Rab GTPase-Activating Protein AS160 Is a Major Downstream Effector of Protein Kinase B/Akt Signaling in Pancreatic β-Cells

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OBJECTIVE—Protein kinase B/Akt plays a central role in β-cells, but little is known regarding downstream Akt substrates in these cells. Recently, Rab GTPase-activating protein AS160, a substrate of Akt, was shown to be involved in insulin modulation of GLUT4 trafficking in skeletal muscle and adipose tissue. The aim of this study was to investigate the expression and potential role of AS160 in β-cells.

RESEARCH DESIGN AND METHODS—AS160 mRNA expression was measured in mouse and human islets and fluorescence-activated cell sorted β-cells and compared in islets from control subjects versus individuals with type 2 diabetes. For knockdown experiments, transformed mouse insulin-secreting MIN6B1 cells were transfected with pSUPER-GFP plasmid encoding a small hairpin RNA against insulin receptor substrate (IRS)-2, AS160, or a negative control. Primary mouse islet cells were transfected with AS160 small interfering RNA.

RESULTS—AS160 was expressed in human and mouse pancreatic β-cells and phosphorylated after glucose stimulation. AS160 mRNA expression was downregulated in pancreatic islets from individuals with type 2 diabetes. In MIN6B1 cells, glucose induced phosphorylation of Akt and AS160, and this was mediated by insulin receptor/IRS-2/phosphatidylinositol 3-kinase independently of changes in cytosolic Ca^{2+}. Knockdown of AS160 resulted in increased basal insulin secretion, whereas glucose-stimulated insulin release was abolished. Furthermore, β-cells with decreased AS160 showed increased apoptosis and loss of glucose-induced proliferation.

CONCLUSIONS—This study shows for the first time that AS160, previously recognized as a key player in insulin signaling in skeletal muscle and adipose tissue, is also a major effector of protein kinase B/Akt signaling in the β-cell. ***Diabetes* 57: 1195–1204, 2008**

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BrdU, 5-bromo-2′-deoxyuridine; ERK1/2, extracellular signal–regulated kinase 1/2; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; hGH, human growth hormone; IRS, insulin receptor substrate; JDRF, Juvenile Diabetes Research Foundation; PI 3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; Rab-GAP, Rab GTPase-activating protein; RNAi, RNA interference; shRNA, small hairpin RNA; TUNEL, transferase-mediated dUTP nick-end labeling.

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Regulation of insulin production is central to mammalian glucose homeostasis, and the loss of functional β-cell mass is common (in varying degrees) to both type 1and type 2 diabetes (1). Molecular signaling pathways that promote β-cell survival and function are thus potential drug targets for the treatment of type 2 diabetes.

Recent studies have demonstrated that proteins involved in the canonical insulin signaling pathway are expressed in human pancreatic β-cells (2). This finding implies an autocrine role for insulin (3) and suggests that dysfunction of the insulin signaling cascade in β-cells may contribute to the compromised insulin secretion of diabetes. β-Cell–specific insulin receptor knockout mice develop age-dependent glucose intolerance, resulting from a progressive decrease in islet volume (4). Mice with targeted knockout of insulin receptor substrate-1 (IRS-1) in β-cells show a decreased insulin content and secretory response to glucose (5), whereas β-cell–specific IRS-2 knockout mice exhibit decreased β-cell mass (6,7).

The signaling cascades by which insulin mediates its effects in its classical target tissues are well established. The main axis involved in GLUT4 vesicular traffic in muscle and adipose tissue is largely within the IRS/phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway, and IRS-1 is indispensable for GLUT4 trafficking in human muscle cells (8). Class I PI 3-kinase docks onto phosphorylated IRS, leading to its activation to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3), a process that has been proposed to mediate the rapid effects of insulin in its target cells (9). PIP3 production activates the Ser/Thr kinase Akt, which is also required for insulin action on GLUT4 translocation in target tissue (10). However, events downstream of Akt had been elusive until recently. In adipocytes and in muscle, a newly discovered protein named Rab GTPase-activating protein (Rab-GAP), AS160, has now been shown to be involved in the insulin signaling pathway (11). This protein, also named AS160/tbc1b4, is phosphorylated on six serine and threonine residues in response to insulin that conforms to the requirements of Akt target motifs, and two of these sites are phosphorylated by Akt in vitro (11). Silencing of AS160 in 3T3-L1 adipocytes prevents insulin action on glucose uptake (12).

Glucose can impact acutely and directly on insulin signaling, but the molecular mechanisms have been only partially elucidated. Short-term glucose action in muscle cells and the L6 line involves retrotranslocation of conventional isoforms of protein kinase C (PKC) from the membrane to the cytoplasm and dissociation from the insulin receptor. This is followed by the transient *trans*-activation

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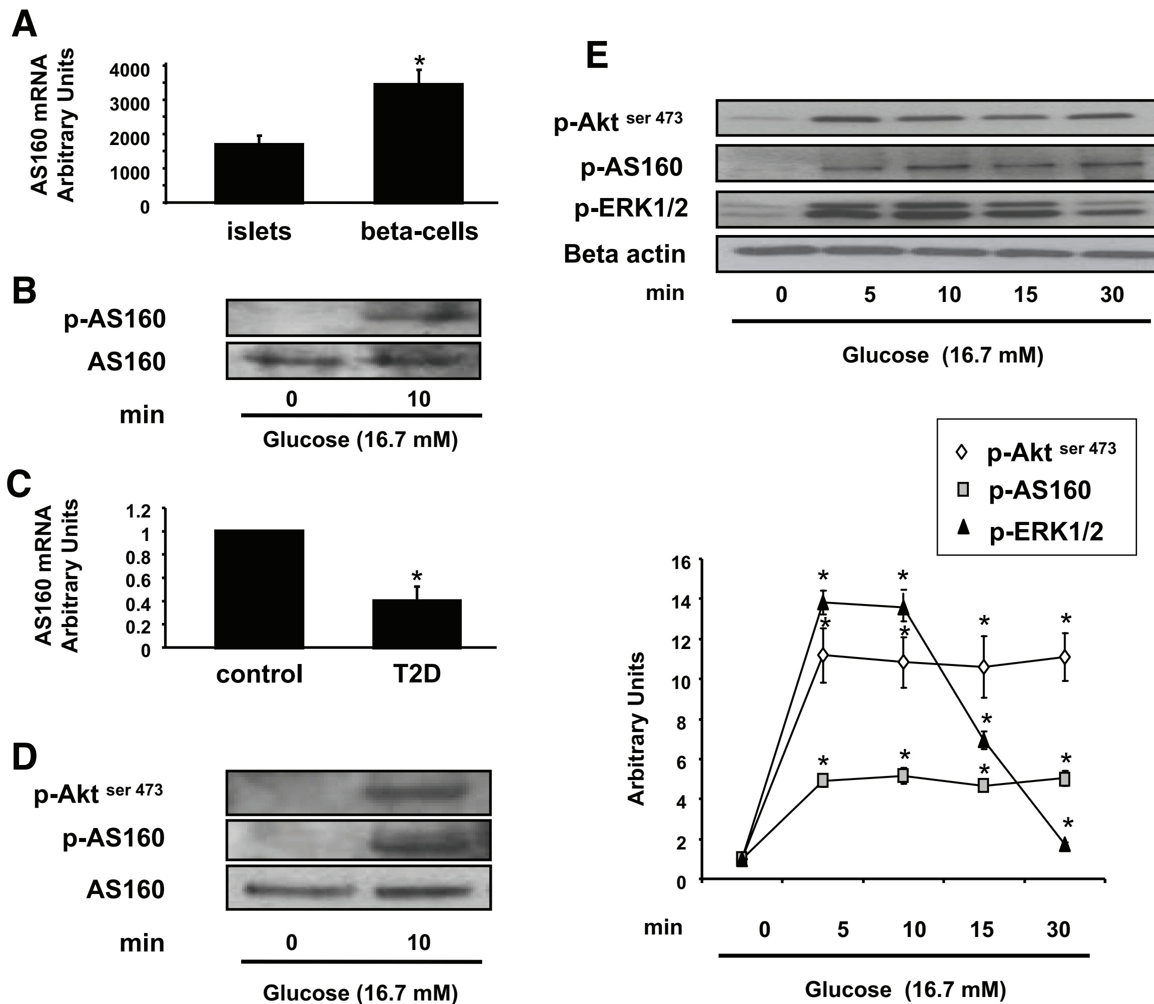

FIG. 1. AS160 is expressed and phosphorylated in β-cells. *A*: AS160 mRNA expression measured by high-density oligonucleotide microarray in human islets versus sorted β-cells. $n = 5$ independent observations; **P* < 0.05. *B*: Western blot (10% acrylamide gel) showing AS160 phosphorylation and protein expression in human islets after 10-min stimulation with 16.7 mmol/l glucose. *C*: AS160 mRNA expression measured by quantitative real-time RT-PCR in human islets from control donors versus type 2 diabetes; $n = 5$ independent observations; **P* < 0.05. *D*: Representative Western blot (10% acrylamide gel) showing Akt Ser473 and AS160 total protein and phosphorylation in primary mouse β-cells after 10-min stimulation with 16.7 mmol/l glucose. *E*: Representative Western blot (10% acrylamide gel) and quantification (below) showing Akt Ser473, AS160, and ERK1/2 phosphorylation in MIN6B1 cells during continuous 30-min stimulation with 16.7 mmol/l glucose. $n = 4$ independent experiments; **P* < 0.05 vs. basal (0 min).

of the insulin receptor tyrosine kinase and a consequent induction of glucose uptake (13). In pancreatic β-cells, although it has been demonstrated that insulin receptor, IRS-2, and Akt are altered in islets isolated from individuals with type 2 diabetes (14,15) and that insulin signaling pathway elements are expressed in human islets, the action of glucose on this pathway and the potential role of AS160 in β-cells remain to be determined.

The current study was therefore designed to investigate whether AS160 is expressed in human and mouse pancreatic β-cells and, if so, to determine its role in glucose action in these cells and the functional consequences of its silencing on glucose-induced insulin secretion as well as β-cell proliferation and survival.

RESEARCH DESIGN AND METHODS

Antibodies and reagents. Diazoxide was purchased from Sigma (St. Louis, MO), and LY-294002 was from Calbiochem (La Jolla, CA). Anti–phospho-Akt (Ser473) and anti–phospho–extracellular signal–regulated kinase 1/2 (ERK1/2) (p42/44 mitogen-activated protein kinase) kinase (Thr202 and Tyr204) were purchased from New England Biolabs (Beverly, MA). Anti–phospho-(Ser/Thr)-AS160, anti-AS160, anti–insulin receptor and anti–IRS-2 were from Cell Signaling Technology (Beverly, MA). Anti-insulin serum was made in the laboratory.

Islets and primary β-cell purification. Islets of Langerhans were isolated by collagenase digestion of pancreas from adult male C57BL/6 mice followed by Ficoll purification based on a method adapted from Rouiller et al. (16). Pancreatic islets were digested with trypsin, and total cells were used for the silencing experiments on primary cells. β-Cells were separated from non–β-cells by autofluorescence-activated cell sorting (FACS) using FACStar-Plus (BD Biosciences, Franklin Lakes, NJ) and a protocol that was adapted from an established method for sorting rat β-cells (17). The sorted cells were used for Western blot analysis of Akt and AS160 expression and phosphorylation after glucose stimulation. Human islets were provided by the Islet Cell Resource Centers of Milan (Italy) and Geneva (Switzerland) with support from the Juvenile Diabetes Research Foundation (JDRF) European islet distribution program. Human β-cells were sorted by FACS after labeling with Newport Green and exclusion of ductal cells and dead cells according to a recently developed method that provides a population comprising >90% β-cells (18).

Detection of apoptosis and proliferation. For MIN6B1 cells, apoptosis was measured by transferase-mediated dUTP nick-end labeling (TUNEL) assay or caspase-3 activation, and proliferation was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation as previously described (19). For primary mouse islets cells, the cells were established in monolayer on dishes coated with 804G-ECM according to established procedures (18). Apoptosis was measured by TUNEL, and proliferation was measured by incorporation of BrdU, in both cases with identification of β-cells by insulin immunofluorescence.

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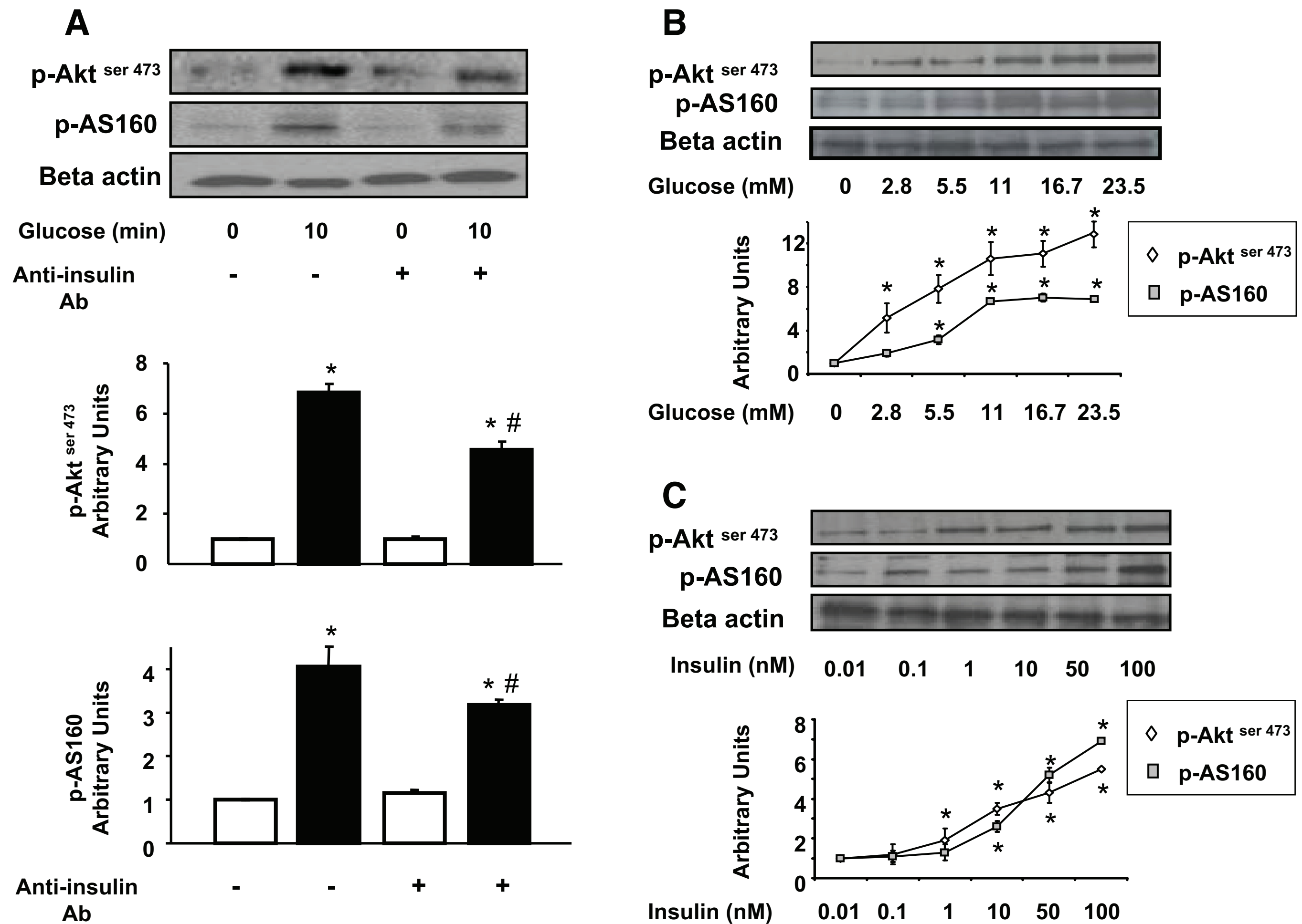

FIG. 2. Glucose and insulin induce Akt and AS160 phosphorylation in MIN6B1 cells. *A*: Western blot (7.5% acrylamide gel) showing that glucose action was only partially insulin dependent. Cells were pretreated for 2 h with anti-insulin antibody and phosphorylation evaluated under basal condition (0 min; □) or stimulated condition for 10 min with 16.7 mmol/l glucose (■) in the continued presence of the antibody. $n = 4$ independent experiments; *$P < 0.05$ vs. basal control; #$P < 0.05$ vs. stimulated control. *B*: Western blot (7.5% acrylamide gel) showing that glucose-induced Akt and AS160 phosphorylation is dose dependent. $n = 4$ independent experiments; *$P < 0.05$. *C*: Western blot (7.5% acrylamide gel) showing that insulin can also stimulate Akt and AS160 phosphorylation in MIN6B1 cells in a concentration-dependent manner. $n = 4$ independent experiments; *$P < 0.05$.

Cell lines and culture conditions. MIN6B1 cells were cultured in complete medium as described previously (20). In general, cells were subcultured to 80% confluency in 10-cm^2 wells and were used up to passage 25.

RNA interference mediated knockdown of endogenous IRS-2 and AS160 by transient transfection. Two 64-bp sequences encoding two different 21-bp-long small hairpin RNAs (shRNAs) specific for either IRS-2 or AS160 were cloned into both pSUPER and pSUPER-GFP plasmids (OligoEngine, Seattle, WA). A similar sequence encoding a nonspecific shRNA without mammalian homology was also cloned and used as a negative control. All different shRNAs were tested for their capacity to knockdown IRS-2 and AS160 protein expression in MIN6B1 cells as follows. Cells were transiently transfected using LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA) with the pSUPER-GFP plasmid containing either one of the shRNAs against IRS-2, AS160, or the negative control shRNA. Cells were cultured for 72 h for RNA interference (RNAi) expression before selection of green fluorescent protein (GFP)-positive cells by FACS. Selected cells were lysed for Western blot analysis of IRS-2 or AS160 levels compared with those of negative control cells, and loading was normalized using β-actin. The shRNA sequence exhibiting the greatest silencing capacity for each protein was used in all subsequent experiments.

Knockdown of AS160 in mouse islet cells was achieved by transfection with small interfering RNA (siRNA) using a method described previously (21). In brief, liposome-siRNA (Lipofectamine 2000 and 100 nmol/l siRNA) was prepared in 200 μl opti–Modified Eagle's Medium as described by the suppliers. The siRNA oligonucleotide was labeled with fluorescein isothiocyanate (FITC). All cells appeared to have been transfected based on their FITC fluorescence.

Growth hormone secretion assays. To study secretion specifically from transfected cells, cotransfection was performed with a human growth hormone (hGH)-expressing vector and the vector of interest (1:3 DNA ratio) and incubated for 72 h before analysis of hGH secretion (used as a surrogate marker for insulin secretion from transfected cells) as previously described (22).

Gene expression. Gene expression in human islets versus sorted human β-cells was detected with HGU133 plus 2.0 Affymetrix microarrays (Affymetrix, Santa Clara, CA). Expression of AS160 mRNA was measured using quantitative real-time PCR analysis as previously described (23), and results were normalized using cyclophilin A.

SDS-PAGE and Western blotting. Protein samples were prepared and immunoblots were analyzed as previously described (8).

mRNA from human islets. mRNA from islets obtained from cadaveric donors without (control subjects) or with previously documented type 2 diabetes was provided by Dr. Piero Marchetti (University of Pisa, Pisa, Italy).

Presentation of data and statistics. Data are presented as means ± SE for three to five independent experiments. Statistical significance for differences was evaluated using ANOVA with post hoc adjustment with $P < 0.05$ considered significant.

RESULTS

AS160 expression in pancreatic islets and primary β-cells. The level of AS160 was twofold higher in sorted human β-cells compared with human islets based on

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cDNA microarray analysis (Fig. 1*A*). Furthermore, AS160 protein was expressed in human islet cells, and stimulation by 16.7 mmol/l glucose for 10 min induced AS160 phosphorylation (Fig. 1*B*). In human islets obtained at death from individuals with documented type 2 diabetes, AS160 mRNA expression was decreased by twofold when compared with control subjects without documented diabetes (Fig. 1*C*).

Glucose-induced Akt and AS160 phosphorylation in MIN6B1 cells. We next analyzed AS160 expression and phosphorylation in a highly enriched population of primary mouse β-cells under basal conditions and after 10-min incubation with 16.7 mmol/l glucose (Fig. 1*D*). Robust and rapid phosphorylation of AS160 in response to glucose led us to investigate the molecular mechanism involved. Because it was not possible to use sorted primary β-cells for this purpose (because of the severe limitations in the amount of material available), we used as a model system the highly differentiated mouse β-cell MIN6 subclone B1 (MIN6B1 cells) that respond to glucose with a robust stimulation of insulin secretion (20). Using MIN6B1 cells, once again 10-min stimulation with glucose led to phosphorylation of AS160, and activation was seen as early as 5 min and was sustained throughout the 30-min incubation with high glucose (Fig. 1*E*). Phosphorylation of Akt followed a similar time course, whereas ERK1/2 phosphorylation was equally rapid in onset, but this was not sustained with a sharp decline between 10 and 30 min of continuous exposure to high glucose (Fig. 1*E*).

It was possible that the glucose-induced increased phosphorylation of Akt and AS160 was mediated by positive feedback due to increased insulin secretion at high glucose (24). To investigate this, and thus to determine whether Akt and AS160 phosphorylation was directly dependent on glucose, MIN6B1 cells were pretreated with anti-insulin antibody and stimulated with glucose for 10 min. Even in the presence of anti-insulin antibody at a titer shown experimentally to adsorb and neutralize >85% of insulin secreted during this period (data not shown), Akt and AS160 phosphorylation was still increased by 10-min exposure to high glucose, after 2 h without glucose, but there was a significant decrease when compared with controls (Fig. 2*A*). Moreover, we measured Akt and AS160 phosphorylation after 10-min incubation with different glucose concentrations (0–23.5 mmol/l) (Fig. 2*B*) or insulin concentrations (0.01–100 nmol/l) without glucose (Fig. 2*C*). We observed that phosphorylation of these two proteins was increased after 10 min with 5 mmol/l glucose and reached a plateau at 11 mmol/l (Fig. 2*B*). Insulin secretion was measured at the end of the 10 min static incubation, with no impact of glucose on secretion observed (7.65 and 7.55 nmol/l insulin measured in the incubation buffer after 10 min at 0 and 23.5 mmol/l glucose, respectively). We cannot exclude, however, that there was a localized increase in the ambient insulin concentration in close proximity to the cells. There was also a direct impact of exogenous insulin, with increased phosphorylation observed at 1 nmol/l and a further concentration-dependent increase (Fig. 2*C*).

Taken together, these results show that Akt and AS160 phosphorylation in our protocol (2-h preincubation of MIN6B1 cells at 0 mmol/l glucose followed by 10-min static incubation with glucose) was in major part due to a direct effect of glucose but that positive feedback from insulin secreted in the incubation buffer in response to the sugar may contribute somewhat.

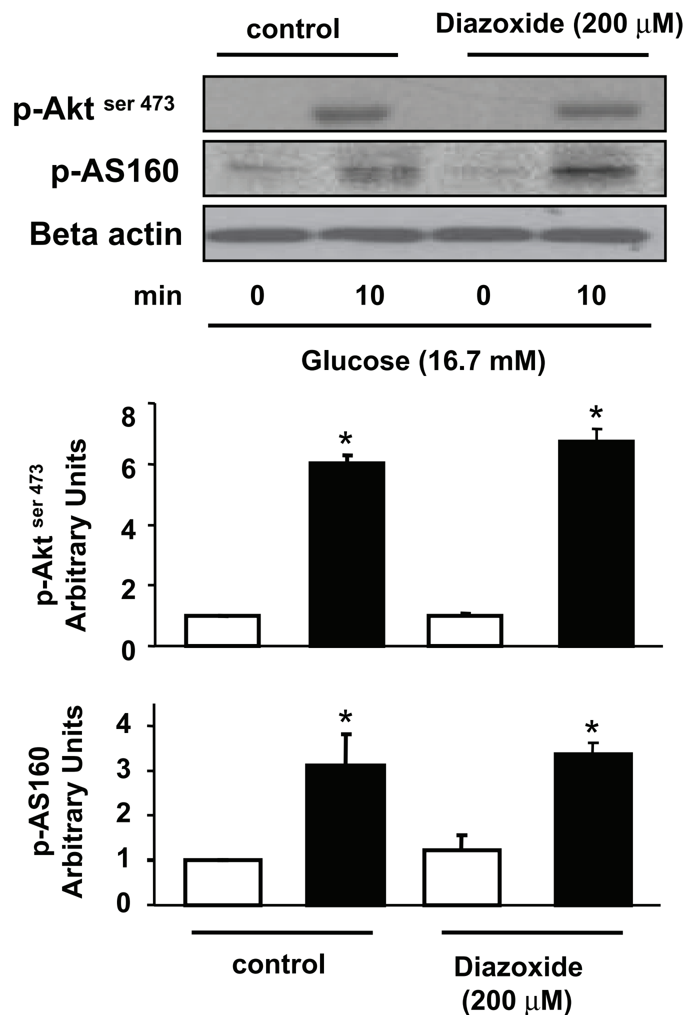

FIG. 3. AS160 phosphorylation is not prevented by diazoxide treatment. Akt Ser473 and AS160 phosphorylation in MIN6B1 cells under basal condition (0 min, □) or after stimulation for 10 min with 16.7 mmol/l glucose (■) in the presence or absence of 200 μmol/l diazoxide. *n* = 4 independent experiments. *P < 0.05 vs. basal control.

Glucose-induced Akt and AS160 phosphorylation in MIN6B1 is Ca^{2+} independent. Some effects of glucose on islet β-cells are Ca^{2+} dependent. The major glucose stimulus-secretion pathway involves its metabolism, increased ATP/ADP, closure of K_{ATP} channels, depolarization, opening of voltage-gated calcium channels, and thus an increase in cytosolic Ca^{2+}. To explore the possible role of increased $[Ca^{2+}]_i$, we examined the effect of an inhibitor of glucose-stimulated Ca^{2+} influx. Diazoxide, a pharmacological agent that opens K_{ATP} channels, thereby abrogating glucose-stimulated Ca^{2+} influx, was without action on glucose-mediated Akt and AS160 phosphorylation (Fig. 3), and this same result was obtained using the calcium-channel blocker SR-7037 (data not shown).

Glucose signal transduction. To explore the role of IRS-2 in glucose action on β-cells, MIN6B1 cells were transiently transfected with a plasmid allowing for expression of GFP and a sequence coding for RNAi against IRS-2 or a scrambled RNAi sequence. Two independent sequences were validated for their ability to silence IRS-2 gene expression. Both RNAi constructs decreased protein expression of IRS-2 by 90% in sorted GFP-positive (transfected) cells (see representative results in Fig. 4*A*). Cells lacking IRS-2 were then treated for 10 min with high

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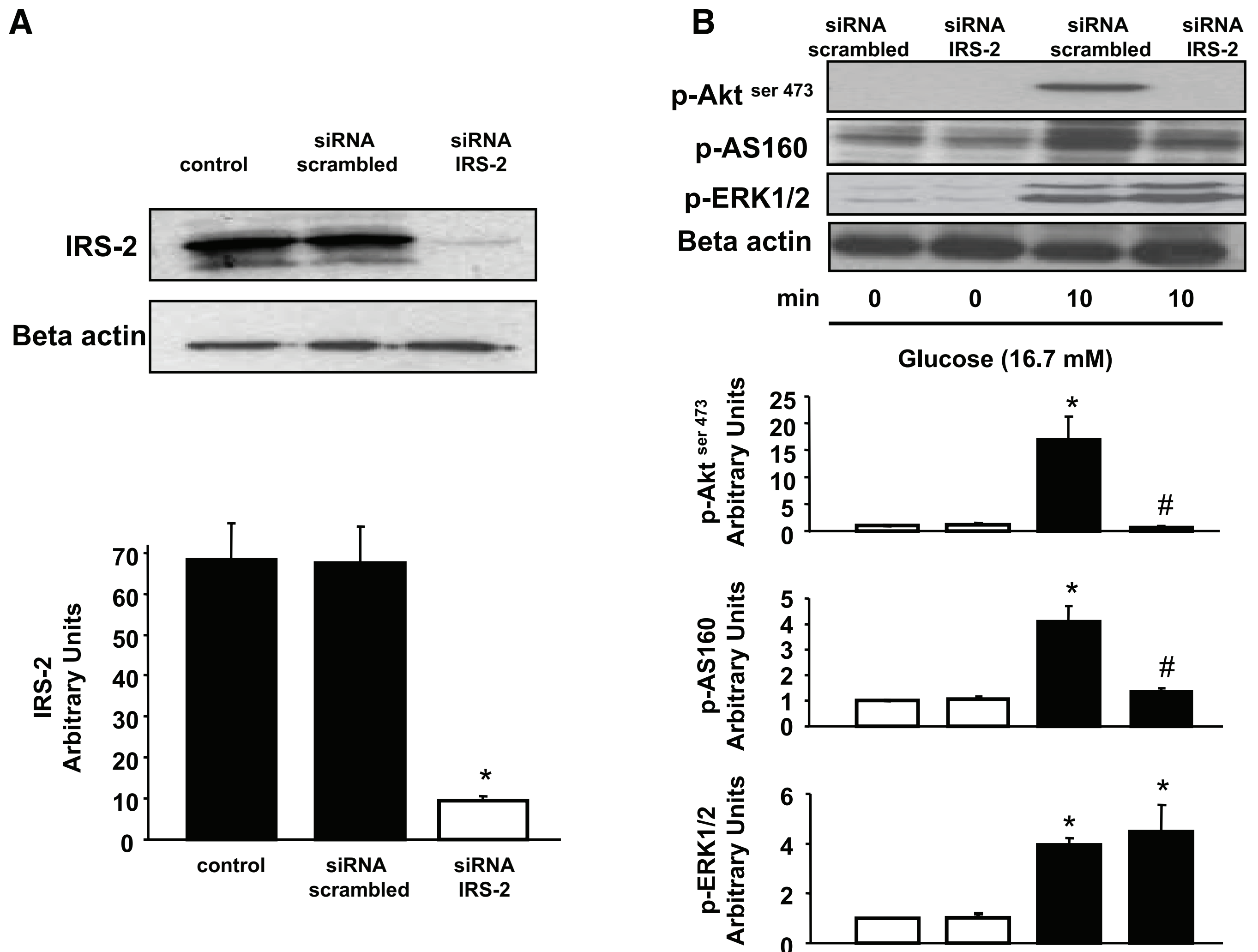

FIG. 4. Glucose action on Akt and AS160 is IRS-2 dependent. *A*: Representative Western blot showing efficiency of IRS-2 knockdown in sorted GFP-positive MIN6B1 cells. Western blots were scanned and quantified and data presented for $n = 4$ independent experiments. *$P < 0.05$ vs. control. *B*: Representative immunoblot (7.5% acrylamide gel) and densitometry highlighting the role of IRS-2 on phosphorylation of Akt Ser473, AS160, and ERK1/2 under basal conditions (□) or after 10-min stimulation with 16.7 mmol/l glucose (■). $n = 4$ independent experiments. *$P <$ 0.05 vs. 0-min control; #$P < 0.05$ vs. 10-min stimulation control.

glucose to study the role of IRS-2 in Akt, AS160, and ERK1/2 phosphorylation (Fig. 4*B*). Depletion of IRS-2 protein in MIN6B1 cells prevented glucose action on Akt and AS160 phosphorylation both acutely (10 min) and after 30 min (not shown), whereas glucose stimulation of ERK1/2 phosphorylation was maintained.

Because IRS-2 is known to interact with PI 3-kinase, MIN6B1 cells were pretreated with LY-294002, a specific inhibitor of PI 3-kinase, to explore the role of that enzyme on glucose action. Inhibition of PI 3-kinase totally prevented glucose action on Akt and AS160 phosphorylation, whereas, as expected, stimulation of ERK1/2 was again not affected (Fig. 5).

Glucose-induced insulin receptor phosphorylation in MIN6B1. It was possible that the glucose-induced increased phosphorylation of Akt and AS160 was mediated by insulin receptor autophosphorylation because we previously demonstrated that it was IRS-2/PI 3-kinase dependent (Figs. 4 and 5). To investigate this and also to determine whether insulin receptor phosphorylation was mainly dependent on glucose, MIN6B1 cells were again pretreated or not with anti-insulin antibody and stimulated with glucose for 10 min. Insulin receptor phosphorylation was remarkably increased by 10-min exposure to high glucose, and similar to the data for Akt and AS160 phosphorylation, addition of anti-insulin serum decreased this signal only modestly (Fig. 6). These results show that glucose can induce insulin receptor phosphorylation independent of insulin while confirming a modest parallel involvement of positive feedback by secreted insulin.

Role of AS160 in glucose action in β-cells. In β-cells, glucose induces insulin secretion, increases cell proliferation, and inhibits apoptosis (25–27). Therefore, we next explored the role of AS160 in these events. MIN6B1 cells were transfected with two different constructs to silence AS160 protein expression as previously shown in 3T3-L1 cells (12). In our model, we obtained a greater knockdown with the construct AS160 no. 2 compared with AS160 no. 1 (80 vs. 30%) (Fig. 7*A*), and it was therefore used for the following experiments. To examine secretion specifically from transfected cells, cotransfected hGH was used as a surrogate marker for insulin secretion (22). AS160-depleted MIN6B1 cells had a fourfold increase in basal secretion (2.8 mmol/l glucose), whereas glucose stimulated secretion was impaired (Fig. 7*A*). These data were confirmed in primary mouse islet cells (Fig. 7*B*) in which

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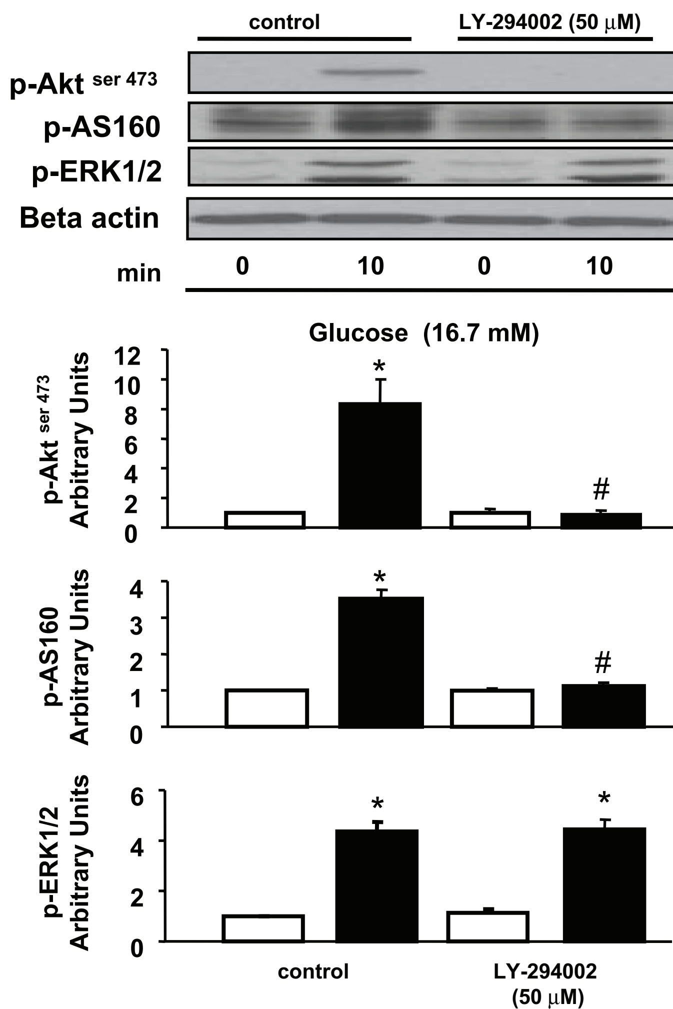

FIG. 5. Glucose action on Akt and AS160 is PI 3-kinase dependent. Representative immunoblot (7.5% acrylamide gel) and densitometry highlighting the role of PI 3-kinase on phosphorylation of Akt Ser473, AS160, and ERK1/2 under basal conditions (□) or after 10-min stimulation with 16.7 mmol/l glucose (■). MIN6B1 cells were pretreated with 50 μmol/l LY-294002 to inhibit PI 3-kinase activation. $n = 4$ independent experiments; *$P < 0.05$ vs. 0-min control; #$P < 0.05$ vs. 10-min stimulation control.

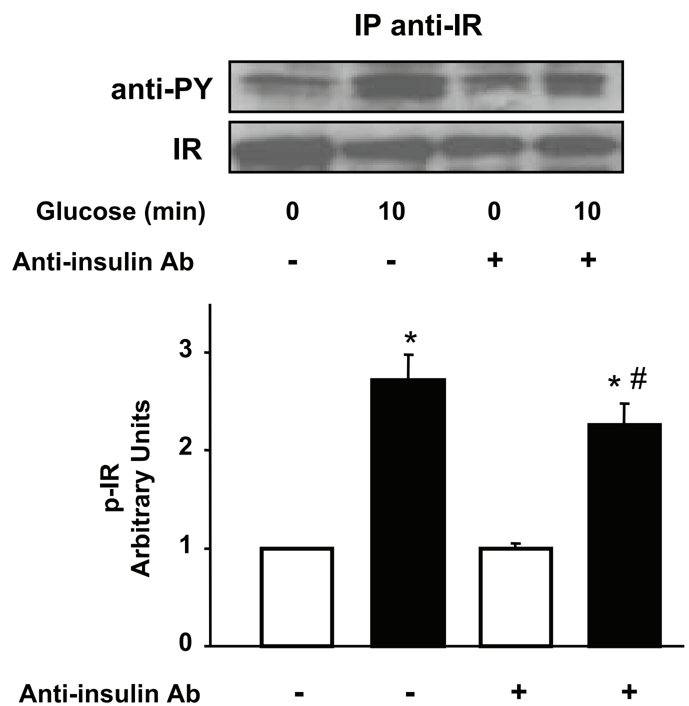

FIG. 6. Glucose induces insulin receptor phosphorylation, and insulin adsorption partially decreases it. Cells were pretreated for 2 h with anti-insulin antibody, and insulin receptor phosphorylation in MIN6B1 cells was evaluated under basal conditions (0 min; □) or after 10-min stimulation with 16.7 mmol/l glucose (■) in the continued presence of the antibody. $n = 4$ independent experiments; *$P < 0.05$ vs. basal control; #$P < 0.05$ vs. stimulated control.

AS160 protein expression was decreased by 40% using siRNA (Fig. 7*B*). Similar results were obtained in MIN6B1 cells lacking both IRS-2 and AS160 protein, whereas cells lacking only IRS-2 had unchanged basal but blunted stimulated secretion (Fig. 7*A*).

We investigated the role of these proteins in β-cell replication. Cell proliferation was quantified by BrdU incorporation in MIN6B1 cells cultured in glucose-free medium versus 16.7 mmol/l glucose (Fig. 7*C*). Proliferation was induced by glucose in cells transfected with scrambled siRNA but not in cells lacking IRS-2 and/or AS160, indicating that both proteins are also required for glucose-stimulated proliferation. We confirmed these results in primary mouse β-cells in which AS160 protein was reduced by 40% using siRNA (Fig. 7*D*).

Finally, to assess cell death, MIN6B1 cells lacking IRS-2 and/or AS160 were cultured for 72 h in either standard (25 mmol/l glucose and 15% FCS) or deprived (5 mmol/l glucose and 1% FCS) condition, the latter having been shown previously to increase apoptosis in these cells (19). Cell death was quantified using either TUNEL assay (28), which detects DNA strand breaks without distinguishing between apoptosis and necrosis, or active caspase-3, a main mediator of apoptosis. In cells transfected with scrambled siRNA, deprived conditions induced a threefold increase in the number of TUNEL or active caspase-3–positive cells when compared with standard conditions (Fig. 8*A* and *B*). Knockdown of IRS-2 and/or AS160 induced apoptosis and activation of caspase-3 (Fig. 8*A* and *B*) after incubation in either standard or deprived conditions, indicating the prosurvival role of both these proteins in β-cells. Similar results were obtained using primary islet cells, with an increase in mouse β-cell death after transfection with AS160 siRNA (Fig. 8*C*).

DISCUSSION

In the present paper, we establish for the first time that AS160 RabGAP is expressed in β-cells and that phosphorylation of this protein is stimulated after just 5 min of glucose stimulation. We further observed that mRNA expression of AS160 was decreased in pancreatic islets from individuals with type 2 diabetes. In MIN6B1 cells, we show that glucose action on Akt and AS160 phosphorylation is insulin receptor/IRS-2/PI 3-kinase dependent. However, increased cytosolic Ca^{2+} was not required for this. In MIN6B1 cells, partial knockdown of AS160 resulted in an increase of basal and a decrease of glucose-stimulated insulin secretion, directly demonstrating a role for AS160 as a key regulator of secretion. Furthermore, β-cells with decreased expression of AS160 and/or IRS-2 display increased apoptosis and are no longer able to proliferate in response to glucose. These findings confirm that Akt phosphorylation after glucose stimulation plays an important role in β-cell function, replication, and survival and

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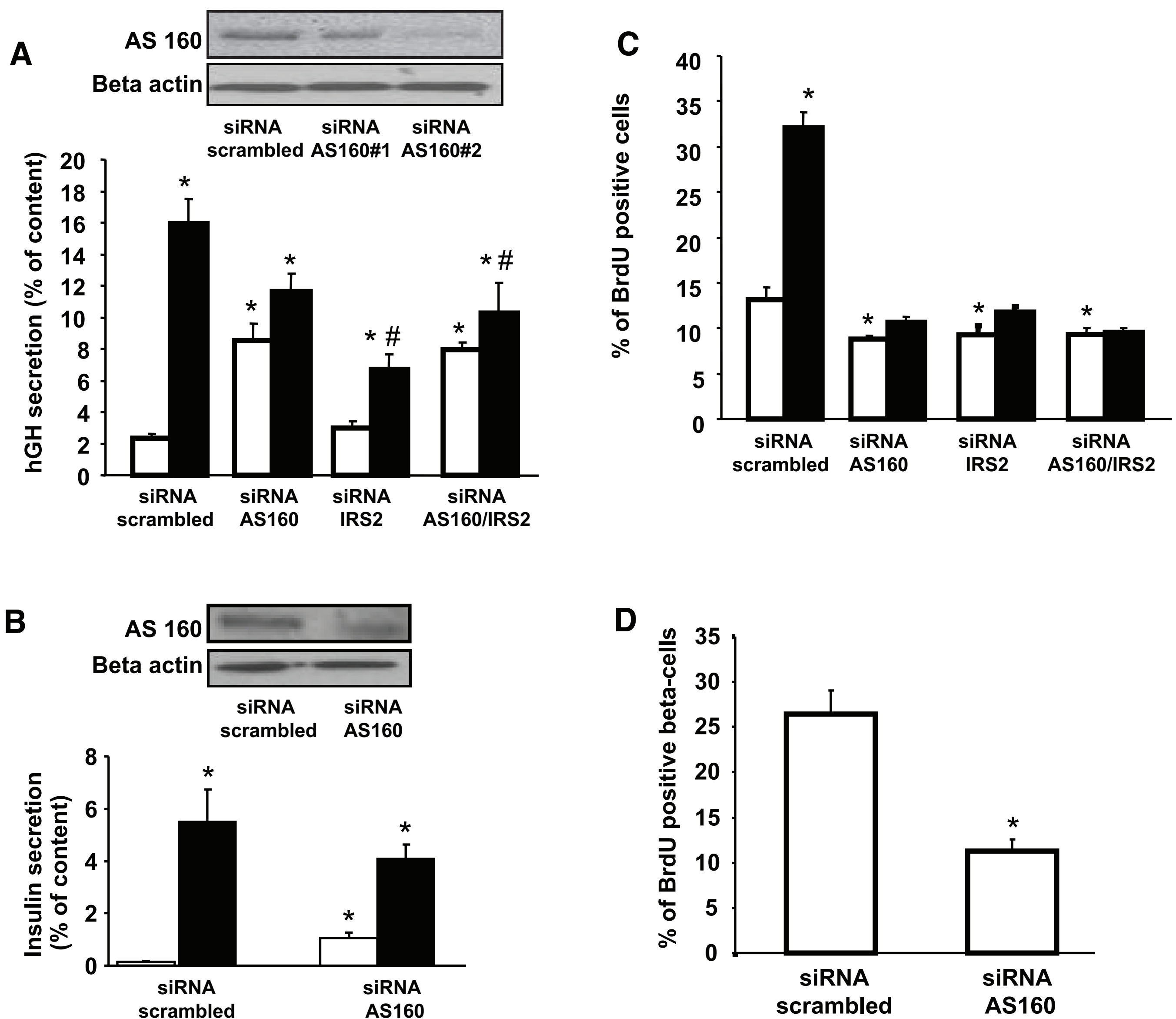

FIG. 7. Role of AS160 in insulin secretion and β-cell proliferation. *A*: Effect of AS160 knockdown on insulin secretion from MIN6B1 cells. hGH secretion was used as a surrogate for insulin secretion from MIN6B1 cells cotransfected with hGH and AS160 siRNA. hGH release was measured from cells incubated for 60 min at 2.8 mmol/l glucose (□, basal secretion) followed by 60 min at 16.7 mmol/l glucose (■, stimulated secretion). *P < 0.05 vs. basal secretion in cells transfected with scrambled siRNA; #P < 0.05 vs. stimulated secretion in cells transfected with scrambled siRNA. *Top panel*: Representative Western blot showing efficiency of AS160 silencing in MIN6B1 cells. *B*: Effect of AS160 knockdown on insulin secretion from primary mouse β-cells. Insulin secretion was measured using mouse primary islet cells transfected or not with AS160 siRNA and incubated for 60 min at 2.8 mmol/l glucose (□, basal secretion) followed by 60 min at 16.7 mmol/l glucose (■, stimulated secretion). *P < 0.05 vs. basal secretion in cells transfected with scrambled siRNA. *Top panel*: Representative Western blot showing efficiency of AS160 silencing in mouse primary islet cells. *C*: Effect of knockdown of AS160 and IRS-2 on MIN6B1 cell proliferation. Proliferation was measured by BrdU incorporation for 2 h under basal conditions (2.8 mmol/l glucose; □) or during 2 h stimulation with 16.7 mmol/l glucose (■). *P < 0.05 over basal from cells transfected with scrambled siRNA. *D*: Effect of AS160 knockdown on proliferation of mouse primary β-cells. Proliferation was measured by BrdU incorporation over 24 h under standard culture conditions (20% FCS, 11.2 mmol/l glucose) with prior transfection with AS160 siRNA or scrambled siRNA. β-Cells were identified by insulin immunofluorescence, and the data are presented as percentage of β-cells that were BrdU positive. *P < 0.05 vs. scrambled siRNA.

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show an important role for AS160 as a downstream effector in these actions.

A recent report has shown that glucose inhibition of apoptosis in pancreatic β-cells is in fact insulin dependent (2). Like our own studies on apoptosis, this earlier study was performed over an extended period of time, and we do not exclude that insulin signaling also plays a major anti-apoptotic role in our situation. The conditions used to induce apoptosis (low glucose/low serum) will also decrease insulin secretion during the experimental period. Similar reasoning leads one to suggest that the effect of glucose on proliferation (measured here in MIN6B1 cells over 2 h) may also be mediated largely by insulin. However, in our short-term studies, the various control experiments indicate that the phosphorylation of Akt and AS160 in response to just 10 min at high glucose was mainly insulin independent. Although understanding the relative contribution of glucose and insulin toward the phosphorylation of protein kinase B/Akt and thus AS160 was not the main purpose of this study, our results are supported by other studies showing that glucose can activate IRS-mediated signal transduction pathways in β-cells indepen-

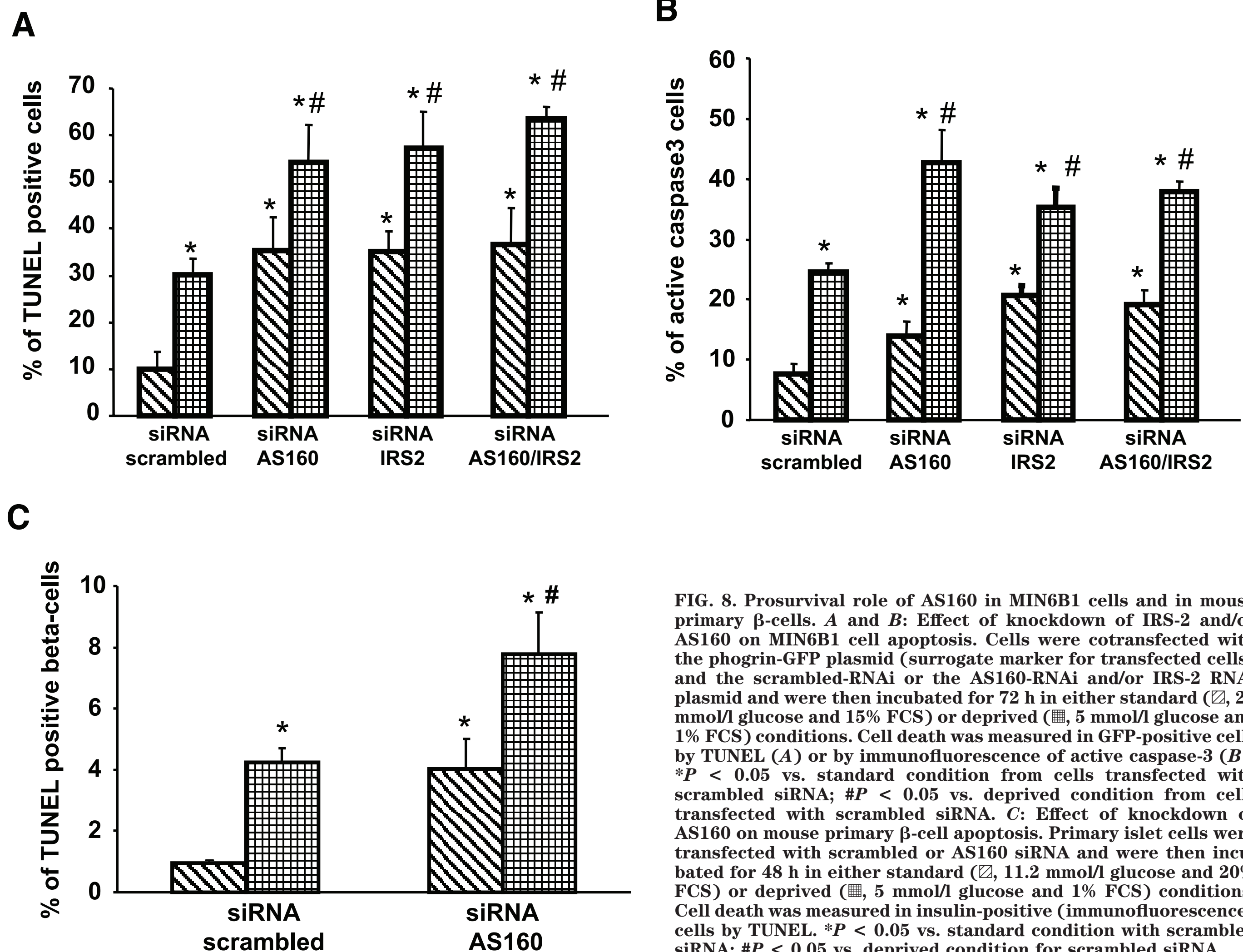

FIG. 8. Prosurvival role of AS160 in MIN6B1 cells and in mouse primary β-cells. *A* and *B*: Effect of knockdown of IRS-2 and/or AS160 on MIN6B1 cell apoptosis. Cells were cotransfected with the phogrin-GFP plasmid (surrogate marker for transfected cells) and the scrambled-RNAi or the AS160-RNAi and/or IRS-2 RNAi plasmid and were then incubated for 72 h in either standard (▨, 25 mmol/l glucose and 15% FCS) or deprived (▦, 5 mmol/l glucose and 1% FCS) conditions. Cell death was measured in GFP-positive cells by TUNEL (*A*) or by immunofluorescence of active caspase-3 (*B*). *P < 0.05 vs. standard condition from cells transfected with scrambled siRNA; #P < 0.05 vs. deprived condition from cells transfected with scrambled siRNA. *C*: Effect of knockdown of AS160 on mouse primary β-cell apoptosis. Primary islet cells were transfected with scrambled or AS160 siRNA and were then incubated for 48 h in either standard (▨, 11.2 mmol/l glucose and 20% FCS) or deprived (▦, 5 mmol/l glucose and 1% FCS) conditions. Cell death was measured in insulin-positive (immunofluorescence) cells by TUNEL. *P < 0.05 vs. standard condition with scrambled siRNA; #P < 0.05 vs. deprived condition for scrambled siRNA.

dent of growth factors (25,29) and that glucose is an important activator of PI 3-kinase (30). Acute glucose action on Akt and AS160 could, however, be due to a transient *trans*-activation of either IGF-R or insulin receptor tyrosine kinase activity as previously proposed in muscle (13). To support this hypothesis, we demonstrated in our study that glucose induces insulin receptor phosphorylation, mainly in an insulin-independent manner. Similar results were obtained in muscle cells, suggesting that glucose can induce protein regulation such as PKC and diacylglycerol kinase, which can in turn activate transient insulin receptor activation (31). Even if the mechanism by which glucose can induce phosphorylation of insulin receptor requires direct investigation in β-cells, the main purpose of our study was to investigate whether glucose (or/and insulin) can induce Akt and AS160 phosphorylation in β-cells and what role, if any, AS160 might have in these cells.

In rodent β-cell lines, IRS-1, -2, -3, and -4 are all present, but IRS-2 appears to be the dominant family member (32–34). Intriguingly, and despite the fact that glucose appears to be able to act directly on Akt and AS160 phosphorylation, this action was totally prevented in MIN6B1 cells lacking IRS-2. In these cells, the mechanism involved in Akt and AS160 phosphorylation differs from skeletal muscle in which AS160 phosphorylation was IRS-1 dependent (8). This could be due to the fact that IRS-1 and -2 are equally expressed in skeletal muscle, whereas IRS-2 is more highly expressed in β-cells, and as just mentioned, the stimulus in our system is predominantly glucose and not insulin. Downstream of IRS-2, induction of PI 3-kinase activity is required for glucose action in β-cells (25,30), and glucose-induced Akt and AS160 phosphorylation was prevented by inhibition of PI 3-kinase. Taken together, these results show that the signaling pathway IRS-2/PI 3-kinase is indispensable for glucose action on Akt and AS160 phosphorylation.

In β-cells, glucose induces insulin secretion and cell proliferation and inhibits apoptosis (25–27). Decreasing expression of AS160 induced an increase of basal insulin secretion, indicating that it is involved in the negative regulation of this particular part of secretion. Our data are supported indirectly by other studies performed in 3T3-L1 adipocytes in which AS160 silencing was followed by an increased translocation of GLUT4 vesicles to the plasma membrane (12). It is currently hypothesized that in the basal state, the GAP activity of AS160 maintains its target Rab in an inactive, GDP-bound form, consequently retaining GLUT4 in intracellular compartment(s) (12,35). On insulin stimulation, Akt phosphorylation of AS160 deactivates its GAP activity, shifting the equilibrium of the target Rab to an active GTP-bound form, thereby relieving an inhibitory effect on GLUT4 trafficking (12). This model proposed for AS160 in adipose tissue and skeletal muscle

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could apply equally to insulin granules in β-cells under basal conditions, as suggested by our results. We are conscious of cell death as a possible confounding factor making interpretation of these functional data less straightforward. Thus, in cells with decreased AS160, apoptosis was dramatically increased even under normal culture conditions. We therefore cannot exclude that the observed increase in basal insulin secretion may be due, in whole or in part, to leakage of insulin from dying/dead cells.

Aside from increasing apoptosis, knocking-down expression of AS160 also impaired glucose stimulation of β-cell proliferation. Similar results were obtained in cells lacking IRS-2. In these cells, activation of Akt after glucose stimulation was impaired. Because Akt is known to play a prosurvival role in β-cells (36,37), we suggest that the increase in apoptosis that we observed in cells lacking IRS-2 was due to an impairment in the Akt signaling pathway. Moreover, in cells lacking AS160, increased apoptosis indicates that Akt/AS160 is the preferred anti-apoptotic pathway.

These documented effects of AS160 in β-cells taken together with decreased AS160 expression in islets from individuals with type 2 diabetes lead to speculation that decreased expression of AS160 may underlie, at least in part, impaired insulin secretion and decreased β-cell mass in type 2 diabetes. However, for these purposes, mRNA was extracted from islets without regard to the cellular makeup of the islets or the cell type(s) expressing AS160. Given that AS160 expression was higher in sorted β-cells than intact human islets, the observed decrease in AS160 expression in islets from cadaveric donors with a clinical history of type 2 diabetes may simply reflect a corresponding decrease in the number of β-cells per islet rather than decreased AS160 expression in each β-cell.

In summary, we show here that Rab-GAP AS160 is expressed in human and mouse pancreatic β-cells, down-regulated in islets from individuals with type 2 diabetes, and phosphorylated after glucose stimulation. We also demonstrate that this protein is involved in β-cell survival, glucose-mediated cell proliferation, and normal regulation of insulin secretion. Studying the pathways regulating AS160 activation may facilitate the discovery of novel targets for the treatment of diabetes and related metabolic disorders.

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