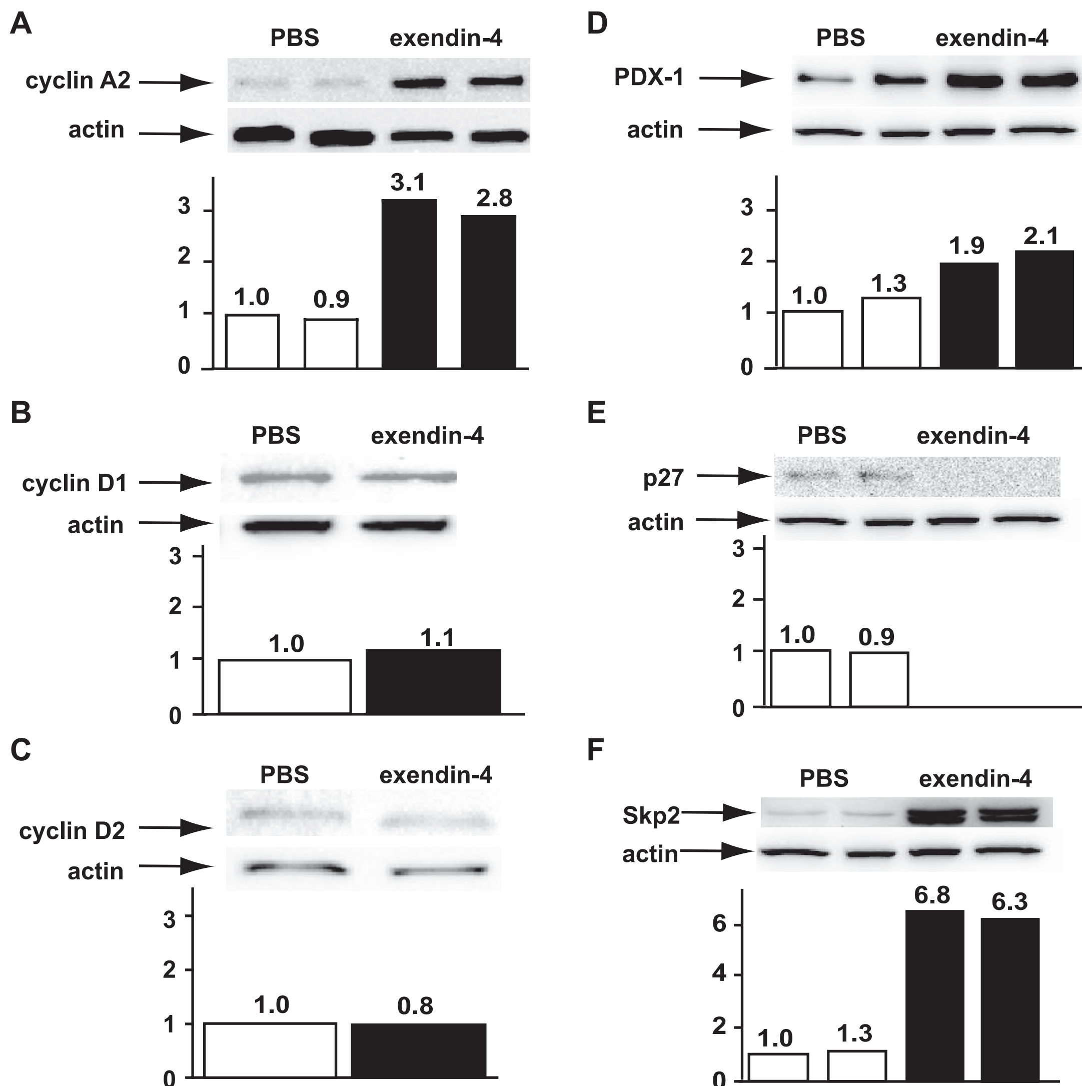

FIG. 2. ***A*****: Exendin-4 treatment increases PDX-1, cyclin A2, and Skp-2 and decreases p27 islet protein levels. Islets isolated from C57Bl/6 mice treated with either vehicle or exendin-4 for 5 days were analyzed for protein levels of cyclin A2 (*A*), cyclin D1 (*B*), cyclin D2 (*C*), PDX-1 (*D*), p27 (*E*), and Skp2 (*F*).** ***B*****: CBP-S436A$^{+/-}$ islets exhibit increased cyclin A2 and decreased p27 but unchanged PDX-1 and Skp2 protein levels. Islets isolated from wild-type and CBP-S436A$^{+/-}$ littermates were analyzed for protein levels of cyclin A2 (*G*), cyclin D1 (*H*), cyclin D2 (*I*), PDX-1 (*J*), p27 (*K*), and Skp2 (*L*). Representative immunoblots are shown together with corresponding immunoblot for actin. Corresponding graphs below the immunoblots depict densitometric analysis of antigen band in relation to the corresponding actin band. Data are normalized to the first control densitometry, which is depicted as 1.0 in the first column of the graph. Numbers indicate absolute value of the corresponding column immediately below them.**

most prominent in β-cell simulation [3,10]), and PDX-1. In cultured islets, exendin-4 stimulation was associated with a rapid (within 2 h) approximate twofold upregulation of cyclin A2 transcript (quantitative RT-PCR) and protein levels. In contrast, PDX-1 transcription and protein levels increased after 4–6 h of exendin-4 stimulation, whereas cyclin D2 levels remained stable (Fig. 3; Table 2). These results indicate that cyclin A2 transcription responds more rapidly than PDX-1 transcription to exendin-4 stimulus.

Exendin-4 stimulates cyclin A2 via cAMP-PKA-CREB and Skp2 via PI 3-kinase in Min6 cells. In transient transfections, 20 nmol/l exendin-4 (for 4 h) stimulated −800-bp cyclin A2-LUC reporter activity by 3.7- ± 0.7-fold (mean ± SE, $P < 0.05$), whereas the CRE mutant of the reporter (−800-bp ΔCRE cyclin A2-LUC) showed no stimulation (Fig. 4). The exendin-4 effect was blocked by the cAMP antagonists H89 and PKI but not by inhibition of the PI 3-kinase and MEK pathways. Constitutively active catalytic subunit of PKA (PKAcs) significantly stimulated −800-bp cyclin A2-LUC reporter activity (4.7- ± 0.4-fold, $P < 0.05$) but not that of the corresponding CRE-mutated reporter. Endogenous cyclin A2 protein levels are stimulated by exendin-4 treatment (see below). CREB overexpression stimulated −800-bp cyclin A2-LUC 9.2- ± 1.1-fold, whereas dominant-negative A-CREB reduced reporter activity to 0.4 ± 0.1 ($P < 0.05$). CREB-CBP (CREB-DIEDML) stimulated cyclin A2-LUC transcription (data not shown). Transfection of CREB and CBP together significantly enhanced reporter activity by 7.5- ± 1.9-fold ($P < 0.05$ vs. control). Replacement of CBP by the CBP-S436A mutant increased transcriptional activity by 25.7- ± 6.6-fold ($P <$ 0.05 vs. control and CBPwt) (Fig. 4).

Based on observations made in CBP-S436A mice, islet Skp2 levels are not stimulated by the cAMP-CREB-CBP pathway. Exendin-4–stimulated Skp2 elevation was prevented by pharmacologic PI 3-kinase (Supplemental Fig. 1) but not by cAMP signaling inhibition, respectively. In summary, in Min6 cells, exendin-4 stimulates cyclin A2 transcription and protein levels by way of cAMP-PKA-

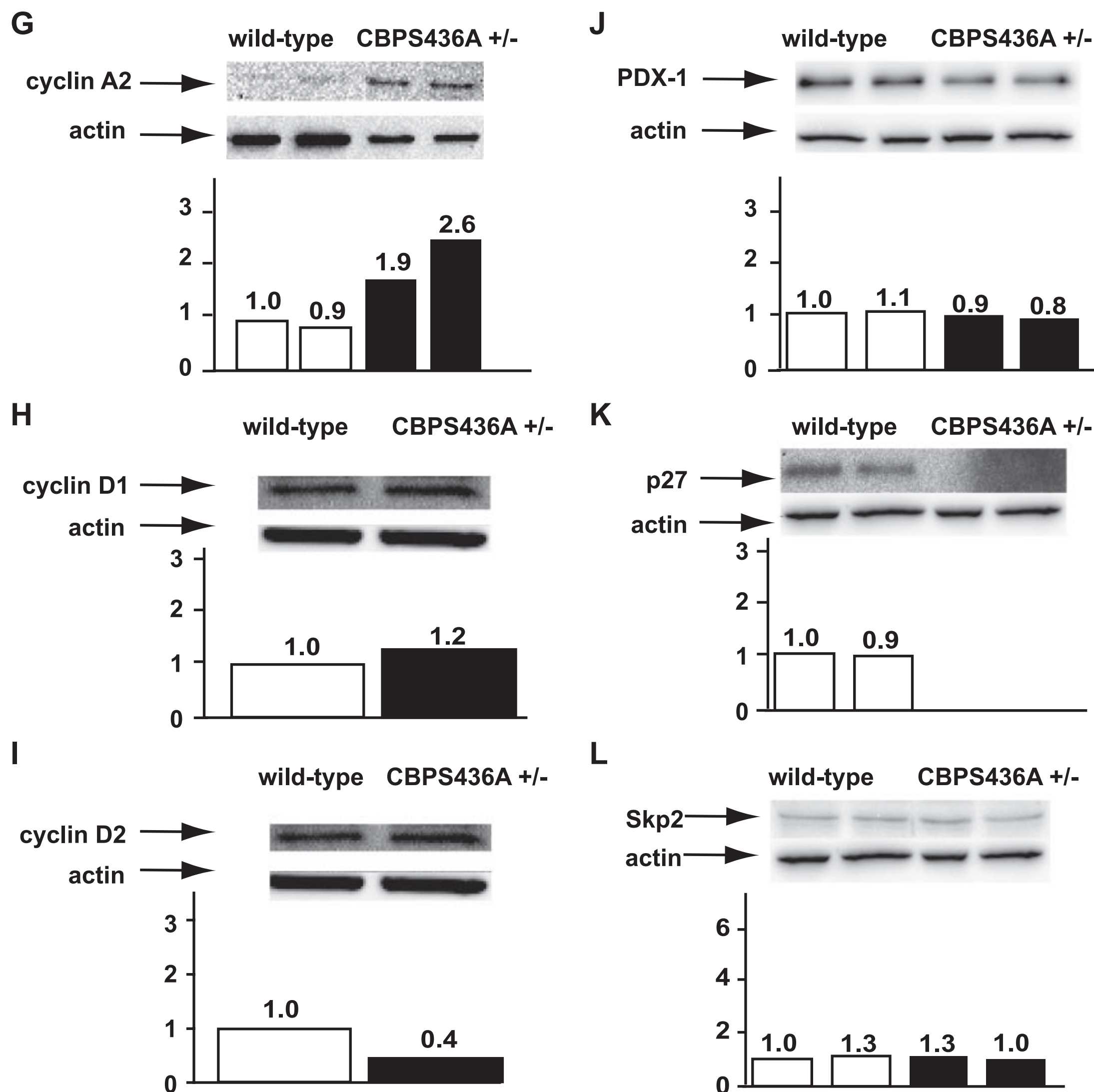

FIG. 2. Continued

CREB-CBP signaling. Skp2 is elevated via the PI 3-kinase pathway, consistent with previous observations (18).

CREB binds to cyclin A2 but not to D1 or D2 promoter CRE. Using ChIP, we examined whether CREB occupies endogenous promoter elements of cyclin A2 and cyclins D1 and D2, which have been reported to contain CRE elements. In exendin-4–stimulated Min6 cells, we detected CREB binding to the mouse CRE in insulin 1 and cyclin A2 promoters but not to CRE of cyclin D1 and D2 promoters (Supplemental Fig. 2). We did not detect any CREB binding in the absence of exendin-4 treatment of Min6 cells. This lack of basal binding may reflect the low

TABLE 1
Relative changes of indicated proteins in total islet extracts in exendin-4–treated (5d) C57Bl/6 mice normalized to vehicle-treated controls and CBP-S436A normalized to wild-type littermate islet extracts

Protein	Exendin-4 versus PBS	P value	CBP-S436A$^{+/-}$ versus wild type	P value
Cyclin A2	2.69 ± 0.17	<0.05	2.02 ± 0.20	<0.05
Cyclin B1	0.90 ± 0.20	NS	0.59 ± 0.16	NS
Cyclin B2	0.87 ± 0.31	NS	1.05 ± 0.31	NS
Cyclin D1	1.26 ± 0.06	NS	0.86 ± 0.15	NS
Cyclin D2	1.00 ± 0.09	NS	0.98 ± 0.31	NS
Cyclin D3	1.11 ± 0.12	NS	0.85 ± 0.03	NS
FoxO1	0.79 ± 0.05	0.05	0.98 ± 0.20	NS
p27	Not detected	<0.05	Not detected	<0.05
Skp2	5.01 ± 0.98	<0.05	0.80 ± 0.21	NS
PDX-1	1.93 ± 0.20	<0.05	0.85 ± 0.03	NS

Data are means ± SE; $n = 4$ for each parameter and group. A significant difference ($P < 0.05$) from the corresponding control is indicated. For details, see text.

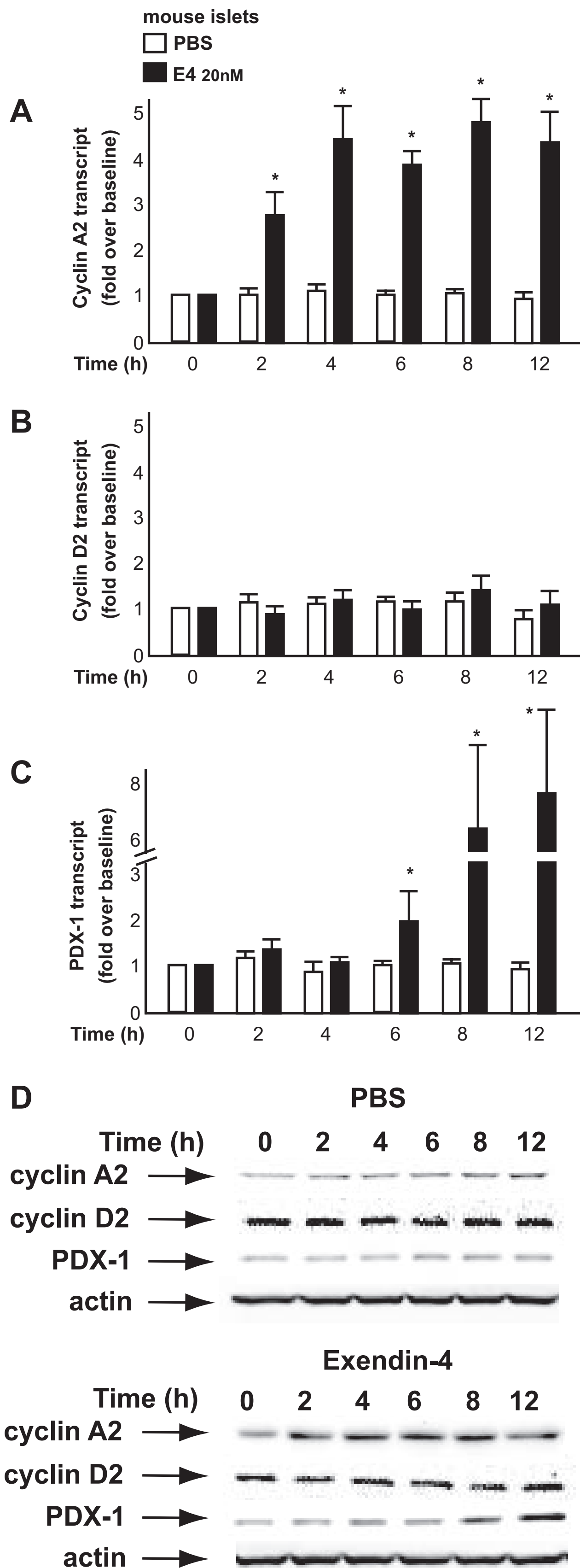

FIG. 3. ***A*: Exendin-4 stimulates cyclin A2 and PDX-1 transcript in ex vivo–cultured mouse islets. Cultured isolated C57Bl/6 mouse islets were exposed to PBS or 20 nmol/l exendin-4 for 0, 2, 4, 6, 8, and 12 h. Transcript levels of cyclin A2 (*A*), cyclin D2 (*B*), and PDX-1 (*C*) were measured by quantitative RT-PCR. Transcripts were normalized to housekeeping gene 36B4 at corresponding time point and are provided in Table 2 in fold change over baseline (time 0 h) as means ± SE of quadruplicate experiments. **P* < 0.05 and statistical significant change. *B*: Exendin-4 stimulates cyclin A2 and PDX-1 protein in ex vivo–cultured mouse islets. Cultured isolated C57Bl/6 mouse islets were exposed to PBS or 20 nmol/l exendin-4 for 0, 2, 4, 6, 8, and 12 h. Representative immunoblots for cyclin A2, cyclin D2, and PDX-1 are shown. Densitometric analysis of relative protein levels normalized to actin at corresponding time points are provided in Table 2 and depicted as fold change over baseline (0 h) as means ± SE of triplicate studies. **P* < 0.05 and statistical significant change.**

sensitivity of the ChIP assay or may indicate that exendin-4 stimulation stabilizes a larger hetero-protein complex (19) surrounding CREB on the CRE elements of insulin-1 and cyclin A2 promoters.

Cyclin A2 knockdown inhibits Min6 proliferation and proliferation stimulation by exendin-4. siRNA transfection achieved ~40% knockdown of cyclin A2 protein (Fig. 5). This resulted in a significantly reduced Min6 proliferation rate (1.14 ± 0.02-fold [cyclin A2 siRNA] vs. 1.36 ± 0.05-fold [scrambled siRNA] expansion in 48 h; $P < 0.05$) (Fig. 5). Cyclin A2 knockdown completely inhibited exendin-4–induced proliferation stimulation in Min6 cells (Fig. 5).

Lentiviral-mediated cyclin A2 elevation stimulates Min6 and primary β-cell proliferation. Infection of Min6 cells with lentivirus expressing cyclin A2 (compared with control virus) resulted in elevated cyclin A2 levels and in increased proliferation (fold increase in cell count within 48 h, 3.9 ± 0.4 vs. 1.5 ± 0.2 in controls, $P < 0.05$) of Min6 cells (Supplemental Fig. 3).

We next tested the effects of cyclin A2 overexpression in freshly isolated C57Bl/6 islets. Lentivirus infection rates were low (~5% of insulin-positive β-cells) but detectable by EGFP expression in cultured islets (Supplemental Fig. 4*A*). Forty-eight hours after exposure to cyclin A2 expressing lentivirus, infected β-cells showed significantly increased frequency of Ki67- or pH3B-positive nuclei compared with β-cells infected with control lentivirus (Fig. 6). Ki67 is positive throughout most of the cell cycle, whereas high phospho-histone 3B levels are restricted to the M phase of the cell cycle. Accordingly, and as expected, Ki67 antigen was detected more frequently than pH3B in cyclin A2–expressing lentivirus-infected cells. Lentivirus overexpression of cyclin A2 in isolated islets resulted in reduced p27 and unchanged Skp2 protein levels (Supplemental Fig. 7; Supplemental Table 4), consistent with findings made in CBP-S436A islets. Taken together, these results indicate that cyclin A2 overexpression results in increased proliferation activity in primary pancreatic β-cells. In contrast to STZ-treated islets, islets infected with cyclin A2 lentivirus for 48 h did not exhibit any detectable apoptosis marker cleaved caspase 3 or in the TUNEL assay (20). In addition, animals treated with exendin-4 and the CBP-S436A$^{+/-}$ animals did not exhibit cleaved caspase 3 (Supplemental Fig. 4). These data indicate that apoptosis is not increased in β-cells with cyclin A2 overexpression.

Homeodomain transcription factor PDX-1 is required for cAMP generation and exendin-4 stimulation of cyclin A2. PDX-1 is required for exendin-4 effects on β-cell proliferation and function (6,8,21). Therefore, we examined the role of PDX-1 in exendin-4–stimulated cyclin A2 expression. siRNA-mediated PDX-1 knockdown for 48 h in Min6 cells had no effect on baseline cyclin A2 levels (1.02 ± 0.6 compared with no siRNA, NS) but prevented significant increase of cyclin A2 by exendin-4 stimulation (from 1.02 ± 0.6 to 1.21 ± 0.1 compared with control).

TABLE 2
Exendin-4 stimulates cyclin A2 and PDX-1 in cultured C57Bl6 mouse islets

	Treatment	Time (h) 0	2	4	6	8	12
Transcript							
Cyclin A2	PBS	1	0.98 ± 0.11	1.09 ± 0.09	1.05 ± 0.04	1.06 ± 0.10	0.99 ± 0.07
Cyclin A2	Exendin-4	1	2.75 ± 0.47*	4.45 ± 0.65*	3.88 ± 0.37*	4.86 ± 0.54*	4.47 ± 0.63*
Cyclin D2	PBS	1	1.09 ± 0.07	1.07 ± 0.05	1.06 ± 0.07	1.05 ± 0.09	0.93 ± 0.10
Cyclin D2	Exendin-4	1	0.99 ± 0.07	1.08 ± 0.09	1.02 ± 0.09	1.18 ± 0.11	1.0 ± 0.15
PDX-1	PBS	1	1.22 ± 0.09	0.99 ± 0.0.07	1.06 ± 0.07	1.05 ± 0.16	0.9 ± 0.16
PDX-1	Exendin-4	1	1.35 ± 0.26	1.06 ± 0.13	2.04 ± 0.67*	6.23 ± 2.88*	7.49 ± 2.97*
Protein							
Cyclin A2	PBS	1	0.96 ± 0.38	1.13 ± 0.39	1.13 ± 0.39	1.14 ± 040	0.15 ± 0.40
Cyclin A2	Exendin-4	1	1.56 ± 0.40*	1.82 ± 0.40*	1.68 ± 0.39*	2.01 ± 0.39*	1.69 ± 0.39*
Cyclin D2	PBS	1	1.09 ± 0.07	1.07 ± 0.05	1.06 ± 0.07	1.05 ± 0.09	0.93 ± 0.10
Cyclin D2	Exendin-4	1	0.99 ± 0.07	1.08 ± 0.09	1.02 ± 0.09	1.18 ± 0.11	1.0 ± 0.15
PDX-1	PBS	1	1.15 ± 0.35	1.02 ± 0.35	1.15 ± 0.35	1.14 ± 0.35	0.15 ± 0.36
PDX-1	Exendin-4	1	1.18 ± 0.37	1.55 ± 0.37	1.67 ± 0.36*	1.97 ± 0.36*	2.67 ± 0.35*

Data are means ± SE. Relative changes of indicated transcripts and immunoblots of indicated proteins in cultured C57Bl/6 islet extracts treated with PBS or 20 nmol/l exendin-4 for 0, 2, 4, 6, 8, and 12 h ($n = 4$ for each parameter and group). Transcripts were normalized to housekeeping gene 36B4 transcript at corresponding time points and results are fold change over baseline (0 h). Exendin-4 stimulates an early (2–4 h) increase in cyclin A2, whereas PDX-1 transcript increases after 6–8 h. Densitometric analysis of relative protein levels normalized to actin at corresponding time points of three independent experiments are shown as fold change over baseline (0 h). Exendin-4 stimulates an early (2–4 h) increase in cyclin A2, whereas PDX-1 levels increase after 6–8 h. *Significant difference ($P < 0.05$) from the corresponding control. For details, see text and Fig. 3.

Furthermore, PDX-1 knockdown prevented exendin-4–stimulated −800-bp cyclin A2-LUC activity. Exendin-4–stimulated cAMP generation was diminished by PDX-1 knockdown. The effect of PDX-1 knockdown on cAMP generation was seen after 48 h and not after 24 h of PDX-1 knockdown (data not shown), suggesting an indirect effect of PDX-1 reduction on cAMP generation and cyclin A2 transcription. PKAcs, which acts downstream of cAMP, rescued −800-bp cyclin A2-LUC activity in Min6 when siRNA-mediated PDX-1 levels and cAMP generation are reduced (Supplemental Fig. 5; Supplemental Table 2).

In primary cultured islets, cyclin A2 knockdown did not influence exendin-4 stimulation of PDX-1 or of cAMP generation. In contrast, PDX-1 knockdown prevented exendin-4 effects on cyclin A2 stimulation, which was also accompanied by reduced exendin-4–induced cAMP generation (Supplemental Fig. 6; Supplemental Table 3).

On exendin-4 binding to the GLP-1 receptor (GLP-1R), a trimer of G-coupled proteins associates to the GLP-1R, of which Gsα is instrumental in generating cAMP via adenylcyclase activation (22). We therefore examined the effects of siRNA-mediated PDX-1 knockdown on GLP-1R and Gsα expression. PDX-1 knockdown reduced both GLP-1R and Gsα transcripts (Supplemental Fig. 8; Supplemental Table 5).

DISCUSSION

The studies herein indicate that in addition to proliferation stimulation via PI 3-kinase–mediated signaling, exendin-4 stimulates cyclin A2 expression in pancreatic β-cells by way of cAMP-PKA-CREB signaling. The effect of exendin-4 on cyclin A2 transcription stimulation depends on the PI 3-kinase–PKB/Akt-FoxO1-PDX-1 pathway, with PDX-1 being necessary for adequate exendin-4–stimulated cAMP generation. Furthermore, in Min6 cells, exendin-4 treatment, via PI 3-kinase–mediated signaling, stimulates Skp2 protein, consistent with previous studies (18). In addition, our studies suggest that upregulation of cyclin A2 levels are sufficient to stimulate β-cell proliferation.

The two in vivo mouse models presented herein, *1*) exendin-4 stimulated pancreatic β-cells and *2*) CBP-S436A$^{+/-}$ knockin mouse models, provide complementary information on cAMP-dependent and -independent cell cycle regulation. The former studies provide a model of incretin trophic effects on β-cells, whereas the latter provide a means to dissect the cAMP-CREB-CBP pathway from other incretin-induced intracellular signaling pathways in the regulation of β-cell proliferation. Our in vivo studies indicate that exendin-4–induced increased β-cell proliferation and mass (Fig. 1) are accompanied by changes in cell cycle regulatory proteins cyclin A2, Skp2, and p27 (Figs. 2 and 3). CBP-S436A mutant mice exhibit hepatic insulin resistance (9), which as stimulus may contribute to the increased β-cell mass. However, as previously shown, β-cells from CBP-S436A$^{+/-}$ mice continue to proliferate ex vivo in cultured islets, indicating that the proliferation has a β-cell autonomous component (9). Our studies in Min6 cells indicate that exendin-4–stimulated cyclin A2 is mediated via cAMP signaling, whereas Skp2, which has been recently been shown to be critical in regulating β-cell proliferation via p27 degradation (3,10), is stimulated independently of cAMP signaling via a PI 3-kinase–mediated pathway. Ex vivo β-cell overexpression of cyclin A2 is associated with stimulated proliferation activity, as assessed by Ki67 (G1, S, M, and G2 phases of the cell cycle [23]) and phospho-histone-3B (M phase [24]) expression without evidence for β-cell apoptosis. Thus, overexpression of cyclin A2 is sufficient to promote β-cell proliferation. These findings are consistent with the observation that cyclin A2 is unique among the cyclins in that it regulates progression at both the G1/S and G2/M checkpoints of mitosis (25) and cyclin A2 overexpression alone in cardiomyocytes stimulates proliferation (26).

A possible additional explanation for increased cyclin A2 levels in exendin-4–treated mouse islets is increased Skp2 (Fig. 2; Table 1), which is reported to bind to and stabilize cyclin A2 levels (27,28). However, CBP-S436A$^{+/-}$

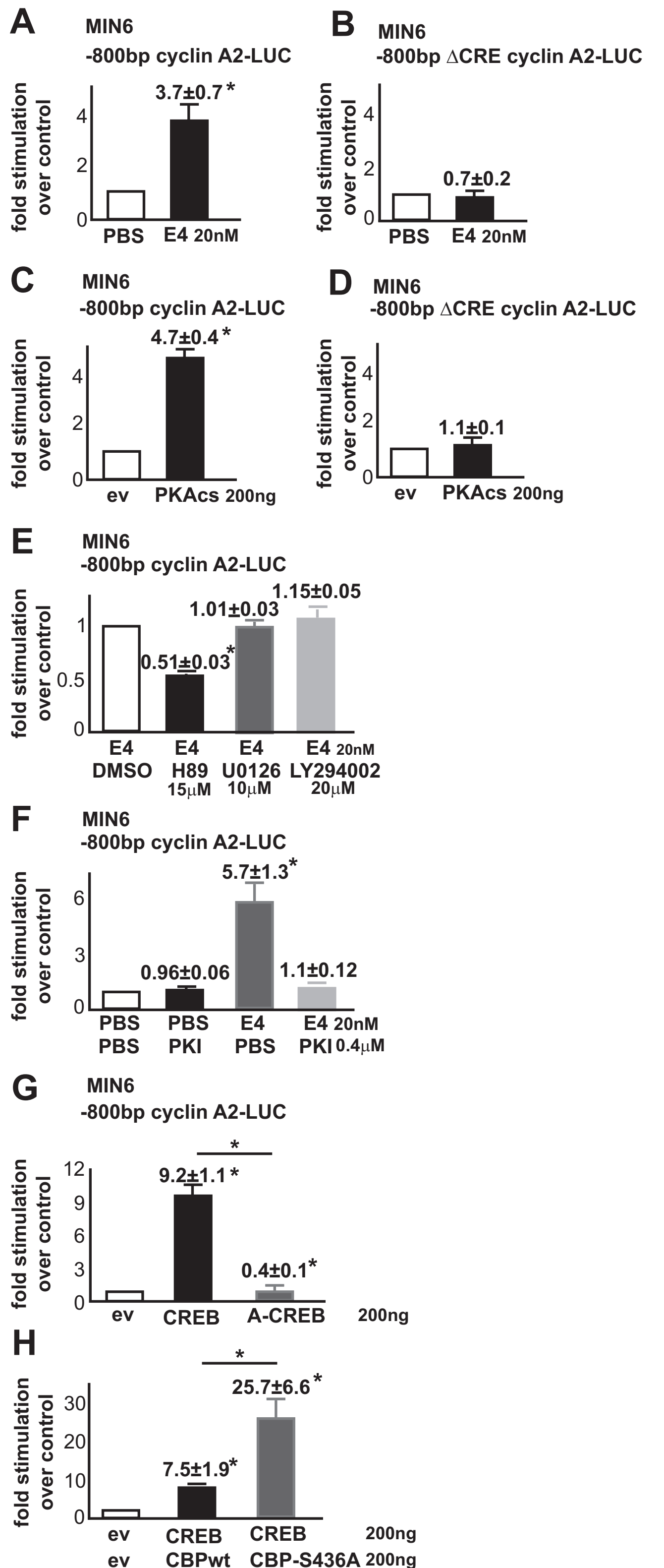

FIG. 4. Exendin-4 stimulates cyclin A2 transcription via the cAMP-PKA-CREB-CBP pathway. Min6 cells transiently transfected with −800-bp 5′-untranslated region of the murine cyclin A2 gene linked to a luciferase cDNA (−800-bp cyclin A2-LUC) or with the same vector carrying a mutation in the CRE (−800-bp ΔCRE cyclin A2-LUC). Exendin-4 (20 nmol/l for 4 h) stimulates the intact reporter (*A*) but not the reporter with the mutant CRE (*B*). Co-transfected expression vector to overexpress active PKAcs stimulates the intact (*C*) but not the CRE mutant promoter-reporter (*D*). Short-term (for 20 min before exendin-4 treatment) pharmacological inhibition of cAMP (H89 and PKI), but not other intracellular pathways (U0126 and LY294002) stimulated by exendin-4, inhibits exendin-4 effects on cyclin A2-LUC activity (*E* and *F*). Co-transfected expression vector to overexpress CREB stimulates and dominant-negative A-CREB suppresses −800-bp cyclin A2-LUC activity (*G*). Co-transfected expression vectors to overexpress CREB and CBP-S4356A mutant stimulate cyclin A2-LUC activity more potently than CREB with wild-type CBP (CBPwt) (*H*). Means ± SE in graphs are provided above the corresponding bars. *Significant difference from the corresponding control with *P* < 0.05.

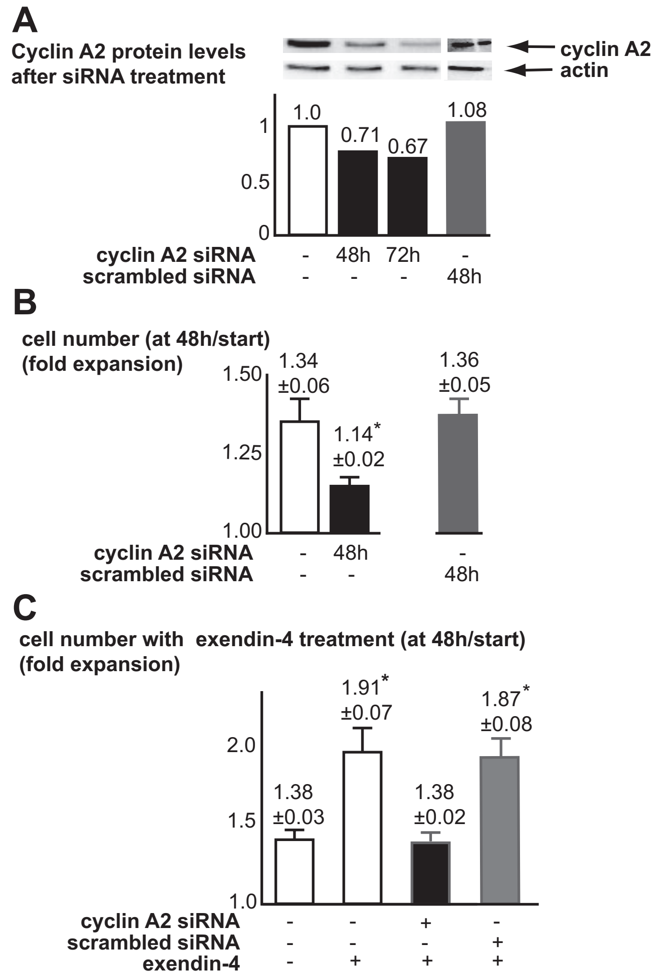

FIG. 5. Knockdown of cyclin A2 in Min6 cells reduces proliferation rate. siRNA-mediated knockdown of cyclin A2 in Min6 cells is ascertained by immunoblot for cyclin A2. A representative immunoblot (*A*, *top*) with densitometric analysis of cyclin A2 relative to actin levels is shown (*A*, *bottom*). Scrambled siRNA does not change cyclin A2 levels (far right column). Data are normalized to the first control densitometry, which is depicted as 1.0 in the first column of the graph. Numbers indicate absolute value of the corresponding column immediately below them. *B*: Proliferation rate of Min6 cells is reduced by siRNA knockdown of cyclin A2, whereas scrambled siRNA does not influence proliferation rates. Graph depicts cell number at 48 h normalized to start (0 h) after no siRNA (left column), cyclin A2-specific (middle column), or scrambled siRNA (right column), respectively. *C*: Proliferation rate of Min6 cells is increased by exendin-4 (4 h) treatment and cyclin A2-specific, but not scrambled siRNA, abolishes the exendin-4 stimulation of cell proliferation. Graph depicts cell number at 48 h normalized to start (0 h) after no exendin-4, exendin-4 only, exendin-4 plus cyclin A2-specific siRNA, or scrambled siRNA, respectively. Means ± SE in graphs are provided above the corresponding bars. *Significant difference from the corresponding control with *P* < 0.05.

animals exhibit β-cell proliferation activity and elevated cyclin A2 without changes in Skp2. In addition, our transient transfection studies in Min6 cells indicate that intracellular cAMP-PKA signaling directly regulates cyclin A2 transcription. On the basis of these observations, we favor direct cAMP-CREB-CBP–responsive activation of the cy-

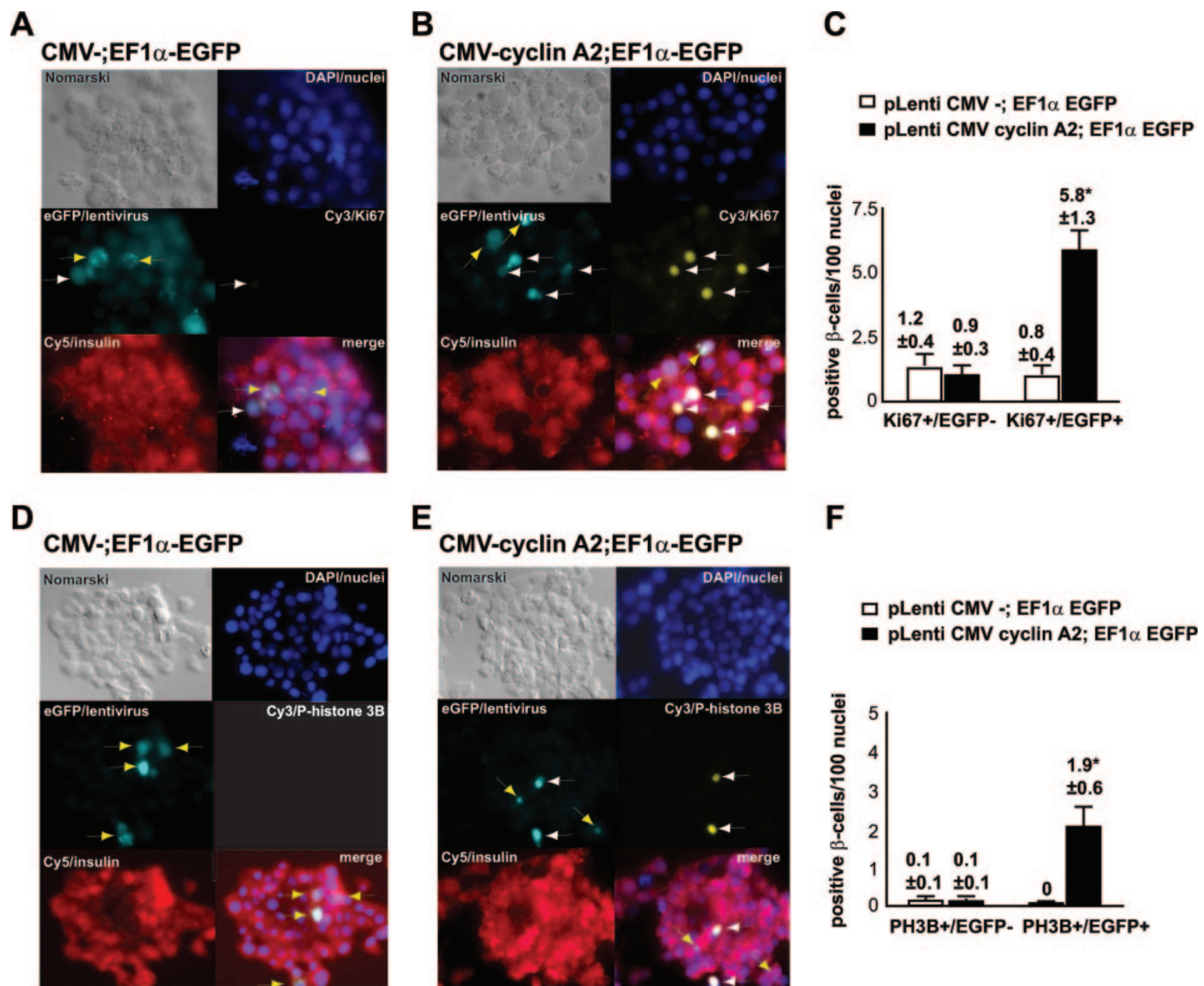

FIG. 6. Lentiviral expression of cyclin A2 in mouse islets stimulates β-cell proliferation. *A–D*: Representative pseudo-colored digitally imaged sections of islets infected for 48 h with control (CMV-; E1fα-EGFP) (*A* and *D*) or cyclin A2 expressing (CMV-cyclin A2; E1fα-EGFP) (*B* and *E*) lentivirus. Sections (Nomarksi image shown in *top left subpanels*, respectively) are co-immunostained and pseudo-colored for nuclei (DAPI, blue; *top right subpanel*), virally expressed EGFP (cyan; *middle left subpanel*), insulin (Cy5, red; *bottom left subpanel*), proliferation markers Ki67 (*A* and *B*) or phospho-histone-3B (*D* and *E*) (yellow; *middle right subpanel*). The *bottom right subpanels* depict the corresponding merged image of the fluorescence channels within the respective panels. White arrows indicate proliferation marker positive and positive lentivirus infection. Yellow arrows indicate proliferation marker negative, lentivirus postive infection. Quantitaive analysis of proliferation markers Ki67 (*C*) or phospho-histone 3B (*F*) in β-cells of mouse islets infected for 48 h with control (CMV-; E1fα-EGFP) or cyclin A2–expressing (CMV-cyclin A2; E1fα-EGFP) lentiviridae. β-Cells infected with cyclin A2–expressing lentivirus (■) show significantly more Ki67 ($Ki67^+/EGFP^+$) and phospho-histone 3B ($Phospho\text{-}H3B^+/EGFP^+$) expression than β-cells infected with control virus (□) or β-cells not infected at all ($Ki67^+/EGFP^-$). Means ± SE in graphs are provided above the corresponding bars. *Significant difference from the corresponding control with $P < 0.05$. (Please see http://dx.doi.org/10.2337/db07-1541 for a high-quality digital representation of this figure.)

clin A2 transcription as the main mechanism underlying elevated cyclin A2 levels during exendin-4 treatment. It is important to note that CBP-S436A mutant transcriptional upregulation is specific for CREB-CBP interaction and does not influence transcription activity of other transcription factors such as FoxO1 or hypoxia-inducible factor 1α (9,12).

The islet p27 reduction (Fig. 2), which is required for cell cycle entry, is common to both mouse models reported here (29). p27 levels are low in the islets of our relatively young mice (Fig. 2), consistent with observations that β-cell p27 increases with age, which prevents β-cell expansion (17). Reduction of p27 levels may occur by multiple pathways, which lead to p27 phosphorylation and phosphorylation-dependent ubiquitination and degradation mediated by Skp2 (rev. in 30). The treatment of C57Bl/6 mice with exendin-4 and studies in Min6 cells indicate that Skp2 elevation likely contributes toward p27 reduction, consistent with previous observations (11). However, in CBP-S436A islets and cyclin A2 overexpression in islets, changes in Skp2 protein levels are not required for p27 reduction (Fig. 2; Supplemental Fig. 7). Formation of cyclin A2–cyclin-dependent kinase (cdk) 1 or A2-cdk 2 complexes lead to p27 phosphorylation and degradation (31), which likely is achieved with the existing Skp2 levels in CBP-S436A β-cells and cyclin A2 overexpression in isolated islets. In summary, p27 levels are most likely reduced by multiple pathways, and as recently reported, in β-cells Skp2 is required for p27 proteosomal degradation (11). The CBP-mutant studies indicate that p27 reduction can be achieved directly or indirectly by nuclear cAMP-CREB-CBP–responsive transcription. Importantly, exendin-4–treated animals required 5 days to achieve an increase in islet size similar to that found in 12-week-old CBP-S436A$^{+/-}$ mice. This time difference may be due in part to elevated Skp2 in exendin-4–treated animals, and as downstream effects, more rapid β-cell proliferation compared with CBP-S436A islets. Although

our studies are consistent with PI 3-kinase–mediated stimulation of Skp2 (18), the exact mechanisms underlying incretin-mediated changes in Skp2 levels in β-cells remain to be elucidated.

β-Cell–specific ablation of PDX-1 markedly reduces GLP-1–induced β-cell proliferation (8), whereas other salient β-cell properties, such as glucokinase activity, mitochondrial metabolism, ATP generation (21), and levels of mRNA transcripts of important proteins such as IRS2, CREB, and transcription factors Nkx2.2 and Isl1, remain intact at normal levels (8,21). Consistent with these observations (5,21), the present findings confirm that PDX-1 expression is required for cAMP generation in pancreatic β-cells. The association between PDX-1 and exendin-4–stimulated cAMP formation and downstream cyclin A2 transcription is supported by the following observations: *1*) PDX-1 knockdown decreases cAMP generation and cyclin A2 activation by exendin-4. The effect of PDX-1 knockdown on cAMP generation was seen after 48 h of PDX-1 knockdown and not after 24 h (data not shown), which may reflect PDX-1 protein half-life and/or an indirect effect of PDX-1. *2*) PDX-1 knockdown reduces GLP-1R and Gsα transcript in β-cells. *3*) PDX-1 knockdown effects can be rescued by the cAMP downstream effector PKAcs. And *4*) PKA inhibition with H89 or PKI supresses exendin-4 stimulation of cyclin A2 transcription when PDX-1 is not knocked down. In cultured isolated islets, which have high PDX-1 levels, transcription of cyclin A2 responds to exendin-4 more rapidly than PDX-1 transcription. These results are consistent with indirect (via IRS2 and FoxO1) exendin-4–stimulated PDX-1 expression (5,32), whereas direct exendin-4-cAMP–mediated cyclin A2 stimulation occurs more rapidly. Although PDX-1 is required for cAMP generation, the studies in CBP-S436A$^{+/-}$ mice argue that augmentation of PDX-1 levels above baseline is not essential for initiating β-cell proliferation (Figs. 1–3).

PDX-1 knockdown is associated with reduced GLP-1R and Gsα transcript in primary islets (Supplemental Fig. 8), consistent with reports of PDX-1–dependent GLP-1R expression (8,21). Our studies extend these observations in that PDX-1 is required for Gsα expression. Studies in cell lines indicate that PDX-1 does not directly regulate GLP-1R or Gsα (33). We therefore speculate that PDX-1 knockdown indirectly reduces GLP-1R and Gsα. Furthermore, the findings herein are consistent with reduced β-cell proliferation in β-cell-specific Gsα knockout mice (22).

In pancreatic β-cells, cyclins D1 and D2 and their interacting partner cdk 4 are essential for postnatal β-cell replication. Mice lacking cyclin D1 and D2 exhibit reduced β-cell mass (3,10). Mice lacking cdk 4 in β-cells exhibit reduced islet mass and diabetes, and mice overexpressing constitutively active cdk 4 in β-cells exhibit increased β-cell proliferation (34,35). The present studies do not exclude a role for D-type cyclins, for cdk 4 (and 6), or for nonexendin-4 extracellular growth stimuli in β-cell proliferation. Our findings suggest a differentiated response of β-cells to extracellular growth factors with cyclin A2 being responsive to the cAMP-PKA-CREB-CBP pathway, whereas the D-type cyclins possibly respond to growth factor stimuli other than cAMP or PI 3-kinase (36). Previous reports have suggested that cyclins D1 and D2 respond to the cAMP pathway in in vitro cell systems (37–39). We do not find any evidence for exendin-4 or CREB-CBP–mediated elevation in D-type cyclins. Thus, on the basis of our results, we conclude that in adult β-cells, exendin-4 likely does not participate in regulating D-type cyclin protein levels.

In summary, the current studies indicate that exendin-4 stimulates cyclin A2 transcription and protein levels via the cAMP-PKA-CREB-CBP pathway. Exendin-4 stimulates Skp2 levels via the PI 3-kinase pathway. Decreased p27 protein levels accompany these changes. Overexpression of cyclin A2 by lentivirus infection in primary pancreatic β-cells is sufficient to stimulate β-cell proliferation activity. Furthermore, our studies provide additional evidence for the central role of homeodomain transcription factor PDX-1 in β-cell proliferation, with PDX-1 being required for cAMP generation.

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