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

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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., J., 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, with 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.

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Stimulation of the insulin and IGF-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 SHPPT2 (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 knock-out 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 NH₂ 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, 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-32P]ATP was supplied by DuPont NEN. Alkaline phosphatase A was supplied by ICN Radiochemicals. Anti-IRS-1 COOH-terminal antibody was 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

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

The increase in 4PS phosphorylation was due to an increase in stoichiometry of phosphorylation, since there was no difference between wild type and IRS-1-deficient mice relative to controls (Fig. 1A, lower panel). Insulin-stimulated tyrosine phosphorylation of 4PS in liver and muscle homogenates were allowed to solubilize at 4 °C for 1 h and centrifuged 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 10⁶ 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 described 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 125I-protein A, and quantitated using a PhosphorImager (Molecular Dynamics). PI 3-kinase assays were performed as described (31).

32P incorporation into PI 3-phosphate was quantified using a PhosphorImager (Molecular Dynamics). Sequential Immunoprecipitations—Supernatants of liver or muscle homogenates from insulin-stimulated mice were precipitated with anti-phosphotyrosine (each in duplicate) and measurement of PI 3-kinase activity. The remaining supernatant was then precipitated with anti-4PS/IRS-2 antibody. Immunoprecipitates were washed, solubilized in Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were probed with anti-4PS/IRS-2 antibodies (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 then precipitated with anti-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. 1A, 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. 1A, 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. 1B). The increase in 4PS phosphorylation was due to an increase in stoichiometry of phosphorylation, since there was no difference in 4PS protein expression as determined by Western blot analysis of liver (Fig. 1C), 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 phosphorylated 4PS in 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.

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.
Insulin also stimulated PI 3-kinase activity in anti-4PS 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. 32P incorporation into PI 3-phosphate was quantified using a PhosphorImage. 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.

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. 2A, center). This protein was phosphorylated following insulin treatment and co-migrated with 4PS from insulin-stimulated myeloid cells (Fig. 2A, 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. 2B). 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. 2C, 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 wild type 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. 3A). 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. 3B). 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. 3C, 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...
Grb2 and 4PS in vivo. Phosphorysine immunoblotting of anti-Grb2 precipitates in wild type animals revealed association of Grb2 with both tyrosine-phosphorylated IRS-1 and IRS-2 following insulin stimulation (Fig. 4A, left). In the IRS-1-deficient mice, Grb2 associated with IRS-2 (Fig. 4A, right). In wild type and IRS-1-deficient mice, Grb2 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. 4B). Alternative Pathways of Signaling—Taken together, these data demonstrate that 4PS is IRS-2 in the IRS-1-deficient mouse and is the dominant alternate substrate of the insulin receptor which is phosphorylated in response to insulin, binds Grb2 and p85, and activates PI 3-kinase (data not shown). Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2. Despite an overall amino acid sequence identity of only 43%, alignment of the data are consistent with the structure of 4PS/IRS-2.
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