A Novel Putative Protein-tyrosine Phosphatase Contains a BRO1-like Domain and Suppresses Ha-ras-mediated Transformation*

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To investigate a potential role of protein-tyrosine phosphatases (PTPases) in myocardial growth and signaling, a degenerate primer-based reverse transcription-polymerase chain reaction approach was used to isolate cDNAs for proteins that contain a PTPase catalytic domain. Among the 16 cDNA clones isolated by reverse transcription-polymerase chain reaction from total neonatal rat cardiomyocyte RNA, one, designated PTP-TD14, was unique. Subsequent isolation and sequencing of a full-length PTP-TD14 cDNA confirmed that it encodes a novel 164-kDa protein, p164PTP-TD14. The C-terminal region contains the PTP-like domain, whereas the N-terminal region shows no homology to any known mammalian protein. However, this region is homologous to a yeast protein, BRO1, that is involved in the mitogen-activated protein kinase signaling pathway. Like BRO1, p164PTP-TD14 contains a proline-rich region with two putative SH3-domain binding sites. By Northern blot analysis, PTP-TD14 is expressed as a 5.3-kilobase pair transcript, not only in neonatal heart but also in many adult rat tissues. When expressed in either COS-7 or NIH-3T3 cells, p164PTP-TD14 localizes to the cytoplasm in association with vesicle-like structures. Expression of p164PTP-TD14 in NIH-3T3 cells inhibits Ha-ras-mediated transformation more than 3-fold. This inhibitory activity is localized to the C-terminal PTPase homology domain, since no inhibition of Ha-ras-mediated focus formation was observed with a PTP-TD14 mutant, in which the putative catalytic activity was presumably inactivated by a point mutation. These findings indicate that PTP-TD14 encodes a novel protein that may be critically involved in regulating Ha-ras-dependent cell growth.

Myocardial growth is dependent upon a variety of extracellular factors, signaling through both receptor protein-tyrosine kinases and G-protein-coupled seven-span membrane receptors (1). A unique feature of myocardial growth is that it switches from a mitotic to a hypertrophic mechanism during ontogeny. The former process is characterized by proliferation of cardiomyocytes, and the latter by an increase in cell volume without DNA synthesis or cell division (2). This switch is associated with terminal differentiation of cardiomyocytes and occurs gradually during heart development, starting during the late embryonic stages and ending a few weeks after birth (3). During this period, gene expression, particularly that involving the cell cycle and signaling, is reprogrammed. For example, expression of a number of receptor protein-tyrosine kinases and other cell cycle components decreases (4, 5).

Concomitant with the switch to hypertrophic growth, cardiac muscle cells show a diminished response to receptor tyrosine kinase-mediated mitogenic stimuli, while maintaining or enhancing their responses to G-protein-coupled stimuli (6, 7). Stimulation of G-protein-coupled receptors can result in cardiomyocyte hypertrophy, even when applied to cells derived from neonatal hearts, and the application of such stimuli to cultured cells has proved to be a useful model for evaluating the biochemical mechanisms of hypertrophy (8, 9). Hypertrophy associated with activation of neonatal rat ventricular myocyte α1-adrenergic receptors has been well studied and appears to involve both Gq- and ras-dependent pathways (10, 11). Mitogen-activated protein kinases may also be involved, as they can be independently induced by both heterotrimeric G-protein- and ras-mediated pathways (12, 13).

The mechanisms underlying the switch from a proliferative to a hypertrophic phenotype, or the potential regulators of the hypertrophic response, however, remain unknown. Since not only serine/threonine kinases but also tyrosine kinases are involved in the development of hypertrophy, it is possible that protein-tyrosine phosphatases (PTPases) may be critically involved in the induction of hypertrophy, or the enhanced G-protein-mediated responsiveness and the diminished receptor tyrosine kinase-mediated effects observed with terminal differentiation. Indeed, PTPases have been demonstrated to both positively and negatively regulate signaling pathways.

To this end, we have used a degenerate primer-based RT-PCR procedure to isolate cDNAs encoding PTPases from neonatal rat cardiomyocytes. The primers used in these studies were synthesized based on the catalytic domains of PTPases. Specifically, these domains each encompass approximate 240 residues that include the consensus signature motif, (V/I)HCXXXR(S/T). Using this approach, four abundantly expressed PTPases were identified, including three that had been isolated previously (PTP-LAR, PTPδ, PTP-PEST) (14–16), and

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The abbreviations used are: PTP, protein-tyrosine phosphate; PTPases, protein-tyrosine phosphatase; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase pair(s).
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a fourth that encoded a novel PTP-like protein, designated PTP-TD14. The full-length PTP-TD14 isolated from a rat brain cDNA library encodes a 164-kDa protein. Although its expression is not confined to cardiomyocytes, it contains a PTP-like catalytic domain at its C-terminal end. In addition, it contains a novel domain with homology to a yeast signaling protein, BRO-1, at its N-terminal end (17). When expressed in NIH-3T3 cells, PTP-TD14 inhibits ras-mediated focus formation. This inhibitory activity resides in the C-terminal PTP-like domain, since a mutant, in which the putative catalytic activity of PTP-TD14 is presumably inactive, fails to inhibit transformation. Although phosphatase activity was not evident with conventional substrates, p164PTP-TD14 may selectively interact with an as yet unidentified 115-kDa protein, which is phosphorylated in response to serum treatment. Thus, PTP-TD14 encodes a novel protein that is likely critically involved in regulating ras-mediated mitogenic activity.

MATERIALS AND METHODS

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) for the Isolation of Partial PTPase cDNAs from Cardiac Muscle Cells—RNA was isolated from primary cultures of purified neonatal rat cardiac myocytes as described previously (8). Oligo(dT)20 primer was used for priming first-strand cDNA synthesis in a RT assay with 3 μg of RNA. One-tenth the volume of the RT assay was used further for degenerate primer-based PCR (see Fig. 1a for details of primers) with 30 thermal cycles: 94 °C for 30 s; 45 °C for 40 s; 71 °C for 45 s. Synthesis of the primers used in the PCR reaction was based on conserved residues in the catalytic domain of PTPases (Fig. 1). PCR products between 350 and 400 base pairs were purified from agarose gels and subjected to a second round of PCR using the same primers and conditions. Second round PCR products around 370 base pairs were purified and inserted into a TA cloning vector (Invitrogen). cDNAs were then sequenced for the identification of PTP-like genes.

Isolation of TD14 cDNAs—A size-fractionated rat brain cDNA library (A ZAP II) was kindly provided by Dr. Lei Yu (University of Cincinnati). The TD14 cDNA fragment isolated by RT-PCR was radiolabeled and used as probe for hybridization library lifts of 1 × 106 plagues. Following standard library screening procedures, three positive clones were isolated, mapped by restriction enzyme digestion and partially sequenced. The longest cDNA was then completely sequenced and analyzed with the MacVector computer program.

Construction of Wild-type and Mutant Expression Plasmids—To develop a PTP-TD14 eukaryotic expression plasmid, the insert from clone 1 was ligated into the vector, pcDNA3 (Invitrogen), at the EcoRI site. To construct a PTP-TD14 mutant lacking the catalytic region of the PTP-like domain, the above construct was digested with NheI, which cuts the cDNA at nucleotide 3440, and XbaI that cuts at the 3′ end of the poly(A) tail. The PCR fragment (pcDNA3 vector and the insert from nucleotides 1 to 3440) from the digestion was ligated into the NheI/XbaI compatible ends to obtain the desired mutant. The nucleotide sequence for a Flag epitope was attached to the 5′ end of the coding region of both the wild-type and mutant PTP-TD14 constructs by overlapping PCR, and verified by sequence analysis. The Cys to Ser mutation on PTP-TD14 was constructed using overlapping PCR with two pairs of primers. One pair amplifies the DNA fragment from the 5′ NheI site to the catalytic motif and the other pair produces the segment from the Cys codon to the Ser codon. The overlapping PCR product was digested by NheI and ligated back to PTP-TD14 at two NheI sites to obtain the mutant, PTP-TD14/S.

Northern Blot Analysis—Northern blot hybridization was performed using standard methodologies and a rat multiple tissue Northern blot (CLONTECH). Two PTP-TD14 cDNA fragments, one from the 5′ end to the HindIII site and the other between two NheI sites near the 3′ end, were radiolabeled and used as probes.

Immunoprecipitation and Microscopy—2 × 106 cells/well in two-well Permanox Chamber slides (Nunc) were transfected by the standard calcium phosphate precipitation method with 3 μg of PTP-TD14F or PTP-TD14Δ DNA and then cultured for 48 h. Cells, washed with phosphate-buffered saline, were then fixed with 2% paraformaldehyde plus 0.1% Triton X-100 at room temperature for 30 min. The fixed cells were then blocked with 5% skimmed milk in phosphate-buffered saline for 30 min, followed by incubation with an anti-Flag antibody (1:200 dilution in blocking buffer) for 45 min at room temperature. After washing, anti-mouse IgG (1:200 dilution in blocking buffer) conjugated with fluorescein isothiocyanate (Silenus Laboratory, Australia) was added, and the cells were incubated for another 0.5 h, washed, and mounted with 1% p-phenylenediamine (1 mg/ml, Sigma) in glycerol. Cells were examined using a UV fluorescence microscope and then photographed.

Focus Formation Assay—Focus formation assays were carried out as described previously (18). 1 × 106 NIH-3T3 cells in a 10-cm diameter dish were transfected with 20 μg of DNA, 1 μg of Ha-ras vector, and 19 μg of PTP-TD14 expression vector or vector only, using the standard calcium phosphate precipitation method. Transfected cells were cultured for 12–15 days until foci appeared. Cells were then washed twice with phosphate-buffered saline and twice with 70% EtOH, stained with xylene cyano FF buffer (saturated in 70% EtOH) for 5 min, and then washed three times with 70% EtOH. Dishes were dried in air for 15 min before counting foci.

Immunoprecipitation and Western Blotting—Immunoprecipitation and Western blotting were performed as described previously with anti-Flag-M2 gel (immunoprecipitation) or antibody (Western blotting) (19). Transfected cells (2 × 105/10-cm dish) were cultured overnight in serum-free (for COS-7) or 3% horse serum (for NIH-3T3)-containing medium, and then stimulated with medium containing 50% fetal bovine serum for 20 min. Cells were then harvested immediately with 1 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 2 mM sodium orthovanadate, 10 mM Tris-HCl, pH 8.0, 1% sodium fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM Tris-HCl, pH 7.5). Cell extracts were precleared with 30 μl of 20% protein A-agarose and then immunoprecipitated with 30 μl of M2 gel. Affinity-purified anti-Flag antibody (IBI) or anti-phosphorylated Tyr antibody (Santa Cruz Biotechnology) was used as the primary antibody at a final concentration of 0.2 mg/ml (1:2000 dilution), and anti-mouse IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) was used as the secondary antibody (1:3000 dilution). After SDS-polyacrylamide gel electrophoresis, proteins were electroblotted onto Immobilon-P (Millipore) and detected using the ECL system (Amersham).

RESULTS

Three hundred clones resulting from RT-PCR amplification of neonatal rat cardiomyocyte RNA were sequenced, and fragments containing PTP-like domains were identified. These 61 cDNA fragments encoded 16 different PTPases (Fig. 1b). The mRNAs of the first four PTases isolated were relatively abundant in cardiac muscle cells, as evidenced by Northern analysis of total RNA prepared from cultured cardiomyocytes (data not shown). However, the others were not detectable by Northern analysis. Fig. 1b shows the relative abundance of different PTases among the PCR-generated cDNAs. The two most abundant cDNAs were PTP-LAR and PTPβ. They encode related receptor-linked PTases with immunoglobulin and fibronectin domains in their extracellular regions and are involved in cell adhesion-dependent signaling pathways. The fourth most abundant clone, PTP-PEST, is a soluble cytoplasmic protein (16, 20, 21), the substrate of which was recently suggested to be p130Cas (22, 23), a Src and Crk-associated protein involved in Src- or Crk-mediated signaling. The third most abundant cDNA encoded a novel PTase, designated PTP-TD14. Northern blot analysis performed with the PTP-TD14 cDNA fragment revealed that, although its expression was not confined to heart, its mRNA was abundant in neonatal rat cardiomyocytes. We, thus, isolated and characterized a full-length cDNA for PTP-TD14.

Specifically, the PTP-TD14 PCR fragment was used to probe a rat brain cDNA library. As shown in Fig. 1c, three clones were isolated, which by sequence analysis are overlapping. The longest, clone 1 (4.8 kb) contains a open reading frame encoding a 164-kDa protein (1494 bp in total) as well as portions of the 5′- and 3′-untranslated regions (Fig. 2a; GenBank™ accession number AF077000). Clone 2 consists of a partial PTP-TD14 cDNA at its 5′ end that is fused to a partial cDNA for Na+/K+ ATPase at the 3′ end. Clone 3 is a partial PTP-TD14 cDNA (nucleotides 1321–4778 of clone 1). With all clones, the nucleotide sequence in the region around that encoding the bound-
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PTP-LAR (1404) KMDPFRNKEIQEQQQ---VHCSSGVGRGCTFF (1527)
PTP-1B (1760) KMDPFRNKEIQEQQQ---VHCSSGVGRGCTFF (1887)

Primer sequences for the degenerate PCR primer sets (bottom) were designed based on these regions. a, a list of PTPases cloned by PCR are ranked by the relative abundance of the PCR products. Those with an asterisk are novel PTP-like cDNAs. c, a diagram of three isolated independent PTP-TD14 cDNAs which are numbered based on the clone 1 sequence, the longest cDNA. Clone 2 is a fusion DNA of the PTP-TD14 and Na⁺/K⁺ ATPase cDNAs. The hatched bar represents a 536-base pair cDNA fragment of the Na⁺/K⁺ ATPase cDNA and is joined with PTP-TD14 at nucleotide 4779 relative to clone 1. Clone 3 is a partial cDNA of PTP-TD14 (nucleotides 1321–4779).

Fig. 1. Molecular cloning of PTPases from neonatal rat cardiac muscle cells. a, a sequence alignment of several PTPase catalytic domains shows consensus sequence regions (top). The degenerate PCR primers (bottom) were designed based on these regions. b, a list of PTPases cloned by PCR are ranked by the relative abundance of the PCR products. Those with an asterisk are novel PTP-like cDNAs. c, a diagram of three isolated independent PTP-TD14 cDNAs which are numbered based on the clone 1 sequence, the longest cDNA. Clone 2 is a fusion DNA of the PTP-TD14 and Na⁺/K⁺ ATPase cDNAs. The hatched bar represents a 536-base pair cDNA fragment of the Na⁺/K⁺ ATPase cDNA and is joined with PTP-TD14 at nucleotide 4779 relative to clone 1. Clone 3 is a partial cDNA of PTP-TD14 (nucleotides 1321–4779).

ary between the N-terminal BRO1 (see below) and C-terminal PTP-like domain is identical. To exclude the possibility that the 5′ end of clone 1, which is not present on clone 3, has resulted from fusion with a distinct gene, the presence of this 5′ region on the PTP-TD14 transcript was evaluated by RT-PCR and by Northern blot analysis (data not shown). This confirmed that the entire open reading frame of clone 1 is indeed, a single transcript.

Computer analysis of the 164-kDa protein, p164PTP-TD14, encoded by PTP-TD14, revealed that it lacks an apparent transmembrane segment, a signal peptide, or a targeting sequence for localization to a discrete intracellular organelle. It is, however, extremely proline-rich (17%), particularly in the midportion that separates two distinct domains (Fig. 2a).

The N-terminal domain shows no homology with any known mammalian protein in either the GenBankTM or Pro-Site data bases. However, it does show a high degree of identity with BRO-1 (Fig. 2b), a yeast protein involved in mitogen-activated protein kinase signaling (17). Like members of the BRO-1 family, PTP-TD14 contains the highly conserved sequence, KNDNFIYHEXV(S/P) (KNDNFIYHEAVP in PTP-TD14), at a position close to the N terminus, as well as two putative SH3 domain-binding sites near the C-terminal end of its BRO1-like domain.

The C-terminal domain of p164PTP-TD14 contains a region that shows homology with the catalytic domains of PTPases and includes the sequence VHCSGSGVGRGTA. This differs by one residue (underlined) from the PTPase catalytic signature motif in which alanine is found instead of the serine (24). Overall, this PTPase domain sequence is rather unique in comparison with the consensus sequences identified from others (Fig. 2c). As shown in Fig. 2c, out of 54 consensus residues there are 13 variations in the PTPase domain of p164PTP-TD14 (double underlined residues in Fig. 2c). To evaluate the phosphatase activity of p164PTP-TD14, it was expressed in COS-7 cells. Immunoprecipitates of the expressed protein were then tested using both a nonspecific PTPase assay in which a Tyr-phosphorylated peptide is the substrate (biotin-DGDFEE-IPEEY(P04)) and in a way that hirudin 53–65, (Boehringer Mannheim), or an assay in which Tyr-phosphorylated myelin basic protein is the substrate. Calfintestinal alkaline phosphatase was used as a positive control for both assays. Phosphatase activity was not observed with either assay (data not shown).

Nevertheless, evidence that p164PTP-TD14 may have the phosphatase activity was obtained from Ha-ras-mediated transformation experiments. In these studies, the wild-type PTP-TD14 or a mutant construct, PTP-TD14Δ, was separately co-transfected with a Ha-ras-expressing plasmid into NIH-3T3 cells. The PTP-TD14Δ mutant lacks residues 1099 to 1316 on the C-terminal end that encompass the putative catalytic domain residues 1139–1316, but retains the entire BRO-1 domain and two putative SH3-domain binding sites (Fig. 3a). The purpose of these experiments was to examine whether p164PTP-TD14 could influence ras-mediated mitogenic activity, since BRO-1 is involved in yeast mitogen-activated protein kinase signaling pathways. As shown in Fig. 3b, expression of PTP-TD14 in NIH-3T3 cells inhibited Ha-ras-mediated foci formation to less than one-third. This inhibitory effect was dependent on the amount of PTP-TD14 cDNA transfected (data not shown) and was independent of variations in transfection efficiency, since these were controlled in all experiments by co-transfection with a green fluorescent protein expression vector. Thus, after normalization for transfection efficiency, PTP-TD14 produced a mean 3.3-fold decrease in Ha-ras-induced foci, compared with cells transformed with Ha-ras alone. This inhibitory effect of p164PTP-TD14 was dependent on its PTP-like domain, since no decrease in foci formation was observed with the PTP-TD14Δ construct. To obtain more confirmative data supporting the above result, a mutant (PTP-TD14Δ5/c/s) was constructed, in which the Cys in the catalytic motif (VHCSGSGVGRGTA) was replaced by Ser. This point mutation resulted in abolishment of phosphatase activity of p164PTP-TD14, it was expressed in COS-7 cells. Immunoprecipitates of the expressed protein were then tested using both a nonspecific PTPase assay in which a Tyr-phosphorylated peptide is the substrate (biotin-DGDFEE-IPEEY(P04)) and in a way that hirudin 53–65, (Boehringer Mannheim), or an assay in which Tyr-phosphorylated myelin basic protein is the substrate. Calfintestinal alkaline phosphatase was used as a positive control for both assays. Phosphatase activity was not observed with either assay (data not shown).

We next examined the expression of wild-type and mutant proteins by immunoprecipitation experiments. In these studies, the wild-type PTP-TD14 or a deletion mutant, PTP-TD14Δ, was separately co-transfected with a Ha-ras-expressing plasmid into NIH-3T3 cells. The PTP-TD14Δ mutant lacks residues 1099 to 1316 on the C-terminal end that encompass the putative catalytic domain residues 1139–1316, but retains the entire BRO-1 domain and two putative SH3-domain binding sites (Fig. 3a). The purpose of these experiments was to examine whether p164PTP-TD14 could influence ras-mediated mitogenic activity, since BRO-1 is involved in yeast mitogen-activated protein kinase signaling pathways. As shown in Fig. 3b, expression of PTP-TD14 in NIH-3T3 cells inhibited Ha-ras-mediated foci formation to less than one-third. This inhibitory effect was dependent on the amount of PTP-TD14 cDNA transfected (data not shown) and was independent of variations in transfection efficiency, since these were controlled in all experiments by co-transfection with a green fluorescent protein expression vector. Thus, after normalization for transfection efficiency, PTP-TD14 produced a mean 3.3-fold decrease in Ha-ras-induced foci, compared with cells transformed with Ha-ras alone. This inhibitory effect of p164PTP-TD14 was dependent on its PTP-like domain, since no decrease in foci formation was observed with the PTP-TD14Δ construct. To obtain more confirmative data supporting the above result, a mutant (PTP-TD14Δ5/c/s) was constructed, in which the Cys in the catalytic motif (VHCSGSGVGRGTA) was replaced by Ser. This point mutation resulted in abolishment of the negative regulation to Ha-ras-mediated transformation (Fig. 3c).
fected either with the wild-type construct, or with the Cys to Ser mutation construct, PTP-TD14c/s (data not shown), which contains the putative catalytic region of the PTP-like domain. To evaluate the subcellular localization of p164PTP-TD14 and the influence of the catalytic region of the PTP-like domain on localization, expression of the wild-type PTP-TD14F and the truncated construct (PTP-TD14F) were evaluated by immunohistochemistry in COS-7 or NIH-3T3 cells. In these studies the wild-type protein was evident in cytoplasmic vesicular structures, and this localization was unaltered by deletion of the catalytic region of the PTP-like domain (Fig. 5). This localization was independent of serum stimulation, since the localization is not affected by culturing cells in serum-free medium or with addition of serum stimulation (data not shown).

The expression of PTP-TD14 was evaluated in various tissues by Northern blot analysis. Both a 3' PTP-TD14 probe that contains the region encoding the PTP-like domain (Fig. 6, upper panel) and an a5' probe that encompasses the BRO-1 domain (data not shown) identified a 5.3-kb transcript. Expression was abundant in most tissues and when normalized for RNA loading (Fig. 6, lower panel), was highest in brain, testis, and kidney, and lowest in skeletal muscle.

**DISCUSSION**

PTPases are a large family of proteins, and the human genome has been estimated to contain as many as 500 such genes (24). Thus far, more than 75 PTPases have been identified in different species, including both receptor-linked and nontransmembrane types. These proteins are involved in various biological processes, including cell growth, differentiation, and transformation (25). For example, PTP1B, a functionally well-defined protein, is implicated as a negative regulator in insulin-mediated cell responses (26–28). Interestingly, this PTPase has recently been proven to suppress v-crk-, v-src-, or v-ras-mediated 3Y1 cell transformation through binding with the SH3-domain of a docking protein, p130CAS (29, 30). A mutation in the SH3-domain binding site of the PTPase resulted in the loss of the binding and suppression activities, indicating that the suppression is not due to a nonspecific PTPase catalytic activity.

The PTPase family is characterized by the presence of a conserved catalytic domain of approximately 240 residues, including the signature motif (I/V)HCXAGXXR(S/T). The receptor-linked PTPases contain, in addition to their extracellular ligand-binding and single transmembrane domains, two catalytic domains located intracellularly on their cytoplasmic tail. However, with some receptor-linked PTPases, phosphatase activity is confined to only one of the two putative catalytic domains (31). In addition, phosphatase activity has not been demonstrated for some receptor-linked PTPases that contain only a single putative catalytic domain. These PTP-like pro-
proteins may therefore have regulatory functions by interacting with specific tyrosine-phosphorylated proteins. In support of this notion, it has been demonstrated that a point mutation in the catalytic domain of PTP-PEST inactivates its phosphatase activity, but does not prevent tight substrate binding (22). In contrast with most receptor-linked PTPases, nontransmembrane PTPases contain only a single putative catalytic domain, with their noncatalytic regions being diverse and likely serving a targeting or regulatory function (32). For example, a recently identified member of the nonreceptor PTPases, MEG2, has its catalytic domain linked to an N-terminal segment with sequence similarity to the cellular retinaldehyde binding protein and to SEC14p of Saccharomyces cerevisiae, which acts as a phosphatidylinositol transfer protein (33). Thus, the N-terminal domain of this PTPase may control the activity of its catalytic domain by binding a lipid moiety.

**FIG. 3.** Effect of PTP-TD14 on oncogenic Ha-ras-mediated NIH-3T3 cell transformation-focus formation. A, a diagram of structures of wild-type PTP-TD14 and the C-terminal deletion mutant, PTP-TD14Δ, which lacks most of the PTP catalytic domain, the pcDNA3 vector, PTP-TD14, and PTP-TD14Δ were separately transfected or co-transfected with Ha-ras into NIH-3T3 cells, as indicated, to examine the effect of PTP-TD14 or PTP-TD14Δ proteins on Ha-ras-mediated foci formation. C, a mutant, in which the Cys1250 was converted to Ser (top panel), was tested by the focus formation experiment (bottom panel). Transformed foci were revealed by xylene staining (see “Materials and Methods”). The dark spots (bottom row dishes of b, or lower panel of c) are xylene-stained foci, and the monolayer cells are stained and shown as background (upper row dishes of b).

**FIG. 4.** p164PTP-TD14 expression and co-immunoprecipitation of a 115-kDa protein with the p122PTP-TD14ΔF. PTP-TD14 and PTP-TD14Δ were, respectively, attached to a Flag epitope at their N terminus (PTP-TD14F and PTP-TD14ΔF) and expressed in COS-7 (a) or NIH-3T3 cells (b). p164PTP-TD14F or p122PTP-TD14ΔF proteins were immunoprecipitated with an anti-Flag gel and analyzed by Western blot analysis with antibodies against the Flag-epitope or phospho-Tyr residues as indicated.

**FIG. 5.** Subcellular localisation of the Flag-PTP-TD14 and Flag-PTP-TD14Δ proteins in COS-7 and NIH-3T3 cells. The immunofluorescently stained PTP-TD14F and PTP-TD14ΔF proteins, which were transiently expressed in COS-7 and NIH-3T3 cells, were detected using anti-Flag M2 antibody.
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Here we report the isolation and initial characterization of a novel putative PTPase. Like MEG2, p164\textsuperscript{PTP-TD14} has a unique N-terminal segment consisting of a BRO1-like domain with two putative SH3-domain binding sites (17). In addition, p164\textsuperscript{PTP-TD14} has a proline-rich central region, and a distinct putative catalytic domain, located in its C-terminal segment, that nonetheless shows critical regions of homology with other PTPases.

The high degree of homology between the N-terminal segment of p164\textsuperscript{PTP-TD14} and the yeast BRO1 family of proteins suggests that it may represent the first identified mammalian homologue of this family. Yeast cells which either possess a mutant form of the BRO1 gene (which presumably produces an homologue of this family. Yeast cells which either possess a BRO1 gene, or lack the BRO1 gene, display phenotypes that they contain a distinct 11-amino acid motif (KDNDFIY-

The putative PTPase catalytic region of p164\textsuperscript{PTP-TD14} shows 34.7% sequence identity with the comparable region of PTP-PEST and contains the sequence VHCSSGVRTGAF. This differs only by an alanine to serine (underlined) substitution from the PTPase signature motif. It is thus tempting to speculate that despite the conservative nature of this substitution, the alanine in this position is critical for catalysis and thus explains the inability to detect phosphatase activity with p164\textsuperscript{PTP-TD14}. In keeping with this notion, in PTPLP, which lacks phosphatase activity, this alanine is not present (35). Similarly, substitution of this alanine in PTP LAR abolishes phosphatase activity. However, in both cases the alanine is replaced by a very nonconservative residue, aspartic acid. Also, the receptor-linked PTPase, PTPε, has an alanine in this position in both of its putative catalytic domains, and yet only one has phosphatase activity. Finally, several dual function PT-Pases, which have both tyrosine and serine/threonine phosphatase activities, retain catalytic activity despite nonconservative substitution of this alanine with either a lysine or arginine (36).

It remains unclear, therefore, if the inability to observe phosphatase activity with p164\textsuperscript{PTP-TD14} is due to the alanine to serine substitution or to other residue differences. For example, an aspartate (Fig. 2c, bold) upstream of the signature motif may be critical. Therefore, substitution of this aspartate with an alanine in the PTPase, PTP-PEST, inactivates its phosphatase activity but does not perturb substrate binding (22). Interestingly, a substitution of the aspartate with a glutamate in PTP1B decreased the catalytic activity more than 600-fold (37).

However, such a mutation is still insufficient to trap its substrate due to a low level of catalytic activity. If the PTPase activity is indeed inactive in p164\textsuperscript{PTP-TD14} it may act, rather, in a regulatory fashion by binding specific tyrosine-phosphorylated proteins. Alternatively, p164\textsuperscript{PTP-TD14} may have a restricted substrate specificity that was not revealed by the two phosphatase assays used to assess its activity. The latter possibility is supported by the finding that the PTPase activity appears to be necessary for suppression of Ha-ras-induced transformation. This was evidenced by assaying a Cys to Ser mutation construct, in which the putative catalytic activity of PTP-TD14 is inactivated. This mutation abolishes the suppression activity for Ha-ras-mediated foci formation. On the other hand, the interacting 115-kDa tyrosine phosphorylated protein was evident in cells expressing a deletion construct lacking the catalytic region of the PTPase domain. p164\textsuperscript{PTP-TD14} may, thus, associate with the tyrosine-phosphorylated 115-kDa protein in vivo, although the association with the 115-kDa protein was undetected in cells expressing the wild-type or Cys to Ser mutation construct. This could be due to a lower level of the binding affinity between the full-length protein of p164\textsuperscript{PTP-TD14} and the 115-kDa protein.

Our ultimate purpose is to identify PTPases involved in the regulation of myocardial cell growth, and to further understand the molecular mechanisms involved. The diverse tissue expression of PTP-TD14 excludes its involvement as a cardiac-specific regulator. However, evidence for its potential involvement in yeast, human, and Aspergillus
cardiomyocyte growth and hypertrophy warrant direct evaluation. Such studies are in progress but are complex since cultured cardiomyocyte cell lines are not available, and with primary culture transfection of cardiomyocytes is of low efficiency. It is evident, however, from the PTPases detected by RT-PCR, that PTP-PEST and PTP-TD14 are abundant cytoplasmic PTPases in neonatal cardiomyocytes. We have found recently that PTP-PEST and PTP-TD14 are abundant cytoplasmic PT-

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