Role of SH-PTP2, a Protein-Tyrosine Phosphatase with Src Homology 2 Domains, in Insulin-Stimulated Ras Activation

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SH-PTP2 is a nontransmembrane human protein-tyrosine phosphatase that contains two Src homology 2 (SH2) domains and binds to insulin receptor substrate 1 (IRS-1) via these domains in response to insulin. The expression of a catalytically inactive mutant of SH-PTP2 (containing the mutation Cys-459→Ser) in Chinese hamster ovary cells that overexpress human insulin receptors (CHO-IR cells) markedly attenuated insulin-stimulated Ras activation. Expression of mutant SH-PTP2 also inhibited MAP kinase activation in response to insulin but not in response to 12-*O*-tetradecanoyl phorbol-13-acetate. In contrast, the insulin-induced association of phosphoinositide 3-kinase activity with IRS-1 was not affected by the expression of inactive SH-PTP2. Furthermore, the expression of mutant SH-PTP2 had no effect on the binding of Grb2 to IRS-1, on the tyrosine phosphorylation of Shc, or on the formation of the complex between Shc and Grb2 in response to insulin. However, the amount of SH-PTP2 bound to IRS-1 in insulin-treated CHO-IR cells expressing mutant SH-PTP2 was greater than that observed in CHO-IR cells overexpressing wild-type SH-PTP2. Recombinant SH-PTP2 specifically dephosphorylated a synthetic phosphopeptide corresponding to the sequence surrounding Tyr-1172 of IRS-1, a putative binding site for SH-PTP2. Additionally, phenylarsine oxide, an inhibitor of protein-tyrosine phosphatases, inactivated SH-PTP2 in vitro and increased the insulin-induced association of SH-PTP2 with IRS-1. These results suggest that SH-PTP2 may regulate an upstream element necessary for Ras activation in response to insulin and that this upstream element may be required for the Grb2- or Shc-dependent pathway. Furthermore, these results are consistent with the notion that SH-PTP2 may bind to IRS-1 through its SH2 domains in response to insulin and dephosphorylate the phosphotyrosine residue to which it binds, thereby regulating its association with IRS-1.

The insulin receptor (IR) belongs to a family of structurally related transmembrane growth factor receptors that exhibit ligand-activated protein-tyrosine kinase activity (15, 38). IR kinase activity is thought to be essential for cellular responses to insulin (7, 9, 14, 27). Activation of the IR kinase induces the rapid autophosphorylation of IR β subunits as well as tyrosine phosphorylation of several cytoplasmic proteins (16, 37). Insulin receptor substrate 1 (IRS-1) (44) is one of the major proteins that undergo tyrosine phosphorylation in response to insulin. IRS-1 contains 20 possible tyrosine phosphorylation sites, and these sites bind to the Src homology 2 (SH2) domains of several proteins, including the 85-kDa subunit of phosphoinositide 3-kinase (PI3-kinase) (4, 52), Grb2 (or Ash) (40, 45), and Nck (21). Recent studies suggest that the binding of the Grb2-Sos complex to tyrosine-phosphorylated IRS-1 or Shc may link IR stimulation to Ras activation (39). In addition, the binding of the 85-kDa subunit of PI3-kinase to tyrosine-phosphorylated IRS-1 is essential for insulin-induced glucose uptake (13) and membrane ruffling (18).

Recently, we and others have molecularly cloned a human protein-tyrosine phosphatase (PTP), SH-PTP2 (also known as PTP1D, PTP2C, SH-PTP3, and SAP-2), that possesses two SH2 domains (1, 2, 12, 25, 50). Syp is the mouse homolog of SH-PTP2 (10). SH-PTP2 binds directly to growth factor receptors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors, in response to stimulation with ligand and undergoes tyrosine phosphoryla-

tion (17, 20). Furthermore, SH-PTP2 also binds via its SH2 domains to IRS-1 in response to insulin (19). The putative binding site of SH-PTP2 on IRS-1 has been suggested to be the YIDL motif that contains Tyr-1172 (43). Although the precise role of SH-PTP2 in signal transduction remains unclear, SH-PTP2 may act as a positive signaling molecule by dephosphorylating phosphotyrosines that negatively regulate signaling mediators such as the Src family of kinases (20). In support of a positive role in growth regulation, the *Drosophila* homolog of SH-PTP2, which is encoded by the *Corkscrew* gene, functions in a conjunction with D-Raf kinase in a positive manner downstream of the *Torso*-encoded receptor tyrosine kinase (31, 32).

To elucidate the role of SH-PTP2 in insulin action, we have now stably expressed either wild-type SH-PTP2 or a catalytically inactive mutant SH-PTP2 in Chinese hamster ovary (CHO) cells that overexpress human insulin receptors (CHO-IR cells) and compared biological responses to insulin in the transfected cells.

MATERIALS AND METHODS

Stable transfection of CHO-IR cells. The full-length human SH-PTP2 cDNA (2.5 kb) was isolated from a KATO-III cDNA library with a 280-bp PCR-amplified fragment corresponding to the PTP domain of SH-PTP2 as a probe (25). The full-length cDNA was inserted into the *EcoRI* site in the pSR α expression vector (originally distributed by N. Arai). A point mutation that changed Cys-459 to Ser was introduced into the SH-PTP2 cDNA by site-directed mutagenesis as described previously (24). The mutant SH-PTP2 cDNA was also cloned into *EcoRI* site of pSR α . CHO-IR cells ($\sim 5 \times 10^5$ cells per

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10-cm-diameter dish) (52), which overexpress human IRs, were transfected with both 10 μ g of pSR α containing SH-PTP2 cDNA and 1 μ g of pHyg, which contains the hygromycin B phosphotransferase gene, by the calcium phosphate precipitation method (48). The cells were cultured in Ham F-12 medium containing hygromycin B (200 μ g/ml; Wako Chemicals, Osaka, Japan) and 10% fetal bovine serum (FBS), and colonies were isolated 14 to 21 days after transfection. Several cell lines expressing wild-type SH-PTP2 (CHO-IR-WT) or a catalytically inactive, mutant SH-PTP2 (CHO-IR-C/S) were identified by immunoblotting cell lysates with polyclonal antibodies to SH-PTP2 as described below.

Antibodies. To generate a polyclonal antiserum to SH-PTP2, we first prepared a cDNA fragment encoding the COOH-terminal region of SH-PTP2 by PCR amplification with a sense primer (5'-TTGGATCCCAGCGCAGGATTGAAGAAGAG CAG; nucleotides 1689 to 1712), an antisense primer (5'-AAGAATTCATCTGAACTTTCTGCTGTTG; nucleotides 1872 to 1895), and the full-length SH-PTP2 cDNA as the template as described previously (24). The amplified PCR fragment was digested with *Bam*HI and *Eco*RI and inserted in frame into the *Bam*HI-*Eco*RI site of pGEX-2T (Pharmacia). Glutathione *S*-transferase (GST) or the GST-SH-PTP2 fusion protein containing the COOH-terminal region of SH-PTP2 was isolated from 0.5 to 2 liters of bacterial culture as described previously (25) and bound to glutathione-Sepharose beads (Pharmacia). Beads were washed twice with 10 ml of 20 mM Tris-HCl (pH 8.0)–100 mM NaCl–1 mM EDTA–0.5% Nonidet P-40 and then incubated with 7 ml of 50 mM Tris-HCl (pH 9.6)–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 (DTT) and then concentrated with Centrprep-10 (Amicon). For immunization, female rabbits were first injected with 0.4 mg of GST-SH-PTP2 fusion protein in complete Freund's adjuvant and then boosted every 2 weeks with 0.2 mg of antigen in incomplete Freund's adjuvant. For affinity purification, 5 mg of either GST or GST-SH-PTP2 protein was mixed overnight at 4°C with 400 mg of cyanogen bromide-activated Sepharose 4B (Pharmacia). After incubation with 30 ml of 1 M Tris-HCl (pH 8.0), the gel was suspended in 1 ml of coupling buffer (0.2 M NaHCO₃ [pH 8.3], 0.5 M NaCl) and washed with 30 ml of elution buffer (100 mM glycine [pH 2.5]). Rabbit antiserum was applied to a column of GST gel. The flowthrough material was then applied to a column of GST-SH-PTP2 gel, which was subsequently washed with 20 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) followed by 20 ml of 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The antibodies bound to the gel were then eluted with 5 ml of elution buffer, and the eluate was combined with 10 ml of 1 M HEPES (pH 8.8).

The rabbit polyclonal antibody to mitogen-activated protein (MAP) kinase, α C92, was prepared against a synthetic peptide corresponding to residues 350 to 367 of rat MAP kinase as described previously (45). The rabbit polyclonal antibodies to human Shc were generated against a GST fusion protein containing the COOH-terminal region of Shc (51). The rabbit polyclonal antibodies to mouse Sos and to mouse Grb2 were obtained from Upstate Biotechnology, Inc. The monoclonal antibody to IRS-1 (1D6) was obtained from the culture medium of hybridoma cells (52); the monoclonal antiphosphotyrosine antibody (PY-20) was from ICN; the monoclonal antibody to Ras (Y13-259) was from the American Type Culture Collection.

Immunoprecipitation and immunoblotting. Confluent (10-cm-diameter plates) transfected CHO-IR cells were incubated in culture medium without FBS for 16 h and then stimulated with 100 nM insulin for the indicated times. In some experiments, cells were incubated with phenylarsine oxide (PAO; Wako Chemicals) for 5 min before stimulation with insulin. After insulin stimulation, the medium was aspirated and cells were immediately washed with ice-cold phosphate-buffered saline and frozen in liquid nitrogen. The cells were then thawed on ice in 1 ml of an ice-cold solution containing 20 mM Tris-HCl (pH 7.6), 140 mM NaCl, 2.6 mM CaCl₂, 1 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium vanadate. The cell lysates were centrifuged at 10,000 $\times$ *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblotting. For immunoprecipitation, cell lysates from one plate were incubated for 4 h at 4°C with monoclonal antibodies to IRS-1 or polyclonal antibodies to Grb2 that had been bound to protein G-Sepharose beads (20 μ l of beads) (Pharmacia). The beads were then washed three times with 1 ml of 50 mM HEPES (pH 7.6)–150 mM NaCl–0.1% Triton X-100, suspended in sodium dodecyl sulfate sample buffer, and boiled for 5 min. Gel electrophoresis and immunoblotting with the ECL detection system (Amersham) were performed as described previously (25).

Determination of Ras-GTP/Ras-GDP ratio. CHO-IR cells were cultured in 6-cm-diameter dishes until a confluent monolayer was achieved. The cells were incubated in the absence of FBS for 20 h in Ham F-12 medium containing 0.1% bovine serum albumin (BSA) and 10 mM HEPES (pH 7.4) and were subsequently labeled for 3 h with 200 μ Ci of ³²P_i (ICN) in 2 ml of phosphate-free RPMI 1640 (Gibco) supplemented with 0.1% BSA and 10 mM HEPES (pH 7.4). After stimulation with the indicated concentrations of insulin for 2 min, the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and 20 μ g of aprotinin per ml. Ras was then immunoprecipitated with anti-Ras monoclonal antibodies, and bound nucleotides were eluted in the presence of nonradioactive carrier. GDP and GTP were resolved by thin-layer chromatography on polyethyleneimine-cellulose plates with 1 M LiCl as the solvent and were detected by autoradiography. Radioactivity in GTP and GDP was determined by scanning the plate with a Fuji BAS 2000 image analyzer. The results were expressed as $[(\text{GTP} \times 2/3)/(\text{GDP} + \text{GTP} \times 2/3)] \times 100$.

Assay for MAP kinase activity. Confluent 6-cm-diameter dishes of cells were incubated in the absence of FBS for 18 h and then stimulated with the indicated concentrations of insulin for 5 min or with 1 μ M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; Sigma) for 10 min. Cells were lysed in 500 μ l of lysis buffer (25 mM Tris-HCl [pH 7.4], 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium PP_i, 0.5 mM EGTA, 10 nM okadaic acid [Wako Chemicals], 1 mM PMSF). The lysates were incubated for 3 h at 4°C with anti-MAP kinase antibody α C92 that had been bound to protein G-Sepharose beads. The immunoprecipitates were washed twice with lysis buffer and suspended in 35 μ l of assay buffer (25 mM Tris-HCl [pH 7.4], 10 mM MgCl₂, 1 mM DTT, 40 μ M ATP, 1 μ Ci of [γ -³²P]ATP, 2 μ M protein kinase inhibitor [Sigma], with 0.5 mM EGTA and myelin basic protein [1 μ g/ μ l; Sigma] as the substrate). After incubation for 10 min at 20°C, the reaction was stopped by adding 10 μ l of stop solution, containing 0.6% HCl, 1 mM ATP, and 1% BSA. Portions (30 μ l) of reaction mixtures were spotted on P-81 paper (Whatman), which was then washed three times with 0.5% phosphoric acid and once with acetone, and the associ-

ated radioactivity was determined by liquid scintillation counter.

Expression and purification of recombinant SH-PTP2. Recombinant full-length SH-PTP2 was generated with the use of the GST fusion protein system. PCR amplification was performed with a sense primer (5'-GGATCCATGACATCGCG GAGATGTTTCA; nucleotides 113 to 136), an antisense primer (5'-AAGAATTCATCTGAACTTTTCTGCTGTTG; nucleotides 1872 to 1895), and the wild-type SH-PTP2 cDNA or SH-PTP2 cDNA with a Cys-459→Ser mutation as the template. The amplified PCR fragment was digested with *Bam*HI and *Eco*RI and inserted in frame into the *Bam*HI-*Eco*RI site of pGEX-2T. The GST fusion protein was expressed and purified essentially as described above. The purified GST-SH-PTP2 fusion protein was incubated at 25°C for 60 min in 4 ml of a solution containing 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, thrombin (25 U/ml; Sigma), and 2.5 mM CaCl₂. Recombinant SH-PTP2 protein was then purified by removal of the cleaved GST via glutathione-Sepharose affinity chromatography (48).

Determination of PTP activity of SH-PTP2. The PTP activity of SH-PTP2 immunoprecipitated with anti-IRS-1 antibodies from transfected CHO-IR cells was assayed with ³²P-labeled Raytide (Oncogene Science) as a substrate as described previously (41, 47). The effect of PAO on the catalytic activity of recombinant SH-PTP2 was assayed with *p*-nitrophenylphosphate (pNPP) as described previously (25).

Dephosphorylation of synthetic phosphopeptides by recombinant SH-PTP2 was determined by incubating 0 to 1 mM phosphopeptide with SH-PTP2 (100 or 6.6 μg/ml) at 30°C for 5 to 20 min in 50 μl of a solution containing 50 mM HEPES (pH 7.1), 150 mM NaCl, 10 mM DTT, and 2 mM EDTA as described previously (42). Released P_i was determined in the reaction mixture with a Phosphor C kit (Wako), and the A₇₅₀ was measured. The amount of released P_i was calculated from a standard curve. All phosphorylated peptides were obtained from Fujiya BioScience Laboratory (Tokyo, Japan). Sequences of phosphopeptides were as follows: EGFRpY992, DADE pYLIPQ; EGFRpY1068, PVPEpYINQS; EGFRpY1086, QNPV pYHNQP; EGFRpY1173, ENAepYLRVA; IRS-1pY46, GPARLEpYYENEKKWRH; IRS-1pY608, LHTDDGpYMP MSPGVAP; IRS-1pY628, RKGNGDpYMPMSPKSVS; IRS-1pY727, LPCTGDpYMNMSpVGDS; IRS-1pY895, SPGE pYVNIEFGS; and IRS-1pY1172, KSLNpYIDLVLK (pY indicates the phosphorylated tyrosine).

PI3-kinase assay. The assay for PI3-kinase activity was performed as previously described (52). Results are expressed as means ± standard errors of three separate experiments, as indicated.

RESULTS

Effect of expression of a catalytically inactive SH-PTP2 on insulin-stimulated Ras and MAP kinase activation. A cysteine residue in the HCSA motif of the PTP domain is highly conserved among various PTPs and is crucial for catalytic activity (11, 46). We therefore generated a mutant SH-PTP2 cDNA encoding a Cys-459→Ser mutation. Expression and purification of a recombinant protein of the mutant SH-PTP2 revealed that the Cys-459→Ser mutation virtually abolished PTP activity toward ³²P-labeled Raytide or pNPP (Table 1). Both the wild-type SH-PTP2 cDNA and the mutant SH-PTP2 cDNA were transfected into CHO-IR cells, and several cell lines overexpressing wild-type (CHO-IR-WT) or catalytically inactive (CHO-IR-C/S) SH-PTP2 were established. The number of hygromycin B-resistant clones obtained in two indepen-

TABLE 1. PTP activity of recombinant SH-PTP2^a

SH-PTP2	Activity obtained with substrate	
	³² P-Raytide (cpm)	pNPP (OD ₄₁₀)
Wild type	17,050	1.25
Mutant	166	0.17

^a Recombinant wild-type SH-PTP2 and mutant SH-PTP2 containing the mutation Cys-459→Ser were generated by the GST fusion protein system. The PTP activity of each recombinant SH-PTP2 was assayed with ³²P-labeled Raytide or with pNPP. The activity observed with assay buffer alone was subtracted from that observed with recombinant SH-PTP2. The results are representative of three separate experiments. OD₄₁₀, optical density at 410 nm.

dent transfections with the vector encoding the mutant SH-PTP2 was consistently lower than that obtained with the vector encoding wild-type SH-PTP2. In addition, a total of 13 clones overexpressing SH-PTP2 among 50 hygromycin B-resistant colonies was obtained after transfection with vector containing a wild-type SH-PTP2 cDNA, whereas only 5 clones overexpressing mutant SH-PTP2 among 44 hygromycin B-resistant colonies were obtained after transfection with the vector containing a mutant-type SH-PTP2 cDNA. These results suggest that expression of a catalytically inactive SH-PTP2 may have a negative effect on cell growth, although a more direct way such as the determination of DNA synthesis is required for studying the growth abilities of cells transfected with a mutant SH-PTP2 cDNA. Of the various cell lines, CHO-IR-WT13, overexpressing wild-type SH-PTP2, and CHO-IR-C/S23 and CHO-IR-C/S46, both overexpressing mutant SH-PTP2, were chosen for further characterization. The extent of expression of exogenous SH-PTP2 was assessed by immunoblotting of cell lysates with antibodies to SH-PTP2 (Fig. 1A). The amount of total SH-PTP2 in CHO-IR-WT13 cells was 10 times that in the parent CHO-IR cells, whereas the amounts in CHO-IR-C/S23 and CHO-IR-C/S46 cells were 5 and 20 times that in CHO-IR cells, respectively.

SH-PTP2 binds to tyrosine-phosphorylated IRS-1 in response to insulin (19). Transfected CHO-IR cells were therefore incubated with or without 100 nM insulin, and cell lysates were then subjected to immunoprecipitation with anti-IRS-1 antibodies and immunoblotting with anti-SH-PTP2 antibodies. The amount of SH-PTP2 that bound to IRS-1 in response to insulin was greater in CHO-IR cells overexpressing wild-type or mutant SH-PTP2 than in control CHO-IR cells (Fig. 1B). The amount of SH-PTP2 bound to IRS-1 in CHO-IR-C/S23 cells was greater than that in CHO-IR-WT13 cells, even though the extent of expression of SH-PTP2 in CHO-IR-C/S23 cells was less than in CHO-IR-WT13 cells. SH-PTP2 has been shown to undergo tyrosine phosphorylation in response to PDGF (10, 17). In contrast, SH-PTP2 that bound to IRS-1 in response to insulin was not phosphorylated on tyrosine residues in any of the CHO-IR cell lines overexpressing SH-PTP2 (data not shown).

We assayed the PTP activity in the anti-IRS-1 immunoprecipitates from transfected CHO-IR cells. The PTP activity in the immunoprecipitate from CHO-IR cells was increased by treatment of the cells with 100 nM insulin (Fig. 1C) and may reflect the catalytic activity of SH-PTP2 associated with IRS-1. In CHO-IR-WT13 cells, the insulin-dependent increase in PTP activity was 100% ± 15% (*n* = 3) of that observed for CHO-IR cells. In contrast, the insulin-dependent increase in PTP activity was completely abolished in CHO-IR-C/S46 cells and was decreased in CHO-IR-C/S23 cells to 40% ± 4% (*n* = 3) of that observed for CHO-IR cells (Fig. 1C). These data suggest that exogenously expressed catalytically inactive SH-PTP2 binds to

FIG. 1. Expression of wild-type and a catalytically inactive SH-PTP2 in CHO-IR cells. (A) Cell lysates (20 μ g) prepared from CHO-IR, CHO-IR-WT13 (WT13), CHO-IR-C/S23 (C/S23), and CHO-IR-C/S46 (C/S46) cells were fractionated by electrophoresis and immunoblotted with antibodies to SH-PTP2. M_r is in thousands. (B) Transfected CHO-IR cells were incubated with or without 100 nM insulin for 5 min at 37°C, and cell lysates were then prepared and subjected to immunoprecipitation with antibodies to IRS-1. The immunoprecipitates were fractionated by electrophoresis and immunoblotted with antibodies to SH-PTP2. M_r is in thousands. (C) Transfected CHO-IR cells were incubated with or without 100 nM insulin for 5 min, and immunoprecipitates prepared with antibodies to IRS-1 were then assayed for PTP activity with 32 P-labeled Raytide as the substrate. The activity observed in unstimulated cells, which was similar in all cell lines, was subtracted from that observed in insulin-stimulated cells. All results are representative of three separate experiments.

IRS-1 in response to insulin and competitively inhibits the insulin-induced binding of endogenous SH-PTP2 in CHO-IR-C/S cells.

SH-PTP2 has been suggested to function as a positive regulator in growth factor-stimulated cell proliferation (20, 32). Insulin, as well as other growth factors, induces mitogenesis via activation of the Ras-Raf-MAP kinase cascade (39, 45). Therefore, we investigated whether the expression of wild-type or catalytically inactive SH-PTP2 alters the effects of insulin on the activation of Ras and MAP kinase in transfected CHO-IR cells. Ras activation in response to insulin was determined by measuring the increase in the ratio of GTP-bound Ras to

GDP-bound Ras. When CHO-IR cells were stimulated with various concentrations of insulin, the percentage of Ras bound to GTP increased in a concentration-dependent manner; Ras activation was maximal at 100 nM insulin (Fig. 2). The stimulatory effect of insulin on Ras activation was slightly greater in CHO-IR-WT13 cells than in CHO-IR cells. In contrast, the insulin-stimulated increase in Ras-GTP was virtually abolished in CHO-IR-C/S46 cells and was markedly reduced in CHO-IR-C/S23 cells. The extent of inhibition of insulin-induced Ras activation in the two CHO-IR-C/S cell lines corresponded to the extent of expression of the catalytically inactive SH-PTP2.

FIG. 2. Effect of expression of wild-type and a catalytically inactive SH-PTP2 on insulin-stimulated Ras activation. Transfected CHO-IR cells labeled with 32 P were stimulated with various concentrations of insulin for 2 min. Ras was then immunoprecipitated with monoclonal antibody Y13-259, and bound nucleotides were eluted and separated by thin-layer chromatography. An autoradiography is shown in panel B. The positions of the nonradioactive GTP and GDP standards and the origin of application are indicated. The radioactivity corresponding to GTP-bound Ras and GDP-bound Ras was measured by image analysis, and Ras-GTP was expressed as a percentage of Ras-GTP plus Ras-GDP (A). All results are representative of three independent experiments. Cell lines are abbreviated as in Fig. 1.

FIG. 3. Effects of insulin and TPA on MAP kinase activity and PI3-kinase activity in transfected CHO-IR cells. (A and B) Transfected CHO-IR cells were stimulated with various concentrations of insulin for 5 min or with 1 μ M TPA for 10 min. Cell lysates were subjected to immunoprecipitation with antibody α C92 to MAP kinase, and MAP kinase activity in immunoprecipitates was assayed with myelin basic protein as the substrate. (B) Open bars, unstimulated cells; closed bars, 100 nM insulin; hatched bars, 1 μ M TPA. (C) Transfected cells were incubated with or without 100 nM insulin for 5 min, and PI3-kinase activity in anti-IRS-1 immunoprecipitates was measured with an *in vitro* kinase assay with phosphatidylinositol as the substrate. ³²P-labeled phosphatidylinositol 3-phosphate (PIP) was detected by thin-layer chromatography and autoradiography. All results are representative of three separate experiments. Cell lines are abbreviated as in Fig. 1.

It is known that the activation of Ras is required for insulin-stimulated MAP kinase activation (39, 45). Therefore, we next examined the effects of insulin on MAP kinase activation in transfected CHO-IR cells. Insulin increased MAP kinase activity in a concentration-dependent manner in CHO-IR cells and CHO-IR-WT cells (Fig. 3A). However, the extent of insulin-induced MAP kinase activation was markedly reduced in CHO-IR-C/S cells; the extent of the reduction again corresponded with the extent of expression levels of mutant SH-PTP2. TPA, a potent activator of protein kinase C, induces MAP kinase activation in a Ras-independent manner (3). In contrast to insulin stimulation of MAP kinase activity, TPA-induced MAP kinase activation was not significantly affected in CHO-IR-C/S cells (Fig. 3B). These results suggest that inhibition of insulin-stimulated MAP kinase activation in CHO-IR-C/S cells is attributable to the inhibition of insulin-stimulated Ras activation rather than to direct inhibition of MAP kinase activity.

The p85 subunit of PI3-kinase associates with IRS-1 in response to insulin, resulting in a stimulation of PI3-kinase activity (4, 52). There was no apparent difference in insulin-stimulated PI3-kinase activities in anti-IRS-1 immunoprecipitates prepared from the various CHO-IR cell lines (Fig. 3C). These results indicate that the expression of a catalytically inactive SH-PTP2 specifically blocks insulin-stimulated Ras activation and subsequent MAP kinase activation.

Effect of mutant SH-PTP2 expression on the insulin-induced association of the Grb2 with IRS-1 and of tyrosine-phosphorylated Shc with Grb2. Tyrosine phosphorylation of IRS-1 is required for Ras activation in response to insulin (51). In addition, Grb2, an adaptor protein that contains a single SH2 domain and two flanking SH3 domains (29), is thought to participate in insulin-stimulated Ras activation (34, 39, 40). Grb2 binds to IRS-1 in response to insulin stimulation and couples tyrosine-phosphorylated IRS-1 to Sos, a guanine nucleotide exchange protein of Ras (39, 40). Because expression of the catalytically inactive SH-PTP2 blocked insulin-induced Ras activation, we next determined the extent of insulin-induced tyrosine phosphorylation of IRS-1 in transfected CHO-IR cells. Lysates of insulin-stimulated cells were subjected to immunoprecipitation with antibodies to IRS-1 and

immunoblotted with antiphosphotyrosine antibodies. The extents of tyrosine phosphorylation of IRS-1 in response to insulin did not differ among the various transfected CHO-IR cell lines (Fig. 4A). The association of Grb2 with IRS-1 after insulin stimulation was also not inhibited in CHO-IR-C/S cells (Fig. 4B). We did not detect complex formation between IRS-1 and Sos in anti-IRS-1 immunoprecipitates of insulin-stimulated CHO-IR cell lysates, although the Grb2-Sos complex was observed in CHO-IR cells (data not shown); this result is consistent with the recent observations of Pronk et al. (34).

Insulin also stimulate the phosphorylation of Shc on tyrosine residues (35). Tyrosine-phosphorylated Shc binds to Grb2, which forms a complex with Sos (34). Thus, Shc is implicated in mediation of insulin-induced Ras activation (34, 39). When CHO-IR cells were stimulated with insulin, the extent of tyrosine phosphorylation of the different isoforms of Shc (p46, p52, and p56 Shc) (30) varied, p52 Shc being the predominant phosphorylated form as described previously (35). The extents of tyrosine phosphorylation of Shc in response to insulin were similar in CHO-IR, CHO-IR-WT, and CHO-IR-C/S cells (data not shown). Furthermore, the formation of an Shc-Grb2 complex in response to insulin was not affected in CHO-IR-C/S cells (Fig. 4C). Thus, our results indicate that expression of a catalytically inactive SH-PTP2 does not affect insulin-induced tyrosine phosphorylation of Shc or the formation of the complex between Grb2 and Shc.

When the insulin-induced association of SH-PTP2 with IRS-1 was monitored as a function of time, the association was shown to be maximal at 1 min after exposure to insulin and had decreased by 5 min in CHO-IR cells (Fig. 4D). This transient association of SH-PTP2 with tyrosine-phosphorylated IRS-1 was more marked in CHO-IR-WT13 cells. However, in CHO-IR-C/S46 cells, the amount of SH-PTP2 associated with IRS-1 continued to increase at 5 min after insulin stimulation (Fig. 4D). In addition, the amount of SH-PTP2 bound to IRS-1 in CHO-IR-C/S46 cells was not changed up to 30 min after insulin stimulation (data not shown).

PTP activity of SH-PTP2 toward phosphopeptides corresponding to IRS-1 and EGF receptor tyrosine phosphorylation sites. The amount of SH-PTP2 bound to IRS-1 in CHO-IR-C/S23 cells was larger than that observed in CHO-IR-WT13

FIG. 4. Effects of insulin on tyrosine phosphorylation of IRS-1 and complex formation between various signaling molecules in transfected CHO-IR cell lines. Transfected CHO-IR cells were incubated with 100 nM insulin for 0, 1, or 5 min. Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to IRS-1 (α IRS-1; A, B, and D) or to Grb2 (α Grb2; C). The immunoprecipitates were fractionated by electrophoresis and immunoblotted with antibodies to phosphotyrosine (PY-20; A), to Grb2 (B), to Shc (α Shc; C), or to SH-PTP2 (α SH-PTP2; D). Cell lines are abbreviated as in Fig. 1. M_r s are in thousands.

cells, even though the extent of expression of SH-PTP2 in CHO-IR-C/S23 cells was lower than that in CHO-IR-WT13 cells (Fig. 1A and B). Furthermore, the amount of wild-type SH-PTP2 associated with IRS-1 decreased 5 min after insulin stimulation, whereas the amount of associated mutant SH-PTP2 did not (Fig. 4D). These results suggest that wild-type SH-PTP2 may bind to IRS-1 in response to insulin and then dephosphorylate the phosphorylated tyrosine residue at the SH-PTP2 binding site on IRS-1. We therefore evaluated the PTP activity of recombinant SH-PTP2 toward various phosphopeptides corresponding to IRS-1 under physiological conditions in vitro. As previously described (42), EGFRpY992, a phosphopeptide corresponding to the sequence surrounding Tyr-992 of the EGF receptor, was an effective substrate for SH-PTP2 (Fig. 5). Among IRS-1 phosphopeptides, IRS-1pY895, which corresponds to the sequence around the putative binding site of Grb2 (43), was the best substrate for SH-PTP2 (K_m , 170 μ M; k_{cat} , 1.44 s^{-1}). In addition, IRS-1pY1172, which corresponds to the sequence around the putative binding site of SH-PTP2 (43), was also an effective substrate for SH-PTP2 (K_m , 240 μ M; k_{cat} , 1.89 s^{-1}). In contrast, IRS-1pY608, which corresponds to the sequence around the binding site of the p85 subunit of PI3-kinase (43), was a poor substrate for SH-PTP2 (K_m , >500 μ M). IRS-1pY46, which does not correspond to a binding site for any known SH2 domain-containing protein, was also a poor substrate (K_m , >500 μ M).

FIG. 5. PTP activity of SH-PTP2 toward phosphopeptides corresponding to IRS-1 and EGF receptor (EGFR) tyrosine phosphorylation sites. Various synthetic phosphopeptides (480 μ M) were incubated with recombinant SH-PTP2 (100 μ g/ml) for 20 min, and the amount of P_i released was assayed. Each value is the mean of duplicate determinations, and the results are representative of three separate experiments.

Effect of PAO on the insulin-induced association of SH-PTP2 with IRS-1 and on the catalytic activity of SH-PTP2. We next examined the effect of PAO, a putative inhibitor of PTPs (26), on the insulin-induced association of SH-PTP2 with IRS-1 in CHO-IR cells. Treatment of CHO-IR cells for 5 min with 25 μ M PAO before stimulation with 100 nM insulin for 5 min increased the insulin-dependent binding of SH-PTP2 to IRS-1 (Fig. 6A). PAO treatment did not affect the insulin-induced association of Grb2 with IRS-1 or the overall tyrosine phosphorylation of IRS-1. PAO inhibited the PTP activity of recombinant SH-PTP2 in vitro in a concentration-dependent manner (Fig. 6B). These results suggest that PAO treatment of CHO-IR cells may inhibit the catalytic activity of SH-PTP2.

DISCUSSION

We have shown that the expression of a catalytically inactive SH-PTP2 inhibited insulin-induced Ras activation, which, in turn, prevented MAP kinase activation in response to insulin. These inhibitory effects of a dominant negative SH-PTP2 were specific for Ras and MAP kinase activation, because PI3-kinase activation in response to insulin was not affected. SH-PTP2 is a human homolog of the *Drosophila* Corkscrew product (12, 31, 32), which has been suggested to be maternally required for normal determination of cell fate in the fly embryo (31, 32). *Corkscrew* functions downstream of *Torso*, a receptor tyrosine kinase that is structurally homologous to the PDGF receptor (32). *Ras1*, a *Drosophila* homolog of mammalian Ras, is also thought to participate in the *Torso* pathway (32). The position of *Corkscrew* relative to *Ras1* is not clear, but overexpression of an activated *Ras1* allele can compensate for the lack of *Corkscrew*, suggesting that the latter may regulate *Ras1*.

FIG. 6. Effects of PAO on the insulin-induced association of SH-PTP2 or Grb2 with IRS-1 and on the catalytic activity of recombinant SH-PTP2 in vitro. (A) CHO-IR cells were incubated in the absence or presence of 25 μ M PAO or vehicle (dimethylsulfoxide [DMSO]) for 5 min and then stimulated with 100 nM insulin for 5 min. Cell lysates were subjected to immunoprecipitation with antibodies to IRS-1 and immunoblotting with antibodies to phosphotyrosine (PY-20), to SH-PTP2 (α SH-PTP2), or to Grb2 (α GRB2). (B) Recombinant SH-PTP2 was incubated with 25 mM pNPP as the substrate for 20 min at 30°C in the presence of vehicle or various concentrations of PAO. Catalytic activity is expressed as the percentage of the control value observed in the absence of PAO or vehicle. Each value is the mean of duplicate determinations, and the results are representative of three separate experiments.

in *Drosophila* embryos (23). The binding of SH-PTP2 to the PDGF receptor, in the absence of association of the receptors with other signaling molecules, such as phospholipase C- γ 1, Ras GTPase-activating protein (GAP), and PI3-kinase, is sufficient for full activation of Ras in response to PDGF (49). Together with our data, this observation suggests that SH-PTP2 may mediate growth factor-induced Ras activation and the subsequent cell proliferation cascade.

A CHO cell line that overexpresses a human IR with a Tyr-960 $\rightarrow$ Ala mutation exhibits impaired tyrosine phosphorylation of IRS-1 and Ras activation in response to insulin (51), suggesting that tyrosine phosphorylation of IRS-1 is essential for insulin-induced Ras activation. We did not observe insulin-induced binding of SH-PTP2 to IRS-1 in CHO-IR cells expressing these mutant IRs (28). Microinjection of antibodies to IRS-1 in cells inhibits the insulin-induced mitogenic response (36). Furthermore, insulin-stimulated Ras activation is markedly inhibited by PAO, suggesting that PAO-sensitive PTP may be important in insulin-stimulated Ras activation (26). We and others (8) have shown that PAO inhibits the catalytic activity of recombinant SH-PTP2 in vitro. In addition, PAO treatment of CHO-IR cells impaired insulin-induced MAP kinase activation (28). These observations indicate that SH-PTP2 is a good candidate for the PAO-sensitive PTP that mediates insulin-induced Ras activation, as previously postulated by Medema et al. (26).

Although our data suggest that SH-PTP2 functions in insulin-stimulated Ras activation, the precise mechanism by which the complex of IRS-1 and SH-PTP2 stimulates Ras activation is unclear. Recent studies suggest that after insulin stimulation, the SH2 domain of Grb2 binds to two tyrosine-phosphorylated proteins: IRS-1 and Shc (34, 39, 40). Because Grb2 forms a complex with Sos (6, 29), the interaction of Grb2 with IRS-1 or Shc may link insulin stimulation to Sos, which increases the percentage of Ras molecules bound to GTP (34, 39, 40). However, the insulin-induced binding of Grb2 to IRS-1 was

similar in both CHO-IR and CHO-IR-C/S cells. In addition, the extent of tyrosine phosphorylation of Shc and the association of Shc with Grb2 in response to insulin were not affected by the expression of the catalytically inactive SH-PTP2. It is possible, therefore, that SH-PTP2 regulates an upstream element necessary for insulin-stimulated Ras activation and that this upstream element is required for the Grb2- or Shc-dependent pathway. We did not detect a complex formation of Sos and IRS-1 in insulin-stimulated CHO-IR cells or transfected CHO-IR cells; the complex of Grb2 and Sos was detected. Furthermore, we could not detect Sos in anti-Shc immunoprecipitates from insulin-stimulated CHO-IR cells (data not shown), although our failure to detect IRS-1-Sos and Shc-Sos complexes may be attributable to inadequate sensitivities of the relevant antibodies. It is possible that SH-PTP2 bound to IRS-1 in response to insulin stimulation directly activates a Sos-like guanine nucleotide exchange protein whose activity is increased by dephosphorylation of phosphorylated tyrosine residues. In fact, we observed that a 120-kDa tyrosine-phosphorylated protein was coimmunoprecipitated with a catalytically inactive SH-PTP2 but not with a wild-type SH-PTP2 (28), suggesting that a 120-kDa tyrosine-phosphorylated protein might be a downstream target of SH-PTP2.

An overexpressed, catalytically inactive SH-PTP2 may bind to not only tyrosine-phosphorylated IRS-1 but also other Ras-related signaling molecules through its SH2 domains, thus affecting Ras activation in response to insulin. However, this possibility appears unlikely because overexpression of wild-type SH-PTP2 slightly enhanced insulin-induced Ras activation. Furthermore, the level of expression of exogenous SH-PTP2 was less in CHO-IR-C/S23 cells than in CHO-IR-WT13 cells, while the inhibition of insulin-stimulated Ras activation was observed in CHO-IR-C/S23 cells. These findings suggest that inhibition of insulin-induced Ras activation by exogenously expressed SH-PTP2 occurs only when the PTP activity of the enzyme is abolished. It is also possible that SH-PTP2

down-regulates GAP activity in the physiological state; thus, the relative activation of GAP that would result from the expression of inactive SH-PTP2 may in turn suppress the increase in GTP-bound Ras in response to insulin. This possibility is worth exploring, although tyrosine phosphorylation of GAP has been suggested not to be a significant upstream regulatory event in insulin-stimulated Ras activation (33). Syp, a mouse homolog of SH-PTP2, undergoes tyrosine phosphorylation and forms a complex with Grb2 and Sos in response to PDGF stimulation, suggesting that Syp may mediate PDGF-stimulated Ras activation by coupling Grb2-Sos to PDGF receptors (22). However, SH-PTP2 bound to IRS-1 in response to insulin is not phosphorylated on tyrosine residues. In addition, we could not detect a complex of SH-PTP2 and Grb2 after insulin treatment of CHO-IR cells (28). Thus, SH-PTP2 may not function as an adaptor protein that couples IRS-1 to the Grb2-Sos complex in insulin signaling.

We have also shown that the association of SH-PTP2 with IRS-1 in CHO-IR cells was maximal 1 min after insulin stimulation and had decreased by 5 min after stimulation. This transient association of SH-PTP2 with tyrosine-phosphorylated IRS-1 was also apparent, but more marked, in CHO-IR cells overexpressing wild-type SH-PTP2. In contrast, the amount of SH-PTP2 bound to tyrosine-phosphorylated IRS-1 in CHO-IR-C/S46 cells had not decreased 5 min after insulin stimulation. Furthermore, although the level of expression of exogenous SH-PTP2 was less in CHO-IR-C/S23 cells than in CHO-IR-WT13 cells, the amount of SH-PTP2 bound to tyrosine-phosphorylated IRS-1 in CHO-IR-C/S23 cells exceeded that observed for CHO-IR-WT13 cells. These results suggest that wild-type SH-PTP2 may dephosphorylate the phosphotyrosine to which it binds through its SH2 domains. The mutant SH-PTP2 may form a more stable complex with tyrosine-phosphorylated IRS-1 because of its lack of PTP activity. SH-PTP2 has been shown to bind to phosphorylated Tyr-1172 of IRS-1 through its amino-terminal SH2 domain (43). A phosphopeptide corresponding to the sequence surrounding Tyr-1172 was shown to be a good substrate for recombinant SH-PTP2 *in vitro*. Furthermore, treatment of CHO-IR cells with PAO, which inhibited the catalytic activity of recombinant SH-PTP2 *in vitro*, increased the amount of endogenous SH-PTP2 bound to IRS-1 in response to insulin. Taken together, our data are consistent with the notion that SH-PTP2 may dephosphorylate the phosphotyrosine on IRS-1 to which it binds and thereby modulate its association with IRS-1. The crystal structure of PTP1B has shown that the catalytic site of PTP1B makes a cleft which recognizes phosphotyrosine residues, and a similar structure is also observed in the structure of SH2 domains (5). Thus, it is possible that the tyrosine-phosphorylated site of IRS-1 binds to the SH2 domains of SH-PTP2 and then slides to the catalytic pocket of SH-PTP2, thereby being dephosphorylated.

In summary, our results suggest that the complex of IRS-1 and SH-PTP2 mediates Ras activation through an unknown mechanism. The phosphotyrosine residue in the binding site of SH-PTP2 on IRS-1 may be one of the targets for the catalytic activity of SH-PTP2.

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