Developmental and Comparative Immunology 76 (2017) 65–76

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Divergent roles of three cytochrome c in CTSB-modulating coelomocyte apoptosis in *Apostichopus japonicus*

Huahui Chen, Miao Lv, Zhimeng Lv, Chenghua Li*, Weiwei Zhang, Xuelin Zhao, Xuemei Duan, Chunhua Jin, Jinbo Xiong, Feng Xu, Ye Li

School of Marine Sciences, Ningbo University, PR China

ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form
20 May 2017
Accepted 21 May 2017
Available online 24 May 2017

Keywords:
Apostichopus japonicus
Cytochrome c
Cathepsin B
Mitochondrial membrane potential
Apoptosis

ABSTRACT

Cytochrome c plays crucial roles in apoptosis and the immune response. We previously demonstrated that cathepsin B from *Apostichopus japonicus* (AjCTSB) induces coelomocyte apoptosis. However, the mechanistic explanation and the regulation of this process have not been investigated. In the present study, we identified three cytochrome c cDNAs from *A. japonicus* (designated Ajcytc1, Ajcytc-1, and Ajcytc-2) using expressed sequence tag- (EST) and RACE-based approaches. The deduced amino acid sequences of the three cytochrome isoforms contained conserved CXXCH motifs, which are involved in binding heme and maintaining proteolytic activity. Time course expression analysis *in vitro* and *in vivo* revealed that the three cytochrome isoforms were induced upon pathogen challenge and LPS exposure. More importantly, *AjCTSB* knockdown by siRNA dramatically increased mitochondrial membrane potential ($\Delta\Psi m$) in a time-dependent manner based on JC-1 fluorescent probe staining. *AjCTSB* knockdown also resulted in decreased expression of these three cytochromes 24 h after siAjCTSB transfection. Functional analysis using isoform-specific siRNAs revealed that *Ajcytc-1*, but not *Ajcytc1* or *Ajcytc-2*, is involved in coelomocyte apoptosis. Moreover, the transcript level of *Ajcaspase-3*, an apoptosis executioner, was also consistently down-regulated upon silencing of *Ajcytc-1* but not *Ajcytc1* or *Ajcytc-2*. Collectively, these results indicate that Ajcytc1, Ajcytc-1, and Ajcytc-2 play distinct roles in mediating the immune response to bacteria according to AjCTSB expression. Moreover, Ajcytc-1 could be released upon dissipation of the $\Delta\Psi m$, which could further trigger coelomocyte apoptosis through the activation of *Ajcaspase-3*.

1. Introduction

Apoptosis is a normal physiological process in immune cells (Strasser et al., 2000; Wyllie, 1986). Moreover, apoptosis plays a critical role in the maintenance of tissues and in cell homeostasis (Opferman and Korsmeyer, 2003; Wyllie et al., 1980), which is also considered as a host defense mechanism against infectious pathogens (Krysko et al., 2008; Sahtout et al., 2001; Xian et al., 2013). Apoptosis is orchestrated through either the extrinsic or intrinsic apoptotic pathway (Savitskaya and Onishchenko, 2015; Tait and Green, 2010). The extrinsic pathway, which is also called the cell death-receptor pathway, is activated by members of the death receptor superfamily, such as Fas (CD95) or TNF-R1 (Siegel et al., 2000). The intrinsic pathway is characterized by mitochondrial dysfunction and the release of caspase activators (Fulda and Debatin, 2006; Jendrossek, 2012), including cytochrome c, followed by the activation of caspase-9 and caspase-3, which elicit the morphologic signs of apoptosis, including membrane blebbing, cell shrinkage, and DNA fragmentation (Hengartner, 2000). Of the events associated with the intrinsic pathway-mediated induction of apoptosis (also referred to as mitochondria-dependent apoptosis), the release of cytochrome c from the mitochondria is the initial signaling event (Li et al., 2004; Reubold and Eschenburg, 2012; Tait and Green, 2010).

The cytochrome c (cytc) family consists of electron transfer proteins, such as cytc and cytc1, which contain one or several heme

* Corresponding author. 818 Fenghua Road, Ningbo University, Ningbo, Zhejiang Province 315211, PR China.
E-mail address: lichenghua@nbu.edu.cn (C. Li).

http://dx.doi.org/10.1016/j.dci.2017.05.018
0145-305X/

C groups that mediate binding through either one or two thioester bonds involving the sulfhydryl groups of cysteine residues (Mavridou et al., 2012, 2013). Cytc proteins are loosely associated with the surface of the inner mitochondrial membrane, which accepts electrons from the cytochrome bc1 complex and transfers electrons to complex IV (Hüttemann et al., 2011). Therefore, cytc is considered an essential component of the mitochondrial electron transport chain. More importantly, in response to bacterial challenge or pathogen infection, cytc is also involved in the initiation of apoptosis (Li et al., 2004). During the early phase of apoptosis, the mitochondrial membrane potential dissipates, and the heme-binding protein cytc becomes detached from the mitochondrial inner membrane, after which it is secreted into the cytoplasm through pores in the outer membrane (Orrenius and Zhivotovsky, 2005). During this process, the release of cytc initiates a chain of events, culminating in the activation of caspase-3 and/or caspase-7, which are responsible for cleaving proteins and inducing apoptosis (Turk et al., 2002a; Green and Kroemer, 2004). Unfortunately, the roles of the different cytc isoforms during the induction of apoptosis remain largely unknown. Moreover, the roles played by the different cytc isoforms during the immune response upon pathogen infection remain poorly understood, especially in non-model invertebrates.

The evolution, structure, and function of cytc in apoptosis have become research focuses in several organisms, especially with respect to their immune-related functions in response to pathogen infection (Allen et al., 2008; Allen, 2011; Brown and Borutaite, 2008; Hüttemann et al., 2011). The invertebrate sea cucumber *Apostichopus japonicus* is an economically important marine species in China (Yuan and Zhao, 2015). However, *A. japonicus* is subject to viral and bacterial infections that dramatically affect survival (Liu et al., 2010). Therefore, understanding the role of apoptosis in the immune response in this species is important. Previous studies have shown that LPS challenge in *A. japonicus* significantly induces coelomocyte apoptosis *in vitro* (Lv et al., 2016; Wang et al., 2016), suggesting that the apoptotic process in coelomocytes could be an important aspect of eliminating deadly pathogens and damaged cells. Furthermore, we previously reported that cathepsin B (CTSB) enhances apoptosis in pathogen-challenged sea cucumber coelomocytes (Chen et al., 2017). In vertebrates, apoptosis is triggered by caspase activation and mitochondrial membrane permeabilization (MMP), which are intimately linked because MMP stimulates caspase activation through the mitochondrial release of several caspase-activating proteins, in particular cytochrome c (Waterhouse et al., 2001). Recent increasing studies have shown that CTSB is one of the major lysosomal cysteine proteases (Deininger et al., 1997; Guicciardi et al., 2004; Morchang et al., 2013) involved in the activation of several caspases to promote the release of proapoptotic mitochondrial factors, which trigger apoptosis via the mitochondria-dependent intrinsic apoptosis pathway (Guicciardi et al., 2000; Foghsgaard et al., 2001; Toomey et al., 2014). Based on these results, we hypothesized that CTSB could induce the loss of mitochondrial membrane potential and thus trigger the release of cytc from the mitochondria into the cytosol to activate caspase-3 and induce sea cucumber coelomocyte apoptosis. To further understand the regulation of apoptotic signaling as it relates to the immune response in the sea cucumber, we cloned three cytochrome c cDNA isoforms, characterized their tissue distribution, and analyzed their expression profiles in response to *Vibrio splendidus* challenge and LPS exposure. Moreover, we analyzed the effect of cytc isoform-specific knockdown on the dissipation of mitochondrial membrane potential and apoptosis by flow cytometry. Our results shed new light on apoptosis signaling through AjCTSB.

2. Materials and methods

2.1. Experimental animals and immune response challenge

The sea cucumbers *Apostichopus japonicus* (weight: 100 ± 15 g) were obtained from the Dalian Pacific Aquaculture Company (Dalian, China) and were acclimatized in 30 L of aerated natural seawater (salinity 28, temperature 16 °C) for three days. For immune challenge experiments, one tank of *A. japonicus* served as the control group, and five test tanks were inoculated with a high density of live *Vibrio splendidus* to a final concentration of 1×10^7 CFU mL^{-1}. The infection dose and sampling points were determined by immune gene expression analysis. The coelomocytes from 5 individuals for the control group and the bacteria-challenged groups were collected at 0, 6, 24, 48, 72, and 96 h. Sea cucumbers were dissected on ice using sterile scissors, and the coelomic fluids were filtered through a 300 Mesh Cell Cribble to remove large tissue debris. The coelomocytes were harvested by syringe and then were centrifuged at 800×*g* at 16 °C for 5 min for the time-course expression analysis. For the spatial expression analysis, coelomocytes and other four tissues, including muscle, tentacles, respiratory trees, and intestines, were collected from control individuals using sterilized scissors and tweezers. These tissues (approx. 100 mg wet weight) were ground into powder under liquid nitrogen using a mortar and pestle. We performed 5 replicates for the experimental group and the control group, and all samples were stored at −80 °C prior to RNA extraction.

2.2. Measurement of mitochondrial membrane potential ($\Delta\Psi m$) after AjCTSB silencing

To evaluate whether AjCTSB modulates apoptosis through mitochondrial-dependent signaling pathways (Guicciardi et al., 2000), the mitochondrial membrane potential was assessed after siAjCTSB transfection. Small interfering RNAs (siRNA) targeting *AjCTSB* were designed and synthesized by GenePharma (Shanghai, China). The detailed sequence information of the siRNAs is shown in Table 1. A control siRNA (negative control, NC) that does not

Table 1
PCR primer and siRNA sequences used in this study.

Primer name	Primer sequence (5′–3′)	Usage
Ajcytc1 3-1	TATGAGGATGGGACGCCAGCAAC	3′ RACE
Ajcytc1 3-2	CCGCCTACTACTTGAAGAGACAC	
Ajcytc-1 3-1	CTCTGGCAAGCACAAACAGGGTC	3′ RACE
Ajcytc-1 3-2	CTCCTCAACCCCAAAGACTACATC	
Ajcytc-2 3-1	TTGACTATGCCAAGGGGTACACGG	3′ RACE
Ajcytc-2 3-2	GTGAGAACAAACAAGATAGACAGGAC	
Ajβ-actin qF	CCATTCAACCCTAAAGCCAACA	Real-time PCR
Ajβ-actin qR	ACACACCGTCTCCTGAGTCCAT	
Ajcytc1 qF	TGACGAGCAAGCAAGAG	Real-time PCR
Ajcytc1 qR	GCCATTCCGATTGCTTGTC	
Ajcytc-1 qF	CTGGCAAGCACAAACAGGGTC	Real-time PCR
Ajcytc-1 qR	TCTTTGGGGTTGAGGAGGTAG	
Ajcytc-2 qF	GAAGCACAAAATGGGTCC	Real-time PCR
Ajcytc-2 qR	GGCATAGTCAAAGTCAGG	
siRNA sequences		
AjCTSB siRNA	GCUAUGUCCGAUCGUUAUUTT AAUAACGAUCGGACAUAGCTT	AjCTSB interference
Ajcytc1 siRNA	GGGAUGAGUUAUACUACAATT UUGUAGUAUAACUCAUCCCTT	Ajcytc1 interference
Ajcytc-1 siRNA	GCACCUGGUUUCUCCUACATT UGUAGGAGAAACCAGGUGCTT	Ajcytc-1 interference
Ajcytc-2 siRNA	CCAAAUCUAUACGCACUUUTT AAAGUGCGUAUAGAUUUGGTT	Ajcytc-2 interference
siRNA control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT	Negative control (NC) for siRNA interference