Journal of Structural Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Small-angle X-ray scattering of calpain-5 reveals a highly open conformation among calpains

Lokesh Gakhar [a,b], Alexander G. Bassuk [c,d], Gabriel Velez [d,e,f], Saif Khan [b], Jing Yang [b], Stephen H. Tsang [g,h], Vinit B. Mahajan [d,e,*]

[a] Department of Biochemistry, University of Iowa, Iowa City, IA, USA
[b] Protein Crystallography Facility, University of Iowa, Iowa City, IA, USA
[c] Department of Pediatrics, University of Iowa, Iowa City, IA, USA
[d] Omics Lab, University of Iowa, Iowa City, IA, USA
[e] Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA, USA
[f] Medical Scientist Training Program, University of Iowa, Iowa City, IA, USA
[g] Barbara and Donald Jonas Laboratory of Stem Cells and Regenerative Medicine and Bernard & Shirlee Brown Glaucoma Laboratory, Edward S. Harkness Eye Institute, Columbia University, New York, NY, USA
[h] Department of Pathology & Cell Biology, College of Physicians & Surgeons, Columbia University, New York, NY, USA

ARTICLE INFO

Article history:
Received 4 December 2015
Received in revised form 22 July 2016
Accepted 26 July 2016
Available online xxxx

Keywords:
CAPN5
ADNIV
SAXS
Protease
Uveitis

ABSTRACT

Calpain-5 is a calcium-activated protease expressed in the retina. Mutations in calpain-5 cause autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV, OMIM#193235). The structure of calpain-5 has not been determined, thus hindering the investigation of its proteolytic targets and pathological role in ADNIV. Herein, we report models of the proteolytic core of calpain-5 (mini-calpain-5) containing two globular domains (termed DIIa-IIb) connected by a short, flexible linker, consistent with small-angle X-ray scattering (SAXS) data. Structural modeling in the absence of calcium suggests that mini-calpain-5 adopts a more open conformation when compared to previously determined structures of other calpain cores. This open conformation, achieved by a rotation of DIIa and DIIb with respect to each other, prevents formation of the active site and constrains the enzyme in an inactivated form. The relative domain rotation of 60–100° we found for mini-calpain-5 (a non-classical calpain) is significantly greater than the largest rotation previously observed for a classical calpain (i.e., 55.0° for mini-calpain-9). Together with our prediction that, in the full-length form, a long loop in DIIb (loop C1), a few residues downstream of the inter-domain linker, likely interacts with the shorter, acidic, inactivating loop on domain-III (DIII), these structural insights illuminate the complexity of calpain regulation. Moreover, our studies argue that pursuing higher resolution structural studies are necessary to understand the complex activity regulation prevalent in the calpain family and for the design of specific calpain inhibitors.

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1. Introduction

CAPN5 encodes calpain-5, a calcium-activated, signaling protease expressed by retinal photoreceptors (Mahajan et al., 2012). Coding mutations in *CAPN5* cause an inherited uveitis called autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV, OMIM #602537) (Mahajan et al., 2012). ADNIV is characterized by progressive, severe intraocular inflammation, photoreceptor degeneration, retinal neovascularization, intraocular fibrosis, and retinal detachment. Otherwise, affected patients have no associated systemic conditions. ADNIV-associated mutations in calpain-5 reside in a flexible loop that gates the active site and are expected to alter enzymatic activity (Wert et al., 2014), likely leading to a gain of function (Mahajan et al., 2012). Since the natural protein targets of calpain-5 proteolysis are unknown, a functional assay for calpain-5 is not currently available. Nevertheless, in general, high calpain activity is associated with retinal and cell degeneration (Azuma and Shearer, 2008; Huang and Wang, 2001; Vanderklish and Bahr, 2000). Accordingly, expressing mutant calpain-5 alleles only in the mouse retina is sufficient to trigger disease (Wert et al., 2014), while deleting the gene (in the knockout mouse) causes no phenotype (Franz et al., 2004).

* Corresponding author at: The University of Iowa Carver College of Medicine, 200 Hawkins Drive, Iowa City, IA 52242, USA.
E-mail address: mahajanlab@gmail.com (V.B. Mahajan).

http://dx.doi.org/10.1016/j.jsb.2016.07.017
1047-8477/Published by Elsevier Inc.

Calpain-5 is the most distant paralog of the calpain family, and a non-classical calpain, but little is known regarding its functional features and natural substrates. Although some substrates were identified for other members of the calpain family, calpain-5 is atypical: it lacks the Ca^{2+}-binding, penta-EF-hand domains that are predicted to play a role in regulating enzymatic activity (Barnes and Hodgkin, 1996; Dear et al., 1997; Matena et al., 1998), dimerization (either homodimerization, as with calpain-3 (Ravulapalli et al., 2009), or heterodimerization, as with the large and small subunits of calpain-1 and -2 with their small subunits, calpain-S1 and calpain-S2), and interaction with the endogenous calpain inhibitor, calpastatin. Instead, calpain-5 has a C2-like domain that is structurally similar to the C2L domain implicated in transient membrane binding (reviewed in Campbell and Davies, 2012). Its closest homolog is calpain-6 (with 46% identity), a calpain without proteolytic activity. Most importantly, calpain-5 has conserved the calcium binding sites in the protease core DI-II, and is therefore expected to undergo a realignment of the active site residues for catalysis in the presence of calcium.

Investigation into proteolysis by calpain-5 has been hampered by the instability of the recombinant protein in purification strategies (Bassuk et al., 2015). Thus, the activity and regulatory mechanisms of calpain-5 remain poorly understood. Nevertheless, key clues about regulation of calpain-5 activity and target specificity were gained from structural models of the calpain catalytic core (Bassuk et al., 2015). This core sequence is highly conserved among the various calpain family members, strongly suggesting that key functions are also highly conserved. Other studies imply that calpains recognize targets via sequences in the proteolytic core (evidenced by yeast two-hybrid screens where 29 of the 37 calpain-1 interactors captured directly contact the proteolytic domain itself) (Jiang et al., 2002). This idea is supported by structural modeling that overlap the various calpains and show the most highly divergent amino acid stretches in the proteolytic domains lie in the flexible loops near the active site where they might mediate specificity (Campbell and Davies, 2012). To gain insights into the relative domain orientation which may modulate activation of the protease, in this study we purified the protease core of calpain-5 (mini-calpain-5) and determined its solution structure by small-angle X-ray scattering (SAXS). We also determined thermostability of mini-calpain-5 in presence and absence of calcium, an inhibitor (leupeptin) and a chelating agent (EGTA). We find that while mini-calpain-5 seems to adopt the typical closed form on addition of calcium, it exists in solution in a highly open form otherwise. Since this open form is quite different from the open form seen for other inactivated calpains, specific inhibitors for calpain-5 could be envisioned that lock the enzyme in this inactivated state where the active site has still not formed. Our thermostability studies show that binding to calcium alone and in presence of the inhibitor leupeptin significantly stabilizes the enzyme as expected. However, binding to leupeptin in the absence of calcium significantly destabilizes the enzyme.

2. Materials and methods

2.1. Homology modeling of mini-calpain-5 based on sequence homology to calpain-9

Homology models for mini-calpain-5 were generated using MODELLER 9.14 (Webb and Sali, 2014), as described previously (Bassuk et al., 2015). Briefly, a BLAST search for mini-calpain-5 against the Protein Database (PDB) returned the structures of the catalytic core of human calpain-9 as the top hit, with a sequence identity of 42%. Other close matches were the catalytic cores of calpain-1, calpain-2, and calpain-8. Homology models of the calpain-5 catalytic core were generated using the structures of calpain-9 (PDB ID 2P0R; calcium and leupeptin bound closed and PDB ID 1ZIV; calcium and beta-mercaptoethanol bound open), calpain-1 (PDB ID 2ARY; calcium-bound closed), calpain-2 (PDB ID 3BOW; calcium and calpastatin bound closed and PDB ID 1DF0; no calcium open), calpain-1/2 hybrid (PDB ID 1QXP; no calcium open) and calpain-8 (PDB ID 2NQA; calcium and leupeptin-bound closed) as templates. The open and closed structures provide distinct orientations of DIIa relative to DIIb and overlap poorly upon alignment (for example, rmsd of 6.6 for 304 C_α atoms for 2P0R and 1ZIV). Ten models were generated using each template, which superimposed well within each group with major variations in the N- and C-terminal regions and minor variations in the loop regions. PyMOL was used to generate all structure figures (Schrödinger Corporation, 2014). Sequence alignments were performed using ClustalW (Thompson et al., 1994).

2.2. Cloning

The catalytic core (DIIa-IIb) of human calpain-5 was cloned into a pUC57 vector with an Xho I restriction site, with an N-terminal thrombin cleavage site, and a C-terminal TEV protease cleavage site followed by a 6xHis tag, as previously described (Bassuk et al., 2015). Calpain constructs were transferred into the pMAL-C5X vector to obtain an N-terminal maltose binding protein (MBP) as a fusion partner that could be cleaved off with thrombin after purification. Sequence of the calpain flanking regions was confirmed by sequencing of constructs. Plasmids were amplified and isolated from DH5α cells and then were transformed into *E. coli* BL21 (DE3).

2.3. Protein purification

For structural studies, samples of mini-calpain-5 were prepared to a purity greater than 95%, as described below. *E. coli* BL21 (DE3) cells expressing mini-calpain-5 were grown in 1 L shake-flask cultures, at 250 rpm in Terrific Broth (Invitrogen), at 37 °C until an OD 600 of 1.0 and then induced with 0.2 mM IPTG. Cells were grown at 16 °C for 19 h, harvested, and centrifuged at 4000*g* for 25 min and pellet frozen at −20 °C in 25 ml of lysis buffer (20 mM Tris, 300 mM NaCl, 1 mM DTT, pH 7.5, one tablet of EDTA-free protease inhibitor (Roche), DNase (Roche)). Cells were lysed using an EmulsiFlex (Avestin) and centrifuged for 50 min. at 120,000*g*. Cell debris was discarded and the supernatant (30 ml) loaded onto a column packed with 12 ml (1CV) amylose resin (New England Biolabs) connected to a BioLogic DuoFlow (BioRad Laboratories Inc., CA). The column was washed with 5–10 CV of wash buffer (20 mM Tris, 300 mM NaCl, pH 7.5) and eluted with 6–8 CV of elution buffer (20 mM Tris, 300 mM NaCl, 10 mM maltose, pH 7.5). Eluted fractions were pooled and concentrated to 6 ml at 4 mg/ml (30 kDa NMWL spin concentrator; Millipore). MBP was removed with thrombin (2 units/mg of protein; thrombin from bovine plasma, T7201, Sigma-Aldrich) at 4 °C for 5 h and the reaction mix passed over Ni-NTA (Qiagen) resin to remove cleaved-off MBP using 6 CV wash buffer (20 mM Tris, 500 mM NaCl, 10 mM Imidazole, pH 7.5). Cleaved and remaining uncleaved mini-calpain-5 was eluted from the column with 5 CV of elution buffer (20 mM Tris, 500 mM NaCl, 200 mM Imidazole, pH 7.5). The eluted protein was concentrated with a 10 kDa NMWL spin concentrator and passed over a Superdex 75 (GE) size-exclusion column to remove residual, uncleaved fusion protein. The column was equilibrated with CD buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM DTT, pH of 7.5), DLS buffer (20 mM Tris-HCl, 300 mM NaCl, pH of 7.5) or SAXS buffer (20 mM Tris-HCl, 150 mM NaCl, 2–5 mM DTT, pH of 7.5) depending on the analysis being performed on the sample.

Please cite this article in press as: Gakhar, L., et al. Small-angle X-ray scattering of calpain-5 reveals a highly open conformation among calpains. J. Struct. Biol. (2016), http://dx.doi.org/10.1016/j.jsb.2016.07.017