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# Evidence That IRS-2 Phosphorylation Is Required for Insulin Action in Hepatocytes*

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**Insulin receptor substrates (IRSs) are tyrosine-phosphorylated following stimulation with insulin, insulin-like growth factors (IGFs), and interleukins. A key question is whether different IRSs play different roles to mediate insulin's metabolic and growth-promoting effects. In a novel system of insulin receptor-deficient hepatocytes, insulin fails to (i) stimulate glucose phosphorylation, (ii) enhance glycogen synthesis, (iii) suppress glucose production, and (iv) promote mitogenesis. However, insulin's ability to induce IRS-1 and gab-1 phosphorylation and binding to phosphatidylinositol (PI) 3-kinase is unaffected, by virtue of the compensatory actions of IGF-1 receptors. In contrast, phosphorylation of IRS-2 and generation of IRS-2/PI 3-kinase complexes are markedly reduced. Thus, absence of insulin receptors selectively reduces IRS-2, but not IRS-1 phosphorylation, and the impairment of IRS-2 activation is associated with lack of insulin effects. To address whether phosphorylation of additional IRSs is also affected, we analyzed phosphotyrosine-containing proteins in PI 3-kinase immunoprecipitates from insulin-treated cells. However, these experiments indicate that IRS-1 and IRS-2 are the main PI 3-kinase-bound proteins in hepatocytes. These data identify IRS-2 as the main effector of both the metabolic and growth-promoting actions of insulin through PI 3-kinase in hepatocytes, and IRS-1 as the main substrate mediating the mitogenic actions of IGF-1 receptors.**

Insulin, IGF-1,[1] and IGF-2, acting through insulin and IGF-1 receptors, promote a wide range of metabolic and growth-promoting functions in typical insulin target cells, such as liver, muscle, and fat, and to a lesser extent in other tissues. The mechanism by which insulin regulates energy metabolism and promotes cell growth has been extensively studied. In recent years, a consensus has emerged that phosphorylation of IRS molecules by the insulin receptor kinase is important for insulin action (1). IRS molecules engage in the formation of signaling complexes with numerous adapter molecules and enzymes via their pY-X-X-M motifs (2–6). Thus, the IRS signaling system provides an elegant explanation for the diversity of insulin signaling (7). Nevertheless, the role of different IRSs in insulin signaling, as well as the role of the numerous additional substrates of the insulin receptor kinase that are distinct from IRS has remained elusive.

Progress in this area has been hampered by the lack of suitable *in vitro* systems in which phosphorylation of individual molecules can be correlated with specific biologic functions. In fact, insulin-responsive cell lines such as 3T3-L1 adipocytes or L6 myoblasts possess an endogenous complement of signaling molecules, so that the effects of individual components can be addressed only by way of overexpression or inhibition experiments. It is significant that much progress in our understanding of the IRS system has derived from studies of the myeloid cell line 32D, which carries a functional knock-out of these molecules (8–10). However, 32D cells may not be representative of classic target tissues of insulin action.

Targeted mutagenesis of genes of the insulin and IGF signaling system in mice has provided clues as to the functional differences among related molecules (11, 12). We and others, for example, have shown that insulin receptors are indeed the master switch of the insulin signaling pathway (13, 14), and that IGF-1 receptors contribute little to metabolic regulation (15–17). Likewise, the phenotype of mice with a genetic ablation of IRS-1 has suggested that IRS-1 plays a more important role in mediating growth than metabolic responses (18, 19). Interestingly, however, combined heterozygosity for an insulin receptor and an IRS-1 null allele triggers synergistic interactions to impair insulin action and causes insulin-resistant diabetes in mice, suggesting that IRS-1 can also affect metabolism (20). On the other hand, mice lacking IRS-2 develop lethal diabetic ketoacidosis as a result of combined insulin resistance and insulin deficiency, indicating that IRS-2 plays a crucial role in the development of mechanisms regulating fuel homeostasis (21).

The derivation of cell lines from mice with targeted mutations provides an important tool to dissect the function of these molecules *in vitro*. For example, Bruning *et al.* (22) have been able to show that IRS-1 and IRS-2 are not functionally interchangeable in mediating various growth-promoting functions of IGF-1 in fibroblasts of IRS-1-deficient mice.

In this study, we have analyzed insulin action in permanent cultures of hepatocytes from mice lacking insulin receptors. We generated these cells using a well established procedure entailing transformation with a temperature-sensitive mutant SV40 virus (23). We asked whether, in the absence of insulin receptors, IGF-1 receptors could mediate the typical metabolic and growth-promoting responses of insulin in hepatocytes. We re-

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[1] The abbreviations used are: IGF, insulin-like growth factor; IRS, insulin receptor substrate; WT, wild type; PI, phosphatidylinositol.

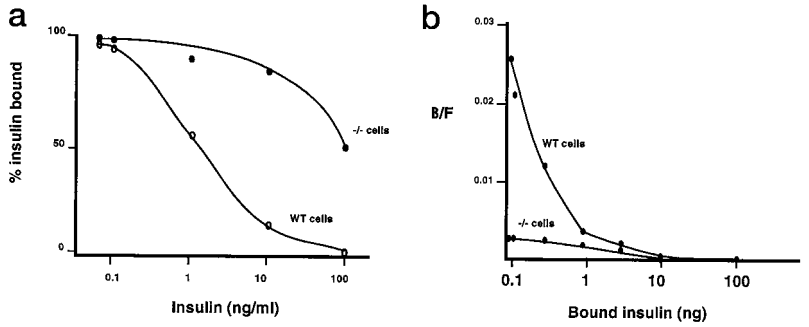


FIG. 1. **$^{125}$I-Insulin binding competition curves for WT and −/− cells.** *a*, binding competition curves. Monolayers were incubated with tracer amounts of $^{125}$I-insulin (20,000 cpm/well) in the presence of increasing amounts of unlabeled hormone. Percent binding in WT cells is ~10%, and in −/− cells ~1%. *b*, Scatchard analysis of binding data. The data presented in *a* were extrapolated according to Scatchard. A typical curvilinear plot is observed in WT cells, whereas a linear plot consistent with binding of insulin to IGF-1 receptors is seen in −/− cells.

port that insulin, acting through the IGF-1 receptor, mediates IRS-1, but not IRS-2 phosphorylation, and that the failure to phosphorylate IRS-2 correlates with the inability of these cells to mediate insulin's characteristic actions. Phosphorylation of gab-1, and association of other phosphotyrosine-containing proteins with the p85 subunit of PI 3-kinase, were similar in normal and insulin receptor-deficient cells. These findings correlate IRS-2 phosphorylation with both the metabolic and growth-promoting actions of insulin, and suggest that individual IRS molecules play a more specific role in signal transduction than previously recognized.

## MATERIALS AND METHODS

*Derivation of Cell Lines*—Livers of transgenic mice lacking insulin receptors and normal controls were isolated between embryonic day 18.5 and postnatal day 1. The livers were digested with collagenase (Worthington), and the resulting cells were plated in 6-cm culture dishes. Transformation with a temperature-sensitive mutant SV40 was performed as described previously (23). Multiple clones were isolated and characterized. To confirm the hepatocyte lineage, albumin secretion in the medium was measured using an enzyme-linked immunosorbent assay. The experiments described in this study were performed on two independent clones of −/− cells, chosen based on high levels of albumin secretion. $^{125}$I-insulin and $^{125}$I-IGF-1 binding were performed according to standard techniques (13). Cells were maintained in α-minimal essential medium supplemented with 1 mM L-glutamine, 200 nM dexamethasone, and 4% fetal calf serum at 33 °C. Preliminary experiments were carried out at both the permissive temperature for viral replication (33 °C) and the nonpermissive temperature (40 °C). However, none of the parameters analyzed in this study was affected by the different temperature (24).

*Immunoprecipitation and Immunoblotting*—Serum-deprived cultures of WT and −/− cells (~80% confluent) were stimulated with insulin at various concentrations for 5 min. Cells were lysed in detergent buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton, phosphatase and protease inhibitors. The lysates were immunoprecipitated with the appropriate antibodies as described. *In vitro* phosphorylation of the glycoprotein fractions isolated from both cell types were carried out as described elsewhere (13). Anti-phosphotyrosine antibodies were purchased from Transduction Laboratories (Lexington, KY), insulin receptor and IGF-1 receptor antibodies from Calbiochem, and anti-IRS-1, anti-IRS-2, anti-Gab-1, and anti-p85 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

*Glucokinase Activity*—Cells were cultured in serum-free medium overnight and incubated in the absence or presence of 100 nM insulin for 3 h. Thereafter, cells were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 0.1 M KCl, 1 mM EDTA, and 2.5 mM β-mercaptoethanol. Following centrifugation at 15,000 rpm for 15 min, the post-mitochondrial supernatant was centrifuged at 180,000 × *g* for 1 h to obtain the cytosolic fraction. Glucokinase activity was measured by a spectrophotometric assay, in the presence of 45 mM Tris-HCl, pH 7.4, 110 mM KCl, 8 mM $MgCl_2$, 0.5 mM NADP, 0.9 unit/ml glucose-6-phosphate dehydrogenase, 100 mM glucose, and ATP (0 or 5 mM). Glucokinase activity was calculated as the ATP-dependent rate of NADPH formation, based on a stoichiometry of 2 mol of NADPH formed per mol of glucose phosphorylated (25).

*Hepatic Glucose Production*—Cells were cultured overnight in α-minimal essential medium supplemented with 0.25% bovine serum albumin, and incubated in glucose- and serum-free α-minimal essential medium supplemented with 16 mM lactate and 4 mM pyruvate in the presence or absence of 100 nM insulin. Aliquots of medium were removed at the indicated time points, and the glucose concentration was measured with a glucose analyzer (Beckman) (26).

*Glycogen Content*—SV40-transformed cells were cultured in serum-free medium as indicated above in the presence or absence of 100 nM insulin. Thereafter, cells were homogenized in 0.6 N $HClO_4$ and centrifuged, and aliquots of the supernatant were incubated with amyloglucosidase in acetate buffer as described previously (27). Released glucose was measured with a glucose analyzer.

*Glycogen Synthase Activity*—Cells were incubated with glucose-free medium supplemented with dialyzed fetal calf serum for 3 h. Insulin was added for 30 min at 37 °C. The reaction was stopped by freezing cells in liquid nitrogen. The reaction was performed as described in buffer containing 40 mM Tris-HCl, 25 mM NaF, 20 mM EDTA, 10 mg/ml glycogen, 7.2 mM UDP-glucose disodium salt, with and without 6.7 mM glucose 6-phosphate, and 0.05 μCi/60 μl [$^{14}$C]UDPG for 25 min at 37 °C (28). The incorporation of [$^{14}$C]UDPG was determined in a liquid scintillation counter. The ratio of glucose 6-phosphate-independent (I) glycogen synthase activity was calculated as shown: % I = I-form/(I- + D-form) × 100.

*DNA Synthesis*—Hepatocytes were grown to near confluence in regular medium, incubated overnight in insulin-free medium, and placed in medium containing increasing concentrations of insulin ($10^{-10}$ to $10^{-7}$ M) or buffer alone for 16 h. Thereafter, the medium was replaced by the same medium supplemented with HEPES (25 mM, pH 7.4) and [$^3$H]thymidine (500 Ci/ml, NEN Life Science Products) for 1 h. Cells were washed three times, solubilized, and precipitated with 20% trichloroacetic acid. Radioactivity was measured in a liquid scintillation counter (28).

*Growth Curves*—Hepatocytes were plated at concentrations of 5 to 50 × $10^4$ cells/ml and allowed to attach to the plates in complete medium. Thereafter, they were cultured for 96 h in regular medium or 1% bovine serum albumin with or without varying concentrations of insulin, IGF-1, or IGF-2. At the end of the incubation, medium containing 2.5% neutral red (Sigma) was added for 2 h, and the absorbance was measured at 570 nm. Cell growth was expressed as percentage of the absorbance values. Basal values are represented by the growth of cells incubated in 1% bovine serum albumin and maximal values by the growth observed in cells incubated with complete medium.

## RESULTS

*Protein Phosphorylation in Insulin Receptor-deficient Hepatocytes*—Initially, we analyzed $^{125}$I-insulin binding to cells derived from insulin receptor-deficient mice (hereafter referred to as −/− cells). Binding competition curves (Fig. 1*a*) and Scatchard analysis (Fig. 1*b*) are consistent with the absence of high affinity insulin binding sites in −/− cells. The residual $^{125}$I-insulin binding ($ID_{50}$ = 100 nM) detected in −/− cells can be attributed to the presence of IGF-1 receptors, as we have previously shown (17). Indeed, the linear Scatchard plot of −/− cells is typical of binding to IGF-1 receptors (Fig. 1*b*). From the intercept of the slope on the horizontal axis of the Scatchard plot, we estimate that WT cells express ~$10^5$ insulin receptors/cell. Previously, we have shown that the levels of expression of IGF-1 receptors are similar in WT and −/− cells (~$10^5$ IGF-1 receptors/cell).

We next examined insulin-induced receptor phosphorylation. Insulin stimulated tyrosine phosphorylation of its receptor in WT cells, but not in −/− cells (Fig. 2*a*). On the contrary, phosphorylation of IGF-1 receptors was similar in both cell

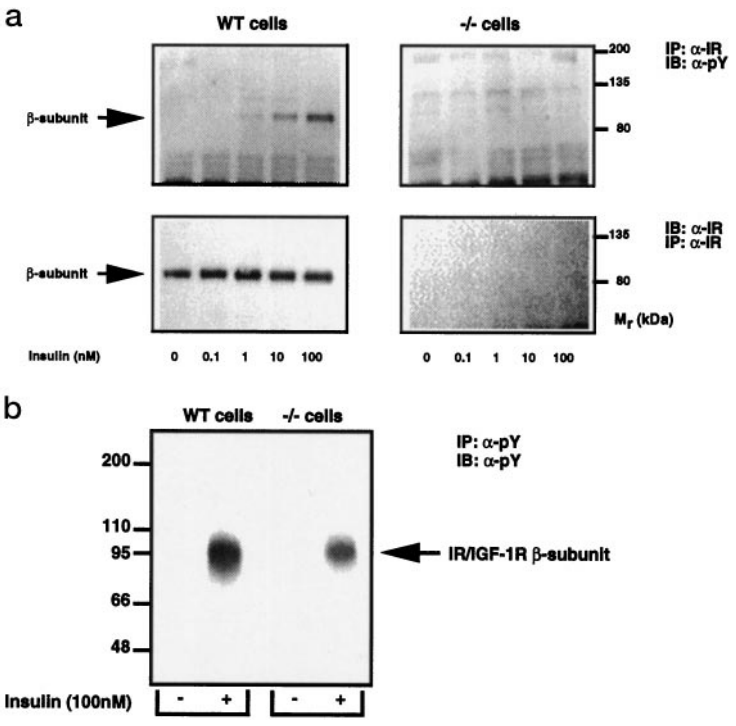


FIG. 2. **Insulin-induced protein phosphorylation.** *a*, Insulin receptors were immunoprecipitated from total cellular lysates of basal or insulin-treated cells, and tyrosine phosphorylation was measured by immunoblotting with anti-phosphotyrosine antibodies. The blots were subsequently stripped and reprobed with anti-insulin receptor antibody (*lower panel*), to normalize phosphotyrosine content to the amount of immunoprecipitable insulin receptors. *b*, insulin-stimulated tyrosine phosphorylation in wheat germ extracts of WT and −/− cells. Glycoproteins were purified from WT and −/− cells using wheat germ agglutinin affinity chromatography, and subjected to *in vitro* phosphorylation with γ-[$^{32}$P]ATP. Following immunoprecipitation with anti-phosphotyrosine antibodies, the immune complexes were resolved on SDS-polyacrylamide gel electrophoresis and phosphotyrosine-containing proteins were detected by autoradiography. The total amount of phosphotyrosine incorporated into the 95-kDa β subunit of insulin and IGF-1 receptors was calculated by scanning densitometry of the autoradiogram (not shown), and is 2-fold higher in WT cells compared with −/− cells.

types, as we have demonstrated in a previous publication (17). To compare the ability of insulin to stimulate tyrosine phosphorylation of the IGF-1 receptor *vis à vis* the insulin receptor, we isolated the glycoprotein fraction from Triton X-100 extracts of WT and −/− cells and subjected it to *in vitro* phosphorylation with γ-[$^{32}$P]ATP in the presence of 100 nM insulin, followed by immunoprecipitation with anti-phosphotyrosine antibodies. The total amount of phosphotyrosine recovered by immunoprecipitation in WT cells extracts was twice the amount recovered in −/− cells (Fig. 2*b*), consistent with the fact that the total number of insulin plus IGF-1 receptors in WT cells is double that of −/− cells. The conclusion of this experiment is that insulin at high doses is equipotent in stimulating phosphate incorporation into insulin and IGF-1 receptors.

*IRS-1 and -2 Phosphorylation and PI 3-Kinase Co-precipitation*—Next, we investigated the ability of insulin to activate intracellular signaling pathways in both cell types. Insulin stimulated tyrosine phosphorylation of IRS-1 in WT and −/− cells to a similar extent. In contrast, tyrosine phosphorylation of IRS-2 was severely blunted in −/− cells compared with WT cells (Fig. 3). Upon tyrosine phosphorylation, IRS molecules bind the regulatory (p85) subunit of PI 3-kinase, leading to increased PI 3-kinase activity. Insulin treatment led to a 3.4-fold increase in the amount of p85 co-precipitated with IRS-1 in WT cells, and to a 5.3-fold increase in −/− cells (Fig. 4*b*). In contrast, the amount of p85 detected in anti-IRS-2 immunoprecipitates was increased 3.3-fold in WT cells, and 1.3-fold in −/− cells (Fig. 4*c*). Thus, insulin can fully activate IRS-1 through IGF-1 receptors, whereas activation of IRS-2 is substantially reduced. The decreased co-precipitation of p85 with IRS-2 suggests that IRS-2-associated PI 3-kinase activity is reduced in −/− cells. In fact, it has been shown that activation of PI 3-kinase by insulin requires binding of tyrosine phosphorylated IRSs to the SH2 domains of p85 (29–31). Thus, this experiment provides insight into activation of PI 3-kinase in response to insulin.

*Investigation of Additional Insulin Receptor Substrates That Associate with p85*—We then addressed whether activation of other molecules important for insulin action is also blunted in −/− cells. Gab-1 is a member of the IRS family, phosphorylation of which is stimulated by both insulin and IGF-1 (6). Tyrosine phosphorylation of gab-1 in insulin-treated WT and −/− cells was similar (data not shown). Two additional members of the IRS family have been described: IRS-3 and IRS-4 (4, 5). Of these, IRS-3 is known to be expressed in liver (32), while no information is available on liver expression of IRS-4 (5). Since we did not have specific antibodies against these two

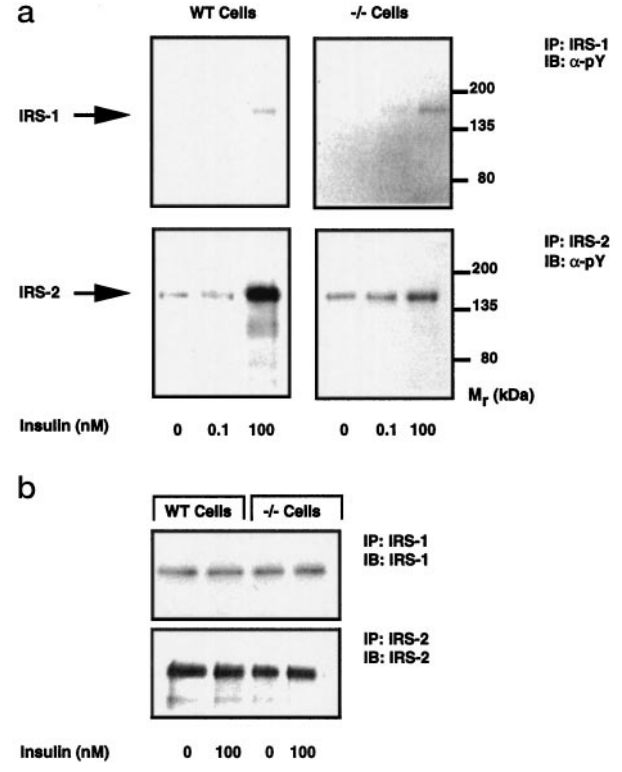


FIG. 3. **Phosphorylation of IRS-1 and IRS-2 in response to insulin.** Lysates from basal and insulin-treated cells were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and blotted with either anti-phosphotyrosine antibody (*a*) or with IRS-1 and IRS-2 antibodies (*b*). A representative experiment is shown.

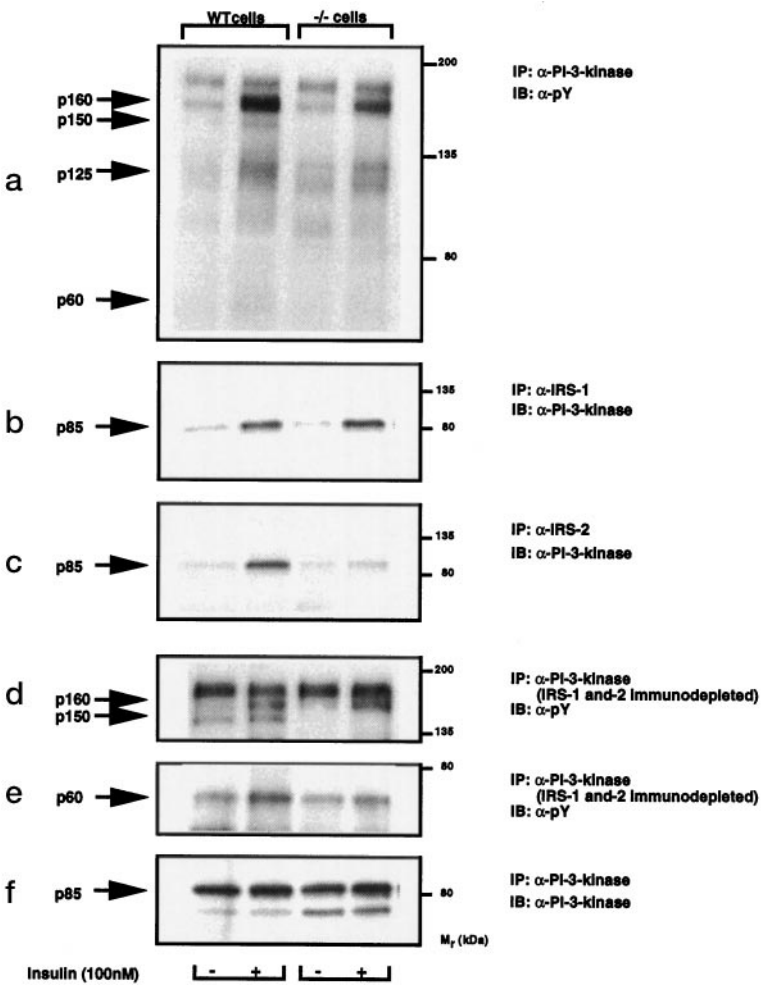


FIG. 4. **Pattern of tyrosine-phosphorylated proteins associated with the PI 3-kinase p85 subunit.** *a*, cell lysates from WT and −/− were immunoprecipitated with p85 antibodies and blotted with anti-phosphotyrosine antibodies. The major bands are identified with *arrowheads*. The p60 band is barely visible on this blot, but could be visualized more readily following immunodepletion of the extracts with IRS-1 and IRS-2 antibodies, as shown in *panel e*. *b* and *c*, cell extracts were immunoprecipitated sequentially with IRS-1 (*b*) and IRS-2 (*c*) antibodies, and blotted with p85 antibody. *d* and *e*, following immunodepletion of IRS-1 and IRS-2 with the relevant antibodies, extracts from WT and −/− cells were immunoprecipitated with anti-p85 antibody and immunoblotted with anti-phosphotyrosine antibody. Two different gels are shown to better visualize the regions of the gel corresponding to 160 kDa (*d*) and 60 kDa (*e*). *f*, as a control for the efficiency of the various immunoprecipitations, extracts were immunoprecipitated and immunoblotted with anti-p85 antibody.

IRSs, we asked whether we could detect them in co-immunoprecipitation assays with anti-p85 antibodies followed by anti-phosphotyrosine immunoblots. In both cell types, the main p85-bound phosphoproteins following insulin treatment are represented by two broad bands at 160–180 kDa and 125 kDa (Fig. 4*a*). Detailed analysis of these patterns leads us to conclude that, among the major p85-associated proteins, only IRS-2 co-immunoprecipitation is significantly impaired in −/− cells compared with WT cells: (i) the p160–180 band can be almost entirely immunodepleted by sequential immunoprecipitation with IRS-1 and IRS-2 antibodies (compare the intensity of the p160 band in Fig. 4, *a* and *d*, with the intensity of the non-insulin-stimulated band present at the top of each gel). A residual p160 band can be detected in extracts immunodepleted of IRS-1 and IRS-2 (Fig. 4*d*). This 160-kDa protein migrates slightly more slowly than IRS-1 and IRS-2, and does not cross-react with either antibody on Western blots. It may represent IRS-4, or a novel member of the IRS family. Co-precipitation of this band is similar in both cell types. A minor band of molecular mass of 150 kDa is also present in WT cells, and completely absent in −/− cells. Co-precipitation of this tyrosine-phosphorylated protein is modestly affected by insulin in WT cells. (ii) Co-precipitation of the p125 band is only slightly decreased in −/− cells (Fig. 4*a*). This band probably contains multiple molecular species, including gab-1. As stated above, phosphorylation of gab-1 is similar in both cell types. (iii) Following immunodepletion of IRS-1 and IRS-2, we observed a 60-kDa band, co-precipitation of which is modestly affected by insulin in WT cells (~2-fold), but not in −/− cells (Fig. 4*e*). It is highly likely that p60 represents IRS-3 (4, 32, 33).

In summary, even though there are multiple differences in the patterns of tyrosine-phosphorylated proteins associated with p85, the most abundant proteins are IRS-1 and IRS-2. Thus, they probably account for most of the PI 3-kinase activity elicited by insulin in these cells. Therefore, IRS-2 is the principle PI 3-kinase-bound protein, phosphorylation of which is significantly impaired in −/− cells.

*Effects of Insulin on Glucose Metabolism Are Blunted in −/− Hepatocytes*—Hepatocytes are an ideal model to study insulin action, because insulin exerts both metabolic and growth-promoting effects in these cells. We asked whether the differences observed in the signaling pathway would be mirrored by changes in the ability of −/− cells to respond to insulin in assays of glucose metabolism. Insulin stimulated glucokinase (Fig. 5*a*) and glycogen synthase activities (Fig. 5*b*), and suppressed glucose production in WT cells (Fig. 5*c*, *left panel*). In −/− cells, basal rates of glucokinase activity and glycogen synthesis, as well as glycogen content (not shown) were decreased compared with WT cells. Glucose production rates, on the other hand, were about 2-fold higher (Fig. 5*c*, *right panel*) in −/− cells. Moreover, insulin was totally ineffective in modulating these metabolic activities in −/− cells, despite the activation of IGF-1 receptors. Thus, IGF-1 receptor-mediated activation of IRS-1 is not sufficient to mediate insulin's metabolic actions in liver.

*Growth-promoting Actions of Insulin*—Insulin has a potent effect to stimulate DNA synthesis and cell growth in hepatocytes (34). In −/− cells, thymidine incorporation in response to insulin was decreased by >80% compared with normal cells, consistent with impaired DNA synthesis (Fig. 6*a*). We also

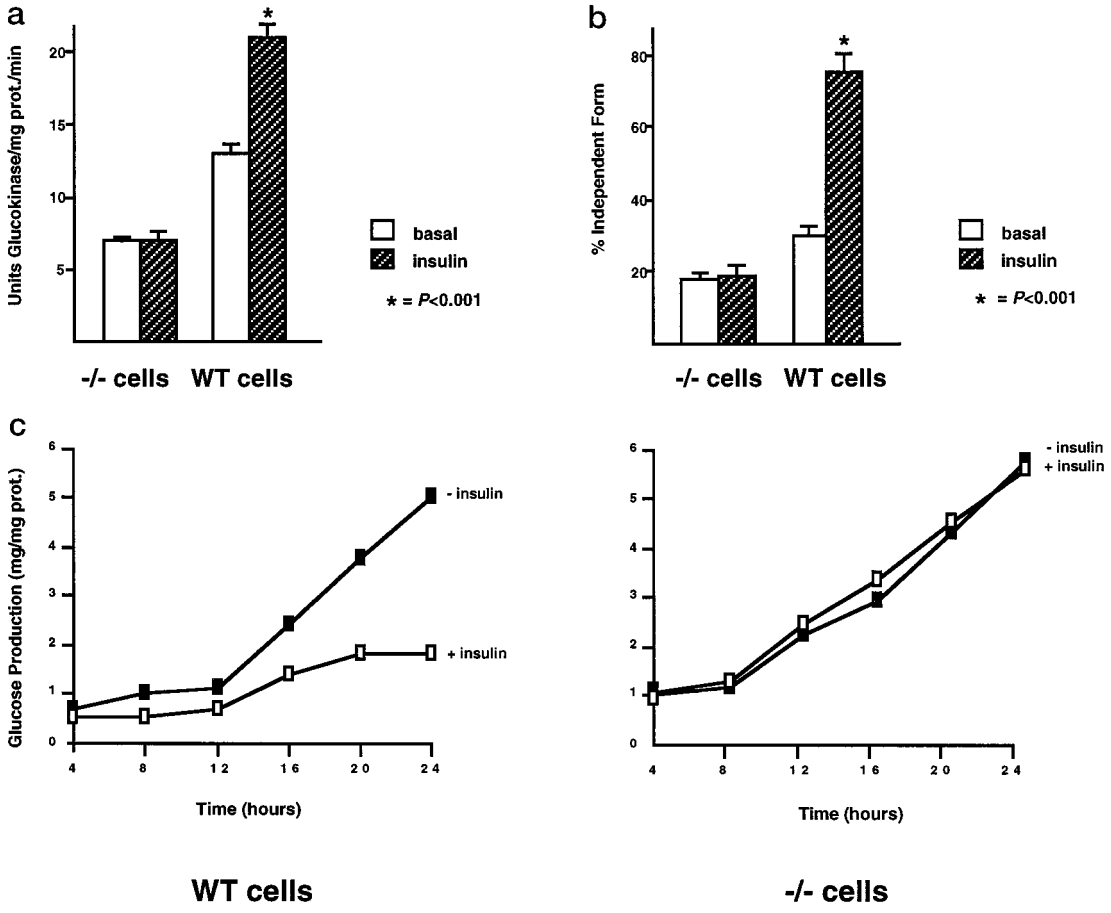


FIG. 5. **Metabolic responses to insulin in hepatocytes.** *a*, glucokinase activity. Glucokinase activity was measured using a spectrophotometric assay based on generation of NADPH in the absence and presence of insulin. *b*, glycogen synthesis. Glycogen synthase activity was measured in insulin-treated WT and −/− cells. The effect of insulin was plotted as percentage of the activity measured in the absence of the allosteric activator glucose 6-phosphate (in both experiments, * = $p < 0.001$). *c*, insulin suppression of glucose production. Glucose production was measured in basal and insulin-treated cells over a period of 24 h. Cells were preincubated in the absence of glucose, and glucose released in the medium was assayed with a glucose analyzer.

studied the ability of WT and −/− cells to replicate (Fig. 6*b*). Both cell types grew at similar rates in the presence of serum. Insulin and IGF-2 were equally effective in stimulating growth of serum-deprived WT cells and ~2-fold more potent than IGF-1. In −/− cells, insulin- and IGF-2-dependent growth was decreased by ~50% ($p < 0.01$), while no differences were observed in IGF-1-dependent growth.

## DISCUSSION

In these investigations, we have analyzed the correlation between IRSs phosphorylation and insulin action in hepatocytes derived from insulin receptor-deficient mice. We provide evidence that impaired activation of IRS-2 in these cells is associated with failure of both metabolic and growth-promoting actions of insulin. An important result of the present study is that ablation of insulin receptors results in a selective loss of IRS-2 phosphorylation, in the absence of detectable changes in IRS-1 phosphorylation. Indirect evidence suggests that additional IRSs are minor components of the IRS signaling system in this cell type. Thus, these data support the notion that signaling from insulin receptors to IRS-2 is required for the characteristic actions of insulin in liver. Since our data were obtained in the context of a physiologic target cell of insulin action, we believe that they add significant new information to our understanding of the insulin signaling system.

The link between impaired phosphorylation of IRS-2 and impaired insulin action is further demonstrated by the decrease in the formation of IRS-2/PI 3-kinase complexes in −/− cells. There is substantial evidence that PI 3-kinase is required for many, if not all, of insulin actions (35–46). While in this study we did not measure PI 3-kinase activity directly, it is well established that insulin activates PI 3-kinase by causing IRSs to bind to the SH2 domains of the p85 subunit (29–31). Thus, there is an excellent correlation between co-precipitation of IRSs with p85 and PI 3-kinase activity. Our analysis of the patterns of tyrosine-phosphorylated proteins detected in p85 immunoprecipitates indicates that the main proteins bound to p85 in hepatocytes are indeed IRS-1 and IRS-2, and therefore account for most of PI 3-kinase activity elicited by insulin in these cells. Furthermore, we have recently been able to show that insulin fails to stimulate Akt activity in −/− cells, which is consistent with an impairment of PI 3-kinase activity[2] (47–50). These data provide support to the notion that impairment of IRS-2 phosphorylation in −/− cells is one of the mechanisms of the failure of insulin action, and not an epiphenomenon.

The conclusion that IRS-2 plays an important role in mediating the metabolic actions of insulin is supported by several lines of independent evidence. IRS-2-deficient mice are diabetic as a result of combined insulin resistance and impaired insulin production (21), while IRS-1-deficient mice are growth retarded, but mildly insulin-resistant (18, 19). Furthermore, normal insulin action in liver of IRS-1-deficient mice is associated with increased phosphorylation of IRS-2 (51). Based on the

[2] B.-C. Park and D. Accili, unpublished observation.

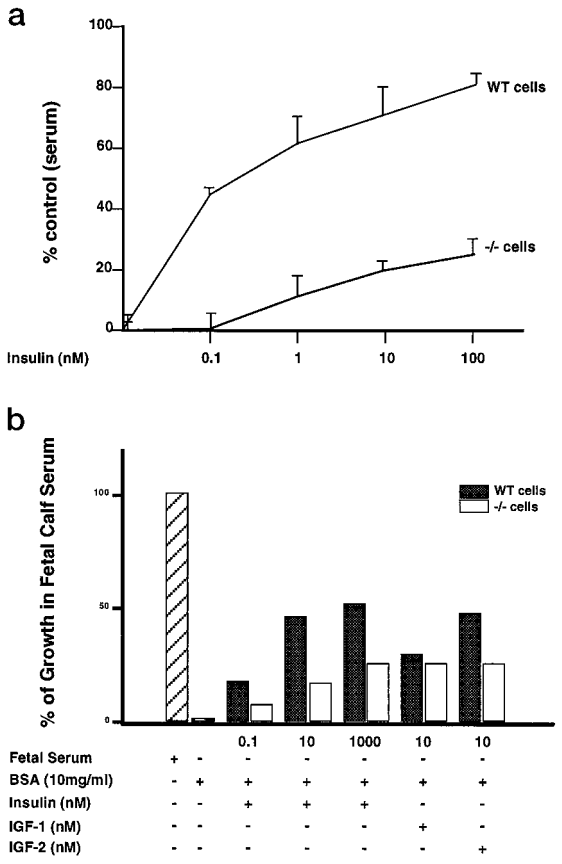


FIG. 6. **DNA synthesis and cell growth.** *a*, thymidine incorporation in the absence or presence of insulin was measured in triplicate. Values are presented as percent of values in the presence of fetal calf serum. *b*, insulin- and IGF-dependent cell growth. WT and −/− cells were incubated for 96 h in medium supplemented with 4% fetal calf serum or 10% bovine serum albumin with and without insulin, IGF-1, and IGF-2 at the concentrations indicated. The mean of three separate experiments is presented. An approximate doubling of cell number per day was observed.

phenotypes of insulin receptor-, IGF-1 receptor-, IRS-1-, and IRS-2-deficient mice (13–15, 18, 19, 21), one has to conclude that IRS-1 is predominantly an IGF-1 receptor substrate, and IRS-2 an insulin receptor substrate. This conclusion is strengthened by the present findings, indicating that insulin receptors are required for optimal IRS-2 phosphorylation. By suggesting that IRS-2 functions primarily as an insulin receptor substrate, our data provide a compelling explanation for the phenotype of mice lacking IRS-2 (21), and corroborate the findings of Bruning *et al.* that IRS-1 and IRS-2 are functionally distinct molecules (22). Substrate selection, however, is likely to be a more complex event *in vivo*, as indicated by studies of mice with combined null mutations of the insulin receptor and IRS-1 genes, which develop insulin resistant diabetes with significantly higher frequency than mice heterozygous for each individual mutation (20).

An important question raised from our studies is why IGF-1 receptors fail to phosphorylate IRS-2 as insulin receptors do. There is ample evidence that IRS-1 and IRS-2 utilize different mechanisms to interact with their receptor partners (52–54). This evidence, however, does not support a different mode of interaction of insulin and IGF-1 receptors with IRS-1 and IRS-2. An alternative possibility is that the subcellular localization of IRS-2 in hepatocytes prevents its efficient phosphorylation by IGF-1 receptors. A similar mechanism has been postulated to explain the differences between epidermal growth factor and insulin signaling (55), but there is no direct evidence that a similar mechanism may be at play in this instance.

The failure of insulin at high doses to activate metabolic responses through IGF-1 receptors is an important finding and deserves further comment. There has been considerable controversy over the ability of IGF-1 receptors to mediate metabolic actions (56). We have previously shown that IGF-1 receptors are weak mediators of metabolic effects in mice lacking insulin receptors (17). We postulated that IGF-1 may enhance peripheral glucose uptake in muscle and decrease hepatic gluconeogenesis. Based on those data, we proposed that IGF-1 may act on hepatic glucose production either directly through IGF-1 receptors, or indirectly through inhibition of glucagon secretion. The failure of IGF-1 receptors to impinge on hepatic glucose metabolism favors an indirect mechanism as a more likely explanation of our previous findings, but further studies comparing gluconeogenetic rates are required.

The impairment of insulin-mediated growth in −/− cells correlated with the loss of insulin receptor-mediated IRS-2 phosphorylation, suggesting that growth-promoting signaling of insulin receptors occurs prevalently through IRS-2. On the other hand, IGF-1-dependent growth occurred normally in −/− cells, indicating that IGF-1 receptors signal through IRS-1, phosphorylation of which is not affected in −/− cells. It is possible that some of the effects of insulin or IGF-1 on growth are mediated by shc through the grb-2/mSOS pathway to mitogen-activated protein kinase (41, 57, 58), although this point remains controversial (59–61). This possibility is currently under investigation. However, it is interesting to note that Bruning and co-workers have shown that IGF-1-dependent growth of IRS-1-deficient fibroblasts is impaired despite normal activation of mitogen-activated protein kinase, and cannot be rescued by IRS-2, consistent with our model in which IGF-1 receptors utilize primarily IRS-1 to mediate their actions on cell growth (22). The impairment of IGF-2-mediated growth in −/− cells is consistent with previous evidence from our laboratory and others that insulin receptors mediate the growth-promoting actions of IGF-2 (62, 63).

In conclusion, our data support a model in which the specificity of insulin signaling in liver is bestowed by the formation of a signaling complex between insulin receptors and IRS-2. It remains to be seen whether a similar mechanism operates in other cell types, for example in insulin-dependent translocation of glucose transporters in skeletal muscle and adipose tissue.

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