Protein-Tyrosine Phosphatase 1B Complexes with the Insulin Receptor in Vivo and Is Tyrosine-phosphorylated in the Presence of Insulin*

(Received for publication, August 6, 1996, and in revised form, October 18, 1996)

Debudatta Bandyopadhyay†, Anasua Kusari‡, Kathleen A. Kenner§, Feng Liu*, Jonathan Chernoff*, Thomas A. Gustafson*, and Jyotirmoy Kusari†††‡‡‡

From the †Department of Physiology and **Molecular and Cellular Biology Program, Tulane University Medical Center, New Orleans, Louisiana 70112-2699, ††Department of Pediatrics, University of California, San Diego, La Jolla, California 92039, ‡‡Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111, and ‡§Department of Physiology, University of Maryland at Baltimore, Baltimore, Maryland 21201

In response to insulin, protein-tyrosine phosphatase 1B (PTPase 1B) dephosphorylates 95- and 160–180-kDa tyrosine phosphorylated (PY) proteins (Kenner, K. A., Anyanwu, E., Olefsky, J. M., and Kusari, J. (1996) J. Biol. Chem. 271, 19810–19816). To characterize these proteins, lysates from control and insulin-treated cells expressing catalytically inactive PTPase 1B (CS) were immunoadsorbed and subsequently immunoblotted using various combinations of phosphotyrosine, PTPase 1B, and insulin receptor (IR) antibodies. Anti-PTPase 1B antibodies coprecipitated a 95-kDa PY protein from insulin-stimulated intact cells, either directly or within a complex involving insulin receptor (IR) subunits. To identify PTPase 1B tyrosine (Tyr) residues that are phosphorylated in response to insulin, three candidate sites (Tyr66, Tyr152, and Tyr153) were replaced with phenylalanine. Replacing Tyr66 or Tyr152 and Tyr153 significantly reduced insulin-stimulated PTPase 1B phosphotyrosine content, as well as its association with the IR. Studies using mutant IRs demonstrated that IR auto-phosphorylation is necessary for the PTPase 1B-IR interaction. These results suggest that PTPase 1B complexes with the autophosphorylated insulin receptor in intact cells, either directly or within a complex involving additional proteins. The interaction requires multiple tyrosine phosphorylation sites within both the receptor and PTPase 1B.

Insulin is a potent metabolic and growth-promoting hormone that has pleiotropic effects at the level of the cell and within the intact organism. Insulin acts on cells to stimulate glucose, protein, and lipid metabolism, as well as RNA and DNA synthesis, by modifying the activity of a variety of enzymes and transport processes (1). As a first step in initiating these responses, insulin binds to its plasma membrane receptor. The insulin receptor is a heterotetrameric protein consisting of two α and two β subunits linked by disulfide bonds to form a β-α-α-β structure. After insulin binding, the insulin receptor undergoes autophosphorylation on tyrosine residues. Auto-phosphorylation increases the tyrosine kinase activity of the insulin receptor, which in turn phosphorylates one or more cellular substrates, leading to a cascade of secondary phosphorylation and dephosphorylation reactions (2).

As the molecular mechanism of insulin action is defined with increasing clarity, so too is our appreciation of the central role played by protein tyrosine phosphorylation. Regulated tyrosine phosphorylation represents a balance of protein-tyrosine kinase (PTKase)1 and protein-tyrosine phosphatase (PTPase) activities. To date, most attempts to assess the role of protein-tyrosine phosphorylation in insulin signal transduction have focused on the action of kinases and thus furnish an incomplete picture of this dynamic process. PTPases can be used as probes to test the role of protein tyrosine phosphorylation, complementing studies performed on the PTKases.

Extensive progress in the identification and characterization of PTPases has been made in recent years (3), partially as a result of our appreciation of the central role played by PTPases in signal transduction (4). PTPase 1B was the first PTPase to be isolated in homogeneous form and sequenced (5, 6). PTPase 1B possesses a catalytic domain characterized by the 11-amino acid sequence motif, (I/V)HCbR(S/T)G. This motif contains cysteine (Cys215) and arginine (Arg221) residues critical for the catalytic activity of the enzyme (7–9). The cDNA sequences for human (10, 11) and rat (8) PTPase 1B predict a protein of 50 kDa with 435 and 432 amino acids, respectively. The conserved phosphatase domain of PTPase 1B is contained within the domain spanning residues 30 to 278. The COOH-terminal noncatalytic extension of the protein serves a regulatory function. The COOH-terminal 35 residues target the enzyme to the cytoplasmic face of the endoplasmic reticulum (12), whereas the preceding 122 residues are predominantly hydrophilic and contain sites for serine phosphorylation. This segment of PTPase 1B is phosphorylated in vivo, and the pattern of phosphorylation is altered in a cell cycle-dependent manner (13). Recently, the crystal structure of the 321-residue (37-kDa) form of human PTPase 1B has been determined, revealing the structural features that provide the protein with its specific enzymatic capacity for phosphotyrosine (14, 15).

The involvement of PTPase 1B in insulin signaling has been

---

1 The abbreviations used are: PTKase, protein-tyrosine kinase; PTPase, protein-tyrosine phosphatase; IR, insulin receptor; IRS, IR substrate; IGF, insulin-like growth factor; FCS, fetal calf serum; TEMED, N,N,N',N''-tetramethylethylenediamine; PAGE, polyacrylamide gel electrophoresis; Grb2, growth factor receptor binding protein 2.
suggested in numerous reports. In initial studies, microinjection of purified placental PTPase 1B into Xenopus oocytes was shown to block insulin-induced S6 peptide phosphorylation and inhibit insulin-induced oocyte maturation (16, 17). Subsequent studies have demonstrated that PTPase 1B is expressed at relatively high levels in insulin-sensitive tissues (18). In clinical studies, we have shown that skeletal muscle biopsies from patients with impaired insulin action contain decreased PTPase 1B protein in comparison with subjects with normal insulin action (19). We have also demonstrated in the rat L6 myotube cell culture system that insulin increases total cellular PTPase activity in a time- and dose-dependent manner. Increased activity is due mainly, if not entirely, to increased PTPase 1B activity, following increased PTPase 1B mRNA and protein expression (20). Recently, we have shown that the overexpression of catalytically active PTPase 1B inhibits insulin-stimulated insulin receptor autophosphorylation, the phosphorylation of insulin receptor substrate (IRS) proteins, and glucose incorporation into glycogen (21). These data suggest that PTPase 1B acts as a negative regulator of insulin signaling. A similar conclusion has also been independently reached by several other laboratories (22, 23).

In this study, we show an in vivo interaction between the insulin receptor and PTPase 1B, using a mutant derivative of PTPase 1B in which the critical active site cysteine residue (Cys²¹⁵) has been mutated to serine (CS). Furthermore, we demonstrate that the interaction of the insulin receptor with PTPase 1B is absolutely dependent upon insulin-stimulated receptor autophosphorylation. Receptor tyrosines Tyr¹¹⁴⁶, Tyr¹¹⁵⁰, and Tyr¹¹⁵¹ are essential for the interaction. PTPase 1B residues Tyr⁶⁶, Tyr¹⁵², and Tyr¹⁵⁸ are important for the insulin-induced tyrosine phosphorylation of PTPase 1B and its association with the receptor complex. Our findings provide new insights into the mechanisms of PTPase 1B action within insulin receptor signaling.

EXPERIMENTAL PROCEDURES

Materials—Insulin and insulin like growth factor-I (IGF-I) were kindly provided by Lilly. Fetal calf serum (FCS), cell culture media, geneticin, gentamicin, and Glutamax were purchased from Life Technologies, Inc. Methotrexate and hygromycin B were from Calbiochem (San Diego, CA). Monoclonal anti-PTPase 1B and anti-IGF-1 receptor antibodies were purchased from Oncogene Science (Uniondale, NY). Monoclonal anti-phosphotyrosine antibody (PY-20), horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY-20H), and polyclonal anti-insulin receptor antibody used for immunoblotting were from Transduction Laboratories (Lexington, KY). Monoclonal anti-insulin receptor antibody (1844) used for immunoprecipitation was generously provided by Dr. Kenneth Siddle (Cambridge, United Kingdom). Rabbit polyclonal anti-PTPase 1B antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Tween 20, protein molecular weight standards, acrylamide, and TEMED were purchased from Bio-Rad. Nonfat dry milk was from Nestle Foods Co. (Glendale, CA). Anti-mouse and anti-rabbit IgGs conjugated with horseradish peroxidase and a enhanced chemiluminescence (ECL) detection system were purchased from Amersham Life Science, Inc. Nitrocellulose membrane was from Schleicher and Schuell. All other reagents were purchased from Sigma and were the highest quality available.

Plasmid Construction—The mutant IR constructs were generated by site-directed mutagenesis using the method of Kunkel et al. (24) with customized primers. The majority of the resultant cDNAs were introduced into the pSR vector (Invitrogen). Detailed cloning procedures are available from (T. A. G.) upon request. The YFF and YYF IR mutants were introduced into the pSRa vector (25) and have been described previously (26). PTPase 1B P2¹¹⁵⁰S,T152,F and PTPase 1B Y2¹¹⁴⁶,S,T152,F were also constructed by standard site-directed mutagenesis techniques (24). Wild-type and mutant cDNAs were subcloned as BamHI/EcoRI fragments into the epoxide-tag expression vector pGL4, which appends the hemagglutinin epitope to the NH₂ terminus (27).

Cell Lines—The cell lines Hirc-M and CIGFR-M used in this study have been described previously (21). Briefly, Hirc-M is a rat 1 fibroblast cell line overexpressing human insulin receptors and CS PTPase 1B. CIGFR-M is a Chinese hamster ovary cell line overexpressing human IGF-I receptors and CS PTPase 1B. Hirc-M cells were propagated in Dulbecco’s minimal media (DMEM) containing 10% FCS, 50 µg/ml gentamicin, 400 µg/ml hygromycin, and 500 nM metothrexate. CIGFR-M were propagated in Ham’s F-12, containing 10% FCS, 50 µg/ml gentamicin, 400 µg/ml hygromycin, and 400 µg/ml genetin.

PTPase 1B Associates with the Insulin Receptor in Vivo

In this study, we show a new insight into the mechanisms of PTPase 1B action within insulin-induced tyrosine phosphorylation of PTPase 1B and insulin receptor signaling.

RESULTS

PTPase 1B Complexes in Vivo with the Activated Insulin Receptor and Is Tyrosine-phosphorylated—We have previously shown that the insulin-stimulated tyrosine phosphorylation of at least two proteins (95 and 160–180 KDa) is reversibly overexpressed of the catalytically active PTPase 1B (21). To further characterize these proteins, we used Hirc-M cells overexpressing human insulin receptors and CS PTPase 1B. The C₂⁵⁵S mutation rendered PTPase 1B enzymatically inactive but still allowed substrate binding. The association of CS PTPase 1B with its substrate is very stable in comparison with active PTPase 1B (30). To identify proteins that interact with PTPase 1B under insulin-stimulated conditions, the lysates from control and insulin-treated cells, used in immunoprecipitation with antibodies against IRS proteins that were immunoprecipitated by the PTPase 1B antibody. Under basal conditions, a single 120-kDa phosphorylated protein was immunoprecipitated by an anti-PTPase 1B antibody or 1:100 anti-insulin receptor antibody) and a 1:4 volume of the 50% anti-mouse IgG agarose slurry for 2 h. The agarose was pelleted by centrifugation at 15,000 × g for 2 min at 4 °C. Pellets were washed five times with ice-cold lysis buffer, dissolved in SDS-PAGE sample buffer, processed by SDS-PAGE, and transferred to nitrocellulose filters. Filters were probed with the indicated antibodies, and bound antibody was visualized using the ECL detection system (Amersham Corp.). To reprobe the blots, the filters were incubated for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS) and then washed two times (10 min each) in room temperature in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). The filters were reprobed with the desired antibodies, and bound antibody was detected as described above.

Cell Lysis, Immunoprecipitation, and Immunoblotting—Cells were serum starved for 16–24 h in appropriate medium with 0.1% FCS before experimental treatment. Treated cells were washed in ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin). The lysates were spun at 15,000 × g for 10 min at 4 °C. The supernatants were removed and assayed for total protein content using the Bradford method (29). After normalization of protein, supernatants were incubated on an end-over-end mixer at 4 °C with antibody (1 µg of anti-PTPase 1B antibody or 1:100 anti-insulin receptor antibody) and a 1:4 volume of the 50% anti-mouse IgG agarose slurry for 2 h. The agarose was pelleted by centrifugation at 15,000 × g for 2 min at 4 °C. Pellets were washed five times with ice-cold lysis buffer, dissolved in SDS-PAGE sample buffer, processed by SDS-PAGE, and transferred to nitrocellulose filters. Filters were probed with the indicated antibodies, and bound antibody was visualized using the ECL detection system (Amersham Corp.). To reprobe the blots, the filters were incubated for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS) and then washed two times (10 min each) in room temperature in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). The filters were reprobed with the desired antibodies, and bound antibody was detected as described above.
PTPase 1B Associates with the Insulin Receptor in Vivo

Phosphorylation of the insulin receptor under basal conditions (Fig. 1). Incubation of intact cells in the absence (−) and presence (+) of 100 nM insulin at 37 °C for 5 min and then collected in lysis buffer. Cell lysates (1 mg) were incubated with monoclonal anti-PTPase 1B antibody (1 μg) for 2 h at 4 °C. Antibody complexes were collected with anti-mouse IgG agarose, and immunoprecipitated proteins were fractionated by 5–12% gradient SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with the monoclonal anti-phosphotyrosine antibody (A). The nitrocellulose membrane used in A was stripped and reprobed with the polyclonal anti-insulin receptor antibody (B). The nitrocellulose membrane used in B was stripped and reprobed with the polyclonal anti-insulin receptor antibody (C). Arrows indicate insulin receptor (IR) and PTPase 1B proteins. This is a representative experiment independently performed five times.

The 180-kDa protein(s) that coprecipitated with both the insulin receptor and PTPase 1B (Figs. 1A, left lane) were serum starved overnight and then incubated in the absence and presence of 100 nM insulin at 37 °C for 5 min. Antibody complexes were collected, fractionated, and immunoblotted as described in Fig. 1. With monoclonal anti-phosphotyrosine antibody (A), polyclonal anti-insulin receptor antibody (B), and polyclonal anti-PTPase 1B antibody (C). Arrows indicate insulin receptor (IR) and PTPase 1B proteins. This is a representative experiment independently performed five times.

These results suggest that in response to IGF-I in vivo, PTPase 1B forms complexes with the autophosphorylated IGF-I receptor. In contrast to our results from insulin-treated cells, we were unable to detect IGF-I-stimulated tyrosine phosphorylation of PTPase 1B. This could result from a lower abundance of tyrosine-phosphorylated PTPase 1B in CIGFR-M cells. It is equally possible that IGF-I fails to stimulate the tyrosine phosphorylation of PTPase 1B. In light of the different biological effects of insulin and IGF-I stimulation, alterations in the tyrosine phosphorylation of receptor-associated PTPase 1B could alter its enzymatic activity or range of substrates, thereby initiating separate signaling pathways for each ligand.

Mapping the Major in Vivo Tyrosine Phosphorylation Sites on PTPase 1B—We have shown above that PTPase 1B is tyrosine-phosphorylated in response to insulin (Figs. 1A and 2A). PTPase 1B may be phosphorylated directly by the activated insulin receptor or by another insulin-inducible protein-tyrosine kinase. It has been shown previously that most protein-
Tyrosine kinases phosphorylate substrates at tyrosine residues that are preceded by acidic amino acids (32). The PTPase 1B coding sequence was examined for such potential tyrosine phosphorylation sites. Only three tyrosine residues fit this consensus; Tyr^{66} (QEDNDY), Tyr^{152} and Tyr^{153} (EDIKSYY). To determine if any of these residues become tyrosine-phosphorylated in the presence of insulin, either Tyr^{66} or Tyr^{152} and Tyr^{153} were replaced with phenylalanine residues. Expression vectors bearing PTPase 1B C215S (CS), C215S, Y66F (YF), or C215S, Y152,153F (YYFF) mutations were coexpressed with the wild-type insulin receptors in COS cells. Twenty-eight h after transfection, COS cells were serum-starved and then incubated in the absence and presence of insulin for 5 min. A fraction of whole-cell lysates from control and insulin-stimulated cells was immunoprecipitated with anti-PTPase 1B antibodies. The immunoprecipitates and remaining cell lysates were then subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-PTPase 1B and anti-insulin antibodies.

As shown in Fig. 4A, expression levels of the various mutant PTPase 1B proteins in COS cells were comparable. Insulin increased the tyrosine phosphorylation of PTPase 1B in cells expressing CS protein (Fig. 4B, lane 2). This is consistent with our above-stated observations (Fig. 1A). However, in cells expressing either YF or YYFF proteins, no phosphorylation of PTPase 1B was observed in response to insulin (Fig. 4B, lanes 4 and 6). To ensure that this result was not due to an inability of the PTPase 1B antibody to precipitate the mutant proteins, PTPase 1B protein levels were measured by Western blot analysis in anti-PTPase 1B immunoprecipitates from cells expressing the various mutant PTPase 1B proteins. As shown in Fig. 4C, the amount of PTPase 1B present in anti-PTPase 1B immunoprecipitates from cells expressing YF or YYFF protein was even higher than from cells expressing CS protein. These results suggest that PTPase 1B residues Tyr^{66}, Tyr^{152}, and/or Tyr^{153} are necessary for insulin-induced phosphorylation of the protein.

\textit{Tyr}^{66} \textit{Tyr}^{152}, and/or \textit{Tyr}^{153} are Essential for the Interaction of PTPase 1B with the Insulin Receptor—We next asked whether replacement of PTPase 1B residues Tyr^{66} or Tyr^{152} and Tyr^{153} with phenylalanine affects its in vivo interaction with the insulin receptor. To address this issue, expression vectors bearing the CS, YF, or YYFF mutations were cotransfected with the wild-type insulin receptors into COS cells. Twenty-eight h after transfection, COS cells were serum-starved and then incubated in the absence and presence of insulin. A fraction of whole-cell lysate from control and insulin-stimulated cells was immunoprecipitated with the anti-PTPase 1B antibody. The remainder of each cell lysate and the immunoprecipitates were then subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with anti-insulin receptor antibody. As shown in Fig. 5A, expression levels of the insulin receptors in COS cells expressing the different mutant PTPase 1B proteins were comparable. Anti-PTPase 1B antibody coprecipitated a considerable amount of the insulin receptor from insulin-stimulated CS-expressing cells (Fig. 5B, lane 2). Significantly less insulin receptor was immunoprecipitated from cells expressing either the YF or YYFF protein (Fig. 5B, lanes 4 and 6). These results indicated that replacement of Tyr^{66}, Tyr^{152}, and/or Tyr^{153} with phenylalanine significantly inhibited the insulin-stimulated \textit{in vivo} association of PTPase 1B with the insulin receptor complex.

Tyrosine Phosphorylation of Insulin Receptor Residues Tyr^{1146}, Tyr^{1150}, and/or Tyr^{1151} Is Required for Complex Formation with PTPase 1B—To identify insulin receptor phosphotyrosine residue(s) essential for complex formation with PTPase 1B, we used several mutant insulin receptors lacking various tyrosine residues. These included: (i) IR ΔCT (ΔCT), in which the receptor COOH-terminal 30 amino acids (containing Tyr^{1156} and Tyr^{1152}, by the numeric assignment of Ulrich et al. (36)) are deleted; (ii) IR Y^{1156,1151}F (YFF), in which the twin tyrosines at positions 1150 and 1151 have been replaced with phenylalanines; (iii) IR Y^{1144}F (FYF), in which tyrosine residue 1146 has been replaced with phenylalanine; and (iv) IR Y^{966}F (YF'), in which tyrosine residue 960 has been replaced with phenylalanine. To investigate whether insulin receptor autophosphorylation is necessary for its interaction with PTPase 1B, we also used an insulin receptor with impaired ligand-stimulated autophosphorylation (AK, (33)). AK receptors contain a K^{1018}A mutation. K^{1018} is a critical residue in the ATP-binding site of the insulin receptor tyrosine kinase domain (34). COS cells were transfected with either wild-type or mutant insulin receptor expression plasmids, plus CS PTPase 1B. Beginning 28 h after transfection, cells were serum-starved overnight and then treated in the absence and presence of insulin for 5 min. A fraction of each cell lysate was immunoadsorbed to anti-PTPase 1B antibodies. The remainder of each cell lysate and the immunoprecipitates were then subjected to SDS-PAGE and analyzed by sequential immunoblotting with anti-insulin receptor and anti-PTPase 1B antibodies.

As shown in Fig. 6A, expression levels of the wild-type and...
various mutant insulin receptor proteins in COS cells were comparable. Expression levels of CS PTPase 1B in these cells were very similar (data not shown). Under insulin-stimulated conditions, anti-PTPase 1B antibody coprecipitated comparable quantities of insulin receptor from cells expressing wild-type insulin receptor, ΔCT, or YF protein (Fig. 6B, lanes 2, 8, and 12). However, very little insulin receptor was immunoprecipitated from cells expressing F tyrosine insulin receptors (Fig. 6B, lane 6), and none was observed in immunoprecipitates from cells expressing either YF or AK receptors (Fig. 6B, lanes 4 and 10). The possibility of variable PTPase 1B immunoprecipitation from the different COS cell lines was excluded after determining that each of the immunoprecipitates contained comparable levels of PTPase 1B protein (data not shown).

Thus, the significant decrease in insulin receptor content in anti-PTPase 1B precipitates from insulin-stimulated cells expressing either the FY, YF, or AK protein was not due to poor precipitation of PTPase 1B by the antibody. Previous reports indicate that insulin-stimulated autophosphorylation of the AK and FYY receptor mutants is approximately 1 and 50%, respectively, of that of the wild-type receptor (30, 31). The decrease in the IR YFF mutant is reduced by more than the IR FYY mutant (35). Taken together, these results also suggest that the phospho-

**FIG. 5.** Replacement of PTPase 1B residues Tyr1146, Tyr1150, and Tyr1151 with phenylalanine significantly inhibits insulin-induced association of the protein with the insulin receptor. COS cells were cotransfected with the expression plasmids pCMV5-IR (IR), pCMV-Y1146F (FY), pCMV-Y1152,1153F (YFF), pCMV-ΔCT (ΔCT), pCMV-A1018K (AK), or pY960F (YF) plus pJ3H-PTPase 1B C215S (CS). Transfected, serum-starved cells were incubated in the absence (+) and presence (+) of 100 nM insulin at 37 °C for 5 min. A, whole-cell lysates were prepared as described under “Experimental Procedures” and immunoblotted, using the polyclonal anti-insulin receptor antibody. B, the cell lysates prepared in A were immunoprecipitated with monoclonal anti-PTPase 1B antibody and immunoblotted, using the polyclonal anti-insulin receptor antibody. The arrow indicates the insulin receptor. This is a representative experiment independently performed four times.

**FIG. 6.** Insulin receptor residues Tyr1146, Tyr1150, and Tyr1151 are essential for its association with PTPase 1B. COS cells were cotransfected with the expression plasmids pCMV5-IR (IR), pCMV-Y1146F (FY), pCMV-Y1152,1153F (YFF), pCMV-ΔCT (ΔCT), pCMV-A1018K (AK), or pY960F (YF) plus pJ3H-PTPase 1B C215S (CS). Transfected, serum-starved cells were incubated in the absence (+) and presence (+) of 100 nM insulin at 37 °C for 5 min. A, whole-cell lysates were prepared as described under “Experimental Procedures” and immunoblotted, using the polyclonal anti-insulin receptor antibody. B, the cell lysates prepared in A were immunoprecipitated with the monoclonal anti-PTPase 1B antibody and immunoblotted, using the polyclonal anti-insulin receptor antibody. The arrow indicates the insulin receptor. This is a representative experiment, independently performed five times.

**DISCUSSION**

Protein-tyrosine phosphorylation plays an important role in regulating many cellular processes. The level of phosphotyrosine in the cell is a balance between the actions of PTKases and PTases. As the role of PTKases in signal transduction is now understood in some detail, increasing attention is being focused on the role of PTases. A key to the physiological roles of PTases is the identity of their in vivo substrates. Recently, substrates of various PTases have been identified using mutant derivatives of these PTases (37–39). PTPase mutants are constructed by replacing the essential catalytic cysteine residue with serine. Mutation of the conserved cysteine renders the protein enzymatically inactive while retaining substrate binding capabilities (30).

To identify the intracellular PTPase 1B substrate(s) within the insulin signaling pathways, we used substrate binding competent, but catalytically inactive, CS PTPase 1B. The experiments described in this report indicate that CS PTPase 1B binds in vivo to a complex containing the activated insulin or IGF-I receptor. CS PTPase 1B also associates in vivo with activated epidermal growth factor and platelet-derived growth factor receptors but not with the CSF-1 receptor. The CSF-1 receptor is similar in structure to the platelet-derived growth factor receptor and belongs to the same subfamily of receptor tyrosine kinases (40). However, the CSF-1 receptor lacks binding sites for the GTPase-activating protein and phospholipase Cγ Src homology 2 domains (40), suggesting that PTPase 1B possibly interacts with proteins through GTPase-activating protein and/or phospholipase Cγ binding sites.

We have shown that PTPase 1B is tyrosine-phosphorylated in response to insulin stimulation. Phospho-amino acid analysis revealed that PTPase 1B is phosphorylated exclusively on serine in unsynchronized HeLa cells (12). Serine phosphorylation is mitosis-specific in HeLa cells and may serve to regulate PTPase 1B enzyme activity within the cell cycle (13). A CS PTPase 1B-GST fusion protein also became heavily tyrosine-phosphorylated when incubated with 3T3/EGFR cell lysates but in a ligand-independent manner (39). Our present studies demonstrate the insulin-dependent tyrosine phosphorylation of PTPase 1B in vivo. The function of this tyrosine phosphorylation is unknown. However, the PTPase activity of Syp (SHPTP2) is modulated by tyrosine/threonine phosphorylation resulting from growth factor activation and in cells transformed by the Rous sarcoma virus (41–45). The in vivo phosphorylation of PTP 1C in response to CSF-1, insulin, or pp60c-src results in 4-fold activation (46–48). Low molecular weight phosphotyrosine-protein phosphatase is tyrosine-phosphorylated by pp60c-src both in vivo and in vitro, correlating with an increase in its catalytic activity (49). Therefore, the insulin-induced tyrosine phosphorylation of PTPase 1B may regulate its phosphatase activity in either a positive or negative manner. Alternatively, it may serve to increase the substrate specificity of PTPase 1B by providing a phosphotyrosine docking
PTPase 1B Associates with the Insulin Receptor in Vivo

Kenneth Siddle

We thank Dr. Jerrold M. Olefsky for providing Rat 1 fibroblast and Chinese Hamster Ovary cells overexpressing human insulin and IGF-I receptors, respectively. We also thank Drs. Kenneth Siddle for providing the monoclonal anti-insulin receptor antibody (1844) and Richard Roth for the YFP and FYF IR constructs.

ACKNOWLEDGMENTS

1. Kahn, C. R., White, M. F., Shoelson, S. E., Backer, J. M., Araki, E., Cheatham, B., Csernely, P., Fell, I., Goldstein, B. J., Huerstas, P., Rothenberg, P. L., Saad, M. J. A., Siddik, K, Sun, X.-J., Wilden, P. A., Yamada, K., and Kahn, S. A. (1993) Recent Prog. Horm. Res. 48, 291–339
2. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
3. Dixon, J. E. (1995) Ann. NY Acad. Sci. 766, 16–22
4. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–481
5. Tunks, N. K., Diltz, C. D., and Fischer, E. H. (1988) J. Biol. Chem. 263, 6722–6730
6. Tunks, N. K., Diltz, C. D., and Fischer, E. H. (1988) J. Biol. Chem. 263, 6731–6737

REFERENCES

site. It should be noted that our findings may be a result of the overexpression of a catalytically inactive PTPase 1B in the presence of high levels of insulin receptor, which appears to result in a stabilized interaction between these proteins, thus favoring phosphorylation of PTPase 1B. However, we also observed insulin-stimulated PTPase 1B tyrosine phosphorylation, albeit to a lesser extent, in untransfected cells (data not shown). These results argue against the possibility that the insulin-induced PTPase 1B phosphorylation is an artifact of overexpression.

In response to insulin, PTPase 1B becomes phosphorylated at Tyr66 and/or Tyr152/153. Replacement of Tyr66 or Tyr152 and Tyr153 with phenylalanine inhibits the insulin-stimulated phosphorylation of PTPase 1B and its association with the insulin receptor. This could be due to incorrect folding or unfolding of PTPase 1B mutants generated by site-directed mutagenesis. However, substitution of Tyr66 or Tyr152 and Tyr153 does not affect the binding of PTPase 1B to the activated epidermal growth factor receptor. Similarly, replacement of Tyr152 and Tyr153 does not alter the epidermal growth factor-stimulated tyrosine phosphorylation of PTPase 1B. These results suggest that replacement of Tyr66 or Tyr152 and Tyr153 may alter the conformation of PTPase 1B in such a manner that specifically inhibits its binding to the insulin receptor, thereby preventing its phosphorylation by the activated insulin receptor tyrosine kinase. Alternatively, the altered PTPase 1B may be unavailable for phosphorylation by insulin-stimulated tyrosine kinases other than the insulin receptor, resulting in inhibition of the association of PTPase 1B with the activated insulin receptor. The mechanism by which these PTPase 1B tyrosine residues may facilitate an interaction with the IR is currently unclear.

It is interesting to note that anti-PTPase 1B antibody coprecipitated some insulin receptors from insulin-stimulated COS cells expressing YF PTPase 1B protein (Fig. 5B, lane 4). However, no tyrosine phosphorylation of the YF protein was observed in these cells in response to insulin (Fig. 4B, lane 4). This could result from a lower abundance of the tyrosine-phosphorylated YF PTPase 1B protein; the phosphotyrosine detection system used may not be sensitive enough to perceive such a low tyrosine phosphorylation level. However, it is also possible that the phosphorylation of Tyr66 of PTPase 1B may not be necessary for complex formation with the insulin receptor. Further studies are required to test these hypotheses.

We have identified PTPase 1B Tyr66 as one of the major insulin-stimulated tyrosine phosphorylation sites in vivo. The sequence surrounding Tyr66, pYINA, conforms to the consensus binding site for the Src homology 2 domain of Grb2 (50). Postreceptor insulin signaling is characterized by the tyrosine phosphorylation of two major intracellular receptor substrates, IRS-1 and Shc. Both tyrosine-phosphorylated proteins associate with Grb2, leading to the activation of Ras and the mitogen-activated protein kinase pathway (51). We have shown previously that PTPase 1B acts as a negative regulator of insulin action (21). Therefore, if PTPase 1B does associate with adapter proteins such as Grb2 following Tyr66 phosphorylation, it is possible that the enzyme may inhibit insulin signaling through the Ras/mitogen-activated protein kinase pathway by competing with IRS-1 and Shc for association with Grb2.

We have shown that the association of PTPase 1B with the insulin receptor is absolutely dependent upon receptor auto-phosphorylation. We have also demonstrated that tyrosine residues within the receptor kinase domain are essential for the interaction. Furthermore, interaction is inhibited in the presence of a phosphopeptide modeled after the kinase domain (DlpYETDpYpYRK)(56). Phosphorylation of kinase domain residues Tyr1146, Tyr1150, and Tyr1151 could create a PTPase 1B-binding site. Alternatively, the phosphorylation of these residues might be necessary for the subsequent tyrosine phosphorylation of alternate PTPase 1B-binding sites or to expose a conformation-dependent binding site.

The structural features of PTPase 1B that enable it to associate with the autophosphorylated insulin receptor are also unclear. PTPase 1B does not contain a recognizable Src homology 2 or PTB domain. It does, however, contain the sequence FKVRES, 19 residues NH2-terminal of catalytically essential Cys215 (8). FKVRES is similar to the consensus FLVRES motif of Src homology 2 domains (52, 53), and might, therefore, mediate its interaction with the insulin receptor. We cannot rule out the additional possibility that the interaction that we have observed in coprecipitation experiments is an indirect one, perhaps mediated by accessory proteins. Additional experiments will be required to answer this question.

These biochemical studies demonstrate that in response to insulin in vivo, PTPase 1B interacts with the autophosphorylated insulin receptor and becomes tyrosine-phosphorylated, possibly by the receptor kinase. Phosphorylation of PTPase 1B might increase its phosphatase activity, which could, in turn, dephosphorylate the insulin receptor and inhibit receptor tyrosine kinase activity. Phosphorylated PTPase 1B could also bind to and sequester effector molecules such as Grb2 from tyrosine-phosphorylated substrates of the insulin receptor to attenuate insulin signaling. Alternatively, PTPase 1B might be phosphorylated by an insulin-stimulated tyrosine kinase other than the insulin receptor, enabling it to bind and dephosphorylate the insulin receptor and thus inhibit insulin signaling. Additional studies are warranted to explore these possibilities.

An unresolved issue is how PTPase 1B, which is localized on the cytoplasmic face of the endoplasmic reticulum (12), might interact with the insulin receptor in the plasma membrane. As one possibility, a fraction of PTPase 1B might be released into the cytosol (22), but previous evidence suggests that this is not the case (21). As another possibility, a fraction of PTPase 1B might associate with cell membranes other than the endoplasmic reticulum, such as the plasma membrane, bringing the PTPase into a more advantageous location for interaction with its endogenous substrates. As a final possibility, activated endosome-associated receptors could be more relevant in insulin signaling than receptors at the plasma membrane (54, 55). Endosome-associated, activated receptors could be rapidly brought into close proximity with PTPase 1B at the endoplasmic reticulum. Subsequent dephosphorylation could inactivate the receptors, preventing further kinase activity. An intriguing aspect of future work will be to determine the precise nature of the interaction between PTPase 1B and the insulin receptor.
