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Cloning, Expression, and Characterization of a Thermostable PAP2L2, a New Member of the Type-2 Phosphatidic Acid Phosphatase Family from *Geobacillus toebii* T-85

Yong ZHANG, Zhenxing YANG, Xiaodong HUANG, Jing PENG, Xiangwei FEI, Shaohua GU, Yi XIE, Chaoneng JI,† and Yumin MAO†

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, P.R. China

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Most members of the type-2 phosphatidic acid phosphatase (PAP2) superfamily are integral membrane phophatases involved in lipid-related signal transduction and metabolism. Here we describe the cloning of a novel gene from *Geobacillus toebii* T-85, encoding a PAP2-like protein, *Gtb* PAP2L2, which contains 212 amino acids and shows a limited homology to other known PAP2s, especially at conserved phosphatase motifs, and a similar six-transmembrane topology structure. This enzyme was expressed, and purified in *Escherichia coli*. Recombinant *Gtb* PAP2L2s from the membrane fractions were solublized with 0.3% (v/v) Triton X-100 and purified by Ni^{2+} affinity chromatography. The purified enzyme showed broad substrate specificity to phosphatidic acid, diacylglycerol pyrophosphate, and lysophosphatidic, but preferred phosphatidic acid and diacylglycerol pyrophosphate *in vitro*. *Gtb* PAP2L2 is a thermal stable enzyme with a half-life of 30 min at 60 °C. The enzyme was strongly inhibited by 1% SDS, 10 mM veranda, and Zn^{2+}, whereas it was independent of the Mg^{2+} ion, and insensitive to N-ethylmaleimide. The purified recombinant *Gtb* PAP2L2 was catalytically active and highly stable, making it ideal as a candidate on which to base further PAP2 structure/function studies.

Key words: type 2 phosphatidic acid phosphatase; thermostability; integral membrane phosphatase; cloning; purification

The type-2 phosphatidic acid phosphatase (PAP2) superfamily is defined by three conserved phosphatase motifs.[1)] Differently from other phosphatases, most members of this family are integral membrane phosphatases catalyzing dephosphorylation of various phospholipids, such as lysophosphatidic acid (LPA), phosphatidic acid (PA), ceramide-1-phosphate (C1P), diacylglycerol pyrophosphate (DGPP), and sphingosine-1-phosphate (S1P), in a Mg^{2+} independent, N-ethylmaleimide (NEM) insensitive manner.[2,3)] All of these phospholipid molecules and the corresponding dephosporylated products (*e.g.*, diacylglycerol, ceramide, and sphingosine) have been found to be potent signaling molecules. On the basis of this and their cellular membrane localizations, these enzymes in the PAP2 family are postulated to play vital roles in lipid signal transduction and metabolism.[4–6)] This class of enzymes has been shown to be involved in the regulation of cell migration and differentiation, *e.g.*, Drosophila Wunen[7)] and rat Dri42[8)] as homologs of PAP2. At least four different PAP2 isoforms have been described in humans, hPAP2a, 2b, 2c, 2d, and all of these enzymes have broad substrate specificity.[9,10)] In addition to these identified eukaryotic PAP2s, a large number of membrane-associated PAP2-like proteins have been found in bacteria. These bacterial membrane proteins, as eukaryotic PAP2 close homologs, have also been found to be lipid phosphate phosphatases.[11,12)]

Despite its apparent importance, relatively little is known as to structural and functional information on this class of integral membrane phosphatases in the PAP2 family. Previously, a critical insight was that PAP2 phosphatase motifs are also found in structure-known soluble fungal vanadium-dependent choroperoxidase[13)] and nonspecial bacterial acid phosphatase.[14)] Subsequently, amino acids sequence comparison, mutational analysis, and extrapolations on the physical organization of the PAP2 family proteins, suggested that three conserved motifs constitute the active sites of the enzymes.[15,16)] But none of the actual structure of

† To whom correspondence should be addressed. Chaoneng JI, Fax: +86-21-65642502; E-mail: chnji@fudan.edu.cn; Yumin MAO, Fax: +86-21-65642502; E-mail: ymmao@fudan.edu.cn

Abbreviations: PAP2, type-2 phosphatidic acid phosphatase; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate; LPA, lysophosphatidic acid; *p*NPP, *p*-nitrophenyl phosphate; NEM, N-ethylmaleimide

membrane-associated PAP2 is available, partly due to tight association with membranes and the low abundance of PAP2 family enzymes, which hampered large-scale purification attempts and related study. In fact, thermostable proteins from thermophilic organisms have been found to be good models for understanding the function and structure of their mesophilic counterparts, and their thermostability would facilitate protein purification and further study.[17] Furthermore, comparative studies of mesophilic and thermophilic protein homologues might provide insight into the mechanisms of protein thermal adaptation as well as protein evolution.

In this study, we cloned a gene coding for a thermostable PAP2 homolog, *Gtb* PAP2L2, from thermophilic *Geobacillus toebii* T-85, and successfully overexpressed, purified, and characterized this novel member of the PAP2 family.

Materials and Methods

Materials. The enzymes used in vector construction were from New England Biolabs (Beijing, China). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Construction of genomic libraries and screening for thermostable PA phosphatase positive clones. Genomic DNA from thermophilic bacterial was prepared by the phenol-chloroform extraction method[18] and partially digested with *Sau*3AI. The DNA fragments, >2 kb, were recovered from agarose DNA gel and ligated into *Bam*HI-digested dephosphorylated plasmid pUC19. *E. coli* DH 5α was transformed with the ligation mixture and plated on Luria-Bertani (LB) agar plates containing 100 μg/ml ampicilline and 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) to form about 10^3 colonies/10-cm dish. Then all the colonies were lifted onto nitrocellulose filter papers, and the filters were placed, colony-side up, on a 10-cm plate and incubated with 3 ml of buffer (0.1 M Tris–HCl, pH 8.5, and 1% Triton X-100) at 70 °C for 5 min. Ten μl of 0.6 M *p*-nitrophenyl phosphate (*p*NPP) (Merck, Darmstadt, Germany) was supplemented and incubated an additional 5 min at 70 °C. Colonies producing phosphatase hydrolyze *p*NPP, releasing *p*NP yellow in color. Corresponding to the original plates, putative positive colonies were found and chosen. And plasmids (termed pTP) from these colonies were prepared and sequenced by submission to the DNA sequencing facility of Unit Gene (Shanghai, China).

Sequence analysis. Homology searches were performed by BLAST at the National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov/BLAST). A hydropathy plot was obtained using the hydropathy analysis program (http://www.tcdb.org/progs/hydro.php). A phylogenetic tree was inferred by the maximum parsimony method using MEGA program version 4.0.

Construction of expression vector and expression of recombinant protein. The *Gtb* PAP2L2 coding sequence without start coden ATG was amplified by polymerase chain reaction (PCR) from plasmid pTP, using specific primers, Pf 5-CTAGCTAGCAACAAATGTCTTCTATCTG-3 and Pr 5-CCGCTCGAGAAATCCATGGCTTTCGTTG-3, containing *Nhe*I and *Xho*I restriction sites respectively (underlined). The resulting PCR product was digested with corresponding restriction enzymes and cloned into the *Nhe*I and *Xho*I sites of pET-28b (Novagen, Madison, WI). The fidelity of the insertion in pET vectors was confirmed by sequencing. In this case, *Gtb* PAP2L2 was expressed with an additional 27 residues, including the N-terminal hexa-his tag sequence. *E. coli* Rosetta (DE3) pLyS harboring the constructed expression plasmid was grown in 1 liter LB medium containing kanamycin (100 mg/l) and chloramphenicol (34 mg/l), and was induced by the addition of 0.5 mM IPTG at OD_{600} 0.6–0.8. After induction for 10 h at 30 °C, the cells were harvested by centrifugation.

Preparation of membrane fraction and purification of Gtb *PAP2L2 fusion protein.* The harvested cell pellet was suspended in a standard buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and ultrasonicated on ice. The lysate was centrifuged at 100,000 × *g* for 50 min at 4 °C. The supernatant was used as the soluble fraction and the pellet as the membrane fraction after resuspension in an equal volume of the standard buffer. Triton X-100 was added to the membrane fraction to a final concentration of 0.3% (v/v). After stirring for 2 h, the mixture was then stored overnight at 4 °C. Crude extract was obtained by centrifugation at 100,000 × *g* for 50 min to remove insoluble materials. Then, the crude extract was incubated at 60 °C for 30 min and centrifuged at 14,000 × *g* for 30 min. The supernatant obtained was batch-bound to 2 ml of pre-equilibrated Ni-NTA Superflow resin (Qiagen, Hilden, Germeny). The material was loaded into a 0.8 × 8 cm chromatography column and washed extensively with washing buffer (0.2% Triton X-100, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with an increasing imidazole concentration (⩽50 mM) to remove unspecifically bound proteins. Finally, the bound enzyme was completely eluted with an elution buffer (0.2% Triton X-100, 250 mM imidazole, NaH_2PO_4, 300 mM NaCl, pH 8.0). Active fractions were pooled and concentrated by ultrafiltration (Amicon-Ultra-15 column, Millipore, MA).

Protein assay. Analytical 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and native polyacrylamide gel electrophoresis (native–PAGE) were performed, gels were stained, and the proteins were visualized using Coomassie brilliant blue R-250.[18] Protein purity was determined by densitometry using a gel documentation system, Gel–Doc EQ (Bio–Rad, Hercules, CA). Recovered protein from the native

gel electrophoresis was also subjected to western blot assay with an anti-hexa-His antibody (Qiagen, Hilden, Germeny). Detection was done using an ECL detection kit (Pierce, Rockford, IL). The protein concentration was determined by BCA protein assay (Pierce) with bovine serum albumin (BSA) as the standard protein.

Enzyme characterization. The substrate specificity of the enzyme was examined using DGPP (8), PA (8), and LPA (18) (Avanti Polar Lipids, Alabaster, AL) by malachite green colorimetric assay.[19] The assay was performed in 94-well surface assay plates (Nunc, Roskilde, Denmark) in 50 µl of assay buffer (0.1 M Tris–HCl, pH 7.5, 0.2% Triton X-100, 10 mM EDTA) containing DGPP, PA, or LPA at 50 µM in the absence and the presence of *Gtb* PAP2L2 at 3.0 µg. The assay plates were placed into a thermal incubation chamber (Yuejin, Shanghai, China), and the reaction was carried out at 65 °C for 5 min. It was stopped with 100 µl of malachite green reagent (Biomol Green, Plymouth Meeting, PA), and the absorbance was read at 620 nm according to the manufacturer's instructions. Kinetic data were analyzed with a surface dilution kinetics model-fitting program according to the Michaelis-Menten equation.[20] A standard assay for phosphatase activity was carried out in 0.5 ml of reaction mixture containing 0.1 M Tris–HCl buffer (pH 7.5), 2.4 mM *p*NPP, and 3.0 µg of the enzyme. After incubation at 65 °C for 5 min, the reaction was terminated by adding an equal volume of 2.0 M NaOH, and the released *p*NP (18,380 $M^{-1}\,cm^{-1}$) was measured at 405 nm using a spectrophotometer (Hitachi, Tokyo). The optimum pH for enzyme activity was determined in 0.1 M acetate buffer (pH 2.5–6.5), 0.1 M Tris–HCl (pH 6.5–9.0), and 0.5 mM diethanolamine (pH 9.0–13.0) buffer at 65 °C with *p*NPP as the substrate. Temperature effects on activity and thermostability were investigated by standard assay at the indicated temperatures. To determine the effects of metal ions and inhibitors, the enzyme was pre-incubated with various divalent ions and inhibitors for 1 h, and then the activity was assayed by standard procedure.

Results and Discussion

Characterization of thermophilic strain T85

Thermophilic bacteria were isolated from a hot spring in China with LB agar media at 65 °C. The bacterial 16S rRNA gene was amplified as described by Marchesi *et al.*[21] The strain T85 rRNA gene partial sequence (GenBank accession no. EF535794) exhibited a high level of homology with those of *Geobacillus toebii* strains, *e.g.*, 99.6% homology with *G. toebii* strain BGSC 99A1, 99.2% homology with *G. toebii* strain DPE7, and 99.0% homology with *G. toebii* strain E26323. Additionally, we analyzed the phenotype of strain T85 by routine methods, including microscopic inspection, Gram staining, optimal growth temperature and pH. Strain T85 was a rod-shaped, not motile Gram-positive bacillus that grew optimally at about 65 °C at pH 7.5. On the basis of morphological, physiological, and 16S rRNA gene characteristics, strain T85 was assigned to *Geobacillus toebii* T85.

Cloning and sequence analysis of Gtb *PAP2L2*

Through activity-based screening of the thermophilic strain T85 genomic library, two clones with apparent phosphatase activity were detected in approximately 10,000 transformed colonies. According to the sequencing results, one of these two clones encoded a putative alkaline phosphatase, but the other one demonstrated an open reading frame (ORF) of 639 bp encoding 212 amino acids, smaller than previously known and predicted PAP2s, varying form 216 to 357 amino acids in length.[1] A search of conserved domain databases of NCBI by RPS-BLAST analysis suggested that the deduced protein contained a single-domain PAP2like2. The following three highly conserved phosphatase motifs were found in the deduced protein sequence: motif 1, K*XXXXXX*RP; motif 2, PSGH; and motif 3, SR*XXXXX*H*XXX*D (Fig. 1A, C). The deduced protein sequence displayed a relatively limited sequence identity with other known eukaryotic PAP2 homologs (<22% identity), whereas a hydropathy analysis of the deduced protein revealed six remarkable hydrophobic regions of sufficient length to span the membrane, indicating the presence of six trans-membrane segments (Fig. 1B). Similar sequence features were also noted in human and other known membrane-bound PAP2 homologs, which suggest that this ORF product is a new member of the PAP2 superfamily. Hence, the ORF was designated the *Gtb* PAP2L2 gene (GenBank accession no. EF535727). Accordingly, the evolutionary relationships among the various PAP2 homologs of human and bacterial origin were analyzed by phylogenetic tree (Fig. 1D). The results confirmed that the *Gtb* PAP2L2 protein is conserved as a novel PAP2 homolog.

Furthermore, we noted that possible orthologs of *Gtb* PAP2L2 are also present in some human pathogens, *e.g.*, several putative membrane-associated phospholipid phosphatases from *Bacillus cereus* (Genbank accession no. NP977723) and *Bacillus anthracis* (Genbank accession no. ZP00391615). Membrane-associated members of the PAP2 family are found across kingdoms, suggesting important roles of these enzymes in cell physiology.

Expression and purification of the recombinant Gtb *PAP2L2 protein*

The recombinant *Gtb* PAP2L2 protein was expressed in *E. coli*. By optimizing the expression conditions, we found significant elevation of thermostable phosphatase activity in the induced sample. Enzymatic activity was detected mainly in prepared membrane fractions, as compared with extremely low activity in the soluble fractions (data not shown). This result is consistent with

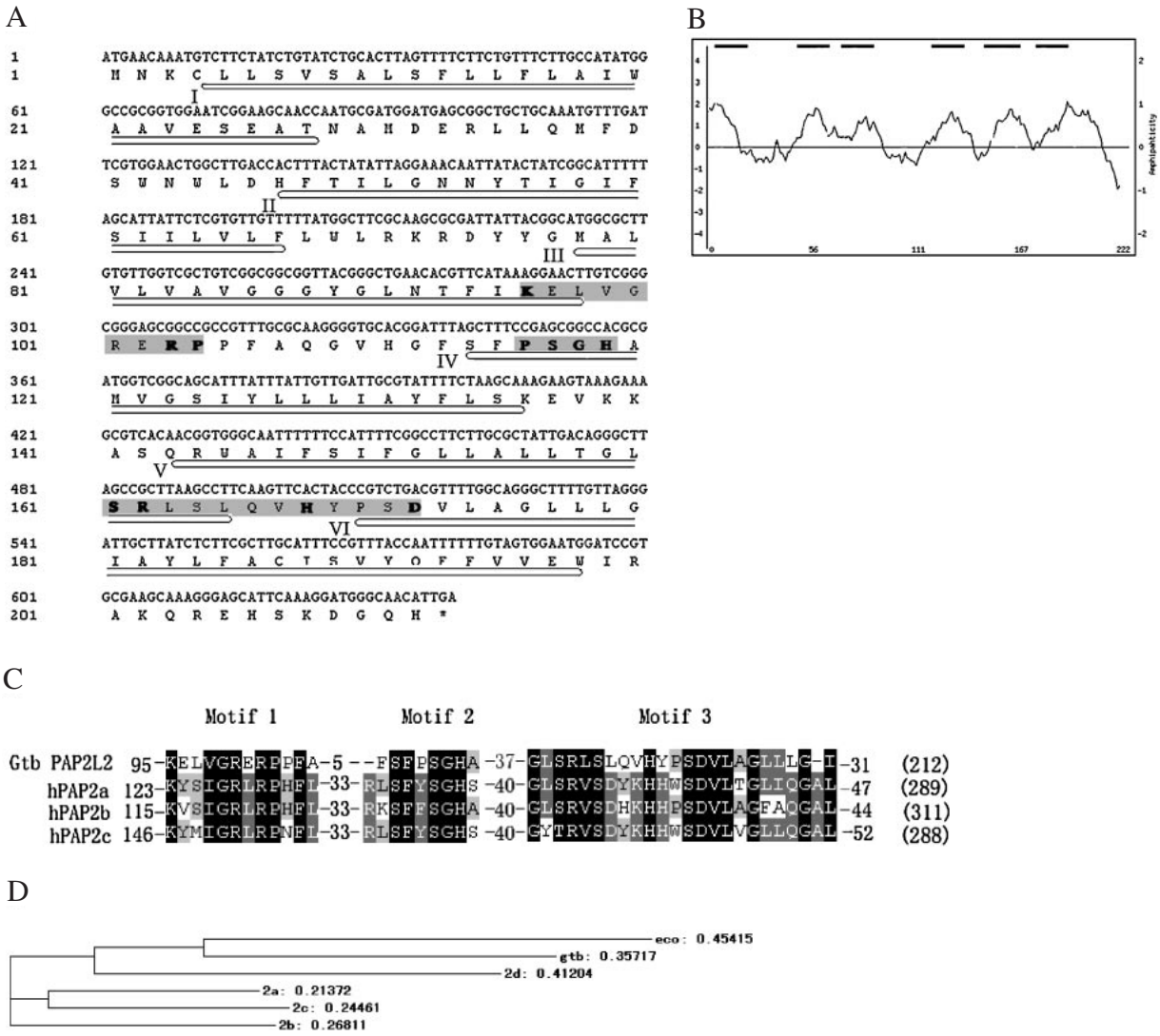

Fig. 1. Sequence Analysis of *Gtb* PAP2L2 Phosphatase.

A, The sequence of *Gtb* PAP2L2. The three conserved phosphatase motifs are in shaded boxes. Predicted transmembrane regions are indicated by the bars under the sequence; B, Hydropathy profile of *Gtb* PAP2L2 amino acid sequence. The hydropathy of *Gtb* PAP2L2 was analyzed by the method of Kyte and Doolittle[22] using a window size of 19 residues; C, Alignments of the three highly conserved type-2 phosphohydrolase motifs of *Gtb* PAP2L2 and several human PAP2s; D, Phylogenetic tree of *Gtb* PAP2L2 and PAP2 homologs. The sequences of several PAP2 family members were aligned by ClustalW. Numbers above the horizontal lines indicate the evolutionary distance between one protein and another. The GenBank accession numbers are NP003702 (2a: hPAP2a), NP003704 (2b: hPAP2b), NP003703 (2c: hPAP2c), NP001032394 (2d: hPAP2d), NP287919 (eco: *E. coli* pgpB), and ABQ12440 (gtb: *Gtb* PAP2L2).

the above membrane protein prediction, and suggests that *Gtb* PAP2L2 was correctly folded and inserted into the plasma membrane of *E. coli*.

In preliminary experiments, we tested several detergents, such as Triton X-100, Na cholate, and CHAPS, to solubilize the recombinant *Gtb* PAP2L2 from the membrane fraction at concentrations from 0 to 2% (data not shown). Finally, most membrane-associated recombinant *Gtb* PAP2L2s were liberated into the supernatant when the membrane fragments were incubated with Triton X-100. Higher concentrations of Triton X-100 released more membrane-associated protein, but resulted in an inhibition of *Gtb* PAP2L2 phosphatase activity and disturbed the resolution of SDS–PAGE. In this experiment, the recombinant *Gtb* PAP2L2 from membrane fragments was effectively solubilized with 0.3% (v/v) Triton X-100. After heat treatment, the majority of thermolabile *E. coli* proteins were removed by centrifugation. Then a single step of Ni^{2+}-chelated affinity chromatography was successful in removing the residual contaminating proteins. The recombinant *Gtb* PAP2L2 was purified to approximately 93% pure, as shown in Fig. 2A. As is often the case with strongly hydrophobic membrane proteins,[23] boiling of protein samples in SDS loading buffer prior on SDS–PAGE often results in significant aggregation of *Gtb* PAP2L2s so that they do not enter the resolving gel. Protein samples were therefore incubated in SDS loading buffer at room temperature before gel loading. The observed protein band migrated to 23.5 kDa

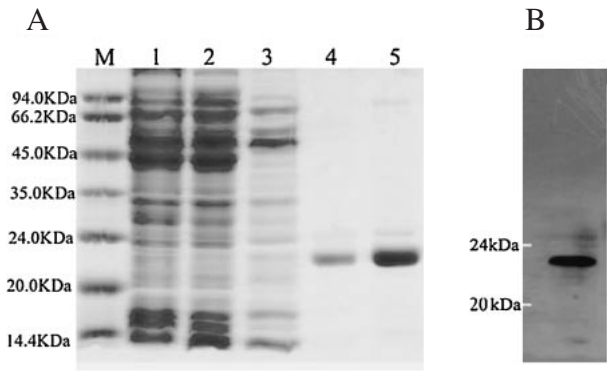

Fig. 2. Electrophoresis and Western Blot Analysis of Purified Recombinant *Gtb* PAP2L2.

A, Analysis of recombinant *Gtb* PAP2L2 expression and purification on 12% SDS–PAGE. Lane M, molecular weight marker; lane 1, total protein of induced bacteria; lane 2, Supernatant of the induced bacteria; lane 3, crude extract after heat treatment at 60 °C for 30 min; lane 4, purified recombinant *Gtb* PAP2L2 protein; lane 5, purified recombinant *Gtb* PAP2L2 after concentration; B, Western blot analysis of purified recombinant protein. The recovered recombinant *Gtb* PAP2L2 protein from native PAGE was further confirmed by Western blotting using the anti-hexa-his tag antibody.

Table 1. Purification of Recombinant *Gtb* PAP2L2 from *E. coli* Cells

	Total proteins (mg)[a]	Total activity (U)[b]	Special activity (U/mg)	Yield (%)	Purification fold
Lysate	224.9	407.4	1.8	100	1
Crude extract	172.2	164.2	1.0	40.3	0.5
Heat treatment[c]	109.8	123.2	1.1	30.2	0.6
Ni-chelation	2.5	81.5	33.3	20.0	18.4

[a]Total protein was determined by BCA assay with BSA as standard.
[b]Activity unit was expressed in μmol/min with *p*NPP as substrate.
[c]Data for heat treatment were taken directly after the heating process, not reflecting that denatured proteins were removed by centrifugation.

Table 2. Kinetic Constants for Recombinant *Gtb* PAP2L2[a]

Substrate	$K_{m(app)}$ (mol %)	$V_{max(app)}$ (units[b]/mg)	V_{max}/K_m (units/mg/mol %)
PA	1.5	0.44	0.29
LPA	2.6	0.10	0.04
DGPP	1.1	0.36	0.32

[a]The concentrations of PA, DGPP, and LPA in the mixed micelles are expressed in terms of surface concentration (in mol %).
[b]Unit of activity defined as μmol/min.

according to a molecular weight marker (Qiagen). This value is smaller than the theoretical value of 26.8 kDa as calculated from the deduced amino acid sequence of recombinant *Gtb* PAP2L2 with the ExPASY proteomics tool.[24] Migration shift is a common feature of membrane proteins.[23] It might be due to the absence of a boiling step unfolding the highly hydrophobic membrane protein in this experiment.

In order further to confirm the identity of the purified recombinant enzyme, the purified samples were subjected to native PAGE at 4 °C. Following electrophoresis, one lane from the gel was stained with Coomassie blue. It showed a single major band. The corresponding gel band on a second lane from the native gel was excised, minced in assay buffer and homogenized, and divided into two fragments. One fragment was detected by Western blot, and a 23.5 kDa band was verified using anti-His_6-tag antibodies (Fig. 2B). In addition, the other fragment was assayed for enzymatic activity, and phosphatase activity was found in the reaction mixture. These results confirmed purified recombinant *Gtb* PAP2 phosphatase. The efficiency of the purification process is shown in Table 1. The typical yield of the purified recombinant *Gtb* PAP2L2 was approximately 2.0 mg per 1 liter culture.

Characterization of recombinant Gtb *PAP2L2*

Since the physiologically relevant substrates for *Gtb* PAP2L2 are still unclear, the enzyme was tested for activity against a variety of phosphorylated lipid substrates. The substrate specificities of the *Gtb* PAP2L2 enzyme for PA, DGPP, and LPA were examined under optimal assay conditions. Kinetic experiments were performed using well-defined phospholipid/Triton X-100 mixed micelles.[20] Acting on detergent solubilized substrates, *Gtb* PAP2L2 exhibited typical surface dilution kinetics, in which the rate of substrate hydrolysis depends on the molar fraction of the substrate in the mixed detergent and lipid micelle (data not shown). Surface dilution kinetics is a model system that mimics the physiological surface of the membrane, where two-dimensional surface interactions occur.[20] The kinetic constants for the enzyme were obtained using the Michaelis-Menten equation, and are summarized in Table 2. *Gtb* PAP2L2 phosphatase catalyzed dephosphorylation of PA, LPA, and DGPP, showing broad substrate specificity, but preferred DGPP and PA ($V_{max}/K_m = 0.29$, 0.04, and 0.32 units/mg/mol % respectively). The K_m values indicate that the affinity of the enzymes for each substrate. The *Gtb* PAP2L2 phosphatase exhibited relatively high affinity for DGPP and PA ($K_m = 1.1$ and 1.5 mol %). Similar K_m values for the PAP2 enzyme hydrolyzing PA was found in human PAP2s ($K_m = 0.04$–3.4 mol %).[25] As a lipid phosphate phosphatase, *Gtb* PAP2L2 differs from the DGPP phosphatases from *E. coli*[26] and from *S. cerevisiae*[27] with respect to substrate specificity. Those DGPP phosphatases displayed about 9-fold greater phosphohydrolase specificity for DGPP than for PA. However, recombinant *Gtb* PAP2L2 phosphatase degraded PA and DGPP at similar rates.

Considering the stability of the tested phospholipids substrates at high temperatures and extreme pH, the activity against *p*-NPP, a general phosphatase substrate, was determined to examine the biochemical properties of *Gtb* PAP2L2 phosphatase. Several membrane-associated

members of the PAP2 family, such as *E. coli* diacylglycerol pyrophosphate phosphatase (DGPPase),[26] yeast DGPPase,[27] and rat PAP2,[28] have been described to be optimally active at pH 6–7. Here, a similar pH-activity profile was found in recombinant *Gtb* PAP2L2, which displayed higher activity at pH 6 to 8, with an optimal pH of 7.5 (Fig. 3A). Negligible phosphatase activity for recombinant *Gtb* PAP2L2 was detected at acidic pH, below pH 5.0, and this clearly distinguished this enzyme from bacterial acid phosphates.[14] The optimal catalytic reaction temperature for the recombinant *Gtb* PAP2L2 was at about 70 °C (Fig. 3B). The purified recombinant *Gtb* PAP2L2, which showed no loss of phosphatase activity at 55 °C after incubation for 1 h, was a thermostable enzyme, as compared to a measured half-life of 55 s at 50 °C for *E. coli* DGPPase.[26] Further analysis indicated that the half-life of recombinant *Gtb* PAP2L2 for thermal inactivation at 60 °C was up to 30 min (Fig. 3C).

The activities of PAP2s from yeast and mammalian cells have been characterized as being Mg^{2+} independent and NEM insensitive.[1,26] The effects of various divalent cations on bacterial *Gtb* PAP2L2 were examined. Mg^{2+} and Ca^{2+} at a concentration of 10 mM had no effect on *Gtb* PAP2L2 phosphatase activity. Cd^{2+}, Co^{2+}, Mn^{2+} exhibited a partly inhibitory effect on activity, whereas the *Gtb* PAP2L2 enzyme lost nearly 100% of its original activity in presence of 10 mM Zn^{2+} (Table 3). These observations corroborate previous findings with other membrane-associated members of the PAP2 family.[27] However, further analysis revealed that the inhibition of Zn^{2+} on *Gtb* PAP2L2 activity was reversible, since the phosphatase activity was completely restored by treatment with EDTA. This reversible inhibitory effect probably reflects a specific interaction of *Gtb* PAP2L2 with Zn^{2+}. It calls for additional study to verify it. Inhibition studies revealed that the enzyme was completely inactivated by ionic detergent 1% SDS, but a non-ionic detergent, 1% Trion X-100, suppressed only about 45.6% of the enzyme activity. Vanadate at 10 mM suppressed of nearly 100% enzyme activity. Molybdate, an analog of phosphate, is recognized as a potent inhibitor of various phosphatases. The activity of *Gtb* PAP2L2 was strongly inhibited by 2 mM molybdate (the residual activity was 10.31%). However, the addition of molybdate at 10 mM to the assay system resulted in a decreased inhibition effect on enzyme activity (46% of activity retained). The reason for this remains unknown. Both inorganic phosphate and pyrophosphate exhibited marked dose-dependence. Slight inhibition of activity was observed with 2 M urea. The measured phosphatase activity of *Gtb* PAP2 before and after EDTA treatment demonstrates that there are no significant differences. The enzyme also exhibited insensitivity to inhibition by thioreactive compounds: NEM and dithiothreitol (DTT). Thus purified recombinant *Gtb* PAP2 phosphatase displayed typical biochemical characteristics of PAP2 homologs.

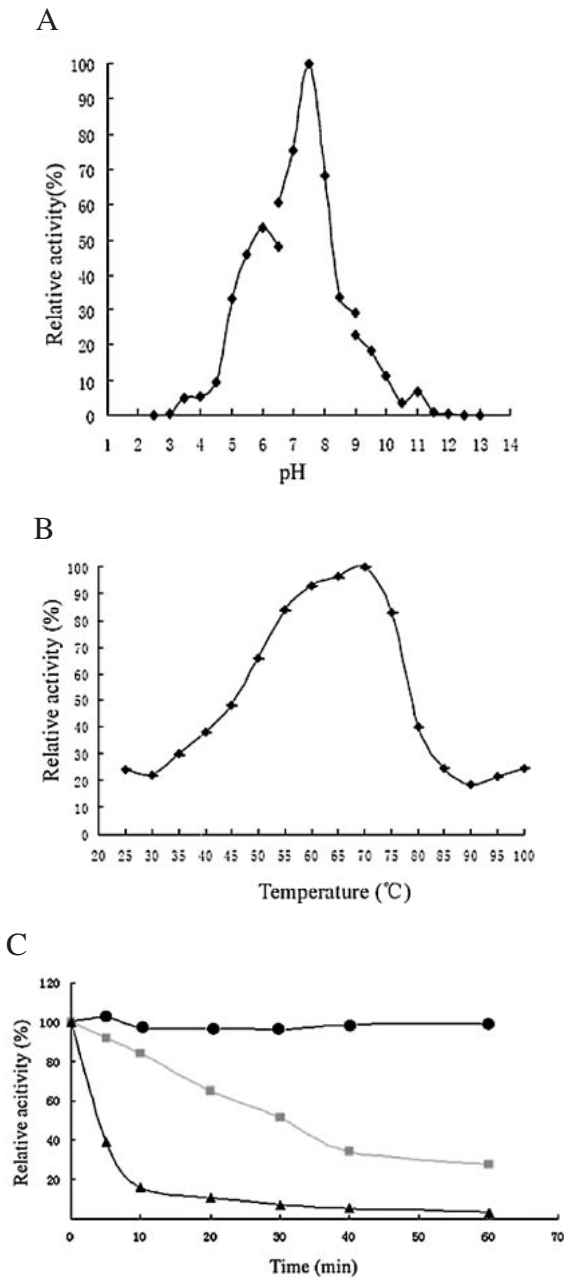

Fig. 3. Activity Profiles of Recombinant *Gtb* PAP2L2.

A, effects of pH on activity. Enzyme activity was determined in the following pH buffers: 0.1 M acetate buffer (pH 2.5–6.5), 0.1 M Tris–HCl (pH 6.5–9.0), and 0.5 mM diethanolamine (pH 9.0–13.0); B, Temperature effects on activity. *Gtb* PAP2L2 phosphatase activity was determined by standard assay from 25 to 100 °C; C, Thermostability. Residual activity was measured by standard assay after incubation of the enzyme at 55 °C (●), 60 °C (■), and 65 °C (▲) for the indicated times; Each data point in above figures represents an average of three determinations with *p*NPP as the substrate.

Conclusion

This study was undertaken to provide a source of large quantities of purified integral membrane phosphatase of the PAP2 family. Facile production and purification of protein is a prelude to in-depth analysis

Table 3. Effect of Various Chemicals on Recombinant *Gtb* PAP2L2 Activity[a]

Metal ions and inhibitors	Concentration	Relative activity (%)
None		100 ± 6.5
Mg^{2+}	10 mM	103.5 ± 4.3
Ca^{2+}	10 mM	101.2 ± 2.4
Zn^{2+}	10 mM	2.0 ± 0.6
Cd^{2+}	10 mM	42.6 ± 1.7
Co^{2+}	10 mM	76.6 ± 2.7
Mn^{2+}	10 mM	80.0 ± 1.1
SDS	1%	0.1 ± 0.5
Triton X-100	1%	45.6 ± 1.3
Inorganic phosphate	1 mM	82.4 ± 1.3
	10 mM	55.8 ± 2.1
Pyrophosphate	1 mM	72.8 ± 6.7
	10 mM	65.5 ± 3.9
Molybdate	1 mM	10.3 ± 4.3
	10 mM	43.6 ± 2.0
Vanadate	1 mM	6.1 ± 0.5
	10 mM	0.2 ± 0.2
Urea	3 M	34.0 ± 0.8
NEM	5 mM	103.1 ± 2.1
DTT	2 mM	96.5 ± 1.7
EDTA	10 mM	104.1 ± 2.1

[a]The enzyme was pre-incubated in absence and the presence of various divalent metal ions and inhibitors for 1 h, and then the effects of the ions and inhibitors on activity were examined by standard procedure. All of the examined metal ions were in the chloride form. Data represent mean ± standard deviation.

of structure and function. In this study, we cloned, expressed, and purified a PAP2-like phosphatase, *Gtb* PAP2L2, from thermophilic *Geobacillus toebii* T-85. As a novel member of the PAP2 superfamily, *Gtb* PAP2L2 was a thermostable protein, exhibiting a half-life of 30 min at 60 °C. To our knowledge, this is the first highly thermostable member of the PAP2 superfamily to be described. Purified recombinant *Gtb* PAP2L2 was catalytically active and highly stable, making it ideal for further structure/function studies.

Acknowledgments

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References

1) Stukey, J., and Carman, G. M., Identification of a novel phosphatase sequence motif. *Protein Sci.*, **6**, 469–472 (1997).
2) Waggoner, D. W., Gomez-Munoz, A., Dewald, J., and Brindley, D. N., Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. *J. Biol. Chem.*, **271**, 16506–16509 (1996).
3) Sigal, Y. J., McDermott, M. I., and Morris, A. J., Integral membrane lipid phosphatases/phosphotransferases: common structure and diverse functions. *Biochem. J.*, **387**, 281–293 (2005).
4) Brindley, D. N., English, D., Pilquil, C., Buri, K., and Ling, Z. C., Lipid phosphate phosphatases regulate signal transduction through glycerolipids and sphingolipids. *Biochim. Biophys. Acta*, **1582**, 33–44 (2002).
5) Sciorra, V. A., and Morris, A. J., Roles for lipid phosphate phosphatases in regulation of cellular signaling. *Biochim. Biophys. Acta*, **1582**, 45–51 (2002).
6) Carman, G. M., and Han, G. S., Roles of phosphatidate phosphatase enzymes in lipid metabolism. *Trends Biochem. Sci.*, **31**, 694–699 (2006).
7) Zhang, N., Zhang, J., Purcell, K. J., Cheng, Y., and Howard, K., The Drosophila protein wunen repels migrating germ cells. *Nature*, **385**, 64–67 (1997).
8) Barila, D., Plateroti, M., Nobili, F., Muda, A. O., Xie, Y., Morimoto, T., and Perozzi, G., The Dri42 gene, whose expression is up-regulated during epithelial differentiation, encodes a novel endoplasmic reticulum resident transmembrane protein. *J. Biol. Chem.*, **271**, 29928–29936 (1996).
9) Hooksa, S. B., Raganb, S. P., and Lyncha, K. R., Identification of a novel human phosphatidic acid phosphatase type 2 isoform. *FEBS Lett.*, **427**, 188–192 (1998).
10) Sun, L. Y., Gu, S. H., Sun, Y. Q., Zheng, D., Wu, Q. H., Li, X., Dai, J. F., Dai, J. L., Ji, C. N., Xie, Y., and Mao, Y. M., Cloning and characterization of a novel human phosphatidic acid phosphatase type 2, *PAP2d*, with two different transcripts PAP2d v1 and PAP2d v2. *Mol. Cell Biochem.*, **272**, 91–96 (2005).
11) Bernard, R., Ghachi, M. E., Mengin-Lecreulx, D., Chippaux, M., and Denizot, F., BcrC from *Bacillus subtilis* acts as an undecaprenyl pyrophosphate phosphatase in bacitracin resistance. *J. Biol. Chem.*, **280**, 28852–28857 (2005).
12) Ghachi, M. E., Derbise, A., Bouhss, A., and Mengin-Lecreulx, D., Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. *J. Biol. Chem.*, **280**, 18689–18695 (2005).
13) Neuwald, A. F., An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases. *Protein Sci.*, **6**, 1764–1767 (1997).
14) Ishikawa, K., Mihara, Y., Gondoh, K., Suzuki, E., and Asano, Y., X-ray structures of a novel acid phosphatase from *Escherichia blattae* and its complex with the transition-state analog molybdate. *EMBO J.*, **19**, 2412–2423 (2000).
15) Toke, D. A., McClintick, M. L., and Carman, G. M., Mutagenesis of the phosphatase sequence motif in diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *Biochemistry*, **38**, 14606–14613 (1999).
16) Zhang, Q. X., Pilquil, C. S., Dewald, J., Berthiaume, L. G., and Brindley, D. N., Identification of structurally important domains of lipid phosphate phosphatase-1: implications for its sites of action. *Biochem. J.*, **280**, 181–184 (2005).
17) Adams, M. W., and Kelly, R. M., Finding and using hyperthermophilic enzymes. *Trends Biotechnol.*, **16**, 329–332 (1998).

18) Sambrook, J., and Russell, D. W., "Molecular Cloning, a Laboratory Manual" 3rd edn., Cold Spring Harbor Press, Cold Spring Harbor (2001).
19) Takeuchi, M., Harigai, M., Momohara, S., Ball, E., Abe, J., Furuichi, K., and Kamatani, N., Cloning and characterization of DPPL1 and DPPL2, representatives of a novel type of mammalian phosphatidate phosphatase. *Gene*, **399**, 174–180 (2007).
20) Carman, G. M., Deems, R. A., and Dennis, E. A., Lipid signaling enzymes and surface dilution kinetics. *J. Biol. Chem.*, **270**, 18711–18714 (1995).
21) Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., Dymock, D., and Wade, W. G., Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.*, **64**, 795–799 (1998).
22) Kyte, J., and Doolittle, R. F., A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132 (1982).
23) Kashino, Y., Separation methods in the analysis of protein membrane complexes. *J. Chromatogr. B*, **797**, 191–216 (2003).
24) Gasteiger, E., Gattiker, A., Hoogland, C., Ivany, I., Appel, R. D., and Bairoch, A., ExPasy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.*, **31**, 3784–3788 (2003).
25) Roberts, R., Sciorra, V. A., and Morris, A. J., Human type 2 phosphatidic acid phosphohydrolases substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. *J. Biol. Chem.*, **273**, 22059–22067 (1998).
26) Dillon, D. A., Wu, W. I., Riedel, B., Wissing, J. B., Dowhan, W., and Carman, G. M., The *Escherichia coli pgpB* gene encodes for a diacylglycerol pyrophosphate phosphatase activity. *J. Biol. Chem.*, **271**, 30548–30553 (1996).
27) Wu, W. I., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M., Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **271**, 1868–1876 (1996).
28) Fleming, I. N., and Yeaman, S. J., Purification and characterization of N-ethylmaleimide-insensitive phosphatidic acid phosphohydrolase (PAP2) from rat liver. *Biochem. J.*, **308**, 983–989 (1995).