Murine protein tyrosine phosphatase-PEST, a stable cytosolic protein tyrosine phosphatase

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We have isolated the murine cDNA homologue of the human protein tyrosine phosphatase PTP-PEST (MPTP-PEST) from an 18.5-day mouse embryonic kidney library. The cDNA isolated has a single open reading frame predicting a protein of 775 amino acids. When expressed *in vitro* as a glutathione S-transferase fusion protein, the catalytic domain (residues 1–453) shows intrinsic phosphatase activity. Reverse transcriptase PCR and Northern-blot analysis show that MPTP-PEST mRNA is expressed throughout murine development. Indirect immunofluorescence in COS-1 cells against a heterologous epitope tag

attached to the N-terminus of MPTP-PEST, together with cellular fractionation and Western-blot experiments from different murine cell lines, indicate that MPTP-PEST is a free cytosolic protein of 112 kDa. Finally, sequence analysis indicates that the C-terminal portion of the protein contains four regions rich in proline, glutamate, serine and threonine, otherwise known as PEST sequences. These are characteristic of proteins that display very short intracellular half-lives. Despite the presence of these motifs, pulse-chase labelling experiments demonstrate that MPTP-PEST has a half-life of more than 4 h.

INTRODUCTION

Protein tyrosine phosphorylation is an important phenomenon by which a wide variety of environmental signals modulate cellular homeostasis. Phosphorylation on tyrosine residues is the result of a highly regulated balance of activity between two antithetic enzymes, protein tyrosine kinases and protein tyrosine phosphatases (PTPases). The latter can be categorized into two different groups on the basis of their substrate specificity [1]. The first group is known as the VH1 subfamily. Members of this dual-specificity subfamily are capable of removing phosphate moieties from phosphoserine, phosphothreonine and phosphotyrosine residues. The second group comprises PTPases that display a unique specificity toward phosphotyrosine residues. PTPases of this group show a broad structural diversity and have been subclassified into either intracellular or transmembrane subfamilies (reviewed in [2]).

Our search for PTPases involved in murine embryogenesis led us to the isolation of a member of the intracellular subfamily of PTPases. This enzyme, which we named MPTP-PEST, has been reported by several groups during the course of our investigation. It has been isolated from murine tissue under the name of P19-PTP [3] or PTPY43 [4] and from human sources as PTPG1 [5] or PTP-PEST [6]. This enzyme displays a ubiquitous pattern of expression in both human and murine tissues and cell lines [3–6]. It is characterized by the presence of a single catalytic domain linked to several PEST motifs. These motifs represent clusters of sequence rich in proline, glutamate, serine and threonine residues (PEST) and were first identified in rapidly degraded proteins [7].

The cellular function of MPTP-PEST has yet to be identified. However, several lines of evidence suggest that it may play an important role in cell growth and differentiation. First, the human gene has recently been mapped to chromosome 7q11.23 which is a common site of chromosomal rearrangement in some malignant melanomas [8]. Second, aberrant transcripts of PTPG1 have been isolated from a human colon cancer cell line [8]. Third, its broad range of expression is also indicative of a fundamental

role in cellular processes. Finally, PTP-PEST was recently shown to be associated with the multifaceted adaptor molecules SHC [9] (A. Charest and M. L. Tremblay, unpublished work). The SHC proteins perform an important function in signalling from various receptor tyrosine kinases to the Ras pathway. Also, SHC has recently been shown to contain a phosphotyrosine-binding domain, termed PTB domain [10], that is different from the conventional SH2 domain. These results demonstrate that PTP-PEST molecules are directly associated with a class of molecules that employ tyrosine phosphorylation as a means of transducing signals and therefore emphasize the possible role of PTP-PEST in signal transduction. To gain further insight into the function and regulation of MPTP-PEST, we have characterized various features of this enzyme. We show that it is an active PTPase that is expressed throughout embryonic development. We also demonstrate that it is a stable protein and that most of it is located in the soluble fraction of the cytoplasm.

MATERIALS AND METHODS

Cell culture and transient transfection

COS-1, Ltk⁺, NIH 3T3, NIH 3T6 and P19 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum at 37 °C in 5 % CO₂. COS-1 cells (between 10⁶ and 10⁷) were electroporated in cold PBS with 20 µg/ml plasmid DNA at 1.2 kV and 25 µF using a Bio-Rad gene pulser.

Oligonucleotides, reverse transcription and PCR

First-strand synthesis reverse transcription of total RNA from whole mouse embryo was performed using Superscript RNase H(–) reverse transcriptase (RT; Gibco-BRL) and random oligomers p(dN)₆ (Pharmacia) or oligod(T)₁₂ (Gibco-BRL) as described by the manufacturer. Subsequent amplification was performed using Vent DNA polymerase (NEB) on a Perkin-

Abbreviations used: PTPase, protein tyrosine phosphatase; pNPP, *p*-nitrophenol phosphate; RT-PCR, reverse transcriptase PCR; RCM-lysozyme, reduced, carboxyamidomethylated and maleylated lysozyme; UTR, untranslated region; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; PDGF, platelet-derived growth factor.

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Elmer Cetus thermal cycler. Oligonucleotides PEST-878s (5'-GCAGTACAAACAAAGGAGCA-3') and PEST-1400 as (5'-AGTGTGTTCCCATCAAAAC-3') were derived from the human PTP-PEST sequence (S.-H. Shen, unpublished work). Oligonucleotides PEST-3 (5'-AGGATGGAGCAAGTGGAG-A-3') and PEST-4 (5'-TGTCCTTTCACTCCCTGCAT-3') were derived from the P19 PTPase sequence [3]. Oligonucleotides PEST-1 (5'-ACTGCCATTTGATCACAGCC-3') and PEST-708 (5'-GGCACATCTTCATGTTCTTGG-3') were derived from the MPTP-PEST sequence.

Molecular cloning and cDNA sequencing

The 18.5-day murine embryonic kidney library was generously provided by Dr. G. Dressler (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI, U.S.A.). Two partial cDNAs (clones 1.2 and 2.8) were isolated using an RT-PCR-generated probe from whole mouse embryo RNA. Full-length cDNAs were reconstituted using RT-PCR methodology as described above. DNA sequencing of both strands was performed with Sequenase (USB) as described by the manufacturer.

Glutathione S-transferase (GST) fusion protein

The phosphatase domain was amplified by Vent DNA polymerase using the cDNA as a template and oligonucleotides PEST-3 and PEST-1400as. The resulting fragment (corresponding to amino acids 1–453) was then subcloned into *Sma*I-digested pGEX-RC (provided by Dr. P. E. Branton, McGill University). In-frame ligations were verified by sequencing. GST fusion proteins were extracted and affinity-purified on glutathione–Sephrose beads (Pharmacia) according to the manufacturer's protocol.

Enzyme assays

For *p*-nitrophenol phosphate (pNPP)-dephosphorylation assays the fusion proteins were incubated in P buffer (25 mM Hepes, pH 7.3, 5 mM EDTA, 10 mM 2-mercaptoethanol) in the presence of 10 mM pNPP at 37 °C. Reactions were terminated at the indicated time points by the addition of an equal volume of 2 M Na₂CO₃; the mixtures were then diluted 5-fold with water, and absorbance was measured at 410 nm. For tyrosine-dephosphorylation experiments, reduced, carboxyamidomethylated and maleylated lysozyme (RCM-lysozyme; provided by Dr. P. E. Branton) was phosphorylated on tyrosine residues as follows: 10 µg of RCM-lysozyme was labelled with 150 µCi of [γ -³²P]ATP (Dupont–NEN) by recombinant Lck tyrosine kinase (provided by Dr. J. Bolen, Bristol-Myers Squibb, Princeton, NJ, U.S.A.) in buffer (50 mM Hepes, pH 7.3, 0.1 mM EDTA, 10 mM MgCl₂, 0.06% 2-mercaptoethanol and 30 µg/ml BSA) at 30 °C overnight. The phosphorylated RCM-lysozyme was separated from unincorporated [γ -³²P]ATP by trichloroacetate precipitation (final concentration 25%) in the presence of 0.2% BSA and 5 mM NaH₂PO₄. After centrifugation (16000 *g*), the pellet was washed several times with cold 20% trichloroacetate/20 mM NaH₂PO₄ and resuspended in a PTPase assay buffer (25 mM Hepes, pH 7.3, 5 mM EDTA, 10 mM dithiothreitol). Dephosphorylation was assayed by mixing a given amount of radio-labelled substrate with the recombinant MPTP-PEST in the PTPase assay buffer, incubated at 25 °C for the indicated time periods and stopped by trichloroacetate precipitation (as described above), and the supernatant (containing released [³²P]P_i) was assayed for radioactivity by liquid-scintillation counting.

HA tag plasmid construct

Plasmid pSM491 (provided by Dr. N. Sonenberg, McGill University) consists of a 9-amino acid triplet repeat HA epitope (YPYDVPDYA) derived from the influenza virus haemagglutinin protein [11–13] inserted into the *Not*I site of pBluescriptII SK (Stratagene). The three HA tags were amplified by PCR with Vent DNA polymerase using pSM491 as template and oligonucleotides H3-ATG (5'-TATCACAAGCTTCCACCATGATCTTTTACCCATACGATGTTCTT-3') and the T7 sequencing primer. The resulting fragment was then digested with *Hind*III and ligated into *Hind*III-digested pRC/CMV (Invitrogen), giving rise to pACTAG-1. pACTAG-2 was derived from the latter simply by digesting with *Xba*I and religating the plasmid on to itself. Inserting the full-length MPTP-PEST cDNA into pACTAG-2 was achieved using recombinant DNA technology. All products were verified by restriction digest pattern and/or DNA sequencing.

Indirect immunofluorescence

pACTAG-2-MPTP-PEST plasmid was transfected by electroporation and transiently expressed in COS-1 cells as described above. At 48 h after transfection, cells were subjected to immunofluorescence microscopy as described elsewhere [14] using the monoclonal antibody 12CA5 as a primary antibody and rhodamine-conjugated goat anti-mouse IgG antibody (Pierce) as a secondary antibody.

Generation of MPTP-PEST antibodies

Polyclonal antibodies were generated in New Zealand White rabbits by the method of Harlow and Lane [15] using MPTP-PEST residues 276–453 fused to GST as the antigen. The bleeding regimen and antibody purification were as described by Harlow and Lane [15]. Immune serum no. 1075 contained a high titre of anti-MPTP-PEST antibodies and was used throughout the experiments.

mRNA detection

Total RNA was isolated from different embryonic stages and Northern-blot analyses was performed as described previously [16]. RT-PCR protocols are described above. ³²P-labelled DNA fragments were prepared using a T7 DNA polymerase random-primed labelling kit (T7 Quick Prime; Pharmacia) according to the instructions of the manufacturer.

Immunoprecipitation of MPTP-PEST

Cells (transfected and non-transfected) were washed several times with PBS and harvested in PBS, centrifuged at 2000 *g* for 5 min at 4 °C and lysed by resuspension in 10 ml of RIPA buffer [50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P40]. In addition, phosphatase and protease inhibitors (5 mM EDTA, 1 mM NaF, 2 mM sodium orthovanadate, 2 mM PMSF, 20 µg/ml aprotinin and 20 µg/ml leupeptin) were added to the RIPA buffer. The lysates were incubated for 10 min on ice and the cell debris was pelleted by centrifugation at 12500 *g* for 20 min at 4 °C. To the supernatants were added 12CA5 monoclonal antibody and protein G–agarose beads (Gibco–BRL), and binding was allowed to proceed for 16 h at 4 °C. The immunoprecipitated protein complexes were washed three times with RIPA buffer and three times in 100 mM Tris/HCl, pH 7.0, containing 200 mM LiCl and 0.1% (v/v) 2-mercaptoethanol. Samples were subsequently subjected to SDS/PAGE.

GCGGCGGCTTCTCTCAGTAGTGTAGGTCT	CTGGGGCGGCACGAGTTGGACTTCCAGAT	GAATTCGCGGCCGCTGTCCAGCTCTGGCG	29
TTTACCACatgcccgtcccagtcgatctg	cttgttcgcctcgtagtaggggaagcgac	CTTGAACCTCTTTGGAGGCTCTCCGCGCG	119
CCGGTCCGCGCCGAGCCGAGCCGCTCG	CGCGGGGAGGGGCGGGCGCGAGCGAG	gctgctctttaagTTGCGCGGGGAGCCCGG	209
GCGGGCGGCGCGGGGGGACGCGGGAGG	ATGGAGCAAGTGGAGATCTGAGGAGTTC	CTGGGGAAGACGGAGCGGGCGCTGCGGCGC	299
GATCACAATGGGGAGGACAACCTTCGCCCCG	M E Q V E I L R R F	ATCCAGAGGGTCCAGGCCATGAAGAGTCCG	389
D H N G E D N F A R	D F M R L R R L S T	I Q R V Q A M K S P	20
GCCACTGGAGAAAAAGAAATGTTAAA	AAGAACAGATATAAGGACATATGCCATTT	AAATATAGAACAGAAAAGATTTATCCACA	479
A T G E K E E N V K	K N R Y K D I L P F	K Y R T E K I Y P T	50
ACTCCATCCCAAGATTTCAGATTATATCAAT	GCAAATTTTATTAAAGGTGTGTATGGGCCA	GATCACAGCCGAGTTAAGTTGACTTTGAAG	569
T P S Q D S D Y I N	A N F I K G V Y G P	D H S R V K L T L K	80
CGGAATACAGTCATAGACTTCTGGAGGATG	ATATGGGAGTATAATGTTGTGATCATCGTG	AAAGCATATGTGGCAACCCAAGGGCTTTT	659
R N T V I D F W R M	I W E Y N V I I V	K A Y V A T Q G P F	110
AAAAAGTGTGAGCGCTACTGGCCTTTGTAT	GGAGAAGATCTCTATAACATTGACCATTT	ATGGCCTGTGAGAAATTTGAGATGGGAAGG	749
K K C E R Y W P L Y	G E D P I T F A P F	M A C R E F E M G R	140
ACCGACTACTTCATCCGAACACTTTTACTT	GAATTTCAAAATGAATCCCGCTCGCTCTAT	AAAATTTCTGTGAAAAATGAACAAGCAAGA	839
T D Y F I R T L L L	E F Q N E S R R L L Y	K I S C E N E Q A R	170
GATGTTCTCTCGTCATTTGATTTCTATCTG	GACATGATAAGCTTAATGAGGAAATACCAA	CAGTTTCATTACGTGAACGTGGCCAGACCAT	929
D V P S S S F D S I L	D M I S L M R K Y Q	Q F H Y V N W P D H	200
TGCAGTCAGGCTGTGGACGAAGGTGCT	ATTGTGTGCCATAGATTACAGTGGAACTTA	GAACATGAAGATGTGCCTATTTGTATTCTAT	1019
C S A G C G R T G A	I C A I D Y T W N L	E H E D V P I C I H	230
AATGTATTTAATTAATAAGAAATGAGA	ACACAGAGGCCTCGGCAGTACAAACAAAG	CTGAAAAGCAGGAAAATTCAGAGGAATTT	1109
N V F N L I Q E M R	T Q R H S A V Q T K	L K A G K I P E E F	260
GCTCAACTGTTTGAAGAAACAGCTACAACG	TATGAATTCATGGAGCAGCAAGATCGCT	GAGCAGTATGAACTTGTTTCATAGGGCTATT	1199
A Q L F E K Q L Q L	Y E I H G A Q K I A	E Q Y E L V H R A I	290
GTCAGTTCCATTGATAGCGAGAAGCAAGAC	TCTCCTCGCCAAAGCCACCGCGGACTCGA	D G N E I T T G T M	320
V S S I D S E K Q D	S P P P K P P R T R	AGTTCCTTGTAGAAAGGGATGCCAAGGAA	1379
GAAATACACGCCACCAGAACCTCACCCG	GTGCCACCATCTGACGCCATCACCTCTCT	S C L V E G D A K E	350
E I L Q P P E P H P	V P P I L T P S P P	TCAGCCTTCCCAACCGTTACCACTGTGTGG	1469
CAGGACAGTGACAGGTACCACCAAAGCCA	GTGCTGCACATGGCCTCACCAGAGCAACAC	S A F P T V T T V W	380
Q D S D R Y H H P K P	V L H M A S P A E Q H	CCAGCCGACCTCAACAGAAGCTATGATAAA	1559
TCAGCGGACCAATGGGGAAAAAGTGAATCA	GCTATTGAGCACATAGATAAAGAGTTAGAG	P A D L N R S Y D K	410
S A D Q W G K S E S	A I E H I D K K L E	CGCAATTTAAGTTTGTAGATTAAAGAAAGTC	1649
CCTCTCCAAGAAGGGCCCAAGTTTGTAT	GGGAACACACTCTTGAATAGGGGACATGCG	R N L S F E I K K V	440
P L Q E G P K S F D	G N T L L N R G H A	ATTAATAATTAATCTGCTTCATCTTCTGTA	1739
GTTGACAGAACCTCTAAACCACAGGAGTTA	AGTGCAGGTGCCCTAAAGGTTGATGATGTA	I K I K S A S S S V	470
V D R T S K P Q E L	S A G A L K V D D V	TCTCAGAAATCTTGC CGCGGACTGTAGTGC	1829
GCTCATTCACACAGAGCTGTGAGTCTGTA	GAGGAGTCCCAGAGCACTACACACACCT	S Q N S C A D C S A	500
A H S H R A A E S S	E E S Q S N S H T P	CCAGGCCAGACTGCTTGCCTCTCGATAAG	1919
AAAGACACGTAACGTGGTCACTTTCATGGA	CCTGAAATGCCACACCTGTACCCGACTCA	P R P D C L P L D K	530
K G H V T W S L H G	P E N A T G P V P D S	CCTGACGGCAAATCCCCAGATAATCATTCT	2009
CAGACTCTGAAAACCGTGAGTTCCACACCC	AATCCACCGCAGAGAGGAGCCACGAT	P D G K S P D N H S	560
Q T L K T V V S T P	N S T A E E E A H D	CCTACAGAGCACCACAACAGCTCCCTCTG	2099
TTGAAAGCTCCCTTCAGCTTTTACCAACCTT	CTTCACTCTGACGACTGGCACTCAGACGGA	L T E H H N S S P L	590
L K A P L S F T N P	L H S D D W H S D G	GGGAGCTCTGATGTTGCTGTGACCGGAAC	2189
AAAATAGCATTTCAACAGCAAGTGGCCACA	GTGTCCTCTCCAGTAGTGTGAGAGTGCT	G S S D G A V T R N	620
K T S I S T A S A T	V S P A S A E S A	TGCCATAGGAGAGTATGCGCATGTCCATT	2279
GCCAGACGGAAGTAGCAGGCACGCCGCAT	TCAGGTGCTGAGAAAGATGCTGATGTTAGT	C H R R V L P M S I	650
A R Q E V A G T P H	S G A E K D A D V S	GAGGAGTCGCTCTCTCTTTACCTGAACGA	2369
ACTCTCTGAGTCTTTGTATTAGCAGATATG	CCTGTAAGACCTGTGAGTGGCATGAACCTCCA	E E S P P P L P E R	680
T P E S F V L A D M	P V R P E W H E L P	AATCAGGAGTGGTCTGAACAAAGGGAATCT	2459
GAAGGCTTGACAACCTCTGGAAATGAAAAA	CATGATGCAGGGGGCATCCACAGAGGCT	N Q E W S E Q R E S	710
E G L T T S G E K	H D A G G I H T E A	TCTGCAGACTCTCCACCTGCTTTTCAGTGAC	2549
AAGAAAGATCAATAACAAAAAGTCCAGCA	GAAGTCACAGATATGGTTTGTGTAATCGC	S A D S P A F S D	740
K K D Q I T K S P A	E V T D I G F G N R	TGTGAAAACCTAAAGGACCAAGAGAGCCA	2639
CCTTCAGAATGGACATGA	TGCAGGGAGTG	C G K P K G P R E P	770
P S E W T *	AAAGGACA		2676
			775

Figure 1 MPTP-PEST cDNA and amino acid sequence

The nucleotide and predicted amino acid sequence of MPTP-PEST are shown. The underlined sequence represents the phosphatase domain. Double-underlined sequences represent PEST motifs. Italicized lower-case letters indicate an open reading frame.

Pulse-chase labelling of MPTP-PEST

Cell monolayers (either Ltk⁻ or transfected COS-1 cells) were washed several times with PBS and starved in methionine/cysteine-free medium (ICN) for 2 h. The cells were then labelled for an additional 3 h with [³⁵S]methionine and [³⁵S]cysteine (Tran³⁵S-label; ICN). Labelled cells were then washed several times with PBS and chased with normal medium for the indicated times after which they were lysed, and MPTP-PEST was immunoprecipitated as described above. MPTP-PEST immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Before exposure of the blot to X-ray film to visualize the metabolically labelled MPTP-PEST proteins, the amount of MPTP-PEST was quantified by Western blotting

using the 1075 polyclonal antibody at a dilution of 1:500, followed by enhanced chemiluminescence (ECL) detection (Amersham).

Subcellular fractionation

Ltk⁻, NIH 3T3 and pACTAG2-MPTP-PEST-transfected COS-1 cells were fractionated as described previously [17], and P19, Ltk⁻ and NIH 3T6 cells were subjected to Triton X-100 extraction [18]. Equal amounts of protein from each fraction were subjected to SDS/PAGE and immunoblotted with the 1075 polyclonal antibody. Anti-[platelet-derived growth factor (PDGF) receptor] antibody was obtained from UBI.

RESULTS

Isolation and structure of the MPTP-PEST

PTP-PEST-specific oligonucleotides, derived from the human PTP-PEST cDNA sequence (S.-H. Shen, unpublished work), were used in an RT-PCR-based approach to isolate a cDNA fragment from 14.5- and 15.5-day mouse embryo mRNA. This 522 bp fragment was then used to probe an 18.5-day embryonic kidney library. Two overlapping clones, coding for the entire cDNA, were isolated. The complete nucleotide and predicted amino acid sequence of MPTP-PEST is depicted in Figure 1. The full MPTP-PEST cDNA sequence contains a single open reading

Figure 2 Demonstration of the intrinsic enzyme activity of the phosphatase domain of MPTP-PEST

Dephosphorylation curves of two substrates, pNPP and RCM-lysozyme, by GST (●, ○), and GST-MPTP-PEST (▲, △) are shown. ▲, ●, A_{410} ; △, ○, $[^{32}\text{P}]\text{P}_i$ released. Inset: Coomassie-Blue-stained gels after SDS/PAGE of the proteins used in the experiment. Lane 1, molecular-mass standards; lane 2, GST; lane 3, GST-MPTP-PEST. The results are representative of at least three different experiments.

Figure 3 Onset of MPTP-PEST gene expression during murine development

(a) RT-PCR of total RNA isolated from embryonic stages (6.5–10.5 days *post coitum*) using oligonucleotides PEST-1 and PEST-708. RNA from each embryonic stage was reverse-transcribed with either random $\text{p}(\text{dN})_6$ oligonucleotides (first lane) or oligo $\text{d}(\text{T})_{12}$ primer (second lane). A control without RT was in the third lane. The arrow indicates the expected size of the PCR product. (b) Northern-blot analysis of MPTP-PEST from later stages of embryonic development (11.5–18.5 days *post coitum*). The arrow indicates the MPTP-PEST message, which is 3.8 kb.

Figure 4 Cellular localization of MPTP-PEST by indirect immunofluorescence

(a) Schematic representation of the triple HA-tagged MPTP-PEST cDNA used to direct transient expression of the MPTP-PEST protein in COS-1 cells. (b) Photomicrographs of COS-1 cells transfected and treated as described in the Materials and methods section. A, C and E, Immunofluorescence microscopy; B, D and F, phase-contrast microscopy. Arrowheads indicate cells that fluoresce in corresponding A, C and E. Photomicrographs in (b) are representative of three different experiments.

Figure 5 Localization of MPTP-PEST by subcellular fractionation studies

(a) Immunoblot analysis of Triton X-100 extraction of P19, Ltk⁻ and NIH 3T6 cells. Cellular components were extracted in a soluble (S) and cytoskeletal fraction (C) as described in the Materials and methods section. Each extract (30 μ g) was separated by SDS/PAGE and immunoblotted with anti-MPTP-PEST polyclonal antibody no. 1075. (b) Coomassie-Blue-stained gel control showing similar amounts of protein in each fraction. The positive (+ve) control refers to 5 μ g of protein extracts from COS-1 cells transiently expressing HA-MPTP-PEST. (c), (d) and (e) Immunoblot analysis of cellular fractions from different sources. Cells were fractionated as described in the Materials and methods section. Protein from each fraction (30 μ g) was subjected to SDS/PAGE and immunoblotted with the anti-MPTP-PEST antibody 1075 (c), anti-(PDGF receptor) antibody (d) and anti-(HA tag) antibody 12CA5 (e). P1, Plasma-membrane and nuclear fraction; S100, cytosolic fraction; P100, particulate fraction. Immunoblot analyses in (c, d and e) are representative of two different experiments.

frame (from nucleotide 330 to 2699) that encodes a protein of 775 amino acids of predicted molecular mass 87 kDa. One of the cDNA clones isolated contains 315 bp of 5'-untranslated region (UTR). This sequence is rich in guanine and cytosine nucleotides and can potentially form secondary structures known to be involved in translational control [19,20]. The 5'-UTR also contains a small open reading frame of 60 nucleotides (Figure 1; lower-case italicized sequence). The N-terminal region of the deduced MPTP-PEST amino acid sequence (from amino acid 55 to 299) contains the catalytic domain. MPTP-PEST catalytic domain contains all of the conserved amino acids found among all tyrosine phosphatases. Overall, the homology between the phosphatase domain of MPTP-PEST and that of other PTPases varies between 30 and 62 %.

Activity of the MPTP-PEST phosphatase domain

To evaluate whether the region defined as the phosphatase domain was capable of intrinsic catalytic activity, a GST fusion protein containing residues 1–453 of the MPTP-PEST protein was constructed (see the Materials and methods section). GST and the GST–MPTP-PEST fusion proteins were affinity-purified on a glutathione–Sepharose matrix, quantified and equal amounts subjected to SDS/PAGE and assayed for enzyme activity (Figure 2). The GST–MPTP-PEST fusion protein readily dephosphorylated the general substrates pNPP and tyrosine ³²P-labelled RCM-lysozyme. As expected, the GST protein was completely devoid of PTPase activity. However, the same fusion protein was not able to dephosphorylate a synthetic tyrosine-phosphorylated peptide derived from an N-terminal region of p34^{cdc2} [21,22] (results not shown).

Expression of MPTP-PEST throughout development

MPTP-PEST has been shown to be ubiquitously expressed [4]. However, its expression during murine development has never been ascertained. Using RT-PCR (Figure 3a) and Northern-blot analysis (Figure 3b), we detected the presence of MPTP-PEST mRNA from day 6.5 *post coitum* onwards. In addition, RNase-protection assays performed on total RNA from embryonic stem cells suggest that MPTP-PEST is expressed as early as the blastocyst stage, i.e. day 3.5 *post coitum* (results not shown).

Cellular localization of MPTP-PEST

In order to narrow the field of potential *in vivo* substrates, we examined the cellular localization of MPTP-PEST by several techniques. The HA epitope (YPYDVPDYA) derived from the haemagglutinin protein of the influenza virus was attached as a three-tandem repeat to the N-terminus of the MPTP-PEST cDNA resulting in the pACTAG-2-MPTP-PEST vector (Figure 4a) (see the Materials and methods section). This vector, when expressed in COS-1 cells, allows the subcellular localization of the HA-MPTP-PEST protein to be tracked by indirect immunofluorescence. At 48 h after transfection, cells were fixed and incubated with the monoclonal antibody 12CA5 which recognizes the HA epitope. Three different fields are shown in Figure 4(b). Neighbouring cells, which are presumably not transfected, display low to relatively non-existent background fluorescence. The micrographs clearly show that the fluorescence staining from the transfected cells, representing the localization of HA-MPTP-PEST, is cytoplasmic. However, the punctate nature of the staining suggests that HA-MPTP-PEST associates or is an integral component of either the cytoskeleton or intracellular membranes such as the endoplasmic reticulum.

Figure 6 Pulse-chase analysis of MPTP-PEST revealing that MPTP-PEST is not rapidly degraded

(a) and (b) Endogenous MPTP-PEST proteins were immunoprecipitated from ^{35}S -labelled Ltk⁻ cells after a chase with unlabelled methionine and cysteine for the indicated time (min). Immunoprecipitates were separated by SDS/PAGE and immunoblotted as described in the Materials and methods section. Western blot (b) allows the quantification of MPTP-PEST proteins present in each sample. (a) Level of ^{35}S -labelled MPTP-PEST immunoprecipitates. The arrowhead indicates MPTP-PEST. Results shown are representative of three different experiments. (c) and (d) COS-1 cells transfected with pACTAG-2-MPTP-PEST and labelled with [^{35}S]methionine/[^{35}S]cysteine were treated as in (a) and (b) except that HA-MPTP-PEST was immunoprecipitated using the 12CA5 monoclonal antibody. Results shown are representative of two different experiments.

To verify the potential association of MPTP-PEST with subcellular structures, a series of subcellular fractionation studies was conducted. First, to determine if MPTP-PEST associates with the cytoskeleton, a differential extraction technique was employed [18]. Adherent cell monolayers were extracted with a buffer that separates cellular components into a Triton X-100-soluble fraction and an insoluble cytoskeleton component. Triton X-100 treatment typically solubilizes membranes allowing the isolation of membrane-bound and cytosolic proteins, leaving the cytoskeleton intact. The latter can therefore be isolated, washed and solubilized. Figure 5(a) clearly shows that MPTP-PEST is present in the soluble (S) fraction of three different murine cell lines but not in the cytoskeletal fraction (C). This suggests that MPTP-PEST is not an integral component of, or does not strongly associate with, the cytoskeleton. As a control for the purity of the fractions, an equal amount of each extract was subjected to SDS/PAGE and then stained with Coomassie Blue. Figure 5(b) shows that each fraction consists of different sets of proteins.

The results obtained from the non-ionic-detergent extraction do not exclude the possibility that MPTP-PEST associates with intracellular membrane structures. To test this, we isolated different cellular fractions and subjected them to Western-blot analysis in order to localize MPTP-PEST. Identical results were obtained with both Ltk⁻ and NIH 3T3 fibroblasts, both of which endogenously express MPTP-PEST proteins (Figure 5c), and COS-1 cells, which transiently express the HA-tagged MPTP-PEST (Figure 5e). Figures 5(c) and 5(e) show that MPTP-PEST is present in the S100 fraction which represents the cytosolic content of the cell. In order to monitor the purity of our

preparations, the Ltk⁻/NIH 3T3 blot was stripped and incubated with an anti-(mouse PDGF receptor) antibody. As shown in Figure 5(d) most of the PDGF receptor immunoreactivity is present in the P1 fraction which is composed of nuclei and plasma membrane. Notice that there is no PDGF receptor immunoreactivity in Ltk⁻ cells. The non-specific bands seen in all three fractions of Figure 5(e) are due to 12CA5 immunoreactive proteins which are detected when large amounts of protein (30 μg) are assayed.

Pulse-chase analysis of MPTP-PEST

As described for the human homologue PTPG1, MPTP-PEST also contains an unusually high content of proline, serine, threonine and glutamate residues within the C-terminal region of the protein. The preponderance of these amino acids is reminiscent of a structure known as PEST motifs [7]. On the basis of the observations of Takekawa et al. [5], we subjected the C-terminal sequence of MPTP-PEST to a PESTFIND algorithm (PC/GENE; Intelligenetics) in order to localize defined regions of PEST sequences. Four regions gave high PEST scores (a score higher than 5 is indicative of potential rapid degradation [7]). Sequence clusters comprised of amino acids 540–553, 565–578, 666–679 and 728–740 (Figure 1, double-underlined sequences) gave PEST scores of 9.78, 11.67, 16.2 and 6.98 respectively. Comparison between the human and mouse PEST motifs demonstrates that only two (565–578 and 666–679) of the four PEST motifs are conserved. Because of the presence of PEST sequences within the C-terminal portion of MPTP-PEST and because proteins harbouring PEST sequences are often thought to be

rapidly degraded [7], we performed pulse-chase experiments to examine the half-life of MPTP-PEST.

Pulse-chase analysis was performed on Ltk⁻ cells and COS-1 cells transiently transfected with pACTAG-2-MPTP-PEST. Cells were starved in methionine- and cysteine-free media, labelled with [³⁵S]methionine/[³⁵S]cysteine, and chased with unlabelled methionine and cysteine for the indicated periods of time at which point MPTP-PEST proteins were immunoprecipitated using anti-MPTP-PEST antibody (Figures 6a and 6b) or anti-(HA tag) antibody (Figures 6c and 6d). The [³⁵S]methionine/[³⁵S]cysteine-labelled immunoprecipitates were separated by SDS/PAGE and transferred to nitrocellulose membranes. The amount of MPTP-PEST protein present in each lane was quantified by Western-blot analysis using the anti-MPTP-PEST antibody 1075 (Figures 6b and 6d). After ECL detection, the blots were completely stripped allowing efficient detection of the ³⁵S-labelled MPTP-PEST proteins using a FUJIX BAS 2000 detection system (Figures 6a and 6c). On densitometric quantification (results not shown) no fluctuation in the level of ³⁵S-labelled MPTP-PEST proteins was observed in immunoprecipitates of Ltk⁻ cells during a chase period of 4 h (Figure 6a). Similarly, 12CA5 immunoprecipitates of COS-1 cells transiently expressing HA-MPTP-PEST also failed to demonstrate that MPTP-PEST is a short-lived protein (Figure 6c). These results indicate that MPTP-PEST is a very stable protein with a half-life of over 4 h.

DISCUSSION

We have isolated the murine homologue of the human PTP-PEST cDNA [6] from an 18.5-day embryonic kidney cDNA library. Alignment of the predicted amino acid sequences of MPTP-PEST with the previously reported murine P19-PTP [3] reveals several discrepancies. One of the major differences lies in a stretch of 118 amino acids contained within residues 296–414 of MPTP-PEST. Nucleotide alignment between MPTP-PEST and P19-PTP cDNAs demonstrates that additional nucleotides in the P19-PTP cDNA sequence are responsible for these observed in-frame shifts (results not shown). DNA sequencing of the regions encompassing the discrepancies at the genomic level (A. Charest, J. Wagner and M. L. Tremblay, unpublished work) indicates that both the nucleotide and amino acid sequences reported in Figure 1 represent the genuine murine PTP-PEST. The overall amino acid sequence identity between MPTP-PEST and its human counterpart is 82.6% (97.9% within the catalytic domain but only 73.8% within the PEST-containing C-terminal domain). Examination of the 5'-UTR sequence (Figure 1) indicates that it is rich in guanine and cytosine nucleotides and thus has the potential of forming stable secondary structures. A second feature of this 315 bp 5'-UTR is the presence of a small open reading frame that may encode a 20-amino acid peptide (Figure 1, nucleotides 129–189). The presence of GC-rich segments and small open reading frames within 5'-UTRs has been shown to influence the efficiency of translation initiation in eukaryotic systems [23]. This represents an additional mechanism through which the function of MPTP-PEST could be regulated.

The predicted molecular mass of MPTP-PEST is 88 kDa. However, Western-blot analysis and immunoprecipitation studies on different murine cell lines indicate that MPTP-PEST migrates as a single 112 kDa band. This molecular mass was confirmed by transient transfection studies of COS-1 cells with the pACTAG-2-MPTP-PEST vector. As expected, the HA-MPTP-PEST protein migrates slightly more slowly than MPTP-PEST protein [Figure 5a, compare murine cell lines with positive control (+ve)] because of the presence of HA tags in the former.

The difference between the predicted and actual molecular mass of MPTP-PEST can probably be attributed to its high proline content which represents 11.1% of the total amino acid content of the protein. Proteins with high proline content are known to display retarded migration on SDS/PAGE. The extensive phosphorylation of MPTP-PEST could also contribute to the retardation of its migration on SDS/PAGE (A. Charest and M. L. Tremblay, unpublished work). Interestingly, Takekawa et al. [8] recently reported that the molecular mass of the human, simian and murine PTP-PEST protein is 88 kDa. This is contradictory to what we have observed. The nature of the phenomenon underlying this inconsistency remains unknown.

When expressed *in vitro* as a GST fusion protein, the phosphatase domain of MPTP-PEST readily dephosphorylated pNPP and tyrosine-phosphorylated RCM-lysozyme (Figure 2) but not a tyrosine-phosphorylated p34^{cdc2} synthetic peptide (results not shown). These results demonstrate that a bacterially expressed MPTP-PEST–GST fusion protein shows specificity in its phosphatase activity towards tyrosine-phosphorylated substrates.

It has been reported that MPTP-PEST is broadly expressed in adult tissues [4]. However, the expression of MPTP-PEST during embryogenesis has not previously been studied. Using a combination of RT-PCR (Figure 3a) and Northern-blot analysis (Figure 3b), we showed that MPTP-PEST mRNA is present as early as day 6.5 *post coitum* of the gestation period. Moreover, RNase-protection experiments, carried out on total RNA isolated from embryonic stem cells which represent embryonic day 3.5 *post coitum*, indicated that MPTP-PEST gene expression can be detected as early as the blastocyst stage of development. These observations, combined with the ubiquitous nature of MPTP-PEST expression in the adult, suggest that MPTP-PEST performs a basic function in mammalian cells.

Because the antibodies available against MPTP-PEST are not sufficiently sensitive to detect the protein by immunofluorescence in non-transfected cells, we utilized the expression system described in Figure 4 to study its cellular localization. Insertion of three HA epitope tags at the N-terminus of MPTP-PEST allowed us to visualize HA-MPTP-PEST by indirect immunofluorescence. Transfected COS-1 cells have been employed extensively in immunofluorescence studies. It has been shown for many different proteins that localization to the appropriate compartments is not altered because of the ectopic expression [14,24,25]. The results from the immunofluorescence (Figure 4b) and cellular fractionation experiments (Figure 5) clearly indicate that MPTP-PEST is a cytoplasmic protein.

Interestingly, Flores et al. [26] recently demonstrated that PTP-PEP, a PTPase with sequence similarity to MPTP-PEST, is located in the nucleus. These authors identified a region of 12 amino acids [⁷⁸⁸SKPKGPRNPPSA⁷⁹⁹] located toward the C-terminus as being responsible for this nuclear translocation. As a similar sequence (⁷⁶²GKPKGPPEPPSE⁷⁷³) is also present in MPTP-PEST, they proposed that this PTPase will also localize to the nucleus. However, our data do not agree with this hypothesis. A possible explanation is the presence of two glutamate residues at positions 769 and 773 of MPTP-PEST. Negatively charged amino acids in the nuclear localization signal are very infrequent, and it has been proposed that such acidic residues counteract the basic amino acid motif present in all nuclear localization signals [27]. Thus these subtle differences could account for the disparity in targeting between MPTP-PEST and PTP-PEP.

PEST sequences were originally defined as being protein motifs that promote rapid degradation [7]. Having localized four of these clusters within the C-terminal region of MPTP-PEST, we

performed pulse-chase experiments to determine if MPTP-PEST, according to the model proposed by Rogers et al. [7], was also a protein with a short half-life. Surprisingly both endogenous MPTP-PEST from Ltk⁻ cells and HA-MPTP-PEST from COS-1 cells transiently transfected with the pACTAG2-MPTP-PEST vector demonstrated very stable half-lives of over 4 h. The lack of conservation of PEST sequences between human and mouse forms suggests that PEST motifs may not be involved in rapid protein degradation for this enzyme. Except for one report by Loetscher et al. [28], the PEST-motif model is solely based on amino acid comparison. In addition, recent data from Pakdel et al. [29] tend to suggest that the PEST hypothesis lacks universality. Interestingly, Flores et al. [26] also demonstrated that the presence of PEST sequences within PTP-PEP does not confer rapid degradation.

The presence of a stable MPTP-PEST free in the cytosol indicates that it could potentially act on any tyrosine-phosphorylated cytoplasmic substrates. We have demonstrated that, although this PTPase contains many PEST motifs, rapid protein degradation does not appear to be a means by which it is regulated. Thus it is likely that the activity of this enzyme is regulated by other mechanisms, such as post-translational modification. Indeed, phosphorylation has been identified in PTP-PEST [30] (A. Charest and M. L. Tremblay, unpublished work) and in several other PTPases [31–33]. The recent observation that MPTP-PEST interacts with SHC proteins suggests another method of regulation of this enzyme [9].

This MPTP-PEST-SHC interaction provides clues to the function of MPTP-PEST. It is possible that, after receptor tyrosine kinase activation, the recruitment of SHC proteins to these receptors may also relocate a fraction of the MPTP-PEST proteins towards tyrosine-phosphorylated signalling molecules and therefore modulate the extent of tyrosine phosphorylation. Under these conditions, MPTP-PEST could act as a modulator of downstream targets such as the Ras pathway. In order to understand the roles and regulation of MPTP-PEST, potential substrates and additional associated proteins of MPTP-PEST will have to be identified.

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