Cyclin-Dependent Kinase 5 Promotes Pancreatic β-Cell Survival via Fak-Akt Signaling Pathways

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OBJECTIVE—Cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 1-like 1 has recently been linked to type 2 diabetes by genome-wide association studies. While CDK5 and its regulatory protein p35 are both expressed and display enzymatic activity in pancreatic β-cells, their precise role in the β-cell remains unknown. Because type 2 diabetes is characterized by a deficit in β-cell mass and increased β-cell apoptosis, we investigated the role of CDK5 in β-cell survival.

RESEARCH DESIGN AND METHODS—We used INS 832/13 cells, rat islets isolated from wild-type or human islet amyloid polypeptide (h-IAPP) transgenic rats, and pancreatic tissue from rats and humans with and without type 2 diabetes and investigated the effect of CDK5/p35 inhibition (by small interfering RNA or by chemical inhibition) as well as CDK5/p35 overexpression on β-cell vulnerability to apoptosis.

RESULTS—CDK5 inhibition led to increased β-cell apoptosis. To identify the mechanisms involved, we examined the phosphorylation state of focal adhesion kinase (Fak)^{Ser732}, a known target of CDK5. Following CDK5 inhibition, the phosphorylation of Fak^{Ser732} decreased with resulting attenuation of phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway. Conversely, CDK5 overexpression increased Fak^{Ser732} phosphorylation and protected β-cells against apoptosis induced by the inhibition of the β-1 integrin signaling pathway. Also, Fak^{Ser732} phosphorylation was less abundant in β-cells in both h-IAPP transgenic rats and humans with type 2 diabetes.

CONCLUSIONS—This study shows that by regulating Fak phosphorylation and subsequently PI3K/Akt survival pathway, CDK5 plays a previously unrecognized role in promoting β-cell survival. *Diabetes* **60:1186–1197, 2011**

Genome-wide association studies have identified a variant of the cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 1-like 1 (CDKAL1) to be associated with type 2 diabetes (1–5). CDK5 is a serine/threonine kinase ubiquitously expressed in mammalian tissues, with a best characterized functional role in the central nervous system (6). CDK5 has no enzyme activity as a monomer, and its activation requires association with a regulatory protein, p35 or p39, abundantly expressed in the brain. CDK5 and its regulatory proteins, p35 and p39, are also expressed in pancreatic β-cells where complexes CDK5/p35 and CDK5/p39 display enzymatic activity (7,8). Type 2 diabetes is

characterized by a deficit in β-cell mass with increased β-cell apoptosis as well as a deficit in β-cell function (9). To date, while a few studies have investigated the potential role of CDK5 in β-cell function (8,10–13), its role in β-cell survival has not been addressed.

Neurons in Alzheimer's disease and β-cells in type 2 diabetes are characterized by endoplasmic reticulum (ER) stress-induced apoptosis associated with formation of misfolded toxic oligomers of locally expressed amyloidogenic proteins (Alzheimer's β-protein [AβP] in brain and islet amyloid polypeptide [IAPP] in β-cells) (14,15). There is an unexplained shared predisposition to type 2 diabetes and Alzheimer's disease while variance in CDK5 and its binding partners is linked to risk for Alzheimer's disease in a high-risk population (16,17). It has been proposed that AβP misfolding, through aberrant proteolytic cleavage of p35 to generate the truncated p25 fragment, induces hyperactivation and mislocalization of CDK5 in neurons that in turn leads to neuronal dysfunction and apoptosis (18).

In the present studies, we first tested the hypothesis that the CDK5 complex acts as an intermediary between IAPP misfolding and β-cell apoptosis. In contrast to AβP, sufficient expression of human-IAPP (h-IAPP) to induce apoptosis did not induce hyperactivation of the CDK5/p35 complex. In contrast, we established that the CDK5/p35 complex is protective in β-cells. Further investigation revealed that in β-cells, the prosurvival mechanism mediated by the CDK5/p35 complex is accomplished by activation of focal adhesion kinase (Fak) and Akt signaling pathways. We conclude that genome-wide association studies linkage of CDKAL1 to type 2 diabetes may reflect the prosurvival properties of the CDK5 complex in β-cells.

RESEARCH DESIGN AND METHODS

Cell culture. Rat insulinoma cell line INS 832/13 (19) was kindly provided by Dr. C. Newgard (Duke University Medical Center, Durham, NC). Cells were grown in RPMI-1640 medium supplemented with 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA), 10% heat-inactivated FBS (Gemini, West Sacramento, CA), and 50 μmol/L β-mercaptoethanol (Sigma, St. Louis, MO) at 37°C in a humidified 5% CO_2 atmosphere.

Transduction experiments. INS 832/13 cells were plated in 6-well plates at 1.0×10^6 cells/well and cultured overnight. Cells were transduced with rodent-IAPP (r-IAPP) or h-IAPP adenoviruses (400 moi [multiplicity of infection], 48 h) in complete RPMI-1640 medium and lysed in NP40 buffer (50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 1 mmol/L dithiothreitol and protease inhibitors [Sigma]).

Islet isolation. Wild-type (WT) and h-IAPP transgenic (HIP) rats were bred and housed at the animal housing facility of the University of California, Los Angeles (UCLA). The UCLA Institutional Animal Care and Use Committee approved all surgical and experimental procedures. The generation of HIP rats has been previously described (20). Islets from WT and HIP rats (Supplementary Table 1) were isolated as described (21).

Roscovitine treatment. Islets from 2-month-old WT rats ($n = 7$) were cultured 24 h in RPMI-1640 medium supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS at 37°C in a humidified 5% CO_2 atmosphere. Islets were treated 48 h with 10 μmol/L roscovitine (Calbiochem, La Jolla, CA) or DMSO (vehicle, Sigma).

Transfection experiments

Small interfering RNA. CDK5 (ACCTACGGAACTGTGTTCAAGGCCA) and Fak (CAGTACTTACTATAAAGCTTCCAAA) small interfering RNAs (siRNAs) were purchased from Invitrogen. p35 (AAGAGGATCGTGGCGGTGTCA) and scramble (AF488) siRNAs were purchased from Qiagen (Valencia, CA). INS 832/13 were seeded in 12-well plates (0.25×10^6 cells/well), cultured overnight, and transfected with 50 nmol/L siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were transduced with r-IAPP or h-IAPP adenoviruses and cultured for an additional 24 h.

Plasmids. CDK5-green fluorescent protein (GFP) (#1346) and p35 (#1347) plasmids were obtained from the plasmid repository Addgene. INS 832/13 were seeded in 12-well plates (0.5×10^6 cells/well), cultured overnight, and transfected for 24 and 48 h with 0.2 μg or 0.4 μg of CDK5 and/or p35 or pCMV-GFP (as control) plasmids according to the manufacturer's instructions.

pBabe/Fak stable lines. INS 832/13 were transduced with pBabe and Fak retroviruses in complete RPMI-1640 medium supplemented with 4 μg/mL protamine sulfate (Sigma) and selected 72-h post-transfection with 0.5 μg/mL puromycin.

Western blotting. Proteins were separated on a 4–12% Bis-Tris NuPAGE gel and blotted onto a nitrocellulose membrane (Whatman, Germany). Membranes were probed overnight at 4°C with CDK5 (DC17), P-Fak^{Ser732}, insulin receptor substrate (IRS) 2 (Millipore/Upstate, St. Louis, MO), C/EBP homologous protein (CHOP) (Sc-575, lot #L057; Santa Cruz Biotechnology, Santa Cruz, CA), p35 (#2680), p35/p25 (#2673), P-Bad^{Ser136}, Bad, P-Akt^{Ser473}, Akt, P-ERK1/2, ERK1/2, Fak, cleaved caspase-3, PARP, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology, Beverly, MA) antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA). Proteins were visualized by enhanced chemiluminescence (Millipore), and protein levels were quantified using the Labworks software (UVP, Upland, CA).

β-1 integrin blockade. INS 832/13 cells were trypsinized and preincubated in suspension for 1 h at 37°C with or without 20 μg/mL β-1 integrin blocking antibody (CD29, Ha2/5, hamster antirat) or hamster IgM as control (G235–1) (BD Bioscience, San Jose, CA) in RPMI-1640 medium supplemented with 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% heat-inactivated FBS, and 50 μmol/L β-mercaptoethanol. Cells were replated in 8-well chamber slides coated with collagene I (BD Bioscience) at 0.15×10^6 cells/well and cultured for 24 h in the presence or absence of antibodies.

Immunofluorescence staining cells and tissue. INS832/13 cells were plated in 4-well chamber permanox slides (Permanox Nunc, Rochester, NY) or 8-well chamber slides coated with collagen. After treatment, cells were fixed 30 min at room temperature in 4% paraformaldehyde.

Rat tissue. Pancreata were obtained from WT, prediabetic HIP and diabetic HIP rats (Supplementary Table 1). Frozen sections were prepared from 4% paraformaldehyde-fixed tissue embedded in optimal cutting temperature compound.

Human tissue. Cases studied are summarized in Supplementary Table 2 and previously described (14). Surgically removed human pancreas specimens were obtained in accordance with UCLA Institutional Review Board approval. Pancreata from type 2 diabetic versus nondiabetic control subjects were fixed overnight in 4% formaldehyde and frozen in optimal cutting temperature compound. Cells and pancreatic sections were permeabilized in 0.4% Triton X-100/TBS and stained as previously described (21).

Propidium iodide staining. INS 832/13 cells were plated as described above. Aliquots of 30 islets isolated from WT rats were cultured in 8-well chamber slides. After treatment, cells or islets were incubated 30 min at 37°C with 50 μg/mL propidium iodide (PI) (Molecular Probes, Eugene, OR) and fixed 30 min at room temperature in 4% paraformaldehyde. Cells (3,000–6,000 per condition) and islets (30 per condition) were imaged using a Leica DM6000 microscope (Leica Microsystems, Wetzlar, Germany). For rat islets, PI positive area was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). In CDK5/p35 overexpression experiments, for each condition, all cells, transfected and nontransfected were counted without distinction and included in the analysis.

Statistical analysis. Data are presented as means ± SEM. Statistical analyses were carried out by Student t test or ANOVA followed by Duncan post hoc test for multiple comparisons using Statistica (Statsoft, Tulsa, OK). A P value of <0.05 was taken as evidence of statistical significance.

RESULTS

Specific inhibition of the complex CDK5/p35 by siRNA does not prevent h-IAPP–induced apoptosis. Apoptosis induced by h-IAPP was assessed in INS 832/13 cells transduced with adenoviruses expressing h-IAPP or the non-amyloidogenic, non-toxic r-IAPP, as control for a comparable burden of protein expression. As described previously, high expression rates of h-IAPP increased caspase-3 cleavage in comparison with r-IAPP–transduced cells (Fig. 1A). To determine whether inhibition of CDK5 would suppress h-IAPP–induced β-cell apoptosis, we transfected INS 832/13 cells with CDK5 siRNA or scramble and transduced these cells with h-IAPP or r-IAPP adenoviruses. CDK5 siRNA decreased CDK5 expression without interfering with p35 expression, but did not decrease h-IAPP–induced apoptosis as determined by cleaved caspase-3 Western blot (Fig. 1B).

Because CDK5 is usually activated by complex formation with p35, we probed the role of the CDK5/p35 complex in h-IAPP–induced apoptosis by the alternative approach of knocking down p35. Despite successful knockdown of p35, cells were not protected against h-IAPP–induced apoptosis (Fig. 1C). Inhibition of the CDK5/p35 complex by either CDK5 or p35 knockdown failed to prevent h-IAPP–induced apoptosis. In contrast, as shown in Fig. 1B and C, inhibition of CDK5/p35 complex in β-cells potentiated h-IAPP toxicity and was sufficient by itself to induce apoptosis in control cells ($P < 0.05$).

We also investigated whether disturbed intracellular Ca^{2+} homeostasis and h-IAPP would induce the cleavage of p35 to generate the p25 truncated fragment and subsequently promote CDK5 hyperactivation in β-cells. Treatment of INS 832/13 cells either with the ER stress inducer thapsigargin or the Ca^{2+} ionophore A23187 induced p35 cleavage (Supplementary Fig. 1A). However, neither p25/p35 ratio, p35, nor CDK5 expression was affected by h-IAPP in INS 832/13 cells (Supplementary Fig. 1B) or in HIP rat islets (Supplementary Fig. 1C) despite the presence of ER stress and apoptosis illustrated by increased CHOP expression and caspase-3 cleavage, respectively. In addition, cytoplasmic and nuclear distribution of CDK5 and p35 was not affected in cells expressing h-IAPP (Supplementary Fig. 2).

Collectively, the absence of effect of CDK5 or p35 knockdown on h-IAPP–induced apoptosis and the absence of effect of high expression rates of h-IAPP sufficient to induce apoptosis on CDK5 or p35 levels in INS 832/13 cells and primary β-cells, lead to the rejection of the stated hypothesis that h-IAPP–induced β-cell apoptosis is mediated by CDK5 hyperactivation.

Specific inhibition of the complex CDK5/p35 by siRNA increases INS 832/13 cell vulnerability to apoptosis. In the data presented in Fig. 1, the loss of CDK5 activity seemed to be sufficient to promote β-cell apoptosis. To confirm and extend this observation, cells were transfected with CDK5 or p35 siRNAs alone, avoiding any bias introduced by the adenoviral transduction. CDK5 siRNA led to a 60% decrease in CDK5 expression ($P < 0.01$) (Fig. 2A). Supporting the results obtained in the previous experiment, CDK5 knockdown led to a threefold increase in caspase-3 cleavage ($P < 0.01$) (Fig. 2A). A similar increase in apoptosis was also observed in cells transfected with p35 siRNA, in which a 50% decrease in p35 levels led to a fourfold increase in cleaved caspase-3 ($P < 0.01$) (Fig. 2B). Apoptosis induction following CDK5 or p35 knockdown was also confirmed by PI staining ($P < 0.01$) (Fig. 2C). Therefore, these results suggest a role of CDK5/p35 activity in β-cell survival.

Loss of CDK5 activity results in the attenuation of the Fak/Akt survival pathway. To further investigate the role of CDK5 in β-cell survival and identify downstream signaling pathways, we examined the effect of CDK5
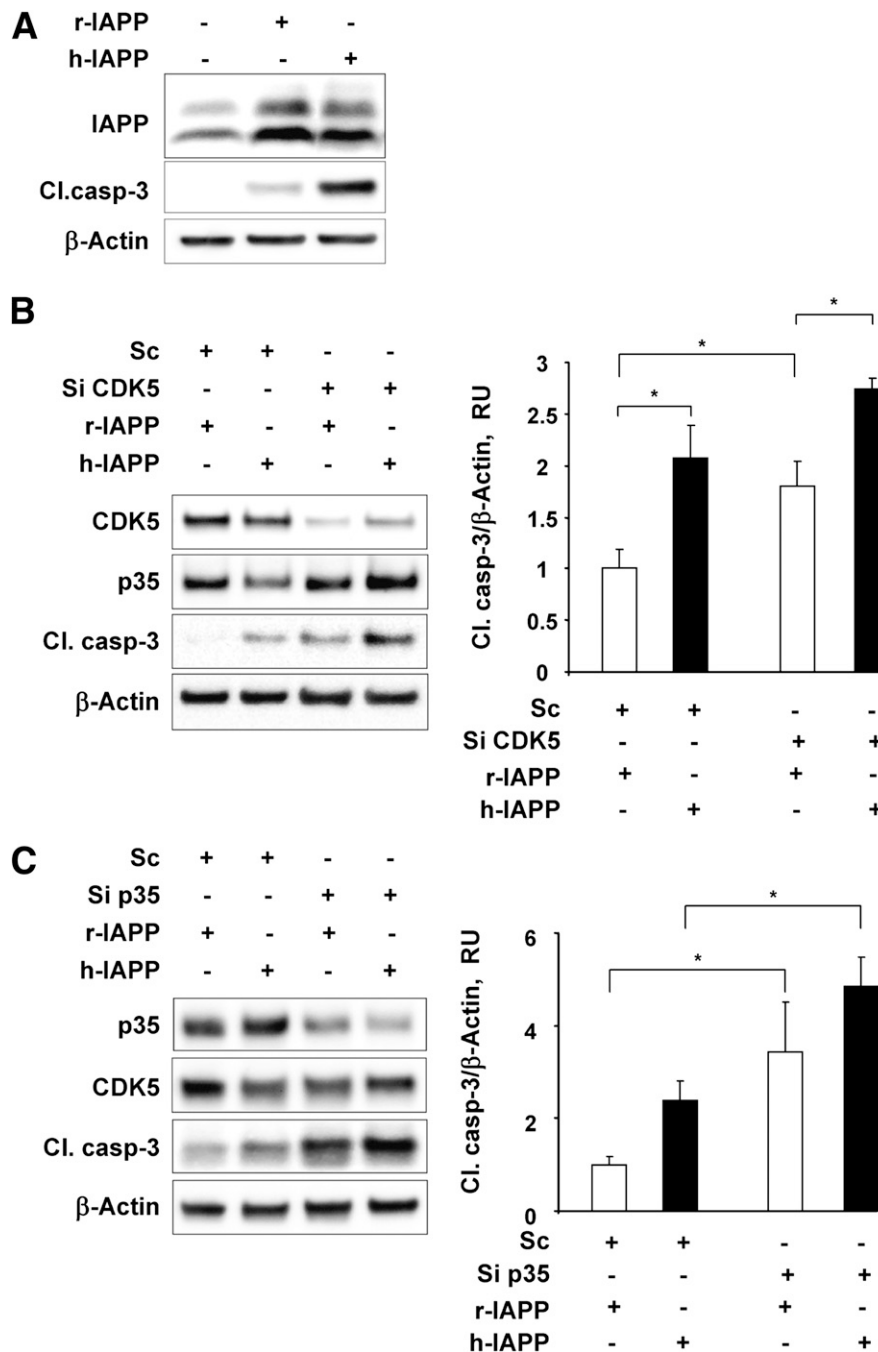
FIG. 1. Specific inhibition of the complex CDK5/p35 by siRNA does not prevent h-IAPP–induced apoptosis. *A*: INS 832/13 cells were transduced at 400 moi for 48 h with r-IAPP or h-IAPP adenoviruses. IAPP expression and cleaved caspase-3 level (Cl. casp-3) to assess apoptosis were examined by Western blot. IAPP image was obtained by grouping different parts of the same gel. *B* and *C*: INS 832/13 cells were transfected for 48 h with scramble (Sc), CDK5 (*B*), or p35 (*C*) siRNAs (50 nmol/L) and transduced with r-IAPP or h-IAPP (400 moi) for the last 24 h of culture. Protein levels of CDK5, p35, and cleaved caspase-3 were analyzed by Western blot (*left panels*). β-Actin was used as loading control. The *right panels* represent the quantification of cleaved caspase-3 levels (*n* = 4). *$P < 0.05$, significant differences. RU, relative units.

inhibition on the phosphorylation of Fak^{Ser732}, identified as CDK5 target in neurons (22). As shown in Fig. 3*A*, CDK5 knockdown led to a 40% decrease in Fak^{Ser732} phosphorylation ($P < 0.05$). This effect also correlated with a decrease in P-Fak^{Tyr397} ($P < 0.05$), suggesting a decrease in Fak kinase activity (Supplementary Fig. 3*A*). Fak is known to regulate prosurvival pathways including ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (23), also involved in β-cell survival (24). In cells transfected with CDK5 siRNA, the level of P-ERK1/2 remained unchanged compared

with control cells (Fig. 3*B*). However, with regard to PI3K/Akt pathway, we found that CDK5 inhibition and subsequently Fak inhibition led to a decrease in P-Akt^{Ser473} (70%, $P < 0.01$). P-Akt^{Ser473} promotes cell survival by phosphorylating the transcription factor cAMP-responsive element-binding protein (CREB). pCREB is known to play a role in regulation of the *IRS2* gene expression that promotes β-cell survival. P-Akt^{Ser473} also increases phosphorylation of Bad^{Ser136}, thereby inhibiting its proapoptotic activity. CDK5 knockdown led to a decrease in IRS2 protein
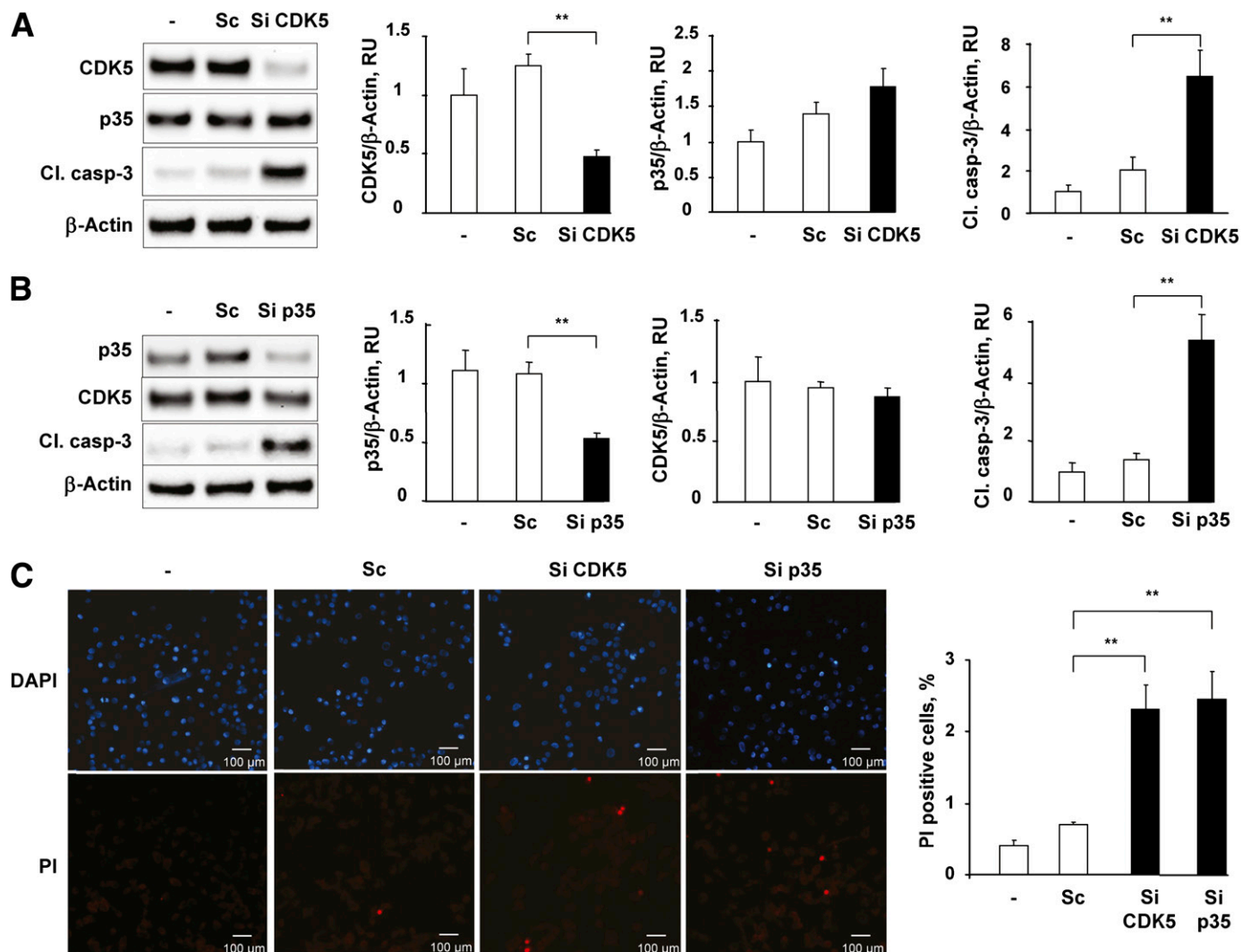
FIG. 2. Specific inhibition of the complex CDK5/p35 by siRNA increases INS 832/13 cell vulnerability to apoptosis. INS 832/13 cells were transfected for 48 h with scramble (Sc), CDK5 or p35 siRNAs (50 nmol/L). *A* and *B*: Protein levels of CDK5, p35, and cleaved caspase-3 were analyzed by Western blot (*left panels*). β-Actin was used as loading control. The *right panels* represent the quantification of CDK5, p35, and cleaved caspase-3 expressions. *C*: Cell death was assessed by PI staining (*n* = 4). **$P < 0.01$, significant differences. RU, relative units. (A high-quality digital representation of this figure is available in the online issue.)

levels and P-Bad^{Ser136} levels that correlated with an increase in apoptosis, therefore supporting an attenuation of Akt signaling pathway ($P < 0.01$) (Fig. 3C).

Roscovitine treatment decreases Fak/Akt survival pathway and induces apoptosis in rat islets. To test whether CDK5 also plays an important role in the survival of primary β-cells, we investigated the effect of CDK5 inhibition in rat islets treated with roscovitine, a chemical inhibitor of CDK5. Roscovitine treatment did not affect the level of ERK1/2 phosphorylation in rat islets but led to a decrease in P-Fak^{Ser732}, P-Akt^{Ser473}, IRS2, and P-Bad^{Ser136} levels ($P < 0.001$) (Fig. 4A). This attenuation in Fak/Akt pathway was accompanied by a 1.6-fold increase in cleaved caspase-3 ($P < 0.01$), confirmed by PI staining ($P < 0.05$) (Fig. 4B and C). A decrease in P-Fak^{Tyr397} was also observed in rat islets following roscovitine treatment, suggesting a decrease in Fak kinase activity ($P < 0.01$) (Supplementary Fig. 3B).

In agreement with the effect of CDK5 knockdown in INS cells, these data in primary cells indicate that decreased CDK5 availability leads to β-cell apoptosis. We then

determined whether Fak was the downstream effector responsible for CDK5 prosurvival activity.

Downstream of CDK5, Fak is directly involved in β-cell survival. To investigate the link between Fak and β-cell survival, we first examined the consequences of Fak knockdown on INS 832/13 cell apoptosis. In common with CDK5 knockdown, we found that the depletion of Fak increased β-cell vulnerability to apoptosis ($P < 0.05$) (Fig. 5A). We then examined whether Fak protects β-cells against apoptosis induced by CDK5 inhibition by overexpressing Fak (or pBabe as a control) in INS 832/13 that were treated (or not) with roscovitine. Under basal conditions, Fak overexpression led to a twofold increase in P-Fak^{Ser732} ($P < 0.001$) (Fig. 5B). The same trend was observed for P-Akt (Fig. 5B). As expected, roscovitine treatment of control cells led to a decrease in P-Fak^{Ser732} that correlated with a decreased P-Akt (Fig. 5B) and an increase in apoptosis (Fig. 5C and D). Although roscovitine also decreased Fak^{Ser732} phosphorylation in Fak-overexpressing cells, Fak phosphorylation was maintained at higher levels than in control cells ($P < 0.001$) (Fig. 5B). Higher levels
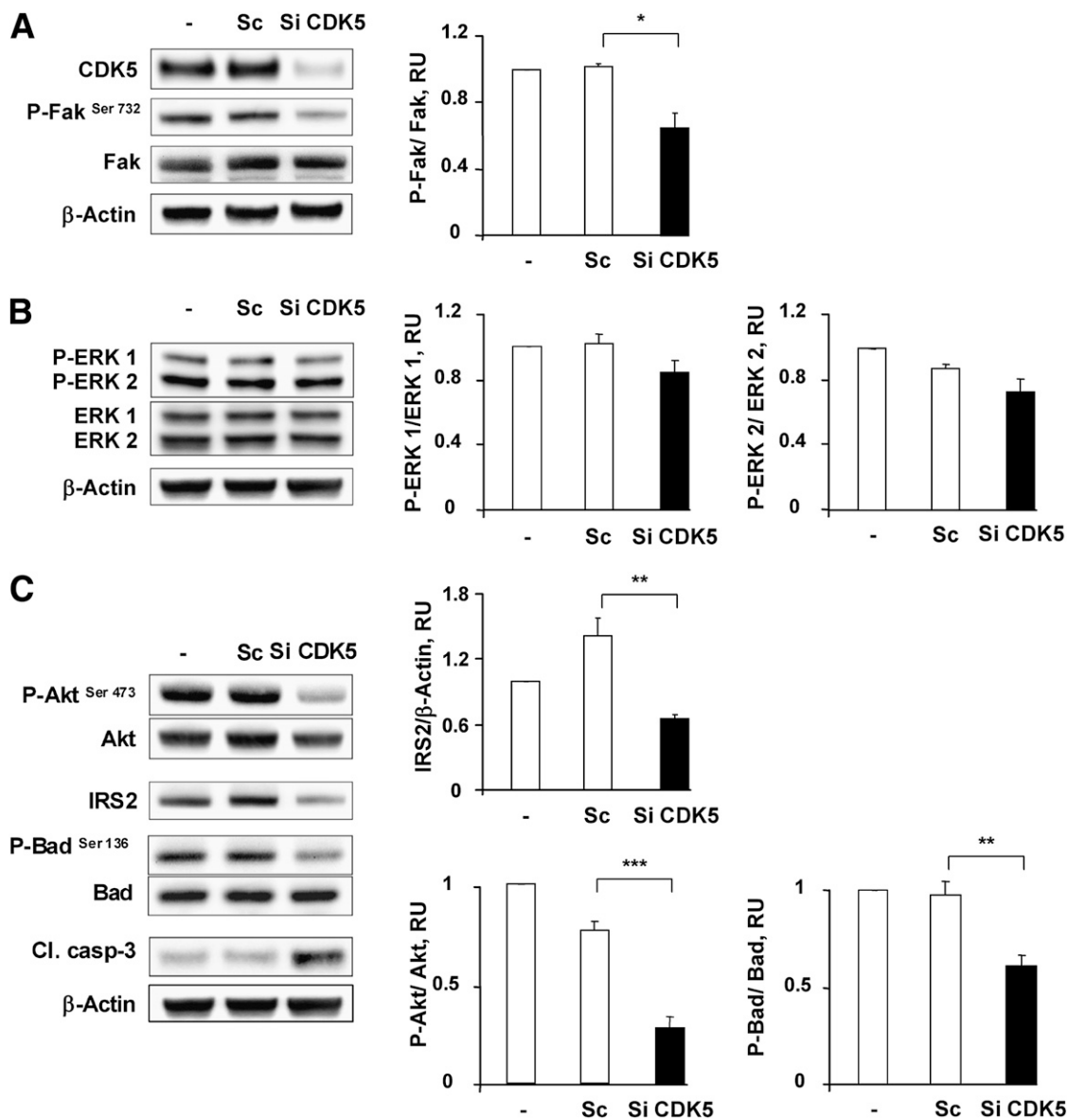
FIG. 3. Loss of CDK5 activity results in the attenuation of the Fak/Akt survival pathway. *A–C*: INS 832/13 cells were transfected for 48 h with scramble (Sc) or CDK5 siRNAs (50 nmol/L). Protein levels of CDK5, Fak, Akt, Bad, ERK1/2, cleaved caspase-3, and phosphorylation levels of Fak^{Ser732}, Akt^{Ser473}, ERK1/2, and Bad^{Ser136} were analyzed by Western blot (*left panels*). β-Actin was used as loading control. The *right panels* represent the quantification of the Western blot (*n* = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences. RU, relative units.

of P-Akt were also present in Fak-overexpressing cells following roscovitine treatment ($P < 0.05$) (Fig. 5*B*) with decreased apoptosis assessed both by staining for cleaved caspase-3 ($P < 0.01$) (Fig. 5*C*) and PI staining ($P < 0.05$) (Fig. 5*D*).

Together, these data suggest that the increase in β-cell apoptosis consistently noted following inhibition of CDK5 expression is at least in part mediated through a decrease in Fak phosphorylation and the subsequent attenuation of Akt survival pathway. To further develop the hypothesis of a prosurvival role of CDK5, we investigated whether CDK5 overexpression would protect β-cells against apoptosis.

CDK5 overexpression protects β-cells against apoptosis induced by the inhibition of β-1 integrin signaling pathway. Fak activation is an early event of the signaling cascade initiated by integrins to promote cell survival (25). It is recognized that integrins, especially the β-1 integrin interactions with extracellular matrix, are critical

for β-cell function and survival. We first examined the effect of inhibition of β-1 integrin signaling on CDK5 activity. While this inhibition did not affect the protein levels of CDK5 and p35, it did result in a decrease in P-Fak^{Ser732} and P-Akt, suggesting a decrease in CDK5/p35 complex activity ($P < 0.05$) (Fig. 6*A*). These effects were also associated with a twofold increase in caspase-3 cleavage ($P < 0.01$) (Fig. 6*B*). We therefore investigated the effect of CDK5 or CDK5/p35 overexpression on β-cell survival following β-1 integrin pathway inhibition. We first confirmed that overexpression of CDK5 or CDK5 in combination with p35 resulted in an increase in CDK5 and p35 protein levels ($P < 0.001$) (Supplementary Fig. 4*A*) and in Fak^{Ser732} phosphorylation levels ($P < 0.05$) (Supplementary Fig. 4*B*). We then investigated the effect of this increased CDK5 activity on β-cell apoptosis induced by β-1 integrin inhibition. As illustrated by PI staining, incubation of INS 832/13 cells with anti–β-1 integrin blocking antibody
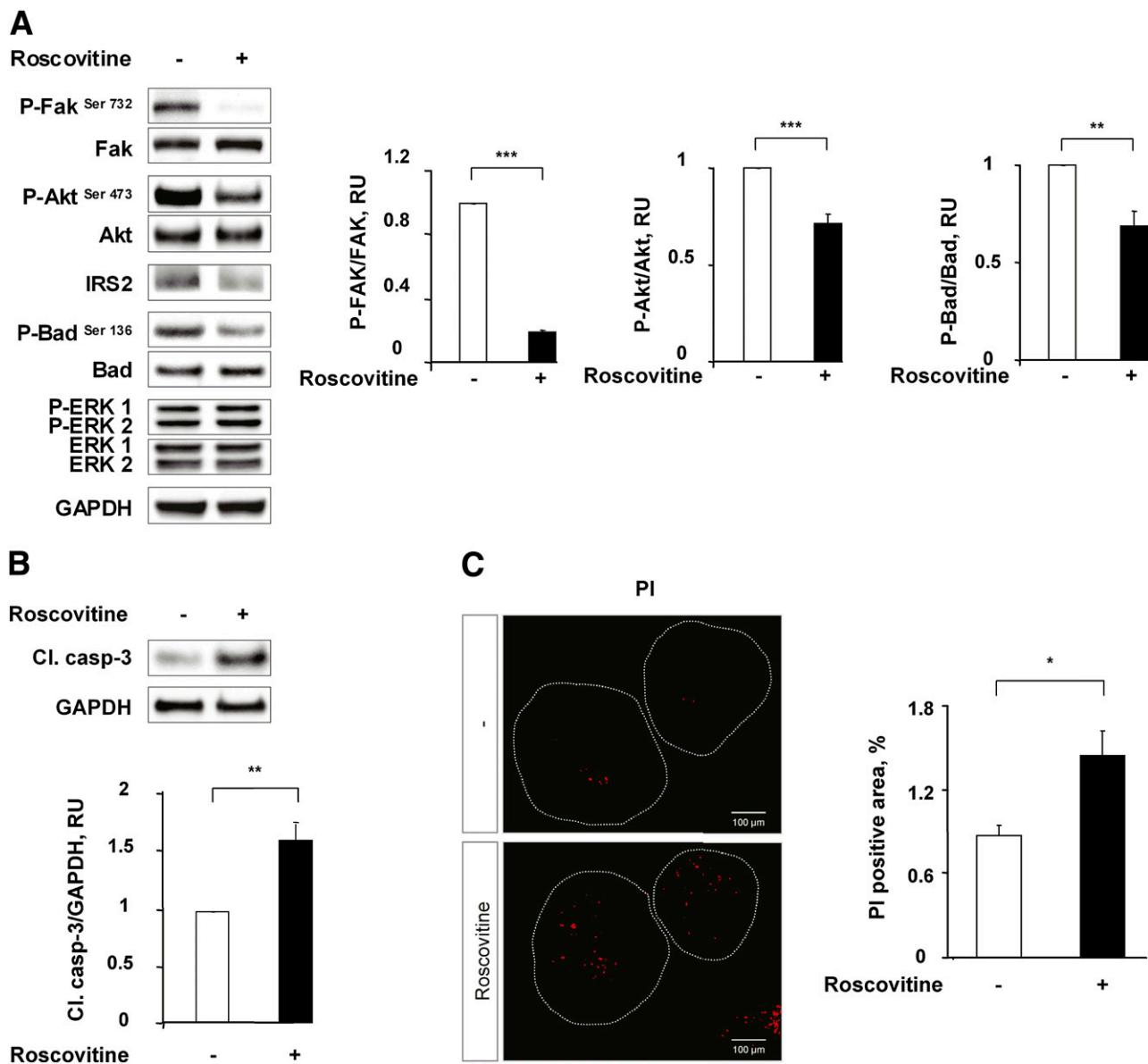
FIG. 4. Roscovitine treatment decreases Fak/Akt survival pathway and induces apoptosis in rat islets. *A–C*: Isolated WT rat islets were cultured in the absence or presence of roscovitine 10 μmol/L for 48 h. *A* and *B*: Protein levels of Fak, IRS2, Akt, Bad, ERK1/2, cleaved caspase-3, and phosphorylation levels of Fak^{Ser732}, Akt^{Ser473}, ERK 1/2, and Bad^{Ser136} were analyzed by Western blot. GAPDH was used as loading control. The graphs represent the quantification of the Western blots. *C*: Islet apoptosis was assessed by PI staining. The graph represents the quantification of the islet area positive for PI, expressed in % ($n = 7$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences. RU, relative units. (A high-quality digital representation of this figure is available in the online issue.)

resulted in a 2.4-fold increase in cell death ($P < 0.001$) (Fig. 6*C*). Both CDK5 and CDK5/p35 overexpression led to a 50% decrease in β-cell death induced by β-1 integrin inhibition in comparison with GFP-transfected cells ($P < 0.001$) (Fig. 6*C*).

Altogether these findings indicate that CDK5 activity, by promoting Fak^{Ser732} phosphorylation, sustains β-cell survival and is able to protect β-cells against apoptosis induced by the inhibition of β-1 integrin signaling pathway. Prior studies have reported that the ERK1/2 signaling pathway is also implicated in the transduction of cell-matrix signaling in β-cells. We have shown in this study that ERK1/2 activity in β-cells was not dependant on CDK5 activity. This could therefore explain why cells overexpressing CDK5 are only partially protected against apoptosis.

To determine if CDK5 activity is also important for β-cell survival in response to other insults, we used thapsigargin-induced apoptosis as a model of ER stress and investigated whether in this context, CDK5 inhibition or activation would affect β-cell survival. In cells transfected with CDK5 siRNA, a sixfold increase in cleaved caspase-3 was observed in the presence of thapsigargin in comparison with cells transfected with Sc ($P < 0.05$) (Supplementary Fig. 5*A*). This indicates that the loss of CDK5 activity enhances β-cell vulnerability to apoptosis induced by thapsigargin. Conversely, overexpression of CDK5, with a resulting increase in P-Fak^{Ser732} resulted in partial protection of β-cells from ER stress-induced apoptosis ($P < 0.05$) (Supplementary Fig. 5*B*), further supporting a prosurvival role of CDK5 activity in β-cells.
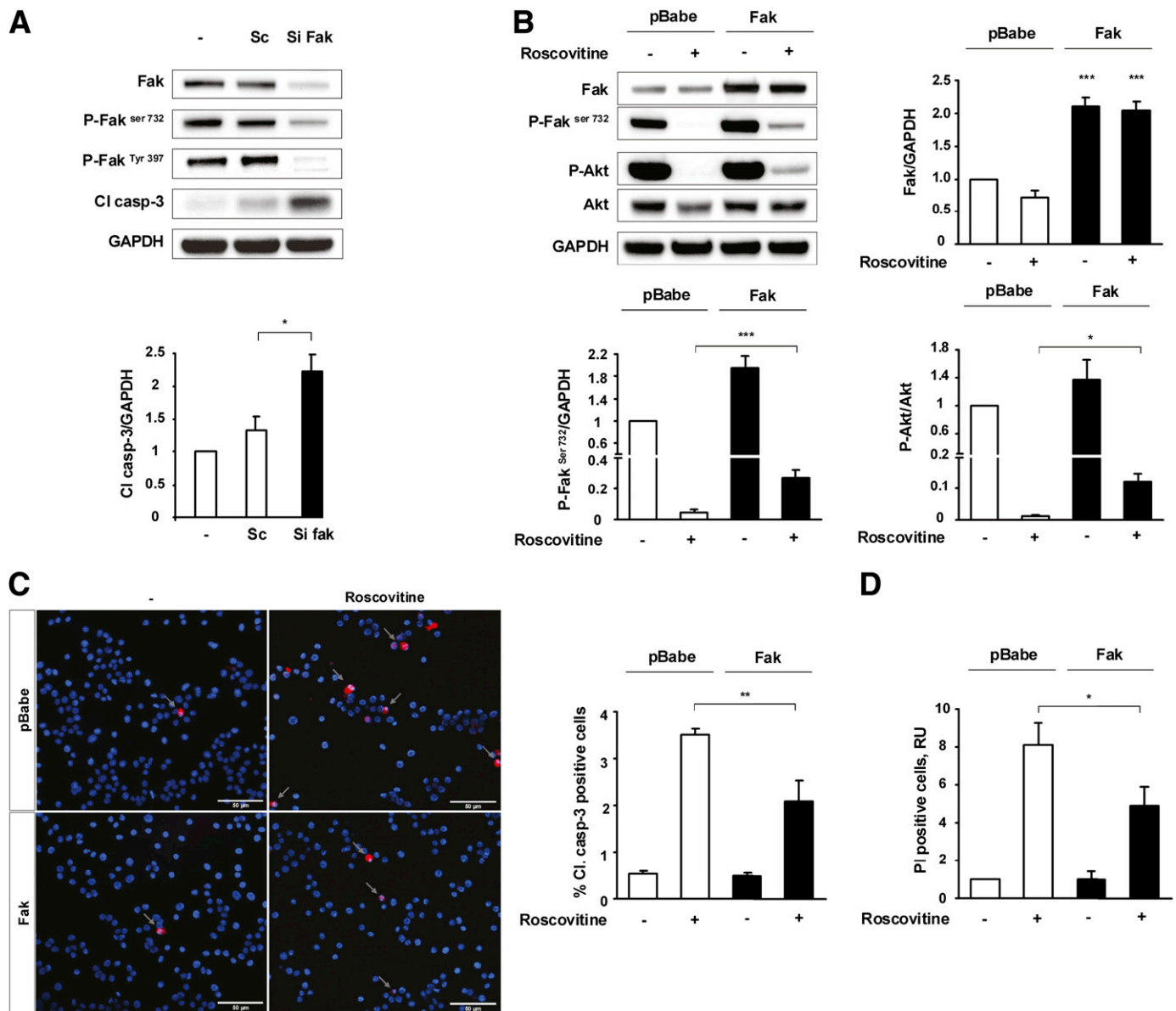
FIG. 5. Downstream of CDK5, Fak is directly involved in β-cell survival. *A*: INS 832/13 cells were transfected with scramble (Sc) or Fak siRNAs (50 nmol/L, 48 h). Protein levels of Fak, P-Fak^{Ser732}, P-Fak^{Tyr397}, and cleaved caspase-3 were analyzed by Western blot. GAPDH was used as loading control. The graph represents the quantification of the Western blot (*n* = 3). *B–D*: pBabe and Fak-INS 832/13 cells were treated with or without roscovitine (40 μm, 24 h). *B*: Fak and Akt protein levels and Fak^{Ser732}, Akt^{Ser473} phosphorylation levels were analyzed by Western blot. GAPDH was used as loading control. The graphs represent the quantification of the Western blots (*n* = 3). *C*: Apoptosis was assessed by immunofluorescence staining using an antibody directed against the cleaved form of caspase-3 (red). Nuclei were stained with DAPI (blue). The graph represents the quantification of the number of cells positive for cleaved caspase-3, expressed in % (*n* = 3). *D*: Cell death was assessed by PI staining (red). The graph represents the quantification of the relative number of PI positive cells compared with control cells (*n* = 3). *P < 0.05, **P < 0.01, ***P < 0.001, significant differences. RU, relative units. (A high-quality digital representation of this figure is available in the online issue.)

These findings indicate that CDK5 plays an important role in promoting β-cell survival in response to β-1 integrin inhibition but also ER stress–induced apoptosis.

Phosphorylation of Fak^{Ser732} is decreased in β-cells in rats with diabetes. Next we investigated whether a deficit in CDK5 activity is associated with the increased β-cell apoptosis present in type 2 diabetes. We examined P-Fak^{Ser732} as an indirect marker of CDK5 activity in a rat model of type 2 diabetes, HIP rats that develop islet pathology comparable to humans with type 2 diabetes (20). Western blotting for P-Fak^{Ser732} was performed on islet lysates obtained from prediabetic and diabetic HIP rats

and age-matched WT rats (Supplementary Table 1). In prediabetic HIP rat islets, the level of P-Fak^{Ser732} was no different from that in WT rat islets (Fig. 7A). In agreement with our previous observation, CDK5 protein levels were also similar in both genotypes. In contrast, in islets isolated from diabetic HIP rats, there was an approximately 50% decrease in P-Fak^{Ser732} in comparison with age-matched WT rat islets (*P* < 0.01) (Fig. 7A). There was also a 27% decrease in the CDK5 protein level in diabetic HIP rat islets (*P* < 0.01).

To confirm that the decreased P-Fak^{Ser732} in diabetic HIP rats was not an artifact of decreased β-cell mass, we
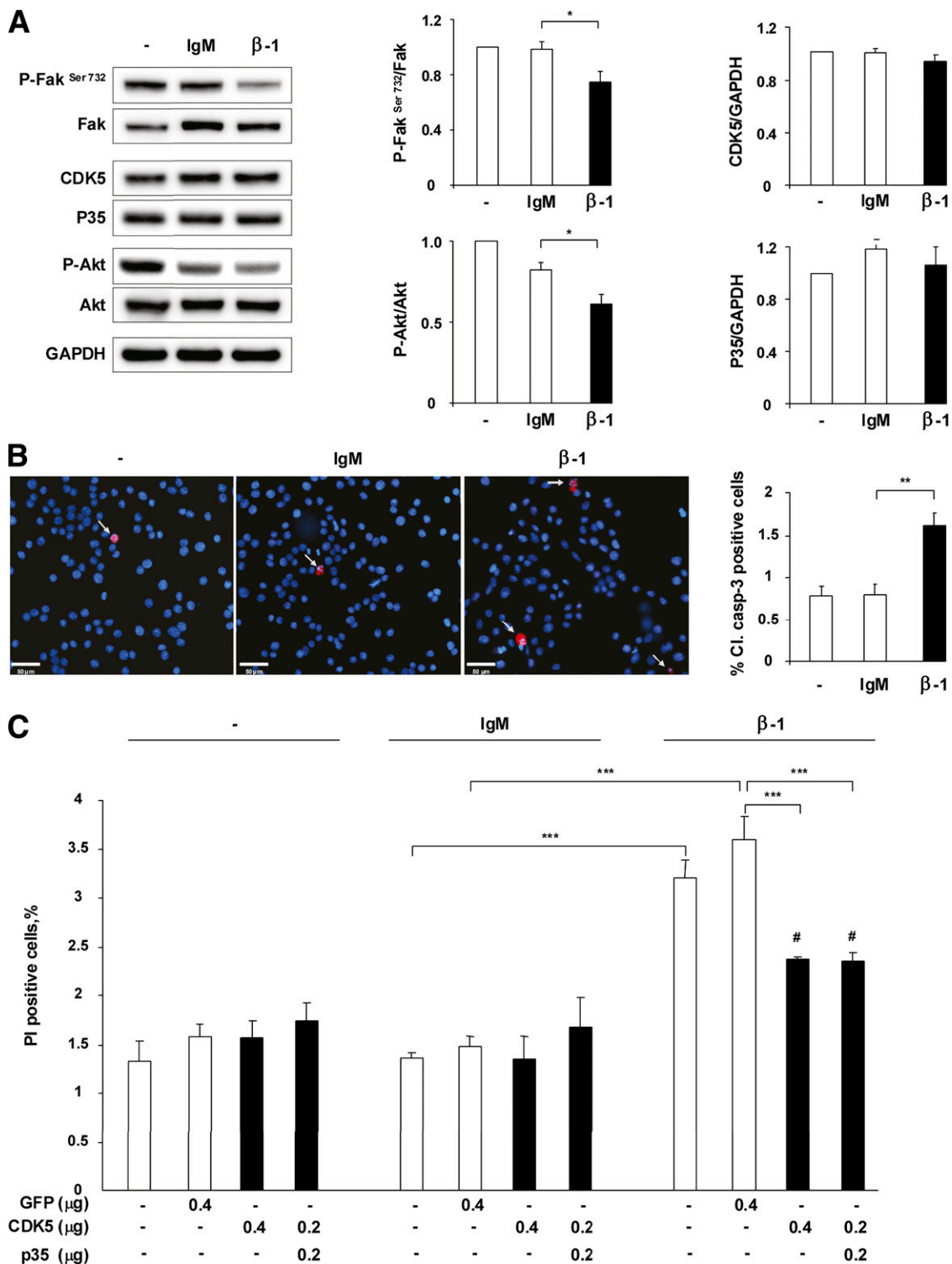
FIG. 6. CDK5 overexpression protects β-cells against apoptosis induced by the inhibition of β-1 integrin signaling pathway. *A* and *B*: INS 832/13 cells were left untreated or incubated with 20 µg/ml of IgM antibody as control or β-1 integrin-blocking antibody for 24 h. *A*: Protein levels of Fak, CDK5, p35, Akt, and phosphorylation levels of Fak^{Ser732}, Akt^{Ser473} were analyzed by Western blot. GAPDH was used as loading control. The graphs represent the quantification of the Western blots ($n = 3$). *B*: Apoptosis was assessed by immunofluorescence staining using an antibody directed against the cleaved form of caspase-3 (red). Nuclei were stained with DAPI (blue). The graph represents the quantification of the number of cells positive for cleaved caspase-3, expressed in % ($n = 3$). *C*: INS 832/13 cells were left untreated or transfected for 24 h with GFP (0.4 µg), CDK5-GFP (0.4 µg), CDK5-GFP (0.2 µg) + p35-myc (0.2 µg). Cells were then incubated with IgM antibody as control or β-1 integrin-blocking antibody for additional 24 h. Cell death was assessed by PI staining ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant differences. #$P < 0.05$, significant differences vs. IgM-treated cells. (A high-quality digital representation of this figure is available in the online issue.)
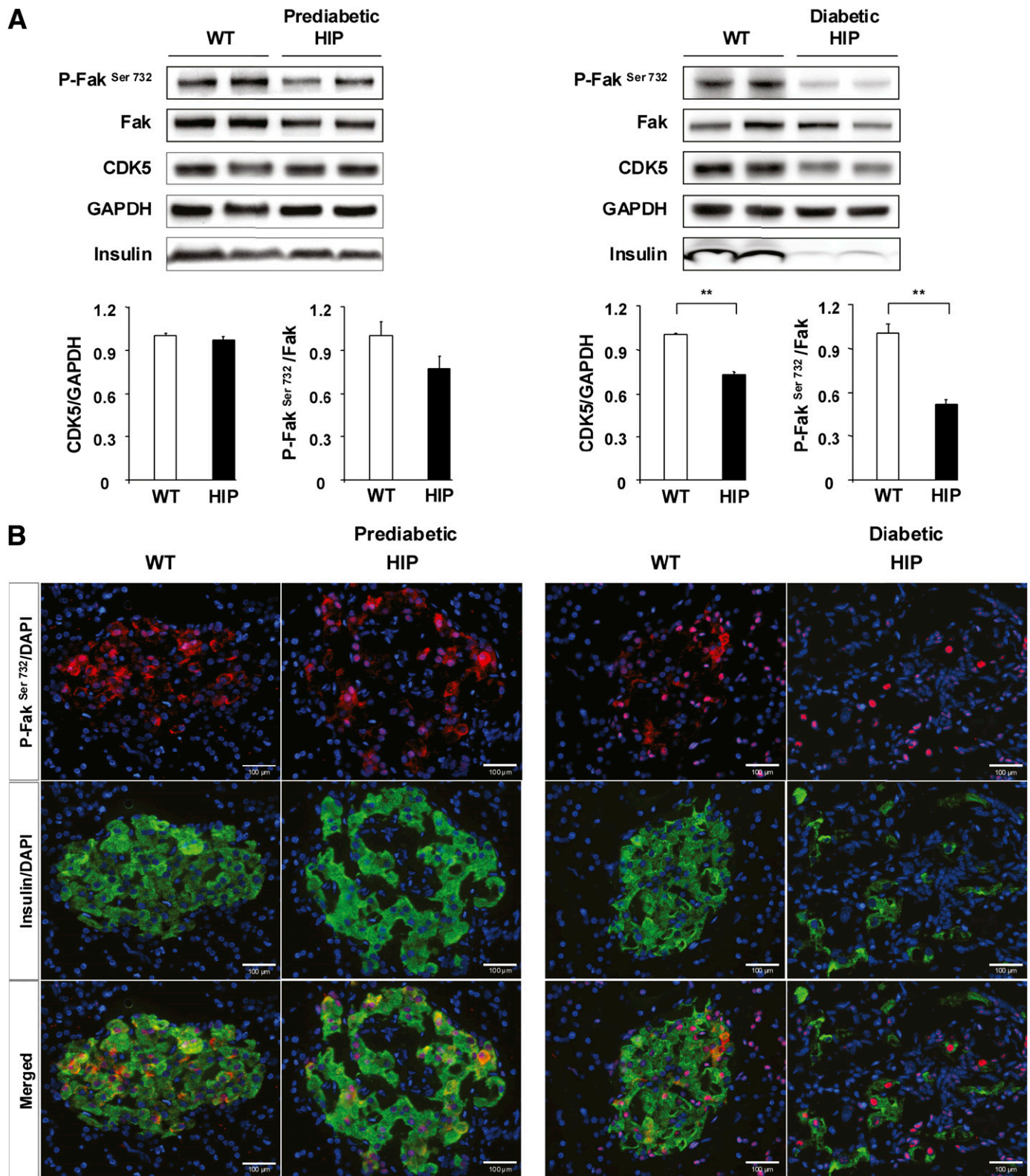
FIG. 7. Phosphorylation of Fak[Ser732] is decreased in β-cells in rats with diabetes. A: The phosphorylation state of Fak[Ser732] and CDK5 protein level were assessed by Western blot using lysates obtained from 4- to 6-month-old WT (*n* = 6) and prediabetic HIP rats (*n* = 6) (*left panel*) and 9- to 11-month-old WT (*n* = 4) and HIP rats with diabetes (*n* = 4) (*right panel*). GAPDH and insulin were used as control. The graphs represent the quantification of the Western blot. **$P < 0.01$, significant differences vs. WT. B: The presence of Fak[Ser732] phosphorylation in β-cells was assessed by immunofluorescence (P-Fak[Ser732], red; Insulin, green; Nuclei, blue) in pancreatic tissue obtained from 4- to 6-month-old WT (*n* = 2) and prediabetic HIP rats (*n* = 2) (*left panels*) and 9- to 11-month-old WT (*n* = 3) and HIP rats with diabetes (*n* = 3) (*right panels*). (A high-quality digital representation of this figure is available in the online issue.)

performed a coimmunostaining for insulin and P-Fak[Ser732] in rat pancreata (Fig. 7B). The results were in agreement with the Western blot data. There was no difference in P-Fak[Ser732] staining between prediabetic HIP and WT rats. However, P-Fak[Ser732] immunostaining was decreased in the cytoplasm of β-cells from diabetic HIP rats in comparison with age-matched WT rats (Fig. 7B).

Moreover, P-Fak[Ser732] staining in β-cells from diabetic HIP rats revealed almost exclusive nuclear localization. Prior studies have reported a nuclear localization of Fak under conditions of cellular stress (26). Therefore, the high level of β-cell apoptosis present in diabetic HIP rats might explain the presence of nuclear P-Fak[Ser732].

Of note, a close parallel was observed between P-Fak[Ser732] and P-Fak[Tyr397] levels in rat β-cells. Indeed, P-Fak[Tyr397] levels were no different to WT controls in prediabetic HIP rat islets but were decreased in HIP rats with diabetes (Supplementary Fig. 6A and B).

In contrast to the decreased P-Fak in β-cells in HIP rats with diabetes, suggesting a deficit in CDK5 activity under these conditions, P-ERK1/2 expression was increased in HIP rat islets with diabetes (Supplementary Fig. 7). This finding implies that CDK5 does not directly regulate the mitogen-activated protein kinase pathway in β-cells.

Phosphorylation of Fak[Ser732] is decreased in β-cells in humans with type 2 diabetes. To determine whether the decrease in P-Fak[Ser732] observed in HIP rats is relevant to humans with type 2 diabetes, pancreata obtained from 4 nondiabetic control subjects and 6 individuals with type 2 diabetes were stained for P-Fak[Ser732]. Analysis of these pancreata revealed a diminished staining in β-cells of individuals with type 2 diabetes (Fig. 8). Quantification of the number of β-cells with cytoplasmic staining showed a 45% decrease in P-Fak[Ser732] in type 2 diabetes ($P < 0.05$). Consistent with the data obtained in the β-cells in the HIP rat, these observations imply that P-Fak[Ser732] is decreased in β-cells in humans with type 2 diabetes, implying either insufficient CDK5 kinase activity or an impaired response to the CDK5 kinase.

DISCUSSION

In this study, we tested the hypothesis that CDK5 hyperactivation contributes to h-IAPP–induced apoptosis. The reasons for testing this postulate were the close similarity between cellular pathology in islets in type 2 diabetes and neurons in Alzheimer's disease, together with the proposed role of CDK5 hyperactivation in AβP-induced neuronal cell apoptosis (15,18,27). The data presented here permit us to comprehensively reject that hypothesis with regard to h-IAPP toxicity in β-cells in type 2 diabetes. Unexpectedly, these studies did uncover a role for CDK5 activity in sustaining β-cell survival by regulating Fak phosphorylation and PI3K/Akt survival pathway.

ER stress and altered calcium homeostasis have been reported in both h-IAPP– and AβP-mediated toxicity (21,28). Neuronal overexpression of AβP induces calpain-mediated cleavage of p35 and the appearance of the p25 truncated fragment that leads to CDK5 hyperactivation and relocalization (29,30). The fact that h-IAPP did not induce p35 cleavage implies that the subcellular compartments disrupted by these misfolded proteins likely differ. Thus, while both IAPP and AβP oligomers have been shown to induce membrane disruption, AβP is localized to the greatest extent at the cell membrane, whereas IAPP is located predominantly within the secretory pathway where the oligomers of IAPP have been shown to form (14). As such, it is perhaps not surprising that the former might have a greater effect to displace CDK5 from the plasma membrane than the latter. In β-cells, ER stress induced by thapsigargin led to a modest cleavage of p35. However, even under those conditions, CDK5 activity was still protective. Also, because p35 levels remained much higher than p25 levels, it seems reasonable to postulate that CDK5/p35 complexes may still prevail over CDK5/p25 complexes. Furthermore, the decreased P-Fak[Ser732] levels observed here following thapsigargin treatment do not support hyperactivation of CDK5. Thus neither thapsigargin nor IAPP-induced ER stress sufficient to induce apoptosis in β-cells promoted CDK5 hyperactivation. By implication,
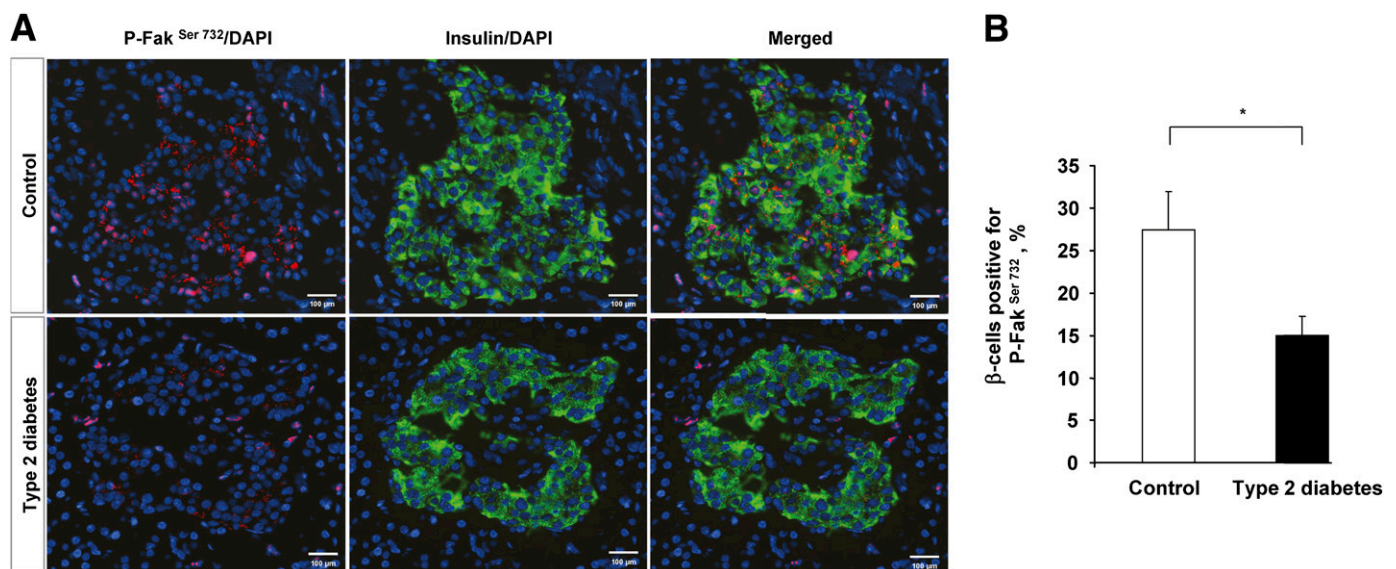
FIG. 8. Phosphorylation of Fak[Ser732] is decreased in β-cells in humans with type 2 diabetes. A: The presence of Fak[Ser732] phosphorylation in β-cell was assessed by immunofluorescence (P-Fak[Ser732], red; Insulin, green; Nuclei, blue) in surgical pancreatic tissue obtained from nondiabetic control subjects (n = 4) and subjects with type 2 diabetes (n = 6). B: The graph represents the quantification of β-cells positive for P-Fak[Ser732], expressed in %. *P < 0.05, significant differences. (A high-quality digital representation of this figure is available in the online issue.)

although CDK5 hyperactivation has been reported to be an important mediator of ER stress-induced apoptosis in neurons, this does not appear to be the case in pancreatic β-cells.

Only a few studies have focused so far on the role of CDK5 in pancreatic β-cells. In INS-1 cells, elevation of extracellular glucose concentrations induces p35 expression and activates the CDK5/p35 complex, which enhances insulin promoter activity (7). Inhibition of CDK5 has been reported to protect INS-1 cells from glucotoxicity (31). Different groups have also studied the role of CDK5 in glucose-stimulated insulin secretion and have reached contradictory conclusions. Studies reported that insulin secretion is enhanced by inhibition of CDK5 (11–13) whereas another group reported that CDK5 promotes insulin exocytosis (8,10). The latter study reported in addition that regulation of insulin exocytosis is specifically mediated by the complex CDK5/p39 and does not involve p35 (8).

Distinct roles in the regulation of CDK5 activity have been attributed to p35 and p39 in the brain using knockout models (32). Loss of p35 seems to be more detrimental to CDK5 function and although p39 expression is upregulated in the brain of $p35^{-/-}$ mice, it is only able to compensate to a limited extent for the loss of p35. To the contrary, the loss of p39 appears to be fully compensated by p35 (32). Similarly, in our study, the increase in apoptosis following p35 knockdown was comparable to that seen with CDK5 knockdown, implying that p39 does not compensate for the loss of p35. This suggests that p35 and p39 may also play different roles in β-cells; p35 may be responsible for a prosurvival activity of CDK5, whereas p39 may be more important in β-cell function (8).

A prosurvival/proapoptotic duality of CDK5 has been noted in neurons. Mice that are deficient in CDK5 die just before or after birth because of an impaired neuro-development and neuron degeneration, suggesting a role of CDK5 in neuron survival (33). In contrast to CDK5/p25 complex associated with apoptosis, CDK5 association with p35 seems to be required for neuronal survival through the activation of ERK1/2 and PI3K/Akt pathways (34,35). In our study, the loss of CDK5 in both INS 832/13 cells and primary β-cells resulted in an attenuation of PI3K/Akt survival pathway and increased β-cell apoptosis. We propose here that the mechanism responsible for this effect involves a decrease in P-Fak^{Ser732} as a consequence of CDK5 inhibition. Fak^{Ser732} has been identified as a target of CDK5 in neurons and implicated in microtubule organization and neuronal migration (22). Fak is generally activated by integrins and growth factors and regulates various signaling pathways related to cytoskeletal organization, cell proliferation, and cell survival (23,25). Integrins and growth factors are widely recognized as important regulators of β-cell function and survival (36–43). However, the role of P-Fak in β-cells remains unknown. In other cell types, Fak interacts with IRSs and PI3K, thereby stimulating growth and survival pathways (44–46). IRS2 and Akt are both major regulators of β-cell survival (24). Our findings suggest that the regulation of Fak activity and subsequently Akt survival pathway may represent a mechanism by which CDK5 promotes β-cell survival.

The deficit in P-Fak^{Ser732} in HIP rats became apparent with development of diabetes, implying that the deficit in P-Fak^{Ser732} is either secondary to and/or may contribute to the decompensation of β-cell function and survival that occurs after the onset of hyperglycemia. Because ER stress and increased β-cell apoptosis are already present in the HIP rat preceding diabetes onset, the deficit in P-Fak^{Ser732} is presumably not simply a consequence of the apoptosis. The integrity of islet morphology, maintained by cell-cell and cell-extracellular matrix interactions, has been reported to be essential for sustaining β-cell survival and function (47–49). Besides increased apoptosis and decreased β-cell function, type 2 diabetes is also characterized by a disruption of islet architecture (27). This disruption occurs at least in part because of the presence of extensive amyloid deposits and membrane disrupting properties of h-IAPP oligomers (14,27,50). Disruption of islet morphology resulting in the disengagement of cell adhesion molecules, integrin receptors, and focal adhesions could contribute to the decrease in the Fak signaling pathway found in rats and humans with type 2 diabetes. On the other hand, given the role of the Fak pathway to foster cell-to-cell communication, the newly reported prosurvival function of the CDK5 complex to promote cell survival through activation of Fak may palliate the actions of extracellular amyloid and cell membrane IAPP oligomers to disrupt cell-to-cell communication in the islet in type 2 diabetes.

In conclusion, there is an as yet unexplained linkage between CDKAL1 and type 2 diabetes in humans. Although further studies will be necessary to determine the exact nature of the CDKAL1 variant and its influence on CDK5, we present data here that reveal a previously unrecognized role of the CDK5 complex in β-cell survival, mediated through activation of the Fak and Akt pathways. These data thus provide a novel mechanism by which variance in CDKAL1 might influence the risk for type 2 diabetes.

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