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$_{1152}$ (HepMUT) 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 HepMUT and L6 skeletal muscle cells expressing the IR$_{1152}$ mutant (L6MUT). At variance, IRS-2 phosphorylation exhibited high basal levels with no further insulin-dependent increase in L6MUT, but almost normal phosphorylation, both basal and insulin-stimulated, in the HepMUT cells. In vitro, IR$_{1152}$ mutant preparations from both the L6MUT and the HepMUT 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

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$_{1152}$→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<sub>1152</sub>) 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 stimulable 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<sub>1152</sub> 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<sub>1152</sub> 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<sub>1152</sub> mutant selectively in liver and not in muscle.

**Materials and methods.**

**RESEARCH AND METHODS**

**Materials and media for tissue culture and the transfection reagent Lipo-**

**fectamine (N-[1-(2,3-diomeooyloxy)-propyl]-N,N,N-trimethylammoniumchloridio/dieoylphosphatidylthetolamine) were purchased from Life Tech-**

**nologies (Grand Island, NY). Electrotransfection reagents were from Bio-Rad**

**(Richmond, VA). Protein A-sepharose beads and sulfo-hydroxysuccinimide**

**long-chain biotin were purchased from Pierce (Rockford, IL). All radio-**

**chemicals, Western blot, and enhanced chemiluminescence (ECL) reagents**

**were from Amersharm (Arlington Heights, IL). Monoclonal Ig2 phosphoty-**

**rosine 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).

**Materials and methods.**

**In vitro 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 · kg⁻¹ · min⁻¹) were infused intravenously for 120 min each, while the glucose concentration was determined with a Beckman glucose analyzer (H9251) at 15-min intervals.

**Metabolic labeling.** For detection of insulin receptor and IRS expression, cells were treated with [¹²⁵I]insulin binding and Western blot analysis with insulin receptor antibodies (29).

**Metabolic labeling.** For detection of insulin receptor α and β subunits, cells were labeled by adding [¹²⁵I]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 M 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 apro- linal, 1 mmol/l pepstatin, 2 mmol/l sodium orthovanadate, 1 mmol/l PMSF, 20 min at 100,000 g (4°C) and precipitated with 2 µg insulin receptor antibo-}

**...
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 IR1152 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 IR1152 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 IR1152 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 [¹²⁵I]insulin binding. Several cell clones were isolated. Two clones expressing 2.8 × 10⁴ (HepWT1) and 0.8 × 10⁴ (HepWT2) wild-type receptors and 2 expressing 2.5 × 10⁴ (HepMUT1) and 1 × 10⁵ (HepMUT2) IR1152 receptors per cell were studied in detail. Based on Scatchard analysis (28) of equilibrium binding data, all these clones displayed dissociation constants (Kₐ) for insulin between 0.8 and 1.4 mmol/l (Table 1). These values are similar to the Kₐ of endogenous insulin receptors measured in hepatocytes from wild-type animals (which express ~10⁵ 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 [³⁵S]methionine. The radio-labeled 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 receptor α- and β-subunits, respectively.

Table 1.

Table of Scatchard analysis of insulin binding data

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>Kₐ (nmol/l)</th>
<th>Receptors/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepWT1</td>
<td>0.8</td>
<td>2.8 × 10⁴</td>
</tr>
<tr>
<td>HepWT2</td>
<td>1.2</td>
<td>0.8 × 10⁴</td>
</tr>
<tr>
<td>HepMUT1</td>
<td>1.4</td>
<td>2.5 × 10⁵</td>
</tr>
<tr>
<td>HepMUT2</td>
<td>1.0</td>
<td>1.0 × 10⁵</td>
</tr>
</tbody>
</table>
receptor α- and β-subunits, respectively (Fig. 2B–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. 2B). Consistently, α and β insulin receptor subunits were undetectable in untransfected hepatocytes from insulin receptor–deficient mice (Fig. 2A, lane A). The rate of insulin receptor biosynthesis was also examined by labeling the cells with [35S]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 IR1152 receptor increased basal levels of both glucokinase and glycogen synthase activities by 15% (HepMUT1) and 40% (HepMUT2) compared with those measured in cells expressing wild-type receptors (P < 0.001 by t test) (Fig. 3A 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 those expressing the wild-type receptors (Fig. 3C). These results are different from our previous findings in L6 skeletal muscle cells and NIH-3T3 cells expressing the IR1152 mutant, in which most insulin metabolic effectors were maximally activated in the basal state and not further stimulatable by insulin (20,22,26).

**IR1152 signaling in hepatocytes.** Our previous observations in L6 skeletal muscle cells indicated that expression of IR1152 induces constitutive phosphorylation of IRS-2 and prevents further activation in response to insulin (20). In HepMUT1 and HepMUT2 cells, IR1152 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. 4A). Insulin (100 nmol/l) elicited no effect on IRS-2 phosphorylation in the untransfected Hep−/− cells. In the HepMUT1 and HepMUT2 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 (HepWT1 and HepWT2 cells). Similar to that of IRS-2, phosphorylation of IRS-1 in the HepMUT1 and HepMUT2 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. 4B). At 100 nmol/l insulin, phosphorylation of IRS-1 in the HepWT1 cells was only partially abolished by virtue of the

**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).
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. 4C). Thus, IR1152 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 IR1152 kinase activity using immobilized IRS-1 as substrate showed a 30% increase versus that of the wild-type receptors (P < 0.001) (Fig. 5A). 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. IR1152 receptors, whether derived from muscle or liver cells, exhibited maximally activated kinase activity toward immobilized liver IRS-2 (Fig. 5B). 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, IR1152 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 IR1152 kinase was accompanied by a specific increase in IR1152 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. 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 [125I]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 [125I]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 IR1152 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.
wild-type (L6WT1) or mutant (L6MUT1) insulin receptor, almost -2 in muscle and liver cells. In L6 cells, whether expressing aim, we first compared the subcellular localization of IRS-1 and IRS-2 in liver cells in the absence of insulin stimulation. To this that the mutant receptor could not effectively interact with intact hepatocytes and muscle cells, we tested the hypothesis from the muscle cells, suggesting that the differential found in the PM fraction. IRS-2 and -1 compartmentalization in the Hep cells, either HepWT1 or HepMUT1, showed no differ- in the Hep cells, either HepWT1 or HepMUT1, showed no differ- variance with IRS-2, at least 75% of IRS-1 was observed in the 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 HepWT1 or HepMUT1, showed no differ- from the muscle cells, suggesting that the differential IR1152 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 × 10^6 and 3.2 × 10^6 wild-type insulin receptors per cell (L6WT1 and L6WT2, respectively), insulin increased receptor internalization by 3-fold, results similar to those in cells expressing only the endogenous complement of insulin receptors (Fig. 7A). In contrast, in L6 cells expressing 1.9 × 10^6 and 3.0 × 10^6 IR1152 receptors per cell (L6MUT1 and L6MUT2, 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. 7B). In the hepatocytes, the internalization increased by 2.5-fold upon insulin exposure, independent of whether the hepatocytes expressed wild-type or mutant receptors. Therefore, IR1152 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. 7C, IRS-1 phosphorylation in the L6WT cells reached comparable levels upon insulin stimulation at both 37°C and 15°C. Decreasing the temperature did not affect the phosphorylation of IRS-1 in the L6MUT 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 IR1152 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 IR1152 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 IR1152, 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 IR1152 patients (19). Thus, IR1152 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 those 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 IR1152 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 IR1152 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, IR1152 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 IR1152 mutant–expressing hepatocytes with the tyrosine phosphatase inhibitor ortho- vanadate 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 IR1152 mutant activity in liver and muscle cells. Finally, in vitro, partially purified IR1152 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 IR1152 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, IR1152 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 IR1152 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, IR1152 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 IR1152. In fact, 1) in L6 muscle cells, inhibition of the constitutive IR1152 mutant internalization by low temperature simultaneously blocks IRS-2 phosphorylation; 2) as at the low temperature, inhibition of IR1152 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 IR1152 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 IR1152 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 IR1152 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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