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Essential Role of Insulin Receptor Substrate-2 in Insulin Stimulation of Glut4 Translocation and Glucose Uptake in Brown Adipocytes*

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Insulin and insulin-like growth factor I signals are mediated via phosphorylation of a family of insulin receptor substrate (IRS) proteins, which may serve both complementary and overlapping functions in the cell. To study the metabolic effects of these proteins in more detail, we established brown adipocyte cell lines from wild type and various IRS knockout (KO) animals and characterized insulin action in these cells *in vitro*. Preadipocytes derived from both wild type and IRS-2 KO mice could be fully differentiated into mature brown adipocytes. In differentiated IRS-2 KO adipocytes, insulin-induced glucose uptake was decreased by 50% compared with their wild type counterparts. This was the result of a decrease in insulin-stimulated Glut4 translocation to the plasma membrane. This decrease in insulin-induced glucose uptake could be partially reconstituted in these cells by retrovirus-mediated reexpression of IRS-2, but not overexpression of IRS-1. Insulin signaling studies revealed a total loss of IRS-2-associated phosphatidylinositol (PI) 3-kinase activity and a reduction in phosphotyrosine-associated PI 3-kinase by 30% ($p < 0.05$) in the KO cells. The phosphorylation and activity of Akt, a major downstream effector of PI 3-kinase, as well as Akt-dependent phosphorylation of glycogen synthase kinase-3 and p70S6 kinase were not affected by the lack of IRS-2; however, there was a decrease in insulin stimulation of Akt associated with the plasma membrane. These results provide evidence for a critical role of IRS-2 as a mediator of insulin-stimulated Glut4 translocation and glucose uptake in adipocytes. This occurs without effects in differentiation, total activation of Akt and its downstream effectors, but may be caused by alterations in compartmentalization of these downstream signals.

Insulin promotes glucose uptake into muscle and adipose tissue through translocation of the Glut4 glucose transporter from an intracellular pool to the plasma membrane. An impairment of the ability of insulin to stimulate glucose uptake in these tissues contributes to the development of type 2 diabetes, hypertension, and cardiovascular disease (1).

Over the past 2 decades, considerable progress has been made defining the upstream signaling mechanisms regulating Glut4 translocation. Activation of the insulin receptor results in tyrosine phosphorylation of several IRS[1] proteins, the most widely distributed of which are IRS-1 and IRS-2 (2). These proteins interact with and activate Src homology 2 domain-containing proteins such as PI 3-kinase, SHP2, and Grb2. The activation of PI 3-kinase has been shown to be essential for insulin-induced Glut4 translocation and glucose uptake (3–7). Several serine/threonine kinases have been implicated in insulin-stimulated glucose transport, including Akt/protein kinase B (8–10) and the atypical forms of protein kinase C, protein kinase Cζ (11, 12) and Cλ (13).

Exactly which IRS protein or proteins are involved in mediating the effect of insulin on glucose transport is unclear. Reduction of IRS-1 using antisense ribozyme (14) or chronic insulin treatment (15) leads to decreased insulin responsiveness of glucose transport, whereas overexpression of IRS-1 in rat adipocytes partially mimics the effect of insulin to stimulate Glut4 translocation (14). Consistent with this, IRS-1-deficient mice show mild insulin resistance (16, 17), and isolated adipocytes and muscle from these animals exhibit a significant decrease in insulin-stimulated glucose uptake (18, 19). On the other hand, disruption of IRS-2 in mice leads to a more profound insulin resistance in muscle and liver, a significantly reduced β-cell mass, and early development of type 2 diabetes (20). However, a physiological role of IRS-2 in insulin-induced glucose transport has not been demonstrated.

In the present study, we have investigated the role of IRS-2 in insulin-stimulated Glut4 translocation and glucose uptake by establishing immortalized brown adipocytes from IRS-2 KO mice and their wild type counterparts. We find that insulin-induced Glut4 translocation and glucose transport are decreased significantly in the IRS-2-deficient cells despite a normal level of cell differentiation. Signaling studies suggest that this may be the result of a decrease in the IRS-2-associated pool of PI 3-kinase and membrane translocation of Akt. Thus, IRS-2 plays a unique role in insulin-induced Glut4 translocation and glucose transport in adipocytes.

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[1] The abbreviations used are: IRS, insulin receptor substrate; HDM, high density microsomal; KO, knockout; LDM, low density microsomal; PI 3-kinase, phosphatidylinositol 3-kinase; PM, plasma membrane; pY, phosphotyrosine;

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EXPERIMENTAL PROCEDURES

Materials—Antibodies used for immunoprecipitation and immunoblotting have been described previously and include anti-IRS-1 (21), anti-IRS-2 (21), antiphosphotyrosine 4G10 (22), anti-insulin receptor (kindly provided by Dr. Bentley Cheatham, Joslin Diabetes Center, Boston), anti-uncoupling protein-1 (Alpha Diagnostic International, San Antonio, TX), anti-PI 3-kinase p85, anti-Akt1, anti-Akt2 (Upstate Biotechnology, Inc., Lake Placid, NY), antiphosphospecific-Akt, antiphosphospecific-glycogen synthase kinase-3, antiphosphospecific-p70S6 kinase (New England Biolabs, Beverly, MA), anti-Akt, anti-peroxisome proliferator-activated receptor-γ, anti-C/EBPα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Glut4 (Chemicon International, Inc., Temecula, CA). Protein A/G-Sepharose was from Amersham Pharmacia Biotech, [γ-^{32}P]ATP from NEN Life Science Products, and ^{125}I-protein A from ICN Biochemicals, Inc. (Costa Mesa, CA). Phosphoinositol was purchased from Avanti Polar Lipids (Alabaster, AL), nitrocellulose from Schleicher & Schuell, thin layer chromatography plates from VWR Scientific (Bridgeport, NJ), and electrophoresis supplies from Bio-Rad. All other supplies were from Sigma Chemical Co.

Cell Isolation and Culture—Brown adipocytes and their precursors were isolated from newborn wild type and IRS-2 KO mice by collagenase digestion (22). Preadipose cells were immortalized by infection with the retroviral vector pBabe encoding SV40T antigen (kindly provided by Dr. J. DeCaprio, Dana Farber Cancer Institute, Boston) and selected with puromycin (1 μg/ml). For differentiation, preadipocytes were grown in culture medium supplemented with 20 nM insulin and 1 nM T3 (differentiation medium). After reaching confluence, cells were cultured for 48 h in differentiation medium further supplemented with 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin. After 4 more days in differentiation medium, cells exhibited a fully differentiated phenotype with massive accumulation of multilocular fat droplets. The stimulation experiments were carried out after culturing the cells in serum-free medium for 16–18 h.

Plasmids and Retroviral Infection of Cells—Full-length human IRS-1 (*Eco*RI-*Sal*I) and murine IRS-2 (*Bam*HI-*Sal*I) fragments described previously (21) were subcloned from the pBabe-puro-vector into the pBabe-bleo-vector (23). Viral ΦNX-packaging cells (24) were cultured and transfected at 70% confluence by calcium phosphate coprecipitation with 15 μg of pBabe-vectors, and viral supernatants were harvested 48 h after transfection. IRS-2-deficient cells were infected at 60% confluence with Polybrene (4 μg/ml)-supplemented virus overnight. Selection with 250 μg/ml of the bleomycin analogue Zeocin (Invitrogen, Carlsbad, CA) was started 48 h after infection.

Oil Red O Staining—Dishes were washed twice with phosphate-buffered saline and fixed by 10% formalin in phosphate-buffered saline for at least 1 h at room temperature. Cells were then stained with a filtered Oil Red O solution (0.5 g of Oil Red O in 100 ml of isopropyl alcohol) for 2 h at room temperature, washed twice with water, and visualized.

Preparation of Subcellular Fractions—After a starvation period of 16–18 h, differentiated brown adipocytes were treated with 100 nM insulin for 20 min, washed twice with ice-cold phosphate-buffered saline, and homogenized in HES buffer (10 mM Hepes, 255 mM sucrose, 1 mM EDTA, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4) using 26 strokes of a 1-ml Teflon-glass homogenizer. The homogenized cells were then subjected to subcellular fractionation to isolate plasma membrane (PM), cytosolic, high density microsomal (HDM), and low density microsomal (LDM) fraction as described previously (25).

Immunoprecipitation and Western Blot Analysis—Cells were harvested in lysis buffer (50 mM Hepes, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4), and lysates were clarified by centrifugation at 12,000 × *g* for 10 min at 4 °C. The protein amount in the supernatants was determined by the Bradford method (26), and equal amounts of protein (100 μg and 500 μg, respectively) were either solubilized directly in Laemmli sample buffer or immunoprecipitated for at least 2 h at 4 °C with the indicated antibodies. Immune complexes were collected by adding 50 μl of a 50% slurry of protein A/G-Sepharose in phosphate-buffered saline for 1 h at 4 °C. After three washes in lysis buffer, immunoprecipitates were solubilized in Laemmli sample buffer. Lysates or immunoprecipitates were boiled for 2 min, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Membranes were blocked in TBS (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.2) containing 3% bovine serum albumin for 30 min and incubated with the appropriate antibody for 2 h. Specifically bound primary antibodies were detected with either peroxidase-coupled secondary antibody and enhanced chemiluminescence or ^{125}I-protein A and autoradiography.

PI 3-Kinase Assays—Cells lysates were obtained as described above. Supernatants containing 500 μg of protein were subjected to immunoprecipitation with the indicated antibodies for 2 h at 4 °C, and the immune complexes were collected by adding 50 μl of 50% protein A-Sepharose in phosphate-buffered saline. After centrifugation, bound complexes were washed twice with phosphate-buffered saline containing 1% Igepal CA-630, twice with 0.5 M LiCl in 0.1 M Tris, pH 7.5, and twice in reaction buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA). Sepharose beads were resuspended in a mixture containing 50 μl of reaction buffer, 10 μl of 100 mM $MgCl_2$, and 10 μl of phosphatidylinositol (2 μg/μl). Reactions were initiated by adding 5 μl of reaction mixture (880 μM ATP, 20 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol)) per tube and stopped after 10 min by adding 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1). The samples were briefly centrifuged, and 50 μl of the lower organic phase was spotted on a silica gel thin layer chromatography plate. The plate was developed in $CHCl_3$:methanol:H_2O:NH_4OH (120:94:23:2.4), dried, exposed to a PhosphorImager screen, and quantitated with a Molecular Dynamics densitometer.

Protein Kinase B/Akt Assay—Protein kinase B/Akt assays were performed as described (27). Briefly, 500 μg of cellular protein was subjected to immunoprecipitation with an Akt-specific antibody, and immunocomplexes were collected with protein G-Sepharose beads. After two washes, the beads were resuspended in 40 μl of reaction buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 40 μM ATP, 3 μCi of [γ-^{32}P]ATP, 5 μg of Crosstide) for 20 min at 30 °C. Aliquots were spotted on P-81 paper, washed, and counted as Cherenkov radiation.

Glucose Uptake Assays—Cells were assayed for glucose uptake essentially as described (28). Differentiated monolayers of brown adipocytes were treated with insulin for the indicated periods of time, after which 50 μl of 2-deoxy-[^{3}H]glucose (0.5 μCi/ml final concentration) was added for an additional 3 min. The incorporated radioactivity was determined by liquid scintillation counting.

Statistical Analysis—Results are indicated as means ± S.E. Unpaired Student's *t* tests were used for analysis of differences between various cell treatments; p values <0.05 are considered significant and <0.01 highly significant.

RESULTS

Immortalized Preadipocytes from Wild Type and IRS-2 KO Mice Can Be Differentiated into Mature Brown Adipocytes—Preadipocytes derived from wild type and IRS-2-deficient mice were differentiated into brown adipocytes using insulin, T3, isobutylmethylxanthine, dexamethasone, and indomethacin as described under "Experimental Procedures." At various days of the differentiation process, cells from both genotypes were either stained with Oil Red O, a fat-specific dye (Fig. 1*A*), or prepared for Western blot analysis of several adipogenic markers (Fig. 1*B*). At day 1 of the differentiation protocol, neither wild type nor IRS-2-deficient preadipocytes showed significant fat staining (Fig. 1*A*). Cells of both genotypes showed a similar increase in fat content over time with maximal amounts at day 10 of differentiation (Fig. 1*A*). The adipogenic markers fatty acid synthase, peroxisome proliferator-activated receptor-γ, and C/EBPα showed a comparable expression pattern during the differentiation process in both wild type and KO cells (Fig. 1*B*). Similar amounts of the thermogenic brown fat-specific uncoupling protein-1 and the insulin-responsive Glut4 were detectable in wild type and IRS-2-deficient adipocytes during differentiation (Fig. 1*B*).

Insulin-stimulated Glucose Uptake Is Impaired in IRS-2-deficient Adipocytes—Basal and insulin-stimulated 2-deoxyglucose uptake were assessed in fully differentiated cells as described under "Experimental Procedures." There was no significant difference in basal glucose transport activity in IRS-2-deficient adipocytes compared with their wild type counterparts (Fig. 2, *A* and *B*). However, in dose response, as well as time course experiments, maximal insulin-stimulated glucose transport was decreased by more than 50% in IRS-2-deficient adipocytes compared with wild type cells ($p < 0.01$) (Fig. 2, *A*

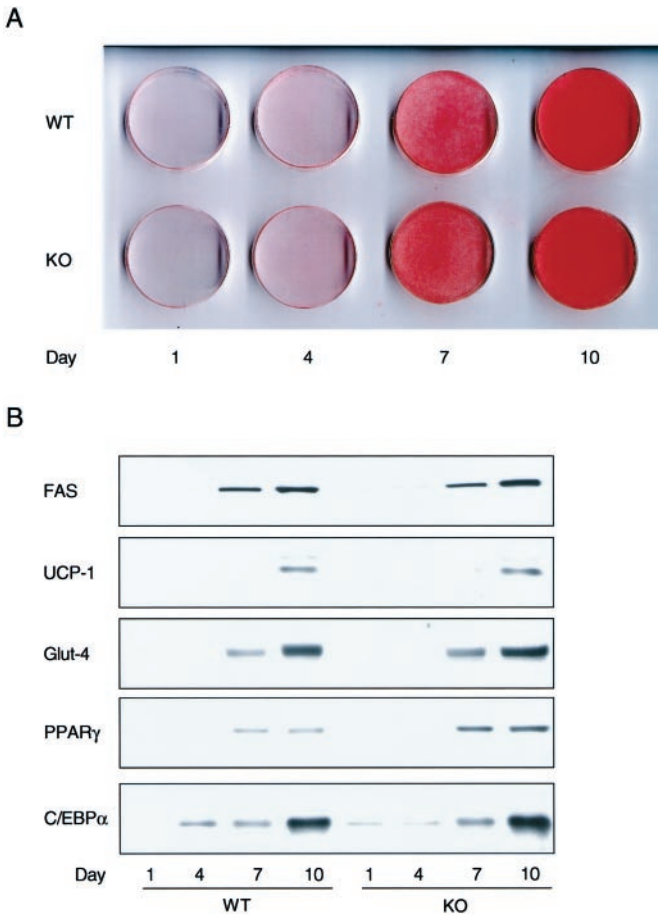

FIG. 1. **Wild type and IRS-2 KO preadipocytes can be differentiated into mature brown adipocytes.** Brown adipose precursor cells were isolated from newborn wild type and IRS-2 KO mice and differentiated into adipocytes as described under "Experimental Procedures." At the indicated days of the differentiation, cells were either stained with Oil Red O (*A*) or lysed (*B*), and various adipogenic markers were determined by immunoblotting with specific antibodies as shown. Results are representative of at least two independent experiments.

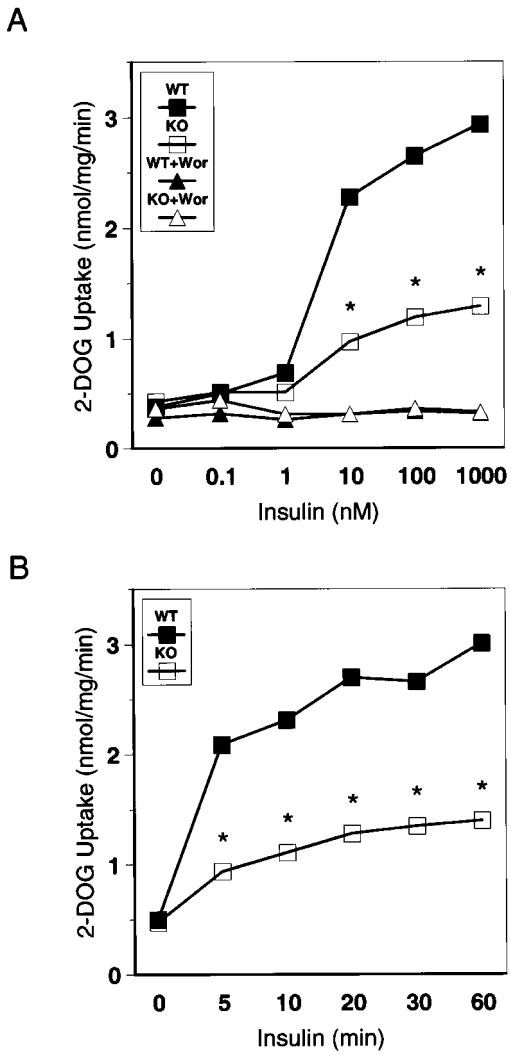

FIG. 2. **Insulin-induced glucose uptake is impaired in IRS-2 KO adipocytes.** Differentiated brown adipocytes from both genotypes were serum starved for 16–18 h. Insulin was added (*A*) at the indicated concentrations for 30 min alone or in combination with the PI 3-kinase inhibitor wortmannin (*Wor*, 100 nM, 30 min pretreatment) or (*B*) at the indicated times (100 nM) prior to exposing cells to [^{3}H]glucose for 3 min. Data are expressed as nmol of glucose taken up/mg of cell protein/min. The graphs present the average of four (*A*) and two (*B*) independent experiments, respectively. * indicates a highly significant difference ($p < 0.01$) comparing wild type (*WT*) and KO cells.

and *B*). In both the wild type and IRS-2 KO adipocytes insulin-induced glucose uptake was suppressed completely by pretreatment of these cells with wortmannin, an irreversible inhibitor of PI 3-kinase (Fig. 2*A*).

Insulin-stimulated Glut4 Translocation Is Decreased in IRS-2-deficient Cells—To determine if the decrease in insulin-stimulated glucose transport was caused by impaired Glut4 translocation from the LDM fraction to the PM, basal and insulin-induced cells were subjected to subcellular fractionation as described under "Experimental Procedures." Western blotting revealed no significant difference in the basal levels of Glut4 in PM and LDM fraction in the two cell genotypes (Fig. 3, *A* and *B*). Upon insulin stimulation, a significant 1.5-fold increase in Glut4 protein could be observed in the PM of wild type adipocytes (Fig. 3*A*). This was associated with a 50% decrease of Glut4 in the LDM fraction (Fig. 3*B*). The translocation of Glut4 to the PM of insulin-induced wild type cells was significantly higher than that in IRS-2-deficient cells, which exhibited only a 1.2-fold increase in the PM and a 15% decrease in the LDM fraction after insulin treatment (Fig. 3, *A* and *B*). Thus, lack of IRS-2 impairs insulin-induced Glut4 translocation, as well as glucose transport, in brown adipocytes.

Expression and Phosphorylation of Insulin Receptor, IRS-1, and IRS-2 in Wild Type and KO Cells—We investigated whether the decrease in insulin-stimulated Glut4 translocation and glucose transport in adipocytes lacking IRS-2 could be linked to changes on different levels of the insulin signaling cascade. There was no detectable difference in insulin receptor protein content or tyrosine phosphorylation between wild type and KO cells in both the basal and insulin-stimulated state (Fig. 4*A*). As expected, no IRS-2 protein was detectable by Western blot in whole cell lysates from IRS-2-deficient adipocytes (Fig. 4*B*). However, IRS-2 was detectable in cells from wild type animals and underwent rapid insulin-induced tyrosine phosphorylation with the maximum at 1 min of stimulation (Fig. 4, *B* and *C*). Western blots probed with anti-IRS-1 antibodies indicated comparable levels of IRS-1 in adipocytes from both genotypes (Fig. 4*B*). Furthermore, no significant difference in IRS-1 tyrosine phosphorylation in the KO cells compared with their wild type counterparts could be observed (Fig. 4*C*).

Preparation of KO Cell Lines Re-expressing IRS-2 and Overexpressing IRS-1—Withers *et al.* (20) have described a secondary IRS-1-associated defect in muscle and liver of IRS-2-deficient mice. Thus, to confirm that the lack of IRS-2 was

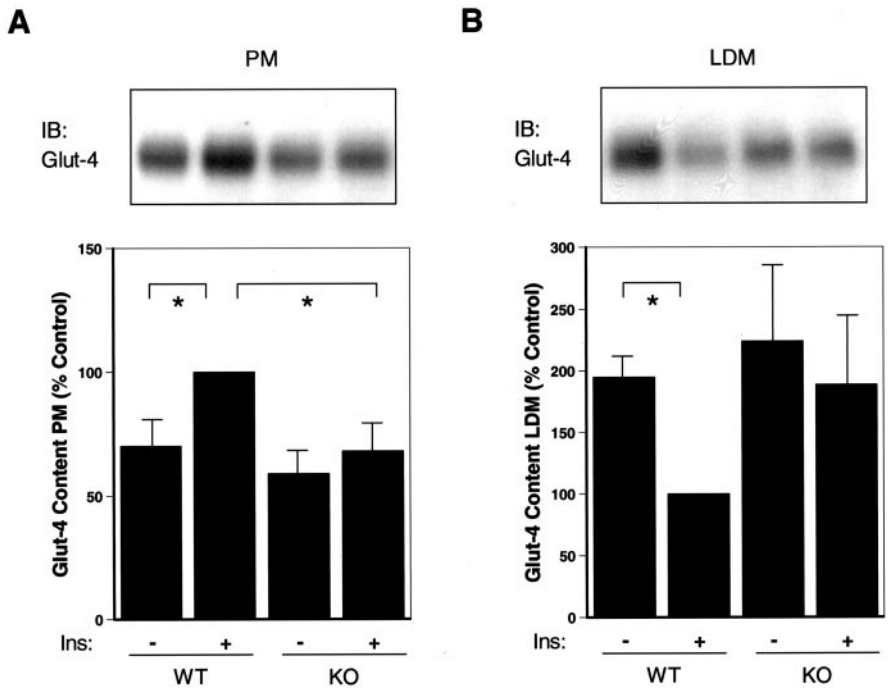

FIG. 3. **Insulin-induced Glut4 translocation is decreased in IRS-2-deficient cells.** Brown adipocytes derived from wild type (*WT*) and IRS-2 KO mice were cultured in serum-free medium (Dulbecco's modified Eagle's medium containing 25 mM glucose) for 16–18 h and then either not treated or stimulated with insulin (*Ins*, 100 nM) for 20 min. Protein lysates were fractionated into PM (*A*) and LDM (*B*) followed by SDS-polyacrylamide gel electrophoresis and Western blotting using a Glut4-specific antibody. A representative experiment and the statistical analysis of four independent experiments with the S.E. are shown. * denotes $p < 0.05$ comparing basal and insulin stimulation in the wild type cells or insulin activation between wild type and KO adipocytes.

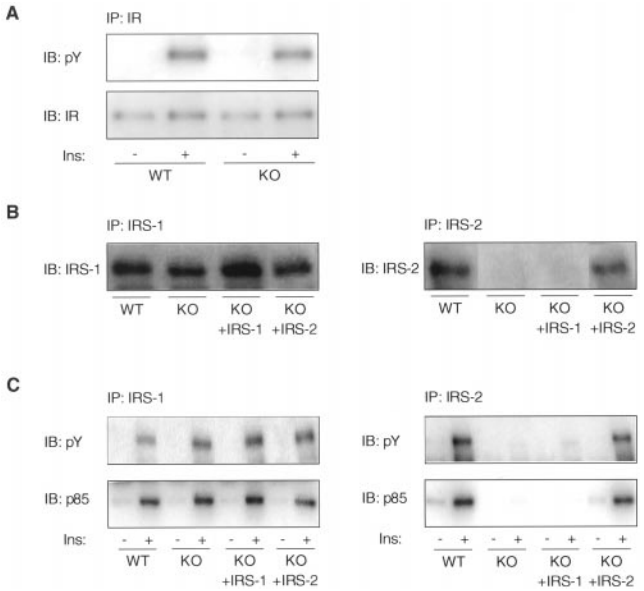

FIG. 4. **Expression and tyrosine phosphorylation of insulin receptor (*IR*), IRS-1, IRS-2, and p85 binding to the IRS in different cell lines.** Wild type (*WT*) and IRS-2-deficient (KO) adipocytes, as well as KO cells overexpressing IRS-1 (KO + IRS-1) or re-expressing IRS-2 (KO + IRS-2), were prepared as described under "Experimental Procedures." After serum starvation for 16–18 h, cells were stimulated with insulin (*Ins*, 100 nM) for 1 min. *A*, protein lysates were subjected to immunoprecipitation with insulin receptor β-subunit-specific antibody followed by immunoblotting with a phosphotyrosine (*upper panel*) or IR (*lower panel*) antibody. *B*, IRS-1 and IRS-2 were immunoprecipitated with specific antibodies followed by SDS-polyacrylamide gel electrophoresis and Western blotting using the same antibodies against each IRS. *C*, immunoprecipitations were performed as described in *B*, and immunoblots were prepared using phosphotyrosine- (*upper panel*) or p85- (*lower panel*) specific antibodies. A representative blot of at least four independent experiments is shown.

responsible for the differences in glucose transport between wild type and KO cells, we used retrovirus-mediated gene transfer to reconstitute the KO cells with IRS-2 or add more IRS-1. After retroviral-mediated gene transfer the level of IRS-2 re-expression in the KO cells was between 50 and 70% of that seen in wild type adipocytes (Fig. 4*B*). After IRS-1 retroviral infection, the IRS-2-deficient cells overexpressed IRS-1 about 1.8-fold above endogenous levels (Fig. 4*B*). Both the re-expressed IRS-2 as well as the overexpressed IRS-1 showed insulin-stimulated phosphorylation in proportion to the increased level of protein (Fig. 4*C*).

Insulin-stimulated Activation of PI 3-Kinase Is Reduced in IRS-2-deficient Adipocytes—Insulin-induced glucose uptake is dependent upon the activation of the enzyme PI 3-kinase, which binds via its p85 regulatory subunit to tyrosine-phosphorylated insulin receptor substrates. Immunoprecipitation of cell lysates with IRS-specific antibodies and immunoblotting with an antibody detecting the p85 subunit indicated an 8-fold and 5-fold stimulation of p85-binding to IRS-1 and IRS-2, respectively, after insulin treatment of wild type adipocytes (Fig. 4*C*). In the IRS-2 KO cells, no p85 binding could be detected in anti-IRS-2 immunoprecipitates, whereas coprecipitation of p85 with IRS-1 was unaltered, consistent with the expression and phosphorylation of these substrates in these cells (Fig. 4*C*). Retroviral re-expression of IRS-2 in KO cells restored insulin-induced p85 binding to IRS-2 in proportion to the level of IRS-2 protein, without affecting the amount of p85 associated with IRS-1 (Fig. 4*C*). IRS-2-deficient adipocytes overexpressing IRS-1 showed an almost 2-fold increase in insulin-induced p85 binding to IRS-1 compared with wild type cells, without having detectable binding of p85 to IRS-2 (Fig. 4*C*).

PI 3-kinase activity assays paralleled the results for p85 association. Thus, IRS-1- and IRS-2-associated PI 3-kinase activities were stimulated 12-fold and 7-fold, respectively, in wild type cells (Fig. 5, *A* and *B*). No IRS-2-associated PI 3-kinase activity was found in IRS-2-deficient adipocytes in either the basal or insulin-stimulated states (Fig. 5, *A* and *B*). Re-expression of IRS-2 in these cells restored this defect in proportion to the level of IRS-2 (Fig. 5, *A* and *B*). As expected, overexpression of IRS-1 had no effect on IRS-2-associated PI 3-kinase activity (Fig. 5, *A* and *B*). Insulin-induced IRS-1-associated PI 3-kinase activity was comparable in wild type and IRS-2-deficient adipocytes consistent with similar expression of IRS-1 in both cell lines, whereas overexpression of IRS-1 in the KO cells was accompanied by a significant 1.5-fold increase of PI 3-kinase activity in anti-IRS-1 immunoprecipitates compared with wild

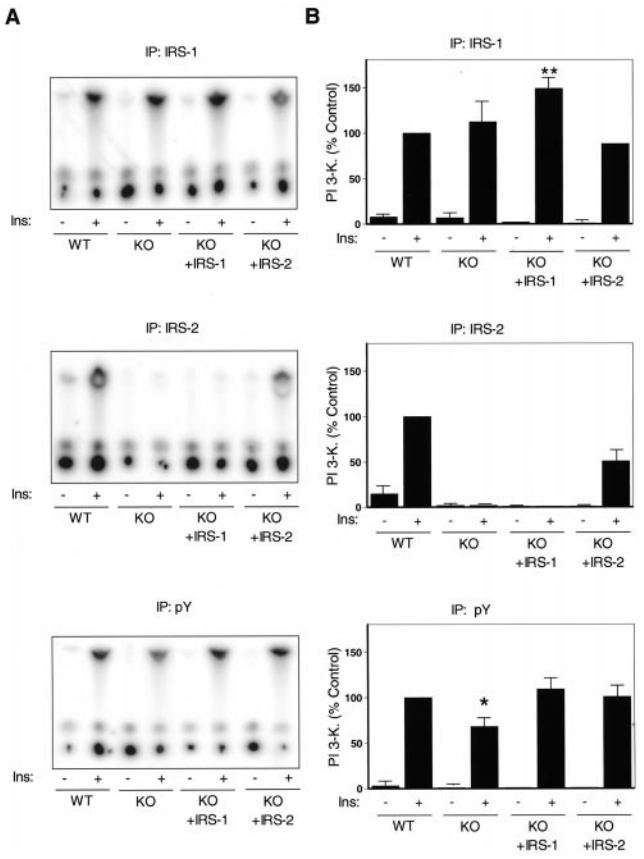

FIG. 5. **Insulin-stimulated PI 3-kinase activation is decreased in IRS-2-deficient adipocytes.** Cell lines designated in Fig. 4 were starved for 16–18 h, and then insulin (*Ins*, 100 nM) was added for 1 min. Lysates were subjected to immunoprecipitation with antibodies against IRS-1 (*top panel*), IRS-2 (*middle panel*), and phosphotyrosine (pY) (*bottom panel*). PI 3-kinase activity in these immunoprecipitates was measured as described under "Experimental Procedures." A representative experiment (*A*) and the statistical analysis with the S.E. (*B*) of at least four independent experiments are shown * indicates $p < 0.05$, ** $p < 0.01$.

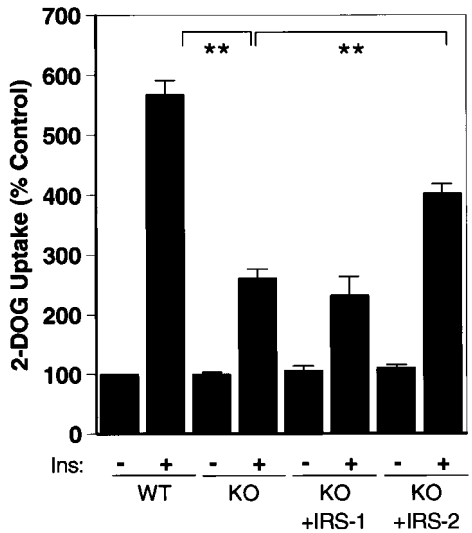

FIG. 6. **Re-expression of IRS-2 but not overexpression of IRS-1 partially reconstitutes insulin-induced glucose uptake in IRS-2 KO adipocytes.** The four different cell lines (designated as in Fig. 4) were serum starved for 16–18 h, and 100 nM insulin (*Ins*) was added for 30 min. 2-Deoxyglucose uptake is presented as a percentage of the basal uptake in wild type cells (100%). The graph presents the average and the S.E. of three independent experiments. ** indicates a highly significant difference ($p < 0.01$).

type cells (Fig. 5, *A* and *B*).

To assess the relative contribution of IRS-2 to total insulin-stimulated PI 3-kinase activation, we determined PI 3-kinase activity in phosphotyrosine (pY) immunoprecipitates. In wild type adipocytes insulin stimulation produced a 30-fold increase of pY-associated PI 3-kinase activity (Fig. 5, *A* and *B*). The level of pY-associated PI 3-kinase activity was decreased by 30% in IRS-2-deficient cells ($p < 0.05$) (Fig. 5, *A* and *B*), indicating that IRS-2 accounts for about 30% of insulin-induced PI 3-kinase activation in differentiated brown adipocytes. Re-expression of IRS-2 and overexpression of IRS-1 in the KO cells were both able to restore this quantitative defect in pY-associated PI 3-kinase (Fig. 5, *A* and *B*).

Re-expression of IRS-2 but Not Overexpression of IRS-1 Partially Reconstitutes Insulin-induced Glucose Uptake in IRS-2-deficient Cells—As both insulin receptor substrates were able to restore total PI 3-kinase activity in IRS-2-deficient adipocytes (Fig. 5, *A* and *B*), we determined whether re-expression of IRS-2 or overexpression of IRS-1 is sufficient to reconstitute insulin-induced glucose transport in these cells. There was no significant difference in basal glucose transport activity in all four cell lines (Fig. 6). Upon stimulation with insulin, wild type adipocytes showed a 6-fold increase in glucose uptake (Fig. 6). This was decreased by 54% in IRS-2-deficient cells (Fig. 6). Re-expression of IRS-2 in IRS-2-deficient cells increased insulin-stimulated glucose uptake 1.5-fold compared with the KO adipocytes and thereby rescued about 50% of glucose uptake lost due to lack of IRS-2 (Fig. 6). By contrast, overexpression of IRS-1 in KO adipocytes was unable to restore glucose uptake in IRS-2-deficient cells, despite its ability to reconstitute pY-associated PI 3-kinase activity fully (Fig. 6).

Insulin-induced Akt Activity and Downstream Signaling Are Not Altered in IRS-2-deficient Adipocytes—The serine/threonine kinase Akt is a downstream target of PI 3-kinase and has been implicated in insulin-stimulated Glut4 translocation and glucose transport. Total Akt content was similar in cell lines from both genotypes (Fig. 7*A*). Furthermore, the protein levels of Akt-1 and Akt-2 were comparable in IRS-2 KO adipocytes and their wild type counterparts as determined by Western blotting using isoform-specific antibodies (Fig. 7*A*). By immunoblotting with a phosphospecific antibody to Akt, we found comparable amounts of activated Akt in both wild type and KO adipocytes at all time points of insulin stimulation (Fig. 7*B*). Consistent with this observation basal and insulin-induced Akt activity were similar in both cell lines (Fig. 7*C*). Furthermore, Akt-dependent phosphorylation of glycogen synthase kinase-3 and p70S6 kinase were comparable between wild type and IRS-2-deficient adipocytes (Fig. 7*D*).

Plasma Membrane-associated Amounts of Akt Are Decreased in IRS-2-deficient Adipocytes upon Insulin Stimulation—We studied whether subcellular distribution of Akt might be different in wild type and KO cells. The majority of Akt was found in the cytosolic fraction in the basal and insulin-stimulated state (Fig. 8*A*). Treatment with insulin induced a similar shift of the cytosolic Akt in both wild type and KO cells, reflecting increased phosphorylation of this protein (Fig. 8*A*). The amount of cytosolic Akt decreased after insulin induction in adipocytes from both genotypes, probably because of translocation of this serine/threonine kinase to different subcellular compartments (Fig. 8*A*). A 1.3-fold increase in Akt content in the PM fraction could be observed in the wild type cells upon insulin stimulation, whereas no increase was detectable in IRS-2-deficient adipocytes (Fig. 8*B*). The amount of Akt in the PM fraction of insulin-induced KO cells was decreased by 50% compared with wild type adipocytes ($p < 0.05$) (Fig. 8*B*). Furthermore, upon insulin stimulation there was a trend ($p = 0.17$) toward de-

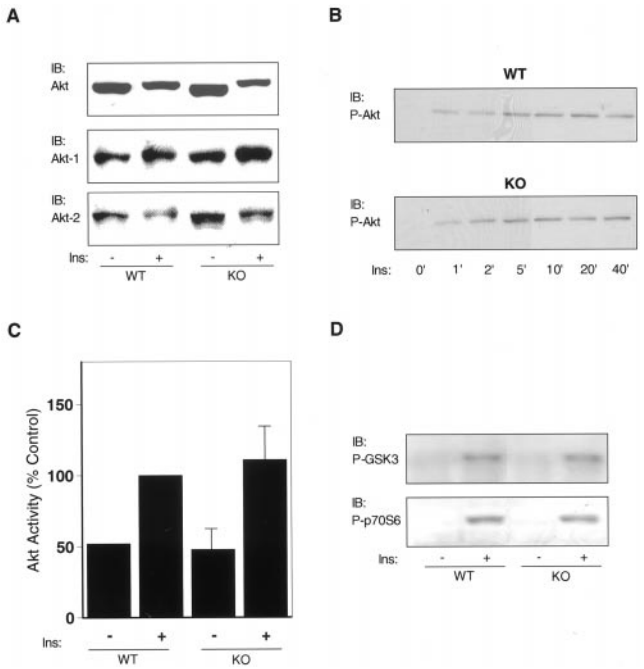

FIG. 7. **Insulin-stimulated activation of Akt and Akt downstream signaling is not altered in IRS-2-deficient cells.** Wild type (*WT*) and IRS-2 KO adipocytes were starved in serum-free medium for 16–18 h. *A*, cells from both genotypes were either not treated or stimulated with insulin (*Ins*, 100 nM) for 5 min. Western blot analysis is shown using antibodies against total Akt (*top panel*), Akt-1 (*middle panel*), and Akt-2 (*bottom panel*). *B*, wild type and KO adipocytes were treated with 100 nM insulin for the indicated periods of time, and immunoblots were prepared using phosphospecific antibody against the active form of Akt. *C*, Akt kinase activity in cells from both genotypes untreated or treated with insulin (100 nM, 5 min) was measured as described under "Experimental Procedures." *D*, wild type and IRS-2-deficient adipocytes were left untreated or stimulated with insulin (100 nM) for 20 min. Western blot analysis is shown using antibodies against phosphoglycogen synthase kinase 3 (*upper panel*) or phospho-p70S6 kinase (*lower panel*). Representative experiments (*A*, *B*, and *D*) or the statistical analysis with the S.E. (*C*) of at least four independent experiments is shown.

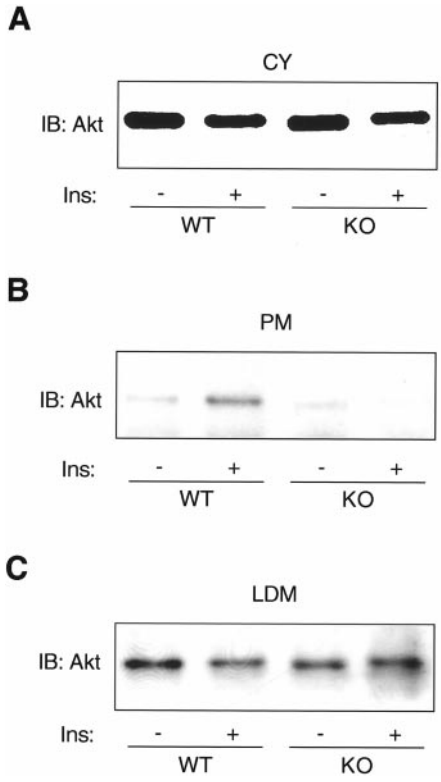

FIG. 8. **Plasma membrane-associated amounts of Akt are decreased in IRS-2-deficient adipocytes upon insulin stimulation.** Wild type (*WT*) and IRS-2-deficient adipocytes were cultured in serum-free medium for 16–18 h and then either not treated or stimulated with insulin (*Ins*, 100 nM) for 20 min. Protein lysates were fractionated into cytosolic (*A*), PM (*B*), and LDM (*C*) followed by Western blotting with an Akt-specific antibody. Representative blots of four independent experiments are shown.

creased Akt amounts in the LDM fraction of wild type cells, whereas no change could be observed in IRS-2-deficient adipocytes (Fig. 8*C*). No significant amount of Akt was detectable in the HDM fraction (data not shown).

DISCUSSION

To obtain a better understanding of the physiological role of the four different IRS proteins, we and other have established mice with a targeted disruption of each IRS gene (16, 17, 20, 29, 30). IRS-1-deficient mice exhibit a phenotype of growth retardation and insulin resistance (16, 17), whereas IRS-2 KO mice are overtly diabetic due to both a β-cell defect and insulin resistance, but they have no growth retardation (20). In contrast, mice with disruption of either IRS-3 or IRS-4 show no significant defect in growth or glucose homeostasis (29, 30), suggesting that these insulin receptor substrates may play specific roles as mediators of very select insulin functions. Recently, a number of studies have examined signaling in cells and tissues derived from IRS-1 KO mice. These studies have suggested a role for IRS-1 in insulin-induced glucose uptake in adipocytes (18) and muscle (19), in insulin-stimulated lipogenesis (31) and protein synthesis (19), and in insulin-like growth factor I-mediated cell growth (21). By comparison, little is known about the function of IRS-2 in isolated cells.

In the current study, we compared insulin signaling in IRS-2 KO and wild type cells utilizing SV40T antigen-immortalized brown adipocytes isolated from neonatal mice of both genotypes. These cells provide an attractive model to study insulin action for several reasons. We have shown that the brown preadipocytes can be differentiated into mature brown adipocytes with an accumulation of multilocular fat droplets as well as expression of adipogenic and thermogenic differentiation markers, including fatty acid synthase and uncoupling protein-1 (this study and Ref. 22). The cells also contain the major elements of the insulin signaling system and exhibit the classical function of insulin in fat, *i.e.* stimulation of glucose transport (this study and Ref. 22). It has further been shown that brown adipocytes express high levels of IRS-1 and IRS-2, unlike other cultured adipose cell models such as 3T3-L1 cells, in which the level of IRS-2 is very low compared with IRS-1 (31). Furthermore, immortalized brown adipocytes can be established from different animal models using a single newborn or late fetal mouse.

Using this system, we find that IRS-2 is not crucial for brown fat cell differentiation, as preadipocytes from both genotypes can be differentiated to a comparable extent into mature brown adipocytes. Furthermore, no differences in content and tyrosine phosphorylation of insulin receptor and IRS-1 could be detected between wild type and IRS-2-deficient adipocytes, whereas, as expected, IRS-2 was not expressed in the KO cells. These findings are in agreement with data obtained in liver and muscle of IRS-2 KO mice (20).

PI 3-kinase activation has been shown to be involved in mediating many of insulin-regulated metabolic effects, including glucose uptake (3), antilipolysis (6), and glycogen synthesis (32). As expected, no IRS-2-associated PI 3-kinase activity was detectable in IRS-2-deficient cells, whereas wild type adipocytes showed a strong 7-fold increase upon insulin stimulation.

This lack of IRS-2-associated function in the KO cells was not compensated for by IRS-1. This is consistent with the findings of Withers *et al.* (20) who showed no IRS-1 up-regulation or compensation of the missing IRS-2-associated PI 3-kinase activity in liver and muscle of IRS-2-deficient mice. In fact, in this study a decrease in insulin-induced IRS-1-associated PI 3-kinase activity in the KO mice compared with wild type animals was observed (20). On the other hand, evidence has been presented that lack of IRS-1 in liver, muscle, and brown fat is compensated for by IRS-2 (31, 33). This apparent difference between IRS-1- and IRS-2-deficient mice might contribute to the more severe phenotype of IRS-2 KO animals.

We also find that overall pY-associated PI 3-kinase activity was decreased by 30% in IRS-2-deficient adipocytes compared with the wild type cells. To determine whether this decrease in insulin-induced overall PI 3-kinase activity in the KO cells might be accompanied by impaired glucose transport we performed glucose uptake assays. These revealed an ~50% decrease of insulin-stimulated glucose transport in IRS-2-deficient adipocytes compared with wild type cells over the entire dose range between 10 nM and 1 μM of insulin. The diminished glucose transport activity in the KO cells could be observed at as early as 5 min of insulin stimulation and was still detectable at 1 h of insulin treatment. To our knowledge, this is the first study to show the physiological relevance of an IRS-2-mediated pathway in insulin-induced glucose uptake. In agreement with our findings Zhou *et al.* (34) showed that overexpression of IRS-2 in rat adipocytes was able to increase insulin-stimulated Glut4 translocation. In contrast to our work Higaki *et al.* (35) could not detect a difference in insulin-induced glucose uptake in isolated muscle of IRS-2 KO mice compared with wild type animals. However, similar amounts of tyrosine-phosphorylated IRS-1 and IRS-2 are detectable in brown adipocytes (data not shown), whereas IRS-2 tyrosine phosphorylation in muscle has been reported to be only 20–30% compared with IRS-1 (19). This difference in the ratio of functional IRS-2 to IRS-1 might well explain the different observations in both tissues. The decrease in insulin-induced glucose transport in the IRS-2-deficient adipocytes is caused by an impairment of Glut4 translocation from the LDM to the PM fraction, in accordance with the view that insulin-stimulated glucose uptake is mediated primarily by Glut4 translocation (36).

We further investigated whether re-expression of IRS-2 or overexpression of IRS-1 in IRS-2-deficient adipocytes might reconstitute impaired insulin-stimulated PI 3-kinase activity, as well as glucose uptake, in the KO cells. Retroviral expression of both substrates was able to reconstitute overall PI 3-kinase activity to the wild type level. However, only IRS-2 re-expression was able to increase insulin-induced glucose uptake in the KO cells significantly. Therefore, it appears that not only the decrease in overall PI 3-kinase activity, but the specific loss of IRS-2-associated PI 3-kinase leads to the impaired insulin-induced glucose uptake in the KO cells. Bruning *et al.* (21) have reported parallel results in IRS-1-deficient 3T3 fibroblasts, which showed decreased insulin-like growth factor I-induced overall PI 3-kinase activity as well as impaired insulin-like growth factor I-stimulated cell growth. In their study, expression of either IRS-1 or IRS-2 was able to reconstitute PI 3-kinase activity to a similar extent, but only re-expression of IRS-1 was able to reconstitute mitogenesis. Both of these studies provide important evidence that IRS-1 and IRS-2 are not simply interchangeable proteins. In this context, it should be noted that the carboxyl-terminal regions of IRS-1 and IRS-2 are rather poorly conserved (35% identity) (37) and that different receptor binding domains such as the kinase regulatory loop binding domain are only present in IRS-2, but not IRS-1 (38). Furthermore, different compartmentalization and trafficking of IRS-1 and IRS-2 might also play a role in the different response of IRS-2-deficient adipocytes to expression of IRS-1 *versus* IRS-2 (39).

The signaling events downstream of PI 3-kinase leading to insulin-induced Glut4 translocation and glucose uptake are at present only partly understood. The protein serine/threonine kinase Akt has been implicated as one downstream target of PI 3-kinase important in insulin-stimulated glucose transport in both adipocytes (8–10) and muscle (27, 40, 41). However, two recent reports could not detect any significant effect of a dominant negative mutant of Akt on insulin-induced Glut4 translocation (13, 42). We found no differences in Akt content, phosphorylation, and activity between the two cell lines. Furthermore, Akt-dependent phosphorylation of p70S6 kinase, as well as phosphorylation of glycogen synthase kinase 3, were comparable in wild type and KO adipocytes. Recently, a report has proposed a role for Akt-2 but not Akt-1 in insulin-induced glucose uptake (43). However, no differences in both isoforms could be detected in IRS-2-deficient cells compared with their wild type counterparts. Recent studies reporting the insulin-induced association of Akt-2 with Glut4-containing vesicles (44) and phosphorylation of its components (45) suggest that subcellular localization of this kinase might be important in the regulation of Glut4 translocation. In fact, we find that Akt content in the PM fraction is significantly reduced in the KO cells compared with wild type adipocytes after insulin stimulation. Furthermore, we observe a trend toward decreased Akt amounts in the LDM fraction of wild type cells upon insulin induction, whereas no change is detectable in IRS-2-deficient adipocytes. These findings raise the possibility that alterations in subcellular localization of Akt rather than in total activation of this kinase might lead to diminished insulin-induced glucose uptake in the KO cells.

In summary, our data implicate a unique role for IRS-2 in insulin-induced Glut4 translocation and glucose uptake in brown adipocytes. Furthermore, we show that IRS-2-deficient cells show decreased overall PI 3-kinase activity and that expression of IRS-2 but not IRS-1 in KO adipocytes reconstitutes insulin-induced glucose uptake. Further work will be needed to clarify the events linking diminished PI 3-kinase activity in IRS-2-deficient cells to decreased Glut4 translocation and glucose uptake.

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