Characterization of a 115-kDa Protein That Binds to SH-PTP2, a Protein-tyrosine Phosphatase with Src Homology 2 Domains, in Chinese Hamster Ovary Cells*

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SH-PTP2, a non-transmembrane-type protein-tyrosine phosphatase with two Src homology 2 domains, was previously shown to play a positive signaling role in the insulin-induced activation of Ras and mitogen-activated protein kinase. SH-PTP2 was shown to associate with a 115-kDa tyrosine-phosphorylated protein (pp115), as well as with insulin receptor substrate 1, in insulin-stimulated Chinese hamster ovary cells that overexpress human insulin receptors (CHO-IR cells). In vivo and in vitro binding experiments revealed that SH-PTP2 bound to pp115 through one or both of its SH2 domains. The pp115 protein was partially purified from insulin-stimulated CHO-IR cells that overexpress a catalytically inactive SH-PTP2 by a combination of immunoaffinity and lectin-affinity chromatography. A monoclonal antibody to pp115 was then generated by injecting the partially purified protein into mice. Experiments with this monoclonal antibody revealed that pp115 is a transmembrane protein with a domain exposed on the cell surface and that it binds to SH-PTP2 in response to insulin. The insulin receptor kinase appeared to phosphorylate pp115 on tyrosine residues both in vivo and in vitro. The extent of tyrosine phosphorylation of pp115 associated with SH-PTP2 was greatly increased in CHO-IR cells that overexpress catalytically inactive SH-PTP2 compared with that observed in CHO-IR cells overexpressing wild-type SH-PTP2. Furthermore, recombinant SH-PTP2 preferentially dephosphorylated pp115 in vitro, indicating that SH-PTP2 may catalyze the dephosphorylation of phosphotyrosine residues in pp115 after it binds to this protein. These results suggest that pp115 may act as a transmembrane anchor to which SH-PTP2 binds in response to insulin. Furthermore, pp115 may be a physiological substrate for both the insulin receptor kinase and SH-PTP2.

We and others have recently cloned a human protein-tyrosine phosphatase (PTP),1 SH-PTP2 (also known as PTP1D, PTP2C, SH-PTP3, and SAP-2), that possesses two Src homology 2 (SH2) domains (1–5). SYP is the mouse homolog of SH-PTP2 (6). SH-PTP2 binds directly to growth factor receptors, such as PDGF receptor, EGF receptor, c-KIT, and the erythropoietin receptor, in response to stimulation with ligand and undergoes tyrosine phosphorylation (2, 6–10). Furthermore, in response to insulin, SH-PTP2 also binds via its SH2 domains to IRS1 and is thereby activated (11–13). CORNSCREW, the Droso phila homolog of SH-PTP2, functions in conjunction with D-Raf kinase downstream of the TORSO receptor tyrosine kinase (14, 15), suggesting a positive role for CORNSCREW in growth regulation by this receptor. SH-PTP2 has been suggested to mediate PDGF-induced Ras activation, because PDGF receptors, in which Tyr1009, the binding site for SH-PTP2, was changed to Phe were not able to activate Ras in response to PDGF (16). In addition, SH-PTP2 plays a positive role in fibroblast growth factor- and activin-mediated mesoderm induction and mitogen-activated protein kinase activation in Xenopus oocytes (17), insulin-induced GLUT1 expression (18), and prolactin receptor-mediated activation of the β-casein gene promoter (19). Thus, SH-PTP2 may play a general role in intracellular signaling elicited by various growth factors and hormones, probably through activation of the Ras pathway.

PDGF stimulates tyrosine phosphorylation of SH-PTP2, which then binds to GRB2, an adapter protein that constitutively forms a complex with SOS, a guanine nucleotide exchange factor for Ras (20, 21). Thus, SH-PTP2 may function as an adapter protein that mediates PDGF-induced Ras activation by coupling the PDGF receptor to the GRB2-SOS complex. However, the significance of the PTP activity of SH-PTP2 in growth factor- or cytokine-induced signal has not been clear.

We and others have shown that the expression of a catalytically inactive SH-PTP2 inhibits insulin-stimulated Ras and mitogen-activated protein kinase activation in CHO cells that overexpress human insulin receptors (CHO-IR cells), suggesting that SH-PTP2 also mediates insulin-induced Ras activation and subsequent DNA synthesis (22–25). Because SH-PTP2 does not undergo tyrosine phosphorylation in response to insulin, it is unlikely to function as an adapter protein that couples IRS1 to the GRB2-SOS complex. Thus, the precise mechanism by which the complex of IRS1 and SH-PTP2 activates Ras is unclear.

The mechanism by which SH-PTP2 mediates Ras activation in response to insulin may be clarified by identification of target proteins for its PTP activity. We have recently shown that a 115-kDa tyrosine-phosphorylated protein, pp115, is co-

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; SH2, Src homology 2; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IRS1, insulin receptor substrate 1; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; ConA, concanavalin; A: GST, glutathione S-transferase; IR, insulin receptor; LCA, Lens culinaris agglutinin; RCA, Ricinus communis agglutinin; IgG, immunoglobulin G.
immunoprecipitated with a catalytically inactive SH-PTP2 to a markedly greater extent than with wild-type SH-PTP2 (26). It is thus possible that pp115 is an endogenous substrate for SH-PTP2 whose dephosphorylation contributes to insulin signaling. In this report, we have further characterized the tyrosine-phosphorylated 115-kDa protein that forms a complex with SH-PTP2 in CHO-IR cells.

MATERIALS AND METHODS

Immunoprecipitation and Immunoblot Analysis—CHO-IR cells that overexpress either wild-type SH-PTP2 (WT13 cells) or a catalytically inactive SH-PTP2 (CS/46 cells) (24) were cultured in Ham’s F-12 medium containing 10% fetal bovine serum. CHO cells that overexpress human PTP-1B (CHO-ER cells) were cultured under identical conditions as CHO-IR cells. Confluent (10-cm plates) CHO-ER or CHO-IR cells were deprived of serum for 16 h and then stimulated with 100 nM insulin or 100 nM EGF, respectively, for various times. Cells were then immediately washed with ice-cold PBS and frozen in liquid nitrogen. The cells were subsequently 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% (v/v) glycerol, 1 mM dithiothreitol, 100 mM glycine (pH 2.7) and 0.1% Nonidet P-40. The homogenate was centrifuged at 100,000 g for 30 min at 4 °C, and the resulting supernatant was referred to as particulate fraction. Vanadate. The suspension was then centrifuged at 100,000 g for 30 min at 4 °C with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. Debris was removed by centrifugation at 10,000 x g for 15 min at 4 °C, and the resulting lysate was subjected to immunoprecipitation and immunoblot analysis. In some experiments, lysates were treated with 1% SDS for 10 min at 95 °C. For immunoprecipitation, cell lysate from one plate was used in a 1 ml reaction with polyclonal antibodies to pp115 that had been bound to protein G-Sepharose beads (2 μg on 20 μl of beads) (Pharmacia). The beads were then washed three times with 1 ml of WG buffer (50 mM Hepes-NaOH (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100), suspended in SDS sample buffer, and boiled for 5 min. SDS-PAGE and immunoblot analysis with the ECL detection system (Amersham) were performed as described previously (5).

Subcellular Fractionation—Cells from one culture dish (10-cm plate) were scraped into 1 ml of ice-cold hypotonic lysis solution (20 mM Hepes-NaOH (pH 7.6), 5 mM sodium pyrophosphate, 5 mM EGTA, and 1 mM MgCl2) containing apronin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate, which were then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 100,000 x g for 60 min and the resulting supernatant was referred to as cytosolic fraction. The pellet was suspended in 1 ml of membrane solubilization buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 5 mM sodium fluoride) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. The suspension was centrifuged at 100,000 x g for 60 min and the resulting supernatant was referred to as particulate fraction. All procedures were performed at 4 °C.

Partial Purification of pp115 and Generation of a Monoclonal Antibody to pp115—Particulate fractions of insulin-stimulated confluent C/S46 cells from a total of 240 culture dishes (10-cm plates) were prepared as described above, and subsequently filtered through a membrane filter (pore size, 0.8 μM) and then subjected to an in vitro binding experiment. Adsorption of cell lysate to the fusion protein was carried out under the same conditions as immunoprecipitation.

Phosphorylation of pp115 by the IRA—Iras were partially purified from lysates of insulin-stimulated or unstimulated CHO-IR cells with WGA-agarose as described previously (29). In vitro phosphorylation of pp115 immunoprecipitated from serum-deprived CHO-IR cells was investigated by incubation for 30 min at 25 °C with WGA-purified IrAs in 50 μl of a solution containing 50 mM Hepes-NaOH (pH 7.6), 3 mM MnCl2, 10 mM MgCl2, 1 mM dithiothreitol, 10 μM ATP, and 2 μCi of [γ-32P]ATP (6000 Ci/mmol) (ICN). The immunoprecipitates were washed with PBS, followed by addition of SDS sample buffer, and boiling. [32P]-Labeled proteins were detected by SDS-PAGE and autoradiography.

Dephosphorylation of pp115 by Recombinant SH-PTP2—Lysates from insulin-stimulated C/S46 cells were subjected to immunoprecipitation with antibodies to SH-PTP2 bound to protein G-Sepharose beads. Portions of the same lysate were separately incubated with ConA-coupled agarose beads. Both types of beads were washed twice with 1 ml of WG buffer, and bound proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Dephosphorylation of phospho-tyrosine-phosphorylated pp115 by recombinant SH-PTP2 was determined by incubating the filters in the absence of presence of recombinant SH-PTP2 (1 or 5 μg/ml) for 20 min at 30 °C in a solution containing 50 mM Hepes-NaOH (pH 7.1), 150 mM NaCl, 10 mM dithiothreitol, and 2 mM EDTA. The filters were then washed for 5 min at room temperature with 500 mM NaCl, 0.5% SDS, and 0.1% Triton X-100, rinsed with PBS, and subjected to immunoblot analysis with PY-20 antibodies as described above.

Other Antibodies and Chemicals—Polyclonal antibodies to SH-PTP2 were generated to the GST fusion protein containing the carboxyl-terminal region of SH-PTP2 as described previously (24). Monoclonal antibodies to SH-PTP2 were generated by immunization of mice with
the GST fusion protein containing full-length SH-PTP2. Monoclonal antibodies to phosphotyrosine (PY-20) were obtained from ICN. *Lens culinaris* agglutinin (LCA)-agarose and *Ricinus communis* agglutinin (RCA)-agarose were obtained from Seikagaku Kougyo.

RESULTS

**Identification of a 115-kDa Tyrosine-phosphorylated Protein That Interacts with SH-PTP2**—CHO-IR cells were incubated in the absence or presence of insulin, lysed, and subjected to immunoprecipitation with antibodies to SH-PTP2. Immunoblot analysis with antibodies to phosphotyrosine revealed that an ~115-kDa tyrosine-phosphorylated protein (pp115) and tyrosine-phosphorylated IRS1 were co-immunoprecipitated with SH-PTP2 from insulin-treated cells (Fig. 1, lanes 1 and 2). The amount of pp115 co-immunoprecipitated with SH-PTP2 in response to insulin was slightly increased in WT13 cells, which overexpress wild-type SH-PTP2, relative to that observed with CHO-IR cells, although pp115 was also detected in WT13 immunoprecipitates in the absence of insulin stimulation (Fig. 1, lanes 3 and 4). In contrast, the amount of pp115 complexed with SH-PTP2 was greatly increased in both insulin-stimulated and unstimulated C/S46 cells, which overexpress a catalytically inactive SH-PTP2 (Fig. 1, lanes 5 and 6). However, these experiments did not reveal whether insulin increases the extent of phosphorylation of pp115 on tyrosine residues or induces complex formation between SH-PTP2 and pp115 that is constitutively tyrosine-phosphorylated.

We next examined the subcellular localization of pp115. Cytosolic and particulate fractions were prepared from insulin-stimulated or unstimulated CHO-IR cells or C/S46 cells and were subjected to immunoprecipitation with antibodies to SH-PTP2. Immunoblot analysis with antibodies to phosphotyrosine showed that pp115 was entirely recovered in the particulate fraction of insulin-stimulated CHO-IR cells, indicating that tyrosine-phosphorylated pp115 might be associated with membrane (Fig. 1, lanes 7–10). In contrast, IRS1 was recovered in both particulate and cytosolic fractions. Probing of the same blot with antibodies to SH-PTP2 revealed that SH-PTP2 was present in the particulate fraction of insulin-stimulated CHO-IR cells but not in that of unstimulated cells. Most SH-PTP2 remained in the cytosolic fraction of insulin-stimulated cells, indicating that only a small fraction of SH-PTP2 might translocate from the cytosol to membranes in response to insulin. The pp115 and IRS1 may be therefore involved in the insulin-induced translocation of SH-PTP2 in CHO-IR cells. The pp115 protein was entirely recovered in the particulate fraction from CHO-IR cells, whereas insulin-stimulated and unstimulated C/S46 cells (Fig. 1, lanes 11–14). In addition, the amount of SH-PTP2 in the particulate fraction was similar to that in the cytosolic fraction, suggesting that some fraction of exogenously expressed inactive SH-PTP2 possibly localized to the particulate fraction in a complex with pp115.

The diffuse nature of the pp115 band on SDS-PAGE also suggested that pp115 might be a heavily glycosylated protein. We subjected a solubilized membrane preparation from C/S46 cells to immunoprecipitation with antibodies to SH-PTP2 and then incubated the precipitated proteins, after elution, with agarose beads coupled to various lectins. The amount of the complex of pp115 and SH-PTP2 recovered was greatest with ConA-coupled beads, with lesser amounts binding to WGA-, RCA-, and LCA-coupled beads (Fig. 2). These results suggest the possibility that pp115 might be a glycosylated protein.

**Role of SH-PTP2 SH2 Domains in Association of SH-PTP2 with pp115**—We next examined whether SH2 domains of SH-PTP2 were involved or not in the complex formation of SH-PTP2 with pp115. Lysates of insulin-stimulated C/S46 cells were subjected to immunoprecipitation with three monoclonal antibodies to SH-PTP2 (M3, M5, and M6), all of which have been shown to recognize the amino-terminal SH2 domain of SH-PTP2.2 Whereas SH-PTP2 was effectively immunoprecipitated by these monoclonal antibodies, the complex of SH-PTP2 with pp115 was not (Fig. 3A, lanes 1–3). IRS1, which is thought to associate with one or both SH2 domains of SH-PTP2, was also not co-immunoprecipitated with SH-PTP2 by these monoclonal antibodies. Polyclonal antibodies to the carboxyl-terminal SH2 domain of SH-PTP2 (pp115) and IRS1, and a 95-kDa tyrosine-phosphorylated protein, which represents the β subunit of the IR, which shows that the complex between SH-PTP2 and pp115 may be mediated directly or indirectly through one or both SH2 domains of SH-PTP2.

We next incubated WT13 or C/S46 cells in the absence or presence of insulin, and then incubated cell lysates with a GST fusion protein containing the SH2 domains of SH-PTP2 or a GST fusion protein containing SH-PTP2 without its SH2 domains. The pp115 protein present in lysates of insulin-stimulated C/S46 cells or WT13 cells bound to the fusion protein containing the SH2 domains but not to that containing the region of SH-PTP2 lacking the SH2 domains (Fig. 3B, lanes 1–5). Similar results were obtained when the cell lysates were denatured by heating in the presence of SDS (Fig. 3B, lanes 6–10), indicating that SH-PTP2 can directly interact with pp115 in vitro.

**Generation of a Monoclonal Antibody That Specifically Recognizes pp115, a Cell Surface Protein That Binds to SH-PTP2 in Response to Insulin**—We generated a monoclonal antibody

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1. T. Noguchi, T. Motozaki, K. Horita, Y. Fujioka, and M. Kasuga, unpublished observations.
specific for pp115 by immunizing BALB/c mice with pp115 partially purified from insulin-stimulated C/S46 cells. Screening of 60 hybridoma wells revealed one clone (4C6) that immunoprecipitated the tyrosine-phosphorylated 115-kDa protein from the membrane fraction of insulin-stimulated C/S46 cells (Fig. 4A). A 180-kDa protein and a 100-kDa protein were non-specifically immunoprecipitated by culture supernatants of all hybridomas tested (data not shown). The 4C6 antibody (Fig. 4B, lanes 3 and 4), but not normal mouse IgG (Fig. 4B, lanes 1 and 2), immunoprecipitated ~115-kDa proteins from insulin-stimulated or unstimulated CHO-IR cells that had been metabolically labeled with [35S]methionine and [35S]cysteine. Furthermore, immunoprecipitates prepared from insulin-stimulated CHO-IR cells with the 4C6 antibody contained a 68-kDa protein, the molecular size of which is consistent with that of SH-PTP2 (Fig. 4B, lane 4). No other protein was specifically immunoprecipitated to a marked extent with the 4C6 antibody. Lysates of insulin-stimulated or unstimulated CHO-IR cells were next subjected to immunoprecipitation with 4C6 antibodies, and the immunoprecipitates were examined by immunoblot analysis with antibodies to SH-PTP2. The amount of SH-PTP2 bound to pp115 was markedly increased in response to insulin (Fig. 4C, lanes 1 and 2). We performed a similar experiment with CHO-ER cells, which express human EGF receptors; EGF stimulation had no effect on the association of SH-PTP2 with pp115 (Fig. 4C, lanes 3 and 4).

Proteins on the surface of CHO-IR cells were biotinylated, and cell lysates were subjected to immunoprecipitation with the 4C6 antibody. The 4C6 antibody (Fig. 4D, lane 2), but not normal mouse IgG (Fig. 4D, lane 1), precipitated biotin-labeled pp115. As a control, WGA-agarose beads were used to precipitate biotinylated IRs (Fig. 4D, lane 3). These results indicate that pp115 is exposed on the surface of CHO-IR cells and is therefore presumably a transmembrane protein.

Effects of Insulin and SH-PTP2 on the Extent of Tyrosine Phosphorylation of pp115—Exposure of WT13 cells to insulin resulted in an increase in the extent of tyrosine phosphorylation of pp115 after 1–5 min; the extent of phosphorylation had decreased again by 30 min (Fig. 5, lanes 1–4). In contrast, tyrosine phosphorylation of pp115 was apparent in the absence of insulin and was markedly increased after 1 min of stimulation with insulin in C/S46 cells; the increase in tyrosine phosphorylation of pp115 continued for up to 30 min after exposure of cells to insulin (Fig. 5, lanes 5–8). The amount of SH-PTP2 associated with pp115 was markedly increased in response to stimulation of WT13 cells with insulin for 1–5 min; the increased association was slightly less marked after 30 min (Fig. 5, lanes 1–4). In contrast, in C/S46 cells, a large amount of SH-PTP2 was associated with pp115 in the absence of insulin stimulation, and the association was increased by insulin stimulation for up to 30 min (Fig. 5, lanes 5–8). These observations suggest that insulin stimulates tyrosine phosphorylation of pp115 and that SH-PTP2, after binding to phosphorylated pp115, may catalyze its dephosphorylation. The catalytically inactive SH-PTP2 in C/S46 cells appears to bind to pp115 but is unable to catalyze its dephosphorylation, resulting in hyper-phosphorylation of pp115. Neither IR nor IRS1 was co-immunoprecipitated with pp115 from WT13 or C/S46 cells (data not shown).

Phosphorylation of pp115 by Activated IR Kinase in Vitro—Since insulin appeared to stimulate the tyrosine phosphorylation of pp115, we next determined whether IR kinase was able to phosphorylate pp115 directly or not. IRs purified by WGA-agarose from insulin-stimulated CHO-IR cells catalyzed the phosphorylation of pp115 that was isolated from serum-
Deprived CHO-IR cells by immunoprecipitation (Fig. 6, lane 3). The band of autophosphorylated IRs was also observed presumably because of insufficient washing of the immune complex. Phosphorylation of pp115 was not apparent on incubation with [γ-32P]ATP and IRs purified from unstimulated CHO-IR cells (Fig. 6, lane 4), indicating that pp115 does not undergo autophosphorylation.

Dephosphorylation of Tyrosine-phosphorylated pp115 by SH-PTP2 in Vitro—Finally, we determined whether SH-PTP2 could catalyze the dephosphorylation of tyrosine-phosphorylated pp115 in vitro. Tyrosine-phosphorylated IRS1 and pp115 were immunoprecipitated with antibodies to SH-PTP2 from insulin-stimulated C/S46 cells (Fig. 7, lanes 1–3). Tyrosine-phosphorylated pp115 and IRs were also precipitated with ConA-agarose beads (Fig. 7, lanes 4–6). Both precipitates were then fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with recombinant SH-PTP2. SH-PTP2 dephosphorylated tyrosine-phosphorylated pp115 in a concentration-dependent manner, but had little effect on the extent of tyrosine phosphorylation of IR or IRS1. These results are consistent with our in vivo observations suggesting that pp115 is a target protein for SH-PTP2-mediated dephosphorylation.

DISCUSSION

In the present study, we have generated a monoclonal antibody to pp115 and have shown that SH-PTP2 binds to tyrosine-phosphorylated pp115 as well as to IRS1 in response to insulin in CHO-IR cells. Both subcellular fractionation and biotinylation of cell surface proteins indicated that pp115 is a membrane-spanning protein with an extracellular domain. Insulin increased the extent of tyrosine phosphorylation of pp115 in intact cells. Furthermore, partially purified IRs phosphorylated pp115 in vitro, indicating that the IR kinase may directly phosphorylate tyrosine residues in the intracellular domain of pp115. Thus, pp115 appears to be a new member of the family of IR kinase substrates, which includes IRS1 and SHC.

FIG. 5. Time course of insulin-induced tyrosine phosphorylation of pp115 and the association of SH-PTP2 with pp115 in WT13 and C/S46 cells. Cells were stimulated with 100 nM insulin for the indicated times, after which whole cell lysates were prepared and subjected to immunoprecipitation with 4C6 monoclonal antibodies. The immunoprecipitates were then subjected to SDS-PAGE and immunoblot analysis with PY-20 or polyclonal antibodies to SH-PTP2.

Deprived CHO-IR cells by immunoprecipitation (Fig. 6, lane 3). The band of autophosphorylated IRs was also observed presumably because of insufficient washing of the immune complex. Phosphorylation of pp115 was not apparent on incubation with [γ-32P]ATP and IRs purified from unstimulated CHO-IR cells (Fig. 6, lane 4), indicating that pp115 does not undergo autophosphorylation.

Dephosphorylation of Tyrosine-phosphorylated pp115 by SH-PTP2 in Vitro—Finally, we determined whether SH-PTP2 could catalyze the dephosphorylation of tyrosine-phosphorylated pp115 in vitro. Tyrosine-phosphorylated IRS1 and pp115 were immunoprecipitated with antibodies to SH-PTP2 from insulin-stimulated C/S46 cells (Fig. 7, lanes 1–3). Tyrosine-phosphorylated pp115 and IRs were also precipitated with ConA-agarose beads (Fig. 7, lanes 4–6). Both precipitates were then fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with recombinant SH-PTP2. SH-PTP2 dephosphorylated tyrosine-phosphorylated pp115 in a concentration-dependent manner, but had little effect on the extent of tyrosine phosphorylation of IR or IRS1. These results are consistent with our in vivo observations suggesting that pp115 is a target protein for SH-PTP2-mediated dephosphorylation.

FIG. 4. Generation and characterization of a monoclonal antibody to pp115. A, screening of a hybridoma clone producing a monoclonal antibody to pp115. The membrane fraction of insulin-stimulated C/S46 cells was subjected to immunoprecipitation with the culture supernatant of hybridoma clone 4C6. The immunoprecipitate was subjected to SDS-PAGE and immunoblot analysis with PY-20. IgG, immunoglobulin G. B, labeling of pp115 with [35S]methionine and [35S]cysteine in CHO-IR cells. CHO-IR cells were metabolically labeled with 35S as described under “Materials and Methods,” deprived of serum, and then incubated in the absence or presence of insulin. Whole cell lysates were prepared and subjected to immunoprecipitation with normal mouse IgG (NMG) or 4C6 monoclonal antibodies. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. C, insulin-induced association of SH-PTP2 with pp115. CHO-IR or CHO-ER cells were incubated in the absence or presence of the appropriate ligand (insulin or EGF, respectively). Immunoprecipitates were prepared from cell lysates with 4C6 antibodies and then subjected to SDS-PAGE and immunoblot analysis with 4C6 antibodies or polyclonal antibodies to SH-PTP2. D, cell surface labeling of CHO-IR cells. Proteins on the surface of intact CHO-IR cells were biotinylated as described under “Materials and Methods.” Whole cell lysates were subjected to precipitation with protein G-Sepharose beads conjugated with either normal mouse IgG (lane 1) or 4C6 antibody (lane 2), or with WGA-conjugated agarose beads (lane 3). The precipitated proteins were then fractionated by SDS-PAGE and transferred to nitrocellulose membranes, and biotin-labeled bands were detected with horseradish peroxidase-conjugated streptavidin.
and pp115 was then determined by immunoblot analysis with PY-20. However, whereas Yamauchi et al. (22, 25, 30) as well as a 43-kDa protein (31). inactive SH-PTP2 results in the hyperphosphorylation of 115–
120-kDa proteins (22, 25, 30) as well as a 43-kDa protein (31). Although we must continue to consider that some other protein than IRS1 may mediate this association. pp115, whereas Yamauchi et al. (32) have demonstrated that EGF stimulates the formation of a complex between SH-PTP2 and a tyrosine-phosphorylated 115-kDa protein in HepG2 cells and NIH 3T3 cells, whereas in our study EGF failed to induce the binding of SH-PTP2 to pp115 in CHO cells overexpressing human EGF receptors. Thus, it is unclear whether the 115-kDa tyrosine-phosphorylated protein in v-Src-transfected rat 3Y1 fibroblasts (33). Because this 115-kDa protein in v-Src-transformed 3Y1 fibroblasts is also membrane-associated and glycosylated, it may be identical to pp115 in CHO cells and therefore represent a common target for both SH-PTP2 and PTP1C.

Our data also suggest that after binding to pp115 in response to insulin, SH-PTP2 catalyzes the dephosphorylation of phosphorylated tyrosine residues in the cytoplasmic domain of pp115. Thus, the extent of tyrosine phosphorylation of pp115 bound to a catalytically inactive SH-PTP2 was greatly increased compared with that of pp115 complexed with wild-type SH-PTP2. The extent of tyrosine phosphorylation of pp115 was maximal 1–5 min after insulin stimulation of WT13 cells. In contrast, the extent of tyrosine phosphorylation of pp115 continued to increase for up to 30 min after exposure of C/S46 cells to insulin. Furthermore, recombinant SH-PTP2 catalyzed the dephosphorylation of phosphotyrosine residues in pp115 but hardly those in IRSs or IRS1. Together, these results indicate that pp115 is a physiological substrate for SH-PTP2. We have previously demonstrated that SH-PTP2 may dephosphorylate the phosphotyrosine residue in its own binding site on IRS1 (24). However, the relative extent of insulin-stimulated association of SH-PTP2 with pp115 in WT13 cells at 30 min appeared greater than that of insulin-stimulated tyrosine phosphorylation of pp115. In addition, treatment of CHO-IR cells with phenylarsine oxide, which inhibits the catalytic activity of recombinant SH-PTP2 in vitro, did not affect the amount of SH-PTP2 bound to pp115 5 min after insulin stimulation, although it did increase the extent of tyrosine phosphorylation of pp115 (data not shown). Thus, it is possible that pp115 possesses multiple tyrosine-phosphorylation sites for the IR kinase and that SH-PTP2 preferentially dephosphorylates phosphotyrosine residues other than that in its binding site. SH-PTP2 does not effectively dephosphorylate the phosphotyrosine residue (Tyr1009) in its binding site on the PDGF receptor; instead, it preferentially dephosphorylates phosphorylated tyrosine residues to which the GTPase-activating protein of Ras or the p85 regulatory subunit of phosphatidylinositol 3-kinase binds in response to PDGF (34).

SH-PTP2 has been suggested to regulate an upstream element necessary for Ras activation in response to insulin (24). Although we have now shown that SH-PTP2 binds to the transmembrane protein pp115 in response to insulin, it remains unclear how pp115 may couple SH-PTP2 to Ras activation. It is possible that pp115 itself may possess SOS-like guanine nucleotide exchange activity that is up-regulated as a result of tyrosine dephosphorylation by SH-PTP2. Alternatively, pp115 may simply act as an anchoring protein to induce SH-PTP2 translocation from the cytosol to the plasma membrane. Thereafter, SH-PTP2 may activate an SOS-like guanine nucleotide exchange protein located near the plasma membrane. Furthermore, if pp115 acts as a docking protein like IRS1, other sig-
naling molecules that contain SH2 domains, in addition to SH-PTP2, may bind to pp115 in response to insulin.

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REFERENCES

1. Freeman, R. M., Jr., Plutzky, J., and Neel, B. G. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11239–11243
2. Vogel, W., Lammers, R., Huang, J., and Ulrich, A. (1993) Science 259, 1611–1614
3. Ahmad, S., Sekiya, M., Miyachi, T., Matsuno, K., Hinoda, Y., Imai, K., and Yachi, A. (1993) FEBS Lett. 314, 335–339
4. Matozaki, T., Suzuki, T., Uchida, T., Inazawa, J., Matsuda, K., Hinoda, Y., Mizuno, H., Sakamoto, C., and Kasuga, M. (1994) J. Biol. Chem. 269, 2075–2081
5. Feng, G.-S., Hui, C.-C., and Pawson, T. (1993) Science 259, 1607–1611
6. Kazlauskas, A., Feng, G.-S., Pawson, T., and Valius, M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6939–6942
7. Lechleider, R. J., Freeman, R. M., Jr., and Neel, B. G. (1993) J. Biol. Chem. 268, 13434–13438
8. Tauchi, T., Feng, G.-S., Marshall, M. S., Shen, R., Mantel, C., Pawson, T., and Broxmeyer, H. E. (1994) J. Biol. Chem. 269, 23206–23211
9. Tauchi, T., Feng, G.-S., Shen, R., Hoatlin, M., Bagby, G. C., Jr., Kabat, D., Lu, L., and Broxmeyer, H. E. (1995) J. Biol. Chem. 270, 5631–5635
10. Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G.-S. (1993) J. Biol. Chem. 268, 11479–11481
11. Sugimoto, S., Wandless, T. J., Shoelson, S. E., Neel, B. G., and Walsh, C. T. (1994) J. Biol. Chem. 269, 13614–13622
12. Perkins, L. A., Larsen, I., and Perrimon, N. (1992) Cell 70, 225–236
13. Perrimon, N. (1993) Cell 74, 219–222
14. Valius, M., and Kazlauskas, A. (1993) Cell 73, 321–334
15. Tang, T. L., Freeman, R. M., Jr., O’Reilly, A. M., Neel, B. G., and Sokol, S. Y. (1995) Cell 80, 473–483
16. Hausdorff, S. F., Bennett, A. M., Neel, B. G., and Birnbaum, M. J. (1995) J. Biol. Chem. 270, 12965–12968
17. Ali, S., Chen, Z., Lebrun, J.-J., Vogel, W., Kharitonenkova, A., Kelly, P. A., and Ulrich, A. (1996) EMBO J. 15, 135–142
18. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J. H., Cooper, J. A., and Schlessinger, J. (1993) Mol. Cell. Biol. 14, 509–517
19. Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7335–7339
20. Milarski, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21239–21243
21. Milarski, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21244–21248
22. Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994) Mol. Cell. Biol. 14, 6674–6682
23. Yamauchi, K., Milarski, K. L., Saltiel, A. R., and Pessin, J. E. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 664–668
24. Matozaki, T., Noguchi, T., Suzuki, T., and Kasuga, M. (1995) Adv. Protein Phosphatases 9, 319–338
25. Hosomi, Y., Shii, K., Ogawa, W., Matsuoka, H., Yoshida, M., Okada, Y., Yokono, K., Kasuga, M., Baba, S., and Roth, R. A. (1994) J. Biol. Chem. 269, 11498–11502
26. Matozaki, T., Sakamoto, C., Suzuki, T., Matsuda, K., Uchida, T., Nakano, O., Wada, K., Nishisaki, H., Konda, Y., Nogao, M., and Kasuga, M. (1992) Cancer Res. 52, 1–7
27. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D., and Kahn, C. R. (1985) Proc. Natl. Acad. Sci. U.S.A. 80, 2137–2141
28. Yamauchi, K., Ribon, V., Saltiel, A. R., and Pessin, J. E. (1995) J. Biol. Chem. 270, 17716–17722
29. Zhao, Z., Tan, Z., Wright, J. H., Dlitz, C. D., Shen, S.-H., Krebs, E. G., and Fischer, E. H. (1995) J. Biol. Chem. 270, 11765–11769
30. Yamauchi, K., and Pessin, J. E. (1995) J. Biol. Chem. 270, 14871–14874
31. Matarzoli, T., Uchida, T., Fujioka, Y., and Kasuga, M. (1994) Biochem. Biophys. Res. Commun. 204, 874–881
32. Klinghoffer, R. A., and Kazlauskas, A. (1995) J. Biol. Chem. 270, 22208–22217