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Different Signaling Roles of SHPTP2 in Insulin-induced GLUT1 Expression and GLUT4 Translocation*

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Insulin activates hexose transport via at least two mechanisms: a $p21^{ras}$-dependent pathway, leading to an increase in the amount of cell surface GLUT1; and a metabolic, $p21^{ras}$-independent pathway, leading to translocation of the insulin-responsive transporter GLUT4 to the cell surface. Following insulin stimulation, SHPTP2, a non-transmembrane protein-tyrosine phosphatase, associates with insulin receptor substrate 1 via its Src homology 2 (SH2) domains. Microinjection of a glutathione *S*-transferase fusion protein encoding the N- and C-terminal SH2 domains of SHPTP2 (GST-NC-SH2) or anti-SHPTP2 antibodies into NIH-3T3 fibroblasts overexpressing the insulin receptor blocks insulin-induced DNA synthesis. Microinjection of either GST-NC-SH2 or anti-SHPTP2 antibodies into 3T3-L1 adipocytes inhibited the insulin-stimulated increase in expression of GLUT1. In contrast, translocation of GLUT4 to the cell surface was unaffected by either GST-NC-SH2 or anti-SHPTP2 antibodies. These data confirm a role for SHPTP2 in insulin-stimulated mitogenesis and indicate that whereas SHPTP2 is necessary for insulin-stimulated expression of GLUT1, it is not required for activation of the metabolic pathway leading to GLUT4 translocation.

Activation of the insulin receptor results in receptor autophosphorylation and subsequent phosphorylation of its substrate, insulin receptor substrate 1 (IRS-1)[1] (1). Tyrosine-phosphorylated IRS-1 then serves to recruit Src homology 2 (SH2) domain-containing proteins, which regulate downstream signaling pathways. These signaling molecules include the $p21^{ras}$ activator complex Grb2/SOS, the p85 subunit of phosphatidylinositol 3-kinase, and the protein tyrosine phosphatase SHPTP2 (1). SHPTP2 associates with IRS-1 (2, 3), as well as with the EGF receptor (3–5) and PDGF receptor (3–5), following stimulation by insulin, EGF, or PDGF, respectively. Upon stimulation of cells with either EGF or PDGF, SHPTP2 becomes tyrosine-phosphorylated (2, 4, 5). In the case of PDGF stimulation, the C-terminal phosphotyrosine residue on SHPTP2 binds Grb2 and may couple the PDGF receptor to the $p21^{ras}$ pathway (6, 7). Microinjection of either the SH2 domains of SHPTP2 or antibodies to SHPTP2 blocks insulin-induced mitogenesis (8). SHPTP2 is not, however, tyrosine-phosphorylated in response to insulin (3), suggesting that the Grb2/SOS association of SHPTP2 is not operative in the insulin signaling pathway.

Recent observations indicate that the phosphatase (PTP) domain of SHPTP2 functions to send a positive signal independent of Grb2/SOS. Overexpression of catalytically inactive SHPTP2 inhibits insulin-induced activation of MAP kinase (9–11), stimulation of c-*fos* reporter gene expression (11) and GTP-loading onto $p21^{ras}$(9), suggesting that SHPTP2 is an upstream mediator of $p21^{ras}$ activation in insulin signaling. The role of the PTP domain of SHPTP2 also extends beyond signaling pathways related to mitogenesis. Injection of catalytically inactive SHPTP2 blocks fibroblast growth factor-induced mesoderm induction in *Xenopus* embryos and prevents completion of gastrulation (12). Association of SHPTP2 through its N-terminal SH2 domain with a phosphotyrosyl (pY) peptide corresponding to its binding site on either the PDGF receptor (13) or IRS-1 (14, 15) leads to a substantial increase in phosphatase activity. This might well provide a signaling mechanism utilized by SHPTP2 that does not rely on recruitment of a Grb2/SOS complex.

Insulin signaling proceeds via at least two divergent pathways. To date, all of the studies examining the role of SHPTP2 in insulin-stimulated signaling have utilized cells in which insulin induces mitogenesis but does not activate physiologically important metabolic pathways. However, the primary function of insulin is to regulate metabolic processes, most strikingly an increase in hexose uptake in adipose tissue and skeletal muscle. In these tissues, insulin rapidly and reversibly augments sugar uptake by promoting the translocation of the "insulin-responsive glucose transporter", GLUT4, from an intracellular compartment to the cell surface (16). In a number of tissue culture cell lines, insulin also increases glucose transport via an alternative pathway, which shows similarity to that used for mitogenesis in most cell types. Stimulation of this pathway results in a modest translocation of the basal transporter, GLUT1, and a larger increase in its expression, mediated primarily by transcriptional activation (17, 18).

The mouse cell line 3T3-L1 has been used as a model system for the study of insulin-stimulated processes (19). They express GLUT1 as well as GLUT4 and respond to insulin by increasing both GLUT1 expression and GLUT4 translocation. We have shown previously that $p21^{ras}$ is necessary for increased GLUT1 expression but is not required for translocation of GLUT4 to the cell surface (20). Since SHPTP2 is required in both mitogenic and non-mitogenic signaling pathways, we asked whether SHPTP2 is required for the insulin-stimulated increase in cell surface expression of glucose transporters.

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[1] The abbreviations used are: IRS-1, insulin receptor substrate 1; SH2, Src homology 2; PTP, phosphatase; pY, phosphotyrosyl; MAP, mitogen-activated protein; DTAF, fluorescein dichlorotriazine; DMEM, Dulbecco's modified Eagle's medium; BrdUrd, 5′-bromo-2′-deoxyuridine; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FBS, fetal bovine serum; GST, glutathione *S*-transferase.

EXPERIMENTAL PROCEDURES

Materials—Crystalline porcine insulin was a gift of Lilly Laboratories. Rhodamine-conjugated donkey anti-rabbit antibodies and fluorescein dichlorotriazine (DTAF)-conjugated donkey anti-rat antibodies were purchased from Jackson Immunologicals (West Grove, PA). Bovine serum albumin used in translocation assays was from Calbiochem. 5-Bromo-2-deoxyuridine (BrdUrd, RPN 201) and mouse-anti-BrdUrd (RPN 202) were purchased from Amersham. MBPras for microinjection studies was purified as described (20). Sheep-anti-MBPras antibodies were purchased from Elmira Biologicals (Iowa City, Iowa). Anti-phosphotyrosine antibody 4G10 was a gift of Dr. Tom Roberts (Dana Farber Cancer Institute, Boston, MA).

Cell Culture—3T3-L1 fibroblasts were grown and differentiated upon confluence as described (21). Adipocytes were maintained in DMEM containing 10% fetal bovine serum and used at 10–20 days post-differentiation. NIH-3T3 fibroblasts stably expressing the human insulin receptor (3T3-HIR) were maintained in DMEM containing 10% calf serum and 200 μg/ml Geneticin (G418).

Protein and Antibody Preparation—A glutathione *S*-transferase fusion protein encoding amino acids 1–251 of SHPTP2 was generated by polymerase chain reaction from the full-length SHPTP2 cDNA using the primers 3′-TCACTATAGGGCGAATTGGGTACC-5′ and 3′-GTTGTCCTAAGGTTTGAA-5′. The resulting PCR product was digested with *Eco*RI and ligated into pGEX-2T to yield GST-NC-SH2. GST-NC-SH2 protein was produced from *Escherichia coli* as described previously (2). In preparation of GST-NC-SH2 protein for microinjection experiments, the fusion protein was eluted from glutathione-Sepharose with 8 M urea. The eluted protein was dialyzed against phosphate-buffered saline using an Amicon microconcentrator and concentrated to 0.5 mg/ml. Antibodies to SHPTP2 were generated against full-length GST fusion protein and purified to a final concentration of 10 mg/ml by Protein G affinity purification. To establish whether GST-NC-SH2 domains bind activated IRS-1, 3T3-L1 adipocytes were serum-starved for 24 h in DMEM + 0.1% FBS, and cells were either stimulated with 500 nM insulin for 5 min or left untreated. Cell lysates were prepared as described previously (2) and incubated with either GST alone or GST-NC-SH2 (4 μg) at 4 °C for 2 h. Glutathione-agarose beads were collected by centrifugation and washed four times in 1%-Nonidet P-40 plus 1 mM sodium orthovanadate. Bound proteins were separated on 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Phosphotyrosine-containing proteins were detected with an anti-phosphotyrosine antibody (4G10) and a donkey anti-mouse horseradish peroxidase-conjugated secondary antibody using Enhanced Chemiluminescence (Amersham Corp.).

Microinjection—3T3-L1 adipocytes were microinjected using an Eppendorf model 5242 injector and a Narishige hydraulic manual micromanipulator. Tips were pulled from filament-containing borosilicate glass (World Precision Instruments) to diameters of 0.2–0.5 mm using a Sachs-Flaming micropipette puller (Sutter, model PC-84). Anti-SHPTP2 antibodies were mixed with MBPras to yield final concentrations of 5 mg/ml antibody and 1 mg/ml marker protein in phosphate-buffered saline. In all experiments, proteins were injected into the cytoplasm of 50–100 cells.

For mitogenesis assays, NIH-3T3 fibroblasts expressing the insulin receptor were serum-starved in DMEM + 0.1% FBS for 24 h. GST-NC-SHPTP2 at a concentration of 0.5 mg/ml and neutralizing antibodies to SHPTP2 at a concentration of 10 mg/ml were microinjected using a Narishige injector model IM200 and a Leitz manual micromanipulator. After approximately 2 h, the cells were stimulated with 1 μM insulin or 10% FBS and BrdUrd was added to a final concentration of 10 μM. The cells were incubated for an additional 18 h, fixed in methanol for 10 min, washed three times with PBS, permeabilized in 0.1% Nonidet P-40, and incubated with a primary antibody to BrdUrd and a fluorescent-labeled secondary antibody. Cells injected with SH2 domains were detected by coinjection of M_r 70,000 fluorescein isothiocyanate-dextran at 5 mg/ml. Antibody-injected cells were identified by staining for the presence of rabbit IgG using a tetramethyl rhodamine isothiocyanate-coupled goat anti-rabbit antibody.

Plasma Membrane Sheet Assay and Immunofluorescence—For GLUT4 translocation sheet assays, cells were injected with either GST-NC-SH2 proteins or polyclonal antibodies to SHPTP2 and were allowed to recover from microinjection for 30 min. They were then incubated in Leibovitz L-15 medium containing 0.2% bovine serum albumin for 2 h and treated with 100 nM insulin for 15 min. Plasma membrane "sheets" were prepared essentially as described (22). Cells to be assayed for GLUT1 expression were incubated for 4 h in DMEM containing 0.5% calf serum prior to injection with GST-NC-SH2 or anti-SHPTP2 antibodies. They were then incubated in the absence or presence of 500 nM insulin for an additional 20 h before preparation of sheets. All plasma membrane sheets were processed for immunofluorescence as described (21) using affinity-purified antibodies to the C terminus of GLUT4 or serum containing antibodies to the C terminus of GLUT1. Antibodies to GLUT1 were a gift of Miles Pharmaceuticals (West Haven, CT). Injected cells were identified by staining with antibodies to MBPras and DTAF-conjugated secondary antibodies. The amount of glucose transporter on the plasma membrane was quantitated by digital image processing as described previously (20).

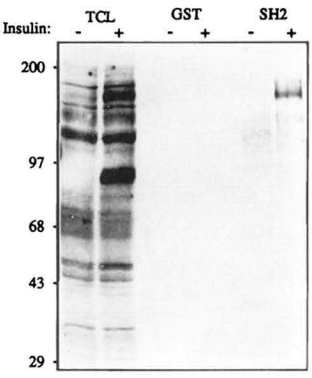

FIG. 1. **SH2 domains of SHPTP2 bind to IRS-1.** GST fusion proteins encoding the N- and C-terminal domains of SHPTP2 (GST-NC-SH2) were bound to glutathione-agarose beads. Either GST alone or GST-NC-SH2 beads were incubated with lysates prepared from 3T3-L1 adipocytes treated with (+) or without (−) insulin for 5 min. Tyrosine-phosphorylated proteins were detected by immunoblotting using 4G10 monoclonal antibody. *TCL*, total cell lysate.

RESULTS AND DISCUSSION

We investigated the role of SHPTP2 in the pathways through which insulin stimulates hexose uptake in cultured adipose cells. For these experiments, two reagents were generated for microinjection into 3T3-L1 adipocytes: a glutathione *S*-transferase (GST) fusion protein encoding the N- and C-terminal SH2 domains of SHPTP2 (GST-NC-SH2), and antibodies against full-length SHPTP2. We first confirmed that GST-NC-SH2 could associate with IRS-1 following insulin stimulation as previously reported (2, 3). Agarose beads bound to GST-NC-SH2 were incubated with lysates prepared from 3T3-L1 adipocytes either left untreated or exposed to insulin for 5 min. The GST-NC-SH2 beads were centrifuged and washed, and bound proteins were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted for the presence of phosphotyrosine (Fig. 1). A single pY protein of 180 kDa corresponding to IRS-1 was specifically precipitated with GST-NC-SH2 but not with GST alone. This result indicates that GST-NC-SH2 associates with IRS-1 and confirms earlier results that binding of the SH2 domains competes with endogenous SHPTP2 (2, 3). Thus, we hypothesized that upon microinjection of GST-NC-SH2 into fibroblasts or adipocytes, GST-NC-SH2 would block association of endogenous SHPTP2 with IRS-1 and prevent propagation of downstream signaling. Moreover, preincubation of GST-NC-SH2 domains with anti-SHPTP2 antibodies (986) inhibited (by approximately 50%) the association of the PDGF receptor with GST-NC-SH2 domains, while preincubation of the GST-NC-SH2 domains with preimmune SHPTP2 antibodies was without effect on PDGF Receptor association.[2] This result suggested that anti-SHPTP2 antibodies had a neutralizing effect on the binding of SHPTP2 SH2 domains to their corresponding phosphotyrosines. We then assessed whether either anti-SHPTP2 antibodies or GST-NC-SH2 had a biological effect on insulin-induced mitogenesis. GST-NC-SH2 and anti-SHPTP2 antibodies were injected into quiescent NIH-3T3

[2] R. M. Freeman and B. G. Neel, unpublished observations.

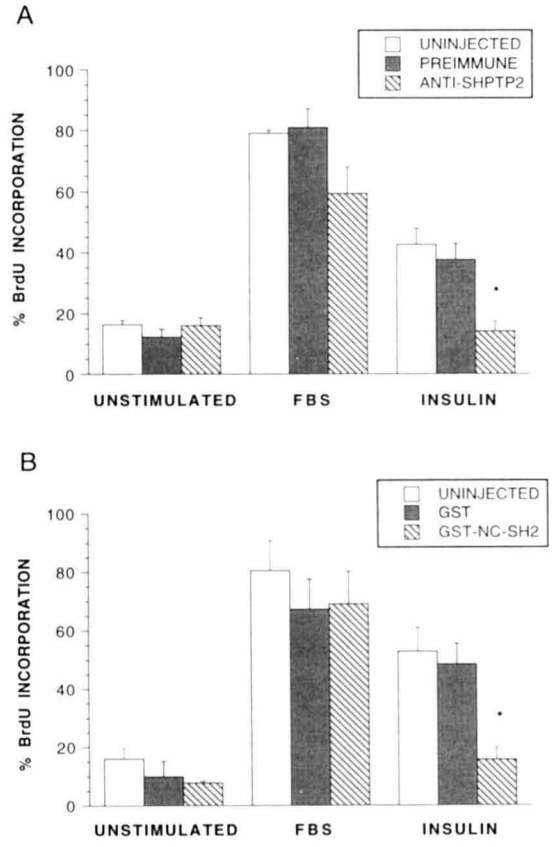

FIG. 2. **GST-NC-SH2 and anti-SHPTP2 antibodies block insulin-stimulated DNA synthesis.** 3T3-HIR cells were serum-starved for 24 h prior to microinjection with GST-NC-SH2 (*A*) or antibodies against SHPTP2 (*B*). The cells were stimulated with 1 μM insulin or 10% FBS, incubated in the presence of BrdUrd for 18 h and fixed in methanol. The fixed cells were stained with a primary antibody to BrdUrd and a fluorescent-labeled secondary antibody, and cells showing nuclear BrdUrd staining were scored positive for entry into S-phase. Results represent the mean ± S.E. of ~200 injected cells obtained from three separate experiments (*, $p < 0.05$).

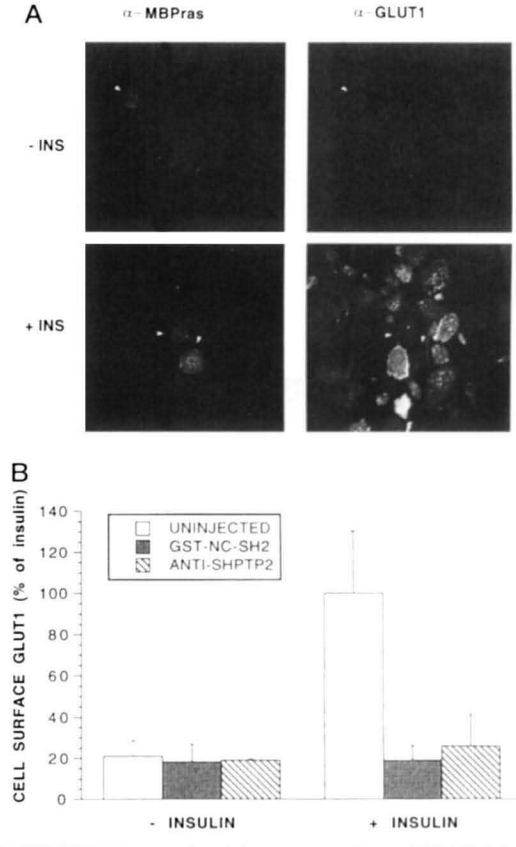

FIG. 3. **SHPTP2 is required for expression of GLUT1 in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were serum-starved for 4 h prior to microinjection with GST-NC-SH2 or antibodies to SHPTP2. The adipocytes were then stimulated with 500 nM insulin and incubated for an additional 20 h before preparation of plasma membrane sheets. The sheets were stained with an antibody to MBPras and a fluorescein-coupled anti-sheep antibody to detect injected cells, and with serum containing antibody to GLUT1 and rhodamine-coupled anti-rabbit antibody for quantitation of GLUT1. *Panel A* shows a representative field of cells injected with GST-NC-SH2 and stained for GLUT1, with *arrowheads* denoting injected cells. Quantitation of GLUT1 in uninjected cells and GST-NC-SH2 or antibody-injected cells from three separate experiments is shown in *panel B*.

cells overexpressing the insulin receptor (3T3-HIR). As shown in Fig. 2, ~45% of the insulin-stimulated and ~80% of the serum-stimulated cells were in S-phase, as compared to ~15% of the unstimulated cells. Microinjection of anti-SHPTP2 antibodies (986) into 3T3-HIR cells blocked their entry into S-phase in response to insulin ($p < 0.05$), but had no effect on serum-induced DNA synthesis (Fig. 2*A*). Similarly, 3T3-HIR cells that were microinjected with GST-NC-SH2 also failed to enter S-phase in response to insulin ($p < 0.05$), whereas the serum-stimulated increase was unaffected (Fig. 2*B*). Neither GST alone nor preimmune SHPTP2 antibodies had an effect on insulin-stimulated mitogenesis. These results indicate that both GST-NC-SH2 and the anti-SHPTP2 antibodies specifically block insulin-induced mitogenesis and that these reagents are not toxic to cell growth. The data confirm the efficacy of these reagents and support previously published studies showing that SHPTP2 is necessary for insulin-stimulated DNA synthesis but is not required for mitogenic induction by serum (8).

Exposure of 3T3-L1 adipocytes to insulin for several hours increases total GLUT1 mRNA and protein as well as the fraction of the hexose carrier on the cell surface (17, 23, 24). We therefore chose to examine the effect of SHPTP2 on the level of cell surface GLUT1 in the absence and presence of chronic insulin exposure. As shown in Fig. 3*A*, treatment of 3T3-L1 adipocytes with insulin for 20 h increases the amount of cell surface GLUT1 by 5-fold. Microinjection of GST-NC-SH2 inhibited expression of GLUT1 in the presence of insulin (Fig. 3*A*) whereas GST or MBPras alone had no effect (data not shown). The relative brightness of injected *versus* uninjected cells was quantified by image processing; the abundance of GLUT1 on plasma membrane sheets is summarized in Fig. 3*B*. GLUT1 expression was also inhibited by microinjection of antibody to SHPTP2 (Fig. 3*B*), while preimmune serum had no effect on GLUT1 distribution (data not shown).

We then addressed whether SHPTP2 is involved in the acute regulation of glucose uptake by insulin. As visualized by the plasma membrane sheet assay, stimulation of 3T3-L1 adipocytes with insulin for 15 min significantly increases the amount of GLUT4 on the plasma membrane (Fig. 4*A*) (22, 25); quantitation of fluorescent staining by image processing indicated that cell surface GLUT4 increased 15-fold in response to insulin (Fig. 4*B*). Microinjection of either GST-NC-SH2 (Fig. 4*A*) or anti-SHPTP2 antibodies was without effect on the distribution of GLUT4 in these cells in the presence or absence of either 100 nM insulin (Fig. 4*B*) or sub-saturating (10 nM) insulin (data not shown). GST, MBPras, and preimmune serum also had no effect on localization of GLUT4 (data not shown).

One of the fundamental problems in understanding the mechanism of insulin action is explaining its specificity in the regulation of metabolism, in spite of significant commonality with other peptide growth factors in terms of the signaling pathways modulated. For example, EGF is a very poor activa-

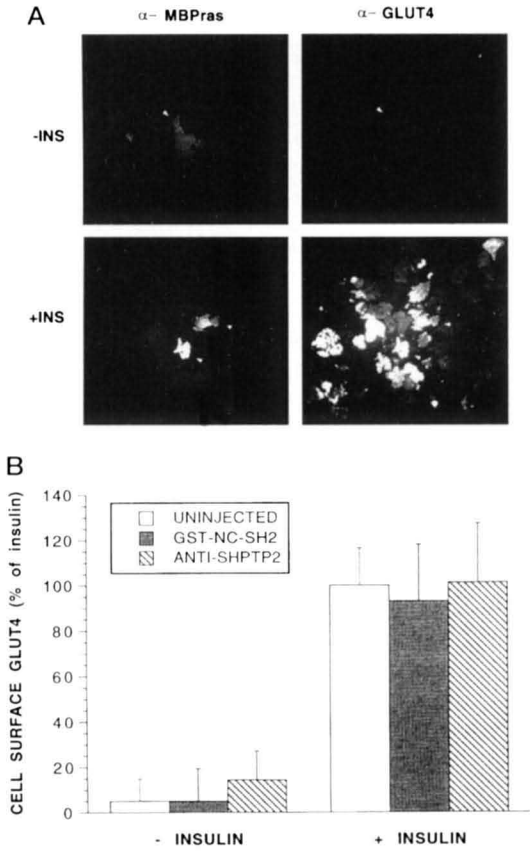

FIG. 4. **SHPTP2 has no effect on translocation of GLUT4 in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were microinjected with GST-NC-SH2 or antibodies to SHPTP2. The adipocytes were serum-starved for 2 h, stimulated with 100 nM insulin, and incubated for an additional 15 min before preparation of plasma membrane sheets. The sheets were stained with an antibody to MBPras and a DTAF-coupled anti-sheep antibody to detect injected cells, and with affinity-purified antibody to GLUT4 and rhodamine-coupled anti-rabbit antibody for quantitation of GLUT4. *Panel A* shows a representative field of cells injected with GST-NC-SH2 and stained for GLUT4, with *arrowheads* denoting injected cells. Quantitation of GLUT4 in uninjected cells and GST-NC-SH2- or antibody-injected cells from three separate experiments is shown in *panel B*.

tor of hexose uptake in 3T3-L1 adipocytes, in spite of equivalent potency to insulin in stimulating MAP kinase (26). Since SHPTP2 is affected quite differently by insulin as compared to PDGF or EGF, in that the former does not promote phosphorylation of the phosphatase, SHPTP2 seemed an attractive target for transducing hormone-specific signals (2–5). Previous studies evaluating the role of SHPTP2 in insulin action have utilized non-differentiated cells in which the most prominent effects of the hormone relate to the initiation of cell growth (8–11). The experiments reported above exclude SHPTP2 as an obligate intermediate in the signaling pathways by which insulin stimulates the translocation of GLUT4, the most important means of rapidly activating hexose uptake into muscle and adipose tissue. The requirement for SHPTP2 for the increase in expression of GLUT1 is consistent both with the critical role of $p21^{ras}$ and Raf-1 in this response, as well as the idea that the increase in transport mediated by GLUT1 represents primarily a component of the mitogenic response (20, 27).

Nonetheless, studies in which catalytically inactive SHPTP2 has been overexpressed in fibroblasts support the idea that the PTP domain is critical for signaling in some insulin stimulated pathways (9–11). Although *in vivo* substrates of SHPTP2 have yet to be identified, current data strongly suggest that SHPTP2 can function as an upstream mediator of $p21^{ras}$.This notion is supported by the observation that, when overexpressed, catalytically inactive SHPTP2 functions as a dominant-inhibitory mutant and abrogates insulin-induced $p21^{ras}$ GTP-loading (9). Moreover, signaling events distal to $p21^{ras}$ such as insulin-induced MAP kinase activation and GLUT1 expression (9, 10), fibroblast growth factor-induced mesoderm induction in *Xenopus* embryos (12), c-*fos* transcription through SRE (25), and EGF-induced MAP kinase transactivation of Elk-1[3] are also inhibited by overexpression of catalytically inactive SHPTP2. We have shown previously that $p21^{ras}$ mediates insulin-stimulated GLUT1 expression but not GLUT4 translocation in 3T3-L1 adipocytes (20). Since SHPTP2 is also necessary for GLUT1 expression but is not required for GLUT4 translocation, our data support published reports suggesting that SHPTP2 is required for processes mediated by $p21^{ras}$. We conclude that SHPTP2 plays a role in insulin-induced transcription of immediate early genes such as GLUT1, but is not required for the metabolic increase in hexose transport mediated by GLUT4 translocation.

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