SH2B1 Enhances Insulin Sensitivity by Both Stimulating the Insulin Receptor and Inhibiting Tyrosine Dephosphorylation of Insulin Receptor Substrate Proteins

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OBJECTIVE—SH2B1 is a SH2 domain-containing adaptor protein expressed in both the central nervous system and peripheral tissues. Neuronal SH2B1 controls body weight; however, the functions of peripheral SH2B1 remain unknown. Here, we studied peripheral SH2B1 regulation of insulin sensitivity and glucose metabolism.

RESEARCH DESIGN AND METHODS—We generated TgKO mice expressing SH2B1 in the brain but not peripheral tissues. Various metabolic parameters and insulin signaling were examined in TgKO mice fed a high-fat diet (HFD). The effect of SH2B1 on the insulin receptor catalytic activity and insulin receptor substrate (IRS)-1/IRS-2 dephosphorylation was examined using in vitro kinase assays and in vitro dephosphorylation assays, respectively. SH2B1 was coexpressed with PTP1B, and insulin receptor–mediated phosphorylation of IRS-1 was examined.

RESULTS—Deletion of peripheral SH2B1 markedly exacerbated HFD-induced hyperglycemia, hyperinsulinemia, and glucose intolerance in TgKO mice. Insulin signaling was dramatically impaired in muscle, liver, and adipose tissue in TgKO mice. Deletion of SH2B1 impaired insulin signaling in primary hepatocytes, whereas SH2B1 overexpression stimulated insulin receptor autophosphorylation and tyrosine phosphorylation of IRSs. Purified SH2B1 stimulated insulin receptor catalytic activity in vitro. The SH2 domain of SH2B1 was both required and sufficient to promote insulin receptor activation. Insulin stimulated the binding of SH2B1 to IRS-1 or IRS-2. This physical interaction inhibited tyrosine dephosphorylation of IRS-1 or IRS-2 and increased the ability of IRS proteins to activate the phosphatidylinositol 3-kinase pathway.

CONCLUSIONS—SH2B1 is an endogenous insulin sensitizer. It directly binds to insulin receptors, IRS-1 and IRS-2, and enhances insulin sensitivity by promoting insulin receptor catalytic activity and by inhibiting tyrosine dephosphorylation of IRS proteins.
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Insulin decreases blood glucose both by promoting glucose uptake into skeletal muscle and adipose tissue and by suppressing hepatic glucose production. In type 2 diabetes, the ability of insulin to reduce blood glucose is impaired (insulin resistance) because of a combination of genetic and environmental factors, resulting in hyperglycemia. Insulin resistance is not only the hallmark but also a determinant of type 2 diabetes.

Insulin binds to and activates the insulin receptor. Insulin receptor tyrosyl phosphorylates insulin receptor substrates (IRS-1, -2, -3, and -4). IRS proteins, particularly IRS-1 and IRS-2, initiate and coordinate multiple downstream pathways, including the phosphatidylinositol 3-kinase/Akt pathway (1). Genetic deletion of IRS-1, IRS-2, or Akt2 causes insulin resistance in mice, indicating that the IRS protein/phosphatidylinositol 3-kinase/Akt2 pathway is required for regulation of glucose homeostasis by insulin (2–5). Insulin receptor and IRS proteins are negatively regulated by various intracellular molecules, including PTP1B, Grb10, Grb14, SOCS1, SOCS3, JNK, PKCθ, S6K, and IKKβ (6–23). The relative contribution of these negative regulators to the progression of insulin resistance has been extensively studied (6–24). However, insulin signaling is likely to also be modulated by positive regulators. In this study, we demonstrate that SH2B1 is a novel endogenous insulin sensitizer.

SH2B1 is a member of the SH2B family of adapter proteins that also includes SH2B2 (APS) and SH2B3 (Lnk). SH2B1 and SH2B2 are expressed in multiple tissues, including insulin target tissues (e.g., skeletal muscle, adipose tissue, liver, and the brain); by contrast, SH2B3 expression is restricted to hematopoietic tissue (25,26). Structurally, SH2B family members have an NH_2-terminal dimerization domain, a central pleckstrin homology domain, and a COOH-terminal Src homology 2 (SH2) domain. The dimerization domain mediates homodimerization or heterodimerization between different SH2B proteins (27). SH2B1 and SH2B2 bind via their SH2 domains to a variety of tyrosine phosphorylated proteins, including JAK2 and insulin receptor, in cultured cells (28). Genetic deletion of SH2B1 results in marked leptin resistance, obesity, insulin resistance, and type 2 diabetes in mice, demonstrating that SH2B1 is required for the maintenance of normal body weight, insulin sensitivity, and glucose metabolism (29–32). Surprisingly, SH2B2-null mice have normal body weight and slightly improved insulin sensitivity (32,33), suggesting that SH2B1 and SH2B2 have distinct functions in vivo. However, it remains unclear whether SH2B1 cell

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autonomously regulates insulin sensitivity in peripheral insulin target tissues because systemic deletion of SH2B1 causes obesity, which may cause insulin resistance in SH2B1-null mice.

We generated a mouse model in which recombinant SH2B1 is specifically expressed in the brain of SH2B1-null mice (TgKO) using transgenic approaches (31). Neuron-specific restoration of SH2B1 corrects both leptin resistance and obesity, suggesting that neuronal SH2B1 regulates energy balance and body weight by enhancing leptin sensitivity (31). Consistent with these conclusions, polymorphisms in the *SH2B1* loci are linked to leptin resistance and obesity in humans (34–36). In this work, we demonstrate that deletion of SH2B1 in peripheral tissues impairs insulin sensitivity independent of obesity in TgKO mice. Moreover, we demonstrate that SH2B1 directly promotes insulin responses by stimulating insulin receptor catalytic activity and by protecting IRS proteins from tyrosine dephosphorylation.

RESEARCH DESIGN AND METHODS

Animal studies. SH2B1 knockout and TgKO mice have been described previously (29,31) and were backcrossed for six generations onto a C57BL/6 genetic background. Mice were housed on a 12-h light/dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan and fed either normal rodent diet (9% fat; Lab Diet) or high-fat diet (HFD; 45% fat; Research Diets) ad libitum with free access to water. Fat content was measured by dual-energy X-ray absorptiometry (Norland Medical System). Blood glucose levels were determined using glucometers (Bayer Corp). Plasma insulin was measured using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem). Glucose tolerance tests (GTT) (2 g D-glucose/kg of body weight) and insulin tolerance tests (ITT) (1 IU/kg of body weight; Eli Lilly) were conducted as previously described (29–31). To analyze insulin signaling, mice (fasted 16 h) were anesthetized with Averin (0.5 g of tribromoethanol and 0.25 g or tert-amyl alcohol in 39.5 ml of water; 0.02 ml/g of body weight) and treated with PBS or human insulin (3 units per mouse; Eli Lilly) via inferior vena cava injection. Five minutes after stimulation, gastrocnemius muscles, liver, and epididymal fat pads were dissected, frozen in liquid nitrogen, and stored at −80°C. Tissues were homogenized in ice-cold lysis buffer (50 mmol/l Tris HCl [pH 7.5], 1.0% NP-40, 150 mmol/l NaCl, 2 mmol/l EGTA, 1 mmol/l Na_3VO_4, 100 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, 1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and extracts were immunoblotted or immunoprecipitated with indicated antibodies. Animal protocols were approved by the University Committee on Use and Care of Animals.

Cell lines and transfection. COS7 and HEK293 cells were grown in DMEM supplemented with 5% bovine serum and transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen). Chinese hamster ovary (CHO^{IR} and $CHO^{IR/IRS-1}$) cells were cultured in Ham's F-12 media supplemented with 8% FBS. Cells were deprived of serum for 16 h in DMEM (COS7 and HEK293) or F-12 (CHO) containing 0.6% BSA before being treated. Primary liver cells were isolated from male mice (8 weeks) by perfusion of the liver with type II collagenase (Worthington Biochem) and plated on collagen-coated plates in M199 containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 2 h, primary cells were rinsed in PBS and cultured for an additional 16 h in Williams' Medium E (Sigma) supplemented with 0.6% BSA, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were conducted as described previously (29,37). Proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences) or ECL (Amersham) and quantified using Odyssey 1.2 software (Li-Cor). Actin, phosphoAkt (Thr^{308}), Akt, insulin receptor β, Myc, Shc, and tubulin antibodies were from Santa Cruz. The phosphotyrosine-specific antibody was from Upstate. The AS160 antibody was from Millipore and phospho Akt substrate antibody was from Cell Signaling. Phospho Akt (Ser^{473}) was from BioSource. SH2B1 and IRS-1 antibodies have been described (14,37).

Insulin receptor kinase assay. Cells were serum deprived for 16 h, treated with insulin, and solubilized in kinase lysis buffer (50 mmol/l Tris HCl [pH 7.5], 0.1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, 1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The insulin receptor was precipitated with wheat germ agglutinin (WGA)-conjugated agarose beads, washed three times in wash buffer (50 mmol/l Tris HCl [pH 7.5], 0.5 mol/l NaCl, 0.1% Triton X-100), and twice in kinase reaction buffer (20 mmol/l HEPES [pH 7.6], 0.1% Triton X-100, 5 mmol/l $MgCl_2$, 100 μmol/l Na_3VO_4). WGA-immobilized proteins were preincubated in kinase reaction buffer supplemented with soluble glutathione *S*-transferase (GST) alone, GST-SH2B1, or GST-SH2 fusion proteins at 37°C. GST-IRS-1 (5–10 μg) and ATP (50 μmol/l) were added to initiate kinase reactions at 37°C. Reactions were stopped by adding SDS-PAGE loading buffer, and reaction mixtures were boiled immediately. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies.

Dephosphorylation assays. Immunopurified proteins were washed in lysis buffer and preincubated with GST-SH2B1 or GST (2 μg) in phosphatase reaction buffer (50 mmol/l Tris-HCl [pH 8.2], 100 nmol/l NaCl, 10 mmol/l $MgCl_2$, 1 mmol/l DTT) for 15 min at room temperature with constant mixing. Alkaline phosphatase (New England Biolabs) was added at the indicated concentration, and the mixtures were incubated an additional 30 min at room temperature. Reactions were stopped by adding SDS-PAGE loading buffer and mixtures were boiled immediately. Proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies.

Statistical analysis. Data are presented as means ± SE. Differences between groups were determined by two-tailed Student's *t* tests or ANOVA. $P < 0.05$ was considered significant.

RESULTS

Loss of peripheral SH2B1 predisposes mice to HFD-induced insulin resistance. We previously generated SH2B1 transgenic (Tg) mice in which the expression of recombinant SH2B1 is controlled by the neuron-specific enolase promoter (31). Tg mice were crossed with SH2B1 knockout mice to generate SH2B1 transgenic/knockout compound mutant (TgKO) mice. In TgKO mice, recombinant SH2B1 is expressed in brain but not in other tissues, including liver, muscle, and adipose tissue (31). Neuron-specific restoration of SH2B1 in TgKO mice fully corrected leptin resistance and obesity and largely rescued the hyperglycemia and insulin resistance observed in SH2B1 null mice, indicating that neuronal SH2B1 indirectly regulates insulin sensitivity and glucose metabolism by controlling adiposity (31).

To determine whether loss of peripheral SH2B1 exacerbates dietary fat–induced insulin resistance, TgKO and wild-type littermates (7 weeks) were fed HFD. Body weight and adiposity were similar between wild-type and TgKO mice fed HFD (Fig. 1*A* and *B*). However, fasting (16 h) blood glucose levels were 1.3-fold higher in TgKO mice than in wild-type mice fed HFD for 16 weeks (Fig. 1*C*). Fasting plasma insulin levels were twofold higher in TgKO mice than in wild-type mice (Fig. 1*D*). To examine insulin sensitivity, GTT and ITT were performed. Blood glucose levels were 23–26% higher in TgKO mice than wild-type mice 15 and 30 min after injection of D-glucose (Fig. 1*E*). Exogenous insulin markedly reduced blood glucose in wild-type but not in TgKO mice during ITT (Fig. 1*F*). These results indicate that loss of peripheral SH2B1 exacerbates HFD-induced insulin resistance, hyperglycemia, and glucose intolerance independent of obesity.

Loss of peripheral SH2B1 impairs insulin signaling in muscle, liver, and adipose tissue in HFD-fed mice. To examine insulin signaling in skeletal muscle, liver, and epididymal fat, mice (7 weeks) were fed HFD for 16 weeks and treated with insulin or PBS vehicle. Insulin markedly stimulated tyrosine phosphorylation of IRS-1 as well as IRS-1 association with p85, the regulatory subunit of the phosphatidylinositol 3-kinase, in skeletal muscle of wild-type mice (Fig. 2*A*). Both IRS-1 phosphorylation and IRS-1-p85 association were markedly reduced in TgKO muscle (Fig. 2*A*). Loss of peripheral SH2B1 also decreased insulin receptor autophosphorylation and impaired the ability of insulin to stimulate Akt phosphorylation on Thr^{308} and Ser^{473} in TgKO muscle (Fig. 2*A*). Insulin-stimulated IRS-1 and Akt Thr^{308} phosphorylation were

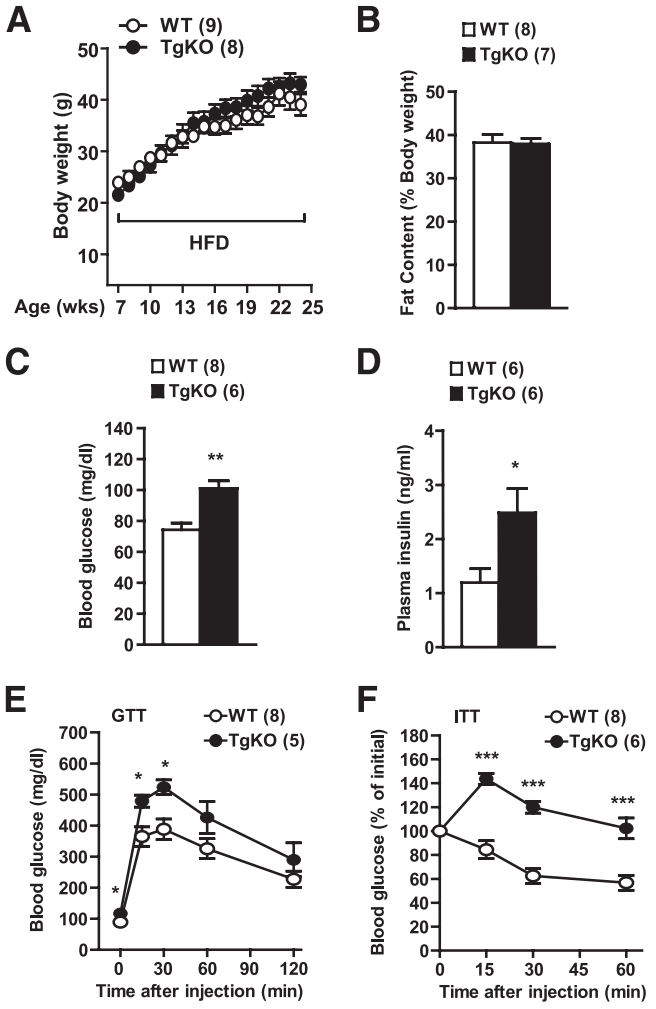

FIG. 1. Peripheral SH2B1 enhances insulin sensitivity in mice. *A–F*: Wild-type (WT) and TgKO male mice (7 weeks) were fed a HFD. *A*: Growth curve. *B*: Body fat content after 16 weeks on HFD. *C*: Fasting (16 h) blood glucose levels and (*D*) plasma insulin levels after 16 weeks on HFD. *E*: GTT performed on mice fed HFD for 16 weeks. *F*: ITT performed on mice fed HFD for 16 weeks. The number of mice is indicated in parenthesis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

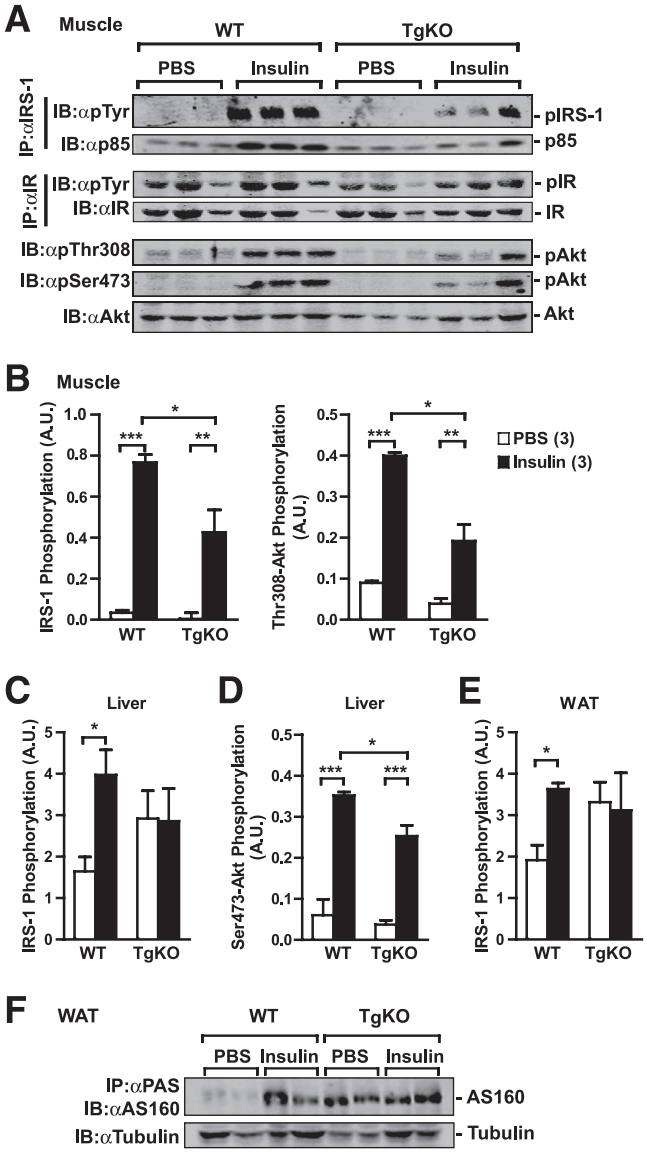

FIG. 2. Deletion of peripheral SH2B1 attenuates insulin signaling in mice. Wild-type (WT) and TgKO males (7 weeks) were fed a HFD for 16 weeks. Mice were fasted for 16 h and treated with PBS or insulin (3 units per mouse). Tissue extracts were prepared 5 min after stimulation. *A*: IRS-1 in muscle extracts was immunoprecipitated with anti–IRS-1 antibody (αIRS-1) and immunoblotted with anti-phosphotyrosine (αpTyr) and αp85 antibodies. Insulin receptor in muscle extracts was immunoprecipitated with αIR and immunoblotted with αpTyr. Muscle extracts were immunoblotted with phospho-specific Akt antibodies against phospho-Thr^{308} (αpThr^{308}) or phospho-Ser^{473} (αpSer^{473}) and αAkt, respectively. *B*: IRS-1 and Akt phosphorylation in (*A*) was quantified by densitometry and normalized to total IRS-1 and Akt protein levels, respectively. *C*: Liver extracts were immunoprecipitated with αIRS-1 and immunoblotted with αpTyr. The same blots were reprobed with αIRS-1. IRS-1 phosphorylation was quantified and normalized to total IRS-1 protein levels. *D*: Liver extracts were immunoblotted with αpSer^{473} and αAkt. Ser^{473} phosphorylation was quantified and normalized to total Akt protein levels. *E*: Epididymal fat (WAT) extracts were immunoprecipitated with αIRS-1 and immunoblotted with αpTyr and reprobed with αIRS-1. IRS-1 phosphorylation was normalized to total IRS-1 protein levels. *F*: WAT extracts were immunoprecipitated with αPAS (anti–phospho-Ser/Thr Akt substrate) and immunoblotted with αAS160. Extracts were also immunoblotted with αtubulin. Three animals were examined for each condition. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

reduced by 44 and 52%, respectively, in TgKO muscle (Fig. 2*B*).

Insulin signaling was also examined in liver and white adipose tissue (WAT) from HFD-fed mice. Relative to wild-type mice, basal IRS-1 phosphorylation was increased in both liver and WAT in TgKO mice; insulin stimulated IRS-1 phosphorylation in these tissues from wild-type but not TgKO mice (Fig. 2*C* and *E*). Akt phosphorylation (Ser^{473}) was also reduced in liver (Fig. 2*D*) and WAT (not shown) in TgKO mice. AS160, a Rab-GAP, is an Akt-substrate involved in GLUT4 vesicle trafficking in adipocytes (38,39). To measure AS160 phosphorylation, WAT extracts were immunoprecipitated with anti–phospho-Ser/Thr Akt substrate antibody and immunoblotted with anti-AS160 antibody. Similar to IRS-1, basal AS160 phosphorylation was increased in adipose tissue from TgKO mice (Fig. 2*F*); however, insulin failed to further stimulate AS160 phosphorylation (Fig. 2*F*). Together, these data indicate that peripheral SH2B1 increases insulin sensitivity in mice by promoting insulin signaling, including the activation of the IRS protein/phosphatidylinositol 3-kinase/Akt pathway, in muscle, liver, and WAT.

SH2B1 cell autonomously promotes insulin signaling via its SH2 domain. To determine whether endogenous SH2B1 directly enhances insulin signaling, primary hepatocyte cultures were prepared from wild-type and SH2B1

knockout littermates, and treated with insulin. SH2B1 was detected in wild-type but not in knockout hepatocytes as expected (Fig. 3*A*). Insulin stimulated tyrosine phosphorylation of insulin receptor and IRS-1, IRS-1 association with p85, and phosphorylation of Akt in wild-type hepatocytes; however, insulin receptor autophosphorylation, IRS-1 phosphorylation, IRS-1 association with p85, and Akt phosphorylation were all reduced in knockout hepatocytes (Fig. 3*A*). Interestingly, SH2B1 deficiency impaired IRS-1 phosphorylation to a greater extent than insulin receptor autophosphorylation. In HEK293 cells, overexpression of SH2B1 markedly increased insulin-stimulated tyrosine phosphorylation of IRS-1; in contrast, a SH2B1 mutant in which the SH2 domain is disrupted because of replacement of Arg^{555} with Glu (R555E) functioned as a dominant negative to inhibit IRS-1 phosphorylation (Fig. 3*B*). To further assess the role of the SH2 domain of SH2B1, IRS-1 and insulin receptor were coexpressed with ΔN504, an NH_2-terminal truncated form of rat SH2B1β (amino acids 504–670) that contained the entire SH2 domain and a minimal number of adjacent amino acids. ΔN504 also promoted IRS-1 phosphorylation in insulin-treated cells (Fig. 3*C*). These data suggest that the SH2 domain of SH2B1 is not only required but also sufficient to promote insulin receptor–mediated phosphorylation of IRS-1.

To determine whether SH2B1 also promotes phosphorylation of other IRSs, Shc was coexpressed with SH2B1 or ΔN504. SH2B1 and ΔN504 enhanced insulin stimulation of Shc phosphorylation to similar levels (Fig. 3*D*). By contrast, SH2B1 stimulated IRS-1 phosphorylation to a higher level than did ΔN504 (Fig. 3*C*), suggesting that SH2B1 promotes phosphorylation of IRS-1 and Shc by different mechanisms.

SH2B1 stimulates insulin receptor catalytic activity through the binding of its SH2 domain to Tyr^{1158} in insulin receptor. SH2B1 directly binds via its SH2 domain to Tyr^{1158} within the activation loop of insulin receptor (40,41). To test whether this interaction modulates insulin receptor activation, CHO^{IR} cells, which stably express insulin receptor, were treated with insulin, and active insulin receptor was purified using WGA-conjugated agarose beads (42). Insulin receptor was then preincubated with purified GST-SH2B1 fusion protein and subsequently subjected to in vitro kinase assays using GST-IRS-1 fusion protein as substrate. Tyrosine phosphorylation of GST-IRS-1 was measured by immunoblotting with anti–phospho-tyrosine antibodies. GST-SH2B1, but not GST alone, dose-dependently stimulated insulin receptor kinase activity as indicated by increased phosphorylation of GST-IRS-1 (Fig. 4*A*). In similar experiments, a GST-SH2 fusion protein prepared by fusing the SH2 domain (amino acids 524–670 of SH2B1β) to GST was preincubated with WGA-purified insulin receptor in vitro kinase assays. The SH2 domain of SH2B1 was sufficient to enhance insulin receptor catalytic activity, stimulating IRS-1 phosphorylation by ~66% (Fig. 4*B*). Additionally, the SH2 domain of SH2B1 also promoted the catalytic activity of insulin receptor immunopurified with an anti–phospho-tyrosine antibody, increasing IRS-1 substrate phosphorylation by ~79% (Fig. 4*C*).

To determine whether Tyr^{1158} in insulin receptor is involved in SH2B1 stimulation of insulin receptor activity, Tyr^{1158} was replaced with Phe (Y1158F). COS7 cells were transiently transfected with insulin receptor or Y1158F and treated with insulin. Insulin stimulated autophosphor-

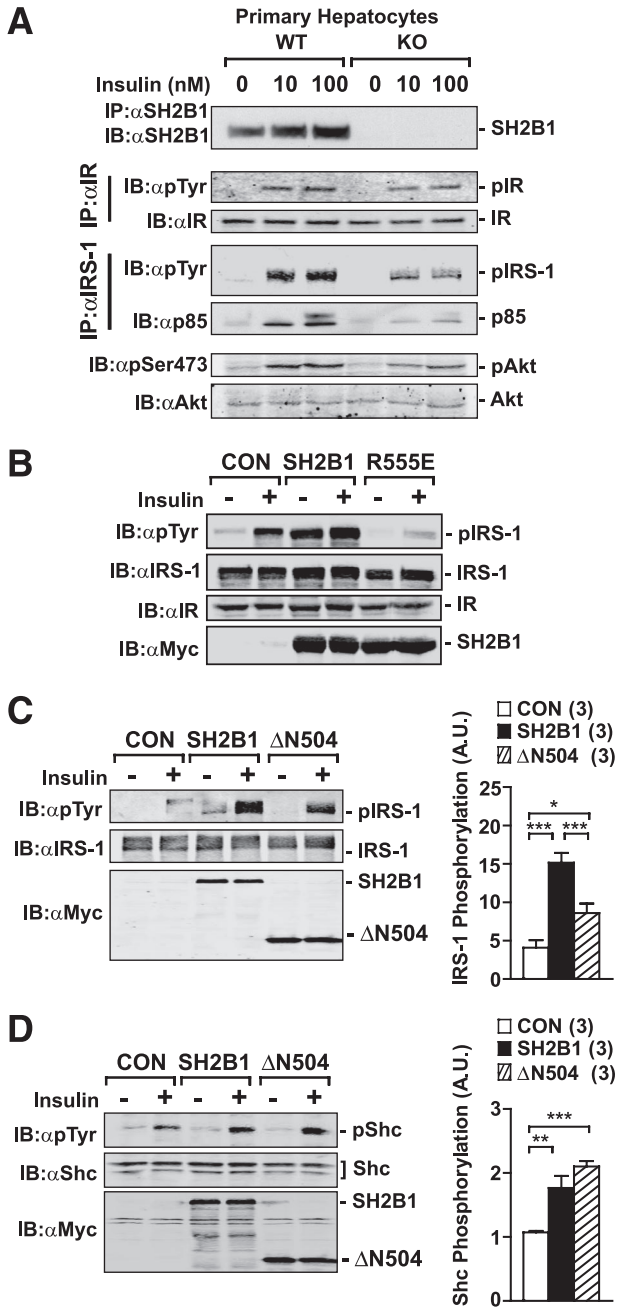

FIG. 3. SH2B1 directly promotes insulin signaling in cells via its SH2 domain. *A*: Primary hepatocyte cultures were prepared from wild-type (WT) or knockout (KO) mice (8 weeks) and treated with 100 nmol/l insulin for 10 min. *Panel 1*: Cell extracts were immunoprecipitated with αSH2B1 and immunoblotted with αSH2B1. *Panels 2 and 3*: Cell extracts were immunoprecipitated with αIR and immunoblotted with αpTyr and αIR. *Panels 4 and 5*: Cell extracts were immunoprecipitated with αIRS-1 and immunoblotted with αpTyr or αp85. *Panels 6 and 7*: Cell extracts were immunoblotted with αSer^{473} and αAkt. *B*: IRS-1 and insulin receptor were transiently coexpressed with empty vector (CON), Myc-tagged SH2B1, or R555E plasmids in HEK293 cells. Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies. *C*: IRS-1 and insulin receptor were transiently coexpressed with empty vector (CON), Myc-tagged SH2B1, or ΔN504 plasmids in HEK293 cells. Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies. IRS-1 phosphorylation was normalized to total IRS-1 protein levels. *D*: Shc and insulin receptor were transiently coexpressed with empty vector (CON), Myc-tagged SH2B1, or ΔN504 plasmids in HEK293 cells. Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies. Shc phosphorylation was normalized to total Shc protein levels. *P < 0.05, **P < 0.01, ***P < 0.001.

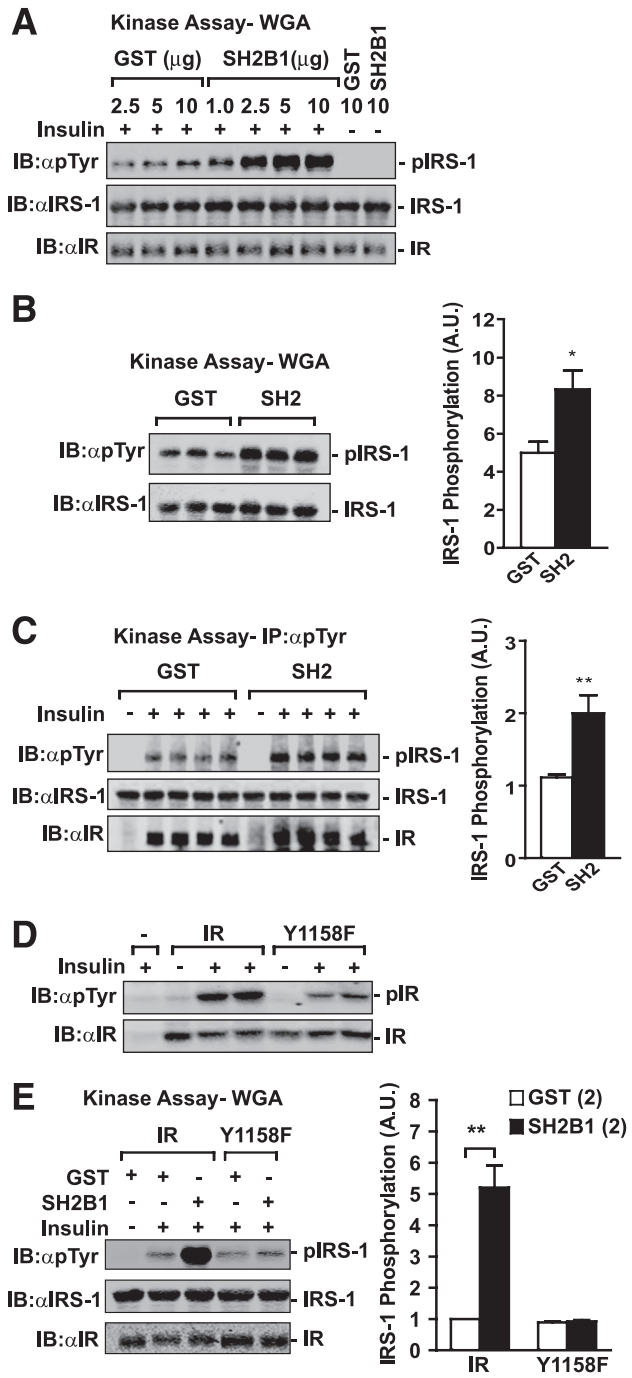

FIG. 4. SH2B1 directly enhances insulin receptor activity in vitro. *A*: CHO^{IR} cells were treated without or with 100 nmol/l insulin for 10 min. Insulin receptor was purified with WGA-agarose beads, preincubated with indicated amounts of GST or GST-SH2B1 fusion protein and subjected to in vitro kinase assays with GST-IRS-1 as substrate for 10 min. Reaction mixtures were immunoblotted with αpTyr, αIRS-1 or αIR. *B*: WGA-purified insulin receptor was preincubated with GST or GST-SH2 fusion protein (5 μg) and subjected to in vitro kinase assays with GST-IRS-1 protein (amino acids 526–859 of rat IRS-1) as substrate for 10 min. IRS-1 phosphorylation was quantified by densitometry and normalized to total GST-IRS-1 levels. *C*: CHO^{IR} cells were treated without or with 100 nmol/l insulin for 10 min. Cell extracts were immunoprecipitated with αpTyr. αpTyr-immunopurified insulin receptor was preincubated with GST or GST-SH2 fusion protein (amino acids 524–670) (5 μg) and subjected to in vitro kinase assays with GST-IRS-1 as substrate for 10 min. Reaction mixtures were immunoblotted with indicated antibodies and IRS-1 phosphorylation was quantified and normalized to total GST-IRS-1 levels. *D*: Wild-type insulin receptor or Y1158F was expressed in COS7 cells. Cells were treated with 100 nmol/l insulin for 10 min. Cell extracts were immunoblotted with αpTyr or αIR. *E*: Wild-type insulin receptor or Y1158F was expressed in COS7 cells and treated with insulin. Wild-type insulin receptor or Y1158F was purified with WGA-agarose beads, preincubated with GST or GST-SH2B1 fusion protein (5 μg), and subjected to in vitro kinase assays with GST-IRS-1 as substrate for 10 min. GST-IRS-1 phosphorylation was normalized to total GST-IRS-1 protein levels. *$P < 0.05$, **$P < 0.01$.

ylation of both insulin receptor and Y1158F, but Y1158F autophosphorylation was reduced (Fig. 4*D*). Y1158F phosphorylated IRS-1 in response to insulin (data not shown), indicating that Y1158F retains the ability to be activated and to phosphorylate its substrates. Insulin receptor and Y1158F were purified using WGA-beads, preincubated with GST-SH2B1, and subjected to in vitro kinase assays. SH2B1 stimulated insulin receptor kinase activity by approximately fivefold; however, SH2B1 was unable to stimulate Y1158F catalytic activity (Fig. 4*E*). Taken together, these data suggest that the physical interaction between the SH2 domain of SH2B1 and Tyr^{1158} in insulin receptor is required and sufficient for stimulation of insulin receptor catalytic activity.

SH2B1 protects IRS proteins against tyrosine dephosphorylation. SH2B1 directly binds to IRS-1 and IRS-2 in vitro (37), and insulin stimulated coimmunoprecipitation of SH2B1 with IRS-1 in cells (Fig. 5*A*). To determine whether this physical interaction inhibits IRS-1 dephosphorylation by phosphatases, CHO$^{IR/IRS-1}$ cells, which stably express insulin receptor and IRS-1, were stimulated with insulin to promote tyrosine phosphorylation of IRS-1. Phosphorylated IRS-1 was immunopurified, preincubated with GST or GST-SH2B1, and subjected to in vitro dephosphorylation assays. IRS-1 bound to GST-SH2B1 but not to GST (data not shown). Alkaline phosphatase dose-dependently dephosphorylated IRS-1 on tyrosines in the GST-pretreated samples; in contrast, alkaline phosphatase was unable to dephosphorylate SH2B1-bound IRS-1 (Fig. 5*B*). Insulin also promoted the association of SH2B1 with IRS-2, and SH2B1 similarly inhibited tyrosine dephosphorylation of IRS-2 (data not shown).

To determine whether SH2B1 inhibits IRS-1 dephosphorylation in cells, IRS-1 was coexpressed with PTP1B (a protein tyrosine phosphatase) in the absence or presence of SH2B1. PTP1B dephosphorylated IRS-1, and SH2B1 dose-dependently attenuated the ability of PTP1B to dephosphorylate IRS-1 (Fig. 5*C*). To determine whether SH2B1 is able to promote IRS-1 phosphorylation without stimulating insulin receptor kinase activity, Y1158F was coexpressed with SH2B1. Although SH2B1 was unable to stimulate Y1158F kinase activity (Fig. 4*E*), SH2B1 still markedly enhanced tyrosine phosphorylation of IRS-1 in Y1158F-expressing cells (Fig. 5*D*). Thus, SH2B1 is likely to augment Y1158F-mediated phosphorylation of IRS-1 by inhibiting IRS-1 dephosphorylation by endogenous protein phosphatase(s).

To determine whether the SH2B1-IRS interaction sterically inhibits the binding of IRS proteins to phosphatidylinositol 3-kinase, IRS-1 and Y1158F were coexpressed with or without SH2B1 in HEK293 cells, and IRS-1-p85 association was examined by coimmunoprecipitation assays. Insulin stimulated coimmunoprecipitation of IRS-1 with p85, the regulatory subunit of phosphatidylinositol 3-kinase; importantly, SH2B1 markedly enhanced insulin-stimulated p85 binding to IRS-1 (Fig. 5*E*). These data indicate that the SH2B1-IRS interaction does not interfere with IRS-phosphatidylinositol 3-kinase interaction but rather increases the IRS-phosphatidylinositol 3-kinase associa-

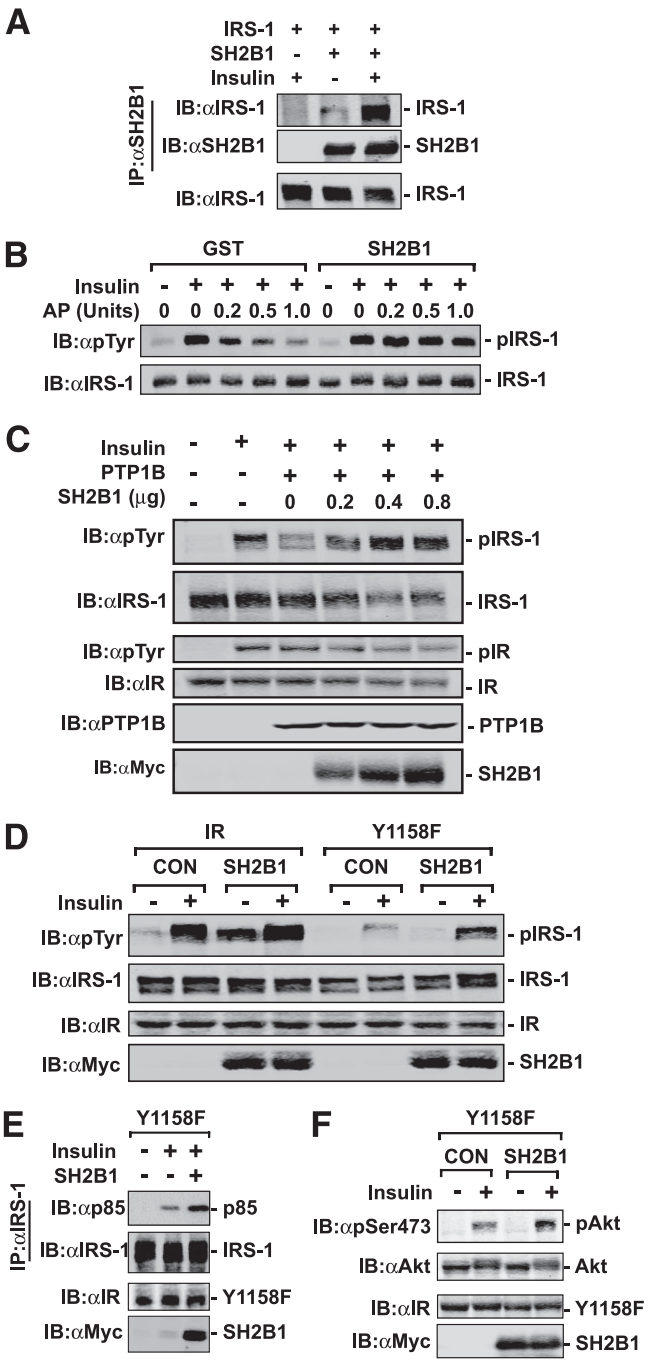

FIG. 5. SH2B1 protects IRS-1 from dephosphorylation. *A*: IRS-1 and SH2B1 were transiently expressed in HEK293 cells. Cell extracts were immunoprecipitated with αSH2B1 and immunoblotted with αIRS-1 and αSH2B1. *B*: CHO$^{IR/IRS-1}$ cells were stimulated with 100 nmol/l insulin for 10 min. IRS-1 was immunoprecipitated with αIRS-1, preincubated with GST or GST-SH2B1 (2 μg), and subjected to in vitro dephosphorylation assays with indicated amounts of alkaline phosphatase for 30 min. Reaction mixtures were immunoblotted with αpTyr and αIRS-1. *C*: Insulin receptor and IRS-1 were transiently expressed with PTP1B (0.1 μg) and increasing amounts of Myc-tagged SH2B1 (0–0.8 μg). Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies. *D*: IRS-1 was expressed with insulin receptor or Y1158F in the absence (CON) or presence of SH2B1 in HEK293 cells. Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies. *E*: IRS-1 (1 μg) and Y1158F (1 μg) plasmids were transiently cotransfected with or without SH2B1 plasmids (0.8 μg) in HEK293 cells. Cells were deprived of serum overnight 48 h after transfection and treated with 100 nmol/l insulin for 10 min. Cell extracts were immunoprecipitated with αIRS-1 and immunoblotted with αp85 and αIRS-1. Extracts were also immunoblotted with indicated antibodies. *F*: IRS-1 and Y1158F were coexpressed with or without SH2B1. Cells were treated with 100 nmol/l insulin for 10 min and extracts were immunoblotted with indicated antibodies.

tion by inhibiting IRS dephosphorylation. Consistent with these observations, SH2B1 also enhanced insulin-stimulated, Y1158F-mediated Akt phosphorylation (Fig. 5*F*). Collectively, these data suggest that, in addition to enhancing insulin receptor catalytic activity via binding to Tyr^{1158}, SH2B1 also promotes activation of the IRS protein/phosphatidylinositol 3-kinase/Akt pathway by inhibiting IRS dephosphorylation.

DISCUSSION

Insulin resistance is the primary risk factor for various metabolic diseases, including type 2 diabetes, nonalcoholic fatty liver disease, dyslipidemia, and cardiovascular disease. The molecular mechanisms underlying insulin resistance are extremely complex and not completely understood. It is commonly accepted that impairments in insulin signal transduction play a key role in the development of insulin resistance. We previously observed that insulin signaling is enhanced by SH2B1 (29). SH2B1 overexpression increases insulin receptor autophosphorylation and tyrosine phosphorylation of IRS-1 and IRS-2 in cultured cells (29,43). Similar observations were independently reported by two other groups (44,45). Additionally, we showed that genetic deletion of SH2B1 results in severe insulin resistance and type 2 diabetes in mice (29). However, SH2B1-null mice are also severely obese because of leptin resistance (30–32), raising the possibility that insulin resistance may be secondary to obesity in SH2B1-null mice. Therefore, it was unclear whether peripheral SH2B1 directly regulates insulin sensitivity in insulin target tissues in vivo.

We generated TgKO mice that express SH2B1 only in the brain but not in peripheral tissues (e.g., liver, muscle, and adipose tissue). Body weight was similar between TgKO and wild-type littermates fed HFD, consistent with our previous conclusion that neuronal SH2B1 controls energy balance and body weight by enhancing leptin sensitivity in the hypothalamus (31). In the current study, we demonstrated that loss of peripheral SH2B1 markedly impaired insulin sensitivity independent of body weight. TgKO mice developed hyperglycemia, hyperinsulinemia, and glucose intolerance to a greater extent than wild-type littermates fed HFD. The ability of exogenous insulin to reduce blood glucose and to stimulate insulin receptor autophosphorylation and phosphorylation of IRS proteins and Akt in muscle, liver, and adipose tissue was significantly reduced in TgKO mice. These results suggest that peripheral SH2B1 serves as an endogenous insulin sensitizer. Insulin signaling has been shown to be attenuated by multiple intracellular signaling molecules (e.g., PTP1B, Grb10, Grb14, SOCS1, SOCS3, JNK, PKCθ, S6K, and IKKβ) that contribute to the development of insulin resistance (6–24). Our data suggest that insulin sensitivity is controlled by a balance between these negative regulators and SH2B1 in insulin target cells.

SH2B1 promotes insulin signaling by stimulating insulin receptor catalytic activity. SH2B1 binds via its SH2 domain to phospho-Tyr^{1158} in the activation loop of insulin receptor (40,41). We showed that bacteria-derived SH2B1 markedly increased the ability of purified insulin receptor to tyrosyl phosphorylate IRS-1 in vitro. In contrast, SH2B1 was unable to stimulate the catalytic activity of Y1158F, an

insulin receptor mutant lacking the binding site for SH2B1. In cells, SH2B1 overexpression promotes insulin receptor autophosphorylation as well as insulin receptor phosphorylation of its substrates (e.g., IRS-1, IRS-2, and Shc). Conversely, deletion of endogenous SH2B1 impaired insulin stimulation of insulin receptor autophosphorylation and IRS-1 phosphorylation in primary hepatocyte cultures. Consistent with these observations, SH2B1 complexes, which are immunoprecipitated from cell extracts, reportedly promote insulin receptor autophosphorylation by reducing the K_m for ATP (45). The same report also concluded that SH2B1 dimerization was required for its stimulation of insulin receptor autophoshorylation, because treatment of cells with dimerization domain peptide mimetics inhibited insulin receptor autophosphorylation and downstream pathways (45). However, the report did not provide evidence showing that the mimetics disrupted SH2B1 dimerization. In contrast, we observed that the SH2 domain alone was sufficient to stimulate insulin receptor catalytic activity in vitro. Moreover, ΔN504, an NH_2-terminal truncated SH2B1 containing the intact SH2 domain but completely lacking both dimerization and pleckstrin homology domains, still markedly enhanced insulin-stimulated tyrosine phosphorylation of both IRS-1 and Shc. Conversely, R555E, a SH2B1 mutant with a defective SH2 domain, inhibited insulin signaling as a dominant negative mutant. These data indicate that the SH2 domain of SH2B1 is both required and sufficient to stimulate insulin receptor kinase activity. Because Tyr^{1158} phosphorylation occurs early in the activation of the insulin receptor kinase (42,46,47), binding of the SH2 domain of SH2B1 to phospho-Tyr^{1158} may stabilize insulin receptor in an active conformation. Alternatively, SH2B1-insulin receptor interaction may facilitate insulin receptor binding to its substrates.

Insulin stimulated the binding of SH2B1 to IRS-1 or IRS-2. Importantly, SH2B1 directly inhibited tyrosine dephosphorylation of IRS-1 and IRS-2 by recombinant phosphatase in vitro and by PTP1B in cultured cells. Although unable to stimulate Y1158F catalytic activity, SH2B1 still enhanced Y1158F-mediated phosphorylation of IRS-1 in cultured cells, presumably by inhibiting IRS-1 dephosphorylation by endogenous tyrosine phosphatase(s). Consistent with these observations, deletion of endogenous SH2B1 impaired tyrosine phosphorylation of IRS-1 to a greater extent than insulin receptor autophosphorylation in primary hepatocyte cultures. Together, these data suggest that the SH2B1-IRS physical interaction inhibits IRS dephosphorylation by tyrosine phosphatases. Interestingly, the SH2B1-IRS interaction did not inhibit the ability of phosphorylated IRS proteins to bind to p85, the regulatory subunit of phosphatidylinositol 3-kinase; in contrast, it enhanced insulin-stimulated IRS-p85 association and subsequent Akt phosphorylation and activation, presumably by protecting IRS proteins against dephosphorylation. These data suggest that SH2B1 does not compete with p85 for the same binding sites in IRS proteins, and that the SH2B1-IRS interaction does not sterically interfere with the IRS-p85 interaction. Therefore, the SH2B1-IRS interaction may selectively block IRS interaction with tyrosine phosphatases, thereby inhibiting IRS dephosphorylation. Alternatively, the SH2B1-IRS interaction may alter IRS conformation so that multiple tyrosine phosphorylation sites, in addition to SH2B1-bound site(s), are resistant to dephosphorylation, but still retain their ability to bind to downstream signaling molecules and activate downstream pathways including the phosphatidylinositol 3-kinase/Akt pathway.

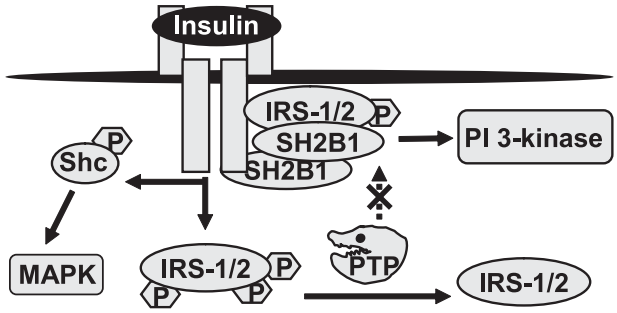

FIG. 6. A model for SH2B1 regulation of insulin signaling. In response to insulin, SH2B1 binds directly to phospho-Tyr^{1158} in insulin receptor via its SH2 domain and stimulates insulin receptor kinase activity, thereby enhancing the activation of multiple signaling pathways downstream of insulin receptor (e.g., the Shc/MAPK and the IRS/phosphatidylinositol 3-kinase pathways). SH2B1 also binds to IRS-1 or IRS-2 and inhibits their dephosphorylation on tyrosines to specifically promote the activation of IRS protein-mediated pathways. Because SH2B1 dimerizes via its dimerization domain, dimerized SH2B1 may further enhance insulin signaling by simultaneously binding to both insulin receptor and IRS-1 to stabilize active insulin receptor with IRS-1 or recruit IRS-1 to insulin receptor. PTP: protein tyrosine phosphatase; *P*: phosphate group.

In conclusion, SH2B1 appears to promote insulin sensitivity in animals by multiple mechanisms (Fig. 6). Neuronal SH2B1 increases insulin sensitivity indirectly by reducing adiposity (31). In muscle, liver, and adipose tissue, SH2B1 binds to insulin receptor and stimulates insulin receptor catalytic activity to globally activate pathways downstream of insulin receptor. SH2B1 binds to both IRS-1 and IRS-2 and protects IRS proteins from tyrosine dephosphorylation, augmenting and/or prolonging IRS protein-mediated pathways. In addition, SH2B1 forms dimers, and each SH2B1 molecule in a SH2B1 dimer may simultaneously bind to insulin receptor and IRS-1 (or IRS-2), thereby stabilizing insulin receptor/IRS-1 (or insulin receptor/IRS-2) complexes. Therefore, SH2B1 and molecules that mimic these functions of SH2B1 are potential therapeutic targets for the treatment of obesity and/or type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.

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