Differential Contribution of Insulin Receptor Substrates 1 Versus 2 to Insulin Signaling and Glucose Uptake in L6 Myotubes*

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Insulin receptor substrates-1 and 2 (IRS-1 and IRS-2) are pivotal in relaying insulin signaling in insulin-responsive tissues such as muscle. However, the precise contribution of IRS-1 vis-à-vis IRS-2 in insulin-mediated metabolic and mitogenic responses has not been compared directly in differentiated muscle cells. This study aimed to determine the relative contribution of IRS-1 versus IRS-2 in these responses, using small interfering RNA (siRNA)-mediated specific gene silencing. In L6 myotubes, transfection of siRNA targeted specifically against IRS-1 (siIRS-1) or IRS-2 (siIRS-2) reduced the cognate protein expression by 70–75%. Insulin-induced ERK phosphorylation was much more sensitive to IRS-2 than IRS-1 ablation, whereas p38MAPK phosphorylation was reduced by 43 or 62% in myotubes treated with siIRS-1 or siIRS-2, respectively. Insulin-induced Akt1 and Akt2 phosphorylation was reduced in myotubes treated with siIRS-1, but only Akt2 phosphorylation was reduced in myotubes treated with siIRS-2. In contrast, siIRS-1 treatment caused a marked reduction in insulin-induced actin remodeling, glucose uptake, and GLUT4 translocation, and siIRS-2 was without effect on these responses. Notably, combined siIRS-1 and siIRS-2, although reducing each IRS by around 75%, caused no further drop in glucose uptake than that achieved with siIRS-1 alone, but abolished p38MAPK phosphorylation. We conclude that insulin-stimulated Akt1 phosphorylation, actin remodeling, GLUT4 translocation, and glucose uptake are regulated mainly by IRS-1, whereas IRS-2 contributes selectively to ERK signaling, and Akt2 and p38MAPK lie downstream of both IRS in muscle cells.

Insulin receptor substrates (IRSs)† mediate diverse metabolic and mitogenic effects of insulin, and dysregulation of IRS expression and activation has been observed in both insulin resistance and diabetes (1–5). To date, six IRS proteins have been identified, but only IRS-1 and IRS-2 are thought to participate in regulation of glucose homoeostasis (6–9). Although IRS-1 and 2 have substantial amino acid similarity (10), there are significant structural differences between them. IRS-2 interacts with the insulin receptor via a phosphotyrosine-binding domain as well as a central domain located between amino acid 591 and 733 which is absent from IRS-1 (11, 12). Furthermore, IRS-1 and IRS-2 differ in their time course of insulin-stimulated tyrosine phosphorylation (13, 14), interaction with the MAPK pathway (15), and intracellular localization (14).

Defining the exact roles of IRS isoforms in insulin-stimulated glucose uptake is not without controversy. In rat adipocytes, overexpression of human IRS-1 increased, whereas elimination of IRS-1 via antisense ribozyme reduced, insulin-stimulated GLUT4 translocation (16). However, other studies observed intact insulin-stimulated GLUT4 translocation when the interaction between insulin receptor and IRS was blocked, although insulin-mediated DNA synthesis and cell growth were reduced (17–19). Furthermore, IRS-1-null mice, although significantly growth-retarded, do not develop overt diabetes, suggesting that IRS-1 plays a more important role in mediating the mitogenic rather than the metabolic effects of insulin (20, 21). When the insulin response of individual tissues was examined, however, phosphatidylinositol 3-kinase activation and glucose uptake were reduced in muscle (22) and adipocytes (23), although the liver preserved normal phosphatidylinositol 3-kinase and MAPK activities (22). These latter results suggested that IRS-1 participates in insulin-stimulated glucose uptake in muscle and adipocytes, whereas in liver, other IRS protein(s) is responsible for insulin action.

IRS-2 was identified as the alternative insulin receptor substrate in the liver and muscle of the IRS-1-null mice (10, 24). Overexpression of IRS-2 in rat adipocytes increased GLUT4 translocation (25), whereas brown adipocytes isolated from IRS-2-null mice showed reduced insulin-stimulated glucose transport and GLUT4 translocation (26). Interestingly, when stimulated with insulin ex vivo, isolated soleus muscles from the IRS-2-null and wild type mice had similar glucose uptake response, albeit with a lower basal glucose transport rate, suggesting that IRS-2 may not be required for insulin-stimulated glucose transport in skeletal muscle (27). Of note, the total GLUT4 expression was decreased in the IRS-2-null mice, yet the insulin-stimulated glucose uptake response was not diminished. A later in vivo study using the hyperinsulinemic-euglycemic clamp, however, showed that although IRS-1 is the major regulator of glucose transport in muscle, IRS-2 also participates in glucose metabolism in muscle, fat, and liver (28). These studies are summarized in Table I. Taken together, studies to date support the requirement of IRS-1 in insulin-stimulated glucose metabolism in muscle, whereas the precise role of IRS-2 in this tissue requires further investigation.

Insulin elicits both mitogenic and metabolic responses, which include activation of the MAPK pathway and gene expression, as well as increasing Akt activity and inducing actin

1 The abbreviations used are: IRS, insulin receptor substrate; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IGF-I, insulin-like growth factor I; α-MEM, α-minimum Eagle’s medium; siIRS-1, IRS-1-specific siRNA; siIRS-2, IRS-2-specific siRNA; siNR, or nonrelevant siRNA control; siRNA, small interfering RNA.

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remodeling. The latter two participate in the regulation of insulin-stimulated GLUT4 translocation and glucose uptake (29–33). The objective of this study was to determine the relative contribution of IRS-1 versus IRS-2 in these responses, using siRNA-mediated specific gene silencing. The transient nature of siRNA-mediated protein reduction minimizes the possibility of compensatory up-regulation of other proteins that may mask the biological consequences of eliminating IRS-1/2. In addition, we made use of the L6-GLUT4myc rat skeletal muscle cell line that stably expresses a myc-tagged GLUT4, where the expression of the GLUT4myc is constant and resistant to manipulations that may affect expression of other proteins (34). This allows us to determine the importance of IRS-1/2 in the regulation of glucose uptake and GLUT4 translocation without the confounding factor of altered GLUT4 expression (34–36), as was observed in the skeletal muscle of IRS-2-null mice.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phospho-specific antibodies to p38MAPK (dual phospho-tyrosine Thr180 and Tyr182), Akt (Thr308 and Ser473), and ERK were purchased from Cell Signaling (Beverly, MA). Polyclonal anti-phosphothreonine, anti-IRS-1, anti-IRS-2, and anti-Rat-Akt2 (immunoadfinity-purified sheep IgG) antibodies (for immunoprecipitation, immunoblotting) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The sequence of the IRS-1 siRNA used was AAC AAG ACA GCU GGU AGG and TCT GGG GAG TCC TGA TTG CAT. The nonrelevant control siRNA sequence was purchased from Dharmacon (Lafayette, CO). SiRNAs targeted against IRS-1 (siIRS-1), IRS-2 (siIRS-2), and nonrelevant control (siNR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IRSs were deprived of serum for 5 h prior to stimulation with insulin.

**Determination of 2-Deoxyglucose Uptake**—2-Deoxyglucose uptake measurements were carried out as described previously (36) for 5 min in HEPES-buffered saline containing 10 μM 2-[14C]deoxyglucose (0.5 μCi/μl) in the absence of insulin. Glucose transport was stopped with ice-cold 0.25 M NaCl containing 1 mM HgCl2. Nonspecific uptake was determined in the presence of 10 μM cytochalasin B and subtracted from all experimental values.

**Reverse Transcription-PCR of Insulin Receptor Isoforms**—Isolation of total RNA and reverse transcription-PCR of insulin receptor from L6 myotubes were performed as described previously (41). The primers used to amplify insulin receptor were: CAT TCA GGA AGA CCT TCG AGG and TCT GGG GAG TCA TGC CAT TGAT.

**Measurement of GLUT4myc Translocation in L6 Myotubes**—Cell surface myc-tagged GLUT4 was quantified by an antibody-coupled fluorometric assay as validated previously (35). Briefly, after a 20-min incubation with insulin at indicated concentrations, confluent myotubes were rinsed with PBS, fixed with 4% paraformaldehyde for 10 min, and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:750) for 45 min, all at 4 °C. Cells were washed six times with phosphate-buffered saline, and 1 ml of OPD reagent was added for 30 min at room temperature. The reaction was stopped with 0.25 ml of 3 N HCl. The supernatant was collected, and absorbance was measured at 492 nm. Nonspecific IgG binding, as measured by a peroxidase-conjugated anti-rabbit IgG and

**Reverse Transfection**—L6 myotubes were transfected with siIRS-2 using a calcium phosphate-based transfection reagent (CellPhect transfection kit, Athera Biosciences), according to the manufacturer’s instructions. Briefly, cells were seeded at 30% confluence in α-MEM supplemented with 10% FBS without antibiotics/antimycotics. On day 2, myotubes were transfected with 50 nM siRNA, and the medium was changed 12–14 h later to α-MEM supplemented with 2% FBS with 1% antibiotics/antimycotics. Cells were transfected again on day 5 with 100 nM siRNA. siRNA transfection had no deleterious effects on either cell viability or fusion of myoblasts to form myotubes.

**Double elimination of IRS-1 and IRS-2** was achieved using the calcium phosphate transfection protocol described above.

**Fluorescence Microscopy**—L6-GLUT4myc myotubes grown on 25-mm-diameter glass coverslips were deprived of serum for 5 h and treated with 0–100 nM insulin for 10 min at 37 °C. Myotubes were fixed with 4% formaldehyde immediately at 4 °C after insulin stimulation and permeabilized in 0.1% (v/v) Triton X-100 for 3 min to preserve actin morphology. Actin filaments were labeled with rhodamine-coupled phalloidin, and immunostaining of IRS-1 or IRS-2 in fixed and permeabilized myotubes was carried out as described previously (39). Primary antibody (IRS-1 and IRS-2 purchased from Upstate Biotechnology) was used at 1:500 dilution. Cells were examined with a Zeiss LSM 510 laser scanning confocal microscope. Acquisition parameters were adjusted to exclude saturation of the pixels. For quantification, such parameters were kept constant among the various conditions compared.

**Reverse Transfection—**L6 myotubes were transfected with 100 nM insulin for 5 min (for IRS-1 studies) or 3 min (for IRS-2 studies), times at which phosphorylation of each isoform peaks (13). IRS-1 and IRS-2 were immunoprecipitated from L6 myotubes as described previously (34, 40). Briefly, 500 μg or 1 mg of protein of whole cell lysates in Triton X-100 were incubated with IRS-1- or IRS-2-specific antibodies at 4 °C overnight under constant rotation, and the antibody-antigen complex was pulled down by protein A/G-Sepharose beads. The pelleted proteins were resolved by SDS-PAGE, and serine and threonine phosphorylation of Akt1 and Akt2 was detected by immunoblotting using anti-phospho-AktSer473 or –Thr308 antibody.

**Immunoprecipitation of Akt1 and Akt2—L6 myotubes were transfected with 200 μg of cell lysates in Triton X-100 containing phosphatase and protease inhibitors (44). The antibody-antigen complex was pulled down by protein A/G-Sepharose beads. The pelleted proteins were resolved by 10% SDS-PAGE, and serine and threonine phosphorylation of Akt1 or Akt2 was detected by immunoblotting using anti-phospho-AktSer473 or –Thr308 antibody.

**Reverse Transfection—**L6 myotubes were transfected with 100 nM insulin for 10 min. We have determined previously that 10 min of stimulation yields maximal phosphorylation of Akt in these cells (44). Immunoprecipitation of Akt1 and Akt2 was performed from 200 μg of cell lysates in Triton X-100 containing phosphatase and protease inhibitors (44). The antibody-antigen complex was pulled down by protein A/G-Sepharose beads. The pelleted proteins were resolved by 10% SDS-PAGE, and serine and threonine phosphorylation of Akt1 or Akt2 was detected by immunoblotting using anti-phospho-AktSer473 or –Thr308 antibody.

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omitting the primary antibody, was subtracted from all experimental values.

Detection of Cellular Protein Expression and Protein Phosphorylation—Whole cell lysates were made from myotubes after incubation with insulin (0–100 nM) for 10 min (as described previously (42)). We have determined previously that 10 min of stimulation yields maximal phosphorylation of Akt and p38MAPK and is also the time shown previously to yield robust ERK phosphorylation in these cells (43). 40 μg of lysates was resolved by SDS-PAGE and transferred onto polyvinylidine difluoride filters, which were incubated with primary antibodies overnight at 4 °C under constant agitation (dilutions: polyclonal anti-IRS-1 or anti-IRS-2, 1:1,000; anti-Akt, anti-ERK, and anti-p38MAPK, 1:500–1:1,000; anti-phospho-Akt-Ser⁴⁷³ or -Thr³⁸³, anti-phospho-ERK, and anti-phospho-p38MAPK, 1:500), followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibody (1:7,500 sheep anti-rabbit antibody, 1:7,500 sheep anti-mouse antibody). Protein was visualized by the enhanced chemiluminescence method and scanned within the linear range using ImageJ software.

RESULTS
Insulin-stimulated Tyrosine Phosphorylation of IRS-2 Is More Insulin-sensitive Than That of IRS-1—We first compared the insulin sensitivity of IRS-1 tyrosine phosphorylation with that of IRS-2. L6-GLUT4myc myotubes were incubated with increasing concentrations of insulin (0.5–100 nM) for 3–5 min, and the results were expressed as a percentage of the response observed at 100 nM insulin. As shown in Fig. 1, insulin-stimulated tyrosine phosphorylation of IRS-2 is more sensitive at low concentrations of insulin than is IRS-1, with an EC₅₀ of 0.5 nM for IRS-2 versus 5–8 nM for IRS-1. A similar difference of insulin sensitivity of IRS-1 and IRS-2 tyrosine phosphorylation was observed in myoblasts (results not shown). Hence, any possible presence of myoblasts in the cultures would be unlikely to be responsible for the marked difference in insulin-sensitivity of IRS-1 and IRS-2.

Akt Phosphorylation Is More Insulin-sensitive Than Phosphorylation of p38MAPK or ERK—Akt, p38MAPK, and ERK have been shown to regulate GLUT4 translocation, glucose uptake, and mitogenic responses, respectively. However, with the exception of Akt, it is unclear whether p38MAPK and ERK are directly downstream of IRS in the insulin signaling pathway. Therefore, we next examined the insulin sensitivities of these enzymes compared with those of IRS-1 and IRS-2. L6 myotubes were treated with increasing concentrations of insulin (0.5–100 nM) for 10 min, and whole cell lysates were prepared. In response to insulin, Akt is phosphorylated at threonine 308 and serine 473, p38MAPK at threonine 180 and tyrosine 182, and ERK at threonine 202 and tyrosine 204. As shown in Fig. 2, insulin-mediated serine phosphorylation of Akt was detected at a lower concentration of insulin than phosphorylation of p38MAPK or ERK, beginning at 0.5, 2, and 5 nM insulin, respectively. Again, the differential sensitivity of these signaling molecules to insulin is unlikely to arise from any residual presence of myoblasts in the culture because, as reported previously, Akt activation and expression (36) and p38MAPK expression (44) did not differ during myogenic differentiation of the culture.

Insulin-induced Actin Structures Are Observed at a Low Concentration of Insulin and Co-localize with IRS-1 but Not IRS-2—One of the earliest responses to insulin is the rearrangement of actin filaments into a cortical mesh. This phenomenon occurs in muscle cells (29, 31, 39, 45), adipocytes (46, 47), and skeletal muscle (48). Actin remodeling is deemed necessary for GLUT4 translocation, but its insulin dose sensitivity was not known. L6 myotubes were incubated with 0.5–100 nM insulin for 10 min and then stained F-actin with rhodamine-phalloidin. Insulin-induced cortical actin structures were observed at 0.5 nM insulin. The remodeled actin structures, clearly visualized on the dorsal plane of the myotubes, became larger and more distinctive with increasing concentrations of the hormone (Fig. 3).

IRS-1 and IRS-2 Participate in Insulin-stimulated Phosphorylation of Akt—The results so far revealed that a low insulin concentration (0.5 nM) stimulated tyrosine phosphorylation of IRS-2, Akt phosphorylation, and actin remodeling, whereas a higher concentration of insulin (≥5 nM) was required to obtain appreciable IRS-1, p38MAPK, and ERK phosphorylation. It is unknown whether these correlations reflect differential regulation of Akt, p38MAPK, ERK activation, and actin remodeling by IRS-2 versus IRS-1. Therefore, we induced gene-specific silencing in L6 myotubes, using siIRS-1 and siIRS-2, to determine their relative contribution in insulin action.

Using siIRS-1 we achieved a 75% reduction in IRS-1 protein expression with little reduction in IRS-2 expression; conversely, siIRS-2 reduced IRS-2 protein by 70% with no effect on IRS-1 expression (Fig. 4A). siRNA-treated L6 myotubes were incubated with submaximal (5 nM) or maximal (100 nM) con-
centrations of insulin for 10 min, and Akt phosphorylation at threonine 308 and serine 473 was determined. The insulin-stimulated (100 nM, 10 min) increase in Akt phosphorylation above basal of siNR-treated cells was designated as the maximal response and expressed as 100%. Akt phosphorylation shown in Fig. 4 reflects the net behavior of IRS-1 and IRS-2 to the major Akt isoforms 1 and 2, siRNA-treated L6 myotubes were incubated without or with 100 nM insulin for 10 min, and Akt1 or Akt2 was individually immunoprecipitated and probed with anti-phospho-Akt antibodies. Fig. 5 shows that reduction of IRS-1 expression led to a blunted insulin-stimulated serine phosphorylation of Akt1 (66 ± 11% versus siNR-treated myotubes) and of Akt2 (55 ± 5% versus siNR-treated myotubes). In contrast, reduction of IRS-2 expression affected only Akt2 phosphorylation (62 ± 8% versus siNR-treated myotubes). Similar observations were made for threonine phosphorylation in all cases (results not shown).

**IRS-1 and IRS-2 Differentially Mediate Insulin-stimulated Phosphorylation of p38MAPK and ERK—Insulin causes activation of ERK and p38MAPK in several cell lines. We therefore examined the contribution of IRS-1 and IRS-2 in relaying the insulin signal to these MAPK pathways. In cells treated with siIRS-1, insulin-stimulated phosphorylation of p38MAPK at submaximal (5 nM) and maximal (100 nM) insulin concentrations was 17 ± 7% and 57 ± 2% of that in cells treated with siNR and stimulated with 100 nM insulin (for 10 min). In cells treated with siIRS-2, insulin-stimulated phosphorylation of p38MAPK at 5 or 100 nM was 25 ± 10% and 38 ± 5% of the maximal response in siNR-treated cells (Fig. 6A). Again, siIRS-1 and siIRS-2 had no effect on p38MAPK protein expression (Fig. 6A).

Interestingly, and in contrast to p38MAPK, IRS-2 appeared to contribute more significantly than IRS-1 to the insulin-stimulated activation of ERK (Fig. 6B). ERK was minimally phosphorylated at 5 nM insulin, whether in cells transfected with siIRS-1, siIRS-2, or siNR. However, at the maximal insulin dose (100 nM), ERK phosphorylation in cells transfected with siIRS-1 was 76 ± 0.6% of the control (siNR-transfected) cells, whereas ERK phosphorylation in siIRS-2-transfected cells was only 15 ± 5% of the controls (Fig. 6B).

**IRS-1 but Not IRS-2 Leads to Insulin-stimulated Glucose Uptake and GLUT4 Translocation—**To our surprise, a 70% reduction in IRS-2 had no effect on insulin-stimulated glucose uptake or GLUT4 translocation at either submaximal (5 nM) or maximal (100 nM) insulin concentrations (Fig. 7B). In contrast,
siRNA-mediated reduction of IRS-1 by 75% significantly reduced insulin-stimulated glucose uptake and GLUT4 translocation (Fig. 7A). Compared with cells transfected with siNR, siIRS-1-treated cells experienced 61 and 51% reductions in insulin-stimulated glucose uptake at submaximal (5 nM) and maximal (100 nM) insulin concentrations, respectively (Fig. 7A). Similar reductions were observed in insulin-stimulated translocation of GLUT4 to cell surface (Fig. 7A).

Unlike the insulin response, basal glucose uptake was not affected by reduction in either IRS-1 or IRS-2, as follows. In the experiments analyzing the effect of siIRS-1, basal glucose uptake was 26 pmol/min/mg of protein in the control siNR-treated myotubes and 30 pmol/min/mg of protein in siIRS-1-treated myotubes measured in parallel. In the siIRS-2 experiments, basal glucose uptake was 15 pmol/min/mg of protein in the control siNR-treated myotubes and 16.5 pmol/min/mg of protein in siIRS-2 myotubes. Likewise, the basal amount of cell surface GLUT4myc was not significantly affected by siIRS-1 or siIRS-2 (for siIRS-1, surface GLUT4myc was 1.23 ± 0.04 relative to siNR, which was assigned a value of 1; for siIRS-2, surface GLUT4myc was 1.06 ± 0.1 relative to siNR).

Consistent with the participation of actin remodeling in insulin-stimulated GLUT4 translocation, a much diminished insulin-induced cortical actin structure was apparent in myotubes treated with siRNA against IRS-1 (Fig. 8A) but not in myotubes treated with siIRS-2 (Fig. 8B). Consistent with these observations, IRS-1 colocalized with the insulin-stimulated actin mesh (39), but no significant colocalization between actin structures and IRS-2 was noted (data not shown).

It is possible that the drop in IRS-2 expression had no effect on insulin-stimulated glucose uptake because IRS-1 substituted for IRS-2 in this metabolic function. To test this possibility, L6 cells were transfected with siRNAs targeted against both IRS-1 and IRS-2. This strategy (siIRS-1/2) achieved 73 and 79% elimination of IRS-1 and IRS-2 protein, respectively, but insulin-stimulated glucose uptake was not further reduced compared with cells transfected with only siIRS-1 (Fig. 9A).

Insulin-stimulated phosphorylation of Akt (Ser473) was reduced to a similar extent in cells transfected with siIRS-1, siIRS-2, or siIRS-1/2 (Fig. 9B, left panel). Thr308 phosphorylation was somewhat lower in cells transfected with siIRS-2 or siIRS-1/2 compared with siIRS-1-transfected cells, but the dif-
Since the identification of IRS-1 and IRS-2, studies in various biological systems, including IRS-1 and IRS-2 knock-out mice, have supported a pivotal role for IRS isoforms in the metabolic and mitogenic effects of insulin (7, 8). However, there are conflicting reports on the precise role of IRSs in insulin-stimulated GLUT4 translocation and glucose transport (16, 18, 26, 28), and to our knowledge, the relative contribution of IRS-1 vis-à-vis IRS-2 in insulin-stimulated glucose uptake and GLUT4 translocation, as well as in Akt and MAPK signaling, has not been compared directly in muscle cells. In this study, we made use of siRNA-mediated gene silencing to knock down specifically IRS-1 or IRS-2 in L6 myotubes. We found that reducing endogenous IRS-1 protein by 70% (without reduction in IRS-2) abated by 55% the insulin-stimulated GLUT4 translocation to the cell surface, with a corresponding drop in insulin-stimulated glucose uptake. Interestingly, reducing endogenous IRS-2 expression by 75% had no effect on insulin-stimulated glucose uptake or GLUT4 translocation (see Table II).

As shown in Fig. 1, insulin-stimulated tyrosine phosphorylation of IRS-2 is more insulin-sensitive than that of IRS-1, and similar but less pronounced differences have been reported earlier (13, 49). Because tyrosine phosphorylation of IRS by insulin requires recruitment to the insulin receptor, this differential insulin sensitivity could potentially be secondary to differential recruitment of IRS-1 and IRS-2 by the two spliced isoforms of the insulin receptor, A or B (50–52). However, by reverse transcription-PCR amplification of mRNA isolated from L6-GLUT4myc myoblasts and myotubes, only isoform A of the insulin receptor was transcribed (data not shown). Therefore, the mechanism for the differential insulin sensitivities of IRS-1 versus IRS-2 tyrosine phosphorylation most likely resides in differences between the two IRS isoforms.

Indeed, although IRS-1 and 2 share between 75% (N-terminal region) and 35% (C-terminal region) amino acid identity, they display significant differences that may contribute to their functional divergence. First, IRS-2 interacts with the insulin receptor and insulin-like growth factor I (IGF-I) receptors via its phosphotyrosine-binding domain as well as a central domain located between amino acids 591 and 733 which is absent from IRS-1. This interaction requires a tyrosine-phosphorylated receptor but it is independent of the NPX(p)(Y)(Tyr) site (11, 12). The presence of this domain in IRS-2 but not in IRS-1 allows IRS-2 to interact with the insulin receptor independently of the phosphotyrosine-binding domain, and insulin-mediated tyrosine phosphorylation of IRS-2 can be eliminated by interfering with insulin receptor-IRS-2 interaction using a peptide that corresponds to this region (53). Second, IRS-1 but not IRS-2 can modulate insulin receptor tyrosine phosphorylation and activity in response insulin, thereby influencing downstream insulin signaling (54). Third, IRS-1 and 2 differ in their intracellular compartmentalization. In 3T3-L1 adipocytes, IRS-1 is more abundant in intracellular membranes than in the cytosol, whereas IRS-2 is more concentrated in the cytosol, and in response to insulin, the association of phosphatidylinositol 3-kinase with IRS-1 in the intracellular membranes is more stable than with IRS-2 (14). Differences in the time course of action of IRS-1 and IRS-2 also occur, where insulin-stimulated tyrosine phosphorylation of IRS-1 is highly sustained and detectable after 60 min, whereas IRS-2 tyrosine phosphorylation peaks around 3 min and becomes essentially undetectable after 10 min of insulin stimulation (13). Taken together, these distinct characteristics of IRS-1 and IRS-2 as well as their differential insulin sensitivities prompted us to investigate their relative roles in insulin signaling, glucose uptake, and GLUT4 translocation.

As became apparent under “Results” and is discussed further below, the cause-and-effect relationship of IRS-1 or IRS-2 to signaling outcomes does not necessarily adhere to correlations of phosphorylation levels of IRS isoforms and the signals in insulin dose-response curves. Hence, we explored the possible cause-effect relationship upon ablation of each IRS. We report that isoform-specific gene silencing can be achieved by trans-
fecting siRNA into L6 myotubes, with >70% reduction of the targeted protein(s) (Fig. 4). The advantage of this approach is that it achieves transient elimination of specific proteins within a time frame where compensatory up-regulation of alternative signaling pathway is less likely than in gene knockout mice. This system also circumvents the confounding effects of metabolic derangements and organ-to-organ cross-talks that occur in IRS-1- and IRS-2-null mice, which may affect the insulin response independently of the direct IRS effects. We found that a 70% loss of endogenous IRS-1 had only a small effect on activation of ERK (Fig. 6B, upper panel). Similar results have been observed in IRS-1-null 3T3 cells, where activation of ERK1 and ERK2 by IGF-I was normal compared with wild type cells (55). SiRNA-mediated reduction of IRS-2, however, reduced insulin-stimulated ERK phosphorylation by 85% (Fig. 6B, lower panel). These observations suggest that IRS-2 may have a more prominent role in mediating insulin-stimulated mitogenic signaling in L6 myotubes, which occurs largely downstream of ERK.

p38MAPK is activated by insulin (42, 56–58), and dysregulation of p38MAPK activity has been observed in both in vitro models of insulin resistance (34) as well as in adipocytes and muscles from diabetic patients (58, 59). We found that siRNA-mediated reduction of either IRS-1 or IRS-2 significantly tapered insulin-stimulated p38MAPK phosphorylation, with IRS-2 having a slightly more pronounced effect. When both IRS-1 and IRS-2 were reduced, p38MAPK phosphorylation was decreased almost additively (Fig. 9C), suggesting that each IRS isoform contributes separately to this activation. To our knowledge, this is the first demonstration that IRS-1 and IRS-2 are upstream of p38MAPK in insulin signaling.

One of the key findings of this study is that elimination of 75% of IRS-2 did not prevent the full stimulation of glucose uptake by insulin, whereas a comparable elimination of IRS-1 halved the hormonal response of glucose uptake (Fig. 7). These results are consistent with findings in muscles isolated from IRS-1- and IRS-2-null mice (22, 27) (see Table II). In contrast to muscle, in adipose tissues both IRS-1 and IRS-2 seem to be important for the metabolic actions of insulin because isolated primary adipocytes from IRS-1-null mice and brown adipocytes...
from IRS-2-null mice have impaired insulin-stimulated GLUT4 translocation and glucose uptake (23, 26). These results again highlight the differential regulation of insulin signaling pathways and the importance of examining the function of signaling intermediates in a tissue-specific manner.

Another major result is the differential sensitivity of Akt isoforms to substantial IRS-1 or IRS-2 elimination. Whereas reducing each by about 70% lowered the net insulin-stimulated Akt phosphorylation by 40–50% (Figs. 4 and 5), there was no further loss of Akt phosphorylation upon combined reduction of both IRS isoforms (Fig. 9). This behavior led us to analyze the response of the two major Akt isoforms, Akt1 and Akt2. This led to the second major observation of this study, that Akt2 is sensitive to reductions in IRS-1 or IRS-2 expression, but only Akt1 is sensitive to a 75% loss of IRS-1. In each case, however, residual Akt-1 or Akt-2 activity remains, revealing that a certain degree of signal amplification occurs through the pathway.

Surprisingly, the status of insulin-stimulated Akt phosphorylation in muscles or adipocytes isolated from IRS-1-null mice has never been reported (22, 23). In undifferentiated L6 myoblasts, however, Pirola et al. (43) found that IRS-2 but not IRS-1 is required for the insulin-stimulated increase in Akt phosphorylation. There are two possible explanations for the difference between that study and the present results. First, regulation of insulin signaling pathway may change as myoblasts differentiate into myotubes. Second, Pirola et al. used a 10-fold higher concentration of insulin than we used in this study. It is possible that at this high dose of insulin, activation of Akt occurs through the IGF-I receptor, such that a decrease in insulin-mediated Akt activation through the insulin receptor via IRS-1 would evade detection. In this scenario, the IGF-1 receptor might have signaled via IRS-2.

The third major observation of this study is that the partial drop in insulin-stimulated Akt phosphorylation (Akt2 isoform) which occurs in siIRS-2-treated cells did not translate into lower insulin-dependent glucose uptake. In contrast, siIRS-1-treated cells displaying similar net reductions in Akt phosphorylation but with reductions in the response of both Akt1 and Akt2 experienced a >50% loss of insulin-stimulated glucose uptake and GLUT4 translocation (Fig. 7). How do we reconcile the impaired Akt phosphorylation in both siIRS-1- and siIRS-2-treated cells with the observation that glucose uptake and the GLUT4 translocation response are only di-
minished in the siIRS-1-treated cells? First, the residual activated Akt may be sufficient to maintain insulin action in siIRS-2-treated cells, provided that IRS-1 and IRS-2 access different pools of Akt. Although there are no studies to this effect, Akt2 is found predominantly at the plasma membrane whereas Akt1 is largely intracellular (62). Second, it is possible that only when a reduction in Akt-1 compounds the reduction in Akt2, glucose uptake is compromised. These results are consistent with the observation that Akt2+/− mice are more insulin-resistant than Akt1+/- mice (64). In adipocytes and Chinese hamster ovary cells, both Akt1 and Akt2 participate in insulin-stimulated glucose transport and GLUT4 translocation, although Akt2 plays a more prominent role (32). Third, inputs other than Akt contribute to GLUT4 translocation. Atypical protein kinase Cs have been shown to regulate glucose transport in brown adipocytes from IRS-2-null mice (63). Although not studied here, it is conceivable that the remaining 25% of IRS-2 in the siIRS-2-treated cells is sufficient to activate a protein kinase C and is responsible for the intact insulin-stimulated glucose transport. Finally, and perhaps most relevant, actin remodeling is crucial for GLUT4 translocation but is independent of Akt activity (30). Cortical actin remodeling was impaired when IRS-1 activation was prevented with a blocking peptide in 3T3-L1 adipocytes (18). As shown in Fig. 8, actin remodeling is downstream of IRS-1 but not of IRS-2. Hence, the defect in actin remodeling caused by IRS-1 silencing may compound the concomitant reduction in activation of Akt1 and Akt2, thereby diminishing GLUT4 translocation and glucose uptake. In the case of IRS-2 silencing, the reduction in Akt2 phosphorylation, in the absence of reduction in actin remodeling, may suffice to sustain normal GLUT4 translocation. This scenario is illustrated along with Table II. Whatever the explanation, it is clear that one cannot assume that there is a linear quantitative pathway from IRS to Akt to GLUT4 translocation without influence of other factors. Taken together, our results suggest that in muscle cells, IRS-1 and IRS-2 participate differentially in the mitogenic and metabolic signals of events downstream of Akt, ERK, and p38MAPK. IRS-1 regulates actin remodeling, Akt1 and Akt2 phosphorylation, GLUT4 translocation, and glucose uptake, whereas IRS-2 regulates Akt2 activation but has minimal contribution to these other functions. Conversely, ERK is primarily downstream of IRS-2, and p38MAPK is equally and complementarily activated via IRS-1 and IRS-2. It must be emphasized that because we only eliminated 70% of IRS-2 using siRNA, it remains possible that IRS-2 contributes to glucose uptake in muscle, although it is unlikely to have a profound effect. Future studies aimed to determine the atypical protein kinase C isoform(s) regulated by IRS-1 and IRS-2 will allow us to understand better the molecular basis of their differential impact on metabolic actions of insulin. From a health perspective, the results presented illustrate the differential sensitivity and resilience of distinct signaling pathways.
pathways to reductions of IRS-1 or IRS-2, which may have implications for patients with variations in this early step in insulin signaling.

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