Extracellular domain splice variants of a transforming protein tyrosine phosphatase α mutant differentially activate Src-kinase dependent focus formation

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The extracellular domains of receptor-type protein-tyrosine phosphatases (PTPs) contain a diverse range of protein modules like fibronectin- or immunoglobulin-like structures. These are frequently expressed in a tissue- and development specific manner as splice variants. The extracellular domain of PTPα is rather short and heavily glycosylated. Two splice variants are known, which it differs by an exon encoding nine amino acids within the extracellular domain. We have analyzed the expression pattern of both variants and found that the smaller form is ubiquitously expressed while the larger form was found at an increased level only in brain, some skeletal muscle and differentiating cells like granule neurons, adipocytes and myotubes. The phosphatase activity of both forms was similar when tested *in vitro* using para-nitrophenylphosphate as a substrate and in a transient expression system with the substrates c-Fyn or c-Src. In a quantitative focus formation assay the capability of the larger form to activate Src-dependent focus formation in intact cells was increased more than twofold whereas the capability to dephosphorylate the insulin receptor in a BHK cell system was similar. We conclude that the two splice variants of PTPα are expressed differentially and regulate c-Src activity in different ways.

Introduction

Protein tyrosine phosphorylation is a major mechanism of cellular signal transduction and is regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Schlessinger 2000; Ostman & Bohmer 2001). There are currently 113 vertebrate PTPs known (Andersen *et al.* 2001), which are either cytoplasmic or membrane-bound enzymes. Alternative splicing is a common feature of receptor type PTPs including CD45 (Johnson *et al.* 1989), PTPλ (Fang *et al.* 1994), PTPδ, PTPσ and LAR (Pulido *et al.* 1995), as well as PTPβ (Krueger *et al.* 1990), GLEPP1 (Aguiar *et al.* 1999) and PTPRR (Chirivi *et al.* 2004). For CD45, alternative splicing of three exons close to the amino-terminus would result in eight isoforms, of which several have been identified (Hermiston *et al.* 2003). A tissue-specific regulation of alternative splicing has been shown (Hathcock *et al.* 1993) and, additionally, an impact of the varying extracellular domains on protein function (Leitenberg *et al.* 1996). More recently, it was reported that the alternatively spliced isoforms homodimerize differentially, resulting in modified protein activity (Xu & Weiss 2002).

The extracellular domains of PTPα and PTPε do not encode characteristic domains but are heavily glycosylated. For PTPα, three splice variants have been described. One variant contains an insert of 36 amino acids in the phosphatase domain (Matthews *et al.* 1990). Further, there exist two isoforms due to an alternative splicing of a 27 bp mini-exon localized in the juxtamembrane extracellular domain. The resulting isoforms have an extracellular domain of 123 or 132 amino acids in the mature protein (Kaplan *et al.* 1990; Krueger *et al.* 1990; Matthews *et al.* 1990; Sap *et al.* 1990) and here it is referred to as small and large isoform or PTPα123 and

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PTPα132, respectively. The tissue-specific expression pattern of the isoforms has not yet been evaluated in detail. Daum *et al.* (1994) found mainly the small form when inspecting three different tissues or cell lines. Analyzing PTPα expression in the major insulin target tissues, Norris *et al.* (1997) detected a general, quite high expression of the smaller isoform whereas the larger isoform was expressed in fat but hardly in other tissues like liver, skeletal muscle and endothelial. However, a possible functional difference of the isoforms has not been looked into.

In the present study, we investigated the differential expression of the isoforms and the difference in their activation potential towards Src kinases and the insulin receptor that are known substrates of PTPα. While PTPα123 was expressed in most tissues, expression of PTPα132 was generally low but up-regulated in some tissues like brain and skeletal muscle. Using focus formation as a cell-based quantitative assay, the larger isoform was more efficient to activate Src-dependent focus formation whereas inactivation of the insulin receptor signal in a different functional and quantitative assay was similar for both isoforms.

Results

Tissue-specific expressions of the PTPα splice variants

Expression analysis of PTPα through Northern blotting using murine and human tissues revealed a ubiquitous expression, with murine brain and kidney having the highest expression levels (Matthews *et al.* 1990; Sap *et al.* 1990) whereas human adult brain showed little expression (Kaplan *et al.* 1990). To verify the expression in human tissues and to evaluate the contribution of the individual PTPα splice variants to total expression, we performed RT-PCR of several tissues and cell lines with primers, which amplified a region covering the alternatively spliced exon (Fig. 1A). Using this approach, we simultaneously detected both isoforms in a single reaction and could directly compare their relative expression. As size controls, PCRs using the cDNAs of human PTPα123 and PTPα132 were performed in parallel. We detected the smaller splice variant PTPα123 as the predominant variant in most sources used, which is similar to the findings of Daum *et al.* (1994). Although mostly at a low level, the amplification product for the larger splice variant, PTPα132, was found at a significantly higher level in human brain and some, but not all, human skeletal muscle derived cDNAs (SKM II and muscle biopsy; Fig. 1B).

With the determination of a higher expression of the larger PTPα isoform in brain and muscle, we noted that both tissues develop through differentiation of precursor cells. Since Fang *et al.* (1996) have described a varying expression of PTPα in the cerebellum, we wanted to investigate a possible time course of isoform-specific expression. To this end, we prepared cerebellum cDNA from rats at different postnatal stages of development for RT-PCR analysis. As expected, we found two PCR fragments, showing that PTPα also occurs with two isoforms in rat. During the first 14 days, the expression of the smaller form remained constant whereas expression of the larger form increased to a similar level as the smaller form (Fig. 1C). The time dependent variation of expression in rat cerebellum suggested an increasingly important role of this splice variant during rat development. This would correlate with the migration and differentiation of granule neurons. Therefore, we tested this hypothesis using primary cultures of granule neurons prepared from rats sacrificed at postnatal day 7. The incubation of these cultures with cytosine-arabinoside arrested the growth of non-neuronal cells, so that more than 95% of the cultured cells belonged to the granule neuron population. One week after preparation, the cultures displayed a dense meshwork of dendritic connections reflecting differentiation of the neurons. At days 1 and 7 after preparation, the cells were lysed and the expression pattern of the PTPα-isoforms analyzed. Figure 1C demonstrates that the granule neuron population expressed increasing amounts of the larger splice variant of PTPα in a differentiation dependent manner.

Since a higher expression of the larger splice variant was also revealed in skeletal muscle, we investigated the expression pattern of PTPα in the murine *in vitro* differentiating C2C12 myoblasts. In addition, 3T3L1 cells were included which are a model cell line for the differentiation to adipocytes. Again, a similar level of the smaller phosphatase splice variant was detected in both cell lines independent of the differentiation status, whereas PTPα132 expression increased during differentiation (Fig. 1D). In summary, both splice variants occur in human, mouse and rat, and the expression of the larger splice variant PTPα132 can increase at specific stages in development or differentiation of some tissues.

PTPα splice variants and their phosphatase activity *in vitro* and towards the insulin receptor

To determine a possible difference in the enzymatic activity of the splice variants, we first used p-nitrophenyl phosphate (pNPP) as a substrate. The splice variants of PTPα were transiently over-expressed in human 293 cells and the lysates directly used for the dephosphorylation assay. In addition, we included PTPα mutants of the carboxyl-terminal tyrosine residue to phenylalanine in

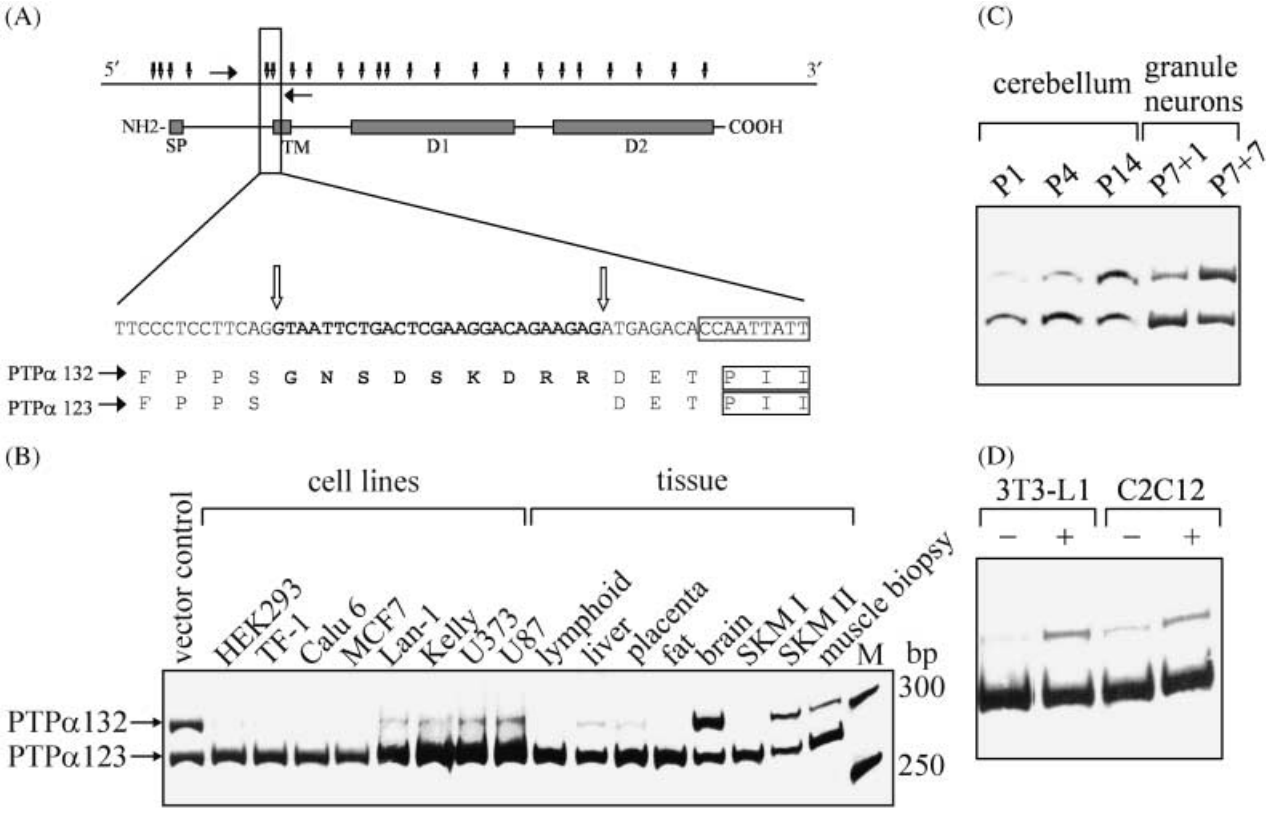

Figure 1 Tissue-specific expression of PTPα splice variants. (A) Schematic representation of PTPα and its alternatively spliced exons. The upper panel shows the exon–intron boundaries based on the published sequence (GenBank Acc. No. HSDJ534B8). Horizontal arrows indicate the position of the primers used for RT-PCR amplification, resulting in fragments of 310 or 283 bp for human RNA. The bottom panel shows the DNA and protein sequence of the alternatively spliced 27 bp miniexon (bold, vertical arrows) including its surrounding sequence. SP, signal peptide; TM, transmembrane segment; D1 and D2, the phosphatase domains. (B) PCR-generated fragments were separated on a 5% polyacrylamide gel and silver stained. Templates included vector controls (left lane), and cDNAs or reverse transcribed RNAs of the indicated human cell lines and tissues (origin of cell lines: 293, kidney; TF-1, bone marrow, erythroblast; Calu 6, lung carcinoma; MCF-7, mammary gland; LAN-1 and Kelly, neuroblastoma; U373 MG and U-87, glioblastoma). M, DNA size marker (bp); SKM, skeletal muscle. (C) RNA from rat cerebellum was isolated at postnatal days 1, 4 and 14. Granule neurons were isolated from rat cerebellum at postnatal day 7 and differentiated *in vitro* for 1 or 7 days. PCR fragments of 302 or 275 bps were separated as described above. P, postnatal day. (D) RNA from 3T3-L1 and C2C12 cells was isolated from undifferentiated (–) or differentiated cells (+). RNA was reverse transcribed and subjected to PCR with species-specific primers yielding fragments of 261 bp and 234 bp. Electrophoretic separation was done as described above.

this assay, since our previous experiments indicated an important role in PTPα function (Lammers *et al.* 2000). As shown in Fig. 2, both splice variants and their mutants of the carboxyl-terminal tyrosine showed similar activity, with the observed minor differences not being statistically significant.

Our previous work has shown that wild-type PTPα132 and the Y798F mutant have a similar activity towards the insulin receptor (Lammers *et al.* 1998). We next investigated a possibly different activity of the two splice variants against the insulin receptor in a functional cellular assay. BHK-cells over-expressing the insulin receptor detach from the cell culture dish upon treatment with insulin. However, this effect can be abrogated by transfection of PTPα (Moller *et al.* 1995). Applying this system, we did not detect a significant difference of the splice variants in the ability to rescue the cells (data not shown).

PTPα splice variants differentially activate the Src family kinases in intact cells

Next, we employed a known protein substrate of PTPα, the Src kinase family member c-Fyn, to investigate the phosphatase activity in intact cells. c-Fyn was transiently

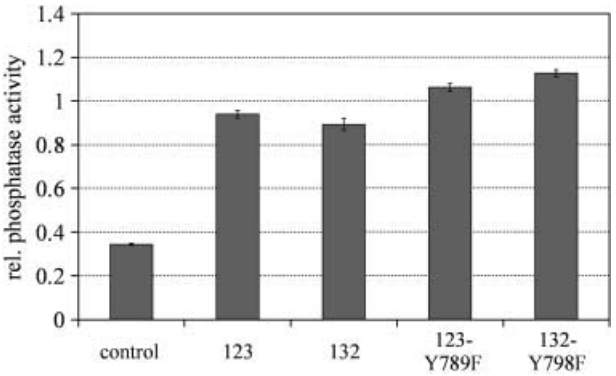

Figure 2 *In vitro* phosphatase activity of PTPα isoforms. 293 cells were transiently transfected with empty plasmid (control), PTPα123, PTPα132, PTPα123-Y789F or PTPα132-Y798F. Cells were lysed and PTP activity was assayed using pNPP as a substrate. The activity was corrected for PTPα expression. The histogram represents an experiment done in quadruplicates and shows the phosphatase activity as means ± SEM.

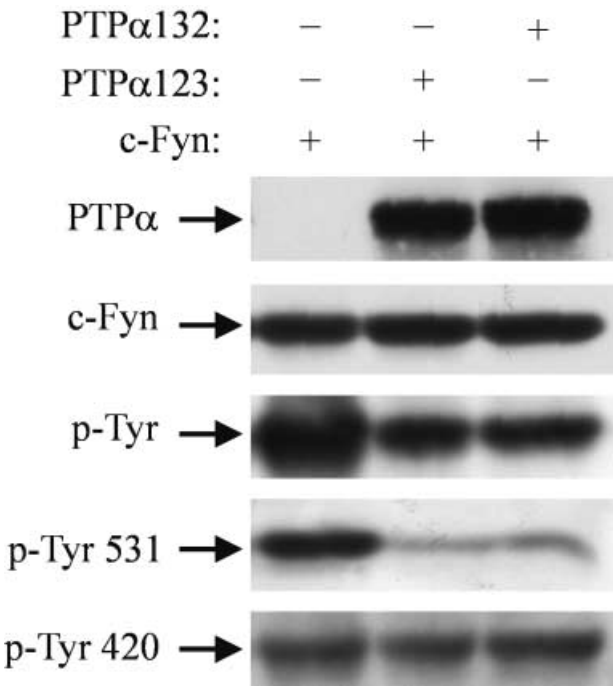

Figure 3 Both isoforms of PTPα dephosphorylate c-Fyn. 293 cells were transfected with 1 μg c-Fyn and 1 μg PTPα123 or PTPα132 expression plasmid as indicated in the figure. Cells were lysed, proteins were separated by SDS-PAGE and analyzed by immunoblotting with the antibodies as indicated.

over-expressed in 293 cells either alone or together with one of the two splice variants of PTPα and cell lysates were analyzed by immunoblotting. The top panels of Fig. 3 show that similar amounts of the splice variants of PTPα and c-Fyn were expressed. Investigating the tyrosine phosphorylation of c-Fyn, we found that overall phosphorylation was reduced upon co-expression of either variant of PTPα. The use of phosphopeptide-specific antibodies to c-Fyn revealed that Y531 at the carboxyl-terminus was specifically dephosphorylated in the presence of either of the two PTPα forms whereas Y420 in the activation loop of c-Fyn remained phosphorylated under these conditions. Using c-Src as a substrate in this system, similar data were obtained (not shown). Thus, under these conditions both splice variants behaved similarly.

We have previously used focus formation as a cell-based assay to determine the activity of different PTPα mutants under physiological conditions. Infection with retroviruses encoding PTPα132-Y798F but not the wild-type phosphatase led to transformation of fibroblast cells stably over-expressing a moderate level of c-Src, indicating that the tyrosine mutant strongly activated c-Src (Lammers *et al.* 2000). We have now employed this assay to determine the activity of the splice variants of PTPα. Previously, Lin *et al.* (1995) have observed a significant anchorage-independent growth and tumorigenicity when murine in contrast to chicken c-Src was over-expressed in NIH3T3 cells. Therefore, we used murine and chicken c-Src in parallel to rule out any effect of PTPα based on the origin of c-Src. PTPα was introduced into the cells by retroviral infection with similar multiplicities of infection for the different isoforms and mutants. Figure 4A shows that both wild-type isoforms, PTPα123 and PTPα132, did not lead to focus formation, whereas PTPα123-Y789F and PTPα132-Y798F activated c-Src (murine origin). Similar data were obtained, when using fibroblasts over-expressing a moderate amount of chicken c-Src (data not shown). As controls, we either infected parental NIH3T3 or used c-Src over-expressing cells, which were not infected with PTPα encoding retroviruses. In both cases, we did not observe any focus formation (data not shown). To quantitatively analyze the effect of the splice variants, we performed a series of five independent experiments that are summarized in Fig. 4B,C. The activation potential of the isoform PTPα132-Y798F exceeded the potential of the isoform PTPα123-Y789F by 181% for murine and 357% for chicken Src, with PTPα123-Y789F representing 100%, respectively. To ensure that expression of both variants was similar, NIH3T3 cells were infected at similar m.o.i. with the corresponding retroviruses, grown to confluence under selection and harvested. A similar expression was detected by Western blotting for

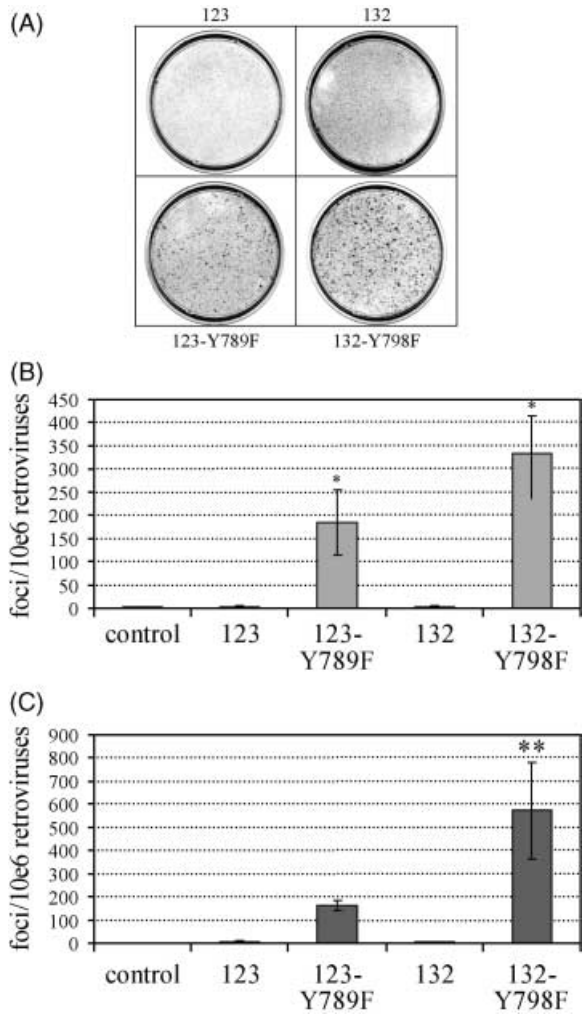

Figure 4 PTPα132-Y798F carries a higher c-Src activation potential. (A) NIH3T3 cells over-expressing murine c-Src were infected with retroviruses encoding the PTPα form indicated. Cells were cultured for 21 days and foci were stained with crystal violet. NIH3T3 cells over-expressing murine (B) or chicken c-Src (C) were infected with retroviruses encoding the indicated PTPα form; control cells were not infected. The histograms represent the average of foci/10e6 retroviruses, given as means ± SEM from five independent experiments. Statistics were done by analysis of variance and Student's *t*-test. The *P* values < 0.05 (*) and < 0.01 (**), as indicated in the figure, were considered to be significant with respect to any other column. The number of foci from control experiments in Fig. 4C as well as SEM from NIH3T3 cells over-expressing chicken c-Src and infected with PTPα132 are too small to be visualized.

both splice variants (Fig. 5C, lower panel). We conclude that the large PTPα isoform more efficiently leads to Src-dependent focus formation, and that the activation is stronger with c-Src derived from chicken.

As a further step, we investigated the activation of c-Src at the molecular level. To this end, we isolated several foci from the focus formation assays described above. After amplification of the cells, lysates were analyzed by immunoblotting using phosphopeptide-specific antibodies. Figure 5A shows a representative set of the c-Src over-expressing cell line and focus derived cell populations (from more than 20 foci analyzed). In control cells expressing murine c-Src, which also serves as a loading control, phosphorylation was found on Y527 but not on Y416. In foci from chicken or murine c-Src expressing cells infected with PTPα132-Y798F, the level of Y527 phosphorylation was reduced and the phosphorylation of Y416 enhanced. The analysis of cells over-expressing PTPα123-Y789F yielded a similar result, showing less phosphorylation of Y527 and enhanced phosphorylation of Y416. Densitometric scanning of the samples shown (Fig. 5B) and analysis of additional foci confirmed that despite of different expression levels of the two PTPα splice variants in the individual foci derived cell lines, the phosphorylation status of c-Src was similar. This was reflected in a Src-kinase assay using parental NIH3T3 cells infected with retrovirus encoding either PTPα isoform. The cells were grown as a pool under selection, and after lysis endogenous Src was immunoprecipitated. One half was assayed with enolase as a substrate, while the other half was directly loaded on the gel to verify similar amounts of Src protein in each immunoprecipitation (Fig. 5C). In addition, the presence of PTPα in the lysate was verified. We conclude that c-Src can be activated by both splice variants; however, the large form has a higher efficiency as represented by the higher number of foci.

One reason for the different behavior could be a difference in the formation of dimers by the two isoforms. To test this, we made use of PTPα extracellular domain mutant P137C, which induces a permanent dimerization due to the formation of stable disulfide bonds (numbering according to Jiang *et al.* (1999) which includes the signal peptide). The authors showed that this mutant had a decreased ability to activate c-Src *in vitro* kinase assays; however, the effect was only shown for the smaller PTPα isoform. To investigate a dimerization-dependent, different focus formation potential of the splice variants, we constructed P137C mutants for both splice variants (PTPα123 P137C,Y789F and PTPα132 P137C,Y798F), generated retroviruses and used them for focus formation assays as above. Interestingly, the number of foci generated by the viruses encoding the additional P137C mutant was similar to the number generated by the PTPα123-Y789F or PTPα132-Y798F form (data not shown). Since this result was unexpected, we performed

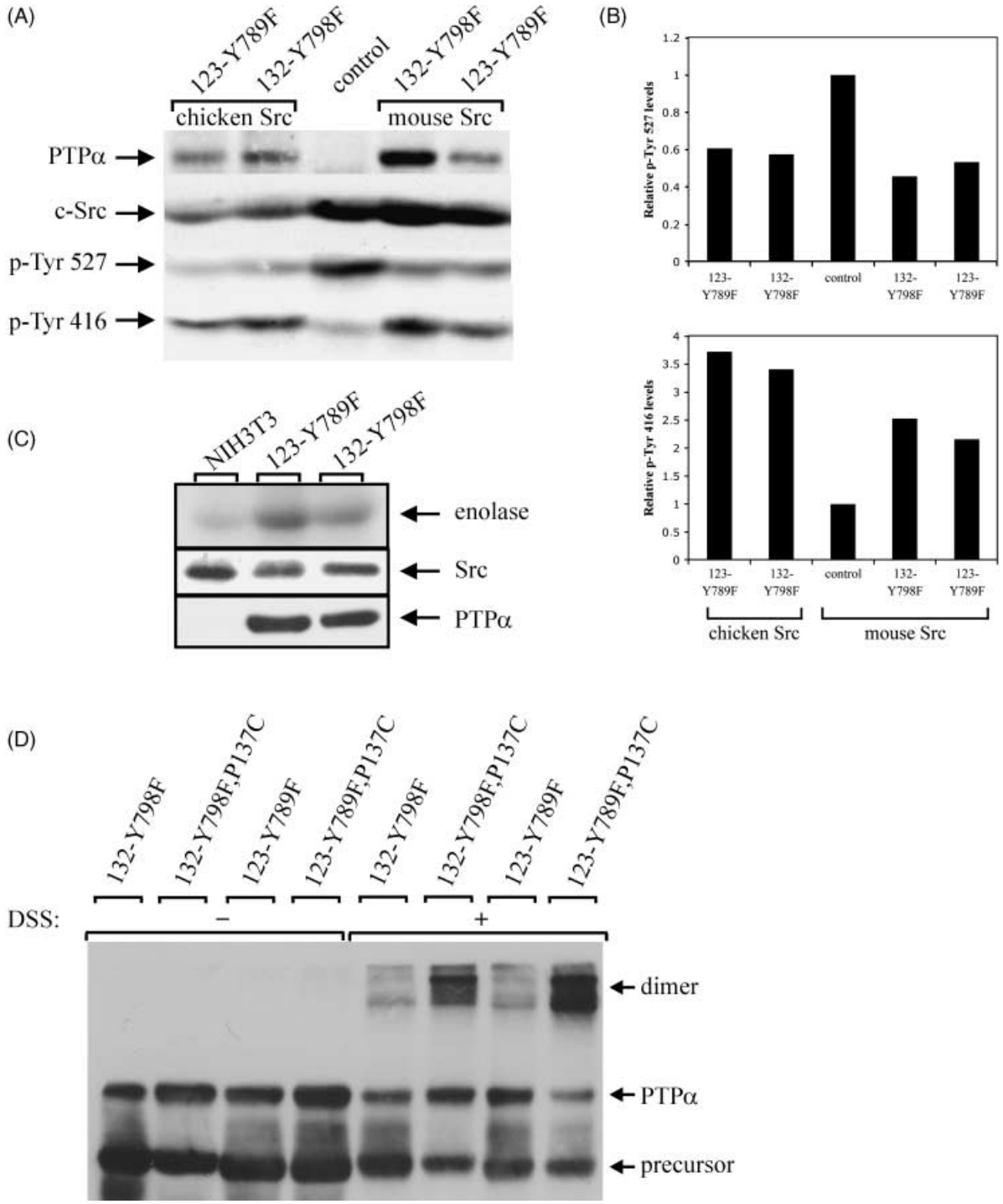

Figure 5 Analysis of c-Src activity. (A) Individual foci were picked, cells expanded and their lysates analyzed by immunoblotting. Data are representative for foci isolated from three independent focus formation assays. control, NIH3T3 cells over-expressing murine c-Src. (B) The relative amount of phosphotyrosine on residues 416 and 527, as shown in A, was quantified. (C) NIH3T3 cells were infected with retrovirus encoding the tyrosine mutant of either splice variant, grown to confluence under selection and Src-kinase activity determined using enolase as a substrate (upper panel). An aliquot of the immunoprecipitated Src is shown in a control blot (middle panel), as is an aliquot of the cell lysate where PTPα is detected (lower panel). (D) The indicated isoforms of PTPα were transiently over-expressed in 293 cells, cells lysed, proteins cross-linked and detected by Western blot analysis. DSS, disuccinimidyl suberate.

a transient expression of the PTPα mutants and analyzed their potential to dimerize by cross-linking of the proteins in the cell lysate. We did not detect a difference between the splice variants, and dimerization was enhanced by the P137C mutant (Fig. 5D). The slightly stronger dimerization that appears for PTPα123-Y789F, P137C is caused by a somewhat higher expression, as becomes apparent on shorter exposures. Thus, phosphatase dimerization and the P137C mutants are not suitable to explain the differential behavior of the splice variants during focus formation.

Discussion

In this study, we have investigated the expression of extracellular splice variants of PTPα and their possible physiological role. One isoform, PTPα123, was ubiquitously and constitutively expressed, whereas a higher expression of the second isoform with an additional exon, PTPα132, was tissue-specific and dependent on the differentiation status. The RT-PCR based expression analysis points towards a possible role for the larger splice variant in brain development. In support of this, in chicken cerebellum the expression of PTPα is enhanced during a comparable stage of development (Fang *et al.* 1996). However, alternative splicing products have not been detected for chicken PTPα, but the described isoform is the orthologue of PTPα132. PTPα expression was also described in zebra fish, especially in the nervous system (van der Sar *et al.* 2001), and the importance of PTPα for retinal development was shown (van der Sar *et al.* 2002). More recently and using PTPα knock-out mice, the role of PTPα in hippocampal neuronal migration and long-term potentiation was described (Petrone *et al.* 2003) as well as a function in learning and other forms of neuroplasticity (Skelton *et al.* 2003). It will be interesting to see whether these functions are isoform dependent.

Alternative splicing during neuronal development was also shown for PTP LAR. There are six alternatively spliced isoforms that differ by small exons ranging from 12 bp (LASE-b), to 27 bp (LASE-c), 33 bp (LASE-a) and 75 bp (LASE-d). The alternatively spliced exons of the LASE-c and LASE-d isoforms also occur in the extracellular domain, and for LASE-c, the expression decreases during CNS development but is up-regulated in NGF-induced PC12 cell differentiation (Zhang & Longo 1995).

In addition to brain, PTPα132 is expressed in some but not all skeletal muscle derived probes. In the C2C12 cell line an increase of PTPα132 correlated with the differentiation from myoblasts to the myotube-like stage. Using multiprobe RNase protection assays Norris *et al.* (1997) detected the highest amounts of this isoform in fat and not in skeletal muscle. A likely explanation for this difference is that PTPα expression is different in various types of skeletal muscle, as we have shown in Fig. 1B. The general importance of PTPα expression for differentiation of skeletal muscle has been demonstrated by Lu *et al.* (2002). In parallel to the analysis of a cellular model for skeletal muscle we investigated PTPα expression in a model for adipose tissue and also found an increased expression of the larger phosphatase form upon differentiation. A function of PTPα in adipose cells was investigated by Cong and co-workers showing that PTPα inhibits translocation of GLUT-4 upon insulin-stimulation (Cong *et al.* 1999). In addition to these tissues, we have recently presented data that both splice variants of PTPα also occur in pancreatic β-cells and that PTPα over-expression can down-regulate insulin secretion (Kapp *et al.* 2003).

Functional analysis of the two splice forms *in vitro* and in a cell based system with the insulin receptor as a substrate revealed no major differences for the PTPα isoforms. This confirms our previous observation that down-regulation of the insulin signal is not affected by mutation of the carboxyl-terminal tyrosine (Lammers *et al.* 1998). Further, in agreement with den-Hertog *et al.* (1994) mutation of the carboxyl-terminal tyrosine to phenylalanine did not change the *in vitro* activity of the phosphatase.

As a second physiological substrate, we have focused on Src family kinases. *In vitro* analyses showed that upon transient over-expression the Src family kinases c-Fyn and c-Src were dephosphorylated by both phosphatase isoforms on the regulatory carboxyl-terminal tyrosine residue. Under these conditions, expression of only the Src kinases revealed that a fraction was constitutively activated and autophosphorylated at Y420, likely because of the limiting amount of endogenous Csk that is not sufficient to phosphorylate the carboxyl-terminal c-Src tyrosine thereby inactivating the kinase. Co-over-expressed PTPα dephosphorylated the carboxyl-terminal tyrosine residue of the inactive fraction, which should lead to autophosphorylation and thus yield an increase in phosphorylation of the activation loop tyrosine residue. We did not detect such an increase; however, it has been reported that PTPα can dephosphorylate both tyrosine residues and thus itself limits the activation range of Src like kinases (den-Hertog *et al.* 1993).

For further characterization of c-Src kinase activity, focus formation as a functional, quantitative and cellular assay was employed. By biochemical analysis of individual foci, we could show that the tyrosine mutants of both

isoforms, PTPα123-Y789F and PTPα132-Y798F, were able to activate c-Src (Fig. 5A). However, as demonstrated by the increased number of foci, the mutant of the larger isoform, PTPα132-Y798F, was more efficient in activating c-Src. Whether this reflects a direct activation of the Src kinase that is not detected in an *in vitro* assay or involves other mechanisms is currently unclear.

Although in the transient over-expression system the wild-type phosphatases efficiently dephosphorylated the Src-family kinases, only the mutants of the carboxyl-terminal tyrosine were able to lead to cellular transformation in NIH3T3 cells, as shown before (Lammers *et al.* 2000). This is in contrast to the proposed phosphotyrosine displacement mechanism of Zheng *et al.* (2000) where the presence of a phosphorylated tyrosine residue at the carboxyl-terminus of PTPα is essential for the dephosphorylation and activation of c-Src. As possible reasons for this strikingly different result we cannot exclude cell type-specific effects, like differences in the expression of other c-Src activity regulating proteins like PKCδ (Brandt *et al.* 2003), Sin (Yang *et al.* 2002) or Srcasm (Seykora *et al.* 2002). However, Yang *et al.* (2002) also demonstrated c-Src activation by the mutant phosphatase, which should not be possible according to the phosphotyrosine displacement mechanism. In addition, these authors found that neurite outgrowth and induction of transin RNA in PC12 cells after EGF stimulation was stronger in mutant than in wild-type PTPα over-expressing cells.

For the tyrosine phosphatase CD45, differential functions of the isoforms also have been described and were explained by a differential homodimerization of the alternatively spliced isoforms (Xu & Weiss 2002). A similar scenario could be possible for PTPα, since dimerization has been shown to regulate its phosphatase activity (Bilwes *et al.* 1996; Blanchetot *et al.* 2002). We therefore employed the constitutively dimerizing PTPαP137C mutant in the focus formation assay. However, we did not find a dimerization related significant difference in foci generation between the splice variants. This result indicates that a differential dimerization potential does not explain the higher efficiency of the larger phosphatase form to activate c-Src.

In conclusion, the splice variants of PTPα are expressed in a tissue and differentiation dependent manner and likely have distinct physiologic roles.

Experimental procedures

Plasmids and antibodies

Human PTPα132 and its mutant PTPα132-Y798F have been described (Kaplan *et al.* 1990; Lammers *et al.* 1998). The isoform PTPα123 was kindly provided by N.P.H. Møller (Bagsværd, Denmark), the mutants of PTPα were generated by standard cloning procedures. For transient expression the pRK5-vector containing a CMV promoter was used. To generate retroviruses, cDNAs were cloned into the vector pLXSN (Clontech).

The monoclonal antibody mab29 is directed against the amino-terminal phosphatase domain of PTPα (kindly provided by N.P.H. Møller). To detect c-Fyn and c-Src, a rabbit polyclonal serum directed against the carboxyl-terminal 15 amino acids was used. For the kinase assay, monoclonal antibody 327 (Calbiochem) was used. Phosphotyrosine was detected with the antibody 4G10 (Upstate), the Phospho-Src (Tyr416) antibody (#2102, Cell Signaling) and the Phospho-Src (Tyr527) antibody (#2105, Cell Signaling). Secondary antibodies were horseradish peroxidase-coupled anti-rabbit or anti-mouse IgGs (Sigma). Proteins were visualized with chemiluminescence (ECL, Amersham Biosciences).

Cell lines and expression analysis

293 cells and BOSC23 cells were grown in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal calf serum and 2 mM L-glutamine. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium with the same supplements and 1 g/L glucose. GP + E cells, C2C12 and 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium with the same supplements and 4.5 g/L glucose.

For differentiation of the mouse 3T3-L1 preadipocytes into adipocytes, the cells were treated with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine for 5–7 days. Differentiation of myoblast C2C12 cells to myotubes was initiated by reducing fetal calf serum in the culture medium to 0.5%. For preparation of primary cerebellar granule neurons, rats were sacrificed at postnatal day 7 (P7) and neurons prepared by dissecting cerebella and mechanically dissociating the cells in the presence of trypsin and DNAse as described previously (Schulz *et al.* 1996). A 5×10^6 cells were plated on polylysine-coated 60-mM dishes and maintained in Eagle's basal medium containing 10% fetal calf serum, 2 mM glutamine, and 20 μg/mL gentamycin. Twenty-four hours after preparation, cytosin-arabinoside was added to the cultures at a final concentration of 10 μM in order to arrest the growth of non-neuronal cells. Cells were lysed for RNA isolation 1 or 8 days later.

Expression analysis

Total RNA was prepared from various cell lines by lysing the cells before reaching confluence in 1 mL of guanidine thiocyanate/phenol-based solution (PeqLab) per 60 mM dish. The RNA was isolated according to the supplier's instructions and dissolved in DEPC-treated water. To assay the quality of the RNA, an aliquot was analyzed by gel electrophoresis. For RT-PCR, 2 μg of total RNA was denatured for 15 min at 65 °C and reverse-transcribed in a total volume of 20 μL using the First Strand Synthesis kit (Roche Diagnostics). Specific PCR reactions were carried out in a total volume of 50 μL containing template (control: 10 ng of each PTPα splice variant cloned into pRK5; cDNA libraries:

100 ng DNA; reverse-transcribed single-stranded cDNA as described above: 5 μL; negative control: 0.5 μg total RNA), 2.5 μM primers (human forward: 5′-AGCAAGCACCAATTCTATAGGC-3′, human reverse: 5′-GTTGGATAAGCGGAAAGAATTG-3′; mouse forward: 5′-CGGAAGCTGGCTTATTGAGAAC-3′, mouse reverse: 5′-CGGAAAGAGTTGGAATGACTCC-3′; rat forward: 5′-CTGATAACCAGTTCACGGATGC-3′, rat reverse: 5′-TGGCCAGAAGTGGTACACTTTG-3′), 0.2 mM nucleotides, 2.5 units Taq polymerase in PCR buffer (PeqLab) with 35 cycles. DNA fragments were separated on a 5% acrylamide gel and silver stained according to standard protocols.

Lysis of cells and blotting

Transfections were performed using the method of Chen & Okayama 1987 and analyzed as described (Lammers *et al.* 1993). Cells derived from foci were lysed in Laemmli buffer, boiled and proteins size-separated by SDS-polyacrylamide (PAGE) gel electrophoresis, transferred to nitrocellulose filters and analyzed by immunoblotting. Densitometric analysis of Western blots was done using the Kodak digital science 1D Image Analysis Software.

For cross-linking, an aliquot of the lysate was treated for 2 h at 4 °C with the cross-linking reagent disuccinimidyl suberate (DSS, Pierce) that was dissolved in dimethyl sulfoxide and used at 0.5 mM. The reactions were terminated by adding Tris buffer, pH 7.5, to a final concentration of 50 mM Tris.

Phosphatase and kinase activity assays

Lysates from transiently transfected 293 cells were diluted with the same volume of phosphate buffered saline. Twenty microliters were incubated at 30 °C with pNPP-assay buffer (62.5 mM NaCl; 12.5 mM DTT; 60 mM K-acetate, pH 5.5) containing 8.67 mM pNPP. Reactions were stopped after 20 min by the addition of NaOH (100 μL, 0.4 M) and the $OD_{405\ nm}$ was measured directly. Experiments were done in quadruplicates. In parallel, aliquots of the lysates were loaded on a SDS-PAGE gel, the gel was blotted and PTPα amounts were quantified using the Kodak digital science 1D Image Analysis Software. The phosphatase activity was corrected for the amount of PTPα present in the lysates, and is therefore referred to as relative phosphatase activity. The *in vitro* kinase assay was performed as described by Yang *et al.* (2002).

Focus formation assay

NIH3T3 cells over-expressing a moderate amount of either murine or chicken c-Src were used as described (Lammers *et al.* 2000). Briefly, 75 000 cells were seeded into a six-well dish and 16 h later infected for 6–7 h with equal amounts of retroviruses (5×10^5 to 10^6) in the presence of 6 μg/mL polybrene. Forty-eight hours later, cells were trypsinized and seeded into a 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).

Inactivation of the insulin signal in BHK cells

BHK cells over-expressing the insulin receptor were transfected and treated as described (Moller *et al.* 1995). After 18 days, the cells were stained with crystal violet and staining evaluated using the TotalLab software from Nonlinear Dynamics.

Statistical analysis

Statistics were done by analysis of variance (ANOVA), followed by Student's *t* tests for unpaired groups. The statistical software package JMP (SAS Institute) was used.

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