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Proteolytic processing of the protein tyrosine phosphatase α extracellular domain is mediated by ADAM17/TACE

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ABSTRACT

The receptor protein tyrosine phosphatase alpha (PTPα) is involved in the regulation of tyrosine kinases like the Src kinase and the insulin receptor. As with other PTPs, its function is determined by alternative splicing, dimerisation, phosphorylation and proteolytical processing. PTPα is cleaved by calpain in its intracellular domain, which decreases its potential to dephosphorylate Src kinase. Here, we demonstrate that PTPα is also processed in the extracellular domain. Extracellular processing was exclusively found for a splice variant containing an extra nine amino acid insert three residues amino-terminal from the transmembrane domain. Processing was sensitive to the metalloprotease-inhibitor Batimastat, and CHO-M2 cells lacking a disintegrin and metalloproteinase 17 (ADAM17; tumor-necrosis-factor α converting enzyme) activity were not able to cleave PTPα. After transient overexpression of ADAM17 and PTPα in these cells, processing was restored, proving that ADAM17 is involved in this process. Further characterization of the consequences of processing revealed that dephosphorylation of the insulin receptor or activation of Src was not affected but focus formation was reduced. We conclude that extracellular proteolytic processing is a novel mechanism for PTPα regulation.

Introduction

The currently known 37 human protein tyrosine phosphatases (PTPs) are involved in the regulation of diverse physiological processes like growth, differentiation or metabolism. Structurally, they can be grouped into transmembrane, receptor-like and non-transmembrane forms. The receptor phosphatases (R-PTPs) have either one or two intracellular phosphatase domains, with the membrane distal domain typically being catalytically inactive (Andersen et al., 2001; Tonks, 2006). PTPα is a receptor phosphatase with two intracellular phosphatase domains (Kaplan et al., 1990; Krueger et al., 1990; Matthews et al., 1990), however, its membrane distal domain has some residual phosphatase activity (Buist et al., 1999). Substrates of PTPα include Src family kinases (Bhandari et al., 1998; Harder et al., 1998; Zheng et al., 1992), the insulin receptor (Møller et al., 1995), the potassium channels Kv1.1, Kv1.2, and Kv2.1 (Gil-Henn et al., 2001; Imbrici et al., 2000; Tsai et al., 1999) as well as the focal adhesion protein p130cas (Buist et al., 2000b). Amongst those substrates, Src family kinases are most prominent and still highly investigated. Mice lacking PTPα have an impaired Src activity (Ponniah et al., 1999; Su et al., 1999) whereas overexpression of PTPα leads to activation of the Src kinase by dephosphorylation of its carboxyl-terminal tyrosine residue and eventually to cell transformation or differentiation (den Hertog et al., 1993; Zheng et al., 1992). More recently, PTPα was shown to regulate integrin-stimulated focal adhesion kinase (FAK) autophosphorylation (Chen et al., 2006; Zeng et al., 2003), the lipid raft protein Cbp/PAG (Maksumova et al., 2005; Vacaresse et al., 2008), and paxillin (Vacaresse et al., 2008) in a Src family kinase dependent manner. In addition, PTPα together with Src is involved in interleukin-1 induced calcium-signaling (Wang et al., 2009).

The extracellular domains of PTPα and the related PTPε are short and heavily glycosylated, which is in contrast to other R-PTPs that have large extracellular domains with a variety of motifs including immunoglobulin or fibronectin type III modules (Tonks, 2006). However, the role of these cell adhesion features is only poorly understood. For many R-PTPs, structural diversity of the extracellular domain is created by alternative splicing. Splice variants of CD45 (Thomas, 1989), LAR (O'Grady et al., 1994), PTPδ (Pulido et al., 1995) and PTPσ (Yan et al., 1993) are expressed in a tissue specific way, but functional differences are still a matter of debate (for CD45, see Tchilian and Beverley, 2006). In addition to a splice variant of PTPα that generates a stop codon in the membrane proximal

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phosphatase domain, two isoforms varying in the extracellular domain were reported (Kaplan et al., 1990; Krueger et al., 1990; Matthews et al., 1990). The proteins differ in a stretch of nine amino acids located in the external membrane proximal stalk region, and are referred to as PTPα132 and PTPα123, respectively. The functional consequence of this structural diversity is a reduced Src activation of the shorter form (PTPα123), as determined in a focus formation assay using Src overexpressing cells and a PTPα containing a mutated carboxyl-terminal tyrosine residue (Kapp et al., 2007). In a different experimental setting, only the non-mutated short form generated foci upon overexpression (Tremper-Wells et al., 2010). Thus, the role of the extracellular domain of PTPα for its efficiency to activate Src is not yet clear.

In addition to generating variance by alternative splicing, several R-PTPs are proteolytically processed. The PTPs LAR, σ, δ, μ and κ occur as two non-covalently linked fragments, which result from the cleavage by a furin-like protease (Aicher et al., 1997, and references therein). Subsequently, some of these PTPs can be further processed by other proteases like ADAM (a disintegrin and metalloproteinase) 10 or 17 and γ-secretase (Anders et al., 2006; Ruhe et al., 2006). IA-2 and IA-2β known as major autoantigens in diabetes mellitus type 1, are also extracellularly cleaved by a furin-like protease (Seidah and Chretien, 1997). More recently, PTPBR7 (Dilaver et al., 2007), and CD45 (Kirchberger et al., 2008) were reported to be processed, and PTPζ is cleaved by plasmin and/or a combination of a metalloproteinase/γ-secretase (Chow et al., 2008a, b). Finally, PTPα and PTPε are cleaved by calpain in the cytoplasmic part. Upon cleavage, the activity to dephosphorylate the membrane associated Src kinase or the potassium channel Kv2.1 is lost or severely impaired, because of translocation of PTPα from the membrane to the cytoplasm (Gil-Henn et al., 2001).

Proteases of the ADAM family are activated by inhibitors of PTPs like peroxovanadate (POV) and phenylarsine oxide (PAO), and by activators of protein kinase C (PKC) like phorbol esters (Merlos-Suarez and Arribas, 1999; Vecchi et al., 1998). We have previously reported about a vanadate induced proteolytic processing of PTPα (Lammers et al., 2000). Here, we have further characterized this processing and found that only the splice variant containing the additional nine amino-acid insert (PTPα132) was cleaved. The ADAM family member ADAM17/TACE (TNF-α converting enzyme) was identified as the responsible protease. Processing of PTPα132 had no effect on regulation of insulin signaling but reduced the potential to activate the Src kinase.

Materials and methods

cDNA constructs and site-directed mutagenesis

cDNAs of the two PTPα splice variants containing (PTPα132) or lacking (PTPα123) a nine amino-acid insert in the extracellular membrane proximal stalk region were cloned into the cytomegalovirus immediate early promoter based expression vector pRK5. Site directed mutagenesis of PTPα132, mutating serines 189 and 213 to alanine, was performed by the single-stranded DNA method of Kunkel et al. (1987) using the oligonucleotides S189A: 5′ GGATAAGCGGAAAGCATTGGAATGGCTCCC and S213A: 5′ TTTCCTGTTGGTGGCTGGGGATCTGGCCAG. The double mutant was generated using standard cloning procedures, and mutations were confirmed by sequence analysis. ADAM17 was cloned from a murine cDNA-library using the primers ADAM17-132: 5′ GCACCTGCTAAGTTGCTTCC and ADAM17-2759: 5′ AGGTTTCCCAGAGAGGTGGT. Mutation of the carboxy-terminal tyrosine or the catalytic cysteine residue has been described earlier (Lammers et al., 1998). The cDNA encoding PTPαΔNT was generated by PCR using the oligonucleotide 5′-XbaI site – haemagglutinin-tag – GGTATTCTGACTCGAAG and cloned into the pRK5 vector. This yields a protein starting with the antibody tag followed by the 9 amino-acids insert of the PTPα132 splice variant in the extracellular domain. Because of the lack of a signal peptide, this protein is located intracellularly. The construct PTPαintra was generated by PCR using the oligonucleotide 5′-BglII-site – GCCATGGCAAGGTTTAAGAAATACAAGCA and cloned into the expression vector pRK5. This generates a PTPα protein starting with the juxtamembrane sequence (M-A-) RFKKY-(PTPα).

Antibodies and cell lines

Mouse monoclonal antibodies 29 and 45 are directed against the first phosphatase domain of PTPα (kind gift of N.P.H. Møller, Bagsværd, Denmark). ADAM17 antibodies were from Santa Cruz (sc-6416). Horseradish peroxidase-coupled secondary antibodies for use in Western blot experiments were from Sigma. BHK, CHO-T, 293, BOSC23 and GP+E cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. Prior to stimulation with the indicated substances, the cells were made quiescent by changing the serum content from 10% to 0.5% for 18–24 h.

Transient expression experiments and protein analysis

Transient expression was performed employing the method of Chen and Okayama (1987). Eighteen hours after the addition of DNA precipitates, cells were washed once and supplied with fresh medium containing 0.5% fetal calf serum. The next day, cells were lysed in 200 μl of lysis buffer/6-well-dish (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl_2$; 1 mM EGTA; 10% (v/v) glycerol; 1% (v/v) Triton X-100; 100 mM NaF; 10 mM $Na_4P_2O_7$; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin), and the lysates pre-cleared by centrifugation at 13,000 × *g* for 4 min at 4 °C. After addition of sample buffer, proteins were boiled for 5 min, size separated by SDS-PAGE, transferred to nitrocellulose filters and analyzed by Western blotting. Bound antibodies were visualized using the ECL system (Amersham Pharmacia Biotech). Alternatively, antibody (1.5–2 μg) and Protein A–sepharose were added to the lysate supernatant, incubated for at least 4 h at 4 °C on a turning wheel, the sepharose beads collected, washed with HNTG (20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% Triton X-100; 10% glycerol; 10 mM NaF; 1 mM sodium orthovanadate) and subjected to immunoanalysis as described above.

In vitro phosphatase activity assay

Lysates from transiently transfected 293 cells were used to dephosphorylate *para*-nitrophenylphosphate as described (Kapp et al., 2007). The phosphatase activity was corrected for the amount of PTPα present in the lysates.

Focus formation assays

BOSC23 cells were transfected with pLXSN plasmids containing the PTPα or ADAM17 cDNAs and the supernatants used to infect GP+E cells. After geneticin selection, cell pools were expanded, supernatants harvested and filter sterilized. To determine the titer, NIH3T3 cells were infected and selected for G-418 resistance. Cell colonies were stained with crystal violet and counted. For focus formation assays, 75,000 NIH3T3 cells overexpressing a moderate amount of c-Src were seeded into a six-well dish and 16 h later infected for 6–7 h with equal amounts of retroviruses (5×10^5–1×10^6) in the presence of 6 μg/ml polybrene. Infection volume was 1.5 ml, equalized with medium and retroviruses without donor DNA. 48 h later, cells were trypsinized and seeded into a

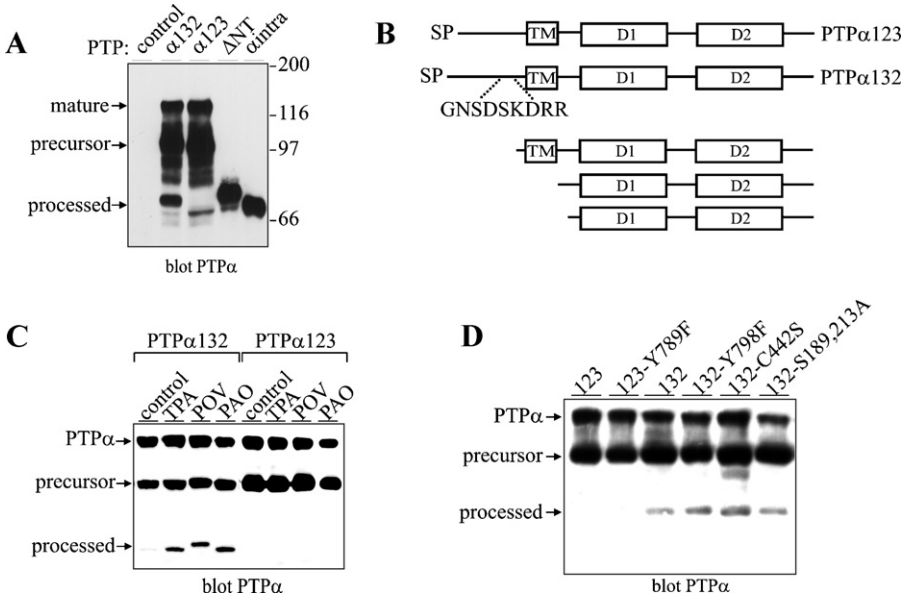

Fig. 1. Proteolytic processing of overexpressed PTPα can be induced by different substances. Two hundred and ninety three cells were transfected with the indicated constructs and 48 h later the cells lysed, proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and PTPα and its cleavage products immunodetected with antibody mab29. (A) PTPα132 is proteolytically processed in the extracellular domain. (B) Schematic representation of PTPα splice variants and their cleavage products. Amino acids 121–129 (GNSDSKDRR) are found only in PTPα132. PTPα123 is processed in the juxtamembrane domain to yield 63 and 68 kDa proteins whereas only PTPα132 can in addition be cleaved in the extracellular juxtamembrane domain to give a 75 kDa product. SP: signal peptide, TM: transmembrane domain; D1: amino-terminal PTP domain; D2: carboxy-terminal PTP domain. (C) Stimulation of proteolytical processing of PTPα132. Cells were transfected and stimulated for 1 h at 37 °C with 400 μM POV, 35 μM PAO, or 1 μM TPA before harvest. (D) Neither PTPα catalytic activity nor phosphorylation is required for processing.

10-cm dish in Dulbecco's modified Eagle's medium (1 g/l glucose) containing 4% fetal calf serum. The medium was changed every other day for 3 weeks, and the cells were stained with crystal violet (0.5% crystal violet in 20% methanol).

Results

PTPα132 but not PTPα123 is processed to generate a 75 kDa protein

In a previous report we have demonstrated that PTPα132 is proteolytically processed upon peroxovanadate (POV) treatment of NIH3T3 cells to generate a 75 kDa protein fragment (Lammers et al., 2000). When investigating the conditions leading to PTPα processing, we noted that upon transient overexpression in 293 cells, some processing occurred constitutively and differed between the two splice variants of the extracellular domain. As shown in Fig. 1A, a strong overexpression generated not only the mature PTPα protein with a molecular weight of 130 kDa but also the 100 kDa precursor was enriched, presumably because of the limited glycosylation capacity of the cells during transient expression (Daum et al., 1994). In addition, proteins between 63 and 75 kDa were generated: for PTPα132 including the nine amino-acid insert a 75 kDa, a 68 kDa (low level) and a 63 kDa protein were detected whereas for the shorter form (PTPα123) only proteins of 68 and 63 kDa were found. To determine the region of cleavage, two truncated forms of PTPα were expressed in parallel and run next to PTPα full length. PTPαΔNT lacks the signal sequence but contains an amino-terminal HA-tag and starts at the nine amino-acid insert of the extracellular domain whereas PTPαintra encodes the complete intracellular domain plus start methionine. Since the 75 kDa protein described above compares to the size of PTPαΔNT, the cleavage site for PTPα132 generating this fragment is likely extracellular and close to the membrane. The 63 kDa protein found with both PTPα forms is smaller than PTPαintra and thus is cleaved in the cytoplasm. It should be identical to the calpain cleavage product of PTPα123 (Gil-Henn et al., 2001). An additional cleavage giving rise to the 68 kDa protein has the size of the PTPαintra and might occur right at the plasma membrane but is seen for PTPα132 better upon longer exposure of the blot (data not shown). A schematic drawing summarizing the proteolytic processing is given in Fig. 1B.

Since the cleavage of PTPα in NIH3T3 cells was stimulated by POV-treatment (Lammers et al., 2000), and considering that POV stimulates matrix metalloproteinases (Dong and Wiley, 2000; Vecchi et al., 1998), our finding that the cleavage site is extracellular, suggests a role of metalloproteinases in processing of PTPα132. To confirm improved processing by POV and other stimulators of metalloproteinase activity, we transfected 293 cells with a low amount of DNA to facilitate the analysis of the processing products. Before harvest, the cells expressing either PTPα132 or PTPα123 were treated with POV, phorbol-12-myristate-13-acetate (TPA, PKC activator) and PAO (phenylarsine oxide). As shown in Fig. 1C, processing was increased after treatment and occurred only in PTPα132. The slightly different molar masses of the processed fragments (about 75 kDa) could be due to a varying degree of phosphorylation induced by the inhibitors. POV treatment increases tyrosine phosphorylation (Buist et al., 2000a) and TPA treatment serine phosphorylation of PTPα (den Hertog et al., 1995; Tracy et al., 1995), respectively. We tested this hypothesis by using two concentrations of POV and saw an intermediate shift of the processed fragment at the lower POV concentration (Fig. S1). In addition to 293 cells, in CHO and BHK cells transiently expressing PTPα, processing was observed upon stimulation with POV (data not shown).

We next analyzed whether TPA dependent serine phosphorylation of PTPα132, phosphorylation at the carboxyl-terminal tyrosine residue or the phosphatase activity were a prerequisite for its cleavage. Therefore, the codons for the two major PKC phosphorylation sites located within the juxtamembrane domain of the phosphatase (Tracy et al., 1995) were mutated to codons for Ala, the codon for Tyr789/798 to Phe and the Cys442 in the membrane proximal PTP domain to Ser. After transient overexpression of the corresponding proteins at a high level and Western blot analysis, no difference to processing of wild type protein was found (Fig. 1D).

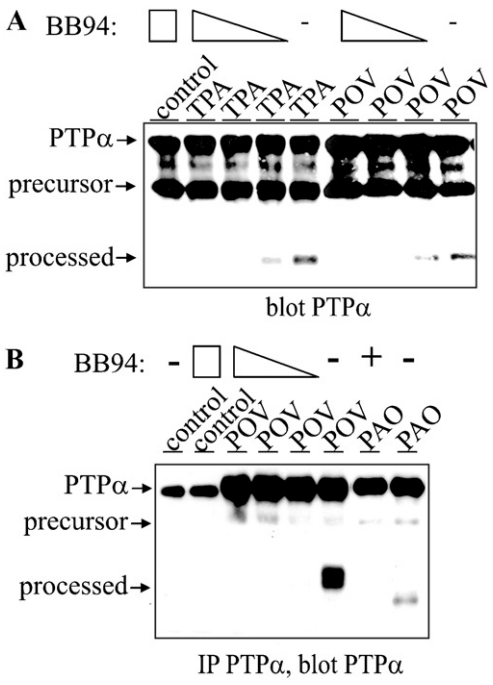

Fig. 2. Extracellular cleavage of PTPα is sensitive to Batimastat. (A) Two hundred and ninety three cells transiently expressing PTPα132 were preincubated for 30 min with 5 μM, 0.5 μM, 50 nM Batimastat (BB94), or vehicle alone, subsequently stimulated with TPA or POV for 1 h and then lysed. After transfer to nitrocellulose filters, proteins were detected by immunoblotting with mab29. (B) BIRα132 cells were either preincubated with decreasing concentrations (5 μM–50 nM) or with 0.5 μM BB94 (+), stimulated with POV for 1 h as indicated and then analyzed as above.

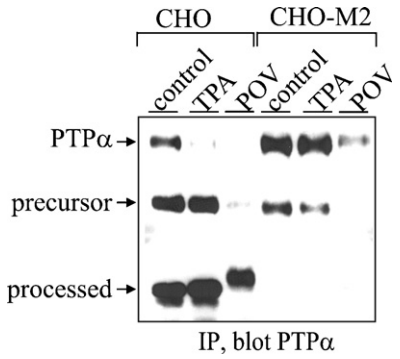

Fig. 3. ADAM17 activity deficient M2 cells are incapable of processing the PTPα extracellular domain. CHO M2 cells or parental CHO cells transiently expressing PTPα132 were left untreated or stimulated with 1 μM TPA or 200 μM POV for 1 h. Cell lysates were immunoprecipitated with anti-PTPα antibody mab45, size separated by SDS-PAGE and proteins analyzed by immunodetection with anti-PTPα mab29.

The metalloprotease inhibitor BB94 (Batimastat) inhibits extracellular PTPα cleavage

Metalloprotease activity results in extracellular cleavage of a heterogeneous group of transmembrane proteins and is inhibited by BB94 (Batimastat) at nanomolar concentrations (Hooper et al., 1997; Turner and Hooper, 1999; Wojtowicz-Praga et al., 1997). To confirm that metalloproteases are involved in PTPα132 processing, 293 cells overexpressing PTPα132 were pre-incubated with BB94 for 30 min (Fig. 2A). BB94 concentrations down to 50 nM almost completely blocked PTPα132 processing upon TPA or POV stimulation. By contrast, BB94 had no effect on intracellular cleavage of PTPα (data not shown). Furthermore, we tested the effect of BB94 in BIR cells (BHK cells overexpressing the insulin receptor; Møller et al., 1995) stably expressing PTPα132 and found that similar concentrations were able to block processing (Fig. 2B).

Extracellular processing of PTPα132 is abolished in ADAM17 activity deficient M2 cells

ADAM17 is a transmembrane protein with well documented metalloprotease activity and cleavage specificity. To investigate if this enzyme is responsible for the extracellular processing of PTPα132, the phosphatase was transiently overexpressed in a mutant CHO cell line (M2) lacking ADAM17 activity (Merlos-Suarez et al., 1998) as well as in the parental cell line that displays normal ADAM17 activity. The cells were either left untreated or stimulated with TPA or POV, lysed, PTPα132 and its fragments immunoprecipitated, and cleavage detected in Western blot analysis. Under these conditions, the parental CHO cell line exhibited considerable constitutive processing of PTPα132 (Fig. 3), and upon TPA-treatment the mature form of the phosphatase was almost completely processed while POV treatment led to complete degradation of precursor and mature protein. For M2 cells, however, no processing of PTPα132 was detectable. Of note, both wild type CHO and the M2 cells showed repeatedly a reduced amount of total PTPα upon POV treatment, indicating an additional proteolytic activity in CHO cells but no processing was detected upon longer exposure of the blot. Thus, the data suggest, that ADAM17 activity is necessary for extracellular cleavage of PTPα132.

ADAM17 cleaves PTPα in 293 and NIH3T3 cells

In order to confirm that ADAM17 is responsible, we investigated the effect upon overexpression of both, ADAM17 and PTPα in 293 cells. For transient transfection of PTPα and ADAM17 we used various amounts of PTPα expression plasmid to avoid transfection induced endogenous protease activity. As shown in Fig. 4A, at low PTPα expression, the proteolytic processing was only detected when ADAM17 was co-expressed. At higher phosphatase expression levels, more protein was processed in the presence of ADAM17. Verifying ADAM17 expression by reblotting revealed that there is already a high level of endogenous ADAM17 (bottom panel). Thus, the cellular stress upon transfection and high expression of a heterologous protein may already be an activator for ADAM17 leading to low constitutive PTPα processing. A similar experiment was also performed in NIH3T3 cells stably expressing PTPα132-Y798F that were infected with retrovirus encoding ADAM17 (Fig. 4B). The PTPα mutant was used because wild type protein cannot be permanently overexpressed in NIH3T3 cells, as was also shown for mouse embryo fibroblasts (Zeng et al., 2003). Again, stimulation with TPA or POV led to processing. Importantly, proteolytic cleavage seems to be specific for the ADAM17 protease since a number of other metalloproteases (MMP1, collagenase3 or the transmembrane types MT1-MMP, ADAM9, ADAM10, ADAM15, ADAM19) were incapable of improving processing of PTPα, when cells were co-transfected with the corresponding expression plasmids (data not shown).

Effect of ADAM17 dependent cleavage on PTPα activity

Calpain induced cleavage of PTPα in the intracellular domain resulted in a reduced activity towards the potassium channel Kv2.1 and loss of activity towards Src (Gil-Henn et al., 2001). We used an *in vitro* and two cellular assays to elucidate the effects of extracellular cleavage on PTPα activity. First, PTPα was overexpressed in 293 cells either alone or in the presence of ADAM17 and the hydrolysis of para-nitrophenylphosphate (pNPP) was photometrically detected. As shown in Fig. 5A, the *in vitro* phosphatase activity was not changed by co-overexpression of ADAM17.

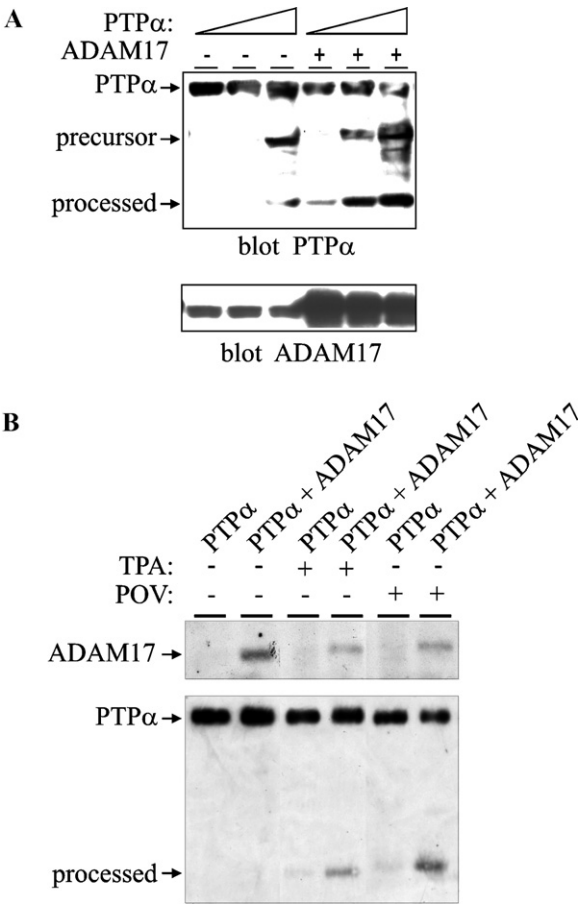

Fig. 4. ADAM17 dependent proteolytic processing of overexpressed PTPα. (A) Two hundred and ninety three cells were transfected with increasing amounts of PTPα132 (0.5, 1.0 and 1.5 μg cDNA) and 1 μg of ADAM17 (as indicated in the figure). Cells were lysed, proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and PTPα and its cleavage products immunoblotted with antibody mab29 or anti-ADAM17 antibodies (bottom panel). (B) A NIH3T3-PTPαY798F cell line was infected with retroviruses encoding ADAM17 and a cell line was established. Parental cells and ADAM17 overexpressing cells were stimulated with TPA (1 μM, 45 min) or POV (200 μM, 30 min) and subsequently lysed. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters and immunoblotted to detect ADAM17 (upper panel). PTPα was immunoprecipitated and detected as described above.

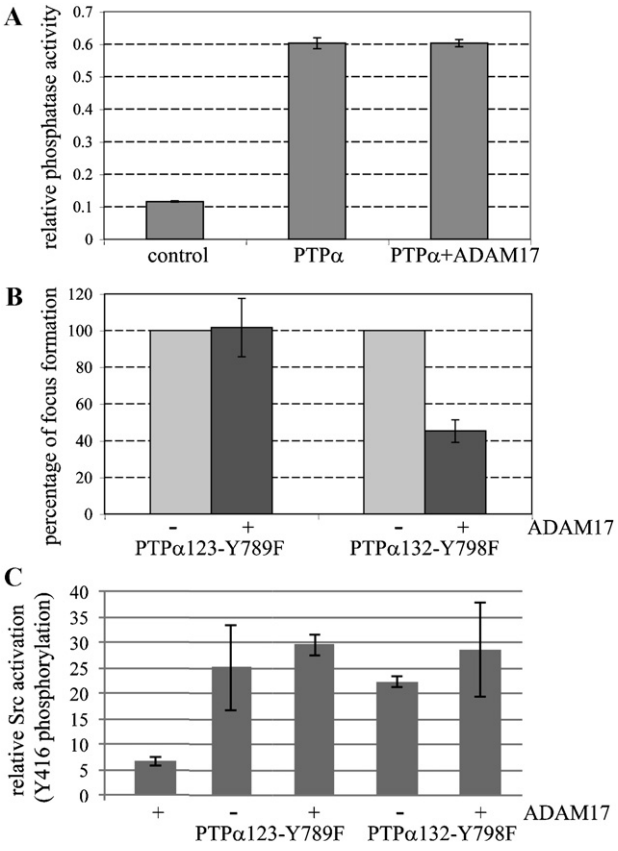

Fig. 5. ADAM17 does not affect PTPα's potential to dephosphorylate pNPP but impairs the PTPα mediated activation of c-Src in a focus formation assay. (A) Lysates of 293 cells transiently expressing PTPα132 alone or together with ADAM17 were used to test the phosphatase activity with pNPP as substrate in a photometric assay. Similar lysates as presented in Fig. 4A were used, showing about 25% (PTPα132) or 30% (PTPα132 + ADAM17) processing. The measured activity was corrected for PTPα expression. The bars represent the phosphatase activity as means ± SEM from two independent experiments, each done in quadruplicate. The SEM from the control cells is too small to be visible. (B) NIH3T3 cells overexpressing c-Src were infected with different forms of PTPα (as indicated in the figure) and without (bright bars) or with ADAM17 encoding retroviruses (dark bars). Cells were grown for 21 days under low serum conditions and then stained with crystal violet. Results of infections with ADAM17 are given as means ± SEM in relation to infections without ADAM17 (100%) and are generated from five independent experiments. (C) NIH3T3 cells were infected with retroviruses encoding either PTPa123-Y789F or PTPa132-Y798F and ADAM17 as indicated. Individual clones were amplified, lysed and used for Western blotting analyses with anti phospho-Src (Y416) antibody and c-Src antibody. The bars represent the relative Src activity (Y416 phosphorylation) from three independent clones as means ± SEM.

In BIR cells overexpressing the insulin receptor, continuous treatment with insulin reduces cell viability (Møller et al., 1995). Since PTPα is a negative regulator of the insulin receptor and upon transfection able to rescue the overexpressing cells from the detrimental insulin effect, we used co-transfection experiments of PTPα and ADAM17 but did not find different rescuing capacities (data not shown). Next, we investigated PTPα activity towards Src and employed focus formation assays. Upon infection of fibroblasts overexpressing a moderate amount of Src with retrovirus encoding PTPα with the mutation of the carboxyl-terminal tyrosine to phenylalanine, activation of Src can be measured as focus formation (Lammers et al., 2000). We used both splice variants in the presence or absence of ADAM17 to infect c-Src overexpressing NIH3T3 cells. As shown in Fig. 5B, the co-infection of ADAM17 did not have any effect on PTPα123-Y789F mediated Src activation. By contrast, we found a reduction to 45% using the mutant PTPα132-Y798F. We concluded from this experiment that ADAM17 dependent processing reduced the PTPα132 dependent activation of Src. However, in an independent approach, we infected NIH3T3 cells with viruses encoding PTPα123-Y789F or PTPα132-Y798F either alone or together with ADAM17. We prepared cell lysates of several clones each and analysed the Src activity using an antibody against phosphorylated Y416 that indicates active c-Src. To our surprise, we did not find a reduced Src activity in the presence of PTPα132-Y798F and ADAM17, suggesting that the effect of ADAM17 overexpression is independent of Src activation (Fig. 5C). This leaves the possibility that PTPα may affect proliferation and loss of contact inhibition in a yet undefined way that is subject to further research.

Discussion

Proteolytical processing and ectodomain shedding is a common mechanism to regulate the signaling activity of a protein. Here, we identified a new cleavage mechanism for PTPα: in addition to the earlier described intracellular cleavage by calpain, a splice variant specific extracellular processing occurs by the protease ADAM17, a

member of the ADAM-family of metalloproteases. This processing had no effects on down regulation of the insulin signal and did not change Src activity, but focus formation was reduced. Since we have shown before that the PTPα132 variant is a stronger Src activator than PTPα123 (Kapp et al., 2007) a negative regulation by ADAM17 would suggest an additional way of regulation of proliferation.

In the recent years, many proteins were identified as substrates of ADAM17. This implicates a role for this protease in a wide range of signaling pathways that include proliferation (EGF-R activation by release of cell surface coupled growth factors), immune regulation (shedding of TNFα) and neuroprotection (processing of the amyloid precursor protein) (Murphy, 2008). Previously, two PTPs have been described as substrates for ADAM17, while the physiological consequences of cleavage have yet not been shown. PTPζ (PTPRz) is expressed in the central nervous system in three isoforms generated by alternative splicing. Chow et al. (2008b) have shown that the transmembrane forms are cleaved in the extracellular space close to the membrane by ADAM17, and that subsequently the γ-secretase complex cleaves within the transmembrane sequence so that an intracellular fragment can translocate to the nucleus. Since we had discovered the 68 kDa processing fragment of PTPα132 that could correspond to an intramembrane cleavage product, we tested a nuclear localization. However, after transient overexpression of PTPα132 no nuclear accumulation was found by immunofluorescence in NIH3T3 cells, not even after phorbol ester stimulation (data not shown). Similarly, the calpain-dependent fragment was not reported to occur in the nucleus (Gil-Henn et al., 2001). The second PTP that is a substrate of ADAM17 is PTP-LAR. Cleavage of this phosphatase also occurred close to the membrane and was dependent on EGF-R and ERK1/2-activation (Ruhe et al., 2006). Different from PTPζ, however, a translocation to the nucleus was not investigated but a degradation of the intracellular fragment with a concomitant decline of PTP activity was found. This may be similar to our findings for PTPα132 in which the presence of ADAM17 reduced focus formation. In addition, we observed the degradation of the processed PTPα132 fragment. An active degradation of the intracellular PTPα fragment is also supported by the fact that cells treated with a proteasome inhibitor contained a higher amount of the 75 kDa protein (data not shown). Thus, ADAM17-dependent processing would rather represent a mechanism to clear PTPα132 from the membrane.

Since ADAM17 in most experimental settings is associated with proliferation it seems odd that its overexpression reduced PTPα and Src dependent focus formation. However, PTPα can act in opposite directions depending on the cellular context. Thus, overexpression of PTPα123 in fibroblasts induced cellular transformation (Zheng et al., 1992) whereas in tumor-derived MCF-7 cells it suppressed transformation (Ardini et al., 2000). PTPα132 is mostly expressed in differentiated cells, as we have shown earlier (Kapp et al., 2007), while ADAM17 is widely expressed (Black et al., 1997). ADAM17 expression could therefore be a regulatory mechanism to counteract PTPα132 dependent proliferation. On the other hand, there may be signaling processes we are not aware of yet that are regulated by ADAM17 mediated cleavage. For example, the shed extracellular domain of PTPα132 could act as a ligand. We have tried to test this hypothesis and were able to generate BHK cell clones that initially expressed a high level of secreted extracellular domain. However, after expansion of cells, expression declined below detection limit, indicating a possible toxicity (data not shown). Further, PTPα has been described as a force-responsive protein that interacts with the extracellular matrix and regulates spreading of colon cancer cells through cytoskeletal contractility (Krndija et al., 2010) The loss of contact to the extracellular matrix after processing may contribute to the reduced focus formation. Alternatively, a complex with other membrane proteins in cis, like contactin (Zeng et al., 1999), could be affected. During neuronal development, other substrates of ADAM17 also interact with contactin, like PTPζ (Bouyain and Watkins, 2010) or Notch (Hu et al., 2003). Of note, PTPα132 is increasingly expressed during neuronal development and can make up to 50% of total PTPα in the cerebellum and granule neurons (Kapp et al., 2007), which is the highest level that we are aware of. In contrast to expression in differentiated cells or tissues, Tremper-Wells et al. (2010) did not find PTPα132 in tumor-derived samples. This is in part in agreement with our own data, detecting no elevated amounts of PTPα132 in primary breast tumor samples although a low level mostly was present (not shown).

Taken together, we have identified a novel mechanism of PTPα proteolytic processing that does not lead to apparent changes in substrate phosphorylation but may induce degradation of the phosphatase. Furthermore, our data have shown that the role of PTPα in cell transformation is not limited to Src-activation. However, this needs further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2012.04.003.

References

Aicher, B., Lerch, M.M., Muller, T., Schilling, J., Ullrich, A., 1997. Cellular redistribution of protein tyrosine phosphatases LAR and PTPsigma by inducible proteolytic processing. J. Cell Biol. 138 (3), 681.

Anders, L., Mertins, P., Lammich, S., Murgia, M., Hartmann, D., Saftig, P., Haass, C., Ullrich, A., 2006. Furin-ADAM 10-, and gamma-secretase-mediated cleavage of a receptor tyrosine phosphatase and regulation of beta-catenin's transcriptional activity. Mol. Cell. Biol. 26 (10), 3917–3934.

Andersen, J.N., Mortensen, O.H., Peters, G.H., Drake, P.G., Iversen, L.F., Olsen, O.H., Jansen, P.G., Andersen, H.S., Tonks, N.K., Møller, N.P.H., 2001. Structural and evolutionary relationships among protein tyrosine phosphatase domains. Mol. Cell. Biol. 21 (21), 7117–7136.

Ardini, E., Agresti, R., Tagliabue, E., Greco, M., Aiello, P., Yang, L.T., Menard, S., Sap, J., 2000. Expression of protein tyrosine phosphatase alpha (RPTPalpha) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth in vitro and in vivo. Oncogene 19 (43), 4979.

Bhandari, V., Lim, K.L., Pallen, C.J., 1998. Physical and functional interactions between receptor-like protein-tyrosine phosphatase alpha and p59fyn. J. Biol. Chem. 273 (15), 8691.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J., Cerretti, D.P., 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385 (6618), 729–733.

Bouyain, S., Watkins, D.J., 2010. The protein tyrosine phosphatases PTPRZ and PTPRG bind to distinct members of the contactin family of neural recognition molecules. Proc. Natl. Acad. Sci. U.S.A. 107 (6), 2443–2448.

Buist, A., Blanchetot, C., den Hertog, J., 2000a. Involvement of the membrane distal catalytic domain in pervanadate-induced tyrosine phosphorylation of receptor protein-tyrosine phosphatase alpha. Biochem. Biophys. Res. Commun. 267 (1), 96.

Buist, A., Blanchetot, C., Tertoolen, L.G., den Hertog, J., 2000b. Identification of p130cas as an *in vivo* substrate of receptor protein-tyrosine phosphatase alpha. J. Biol. Chem. 275 (27), 20754.

Buist, A., Zhang, Y.L., Keng, Y.F., Wu, L., Zhang, Z.Y., den Hertog, J., 1999. Restoration of potent protein-tyrosine phosphatase activity into the membrane-distal domain of receptor protein-tyrosine phosphatase alpha. Biochemistry 38 (3), 914.

Chen, C., Okayama, H., 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7 (8), 2745–2752.

Chen, M., Chen, S.C., Pallen, C.J., 2006. Integrin-induced tyrosine phosphorylation of protein-tyrosine phosphatase-alpha is required for cytoskeletal reorganization and cell migration. J. Biol. Chem. 281 (17), 11972–11980.

Chow, J.P., Fujikawa, A., Shimizu, H., Noda, M., 2008a. Plasmin-mediated processing of protein tyrosine phosphatase receptor type Z in the mouse brain. Neurosci. Lett. 442 (3), 208–212.

Chow, J.P., Fujikawa, A., Shimizu, H., Suzuki, R., Noda, M., 2008b. Metalloproteinase- and gamma-secretase-mediated cleavage of protein-tyrosine phosphatase receptor type Z. J. Biol. Chem. 283 (45), 30879–30889.
Daum, G., Regenass, S., Sap, J., Schlessinger, J., Fischer, E.H., 1994. Multiple forms of the human tyrosine phosphatase RPTP alpha. Isozymes and differences in glycosylation. J. Biol. Chem. 269 (14), 10524.
den Hertog, J., Pals, C.E., Peppelenbosch, M.P., Tertoolen, L.G., de Laat, S.W., Kruijer, W., 1993. Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. EMBO J. 12 (10), 3789.
den Hertog, J., Sap, J., Pals, C.E., Schlessinger, J., Kruijer, W., 1995. Stimulation of receptor protein-tyrosine phosphatase alpha activity and phosphorylation by phorbol ester. Cell Growth Differ. 6 (3), 303.
Dilaver, G., van de Vorstenbosch, R., Tarrega, C., Rios, P., Pulido, R., van Aerde, K., Fransen, J., Hendriks, W., 2007. Proteolytic processing of the receptor-type protein tyrosine phosphatase PTPBR7. FEBS J. 274 (1), 96–108.
Dong, J., Wiley, H.S., 2000. Trafficking and proteolytic release of epidermal growth factor receptor ligands are modulated by their membrane-anchoring domains. J. Biol. Chem. 275 (1), 557–564.
Gil-Henn, H., Volohonsky, G., Elson, A., 2001. Regulation of protein-tyrosine phosphatases alpha and epsilon by calpain-mediated proteolytic cleavage. J. Biol. Chem. 276 (34), 31772.
Harder, K.W., Møller, N.P.H., Peacock, J.W., Jirik, F.R., 1998. Protein-tyrosine phosphatase alpha regulates Src family kinases and alters cell-substratum adhesion. J. Biol. Chem. 273 (48), 31890.
Hooper, N.M., Karran, E.H., Turner, A.J., 1997. Membrane protein secretases. Biochem. J. 321 (Pt 2), 265–279.
Hu, Q.D., Ang, B.T., Karsak, M., Hu, W.P., Cui, X.Y., Duka, T., Takeda, Y., Chia, W., Sankar, N., Ng, Y.K., Ling, E.A., Maciag, T., Small, D., Trifonova, R., Kopan, R., Okano, H., Nakafuku, M., Chiba, S., Hirai, H., Aster, J.C., Schachner, M., Pallen, C.J., Watanabe, K., Xiao, Z.C., 2003. F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. Cell 115 (2), 163–175.
Imbrici, P., Tucker, S.J., D'Adamo, M.C., Pessia, M., 2000. Role of receptor protein tyrosine phosphatase alpha (RPTPalpha) and tyrosine phosphorylation in the serotonergic inhibition of voltage-dependent potassium channels. Pflugers Arch. 441 (2–3), 257.
Kaplan, R., Morse, B., Huebner, K., Croce, C., Howk, R., Ravera, M., Ricca, G., Jaye, M., Schlessinger, J., 1990. Cloning of three human tyrosine phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain. Proc. Natl. Acad. Sci. U.S.A. 87 (18), 7000.
Kapp, K., Siemens, J., Weyrich, P., Schulz, J.B., Haring, H.U., Lammers, R., 2007. Extracellular domain splice variants of a transforming protein tyrosine phosphatase alpha mutant differentially activate Src-kinase dependent focus formation. Genes Cells 12 (1), 63–73.
Kirchberger, S., Majdic, O., Bluml, S., Schrauf, C., Leitner, J., Gerner, C., Paster, W., Gundacker, N., Sibilia, M., Stockl, J., 2008. The cytoplasmic tail of CD45 is released from activated phagocytes and can act as an inhibitory messenger for T cells. Blood 112 (4), 1240–1248.
Krndija, D., Schmid, H., Eismann, J.-L., Lother, U., Adler, G., Oswald, F., Seufferlein, T., von Wichert, G., 2010. Substrate stiffness and the receptor-type protein phosphatase alpha regulate spreading of colon cells through cytoskeletal contractility. Oncogene 29, 2724–2738.
Krueger, N.X., Streuli, M., Saito, H., 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. EMBO J. 9 (10), 3241.
Kunkel, T.A., Roberts, J.D., Zakour, R.A., 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154, 367–382.
Lammers, R., Møller, N.P.H., Ullrich, A., 1998. Mutant forms of the protein tyrosine phosphatase a show differential activities towards intracellular substarates. Biochem. Biophys. Res. Commun. 242, 32–38.
Lammers, R., Lerch, M.M., Ullrich, A., 2000. The carboxyl-terminal tyrosine residue of protein-tyrosine phosphatase alpha mediates association with focal adhesion plaques. J. Biol. Chem. 275 (5), 3391.
Maksumova, L., Le, H.T., Muratkhodjaev, F., Davidson, D., Veillette, A., Pallen, C.J., 2005. Protein tyrosine phosphatase alpha regulates Fyn activity and Cbp/PAG phosphorylation in thymocyte lipid rafts. J. Immunol. 175 (12), 7947–7956.
Matthews, R.J., Cahir, E.D., Thomas, M.L., 1990. Identification of an additional member of the protein-tyrosine-phosphatase family: evidence for alternative splicing in the tyrosine phosphatase domain. Proc. Natl. Acad. Sci. U.S.A. 87 (12), 4444–4448.
Merlos-Suarez, A., Arribas, J., 1999. Mechanisms controlling the shedding of transmembrane molecules. Biochem. Soc. Trans. 27 (2), 243–246.
Merlos-Suarez, A., Fernandez-Larrea, J., Reddy, P., Baselga, J., Arribas, J., 1998. Pro-tumor necrosis factor-alpha processing activity is tightly controlled by a component that does not affect notch processing. J. Biol. Chem. 273 (38), 24955–24962.
Møller, N.P., Møller, K.B., Lammers, R., Kharitonenkov, A., Hoppe, E., Wiberg, F.C., Sures, I., Ullrich, A., 1995. Selective down-regulation of the insulin receptor signal by protein-tyrosine phosphatases alpha and epsilon. J. Biol. Chem. 270 (39), 23126.
Murphy, G., 2008. The ADAMs: signalling scissors in the tumour microenvironment. Nat. Rev. Cancer 8 (12), 929–941.
O'Grady, P., Krueger, N.X., Streuli, M., Saito, H., 1994. Genomic organization of the human LAR protein tyrosine phosphatase gene and alternative splicing in the extracellular fibronectin type-III domains. J. Biol. Chem. 269 (40), 25193.
Ponniah, S., Wang, D.Z.M., Lim, K.L., Pallen, G.J., 1999. Targeted disruption of the tyrosine phosphatase PTP alpha leads to constitutive downregulation of the kinases Src and Fyn. Curr. Biol. 9 (10), 535.
Pulido, R., Krueger, N.X., Serra, P.C., Saito, H., Streuli, M., 1995. Molecular characterization of the human transmembrane protein-tyrosine phosphatase delta. Evidence for tissue-specific expression of alternative human transmembrane protein-tyrosine phosphatase delta isoforms. J. Biol. Chem. 270 (12), 6722.
Ruhe, J.E., Streit, S., Hart, S., Ullrich, A., 2006. EGFR signaling leads to downregulation of PTP-LAR via TACE-mediated proteolytic processing. Cell. Signal. 18 (9), 1515–1527.
Seidah, N.G., Chretien, M., 1997. Eukaryotic protein processing: endoproteolysis of precursor proteins. Curr. Opin. Biotechnol. 8 (5), 602–607.
Su, J., Muranjan, M., Sap, J., 1999. Receptor protein tyrosine phosphatase alpha activates Src-family kinases and controls integrin-mediated responses in fibroblasts. Curr. Biol. 9 (10), 505.
Tchilian, E.Z., Beverley, P.C., 2006. Altered CD45 expression and disease. Trends Immunol. 27 (3), 146–153.
Thomas, M.L., 1989. The leukocyte common antigen family. Annu. Rev. Immunol. 7, 339–369.
Tonks, N.K., 2006. Protein tyrosine phosphatases: from genes, to function, to disease. Nat. Rev. Mol. Cell Biol. 7 (11), 833–846.
Tracy, S., van der Geer, P., Hunter, T., 1995. The receptor-like protein-tyrosine phosphatase, RPTP alpha, is phosphorylated by protein kinase C on two serines close to the inner face of the plasma membrane. J. Biol. Chem. 270 (18), 10587.
Tremper-Wells, B., Resnick, R.J., Zheng, X., Holsinger, L.J., Shalloway, D., 2010. Extracellular domain dependence of PTPalpha transforming activity. Genes Cells 15 (7), 711–724.
Tsai, W., Morielli, A.D., Cachero, T.G., Peralta, E.G., 1999. Receptor protein tyrosine phosphatase alpha participates in the m1 muscarinic acetylcholine receptor-dependent regulation of Kv1.2 channel activity. EMBO J. 18 (1), 109.
Turner, A.J., Hooper, N.M., 1999. Role for ADAM-family proteinases as membrane protein secretases. Biochem. Soc. Trans. 27 (2), 255–259.
Vacaresse, N., Moller, B., Danielsen, E.M., Okada, M., Sap, J., 2008. Activation of c-Src and Fyn kinases by protein-tyrosine phosphatase RPTPalpha is substrate-specific and compatible with lipid raft localization. J. Biol. Chem. 283 (51), 35815–35824.
Vecchi, M., Rudolph, O.L.A., Brown, C.L., Dempsey, P.J., Carpenter, G., 1998. Tyrosine phosphorylation and proteolysis. Pervanadate-induced, metalloprotease-dependent cleavage of the ErbB-4 receptor and amphiregulin. J. Biol. Chem. 273 (32), 20589.
Wang, Q., Rajshankar, D., Branch, D.R., Siminovitch, K.A., Herrera Abreu, M.T., Downey, G.P., McCulloch, C.A., 2009. Protein-tyrosine phosphatase-alpha and Src functionally link focal adhesions to the endoplasmic reticulum to mediate interleukin-1-induced Ca^{2+} signaling. J. Biol. Chem. 284 (31), 20763–20772.
Wojtowicz-Praga, S.M., Dickson, R.B., Hawkins, M.J., 1997. Matrix metalloproteinase inhibitors. Invest. New Drugs 15 (1), 61–75.
Yan, H., Grossman, A., Wang, H., D'Eustachio, P., Mossie, K., Musacchio, J.M., Silvennoinen, O., Schlessinger, J., 1993. A novel receptor tyrosine phosphatase-sigma that is highly expressed in the nervous system. J. Biol. Chem. 268 (33), 24880–24886.
Zeng, L., D'Alessandri, L., Kalousek, M.B., Vaughan, L., Pallen, C.J., 1999. Protein tyrosine phosphatase alpha (PTPalpha) and contactin form a novel neuronal receptor complex linked to the intracellular tyrosine kinase fyn. J. Cell Biol. 147 (4), 707.
Zeng, L., Si, X., Yu, W.P., Le, H.T., Ng, K.P., Teng, R.M., Ryan, K., Wang, D.Z., Ponniah, S., Pallen, C.J., 2003. PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. J. Cell Biol. 160 (1), 137–146.
Zheng, X.M., Wang, Y., Pallen, C.J., 1992. Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. Nature 359 (6393), 336.