Communication

Protein-tyrosine Phosphatase 1D Modulates Its Own State of Tyrosine Phosphorylation*

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The insulin receptor-mediated signal transduction pathway involves insulin receptor substrate 1 and a variety of proteins containing Src homology-2 (SH2) domains, such as phosphatidylinositol 3-kinase, Grb2, and protein-tyrosine phosphatase 1D (PTP1D). Upon insulin stimulation of baby hamster kidney cells overexpressing the IR, the catalytically inactive mutant of PTP1D, C463A, becomes tyrosine-phosphorylated and coprecipitates with Grb2. Tyrosine phosphorylation of this mutant is significantly reduced when wild type PTP1D is coexpressed. Substitution of tyrosine residues 546 and 584 with phenylalanine abrogates tyrosine phosphorylation of the catalytically inactive mutant and abolishes its interaction with Grb2.

We show here that the catalytically inactive mutant of PTP1D, C463A (32), becomes phosphorylated on tyrosine and coprecipitates with Grb2 after insulin treatment in BHK-IR cells. Furthermore, we demonstrate that tyrosines at positions 546 and 584 are responsible for this phosphorylation and that substitution of these residues with phenylalanine abolishes Grb2 association. Coexpression of wild type PTP1D and the catalytically inactive mutant leads to significantly reduced tyrosine phosphorylation of the latter, suggesting that PTP1D may modulate its own tyrosine phosphorylation state. Our findings provide new insights into mechanisms of PTP1D action within the insulin receptor signal.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasmid Constructs—Protein A- and thal-tahione-Sepharose were purchased from Pharmacia Biotech Inc. All other reagents were obtained from Sigma.

Antibodies used were affinity-purified rabbit polyclonal anti-PTP1D antibody raised against a C-terminal PTP1D peptide (amino acids 546–584) (31), mouse monoclonal anti-PTP1D antibody (Transduction Laboratories), mouse monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.), mouse monoclonal anti-Grb2 antibody (Upstate Biotechnology, Inc.), and mouse monoclonal anti-hemagglutinin antibody 12CA5 (Boehringer Mannheim). As secondary antibodies, goat anti-mouse or anti-rabbit conjugates (Bio-Rad) were used. For immunoblot detection, the ECL system from Amersham was utilized. Stripping and reprobing of blots were performed according to the manufacturers’ recommendations.

The construction of cytomegalovirus promoter-based expression plasmids for PTP1D has been described previously (11, 33).

Cell Culture, Transient Expression, and Lysis Procedure—BHK-IR cells (34) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 1 × 10⁶ cells were seeded per six-well dish and transfected 24 h later with 4 μg of DNA (cotransfections: 2 μg of each plasmid) using the calcium precipitation method (35). After 16-h starvation (Dulbecco's modified Eagle's medium with 0.5% fetal calf serum), cells were stimulated with insulin (5 × 10⁻⁹ M) for different time periods (see figures), lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin. After 10 min, cells were harvested and centrifuged at 4°C for 15 min at 13,000 rpm. Aliquots of the supernatant were directly subjected to SDS-polyacrylamide gel electrophoresis or further analyzed by immunoprecipitation.

Immunoprecipitation and Western Blotting—For immunoprecipitation, cell lysates were incubated for 4 h at 4°C with polyclonal anti-PTP1D antibody or with monoclonal anti-hemagglutinin antibody that had been bound to protein A-Sepharose beads (30 μl of beads). The beads were then washed three times with 1.0 ml of buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin (HNTG buffer), suspended in SDS sample buffer, boiled for 5 min, and subjected to gel electrophoresis.

Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and immunoblotted.

Grb2-GST Fusion Protein Purification and Binding Assay—The pGEX-Grb2 construct containing the complete SH2 and C-terminal SH3 domain (amino acids 14–217) was kindly provided by Dr. Reiner Lammers. After induction of transformed (pGEX-Grb2) DH5α (Stratagene) culture with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h, bacteria were lysed, sonicated, and centrifuged.

For binding assays, purified Grb2-GST (glutathione S-transferase fusion protein) was coupled to glutathione-Sepharose. Approximately 2 μg of Grb2-GST was incubated with 10 μl of glutathione-Sepharose beads and rotated for 2 h at 4°C. Beads were then washed three times with 1 ml of HNTG buffer, and Grb2-coupled beads were incubated with BHK-IR lysates for 4 h at 4°C.

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Preparation of PTP1D Mutant Expression Plasmids—PTP1D mutants containing a Cys to Ala substitution at position 463, and Tyr to Phe substitutions at positions 546 and 584, were generated using the site-directed mutagenesis protocol of Kunkel (36). Oligonucleotide primers were as follows: 5′-TGGTGACGGCCAGGCTC-3′ (C463A), 5′-AGAATACATATGTGTGAATCTCCGCCC-3′ (Y546F), and 5′-CAGTGTTCAGAAGACTCTAGCA-3′ (Y546F). The cDNA of PTP1D C463A was tagged with the hemagglutinin epitope at the C terminus using the following PCR primer: 5′-CCGGTGACGACTACGGTATCTGTCGCGGACATCGTACGGGTATCTGAAACTTTTCTGCTG-3′. The tagged 9 amino acids were N-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-C.

RESULTS AND DISCUSSION

After stimulation with growth factors, the SH2-containing protein-tyrosine phosphatase 1D associates with various tyrosine kinases and becomes tyrosine-phosphorylated (11, 15, 18). PTP1D association with the IR has been observed in vitro (19–22) and in vivo, but tyrosine phosphorylation of the phosphatase has not yet been detected in intact cells (10, 28). To investigate the role of PTP1D in the insulin signal, we transiently expressed the wild-type phosphatase and the catalytically inactive mutant C463A in BHK-IR cells. We found that overexpressed PTP1D was weakly phosphorylated on tyrosine after insulin stimulation (Fig. 1, lanes 6–10). Surprisingly, the PTP1D phosphorylation state was considerably higher when the C463A mutant was used (Fig. 1, lanes 11–15). Reprobing with anti-PTP1D antibody demonstrated that comparable amounts of protein were present in all relevant lanes (Fig. 1, lower panel).

To determine the identity of the phosphorylated tyrosine residues, we generated tyrosine mutations in the inactive form of PTP1D using standard in vitro mutagenesis techniques. These Tyr/Phe mutants were then transiently expressed in BHK-IR cells. A single Tyr/Phe substitution at position 546 of the catalytically inactive phosphatase resulted in a significant decrease in phosphotyrosine content, as shown in Fig. 2 (upper panel, lane 10). The combination of two tyrosine substitutions in the C-terminal tail (Y546F, Fig. 2, lower panel) abolished beyond detection tyrosine phosphorylation of the inactive form of PTP1D (Fig. 2, upper panel, lane 8). Reprobing with anti-PTP1D antibody demonstrated again that the observed differences were not due to different levels of protein expression (Fig. 2, middle panel).

To investigate the functional significance of the identified tyrosine phosphorylation sites, we incubated aliquots of the transfected BHK-IR lysates with a Grb2-GST fusion protein and determined the interaction with the different PTP1D forms by coprecipitation. As indicated in Fig. 3, the amount of associated catalytically inactive PTP1D C463A was markedly increased in comparison with wild type PTP1D. Substitution of tyrosine 546 with phenylalanine (Y546F) reduced this association dramatically and mutation of both tyrosine residues in 1D (Y546F/S584F) abolished the interaction between Grb2 and PTP1D.

The observed high level of tyrosine phosphorylation in the C463A mutant in contrast to wild type PTP1D suggested the possibility that the phosphatase activity of PTP1D may modulate its own state of tyrosine phosphorylation. To investigate this possibility further, we coexpressed the catalytically active and inactive forms of PTP1D in BHK-IR cells. In order to be able to distinguish between the two forms, the C463A mutant was modified with a hemagglutinin (HA) tag at the C terminus, permitting selective immunoprecipitation of this form of PTP1D with an anti-HA antibody. The coexpression of tagged and untagged inactive mutants served as a control. To verify the specificity of the hemagglutinin antibody, the untagged C463A variant was first expressed alone. As shown in Fig. 4 (lanes 6 and 8), tyrosine phosphorylation of the HA-tagged, inactive C463A-HA was clearly lower when coexpressed with wild type PTP1D rather than with the untagged inactive PTP1D mutant. These results demonstrate dephosphorylation of the tagged C463A-HA mutant by active PTP1D and therefore suggest an intermolecular transdephosphorylation mechanism. We cannot, however, rule out the possibilities of at least a partial contribution of an intramolecular mechanism or the involvement of other protein-tyrosine phosphatases of un-
The hemagglutinin-tagged, catalytically inactive PTP1D mutant, C463A-HA, was coexpressed together with untagged, catalytically active (lanes 1 and 2) and the untagged, catalytically inactive mutant alone (lanes 3 and 4) served as controls. Following 16 h of starvation, cells were stimulated for 15 min with $5 \times 10^{-7} \text{M}$ insulin (lanes 2, 4, 6, and 8) or left unstimulated (lanes 1, 3, 5, and 7). After cell lysis, the tagged PTP1D protein was precipitated with anti-hemagglutinin antibody, and the presence of its tyrosine phosphorylation targets was detected by immunoblot analysis with monoclonal anti-PTP1D antibody (upper panel). The same blot was reprobed with monoclonal anti-PTP1D antibody (lower panel). IgG, immunoglobulin heavy chain.
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