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### Rapid Publication

Insulin receptor substrate–1 (IRS-1) is pivotal in mediating the actions of insulin and growth factors in most tissues of the body, but its role in insulin-producing  $\beta$  islet cells is unclear. Freshly isolated islets from *IRS-1* knockout mice and SV40-transformed IRS-1–deficient  $\beta$ -cell lines exhibit marked insulin secretory defects in response to glucose and arginine. Furthermore, insulin expression is reduced by about 2-fold in the IRS-1–null islets and  $\beta$ -cell lines, and this defect can be partially restored by transfecting the cells with IRS-1. These data provide evidence for an important role of IRS-1 in islet function and provide a novel functional link between the insulin signaling and insulin secretion pathways.

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# Altered function of insulin receptor substrate-1-deficient mouse islets and cultured $\beta$ -cell lines

Rapid  
PUBLICATION

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Insulin receptor substrate-1 (IRS-1) is pivotal in mediating the actions of insulin and growth factors in most tissues of the body, but its role in insulin-producing  $\beta$  islet cells is unclear. Freshly isolated islets from *IRS-1* knockout mice and SV40-transformed IRS-1-deficient  $\beta$ -cell lines exhibit marked insulin secretory defects in response to glucose and arginine. Furthermore, insulin expression is reduced by about 2-fold in the IRS-1-null islets and  $\beta$ -cell lines, and this defect can be partially restored by transfecting the cells with IRS-1. These data provide evidence for an important role of IRS-1 in islet function and provide a novel functional link between the insulin signaling and insulin secretion pathways.

vivo experiments of islet function in mice with homozygous and heterozygous mutations in the IRS-1-null allele, as well as in vitro studies on isolated islets and SV40-transformed  $\beta$ -cell lines derived from IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> mice. These studies indicate that IRS-1 deficiency leads to a decrease in insulin content of  $\beta$  cells and reduced response to glucose and arginine without a reduction in  $\beta$ -cell mass. Thus, the IRS proteins and insulin receptor play unique roles in  $\beta$ -cell development or function.

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## Introduction

The insulin receptor and its principal substrates, insulin receptor substrate-1 (IRS-1) and IRS-2, are distributed in most mammalian cells (1, 2). The presence and significance of these molecules in insulin sensitive tissues have been extensively studied (3–5), but their roles in other tissues, such as brain, endothelial cells, and pancreatic islet cells, are less clear. Mice made deficient for IRS-1 show marked growth retardation, mild insulin resistance, hyperinsulinemia, and an increase in  $\beta$ -cell mass (6–9). Combined defects in the insulin receptor and IRS-1 in double heterozygous knockout mice result in even more severe insulin resistance and massive  $\beta$ -cell hyperplasia (10). In spite of this, by 6 months of age, 50% of these mice develop diabetes. By contrast, IRS-2 knockout mice develop hyperinsulinemia and diabetes early and display  $\beta$ -cell failure secondary to islet hypopla-

sia. Recently, we have shown that the insulin receptor also has an important functional role in the pancreatic  $\beta$ -cell in the maintenance of glucose homeostasis in vivo (11). Indeed, mice with a tissue-specific knockout of insulin receptors in  $\beta$ -cells ( $\beta$ IRKO) lose their ability to respond adequately to a glucose stimulus and manifest progressive glucose intolerance. In addition, other recent studies indicate that insulin positively regulates transcription of its own gene (12, 13) and stimulates vesicle exocytosis from isolated  $\beta$  cells (14). Collectively, these studies indicate a role for the insulin-signaling pathway in the regulation of  $\beta$ -cell function and suggest complex interactions between the insulin receptor substrates IRS-1 and IRS-2 and the insulin receptor in influencing islet growth and function.

To study the specific role of IRS-1 in endocrine pancreatic function, in the present study we have performed in

## Methods

**Animals.** IRS-1 knockout mice (6) were maintained on the original 129Sv/C57Bl hybrid background at Taconic Farms (Germantown, New York, USA) and were shipped to the Joslin Animal Facility before experiments. All mice were kept on a 12/12-hour light/dark cycle with ad libitum access to water and chow. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center.

**Glucose tolerance tests and acute insulin release experiments.** In vivo experiments were performed as described previously (11). Briefly, overnight fasted mice were injected intraperitoneally with glucose (2 g/kg body weight) for glucose tolerance testing (GTT); glucose (3 g/kg body weight) for acute insulin release or L-arginine (0.3 g/kg body weight in 0.2 M PBS) for acute insulin release. Whole blood was collected from the tail vein at 0, 15, 30, 60, and

120 minutes (for GTT studies) or at 0, 2, and 5 minutes (for acute insulin release studies), and glucose was measured using an automatic glucometer (One Touch Lifescan, Johnson and Johnson, Milpitas, California, USA). For acute insulin release experiments, whole blood was collected in chilled heparinized tubes and centrifuged and the plasma was stored at  $-20^{\circ}\text{C}$  for subsequent insulin RIA.

**Islet isolation and insulin secretion.** Islets were isolated essentially as described previously (15) using the intraductal collagenase technique. Islets were hand picked under a stereomicroscope (Stereozoom GZ7, Leica, Buffalo, New York, USA) and used for RNA extraction. For secretion experiments, islets of similar sizes (100–150  $\mu\text{m}$ ) were hand picked from wild-type (WT), IRS-1<sup>+/-</sup>, and IRS-1<sup>-/-</sup> mice, and large islets (> 200  $\mu\text{m}$ ) from IRS-1<sup>-/-</sup> mice were grouped separately. All experiments were carried out with islets from a single harvest pool. Islets were washed in DMEM with FBS and cultured for 48 hours at  $37^{\circ}\text{C}$ . Secretion experiments were carried out in 12-well plates (15–25 islets per well) in DMEM. After incubation, the media was collected, centrifuged, and stored at  $-20^{\circ}\text{C}$  for RIA of insulin or glucagon. The islets were extracted in acid ethanol at  $4^{\circ}\text{C}$  (15) and stored at  $-20^{\circ}\text{C}$  for measurement of insulin content by RIA.

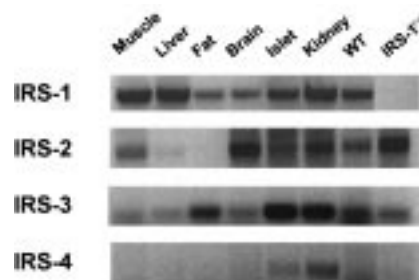
**Assessment of the presence of IRS proteins in mouse islets using RT-PCR.** RNA was prepared from several mouse tissues using the Ultraspec RNA isolation kit (Biotech, Houston, Texas, USA). The RT reaction was carried out using a RT-PCR kit (Pharmacia Biotech, Uppsala, Sweden) with 4  $\mu\text{g}$  of total RNA, first strand mix, and a random hexamer primer. The RNA was heated to  $65^{\circ}\text{C}$  for 5 minutes, quenched on ice and incubated at  $37^{\circ}\text{C}$  for 1 hour. Three microliters of the RT reaction was used in a total volume of 50  $\mu\text{l}$  in the PCR for IRS-1, IRS-2, IRS-3, and IRS-4. All PCR conditions included a final cycle of  $72^{\circ}\text{C}$  for 10 minutes (for IRS-1, -2, and -3) or 5 minutes (for IRS-4). Detail of the primers are available on request.

**Preparation of IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup>  $\beta$ -cell lines and insulin secretion.** Briefly, IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> mice were bred

with mice expressing the SV40 T antigen (RIP1-Tag2) via the  $\beta$ -cell specific insulin promoter (16). Tumors from 12- to 14-week-old RIP1-Tag2/IRS-1<sup>+/-</sup> and RIP1-Tag2/IRS-1<sup>-/-</sup> mice were manually dissected and placed in DMEM supplemented with FCS, penicillin, and streptomycin. The tumor capsule was disrupted, and the cells were gently dispersed. Tumor cells were purified by gravity sedimentation and seeded in a 48-well plate containing DMEM (16). IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup>  $\beta$  cells were grown to confluence and split 1:3 in DMEM supplemented with FBS. For secretion experiments, cells were plated at a density of  $1 \times 10^5$  to  $2 \times 10^5$  cells per well in 24-well plates containing 1 mL of DMEM medium supplemented with FBS. The remainder of the procedure for secretion experiments has been described elsewhere (15).

**Re-expression of IRS-1 in IRS-1<sup>-/-</sup> cells and Western blotting.** IRS-1 was re-expressed in IRS-1<sup>-/-</sup> cells using the Lipofectamine reagent (GIBCO BRL, Gaithersburg, Maryland, USA). Briefly, 4  $\mu\text{g}$  of the IRS-1 cDNA was mixed with Lipofectamine in Optimum I medium and incubated at room temperature for 30 minutes. IRS-1<sup>-/-</sup>  $\beta$  cells were washed and incubated at  $37^{\circ}\text{C}$  for 10 minutes. The DNA-Lipofectamine mix was added to the cells and incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Four hours later, the DNA-Lipofectamine mix was replaced with fresh DMEM medium supplemented with FBS. The medium was changed overnight, and confluent cells were split at 1:3. Cells were selected using puromycin (0.2 mg/mL). Experiments were carried out at passages 3–7 cells. Western blotting was carried out as described previously (10) using cells grown to confluence. After extraction, the protein content was measured by the Bradford method. The extract was resolved in SDS polyacrylamide gels and immunoprecipitation was carried out as described previously (10).

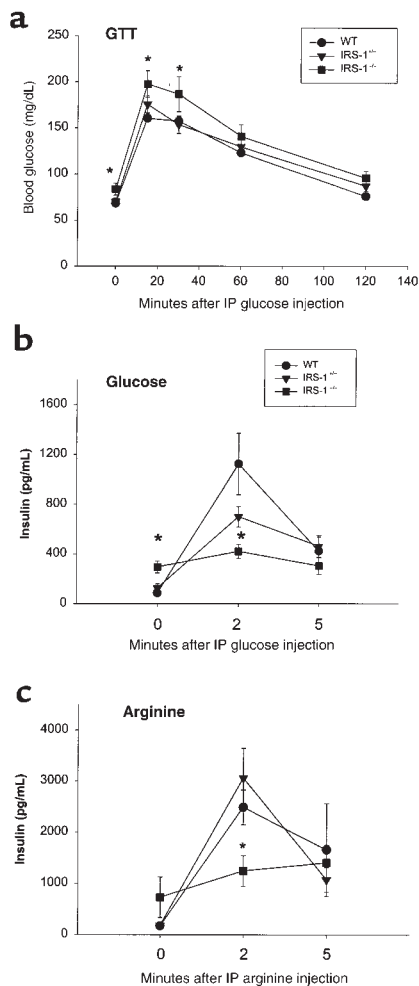
**Pancreatic, islet, and cellular insulin content.** To assess insulin content, the pancreas was removed rapidly, homogenized, and extracted in acid ethanol overnight at  $4^{\circ}\text{C}$ . A similar protocol was used for measurement of insulin content in isolated islets and cultured  $\beta$  cells. All extracts were stored at



**Figure 1** RT-PCR analysis of IRS protein expression in mouse tissues. PCR for IRS-1, -2, -3, and -4 was performed on RNA prepared from various mouse tissues including WT and IRS-1<sup>-/-</sup> islets. The sizes of the bands specific for the IRS proteins were 425 bp (IRS-1), 515 bp (IRS-2), 200 bp (IRS-3), and 350 bp (IRS-4).

$-20^{\circ}\text{C}$  for subsequent insulin RIA. For preparation of DNA, islets were placed in 0.5 mL eppendorf tubes containing 50 mM Tris, 100 mM EDTA, 0.5% SDS and 5  $\mu\text{l}$  of a 5 mg/mL solution of proteinase K. Samples were extracted with phenol and phenol/chloroform followed by precipitation with 3 M sodium acetate and 100% ethanol.

**Morphological analysis of pancreas and electron microscopy of islets.** Pancreata were rapidly dissected, weighed, fixed in Bouin's solution for 4 hours, transferred to 10% buffered formalin for 48 hours, and then placed in PBS, pH 7.4. Paraffin-embedded pancreata were sectioned and stained either for  $\beta$  cells using guinea pig anti-human insulin antibody (1:200; Linco Research Inc., St. Charles, Missouri, USA) or for non- $\beta$  cells using a cocktail of antibodies (anti-glucagon, 1:3,000, Linco; antipancreatic polypeptide, 1:3,000, gift from S. Bonner-Weir; and antismatostatin, 1:300, Incstar, Stillwater, Minnesota, USA) incubated overnight at  $4^{\circ}\text{C}$ . Secondary antibodies (for  $\beta$  cells, goat anti-guinea pig, 1:200; and for non- $\beta$  cells, goat anti-rabbit, 1:200; Miles-Yeda, Rehovot, Israel) were incubated for 3 hours at room temperature. The remainder of the procedure has been described elsewhere (10). Sections were analyzed using a AX70 Olympus microscope using a Phase 3 Imaging system (Image Pro 4.0, Media Cybernetics L.P., Santa Clara, California, USA). Electron microscopy of isolated islets was carried out as described previously (11) using a



**Figure 2**

Impaired glucose tolerance and reduced acute phase insulin release in IRS-1<sup>-/-</sup> mice. Intraperitoneal (IP) injection of (a and b) glucose (3 g/kg body weight) or (c) L-arginine (0.3 g/kg body weight) shows an impaired glucose tolerance and a reduced insulin response in IRS-1<sup>-/-</sup> mice compared with age-matched WT controls. Whole blood was collected from tail vein samples at the indicated times for insulin measurements. Values are means ± SEM (n = 4-6). \*P < 0.05 versus WT.

erwise indicated are presented as means ± SEM. Data were analyzed using an unpaired Student's *t* test, and the null hypothesis was rejected at the 0.05 level.

**Results**

*Presence of IRS proteins in islets and the physiology of IRS-1-deficient mice.* Using RT-PCR with specific primers, all 4 IRS proteins (IRS-1, -2, -3, and -4) could be detected in islet RNA from normal mice (Figure 1). IRS-1, IRS-2, and IRS-3 were also observed in most mouse tissues tested, whereas IRS-4 had a limited distribution including islet tissue, kidney, and brain. As expected, no IRS-1 mRNA could be detected in IRS-1 knockout mouse islets.

As previously reported (6), IRS-1<sup>-/-</sup> mice were approximately 50% smaller compared with both WT and IRS-1<sup>+/-</sup> mice (data not shown) and showed an approximately 50% decrease in circulating leptin levels (1.31 ± 0.12 ng/mL for WT versus 0.7 ± 0.1 ng/mL for IRS-1<sup>-/-</sup>; n = 10-12; P < 0.01), reflecting a decrease in fat mass. IRS-1<sup>-/-</sup> mice showed only mildly impaired glucose tolerance (6, 8) and higher fasting blood glucose levels (Figure 2a). Fed blood glucose levels were not different in the 3 groups (data not shown). Fasting insulin and C-peptide levels were approximately 2-fold and 4-fold higher in the IRS-1<sup>-/-</sup> mice than in WT controls (insulin: 136.0 ± 12.5 pg/mL for WT versus 294.0 ± 47.4 pg/mL for IRS-1<sup>-/-</sup>; C-peptide: 135.9 ± 18.9 pM for

WT versus 418.9 ± 66.6 pM for IRS-1<sup>-/-</sup>; n = 8-12; both, P < 0.01) suggesting an increased basal secretion by islet β cells. Despite this increase in basal insulin, acute insulin release as measured at 2 minutes in response to glucose was reduced by 77% in the IRS-1<sup>-/-</sup> mice when compared with WT mice (P < 0.01; Figure 2b). Furthermore, the potentiation of the glucose-stimulated insulin release after intraperitoneal L-arginine injection was also lower in the IRS-1<sup>-/-</sup> mice (Figure 2c). IRS-1<sup>+/-</sup> mice showed intermediate insulin levels between the knockout and WT mice. These results suggest that IRS-1 deficiency alters the insulin secretory response and indicate a functional insulin signaling pathway in the β cell.

*Morphology of pancreatic sections from IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> mice.* Assessment of islet size in pancreata from the different groups was carried out by analysis of sections stained with a cocktail of antibodies to the non-β cells (17). At least 400 islets were analyzed from multiple sections from each genotype in 4-month-old mice, and the mean

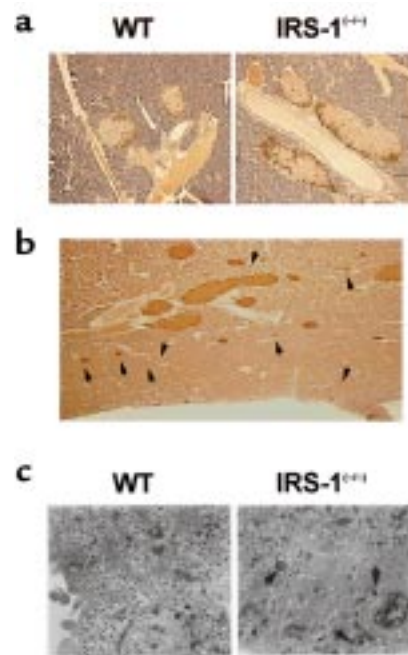
Philips 301 Transmission Electron Microscope (Philips Electron Optics-FEI, Millersville, Maryland, USA).

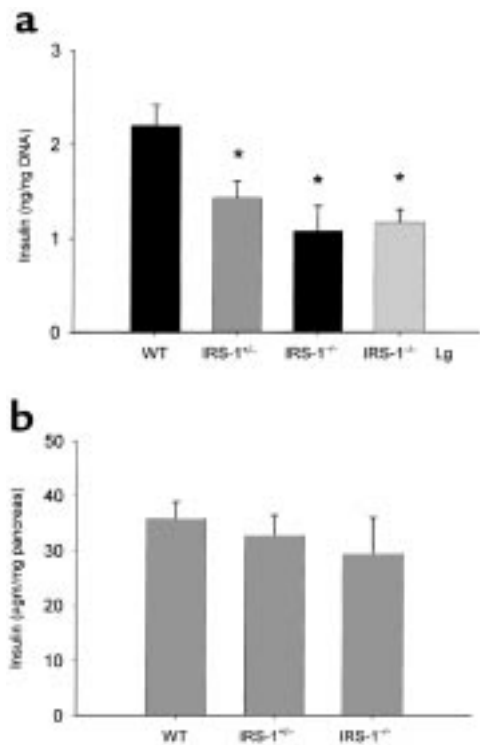
*RIA.* Insulin secretion in culture media and insulin content in extracts were measured by RIA using a Linco kit. Glucagon was measured in culture media collected in tubes containing Trasylol (10,000 KIU/mL) using an RIA kit obtained from Linco. Plasma C-peptide and leptin levels were measured using RIA kits (Linco).

*Statistical analysis.* All data unless oth-

**Figure 3**

Islet morphology and electron microscopy. (a) Sections of pancreas were stained for non-β cells using a cocktail antibody as described in Methods. Representative sections of pancreas from WT and IRS-1<sup>-/-</sup> mice show an approximately 2-fold increase in islet size in the latter. (b) Representative section of pancreas from an IRS-1<sup>-/-</sup> mouse stained for insulin as described in Methods shows insulin staining of β cells in the islets. Spotty insulin staining can be observed throughout the exocrine pancreas, indicating the presence of insulin-containing cells. (c) Electron microscopic examination of β cells in islets from IRS-1<sup>-/-</sup> mice was performed as described. A scant electron dense granulation pattern was observed in IRS-1<sup>-/-</sup> β cells compared with WT cells. An increased number of autophagic vacuoles (arrows) were observed in individual IRS-1<sup>-/-</sup> β cells. A representative photograph is shown. ×9,690.





**Figure 4**

Islet and pancreatic insulin content. (a) Insulin content expressed per nanogram of DNA is reduced in islets isolated from IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> mice. “Large” (Lg) islets show a reduced content compared with WT islets but were comparable to the normal islets isolated from IRS-1<sup>-/-</sup> mice. Values are means ± SEM ( $n = 35\text{--}50$  islets each from 3 mice of each genotype). \* $P < 0.05$  versus WT. (b) Total pancreatic insulin content is similar among the 3 groups. Pancreata were homogenized in acid ethanol as described in Methods, and insulin was measured by RIA. Values are means ± SEM ( $n = 3$ ).

islet size was measured using a calibrated micrometer. Sections from IRS-1<sup>-/-</sup> pancreas showed 3 populations of islets. About 54% were “normal-sized” islets ( $118 \pm 12 \mu\text{m}$ ) comparable to WT ( $112 \pm 10 \mu\text{m}$ ), whereas 31% were “large” islets; i.e., approximately 2-fold greater in size ( $243 \pm 11 \mu\text{m}$ ) than the WT islets (Figure 3a). In addition, about 15% of islets in IRS-1<sup>-/-</sup> pancreas were small ( $\sim 75 \mu\text{m}$ ) in comparison to less than 4% in the WT. Insulin-staining in the IRS-1<sup>-/-</sup> islets revealed a spotty distribution throughout the exocrine pancreas, indicating insulin-producing cells (Figure 3b). Electron microscopic examination of  $\beta$  cells in WT islets showed electron-dense insulin-containing granules. By contrast, the granules in  $\beta$  cells from IRS-1<sup>-/-</sup> mice were smaller and distinctly sparse (Figure 3c). In addition IRS-1<sup>-/-</sup>  $\beta$  cells displayed autophagic vacuoles not evident in WT  $\beta$ -cells (Figure 3c). Similar data were obtained from 3 different 4-month-old mice.

**Reduced insulin content in islets of IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> mice.** Insulin content was measured in acid-ethanol extracts of size-matched islets isolated from WT, IRS-1<sup>+/-</sup>, and IRS-1<sup>-/-</sup> mice, as well as the whole pancreas. In isolated islets, insulin content was reduced in

the heterozygous and homozygous knockout mice by approximately 34% and approximately 51%, respectively (Figure 4a). Even the large islets isolated from IRS-1<sup>-/-</sup> mice had lower ( $\sim 47\%$ ) insulin content than the WT islets. On the other hand, total pancreatic insulin content (expressed per milligram of wet weight of pancreas) was similar among the 3 groups, suggesting that the insulin content in the pancreas is likely made up by the greater proportion of large islets in the IRS-1-deficient mice (Figure 4b).

**Insulin secretion is blunted in IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> islets, whereas glucagon secretion is unaltered.** To assess the glucose-stimulated insulin secretion pattern in vitro, cultured islets were subjected to a glucose dose response. Blunted insulin secretion was observed in both IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> islets when compared with size-matched islets from WT mice and was particularly prominent at higher glucose concentrations (8.3–22.2 mM) (Figure 5a). The reduced response was evident even when corrected for insulin content, indicating alterations at the level of glucose sensitivity. In time-course experiments, islets from the IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> mice showed a diminished insulin secretion pattern from 0 to 60 minutes (Figure 5b). Taken together, these data demonstrate a lower insulin secretory response and altered kinetics to glucose stimulation in IRS-1<sup>-/-</sup> islets.

Measurement of glucagon in the culture media in the incubation experiments showed an approximately 45% suppression of glucagon secretion in all 3 groups, in response to a physiological glucose concentration (8.3 mM), suggesting a normal  $\alpha$ -cell secretory function (Figure 5c).

**Insulin content and secretion in  $\beta$ -cell lines.** To assess the influence of IRS-1 deficiency specifically in  $\beta$  cells, cell lines were derived from IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> mice (see Methods). As in freshly isolated islets, insulin content was approximately 34% lower in the IRS-1<sup>+/-</sup> cells and approximately 55% lower in the IRS-1 knockout cells compared with WT cells. Similar results were obtained from 2 different mice of each genotype (data not shown). When the IRS-1<sup>-/-</sup> cells were transiently transfected with IRS-1 cDNA, the level of expression of IRS-1 protein was restored to 40% of that in WT cells and was accompanied by an increase in insulin content to levels similar to the heterozygote cell line (Figure 6a). These data are consistent with a direct role for this protein in maintenance of insulin content.

As in isolated islets, glucose-stimulated insulin secretion was also reduced in the IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup>  $\beta$ -cell lines. The secretory responses were significantly lower even after correcting for the approximately 30–50% lower insulin content, suggesting a defect in the ability of the  $\beta$  cells to respond adequately to glucose stimulation (Figure 6b). In addition, the IRS-1-null  $\beta$  cells showed a shift in the glucose sensitivity curve, compared with isolated islets, with higher glucose concentrations being required to produce an increase in insulin secretion. This pattern of lower glucose sensitivity is typical of  $\beta$ -cell lines (16, 18–20) and was similar in IRS-1<sup>+/-</sup> and IRS-1<sup>-/-</sup> and WT cells. A similar blunted secretion pattern was apparent in response to L-arginine, mimicking the observations in the islet studies (Figure 6c). Taken together these data indicate a defect in the insulin secretory response to multiple stimuli in  $\beta$  cells lacking IRS-1.

## Discussion

Type 2 diabetes is characterized by peripheral insulin resistance followed by a failure of compensation by the pancreatic  $\beta$  cell and, ultimately, frank hyperglycemia (21, 22). Traditionally, these have been discrete pathophysiological lesions; however, it is increasingly clear that the insulin-signaling cascade may play an important role in the functioning of the islet/ $\beta$  cell (9, 11–14, 23–25). Previous work (9, 24, 26) and the results of the present study indicate that the IRS proteins are present in pancreatic islets and that the  $\beta$ -cells lacking IRS-1 or IRS-2 show altered function and/or growth. In addition, our recent demonstration of altered glucose sensing in a  $\beta$ -cell tissue-specific knockout of the insulin receptor suggests that insulin resistance at the level of the  $\beta$  cell itself may contribute to the failure of pancreatic compensation, thereby suggesting a unifying theory for the pathogenesis of type 2 diabetes (11, 27).

In the present study, we have demonstrated that although IRS-1 knockout mice appear to have a normal  $\beta$ -cell hyperplastic response to insulin resistance (7, 10), IRS-1 deficiency leads to decreased insulin content of  $\beta$  cells and a reduced acute phase secretion to both glucose and L-arginine. The diminished glucose-stimulated insulin secretion likely contributes to the glucose intolerance observed in the IRS-1<sup>-/-</sup> mice. When arginine is used as a stimulant, there is also a reduced insulin response in the IRS-1<sup>-/-</sup> mice. Although the precise mechanism of action of arginine is poorly understood, the reduced response to both glucose and arginine suggests that the lack of IRS-1 in the  $\beta$  cell impairs a

final step in the insulin secretory process. This phenotype is different from those both in the  $\beta$ -cell-specific insulin receptor knockout that influences glucose-stimulated insulin release (11), and in the IRS-2 knockout that manifests no secretory defects but altered  $\beta$ -cell growth (28). It is notable that in addition to the insulin receptor, IRS-1 acts as a substrate for IGF-1, prolactin, and growth hormone receptors (29–31). The presence of these receptors in insulin-producing cells (2, 32–34) indicates they may potentially activate IRS-1 in islets. Similarly, as several cytokines and growth factors activate IRS-2 (35), it is possible that this protein is a substrate for the growth-promoting IGF-1 receptor in the  $\beta$  cells (25). The reduced number of insulin-positive cells in islets isolated from mice heterozygous for the IGF-1 receptor and homozygous for IRS-2 suggests that IGF-1 receptor-mediated  $\beta$ -cell growth is likely mediated by IRS-2 (36). Taken together, these data suggest that insulin receptors and IGF-1 receptors, as well as IRS-1 and IRS-2, each have distinct functions in the islet/ $\beta$ -cells.

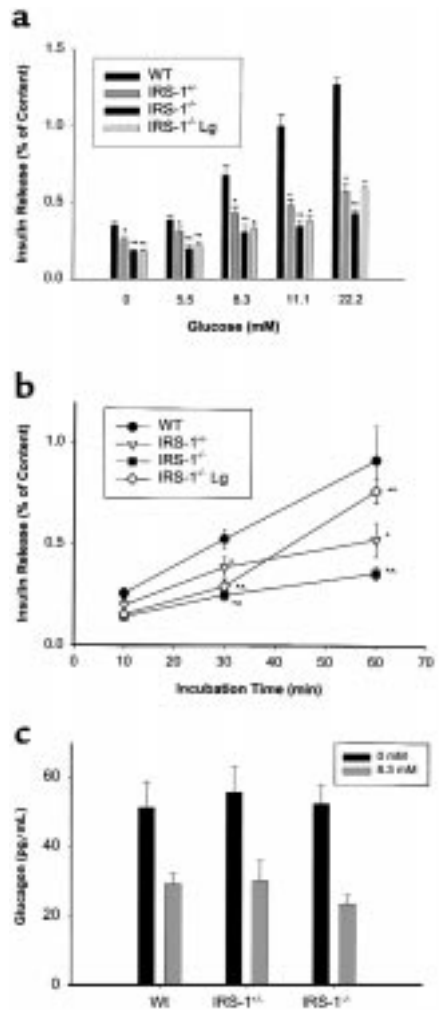
Although it is clear from our *in vivo* experiments that the acute insulin secretory pattern is blunted, basal circulating insulin levels are higher in the IRS-1<sup>-/-</sup> mice than in controls. This mild hyperinsulinemia is due to increased secretion, as evidenced by increased C-peptide levels, and may be due to 2 factors. First, IRS-1<sup>-/-</sup> mice are insulin resistant (6, 8), and this by itself may cause hyperinsulinemia due to normal feedback mechanisms (11, 12, 14). In addition, the circulating leptin levels are 50% lower in the growth-retarded IRS-1<sup>-/-</sup> mice, similar

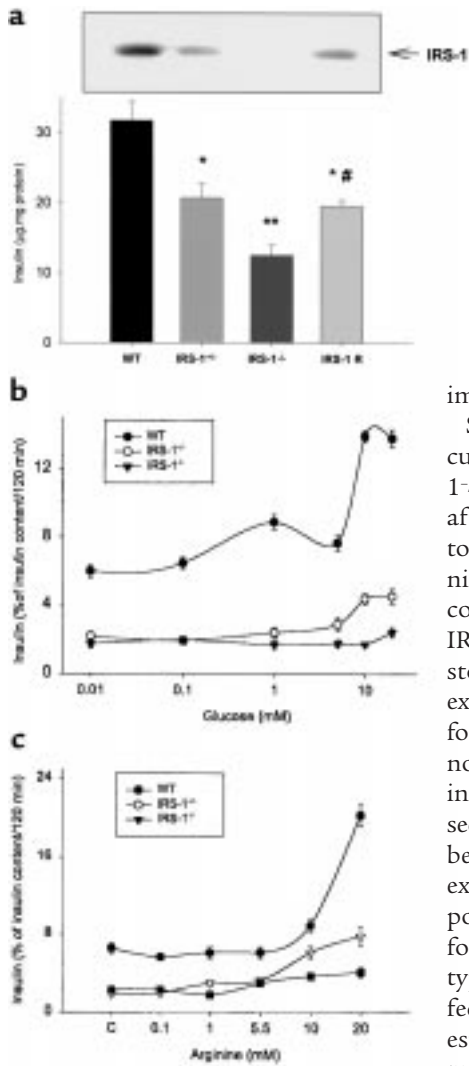
to obese individuals carrying a variant of IRS-1 (37). Leptin has been shown to suppress insulin secretion *in vivo* and *in vitro* (15, 38) and may result in the removal of a tonic insulin inhibitory effect resulting in unrestrained insulin secretion.

To define the nature of the insulin secretory defect in the absence of this and other *in vivo* feedback mechanisms, functional studies were conducted on isolated islets. This also enabled us to compare directly islet function in islets of similar size. Although small and large islets could be detected in the IRS-1<sup>-/-</sup> mice, the insulin content in both of these types of islets was lower than in WT islets. Because the IRS-1<sup>-/-</sup> mice are growth retarded, the islet size/body mass ratio is actually greater in these mice when compared with WT controls. This factor and a greater proportion of large islets and a smaller total blood volume

## Figure 5

Reduced dose- and time-dependent glucose-stimulated insulin secretion in IRS-1-deficient islets. (a) Insulin secretion in response to glucose shows a dose-dependent decrease in insulin secretion in size-matched islets isolated from IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> mice compared with WT controls. Insulin secretion is expressed as a percent of the islet insulin content. Values are means  $\pm$  SEM ( $n = 4-6$ ). \* $P < 0.05$  versus WT;  $\wedge P < 0.01$  versus IRS-1<sup>-/-</sup>. (b) Incubation of islets in the presence of 8.3 mM glucose showed a decreased insulin secretion in IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> islets compared with WT islets up to 60 minutes. Insulin secretion in IRS-1<sup>-/-</sup> was lower even when compared with IRS-1<sup>+/-</sup> islets at 30 and 60 minutes. Values are means  $\pm$  SEM ( $n = 4-6$ ). \* $P < 0.05$  versus WT;  $\wedge P < 0.02$  versus IRS-1<sup>+/-</sup>; \*\*\* $P < 0.05$  versus IRS-1<sup>-/-</sup> or IRS-1<sup>+/-</sup>. (c) Incubation of islets in 8.3 mM glucose showed a suppression of glucagon in all 3 groups. No significant differences in suppression could be observed between islets isolated from WT, IRS-1<sup>-/-</sup>, and IRS-1<sup>+/-</sup> mice. Values are means  $\pm$  SEM ( $n = 4-6$ ).





**Figure 6**

Insulin content is partially restored in IRS-1 re-expressing cultured  $\beta$ -cells. (a) A reduced insulin content is observed in IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> cells compared with WT cells. Re-expression of IRS-1 in IRS-1<sup>-/-</sup> cells (upper panel) shows a partial restoration of insulin content and is significantly higher than IRS-1<sup>-/-</sup> cells (lower panel). Values are means  $\pm$  SEM ( $n = 6-7$ ). \* $P < 0.05$  versus WT; \*\* $P < 0.01$  versus WT; # $P < 0.05$  versus IRS-1<sup>-/-</sup>. Insulin secretion is blunted in response to increasing concentrations of (b) glucose and (c) L-arginine in IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup>  $\beta$ -cell lines. Results are expressed as a percent of the cellular insulin content. Values are means  $\pm$  SEM ( $n = 6$ ).  $P < 0.05$  IRS-1<sup>+/-</sup> or IRS-1<sup>-/-</sup> versus WT at all concentrations;  $P < 0.05$  IRS-1<sup>+/-</sup> versus IRS-1<sup>-/-</sup> at all concentrations except 0.01 and 0.1 mM glucose and 0.1 and 5.5 mM L-arginine.

iments as considered in our study.

Similar results were obtained with cultured  $\beta$ -cell lines derived from IRS-1<sup>-/-</sup> and IRS-1<sup>+/-</sup> mice. Furthermore, after transfection of the IRS-1<sup>-/-</sup> cells to restore IRS-1 protein, there is a significant increase in insulin content consistent with an important role for IRS-1 in the regulation of insulin stores in the  $\beta$  cells. Interestingly, expression of a mutant IRS-1 (glycine for arginine in codon 972) in rat insulinoma cells has been reported to result in a lower insulin content and a lower secretory response to glucose and glibenclamide compared with cells expressing WT IRS-1 (39). Indeed, polymorphisms in IRS-1 occur at a 2-fold higher frequency in patients with type 2 diabetes compared with unaffected individuals (40-43). It is interesting that carriers of this mutation manifest low serum insulin and C-peptide levels (41). The results presented in this study suggest that the presence of altered IRS-1 molecules contribute to the insulin deficiency in these patients.

The exact molecular consequences of IRS-1 deficiency that lead to the altered insulin content and secretion need further investigation. We and others (24) have shown that glucose stimulates phosphorylation of IRS-1 in islets. In addition, this promotes IRS-1 association with the regulatory subunit of PI 3-kinase (24) and activates pathways involved in  $\beta$ -cell proliferation (25). Expression of the mutant IRS-1 in rat insulinoma cells showed a decrease in binding of the p85 subunit of PI 3-kinase (39). PI 3-kinase activity has been implicated in multiple vesicle trafficking roles and in insulin and growth factor-regulated gene expression (44-48). We are currently performing single-cell amperometric experiments to dissect the

mechanism of the loss of glucose-stimulated insulin secretion in IRS-1<sup>-/-</sup> cells. Preliminary data show a delayed rise in Ca<sup>2+</sup> in response to glucose consistent with the data in IRS-1-deficient islets and cultured  $\beta$ -cell lines (data not shown). It is therefore conceivable that the insulin receptor/IRS-1/PI 3-kinase pathway is involved in islet secretory function and insulin gene expression.

The presence of a  $\beta$ -cell defect is thought to be one of the prerequisites for full development of type 2 diabetes. However, defining the temporal onset of islet decompensation and localizing the functional defects in humans has been difficult. The IRS-1 knockout mouse provides an excellent genetic model in that it exhibits early insulin resistance but manifests only impaired glucose tolerance and does not become overtly diabetic. The demonstrable detectable defect in islet function, especially clear in vitro, therefore implicates confounding systemic factors that mask the islet dysfunction. One interesting line of approach would be to investigate the IRS-1 knockout mice on other genetic backgrounds, as polymorphic modifier genes could well alter the phenotypes and produce frank diabetes. In summary, the present data provide novel evidence of a role for IRS-1 in an insulin-signaling cascade that links it to insulin content and secretion in the islet  $\beta$  cell, a linkage that may be disrupted in patients with type 2 diabetes manifesting insulin resistance.

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contribute in part to the mildly higher circulating insulin in the knockout mice. When incubated in vitro, IRS-1-deficient islets secrete lower insulin in the basal state. In vitro glucose stimulation showed a diminished release in islets isolated from IRS-1<sup>+/-</sup> and even more so with IRS-1<sup>-/-</sup> mice, indicating a genetic dose response. These secretory responses were lower even after correcting for the lower insulin content, indicating a secretory defect in the islets lacking IRS-1. An earlier study by Terauchi et al. (7) also reported a reduced basal insulin release and consistently lower secretory responses to increasing concentration of glucose in batch-incubated and perfused islets isolated from IRS-1<sup>-/-</sup> mice compared with WT controls. However, no reference is made as to whether islet size is taken into consideration in these exper-

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