The Reciprocal Stability of FOXO1 and IRS2 Creates a Regulatory Circuit that Controls Insulin Signaling

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The transcription factor FoxO1 links the phosphatidylinositol 3-kinase (PI 3-kinase) → Akt cascade to gene expression that regulates cell growth, survival, and metabolism. The receptors for insulin and IGFs factors are linked to this pathway through tyrosine phosphorylation of insulin receptor substrates—Irs1, 2, 3, and 4. However, it is unclear why Irs2 signaling predominates in certain tissues, including pancreatic β cells, dermal fibroblasts, photoreceptors, central neurons, and metastatic mammary tumor cells. We used wild-type mouse embryo fibroblasts (MEFs) and Irs1−/− or Irs2−/− MEFs—to establish the relation between Irs1, Irs2, and FoxO during insulin signaling. PI 3-kinase associated with Irs1 and Irs2 during insulin stimulation of wt MEFs, which strongly promoted Akt and FoxO phosphorylation, led to FoxO nuclear exclusion and degradation. However, insulin failed to activate the Akt→FoxO cascade in Irs2−/− MEFs because Irs1 expression was reduced in these cells, and p110α-PI 3-kinase was inefficiently activated during recruitment by Irs1. By contrast, insulin stimulation of Irs1−/− MEFs caused FoxO degradation, not only because Irs2 expression increased but also because Irs2 efficiently activated p110α→Akt cascade. Importantly, prolonged insulin stimulation restored FoxO1 expression in wild-type or Irs1−/− MEFs because Irs2 was degraded and Irs1 alone failed to activate sufficient p110α to promote the Akt→FoxO cascade. Inhibition of Irs2 degradation with rapamycin caused persistent FoxO degradation even during prolonged insulin stimulation. The dynamic relation between Irs2 and FoxO expression, compared with the subordinate role of Irs1, can explain the dominant role of Irs2 in metabolic regulation. (Molecular Endocrinology 20: 3389–3399, 2006)

INSULIN AND ITS homologs control cell growth, survival, and metabolism by activating signaling cascades that regulate enzyme activity and gene expression (1–3). FoxO1 is a forkhead winged/helix transcription factor that mediates many effects of insulin upon gene expression downstream from phosphatidylinositol 3-kinase (PI 3-kinase)→Akt cascade (4–9). Genetic studies from Caenorhabditis elegans and Drosophila show that inhibition of insulin signaling promotes the nuclear function of the FoxO1 orthologs, which stimulates nutrient storage, diminishes fertility, and increases animal longevity (10–12). In mammals, nuclear FoxO1 accumulates during insulin resistance, which dysregulates nutrient homeostasis by promoting hepatic gluconeogenesis (7, 13, 14); inhibiting adipocyte differentiation (15); and impairing compensatory β-cell function (16). By contrast, the suppression of FoxO1 expression can reverse the effects of insulin resistance and restore glucose tolerance in diabetic mice, including Irs2−/− mice (17). Thus, suppression of FoxO1 by Irs2 signaling plays an important physiological role in nutrient homeostasis.

The insulin and IGF-I receptors phosphorylate many cellular proteins, but the insulin receptor substrate (Irs) proteins play a central role in all cells. Irs1 and Irs2 are the principal IRS proteins that link the receptor tyrosine kinases for insulin or IGF-I to the PI 3-kinase→Akt and Ras→RasErk cascades (18). Despite the expectation that Irs1 and Irs2 serve similar regulatory functions, experimental results in genetically altered mice reveal important differences (19–21). Irs1−/− mice are small with mild glucose intolerance that never progresses to diabetes owing to compensatory β-cell growth (22). By contrast, dysregulated Irs2 signaling has profound effects upon metabolic and cell-based disease (23). Metabolic dysregulation is severe in Irs2−/− mice owing to excessive gluconeogenesis, decreased hepatic glycogen synthesis, and unpressed plasma free fatty acid/glycerol levels during the hyperinsulinemia (24). Irs2−/− mice progress steadily toward diabetes owing to insufficient compensatory insulin secretion (19).

Regardless of these distinctions, early work shows that insulin and IGF-I promote the association of PI 3-kinase with Irs1 and Irs2 in many cells and cell-based assays. Moreover, assays with purified components show that tyrosine phosphorylated Irs1—or phosphopeptides derived from Irs1—can activate the

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Abbreviations: Ct, Cycle threshold; Irs, insulin receptor substrate; MEFs, mouse embryo fibroblasts; moi, multiplicity of infection; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; TSC, tuberous sclerosis complex.

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PL 3-kinase; a similar function is anticipated for Irs2 (25). This shared function is revealed in liver, where Irs1 and Irs2 contribute significantly to the activation of the PI 3-kinase—Akt—FoxO1 cascade (26). However, in other tissue backgrounds Irs2 plays a dominant role—including pancreatic β-cell growth and survival (19, 27); brain growth during development (28); photoreceptor survival immediately after birth (29); mammary tumor metastasis (30); and skin differentiation and growth (31). The mechanisms that promote differential signaling through Irs1 and Irs2 are poorly understood.

We used mouse embryo fibroblasts (MEFs) generated from Irs1−/− or Irs2−/− mice to investigate the differential regulation of the PI 3-kinase—Akt—FoxO1 cascade by Irs1 and Irs2. These cells are useful for this work because both Irs proteins are expressed naturally, so confusion caused by the overexpression of recombinant proteins can be avoided. Using these cells, we find that Irs2 is a stronger activator of p110α than Irs1, which leads to Irs2-mediated inactivation of FoxO1 that cannot be achieved by endogenous Irs1. Because prolonged insulin stimulation promotes Irs2 degradation in MEFs (32), our results show how Irs2 signaling can have a dominant and dynamic effect upon metabolism and cell growth.

RESULTS

Differential Tyrosine Phosphorylation of Irs1 and Irs2 in MEFs

We used MEFs generated from wild-type (wt) mice or from Irs1−/− or Irs2−/− mice to establish the contribution of each Irs-protein to insulin signaling. Quantitative RT-PCR was used to quantify the copies of Irs1 and Irs2 mRNA in each cell line. In wt MEFs, Irs1 mRNA was 6-fold higher than Irs2 mRNA (Fig. 1A). By comparison, Irs2 mRNA increased 3-fold in Irs1−/− MEFs, whereas Irs1 mRNA decreased 3-fold in Irs2−/− MEFs (Fig. 1A).

Specific immunoblotting was used to estimate the relative change in Irs1 and Irs2 protein levels in each cell line after 18 h incubation in serum-free DMEM: the relative level of Irs1 protein decreased 30% in Irs2−/− MEFs, but the relative amount of Irs2 protein increased 50% in Irs1−/− MEFs (Fig. 1A).

To assess tyrosine phosphorylation of the Irs proteins, serum-starved MEFs were stimulated for 20 min with 100 nM insulin. Insulin stimulated Irs1 tyrosine phosphorylation in wt and Irs2−/− MEFs, whereas insulin stimulated tyrosine phosphorylation of Irs2 in wt and Irs1−/− MEFs (Fig. 1, B and C). During insulin stimulation of wt MEFs, specific tyrosine phosphorylation of Irs1 (pIrs1/Irs1) was 2-fold greater than that of Irs2 (Fig. 1, B and C). By comparison, the specific tyrosine phosphorylation of Irs1 was reduced 50% in Irs2−/− MEFs, whereas the specific tyrosine phosphorylation of Irs2 was increased 3-fold in Irs1−/− MEFs (Fig. 1, B and C). Thus, based upon tyrosine phosphorylation and total Irs1 or Irs2 were analyzed by Western blot in three different experiments. The signal intensity from the proteins scanned from an Epson Perfection 4990 photo scanner was quantified using ImageQuant TL2003 (Amersham Biosciences). For RNA assay, equal amounts of RNA from wt, Irs1−/−, and Irs2−/− MEFs incubated with starvation medium for 18 h were immunoprecipitated, and total Irs1 and Irs2 were analyzed by Western blot in three different experiments. The signal intensity from the proteins scanned from an Epson Perfection 4990 photo scanner was quantified using ImageQuant TL2003 (Amersham Biosciences). For RNA assay, equal amounts of RNA from wt, Irs1−/−, and Irs2−/− MEFs that were starved for 18 h were subjected to Q-PCR and relative abundance of RNA were determined by ∆∆Ct in three different experiments. Amplification efficiency of Irs1, Irs2, and cyclophilin were 0.8 ± 0.01, 0.8 ± 0.02, and 1 ± 0.01, respectively. B. Equal amounts of cell lysate protein from wt, Irs1−/−, or Irs2−/− MEFs incubated with or without 100 nM insulin for 20 min were immunoprecipitated with Irs1 (B) or Irs2 (C) antibody, and the tyrosine phosphorylation and total Irs1 or Irs2 were analyzed by Western blot and quantitatively calculated by ImageQuant TL2003 in three different experiments.

Fig. 1. The Relative Abundance of Irs1 and Irs2 and Their Differential Signaling by Insulin in MEFs

A, For protein assay, equal amounts of cell lysate protein (500 µg) from wt, Irs1−/−, or Irs2−/− MEFs incubated with starvation medium for 18 h were immunoprecipitated, and total Irs1 and Irs2 were analyzed by Western blot in three different experiments. The signal intensity from the proteins scanned from an Epson Perfection 4990 photo scanner was quantified using ImageQuant TL2003 (Amersham Biosciences). For RNA assay, equal amounts of RNA from wt, Irs1−/−, and Irs2−/− MEFs that were starved for 18 h were subjected to Q-PCR and relative abundance of RNA were determined by ∆∆Ct in three different experiments. Amplification efficiency of Irs1, Irs2, and cyclophilin were 0.8 ± 0.01, 0.8 ± 0.02, and 1 ± 0.01, respectively. B. Equal amounts of cell lysate protein from wt, Irs1−/−, or Irs2−/− MEFs incubated with or without 100 nM insulin for 20 min were immunoprecipitated with Irs1 (B) or Irs2 (C) antibody, and the tyrosine phosphorylation and total Irs1 or Irs2 were analyzed by Western blot and quantitatively calculated by ImageQuant TL2003 in three different experiments.
phosphorylation, IRS2 was most active in IRS1−/− MEFs, whereas IRS1 was most active in wt MEFs.

**Regulation of the PI 3-Kinase Cascade by IRS1 and IRS2**

Next, we investigated PI 3-kinase activity in specific IRS1 or IRS2 immunoprecipitates from wt MEFs, IRS1−/− or IRS2−/− MEFs. Each cell line was incubated for 18 h in serum-free DMEM before insulin stimulation (100 nM) for 5 or 15 min. PI 3-kinase activity was determined by incubating IRS1 or IRS2 immunoprecipitates with [γ-32P]ATP (Fig. 2A). Insulin increased 5-fold the production of [32P]PI-3P by IRS1 or IRS2 immunoprecipitates from wt MEFs (Fig. 2A). Although the specific tyrosine phosphorylation of IRS2 (pIRS2/IRS2) was less than that of IRS1, the associated PI 3-kinase activity was approximately equal (Fig. 2A and B). By comparison, the

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**Fig. 2. IRS2 Is a Major Regulator for p110α-PI 3-Kinase Cascade in Insulin Action**

A, IRS1, IRS2, or phosphotyrosine (pY) was immunopurified from 500 μg cell lysate proteins of MEFs stimulated with or without 100 nM insulin for 5 and 15 min, its associated-PI 3-kinase was measured by the generation of PI-3P in three different experiments, and the representative data are shown. B, The ratio of pIRS1/IRS1 or pIRS2/IRS2 was plotted with the IRS1- or IRS2-associated PI 3-kinase activity measured from three different experiments. The arrow indicates the direction of changes from wt to mutant MEFs. C, PI 3-kinase catalytic subunit p110α was immunopurified from equal amounts of cell lysate protein of MEFs and its kinase activity was measured by generation of PI-3P in three different experiments. The kinase activities of the PI 3-kinase were quantitatively analyzed using the Typhoon 9421 scanner and ImageQuant. The fold changes in kinase activity compared with that of the control and the representative data are shown. D, Akt1 was immunoprecipitated (IP) from 500 μg cell lysate proteins of wt, IRS1−/−, and IRS2−/− MEFs treated with or without 100 nM insulin for a period as indicated. The phosphorylation of Akt1 at Thr308 and Ser473, as well as the total Akt1 was analyzed by Western blot. E, Same as that of panel C except that Akt2 was immunoprecipitated and the phosphorylation at Thr309 and total Akt2 were analyzed. F, IRS1−/− or IRS2−/− MEFs were treated with 50 ng/ml PDGF-BB for 30 min and cell extracts were immunoblotted with αFoxO1, αpAktT308, and αp85.

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and Irs1 3-kinase activity associated with tyrosine phosphorylation, not increased in p110
rapamycin (mTOR) complex (33, 34). After 5 or 15 min of insulin stimulation of Irs2
in Akt2). Thr308/Thr309 is phosphorylated by the PI-3kinase-dependent kinase (PDK1), whereas Ser 473/Ser474 is phosphorylated by the rictor-mammalian target of
because PDGF reduced FoxO1 protein levels in both Irs1−/− and Irs2−/− MEFs (Fig. 3A). Consistent with these results, FoxO1 protein level was reduced in wt or Irs1−/− MEFs during insulin stimulation; however, insulin failed to reduce FoxO1 protein levels in Irs2−/− MEFs (Fig. 3A). The mechanism for FoxO1 degradation was functional in both cell lines because PDGF reduced FoxO1 protein levels in both Irs1−/− and Irs2−/− MEFs (Fig. 2F). Therefore, the effect of insulin to reduce FoxO1 protein level depended upon Irs2 signaling.

Previous work shows that phosphorylated FoxO1 is degraded in proteasomes (35). Consistent with this mechanism, 26S proteasome inhibitors—lactacystin or MG132—inhibited the loss of FoxO1 from insulin-stimulated wt MEFs (Fig. 3B). These results confirmed that the loss of FoxO1 during insulin stimulation involved proteasome-mediated degradation. Insulin also promoted the degradation of FoxO4 in wt and Irs1−/− MEFs, but not in Irs2−/− MEFs (Fig. 3C). Insulin-stimulated FoxO1 and FoxO4 degradation in wt and Irs1−/− MEFs was consistent with the stronger PI 3-kinase→Akt cascade—monitored by S473Akt1 phosphorylation—in cells expressing Irs2 (Fig. 3C).

The phosphorylation of FoxO1 also promotes its exclusion from the nucleus (9). To determine whether FoxO1 nuclear exclusion was regulated differently in the Irs1−/− or Irs2−/− MEFs, nuclear and cytoplasmic proteins were extracted from Irs1−/− or Irs2−/− MEFs under serum-free condition, or after incubation with 100 nM insulin for 30 min. Under basal conditions, 40% of the FoxO1 protein was detected in nuclear histone H1-containing fractions, whereas 60% of the FoxO1 was detected in cytoplasmic fractions (Fig. 3D). Insulin treatment of Irs1−/− MEFs decreased total FoxO1 by more than 50%, and almost completely depleted nuclear FoxO1 (Fig. 3D); a similar effect of insulin was observed in wt MEFs (data not shown). By contrast, insulin treatment of Irs2−/− MEFs barely changed the levels of FoxO1, and the nuclear FoxO1 was unaltered (Fig. 3D). Taken together, these data show that Irs2 signaling was required to decrease nuclear FoxO1 and promote its degradation during insulin stimulation of MEFs.

Finally, we investigated the role of PI 3-kinase and mTOR in the regulation of FoxO1 degradation. Insulin (100 nM, 30 min) stimulated S473Akt1 and p70SK phosphorylation, and promoted T24FoxO1 phosphorylation and degradation (Fig. 3E). Treatment of wt MEFs with LY294002 (20 μM) for 30 min inhibited insulin-stimulated phosphorylation of Akt, p70SK and FoxO1T24—and prevented FoxO1 degradation (Fig. 3E). By contrast, rapamycin only inhibited insulin-stimulated phosphorylation of p70SK phosphorylation, whereas Akt and FoxO1 phosphorylation—and FoxO1 degradation—occurred normally (Fig. 3E). Taken together, these

Irs2 Promotes Phosphorylation and Degradation of FoxO Transcription Factors

Growth factor treatment or constitutively active Akt promotes phosphorylation and degradation of FoxO1 in fibroblasts (35). As expected, insulin treatment for 30 min stimulated FoxO1 phosphorylation in wt MEFs, as monitored by immunoblotting with antibodies against pS253FoxO1 (Fig. 3A). Insulin also strongly stimulated pS253FoxO1 phosphorylation in Irs1−/− MEFs, but insulin had no detectable effect in Irs2−/− MEFs (Fig. 3A). With these results, FoxO1 protein level was reduced in wt or Irs1−/− MEFs during insulin stimulation; however, insulin failed to reduce FoxO1 protein levels in Irs2−/− MEFs (Fig. 3A). The mechanism for FoxO1 degradation was functional in both cell lines because PDGF reduced FoxO1 protein levels in both Irs1−/− and Irs2−/− MEFs (Fig. 2F). Therefore, the effect of insulin to reduce FoxO1 protein level depended upon Irs2 signaling.

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Recombinant Irs1 or Irs2 Rescues FoxO1 Degradation in Irs2−/− MEFs

Recombinant Irs1 or Irs2 was expressed in Irs2−/− MEFs to determine whether insulin-stimulated FoxO1 degradation could be restored. Irs2−/− MEFs were infected with a low [20 multiplicity of infection (moi)] or high dose (50 moi) of adenovirus encoding the Irs1 or Irs2 cDNA under the control of the cytomegalovirus promoter (36). This infection produced a graded expression of recombinant Irs1 or Irs2 protein in the Irs2−/− MEFs (data not shown). Consistent with previous results, insulin stimulation (30 min) of uninfected Irs2−/− MEFs failed to promote T308Akt1 phosphorylation or FoxO1 degradation (Fig. 4A). By contrast, a low and high expression of recombinant Irs2 promoted a graded phosphorylation of T308Akt1 and degradation of FoxO1 after 30 min of insulin stimulation (Fig. 4A). Interestingly, recombinant Irs1 had a similar graded effect upon T308Akt1 phosphorylation and FoxO1 degradation (Fig. 4B). Thus, overexpression of recombinant Irs1 or Irs2 promoted sufficient PI 3-kinase Akt signaling in Irs2−/− MEFs to mediate FoxO1 degradation during insulin stimulation.

Raptor-mTOR Inhibits FoxO1 Degradation during Chronic Insulin Stimulation

The acute degradation of FoxO1 measured after 30 min of insulin stimulation was identical in wt or Irs1−/− MEFs (IC50 8–10 nM insulin), but barely degraded in Irs2−/− MEFs—even at the highest insulin concentrations tested (Fig. 5A). During 6 h of continuous insulin stimulation, FoxO1 protein expression returned to basal levels in wt or Irs1−/− MEFs, suggesting that the insulin signal was attenuated by Irs2 degradation (Fig. 5B). Consistent with this hypothesis, withdrawal of

Fig. 3. Irs2 Promotes the Phosphorylation and Degradation of FoxO Transcription Factors

A, FoxO1 was immunoprecipitated (IP) from 500 μg cell lysate proteins of wt, Irs1−/−, and Irs2−/− MEFs treated with or without 100 nM insulin (Ins) for 30 min. The total FoxO1 and the phosphorylation at Ser253 were analyzed by Western blot. B, Lactacystin MG132 and p85. C, Equal amounts of cell lysate protein from wt, Irs1−/−, and Irs2−/− MEFs treated with or without 100 nM insulin for 30 min were analyzed by immunoblotting using antibodies for FoxO1, FoxO4, pAKTS473, and p85. D, Irs1−/− or Irs2−/− MEFs were starved for 24 h and then treated with or without 100 nM insulin for 30 min. The nuclear (N) and cytoplasmic proteins (C) were extracted from the cells and immunoblotted with αFoxO1, αHistone H1, and αtubulin. E, Before 100 nM insulin stimulation for 30 min, wt MEFs were pretreated for 30 min with 20 μM LY 294002 (LY), or 100 nM rapamycin (Rap). One hundred-microgram cell lysate proteins were immunoblotted with αFoxO1, αpAktS473, αpFoxO1T24, αpS6K738, and αAkt.
insulin for 2.5 h—which restores Irs2 expression (32)—also restored the ability of insulin to stimulate degradation of FoxO1 (Fig. 5B). Another way to confirm that persistent Irs2 signaling promotes FoxO1 degradation was by enforced expression of recombinant Irs2 in wt MEFs. The expression of recombinant Irs2 in wt MEFs prevented the regeneration of FoxO1 during 6 h of insulin stimulation (Fig. 5C).

To confirm whether the expression of Irs2 protein inversely modulates the degradation of FoxO1 during continuous insulin stimulation, we treated the MEFs cell lines with rapamycin to inhibit Irs2 degradation (32). The wt MEFs were treated with or without 100 nM rapamycin for 30 min, and then with insulin (100 nM) for 0.5, 4, and 6 h. FoxO1 was acutely degraded more than 90% after 30 min of insulin stimulation whether or not rapamycin was present (Fig. 5D). During 6 h of continuous insulin stimulation in the absence of rapamycin, Irs2 protein expression and Akt phosphorylation were progressively lost, and FoxO1 protein returned to its basal level (Fig. 5D). By comparison, rapamycin prevented the degradation of Irs2 and sustained Akt phosphorylation during 6 h of insulin stimulation (Fig. 5D). Moreover, rapamycin also prevented the reexpression of FoxO1 during continuous insulin stimulation.

To confirm that rapamycin did not directly promote FoxO1 degradation, Irs1/H11002 or Irs2/H11002 MEFs were incubated with 100 nM rapamycin for 6 h before 2 h insulin stimulation—which restores Irs2 expression (32)—also restored the ability of insulin to stimulate degradation of FoxO1 (Fig. 5B). Another way to confirm that persistent Irs2 signaling promotes FoxO1 degradation was by enforced expression of recombinant Irs2 in wt MEFs. The expression of recombinant Irs2 in wt MEFs prevented the regeneration of FoxO1 during 6 h of insulin stimulation—unless wortmannin was added to inhibit the PI 3-kinase (Fig. 5C).

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insulin stimulation. Because rapamycin inhibits insulin-stimulated degradation of Irs2, it augmented Akt phosphorylation in Irs1−/− MEFs during 6 h of continuous insulin stimulation. By comparison, rapamycin had no effect upon Akt phosphorylation in Irs2−/− MEFs (Fig. 5E). Consistent with these results, 6 h of continuous insulin stimulation promoted FoxO1 degradation in rapamycin-treated Irs1−/− MEFs, but not in rapamycin-treated Irs2−/− MEFs (Fig. 5E). Thus, rapamycin did not promote FoxO1 degradation in the absence of Irs2.

To confirm that rapamycin did not interfere with de novo synthesis of FoxO1 needed for reexpression during prolonged growth factor stimulation, we tested the effect of rapamycin during PDGF stimulation. PDGF→Akt→FoxO1 signaling is independent of Irs2; however, the cascade is desensitized by degradation of the internalized PDGF receptor (37). Consistent with this mechanism, PDGF stimulation for 30 min strongly increased Akt phosphorylation and FoxO1 was largely degraded (Fig. 5F). However, after 6 h of continuous PDGF stimulation, Akt phosphorylation was largely lost and FoxO1 protein expression approached the basal level (Fig. 5F). Because rapamycin did not prevent the desensitization of the PDGF→Akt signal after 6 h of PDGF stimulation, FoxO1 protein expression also returned to basal level in the presence of rapamycin (Fig. 5F). These results show that rapamycin did not prevent the de novo synthesis of FoxO1 protein in wt MEFs.

**DISCUSSION**

Our experiments provide genetic and biochemical evidence that Irs2 is the major activator of the PI 3-kinase→Akt cascade in MEFs during insulin stimulation. Although both Irs1 and Irs2 bind PI 3-kinase, only Irs2 efficiently activates p110α in our experiments. Consequently, Irs2 has a greater potential to recruit PI 3-kinase into the insulin signaling cascade, activate Akt, and inactivate FoxO transcription factors. Irs1 can also activate Akt→FoxO signaling, but a higher level of Irs1 appears to be required to activate the PI 3-kinase→Akt cascade because endogenous Irs1 in MEFs does not efficiently activate p110α during its association with the p85 regulatory subunit. In ordinary MEFs, Irs1 is expressed and phosphorylated at a higher level than Irs2, but both proteins recruit about the same level of PI 3-kinase activity into the insulin signaling cascade—because PI 3-kinase is activated during association with Irs2. However, in Irs2−/− MEFs, Irs1 fails to bring sufficient PI 3-kinase into the insulin signaling cascade—in part because Irs1 expression is suppressed—but also because p110α is not activated. Thus, Akt is poorly activated and FoxO1 is not inactivated in Irs2−/− MEFs. This desensitization is also apparent in wt MEFs when Irs2 is degraded during prolonged insulin stimulation, and the insulin signal is mediated largely through Irs1. The deletion of Irs1 does not dysregulate Akt→FoxO signaling, and might actually improve it, because Irs2 expression increases and PI 3-kinase is more strongly activated by Irs2—especially when it no longer competes with Irs1.

Irs1 and Irs2 can have redundant signaling functions in many cells and tissues, but have distinct functions in others (19, 29, 31, 36, 38). In this study, MEFs from the Irs1−/− or Irs2−/− mice to show that Irs2 is the principal regulator of the PI 3-kinase→Akt cascade during insulin stimulation—which is essential for the phosphorylation and degradation of FoxO1. The dominant role of Irs2 emerges in MEFs because insulin weakly stimulates the degradation of FoxO1 in the absence of Irs2, whereas insulin stimulated phosphorylation and degradation of FoxO1 occurs efficiently without Irs1. This relation between Irs1 and Irs2 in MEFs is similar to that observed in pancreatic β-cells and the INS-1 β-cell line, retinal photoreceptors, and dermal fibroblasts (27, 29, 31, 36). By comparison, the consequences of Irs2 inactivation might be less obvious in tissues where Irs1 signaling plays a greater role—such as hepatocytes (26, 38). The deletion of Irs1 or Irs2 in hepatocytes partially reduces Akt phosphorylation and FoxO1 inactivation during insulin stimulation, but the deletion of both proteins is necessary to completely desensitize insulin signaling. The exact nature of the signaling cascade will depend upon the relative expression of Irs1 and Irs2, and tissue-specific regulation owing to the exact serine and threonine phosphorylation status of Irs1 and Irs2.

Type 2 diabetes emerges when nutrient homeostasis is dysregulated by peripheral insulin resistance and pancreatic β-cell failure (1, 39). Cell-based and mouse-based strategies have provided considerable insight into the mechanisms that cause insulin resistance. Although our current view of insulin signaling emerged from the discovery of Irs1, mouse experiments point to Irs2 as a principal regulator of nutrient homeostasis (40). Work with genetically altered mice reveals that Irs2 signaling is a common molecular link between central and peripheral nutrient homeostasis, and β-cell growth and survival (1). Consequently, dysregulation of Irs2 signaling—especially in β-cells and parts of the brain—causes obesity that progresses to diabetes unless normal β-cell function can be restored (19). Although multiple genetic loci and environmental stress contribute to metabolic disease and its progression to diabetes, the Irs2 branch of the insulin/IGF-I signaling cascade appears to play a central role. The dominant role of Irs2→FoxO1 cascade revealed in our experiments can explain at least in part why Irs2 signaling plays a critical role in nutrient homeostasis.

Although FoxO1 expression in MEFs is almost completely depleted 30 min after insulin stimulation, it returns to the basal level when Akt activity is attenuated—whether or not insulin is removed from the medium. Our results show that the principal mechanism inhibiting FoxO1 degradation in MEFs during chronic insulin stimulation involves the loss of Irs2→PI3K.
3-kinase signaling. This mechanism could be dysregulated at many steps. Hyperactivation or overexpression of Irs1 relative to Irs2 could diminish the impact of regulation of the Irs2 branch of the signal and cause persistent inactivation of FoxO1 that leads to extreme insulin sensitivity. Overexpression of Irs2 can also bypass the regulatory mechanisms. Dysregulation of the molecular mechanisms that recruit ubiquitin ligases to FoxO1 or Irs2 could also disrupt the regulatory circuit.

The importance of differential regulation of Irs1 and Irs2 is illustrated in MEFs harboring mutations in the tuberous sclerosis complex, Tsc1/Tsc2 (41). Tsc1/Tsc2 complex is a Rheb-GTPase that inactivates Rheb and inhibits mTOR (42). Mutations in Tsc1/Tsc2 activate mTOR because Rheb is never inactivated, but severe insulin resistance develops because both Irs1 and Irs2 are degraded (41). This mechanism can explain in part why hamartomas accumulate protein and grow large—but rarely metastasize (43). Under ordinary circumstances, exercise or caloric restriction is expected to reduce mTOR activity, which can stabilize Irs-protein levels and promote insulin sensitivity that facilitates FoxO1 phosphorylation and degradation (43).

The role of the rapamycin-sensitive mTOR complex in insulin action is complicated because it mediates the effect of insulin upon protein synthesis, whereas promoting Ser/Thr-phosphorylation and degradation of the Irs-proteins in some but not all cells (44). The mTOR cascade can directly stimulate Irs protein phosphorylation through down stream effectors like the S6K (45, 46). However, mTOR signaling also inhibits the activity of phosphoprotein phosphatase 2A that can have nonspecific effects upon Irs protein phosphorylation that is mediated by other kinases (47, 48). In this case, the effects of mTOR upon Irs1 or Irs2 signaling might depend upon the pattern of Ser/Thr-phosphorylation that develops through the activity of other cell-specific kinases. Experiments with INS-1 cells suggest that Ser/Thr-phosphorylation of Irs2 is mediated by kinases that are regulated by nutrients (glucose, amino acids, or fatty acids), proinflammatory cytokines (TNFα, IL6, IL1β), or chronic insulin stimulation (36). The activation of mTOR augments the phosphorylation of Irs2 and promotes its degradation, which impairs β-cell-specific function (36). Thus, β-cells appear to display similar regulatory mechanism revealed by our experiment with MEFs. Understanding the role of Ser/Thr-phosphorylation in these cells and others might provide a rational basis to treat or cure diabetes.

In summary, insulin-stimulated Irs2 signaling plays a central role to reduce nuclear FoxO1, which is mediated by the PI 3-kinase—Akt cascade. Because Irs2 is a better activator than Irs1 for P110α, Irs2 has a dominant role in the recruitment of PI 3-kinase into the insulin signaling cascade (Fig. 6). By contrast, PI 3-kinase—mTOR signaling promotes Irs2 degradation that protects FoxO from insulin-stimulated degradation, at least in MEFs (33) (Fig. 6). Because Irs2 gene expression can be increased by nuclear FoxO1 (49), this mechanism can explain how the expression of the Irs2 increases rapidly after FoxO1 returns to the nucleus; however, attenuation of the mTOR cascade would be necessary before Irs2 protein accumulates. Thus, mTOR modulates the Irs2—PI 3-kinase—Akt—FoxO1—Irs2 regulatory circuit by controlling Irs2 protein levels (Fig. 6). Because the activity of FoxO1 on the Irs2 promoter can be inhibited by Srebp1c, nutrient excess can have a dual effect to suppress Irs2 transcription and protein stability (49). Therefore, dysregulation of the Irs2 branch of the insulin/IGF signaling cascade can explain the close association between nutrient excess and metabolic disease that progresses to diabetes.

**MATERIALS AND METHODS**

Reagents

Human insulin was purchased from Sigma (St. Louis, MO). The enhanced chemiluminescence detection system was
from Amershams Pharmacia Biotech (Piscataway, NJ). Lipo- 
fectamine 2000 was from Invitrogen (Carlsbad, CA). MG132, 
lactacystin, rapamycin, LY294002, wortmannin, and Nonidet 
P-40 were from Calbiochem (La Jolla, CA). Polyclonal anti- 
bodies against FoxO1, FoxO4 (AFX) and Akt2, and phos- 
phospecific antibodies against FoxO1S256 and FoxO1T24 and, 
AktS473 and AktT308 were obtained from Cell Signaling Tech- 
nology, Inc. (Beverly, MA). FoxO1 antibody used for immu-
noprecipitation, polyclonal antibodies against histone-H1, 
Akt1 and monoclonal antibodies against tubulin were from 
Santa Cruz Inc. (Santa Cruz, CA); anti-p110α-Pl 3-kinase 
subunit was from Upstate (Lake Placid, NY); polyclonal anti-
bodies were prepared in our laboratory against full-length rat 
leupeptin. After centrifugation at 14,000 × g for 10 min at 4 °C, 
the supernatant was boiled for 5 min in SDS-PAGE sample 
buffer [50 mM Tris-Cl (pH 6.8), 2% sodium dodecyl sulfate, 
10% glycerol, 2% β-mercaptoethanol, and 0.004% bromo- 
phenol blue] and separated by 7.5% SDS-dodece PAGE. In immu-
noprecipitation experiments, the supernatant was incubated with 
the indicated primary antibody on ice for 2 h, and the 
immune complex was collected on protein A-agarose during 
1 h incubation at 4 °C. The beads were washed three times with 
buffer containing 50 mM Tris (pH 7.5), 1% Nonidet P-40, 150 
mM NaCl, 2 mM EDTA, and boiled for 5 min in SDS-PAGE 
sample buffer. The solubilized proteins were separated by 
SDS-PAGE, transferred to nitrocellulose membrane (Amer-
shams Pharmacia Biotech), and detected by immunoblotting 
with the indicated antibody using enhanced chemilumines-
cence. Some membranes were subsequently incubated at 55 
°C for 30 min in stripping buffer—100 mM β-mercaptoethanol, 
2% sodium dodecyl sulfate, 62.5 mM Tris-Cl (pH 6.7)—to 
prepare them for a second round of immunoblotting.

PI 3-Kinase Activity

In vitro phosphorylation of PI was performed with immuno-
precipitated proteins using anti-Irs1, anti-Irs2, anti-p110α-Pl 
3-kinase, or antiphosphotyrosine antibody. The immunocom-
plexes were washed three times with PBS (pH 7.4) containing 
1% Nonidet P-40, and 0.1 mM sodium orthovanadate; 0.1 mM 
Tris-Cl (pH 7.5) containing 0.5 mM LiCl and 0.1 mM sodium 
orthovanadate; and 10 mM Tris-Cl (pH 7.5) containing 100 mM 
NaCl, 1 mM EDTA, and 0.1 mM sodium orthovanadate. The 
immunoprecipitates were then incubated for 15 min at 22 °C 
with 100 μCi/ml [γ-32P]ATP and 0.1 mg/ml sonicated PI in 75 
μl of 10 mM Tris-Cl (pH 7.5) containing 100 mM NaCl, 1 mM 
EDTA, and 10 μg MglCl2. The reaction was stopped with 20 
μl of 8% HCl and 160 μl of CHCl3/MEOH (1:1, vol/vol). After 
centrifugation for 5 min, the organic phase was collected and 
spotted on a silica gel thin-layer chromatography plate. The 
plate was developed in CHCl3/MEOH/H2O/NH4OH (100:78: 
19:3:3:3), dried, and visualized by autoradiography. The sig- 
nal intensities were quantitatively analyzed with typhoon 
9410 Imager (Amershams Biociences).

Quantitative Real-Time PCR

Total cellular RNA from MEFs was prepared with Trizol re-
agent (Invitrogen), and 1 μg RNA was used for cDNA syn-
thesis with the iScript c DNA synthesis kit (Bio-Rad, Hercules, 
CA). PCR was in reaction with IQ cyber green super mix and 
performed on the iCycler (Bio-Rad). The specificity of primers 
was verified by BLAST analysis of the mouse genome, visu-
alization of RT-PCR products after agarose gel electrophore-
sis, and by melting point analysis of PCR products. Sequence 
for primers used in this study are Irs1: sense 5′-CGGGTG-
GTGCACAAATGG-3′, antisense 5′-GCCACTGGTGAGG-
TATTCCATAGC-3′; Irs2: sense 5′-ACTTCCACATGCTCC-
ACTGCT-3′, antisense 5′-GGCTTTGAGGTTCCAGAT- 
AG-3′; and Cyclophilin: sense 5′-CTAAGACATAAGTCCG-
TGACATTTG-3′, antisense 5′-TGCCATACGCGTCTCAG-
TGT-3′. PCR was carried out under the following program: 
1 cycle at 95 C for 3 min; 45 cycles at 95 C, 20 sec and 60 C 
for 1 min; 1 cycle at 95 C for 1 min and 65 C for 1 min; 80 
cycles at 55 C for 10 sec and increase set point temperature 
after cycle 2 by 0.5 C. The melt curve data were collected and 
analyzed. The relative mRNA abundance normalized to cy-
toplasmic RNA levels was determined using ΔΔCt (cycle 
threshold) method after amplification. Data are represented 
as mean ± SD. PCR amplification efficiency of the Irs1, Irs2, 
or cyclophilin gene was determined using Ct slop method.
This assay involved in generating a dilution serious of the 
target template and determining the Ct value for each dilu-
ion. A 4-log dilution arrange in triplicate was generated using

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5-fold serial dilutions of the target PCR product (1:1, 1:4, 1:16, 1:64, 1:128). Each of these dilutions was subjected to real-time PCR amplification. The Ct values obtained over this 4-log dilution range were plotted against cDNA concentration and the amplification efficiency was calculated from the slope of the graph using the equation: \( Ex = 10^{-\frac{1}{slope}} - 1 \).

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