



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 (PTP α) 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. PTP α is cleaved by calpain in its intracellular domain, which decreases its potential to dephosphorylate Src kinase. Here, we demonstrate that PTP α 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 PTP α . After transient overexpression of ADAM17 and PTP α 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 PTP α regulation.

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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). PTP α 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 PTP α 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 PTP α have an impaired Src activity (Ponniah et al., 1999; Su et al., 1999) whereas overexpression of PTP α 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, PTP α 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 (Vacaressse et al., 2008) in a Src family kinase dependent manner. In addition, PTP α together with Src is involved in interleukin-1 induced calcium-signaling (Wang et al., 2009).

The extracellular domains of PTP α and the related PTP β 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), PTP δ (Pulido et al., 1995) and PTP σ (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 PTP α 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 PTP α 132 and PTP α 123, respectively. The functional consequence of this structural diversity is a reduced Src activation of the shorter form (PTP α 123), as determined in a focus formation assay using Src overexpressing cells and a PTP α 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 PTP α 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-2 β 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 PTP ζ is cleaved by plasmin and/or a combination of a metalloproteinase/ γ -secretase (Chow et al., 2008a, b). Finally, PTP α and PTP ϵ 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 PTP α 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 PTP α (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 (PTP α 132) was cleaved. The ADAM family member ADAM17/TACE (TNF- α converting enzyme) was identified as the responsible protease. Processing of PTP α 132 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 PTP α splice variants containing (PTP α 132) or lacking (PTP α 123) 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 PTP α 132, 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' GGATAAGCGAAAGCATTGGAATGGCTCC and S213A: 5' TTTCTGTGGTGGCTGGGGATCTGGCCAG. 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' AGGTTTCCCA-GAGAGGTGGT. Mutation of the carboxy-terminal tyrosine or the catalytic cysteine residue has been described earlier (Lammers et al., 1998). The cDNA encoding PTP α Δ NT 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 PTP α 132 splice variant in the extracellular domain. Because of the lack of a signal peptide, this protein is located intracellularly. The construct PTP α intra was generated by PCR using the oligonucleotide 5'-BglII-site – GCCATGGCAAGTT-TAAGAAATACAAGCA and cloned into the expression vector pRK5. This generates a PTP α protein starting with the juxtamembrane sequence (M-A-) RFKKY-(PTP α).

Antibodies and cell lines

Mouse monoclonal antibodies 29 and 45 are directed against the first phosphatase domain of PTP α (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 μ l of lysis buffer/6-well-dish (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA; 10% (v/v) glycerol; 1% (v/v) Triton X-100; 100 mM NaF; 10 mM Na₄P₂O₇; 1 mM Na₃VO₄; 1 mM phenylmethylsulfonyl fluoride; 10 μ g/ml aprotinin), and the lysates pre-cleared by centrifugation at 13,000 \times 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 μ g) 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 PTP α present in the lysates.

Focus formation assays

BOSC23 cells were transfected with pLXSN plasmids containing the PTP α or ADAM17 cDNAs and the supernatants used to infect GP+E cells. After genetic 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 μ g/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

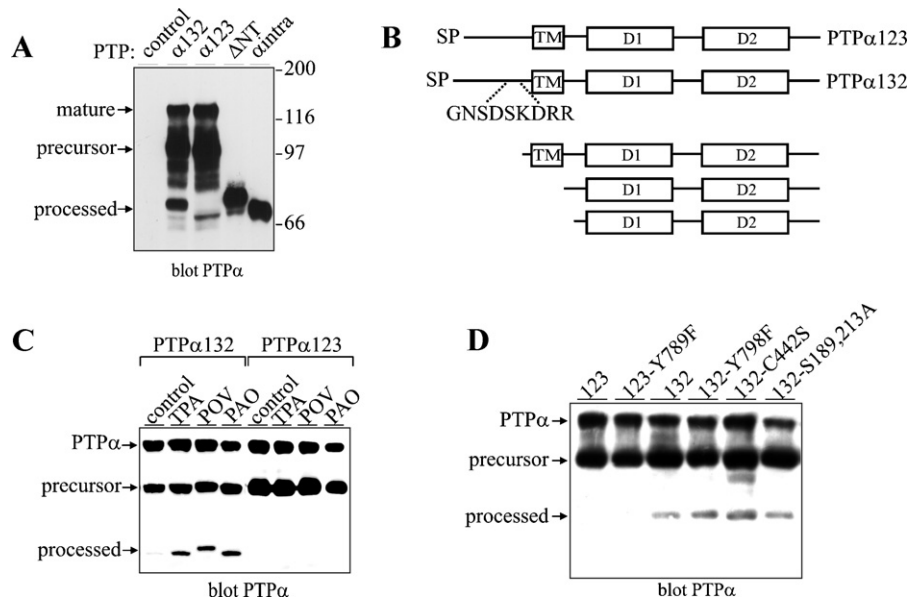


Fig. 1. Proteolytic processing of overexpressed PTP α can be induced by different substances. Two hundred and ninety three cells were transfected with the indicated constructs and 48 h later the cells lysed, proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and PTP α and its cleavage products immunodetected with antibody mab29. (A) PTP α 132 is proteolytically processed in the extracellular domain. (B) Schematic representation of PTP α splice variants and their cleavage products. Amino acids 121–129 (GNSDSKDRR) are found only in PTP α 132. PTP α 123 is processed in the juxtamembrane domain to yield 63 and 68 kDa proteins whereas only PTP α 132 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 proteolytic processing of PTP α 132. Cells were transfected and stimulated for 1 h at 37 °C with 400 μ M POV, 35 μ M PAO, or 1 μ M TPA before harvest. (D) Neither PTP α 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

PTP α 132 but not PTP α 123 is processed to generate a 75 kDa protein

In a previous report we have demonstrated that PTP α 132 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 PTP α 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 PTP α 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 PTP α 132 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 (PTP α 123) only proteins of 68 and 63 kDa were found. To determine the region of cleavage, two truncated forms of PTP α were expressed in parallel and run next to PTP α full length. PTP α Δ NT lacks the signal sequence but contains an amino-terminal HA-tag and starts at the nine amino-acid insert of the extracellular domain whereas PTP α intra encodes the complete intracellular domain plus start methionine. Since the 75 kDa protein described above compares to the size of PTP α Δ NT, the cleavage site for PTP α 132 generating this fragment is likely extracellular and close to the membrane. The 63 kDa protein found with both PTP α forms is smaller than PTP α intra and thus is cleaved in the cytoplasm. It should be identical to the calpain cleavage product of PTP α 123 (Gil-Henn et al., 2001). An additional cleavage giving rise

to the 68 kDa protein has the size of the PTP α intra and might occur right at the plasma membrane but is seen for PTP α 132 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 PTP α 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 PTP α 132. 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 PTP α 132 or PTP α 123 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 PTP α 132. 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 PTP α (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 PTP α , processing was observed upon stimulation with POV (data not shown).

We next analyzed whether TPA dependent serine phosphorylation of PTP α 132, 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).

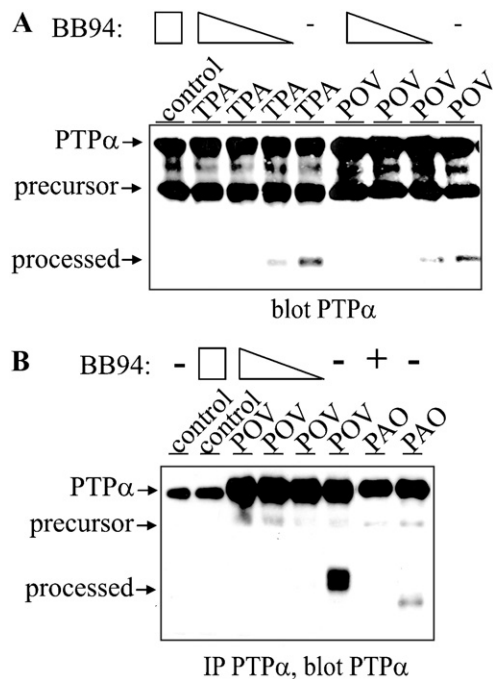


Fig. 2. Extracellular cleavage of PTP α is sensitive to Batimastat. (A) Two hundred and ninety three cells transiently expressing PTP α 132 were preincubated for 30 min with 5 μ M, 0.5 μ M, 50 nM Batimastat (BB94), or vehicle alone, subsequently stimulated with TPA or POV for 1 h and then lysed. After transfer to nitrocellulose filters, proteins were detected by immunoblotting with mab29. (B) BIR α 132 cells were either preincubated with decreasing concentrations (5 μ M–50 nM) or with 0.5 μ M BB94 (+), stimulated with POV for 1 h as indicated and then analyzed as above.

The metalloprotease inhibitor BB94 (Batimastat) inhibits extracellular PTP α 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 PTP α 132 processing, 293 cells overexpressing PTP α 132 were pre-incubated with BB94 for 30 min (Fig. 2A). BB94 concentrations down to 50 nM almost completely blocked PTP α 132 processing upon TPA or POV stimulation. By contrast, BB94 had no effect on intracellular cleavage of PTP α (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 PTP α 132 and found that similar concentrations were able to block processing (Fig. 2B).

Extracellular processing of PTP α 132 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 PTP α 132, 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, PTP α 132 and its fragments immunoprecipitated, and cleavage detected in Western blot analysis. Under these conditions, the parental CHO cell line exhibited considerable constitutive processing of PTP α 132 (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

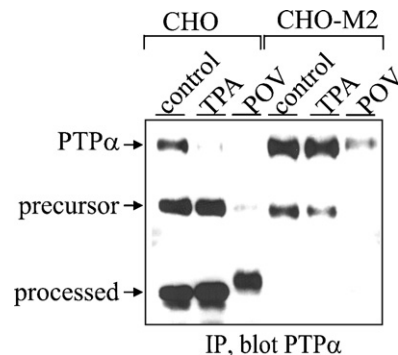


Fig. 3. ADAM17 activity deficient M2 cells are incapable of processing the PTP α extracellular domain. CHO M2 cells or parental CHO cells transiently expressing PTP α 132 were left untreated or stimulated with 1 μ M TPA or 200 μ M POV for 1 h. Cell lysates were immunoprecipitated with anti-PTP α antibody mab45, size separated by SDS-PAGE and proteins analyzed by immunodetection with anti-PTP α mab29.

processing of PTP α 132 was detectable. Of note, both wild type CHO and the M2 cells showed repeatedly a reduced amount of total PTP α 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 PTP α 132.

ADAM17 cleaves PTP α in 293 and NIH3T3 cells

In order to confirm that ADAM17 is responsible, we investigated the effect upon overexpression of both, ADAM17 and PTP α in 293 cells. For transient transfection of PTP α and ADAM17 we used various amounts of PTP α expression plasmid to avoid transfection induced endogenous protease activity. As shown in Fig. 4A, at low PTP α 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 PTP α processing. A similar experiment was also performed in NIH3T3 cells stably expressing PTP α 132-Y798F that were infected with retrovirus encoding ADAM17 (Fig. 4B). The PTP α 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 PTP α , when cells were co-transfected with the corresponding expression plasmids (data not shown).

Effect of ADAM17 dependent cleavage on PTP α activity

Calpain induced cleavage of PTP α 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 PTP α activity. First, PTP α 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.

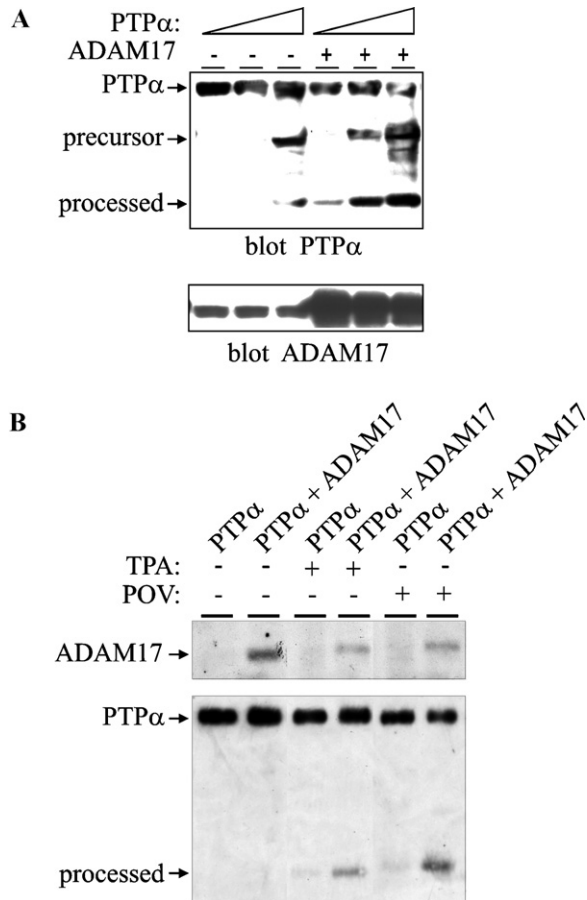


Fig. 4. ADAM17 dependent proteolytic processing of overexpressed PTP α . (A) Two hundred and ninety three cells were transfected with increasing amounts of PTP α 132 (0.5, 1.0 and 1.5 μ g cDNA) and 1 μ g of ADAM17 (as indicated in the figure). Cells were lysed, proteins separated by SDS-PAGE, transferred to nitrocellulose membranes and PTP α and its cleavage products immunoblotted with antibody mab29 or anti-ADAM17 antibodies (bottom panel). (B) A NIH3T3-PTP α Y798F 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 μ M, 45 min) or POV (200 μ M, 30 min) and subsequently lysed. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters and immunoblotted to detect ADAM17 (upper panel). PTP α was immunoprecipitated and detected as described above.

In BIR cells overexpressing the insulin receptor, continuous treatment with insulin reduces cell viability (Møller et al., 1995). Since PTP α 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 PTP α and ADAM17 but did not find different rescuing capacities (data not shown). Next, we investigated PTP α activity towards Src and employed focus formation assays. Upon infection of fibroblasts overexpressing a moderate amount of Src with retrovirus encoding PTP α 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 PTP α 123-Y789F mediated Src activation. By contrast, we found a reduction to 45% using the mutant PTP α 132-Y798F. We concluded from this experiment that ADAM17 dependent processing reduced the PTP α 132 dependent activation of Src. However, in an independent approach, we infected NIH3T3 cells with viruses encoding PTP α 123-Y789F or PTP α 132-Y798F either alone or together with ADAM17. We prepared cell lysates

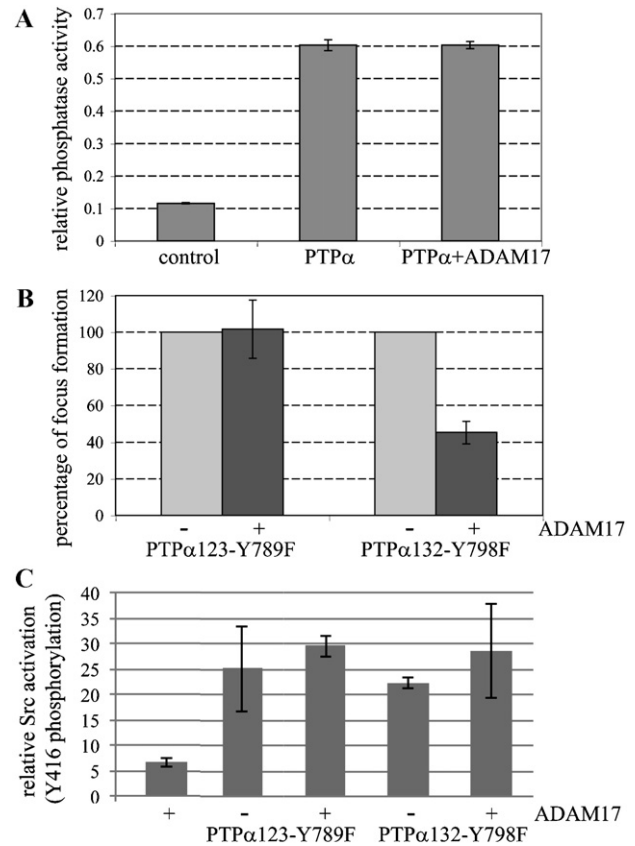


Fig. 5. ADAM17 does not affect PTP α 's potential to dephosphorylate pNPP but impairs the PTP α mediated activation of c-Src in a focus formation assay. (A) Lysates of 293 cells transiently expressing PTP α 132 alone or together with ADAM17 were used to test the phosphatase activity with pNPP as substrate in a photometric assay. Similar lysates as presented in Fig. 4A were used, showing about 25% (PTP α 132) or 30% (PTP α 132 + ADAM17) processing. The measured activity was corrected for PTP α expression. The bars represent the phosphatase activity as means \pm 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 PTP α (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 \pm SEM in relation to infections without ADAM17 (100%) and are generated from five independent experiments. (C) NIH3T3 cells were infected with retroviruses encoding either PTP α 123-Y789F or PTP α 132-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 \pm SEM.

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 PTP α 132-Y798F and ADAM17, suggesting that the effect of ADAM17 overexpression is independent of Src activation (Fig. 5C). This leaves the possibility that PTP α 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 PTP α : 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 PTP α 132 variant is a stronger Src activator than PTP α 123 (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 TNF α) 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. PTP ζ (PTPR ζ) 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 PTP α 132 that could correspond to an intramembrane cleavage product, we tested a nuclear localization. However, after transient overexpression of PTP α 132 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 PTP ζ , 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 PTP α 132 in which the presence of ADAM17 reduced focus formation. In addition, we observed the degradation of the processed PTP α 132 fragment. An active degradation of the intracellular PTP α 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 PTP α 132 from the membrane.

Since ADAM17 in most experimental settings is associated with proliferation it seems odd that its overexpression reduced PTP α and Src dependent focus formation. However, PTP α can act in opposite directions depending on the cellular context. Thus, overexpression of PTP α 123 in fibroblasts induced cellular transformation (Zheng et al., 1992) whereas in tumor-derived MCF-7 cells it suppressed transformation (Ardini et al., 2000). PTP α 132 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 PTP α 132 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 PTP α 132 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, PTP α has been described as a force-responsive protein that interacts with the extracellular matrix and regulates spreading of colon cancer cells through cytoskeletal contractility (Krnjija 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 PTP ζ (Bouyain and Watkins, 2010) or Notch (Hu et al., 2003). Of note, PTP α 132 is increasingly expressed during neuronal development and can make up to 50% of total PTP α 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 PTP α 132 in tumor-derived samples. This is in part in agreement with our own data, detecting no elevated amounts of PTP α 132 in primary breast tumor samples although a low level mostly was present (not shown).

Taken together, we have identified a novel mechanism of PTP α 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 PTP α 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>.

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