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Research Article

The PREPL A protein, a new member of the prolyl oligopeptidase family, lacking catalytic activity

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Abstract. The PREPL (previously called KIAA0436) gene encodes a putative serine peptidase from the prolyl oligopeptidase family. A chromosomal deletion involving the PREPL gene leads to a severe syndrome with multiple symptoms. Homology with oligopeptidase B suggested that the enzyme cleaves after an arginine or lysine residue. Several PREPL splice variants have been identified, and a 638-residue variant (PREPL A) was expressed in *Escherichia coli* and purified. Its secondary structure was similar to that of oligopeptidase B, but differential-scanning calorimetry indicated a higher conformational stability. Dimerization may account for the enhanced stability. Unexpectedly, the PREPL A protein did not cleave peptide substrates containing a P1 basic residue, but did slowly hydrolyse an activated ester substrate, and reacted with diisopropyl fluorophosphate. These results indicated that the catalytic serine is a reactive residue. However, the negligible hydrolytic activity suggests that the function of PREPL A is different from that of the other members of the prolyl oligopeptidase family.

Key words. KIAA0436; oligopeptidase B; protein expression and isolation; hydrolytic activity; dimerization; denaturation.

A unique syndrome has been reported characterized by cystinuria, neonatal seizures, hypotonia, somatic and developmental delay, facial dysmorphism and lactic acidaemia. The molecular basis of this disorder was a deletion of 179,311 bp on chromosome 2p21 (previously 2p16), which included the type I cystinuria gene SLC3A1, the protein phosphatase 2C gene, an unidentified gene KIAA0436 (presently approved gene symbol PREPL, prolyl endopeptidase-like) and several additional transcripts represented by ESTs [1]. A comprehensive verification of all ESTs and transcripts in the genomic interval of the PREPL gene identified several splice variants predicted to produce four different proteins (PREPLs A, C, D and E) [2]. (PREPL A is named PREPL A_B in Parvari et al. [2]). Here we report on one protein product of the splice variants, named PREPL A, represented by a full-length cDNA prepared by the oligo-capping method. The gene was cloned by the NEDO human cDNA sequencing project (Acc. No. at Entrez NCBI: AK025649) and supported by six ESTs (BG704689, BG717492, AL602657, AL603269, AL710407 and BQ231866). The initiator methionine of this isoform is preceded by a stop codon, indicating that this protein is complete. The initiator methionine is in a strong Kozak consensus sequence (AACATGG, the ATG start codon in the context RNNatgG, where R is a purine), obeying the rule that the translational start site in vertebrate mRNA nearly

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always conforms to the consensus sequence [3]. This splice variant was also identified in oran-gutan, rat and mouse (97%, 92% and 91% identity, respectively; fig. 7B in Parvari et al. [2]).

PREPL A displays sequence homology with the serine peptidases of the prolyl oligopeptidase family (S9 of clan SC). Members of the family include dipeptidyl peptidase IV, oligopeptidase B, acylaminoacyl peptidase and the prototype prolyl oligopeptidase, all of which are of physiological and pharmacological importance [4]. The strongest homology was found with oligopeptidase B (EC 3.4.21.83). PREPL A is expressed in many tissues, including brain, testis, kidney, liver, colon, muscle and lung (fig. 8 in Parvari et al. [2] and the GNF Expression Atlas track at the UCSC genome browser in Human: http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr2:44457553-44500274&hgsid=42213428&knownGene=pack&hgFind.matches=AB007896).

The three-dimensional structure determination of prolyl oligopeptidase provided the first structural information about these enzymes. It was shown that the carboxyl-terminal peptidase domain of prolyl oligopeptidase displayed an α/β hydrolase fold, and its catalytic triad (Ser554, His680, Asp641) was covered by the central tunnel of an unusual β propeller [5]. This domain made the enzyme an oligopeptidase by excluding large structured peptides from the active site. The peptidase domain is built up of residues 1–72 and 428–710, and the residues between these two portions constitute the propeller domain. The propeller domain is based on a sevenfold repeat of four-stranded antiparallel β sheets, which are twisted and radially arranged around their central tunnel.

Oligopeptidase B is a cytosolic enzyme found in Gram-negative bacteria and trypanosomes [6–8]. Enzyme activity has been linked to the virulence of bacterial and eukaryotic pathogens [9], making it a potential therapeutic target. Oligopeptidase B hydrolyses peptides exclusively at the carboxyl side of arginine or lysine residues, but cleavage is faster after two adjacent basic residues. Here we concentrate on PREPL A and demonstrate that though the catalytic triad and the S1 subsites of PREPL A and oligopeptidase B are similar, PREPL A dispenses with peptidase activity using a broad range of substrates containing P1 basic residues.

Materials and methods

Expression and purification of PREPL A. The PREPL A cDNA was cloned and provided by the Kazusa DNA Research Institute (accession at Entrez NCBI: AB007896). The clone was inserted from the first 5' methionine to the last amino acid (phenylalanine, position 638) into the *Bam*HI (5') and *Eco*RI (3') sites of the pGEX2T vector (Pharmacia), which produced the protein with a glutathione-S-transferase (GST)-tag that could be cleaved with thrombin. The gene was expressed in the *Escherichia coli* BL21(DE3)pLysS strain grown in six 500-ml LB cultures containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 37 °C. When the OD_{600} reached a value of about 0.2, an additional 100 μg/ml ampicillin was added. At an OD_{600} of about 0.6, a new portion of ampicillin and 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) were added. The cultures were incubated overnight at 26 °C. The cells were collected by centrifugation and sonicated in 100 ml of ice-cold 20 mM phosphate buffer, pH 7.3, containing 0.1 M NaCl. The sonicate was centrifuged at 20,000 g, and Triton X-100 (1% final concentration) was added to the supernatant to increase the solubility of the fusion construct. Purification of the GST-protein was carried out according to the batch/column protocol of Pharmacia, so that 4 ml of glutathione Sepharose-4B affinity adsorbent was added to the clear sonicate, mixed gently for 1 h and then poured into a column. After a thorough washing with the phosphate buffer used for the sonication, the elution was repeated four to five times with 10 mM reduced glutathione in 50 mM Tris.HCl, pH 8.0. The eluted protein solutions were combined, concentrated on an Amicon PM30 membrane up to 2 mg/ml protein concentration, and the glutathione was washed out with 50 mM phosphate buffer, pH 7.6, containing 1 mM ethylenediaminetetraacetic acid (EDTA). The GST-PREPL A protein concentration was determined at 280 nm, using $M_r = 99640$ and $A_{280}(0.1\%) = 1.24$. The yield of the GST-tagged protein was 40–50 mg. To eliminate the GST-tag, 2-mercaptoethanol was added to the solution to a final concentration of 2 mM, and the fusion protein was digested with thrombin. After complete digestion controlled by SDS polyacrylamide gel electrophoresis, the untagged protein was separated from thrombin and GST by FPLC chromatography on a MonoQ column (Pharmacia). The chromatography was performed in 20 mM phosphate buffer, pH 7.2, containing 1 mM 2-mercaptoethanol. The GST-tag eluted in the flow-through fraction. The PREPL A protein was eluted with a linear gradient of NaCl (0–1.0 M) and concentrated using an Amicon PM30 membrane. The pure PREPL A tends to precipitate above 2 mg/ml concentration. The concentration of the monomer was calculated from the absorbance at 280 nm using an M_r value of 73,352 and $A_{280}(0.1\%)$ of 1.13.

Preparation of oligopeptidases. The overexpression and purification of oligopeptidase B from *E. coli* [10] and porcine prolyl oligopeptidase [11] were described earlier.

Activity measurements. Samples were prepared in 50 mM phosphate buffer, pH 7.0, containing 0.7 μM protein and 20 μM 4-methylumbelliferyl-p-guanidinobenzoate (MUGB), and the initial rates of the reactions were re-

corded with a Cary Eclipse fluorescence spectrometer using 323 and 446 nm excitation and emission wavelengths (5-nm bandwidth each), respectively. Due to the slow reactions with PREPL A and prolyl oligopeptidase (about 2 h), the fluorescence data were registered every 5 min to omit the photodecomposition of the fluorophore. The reaction of oligopeptidase B was continuously measured for a few minutes. The spontaneous hydrolysis of MUGB was taken into account.

The reaction of PREPL A with diisopropyl-fluorophosphate (DIFP) was performed in 15 µl volume, using 40 µM protein and 940 µM DIFP and incubated for 0.5 and 2 h in 200 µl capped PCR tubes at 25 °C. The remaining activity was determined for oligopeptidase B and PREPL A with the MUGB substrate and for prolyl oligopeptidase with benzyloxycarbonyl (Z)-Gly-Pro-Nap (2-naphthylamide) at 340 and 410 nm excitation and emission wavelengths, respectively.

Gel chromatography. A 100-µl of sample (10 µM) was loaded on a Superose 12 column, which was equilibrated with 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, and 1 mM 1,4-dithioerythritol (DTE).

Differential scanning calorimetry. Calorimetric measurements were carried out with a Microcal VP-DSC differential scanning calorimeter. Denaturation curves were recorded between 25 and 80°C, at a pressure of 2.5 atm using a scan rate of 60°C/h. The protein concentration was 1.5–2.5 µM (0.1–0.2 mg/ml) in 50 mM phosphate buffer, pH 8.0, containing 0.5 mM EDTA.

Circular dichroism. Measurements were carried out in 20 mM phosphate buffer, pH 8.0, with a Jasco J-720 CD spectropolarimeter at 25 °C, using thermostated cuvettes of 0.1-cm path lengths. Protein concentration was typically 0.1 mg/ml. Spectra were acquired between 200 and 250 nm at a scan speed of 20 nm/min, averaging three scans. For all CD spectra, the baseline of the buffer was subtracted. Assignments to the secondary structural elements for prolyl oligopeptidase were taken from PDB code 1h2w. The CD spectra analyses were performed with the CDPro software package http://lamar.colostate.edu/~sreeram/CDPro).

1-Chloro-2,4-dinitrobenzene assay. The GST-tagged protein was identified during purification by measuring the reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione, which was proportional to the GST concentration. The reaction was followed by the increase in absorbance at 340 nm in 0.1 M potassium phosphate buffer, pH 6.5, containing 1 mM CDNB and 1 mM glutathione. Slow enzymatic rates were corrected for the spontaneous reaction.

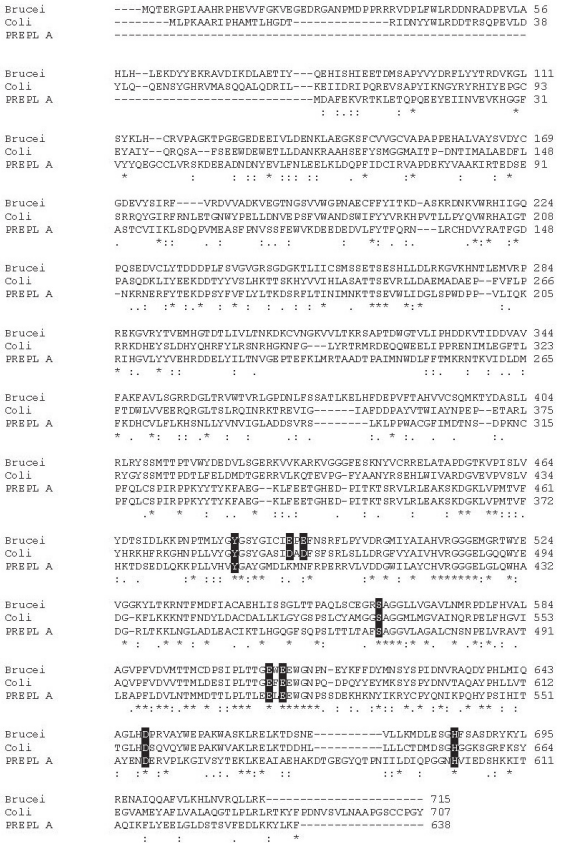

Figure 1. Amino acid sequence alignment for PREPL A, and oligopeptidase B from *T. brucei* and *E. coli.*

Results and discussion

Structural relationship between PREPL A and oligopeptidase B. PREPL A exhibits higher homology to oligopeptidase B (BAA01750, 25.5% identity with the *E. coli* enzyme) than to porcine prolyl oligopeptidase (A37942, 19.1% identity) for which the three-dimensional structure is known. Figure 1 shows the alignment (ClustalW) of PREPL A (AB007896) with two orthologues of oligopeptidase B, one from *Trypanosoma brucei* (AAC80459) and the other from *E. coli* (BAA01750). The N terminus of prolyl oligopeptidase, as well as that of oligopeptidase B as indicated by model building [12], contains a short two-stranded antiparallel β sheet and two long α helices, which are connected to the C-terminal catalytic domain [5]. These structural elements are practically absent from the PREPL A variant.

The catalytic triads of oligopeptidase B from *T. brucei* (Ser563, Asp 648, His683) and *E. coli* (Ser532, Asp617, His652) are aligned with the corresponding amino acids (Ser470, Asp556, His601 and Ser559, Asp645, His690) of the PREPL A (fig. 1). Tyr482 and Tyr451 of oligopeptidase

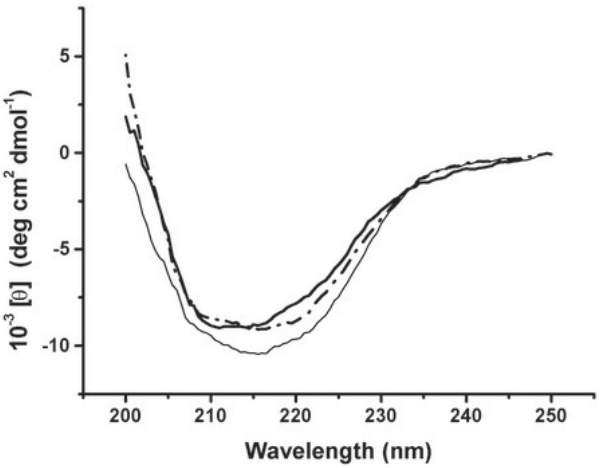

Figure 2. Far-UV CD spectra of PREPL A (dash-dotted line), oligopeptidase B from *E. coli* (thin line) and porcine prolyl oligopeptidase (thick line).

Table 1. Secondary-structure fractions for oligopeptidases.

Protein	Method	Helix	β strand	Turn	Unordered
POP	DSSP (PDB)	0.224	0.292	0.224	0.261
	CDSSTR	0.153	0.298	0.220	0.332
	CONTILL	0.165	0.299	0.219	0.317
	SELCON3	0.174	0.322	0.219	0.298
OPB	CDSSTR	0.171	0.280	0.209	0.343
	CONTILL	0.161	0.256	0.218	0.365
	SELCON3	0.239	0.265	0.228	0.286
PREPL A	CDSSTR	0.190	0.279	0.216	0.313
	CONTILL	0.206	0.293	0.216	0.285
	SELCON3	0.203	0.295	0.220	0.291

POP, prolyl oligopeptidase; OPB, oligopeptidase B. The data obtained with the DSSP method refer to the crystallographic data of prolyl oligopeptidase. The secondary structures are classified according to Sreeramaa et al. [15]. The other three methods show the far-UV CD spectra analyses with the CDPro software package. The helix and the strand columns both include the sum of the regular and distorted fractions.

B from *T. brucei* and *E. coli*, respectively (Tyr473 of prolyl oligopeptidase), are part of the oxyanion-binding site that stabilizes the transition state of the reaction. Tyr390 in PREPL A corresponds to this catalytically important residue. However, there appear to be differences in the substrate specificity of the two enzymes. Oligopeptidases B of *T. brucei* and *E. coli* have a pair of acidic residues at the S1 recognition site (Glu607, Glu609 and Glu576, Glu578, respectively), which is specific for basic amino acid residues (fig. 1) [13, 14]. In PREPL A, there is a pair of glutamic acid residues (Glu514 and Glu516) at the equivalent position, suggesting the enzyme is also specific for basic P1 amino acids. Oligopeptidase B hydrolyses peptides exclusively at the carboxyl side of arginine or lysine residues, but the cleavage is considerably faster after two adjacent basic residues. Oligopeptidase B has an additional pair of acidic residues (Glu490 and Glu492 in *T. brucei*, and Asp459 and Asp461 in *E. coli*) forming the S2 subsite for recognition of a P2 basic residue [13, 14]. The equivalent acidic S2 residues are not present in PREPL A (fig. 1), suggesting a substrate specificity that is similar to but distinct from oligopeptidase B, such that it constitutes a new member of the prolyl oligopeptidase family.

The far-UV CD spectrum of proteins is characteristic of their secondary structure. Figure 2 shows that the CD spectra of PREPL A, oligopeptidase B from *E. coli* and porcine prolyl oligopeptidase are similar. The structural elements calculated from the spectra are compiled in table 1. The data confirm the conformational relationship among the three proteins.

The catalytic activity of PREPL A. The similarity of the S1 subsite of PREPL A to that of oligopeptidase B suggested that the enzyme exhibits specificity for P1 basic residues. We examined several substrates containing Arg and Lys residues, including simple substrates like benzoyl (Bz)-Arg-Nan (4-nitroanilide), Bz-Arg-Nap, Z-Arg-Arg-Amc (7-amido-4-methylcoumarin) and intrinsically fluorescent substrates that have 2-aminobenzoyl (Abz) and 4-nitrophenylalanyl [Phe(NO_2)] as fluorescent donor and acceptor/quencher, respectively. These substrates were Abz-Arg-Arg-Phe(NO_2)-Ala-NH_2, Abz-Ala-Ala-Arg-Arg-Phe(NO_2)-Ala-NH_2, Abz-Thr-Arg-Arg-Phe(NO_2)-Ser-Leu-NH_2, Abz-Thr-citrulline-Arg-Phe(NO_2)-Ser-Leu-NH_2, Abz-Thr-Lys-Lys-Phe(NO_2)-Ser-Leu-NH_2 and Abz-Gly-Phe-Arg-Pro-Phe(NO_2)-Arg-Ala. However, none of these peptides was hydrolysed during several hours of incubation. Therefore, we examined MUGB, an activated ester substrate, used for the-active site titration of trypsin, also specific for P1 Arg and Lys residues. A very low reaction rate was found with PREPL A, 540 times lower than that observed with oligopeptidase B. An even lower rate was obtained with prolyl oligopeptidase, not specific for basic residues (table 2). We also examined the reaction of Bz-Arg-ethyl ester by PREPL A, but this simple alkyl ester was not hydrolysed either. There was also no cleavage in the substrates specific for prolyl oligopeptidase and chymotrypsin. These results indicated that PREPL A had an active serine residue, but its catalytic machinery was damaged or its specificity was outside the range of the substrates examined.

Interestingly, the fast reaction of oligopeptidase B with MUGB was not stoichiometric, as in the case of trypsin, but the substrate was completely hydrolysed, providing

Table 2. Activity and denaturation of oligopeptidases.

Protein	MUGB*	0.5 hour+	2 hours+	k (min^{-1})‡
PREPL A	1	45	35	0.43±0.04
OPB	540	10	0	0.42±0.03
POP	0.33	0	0	6.2±0.9, 0.25±0.03

* Relative activities from initial rate measurements.
+ Remaining activities (%) after incubation with DIFP for the given time period.
‡ Denaturation rate constants in 4 M urea. The denaturation rate of prolyl oligopeptidase conforms to a double exponential curve.

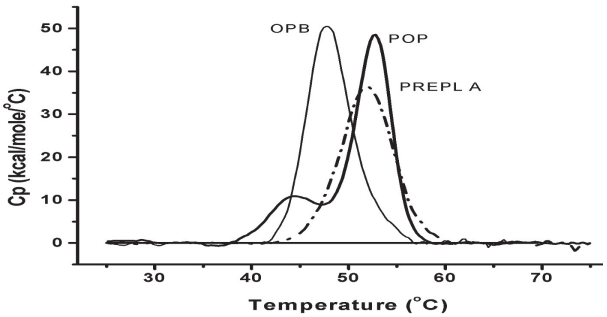

Figure 3. DSC scans of PREPL A, *E. coli* oligopeptidase B (OPB) and porcine prolyl oligopeptidase (POP).

K_m = 3.3 μM and k_{cat}/K_m = 37 $mM^{-1}\ s^{-1}$, calculated from the Michaelis parameters. The rate constant measured under first-order conditions (MUGB = 0.2 μM) and divided by the enzyme concentration gave a k_{cat}/K_m = 46 $mM^{-1}\ s^{-1}$. The reaction of PREPL A was not stoichiometric either. Using the same enzyme concentration (0.7 μM), the initial rates were identical at 10, 20 and 40 μM MUGB concentrations, indicating that the PREPL A was saturated at the substrate concentrations employed. On the other hand, the increase in initial rates was proportional with the increase in the enzyme concentration (0.15, 0.30, 0.69 and 1.25 μM), as expected.

DIFP is known to inhibit serine peptidases. Table 2 shows that PREPL A reacts with DIFP but at a much lower rate than oligopeptidase B and, in particular, prolyl oligopeptidase, which after 2-min incubation retains only 2% activity. This again shows an enhanced nucleophilic reactivity of the catalytic serine of PREPL A and confirms that the hydrolysis of the activated ester substrate is an enzymatic reaction.

Conformational stability of PREPL A. The stability of PREPL A protein was determined with several independent denaturation methods, using high temperature, acidic and alkaline pH, and denaturation with urea. (i) The pH-induced unfolding was determined fluorimetrically in a wide pH range. The internal fluorescence of PREPL A decreased at moderate acidic and alkaline pH values, giving rise to a profile similar to that of oligopeptidase B [10] and prolyl oligopeptidase [11]. Specifically, the conformation of PREPL A did not change in the pH range 5.5–9.5. The fluorescence intensity only diminished outside this range. (ii) The thermal stability of PREPL A was determined with differential scanning calorimetry (DSC). The protein exhibited a higher T_m value (51.8 °C) than oligopeptidase B (47.7 °C), and both proteins gave a single peak, whereas prolyl oligopeptidase displayed a more complex temperature scanning profile with two T_m values (44.6 and 52.8 °C) (fig. 3). (iii) Denaturation of PREPL A in the presence of 4 M urea followed first-order kinetics and produced a similar rate constant to that observed with oligopeptidase B. However, prolyl oligopeptidase exhibited a double exponential curve, having a fast and a slow phase (table 2). Such a difference was also found in the DSC measurements, where prolyl oligopeptidase displayed two T_m values. (iv) We anticipated that PREPL A would be less stable than prolyl oligopeptidase or oligopepidase B because of the missing N terminus. However, this was not verified by the denaturation experiments. Apparently, two PREPL A molecules might associate at their open surface, providing a more stable dimer structure. Indeed, size exclusion chromatography on a Superose 12 column clearly demonstrated that PREPL A is a dimer with a molecular mass of 145.5 kDa, as obtained from the calibration curve (SIGMA Molecular Weight Market Kit, 12.4–200 kDa). The molecular mass calculated for the monomer is 73,352 Da, which was absolutely absent from the size exclusion chromatogram.

Conclusion. While PREPL A belongs to the prolyl oligopeptidase family and obviously expresses in different tissues, it does not exhibit an appreciable hydrolytic activity versus substrates containing a P1 basic residue. Amino acid sequence homology clearly indicates that PREPL A is more related to oligopeptidase B than to prolyl oligopeptidase. Therefore, OPBL (oligopeptidase B-like) would be a more appropriate acronym for the gene than the recently proposed PREPL (prolyl oligopeptidase-like). Although the specificities of the S1 binding subsites for P1 basic residues are identical in PREPL A and oligopeptidase B, the S2 sites are different, indicating that the two enzymes have distinct specificities. PREPL A also differs from oligopeptidase B, as it is a dimer rather than a monomer. The insignificant activity of the dimer suggests that its function might be different from the known enzymes of the prolyl oligopeptidase family and its biological role remains to be established.

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