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Original Article

Pleiotropic effects of zebrafish Protein-Tyrosine Phosphatase-1B on early embryonic development

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ABSTRACT Protein tyrosine phosphorylation is an important mechanism of eukaryotic cell signalling which is regulated by protein-tyrosine kinases and protein-tyrosine phosphatases. Here we report the molecular cloning of the first zebrafish protein-tyrosine phosphatase, zf-PTP-1B, the homologue of human PTP-1B. Zf-PTP-1B was catalytically active and localised to the endoplasmic reticulum, like human PTP-1B. Zf-PTP-1B was maternally expressed in zebrafish embryos, and low ubiquitous expression was detected up to day 7 of development. Microinjection of zf-PTP-1B RNA induced pleiotropic, but reproducible developmental defects. Evaluation of the live embryos at 24 h post fertilisation indicated that zf-PTP-1B induced defects in somite formation. The phenotype was dependent on protein-tyrosine phosphatase activity of zf-PTP-1B, since embryos injected with catalytically inactive zf-PTP-1B-C213S developed normally. Co-injection of wild type and inactive zf-PTP-1B led to a rescue of the zf-PTP-1B-induced phenotype, suggesting that zf-PTP-1B-C213S had dominant negative activity. The zf-PTP-1B-induced phenotype suggests that proper tyrosine phosphorylation of key proteins is essential for early development, most notably somitogenesis.

KEY WORDS: *protein-tyrosine phosphatase, zebrafish, embryogenesis, somitogenesis, tyrosine (de)phosphorylation*

Introduction

Protein phosphorylation on tyrosine residues plays a pivotal role in cellular processes like proliferation and differentiation. The level of tyrosine phosphorylation is regulated by the antagonising actions of two large families of proteins, the protein-tyrosine kinases (PTKs) and the protein-tyrosine phosphatases (PTPs). The PTP-family can be subdivided into two broad categories: the cytosolic PTPs and the transmembrane, receptor-like PTPs (RPTPs) (Neel and Tonks, 1997). All PTPs have at least one conserved PTP domain, the catalytic module containing the enzymatic activity of the protein. A highly conserved region within the PTP domain, the PTP-fingerprint sequence (Fischer *et al.*, 1991), contains a cysteine residue, which is absolutely conserved and essential for PTP activity (Guan and Dixon, 1991a; Pot and Dixon, 1992; Denu *et al.*, 1996).

Several PTPs have been identified to play an important role in development (Van Vactor, 1998; Van Vactor *et al.*, 1998; den Hertog, 1999). For instance, *Drosophila* Corkscrew (Csw) and its vertebrate homologue Shp-2 are crucial for early embryonic development. *Drosophila* mutants with mutations in *csw* have defects in cell fate determination at the termini of the embryo and are zygotic lethal (Perkins *et al.*, 1992). Homozygous mice with a targeted mutation in Shp-2 fail to gastrulate properly and die around mid-gestation (Arrandale *et al.*, 1996; Saxton *et al.*,1997). Furthermore, overexpression of dominant negative Shp-2 in *Xenopus* causes severe posterior truncations (Tang *et al.*, 1995). RPTPs play an important role in development as well. RPTPα is involved in neuronal differentiation, since overexpression in P19 embryonal carcinoma cells changes the differentiation fate of these cells towards neuronal differentiation (den Hertog *et al.*, 1993). Furthermore, three *Drosophila* RPTPs, DLAR, DPTP69D and DPTP99A, are essential for proper axon pathfinding and muscle innervation, since in mutant flies with mutations in these RPTPs, growth cones do not reach or bypass their muscle targets (Desai *et al.*, 1996, 1997; Krueger *et al.*, 1996). Recently, the RPTP Clr-1 was identified in *C. elegans*, and mutations in *clr-1* resulted in the dramatic *clear* phenotype that is characterised by

Abbreviations used in this paper: PTK, Protein-Tyrosine Kinase; (R)PTP, (Receptor) Protein-Tyrosine Phosphatase; ER, endoplasmic reticulum; IR, Insulin Receptor; EGFR, Epidermal Growth Factor Receptor; hpf, hours post fertilisation; GST, Glutathione-S-transferase; pNPP, p-nitrophenyl phosphate; AP, anterior to posterior; Shh, sonic hedgehog; GFP, Green Fluorescent Protein.

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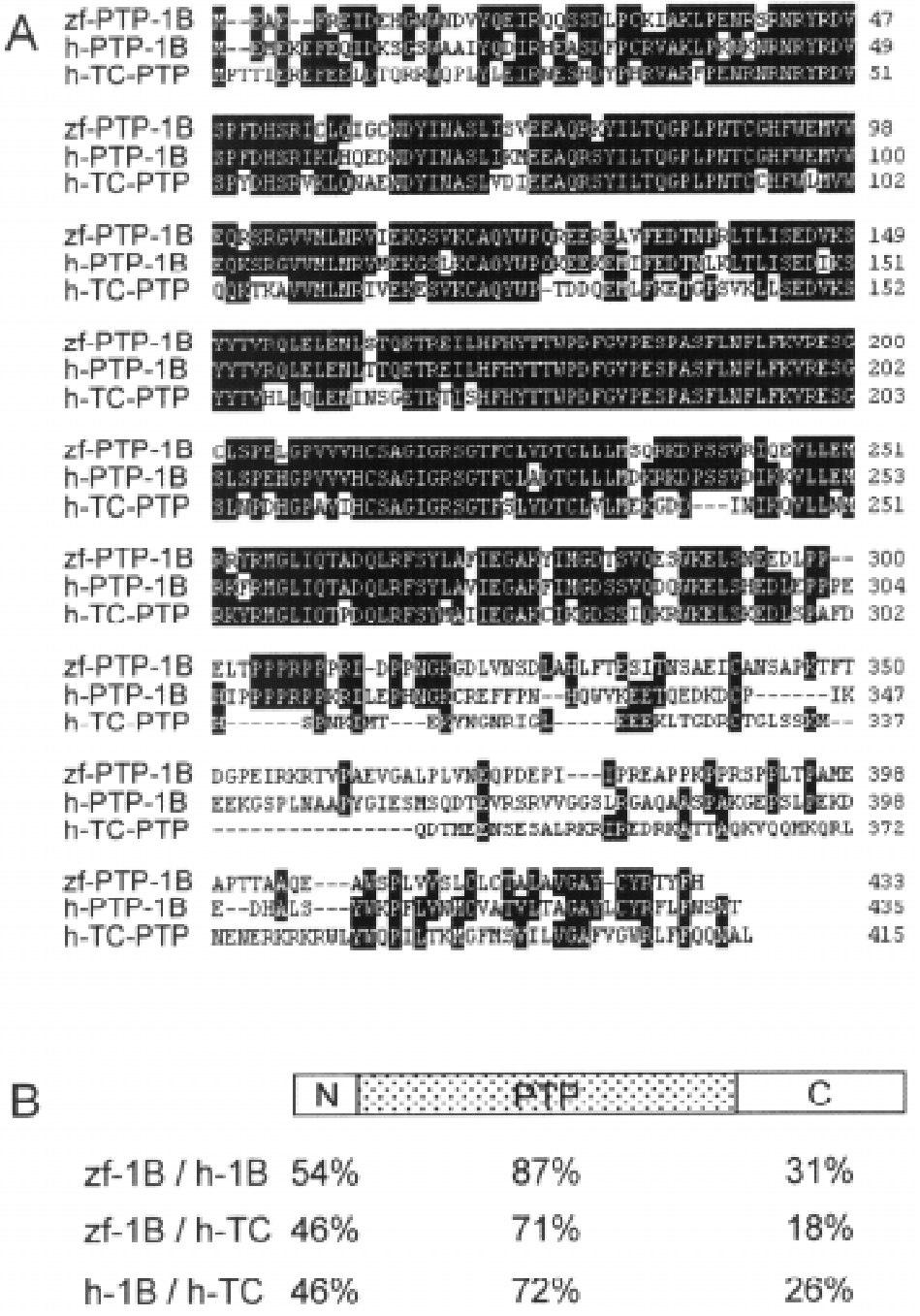

Fig. 1. Sequence analysis of zf-PTP-1B. (A) *Sequence alignment of zebrafish PTP-1B and the closely related human PTPs, PTP-1B (Chernoff et al., 1990) and TC-PTP (Cool et al., 1989). The amino acid sequences are numbered on the right and identical amino acids are boxed.* **(B)** *Homologies of three PTP-1B subdomains, the N-terminal part (N) the PTP domain (PTP) and the C-terminal part (C). The percentage amino acid identity is depicted for zf-PTP-1B and human PTP-1B (zf-1B/h-1B), for zf-PTP-1B and human TC-PTP (zf-1B/h-TC) and for human PTP-1B and human TC-PTP (h-1B/h-TC).*

extremely short, immobile and infertile animals of which the pharynx and intestine appear to float within the pseudocoelom (Kokel *et al.*, 1998).

We are interested in the role of PTPs in vertebrate development. As a model system, we use the zebrafish, *Danio rerio*, and here we report the molecular cloning of the zebrafish homologue of human PTP-1B. Human PTP-1B was the first PTP that was purified to homogeneity (Tonks *et al.*, 1988). Protein sequencing and cloning indicated that human PTP-1B is a cytosolic PTP of 435 amino acids, containing one PTP domain (Chernoff *et al.*, 1990). A C-terminal hydrophobic sequence localises the protein to the cytosolic face of the endoplasmic reticulum (ER)(Frangioni *et al.*, 1992). Calpain-mediated cleavage of the C-terminal hydrophobic region results in translocation of the N-terminal fragment, including the entire catalytic domain, to the cytoplasm which allows dephosphorylation of cytoplasmic proteins. Therefore, calpain-mediated proteolysis of PTP-1B may regulate PTP-1B-mediated dephosphorylation (Frangioni *et al.*, 1993).

Relatively little is known about the function of PTP-1B in development. The recently described PTP-1B knock-out did not display developmental defects, but PTP-1B$^{-/-}$ mice were resistant to weight gain and remained insulin resistant on a high fat diet, a

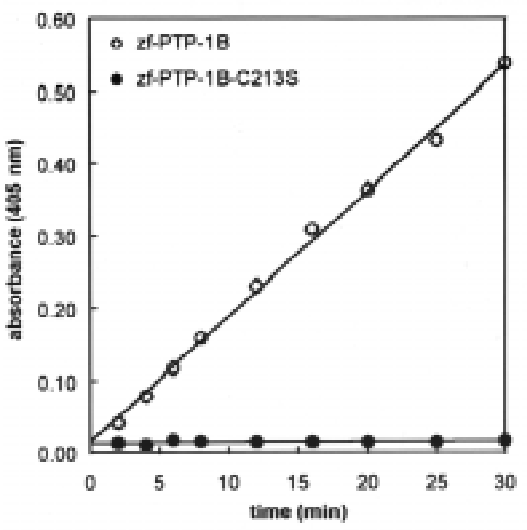

Fig. 2. PTP activity of zf-PTP-1B *in vitro*. *Equal amounts of GST-zf-PTP-1B (○) and GST-zf-PTP-1B-C213S (●) were subjected to a PTP assay, using p-nitrophenol phosphate as a substrate. Reactions were stopped after the times indicated and the amount of p-nitrophenol product was determined colorimetrically in quadruplicate by measuring the absorption at 405 nm.*

phenotype that is reminiscent of type 2 diabetes (Elchebly *et al.*, 1999). Apparently, PTP-1B is not required for early development of the mouse. It is not clear whether PTP-1B is differentially expressed during vertebrate development, but PTP-1B mRNA was detected in the fetal rat brain by RT-PCR (Shock *et al.*, 1995). In the adult, PTP-1B mRNA is widely expressed, in that PTP-1B mRNA was detected in human placenta, adult liver, heart, lung, kidney, epidermis and brain (Chernoff *et al.*, 1990). Functional studies with PTP-1B indicate that micro-injection of human PTP-1B protein in *Xenopus* oocyte delays oocyte maturation (Tonks *et al.*, 1990). Recently, the insulin receptor (IR) and the epidermal growth factor receptor (EGFR), two receptor PTKs, were identified as specific PTP-1B substrates (Flint *et al.*, 1997; Elchebly *et al.*, 1999).

Here we report the isolation of a cDNA encoding zf-PTP-1B, which was catalytically active and localised to the ER. Zf-PTP-1B was ubiquitously expressed during zebrafish development. Overexpression of zf-PTP-1B led to pleiotropic defects during early embryonic development, which were dependent on zf-PTP-1B activity. Co-injection of catalytically inactive zf-PTP-1B-C213S with zf-PTP-1B rescued the zf-PTP-1B-induced phenotype, suggesting that zf-PTP-1B-C213S acted dominant negatively over wild type zf-PTP-1B. The most prominent features of the zf-PTP-1B-induced phenotype were gastrulation defects and variable defects in somitogenesis, suggesting that zf-PTP-1B-mediated dephosphorylation of key proteins severely affected these developmental events.

Results

Cloning of zebrafish PTP-1B

Two degenerate primers directed at conserved PTP-sequences and oligo-dT primed cDNA from 9.6 hpf zebrafish embryos were used to generate PCR fragments of 370bp that were cloned and sequenced. With this strategy we identified four unique fragments encoding PTPs, which were used to screen a 1-48 hpf zebrafish cDNA library. Seventeen positive clones were obtained, of which clone 1B-2.2 was sequenced to completion. The open reading frame of clone 1B-2.2 encoded a protein of 433 amino acids. Extensive database searches indicated that this protein is most homologous to human PTP-1B. The overall sequence identity is 60% (Fig. 1), and the homology in the PTP domain of these proteins is even 87% (Fig. 1B). For comparison, we have aligned this zebrafish PTP with human PTP-1B and T-cell PTP, two highly homologous human PTPs (Fig. 1A). The sequence identities between these three PTPs illustrate that our zebrafish PTP clone is more homologous to PTP-1B than to T-cell PTP (Fig. 1B). Based on extensive database searches, we conclude that the zebrafish PTP we have cloned encodes the homologue (orthologue) of human PTP-1B and therefore we designated our clone zf-PTP-1B.

Zf-PTP-1B is catalytically active

The PTP activity of zf-PTP-1B was determined in order to establish that zf-PTP-1B is a bona fide PTP. A fragment of wild type zf-PTP-1B, encompassing the entire PTP domain (residues 1-280), was bacterially expressed as a glutathione S-transferase (GST) fusion protein and purified. GST-zf-PTP-1B had the expected size of 57 kDa as confirmed by SDS-polyacrylamide gel electrophoresis (data not shown). GST-zf-PTP-1B was catalytically active in an *in vitro* PTP assay with p-nitrophenyl phosphate (pNPP) as a substrate (Fig. 2). As a control, we tested zf-PTP-1B with a catalytic site Cys→Ser mutation, GST-zf-PTP-1B-C213S,

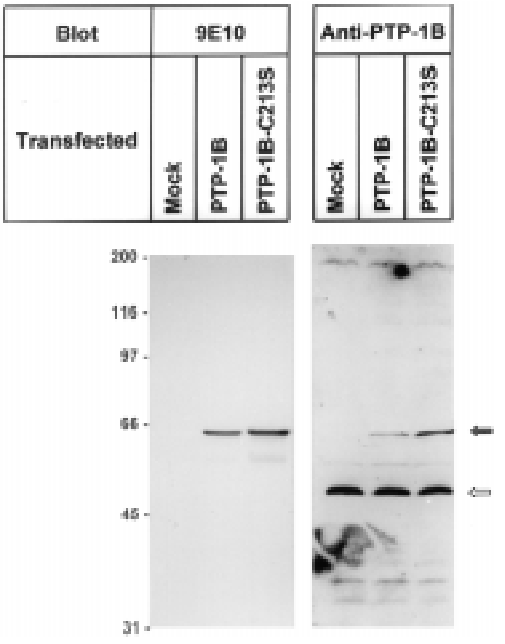

Fig. 3. Clone 1B-2.2 encodes full-length zf-PTP-1B. *Human embryonic kidney 293 cells were transfected with expression vector alone (Mock) or with a CMV-driven expression vector for Myc-tagged zf-PTP-1B (PTP-1B) or mutant zf-PTP-1B with a mutation in the catalytic site cysteine (PTP-1B-C213S). The cells were lysed in Laemmli sample buffer and aliquots were run on a 12.5% SDS-polyacrylamide gel. The gels were blotted onto Immobilon and parallel blots were probed with the anti-Myc tag MAb 9E10, or anti-human PTP-1B (Anti-PTP-1B), as indicated. The blots were developed using enhanced chemiluminescence (ECL). Immunoblots are depicted with the relative molecular mass (M_r) of marker proteins in kDa on the left. The position of Myc-tagged zf-PTP-1B is indicated with a hatched arrow, and endogenous human PTP-1B with an open arrow.*

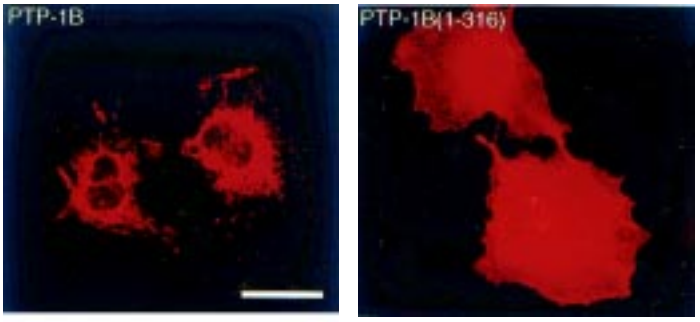

Fig. 4. ER localisation of zf-PTP-1B. *Cos-7 cells were transfected with Myc-tagged zf-PTP-1B (PTP-1B) or C-terminally truncated zf-PTP-1B (residues 1-316). Indirect immunofluorescence was done using the anti-Myc tag MAb, 9E10. Representative micrographs are depicted. Bar, 20 μm.*

for PTP activity in parallel with wild-type GST-zf-PTP-1B, using equal amounts of enzyme. As expected, GST-zf-PTP-1B-C213S did not display any PTP activity, using pNPP as a substrate (Fig. 2). These results demonstrate that wild-type zf-PTP-1B is a bona fide PTP with catalytic activity.

ER localisation of zf-PTP-1B

In order to determine its subcellular localisation, zf-PTP-1B was expressed in eukaryotic cells. First, we analysed expression of transiently transfected zf-PTP-1B by immunoblotting. We generated a mammalian expression vector, driven by the cytomegalovirus promoter, which encoded zf-PTP-1B fused at the N-terminus to a tandem repeat of five Myc-epitope tags (MT-zf-PTP-1B). MT-zf-PTP-1B and a similar construct encoding catalytically inactive MT-zf-PTP-1B-C213S were transfected into human embryonic kidney 293 cells. Immunoblotting of total cell lysates using an anti-Myc MAb (9E10) demonstrated that both wild-type and inactive MT-zf-PTP-1B migrated in SDS-polyacrylamide gels with an apparent M_r of 65 kDa (Fig. 3). Parallel blots were probed with an anti-human PTP-1B antibody. Endogenous human PTP-1B was detected as a 50 kDa protein, while the transfected MT-zf-PTP-1B and MT-zf-PTP-1B-C213S had an apparent M_r of 65 kDa, running at the same position as the proteins detected with 9E10 (Fig. 3).

Human PTP-1B localised to the ER through a hydrophobic region in its C-terminus (Frangioni *et al.*, 1992). To investigate whether zf-PTP-1B also localised to the ER through its hydrophobic C-terminus, we transfected pCMV-MT-zf-PTP-1B and a construct lacking the hydrophobic C-terminus, pCMV-MT-zf-PTP-1B (1-316), into COS-7 cells. Immunoblotting indicated that full length and C-terminally truncated MT-zf-PTP-1B (1-316) were expressed at similar levels (data not shown). Indirect immuno-fluorescence using anti-Myc MAb 9E10 indicated that full-length MT-zf-PTP-1B localised to the ER (Fig. 4). The staining pattern was highly reminiscent of the pattern described for human PTP-1B (Frangioni *et al.*, 1992). By contrast, truncated MT-zf-PTP-1B (1-316) was diffusely dispersed throughout the cytoplasm (Fig. 4). These results indicate that zf-PTP-1B, like human PTP-1B, localised to the ER through the C-terminal hydrophobic region.

Constitutive expression of zf-PTP-1B during embryonic development

We investigated the expression pattern of zf-PTP-1B by whole-mount *in situ* hybridisation and by Northern blotting. Whole-mount *in situ* hybridisation indicated that zf-PTP-1B expression was ubiquitous, but low during early zebrafish development. Detectable signals were only obtained after very long staining, and no differential expression was detected (data not shown). Therefore, we used Northern blotting to determine whether zf-PTP-1B expression was regulated in time. Again the signals were relatively weak (Fig. 5), as compared to expression of other genes (data not shown). Zf-PTP-1B expression was detectable in the 128 cell stage (2.2 hpf), i.e. before zygotic transcription is activated, indicating that zf-PTP-1B is maternally expressed. A second band was detected in 2.2 hpf old embryos, which may be due to alternative splicing, or to cross-hybridisation with another gene. This additional band was not detected in older zebrafish embryos. Comparable zf-PTP-1B expression levels were detected as a single band from 6 hpf until 7 dpf (Fig. 5), demonstrating that zf-PTP-1B is not differentially expressed during early zebrafish development. Taken together, the Northern blotting and *in situ* hybridisation experiments indicate that zf-PTP-1B is expressed ubiquitously at low levels throughout the embryo during embryonic and early larval development.

Overexpression of zf-PTP-1B induced pleiotropic phenotypes

To determine the effects of overexpression of zf-PTP-1B on zebrafish development, we injected *in vitro* transcribed RNA, encoding MT-zf-PTP-1B or catalytically inactive MT-zf-PTP-1B-C213S into embryos at the 2- to 4-cell stage and followed their subsequent development. Morphological examination of the embryos injected with zf-PTP-1B revealed that defects were detectable in some embryos from 6 hpf onwards. Approximately 10% of the zf-PTP-1B-injected embryos did not complete gastrulation and died. The morphology of the embryos that survived the first 24 h of development was examined and these embryos were subjected to *in situ* hybridisation analysis. Morphologically, 40% of the embryos displayed pleiotropic, but reproducible phenotypes (Fig. 6; Table 1). These phenotypes varied in severity (Fig. 6D-I), and the most severely affected embryos resembled a clump of cells with some anterior (eyes) and/or posterior (tail) structures still detectable. Mildly affected embryos had apparently split hindbrain regions,

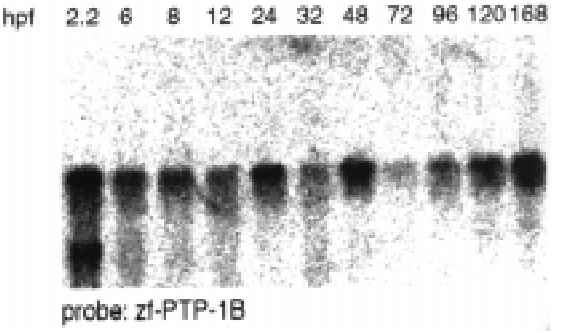

Fig. 5. Zf-PTP-1B mRNA is constitutively expressed during embryonic development. *RNA was isolated from zebrafish embryos at the indicated times (hours post fertilisation, hpf; 2.2 hpf represents 128 cell stage), and fractionated on a 0.8% formaldehyde-agarose gel (15 μg total RNA per lane), which was stained with ethidium bromide to monitor equal loading (not shown). The RNA was transferred to a nitrocellulose blot, and hybridised to a zf-PTP-1B specific probe. Radioactivity on the blot was visualised using a Phosphor Imager.*

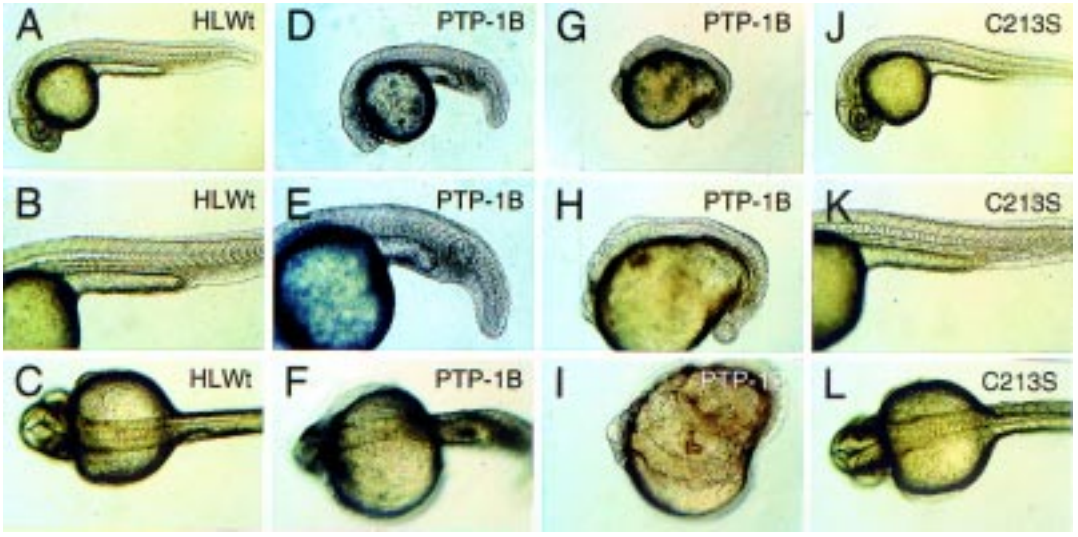

Fig. 6. Zf-PTP-1B overexpression induced pleiotropic, but reproducible phenotypes in zebrafish embryos. *Phenotypes were scored at 24 hpf after injection of RNA, encoding Myc-tagged zf-PTP-1B or non-functional zf-PTP-1B-C213S, in one of the blastomeres of 2- to 4-cell stage embryos. Lateral views* **(A,B,D,E,G,H,J,K)** *and dorsal views* **(C,F,I,L)** *of representative, live embryos are depicted.* **(A-C)** *Non-injected wild type (HLWt) embryos.* **(D-I)** *Embryos injected with 250 pg zf-PTP-1B mRNA, which induced a mild phenotype* **(D-F)** *or a more severe phenotype* **(G-I)**. **(J-L)** *Embryos injected with 250 pg of zf-PTP-1B-C213S.*

sometimes extending into the spinal cord, together with lack of eyes, cyclopic eyes, fused or normal eyes, U-shaped somites and shortened, posteriorly truncated anterior to posterior (AP) axes (Fig. 6G-I). The embryos displaying split hindbrains had a single notochord, which was located asymmetrically to one side of the embryo. Furthermore, milder phenotypes were observed displaying a downwardly kinked tail (Fig. 6D,E) and some malformed or fused somites (Fig. 6E,F). Control embryos injected with Green Fluorescent Protein (GFP) RNA developed normally with a small percentage of defects, which are not related to the zf-PTP-1B induced phenotype (Table 1).

In order to establish whether PTP activity was required for the zf-PTP-1B-induced phenotype, embryos were injected with RNA encoding catalytically inactive zf-PTP-1B-C213S. Morphological examination of these embryos demonstrated that they did not display significant developmental defects (Fig. 6J-L, Table 1). These results demonstrate that PTP activity is essential for the observed defects in zf-PTP-1B injected embryos.

All zf-PTP-1B injected embryos that were scored as phenotypically abnormal displayed abnormal somitogenesis. To visualise these somite abnormalities in more detail, whole-mount *in situ* hybridisation was performed on 24 hpf embryos, using a myoD-specific probe (Weinberg *et al.*, 1996). In control embryos, the myoD marker showed the typical segmented pattern of staining of the chevron-shaped somites (Fig. 7A,B)(Weinberg *et al.*, 1996). MyoD-staining of some embryos injected with zf-PTP-1B revealed somites that are irregular, smaller than usual and fused (Fig. 7D,E). Other zf-PTP-1B injected embryos had U-shaped instead of chevron-shaped somites (Fig. 7G), and even somites that were missing altogether on one side (Fig. 7H). These myoD *in situ* hybridisations illustrate that injection of zf-PTP-1B caused various abnormalities in somite formation. In contrast, zf-PTP-1B-C213S injected embryos did not display defects in somitogenesis, as judged by the myoD-marker (data not shown), again demonstrating that catalytic activity is essential for the observed phenotype.

Midline signalling from the notochord plays a crucial role in somitogenesis (van Eeden *et al.*, 1996). One of the key players in signalling from the midline is Sonic hedgehog (Shh), which is expressed in the notochord and the floor plate (Krauss *et al.*, 1993; Müller *et al.*, 1999), and which patterns the neighbouring tissues, such as the somites (Schauerte *et al.*, 1998). Since zf-PTP-1B induced defects in somite formation, we investigated shh expression in 16 hpf old embryos. Shh expression in the posterior notochord and floor plate was not affected by zf-PTP-1B injection (Fig. 7C,F,I), albeit development of some embryos was retarded, and therefore shh was mainly detected in the notochord of these embryos (cf. Fig. 7F). Shh expression in zf-PTP-1B-injected embryos, together with the general presence of the notochord in all categories of phenotypes, suggests that midline signalling is not affected severely in these embryos. These results suggest that it is unlikely that the zf-PTP-1B-induced defects are a direct consequence of abnormal signalling from the axial mesoderm. The observed phenotypes are dependent on zf-PTP-1B catalytic activity, since catalytically inactive zf-PTP-1B-C213S did not interfere with normal development.

Rescue of zf-PTP-1B induced defects by mutant zf-PTP-1B-C213S

Catalytically inactive PTP mutants (catalytic site Cys→Ser/Ala) may have dominant negative activity by binding to their substrates, thereby protecting them from dephosphorylation (Bliska *et al.*, 1992). For instance, Shp-2 with a catalytic site Cys→Ser

TABLE 1

ZF-PTP-1B, BUT NOT ZF-PTP-1B-C213S, INDUCED A PLEIOTROPIC PHENOTYPE IN ZEBRAFISH EMBRYOS

RNA injected	Number of experiments	Number of embryos	% Normal	% Developmental defects	%Dead
PTP-1B	7	301	48	42	10
PTP-1B-C213S	4	141	86	10	4
GFP	2	57	88	12	0

Embryos were injected with 250 pg RNA in one of the blastomeres at the 2- to 4-cell stage and scored for phenotypes at 24 hpf. The phenotypes of the zf-PTP-1B-injected embryos varied in severity but all showed abnormal somitogenesis. The developmental defects that were observed in the zf-PTP-1B-C213S- and GFP-injected embryos were non-specific and unrelated to the zf-PTP-1B induced phenotype.

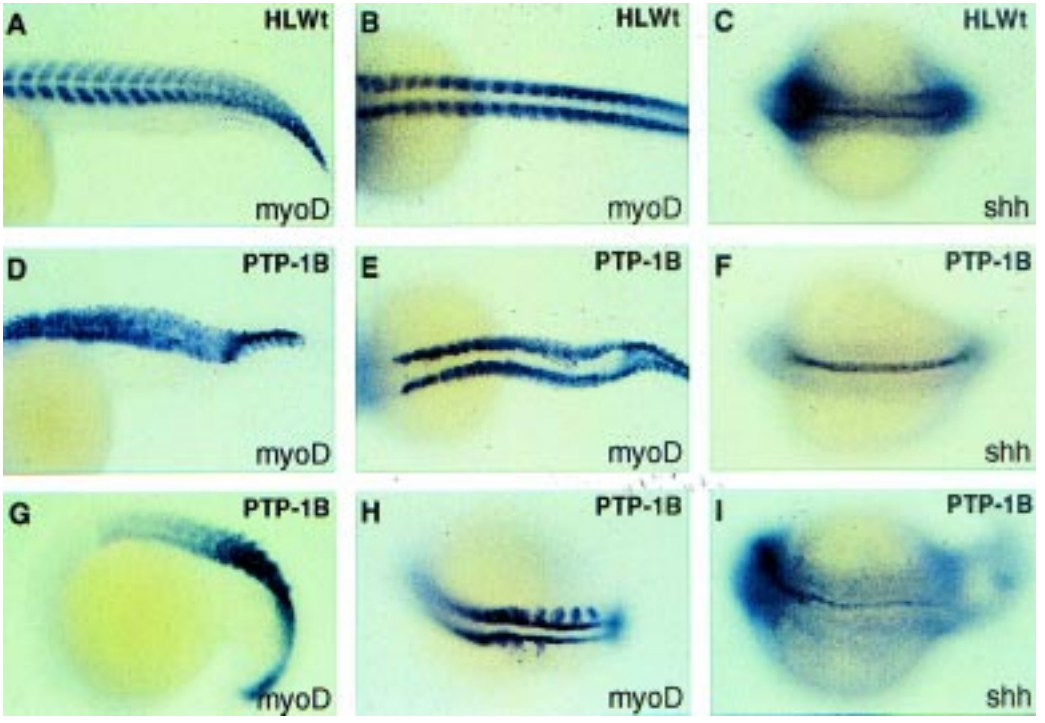

Fig. 7. Zf-PTP-1B overexpression induced abnormal somitogenesis. *Whole-mount in situ hybridisation, using a myoD probe that specifically stains somites and adaxial mesoderm, was done on 24 hpf zebrafish embryos. Lateral views* **(A,D,G)** *and dorsal views* **(B,E,H)** *are depicted (anterior to the left) of a control zebrafish embryo (HLWt)* **(A,B)**, *and of two representative embryos that were injected at the 2- to 4-cell stage with 250 pg RNA encoding Myc-tagged zf-PTP-1B (PTP-1B)* **(D,E,G,H)**. *In addition, a shh probe was used to label a 16 hpf control embryo* **(C)** *or zf-PTP-1B-injected* **(F,I)** *embryos. Dorsal views (anterior to the left) are depicted of the shh-stained embryos.*

mutation has a dominant negative effect on interferon α/β-induced gene expression (David *et al.*, 1996). In contrast, it has been demonstrated that the dual specificity phosphatase, MKP-1, with a catalytic site Cys→Ser mutation has similar effects as wild type MKP-1 presumably due to sequestering and inactivation of its substrate, MAP kinase (Sun *et al.,* 1993). We investigated the dominant negative potential of zf-PTP-1B-C213S on the wild type zf-PTP-1B-induced phenotype during zebrafish embryonic development. To this end, we co-injected embryos with zf-PTP-1B RNA and zf-PTP-1B-C213S RNA in a 1:1 ratio. At 24 hpf 94% of the injected embryos had developed completely normally (Figs. 8,9). Reduction of the amount of co-injected zf-PTP-1B-C213S led to an increase in the number of abnormal embryos scored at 24 hpf (Fig. 9). As a control, we co-injected GFP with zf-PTP-1B. At 24 hpf, a similar percentage (40%) of the embryos displayed the same pleiotropic phenotypes as found with zf-PTP-1B alone (Table 1; Fig. 9). These results demonstrate that zf-PTP-1B-C213S specifically rescued the zf-PTP-1B-induced phenotype and therefore acted in a dominant negative fashion over overexpressed zf-PTP-1B.

Discussion

Here we report the molecular cloning of the first PTP from zebrafish, zf-PTP-1B. Zf-PTP-1B is ubiquitously expressed at low levels during development. Functional experiments demonstrated that overexpression of zf-PTP-1B in zebrafish embryos induced defects in gastrulation and somitogenesis, indicating that proper tyrosine phosphorylation of key proteins is essential for these developmental processes.

Four types of evidence indicate that the zebrafish PTP we describe here is the homologue of human PTP-1B. Firstly, extensive database searches indicate that zf-PTP-1B is most homologous to human PTP-1B, illustrated by the fact that zf-PTP-1B is more homologous to human PTP-1B than to human PTP-1B's closest relative, human TC-PTP (Fig.1). Secondly, zf-PTP-1B was catalytically active in an *in vitro* PTP assay, using pNPP as a substrate (Fig. 2). Thirdly, zf-PTP-1B was recognised by an anti-human PTP-1B antibody on immunoblots (Fig. 3). Finally, zf-PTP-1B localised to the ER, like human PTP-1B, which was dependent on the C-terminal hydrophobic region (Fig. 4). Therefore, we conclude that the PTP we have identified is the zebrafish homologue of human PTP-1B.

Little is known about PTP-1B expression during development. In the adult, human PTP-1B is widely expressed in all tissues that were tested (Chernoff *et al.*, 1990). Rat PTP-1B is expressed in the fetal brain (Shock *et al.*, 1995), but no expression patterns nor changes in expression of PTP-1B during embryonic development have been reported. Here we demonstrate that zf-PTP-1B is maternally expressed, and that zf-PTP-1B is ubiquitously expressed at low levels during development, which is consistent with ubiquitous expression of PTP-1B in the adult.

Overexpression of zf-PTP-1B during early development by microinjection of zf-PTP-1B RNA induced pleiotropic phenotypes, as evaluated at 24 hpf. The phenotypes varied from gastrulation defects to relatively subtle defects in somite induction and patterning. In contrast, injection of catalytically inactive zf-PTP-1B-C213S resulted in embryos that developed normally, as judged by morphology and by expression of the somite-specific marker myoD, demonstrating that the effects on embryonic development of wild type zf-PTP-1B overexpression are dependent on the PTP activity of zf-PTP-1B.

Microinjection of zf-PTP-1B did not induce a phenotype in all injected embryos and the severity of the phenotype varied from embryo to embryo. We have monitored the presence of MT-zf-PTP-1B and MT-zf-PTP-1B-C213S protein by whole-mount immu-

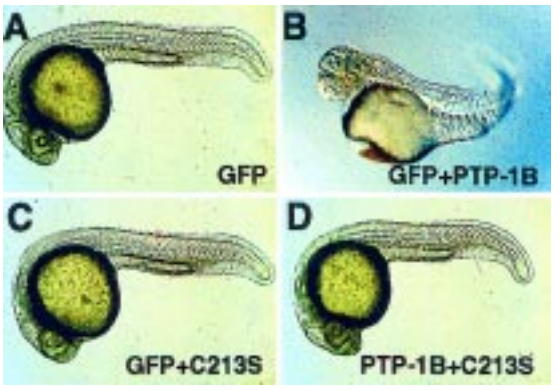

Fig. 8. Rescue of the zf-PTP-1B-induced phenotype by co-injection of zf-PTP-1B-C213S. *Zebrafish embryos were (co-)injected at the 2- to 4-cell stage and lateral views of representative live embryos, photographed at 24 hpf, are depicted. The embryos were injected with 250 pg RNA encoding GFP as a control* **(A)**, *with 125 pg GFP RNA and 125 pg zf-PTP-1B RNA* **(B)**, *with 125 pg GFP RNA and 125 pg zf-PTP-1B-C213S RNA* **(C)** *or with 125 pg zf-PTP-1B RNA and 125 pg zf-PTP-1B-C213S RNA* **(D)**.

nohistochemistry at 24 hpf using the anti-Myc tag MAb 9E10. Approximately 90% of the injected embryos expressed MT-zf-PTP-1B or MT-zf-PTP-1B-C213S. However, the localisation and extent of expression varied from embryo to embryo (data not shown). Embryos injected with GFP RNA showed comparable results with regard to localisation and extent of expression, in that in general 90% of the embryos expressed GFP in 25-50% of the cells (data not shown). Therefore, the fact that not all of the injected embryos displayed a phenotype is likely due to differences in levels or localisation of overexpressed zf-PTP-1B. Furthermore, the severity of the phenotype is conceivably also linked to localisation and level of overexpressed zf-PTP-1B.

Zf-PTP-1B with a catalytic site Cys→Ser mutation displayed dominant negative activity upon co-injection with wild type zf-PTP-1B. Co-injection of increasing amounts of zf-PTP-1B-C213S progressively reduced the percentage of embryos with a phenotype, and co-injection in a 1:1 ratio almost completely rescued the zf-PTP-1B-induced phenotype. Dominant negative activity of PTPs with mutations in the catalytic site is not unprecedented, since both deletion of the critical catalytic core, as well as mutation of the catalytic site cysteine in Shp-2 led to dominant negative effects (Tang *et al.*, 1995; David *et al.*, 1996). The fact that dominant negative zf-PTP-1B-C213S did not induce a phenotype by itself suggests that zf-PTP-1B is dispensable for early zebrafish development, which is consistent with the lack of developmental defects in PTP-1B$^{-/-}$ mice (Elchebly *et al.*, 1999).

It is noteworthy that only a few reports describe overexpression of PTPs in vertebrates. Microinjection of PTP-1B RNA in vertebrate embryos has not been reported before, but microinjection of PTP-1B protein into *Xenopus* oocytes delayed insulin-induced maturation, while no effects on development were reported (Tonks *et al.*, 1990). Tang *et al.* (1995) described overexpression of Shp-2 in *Xenopus* embryos, which did not affect development significantly. In contrast, overexpression of catalytically inactive, dominant negative Shp-2 induced defects in mesoderm development (Tang *et al.*, 1995). Targeting of Shp-2 exon 3 in mice was aimed at deletion of Shp-2, but instead led to expression of a truncated form of Shp-2, which lacked part of its C-terminal SH2 domain (Saxton *et al.*, 1997). The PTP domain was still intact and Shp-2Δexon-3 displayed PTP activity. Homozygous Shp-2$^{exon3-/-}$ mice failed to gastrulate properly and died around mid-gestation. Since Shp-2Δexon-3 still was expressed in Shp-2$^{exon3-/-}$ mice, it might be involved in the phenotype. However, it is unlikely that Shp-2Δexon-3 by itself induced a phenotype, since heterozygous Shp-2$^{exon3+/-}$ mice were normal (Saxton *et al.*, 1997). It remains to be determined whether overexpression of other PTPs will induce similar phenotypes in zebrafish embryos as zf-PTP-1B. Interestingly, overexpression of the membrane proximal PTP domain of Receptor Protein-Tyrosine Phosphatase α (RPTPα), which contains the majority of the catalytic activity of RPTPα, induced similar phenotypes as zf-PTP-1B in zebrafish embryos (A.M. van der Sar and J. den Hertog, unpublished observation), suggesting that elevation of PTP activity is sufficient to induce developmental defects.

The developmental mechanism by which overexpressed zf-PTP-1B affects zebrafish embryogenesis remains to be determined. In the most severe cases, the zf-PTP-1B-injected embryos did not complete gastrulation, and died within 24 h. Most of the embryos that survived the first 24 h displayed defects in somitogenesis. Some zf-PTP-1B-injected embryos lacked properly segmented paraxial mesoderm (Fig. 7D). In other embryos, myoD-staining was absent unilaterally (Fig. 7H), suggesting that the paraxial mesoderm was missing, while adaxial myoD labelling was still present. The variability in the severity of the phenotypes may be due to the level and localisation of overexpressed zf-PTP-1B within the embryos (see above). Midline signalling from the notochord plays an important role in patterning of the somites (van

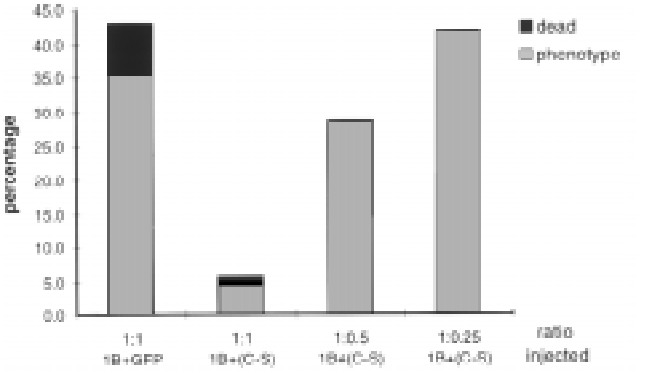

Fig. 9. Dose-dependent rescue of the zf-PTP-1B-induced phenotype by zf-PTP-1B-C213S. *Zebrafish embryos were co-injected at the 2- to 4-cell stage with 125 pg zf-PTP-1B RNA together with 125 pg GFP RNA as a control (1B+GFP), or with decreasing amounts of zf-PTP-1B-C213S RNA (1B+C-S). The relative amount of co-injected zf-PTP-1B-C213S RNA is indicated as the ratio of wild-type and mutant PTP-1B RNA, in that 125 pg is a 1:1 ratio, 62 pg a 1:0.5 ratio and 31 pg a 1:0.25 ratio. The total amount of injected RNA was adjusted to 250 pg in the latter two cases with GFP RNA. The embryos were scored for phenotypes at 24 hpf. Although the severity of the phenotypes varied, all embryos that were scored positive for a phenotype displayed defects in somitogenesis. The percentage of embryos with a phenotype is depicted (hatched bars) as well as the percentage dead embryos (black bars). The percentages were calculated from four independent experiments (1B+GFP and 1B+C-S, 1:1 ratio, with 196 and 264 embryos, respectively). The percentages of the 1B+C-S co-injections, 1:0.5 and 1:0.25 ratio, were calculated from two independent experiments with 72 and 58 embryos, respectively.*

Eeden *et al.*, 1996; Schauerte *et al.*, 1998). It is noteworthy that shh expression, a marker for axial mesoderm, was not affected in zf-PTP-1B-injected embryos (Fig. 7C,F,I), suggesting that deficiencies in somitogenesis are not a consequence of an aberrant midline signal in zf-PTP-1B-injected embryos. Moreover, whole-mount *in situ* hybridisation of zf-PTP-1B-injected tailbud stage embryos using an axial riboprobe displayed a normal axial expression domain in the axial mesoderm (data not shown). Zf-PTP-1B may somehow interfere with receipt or transduction of the midline signal by the paraxial mesoderm. It is tempting to speculate that (receptor) PTKs are involved and that zf-PTP-1B mediated dephosphorylation interferes with PTK-mediated signalling.

The zf-PTP-1B-induced phenotype is dependent on PTP activity, indicating that zf-PTP-1B-mediated dephosphorylation of substrates is involved. Several substrates of mammalian PTP-1B have been identified, including the IR and the EGFR, two RPTKs (Flint *et al.*, 1997; Elchebly *et al.*, 1999). Whether the zebrafish homologues of the human IR and EGFR are substrates of zf-PTP-1B remains to be determined. However, it is unlikely that the zf-PTP-1B-induced phenotype is due to specific dephosphorylation of the EGFR, since the EGFR is involved in epithelial proliferation and differentiation in mammals (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995), which is not consistent with the zf-PTP-1B-induced phenotype. However, dephosphorylation of other substrates may be involved in the zf-PTP-1B-induced phenotype. Several unidentified potential substrates bound to human PTP-1B in substrate trapping procedures, and the oncoprotein $p210^{bcr\text{-}abl}$ was recently identified as a PTP-1B substrate as well (Flint *et al.*, 1997; Lamontagne *et al.*, 1998). It will be interesting to see whether these substrates are involved in somitogenesis *in vivo*.

In conclusion, here we describe the molecular cloning of the first zebrafish PTP, zf-PTP-1B. Overexpression of zf-PTP-1B led to pleiotropic phenotypes, with the most prominent defects in gastrulation and somite formation, which may be caused by dephosphorylation of key substrates in early stages of development. Apparently, proper tyrosine phosphorylation of key proteins is essential for early development.

Materials and Methods

Fish and embryos

Zebrafish were kept at 27.5ºC. Embryos were obtained by natural matings and cultured routinely in embryo medium at 28.5ºC. Staging of the embryos was done according to The Zebrafish Book (Westerfield, 1993).

Cloning of zebrafish PTP-1B

Fragments of cDNAs, encoding PTPs, were obtained by PCR using cDNA prepared from 9.6 h post fertilisation (hpf) zebrafish embryos. The two degenerate oligonucleotides directed against conserved PTP sequences that were used are:

S1 5' GCGGAATTCTT(T/C)TGGI(A/T/G)IATG(G/A)TNTGG 3'
AS1 5' CGCGGATCCCCNGCI(G/C)(A/T)(A/G)CA(A/G)TGIAC 3'

The PCR fragments were cloned and sequenced and four fragments encoding zf-PTPs were selected, labelled to high specific activity with α-[^{32}P]-dCTP using a multiprime labelling kit (Amersham) and used as probes to screen $1x10^6$ phages of a λZAP library prepared from 1-48 hpf zebrafish embryos (kind gift of M. Petkovich). Hybridisations, selection of positive clones and plasmid excision was done according to standard protocols. After characterisation of the positive clones by partial sequence analysis, the 1B-2.2 clone was subcloned and sequenced. Nucleic acid sequencing was performed on an Applied Biosystems automated sequencer with chemicals supplied by the manufacturer. Blast searches were done to compare cloned sequences with sequences in databases, and DNASTAR software was used to generate alignments.

Plasmids and site-directed mutagenesis

For site directed mutagenesis and construction of plasmids the following oligonucleotides were used:

1B-C213S 5' GTGGTCCACAGCAGCGCTGGC 3'
1B-1 5' GCGCCATGGAAGCCGAGTTTCGG 3'
1B-280 5' CGCCTCGAGTCACATGATGTAGTTGGC 3'
1B-316 5' CGCCTCGAGTCAGTTAGGAGGGTCTAT 3'

Site directed mutagenesis was done on the complete zf-PTP-1B cDNA in pBluescript SK- (Stratagene) with the oligonucleotide 1B-C213S. The mutation was verified by sequencing. Deletion mutants were generated by PCR, using 1B-1, 1B-280, 1B-316 and T7. The PCR fragments were digested with NcoI and XhoI (deletion mutants) or NcoI and XbaI (full length) and inserted into NcoI/XhoI or NcoI/XbaI digested pGEX-KG (Guan and Dixon, 1991b) for prokaryotic expression or into NcoI/XhoI or NcoI/XbaI digested pCS2+MT (Rupp *et al.*, 1994) for *in vitro* transcription or transfection into eukaryotic cells.

GST-fusion proteins and PTP assay

Bacterial cultures were grown overnight and GST-fusion proteins were purified with glutathione-agarose beads (Sigma) exactly as described (Guan and Dixon, 1991b). The catalytic activity was determined by adding aliquots of the purified GST-fusion proteins to 200 µl PTP buffer (20 mM MES pH 6.0 , 1 mM DTT, 1 mM EDTA, 150 mM NaCl and 10 mM pNPP). After incubation at 30ºC, 1 ml 1 N NaOH was added to stop the reaction and the amount of product p-nitrophenol was determined in quadruplicate by measuring the absorbance at 405 nm (Buist *et al.*, 1999).

Cells, transfections, immunoblotting and immunofluorescence

Two hundred and ninety three human embryonic kidney cells and COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7.5% bovine calf serum. Transient transfections were done exactly as described (den Hertog *et al.,* 1993). After transfection, the cells were lysed and aliquots were loaded on an SDS-polyacrylamide gel. The material was transferred to Immobilon (Millipore) by semi-dry blotting, and the blots were incubated overnight at 4ºC in blocking buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 5% non-fat milk), and subsequently in blocking buffer containing rabbit polyclonal anti-human PTP-1B antibody (kind gift of Ben Neel) or mouse anti-Myc tag antibody (9E10, Santa Cruz) for 1.5 h at 25ºC. Following extensive washing, incubation for 1 h at 25ºC with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and extensive washing, the blots were developed by enhanced chemiluminescence (ECL).

Transiently transfected COS-7 cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 0.5% BSA, the cells were incubated with anti-Myc tag MAb (9E10) in 0.5% BSA. Following extensive washing the cells were incubated with TRITC-conjugated secondary anti-mouse antibody.

Northern blotting and whole-mount in situ hybridisation

Northern blotting was done according to standard protocols, using 15 µg total RNA per lane, formaldehyde-containing 0.8% agarose gels, and a multiprime (Amersham) labelled zf-PTP-1B-specific probe. For *in situ* hybridisation, we used probes specific for myoD (Weinberg *et al.*,1996) and shh (Krauss *et al.*, 1993) and a fragment containing part of the coding region (residues 1-320) of zf-PTP-1B. Digoxigenin-labelled sense and antisense probes were prepared by *in vitro* transcription, using appropriate polymerases and a nucleotide mix (Boehringer Mannheim). Embryos were dechorionated mechanically and fixed overnight at 4ºC in 4% paraformaldehyde in PBS. Whole-mount *in situ* hybridisation was done essentially as described (Thisse *et al.*, 1993).

Microinjections

Microinjection needles were pulled on a Sytter pipette puller model P-80/PC using 1 mm borosilicate glass capillaries (Clark Electromedical Instruments) and broken under a microscope to obtain approximately 5 μm tips. RNA was synthesised *in vitro*, using pCS2+MT-zf-PTP-1B (or -zf-PTP-1B-C213S) as template. The indicated quantities of RNA (routinely 250 pg) in nuclease free H_2O were injected in a volume of 1 nl into the blastomeres of 2- to 4-cell stage embryos, using a Narishige nitrogen-pressure injection apparatus and micromanipulator.

Note added in proof: The EMBL database accession number of zf-PTP-1B is Y14427.

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