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Identification of p130^{cas} as an *in Vivo* Substrate of Receptor Protein-tyrosine Phosphatase α*

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We have employed a substrate trapping strategy to identify physiological substrates of the receptor protein-tyrosine phosphatase α (RPTPα). Here we report that a substrate-trapping mutant of the RPTPα membrane proximal catalytic domain (D1), RPTPα-D1-C433S, specifically bound to tyrosine-phosphorylated proteins from pervanadate-treated cells. The membrane distal catalytic domain of RPTPα (D2) and mutants thereof did not bind to tyrosine-phosphorylated proteins. The pattern of tyrosine-phosphorylated proteins that bound to RPTPα-D1-C433S varied between cell lines, but a protein of approximately 130 kDa was pulled down from every cell line. This protein was identified as p130^{cas}. Tyrosine-phosphorylated p130^{cas} from fibronectin-stimulated NIH3T3 cells bound to RPTPα-D1-C433S as well, suggesting that p130^{cas} is a physiological substrate of RPTPα. RPTPα dephosphorylated p130^{cas} *in vitro*, and RPTPα co-localized with a subpopulation of p130^{cas} to the plasma membrane. Co-transfection experiments with activated SrcY529F, p130^{cas}, and RPTPα or inactive, mutant RPTPα indicated that RPTPα dephosphorylated p130^{cas} *in vivo*. Tyrosine-phosphorylated epidermal growth factor receptor was not dephosphorylated by RPTPα under these conditions, suggesting that p130^{cas} is a specific substrate of RPTPα in living cells. In conclusion, our results provide evidence that p130^{cas} is a physiological substrate of RPTPα *in vivo*.

Protein-tyrosine kinases (PTKs)[1] and protein-tyrosine phosphatases (PTPs) regulate the level of tyrosine phosphorylation of target proteins in cells, thereby regulating many important eukaryotic cell-signaling pathways. PTPs catalyze the hydrolysis of phosphoryl groups on Tyr residues in proteins. Each member of the PTP family contains one or two conserved PTP domains of approximately 240 amino acids including the signature motif (I/V)HC*X*AG*XX*R(S/T)G (1). These PTP domains are not only conserved in sequence but also in structure (2–8). The PTP family can be subdivided based on structural differences into receptor-like (RPTP) and cytosolic proteins (9). RPTPs, with CD45 as the founding member (10), consist of an extracellular domain, a single membrane spanning domain, and a cytoplasmic domain. Most RPTPs contain two tandemly repeated PTP domains in their cytoplasmic domain. Interestingly, for all RPTPs with two PTP domains, the majority of the catalytic activity resides within the membrane proximal PTP domain (D1), whereas the membrane distal domain, D2, displays little or no catalytic activity. Inactivation of RPTPα-D1 or CD45-D1 is sufficient to abolish their biological activities, indicating that PTP activity in D1, but not D2, is essential for the function of RPTPs (11, 12). D2s are conserved in sequence and in structure (6, 13),[2] but all D2s lack residues essential for catalysis, suggesting an important role for D2s in processes other than catalysis (6, 14, 15).

To elucidate the function of PTPs *in vivo*, it is essential to know their natural substrates. To identify substrates of PTPs, "substrate trapping" mutants have been designed. For instance, mutation of the catalytic site Cys to Ala in YopH, a *Yersinia* PTP, resulted in a mutant that bound substrates but was no longer able to dephosphorylate them, making this an excellent tool for identifying potential substrates (16). Since then, other mutants have been found to bind substrates. For instance, mutation of the highly conserved Asp residue from the "WpD" loop that functions as a general acid to facilitate cleavage of the scissile P–O bond in the substrate generated an efficient substrate-trapping mutant. In fact, for PTP-PEST and PTP1B, these Asp mutants were shown to be even more efficient substrate-trapping mutants than the catalytic site Cys mutants (17, 18). By now, substrate-trapping mutants of many non-receptor PTPs have successfully been used to identify substrates (17–25).

RPTPα is a transmembrane PTP with a short heavily glycosylated extracellular domain and two cytoplasmic PTP domains. Several potential physiological substrates of RPTPα have been identified. Expression of RPTPα was shown to interfere with insulin receptor (IR) signaling (26). Other reports have shown that RPTPα interferes with insulin-induced prolactin gene expression and GLUT4 translocation to the membrane (27, 28). Whether direct dephosphorylation of the IR by RPTPα mediates the effects on the IR-signaling pathway remains to be determined definitively. RPTPα associates with the Kv1.2 potassium channel in response to activation of the m1 muscarinic acetylcholine receptor, a G protein-coupled receptor. m1 muscarinic acetylcholine receptor activation regulates a tyrosine kinase that phosphorylates the Kv1.2 potassium channel, thereby suppressing the current generated by this channel. RPTPα recruitment to the Kv1.2 channel reverses the

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; RPTPα, receptor protein-tyrosine phosphatase α; D1, membrane proximal domain; D2, membrane distal domain; IR, insulin receptor; FN, fibronectin; EGF, epidermal growth factor; EGFR, EGF receptor; YFP, yellow fluorescent protein; Tyr(P), phosphotyrosine; GST, glutathione *S*-transferase; HA, hemagglutinin; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; WT, wild type; PAGE, polyacrylamide gel electrophoresis.

[2] A. Bilwes and J. Noel, personal communication.

tyrosine kinase-induced phosphorylation and suppression of Kv1.2, suggesting that the Kv1.2 potassium channel may be a direct substrate of RPTPα in cells (29).

The PTK Src is the most clearly defined target of RPTPα (11, 30). RPTPα dephosphorylates and activates Src *in vitro* and *in vivo* (11, 30–32). Murine Src is phosphorylated on Tyr^{529}. The crystal structure of Src and Src family members demonstrated that the SH2 domain of Src binds to phosphorylated Tyr^{529}, thereby blocking the catalytic site of the kinase (Ref. 33 and references therein). Recently, a phosphotyrosine displacement mechanism was proposed to underlie RPTPα-mediated dephosphorylation of Src Tyr(P)^{529} (34). The C-terminal RPTPα Tyr(P)^{789} binds the Src SH2 domain, thereby displacing intramolecular Src SH2-Tyr(P)^{529} binding and allowing dephosphorylation and thus activation of Src. This model is consistent with the finding that RPTPα associates with Src family members independently of RPTPα activity (35, 36). RPTPα knockout mice show an increase in Src Tyr^{529} phosphorylation and a decrease in Src activity, providing strong support for the finding that Src is an *in vivo* substrate of RPTPα (31, 32). Similarly, targeted disruption of RPTPα showed a decrease in activity of the Src family member Fyn (31, 32).

We set out to identify physiological substrates of RPTPα, using substrate-trapping mutants. Here, we report that a substrate-trapping mutant of RPTPα specifically bound to tyrosine-phosphorylated p130^{cas} from pervanadate-treated and fibronectin-stimulated cells. The interaction with RPTPα depended on the tyrosine phosphorylation state of p130^{cas}. Furthermore, RPTPα dephosphorylated p130^{cas} *in vitro* and *in vivo*. Analysis of the subcellular localization of p130^{cas} and RPTPα by (immuno)fluorescence demonstrated that some, but not all, p130^{cas} co-localized with RPTPα at the plasma membrane. Our results demonstrate that tyrosine-phosphorylated p130^{cas} is a substrate of RPTPα.

MATERIALS AND METHODS

Cells and Transfections—NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Life Technologies, Inc.). HepG2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. SK-N-MC neuroepithelioma and FaO cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DF) medium supplemented with 10% fetal calf serum. P19 EC, COS-7, and F9 cells were cultured in DF supplemented with 7.5% fetal calf serum.

Pervanadate treatment of the cells was done for 30 min by addition of 1 mM orthovanadate and 1 mM H_2O_2, generating pervanadate, directly into the medium of nearly confluent cells. Fibronectin stimulation was done essentially as described (37). Serum-starved NIH3T3 cells were removed from the dish gently using EDTA, allowed to recover in medium supplemented with 0.5% bovine calf serum for 30 min, and replated onto dishes that had been coated with fibronectin (10 μg/ml, overnight, 4 °C). The cells were harvested after 30 min. Transient and stable transfection of SK-N-MC and P19 EC cells was done using calcium phosphate precipitation, exactly as described (11).

Plasmids and Site-directed Mutagenesis—A cytomegalovirus promoter-driven expression vector for activated murine Src, SrcY529F, an expression vector for GST-p130^{cas}, and an expression vector for human EGFR have been described (38–40). SV40-driven expression vectors for expression of RPTPα or mutants have also been described (39). A hemagglutinin epitope tag was cloned to the N-terminal side of Asn^{20} in RPTPα (41). Site-directed mutagenesis was done on pSG-RPTPα or on pSG-HA-RPTPα. Mutations were verified by sequencing. The oligonucleotides that were used for site-directed mutagenesis were as follows: RPTPα-D401A, 5′-AGCTGGCCAGCCTTTGGGGTG; RPTPα-C433S, 5′-ACCTGCACTGGAGTGGACCAC; RPTPα-R438K, 5′-GCAGGTGTAGGGAAAACTGGCACCTTTG; RPTPα-C723S, 5′-CCCGGCACTGGAGTGGTGCACAGT; and RPTPα-R729K, 5′-GGGGCAGGAAAGACAGGAACC.

The RPTPα-YFP fusion protein was generated by introduction of a *Bgl*II site at position 516 in RPTPα by site-directed mutagenesis. Subsequently, a polymerase chain reaction-generated *Bgl*II fragment encoding yellow fluorescent protein (the kind gift of Roger Y. Tsien) was inserted in RPTPα at position 516, thus replacing RPTPα-D2. Expression vectors for bacterial expression of RPTPα glutathione *S*-transferase fusion proteins were made as described (42), using pGEX-KG (43). The expression vectors for GST-RPTPα-D1 contained residues 167–503, GST-RPTPα-D2 contained residues 504–793, and full-length bacterial RPTPα contained residues 167–793 (numbering according to Sap *et al.* (44)). The expression vector for bacterial expression of zebrafish PTP1B-C213S containing residues 1–282, corresponding to residues 1–284 in human PTP1B, was made as described by van der Sar *et al.* (45).

Recombinant Enzymes—The glutathione *S*-transferase fusion proteins were purified essentially as described (43). The fusion proteins were not cleaved off the beads but were eluted with 10 mM reduced glutathione in 50 mM Tris, pH 8.0, 10% glycerol, 10 units/ml aprotinin, and 1 μM PMSF. The fusion proteins were dialyzed against TBS (50 mM Tris, pH 8.0, 150 mM NaCl) and rebound to glutathione-agarose beads. This elution and rebinding to glutathione-agarose beads greatly reduced contamination with bacterial proteins.

GST Pull-down Experiments, Immunoprecipitation, and Immunoblotting—For GST pull-down experiments, cell lysates were made as described (18). Briefly, NIH3T3 cells were lysed in lysis buffer A (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 5 mM iodoacetic acid, 10 units/ml aprotinin, 1 μM PMSF), and all other cells were lysed in lysis buffer B (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM iodoacetic acid, 1 mM orthovanadate, 10 units/ml aprotinin, 1 μM PMSF), incubated at 4 °C for 30 min, and 10 mM dithiothreitol added to inactivate any unreacted iodoacetic acid. The lysates were incubated with GST fusion proteins coupled to glutathione-agarose beads overnight at 4 °C. The beads were washed four times with RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM Na_2HPO_4, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 unist/ml aprotinin, 1 μM PMSF), resuspended in Laemmli sample buffer, and boiled for 5 min, and the samples were loaded onto SDS-polyacrylamide gels.

For immunoprecipitations, nearly confluent cells were lysed in CLB (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 units/ml aprotinin, 1 μM PMSF, 200 μM sodium orthovanadate). Immunoprecipitation of HA-RPTPα was done by incubation with anti-hemagglutinin epitope tag antibody (mAb 12CA5) and protein A-Sepharose (Amersham Pharmacia Biotech). The EGFR was immunoprecipitated using mAb 108.1 (46). GST-p130^{cas} was precipitated by incubation with glutathione-agarose beads for 3 h at 4 °C. The beads were washed four times with RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM Na_2HPO_4, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 units/ml aprotinin, 1 μM PMSF), resuspended in Laemmli sample buffer, and boiled for 5 min, and the samples were loaded onto SDS-polyacrylamide gels.

For immunoblotting analysis the material on the polyacrylamide gels was transferred to Immobilon (Millipore, Bedford, MA) by semi-dry blotting for 2 h at 0.8 mA/cm^2 gel in transfer buffer (50 mM Tris, 40 mM glycine, 0.0375% SDS, 20% methanol). Following transfer, the blots were incubated in blocking buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% bovine serum albumin) for 1 h at 22 °C and in blocking buffer containing anti-Tyr(P) antibody PY20 (Transduction Laboratories, Lexington, KY) for 1.5 h at 22 °C. For immunoblotting with anti-p130^{cas} (Transduction Laboratories, Lexington, KY), with 12CA5, or affinity-purified anti-RPTPα antiserum 5478, blocking buffer contained 5% non-fat milk instead of bovine serum albumin. The filters were washed four times in TBS-T (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated for 1 h at 22 °C with horseradish peroxidase-conjugated anti-mouse antibody or horseradish peroxidase-conjugated protein A (Transduction Laboratories, Lexington, KY) in TBS-T. The filters were washed four times in TBS-T and enhanced chemiluminescence (ECL) detection was performed. Before reprobing with different primary antibodies, the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 45 min at 70 °C.

In Vitro Dephosphorylation Assay—For *in vitro* dephosphorylation assays, SK-N-MC cells were transiently transfected with GST-p130^{cas} together with SrcY529F. GST-p130^{cas} was precipitated from these cells with glutathione-agarose beads; the beads were washed 3 times in RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM Na_2HPO_4, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 units/ml aprotinin, 1 μM PMSF) and two times in succinate buffer (50 mM succinate, pH 6.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The beads were incubated with full-length GST-RPTPα in succinate buffer for different times at 30 °C, resuspended in Laemmli

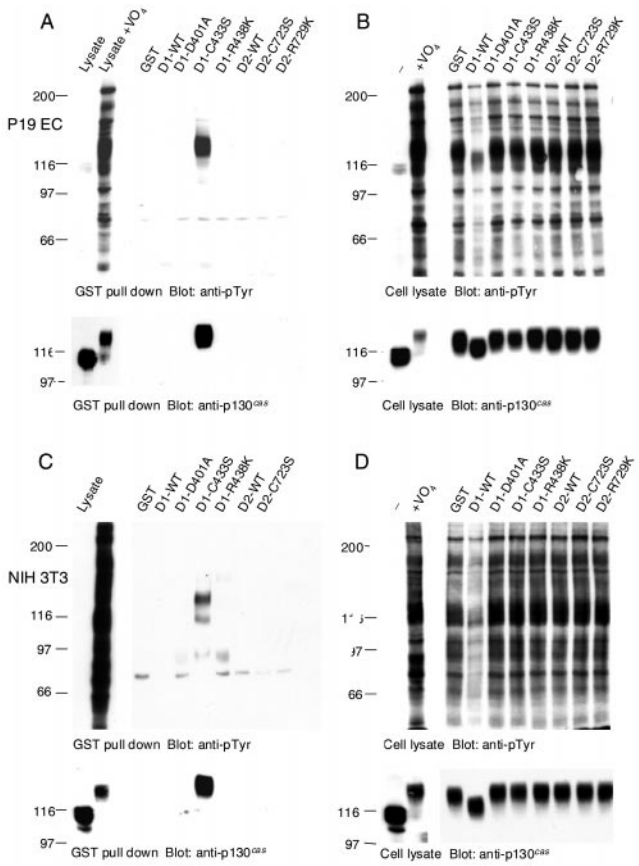

FIG. 1. **Interaction of RPTPα-D1-C433S with p130^{cas}.** P19 EC cells (*A* and *B*) or NIH3T3 cells (*C* and *D*) were treated with 1 mM orthovanadate and 1 mM H_2O_2 for 30 min. The cells were lysed, and the lysate was incubated with equal amounts (10 μg) of GST, GST-RPTPα-D1, D1-D401A, D1-C433S, D1-R438K, D2-wild type, D2-C723S, or D2-R729K. The associated proteins together with an aliquot of the lysate of pervanadate-treated (*Lysate + VO_4*) or untreated (*Lysate*) cells were analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) (PY20) (*top panel*) and anti-p130^{cas} (*bottom panel*) antibodies. Aliquots of the lysates from pervanadate-treated P19 EC cells (*B*) and NIH3T3 cells (*D*) following incubation with the different fusion proteins and removal of the GST-agarose beads were run on SDS-PAGE gels. For comparison, lysates of pervanadate-treated (*+VO_4*) or untreated (−) cells that were not incubated with GST fusion proteins were included. The gels were blotted, and the blots were analyzed by immunoblotting with anti-Tyr(P) antibodies (PY20) (*top panel*) and anti-p130^{cas} antibodies (*bottom panel*). The molecular sizes (in kDa) of marker proteins are indicated on the *left*.

sample buffer, and boiled for 5 min, and the samples were loaded onto SDS-polyacrylamide gels.

Indirect Immunofluorescence—For immunofluorescence labeling, cells were seeded on glass coverslips, and at the appropriate time the cells were fixed with 2% paraformaldehyde in PBS. After washing with PBS the cells were permeabilized with 0.1% Triton X-100 in PBS for 45 s. After washing the coverslips were blocked in 5% bovine serum albumin in PBS for 1 h. Incubation of the coverslips with anti-neurofilament or anti-p130^{cas} antibodies and CY3-conjugated secondary antibodies was done exactly as described previously (38).

RESULTS

RPTPα-D1-C433S Specifically Bound p130^{cas}—In this study we set out to identify substrates of RPTPα. Specific substrates of several non-receptor PTPs have been identified successfully in the past using substrate-trapping mutants (17–19, 24, 25, 47). We used a similar substrate trapping approach to identify physiological substrates of RPTPα. P19 EC cells were treated with pervanadate, which induced a strong increase in Tyr(P) content of many proteins (Fig. 1*A, left two lanes*). Bacterially expressed GST fusion proteins containing either the N-terminal or the C-terminal catalytic domain of RPTPα (D1 or D2, respectively) or mutants thereof were purified and incubated with lysates from pervanadate-treated P19 EC cells. Proteins from the lysate that associated with the fusion proteins were then analyzed by immunoblotting with anti-Tyr(P) mAb (PY20) (Fig. 1). A major Tyr(P)-containing protein of approximately 130 kDa (p130) bound specifically to GST-RPTPα-D1 with the catalytic site Cys mutated to Ser (D1-C433S). p130 is the most prominent phosphotyrosyl protein in the lysate from pervanadate-treated P19 EC cells. Nevertheless, it is clear that RPTPα-C433S bound preferentially to p130 (Fig. 1*A*, and not shown overexposures of the blot in Fig. 1*A*). No tyrosine-phosphorylated proteins were detected to bind to wild type GST-RPTPα-D1 or GST alone (Fig. 1*A*).

Substrate-trapping mutants of PTP1B and PTP-PEST in which the general base/general acid Asp was mutated to Ala were shown to be even more efficient substrate-trapping mutants than the catalytic site Cys mutants (17, 18). RPTPα-D1 with the corresponding Asp to Ala mutation, GST-D1-D401A, did not bind any Tyr(P)-containing proteins from the lysate (Fig. 1*A*). This suggests that there are differences in substrate binding between the Asp mutants in PTP1B or PTP-PEST and RPTPα-D1. It has been shown for PTP1B that mutation of the invariant Arg in the signature motif, Arg^{221} in PTP1B, almost completely abolished catalytic activity (17). Similarly, GST-RPTPα-D1 with the corresponding mutation, D1-R438K, had no detectable PTP activity in *in vitro* PTP assays toward any of the substrates tested (data not shown). No tyrosine-phosphorylated proteins were detected to bind to GST-D1-R438K (Fig. 1*A*). Since p130 bound specifically to GST-D1-C433S and not to D1-D401A or D1-R438K, we conclude that p130 is not merely a RPTPα-binding protein but a *bona fide* substrate.

Similar to the experiments with RPTPα-D1, substrate trapping experiments were performed with RPTPα-D2 and mutants thereof. No Tyr(P)-containing proteins bound to wild type D2 nor to GST-D2-C723S nor to any other D2 mutant (Fig. 1*A*). Taken together, substrate-trapping mutants of RPTPα-D2 did not bind Tyr(P)-containing proteins.

The apparent molecular weight of the protein that bound to RPTPα-D1-C433S prompted us to test whether this protein was p130^{cas}. The blot depicted in Fig. 1*A* was stripped and reprobed with anti-p130^{cas} antibody (Fig. 1*A, bottom panel*). The substrate-trapping mutant GST-D1-RPTPα-C433S bound to a single protein detected by the anti-p130^{cas} antibody. This protein co-migrated exactly with p130^{cas} in lysates from pervanadate-treated cells. p130^{cas} in lysates from untreated cells migrated faster in the gel than p130^{cas} in lysates from pervanadate-treated cells (Fig. 1*A, 1st two lanes, bottom panel*), consistent with reports that phosphorylation of p130^{cas} induced a mobility shift in SDS-PAGE gels (48). p130^{cas} did not bind to wild type RPTPα-D1, D1-D401A, D1-R438K, or GST alone, demonstrating that p130^{cas} binding is specific for the D1-C433S substrate-trapping mutant. p130^{cas} did not bind to GST-D2 or any of its mutants either. In conclusion, p130 from pervanadate-treated P19 EC cells that bound to GST-D1-C433S is p130^{cas}, and p130^{cas} bound specifically to the substrate-trapping mutant RPTPα-D1-C433S.

Aliquots of the remaining P19 EC cell lysates were analyzed by immunoblotting using anti-Tyr(P) antibody PY20 following the pull-down procedure, using the different GST fusion proteins (Fig. 1*B*). Incubation of the lysate with GST-RPTPα-D1-WT reduced the Tyr(P) content of all the Tyr(P)-containing proteins in the lysate, as detected with anti-Tyr(P) antibody, PY20. Importantly, phosphorylation of the highly phosphorylated p130 was greatly reduced. Incubation with GST or the other GST fusion proteins had no effect on the Tyr(P) levels of

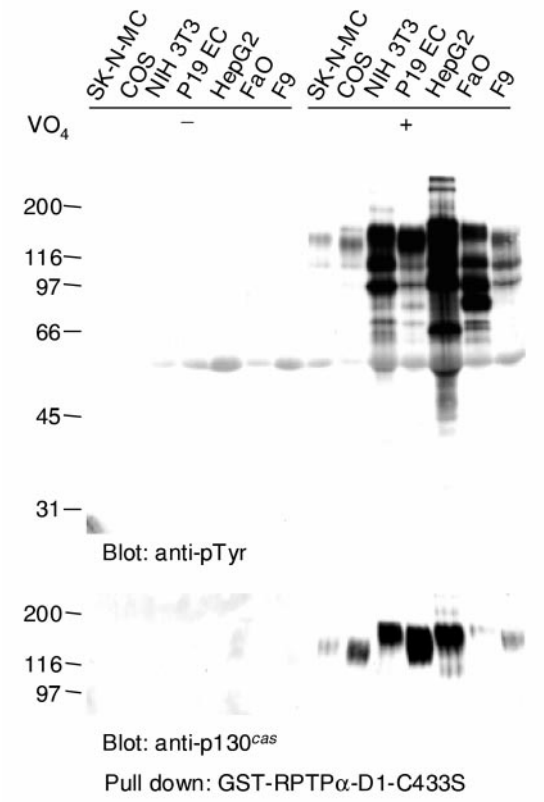

FIG. 2. **RPTPα-D1-C433S substrate trapping in lysates from various cell types.** SK-N-MC, COS-7, NIH3T3, P19 EC, HepG2, FaO, and F9 cells were treated with 1 mM orthovanadate and 1 mM H_2O_2 for 30 min or were left untreated. The cells were lysed, and the lysate was incubated with equal amounts of GST-RPTPα-D1-C433S. The associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) (*top panel*) or anti-p130^{cas} (*bottom panel*) antibodies. The molecular sizes (in kDa) of marker proteins are indicated on the *left*.

the proteins in the lysate, except for p130 in lysate incubated with GST-D1-C433S that showed a significant reduction. Stripping the blot and reprobing it with anti-p130^{cas} antibodies showed that the highly phosphorylated 130-kDa protein was p130^{cas} (Fig. 1*B, bottom panel*). The amount of p130^{cas} in the lysate was reduced after pull down with GST-RPTPα-D1-C433S, which was consistent with the removal of p130^{cas} from the lysate by the pull-down procedure. Incubation of the lysate from pervanadate-treated P19 EC cells with GST-RPTPα-D1-WT induced a shift down of all p130^{cas} (Fig. 1*B, bottom panel*), suggesting that none of the slower migrating tyrosine-phosphorylated p130^{cas} remained, and thus that p130^{cas} tyrosine dephosphorylation was complete. p130^{cas} did not shift down to the same level as p130^{cas} from untreated cells, which is probably due to residual serine/threonine phosphorylation. In conclusion, GST-RPTPα-D1-WT preferentially dephosphorylated p130^{cas}, suggesting that dephosphorylation of p130^{cas} by RPTPα is specific.

Similar results were obtained using NIH3T3 cells instead of P19 EC cells. GST-RPTPα-D1-C433S specifically bound to a 130-kDa protein that was identified as p130^{cas} (Fig. 1*C*). In addition, GST-RPTPα-D1-WT preferentially dephosphorylated p130^{cas} from pervanadate-treated NIH3T3 cell lysates (Fig. 1*D*). In addition to p130^{cas}, GST-RPTPα-D1-C433S bound three other phosphotyrosyl proteins from NIH3T3 cells, a doublet of approximately 110 kDa and a protein of 85 kDa. The 110-kDa proteins did not bind to any of the other GST fusion proteins, suggesting that the 110-kDa protein is a *bona fide* substrate for RPTPα too. The 85-kDa protein, however, bound to GST-RPTPα-D1-D401A and -R438K with similar efficiencies as to

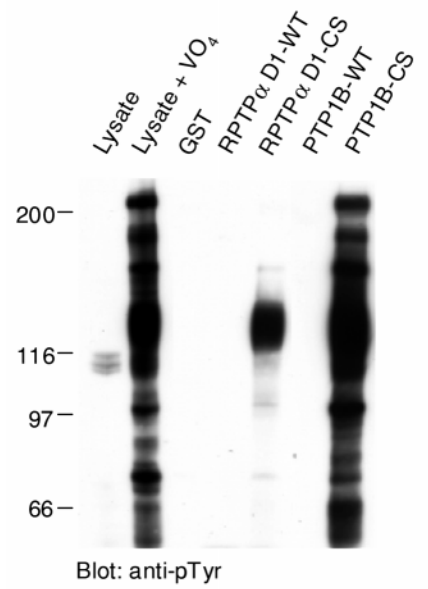

FIG. 3. **RPTPα, but not PTP1B, interacts almost exclusively with p130^{cas}.** P19 EC cells were treated with 1 mM orthovanadate and 1 mM H_2O_2 for 30 min. The cells were lysed, and the lysate was incubated with equal amounts of GST-RPTPα-D1, D1-C433S, GST-PTP1B, or PTP1B-C213S. The associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) antibodies. The molecular sizes (in kDa) of marker proteins are indicated on the *left*.

-C433S, suggesting that the 85-kDa protein bound to RPTPα-D1, independently of the catalytic site. In conclusion, tyrosine-phosphorylated p130^{cas} from P19 EC cells and NIH3T3 cells specifically bound to RPTPα-D1-C433S.

Interaction of RPTPα-D1-C433S with p130^{cas} from Different Cell Types—To determine whether p130^{cas} binding to RPTPα-D1-C433S was cell type-specific, we incubated GST-RPTPα-D1-C433S with cell lysates from a range of different cells treated with pervanadate (Fig. 2). GST-RPTPα-D1-C433S bound to p130^{cas} from all cell types we tested. Furthermore, as seen for NIH3T3 cells, GST-RPTPα-D1-C433S bound three additional proteins of 85 and 110 kDa from F9, FaO, and HepG2 cells. GST-RPTPα-D1-C433S bound an additional phosphotyrosyl protein from HepG2 cell lysate of about 70 kDa and one from FaO cell lysate of about 80 kDa that were not seen in the other cells. Long exposures demonstrated that little or no Tyr(P)-containing proteins were detected to bind to GST-RPTPα-D1-C433S from cells that were not treated with pervanadate (Fig. 2 and data not shown). Both GST-RPTPα-D1-WT and GST alone failed to bind to any tyrosine-phosphorylated protein from the different cell lysates (data not shown). In conclusion, RPTPα-D1-C433S associated with tyrosine-phosphorylated p130^{cas} from all cell types tested.

Specific Interaction of RPTPα-D1 with p130^{cas}—Next, we compared the specificities of RPTPα-D1 and PTP1B. GST fusion proteins of RPTPα-D1 and of wild type PTP1B and PTP1B-C213S were incubated with lysate from pervanadate-treated P19 EC cells. GST-RPTPα-D1-C433S bound predominantly one protein from P19 EC cell lysate (Fig. 3). As reported before (17) the pattern of the Tyr(P)-containing proteins bound by the PTP1B substrate-trapping mutant strongly resembled that of the input lysate, indicating that the PTP1B substrate-trapping mutant bound most phosphotyrosyl proteins from the lysate with similar efficiencies. No tyrosine-phosphorylated proteins were detected to bind to the wild type fusion proteins of RPTPα-D1 and PTP1B. In conclusion, the PTP1B substrate-trapping mutant showed little or no substrate specificity, whereas RPTPα-D1-C433S bound predominantly to one protein from P19 EC cells.

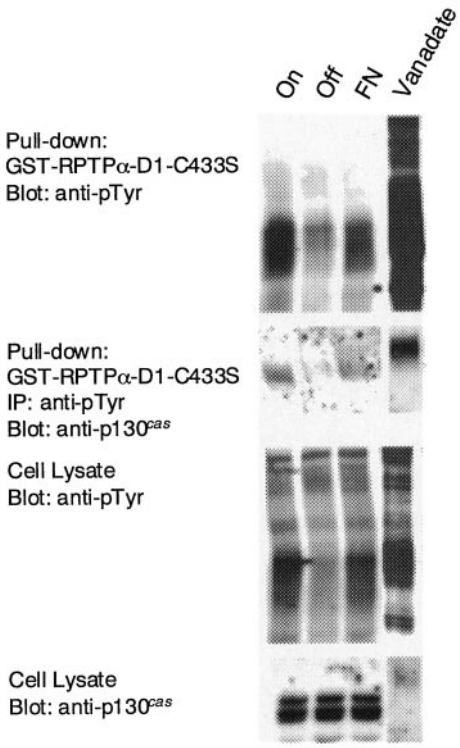

FIG. 4. **RPTPα-D1-C433S substrate trapping of fibronectin-stimulated p130^{cas}.** Serum-starved NIH3T3 cells were left untreated (*On*), taken off the dish with EDTA (*Off*), or replated on fibronectin-coated (*FN*) dishes for 30 min as described under "Materials and Methods." As a positive control, NIH3T3 cells were stimulated for 30 min with 1 mM orthovanadate and 1 mM H_2O_2. The cells were lysed, and pull downs were performed with GST-RPTPα-D1-C433S. Putative substrates were analyzed by SDS-PAGE and immunoblotting, using anti-Tyr(P) mAb PY20 (*top panel*). To establish that tyrosine-phosphorylated p130 in the *top panel* was p130^{cas}, proteins were eluted from the glutathione-agarose beads following the pull down and immunoprecipitated (*IP*) using anti-Tyr(P) mAb PY20. These immunoprecipitates were analyzed by SDS-PAGE and immunoblotting, using anti-p130^{cas} antibodies (*second panel*). Total cell lysates were analyzed in parallel to monitor Tyr(P) (*3rd panel*) and p130^{cas} levels (*bottom panel*). Exposure times were 30 min for the anti-Tyr(P) blots, except for the vanadate lanes (30 s), 2 min for the p130^{cas} blot of the pull downs, except for the vanadate lane (30 s), and 30 s for the p130^{cas} blot of the cell lysates. Note the shift in p130^{cas} in vanadate-treated cells. Highly tyrosine-phosphorylated p130^{cas} is poorly recognized by the anti-p130^{cas} antibody (*bottom panel*) (*cf.* Fig. 5).

Binding of p130^{cas} from Fibronectin-stimulated Cells to GST-RPTPα-D1-C433S—Pervanadate treatment of cells induced massive tyrosine phosphorylation of many proteins, including p130^{cas}. In order to investigate whether GST-RPTPα-D1-C433S recognized p130^{cas} under physiological conditions, we stimulated NIH3T3 fibroblasts by plating them on fibronectin (FN), a well known stimulus for p130^{cas} tyrosine phosphorylation (49–51). Hardly any p130^{cas} tyrosine phosphorylation was detected in cells that were detached from the substratum for 30 min, and replating of the cells on FN rapidly led to recovery of p130^{cas} phosphorylation (Fig. 4), consistent with previous reports (37). GST-RPTPα-D1-C433S pull-down experiments showed that tyrosine-phosphorylated p130^{cas} from FN-stimulated cells, but not unphosphorylated p130^{cas} from detached cells, was recognized by RPTPα-D1-C433S (Fig. 4). Long exposures are depicted in Fig. 4, as compared with Figs. 1 and 2, leading to detection of p130^{cas} from control cells as well. Pull down of p130^{cas} from pervanadate-treated cells was much more efficient, due to much higher levels of p130^{cas} tyrosine phosphorylation. These results demonstrate that not only pervanadate- but also fibronectin-stimulated tyrosine-phosphorylated p130^{cas} bound to RPTPα-D1-C433S, suggesting that p130^{cas} is a physiological substrate of RPTPα.

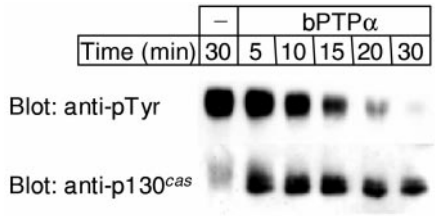

FIG. 5. **p130^{cas} dephosphorylation by RPTPα *in vitro*.** SK-N-MC cells were transiently transfected with GST-p130^{cas} together with SrcY529F. The cells were lysed, and GST-p130^{cas} was purified, aliquoted, and incubated with purified bacterially expressed PTPα encoding the complete cytoplasmic domain of RPTPα (*bPTPα*) for 5, 10, 15, 20, or 30 min, or with GST (−) for 30 min at 30 °C. The samples were analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) antibodies (PY20) (*top panel*) or anti-p130^{cas} antibodies (*bottom panel*). Note that tyrosine-phosphorylated p130^{cas} is poorly recognized by the anti-p130^{cas} antibody (*cf. lane 1* and *6*).

p130^{cas} Is a Substrate of RPTPα in Vitro—We tested the capacity of RPTPα to dephosphorylate p130^{cas} *in vitro*. As a source of tyrosine-phosphorylated p130^{cas}, we used transiently transfected SK-N-MC cells co-expressing GST-p130^{cas} and SrcY529F. Tyrosine-phosphorylated GST-p130^{cas} was purified from these cells, pooled, aliquoted, and incubated with a limited amount of purified bacterially expressed RPTPα for different periods. The Tyr(P) content of p130^{cas} was analyzed by immunoblotting with an anti-Tyr(P) mAb (PY20). Incubation of p130^{cas} with bacterially expressed RPTPα resulted in complete dephosphorylation of p130^{cas}, which was accompanied by a shift down of p130^{cas}, due to dephosphorylation (Fig. 5). p130^{cas} did not shift down to prestimulation levels, which was presumably due to residual serine/threonine phosphorylation. Incubation of p130^{cas} without RPTPα had no effect on the phosphorylation state of p130^{cas} (Fig. 5). In conclusion, RPTPα completely dephosphorylated p130^{cas} *in vitro*.

p130^{cas} and RPTPα Co-localized at the Membrane—For RPTPα to dephosphorylate p130^{cas} *in vivo*, both proteins must co-localize subcellularly. To examine subcellular localization of RPTPα and p130^{cas}, SK-N-MC cells, plated on glass coverslips, were transiently transfected with RPTPα in which D2 was replaced by yellow fluorescent protein (YFP). p130^{cas} was visualized by indirect immunofluorescence using anti-p130^{cas} antibodies. Confocal microscopy demonstrated that p130^{cas} localized to the cytoplasm and to the plasma membrane (Fig. 6). RPTPα-YFP localized predominantly to the plasma membrane. The combined image of RPTPα-YFP and p130^{cas} demonstrated that p130^{cas} and RPTPα co-localized at the membrane. These results show that RPTPα co-localized with a subpopulation of p130^{cas}, suggesting that RPTPα is in the right subcellular location to dephosphorylate p130^{cas}.

p130^{cas} Is a Substrate of RPTPα in Vivo—Finally, we investigated whether p130^{cas} was an *in vivo* substrate of RPTPα. SK-N-MC cells were transiently transfected with GST-p130^{cas} together with expression vector, wild type HA-RPTPα, or HA-RPTPα-C433S. The cells were co-transfected with active Src (SrcY529F), strongly increasing tyrosine phosphorylation of GST-p130^{cas} (Fig. 7*A*). Co-expression of wild type RPTPα in these cells significantly reduced tyrosine phosphorylation of p130^{cas}, which was accompanied by a shift in migration (Fig. 7*A*). Basal level p130^{cas} tyrosine phosphorylation in cells that were not co-transfected with active Src was not affected significantly by co-expression of RPTPα. Analysis of the Tyr(P) content of proteins in the lysates of cells co-transfected with SrcY529F and RPTPα indicated that not all proteins were dephosphorylated upon co-transfection of RPTPα (Fig. 7*A, bot-*

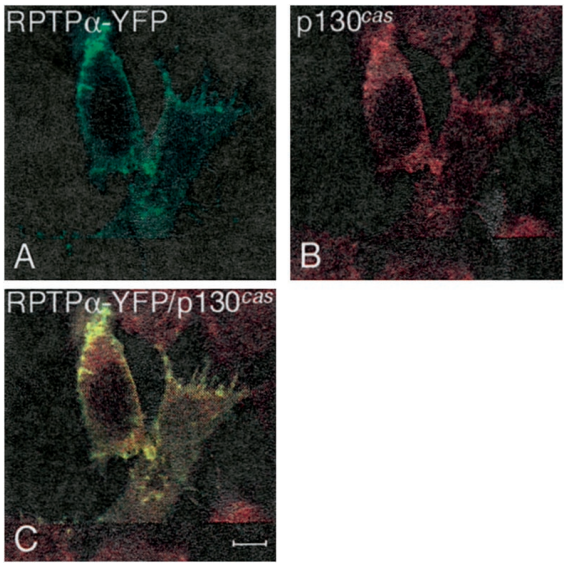

FIG. 6. **Subcellular co-localization of p130^{cas} and RPTPα.** SK-N-MC cells were plated on glass coverslips and transiently transfected with RPTPα-YFP. The cells were labeled with anti-p130^{cas} antibodies and CY3-coupled anti-mouse secondary antibodies. Confocal microscopy was used to determine the subcellular localization of RPTPα-YFP (*green labeling* in *A*), p130^{cas} (*red labeling* in *B*), and sites of co-localization of RPTPα and p130^{cas} (*yellow labeling* in *C*). *Bar,* 10 μm.

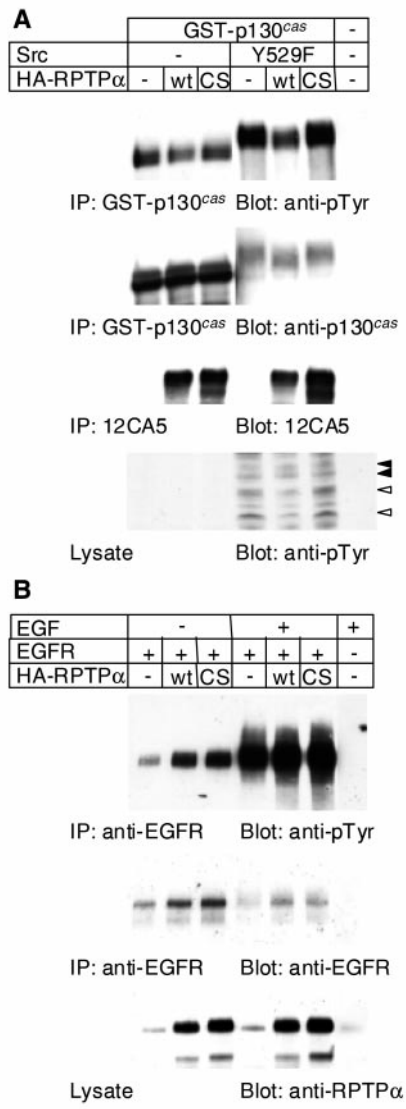

FIG. 7. **Dephosphorylation of p130^{cas} but not EGFR by RPTPα *in vivo*.** *A,* SK-N-MC cells were transiently transfected with GST-p130^{cas} together with expression vector pSG-5 (−), pSG-HA-RPTPα (*wt*), or pSG-HA-RPTPα-C433S (*CS*). The cells were co-transfected with the expression vector pSG-5 (−) or SrcY529F. The cells were lysed, and GST-p130^{cas} was precipitated and analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) mAb (PY20) or anti-p130^{cas} antibodies. The *left part* of the anti-p130^{cas} blot (*2nd panel* from the *top*) was exposed for 10 s, and the *right part* was exposed for 2 min, since tyrosine phosphorylation reduced detection of p130^{cas} tremendously (*cf.* Fig. 5). HA-RPTPα protein expression levels were monitored by immunoprecipitation with 12CA5 mAb and immunoblotting with 12CA5 mAb (*3rd panel*). Tyr(P) levels in the lysates were determined by immunoblotting, using anti-Tyr(P) mAb, PY20. A part of the gel (between 66 and 97 kDa) is shown in the *bottom panel,* and Tyr(P)-containing bands that are not affected by RPTPα are indicated with *black arrowheads*, whereas proteins that are less phosphorylated upon co-transfection of RPTPα, like p130^{cas}, are indicated with *open arrowheads*. *B,* SK-N-MC cells were transiently transfected with an expression vector for the human EGFR, together with expression vector pSG-5 (−), pSG-HA-RPTPα (*wt*), or pSG-HA-RPTPα-C433S (*CS*). The cells were stimulated with EGF (10 ng/ml) for 5 min, or left untreated. The EGFR was immunoprecipitated and analyzed by anti-Tyr(P) and anti-EGFR immunoblotting (*top two panels*). SK-N-MC cells do not express endogenous EGFR (*middle panel*). The expression of co-transfected (mutant) RPTPα was monitored by immunoblotting, using an anti-RPTPα antibody.

tom panel), suggesting that RPTPα did not merely dephosphorylate all tyrosine-phosphorylated proteins. As a control, we investigated whether RPTPα dephosphorylated tyrosine-phosphorylated EGFR. The EGFR was co-transfected with control vector, HA-RPTPα, or HA-RPTPα-C433S, and the cells were stimulated with EGF or left untreated. Co-transfection of RPTPα did not reduce EGF-induced EGFR tyrosine phosphorylation (Fig. 7*B*, *cf. wt* and *CS lanes*). Overexpression of the EGFR at high levels led to ligand-independent basal level tyrosine phosphorylation of the EGFR, which was not reduced by co-transfection of RPTPα either. The apparent increase in EGFR tyrosine phosphorylation upon co-transfection of (mutant) HA-RPTPα was due to differences in expression levels (Fig. 7*B*, *cf. top* and *middle panels*). Taken together, RPTPα co-expression in SrcY529F-expressing cells led to a reduction in p130^{cas} tyrosine phosphorylation, strongly suggesting that RPTPα dephosphorylated p130^{cas} *in vivo*. Moreover, RPTPα co-expression did not reduce EGFR tyrosine phosphorylation, suggesting that RPTPα displays substrate specificity in living cells.

DISCUSSION

In order to understand the function of PTPs, it is essential to know the identity of their substrates. We have used a substrate trapping procedure to identify substrates of RPTPα, a transmembrane PTP with a short extracellular domain. RPTPα-D1-C433S bound specifically to tyrosine-phosphorylated proteins from pervanadate-treated cells. One of these proteins was identified as p130^{cas}, and RPTPα-D1-C433S bound tyrosine-phosphorylated p130^{cas} from fibronectin-stimulated cells as well, suggesting that p130^{cas} is a physiological substrate of RPTPα. RPTPα-D1 preferentially dephosphorylated p130^{cas} from pervanadate-treated P19 EC and NIH3T3 cell lysates (Fig. 1, *B* and *D*). This preference was surprising, since previously we demonstrated that RPTPα displayed only modest selectivity toward peptide substrates *in vitro* (42). In living cells, p130^{cas}, but not EGFR tyrosine phosphorylation, was reduced upon co-transfection of RPTPα (Fig. 7). These results suggest that RPTPα displays substrate specificity *in vivo*.

Substrate trapping experiments with PTP-PEST and PTP1B demonstrated that a substrate-trapping mutant, in which the Asp residue that functions as general base in catalysis was mutated to Ala, displayed much higher affinity for Tyr(P)-containing proteins than catalytic site Cys to Ser mutants (18).

RPTPα-D1 with the similar Asp to Ala mutation (RPTPα-D401A) did not bind any Tyr(P)-containing protein from pervanadate-treated P19 EC cells or NIH3T3 cells (Fig. 1), demonstrating that the WpD motif Asp mutants are not always the best substrate-trapping mutants.

p130^{cas} is not the only substrate of RPTPα, since several other tyrosine-phosphorylated proteins from different cell lines bound to RPTPα-D1-C433S. For instance, RPTPα-D1-C433S bound, next to p130^{cas}, three other proteins, a doublet of 110 kDa and a protein of 85 kDa from NIH3T3 cells. The p85 protein bound to RPTPα-D401A and RPTPα-D1-R438K as well, suggesting that binding to p85 was independent of the catalytic site. p85 was not detected to bind to wild type RPTPα-D1, which may be due to dephosphorylation of p85 by RPTPα-D1. Perhaps p85 is not only a binding protein but also a substrate of RPTPα. Identification of p85 will facilitate rigorous testing of the interaction between p85 and RPTPα. The p110 protein specifically bound to RPTPα-D1-C433S and not to any of the other GST fusion proteins, indicating that p110 is an additional RPTPα substrate. The apparent molecular weight of p110 and p85 suggested that these proteins might be phosphatidylinositol 3-kinase. However, antibodies directed at the p110 subunit of phosphatidylinositol 3-kinase did not recognize the 110-kDa substrate of RPTPα (data not shown), suggesting phosphatidylinositol 3-kinase is not a substrate of RPTPα. Currently, we are trying to identify p110 and p85 by protein purification and microsequencing.

Other substrates previously described for RPTPα include the related PTKs Src and Fyn. RPTPα activates Src *in vitro* and *in vivo* by dephosphorylating the inhibiting Tyr(P)^{529} (11, 30–32). Rat embryo fibroblasts, P19 EC cells, and A431 cells overexpressing RPTPα showed an increase in Src activity (11, 30, 36). In RPTPα$^{-/-}$ cells Src and Fyn activity is reduced (31), providing strong support that these PTKs are substrates of RPTPα. By using the substrate trapping experiments, we did not pull down proteins with apparent molecular weights that correspond to Src and Fyn. This may be due to poor binding of RPTPα-D1-C433S to Src and Fyn. Moreover, failure to detect Src and Fyn in RPTPα-D1-C433S pull downs may be due to poor recognition of the C-terminal regulatory Tyr(P) in Src and Fyn by the anti-Tyr(P) antibody, PY20.

For all RPTPs with two PTP domains, the majority of the catalytic activity resides within D1, whereas D2 displays little or no catalytic activity. D2s are thought to have a more regulatory role. The existence of naturally occurring inactive PTP domains, such as D2s, has led to the suggestion that RPTP-D2s function as Tyr(P)-binding modules (52). Here we demonstrate that GST-RPTPα-D2 did not bind any Tyr(P)-containing proteins from pervanadate-treated cells (Fig. 1). GST-RPTPα-D2 with the catalytic site Cys mutated to Ser (C723S) or another inactive D2 mutant (R729K) did not bind Tyr(P)-containing proteins either. RPTPα-D2 lacks the highly conserved Tyr in the KNRY motif (14) that interacts with the main chain atoms and the aromatic ring of the substrate Tyr(P) (53). Introduction of this conserved Tyr in RPTPα-D2 (V555Y) did not restore Tyr(P) binding (data not shown). We conclude that it is unlikely that RPTPα-D2 by itself is a Tyr(P)-binding module. However, we cannot exclude the possibility that RPTPα-D2 cooperates with RPTPα-D1 in binding to substrates.

Other PTPs, like PTP1B, PTP-PEST, and LAR, have been found to dephosphorylate p130^{cas} as well (18, 54, 55). Both PTP1B and PTP-PEST have been found to associate with and dephosphorylate p130^{cas} (18, 54, 56, 57). Interaction of these PTPs with p130^{cas} is mediated by a proline-rich region in PTP1B or PTP-PEST and the SH3 domain of p130^{cas} (54, 56, 58). The p130^{cas} SH3 domain-PTP-PEST interaction is not required for the interaction of PTP-PEST with p130^{cas}, since substrate-trapping mutants of the PTP-PEST catalytic domain bound to p130^{cas} in the absence of the proline-rich region (18). RPTPα contains a proline-rich region (PPLP, residues 210–213), but this region is not sufficient for p130^{cas} binding, since p130^{cas} did not bind to wild type RPTPα nor the inactive mutants D401A and R438K that all contained the proline-rich region (Fig. 1).

p130^{cas} is part of focal adhesion complexes. Turnover of focal adhesion complexes is essential for cell movement and outgrowth of cell extensions. Interfering with PTP1B, PTP-PEST, or RPTPα resulted in altered cell motility (18, 32, 58–61), suggesting that these PTPs are somehow involved in regulating signaling of focal adhesion complexes. Regulation of p130^{cas} tyrosine phosphorylation by these PTPs may be the mechanism that underlies involvement of these PTPs in cell motility.

Here, we demonstrate that tyrosine-phosphorylated p130^{cas} is a substrate of RPTPα. Activated SrcY529F-induced tyrosine phosphorylation of p130^{cas} is clearly reduced in cells that express active RPTPα (Fig. 7). Src is negatively regulated by phosphorylation in its C terminus (Tyr^{529}) and positively regulated by phosphorylation of Tyr^{416}. RPTPα dephosphorylates both Tyr(P)^{529} and Tyr(P)^{416} in Src (11). We used an excess of SrcY529F in our experiments, and therefore the effects of RPTPα on SrcY529F activity via dephosphorylation of Tyr(P)^{416} are negligible, which is illustrated by the fact that co-expression of RPTPα did not affect tyrosine phosphorylation of all proteins in the lysate (Fig. 7*A, bottom panel*). Therefore, the effect of RPTPα on p130^{cas} tyrosine phosphorylation is due to dephosphorylation of p130^{cas}, not to reduced activity of SrcY529F.

The interaction between RPTPα and p130^{cas} may be complex. Src and Fyn are substrates of RPTPα, and these PTKs are activated by dephosphorylation of the C-terminal regulatory Tyr(P). Active Src and Fyn in turn phosphorylate p130^{cas} (48, 62). Therefore, RPTPα may have a dual effect on p130^{cas} tyrosine phosphorylation. RPTPα may dephosphorylate p130^{cas} directly, and RPTPα may induce enhanced phosphorylation of p130^{cas} via activation of Src and Fyn. Overexpression of RPTPα in the absence of activated SrcY529F did not affect p130^{cas} tyrosine phosphorylation significantly (Fig. 6), which may be due to the dual effect of RPTPα.

In conclusion, we provide evidence that RPTPα dephosphorylatyed p130^{cas} *in vitro* and in vivo. p130^{cas} contains many putative tyrosine phosphorylation sites. It remains to be determined whether all of these sites are substrates for RPTPα or for any other PTP. Different PTPs may dephosphorylate different Tyr(P) sites in p130^{cas}. We are currently investigating this interesting possibility.

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Identification of p130 *cas* as an *in Vivo* Substrate of Receptor Protein-tyrosine Phosphatase α

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