CLONING, EXPRESSION AND MUTATIONAL ANALYSIS OF SH-PTP2, HUMAN PROTEIN-TYROSINE PHOSPHATASE [1]

Lison **BASTIEN**, Chidambaram **RAMACHANDRAN**†, Susana **LIU**† and Mohammed **ADAM***

Departments of Molecular Biology and †Biochemistry, Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Quebec, Canada H9R 4P8

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Summary: A human cDNA clone encoding a nonreceptor protein-tyrosine-phosphatase (PTP) has been isolated and sequenced. The 2.1 kilobase pair cDNA encodes for a 593 amino acid protein that contains a single tyrosine phosphatase catalytic domain at the C-terminus. At the N-terminus the protein has two adjacent copies of Src homology region (SH2 domain) which show 61% and 73% identity at the amino acid level to the SH2 domains of the human PTP1C and Drosophila *corkscrew* protein, respectively. The overall homology between SH-PTP2 and PTP1C or to *corkscrew* protein is 58%. When this protein (or its catalytic domain) was expressed in *E. coli* as a glutathione-S-transferase fusion protein tyrosine-phosphatase activity was detected in bacterial cell extracts. Site-directed mutation made at the conserved cysteine (459) residue to serine within the highly conserved VHCXAGXXR sequence in the PTP catalytic domain resulted in complete loss of enzymatic activity demonstrating the importance of this cysteine residue in catalysis. Northern blot analysis showed that SH-PTP2 is expressed as a 6.5 kilobase mRNA in a number of fetal and adult human tissues and cell lines. The highest levels of its mRNA were detected in fetal brain and in adult heart tissue. The identification of SH-PTP2 along with PTP1C and *corkscrew* protein suggest that there exist a family of nonreceptor PTP containing SH2-domain which will participate in specific signal transduction pathways involving tyrosine phosphorylation-dephosphorylation. © 1993 Academic Press, Inc.

Protein-tyrosine-phosphorylation plays a critical role in regulating several fundamental physiological cellular processes such as cellular proliferation and differentiation, cell cycle, gene expression, signal transduction by hormones and growth factors and pathological processes such as oncogenesis (1-4). The tyrosyl phosphorylation state of a protein in cells reflects the balance

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*To whom correspondence should be addressed. Fax: 514-428-8615.

Abbreviations: PTP, protein-tyrosine phosphatase; SH2, Src homology region 2; GST, glutathione-S-transferase; RT-PCR, reverse-transcription polymerase chain reaction; RCM lysozyme, reduced, carboxamidomethylated and maleylated lysozyme.

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between the effects of two reactions namely phosphorylation and dephosphorylation. Phosphorylation is catalyzed by protein tyrosine kinases (PTKs) whereas dephosphorylation is catalyzed by protein-tyrosine-phosphatases (PTPs). Substantial progress has been made in understanding the PTKs, however only recently has increased attention been focused on PTPs. With purification and sequencing of the human placental PTP1B, now at least 15 different members of this family have been identified (5-7). It has been suggested that some PTPs may be tumour suppressor genes (8), where as others may be oncogenes (9).

The PTPs family is broadly divided into two classes. The first class includes low molecular weight intracellular enzymes typified by PTP1B and includes TCPTP, PTP1C, PTPH1, PTP-MEG, PTP-MEG2 and STEP. This class of enzymes is characterized by a single catalytic domain of about 300 amino acids and a variable amino or carboxyl terminal regulatory domain (5-7). The second class of PTPs are receptor like transmembrane proteins and includes CD45, LAR, PTPα, β, γ, δ, ε, ζ. This class of enzymes is characterized by two homologous intracellular catalytic domains (except in the case of PTPβ which has a single domain) and a variable extracellular domain (5-7). The extracellular domain of some members of this class of enzymes shows homology to either Ig-like, fibronectin 3 like repeats or to carbonic anhydrase (10). The ligands, if any, for these receptor like PTPs are not known.

To identify other members of the family of PTPs in human skeletal muscle, we performed reverse transcription-polymerase chain reaction (RT-PCR) amplification using human skeletal muscle RNA as template and with oligonucleotide primers corresponding to the conserved PTP catalytic domain. We report here the sequence of a novel widely expressed PTP that has the hallmarks of the intracellular class of PTPs. It has two SH2 domains on the amino terminus and is different from PTP1C in its tissue distribution.

MATERIALS AND METHODS

Human monocyte (THP-1) and T-cell (CCRF-CEM) cell lines were obtained from ATCC. The human monocyte (THP-1) and T-cell (Jurkat) cDNA libraries were purchased from Clontech. The human skeletal muscle cDNA library was purchased from Stratagene. The Reverse-transcription polymerase chain reaction (RT-PCR) kit was purchased from Perkin-Elmer Cetus. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. Standard DNA manipulations and cloning procedures were done according to Sambrook *et al.* (11).

Isolation of RNA and Northern Analysis; RNAs were either obtained from Clontech or prepared from cell lines by the guanidinium isothiocyanate procedure (12). RNA was electrophoresed on a 0.8% agarose gel containing formaldehyde and transferred to Hybond N membrane (Amersham). The membranes were UV crosslinked and hybridized at 42°C with a radiolabelled human SH-PTP2 cDNA for 20 h as described (13).

Reverse Transcription Polymerase Chain Reaction (RT-PCR): Total RNA from human skeletal muscle was converted to cDNA by random hexamer primers and M-MLV reverse

transcriptase. This cDNA was used as a template for subsequent amplification with *Taq*. DNA polymerase and degenerate primers 1 (sense) and 2 (antisense). The primers 1 and 2 were synthesized based on the conserved amino acid sequences GSDYINA (5'-GGITCIGA(T/C)TA (T/C)ATIAA(T/C)GC-3', primer 1) and KCDQYWP (5'-GGCCA(A/G)TA(T/C)TGI(G/T)C(G/A) CAT(T/C)TT-3', primer 2) within the catalytic domains of PTPs. The PCR was carried out in a Perkin-Elmer Cetus DNA thermal cycler using 35 cycles. The PCR cycles consisted of denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 2 min. The PCR products obtained, between 190-230 base pairs (bp) were cloned into the EcoRV site of pBluescript sk(+) phagemid (Strateagene) and sequenced.

Isolation of Human SH-PTP2 cDNA Clone: The human skeletal muscle cDNA library (~$1x10^6$ plaques) was screened with a ^{32}P-labelled 223 bp PCR fragment, representing a novel PTPs, identified by sequence comparison to other PTPs. Subsequently a human monocyte (THP-1) cDNA library and a human T-cell (Jurkat) cDNA library were screened with a radiolabelled partial cDNA clone isolated from the human skeletal muscle cDNA library using standard procedures (11). To obtain full length SH-PTP2 coding sequences two partial overlapping clones from the monocyte library and one partial overlapping clone from the T-cell library were isolated and subcloned to obtain the full length clone. Briefly, the DNA fragment from clone PTP THP-7A (containing the 5' end, Fig. 1) was subcloned into clone PTP THP-24 (containing the two SH2 domains and the PTP catalytic domain, Fig. 1). Finally, the 3' end of the cDNA was subcloned from clone PTP Jurkat-13 into PTP THP-7A-24, resulting in a full length clone containing the complete coding sequence for SH-PTP2. Nucleotide sequences for all the clones were determined on both strands using the Pharmacia T7 DNA sequencing kit.

Site-Directed Mutagenesis: Site-directed mutagenesis of SH-PTP2 was carried out using the Amersham oligonucleotide-directed for mutagenesis system. The mutation was confirmed by DNA sequencing.

Expression of PTP in Escherichia coli and PTP Assay: The full length SH-PTP2 coding sequence (bases 162-2099), the catalytic domain (bases 779-2099) of SH-PTP2 coding sequence and the Cys 459/Ser mutant SH-PTP2 were subcloned into pGEX-2T (Pharmacia) to generate glutathione-S-transferase (GST) fusion proteins. For protein expression, *E. coli* strain XL-I blue, transformed with pGEX-2T containing the full length, truncated and Cys 459/Ser mutant form of SH-PTP2 cDNA, was grown in 2X YT medium at 37°C to an OD_{600} of 0.8 to 1 and was cooled to 25°C, and induced with 0.1 mM isopropyl *b*-D-galactopyranoside (IPTG) for 18 hr at 25°C. The bacterial cell pellet was washed with PBS and suspended in 1/10th volume of 20 mM Hepes, pH 7.4, 1 mM EDTA, 5 mM DTT, 1 mM Nα-Benzoyl-L-arginine ethyl ester (BAEE), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μg/ml lysozyme and then incubated on ice for 10 min. The bacterial suspension was sonicated on ice for two 15 second periods with 1 min interval. The lysis was completed by adding 10% Triton X-100 solution to a final concentration of 1% and the extract was centrifuged at 10,000 x g for 15 min at 4°C. An aliquot of the supernatant fraction was added to a reaction mixture containing 25 mM imidazole, pH 7.0, 45 mM EDTA, 200 μg/ml BSA and 2 μM ^{32}P-labelled RCM lysozyme (14). The incubation temperature was 25°C and at the times indicated in the figure trichloroacetic acid (final concentration 25%) and BSA (final concentration 2.5 mg/ml) were added. The mixture was incubated on ice for 10 min and centrifuged at 4°C for 10 min at 15,800 x g. An aliquot of the supernatant was counted using 10 ml Aquasol in a liquid scintillation counter.

RESULTS

Cloning of SH-PTP2: In order to identify novel PTP genes in skeletal muscle, we performed RT-PCR amplification using human skeletal muscle RNA as template and degenerate oligonucleotide primers that were deduced from highly conserved residues within the PTP

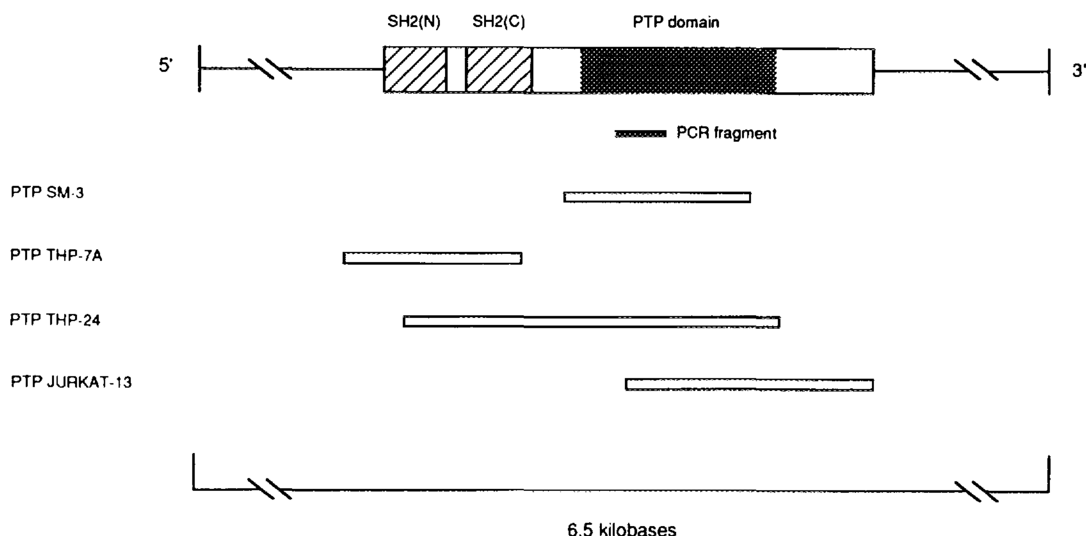

Figure 1. Structure of the full-length SH-PTP2 cDNA, various partial cDNA clones and the novel PCR fragment. Thin lines indicate 5' and 3' noncoding regions. Black box indicates the PTP catalytic domain and the hatched regions indicate the SH2 domains. The thin open box indicates the size and the order of various cDNA clones. SM-3 clone was isolated from skeletal muscle library, THP-7A and THP-24 clones were isolated from monocyte library and Jurkat-13 clone was isolated from T-cell library.

catalytic domains. The products of these reactions resulted in two major fragments, corresponding to sizes of 191-base-pairs and 223-base-pairs (data not shown). These fragments were isolated, cloned and sequenced. Sequence analysis revealed the presence of a number of different known PTP clones including PTPH1, TCPTP, PTP1B, CD45, PTPα (5-7) and one previously unidentified clone, named SH-PTP2, which showed sequence similarities but was not identical to any previously cloned PTPs. Using this SH-PTP2 fragment as a hybridization probe, a human skeletal muscle cDNA library was screened and a partial SH-PTP2 clone (SM-3) was identified. Based on Northern analysis (see below) we subsequently screened human monocyte (THP-1) and T-cell (Jurkat) cDNA libraries using SM-3 as probe. Two overlapping clones from a monocyte cDNA library (THP-7A and THP-24) and one overlapping clone from a T-cell library (Jurkat-13) provided the complete coding region for the SH-PTP2 gene. Figure 1 shows the structure and relative sizes of respective human SH-PTP2 overlapping cDNA clones. Although the entire protein coding region is predicted from the composite structure of the cDNAs a substantial portion (~4.3 kilobases) of the 5' and/or 3' noncoding sequence has not been cloned, as predicted from Northern analysis (see below).

The cDNA sequence and the deduced amino acid sequence of SH-PTP2 are shown in Figure 2. The nucleotide sequence contains a 153 bp 5' untranslated leader, that is GC rich (77%) and contains an out-of-frame upstream ATG immediately followed by a TGA termination

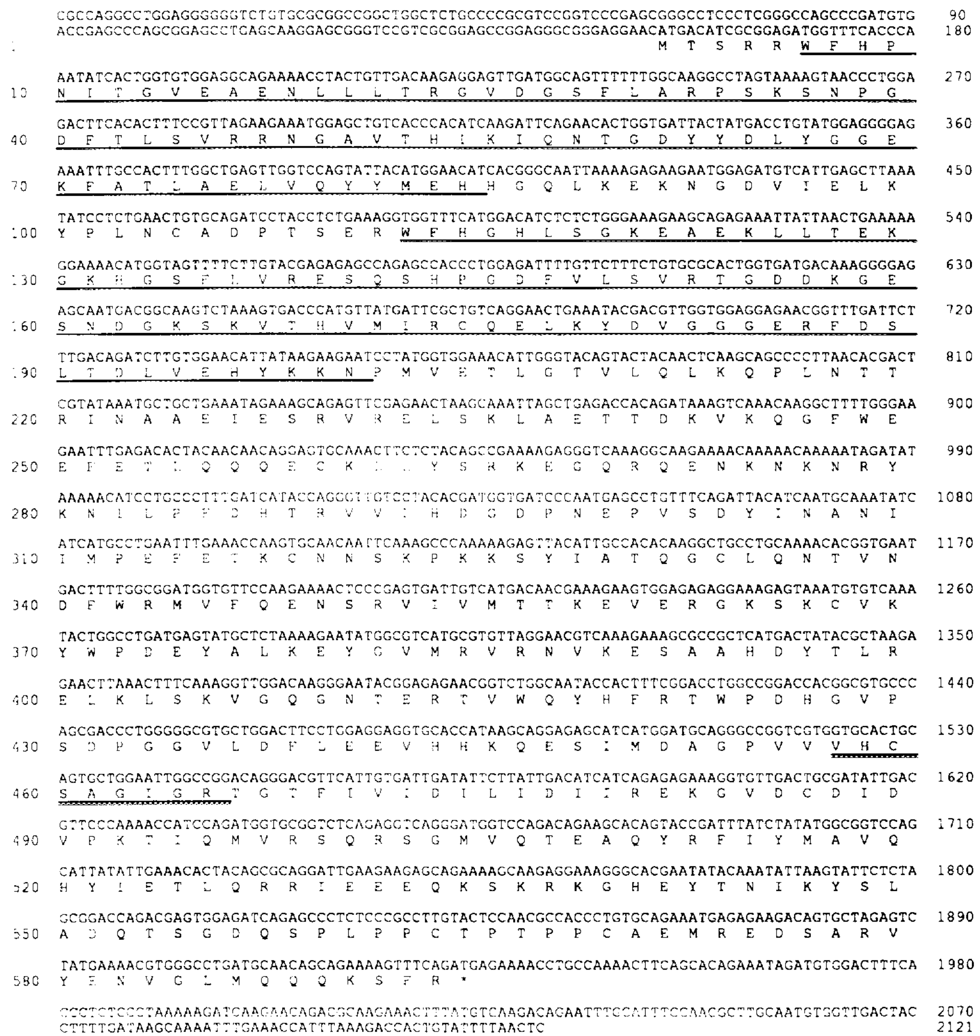

Figure 2. Nucleotide and predicted amino acid sequences of the cDNA encoding the human SH-PTP2. Amino acid residues are numbered on the left; nucleotide positions are numbered on the right. The two SH2 domains are underlined. The amino acid consensus sequence (VHCXAGXXR) found in all tyrosine phosphatases is double underlined. An asterisk denotes the stop codon.

codon. The second ATG at position 154, has a reasonably good consensus sequence for translation initiation (AACATGA) (15) with an in-frame upstream stop codon. The open reading frame terminates at position 1932 bp followed by a TGA termination codon and a 3' untranslated region. The open reading frame of SH-PTP2 encodes a protein of 593 amino acids with a calculated molecular weight of 68.0 kd. Analysis of the deduced protein sequence shows no signal sequence or hydrophobic transmembrane regions suggesting that the protein is cytoplasmic. A single conserved tyrosine phosphatase catalytic domain with a highly conserved sequence VHCXAGXXR which is thought to form part of the dephosphorylation catalytic site is present

A. Alignment of SH2 domains

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                  *            *            *
SH2-PTP-2(N)    6 WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNG..........AVTHIKIQNTGDYYDLYGGEKFATLAELVQYYMEH
SH2-PTP-2(C)  112 WFHGHLSGKEAEKLLTEKGKHGSFLVRESQSHPGDFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFDSLTDLVEHYKKN
PTP1C(N)        6 WFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGD..........QVTHIRIQNSGDFYDLYGGEKFATLTELVEYYTQQ
PTP1C(C)      112 WYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPL.RVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKT
csw(N)          6 WFHPTISGIEAEKLLQEQGFDGSFLARLSSSNPGAFTLSVRRGN..........EVTHIKIQNNGDFFDLYGGEKFATLPELVQYYMEN
csw(C)        111 WFHGNLSGKEAEKLILERGKNGSFLVRESQSKPGDFVLSVRTDD..........KVTHVMIRWQDKKYDVGGGESFGTLSELIDHYKRN
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B. Alignment of core PTP domains

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SH2-PTP-2  267 GQRQENKNKNRYKNILPFDHTRVVLHDGDPNEPVSDYINANIIMPEFETKCNNSKPKKSYIATQGCLQ....NTVNDFWRMVFQENSRVI
PTP1C      267 GQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINANYIKNQLLGPDENA...KTYIASQGCLEA....TVNDFWQMAWQENSRVI
csw        247 GYKQENRLKNRYRNILPYDHTRVKLLDVEHSVAGAEYINANYI.(150 aa insert).KTYIATQGCLLTQQVNTVTDFWNMVWQENTRVI

SH2-PTP-2  353 VMTTKEVERGKSKCVKYWPDEYALKEYGVMRVRNVKESAAHDYTLRELKLSKVGQGNTERTVWQYHFRTWPDHGVPSDPGGVLDFLEEV
PTP1C      350 VMTTREVEKGRNKCVPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFLDQI
csw        475 VMTTKEYERGKEKCARYWPDEGRSEQFGHARIQCVSENSTSDYTLREFLVSWRDQPA..RRIFHYHFQVWPDHGVPADPGCVLNFLQDV
                                 ♦♦♦♦♦♦♦♦♦
SH2-PTP-2  442 HHKQESIMDAGPVV....VHCSAGIGRTGTFIVIDILIDIIREKGVDCDIDVPKTIQMVRSQRSGMVQTEAQYRFIYMAVQHYIETLQR
PTP1C      439 NQRQESLPHAGPII....VHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKK
csw        562 NTRQSHLAQAGEKPGPICVHCSAGIGRTGTFIVIDMILDQIVRNGLDTEIDIQRTIQMVRSQRSGLVQTEAQYKFVYYAVQHYIQTLIA
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Figure 3. Comparison of the human SH-PTP2, SH2 and PTP domain amino acid sequences with human PTP1C and Drosophila *corkscrew* protein. (A) Amino acid alignment of the SH2 domains. Boldface type indicates amino acid identity conserved between SH-PTP2, PTP1C and *corkscrew* protein. Invariant residues are indicated by asterisk and open circles denote basic amino acids that might participate in interactions with phosphotyrosine. (B) Amino acid alignment of PTP domains. Boldface type indicates amino acid identity conserved between SH-PTP2, PTP1C and *corkscrew* protein. The highly conserved amino acid sequences which might be essential for PTP catalytic activity is indicated by diamonds.

at the C-terminus of this protein as in known PTPs (6). At the N-terminus, the protein also contains two adjacent copies of Src homology (SH2) domains similar to Src and Src-linked tyrosine kinases (16). The overall structure of the SH-PTP2 is similar to recently identified mammalian phosphatase PTP1C (17) and Drosophila *corkscrew* protein (18). The PTPs domains of SH-PTP2 shares the highest homology with PTP domains of PTP1C (60% identity) and *corkscrew* protein (58% identity, excluding the 150 amino acid insert in the PTP domain) (Figure 3B). The two SH2 domains in SH-PTP2 are most homologous to the corresponding SH2 domains of *corkscrew* protein (73% identity) and PTP1C (61% identify), and when conserved amino acids are taken into account they have 80% and 72% hemology, respectively (Figure 3A). Finally the overall homology of SH-PTP2 is 58% identical to PTP1C and 57% identical to *corkscrew* protein. The cloning of this novel SH2 domain containing PTP suggests that SH-PTP2, PTP1C and *corkscrew* protein belong to a subfamily of PTPs which contain both PTP catalytic domain and non-catalytic SH2 domains.

Bacterial expression and PTP activity: To confirm that SH-PTP2 encodes an active PTP, the SH2-PTP2 full length protein or the PTP catalytic domain coding sequence was cloned

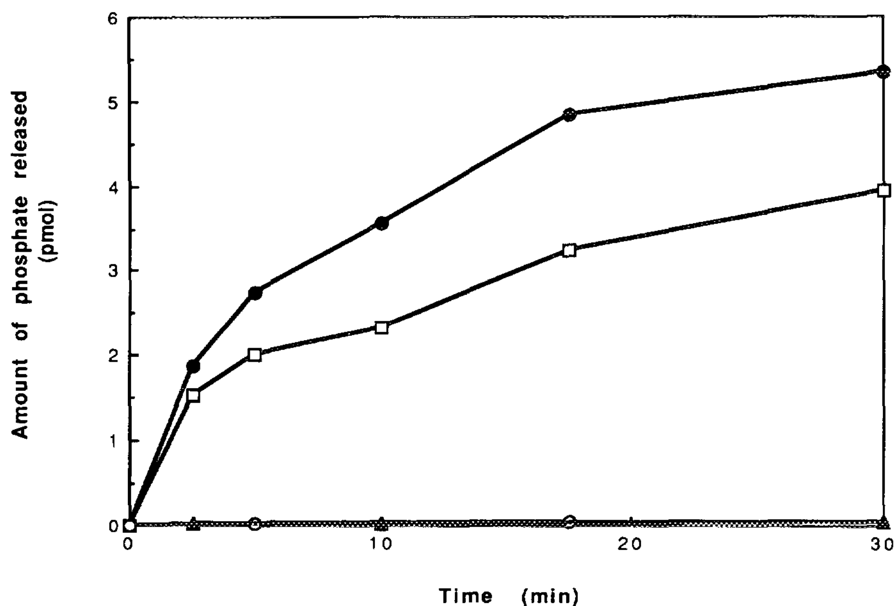

Figure 4. Time course of dephosphorylation of RCM lysozyme. Bacterial extracts from *E. coli* expressing SH-PTP2-GST fusion protein (pGEX-SH-PTP2, □—□), the PTP catalytic domain of SH-PTP2 (pGEX-PTP-2, ●—●), the mutant Cys 459/Ser of SH-PTP2 (pGEX-SH-PTP2 Cys/Ser, ▲—▲) or the GST alone (pGEX, O—O) were incubated with ^{32}P-labelled RCM lysozyme and at time points indicated in the figure dephosphorylation was measured as described in materials and methods. The amount of phosphate released per µl of extract is indicated.

into the bacterial expression vector pGEX-2T. Predicted 95.5 kd and 72 kd recombinant GST-fusion proteins were detected on SDS/PAGE when transformed *E. coli* were induced with IPTG (data not shown). Extracts prepared from IPTG-induced *E. coli* expressing recombinant proteins were tested for tyrosine-phosphatase activity, using ^{32}P-phosphotyrosine containing RCM lysozyme as a substrate. Figure 4 shows the time course of dephosphorylation of RCM lysozyme by bacterial cell extracts. Tyrosine phosphatase activity was detected in extracts containing either the full length SH2-PTP2 GST fusion protein or the catalytic domain of SH2-PTP2 GST fusion protein, whereas no activity was detected in extracts containing GST alone. The dephosphorylation of RCM lysozyme was inhibited by vanadate.

Comparison of deduced amino acid sequences of the SH-PTP2 with other PTPs revealed the presence of the conserved residues VHCXAGXXR within the PTPs domain, which may be important for tyrosine phosphatases catalytic activity. This sequence includes the catalytically essential cysteine (14). Site-directed mutagenesis was used to introduce serine into this position in the protein. This mutant Cys 459/Ser was expressed in the bacterial expression system and tested for tyrosine-phosphatase activity as described above for the wild type protein. As seen from figure 4 the mutation of Cysteine 459 to serine completely abolished the phosphatase

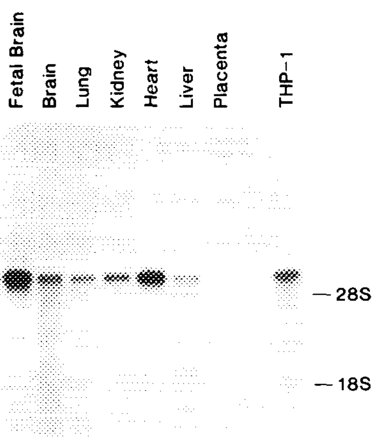

Figure 5. Tissue distribution of SH-PTP2 expression. Northern blot analysis of poly (A)$^+$ RNA (4 μg per lane) from various human tissues and human monocytes cell line (THP-1, 10 μg of total RNA) is shown. The blot was hybridized with ^{32}P-labelled SH-PTP2 cDNA. The hybridization temperature was 42°C in 50% formamide and the final wash was in 1 x SSC at 55°C. Autoradiogram was exposed for 2 days with intensifying screens. Positions of 28S (4.7 kb) and 18S (1.9 kb) ribosomal RNAs are marked. The amount of RNA loaded per lane was equal for all human tissues as indicated by the ethidium bromide-stained gel (not shown).

activity. This suggests that cysteine 459 residue is essential for the enzymatic activity and may play an important role in the catalytic process of the enzyme.

Expression of SH-PTP2 in human tissues: Northern blot analysis of poly (A) RNA from different tissues revealed that the SH-PTP2 gene encodes a ~6.5 kb mRNA and is expressed at some level in all tissues examined. Since the cloned cDNA is 2.1 kb long, the full-length transcript probably contains a considerably longer 5' and/or 3' untranslated sequence. A single 6.5 kb transcript was present in each of the human tissues examined. The highest expression was found in the fetal brain and the heart in adult tissues (Figure 5). SH-PTP2 mRNA was also detected in all the cell lines examined, including human monocyte THP-1 (Figure 5), U937, HL-60, K562, T-cell (Jurkat) and HEPG2 cells (data not shown). This wide tissue distribution of SH-PTP2 expression is in contrast to PTP1C expression which is mainly expressed in haematopoietic cells.

DISCUSSION

We have described a cDNA encoding a non-transmembrane PTP designated as SH2-PTP2. It encodes a 593 amino acid protein containing a single C-terminal catalytic domain and two adjacent N-terminal SH2 domains. The overall structure of SH2-PTP2 is very similar to the human PTP1C (17) and Drosophila *corkscrew* protein (18). Both the full length and the catalytic domain when expressed in bacteria have tyrosine phosphatase activity, however, mutant cysteine

459/Ser is completely inactive suggesting that cysteine 459 is essential for catalytic activity. Northern blot analysis reveals a single 6.5 kb message. In contrast to PTP1C which is restricted to haematopoietic cells, SH2-PTP2 has a wider tissue distribution. Highest amount of mRNA is present in fetal brain and adult heart. Thus SH2-PTP2, PTP1C, and *corkscrew* protein form a subfamily of non-transmembrane PTPs containing non-catalytic SH2 domains.

SH2 domains (Src-homology domain 2) were originally identified as a non-catalytic domain present in the intracellular PTK Src, that was essential for oncogenicity (3). They are approximately 100 amino acids in length and are now known to be present in a wide variety of regulatory proteins that bind specifically to phosphotyrosine containing motifs (reviewed in 16). This SH2 domain phosphotyrosine motif interaction brings about targeting of signal transduction molecules like $p21^{ras}$GTPase-activating protein (GAP), phosphatidylinositol 3'-kinase (P13K) and phospholipase C(PLC)-γ1 to activated (tyrosine phosphorylated) growth factor receptor tyrosine kinases, thereby promoting specific tyrosyl phosphorylation of these molecules resulting in alteration of their function (19). In addition some SH2 domains have been shown *in vitro* to protect certain phosphotyrosyl residues from dephosphorylation by PTPs (20).

SH2 domain containing proteins are divided into two classes, those having catalytic functions like GAP, PLCγ1, Src and those that function simply as adaptor molecules like the p85 subunit of P13K. SH2 domain containing PTPs belong to the first class of protein having enzymatic activity in the molecule. The SH2 domain of SH2-PTP2 has all of the conserved residues typical for binding to phosphotyrosine motifs in protein. (See Figure 3A, 16).

The function of the SH2 domain in SH2 domain containing PTPs is not known. One possibility is that the SH2 domain serves as a localization signal for these PTPs to phosphotyrosine containing substrates. However it seems unlikely as all other PTPs do not have any such domain and are fully functional. The more likely possibility is that the SH2 domain brings about dephosphorylation site specificity. All of the isolated PTPs (except the cdc 25 family) have extremely high activities at least with a variety of artificial substrates. Although they are specific for the dephosphorylation of phosphotyrosyl residues (except the cdc 25 family and Yersinia PTP) they appear to have a very broad substrate selectivity (5-7). The SH2 domain of SH2-PTPs may bring about site selective dephosphorylation of phosphotyrosyl residues not only by protecting certain phosphotyrosyl residues from dephosphorylation, but also may promote dephosphorylation of phosphotyrosyl residues at a different site in the same protein by positioning that residue close to or at the catalytic site. It will be of considerable interest to identify the natural substrates for these PTPs and to investigate the role of the SH2 domain.

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Note added in proof: During preparation of this manuscript, five other reports describing SH-PTP2 have emerged: Feng et al (21), Vogel et al (22), Freeman et al (23), Ahmad et al (24) and Adachi et al (25). However,none of the reports addressed any mutational analysis studies of the protein.

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