Insulin Receptor Substrate 2-mediated Phosphatidylinositol 3-kinase Signaling Selectively Inhibits Glycogen Synthase Kinase 3β to Regulate Aerobic Glycolysis*

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Justine Landis and Leslie M. Shaw

From the Department of Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Background: Insulin receptor substrate 2 (Irs-2)-mediated signaling by the insulin and insulin-like growth factor 1 receptors regulates metabolism.

Results: Direct activation of PI3K by Irs-2 inhibits glycogen synthase kinase 3β (Gsk-3β) to stimulate glucose uptake and aerobic glycolysis.

Conclusion: Irs-1 and Irs-2 regulate distinct subsets of Akt effectors.

Significance: The Irs-2-mediated PI3K signaling pathway could be targeted to inhibit tumor metabolism.

Insulin receptor substrate 1 (IRS-1) and IRS-2 are cytoplasmic adaptor proteins that mediate the activation of signaling pathways in response to ligand stimulation of upstream cell surface receptors. Despite sharing a high level of homology and the ability to activate PI3K, only IRS-2 positively regulates aerobic glycolysis in mammary tumor cells. To determine the contribution of Irs-2-dependent PI3K signaling to this selective regulation, we generated an Irs-2 mutant deficient in the recruitment of PI3K. We identified four tyrosine residues (Tyr-649, Tyr-671, Tyr-734, and Tyr-814) that are essential for the association of PI3K with Irs-2 and demonstrate that combined mutation of these tyrosines inhibits glucose uptake and lactate production, two measures of aerobic glycolysis. IRS-2-dependent activation of PI3K regulates the phosphorylation of specific Akt substrates, most notably glycogen synthase kinase 3β (Gsk-3β). Inhibition of Gsk-3β by Irs-2-dependent PI3K signaling promotes glucose uptake and aerobic glycolysis. The regulation of unique subsets of Akt substrates by Irs-1 and Irs-2 may explain their non-redundant roles in mammary tumor biology. Taken together, our study reveals a novel mechanism by which IRS-2 signaling preferentially regulates tumor cell metabolism and adds to our understanding of how this adaptor protein contributes to breast cancer progression.

The insulin receptor substrate (IRS)² proteins are cytoplasmic adaptor proteins that organize signaling complexes downstream of cell surface receptors (1). Originally discovered as substrates of the insulin receptor, they function as adaptor proteins for additional surface receptors, including the closely related insulin-like growth factor 1 receptor (IGF-1R) (2, 3). Upon ligand stimulation, the IRS proteins are recruited to activated receptors where they are phosphorylated on tyrosine residues within their C termini, generating binding sites for the recruitment of downstream signaling effectors, including PI3K, growth factor receptor-bound protein 2 (GRB-2), and Src homology 2 domain-containing protein-tyrosine phosphatase 2 (SHP-2) (4). Following receptor activation, the combinatorial recruitment of these effectors by IRS-1 and IRS-2 is required for amplification of signaling cascades that regulate changes in cellular behavior. IRS-1 and IRS-2 are expressed ubiquitously and are the primary mediators of insulin-dependent mitogenesis and glucose metabolism in most cell types (1). Although they share considerable homology, in vitro cell line studies and distinct phenotypes of the Irs-1 and Irs-2 knockout mice confirm that these proteins do not function in a redundant manner in normal cell biology or development (5–10).

IRS-1 and IRS-2 also play divergent roles in breast cancer. Stimulation of human breast carcinoma cell lines expressing only IRS-1 with IGF-1 increases their proliferation, whereas stimulation of cells expressing only IRS-2 promotes cell migration (11, 12). These differences in IRS function have also been demonstrated in vivo using the mouse mammary tumor virus-polyoma virus middle T antigen (MMTV-PyMT) mouse model of mammary tumor progression. Specifically, in mice expressing PyMT, mammary tumor metastasis is diminished significantly in the absence of IRS-2 expression, and IRS-1 does not compensate for this loss (13). In fact, PyMT: IRS-1−/− tumors have elevated expression and tyrosine phosphorylation of IRS-2, and these tumors are more metastatic when compared with their WT counterparts (14). Cells derived from PyMT: IRS-2−/− tumors are also significantly less invasive and display decreased aerobic glycolysis relative to PyMT:WT or PyMT: IRS-1−/− tumor cells in vitro (13, 15). Distinct functions for IRS-1 and IRS-2 in human breast tumors are also indicated by their unique intracellular localization patterns. Tumor cells express both IRS-1 and IRS-2 in the cytoplasm, whereas IRS-1 also localizes to the nucleus and IRS-2 to the cell membrane (16, 17). Nuclear localization of IRS-1 correlates with an increased...
response to tamoxifen and improved patient survival, whereas IRS-2 cell membrane localization correlates with decreased overall patient survival (16, 18). Differential intracellular compartmentalization may contribute to the ability of the IRS proteins to regulate distinct tumor cell functions.

The IRS proteins share their highest degree of homology in their N-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains, which mediate their interactions with upstream receptors (19–22). The C termini of the IRS proteins are less conserved, and it is these C-terminal differences that likely confer upon the IRS proteins their divergent functions through specific interactions that impact localization and signaling (4). With regard to signaling, activation of the PI3K/mechanistic target of rapamycin (mTor) pathway is enhanced in PyMT::Irs-1/−/− tumors that express only IRS-2 and is significantly lower in tumors that lack IRS-2 expression, indicating that IRS-1 does not compensate fully for the activation of this pathway in vivo (14, 15). Similarly, in vitro, mammary tumor cells derived from PyMT::Irs-1/−/− tumors have enhanced PI3K/mTor signaling, and this increased activity is dependent upon IRS-2 (14). The PI3K signaling pathway is one of the most commonly mutated pathways in cancer, including breast cancer (23, 24). PI3K itself is an oncogene, and activating mutations have been observed in many types of cancer (25). In tumors that do not harbor PI3K mutations, other components of this signaling pathway, such as the lipid phosphatase PTEN (phosphatase and tensin homology) and AKT, are often mutated so that PI3K signaling is enhanced (25, 26). One of the major contributions of the PI3K/AKT signaling pathway to tumors is the regulation of metabolic pathways that promote aerobic glycolysis, a hallmark of cancer (27, 28). The overall goal of this study was to establish the contribution of IRS-2-mediated PI3K activation to mammary tumor cell metabolism and to determine the mechanism by which IRS-2/PI3K signaling preferentially regulates aerobic glycolysis.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents—IRS-2/−/− mouse mammary tumor cell lines were isolated from MMTV-PyMT: IRS-2/−/− mice as described previously (13). IRS-2/Wt and IRS-1/Wt/IRS-2/Wt mammary tumor cells were isolated from female FVB MMTV-PyMT:: IRS-2/Wt and FVB MMTV-PyMT:: IRS-1/Wt/IRS-2/Wt mice, respectively, using a similar procedure. IRS-1/Wt and IRS-2/Wt mice were a gift from Morris White (Children’s Hospital, Boston, MA) (29, 30). All cell lines were maintained in low-glucose (1g/liter) DMEM (Invitrogen) containing 10% FBS (Sigma). PyMT::IRS-2/Wt and PyMT::IRS-1/Wt/IRS-2/Wt cells were infected with adenoviruses containing either GFP or GFP and Cre recombinase (Gene Transfer Vector Core, University of Iowa) at a multiplicity of infection of 10, and GFP-positive cells were isolated by FACS. The IRS-1/−/−/ IRS-2/−/− double null cells were subcloned after Cre infection and sorting to isolate cells with a complete knockout of both IRS-1 and IRS-2.

The murine pCMV-His-IRS-2 construct was provided by Morris White (Children’s Hospital, Boston, MA). Murine IRS-2 was subcloned into the pExchange-Puro vector (Stratagene). Tyrosine residues in IRS-2 were mutated to phenylalanine using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. Mutagenesis primers were purchased from Integrated DNA Technologies (Y538F, 5′ GGGCGAACTCTATGGTTCTAGGATGC 3′ and 3′ GTCTATCCATGCTCATGAACCCAT AGATG 5′; Y628F, 5′ CCCCACCAAGAGA CTGGAGACAT -TGAG 3′ and 3′ CTCAATG TCTCCAAATGCTCTGGGTAAG 5′; Y649F, 5′ GGCAGATGCTGCCCCTGCAGAATGG 3′; Y734F, 5′ CGCCAAGACATGGTGTTCCATGTCATGC 5′; Y758F, 5′ CCCCCACGGGACTT CCCTCAACATGTTCC 3′ and 3′ GGAGGACA TGGGAGCCTCCATGC 5′; Y765F, 5′ TCCCT CAACATGTCCCCTAGC 3′; Y782F, 5′ TCGGTGAGCCTCCATGC 5′; Y814F, 5′ CACAGGAGCAATGACCAGTTTGCAT’CAGACG 3′ and 3′ GTCTGA GAGACATGTCCAGCTGCGT 5′). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. For stable selection, cells were grown in puromycin (100 μg/ml, Fisher).

The following antibodies were used for immunoprecipitation or immunoblotting: IRS-1 (Bethyl Laboratories), IRS-2 (immunoblot, Calbiochem; immunoprecipitation, Bethyl Laboratories), p85 (Millipore), PY99 (Santa Cruz Biotechnology), Glut1 (Abcam), and a6 integrin subunit (a gift from Anne Cress, University of Arizona). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Labs. All other antibodies were obtained from Cell Signaling Technologies.

**Immunoprecipitation and Immunoblotting—**Cells were serum-starved for 4 h in 0.1% BSA/DMEM (1g/liter glucose) and then stimulated with IGF-1 (100 ng/ml) or insulin (100 ng/ml) for the times listed in the figure legends. Cells were solubilized on ice for 20 min in lysis buffer (50 ml Tris (pH 7.4) containing 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitors (Complete Mini, Roche)). Insoluble material was removed by centrifugation at 14,000 rpm for 10 min at 4 °C.

Aliquots of cell extracts containing equivalent amounts of total protein were incubated under constant agitation at 4 °C overnight with IRS-1- or IRS-2-specific antibodies and protein A-Sepharose beads (GE Healthcare). Immune complexes were washed with lysis buffer and incubated with Laemmli sample buffer containing β-mercaptoethanol for 5 min at 95 °C. Immune complexes as well as aliquots of cell extracts containing equal amounts of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 1X TBST buffer (50 mM Tris (pH 7.5) containing 0.15 M NaCl and 0.1% Tween 20) containing 5% (w/v) Carnation dry milk. Membranes were then incubated overnight at 4 °C in blocking buffer containing primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature in blocking buffer, and proteins were then detected using enhanced chemiluminescence (Pierce and Bio-Rad). For phospho-antibodies, the blocking buffer contained 5% BSA (w/v) (Sigma).
Lactate and Glucose Uptake Assays—Cells were grown in 24-well plates to near confluence, washed with PBS, and then incubated with 0.1% BSA/DMEM (1g/liter glucose) supplemented with IGF-1 (20 ng/ml) for 24 h with or without the addition of the Gsk-3β inhibitor SB 216763 (10 μM, Sigma). Lactate levels in the conditioned media were measured using a lactate assay kit (Triinity Biotech), and glucose levels were measured using a glucose assay kit (Sigma) according to the instructions of the manufacturer. Total cellular protein per well was quantified using a Bradford assay (Bio-Rad), and lactate production and glucose uptake were expressed as a rate measurement (millimolar/milligram/hour) normalized to protein content.

Cell Surface Biotinylation—Cells were grown to near confluence and then incubated in 0.1% BSA/DMEM (1g/liter glucose) with or without IGF-1 (20 ng/ml) or SB 216763 (10 μM) for 24 h. Following two washes with cold PBS, cells were biotinylated at 4 °C for 30 min using EZ-Link™ Sulfo-NHS-SS-Biotin (0.5 mg/ml, Pierce Biotechnology). Biotin was removed, and the cells were incubated in a 50 mM Tris buffer (pH 7.4) for 5 min to quench any residual biotin. Cells were washed with cold PBS and solubilized on ice with a 50 mM Tris buffer containing 0.15 M NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitors (Complete Mini, Roche). Nuclear contaminates were removed by centrifugation at 14,000 rpm for 10 min at 4 °C.

Aliquots of cell extracts containing equal amounts of protein were incubated with Neutravidin agarose beads (Pierce Biotechnology) with constant agitation overnight at 4 °C. Pulldown complexes were washed with the lysis buffer described above and incubated with Laemmli sample buffer containing β-mercaptoethanol for 5 min at 95 °C.

RESULTS

Identification of Tyrosine Residues within Irs-2 That Contribute to the Recruitment of PI3K—PI3K is activated by the interaction of SH2 domains within the p85 regulatory subunit with phosphotyrosine residues in upstream receptors or adaptor proteins (31–35). To understand how Irs-2 activates PI3K signaling, we initially sought to identify specific tyrosine residues that participate in the recruitment of PI3K. Tyrosines located within consensus PI3K binding motifs (YXXM) were mutated to phenylalanine to prevent phosphorylation while maintaining their ability to recruit PI3K (Tyr-628 and Tyr-758) were also mutated in an Irs-2 proteomics phosphopeptide screen to be capable of binding to PI3K. Tyrosines that individually contributed to the association of Irs-2 with p85 (Tyr-649, Tyr-671, and Tyr-814); Irs-2 Y4F, which added an additional mutation at Tyr-734 to Y3F; and Irs-2 Y5F, which added an additional mutation at Tyr-538 to Y4F (Fig. 1A). The level of tyrosine phosphorylation and association with p85 that was observed for the Irs-2 Y3F mutant was only marginally lower than that observed for the individual Y671F and Y814F Irs-2 mutants (Fig. 2, A and B). Additional mutation of Tyr-734 significantly reduced the level of p85 association when compared with Irs-2 Y3F, indicating that Tyr-734 participates in the interaction of Irs-2 with PI3K (Fig. 2, A and C). The reason why protein expression can be obtained when this tyrosine is mutated in conjunction with other tyrosine mutations, whereas mutation of this tyrosine alone leads to protein instability, remains to be determined. Additional mutation of Tyr-538 (Irs-2 Y5F) did not provide a further significant reduction in tyrosine phosphorylation or p85 association, confirming that this tyrosine residue does not contribute to the interaction of Irs-2 with PI3K (Fig. 2, D–F).

Irs-2-dependent Activation of PI3K Stimulates Aerobic Glycolysis—To establish a model system with which to study Irs-2-dependent PI3K activation and its role in the regulation of aerobic glycolysis, we derived cell lines from PyMT: Irs-2fl/fl mammary tumors and acutely deleted Irs-2 expression in vitro by transient adenoviral infection of Cre recombinase. These PyMT: Irs-2−/− cells were stably transfected to express either empty vector, WT Irs-2, or the Irs-2 Y5F mutant at equivalent protein levels compared with the parental population (Irs2fl/fl) (Fig. 3A). Similar to the results obtained after transient expression of Irs-2 Y5F, a significant, but not total, reduction in Irs-2 tyrosine phosphorylation and association with PI3K was observed when compared with WT Irs-2 (76 and 86% reduction, respectively) (Fig. 3, B and C). PyMT: Irs-2−/− cells expressing an empty vector and PyMT: Irs-2−/− cells expressing an empty vector, WT Irs-2, or Irs-2 Y5F were assayed for their level of aerobic glycolysis. As shown in Fig. 3, D and E, IGF-1-stimulated glucose uptake and lactate production were reduced by nearly 40% in the absence of Irs-2, and these activities were...
rescued by restoring WT Irs-2 expression. However, expression of Irs-2 Y5F did not restore glucose uptake or lactate production to the levels observed in WT Irs-2-expressing cells, establishing the requirement for direct PI3K activation by Irs-2 in the regulation of aerobic glycolysis (Fig. 3, D and E).

Identification of Irs-2/PI3K-dependent Akt Effectors—Although both Irs-1 and Irs-2 are capable of recruiting PI3K and stimulating the activation of Akt, which plays an important role in regulating metabolism, Irs-2 preferentially regulates aerobic glycolysis in mammary tumor cells (15, 35, 37, 40). To further investigate the mechanism of this metabolic regulation, we sought to identify downstream signaling effectors of Akt that are selectively activated by Irs-2-dependent signaling. Loss of Irs-2 expression did not diminish the overall level of IGF-1-

FIGURE 1. Identification of tyrosine residues that mediate Irs-2 recruitment of PI3K. A, schematic of WT Irs-2 and Irs-2 tyrosine mutants. Individual tyrosines that were mutated to phenylalanine are indicated for each mutant construct. B–E, Irs-2−/− cells were transiently transfected with WT Irs-2 or the individual tyrosine mutant constructs and stimulated with IGF-1 (100 ng/ml) (B and C) or insulin (100 ng/ml) (D and E) for 10 min. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated (IP) with Irs-2-specific antibodies, and the immune complexes were immunoblotted with antibodies that recognize either phosphotyrosine (p-Tyr) or p85α. The p-Tyr blots were stripped and reprobed for Irs-2. The data shown in the graphs represent the mean ± S.E. of four (B), three (C and D), or two (E) independent experiments. NT, mock transfection. *, p < 0.05 relative to WT Irs-2.

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stimulated Akt activation, as assessed by phosphorylation on either Ser-473 or Thr-308 (Fig. 4A). Similarly, restoring WT Irs-2 or Irs-2 Y5F expression in Irs-2−/− cells also did not significantly alter phosphorylation of Akt on either site relative to the level of phosphorylation observed in the vector control, Irs-2−/− cells (Fig. 4, B–D). This lack of impact on signaling is likely explained by increased expression of Irs-1 in the Irs-2−/− cells, which compensates for the activation of PI3K and Akt.

To assay for Irs-2-specific Akt activation independent of Irs-1, WT Irs-2 and Irs-2 Y5F were expressed in cells that lack

FIGURE 2. Generation of a PI3K-binding deficient Irs-2 mutant. Irs-2−/− cells were transiently transfected with WT Irs-2 or Irs-2 multiple-tyrosine mutant constructs (Y3F, Y4F, and Y5F) and stimulated with IGF-1 (100 ng/ml) (A–C) or insulin (100 ng/ml) (D–F) for 10 min. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated (IP) with Irs-2-specific antibodies, and the immune complexes were immunoblotted with antibodies that recognize either p-Tyr or p85α. The p-Tyr blots were stripped and reprobed for Irs-2. The data shown in the graphs represent the mean ± S.E. of three independent experiments. NT, mock transfection. *, p < 0.05 relative to WT Irs-2; **, p < 0.05 relative to Irs-2 Y3F.

FIGURE 3. Irs-2-dependent PI3K signaling regulates aerobic glycolysis. A, stable cell lines were generated of PyMT::Irs-2fl/fl cells expressing an empty vector (EV) and PyMT::Irs-2−/− cells expressing an EV, WT Irs-2 (WT), or Irs-2 Y5F (Y5F). Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-2 and tubulin. B and C, cells were serum-deprived and then stimulated with IGF-1 (100 ng/ml) for 10 min. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated (IP) with Irs-2-specific antibodies, and the immune complexes were immunoblotted with antibodies that recognize either p-Tyr or p85a. The p-Tyr blots were stripped and reprobed for Irs-2. The data shown in C represent the mean ± S.E. of five independent experiments. *, p < 0.0001 relative to WT Irs-2; **, p < 0.0001 relative to WT Irs-2. D and E, cells were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20 ng/ml) for 24 h. Glucose uptake (D) and lactate production (E) were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (millimolar/milligram/hour) and represent the mean ± S.E. of five independent experiments. *, p < 0.02 relative to WT Irs-2; **, p < 0.02 relative to Irs-2fl/fl; N.S., not significant.
FIGURE 4. Signaling downstream of a PI3K binding-deficient Irs-2 mutant. A–D, PyMT::Irs-2fl/fl cells expressing an EV and PyMT::Irs-2H11002/WT Irs-2H11002 cells expressing an EV, WT Irs-2, or Irs-2 Y5F were stimulated with IGF-1 (100 ng/ml) for 10 min. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr-1135/1136), Igf-1R, p-Akt (Thr-308, Ser-473), Akt, p-MAPK (Thr-202/Tyr-204), MAPK, and tubulin. The data shown in C and D represent the mean ± S.E. of four independent experiments.

E, stable cell lines of PyMT::Irs-1H11002/H11002/Irs-2H11002/Irs-2H11002/Irs-2H11002 cells expressing an EV, WT Irs-2, or Irs-2 Y5F were generated. Aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, and tubulin. F and G, PyMT::Irs-1H11002/Irs-2H11002 cells expressing an EV, WT Irs-2, or Irs-2 Y5F were stimulated with IGF-1 (100 ng/ml) for 10 min. F, aliquots of cell extracts that contained equivalent amounts of total protein were immunoprecipitated (IP) with Irs-2-specific antibodies, and the immune complexes were immunoblotted with antibodies that recognize either p-Tyr or p85α. The p-Tyr blots were stripped and reprobed for Irs-2. The data shown in the graph represent the mean ± S.E. of three independent experiments. *, p < 0.0002 relative to WT Irs-2. G, aliquots of cell extracts that contained equivalent amounts of total protein were immunoblotted with antibodies that recognize Irs-1, Irs-2, pIgf-IR (Tyr-1135/1136), Igf-1R, pS6k (Thr-389), S6k, p-Akt (Thr-308, Ser-473), Akt, and tubulin. H and I, PyMT::Irs-1H11002/Irs-2H11002 cells expressing an EV, WT Irs-2, or Irs-2 Y5F were grown in 0.1% BSA/DMEM supplemented with IGF-1 (20 ng/ml) for 24 h. Glucose uptake (H) and lactate production (I) were measured and normalized to total protein concentration. The data shown are expressed as a rate measurement (millimolar/milligram/hour) and represent the mean ± S.E. of three independent experiments. *, p < 0.003 relative to an EV.
expression of both Irs-1 and Irs-2 (Fig. 4E). This Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> mammary tumor cell line was generated from PyMT::Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> mammary tumor cells after transient infection with adenoviral Cre recombinase. As we observed with the PyMT::Irs-2<sup>−/−</sup> cells (Fig. 3, B and C), Irs-2 Y5F tyrosine phosphorylation and association with PI3K was significantly, but not completely, reduced in Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> cells (Fig. 4F). Akt was not activated in response to IGF-1 stimulation in the double null cells, which is in keeping with previous reports that the Irs proteins are required for PI3K activation downstream of the IGF-1 and insulin receptors (Fig. 4G) (29, 41). In the absence of background Irs-1 signaling, Akt phosphorylation was diminished in the Irs-2 Y5F-expressing cells relative to cells expressing WT Irs-2 (Fig. 4G). Phosphorylation of p70-S6 kinase, which we showed previously was activated preferentially by Irs-2, was also reduced (Fig. 4G) (14). Phosphorylation of the Igf-1R was similar in all cells examined, indicating that neither Irs expression nor the ability of Irs-2 to activate PI3K is required for upstream receptor activation (Fig. 4, A, B, and G). Importantly, glucose uptake and lactate production were enhanced significantly upon expression of WT Irs-2, but not Irs-2 Y5F, in Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> cells (Fig. 4, H and I).

Akt phosphorylates a large number of effectors, a subset of which has been implicated in the regulation of aerobic glycolysis (40, 42). To identify Irs-2-dependent Akt substrates, Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> cells expressing either empty vector, WT Irs-2, or Irs-2 Y5F were stimulated with IGF-1, and total cell extracts were immunoblotted with a phospho-Akt substrate-specific (RXXpS/T) antibody (Fig. 5A) (43). Although many of the detected bands were common between the three cell lines, some IGF-1-dependent differences were observed. In particular, one protein with a molecular mass of ~45 kDa showed higher levels of phosphorylation in cells expressing WT Irs-2 relative to vector control cells or cells expressing Irs-2 Y5F (Fig. 5A). We surmised that this protein could be Gsk-3β on the basis of the molecular weight and its known involvement in the regulation of glucose metabolism (44). Phosphorylation of Gsk-3β Ser-9, the Akt-dependent phosphorylation site in Gsk-3β, increased significantly in Irs-1<sup>−/−</sup>/Irs-2<sup>−/−</sup> cells expressing WT Irs-2 relative to vector controls, and this increase was not observed in the same cells expressing Irs-2 Y5F (Fig. 5B) (45). Similar results were observed using Irs-2<sup>−/−</sup> cells expressing either an empty vector, WT Irs-2, or Irs-2 Y5F. Although Irs-1 is expressed in these cells, Gsk-3β phosphorylation in response to IGF-1 stimulation is predominantly dependent on Irs-2 expression and its ability to activate PI3K (Fig. 5, C and D), and Irs-1-dependent signaling is unable to compensate for this phosphorylation.

Irs-2 Regulates Aerobic Glycolysis through Inhibition of Gsk-3β—To determine whether Irs-2-specific activation of PI3K signaling leads to inhibition of Gsk-3β activation, thereby relieving its inhibition of glycolysis, glucose uptake and lactate production were measured in the presence or absence of the Gsk-3β inhibitor SB 216763. Inhibition of Gsk-3β rescued glucose uptake and lactate production in the Irs-2<sup>−/−</sup> vector control and Irs-2 Y5F expressing cells to the level observed in cells expressing WT Irs-2 (Fig. 6, A and B). Previous studies have reported that Gsk-3β regulates aerobic glycolysis at the level of glucose uptake either by regulating Glut1 protein expression, promoting Glut1 retention at the cell membrane, or increasing glucose transporter activity (46, 47). To address the mechanism by which Irs-2/Gsk-3β regulates glucose uptake, we first
assessed total Glut1 expression. Glut1 protein expression levels were equivalent in cells that lack Irs-2 expression and express WT Irs-2 or Irs-2 Y5F (Fig. 6, C and D). Additionally, total Glut1 expression was not altered by Gsk-3β inhibition (Fig. 6, C and D). Irs-2−/− cells expressed significantly less Glut1 on the cell surface compared with Irs2fl/fl cells and Irs-2−/− cells with restored WT Irs-2 expression, confirming our previous data that Irs-2 regulates Glut1 surface expression (Fig. 6C) (15). However, Glut1 surface levels in cells expressing Irs-2 Y5F were equivalent to that observed for WT Irs-2, suggesting that direct activation of PI3K by Irs-2 is not required for regulating Glut1 expression on the cell surface (Fig. 6D). Moreover, Gsk-3β inhibition did not significantly increase Glut1 surface levels (Fig. 6E). Therefore, the regulation of Gsk-3β by Irs-2-dependent PI3K signaling likely impacts glucose uptake through a mechanism independent of Glut1 surface expression.

**DISCUSSION**

In this study, we investigated the mechanism by which Irs-2 recruits and activates PI3K and demonstrated the requirement of Irs-2-dependent PI3K signaling for breast carcinoma cell metabolism. Our work identifies specific tyrosine residues...
within Irs-2 that are essential for its association with PI3K and provides a mechanistic basis for the amplification of PI3K signaling downstream of this adaptor protein. Irs-2-dependent PI3K signaling promotes glucose uptake and lactate production, two measures of aerobic glycolysis. The regulation of these functions is specific for Irs-2 because genetically identical mammary tumor cells expressing only Irs-1, which can also activate PI3K, are diminished in their glycolytic capacity. Although global Akt activation was not diminished in the absence of Irs-2-dependent PI3K activation, a reduction in the phosphorylation of specific Akt substrates was observed, indicating that Irs-1 and Irs-2 regulate unique subsets of Akt effectors. Inhibition of one of these Irs-2-specific Akt effectors, Gsk-3β, is required for the regulation of glucose uptake and lactate production. In summary, our data reveal a novel mechanism by which Irs-2 signaling preferentially regulates tumor cell metabolism and adds to our understanding of how this adaptor protein contributes to breast cancer progression.

Aerobic glycolysis is a hallmark of aggressive tumor cells that supports rapid proliferation, survival, and invasion (28, 48). The importance of this altered tumor cell metabolism is underscored by the fact that aerobic glycolysis is regulated by many oncogenic and tumor suppressor pathways that promote tumor progression. We have reported previously that signaling through Irs-2 regulates mammary tumor cell glycolysis through the control of Glut1 surface expression, which was dependent upon PI3K and mTor pathway activation (15). We now provide evidence that the direct activation of PI3K by Irs-2 is required for regulating aerobic glycolysis and demonstrate that specific downstream effectors of this pathway control glucose uptake, the rate-limiting step in glycolysis. Glut1 surface expression is decreased in cells lacking Irs-2 but not in cells expressing an Irs-2 mutant with a significantly reduced ability to stimulate PI3K signaling, indicating that Irs-2-dependent PI3K activation is not required for Glut1 surface localization. However, it is possible that the low level of Irs-2/PI3K/Akt signaling retained by the Irs-2 Y5F mutant (~15%) is sufficient to regulate Glut1 surface localization. Generation of an Irs-2 mutant that is completely deficient in PI3K recruitment is required to address this potential mechanism of regulation. Of note, inhibition of Gsk-3β activity rescues glucose uptake but does not increase Glut1 expression on the cell surface. Taken together with our previous study, which implicated mTor1c1 in the regulation of Glut1 surface expression, our data support that Irs-2 coordinates the activation of signaling pathways to promote Glut1 expression on the cell surface and stimulate optimal uptake of glucose and aerobic glycolysis (15). Given that Irs-2 is a hypoxia-regulated gene and that its expression is required to sustain Akt activation in hypoxic environments, Irs-2-dependent PI3K signaling likely plays a key role in controlling glucose uptake and glycolytic metabolism in both normoxic and hypoxic tumor microenvironments (49).

We identified four individual tyrosine residues within Irs-2 that are required for the recruitment and activation of PI3K in response to both IGF-1 and insulin stimulation. These tyrosines have been reported previously to be phosphorylated in response to insulin stimulation, but their involvement in recruiting PI3K has not been assessed (50). Likewise, each of these tyrosines has been shown to have the potential to bind to PI3K in a phosphopeptide pulldown proteomics screen (39). However, this screen also implicated Tyr-628 and Tyr-758, which do not contribute to PI3K association with Irs-2 upon stimulation of mammary tumor cells with either IGF-1 or insulin. A previous study reported that Tyr-628 inhibits Irs-2 tyrosine phosphorylation in response to insulin but not IGF-1 stimulation (51). We did not observe any difference in the phosphorylation of Irs-2 in response to either insulin or IGF-1 stimulation when this site was mutated, suggesting a cell context-dependent role for this regulation. Combined mutation of all of the tyrosines located within canonical PI3K binding motifs did not completely inhibit PI3K association with Irs-2 or downstream activation of Akt signaling. Although the tyrosines we identified play a dominant role in recruiting PI3K (>90%), additional tyrosine residues within Irs-2 may recruit PI3K through a non-canonical interaction. Additionally, PI3K recruitment may occur through an indirect interaction with another binding protein. A rigorous study of Irs-2 interacting proteins will be necessary to identify these alternative mechanisms of PI3K recruitment.

The regulatory subunit of PI3K, p85, contains two SH2 domains that must be engaged by phosphorylated tyrosines to induce a conformational change, leading to inhibition and activation of the p110 catalytic subunit (31). Although binding to one SH2 domain can partially activate PI3K, binding of both SH2 domains promotes maximal kinase activity. We implicated four tyrosines in the association of Irs-2 with PI3K and, on the basis of the primary sequence alone, Tyr-649/Tyr-671 and Tyr-734/Tyr-814 could be predicted to form docking sites for two tandem p85 SH2 domains. However, Irs-2 is an intrinsically disordered protein with minimal structural information available to guide predictions of physical proximity (52). Therefore, additional potential pairings are possible both in cis and trans, given that the IGF-1 and insulin receptors are dimeric and that two Irs proteins can be recruited simultaneously (53). When comparing Irs-1 with Irs-2, only two of the four tyrosines implicated in Irs-2-dependent PI3K activation have corresponding residues within Irs-1. Tyr-612 and Tyr-628 (human numbering) in Irs-1, which correspond to Tyr-649 and Tyr-671 in Irs-2, mediate full PI3K recruitment, suggesting that only a single PI3K heterodimer is recruited to Irs-1 (54). Tyr-734 and Tyr-814 do not share homologous sites within Irs-1, and individual mutation of Tyr-814 had the most significant impact on the association of Irs-2 with PI3K. Therefore, Tyr-734 and Tyr-814 may be determining factors in the enhanced activation of PI3K by Irs-2.

Our study reveals a novel mechanism for how Irs-1 and Irs-2 differentially regulate tumor cell metabolism and contribute to breast cancer progression. Both Irs-1 and Irs-2 can recruit and activate PI3K to promote Akt signaling when stimulated with IGF-1, but the functional outcomes that result from this activation are different. Irs-1 and Irs-2 have distinct subcellular localization patterns in human tumors, with Irs-1 localized to both the cytoplasm and nucleus and Irs-2 to the cytoplasm and at the cell membrane (16, 17). Irs-2 membrane localization is a predictor of reduced breast cancer patient overall survival, whereas Irs-1 nuclear localization is associated with tumors that are
more differentiated and non-metastatic and more sensitive to tamoxifen response (16, 18). These studies reveal that differences in the localization of IRS-1 and IRS-2 impact outcomes in human cancer and point toward localization as being a key determinant of the function of these adaptor proteins. Localizing to distinct intracellular compartments would not only determine access to unique subsets of downstream effectors but also to the substrates of these effectors to impact cell function. With regard to IRS-2, we hypothesize that activation of Akt at the plasma membrane would target inhibition of Gsk-3β, where it could regulate glucose uptake. We identify Gsk-3β as one Akt target that is regulated preferentially by IRS-2-dependent PI3K signaling. Additional Akt effectors that are regulated selectively by this signaling pathway may also play a role in metabolism and other functions regulated by IRS-2. In this regard, Foxo1 is preferentially regulated by IRS-2 because PI3K is not sufficiently activated by IRS-1 to support this regulation (55). The PI3K kinase signal is likely enhanced when activated through IRS-2 because it has a greater potential for PI3K recruitment.

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