Identification of sirm, a Novel Insulin-regulated SH3 Binding Protein That Associates with Grb-2 and FYN*

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We have previously developed a mouse model of insulin-resistant diabetes by targeted inactivation of the insulin receptor gene. During studies of gene expression in livers of insulin receptor-deficient mice, we identified a novel cDNA, which we have termed sirm (Son of Insulin Receptor Mutant mice). sirm is largely, albeit not exclusively, expressed in insulin-responsive tissues. Insulin is a potent modulator of sirm expression, and sirm mRNA levels correlate with tissue sensitivity to insulin. The product of the sirm gene is a serine/threonine-rich protein with several proline-rich motifs and an NPXY motif, conforming to the consensus sequence recognized by the phosphotyrosine binding domains of insulin receptor substrate and Shc proteins. However, Sirm bears no extended homologies with other known proteins. Based on the sequences of the proline-rich domains, we sought to determine whether Sirm binds to the SH3 domains of FYN and Grb-2. We demonstrate here that Sirm binds to FYN and Grb-2 in 3T3-L1 adipocytes and that insulin treatment results in the dissociation of the Sirm-FYN and Sirm-Grb-2 complexes. We also show that Sirm is a substrate for the kinase activity of FYN in vitro. Based on the patterns of expression of sirm, its regulation by insulin, and the interactions with molecules in the insulin signaling pathway, we surmise that Sirm plays a role in modulating tissue sensitivity to insulin.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U59739.

The abbreviations used are: ir, insulin receptor; Grb-2, growth factor receptor binding protein-2; BSA, bovine serum albumin; SH3, src homology 3 domain; IGF-1, insulin-like growth factor type 1; IRS, insulin receptor substrate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; EST, expressed sequence tags; nt, nucleotide(s).

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‡ The abbreviations used are: ir, insulin receptor; Grb-2, growth factor receptor binding protein-2; BSA, bovine serum albumin; SH3, src homology 3 domain; IGF-1, insulin-like growth factor type 1; IRS, insulin receptor substrate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; EST, expressed sequence tags; nt, nucleotide(s).
incubated for 20 min. Three additional washes were performed in PBS/Tween/Triton, followed by detection of chemiluminescence using ECL (Amersham Corp.).

RNA Analysis—Total RNA was isolated from mouse livers and hind limb skeletal muscles or from SV40-transformed hepatocytes using a guanidinium isothiocyanate/acid phenol extraction procedure. Reverse transcription-PCR was performed as described previously. PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min for 30 cycles. PCR products were analyzed by gel electrophoresis. For Northern analysis, poly(A)⁺-enriched RNA was obtained by oligo-(dT) affinity chromatography. 2–3 μg of poly(A)⁺ RNA were size-fractionated on denaturing 1% agarose, 2.2 M formaldehyde gels and transferred to nitrocellulose membranes. The filters were probed with random-primed sirm cDNA. Ad-

Fig. 1. A, alignment of mouse IGF-1 receptor and sirm sequences. This figure shows the partial homology between the primers used to screen for IGF-1 receptor-related sequences and the sirm sequence. There is 66 and 57% identity between the upstream and downstream IGF-1 receptor primers and the relevant sirm sequences. Of note is also the virtual identity (10 out of 11 matches) at the 3' end of the upstream primer. B, schematic diagram of the Sirm protein and cloning strategy. Represented from top to bottom: *, a diagram of the Sirm protein with potential tyrosine phosphorylation sites; ‡, proline-directed serine phosphorylation sites; vertical bars, proline-rich sequences; a diagram of the GST-Sirm used for in vitro phosphorylation assays; the location of the peptide used to raise antibodies; a diagram of the cDNA clone, horizontal bar, the sequence of which is shown in C, with the location of the coding sequence; the location of Marathon cDNA clones used to obtain the 5' and 3' end of the coding sequence; the cDNAs isolated from library screening and RT-PCR; the original sirm PCR product, with the primers used to identify sirm; and the in vitro translation product of the sirm cDNA. The sequence of the PCR primers is reported in A. The sequence NPNY (amino acids 34–37) is indicated above the bar. C, sequence of mouse sirm complementary DNA. A consensus sequence was developed using the Autoassembler software from a combination of cloned cDNA clones and amplified products from Marathon-ready cDNA. The translation start and stop codons, the putative polyadenylation signals, and some of the major structural motifs are indicated in bold.
Additionally, a mouse multiple tissue, an embryonic Northern blot, as well as a zoo blot (CLONTECH, Palo Alto, CA) were used to confirm the identity of the various clones isolated, to study the distribution of the sirm transcripts, and to analyze the presence of sirm gene homologues in other species. Hybridization was performed according to standard procedures.

Cloning of Full-length Sirm cDNA—Initially, a random primed sirm fragment obtained by RT-PCR amplification of ir2/2 liver RNA was used to probe 10^6 plaques of mouse embryo and mouse fetal heart cDNA libraries (CLONTECH). Hybridization and plaque purification of individual positive clones were carried out according to standard methods. However, only partial cDNA clones could be isolated from these libraries. To isolate full-length clones, mouse heart Marathon-ready cDNA (CLONTECH) was amplified using primers derived from the 5' and 3' end of the available sequence. Amplification was carried out as suggested by the manufacturer, with a mixture of Klenow and Taq polymerase. Amplified products were subcloned into a PCR II vector (Invitrogen, Carlsbad, CA) and sequenced using cycle sequencing with fluorescent dideoxy terminators (Applied Biosystems, Foster City, CA) on 373 and 377 Applied Biosystems sequencers. Each partial clone was hybridized with multiple tissue Northern blots as well as Northern blots of ir2/2 mice to confirm that amplified sequences derived from the same original transcripts. Multiple subcloned fragments were analyzed to confirm the nucleotide sequence. Sequence alignment was carried out using computer software, and the results were compared with other known sequences in the literature.

*Fig. 1—continued*
using the Autoassembler software (Applied Biosystems) to develop a
consensus sequence. Ambiguities were further resolved by visual in-
spection of electropherograms.

Expression of GST-sirm Fusion Products—An 1800-nt fragment of
the mouse sirm cDNA was amplified from Marathon-ready mouse heart
cDNA. The upstream primer 5′-GAA TTC TCC AGC TGG CAC
ATG AAC AAC AGT3′ (nt 949–972) contained an additional
EcoRI site in frame with the ATG initiation codon (bold face). The downstream
primer spanned nt 2924–2901. The amplified product was digested
with EcoRI to generate a 1590-nt fragment, which was subsequently
subcloned into the EcoRI site of pGEX2T (Pharmacia Biotech Inc.). The
amplified fragment was entirely sequenced to rule out nucleotide sub-
stitutions during the amplification procedure. Bacterial cultures were
obtained as indicated by the manufacturer, and the recombinant GST-
Sirm fusion protein was isolated by affinity chromatography using
glutathione-Sepharose beads.

Tissue Isolation—Whole E13.5 and 18.5 mouse embryos, or livers,
hind limb muscles, hearts, epididymal fat pads, and kidneys derived
from adult mice were finely minced and homogenized using a glass-
glass pestle in 1 mM NaHCO3 containing a mixture of protease inhibi-
tors (Boehringer Mannheim). 20 μg of whole tissue extracts were pre-
pared for SDS-gel electrophoresis as described (2).

Subcellular Fractionation—In some experiments, subcellular frac-
tions were obtained from cultures of 3T3-L1 cells or from mouse skeletal
muscle. After resuspending the cells or tissue in 1 mM NaHCO3 buffer
as described above, nuclei were removed by a low speed centrifugation
(1000 × g) for 10 min, followed by high speed centrifugation (20,000 ×
g) to pellet the microsomal fraction and separate the cytosol. The crude
microsomal homogenate was solubilized in 50 mM HEPES (pH 7.6) 150
mM NaCl containing 1% Triton X-100 and protease inhibitors, followed
by centrifugation at 100,000 × g to remove the insoluble material.

Insulin Stimulation of 3T3-L1 Cells and SV40-transformed Hepato-
cytes—3T3-L1 cells were differentiated as described (5). Cultures of
differentiated 3T3-L1 cells or confluent SV40-transformed hepatocytes
derived from ir−/− mice or from ir+/− mice were grown in 100-mm Petri
dishes as described. Following incubation overnight in 1% dialyzed fetal
bovine serum (3T3-L1) or for 4 h in 1% insulin-free BSA (SV40-trans-
formed hepatocytes), cells were incubated in the presence or absence of
100 nM insulin for various lengths of time at 37 °C. Thereafter, cells
were frozen in liquid N2 and thawed in solubilization buffer (1% Triton
X-100 in 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 4 mM sodium
orthovanadate, 1 mM EDTA, 4 mM sodium pyrophosphate). For RNA
analysis studies, SV40-transformed hepatocytes were incubated with
insulin (1 or 100 nM) for 4 h, frozen in liquid N2, and thawed for RNA
extraction as outlined above.

In vitro transcription/translation was performed using a coupled
rabbit reticulocyte system (Promega, Madison WI) according to the
manufacturer’s instructions.

GST Fusion Protein Binding Assay—The GST fusion proteins used in
these studies are as follows: mouse c-Abl type IV SH3 domain (amino

**FIG. 2.** Alignments of Sirm proline-rich domains with consensus motifs that bind the SH3 domains of FYN and Grb2. A, consensus sequences for class I and class II SH3 binding; B, alignment of two potential SH3 binding domains (amino acids 231–240 and 332–337) in the Sirm sequence with the consensus Src, FYN, and Grb2 SH3 bind-
ing sites. C, alignment of the proline-rich domain encoded by amino acids 231–240 of Sirm with the SH3 binding domains of CR16 (15), SH3 BP-2 (16), Ras GAP-SH3 (17), and Abi-2 (18).

**FIG. 3.** Sirm mRNA levels are increased in mice lacking insulin receptors. RNAs were isolated from newborn mouse muscle (lanes 1–3) and liver (lanes 4–6). The blot was hybridized with a sirm cDNA and reprobed with an actin probe to normalize for RNA content. Scanning densitometry was employed to quantitate levels of sirm mRNA. Quantitative representation of the data is presented at the bottom of the figure. In each set of tissues, the highest levels of sirm mRNA were normalized to 100%. For each lane, RNAs from 3 to 5 animals were pooled. 2 μg of poly(A)+ were applied to lanes 1–3, whereas 3 μg of poly(A)+ were applied to lanes 4–6.
acids 84–138) (6), the mouse phosphatidylinositol 3-kinase p85 SH3 domain (amino acids 1–80) (7), the human FYN SH3 domain (amino acids 84–148) (8), and the full-length mouse Grb2 (Upstate Biotechnology). Confluent monolayers of SV40-transformed murine hepatocytes in two 175-cm² flasks were solubilized in 5 ml of 1% Triton buffer. 5 µg of recombinant GST fusion protein in 0.05 ml were added to the extracts and allowed to bind for 1 h at 4 °C on a rotating wheel. Thereafter, the material was pelleted by centrifugation and washed in 10 ml of 0.1% Triton buffer four times. The final pellet was prepared for electrophoresis as described above.

In Vitro Kinase Activity—2 units of purified FYN kinase (Upstate Biotechnology) were incubated with 1 unit of denatured rabbit muscle enolase (Sigma) in the presence of GST, or GST-Sirm, or with various concentrations of peptides corresponding to Sirm amino acid sequences 224–246 (peptide 1), 331–339 (peptide 2), and 29–40 (peptide 3). Thereafter, a phosphorylation buffer containing 50 mM HEPES (pH 7.4), 5 mM MgCl₂, and 10 µCi of [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products) was added, and the reaction was incubated at room temperature for 30 min. The reaction was stopped by adding Laemmli sample buffer, and the products were analyzed by SDS-PAGE followed by autoradiography. One of two experiments is shown.

RESULTS

Cloning of Sirm—We had previously shown that insulin stimulation of liver extracts derived from ir⁻/⁻ mice results in tyrosine phosphorylation of a protein of apparent mass of ~75 kDa that is not observed in ir⁺/⁺ mice (see Fig. 3B in Ref. 2). We questioned whether this protein may share sequence similarity with the insulin receptor or its substrates. To this end, we carried out RT-PCR amplification from ir⁻/⁻ and ir⁺/⁺ mice liver RNA using several sets of primers, the sequences of which were derived from mouse insulin and IGF-1 receptors, as well as insulin receptor substrate-1 (IRS-1). A set of primers designed to amplify the extracellular domain of the IGF-1 receptor consistently gave rise to an additional band of slightly larger size than predicted (Fig. 1A). This band was unique to ir⁻/⁻ liver RNA and was never observed in amplification experiments with ir⁺/⁺ mouse liver RNA (data not shown). The band was isolated, sequenced, and used as a hybridization probe to isolate clones containing the sirm coding sequence. By using a combination of cDNA library screening and cDNA amplification techniques, we cloned a 3661-nt cDNA, with an open reading frame of 1800 nucleotides, predicted to encode a 600-amino acid polypeptide. The 5’ end of the cDNA was isolated from mouse heart Marathon cDNA (CLONTECH). Two different clones were isolated, possibly as the result of two different transcription start sites. The longest cDNA contains 962 nt of 5’-untranslated sequence; the shorter one contains 620 nt. Two non-canonical ATTAAA polyadenylation signals are found 24 and 160 nt downstream of a putative ochre termination codon. In vitro translation of a full-length sirm cDNA clone in a rabbit reticulocyte lysate yielded a peptide of 81 kDa (Fig. 1B).

The predicted amino acid sequence of Sirm (Fig. 1C) bears weak homology (20% identity in a 511-amino acid overlap) to the KSP region of the unc-89 gene product in Caenorhabditis elegans (9) and
to the open reading frame K08E7.5 on chromosome IV of C. elegans (21% identity in a 598-amino acid overlap) (10). The KSP region of the unc-89 gene is serine- and threonine-rich and is thought to represent a potential target for phosphorylation by cell cycle-de- pendent kinases. We did not detect any homology to known mam- malian proteins. However, analysis of the data base of expressed sequence tags revealed that sirm is virtually identical to two hu- man ESTs. The first EST corresponds to a skeletal muscle cDNA (Z19503/HSB35G062); the second one to a beta cell library cDNA (T10445) (11).

The longest sirm open reading frame is predicted to encode a polypeptide of 69,000 Da (Fig. 1 C). Hydropathy profile analysis indicates that Sirm lacks a hydrophobic sequence long enough to serve as a transmembrane domain. The sequence is enriched in serines and threonines (20% of all amino acids) and in basic residues (17% arginines, lysines, and histidines). There are five potential —SP— phosphorylation sites by proline-directed ser- ine kinases at amino acid residues 76, 189, 231, 418, and 438. Three potential sites of tyrosine phosphorylation are present at positions 37, 56, and 81. Tyr37 is of interest in that it conforms to an NPXpY motif found in receptor tyrosine kinases and is thought to serve as a binding site for the phosphotyrosine binding domains of IRS and Shc molecules upon phosphoryla- tion (12). Another interesting feature of Sirm is the presence of four proline-rich motifs, conforming to the SH3 binding consen- sus PpXpPLP (13). Of these, sequences NPLPTTPKR (amino acids 328–336) and PLPLLPSK (amino acids 240 to 233, in reverse orientation) share homology with the Src and Grb2 SH3 con- sensus binding site RPLPPLP (14) (Fig. 2 B). The sequence between amino acids 224 and 246 conforms to a consensus PCXXSPLLP sequence, where C indicates a hydrophobic amino acid, found in the following four known SH3 domain-binding proteins: CR16, a protein encoded by a brain-specific mRNA regulated by glucocorticoids (15); SH3 BP2, a protein

Fig. 6. A, tissue distribution of immunoreactive Sirm protein. Different mouse tissues, indicated on the top, were solubilized in NaHCO3 buffer containing protease inhibitors. 15 μg of proteins were applied to the lanes containing muscle, heart, fat, and embryo extracts; 40 μg were applied to the lanes containing liver and kidney extracts. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with anti-Sirm antibody (left panel) in the absence or presence of 1 μM competing peptide (middle panel). Preimmune serum was also used as control and is shown in the right-hand panel. B, subcellular localization of Sirm. Tissue extracts of mouse skeletal muscle and 3T3-L1 adipocytes were fractionated into a cytosolic and a membrane-enriched fraction (see “Experimental Procedures”). As a control, nuclei (nuc) were collected by low speed centrifugation during the preparation of muscle extracts and are shown in the 1st two lanes on the left. The amounts of protein extracts applied were the following: 15 μg of muscle nuclei, 15 μg of muscle and 3T3-L1 membrane (m) and cytosol (c), 40 μg of liver cytosol (ir-r mice), 80 μg of liver cytosol (ir-r mice), 10 μg of heart cytosol (ir-r, mice), and 10 μg of heart cytosol (ir-r mice). Proteins from each fraction were analyzed by SDS-PAGE followed by immunoblotting with anti-Sirm antibody. WT, wild-type

Insulin-regulated Grb2- and FYN-binding Protein

Fig. 7. Association of Sirm with Grb2 and with SH3 domains of p85α, Abl, and FYN. Left panel, GST fusion proteins encoding the SH3 domains of Abl, FYN, p85α, Abl, and FYN. The resulting pellets were immunoblotted with an anti-Sirm antibody. Right panel, in vitro translated Sirm protein (as shown in Fig. 1 B) was incubated with the same amount of Abl, Grb2, FYN, and wild-type GST fusion protein as shown in the left panel, in the absence or presence of 1 μM competing peptide. The peptide sequences are as follows: peptide 1, IPLIKSPLLPTPKS; peptide 2, NPLPTTPKR.
Fig. 8. Sirm binds to FYN and Grb-2 in 3T3-L1 cells. Detergent extracts of undifferentiated or differentiated 3T3-L1 cells were incubated with various antibodies, as indicated on the top of each panel. The immune complexes were centrifuged for 5 min at 14,000 rpm and washed as described under "Experimental Procedures." The samples were prepared for electrophoresis and analyzed by immunoblotting with an anti-Sirm antibody.

that binds to the Src, Nck, Grb-2, and Abl SH3 domains (16); the Ras-GAP SH3 binding protein (17); and Abi-2, a protein cloned based on its ability to bind with high affinity to the SH3 domain of Abl (18). It should be noted, however, that the proline-rich domains of SH3 BP-2 and Abi-2 that are homologous to Sirm are not the same as those implicated in Abl binding (16, 18).

Sirm mRNA Is Overexpressed in Liver and Skeletal Muscle of Mice Lacking Insulin Receptors—sirm was originally detected in RNA derived from ir−/− mice. Next, we asked whether failure to detect sirm in the same assay in ir+/− mice could be due to overexpression of sirm in ir−/− mice compared with normal littersmates. Northern blotting analysis was performed on pooled RNAs derived from skeletal muscle and liver of three to five ir−/−, ir+/−, and ir+/+ mice (Fig. 3). In liver of ir−/− mice, sirm mRNA levels were 3-fold higher than in ir+/− mice (Fig. 3, lanes 4 and 6) after correcting for the amount of actin mRNA (lower panel), whereas in skeletal muscle they were 40% higher (lanes 1 and 3). Intermediate variations were observed in ir+/− mice (lanes 2 and 4).

Expression of Sirm Correlates with Tissue Sensitivity to Insulin—To determine further whether expression of sirm is under regulation by insulin, we studied the effect of insulin on sirm expression in SV40-transformed hepatocytes derived from ir−/− and ir+/− mice. Cells derived from ir−/− mice are devoid of insulin receptors but possess about 50,000 IGF-1 receptors/cell.2 After treatment with insulin (1 or 100 nM), RNA was isolated and analyzed by Northern blotting with a 32P-labeled sirm cDNA (Fig. 4). 4 h treatment of normal cells with 1 nM insulin led to a 90% decrease of sirm expression, and 100 nM insulin led to a 90% decrease (Fig. 4, lanes 1–3). In contrast, in ir−/− cells, 1 nM insulin decreased sirm mRNA levels by 20% and 100 nM insulin by 45% (Fig. 4, lanes 4–6). Thus, expression of the sirm gene is decreased following prolonged exposure of cells to insulin. The ID50 for insulin inhibition of sirm expression is ~1 nM in ir+/− cells and ~100 nM in ir−/− cells. This observation suggests that insulin regulates sirm expression in ir−/− cells by binding to IGF-1 receptors. In fact, insulin would be predicted to bind to IGF-1 receptors with about 100-fold lower affinity compared with insulin receptors.

Sirm Is Prevalently Expressed in Insulin-responsive Tissues—We performed a tissue survey of sirm gene expression (Fig. 5). During mouse embryonic development, sirm expression peaks in mid-late gestation (E11–15), to then decline prior to birth (left panel). In adult mouse, sirm is most abundant in skeletal muscle and heart (Fig. 5, middle panel). Additionally, sirm is expressed in kidney, brain, and lung. Although not clearly visible on this exposure of the blot, sirm mRNA can be detected in liver, as demonstrated in Figs. 3 and 4. Expression of sirm is associated with adipocyte differentiation of 3T3-L1 cells (Fig. 5, right panel). Furthermore, sirm is identical to EST T10445, which was isolated from a pancreatic islet library (11, 19). Thus, sirm is expressed in virtually all insulin-responsive tissues and, at lower levels, in other organs. Similar expression patterns were detected using Northern analysis of human RNAs (data not shown). A zoo blot (CLONTECH) was hybridized to a 32P-labeled mouse sirm cDNA. Results indicate that homologues of the mouse sirm gene are present in human, rat, and yeast (data not shown).

Subcellular Distribution of Sirm and Quantification of the Protein Product in Mice—We raised a polyclonal antiserum against the peptide sequence corresponding to amino acids 136–149 of Sirm. The antiserum detected an immunoreactive species of ~90 kDa in several mouse tissues, which is specifically competed by the peptide used for affinity purification of the antiserum (Fig. 6A, compare left and middle panel). The same band is not present when preimmune serum is used for immunoblotting (Fig. 6A, right panel). The tissue distribution of immunoreactive Sirm protein correlates well with patterns of mRNA expression.

We next examined the subcellular localization of Sirm (Fig. 6B). Extracts of skeletal muscle and 3T3-L1 adipocytes were separated into cytosolic and membrane-enriched fractions. In skeletal muscle, immunoreactive Sirm was detected exclusively in the cytosolic fraction. In contrast, in 3T3-L1 adipocytes, a fraction of Sirm immunoreactivity corresponding to about 25% of the total was membrane-associated (Fig. 6B). Short term (5 min) stimulation with 100 nM insulin did not affect the subcellular distribution of Sirm (Fig. 6B, compare −insulin and +insulin lanes).

We also compared the levels of Sirm immunoreactivity in livers and hearts of ir−/− and ir+/− mice (Fig. 6B, compare WT and −/− lanes), but we failed to detect the difference predicted on the basis of the Northern blot shown in Fig. 3. It is possible that the discrepancy between levels of mRNA and immunoreactivity is due to impaired recovery of immunoreactive Sirm protein in insulin receptor-deficient mice caused by their advanced metabolic derangement.

Sirm Binds to Grb-2 and to the SH3 Domain of FYN in Vitro—The amino acid sequence of Sirm contains four proline-rich domains that could potentially serve as binding sites for SH3-containing proteins. Based on the homology between these domains and the known binding specificities of Grb-2 and FYN (Fig. 2B), we investigated whether Sirm could bind to Grb-2 or FYN SH3-GST fusion proteins in vitro. Furthermore, in view of the consensus sequence among Sirm (amino acids 224–246) and several Abl-binding proteins, we also included the Abl SH3-GST protein in these experiments, as well as the p85α SH3-GST as a negative control. GST fusion proteins containing the SH3 domains of the p85α subunit of phosphatidylinositol 3-kinase (7), Abl (6), FYN (8), and full-length Grb-2 were expressed in Escherichia coli, purified, and incubated with detergent extracts of SV40-transformed hepatocytes. Immunoblotting of the resulting gels with the Sirm antibody showed that Sirm binds to GST-Grb2 and GST-FYN SH3 but not to the SH3 domains of p85α or Abl (Fig. 7, left panel). To characterize better the nature of this interaction, we incubated in vitro translated Sirm with GST fusion proteins in the absence or presence of two proline-rich peptides derived from the Sirm sequence (Fig. 7, right panel). Sirm binding to Grb-2 was

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Sirm-derived peptides altered phosphorylation of enolase by FYN. Furthermore, FYN catalyzed the phosphorylation of GST-Sirm (lane 3). We have also shown that Sirm can serve as a substrate for the insulin and IGF-1 receptor tyrosine kinases in vitro (not shown).

**DISCUSSION**

In this paper, we report the identification and a preliminary characterization of a novel gene, which we have termed *sirm*. Even though the identification of *sirm* was the serendipitous by-product of a search to identify proteins uniquely expressed by insulin receptor-deficient mice, several features of the *sirm* gene prompted us to investigate it further. First, insulin is a potent regulator of *sirm* mRNA expression, so that *sirm* mRNA is up-regulated in conditions of increased tissue sensitivity to insulin, such as differentiation of 3T3-L1 cells and diabetic ketoacidosis, whereas it is down-regulated by prolonged insulin treatment. Interestingly, the highest levels of *sirm* expression are found in insulin-sensitive tissues, such as skeletal muscle, heart, fat, kidney, and liver. Indirect evidence indicates that *sirm* is also expressed in pancreatic beta cells, since an EST with virtual sequence identity to *sirm* was detected in a beta cell library. Interestingly, we have localized the human homologue of *sirm* to chromosome 15q15, in the genetic interval between D15S118 and D15S123, which corresponds to 32–45 centimorgans on the physical map of chromosome 15.3

This region contains marker D15S102. In studies of Mexican American sibling pairs with non-insulin-dependent diabetes mellitus, Hanis *et al.* (23) reported a significant departure from the expected frequency of allele sharing at this marker. Further studies will be necessary to determine whether this region contains a diabetes-susceptibility locus; however, the co-localization of SIRM to this region is intriguing.

The sequence of Sirm contains a number of interesting features that suggest clues as to its function. In this paper, we have shown that Sirm can interact with proteins containing SH3 domains through its proline-rich motifs. In 3T3-L1 cells, Sirm associates with the Src family tyrosine kinase FYN. Binding of Sirm to FYN was predicted on the basis of a consensus binding site for the FYN SH3 domain (Fig. 2). Following immunoprecipitation with an anti-Sirm antibody, no Sirm could be detected in differentiated 3T3-L1 cells under basal conditions. However, insulin treatment resulted in the complete disappearance of FYN-bound Sirm, indicating that FYN is indeed complexed with Sirm in 3T3-L1 cells and that insulin treatment causes dissociation of the Sirm-FYN complexes. Likewise, Sirm could be detected in Grb-2 immunoprecipitates under basal conditions but not following insulin treatment (Fig. 8C). No Sirm immunoreactivity is present following immunoprecipitation with nonimmune serum (Fig. 8D). Control immunoblotting experiments with Grb-2 and FYN antibodies indicate that the same amount of protein is present in all lanes (not shown).

**Sirm Is a Substrate for the Kinase Activity of FYN in Vitro, the Failure of Sirm to Modulate FYN Kinase**—The activity of Src family kinases, such as FYN, is regulated through interactions of cellular proteins with their SH2 and SH3 domains. For example, deletion of either domain leads to constitutive activation of the Ab1 or Src kinases (20–22). We wanted to determine whether Sirm could modulate FYN kinase activity through SH3 domain interactions. GST-Sirm (amino acids 1–535) was incubated with partially purified FYN and [γ-32P]ATP (Fig. 9), and the kinase activity of FYN was measured using enolase as a substrate in the presence or absence of various concentrations of the two proline-rich peptides that interact with FYN (Fig. 9, lanes 4–9). Neither GST-Sirm (amino acids 1–535) nor

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cells compared with their undifferentiated counterpart. On the other hand, the identification of Sirm as an adipocyte-specific substrate of FYN raises the interesting possibility that Sirm may participate in adipocyte-specific functions of FYN.

The binding of Sirm to Grb-2 is also consistent with the known specificity of the Grb-2 SH3 binding domain (Fig. 2B) (28). The dissociation of Sirm from Grb-2 follows the same time course and dose responsiveness as insulin-induced dissociation of Grb-2 from son-of-sevenless (29, 30). This dissociation has been invoked as a possible mechanism underlying the transient nature of insulin's effect to activate Ras. By analogy, one could envision that Sirm links Grb-2 to downstream effectors and that this effect is lost upon prolonged insulin stimulation.

The evidence presented in this paper indicates that Sirm is a novel adapter protein, which can function in a variety of cell types by binding the Src family kinase FYN and Grb-2. Sirm expression appears to correlate with the ability of organs and cells to respond to insulin, and several of the intracellular targets of Sirm have been implicated in signaling downstream of the insulin receptor. We would like to tentatively propose that Sirm plays a role in modulating insulin action in insulin-sensitive tissues via its association with signaling molecules. Further studies required to conclusively test this hypothesis are under way.

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