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cDNA CLONING OF A NOVEL PROTEIN TYROSINE PHOSPHATASE WITH HOMOLOGY TO CYTOSKELETAL PROTEIN 4.1 AND ITS EXPRESSION IN T-LINEAGE CELLS[1]

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Summary. Reversible tyrosine phosphorylation plays important regulatory roles in various cellular events including the differentiation and function of lymphocytes. Here we report the cDNA cloning of a non-receptor type protein tyrosine phosphatase, PTP36, which is expressed in murine thymus. PTP36 was a new member of a tyrosine phosphatase subfamily defined by MEG-01 and PTPH1, which had a C-terminal phosphatase domain as well as an N-terminal domain with homology to cytoskeletal-associated proteins like band 4.1, ezrin, and talin. In addition, we found a putative SH3-binding motif in PTP36 but not in MEG-01 or PTPH1. PTP36 was expressed in cells of both hematopoietic and non-hematopoietic origins. In thymocytes subpopulations, PTP36 was preferentially expressed in double positive stage cells. The change of PTP36 expression level along with T cell maturation suggests its involvement in the regulation of T cell development.

The importance of tyrosine phosphorylation by PTK (protein tyrosine kinase) has been well established in various cellular events. Comparing with PTK, much less is known about the role of PTP (protein tyrosine phosphatase), which has potential regulatory functions through negatively controlling the tyrosine phosphorylation level (reviewed in (1)). In the activation or differentiation of T lymphocytes, at least one PTP, CD45, is indispensable (2). However, the family of PTPs has been growing rapidly and there is little information about the role of other PTPs. Therefore we screened the PTPs expressed in lymphoid organs by PCR with degenerative primers. Here we report the cDNA cloning of a non-receptor PTP, PTP36, which is a new member of a PTP subfamily defined by MEG-01 (3) and PTPH1 (4). N-terminal domain of PTP36 has homology to cytoskeletal-associated proteins like band 4.1 (5), ezrin (6), and talin (7), suggesting its localization at membrane or cytoskeleton. In developing thymocytes, PTP36 was transiently expressed in DP stage cells and this might reveal its involvement in T cell development.

[1] The accession number for the sequence of PTP36 is D31842.

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Abbreviations: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PCR, polymerase chain reaction; DP, double positive; DN, double negative; SP, single positive.

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Materials and Methods

Materials. CB-17 scid mice and C57BL/6 mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and Shizuoka Experimental Animal Laboratory (Hamamatsu, Japan), respectively. The thymic stroma cell line, MRL104.8a, was established in our laboratory (8). R9 and R4L2 thymoma cell lines were kindly provided by Dr. H. Yagita, Department of Immunology, Juntendo University School of Medicine.

DNA amplification and cDNA cloning. First-strand cDNA was synthesized from total RNA of day 14 fetal thymus, and was amplified by PCR. Degenerative oligonucleotide primers used were; PTP-A, 5'-CCGCATATG-GA(T/C)-TT(C/T)-TGG-(C/A)(G/T/A)N-ATG-(A/G)TN-(T/G)(T/G)N-(G/C)A-3' (sense), and PTP-B, 5'-CCGCAAGCTTT-CG-CCC-(G/A)A(C/T)-NCC-NGC-NC(T/A)-(G/A)CA-(G/A)TG-3' (antisense). Amplified cDNAs were cloned in a plasmid vector and identified by DNA sequencing. A plasmid clone containing the partial cDNA of a novel PTP was used as a probe and P36-1 was isolated from CB-17 scid thymus library. Further 5' sequence was isolated by anchor-PCR as described (9). Anchor primer and specific antisense primers used to isolate P36-8, -9, -10-5, and -10-12 were; NotI-dT, ATTCGCGGCCGCT$_{17}$(A/G/C); PTP36-B1, ACCTTCCGAGTTACCAGATCAGGGCTGCT; PTP36-B2, GACATGCATGGGAGGCAAGATATAC; PTP36-B3, CAGGTAATACTGATATCTTGTGGC. Finally, PTP36-B2 and PTP36-A2, ATCGAGTGCACGCTGTCGGTGGAAA, were used to isolate P36-11-1 and -2, and PTP36-B1 and PTP36-A3, TCTCGGTTGTTTACCACTCGGCACA were used to isolate P36-12-3 and -16.

DNA sequencing. Nested sets of deletion mutants were generated (10) and sequenced by the dideoxy chain-termination method, using a Taq dye primer cycle sequencing kit and ABI 373A DNA sequencer (Applied Biosystems). Sequence analysis or comparison was done using NCBI E-mail server, FASTA and BLAST E-mail server (11) on GenomeNet, and molecular analysis systems in DNA data bank of Japan (DDBJ).

Preparation of DN, DP, and SP thymocytes. DN, DP, and SP thymocytes were separated as described (12, 13). The purity of separated cells was tested by flow cytometry after staining with anti-CD4 and anti-CD8 monoclonal antibody as described (12).

RNA analysis. Total RNA was prepared by the acidic guanidium isothiocyanate-phenol-chloroform extraction method (14). An aliquot of each RNA sample was electrophoresed on 1% agarose gel and intactness of the RNA was confirmed by visualizing 18S and 28S rRNA bands. RNAase protection assay was done as described (15) with minor modifications. Briefly, labeled RNA (1 to 2 X 10^5 cpm) was hybridized to total RNA samples (5 to 10 μg) in 15 μl hybridization buffer. After hybridization for 12 to 20 hr at 50°C, samples were digested with RNAase A and T1 at 37°C for 60 min and separated on 6% polyacrylamide / 7 M urea gel. Data were visualized by autoradiography.

Results

cDNA cloning of a protein tyrosine phosphatase expressed in thymus. To search for PTPs expressed in the murine lymphoid organs, we amplified partial cDNA of PTPs by PCR with degenerative primers. The amplified cDNA fragments were identified by DNA sequencing and we found several cDNA clones that defined novel murine PTPs. Here we report the structure and the expression of one of these PTPs, PTP36.

Using the partial PCR fragment as a probe, we isolated a cDNA clone, P36-1, from a scid thymus cDNA library (Figure 1). There was no stop codon preceding the first methionine codon in the longest open reading frame of P36-1, suggesting the extension of coding region further in the 5' direction. We could not isolate cDNA clones containing more 5' sequence from the library. Therefore we did anchor-PCR and obtained P36-8, -9, -10-5, and -10-12. Since the substantial error rate of *Taq* DNA polymerase had been reported, we did additional PCR based on the sequence of the anchor-PCR fragments and got P36-11-1, -11-2, -12-3, and -12-16.

Sequence analysis of PTP36. The consensus nucleotide sequence of the nine overlapping cDNA clones and deduced amino acid sequence are shown in Figure 2. The nucleotide sequence

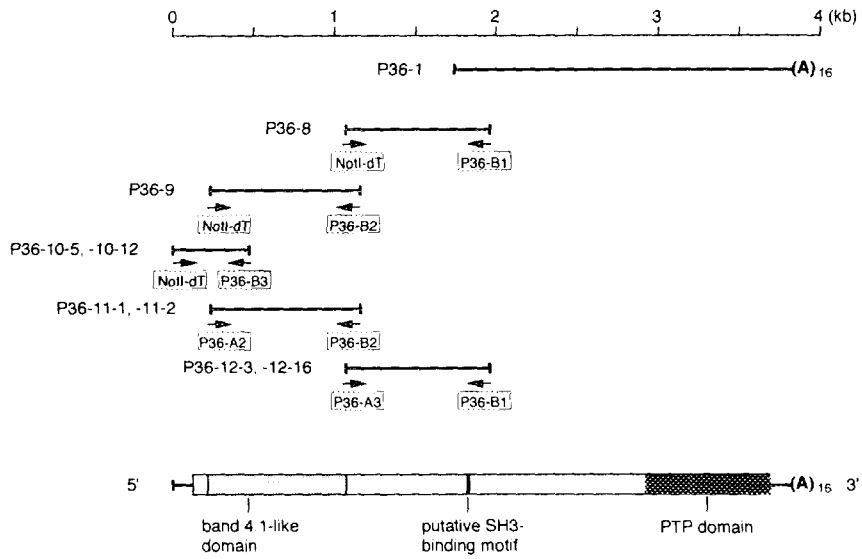

Figure 1. ***Overlapping cDNA clones of PTP36.*** Nine overlapping clones and the predicted structure of PTP36 are shown. The clone P36-1 was isolated from scid thymus cDNA library. P36-8 to -12 were obtained by PCR. Primers used were shown below the each PCR clone.

derived from PCR fragments was confirmed by sequencing several independent clones. At least two independent PCR clones had identical nucleotide at each position.

The longest open reading frame of PTP36 encoded a protein of 1189 amino acids. There was an in-frame stop codon (at nucleotides 44-46) 5' to the first ATG. PTP36 had one putative tyrosine phosphatase domain on its C-terminal end (Figure 2). For the amino acid sequence of the rest part of the molecule, NCBI data base search using BLAST algorithm (11) revealed similarity of amino acid 22 to 241 of PTP36 to N-terminal domains of several cytoskeletal-associated proteins including band 4.1, ezrin, and talin. This structural feature of PTP36 resembled that of two previously reported PTPs, MEG-01 and PTPH1, which were cloned from HUVEC and HeLa cell cDNA library, respectively. (3, 4). PTP-BAS, another PTP with band 4.1-like domain, had somewhat different structure (16). Unlike PTP36, the band 4.1-like domain of PTP-BAS was not on its N-terminus and PTP-BAS has GLGF repeats not found in PTP36, PTPH1, and MEG-01.

The sequence similarity of PTP36 to MEG-01 or PTPH1 was lower than that of MEG-01 to PTPH1 (Table 1). In fact, the similarity of PTP36 to MEG-01 or PTPH1 was marginal in the spacer region connecting N-terminal band 4.1-like domain and C-terminal PTP domain. In this spacer region of PTP36, we found two proline-rich sequences (Figure 2). The sequence from residues 565 to 571, RPPPPYP, matched to the reported SH3-binding site consensus, XpφPpXP (17). The binding of PTP36 to SH3 remains to be determined experimentally.

Distribution of PTP36. Tissue distribution of PTP36 was studied by RNAase protection assay using radiolabeled antisense RNA probe covering a part of PTP domain (Figure 3). The expression of PTP36 was detectable in thymus, kidney, and spleen, weakly detectable in bone marrow and brain, whereas almost undetectable in liver. In cell lines, PTP36 was expressed at a high level in thymic stroma (MRL104.8a and BMST), and fibroblast (BALB3T3) cell lines.

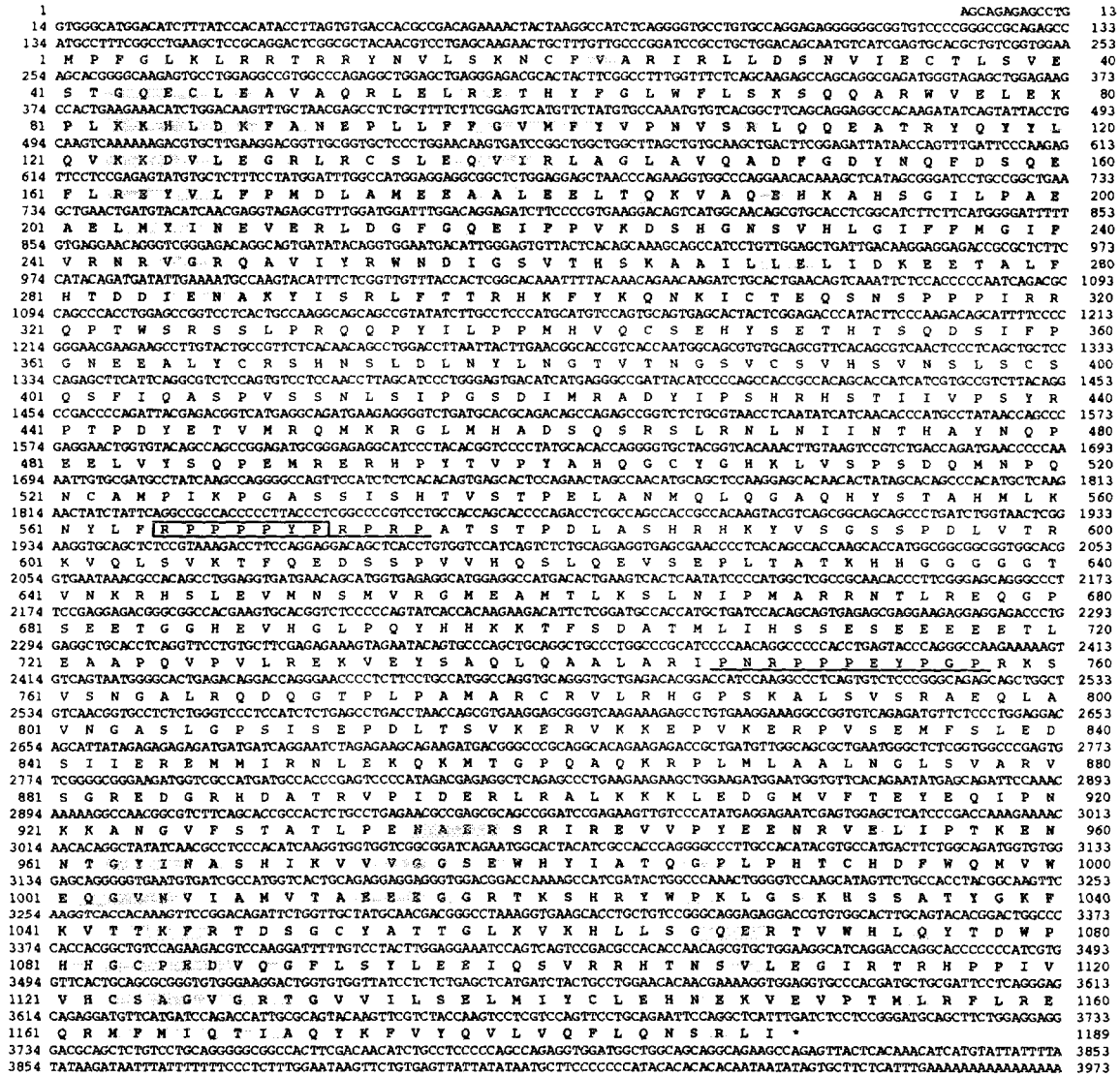

Figure 2. ***Nucleotide and predicted amino acid sequence of PTP36.*** The approximate locations of N-terminal domain with homology to cytoskeletal proteins and C-terminal PTP domains are shaded. Two proline-rich sequences are underlined. Putative SH3-binding motif is boxed.

Weaker level of expression was also detectable in some T-lineage (R4L2, EL4, R9 and 2B4), myeloid (WEHI3), and B-lineage (LK) cell lines. The expression within thymocytes subpopulations was analyzed further. Thymocytes were separated into three fractions, namely, most immature DN ($CD4^-CD8^-$), more mature DP ($CD4^+CD8^+$), and most mature SP ($CD4^+CD8^-$ and $CD4^-CD8^+$) cell fractions. The purity of each fraction was analyzed by flow cytometry and was 94%, 89%, and 89%, respectively. PTP36 expression was detectable in DP cell fraction but only marginal in DN or SP cell fractions (Figure 4), suggesting developmentally regulated expression of PTP36 in the T cell maturation process.

Table 1: Comparison of amino acid sequences of PTP36, MEG-01, and PTPH1

	Whole		N-domain		N to PTP		PTP-domain	
	PTP36	MEG-01	PTP36	MEG-01	PTP36	MEG-01	PTP36	MEG-01
MEG-01	26	-	27	-	14	-	41	-
PTPH1	28	52	30	55	19	42	40	63

Amino acid sequences were compared and identities were expressed as %. Whole, whole molecule; N-domain, N-terminal band 4.1-like domain; N to PTP, the sequence lying between N-terminal band 4.1-like and C-terminal PTP domains; PTP domain, C-terminal PTP domain.

Discussion

We have cloned the cDNA of a novel PTP, PTP36, from murine thymus. PTP36 has the structural features similar to MEG-01 and PTPH1 (3, 4), *i.e.* a C-terminal PTP domain and an N-terminal band 4.1-like domain. These three PTPs defines a subfamily of non-receptor PTP. The sequence similarity of PTP36 to MEG-01 or PTPH1 was lower than that of MEG-01 to PTPH1.

The physiological roles of these PTPs are so far unknown. The N-terminal motif found in these PTPs has potential function to localize these PTPs to plasma membrane or cytoskeleton. Considering the association of many PTKs with membrane or cytoskeleton, it is possible that PTP36 regulates cellular events through negatively controlling phosphorylation level at such sites.

In addition, we found a sequence motif for the SH3-binding site (17) in the spacer region of PTP36. It is possible that PTP36 interact with SH3-containing signal transducing molecules directly through this motif. The sequence similarity of PTP36 to MEG-01 or PTPH1 was only marginal in this region and we failed to find this motif in MEG-01 and PTPH1. This might indicate a unique role of PTP36 distinct from MEG-01 or PTPH1.

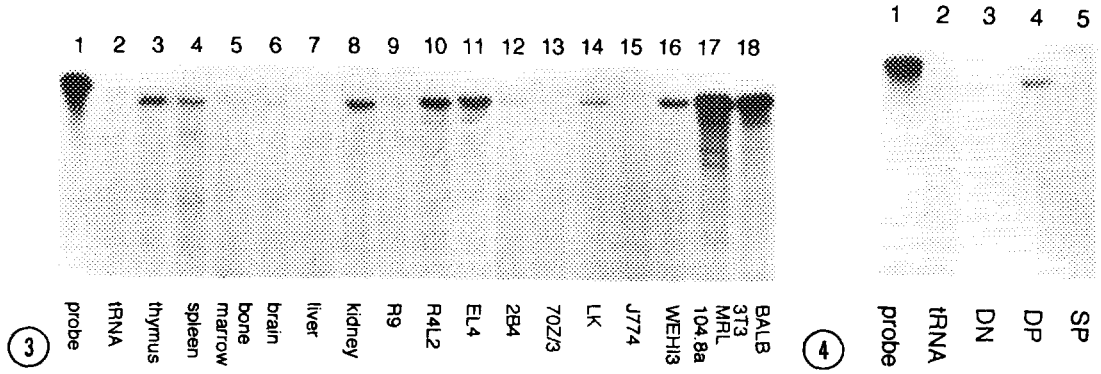

Figure 3. ***RNAase protection assay of the expression of PTP36.*** 10 μg of total RNA from indicated organs of adult C57BL/6 mice or cell lines was used. For each RNA sample, an aliquot was electrophoresed on 1% agarose gel and intactness of the RNA was confirmed by visualizing 18S and 28S rRNA bands (data not shown). The probe arose from the sequence coding a part of PTP domain (nucleotides 3113 to 3509). The size of undigested probe is 475 nt and protection of PTP36 transcripts yields 397 nt fragment. 200 cpm of undigested probe (lane 1) and yeast tRNA (lane 2) were included as controls.

Figure 4. ***Expression of PTP36 in DN, DP and SP thymocytes subpopulations.*** 5 μg of total RNA from DN, DP, and SP fractions of C57BL/6 mice thymocytes was analyzed as described in the legend of Figure 3.

The expression of PTP36 in thymus, where most maturation events of T cell are taken place, is intriguing. We detected the expression of PTP36 in thymic stroma cells as well as in T-lineage cells. In the T cell maturation process, the importance of protein tyrosine phosphorylation was shown by, for example, manipulating the expression level of a PTK, $p56^{lck}$ (18, 19, 20). In thymus, immature DN cells maturate into SP cells through DP stage. PTP36 was expressed transiently in DP stage cells and this suggests the potential regulatory role of PTP36 in T cell development.

PTP36 is clearly not the only PTP whose expression is developmentally regulated. Recently, we have cloned another PTP, PTPT9, which is preferentially expressed in DN cells (manuscript submitted). The role of these PTPs in T cell development remains to be elucidated.

Acknowledgments

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