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4PS/Insulin Receptor Substrate (IRS)-2 Is the Alternative Substrate of the Insulin Receptor in IRS-1-deficient Mice*

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Mary-Elizabeth Patti, Xiao-Jian Sun‡, Jens C. Bruening§, Eiichi Araki¶, Myra A. Lipes‖, Morris F. White, and C. Ronald Kahn**

From the Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

Insulin receptor substrate-1 (IRS-1) is the major cytoplasmic substrate of the insulin and insulin-like growth factor (IGF)-1 receptors. Transgenic mice lacking IRS-1 are resistant to insulin and IGF-1, but exhibit significant residual insulin action which corresponds to the presence of an alternative high molecular weight substrate in liver and muscle. Recently, Sun *et al.* (Sun, X.-J, Wang, L.-M., Zhang, Y., Yenush, L. P., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) *Nature* 377, 173–177) purified and cloned 4PS, the major substrate of the IL-4 receptor-associated tyrosine kinase in myeloid cells, which has significant structural similarity to IRS-1. To determine if 4PS is the alternative substrate of the insulin receptor in IRS-1-deficient mice, we performed immunoprecipitation, immunoblotting, and phosphatidylinositol (PI) 3-kinase assays using specific antibodies to 4PS. Following insulin stimulation, 4PS is rapidly phosphorylated in liver and muscle, binds to the p85 subunit of PI 3-kinase, and activates the enzyme. Insulin stimulation also results in the association of 4PS with Grb 2 in both liver and muscle. In IRS-1-deficient mice, both the phosphorylation of 4PS and associated PI 3-kinase activity are enhanced, without an increase in protein expression. Immunodepletion of 4PS from liver and muscle homogenates removes most of the phosphotyrosine-associated PI 3-kinase activity in IRS-1-deficient mice. Thus, 4PS is the primary alternative substrate, *i.e.* IRS-2, which plays a major role in physiologic insulin signal transduction via both PI 3-kinase activation and Grb 2/Sos association. In IRS-1-deficient mice, 4PS/IRS-2 provides signal transduction to these two major pathways of insulin signaling.

Stimulation of the insulin and IGF-1[1] receptor tyrosine kinases results in rapid autophosphorylation and subsequent phosphorylation of cytoplasmic substrates. A major substrate of the insulin receptor is IRS-1, a cytoplasmic protein of 160–185 kDa on SDS-PAGE (1–3). Following insulin/IGF-1 stimulation, IRS-1 is rapidly phosphorylated on multiple tyrosines (4). This results in docking of several SH2 domain proteins, including: the p85 subunit of PI 3-kinase (5–8), an upstream element in insulin-stimulated glucose transport and activation of p70 S6 kinase (9, 10); Grb 2, an adapter molecule linking IRS-1 to activation of Ras and mitogen-activated protein kinase (11–13); and the tyrosine phosphatase SHPTP2 (14, 15). Insulin and IGF-1 receptors can also phosphorylate other cytoplasmic proteins. These include Shc, a cytoplasmic protein which binds to Grb 2 (16), a p62 protein which associates with Ras-GAP (17), and a 55–60-kDa protein which associates with PI 3-kinase (18, 19).

Abundant evidence from *Xenopus* oocytes (20), cell culture systems (21–23), and animal models (24, 25) has demonstrated the central role of IRS-1 in mediating downstream effects of insulin and IGF-1. Recently, we (26) and others (27) have shown that mice made IRS-1-deficient by targeted gene knockout exhibit hyperinsulinemia, glucose intolerance, and marked growth retardation. However, IRS-1-deficient mice continue to exhibit some insulin-stimulated glucose disposal and phosphotyrosine-associated PI 3-kinase activation, suggesting the presence of an IRS-1-independent pathway of signaling. Immunoblots from both liver and muscle tissue of IRS-1 (−/−) animals reveal a ~180-kDa protein (tentatively designated IRS-2) which is tyrosine-phosphorylated within 1 min of insulin stimulation and binds to PI 3-kinase, but is not immunoreactive with anti-IRS-1 antibodies (26).

A candidate protein for IRS-1-independent signal transduction is 4PS, a protein of ~180 kDa initially observed as the primary substrate of the interleukin 4 receptor-associated tyrosine kinase (28). In myeloid progenitor cells, 4PS is rapidly phosphorylated in response to IL-4 or insulin, binds p85, and activates PI 3-kinase. In myeloid cells which lack 4PS, overexpression of IRS-1 can restore sensitivity to IL-4 and insulin (29). This functional similarity between 4PS and IRS-1 has been confirmed by the recent cloning of 4PS, which reveals an IRS-1-like molecule with multiple conserved tyrosine phosphorylation sites, as well as several homologous domains near the NH_2 terminus (30). The similarity between these properties of 4PS and the alternative substrate in IRS-1-deficient mice, as well as the fact that IL-4 action is normal in IRS-1 (−/−) animals,[2] suggested to us that 4PS might be the alternative substrate for insulin action in the IRS-1-deficient mouse.

EXPERIMENTAL PROCEDURES

Materials—Reagents for SDS-PAGE and immunoblotting were from Bio-Rad. [γ-^{32}P]ATP was supplied by DuPont NEN. ^{125}I-Protein A was supplied by ICN Radiochemicals. Anti-IRS-1 COOH-terminal antibodies were raised in rabbits using a synthetic peptide derived from the rat carboxyl-terminal sequence (amino acids 1221–1235) and protein A-purified. Two specific antibodies to 4PS were raised to amino acid sequences 618–747 and 976-1094, respectively. Anti-phosphotyrosine

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‡ Mary K. Iacocca Fellow.

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¶ Current address: Dept. of Metabolic Medicine, Kumamoto University Medical School, Kumamoto 860, Japan.

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** To whom correspondence should be addressed: Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. Tel.: 617-732-2635; Fax: 617-732-2593.

[1] The abbreviations used are: IGF, insulin-like growth factor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; IL, interleukin.

[2] M.-E. Patti and C. R. Kahn, manuscript in preparation.

monoclonal antibodies (4G10) and anti-p85 polyclonal antibodies were from Upstate Biotechnology, Inc. Anti-Grb-2 antibodies were from Santa Cruz Biotechnology. Sodium pentobarbital was from Abbott Laboratories. Human insulin was from Lilly. Phosphoinositol was from Avanti. All other chemical reagents were from Sigma.

Animals—Animal care was provided in accordance with Public Health Service and institutional guidelines. Mouse genotypes were determined as described (26). IRS-1-deficient mice and age-matched wild type control mice were fasted for 12 h prior to the experiment. Mice were anesthetized with 115 mg/kg of sodium pentobarbital injected intraperitoneally. Adequacy of anesthesia was assured by loss of pedal reflexes and response to tail pinching. 5 units (0.2 mg) of regular human insulin or its diluent were injected as a bolus into the inferior vena cava. The liver, gastrocnemius, and quadriceps muscles were removed at 1.5, 3, and 4 min, respectively, following insulin injection and homogenized at 4 °C as described (26). Liver and muscle homogenates were allowed to solubilize at 4 °C for 1 h and clarified by centrifugation at 277,000 × *g* for 1 h.

Cell Culture—FDC-P2 myeloid cells were grown in RPMI containing 10% fetal calf serum and 5% IL-3 (WEHI supernatant) to a density of 10^6 cells/ml. Cells were incubated in media with 0.5% bovine serum albumin and 50 μM vanadate for 2 h, stimulated with 100 nM insulin for 10 min, lysed in extraction buffer (as above), and centrifuged at 10,000 × *g* for 15 min. Supernatants were processed as for tissue homogenates.

Immunoprecipitation, Immunoblotting, and PI 3-Kinase Assays—Supernatants of tissue homogenates containing 5 mg of protein were immunoprecipitated overnight with the indicated antibody. Immune complexes were collected with 80 μl of a 50% slurry of protein A-Sepharose. Immunoprecipitates were washed, solubilized in Laemmli sample buffer, and separated using 6% SDS-PAGE. Proteins were transferred to nitrocellulose, probed with the indicated antibody, detected with ^{125}I-protein A, and quantitated using a PhosphorImager (Molecular Dynamics). PI 3-kinase assays were performed as described (31). ^{32}P incorporation into PI 3-phosphate was quantified using a PhosphorImager (Molecular Dynamics).

Sequential Immunoprecipitations—Supernatants of liver or muscle homogenates from insulin-stimulated mice (two per group) were immunoprecipitated with anti-4PS/IRS-2 antibody. Immune complexes were collected with protein A-Sepharose, whereas supernatants were reprecipitated with anti-4PS/IRS-2 antibody. Following four rounds of anti-4PS/IRS-2 immunoprecipitation, resulting supernatants were immunoprecipitated with anti-p85 antibody. Immune complexes from each step were solubilized in Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were immunoblotted with anti-phosphotyrosine 4G10. For sequential PI 3-kinase assays, equal volume aliquots of supernatant from each precipitation step were collected for subsequent precipitation with either anti-4PS/IRS-2 or anti-phosphotyrosine antibodies (each in duplicate) and measurement of PI 3-kinase activity. The remaining supernatant was again precipitated with 4PS/IRS-2 for use in subsequent steps.

RESULTS AND DISCUSSION

4PS Is Tyrosine-phosphorylated following in Vivo Insulin Stimulation—4PS, the substrate of the IL-4 receptor-associated tyrosine kinase, shares many functional and structural characteristics with IRS-1 (30). To determine if 4PS is a physiologic insulin receptor substrate and, more specifically, the 180-kDa alternative substrate observed in IRS-1-deficient mice, liver and muscle homogenates from insulin-stimulated wild type and IRS-1-deficient mice were immunoprecipitated with anti-IRS-1 or two different specific anti-4PS antibodies and separated by SDS-PAGE. As shown previously (26), tyrosine-phosphorylated IRS-1 was detected in anti-IRS-1 immunoprecipitates from wild type animals, but not in tissues from the knockout animals (Fig. 1*A*, *upper panel*). In contrast, phosphotyrosine blotting of anti-4PS immunoprecipitates demonstrated that insulin stimulation of both wild type and IRS-1-deficient mice resulted in rapid phosphorylation of 4PS in liver and skeletal muscle (Fig. 1*A*, *lower panel*). Quantitation of multiple experiments demonstrated 30 and 63% increases in insulin-stimulated tyrosine phosphorylation of 4PS in liver and muscle of IRS-1-deficient mice relative to controls (Fig. 1*B*). The increase in 4PS phosphorylation was due to an increase in stoichiometry of phosphorylation, since there was no difference

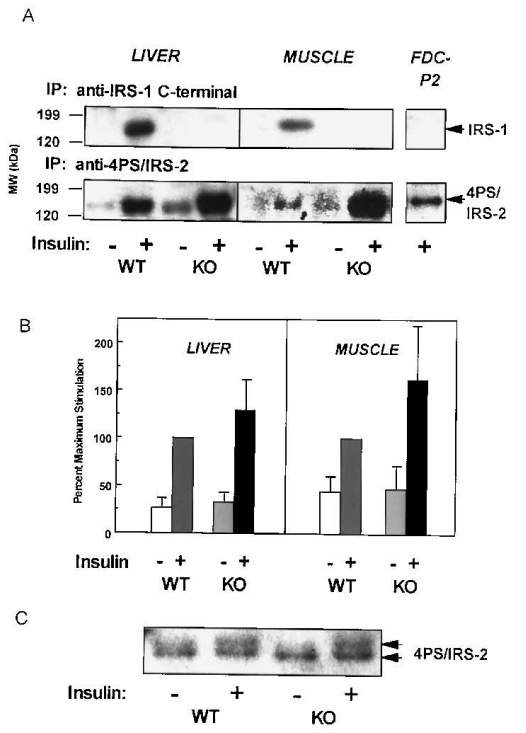

FIG. 1. **Insulin-stimulated phosphorylation of 4PS/IRS-2 in the wild type (IRS-1 +/+) and knockout (IRS-1 −/−) mouse.** *A*, phosphotyrosine immunoblots of IRS-1 and 4PS/IRS-2 immunoprecipitates. Extracts of liver or muscle from mice treated with diluent or insulin *in vivo* or myeloid precursor FDC-P2 cells treated with insulin *in vitro* were immunoprecipitated with anti-IRS-1 COOH-terminal (*upper panel*) or anti-4PS/IRS-2 (*lower panel*) antibody, separated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. The positions of IRS-1 and 4PS/IRS-2 are indicated by the *arrows*. The migration of phosphorylated 4PS from liver homogenates was similar to that of 4PS from insulin-treated FDC-P2 cells; the migration of phosphorylated 4PS in muscle was slightly more retarded, suggesting differences in phosphorylation state. For liver and muscle, each lane represents tissue from one animal; each blot is representative of at least three independent experiments. *B*, quantitation of 4PS/IRS-2 phosphorylation from phosphotyrosine immunoblots of anti-4PS/IRS-2 precipitates. Insulin-stimulated phosphorylation in wild type IRS-1 (+/+) animals was assigned a relative value of 100%. Data are mean ± S.E. for four independent experiments. *C*, anti-4PS/IRS-2 immunoblot of 4PS/IRS-2 immunoprecipitates. Supernatants of liver homogenates from mice treated with diluent or insulin *in vivo* were immunoprecipitated with anti-4PS/IRS-2, separated by SDS-PAGE, and immunoblotted with anti-4PS/IRS-2 antibodies.

in 4PS protein expression as determined by Western blot analysis of liver (Fig. 1*C*), skeletal muscle, adipose tissue, brain, heart, lung, kidney, and lymphoid cells (data not shown).

4PS Associates with and Activates PI 3-Kinase in Insulin-stimulated Mice—Previous studies in cultured cells have demonstrated that phosphorylated IRS-1 and phosphorylated 4PS can bind to and activate PI 3-kinase (5–8, 28), and, in the case of IRS-1, this appears to link insulin action to stimulation of glucose transport (9, 10). Similarly, in control mice, the p85 subunit of PI 3-kinase associated with phosphorylated IRS-1 following insulin stimulation (Fig. 2*A*, *left*). This was associated with a 4.6-fold increase in IRS-1-associated PI 3-kinase activity in liver and a 9.5-fold increase in muscle (Fig. 2*C*, *left*). Not surprisingly, IRS-1-deficient animals demonstrated no insulin-stimulated IRS-1-associated PI 3-kinase. However, PI 3-kinase activity was present in anti-phosphotyrosine immunoprecipitates in IRS-1-deficient mice (Fig. 2*C*, *middle*), indicating that a non-IRS-1 phosphotyrosine protein was responsible for activating PI 3-kinase following insulin stimulation. Also, as noted previously (26), in IRS-1 (−/−) mice this correlated with the

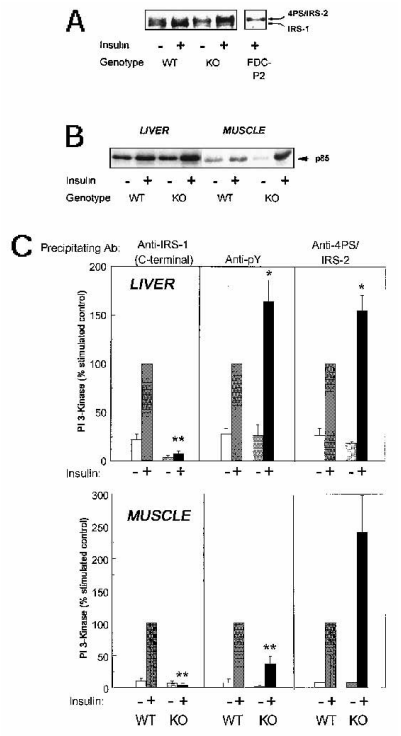

FIG. 2. **Insulin-stimulated association of 4PS/IRS-2 with the p85 subunit of PI 3-kinase and activation of PI 3-kinase activity.** *A*, phosphotyrosine Western blot of anti-p85 immunoprecipitates from liver of wild type (IRS-1 +/+) and knockout (IRS-1 −/−) mice. The migration of IRS-1 and IRS-2 is indicated by the *arrows*. *B*, p85 Western blot of anti-4PS/IRS-2 immunoprecipitates from liver and muscle of wild type and knockout mice. *C*, IRS-1, phosphotyrosine, and 4PS/IRS-2-associated PI 3-kinase activity in liver (*upper panel*) and muscle (*lower panel*) of wild type and knockout mice. Tissue homogenates were immunoprecipitated with anti-IRS-1 COOH-terminal, anti-4PS/IRS-2, or anti-phosphotyrosine 4G10, followed by PI 3-kinase assays. ^{32}P incorporation into PI 3-phosphate was quantified using a PhosphorImager. Data are the mean ± S.E. of three independent experiments and are expressed as activity relative to the insulin-stimulated wild type animals (assigned a value of 100%). *, $p < 0.05$; **, $p < 0.001$.

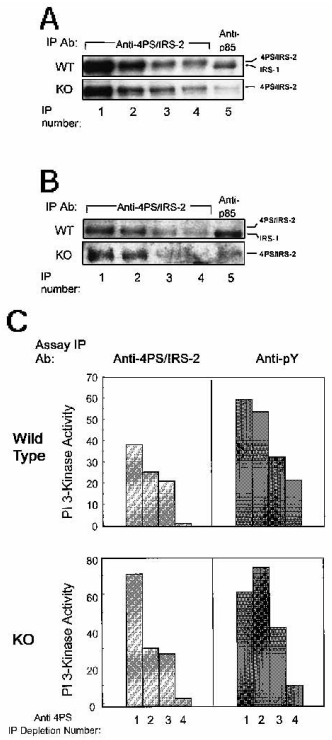

FIG. 3. **Sequential immunodepletion of 4PS/IRS-2 from tissue homogenates: *A* and *B*, anti-phosphotyrosine 4G10 immunoblot of liver (*A*) or muscle (*B*) homogenates from insulin-stimulated mice subjected to four rounds of sequential anti-4PS/IRS-2 immunoprecipitation followed by anti-p85 immunoprecipitation as described under "Experimental Procedures."** The migration of residual IRS-2 (in both wild type and IRS-1 knockout animals) and of residual IRS-1 (in wild type animals only) is indicated by the *arrows*. *C*, PI 3-kinase activity with 4PS/IRS-2 immunodepletion in muscle of insulin-stimulated wild type and knockout mice. PI 3-kinase activity was measured in both anti-IRS-2 (*left panels*) and anti-phosphotyrosine (*right panels*) immune complexes following each of four successive immunoprecipitations with anti-4PS/IRS-2 antibody. Data are the means of duplicate assays performed after each immunodepletion step and are expressed in arbitrary PhosphorImager units.

appearance of another tyrosine-phosphorylated protein (designated IRS-2) in anti-p85 precipitates which migrated just above the position of IRS-1 in SDS gels (Fig. 2*A*, *center*). This protein was phosphorylated following insulin treatment and co-migrated with 4PS from insulin-stimulated myeloid cells (Fig. 2*A*, *right*).

Direct immunoblots of anti-4PS precipitates revealed that p85 also associated with 4PS in an insulin-stimulated fashion in both liver and muscle (Fig. 2*B*). Again, p85 association with 4PS was enhanced in the IRS-1-deficient mice despite equivalent total p85 protein expression (data not shown). Precipitation of the same extracts with anti-4PS antibodies showed that insulin also stimulated PI 3-kinase activity in anti-4PS immunocomplexes in both wild type and knockout animals (Fig. 2*C*, *right*). The magnitude of the 4PS-associated PI 3-kinase activation was 55 and 141% greater in liver and muscle, respectively, of knockout than in control animals, consistent with the enhanced phosphorylation of 4PS in the absence of IRS-1.

4PS Is the Dominant Phosphoprotein Interacting with PI 3-Kinase in IRS-1-deficient Mice—To determine if 4PS was the only, or at least the major, alternative substrate of the insulin receptor which binds to PI 3-kinase, we performed sequential immunoprecipitation with anti-4PS antibodies followed by phosphotyrosine blotting and PI 3-kinase assays. In liver homogenates of insulin-stimulated wild type mice, four rounds of immunoprecipitation resulted in depletion of 89% of the 4PS phosphoprotein (Fig. 3*A*). Subsequent precipitation of the remaining extract with anti-p85 demonstrated that tyrosine-phosphorylated IRS-1 was still available to bind p85. In contrast, following immunodepletion of 4PS from liver of IRS-1-deficient mice, no other tyrosine-phosphorylated proteins were detected. Sequential immunodepletion of 4PS was quantitatively more complete in muscle (Fig. 3*B*). Again, residual tyrosine-phosphorylated IRS-1 was noted following 4PS depletion in wild type mice, but no other phosphoproteins were seen in the IRS-1-deficient mice. PI 3-kinase activity associated with both 4PS and phosphotyrosine-containing proteins was also assayed following each round of immunodepletion. As expected, 4PS-associated PI 3-kinase activity declined progressively with 4PS immunodepletion in tissues of both wild type and knockout mice (Fig. 3*C*, *left*). Phosphotyrosine-associated PI 3-kinase activity also declined as 4PS was depleted. This decline was nearly complete in muscle of the IRS-1 (−/−) mice, reflecting the significant contribution of 4PS to the phosphoprotein pool. In contrast, residual phosphotyrosine-associated PI 3-kinase was seen in the wild type animals due to the presence of IRS-1 and its contribution to PI 3-kinase activation.

4PS Associates with Grb 2 following Insulin Stimulation—The adapter protein Grb 2 has been shown to link both IRS-1 and Shc to activation of Ras and mitogen-activated protein kinase (32). Since the amino acid sequence of 4PS predicts a tyrosine phosphorylation motif which could serve as a Grb 2 binding site (30), we sought evidence for direct insulin-stimulated association between

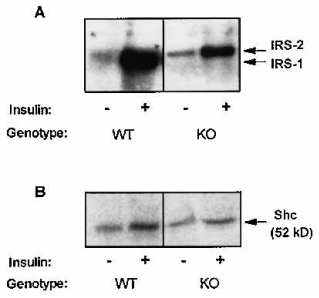

FIG. 4. **Insulin-stimulated association of Grb 2 with IRS-1, IRS-2, and Shc *in vivo*.** *A*, phosphotyrosine immunoblots of anti-Grb 2 immunoprecipitates from liver of wild type (IRS-1 +/+) and knockout (IRS-1 −/−) mice stimulated *in vivo* with insulin for 1.5 min. The migration of IRS-1 and IRS-2 is indicated by the *arrows*. *B*, anti-Shc immunoblot of anti-Grb 2 immunoprecipitates from liver of wild type and knockout mice stimulated *in vivo* with insulin for 1.5 min. The migration of the 52-kDa Shc isoform is indicated by the *arrow*.

Grb 2 and 4PS *in vivo*. Phosphotyrosine immunoblotting of anti-Grb 2 precipitates in wild type animals revealed association of Grb 2 with both tyrosine-phosphorylated IRS-1 and IRS-2 following insulin stimulation (Fig. 4*A*, *left*). In the IRS-1-deficient mice, Grb 2 associated with IRS-2 (Fig. 4*A*, *right*). In wild type and IRS-1-deficient mice, Grb 2 also associated with Shc in an insulin-dependent fashion; the magnitude of this insulin-stimulated association was similar in wild type and IRS-1-deficient animals (Fig. 4*B*).

Alternative Pathways of Signaling—Taken together, these data demonstrate that 4PS is IRS-2 in the IRS-1-deficient mouse and is the dominant alternative substrate of the insulin receptor which is phosphorylated in response to insulin, binds Grb 2 and p85, and activates PI 3-kinase in tissues of these animals. These functional data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the IRS-1 and 4PS/IRS-2 sequences reveals several important similarities. These include three regions of homology within the NH_2 terminus encoding the pleckstrin homology and phosphotyrosine binding domains. More importantly, 4PS possesses 22 potential tyrosine phosphorylation sites, of which 14 show identity or near identity to those present in IRS-1, including phosphorylation sites which can bind SH2 domains of PI 3-kinase, Grb 2, and SHPTP2 (30). Furthermore, studies in cultured cells have shown that 4PS can substitute for IRS-1 in signaling to PI 3-kinase and stimulating mitogenesis. Thus, 4PS/IRS-2 has the potential to link insulin signaling to both PI 3-kinase and Ras activation, in a manner similar to IRS-1.

The present study also shows that 4PS/IRS-2 may play a major role in early steps of insulin signal transduction in normal target tissues of intact animals. The exact contribution of 4PS/IRS-2 in comparison with IRS-1 in propagating the downstream insulin signal is difficult to determine due to the differing efficiency of immunoprecipitation with antibodies to IRS-1 and 4PS/IRS-2. Given the 50% reduction in insulin-stimulated glucose disposal in the IRS-1-deficient mice *in vivo* and decreased phosphotyrosine-associated PI 3-kinase activity in muscle (26), we would predict that 4PS/IRS-2 may contribute up to 50% of total insulin-stimulated activation of PI 3-kinase and downstream glucose transport in skeletal muscle. The enhanced phosphotyrosine-associated PI 3-kinase activity observed in liver relative to muscle, despite similar magnitude of insulin stimulation of 4PS/IRS-2-associated PI 3-kinase, may reflect a greater abundance of 4PS/IRS-2 protein in liver as compared with muscle (data not shown) or, alternatively, the presence of additional phosphoproteins which may play a role in insulin signaling in liver. Although we have been unable to detect additional phosphoproteins in either liver or muscle thus far, we cannot entirely exclude their presence given limitations in sensitivity at the protein level. However, IRS-1-deficient mice show no difference in insulin-stimulated Shc phosphorylation and exhibit no other phosphoproteins, such as the p55–p60 proteins described by others (18, 19), which bind to or activate PI 3-kinase (data not shown).

In summary, 4PS, initially identified as the IL-4 receptor-associated substrate, plays a role in normal insulin signaling in physiologically relevant tissues and helps to rescue the IRS-1-deficient mouse. Thus, 4PS/IRS-2 is a common substrate of two physiologically diverse growth factor receptors. Further investigation will attempt to clarify how specificity for metabolic *versus* cytokine downstream signaling is achieved.

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