Role of the Insulin Receptor Substrate 1 and Phosphatidylinositol 3-Kinase Signaling Pathway in Insulin-Induced Expression of Sterol Regulatory Element Binding Protein 1c and Glucokinase Genes in Rat Hepatocytes

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The mechanism by which insulin induces the expression of the sterol regulatory element binding protein 1c (SREBP-1c) and glucokinase genes was investigated in cultured rat hepatocytes. Overexpression of an NH_2-terminal fragment of IRS-1 that contains the pleckstrin homology and phosphotyrosine binding domains (insulin receptor substrate-1 NH_2-terminal fragment [IRS-1N]) inhibited insulin-induced tyrosine phosphorylation of IRS-1 as well as the association of IRS-1 with phosphatidylinositol (PI) 3-kinase activity, whereas the tyrosine phosphorylation of IRS-2 and its association with PI 3-kinase activity were slightly enhanced. The equivalent fragment of IRS-2 (IRS-2N) prevented insulin-induced tyrosine phosphorylation of both IRS-1 and IRS-2, although that of IRS-1 was inhibited more efficiently. The insulin-induced increases in the abundance of SREBP-1c and glucokinase mRNAs, both of which were sensitive to a dominant-negative mutant of PI 3-kinase, were blocked in cells in which the insulin-induced tyrosine phosphorylation of IRS-1 was inhibited by IRS-1N or IRS-2N. A dominant-negative mutant of Akt enhanced insulin-induced tyrosine phosphorylation of IRS-1 (but not that of IRS-2) and its association with PI 3-kinase activity, suggesting that Akt contributes to negative feedback regulation of IRS-1. The Akt mutant also promoted the effects of insulin on the accumulation of SREBP-1c and glucokinase mRNAs. These results suggest that the IRS-1–PI 3-kinase pathway is essential for insulin-induced expression of SREBP-1c and glucokinase genes. *Diabetes* 51:1672–1680, 2002

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FBS, fetal bovine serum; GK, glucokinase; GSK, glycogen synthase kinase; HA, hemagglutinin epiptope; IRS, insulin receptor substrate; IRS-1N, IRS-1 NH_2-terminal fragment; KRLB, kinase regulatory loop binding; MOI, multiplicity of infection; PFU, plaque-forming unit; PH, pleckstrin homology; PI, phosphatidylinositol; PTB, phosphotyrosine binding; SREBP-1c, sterol regulatory element binding protein 1c.

Impairment of the mechanism by which insulin reduces the blood concentration of glucose is an important feature of type 2 diabetes (1). However, the pathogenesis of such insulin resistance and the specific steps at which insulin action is affected are not fully understood (1). Although insulin activates various signaling molecules in its target cells, the signaling pathways mediated by insulin receptor substrate (IRS) and phosphatidylinositol (PI) 3-kinase are thought to play a central role in the metabolic actions of insulin (2,3), suggesting that disturbance of these pathways may contribute to the development of insulin resistance. Indeed, disruption of the genes for IRS-1 or IRS-2, both of which are expressed in many tissues and cells (4), was shown to result in insulin resistance in mice (5–8).

Although the structures of the two proteins are highly similar, mice deficient in IRS-1 or IRS-2 exhibit distinct phenotypes. For example, IRS-1–deficient mice compensate for the impairment in insulin action by increasing the secretion of insulin and therefore do not develop diabetes (5,6). In contrast, mice deficient in IRS-2 fail to develop such a compensatory response of pancreatic islets, probably because of an inability of their β-cells to proliferate or differentiate (7,8). Clamp studies indicated that insulin resistance in IRS-1–deficient mice is attributable mainly to a reduced effect of insulin on glucose metabolism in peripheral tissues, whereas IRS-2–deficient mice manifest multiple defects in insulin action in the liver and peripheral tissues (9). Moreover, in vitro studies have revealed that defects in IRS-1– or IRS-2–dependent signaling result in distinct effects on cellular functions (10–13), suggesting that IRS-1 and IRS-2 may not only be redundant but rather are selectively linked to specific actions of insulin.

Sterol regulatory element binding protein 1c (SREBP-1c) belongs to a family of transcription factors that regulate the expression of genes for various lipogenic enzymes (14). Moreover, SREBP-1c is also implicated in regulation of the expression of both glucokinase (GK) and PEPCK genes (15,16), suggesting that it participates in control of glucose metabolism in the liver. Insulin stimulates expression of the SREBP-1c gene in primary cultured hepato-

cytes (15,17). The observation that insulin-induced expression of the SREBP-1c gene was prevented by a pharmacological inhibitor of PI 3-kinase (18,19) further suggests that this latter enzyme mediates this effect of insulin.

To further characterize the molecular mechanism by which insulin induces expression of the SREBP-1c gene, we investigated the relative contributions of IRS-1– and IRS-2–dependent signaling to this process in primary cultured hepatocytes. For this study, we examined the effects of both an IRS-1 mutant that comprises only the NH_2-terminal region of the protein and a dominant-negative mutant of the protein kinase Akt. The expression of these two mutant proteins was shown to attenuate selectively and to potentiate, respectively, the insulin-induced increase in IRS-1–associated PI 3-kinase activity. Our results demonstrate that the IRS-1–PI 3-kinase signaling pathway is essential for the insulin-induced expression of both SREBP-1c and GK genes.

RESEARCH DESIGN AND METHODS

Adenovirus vectors. Adenovirus vectors encoding a dominant-negative mutant of PI 3-kinase (AxCAΔp85) (20), a constitutively active form of PI 3-kinase (AxCAMyr-p110) (21), and an Akt mutant in which Thr^{308} and Ser^{473} are replaced by alanine (AxCAAkt-AA) (22) were described previously. A control adenovirus encoding β-galactosidase was provided by I. Saito. Complementary DNAs encoding NH_2-terminal portions of human IRS-1 (amino acids 2–401) (IRS-1N) and mouse IRS-2 (amino acids 2–430) (IRS-2N) tagged at their NH_2-termini with the hemagglutinin epiptope (HA) were synthesized with the use of PCR. Adenovirus vectors encoding HA-tagged IRS-1N or IRS-2N (AxCAIRS-1N and AxCAIRS-2N, respectively) were generated with the use of an adenovirus expression kit (Takara, Tokyo) as described previously (22).

Hepatocyte culture and Northern blot analysis. Hepatocytes were isolated from the liver of male Wistar rats (body mass 150–250 g) with the use of collagenase (23). The isolated hepatocytes were seeded at a density of 2.6×10^6 cells per 60-mm dish (coated with rat collagen-I) in medium A (medium 199 containing Earle's salt, penicillin [100 units/ml], and streptomycin [100 μg/ml]) supplemented with 5% (vol/vol) fetal bovine serum (FBS), 100 nmol/l dexamethasone, 1 nmol/l insulin, and 100 nmol/l triiodothyronine. At 8–12 h after seeding, hepatocytes were infected with adenovirus vectors by incubation for 2 h at 37°C in medium A supplemented with 5% FBS and the indicated amounts of virus. The virus-containing medium was then aspirated, and the cells were incubated for an additional 6–8 h in medium A containing 5% FBS. For Northern blot analysis of SREBP-1c mRNA, cells infected (or not) with adenovirus vectors were incubated in medium A (serum free) for 16 h and then treated with 100 nmol/l insulin in the same medium for 6 h; for analysis of GK mRNA, the cells (infected or not) were incubated with 100 nmol/l insulin in medium A for 18 h. After insulin treatment, total RNA was extracted from the cells with the use of an RNeasy kit (Qiagen), and portions (20 μg) of the isolated RNA were subjected to Northern analysis with ^{32}P-labeled probes prepared with a BcaBEST labeling kit (Takara). Probes for SREBP-1c (24) and GK (25) mRNAs were provided by H. Shimano and T. Noguchi, respectively. Autoradiograms of Northern blots were visualized with a BAS2000 image analyzer (Fuji, Tokyo).

Immunoblot analysis and assays of PI 3-kinase and Akt activity. For immunoblot analysis and assays of PI 3-kinase and Akt activity, hepatocytes infected (or not) with adenovirus vectors were incubated in medium A (serum free) for 16 h, treated (or not) with 100 nmol/l insulin for the indicated times, and lysed. The cell lysates were then subjected to immunoprecipitation or immunoblot analysis. Antibodies to glycogen synthase kinase (GSK)-3β were obtained from Transduction Laboratories. Antibodies to IRS-1, IRS-2, Akt2, or phosphotyrosine (4G10) were obtained from Upstate Biotechnology, and those specific for phospho-Ser^{473} forms of Akt or phosphorylated forms of GSK3 were from Cell Signaling. PI 3-kinase activity in immunoprecipitates prepared with antibodies to IRS-1 or IRS-2 was assayed as described (20), and Akt activity in immunoprecipitates with antibodies to Akt2 was assayed with the use of Crosstide peptide as a substrate as described (26).

RESULTS

Effects of a dominant-negative mutant of PI 3-kinase on insulin-induced expression of SREBP-1c and GK genes. In primary cultured hepatocytes, insulin induced a marked increase in the amount of PI 3-kinase activity associated with IRS-1 or IRS-2 immunoprecipitates (Fig. 1*A*). Infection of the cells with an adenovirus vector encoding a dominant-negative mutant of PI 3-kinase (AxCAΔp85) resulted in almost complete inhibition of the insulin-induced increase in the amount of PI 3-kinase activity that coprecipitated with IRS-1 or IRS-2. Cells that had been infected (or not) with AxCAΔp85 were then incubated in the absence or presence of insulin, after which total RNA was isolated and subjected to Northern blot analysis with a fragment of SREBP-1c cDNA as a probe. Although this probe reacts with both SREBP-1a and SREBP-1c mRNAs, insulin increases the amount of SREBP-1c mRNA, but not that of SREBP-1a mRNA, in primary cultured hepatocytes (17); the insulin-induced increase in the hybridization signal intensity apparent with this probe for these cells therefore reflects the increase in the abundance of SREBP-1c mRNA. Whereas insulin induced a marked increase in the amount of SREBP-1c mRNA in noninfected hepatocytes (Fig. 1*B*), this effect was inhibited in a multiplicity of infection (MOI)-dependent manner in the cells infected with AxCAΔp85 (Fig. 1*B*). Given that SREBP-1c is thought to regulate expression of the GK gene (15), we also examined the effect of Δp85 on the insulin-induced expression of this gene. The increase in the amount of GK mRNA triggered by insulin that was apparent in noninfected hepatocytes was inhibited in an MOI-dependent manner in the cells infected with AxCAΔp85 (Fig. 1*C*). Infection of hepatocytes with AxCAMyr-p110, an adenovirus vector that encodes a constitutively active form of PI 3-kinase, resulted in an increase in the abundance of both SREBP-1c and GK mRNAs in the absence of insulin (Fig. 1*B* and *C*). These results indicate that PI 3-kinase is required for the insulin-induced expression of SREBP-1c and GK genes.

Effects of an NH_2-terminal fragment of IRS-1 on insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2. The NH_2-terminal regions of IRS proteins contain pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, both of which have been suggested to contribute to the interaction between IRS and the insulin receptor (27,28). We investigated the effects of an NH_2-terminal portion of IRS-1 (IRS-1N) that contains the PH and PTB domains on insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2. Infection of hepatocytes with an adenovirus vector encoding IRS-1N (AxCAIRS-1N) resulted in an MOI-dependent increase in the abundance of the mutant protein (Fig. 2*A*). The expression of IRS-1N did not affect the abundance of IRS-1 and IRS-2 in the cells (Fig. 2*B* and *C*). Expression of this mutant protein did, however, result in an MOI-dependent inhibition of insulin-induced tyrosine phosphorylation of IRS-1. In contrast, tyrosine phosphorylation of IRS-2 was slightly increased by IRS-1N. IRS-1N did not affect insulin-induced tyrosine phosphorylation of the insulin receptor, and a control virus encoding β-galactosidase had no effect on the insulin-induced tyrosine phosphorylation of IRS-1 or IRS-2 at an MOI of up to 30 plaque-forming units (PFUs)/cell

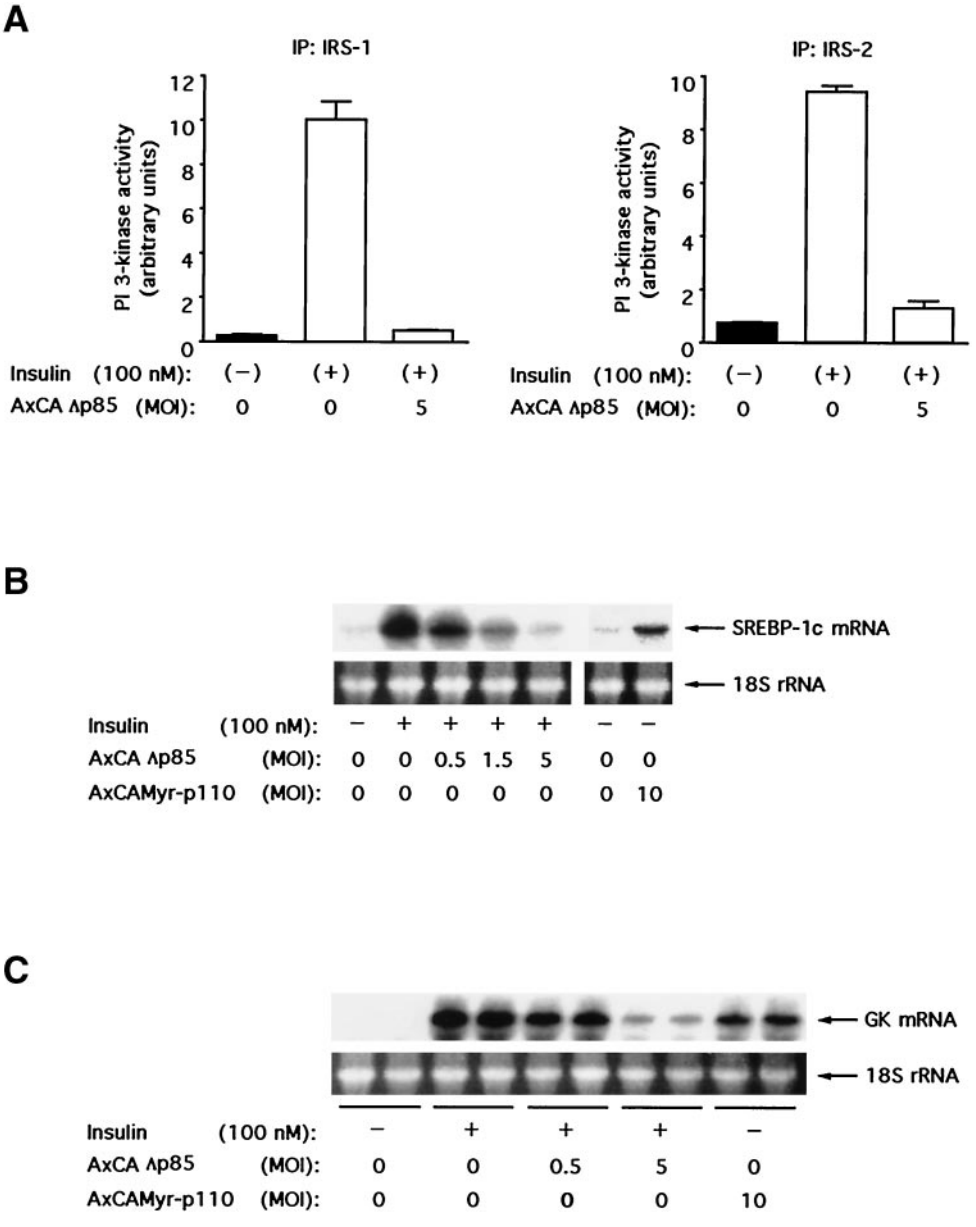

FIG. 1. Effects of a dominant-negative mutant of PI 3-kinase (Δp85) on the insulin-induced expression of SREBP-1c and GK genes. *A*: Effects of Δp85 on the insulin-induced increase in the amount of PI 3-kinase activity associated with IRS-1 or IRS-2. Hepatocytes infected (or not) with AxCAΔp85 at an MOI of 5 PFUs per cell were incubated in the absence or presence of 100 nmol/l insulin for 1 min and then lysed. Cell lysates were subjected to immunoprecipitation (IP) with antibodies to IRS-1 (left panel) or IRS-2 (right panel), and the resulting precipitates were assayed for PI 3-kinase activity. Data are expressed in arbitrary units and are means ± SE of values from three independent experiments. *B* and *C*: Effects of Δp85 and Myr-p110 on the insulin-induced increases in the abundance of SREBP-1c and GK mRNAs. Hepatocytes infected with AxCAΔp85 or AxCAMyr-p110 at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin, as described in RESEARCH DESIGN AND METHODS. Total RNA was then isolated from the cells and subjected to Northern blot analysis of SREBP-1c (*B*) or GK (*C*) mRNAs; samples from duplicate incubations were analyzed (*C*). The amount of 18S rRNA was visualized by staining with ethidium bromide. Data are representative of at least three independent experiments.

(data not shown). The insulin-induced increases in the amount of PI 3-kinase activity associated with IRS-1 and IRS-2 were inhibited by >90% and increased by ~30%, respectively, as a result of infection of hepatocytes with AxCAIRS-1N at an MOI of 10 PFUs/cell (Fig. 2*D*). These results thus indicate that IRS-1N inhibits the association of PI 3-kinase with IRS-1 by attenuating the tyrosine phosphorylation of endogenous IRS-1.

Effects of IRS-1N on insulin-induced expression of SREBP-1c and GK genes and phosphorylation of Akt. On the basis of our observation that IRS-1N selectively inhibited the IRS-1–PI 3-kinase signaling pathway, we next investigated the contribution of this pathway to the insulin-induced expression of the SREBP-1c gene with the use of IRS-1N. Infection of hepatocytes with AxCAIRS-1N resulted in an MOI-dependent inhibition of the insulin-induced increase in the abundance of SREBP-1c mRNA (Fig. 3*A*); this effect of insulin was almost completely blocked at an MOI of 10 PFUs/cell, a virus dose sufficient to inhibit the insulin-induced increase in IRS-1–associated PI 3-kinase activity by >90% (Fig. 2*D*). The insulin-induced increase in the amount of GK mRNA was also similarly inhibited by IRS-1N in a dose-dependent manner (Fig. 3*B*). Infection of the cells with a control adenovirus encoding

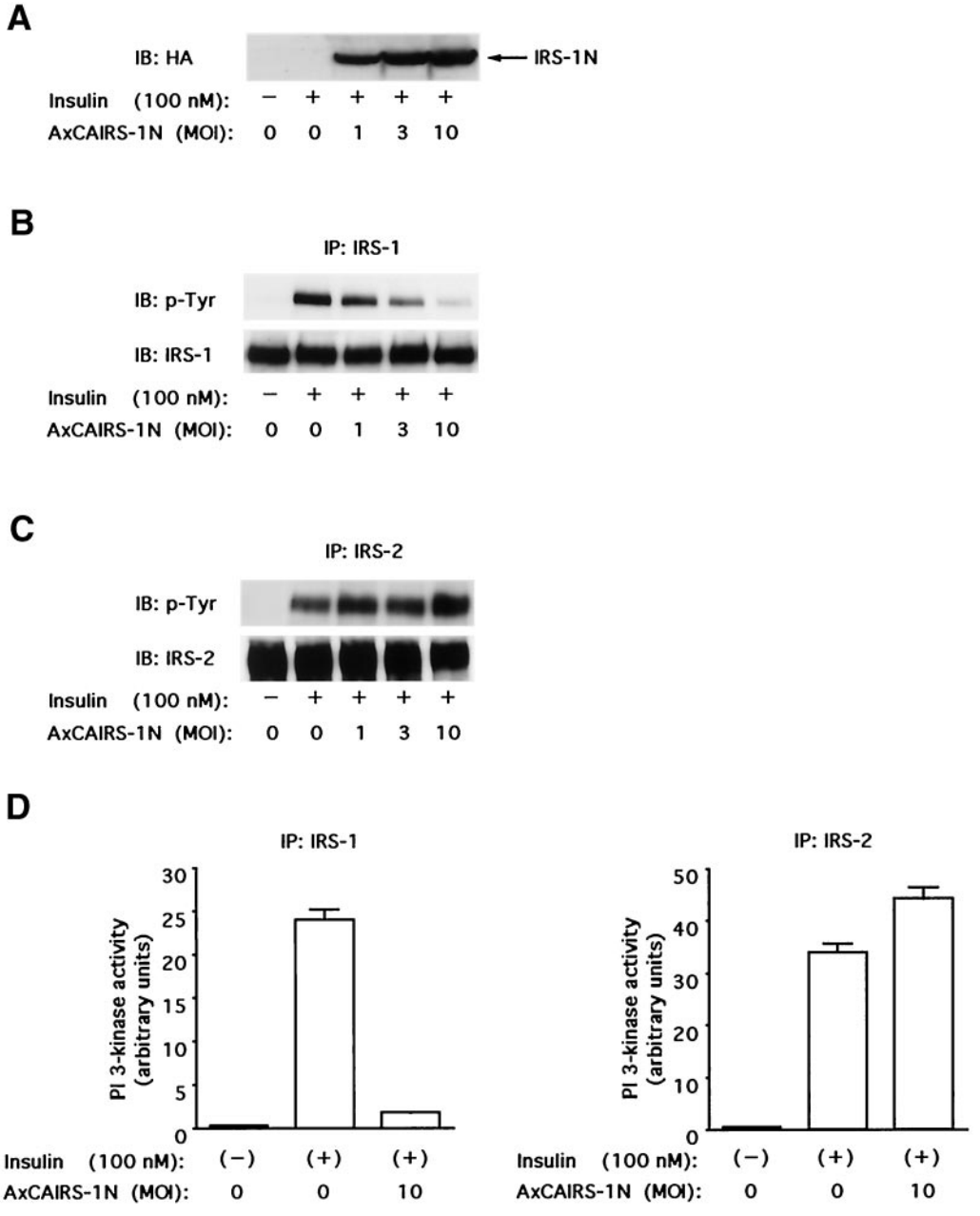

FIG. 2. Effects of an NH_2-terminal fragment of IRS-1 (IRS-1N) on the insulin-induced tyrosine phosphorylation of, and association of PI 3-kinase activity with, IRS-1 and -2. Hepatocytes infected with AxCAIRS-1N at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin for 1 min and then lysed. The cell lysates were either subjected directly to immunoblot analysis (IB) with antibodies to HA (*A*) or subjected to immunoprecipitation (IP) with antibodies to IRS-1 (which do not recognize IRS-1N) (*B* and *D*) or to IRS-2 (*C* and *D*); the resulting precipitates were then either subjected to immunoblot analysis with antibodies to phosphotyrosine (*B* and *C*, upper panels), IRS-1 (*B*, lower panel), or IRS-2 (*C*, lower panel), or assayed for PI 3-kinase activity (*D*). Data in *A–C* are representative of three independent experiments, and those in *D* are means ± SE of values from three independent experiments.

β-galactosidase did not affect the abundance of SREBP-1c or GK mRNAs at an MOI of up to 30 PFUs/cell (data not shown). In contrast, the insulin-induced phosphorylation of Akt, which is thought to be essential for the activation of this kinase, was not affected by expression of IRS-1N, even at an MOI of 10 PFUs/cell, as assessed by immunoblot analysis with antibodies specific for phosphorylated forms of the enzyme (Fig. 3*C*). These results suggest that the insulin-induced expression of SREBP-1c and GK genes depends on the IRS-1–PI 3-kinase pathway, whereas inhibition of this signaling pathway does not prevent the insulin-induced phosphorylation of Akt.

Effects of a dominant-negative mutant of Akt on the insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2 and expression of SREBP-1c and GK genes. We next investigated the effects of a dominant-negative mutant of Akt (Akt-AA), which has been shown to inhibit the insulin-induced activation of Akt as well as various metabolic actions of insulin (21,22,29), on insulin-induced expression of SREBP-1c and GK genes. Hepatocytes were infected (or not) with an adenovirus vector encoding Akt-AA (AxCAAkt-AA), incubated in the absence or presence of insulin, and then lysed. Cell lysates were subjected to immunoprecipitation with antibodies to Akt2, and the

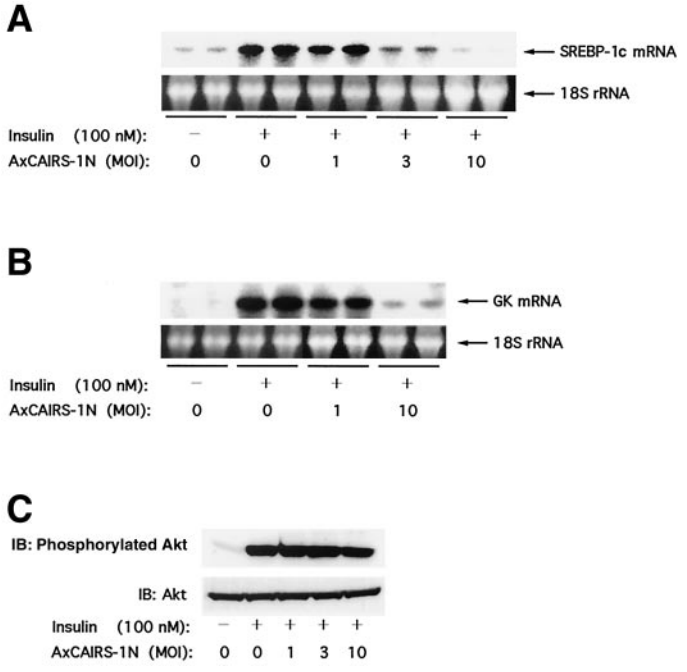

FIG. 3. Effects of IRS-1N on the expression of SREBP-1c and GK genes and the phosphorylation of Akt induced by insulin. *A* and *B*: Hepatocytes infected with AxCAIRS-1N at the indicated MOI were incubated in the absence or presence of insulin. Total RNA was then prepared from the cells and subjected to Northern blot analysis of SREBP-1c mRNA (*A*) or GK mRNA (*B*). Data are shown for duplicate incubations. *C*: Cells infected with AxCAIRS-1N at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin for 10 min and then lysed. Cell lysates were subjected to immunoblot analysis (IB) with antibodies to phosphorylated forms of Akt (upper panel) or Akt (lower panel). All data are representative of at least three independent experiments.

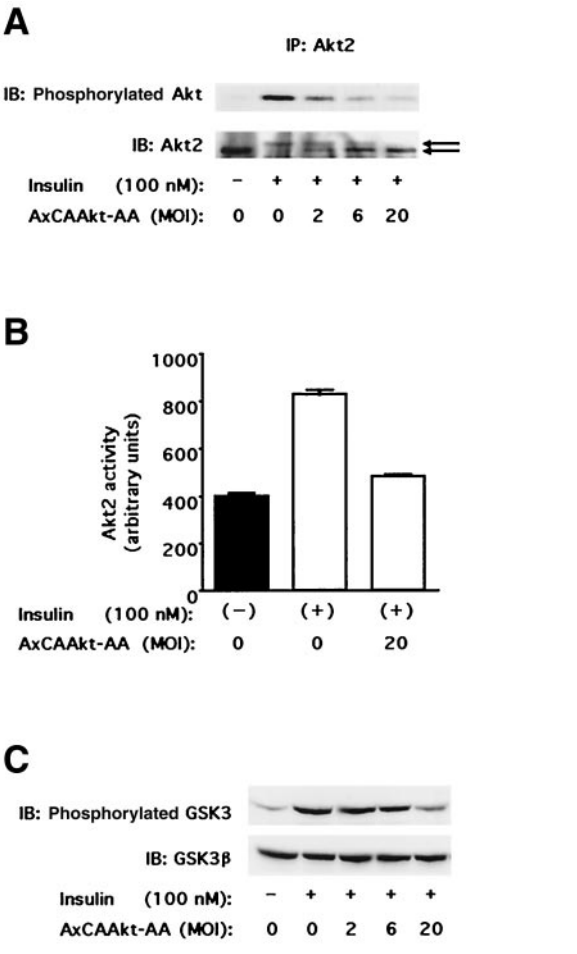

FIG. 4. Effects of a dominant-negative mutant of Akt (Akt-AA) on the insulin-induced activity of Akt and phosphorylation of GSK3β. Hepatocytes infected with AxCAAkt-AA at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin for 10 min and then lysed. *A* and *B*: Cell lysates were subjected to immunoprecipitation (IP) with antibodies to Akt2, and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to phosphorylated forms of Akt (*A*, upper panel) or to Akt2 (A, lower panel), or they were subjected to kinase assay for Akt2 activity (*B*). Arrows indicate higher-mobility (nonphosphorylated) and lower-mobility (phosphorylated) forms of Akt2 (*C*). The cell lysates were subjected directly to immunoblot analysis with antibodies to phosphorylated forms of GSK3 (upper panel) or GSK3β (lower panel). Data in *A* and *C* are representative of three independent experiments and in *B* are means ± SE of values from three experiments.

resulting precipitates were subjected to immunoblot analysis with antibodies to Akt2 or phosphorylated Akt. Because the Akt-AA cDNA was constructed from rat Akt1 cDNA, the Akt2-specific antibodies did not recognize the Akt-AA protein. Expression of Akt-AA resulted in inhibition of the insulin-induced phosphorylation of Akt2, as revealed both by immunoblot analysis with the antibodies specific for the phosphorylated protein and by the mobility shift detected with antibodies to Akt2 (Fig. 4*A*), the latter of which also reflects phosphorylation status. Moreover, infection of the cells with AxCAAkt-AA at an MOI of 20 inhibited insulin-induced activity of endogenous Akt2 by ~90% (Fig. 4*B*). The insulin-induced phosphorylation of GSK3β, which is a direct substrate of Akt (30), was also inhibited by Akt-AA (Fig. 4*C*). These results suggest that Akt-AA exerts a dominant-negative effect on the insulin-induced activation of Akt as well as on signaling downstream of this protein in primary cultured hepatocytes. However, expression of Akt-AA did not affect the insulin-induced increases in the abundance of SREBP-1c and GK mRNAs; rather, Akt-AA increased the amounts of both mRNAs in a dose-dependent manner (Fig. 5).

The abundance of IRS-1 and IRS-2 was not affected by Akt-AA (Fig. 6*A* and *B*). Although insulin-induced tyrosine phosphorylation of IRS-1 was enhanced in cells expressing Akt-AA, that of IRS-2 was slightly decreased. Furthermore, the amount of PI 3-kinase activity associated with IRS-1 was increased, whereas that associated with IRS-2 was slightly reduced in cells expressing Akt-AA (Fig. 6*C*). These results thus suggest that inhibition of Akt activity promoted the association of PI 3-kinase with IRS-1 by increasing the extent of tyrosine phosphorylation of IRS-1, consistent with the recent observation that Akt contributes to a negative feedback pathway directed at IRS-1 (31).

Effects of an NH_2-terminal fragment of IRS-2 on insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2, phosphorylation of Akt, and expression of SREBP-1c and GK genes. Finally, we investigated the effects of an NH_2-terminal portion of IRS-2 that contains the PH and PTB domains (IRS-2N) on insulin action. Infection of hepatocytes with an adenovirus vector encoding IRS-2N (AxCAIRS-2N) resulted in an MOI-dependent increase in the abundance of the mutant protein (Fig. 7*A*). Expression of IRS-2N resulted in a dose-dependent inhibition of the insulin-induced tyrosine phosphorylation of both IRS-1 and IRS-2 without an effect on the abundance of

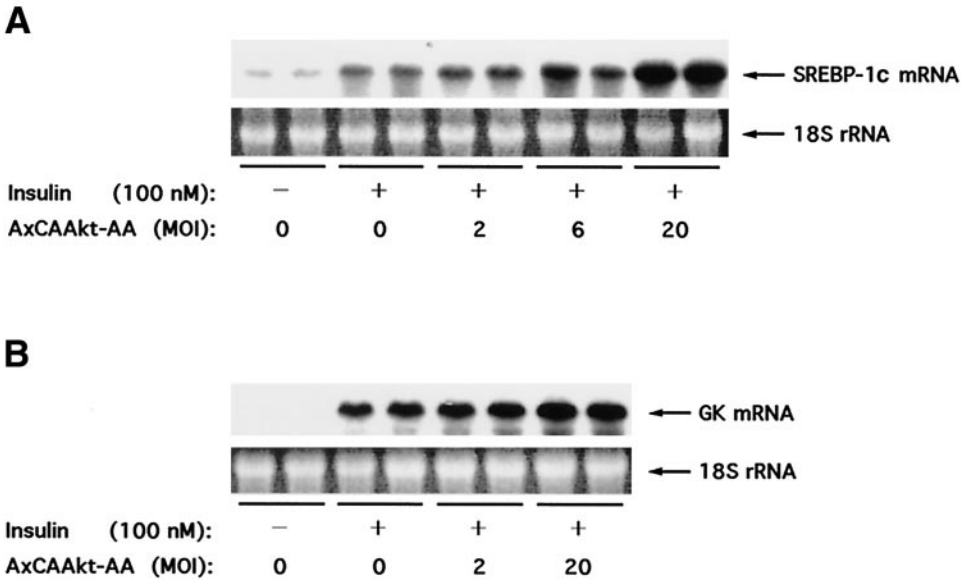

FIG. 5. Effects of Akt-AA on insulin-induced expression of SREBP-1c and GK genes. Hepatocytes infected with AxCAAkt-AA at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin, after which total RNA was extracted and subjected to Northern blot analysis of SREBP-1c mRNA (*A*) or GK mRNA (*B*). Data are shown for duplicate incubations and are representative of at least three independent experiments.

these proteins (Fig. 7*B* and *C*). However, the tyrosine phosphorylation of IRS-1 was inhibited more efficiently by IRS-2N than was that of IRS-2; whereas tyrosine phosphorylation of IRS-2 was not affected in cells infected with AxCAIRS-2N at an MOI of 1 PFU/cell, that of IRS-1 was markedly inhibited.

Infection of hepatocytes with AxCAIRS-2N at an MOI of 1 PFU/cell, a virus dose sufficient to inhibit insulin-induced tyrosine phosphorylation of IRS-1, did not inhibit insulin-induced phosphorylation of Akt (Fig. 8*A*). However, in cells infected with AxCAIRS-2N at an MOI of 10 or 30 PFUs/cell, the insulin-induced phosphorylation of Akt was inhibited in a dose-dependent manner. Insulin-induced phosphorylation of GSK3β was inhibited by the expression of IRS-2N (Fig. 8*B*), and the dose-response relation of inhibition was similar to that apparent for

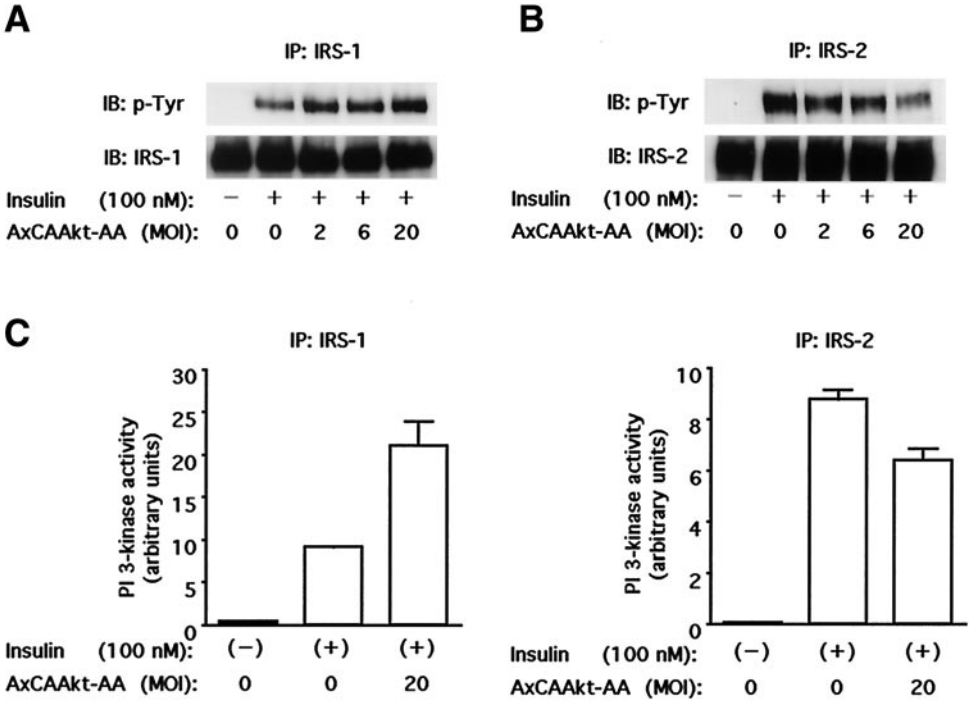

FIG. 6. Effects of Akt-AA on the insulin-induced tyrosine phosphorylation of, and association of PI 3-kinase activity with, IRS-1 and IRS-2. Hepatocytes infected with AxCAAkt-AA at the indicated MOI were assayed for insulin-induced tyrosine phosphorylation of IRS-1 (*A*) and IRS-2 (*B*) as well as for PI 3-kinase activity associated with these proteins (*C*), as described in the Fig. 2 legend. Data in *A* and *B* are representative of three independent experiments, and those in *C* are means ± SE of values from three independent experiments. IB, immunoblot analysis; IP, immunoprecipitation. p-Tyr, phospho-tyrosine

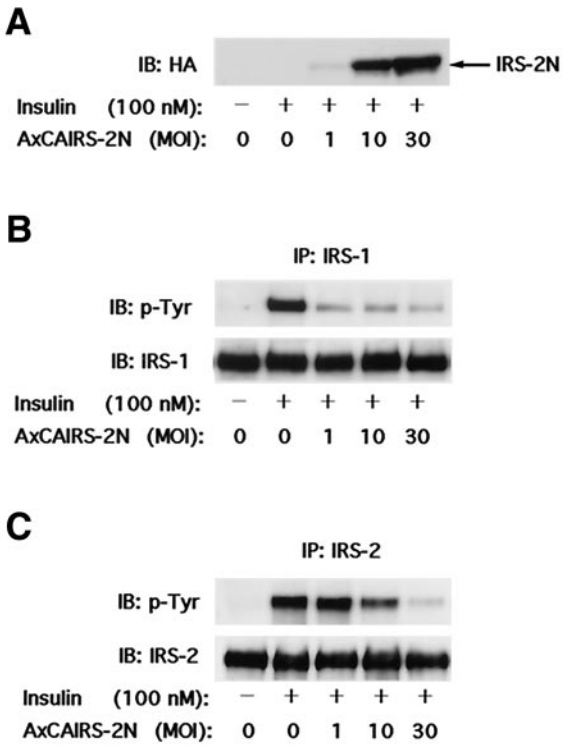

FIG. 7. Effects of an NH_2-terminal fragment of IRS-2 (IRS-2N) on insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2. Hepatocytes infected with AxCAIRS-2N at the indicated MOI were subjected to immunoblot analysis (IB) with antibodies to HA (*A*) or assayed for insulin-induced tyrosine phosphorylation of IRS-1 (*B*) and IRS-2 (*C*), as described in the Fig. 2 legend. All data are representative of at least three independent experiments. IP, immunoprecipitation.

insulin-induced phosphorylation of Akt and of IRS-2. The insulin-induced increases in the abundance of SREBP-1c and GK mRNAs were almost completely inhibited by AxCAIRS-2N at an MOI of 1 PFU/cell (Fig. 8*C* and *D*), a virus dose sufficient to inhibit tyrosine phosphorylation of IRS-1 but insufficient to inhibit that of IRS-2.

DISCUSSION

The NH_2-terminal portion of IRS is thought to participate in the interaction of IRS with the insulin receptor (27,28). We have now shown that an NH_2-terminal fragment of IRS-1 that contains the PH and PTB domains (IRS-1N) prevented the insulin-induced tyrosine phosphorylation of IRS-1, but not that of IRS-2, when expressed in primary cultured hepatocytes. An equivalent fragment of IRS-2 (IRS-2N) prevented the insulin-induced tyrosine phosphorylation of both IRS-1 and IRS-2, although that of IRS-1 was inhibited more efficiently than that of IRS-2. These results are consistent with the notion that the affinity of the NH_2-terminal portion of IRS-2 for the insulin receptor is higher than that of the equivalent portion of IRS-1. Sawka-Verhelle et al. (32) have identified a region of IRS-2 that has no counterpart in IRS-1. This region, termed the kinase regulatory loop binding (KRLB) domain, also binds directly to the insulin receptor (32). It is thus possible that, whereas IRS-1 binds to the insulin receptor solely through its NH_2-terminal portion, IRS-2 interacts with the receptor through both the NH_2-terminal portion and the KRLB domain. Although it remains unclear why IRS-1N slightly enhances the tyrosine phosphorylation of IRS-2 induced by insulin, overexpression of the KRLB domain inhibited the binding of IRS-2 to the insulin receptor and, concomitantly, enhanced that of IRS-1 (33). IRS-1 and IRS-2 might therefore compete with each other for binding to the insulin receptor; therefore, a decrease in the binding of one of these proteins to the receptor may result in a reciprocal increase in the binding of the other.

We have also shown that the insulin-induced increases

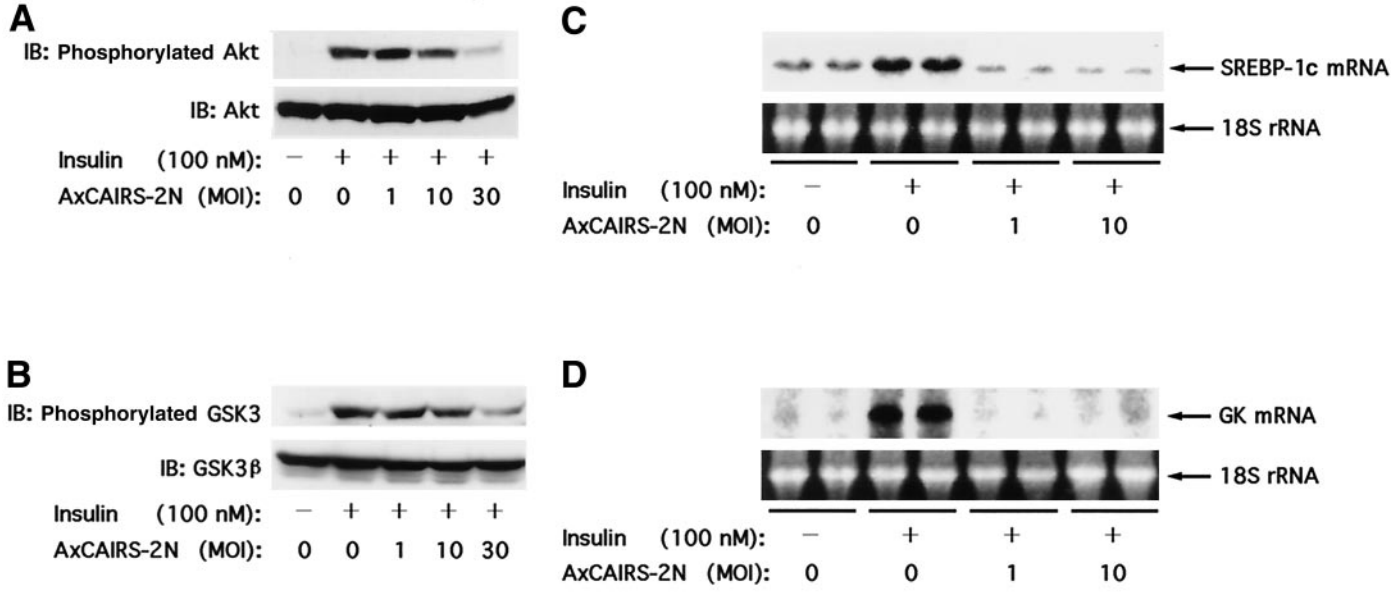

FIG. 8. Effects of IRS-2N on the phosphorylation of Akt and GSK3β as well as on the expression of SREBP-1c and GK genes induced by insulin. *A* and *B*: Hepatocytes infected with AxCAIRS-2N at the indicated MOI were incubated in the absence or presence of 100 nmol/l insulin for 10 min and then lysed. Cell lysates were subjected to immunoblot analysis (IB) with antibodies to phosphorylated Akt or Akt2 (*A*) or with those specific for phosphorylated GSK3 or GSK3β (*B*). *C* and *D*: Hepatocytes infected with AxCAIRS-2N at the indicated MOI were incubated in the absence or presence of insulin, after which total RNA was isolated and subjected to Northern blot analysis of SREBP-1c mRNA (*C*) or GK mRNA (*D*). All data are representative of at least three independent experiments.

in the abundance of SREBP-1c and GK mRNAs, both of which are mediated by PI 3-kinase, were blocked in cells in which the insulin-induced tyrosine phosphorylation of IRS-1 (but not that of IRS-2) was inhibited by expression of either IRS-1N or a small amount of IRS-2N. These results suggest that the IRS-1–PI 3-kinase pathway is essential for insulin-induced expression of the SREBP-1c and GK genes, whereas the IRS-2–PI 3-kinase pathway alone is not sufficient to mediate these effects of insulin. In contrast, the insulin-induced phosphorylation of Akt was not affected by IRS-1N, indicating that IRS-1 is responsible for some, but not all, of the PI 3-kinase–dependent actions of insulin in hepatocytes. Recent studies have revealed that defects in IRS-1– or IRS-2–dependent signaling result in distinct effects on cellular functions. For example, ablation of the IRS-1 gene in immortalized brown preadipocytes or embryonic fibroblasts markedly impaired the ability of these cells to acquire adipocyte characteristics, whereas a deficiency of IRS-2 in these cells had little effect on this ability (12,13). In contrast, the attenuation of insulin-stimulated glucose uptake apparent in IRS-2–deficient brown adipocytes was partly reversed by expression of IRS-2 but not by that of IRS-1 (11). These data, together with our present results, suggest that, despite their structural and biochemical similarities, IRS-1 and IRS-2 are not completely redundant, but rather are selectively linked to specific actions of insulin. The mechanisms by which each IRS protein contributes differentially to various actions of insulin in the same cells remain to be determined. The relative abundance and subcellular localizations of IRS-1 and IRS-2 as well as their interactions with specific molecules and the time courses of their upregulation or downregulation may be important determinants of their distinct contributions to insulin actions.

Shimomura et al. (34) showed that the expression of IRS-2 and the phosphorylation of Akt are markedly reduced, whereas the expression of SREBP-1c is increased in the liver of lipodystrophic or leptin-deficient (*ob*/*ob*) mice, both of which exhibit whole-body insulin resistance and chronic hyperinsulinemia. These investigators concluded that insulin resistance (impaired activation of Akt) and hypersensitivity to insulin (increased expression of SREBP-1c) exist concomitantly in the liver of these mice. This apparently paradoxical phenomenon may be explained if the IRS-1–PI 3-kinase and IRS-2–PI 3-kinase pathways diverge in hepatocytes, with the former pathway being more closely linked to regulation of the SREBP-1c gene, as demonstrated in the present study.

Expression of a dominant-negative mutant of Akt (Akt-AA) enhanced the insulin-induced tyrosine phosphorylation of IRS-1 in hepatocytes. Evidence suggests that PI 3-kinase–Akt signaling contributes to serine phosphorylation of IRS-1 and, in turn, attenuates tyrosine phosphorylation of IRS-1 (31). Expression of Akt-AA thus likely disrupted an Akt-mediated negative feedback pathway leading to IRS-1, resulting in upregulation of tyrosine phosphorylation of IRS-1. Given that Akt-AA did not augment IRS-2–PI 3-kinase signaling, Akt-mediated negative feedback does not appear to be directed at IRS-2.

Insulin induced the expression of SREBP-1c and GK genes in cells expressing Akt-AA. Moreover, the effects of insulin on the expression of these genes were actually enhanced in these cells. These results are also consistent with the observation that the expression of SREBP-1c was increased, whereas the insulin-induced activation of Akt was inhibited in the liver of insulin-resistant mice (34). Although expression of an active form of Akt has been shown to increase the abundance of SREBP-1c mRNA in hepatocytes (18), it is likely that Akt signaling, at least that mediated through Akt2, is not required for the insulin-induced expression of SREBP-1c and GK genes. The enhanced tyrosine phosphorylation of IRS-1, but not of IRS-2, as well as the increased expression of SREBP-1c and GK genes induced by insulin in Akt-AA–expressing cells, also support our hypothesis that the IRS-1–PI 3-kinase pathway plays a major role in mediating the insulin-induced expression of these genes.

Although IRS-1N did not prevent insulin-induced phosphorylation of Akt, expression of IRS-2N at a level sufficient to inhibit insulin-induced tyrosine phosphorylation of both IRS-1 and IRS-2 also blocked insulin-induced phosphorylation of Akt. One possible explanation for these results is that the IRS-2–PI 3-kinase pathway, but not the IRS-1–PI 3-kinase pathway, is responsible for insulin-induced phosphorylation of Akt. This notion is consistent with the observation that long-term exposure of hepatocytes to insulin results in marked decreases in both the amount of IRS-2 and the extent of insulin-induced phosphorylation of Akt, with no effect on the amount of IRS-1 (34). Another possibility is that the insulin-induced phosphorylation of Akt is mediated through both IRS-2– and IRS-1–dependent pathways and that the inhibition of only one pathway is not sufficient to prevent this effect. Regardless, the IRS-2–PI 3-kinase pathway appears to participate in signaling leading to Akt in hepatocytes. Given that the insulin-induced phosphorylation of Akt was shown to be reduced in brown preadipocytes derived from IRS-1–deficient mice, whereas IRS-2–associated PI 3-kinase activity was increased in these cells (35), the relative contributions of IRS-1 and IRS-2 to the activation of Akt may thus differ in different cell types.

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REFERENCES

1. Kahn CR: Diabetes: causes of insulin resistance. *Nature* 373:384–385, 1995
2. White MF: The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182:3–11, 1998
3. Ogawa W, Matozaki T, Kasuga M: Role of binding proteins to IRS-1 in insulin signalling. *Mol Cell Biochem* 182:13–22, 1998
4. Sun XJ, Pons S, Wang LM, Zhang Y, Yenush L, Burks D, Myers MG Jr, Glasheen E, Copeland NG, Jenkins NA, Pierce JH, White MF: The IRS-2 gene on murine chromosome 8 encodes a unique signaling adapter for insulin and cytokine action. *Mol Endocrinol* 11:251–262, 1997
5. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B III, Johnson RS, Kahn CR:

Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186–190, 1994
6. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, Skekihara H, Yoshioka S, Horikoshi H, Furuta Y, Ikawa Y, Kasuga M, Yazaki Y, Aizawa S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182–186, 1994
7. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900–904, 1998
8. Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T: Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes* 49:1880–1889, 2000
9. Previs SF, Withers DJ, Ren JM, White MF, Shulman GI: Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *J Biol Chem* 275:38990–38994, 2000
10. Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P, Accili D: Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *J Biol Chem* 273:17491–17497, 1998
11. Fasshauer M, Klein J, Ueki K, Kriauciunas KM, Benito M, White MF, Kahn CR: Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. *J Biol Chem* 275:25494–25501, 2000
12. Fasshauer M, Klein J, Kriauciunas KM, Ueki K, Benito M, Kahn CR: Essential role of insulin receptor substrate 1 in differentiation of brown adipocytes. *Mol Cell Biol* 21:319–329, 2001
13. Miki H, Yamauchi T, Suzuki R, Komeda K, Tsuchida A, Kubota N, Terauchi Y, Kamon J, Kaburagi Y, Matsui J, Akanuma Y, Nagai R, Kimura S, Tobe K, Kadowaki T: Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation. *Mol Cell Biol* 21:2521–2532, 2001
14. Brown MS, Goldstein JL: The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331–340, 1997
15. Foretz M, Guichard C, Ferre P, Foufelle F: Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc Natl Acad Sci U S A* 96:12737–12742, 1999
16. Chakravarty K, Leahy P, Becard D, Hakimi P, Foretz M, Ferre P, Foufelle F, Hanson RW: Sterol regulatory element-binding protein-1c mimics the negative effect of insulin on phosphoenolpyruvate carboxykinase (GTP) gene transcription. *J Biol Chem* 276:34816–34823, 2001
17. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL: Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:13656–13661, 1999
18. Fleischmann M, Iynedjian PB: Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/cAkt. *Biochem J* 349:13–17, 2000
19. Azzout-Marniche D, Becard D, Guichard C, Foretz M, Ferre P, Foufelle F: Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes. *Biochem J* 350:389–393, 2000
20. Sakaue H, Ogawa W, Takata M, Kuroda S, Kotani K, Matsumoto M, Sakaue M, Nishio S, Ueno H, Kasuga M: Phosphoinositide 3-kinase is required for insulin-induced but not for growth hormone- or hyperosmolarity-induced glucose uptake in 3T3–L1 adipocytes. *Mol Endocrinol* 11:1552–1562, 1997
21. Kitamura T, Kitamura Y, Kuroda S, Hino Y, Ando M, Kotani K, Konishi H, Matsuzaki H, Kikkawa U, Ogawa W, Kasuga M: Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. *Mol Cell Biol* 19:6286–6296, 1999
22. Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U, Kasuga M: Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* 18:3708–3717, 1998
23. Berry MN, Friend DS: High yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* 43:506–520, 1969
24. Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL: Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* 98:1575–1584, 1996
25. Matsuda T, Noguchi T, Yamada K, Takenaka M, Tanaka T: Regulation of the gene expression of glucokinase and L-type pyruvate kinase in primary cultures of rat hepatocytes by hormones and carbohydrates. *J Biochem (Tokyo)* 108:778–784, 1990
26. Matsumoto M, Ogawa W, Hino Y, Furukawa K, Ono Y, Takahashi M, Ohba M, Kuroki T, Kasuga M: Inhibition of insulin-induced activation of Akt by a kinase-deficient mutant of the ϵ isozyme of protein kinase C. *J Biol Chem* 276:14400–14406, 2001
27. Eck MJ, Dhe-Paganon S, Trub T, Nolte RT, Shoelson SE: Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell* 85:695–705, 1996
28. Myers MG Jr, Grammer TC, Brooks J, Glasheen EM, Wang LM, Sun XJ, Blenis J, Pierce JH, White MF: The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. *J Biol Chem* 270:11715–11718, 1995
29. Takata M, Ogawa W, Kitamura T, Hino Y, Kuroda S, Kotani K, Klip A, Gingras AC, Sonenberg N, Kasuga M: Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). *J Biol Chem* 274:20611–20618, 1999
30. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–789, 1995
31. Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB: A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc Natl Acad Sci U S A* 98:4640–4645, 2001
32. Sawka-Verhelle D, Tartare-Deckert S, White MF, Van Obberghen E: Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified domain comprising amino acids 591–786. *J Biol Chem* 271:5980–5983, 1996
33. Oriente F, Formisano P, Miele C, Fiory F, Maitan MA, Vigliotta G, Trencia A, Santopietro S, Caruso M, Condorelli G, Van Obberghen E, Beguinot F: Insulin receptor substrate (IRS)-2 phosphorylation is necessary for PKCζ activation by insulin in L6 cells. *J Biol Chem* 276:37109–37119, 2001
34. Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL: Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol Cell* 6:77–86, 2000
35. Valverde AM, Kahn CR, Benito M: Insulin signaling in insulin receptor substrate (IRS)-1-deficient brown adipocytes: requirement of IRS-1 for lipid synthesis. *Diabetes* 48:2122–2131, 1999