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Short communication

Defining the regulatory and inhibitory elements within the prodomain of CsCF-6, a cathepsin F cysteine protease of *Clonorchis sinensis*

Jung-Mi Kang, Hye-Lim Ju, Woon-Mok Sohn, Byoung-Kuk Na*

Department of Parasitology and Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju 660-751, Republic of Korea

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ABSTRACT

CsCF-6 is a member of the multigene family of cathepsin F cysteine proteases of *Clonorchis sinensis*. Similar to other papain family proteases, CsCF-6 is synthesized as a proenzyme and is converted to the mature form by autocatalytic removal of the prodomain. Here, we analyzed the regulatory and inhibitory elements within the CsCF-6 prodomain to understand the regulatory mechanism of CsCF-6 by its prodomain. The CsCF-6 prodomain played an essential role in the folding of CsCF-6. Particularly, the ERFNAQ motif within the prodomain was essential, and the minimum segment required for this event was the C-terminal part of the prodomain, including Asn^{58} and downstream residues. The CsCF-6 prodomain effectively inhibited CsCF-6, in which the ERFNAQ motif played a critical role in the inhibition, but the GTFD motif was also required for complete inhibition of CsCF-6. The CsCF-6 prodomain showed broad inhibitory activity against several cysteine proteases. These results suggest that the CsCF-6 prodomain plays bi-functional roles in correct folding and inhibition of its cognate enzyme.

Most of papain family cysteine proteases are synthesized as inactive zymogens that consist of a signal peptide, a prodomain, and a mature domain that possesses catalytic activity. The zymogens are converted to active mature forms by proteolytic cleavage of the proregion, which is mediated by other proteases or by an autocatalytic processing event. The proregion of these enzymes is essential for several important physiological functions such as proper folding and correct targeting of the enzymes during maturation and regulation of the mature enzymes [1–3].

Papain family cysteine proteases are widely expressed in helminth parasites and their essential roles in parasite physiology and host-parasite interactions have been studied extensively [4–8]. However, only a few studies have been done on the nature of prodomains in cysteine proteases of helminth parasites. The proregions of *Fasciola hepatica* and *F. gigantica* cathepsin Ls are required for folding of parental enzymes [9,10]. The propeptide of *F. hepatica* cathepsin L selectively inhibits its cognate enzymes but does not inhibit mammalian cathepsins L, K and B, or papain [11]. These results suggest that cysteine proteases of helminth parasites are regulated by their prodomains, and, therefore, understanding the functional nature of the helminth parasite cysteine protease prodomain would be helpful to gain insight into the nature and biology of the enzymes and also may pave the way for the design of new selective inhibitors that can be applicable for drug development.

Cathepsin Fs of *Clonorchis sinensis* (CsCFs) are multigene family cysteine proteases consisting of 12 different genes [8]. They are synthesized in the parasite's intestine, actively secreted into the intestinal lumen, and mainly associated with nutrient-acquisition by the parasite [6,8]. These enzymes are major components of excretory and secretory products (ESP) of the parasite and this also suggests their possible extracorporeal roles in host-parasite interactions. Similar to other mammalian papain family cysteine proteases, CsCFs are synthesized as proenzymes and are converted to active, mature forms by removal of the prodomain via autocatalytic processing at an acidic pH [6,8]. Considering their important roles in parasite physiology and their potential as drug targets, it would be interesting to investigate the nature and functional features of the prodomain of the enzymes for regulatory and inhibitory elements.

To investigate the role of the CsCF-6 prodomain in the folding process of its cognate enzyme, five expression constructs (EF1–EF5), each containing the entire mature domain with a different proregion lengths (Fig. 1A), were amplified by polymerase chain reaction (PCR), cloned into the pQE-30 vector (Qiagen, CA, USA), and transformed into *Escherichia coli* M15 [pREP4] (Qiagen), respectively. Expression of each recombinant protein was induced with 1 mM isopropyl-1-thiol-β-D-galactopyranoside (IPTG) at 37 °C for 3 h. The *E. coli* cells were collected and suspended in 8 M urea lysis buffer. The recombinant proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen) and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The purified proteins were refolded, activated, and concentrated as described

* Corresponding author. Tel.: +82 55 772 8102; fax: +82 55 772 8109.
E-mail addresses: bkna@gnu.ac.kr, caunbk@hanmail.net (B.-K. Na).

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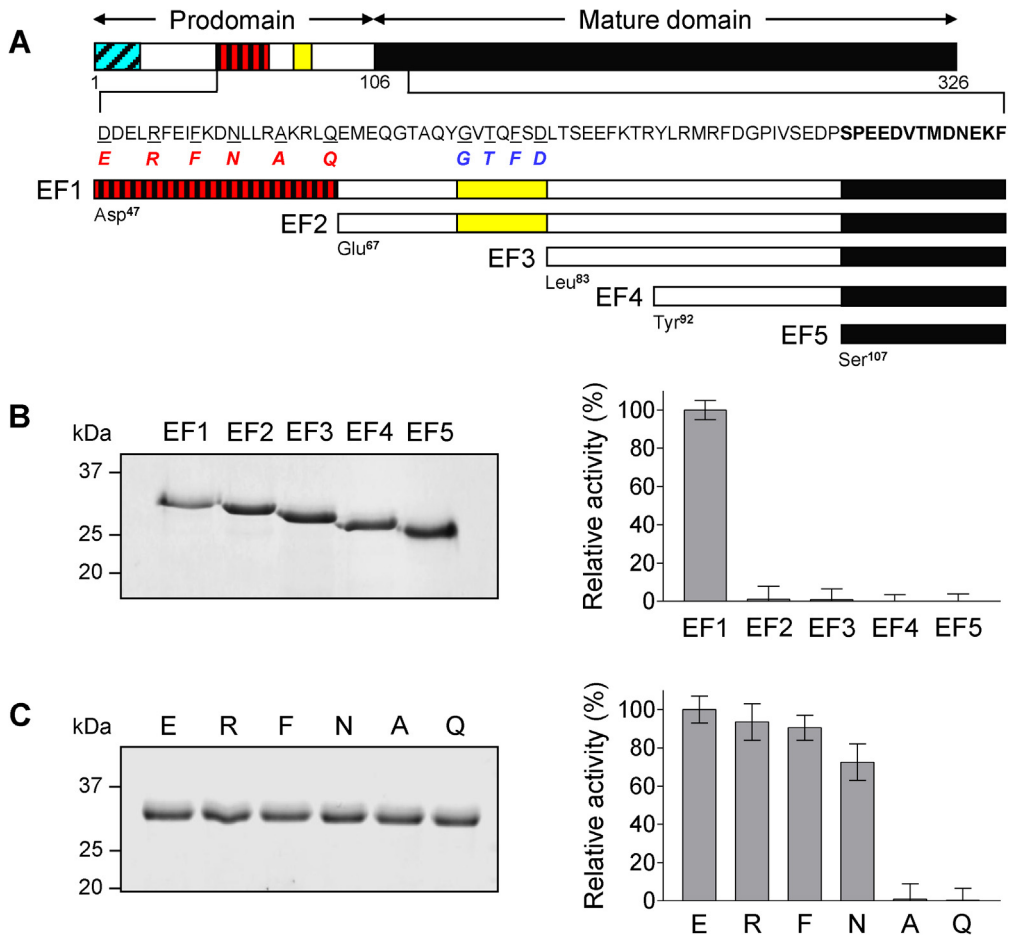

Fig. 1. Determination of the CsCF-6 prodomain regulatory element for folding. (A) Schematic for expression constructs. (B) Expression of recombinant proteins and enzyme activity assay. (C) The ERFNAQ motif region was further segmented to define the minimum length of prodomain required for CsCF-6 folding. All assays were performed triplicate and the mean and standard deviation (SD) were calculated.

previously [6]. To evaluate the folding efficacy of each protein, enzyme activity was assayed with benzyloxycarbonyl-L-leucyl-L-arginine 4-methyl-coumaryl-7-amide (Z-LR-MCA; Peptide International, KY, USA) as a substrate with a method described previously [6]. The release of fluorescence was monitored for 20 min at room temperature with a Fluoroskan Ascent FL (Thermo, Vantaa, Finland) at an excitation wavelength of 355 nm and emission wavelength of 460 nm. EF1 activity was chosen as 100% folding efficacy, as it has been demonstrated to be refolded correctly and to show enzyme activity [6]. The folding efficacies of EF2–EF5 were calculated by comparing their activities with that of EF1. To further define the minimum element of the prodomain that is essentially required for folding of CsCF-6, six different constructs spanning the entire mature domain and a segmented ERFNAQ motif (E, R, F, N, A, and Q) were amplified, cloned into the pQE-30 vector, and transformed into *E. coli* M15 [pREP4], respectively. The recombinant proteins were expressed, purified and refolded as described above and their activities were analyzed. All assays were performed triplicate and the mean and standard deviation (SD) were calculated.

To analyze the nature of CsCF-6 prodomain as an inhibitory element, a series of CsCF-6 prodomain fragments (PF1–PF6) were amplified (Fig. 2A), cloned into the pQE-30 vector, and transformed into *E. coli* M15 [pREP4], respectively. Expression of each recombinant protein was induced with 1 mM IPTG at 37 °C for 3 h, and the *E. coli* cells were collected and suspended in native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were sonicated on ice and the supernatant was collected by centrifugation at 4 °C for 20 min at 12,000 × *g*. The recombinant protein was purified by Ni-NTA column and the purity of each recombinant protein was confirmed by 15% SDS-PAGE. The inhibitory activity of each CsCF-6 prodomain fragment on mature CsCF-6 was analyzed. The active mature CsCF-6 was prepared as described previously [6]. Mature CsCF-6 (20 nM) was incubated with different concentrations (0–100 nM) of each prodomain fragment in 50 mM sodium phosphate (pH 6.0) for 30 min at room temperature. Then, the substrate (Z-LR-MCA) was added to each mixture, and the residual enzyme activity was measured as described above. The data were normalized between 0 and 100% activity, and the K_i values were calculated by non-linear regression using GraphPad Prism 5.0 Software (CA, USA).

The inhibitory activity of the CsCF-6 prodomain on other related cysteine proteases, including CsCF-4, CsCF-11, human cathepsin B (Sigma, MO, USA), human cathepsin L (Sigma), and papain (Sigma), was also analyzed. Recombinant CsCF-4 and CsCF-11 were prepared as described previously [8]. The substrate and assay buffer for each enzyme were as follows. CsCF-4 and CsCF-11: Z-LR-MCA, 50 mM sodium acetate (pH 6.0); human cathepsin B: Z-L-arginyl-L-arginine-MCA (Z-RR-MCA), 50 mM sodium acetate (pH 6.0); human cathepsin L and papain: Z-L-phenylalaninyl-L-arginine-MCA (Z-FR-MCA), 50 mM sodium acetate (pH 6.0). In all assay experiments, 10 mM dithiothreitol was included in the assay buffers. The concentration of cysteine proteases used in this study was determined by active site titration with *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane (E-64) [8]. All assays were performed in triplicate with two replications and the mean and SD were calculated.