Down-regulation of Insulin Signaling by Protein-tyrosine Phosphatase 1B Is Mediated by an N-terminal Binding Region*

Received for publication, February 9, 2000, and in revised form, April 27, 2000
Published, JBC Papers in Press, May 11, 2000, DOI 10.1074/jbc.M001063200

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Protein-tyrosine phosphatases (PTPs) play a major role in regulating insulin signaling. Among the PTPs that regulate this signaling pathway, PTP1B plays an especially prominent role. PTP1B inhibits insulin signaling and has previously been shown to bind to the activated insulin receptor (IR), but neither the mechanism nor the physiological importance of such binding have been established. Here, we show that a previously undefined region in the N-terminal, catalytic half of PTP1B contributes to IR binding. Point mutations within this region of PTP1B disrupt IR binding but do not affect the catalytic activity of this phosphatase. This binding-defective mutant of PTP1B does not efficiently dephosphorylate the IR in cells, nor does it effectively inhibit IR signaling. These results suggest that PTP1B targets the IR through a novel binding element and that binding is required for the physiological effects of PTP1B on IR signal transduction.

The control of insulin signaling is complex, involving the coordinated action of both positive and negative regulatory proteins. The role of negative regulators, which serves to blunt or terminate insulin signals, is not yet as well understood as the role of those that promote insulin’s actions. Among the negative regulatory factors, protein-tyrosine phosphatases (PTPs)\(^1\) play a prominent role. After stimulation, the insulin receptor (IR) \(\beta\) subunit is rapidly dephosphorylated by one or more PTPs, returning to basal phosphotyrosine levels within a few minutes (1). This dephosphorylation inactivates the IR and thereby terminates the insulin signal.

Several PTPs have been implicated in IR tyrosine dephosphorylation (1, 2). These include the transmembrane enzymes LAR and PTPs and the cytosolic enzymes SHP2 and PTP1B (1, 2). The evidence that PTP1B is a key physiological regulator of insulin signaling is especially compelling. First, injection of PTP1B into Xenopus oocytes impedes insulin-stimulated maturation (3). Second, overexpression of PTP1B in mammalian cells suppresses insulin signals (4–6), whereas inhibition of this PTP enhances insulin signals (7, 8). Third, PTP1B binds to the IR and efficiently dephosphorylates it in vitro (6, 9, 10). Finally, deletion of the ptp1B gene in mice causes marked insulin sensitivity and prolonged IR autophosphorylation (11). These results show that PTP1B plays an important role in down-regulating insulin signaling.

Although mutant, substrate-trap forms of PTP1B associate with the IR, the wild-type PTP does not coimmunoprecipitate with this or other receptor tyrosine kinases (RPTKs). The means by which PTP1B selects this substrate are not known. PTP1B does not contain any obvious targeting motifs such as src homology 2 or phosphotyrosine binding domains, which might direct this enzyme to the IR. Furthermore, whatever the mechanism, a requirement for PTP1B/IR association in suppressing insulin signaling has not been demonstrated. We therefore sought to clarify the issues of how PTP1B binds to the IR and if such binding is relevant to its function.

In this work, we identify an IR targeting motif in the N-terminal half of PTP1B and show that the elimination of this motif does not affect catalytic activity of PTP1B but does inhibit the ability of this phosphatase to regulate insulin signaling. These results suggest that the substrate specificity of PTP1B is imparted by a unique region outside the catalytic core of this enzyme and that manipulating this region might selectively impact insulin signaling.

**EXPERIMENTAL PROCEDURES**

Materials—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum. The monoclonal anti-hemagglutinin antibody 12CA5-J was obtained from Babco. Monoclonal anti-phosphotyrosine PY20, polyclonal anti-IR, and monoclonal anti-MAPK (mitogen-activated protein kinase) antibody were purchased from Transduction Laboratories. The monoclonal anti-GST (glutathione S-transferase) antibody was obtained from Santa Cruz Biotechnology, Inc. Anti-Akt antibody was obtained from Santa Cruz Biotechnology, Inc., and anti-phospho-Akt (Ser-473) antibody was purchased from New England BioLabs. Anti-active MAPK polyclonal antibody was purchased from Promega. \(^{18}\)P[ATP and \(^{3}H\)Deoxyglucose were purchased from NEN Life Science Products. All chemical reagents were purchased from Sigma.

Expression Plasmids—The mammalian expression vector p3H-PTP1B and its derivatives have been described previously (12, 13). pCMV6-GST was made by cloning a SalI/XhoI polymerase chain reaction fragment containing the GST sequence from pGEX-2T (14) into the mammalian expression vector pCMV6 (15). Truncated and internally deleted forms of PTP1B were constructed by polymerase chain reaction mutagenesis (13, 16). Mutations were confirmed by sequence analysis. Truncated and internally deleted pCMV6-GST-PTP1B constructs were made by subcloning BamHI/EcoRI fragments of PTP1B from pGEX-2T into the pCMV6-GST vector.

Purification of pGEX2T-PTP1B Fusion Proteins—Various truncated and internally deleted pGEX2T-PTP1B transformants were grown in...
LB containing 100 μg/ml ampicillin overnight at 37 °C in a shaking incubator. The cultures were then diluted 1:10 and grown for 1 h at 37 °C. Isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.5 mM, and the cultures were incubated further for 4 h. The cultures were spun down at 5500 rpm for 10 min at 4 °C. The pellet was washed twice with 0.15 M NaCl, and the supernatant was filtered using a 0.45-μm filter and applied to a glutathione Sepharose-4B column. The column was washed with 10 ml of phosphate-buffered saline (PBS) plus 10 mM dithiothreitol, then bound proteins were eluted with 0.1 M Tris-HCl, pH 7.5, 20 mM dithiothreitol and 20 mM glutathione. The yield of the protein was estimated by measuring the absorbance at 280 nm (assuming 1 absorbance unit = 0.5 mg/ml).

In Vitro Binding Assay—Insectins receptors were partially purified using wheat germ agglutinin-agarose (4) from HircB cells (17). The partially purified insectins receptors were activated and phosphorylated by incubating the receptor preparation with insulin at 4 °C for 30 min and then in the presence of manganese chloride, ATP, and sodium vanadate at 4 °C for another 10 min. The phosphorylated IR was then incubated at 4 °C overnight with 10 μl of different truncated or internally deleted forms of PTP1B. 20 μl of 50% glutathione-Sepharose beads was added to the sample and incubated further at 4 °C for 45 min. It was then washed five times with 1 ml of PBS. The beads were resuspended in 20 μl 1× SDS sample buffer. The samples were fractionated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and probed with anti-IR antibodies.

Transient Transformation—COS-7 were grown to 80% confluency in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and transfected with truncated and internally deleted forms of either pCMV6-GST-PTP1B or pJ3H bearing full-length wild-type PTP1B or harboring different point mutations alone or together with pCMV5-IR, using Lipofectamine PLUS reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. 48 hr after transfection, the cells were harvested for analysis.

Immunoprecipitation and Immunoblot—COS-7 cells were transiently transfected with either truncated and internally deleted forms of pCMV6-GST-PTP1B or pJ3H-bearing wild-type PTP1B or harboring different point mutations together with pCMV5-IR, as indicated in the text and figure legends. Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM β-glycerol phosphate) containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonl fluoride, and 10 μg/ml aprotinin. Lysate protein concentrations were estimated using bicinchoninic acid (Pierce). For immunoprecipitation, 1 mg of cell lysate was immunoprecipitated with 2 μg of anti-hemagglutinin antibody or anti-GST antibodies. As in the in vitro study, the N terminus of PTP1B contains an element that is required for association with the IR. We first attempted to determine the identity of the binding elements involved in the association of PTP1B with the IR. We first assessed the ability of truncated and internally deleted forms of PTP1B to associate with the IR in vitro. Purified GST or GST-PTP1B fusion proteins were incubated in the presence of activated and phosphorylated IR. The beads were then bound to glutathione-Sepharose and extensively washed. The presence or absence of associated IR was determined by immunoblotting. Because full-length (435 amino acids) PTP1B is difficult to produce in Escherichia coli, we tested the ability of a nearly full-length form of this protein, lacking only the 32-amino acid C-terminal hydrophobic domain (21, 22) as well as a progressive series of C-terminal truncations and internal deletions, to bind the IR. As expected, substrate-trap forms of PTP1B (in which the essential cysteine 215 residue is changed to serine) bind the IR (lanes 6–9). This binding occurs even if the N-terminal half of the enzyme is absent, as in the construct PTP403-CS ΔN193 (Fig. 1, lane 8). However, a protein comprising the N-terminal 193 amino acids of PTP1B alone readily binds the IR (lane 4), whereas a more extensive truncation, PTP100 (lane 3), does not. These data indicate that a substrate-trap form of PTP1B can stably associate with the IR, but that an element within the N-terminal half of this phosphatase, including residues 100 and 193, independently contributes to IR binding.

The N terminus of PTP1B Binds the IR in Vivo—To test the ability of the N terminus of PTP1B to bind the IR in cells, we carried out coimmunoprecipitation experiments. COS-7 cells were transiently transfected with expression vectors bearing various truncated and internally deleted forms of GST-PTP1B and IR. The cells were lysed in Nonidet P-40 lysis buffer, and glutathione-Sepharose beads were added to the cell lysate. After incubating for 1 h at 4 °C, the samples were washed extensively with lysis buffer, boiled in SDS sample buffer, electrophoresed on 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. Immunoblots were developed using the following protocol: The cells were serum starved for 2–4 h and then washed twice with serum-free, glucose-free DMEM. Cells were then incubated with 100 nM insulin in glucose-free DMEM for 15 min at 37 °C. 10 μl of 2-deoxyglucose mix (20 μl of 1 ml/ml [2-3H]deoxy-glucose, 30 μl of 100 mM 2-deoxy-γ-glucose, and 550 μl of glucose-free DMEM) was added to the cells and incubated for 10 min at 37 °C in a shaking water bath. The media was aspirated quickly after placing the cells on ice and washed twice with ice-cold PBS. The cells were solubilized by adding 400 μl of 1 N NaOH per well for 30 min at 37 °C. 50 μl of concentrated HCl was then added to each well, and the solution was transferred to a scintillation vial. 5 ml of Instagel plus was added to the samples, and the radioactivity was measured using a liquid scintillation counter.

RESULTS

Mapping the Insulin Receptor Binding Elements within PTP1B—Catalytically inactive, substrate-trap forms of PTP1B have previously been shown to bind activated RPTKs such as the epidermal growth factor, the platelet-derived growth factor, and the insulin receptor (10, 16, 19, 20). Because several PTPs contain specific substrate-targeting domains (see “Discussion”), we attempted to determine the identity of the binding elements involved in the association of PTP1B with the IR. We first assessed the ability of truncated and internally deleted forms of PTP1B to associate with the IR in vitro. Purified GST or GST-PTP1B fusion proteins were incubated in the presence of activated and phosphorylated IR. The beads were then bound to glutathione-Sepharose and extensively washed. The presence or absence of associated IR was determined by immunoblotting. Because full-length (435 amino acids) PTP1B is difficult to produce in Escherichia coli, we tested the ability of a nearly full-length form of this protein, lacking only the 32-amino acid C-terminal hydrophobic domain (21, 22) as well as a progressive series of C-terminal truncations and internal deletions, to bind the IR. As expected, substrate-trap forms of PTP1B (in which the essential cysteine 215 residue is changed to serine) bind the IR (lanes 6–9). This binding occurs even if the N-terminal half of the enzyme is absent, as in the construct PTP403-CS ΔN193 (Fig. 1, lane 8). However, a protein comprising the N-terminal 193 amino acids of PTP1B alone readily binds the IR (lane 4), whereas a more extensive truncation, PTP100 (lane 3), does not. These data indicate that a substrate-trap form of PTP1B can stably associate with the IR, but that an element within the N-terminal half of this phosphatase, including residues 100 and 193, independently contributes to IR binding.

The N terminus of PTP1B Binds the IR in Vivo—To test the ability of the N terminus of PTP1B to bind the IR in cells, we carried out cotransfection experiments. COS-7 cells were transiently transfected with expression vectors bearing various truncated and internally deleted forms of GST-PTP1B and IR. The cells were lysed in Nonidet P-40 lysis buffer, and glutathione-Sepharose beads were added to the cell lysate. After incubating for 1 h at 4 °C, the samples were washed extensively with lysis buffer, boiled in SDS sample buffer, electrophoresed on 10% SDS-PAGE, and blotted with anti-IR and anti-GST antibodies. As in the in vitro experiments, substrate-trap forms of PTP1B associate with the IR (Fig. 2, lanes 3 and 4). In addition, we also found that the N-terminal half of PTP1B by itself stably associates with the IR (lane 2), whereas a shorter form comprising only the N-terminal 100 residues (lane 1), does not. These results confirm the previous in vitro data that the region in PTP1B between residues 100 and 193 contains an element that is required for association with the IR.

PTP1B Tyrosine Residues 152 and 153 Are Required for Association with the IR—In a previous work, we showed that tyrosine residues 152 and 153 in the N terminus of PTP1B are phosphorylated by the IR in vitro (10). To determine if the
presence of these tyrosines contributes to IR binding, we asked whether a mutant form of the N terminus of PTP1B lacking these residues still associated with this RPTK. COS-7 cells were transiently cotransfected with an expression vector bearing IR and the indicated forms of GST-PTP1B. GST-PTP1B was isolated using glutathione-Sepharose beads, and the presence or absence of associated IR was determined by immunoblot. In A, constructs encoding the N-terminal 193 amino acids of PTP1B were used; in B, full-length PTP1B constructs were used.

FIG. 3. Tyrosines 152 and 153 on PTP1B contribute to association of PTP1B with the IR. COS-7 cells were cotransfected with expression vectors bearing IR and the indicated forms of GST-PTP1B. GST-PTP1B was isolated using glutathione-Sepharose beads, and the presence or absence of associated IR was determined by immunoblot. In A, constructs encoding the N-terminal 193 amino acids of PTP1B were used; in B, full-length PTP1B constructs were used.

Tyrosines 152 and 153 on PTP1B contribute to the interaction of the N terminus of this phosphatase with the IR.

We next examined the role of these tyrosines in association of full-length PTP1B with the IR. Wild-type PTP1B does not detectably associate with the IR (data not shown). However, a substrate-trap form of full-length PTP1B-DA (PTP1B-CS) (20) readily associates with the IR (Fig. 3B). Mutation of tyrosines 152 and 153 in this form of PTP1B reduces this binding by nearly 60%. These results suggest that these N-terminal tyrosine residues make an important contribution to stabilizing IR binding.

IR Binding Is Required for the Inhibitory Effects of PTP1B on Insulin Signaling—If PTP1B tyrosines 152 and 153 contribute to IR binding, and if such binding is physiologically important, then altering these residues should affect the ability of PTP1B to down-regulate insulin signaling. Because these residues lie within the catalytic domain of the enzyme, we needed to assess the effect of these mutations on PTP1B catalytic function before carrying out other functional assays. As shown in Fig. 4, wild-type PTP1B and the PTP1B-YF mutant dephosphorylate the artificial substrate p-nitrophenol phosphate with essentially identical kinetics ($K_m = 6.1–6.3$ and $5.8–6.0$ mM, for wild-type PTP1B and PTP1B-YF, respectively) (data not shown). PTP1B bearing a key substitution in the catalytic domain (PTP1B-CS) displays no activity, demonstrating that the phosphatase activity measured in these assays is due to recombinant PTP1B protein rather than to contaminants. As the wild-type and IR binding-defective version of PTP1B behave identically in this activity assay, any differences in the effects of these two forms of PTP1B on insulin signaling can be attributed to differences in their ability to bind the IR rather than to impaired catalytic function.

Having established that the relevant mutations do not perturb catalytic function, we next cotransfected COS-7 cells with an IR expression vector plus expression vectors bearing wild-

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**Fig. 1. In vitro binding of PTP1B to the IR.** 10 μg of purified GST or GST-PTP1B proteins were incubated with partially purified, activated IR. Glutathione-Sepharose 4B beads were added to the mixture. The beads were washed extensively and analyzed by immunoblot. A, PTP1B constructs. YY denotes tyrosine residues 152 and 153 and C denotes cysteine residue 215. B, anti-IR immunoblot. C, anti-GST immunoblot. Lane 1, positive control for IR.

**Fig. 2. In vivo binding of PTP1B to the IR.** COS-7 cells were cotransfected with expression vectors bearing IR and the indicated forms of GST-PTP1B. GST-PTP1B fusion proteins were isolated on glutathione-Sepharose beads. Following extensive washing, the presence or absence of associated IR was determined by immunoblot (top). The expression of the various GST-PTP1B fusion proteins was comparable (bottom).
type PTP1B or PTP1B-YF. 48 h after transfection, the cells were treated with insulin for 10 min and lysed, and the IR was immunoprecipitated and analyzed for autophosphorylation. Quantification of IR β subunit phosphotyrosine content from immunoblots was performed by densitometry. Expression of wild-type PTP1B reduced IR autophosphorylation by 25%, whereas expression of the binding-defective PTP1B-YF mutant reduced IR autophosphorylation by only 10% (Fig. 5). The results of this experiment show that the effect of PTP1B on IR autophosphorylation correlates with its ability to bind to this RPTK.

We next examined the effects of wild-type and mutant PTP1B on downstream insulin-stimulated signaling events. COS-7 cells were cotransfected with an IR expression vector plus expression vectors bearing either wild-type PTP1B, a catalytically inactive mutant (PTP1B-CS), or the binding mutant PTP1B-YF. Lysates were probed by immunoblot for the presence of tyrosine-phosphorylated IR, activated Akt, and activated MAPK. As with the IR β subunit, expression of wild-type PTP1B inhibited activation of Akt and MAPK, whereas the catalytically defective PTP1B mutant and the binding-defective PTP1B-YF mutant had a weaker effect (Fig. 6). Similar findings were made with regard to PI3K activity (Fig. 7). Expression of wild-type PTP1B decreased PI3K activity about 60%, whereas the catalytically inactive CS mutant had no inhibitory effect, and the YF binding mutant decreased PI3K activity by only about 20%. Thus, the ability of PTP1B to complex with the IR correlates with its ability to inhibit multiple insulin-stimulated signaling pathways.

Finally, we also assessed the effects of wild-type and mutant PTP1B expression on insulin-stimulated glucose uptake. We cotransfected COS-7 cells with IR plus empty vector or an expression vector bearing wild-type PTP1B or PTP1B-YF. Glucose uptake was then measured following stimulation by insulin. Expression of wild-type PTP1B reduced both basal and insulin-stimulated glucose uptake by about 60% (Fig. 8). In contrast, expression of the binding-defective PTP1B-YF mutant had a lesser effect, inhibiting insulin-stimulated glucose uptake by about 30%. Because this mutant is catalytically competent, these results suggest that PTP1B must associate with the IR to efficiently down-regulate the activity of this RPTK.

**DISCUSSION**

The strength and duration of insulin signaling is achieved in large part by the coordinated actions of tyrosine kinases and phosphatases. PTP1B plays a major role in this regulation, as reflected by the effects of deleting the *ptp1b* gene in mice (11). Here we show that PTP1B targets the IR by means of an N-terminal binding region and that deleting or mutating certain residues in this region impacts the ability of PTP1B to regulate IR autophosphorylation and downstream signaling. PTP1B has previously been shown to bind the IR *in vitro* and by coimmunoprecipitation from transfected cells. Most of these experiments used substrate-trap PTP mutants, which stabilize the interaction of the catalytic domain of the PTP with its
substrate. Although the catalytic domains of some PTPs apparently have intrinsic substrate specificity (23), many PTPs have in addition distinct binding elements that may affect the selection of substrates. These binding elements include SH2 domains (in Shp1 and -2) (reviewed in Ref. 24), proline-rich motifs (PTP1B and members of the PTP-PEST family) (13, 23, 25–33), PDZ domains (PTP-BL) (34–36), and MAPK-binding elements (PTP-SL, PTP-STEP, and various dual specificity MAPK phosphatases) (37–39). In the case of PTP1B, the C-terminal proline-rich motif, which directs binding to SH3-containing proteins such as p130Cbl (13, 40), is unlikely to be involved in the binding of this phosphatase to the IR or other RPTKs, because these proteins lack SH3 domains. Instead, our results indicate that a different part of the PTP1B, located in the N-terminal half of this molecule, is involved in IR binding. A similar region is also found in the closely related TC-PTP, but not in any other proteins in the current data base. Thus, this region may define a novel binding element that recognizes phosphotyrosine residues in the IR and perhaps in related RPTKs. Interestingly, the tyrosine residues in PTP1B implicated in IR binding are phosphorylated by this RPTK in vitro (10) but not by the epidermal growth factor receptor (16). Thus, the association of PTP1B with the IR may be regulated by tyrosine phosphorylation.

Much of the N-terminal portion of PTP1B that is required for IR binding is poorly conserved in other PTPs. Only the closely related TC-PTP has a substantially similar sequence in this region (41). Interestingly, like PTP1B, overexpression of TC-PTP also inhibits IR autophosphorylation (42), and substrate-trap forms of TC-PTP have been shown to bind certain RPTKs such as the epidermal growth factor receptor (43). Tyrosines 152 and 153 are located at the junction between the β9 and β10 strands in PTP1B (44) and are exposed on the surface of the molecule. These crystallographic data, together with our interaction mapping results and the fact that much of the sequence flanking these tyrosines is unique to PTP1B and TC-PTP, suggest that this region contacts the IR. If so, the contact sites may include phosphotyrosyl epitopes on the IR, because mutational analysis of this RPTK has shown that PTP1B binding is correlated with phosphorylation on tyrosine residues 1146, 1150, and 1151 (10).

We show here that mutation of tyrosines 152 and 153 in PTP1B affect the ability of PTP1B to bind the IR and decrease the effects of this enzyme on IR signaling. However, wild-type PTP1B does not detectably associate with the IR. Because tyrosines 152 and 153 are required for the association of the isolated N terminus of PTP1B with the IR and affect the stability of interaction of the full-length substrate-trap form of PTP1B with the IR, we suggest that these residues are involved in directing wild-type PTP1B to the IR in vivo but that the association between these proteins is normally transient and unstable. Once the IR is dephosphorylated, the interaction between wild-type PTP1B and the IR is presumably too weak to be maintained during immunoprecipitation. A similar situation may exist for other PTPs, such as PTP-PEST, which contains a binding element for p130Cbl but nevertheless does not communoprecipitate with this protein.

When overexpressed, PTP1B negatively regulated both
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RPTK and integrin signaling. The effects on the latter pathway are likely mediated by SH3-dependent interactions of PTP1B with focal adhesion proteins such as p130Cas and Fak (13, 40, 45). In this case, these focal adhesion proteins are targeted by a C-terminal proline-rich motif in PTP1B. In contrast, we show here that the interactions of PTP1B with the IR are mediated by a distinct, N-terminal binding region in this phosphatase. These results suggest that the regulation of insulin versus integrin signaling by PTP1B is separable.

Acknowledgments—We thank Mary Ann Sells, Erica Golemis, and Tom Coleman for reviewing this manuscript.

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