Insect Biochemistry and Molecular Biology 43 (2013) 936–946

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Inhibition of host cell encapsulation through inhibiting immune gene expression by the parasitic wasp venom calreticulin

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ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form
26 July 2013
Accepted 29 July 2013

Keywords:
Calreticulin
Venom
Parasitoid
Encapsulation

ABSTRACT

Parasitoid wasps inject venom into the host to protect their offspring against host immune responses. In our previous study, we identified a calreticulin (CRT) in *Pteromalus puparum* venom. In this study, we expressed the wild-type and the coiled-coil domain deletion mutant *P. puparum* calreticulins (PpCRTs) in *Escherichia coli* and prepared polyclonal antibody in rabbit against PpCRT. Western blot analysis showed that PpCRT protein was not only present in the venom but also in all the tissues tested. Real time PCR results indicated that PpCRT mRNA was highly expressed in the venom gland. The transcript level of PpCRT in the venom gland was peaked at 2 days post-eclosion, while the PpCRT protein in the venom was maintained at a constant level. Both recombinant wild-type and mutant PpCRT proteins could bind to the surface of *P. puparum* eggs. Recombinant PpCRT inhibited hemocyte spreading and cellular encapsulation of the host *Pieris rapae in vitro*, and the coiled-coil domain is important for the inhibitory function of PpCRT. Immunocytochemistry results showed that PpCRT entered *P. rapae* hemocytes, and the coiled-coil domain played a role in this process. After injection of recombinant PpCRT into *P. rapae* pupae, real time PCR results showed that PpCRT inhibited transcript levels of host encapsulation-related genes, including calreticulin and scavenger receptor genes. In conclusion, our results suggest that *P. puparum* venom protects its offspring against host cellular immune responses via its functional component PpCRT to inhibit the expression of host cellular response-related genes.

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1. Introduction

Parasitoids are natural enemies of numerous insects and can serve as important biological control agents in agricultural ecosystem. Endoparasitoids oviposit their eggs into the hemocoel of their insect hosts, where their offspring continue to grow and develop. Encapsulation is an important cellular response in insect innate immunity, and it is triggered when insects are infected with some large invaders such as the eggs of parasitic wasps. During encapsulation, hemocytes first adhere to the surface of large invaders and then spread to form the layers of overlapping cells, which are ultimately accompanied by melanization (Lavine and Strand, 2002; Strand, 2008). For parasitoids, in order to allow the eggs or larvae to grow and develop inside their hosts, they have developed “passive” and “active” strategies to avoid or inhibit host immune reactions (Pennacchio and Strand, 2006). Some parasitoid eggs or their larvae possess unique surface features to passively evade host hemocyte encapsulation (Corley and Strand, 2003). Some other parasitoids can actively interfere with host immune defenses using venom, polydnaviruses (PDVs), virus-like particles (VLPs), teratocytes, ovarian proteins and parasitoid larval secretions (Asgari and Rivers, 2011; Fang et al., 2010; Moreau and Guillot, 2005; Pennacchio and Strand, 2006). In endoparasitoid species, venom proteins are predominately involved in the regulation of host immune responses or development, alone or in combination with other factors (Asgari and Rivers, 2011; Fang et al., 2011). Our previous results have demonstrated that parasitoid venom as a whole agent could disrupt host immune responses (Fang et al., 2010, 2011), however, there are only a few reports regarding the characterizations and functional studies of different venom components (Asgari and Rivers, 2011; de Graaf et al., 2010). For examples, A Rho-GAP domain venom protein (P4) from *Leptopilina boulardi*, calreticulin from *Costesia rubecula* venom, 30 kDa venom

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0965-1748/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2013.07.010

protein (VPr3) from *Pimpla hypochondriaca*, and a 24.1 kDa venom protein (Vn11) from *Pteromalus puparum* have been identified as the functional components to disrupt host cellular immunity (Colinet et al., 2007; Labrosse et al., 2005; Richards and Dani, 2008; Wu et al., 2008; Zhang et al., 2006). A 50 kDa glycoprotein (Vn50) and a 4.6 kDa peptide (Vn4.6) from *C. rubecula* venom, a serpin and an extracellular SOD from *L. boulardi* venom have also been shown to inhibit melanization of their host hemolymph (Asgari et al., 2003a, 2003b; Colinet et al., 2009, 2011).

Calreticulin (CRT) is a 46-kDa chaperone protein first isolated from the endoplasmic reticulum (ER) of rabbit skeletal muscle cells, and it is composed of three structurally and functionally distinct domains (Michalak et al., 1999). Based on the results of protein localization, calreticulin is localized in the intracellular and extracellular compartments, and is also present on the cell surface (Gold et al., 2010). Calreticulin is a multifunctional protein participating in wound healing, immune response, fibrosis and other physiological processes (Gold et al., 2010). Calreticulin has been identified in mammals, invertebrates and plants but not in yeast or prokaryotes. In insect species, calreticulin was first reported in *Drosophila melanogaster*, and it is involved in the olfactory system and phagocytosis of apoptotic cells (Kuraishi et al., 2007; Stoltzfus et al., 2003). *Pieris rapae* (Lepidoptera: Pieridae, a small cabbage white butterfly) calreticulin (PrCRT) was demonstrated to enhance cellular encapsulation *in vitro*, and the N-domain of PrCRT was required for encapsulation (Wang et al., 2012). In parasitoids, calreticulin was first isolated from the expression products of polydnavirus and was subsequently discovered in the venom fluid of *C. rubecula* (Asgari et al., 2003c; Zhang et al., 2006). Since then, cDNA sequences encoding calreticulins have been successively identified from other parasitoids, including *Microctonus hyperodae*, *Microctonus aethiopoides*, *Nasonia vitripennis* and *P. puparum* (Crawford et al., 2008; de Graaf et al., 2010; Zhu et al., 2010). To date, only the function of venom calreticulin from *C. rubecula* (CrCRT) was previously determined. CrCRT was present in all examined tissues of female wasps, including ovaries, venom gland, venom reservoirs and gut, and it could inhibit hemocyte spreading and cellular encapsulation *in vitro* (Zhang et al., 2006). However, the mechanism by which the venom calreticulin inhibits encapsulation remains unclear.

P. puparum (Hymenoptera: Pteromalidae) is a gregarious pupal endoparasitoid with a wide host range and prefers to parasitize the pupae of certain papilionid and pierid butterfly species (Dweck, 2009; Takagi, 1985). It is the most predominant pupal parasitoid of the small white butterfly, *P. rapae*, with a parasitism rate greater than 90% in the fields of cruciferous vegetables in China (Hu, 1984). *P. puparum* injects venom, the key virulence factor, into its hosts to manipulate their immune system during oviposition. *P. puparum* and its pupal host *P. rapae* comprise a model system for studying the influence of venom on host biology in non-PDV systems (Fang et al., 2010). Previous studies have successfully identified a calreticulin in *P. puparum* venom (PpCRT) using a 2D/MS method (Zhu et al., 2010). However, the function of PpCRT is still unclear. In the present study, we mainly determine the function of PpCRT, including its effect on host (*P. rapae*) immune-related genes. In addition, we for the first time identified a coiled-coil domain in PpCRT and discovered that all calreticulins contain a coiled-coil domain, which is also present in a viral protein CrV1 from *C. rubecula*. Coiled-coil domain is a typical structural motif in proteins for oligomer formation. The coiled-coil domain in the viral protein CrV1 is essential for CrV1 to bind and to be uptaken by hemocytes, facilitating the fusion of viral and cellular membranes (Asgari and Schmidt, 2002). Therefore, we also analyzed the effect of the coiled-coil domain on PpCRT function by constructing a deletion mutant of PpCRT. Our results further reveal the mechanism of calreticulin from parasitoid venom in inhibition of host encapsulation.

2. Materials and methods

2.1. Insect rearing

Parasitoid *P. puparum* and host *P. rapae* were maintained as described previously and used in all experiments (Fang et al., 2010). Briefly, *P. rapae* larvae were fed fresh cabbage leaves at 25 ± 1 °C with a photoperiod of 10:14 h (light : darkness) until they pupated. Newly pupated hosts were exposed to 3-day old mated female wasps of *P. puparum*. Then, parasitized *P. rapae* pupae were maintained at 25 ± 1 °C with a photoperiod of 10:14 h (light : darkness). Once emerged, *P. puparum* adults were collected and held together in glass vials and fed with 50% (v/v) honey solution absorbed on cotton under the same conditions.

2.2. Expression and purification of recombinant wild-type (mature) PpCRT and the coil-coiled domain deletion mutant PpCRT

To construct pET28a-wild type PpCRT expression vector, we employed a PCR strategy to amplify *PpCRT* cDNA encoding the mature protein (residues 19–403) using primers PpCRT-SP and PpCRT-AP (Table 1). *PpCRT* cDNA lacking the coiled-coil domain ($PpCRT^{\Delta 331-368}$) was constructed by overlapping PCR extension method (Heckman and Pease, 2007). Briefly, the 5′-end part of *PpCRT* cDNA (encoding residues 19–330) was generated using primer PpCRT-SP and PpCRT-mR by PCR, and the 3′-end part (encoding residues 369–403) was produced using primers PpCRT-mF and PpCRT-AP (Table 1). The two PCR products were isolated purified and quantified. Then, the two fragments were used as the template to generate the coiled-coil domain deletion mutant PpCRT using primers PpCRT-SP and PpCRT-AP. The PCR products were digested by *BamH*I and *Xho*I and then ligated to a *BamH*I/*Xho*I digested pET28a vector to transform competent *Escherichia coli* BL21 (DE3) cells. The inserted gene sequence was confirmed by bidirectional sequencing. The positive clones containing the inserts were selected and incubated at 30 °C in LB medium containing kanamycin (50 μg/ml) until $OD_{600} = 0.6$. Recombinant protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and the temperature was shifted to 28 °C. After incubation for another 5 h, bacteria were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, lysed by sonication and centrifuged. The recombinant proteins in the supernatants were loaded onto a previously equilibrated Ni-NTA affinity column (Novagen, New Jersey, USA) and then purified following the manufacturer's protocol.

2.3. Preparation and purification of PpCRT polyclonal antibody

The recombinant PpCRT (wild-type) protein was separated by 12% SDS-polyacrylamide gel electrophoresis under reducing conditions, and the gel slice containing recombinant PpCRT was cut out. The protein in the gel was eluted by electrodialysis in a dialysis tube and used as an antigen for immunization in rabbits. The immunization procedure was carried out by the Animal Experimental Center in Zhejiang Chinese Medical University. An antibody to PpCRT was purified from antiserum using the Montage Antibody Purification kit with PROSEP-A media (Millipore, Billerica, MA), following the manufacturer's protocol.

2.4. SDS-PAGE and immunoblot analysis

Protein quantification was determined by the Bradford assay. Total proteins from each sample were subjected to 12% SDS-PAGE, and the proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma, St. Louis, MO) by a semi-dry electrophoretic transfer system (Bio-Rad, Hercules, CA). The membranes

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