Subcellular Localization of Insulin Receptor Substrate Family Proteins Associated With Phosphatidylinositol 3-Kinase Activity and Alterations in Lipolysis in Primary Mouse Adipocytes From IRS-1 Null Mice

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To clarify the roles of insulin receptor substrate (IRS) family proteins in phosphatidylinositol (PI) 3-kinase activation and insulin actions in adipocytes, we investigated the intracellular localization of IRS family proteins and PI 3-kinase activation in response to insulin by fractionation of mouse adipocytes from wild-type and IRS-1 null mice. In adipocytes from wild-type mice, tyrosine-phosphorylated IRS-1 and IRS-2, which were found to associate with PI 3-kinase in response to insulin, were detected in the plasma membrane (PM) and low-density microsome (LDM) fractions. By contrast, tyrosine-phosphorylated IRS-3 (pp60), which was found to associate with PI 3-kinase, was predominantly localized in the PM fraction. In adipocytes from IRS-1–null mice, insulin-stimulated PI 3-kinase activity in anti-phosphotyrosine (αPY) immunoprecipitates in the LDM fraction was almost exclusively mediated via IRS-2 and was reduced to 25%; however, insulin-stimulated PI 3-kinase activity in the PM fraction was primarily mediated via IRS-3 and was reduced to 60%. To determine the potential functional impact of the distinct subcellular localization of IRSs and associating PI 3-kinase activity on adipocyte-specific metabolic actions, we examined lipolysis in IRS-1 null mice. The level of isoproterenol-induced lipolysis was increased 5.1-fold in adipocytes from IRS-1 null mice as compared with wild-type mice. Moreover, hormone-sensitive lipase (HSL) protein was increased 4.3-fold in adipocytes from IRS-1–null mice compared with wild-type mice, and HSL mRNA expression was also increased. The antilipolytic effect of insulin in IRS-1 null adipocytes, however, was comparable to that in wild-type mice. Thus, discordance between these two insulin actions as well as the transcriptional and translational effect (HSL mRNA and protein regulation) and the PM effect (antilipolysis) of insulin may be explained by distinct roles of both PI 3-kinase activity associated with IRS-1/IRS-2 and PI 3-kinase activity associated with IRS-3 in insulin actions related to their subcellular localization. *Diabetes* 50:1455–1463, 2001

Insulin stimulates the tyrosine phosphorylation (via the tyrosine kinase of the insulin receptor) of several endogenous substrates, such as insulin receptor substrate (IRS) family proteins, Grb2-associated binder-1, and Shc (1–11). These proteins interact through their phosphotyrosine residues with Src homology-2 (SH2) domain–containing proteins, such as the regulatory 85-kDa subunit (p85) of phosphatidylinositol (PI) 3-kinase, Grb2, Crk, and SHPTP2 (10–15). Among these, PI 3-kinase activity has been shown to be required for metabolic actions of insulin, such as glucose transport, glycogen synthesis, and antilipolysis (16–18). The IRS family proteins IRS-1, -2, -3, and -4 contain a conserved pleckstrin homology (PH) domain and a phosphotyrosine-binding (PTB) domain that interacts with a phosphorylated NPXY motif in the insulin receptor at the NH_2-terminus (3–9).

IRS-1 has been shown to be the major substrate of the insulin receptor kinase, and we and others have demonstrated that IRS-1 null mice do in fact exhibit insulin resistance (19,20). We have also shown that insulin-induced PI 3-kinase activity in anti-phosphotyrosine (αPY) immunoprecipitates, glucose transport, and GLUT4 translocation in adipocytes from IRS-1 null mice were decreased to ~50% of those of wild-type mice (21). IRS-2 was identified as a 190-kDa protein that is rapidly tyrosine-phosphorylated in response to insulin and that partially compensates for the lack of IRS-1 in various insulin actions (20,22). For example, IRS-2, like IRS-1, is capable of participating in insulin signal transduction pathways, leading to the recruitment of GLUT4 (23). IRS-3 was first described in rat adipocytes as a 60-kDa protein that is rapidly tyrosine-phosphorylated in response to insulin, and it is considered to be a direct substrate of the

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αIRS, anti-IRS antibody; αPY, anti-phosphotyrosine; BSA, bovine serum albumin; CYTO, cytosol; GST, glutathione S-transferase; HSL, hormone-sensitive lipase; IRS, insulin receptor substrate; KRBH, Krebs-Ringer bicarbonate–HEPES; HDM, high-density microsome; LDM, low-density microsome; NEFA, nonesterified fatty acid; PCR, polymerase chain reaction; PDE3B, phosphodiesterase 3B; PH, pleckstrin homology; PI, phosphatidylinositol; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; PTB, phosphotyrosine-binding; SH2, Src homology-2; TOTAL, total lysates.

TABLE 1
Distribution of marker enzymes in subcellular fractions of mouse adipocytes

Fraction	5′-Nucleotidase ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)		Galactosyl transferase ($nmol \cdot 2\ h^{-1} \cdot mg^{-1}$)	
	Basal	Insulin	Basal	Insulin
TOTAL (homogenates)	0.23 ± 0.05	0.25 ± 0.05	4.59 ± 0.68	5.56 ± 0.47
PM	1.47 ± 0.20	1.80 ± 0.33	17.43 ± 1.99	16.23 ± 0.58
HDM	0.24 ± 0.05	0.22 ± 0.10	18.07 ± 1.58	19.87 ± 2.70
LDM	0.15 ± 0.04	0.21 ± 0.06	31.50 ± 2.37	28.03 ± 2.88

*Data are means ± SE of three separate experiments. Mouse adipocytes were fractionated, and 5′-nucleotidase, a plasma membrane marker, and galactocyl transferase (UDP-galactose, or *N*-acetylglucosamine galactosyl transferase), a Golgi apparatus marker, were measured in various cellular fractions.

activated insulin receptor (5,6,21,24–26). IRS-3 can bind and activate PI 3-kinase in response to insulin, and it was found to be the major PI 3-kinase–binding substrate in IRS-1 null mice (21,24). It has been reported that IRS-3 is tyrosine-phosphorylated more rapidly than IRS-1/IRS-2, thereby more rapidly activating PI 3-kinase activity in response to insulin (24); it has also been suggested that IRS-3 may play a unique role in mediating insulin-stimulated metabolic actions in adipocytes.

Although IRS family proteins share extensive homology and can bind and activate similar sets of signaling molecules, the distinct functions of the IRS family of proteins remain unknown. In this study, we investigated the localization of tyrosine-phosphorylated IRS family proteins that became associated with PI 3-kinase in response to insulin, and we studied the insulin-induced PI 3-kinase activity of each fraction of adipocytes from both wild-type and IRS-1 null mice. Both IRS-1 and IRS-2 were detected in all of the fractions and were associated with PI 3-kinase in response to insulin. By contrast, IRS-3 was predominantly localized in the plasma membrane (PM), not in the low-density microsome (LDM) fraction, and it contributed to insulin-induced PI 3-kinase activation in the PM fraction.

Several insulin actions are controlled by PI 3-kinase activation in adipocytes. To determine the potential functional impact of the distinct subcellular localization of IRSs and associating PI 3-kinase activity on adipocyte-specific metabolic actions, we examined lipolysis in IRS-1 null mice. The antilipolytic effect of insulin can be inhibited by wortmannin, a PI 3-kinase inhibitor (16), and insulin can regulate gene transcription and protein synthesis via PI 3-kinase activation (17). In IRS-1 null mice, the basal level of lipolysis and isoproterenol-induced lipolysis were increased because of increased hormone-sensitive lipase (HSL) mRNA and protein levels. The antilipolytic effect of insulin, however, was unaffected in IRS-1 null mice. Thus, discordance between these two insulin actions may be explained by the distinct roles of PI 3-kinase activity associated with IRS-1/IRS-2 and with IRS-3 in insulin actions related to their subcellular localization.

RESEARCH DESIGN AND METHODS

Materials. We purchased fraction V bovine serum albumin (BSA) from Intergen (Purchase, NY). Collagenase type II, porcine insulin, PI, adenosine deaminase, and (-)-N^6-(2-phenylisopropyl)-adenosine were from Sigma Chemical (St. Louis, MO). The monoclonal αPY antibody was purchased from Transduction Laboratories (Lexington, KY) or Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibody against a 14–amino acid peptide corresponding to the COOH-terminal sequence (YASINFQKQPEDRQ) of rat IRS-1 (αIRS-1), rabbit polyclonal antibody against a glutathione S-transferase (GST) fusion protein containing a polypeptide corresponding to amino acids 976–1094 of mouse IRS-2 (αIRS-2), and anti-p85 polyclonal antibody against a full-length p85-GST fusion protein containing the NH_2-terminal SH2 domain of p85α ($\alpha p85^{PAN}$) were purchased from Upstate Biotechnology. ^{125}I-protein A and [γ-^{32}P]ATP were purchased from Amersham International (Buckinghamshire, U.K.). Silicagel 60 plates were purchased from Merck (Darmstadt, Germany). Horseradish peroxidase–linked protein A and enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham International.

Isolation of mice adipocytes. Isolated adipocytes were prepared from the paraepididymal and parametrial fat pads of fed male and female wild-type (C57BL/6) and IRS-1 null mice (19) (body weight 20–25 g; 2–3 months old). Adipocyte numbers were normalized by total protein content or cell volume. Isolated fat cells were incubated in Krebs-Ringer bicarbonate–HEPES (KRBH) buffer containing 5.0% BSA, 120 mmol/l NaCl, 4 mmol/l KH_2PO_4, 1 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 10 mmol/l $NaHCO_3$, and 30 mmol/l HEPES, pH 7.4) by collagenase digestion (3.3 mg/ml) for 1 h at 37°C in a metabolic shaker. After incubation, the cells were strained through mesh, washed three times with KRBH buffer containing 0.5% BSA, and resuspended in the same buffer at a final concentration of <50%.

Stimulation of adipocytes with insulin. The cell suspension was incubated with 100 nmol/l insulin in KRBH buffer containing 0.5% BSA for 5 min at 37°C in a metabolic shaker. To stop insulin stimulation, the cells were washed twice with ice-cold BSA-free KRBH buffer.

Preparation of the subcellular fractions of adipocytes. Subcellular fractions were prepared as previously described (26–28), with some modifications. In brief, cells were suspended in 6 volumes (vol/vol) of homogenization buffer containing 20 mmol/l Tris-HCl, pH 7.4, 250 mmol/l sucrose, 5 mmol/l EGTA, 5 mmol/l EDTA, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, 10 mmol/l NaPPi, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, and 10 mg/ml aprotinin. They were then homogenized by 10 strokes in a Teflon pestle (Thomas Scientific, Swedesboro, NJ) at 17°C. Homogenates, or total lysates (TOTAL), were centrifuged for 15 min at 18,000*g* at −4°C to remove the fat cake. The PM-rich fraction was prepared as follows. The pellet was suspended in homogenizing buffer and centrifuged for 20 min at 100,000*g* on a sucrose cushion (1.12 mol/l sucrose, 20 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA). The PMs, collected at the interface, were resuspended in homogenizing buffer and centrifuged at 60,000*g* for 10 min. The 18,000*g* supernatant was centrifuged for 30 min at 48,000*g*, yielding a pellet of the high-density microsome (HDM) fraction. The 48,000*g* supernatant was centrifuged at 400,000*g* for 60 min, yielding a pellet of the LDM fraction and a supernatant containing the cytosol (CYTO). All procedures were carried out at 4°C except the homogenizing step, and the membranes were used immediately for assay of PI 3-kinase activity or for immunoprecipitation. The distribution of marker enzymes (5′ nucleotidase for the PM and galactosyl transferase [UDP-galactose, or *N*-acetylglucosamine galactosyltransferase] for the Golgi apparatus) in the different cellular fractions was assayed as described previously by Simpson et al. (27) (Table 1). 5′-Nucleotidase was sixfold more concentrated in the PM fraction than in the LDM and HDM fractions. The concentration of galactosyl transferase in the LDM fraction was increased by 70% compared with the HDM fraction, and it was increased by 80% compared with the PM fraction. The quality of the fractionation is similar to that reported by Simpson et al. (27), except that the ratio of galactosyl transferase in the PM–to–galactosyl transferase in the LDM was slightly higher under our experimental conditions. Judging from the distribution of marker enzymes, the results of the fractionation were concluded to be acceptable, although there was some cross-contamination.

Immunoprecipitation. For the immunoprecipitation experiments, mice were treated as previously described (21,26,28). In brief, adipose tissue was

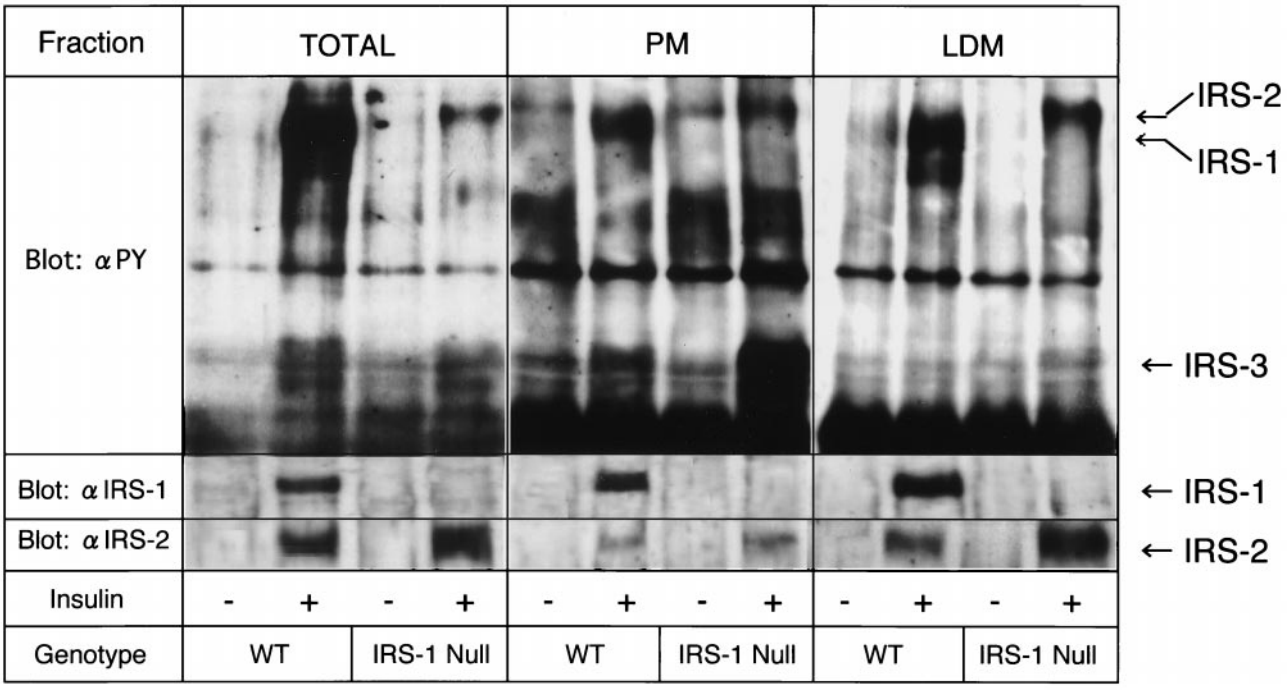

FIG. 1. Intracellular localization of insulin-induced tyrosine-phosphorylated IRS family proteins associated with PI 3-kinase in adipocytes from wild-type and IRS-1 null mice. After treatment with 10^{-7} mol/l insulin for 5 min, isolated adipocytes from wild-type and IRS-1 null mice were homogenized and fractionated. TOTAL, PM, and LDM were immunoprecipitated with $\alpha p85^{PAN}$ and subjected to SDS-PAGE, followed by immunoblotting with αPY (upper panel), αIRS-1 (middle panel), and αIRS-2 antibody (lower panel). The bands corresponding to IRS-1, -2, and -3 are indicated. WT, wild-type.

excised and homogenized in ice-cold buffer A (25 mmol/l Tris-HCl, pH 7.4, 10 mmol/l Na_3VO_4, 10 mmol/l NaPPi, 100 mmol/l NaF, 10 mmol/l EDTA, 10 mmol/l EGTA, and 1 mmol/l PMSF), and adipocytes were solubilized with ice-cold buffer A containing 1% nonylphenoxy polyethoxy ethanol (TOTAL). After subcellular fractionation of some of the samples, the PM or LDM pellets were solubilized in resuspension buffer (20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l Na_3VO_4, 10 mmol/l NaPPi, 100 mmol/l NaF, 5 mmol/l EDTA, 5 mmol/l EGTA, 10% glycerol, 1 mmol/l PMSF, 10 mg/ml leupeptin, and 10 mg/ml aprotinin) containing 1% Triton X-100 and incubated for 30 min at 4°C with shaking. Each fat-free lysate (TOTAL, PM, or LDM) was prepared by centrifugation (15,000 rpm, 20 min, 4°C). Lysates containing equal amounts of total protein (~30–100 μg) were incubated with the indicated antibody for 1 h at 4°C and then with protein G–Sepharose for 1 h at 4°C. The beads were washed three times with homogenization buffer or suspension buffer containing 1% Triton X-100, and the immunoprecipitated proteins were solubilized with Laemmli's sample buffer. In the immunodepletion experiments, the homogenate (TOTAL) and each subcellular fraction (PM and LDM) were immunoprecipitated with αIRS-2, and the supernatant after αIRS-2 immunodepletion was further incubated overnight with the indicated antibody and protein G–Sepharose beads.

Immunoblotting. Samples were separated on polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with each antibody. The blots were incubated with horseradish peroxidase–linked protein A or ^{125}I-protein A, and the bands were detected by enhanced chemiluminescence (Amersham International) or with a BAS 2000 system (Fuji Film, Kanagawa, Japan).

PI 3-kinase assay. Adipocytes were incubated in the presence or absence of insulin, homogenized with a Teflon pestle (Thomas Scientific), and subjected in some cases to fractionation as described above. Membrane fractions (PM or LDM) were suspended in suspending buffer. PI 3-kinase was immunoprecipitated with the indicated antibody and protein G–Sepharose beads overnight. The immunoprecipitates were washed three times with homogenization or resuspension buffer containing 1% Triton X-100 and washed three more times with PI 3-kinase reaction buffer (20 mmol/l Tris-HCl, pH 7.4, 100 mmol/l NaCl, 1 mmol/l Na_3VO_4, and 0.5 mmol/l EGTA). The reaction was initiated by adding 50 μl PI 3-kinase reaction buffer containing 20 mmol/l $MgCl_2$, 20 μmol/l ATP, 5 μCi [γ-^{32}P]ATP, and 0.2 mg/ml PI to the immunoprecipitates. After incubation at 25°C for 20 min, the reaction was terminated by the addition of 100 μl chloroform, and the organic phase was separated by centrifugation and washed three times with methanol and 1 mol/l HCl (1:1 ratio). The lipids were spotted onto a Silicagel 60 plate and developed in chloroform, methanol, 28% ammonium hydroxide, and water (43:38:5:7 ratio). The phosphorylated lipids were visualized and evaluated by autoradiography.

Lipolysis and antilipolytic effect of insulin. A 10% cell suspension (10^4–10^5 cells/ml) was prepared in KRBH buffer containing 1 U/ml adenosine deaminase and 10 μmol/l (-)-N^6-(2-phenylisopropyl)-adenosine and then incubated. Cells were stimulated with 1 μmol/l isoproterenol for 30 min at 37°C with shaking to evaluate both basal and isoproterenol-induced lipolysis. The antilipolytic effect of insulin was measured by adding 0.5 or 1 nmol/l insulin to the cell suspension for 15 min at 37°C and then stimulating with 1 μmol/l isoproterenol for 30 min at 37°C. The reactions were terminated by spinning the cells through dinonyl phthalate oil. Nonesterified fatty acid (NEFA) released into medium was measured with a commercial kit (Wako Chemicals, Osaka, Japan).

Quantity of HSL. Lysates containing an equal amount of total protein (~15–50 μg) were solubilized with Laemmli's sample buffer, separated on polyacrylamide gels, and transferred onto nitrocellulose. Immunoblotting was performed using rabbit polyclonal anti-rat HSL–fusion protein antibodies, as described previously (29). Immunoblots were visualized with enhanced chemiluminescence, and the relative amount of immunodetectable HSL contained in each lane was determined by scanning.

RNA isolation and measurement of HSL expression. Total cellular RNA was extracted from freshly isolated adipocytes by acid guanidine phenol/$CHCl_3$ extraction as previously described (30). Equal aliquots of total RNA in adipocytes from wild-type and IRS-1 null mice were pooled (15 μg), denatured with formaldehyde and formamide, and subjected to Northern blot analysis with a cDNA probe for mouse HSL prepared by reverse transcriptase–polymerase chain reaction (PCR) with mouse epididymal fat total RNA as the template. The PCR primers used were sense primer 5′-ACCTGAGGCCTTT-GAGATGCCACTC-3′ and antisense primer 5′-CACTCCATAGGCTGCTGC-CCGAAG-3′ (31). Hybridizations were performed by using this probe as described previously (30). The resulting bands were quantified by exposure of the filter to a BioImaging Analyzer with a BAS 2000 system, and the results were normalized to the signal generated from 28s rRNA and 18s rRNA.

RESULTS

Intracellular localization of insulin-induced tyrosine-phosphorylated IRS family proteins associated with PI 3-kinase in primary mouse adipocytes. To investi-

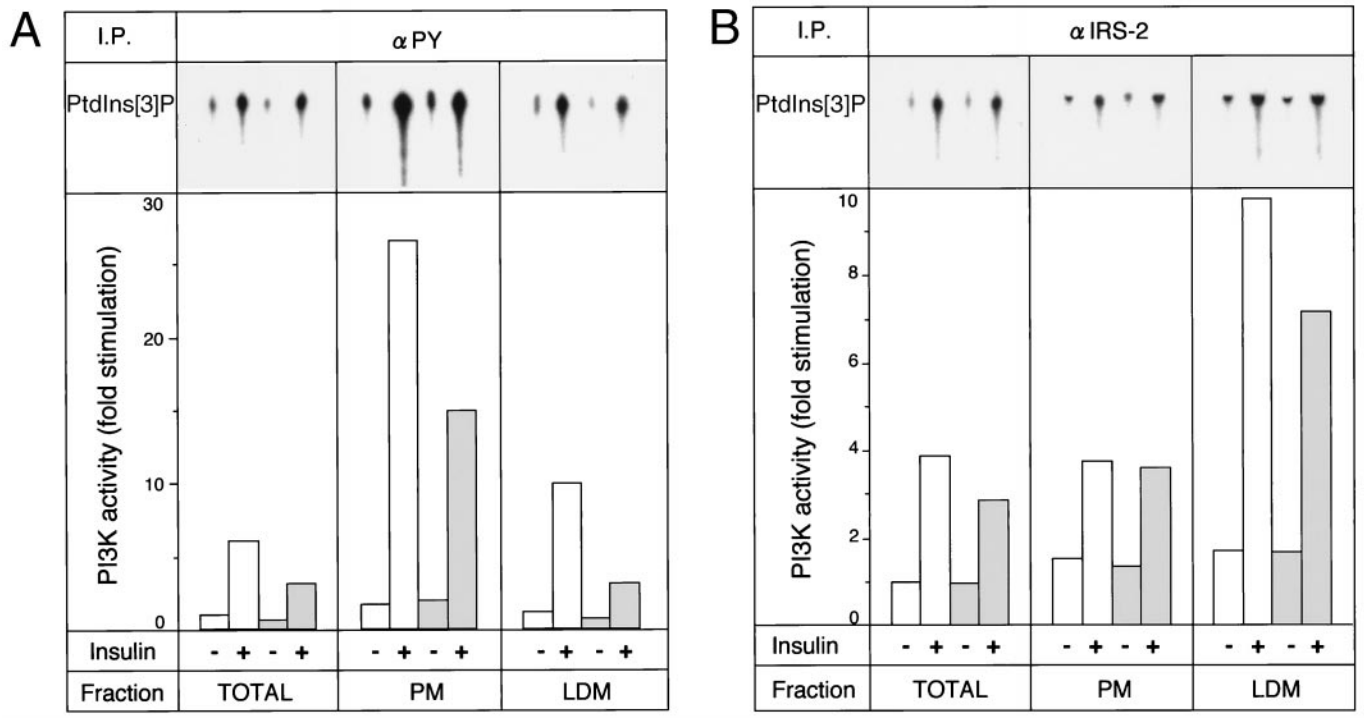

FIG. 2. Insulin-induced PI 3-kinase activity in αPY immunoprecipitates (*A*) and αIRS-2 immunoprecipitates (*B*) in adipocytes from wild-type and IRS-1 null mice. After treatment with 10^{-7} mol/l insulin for 5 min, isolated adipocytes from wild-type (□) and IRS-1 null mice (▨) were homogenized and fractionated. A 30-μg sample of the homogenate (TOTAL) and of each subcellular fraction (PM and LDM) was immunoprecipitated with each antibody. PI 3-kinase activity in the immunoprecipitates was measured as described in RESEARCH DESIGN AND METHODS. The top panel is a representative autoradiogram of the thin-layer chromatograph showing the products of PI 3-kinase (PtdIns[3]P) in each fraction. The data obtained are the means of duplicate assays and have been converted to a percentage of the unstimulated value of adipocytes from wild-type mice. PI3K, PI 3-kinase; WT, wild-type.

gate the intracellular distribution of insulin-induced IRS family proteins, we fractionated mouse adipocytes from wild-type and IRS-1 null mice before and after insulin stimulation, subjected each fraction to immunoprecipitation with $\alpha p85^{PAN}$, and then immunoblotted the precipitates with αPY (Fig. 1, upper panel). A 180-kDa tyrosine-phosphorylated protein associated with PI 3-kinase was detected in the TOTAL, PM, and LDM fractions in wild-type mice in response to insulin (Fig. 1, upper panel). This protein was identified as IRS-1 because it was recognized by αIRS-1 antibody (Fig. 1, middle panel) and was not detected in IRS-1 null mice. A 190-kDa tyrosine-phosphorylated protein associated with PI 3-kinase was detected in response to insulin in all of the fractions from IRS-1 null mice (Fig. 1, upper panel). This protein was identified as IRS-2 because it was recognized by αIRS-2 antibody (Fig. 1, lower panel) and was detected in both wild-type and IRS-1 null mice. An insulin-induced 60-kDa tyrosine-phosphorylated protein (presumably IRS-3) associated with PI 3-kinase was shown to be predominantly localized in the PM fraction of both wild-type and IRS-1 null mice but only slightly localized in their LDM fraction (Fig. 1, upper panel). No tyrosine phosphorylation of the p85 molecule was detected under our experimental conditions (Fig. 1, upper panel).

PI 3-kinase activity in αPY, αIRS-1, and αIRS-2 immunoprecipitates of adipocyte subcellular fractions. To examine the contribution of IRS family proteins (IRS-1, -2, and -3) to insulin-stimulated PI 3-kinase activation, PI 3-kinase activity was measured in the αPY immunoprecipitates in subcellular fractions of adipocytes from wild-type and IRS-1 null mice. PI 3-kinase activity in the αPY immunoprecipitates in the TOTAL fraction from IRS-1 null mice was ~50% of the activity in wild-type mice (Fig. 2*A*), a finding consistent with the results we reported previously (21). We then fractionated the adipocytes and measured the PI 3-kinase activity associated with IRS family proteins. Insulin increased PI 3-kinase activity in the αPY immunoprecipitates in the PM and LDM fractions of wild-type mice (15.2-fold and 8.4-fold, respectively). In the IRS-1 null mice, insulin-induced PI 3-kinase activity associated with IRS family proteins was decreased to 60% in the PM fraction and 25% in the LDM fraction compared with wild-type mice.

To examine the contribution of IRS-1 and IRS-2 to insulin-stimulated PI 3-kinase activation, PI 3-kinase activity was measured in the αIRS-1 and αIRS-2 immunoprecipitates in subcellular fractions of adipocytes from wild-type and IRS-1 null mice. As predicted, insulin-stimulated PI 3-kinase activity via IRS-1 was detected in all of the fractions from wild-type mice but not in those from IRS-1 null mice (data not shown). Insulin increased PI 3-kinase activity in αIRS-2 immunoprecipitates in the TOTAL, PM, and LDM fractions of wild-type mice (3.9-fold, 2.5-fold, and 5.8-fold, respectively) (Fig. 2*B*). Insulin-induced PI 3-kinase activity associated with IRS-2 in IRS-1 null mice was detected in all fractions (3.0-fold, 2.6-fold, and 4.3-fold in TOTAL, PM, and LDM fractions, respectively), and it was not significantly different from the values in wild-type mice.

Intracellular localization of insulin-induced tyrosine-phosphorylated IRS-3 and associating PI 3-kinase

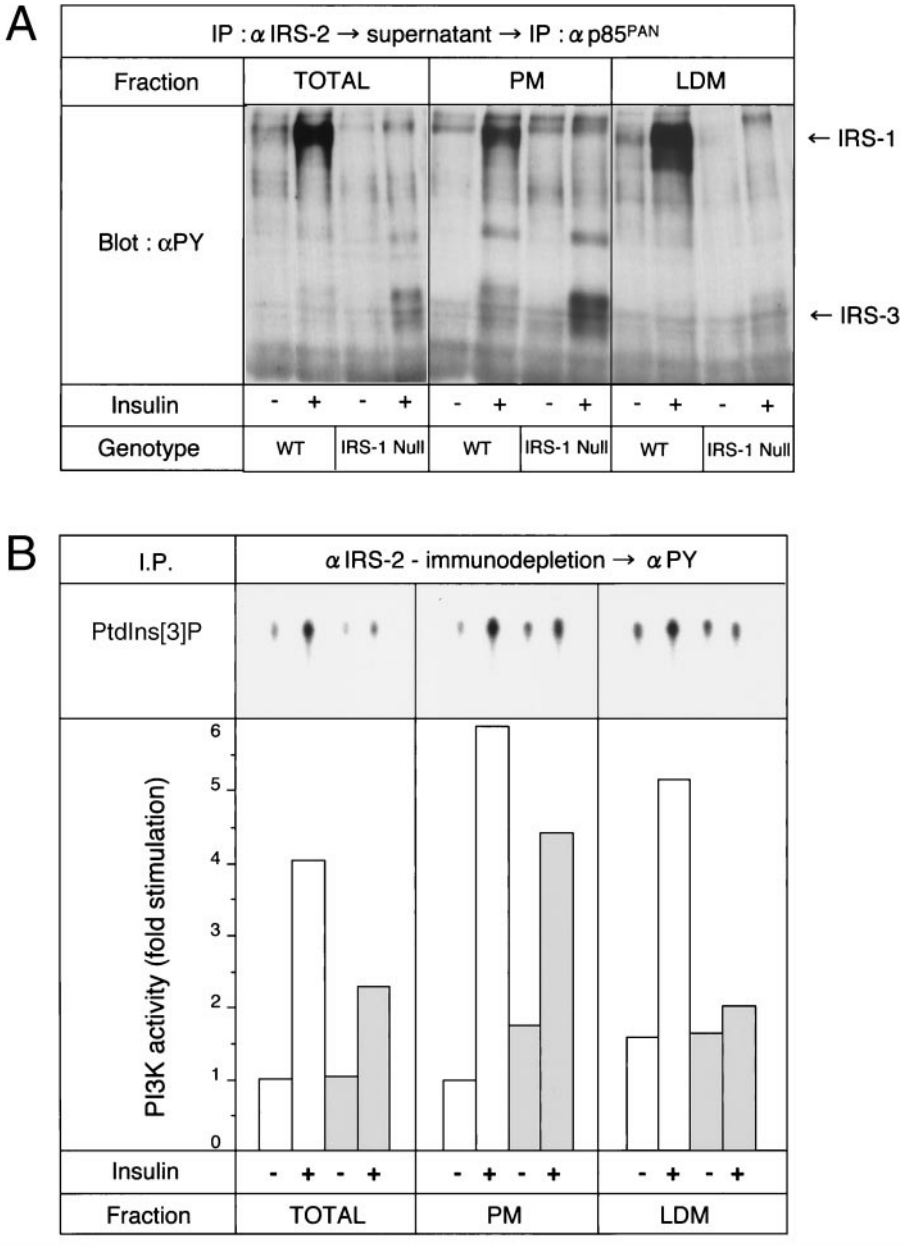

FIG. 3. ***A*****: Intracellular localization of insulin-induced tyrosine-phosphorylated IRS family proteins associated with PI 3-kinase of the supernatant after αIRS-2 immunodepletion in adipocytes from wild-type and IRS-1–null mice. In this experiment, after insulin treatment (10^{-7} mol/l, 5 min), a sample of homogenate (TOTAL) and of each subcellular fraction (PM and LDM) was immunoprecipitated with αIRS-2. After αIRS-2 immunodepletion, the supernatant was immunoprecipitated with αp85^{PAN}, subjected to SDS-PAGE, and immunoblotted with αPY.** ***B*****: Insulin-induced PI 3-kinase activity in αPY-immunoprecipitates of supernatant after αIRS-2 immunodepletion of adipocytes from wild-type (□) and IRS-1 null (▨) mice. In this experiment, after insulin treatment (10^{-7} mol/l, 5 min), a sample of homogenate (TOTAL) and of each subcellular fraction (PM and LDM) was immunoprecipitated with αIRS-2. After αIRS-2 immunodepletion, the supernatant was immunoprecipitated with αPY, and PI 3-kinase activity was measured in the immunoprecipitates. The top panel is a representative autoradiogram of the thin-layer chromatogram showing the products of PI 3-kinase (PtdIns[3]P) in each fraction. The data obtained are the means of duplicate assays and have been converted to percentages of the unstimulated value of adipocytes from wild-type mice. PI3K, PI 3-kinase; WT, wild-type.**

activity in adipocytes from IRS-1 null mice. To determine the relative contribution of IRS-1 and IRS-2 in insulin-stimulated PI 3-kinase activation in the PM and LDM fractions of adipocytes from IRS-1 null mice, tyrosine-phosphorylated IRS-3 associated with PI 3-kinase after αIRS-2 immunodepletion was investigated in each fraction. The efficiency of αIRS-2 immunodepletion was estimated to be nearly 100% because of the disappearance of a 190-kDa tyrosine-phosphorylated protein (Fig. 3*A*), an observation that was further confirmed by the complete disappearance of IRS-2 with αIRS-2 immunoblotting (data not shown). In wild-type mice, IRS-1 was identified as one of the major PI 3-kinase binding substrates in response to insulin in all fractions, including the LDM fraction. IRS-3 was identified as a PI 3-kinase binding substrate only in the PM fraction of wild-type mice. In IRS-1 null mice, tyrosine phosphorylation of IRS-3 associated with PI 3-kinase was increased in the PM fraction. Although slight tyrosine phosphorylation of IRS-3 was detected in the LDM fraction of IRS-1 null mice, it was likely due to cross-contamination (Table 1).

PI 3-kinase activity associated with IRS-3 was measured after αIRS-2 immunodepletion of each of the subcellular fractions of adipocytes from IRS-1 null mice. The samples used in this experiment were the same as those shown in Fig. 3*A*. In wild-type mice, insulin stimulated PI 3-kinase activity in the TOTAL, PM, and LDM fractions (4.0-fold, 6.0-fold, and 3.3-fold, respectively), and the stimulation was presumably mediated by IRS-1 and -3. In IRS-1 null mice, insulin stimulated PI 3-kinase activity in the TOTAL and PM fractions (2.2-fold and 2.5-fold, respectively) (Fig. 3*B*), and the stimulation was presumably mediated by IRS-3. However, insulin failed to cause activation of PI 3-kinase in the LDM fraction of adipocytes from IRS-1 null mice (Fig. 3*B*), suggesting little insulin stimulation of PI 3-kinase activation via IRS-3 in the LDM fraction of adipocytes from IRS-1 null mice. This finding was consistent with the absence of IRS-3 in the LDM fraction (Fig. 3*A*) and

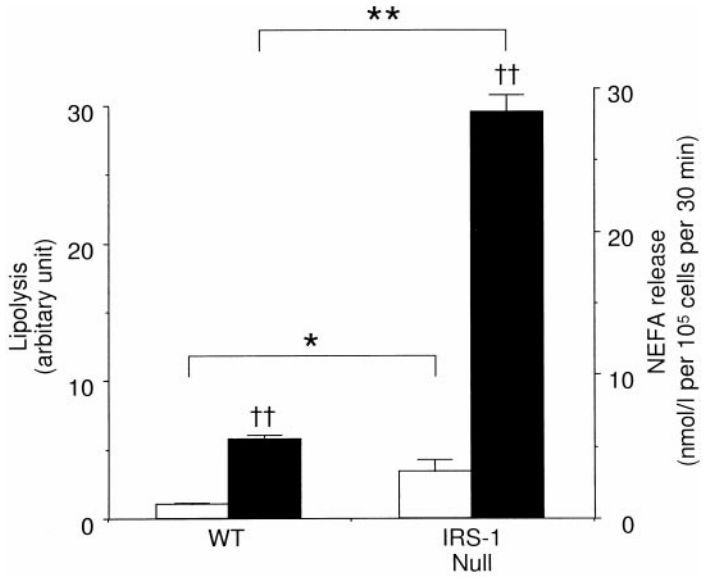

FIG. 4. Lipolysis in adipocytes from wild-type and IRS-1 null mice. Adipocytes from wild-type and IRS-1 null mice were incubated either in the absence (□) or presence (■) of 1 μmol/l isoproterenol (basal or ispotroterenol-stimulated, respectively). Data are the means ± SE of four separate experiments. WT, wild-type. *$P < 0.05$ and **$P < 0.01$ vs. wild-type; and †$P < 0.05$ and ††$P < 0.01$ vs. basal.

suggested that insulin-induced activation of PI 3-kinase in the LDM fraction may be mediated mainly by IRS-2 in the absence of IRS-1.

Increased lipolysis and unaffected antilipolytic effect of insulin in IRS-1 null mice. To determine the potential functional impact of the distinct subcellular localization of IRSs and associating PI 3-kinase activity on adipocyte-specific metabolic actions, we examined lipolysis in IRS-1–null mice. We first measured NEFA release into the medium to examine lipolysis in adipocytes from both mouse genotypes. The basal rate of lipolysis in adipocytes from IRS-1 null mice was increased 3.4-fold compared with wild-type mice ($P < 0.05$), and isoproterenol-stimulated lipolysis was increased 5.1-fold ($P < 0.01$) (Fig. 4). It is thought that lipolysis is primarily mediated by HSL (32), although there may be additional pathways (33). To clarify the mechanism whereby lipolysis is increased in IRS-1–deficient adipocytes, we measured the immunoreactive HSL protein in isolated adipocytes with an antibody against HSL protein. The result showed a 4.3-fold increase in the amount of the immunoreactive HSL protein in IRS-1 null mice compared with wild-type mice ($P < 0.05$) (Fig. 5*A*), and the level of HSL mRNA in IRS-1 null mice was also increased 1.4-fold compared with wild-type mice ($P < 0.05$) (Fig. 5*B*).

Next, we investigated the antilipolytic effect of insulin in adipocytes from wild-type and IRS-1 null mice. The antilipolytic effect of insulin was measured with 0.5 nmol/l and 1 nmol/l insulin because these concentrations lie within the physiological range of plasma insulin concentrations in wild-type and IRS-1 null mice (19,34). A statistically significant antilipolytic effect of insulin was observed in adipocytes from both wild-type mice and IRS-1 null mice (Fig. 6), but the extent of inhibition of lipolysis by insulin was similar between the two mouse genotypes; with 0.5 nmol/l insulin, we found 17.2 and 13.0% inhibition in wild-type and IRS-1 null mice, respectively, and with 1 nmol/l insulin, we found 20.7 and 24.6% inhibition in wild-type and IRS-1 null mice, respectively (Fig. 6).

DISCUSSION

We have clarified the intracellular localization of IRS family proteins associated with PI 3-kinase in mouse adipocytes and their activity in response to insulin. In adipocytes from wild-type mice, tyrosine-phosphorylated IRS-1 and IRS-2 were distributed in the PM and LDM fractions, whereas IRS-3 was predominantly localized in the PM fraction (Fig. 1). Insulin stimulated PI 3-kinase activity via all three proteins (IRS-1, -2, and -3) in the PM fraction of wild-type adipocytes and via IRS-1 and IRS-2 in the LDM fraction (Figs. 2 and 3*B*). In adipocytes from IRS-1 null mice, insulin-stimulated PI 3-kinase activity in αPY immunoprecipitates was reduced by 69% in the LDM fraction because of extremely low tyrosine-phosphorylation of IRS-3, despite tyrosine-phosphorylation of IRS-2, and it was reduced to 60% in the PM fraction because of a substantial amount of tyrosine-phosphorylation of IRS-3. These results were consistent with the subcellular localization of IRS-1, -2, and -3. In addition, insulin-stimulated

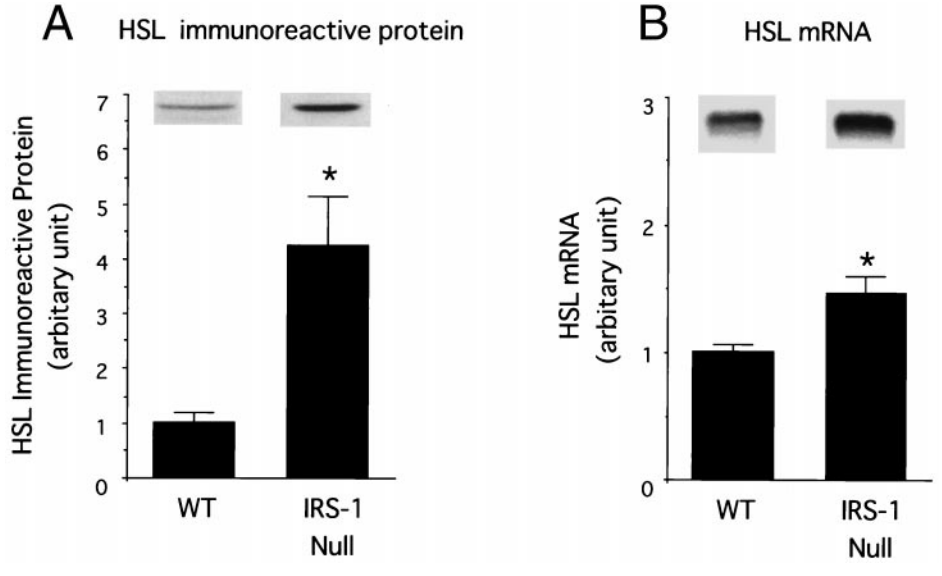

FIG. 5. *A*: Immunoreactive HSL protein in adipocytes from wild-type and IRS-1 null mice. Data are the means ± SE of three separate densitometric intensity values for immunoblots of HSL in isolated adiopocytes from wild-type and IRS-1 null mice. The upper panel is a representative immunoblot of HSL protein from wild-type and IRS-1 null mice. *$P < 0.05$ vs. wild-type. *B*: HSL mRNA in adipocytes from wild-type and IRS-1 null mice. Data are the means ± SE of three separate densitometric intensity values for HSL mRNA in isolated adiopocytes from wild-type and IRS-1 null mice. The scans of 18s rRNA and 28s rRNA demonstrated equivalent amounts of mRNA in each lane (data not shown). The upper panel is a representative blot of HSL mRNA from wild-type or IRS-1 null mice. WT, wild type. *$P < 0.05$ vs. wild-type.

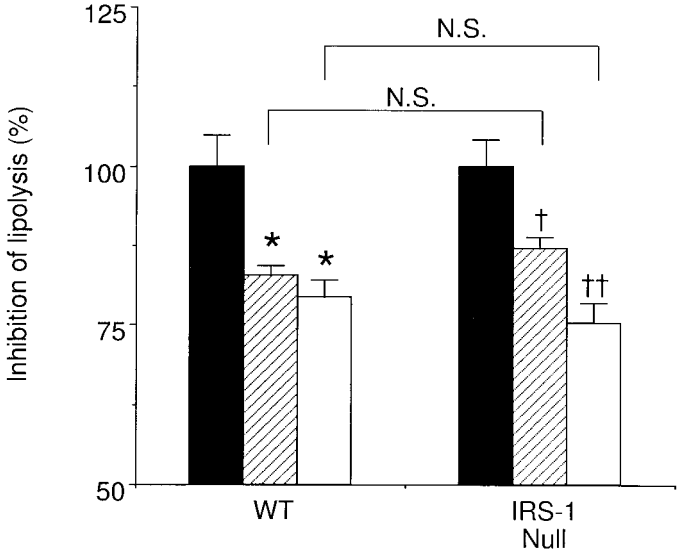

FIG. 6. Antilipolytic action of insulin in adipocytes from wild-type and IRS-1 null mice. The antilipolytic effect of insulin: inhibition of lipolysis in the absence (■) or presence of 0.5 nmol/l (▨) or 1 nmol/l (□) insulin plus 1 μmol/l isoproterenol was measured as described in RESEARCH DESIGN AND METHODS. Data are the means ± SE of four separate experiments. N.S., not significantly different; WT, wild type. *$P < 0.05$ vs. isoproterenol-stimulated (wild-type mice); †$P < 0.05$ and ††$P < 0.01$ vs. isoproterenol-stimulated (IRS-1 null mice).

PI 3-kinase activity in αPY immunoprecipitates after IRS-2 immunodepletion in the PM fraction from IRS-1 null mice was stimulated several-fold by insulin, but it was only barely stimulated by insulin in the LDM fraction (Fig. 3*B*).

Our observations confirmed the earlier findings of Ruderman's group that IRS-1 and pp60 (now referred to as IRS-3) play a role in activating PI 3-kinase in response to insulin in the LDM and PM fractions, respectively, by using αPY immunoprecipitates of rat adipocytes (26,28). However, IRS-2 had not been identified at that time, and thus the role and subcellular localization of IRS-2 in adipocytes was not specifically addressed. In the present study, we measured PI 3-kinase activity in anti–IRS-2 antibody immunoprecipitates in the PM and LDM of extracts of adipocytes from wild-type mice to evaluate PI 3-kinase activity specifically associated with IRS-2. We also measured PI 3-kinase activity in PM and LDM of extracts from IRS-1 null mice after immunodepleion by anti–IRS-2 antibody to evaluate PI 3-kinase activity associated with IRS-3. As a result, we demonstrated that not only IRS-1 but also IRS-2 plays a major role in activating PI 3-kinase in response to insulin in the LDM fraction and that IRS-3 plays a major role in the PM fraction.

Clark et al. (35) recently reported that IRS-1 is localized in the cytoskeleton fraction, which corresponds to the LDM fraction under our experimental conditions; thus, our finding that insulin activated PI 3-kinase in the LDM fraction is consistent with their report (35). In addition, insulin also activated PI 3-kinase in the PM fraction under our experimental conditions (Figs. 1–3); this observation is in accordance with a recent study showing that IRS-1 (and probably IRS-2) is translocated from the intracellular fractions to the PM fraction in response to insulin (36,37), a finding that we ourselves have recently confirmed (Y.K. and T.K., unpublished data). It was also recently shown that IRS-3 is more rapidly tyrosine-phosphorylated in response to insulin than IRS-1 or -2 (24). Taken together, these findings raise the possibility that both IRS-1 and -2 play roles that are different from IRS-3 in a variety of insulin actions. Given that specific alterations of expression of IRSs have been reported in insulin-resistant states (38,39), the specific subcellular localization of IRSs may be associated with specific metabolic derangements.

PI 3-kinase plays important roles in the regulation of insulin actions (e.g., glucose transport, antilipolysis, protein synthesis, and gene transcription) (16–18), and it has been proposed that activation of PI 3-kinase in the LDM fraction is important for insulin-stimulated GLUT4 translocation and glucose transport in adipocytes (40–42). In this regard, the results of the present study suggest that both IRS-1 and -2 play a major role in mediating activation of PI 3-kinase in response to insulin in the LDM fraction of adipocytes from wild-type and that IRS-2 does so in the LDM fraction of adipocytes from IRS-1 null mice. We have previously shown that insulin-stimulated glucose transport is decreased by 50% in adipocytes from IRS-1 null mice (21). If insulin-stimulated glucose transport is indeed mediated by activated PI 3-kinase in the LDM fraction, it seems likely that the remaining glucose transport in adipocytes from IRS-1 null mice may be dependent on PI 3-kinase activation associated with IRS-2 in this fraction. It is possible, however, that PI 3-kinase activation mediated via IRS-3 in the PM fraction contributes to the translocation of GLUT4. Indeed, increased translocation of GLUT4 has been demonstrated in rat adipose cells that overexpress IRS-3 (43). A more recent report, however, has shown that insulin-stimulated glucose transport in adipocytes from IRS-3–null mice is the same as in wild-type mice (44). Thus, it seems likely that IRS-3 may not play a role in glucose transport because of its PM localization.

We next studied the antilipolytic effect of insulin as a representative PM effect of insulin that may more closely parallel the PI 3-kinase activity associated IRS-3. We also studied the transcriptional and translational regulation of adipocyte specific enzymes (e.g., HSL) by insulin as representative ribosomal and nuclear effects of insulin that may more closely parallel the PI 3-kinase activity associated with IRS-1 and -2. The results showed that both immunoreactive HSL protein and HSL mRNA were increased in adipocytes from IRS-1 null mice compared with wild-type mice (Fig. 5), and that this led to an increase in both basal and isoproterenol-stimulated lipolysis (Fig. 4). Insulin deficiency induced by streptozotocin has also been reported to increase expression levels of HSL, the key regulator of lipolysis, via pretranslational mechanisms in rat adipocytes (45). Thus, it seems likely that insulin inhibits the expression of the HSL gene and decreases the amount of HSL protein. In the PM fraction, PI 3-kinase activity in IRS-1 null mice was mediated via IRS-3 and was reduced only modestly (60%); however, in the LDM fraction, it was almost exclusively mediated via IRS-2 and was severely reduced (25%). Because of this, a marked increase in lipolysis and HSL protein level may be explained by a reduction of PI 3-kinase activity in the latter fraction. Thus, IRS-1 and -2 but not IRS-3 may play a uniquely important role in this action. The precise mechanism by which insulin regulates HSL should be further clarified.

The antilipolytic effect of insulin in adipose tissue

appears to be one of the downstream actions of insulin signaling because antilipolysis is inhibited by the PI 3-kinase inhibitor wortmannin (16). It was surprising that the antilipolytic effects of insulin actions were preserved in adipocytes from IRS-1 null mice. This was in marked contrast to the impaired insulin action in IRS-1 null mice with regard to glucose transport and the regulation of HSL transcription and translation. Thus, it seems possible that PI 3-kinase activity in a specific subcellular fraction is important for this action of insulin. Activation of the membrane-associated phosphodiesterase 3B (PDE3B) has been reported to be the major mechanism whereby insulin antagonizes catecholamine-induced lipolysis (33). Because PI 3-kinase activation in the PM fraction was relatively preserved in IRS-1 null mice (primarily due to PI 3-kinase activity associated with IRS-3), activation of membrane-associated PDE3B may be mediated through the remaining PI 3-kinase activity, mainly via IRS-3 in the PM fraction. Further study will be needed to clarify these points.

In summary, we have provided evidence that IRS-1, -2, and -3 are all present in distinct intracellular fractions of mouse adipocytes and that there are distinct effects of IRS-1 deficiency on insulin's actions. These findings raise the interesting possibility that each of the actions of insulin may be linked to distinct IRS family proteins and/or the activation of PI 3-kinase in distinct subcellular fractions.

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REFERENCES

1. Kasuga M, Karlsson FA, Kahn CR: Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. *Science* 215:185–187, 1982
2. White MF, Maron R, Kahn CR: Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature* 318:183–186, 1985
3. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73–77, 1991
4. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG Jr., Glasheen E, Lane WS, Pierce JH, White MF: Role of IRS-2 in insulin and cytokine signalling. *Nature* 377:173–177, 1995
5. Laven BE, Lienhard GE: The insulin-elicited 60-kDa phosphotyrosine protein in rat adipocytes is associated with phosphatidylinositol 3-kinase. *J Biol Chem* 268:5921–5928, 1993
6. Laven BE, Lane WS, Lienhard GE: The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* 272:11439–11443, 1997
7. Laven BE, Fantin VR, Chang ET, Lane WS, Keller SR, Lienhard GE: A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J Biol Chem* 272:21403–21407, 1997
8. Fantin VR, Sparling JD, Slot JW, Keller SR, Lienhard GE, Lavan BE: Characterization of insulin receptor substrate 4 in human embryonic kidney 293 cells. *J Biol Chem* 273:10726–10732, 1998
9. White MF, Kahn CR: The insulin signalling system. *J Biol Chem* 269:1–4, 1994
10. Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJ: A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature* 379:560–564, 1996
11. Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers MG Jr., Backer JM, Ullrich A, White MF, Schlessinger J: The SH2-SH3 domain-containing protein Grb-2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. *EMBO J* 12:1929–1936, 1993
12. Backer JM, Myers MG Jr, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skonik EY, Schlessinger J, White MF: Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* 11:3469–3479, 1992
13. Kasuga M: Role of PI 3-kinase and SH-PTP2 in insulin action. *Diabet Med* 13:S87–S89, 1996
14. Kuhne MR, Pawson T, Lienhard GE, Feng GS: The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. *J Biol Chem* 268:11479–11481, 1993
15. Beitner-Johnson D, Blakesley VA, Shen-Orr Z, Jimenez M, Stannard B, Wang LM, Pierce J, LeRoith D: The proto-oncogene product c-Crk associates with insulin receptor substrate-1 and 4PS. *J Biol Chem* 271:9287–9290, 1996
16. Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J Biol Chem* 269:3568–3573, 1994
17. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR: Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 14:4902–4911, 1994
18. Yamamoto-Honda R, Tobe K, Kaburagi Y, Ueki K, Asai S, Yachi M, Shirouzu M, Yodoi J, Akanuma Y, Yokoyama S, Yazaki Y, Kadowaki T: Upstream mechanisms of glycogen synthase activation by insulin and insulin-like growth factor-I. *J Biol Chem* 270:2729–2734, 1997
19. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, Sekihara H, Yoshioka S, Horikoshi H, FurutaY, Ikawa Y, Kasuga M, Yazaki Y, Aizawa S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182–186, 1994
20. Araki E, Lipes MA, Patti ME, Brüning JC, Haag B III, Johnson RS, Kahn CR: Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186–190, 1994
21. Kaburagi Y, Satoh S, Tamemoto H, Yamamoto-Honda R, Tobe K, Ueki K, Yamauchi T, Kono-Sugita E, Sekihara H, Aizawa S, Cushman SW, Akanuma Y, Yazaki Y, Kadowaki T: Role of insulin receptor substrate-1 and pp60 in the regulation of insulin-induced glucose transport and GLUT4 translocation in primary adipocytes. *J Biol Chem* 272:25839–25844, 1997
22. Tobe K, Tamemoto H, Yamauchi T, Aizawa S, Yazaki Y, Kadowaki T: Identification of a 190-kDa protein as a novel substrate for the insulin receptor kinase functionally similar to insulin receptor substrate-1. *J Biol Chem* 270:5698–5701, 1995
23. Zhou L, Chen H, Lin CH, Cong LN, McGibbon MA, Sciacchitano S, Lesniak MA, Quon MJ, Taylor SI: Insulin receptor substrate-2 (IRS-2) can mediate the action of insulin to stimulate translocation of GLUT4 to the cell surface in rat adipose cells. *J Biol Chem* 272:29829–29833, 1997
24. Smith-Hall J, Pons S, Patti ME, Burks DJ, Yenush L, Sun XJ, Kahn CR, White MF: The 60 kDa insulin receptor substrate functions like an IRS protein ($pp60^{IRS3}$) in adipose cells. *Biochemistry* 36:8304–8310, 1997
25. Momomura K, Tobe K, Seyama Y, Takaku F, Kasuga M: Insulin-induced tyrosine-phosphorylation in intact rat adipocytes. *Biochem Biophys Res Commun* 155:1181–1186, 1988
26. Kelly KL, Ruderman NB: Insulin-stimulated phosphatidylinositol 3-kinase: association with a 185-kDa tyrosine-phosphorylated protein (IRS-1) and localization in a low-density membrane vesicle. *J Biol Chem* 268:4391–4398, 1993
27. Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW: Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochim Biophys Acta* 763:393–407, 1983
28. Kelly KL, Ruderman NB, Chen KS: Phosphatidylinositol-3-kinase in isolated rat adipocytes. *J Biol Chem* 267:3423–3428, 1992
29. Kraemer FB, Patel S, Saedi MS, Sztalryd C: Detection of hormone-sensitive lipase in various tissues. I. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies. *J Lipid Res* 34:663–671, 1993
30. Kraemer FB, Tavanger K, Hoffman AR: Developmental regulation of hormone-sensitive lipase mRNA in the rat: changes in steroidogenic tissues. *J Lipid Res* 32:1303–1310, 1991
31. Li Z, Sumida M, Birchbauer A, Schotz MC, Reue K: Isolation and characterization of the gene for mouse hormone-sensitive lipase. *Genomics* 24:259–265, 1994
32. Carey GB: Mechanisms regulating adipocyte lipolysis. *Adv Exp Med Biol* 441:157–170, 1998

33. Osuga J, Ishibashi S, Oka T, Yagyu H, Tozawa R, Fujimoto A, Shionoiri F, Yahagi N, Kraemer FB, Tsutsumi O, Yamada N: Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc Natl Acad Sci U S A* 97:787–792, 2000
34. Terauchi Y, Iwamoto K, Tamemoto H, Komeda K, Ishii C, Kanazawa Y, Asanuma N, Aizawa T, Akanuma Y, Yasuda K, Kodama T, Tobe K, Yazaki Y, Kadowaki T: Development of non-insulin-dependent diabetes mellitus in the double knockout mice with disruption of insulin receptor substrate-1 and β cell glucokinase gense. *J Clin Invest* 99:861–866, 1997
35. Clark SF, Martin S, Carozzi AJ, Hill MM, James DE: Intracellular localization of phosphatidylinositide 3-kinase and insulin receptor substrate-1 in adipocytes: potential involvement of a membrane skeleton. *J Cell Biol* 140:1211–1225, 1998
36. Dhe-Paganon S, Ottinger EA, Nolte RT, Eck MJ, Shoelson SE: Crystal structure of the pleckstrin homology-phosphotyrosine binding (PH-PTB) targeting region of insulin receptor substrate 1. *Proc Natl Acad Sci U S A* 96:8378–8383, 1999
37. Razzini G, Ingrosso A, Brancaccio A, Sciacchitano S, Esposito DL, Falasca M: Different subcellular localization and phosphoinositides binding of insulin receptor substrate protein pleckstrin homology domains. *Mol Endcrinol* 14:823–836, 2000
38. Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH, Smith U: Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 94:4171–4175, 1997
39. Friedman JE, Ishizuka T, Shao J, Huston L, Highman T, Catalano P: Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese woman with gestational diabetes. *Diabetes* 48:1807–1814, 1999
40. Ricort JM, Tanti JF, Van Obbergen E, Le Marchand-Brustel YL: Different effects of insulin and platelet-derived growth factor on phosphatidylinositol 3-kinase at the subcellular level in 3T3–L1 adipocytes. *Eur J Biochem* 239:17–22, 1996
41. Nave BT, Haigh RJ, Hayaward AC, Siddle K, Shepherd PR: Compartment-specific regulation of phosphoinositide 3-kinase by platelet-derived growth factor and insulin in 3T3–L1 adipocytes. *Biochem J* 318:55–60, 1996
42. Holman GD, Kasuga M: From receptor to transporter: insulin signalling to glucose transport. *Diabetologia* 40:991–1003, 1997
43. Zhou L, Chen H, Xu P, Cong LN, Sciacchitano S, Li Yunhua, Graham D, Jaccobs AR, Taylor SI, Quon MJ: Action of insulin receptor substrate-3 (IRS-3) and IRS-4 to stimulate translocation of GLUT4 in rat adipose cells. *Mol Endcrinol* 13:505–514, 1999
44. Liu SCH, Wang Q, Lienhard GE, Keller SR: Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. *J Biol Chem* 274:18093–18099, 1999
45. Sztalryd C, Kraemer FB: Regulation of hormone-sensitive lipase in streptozotocin-induced diabetic rat. *Metabolism* 44:1391–1396, 1995