Identification of p130\textsuperscript{cas} as an \textit{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\textsuperscript{cas}. Tyrosine-phosphorylated p130\textsuperscript{cas} from fibroblast-stimulated NIH3T3 cells bound to RPTPα-D1-C433S as well, suggesting that p130\textsuperscript{cas} is a physiological substrate of RPTPα. RPTPα dephosphorylated p130\textsuperscript{cas} \textit{in vitro}, and RPTPα co-localized with a subpopulation of p130\textsuperscript{cas} to the plasma membrane. Co-transfection experiments with activated SrcY529F, p130\textsuperscript{cas}, and RPTPα or inactive, mutant RPTPα indicated that RPTPα dephosphorylated p130\textsuperscript{cas} \textit{in vivo}. Tyrosine-phosphorylated epidermal growth factor receptor was not dephosphorylated by RPTPα under these conditions, suggesting that p130\textsuperscript{cas} is a specific substrate of RPTPα in living cells. In conclusion, our results provide evidence that p130\textsuperscript{cas} is a physiological substrate of RPTPα \textit{in vivo}.

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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)HCXXGXXR/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 RPTPα (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 \textit{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 \textit{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 phosphatases; 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 Tyr529. The crystal structure of Src and Src family members demonstrated that the SH2 domain of Src binds to phosphorylated Tyr529, 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)529 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 Tyr529 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 p130cas from pervanadate-treated and fibronectin-stimulated cells. The interaction with RPTPα depended on the tyrosine phosphorylation state of p130cas. Furthermore, RPTPα dephosphorylated p130cas in vitro and in vivo. Analysis of the subcellular localization of p130cas and RPTPα by (immuno)fluorescence demonstrated that some, but not all, p130cas co-localized with RPTPα at the plasma membrane. Our results demonstrate that tyrosine-phosphorylated p130cas is a substrate of RPTPα.

**MATERIALS AND METHODS**

**Cells and Transfections—**NH3T3 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 FaOs 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 H2O2 generating pervanadate, directly into the medium of nearly confluent cells. Fibronectin stimulation was done essentially as described (37). Serum-starved NH3T3 cells were removed from the dish gently using EMDA, 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 strong support for the finding that Src is an in vivo substrate of RPTPα.

**GST Pull-down Experiments, Immunoprecipitation, and Immuno blotting—**For GST pull-down experiments, cell lysates were made as described (38). 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 iodoacetate 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 iodoacetate acid, 10 units/ml aprotinin, 1 μM PMSF), incubated at 4 °C for 30 min, and 10 units/ml aprotinin added to inactivate any active cysteine protease. 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 NaHPO4, 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 immunoprecipitations, nearly confluent cells were lysed in CLB (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 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 (40). GST-p130cas was precipitated by incubation with glutathione-agarose beads 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 units/ml aprotinin, 1 μM PMSF), and the samples were loaded onto SDS-polyacrylamide gels.

**For immunoblotting analysis the material on the polyacrylamide gels was transferred to Immobilon membranes and blocked in TBS containing 5% non-fat milk. The filters were washed four times in TBS-T and blotted to IB for 2 h at 0.8 mA/cm2 gel in transfer buffer (50 mM Tris, 40 mM glycine, 0.0375% SDS, 20% methanol). Following transfer, the filters were incubated in blocking buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM NaHPO4, 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.

**In vitro Dephosphorylation Assay—**For in vitro dephosphorylation assays, SK-N-MC cells were transiently transfected with GST-p130cas together with SrcY529F. GST-p130cas was precipitated from these cells with glutathione-agarose beads; the beads were washed three times in RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM NaHPO4, 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...
p130Cas Is a Substrate of RPTPα

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-p130cas antibodies and CY3-conjugated secondary antibodies was done exactly as described previously (38).

RESULTS

RPTPα-D1-C433S Specifically Bound p130cas—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. 1A, 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. 1A). 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. 1A, and not shown overexposures of the blot in Fig. 1A). No tyrosine-phosphorylated proteins were detected to bind to wild type GST-RPTPα-D1 or GST alone (Fig. 1A).

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. 1A). 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, Arg422 in PTP1B, almost completely abolished catalytic activity (17). Similarly, GST-RPTPα-D1 with the corresponding mutation, D1-R438K, had no detectable PTP activity 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. 1A). Since p130 bound specifically to GST-D1-C433S and not to D1-D401A or D1-R438K, we conclude that p130 is not merely a Tyr(P)-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. 1A). 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 p130cas. The blot depicted in Fig. 1A was stripped and reprobed with anti-p130cas antibody (Fig. 1A, bottom panel). The substrate-trapping mutant GST-D1-RPTPα-C433S bound to a single protein detected by the anti-p130cas antibody. This protein co-migrated exactly with p130cas in lysates from pervanadate-treated cells. p130cas in lysates from untreated cells migrated faster in the gel than p130cas in lysates from pervanadate-treated cells (Fig. 1A, Ist two lanes, bottom panel), consistent with reports that phosphorylation of p130cas induced a mobility shift in SDS-PAGE gels (48). p130cas did not bind to wild type RPTPα-D1, D1-D401A, D1-R438K, or GST alone, demonstrating that p130cas binding is specific for the D1-C433S substrate-trapping mutant. p130cas 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 p130cas and p130cas 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. 1B). 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...
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Interaction of RPTPα-D1-C433S with p130
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with 1 mM orthovanadate and 1 mM H2O2. The cells were lysed, and pull-downs were performed with GST-RPTPα-D1-C433S. The combined image of RPTPα-D1-C433Ssubstrate 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 H2O2. 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*cas in the top panel was p130*cas, proteins were eluted from the glutathione-agarose beads following the pull down and immunoprecipitation (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 substrate 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.

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. 7A). 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. 7A). 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. 7A, bot-
RPTPα was stimulated with EGF or left untreated. Co-transfection of HA-RPTPα with EGFR tyrosine phosphorylation upon co-transfection of mutant (μ-mut) HA-RPTPα was due to differences in expression levels (Fig. 7B, 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 demonstrated 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. 7B, 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. 7B, cf. top and middle panels). Taken together, RPTPα co-expression in SrcY529F-expressing cells led to a reduction in p130cas tyrosine phosphorylation, strongly suggesting that RPTPα dephosphorylated p130cas 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 p130cas, and RPTPα-D1-C433S bound tyrosine-phosphorylated p130cas from fibronectin-stimulated cells as well, suggesting that p130cas is a physiological substrate of RPTPα. RPTPα-D1 preferentially dephosphorylated p130cas 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, p130cas, 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 per- vanadate-treated P19 EC cells or NIH3T3 cells (Fig. 1), demonstrating that the WpD motif Asp mutants are not always the best substrate-trapping mutants.

p130cas 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 p130cas, 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 phosphatidylinosi- tol 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 phosphatidylinosi- tol 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 activated 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 corre- spond 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 cooperates with RPTPα-D1 in binding to substrates.

Other PTPs, like PTP1B, PTP-PEST, and LAT, have been found to dephosphorylate p130cas as well (18, 54, 55). Both PTP1B and PTP-PEST have been found to associate with and dephosphorylate p130cas (18, 54, 56, 57). Interaction of these PTPs with p130cas is mediated by a proline-rich region in PTP1B or PTP-PEST and the SH3 domain of p130cas (54, 56, 58). The p130cas SH3 domain-PTP-PEST interaction is not required for the interaction of PTP-PEST with p130cas, since substrate-trapping mutants of the PTP-PEST catalytic domain bound to p130cas in the absence of the proline-rich region (18). RPTPα contains a proline-rich region (PLLP, residues 210– 213), but this region is not sufficient for p130cas binding, since p130cas did not bind to wild type RPTPα nor the inactive mutants D401A and R438K that all contained the proline-rich region (Fig. 1).

p130cas is part of focal adhesion complexes. Turnover of focal adhesion complexes is essential for cell movement and out- growth 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 p130cas tyrosine phosphorylation by these PTPs may be the mechanism that underlies involvement of these PTPs in cell motility.

Here, we demonstrate that tyrosine-phosphorylated p130cas is a substrate of RPTPα. Activated SrcY529F-induced tyrosine phosphorylation of p130cas is clearly reduced in cells that express active RPTPα (Fig. 7). Src is negatively regulated by phosphorylation in its C terminus (Tyr416) and positively regulated by phosphorylation of Tyr416. 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. 7A, bottom panel). Therefore, the effect of RPTPα on p130cas tyrosine phosphorylation is due to dephosphorylation of p130cas, not to reduced activity of SrcY529F.

The interaction between RPTPα and p130cas 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 p130cas (48, 62). Therefore, RPTPα may have a dual effect on p130cas tyrosine phosphorylation. RPTPα may dephosphorylate p130cas directly, and RPTPα may induce enhanced phosphorylation of p130cas via activation of Src and Fyn. Overexpression of RPTPα in the absence of activated SrcY529F did not affect p130cas tyrosine phosphorylation significantly (Fig. 6), which may be due to the dual effect of RPTPα.

In conclusion, we provide evidence that RPTPα dephosphorylated p130cas in vitro and in vivo. p130cas contains many putative tyrosine phosphorylation sites. It remains to be deter- mined whether all of these sites are substrates for RPTPα or for any other PTP. Different PTPs may dephosphorylate different Tyr(P) sites in p130cas. We are currently investigating this interesting possibility.

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