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# A Novel Membrane Glycoprotein, SHPS-1, That Binds the SH2-Domain-Containing Protein Tyrosine Phosphatase SHP-2 in Response to Mitogens and Cell Adhesion

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**Protein tyrosine phosphatases (PTPases), such as SHP-1 and SHP-2, that contain Src homology 2 (SH2) domains play important roles in growth factor and cytokine signal transduction pathways. A protein of ~115 to 120 kDa that interacts with SHP-1 and SHP-2 was purified from v-*src*-transformed rat fibroblasts (SR-3Y1 cells), and the corresponding cDNA was cloned. The predicted amino acid sequence of the encoded protein, termed SHPS-1 (SHP substrate 1), suggests that it is a glycosylated receptor-like protein with three immunoglobulin-like domains in its extracellular region and four YXX(L/V/I) motifs, potential tyrosine phosphorylation and SH2-domain binding sites, in its cytoplasmic region. Various mitogens, including serum, insulin, and lysophosphatidic acid, or cell adhesion induced tyrosine phosphorylation of SHPS-1 and its subsequent association with SHP-2 in cultured cells. Thus, SHPS-1 may be a direct substrate for both tyrosine kinases, such as the insulin receptor kinase or Src, and a specific docking protein for SH2-domain-containing PTPases. In addition, we suggest that SHPS-1 may be a potential substrate for SHP-2 and may function in both growth factor- and cell adhesion-induced cell signaling.**

SHP-2 (also named SH-PTP2, PTP1D, Syp, PTP2C, SH-PTP3, and SAP-2) (1–3, 9, 10, 28, 56), is a non-transmembrane protein tyrosine phosphatase (PTPase) that contains two Src homology 2 (SH2) domains (17) and is thought to participate in intracellular signaling in response to various growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin (26, 27). SHP-2 binds to tyrosine-phosphorylated PDGF receptors in response to PDGF (9, 15, 22) and also to tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) in response to insulin (20). In addition, it has been demonstrated that expression of a catalytically inactive SHP-2 inhibited the activation of RAS and mitogen-activated protein (MAP) kinase in response to insulin in a dominant negative manner (33). The expression of a catalytically inactive SHP-2 also inhibited the activation of MAP kinase in response to insulin (31, 61) or fibroblast growth factor (50), indicating that SHP-2 mediates growth factor stimulation of the RAS-MAP kinase cascade that leads to DNA synthesis (59). It has also been suggested that SHP-2 may play an important role in EGF-stimulated MAP kinase activation and mitogenesis (4, 62). However, the mechanism by which SHP-2 mediates RAS-MAP kinase cascade activation in response to insulin or other mitogens is largely unknown. Furthermore, the site at which SHP-2 may act in the RAS-MAP kinase cascade is still controversial. In contrast to our previous observation for Chinese hamster ovary (CHO) cells that overexpress human insulin receptors (IRs) (CHO-IR cells) (33), Sawada et al. have reported that expression of a catalytically inactive SHP-2 inhibited the activation of MEK and Raf-1 kinase in response to insulin and had no detectable effect on insulin-induced activation of RAS in NIH 3T3 cells overexpressing human IRs (39). It has been suggested that *Corkscrew* (the putative *Drosophila* homolog of SHP-2) may be required upstream for *Ras1* activation or that it functions in conjunction with *Ras1* during *Sevenless* receptor tyrosine kinase signaling (12). Thus, the identification of a phosphorylated substrate of SHP-2 is essential for understanding the SHP-2-mediated signaling pathway.

It has recently been shown that insulin induces tyrosine phosphorylation of an ~115-kDa membrane glycoprotein, pp115, and subsequent association of SHP-2 with pp115 in CHO-IR cells (32). The extent of tyrosine phosphorylation of pp115 was greatly increased, relative to that in CHO-IR cells, in CHO-IR cells that also overexpress catalytically inactive SHP-2 (27, 32). The lack of PTPase activity of the mutant SHP-2 may result in its forming a stable complex with tyrosine-phosphorylated pp115, suggesting that pp115 may be a physiological substrate for SHP-2 and that it may mediate SHP-2 signaling to downstream components. It has also been reported that transient expression of a catalytically inactive SHP-2 induces hyperphosphorylation of a 120-kDa protein which binds to the fusion protein containing SH2 domains of SHP-2 in NIH 3T3 cells overexpressing human IRs (31). SHP-2 has also been demonstrated to bind to a tyrosine-phosphorylated 115-kDa protein in response to insulin (63) or EGF (62), while this 115-kDa protein did not bind to a fusion protein containing SH2 domains of SHP-2 (63).

It has also been demonstrated that SHP-1, another SH2-domain-containing PTPase (1, 30, 37, 43, 64), formed a complex with an ~120-kDa tyrosine-phosphorylated protein, pp120, when SHP-1 was overexpressed in v-*src*-transformed rat fibroblasts (SR-3Y1 cells) (29). SHP-1 is expressed predominantly in hematopoietic cells and is thought to play an inhibitory role in cytokine-stimulated proliferation of these cells (16,

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26, 44, 51, 65). Although the physiological substrate of SHP-1 remains to be identified, pp120 is a potential candidate.

We have now purified pp120 from SR-3Y1 cell membranes and cloned its cDNA. Because pp120 is a potential substrate for SHP-1 and SHP-2, we named this protein SHPS-1 (SHP substrate 1). SHPS-1 possesses immunoglobulin (Ig)-like domains in its putative extracellular region and potential binding sites for the SH2 domains of SHP-1 and SHP-2 in its cytoplasmic region. In addition, we show that SHP-2 binds to tyrosine-phosphorylated SHPS-1 in intact cells in response to mitogens and cell adhesion.

## MATERIALS AND METHODS

**Cells and antibodies.** 3Y1 rat fibroblast cells and SR-3Y1 cells were obtained from the Japanese Cancer Research Resources Bank. SR-3Y1 cells that overexpress either SHP-1 (SR-3Y1-P cells) or SHP-1 lacking SH2 domains (SR-3Y1-C cells) were generated as previously described (29). CHO-IR cells that overexpress either wild-type SHP-2 (CHO-SHP-2-WT cells) or catalytically inactive SHP-2 (CHO-SHP-2-C/S cells) were generated as previously described (33).

To generate polyclonal antibodies to SHPS-1, we first prepared a cDNA fragment encoding the putative cytoplasmic region of SHPS-1 by PCR amplification with a sense primer (5′-TTGGATCCAAGAAAGCCAAGGGCTCAAC TTCT; nucleotides [nt] 1246 to 1269), an antisense primer (5′-AAGAATTCTC ACTTCCTCTGGACTTGGACACT; nt 1552 to 1575), and the full-length rat SHPS-1 cDNA as a template, as described previously (33). The amplified PCR fragment was inserted in frame into the *Bam*HI and *Eco*RI site of pGEX-2T (Pharmacia), and the glutathione-*S*-transferase (GST) fusion protein containing the cytoplasmic region of SHPS-1 was expressed in *Escherichia coli* and purified. Female rabbits were injected with the GST fusion protein, and polyclonal antibodies were affinity purified with CNBr-activated Sepharose beads (Pharmacia) coupled to the GST–SHPS-1 protein as described previously (33).

Monoclonal antibody to pp115 of CHO cells, 4C6, was generated by injecting mice with pp115 partially purified from CHO-IR cells as described elsewhere (32). This monoclonal antibody reacts well with pp115 of CHO cells but poorly with the corresponding protein of other species, such as rats or mice.

Rabbit polyclonal antibodies to SHP-1 were generated against a synthetic peptide corresponding to the COOH-terminal region of SHP-1 as described previously (52). Rabbit polyclonal antibodies to SHP-2 were generated against a GST fusion protein containing the COOH-terminal region of SHP-2 as described previously (33). The PY-20 monoclonal antibody to phosphotyrosine was obtained from Transduction Laboratories. Monoclonal antibody 9E10, to MYC epitope tag, was purified from the ascites of mice injected with MYC1-9E10 hybridoma cells that were obtained from American Type Culture Collection. Monoclonal antibody to v-Src (Ab-1) was obtained from Oncogene Science.

**Immunoprecipitation and immunoblot analysis.** SR-3Y1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Confluent cells (10-cm-diameter plates) were washed with phosphate-buffered saline (PBS) and immediately frozen in liquid nitrogen. The cells were then lysed on ice in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl [pH 7.6], 140 mM NaCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40, 10% [vol/vol] glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium vanadate. The lysates were centrifuged at 10,000 × *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis. Supernatants were incubated for 4 h at 4°C with various antibodies bound to protein G-Sepharose beads (2 μg of antibody on 20 μl of beads) (Pharmacia) in the presence or absence of the immune antigen. For in vitro binding experiments, the supernatants prepared from SR-3Y1 cells were also incubated with various GST fusion proteins immobilized on glutathione-Sepharose beads (Pharmacia). The beads were then washed twice with 1 ml of WG buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.6], 150 mM NaCl, 0.1% Triton X-100) and resuspended in sodium dodecyl sulfate (SDS) sample buffer. Gel electrophoresis and immunoblot analysis with PY-20 or other antibodies and an enhanced chemiluminescence detection kit (Amersham) were performed as described previously (29, 33).

SR-3Y1 cells were also incubated in the absence or presence of tunicamycin (5 μg/ml) (Sigma) for 48 h at 37°C. Solubilized membrane fractions were prepared as described below and incubated with GST–SHP-1 fusion protein immobilized on beads. The bound proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with PY-20.

**Expression and purification of recombinant PTPases.** Recombinant full-length SHP-1 was generated with the use of the GST fusion protein system. PCR amplification was performed with a sense primer (5′-ATTGAATTCTGTCCCG TGGGTGGTTTCAC; nt 256 to 279), an antisense primer (5′-ACCGAATTCT CACTTCCTCTTGAGGGAACC; nt 2029 to 2049), and the full-length SHP-1 cDNA as a template (53). In addition, an SHP-1 cDNA lacking the sequence encoding the SH2 domains was constructed by PCR amplification of the portion of the full-length cDNA coding for the PTPase domain and the COOH-terminal region; full-length cDNA served as the template, the sense primer was 5′-TTA GAATTCACCATGAACTTGCACCAGCGTCTGGAA (nt 1033 to 1053), and the antisense primer was 5′-TATGAATTCTCACTTCCTCTTGAGGGAACC (nt 2029 to 2049). The amplified PCR fragments were digested with *Bam*HI and *Eco*RI and inserted in frame into the *Bam*HI and *Eco*RI site of pGEX-2T. The GST fusion protein containing full-length SHP-1 (GST–SHP-1) or that containing SHP-1 lacking SH2 domains (GST–SHP-1ΔSH2) was expressed in *E. coli* (10-ml culture) as described previously (53), and the cells were immobilized on glutathione-Sepharose beads, which were then subjected to in vitro binding experiments. Incubation of cell lysate supernatants with the beads was performed under the same conditions as for immunoprecipitation.

GST–SHP-1 was also isolated from 0.5 to 2 liters of bacterial culture as described previously (33) and bound to glutathione-Sepharose beads. The beads were washed twice with 10 ml of NETN lysis solution (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and the bound proteins were then eluted by incubation with 7 ml of a solution containing 50 mM Tris-HCl (pH 8.0) and 10 mM glutathione (Sigma) for 30 min at 4°C. Proteins eluted from the beads were dialyzed overnight against 1 liter of a solution containing 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol and then concentrated with a Centriprep-30 filtration cell (Amicon).

Expression and purification of GST fusion proteins containing full-length SHP-2 (GST–SHP-2), the SH2 domains of SHP-2 (GST–SHP-2-SH2), or SHP-2 lacking SH2 domains (GST–SHP-2ΔSH2) were performed as previously described (33).

**Subcellular fractionation.** Confluent cells (10-cm-diameter plate) were washed with ice-cold PBS, frozen in liquid nitrogen, scraped into 1 ml of ice-cold hypotonic lysis solution, which consisted of 20 mM HEPES-NaOH (pH 7.6), 5 mM $NaPP_i$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1 mM $MgCl_2$, containing aprotinin (10 μg/ml), 1 mM PMSF, and 1 mM sodium vanadate, and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the resulting supernatant was referred to as the cytosolic fraction. The pellet was suspended in 1 ml of membrane solubilization solution (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 150 mM NaCl, 1 mM $MgCl_2$) supplemented with 1 mM PMSF and 1 mM sodium vanadate. The suspension was centrifuged at 100,000 × *g* for 60 min, and the resulting supernatant was referred to as the solubilized membrane fraction. All procedures were performed at 4°C.

**Affinity purification of pp120.** Confluent SR-3Y1 cells from a total of 100 culture dishes (175 $mm^2$) were washed with ice-cold PBS and immediately frozen in liquid nitrogen. Subsequent procedures were performed at 4°C unless indicated otherwise. The cells were scraped with a rubber policeman on ice into a total volume of 150 ml of hypotonic lysis solution containing aprotinin (10 μg/ml), 1 mM PMSF, and 1 mM sodium vanadate. After addition of NaCl to a final concentration of 500 mM, the cells were homogenized with a Dounce homogenizer (100 strokes). The homogenates were then centrifuged at 100,000 × *g* for 60 min, and the resulting pellets were resuspended in 30 ml of Triton lysis solution (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol) supplemented with 1 mM PMSF and 1 mM sodium vanadate. The suspension was again homogenized with a Dounce homogenizer (100 strokes) and centrifuged at 100,000 × *g* for 60 min, and the resulting supernatant was passed through a 0.8-μm-pore-size membrane filter (Amicon). The filtrate was incubated with 100 μl of CNBr-activated Sepharose beads coupled with the GST–SHP-1 fusion protein (~1 μg of protein coupled to 1 μl of beads) in two 15-ml tubes with gentle rotation for 4 to 12 h. The beads were washed with Triton lysis solution in the 15-ml tubes, transferred to a 1.5-ml Eppendorf tube, and washed with 1 ml of Triton wash buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM EDTA, and 500 mM NaCl, with rotation, for 1 to 2 h at 25°C. The bound proteins were then eluted with 500 μl of SDS elution buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 0.1% SDS) at 25°C. After the addition of $MnCl_2$ to a final concentration of 1 mM, the eluate was incubated for 2 h with 100 μl of agarose beads coupled with concanavalin A (ConA) (Seikagaku Kogyo Co.). The beads were washed twice with 1 ml of Triton wash buffer, and the bound proteins were eluted with 500 μl of Tween buffer (10 mM Tris-HCl [pH 7.5], 0.1% Tween 20, 150 mM NaCl) containing 200 mM α-methyl-D-mannoside (Wako). The eluted proteins were then precipitated with 10% (wt/vol) trichloroacetic acid, washed once with cold (−20°C) acetone, and dried with a Speed-Vac concentrator.

In a separate experiment, a solubilized membrane fraction of SR-3Y1 cells was incubated with GST–SHP-1 immobilized on glutathione-Sepharose beads; thereafter, the beads were washed and the bound proteins were eluted with SDS elution buffer as described above. The eluted proteins were then incubated with agarose beads conjugated with wheat germ agglutinin (WGA), *Lens culinaris* agglutinin (LCA), or *Ricinus communis* agglutinin (RCA) (Seikagaku Kogyo Co.) under the same conditions used for ConA. The proteins bound to the lectin-coupled beads were separated by SDS-PAGE and subjected to immunoblot analysis with PY-20.

**Separation of pp120 by SDS-PAGE and internal amino acid sequencing.** Affinity-purified pp120 was dissolved in SDS sample buffer, subjected to SDS-PAGE in a 7.5% gel, and transferred to a polyvinylidene difluoride filter (ProBlott; Applied Biosystems). Proteins were visualized by being stained with 0.1% Ponceau S in 1% acetic acid, and the band corresponding to pp120 was excised for amino acid sequence analysis. Confluent SR-3Y1 cells from a total of 1,000

culture dishes (175 $mm^2$) were required to obtain sufficient pp120 for amino acid sequencing.

After reduction and S-carboxymethylation, the immobilized protein on the membrane filter was digested with 0.2 pmol of *Achromobacter* protease I (lysylendopeptidase) (Wako), essentially as described previously (13). Generated peptides were separated by high-performance liquid chromatography with a Wakosil-II AR octyldecyl silane column (2.0 by 150 mm) (Wako). Amino acid sequencing of the peptides was performed with a gas-phase sequencer (model PPSQ-10; Shimazu). The resultant phenylthiohydantoin derivatives were identified by isocratic high-performance liquid chromatography as described previously (13).

**Cloning of SHPS-1 cDNAs.** Eight amino acid sequences obtained from the 12 pp120 peptides isolated were used to design degenerate oligonucleotide primers for PCR. DNA fragments were amplified by PCR with all possible combinations of degenerate primers and rat brain cDNA (Clontech) as a template. PCR was performed for three cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1.5 min, and extension at 72°C for 2 min, followed by 37 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min. A 168-bp PCR fragment (nt 289 to 456 of the cDNA sequence) was obtained with a sense primer [5′-TT(T/C)ATIG GIGGIGA(A/G)CA(T/C)TT] derived from the peptide AP-11 and an antisense primer [5′-TTIAT(C/T)TCIGT(G/A)TCIGG(C/T)TC] derived from the peptide AP-6. This fragment was chosen as a screening probe because it contained two amino acid sequences in the same coding frame as determined by sequence analysis. A rat 3Y1 cell ZAP-II cDNA library (kindly provided by H. Nojima) was then screened as previously described (28), and the nucleotide sequence of the longest cDNA clone was determined in both directions by dideoxy termination methods with a Sequencing Pro kit (TOYOBO). Screening of a mouse brain ZAP-II cDNA library (Stratagene) and a human brain λgt10 cDNA library (Clontech) with $^{32}$P-labeled rat SHPS-1 cDNA as a probe yielded a 3.0-kb mouse full-length SHPS-1 cDNA and a 1.6-kb human partial SHPS-1 cDNA, both of which were isolated and sequenced.

**Northern (RNA) blot analysis.** A rat multiple-tissue Northern blot (Clontech) containing 2 μg of poly(A)$^+$ RNA was hybridized consecutively with a $^{32}$P-labeled 3.7-kb cDNA fragment of rat SHPS-1 cDNA and a $^{32}$P-labeled mouse β-actin cDNA probe.

**Transfection of SHPS-1 cDNA.** Full-length rat SHPS-1 cDNA was inserted into the *Eco*RI site in the pSRα expression vector (pSRα-SHPS-1). To construct a pSRα expression vector encoding MYC epitope-tagged SHPS-1 (pSRα-SHPS-1-MYC), we performed PCR with a pBluescript vector (Stratagene) containing full-length rat SHPS-1 cDNA as a template, T3 primer as the sense primer, and the antisense primer 5′-GTGAATTCTCAGAGGTCTTCTTCCGATATCAGC TTCTGTTC, which results in deletion of the natural termination codon of SHPS-1 cDNA and addition of another sequence encoding the amino acids EQKLISEEDL followed by a termination codon and an *Eco*RI site. The amplified DNA fragment was digested with *Eco*RI and inserted into the *Eco*RI site of pSRα, yielding pSRα-SHPS-1-MYC.

Semiconfluent SR-3Y1 cells cultured in DMEM supplemented with 10% FBS were transfected with 10 μg of pSRα-SHPS-1 with the use of Lipofectamine (Gibco). Cells were harvested 2 days after transfection and lysed as described above for immunoblot analysis with antibodies to SHPS-1.

CHO-IR cells (~$5 \times 10^5$ cells per 10-cm-diameter dish) were transfected with both 10 μg of pSRα-SHPS-1-MYC and 1 μg of pHyg, which contains the hygromycin B phosphotransferase gene, by the calcium phosphate precipitation method (33). The cells were cultured in Ham's F-12 medium containing hygromycin B (200 μg/ml) (Wako) and 10% FBS, and colonies were isolated 14 to 21 days after transfection. Several cell lines expressing MYC epitope-tagged SHPS-1 (CHO-IR-SHPS-1) were identified by immunoblotting cell lysates with polyclonal antibody to SHPS-1 as described above.

**Stimulation of cells with mitogens and cell adhesion.** CHO-IR cells or CHO-IR-SHPS-1 cells cultured in Ham's F-12 medium containing 10% FBS were deprived of serum for 16 h and then stimulated with 100 nM insulin for 5 min. Confluent Rat-1-IR cells cultured in DMEM supplemented with 10% FBS were also deprived of serum and stimulated with mitogens. After stimulation, the culture medium was aspirated and the cells were immediately washed with ice-cold PBS and frozen in liquid nitrogen. Cell lysates were prepared and subjected to immunoprecipitation and immunoblot analysis as described above.

For adhesion experiments, CHO-IR cells were treated with PBS containing 1 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$ or with PBS without $Ca^{2+}$ and $Mg^{2+}$ for 10 min as described previously (24), after which lysates were prepared. Confluent CHO-IR cells were also detached by treatment with 0.05% trypsin and 0.02% EDTA, washed three times with serum-free Ham's F-12 medium, plated on polystyrene dishes which were coated with fibronectin (Falcon) or uncoated, and incubated at 37°C for 1 h in serum-free Ham's F-12 medium as described previously (35). Bound cells were then lysed and subjected to immunoprecipitation and immunoblot analysis.

**In vitro phosphorylation of the cytoplasmic portion of SHPS-1 by IRs and its binding to SH2 domains of SHP-2.** IRs were partially purified from lysates of insulin-stimulated CHO-IR cells with WGA-agarose as described previously (14). The GST fusion protein containing the cytoplasmic region of SHPS-1, purified as described above (~1 μg), was incubated for 30 min at 25°C with partially purified IRs immobilized on WGA-agarose beads in 50 μl of a solution containing 50 mM HEPES-NaOH (pH 7.6), 3 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μM ATP. The supernatant of the reaction mixture was separated from WGA-agarose beads by centrifugation at 10,000 × *g* for 5 min. Thereafter, the supernatant was incubated with 10 μl of CNBr-activated Sepharose beads coupled with a GST protein or a GST fusion protein containing SH2 domains of SHP-2 (~0.5 μg of protein coupled to 1 μl of beads) for 4 h at 4°C. The beads were washed three times with 1 ml of WG buffer, and the proteins bound to GST protein beads were separated by SDS-PAGE and subjected to immunoblot analysis with PY-20.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D85183.

## RESULTS

**The pp120 protein that binds to SHP-1 in SR-3Y1 cells is a membrane glycoprotein.** As has been shown previously (29), an ~120-kDa tyrosine-phosphorylated protein, pp120, was coimmunoprecipitated with tyrosine-phosphorylated SHP-1 in SR-3Y1-P cells (Fig. 1A, lane 2). The pp120 protein did not form a complex with an SHP-1 protein lacking SH2 domains in SR-3Y1-C cells (Fig. 1A, lane 3). On incubation of SR-3Y1 cell lysates with a GST fusion protein containing either full-length SHP-1 (GST–SHP-1) or SHP-1 lacking SH2 domains (GST–SHP-1ΔSH2), a 120-kDa tyrosine-phosphorylated protein bound to GST–SHP-1 but not to GST alone or GST–SHP-1ΔSH2 (Fig. 1A, lanes 4 to 6). These data suggest that SHP-1 forms a complex with pp120 both in vivo and in vitro, presumably through its SH2 domains.

To determine the subcellular localization of pp120, we prepared cytosolic and solubilized membrane fractions of SR-3Y1-P cells. Each fraction was subjected to immunoprecipitation with antibodies to SHP-1 and immunoblot analysis with the PY-20 monoclonal antibody to phosphotyrosine. Src, which has been known to associate with the cell membrane, was recovered in the membrane fraction (Fig. 1B, lanes 5 and 6). The pp120 protein was recovered exclusively in the membrane fraction (Fig. 1B, lanes 1 and 2). The same result was obtained when subcellular fractions prepared from SR-3Y1 cells were incubated with GST–SHP-1 and the bound proteins were subjected to analysis with PY-20 (Fig. 1B, lanes 3 and 4).

The diffuse nature of the pp120 band on gel electrophoresis suggested that pp120 might be a glycosylated protein. When pp120 bound to GST–SHP-1 was eluted and then incubated with various lectins coupled to agarose beads, the amount of pp120 recovered was greatest with ConA- or WGA-coupled beads, with lesser amounts binding to LCA- or RCA-coupled beads (Fig. 1C). In addition, incubation of SR-3Y1 cells with tunicamycin (5 μg/ml) for 48 h reduced the apparent molecular size of pp120 from 120 to ~60 kDa (Fig. 1D). These results thus suggest that pp120 may be a membrane-associated glycoprotein.

**SHP-2 binds to pp120 in vitro.** Because SHP-2 is structurally similar to SHP-1, it is possible that SHP-2 also binds to pp120 in SR-3Y1 cells. A 120-kDa tyrosine-phosphorylated protein present in the solubilized membrane fraction of SR-3Y1 cells bound to GST–SHP-2 as well as to GST–SHP-1 (Fig. 2, lanes 1 and 2). In addition, pp120 bound to a GST fusion protein containing the SH2 domains of SHP-2 (GST–SHP-2-SH2) but not to GST–SHP-2 fusion protein lacking the SH2 domains (GST–SHP-2ΔSH2) (Fig. 2, lanes 3 and 4), suggesting that the SH2 domains of SHP-2 may mediate the association of SHP-2 with pp120.

**Affinity purification and amino acid sequence analysis of pp120.** Given that pp120 from SR-3Y1 cells was shown to bind to both GST-SHP-1 and ConA, we attempted to purify this protein from SR-3Y1 cell membranes by taking advantage of these properties. SR-3Y1 cells from 100 culture dishes (175 $mm^2$) were scraped into a hypotonic solution. After addition of

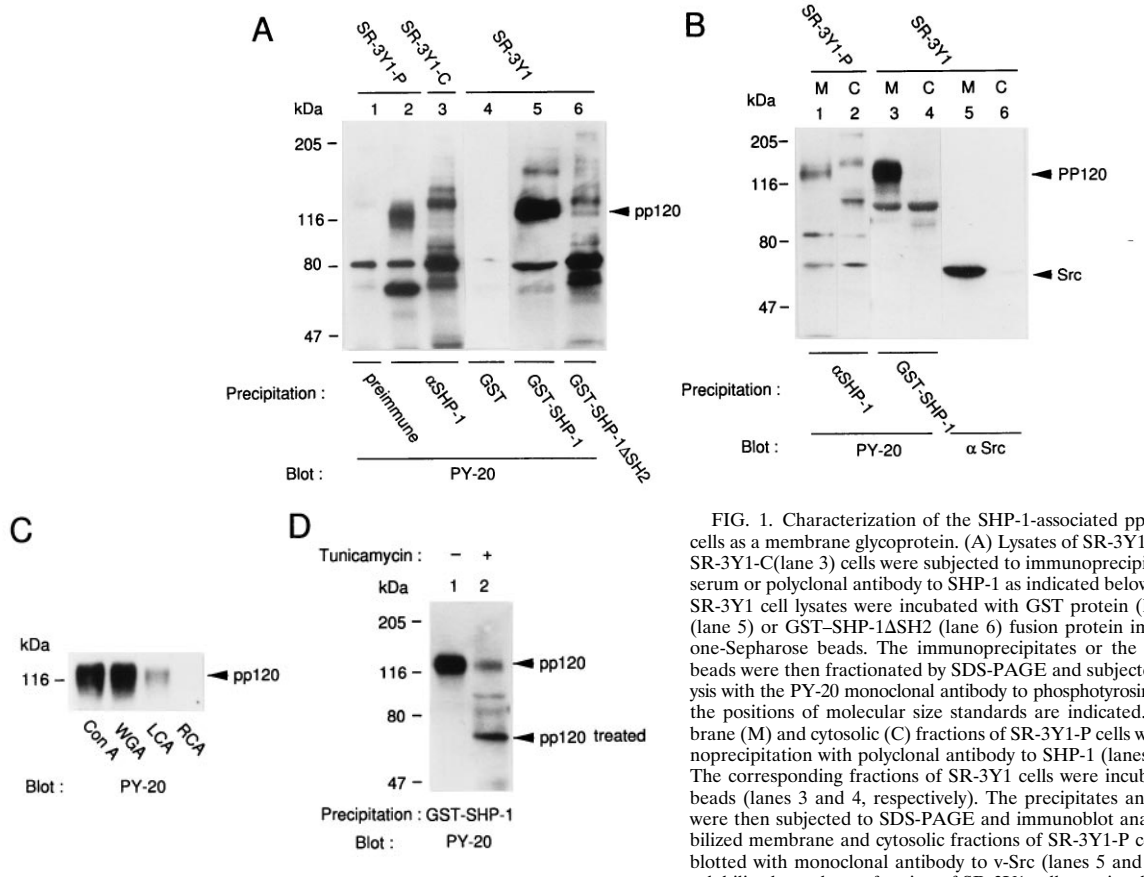


FIG. 1. Characterization of the SHP-1-associated pp120 protein in SR-3Y1 cells as a membrane glycoprotein. (A) Lysates of SR-3Y1-P (lanes 1 and 2) or of SR-3Y1-C(lane 3) cells were subjected to immunoprecipitation with preimmune serum or polyclonal antibody to SHP-1 as indicated below the lanes. In addition, SR-3Y1 cell lysates were incubated with GST protein (lane 4) or GST–SHP-1 (lane 5) or GST–SHP-1ΔSH2 (lane 6) fusion protein immobilized on glutathione-Sepharose beads. The immunoprecipitates or the proteins bound to the beads were then fractionated by SDS-PAGE and subjected to immunoblot analysis with the PY-20 monoclonal antibody to phosphotyrosine. The pp120 band and the positions of molecular size standards are indicated. (B) Solubilized membrane (M) and cytosolic (C) fractions of SR-3Y1-P cells were subjected to immunoprecipitation with polyclonal antibody to SHP-1 (lanes 1 and 2, respectively). The corresponding fractions of SR-3Y1 cells were incubated with GST–SHP-1 beads (lanes 3 and 4, respectively). The precipitates and bead-bound proteins were then subjected to SDS-PAGE and immunoblot analysis with PY-20. Solubilized membrane and cytosolic fractions of SR-3Y1-P cells were also immunoblotted with monoclonal antibody to v-Src (lanes 5 and 6, respectively). (C) A solubilized membrane fraction of SR-3Y1 cells was incubated with GST–SHP-1 immobilized on glutathione-Sepharose beads. The beads were washed and the bound proteins were eluted with SDS elution buffer as described in Materials and Methods. The eluted proteins were then incubated with agarose beads conjugated with ConA, WGA, LCA, or RCA. The proteins bound to the lectin-coupled beads were separated by SDS-PAGE and subjected to immunoblot analysis with PY-20. (D) SR-3Y1 cells were incubated in the absence or presence of tunicamycin (5 μg/ml) for 48 h at 37°C. Solubilized membrane fractions were prepared and incubated with GST–SHP-1 immobilized on beads. The bound proteins were subjected to SDS-PAGE and immunoblot analysis with PY-20.

500 mM NaCl, which markedly reduced the amount of proteins associated with membranes, the cells were homogenized and separated into cytosolic and membrane fractions. The membrane fraction was solubilized in a buffer containing 1% Triton X-100 and then incubated with GST–SHP-1 immobilized on CNBr-activated Sepharose beads. Of several conditions tested for elution of pp120 from the GST–SHP-1 beads, we chose a solution containing 0.1% SDS and 0.1% Triton X-100 at 25°C because it resulted in efficient and the most specific elution of pp120. We have also used immobilized GST–SHP-2 to purify pp120; however, the results were inferior to those obtained with GST–SHP-1 because GST–SHP-2 binds many more proteins than GST–SHP-1, as revealed by silver staining (data not shown). The proteins eluted from GST–SHP-1 beads were then incubated with agarose beads conjugated with ConA. After extensive washing of the beads, bound proteins were eluted with α-methyl-D-mannoside. The proteins were then precipitated with trichloroacetic acid, separated on a 7.5% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane filter, and stained with Ponceau S. A major band corresponding to pp120 and several minor bands were detected (Fig. 3A).

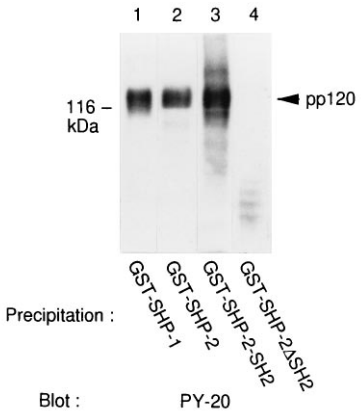


FIG. 2. Binding of SHP-2 to pp120 in vitro. The solubilized membrane fraction of SR-3Y1 cells was incubated with the indicated GST fusion proteins immobilized on beads. The bound proteins were then subjected to SDS-PAGE and immunoblot analysis with PY-20.

We repeated the purification procedure a total of 10 times to obtain sufficient protein for amino acid sequence analysis. We finally accumulated ~5 μg of purified pp120, as estimated by the intensity of the band stained with Ponceau S. The purified protein was then subjected to internal amino acid sequencing. More than 20 peptide fractions were obtained after digestion of pp120 with *Achromobacter* protease I (Fig. 3B), and nine single and three mixed sequences were determined (Table 1). Searching the Swiss Prot database with the BLAST program

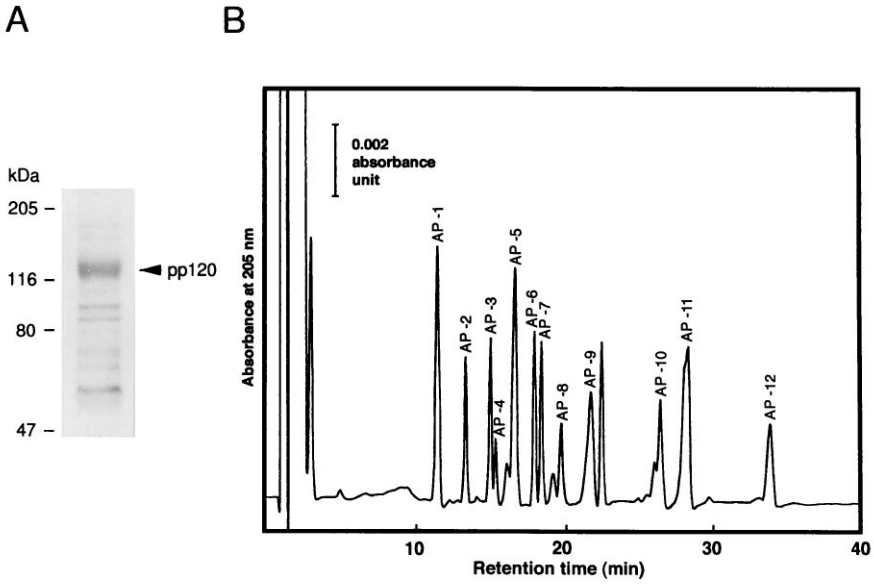


FIG. 3. Purification and protease digestion of pp120. (A) The pp120 protein was isolated from 100 culture dishes of confluent SR-3Y1 cells by a two-step affinity purification protocol as described in Materials and Methods. The purified protein was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Ponceau S. The band corresponding to pp120 is indicated. (B) Purified pp120 was digested with *Achromobacter* protease I and the resulting peptides were separated by reversed-phase high-performance liquid chromatography. Elution of the peptides was monitored by $A_{205}$. The amino acid sequences of the peptides in peaks AP-1 to AP-12 were determined by phenylthiohydantoin derivative analysis.

revealed that none of the peptide sequences was identical to listed sequences, indicating that pp120 is a previously uncharacterized protein. Because it is a potential target for SH2-domain-containing PTPases, we designated pp120 SHPS-1, for SHP substrate 1.

**Cloning of SHPS-1 cDNA.** The amino acid sequences obtained from purified SHPS-1 were used to design degenerate oligonucleotide primers for the PCR. Screening of a rat 3Y1 cell ZAP-II cDNA library with a 168-bp $^{32}$P-labeled PCR DNA fragment as a probe yielded a full-length SHPS-1 cDNA. The cloned SHPS-1 cDNA comprised 3,728 nt and contained a single open reading frame encoding a protein of 509 amino acids (nt 46 to 1572) (Fig. 4A). The first ATG codon (nt 46 to 48) matched the Kozak consensus sequence (19) for a translation initiation site and was presumed to be the translation initiation codon. The 3′ noncoding region of the cDNA contains a typical polyadenylation signal (AATAAA) followed by a poly(A) tail. The predicted amino acid sequence revealed that residues 344 to 368 are highly hydrophobic, indicating that SHPS-1 is a transmembrane protein, as expected. In addition, the $NH_2$-terminal 28 amino acids are also hydrophobic and likely constitute a signal peptide (57). The entire putative extracellular region of SHPS-1 contains three homologous Ig-like domains, indicating that SHPS-1 is a member of the Ig superfamily. Three types of Ig superfamily domain, V, C1, and C2, have been differentiated (58). BLAST sequence analysis of the Swiss Prot database revealed that the $NH_2$-terminal Ig-like domain of SHPS-1 is homologous to an Ig V domain, whereas the second and third SHPS-1 domains resemble an Ig C1 domain. The extracellular region of SHPS-1 also contains 15 potential N-linked glycosylation sites (NXS or NXT, where X is any amino acid), consistent with the observation that SHPS-1 is highly glycosylated. In the cytoplasmic region of SHPS-1, four tyrosine residues followed by XX(L/V/I) sequences (Y408ADL, Y432ASIE, Y449ADL, and Y473ASV) represent potential tyrosine phosphorylation sites. Several putative serine-threonine phosphorylation sites are also present in this region. The sequences downstream of the potential tyrosine phosphorylation sites of SHPS-1 correspond well to binding sites for SHP-2 and SHP-1 (7, 16, 45, 46, 48). BLAST analysis showed that the sequences surrounding Tyr-408 and Tyr-449 of SHPS-1 are similar to the sequence surrounding Tyr-1172 of IRS-1 (Fig. 4B). In addition, the sequences surrounding Tyr-432 and Tyr-473 in SHPS-1 resemble that surrounding Tyr-1222 of IRS-1. Furthermore, the sequences surrounding Tyr-408 and Tyr-449 of SHPS-1 resemble each other, as do those surrounding Tyr-432 and Tyr-473 (Fig. 4B).

TABLE 1. Amino acid sequences of peptides derived from purified pp120[a]

| Peptide | Sequence |
|---|---|
| AP-1 | (K) GSTSSTRLHEp |
| | FYPK |
| AP-2 | (K) WLK |
| AP-3 | (K) WFK |
| AP-4 | (K) KPAPRVXE |
| AP-5 | (K) SXVSYXISs |
| | PSSPEVsXP |
| AP-6 | (K) GIVEPDTEIK |
| AP-7 | (K) ELSHLETtISs |
| AP-8 | (K) ITQqPLTPASXV |
| AP-9 | (K) SYGFSPRXIXLk |
| AP-10 | (K) SVSVAAGDSATLXCTVSsL |
| AP-11 | (K) GEGQNRSPIYSFIGGEHFPrI |
| AP-12 | (K) LSPEDIHSRVICEVAHVXle |

[a] Peptides generated by digestion of purified pp120 with *Achromobacter* protease I were subjected to $NH_2$-terminal sequence analysis. Designations of peptide fragments correspond to the absorbance peaks in Fig. 3B. Where two sequences are shown for the same peak, the residues could not be assigned unambiguously to the major or minor sequence because of their equal yields. Residues in lowercase letters represent assignments of less than full confidence. Positions for which no assignment was possible are indicated by X. The $NH_2$-terminal K in parentheses was deduced from the substrate specificity of the protease.

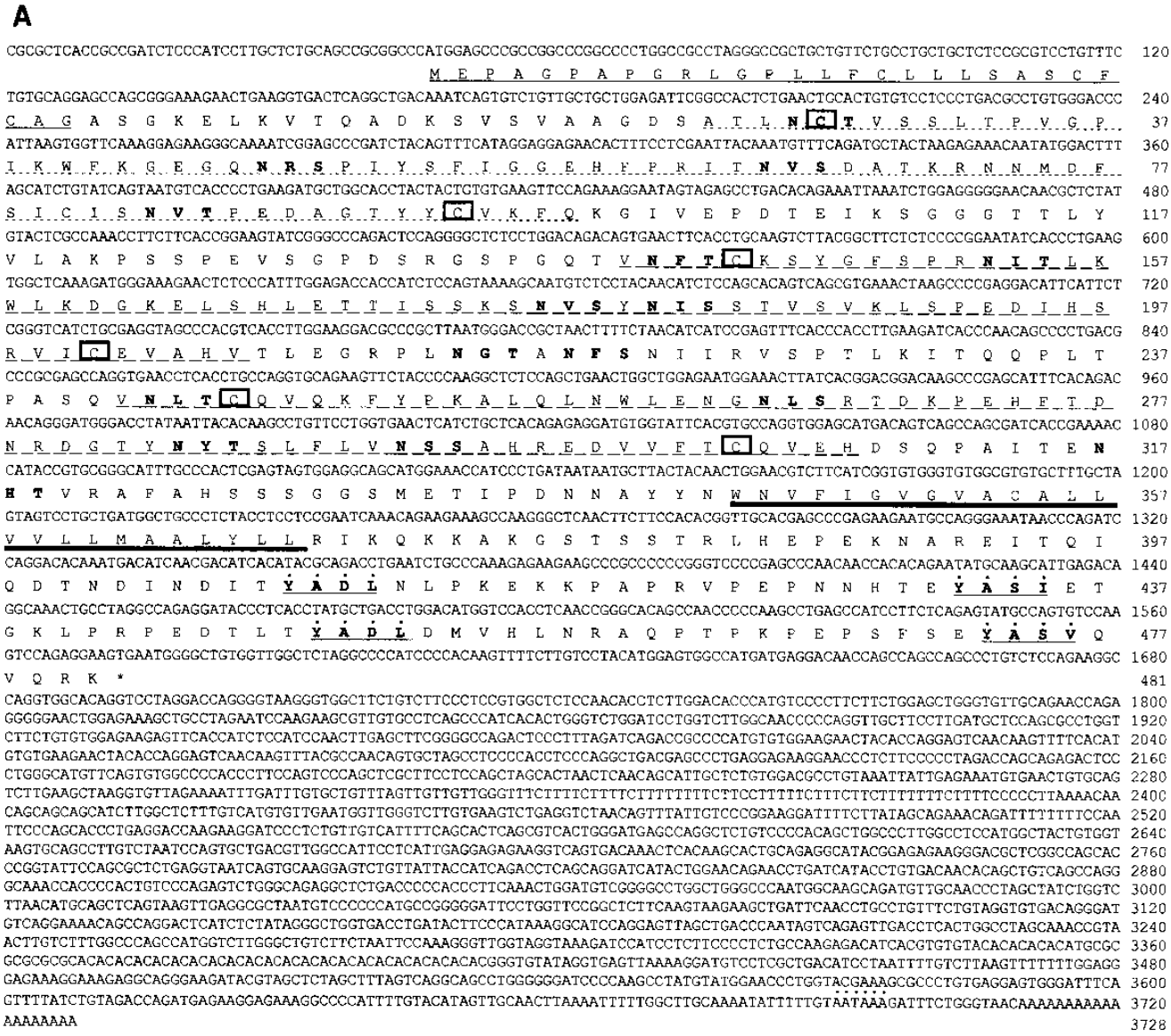

FIG. 4. (A) Nucleotide sequence and deduced amino acid sequence of rat SHPS-1 cDNA. The putative signal peptide (solid underline), the transmembrane region (boldface underline), the AATAAA box close to the polyadenylated 3′ end of the cDNA (dots), the Ig-like domains (broken lines); (the Ig V-like domain, [small broken line] and the Ig C1-like domains [large broken lines]), cysteine residues that potentially form disulfide bonds in the Ig-like domains (boxes), the putative binding sites for SH2 domains of PTPases (boldface with underlines and small dots), and potential sites of N-linked glycosylation (boldface) are indicated. Potential serine-threonine phosphorylation sites include Ser-381, Thr-437, and Thr-463 for protein kinase C [(S/T)X(R/K)] and Thr-407, Thr-448, and Ser-469 for casein kinase II [(S/T)XX(E/D)]. The numbers to the right of each row refer to nucleotide position (upper sequence) or to amino acid position (lower sequence) in the predicted mature SHPS-1 protein. (B) Alignment of the amino acid sequences containing the putative SH2-domain binding sites of rat SHPS-1 with those of rat IRS-1 (47) and with each other. The tyrosine residue in each sequence is indicated in parentheses. Residues shared by sequences (single-letter codes between two sequences) and similar amino acids (plus signs) are indicated. The alignments were performed by using the BLAST program and the Swiss Prot database. (C) Alignment of the deduced amino acid sequences of the putative cytoplasmic portions of rat, mouse, and human SHPS-1. Residues that are identical in all species (boxed), the putative binding sites for SH2 domains of PTPases (boldface), and gaps introduced for optimal alignment (hyphens) are indicated.

In addition to the rat SHPS-1 cDNA, we also cloned mouse and human SHPS-1 cDNAs. The four putative SH2-domain binding sites, consisting of a tyrosine residue and the following three amino acids, are completely conserved, and the overall cytoplasmic region is highly conserved among the putative SHPS-1 proteins from these three species (Fig. 4C). Detailed characterization of the human and mouse SHPS-1 genes will be given elsewhere. No sequences corresponding to known catalytic domains were detected in the cytoplasmic region of SHPS-1.

Northern blot analysis revealed that the rat SHPS-1 mRNA is ~4.2 kb and was present in all tissues examined, being most abundant in brain, lung, and spleen tissues (Fig. 5).

**SHPS-1 is a substrate for the IR kinase and forms a complex with SHP-2 in response to insulin.** We have generated a polyclonal antibody to a GST fusion protein containing the cytoplasmic region of rat SHPS-1 (αSHPS-1). This antibody recognized SHPS-1 purified from SR-3Y1 cell membranes (Fig. 6A, lane 1). A 120-kDa protein was also detected in the immunoblot analysis with this antibody of cell lysates prepared

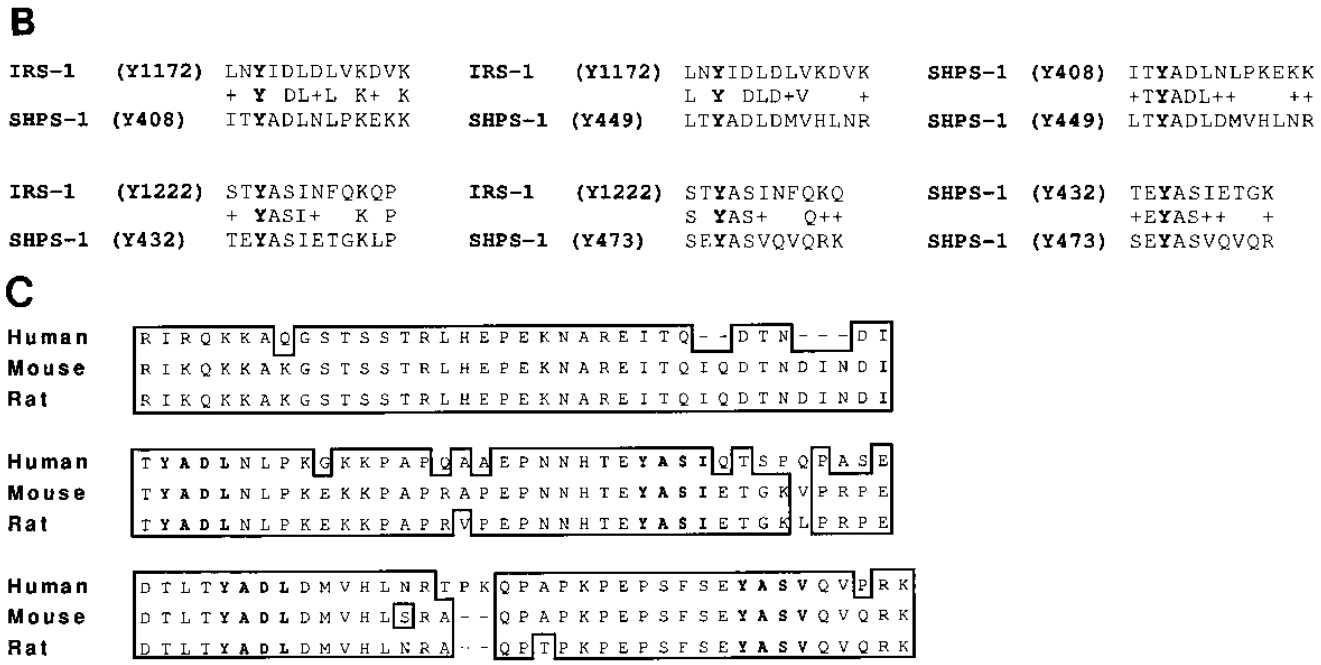


FIG. 4—*Continued.*

from 3Y1 cells and SR-3Y1 cells (Fig. 6A, lanes 2 and 3). The 120-kDa protein was immunoprecipitated with αSHPS-1 polyclonal antibody but not with preimmune serum from 3Y1 cell lysates (Fig. 6A, lanes 4 and 5). The immunoprecipitation of the 120-kDa protein was significantly inhibited by incubation of antibody with a GST fusion protein containing the cytoplasmic region of SHPS-1 (Fig. 6A, lane 6). Thus, these data indicate that the polyclonal antibody αSHPS-1 specifically recognized rat 120-kDa SHPS-1. We have found that αSHPS-1 immunoprecipitated less SHPS-1 from SR-3Y1 cell lysates than from 3Y1 cells (Fig. 6A, lane 7). Since αSHPS-1 polyclonal antibody was generated against a GST fusion protein containing the cytoplasmic region of SHPS-1, it is possible that αSHPS-1 polyclonal antibody may not be able to immunoprecipitate the tyrosine-phosphorylated form of SHPS-1 or the SHPS-1 complexed with SHP-2 in SR-3Y1 cells. Since we purified SHPS-1 from SR-3Y1 cells, we next transfected SR-3Y1 cells with cDNA of SHPS-1. The transfection of the SR-3Y1 cells yielded a polypeptide of ~120 kDa that was specifically recognized by αSHPS-1 polyclonal antibody (Fig. 6B, lane 2). Thus, these results indicate that our cloned SHPS-1 cDNA encodes SHPS-1 originally purified from SR-3Y1 cells.

We have shown that insulin stimulates both the tyrosine phosphorylation of an ~115-kDa membrane glycoprotein, pp115, and the subsequent association of SHP-2 with pp115 in CHO-IR cells (32). In addition, the extent of tyrosine phosphorylation of pp115 is markedly increased in CHO-IR cells overexpressing a catalytically inactive SHP-2, and the formation of a more stable complex of pp115 with the mutant protein than with wild-type SHP-2 was observed (27, 32). We have subsequently generated a monoclonal antibody, 4C6, which specifically recognizes pp115 of CHO cells and can be used for both immunoprecipitation and immunoblotting of pp115 (32). When lysates of unstimulated CHO-IR cells were subjected to immunoprecipitation with 4C6 monoclonal antibody, the precipitated 115-kDa protein was detectable by immunoblot analysis with αSHPS-1 polyclonal antibody to rat SHPS-1 (Fig. 7A, lane 2). Lysates prepared from unstimulated CHO-SHP-2-C/S cells, in which a catalytically inactive SHP-2 forms a stable complex with tyrosine-phosphorylated pp115 (27, 32), were also immunoprecipitated with normal mouse IgG or 4C6 monoclonal antibody to pp115 (Fig. 7B, lanes 1 and 2). The resulting supernatants from the first immunoprecipitation were then subjected to a second round of immunoprecipitation with polyclonal antibody to SHP-2. When the second immunoprecipitate was in turn subjected to immunoblot analysis with αSHPS-1 polyclonal antibody, the amount of pp115 complexed with SHP-2 was significantly decreased by the first immunoprecipitation with 4C6 monoclonal antibody (Fig. 7B, lanes 1 and 2). These results suggest that polyclonal antibody to SHPS-1 recognizes pp115 which is immunoprecipitated with 4C6 monoclonal antibody from CHO-IR cells.

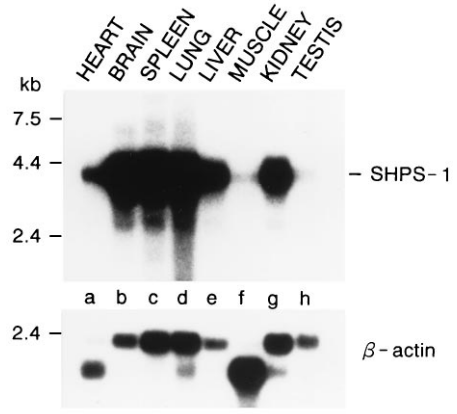


FIG. 5. Tissue distribution of rat mRNA. Poly(A)$^+$ RNAs (2 μg) from various rat tissues were subjected to Northern blot analysis with $^{32}$P-labeled full-length rat SHPS-1 cDNA (upper panel) and, subsequently, $^{32}$P-labeled mouse β-actin cDNA (lower panel) as probes. The positions of molecular size standards are shown on the left.

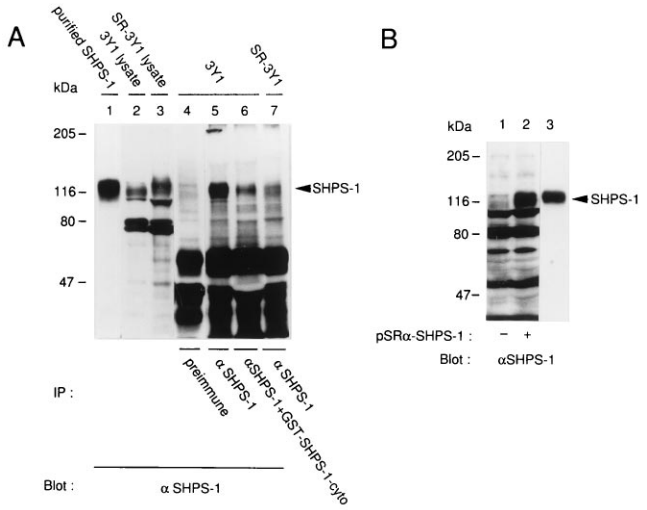


FIG. 6. Transient expression of SHPS-1. (A) The pp120 protein was purified from SR-3Y1 cell membranes as described in the legend to Fig. 3A. Purified pp120 (lane 1) or cell lysates prepared from 3Y1 cells (lane 2) and SR-3Y1 cells (lane 3) were subjected to immunoblot analysis with polyclonal antibody to SHPS-1. Cell lysates prepared from 3Y1 cells (lanes 4 to 6) or SR-3Y1 cells (lane 7) were subjected to immunoprecipitation (IP) with preimmune serum and αSHPS-1 polyclonal antibody as indicated below the lanes. Immunoprecipitation was carried out in the presence of 1 μg of GST fusion protein containing the cytosolic domain of SHPS-1 (GST–SHPS-1-cyto) (lane 6). The immunoprecipitates were then fractionated by SDS-PAGE and subjected to immunoblot analysis with αSHPS-1. (B) Cell lysates prepared from nontransfected SR-3Y1 cells (lane 1) or SR-3Y1 cells transfected with a pSRα vector containing the full-length rat SHPS-1 cDNA (pSRα-SHPS-1) (lane 2), as well as purified pp120 protein from SR-3Y1 cells (lane 3), were subjected to immunoblot analysis with polyclonal antibody to SHPS-1.

Lysates of CHO-IR cells expressing wild-type SHP-2 (CHO-SHP-2-WT cells) or a catalytically inactive SHP-2 (CHO-SHP-2-C/S cells) were subjected to immunoprecipitation with antibody to SHP-2 followed by immunoblot analysis with polyclonal antibody to SHPS-1. The amount of SHP-2 immunoprecipitated from CHO-SHP-2-C/S cells was approximately twice as great as that from CHO-SHP-2-WT cells (Fig. 7C, lanes 1 and 2), because the level of expression of SHP-2 in CHO-SHP-2-C/S cells was higher than that of CHO-SHP-2-WT cells (33). However, SHPS-1 was shown to associate to a much greater extent with SHP-2 in CHO-SHP-2-C/S cells than in CHO-SHP-2-WT cells (Fig. 7C, lanes 1 and 2). Incubation of CHO-IR cells that overexpress SHPS-1 containing a MYC epitope tag (SHPS-1-MYC) with 100 nM insulin for 5 min induced both tyrosine phosphorylation of SHPS-1-MYC and the association of SHP-2 with SHPS-1-MYC (Fig. 7D, left panel); similar amounts of SHPS-1-MYC were immunoprecipitated with the 9E10 monoclonal antibody to MYC tag under the two conditions (Fig. 7D, right panel). Together, these results strongly suggest that pp115 previously identified in CHO cells (27, 32) may be SHPS-1.

When lysates prepared from insulin-stimulated or -unstimulated CHO-IR cells were immunoblotted with PY-20, insulin stimulation of CHO-IR cells resulted in a marginal but significant tyrosine phosphorylation of ~115-kDa protein (Fig. 8A, lane 2) that corresponded to the tyrosine-phosphorylated pp115 (Fig. 8A, lane 4). The extent of insulin-dependent tyrosine phosphorylation of pp115 was much weaker than that of IRS-1, a major substrate for IRs, in the immunoblot analysis with PY-20 of cell lysates.

The partially purified, activated IRs tyrosine phosphorylated the GST fusion protein containing the cytoplasmic portion of SHPS-1 but not GST protein alone (data not shown), suggesting that SHPS-1 may be a direct substrate for the IR kinase in vitro. Furthermore, when tyrosine-phosphorylated GST fusion protein containing the cytoplasmic portion of SHPS-1 was incubated with either GST alone or GST-SH2 domains of SHP-2, the tyrosine-phosphorylated fusion protein bound to GST-SH2 domains of SHP-2 but not to GST (Fig. 8B, lanes 1 and 2). This result suggests the possibility that SHP-2 may directly bind to tyrosine-phosphorylated SHPS-1 through SH2 domains of SHP-2.

**Mitogens and cell adhesion induce tyrosine phosphorylation of SHPS-1 and its association with SHP-2.** We next tested whether other mitogens in addition to insulin also induce tyrosine phosphorylation of SHPS-1 and its subsequent association with SHP-2 in Rat-1 fibroblasts that overexpress human IRs (Rat-1-IR cells). The reason we used Rat-1 cells in this experiment is that Rat-1-IR cells can respond to various mitogens such as serum, lysophosphatidic acid (LPA), and insulin (5). Even in unstimulated cells, both tyrosine phosphorylation of SHPS-1 and association of SHP-2 with SHPS-1 were apparent (Fig. 9A). Incubation of serum-starved Rat-1-IR cells with insulin, serum, or LPA, all of which induce MAP kinase activation in these cells (5), increased the extent of both tyrosine phosphorylation of SHPS-1 and association of SHPS-1 with SHP-2 (Fig. 9A). The ~180-kDa tyrosine-phosphorylated protein coimmunoprecipitated with SHP-2 from insulin-stimulated Rat-1-IR cells was found to be IRS-1 (Fig. 9A, left panel) when the same filter was reprobed with antibody to IRS-1 (data not shown), while the ~180-kDa unknown protein that reacted with polyclonal antibody to SHPS-1 was coimmunoprecipitated with SHP-2 from serum-stimulated Rat-1-IR cells (Fig. 9A, right panel).

We hypothesized that the tyrosine phosphorylation of SHPS-1 and its association with SHP-2 in unstimulated cells might be attributable to the effects of cell adhesion. Since 4C6 monoclonal antibody was able to effectively immunoprecipitate SHPS-1 which is tyrosine phosphorylated and complexed with SHP-2, we used CHO-IR cells for following experiments. When CHO-IR cells were incubated for 10 min with PBS free of both $Ca^{2+}$ and $Mg^{2+}$, the cells became round but remained tightly bound to the polystyrene plates. This treatment of cells was reported previously to reduce cell-substrate adhesion and the extent of tyrosine phosphorylation of cellular proteins (24). This treatment of CHO-IR cells also induced a marked decrease in the extent of tyrosine phosphorylation of the $pp125^{FAK}$ focal adhesion kinase (40) (data not shown), indicating that it may reduce the number of focal contacts of CHO-IR cells. The extent of tyrosine phosphorylation of SHPS-1 and the amount of SHP-2 bound to SHPS-1 were both markedly reduced in CHO-IR cells exposed to $Ca^{2+}$- and $Mg^{2+}$-free PBS compared with those of control CHO-IR cells (Fig. 9B, left panel, lanes 1 and 2); similar amounts of $pp115^{SHPS-1}$ were immunoprecipitated with the 4C6 monoclonal antibody under the two conditions (Fig. 9B, right panel, lanes 1 and 2). Adherence of cells to cell matrix proteins such as fibronectin increases the numbers of focal contacts and the extent of tyrosine phosphorylation of various proteins associated with such contacts (40). We detached CHO-IR cells from culture dishes by treatment with 0.05% trypsin and 0.02% EDTA and then plated the cells in polystyrene dishes which were coated with fibronectin or uncoated. Adherence of CHO-IR cells to fibronectin induced a marked increase in both the extent of tyrosine phosphorylation of SHPS-1 and the amount of SHP-2 bound to SHPS-1 (Fig. 9B, left panel, lanes 3 and 4).

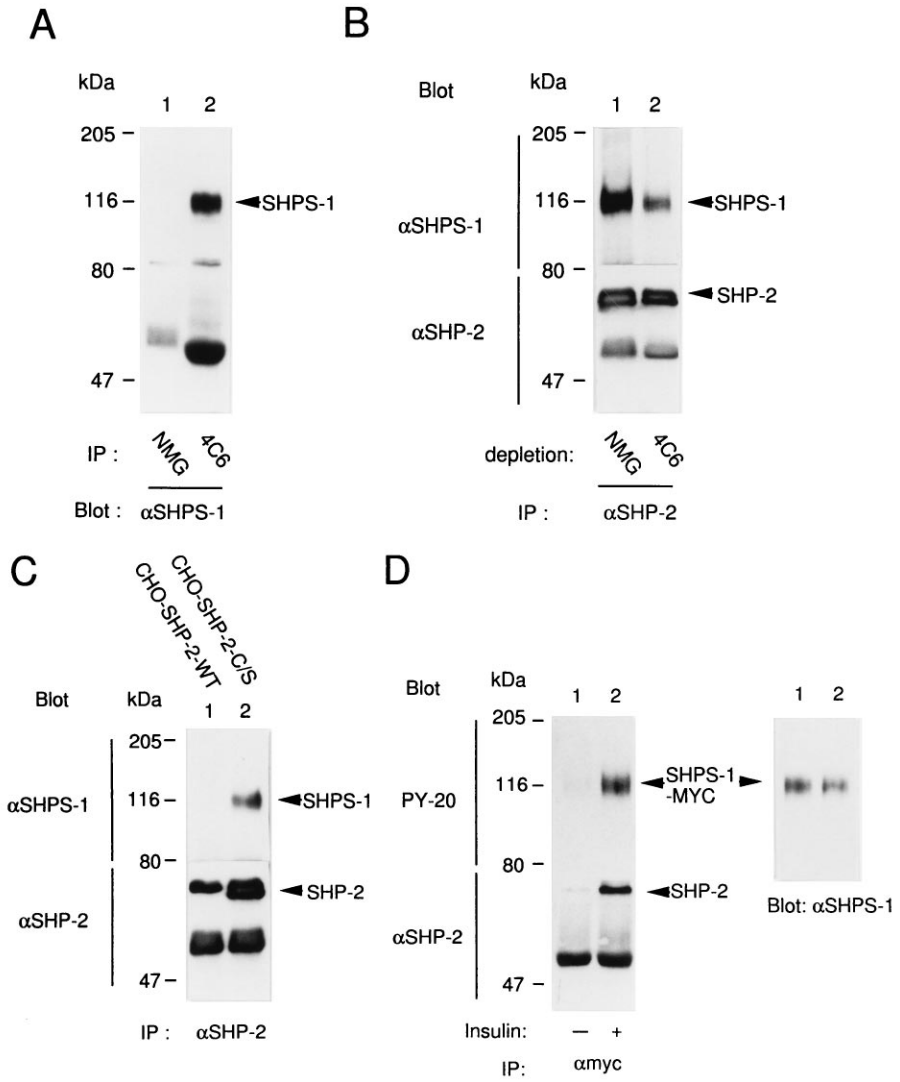


FIG. 7. The pp115 of CHO cells may be SHPS-1. (A) Lysates prepared from CHO-IR cells were subjected to immunoprecipitation (IP) with normal mouse IgG (NMG) (lane 1) or 4C6 monoclonal antibody to pp115 (lane 2). The immunoprecipitates were in turn subjected to immunoblot analysis with polyclonal antibody to SHPS-1. (B) Lysates prepared from unstimulated CHO-SHP-2-C/S cells were immunoprecipitated with normal mouse IgG (lane 1) or 4C6 monoclonal antibody to pp115 (lane 2). The resulting supernatants from the first immunoprecipitation in lanes 1 and 2 were then subjected to a second round of immunoprecipitation with polyclonal antibody to SHP-2. The immunoprecipitates were in turn subjected to immunoblot analysis with polyclonal antibodies to SHPS-1 (top) and SHP-2 (bottom). (C) Lysates prepared from CHO-SHP-2-WT (lane 1) or CHO-SHP-2-C/S (lane 2) cells were subjected to immunoprecipitation with polyclonal antibody to SHP-2. The immunoprecipitates were in turn subjected to immunoblot analysis with polyclonal antibodies to SHPS-1 (top) and SHP-2 (bottom). (D) CHO-IR cells that overexpress SHPS-1 tagged with a MYC epitope (SHPS-1-MYC) (lanes 1 and 2) were incubated in the absence (−) or presence (+) of 100 nM insulin for 5 min as indicated. Cell lysates were then prepared and subjected to immunoprecipitation with antibody to MYC and immunoblot analysis with PY-20 (left panel, top) and antibody to SHP-2 (left panel, bottom). The upper portion of the blot was reprobed with polyclonal antibody to SHPS-1 (right).

## DISCUSSION

The biochemical characterization and molecular cloning of SHPS-1 have demonstrated that it is a transmembrane glycoprotein that contains three Ig-like domains in the extracellular region and four putative binding sites for SH2-domain-containing PTPases in the cytoplasmic region. In SR-3Y1 cells, SHPS-1 is presumably tyrosine phosphorylated by the v-*src*-encoded kinase and forms a complex with overexpressed SHP-1 through the SH2 domains of SHP-1. It has recently been shown that the extent of tyrosine phosphorylation of pp115, a membrane glycoprotein, was greatly increased, relative to that in CHO-IR cells, in CHO-IR cells that also overexpress catalytically inactive SHP-2 (26, 27, 32). We have also demonstrated that insulin stimulates tyrosine phosphorylation of pp115 and subsequent association of SHP-2 with pp115 (32). Although we purified pp120 and cloned its cDNA from rat SR-3Y1 cells, the biochemical characteristics of SHPS-1 and pp115 seem to be almost identical; the two are similar in molecular size, membrane glycoproteins, and tyrosine-phosphorylated proteins bound to the SH2 domains of SHP-2. Furthermore, the anti-SHPS-1 polyclonal antibody that was raised against the GST-cytosolic domain of SHPS-1 recognized pp115 immunoprecipitated with 4C6 monoclonal antibody to pp115 from CHO-IR cells. This monoclonal antibody has recently been generated against the partially purified pp115 from CHO-IR cells expressing catalytically inactive mutant SHP-2 (32). In addition, when the lysates of CHO-SHP-2-WT cells or CHO-SHP-2-C/S cells were subjected to immunoprecipitation

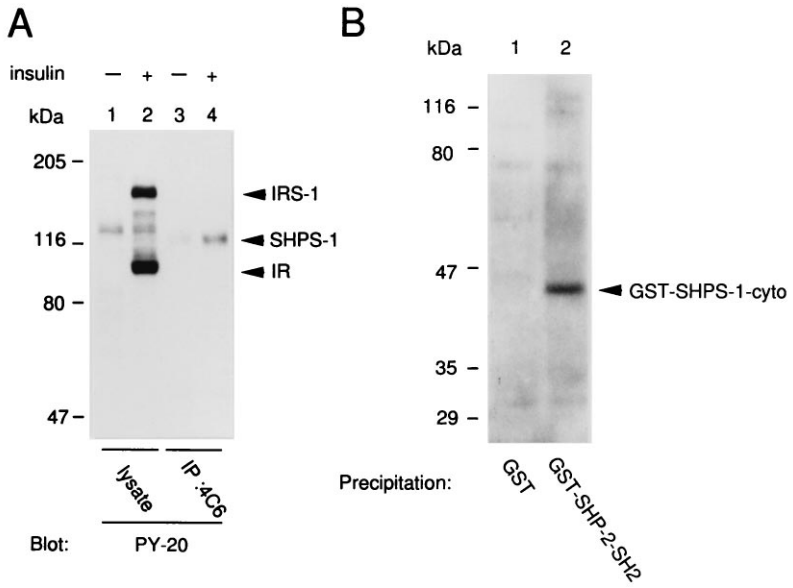


FIG. 8. Insulin and IR kinase tyrosine phosphorylates SHPS-1. (A) CHO-IR cells were incubated with (lanes 2 and 4) or without (lanes 1 and 3) 100 nM insulin for 5 min. Cell lysates were then prepared and subjected to immunoblot analysis with PY-20 (lanes 1 and 2). The cell lysates were also subjected to immunoprecipitation (IP) with 4C6 monoclonal antibody and immunoblot analysis with PY-20 (lanes 3 and 4). (B) The GST fusion protein containing the cytoplasmic portion of SHPS-1 (GST–SHPS-1-cyto) was incubated in the presence of 10 μM ATP with IRs purified with WGA-agarose from insulin-stimulated CHO-IR cells as described in Materials and Methods. Phosphorylated GST fusion protein containing the cytoplasmic portion of SHPS-1 was then incubated with CNBr-activated Sepharose beads coupled with GST alone (lane 1) or GST-SH2 domains of SHP-2 fusion proteins (lane 2). The bound proteins were then subjected to SDS-PAGE and were detected by immunoblotting with PY-20.

with antibody to SHP-2 followed by immunoblot analysis with polyclonal antibody to SHPS-1, SHPS-1 was shown to associate to a much greater extent with SHP-2 in CHO-SHP-2-C/S cells than in CHO-SHP-2-WT cells. When MYC-tagged SHPS-1 was overexpressed in CHO-IR cells, insulin induced both tyrosine phosphorylation of MYC-tagged SHPS-1 and the subsequent binding of SHP-2 to MYC-tagged SHPS-1. Together, these results strongly suggest that SHPS-1 and the pp115 that was previously identified in CHO cells (32) are likely to be the same. WGA-purified activated IRs phosphorylated the recombinant cytoplasmic portion of SHPS-1 in vitro. Thus, SHPS-1 appears to be a direct substrate for tyrosine kinases such as Src and the IR kinase.

Milarski and Saltiel have demonstrated that transient expression of a catalytically inactive SHP-2 induces hyperphosphorylation of 120-kDa protein which binds to SH2 domains of SHP-2 in NIH 3T3 cells overexpressing human IRs (31). However, this tyrosine-phosphorylated 120-kDa protein could not be detected by immunoprecipitation with an SHP-2 antibody (31). Yamauchi and Pessin have reported that SHP-2 binds to tyrosine-phosphorylated 115-kDa protein in response to insulin (63) or EGF (62). Yamauchi et al. have also demonstrated that the extent of tyrosine phosphorylation of a 115-kDa protein was greatly increased, relative to that in CHO-IR cells, in CHO-IR cells that overexpress catalytically inactive SHP-2 (63). However, they reported that they did not observe any precipitation of a 115-kDa protein from cell extracts with the SH2 domains of SHP-2 fusion protein (63). Thus, there appear to exist different types of tyrosine-phosphorylated 115- to 120-kDa proteins which interact with SHP-2, and further efforts will be required to characterize each of these proteins.

In vitro binding studies with a phosphotyrosyl peptide library have suggested that the SH2 domains of SHP-2 bind to phosphopeptides that contain the sequence motif pYXXL/V/I (pY, phosphorylated tyrosine) (45). In fact, SHP-2 was subsequently shown to bind to pY1009TAV of the PDGF β receptor (15, 22) and to pY1172IDL or pY1222ASI of IRS-1 (36, 48) in a ligand-dependent manner. The cytoplasmic region of SHPS-1 contains four YXX(L/V/I) sequences, Y408ADL, Y432ASIE, Y449ADL, and Y473ASV. Thus, the SH2 domains of SHP-2 may bind to one or more phosphorylated tyrosine residues in the cytoplasmic region of SHPS-1. The sequences surrounding Tyr-408 and Tyr-449 of SHPS-1 are similar to that surrounding Tyr-1172 of IRS-1, whereas the sequences surrounding Tyr-432 and Tyr-473 of SHPS-1 are homologous to that surrounding Tyr-1222 of IRS-1. The $NH_2$-terminal and COOH-terminal SH2 domains of SHP-2 are thought to bind to Y1172IDL and Y1222ASI of IRS-1, respectively, in response to insulin stimulation (8, 36). Thus, it is possible that one SHP-2 molecule may bind to the two $NH_2$-terminal SH2 binding sites (Y408ADL and Y432ASI) of SHPS-1 and another SHP-2 molecule may bind to the two COOH-terminal SH2 binding sites (Y449ADL and Y473ASV) of SHPS-1. The SH2 domains of SHP-1 have been suggested to bind to phosphopeptides that contain the motif pYXXL, which is very close to that of SHP-2 (7, 46). Thus, SHP-1 may bind to tyrosine-phosphorylated SHPS-1 when SHP-1 is overexpressed in SR-3Y1 cells. However, since the physiological functions of these two SHP proteins were quite different (26), these two proteins may not bind to the same phosphorylated tyrosine residue(s) of SHPS-1.

We have recently shown that both the extent of tyrosine phosphorylation of $pp115^{SHPS-1}$ and the association of SHP-2 with $pp115^{SHPS-1}$ are maximal at 1 to 5 min after insulin stimulation of CHO-IR cells (32). In contrast, the extent of tyrosine phosphorylation of $pp115^{SHPS-1}$ continued to increase for up to 30 min after stimulation of CHO-SHP-2-C/S cells, presumably because of a lack of PTPase activity of mutant SHP-2. In addition, we have shown that recombinant SHP-2 effectively dephosphorylates tyrosine-phosphorylated SHPS-1, which was prepared from SR-3Y1 cell membranes, in vitro (11). Thus, we

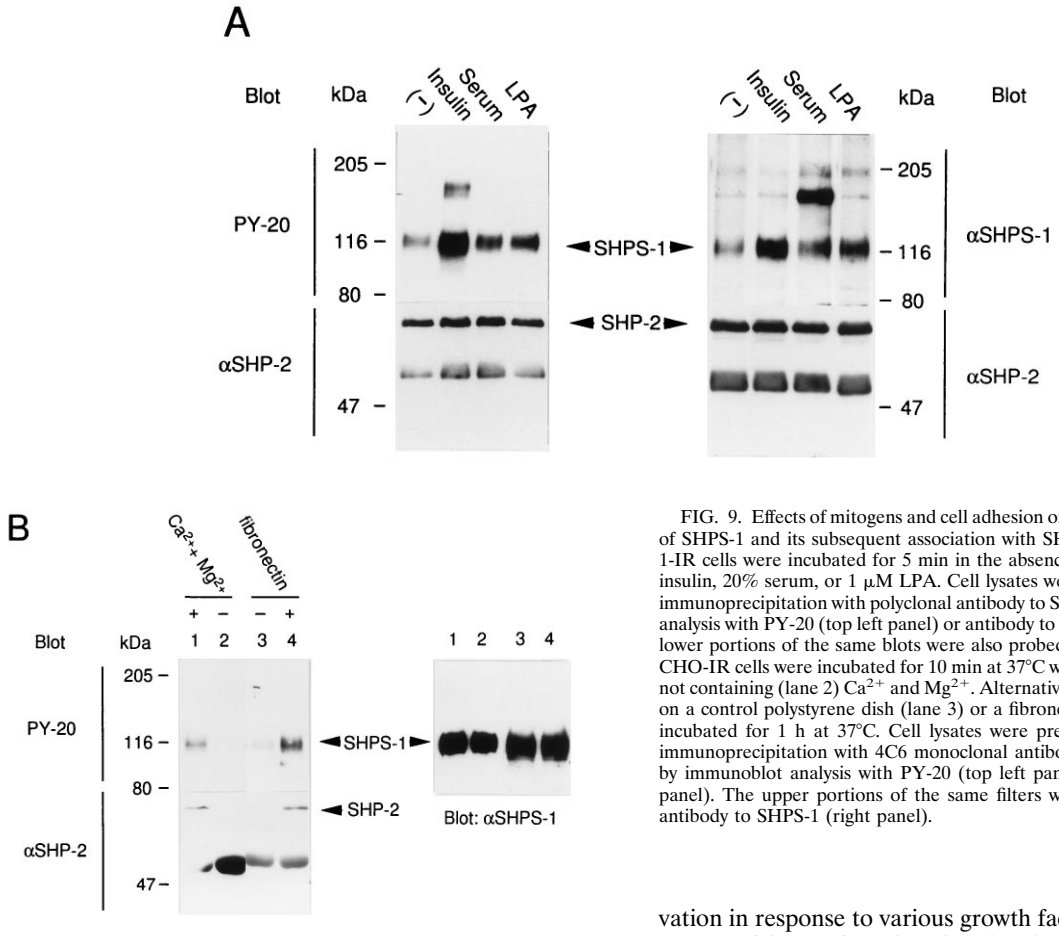


FIG. 9. Effects of mitogens and cell adhesion on the tyrosine phosphorylation of SHPS-1 and its subsequent association with SHP-2. (A) Serum-starved Rat-1-IR cells were incubated for 5 min in the absence (−) or presence of 100 nM insulin, 20% serum, or 1 μM LPA. Cell lysates were prepared and subjected to immunoprecipitation with polyclonal antibody to SHP-2 followed by immunoblot analysis with PY-20 (top left panel) or antibody to SHPS-1 (top right panel). The lower portions of the same blots were also probed with antibody to SHP-2. (B) CHO-IR cells were incubated for 10 min at 37°C with PBS containing (lane 1) or not containing (lane 2) $Ca^{2+}$ and $Mg^{2+}$. Alternatively, CHO-IR cells were plated on a control polystyrene dish (lane 3) or a fibronectin-coated dish (lane 4) and incubated for 1 h at 37°C. Cell lysates were prepared and then subjected to immunoprecipitation with 4C6 monoclonal antibodies to $pp115^{SHPS-1}$ followed by immunoblot analysis with PY-20 (top left panel) or to SHP-2 (bottom left panel). The upper portions of the same filters were reprobed with polyclonal antibody to SHPS-1 (right panel).

propose a model that SHP-2 may dephosphorylate one or more phosphotyrosine residues of SHPS-1 after interaction of the two proteins in response to insulin stimulation. However, further efforts will be required to clarify whether SHPS-1 is a physiological substrate for SHP-2 in a growth factor-mediated signal transduction pathway.

We have shown that stimulation with serum or LPA also induced tyrosine phosphorylation of SHPS-1 and its subsequent association with SHPS-1. LPA, the receptor for which is coupled to a G protein, stimulates the RAS-MAP kinase pathway (18, 55) and also induces the tyrosine phosphorylation of several proteins, including $pp125^{FAK}$ (42) and SHC (54). Thus, SHPS-1 appears to be a new target protein for tyrosine phosphorylation induced by LPA, although the mechanism by which LPA achieves this effect is not known. We further showed that cell adhesion to fibronectin induces tyrosine phosphorylation of SHPS-1 and the binding of SHP-2 to tyrosine-phosphorylated SHPS-1, effects presumably mediated by an integrin-coupled pathway (23). Engagement of cell surface integrins rapidly stimulates the tyrosine phosphorylation of several intracellular proteins, including paxillin, tensin, FAK, and $p130^{CAS}$ (35, 40). These proteins are also tyrosine phosphorylated in v-*src*-transformed cells (35, 40), suggesting the possibility that Src family kinases or FAK might mediate the cell adhesion-induced tyrosine phosphorylation of SHPS-1. Both integrin-mediated cell adhesion and LPA activate RAS and MAP kinase in a manner independent of protein kinase C (6, 41). Because SHP-2 may mediate RAS and MAP kinase activation in response to various growth factors, the association of SHP-2 with tyrosine-phosphorylated SHPS-1 may contribute to LPA- or integrin-induced RAS and MAP kinase activation.

Rat SHPS-1 mRNA was present in all tissues examined, with the greatest abundance in brain, lung, and spleen tissues. Human SHPS-1 mRNA was also detected in the thymus, leukocytes, and the intestine, in addition to the rat tissues tested; again, human SHPS-1 mRNA was most abundant in the brain (60). The tissue distribution of SHPS-1 mRNA resembles that of SHP-2 mRNA rather than that of SHP-1 mRNA, the latter being mostly restricted to hematopoietic tissues (26, 64). Both SHPS-1 and SHP-2 (49) are highly expressed in the brain, suggesting the possible role of SHPS-1 in SHP-2-mediated signal transduction in the brain. In contrast, the level of expression of SHPS-1 in skeletal muscle is relatively low, as shown in Fig. 5, while the level of expression of SHP-2 in the same tissue in fact is high (10, 49). This indicates that SHPS-1 may not be important for the function of SHP-2, and an alternative target for SHP-2 may exist in the skeletal muscle. SHP-2 has previously been shown to be expressed in the mouse embryo (9). SHPS-1 mRNA was also detected together with SHP-2 mRNA in early-stage mouse embryos, in which SHP-1 mRNA was virtually undetectable (60), indicating that SHP-2 and SHPS-1 might function simultaneously during embryogenesis.

We discovered that the human SHPS-1 cDNA appears to be the same as the CCA53 cDNA, which was recently cloned by direct screening of a human brain cDNA library with an oligonucleotide containing CCA repeats (25). However, the function of CCA53 cDNA-encoded protein was not identified. Expansion of trinucleotide repeats is responsible for several hereditary neurological disorders, including myotonic dystro-

phy, fragile X syndrome, and Huntington's disease (21, 38). Given that human SHPS-1 mRNA is also abundant in the brain (60), the expansion of the triplet repeats in the SHPS-1 gene may underlie some neurological disease.

It has previously been demonstrated that SHP-2 may regulate an upstream element necessary for RAS activation in response to insulin (33), while the place of SHP-2 during the activation of the RAS-MAP kinase cascade in response to insulin is still controversial (12, 39). Although we have now clarified the structure of SHPS-1, to which SHP-2 binds, it remains unclear how SHPS-1 may couple SHP-2 to RAS activation. No known catalytic domain was detected in the cytoplasmic region of SHPS-1. In the preliminary experiment, neither Grb2, Shc, nor GAP bound to SHPS-1 in response to insulin in CHO-IR cells (34). Thus, SHPS-1 may simply act as a docking protein, similar to IRS-1, and induce translocation of SHP-2 from the cytosol to the plasma membrane in response to mitogens and cell adhesion. Approximately 2% of the total SHP-2 was recovered in the SHPS-1 immunoprecipitates with 4C6 monoclonal antibody when CHO-IR cells were stimulated with insulin (34). However, it is possible that the minor fraction of SHP-2 may be enough to mediate its downstream signals. After binding to SHPS-1, SHP-2 may dephosphorylate and dissociate from SHPS-1, in the process possibly activating an SOS-like guanine nucleotide exchange protein near the plasma membrane by catalyzing its tyrosine dephosphorylation. In addition, it remains to be determined whether SHP-1 also binds to SHPS-1 in response to cytokines in hematopoietic cells and plays a role in cytokine signal transduction.

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