Tyrosine Dephosphorylation and Deactivation of Insulin Receptor Substrate-1 by Protein-tyrosine Phosphatase 1B

POSSIBLE FACILITATION BY THE FORMATION OF A TERNARY COMPLEX WITH THE GRB2 ADAPTOR PROTEIN*

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Regulation of the steady-state tyrosine phosphorylation of the insulin receptor and its postreceptor substrates are essential determinants of insulin signal transduction. However, little is known regarding the molecular interactions that influence the balance of these processes, especially the phosphorylation state of postinsulin receptor substrates, such as insulin receptor substrate-1 (IRS-1). The specific activity of four candidate protein-tyrosine phosphatases (protein-tyrosine phosphatase 1B (PTP1B), SH2 domain-containing PTPase-2 (SHP-2), leukocyte common antigen-related (LAR), and leukocyte antigen-related phosphatase) (LRP) toward IRS-1 dephosphorylation was studied using recombinant proteins *in vitro*. PTP1B exhibited the highest specific activity (percentage dephosphorylated per μg per min), and the enzyme activities varied over a range of 5.5×10^3. When evaluated as a ratio of activity *versus* IRS-1 to that *versus* p-nitrophenyl phosphate, PTP1B remained significantly more active by 3.1–293-fold, respectively. Overlay blots with recombinant Src homology 2 domains of IRS-1 adaptor proteins showed that the loss of IRS-1 binding of Crk, GRB2, SHP-2, and the p85 subunit of phosphatidylinositol 3′-kinase paralleled the rate of overall IRS-1 dephosphorylation. Further studies revealed that the adaptor protein GRB2 strongly promoted the formation of a stable protein complex between tyrosine-phosphorylated IRS-1 and catalytically inactive PTP1B, increasing their co-immunoprecipitation from an equimolar solution by 13.5 ± 3.3-fold ($n = 7$; $p < 0.01$). Inclusion of GRB2 in a reaction mixture of IRS-1 and active PTP1B also increased the overall rate of IRS-1 tyrosine dephosphorylation by 2.7–3.9-fold ($p < 0.01$). These results provide new insight into novel molecular interactions involving PTP1B and GRB2 that may influence the steady-state capacity of IRS-1 to function as a phosphotyrosine scaffold and possibly affect the balance of postreceptor insulin signaling.

Cellular protein-tyrosine phosphatases (PTPases)[1] play a crucial role in maintaining the steady-state phosphotyrosine content of proteins in the insulin action pathway (1, 2). Much of the available evidence characterizing the role of PTPases in insulin signaling has focused on the regulation of the activation state of the insulin receptor itself, where several PTPases, in particular the intracellular enzyme PTP1B and the transmembrane homologs LAR and LRP (RPTP-α), have been postulated to have a regulatory function (3–6). However, the identity of PTPase(s) that regulate the tyrosine phosphorylation state of substrates of the insulin receptor kinase, such as IRS-1, has not been determined.

IRS-1 is phosphorylated on multiple tyrosine residues by the activated insulin receptor kinase and serves as a docking site or scaffold for a series of adaptor proteins that possess Src homology 2 (SH2) domains (7). These proteins bind to specific phosphotyrosine sites on IRS-1, become activated, and transmit the downstream signal from the insulin receptor to various metabolic and mitogenic pathways in the cell. Among these proteins are the p85 subunit of phosphatidylinositol 3-kinase, the intracellular PTPase SHP-2, and the adaptor proteins Nck, Crk, and GRB2, which also contain additional domains for interaction with other signaling molecules (7, 8). Since the function of IRS-1 in signal transduction is determined by the steady-state phosphorylation of individual tyrosine residues involved in binding to docking proteins, a major mechanism for the regulation of postreceptor signaling involves the overall dephosphorylation of IRS-1 by cellular PTPases as well as by the dephosphorylation of specific tyrosine phosphorylation sites, which would more closely regulate individual signaling pathways.

In the present study, we investigated the potential role of four candidate PTPases that are widely expressed in insulin-sensitive tissues in the regulation of the tyrosine phosphorylation state of IRS-1, including the intracellular enzymes PTP1B and SHP-2 and the transmembrane enzymes LAR and LRP. Recombinant IRS-1, expressed in Sf9 cells and phosphorylated by purified insulin receptors, was used as a substrate *in vitro* for dephosphorylation by recombinant PTPases from a bacterial expression system. The PTPases were tested for their relative activities against the overall tyrosine dephosphorylation of IRS-1. In addition, the dephosphorylation of specific phosphotyrosine adaptor sites by individual PTPases was evaluated by studying their effect on the binding of purified recombinant SH2 domains of the docking proteins SHP-2, p85, Crk, and GRB2. Since PTP1B has recently been shown to interact

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[1] The abbreviations used are: PTPase, protein-tyrosine phosphatase; PTP1B, protein-tyrosine phosphatase 1B; BSA, bovine serum albumin; GST, glutathione S-transferase; IRS-1, insulin receptor substrate-1; LAR, leukocyte common antigen-related; LRP, leukocyte antigen-related phosphatase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; pNPP, p-nitrophenyl phosphate; RPTP-α, receptor-type PTPase α; SH2, Src homology 2.

directly with the GRB2 adaptor protein (9), in further studies we also examined the effect of GRB2 on protein complex formation between IRS-1 and PTP1B and the potential role of this protein complex in the rate and specificity of the IRS-1 tyrosine dephosphorylation catalyzed by PTP1B.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified polyclonal antibodies to PTP1B (N-terminal amino acids 1–158) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibody to IRS-1 (JD 159) was generated to the baculovirus-expressed recombinant protein. Antibodies to phosphotyrosine were prepared as described by Kamps (10).

Isolation of Recombinant Proteins—Rat IRS-1 was produced in the baculovirus expression system and purified by fast protein liquid chromatography gel permeation chromatography (11). Recombinant PTPases were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins in the pGEX-KG vector system and cleaved with thrombin (12). The PTP1B and SHP-2 enzymes were full-length, and the LAR and LRP enzymes contained the entire tandem cytoplasmic domains. GST fusion proteins containing the SH2 domains of Crk, SHP-2 (tandem domains), GRB2, and p85 (N-terminal) were also expressed in *E. coli* and purified by glutathione-agarose affinity chromatography. The GST fusion protein containing GRB2 was expressed in the pGEX-3X vector (Amersham Pharmacia Biotech) and cleaved with factor Xa protease. Protein was measured by the method of Bradford (13), and electrophoretic protein migration was calibrated with prestained molecular size standards from Bio-Rad.

Tyrosine Phosphorylation and Dephosphorylation of IRS-1—Purified, recombinant IRS-1 was phosphorylated *in vitro* using insulin receptor purified by wheat germ agglutinin-lectin chromatography from Chinese hamster ovary cells transfected with the human insulin receptor cDNA as described (11, 14), and the reaction was quenched by the addition of excess EDTA (15). Purified insulin receptors were activated by a 15-min preincubation with insulin in phosphorylation buffer containing ATP, followed by the addition of recombinant IRS-1 for an additional 6 h at room temperature. Phosphorylated IRS-1 (12.5 μg/ml) was incubated with varying amounts of each recombinant PTPase (typically, 0.05 μg/ml PTP1B, 1 μg/ml SHP-2, 15 μg/ml LAR, or 30 μg/ml LRP). Aliquots were removed at 0, 2, 5, 10, 30, and 60 min, and the reaction was quenched by the addition of Laemmli sample buffer for SDS gel electrophoresis.

Immunoblotting Studies and Adaptor Protein Overlay Blotting—Samples were separated by electrophoresis on 7.5% polyacrylamide gels containing SDS and then transferred to nitrocellulose as described previously (3). Following transfer, membranes were blocked with 2% (w/v) bovine serum albumin in TBST buffer (150 mM NaCl, 0.5% (v/v) Tween 20, 0.2% (w/v) sodium azide in 250 mM Tris-HCl, pH 7.5). For anti-phosphotyrosine blots, the membranes were incubated with monoclonal PY-20 anti-phosphotyrosine antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by rabbit anti-mouse IgG (Sigma) or with polyclonal anti-phosphotyrosine antibodies generated in our laboratory (10). Other determinations were performed with immunoblotting with antibodies to IRS-1 or to GRB2 (Santa Cruz Biotechnology). For the SH2-GST fusion protein overlay ("adaptor protein") blots, membranes were incubated with the purified SH2 domain-GST fusion proteins diluted in blocking buffer, followed by anti-GST antibodies (Santa Cruz Biotechnology). In each case, signal was detected by binding of ^{125}I-protein A to the membranes, and the intensity was quantitated on a PhosphorImager using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

PTPase Assays—Activity of the recombinant phosphatases toward simple substrates was determined using *p*-nitrophenyl phosphate (pNPP) as well as a synthetic monophosphotyrosyl-containing peptide (Upstate Biotechnology, Inc., Lake Placid, NY). Hydrolysis of pNPP was performed at 37 °C for 10–30 min, the reactions were quenched by the addition of 1 M NaOH, and the absorbance at 410 nm was determined. Hydrolysis of the phosphotyrosyl peptide was performed for 2–20 min at 25 °C. Phosphate release was determined by a modified malachite green assay, using materials provided by the manufacturer. For each substrate, specific activity (pmol of product/min/μg of protein) was determined from the initial linear reaction period.

Protein Complex Formation—In order to demonstrate protein association between IRS-1, GRB2, and PTP1B by co-immunoprecipitation, certain experimental obstacles were overcome. It was impossible to visualize PTP1B by immunoblotting following immunoprecipitation, since the antibody heavy chain reacting with the ^{125}I-protein A reagent co-migrated at the position of PTP1B (~50 kDa). This artifact could not be resolved on nonreducing gels or gradient gels. Also, the background of nonspecific adsorption of IRS-1 in the immune complex mixture was virtually eliminated by preincubation of the protein A beads in PBS containing 1% (w/v) BSA and including this as a blocking solution during the protein incubations and immunoprecipitation mixtures with the addition of 0.1% (v/v) Triton X-100. A typical experiment contained 2 μg of tyrosine-phosphorylated IRS-1, 2.6 μg of PTP1B, and 1.8 μg of GRB2 as a fusion protein with glutathione *S*-transferase, in a volume of 1.2 ml of PBS containing 1% (w/v) BSA. Where indicated, the enzymatic activity of PTP1B was abolished by treatment with 20 mM *N*-ethylmaleimide at room temperature for 2 h and verified by a complete lack of catalytic activity in the hydrolysis of *p*-nitrophenyl phosphate. After incubation for 1 h at room temperature, Triton X-100 was added to 0.1% (v/v), 40 μl of a 50% (v/v) suspension of Trisacryl protein A beads were added that had been previously pretreated with 1% (w/v) BSA in PBS, and the sample was incubated on a rotating wheel for 1.5 h at 4 °C. The beads from the preclearing procedure were removed by centrifugation, and appropriate antibodies were added to the supernatants for immunoprecipitation, followed by incubation for 2 h at 4 °C. Following the addition of 40 μl of a 50% (v/v) suspension of pretreated Trisacryl protein A beads, the sample was incubated again with rotation for 1.5 h at 4 °C. The beads were washed twice with 1 ml of ice-cold 0.1% (v/v) Triton X-100 in PBS and resuspended in Laemmli gel sample buffer and boiled prior to electrophoresis, followed by transfer to nitrocellulose filters and immunoblotting with anti-IRS-1 antibodies.

RESULTS

Specific Activity of Recombinant PTPases toward IRS-1—The relative activity of four candidate PTPases toward the overall tyrosine dephosphorylation of IRS-1 *in vitro* was evaluated using recombinant IRS-1 purified from an Sf9 cell expression system that was phosphorylated on tyrosine residues with recombinant insulin receptors. IRS-1 phosphotyrosine content was monitored by immunoblotting with anti-phosphotyrosine antibodies. In control experiments with no added PTPase, there was negligible change in the phosphotyrosine content of IRS-1 or IRS-1 protein content in the reaction mixture when incubated in buffer alone for the duration of the assay, indicating that the loss of the IRS-1 phosphotyrosine signal was not the result of contaminating enzyme activities (Figs. 1 and 2).

Each of the recombinant PTPases dephosphorylated IRS-1 in a time-dependent fashion over the time course of the assay (Figs. 1 and 2). However, the four enzymes differed significantly in their specific activity toward IRS-1 (percentage of tyrosine dephosphorylation/min/μg of enzyme protein). PTP1B exhibited 2500–5500 times more activity than LRP or LAR, respectively, and 12 times more activity than SHP-2 (Fig. 2). To correct for potential differences in enzyme activity toward simple substrates, the phosphatase activity of each PTPase toward IRS-1 was also compared with its activity toward a synthetic phosphotyrosine-containing peptide and toward pNPP. The ratio of IRS-1-specific activity *versus* the activity toward the simple substrates for each of the PTPases tested is shown in Fig. 3. Compared with LAR and LRP, PTP1B was significantly more active *versus* either simple substrate, with activity ratios on the order of 65–293 times higher. The ratio of PTP1B activity toward IRS-1 *versus* the phosphopeptide substrate was similar to the results with SHP-2; however, PTP1B was still 3.1 times more active than SHP-2 when the IRS-1 dephosphorylating activity was compared with the activity *versus* pNPP as substrate.

Dephosphorylation of Specific Phosphotyrosine Sites on IRS-1 by PTPases—In order to evaluate whether there were specific regional differences in the activity of the candidate PTPases on the dephosphorylation of IRS-1, we monitored for changes in the ability of a series of specific SH2 domain-containing adaptor proteins to bind to the IRS-1 following dephosphorylation by IRS-1 using an overlay blot technique. In this method, samples of IRS-1 following dephosphorylation by the
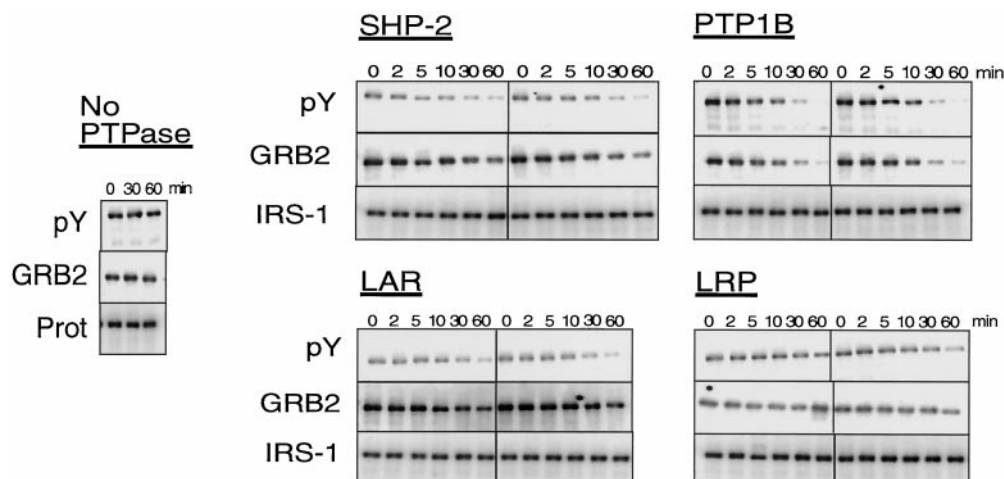
F𝕚𝔾. 1. **Time course of IRS-1 tyrosine dephosphorylation by recombinant PTPases *in vitro* and loss of binding of the SH2 domain of the adaptor protein GRB2.** IRS-1 was tyrosine-phosphorylated *in vitro* by partially purified insulin receptors and incubated with candidate recombinant PTPases (SHP-2, PTP1B, LAR, and LRP) for the indicated amount of time. The phosphor image shows a composite of the phosphotyrosine content of IRS-1 in the sample determined by immunoblotting (*pY*), a representative adaptor protein "overlay" blot measuring the remaining binding capacity for a GRB2 SH2 domain-GST fusion protein (*GRB2*), and the amount of IRS-1 protein remaining in the sample (*IRS-1*) as described under "Experimental Procedures." Results from a sample incubated without added PTPase enzyme are shown on the *left* (*No PTPase*).

candidate PTPases were subjected to electrophoresis and transferred to nitrocellulose filters as for the immunoblotting studies noted above. Adaptor protein binding was then measured by incubating individual GST fusion proteins containing recombinant SH2 domains with the membranes, and the signal was visualized by incubation with anti-GST antibodies and ^{125}I-protein A followed by autoradiography. The proteins tested in the overlay blot method included the SH2 domains for the p85 subunit of phosphatidylinositol 3-kinase, SHP-2, GRB2, and Crk.

In control experiments, as for the anti-phosphotyrosine immunoblotting, there was no significant change in SH2 domain-containing fusion protein binding in the samples that were not subjected to dephosphorylation with the candidate PTPases (Figs. 1 and 4). Also, anti-IRS-1 immunoblotting on samples for each time course of dephosphorylation did not show any degradation or loss of IRS-1 protein during the incubations (Fig. 1), confirming that loss of IRS-1 phosphotyrosine content was due to dephosphorylation and not proteolysis. The overlay blots with recombinant domains of IRS-1 adaptor proteins showed that the loss of binding of the SH2 domains of Crk, GRB2, SHP-2, and the p85 subunit of phosphatidylinositol 3-kinase paralleled the rate of overall *in vitro* dephosphorylation of IRS-1 by each of the PTPases, without evidence for regional or site specificity in this process (Fig. 4).

Binding of PTP1B to the GRB2 Adaptor Protein—Previous work reported by Liu *et al.* (9) demonstrated that PTP1B formed a protein complex through the proline-rich sequences of its C terminus with a number of SH3 domain-containing adaptor proteins, including GRB2, a well known docking protein for IRS-1 (16, 17). Since our studies showed that PTP1B was relatively more active in the tyrosine dephosphorylation of IRS-1, we tested whether GRB2 could mediate an association between PTP1B and IRS-1 and potentially have functional effects on the rate or specificity of IRS-1 dephosphorylation by PTP1B.

Initially, we reconfirmed the finding that PTP1B and GRB2 interacted *in vitro*. PTP1B (43 nM) was incubated with GRB2 (either as the native full-length protein (56 nM) or as a GST fusion protein (28 nM) in PBS containing 1% (w/v) BSA to block nonspecific protein adsorption. Immunoprecipitation with anti-PTP1B antibodies followed by immunoblotting with anti-GRB2 antibodies revealed a stable interaction between these two

proteins (Fig. 5). Quantitation of the protein association resulting from immunoprecipitation under these incubation and washing conditions revealed that between 2.9 and 7.1% of the GRB2 in the initial reaction mixture was co-isolated with PTP1B.

GRB2 Mediates Complex Formation between IRS-1 and PTP1B—Since GRB2 has been shown in a number of previous studies to associate with tyrosine-phosphorylated IRS-1, we then tested the hypothesis that this adaptor protein could mediate the formation of a ternary protein complex between IRS-1 and PTP1B. In these studies, the background of nonspecific adsorption of IRS-1 in the immunoprecipitation samples was minimized by elaborate blocking methods indicated above. In addition, to prevent the tyrosine dephosphorylation of IRS-1 during the studies on protein complex formation, these experiments employed PTP1B that had its catalytic activity inactivated by incubation with the sulfhydryl alkylating agent *N*-ethylmaleimide (see "Experimental Procedures").

Under the relatively stringent incubation and washing conditions, significant protein-protein interactions between tyrosine-phosphorylated IRS-1 and PTP1B were strongly potentiated by co-incubation with the GRB2 adaptor protein (Fig. 6). This interaction was entirely dependent on the presence of GRB2 as well as the tyrosine phosphorylation of IRS-1, since the inclusion of unphosphorylated IRS-1 was unable to mediate this interaction (Fig. 6, *right*). Quantitation of the abundance of complexed proteins in the immunoprecipitates indicated that the presence of GRB2 increased the co-immunoprecipitation of IRS-1 with PTP1B from the equimolar incubation by 13.5 ± 3.3-fold ($n = 7$; $p < 0.01$).

GRB2-mediated Protein Association between PTP1B and IRS-1 Leads to Enhanced IRS-1 Dephosphorylation—The inclusion of GRB2 in a dephosphorylation reaction between IRS-1 and enzymatically active PTP1B increased the initial rate of IRS-1 tyrosine dephosphorylation by an average of 2.7–3.9-fold ($p < 0.01$; Fig. 7). This effect was apparent from studies using either anti-phosphotyrosine immunoblotting of IRS-1 following SDS-gel electrophoresis or by a slot-blot technique using aliquots of the dephosphorylation reaction mixture over a similar time course.

Assessment of SH2 Adaptor Protein Binding following Dephosphorylation of IRS-1 by PTP1B in the Presence of GRB2—We hypothesized that because of the formation of a
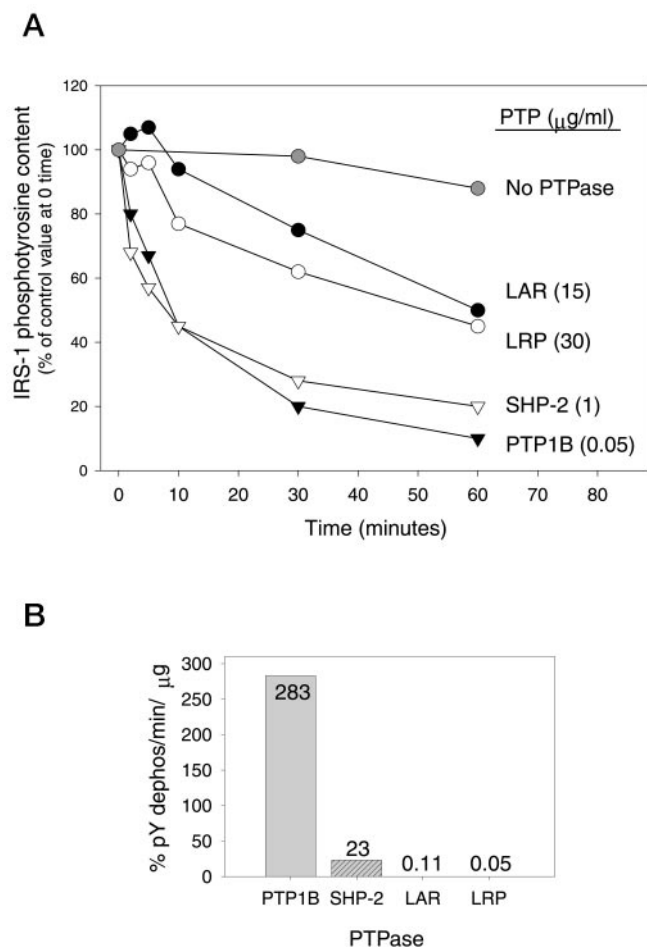
Dephosphorylation of IRS-1 by PTP1B

A

B

FIG. 2. **Quantitation of the time course of IRS-1 tyrosine dephosphorylation by recombinant PTPase enzymes.** *A*, results tabulated from three replicate experiments performed as shown in Fig. 1 and presented for the control sample with no added enzyme (*No PTPase*) as well as results with LAR, LRP, SHP-2, and PTP1B. The amount of recombinant enzyme added to each incubation is indicated in *parentheses*, ranging from 0.05 μg/ml for PTP1B to 30 μg/ml for LRP. Note that the amount of PTP1B used in these experiments was 20, 600, and 300 times less than that employed for SHP-2, LRP, and LAR to elicit the degree of IRS-1 dephosphorylation shown. *B*, specific activities for the tyrosine dephosphorylation of IRS-1 by recombinant PTPases *in vitro*. The initial rate of the dephosphorylation reaction was estimated from data shown in *A*, and the specific activity was determined per amount of enzyme protein added to the reaction mixture.
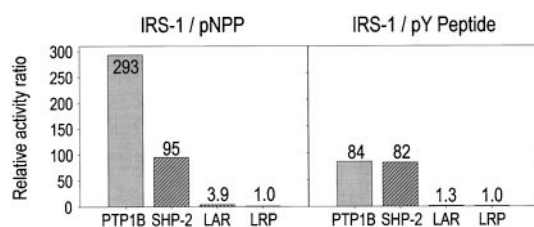

FIG. 3. **Activity ratios of the *in vitro* tyrosine dephosphorylation of IRS-1 by recombinant PTPases *versus* hydrolysis of pNPP and a phosphotyrosine-containing peptide (*pY*).** The initial rate of the phosphatase reaction with pNPP or the phosphotyrosine-containing peptide was determined as described under "Experimental Procedures." The ratios of the specific PTPase activity for the enzyme proteins as shown in Fig. 2 to the activity in the pNPP and the phosphotyrosine-containing peptide assay are shown in the *left* and *right panels*, respectively.

ternary protein complex, inclusion of GRB2 in the IRS-1 dephosphorylation reaction might alter the specificity of PTP1B toward the rate of hydrolysis of certain phosphotyrosine residues. This possibility was evaluated by performing the adaptor
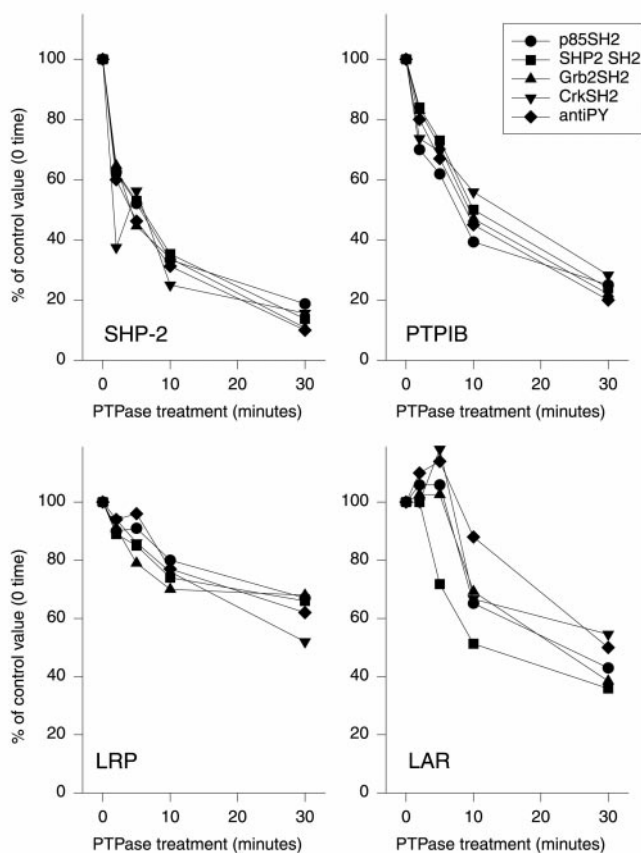

FIG. 4. **Binding of SH2 domain-containing adaptor proteins to IRS-1 following dephosphorylation *in vitro* by recombinant PTPases.** Representative primary data and methods used to determine SH2 domain binding are shown in Fig. 1. IRS-1 was dephosphorylated *in vitro* with recombinant PTPases, subjected to SDS gel electrophoresis, and transferred to nitrocellulose filters. IRS-1 phosphotyrosine content was determined by anti-phosphotyrosine immunoblotting (*antipY*), and adaptor protein SH2 domain binding (p85, SHP-2, GRB2, and Crk) to dephosphorylated IRS-1 was evaluated by the overlay technique using GST fusion proteins followed by anti-GST antibodies and ^{125}I-labeled protein A. The amount of radioactivity remaining on the blot was determined by PhosphorImager analysis. Results from control samples incubated without added PTPase enzyme demonstrated negligible changes in the binding of the adaptor proteins over the time course of the incubation (not shown). The mean of 3–5 determinations is shown for each condition tested.

protein-blot overlay technique following gel electrophoresis and Western protein transfer or after the preparation of a slot-blot of the time course of dephosphorylated IRS-1 samples. While the presence of GRB2 significantly increased the overall dephosphorylation of IRS-1 in these studies, these techniques did not detect an change in the relative ability of PTP1B to dephosphorylate the phosphotyrosine residues on IRS-1 that mediate the binding of the specific SH2 adaptor proteins tested, including GRB2, SHP-2, p85, and Crk under the *in vitro* conditions employed (data not shown).

DISCUSSION

Recent studies have provided strong evidence supporting a key role for protein-tyrosine phosphatases in the steady-state regulation of reversible tyrosine phosphorylation events in the insulin action pathway (1, 2, 4). The available data have indicated that a number of candidate PTPases, in particular the intracellular enzyme PTP1B and the transmembrane homologs LAR and LRP (RPTP-α), have important regulatory effects on insulin signaling. In most studies, these PTPases have been shown to exert a negative regulation on insulin action that occurs proximally in the signal transduction pathway, affecting
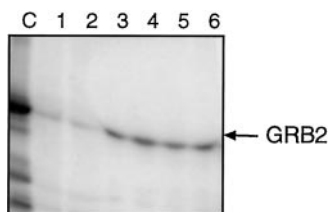
FIG. 5. **Co-immunoprecipitation of GRB2 with PTP1B following incubation of the recombinant proteins *in vitro*.** As described under "Experimental Procedures," PTP1B (inactivated by incubation with *N*-ethylmaleimide) and full-length GRB2 (cleaved from a fusion construct with GST) were incubated for 1 h at room temperature. For quantitation of the amount of GRB2 immunocomplexed with PTP1B, an aliquot of the incubation mixture was incubated with anti-PTP1B antibodies and adsorbed onto Trisacryl-protein A beads prior to separation on an SDS gel containing 7.5% (w/v) polyacrylamide, transfer to nitrocellulose, and Western blot analysis performed using polyclonal anti-GRB2 antibodies. The content of the *lanes* on the gel is as follows. *Lane C*, recombinant GRB2 as position marker; *lanes 1* and *2*, aliquot immunoprecipitated with anti-PTP1B antibodies; *lanes 3* and *4*, aliquot of the initial incubation mixture prior to immunoprecipitation to quantitate the starting amount of GRB2; *lanes 5* and *6*, aliquot of the supernatant following immunoprecipitation, representing the amount of GRB2 remaining after the removal of PTP1B. The migration of the GRB2 at ~25 kDa is shown.
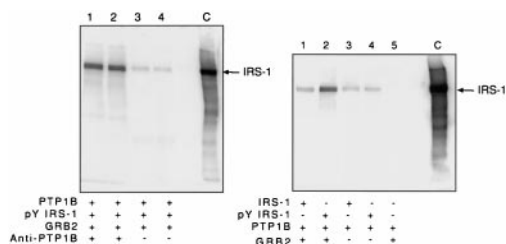

FIG. 6. **Formation of a ternary complex involving catalytically inactive PTP1B, GRB2, and tyrosine-phosphorylated IRS-1 *in vitro*.** Incubations of recombinant proteins were performed as described under "Experimental Procedures" using incubation mixtures containing the indicated reaction components. Following the immunoprecipitation of PTP1B with specific antibodies, the samples were subjected to electrophoresis on SDS gels containing 7.5% (w/v) polyacrylamide and immunoblot analysis with antibodies to IRS-1. The *left panel* shows a representative gel indicating the low background of nonspecific IRS-1 adsorption under the immunoprecipitation and washing conditions employed (*lanes 3* and *4*) and the co-immunoprecipitation of IRS-1 with anti-PTP1B antibodies (*lanes 1* and *2*). The *right panel* shows a typical experiment indicating the dependence of IRS-1 co-immunoprecipitation with PTP1B on the presence of GRB2 (*lanes 1* and *2 versus lanes 3* and *4*) and the prior tyrosine phosphorylation of IRS-1 (*lane 2 versus lane 1*). *Lane C*, recombinant IRS-1 as a position marker. The migration of phosphorylated IRS-1 at ~180 kDa is shown.

FIG. 7. *A*, time course of tyrosine dephosphorylation of IRS-1 by PTP1B *in vitro* in the presence and absence of GRB2. Tyrosine-phosphorylated IRS-1 was added to a reaction mixture containing recombinant PTP1B without GRB2 (*right panel*) and with GRB2 (*left panel*) and incubated for the indicated period of time. Similar to results shown in Fig. 1 and described under "Experimental Procedures," the phosphor image shows the phosphotyrosine content of IRS-1 in the sample determined by immunoblotting. *B*, quantitation of the initial time course of IRS-1 tyrosine dephosphorylation by PTP1B in the presence and absence of GRB2. Results were tabulated from replicate experiments performed as shown in Fig. 8 and presented for the initial 5 min of the incubation. The phosphotyrosine content of IRS-1 relative to the initial sample was determined by PhosphorImager analysis of the immunoblots.

from IRS-1 adaptor proteins to the dephosphorylated IRS-1.

Among four candidate PTPases, PTP1B, SHP-2, LAR, and LRP, the intracellular enzymes PTP1B and (to a lesser extent) SHP-2 were more active toward the overall *in vitro* tyrosine dephosphorylation of IRS-1 than the cytoplasmic domains of the transmembrane enzymes LAR and LRP. The relative activities of these candidate enzymes were maintained when assessed either by actual specific activity or when enzyme activity was related to the dephosphorylation rate against pNPP or a representative phosphotyrosine-containing peptide substrate. The loss of binding of the SH2 domains from the p85 subunit of phosphatidylinositol 3-kinase, Crk, GRB2, and SHP-2 paralleled the overall rate of loss of IRS-1 dephosphorylation for each of the four PTPases examined. Thus, the general order of activity in the dephosphorylation of IRS-1 of PTP1B > SHP-2 ≫ LAR, LRP also followed the specific activity of these enzymes toward each of the SH2 adaptor binding sites, and regional specificity for any of the recombinant enzymes was not detected under the *in vitro* conditions and methods employed.

These findings are interesting in comparison with our previous data on the *in vitro* activity of recombinant PTPases toward the insulin receptor. Although PTP1B, LRP, and LAR were each active against the overall tyrosine dephosphorylation of the insulin receptor, we found that the cytoplasmic domain of LAR had a 3–4-fold higher activity toward the dephosphorylation and deactivation of the insulin receptor kinase domain *in vitro* when compared with PTP1B or LRP (15, 18). In combina-

the activation state of the insulin receptor itself. Experimental systems using intact cells have demonstrated parallel changes in the tyrosine phosphorylation state of high molecular mass substrates of the receptor kinase, including IRS-1. Since these PTPases reduce or abolish the activity of the insulin receptor kinase, it has been difficult to discern whether specific PTPases also independently influence the phosphotyrosine content of IRS-1 or whether the observed changes in IRS-1 phosphorylation simply occur secondarily, reflecting the inactivation of the cellular insulin receptor.

In order to provide initial data to help identify the PTPase(s) that regulate the tyrosine phosphorylation state of IRS-1, in the present work we examined the activity of four candidate PTPases (PTP1B, SHP-2, LAR, and LRP) toward the dephosphorylation of IRS-1 *in vitro*. In addition to assessing the overall rate of loss of IRS-1 phosphotyrosine content during the incubation with PTPases, we also evaluated the regional specificity of IRS-1 dephosphorylation by an overlay blot technique that measures the loss of binding of recombinant SH2 domains
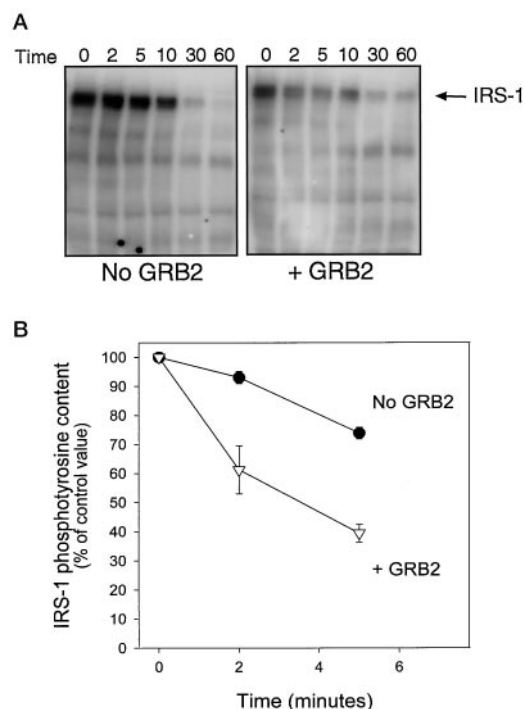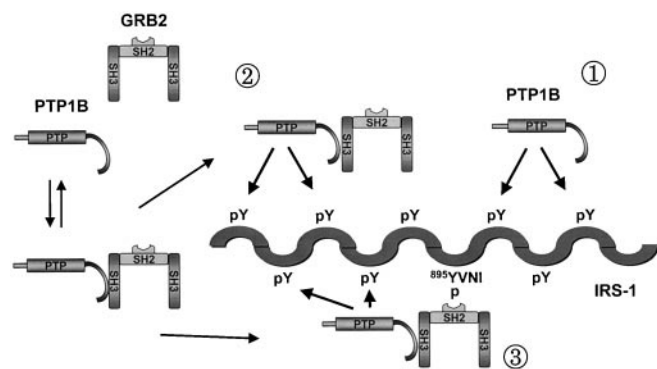
FIG. 8. **Scheme of potential mechanisms that may influence the interaction between GRB2 and PTP1B with subsequent effects on IRS-1 dephosphorylation.** As discussed, the binding of GRB2 to both PTP1B and to IRS-1 and the formation of a ternary complex among them offers a number of contact sites that may mediate regulatory interactions between these signaling proteins. These possible interactions include direct dephosphorylation of IRS-1 by PTP1B without complex formation involving GRB2 (*1*); dephosphorylation of IRS-1 by PTP1B complexed with GRB2, which may exhibit altered reactivity or site-specificity *in vivo* (*2*); or dephosphorylation of IRS-1 in a ternary complex involving PTP1B bound to GRB2 that is docked to the phosphotyrosine site on IRS-1 at residue 895 (*3*). These several models are testable in further experiments using intact cell systems.

tion with the present work, these findings suggest that various aspects of the regulation of insulin signaling at the receptor and postreceptor levels are likely to be under the control of different PTPases.

Increasingly, novel protein complexes have been identified and characterized to play an important regulatory role in signal transduction in systems involving kinases and phosphatases (19). PTP1B has recently been shown to interact directly with the GRB2 adaptor protein (9). We confirmed the interaction between PTP1B and GRB2, demonstrating a significant co-immunoprecipitation of these two proteins, involving up to 7% of the GRB2 present, under relatively stringent conditions that were necessary to avoid nonspecific adsorption of IRS-1 in the immunoprecipitation steps in subsequent experiments. We further demonstrated that GRB2 can mediate the stable formation of a ternary complex between itself, IRS-1, and PTP1B, increasing the amount of IRS-1 co-immunoprecipitated with antibodies to PTP1B by an average of 13.5-fold. The potential functional role of this ternary protein complex was further established by demonstrating that the initial rate of the overall tyrosine dephosphorylation of IRS-1 by PTP1B was increased by an average of up to 3.9-fold in the presence of GRB2 *in vitro*.

PTP1B was the first PTPase to be shown to have a major effect on the regulation of insulin signaling. Initially, Cicirelli *et al.* (20) and Tonks *et al.* (21) showed that microinjection of the catalytic core of a truncated PTP1B enzyme into *Xenopus* oocytes blocked insulin-stimulated S6 kinase activation and retarded insulin-induced oocyte maturation. Subsequently, additional evidence that PTP1B plays a role as a negative regulator of insulin action has been provided by studies in transfected cells overexpressing the active PTPase (22, 23) and in our laboratory using cells loaded with inhibitory antibodies to block the catalytic activity of PTP1B *in situ* (3). Catalytically inactive site-directed mutants of PTP1B have also been shown to physically associate with the activated insulin receptor in intact cells and to have dominant negative effects on insulin signaling *in situ* (24–26). A global PTP1B knock-out mouse model recently provided strong evidence for an essential physiologic role for PTP1B in the negative regulation of insulin action (4). Studies of insulin and glucose dynamics revealed a heightened level of insulin sensitivity with enhanced insulin receptor autophosphorylation and insulin-stimulated IRS-1

phosphorylation in skeletal muscle and liver tissue.

GRB2, a relatively abundant cytosolic protein, is a well characterized binding partner for IRS-1 that interacts with a specific phosphorylated residue at Tyr-895 (7, 11). In particular, the association of GRB2 with tyrosine-phosphorylated IRS-1 is a major mechanism linking insulin signaling to the Ras pathway (27 ,28) and downstream effects in the insulin action pathway (29). The interaction between GRB2 and PTP1B and the functional effects on IRS-1 dephosphorylation described in the present work have implications for the regulation of specific actions of insulin distal to IRS-1 in intact cells and tissues.

In addition to its binding to PTP1B, GRB2 has also been shown to associate with other PTPases in a manner that may influence the regulation of these enzymes or mediate their networking with other signaling pathways. For example, GRB2 binds by a multisite interaction with tyrosine-phosphorylated LRP (RPTP-α), and this interaction can also mediate the activation by GRB2 of signaling through the Sos pathway (30, 31). In addition, GRB2 associates with SH2 domain-containing PTPases in a variety of cell types (32–34) as well as with PTP-PEST (35). These data suggest that GRB2 may have an important cellular role in the regulation of PTPase activities as well as their association with other signaling proteins with potentially important effects on substrate localization or complex formation that influences the rate of dephosphorylation of various PTPase substrates.

In conclusion, these data suggest that PTP1B may play an important role in regulating the postreceptor insulin signaling pathway, by modulating the steady-state balance of the tyrosine phosphorylation of IRS-1 and the docking of SH2 domain-containing adaptor proteins to IRS-1. In intact cells, additional regulatory mechanisms may have an important influence on the molecular interactions between the insulin receptor substrate proteins and cellular PTPases. Compartmentalization of several proteins in the insulin signaling cascade has recently been demonstrated, and dynamic changes can occur with insulin stimulation that may influence protein interactions, including those involving PTPases (36–38). Nevertheless, the present studies provide a framework for further investigation into the role of PTP1B in the regulation of the *in vivo* tyrosine phosphorylation state of IRS-1 in intact cells, to determine how variations in the activity of PTP1B as well as its complex formation with IRS-1 and GRB2 may affect specific post-IRS-1 signals in the insulin action pathway (Fig. 8).

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