It is well known that insulin receptor substrates (IRS) act as a mediator for signal transduction of insulin, insulin-like growth factors, and several cytokines. To identify proteins that interact with IRS and modulate IRS-mediated signals, we performed yeast two-hybrid screening with IRS-1 as bait. Out of 109 cDNA-positive clones identified from a human placental cDNA library, two clones encoded 53BP2, p53-binding protein 2 (53BP2S), a short form splicing variant of the apoptosis-stimulating protein of p53 that possesses Src homology region 3 domain, and ankyrin repeats domain, and had been reported to interact with p53, Bcl-2, and NF-κB. Interaction of 53BP2S with IRS-1 was confirmed by glutathione S-transferase pull-down and co-immunoprecipitation assays in COS-7 cells and 3T3-L1 adipocytes. The Src homology region 3 domain and ankyrin repeats domain of IRS-1 were required for its interaction with 53BP2S. In CHO-C400 cells, expression of 53BP2S reduced insulin-stimulated IRS-1 tyrosine phosphorylation with a concomitant enhancement of IRS-2 tyrosine phosphorylation. In addition, the amount of the phosphatidylinositol 3-kinase regulatory p85 subunit associated with tyrosine-phosphorylated proteins, and activation of Akt was enhanced by 53BP2S expression. Although 53BP2s also enhanced Akt activation in 3T3-L1 adipocytes, insulin-induced glucose transporter 4 translocation was markedly inhibited in accordance with the amount of the phosphatidylinositol 3-kinase regulatory p85 subunit associated with tyrosine-phosphorylated proteins, and activation of Akt was enhanced by 53BP2S expression. In addition, the amount of the phosphatidylinositol 3-kinase regulatory p85 subunit associated with tyrosine-phosphorylated proteins, and activation of Akt was enhanced by 53BP2S expression. It is well established that insulin and IGFs display a variety of bioactivities, including induction of growth promotion, differentiation, and metabolic function. Insulin or IGFs bind to the extracellular subunits of their respective receptors and induce intramolecular conformational changes resulting in the activation of the β subunit intrinsic tyrosine kinase activity (1, 2). Activated receptor kinases phosphorylate several intracellular substrates, including insulin receptor substrates (IRs), Shc, Cbl, or Crk. Tyrosine phosphorylation of these substrates leads to their binding to several intermediate signaling molecules containing SH2 domains. In particular, the binding of the p85 regulatory subunit of PI 3-kinase to IRSs results in the recruitment and activation of the p110 catalytic subunit (3, 4). The interaction of Grb2 with tyrosine-phosphorylated IRS and Shc leads to activation of the small GTP-binding protein Ras through the plasma membrane recruitment of the guanyl nucleotide exchange factor SOS (3, 5, 6). These interactions result in activation of PI 3-kinase cascade and Ras-MAPK cascade, respectively (7, 8). It has become clear that activation of these two cascades play important roles in a variety of insulin or IGF actions.

Insulin is well known to induce translocation of glucose transporter 4 (Glut4), which is expressed in muscle and adipose tissue, from multiple intracellular compartments to the plasma membrane, leading to an enhancement of glucose uptake. It is well established that activation of PI 3-kinase activity, generation of the PI 3,4,5-trisphosphate, activation of the downstream effector Akt, and subsequent phosphorylation of Akt substrate, AS160, are necessary events required for the insulin stimulation of Glut4 translocation and glucose uptake (9–17). Thus, the IRS family proteins play essential roles as intermediate mediators for this signal transduction pathway central to the biological actions of both insulin and IGF-I receptors.

Four members of IRS family proteins (IRS1–4) have been identified to date (8). These IRS family proteins share two highly homologous amino-terminal regions, the pleckstrin

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* This work was supported in part by Grant-in-aid for International Joint Research 08044193 (to S.-I. T.), Grant-in-aid for Scientific Research A 16208028 (to S.-I. T.) from the Ministry of Education, Science, and Culture of Japan, and by the Program for Promotion of Basic Research Activities for Innovative Biosciences (to F. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: IGF, insulin-like growth factor; 53BP2, p53-binding protein 2; ASP2P, apoptosis-stimulating protein of p53; IRS, insulin receptor substrate; PH, pleckstrin homology; PTB, phosphotyrosine binding; SH, Src homology region; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GST, glutathione S-transferase; HEK, human embryonic kidney; PI 3-kinase, phosphatidylinositol 3-kinase; IPTG, isopropyl β-D-thiogalactopyranoside; Bcl-2, B cell lymphoma/leukemia-2; Glut4, glucose transporter 4; NF-κB, nuclear factor-κB; GFP, green fluorescent protein; PI 3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; RT, reverse transcription; ERK, extracellular signal-regulated kinase.
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homology (PH) domain and the phosphotyrosine binding (PTB) domain, which play important roles in the interaction with receptor tyrosine kinase. However, the carboxyl-terminal region is not conserved except for the tyrosine residues possibly phosphorylated by the receptor tyrosine kinases (8).

In addition to tyrosine phosphorylation, there are a multiple putative serine/threonine (Ser/Thr) phosphorylation sites in the carboxyl-terminal region, several of which are reported to be involved in the modulation of insulin-induced IRS tyrosine phosphorylation and play important roles in modulation of insulin or IGF signals (18–24). For example, in 3T3-L1 adipocytes pretreatment with tumor necrosis factor-α reduced insulin-induced glucose uptake through an impairment of insulin-stimulated IRS-1 tyrosine phosphorylation (25–27). In contrast, we found that in rat FRTL-5 thyroid cells, chronic pretreatment with thrytrotpin markedly potentiated DNA synthesis in response to IGF-1 (28, 29). Detailed analyses showed that thrytrotpin pretreatment enhanced IGF-1-induced IRS-2 tyrosine phosphorylation, resulting in the augmentation of IGF-1 signals (30, 31). Nevertheless, in both experimental model systems, in vitro phosphorylation assays demonstrated that Ser/Thr phosphorylation and association of some proteins with IRS are related to alterations of IRS tyrosine phosphorylation (26). Thus the identification of IRS-associated proteins is an essential prerequisite for understanding the alteration of IRS tyrosine phosphorylation. Thus, this study was undertaken to isolate proteins that interact and modulate IRS-mediated signals. By using yeast two-hybrid screening, we have cloned a cDNA that encodes a protein known as 53BP2S, a short form cDNA that encodes a protein known as 53BP2S, a short form

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS), and Hanks’ buffered saline solution were purchased from Nissui (Tokyo, Japan). Calf serum and fetal bovine serum were obtained from JRH Bioscience (Tokyo, Japan). Penicillin and streptomycin were purchased from Ban’yu Pharmaceutical Co. (Tokyo, Japan). Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan) generously provided leupeptin and pepstatin. The phosphotyrosine monoclonal antibodies PY20 and 4G10 were from Sigma and ICN (Irvine, CA). Polyclonal IRS-1 and IRS-2 antibodies were prepared by immunizing rabbits with synthetic peptides as reported previously (30). GFP polyclonal antibody was purchased from BD Biosciences. The FLAG M2 monoclonal antibody and HA antibody were from Sigma. Phospho-Akt-specific antibody (Ser-473) and phospho-ERK-specific antibody were from Cell Signaling (Beverly, MA). The 53BP2 antibody was purchased from BD Biosciences. The monoclonal ASPP2 antibody clone DX54.10 was from Sigma. The Myc and AS160 antibodies were purchased from Upstate (Charlottesville, VA). Phospho-AS160 (Thr-642)-specific antibody was from BioSource (Camarillo, CA). LY294002 was obtained from Sigma. Alexa Fluor 596 anti-

mouse IgG and Alexa Fluor 488 anti-mouse IgG were purchased from Molecular Probes (Eugene, OR). All dishes, plates, and flasks were obtained from IWAKI (Tokyo, Japan). Other chemicals were of the reagent grade and available commercially.

Cell Culture of COS-7, CHO-C400, and 3T3-L1 Cells—COS-7 cells were provided by Dr. Hiroshi Kataoka (Graduate School of Frontier Sciences, the University of Tokyo, Tokyo, Japan) and CHO-C400 cells were kind gifts from Dr. Minoru Yoshida (RIKEN, Saitama, Japan). COS-7 or CHO-C400 cells were maintained at 37 °C in a humidified 5% CO2-controlled atmosphere in DMEM supplemented with 10% fetal bovine serum, 0.1% NaHCO3, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.5 μg/ml amphotericin B (Sanko, Tokyo, Japan). Murine 3T3-L1 preadipocytes were purchased from the American Type Tissue Culture Collection. 3T3-L1 preadipocytes were cultured in DMEM containing 10% calf serum at 37 °C in a 5% CO2 atmosphere and induced to differentiate into adipocytes as described previously (32).

Plasmid Construction—We obtained pBS-Bbp, containing a short form splicing variant of ASPP2, and 53BP2S (residues 123–1128 of ASPP2), a kind gift from Dr. Louie Naumovski (Stanford University School of Medicine, Stanford, CA) (33). IRS-1 cDNA was a kind gift from Dr. Takashi Kadowaki (Graduate School of Medicine, the University of Tokyo, Tokyo, Japan). IRS-1 cDNA containing full-length open reading frame was amplified by PCR using two primers, 5’-GGGGCATATGGCGAGCCCTTCGGGATA-3’ and T7 primer. The PCR product was digested by NdeI and BamHI and was cloned into the NdeI-BamHI site of the pAS2-1 vector (BD Biosciences). The resulting plasmid was named pAS-IRS-1 and used for two-hybrid screening as bait. pGEX vectors were used for expression of fusion proteins with GST in Escherichia coli. By digesting pACT-53BP2S, the EcoRI-PstI, PstI-EcoRI, or EcoRI-EcoRI fragment, which encodes only ankyrin repeats, only the SH3 domain, or both domains, was cloned into the pGEX vector in-frame, yielding pGEX-ANK, pGEX-SH3, or pGEX-53BP2S, respectively. These plasmids were used for expression and purification of fusion proteins with GST in E. coli. pACT-ANK and pACT-SH3 were constructed in pACT2 vector (BD Biosciences) by the same way as pGEX-ANK and pGEX-SH3. pIRS-3 and pGFP-IRS-4 were constructed as described before (34). pIRS-2 was constructed as follows. Briefly, EcoRI fragment containing the full length of IRS-2 was cloned into pcDNA3. pFLAG-IRS-1, which expresses FLAG-tagged IRS-1, was constructed as follows. Full length of IRS-1 open reading frame was amplified by PCR using two primers, 5’-CCCGCATATTCATGCATGCGAGCAGCTTCCGG-3’ and T3 primers. The EcoRV-BamHI fragments of the PCR product was cloned into pCMV-FLAG-2 vector-in-frame. Convenient restriction enzymes were used to construct the plasmids expressing some deletion mutants of IRS-1 fused with GFP. pGFP-D13 or pGFP-D14, which is a plasmid expressing IRS-1 D13 or IRS-1 D14 deletion mutant fused with GFP, respectively, was constructed as follows. IRS-1 fragment encoding the amino acid residues 660–861 or 750–861 was amplified by PCR using two primers, 5’-AGATGAGAAGGCTTGCCGAGGTTCCAG-3’ and T3 primer or 5’-CCAGAAGCCAGCCCAGCCAAGCC-3’ and T3 primer. Amplified fragments were digested by HindIII and BamHI and
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cholesterol; the cells were then allowed to adhere to tissue culture dishes for 24 h, and the adipocytes were then serum-starved for 2 h before experiments. In some experiments, the electroporated adipocytes were seeded on coverslips.

Purification of GST Fusion Proteins—pGEX plasmid was transformed into *E. coli* BL21 (DE3) pLysS. Isopropyl β-D-thiogalactopyranoside was added to 1 mM in the final concentration, and the expression of GST fusion protein was induced overnight at 26°C. Cells were harvested and resuspended in PBS with 1% Triton X-100 and lysed by sonication three times for 30 s on ice. The lysates were centrifuged, and supernatant was added to the glutathione-Sepharose column pre-equilibrated in the PBS buffer. The column was washed with PBS three times, and the GST fusion proteins were eluted by elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). Eluted solution was fractionated by 1 ml. Each fraction was subjected to protein assay using protein assay kit (Bio-Rad). The most concentrated fraction was used for experiments.

GST Pulldown Assay—COS-7 cells were transfected with expression plasmids as described above. Two days after transfection, cells were harvested by cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 100 kallikrein-inactivating units/ml aprotinin, 20 mg/ml phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin, 5 mg/ml pepstatin). The lysates were centrifuged at 14,000 × g for 20 min at 4°C. The supernatant was subjected to protein assay using the protein assay kit. Cell lysates (1 mg of protein) were incubated with 100 pmol of purified GST, GST-53BP2S, GST-ANK, or GST-SH3 fusion protein at 4°C for 2.5 h. Forty μl of glutathione-Sepharose beads (50% (v/v)) was then added, and incubation was continued for additional 2.5 h. Sepharose beads were collected by centrifuge and washed three times with washing buffer containing 50 mM Tris-HCl, 1 mM EDTA, and 0.1% Triton X-100. Bound proteins were subjected to SDS-PAGE, transferred to nylon membrane, and immunoblotted with the indicated antibody.

Analysis of 53BP2S Expression in 3T3-L1 Adipocytes—3T3-L1 adipocytes were induced to differentiate as described above. On 0, 2, 4, 6, and 8 days after differentiation, total cellular RNA was isolated by the TRizol reagent according to the manufacturer’s protocol (Invitrogen). First strand cDNA was synthesized from 0.2 μg of total RNA with oligo(dT) primers using the SuperScript II RT-PCR kit (Invitrogen). To determine expression of 53BP2S mRNA, first strand cDNA was subjected to PCR. Two specific primers for 53BP2S, 5'-ACGCCCAGTTGGCTGATAAACG-3' and 5'-CCAGCATTGTTGCTGGCCGCC-3', were used for PCR. The 36B4 gene was used as the internal control: 5'-AAGCGGCTTCCGAGTGGCT-3' (sense) and 5'-CCGAGGGCAGAGTGGTGT-3' (antisense).

Analyses of Insulin Signaling in CHO-C400—CHO-C400 cells transfected with pEGFP-53BP2S were grown to confluence, and the quiescent cells were stimulated with insulin (100 nM) for indicated times. Cell extracts were prepared in lysis buffer, and 1 mg of total lysate protein was used for immunoprecipitation with IRS-1, IRS-2, or 4G10 antibody. Precipitates were separated by 8% SDS-PAGE and immunoblotted with 4G10 or p85 antibody. One hundred μg of total cell lysates were separated by 12% SDS-PAGE and immunoblotted with phos-
pho-Akt-specific antibody (Ser-473) or with phospho-ERK antibodies.

**Immunofluorescence Analysis**—Electroporated 3T3-L1 adipocytes were washed once with PBS and fixed/permeabilized with a solution containing 3.7% formaldehyde and 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed with PBS and incubated with blocking buffer (1% bovine serum albumin and 5% donkey serum in PBS) for 1 h at room temperature, and primary antibodies (1:200 for anti-Myc, 1:100 for phospho-Akt-specific antibody (Ser-473), or 1:100 for FLAG antibody) were added for 1 h at room temperature. The samples were again washed with PBS, incubated with a secondary antibody conjugated to Texas Red (1:100 dilution), Alexa Fluor 488 (1:1000 dilution), or Alexa Fluor 596 (1:1000 dilution) for 40 min and washed, and the coverslips were mounted on Vectashield for visualization using a Zeiss LSM510 confocal microscope.

**Assay of the Glut4 Translocation to the Plasma Membrane**—Fully differentiated 3T3-L1 adipocytes were transfected with pEGFP vector or pEGFP-53BP2S along with pGlut4-myc by

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**TABLE 1**

Interaction of 53BP2S with IRS proteins in the yeast two-hybrid system

cDNAs fused with the DNA binding domain or cDNAs fused with the activation domain are shown in the DNA binding domain column or the activation domain, column respectively. Yeast strains used for the β-galactosidase assay are shown in the strains column. + means that we could observe the interaction in the galactosidase assay, and − means that we could not observe the interaction.

| Expression vector | DNA binding domain | Activation domain | Strains | β-Galactosidase assay |
|-------------------|--------------------|--------------------|---------|----------------------|
| IRS-1             | None               | 53BP2S (758–1005)  | Y190    | +                    |
| IRS-2             | 53BP2S (758–1005)  | Y190               | +       |
| IRS-3             | Ankyrin            | Y190               | −       |
| IRS-4             | SH3                | Y190               | −       |
| IGF-IR            | 53BP2S (758–1005)  | L40                | −       |

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**FIGURE 1. Interaction of 53BP2S with IRSs in the GST pulldown assay.** A, schematic structure of ASPP2 and 53BP2S proteins. Two boxes show ankyrin repeat domains and SH3 domain, respectively. Below the structure, arrow indicates the deletion mutants used for two-hybrid assay or GST precipitation assay. Direction of arrow indicates the open reading frame. These mutants were fused with Gal4 DNA binding domain or fused with GST, and interaction with IRS-1 was analyzed by the two-hybrid assay or the pulldown assay. a.a, amino acid. B, three GST-53BP2S deletion series, including only ankyrin repeats (GST-ANK), only SH3 domain (GST-SH3), or both (GST-53BP2S), were purified from E. coli. Purified proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. C, cell lysate of COS-7 expressing FLAG-tagged IRS-1, intact IRS-2, intact IRS-3, or GFP-tagged IRS-4 was incubated with purified GST or GST-53BP2S. Proteins precipitated by glutathione-Sepharose beads were subjected to immunoblotting (IB) with indicated antibodies. Input represents an aliquot corresponding to 1% of the lysates used in each binding reaction. These are representative immunoblots independently performed three times. D, interaction of 53BP2S mutants with IRS-1 was analyzed in pulldown assay. Cell lysate of COS-7 expressing FLAG-tagged IRS-1 was incubated with purified GST, GST-53BP2S, GST-ANK, or GST-SH3. Proteins precipitated by glutathione-Sepharose beads were subjected to immunoblotting with anti-FLAG antibody. Input represents an aliquot corresponding to 3% of the lysates used in each binding reaction. These are representative immunoblots independently performed three times.
electroporation. Twenty four hours later, cells were serum-starved for 2 h followed by stimulation with or without insulin (100 nM) for 20 min. Cells were fixed without permeabilization and incubated with a Myc antibody. The ratio of Glut4 translocation was determined by comparison of the total Myc fluorescence intensity with the total GFP intensity.

Statistical Analysis—Results are expressed as means ± S.E. For comparison, the data were analyzed by analysis of variance followed by Student’s t-test, and the difference was considered significant at p < 0.05.

RESULTS

Identification of Proteins That Interact with Insulin Receptor Substrate-1—To identify proteins that interact with IRS-1, the full-length rat IRS-1 cDNA was fused with the Gal4 DNA binding domain in pAS2-1 vector and used as bait for a two-hybrid screening. We screened a human placental cDNA library fused with Gal4 activation domain in pACT2, and from 1 × 10^6 clones, 109 IRS-1 interacting candidates were identified. Among them, 33 clones included cDNAs encoding 14-3-3 isoforms, which were previously shown to interact with IRS-1 or IRS-2 (36). In addition to the various clones identified, two contained the same insert, a region of ASPP2 cDNA sequence corresponding to amino acid residues 881–1128. ASPP2 is an 1128-amino acid protein that consists of four ankyrin repeats domain and an Src homology (SH) 3 domain in the carboxyl-terminal region (Fig. 1A). ASPP2 was originally isolated as Bbp (33, 37). Recently, it was reported that ASPP2 is a 1005-amino acid protein and that ASPP2 contained an additional 123 amino acids to the amino terminus of Bbp. In addition, ASPP2 is an alternative splicing variant of ASPP2, and they proposed that we call this splicing variant ASPP2S (38). In this study, we used ASPP2S as an ASPP2 splicing variant.

53BP2S Interacts with IRS Family Proteins in Vitro—The clones isolated in the two-hybrid screen contained the carboxyl-terminal region that includes the SH3 domain and ankyrin

FIGURE 2. Interaction of 53BP2S with IRS-1 deletion mutants in the GST pulldown assay. Schematic structure of IRS-1 protein is shown. Two boxes represent PH or PTB domain, respectively. Boxes above the IRS-1 structure indicate the 53BP2S binding domains. Below the IRS-1 structure, constructs of some deletion mutants (D1–D14) are shown. Each deletion mutant was fused with GFP at the amino-terminal end. Plasmids expressing each deletion mutant of IRS-1 (D1–D14) were transfected into COS-7. Cell lysate of COS-7 expressing each deletion mutant was incubated with purified GST or GST-53BP2S. Proteins precipitated by glutathione-Sepharose beads were subjected to immunoblotting with anti-GFP antibody. The results of each pulldown assay are shown on the right side of the deletion constructs. Input represents an aliquot corresponding to 1% of the lysates used in each binding reaction.
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FIGURE 3. Effects of tyrosine phosphorylation of IRS-1 on interaction between IRS-1 and 53BP2S. A, left panel, total RNA was extracted from 3T3-L1 preadipocytes. Using this total RNA, first strand cDNA was synthesized with (RT+) or without (RT−) SuperScript2. PCR was carried out using these first strand cDNA as templates. PCR condition was as follows: 30 cycles of sequential incubation at 94 °C for 30 s, 50 or 55 °C for 60 s, and 72 °C for 60 s followed by final extension at 72 °C for 5 min. Right panel, total RNA was extracted from 3T3-L1 cells at differentiation stage 0, 2, 4, 6, and 8 day. PCR condition was 25 cycles of sequential incubation at 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s for both 53BP2S and 36B4. These are representative data independently performed five times. B, cell lysates of fully differentiated 3T3-L1 adipocytes were immunoprecipitated (IP) by IRS-1 antibody or preimmune serum. Total cell lysates of HEK293 cells or 3T3-L1 adipocytes and immunoprecipitants by IRS-1 antibody were immunoblotted (IB) with anti-ASPP2 antibody (Sigma) or anti-IRS-1 antibody. Input represents an aliquot corresponding to 1% of the lysates used in immunoprecipitation assay. C, cell lysate of 3T3-L1 adipocytes or stimulated with insulin (100 μM) for 2 min was incubated with purified GST or GST-53BP2S. Proteins precipitated by glutathione-Sepharose beads were detected by anti-IRS-1 antibody. Input represents an aliquot corresponding to 10% of the lysates used in each binding reaction. These are representative immunoblots independently performed three times. D, pEGFP or pEGFP-53BP2S was transiently transfected into 3T3-L1 adipocytes by electroporation. Cells were serum-starved for 2 h followed by stimulation with insulin or without insulin for 2 min. Cell lysates were immunoprecipitated by IRS-1 antibody or preimmune serum and immunoblotted with anti-53BP2S antibody.

repeats. We used a β-galactosidase assay to assess the ability of 53BP2S (amino acid residues 758–1005) to interact with IRS-1 (Table 1). pAS-IRS-1, which expresses IRS-1-DB hybrid (Gal4 DNA binding domain), or pACT-53BP2S, which expresses 53BP2S-AD hybrid (Gal4 activation domain), did not by itself activate lacZ transcription. When pAS-IRS-1 was transformed into Y190 along with pACT-53BP2S, transfectants turned blue in the β-galactosidase assay, indicating that IRS-1 and 53BP2S specifically interact with each other (Table 1).

To confirm the interaction of 53BP2S with IRS-1 by the two-hybrid assay, 53BP2S was tested in a GST pulldown assay. Isolated 53BP2S cDNA (amino acid residues 758–1005) fused with GST (GST-53BP2S) was constructed in a pGEX vector. GST-53BP2S protein or GST alone was then expressed and purified from E. coli (Fig. 1B). Cell lysates isolated from COS-7 cells expressing full-length FLAG tagged IRS-1 (FLAG-IRS-1) were incubated with purified GST-53BP2S or GST only. Protein complexes precipitated with glutathione-Sepharose beads were separated by SDS-PAGE, and FLAG-IRS-1 bound to GST-53BP2S was detected by immunoblotting with the FLAG antibody. FLAG-IRS-1 associated with GST-53BP2S but not with GST alone (Fig. 1C), indicating that 53BP2S specifically associates with IRS-1 in vitro as well as in the yeast two-hybrid assay.

Four members of IRS family proteins, IRS-1, IRS-2, IRS-3, and IRS-4, have been identified to date. Among them, PH and PTB domains are highly conserved, but other regions are divergent except for the tyrosine residues possibly phosphorylated by receptor tyrosine kinases (2). Fig. 1C shows that IRS-2, IRS-3, and GFP-tagged IRS-4 could also interact with 53BP2S (Fig. 1C).

Interaction of IRS-1 with 53BP2S Requires Both Ankyrin Repeats and SH3 Domain of 53BP2S—To investigate which region of 53BP2S is required for the interaction with IRS-1, two-hybrid β-galactosidase and GST pulldown assays were carried out. pACT–ANK or pACT–SH3 was constructed to contain only ankyrin repeats or only the SH3 domain in pACT2 vector, respectively (Fig. 1A). In the two-hybrid system, neither interaction between IRS-1 and the ankyrin repeats nor between IRS-1 and the SH3 domain was detectable by the β-galactosidase assay (Table 1). Similarly, we examined the precipitation of IRS-1 using an ankyrin repeat (GST–ANK) and an SH3 domain fusion protein (GST–SH3), respectively (Fig. 1B). Neither GST–ANK nor GST–SH3 could pull down FLAG-tagged IRS-1 (Fig. 1D), indicating that both the ankyrin repeats and the SH3 domain are required for the interaction with IRS-1. Consistent with these data, the interaction of 53BP2S with p53 and Bcl-2 is also required for both the ankyrin repeats and SH3 domain (33, 37, 39).

53BP2S Interacts with PTB Domain and a Central Region (Amino Acid Residues 750–861) of IRS-1—To identify regions of IRS-1 that are required for the interaction with 53BP2S, we examined IRS-1 deletion mutants fused with GFP (D1–D14), and pulldown assays were performed for each mutant (Fig. 2). An IRS-1 mutant that contains only the PH domain (D8) could not, whereas a mutant that contains the PTB domain (D9) could interact with GST-53BP2S (amino acid residues 758–1005). These data indicate that the IRS-1 PTB domain, but not the PH domain, is sufficient for the interaction with 53BP2S. Surprisingly, a mutant in which PH and PTB domains were both deleted (D5) still interacted with 53BP2S. More detailed analyses identified another 53BP2S binding domain, a central region containing 112 amino acid residues (750–861), that was sufficient for the interaction with IRS-1 (Fig. 2).

Endogenously Expressed 53BP2S Interacts with IRS-1 in 3T3-L1 Adipocytes—We next investigated the interaction of endogenously expressed 53BP2S with IRS-1. To confirm the expression of 53BP2S in 3T3-L1 cells, RT-PCR was carried out using total cellular RNA from 3T3-L1 preadipocytes. 53BP2S cDNA fragment was not amplified from first strand cDNA without SuperScript2 (RT−), whereas 53BP2S cDNA fragment
was amplified when PCR was carried out using first strand cDNA with SuperScript2 (RT+), indicating that 53BP2S mRNA was expressed in 3T3-L1 preadipocytes (Fig. 3A). RT-PCR confirmed the expression of 53BP2S in differentiated 3T3-L1 adipocytes. The expression of 53BP2S was increased by differentiation of 3T3-L1 adipocytes, suggesting that 53BP2S plays an important role in differentiated 3T3-L1 cells (Fig. 3A). Immunoblotting of 3T3L1 adipocyte extracts demonstrated the presence of a specific protein band that migrated identical human ASPP2 expressed in human embryonic kidney cell HEK293 cells (Fig. 3B). More importantly, immunoprecipitation of endogenous IRS-1 resulted in the co-immunoprecipitation of the endogenous 53BP2S in 3T3-L1 adipocytes (Fig. 3B). These data demonstrate that 53BP2S and IRS-1 directly interact in vivo.

**53BP2S Interacts with IRS-1 Not through Recognition of Phosphotyrosine Residues**—Because yeast has few tyrosine kinases, the expressed IRS-1 is not tyrosine-phosphorylated. To investigate whether the interaction of IRS-1 with 53BP2S could be modulated by IRS1 tyrosine phosphorylation, precipitation assays using phosphorylated IRS-1 or nonphosphorylated IRS-1 was carried out. Briefly, cell lysates of quiescent or insulin-stimulated 3T3-L1 adipocyte cells were incubated with GST-53BP2S (amino acid residues 758–1005) or GST alone. Endogenous IRS-1 protein bound to GST-53BP2S was visualized by immunoblotting with anti-IRS-1 antibody. As expected, IRS-1 proteins derived from quiescent 3T3-L1 adipocyte cells were precipitated by GST-53BP2S (Fig. 3C). IRS-1 proteins precipitated in lysates isolated from either quiescent or insulin-stimulated cell did not display any immunoreactivity against a phosphotyrosine-specific antibody. In addition, the amount of IRS-1 co-precipitated by 53BP2S was markedly decreased following insulin stimulation by immunoblotting with an IRS-1 antibody (Fig. 3C). Taken together, these data indicate that formation of a 53BP2S-IRS-1 complex is inhibited by insulin stimulation, most likely because of IRS-1 tyrosine phosphorylation.

To evaluate the mechanism of interaction between IRS-1 and exogenously expressed 53BP2S in adipocytes, fully differentiated 3T3-L1 adipocytes were electroporated with pEGFP or the pEGFP-53BP2S expression vectors. Electroporated cells were starved for 2 h, and cell lysates were immunoprecipitated with an IRS-1 antibody. As observed in the GST pulldown assays, 53BP2S was co-immunoprecipitated with IRS-1 (Fig. 3D). In addition, insulin stimulation resulted in a decreased amount of 53BP2S that co-immunoprecipitated with IRS-1 (Fig. 3D). These data are consistent with the GST pulldown results and further document that post-translational modification of IRS-1, possibly tyrosine phosphorylation, inhibited the interaction between IRS-1 and 53BP2S.

**Effect of 53BP2S Expression on Insulin Signals in CHO-C400 Cells**—To examine biological function of 53BP2S in insulin/IGF-1 signaling mediated by IRS proteins, we assessed the effect of 53BP2S expression on insulin signaling. GFP or GFP-53BP2S expression vectors were introduced into CHO-C400 cells by calcium phosphate transfection, and the activation of signaling targets in response to insulin was assessed. Expression of 53BP2S resulted in an increase in insulin-stimulated IRS-2 tyrosine phosphorylation, whereas IRS-1 tyrosine phosphorylation was decreased (Fig. 4B). In addition, the amount of IRS-1-associated p85 PI 3-kinase was decreased in 53BP2S-transfected cells, whereas the amount of p85 PI 3-kinase in IRS-2-immunocomplex was enhanced compared with GFP-transfected cells (Fig. 4A). To measure the total amount of p85 PI 3-kinase associated with total tyrosine-phosphorylated proteins, whole cell lysates were immunoprecipitated with phosphotyrosine antibody (4G10), and the amount of p85 PI 3-kinase in the immunocomplex was measured. Tyrosine phosphorylation of proteins at 180 kDa was enhanced by 53BP2S expression. Similarly, the amount of p85 PI 3-kinase immunoprecipitated by 4G10 was enhanced by 53BP2S overexpression (Fig. 5A). These data suggested that PI 3-kinase activation was elevated in 53BP2S-expressing cells. Because Akt activation was induced by PI 3-kinase, we next determined Akt activation in 53BP2S-expressing cells. It is well known that full activation of Akt kinase activity requires PDK1-dependent phosphorylation of threonine 308 followed by PDK2 phosphorylation on serine 473 (40). Consistent with an increase in the amount of p85 PI 3-kinase associated with tyrosine-phosphorylated proteins, insulin-stimulated Akt serine 473 phosphorylation was enhanced by GFP-53BP2S expression without any change in ERK activation (Fig. 5B). These results indicate...
that the enhancement of IRS-2 tyrosine phosphorylation and association of p85 PI 3-kinase with IRS-2 compensated for the reduction of IRS-1 tyrosine phosphorylation and p85 PI 3-kinase association with IRS-1 in these cells. The net effect results in a 53BP2S enhancement of Akt activation.

Effect of 53BP2S Expression on PI 3,4,5-P3 Production and Akt Activation in 3T3-L1 Adipocytes—To determine the effect of 53BP2S expression on insulin signals in 3T3-L1 adipocytes, we measured the insulin stimulation of PI 3-kinase pathway activation in 53BP2S-expressing cells. We took advantage of the pleckstrin homology (PH) domain of Grp1 fused to GFP 

In FLAG-tagged 53BP2S-expressing cells, the plasma membrane staining of Grp-PH-GFP could be observed, indicating that PI 3,4,5-P3 was normally produced in response to insulin even in 53BP2S-expressing cells (Fig. 6). Taken together, these data suggested that PI 3-kinase was activated in 53BP2S-expressing cells.

To investigate the effect of 53BP2S expression on Akt activation in vivo, we established the single cell assay of Akt activation. At first, adipocyte cells were stained with phospho-Akt-specific antibody (Ser-473) with or without insulin stimulation. We found the nuclei staining, which is independent of insulin stimulation, but we also observed staining on the plasma membrane, which is dependent of insulin stimulation (Fig. 7A). The plasma membrane labeling was specific for Akt activation, as it was prevented by the PI 3-kinase-specific inhibitor LY294002 (Fig. 7A). Although the number of cells displaying the Akt staining on the plasma membrane under basal conditions was essentially 0%, insulin stimulation resulted in greater than 90% of the cells phospho-Akt-positive. Also in 53BP2S-expressing cells, insulin-induced phospho-Akt staining was normally observed (Fig. 7B). In this assay, we could not detect the enhancement of insulin-induced Akt phosphorylation by 53BP2S expression. To compare the relative effect of 53BP2S expression on the extent of Akt, we co-transfected 3T3-L1 adipocytes with 50 µg of pHA-Akt2 and 200 µg of pGFP or 50 µg of pHA-Akt2 and 200 µg of pGFP-53BP2S. In this way, most cells expressing HA-Akt2 were expected to also express GFP or GFP-53BP2S. Cell lysates were prepared from these transfectants and immunoprecipitated with the HA antibody, followed by immunoblotting with phospho-Akt-specific antibody (Ser-473). As shown in Fig. 7C, HA-Akt2 was activated following insulin stimulation that was enhanced by 53BP2S expression.

Expression of 53BP2S Inhibits the Glut4 Translocation Induced by Insulin in 3T3-L1 Adipocytes—Because insulin-stimulated Glut4 translocation is an important readout for insulin signaling, we next examined the effect of 53BP2S in the 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were transfected with plasmid expressing GFP and Glut4-myc or GFP-53BP2S and Glut4-myc. Twenty four hours later, cells were serum-starved for 2 h, followed by stimulation with insulin.
translocation of Glut4 using a double-labeled Glut4 reporter demonstrating that 53BP2S specifically inhibited insulin signal downstream readout of Glut4 translocation was inhibited. To confirm this apparent inhibition of insulin-stimulated Glut4 translocation by 53BP2S, we next quantified the extent of Glut4 translocation using a double-labeled Glut4 reporter (Glut4-myc-eGFP). Briefly, 3T3-L1 adipocytes were electroporated with 200 µg of pFLAG vector or 200 µg of pFLAG-53BP2S along with 50 µg of pGFP to induce Glut4 translocation. Cells were fixed without permeabilization and incubated with Myc antibody. We assessed the relative Myc fluorescent intensity in comparison with the total cell GFP fluorescence. The results further demonstrate that 53BP2S-expressing cells have a marked attenuation of insulin-stimulated Glut4 translocation (Fig. 8C).

Because 53BP2S expression enhanced Akt activation and the downstream readout of Glut4 translocation was inhibited, 53BP2S must affect another target in the Glut4 translocation pathway. Recently it was reported that insulin induced phosphorylation of AS160 (Rab-GAP), one of Akt substrate, followed by activation of Rab10 was required for Glut4 translocation in 3T3-L1 adipocytes. To evaluate the effect of 53BP2S expression on the AS160 phosphorylation, we co-transfected 3T3-L1 adipocytes with 50 µg of pFLAG-AS160 and 200 µg of pGFP or 50 µg of pFLAG-AS160 and 200 µg of pGFP-53BP2S. Cell lysates were prepared from and immunoprecipitated with the FLAG antibody, followed by immunoblotting with phosho-AS160-specific antibody (Thr-642). As shown in Fig. 8D, FLAG-AS160 was phosphorylated following insulin stimulation, and this phosphorylation was repressed by 53BP2S expression.

### DISCUSSION

This study was undertaken to identify proteins, which interact with IRS and modulate insulin signaling. Using yeast two-hybrid screens, we isolated 53BP2S as one of IRS-interacting proteins. The interaction between 53BP2S and IRS proteins was confirmed by using both pulldown and co-immunoprecipitation binding assays. Expression of 53BP2S reduced insulin-induced IRS-1 tyrosine phosphorylation with a concomitant enhancement of IRS-2 tyrosine phosphorylation in CHO-C400 cells. In addition, Akt activation was enhanced by 53BP2S expression. Consistent with these data, Akt activation was enhanced by 53BP2S expression also in 3T3-L1 adipocytes; however, Glut4 translocation to plasma membrane in response to insulin was significantly inhibited by 53BP2S expression.

Previous studies demonstrated that chronic tumor necrosis factor-α pretreatment inhibited insulin-induced IRS-1 tyrosine phosphorylation, leading to a decrease in insulin sensitivity (26). Previously, we reported that chronic thrytropin pretreatment enhances the IGF-1-induced IRS-2 tyrosine phosphorylation, leading to augmentation of IGF-1-induced DNA synthesis (28–31). Although multiple studies have demonstrated that post-translational modification, such as Ser/Thr phosphorylation, plays important roles in modulating IRS tyrosine phosphorylation (18–24), we have also observed that various IRS-associated proteins can have dramatic effects on IRS phosphorylation. These data indicated the possibility that IRS-associated proteins play important roles in modulation of insulin/IGF bioactivities.

In this study, we identified the interaction regions of IRS-1, PTB domain, and the central region with 53BP2S. Although PTB domain is highly homologous among IRS family proteins, the central region is a unique sequence of IRS-1 compared with other IRS members, suggesting that the binding mechanism of 53BP2S with IRS-1 or IRS-2 could be different. This might account for the opposite effect of 53BP2S on tyrosine phosphorylation of IRS-1 and IRS-2. On the other hand, we also identified the interaction region of 53BP2S. Both SH3 and ankyrin repeat domains were required for association, indicating that SH3 and ankyrin repeats domains cooperatively form the conformational structure that is important for this interaction.

The interaction domains of IRS-1 with 53BP2S did not contain the putative tyrosine phosphorylation sites, and this interaction was clearly detected in the basal state. These data suggest that 53BP2S interacts with IRS-1 in the absence of tyrosine phosphorylation and that 53BP2S interaction modulates IRS tyrosine phosphorylation mediated by the insulin receptor tyrosine kinase. Thus, 53BP2S is a likely candidate for a protein

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**FIGURE 6. Effects of 53BP2S expression on phosphatidylinositol 3,4,5-trisphosphate production in 3T3-L1 adipocytes.** Fully differentiated 3T3-L1 adipocytes were transfected with 50 µg of pFLAG and 200 µg of pGFP or 50 µg of pFLAG-53BP2S and 200 µg of pGFP-53BP2S by electroporation as described under “Experimental Procedures.” IF, immunofluorescence. After a 2-h serum starvation, quiescent cells were stimulated with insulin (100 nM) for 5 min. Cells were immunostained with anti-FLAG antibody. Transfected Grp-PH-GFP was visualized in the confocal microscopy in green. FLAG-53BP2S protein was visualized by Texas Red in red.

| Transfection | pFLAG (50µg) | pGFP (200µg) | pFLAG-53BP2S (50µg) | pGFP-53BP2S (200µg) |
|--------------|-------------|-------------|--------------------|--------------------|
| Basal        | GFP         | IF: vFLAG   | GFP                | IF: vFLAG           |
| Insulin (100nM) | GFP         | IF: vFLAG   | GFP                | IF: vFLAG           |
that plays important roles in the modulation of insulin/IGF signals.

We have also observed that insulin stimulation decreased the interaction between 53BP2S and IRS. Although we were unable to observe a distinct translocation of 53BP2S by insulin stimulation, a small amount of 53BP2S and IRS-1 did appear to undergo changes in subcellular distribution. Consistent with this model, GFP-53BP2S was localized on the plasma membrane and partially co-localized with IRS-1 but was strongly co-localized with actin (data not shown). Clark et al. (41) suggested that IRS proteins may associate with the actin cytoskeleton. In addition, it was reported recently (42) that the caveolin-actin structure is very important for the Glut4 translocation to the plasma membrane. Thus, it is formally possible that 53BP2S overexpression disturbs the caveolin-actin structure in 3T3-L1 adipocytes thereby impairing Glut4 translocation.

It is also well established that activation of PI 3-kinase, generation of the PI 3,4,5-P3, and subsequent activation of Akt and phosphorylation of AS160 are necessary for the insulin-induced Glut4 translocation and glucose uptake (9–15). There are several possible mechanisms that could account for insulin-stimulated Akt activation but with reduced AS160 phosphorylation and reduced Glut4 translocation in GFP-53BP2S-expressing cells. First, because 53BP2S reduced insulin-stimulated IRS-1 tyrosine phosphorylation, it is possible that PI 3-kinase associated with IRS-1 or IRS-2 facilitates different roles in Glut4 translocation. In this regard, several studies have suggested that IRS-1 and IRS-2 are not functionally identical and therefore could result in differential spatial localization of PI 3-kinase (43–45). Similarly, studies using small interfering RNA-mediated gene silencing suggested a different function between IRS-1 and IRS-2 in L6 myotube cells (46). Alternatively, Glut4 translocation may require both PI 3-kinase and Akt isoform specificity. For example, several studies have observed that Akt2 is the predominant family member required for insulin-stimulated Glut4 translocation and glucose uptake (47–50). Similarly, knockouts of the

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**FIGURE 7. Effects of 53BP2S expression on Akt activation in 3T3-L1 adipocytes.** A, fully differentiated 3T3-L1 adipocytes were serum-starved for 2 h. Cells were incubated with or without LY294002 (100 μM) for 15 min prior to stimulation with or without insulin (100 nM) for 2 min. Each cell was stained with anti-phospho-Akt-specific antibody (Ser-473) and visualized in green. B, fully differentiated 3T3-L1 adipocytes were transfected with 50 μg of pGFP or 50 μg of pGFP-53BP2S by electroporation as described under “Experimental Procedures.” After a 2-h serum starvation, quiescent cells were stimulated with insulin (100 nM) for 2 min. Cells were immunostained with anti-phospho-Akt-specific antibody (Ser-473), and the ratio of the cells displaying Akt staining on the plasma membrane. Transfected GFP or GFP-53BP2S was visualized in the confocal microscope in green. Activated Akt was visualized by Texas Red in red. These are representative data independently performed three times. IF, immunofluorescence. C, fully differentiated 3T3-L1 adipocytes were transfected with 200 μg of pGFP and 50 μg of pHA-Akt2 or 200 μg of pEGFP-53BP2S and 50 μg of pHA-Akt2 by electroporation. After 18 h of serum starvation, quiescent cells were stimulated with insulin (100 nM) for the indicated time. Cell lysates were prepared from each cells and immunoprecipitated (IP) by anti-hemagglutinin antibody. Immunoprecipitants were separated by SDS-PAGE and immunoblotted (IB) with anti-Akt antibody or anti-phospho-Akt-specific antibody (Ser-473). These are representative immunoblots independently performed three times.
p85 PI 3-kinase regulatory subunit result in the preferential association of the catalytic subunit with p50 and enhanced insulin sensitivity (51). Thus, adequate cellular distribution or isomorph-selective activation of IRS, PI 3-kinase, and Akt could be disturbed by overexpression of 53BP2S. This disturbance could account for the inability of activated Akt to phosphorylate AS160. In addition, several studies have suggested the presence of additional signaling pathways that may function in concert with IRS-1/PI 3-kinase signaling through caveolin-enriched lipid raft microdomains (52, 53). However, we have not assessed the effect of 53BP2S on lipid raft-dependent signaling, there is no evidence that the IRS/PI 3-kinase functions through these microdomains (52). However, it is more likely that 53BP2S inhibition could occur by blocking additional IRS-1-mediated signals. In addition, we could not rule out the possibility that 53BP2S inhibited the Akt downstream pathway required for Glut4 translocation independent of modulation of IRS-mediated insulin signals.

53BP2S and ASPP2 protein has been also isolated as a binding partner of many proteins, including p53, Bcl2, NF-κB, PP1, and APLC (33, 37, 39, 54, 55), suggesting that 53BP2S/ASPP2 is involved in apoptotic pathways. IGF-I and IRS-1 are also well known to have anti-apoptotic activity. Ueno et al. (56) showed that IRS-1 interacts with Bcl-2 and has an anti-apoptotic effect. It is possible that Bcl-2 and 53BP2S/ASPP2 compete each other for interacting with IRS and regulate anti-apoptotic role IRS proteins. Whether the interaction between IRS-1 and 53BP2S/ASPP2 is involved in apoptosis awaits further study.

In summary, we have found that 53BP2S protein directly interacts with IRS family proteins both in pulldown and co-immunoprecipitation assays dependent of IRS tyrosine phosphorylation, and we have identified the specific binding regions responsible. Importantly, 53BP2S function to regulate IRS isoform tyrosine phosphorylation induced by insulin, resulting in modulation of insulin signals. These lead to attenuation of Glut4 translocation to the plasma membrane by as of yet unknown mechanisms.

Acknowledgments—We thank Dr. Takashi Kadowaki (Graduate School of Medicine, the University of Tokyo, Tokyo, Japan) for the kind gift of rat IRS-1 cDNA and Dr. Louie Naumovski (Stanford University School of Medicine, Stanford, CA) for the kind gift of 53BP2S cDNA. We thank Dr. Tomoichiro Asano (Graduate School of Frontier Sciences, the University of Tokyo, Japan) for the kind gift of COS-7 cells and Dr. Minoru Yoshida (RIKEN, Saitama, Japan) for the kind gift of CHO-C400 cells. We also express our appreciation to Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan) for leupeptin and pepstatin.

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