Direct Binding of the Proline-rich Region of Protein Tyrosine Phosphatase 1B to the Src Homology 3 Domain of p130Cas

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Feng Liu‡, David E. Hill$ and Jonathan Chernoff¶

From the ‡Chemistry Department, Temple University, Philadelphia, Pennsylvania 19122, $Oncogene Research Products, Cambridge, Massachusetts 12142, and ¶Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Protein tyrosine phosphatase 1B (PTP1B) is an abundant intracellular enzyme that is thought to act as a negative regulator of certain signaling pathways. The C terminus of PTP1B contains two proline-rich regions which conform to the canonical class II Src homology 3 domain binding motif, Pro-X-X-Pro-X-Arg. In this study, we establish that PTP1B interacts with Crk, Grb2, and p130Cas in vitro and with at least one of these, p130Cas, in intact cells. The interaction of PTP1B and p130Cas is independent of tyrosine phosphorylation, but can be disrupted by replacing two critical proline residues in the proline-rich domain of PTP1B between amino acids 301 and 315. When wild-type PTP1B is expressed in 3Y1-v-crk cells, p130Cas shows substantial dephosphorylation, whereas the proline mutant does not have this effect. In 3Y1 and 3Y1 v-crk-transformed fibroblasts, almost all of the total PTP1B and about 40% of total p130Cas co-sediment with membranes composed primarily of endoplasmic reticulum. These results suggest that the proline-rich domain between amino acids 301 and 315 in PTP1B binds Src homology 3-containing proteins and that p130Cas may be a physiological target of this phosphatase in cells.

Protein tyrosine phosphatases (PTPs) play a critical role in regulating a wide variety of intracellular signaling processes (1). In vitro, most PTPs display a broad substrate specificity, raising a question as to how these enzymes recognize their appropriate targets in cells. It has been suggested that differential location may account at least in part for substrate selection by PTPs (2). Many PTPs contain targeting motifs, which direct localization to particular sites within the cell, and such restricted location may limit access to substrates (2, 3). In addition, some PTPs, such as Shp-1 and -2, contain Src homology 2 (SH2) domains, which direct binding to specific phosphotyrosine-containing proteins (4). However, the mechanisms by which most other PTPs select their targets remain unclear.

PTP1B is the prototype of nontransmembrane PTPs and has served as a useful model for these enzymes. It was originally identified as the major PTP activity in human placenta (5, 6). Subsequent studies have shown that PTP1B is a ubiquitous and abundant enzyme, suggesting that it plays a general role in controlling cellular function (7, 8). PTP1B localizes to the endoplasmic reticulum (ER) via its 35-amino acid C-terminal sequence, with its phosphatase domain oriented toward the cytoplasm (9). Although the mechanism by which PTP1B is regulated is not understood, PTP1B undergoes cell cycle-regulated serine phosphorylation as well as alternative splicing (10–12). In addition, in some cell types, PTP1B undergoes Ca2+-dependent proteolysis, releasing a C-terminal truncated, soluble form of the enzyme, which may then act on previously inaccessible substrates (8). Overexpression of artificially generated C-terminal truncations of PTP1B and the related enzyme T-cell PTP (13, 14) has profound effects on cell proliferation that are not seen with the full-length molecule, again suggesting that appropriate location of PTPs is critical to their function.

The physiological substrates of PTP1B have not been identified. In an attempt to identify candidate substrates, we noted that PTP1B contains two proline-rich domains, which fit the consensus sequence for class II SH3 domain binding motifs (15, 16). In this study, we tested whether these proline-rich domains could direct PTP1B to recognize potential SH3-containing substrates. We found that PTP1B selectively binds to SH3 domains derived from Grb2, Crk, and p130Cas in vitro. The binding between PTP1B and p130Cas was confirmed in vivo and found to be mediated through one of the two proline-rich domains on PTP1B. This interaction is independent of p130Cas tyrosine phosphorylation levels. In v-crk-transformed fibroblasts, overexpression of wild-type PTP1B, but not a mutant form unable to bind p130Cas, results in dephosphorylation of p130Cas, suggesting that PTP1B might use its proline-rich domain to recognize and dephosphorylate this protein. Like PTP1B, a substantial amount of p130Cas co-sediments with ER membranes. Thus, the proline rich domains on PTP1B may direct this enzyme to some of its targets in cells.

**EXPERIMENTAL PROCEDURES**

Materials—3Y1 and 3Y1 v-crk-transformed cells, as well as glutathione S-transferase (GST) fusion proteins containing SH3 domains from Abl, Arg, Crk1, Crk2, Eps8, Fyn, Gap, Grb2C, Grb2N, Nck, phospholipase Cγ, p130Cas, and Src, respectively, were provided by Gary Kruh (Fox Chase Cancer Center). 3Y1 and 3Y1 v-crk-transformed cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. Wild-type and mutant forms of PTP1B were subcloned as BamHI-EcoRI fragments into pGEX-2T and, GST-PTP1B fusion proteins were made and purified by standard methods (17). The monoclonal anti-hemagglutinin (HA) antibody 12CA5 was obtained from Babco. Monoclonal anti-PTP1B antibody FG6 was obtained from Oncogene Science. Monoclonal antiphosphotyrosine 20, anti-p130Cas, and

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anti-Fak antibodies were purchased from Transduction Laboratories, and polyclonal anti-p130Cas antibody was from Santa Cruz Biotechnology, Inc.

Expression Plasmids—A catalytically inactive, C215S (CS) mutant of PTP1B was constructed by site-directed mutagenesis using a standard technique (18). Truncated and internally deleted forms of PTP1B (PTP1B-321 and PTP1B-403[320–329]) were constructed by polymerase chain reaction mutagenesis. A P309A/P310A (PA) mutant of PTP1B was made by the unique site elimination method of Deng and Nickoloff (19). Mutations were confirmed by sequence analysis. pJ3H-PTP1B constructs were made as described previously (20). These plasmids express an N-terminal HA-tagged PTP1B.

Transient Transfection—COS1 cells were grown to 80% confluence in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and transfected with pJ3H-PTP expression plasmids using LipofectAMINE (Life Technologies, Inc) according to the manufacturer’s recommendations. Forty-eight hours after transfection, the cells were harvested for analysis. 3Y1 and 3Y1-v-crk cells were grown to 40% confluence in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and transfected with expression plasmids using a calcium phosphate precipitation method (21). Forty-eight hours after transfection, the cells were harvested for analysis.

In Vitro Binding Assays—Transfected COS1 cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mM NaF, and 10 mM β-glycerophosphate) containing 1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride, and 10 µM leupeptin. Lysate protein concentrations were measured using BCA (Pierce). 500 µg of total cell lysates were incubated with 5 µl of GST SH3 domain fusion protein beads at 4°C for 2 h. The beads were washed three times with Nonidet P-40 lysis buffer and then boiled in SDS sample buffer. The samples were fractionated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-HA antibodies 12CA5. Signals were developed by chemiluminescence (Pierce). For 3Y1 and 3Y1-v-crk-transformed cells, 500 µg of cell lysate were incubated with 10 µl of GST PTP1B fusion protein beads. The samples were separated by 6% SDS-PAGE and analyzed by immunoblot using anti-p130Cas antibodies.

Immunoprecipitation and Immunoblot—3Y1 and 3Y1-v-crk cells were transiently transfected with either pJ3H alone or pJ3H bearing PTP1B, CS-PTP1B, or PA-PTP1B. Cells were lysed in Nonidet P-40 lysis buffer. For immunoprecipitation, 1 mg of cell lysates was immunoprecipitated with 2 µg of anti-HA antibody 12CA5. Signals were developed by chemiluminescence.

Enzyme Assay—GST-PTP1B and GST-PA-PTP1B activity was measured using the increase in absorbance at 405 nm at 30-s intervals. The nonenzymatic hydrolysis of para-nitrophenol phosphate was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of enzyme.

RESULTS

PTP1B Binds Selectively to SH3 Domain Fusion Proteins in Vitro—PTP1B contains two proline-rich motifs that fit the con-
sensus sequence for SH3 binding (15, 16). In an attempt to determine whether PTP1B binds to proteins containing SH3 domains, we assessed the ability of this phosphatase to associate with a variety of SH3-containing proteins in vitro. Purified GST-SH3 domain fusion proteins were immobilized on glutathione-Sepharose beads and then incubated with lysates from COS cells expressing HA-tagged PTP1B. The proteins adsorbed by the GST fusion proteins were analyzed by anti-HA immunoblot (Fig. 1A). Among the constructs tested, only the SH3 domains derived from Grb2 (both N- and C-terminal SH3 domains), Crk, and p130Cas bound detectable amounts of PTP1B. Similar results were obtained with nontagged PTP1B (data not shown). The SH3 domains from Crk and Grb2 are known to bind class II SH3 ligands (Pro-X-Pro-X-Pro-X-Arg) (26), whereas the preferred ligands for p130Cas are unknown. We also identified Crk as a PTP1B-binding protein using a yeast interaction trap screen (27), with PTP1B as bait and a HeLa cDNA library as source of interacting proteins. As the SH3 domain derived from p130Cas consistently bound more PTP1B than any other SH3 domain tested in vitro (as assessed by densitometry of immunoblots), we investigated this interaction in more detail.

**PTP1B Binds p130Cas in a Phosphotyrosine-independent Manner in Vitro**—To determine whether tyrosine phosphorylation of p130Cas affects its binding to PTP1B, we analyzed the ability of GST-PTP1B to bind p130Cas from 3Y1 cells and tyrosine-phosphorylated p130Cas from 3Y1-v-crk cells bound about equally well to the GST-PTP1B fusion protein, indicating that this association is phosphotyrosine-independent.

**The p130Cas Binding Element on PTP1B Requires Prolines**—There are two proline-rich domains in PTP1B. One (PPPEHIPPPPRPPKR) is located from amino acids 301 to 315, the other (SPAKEPEPSLPEK) spans amino acids 386–397 (Fig. 3A). Both of these proline-rich motifs contain the consensus for class II SH3 binding ligands (PXXPXR/K) (16). To determine whether either of these regions mediates binding to p130Cas, we tested the binding properties of PTP-321, which lacks the second (more C-terminal) proline-rich domain, and PTP-403D300–320, which lacks the first (more N-terminal) proline-rich domain (Fig. 3A). Anti-HA immunoblotting revealed that the p130 Cas SH3 domain binds to PTP321 but not to PTP403D300–320, indicating that the first proline-rich domain is required for binding to p130Cas (Fig. 3, B and C). To further characterize the binding elements, we made point mutations within this proline-rich domain, replacing proline residues 309 and 310 with alanine. Based on the crystal structure of SH3 ligands, these mutations are predicted to prevent formation of a left-handed proline-containing helix required for interaction with SH3 domains (15). This mutant form of PTP1B (PA-PTP) fails to bind the p130Cas SH3 domain (Fig. 3D). These results indicate that the association of PTP1B with p130Cas is likely to be based on an interaction between the more N-terminal of the two PTP1B domains.
proline-rich regions to the p130Cas SH3 domain.

PTP1B Interacts with p130Cas in Cells—To investigate potential interactions between PTP1B and p130Cas in intact cells, we asked whether p130Cas co-immunoprecipitates with PTP1B from 3Y1-v-crk cells. Anti-PTP1B immunoprecipitates contain a ~130-kDa band that co-migrates with and is recognized by anti-p130Cas antibodies (Fig. 4). This band is not seen in control immunoprecipitates. The p130 protein that co-immunoprecipitates with PTP1B is tyrosine-phosphorylated, as is authentic p130Cas. These data strongly suggest that endogenous PTP1B associates with p130Cas in 3Y1-v-crk cells.

To further investigate the nature of the PTP1B-p130Cas interaction in cells, 3Y1 and 3Y1-v-crk cells were transiently transfected with various PTP1B expression plasmids, and the cells were lysed and immunoprecipitated with anti-HA antibodies. The immunocomplexes were separated by SDS-PAGE and analyzed by immunoblot using anti-Cas antibodies. Both wild-type (WT) PTP1B and enzymatically inactive CS-PTP1B bind to p130Cas (Fig. 5). The binding of both WT- and CS-PTP1B to p130Cas suggests that the interaction between PTP1B and p130Cas is phosphotyrosine-independent. As in the in vitro experiments, PA-PTP1B fails to bind to p130Cas, indicating that prolines 309 and 310 on PA-PTP1B are not required for binding to this protein in both cell types as well.

Expression of PTP1B in 3Y1-v-crk Cells Causes Tyrosine Dephosphorylation of p130Cas—The ability of PTP1B to bind to p130Cas suggests that this protein might be a physiological target for PTP1B. To examine this possibility, 3Y1-v-crk cells were transiently transfected with expression vectors bearing either (a) no insert, (b) WT-PTP, (c) CS-PTP, or (d) PA-PTP. Anti-HA immunoblot analysis indicates that all the PTP constructs were expressed equally (Fig. 6C). Cell lysates were immunoprecipitated with anti-p130Cas antibodies, separated by 7% SDS-PAGE, and probed with antiphosphotyrosine antibodies to determine whether PTP1B expression affected p130Cas tyrosine phosphorylation (Fig. 6A). The blots were then stripped and reprobed with anti-p130Cas antibodies to ensure that the immunoprecipitates contained equal levels of this protein (Fig. 6B). The level of tyrosine-phosphorylated p130Cas is substantially reduced (about a 3–4-fold decrease from five independent experiments, as assessed by densitometry) in 3Y1-v-crk cells transfected with WT-PTP compared with those transfected with either inactive PTP (CS-PTP) or the p130Cas binding mutant (PA-PTP). Although we cannot exclude an indirect effect of PTP1B on p130Cas tyrosine phosphorylation levels (e.g. by inactivating a tyrosine kinase that acts on p130Cas, such as Src or Fak), these data are consistent with a direct enzyme-substrate relationship between these two proteins.

Besides p130Cas, three other prominent phosphotyrosyl proteins, which migrate at about 120, 90, and 65 kDa on SDS-PAGE, are apparent in 3Y1-v-crk cells. Two of these (pp120 and pp65) are also partially dephosphorylated in cells expressing WT-PTP but not PA-PTP. These proteins may represent additional binding partners or substrates for PTP1B in 3Y1-v-crk cells. Although we do not know the identity of these proteins, the 120-kDa band may represent an isoform of p130Cas (28) or the related protein Hef1 (29). The ~90-kDa phosphotyrosyl protein is not affected by PTP1B expression, indicating that this phosphatase does not indiscriminately dephosphorylate all potential substrates within the cell.

To exclude the possibility that PA-PTP fails to affect the tyrosine phosphorylation of p130Cas due to a reduction in overall phosphatase activity, we compared the enzymatic activity of WT- and PA-PTP. The activities of GST fusions with WT- and PA-PTP were measured against the phosphophoester paranitrophenyl phosphate (Fig. 6D). The WT- and PA-PTP mutant have similar activity profiles, indicating that this mutation does not overtly affect the ability of this enzyme to dephospho-
PTP1B Binds to p130Cas

TABLE I
Distribution of subcellular compartment markers after cell fractionation

| Marker                          | Total | S100       | 0.25–1.2 M interface | 1.2–2.0 M interface | P1 | Recovery |
|---------------------------------|-------|------------|----------------------|---------------------|----|----------|
| Protein (mg)                    | 28.0  | 20.0       | 0.41                 | 1.09                | 4.3| 94       |
| Lactate dehydrogenase (relative specific activity) | 1.0  | 1.2       | 0.0                  | 0.0                 | 0.1| 100      |
| 5'-Nucleotidase (relative specific activity) | 1.0  | 0.1       | 8.7                  | 2.5                 | 1.1| 97       |
| NADPH cytochrome c reductase (relative specific activity) | 1.0  | 0.0       | 2.2                  | 7.5                 | 1.2| 98       |
| DNA (relative DNA content)      | 1.0  | 0.1       | 0.3                  | 0.2                 | 4.0| 101      |

FIG. 7. Subcellular fractionation of PTP1B and p130Cas. Equal amounts of protein from each subcellular fraction were separated by 7% SDS-PAGE and immunoblotted with anti-PTP1B (PG6), anti-p130Cas (polyclonal), or anti-Fak antibodies. Subcellular fractions are total, S100 (cytosol), 0.25–1.2 M sucrose (Golgi and plasma membrane), 1.2–2.0 M sucrose (endoplasmic reticulum), and P1 (nuclei and unbroken cells).

In this study, we demonstrate that PTP1B binds to three SH3-containing proteins in vitro and at least one of these proteins, p130Cas, in intact cells. This binding is mediated by the proline-rich domain between residues 301 and 315 on PTP1B, and point mutations within this region abolish binding to p130Cas and also inhibit the ability of PTP1B expression to promote p130Cas tyrosine dephosphorylation in cells. These data suggest that p130Cas may be a physiological substrate for PTP1B.

To our knowledge, only two other PTPs have been shown to bind to an SH3-containing protein, and neither of these is mediated by a polyproline-SH3 interaction. PTP-PEST associates with the SH3-containing adapter protein She, but this association appears to be mediated by an NPLH sequence in PTP-PEST to the phosphotyrosine binding domain on Shc (32). Similarly, the PTPs Shp-1 and -2 bind to Src, but the interaction is thought to be mediated by the SH2 domain on Shp to tyrosine autophosphorylation sites on Src (33, 34). T-cell-PTP, which is closely related to PTP1B and is also located in the ER (35), has no proline-rich motifs and does not bind p130Cas.5 It is therefore possible that these two PTPs have distinct cellular functions based on the ability to interact with different substrates.

Many of the known posttranslation modifications of PTP1B occur at its C terminus. This region mediates binding to the ER (9), is the site of mitotic serine phosphorylations (10, 11), is affected by alternative splicing (12), and is subject to proteolytic cleavage in response to certain stimuli (8). The proline-rich motif that is required for p130Cas binding also resides in this region, just C-terminal to the PTP domain. The reported alternatively spliced and proteolytic cleavage forms of PTP1B do not alter this proline-rich motif between amino acids 301 and 315. We are currently testing whether the mitotic phosphorylation of PTP1B, which occurs at serines 352 and 386, affect its association with p130Cas.

p130Cas was initially described as a highly tyrosine-phosphorylated 130-kDa protein in v-crk-transformed (28, 36, 37) and v-src-transformed (38, 39) fibroblasts. In REF52 cells, p130Cas is concentrated at focal adhesions (40), in which it may act as asubstrate for PTP1B.
platform for the assembly of integrin-activated signaling molecules (28, 40–44). In other cell types, p130Cas has been reported to reside in the nucleus (38) and cytosol (27). Our data demonstrate that in 3Y1-v-crk cells, in which p130Cas is heavily tyrosine-phosphorylated, a substantial fraction of this protein co-purifies with ER membranes. It is interesting to note that Sakai et al. (28) reported that p130Cas moves from the cytosol to particulate fractions following tyrosine phosphorylation. As the fractionation procedure used by these authors did not distinguish plasma membrane from other membrane fractions, our results are consistent with their observations. Although most of the tyrosine phosphorylation-dependent signaling complexes described to date assemble at the plasma membrane, we observed almost no p130Cas protein in immunoblots from the fraction of plasma membranes that we prepared. The aminoterminal region of p130Cas consists of several potential tyrosine phosphorylation sites (28), and hence it is unlikley that its ER localization represents a consequence of its tyrosine phosphorylation state. Our inability to detect p130Cas in the plasma membranes may be due to its limited abundance and/or its instability in this compartment (43).

In vertebrate cells, a number of signaling proteins have been localized to the ER. Among these are protein kinases, such as Ltk (45) and the α and δ isoforms of protein kinase C (46, 47), PTPs such as PTP1B (9) and T-cell-PTP (31), adaptor proteins such as Shc (48), and perhaps “docking” proteins such as crk (49). It is not clear whether such proteins are unique to the ER or are also distributed to other membranes.

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33.报告期内p30Cas在免疫印迹中未被检测到，表明它在细胞膜中组装。我们注意到Sakai et al. (28)报告p30Cas在细胞膜中移动到含蛋白磷酸化的信号复杂体中。这一现象是p30Cas是否在细胞膜中稳定地组装。如上述所述，p30Cas的氨基端含有多个潜在的酪氨酸磷酸化位点（28），因此这种ER的定位可能不是由于其磷酸化状态引起的。我们的结果表明p30Cas在细胞膜中可能被招募到信号复杂体中，这与信号的正常调节有关。