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 NH₂-terminal fragment of IRS-1 that contains the pleckstrin homology and phosphotyrosine binding domains (insulin receptor substrate-1 NH₂-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

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 hepatocytes.

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FBS, fetal bovine serum; GK, glucokinase; GSK, glycogen synthase kinase; HA, hemagglutinin epitope; IRS, insulin receptor substrate; IRS-1N, IRS-1 NH₂-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.
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 NH2-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.

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. 1A). Infection of the cells with an adenovirus vector encoding a dominant-negative mutant of PI 3-kinase (AxCA Δp85) 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 AxCA Δp85 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. 1B), this effect was inhibited in a multiplicity of infection (MOI)-dependent manner in the cells infected with AxCA Δp85 (Fig. 1B). 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 AxCA Δp85 (Fig. 1C). Infection of hepatocytes with AxCA Myr-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. 1B 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 NH2-terminal fragment of IRS-1 on insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2. The NH2-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 NH2-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. 2A). The expression of IRS-1N did not affect the abundance of IRS-1 and IRS-2 in the cells (Fig. 2B 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.
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. 2D). 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. 3A); 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. 2D). The insulin-induced increase in the amount of GK mRNA was also similarly inhibited by IRS-1N in a dose-dependent manner (Fig. 3B). Infection of the cells with a control adenovirus encoding...
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. 3C). 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
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. 4A), 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. 4B). The insulin-induced phosphorylation of GSK3β, which is a direct substrate of Akt (30), was also inhibited by Akt-AA (Fig. 4C). 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. 6A 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. 6C).

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 NH₂-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 NH₂-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. 7A). 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
these proteins (Fig. 7B and C). However, the tyrosine phosphorylation of IRS-1 was inhibited more efficiently by IRS-2N than 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. 8A). 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. 8B), and the dose-response relation of inhibition was similar to that apparent for...
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. 8C and D), a virus dose sufficient to inhibit tyrosine phosphorylation of IRS-1 but insufficient to inhibit that of IRS-2.

**DISCUSSION**

The NH2-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 NH2-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 NH2-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 NH2-terminal portion, IRS-2 interacts with the receptor through both the NH2-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
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
5. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B III, Johnson RS, Kahn CR:


Shimomura I, Matsuda T, Hamre RR, 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