The IR_{1152} Mutant Insulin Receptor Selectively Impairs Insulin Action in Skeletal Muscle but Not in Liver

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In patients harboring the IR_{1152} mutant insulin receptor, hepatic glucose production was normally suppressed by insulin. Hepatocytes without the insulin receptor gene and expressing IR_{1152} (Hep_{MUT}) also showed normal insulin suppression of glucose production and full insulin response of glycogen synthase. In contrast, expression of the IR_{1152} mutant in skeletal muscle maximally increased glucose uptake and storage, preventing further insulin stimulation. IRS-1 phosphorylation was normally stimulated by insulin in both intact Hep_{MUT} and L6 skeletal muscle cells expressing the IR_{1152} mutant ($L6_{MUT}$). At variance, IRS-2 phosphorylation exhibited high basal levels with no further insulin-dependent increase in $L6_{MUT}$ but almost normal phosphorylation, both basal and insulin-stimulated, in the Hep_{MUT} cells. In vitro, IR_{1152} mutant preparations from both the $L6_{MUT}$ and the Hep_{MUT} cells exhibited increased basal and no insulin-stimulated phosphorylation of IRS-2 immobilized from either muscle or liver cells. IR_{1152} internalization in liver and muscle cells closely paralleled the ability of this mutant to phosphorylate IRS-2 in vivo in these cells. Block of receptor internalization (wild-type and mutant) in the liver and muscle cells also inhibited IRS-2, but not IRS-1, phosphorylation. Thus, the mechanisms controlling insulin receptor internalization differ in liver and skeletal muscle cells and may enable IR_{1152} to control glucose metabolism selectively in liver. In both cell types, receptor internalization seems necessary for IRS-2 but not IRS-1 phosphorylation. *Diabetes* 49:1194–1202, 2000

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α-MEM, α-minimum essential medium; BSA, bovine serum albumin; Cy, cytosolic; ECL, enhanced chemiluminescence; HGP, hepatic glucose production; IM, internal membrane; IRS, insulin receptor substrate; PI, phosphatidylinositol; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; UDPG, uridine 5′-diphosphate-glucose.

In all tissues, insulin activation of the receptor kinase determines phosphorylation and subsequent activation of several intracellular substrates (1,2). These include the multisite docking proteins insulin receptor substrate (IRS)-1 and -2 and other low-molecular-weight species such as Shc. The function of all these substrates is to reversibly bridge the activated insulin receptors to a variety of distal pathways (1–3). These downstream pathways are entered through substrate activation of at least 2 major signaling complexes, which include Ras and phosphatidylinositol (PI)-3 kinase (4–7). Some of these early events leading the insulin signal to its cellular effectors occur at the level of the plasma membrane (8,9). Others may follow the internalization of the insulin receptor kinase into the endosomal apparatus of the cell (10,11). However, the cellular compartment where IRS phosphorylation and the subsequent engagement of the major signaling complexes occur is still debated.

At least 2 factors contribute to the complexity of this signaling network and make it difficult to dissect the mechanisms responsible for each insulin effect in the different tissues. One is the large number of individual events and molecules that are involved. Second, the role of most known steps in insulin action may vary in different tissues because of different subcellular compartmentalization or different regulation of key signaling molecules (12,13). For instance, control of insulin receptor endocytosis is based on distinct molecules in liver and other major targets of insulin action (9,14,15). In liver and muscle tissues and in adipocytes, IRS-1 and IRS-2 exhibit different distribution and regulation (16,17). Furthermore, different isoforms of the PI-3 kinase regulatory subunit are expressed in liver, fat, and skeletal muscle (18), suggesting that regulation of this key signaling complex occurs differently in the major targets of insulin action. How this diversity in the signaling cascade affects the different responsivity of the major target tissues to insulin has not been elucidated.

In a family of individuals with type 2 diabetes, we have identified a mutation in the insulin receptor gene leading to the substitution of $Arg_{1152} \rightarrow Gln$ in the kinase regulatory region of the receptor (19). In vitro, this defect increases insulin receptor binding to the kinase regulatory loop binding domain of IRS-2 and maximally activates receptor phosphorylation of this substrate, preventing further activation by insulin (20). Compared with that of IRS-2, phosphorylation of IRS-1 by this

mutant receptor (IR_{1152}) exhibited only a modest basal increase and full insulin sensitivity (20). In fibroblasts from patients expressing the mutant receptor, the metabolic effects of insulin were also maximally activated and not further stimulatable by insulin (21). Consistently, in vivo studies in the forearm from these patients and studies with skeletal muscle cells transfected with the mutant receptor revealed that the IR_{1152} mutant constitutively increases glucose disposal but impairs insulin action in this tissue (22). Despite the presence of severe insulin resistance in the skeletal muscle, however, good glycemic control is maintained by low doses of insulin (~30 U/day) in these patients. These findings raise the possibility that the mutant receptor may affect insulin sensitivity differently in skeletal muscle and the other tissues playing a role in insulin-regulated glucose homeostasis.

To address this issue, we expressed IR_{1152} in hepatocytes from mice featuring the knockout of the insulin receptor gene. We found that, in both liver and skeletal muscle cells, insulin receptor internalization is not required for activating IRS-1, although it is necessary for inducing IRS-2 phosphorylation and insulin action on glucose metabolism. The mechanisms controlling receptor internalization appear to differ in liver and skeletal muscle and enable insulin control of glucose metabolism through the IR_{1152} mutant selectively in liver and not in muscle.

RESEARCH DESIGN AND METHODS

Materials. Media and sera for tissue culture and the transfection reagent Lipofectamine (*N*-[1-(2,3-diomeoyloxy)-propyl]-*N*,*N*,*N*-trimethylammoniumchloride/dioleoylphosphatydilethanolamine) were purchased from Life Technologies (Grand Island, NY). Electrophoresis reagents were from Bio-Rad (Richmond, VA). Protein A-sepharose beads and sulfo-hydroxysuccinimide long-chain biotin were purchased from Pierce (Rockford, IL). All radiochemicals, Western blot, and enhanced chemiluminescence (ECL) reagents were from Amersham (Arlinghton Heights, IL). Monoclonal Ig2 phosphotyrosine antibodies (catalog number 05-321) and monoclonal IRS-1 antibodies (catalog number 06-248) were from Upstate Biotechnology (Lake Placid, NY). Polyclonal IRS-2 antibodies (catalog number sc-1555) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal insulin receptor antibodies (catalog number GR07) from Oncogene Science (Manhasset, NY). All other reagents were from Sigma (St. Louis, MO).

In vivo studies. To evaluate hepatic insulin sensitivity, a dose-response study for the suppressive effect of insulin on hepatic glucose production (HGP) was performed as described (23). Three different doses of insulin (0.5, 2, and 5 $mU \cdot kg^{-1} \cdot min^{-1}$) were infused intravenously for 120 min each, while blood glucose was maintained constant at normoglycemic levels by means of a variable glucose infusion. A primed (30 µCi) continuous (0.3 µCi/min) infusion of 3-[^{3}H]glucose (New England Nuclear, Boston, MA) was started 120 min before the start of insulin infusion and maintained for the entire study period. HGP was calculated using the Steele equation (24). This model is known to give frequently negative numbers at high insulin levels, and we took the negative numbers to indicate nil HGP.

Cell culture and transfection. The L6 cell clones, both those expressing the IR_{1152} mutant and those expressing wild-type human insulin receptors, and culture method used in the present work have been characterized and reported (22). The hepatocytes from insulin receptor knockout mice ($Hep_{-/-}$ cells) have also been previously characterized (25). In the present study, the hepatocytes were maintained in α-minimum essential medium (α-MEM) supplemented with 1 mmol/l L-glutamine, 200 nmol/l dexamethasone, and 5% fetal calf serum and transfected with either IR_{1152} or the wild-type insulin receptor cDNA (22) using the calcium phosphate method (26). For selection, hygromycin was used at the effective concentration of 0.6 mg/ml. Individual hygromycin-resistant clones were isolated and screened by [^{125}I]insulin binding (27,28) and Western blot analysis with insulin receptor antibodies (29).

Metabolic labeling. For detection of insulin receptor α and β subunits, confluent monolayers of hepatocytes were incubated in methionine-free α-MEM supplemented with 0.25% bovine serum albumin (BSA) for 3 h. The cells were labeled by adding [^{35}S]methionine (1,000 Ci/mmol, 50 µCi/ml) for 16 h in the same medium supplemented with 10% fetal calf serum and 1 mmol/l glutamine. The cells were then rinsed and solubilized in 0.5 ml S-buffer (50 mmol/l HEPES, pH 7.4, 5 mmol/l EDTA, 5 mmol/l EGTA, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 50 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 µg/ml pepstatin). Cell lysates were clarified by centrifugation for 20 min at 100,000*g* (4°C) and precipitated with 2 µg insulin receptor antibodies. Precipitated receptors were washed with 60 volumes of 50 mmol/l HEPES, pH 7.4, 0.1% Triton X-100, 150 mmol/l NaCl, and separated by SDS-PAGE. The gels were treated with Enhance (Du Pont-NEN, Florence, Italy) for 30 min, and radiolabeled receptors were detected by autoradiography.

Glucokinase, glycogen synthase assays, and liver glucose output studies. For quantitation of glucokinase activity, the hepatocytes were deprived of serum for 18 h and exposed to 100 nmol/l insulin for 30 min. The cells were subsequently homogenized at 4°C in 20 mmol/l Tris-HCl, pH 7.4, 250 mmol/l sucrose, 80 mmol/l KCl, 5 mmol/l EDTA, 4 mmol/l $MgCl_2$, 2.5 mmol/l dithiothreitol, 0.125 mmol/l PMSF, 2.5 µg/ml aprotinin, and 1 µg/ml pepstatin. The homogenates were centrifuged at 12,000*g* for 15 min and the supernatants further centrifuged at 180,000*g* for 60 min. Glucokinase activity was quantitated in the final supernatants as described (25). Briefly, cell extracts were incubated in a reaction mixture containing 45 mmol/l Tris-HCl, pH 7.5, 110 mmol/l KCl, 8 mmol/l $MgCl_2$, 0.5 mmol/l NADP, 0.9 U/ml glucose-6-phosphate dehydrogenase, and either 0.5 or 100 mmol/l glucose in the absence or the presence of 0.5 mmol/l ATP. Enzyme activity was then estimated as the difference between the ATP-dependent rates of NADPH generation at 100 and 0.5 mmol/l glucose. In this assay, 2 mol NADPH corresponds to 1 mol phosphorylated glucose. Enzyme activity is expressed in units per minute (1 unit equals 1 µmol NADPH generated).

For the glycogen synthase assay, liver cells were preincubated at 37°C in HEPES buffer, pH 7.8, for 3 h before the assay as described (22). The cells were then stimulated with 100 nmol/l insulin, resuspended in 10 mmol/l EDTA, and sonicated for 10 s at 300 W. The cell suspension was centrifuged for 10 min at 2,000 rpm, and 20-µl aliquots of the supernatants (20 µg cell proteins) were added to 60 µl of a reaction mixture containing 40 mmol/l Tris-HCl, pH 7.8, 25 mmol/l NaF, 20 mmol/l EDTA, 10 mg/ml glycogen, and 7.2 mmol/l uridine 5′-diphosphate-glucose (UDPG) in the absence or the presence of 6.7 mmol/l glucose-6-phosphate. The incubation was prolonged for 20 min at 30°C and then terminated by spotting on filter paper followed by precipitation with ice-cold ethanol and detection of radioactivity in a Beckman scintillation counter.

For quantitation of glucose production, liver cells were preincubated overnight in α-MEM supplemented with 0.25% BSA and then further incubated in glucose-free, serum-free medium supplemented with 16 mmol/l lactate and 4 mmol/l pyruvate in the absence or the presence of 100 nmol/l insulin. Aliquots of the medium were removed at the indicated time points, and the glucose concentration was determined with a Beckman glucose analyzer as described (25).

Preparation of subcellular fractions. L6 and Hep cells were starved of serum for 18 h in medium supplemented with 2% BSA. The cells were then washed with buffer A (20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 255 mmol/l sucrose, 1 mmol/l PMSF, 10 mmol/l NaF, 100 µmol/l Na_3VO_4, 1 mmol/l NaP_2O_7, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). Cell fractions were prepared as described by Simpson et al. (30). Briefly, the cells were homogenized in buffer A using 15 strokes of a Teflon-glass homogenizer and centrifuged at 16,000*g* for 20 min at 4°C. Pellets were applied on a 1.12 mol/l sucrose cushion and further centrifuged at 100,000*g* for 70 min for preparation of the plasma membrane fraction. Supernatants were further centrifuged at 212,000*g* for 20 min for preparation of the internal membrane (pellet) and cytosolic (supernatant) fractions.

IRS detection and phosphorylation. Intact cells were exposed to 100 nmol/l insulin for 5 min at 37°C and solubilized in TAT buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 1 mmol/l Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 mmol/l NaF, and 1 mmol/l PMSF), as described (20). Cell lysates were then precipitated with IRS-1 or -2 antibodies for 18 h at 4°C, followed by treatment with protein A-sepharose beads. The immunocomplexes were reduced in Laemmli buffer, separated by SDS-PAGE, and subjected to Western blotting as described (20). Blots were probed with phosphotyrosine antibodies and analyzed by autoradiography. Quantitation was achieved by laser densitometry of the bands on the autoradiographs.

To investigate IRS phosphorylation in vitro, the cells were lysed with TAT buffer and precipitated with IRS-1 or -2 antibodies as described above. Precipitated proteins were then incubated with protein A-sepharose, and the proteins immobilized on the beads were further incubated with either wild-type or IR_{1152} insulin receptors as indicated (250 fmol/assay). Phosphorylation reactions were initiated by adding 2 µmol/l CTP, 2 µmol/l ATP, 10 mmol/l HEPES, pH 7.4, 0.02% Triton X-100, 5 mmol/l $MnCl_2$, 7 mmol/l $MgCl_2$, and 0.02 µmol/l [γ-^{32}P]ATP. The incubation was prolonged for 30 min at 22°C as described (20). Phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography.

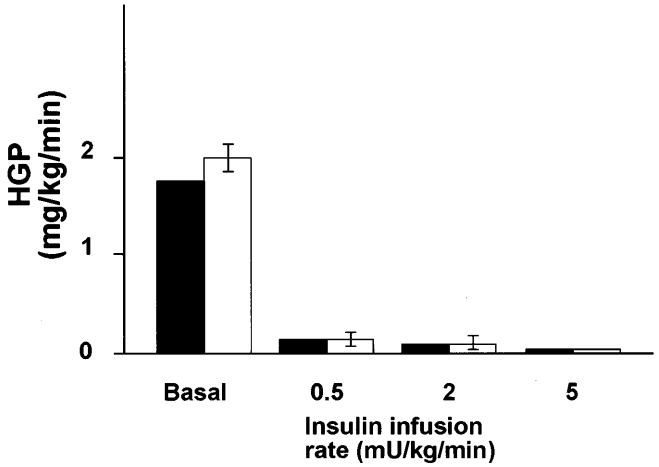

FIG. 1. Hepatic glucose production in the IR_{1152} patient and in nondiabetic individuals. The rate of hepatic glucose production was calculated in the IR_{1152} patient (■; mean values obtained in 2 independent studies) and in nondiabetic individuals (n = 8) (□; mean values ± SD) in the basal state and during 0.5, 2, and 5 mU · kg^{-1} · min^{-1} euglycemic insulin clamp studies as indicated. These values differed from each other by <10%.

Insulin receptor internalization. The internalization of biotinylated insulin receptors was investigated as described (15). Briefly, cells were incubated with 0.5 mg/ml biotin at 4°C and further incubated at 37°C for 20 min in the absence or presence of 100 nmol/l insulin. The cells were then treated with Pronase (2.5 mg/ml) for 1 h at 4°C and solubilized in 50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l PMSF, and a mixture of protease inhibitors. The lysates were immunoprecipitated with insulin receptor antibodies, and the receptors were analyzed by Western blotting as described (31). Detection of internalized receptors was achieved by ECL according to the manufacturer's instructions and quantitation of the autoradiographs by laser densitometry.

RESULTS

In vivo studies. As shown in Fig. 1, the IR_{1152} patient showed a normal rate of HGP (1.7 mg · kg^{-1} · min^{-1}) in the basal state, as well as normal response to stepwise insulin infusion. The values of HGP in the IR_{1152} subject were not different from those of a control group of nondiabetic individuals. The finding of a complete suppression of HGP at the lower insulin infusion rate indicated that liver sensitivity to insulin was well preserved in the patient with the mutant receptor. In this patient, total body glucose metabolism was 3, 5.5, and 7 mg · kg^{-1} · min^{-1} at 0, 2, and 5 mU · kg^{-1} · min^{-1} insulin infusion, respectively (data not shown).

Expression and function of mutant receptors in hepatocytes. To further analyze IR_{1152} function in liver, we stably transfected hepatocytes from insulin receptor–deficient mice ($Hep_{-/-}$) (25,32) with either wild-type or mutant receptor cDNAs. Clonal cell lines were then screened for expression of transfected receptors by [^{125}I]insulin binding. Several cell clones were isolated. Two clones expressing 2.8 × 10^4 (Hep_{WT1}) and 0.8 × 10^5 (Hep_{WT2}) wild-type receptors and 2 expressing 2.5 × 10^4 (Hep_{MUT1}) and 1 × 10^5 (Hep_{MUT2}) IR_{1152} receptors per cell were studied in detail. Based on Scatchard analysis (28) of equilibrium binding data, all these clones displayed dissociation constants (K_d) for insulin between 0.8 and 1.4 nmol/l (Table 1). These values are similar to the K_d of endogenous insulin receptors measured in hepatocytes from wild-type animals (which express ~10^5 endogenous insulin receptors per cell).

To ensure that insulin receptor was properly processed and transported to the cell surface, extracts were prepared from cells metabolically labeled with [^{35}S]methionine. The radiolabeled insulin receptors were then immunoprecipitated with insulin receptor antibodies. In all the cell lines expressing transfected wild-type and mutant receptors, these antibodies immunoprecipitated 2 proteins migrating at molecular weight 130,000 and 92,000, which correspond to the insulin

TABLE 1
Results of Scatchard analysis of insulin binding data

Cell clone	K_d (nmol/l)	Receptors/cell
Hep_{WT1}	0.8	2.8 × 10^4
Hep_{WT2}	1.2	0.8 × 10^5
Hep_{MUT1}	1.4	2.5 × 10^4
Hep_{MUT2}	1.0	1.0 × 10^5

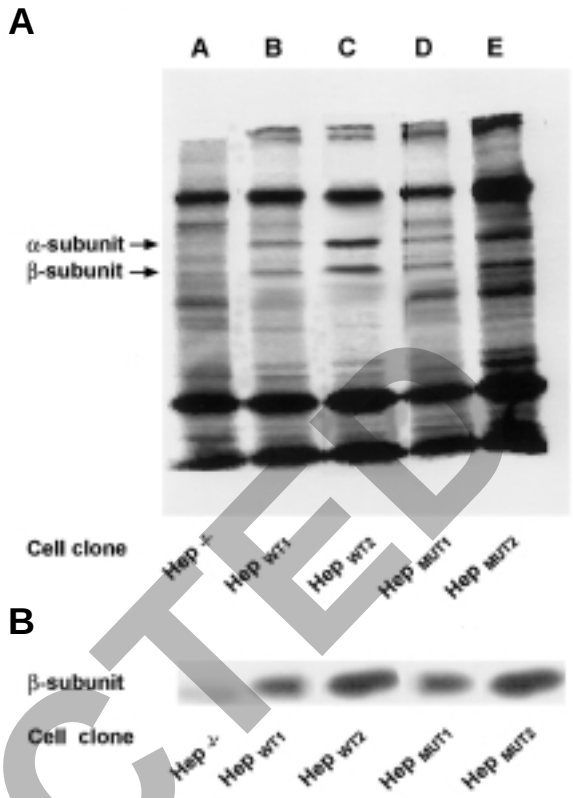

FIG. 2. Immunoprecipitation of transfected insulin receptors from liver cells. *A*: Cell labeling with [^{35}S]methionine. Parental (untransfected) $Hep_{-/-}$ cells and individual clones of cells transfected with either wild-type (Hep_{WT1}, Hep_{WT2}) or mutant (Hep_{MUT1}, Hep_{MUT2}) IR_{1152} receptors were labeled for 16 h with [^{35}S]methionine as described in RESEARCH DESIGN AND METHODS. Cell extracts were prepared and immunoprecipitated with specific insulin receptor antibodies. Immunoprecipitates were subjected to 7.5% SDS-PAGE under reducing conditions and analyzed by autoradiography. Arrows indicate positions of the 130-kDa α-subunits and the 92-kDa β-subunits. The autoradiograph shown was exposed at –70°C for 48 h. *B*: The cells were exposed to 100 nmol/l insulin and lysed, and cell proteins were separated by reducing SDS-PAGE, blotted with phosphotyrosine antibodies, and analyzed by autoradiography. The arrow indicates insulin receptor β-subunits. The autoradiographs shown are representative of 4 (*A*) and 3 (*B*) independent experiments.

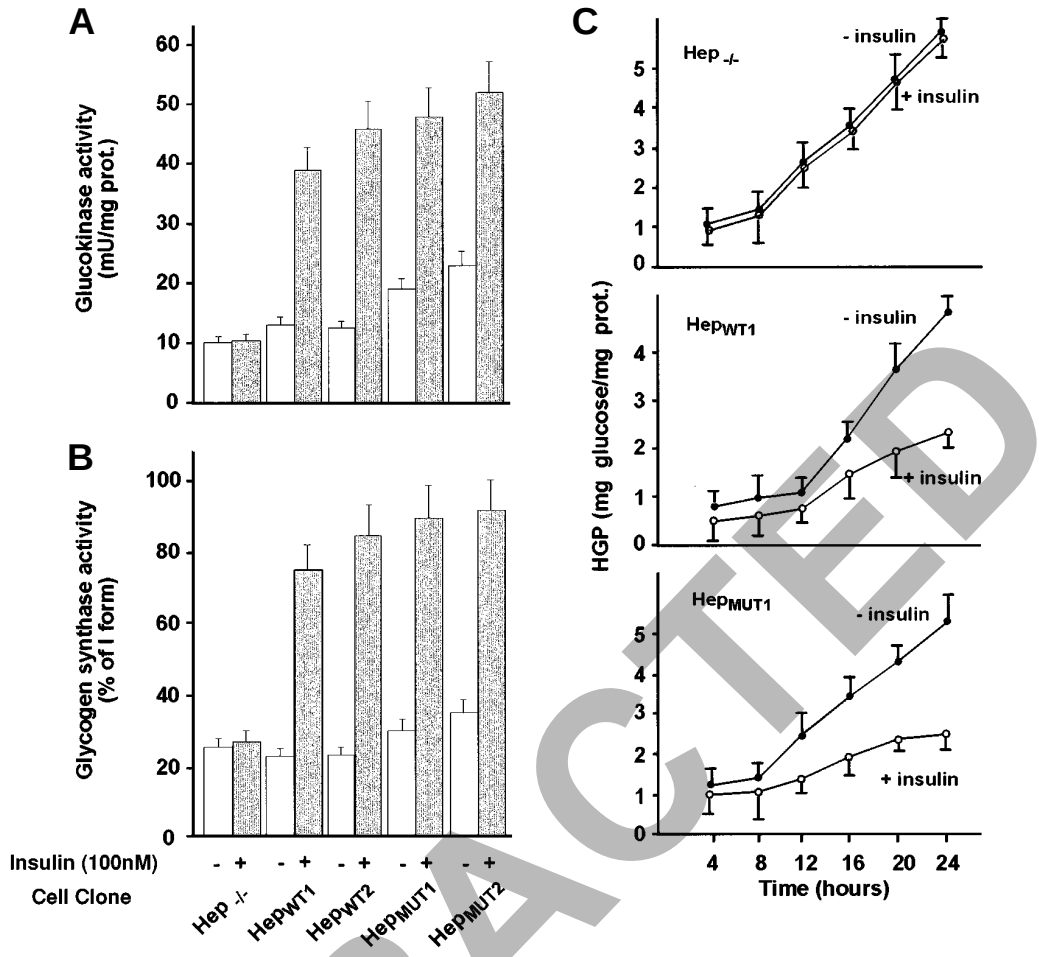

FIG. 3. Insulin action in liver cells. The cells were incubated in the absence or the presence of 100 nmol/l insulin as indicated and then assayed for glucokinase activity (*A*), glycogen synthase activity (*B*), or glucose production (*C*) as described in RESEARCH DESIGN AND METHODS. Each bar or data point represents the mean ± SD of triplicate determinations in 3 (glucokinase and glycogen synthase activities) and 4 (glucose production) independent experiments. Statistical significance of the differences was tested by unpaired *t* test (values given in the text).

receptor α- and β-subunits, respectively (Fig. 2*B–E*). Based on laser densitometry, the intensity of these bands correlated well with the amount of cell-surface receptors as measured by insulin binding (Table 1) or Western blot (Fig. 2*B*). Consistently, α and β insulin receptor subunits were undetectable in untransfected hepatocytes from insulin receptor–deficient mice (Fig. 2*A*, lane A). The rate of insulin receptor biosynthesis was also examined by labeling the cells with [^{35}S]methionine for 15 min followed by a chase for varying lengths of time. Both wild-type and mutant receptors were synthesized at approximately the same rate with no significant difference from the endogenous receptors (data not shown).

In the hepatocytes from insulin receptor–deficient mice, expression of the constitutively active IR_{1152} receptor increased basal levels of both glucokinase and glycogen synthase activities by 15% (Hep_{MUT1}) and 40% (Hep_{MUT2}) compared with those measured in cells expressing wild-type receptors ($P < 0.001$ by *t* test) (Fig. 3*A* and *B*). Insulin stimulation (100 nmol/l) of glucokinase and glycogen synthase was comparable in cells expressing the mutant and wild-type receptors, whereas no effect was detectable in the untransfected $Hep_{-/-}$ cells. Similarly, insulin elicited no suppression of glucose production in the $Hep_{-/-}$ cells but inhibited glucose production to a similar extent in hepatocytes expressing the mutant and in those expressing the wild-type receptors (Fig. 3*C*). These results are different from our previous findings in L6 skeletal muscle cells and NIH-3T3 cells expressing the IR_{1152} mutant, in which most insulin metabolic effectors were maximally activated in the basal state and not further stimulatable by insulin (20,22,26).

IR_{1152} signaling in hepatocytes. Our previous observations in L6 skeletal muscle cells indicated that expression of IR_{1152} induces constitutive phosphorylation of IRS-2 and prevents further activation in response to insulin (20). In Hep_{MUT1} and Hep_{MUT2} cells, IR_{1152} receptors increased basal phosphorylation of IRS-2 by only 20 and 40%, respectively, above the levels of cells expressing similar numbers of wild-type receptors ($P < 0.001$ by *t* test) (Fig. 4*A*). Insulin (100 nmol/l) elicited no effect on IRS-2 phosphorylation in the untransfected $Hep_{-/-}$ cells. In the Hep_{MUT1} and Hep_{MUT2} cells, however, insulin stimulated phosphorylation of IRS-2 by 2.5- and 3-fold, respectively, results similar to those in cells expressing wild-type insulin receptors (Hep_{WT1} and Hep_{WT2} cells). Similar to that of IRS-2, phosphorylation of IRS-1 in the Hep_{MUT1} and Hep_{MUT2} cells exhibited a basal increase of 15–40% above that of control cells, increasing by 3-fold in response to insulin, as in the control cells (Fig. 4*B*). At 100 nmol/l insulin, phosphorylation of IRS-1 in the $Hep_{-/-}$ cells was only partially abolished by virtue of the

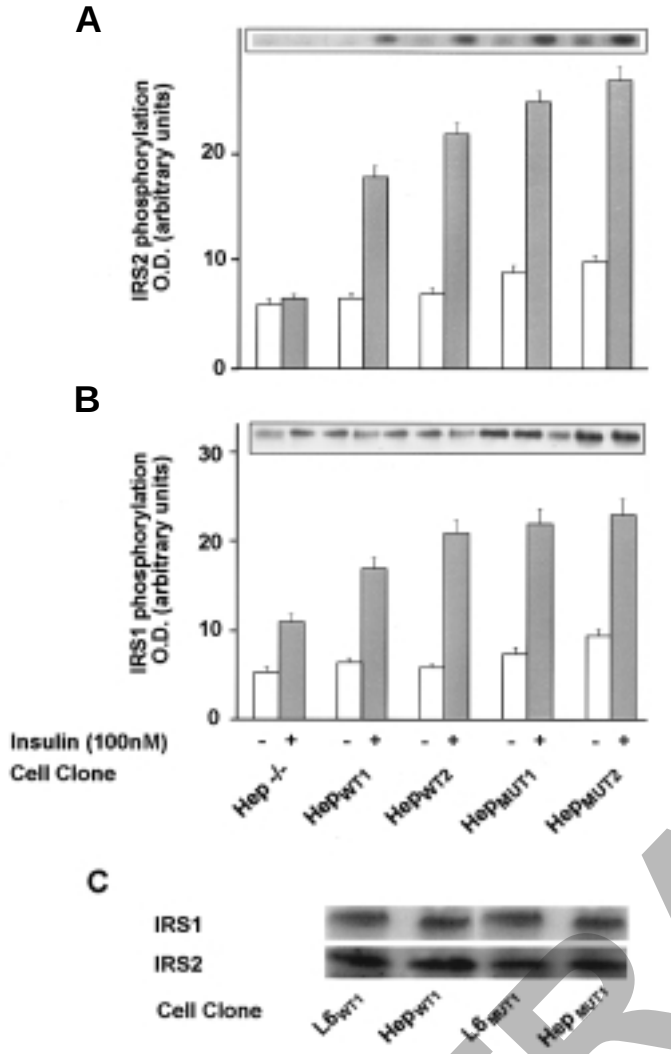

FIG. 4. IRS phosphorylation in intact liver cells. Untransfected cells and cells expressing the wild-type or mutant insulin receptors were stimulated with 100 nmol/l insulin for 5 min and solubilized as described in RESEARCH DESIGN AND METHODS. Cell lysates were then precipitated with specific IRS-2 (*A*) or IRS-1 (*B*) antibodies and blotted with phosphotyrosine antibodies. Detection of the bands was achieved by [^{125}I]protein A and autoradiography. Each bar represents the mean ± SD of values from 3 independent experiments. Those shown in the insets are representative autoradiographs. Detection of total IRS levels in cell lysates (*C*) was achieved by separating cell proteins by SDS-PAGE followed by blotting with IRS-1 or IRS-2 antibodies. The IRS were then revealed with [^{125}I]protein A and autoradiography. The autoradiograph shown is representative of 3 independent experiments. O.D., optical density.

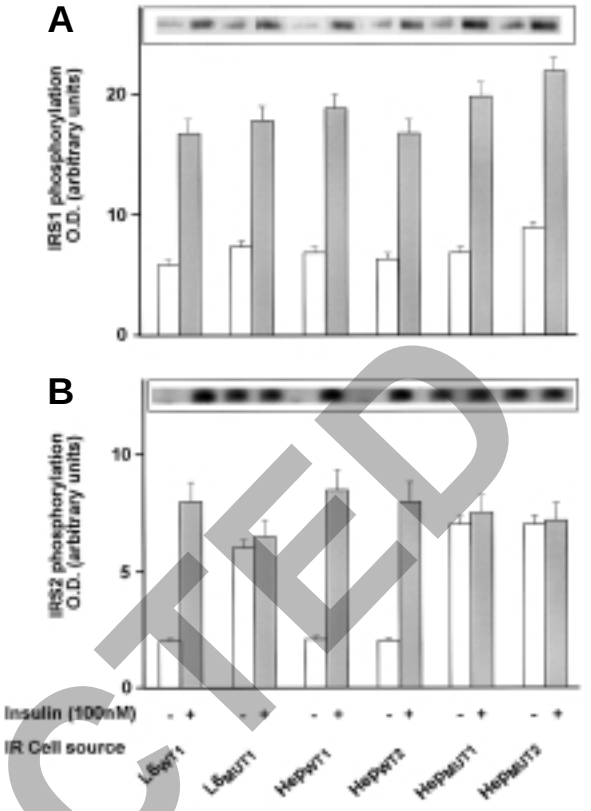

FIG. 5. In vitro phosphorylation of IRS by insulin receptors from hepatocytes and L6 cells. IRS-1 (*A*) and IRS-2 (*B*) from Hep$_{-/-}$ cells were immobilized in protein A-sepharose complexed to specific IRS antibodies as described in RESEARCH DESIGN AND METHODS. Wild-type and IR$_{1152}$ insulin receptors were purified from either the L6 or the Hep cells and incubated with 100 nmol/l insulin for 30 min. The incubation proceeded further in the presence of the immobilized IRS. The phosphorylated IRS were finally separated by SDS-PAGE and analyzed by autoradiography. Quantitation was achieved by laser densitometry of the autoradiographs. Each bar represents the mean ± SD of values from 4 independent experiments. Representative autoradiographs are shown in the insets. IR, insulin receptor; O.D., optical density.

IGF-I receptor complement of the cells, as we have reported (25). The total levels of IRS-2 and -1 were identical in all the liver cell clones and very comparable to those in the L6 skeletal muscle cells (Fig. 4*C*). Thus, IR$_{1152}$ could phosphorylate IRS-2 in response to insulin and evoke insulin-dependent metabolic responses almost normally in the liver but not in the skeletal muscle cells, whereas IRS-1 phosphorylation by the mutant receptor was similar to that attained by the wild-type receptors in both cell types.

To further explore these differences, we tested the hypothesis that they were caused by differences in the properties of the receptor when expressed in the various cell types. We therefore analyzed liver IRS phosphorylation in vitro using partially purified insulin receptor preparations from L6 muscle cells and liver cells. With both cell types, basal IR$_{1152}$ kinase activity using immobilized IRS-1 as substrate showed a 30% increase versus that of the wild-type receptors ($P <$ 0.001) (Fig. 5*A*). However, in the presence of insulin, the kinases of both wild-type and mutant receptors were activated by 2.5-fold, independent of the cell source. IR$_{1152}$ receptors, whether derived from muscle or liver cells, exhibited maximally activated kinase activity toward immobilized liver IRS-2 (Fig. 5*B*). No further IRS-2 phosphorylation occurred in the presence of insulin with the mutant receptors. Compared with the mutant, the wild-type receptors showed 2.5-fold lower basal but fully insulin-stimulatable kinase activities toward IRS-2. Thus, as opposed to the intact cells in vitro, IR$_{1152}$ from both liver and muscle cells showed constitutively activated kinase activity toward liver IRS-2 and no sensitivity to insulin. As previously shown with muscle cell IRS-2 (20), this constitutive activation of IR$_{1152}$ kinase was accompanied by a specific increase in IR$_{1152}$ interaction with the kinase regulatory loop binding domain of IRS-2, with no change in the ability of the mutant receptor to bind the IRS-2 phosphotyrosine binding domain (data not shown).

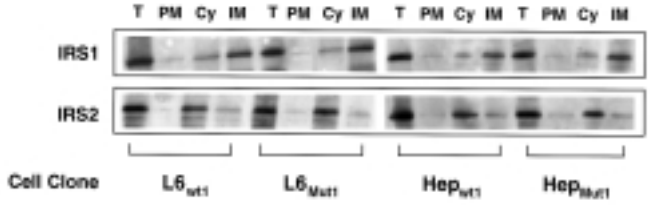

FIG. 6. IRS localization in subcellular fractions from L6 and Hep$_{-/-}$ cells. PM, IM, and Cy were obtained from the cells as described in RESEARCH DESIGN AND METHODS. All samples, including the total cell lysates (T), were resuspended in buffer A to a final concentration of 1 mg protein/ml before being used. Proteins in each fraction were separated by SDS-PAGE and blotted with IRS-1 or IRS-2 antibodies as indicated. The autoradiograph shown is representative of 3 independent experiments.

IR$_{1152}$ internalization in muscle and liver cells. To further address the differences in IRS-2 phosphorylation by IR$_{1152}$ in intact hepatocytes and muscle cells, we tested the hypothesis that the mutant receptor could not effectively interact with IRS-2 in liver cells in the absence of insulin stimulation. To this aim, we first compared the subcellular localization of IRS-1 and -2 in muscle and liver cells. In L6 cells, whether expressing wild-type (L6$_{WT1}$) or mutant (L6$_{MUT1}$) insulin receptor, almost 80% of IRS-2 was cytosolic (Cy) (Fig. 6). Only 15% of IRS-2 was found to be associated with the internal membrane (IM) compartment and very little with the plasma membrane (PM). At variance with IRS-2, at least 75% of IRS-1 was observed in the IM compartment with only 20% in the Cy. Almost no IRS-1 was found in the PM fraction. IRS-2 and -1 compartmentalization in the Hep cells, either Hep$_{WT1}$ or Hep$_{MUT1}$, showed no difference from the muscle cells, suggesting that the differential IR$_{1152}$ phosphorylation of IRS-2 occurring in these 2 cell types is not dependent on diversity in substrate localization. Next, we compared the ability of the receptor to internalize in liver and muscle cells. In 2 clones of L6 cells expressing 1.8×10^4 and 3.2×10^4 wild-type insulin receptors per cell (L6$_{WT1}$ and L6$_{WT2}$, respectively), insulin increased receptor internalization by 3-fold, results similar to those in cells expressing only the endogenous complement of insulin receptors (Fig. 7*A*). In contrast, in L6 cells expressing 1.9×10^4 and 3.0×10^4 IR$_{1152}$ receptors per cell (L6$_{MUT1}$ and L6$_{MUT2}$, respectively), the basal internalization of receptors was constitutively activated to levels comparable to those achieved in the control cells only after insulin exposure. No further increase in receptor internalization occurred in these cells after exposure to insulin, results similar to those with IRS-2 phosphorylation. In contrast to L6 cells, liver cells expressing mutant receptors showed only a 20% increased basal receptor internalization compared with those expressing wild-type receptors ($P < 0.001$) (Fig. 7*B*). In the hepatocytes, the internalization increased by 2.5-fold upon insulin exposure, independent of whether the hepatocytes expressed wild-type or mutant receptors. Thus, IR$_{1152}$ internalization appeared to be differently controlled in hepatocytes and muscle cells and closely paralleled the phosphorylation of IRS-2.

To verify whether IRS phosphorylation depends on receptor internalization in these cells, we compared IRS-1 and -2 phosphorylations at 37°C and 15°C, when receptor internalization is enabled or blocked, respectively. As shown in Fig. 7*C*, IRS-1 phosphorylation in the L6$_{WT}$ cells reached comparable levels upon insulin stimulation at both 37°C and

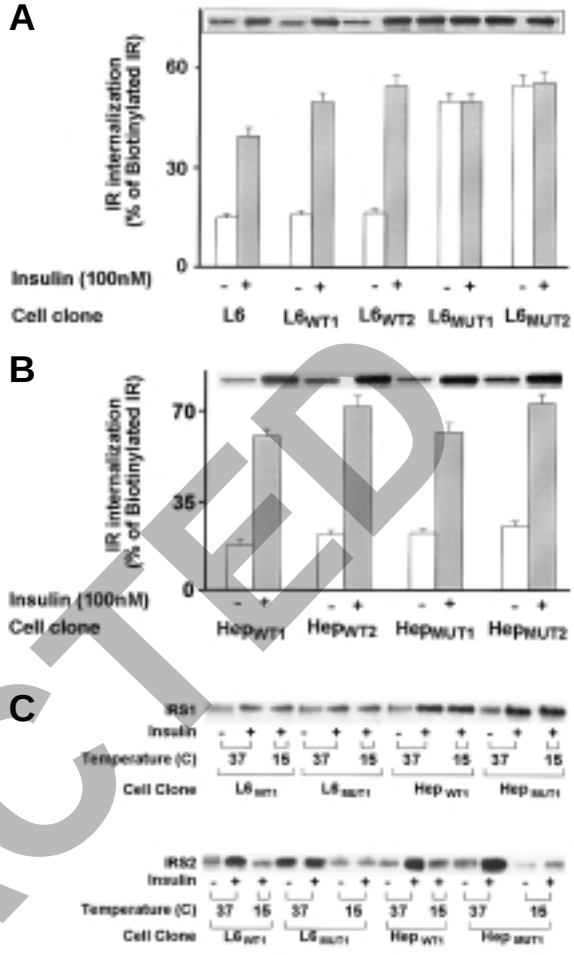

FIG. 7. Insulin receptor internalization and IRS phosphorylation in L6 and Hep$_{-/-}$ cells. After labeling of cell-surface proteins with biotin, monolayers of L6 (*A*) or Hep cells (*B*) were incubated at 37°C for 20 min in the absence or the presence of 100 nmol/l insulin. After incubation with Pronase, the cells were lysed and immunoprecipitated with anti–insulin receptor antibodies. Precipitated receptors were detected by streptavidin blotting and ECL as described in RESEARCH DESIGN AND METHODS. Internalized insulin receptors are plotted as percent of the total biotinylated receptors in lysates from cells not treated with pronase. Each bar is the mean ± SD of duplicate determinations in 4 (*A*) and 5 (*B*) independent experiments. *C*: L6 and Hep cells were incubated at 37°C or 15°C for 20 min in the absence or the presence of 100 nmol/l insulin as indicated in the figure. The cells were then harvested for detection of IRS-1 and IRS-2 phosphorylation as described in Fig. 4. The autoradiograph shown is representative of 3 independent experiments and was obtained by exposing the filters for 36 h at –70°C. IR, insulin receptor.

15°C. Decreasing the temperature did not affect the phosphorylation of IRS-1 in the L6$_{MUT1}$ cells either. Very similar findings were also attained in the liver cells, both those expressing the wild-type and those expressing the mutant receptors, suggesting that IRS-1 phosphorylation by the insulin receptor in these cells is not dependent on receptor internalization. In the case of IRS-2, phosphorylation by the wild-type as well as by the mutant receptors was decreased by 3-fold at 15°C compared with 37°C. This decrease occurred in both muscle and liver cells. Almost identical results were obtained by inhibiting receptor endocytosis through exposure of the cells to hypertonic media or trans-

fection of a dominant-interfering mutant of dynamin (8) (data not shown). Thus, in muscle as well as liver cells, the inhibition of receptor internalization was accompanied by impairment of IRS-2, but not IRS-1, phosphorylation.

DISCUSSION

The ability of tissues to respond to insulin has long been recognized to be determined by the nature of the distal effectors of insulin action present in each individual cell (1,2,12,13). More recently, evidence became available that the proximal events in the insulin signaling machinery may also feature tissue-specific properties (12–15). For instance, p120, an IRS involved in the internalization of the insulin receptor, is selectively expressed in liver and kidney (14,15,33), and IRSs appear to be differently distributed and regulated in the insulin target tissues (16,17). However, the extent to which differences in these early signaling mechanisms generate diversity in insulin action in the different tissues, as well as the relevance of this diversity to conditions of impaired insulin action, is far from understood. In the present report, we have addressed these issues by investigating insulin signaling through the IR_{1152} mutant receptor in cultured liver and skeletal muscle cells, since these tissues represent 2 major sites of insulin action.

Previous in vivo studies showed that muscle glucose disposal in response to insulin is markedly reduced in individuals expressing the IR_{1152} mutant receptor (22). In the present work, we demonstrate that the suppressive effect of insulin on HGP occurs normally in these patients. Consistent with these findings, total body glucose disposal was not markedly impaired, suggesting that the modest decrease in insulin sensitivity detected in these subjects at the whole body level is compounded by severe insulin-resistance in skeletal muscle and normal insulin action in the liver. In hepatocytes from insulin-receptor knockout mice expressing IR_{1152}, insulin is also able to suppress glucose production and stimulate glycogen synthesis to an extent similar to that in hepatocytes expressing an equal number of wild-type receptors. In contrast, in L6 skeletal muscle cells expressing the mutant receptor, glycogen synthesis was maximally activated in the basal state, preventing further insulin-dependent increase (20,22), similar to forearm glucose uptake in the IR_{1152} patients (19). Thus, IR_{1152} generates a muscle-specific condition of insulin resistance that does not occur in liver. To our knowledge, no other insulin receptor mutation has been reported to cause a tissue-specific condition of insulin resistance. It is noteworthy that a close parallelism exists between our in vivo and in vitro studies. In fact, in both models, the presence of this mutation is associated with severe insulin resistance in skeletal muscle but preserved insulin action at the hepatic level.

In both L6 muscle cells and hepatocytes expressing the mutant IR_{1152} receptor, IRS-1 phosphorylation occurred at very low levels in basal conditions and appeared to be fully activated after insulin exposure, as in cells expressing wild-type receptors. In contrast to IRS-1, IRS-2 exhibited only a slight increase in phosphorylation in the liver cells and maintained full insulin sensitivity for phosphorylation but, as we previously reported, was constitutively phosphorylated and unresponsive to insulin in the muscle cells (20). The different ability of the IR_{1152} mutant to phosphorylate IRS-2 in intact liver and skeletal muscle cells did not depend on substrate saturation of the receptor kinase in the muscle cells. Hence, IR_{1152} and IRS-2 were expressed at comparable levels in both hepatocytes and muscle cells, and still, insulin was able to increase IRS-2 phosphorylation selectively in the former and not in the latter cell type. Pretreatment of IR_{1152} mutant–expressing hepatocytes with the tyrosine phosphatase inhibitor orthovanadate did not abolish insulin activation of IRS-2 by the mutant receptor (data not shown). Thus, rapid IRS-2 tyrosine dephosphorylation by liver-specific phosphatases may not account for the different IR_{1152} mutant activity in liver and muscle cells. Finally, in vitro, partially purified IR_{1152} mutant preparations from both hepatocytes and muscle cells exhibited constitutive kinase activation toward immobilized IRS-2 as well as lack of further insulin-dependent activation. These features of the receptors from each of the 2 cell sources did not change whether their kinase was tested toward IRS-2 preparations from either the hepatocytes or the muscle cells. This indicated that the differential IRS-2 phosphorylation by the IR_{1152} mutant observed in the L6 cells and the hepatocytes was not dependent on differences intrinsic to the receptor or to IRS-2 occurring in one cell type and not in the other.

Interestingly, however, upon expression in the L6 muscle cells, IR_{1152} internalized at a very high rate, a result similar to that previously observed in NIH-3T3 fibroblasts (34). Exposure of these cells to insulin does not further increase the internalization of the mutant receptor, as was the case for IRS-2 phosphorylation. In contrast, in hepatocytes, the IR_{1152} mutant exhibited a low basal rate of internalization, comparable to that of wild-type insulin receptors, and increased by 2-fold following insulin binding, as in the case of wild-type receptors. Thus, IR_{1152} mutant internalization is differently controlled in liver and muscle cells and closely parallels IRS-2 phosphorylation in these cells. The same correlations between IRS-2 phosphorylation and IR internalization were also observed in C2C12 myocytes and in Fao and H35 liver cell models (not shown). Therefore, we propose that the constitutive internalization is responsible for the changes in IRS-2 phosphorylation occurring in L6 cells upon expression of IR_{1152}. In fact, *1*) in L6 muscle cells, inhibition of the constitutive IR_{1152} mutant internalization by low temperature simultaneously blocks IRS-2 phosphorylation; *2*) as at the low temperature, inhibition of IR_{1152} internalization through hypertonic media exposure (35) or transfection of a dominant-interfering mutant of dynamin (8) also blocked IRS-2 phosphorylation (data not shown); and *3*) the different IRS-2 phosphorylation in hepatocytes and L6 cells expressing the IR_{1152} mutant was not accounted for by a different compartmentalization of the substrate in the 2 cell types. Treatments of cells at low temperature or with hypertonic media are well known to inhibit coated pit-mediated internalization of several receptors, other than those for insulin, which do not interact with IRS-2 (35). Therefore, we consider it less likely that the constitutive IRS-2 phosphorylation increases receptor internalization in IR_{1152} cells. Additional work is in progress in our laboratory to further address this issue.

IRS-1 and IRS-2 are intracellular proteins (2,3,16). In 3T3-L1 adipocytes, they translocate from internal membrane to cytosolic compartments after insulin exposure of the cells (16). In the case of IRS-1, the intracellular compartment where its phosphorylation occurs appears to be in close proximity to the plasma membrane. In fact, phosphorylation of IRS-1 appears to be independent of insulin receptor endocytosis and initiated activation of the insulin receptor localized on the plasma membrane (9,10,36). Here, we show that phosphorylation of IRS-2 in liver and muscle cells requires the internalization of the active

insulin receptor kinase. Therefore, phosphorylation of IRS-2 may occur in a compartment of the cell distinct from that where phosphorylation of IRS-1 is accomplished.

In conclusion, we have shown that a single genetic defect is able to affect insulin signaling and action differently in liver and in skeletal muscle. Hence, in the IR_{1152} patients, the preserved hepatic insulin sensitivity is likely to limit the metabolic derangement expected on the basis of the severe degree of insulin resistance in skeletal muscle. In addition, we have provided evidence that receptor internalization, an early event following insulin binding, is necessary for enabling IRS-2, but not IRS-1, phosphorylation in these cells.

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