Insulin Signaling in Mice Expressing Reduced Levels of Syp*

(Received for publication, February 14, 1996, and in revised form, June 6, 1996)

J oanne M. Arrandale, Ann Gore-Willse, Sandra Rocks, J iang-Ming Ren, J ian Zhu, Ann Davis, J ames N. Livingston, and Daniel U. Rabin‡

From the Bayer Corporation, Pharmaceutical Division, Metabolic Disorders Research, West Haven, Connecticut 06516

Syp is a protein tyrosine phosphatase implicated in insulin and growth factor signaling. To evaluate the role of syp in insulin's regulation of plasma glucose, we generated knockout mice. Homozygous knockout mice died prior to day 10.5 of embryonic development. Hemizygous mice express half the levels of syp protein compared with their wild type littermates but do not display any gross morphological changes. Total body weight (age 2–10 weeks) and plasma insulin and glucose levels both in fasting and glucose-challenged states were comparable in the wild type and the hemizygous mice. No differences were observed in insulin-induced glucose uptake in soleus muscle and epididymal fat; insulin inhibition of lipolysis was also similar. We injected insulin into the portal vein of the mice to examine upstream events of the insulin signaling cascade. Tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) from hemizygous tissue was similar to that of wild type tissue. Association of the p85 subunit of phosphatidylinositol 3-kinase to IRS-1 increased an average of 2-fold in both groups. We did not observe an increase of IRS-1/syp association after insulin administration, but we did note a significant basal association in both wild type and hemizygous tissue. Our results do not support a major role for syp in the acute in vivo metabolic actions of insulin.

*Syp, a protein tyrosine phosphatase (PTPase), was identified by a number of groups (1–4), leading to its numerous names: syp, SHPTP2, PTP1D, and PTP2C. Syp is a nonreceptor PTPase that possesses two related Src homology 2 segments (SH2 domain) in tandem at its NH₂-terminal end, upstream from the catalytic domain. SH2 domains bind to phosphorylated residues in activated receptors or other proteins. This binding is a mechanism that allows the SH2 domains of syp to bind to a protein and allows it to hydrolyze exposed phosphotyrosines in the same protein or other nearby proteins. In addition to this docking function, Pluskey et al. (5) report that the SH2 domains may function to repress syp activity in the soluble form of the enzyme. They find that simultaneous occupancy of both SH2 domains in syp by a tethered peptide with two IRS-1-derived phosphorylation sites leads to potent stimulation (37-fold) of its phosphatase activity. Monomeric phosphopeptides stimulate activity only 9–16-fold. These studies suggest that whenever the SH2 domains bind a phosphorylated target, the enzyme's catalytic domain is exposed and the enzyme becomes active.

Syp is ubiquitously expressed (6) and has been implicated in insulin (7, 8), growth factor (9–12), and T cell receptor signaling (13). Syp associates with the platelet-derived growth factor and epidermal growth factor receptors upon ligand binding, but it does not associate with the insulin receptor (IR) upon insulin binding (7). Rather, it binds to IRS-1 through its SH2 domains. Kuhne et al. (7) have demonstrated an enhanced syp PTPTase activity with insulin, and Sun and co-workers (14) have determined that syp associates with IRS-1 at phosphotyrosine 1172, but they did not examine its activity after binding.

Recently, numerous groups (8, 11, 12, 15) have published reports that syp may play an important role in insulin action. Microinjection of a GST-fusion protein encoding both SH2 domains of syp (GST-SH2) or anti-syp antibodies inhibits the insulin induction of DNA synthesis; the percent bromodeoxyuridine incorporation in the presence of GST-SH2 or anti-syp antibodies is comparable to non-insulin-stimulated levels (9, 12). Microinjection of the above proteins has no effect on serum-induced DNA synthesis. In similar microinjection studies, GST-SH2 and anti-syp antibodies inhibit the insulin-stimulated rise in expression of GLUT1 protein (12). In addition, when catalytically inactive syp (C459S) is expressed in NIH3T3 cells (11) or Chinese hamster ovary IR-overexpressing cells (8), the insulin-dependent tyrosine phosphorylation and activation of mitogen-activated protein kinase (MAP) are attenuated, whereas tyrosine phosphorylation of IRS-1 and Shc is unaffected (11). Effects of the mutant appear to be specific to insulin signaling since it had no effect on 12-O-tetradecanoylphorbol-13-acetate activation of MAP kinase (8). This same mutant also blocks insulin-induced activation of MAP/ERK andraf-1 kinase but not of p21ras (14) and does not inhibit the binding of Grb2 to IRS-1 or the formation of a Shc-Grb2 complex (8), suggesting that syp acts on proteins immediately downstream of p21ras. Together, these studies favor syp as being an important positive mediator of insulin signal transduction.

Although some progress has been made identifying the functions of syp in insulin and growth factor signaling, all of the studies have been performed in vitro and in cell cultures. For this reason, we were interested in studying syp in whole animal models. We generated syp knockout mice and examined several actions of insulin. Homozygous animals were not viable and died by day 10.5 of embryonic development. Hemizygous mice, however, express half the normal amount of syp protein in all organs examined and were used for subsequent experiments. Here we show that animals expressing reduced levels of syp retain their acute metabolic response to insulin.

21353
EXPERIMENTAL PROCEDURES
Genomic Screening and Vector Construction

The mouse syp gene was cloned from a mouse genomic library 129SVJ (Stratagene) using the entire coding region of human SHPTP2 cDNA as a probe. Low stringency and nonradioactive Genius"™ (Boehringer Mannheim) hybridization techniques were utilized (16). Four overlapping 5' clones spanning 26 kb and two overlapping 3' clones spanning 20 kb were obtained. All six clones were subcloned to Blue-script SK* (Stratagene) and analyzed by restriction analysis, Southern analysis, and sequencing.

The knockout cassette was constructed as follows. First, a 4-kb NotI-SpeI fragment was subcloned from a 5' genomic clone to a neomycin cassette containing the neomycin resistance gene driven by the pPol II long promoter so that neomycin was positioned in the antisense orientation. A 4.5-kb BamHI fragment was subcloned from a genomic clone to pUC19. A 2.1-kb XhoI fragment containing the thymidine kinase gene driven by the MCI promoter was subcloned to a single XhoI site in the BamHI fragment. A 6.6-kb SalI fragment containing the 3'-flanking region and thymidine kinase resistance gene was subcloned to the BSSS-XhoI site. A 5.8-kb SalI fragment containing the 5'-flanking region and the neomycin resistance gene was subcloned to the SalI site on this last vector.

Generation of Syp Mutant ES Cell Lines

The G1 cell line2 was grown according to Robertson (17). Twenty-five μg of the targeting plasmid, linearized with NodI, was transfected into 1 x 10^7 G1 cells in 5 ml of phosphate-buffered saline and actinomycin (Bio-Rad) at 230 volts/500 microfarads. The cells were plated on 21354 mM Tris, pH 7.5, 50 mM EDTA, 100 mM NaCl, 5 mM dithiothreitol, 0.5 mM ethanol, the DNA was dissolved in 10 mM Tris, pH 7.4, 1 mM EDTA, and prehybridized for several hours at 65°C in hybridization solution containing 270 μg/ml G418 and 0.2 μM 5-FIAU. At 10 days, 400 individual colonies were picked and grown in 96-well plates with feeder cells and no selection. After reaching confluence, the cells were split. One plate was frozen for future recovery, and the other was used to prepare DNA for mini-Southern analysis (18).

Generation of Mouse Carrying the Mutated Allele

Blastocysts were harvested from C57BL/7 mice approximately 3.5 days postcoitus. These were injected with targeted ES cells as described (19, 20) under a Zeiss Axiovert 135 and Narishige micromanipulators. Injected blastocysts were implanted into the uteri of pseudopregnant ICR females. Chimeric progeny were identified by coat color, and male chimeras were mated to Black Swiss females (Taconic). Progeny with agouti coat color were examined for transmission of the targeted allele by Southern blotting of tail DNA (see below). Chimeras were bred to 129Svev females to maintain inbred breeding stocks.

Southern Analysis of DNA from ES Cells and Mouse Tails

ES cells were pelleted and rinsed twice with phosphate-buffered saline. Cells were lysed overnight at 55°C in 50 mM Tris, pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, and 0.8 mg/ml proteinase K (Boehringer Mannheim). Tails from 10- to 14-day-old mice were cut approximately 1 cm from the tips and were incubated overnight at 65°C in 50 mM Tris, pH 7.5, 50 mM EDTA, 100 mM NaCl, 5 mM dithiothreitol, 0.5 mM spermidine, 1% SDS, and 0.6 mg/ml proteinase K DNA. ES cells and tails were precipitated with an equal volume of isopropyl alcohol and spotted onto a flame-sealed microcassette. After rinsing in ethanol, the DNA was dissolved in 10 mM Tris, pH 7.4, 1 mM EDTA and subjected to restriction enzyme digestion followed by electrophoresis on 0.7% agarose gels.

Gels were pretreated and transferred overnight as described (18). Membranes were cross-linked in a UV Stratalinker 2400 (Stratagene) and prehybridized for several hours at 65°C in hybridization solution (1.5 x SSPE, 1% SDS, 0.5% nonfat dry milk, and 0.2 mg/ml denatured salmon sperm DNA). Hybridizations were performed overnight at 65°C in fresh hybridization solution containing the radiolabeled probe (labeled using T7 QuickPrime Kit from Pharmacia). The following day, membranes were washed three times in 2 x SSC, 0.1% SDS (twice for 5 min at room temperature and once at 65°C for 30 min).

Plasma Insulin and Glucose Levels

Seven-week-old male mice were fasted 18 h before being bled via the retro-orbital sinus using a heparinized capillary tube. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments), and plasma insulin was measured by double antibody radioimmunoassay using rat insulin as standard (Insulin RIA Kit, Linco Research).

Oral glucose tolerance tests were done on fasted 8-week-old mice. Glucose (1 g/kg of body weight) was delivered as a 10% solution by oral gavage. Blood was sampled after 30 min and analyzed for glucose and insulin levels.

Glucose Transport Activity

Soleus Muscle—Soleus muscles were isolated from 8-week-old male mice. Muscles were placed in 2 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB) including 8 mM glucose, 32 mM mannitol, 0.1% radioimmunoassay grade bovine serum albumin, and 2.5 mM insulin and incubated with shaking at 35°C for 30 min. Control muscles were incubated under the same conditions without insulin. The gas phase in the flasks was 95% O2, 5% CO2.

After the initial incubation period, the muscles were transferred to flasks containing 2 ml of KHB with 40 mM mannitol and incubated with shaking for 30 min at 29°C to remove glucose. Glucose transport activity was measured with the glucose analog 2-deoxy-D-glucose as described previously (21). Briefly, the muscles were incubated for 20 min at 29°C in 1.5 ml of KHB containing 1 mM 2-deoxy-[1,2-3H]glucose (190 μCi/mmol, DuPont NEN) and 39 mM [U-14C]mannitol (3.9 μCi/mmol, DuPont NEN), and if insulin was present in the previous incubations, it was also included during the rinse and final incubation. Adipocytes—Adipocytes from epididymal fat were isolated from 7- to 8-week-old male mice by collagenase treatment as described previously (22). To measure glucose uptake, fat cells (2 x 10^6 cells/ml) were incubated in the presence of varying insulin concentrations (0–16 ng/ml, 0.1 mM d-glucose, and 0.1 μCi of [3H]glucose for 2 h at 37°C. Toluene-based scintillation mixture was added directly to the cells. The mixture was vortexed and the 3H-labeled lipid was allowed to separate into the scintillant phase. Samples were then counted.

Antilipolysis

Adipocytes from epididymal fat were isolated from 8- to 10-week-old mice as described above. Lipolysis was initiated with the addition of 10 μM forskolin to cells to achieve maximal effects. Increasing concentrations of insulin (0–3.0 ng/ml) were added to the cells which were incubated at 37°C for 30 min. Levels of glycerol, a product of triglyceride hydrolysis released into the media, were measured in a colorimetric assay to assess the extent of lipolysis. This involved the addition of triglyceride (GDO-Trinder) reagent A (Sigma) to an aliquot of above cells and measuring the optical density at 540 nm.

Portal Vein Injections

Male mice were anesthetized with a solution containing 10 mg/ml ketamine and 1 mg/ml xylazine at 0.15 mg/10 g of mouse body weight. Twenty μg of insulin (Sigma) or vehicle was injected into the portal vein of mice. After 5 min, tissues were dissected and frozen in liquid N2.

Tissue extracts (0.5 mg of tissue/ml of extraction buffer) were prepared by homogenization in 150 mM NaCl; 10 mM Tris, pH 8.0; 1 mM EDTA; 1% Triton X-100; 20 mM sodium azide; 25 mM benazamide; 1 mM phenylmethylsulfonyl fluoride; 5 mM pepsinogen; 25 μg/ml each of pepstatin, leupeptin, antipain, and chymostatin. Extracts were chilled on ice for approximately 1 h prior to centrifugation.

Immunoprecipitations and Western Blots

Total protein concentration in the tissue extracts was determined using the BCA protein assay reagent (Pierce). Two to 5 mg of total protein was used for immunoprecipitations with the following antibodies: rabbit anti-iR1-1 antiseraum2 or mouse anti-phosphotyrosine (pY70, Transduction Laboratories). Immunocomplexes were precipitated with protein G-Sepharose 4 Fast Flow (Pharmacia) and washed three times in Tris-buffered saline. Proteins were eluted with 2 x loading buffer (100 mM Tris, pH 6.8, 2% β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol, final) and boiled for 5 min. Proteins were resolved in precast 10–20% Tris-Tricine gradient gels (Novex). For Western blotting, proteins were transferred onto polyvinylidene difluoride filter paper and incubated with primary antibodies: mouse anti-SHP2 (Transduction Laboratories), rabbit anti-insulin receptor (aRIR, Ref. 24), rabbit anti-phosphotyrosine (Upstate Biotechnology), and rabbit anti-iR1-1 antiseraum (Bayer). Antigens were visualized with alkaline phosphatase-conjugated secondary antibodies (DAKO) and 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (Kirkegaard and Perry Laboratories Inc.).

2 Bayer Corp., unpublished data.

Downloaded from http://www.jbc.org/ by guest on July 21, 2018
Correct targeting of the neo replacement sequence to the syp gene in ES cells was assessed by mini-Southern analyses using genomic sequences of syp (3 kb) through homologous recombination. Exon 2 and flanking intron sequences of syp were replaced with the sequences of neomycin (1.8 kb) replaced exon 2 and flanking intron. The length of the intron between exons 1 and 2 was estimated by polymerase chain reaction to be ~10 kb. Panel B, the table outlines the expected size bands for the digests and the probes used to identify correctly targeted ES cells (probes A and B) and genotype mice (probes C). The star symbol indicates that the probe hybridizes to the neo sequence that is not found in ES cells. The plus sign indicates hemizygous mice having two bands at 6.4 and 7.5 kb.

**RESULTS AND DISCUSSION**

Exon 2 and flanking intron sequences of syp were replaced with the sequences of neomycin to knockout an allele of syp. Correct targeting of the neo replacement sequence to the syp gene in ES cells was assessed by mini-Southern analyses using two different probes (Fig. 1A). Probe A hybridizes outside the area of homologous recombination to exon 4. When DNA is digested with KpnI, probe A will hybridize to two bands (13.2 and 16.4 kb) in correctly targeted clones and a single band (13.2 kb) in wild type clones (Fig. 1B). Probe B hybridizes inside the area of homologous recombination to the neo sequence. When DNA is digested with SspI, this probe identifies a single band at 6.3 kb in targeted clones. Mini-Southern analyses using these probes identified properly targeted ES cell clones (Fig. 2A). Genotypes of mice were determined by mini-Southern analysis of tail DNA using restriction enzyme SpeI and probe C (Fig. 1A). Bands at 7.5 and 6.4 kb are diagnostic of wild type and knockout alleles, respectively (Fig. 1B). Illustrative mini-Southern blots are presented in Fig. 2B.

Eighty-five pups were born from 10 hemizygous breeding pairs. Thirty-nine percent were wild type, 61% were hemizygous, but none was homozygous. Litter size from our hemizygous breeding pairs averaged 6.2 ± 2.0, compared with 10.8 ± 2.0 for hemizygous male × wild type female crosses. Examination of embryos at days 10.5 (n = 39) and 12.5 (n = 24) revealed that homozygous embryos die prior to day 10.5 of development. At this stage, 44% of the embryos were resorbed; all viable embryos were either wild type or hemizygous (data not shown). Enough DNA was isolated from one resorbed embryo to permit genotyping, and it was found to be homozygous. This is consistent with the observations of Tang and co-workers (10) who found that injection of a dominant mutant of syp RNA into one or two-cell stage Xenopus embryos resulted in severe posterior truncations.

The cause of homozygote embryonal death is not known. Syp is expressed in ES cells, which are representative of day 5 embryos. Several syp interacting receptors (epidermal growth factor receptor (25), platelet-derived growth factor receptor (26) and endothelial receptor kinase TEK (27)) are diversely expressed in the embryo, but how these molecules, others, or combinations thereof are involved is not clear.

Hemizygous animals did not display any gross alterations of phenotype compared with wild type littermates. Body weight between age 10–70 days of both hemizygous and wild type males and females also did not differ. Necropsy and histological studies were unremarkable.

It was possible that syp hemizygotes were compensating and producing normal levels of syp protein. This was examined by Western blotting. Fig. 3 compares syp levels in brain, fat, heart, kidney, liver, skeletal muscle, and thymus in hemizygous and wild type mice. Syp levels were decreased by 40–50% in all hemizygous tissue examined. Similar results were obtained when a rabbit anti-syp antibody against the carboxyl terminus of syp was used (not shown). Reductions of syp were also observed in lung and spleen (46 and 39%, respectively). Thus, despite their normal appearance the syp hemizygote mice expressed half the normal amount of the enzyme.

A series of metabolic parameters was examined to see if reducing syp altered metabolic insulin signaling. Fasting plasma insulin and plasma glucose levels were similar in wild type and hemizygous animals (Table I). There was no significant difference between these levels 30 min after an oral glucose challenge. It was possible that there were tissue differences in meta-
bolic insulin action, so muscle and adipose tissue were tested. Insulin-stimulated 2-deoxyglucose uptake was assayed in isolated soleus muscle. Insulin at 2.5 ng/ml increased glucose uptake 2.5-fold and 2.8-fold in wild type and hemizygous muscles, respectively. Both basal and stimulated levels of 2-deoxyglucose uptake were comparable between the two groups (Fig. 4).

Insulin-stimulated glucose uptake was also measured in adipocytes isolated from epididymal fat pads of 7–8-week-old male mice. Stimulated glucose uptake in wild type and hemizygous adipocytes was not significantly different over a range of insulin concentrations (Fig. 5). Basal cpm of $[^{3}H]$glucose transport in wild type and hemizygous adipocytes averaged 958 and 955 cpm, respectively (four experiments). The $ED_{50}$ for insulin stimulation was $0.77 \pm 0.18$ ng/ml for wild type and $0.52 \pm 0.2$ ng/ml for hemizygotes. At a maximal insulin concentration of 16 ng/ml, insulin induced a $5.2 \pm 1.8$ ($n = 4$)-fold increase in uptake in wild type adipocytes and a $4.2 \pm 0.9$-fold increase in uptake in hemizygous adipocytes.

**Table I**

|                | Body weight | Plasma glucose (mg/dl) | Plasma insulin (ng/ml) |
|----------------|-------------|------------------------|------------------------|
| **Wild type**  |             |                        |                        |
| Basal          | 26.1 ± 2.1  | 111 ± 18               | 1.44 ± 1.14            |
| OGGTT          | 27.6 ± 3.0  | 198 ± 9*               | 0.64 ± 0.22            |
| **Hemizygous** |             |                        |                        |
| Basal          | 27.6 ± 3.0  | 114 ± 18               | 1.03 ± 0.99            |
| OGGTT          | 224 ± 17*   | 0.65 ± 0.15            |                        |

**Fig. 3.** Syp levels in hemizygous and wild type mice. Six μg of total protein from organ extracts was loaded onto each lane of a 10–20% Tricine gradient gel. Proteins were transferred onto polyvinylidene difluoride filter paper, incubated with anti-SHPTP2 antibodies (1:500), and visualized with alkaline phosphatase-conjugated secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Panel A, Western blot. Lanes 1, wild type; lanes 2, hemizygous. Panel B, a Western blot from panel A was quantitated by reflectance optical densitometry using a Bio-Rad GS-670 densitometer.

**Fig. 4.** Insulin-stimulated glucose uptake in soleus muscle. Soleus muscles were isolated from 8-week-old male mice and incubated in the presence or absence of 2.5 ng/ml insulin as described under “Experimental Procedures.” $n$ indicates the number of individual muscles per condition; $T$ bars show the S.E.
hemizygous adipocytes (not significant \( p < 0.05 \)).

In addition to inducing glucose uptake in muscle and fat, insulin inhibits lipolysis in fat. Maximal lipolysis in adipocytes was initiated with 10 \( \mu M \) forskolin, and concentrations of insulin up to 3.0 ng/ml were added to inhibit fat breakdown. As shown in Fig. 6, insulin inhibits glycerol release in both wild type and hemizygous adipocytes to the same extent. Similar maximal levels of glycerol release in wild types and hemizygotes were observed with 10 \( \mu M \) forskolin.

To summarize the metabolic effects of reducing syp levels in whole animals and primary tissue, we observed no difference in wild type and hemizygous syp mice in terms of body weight, plasma insulin, and glucose levels during fasting or after a glucose challenge, insulin-stimulated glucose uptake in soleus muscle and adipocytes, and insulin-inhibited lipolysis in adipocytes. Because of the considerable literature implicating syp in the insulin signaling cascade, we examined wild type and hemizygous mice for IR and IRS-1 phosphorylation, and IRS-1 association in response to insulin. Wild type and hemizygous male mice received an injection of 20 \( \mu g \) of insulin or vehicle into the portal vein. After a 5-min stimulation, tissues were removed and processed as described above. Total protein concentration was determined, and equal protein amounts from each sample were used for immunoprecipitations. IRS-1 and tyrosyl-phosphorylated proteins were immunoprecipitated from the tissue extracts and immunoblotted with antibodies against syp, IR, IRS-1, or the p85 subunit of phosphatidylinositol 3-kinase. Results for skeletal muscle are presented in Fig. 7.

Insulin reached the muscle, bound to the IR, and initiated the signaling cascade since IR and IRS-1 tyrosine phosphorylation increased in response to the hormone. The extent of phosphorylation on the IR and IRS-1 was similar between the wild type and hemizygous tissue (Fig. 7A). An association of p85 with IRS-1 was also observed in the mouse muscle tissue; both wild type and hemizygous mice showed an average of a 2-fold increase in the association (Fig. 7B).

Having established that the total levels of syp are reduced in the hemizygotes but that insulin metabolic signaling is normal, it was necessary to determine whether the association of syp with IRS-1 is altered under basal and insulin-stimulated conditions. If the association is the same in the hemizygous and wild type animals, it could be concluded that there is an excess of syp for insulin signaling through IRS-1, and the hemizygotes shed no light on the involvement of syp in the insulin signaling cascade. If, on the other hand, the syp/IRS-1 association is reduced, the role of syp as an important player in insulin metabolic signaling would be in question. We found that levels of syp precipitated with IRS-1 antibodies were reduced in hemizygous tissue compared with wild type tissue, indicating that less syp associated with IRS-1 in the hemizygotes compared with the wild types (Fig. 7B). Equal levels of IRS-1 were immunoprecipitated from each of the extracts and were not affected by acute insulin stimulation.

Previous reports (7, 8) showed undetectable basal association of syp/IRS-1 and massive stimulation of the association by insulin. Syp and IRS-1 were associated under basal conditions, without exogenous insulin stimulation, and we did not observe a significant elevation of syp/IRS-1 association in response to insulin (Fig. 7B). Our finding may reflect the presence of low plasma insulin and basal phosphorylation of IRS-1 in the mice at the onset of the experiment rather than the insulin-starved conditions possible in cell culture experiments. The animals, however, responded well to the injected insulin as seen by the large increases of IR and IRS-1 tyrosine phosphorylation.
There was also a 2-fold increase of IRS-1/p85 association in response to insulin (Fig. 7B), in agreement with other reports (28). This shows that injection of insulin into the portal vein is initiating signaling. Nevertheless, we did not observe the induction of syp/IRS-1 association by insulin.

These insulin injection experiments demonstrate that (a) although insulin stimulated IR and IRS-1 phosphorylation and p85 binding to IRS-1, syp association to IRS-1 is not affected in wild type or hemizygous mice; (b) IRS-1 is tyrosine phosphorylated in the basal state, and syp binds to it; and (c) less syp binds to IRS-1 in the hemizygotes than in the wild type muscle. No difference in the stimulated tyrosine phosphorylation of IRS-1 does not support the predictions of Kühne et al. (29) that syp is the PTPase responsible for the dephosphorylation of IRS-1 in vivo, but is consistent with another study by the same group (30) where they overexpressed normal syp and a catalytically inactive syp in kidney 293 cells. Here they found no differences among the cell lines in the extent of insulin-induced tyrosine phosphorylation of 160- and 100-kDa proteins, or the rate of their dephosphorylation upon insulin withdrawal. It is possible, however, that intracellular segregation of syp with these signaling molecules could overcome the overall protein deficiency of the hemizygotes, or more than a 50% reduction is needed to see any differences in phosphorylation.

The data presented in this study do not support syp as a significant factor in the metabolic action of insulin. If syp binds to IRS-1 rapidly and if the complex concentration in the hemizygotes is significantly greater than the threshold required for signaling, it is formally possible that the experiments presented in this study would not detect altered insulin signaling with reduced syp. However, one would expect that the level of complex we observe would lead to changes in the metabolic parameters if the complex were pivotal for insulin metabolic signaling. Although some studies suggest that the association of syp and IRS-1 is a critical step in insulin signal transduction, Yamauchi et al. (31) discovered that only a small percentage of phosphorylated IRS-1 (4%) is associated with syp, and only 2% of total syp associates with IRS-1. They also showed that the major syp-binding protein in response to insulin in Chinese hamster ovary cells overexpressing IR is pp115, pp115 may be a substrate for syp since expression of catalytically inactive syp results in a decrease of common precipitated tyrosine-phosphorylated pp115.

Milarski and Saltiel (11) described similar observations when overexpressing catalytically inactive syp in NIH3T3 cells: the major syp-binding protein was a 120-kDa protein (pp120). The identity of pp115 and pp120 and their relevance to signal transduction are currently unknown; however, a 125-kDa focal adhesion kinase has been shown to be a substrate for syp upon insulin stimulation (32).

In contrast to the acute metabolic effects of insulin, evidence for the importance of syp in the mitogenic actions of insulin is growing. Milarski and Saltiel (11) have reported that overexpression of catalytically inactive syp attenuates the insulin-dependent tyrosine phosphorylation and activation of MAP kinase. Similarly, Xiao et al. (9) have observed a dramatic decrease in insulin-induced bromodeoxyuridine incorporation following microinjection of a syp antibody or syp GST-SH2 fusion protein in rat fibroblasts, and Noguchi et al. (8) concluded from their studies that syp regulates an upstream element necessary for insulin-induced ras activation. Furthermore, Hausdorff and co-workers (12) showed in 3T3L1 adipocytes that syp is not required for insulin-stimulated GLUT4 translocation, but is necessary for insulin-stimulated expression of GLUT1. These studies point to syp as a positive mediator of insulin-induced mitogenesis.

In conclusion, our hemizygous syp mice that have half the normal levels of syp have normal acute insulin metabolic effects. Fasting plasma insulin and glucose levels, oral glucose tolerance, insulin-stimulated glucose uptake in fat and muscle, as well as antilipolysis in adipocytes all appear normal. Efforts are under way to use the hemizygous mice to study insulin-stimulated mitogenesis in whole animal models.

Acknowledgments—We thank Lynn Lemoine and Laurel Sweet for the generous supply of anti-IRS-1 antisera. Jeffrey Shapiro for providing the SHPTP2 cDNA probe, Margit MacDougall for performing the portal vein injections, and Mary Tyler for transfecting and growing the embryonic stem cell clones. Yaxin Li assisted with the muscle experiment and the oral glucose tolerance test.

REFERENCES
1. Feng, G.-S., Hu, C.-C., and Pawson, T. (1993) Science 259, 1607–1611
2. Freeman, R. M., Jr., Plutzsky, J., and Neel, B. G. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11239–11243
3. Vogel, W., Lammers, R., Huang, J., and Ulrich, A. (1993) Science 259, 1611–1614
4. Ahmad, S., Banville, D., Zhao, Z., Fischer, E. H., and Shen, S.-H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2137–2141
5. Pluskey, S., Wandlest, T. J., Walsh, C. T., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 2897–2900
6. Bastien, L., Ramachandran, C., Liu, S., and Adam, M. (1993) Biochem. Biophys. Res. Commun. 196, 124–133
7. Kuhné, M. R., Pawson, T., Lienhard, G. E., and Feng, G.-S. (1993) J. Biol. Chem. 268, 11479–11483
8. Noguchi, T., Matzaki, T., Harita, K., Fugikusa, Y., and Kasuga, M. (1994) Mol. Cell. Biol. 10, 6674–6682
9. Xiao, S., Ross, D. W., Sasaoka, T., Maegawa, H., Burke, T. R., Jr., Roller, P. P., Shoelson, S. E., and Dietsky, J. M. (1994) J. Biol. Chem. 269, 21244–21248
10. Tang, T. L., Freeman, R. M., Jr., O’Reilly, A. M., Neel, B. G., and Sokol, S. Y. (1995) Cell 80, 473–483
11. Milarski, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21239–21243
12. Hausdorff, S. F., Bennett, A. M., Neel, B. G., and Birnbaum, M. J. (1995) J. Biol. Chem. 270, 12965–12968
13. Marengère, L. E. M., Waterhouse, P., Duncan, G. S., Mittrucker, H.-W., Feng, G.-S., and Mak, T. W. (1996) Science 272, 1170–1173
14. Sun, X.-J., Crimmins, D. L., Myers, M. G., Miralpeix, M., and White, M. F. (1993) Mol. Cell. Biol. 13, 7418–7428
15. Sawada, T., Milarski, K. L., and Saltiel, A. R. (1995) Biochem. Biophys. Res. Commun. 214, 737–743
16. Yoo-Warrren, H., Gore-Willsie, A., Hancock, N., Hull, J., McCaleb, M., and Livingston, J. N. (1994) Biochem. Biophys. Res. Commun. 205, 347–353
17. Robertson, E. J., Jr. (ed) (1987) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, pp. 19–49, IRL Press, Oxford
18. Ramirez-Solis, R., Rivera-Perez, J., Wallace, J. D., Wims, M., Zheng, H., and Bradley, A. (1992) Anal. Biochem. 201, 331–335
19. Robinson, C. B., Book, M., and Locasale, J. W. (1995) J. Biol. Chem. 270, 12965–12968
20. Wang, R., Mclachlan, M., and Lienhard, G. E. (1994) J. Biol. Chem. 269, 17483–17486
21. Kuhné, M. R., Zhao, Z., Rowsle, J., Lavan, B. E., Shen, S.-H., Fischer, E. H., and Lienhard, G. E. (1994) J. Biol. Chem. 269, 15336–15337
22. Kuhné, M. R., Zhao, Z., and Lienhard, G. E. (1996) Biochem. Biophys. Res. Commun. 211, 190–197
23. Yamauchi, K., Ribon, V., Saltiel, A. R., and Pessin, J. E. (1995) J. Biol. Chem. 270, 17716–17722
24. Yamauchi, K., Milarski, K. L., Saltiel, A. R., and Pessin, J. E. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 664–668
Insulin Signaling in Mice Expressing Reduced Levels of Syp
Joanne M. Arrandale, Ann Gore-Willse, Sandra Rocks, Jiang-Ming Ren, Jian Zhu, Ann Davis, James N. Livingston and Daniel U. Rabin

J. Biol. Chem. 1996, 271:21353-21358.
doi: 10.1074/jbc.271.35.21353

Access the most updated version of this article at http://www.jbc.org/content/271/35/21353

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 20 of which can be accessed free at http://www.jbc.org/content/271/35/21353.full.html#ref-list-1