Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structural basis for specific calcium binding by the polycystic-kidney-disease domain of *Vibrio anguillarum* protease Epp

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ARTICLE INFO

Article history:
Received 9 September 2018
Accepted 17 September 2018
Available online xxx

Keywords:
Vibrio anguillarum
Polycystic-kidney-disease domain
Protease maturation
Ca^{2+}-binding site
Protein stability

ABSTRACT

Extracellular proteases are often produced as pre-pro-enzyme and then undergo multiple processing steps to mature into the active form. The protease Epp, a virulent factor of *Vibrio anguillarum*, belongs to this family. Its maturation might be regulated by Ca^{2+} via its polycystic kidney disease (PKD) domain, but the molecular mechanism is unknown. Herein, we report the crystal structure of the first PKD domain from *V. anguillarum* Epp (Epp-PKD1) and its specific Ca^{2+}-binding capacity. Epp-PKD1 exists as a monomer, consisting of seven β-strands which form two β-sheets stacking with each other. One Ca^{2+} is bound by the residues Asn3, Gln4, Asp27, Asp29, Asp68 and a water molecule with a pentagonal bipyramidal geometry. Incubating the *apo* Epp-PKD1 with Ca^{2+} but not Mg^{2+}, Mn^{2+}, or Zn^{2+}, enhances the thermal and chemical stability of Epp-PKD1, indicating its specific binding to Ca^{2+}. Epp-PKD1 shares high similarity in both sequence and overall structure with that of *Vibrio cholerae* PrtV, a homologous protease of Epp, however, they differ in the oligomeric state and local structure at the Ca^{2+}-binding site, suggesting maturation of PrtV and Epp might be differently regulated by Ca^{2+}. Likely, proteases may take advantage of the structural diversity in PKD domains to tune their Ca^{2+}-regulated maturation process.

1. Introduction

Extracellular proteases are important virulent factors for pathogens [1,2]. Very often, they are produced as inactive pre-pro-enzymes and must undergo multiple processing steps to mature into the active form. For examples, LasB of *Pseudomonas aeruginosa*, Vvp of *Vibrio vulnificus*, EmpA of *Vibrio anguillarum*, and PrtV of *Vibrio cholerae*, all belong to such proteases [2–4]. Interestingly, Ca^{2+} can play a regulatory role in the maturation step of some proteases, such as the PrtV of *V. cholerae* [3,4].

The PrtV pre-pro-enzyme contains the signal peptide, the N-terminal domain, the M6 metalloprotease domain, followed by two polycystic-kidney-disease (PKD) domains [3,5]. Although the PKD domain was originally identified in the human PKD1 gene encoding glycoprotein polycystin-1 [6], it is universal and found in chitinases, cellulases, collagenases, and proteases for the interaction between protein and protein or protein and polysaccharide [3,5,7,8]. In PrtV, the functions of the PKD domains are not fully understood, but removal of both PKD domains is required for the maturation of PrtV [3–5]. Ca^{2+} can bind to the first PKD domain of PrtV (PrtV-PKD1) and regulate the maturation process by controlling the domain linker flexibility in the PrtV pro-enzyme [4,5]. This PKD domain mediated Ca^{2+} regulation is an important step in the maturation of PrtV.

V. anguillarum is an important pathogen of vibriosis that causes high mortality of infected fish as well as human [1,2,9]. The zinc metalloprotease EmpA is an important virulence factor of *V. anguillarum* [2]. Interestingly, its maturation is aided by another protease, called EmpA-processing protease (Epp) [2]. In fact, Epp is homology of *V. cholera* PrtV with similar domain organization [2,3] and it also contains a PKD domain that is highly homologous to PrtV-PKD1, suggesting Epp undergoes a similar Ca^{2+}-regulated maturation process to that of PrtV [2,3]. Interestingly, sequence alignment reveals that the amino-acid residues involved in Ca^{2+}-binding site are different between them (Fig. 1A), suggesting Ca^{2+}-regulation for maturation may vary in these proteases. To better understand the role of Ca^{2+}-regulation in the maturation of

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https://doi.org/10.1016/j.bbrc.2018.09.108
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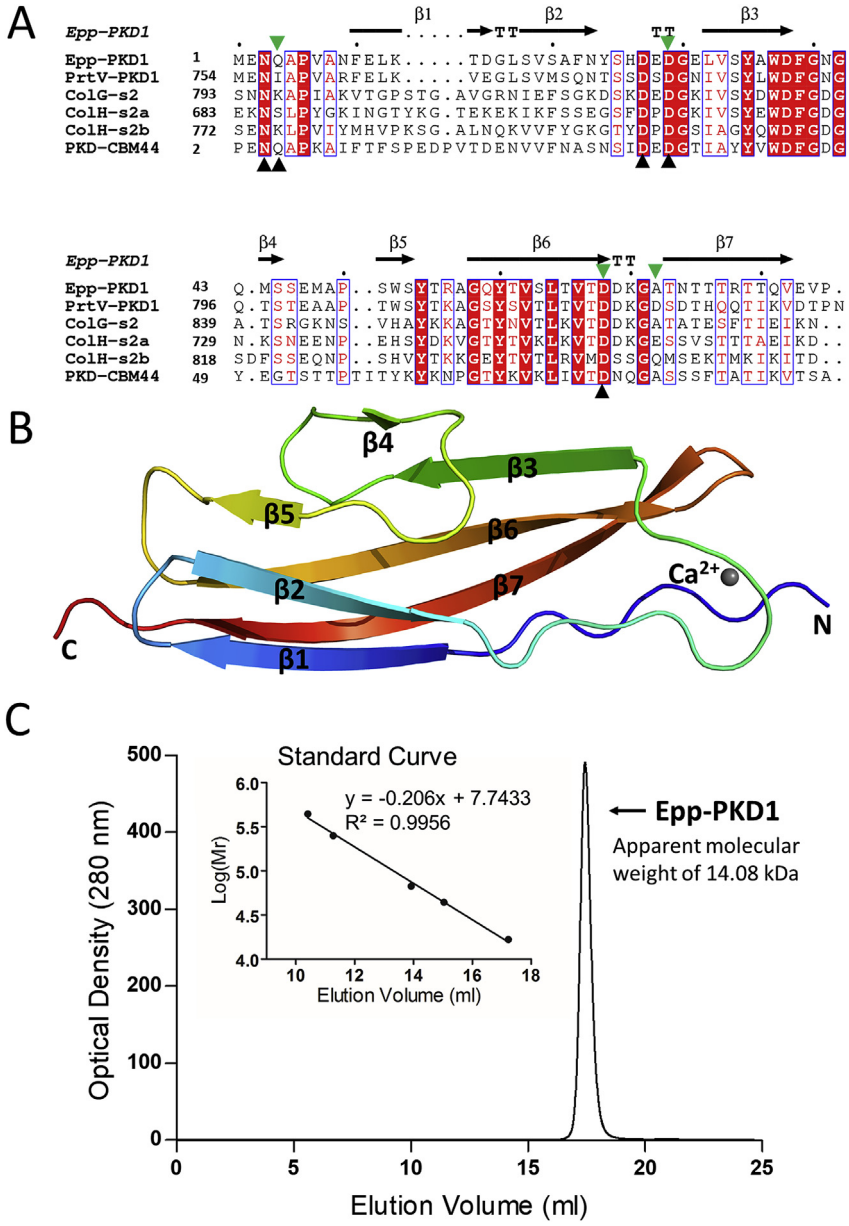

Fig. 1. Sequence and overall structure of Epp-PKD1. (A) Structure-based sequence alignment of Epp-PKD1 with five other PKD domains, including PrtV-PKD1 (4L9D), ColG-s2 (4AQO), ColH-s2a (4U7K), ColH-s2b (4JGU), PKD-CBM44 (2C4X). Red background indicates sequence identity; red letters indicate sequence similarity. The β-sheets are indicated in the upper side. Residues in the Ca^{2+}-binding sites of Epp-PKD1, ColG-s2, ColH-s2a, ColH-s2b and PKD-CBM44 are labelled with black triangles, and residues in the Ca^{2+}-binding site of PrtV-PKD1 are highlighted by green triangles. (B) Ribbon representation of Epp-PKD1 structure. The structure is colored rainbow. The calcium ion is indicated. The strands are shown as β1 to β7. (C) Gel-filtration chromatography of Epp-PKD1 eluted from Superdex 200 Increase. Standard curve of molecular mass was used to estimate the apparent molecular weight of Epp-PKD1 (14.08 kDa). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

complex proteases, we subject the PKD1 domain of Epp (Epp-PKD1) to structural study and compared it to PrtV-PKD1.

In the present study, we report the crystal structure of Epp-PKD1, in which the Ca^{2+}-binding site and oligomeric state are distinct from that in PrtV-PKD1. We also show that Ca^{2+}-binding specifically stabilizes Epp-PKD1 conformation.

2. Materials and methods

2.1. Cloning, protein expression and purification

The gene encoding Epp-PKD1 (comprising Gln755-Pro838 of Epp) was amplified using *V. anguillarum* genomic DNA as template by polymerase chain reaction (PCR) with primers (forward, 5′-AAACCATGGAAAACCAAGCTCCTGTCGCT-3′; reverse, 5′-AAACTCGAGTTAAGGCACTTCAACCTGTGT-3′). The purified PCR product was cloned into pETM11 vector (EMBL) using the *Nco*I and *Xho*I restriction sites and the recombinant protein product was expected to carry an N-terminal 6×His tag and a tobacco etch virus (TEV) protease site. The expression plasmid was then transformed into *Escherichia coli* Rosetta (DE3) strain. Cells were grown in Luria-Bertani broth at 37 °C with a shaking speed of 150 rpm. When the $OD_{600\ nm}$ value was about 0.6, the cells were induced by 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), with further culturing at 16 °C for 16 h. Cells were harvested by centrifugation and then lysed by sonication in the lysis buffer (50 mM Tris-HCl (pH

Please cite this article in press as: P. Li, et al., Structural basis for specific calcium binding by the polycystic-kidney-disease domain of *Vibrio anguillarum* protease Epp, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.108