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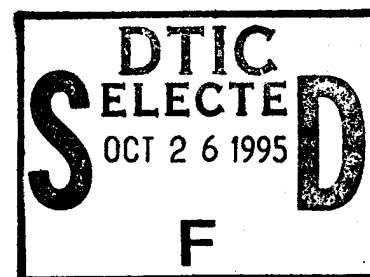
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13. ABSTRACT (Maximum 200 words) <p>During the past funding period, we have made excellent progress in our overall objectives to determine the signaling mechanisms of the protein tyrosine-specific phosphatase, SHPTP2. These current studies have resulted in our demonstration that SHPTP2 is a required positive effector in mediating both insulin and EGF receptor signaling events leading to the activation of ras and the ERK pathway. Since, activation of the ras/raf/MEK/ERK pathway is sufficient for mitogenesis, these data demonstrate that SHPTP2 is an important upstream mediator for growth factor stimulated mitogenesis. In order to identify the specific associated proteins and/or direct substrates for SHPTP2 activity, we have successfully performed a series of co-immunoprecipitation studies with carboxyl terminal SHPTP2 antibodies. These studies have identified a novel 115 kDa protein that is tyrosine phosphorylated and associated with SHPTP2 following insulin and EGF stimulation but not PDGF. This pp115 protein is the predominant SHPTP2 binding protein which appears to function both as a substrate as well as a docking protein based upon expression studies using a mutant SHPTP2 in which the catalytic cysteine was replaced with a serine residue. During the next budget period, we will be attempting to purify and further characterize this pp115 protein in order to determine its function as an upstream activator of ras.</p>				
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FOREWORD

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A) INTRODUCTION:

Growth factor receptors are a family of ligand-stimulated transmembrane tyrosine-specific protein kinase that phosphorylate themselves as well as intracellular substrates on specific tyrosine residues (1,2). In the case of the EGF receptor, autophosphorylation of the EGF and PDGF receptors results in the formation of recognition signals for the binding of specific SH2 (src homology 2) domain containing proteins (3,4). In contrast, autophosphorylation of the insulin and IGF1 receptors does not generate these binding motifs but instead activates their substrate kinase activity to phosphorylate intermediate docking proteins such as IRS1 and IRS2 (5-7). The interaction of IRS1/2 or receptors with multiple SH2 domain containing signaling proteins provides a mechanism by which growth factors can modulate the function of several distinct pathways. For example, activation of phosphatidylinositol (PI) 3-kinase activity occurs upon the direct association of the p85 subunit of the PI 3-kinase with tyrosine phosphorylated IRS1 or growth factor receptors (8-10). It has also been suggested that the growth factor activation of ras function results from the interaction and/or appropriate targeting of the guanylnucleotide exchange factor SOS with the tyrosine phosphorylated substrate Shc (11-13). This is thought to occur via the constitutive association of the GRB2 adapter protein SH3 (src homology 3) domains with the carboxyl terminal domain of SOS (GRB2/SOS complex) and subsequent binding of the GRB2 SH2 domain to tyrosine phosphorylated receptors and/or Shc.

Recently, several groups have identified an ubiquitously expressed 68 kDa tyrosine-specific phosphatase, SHPTP2 (also termed Syp, PTP1D, SHPTP3, PTP2C or PTPL1), that contains two amino terminal SH2 domains and a carboxyl catalytic domain (14-19). The SH2 domains of this phosphatase mediate the binding of SHPTP2 to the tyrosine phosphorylated epidermal growth factor receptor, platelet-derived growth factor receptor and insulin receptor substrate 1 (IRS1) resulting in the activation of protein tyrosine-specific phosphatase activity (20-22). Although protein tyrosine phosphatase activity is generally thought to function as the inactivating arm of receptor kinase signaling pathways, in the case of T cell receptor signaling, the CD45 tyrosine protein phosphatase dephosphorylates the inhibitory carboxyl-terminal tyrosine phosphorylation site on fyn and/or lck (23-26). The subsequent relief of this kinase inhibition is an essential requirement for T cell receptor-mediated biological responses (27,28). Similarly, SHPTP2 is the mammalian homologue of the *Drosophila* SH2 domain-containing protein tyrosine phosphatase, termed *corkscrew* (29). Genetic epistasis experiments have also demonstrated that *corkscrew* is

an essential gene for the appropriate development of anterior/posterior structures during embryogenesis, functioning downstream of the *torso* tyrosine kinase, a homologue of the mammalian PDGF receptor, and upstream of *polehole*, a homologue of the mammalian raf serine/threonine kinase (30-32). Similarly, recent studies have demonstrated that SHPTP2 protein tyrosine-specific phosphatase plays an important positive role in growth factor tyrosine kinase downstream signaling. Microinjection of SHPTP2 specific antibodies was observed to block insulin-stimulated DNA synthesis and expression of dominant-interfering SHPTP2 mutants inhibited activation of mitogen-activated protein kinase, *c-fos* transcription, DNA synthesis and fibroblast growth factor-stimulated *Xenopus* oocyte mesoderm induction (33-37).

These data have provided substantial evidence demonstrating a positive signaling role for SHPTP2 in mediating tyrosine kinase growth factor receptor action. To identify potential physiological targets for SHPTP2, we have begun to identify the physiological targets of SHPTP2 function in order to determine its role in mediating ras activation and subsequent stimulation of the Raf/MEK/ERK pathway leading to mitogenesis.

B) BODY:

1. Experimental Procedures:

Cell culture - Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) were isolated and maintained in minimal Eagle's medium containing nucleotides plus 10% fetal bovine serum as previously described (38). HepG2 and 3T3L1 pre-adipocytes were obtained from the American Type Tissue Culture collection and were maintained in Dulbecco's modified Eagle's medium 10% fetal bovine serum. The 3T3L1 cells were differentiated into adipocytes by replacement with fresh medium containing 10% fetal bovine serum, 0.25 μ M dexamethasone, 500 μ M isobutylmethylxanthine and 1 μ g/ml insulin for 3 days. NIH 3T3 cells over-expressing human EGF receptor (3T3/ER) were obtained from Dr. John Koland (The University of Iowa). 3T3/ER cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The human Jurkat T cell line and TCR monoclonal antibody (OKT3) were provided by Dr. Gary Koretzky (The University of Iowa). The Jurkat T cells were maintained in RPMI 1640 containing 10% fetal bovine serum.

Expression plasmids - The myc epitope (MEQKLISEEDL) tag was introduced into the amino terminus of SHPTP2 by cloning an oligonucleotide linking upstream of the initiation methionine codon. This domain was introduced

both in the wild type SHPTP2 cDNA (myc-WT-SHPTP2) and in the catalytic inactive SHPTP2 mutant in which cysteine 459 was replaced with serine (myc-C/S-SHPTP2) by site-directed mutagenesis. These cDNAs were then subcloned into the high efficiency mammalian expression vector, CLDN (39).

CaPO₄ transfection - Cells were transiently transfected with 1 μ g of a serum response element driven luciferase reporter plasmid (SRE-Luc), 2 μ g of a Rous sarcoma virus promoter driven β -galactosidase reference plasmid (RSV- β Gal) and 5 μ g of various protein tyrosine-specific phosphatase cDNAs cloned into the mammalian expression vector, CLDN as previously described (35). The total amount of transfected DNA was maintained at 23 μ g with the empty CLDN vector. Eighteen h following transfection the cells were serum starved for 12 h, followed by the addition of 100 nM insulin or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 6 h before preparation of cell extracts and assayed for luciferase and β -galactosidase activity.

Transfection by electroporation - In order to obtain a high degree of transfection efficiency necessary for immunoprecipitation and Western blotting of whole cell extracts, CHO/IR cells were electroporated with a total of 40 μ g of plasmid DNA at 340 volts and 960 μ F as previously described (39). Under these conditions, approximately 80-95% of the viable cell population were functionally transfected as assessed by *in situ* β -galactosidase staining. Thirty-six h following transfection the cells were serum starved for 6 h and either untreated or incubated for 5 min in the presence of 100 nM insulin or 100 ng/ml PDGF prior to the preparation of whole cell lysates.

Western blot analysis of whole cell extracts - Whole cell extracts were prepared by detergent solubilization in a lysis buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 2 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10 μ g/ml aprotinin and 1.5 mM pepstatin) for 1 h at 4°C. The resultant cell extracts were subjected to Western blotting using an amino terminal SHPTP2 antibody (Transduction Lab), a myc epitope tag antibody (9E10, Santa Cruz) or phosphotyrosine antibody (PY20-HRP, Santa Cruz) and visualization with the Enhanced Chemical Luminescence (ECL) detection system (Amersham).

Immunoprecipitations - Immunoprecipitation of the whole cell lysates were performed by a 5-fold dilution of the detergent solubilized cell extracts (lysis buffer without Triton X-100) and incubation with 4 μ g of a carboxyl terminal SHPTP2 polyclonal antibody (Santa Cruz), an EGF

receptor monoclonal antibody, LA1 (Upstate Biotechnology), a monoclonal antibody specific for the myc epitope tag (9E10, Santa Cruz), a polyclonal Grb2 antibody (Santa Cruz), a Crk monoclonal antibody (Transduction Laboratories), a Nck monoclonal antibody (Upstate Biotechnology Inc.), or an IRS1 rabbit polyclonal anti-serum (a kind gift from Dr. Gus Lienhard, Dartmouth Medical School, NH) for 2 h at 4°C. The primary polyclonal and monoclonal antibodies were incubated with protein A-agarose or protein G plus-agarose, respectively for 1 h at 4°C. The resulting immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotted as described above.

MAP kinase activity - MAP kinase activity from cell lysates was determined as previously described (35). Briefly, 10 µl of cell extracts were incubated with 0.2 mg/ml microtubule-associated protein (MAP-2) for 10 min at 30°C in 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 10 mM MgCl₂, and 40 µM [γ -³²P]ATP (1 µCi) in a total volume of 25 µl. The reaction was terminated by the addition of SDS sample buffer and the phosphorylated MAP-2 was resolved by SDS-polyacrylamide gel electrophoresis. Incorporated radioactivity was determined by excision of the MAP-2 band followed by scintillation counting.

2. Results:

To examine the role of SHPTP2 in growth factor receptor tyrosine kinase downstream signaling, we initially utilized Chinese hamster ovary cells expressing high levels of the human insulin receptor. We choose these cells as our initial model, since they can be transiently transfected quantitatively with various cDNAs of interest by electroporation. In these studies, we examined the effect of SHPTP2 on mediating c-fos gene expression, detected by co-transfection with a c-fos serum response element (SRE) driving the luciferase (Luc) reporter gene (SRE-Luc). Expression of full length SHPTP2 had no significant effect on insulin stimulation of SRE-Luc activity whereas expression of the SH2 domains of SHPTP2 inhibited expression. The inhibitory effect of the SH2 domains was specific as the src SH2 domain was without effect. Further, expression of a SHPTP2 catalytic point mutant in which cysteine 459 was replaced with serine (SHPTP2C/S) also inhibited c-fos expression. As controls, expression of either the wild type or catalytic point mutant of another protein tyrosine-specific phosphatase (PTP1B) was without effect. Thus, these data indicate that SHPTP2 functions as a positive effector in insulin signaling.

We next characterized the signaling pathway that required SHPTP2 function to mediate c-fos transcription. Since the ERK pathway is an established route for the activation of c-fos expression, we next determined the effect of SHPTP2 on MAP kinase (ERK) activity. Expression of either the SH2 domains of SHPTP2 or the catalytic mutant SHPTP2C/S resulted in a marked inhibition of insulin-stimulated MAP kinase activity. In contrast, the dominant-interfering SHPTP2 mutants did not block SRE-Luc activation by v-Ras. These data indicate that, in a linear model of signaling pathways, SHPTP2 functions upstream of Ras.

Having established that SHPTP2 is a required positive effector of receptor tyrosine kinase downstream signaling events leading to the Ras/Raf/MEK/ERK pathway, we performed co-immunoprecipitation experiments to identify potential targets of SHPTP2 function. Initial experiments using an amino terminal SHPTP2 antibody failed to detect any co-immunoprecipitated proteins. This was probably due to the antibody interfering with the amino terminal SH2 domains. However, when we performed co-immunoprecipitations with a carboxyl terminal SHPTP2 antibody, we detected the specific co-immunoprecipitation of a diffuse 115 kDa tyrosine phosphorylated protein. This 115 kDa band was tyrosine phosphorylated in response to both insulin and EGF but not PDGF in numerous cell lines. Interestingly, pp115 was found to be the predominant SHPTP2 associated tyrosine phosphorylated protein whereas the EGF receptor and IRS1 association only accounted for a small fraction of the total SHPTP2 pool. Thus, pp115 appears to be an important component of SHPTP2 function.

Currently, we are attempting to identify the nature of the pp115. We have attempted to identify this species by immunoprecipitation and Western blotting using antibodies directed against known tyrosine phosphorylated proteins of this approximate molecular weight such as FAK, c-Cbl, c-Dbl, Tyk2, Jak1, Jak2, Jak3, Stat3, p120src substrate and rasGAP. However, these antibodies did not cross react with pp115. We therefore believe that pp115 is a novel protein intimately involved in the upstream regulation of Ras function through SHPTP2.

In the upcoming funding period, we are attempting to approach to further our understanding of SHPTP2 function and its relationship to pp115. First, we are planning to express the dominant interfering mutant (SHPTP2C/S) in transgenic mice to determine the *in vivo* function of SHPTP2 in downstream signaling. Secondly, we are in the process of purifying sufficient quantities of pp115 to obtain amino acid sequence information and/or production of antibodies for cDNA cloning of this protein.

C) CONCLUSIONS:

Over the past several years substantial progress has been made in elucidating various intracellular signaling events mediating positive protein-protein interactions by tyrosine kinase receptor activation. On the other hand, the processes that mediate the inactivation of these events through tyrosine dephosphorylation remain less defined. The tyrosine phosphatase SHPTP2 is characteristic of a family of related cytosolic enzymes that contain two amino terminal SH2 domains (14-19). This phosphatase is ubiquitously expressed and is regulated by its association with a variety of tyrosine phosphorylated receptors and receptor substrates through engagement of its SH2 domains. For example, SHPTP2 has been observed to associate with tyrosine phosphorylated IRS1 resulting in the stimulation of catalytic activity (40-42). Several laboratories have also demonstrated that expression of a catalytically inactive SHPTP2 protein inhibits the insulin-stimulation of the MAP kinase pathway thereby blocking c-fos transcription and mitogenesis (33-35). Thus, SHPTP2 appears to serve an essential role in the growth promoting actions of insulin in a fashion analogous to the *drosophila* homologue of SHPTP2, *corkscrew*, which is required for normal anterior/posterior development (29-31).

Even though these data suggest that SHPTP2 is a required positive effector of tyrosine kinase signaling, its physiological targets have not been identified. Based upon *in vitro* studies, it was suggested that the insulin-stimulated association of SHPTP2 with IRS1 functioned to dephosphorylate the tyrosine phosphorylated IRS1 protein (43). However, expression of either the wild type or a catalytic inactive SHPTP2 mutant did not significantly effect the insulin-stimulated tyrosine phosphorylation state of IRS1 (33-35). Consistent with these reports, we have observed that only a very small fraction (< 4%) of the total pool of tyrosine phosphorylated IRS1 was associated with SHPTP2 following insulin stimulation of 3T3L1 adipocytes. Although we have been unable to quantitatively immunoprecipitate IRS1 from 3T3L1 cell extracts, semi-quantitative analysis also indicated that only a very small fraction (< 2%) of the total SHPTP2 pool was co-immunoprecipitated with tyrosine phosphorylated IRS1. Taken together, these data suggest that IRS1 was not the predominant target of SHPTP2 binding and/or function in 3T3L1 adipocytes.

To identify the insulin-stimulated substrate(s) and/or protein(s) associated with SHPTP2, we screened cell extracts for tyrosine phosphorylated proteins that were co-immunoprecipitated with a SHPTP2 antibody. In this manner, we have identified a predominant tyrosine

phosphorylated band in the 115 kDa region of SDS gels that was co-immunoprecipitated with SHPTP2 from insulin and EGF-stimulated CHO/IR and 3T3L1 adipocytes. Similar to insulin stimulation, pp115 was also the predominant EGF-stimulated tyrosine phosphorylated protein associated with SHPTP2 with the EGF receptor only accounting for a minor fraction of the bound SHPTP2. Interestingly, the tyrosine phosphorylation and/or association of this broad pp115 band with SHPTP2 was not observed following PDGF stimulation. This finding is consistent with the ability of the PDGF receptor to directly tyrosine phosphorylate SHPTP2 whereas activation of either the insulin or EGF receptors does not

In addition to its function as the predominant SHPTP2 tyrosine phosphorylated binding protein, pp115 also appears to be an *in vivo* substrate for the SHPTP2 phosphatase. This speculation is consistent with the large increase in the amount of tyrosine phosphorylated pp115 that was co-immunoprecipitated with SHPTP2 following expression of the catalytically inactive SHPTP2 mutant. However, it should be noted that in these studies we were not able to distinguish between the amount of pp115 bound to SHPTP2 versus the extent of tyrosine phosphorylation.

Interestingly, the pp115 protein was highly diffuse, perhaps representing heterogeneity at the level of phosphorylation or alternatively the presence of multiple, closely related molecular weight phosphoproteins. In some gels, this region appeared to be composed of several closely spaced but discrete protein bands. However, this separation was quite variable, and we have not yet found a gel system to reproducibly separate these bands. Since a variety of proteins in the $M_r = 115-125$ kDa range have been shown to be tyrosine phosphorylated, we attempted to identify these components by immunoprecipitation with known antibodies. However, we were unable to co-immunoprecipitate any insulin-stimulated tyrosine phosphorylated proteins with antibodies directed against Jak1, Jak2, Jak3, Tyk2, STAT2, rasGAP, c-Dbl, c-Cbl or the p120 src substrate. In addition, Grb2 and Crk have been observed to co-immunoprecipitate several tyrosine phosphorylated proteins in this molecular weight range following T cell activation (44,45). However, co-immunoprecipitation with Grb2, Crk and Nck antibodies were also unsuccessful in identifying any insulin-stimulated tyrosine phosphorylation proteins in this molecular weight range.

Recently, a tyrosine phosphorylated 120 kDa SHPTP2-associated protein was detected in NIH 3T3 cells expressing high levels of the human insulin receptor (34). However, the pp115 protein identified in this study has distinctly different properties. For example, the

previously described 120 kDa protein could not be detected by immunoprecipitation with a SHPTP2 antibody. In addition, the tyrosine phosphorylated 120 kDa protein was only observed in cells expressing a catalytically inactive SHPTP2 mutant by precipitation with an amino terminal SHPTP2 SH2 domain fusion protein. In contrast, the pp115 protein identified in the current study was readily detected by co-immunoprecipitation with a SHPTP2 antibody in extracts from both control and wild type SHPTP2 expressing cells. Furthermore, we have not observed any precipitation of pp115 from either CHO/IR or 3T3L1 adipocyte cell extracts with the SHPTP2 SH2 fusion protein (data not shown).

In summary, we have identified the predominant insulin and EGF-stimulated tyrosine phosphorylated SHPTP2 associated protein(s) as a diffuse pp115 band. We speculate that pp115 functions as a direct substrate of the insulin receptor kinase, that subsequently associates with SHPTP2. In addition, the increased association and tyrosine phosphorylation of pp115 observed in cells expressing a catalytically inactive SHPTP2 mutant strongly suggests that pp115 functions as an *in vivo* substrate for the SHPTP2 phosphatase activity. The fact that SHPTP2 appears to be an essential upstream effector for insulin and EGF-dependent stimulation of MAP kinase signaling also argues for a role of pp115 in this pathway. Clarification of these issues and identification of the physiological function of pp115 will require the molecular cloning and isolation of pp115 specific antibodies.

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Epidermal Growth Factor-induced Association of the SHPTP2 Protein Tyrosine Phosphatase with a 115-kDa Phosphotyrosine Protein*

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Epidermal growth factor (EGF) stimulation of HepG2 and NIH 3T3 cells expressing high levels of the human EGF receptor (3T3/ER) resulted in the tyrosine phosphorylation of a 115-kDa protein that was co-immunoprecipitated with the Src homology 2 (SH2) domain containing protein tyrosine phosphatase, SHPTP2. In contrast, activation of the EGF receptor resulted in a relatively low level (<1%) of the total SHPTP2 pool associated with the tyrosine-autophosphorylated EGF receptor itself. Similarly, quantitative immunoprecipitations also demonstrated that only trace amounts of the total EGF receptor pool were associated with SHPTP2. Further, activation of the EGF receptor did not result in any significant tyrosine phosphorylation of SHPTP2 and/or the association of the 115-kDa protein with Grb2. In comparison, activation of Jurkat cells with a T cell receptor agonist monoclonal antibody resulted in the co-immunoprecipitation of a 120-kDa tyrosine-phosphorylated protein with Grb2 and a 105-kDa protein with SHPTP2. Thus, these data have identified the 115- and 105-kDa proteins as the predominant SHPTP2-associated phosphotyrosine proteins in EGF- and T cell receptor-activated cells, respectively.

Protein phosphatases are typically thought to function as the inactivation arms of protein kinase-mediated signaling pathways. However, there are several recent examples whereby protein tyrosine-specific phosphatases play an essential positive signaling role. For example, in T cell receptor signaling, the CD45 protein tyrosine-specific phosphatase dephosphorylates the inhibitory carboxyl-terminal tyrosine phosphorylation site on Fyn and/or Lck allowing for activation of tyrosine protein kinase activity (1–3). Similarly, recent studies have demonstrated that SHPTP2 protein tyrosine-specific phosphatase plays an important positive role in tyrosine kinase downstream signaling. Microinjection of SHPTP2-specific antibodies was observed to block insulin-stimulated DNA synthesis and expression of dominant interfering SHPTP2 mutants inhibited activation of mitogen-activated protein kinase, *c-fos* transcription, DNA synthesis, and fibroblast growth factor-stimulated

Xenopus oocyte mesoderm induction (4–8).

These data have provided substantial evidence demonstrating a positive signaling role for SHPTP2 in mediating tyrosine kinase growth factor receptor action. To identify potential physiological targets for SHPTP2, previous studies have observed that the SH2 domains of SHPTP2 result in the targeting to the tyrosine-phosphorylated epidermal growth factor (EGF)¹ receptor, platelet-derived growth factor receptor, and insulin receptor substrate-1 (IRS1) (9–13). In addition, it has been reported that tyrosine-phosphorylated IRS1 functions as a substrate for SHPTP2 *in vitro* (14). However, the tyrosine dephosphorylation of IRS1 is difficult to reconcile with a positive effector role for SHPTP2 tyrosine phosphatase activity, and a quantitative assessment of these associations has not yet been determined. In this paper, we have determined that only a small fraction of SHPTP2 associates with the EGF receptor and that a 115-kDa tyrosine-phosphorylated protein (pp115) is the major SHPTP2 binding protein in EGF-stimulated cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HepG2 cells were obtained from the American Type Tissue Culture collection and were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. NIH 3T3 cells over-expressing human EGF receptor (3T3/ER) were obtained from Dr. John Koland (University of Iowa). 3T3/ER cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The human Jurkat T cell line and TCR monoclonal antibody (OKT3) were provided by Dr. Gary Koretzky (University of Iowa). The Jurkat T cells were maintained in RPMI 1640 containing 10% fetal bovine serum.

Immunoprecipitations and Western Blot Analysis—Whole cell extracts were prepared by detergent solubilization in lysis buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 2 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10 μ g/ml aprotinin, and 1.5 mM pepstatin) for 1 h at 4 °C. The resultant cell extracts (500 μ g) were diluted 5-fold using the lysis buffer without Triton X-100 and incubated with 4 μ g of a carboxyl-terminal SHPTP2 polyclonal antibody (Santa Cruz) or an EGF receptor monoclonal antibody, LA1 (Upstate Biotechnology), for 2 h at 4 °C. The primary polyclonal and monoclonal antibodies were incubated with Protein A-agarose or Protein G PLUS-agarose, respectively, for 1 h at 4 °C. The resulting immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis. The whole cell extracts and the immunoprecipitates were then subjected to Western blot analysis using an amino-terminal SHPTP2 antibody (Transduction Laboratories), an EGF receptor antibody (Transduction Laboratories), or a phosphotyrosine antibody (PY20-HRP, Santa Cruz) and visualized with the enhanced chemical luminescence (ECL) detection system (Amersham Corp.).

RESULTS AND DISCUSSION

We and others have recently observed that SHPTP2 functions as a positive mediator in the regulation of tyrosine kinase receptor downstream signaling (4–8). To identify potential targets for this phosphatase, we assessed the presence of phosphotyrosine-containing proteins that associated with SHPTP2 (Fig. 1). Stimulation of NIH 3T3/ER cells with 100 ng/ml EGF for 5 min resulted in a marked increase in the tyrosine phosphorylation of the EGF receptor (ER) following immunoprecipitation with an ER-specific antibody (Fig. 1A, lanes 1 and 2). In contrast, immunoprecipitation of SHPTP2 primarily resulted in the co-immunoprecipitation of a 115-kDa protein and another band migrating at approximately 55 kDa (Fig. 1A, lanes

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¹ The abbreviations used are: EGF, epidermal growth factor; ER, epidermal growth factor receptor; IRS1, insulin receptor substrate-1; TCR, T cell antigen receptor.

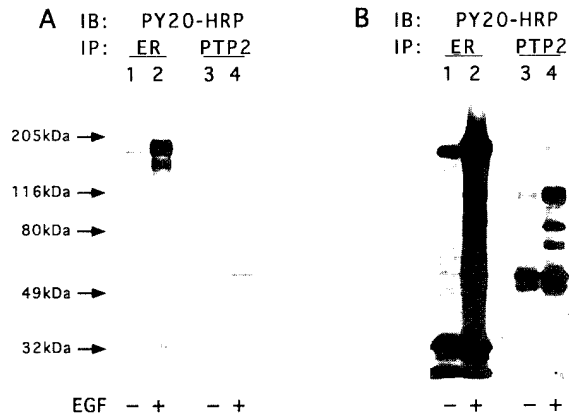


FIG. 1. Identification of pp115 as the predominant tyrosine-phosphorylated protein associated with SHPTP2 following EGF stimulation of 3T3/ER cells. NIH 3T3/ER cells were incubated in the absence (*lanes 1 and 3*) or presence (*lanes 2 and 4*) of 100 ng/ml EGF for 5 min at 37 °C. Whole cell detergent lysates were prepared and immunoprecipitated with an ER-specific monoclonal antibody (*ER, lanes 1 and 2*) or with a SHPTP2-specific polyclonal antibody (*lanes 3 and 4*) as described under "Experimental Procedures." The immunoprecipitates were then subjected to phosphotyrosine immunoblotting using the PY20-HRP antibody and visualized by the ECL method for 5 s (*A*) or for 2 min (*B*). *IB*, immunoblot; *IP*, immunoprecipitate.

3 and 4). Surprisingly, at this exposure level there was no detectable co-immunoprecipitation of the tyrosine-phosphorylated ER. To assess the presence of the ER in the SHPTP2 immunoprecipitates, *panel A* was overexposed (*Fig. 1B*). At this exposure level, we were able to detect a small amount of an EGF-stimulated phosphotyrosine band that co-migrated with the ER in the SHPTP2 immunoprecipitates (*Fig. 1B, lanes 3 and 4*). In addition to the predominant EGF-stimulated 115-kDa protein, several other lower molecular weight bands were detected. These additional SHPTP2-associated phosphotyrosine-containing bands probably represent degradation products of the 115-kDa protein.

Since previous studies have demonstrated that SHPTP2 can associate with EGF-stimulated tyrosine-phosphorylated ER (10), we next determined the relative extent of SHPTP2 binding compared with the total cellular pool of ER and SHPTP2 (*Fig. 2*). Control and EGF-stimulated NIH 3T3/ER cells were initially immunoprecipitated with the SHPTP2 antibody and subjected to Western blotting with the ER antibody (*Fig. 2A, lanes 1 and 2*). Under these conditions, we were unable to detect any significant level of ER protein by ER Western blotting. In contrast, when the supernatant from the initial SHPTP2 immunoprecipitate was subjected to a second round of immunoprecipitation using the ER antibody, a strong positive ER Western blotting signal was readily observed (*Fig. 2A, lanes 3 and 4*). It should be noted that the apparent increase in the molecular weight of the ER following EGF stimulation (*Fig. 2A, lane 4*) compared with unstimulated cells (*Fig. 2A, lane 3*) reflects a decrease in electrophoretic mobility due to the phosphorylation of the ER.

To demonstrate that the SHPTP2 antibody was effective, the initial SHPTP2 immunoprecipitation was Western blotted for the presence of SHPTP2 (*Fig. 2A, lanes 5 and 6*). Under these conditions, the SHPTP2 antibody quantitatively immunoprecipitated 100% of the total SHPTP2 protein pool present in the whole cell detergent extracts (data not shown). In addition, the supernatant from the initial SHPTP2 immunoprecipitation was then subjected to immunoprecipitation with the ER antibody. As expected, the immunoprecipitation of ER from the initial SHPTP2-cleared supernatant was unable to immunoprecipitate any detectable SHPTP2 protein (*Fig. 2A, lanes 7 and 8*).

In a complementary approach, control and EGF-stimulated

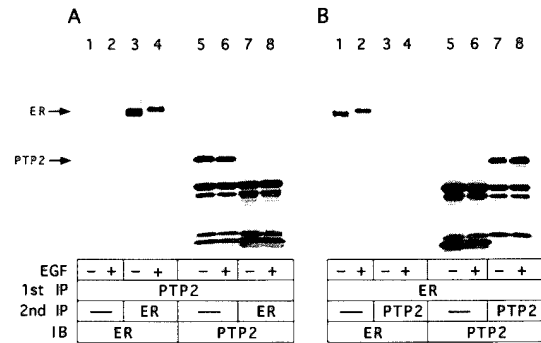


FIG. 2. Determination of the relative amount of ER associated with SHPTP2. 3T3/ER cells were either left untreated (*lanes 1, 3, 5, and 7*) or incubated with 100 ng/ml EGF (*lanes 2, 4, 6, and 8*) for 5 min at 37 °C. Whole cell detergent lysates were prepared and subjected to immunoprecipitation with the SHPTP2 polyclonal antibody (*A*) or with the ER monoclonal antibody (*B*) as described under "Experimental Procedures." The resulting supernatants from the initial SHPTP2 immunoprecipitation in *panel A* were subjected to a second round of ER immunoprecipitation (*lanes 3, 4, 7, and 8*). The resulting supernatants from the initial ER immunoprecipitation in *panel B* were subjected to a second round of SHPTP2 immunoprecipitation (*lanes 3, 4, 7, and 8*). The immunoprecipitates were then Western blotted with an ER-specific antibody (*lanes 1-4*) or with a SHPTP2 specific antibody (*lanes 5-8*). *IB*, immunoblot; *IP*, immunoprecipitate.

NIH 3T3/ER cells were initially immunoprecipitated with an ER antibody and subsequently subjected to Western blotting with the ER antibody (*Fig. 2B, lanes 1 and 2*). Under these conditions the ER antibody was able to immunoprecipitate greater than 95% of the total pool of ER protein present in the whole cell detergent extracts (data not shown). However, there was no detectable ER co-immunoprecipitated SHPTP2 protein (*Fig. 2B, lanes 5 and 6*). Similarly, SHPTP2 immunoprecipitation of the ER-cleared supernatants did not result in the co-immunoprecipitation of the ER (*Fig. 2B, lanes 3 and 4*) but did immunoprecipitate the SHPTP2 protein (*Fig. 2B, lanes 7 and 8*). Thus, these data indicated that essentially an undetectable level of the total SHPTP2 protein pool was associated with the ER. Similarly, the total amount of the ER associated with SHPTP2 was also relatively low. The fact that a detectable, albeit small amount, of tyrosine-phosphorylated ER was co-immunoprecipitated with SHPTP2 (*Fig. 1*) probably reflects a greater sensitivity of the PY20-HRP antibody compared with the SHPTP2 and ER antibody used in the Western blots (*Fig. 2*). Nevertheless, all these data were consistent with the ER playing a minor role in the association of SHPTP2 whereas the predominant SHPTP2 binding phosphotyrosine protein in EGF-stimulated 3T3/ER cells was apparently the 115-kDa species.

To determine if the EGF-stimulated association of SHPTP2 with the tyrosine-phosphorylated 115-kDa protein was unique to NIH 3T3 cells genetically engineered to express high levels of the human ER, we directly compared the 3T3/ER cells with the human hepatoblastoma cell line, HepG2 (*Fig. 3*). Phosphotyrosine immunoblotting of whole cell detergent extracts demonstrated the EGF-stimulated tyrosine phosphorylation of the ER and proteins of approximately 55 and 42 kDa in the 3T3/ER cells (*Fig. 3A, lanes 1 and 2*). The 55- and 42-kDa proteins most likely reflect the tyrosine phosphorylation of Shc and ERK2. In any case, EGF treatment of HepG2 cells resulted in a significantly weaker tyrosine phosphorylation of the ER, consistent with the lower levels of the ER in the HepG2 cells compared with the 3T3/ER cell line (*Fig. 3A, lanes 3 and 4*). As previously observed, SHPTP2 immunoprecipitation of extracts from EGF-stimulated 3T3/ER cells resulted in the predominant co-immunoprecipitation of the 115-kDa phosphotyrosine-containing protein with a relatively low amount of the tyrosine phosphorylated ER (*Fig. 3B, lanes 1 and 2*). Similarly, EGF stimulation

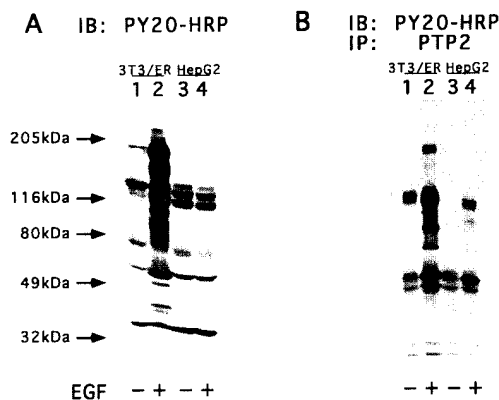


FIG. 3. Comparison of EGF-stimulated tyrosine phosphorylation and association of SHPTP2 with pp115 in 3T3/ER and HepG2 cells. NIH 3T3/ER and HepG2 cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 ng/ml EGF for 5 min at 37 °C. A, whole cell detergent lysates were prepared and directly subjected to Western blotting using the phosphotyrosine antibody PY20-HRP. B, the detergent lysates prepared in A were immunoprecipitated with the SHPTP2 polyclonal antibody and subjected to Western blotting using the phosphotyrosine antibody PY20-HRP. IB, immunoblot; IP, immunoprecipitate.

of the HepG2 cells also demonstrated that the predominant SHPTP2 co-immunoprecipitated phosphotyrosine-containing protein was pp115 (Fig. 3B, lanes 3 and 4). As expected, a small amount of tyrosine-phosphorylated ER was detected in the SHPTP2 immunoprecipitate of HepG2 cell extracts (Fig. 2B, lane 4) when the gel was overexposed (data not shown). These data demonstrate that the EGF-stimulated tyrosine phosphorylation and association of pp115 with SHPTP2 do not result from high level of ER expression and also occur in a typical EGF-responsive cell line.

Recently it was reported that activation of the T cell antigen receptor (TCR) resulted in the tyrosine phosphorylation of proteins in the 116-kDa range that were associated with the small adapter proteins Crk and Grb2 (15, 16). To determine if the SHPTP2-associated 115-kDa protein was potentially related to these proteins, we compared the effect of EGF on HepG2 cells with that of TCR activation on Jurkat T cells (Fig. 4). As expected, EGF stimulation of HepG2 cells resulted in the co-immunoprecipitation of the 115-kDa tyrosine-phosphorylated protein with the SHPTP2 antibody (Fig. 4A, lanes 1 and 2). Incubation of Jurkat cells with an activating TCR antibody (OKT3) resulted in the predominant tyrosine phosphorylation of a 105-kDa protein that was co-immunoprecipitated with the SHPTP2 antibody (Fig. 4A, lanes 3 and 4). The other major band observed at approximately 25 kDa following TCR activation represents the light chain of the OKT3 monoclonal antibody. We next assessed whether the SHPTP2-associated proteins were also bound to Grb2 by co-immunoprecipitation with a Grb2 antibody (Fig. 4B). Immunoprecipitation of Grb2 from EGF-stimulated HepG2 cells demonstrated the presence of tyrosine-phosphorylated 52- and 46-kDa proteins, which probably represent the Shc proteins (Fig. 4B, lanes 1 and 2). Due to the relatively low level of ER in these cells, the tyrosine-phosphorylated ER was only observed when the gel was overexposed (data not shown). Importantly, the SHPTP2-associated 115-kDa protein was not co-immunoprecipitated with Grb2. However, as previously reported (15, 16), activation of the TCR resulted in the Grb2 co-immunoprecipitation of a 120-kDa tyrosine-phosphorylated band (Fig. 4B, lanes 3 and 4). This band did not co-migrate with the SHPTP2-associated 105-kDa protein observed in these cells. Thus, the 115- and 105-kDa SHPTP2-associated proteins were unrelated to the previously described Grb2-associated tyrosine-phosphorylated protein.

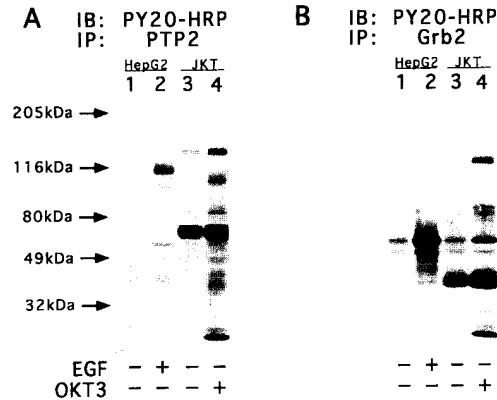


FIG. 4. Comparison of SHPTP2- and Grb2-associated phosphotyrosine proteins in HepG2 and Jurkat T cells. HepG2 cells were incubated in the absence (lane 1) or presence (lane 2) of 100 ng/ml EGF for 5 min at 37 °C. Jurkat T cells (JKT) were incubated in the absence (lane 3) or presence of the TCR agonist antibody OKT3 (lane 4). Whole cell detergent lysates were prepared and were either immunoprecipitated with a SHPTP2 antibody (A) or with a Grb2 antibody (B). The immunoprecipitates were then subjected to Western blotting using the phosphotyrosine antibody PY20-HRP. IB, immunoblot; IP, immunoprecipitate.

Furthermore, we have attempted to identify these components by immunoprecipitation with known antibodies against tyrosine-phosphorylated proteins in this molecular weight range. However, we have been unable to co-immunoprecipitate SHPTP2 with any EGF-stimulated tyrosine-phosphorylated proteins using antibodies directed against Jak1, Jak2, Jak3, Tyk2, STAT2, FAK, rasGAP, c-Dbl, c-Cbl, or the p120 Src substrate (data not shown).

In summary, the data presented in this paper demonstrate that a protein of 115 kDa is a significant EGF-stimulated SHPTP2-associated tyrosine-phosphorylated protein in NIH 3T3/ER and HepG2 cells. Although SHPTP2 can bind to the autophosphorylated ER, the extent of this association is relatively minor and only accounts for a small fraction of the total cellular ER and SHPTP2 pool. Since we have not yet identified the nature of the 115-kDa protein and currently no antibodies are available, we have not been able to determine the relative extent of SHPTP2 association with pp115. However, based upon the tyrosine phosphorylation signal, we speculate that this protein is both a substrate for the ER and is the major SHPTP2 binding protein. In addition, an apparently related 105-kDa protein was detected in Jurkat T cells, and neither the 115- nor 105-kDa proteins were found to associate with Grb2. Clearly, the identification of the physiological function of pp115 is an important issue necessary to determine the molecular role of SHPTP2 in ER signaling.

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Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling

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ABSTRACT SHPTP2 is a ubiquitously expressed tyrosine-specific protein phosphatase that contains two amino-terminal Src homology 2 (SH2) domains responsible for its association with tyrosine-phosphorylated proteins. In this study, expression of dominant interfering mutants of SHPTP2 was found to inhibit insulin stimulation of *c-fos* reporter gene expression and activation of the 42-kDa (Erk2) and 44-kDa (Erk1) mitogen-activated protein kinases. Cotransfection of dominant interfering SHPTP2 mutants with *v-Ras* or *Grb2* indicated that SHPTP2 regulated insulin signaling either upstream of or in parallel to *Ras* function. Furthermore, phosphotyrosine blotting and immunoprecipitation identified the 125-kDa focal adhesion kinase (pp125^{FAK}) as a substrate for insulin-dependent tyrosine dephosphorylation. These data demonstrate that SHPTP2 functions as a positive regulator of insulin action and that insulin signaling results in the dephosphorylation of tyrosine-phosphorylated pp125^{FAK}.

The insulin receptor is a ligand-stimulated transmembrane protein-tyrosine kinase that phosphorylates itself as well as intracellular substrates on specific tyrosine residues (1, 2). One proximal intracellular target for the kinase-activated insulin receptor has been identified as a 185-kDa protein, termed insulin receptor substrate 1 (IRS1) (3, 4). This molecule contains several insulin receptor-specific tyrosine phosphorylation sites that provide recognition signals for the binding of specific Src homology 2 (SH2) domain-containing proteins (5, 6). The interaction of IRS1 with signaling proteins containing multiple SH2 domains provides a mechanism by which insulin can modulate the function of several distinct pathways. For example, activation of phosphatidylinositol (PI) 3-kinase activity occurs upon the association of the p85 subunit of the PI 3-kinase with tyrosine-phosphorylated IRS1 (7–9). It has been suggested that the insulin activation of *Ras* function results from the interaction and/or appropriate targeting of the guanine nucleotide exchange factor *Sos* with IRS1 (6, 10). This is thought to occur via the constitutive association of the Src homology 3 (SH3) domains of the adapter protein *Grb2* with the C-terminal domain of *Sos* (*Grb2/Sos* complex) and subsequent binding of the *Grb2* SH2 domain to tyrosine-phosphorylated IRS1 (10, 11).

Recently, several groups have identified a ubiquitously expressed 68-kDa tyrosine-specific protein phosphatase, SHPTP2 (also termed *Syp*, *PTP1D*, *SHPTP3*, *PTP2C*, or *PTPL1*), that contains two N-terminal SH2 domains and a C-terminal catalytic domain (12–17). The SH2 domains of this phosphatase mediate the binding of SHPTP2 to tyrosine-phosphorylated epidermal growth factor receptor, platelet-derived growth factor receptor, and IRS1, resulting in the activation of protein-tyrosine-phosphatase activity (18–21). Although protein tyrosine phosphatase activity is generally thought to function as the inactivating arm of receptor kinase

signaling pathways, in the case of T-cell receptor signaling, the CD45 protein-tyrosine-phosphatase is essential for downstream biological responsiveness (22, 23). Here we demonstrate that the SHPTP2 protein tyrosine-specific phosphatase functions as part of the positive signaling pathway mediating the insulin activation of mitogen-activated protein kinase (MAP kinase) and hence *c-fos* transcription. In addition, we have observed that insulin stimulation results in the tyrosine dephosphorylation of the focal adhesion kinase (FAK) pp125^{FAK}.

EXPERIMENTAL PROCEDURES

Transfection of CHO/IR Cells by the Calcium Phosphate Method. Chinese hamster ovary cells expressing high levels of the insulin receptor (CHO/IR cells) were transiently transfected as described (24) with 1 μ g of a serum response element-driven luciferase reporter plasmid (SRE-Luc), 2 μ g of a Rous sarcoma virus promoter-driven β -galactosidase reference plasmid (RSV- β Gal), and 5 μ g of the mammalian expression vector CLDN carrying various protein-tyrosine-phosphatase cDNAs. The total amount of transfected DNA was maintained at 23 μ g with the empty CLDN vector. Eighteen hours after transfection the cells were serum starved for 12 hr. Then 100 nM insulin or phorbol 12-myristate 13-acetate (PMA) was added for 6 hr before cell extracts were prepared and assayed for luciferase and β -galactosidase activity.

Transfection of CHO/IR Cells by Electroporation. To obtain the high degree of transfection efficiency necessary for immunoprecipitation and Western blotting of whole cell extracts, CHO/IR cells were electroporated with 40 μ g of plasmid DNA at 340 V and 960 μ F. Under these conditions \approx 25% of the cells remained viable and 80–95% of the surviving cells were transfected as determined by parallel transfection with the cytomegalovirus promoter-driven plasmid CMV- β Gal (K.Y. & J.E.P., unpublished work). Thirty-six hours after transfection the cells were serum starved for 6 hr before 100 nM insulin was added for 5 min.

Western Blot Analysis of Whole Cell Extracts. Whole cell extracts were prepared by detergent solubilization in a modified RIPA buffer [20 mM Hepes, pH 7.4/1% (vol/vol) Triton X-100/0.1% (wt/vol) SDS/0.1% (wt/vol) sodium deoxycholate/2 mM EDTA/100 mM NaF/10 mM Na₄P₂O₇/2 mM Na₃VO₄/1 mM phenylmethylsulfonyl fluoride/10 μ M leupeptin/1.5 μ M pepstatin containing aprotinin at 10 μ g/ml] for 1 hr at 4°C. The resultant cell extracts were subjected to Western blotting with a MAP kinase antibody (Zymed) or a phosphotyrosine antibody (α PY, kindly provided by Peter A. Wilden, University of Missouri).

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Abbreviations: FAK, focal adhesion kinase; IRS1, insulin receptor substrate 1; MAP-2, microtubule-associated protein 2; MAP kinase, mitogen-activated protein kinase; PI, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate; SH2, Src homology 2; SRE, serum response element.

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MAP Kinase Activity. MAP kinase activity from cell lysates was determined as described (25). Briefly, 10 μ l of cell extract was incubated for 10 min at 30°C with microtubule-associated protein (MAP-2) at 0.2 mg/ml in 50 mM Tris-HCl, pH 7.4/2 mM EGTA/10 mM MgCl₂/40 μ M [γ -³²P]ATP [1 μ Ci (= 37 kBq)] in a total volume of 25 μ l. The reaction was terminated by the addition of SDS sample buffer and the phosphorylated MAP-2 was resolved by SDS/PAGE. Incorporated radioactivity was determined by excision of the MAP-2 band followed by scintillation counting.

Immunoprecipitation of FAK. Following electroporation and detergent solubilization (RIPA) as described above, the cell extracts were heated at 100°C for 5 min in the presence of 1% SDS. The samples were then diluted 10-fold (10 mM Tris, pH 7.4/1% Triton X-100/0.5% Nonidet P-40/150 mM NaCl/2 mM EDTA/0.2 mM Na₃VO₄/0.2 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with 4 μ g of a FAK monoclonal antibody (α FAK, Transduction Laboratories, Lexington, KY). The immunoprecipitates were subjected to SDS/PAGE and Western blotting using either the 4G10 phosphotyrosine monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) or the FAK monoclonal antibody.

RESULTS

To assess the role of the SHPTP2 protein-tyrosine-phosphatase in mediating insulin signaling, we examined the insulin activation of *c-fos* transcription by using a SRE-Luc reporter gene (Fig. 1). The *c-fos* SRE is highly sensitive to transcriptional activation by insulin and serves as a convenient downstream assay for insulin signaling (24, 27, 28). In CHO/IR cells, insulin-stimulated SRE-Luc activity was \approx 10-fold in control cells and was not affected by expression of the wild-type SHPTP2 (PTP2). In contrast, expression of either a point mutant in which the essential catalytic cysteine residue was replaced with serine (C/SPTP2) or the SH2 domains of SHPTP2 (PTP2SH2) markedly inhibited the insulin stimulation of SRE-Luc reporter activity. This inhibition of insulin signaling was specific, in that expression of the *c-Src* SH2 domain (*srcSH2*) did not affect insulin stimulation of SRE-Luc activity. In addition, expression of the SHPTP2 mutants had no effect on the ability of PMA to increase SRE-Luc

expression (Fig. 1A). The dominant interfering action of the SHPTP2 mutants was not a general property of all protein-tyrosine-phosphatases (Fig. 1B). Expression of another wild-type protein-tyrosine-phosphatase, PTP1B, or a PTP1B phosphatase-negative mutant (C/SPTP1B) had no significant effect on insulin stimulation of SRE-Luc reporter gene activity.

The Ras/MAP kinase pathway is one of the major routes responsible for growth factor activation of the *c-fos* SRE (reviewed in refs. 29–31). Thus, to determine whether the inhibition of insulin-stimulated SRE-Luc activity by the dominant interfering SHPTP2 mutants occurred via a blockade of MAP kinase activation, we performed MAP kinase Western blots on extracts from control and insulin-treated cells (Fig. 2A). The predominant insulin-stimulated MAP kinase isoform in CHO/IR cells is the 42-kDa MAP kinase (Erk2), with relatively lower levels of the 44-kDa MAP kinase (Erk1). Subsequent to insulin treatment, both Erk1 and Erk2 displayed the typical mobility shift due to tyrosine and threonine phosphorylation (32–34) in both control transfected cells (Fig. 2A, lanes 1 and 2) and cells transfected with wild-type SHPTP2 (lanes 3 and 4). In contrast, expression of either dominant interfering SHPTP2 mutant prevented the formation of the slower-mobility Erk1 and Erk2 species in the presence of insulin (Fig. 2A, lanes 5–8).

To determine whether this inhibition of the insulin-stimulated MAP kinase gel shift directly reflected changes in MAP kinase activity, we next examined MAP-2 phosphorylation from control and SHPTP2 transfected CHO/IR cells (Fig. 2B). Control and wild-type SHPTP2-transfected cells displayed an \approx 4-fold insulin stimulation of MAP kinase activity. In contrast, expression of the C/SPTP2 and PTP2SH2 mutants inhibited the insulin stimulation of MAP kinase activity, resulting in only 2.3- and 1.8-fold increases, respectively. The incomplete inhibition of insulin-stimulated MAP kinase activation was in agreement with the partial inhibition of insulin-stimulated SRE-Luc activity achieved under these transient transfection conditions (Fig. 1). Together, these data demonstrate that at least one function of the SHPTP2 tyrosine-specific phosphatase is to transmit a positive insulin signal that results in the activation of MAP kinase activity.

Since MAP kinase has also been established as a downstream target of Ras (32–37), we next addressed the relation-

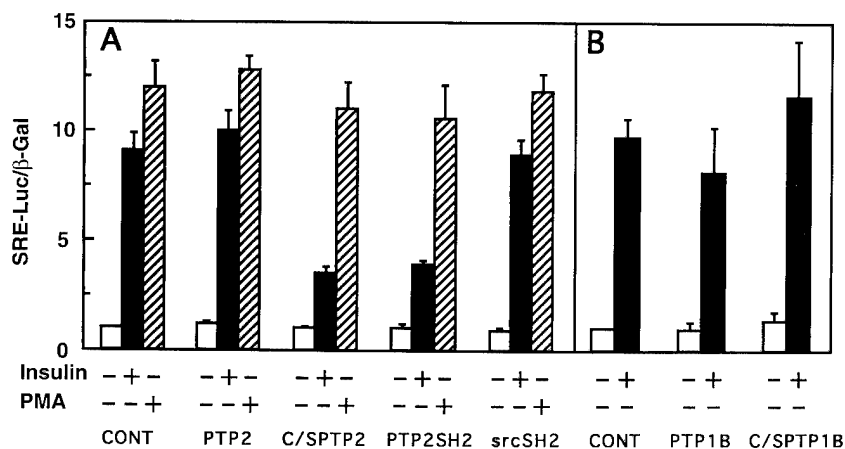


FIG. 1. Expression of dominant interfering SHPTP2 mutants specifically inhibits insulin stimulation of *c-fos* transcription through the SRE. (A) CHO/IR cells were transiently transfected (calcium phosphate method) with the SRE-Luc and RSV- β Gal reporter plasmids plus 5 μ g of the empty CLDN vector (CONT) or of CLDN vector containing cDNA encoding wild-type SHPTP2 (PTP2), SH2 domains of SHPTP2 (PTP2SH2), an SHPTP2 mutant in which the catalytic cysteine-459 was replaced with serine (C/SPTP2), or the SH2 domain of *Src* (*srcSH2*). The cells were then serum starved and either left untreated (open bars) or stimulated with 100 nM insulin (solid bars) or 100 nM PMA (hatched bars) as described under *Experimental Procedures*. (B) CHO/IR cells were transiently transfected with the SRE-Luc and RSV- β Gal reporter plasmids plus 5 μ g of the empty CLDN vector (CONT) or CLDN containing cDNA encoding wild-type PTP1B (PTP1B) or the PTP1B mutant in which the catalytic cysteine-215 was replaced with serine (C/SPTP1B) (26). Basal (open bars) and insulin (solid bars) stimulation of SRE-Luc reporter gene activity was then determined as described above. The amount of SRE-Luc activity determined in cell extracts was normalized for expression of RSV- β Gal. Data are presented as means with SEM from four independent experiments each performed in triplicate.

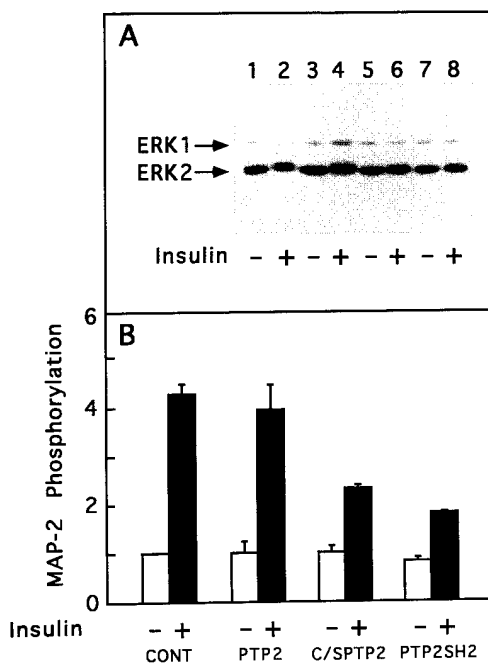


FIG. 2. SHPTP2 functions upstream of MAP kinase in mediating the insulin stimulation of SRE-Luc activity. (A) CHO/IR cells were transfected by electroporation with 40 μ g of the empty CLDN vector (CONT; lanes 1 and 2) or CLDN containing cDNA encoding wild-type SHPTP2 (PTP2; lanes 3 and 4), SHPTP2 mutant in which the catalytic cysteine-459 was replaced with serine (C/SPTP2; lanes 5 and 6) or the SH2 domains of SHPTP2 (PTP2SH2; lanes 7 and 8). The transfected cells were then either left untreated (lanes 1, 3, 5, and 7) or incubated with 100 nM insulin for 5 min (lanes 2, 4, 6, and 8) before isolation of total cell extracts (25 μ g) and Western blotting with a MAP kinase (Erk) antibody. To observe the mobility shift between the nonphosphorylated and phosphorylated forms of Erk1 and Erk2, the cell extracts were separated under reducing conditions in an SDS/polyacrylamide gel (1.5 mm thick, 32 cm long). Shown is an anti-Erk Western blot that is representative of four independent blots. (B) Whole cell lysates from control (open bars) and insulin-treated (solid bars) cultures were prepared as described in A. The samples were then assayed for MAP-2 substrate kinase activity. Data are presented as means with the range of values from two independent experiments. To compare these experiments, the data were normalized to the activity observed in the control cell extracts in the absence of insulin.

ship between SHPTP2 function and that of Ras activation. As observed in Fig. 1, insulin stimulated SRE-Luc activity \approx 10-fold (Fig. 3A). Similarly, expression of oncogenic viral Ras (v-Ras) also increased SRE-Luc activity compared with control cells. Furthermore, insulin treatment of cells transfected with v-Ras increased SRE-Luc reporter activity 22-fold compared with the 16-fold increase in v-Ras-transfected cells in the absence of insulin ($P < 0.05$). Although expression of the C/SPTP2 point mutant partially inhibited insulin stimulation of SRE-Luc activity ($P < 0.05$), this mutant had no effect on v-Ras stimulation of the reporter gene (Fig. 3A, compare bars 5 and 7). However, the dominant interfering C/SPTP2 point mutant did reduce the additional effect of insulin in cells which were both transfected with v-Ras and treated with insulin (compare bars 6 and 8; $P < 0.05$). These data suggest that SHPTP2 functions either upstream of or in a pathway parallel to Ras.

Recent studies have suggested that the adapter protein Grb2 can function as an upstream activator of Ras by complexing with and appropriately targeting the guanine nucleotide exchange factor Sos to Ras (38–42). Consistent with these results, expression of Grb2 enhanced insulin stimulation of SRE-Luc activity \approx 50% ($P < 0.05$; Fig. 3B), most likely by an increased coupling efficiency of Sos to tyrosine-phosphory-

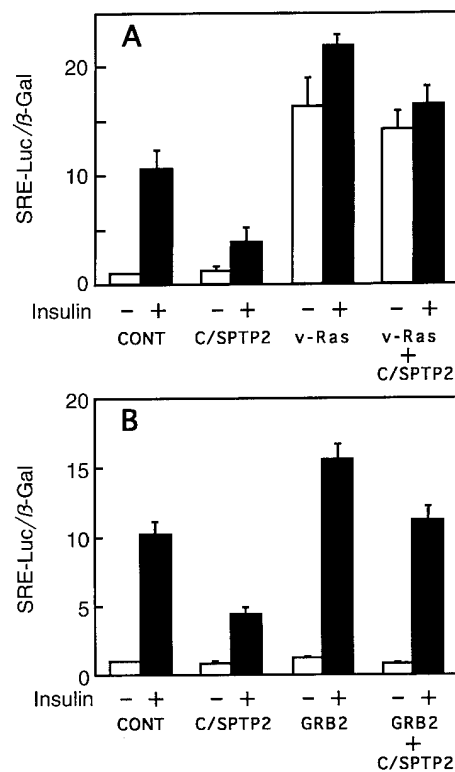


FIG. 3. Relationship of SHPTP2, Ras, and Grb2 function in mediating SRE-Luc activation. (A) CHO/IR cells were transiently transfected (calcium phosphate method) with the SRE-Luc and RSV- β Gal reporter plasmids plus 5 μ g of the empty CLDN vector (CONT) or CLDN with cDNA for the SHPTP2 mutant in which the catalytic cysteine-459 was replaced with serine (C/SPTP2), constitutively activated Ras (v-Ras), or constitutively activated Ras plus the SHPTP2 point mutant (v-Ras plus C/SPTP2). (B) CHO/IR cells were transiently transfected (calcium phosphate method) with the SRE-Luc and RSV- β Gal reporter plasmids plus 5 μ g of the empty CLDN vector (CONT) or CLDN with cDNA for C/SPTP2, the small adapter molecule Grb2, or Grb2 plus the SHPTP2 point mutant. Basal (open bars) and insulin (solid bars) stimulation of SRE-Luc reporter gene activity was then determined as described in Fig. 1. Data are presented as means with SEM from three independent experiments each performed in triplicate.

lated IRS1 or Shc (10, 11, 43, 44). Although expression of the C/SPTP2 mutant significantly reduced the insulin stimulation of SRE-Luc activity ($P < 0.05$), coexpression of the mutant phosphatase with Grb2 restored SRE-Luc activity to the level observed in control cells treated with insulin alone. The ability of Grb2 to partially circumvent the C/SPTP2 inhibition of downstream signaling in the presence of insulin further suggests that Grb2 may couple to the Ras/MAP kinase pathway via a pathway parallel to that used by SHPTP2.

To identify potential targets of SHPTP2 responsible for insulin downstream signaling, we analyzed phosphotyrosine Western blots of whole cell extracts from basal and insulin-stimulated CHO/IR cells. Control transfected cells displayed an insulin stimulation of IRS1, insulin receptor β subunit, and pp60 (Fig. 4, lanes 1 and 2). In addition, two bands in the vicinity of 50 kDa were also tyrosine phosphorylated and probably represent the 46- and 52-kDa forms of Shc (44). Similarly, insulin stimulation of cells transfected with the wild-type SHPTP2 (Fig. 4, lanes 3 and 4), the C/SPTP2 point mutant (lanes 5 and 6), or the PTP2SH2 domain (lanes 7 and 8) also resulted in the equivalent tyrosine phosphorylation of the insulin receptor β subunit, pp60, and the two \approx 50-kDa proteins. Thus, SHPTP2 does not function in the inactivation (dephosphorylation) of these tyrosine phosphorylation events under these conditions (5 min of insulin treatment). However,

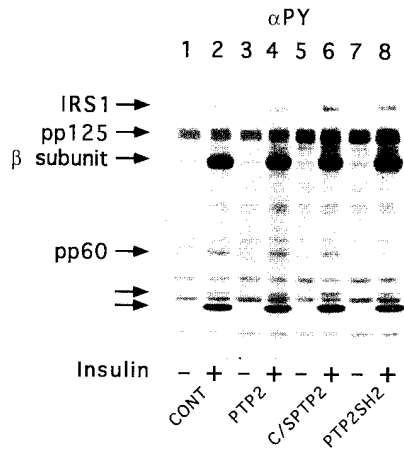


FIG. 4. Phosphotyrosine Western blot analysis of total cell lysates following insulin stimulation of CHO/IR cells expressing various SHPTP2 cDNAs. CHO/IR cells were transfected by electroporation with 40 μ g of the empty CLDN vector (CONT; lanes 1 and 2) or CLDN with cDNA for wild-type SHPTP2 (PTP2; lanes 3 and 4), SHPTP2 mutant in which the catalytic cysteine-459 was replaced with serine (C/SPTP2; lanes 5 and 6), or the SH2 domains of SHPTP2 (PTP2SH2; lanes 7 and 8). The transfected cells were then either left untreated (lanes 1, 3, 5, and 7) or incubated with 100 nM insulin for 5 min (lanes 2, 4, 6, and 8) before isolation of total cell extracts and Western blotting with a phosphotyrosine antibody (α PY). Shown is a representative phosphotyrosine Western blot selected from three independent transfection experiments. The identification of the insulin receptor β subunit and IRS1 was based upon electrophoretic mobility compared with known samples.

there was a small but consistent increase in the extent of insulin-stimulated tyrosine phosphorylation of IRS1 from cells transfected with the dominant interfering SHPTP2 mutants (Fig. 4, compare lanes 6 and 8 with lanes 2 and 4). Although the increase in IRS1 tyrosine phosphorylation was relatively small, these data are consistent with a recent report that SHPTP2 can dephosphorylate IRS1 *in vitro* (45). In addition, expression of either the dominant interfering C/SPTP2 point mutant (Fig. 4, lanes 5 and 6) or the PTP2SH2 domain (lanes 7 and 8) resulted in increased tyrosine phosphorylation of diffuse bands at 120–130 kDa compared with the control cells (Fig. 4, lanes 1 and 2) or cells transfected with the wild-type SHPTP2 (lanes 3 and 4).

In attempts to identify the components of these diffuse protein bands, we performed selective immunoprecipitation and phosphotyrosine Western blotting of several known tyrosine-phosphorylated proteins in this molecular size range. These data demonstrated that these bands were not the Ras GTPase-activating protein or the Src kinase pp120 substrate (data not shown). In contrast, immunoprecipitation with an antibody directed against the 125-kDa FAK followed by phosphotyrosine Western blotting directly demonstrated that expression of the dominant interfering C/SPTP2 mutant resulted in a basal increase in pp125^{FAK} tyrosine phosphorylation (Fig. 5A, compare lanes 1 and 3). The increased labeling in the C/SPTP2-transfected cells is consistent with a role for SHPTP2 in the tyrosine dephosphorylation of pp125^{FAK}. Surprisingly, however, insulin treatment induced tyrosine dephosphorylation of pp125^{FAK} in both control cells (Fig. 5A, lane 2) and C/SPTP2-transfected cells (lane 4). Quantitation of these data by laser scanning densitometry indicated that insulin treatment of control cells decreased the tyrosine phosphate content of pp125^{FAK} by $66 \pm 5\%$ compared with the basal state. Although expression of C/SPTP2 increased (≈ 2 -fold) pp125^{FAK} tyrosine phosphorylation compared with control cells in the basal state, insulin treatment resulted in a similar decrease in pp125^{FAK} tyrosine phosphate content ($72 \pm 3\%$). These alterations were specifically due to changes in

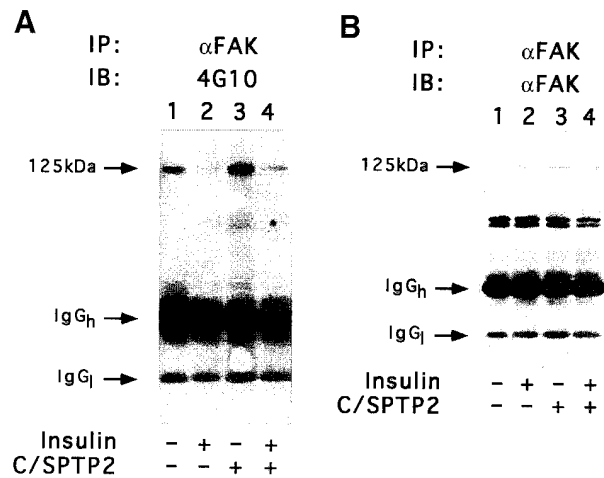


FIG. 5. Identification of pp125^{FAK} as a phosphotyrosine-containing protein that is specifically dephosphorylated by insulin. CHO/IR cells were transfected by electroporation with 40 μ g of the empty CLDN vector (CONT; lanes 1 and 2) or CLDN with cDNA for the SHPTP2 mutant in which the catalytic cysteine-459 was replaced with serine (C/SPTP2; lanes 3 and 4). The cells were either left untreated (lanes 1 and 3) or incubated with 100 nM insulin for 5 min (lanes 2 and 4) before isolation of total cell extracts and immunoprecipitation with a FAK antibody (α FAK). The samples were then subjected to Western blotting with either the 4G10 phosphotyrosine antibody (A) or the FAK antibody (B). The 4G10 phosphotyrosine Western blot analysis presented in A was independently performed four times and the FAK Western blot analysis presented in B was independently performed twice. IP, immunoprecipitation; IB, immunoblot; IgG_h and IgG_l, IgG heavy and light chains.

pp125^{FAK} tyrosine phosphorylation, since direct Western blotting using a pp125^{FAK} antibody demonstrated identical levels of FAK protein immunoprecipitated under these conditions (Fig. 5B).

DISCUSSION

Protein phosphatase activity has generally been thought to function as the inactivating portion of signaling pathways by reversing the stimulatory phosphorylation event. However, phosphatase activity has been shown to play an important positive signaling role in the case of T-cell receptor signaling (22, 23). In this regard, the data presented in this study suggest that the SHPTP2 protein-tyrosine-phosphatase plays an essential role in insulin signal propagation from the insulin receptor to MAP kinase and *c-fos* transcription. This was detected as an inhibition of insulin-stimulated MAP kinase gel shift and partial inhibition of MAP kinase activity and SRE-Luc expression in cells expressing inactive SHPTP2 mutants. This partial inhibition of downstream signaling reflects either an incomplete blockade of endogenous SHPTP2 function or the presence of alternative signaling pathways leading to MAP kinase activation. However, the extent of inhibition of MAP kinase activity was in proportion to the reduction in insulin-stimulated SRE-Luc expression. This is consistent with the MAP kinase functioning as an upstream kinase for the nuclear transcription factors SRF and p62^{TCF}, which mediate SRE responsiveness (for review, see ref. 46).

The requirement for SHPTP2 function as a positive effector in tyrosine kinase receptor downstream signaling is analogous to genetic epistasis studies in *Drosophila* demonstrating that the torso tyrosine kinase (homologue of the platelet-derived growth factor receptor) functions upstream of the SHPTP2 homologue corkscrew. Corkscrew, in turn, provides an essential upstream function for the *Drosophila* homologue of mammalian Raf, 1(1)pole hole (D-Raf), that is necessary for appropriate anterior/posterior embryo development (47–50).

Insulin has been well documented to increase tyrosine phosphorylation of numerous proteins, including the insulin receptor itself, IRS1, pp60, and Shc (for reviews, see refs. 5 and 6). However, we have identified pp125^{FAK} as a target protein for insulin-dependent tyrosine dephosphorylation. Since insulin stimulated pp125^{FAK} tyrosine dephosphorylation in both control and C/SPTP2-expressing cells, pp125^{FAK} is probably not a direct substrate for SHPTP2. However, the level of tyrosine-phosphorylated pp125^{FAK} following insulin stimulation of C/SPTP2-transfected cells was similar to that observed in the basal state of control transfected cells. Thus, it is formally possible, although unlikely, that the insulin-induced pp125^{FAK} tyrosine dephosphorylation in the cells expressing C/SPTP2 did not occur to a sufficient extent to generate an insulin-mediated downstream signal for MAP kinase and *c-fos* transcriptional activation.

In any case, the cellular signaling role of pp125^{FAK} in growth factor action has not been elucidated to date. However, pp125^{FAK} appears to be an important integration point for the actions of G-protein-coupled receptors, the non-tyrosine kinase integrin receptor family members, and receptor and nonreceptor tyrosine kinases (for review, see ref. 51). Activation of these signaling pathways increases pp125^{FAK} tyrosine phosphorylation, which has been directly correlated with an increased formation of actin stress fibers (filamentous actin). In contrast, we have observed that the insulin-induced tyrosine dephosphorylation of pp125^{FAK} correlates with a marked decrease in the amount of actin stress fibers (data not shown). Thus, the unique ability of the insulin receptor tyrosine kinase to decrease pp125^{FAK} tyrosine phosphorylation suggests an important role for pp125^{FAK} in the divergent signaling pathways between insulin and other growth factor signaling systems.

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